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National University of Ireland, Galway

Ryan Institute, Environmental, Marine and Energy Research

Irish Seaweed Research Group

**Diversity, species concepts and phylogenetic  
relationships of some marine algae in a perspective  
of Biodiscovery**

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Supervisors: Prof. Mark Johnson, Dr. Fabio Rindi

February 2013

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## Declaration of Author's contribution

### Declaration of Author's contribution

Chapter B2: Mónica Moniz and Fabio Rindi designed the study. Mónica Moniz carried out lab work. Michael D. Guiry collected the most relevant samples in Tasmania. Mónica Moniz and Fabio Rindi maintained algal cultures. Mónica Moniz performed sequence alignment and data analysis. Mónica Moniz wrote the manuscript but all authors revised and approved the final version of it. It was accepted and published as: Moniz, M.B.J.; Rindi, F. and Guiry, M.D. (2012) Phylogeny and taxonomy of Prasiolales (Trebouxiophyceae, Chlorophyta) from Tasmania, including *Rosenvingiella tasmanica* sp. nov. *Phycologia*. 51(1):89-97.

Chapter B3: Mónica Moniz and Fabio Rindi designed the study. Mónica Moniz carried out lab work. Mónica Moniz and Fabio Rindi maintained algal cultures. Phil Novis and Paul Broady contributed with some of the most important samples and key information for the amplification of *psaB*. Mónica Moniz performed sequence alignment and data analysis. Mónica Moniz wrote the manuscript but all authors revised and approved the final version of it. It was accepted and published as: Moniz, M.B.J.; Rindi, F.; Novis, P.M.; Broady, P.; Guiry, M.D. (2012) Molecular phylogeny of Antarctic Prasiola (Prasiolales, Trebouxiophyceae) reveals extensive cryptic diversity. *Journal of Phycology*. 48(4):940-950.

Chapter B4: Mónica Moniz and Fabio Rindi designed the study. Mónica Moniz and Fabio Rindi maintained algal cultures. Some samples were sent to Dr. Gary Saunders lab for DNA extraction, and amplification and sequencing of the gene *tufA*. Mónica Moniz performed sequence alignment and data analysis. It will be submitted as publication co-authored by Fabio Rindi, Sandra Lindstrom and Gary Saunders.

Chapter C2: Mónica Moniz and Fabio Rindi designed the study. Mónica Moniz identified all samples with the supervision of Fabio Rindi. Mónica Moniz maintained algal cultures. Elena Maggi and Patrick Collins advised on and performed some of the statistical treatment. Mónica Moniz wrote the manuscript but all authors revised and approved the final version of it. A shortened version of this chapter was published as: Moniz M.B.J.; Rindi F.; Stephens K.; Maggi E.; Collins P.; McCormack G.P. (2013) Composition and temporal variation of the

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algal assemblage associated with the haplosclerid sponge *Haliclona indistincta* (Bowerbank). *Aquatic Botany*: in press (online publication 20 December 2012).

Chapter C3: Mónica Moniz, Carsten Wolff and Grace McCormack designed the study. Mónica Moniz carried out lab work, with the exception of sponge cell separation performed by Carsten Wolff. Simon Travers provided assistance with data analysis. Fabio Rindi contributed to the interpretation of the results. It will be submitted co-authored by Carsten Wolff, Simon Travers, Fabio Rindi and Grace McCormack.

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## Summary

### Summary of contents

The overall goal was to investigate the genetic diversity, phylogenetic relationships and taxonomy of some groups of marine algae of particular interest from a taxonomic and biogeographic point of view in a perspective of biodiscovery.

The order Prasiolales was chosen because its evolutionary history and classification are still in need of clarification. The results add substantial taxonomic and biogeographic coverage to previous studies and other molecular markers (*rbcL*, *psaB* and *tufA*). Two new species were described: *Rosenvingiella tasmanica* M.B.J.Moniz, Rindi & Guiry from Tasmania and *Prasiola glacialis* M.B.J.Moniz, Rindi, Novis, Broady & Guiry from Antarctica. Some records were reassessed: samples previously classified as *R. polyrhiza* (Rosenvinge) P.C.Silva from Australia was referred to *R. constricta* (Setchell & N.L.Gardner) P.C.Silva and samples formerly classified as *Prasiola crispa* (Lightfoot) Kützing are in fact *P. borealis* M.Reed. Antarctic cryptic species that were previously confused under *P. crispa* include genuine *P. crispa*, *P. antarctica* Kützing resurrected as an independent species and the new species *P. glacialis*. Marine samples of *Prasiola* from North Atlantic and the northwestern Pacific were sequenced to help clarifying species circumscriptions in this group. Results support the conspecificity of *P. stipitata* Suhr ex Jessen and *P. meridionalis* Setchell & N.L.Gardner.

To further understand the origin of bioactives produced by sponges and their symbionts, eukaryotes associated with the sponge *Haliclona indistincta* were investigated using morphological observations and next generation sequencing (NGS). Morphological investigation revealed the presence of 66 algal species, mostly filamentous, which colonized the surface of the sponge and did not penetrate deeply into it. In the course of one year, this community varied on the temporal scales of season and sampling date. In the NGS study, focus was given to the eukaryotic diversity associated with the sponge often ignored in similar studies. Data shows a high biodiversity of operational taxonomic units (OTUs) often represented by unique sequences. Possible fungal and dinoflagellate

## Acknowledgments

symbionts were present but diatoms were underrepresented compared to seawater perhaps due to active protection by the sponge. Divergence in the sponge rRNA 18S gene suggests the presence of multiple copies.

**Part A: Introduction**

**A1) Diversity, Classification and Species concepts in Marine Macroalgae**

The history of marine macroalgae classification evolved in parallel with the observation tools available and with the philosophical thinking and revolutionizing definitions of species and ancestry. Definition and circumscription of species represent one of the most important aspects in the classification of all groups of organisms. So far, in the history of systematics, no less than 26 species concepts have been proposed (Wilkins 2002). Far from being a purely academic exercise, the species concept has important practical repercussions, with financial, legal, biological and conservation implications (Frankham et al. 2012).

There is significant confusion between what a species is (species concept) and what delimits a species allowing its identification. De Queiroz (2007) defines species as a segment of separately evolving metapopulation lineages (i.e. any given species is but one of many segments that make up a species level lineage). In the next paragraphs, I will focus on what De Queiroz (2007) called secondary species criteria, i.e. the characteristics/boundaries used to define a species that arrive (if they do) at different points during speciation. I will list how species of algae have been defined and circumscribed through time.

The first detailed descriptions and classifications of seaweeds were based on the morphological features observed by unaided eye and light microscopy. Characteristics such as colour, size, shape of thallus, branching pattern, manner of attachment to the substratum, shape and number of chloroplasts and presence of certain structures like pyrenoids were used not only to identify species but also to infer degrees of relationship. The development of new microscopical techniques such as Scanning Electron Microscopy (SEM) or Transmission Electron Microscopy (TEM), which became widespread in the 1970s, introduced the use of ultrastructural characters in algal taxonomy and systematics. Characters such as ultrastructure of the flagellar apparatus, structure of pit connections and plasmodesmata, arrangement of thylakoids in the chloroplasts, and details of mitosis and cell division were used to characterize algal taxa and derive

## Part A: Introduction

relationships (Mattox and Stewart 1984, Melkonian 1982, Melkonian 1984, O'Kelly and Floyd 1984a, O'Kelly and Floyd 1984b, Picket-Heaps and Marchant 1972, van den Hoek et al. 1988).

The morphological species concept mentioned above was the first species concept to be used. It was utilised by the first scientists who described species of algae (Linnaeus 1753, 1759) and was essentially the foundation of the classification system used by Aristotle. To date, the vast majority of the algal species known has been described based on this concept. The morphological species concept states that a species (or morphospecies) is the smallest group of organisms that can be defined by structural characters and is (to a certain degree) easy to distinguish (Cronquist 1978, Graham et al. 2009, Grant 1981, Wilkins 2002). The difficulty with this concept is to objectively decide how much morphological difference is required for a group of organisms to be considered a separate species. At the same time, it is well known that the morphology and previously considered diagnostic characters of many marine algae are affected by strong phenotypic plasticity and may vary in relation to seasonal environmental factors such as temperature (Kubler and Dudgeon 1996), wave intensity (Fowler-Walker et al. 2006) and interactions such as herbivory (Diaz-Pulido et al. 2007); this is a particularly serious problem with morphologically simple algae. Recognition of the necessity of understanding whole life cycles has been a breakthrough in many species with some famous examples being *Asparagopsis armata* Harvey, for which the two phases of the life cycle were previously considered two species (Chihara 1962) and the genus *Porphyra* Agardh, for which the comprehension of its life cycle allowed for successful cultivation (Drew 1949).

In the last century, other species concepts have emerged. A popular one is the biospecies, or biological species concept, which was developed by Mayr (1942). Following this concept, species are considered groups of actually or potentially interbreeding natural populations which are reproductively isolated from other such groups (Mayr 1942). Other authors had previously remarked how important sexual isolation is in defining a species. For example, the first author to

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call attention to the necessity of applying the biological concept of species to an algal group (diatoms) seems to be Comber (1897). This author considered that species and varieties were separated by the level of differentiation of the sexual elements, which in the case of species, would lead to reproductive isolation. Comber made clear that, at the time, sexuality and reproductive isolation were not well understood and it would have been difficult to apply this concept to any classification. Consequently, he suggested that the criterion for distinction at specific level should be the presence or absence of a continuous series of morphological intermediates (Mann 1999).

Some cases of successful application of the biospecies concept to marine macroalgae have been described. Guiry (1992) focused on cases of red algae giving examples of *Aglaothamnion westbrookiae* Rueness & L'Hardy-Halos, *Gracilaria tikvahiae* McLachlan and *Gigartina teedii* (Mertens ex Roth) J.V. Lamouroux as species with populations that are morphologically indistinguishable and interbreed with each other throughout their geographical ranges. Using interbreeding experiments, Guiry and West (1983) used this concept to resolve the taxonomy of *Gigartina stellata* (Stackhouse) Batters (= *Mastocarpus stellatus* (Stackhouse) Guiry) in the North Atlantic. Coleman (1977) found 20 distinct mating complexes within the green volvocine *Pandorina morum* Bory.

There are several criticisms to this concept that range from practicality aspects to application to real cases. Practically, it is often very hard to verify sexual behaviour or demonstrate that progeny is fertile (Graham et al. 2009). Also, this concept cannot be used in several groups of unicellular or few-celled algae, especially green algae (Trebouxiophyceae, Klebsormidiophyceae, Chlorokybophyceae, some prasinophytes) that don't have sexual reproduction or for which sexual reproduction has never been documented (for a recent review see Leliaert et al. 2012). Some authors do not support this concept since they regard sexuality as a primitive characteristic. These authors state that there is evidence for algae and other organisms which show considerable evolutionary divergence, but are still capable of interbreeding (John and Maggs 1997). One example is clones of temporarily named *Chlamydomonas reinhardtii* Dangeard which are

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crossed with an authenticated clone and are able to interbreed, but show big divergence in various characteristics such as heavy metal tolerance, protein composition, mitochondrial DNA length, and nuclear and organelle DNA restriction fragment length polymorphisms (Spanier et al.1992).

For cases such as uniparental asexual and parasexual organisms, another concept has been introduced, the agamospecies (Cain 1954), also known as microspecies (Grant 1981). In this case, species are groups with one parental line.

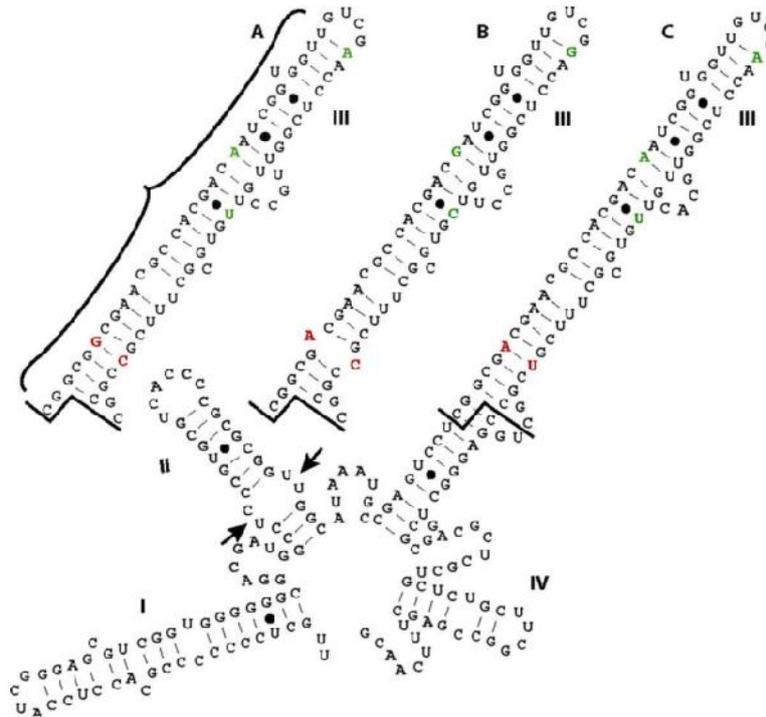
In order to integrate geological time and fossil records into any concept, the successional species concept (George 1956, Simpson 1961) was defined. This would see a species as a changing biological species viewed in the perspective of geological time. Organisms belonging to a successional species are members of the same lineage living in different time periods and possibly possessing different morphological characters. The decision to draw a dividing line is arbitrary, often decided by gaps in the fossil record.

An attempt to join the three previous concepts (the biological, agamospecies and successional species) has yielded the concept of the evolutionary species (sensu Simpson 1961). An evolutionary species a) is a lineage, i.e. an ancestral-descendant sequence of populations existing in space and time, b) evolves separately from other such lineages, c) has its own particular ecological niche in a biotic community and d) has its own evolutionary role during the course of its history. This concept solves the common problem of hybridization in plants and algae since, what is important is not whether two species hybridize but whether or not they lose their distinct ecological and evolutionary roles. If these species do not merge despite some cases of hybridization, then they are separate species (Simpson 1961).

The appearance of genetics and genetic markers has introduced new concepts and reshaped older ones. One example of a new concept is the genetic species (Dobzhansky 1950, Mayr 1969, Simpson 1943) where a species is a group of organisms that have a common gene pool (Wilkins 2002). Another, more recent one is the Compensatory Base Change (CBC) species definition (Coleman 2009).

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This concept is based on the secondary structure of the Internal Transcribed Spacer 2 (ITS2) of the ribosomal DNA. Based on data available for some algal, plant and animal groups, Coleman (2009) concluded that (1) organisms that differ by one CBC in the highly conserved region of the 5'-side of ITS2 helix III are completely unable to cross and (2) identity for the entire ITS2 correlates with significant interbreeding potential.



**Fig.1** Secondary structure of ITS2, and comparisons of helix III among examples from the apple subfamily Maloideae, (A) *Amelanchier*, (B) *Mespilus* and (C) *Photinia*. The entire ITS2 structure of *Photinia* is shown to illustrate the typical ITS2 secondary structure of eukaryotes; hallmark characteristics are marked in helix II by arrowheads (pyrimidine-pyrimidine bulge) and in helix III by a bracket (5' 30 most conserved nucleotide positions). Helix III pairing variants among species of the family include one CBC (red) and three hemi-CBCs (green). *Amelanchier* fails to cross with any of the several genera that differ by the CBC. Taken from Coleman (2009).

And so “where a CBC in the critical 30 nucleotide section of helix III has appeared, the clade of organisms defined by that CBC very likely contains at least one, and perhaps a very small number of Z clades, several *biological species*, and one or more morphological species” (Coleman 2009). There have already been

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applications of this concept (Amato et al. 2007, Behnke et al. 2004, Müller et al. 2007), although cases in which this concept does not work have also been documented; Caisova et al. (2011) demonstrated that in the order Ulvales (Ulvophyceae) the presence of a CBC was not linked to any particular taxonomic level and most CBC “clades” sensu Coleman were paraphyletic. A CBC clade may therefore still encompass several biological species and thus, may require later revision. Moreover, it is not the most practical of concepts from a functional point of view. The ribosomal cistron occurs in hundreds of thousands of copies, which can constitute up to 10% of the nuclear genome (Smith et al. 2010), and different copies of ITS may be present in different populations, different specimens or sometimes even within the same individual thallus. Thus, ITS2 sequences often cannot be sequenced directly from the PCR product, but require cloning. The reconstruction of the secondary structure of ITS2 may not be straight forward, although some software programs for secondary structure predictions are now widely available (Schultz and Wolf 2009).

One example of reformulation of a previously used concept is the case of the Phylogenetic species concept. This concept has been formulated in different forms (Cracraft 1983, Eldredge and Cracraft 1980, Nixon and Wheeler 1990); in the most widespread interpretation, the phylopecies, also known as an autapomorphic species, is the smallest group of organisms that exhibit at least one synapomorphy, i.e. a shared derived character (Nelson and Platnick 1981). It is the most commonly used concept in the definition of new species in the modern age. Originally, the characters used were morphological, ultrastructural or biochemical (e.g. pigment composition). With the development of molecular systematics, molecular synapomorphies have become widely used. Nowadays, most new species of algae are described as phylogenetic species that form monophyletic groups in molecular phylogenies, usually supported by morphological synapomorphies.

Another way to delineate species which is becoming more common is utilising coalescent methods. As Knowles and Carstens (2007) explain: the relationship between the gene trees and the species history is modelled probabilistically. This means that a coalescent framework is used to calculate

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gene-tree probabilities, under a particular history, and estimate the likelihood that speciation has occurred (Knowles and Carstens 2007 and references therein).

These methods allow the delineation of species even when there is widespread incomplete lineage sorting and discordance of loci. This way, even very recently derived species can be identified. The advantages are that a model-based approach avoids biases in species detection arising from when and how speciation occurred and takes into account the high stochastic variance of genetic processes.

Several phycologists have discussed the particularities of species concepts in algae or specific groups of algae (Guiry 1992, 2012, John and Maggs 1997, Mann 1999). Guiry (1992) states that for most organisms we do not have information on breeding capabilities and thus the morphological or genetic definition is the default taxonomic base. He adds that although a consensus on a single, universal species concept amongst biologists is not essential, it would be helpful from the viewpoint of communication to know what exactly is meant by the term species. If we accept that evolution is taking place continuously, we must expect that there will be gradations of any particular criterion we choose to define. We should also not expect that breeding isolation will result in convenient, recognizable morphological characters purely for our convenience (Guiry 1992, Mann 1999).

The development of molecular systematics that has taken place in the last 40 years has substantially reshaped species definitions in all algal groups, showing numerous cases in which morphological features are inadequate to identify algal species (e.g. for the genus *Porphyra*, Broom et al. 2002 ). Numerous studies have revealed cryptic diversity in algae, including genera and species occurring in Ireland (Guiry 2012). Recently, the application of likelihood methods based on coalescent models has been used to redefine species of the *Boodlea* complex comprising the marine green algal genera *Boodlea*, *Cladophoropsis*, *Phyllocladon* and *Struveopsis* (Leliaert et al. 2009). Incongruence between traditional and phylogenetic species definition made the authors suggest encompassing all species under one genus, *Boodlea*. This study is also an example of how using the nrDNA internal transcribed spacer (ITS) causes problems when

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using the general mixed Yule-coalescent (GMYC) model. Ancestral ITS polymorphism caused by incomplete coalescence blurs the transition between species-level and population-level branching processes. Incomplete coalescence occurs when speciation has been more rapid than concerted evolution of the multiple rDNA repeats (Leliaert et al. 2009).

Using the different species concepts mentioned above, diversity discovery can be accomplished in different ways. During this Ph.D., two main strategies were followed, which confer a dual quality to this thesis. The first strategy was to investigate an algal taxon, the order Prasiolales, studying its evolutionary history and the possible existence of undescribed species, focusing on geographical regions in which these algae have not been examined using molecular tools. This group is particularly suitable for species discovery since its simple morphology can hide cryptic diversity and the south hemisphere has been undersampled. Also its evolutionary history is still not fully understood. The other strategy was to focus on an algal community associated with an understudied environment, the algal epibionts of the sponge *Haliclona indistincta* of the west coast of Ireland, in order to examine its species diversity and find out what species-level associations occur.

### **A2) Seaweed diversity in Ireland and the importance of marine macroalgae in biodiscovery research**

Considering the length of its marine coastline (6437 Km, World Resources Institute 2012) and its limited latitudinal range (from Malin Head, 55°23' N, to Mizen Head 51°26' N) Ireland is surprisingly species-diverse in marine macroalgal biodiversity. Ca. 6% of species existing worldwide live in Ireland, which is a much higher percentage compared to the terrestrial flora (optimistic calculations say it represents 0.25% of worldwide diversity, Guiry 2012). Five hundred and seventy species were catalogued recently for the Irish seaweed flora, belonging to 11 classes, 25 orders and 91 families (Guiry 2012). The highest number belongs to Rhodophyta (303), followed by Phaeophyceae (161), Chlorophyta (93) and Xanthophyceae (13, Guiry 2012).

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The seaweed flora of the west coast of Ireland is more diverse than that of other coasts because of the diversity of habitats, clearer water, and a relative paucity of large sandy stretches compared to other parts of the Irish shoreline. The occurrence of considerable stretches of porous shales and limestones in the West. These two rock types provide two types of habitats which allow for different species needs. Shales are soft and naturally erode to provide crevices under the harder limestone and the limestone provides a solid attachment (Hardy and Guiry 2006, Guiry 2012, Guiry pers. comm.).

Well known hotspots for marine algae, and other marine organisms, are the marine reserve of Lough Hyne, Co. Cork, the area of Finavarra, Co. Clare, and Clare Island, Co. Mayo (Rindi and Guiry 2004). These areas have two common features which make them interesting sites for research: they have physical conditions which promote marine biodiversity and they have been thoroughly studied. The shore in Finavarra is very diverse, encompassing limestone banks with irregular surfaces, exposed and partially or completely covered by coarse sand inter-mixed with sandy stretches, and has intermediate wave exposure conditions (appendix F1). Lough Hyne is a marine lake 1km long and  $\frac{3}{4}$  km wide and is connected to the Atlantic Ocean via Barloge Creek, by a narrow tidal channel, known as the Rapids. Tidal flows from the Atlantic fill Lough Hyne twice a day, running over the Rapids at up to 16 km per hour, and create in the lake a unique habitat of warm oxygenated seawater. Due to these special characteristics, the area was designated as Ireland's first Marine Nature Conservation Reserve. Clare Island is located 5 km off the shore of County Mayo. It is characterized by high habitat diversity and exposure conditions ranging from extremely exposed to sheltered. Clare Island has been the focus of two major natural history investigations promoted by the Royal Irish Academy, in which the seaweed flora was studied with great detail (Cotton 1912, Rindi and Guiry 2004).

Rindi (2008) stated that many seaweed species which have been reported to have bioactivity relevant for the pharmaceutical industry were found in Ireland. Reports of bioactivity for seaweeds collected in Ireland are detailed in Table 1. However, Rindi (2008) also pointed out that many reports mention species or

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groups of species, e.g. *Chlorella* M. Beijerinck, *Laurencia* J.V.Lamouroux and *Gigartina* Stackhouse, whose taxonomic classifications were later rearranged and now, without access to the original material, it may be impossible to know with certainty the identity of the material examined. This is an issue that is raised over and over again in biological exploitation of marine organisms. A correct identification of the specimens screened is essential; a misidentification would result in an incorrect selection of the algae containing the targeted molecule of interest, with the likely consequence of a considerable waste of time and financial resources. Furthermore, incorrect identifications will lead to misinterpretations of published studies, spreading confusion and misleading future work (Rindi et al. 2011). Identification of algae is a complex task that requires the expertise of skilled taxonomists and that involves several problematic aspects, which have become increasingly evident in recent years (see appendix F1).

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**Table 1:** Details on species reported to show bioactivity collected in Ireland.

Species	Location	Activity	Reference
21 species of brown algae, among which the most active are <i>Halidrys siliquosa</i> , <i>Bifurcaria bifurcata</i> and <i>Cystoseira tamariscifolia</i>	South coast of England and the west coast of Ireland (Finavarra, and Fanore, Co. Clare)	Antiprotozoal and antimycobacterial activity	Spavieri et al. 2010
23 species of red algae, among which the most active are <i>Corallina officinalis</i> , <i>Ceramium virgatum</i> , <i>Porphyra leucosticta</i> and <i>Calliblepharis jubata</i>	South coast of England and the west coast of Ireland (Finavarra, and Fanore, Co. Clare)	Antiprotozoal and antimycobacterial	Allmendinger et al. 2010
A collection of Laminariales species, the most active being <i>Laminaria</i> and <i>Saccharina</i> species, <i>Postelsia palmaeformis</i> , <i>Pseudochorda nagaii</i> and <i>Akkesiphycus lubricus</i>	West coast of Ireland	Presence of trigonelline and other betaines	Blunden et al. 2012
<i>Ascophyllum nodosum</i> , <i>Pelvetia canaliculata</i> , <i>Fucus serratus</i> and <i>F. vesiculosus</i>	Finavarra, Co. Clare.	Anti-oxidant	Quéguineur et al. 2012
<i>Fucus serratus</i> , <i>F. vesiculosus</i> , <i>Pelvetia canaliculata</i> , <i>Ascophyllum nodosum</i> , <i>Halidrys siliquosa</i> , <i>Bifurcaria bifurcata</i> , <i>Dictyota dichotoma</i> and <i>Halopithys incurva</i>	East coast of Ireland	Molluscicidal	Patel et al. 2008
<i>Ulva lactuca</i>	Baginbun Head, Wexford	Antibacterial	Tan et al. 2012

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Problems related to taxonomic identification need to be considered carefully in any bioactivity investigation, and two basic procedures should be adopted: (1) voucher specimens should be deposited in reliable repositories (such as public herbaria, museums or culture collections); and, (2) DNA sequences should be produced for samples with valuable properties and deposited in public repositories (such as GenBank), so that they are publicly available (see appendix F1).

### **A3) Aims of the work**

The overall goal of the project was to investigate the genetic diversity, phylogenetic relationships and taxonomy of some groups of marine algae of particular interest from a taxonomic or biogeographic point of view.

The order Prasiolales (Trebouxiophyceae, Chlorophyta) was chosen as the focus of the taxonomic and phylogenetic studies because it is a particularly interesting taxon and its evolutionary history and classification are still in need of clarification. This group not only has the uncommon characteristic of encompassing marine, terrestrial and freshwater species but it also exhibits a very unusual sexual behaviour (Rindi 2007). These characteristics, together with a simple morphology, make it a good candidate for molecular systematics and for the investigation of interesting evolutionary questions such as the conquest of different habitats, the origin and maintenance of sexual reproduction, species concepts in organisms with simple morphology.

Sponges are well known to be a rich source of bioactives (Gademann and Kobylinska 2009, García et al. 2012, Laport et al. 2009). It has been demonstrated that, often, this activity comes from the prokaryotes living in association with the sponge (e.g. Rützler 1990, Simister et al. 2012, Taylor et al. 2007, Trautman and Hinde 2002). However, very little has been reported on the algal eukaryotic community associated with sponges (e.g. Davis and White 1994). It is reasonable to speculate that this community can itself be a possible rich source of bioactivity.

## Part A: Introduction

Specific objectives were the following:

### 1) Taxonomy, phylogeny and biogeography:

- To clarify species concepts in Prasiolales using a combination of molecular, morphological and ecological data (Section B);
- To examine the phylogenetic relationships between Prasiolales of the northern hemisphere and of the southern hemisphere (Chapters B2 and B3);
- To test the use of different molecular markers for DNA barcoding in the Prasiolales (Chapter B4).

### 2) Algal flora associated with sponges of the Irish shores:

- To examine composition and seasonal dynamics of the algal assemblage colonizing the sponge *Haliclona indistincta* at Corranroo, Co. Galway (Chapter C2);
- To clarify the taxonomic identity of selected algal epibionts by isolation in culture and to characterise the existent molecular diversity using Next Generation Sequencing methods (Chapter C3).



## Part B: Prasiolales

### B1) Introduction

Species of Prasiolales have long been known by phycologists. Descriptions of algae referable to this group are reported in several early treatments (Greville 1826, Jessen 1848, Kützing 1845, 1849, Lightfoot 1777). The first species of this order to be described was *Prasiola crispa*, by Lightfoot in 1777 as *Ulva crispa*. Meneghini (1838) erected the genus *Prasiola* to include a number of species previously placed in *Ulva* (among which *Prasiola crispa*), but did not formally state the binomial name of species to be included in this new genus. Kützing (1843) was the first to state the binomial name *Prasiola crispa* and also included *P. furfuracea* in this genus. Jessen, in 1848, increased the number of species belonging to the genus, together with the most complete and detailed descriptions of the genus and species. This group was formally described as a new order by West and Fritsch (1927).

This order comprises green algae with thalli consisting of uniseriate filaments, pluriseriate pseudoparenchymatous axes, packet-like colonies or monostromatic blades (polystromatic when reproductive), which may be basally attached by rhizoids. Cells are small, uninucleate, and in foliose taxa usually arranged in regular rows and often circumscribed in square to rectangular groups separated by thicker walls, with a single stellate chloroplast containing a single pyrenoid (Rindi 2007, Womersley 1984). Ultrastructural details are only known for some species, which I list here: In *Prasiola stipitata*, the cell wall consists of inner and outer cellulose layers separated by mucopolysaccharide matrix (Friedman 1959, Takeda et al. 1967). In *Prasiola meridionalis*, *P. stipitata* and *Rosenvingiella constricta*, the flagellar apparatus has a 180° rotational symmetry and counter-clockwise absolute orientation of the major components. The sperm possesses a 9+1 microtubule configuration in the flagellar axoneme, which is an uncommon feature (O'Kelly et al. 1989).

This order includes only one family, Prasiolaceae, and it is one of the few algal groups with marine, freshwater and terrestrial members. The four genera

## Part B: Prasiolales

currently accepted are *Prasiola* (C. Agardh) Meneghini, *Rosenvingiella* P.C. Silva, *Prasiococcus* Vischer and *Prasiolopsis* Vischer. *Prasiola* is the most speciose genus, with 35 valid species registered in AlgaeBase (Guiry and Guiry 2012). *Rosenvingiella* has five (Guiry and Guiry 2012), *Prasiococcus* and *Prasiolopsis* only one (Guiry and Guiry 2012). Contrary to what was reported in some previous studies, it is now known that *Trichophilus* does not belong to the Prasiolales (Suutari et al. 2010). Previous erroneous reports of *Trichophilus* in the Prasiolales (Friedl et al. 1997) were due to a culture in the culture collection of algae at Goettingen University (SAG) labelled as *Trichophilus*, which was either swapped or contaminated with *Prasiolopsis* (Suutari et al. 2010).

Monophyly of Prasiolales was first demonstrated molecularly by Sherwood et al. (2000), with *rbcL* gene sequences for two genera and five species, and 18S rRNA gene sequences for four species. Naw and Hara (2002) and Friedl and O'Kelly (2002) confirmed its placement in the class Trebouxiophyceae, which was later confirmed by other authors (Friedl and O'Kelly 2002, Karsten et al. 2005). The class Trebouxiophyceae is one of the derived groups of the chlorophytan lineage of the Viridiplantae, together with the early diverging Chlorodendrophyceae and two other major classes, the Ulvophyceae and the Chlorophyceae (Leliaert et al. 2012). The relationships between these classes are still very much debated and require multi-gene, multi-family analyses (Leliaert et al. 2012). Trebouxiophyceae consists of an ecologically and morphologically very diverse assemblage of algae as Chlorellales, Trebouxiales, Microthamniales, Prasiolales, and several other clades that have yet to receive a formal name (references in Leliaert et al. 2012).

Prasiolales is distributed in cold-temperate and polar regions. The only species found in countries with warmer climates are freshwater species of *Prasiola* found at high altitudes in cold streams (Hu and Wei 2006, Rodríguez and Jiménez 2005) Therefore, many physiological studies using species from this family have focused on adaptations to freezing and UV exposure. For example, *P. crista* and several species related to it were found to contain high concentrations of a unique UV-absorbing compound with an absorption maximum at 324 nm that

## Part B: Prasiolales

was characterized as a putative mycosporine-like aminoacid which is the most common cryopreservant in marine organisms (Groeniger and Haeder 2002, Hoyer et al. 2001, Karsten et al. 2005). It has also been demonstrated that species from Antarctica have an increase of proline during the summer months which protects the cells' photosynthetic apparatus from UV-damage (Jackson and Seppelt 1995).

Some species of *Prasiola* are also used for practical purposes. For example, the freshwater species *Prasiola japonica*, known as *kawanori* in Japan, and dried *P. yunnanica* in China (Johnston 1970) are consumed as food. In Nepal, a species of this genus is incorporated as a protein source in other foods like *Khulu-Simali* bread (Malla and Shakya 2004) and in Mexico *P. mexicana*, known as Nitla, is used as a cough suppressant and to halt nosebleeds medication (Godínez et al. 2001).

In Ireland, Prasiolales is represented by six species, *Prasiola crispa*, *P. calophylla*, *P. stipitata*, *P. furfuracea*, *Rosenvingiella radicans* and *R. polyrhiza* (Rindi et al. 1999, Rindi 2007). *Prasiola crispa*, *P. calophylla*, *P. furfuracea* and *R. radicans* occur in supralittoral and terrestrial habitats, including damp spots in urban environments where it is common for dogs to urinate (Rindi et al. 1999, 2004). *Rosenvingiella radicans* is tolerant to a wide range of salinity, growing in both freshwater and marine media (Rindi et al. 2004). *Rosenvingiella polyrhiza* and *P. stipitata* are marine species and occur in the upper intertidal zone.

Molecular studies carried out in recent years have considerably advanced our knowledge on the taxonomy and systematics of the Prasiolales, clarifying some important problems. In particular, molecular data have confirmed the separation of the genera *Prasiola* and *Rosenvingiella* (Rindi et al. 2004), which has been uncertain for a long time (Bravo 1965, Edwards 1975, Waern 1952). Molecular investigations, however, have highlighted difficulties in species delineation, especially for the marine *Prasiola* species (Rindi et al. 1999, Rindi et al. 2004, Rindi et al. 2007). This is also an interesting group in terms of evolution since its species have a variety of life histories and it has organisms conquering very different habitats.

The main goals of this project were to understand better the evolutionary history

## Part B: Prasiolales

of this group including taxa from the southern hemisphere and to reassess the delineation of species within this group worldwide (with special focus on marine *Prasiola*).

**B2) Phylogeny and taxonomy of *Prasiolales* (Trebouxiophyceae, Chlorophyta) from Tasmania, including *Rosenvingiella tasmanica* sp. nov.**

***Abstract***

The order Prasiolales includes trebouxiophycean green algae widely distributed in polar and cold temperate regions. Molecular data produced in recent years have shed considerable light on the phylogeny and genetic diversity of this group. Most of the information available for the order, however, has been obtained for the northern hemisphere; information for the southern hemisphere is comparatively scant. Collections of Prasiolales were obtained from coastal sites in southern and eastern Tasmania and studied by microscopic examination, culture experiments and molecular analyses based on *rbcL* sequences. The results led to the discovery of a new species, *Rosenvingiella tasmanica*, which represents a previously unknown lineage within the genus *Rosenvingiella*. Culture observations and molecular data showed that collections from Tasmania previously identified as *R. polyrhiza* must be referred to *R. constricta*. This is the first record of this species for the southern hemisphere and outside of Eurasia and North America. In the same way, the molecular data revealed that the alga formerly known in Tasmania as *Prasiola crispa* is in fact referable to *P. borealis*. This organism was found both as a free-living alga and in a lichenized form similar to the original collections from North America. The *rbcL* gene sequence comparisons indicate a high genetic similarity between the *Prasiolales* of Tasmania and those of Pacific North America.

***Key words***

Australia, Biogeography, Phylogeny, *Prasiola*, *Prasiolales*, *rbcL*, *Rosenvingiella*, Tasmania, Taxonomy, Trebouxiophyceae

***Introduction***

The green algal class Trebouxiophyceae includes a large assemblage of unicellular and multicellular microchlorophytes distributed in virtually every type of terrestrial and aquatic habitat and often involved in lichen symbioses (Lewis and McCourt 2004). The

## Part B: Prasiolales

order Prasiolales represents one of the most ecologically differentiated lineages among Trebouxiophyceae because it includes taxa distributed in marine, freshwater and terrestrial habitats in polar and cold-temperate regions (Ettl and Gärtner 1995, John 2002, Rindi 2007). This order includes algae consisting of uniseriate filaments, ribbonlike thalli, expanded monostromatic blades, packet-like colonies and pseudoparenchymatous axes (Ettl and Gärtner 1995, Knebel 1935), characterized by an unusual combination of morphological and ultrastructural characters that made their phylogenetic position uncertain for a long time (Hoek et al. 1995, O'Kelly et al. 1989, Sherwood et al. 2000). As currently circumscribed, Prasiolales includes one family (the Prasiolaceae) and about 35 species belonging to the genera *Prasiococcus* Vischer, *Prasiola* Meneghini, *Prasiolopsis* Vischer and *Rosenvingiella* P.C. Silva (Guiry and Guiry 2010). The genus *Trichophilus* Webervan Bosse, which was formerly believed to belong to this group, was recently shown to represent a separate lineage in the class Ulvophyceae. Suutari et al. (2010) showed that the culture SAG84.81, from which the sequences EF203012 and AY762601 were obtained, does not represent the genuine *T. welckeri* and is a prasiolalean alga probably referable to *Prasiolopsis*.

Molecular studies published in the last 10 years have robustly established the placement of Prasiolales within Trebouxiophyceae (Dariencko et al. 2010, Karsten et al. 2005) and have greatly advanced our understanding of the phylogeny and diversity of this group (Perez-Ortega et al. 2010, Rindi et al. 2004, 2007, Sherwood et al. 2000). Molecular data have partially confirmed conclusions based on morphology but have also led to some unexpected results and have revealed a high level of cryptic diversity in some taxa (Perez-Ortega et al. 2010, Rindi et al. 2004, 2007). Most of the taxonomic and distributional information (as well as molecular data) currently available for Prasiolales has been produced in the northern hemisphere, mainly because scientists interested in the systematics of this group are based primarily in northern geographical regions. In general, the information available for these algae in the southern hemisphere is much more limited, with the remarkable exception of Antarctic *Prasiola crispa* (Lightfoot) Kützing (which is one of the best-studied terrestrial algae from ecological and physiological perspectives; e.g., Hoyer et al. 2001, Kosugi et al. 2010, Kovacik and Batista Pereira 2001, Lud et al. 2001). Records of Prasiolales, particularly marine species, are available from several regions of the southern hemisphere (Adams 1994, Boraso de Zaixso 2002, Ramírez and Santelices 1991, Womersley 1956, 1984). With

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few exceptions, however, the details reported on the biology of these organisms are limited. To date, relatively few sequences of samples from the southern hemisphere are available in GenBank (see Materials and Methods).

In 2009 and 2010 specimens of Prasiolales from several coastal sites in Tasmania were collected by one of us (MDG) and received from Dr Fiona Scott. Morphologically, the algae corresponded to the descriptions of *P. crista* and *Rosenvingiella polyrhiza* (Rosenvinge) P.C. Silva given by Womersley (1984) in his monograph of the green seaweeds of southern Australia. However, culture studies and molecular investigations based on sequences of the *rbcL* gene led to unexpected results, including the discovery of an undescribed species. These results are presented here.

### ***Material and Methods***

#### *Field collections and morphological examination*

Collections of Prasiolales were made from the supralittoral and uppermost littoral zone at several sites on the eastern and southern shore of Tasmania (details in Table 1). The material was placed in sealed Ziploc bags and mailed to the laboratory in Ireland, where it was inspected and examined by light microscopy. Microphotographs were taken with a Nikon DXM1200 digital camera (Nikon Corporation, Tokyo, Japan). Voucher specimens were deposited in the Phycological Herbarium of the National University of Ireland, Galway (GALW).

#### *Culture studies*

Unialgal cultures of the prasiolalean algae identified were established either from vegetative fragments or from released reproductive bodies (gametes or aplanospores) using Von Stosch enriched seawater medium (VS5) modified following Guiry and Cunningham (1984) or Jaworski's medium (JM, both liquid and agarized; Tompkins et al. 1995). Growth and reproduction of the strains isolated were examined at 10°C and 15°C, 20–40  $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ , 14:10 h light:dark. The experiments were carried out in Sanyo MLR-351 culture chambers (Sanyo Electric Co., Osaka, Japan) using plastic dishes containing approximately 30 ml of medium (Bibby Sterilin, Stone, U.K.). The culture medium was replaced every 10–15 days.

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### *Molecular studies*

Genomic DNA was extracted from field and cultured material using the Qiagen DNeasy Plant Mini Kit (Qiagen, Crawley, U.K.). A Techne TC-3000 thermal cycler (Techne, Stone, U.K.) was used for polymerase chain reaction (PCR) amplification of the *rbcl* gene. PCR was performed as two overlapping fragments using the primers RH1 (Zurawski and Clegg 1987) and RT1134 (Rindi et al. 2008). Each 50  $\mu$ l reaction consisted of 34.7  $\mu$ l of HyPure Cell Culture Grade Water (Thermo Scientific, South Logan, Utah), 5  $\mu$ l of 103 OptiBuffer, 2  $\mu$ l of dNTP Mix, 1  $\mu$ l of 50 mM MgCl<sub>2</sub> solution, 0.3  $\mu$ l of BIO-X-ACT Short DNA polymerase (Bioline, London, U.K.), 1  $\mu$ l of each primer mix (100 mM) and 5  $\mu$ l of DNA sample. The PCR protocol used for the amplification was the following: an initial denaturation step at 94°C for 2 min followed by 40 cycles with denaturation at 94°C for 1 min, annealing at 55°C for 1 min and elongation at 72°C for 2 min, with a final elongation step at 72°C for 3 min. PCR products were examined under UV light on 1.2% agarose gels stained with SYBR Safe DNA stain (Invitrogen, Carlsbad, California). The amount of DNA in PCR products was quantified visually on agarose gels using HyperLadder II (Bioline) as reference and the products of successful reactions were purified using the Qiagen MinElute Gel Extraction Kit (Qiagen). PCR products of appropriate length, yield and purity were custom-sequenced by GATC Biotech AG (Konstanz, Germany).

The *rbcl* dataset consisted of 41 taxa for which 918 bps (corresponding to positions 193–1110 of the complete sequence of *Chlorella vulgaris* AB001684; Wakasugi et al. 1997) were aligned using ClustalW (Thompson et al. 1994). Besides the samples from Tasmania, most of the sequences of prasiolalean taxa available in GenBank (*Prasiococcus*, *Prasiola*, *Prasiolopsis*, *Rosenvingiella*, “*Trichophilus welckeri*” EF203012) were included in the alignment. All sequences obtained from samples collected in the southern hemisphere (*Prasiola calophylla* EF589145, EU380573, EU380546; *P. crispera* EF589146, GQ423923, GQ423928; *Prasiola sp.* AF387111; *Prasiola sp.* FN668957 and FN668958) were used in the phylogenetic analyses. Phylogenetic trees were rooted using as outgroup taxa *Chlorella vulgaris* AF499684, *Stichococcus bacillaris* AM260442 and *Trebouxia anticipata* AF189069. These are trebouxiophycean taxa closely related to Prasiolales, which were shown to be suitable outgroups in previous studies (Rindi et al. 2004, 2007). The aligned dataset was analyzed using Maximum Likelihood (ML) in Treefinder (version October 2008, Jobb

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2008) and PhyML (Guindon and Gascuel 2003). Bayesian inference (BI) analysis was performed using MrBayes 3.04 (Huelsenbeck and Ronquist 2001). The Treefinder and BI analyses were performed on a partitioned dataset, applying separate models to each partition (first, second and third codon position of *rbcL*). The evolutionary model for the PhyML and BI analyses was selected using jModelTest (Posada 2008) under the Akaike Information Criterion. For the Treefinder analysis, the models selected by Treefinder under the corrected Akaike Information Criterion (AICc) were applied. For PhyML and Treefinder, nodal support was assessed by nonparametric bootstrap analysis with 1000 resamplings. The BI analysis was performed using the priors set as default in MrBayes; the parameters were unlinked and allowed to vary across partitions. Four Monte Carlo Markov chains were run for  $4 \times 10^6$  generations, with tree sampling every 100 generations. Convergence of the two runs and burn-in phase were assessed using the sump command and plotting the likelihood scores versus the number of generations. The 50% majority-rule tree was built using the last 10,000 trees sampled.

**Table 1.** Details of collections.

Taxon	Date	Site	Habitat	Herbarium	<i>rbcL</i> GenBank accession number
<i>Prasiola borealis</i>	31 Oct. 2009	Fossil Island, Pirates Bay	Upper shore; on rocks in lichen zone	GALW015588	JF949724
<i>P. borealis</i>	2 Nov. 2009	Fortescue Bay	On large flat rocks in the splash zone used by seabirds for dropping molluscs to break shells	GALW015589	Sequence identical to previous sample
<i>P. borealis</i>	18 Jul. 2010	Lady Bay (43°40701 S, 147°01857 E)	Upper shore, in the vicinity of a well-splattered bird nest	GALW015590	JF949723
<i>Rosenvingiella constricta</i>	31 Oct. 2009	Fossil Island, Pirates Bay	Forming large green patches on rock around and above the high-water mark	GALW015591	JF949725
<i>R. tasmanica</i>	2 Nov. 2009	Fortescue Bay	Forming bright green patches mixed with <i>P.</i> <i>borealis</i>	GALW015592	JF949726

## **Results**

### *Prasiola borealis* Reed

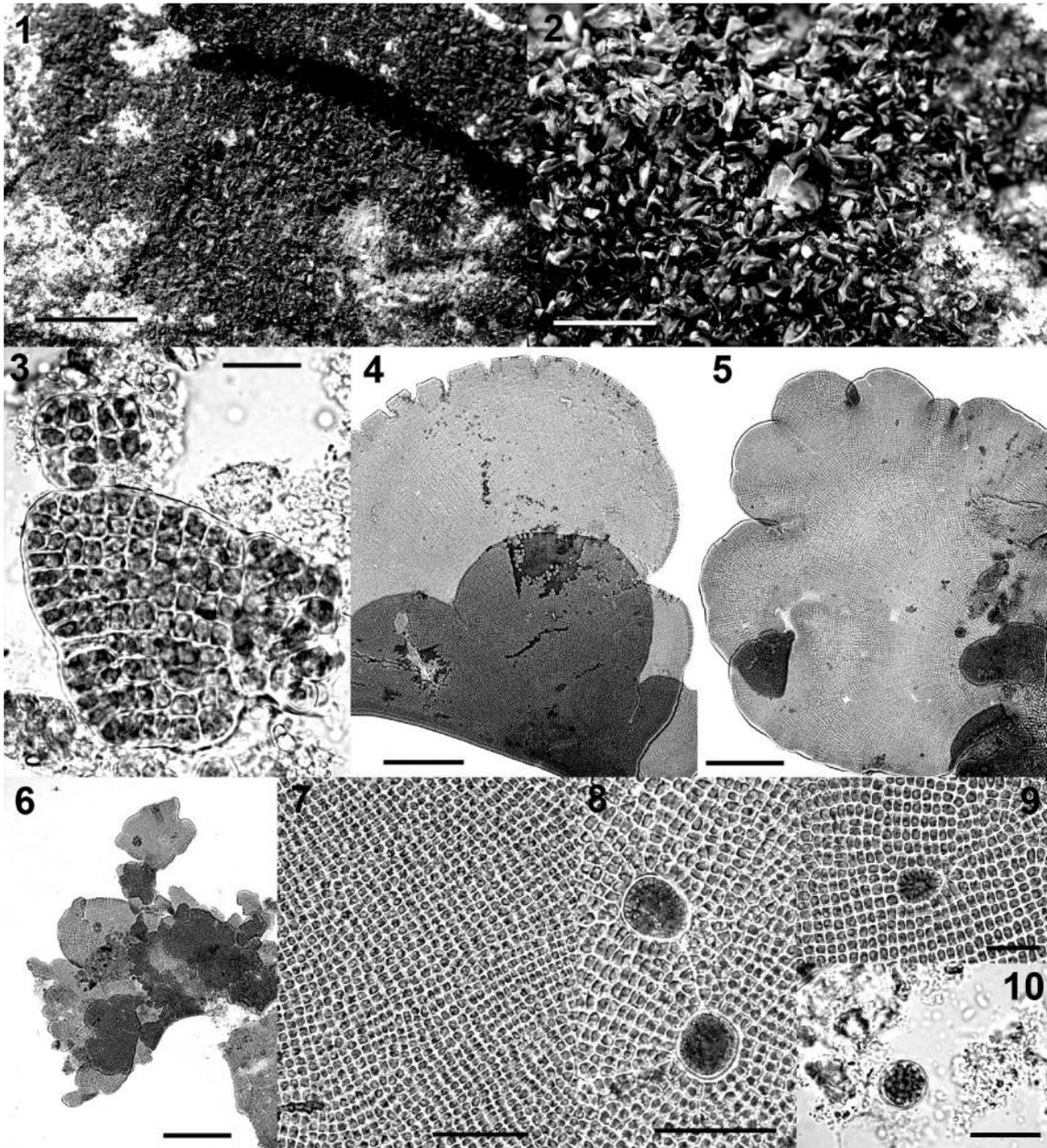
This alga occurred on the shores of Tasmania in two different forms: as a free-living independent organism (collections from Pirates Bay and Fortescue Bay) and in a lichenized form with the thallus enveloped by a coating of fungal hyphae (collection from Lady Bay). It formed dense populations in the supralittoral and uppermost littoral zone, particularly on bird-roosting rocks at wave-exposed sites (Figs 1, 2) or on large flat rocks (Fortescue Bay) in the splash zone used by seabirds for dropping molluscs to break shells. At the Pirates Bay site, the swards occurred in the lichen zone (mainly orange and yellow lichens). The free-living thalli consisted of small bushy or branched algae up to 5–6 mm wide, bright to dark green, reminiscent of small lettuce plants. The thalli were formed by numerous blades arising from a common holdfast, without a distinct stipe or with a very short stipe. The holdfast consisted of a small basal callus formed by adhesive extracellular substances; no rhizoidal outgrowths were observed. Young blades were circular, orbicular or polygonal, with entire margins (Fig. 3). Following subsequent growth, the margins became lobed and the blades assumed a shape ranging from rounded or orbicular to deeply incised and irregularly branched (Figs 4–6). Old blades became irregular in shape, very curled, crisped and folded. Short incisions perpendicular to the margin of the blades were observed in many specimens (Fig. 4). The blades were 20–36  $\mu\text{m}$  thick and in surface view showed a uniform appearance, without evident thickened lines or areolae (Fig. 7). The cells were arranged in regular vertical and horizontal rows (except for parts near the holdfast and occasionally near the margins, where the arrangement was more irregular), 4–10  $\mu\text{m}$  long and wide. Reproduction appeared to take place by production of akinetes. In the collection from Fortescue Bay blades observed in surface view bore some circular or oval cells 20–40  $\mu\text{m}$  in diameter, much larger than the normal vegetative cells (Figs 8, 9). In some of these the cytoplasm appeared to be in division phase (Fig. 9). It is likely that these cells become akinetes and, on maturation, aplanosporangia. Release of aplanospores was not observed; however, many single cells (5–6  $\mu\text{m}$  in diameter) that were probably released aplanospores close to germination (Fig. 10) were observed in the collections from Pirates Bay and Fortescue Bay.

The lichenized form, which was collected at Lady Bay, consisted of small aggregates with habit varying from bushy to very irregular, up to 1 cm wide. The habit

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of the specimens varied considerably in relation to the developmental stage of the thallus and the extent of the fungal infestation. Some thalli almost devoid of fungi were found; however, most specimens were more or less heavily infected. The development of the fungus caused major modifications in the morphological structure of the alga. Young specimens, in which the fungus was absent or little developed, were similar to free-living plants. The development of the fungus started in the basal parts of the alga; fungal spores could be observed in living and dead algal cells in the basal parts of the blades (Fig. 11). Blades colonized by the fungus gradually developed areolation, which was not seen in free-living thalli. The fungus subsequently developed to such an extent that in old specimens the algal cells were eventually surrounded by a continuous layer of fungal hyphae. Blades of lichenized specimens were considerably thicker than in the free-living form (40–80  $\mu\text{m}$ ). When the infection became substantial, the structure of the blade was altered and in section it appeared distromatic or polystromatic (Figs 12, 13). In surface view the cells were clustered in groups of four, reminiscent of the terrestrial genera *Desmococcus* and *Apatococcus* (Fig. 14); their size was also generally larger than in uninfected blades (5–18  $\mu\text{m}$  in length and width). The algal blades did not produce any stipes. However, old thalli appeared stipitate because the fungal hyphae grew producing a compact stipelike structure, in which some algal cells were interspersed (Fig. 15). No akinetes or other specialized reproductive structures were observed in the lichenized form.

In culture, *P. borealis* was grown successfully from vegetative fragments. It grew both in VS5 and JM, although overall it showed a preference for JM (particularly on agarized medium). Cultured specimens formed crisped blades densely packed, with irregular habit, generally larger than field-collected material; apart from this, their morphology was similar to that of uninfected field specimens. Vegetative growth was the only form of propagation observed in culture. Akinetes or other specialized reproductive structures were not produced.



**Figs 1–10.** *Prasiola borealis* (GALW015588 and GALW015589).

**Fig. 1.** Habitat of a natural population on supralittoral rock (Fortescue Bay). Scale bar = 5cm.

**Fig. 2.** Close-up view of a natural population (Fossil Island, Pirates Bay). Scale bar = 5cm.

**Fig. 3.** Detail of a juvenile blade. Scale bar = 30  $\mu$ m.

**Fig. 4.** Habit of a blade with marginal incisions. Scale bar = 200  $\mu$ m.

**Fig. 5.** Habit of blade with lobed margin. Scale bar = 200  $\mu$ m.

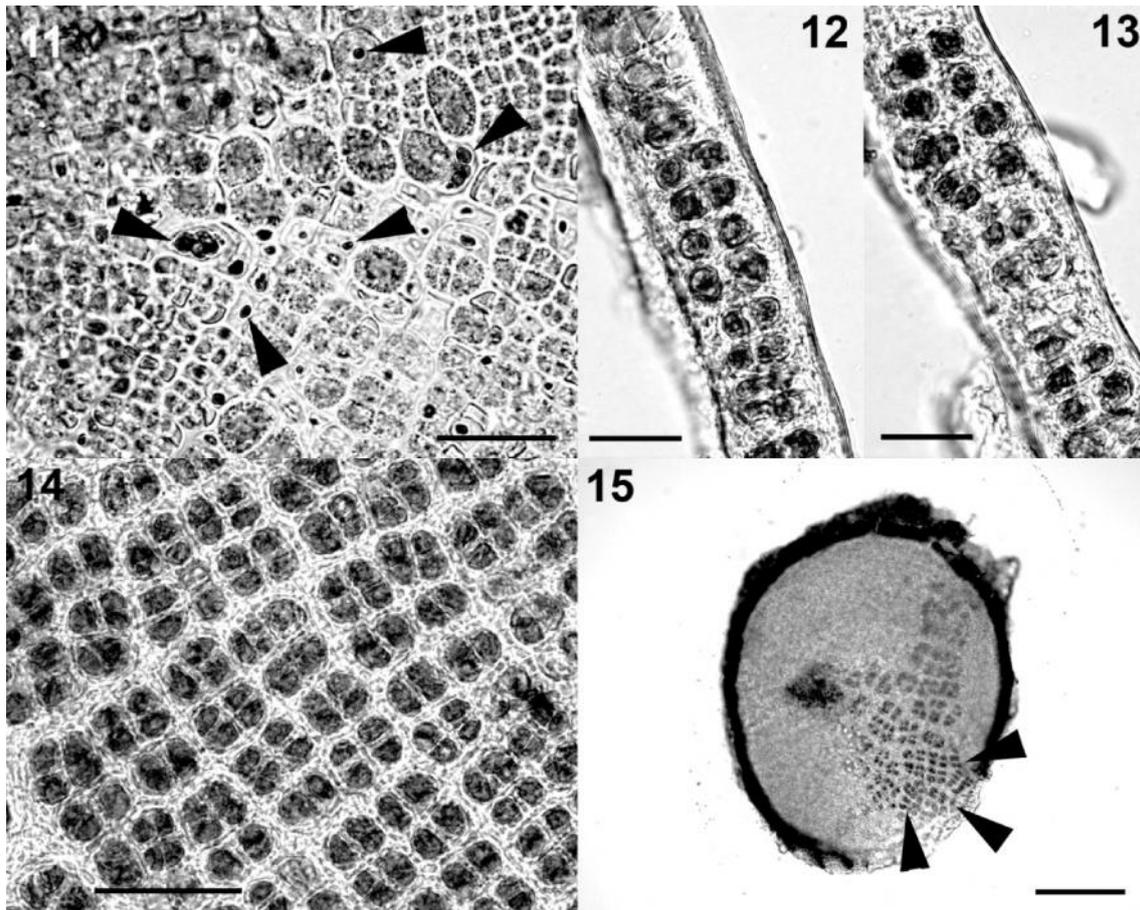
**Fig. 6.** Habit of a dissected blade. Scale bar = 200  $\mu$ m.

**Fig. 7.** Surface view of blade. Scale bar = 50  $\mu$ m.

**Fig. 8.** View of blade surface showing two presumptive akinetes. Scale bar = 50  $\mu$ m.

**Fig. 9.** Detail of a presumptive akinete in which the cytoplasmic contents are beginning to divide. Scale bar = 30  $\mu$ m.

**Fig. 10.** Detail of a presumptive aplanospore. Scale bar = 10  $\mu$ m.



**Figs 11–15.** *Prasiola borealis*. Lichenized form (GALW015590).

**Fig. 11.** Detail of the basal parts of a thallus at an initial stage of fungal infection. Fungal spores can be seen inside living and dead algal cells (spores are the black dots indicated by arrowheads). Scale bar = 20  $\mu\text{m}$ .

**Fig. 12.** Cross section of a lichenized thallus showing two layers of algal cells. Scale bar = 30  $\mu\text{m}$ .

**Fig. 13.** Cross section of a lichenized thallus showing thickened blade with two layers of algal cells. Scale bar = 30  $\mu\text{m}$ .

**Fig. 14.** Surface view of lichenized thallus, showing cells clustered in groups of 4. Scale bar = 50  $\mu\text{m}$ .

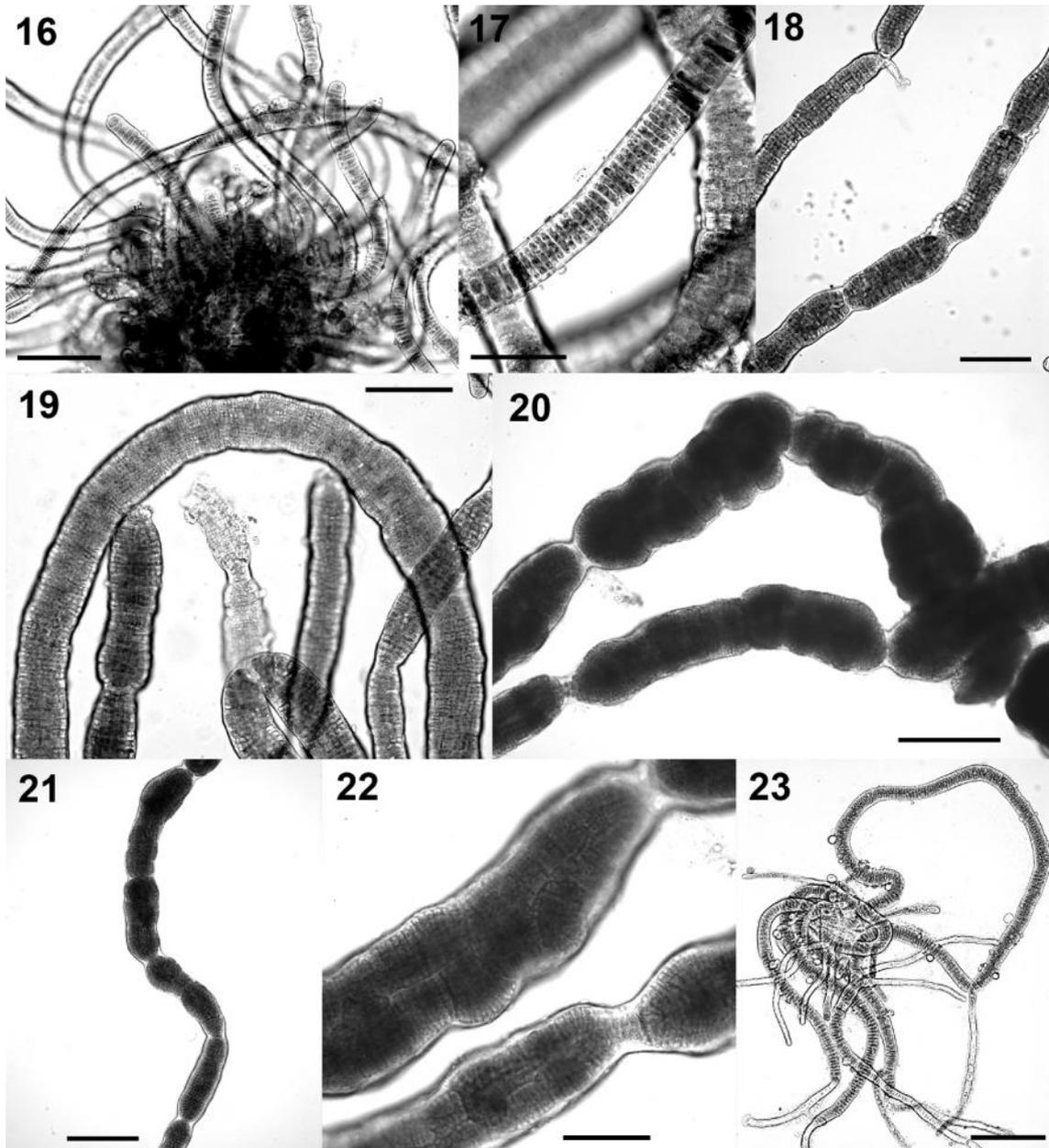
**Fig. 15.** Section of the basal, stipe-like portion of a lichenized thallus. Groups of algal cells (arrowheads) are interspersed among the fungal hyphae. Scale bar = 200  $\mu\text{m}$ .

*Rosenvingiella constricta* (Setchell and N.L. Gardner) P.C. Silva

This species was collected on Fossil Island, Pirates Bay, where it formed large green patches on rock around and above the high-water mark, and like *P. borealis*, was more associated with the yellow lichen zone. This alga consisted of densely entangled tufts forming thick filamentous masses, often twisted and ropelike. Juvenile thalli consisted of unbranched uniseriate filaments up to 300  $\mu\text{m}$  long (Fig. 16). In adult specimens, the basal parts remained uniseriate; they were formed by cells 18–22  $\mu\text{m}$  wide, with a thick

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cell wall, 0.3–0.6 as long as wide (Fig. 17). The uniseriate filaments produced unicellular colourless rhizoids that developed as prolongations of vegetative cells; these were formed either singly or in series in adjacent cells and acted as structures of attachment to the substratum. The erect parts of the thallus consisted of cylindrical pseudoparenchymatous axes 30–80  $\mu\text{m}$  wide, slightly constricted at intervals (Fig. 18). In surface view the cells in the erect parts were square or rectangular, 10–15  $\mu\text{m}$  in length and width; however, in some parts of the thallus (which were presumably close to reproductive maturation) they were smaller (5–8  $\mu\text{m}$ ) (Fig. 19). This alga grew well in culture in VS5 at 10°C and 15°C. Vegetative fragments of uniseriate filaments placed in culture produced unicellular rhizoids that anchored them to the bottom of the dishes. The filaments divided anticlinally and became pluriseriate, eventually developing into the pseudoparenchymatous axes typical of the field specimens. Cultured specimens, however, became larger (up to 180  $\mu\text{m}$  broad) and showed more marked constrictions than field-collected material, with the typical morphology of *R. constricta* (Figs 20, 21). The rhizoids were more abundant and considerably longer than in field specimens (up to 300  $\mu\text{m}$ ); they were occasionally branched. After 8–10 weeks in culture the thalli became reproductive. The pseudoparenchymatous axes disintegrated, releasing numerous spherical reproductive cells (12–18  $\mu\text{m}$  in diameter) that adhered to the bottom of the dishes and germinated producing new thalli. Details of reproduction could not be observed; based on studies from other populations of *Rosenvingiella*, however, we believe that the reproductive cells were zygotes. It is likely that the pluriseriate axes acted as gametangia forming both male and female gametes and that, on release, flagellate male gametes fertilized the egg cells; the zygotes then germinated into new thalli.



**Figs 16–23.** *Rosenvingiella constricta* (GALW015591).

**Fig. 16.** Habit of juvenile specimens in the field. Scale bar = 100 µm.

**Fig. 17.** Detail of basal, uniseriate filaments. Scale bar = 50 µm.

**Fig. 18.** Habit of pluriseriate axes. Scale bar = 100 µm.

**Fig. 19.** Habit of pluriseriate axes that appear close to reproductive maturity. Scale bar = 100 µm.

**Fig. 20.** Habit of thalli grown in culture. Scale bar = 200 µm.

**Fig. 21.** A culture-grown thallus with a constricted pluriseriate axis. Scale bar = 200 µm.

**Fig. 22.** Detail of pluriseriate axes of culture-grown thalli. Scale bar = 100 µm.

**Fig. 23.** A juvenile thallus grown in culture. Note the numerous unicellular rhizoids (colourless prologations issued from the filaments). Scale bar = 100 µm.

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Juvenile thalli were uniseriate and adhered to the substratum by a long basal rhizoid (Fig. 22). The filaments grew producing occasionally false branches and issued numerous unicellular rhizoids (Fig. 23). With subsequent growth the thalli assumed the same morphology as the parent plants and eventually reproduced, presumably with the same mechanism. Interestingly, individual specimens separated from other thalli were able to reproduce and release viable zygotes. This suggests that this alga was capable of either self-fertilization or parthenogenesis.

*Rosenvingiella tasmanica* Moniz, Rindi et Guiry sp. nov.

Filamentous algae bearing abundant rhizoids. Filaments uniseriate, rarely pluriseriate, unbranched, 20–26 µm broad; cells 1/3–1/6 as long as wide, with thick cell wall.

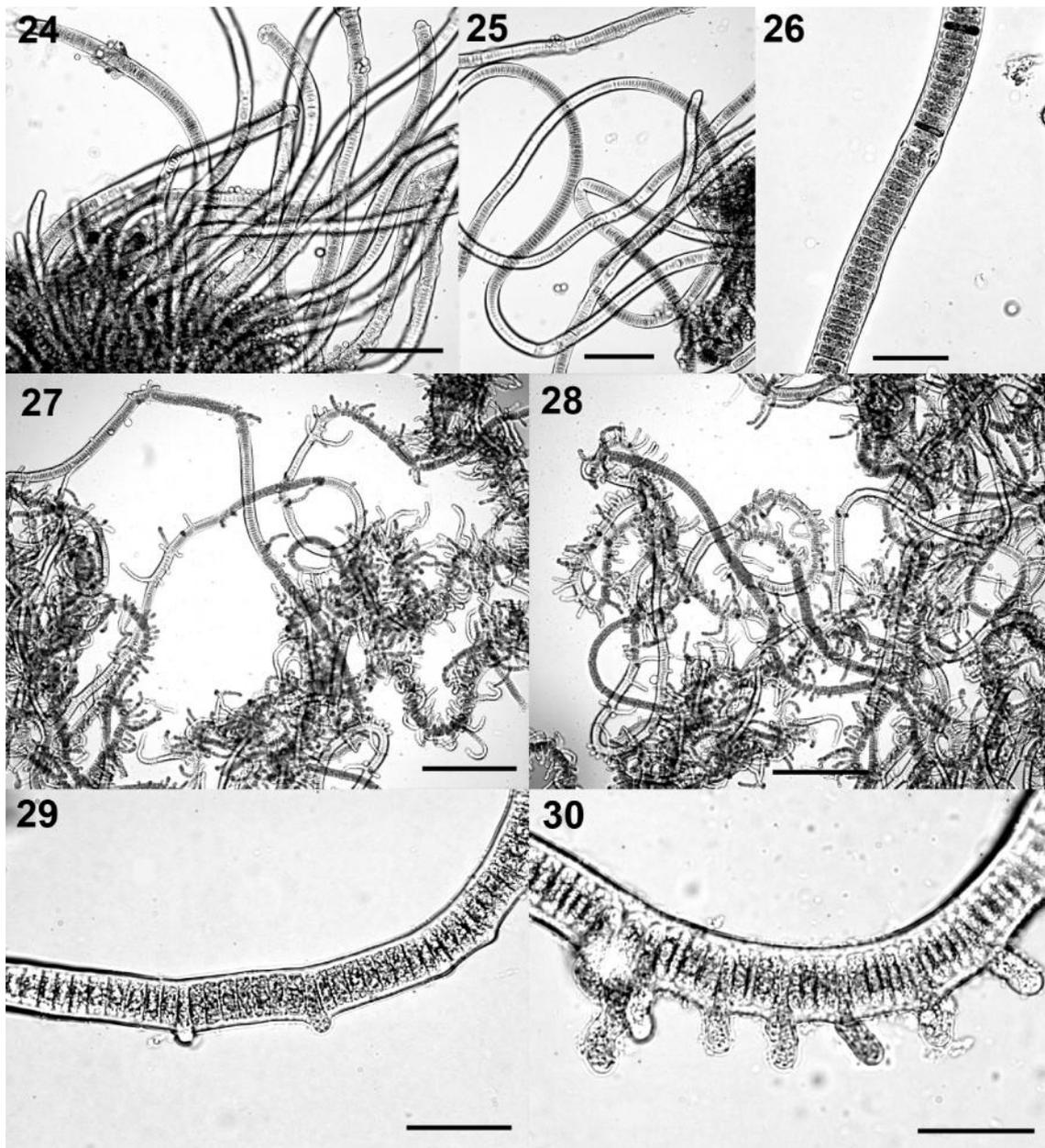
Rhizoids unicellular, up to 150 µm long. Sexual reproduction unknown. It differs from the other species of *Rosenvingiella* by the thickness of the filaments and the *rbcL* sequence. It occurs on the shores of southern Tasmania.

HOLOTYPE SPECIMEN: GALW015592. Collected by Michael Guiry on 2 November 2009. Isotype specimen in BM.

ETYMOLOGY: The specific epithet refers to the geographical region in which the alga was found.

TYPE LOCALITY: Fortescue Bay, Tasmania, Australia (43°89' 24.440 S; 147° 58' 5.250 E). Supralittoral zone; mixed with *P. borealis* on a flat rock used by seabirds for dropping marine molluscs to break their shells, with abundant bird faeces, semi-exposed to wave action.

*Rosenvingiella tasmanica* was collected at Fortescue Bay, where it formed some bright green patches mixed with *P. borealis* in the supralittoral zone. In the field collection it consisted of robust uniseriate filaments of variable length, 20–26 µm wide (Figs 24–26) attached to the substratum by unicellular rhizoids that remained in open connection with the cell from which they were issued. No sexual or asexual reproductive structures were observed. This alga was isolated into culture without difficulty, where it grew well in VS5 at 10°C and 15°C. Cultured material produced thick mats and showed a similar habit to that of field collections (Figs. 27, 28).



**Figs 24–30.** *Rosenvingiella tasmanica* (holotype GALW015592).

**Fig. 24.** Basal parts of field-collected specimens. Scale bar = 100 µm.

**Fig. 25.** Habit of field-collected specimens. Scale bar = 100 µm.

**Fig. 26.** Detail of a filament. Scale bar = 40 µm.

**Fig. 27.** Habit of culture-grown material. Note the abundant production of rhizoids. Scale bar = 200 µm.

**Fig. 28.** Habit of culture-grown material. Note the abundant production of rhizoids. Scale bar = 200 µm.

**Fig. 29.** Detail of a culture-grown thallus with unicellular rhizoids. Scale bar = 40 µm.

**Fig. 30.** Detail of a culture-grown thallus with unicellular rhizoids produced in short series. Scale bar = 40 µm.

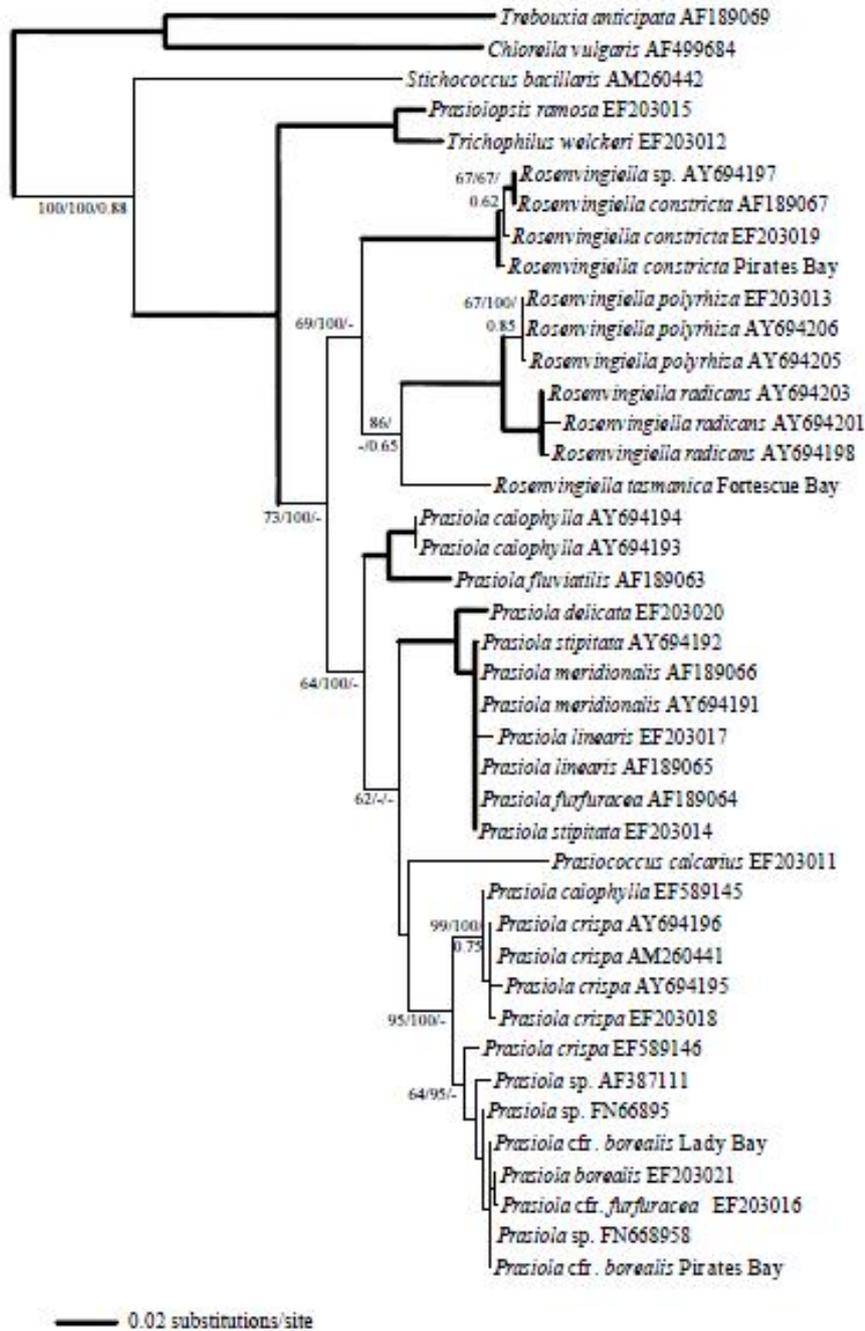
The main difference was the profuse production of unicellular rhizoids, which were issued in large numbers, either singly or in multiple series from adjacent or close cells

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(Figs. 29, 30). Rhizoids were also longer than in field specimens, reaching 120-150  $\mu\text{m}$  in length (Figs. 27, 28). Vegetative fragmentation was the only form of reproduction observed and once again no gametangia or sporangia were produced.

### *Molecular phylogeny*

The results of the molecular analyses produced a phylogeny in agreement with previous studies on Prasiolales in which the *rbcL* gene was used (Rindi et al. 2004, 2007) (Fig. 31). The samples of *Prasiola* from Tasmania had identical *rbcL* sequences, suggesting that the free-living form from Pirates Bay and Fortescue Bay was the algal symbiont of the lichenized form occurring in Lady Bay. Our *Prasiola* sequences were also identical to the only sequence of a sample from Tasmania currently available in GenBank [FN668958; a sample from Fishers Point reported by Perez-Ortega et al. (2010) as *Prasiola* MAF-Lich 16295, phycobiont of *Mastodia tessellata* (J.D. Hooker and Harvey) J.D. Hooker and Harvey]. These sequences differed by only 1 bp from a sample from Helby Island, British Columbia, identified as *P. borealis* by Rindi et al. (2007). The sample of *Rosenvingiella* from Pirates Bay was recovered with high statistical support as sister taxon to a clade formed mainly by specimens of *R. constricta* from Pacific North America, from which it differed by 3 to 6 bp. *Rosenvingiella tasmanica* formed an evolutionary lineage not found in previous phylogenetic studies on the Prasiolales. It was recovered as sister taxon to a well-supported clade formed by *R. polyrhiza* and *R. radicans* (Kützing) Rindi, McIvor and Guiry. This relationship, however, was highly supported only in the PhyML analysis.



**Fig. 31.** Phylogram inferred from ML analysis of the *rbcL* gene in the Prasiolales (obtained with PhyML; ln(L) 5 24280.8). From left to right (or from top to bottom, above and below branches) numbers marked at the nodes indicate ML bootstrap (PhyML), ML bootstrap (Treefinder) and Bayesian posterior probabilities. Bootstrap values lower than 60% and posterior probabilities lower than 0.6 are not shown. Thickened lines indicate branches highly supported under all inference methods (>80% BP support and >0.95 PP). BP 5 bootstrap support; PP 5 posterior probabilities.

### **Discussion**

The characterization of *R. tasmanica* is the most significant discovery of this study. The morphological habit of this alga fits well with the features of the genus *Rosenvingiella*, particularly in the production of unicellular rhizoids singly or in series (Kornmann and Sahling 1974, Rindi 2007, Setchell and Gardner 1920a, Silva 1957, 1974). Inclusion in this genus was confirmed by our molecular analyses, although the support was variable depending on the phylogenetic method and software used. Three species are currently included in *Rosenvingiella*: *R. constricta* (Setchell & N.L.Gardner) P.C.Silva, *R. polyrhiza* (Rosenvinge) P.C.Silva (generitype) and *R. radicans* (Kützing) Rindi, L.McIvor & Guiry (Guiry and Guiry 2010). The first two are easily distinguished from *R. tasmanica* because at maturity their thallus consists mostly of thick pseudoparenchymatous axes (Rindi 2007, Rosenvinge 1893, Setchell and Gardner 1920a); these are not produced in *R. tasmanica* either in field or cultured specimens. *Rosenvingiella radicans* differs from *R. tasmanica* in its narrower filaments (7–20 µm wide, mostly 8–14 µm compared with 20–26 µm wide; Rindi et al. 2004, Rindi 2007). Furthermore, rhizoids in *R. radicans* occur singly or in pairs but never in series, as is common in *R. tasmanica* (as well as *R. polyrhiza*), and do not reach the same length (in *R. radicans* they are no longer than about 50 µm).

Our findings of *Rosenvingiella constricta* and *P. borealis* represent new records for Australia. For *R. constricta* this is the first record in the southern hemisphere and from a continent other than North America and Eurasia. Rather than new discoveries, however, these records are the result of a taxonomic reassessment of algae whose presence in Tasmania was already known. They correspond to the algae identified as *R. polyrhiza* and *P. crispa* by Womersley (1984). From a morphological point of view, Womersley's identification of Tasmanian *Rosenvingiella* as *R. polyrhiza* is indisputable, as field-collected specimens show good correspondence with Rosenvinge's (1893) original material and other descriptions of this species (Rindi et al. 2004, Rindi 2007, Setchell and Gardner 1920a). However, the *rbcL* analyses revealed that the Tasmanian alga is closely related to *R. constricta* from Pacific North America, and our culture observations strongly suggest that the same entity is involved. *Rosenvingiella constricta* was originally separated from *R. polyrhiza* on the basis of the larger size of the pluriseriate axes, the presence of constrictions and much longer rhizoids (Gardner 1917, Setchell and Gardner 1920a). Once placed in culture, the material from Pirates Bay

assumed a morphology that in fact corresponded much more to *R. constricta* than to *R. polyrhiza*: the size of the pluriseriate axes became considerably larger, the constrictions were much more evident and the rhizoids were longer. Morphological plasticity related to environmental conditions is a feature typical of many green microalgae, particularly members of the class Trebouxiophyceae (Bock et al. 2010, Krienitz et al. 2010, Luo et al. 2006) and Prasiolales is no exception. Cases in which striking changes of morphology take place from the field to culture conditions have been already reported for some prasiolalean species, such as *P. calophylla* (Carmichael ex Greville) Kützing (Kornmann and Sahling 1974, Rindi et al. 2004) and *P. velutina* (Lyngbye) Trevisan (Lokhorst and Star 1988). Thus, these changes in *Rosenvingiella* from Tasmania, even though unexpected, are not particularly surprising. Based on our culture observations and the molecular evidence, we conclude that *R. constricta* represents the correct name for the Tasmanian *Rosenvingiella*. The alga that we are designating here as *P. borealis* corresponds perfectly in terms of morphology with the entity identified by Womersley (1984) as *P. crispa*. Our molecular analyses, however, indicate that this alga is genetically different from European samples of *P. crispa* and is more closely related to prasiolalean algae from other regions, in particular *P. borealis* from British Columbia (Canada). This is supported also by the results of Perez-Ortega et al. (2010), who obtained an identical *rbcL* sequence for a sample identified as *Mastodia tessellata* collected at a different locality in Tasmania (Fishers Point). In its typical foliaceous form, *P. crispa* [originally described as *Ulva crispa* by Lightfoot (1777) on the basis of collections from Scotland], is one of the most easily recognized species of terrestrial green algae. However, this species is also well known to encompass a large variety of morphological habits, ranging from uniseriate filaments (Hormidium form) to narrow ribbon-like blades (Schizogonium form) to expanded blades (Ettl and Gärtner 1935, Kobayasi 1967, Kovacik and Batista Pereira 2001, Printz 1964), which has given rise to great uncertainties in the delimitation of this species. Molecular data have contributed to solving some major problems, notably the confusion between *P. crispa* and *R. radicans* (Rindi et al. 2004). In spite of this, the complex of organisms related to *P. crispa* still represents a difficult group, for which a definitive taxonomic reassessment based on new molecular data is clearly needed. In previous investigations in which molecular data were used, specimens of *P. crispa* from Europe (which can be assumed to represent the authentic *P. crispa*) had almost identical *rbcL* sequences and formed a strongly supported clade (in the present study the sequence *P. crispa* EF589146 is separated

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from the other sequences of *P. crispera* and occurs basally to the *P. borealis* clade; we could not examine this sample, for which a taxonomic reassessment would be desirable). Our *Prasiola* from Tasmania does not belong to this clade and appears morphologically distinct from European *P. crispera* with respect to several characters. Whereas blades of European *P. crispera* are typically devoid of clearly defined attachment structures (Ettl and Gärtner 1995, Printz 1964, Rindi 2007), in the Tasmanian *Prasiola* a clear holdfast is present. The habit of the specimens is generally quite different; in the alga from Tasmania the blades are deeply entangled to produce aggregates resembling small lettuce plants up to 5–6 mm broad; whereas, well-developed thalli of *P. crispera* consist of irregular blades that may reach 5–6 cm or larger in width (Knebel 1935, Rindi 2007). In *P. crispera* the blades often show thickened lines and evident areolae; in free-living specimens of Tasmanian *Prasiola* this is not the case, although areolation gradually develops in lichenized specimens. Based on the molecular evidence and these morphological differences, we believe that *P. crispera* is not the correct name for the Tasmanian alga. *Prasiola borealis* was described by Reed (1902) to accommodate lichenized specimens of *Prasiola* collected at Unalaska and Kodiak Islands (Alaska). Our alga from Tasmania differs from Reed's (1902) description in some characters, namely thicker fronds and absence of fungal perithecia (Reed 1902, Setchell and Gardner 1920a); however, it also shares several similarities (habit, thallus size and cell size, and loss of organization with increased fungal contamination). Pending availability of more molecular data for *P. borealis*, we provisionally refer the Tasmanian alga to this taxon. So far, the only sequence deposited in GenBank under *P. borealis* was obtained from specimens collected by one of us (MDG) at Helby Island, Barkley Sound, Vancouver, British Columbia. The material sequenced (by Rindi et al. 2007) was morphologically in agreement with the description of *P. borealis* and differs by only 1 bp from the Tasmanian *Prasiola*. It would be highly desirable to sequence specimens of *P. borealis* from Alaska (particularly Reed's original collections or collections from the same locality) and clarify their relationships with *P. crispera* (which has not been fully possible here, due to the low support of some nodes). Our results and those of Perez-Ortega et al. (2010) suggest that a taxonomic re-evaluation of the species of *Prasiola* involved in symbioses with fungi is necessary. It is generally believed that the species involved in these interactions is *P. crispera* (Knebel 1935, Kobayasi 1967, Kovacic and Batista Pereira 2001, Kützing 1849, Ricker 1987). However, the molecular information produced in this and other studies (Perez-Ortega et al. 2010, Rindi et al.

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2007) indicates that *P. borealis* is the entity involved. In general, our results suggest that the taxonomy and biogeography of the Prasiolales still have major surprises in store and represent an exciting subject of investigation. Regions of the world that have not been explored in detail might host other undescribed taxa; we suggest that the southernmost parts of America and isolated islands located at great distance from major land bodies are good candidates for the discovery of new taxonomic entities. The great genetic similarity of the Tasmanian Prasiolales with taxa from Pacific North America is a fascinating aspect that indicates that the biogeography of these organisms is more complex than previously believed. In the course of the geological history of our planet, Australia and North America have always been located at great distance from each other, and the close genetic relatedness of organisms distributed in these regions is not an expected outcome. It would also be very interesting to examine the relationships between Prasiolales of southern Australia and the Prasiolales of northwestern Pacific regions (China, Japan, Korea, Taiwan, Pacific Russia) but at the moment this is prevented by the lack of molecular data for specimens from these regions. Further data and better information on the dispersal mechanisms in these algae will help clarify these problems.

### ***Acknowledgments***

I am very grateful to Fiona Scott for the collection of *Prasiola* from Lady Bay, pictures and useful information, and to Dr and Mrs G.T. Kraft for encouraging and facilitating MDG's Tasmanian fieldtrip.



### **B3) Molecular phylogeny of Antarctic *Prasiola* (Prasiolales, Trebouxiophyceae) reveals extensive cryptic diversity**

#### ***Abstract***

Trebouxiophytes of the genus *Prasiola* are well known in Antarctica, where they are among the most important primary producers. Although many aspects of their biology have been thoroughly investigated, the scarcity of molecular data has so far prevented an accurate assessment of their taxonomy and phylogenetic position. Using sequences of the chloroplast genes *rbcL* and *psaB*, we demonstrate the existence of three cryptic species that were previously confused under *Prasiola crispa* (Lightfoot) Kützing. Genuine *P. crispa* occurs in Antarctica; its presence was confirmed by comparison with the *rbcL* sequence of the type specimen (from the Isle of Skye, Scotland). *Prasiola antarctica* Kützing is resurrected as an independent species to designate algae with gross morphology identical to *P. crispa* but robustly placed in a separate lineage. The third species is represented by specimens identified as *P. calophylla* (Carmichael ex Greville) Kützing in previous studies, but clearly separated from European *P. calophylla* (type locality: Argyll, Scotland); this alga is described as *P. glacialis* sp. nov. The molecular data demonstrated the presence of *P. crispa* in maritime and continental Antarctica. *P. antarctica* was recorded from the Antarctic Peninsula and South Shetland Islands, and *P. glacialis* from the Southern Ocean islands and coast. Such unexpected cryptic diversity highlights the need for a taxonomic reassessment of many published Antarctic records of *P. crispa*. The results also provide new insights into the evolution of Prasiolales, suggesting that this group has a terrestrial origin and has colonized marine habitats once and freshwater habitats in multiple independent events.

#### ***Key words***

Antarctica, cryptic diversity, molecular phylogeny, *Prasiola*, Prasiolales, terrestrial algae

#### ***Abbreviations***

BI, Bayesian inference; BM, British Museum of Natural History; L, Rijksherbarium Leiden; ML, Maximum Likelihood; NJ, Neighbor Joining; *psaB*, P700 chlorophyll a-

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apoprotein A2 gene; *rbcL*, large subunit of ribulose-1,5-bisphosphate carboxylase oxygenase gene.

### ***Introduction***

Understanding diversity and biogeography of green algae has traditionally been a major challenge. Molecular data produced in the last 15 years have considerably reshaped our view of these organisms, revealing a far greater genetic diversity than their simple morphology suggests (Leliaert et al. 2012, Pröschold and Leliaert 2007). The circumscription of many freshwater and terrestrial taxa has been substantially modified based on molecular evidence (e.g. Darienko et al. 2010, Fucikova et al. 2011a, Mikhailyuk et al. 2008, Novis et al. 2010, Škaloud and Peksa 2010). Although considerable progress has been made, there is a general consensus that the diversity of green microalgae at all levels is still far from fully understood; in particular, new evolutionary lineages continue to be discovered and new species continue to be described. Little-explored geographical regions hold the best potential for the discovery of new taxa, especially if they exhibit extreme environmental features (Rindi et al. 2011). Polar environments can be included in this category.

With only 0.32% of its surface ice-free (Chown and Convey 2007), Antarctica is the coldest, windiest and driest continent. Three biogeographic zones are recognized in Antarctica and neighboring regions (Convey 2007): the Sub-Antarctic, Maritime Antarctic and Continental Antarctic. The terrestrial ecosystems and climatic characteristics of these three zones are distinctly different. The Continental Antarctic zone is subjected to harsher conditions and more extreme temperatures, and is characterized by lower species diversity than the other two zones (Convey 2007). Due to its extreme characteristics and limited human impact, Antarctica has attracted considerable interest from biologists. However, logistical difficulties related to sampling and other fieldwork activities have been an impediment to increasing knowledge of its biological diversity. Overall, non-marine algae have been well studied (e.g., Adams et al. 2006, Broady 1996, and references therein; 2005, Büdel et al. 2008, Casamatta et al. 2005, De los Ríos et al. 2007, De Wever et al. 2009, Hughes et al. 2004, Jungblut et al. 2005, Komárek et al. 2008, Laybourn-Parry and Pearce 2007, Mataloni et al. 2005, Morgan-Kiss et al. 2005, Van de Vijver and Beyens 1999).

Trebouxiophyceae green algae of the genus *Prasiola* are among the best known

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Antarctic algae and at many terrestrial and supralittoral sites represent the most important primary producers (Convey 2007, Ková ik and Pereira 2001, Wiencke and Clayton 2002). The species most commonly reported is *Prasiola crispa* (Lightfoot) Kützing. This nitrophilic alga typically grows on moist soils that are fertilised by bird guano, for instance within and adjacent to penguin rookeries (Graham et al. 2009). It tolerates repeated freeze/thaw cycles in spring and fall, freezing over winter, and high levels of UV radiation during summer (Jackson and Seppelt 1995, 1997, Kosugi et al. 2010, Lud et al. 2001). Amino-acids such as proline serve as cryoprotectants, and mycosporine-like amino-acids absorb UV (Hoyer et al. 2001, Jackson and Seppelt 1997, Karsten et al. 2005).

Detailed investigations on the taxonomy of Antarctic representatives of *Prasiola* are virtually non-existent in the recent literature. *P. crispa* was first described by Lightfoot (1777) as *Ulva crispa* based on material from the Isle of Skye, Scotland, but since then has been reported as cosmopolitan in cold-temperate and polar areas, including all three Antarctic zones. Some early authors described a number of *Prasiola* taxa from Antarctica and cold temperate regions of the Southern Hemisphere: *Prasiola antarctica* (Kützing 1849; type locality: Ross Island ), *P. rothii* var. *falklandica* (Kützing 1849; type locality: Falkland Islands), *P. georgica* (Reinsch 1890a; type locality: South Georgia) and *P. crispa* var. *aspera* (West and West 1911; type locality: Cape Royds, Ross Island). These taxa were separated from *P. crispa* based on morphological characters of doubtful value (arrangement of cells, thickness of the intercellular spaces, texture of the surface). In the subsequent literature they have been regarded as synonyms or subspecific taxa of *P. crispa* (Kobayasi 1967, Ricker 1987). *P. antarctica* was treated as a subspecies of *P. crispa* by Knebel (1936), which has since become the accepted taxonomic treatment for Antarctic specimens. Kobayasi (1967), however, concluded that the morphological differences between *P. crispa* subsp. *antarctica* and *P. crispa* from the Northern Hemisphere were not sufficiently reliable to justify their separation. The terrestrial *Prasiola calophylla* (Carmichael ex Greville) Kützing was originally described from Argyll, Scotland (Greville 1826) and has been reported in cold-temperate regions of the Northern Hemisphere (Guiry and Guiry 2011). Several Antarctic records are available for this species (e.g. Broady 1989 and references therein, Fritsch 1912, Mataloni et al. 2005, Schofield and Ahmadjian 1972). In a field study conducted on Ross Island and in Victoria Land and Marie Byrd Land, Broady (1989) referred to this species all specimens of *Prasiola* collected from streams and

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water-flushed ground distant from fertilization by birds.

Lichenized forms of *Prasiola* are also known from Antarctica. These have generally been referred to *Mastodia tessellata* (J.D. Hooker and Harvey) J.D. Hooker and Harvey (Kováčik and Pereira 2001), originally described from Kerguelen as *Ulva tessellata* by Hooker and Harvey (1845). Subsequently this organism has either been regarded as a lichenized form of *P. crispa* and put in synonymy with it, or classified separately as a lichen (e.g. Ricker 1987). However, its taxonomic identity has been questioned by recent molecular data (Pérez-Ortega et al. 2010) which showed that the alga involved in the *M. tessellata* symbiosis is more closely related to *Prasiola borealis* Reed than to *P. crispa*.

In recent years we have obtained several samples of *Prasiola* from Maritime and Continental Antarctica together with additional samples of *Prasiola* from other regions for which no molecular data were previously available. We examined their morphology and investigated their phylogenetic relationships using sequences of the chloroplast genes *rbcL* and *psaB*. The results, which are presented here, reveal a previously unsuspected cryptic diversity and show that the taxonomy of Antarctic *Prasiola* is more complex than previously realized.

### ***Materials and Methods***

#### *Collections and morphological studies*

Collections of Prasiolales were either made by the authors or obtained from collaborators (details in Table 1). The specimens examined and newly sequenced in this study were primarily from Maritime and Continental Antarctica, but additional specimens from other geographical regions (California, China, Czech Republic, England, Ireland, central Mexico, New Brunswick, Newfoundland, Norway, Tasmania and Washington State) were included. The specimens were placed in sealed containers and conserved mostly as silica-dried or frozen material. Microscopic examination was performed either on fresh or rehydrated material. Voucher specimens were deposited in the Phycological Herbarium of the National University of Ireland, Galway (GALW) and in the Landcare Research Allan Herbarium, Christchurch, New Zealand (CHR). Also examined were additional specimens of critical taxonomic importance deposited in other herbaria (BM and L).

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### *Molecular studies*

Overall, 51 new sequences (22 *rbcL* and 29 *psaB*) were generated in this study. Total genomic DNA was extracted from the samples listed in Table 1 using the Qiagen DNeasy Plant Mini Kit (Qiagen, Crawley, UK) according to the manufacturer's instructions. A Techne TC-3000 thermal cycler (Techne, Stone, UK) was used for all PCR amplifications. PCR of the *psaB* and *rbcL* genes was performed as two overlapping fragments (one forward and one reverse) for each gene. Ca. 1100 bp of the *rbcL* gene were amplified in most cases using the primers RH1 (Manhart 1994) and RT1134 (Rindi et al. 2008). In some cases, the reverse primer *rbcLQ* (Zechman 2003) was used with better results. Each 50  $\mu\text{L}$  reaction consisted of 34.7  $\mu\text{L}$  of HyPure Cell Culture Grade Water (Thermo Scientific, South Logan, UT, USA), 5  $\mu\text{L}$  of 10x OptiBuffer, 2  $\mu\text{L}$  of dNTP Mix, 1  $\mu\text{L}$  of 50 mM  $\text{MgCl}_2$  solution, 0.3  $\mu\text{L}$  of BIO-X-ACT Short DNA polymerase (Bioline, London, UK), 1  $\mu\text{L}$  of each primer mix (100  $\mu\text{M}$ ) and 5  $\mu\text{L}$  of DNA sample. The PCR protocol used for the amplification was the following: an initial denaturation step at 94°C for 2 min followed by 40 cycles with denaturation at 94°C for 1 min, annealing at 55°C for 1 min and elongation at 72°C for 2 min, with a final elongation step at 72°C for 3 min. For the specimen P52 (type specimen of *Prasiola crispa*), a nested PCR was performed with the primers RH1 and *rbcLQ* used as described above but with an annealing temperature of 53°C, followed by a second round of PCR with the primers R320 (Nozaki et al. 1995) and RT1134 using a concentration gradient of the previous PCR- resulting solution as DNA template. Using 0.5  $\mu\text{L}$  and 1  $\mu\text{L}$  yielded the best results. Ca. 1400 bp of the *psaB* gene were amplified using the two pairs of primers pp1F and pp3R and pp2F and pp4R (Novis et al. 2010). The PCR protocol was the same as for the *rbcL* gene. PCR products were visualized in 1.2% agarose gels stained with SYBR Safe DNA stain (Invitrogen, Carlsbad, CA, USA). The amount of DNA in PCR products was quantified visually on agarose gels using HyperLadder II (Bioline) as reference. Cleaning of the products was performed using the Qiagen MinElute Gel Extraction Kit (Qiagen) following the manufacturer's specifications. PCR products of appropriate length, yield and purity were custom-sequenced by GATC Biotech AG (Konstanz, Germany).

### *Sequence alignment and phylogenetic analyses*

Electropherograms for both genes were inspected with BioEdit version 7.0.5.3. All sequences included in the *psaB* and *rbcL* alignments were based on high-quality bidirectional readings. Besides the new sequences produced in this study, all *psaB*

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sequences and most *rbcL* sequences of Prasiolales available in GenBank were included in the alignments. In order to reduce computation time, we omitted some GenBank *rbcL* sequences when these were identical or very similar to some sequences used (as already verified in previous studies, Rindi et al. 2004, 2007). The *psaB* alignment consisted of 33 sequences for which 1368 bp (corresponding to the positions 277-1644 of the complete *psaB* sequence of *Chlorella vulgaris* AB001684) (Wakasugi et al. 1997) could be aligned unambiguously. The *rbcL* alignment consisted of 62 sequences for which 1104 bp (corresponding to positions 103-1206 of *Chlorella vulgaris* AB001684) were used. Alignment was performed using ClustalW (Thompson et al. 1994) as implemented in BioEdit and refined by eye. The analyses performed on the separate datasets showed high congruence of the *psaB* and *rbcL* phylogenetic signals, recovering equivalent topologies and similar support values. In consideration of this, it was decided to combine the two alignments in a concatenated dataset, which included 25 taxa for which both genes were sequenced successfully. The concatenated alignment was 2472 bp long.

**Table 1.** Details of collections examined in the study.

Species	Sample	Collection information	Herbarium number	<i>rbcL</i> GenBank accession number	<i>psaB</i> GenBank accession number
<i>Prasiola antarctica</i>	P18	King George Island, South Shetland Islands, Antarctic Sea. Han-Gu Choi, 30 January 2010. In penguin nesting area. Sample CH2461.	GALW015710	JQ669718	JQ669683
<i>P. antarctica</i>	P30	Area behind Palmer Station, Antarctica (64° 46.450' S; 64° 02.998' W). Charles D. Amsler, 17 March 2010.	GALW015711	JQ669720	JQ669703
<i>P. antarctica</i>	P31	Palmer Station, Antarctica, background (64° 46.492' S; 64° 02.924' W). Charles D. Amsler, 4 April 2010.	GALW015712	JQ669721	
<i>P. antarctica</i>	P17	Amsler Island, Antarctic Peninsula, Antarctica (64° 45.670' S; 64° 05.027' W). Charles D. Amsler, 16 March 2008. On granite rock.	GALW015713	JQ669712	
<i>P. antarctica</i>	P45	Type specimen. Mount Erebus, Ross Island. Marked "K. Suringar, in 1843" but exact date not readable (Kützing's	L 0834589		

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		handwriting). 64°135 S, 57°E.			
<i>P. borealis</i>	P4	Fossil Island, Pirates Bay, Tasmania, Australia. Michael Guiry, 31 October 2009. Upper shore, on rocks in lichen zone. Details in Chapter B2.	GALW015588	JF949724	JQ669689
<i>P. borealis</i>	P20	Lady Bay, Tasmania, Australia (43°40'01 S, 147°01'57 E). Fiona Scott, 18 July 2010. Upper shore, in the vicinity of a well- splattered bird nest. Details in Chapter B2.	GALW015590	JF949723	JQ669702
<i>P. calophylla</i>	P41	Salthill, Galway, Ireland. Fabio Rindi, September 2010. Forming dark green patch at the base of lamp pole on road sideway.	GALW015716	JQ669726	JQ669706
<i>P. crispa</i>	P25	Garwood Valley, McMurdo Dry Valleys, Antarctica; Paul Broady. Valley-side stream, green ribbons wrapped around stones. Sample G96 Paul Broady.	GALW015717	JQ669709	
<i>P. crispa</i>	P13	Upper Garwood Valley, Antarctica. Phil Novis, 28 January 2003. Irrigated shingle fan East of Lake Colleen, 364 m a.s.l. Sample 34 by Phil Novis.	Hancock Herbarium	JQ669715	JQ669678
<i>P. crispa</i>	P21	Galway City, Ireland. Fabio Rindi, May 2010. Space along canal on the back side of Róisín Dubh pub. On tarred concrete, mixed with mosses and grass.	GALW015718	JQ669711	JQ669705
<i>P. crispa</i>	P23	Sæter Øya, near Ona, Norway; Fabio Rindi, 28 June 2004. Small offshore island; supralittoral zone, on the top of the island.	GALW015335	JQ669714	
<i>P. crispa</i>	P24	Prague, Czech Republic; Jiří Neustupa. Sample originally isolated in culture from soil.	GALW015719	JQ669713	JQ669688
<i>P. crispa</i>	P28	Marshall Valley, Antarctica (78° 03.806' S; 164° 02.714' E). Phil Novis, 24 January 2003. Irrigated shingle fan below glacier, 474 m a.s.l. Sample 29 by Phil Novis.	Hancock Herbarium	JQ669707	
<i>P. crispa</i>	P29	Lake Colleen, Upper Garwood Valley, Antarctica (78° 01.398' S; 16° 55.291' E). Phil Novis.	Hancock Herbarium	JQ669716	JQ669679

Part B: Prasiolales

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		Irrigated shingle fan East of Lake Colleen, 364 m a.s.l. Sample 35 by Phil Novis.			
<i>P. crispa</i>	P32	Torgersen Island, Antarctica (64° 46.255' S; 64° 04.531' W). Charles D. Amsler, 17 March 2010.	GALW015720	JQ669722	JQ669704
<i>P. crispa</i>	P34	Durham, England, U.K. Fabio Rindi, 9 July 2002. Forming loose masses on concrete surface at the base of wall in car park.	GALW014232		JQ669695
<i>P. crispa</i>	P42	The Neck, Sawnders Island, Antarctica. Frithjof Küpper, 1 November 2010.	GALW015722	JQ669727	JQ669682
<i>P. crispa</i>	P43	King George Island, South Shetland Islands, Antarctic Sea. Han-Gu Choi, 17 January 2010. Penguin nesting area. CH2227.	GALW015723	JQ669723	JQ669685
<i>P. crispa</i>	P44	King George Island, South Shetland Islands, Antarctic Sea; Han-Gu Choi, 17 January 2010. Penguin nesting area. Sample CH2228.	GALW015724	JQ669724	JQ669684
<i>P. crispa</i>	P48	King George Island, South Shetland Islands, Antarctic Sea. Han-Gu Choi, 17 January 2010. Penguin nesting area. Sample CH2229.	GALW015725	JQ669717	JQ669681
<i>P. crispa</i>	P52	Isle of Skye, Argyll, Scotland, U.K. "Upon the ground in shady places, at the foot of walls and houses" (Lightfoot's handwriting). Holotype specimen.	British Natural History Museum (BM)	JQ669725	
<i>P. glacialis</i>	P22	Cape Royds, Ross Island, Antarctica. Paul Broady. Slight trickle from small pond; abundant growth of <i>P. calophylla</i> , associated with blue-green mat of cyanobacteria. Sample 186 Paul Broady.	GALW015714	JQ669728	JQ669680
<i>P. glacialis</i>	P27	Garwood Valley, McMurdo Dry Valleys, Antarctica. Paul Broady, 2 January 1987. Slight stream fed by melt from an up-slope, ice field; growing intermingled with <i>Nostoc commune</i> colonies. Sample G105 Paul Broady. Designated as holotype specimen.	GALW015715	JQ669710	

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Part B: Prasiolales

<i>P. meridionalis</i>	P10	Bodega Marina Lab, California, U.S.A. (38° 19.102' N; 123° 04.416' W). Sandra Lindstrom, 16 February 2010. High/supratidal bedrock.	GALW015726	Identical to JQ669729	JQ669701
<i>P. meridionalis</i>	P7	Deadman Bay, Washington State, U.S.A. Sandra Lindstrom, 11 December 2009.	GALW015727	Identical to JQ669729	JQ669691
<i>P. mexicana</i>	P46	Central Region of Mexico. Rocío Ramírez Rodríguez and Javier Carmona Jiménez. In mountain stream.	GALW015728	JQ669719	JQ669687
<i>P. stipitata</i>	P26	Plymouth, England, U.K. Fabio Rindi, 2 February 2010. Upper intertidal zone; rocks on shore in front of National Aquarium.	GALW015729	Identical to JQ669729	JQ669692
<i>P. stipitata</i>	P38	Long Eddy Point, Grand Manan, Bay of Fundy, New Brunswick, Canada. Gary W. Saunders, 31 March 2007.	GWS005964	JQ669729	JQ669694
<i>P. stipitata</i>	P39	Rocky Harbour, Bonne Bay, Newfoundland, Canada. Line Le Gall, Hana Kucera and J. Utge, 13 July 2006.	GWS007186		JQ669696
<i>P. stipitata</i>	P40	Claddagh, Galway, Ireland. Fabio Rindi, 20 May 2010. Forming green patches on rock at the high water mark.	GALW015730	Identical to JQ669729	JQ669693
<i>P. stipitata</i>	P49	Swallow Tail Lighthouse, Grand Manan, New Brunswick, Canada (44° 75' 18 N 66° 73' 15 W). Gary Saunders, 27 November 2005. On rock, uppermost with guano.	GWS003545		JQ669699
<i>P. stipitata</i>	P50	Eastport, Newfoundland, Canada. Line Le Gall, Hana Kucera, Dan McDevit and J. Utge, 18 July 2006.	GWS007406	JQ669730	JQ669697
<i>P. stipitata</i>	P51	Green Point, Bonne Bay, New Foundland, Canada; Gary Saunders, 18 July 2006. (49° 68' 198 N 57° 96' 275W).	GWS007257		JQ669698
<i>P. yunnanica</i>	P16	Qing Bi Creek, Chanshan Mountains, near Dali, China. Stefano Draisma. Stream at 2600 m a.s.l. Sample Stefano Draisma 0912008.	GALW015731	JQ669708	JQ669686

## Part B: Prasiolales

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<i>Rosenvingiella constricta</i>	P3	Fossil Island, Pirates Bay, Tasmania, Australia. Michael Guiry, October 2009. Forming large green patches on rock around and above the high-water mark. Details in Chapter B2.	GALW015591	JF949725	JQ669690
<i>R. tasmanica</i>	P6	Fortescue Bay, Tasmania, Australia. Michael Guiry, November 2009. Forming bright green patches mixed with <i>Prasiola borealis</i> . Details in Chapter B2.	GALW015592	JF949726	JQ669700

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Phylogenetic trees were outgroup-rooted following the recommendations of Verbruggen and Theriot (2008) after some preliminary analyses in which different taxa were tested as possible suitable outgroups. For *rbcL* the outgroup taxa used were: *Diplosphaera* sp. AM260445, *Diplosphaera mucosa* AM260444, uncultured *Chlorella* AM260443 and *Stichococcus bacillaris* AM260442; for *psaB*: *Microthamnion kuetzingianum* EU380579 and *Stichococcus* sp. GQ423929. For the concatenated dataset *Stichococcus* sp., for which the *psaB* sequence GQ423929 and the *rbcL* sequence EF589147 were obtained from the same sample, was used.

Neighbor Joining (NJ) analysis was performed on the aligned datasets in SeaView (Gouy et al. 2010). Phylogenetic analyses were performed using Maximum Likelihood (ML) in Treefinder version March 2011 (Jobb 2011) and PhyML (Guindon and Gascuel 2003); Bayesian analyses (BI) were performed using MrBayes 3.04 (Huelsenbeck and Ronquist 2001). The NJ analysis was performed on uncorrected p-distances. The ML Treefinder and BI analyses were performed on partitioned datasets, with three partitions corresponding to the first, second and third codon positions of the *psaB* and *rbcL* genes. The same partition was used in the concatenated dataset; Verbruggen et al. (2010) recommended this strategy for the analysis of protein-coding plastid genes (such as *psaB* and *rbcL*). The parameters for the PhyML ML and BI analyses were chosen after selection of the appropriate evolutionary model with jModelTest (Posada and Crandall 1998) under the Akaike Information Criterion. For the Treefinder ML analysis, the models selected by Treefinder under the corrected Akaike Information Criterion (AICc) were applied. For the NJ and ML Treefinder analyses, nodal support was assessed by non-parametric bootstrap analysis with 1000 resamplings. The BI analysis was performed using the priors set as default in MrBayes; the parameters were unlinked and allowed to vary across partitions. Four Monte Carlo

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Markov chains were run for  $5 \times 10^6$  generations, with tree sampling every 1000 generations. It was assumed that convergence of the two runs was reached when the average standard deviation of split frequencies between the two runs was lower than 0.01; this was further verified using the sump command and plotting the likelihood scores versus the number of generations. The first 100 samples were discarded as burn-in and the remainder trees were used to build the 50% majority-rule trees.

### **Results**

#### *Molecular phylogeny*

The *rbcL* and *psaB* genes showed a congruent phylogenetic signal in the datasets analyzed. Phylogenetic analyses performed on the two genes and the concatenated alignment produced trees with equivalent topologies in which identical clades were recovered (Figs. 1, 2 and 3). Statistical support varied among lineages and was generally higher for the terminal nodes than for the internal nodes, with higher values in the concatenated dataset.

The Antarctic specimens of *Prasiola* sequenced in this study occurred in three well-supported clades that we consider referable to different species. These will now be referred to as the *Prasiola antarctica*, *P. glacialis* and *P. crispa* clades (Figs. 1, 2 and 3). These clades belonged to a large monophyletic group of *Prasiola* taxa distributed in both hemispheres and occurring in a range of subaerial habitats. In the *rbcL* phylogeny this group was sister to *Prasiococcus calcarius* (J.B. Petersen) Vischer with moderate support, but its position was not resolved in the *psaB* and concatenated phylogenies. It was, however, recovered with moderate to high support in all three phylogenies. The positions of the three clades within this group were identical in all analyses. The *Prasiola antarctica* clade, which included algae collected from the Antarctic Peninsula and King George Island, had a sister relationship to a lineage containing the other two clades. The *Prasiola glacialis* clade, formed by specimens collected from non-permanent freshwater habitats in Continental Antarctica (Ross Island and Garwood Valley), was placed in intermediate position between *Prasiola borealis* M. Reed and *P. crispa* and *P. mexicana* J. Agardh. The *Prasiola crispa* clade included the majority of the Antarctic specimens of *Prasiola*, together with many samples of *P. crispa* from the Northern Hemisphere. In the *rbcL* phylogeny this clade included the sequence of the type specimen of *P. crispa* [described by Lightfoot (1777) from the Isle of Skye,

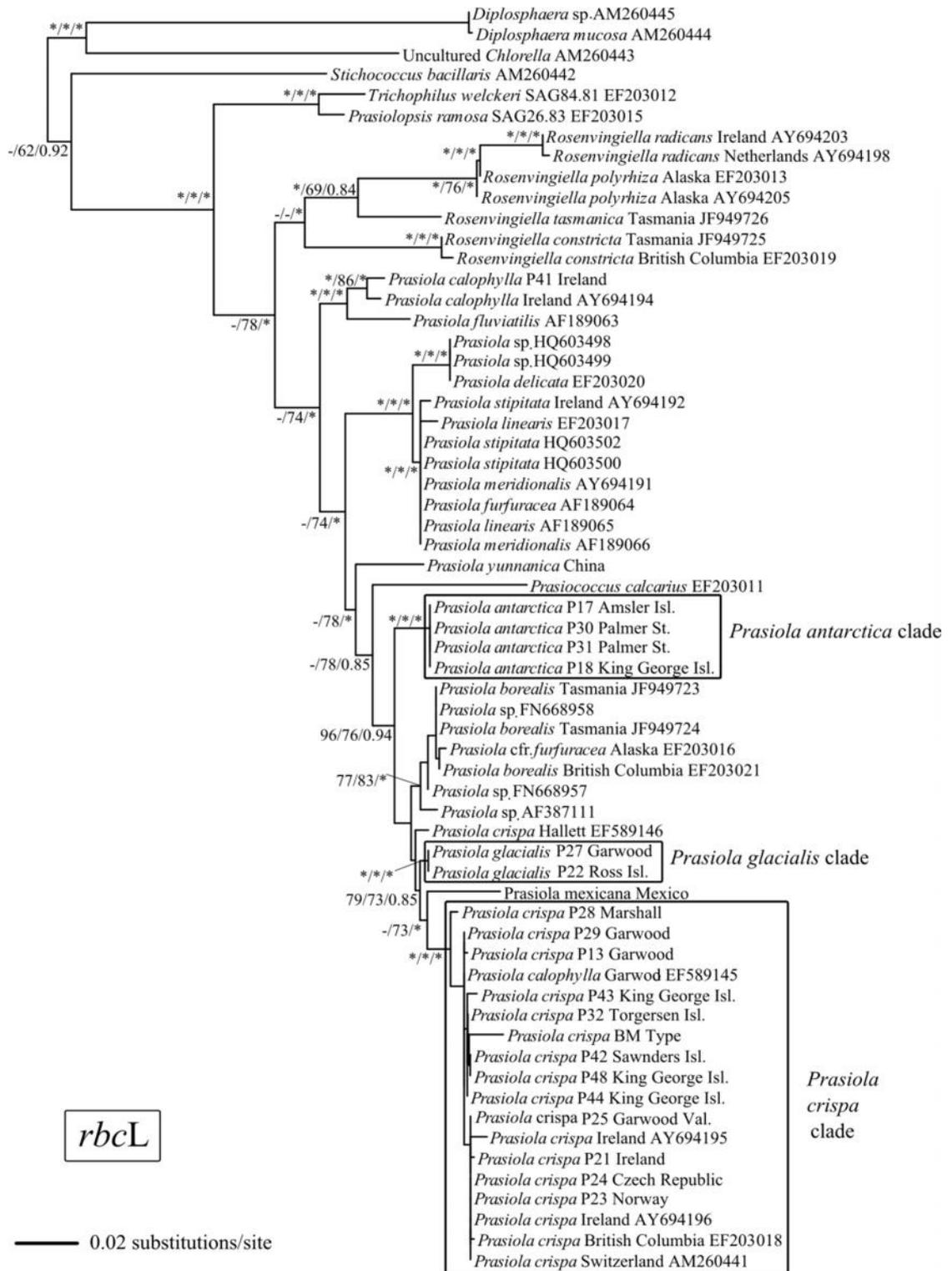
## Part B: Prasiolales

Scotland; type in BM]. The *rbcL* sequence of the type specimen diverged 5 to 9 bp from the other sequences in this clade, whereas the pairwise difference among other sequences ranged from 0 to 5 bp within the same comparable region. Within this clade, *psaB* sequences diverged between 0 and 7 bp.

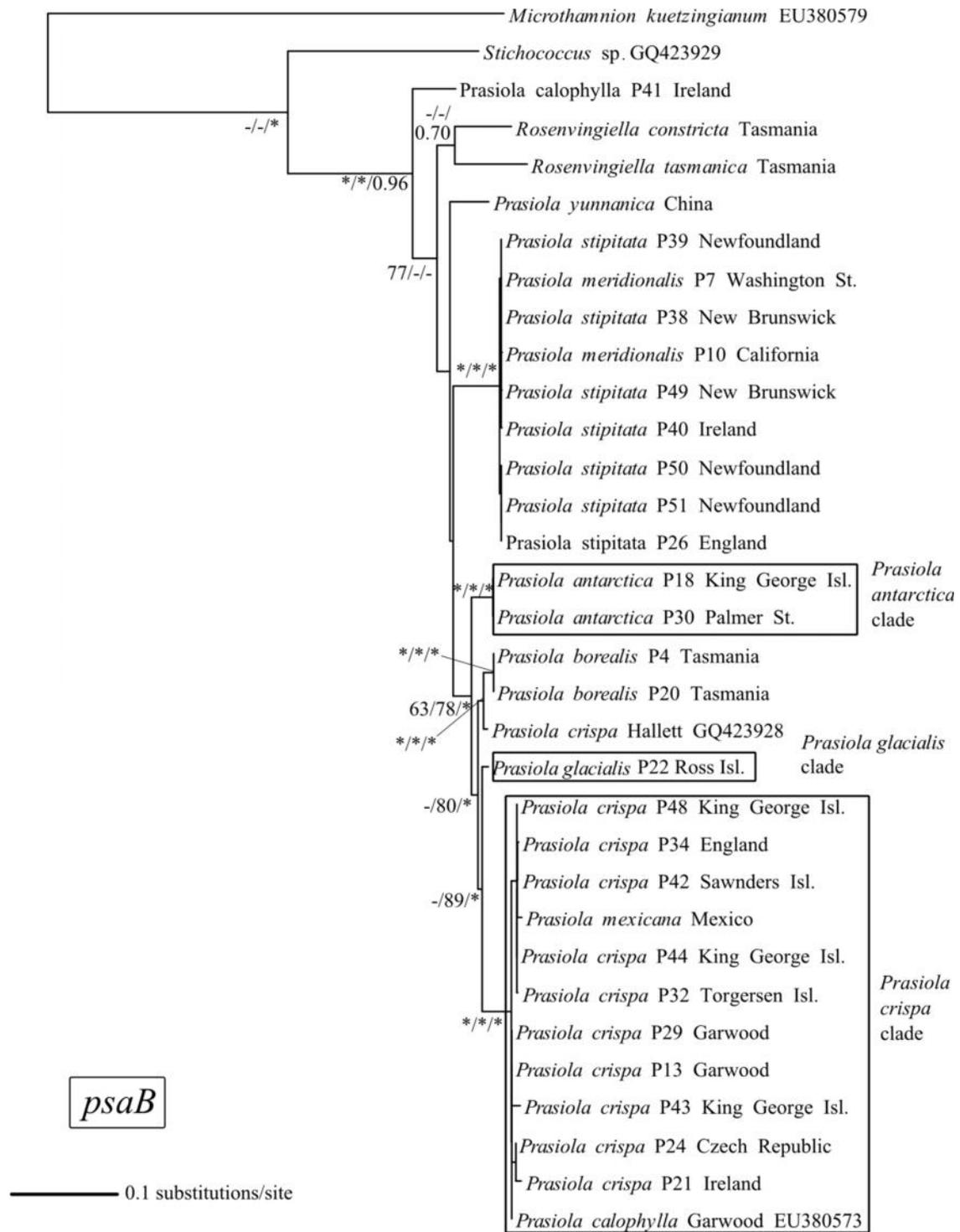
The *rbcL* sequences in the *P. antarctica* clade were identical and differed by 25 bp from the *P. crispa* type material, despite having identical gross morphology. The two *psaB* sequences of the *P. antarctica* clade were also identical and differed between 29 and 32 bp from the sequences of the *P. crispa* clade within the same comparable region. The *rbcL* sequences of *P. glacialis* were identical and diverged 38 to 42 bp from European specimens of *P. calophylla* and 8 to 13 bp from *P. crispa*; the *psaB* sequence of *P. glacialis* differed by 97 bp from *P. calophylla* and by 29-35 bp from *P. crispa*.

The present study produced the first *rbcL* and *psaB* sequences for two freshwater species of *Prasiola*, namely *P. mexicana* and *P. yunnanica* C.-C. Jao. *Prasiola mexicana* was sister to *P. crispa* with moderate support in the *rbcL* phylogeny and high support in the concatenated phylogeny, whereas it was merged into the *P. crispa* clade in the *psaB* phylogeny. *Prasiola yunnanica* formed a separate lineage without any clear affinity to any of the prasiolalean lineages currently known; its position remained unresolved in all phylogenetic trees.

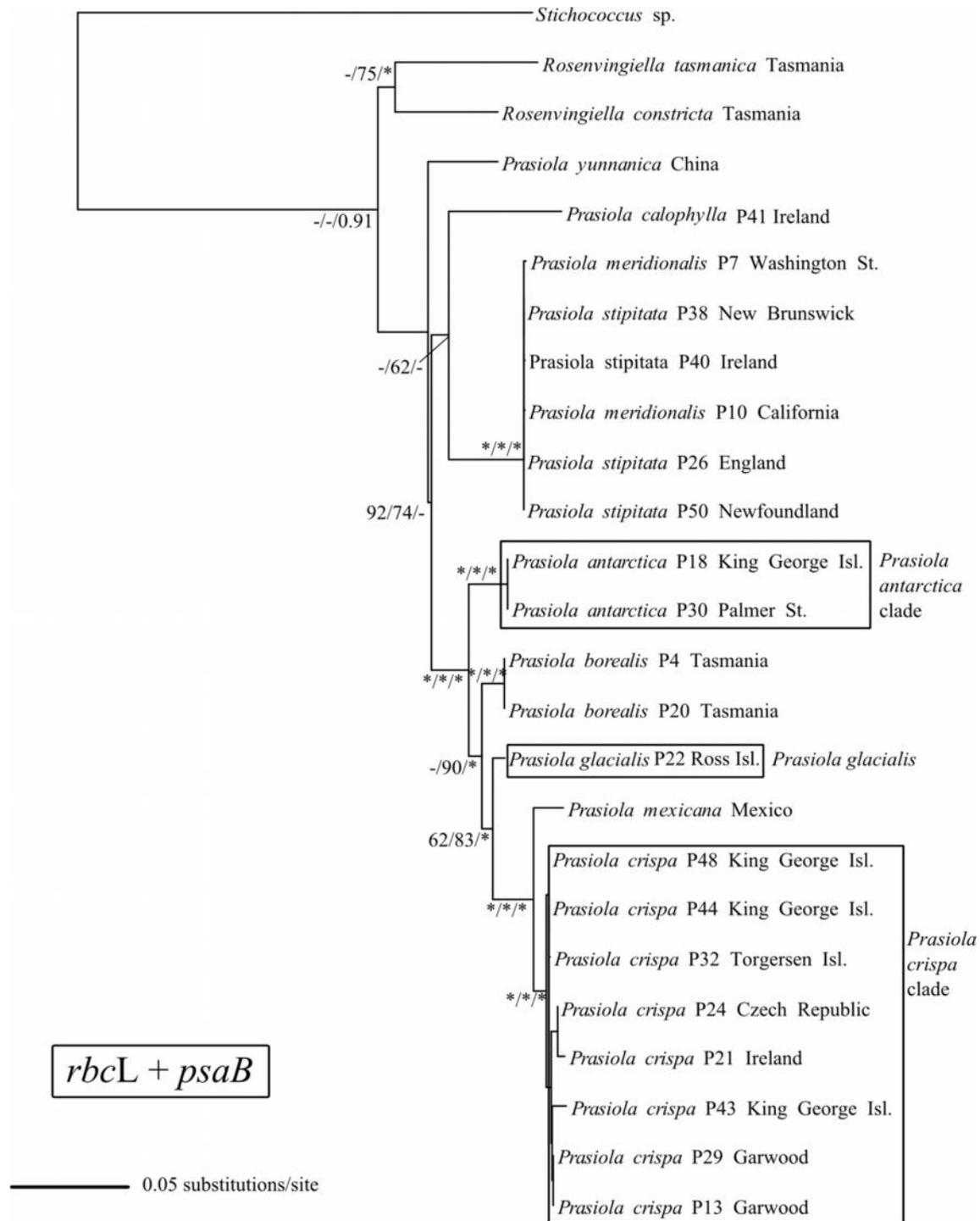
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**Fig 1.** Phylogram inferred from ML analysis of the *rbcL* gene in *Prasiola* and closely related taxa (obtained with PhyML). From left to right numbers marked at the nodes indicate NJ bootstrap, ML bootstrap (Treefinder) and Bayesian posterior probabilities. Models selected by Treefinder: TN for first codon positions, HKY for second codon positions, J2 for third codon positions. Settings applied for BI: nst=6 for all partitions, rates set to gamma for codon positions 1 and 2 and invgamma for codon positions 3. Asterisks indicate BP values  $\geq 90\%$  and PP  $\geq 0.95$ . BP values lower than 60% and PP lower than 0.7 are not shown.



**Fig. 2.** Phylogram inferred from ML analysis of the *psaB* gene in *Prasiola* and closely related taxa (obtained with PhyML). From left to right numbers marked at the nodes indicate NJ bootstrap, ML bootstrap (Treefinder) and Bayesian posterior probabilities. Models selected by Treefinder: J3 for first codon positions, HKY for second codon positions, J3 for third codon positions. Settings applied for BI: nst=6 for all partitions, rates set to gamma for codon positions 1 and invgamma for codon positions 2 and 3. Asterisks indicate BP values  $\geq 90\%$  and PP  $\geq 0.95$ . BP values lower than 60% and PP lower than 0.7 are not shown.



**Fig. 3.** Phylogram inferred from ML analysis of the concatenated dataset *rbcL-psaB* in *Prasiola* and closely related taxa (obtained with PhyML). From left to right numbers marked at the nodes indicate NJ bootstrap, ML bootstrap (Treefinder) and Bayesian posterior probabilities. Models selected by Treefinder: J3 for first codon positions, HKY for second codon positions, GTR for third codon positions. Settings applied for BI: nst=6 for all partitions, rates set to invgamma for codon positions 1 and gamma for codon positions 2 and 3. Asterisks indicate BP values  $\geq 90\%$  and PP  $\geq 0.95$ . BP values lower than 60% and PP lower than 0.7 are not shown.

*Morphology, ecology and distribution*

The morphological features of the Antarctic specimens examined in this study are summarized in Table 2. The three *Prasiola* species occurring in Antarctica form a complex of cryptic taxa that cannot be distinguished in terms of gross morphology. Additionally, *P. crispera* and *P. antarctica* share the same type of habitat (soil and rock at sites subject to enrichment in organic nitrogen, especially guano in penguin rookeries), which further complicates their identification. Remarkably, in certain locations, *P. antarctica* and *P. crispera* occurred in close proximity of each other; the samples *P. antarctica* P18 and *P. crispera* P43, P44 and P48 were collected a few tens of meters apart in a penguin rookery on King George Is. On the other hand, specimens of *P. glacialis* were collected in non-permanent freshwater habitats distant from fertilization by birds.

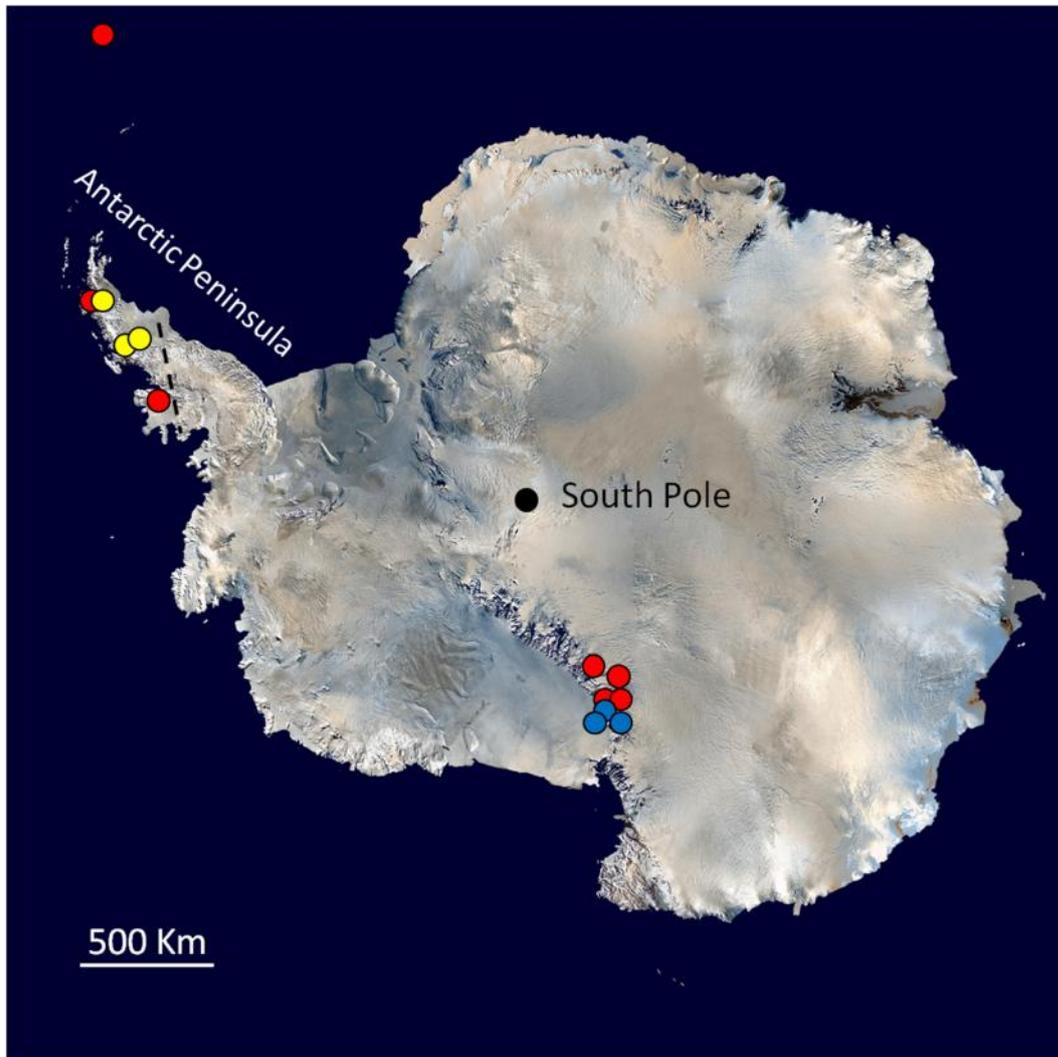
*Prasiola crispera* is widely distributed in Continental Antarctica (Upper Garwood Valley and Marshall Valley) and Maritime Antarctica (Torgersen Is., Sawnders Is. and King George Is.) (Fig. S1). The thalli usually consisted of monostromatic blades with expanded or ribbon-like (Fig. 4a) habit. Large blades became curled or deeply folded, and the aggregation of numerous blades produced dense leafy mats up to 5 cm long and wide. Some collections, however, consisted mostly of uniseriate filaments (*Hormidium* stages) or narrow ribbon-like blades (*Schizogonium* stages) (Fig. 4b). In some samples (e.g., P25 from Garwood Valley) a complete morphological range encompassing every stage from uniseriate filaments to expanded blades was observed. The blades were monostromatic, 18-23  $\mu\text{m}$  in thickness; in section their surface was usually smooth, but appeared corrugated in some samples. The cells were arranged in regular rows and the occurrence of areolae showed great variation among samples. Whereas clear areolae were observed in some samples (e.g. P28 from Marshall Valley and P32 from Torgersen Is.), other specimens lacked areolation (e.g. P44 and P48 from King George Is.). In surface view the cells were 4-16  $\mu\text{m}$  long and wide (Fig. 4c), again with some variation among different specimens. The specimens from Continental Antarctica had generally smaller cells than specimens from other locations (4-8  $\mu\text{m}$  in width and length, mainly 4-6  $\mu\text{m}$ ). No akinetes, aplanospores or other differentiated cells were observed.

Specimens referred to *Prasiola antarctica* were collected at Palmer Station (Antarctic Peninsula), on Amsler Is. and on King George Is. (South Shetland Is.) (Fig. S1). This alga formed thick, dense, dark green masses not attached to the substratum, up to several cm long and wide, formed by many blades with irregular habit. The blades

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were expanded, curled or deeply folded (Fig. 4d), devoid of attachment structures. Cells were 4-14  $\mu\text{m}$  long and wide, and arranged in clear areolae with thickened borders, which included groups of 16, 32, 64 or more cells (Fig. 4e). Akinetes, aplanospores or other differentiated cells were not observed. Filamentous stages were not observed, even though in some blades the margins formed proliferations similar to narrow ribbons (Fig. 4f). The blade was monostromatic, 15-30  $\mu\text{m}$  thick (mainly 20-25  $\mu\text{m}$ ); the cells in section were 14-16  $\mu\text{m}$  tall. The surface of the blade was smooth or slightly undulated and appeared equally thickened on both sides of the thallus.

*Prasiola glacialis* was collected at Cape Royds, Ross Is., and Garwood Valley in the McMurdo Dry Valleys (Fig. S1). Broady (1989) considered this alga a foliose ecophene of *Prasiola calophylla*, with expanded blade seemingly identical to *P. crispa*. It occurred in small streams or on the surface of ice, soil and gravel irrigated by slow flows of melt water, often mixed with mats of filamentous cyanobacteria including *Nostoc*. The thallus consisted of monostromatic blades with the habit of large ribbons or leafy, expanded blades similar to *P. crispa* (Figs 4, g and h). In section, the blade was 12-24  $\mu\text{m}$  thick (mainly 18-20) with a slightly irregular surface that did not show marked corrugations. In surface view the cells were 4-8  $\mu\text{m}$  long and wide (Fig. 4i). Some thickened lines were present and areolae could be observed in some parts of the thallus; they were, however, not very evident and appeared to be completely lacking in other parts of the same blades. Aplanosporangia were not observed.

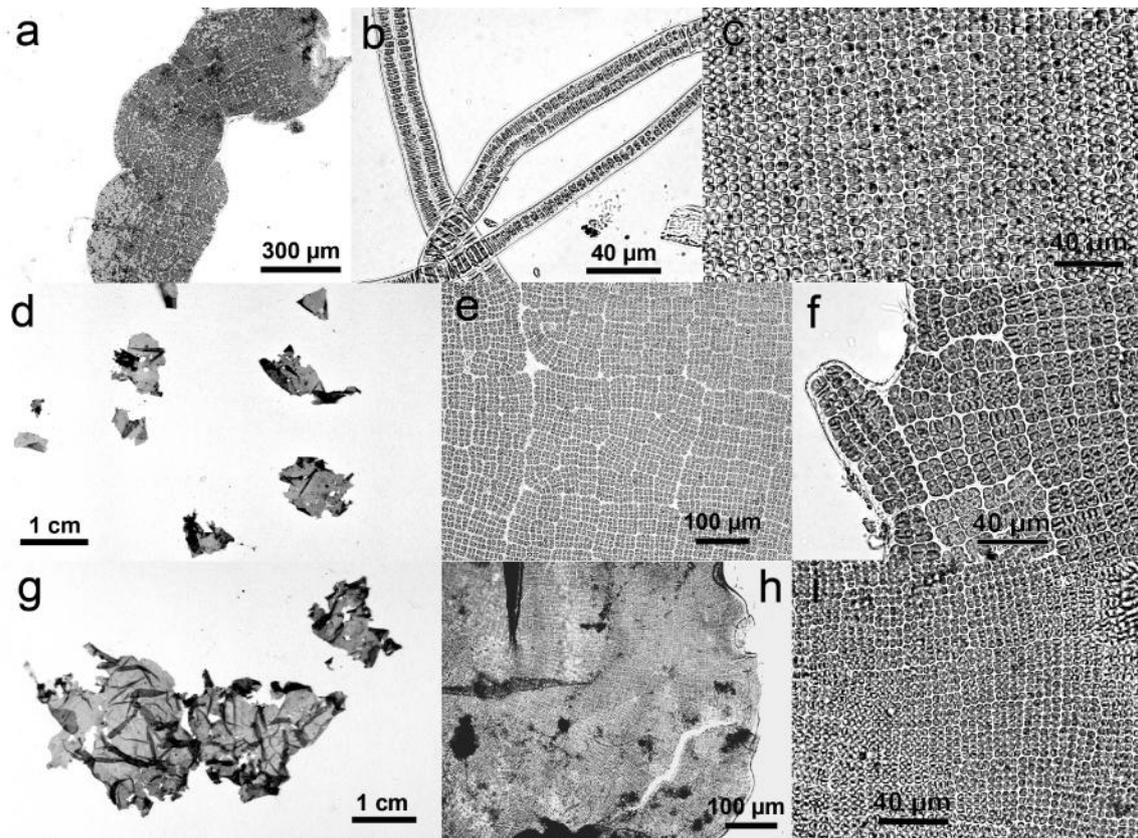


**Fig. S1.** Map showing the collection sites for the Antarctic specimens of *Prasiola* examined in this study. Collections of *Prasiola antarctica* are indicated by yellow dots, *P. crispera* by red dots and *P. cf. calophylla* by blue dots. Dotted line showing the “Gressitt Line” as described by Chown and Convey (2007).

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**Table 2.** Summary of morphological data for specimens of *Prasiola* belonging to the *Prasiola antarctica*, *Prasiola glacialis* and *Prasiola crispa* clades examined in this study. Numbers indicate mean  $\pm$  standard deviation (n=10 for cell width and length; n=6 for blade thickness and filament width).

Species	Sample	Location	Habit	Cell width ( $\mu\text{m}$ )	Cell length ( $\mu\text{m}$ )	Blade thickness ( $\mu\text{m}$ )	Blade texture	Areolae	Filament width
<i>P. antarctica</i>	P17	Amsler Island	Blade	5.3 $\pm$ 0.88	7.17 $\pm$ 1.41	20-28 (20-25)	Smooth	Present	NA
<i>P. antarctica</i>	P18	King George Island	Blade	9.2 $\pm$ 1.21	11.6 $\pm$ 1.35	20-30 (20-25)	Slightly undulate	Clear	NA
<i>P. antarctica</i>	P30	Palmer Station	Blade	6 $\pm$ 0	7.6 $\pm$ 1.51	15-24 (20)	Slightly undulate	Clear	NA
<i>P. antarctica</i>	P31	Palmer Station	Blade	6.1 $\pm$ 0.57	8 $\pm$ 1.05	20-25	Slightly undulate	Very clear	NA
<i>P. antarctica</i>	P45	Mount Erebus	Blade	7.6 $\pm$ 1.7	10 $\pm$ 2.03	20-23	Corrugated	Very clear	NA
<i>P. glacialis</i>	P22	Ross Island	Blade	5.38 $\pm$ 0.96	5.85 $\pm$ 1.07	14-24		Not clear	NA
<i>P. glacialis</i>	P27	McMurdo Dry Valleys	Blade	6.5 $\pm$ 1.24	6 $\pm$ 1.5	12-22	Slightly undulate	Clear	NA
<i>P. crispa</i>	P13	Upper Garwood Valley	Filaments	NA	NA	NA	NA	NA	15.83 $\pm$ 1.17
<i>P. crispa</i>	P21	Galway City	Blade	7.8 $\pm$ 1.97	10.13 $\pm$ 2.53	18-22	Smooth	Absent	NA
<i>P. crispa</i>	P23	Ona	Blade	7.13 $\pm$ 1.6	9.8 $\pm$ 1.15	18-21	Smooth	Clear	NA
<i>P. crispa</i>	P24	Prague	Filaments	NA	NA	NA		NA	16 $\pm$ 1.15
<i>P. crispa</i>	P25	McMurdo Dry Valleys	Mixture	NA	NA	NA	Smooth	NA	14.3 $\pm$ 1.25
<i>P. crispa</i>	P28	Marshall Valley	Ribbon-like	4.75 $\pm$ 1.42	6.41 $\pm$ 1.5			Clear	NA
<i>P. crispa</i>	P29	Lake Colleen	Mixture	4.7 $\pm$ 0.82	6.3 $\pm$ 0.82		Smooth	Not clear	14 $\pm$ 1.26
<i>P. crispa</i>	P32	Torgersen Island	Blade	5.7 $\pm$ 1.16	7.5 $\pm$ 1.43	20-23	Smooth	Clear	NA
<i>P. crispa</i>	P34	Durham	Blade	7.12 $\pm$ 1.12	8.87 $\pm$ 1.46	14-20	Corrugated	Absent	NA
<i>P. crispa</i>	P42	Sawnders Island	Filaments	NA	NA	NA	NA	NA	13.7 $\pm$ 2.26
<i>P. crispa</i>	P43	King George Island	Blade	6.3 $\pm$ 0.95	9 $\pm$ 2.21	20-22	Smooth	Not clear	NA
<i>P. crispa</i>	P44	King George Island	Blade	5.3 $\pm$ 0.67	7.6 $\pm$ 1.43	18-21 (19-20)	Smooth	Absent	NA
<i>P. crispa</i>	P48	King George Island	Blade	6.12 $\pm$ 1.36	8.81 $\pm$ 2.43	15-22	Smooth	Absent	NA
<i>P. crispa</i>	P52	Isle of Skye	Mixture	7 $\pm$ 1.38	8.7 $\pm$ 1.78	18-20	Smooth	Not clear	13.75 $\pm$ 1.03



**Fig. 4.** Morphology of Antarctic *Prasiola* species. *Prasiola crispera* (a-c): (a) habit of a ribbon-like blade (sample P28); (b) habit of uniseriate and biseriolate filaments (sample P29); (c) surface view of a blade lacking areolation (sample P44). *Prasiola antarctica*: (d) habit (sample P18); (e) surface view of a blade showing evident areolation (sample P18); (f) detail of a blade, showing marginal proliferation (sample P30). *Prasiola glacialis*: (g) habit (holotype, sample P27); (h) detail of a blade, showing slightly undulated margin (sample P22); (i) surface view of a blade (sample P22).



**Fig. 5.** Morphology of type specimens of *Prasiola antarctica* and *Prasiola crispa*. *Prasiola antarctica* (L 0834589): (a) habit of blade margin in surface view; (b) detail of blade, showing the corrugated surface; (c) detail of blade surface showing thickenings and very clear areolae. *Prasiola crispa* (BM): (d) detail showing blade and uniseriate filaments mixed; (e) detail of blade in surface view showing some thickened lines but no clear areolae; (f) detail of a blade, showing smooth surface.

## ***Discussion***

### *Diversity*

This is the first study to explore in detail the molecular diversity of Antarctic *Prasiola*. Sequences are currently available in GenBank for only two Antarctic representatives of this genus, a specimen referred to *P. calophylla* from Garwood Valley and a specimen referred to *P. crispa* from Cape Hallett for which *atpB*, *psaB* and *rbcL* sequences were produced by one of us (PMN). The three clades containing Antarctic *Prasiola* are nested within a large and robustly supported group of *Prasiola* strains distributed in freshwater and terrestrial habitats in both hemispheres. This group was already recovered with high support in previous *rbcL* phylogenies (Rindi et al. 2004, 2007) and the new data presented here allow for an improved characterization. Within this group, *P. antarctica* is sister to a lineage formed by all other taxa, whereas *P. glacialis* and *P. crispa* are merged in this lineage. *P. mexicana*, for which the first *rbcL* and *psaB* sequences have been produced in this study, also belongs to this group. We propose that the appropriate taxonomic solution is to regard the three clades with Antarctic specimens as separate species. The alternative, the application of the name *Prasiola crispa* to the whole group, would lead to major (and in our opinion unnecessary) difficulties in species circumscription. Besides Antarctic *Prasiola*, this group includes *Prasiola borealis* and *P. mexicana*, two species that differ substantially from *P. crispa* both in terms of morphology and habitat (Printz 1964, Reed 1902, Rodríguez and Jiménez 2005, Rodríguez et al. 2007, Setchell and Gardner 1920a, 1920b, chapter B2). These have always been considered independent species and to merge them into *P. crispa* would lead to a circumscription of this species that in our opinion would be unacceptably broad.

The discovery of three species that are genetically distinct but exhibit a virtually identical morphology represents just a new addition to the many cases of cryptic diversity revealed in freshwater and terrestrial microchlorophytes (e.g. Fucikova et al. 2011b, Krienitz et al. 2011a, 2011b, Luo et al. 2010, Rindi et al. 2011, Škaloud and Peksa 2010). It is noteworthy that recent studies have also revealed a high genetic diversity in other Antarctic green microalgae. De Wever et al. (2009) found a high phylodiversity in chlorophycean and trebouxiophycean algae from lacustrine habitats in Maritime and Continental Antarctica. This situation is in remarkable contrast with marine species of *Prasiola*, for which previous studies showed very low genetic diversity despite considerable variation in morphology and life histories (Rindi et al.

2004, 2007, Rindi 2010).

Antarctic *Prasiola* has been the subject of some major physiological investigations (Bock et al. 1996, Jackson and Seppelt 1995, 1997, Kosugi et al. 2010, Lud et al. 2001). In consideration of the cryptic diversity demonstrated here, the taxonomic identity of the material used in these studies needs to be reassessed. It is also critical that samples to be used for similar work in the future are identified using molecular data. The genetic diversity of the three Antarctic clades might be linked to biochemical and physiological differences, which could potentially lead to misinterpretations of results.

#### *Taxonomy and nomenclature*

Most Continental and Maritime Antarctic specimens sequenced in this study belong to *Prasiola crispa*. This has been assessed unambiguously because the *rbcL* sequence of the type specimen of *Ulva crispa* [basionym of *P. crispa*; Lightfoot (1777)] is robustly positioned in this clade. This species occurred in all surveyed areas, indicating an extensive distribution in Antarctica (Fig. S1). The sequences of Antarctic specimens of *P. crispa* are identical or very similar to those of *P. crispa* from the Northern Hemisphere, which confirms the cosmopolitan distribution of this species in polar and cold temperate zones.

For the other two clades containing Antarctic specimens of *Prasiola* an unambiguous nomenclatural assessment is more difficult. For the clade sister to the lineage containing *P. borealis*, *P. glacialis*, *P. mexicana* and *P. crispa*, after examining the possible nomenclatural alternatives available, we provisionally propose the use of the name *Prasiola antarctica* Kützing as an independent species. *Prasiola antarctica* was described by Kützing (1849) based on material collected by J.D. Hooker on the 6<sup>th</sup> of January 1843 on moist stones from moraines at the lower slopes of Mount Erebus, Ross Island. This alga was characterized in the original description as consisting of small rounded or oblong blades, with smooth to crenulate margins, and cells grouped in very distinct areolae (Kützing 1849, p. 473). The type specimen, L 0834589, which we examined (Figs 5, a, b and c), agrees well with the description. In the subsequent literature this species has been mostly considered either conspecific with *P. crispa* or a subspecies of it, namely *P. crispa* subsp. *antarctica* (Kützing) Knebel. In this expanded circumscription, the type of *P. antarctica* is considered part of a continuum of forms that includes the majority of Antarctic specimens of *P. crispa*, in which the cells are consistently arranged in clear areolae and the blade has a thickened, corrugated surface

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(the characters that Knebel (1936) used to separate *P. crispa* subsp. *antarctica* from *P. crispa* of the Northern Hemisphere). Our results suggest that the taxonomic utility of these features is very limited, as they are affected by obvious intraspecific variation (Table 2). Further studies will be necessary to establish whether other characters may be more useful for the intraspecific taxonomy and the delimitation of subspecific taxa within *P. crispa*. Pending such data, our specimens of the *P. antarctica* clade show good correspondence with the general circumscription of *P. crispa* subsp. *antarctica* available in the literature (Knebel 1936, Kovářík and Pereira 2001, Wiencke and Clayton 2002) and we presently consider this taxon an appropriate taxonomic attribution for them.

*Prasiola rothii* var. *falklandica* was described by Kützing (1849, p. 473 as “*β falklandica*”) for material from the Falkland Islands and subsequently elevated to species status as *Prasiola falklandica* (Kützing) Kützing by Kützing (1855, p. 13). Applying this name to any of our material is very difficult as Kützing (1849, 1855), as it was customary for him, provided only a cursory description and limited illustrations which are insufficient to discriminate it from *P. crispa* and similar forms. In the subsequent literature this name has been mostly ignored, presumably because it has been considered to be a form of *P. crispa*. It has not been reported from Antarctica, and its taxonomic identity remains uncertain and in need of reassessment. *Prasiola georgica*, described by Reinsch (1890a, p. 355) from cliffs in South Georgia, has a gross morphology similar to our specimens and is another potential nomenclatural candidate but the cell size reported in the original description (1.6-2 µm) is considerably smaller than in any of the specimens that we examined.

Additional studies will be necessary for definitive nomenclatural and taxonomic decisions. Unfortunately, an insurmountable limitation that prevents a taxonomic reassessment of *P. antarctica* and *P. falklandica* is the impossibility of obtaining sequences from the type specimens. Both specimens consist of a very limited amount of material and the Leiden Rijksherbarium (L), which hosts Kützing’s herbarium, understandably does not allow destructive sampling. New collections from the type localities are therefore essential to clarify their identities.

The alga that we are describing here as *Prasiola glacialis* sp. nov. has been studied in detail by one of us (Broady 1989). Examination of the morphology and distribution of *Prasiola* in freshwater habitats on Ross Island and in Victoria Land and Marie Byrd Land showed a mixture of three different morphotypes (uniserial

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filaments, narrow ribbons, and expanded blades) that were regarded as different developmental forms of *P. calophylla*. The specimens that we sequenced correspond to the expanded blade morphotype and in the molecular phylogenies are more closely related to *P. crispa* than to *P. calophylla* from the Northern Hemisphere (in agreement with their morphology). Specimens of *P. calophylla* for which molecular data are currently available were sequenced by Rindi et al. (2004, 2007). These samples correspond morphologically to the original description (Carmichael in Greville 1826) and were collected from the identical habitats in Ireland as described by Carmichael (“On a block of stone near the Clergyman’s house on the Island of Lismore. October”). The Island of Lismore lies in Lough Linnhe east of Mull in Argyll, Scotland, where the environmental conditions are very similar to the west of Ireland. For this reason, we believe that the Irish specimens are representative of the genuine *P. calophylla* and that the Antarctic specimens should be referred to a different species. In this study it has not been possible to sequence thalli with filamentous and ribbon-like habit as reported by Broady (1989); future investigations might indeed show that *Prasiola calophylla* is the correct name for these forms. Leafy forms are much more similar to *P. crispa* and *P. antarctica* and are very difficult or impossible to separate from these species on a strictly morphological basis. However, in consideration of the fact that they are genetically differentiated from these species and in the analyses they are consistently recovered in a separate clade, we consider justified their attribution to a new species.

*Prasiola glacialis* M. B. J. Moniz, Rindi, Novis, Broady et Guiry, sp. nov.

DESCRIPTION: Thallus formed by monostromatic blades with the habit of large ribbons or leafy, expanded blades up to 5 cm long and wide, bright green in color. In section the blade is 12-24  $\mu\text{m}$  thick (mainly 18-20  $\mu\text{m}$ ), with a slightly irregular surface devoid of of marked corrugations. In surface view the cells are 4-8  $\mu\text{m}$  long and wide. Thickened lines and areolae may be present but they are not well marked and may be completely absent in some parts of the thallus. Reproductive structures not observed. It is similar in habit to *Prasiola crispa* and *P. antarctica*, but it differs from these species in the *rbcL* and *psaB* sequences and the type of habitat (it occurs on surfaces characterized by slowly flowing water, not associated with nutrient enrichment).

ETYMOLOGY: the specific epithet refers to this taxon’s association with ice; this species occurs in small streams or on the surface of ice, soil and gravel irrigated by slow flows of melting ice.

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HOLOTYPE SPECIMEN: GALW015715 (Fig. 4g). Collected by Paul Broady, 2 January 1987.

TYPE LOCALITY: Garwood Valley, McMurdo Dry Valleys, Antarctica. In a small stream fed by melt from an up-slope, ice field; growing intermingled with *Nostoc commune* colonies.

At present it is not possible to indicate clear-cut morphological characters useful for the separation of the three clades of Antarctic *Prasiola*. To some extent, *P. glacialis* may be differentiated from *P. antarctica* and *P. crispa* on ecological grounds. *P. glacialis* was collected from habitats not experiencing nutrient enrichment, whereas the other two species were associated with high levels of organic nitrogen (typically in the form of guano produced by penguin populations). Furthermore the specimens of *P. glacialis* sequenced in this study occurred in conditions of full submersion or contact with liquid water, whereas *P. antarctica* and *P. crispa* were mostly subaerial algae. This distinction, however, is probably of very limited significance; all Antarctic representatives of *Prasiola* live in conditions that can be considered as intermediate between fully aquatic and fully terrestrial and are probably subjected to a fluctuating exposure to water and air.

*Prasiola crispa* and *P. antarctica* (as *P. crispa* subsp. *antarctica*) were separated by Knebel on the basis of vegetative morphology: areolation and thick spaces between cells (absent in *P. crispa*, present in *P. crispa* subsp. *antarctica*) and habit of the blade (smooth in *P. crispa*, thick with rough surface in *P. crispa* subsp. *antarctica*). Our results (see Table 2) indicate that these characters cannot be used to separate the *P. crispa* clade and the *P. antarctica* clade. There is considerable variation between populations and even between different thalli within the same population, especially in *P. crispa*, in which the occurrence of areolae shows inter-population and inter-individual variation. This is particularly evident in a population at a terrestrial site in Galway City, Ireland, which has been studied for many years (Rindi, personal observation). We agree with Kobayasi (1967) that the taxonomic significance of these characters is very limited and that they cannot be used for separation of taxa within *Prasiola*. At present, the only morphological character that we consider potentially useful for the separation of the *P. crispa* and *P. antarctica* clades is the presence of uniseriate filaments, the so-called *Hormidium* stages. It has long been known that *P. crispa* may produce this developmental form (Ettl and Gärtner 1995, Knebel 1936, Kobayasi 1967, Ricker 1987, Rindi et al. 2004) which can be observed also in

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Lightfoot's type specimen in BM (Fig. 5, d, e and f). Uniseriate filaments were present in several samples of the *P. crisper* clade (and in some they were the dominant form) but did not occur in any specimen of the *P. antarctica* clade. The stability of this character will have to be confirmed by examination of additional samples of *P. antarctica*, but it is noteworthy that so far *P. crisper* is the only species of *Prasiola* for which this type of growth can be confirmed.

Further distributional information might also provide valuable insights.

Molecular studies performed in recent years have produced evidence for some groups of organisms (e.g., nematodes, mites, bryophytes) of a major biogeographical discontinuity between the Antarctic Peninsula and the rest of the continent, named by Chown and Convey (2007) the "Gressitt Line". To date specimens belonging to the *P. antarctica* clade have been collected only from the Antarctic Peninsula and islands of Maritime Antarctica, and it would be interesting to establish whether this group occurs in the rest of the continent.

### *Evolution of the Prasiolales*

The results of our study have led to new major insights into the evolution of the Prasiolales. Due to an unusual combination of morphological and ultrastructural characters, the phylogenetic position of this order was uncertain for a long time (e.g. van den Hoek et al. 1995). Molecular data produced in recent years, however, have robustly placed the order in Trebouxiophyceae (Darienkov et al. 2010, Karsten et al. 2005, Sherwood et al. 2000), a green algal class largely composed of terrestrial microchlorophytes (Leliaert et al. 2012). In recent molecular phylogenies Prasiolales form a well-supported clade, the closest relatives of which are all subaerial unicells, such as *Stichococcus*, *Pabia* and *Pseudochlorella* (Darienkov et al. 2010, Karsten et al. 2005, Pröschold and Leliaert 2007). It is therefore reasonable to postulate that in Prasiolales the subaerial lifestyle is the ancestral condition and the presence of these algae in marine and freshwater habitats is the result of a secondary recolonization of aquatic environments. This type of ecological distinction is not always straightforward, as a continuum often exists between fully terrestrial and fully aquatic environments. This is particularly the case in Antarctica, where the presence of liquid water is linked to ice melting, with the result that Antarctic *Prasiola* occurs in habitats that are in fact alternately aquatic and terrestrial. There are, however, other members of Prasiolales that can be regarded as genuinely subaerial organisms, such as *Prasiolopsis ramosa* Vischer, *Rosenvingiella radicans* (Kützing) Rindi, McIvor and Guiry and *Prasiola calophylla*;

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these algae occur in habitats that are constantly exposed to air (e.g. tree bark, bases of urban walls, concrete poles, natural rocks; Kornmann and Sahling 1974, Ettl and Gärtner 1995, Rindi et al. 2004). Conversely, species of *Prasiola* from western North America (*P. mexicana*, *P. nevadensis* Setchell and N.L. Gardner, Setchell and Gardner 1920a, 1920b, Printz 1964) and eastern Asia (e.g. *P. elongata* Hu, *P. japonica* Yatabe, *P. formosana* Okada, *P. tibetica* C.-C. Jao, *P. yunnanica*; Jao 1947, Hu and Wei 2006) are fully freshwater organisms, which only occur completely submerged in permanent streams.

For *Prasiola*, the recolonization of marine environments appears to have taken place only once, as all marine species for which molecular data are available (the North Atlantic *P. stipitata* Suhr ex Jessen and the North Pacific *P. delicata* Setchell and N. L. Gardner, *P. linearis* C.-C. Jao and *P. meridionalis* Setchell and N. L. Gardner) occur together in a single, well-supported clade. This transition appears to be evolutionarily recent, as suggested by the great sequence similarity among marine strains belonging to different species and distributed in widely separate geographical regions (Rindi et al. 2007; the present study). By contrast, our results show that the transition to freshwater habitats has taken place several times independently. Molecular data have been available so far for few strictly freshwater taxa [an *rbcL* sequence for *P. fluviatilis* (Sherwood et al. 2000); 18S rDNA sequences for *P. mexicana* (Sherwood et al. 2000) and *Prasiola* sp. (Naw and Hara 2002)]. This has made it impossible to draw generalizations about their evolution. The new sequences produced here for *P. mexicana* and *P. yunnanica* show that these taxa represent unrelated lineages. The case of *P. yunnanica*, known only from south-eastern China (Hu and Wei 2006, Jao 1947), is particularly interesting. This species represents an individual lineage without close affinities to any other species of *Prasiola*. Nine freshwater taxa of *Prasiola* have been described from eastern Asia (Guiry and Guiry 2011) and it would be particularly interesting to establish whether they all belong to the same lineage as *P. yunnanica*. A major limitation is represented by the logistical difficulty of obtaining samples, as they mostly occur in remote habitats such as high mountain streams.

In conclusion, we demonstrate a high cryptic diversity of the genus *Prasiola* in Antarctica. Further studies on the distribution and ecology of the three cryptic species might lead to further surprising discoveries on these widespread, but poorly understood organisms.

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#### **B4) Species boundaries in marine species of *Prasiola***

##### ***Abstract***

The order Prasiolales has the unique characteristic of having species living in terrestrial, freshwater and marine habitats. Previous molecular work has helped to understand species boundaries and phylogenetic relationships between species but some problems still remain namely the delimitations of some marine species. Collections of Prasiolales were obtained from the east and west North-Atlantic coasts and the west coast of the North-Pacific. Molecular analyses based on *tufA* sequences add to previous datasets to further clarify the delimitations within the marine group. The results support the conspecificity of *Prasiola stipitata* and *Prasiola meridionalis*.

##### ***Key words***

Cryptic diversity, marine algae, molecular phylogeny, *Prasiola*, Prasiolales.

##### ***Introduction***

The order Prasiolales is characteristic in that it is one of the few algal taxa including species from marine, terrestrial and freshwater habitats. In the past decade and a half, many new molecular data have contributed to understanding its phylogenetic relationships (Friedl and O'Kelly 2002, Karsten et al. 2005, Naw and Hara 2002, Rindi et al. 2007, Sherwood et al. 2000, chapter B3). However, the taxonomy of this group is not yet definitively assessed and there are still some unsettled questions concerning the circumscriptions of some species.

One of the main problems is the definition of the boundaries of the marine species of this group. In the case of Prasiolales, the marine species are not strictly aquatic; the habitat occupied by these algae includes the supratidal and upper intertidal zone of rocky shores, with species being completely submerged only for short periods of time (Guiry and Guiry 2012, Rindi 2007, Rindi et al. 2007). The species of *Prasiola* that are considered marine are the North Atlantic *P. stipitata* Suhr ex Jessen, the North Pacific *P. delicata* Setchell and N. L. Gardner, *P. linearis* C.-C. Jao and *P. meridionalis* Setchell and N. L. Gardner, the New Zealand and Australian *Prasiola novaeseelandiae*

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S.Heesch & W.A.Nelson and the probably cosmopolitan *Prasiola borealis* M.Reed. There are three other species (*P. mauritiana* Børgesen, *P. fangschngensis* Luan & Ding and *P. volcanica* Luan & Ding) reported from marine habitats. These will not be considered here since there are no molecular data available and doubts have been cast on their identity (Rindi et al. 2007). The original descriptions of these species (Børgesen 1946, Luan et al. 2009) suggest that these may be misidentifications of species of *Monostroma* or other ulvophycean chlorophytes.

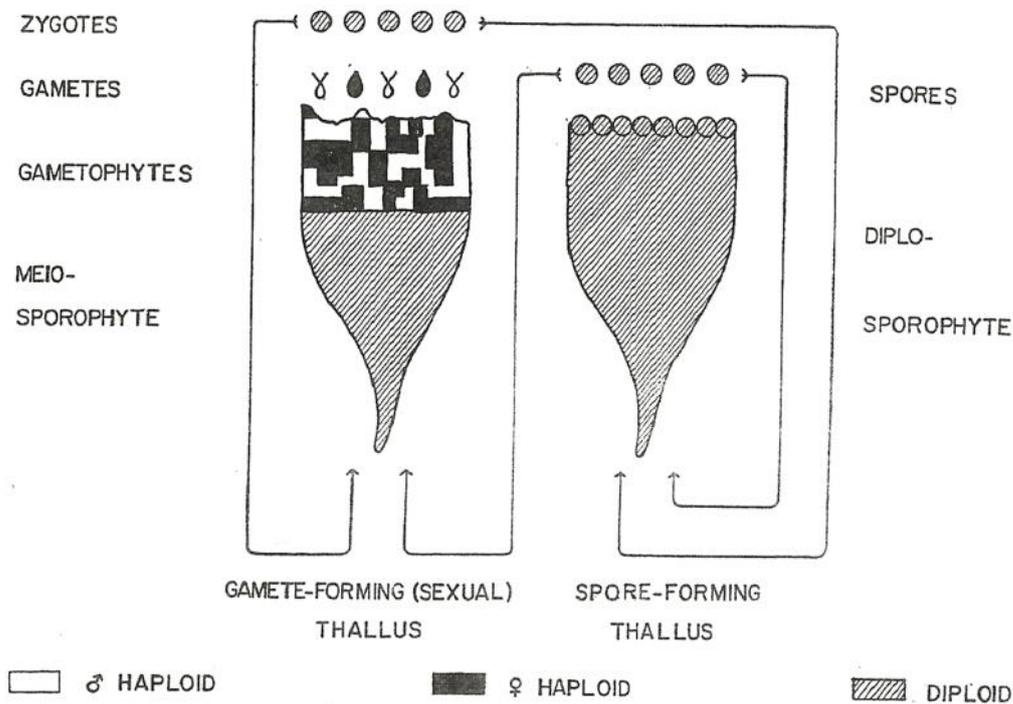
*Prasiola stipitata* and *P. meridionalis* have the largest geographical distribution (Hardy and Guiry 2006). Both species consist of tufts of monostromatic blades (non-reproductive thallus), in a variety of shapes from linear, lanceolate, wedge-shaped, tongue-shaped, fan-shaped, spoon-shaped, ovoid, or irregular (Rindi 2007). They are common species and known to grow on rocks or other hard surfaces, in the supralittoral and upper intertidal zones. These algae form well-developed populations at sites fertilized by seabird guano, where sea birds rest or build their nests. *Prasiola stipitata* was first described in 1848 by Jessen from Sandweick, Schleswig in Germany (Jessen 1848). Its distribution includes the entire Atlantic European coast from Iceland to the North of Spain and the Atlantic North American coast from Newfoundland to North Carolina (Guiry and Guiry 2012). *Prasiola meridionalis* was described by Setchell and Gardner (1920a) using specimens from Neah Bay in Washington State, USA. These authors reported this species as widespread on the U.S. Pacific coast, ranging from Friday Harbour (Washington State) to California. Its known distribution spans from Alaska to central California (Guiry and Guiry 2012). Records from the Atlantic are scanty (e.g. Pedersen 2011 for Greenland) and uncertain, even in relation to the unclear separation between *P. stipitata* and *P. meridionalis*.

The life cycle represents one of the most interesting aspects of the biology of these species and has been substantially studied (Cole and Akintobi 1963, Friedmann 1959, 1963, 1969). Although the morphology of the gamete-producing structures is different in *P. stipitata* and *P. meridionalis*, the life cycle of these species is considered identical. This is either regarded as heteromorphic diplohaplontic or diplontic depending how one interprets it (Van den Hoek et al. 1995).

Two types of thalli are recognized: gamete-bearing and spore-bearing. Both gamete-bearing and spore-bearing thalli are diploid. Vegetative meiosis takes place in

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the upper edge of the gamete-bearing thallus (Fig.1), with consequential rounds of mitosis in the haploid tissue previously produced (Friedmann 1959, Rindi 2007, van den Hoek et al. 1995). Half of this haploid tissue will mature to cells which produce spermatozooids and the other half will mature into cells which produce eggs (Fig.1). The zygote resulting from fertilization is uniflagellate and can swim until it attaches to a stable substratum. The zygote germinates into a new thallus. If one considers the haploid gametangial parts a gametophytic generation, then the life cycle is heteromorphic and diplohaplontic. If, on the other hand, one considers the gamete-producing cells as gametangia, the life cycle is diplontic (Rindi 2007).



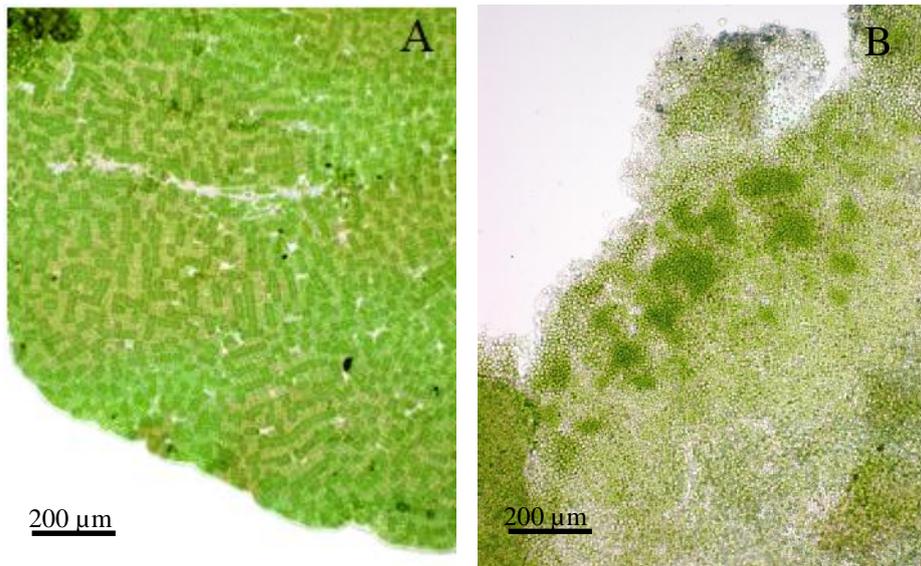
**Fig.1** Diagram of the life history of *Prasiola stipitata* adapted from Friedmann (1959).

Both of these cases are infrequent in green algae, with diplohaplontic life cycles occurring in Ulvophyceae, Cladophorophyceae and Trentepohliophyceae as well as “plants” Bryophyta and Tracheophyta, and diplontic life cycle cases not known (Van den Hoek et al. 1995).

These two species were considered possibly conspecific first by Bravo (1965) and other studies (Rindi et al. 2007 and chapter B3) have shown genetic evidence for this. So far, however, there are still doubts that have led to the maintenance of two separate species. In *P. stipitata* the mature gametangial parts have a typical patchwork habit, with dark green parts producing female gametes and light green parts producing

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male gametes (Fig.2A). This has never been observed in *P. meridionalis*, where the patches are irregular (Fig.2B) and similar to the sporangial parts in *P. stipitata*. No intermediate forms have so far been found even in a large range of conditions (Rindi et al. 2007). The biogeographic evidence available also indicates a sharp separation in the northern hemisphere, with *P. stipitata* confined to the Atlantic Ocean and *P. meridionalis* to the Pacific Ocean (Guiry and Guiry 2012).



**Fig.2** Comparison of morphology between gamete-bearing thallus of *Prasiola stipitata* (A) and of *Prasiola meridionalis* (B).

*Prasiola linearis*, although described from grasses in the upper littoral shore, has an identical *rbcL* sequence to *P. stipitata* and *P. meridionalis* (Rindi et al. 2007), but other differences separate it from these two species. Culture studies conducted on *P. linearis* revealed an entirely asexual life cycle consisting of a repetition of the same morphological phase reproducing by aplanospores (Rindi 2010). It also has a characteristic and fairly constant linear shape of the blade not found in other marine species and its fronds can grow to larger sizes (10–15 cm tall). No forms of this species similar *P. stipitata* or *P. meridionalis* have been observed in a range of culture conditions (Rindi 2010).

The other two known marine species, *Prasiola furfuracea* and *P. delicata*, share a similar morphology. They are small, rounded, wedge-shaped or spoon-shaped blades, supported by a short stipe (Kornmann and Sahling 1974, Setchell and Gardner 1920b,

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Yamada and Kurogi 1974). They also reproduce solely asexually (Kornmann and Sahling 1974, Yamada and Kurogi 1974). Although, using *rbcL* phylogenies, the divergence of *P. delicata* from the rest of the marine species is quite low (<0.8%), this divergence is still higher than the divergence between the rest of the marine species (Rindi et al. 2007).

Since only a low number of samples and only one (Rindi et al. 2007) or maximum two markers (Chapter B3) have been used and life cycle and morphological information do not support joining all these species into one, no synonymization has been suggested yet. In this study, another marker was used to further investigate this problem. The plastid gene encoding the elongation factor TU (*tufA*) has been used in green algae at least since 2002 (Famá et al. 2002), when it was used to resolve the molecular phylogeny of some *Caulerpa* spp. In the mean time, it was used for inferring phylogenies or for species identification in green algal taxa as varied as *Halimeda* (Verbruggen et al. 2006, 2007), *Pseudomuriella* (Fučíková et al. 2011b), *Acrochaete* (Rinkel et al. 2012) and several coccoid green algae (Fučíková et al. 2011a). In 2010, two independent studies, after evaluating various commonly used markers, agreed that *tufA* in conjunction with another marker, namely *rbcL* was a good candidate for the DNA barcoding in freshwater and marine green algae, with the exception of the charophytes (Hall et al. 2010, Saunders and Kucera 2010). In the latter taxon, there have been some examples where the gene migrated to the nucleus while still preserving a copy in the chloroplast, which leads to problems of paralogy, pseudogenes and other bias (Baldauf et al. 1990). In the chlorophytes, this gene has the highest universality, the lowest contamination rate and the highest sequence quality, followed closely by the *rbcL* gene. In most cases the “barcode gap”, i.e. the separation between the number intraspecific bp differences and heterospecific was also higher than *rbcL* (Saunders and Kucera 2010). These features make this gene a good candidate to further shed light in the separation of the marine *Prasiola* species.

### **Materials and Methods**

#### *Collections and morphological studies*

Collections of Prasiolales were either made by the authors or obtained from collaborators (details in Table 1). The specimens examined and newly sequenced in this

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study were primarily from the Maritime Provinces in Canada and North Europe, but additional specimens from other geographical regions (already sequenced for the *rbcL* and *psaB* genes previously, see chapters B2 and B3) were included. The specimens were placed in sealed containers and conserved mostly as silica-dried or frozen material. Microscopic examination was performed either on fresh or rehydrated material. Voucher specimens were deposited in the Phycological Herbarium of the National University of Ireland, Galway (GALW).

### *Molecular studies*

Overall, 30 new *tufA* sequences were generated in this study. Extraction of total genomic DNA was carried out using the protocol from Saunders (1993) with modifications (Saunders 2008). Primers and PCR protocol were as described in Saunders and Kucera (2010). A 96 well plate was sent to Nanuq (McGill University and Genome Quebec) where cleaning of PCR products and sequencing were conducted using their standard procedures. Sequencing was performed using the Sanger method.

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**Table 1.** Details of collections examined in the study.

<i>Species</i>	Sample	Collection information	Herbarium number	<i>tufA</i> GenBank accession number	<i>rbcL</i> GenBank accession number	<i>PsaB</i> GenBank accession number
<i>Prasiola antarctica</i>	P30	Area behind Palmer Station, Antarctica (64° 46.450' S; 64° 02.998' W). Charles D. Amsler, 17 March 2010.	GALW15711	To be deposited	JQ669720	JQ669703
<i>P. antarctica</i>	P31	Palmer Station, Antarctica, background (64° 46.492' S; 64° 02.924' W). Charles D. Amsler, 4 April 2010.	GALW15712	To be deposited	JQ669712	-
<i>P. borealis</i>	F13	Porlier Pass, Galiano Island, BC, Canada; Louis Hanic, 30 <sup>th</sup> August 2004.	GALW15437	To be deposited	-	-
<i>P. borealis</i>	P4	Fossil Island, Pirates Bay, Tasmania, Australia. Michael Guiry, 31 October 2009. Upper shore, on rocks in lichen zone. Details in chapter B2.	GALW15588	To be deposited	JF949724	JQ669689
<i>P. borealis</i>	P33	Helby Island, Barkley Sound, BC, Canada; Michael Guiry, 3 May 2004.	GALW15301	To be deposited	-	-
<i>P. borealis</i>	P62	Rowing Marma, Galway, Ireland; Michael Guiry, 6 June 2011. On rocks just above the water mark.	GALW15790	To be deposited	-	-
<i>P. borealis</i>	P57	Deep Harbor; Sandra C. Lindstrom, 25 May 2009.	GALW15791	To be deposited	-	-
<i>P. borealis</i>	F11	Seldovia Point, Alaska, USA; Susan M. Saupe, 1 July 2003.	GALW15792	To be deposited	-	-
<i>P. calophylla</i>	P41	Salthill, Galway, Ireland. Fabio Rindi, September 2010. Forming dark green patch at the base of lamp pole on road sideway.	GALW015716	To be deposited	JQ669726	JQ669706
<i>P. calophylla</i>	P61	Corrib river, Galway, Ireland; Michael Guiry, 6 June 2011. On rocks just above the water mark.	GALW15793	To be deposited	-	-
<i>P. cf. delicata</i>	P56	Pearse Island, BC, Canada; Sandra C. Lindstrom, 24 May 2009.	GALW15795	To be deposited	-	-
<i>P. crispa</i>	P43	King George Island, South Shetland Islands, Antarctic Sea. Han-Gu Choi, 17	GALW15723	To be deposited	JQ669723	JQ669685

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		January 2010. Penguin nesting area. CH2227.				
<i>P. crispa</i>	P65	Gothenburg, Sweden; Michael Guiry, 2 June 2012. Soil near Plaza Elite Hotel.	GALW15796	To be deposited	-	-
<i>P. novaezelandiae</i>	P2	Marjoribanks Street, Wellington, New Zealand; Michael Guiry, 3 November 2009.	GALW15797	To be deposited	To be deposited	To be deposited
<i>Prasiola</i> sp.	P10	Bodega Marina Lab, California, USA; Sandra C. Lindstrom, 16 February 2010, 38° 19102N 123°04416E.	GALW15726	To be deposited	To be deposited	To be deposited
<i>P. stipitata</i>	F26	Reykjavik; Iceland; Stefan Kraan, December 2003.	GALW15798	To be deposited	-	-
<i>P. stipitata</i>	P26	Plymouth, England, U.K. Fabio Rindi, 2 February 2010. Upper intertidal zone; rocks on shore in front of National Aquarium.	GALW15729	To be deposited	Identical to JQ669729	JQ669697
<i>P. stipitata</i>	P40	Claddagh, Galway, Ireland; Fabio Rindi, 20 May 2010. Forming green patches on rock at the high water mark.	GALW15730	To be deposited	Identical to JQ669729	JQ669693
<i>P. stipitata</i>	P54	Finnøy, Norway; Fabio Rindi, 27 July 2004. On rock at high water mark.	GALW15337	To be deposited		-
<i>P. stipitata</i>	P55	Duggerna rock, Kilkee, Co, Clare, Ireland; Mónica Moniz and Fabio Rindi, 2 March 2010.	GALW15799	To be deposited	-	-
<i>P. stipitata</i> subsp. <i>meridionalis</i>	F12	Mouth of Amalik Bay, Katmai National Park, Alaska, USA; Sandra C. Lindstrom, 13 June 2003.	GALW15800	To be deposited	-	-
<i>P. stipitata</i> subsp. <i>meridionalis</i>	F21	Newport, Oregon, USA; Fabio Rindi, 15 July 2003, jetty under bridge.	GALW14422	To be deposited	-	-
<i>P. stipitata</i> subsp. <i>meridionalis</i>	F30	Friday Harbour, Washington State, USA; Michael Guiry, 29 April 2004.	GALW15803	To be deposited	-	-
<i>P. stipitata</i> subsp. <i>meridionalis</i>	F31	False Creek Yacht Club, Vancouver, BC, Canada; Michael Guiry, 22 April 2004.	GALW15303	To be deposited	-	-

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<i>P. stipitata</i> subsp. <i>meridionalis</i>	P7	Deadman Bay, Washington state, USA; Sandra C. Lindstrom, 11 December 2009.	GALW15727	To be deposited	Identical to JQ669729	JQ669691
<i>P. stipitata</i> subsp. <i>meridionalis</i>	P8	Humbolt Bay, California, USA; Sandra C. Lindstrom, 15 February 2010, Seaward end of North jetty; north side, top of supratidal stanchion, 40° 46'14.8"N 124° 14'35.9"E.	GALW15801	To be deposited	-	-
<i>P. yunnanica</i>	P16	Qing Bi Creek, Chanshan Mountains, near Dali, China. Stefano Draisma. Stream at 2600 m a.s.l. Sample Stefano Draisma 0912008.	GALW15731	To be deposited	JQ669708	JQ669686
<i>Rosenvingiella constricta</i>	F01	Porlier Pass, Galiano Island, BC, Canada; Louis Hanic.	GALW15433	To be deposited	-	-
<i>R. radicans</i>	F24	Ona, Norway; Fabio Rindi, June 2004.	GALW15802	To be deposited	-	-
<i>R. radicans</i>	F25	Reykjavik; Iceland; Stefan Kraan, 1 December 2003; base of urban wall.	GALW15260	To be deposited	-	-

*Sequence alignment and phylogenetic analyses*

Electropherograms were inspected with BioEdit version 7.0.5.3. All sequences included in the alignments were based on high-quality bidirectional readings. Besides the new sequences produced in this study, all *tufA* sequences of Prasiolales available in GenBank database were included in the alignments. The *tufA* alignment consisted of 30 sequences for which 752 bp could be aligned unambiguously. Alignment was performed using ClustalW (Thompson et al. 1994) as implemented in BioEdit and refined by eye. Since the resulting tree agreed with previous data, it was also decided to combine the alignment with *rbcL* and *psaB* alignments obtained in the previous chapter in a concatenated dataset, which included 13 taxa. All samples were successfully amplified for the three genes with the exception of the outgroup *Stichococcus* which was amplified for only *psaB* and *rbcL*, *P. antarctica* P30 which was only amplified for *tufA* and *rbcL* and *P. antarctica* P31 only amplified for *rbcL* and *psaB*. The concatenated alignment was 3225 bp long.

Phylogenetic trees were outgroup-rooted following the recommendations of Verbruggen and Theriot (2008) after some preliminary analyses in which different taxa were tested as possible suitable outgroups. The outgroup taxa used for the *tufA* tree were *Parachlorella kessleri* FJ968741.1 and *Chlorella vulgaris* AB0011684, and the outgroup taxa used for the concatenated tree were *Chlorella vulgaris* (complete genome NC001865) and *Stichococcus* sp. (*rbcL* sequence EF589147 and *psaB* sequence GQ423929).

Phylogenetic analyses were performed using Maximum Likelihood (ML) in Treefinder version March 2011 (Jobb 2011) and PhyML (Guindon and Gascuel 2003) in Seaview (Galtier et al. 1996); Bayesian analyses (BI) were performed using MrBayes 3.04 (Huelsenbeck and Ronquist 2001). The ML Treefinder and BI analyses were performed on partitioned datasets, with three partitions corresponding to the first, second and third codon positions of the genes. Verbruggen (2010) recommended this strategy for the analysis of protein-coding plastid genes (such as *tufA*). The parameters for the PhyML ML and BI analyses were chosen after selection of the appropriate evolutionary model with jModelTest (Posada and Crandall 1998) under the Akaike Information Criterion.

For the Treefinder ML analysis, the models selected by Treefinder under the corrected Akaike Information Criterion (AICc) were applied. For the PhyML and ML Treefinder analyses, nodal support was assessed by non-parametric bootstrap analysis with 1000 resamplings. The BI analysis was performed using the priors set as default in MrBayes; the parameters were unlinked and allowed to vary across partitions. Four Monte Carlo Markov chains were run for  $2 \times 10^6$  generations, with tree sampling every 1000 generations. It was assumed that convergence of the two runs was reached when the average standard deviation of split frequencies between the two runs was lower than 0.01; this was further verified using the sump command and plotting the likelihood scores versus the number of generations. The first 100 samples were discarded as burn-in and the remainder trees were used to build the 50% majority-rule trees.

## **Results**

### *Interspecific divergences and molecular phylogeny*

The gene *tufA*, as with other genes, showed no genetic separation between the Atlantic *P. stipitata* and Pacific *P. meridionalis* (Table 2). However, it showed a higher divergence than *rbcL* and *psaB* (where information is available) between the clade *P. stipitata* / *P. meridionalis* and *P. delicata*, *P. borealis* and sample P10.

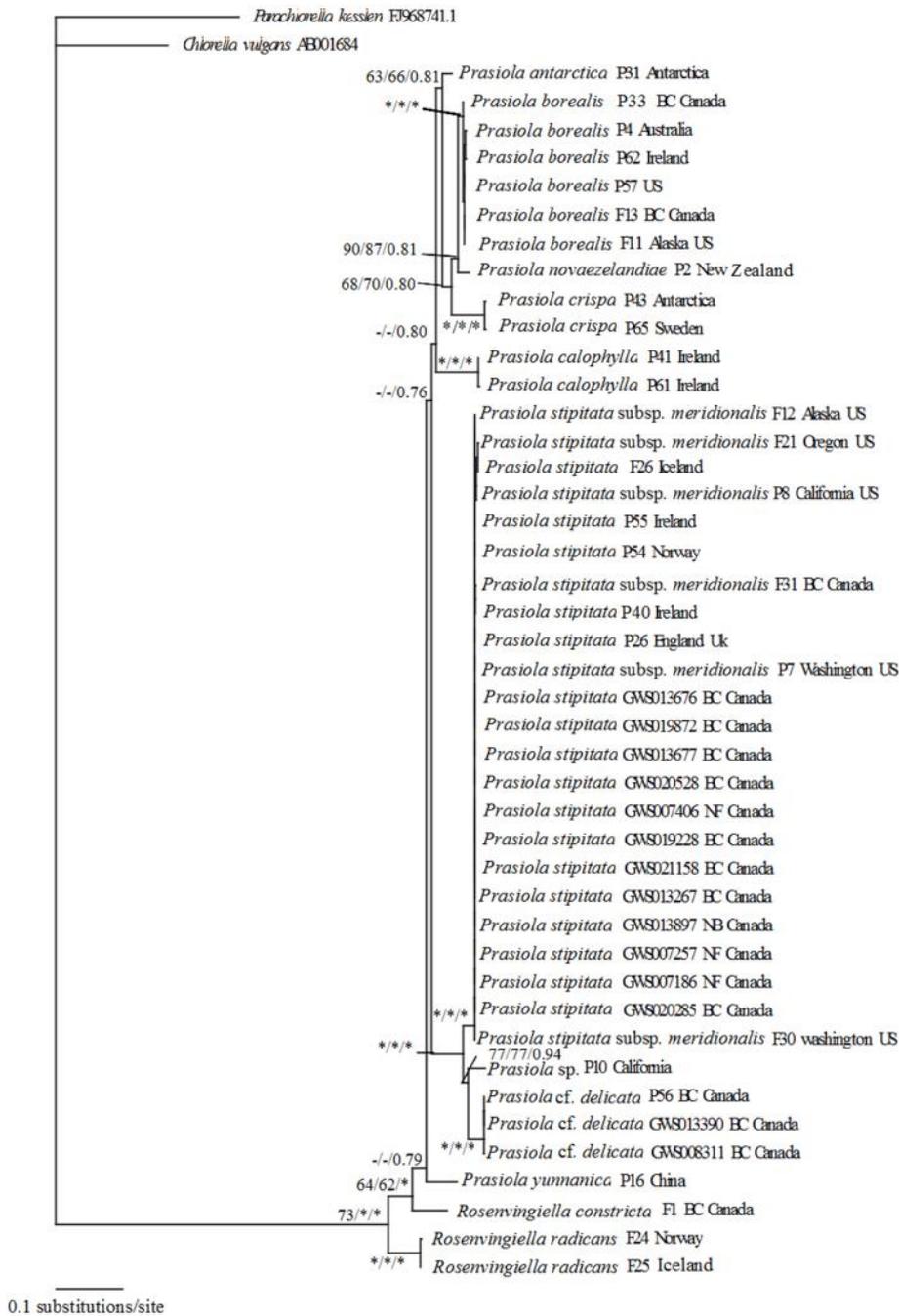
The *tufA* tree (Fig.2) topology agreed with previous studies based on *rbcL* and *psaB* (see chapter B2 and B3, Rindi et al. 2007). Furthermore, as with previous datasets (see chapter B2 and B3, Rindi et al. 2007), statistical support varied among lineages and was generally higher for the terminal nodes than for the internal nodes (Fig.2). The concatenated tree, although with fewer samples, had higher support in the internal nodes (Fig.3).

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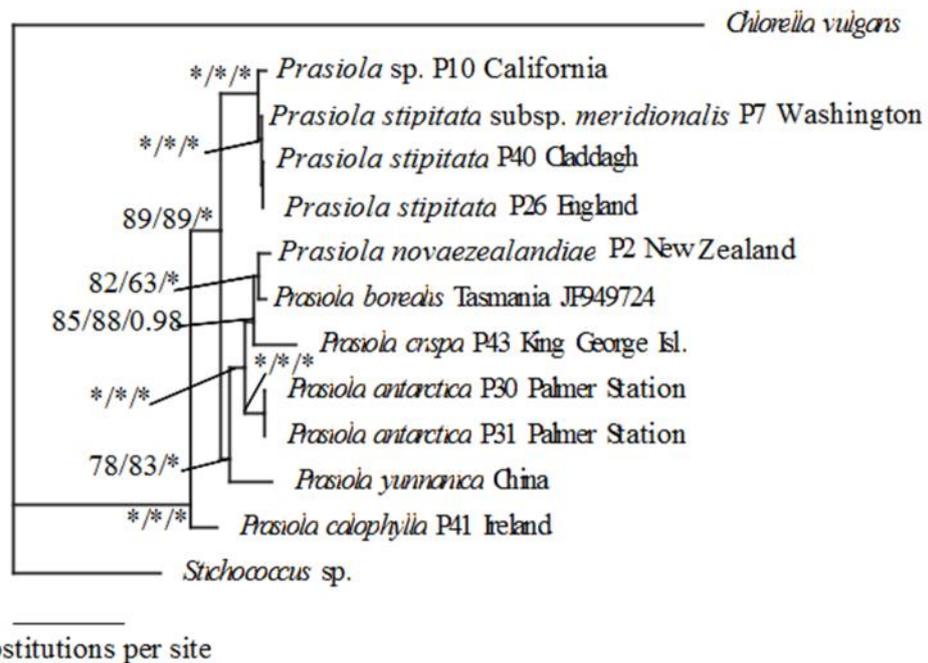
**Table 2.** Percentage of bp differences between marine *Prasiola* species under discussion for the three markers. Top row *rbcL* (879bp), mid row *psaB* (1368bp) and bottom row *tufA* (379bp). The lengths of the sequences were chosen to minimize the amount of missing data. After each gene, the number of samples representing each species is shown. N.A. = not applicable; N.I. = no information available.

	<i>P. stipitata</i>	<i>P. stipitata</i> subsp. <i>meridionalis</i>	<i>P. delicata</i>	<i>P. linearis</i>	<i>P. borealis</i>	P10
<i>P. stipitata</i>						
<i>rbcL</i> (9)	0-0.01					
<i>psaB</i> (6)	0-0.01					
<i>tufA</i> (17)	0					
<i>P. stipitata</i>						
subsp	0	0				
<i>meridionalis</i>	0-0.01	0-0.01				
<i>rbcL</i> (5)	0	0				
<i>psaB</i> (3)						
<i>tufA</i> (6)						
<i>P. delicata</i>						
<i>rbcL</i> (1)	0.01-0.02	0.01-0.02	N.A.			
<i>psaB</i> (0)	N.I.	N.I.	N.I.			
<i>tufA</i> (3)	0.05	0.05	0			
<i>P. linearis</i>						
<i>rbcL</i> (2)	0-0.03	0-0.03	0.01-0.04	0-0.03		
<i>psaB</i> (0)	N.I.	N.I.	N.I.	N.I.		
<i>tufA</i> (0)	N.I.	N.I.	N.I.	N.I.		
<i>P. borealis</i>						
<i>rbcL</i> (1)	0.04	0.04	0.04	0.04-0.07	N.A.	
<i>psaB</i> (0)	N.I.	N.I.	N.I.	N.I.	N.I.	
<i>tufA</i> (4)	0.08-0.10	0.08-0.10	0.08-0.10	N.I.	0-0.02	
Sample P10						
<i>rbcL</i> (1)	0	0	0.01	0	0-0.04	N.A.
<i>psaB</i> (1)	0-0.01	0	N.I.	N.I.	N.I.	N.A.
<i>tufA</i> (1)	0.04	0.04-0.05	0.04	N.I.	0.08-0.09	N.A.

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**Fig 3.** Phylogram inferred from ML analysis of the *tufA* gene in *Prasiola* and closely related taxa (obtained with Treefinder). From left to right numbers marked at the nodes indicate PhyML bootstrap, ML bootstrap (Treefinder) and Bayesian posterior probabilities. Models selected by Treefinder: J2 for first codon positions, J1 for second codon positions, HKY for third codon positions. Settings applied for BI: nst=6 for all partitions, rates set to gamma for all codon positions. Asterisks indicate BP values  $\geq 90\%$  and PP  $\geq 0.95$ . BP values lower than 60% and PP lower than 0.7 are not shown.



**Fig. 4.** Phylogram inferred from ML analysis of a concatenated dataset *rbcL-psaB-tufA* in *Prasiola* and closely related taxa (obtained in Treefinder). From left to right numbers marked at the nodes indicate PhyML bootstrap, ML bootstrap (Treefinder) and Bayesian posterior probabilities. Models selected by Treefinder: TN for first codon positions, J2 for second codon positions, J1 for third codon positions. Settings applied for BI: nst=6 for all partitions, rates set to gamma for all codon positions. Asterisks indicate BP values  $\geq 90\%$  and PP  $\geq 0.95$ . BP values lower than 60% and PP lower than 0.7 are not shown.

Marine *Prasiola* species occurred in two clades. One was subdivided into the subclade *P. novaezealandiae* and the subclade *P. borealis*. The other is divided into three subclades: a) the clade formed by samples GWS008311, GWS013390 and P56 which we provisionally consider *P. cf. delicata*; b) a clade with only one sample from California, P10; c) a *P. stipitata* / *P. stipitata* subsp. *meridionalis* clade which, with 22 samples, was the best represented in this study (Table 1, Fig.3). *Prasiola novaezealandiae* had a sister relationship to the *P. cf. borealis* clade with good support in the *tufA* tree (Fig.3) but lower in the concatenated tree for ML (Fig.4). The group formed by these species was sister to *P. crista* albeit with lower support in the *tufA* tree (Fig.3) but higher in the concatenated tree (Fig.4). *Prasiola antarctica* was sister to the clade formed by the previous species with low support in the *tufA* tree (Fig.3) but with high support in the concatenated tree (Fig.4). *Prasiola calophylla*'s and *P. yunnanica*'s positions were not resolved,

with the exception of the Bayesian analysis, in the *tufA* tree (Fig.3). However they came resolved in the concatenated tree analysis (Fig.3): *Prasiola yunannica* as sister to the clade formed by *P. antarctica*, *P. crispa* and *P. novaezealandiae* / *P. borealis*, and *P. calophylla* as sister to all other *Prasiola* species. The genus *Rosenvingiella* was not monophyletic in the *tufA* tree (Fig.3) and was not represented in the concatenated tree (Fig.4).

## ***Discussion***

### *Taxonomy*

The most important outcome of this study is that the three genes used do not separate the Atlantic *P. stipitata* and the Pacific *P. meridionalis* (Table 2, Fig.3 and 4). In fact, even the more variable *tufA*, which shows higher divergence among other marine green species, shows zero bp differences among samples referred to these two species. In another example of a well sampled species within this genus, *P. crispa*, similar pairwise differences were found between samples from Europe, British Columbia in Canada and Antarctica for the *rbcL* gene (Chapter B3) and for European and Antarctic samples for the *tufA* gene (this study). These results agree with previous studies (Rindi et al. 2007, Chapter B3). One of the samples (F30) used here was also collected in the type locality of *P. meridionalis* which adds further support to this conclusion (Table 1). It is therefore proposed to consider *P. meridionalis* and *P. stipitata* conspecific; since the latter has nomenclatural priority (1848), *P. meridionalis* is reduced to subspecific taxon of *P. stipitata*. The fact that they share a similar life cycle further grants evidence to this result (Rindi 2007 and references within). A new combination follows:

*Prasiola stipitata* subsp. *Meridionalis* (Setchell & N.L. Gardner) comb. nov.

BASIONYM: *Prasiola meridionalis* Setchell & N.L. Gardner, *Univ. Calif. Publ. Bot.*, vol. 7: 291, pl. 25: fig. 2, 1920

## Part B: Prasiolales

SYNTYPE LOCALITIES: Neah Bay, Washington, U.S.A. Holotype: N.L. Gardner 3824; UC.

SPECIES DESCRIPTION: Non-reproductive thallus formed by monostromatic blades with spoon-shaped, ovoid, wedge-shaped or irregular habit. Blades up to 1 cm long and 5 mm wide, attached to the substratum by a multicellular holdfast and consisting of a pluriseriate stipe of variable length, 300  $\mu\text{m}$  wide, expanding into an expanded blade. Tissue portions producing spores can be up to 4 cell layers thick and portions producing gametes up to 8 cells thick; cells in surface view are square or rectangular, 5-12  $\mu\text{m}$  wide and long, usually arranged in regular transverse and longitudinal rows. Reproductive tissue shows variegated irregular pattern. Aplanospores are spherical and their diameter varies between 10 and 20  $\mu\text{m}$ . Sexual reproduction by biflagellate male gametes and non-flagellate female gametes produced following somatic meiosis in packets in different areas of the same diploid blade; Asexual reproduction by aplanospores produced by spore-bearing blades.

DISTRIBUTION AND ECOLOGY: This is a subspecies that lives in the supralittoral and upper intertidal zones, particularly abundant on rocks covered by bird guano, concrete or boulders. Guano is its major source of organic nitrogen. It occurs in the eastern Pacific, its distribution extends from Alaska to central California.

The clade that we indicate here as *P. cf. delicata* is separated from the *P. stipitata* clade with high support (Fig.2). The samples forming this clade were sequenced by other authors and we did not have the possibility to examine their morphology. The position of this clade, however, corresponds to a clade recovered in *rbcL* phylogenies in which a sample of *P. delicata* from British Columbia (identified by Rindi et al. 2007) was included. All these samples were collected from British Columbia and *P. delicata* was originally described from Sitka, Alaska. New collections from this locality would be useful to confirm the attribution of this clade to this species.

The identity of the sample P10 (from Bodega, California) is problematic. This is somewhat puzzling because its *rbcL* and *psaB* sequences are identical to

all *P. stipitata* and *P. stipitata* subsp. *meridionalis* samples (as seen in Chapter B3), whereas in the *tufA* phylogeny it falls separately (Fig. 3 and 4). It seems sister to *P. delicata* in the *tufA* phylogeny albeit with a bootstrap that can be improved with more sampling. Also, there are no morphological characteristics that justify its separation except maybe for the stipe being only visible in young blades and sometimes being uniseriate (data not shown). More sampling is required to further understand whether this is a haplotype of one of the marine species and maybe a group in the process of speciation.

The clade including the samples P4 and P33 is referred to as *P. borealis*, since these samples were previously identified as *P. borealis* (Rindi et al. 2007, Discussion chapter B2). As discussed in chapter B2, sequences of samples from the type locality (Unalaska or Kodiak Islands in Alaska) (since there is no type specimen) are not yet available. This species was described to accommodate lichenized forms of *Prasiola*. The samples used in this study were not associated with lichens, with the exception of P4 as described before. However, it seems this species is present commonly in the lichen and free form (chapter B2). If this classification is correct, this study shows a much wider geographical distribution than previously thought including Ireland, British Columbia, Alaska and Canada. Our record from Tasmania and almost identical *rbcL* sequences produced by Pérez-Ortega et al. (2010) for material from Tierra del Fuego and Tasmania suggest that this alga is also widespread in the southern hemisphere.

One last note is the addition of a freshwater specimen of *P. calophylla* P61 from the upper water mark of a river (Fig.3 and Table 1). This confirms that this species, originally described from a strictly subaerial habitat (a block of stone near the Clergyman's house on the Island of Lismore, Scotland; Greville 1826), grows also in habitats that can be considered freshwater.

#### *Evolution of Prasiolales*

The addition of a new marker has produced a more resolved phylogeny than previous studies. Future studies should expand the taxon sampling in the concatenated *psaB-rbcL-tufA* dataset; it is clear, however, that the combination of

these genes produces a substantial increase in the nodal support, even in the internal nodes (Fig.4). It is now clear that marine species appear in two well resolved, separate clades. One is exclusively marine with *P. stipitata* subspp. (and presumably *P. linearis*, which could not be sequenced in this study); the other includes *P. borealis* and *P. novaezelandiae*, and is more closely related to terrestrial species, such as *P. antarctica* and *P. crispa*. The information currently available tends to support the idea that distribution in terrestrial habitats represents the ancestral condition in the Prasiolales (Dariencko et al. 2010, Chapter B3). Therefore it seems that in their evolutionary history, Prasiolales have conquered the upper littoral habitats, at least, twice. Once, giving rise to *P. stipitata* subspp. (Fig.3 and Fig.4) and probably *P. delicata* (Fig.3) and the other giving rise to *P. borealis* and *P. novaezelandiae* (Fig.3 and Fig.4).

Based on the information available, the species *P. calophylla* seems to be the earliest-diverging *Prasiola*, which adds evidence that the first forms of Prasiolales were terrestrial and with preference for freshwater habitats. The position of another freshwater species, *Prasiola yunnanica*, is better resolved in this study than before (Chapter B3). It seems to be sister to the clade that includes the terrestrial *P. crispa* and *P. antarctica* and the marine *P. borealis* and *P. novaezelandiae*. This clade should also include the freshwater *P. mexicana* (see chapter B3).

#### *Considerations for DNA barcoding*

This study also adds one more example of gene comparison for green algal barcodes. The results are in accordance with Saunders and Kucera (2010) and Hall et al. (2010) showing that *tufA* is at least as reliable and as universal as commonly used *rbcL* and with a bigger “barcode gap”. Of the 42 samples that were extracted and sequenced, 11 did not amplify and six had both trace files showing slightly lower quality, although still possible to use. These last sequences were shorter and defined the length of the alignment. In our experience, *rbcL* sequencing within this group does not pose so many sequencing problems but since this extraction, amplification and sequencing was performed as a 96 plate bulk, it might pose more problems. When Saunders and Kucera (2010) compared *tufA* and *rbcL* using

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the same methodology, *tufA* performed better. *PsaB* showed the same resolving power as *rbcL* but it requires two sets of primers to amplify the whole gene, which increases the difficulty. For some samples, only one strand could be amplified successfully.

In this study, *tufA* confirmed previous studies done on Prasiolales and further revealed possible hidden cryptic diversity (the case of P10). The only disadvantage, when compared to *rbcL*, is that this is the first large dataset of sequences that will be available in GenBank, which is lower than the large number of sequences available for *rbcL*. Of course, more sequencing efforts will resolve this in the future. Therefore *tufA* shows good potential as a barcode for this genus.

In conclusion, this study has helped clarify the delimitation of the species *Prasiola stipitata* and the relationships within Prasiolales. It also draws attention to potential hidden diversity as exemplified by sample P10. Furthermore, it is one more example of *tufA* usage as a good chlorophytes barcode.

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## Part C: Seaweeds and Sponges

### C1) Introduction

Co-existing in a multitude of marine and freshwater habitats, sponges and algae interact in many different ways and may form associations with mutual benefits, which in some cases develop to the extent of full symbioses (Trautman and Hinde 2002, Wilkinson 1992). Symbioses between algae and sponges may result in very effective partnerships, of much higher ecological success than the separate free-living organisms (Carballo and Avila 2004). When the algal partner involved is a benthic macroalga, the benefit for the sponge is usually mechanical support, as the thallus of the alga acts as a substitute for skeletal fiber (Avila et al. 2007, Calcinaï et al. 2006, Carballo et al. 2006, Enriquez et al. 2007, Rützler 1990), or physical protection (Mercurio et al. 2006). In other cases the interaction between alga and sponge proceeds to the level of a deep metabolic integration, e.g. the red alga *Ceratodictyon spongiosum*, which is constantly associated with *Haliclona cymiformis* (Trautman et al. 2000, Trautman and Hinde 2002). The alga gets most of its nitrogen requirements directly from the sponge and contributes marginally to the sponge trophic supply (Davy et al. 2002). Recent evidence indicates also that some species of macroalgae that occur as epibionts on sponges may contribute to their dispersal (Avila et al. 2012).

Despite the great ecological significance of positive interactions, neutral or negative interactions are a more common case (Cebrian and Uriz 2006, Konar and Iken 2005, Lopez-Victoria et al. 2006). Some species of seaweeds may settle on sponges and grow as epibionts on them. For the epibiont, fouling can be an advantageous conquest of a new position for colonization, ensuring a hydrodynamically favourable situation, which can bring a higher nutrient supply and a more efficient removal of wastes (Trautman and Hinde 2002, Wahl 1989). A more exposed habitat also ensures access to higher irradiance, essential for photosynthesis. On the other hand, epibiotic algae may suffer from potentially toxic exudates from the basibiont or get dislodged due to normal mortality, physical disturbance and predation or by shrinkage or growth of the sponge

(Trautman and Hinde 2002, Wahl 1989). There are a number of possible disadvantages for the sponge, including competition for organic nutrients, impediment of uptake and excretion, indirect damage by grazers (Konar and Iken 2005, Lopez-Victoria et al. 2006, Wahl 1989), and increased brittleness and therefore breakage in high turbulence environments.

Past studies have suggested that certain sponges and seaweeds are more likely to establish associations than others. Among algae, species of *Acrochaetium* (Acrochaetiales; Boney and White 1967, Dawson 1953), *Ptilophora* (Gelidiales; Tronchin et al. 2006) and *Jania* (Corallinales; Carballo and Avila 2004, Enriquez et al. 2009, Rützler 1990,) show a frequent tendency to establish associations with sponges, whereas species of *Ceratodictyon*, *Codiophyllum* and *Thamnoclonium* (Halymeniales) are only known in alga-sponge associations (Huisman et al. 2011, Scott et al. 1984). Among sponges, most of the intimate associations known have been described from the order Haplosclerida (Demospongiae) and more than 50% of the associations described in the order involve species of the family Chalinidae (Avila et al. 2007).

Despite the numerous studies concerning interactions among individual algae and sponges, detailed information about the whole algal assemblage associated with a certain sponge species is virtually non-existent. The information available in this regard is scanty and based on low taxonomic resolution (e.g. Davis and White 1994). Given the widespread co-occurrence of sponges and seaweeds in littoral habitats, such lack of published information is surprising. This is possibly due to the fact that, at a visual inspection, many species of sponges appear free of epibionts. It is well known that sponges produce many secondary metabolites with biological functions (Blunt et al. 2011, Dobretsov et al. 2005, Taylor et al. 2007,), including defence against epibionts and competitors; so it is probably assumed that sponges do not host a diverse assemblage of epibionts. Conversely, a few investigations are available on the diversity of sponge assemblages associated with individual seaweed species (e.g. Avila et al. 2009, Tronchin et al. 2006).

With this project, we wanted to contribute to the understanding of what

## Part C: Seaweeds and Sponges

types of interactions exist between sponges and algae in the Northern hemisphere, by starting with the west coast of Ireland.



**C2) Composition and temporal variation of the algal assemblage associated with the haplosclerid sponge *Haliclona indistincta* (Bowerbank)**

***Abstract***

Although interactions between seaweeds and sponges have been studied in detail, general information concerning the whole algal assemblage associated with a sponge species is virtually non-existent. We present here the first study in which the algal community associated with a sponge, *Haliclona indistincta* (Bowerbank), was examined in detail. In the period October 2009-September 2010, the seaweed assemblage epibiontic on *H. indistincta* at a site on the Irish West coast was composed of 66 algal taxa (48 red algae, 7 green algae, 11 brown algae). The red algae *Gelidium spinosum* and *Rhodothamniella floridula* were the only epibionts associated with *H. indistincta* for the whole annual cycle. Most of the algal epibionts were filamentous species, which colonized the surface of the sponge and did not penetrate deeply into it. The algal assemblage was most abundant and species-diverse in the period late winter-spring; multivariate analyses revealed a significant variation of the community on the temporal scale of season and sampling date (weeks to months). The results indicate that the algal communities associated with sponges may be very diverse, showing that this type of assemblage deserves further detailed studies.

***Key words***

Epibiosis, *Haliclona indistincta*, marine algae, temporal variation, sponges.

***Introduction***

Macroalgae and sponges are ubiquitous in all marine coastal ecosystems where stable substrata exist. Given their widespread co-occurrence, these organisms may interact in many different ways, including complete symbiosis, mutualism, neutral tolerance, competition and parasitism. Positive interactions have been studied in

detail and involve benefits such as mechanical support for the sponge (Calcinai et al. 2006, Carballo et al. 2006), metabolic integration and supply of nutrients to the alga (Davy et al. 2002, Trautman and Hinde 2002), physical protection for the sponge (Mercurio et al. 2006) and facilitation for the dispersal of the sponge (Avila et al. 2012). Neutral or negative interactions such as epibiosis and competition are a more common but less studied case (Konar and Iken 2005, Cebrian and Uriz 2006, Lopez-Victoria et al. 2006). Some species of seaweeds may settle on sponges and grow as epibionts on them. For the algal epibiont, settlement on a sponge can represent the conquest of a favourable position for growth, ensuring a hydrodynamically favourable situation, which can bring a higher nutrient supply and a more efficient removal of wastes (Wahl 1989). A more exposed habitat also ensures access to more light, essential for photosynthesis. On the other hand, epibiontic algae may suffer from potentially toxic secondary metabolites from the basibiont or get dislodged due to normal mortality, physical disturbance and predation or by shrinkage or growth of the sponge (Wahl 1989, Trautman and Hinde 2002). There are a number of possible disadvantages for the sponge, including competition for organic nutrients, impediment of uptake and excretion, indirect damage by grazers (Wahl 1989, Konar and Iken 2005, Lopez-Victoria et al. 2006) and increased brittleness and therefore breakage in high turbulence environments.

To date, the studies that have considered sponges and macroalgae in conjunction have focused on individual species, most frequently a species of alga and a species of sponge forming a strict association. A few investigations are available on the diversity of sponge assemblages associated with individual seaweed species (e.g., Tronchin et al. 2006, Avila et al. 2009). Conversely, detailed information about the whole algal assemblage associated with a certain sponge species is virtually non-existent. The information available in this regard is scant and based on low taxonomic resolution (e.g., Davis and White 1994), quite possibly due to the fact that many species of sponges look superficially free of epibionts. It is well known that sponges produce many secondary metabolites with biological functions (Dobretsov et al. 2005, Taylor et al. 2007), including defence against epibionts and competitors; so it is probably assumed that sponges do not

host a diverse assemblage of epibionts.

The first study examining in detail the diversity and temporal variation of the algal community associated with a sponge species is presented here. We examined the epibiotic assemblage of *Haliclona indistincta* (Bowerbank) at a site on the West Coast of Ireland. The genus *Haliclona* has been reported in frequent association with seaweeds (Boney and White 1967, Tronchin et al. 2006, Avila et al. 2009) and this species was therefore considered a good candidate to host a rich and diverse vegetation of epibiotic algae.

### ***Materials and methods***

#### *Study site*

Sampling was performed at Corranroo, (53° 9' 6N, 9° 0' 59W), Galway Bay, Ireland, at low tide. At this site, *Haliclona indistincta* is one of the dominant sponge species and forms a large population in the shallow subtidal zone. The site is a tidal inlet formed by a channel 50 m wide and is located approximately 7 km from the open part of Galway Bay. The rocky substratum consists of a mixture of granite and limestone. Due to its configuration, the site is subjected to wave-induced turbulence and strong tidal currents, with tidal amplitude ranging from about 5 m at spring tides to 2.5 m at neap tides. The approximate range of water surface temperature in the study area is historically 5.5°C in January/February to 16°C in July (Guiry and Cunningham 1984).

#### *Sampling procedure*

Sampling was carried out in the course of an annual cycle (from October 2009 to September 2010) in an area of approximately 2,000 m<sup>2</sup> in extent. For data analysis, the period from October 2009 to December 2009 was considered autumn; from January 2010 to March 2010, winter; from April 2010 to June 2010, spring; and from July 2010 to September 2010, summer. Thirty individuals of *H. indistincta* were collected on three sampling dates selected randomly within each season (listed in Table S1). Samples were collected manually at low tide, removing sponge individuals in their entirety with a sharp knife. Each sponge individual was placed in a sealed plastic bag, which was kept in a cooler during

transportation to the laboratory. In the laboratory, all visible algal epibionts (including those partly embedded in the tissue) were removed and identified immediately or frozen for subsequent identification. In order to make the data as comparable as possible, care was taken to collect for the study only sponges at least 10 cm in length and width.

Algal specimens were examined by light microscopy and identified based on morphological features. Voucher specimens for most of the species identified were deposited in the Phycological Herbarium of the National University of Ireland, Galway (GALW).

#### *Data analysis*

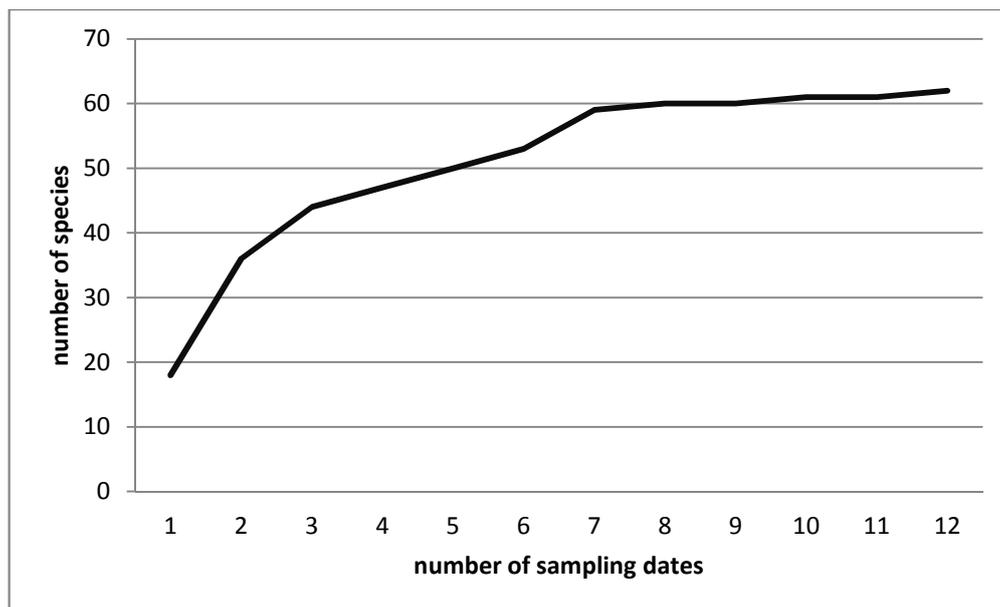
Due to the irregular shape of the sponge and the varied surface area of different sponge individuals, it proved highly challenging to standardize the abundance of the algal epibionts in terms of percentage cover. The small size of many epibionts also made it impractical to use biomass as a meaningful indicator of abundance. It was therefore decided to analyse the species richness as total number of species in each sample, while the structure and temporal variation of the community were analysed in terms of presence/absence data. Because many species were only found in a relatively small number of samples, multivariate analyses were performed both on the complete species dataset and on a reduced dataset of 15 species (the most frequent and visually abundant).

Species richness data were analysed by ANOVA (Underwood 1997), while multivariate data by means of permutational multivariate analysis of variance (PERMANOVA, Anderson 2001) based on Jaccard dissimilarities (Legendre and Legendre 1998). In both cases a nested design was used, and there were two factors: Season (4 levels, fixed) and Date (3 levels, random, nested within Season). Patterns of multivariate data were visualized by means of nonmetric multidimensional scalings (nMDS). The centroids of the 30 replicate sponges on each date were used to display differences among dates and seasons. Centroids were calculated from the full set of principal coordinates obtained from the Jaccard dissimilarity matrix. Centroids and distances among them were obtained using the computer program PCO.exe (Anderson 2003); nMDS plots were generated with PRIMER (Clarke and Gorley 2006).

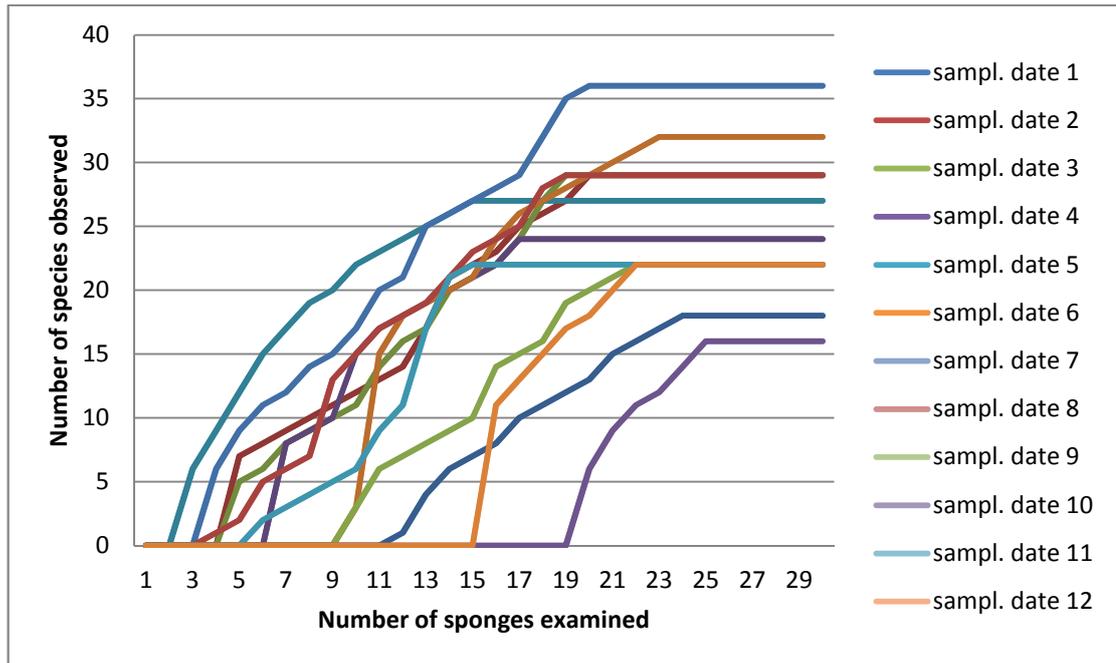
### **Results**

Overall, 66 algal epibionts were recorded in the course of the study (Table 1). These consisted of seven green algae (Chlorophyta), 11 brown algae (Phaeophyceae) and 48 red algae (Rhodophyta). About 9% of the taxa could not be identified reliably and it was therefore preferred not to determine them to species level. These were mainly algae belonging to morphologically simple genera (such as the green *Cladophora* and *Ulva*), whose identification is based on a limited set of morphological characters (some of which are known to be affected by morphological plasticity).

The whole algal diversity present in each sampling date and during the whole year seems to have been captured with this sampling design. All rarefaction curves, representing the number of species found after 12 sampling dates (Fig. 1) and found in the 30 sponges collected in each sampling date (Fig. 2.) approach asymptotes.



**Fig. 1.** Rarefaction curve based on number of species observed per number of sampling dates.



**Fig. 2.** Rarefaction curve based on number of species observed in each sampling date per number of

At all times in the course of the study, most of the algal taxa found on *H. indistincta* (60%) belonged to the division Rhodophyta (Tables 1 and 2). Except for the first sampling date of summer (date 10), the majority of the sponge individuals collected supported one or more algal epibionts. The highest numbers of sponge individuals with epibiontic algae were found in winter and spring whereas the lowest were found in summer (Table 2). The mean number of algal taxa per sponge individual (not considering individuals devoid of epibionts) was highest in spring, particularly early spring (Table 2). The highest number of taxa found on a single individual sponge (12 taxa) was recorded on dates 6 and 7, which correspond to late winter and early spring.

Part C: Seaweeds and Sponges

**Table 1.** Floristic list of the algal species found on *Haliclona indistincta* showing their presence/absence for each sampling date. Full names including authorities are available in AlgaeBase ([www.algaebase.org](http://www.algaebase.org)). Labels between brackets indicate morphological groups: (AC) = articulated corallines; (CC) = corticated seaweeds with cylindrical thallus; (F) = filamentous algae; (FC) = corticated seaweeds with flattened thallus; (L) = leafy algae; (LM) = thick leathery macrophytes

Taxa	1	2	3	4	5	6	7	8	9	10	11	12
	Autumn		Winter			Spring			Summer			
Chlorophyta (green algae)												
<i>Bryopsis plumosa</i> (F)		*						*		*	*	
<i>Cladophora</i> spp. (F)	*	*	*	*	*	*	*	*	*	*	*	*
<i>Chaetomorpha</i> sp. (F)	*	*					*	*				
<i>Derbesia marina</i> (F)												*
<i>Monostroma grevillei</i> (L)						*						
<i>Ulva</i> spp. ( <i>Ulva</i> -morphology) (L)	*	*	*	*	*	*	*	*	*	*	*	*
<i>Ulva</i> spp. ( <i>Enteromorpha</i> -morphology) (F)	*	*	*	*			*	*	*	*	*	*
Phaeophyceae, Ochrophyta (brown algae)												
<i>Asperococcus fistulosus</i> (CC)							*			*		
<i>Cystoseira</i> cfr. <i>nodicaulis</i> (LM)							*	*	*			
<i>Colpomenia peregrina</i> (CC)							*	*		*		*
<i>Dictyota dichotoma</i> (FC)				*	*		*	*	*		*	*
<i>Ectocarpus</i> sp. (F)	*						*	*	*			
<i>Halidrys siliquosa</i> (LM)						*						
<i>Hinckesia secunda</i> (F)						*		*				
<i>Petalonia fascia</i> (L)										*		
<i>Punctaria latifolia</i> (L)							*					
<i>Saccharina latissima</i> (LM)			*			*	*	*	*		*	*
<i>Sphacelaria cirrosa</i> (F)	*	*			*	*	*	*	*		*	
Rhodophyta (red algae)												
<i>Aglaothamnion gallicum</i> (F)	*				*	*						
<i>Anotrichium barbatum</i> (F)				*		*						
<i>Anthithamnion</i> sp. (F)		*										
<i>Apoglossum ruscifolium</i> (L)		*	*	*	*	*						
<i>Asparagopsis armata</i> ( <i>Falkenbergia</i> phase) (F)	*											
<i>Audouinella</i> sp. (F)	*											
<i>Bonnemaisonia hamifera</i> ( <i>Trailliella</i> phase) (F)					*							
<i>Boergeseniella fruticulosa</i> (CC)		*	*	*	*		*	*	*	*	*	*
<i>Calliblepharis jubata</i> (FC)		*	*			*					*	
<i>Callophyllis laciniata</i> (FC)						*		*				
<i>Champia parvula</i> (CC)		*	*	*	*	*	*		*		*	*
<i>Chondrachantus acicularis</i> (CC)		*						*				
<i>Chondrus crispus</i> (FC)		*	*		*	*	*	*			*	
<i>Chylocladia verticillata</i> (CC)							*		*			
<i>Ceramium ciliatum</i> (F)	*	*	*				*	*				*
<i>Ceramium echionotum</i> (F)		*	*	*	*	*	*	*	*	*	*	*
<i>Ceramium</i> cfr. <i>pallidum</i> (F)			*	*				*	*			*
<i>Ceramium virgatum</i> (F)				*		*	*	*	*			
<i>Compsothamnion thuyoides</i> (F)								*				
<i>Corallina</i> sp. (AC)			*	*	*	*	*		*		*	*
<i>Cystoclonium purpureum</i> (CC)			*	*			*		*			
<i>Cryptopleura ramosa</i> (FC)		*	*			*	*					
<i>Dasya</i> sp. (F)	*				*	*	*					
Delesseriaceae spp. (L)		*		*	*	*	*	*	*	*	*	*
<i>Furcellaria lumbricalis</i> (CC)			*			*						
<i>Gastroclonium ovatum</i> (CC)		*	*	*	*	*	*			*	*	*

Part C: Seaweeds and Sponges

Taxa	1	2	3	4	5	6	7	8	9	10	11	12
	Autumn			Winter			Spring			Summer		
<i>Gelidium spinosum</i> (CC)	*	*	*	*	*	*	*	*	*	*	*	*
<i>Griffithsia corallinoides</i> (F)	*		*	*	*	*					*	*
<i>Heterosiphonia japonica</i> (F)	*	*	*	*	*	*	*	*	*			*
<i>Heterosiphonia plumosa</i> (F)		*	*	*	*	*	*			*		
<i>Hypoglossum hypoglossoides</i> (L)	*	*						*				
<i>Jania rubens</i> (AC)			*									
<i>Laurencia obtusa</i> (CC)			*									
<i>Lomentaria articulata</i> (CC)		*		*		*	*	*				
<i>Lomentaria clavellosa</i> (CC)	*	*	*		*	*	*		*		*	
<i>Lomentaria</i> sp. (CC)						*	*					
<i>Monosporus pedicellatus</i> (F)		*				*						
<i>Nitophyllum punctatum</i> (L)						*					*	
<i>Osmundea pinnatifida</i> (CC)					*	*	*	*	*			*
<i>Plocamium</i> sp. (FC)		*	*									*
<i>Polysiphonia brodiei</i> (F)					*							
<i>Polysiphonia elongata</i> (CC)		*	*	*	*	*	*	*		*	*	*
<i>Polysiphonia stricta</i> (F)							*					
<i>Pterosiphonia parasitica</i> (F)						*						
<i>Pterosiphonia pennata</i> (F)	*											
<i>Rhodomela confervoides</i> (CC)			*									
<i>Rhodophyllis divaricata</i> (L)		*	*				*			*	*	
<i>Rhodothamniella floridula</i> (F)	*	*	*	*	*	*	*	*	*	*	*	*

**Table 2.** Variation of some parameters of the structure of the epibiotic algal community of *Haliclona indistincta* in different seasons. Where appropriate, data are expressed as means  $\pm$  standard errors.

	Autumn	Winter	Spring	Summer
Number of Chlorophyta (green algae)	5	3	5	5
Number of Phaeophyceae (brown algae)	4	5	9	6
Number of Rhodophyta (red algae)	36	33	29	20
Number of sponge individuals with 1 algal taxon per sampling date	23.7 $\pm$ 4 (n=3)	24 $\pm$ 3 (n=3)	25 $\pm$ 3.4 (n=3)	17 $\pm$ 7.2 (n=3)
Number of algal taxa per sponge individual	3.5 $\pm$ 2.7 (n=71)	3.1 $\pm$ 2.5 (n=73)	3.8 $\pm$ 3.2 (n=75)	2.1 $\pm$ 2.7 (n=51)

The only two species recorded on all sampling dates were the red seaweeds *Rhodothamniella floridula* and *Gelidium spinosum* (Table 1). Whereas specimens of *Cladophora* and leafy *Ulva* (Chlorophyta) were also recorded throughout the study period, these genera probably included several different species; it is likely that *C. hutchinsiae* and *U. lactuca* were the two most common. *Ceramium echionotum* was also very common, occurring as an epibiont of *H. indistincta* on all sampling dates except the first (Table 1). In general, many algal taxa occurred on a relatively low number of sponge individuals. Many epibionts, especially filamentous species, were small-sized and/or in juvenile form. Usually,

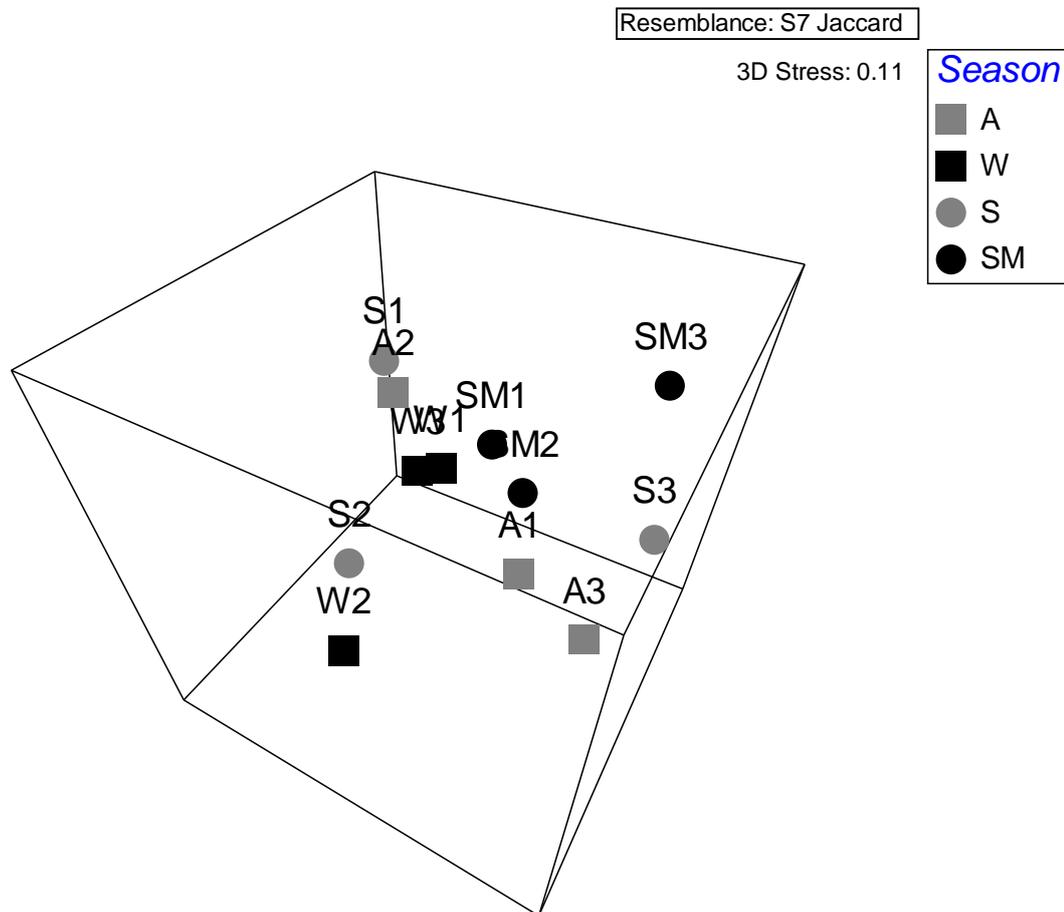
filamentous and leafy algae grew on the surface of the sponge and did not penetrate deeply into it. Conversely, some corticated species (in particular *G. spinosum*) were often embedded in the tissues of the sponge, sometimes to the extent that only a limited part of the alga could be observed externally. In the case of some leathery macrophytes (*Cystoseira* cfr. *nodicaulis* and *Saccharina latissima*), articulated corallines (*Corallina* sp.) and corticated species (*Gastroclonium ovatum*, *Osmundea pinnatifida* and *Polysiphonia elongata*) the relationship established appeared to be coalescence rather than epibiosis. The sponge seemed to have gradually grown around the holdfast or stipe of the alga, surrounding it almost completely.

**Table 3.** Results of the PERMANOVA analyses performed on presence and absence of the 15 most common species and morphological groups for the factors Season and Date. The test is based on Jaccard dissimilarities, using 999 permutations.

Source	Df	SS	MS	Pseudo-F	<i>P</i> values
Presence/absence (15 most common species)					
Season	3	42651	14217	1.6495	0.01
Date (Season)	8	68952	8619	2.2181	0.001

The ANOVA performed on species richness showed only a significant effect of the date of sampling ( $MS = 3.228$ ,  $F = 6.01$ ,  $p < 0.0001$ ; transformation:  $\ln(x+1)$ ), with no differences among seasons ( $MS = 4.908$ ,  $F = 1.52$ ,  $p > 0.2$ ; transformation:  $\ln(x+1)$ ). The results of the PERMANOVA performed on presence/absence data of the 15 most common species showed significant effects of both season and sampling date (Table 3). The same results were obtained when the whole species dataset was analysed (results not shown). The nMDS plot for the reduced species dataset reflected this pattern, showing that some dates were clearly separated from the others, even within the same season (Fig. 1). In terms of seasonality, a separation between summer and winter and between summer and autumn were the most apparent patterns (Fig. 1). *C. echionotum*, *Cladophora* spp., *Heterosiphonia japonica*, *Heterosiphonia plumosa* and *Corallina* sp. were more frequently present in winter than summer; *Cladophora* spp., *H. japonica*, *H. plumosa*, *R. floridula*, *Boergeseniella fruticulosa*, *Ulva* spp. (blade forms) and

*Ulva* spp. (*Enteromorpha* forms) were more frequently present in autumn than summer (Complementary Fig. 1).



**Fig. 1.** Frequency of the 15 most common species in the four seasons (as number of presences per sample on total number of samples within each season).

### *Discussion*

With 66 taxa recorded, the algal community associated with *H. indistincta* was unexpectedly diverse, even though comparisons with other sponge species are impossible due to the almost complete absence of published data. Apart from a few exceptions (such as *Anotrichium barbatum*), the algal species recorded are widespread on the shores of Ireland and Britain, where they are usually epilithic, epiphytic on larger seaweeds or epizooic on mussels and other shellfish. Only one, *Derbesia marina*, has been previously reported as an epibiont of sponges (Brodie et al. 2007). In the area of Galway Bay the taxa reported are generally common,

and we observed many of them growing on rock or larger seaweeds close to *H. indistincta* at the study site. For many species, especially filamentous, the presence on *H. indistincta* was occasional; these algae were found in small amounts, on a few sampling dates and on a small number of sponge individuals. In many cases they did not penetrate deeply into the body of *H. indistincta*, suggesting that they were serendipitous, opportunistic colonizers of the sponge surface. This appeared to be also the case for leafy species of *Ulva*, which reached some of the highest cover values observed in the assemblage in spring.

Interestingly, the two seaweeds most frequently found on *H. indistincta* are the red algae *Rhodothamniella floridula* and *Gelidium spinosum*. *Rhodothamniella floridula* is well known as a perennial epilithic alga with sand-binding capacities (Dixon and Irvine 1995, Bunker et al. 2010) and forms thick cushions on rocky bottoms partially covered by sand; although widespread on the shores of northern Europe, it has not been previously reported as an epibiont of sponges. The gross morphology of *R. floridula* is identical to that of another widespread group of rhodophytes, the order Acrochaetiales, which has species among the most common red algal endobionts. It is unclear whether the interaction established offers any benefits for the host organism (because of their small size and usually low biomass, *Acrochaetium*-like algae are unlikely to affect the host either positively or negatively). Considering that in these algae the tendency to establish endobiotic relationships is widespread and the taxonomic range of host organisms is wide, the interaction is probably beneficial for the alga. Due to their thin and delicate thallus, *Acrochaetium*-like algae may be easily consumed by grazers or damaged mechanically by larger sessile organisms located nearby. Epibiotic growth on a sponge can provide the alga with mechanical and chemical protection against grazers and competitors, as well as a strategic positioning which allows the alga to benefit from water flows bringing nutrients or nitrogen supplements from the sponge. Sponges are known to produce a wide range of biologically active compounds (Taylor et al. 2007, Blunt et al. 2011) with multiple functions, including deterrents against grazers. In the case of *Acrochaetium* sp. and the sponge *Mycale laxissima* (Rützler 1990) TEM observations suggested that spongin was deposited onto the algal cell wall during simultaneous growth of

seaweed and sponge. It would be interesting to understand whether this also happens with *H. indistincta* and *R. floridula*.

Among all seaweeds recorded, *Gelidium spinosum* was the species that established the closest relationship with *H. indistincta*. *G. spinosum* is a member of the red algal order Gelidiales, a group which includes another genus strictly associated with sponges, *Ptilophora*. The majority of *Ptilophora* species are commonly found with sponge epiphytes and some of them are consistently covered by extensive coatings of epiphytic sponges (Tronchin et al. 2006). Many species of *Ptilophora* produce proliferations of various shape and size on the surface of the thallus. After examining a large number of *Ptilophora* specimens from several geographic regions, Tronchin et al. (2006) concluded that the presence of surface proliferations probably facilitates settlement and growth of the sponges. Sponges and *Ptilophora* appear structurally related in that the sponge probably exploits a niche habitat provided by the alga, since sheltered microhabitats located at the axils of the proliferations may represent favourable habitats for the settlement of sponge larvae (Tronchin et al. 2006). This case has been also suggested for other red algae producing superficial proliferations and supporting epiphytic sponges, such as the rhodomelacean genera *Epiglossum* and *Osmundaria* (Phillips 2002). *G. spinosum* does not produce superficial proliferations similar to those of *Ptilophora*. However, well-developed specimens of *G. spinosum* have a pinnate habit, with lateral branches arranged in regular rows on the sides of the main axes. In a study of the sponge assemblages associated with *Sargassum* spp. in the south-western gulf of California, Avila et al. (2009) remarked that a selective larval colonization could possibly explain the distribution patterns observed, since it is known that larvae of many marine invertebrates can locate and settle selectively in physical microrefuges (Avila and Carballo 2006).

Temporal variation in the structure of the community was observed at season level and, most evidently, at sampling date level. When Davis and White (1994) looked to autumn's versus spring's epibiotic community of several sponge species in south-eastern Australia, these authors found that *Darwinella australiensis* and *Euryspongia* sp. were always dominated by non-calcareous

algae, whereas *Chondrilla australiensis*, *Clathria* sp. and *Callyspongia* sp. showed an increase in algal fouling in spring. The authors did not offer an explanation for this difference except that in general senescent basibionts tend to be more fouled. In the current study, the number of individuals fouled decreased in the summer (Table 2), but the variation between other times of the year was negligible. All sponges collected were fully-developed specimens, and therefore senescence and new generation of sponges are not explanations for the lower number of sponges fouled in summer. Further studies are needed to understand why not all sponges were fouled and why this decrease. In general, the significant variability among seasons is probably due to differences in abundance of filamentous taxa (in particular *Cladophora* spp., *Heterosiphonia japonica* and *Heterosiphonia plumosa*). The fact that filamentous species were the most common may also explain why small scale temporal variation (weeks and months) was more significant than seasonal variation. Filamentous seaweeds usually have a quick growth, high inputs of propagules and quick life cycles with rapid transitions between different phases. The seasonal variance in the recruitment of foulers is probably explained by the seasonal variation in the phenology of the surrounding vegetation (Davis and White 1994), since the life cycle of the epibiontic seaweeds is highly dependent on factors related to seasonality, such as day length, water temperature and amount of nutrients in the water column (Guiry and Cunningham 1984).

Overall, the results indicate that the algal communities associated with sponges may be very diverse and include associations that are not immediately observable with a superficial observation restricted to a single sampling time. Further studies extended to other sponge species and conducted on longer time spans will allow drawing more general conclusions on the dynamics of these little-known assemblages.

### ***Acknowledgments***

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suggestions.

**Table S1.** Detail of collection dates

<b>Sampling date</b>	<b>Date</b>	<b>Season</b>
1	8 October 2009	Autumn
2	6 November 2009	Autumn
3	15 December 2009	Autumn
4	29 January 2010	Winter
5	18 February 2010	Winter
6	18 March 2010	Winter
7	29 April 2010	Spring
8	25 May 2010	Spring
9	24 June 2010	Spring
10	16 July 2010	Summer
11	16 August 2010	Summer
12	9 September 2010	Summer

### **C3) Metabarcoding analysis of the epibiotic communities associated with *Haliclona indistincta* (Bowerbank)**

#### ***Abstract***

Recent next generation sequencing (NGS) studies have focused mostly on the bacterial epi and endobionts of sponges, but the eukaryotes have not been given the same consideration. Following a morphological analysis of the macroalgal epibiotic diversity of the sponge *Haliclona indistincta*, a NGS approach was used to explore the missed diversity of algal and animal organisms living in and on the sponge. The results allowed for a better resolution at species level of some previously reported species and information on unreported species. Data showed a high biodiversity of operational taxonomic units (OTUs) represented often by unique sequences. Possible fungal and dinoflagellate symbionts are pinpointed. Diatoms were underrepresented compared to seawater perhaps due to active protection by the sponge. Divergence in the sponge rRNA 18S gene suggests the presence of multiple copies.

#### ***Key words***

Endobionts, epibionts, *Haliclona indistincta*, Next Generation Sequencing, sponge, 16S rRNA gene, 18S rRNA gene.

#### ***Introduction***

When trying to characterize prokaryote diversity, molecular techniques have become essential mostly because it is not possible to culture most prokaryotes in laboratory conditions. Traditional microscopic isolation methods only detect 0.001% to 15% of the total of visible cells depending on the ecosystem type (Spring et al. 2000). Culturing in different types of selective media was an essential step when characterising such organisms until identification by PCR amplification and sequencing of the 16S rRNA ribosomal RNA gene became routine (Snyder et al.2009, Spring et al. 2000). Although this was an important

technical revolution, it remains laborious especially when focusing on complex prokaryote communities, often requiring a cloning step. More recently, next generation sequencing methods have removed the need for cloning, speeding up the process substantially and increasing the amount of data generated (Snyder et al. 2009, Spring et al. 2000). This has allowed the discovery of many rare or unculturable species.

It is also accepted that for unicellular eukaryotic communities molecular approaches can improve the resolution of species identification, in particular by uncovering rare ones (e.g. Moreira and López-García 2002, Richards and Bass 2005, Takishita et al. 2007). Although there has been an increase in the number of studies which use next generation sequencing (NGS) methods to study prokaryotic communities (Fry et al. 2008, Simister et al. 2012, Snyder et al. 2009, Webster et al. 2010), few investigations have focused so far on eukaryotic communities (Creer et al. 2010, Dunthron et al. 2012, Jumponen et al. 2010, Stoeck et al. 2010). Due to the lower number of studies there is a lack of understanding on how these techniques reflect the real diversity present. Medinger et al. (2010) compared microscopic observations, traditional Sanger sequencing and 454 NGS techniques to describe seasonal changes in a protist community residing in an oligotrophic freshwater habitat. They concluded that although NGS data tended to overestimate the quantities of dinoflagellates, for example, they more accurately reflected the frequency shifts in abundance between seasonal samples, and were superior in the identification of rare species. Overestimation of quantity may be due to the fact that some organisms, such as dinoflagellates, have high numbers of copies of the small ribosomal subunit (SSU), the gene most utilised for this approach (Dyal et al. 1995, Zhu et al. 2005). However NGS can identify resting stages of protists that are generally missed or impossible to identify by morphological observations and, in some cases, rather than being overestimated some specific taxa are usually underrepresented. Diatoms, for example have had a low representation in certain NGS studies when compared to morphological studies, presumably due to the difficulty in breaking their silica frustules and subsequent DNA extraction, and flagellates are known to have a very low number of copies of the SSU gene making it difficult to amplify by PCR

(Medinger et al. 2010, Stoeck et al. 2010, Zhu et al. 2005). Discrepancies between morphological and NGS-based measurements of diatoms can be explained by the 10-30% loss of cell frustules during the preservation of samples for morphological analyses (Sime-Ngando and Grolière 1991). Other research groups also highlight the advantages of NGS for protist and meiofaunal diversity studies but recommend a comparison with “traditional” techniques for better interpretation of the obtained information (Creer et al. 2010, Stoeck et al. 2010, Lara and Acosta-Mercado 2012).

As sessile aquatic organisms, sponges become important micro-habitats and communities harbouring bacteria, algae and other small organisms (Wahl 1989, 2008, chapter C2). Not being able to escape their predators, many rely on the production of secondary metabolites as means of biological defence. These compounds can be used to deter settlement of various organisms, including ascidian larvae (Davis et al. 1991), avoid predation by fish (Chanas et al. 1997), compete with other organisms for space including corals by preventing their growth (Sullivan et al. 1983), inhibit bacterial and viral growth (Bobzin and Faulkner 1992, Bokesch et al. 2002) and also in competition with other sponges (Thacker et al. 1998). From an industrial point of view, these compounds can have several applications, serving as antimicrobial (Donia and Hamman 2003, Ereskovsky et al. 2005), anti-viral (e.g. Bokesch et al. 2002) and cytotoxic (used in cancer research) (e.g. Proksch et al. 2002, Donia et al. 2003). Other useful characteristics include anticoagulant, anti-diabetic, anti-inflammatory, and anti-tuberculosis properties (Mayer et al. 2005). There have been a few cases in which a natural product first attributed to a sponge was later shown to be produced by microorganisms living in symbiosis with the sponge host. Some examples include the production of diketopiperazines previously ascribed to the host sponge *Tedania ignis* Duchassaing & Michelotti, which is now attributed to the symbiotic bacterium *Micrococcus* sp. (Stierle et al 1988). The production of brominated biphenyl ethers initially attributed to the sponge *Dysidea* sp. is now known to be due to the bacterium *Vibrio* sp. (Elyakov et al. 1991). Although it is still unresolved whether in most cases the producer of the compounds is the sponge or the symbiont (Faulkner et al. 1999, Lee et al. 2000), it is suspected that more

bioactive natural products will be discovered from sponge-symbiotic microorganisms. This can be expected in the following cases: when one sponge species contains different classes of metabolites; when taxonomically different sponges contain the same metabolite; when free-living microorganisms produce identical or similar metabolites and or when the metabolite concentrations are exceedingly low (Lee et al. 2001). The advantage of the secondary metabolites being produced by a symbiont, from a point of view of biodiscovery, is that this organism may be easier to culture in larger quantities than sponges, making the study of the endo- and epibiont community of a sponge a very important step in screening programmes.

Most studies that focus on sponge epibionts or symbionts focus on the bacteria living in the sponge but tend to disregard eukaryotes (e.g. Wilkinson and Fray 1979, Shmitt et al. 2007, Taylor et al. 2007, Webster et al. 2010). However, there are some cases where a eukaryotic symbiont was found to be the secondary metabolite producer. For example, the dinoflagellate *Prorocentrum lima* (Ehrenberg) F.Stein was found to produce okadaic acid, first isolated from the host sponge *Halichondria okadai* Kadota (Kobayashi et al.1993). Another group of eukaryotes that has shown potential in production of secondary metabolites are sponge-associated fungi (Proksch et al.2008). As discussed in the previous chapter, most studies describing algal-sponge interactions tend to focus on one to one species interactions where a sponge and an alga form a close association (e.g. Frost et al. 1997, Trautman et al. 2000, 2003, Carballo and Avila 2004) or on the sponge community living on one seaweed, usually large seaweeds (e.g., Tronchin et al. 2006, Avila et al. 2009). Apart from the study described in chapter C2, only one other published work has described the major algal epibionts present in several sponges and only in autumn and in spring (Davis and White 1994). Chapter C2 was the first study that described the whole macro-algal community associated with a sponge and to show temporal variation of this community during a whole year. Animal epibionts have also been documented for the same sponge species at different times of the year. In total 20 species were recorded, mostly crustaceans and annelids, with some sea stars, a nematode and a gastropod also recorded (S. Murphy, NUIG, unpublished). Within the main phyla the best

represented orders were Amphipoda (Crustaceans) and Phyllodocida (Polychaetes) (S. Murphy, NUIG, unpublished). The numbers of species recorded are probably an underestimate since the material was collected for another purpose and some animals may have escaped the sponge or been otherwise lost between collection on the shore and the sponge preservation (S. Murphy, NUIG, unpublished). However, in both studies, the focus was on biodiversity that was visible and therefore possible to identify by morphological characters. All the microbial community was missed and some of the macro-organisms could not be fully identified since they were in a juvenile form, or only parts rather than the full organism had been recovered. Furthermore, the organisms studied were only the epibionts and, to some extent, organisms that were partially embedded in the sponge tissues. Organisms deeply embedded in the sponge, living in microenvironments within the sponge tissues or living inside the cells were possibly missed. In order to obtain a detailed characterization of the biotic community associated with *Haliclona indistincta* Bowerbank, a full investigation of the existing community of eukaryote and prokaryote organisms in this sponge was performed using NGS 454 sequencing. This chapter focus primarily on the eukaryotic community.

### ***Materials and Methods***

#### *Sampling and isolation of DNA*

Seventy-three grams (wet weight) of *H. indistincta* (one organism) and 2 l of seawater surrounding the sponge were collected in May 2011, at low tide, in Corranroo, Co. Clare, Ireland (53° 9' 6N, 9° 0' 59W). The seawater collected *in situ* was filtered with a Whatman GF/C (1.2µm) using a 0.2 µm filter. The accumulated retentate on the filters, were used to represent the microflora and fauna of the seawater environment surrounding the sponge and subsequently referred to as the sea water sample.

In a laminar flow hood all visible sponge epibionts and water flushed out from the sponge were transferred to collection tubes and immediately frozen

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together as the epibiotic sample. The remaining sponge material was aseptically rinsed in calcium and magnesium-free artificial seawater (CMF-ASW), pH 7.4, to remove natural seawater and thus prevent re-aggregation of cells once extracted. The material was then scalpel-cut into pieces of less than 5 mm<sup>3</sup> and kept in CMF-ASW (ratio of at least 1ml/2g tissue) and Proteinase K (final concentration 2.5 mg/ml) at 4°C for 30 minutes. The sponge-tissue was then squeezed through a fine nylon mesh (50 µm) and the resulting cell suspension was centrifuged in two 50 ml Falcon™ tubes at 600 g and 4°C for 5 min in a Sorvall™ swing-bucket centrifuge. The pellet was re-suspended in fresh CMF-ASW and centrifuged at 600 g and 4°C for another 5 min (modified after Thompson et al. 1984, Garson et al. 1992 and Flowers et al. 1998).

The cells were fractionated according to density via centrifugation at 600 g for 30 min at ambient temperature across a discontinuous Ficoll™ gradient at 26, 21, 19, 14, 10, and 5% (w/v) respective concentrations in CMF-ASW, modified after Garson et al. (1992). The bands of cells that accumulated at the density interfaces were carefully isolated individually by glass-pipette, washed twice with CMF-ASW to remove Ficoll and then again pelleted at 600 g and 4°C for 5 minutes. The supernatant was re-centrifuged in eppendorf tubes at 13x10<sup>3</sup> g (bench microcentrifuge, ThermoFisher) for 15 minutes and the resulting pellet retained. Samples of the cell pellet were taken for light microscopy and each pellet was subsequently re-suspended in 2ml CMF-ASW.

Genomic DNA was extracted from the eight resulting Ficoll fractions as well as the epibiont and seawater samples using a PowerSoil® DNA Isolation Kit (MoBio). DNA from the Ficoll fractions were pooled into two separate samples according to predominant cell type, resulting in sponge and bacteria DNA aliquots complementing the epibiont and seawater DNA aliquots.

### *Amplification of targeted regions and new generation sequencing*

A TC-3000 thermal cycler (Techne) was used for all PCR amplifications. To generate NGS data from the eukaryotic component of the community residing within *H. indistincta*, PCR amplification of a 400 bp fragment of the 18S rRNA

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gene was performed. The forward primer was G03 (GTC TGG TGC CAG CAG CCG CGG) (Harper and Saunders, 2002). The reverse primer was designed to contain the GS FLX Titanium primer B, the Multiplex Identifier (MID) and our specific region primer G04R (ATC CAA GAA TTT CAC CTC TG), adapted from Harper and Saunders (2002). The region amplified included the highly variable V4 region, which has been established as being comparable to amplifying the whole SSU region when accessing diversity (Dunthorn et al. 2012).

PCR amplification was carried out on each of the four DNA samples (i.e. from seawater, epibiont, sponge and bacteria cells) ten times using the following protocol: 30  $\mu$ l reaction consisted of 21.2  $\mu$ l of HyPure Cell Culture Grade Water (Thermo Scientific, South Logan, Utah), 3  $\mu$ l of 10x OptiBuffer, 0.6  $\mu$ l of dNTP Mix, 0.9  $\mu$ l of 50 mM MgCl<sub>2</sub> solution, 0.18  $\mu$ l of Platinum Taq (Biosciences), 0.6  $\mu$ l of each primer mix (100  $\mu$ M) and 2  $\mu$ l of DNA sample. The protocol used was the following: an initial denaturation step at 94°C for 3 min followed by 30 cycles with denaturation at 94°C for 30 s, annealing at 55°C for 45 s and elongation at 72°C for 1 min, with a final elongation step at 72°C for 10 min.

Amplification of ca 500 bp of the variable V3 region of the 16S rRNA gene from bacteria and archaea were carried out as above adding MID to the following primers: F63 (CAGGCCTAACACATGCAAGTC, Marchesi et al. 1998) and 518R (ATTACCGCGGCTGCTGG, Marques et al. 1998) for bacteria and using two forward primers A2Fa (TTCCGGTTGATCCYGCCGGA, Barns et al. 1994) and N3F (TCCCGTTGATCCTGCG, Baker et al. 2003) and reverse primer A571R (GCT ACG GNY SCT TTA RGC, Baker et al. 2003) for two different groups of archaea. Also, for archaea, a slight modification to the amplification protocol was required after unsuccessful amplification using the previous protocol: A first step with non-tagged primers of 25 cycles equal to the ones described above followed by ten more cycles with tagged primers and a new aliquot of dNTP Mix solution.

PCR products were visualized on 1.2% agarose gels stained with SYBR Safe DNA stain (Invitrogen). The amount of DNA in PCR products was quantified visually on agarose gels using HyperLadder II (Bioline) as a reference

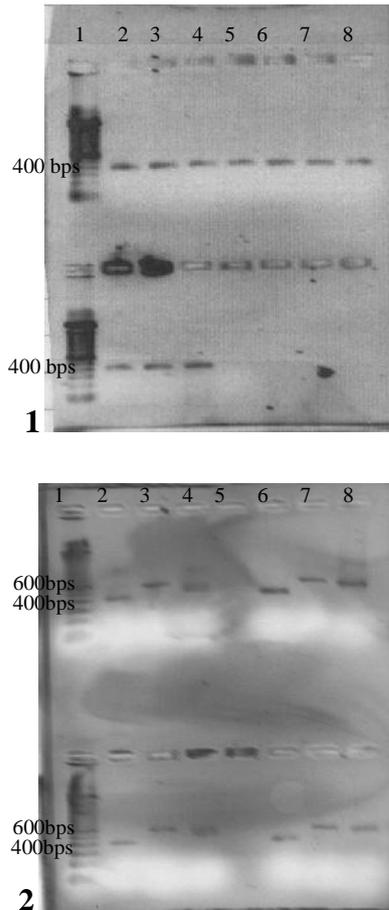
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and also calculated via a spectrophotometer (Nanodrop). In a first step, all 10 amplicons for the same sample were confirmed to be equimolar and then combined (Fig. 1). The combined PCR amplicons were purified using the MinElute Gel Extraction Kit (Qiagen). After purification the amount of DNA was verified once again as above (Fig.2 and Table 1) to ensure equimolarity of the different organismal amplicons (bacteria, archaea and eukaryotes) that had been amplified from each DNA sample (i.e. seawater, epibionts, sponge and bacteria). LGC required 1µg of DNA per sample, therefore it was prepared to contain 333ng of bacterial and archaeal 16S rRNA and eukaryotic 18S rRNA genes.

**Table 1.** Concentration of DNA from combined and purified PCR samples calculated by spectrophotometry Nanodrop. Archaeal 16S rRNA concentrations include both PCR reaction samples, resulting from the two sets of primers used, combined. All samples were 200 µl.

Sample type/sequence type	Concentration (ng/µl)
Sea water/ 18S rRNA	15
Sea water /bacterial 16S rRNA	20
Sea water/ archaeal 16S rRNA	16
Epibionts/18S rRNA	10
Epibionts/bacterial 16S rRNA rRNA	11
Epibionts/archaeal 16S rRNA	12
Sponge cells fraction/18S rRNA	22
Sponge cellsfraction/bacterial 16S rRNA	33
Sponge cells fraction/archaeal 16S rRNA	15
Bacterial cells fraction/18S rRNA	11
Bacterial cells fraction/bacterial 16S rRNA	14
Bacterial cells fraction/archaeal 16S rRNA	12

The four samples comprising of purified PCR products of appropriate length, yield and purity were sent to LGC Genomics (Berlin, Germany). There, all samples were reamplified in order to add GS FLX Titanium primer A to the forward primers which is essential in this type of sequencing. Afterwards, Unidirectional Sequencing of Amplicon Libraries Using GS FLX Titanium emPCR Kits was performed on half of a plate.



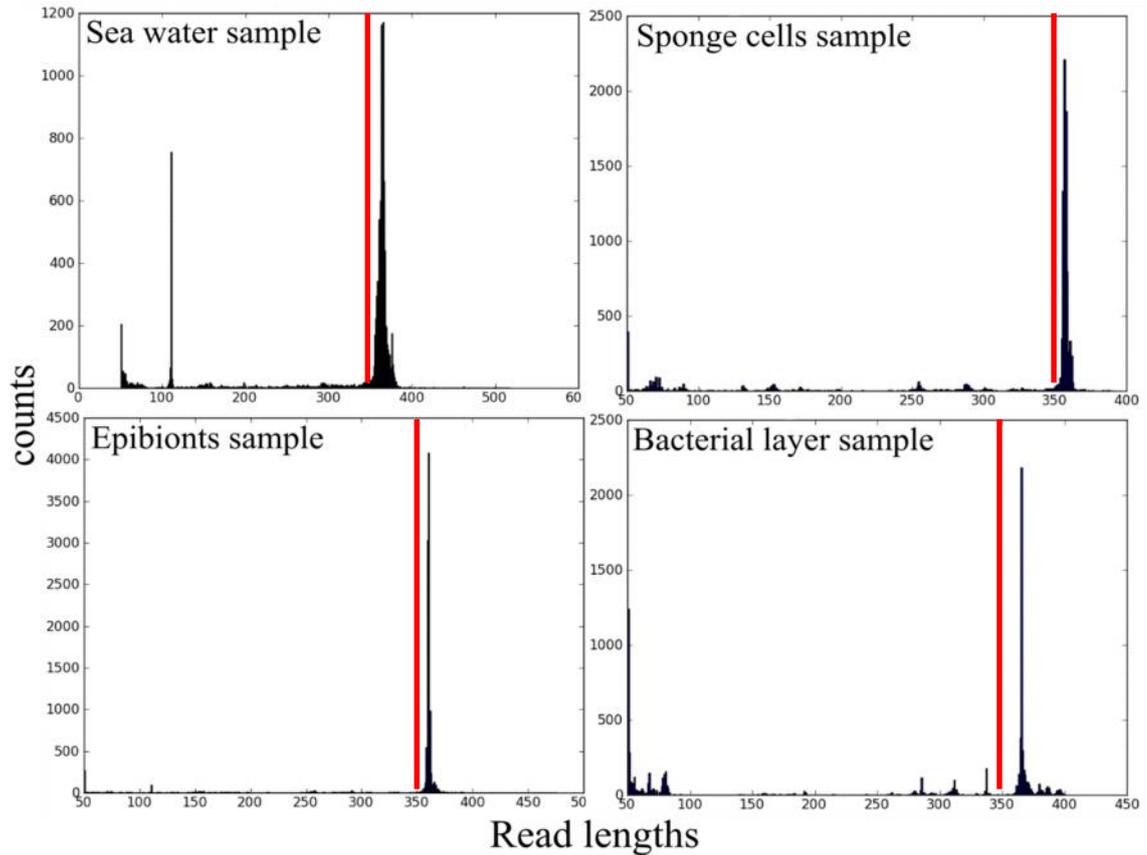
**Fig. 1 and 2 Visualization and quantification gels:** Fig. 1. 1.2% gel visualization of 10 PCR 18S rRNA gene amplicons for the epibionts sample as an example. Lane 5, row 2 of gel shows negative control. Fig. 2. 1.2% gel visualization of combined and purified amplicons. Lane 2-8 in row 1 shows sea water and sponge cell fraction amplicons (3+3). Lane 2-8 in row 2 shows the epibionts' and bacterial cells fraction amplicons (3+3). Each sample was amplified with eukaryotic, archaeal and bacterial primers.

### *Software treatment*

Using the unique MID tags that were attached to the PCR primers, the resulting sequences were separated by organism type (eukaryotic 18S rRNA, bacterial 16S rRNA and archaeal 16S rRNA) and sample type (seawater, epibiont, sponge, bacteria) yielding 12 datasets. Quality control and analysis were performed independently for the reads corresponding to each sample. Data were trimmed using QTrim (<http://hiv.sanbi.ac.za/software/qtrim>) to remove sequences of low quality according to Phred scores. All sequences less than 50bps were removed

and the remainder were compared against the GenBank database (using BLAST) to place an initial organismal type identity to each of the reads. A histogram showing read length vs read frequency (Fig. 3) was used to choose the sequences employed for alignment assembly and subsequent analysis. In the case of the 18S rRNA gene amplicons, all sequences of length greater than 350 bps were selected and in the case of 16S rRNA gene amplicons, all sequences of length greater than 400 bps were selected. All selected sequences were trimmed to 350 bps and 400 bps respectively and redundant sequences were identified and counted. Two further datasets were generated from each of the 12 sequence datasets; one containing all sequences (redundant) and one containing only unique sequences (non redundant). Chimeras were identified as sequences with a query coverage of less than 75% in the BLAST search and were disregarded.

All sequences from the redundant dataset sequences were aligned in the Ribosomal Database Project's Pyrosequencing Pipeline (RDP, <http://pyro.cme.msu.edu/index.jsp>) aligner tool using the default sets. Alignments were used to produce cluster files using default step 1.0 and 3% maximum distance in the same pipeline. This means that OTUs were defined by a 97% cut-off. Also in RDP, the resulting cluster files were used to calculate Shannon and Chao1 and Jaccard and Sørensen indexes, which predict the number of OTUs for each sample and rarefaction curves, which show the number of OTUs for each sample.



**Fig.3.** Output 18S rRNA sequence read lengths versus number of sequence reads for each sample, after quality trimming and removal of sequences <50bps. Red lines represent the cut-off for 18S rRNA sequences, 350 bps. This was chosen to include the majority of sequences.

### *Phylogentic analysis*

Using the non-redundant algal, sponge and other animal sequences, alignments were performed using ClustalW (Thompson et al. 1994) as implemented in BioEdit and refined by eye. Distance matrices were calculated on the bases of p-value and the alignment was collapsed to only contain sequences differing by more than 3%. These were considered the OTUs existent for each subset.

Phylogenetic analyses were performed on the aligned datasets using Maximum Likelihood (ML) in Treefinder version March 2011 (Jobb 2011) using the models selected by Treefinder under the corrected Akaike Information Criterion (AICc) were applied. The models selected were J1, for algae, and for

GTR, for animal and sponge. For the ML analyses of the algal alignment, nodal support was assessed by non-parametric bootstrap analysis with 1000 resamplings.

## **Results**

### *PCR results and Sequence quality*

The 18S rRNA and bacterial 16S rRNA genes were easily amplified for all sample types. The archaeal amplicons were the most difficult to obtain and even after applying the specific protocol for archaea (see Methods), the primer pair N3F and A5710R failed to amplify archaea in the bacterial cell sample. In this sample only the primer pair A2Fa and A5710R allowed successful amplification. Despite the difficulty with amplification of archaeal genes ultimately the lowest quantity of amplicon DNA was produced from the eukaryotic 18 rRNA gene from the bacterial cells and the epibiont sample followed by the bacterial 16S rRNA amplicons from the epibiont sample. The largest quantity of DNA was retrieved from the bacterial 16S rRNA and eukaryotic 18S rRNA amplicons from the sponge cells and the bacterial 16S rRNA amplicons from the seawater sample (Table 1).

In total, 198,057 raw sequences were obtained (Table 2). The epibiont sample yielded most sequences (55,776) and the bacterial cells the fewest (37,221, Table 2). The organism type that yielded the most sequences depended on the sample type, with 18S rRNA amplicons dominating for the seawater and sponge cell samples. The bacterial 16S rRNA had the largest number of reads for the epibiont sample and the archaeal 16S rRNA amplicons yielded the largest number of reads in the bacterial cells fraction. After eliminating all sequences smaller than 50 bps and with low quality, the biggest reduction of numbers were eukaryote reads from all samples, with the highest being the removal of 39% of sequences from the sponge cell sample (Table 2). Bacterial and archaeal reads had very similar reductions with the highest again being from the sponge cell sample (~5%). Archaeal and bacterial 16S rRNA sequences were of very good quality with very few being removed due to poor quality or short reads (Table 2). There

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was no obvious relationship between quantity of DNA in amplicons and number of reads returned.

The bacterial 16S rRNA datasets had the highest number of unique sequences for all four samples except for the bacterial cell fraction (18S rRNA sequences had the highest number here, Table 2). After checking for redundancy and selecting only unique sequences, the most redundant set was the 18S rRNA sequences from the epibiota, and the sponge and bacterial cells with around 65% of sequences being repeats. The least redundant were the archaeal 16S rRNA sequences in the bacterial cell fraction (2% redundancy) followed by the archaeal 16S rRNA sequences in the sponge cell sample (22% redundancy).

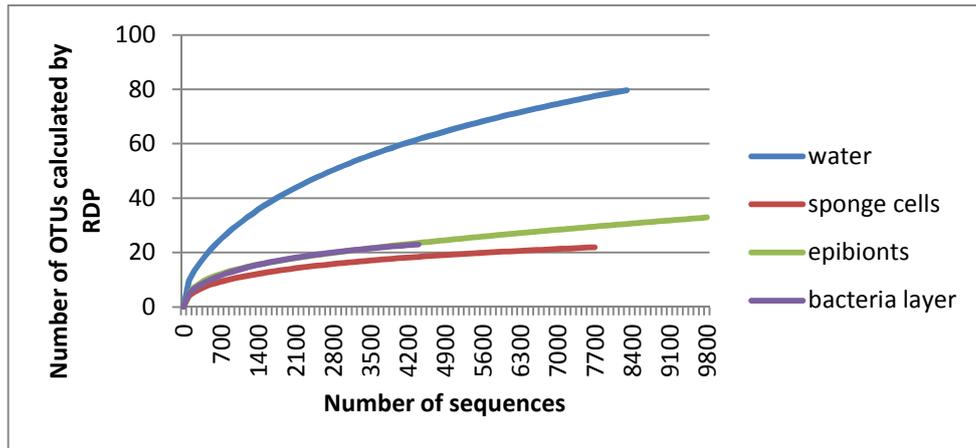
**Table 2:** Number of sequences obtained initially and through the various sequence treatments. The number of sequences after trimming represent all sequences 350bps or longer for eukaryots and 400bps or longer for archaeal and bacterial 16S.

Sample type	Amplicons	# sequences obtained	# sequences >50bp and of good quality	# sequences after trimming (>350bp or >400bps )	# unique sequences (non-redundant set)
Sea water	18S rRNA	21,017	13,495	8,382	6,028
	Archaeal 16S rRNA	15,596	15,277	9,811	7,191
	Bacterial 16S rRNA	15,868	15,527	11,919	8,133
Epibionts	18S rRNA	18,727	14,287	9,872	3,292
	Archaeal 16S rRNA	16,632	16,501	11,613	6,109
	Bacterial 16S rRNA	20,417	20,193	11,794	8,741
Sponge cells fraction	18S rRNA	20,210	12,404	7,745	2,791
	Archaeal 16S rRNA	15,686	14,847	7,410	5,833
	Bacterial 16S rRNA	16,683	15,984	8,232	5,800
Bacterial cells fraction	18S rRNA	14,132	11,996	4,422	1,538
	Archaeal 16S rRNA	14,665	14,381	200	198
	Bacterial 16S rRNA	8,424	8,278	1,100	832

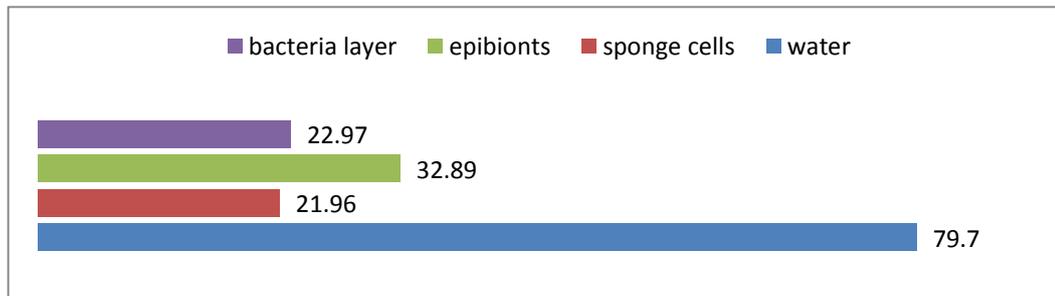
*Diversity of eukaryotic epi- and endobionts of Haliclona indistincta*

From all types of samples (epibiont, sponge and bacterial cells) 7621 unique 18S rRNA gene sequences were obtained (Table 2) of length >350 bp. Of these only six were initially flagged as potentially chimera sequences. Despite the high diversity of eukaryotic sequences, with rarefaction curves approaching asymptotes (Fig. 4), the total number of operational taxonomic units (OTUs) detected at 97% sequence similarity in RDP was ca. 23 in the bacterial, 22 in the sponge cells and 33 in the epibionts; all lower numbers than the 80 OTUs found in the water (Fig. 5). The Shannon-Chao1 richness estimates are slightly higher predicting 25 OTUs for the bacterial cell fraction, 32.5 OTUs for sponge cell fraction, 59.25 OTUs for the epibionts and 94 OTUs for the water column sample. The majority of the OTUs appeared only once (Fig. 6). The OTU represented by the largest number of sequences, in all three samples, was identified as the sponge *Amphimedon compressa* by BLAST against the GenBank database (more details below). In the seawater sample some BLAST accession numbers were represented by more than 100 unique sequences. These included five diatoms (top hits identified as *Skeletonema dohrnii*, 89-100% identity, *Thalassiosira oceanica*, 90-99% identity, *Cerataulina pelagica*, 89-100% identity), and two unidentified in BLAST. They also included two unidentified dinoflagellates, two unidentified picoeukaryotes, an unidentified cryptophyte, two unidentified prasinophyte green algae (top hits identified as Pyramimonadales 91-100% identity, and *Micromonas* sp., 90-100% identity), a cnidarian (top hit identified as *Aurelia* sp. 94-100% identity), a sponge (top hit identified as *Hymeniacidon* sp. 90-100% identity), an unidentified metazoan, an unidentified cercozoan, and two unidentified eukaryotes appearing most often. The seawater sample also had the largest number of possible chimera 18S rRNA gene sequences (164).

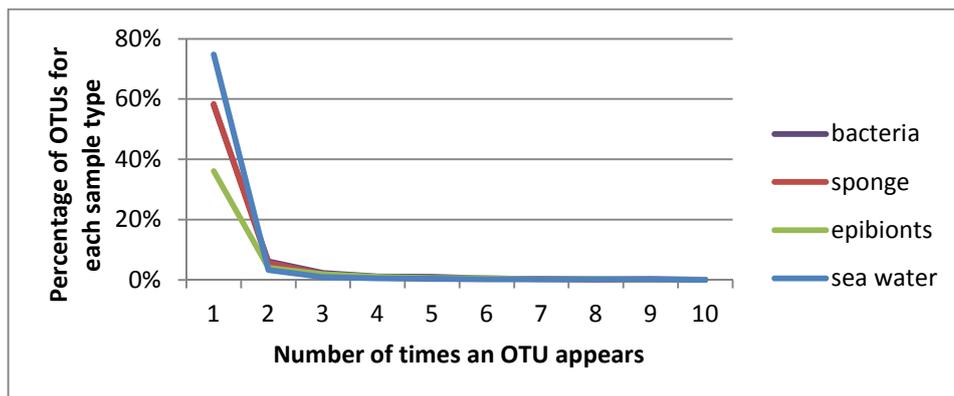
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**Fig. 4.** Rarefaction curves based on operational taxonomic units at a 97% sequence similarity threshold for each sample calculated in RDP.



**Fig. 5.** Number of OTUs with a cut-off of 97% calculated by RDP in this study for each sample.



**Fig.6.** Rank abundance curves based on OTUs at a 97% sequence similarity (as calculated by RDP) for each sample. Only first 10 first ranks are displayed.

*Diversity of photosynthetic epi- and endobionts of Haliclona indistincta*

Of the 94 OTUs (calculated using p-distance matrices, with minimum of 3% difference) found in the three sponge sample types (epibionts, sponge and bacterial cells) 27 were from the following Phyla: Chlorophyta (9), Ochrophyta (5), Rhodophyta (3) and Myzozoa (8). Most of them (20) were found in the epibiont sample with the other samples each having three unique sequences (Fig.7 and 8).

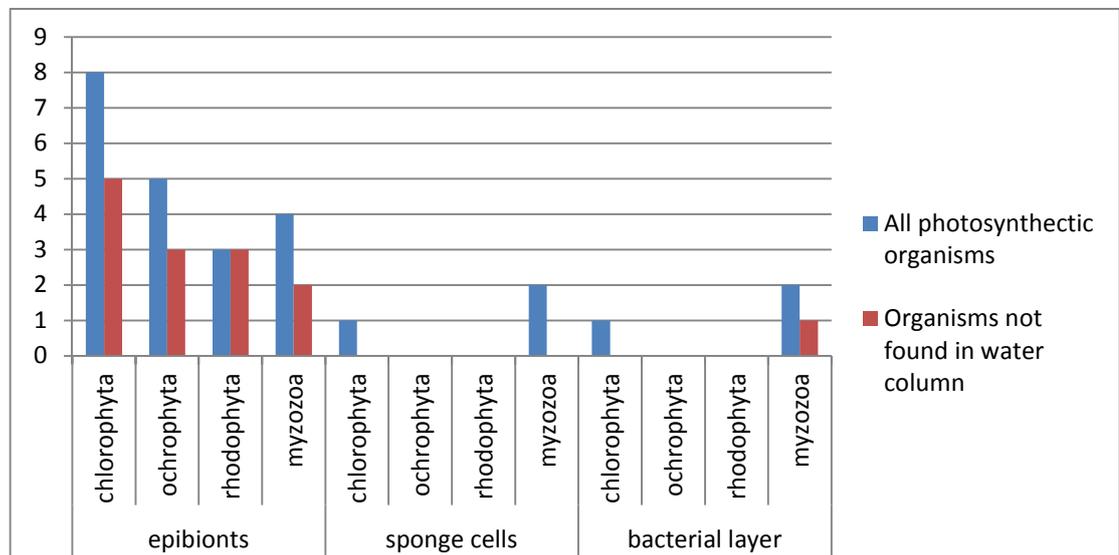
The chlorophytes were represented by one OTU from the bacterial cells fraction, in which only one sequence was found to have closest identity to *Micromonas* via BLAST, and one OTU identified via BLAST as unidentified Prasinophyceae in the sponge cells fraction, also represented by one sequence (Table 4). The epibionts sample yielded three OTUs for which *Ulvaria fusca* (Wittrock) Vinogradova was the top hit via BLAST (represented by nine sequences), others included *Ulva compressa* Linnaeus (only one sequence), *Umbraulva japonica* (Holmes) Bae & I.K.Lee (represented by two sequences), *Pterosperma cristatum* Schiller (only one sequence) and two unidentified OTUs represented by two and one sequences respectively (Table 4, Fig. 8). Of the chlorophytes found, *U. compressa*, *U. fusca* and one unidentified OTU were not found in the seawater sample (Fig. 8).

Overall, eight OTUs classified as dinoflagellates were found in the sponge, with four being present in the epibiontic sample, two in the bacterial cells and two more in the sponge cells (Fig.7 and 8). All these OTUs were represented by unique sequences. Of these, two that were present in the epibionts sample (with closest similarity to *Protoberidinium conicum* (Gran) Balech, and an unidentified eukaryote in GenBank) and one that was present in the bacterial cell fraction (unidentified via BLAST) were not found in the water column (Fig.7 and 8, Table 4).

The other algal phyla were all restricted to the epibiont sample and included, red seaweeds including filamentous (closest similarity via BLAST were *Rhodothamniella floridula* (Dillwyn) Feldmann and *Corallina officinalis*

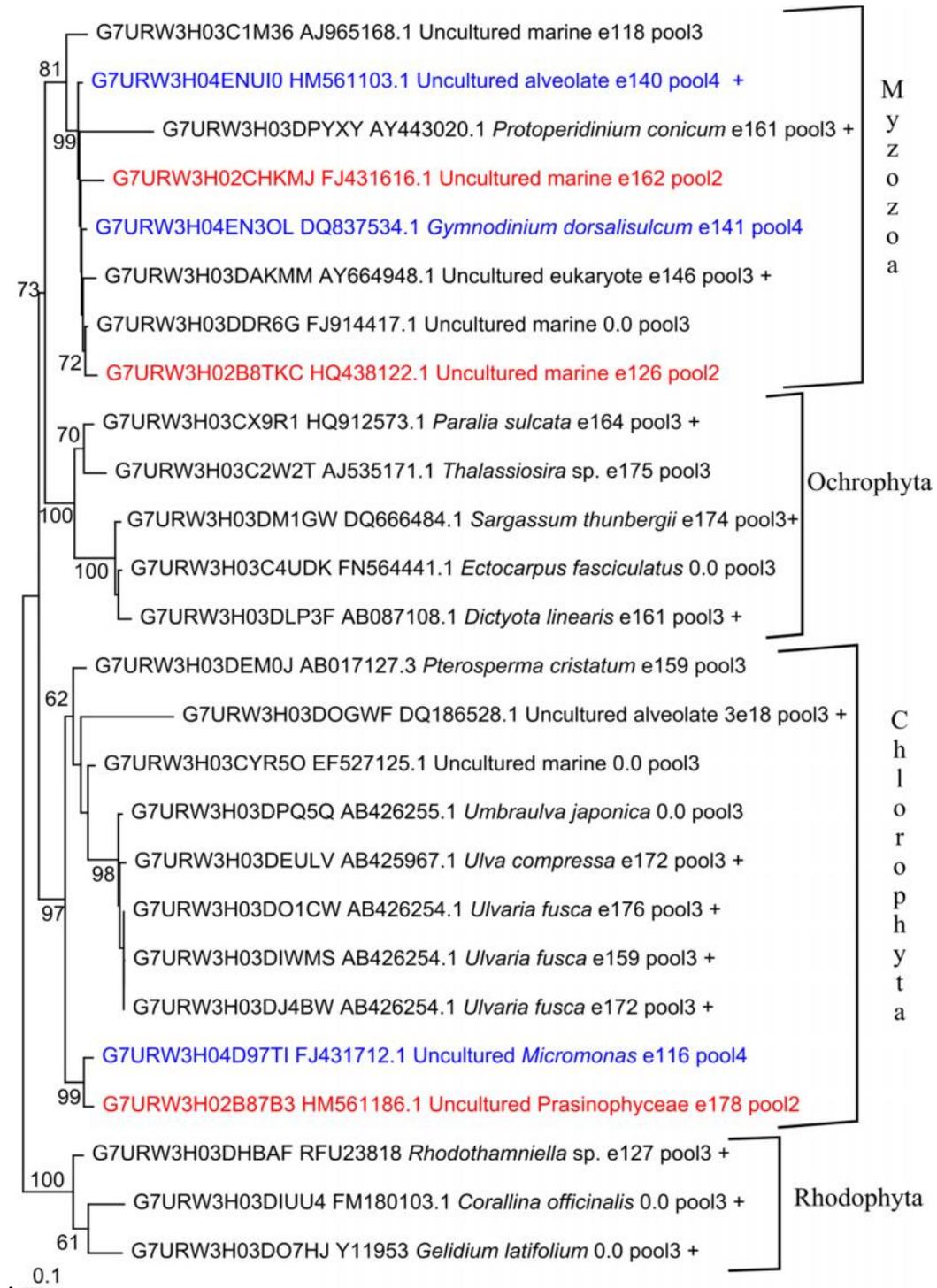
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Linnaeus, both represented by one sequence) and corticated (top hit *Gelidium latifolium* Bornet ex Hauck represented by one sequence), brown seaweeds (top hits *Sargassum thunbergii* (Mertens ex Roth) Kuntze, represented by five sequences, *Dictyota linearis* (C.Agardh) Greville, represented by one and *Ectocarpus fasciculatus* Harvey, represented by four sequences) and diatoms (top hits as *Thalassiosira* sp. and *Paralia sulcata* (Ehrenberg) Cleve, both represented by one sequence, Fig.7 and 8, Table 4). Of these, all the red seaweeds and those similar to *S. thunbergii* and *D. linearis* and *P. sulcata* were not present in the seawater sample (Fig.7 and 8). The phylogenetic tree shows good support for most branches and it is in accordance to known systematic data so far.



**Fig. 7.** Taxonomic distribution of OTUs (as calculated by p-distance with minimum 3% difference). Blue bars represent all photosynthetic eukaryotes found and red bars represent the organisms present only in the referred sample and not in the seawater sample

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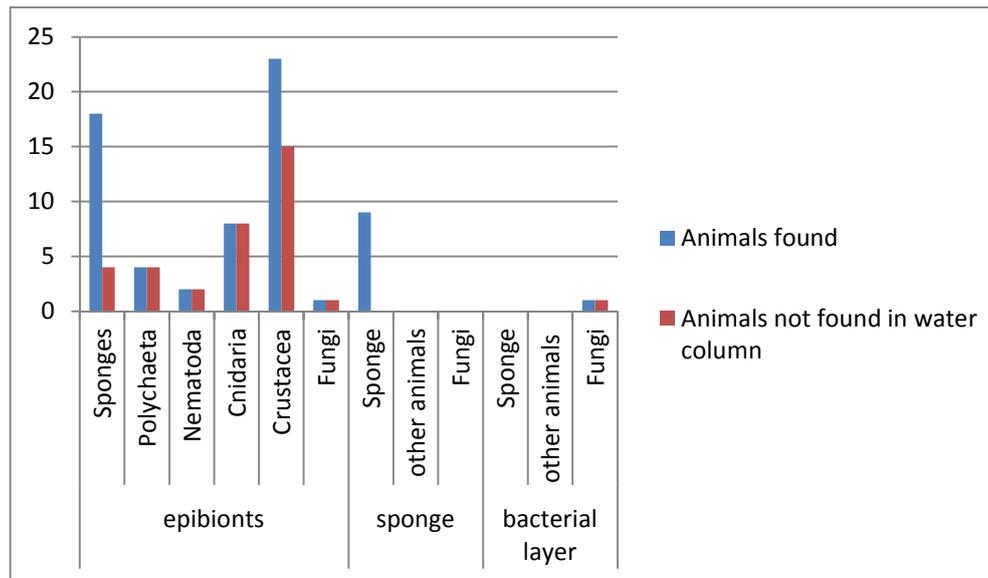
**Fig.8.** Maximum Likelihood tree summarizing which algal OTUs (as calculated by p-distance with minimum 3% difference) were found in the various sponge samples. Each unique sequence is identified by: a code; the GenBank number of the top hit; the name of the species associated with the GenBank number; the e score and the pool in which was found. Pool 2 (in red) represents the sponge cells fraction, pool 3 (in black) represents the epibionts and pool 4 (in blue) represents the bacterial cells fraction. Major algal phyla are indicated on the right. Numbers on branches represent bootstrap support.

**Table 4.** Algal OTUs indicated according to the top hit in Blast, the name and GenBank code of the top hit phyla. Corresponding percentage identities as calculated by BLAST and Phylla are also indicated.

OTU	% Identity	Phyllum
G7URW3H02B87B3_HM561186.1_Uncultured_Prasinophyceae	98%	Chlorophyta
G7URW3H03CYR50_EF527125.1_Uncultured_marine	99%	Chlorophyta
G7URW3H03DEULV_AB425967.1_Ulva_compressa	98%	Chlorophyta
G7URW3H03DIWMS_AB426254.1_Ulvaria_fusca	96%	Chlorophyta
G7URW3H03DJ4BW_AB426254.1_Ulvaria_fusca	98%	Chlorophyta
G7URW3H03DOICW_AB426254.1_Ulvaria_fusca	98%	Chlorophyta
G7URW3H03DOGWF_DQ186528.1_Uncultured_alveolate	79%	Chlorophyta
G7URW3H03DPQ5Q_AB426255.1_Umbraulva_japonica	98%	Chlorophyta
G7URW3H04D97TI_FJ431712.1_Uncultured_Micromonas	92%	Chlorophyta
G7URW3H02B8TKC_HQ438122.1_Uncultured_marine	92%	Myzozoa
G7URW3H02CHKMJ_FJ431616.1_Uncultured_marine	96%	Myzozoa
G7URW3H03C1M36_AJ965168.1_Uncultured_marine	92%	Myzozoa
G7URW3H03DAKMM_AY664948.1_Uncultured_eukaryote	94%	Myzozoa
G7URW3H03DDR6G_FJ914417.1_Uncultured_marine	99%	Myzozoa
G7URW3H03DPYXY_AY443020.1_Protoperidinium_conicum	96%	Myzozoa
G7URW3H04EN3OL_DQ837534.1_Gymnodinium_dorsalisulcum	94%	Myzozoa
G7URW3H04ENU10_HM561103.1_Uncultured_alveolate	95%	Myzozoa
G7URW3H03C2W2T_AJ535171.1_Thalassiosira_sp.	97%	Ochrophyta
G7URW3H03C4UDK_FN564441.1_Ectocarpus_fasciculatus	99%	Ochrophyta
G7URW3H03CX9R1_HQ912573.1_Paralia_sulcata	96%	Ochrophyta
G7URW3H03DEM0J_AB017127.3_Pterosperma_cristatum	96%	Ochrophyta
G7URW3H03DLP3F_AB087108.1_Dictyota_linearis	98%	Ochrophyta
G7URW3H03DM1GW_DQ666484.1_Sargassum_thunbergii	98%	Ochrophyta
G7URW3H03DHBAF_RFU23818_Rhodothamniella_floridula	93%	Rhodophyta
G7URW3H03DIUU4_FM180103.1_Corallina_officinalis	99%	Rhodophyta
G7URW3H03DO7HJ_Y11953_Gelidium_latifolium	99%	Rhodophyta

#### *Diversity of non-photosynthetic epi- and endobionts of Haliclona indistincta*

Of the 94 OTUs (calculated using p-distance matrixes, with minimum of 3% difference) found in the three sponge sample types (epibionts, sponge and bacterial cells) 67 were from the following groups: sponges (27), non-sponge animals (38) and fungi (2, Fig. 9). The fungal OTUs were present in the epibionts sample (with top hit identified as *Magnaporthe salvinii* (Catt.) R. A. Krause & R. K. Webster, 97% identity, only 1 sequence) and in the bacterial cells fraction (with top hit identified as a Basidiomycota species, 94% identity, only 1 sequence). Neither species were not found in the water column.



**Fig. 9.** Taxonomic distribution of OTUs (as calculated by p-distance with minimum 3% difference). Blue bars represent all non-photosynthetic eukaryotes found and red bars represent the organisms present only in the referred sample and not in the seawater sample.

All animals (excluding sponges) were found only in the epibionts sample and not in the other sponge sample types (Fig. 9 and 10). The animal groups found were Polychaeta (4 OTUs), Nematoda (2 OTUs), Cnidaria (8 OTUs), Crustacea (24 OTUs, Fig. 9 and 10). Of these, some were not found in the water column; these include all four polychaetes, the two nematodes, six cnidarians (with top hits of *Hydractinia echinata* Fleming, *Dipurena gemnifera* Forbes, *Sarsia princeps* Haeckel, and three *Turritopsis* sp., see Table 5) and 15 maxillopods (three with top hit of *Paramphiascella fulvofasciata* Rosenfield and Coull, three of *Argestigens* sp., two of *Ameira scotti* Sars, *Cancricola plumipes* Humes, *Atheyella crassa* Sars, *Dactylopusia* sp, *Harpacticus* sp., *Euryte* sp., *Itunella muelleri* Gagern, *Diarthrodes* sp. and *Aphotoponthius mammilatus* Humes, Fig. 10, Table 5). Most OTUs were represented by fewer than five sequences, with the exceptions of the cnidareans *Garveia grisea* Motz-Kossowska OTU G7URW3H03CZK9I with 33 sequences and *Turritopsis* sp OTU G7URW3H03DNV9H with 42 sequences; and the copepods *P. fulvofasciata* OTU G7URW3H03C8HYO with 53 sequences, *Dactylopusia* sp. with 16 sequences, *Diarthrodes* sp. with 15 sequences, *Stenopontius* sp. OTU G7URW3H03DNMK5 with 140 sequences.

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**Fig. 10.** Maximum Likelihood tree summarizing non-sponge animal OTUs (as calculated by p-distance with minimum 3% difference). Each OTU is identified by a code, the GenBank number of the top hit, the name of the species associated with the GenBank number, the E score and the pool in which was found. In this case all OTUs were found in pool 3 (in black) the epibionts sample. Major animal groups are indicated on the right. No bootstrap was calculated.

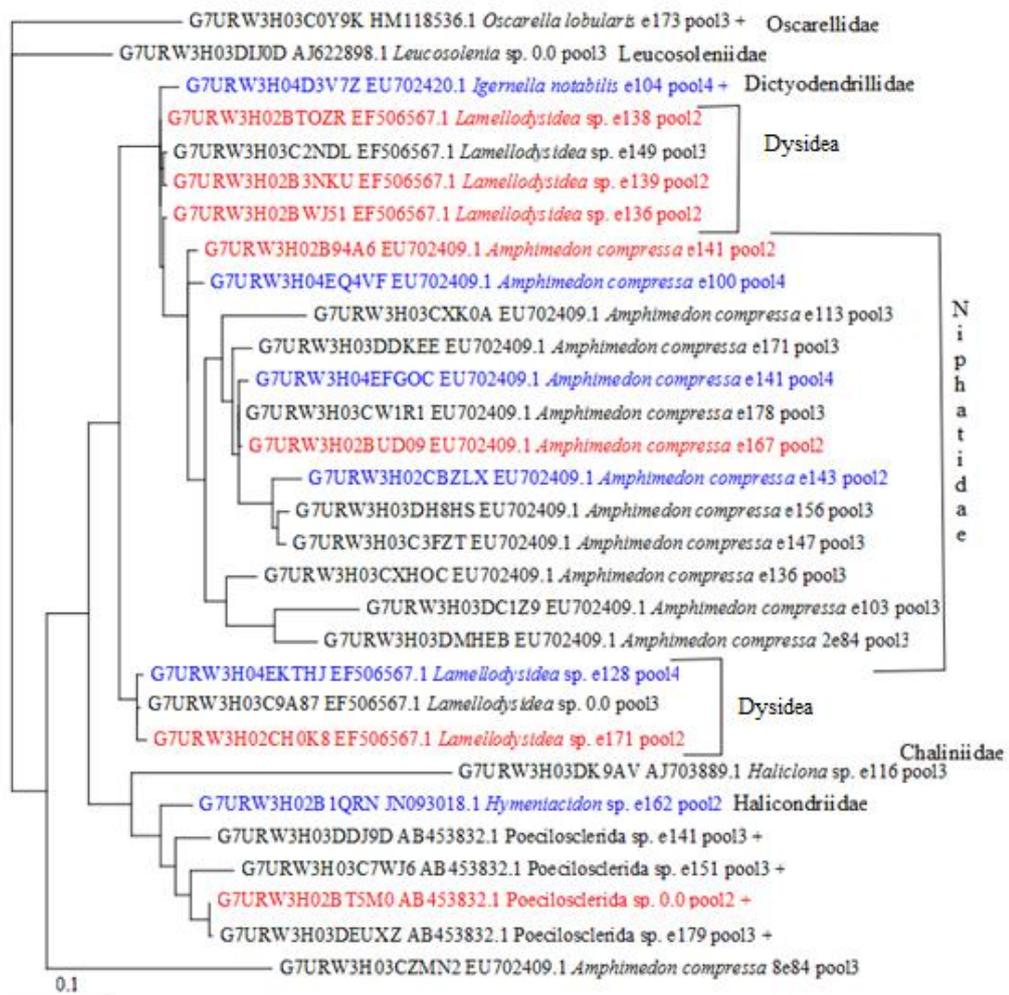
Part C: Seaweeds and Sponges

**Table 5.** Non-sponge animal OTUs indicated according to the top hit in Blast, the name and GenBank code of the top hit phyla. Corresponding percentage identities as calculated by BLAST and Class are also indicated.

OTUs	Class / Order	Identities percentages
G7URW3H03CYY3W FJ153780.2 Uncultured eukaryote		94%
G7URW3H03C4XFK HM564614.1 <i>Syringolaimus</i> sp	Adenophorea / Enoplida	95%
G7URW3H03DHQ8O EF659823.1 Uncultured <i>Rhabdolaimus</i>	Adenophorea / Chromadorida	82%
G7URW3H03CZK9I EU272632.1 <i>Garveia grisea</i>	Hydrozoa / Anthoathecata	98%
G7URW3H03DLJGR EU305504.1 <i>Turritopsis</i> sp.	Hydrozoa / Anthoathecata	96%
G7URW3H03DDNUB EU876573.1 <i>Dipurena gemmifera</i>	Hydrozoa / Anthoathecata	96%
G7URW3H03DKQJM EU305504.1 <i>Turritopsis</i> sp.	Hydrozoa / Anthoathecata	94%
G7URW3H03DL76D AY920763.1 <i>Hydractinia echinata</i>	Hydrozoa / Anthoathecata	96%
G7URW3H03DNNV9H EU305504.1 <i>Turritopsis</i> sp.	Hydrozoa / Anthoathecata	98%
G7URW3H03DOBCH EU876575.1 <i>Sarsia princeps</i>	Hydrozoa / Anthoathecata	97%
G7URW3H03DQXU AY626996.1 <i>Euryte</i> sp.	Maxillopoda / Cyclopoida	99%
G7URW3H03C0NYZ EU380303.1 <i>Ameira scotti</i>	Maxillopoda / Harpacticoida	95%
G7URW3H03C3D8O EU380293.1 <i>Paramphiascella fulvofasciata</i>	Maxillopoda / Harpacticoida	93%
G7URW3H03C3HUL EU380310.1 <i>Eurycletodes laticauda</i>	Maxillopoda / Harpacticoida	90%
G7URW3H03C6MII L81938.1 <i>Cancrincola plumipes</i>	Maxillopoda / Harpacticoida	89%
G7URW3H03C70GR EU380309.1 <i>Itunella muelleri</i>	Maxillopoda / Harpacticoida	93%
G7URW3H03C7HRJ EU380295.1 <i>Dactylopusia</i> sp.	Maxillopoda / Harpacticoida	90%
G7URW3H03C8GUZ EU380293.1 <i>Paramphiascella fulvofasciata</i>	Maxillopoda / Harpacticoida	89%
G7URW3H03C8HYO EU380293.1 <i>Paramphiascella fulvofasciata</i>	Maxillopoda / Harpacticoida	96%
G7URW3H03DEIGD L81938.1 <i>Cancrincola plumipes</i>	Maxillopoda / Harpacticoida	94%
G7URW3H03DEBXU EU380307.1 <i>Attheyella crassa</i>	Maxillopoda / Harpacticoida	93%
G7URW3H03DGU2X EU380306.1 <i>Argestigens</i> sp.	Maxillopoda / Harpacticoida	94%
G7URW3H03DIP9X EU380309.1 <i>Itunella muelleri</i>	Maxillopoda / Harpacticoida	94%
G7URW3H03DLYBF EU380306.1 <i>Argestigens</i> sp.	Maxillopoda / Harpacticoida	90%
G7URW3H03DMMN9 EU380285.1 <i>Harpacticus</i> sp.	Maxillopoda / Harpacticoida	99%
G7URW3H03DORDK EU380297.1 <i>Diarthrodes</i> sp.	Maxillopoda / Harpacticoida	94%
G7URW3H03DPLL3 EU380306.1 <i>Argestigens</i> sp.	Maxillopoda / Harpacticoida	89%
G7URW3H03DNT9R EU380303.1 <i>Ameira scotti</i>	Maxillopoda / Harpacticoida	96%
G7URW3H03DATG8 DQ538508.1 <i>Aphotopontius mammillatus</i>	Maxillopoda Siphonostomatoida	92%
G7URW3H03DJ1N7 AY627022.1 <i>Stenopontius</i> sp.	Maxillopoda Siphonostomatoida	90%
G7URW3H03DN9HI AY627022.1 <i>Stenopontius</i> sp.	Maxillopoda Siphonostomatoida	91%
G7URW3H03DNMK5 AY627022.1 <i>Stenopontius</i> sp.	Maxillopoda Siphonostomatoida	94%
G7URW3H03DF3KW EF123886.1 <i>Exogone naidina</i>	Polychaeta / Phyllococida	92%
G7URW3H03DHXPU AY996083.1 <i>Eumida sanguinea</i>	Polychaeta / Phyllococida	99%
G7URW3H03DPWTX AY611455.1 <i>Polydora giardi</i>	Polychaeta / Spionida	97%
G7URW3H03DAV7E AY894300.1 <i>Lepidonotus squamatus</i>	Polychaeta / Phyllococida	98%

The sponge families which were found were Chaliniidae (1 OTU), Halichondriidae (1 OTU), an unidentified poecilosclerid (4 OTUS), Dysidea (7 OTUs), Niphatidae (14 with 13 grouping together), Oscarelliidae (1 OTU) and Leucosoleniidae (1 OTU, Fig. 11). Of only the these, reads with closest identity Poecilosclerida, *Oscarella lobularis* Schmidt and *Igernella notabilis* Duchassaing & Michelotti OTUs were not found in the water column (Fig. 11, Table 6). All OTUs were represented by one sequence with the exception of Poecilosclerida G7URW3H02BT5M0 being represented by 30 and the OTU *Amphimedon compressa* Duchassaing G7URW3HO2BUD09 being represented by more than 7000 sequences. *Amphimedon compressa* comprised 14 OTUs (13 grouping together) distributed between all sponge sample types (Table 6).

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**Fig. 11.** Maximum Likelihood tree summarizing sponge OTUs (as calculated by p-distance with minimum 3% difference). Each OTU is identified by a code, the GenBank number of the top hit, the name of the species associated with the GenBank number, the E score and the pool in which it was found. Pool2 (in red) represents the sponge cells fraction, pool 3 (in black) the epibionts and pool 4 (in blue) the bacterial cells fraction. Sponge family names are indicated on the right. No bootstrap was calculated.

**Table 6.** Sponge OTUs and their identity percentages as calculated by BLAST.

OTUs	Identity percentages
G7URW3H02B94A6_EU702409.1_Amphimedon_compressa	94%
G7URW3H04EQ4VF_EU702409.1_Amphimedon_compressa	89%
G7URW3H03CXK0A_EU702409.1_Amphimedon_compressa	91%
G7URW3H03DDKEE_EU702409.1_Amphimedon_compressa	97%
G7URW3H04EFGOC_EU702409.1_Amphimedon_compressa	94%
G7URW3H03CW1R1_EU702409.1_Amphimedon_compressa	94%
G7URW3H02BUD09_EU702409.1_Amphimedon_compressa	97%
G7URW3H02CBZLX_EU702409.1_Amphimedon_compressa	94%
G7URW3H03DH8HS_EU702409.1_Amphimedon_compressa	95%
G7URW3H03C3FZT_EU702409.1_Amphimedon_compressa	95%
G7URW3H03CXHOC_EU702409.1_Amphimedon_compressa	93%
G7URW3H03DC1Z9_EU702409.1_Amphimedon_compressa	88%
G7URW3H03DMHEB_EU702409.1_Amphimedon_compressa	86%
G7URW3H03DK9AV_AJ703889.1_Haliclona_sp.	97%
G7URW3H02B1QRN_JN093018.1_Hymeniacion_sp.	96%
G7URW3H04D3V7Z_EU702420.1_Igernella_notabilis	90%
G7URW3H03C9A87_EF506567.1_Lamellodysidea_sp.	98%
G7URW3H04EKTHJ_EF506567.1_Lamellodysidea_sp._	94%
G7URW3H02CH0K8_EF506567.1_Lamellodysidea_sp.	97%
G7URW3H02BWJ51_EF506567.1_Lamellodysidea_sp.	93%
G7URW3H02BTOZR_EF506567.1_Lamellodysidea_sp.	94%
G7URW3H03C2NDL_EF506567.1_Lamellodysidea_sp.	94%
G7URW3H02B3NKU_EF506567.1_Lamellodysidea_sp.	93%
G7URW3H03DIJ0D_AJ622898.1_Leucosolenia_sp.	98%
G7URW3H03C0Y9K_HM118536.1_Oscarella_lobularis	97%
G7URW3H03DDJ9D_AB453832.1_Poecilosclerida_sp.	93%
G7URW3H03C7WJ6_AB453832.1_Poecilosclerida_sp.	94%
G7URW3H02BT5M0_AB453832.1_Poecilosclerida_sp.	98%
G7URW3H03DEUXZ_AB453832.1_Poecilosclerida_sp.	98%

### Discussion

*Comparing this study with previous epibiont morphological studies from this sponge*

In the previous chapter, the whole visible epibiont macroalgal community was examined for this sponge (C2). The two most prevalent species reported in the previous chapter (C2) were *R. floridula* and *G. spinosum*. *Rhodothamniella floridula* was also the name of a top hit found, in the NGS study, in the epibionts sample (Fig.8). Although there was only one sequence with 93% identity, it is highly probable that this is the sequence of the species which was found in most of the samples collected in chapter C2. It might be that the sequence deposited in Genbank was misidentified by the authors. This GenBank sequence belongs to culture CCAP1360/6, which doesn't seem to exist anymore in the culture

collection.

The *Gelidium* species which appeared here was a *G. latifolium*, with 99% identity (Fig.8) which is the name previously used for *G. spinosum*. So it is more than likely that this sequence belongs to the species we have identified as such in the previous chapter (C2). In both cases it is surprising that only one sequence with good quality was retrieved for the species' which were found to be frequently associated with this sponge (C2). In general, algae OTUs were amplified in a much lower number than animal OTUs. These primers were tested previously to this study on different types of algae and animals and they worked. They were originally designed to sequence red seaweeds. In the seawater samples, some of the best represented OTUs in numbers of sequences were algae, with five diatoms, two dinoflagellates, two unidentified picoeukaryotes, unidentified cryptophytes and two unidentified chlorophytes having more than 100 sequences. Perhaps, the DNA extraction of the epibionts and sponge cell fraction was biased in that the animal and sponge DNA was recovered in better and larger quantities than that of the algae DNA.

The other macroalgae identified in this study were three green algae (Chlorophyta) and three brown macro-algae (Ocrophyta). The three green algae OTUs were identified by top hits with names of species which were not found in the previous study (C2). However *Ulvaria fusca* and *Ulva compressa* are both species common to the Irish coast and hard to identify morphologically. These names potentially hide more diversity, since *Ulvaria fusca* was the top hit of three different OTUs with different identity percentages from 96 to 98%. *Ulva*-like organisms were visible in the sponge material, so these sequences are likely to represent organisms which were in fact present on the sponge. In the previous study, it was impossible to identify this group to the species level. And, again, using this marker did not allow identification of the species, but it allowed understanding of the possible diversity within this group existent in one sponge specimen.

The brown macro-algae species (Ocrophyta) was identified by the following top hits species names: *Sargassum thumbergii*, which was not found in

the previous chapter and does not exist in Ireland. Since it is one of the few species of seaweed represented in the epibionts sample with multiple sequences, it seems certain that a congener (the identity was only 98%) was present on this sponge. Probably it was *S. muticum* (Yendo) Fensholt which is a known invasive species and is common in this region. *Ectocarpus fasciculatus* is common on the west coast of Ireland (Guiry and Guiry 2013). It is reasonable to assume that this is one of the *Ectocarpus* spp. which it was not possible to identify morphologically to the species level in the previous study (C2), especially since it has a high identity percentage. *Dictyota linearis* is more common in the south of Europe. This is not *D. dichotoma*, a species found in chapter C2, since this species has an available 18S rRNA sequence in Genbank and this sequence only comes as 97% identical. However it could represent another *Dictyota* species which has not yet been sequenced for this gene.

The morphological identification of visible animals associated with this sponge species was attempted by Murphy (unpublished). There were only three phyla that were both found in this NGS study and in the morphological observation study: the polychaetes, crustaceans and nematodes (Fig. 10). Murphy (unpublished) found at least seven different polychaete species, with two possibly being the same as the OTUs found in this study: *Polydora cornuta* Bosc and *Lepidonotus clava* Montagu. There are no 18S rRNA sequences of *P. cornuta* available in GenBank and the percentage identity of the OTU sampled in *H. indistincta* in this study to the *P. giardi* GenBank sequence was 97%, which would be consistent with the sequence sampled being a congener. *Lepidonotus clava* does have an 18S rRNA sequence available in GenBank and the read generated from this study did not return this in the BLAST search. The read had a percentage identity of 98% to the *L. squamatus* sequence which was generated from a specimen from the United States which would mean that this was either *L. clava* or a very close species.

Only one unidentified nematode was found by Murphy (unpublished), whereas in this study, two were found. It is unlikely that the species reported here is a *Rhabdolaimus* sp. due to its low identity percentage but the other OTU is likely to

be a *Syringolaimus* sp. The two groups that were missed by Murphy (unpublished) and found here were Hydrozoans and Maxillopoda (although another crustacean group was found, malacostraca) (Fig. 10). Maxillopoda included the most variety of OTUs (23). Some of them have low identity percentages and so it is not possible to assess whether all OTUs belong to the order Copepoda. The OTUs which can be identified with more certainty are *Harpacticus* sp. and *Euryte* sp.

Copepodes and hydrozoans are very small, with copepods generally varying between 1 and 2 mm and hydrozoans, especially in the polyp phase, varying between 1 mm and 10 cm (Barnes 1982). Therefore it is not strange for an inexperienced person to have missed them. In fact, when identifying the seaweeds associated with the sponge in the previous study, we observed many organisms that potentially could appear to be seaweed to an inexperienced person but were in fact hydrozoans. Murphy (unpublished) observed a gastropod, two starfish and three malacostracan crustaceans which were not identified in this study. Nonetheless, these were only seen sporadically in one sample each and it is possible that these species are not usually associated with the sponge. Many OTUs were not found in the water column. Nonetheless this is not surprising since no animals were visible in the 2 L water sample and many of these organisms are benthic with the exception of perhaps the nematodes.

This study has demonstrated that there is a surprising diversity of animals living on this sponge. In another study in which the sponge *Halichondria panacea* was collected during a period of two years and the associated fauna was registered, species of nematodes, annelids, crustaceans, pyenogonids, echinoderms and fish were found, but none were sponge specific (Frith 1976). Murphy (unpublished) observed some seasonality in the species collected, with more diversity and higher numbers found in winter months. He suggests that animals could use the sponge as shelter. The sponge in this study was collected in May and so exposed to milder weather conditions than in winter. Nonetheless, many species seemed to use the sponge collected here for the NGS study as a habitat. The fact that these sponges have a high diversity of algae could mean that

these animals are just associated with the sponge because they use the different types of algae as food.

One group which was not previously investigated for sponge species were associated fungi (not present in surrounding seawater). Two fungal OTUs were found associated with this sponge. One in the epibiont sample, with top hit identified as *Magnaporthe salvinii* and another in the bacterial cells fraction (with top hit identified as Basidiomycota species). *Magnaporthe salvinii* is a fungal plant pathogen and therefore not marine (Gutierrez and Mazanti 2012). It would have to be another phylogenetically close relative since the percentage identity was 97%. Due to the fact that this OTU was found in the epibiont sample, it could have been associated with one of the other epibionts. Neither species was found in the water column and it would be interesting to further identify them. Although the 18S rRNA gene is also used as a barcode for fungi (Mahé et al. 2012), ITS is considered the barcoding gene for this group (Toju et al. 2012) and has more sequences than 18S rRNA already deposited, with around one million at the time of writing (GenBank). The basidiomycote could be an endosymbiont of the sponge. Microscopic observations would be required to understand the size of this organism. It could also be that spores of the fungus have been filtered from the water entering the sponge tissues, thus having no active role in the biology of the sponge. However, they were evidently not present in high numbers in the water column; otherwise they would have been detected.

Recently, a research group recovered 17 genera of fungi from the sponges *Clathrina luteoculcitella* and *Holoxea* sp. with at least one genus belonging to the phylum Basidiomycota. Of these, eight were novel marine fungi, 111 strains were culturable and one strain had a broad anti-bacterial spectrum (Ding et al. 2011). Fungi found in sponges are thought to be involved in a variety of ecological functions, including production of secondary metabolites that can be self-beneficial and beneficial to their hosts (Paz et al. 2010 and references within). Some of the chemicals produced by these microorganisms have been shown to exhibit anti-predation, anti-competition and anti-fouling capabilities (Hay 1996, Lopanik et al. 2004). Understandably, the number of studies of sponge-associated

fungi has increased in recent years with new natural products being found (e.g Bugni and Ireland 2004, Jadulco et al. 2002, 2004, Morrison- Gardiner 2002).

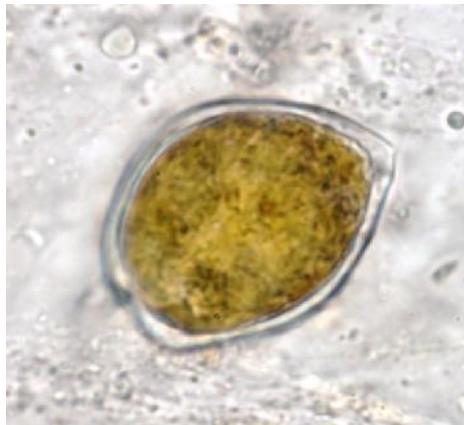
*Microscopic organisms associated with this sponge*

The previous study did not focus on microalgae, although these can have important roles in the ecology of the sponge, as food (Duckworth et al. 2003, Duckworth and Pomponi 2005), as symbionts (Lee et al. 2001) responsible for secondary metabolite production, or simply as foulers (Wahl 1989, Cerrano et al. 2000).

Secondary metabolites isolated from sponges have been found to be produced by dinoflagellates living within the sponge. For example, okadaic acid was initially isolated and characterized from the sponges *Halichondria okadai* and *H. melanodocia* (Tachibana et al. 1981). Now, its source and that of its analogs is accepted to come from dinoflagellates belonging to the genera *Prorocentrum* and *Dinophysis* (Yasumoto *et al.*, 1987; Zhou and Fritz, 1994; McLachlan *et al.*, 1997). The function of this acid in sponges is not well understood. Studies by Wiens *et al.* (2003) have provided evidence for at least two putative roles of OA within the sponge *Suberites domuncula*. At low concentrations it acts as anti-bacterial, at higher concentrations it acts as an apoptogen. Further studies suggested that it may serve as a defense molecule by inducing apoptosis in symbiotic or parasitic annelids. It has been hypothesized that because *Prorocentrum* spp. are generally associated with benthic organisms such as seagrasses and macroalgae, grazers foraging on these items may incidentally ingest the microalgae while feeding and thus be exposed to OA (Landsberg *et al.*, 1999).

Of all the groups sequenced in this study, the dinoflagellates have the most potential to include true symbionts. In this study, at least eight OTUs were found which belonged to dinoflagellates. Only two give a suggestion of what species they could represent: *Protoperidinium conicum* and *Gymnodinium dorsalisulcum*. Unfortunately many of the available 18S rRNA sequences for dinoflagellates are of unidentified species and so this is not a very good barcoding gene. Nonetheless,

it is possible to see that there is diversity of dinoflagellates available in this sponge and four of them are endobionts found amongst the sponge cells and the bacterial cells. *Gymnodinium dorsalisulcum* is about 40 µm in radius (Encyclopedia of life, <http://eol.org>) and sponge cells are about 1-2µm (Stephens submitted), so it is more probable that these dinoflagellates occur between the cells and do not live intracellularly. In a microscopic observation of sponge tissue collected from this sponge (performed only once) a high density of dinoflagellate cells was observed. They appeared to be *Prorocentrum micans* and they were also visible between the cells (Fig. 12). None of the dinoflagellate species, found in the NGS study, belong to same order of *P. micans* and so this species was not captured here. The sponge which showed *P. micans* was sampled in October and the sponge used in this study was sampled in May; this could explain the different dinoflagellate species identified. As seen in the previous chapter there is some temporal variation of the macroalgal community associated with the sponge, so similar variation can also be expected for the microalgae.



**Fig. 12** Dinoflagellate observed in a sponge cell smear collected from *H. indistincta* collected in October 2009. At the time, hundreds of cells of apparently the same species were observable in between the sponge cells.

To confirm which species were associated with this sponge, further microscopic observations with *in situ* hybridisation and sequencing of other markers such as the Internal Transcribed Spacer (ITS) would be necessary. Also, although a positive bias towards this group has been reported, since these species contain multiple copies of 18S rRNA (Dyal et al. 1995, Zhu et al. 2005), this bias

has not been seen in this study, as once again, most OTUs were only present once. It is important to pursue the identification of these organisms and understand whether they are really present and whether they are occasional associations or true symbionts. One possible way to do this would be to use specific primers for this group and by qPCR quantify the diversity present in various samples from different seasons.

Two diatom OTUs were identified in the epibiont sample. Curiously, these type of organisms although of similar size to dinoflagellates and green unicellular algae found in the other samples, were not found either in the sponge or bacterial cells fractions (Fig. 8). Large numbers of reads from putative *Paralia sulcata* and a *Thalassiosira* species, as well as other diatom species were found in the water column. The two organisms are centric diatoms and therefore commonly found in the water column along the Irish coast (Guiry and Guiry 2013). It is possible that this sponge actively protects itself against this group or as stated in the introduction, there is a negative bias in the extraction of diatom DNA. This last hypothesis would not explain why there were at least five diatom species with more than 100 sequences in the seawater sample. Bioactives derived from sponges have already been proven to be effective anti-foulers against diatoms (Dobretsov et al. 2005, Ortlepp et al. 2008).

At least three single cell green algae OTUs were found in the three sponge samples. The three are all species or groups referable to the assemblage of unicellular green algae called prasinophytes, the most primitive representatives of the chlorophyten lineage of Viridiplantae (Leliaert et al. 2012). These algae are common members of the marine phytoplankton, even though their abundance may be underestimated due to their small size. Members of *Micromonas* (which was a top hit for one of the sequences recovered) belong to the picoplankton (<2 µm) and have become popular model organisms due to the extreme reduction of their cell structure and genome (Brussaard et al. 2004).

### *Sponge OTUs*

Attempts to generate quality DNA sequences of the 18S rRNA and mitochondrial *cox 1* genes from the cushion form of this sponge have failed so far (Murphy,

unpublished; Stephens, unpublished). Usually evidence of a mixed template is returned (McCormack, personal communication; Murphy, unpublished). Even cloning has failed to provide good quality mt *cox1* sequences with all retrieved sequences being non sponge in origin (McCormack personal communication). Since no other 18S rRNA gene sequences are available for *H. indistincta*, the one recovered here would be the first sequence of this gene for this species. The percentage identity varied between 86 and 97%, which are percentages ranging from the ones found between different genera to between close species. However, the read with highest frequency is the true sequence for this sponge. We might expect one congener to be present (*H. viscosa*) but otherwise no other sponge collected in Ireland in the west coast is closely related to *H. indistincta*. Therefore, most likely the sequence divergence represents intragenomic variability in 18S rRNA. There has not been any report of intragenomic variability in 18S rRNA in sponges but there have been cases of other animals such as cephalopods and a platyhelminth (Bonnaud et al. 2002 and references within), and it is known to occur with ITS rRNA (Redmond and McCormack 2009, Worheide et al. 2004). This variability can also be explained by errors during PCR and sequencing and PCR chimaera formation as explained below.

Other sponge OTUs were found in the epibiotic, sponge cell and bacterial cells fractions. Some of these were not present in the water column. These sequences of different sponges could come from larvae, sperm or dead tissue of other sponges present in the area but missed in the water column sample. Sponges reproduce by sperm being carried in to the sponge from the water column and fertilising an internal egg. So, sperm could be present in the water column. However this sponge and the seawater were collected in May, which does not correspond to the reproductive period known for this species (Stephens unpublished). It should be remarked, however, that many sponge life cycles are not known. More probably the origin of this DNA is due to fragments of sponges, since these organisms can reproduce by fragmentation or can be attacked by predators.

*Comparison with other eukaryotic NGS studies*

Other eukaryotic NGS studies have targeted meiofaunal organisms and fungi from diverse habitats such as tropical forest, marine environments (references within Creer et al. 2010, Stoeck et al. 2010) and one single tree (Jumponen et al. 2010). Meiofaunal organisms include animals that pass through a 0.5-mm sieve but are retained on 25-65 mm sieves (Creer et al. 2010). We can consider that most animals sequenced here are included in this category and so it is possible to compare these studies. As in here, all these studies defined cut-offs ranging from 95 to 99% to define their OTUs (Creer et al. 2010, Jumponen et al. 2010, Stoeck et al. 2010). With the exception of the fungi NGS study which used ITS rRNA (Jumponen et al. 2010), all studies have used some part of 18S rRNA as the marker of choice (references within Creer et al. 2010, Stoeck et al. 2010).

Interestingly, high percentages of sequences only appeared once in the various studies like 57% in the study using ITS rRNA for fungi (Jumponen et al. 2010), 75% using the V4 region of 18S rRNA for anoxic marine eukarya and 68% using the V9 region for the same group (Creer et al. 2010). This is in accordance with what we have seen in this study with an approximate 40% of OTUs being represented by one sequence for the sponge cell and bacteria cell fractions and epibionts. In the seawater sample only 4% of accession numbers were represented by one sequence. It is still debatable whether these proportions refer to large fractions of rare genotypes, whether these samples are represented in low numbers due to some DNA extraction and PCR bias or whether these are artificial OTUs.

There are three potential sources of error: the combined effects of nucleotide misincorporation and read errors during PCR and sequencing, PCR chimaera formation, and intragenomic polymorphism among multiple copies of the rRNA cistron within a single nucleus (Stoeck et al. 2010). Taking as an approximation the calculations of Stoeck et al. (2010), one can have a minimum of 0.676 nucleotide changes due to error per V4 sequence read (of 270 and 200 bp average length). 454 sequencing error rates can be higher in gene regions where homopolymers (runs of the same nucleotide) occur. Our study didn't seem to have a large number of chimaeras compared to others (35 to 49%, Cree et al. 2010) but

our initial values may possibly be an under-representation and we are still evaluating more stringent ways to identify chimaeras. Some studies report a more similar percentage compared to us, 0.4% for recent control experiments on artificial nematode communities (Porazinska et al. 2009). Therefore, it is difficult to assess how much is artifact error and how much is biological diversity.

Bias in numbers of certain groups of organisms caused by DNA extraction, amplification and sequencing should also be taken into account when interpreting diversity results in NGS studies. Larger cells may have higher levels of intragenomic polymorphism than smaller cells, as has been observed in large-celled Foraminifera (Pawlowski et al. 2002). The taxonomic groups producing the highest number of unique tags in the marine anoxic samples were the dinoflagellates and chlorophytes, which are known to contain many large-celled taxa, and have been shown to contain many rRNA cistron copies (Zhu et al. 2005). The same is true for diatoms, in which intragenomic SSU polymorphism levels of c. 0.5% -2% have been reported (e.g. Alverson and Kolnick 2005). In our study diatoms were present in low numbers in and on the sponge but not in the seawater, reinforcing that the numbers of diatom OTUs associated with the sponge have a biological significance. In the meiofaunal study in a marine environment (Cree et al. 2010), certain animal groups were missed altogether like Cnidaria, Nemertea, Rotifera, Brachiopoda and Echinodermata. They suggest that with the exception of cnidarians, these should have amplified if genomic DNA was available in the PCR reaction. Therefore, these phyla were either not present in these samples or competitive PCR interactions (von Wintzingerode et al. 1997) may have prevented amplification of the missing phyla. We also did not sequence some of these groups but since they were not observed or only observed sporadically in the morphological study, we may assume they were not present. Cree et al. 2010 also report very few fungal sequences which they explain by a lack of suitability for the primers used. This may also be the case here.

An analysis of all available 16S and 18S rRNA sequences obtained from sponges (from Sanger sequencing projects) revealed that of 95 organisms which included diatoms, dinoflagellates and fungi, 14% belonged to sponge-specific

clusters (Simister et al. 2012). These are the groups which have more potential to be symbionts and focus should be given to these groups. As mentioned above, fungi are receiving special attention from a biotechnological point of view and therefore it would be important to identify and quantify the potential fungi OTUs found here.

### *Conclusion*

This study has contributed to the understanding of the eukaryotic diversity associated with *H. indistincta*. It builds on previous studies (C2, Murphy unpublished), by further identifying certain species, increasing previous known associated diversity, and presenting eukaryotic groups such as microalgae and fungi that were not analysed before. As mentioned in the previous chapter (C2), the sponge was collected in a very diversity-rich area in terms of algae but also in terms of marine animals and it is not surprising that these organisms foul this sponge. From a Biodiscovery and ecological point of view, it is important to understand not only the extent of the diversity associated with the sponge but also the extent of the associations. Are they fouling or in some cases symbioses? It seems that the dinoflagellate and the fungi groups stand out as meriting further investigation as possible symbionts.

However, it is disappointing to have such low number of sequences even of those species recorded in the area and in the previous chapter. If we take the purpose of metagenomic studies, like this, to be to unravel the genetic diversity of the community associated with the sponge, then we can consider this to be a successful study. As Creer et al. (2010) explain, without relating species to sequences, the approach will remain analogous to the phylotype approach adopted for microbial organisms. Associating species to OTUs is not going to be easy or universally agreed, but bioinformatic sequence comparisons (such as the ones we do here) with more complete databases will eventually become routine. Many questions remain as to the benefits of this technique when trying to answer how much diversity exists and what types of associations are present. Having to choose a universal primer for eukaryotes made species identification very difficult and sometimes impossible, not only because 18S rRNA is a conservative marker at

species level but because there are still many species of marine algae and animals for which this gene has not been sequenced (or, in some cases, it was sequenced in specimens which were not identified at species or genus level). Furthermore, whole DNA extraction may have caused bias in the representation of the groups sequenced in higher amounts, with evidence of an underrepresentation of algae and fungi. Although there was often just one sequence with good quality and long enough to pass our selection criteria, these are likely to represent real organisms since our sequence length improves the chances of sequence error being negligible. More sequences of these OTUs might have been overlooked due to the fact that they were too short or their quality was too low. Other studies focusing on certain phyla or orders, with more appropriate specific markers and using other techniques such as qPCR or RNA seq (sequencing of RNA present) could provide more clues to the extent of the prevalence of these organisms associated with the sponge

## **Part D: Discussion and Future work**

### **D1) Discussion**

So far, bioprospecting has been for the most part synonymous with screening since most research has focused on the biological activities of the compounds found and, at most, on the mechanism of action for molecules that are promising in the context of pharmaceuticals or antifouling (Pereira and Costa-Lotufo 2012). However, in biodiscovery programmes, taxonomy can provide further dimensions which are essential for the success of such programmes. The taxonomist has two important roles: 1) to identify species of potential interest to the programme and 2) later on, to advise in the exploration and conservation strategies for the targeted species. In fact, supply is a determinant factor in the development of pharmaceuticals using marine secondary metabolites and this is one of the reasons why after forty years, only few marine-derived compounds are in use for treatment of human diseases (Pereira and Costa-Lotufo 2012).

In part C, especially chapter C3, the literature shows how it is relevant to discuss which organisms are the producers of potentially economically important bioactives (Paul et al. 2007). Sponges as well as other sessile invertebrates harbour an abundant bacterial and archaeal assemblage (reviewed in Webster and Taylor 2012) as well as a potentially diverse flora and fauna as shown here (part C). This presents a challenge in identifying species-specific associations and original producers of the bioactives in question. In chapter C2, at least two algal species seemed to favour being, or be favoured as, epibionts (*Gelidium spinosum* and *Rhodothamniella floridula*), potentially providing structure and support for the sponge. And in chapter C3, four dinoflagellate and two fungal OTUs were reported all of which had the potential of living symbiotically with the sponge, and to be of interest in a screening programme.

The choice of which species concepts are used by the researcher can have a large impact in either screening or conservation studies (Brodie et al. 2009, Frankham et al. 2012). In conservation studies, it is more common that authors refer to which species concept has been used (e.g. Brodie et al. 2009, Pena and Barbara 2010, Hoetjes and Carpenter 2010). That is not the case in

biotechnological research (e.g. Lordan et al. 2011 and references within). There are, currently, at least 26 definitions of species at the biologist's disposal (Wilkins 2002). Frankham and colleagues (2012) have tested how four different species concepts can lead to different strategies of conservation and ultimately to the success or failure in recovering threatened species. They concluded that species concepts which have very broad definitions, e.g. morphological concept in the case of cryptic species, or the genetic species concept using a marker which is too conserved, may lead to outbreeding depression when populations are crossed. Outbreeding depression is a term that includes any deleterious consequences of crossing on mating preference, pre- or post-zygotic reproductive fitness (Frankham et al. 2010, 2011).

In contrast, concepts that split excessively, e.g. the genetic concept while using only an excessively divergent marker, may endanger rescue strategies of small inbred populations with low genetic diversity. Frankham and colleagues (2012) advise using the criterion of reproductive isolation (pre- and/or post-zygotic) included in the biological species concept or more recently the differential fitness concept (Hausdorf 2011) as much as possible in conservation strategies. Conversely, the use of the diagnostic phylogenetic or the taxonomic species concepts should be avoided. As mentioned in the Introduction (A1), the biological species concept is not easy, and sometimes it is even impossible, to be applied when the target of research are algae. However, most importantly, the species definition being used should always be clearly mentioned (Frankham et al. 2012).

Conservation initiatives concerning algae can have two types of approach, either to preserve individual species or preserve individual sites where either rare or threatened algae species occur, and/or endemic or species of high botanical value exist (Brodie et al. 2009). In the first case, knowing the biology of each individual species is essential. Also, it is very rare that species of algae are known to be endangered or even to have gone extinct (Millar 2003). If choosing to preserve sites the question arises of whether that is enough to guarantee the survival of as much diversity as possible (Brodie et al. 2009). In Europe, the Important Plant Area (IPA) programme (Anderson 2002) was developed to

identify and protect a network of the best sites for conservation. The criteria used for choosing sites are related to botanical richness, important assemblages of rare and/or threatened and/or endemic species and vegetation of high botanic value. In the United Kingdom, the first IPAs were defined including nine sites of international importance for marine algae and six freshwater sites of international importance for desmids (Brodie et al. 2007). The herbarium collection of the Natural History Museum London (BM) was used in this work (Brodie et al. 2007). This is an example of a very practical use of important algal culture collections. No matter which strategy is used in species discovery or conservation, the role of taxonomy is extremely important and the choice of which species concepts are used can have very visible consequences.

The research on the epibiotic macroalgal (chapter C2) community associated with *Haliclona indistincta* has revealed a higher diversity than previously known, so, it suggests that in conservation plans even assemblages of this type should be considered. In this case, the morphological species concept already contributed to evaluate the diversity of this micro-habitat and the extent to which it varied seasonally. This study was complemented by the NGS information and other morphological studies (Murphy unpublished). Macroscopically, this seaweed biodiversity is coupled with a rich animal diversity (chapter C3, Murphy unpublished). Microscopically, certain organism groups can be found associated with this sponge that may not be detected in a routine 2L seawater collection as demonstrated in the NGS study (chapter C3). In this case a more target oriented study with group specific markers would provide more information on what species are present, but a more general marker such as the one used in this research (Chapter C3) already demonstrates the extent of the diversity present.

More and more studies have demonstrated that studies that use exclusively morphological concepts to draw seaweed (or algae in general) inventories may underestimate the diversity present. Until the research reported here and similar studies (part B, Heesch et al. 2012), the diversity of Prasiolales in the south hemisphere was greatly underestimated. As an example, the most researched alga in Antarctica in physiological studies (Kováčik and Pereira 2001, Wiencke and Clayton 2002, Convey 2007) is *P. crispata*. This species was considered the only

one present in Antarctica, since, until now only the morphological concept had been applied. Using two different genetic markers, it is now apparent that three species are present in Antarctic habitats (*P. crispa* / *P. antarctica* / *P. glacialis*).

The Prasiolales are, in fact, a very good example of how using just one species concept, even within a group, can mislead researchers as to the extent of diversity present. In the cases referred above but also in *Rosenvingiella radicans* and filamentous forms of *P. crispa*, the species are morphologically indistinguishable. However, two groups previously considered here as two different species (*P. meridionalis* and *P. stipitata*) are in fact the same species (Chapter C4).

In the same way, using certain molecular markers as species barcodes will underestimate the existent Prasiolales diversity, namely the nuclear marker 18S (Rindi et al. 2007). Chloroplastic markers, especially *tufA* (chapter B4) seem to be much better barcodes for this group, as demonstrated here for the first time for Trebouxiophyceae. Usually nuclear markers such as 18S and ITS are used for this class (Bock et al. 2010, Demchenko et al. 2012, Krienitz et al. 2011a, 2011b). *TufA* so far has been used mostly by marine phycologists and applied to Bryopsidophyceae (e.g. Dijou et al. 2012, Fucikova et al. 2011b) and Ulvophyceae (e.g. Rinkel et al. 2012, Wolf et al. 2012).

Prasiolales is just one more group of green algae for which its simple morphology hides a molecular, physiological and or ecological diversity which in the latter years has been uncovered especially using different molecular markers. Whether previous taxonomic studies did not use all potential morphological characters to separate species or other taxonomic levels (pseudo-cryptic species) or the morphological species concept is not appropriate in that group (cryptic species s.s.), there are several such cases in green algae. *Pseudo-chlorodesmis* has one of the simplest morphologies among bryopsidalean algae, with a diminutive siphon that may be branched but only to a small degree. This simple morphology was described as a life stage of more complex species belonging to the genera *Halimeda* and *Botryodesmis* (Verbruggen et al. 2009 and references within). A multilocus (*rbcL*, *tufA* and 18S) study revealed that the morphological complex

of *Pseudo-chlorodesmis* hides cryptic diversity exceeding not only the genera level but also the family one (Verbruggen et al. 2009). *Ulva* is another example where simple morphology often leads to an underestimation of species present as exemplified by our incapacity to identify *Ulva* like species in chapter C2, or a recent study which showed the Mediterranean to be more rich in variety within this genus (Wolf et al. 2012). To cite other examples (not exhaustively), I can mention the genus *Chladophora* (Leliaert et al. 2007), *Chlorodesmis* (Kooistra 2002), *Pseudomuriella* (Fucík et al. 2011a, b).

Summarizing, my research has contributed to the following conclusions: The order Prasiolales is more diverse and has a more complex biogeography than generally appreciated. In particular, the Prasiolales of the southern hemisphere are more diverse than previously thought, especially in usually perceived diversity-poor Antarctica (B1). The phylogeny of marine species is also more complex than studies had shown before, with more than one clade including species with this habitat preference. Also, groups separated geographically and with different morphology of sexual tissues were shown to be the same species (B3). After three markers being studied, *tufA* is recommended to be included in future studies where species definition is required for this group (B3).

The algal flora associated with sponges of the Irish shores, especially *Haliclona indistincta*, is diverse and varies seasonally (C1). Common and constant associations exist and although they may not be called, at this time, symbiotic, merit more attention. New techniques like Next Generation Sequencing (C2) and common techniques like culturing (appendix F2) are important in the understanding and discovery of sponge associated algal diversity.

## **D2) Future work**

This section summarizes important steps that should follow my research: There are certainly still some unresolved clades in the *Prasiolales*, for example other marine species of *Prasiola* other than *P. stipitata* subspecies such as *P. delicata* and *P. furfuracea* which would benefit from more samples and coverage being sequenced. Preliminary molecular tests of samples from Svalbard promise to reveal an undescribed lineage. Also, more sampling and markers would help

resolve the phylogenetic position of Prasiolales within the class Trebouxiophyceae, since internal nodes of existent trees tend to not be resolved (Friedl & Rybalka 2012; Hodac *et al.* 2012). New studies should include some unicellular and colonial genera such as *Koliella*, *Pseudochlorella*, *Desmococcus*, *Diplosphaera*, *Pabia*, *Pseudochlorella* and *Stichococcus*.

A project that will contribute to a better understanding of this genus will be the sequencing and annotation of the genome of the strain of *Prasiola crispa* (Trebouxiophyceae, Chlorophyta) from the Falkland Islands which I have used in the chapter B3. This project will run at the same time as others included for example in the SFI funded Green Algal Tree of Life, which are also focusing in transcriptomics and this species. This shows that this species can soon become an important model for the Prasiolales and Trebouxiophyceae, which would benefit the study of a group which still raises so many questions.

This type of project could elucidate different aspects which make this group so unique. For example, the origin of multicellularity in *Prasiola* and in other genera of Prasiolales: This clade comprises species with diverse morphologies, forming e.g. multiseriate filaments or blades. However, it seems that the closest relatives of Prasiolales are single-celled coccal microalgae such as *Stichococcus* spp (Sherwood *et al.* 2000). Furthermore, with the exception of the enigmatic basal Palmophyllales, all the prasinophyte ancestors of the core chlorophyta are single-celled (Leliaert *et al.* 2012). In the Trebouxiophyceae, coccal single cells are the most common growth form and complex thallus forms are rarely found. Conversely, the two other classes of core chlorophytes (Ulvophyceae and Chlorophyceae) are morphologically more diversified and include more complex forms (Cocquyt *et al.* 2010). Comparative studies with the colonial *Volvox* (Chlorophyta) genome would be probably of great interest. *Volvox*, a genus of Chlorophyceae, includes species that form spherical colonies in which cells interact closely and already show some level of specialization. For this reason, this genus is used in studies which try to understand the transition from unicellularity to multicellularity (Kirk 2005).

The genomic data from *Prasiola* have a great potential to shed light on the

adaptations of green algae to terrestrial habitats. From the ecological point of view the genus *Prasiola* is one of the very few algal taxa that include species living in terrestrial, freshwater and marine habitats. The fact that it belongs to a mostly terrestrial class (the Trebouxiophyceae) suggests that the marine and freshwater species represent an almost unique case of algae moving back from land to water. Future comparative data for aquatic species (e.g., the marine *Prasiola stipitata*) may lead to great insights into the genetic rearrangements associated with transfers between different types of habitats.

Finally, *Prasiola crispa* is an interesting subject to look for sex-related genes (genes for meiotic apparatus and flagellar proteins). Until recent evidence for cryptic sex revealed by the *Chlorella* genome (Blanc et al. 2010), the Trebouxiophyceae have been assumed to be mostly asexual. Surprisingly, even a small non-motile alga such as *Chlorella* can possess all known genes for meiotic apparatus and about 1/3 of the genes for flagella that were found in *Chlamydomonas* (Blanc et al. 2010). Sexual reproduction has not been observed in *P. crispa*, but it is well documented for its close aquatic relatives *P. stipitata* and *P. japonica*. It would be therefore very interesting to verify if *P. crispa* also has genes which may be directly or indirectly related to sexual reproduction (e.g. meiosis, flagellar proteins).

Due to the locality where this species was collected, it would be interesting to seek genes that are responsible for adaptation to an extremely cold climate. Recently, the genome of the polar microalga *Coccomyxa subellipsoidea* showed that this eukaryote follows cold adaptation strategies that are similar to cold adapted prokaryotes such as increased fluidity of the cell membrane, reduction of freezing point of cytoplasm and stabilization of macromolecules and protection against reactive oxygen species (Blanc et al. 2012).

This project would add new genomic data for addressing diverse questions on evolution of green algae. A search in GenBank in January 2013 showed seven ongoing genome projects for the trebouxiophytes (*Botryococcus braunii*, *Chlorella pyrenoidosa*, *C. vulgaris*, *C. variabilis*, *Coccomyxa subellipsoidea*, *Nannochloris*, *Trebouxia* sp.). In case of successful assembly of the plastid genome, multigene phylogenies can be reconstructed as an attempt to clarify the

phylogenetic structure of the Trebouxiophyceae and the whole "core" group of Chlorophyta.

On the sponge algal community research, we only now scratched the surface. I am convinced that not only will we understand the ecology and roles of each partner better, but this will also have repercussions in Biodiscovery projects in the understanding of what is producing certain bioactives, where, and under which conditions. Species like *Rhodothamniella floridula* and *Gelidium spinosum* merit more studies to further understand their role in the life of the sponge and vice-versa. As referred in chapter C2, one avenue would be TEM studies to see if spongin is deposited onto the algal cell wall during simultaneous growth of seaweed and sponge. Other genetic markers would confirm if these species differ in any way molecularly from species not known to be so closely associated with sponges.

As stated in chapter C3 more specifically targeted primer pairs, especially for dinoflagellates and the fungi would enlighten if these are in fact symbionts, and which species they are. This, coupled with QPCR and *in situ* hybridization could give an idea of the quantities of cells or hyphae present per sponge. Finally, it would be important to see if diatoms are in fact present inside the sponge, and if not, why not.

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## **Part F: Appendix**

### **F1) Marine Bioactivity in Irish Waters**

Margaret Rae, Helka Folch, Mónica B.J. Moniz, Carsten W. Wolff, Grace P. McCormack, Fabio Rindi and Mark P. Johnson

#### ***Abstract***

In 2009, the Marine Biodiscovery Laboratory was set-up at the Marine Institute with funds from the Marine Institute and the Beaufort Marine Biodiscovery Research Programme. The Marine Biodiscovery Laboratory has already processed over 130 marine specimens from coastal zones and from the Deep Sea ( > 3000 m) within the Marine Irish Exclusive Economic Zone. Beaufort Biodiscovery funded taxonomists are involved in species identification and elucidation of evolutionary relationships. The project approach links sampling, systematics, extraction, microbial metagenomics and biomaterials.

The Laboratory consists of approximately 56m<sup>2</sup> including an extraction and a bioassay suite. The Laboratory samples and assesses marine biological diversity geared towards developing natural products for drug discovery, advanced material applications and bio-medical devices. Samples are tracked from sample log-in to right through to extraction and bioassay using a customised Marine Biodiscovery Database. The extraction procedure is described along with the anti-bacterial bioassay selected for routine use.

The Marine Biodiscovery Database manages the data generated and links the data collected by the project's stakeholders to existing biodiversity, genetic and chemical resources. The system uses in-house developed software tools to merge biodiversity data collected with other MI resources and external databases and for the data mining and visualisation of biogeographical, genetic and chemical information aimed at the identification of potential biodiversity and bioactivity “hotspots”.

### ***Introduction***

In 2007, the Department of Communications, Marine and Natural Resources awarded an all-island consortium with funding of €7.25 million to develop and implement Marine Biodiscovery Research over the period 2008-2015. This initiative was called the Beaufort Marine Biodiscovery Research programme. Under this programme, three universities along with the Marine Institute are collaborating to establish an initial research capacity to meet the objectives of Ireland's National Marine Biotechnology Programme. Marine Biodiscovery Research is an important component of the Marine Knowledge, Research and Innovation Strategy for Ireland 2007-2013 (**Sea Change**), which identified marine biodiscovery and biotechnology as areas of development for the country. The three universities involved are the National University of Ireland Galway (NUIG) - Ryan Institute and the School of Chemistry, Queen's University Belfast (QUB) – School of Biological Sciences and University College Cork (UCC) – Environmental Research Institute. In the sections below we outline features of Ireland's marine biodiversity, issues related to sampling and identification of species and how a programme of sample extraction and screening is building up a knowledge base of the national resource.

### ***Ireland's Marine Environment***

The marine coastline of Ireland is approximately 7,000 km long and represents one of the most habitat-diverse shorelines in Europe. Such habitat diversity produces ideal conditions for the establishment of a high biological diversity, which is reflected by a high number of marine organisms for an island that encompasses a relatively modest latitudinal range. There are estimated to be 80,000 species across marine, freshwater and terrestrial habitats in Ireland (Guiry and Guiry 2011). The geographic distribution of marine species is best known in coastal waters and less so offshore.

### ***Algal Biodiversity***

The benthic algal flora of Ireland includes approximately 600 species of blue-green (Cyanophyta), green (Chlorophyta), brown (Phaeophyceae) and red algae (Rhodophyta) (Guiry and Guiry 2011). It is largely similar to the seaweed floras

of other North Atlantic regions located at comparable latitudes and there are no species that are currently known to be endemic to Ireland. Overall, it is a well-preserved flora; there is no evidence of species that have disappeared and the number of introduced species is limited, if compared with many other European regions. Well-established introductions include the red algae *Asparagopsis armata* (known in Ireland since 1940), *Bonnemaisonia hamifera* (since around 1890) and *Polysiphonia harveyi* (since 1990), and the green algae *Codium fragile* (since 1941) and *Codium fragile* subsp. *atlanticum* (since 1808). These are widely introduced species at a global level and in Ireland are nowadays common members of the seaweed flora. The brown alga *Sargassum muticum* is a more recent arrival, having appeared in 2002; since then it has been constantly spreading and at many sites is showing a high invasive potential.

In general the diversity and many aspects of the biology of Irish marine algae are well known, due to a long history of studies spanning over two centuries (Harvey WH 1841-1857). Work carried out in recent decades has led to a deep understanding of distributional patterns, ecology and physiological responses for many species (Guiry and Hession 1998, Hardy and Guiry 2003, Rindi and Guiry 2004). DNA sequences and other molecular data became available in the last 20 years and have a major impact on the knowledge of Irish seaweeds. A detailed genetic characterization has become possible for numerous species, leading to taxonomic reassessments, discovery of cryptic taxa (McIvor et al., 2001), reconstruction of evolutionary histories and clarification of the origin of some introduced species.

In terms of distribution, the eastern and southeastern shores are characterized by a lower diversity and abundance in the seaweed vegetation than the other parts of the Irish coastline. The reason for this is related to physical factors. Marine benthic algae require the presence of rock or some other stable substratum to settle and produce viable populations; since most of the eastern and southeastern shores are sandy, the conditions are generally unsuitable for the establishment of seaweed assemblages. On rocky shores there is a general pattern for higher species richness towards the southwest, as seen in Great Britain (Blight

et al. 2009), albeit some local variation may partially obscure this trend. Areas that are known to host a high diversity are the marine reserve of Lough Hyne, Co. Cork, the area of Finavarra, Co. Clare, and Clare Island, Co. Mayo. It is likely that Lough Hyne and Finavarra represent actual biodiversity hotspots for seaweeds. Lough Hyne has a peculiar hydrological regime that promotes a high diversity for many marine organisms and is considered a biodiversity hotspot for many groups, not only algae (e.g., 77 species of sponges; Bell, 2007). Finavarra has a very diverse shore, in which limestone banks with a heterogeneous surface are mixed with sandy stretches and portions of rock partially or completely covered by coarse sand; the area has also intermediate conditions of wave exposure, which are generally the best for the settlement and growth of benthic algae. The combination of these factors makes Finavarra an ideal area for high algal diversity, and a peak value of 336 algal entities has been recorded for a  $10 \times 10$  km grid square. It is clear, however, that the high diversity of these three areas reflects also the fact that they have been thoroughly investigated. Lough Hyne and Finavarra are located in proximity of research-focused institutions (UCC and NUIG, respectively) and host important field stations actively used by marine scientists. Clare Island has been the focus of two major natural history investigations promoted by the Royal Irish Academy. The original Survey of Clare Island produced a very detailed report in which 224 species were recorded (Cotton, 1912); the new survey, conducted with similar criteria almost a century later, has revealed an almost identical scenario and recorded 223 species (Rindi and Guiry, 2004). It is expected that detailed floristic surveys conducted at other sites, especially along the western and south-western shores, would reveal comparable species diversities.

#### *Invertebrate Biodiversity*

Currently, the invertebrate focus of the marine biodiscovery programme is on the sponges (Phylum Porifera) given their importance in the search for novel compounds of interest and the taxonomic expertise available amongst the partners. Published studies on the biodiversity of sponges in Ireland are focused on a few geographical localities mostly located on the west and south west of the

country. As with the algae (above) areas known to harbour a high sponge diversity are Lough Hyne, and west Cork (van Soest et al., 1981 (60 species); Bell and Barnes, 2000 (77 species) and Clare Island, Co. Mayo (Stephens, 1912 (75 species). Further sponge diversity has been recorded in Connemara, Co. Galway with 66 species described, 12 of which were new to Ireland (Konnecker, 1973). Van Soest (1994) recorded 348 sponge species for the boreal East Atlantic and 445 for the East Atlantic while Van Soest and Weinberg (1980) recorded 219 sponge species from Irish waters. However, an exceptionally high biodiversity of 343 sponge species were recorded in Rathlin Island in the North Eastern part of the Island (Picton and Goodwin, 2007). Additional information on the presence of sponges in other areas can be found on online sources and in unpublished reports (e.g. the encyclopedia of marine life- <http://www.habitas.org.uk/marinelife/>; the national biodiversity network <http://www.nbn.org.uk/>).

Subtidal species are best described, as many of the intertidal species are small and more difficult to identify. Some groups, like the intertidal *Haliclona*'s have had little attention and likely contain far greater number of species than currently reported for Ireland. Some representatives of this group (species of *Haliclona*, and related species in the Order Haplosclerida) are important producers of bioactive compounds, grow to a reasonable size and are therefore worth investigation. Representative members of other sponge Orders of even greater importance from a bioactivity viewpoint (e.g. Halichondrida, Hadromerida, Dictyoceratida) are also present and accessible. It is well recognized that the microbial and algal inhabitants of sponges are often responsible for the production of the bioactive in question and in this Programme research focus is also placed on determining the eukaryotic and prokaryotic biodiversity associated with species of suspected importance using a combination of taxonomic identification with bulk sequencing and next-generation sequencing approaches.

#### *Offshore Biodiversity*

The distribution of biodiversity offshore is less well characterised; a situation that reflects the logistic restrictions on extensive benthic sampling and the large potential survey area (the Exclusive Economic Zone (EEZ) is estimated as

410,534 km<sup>2</sup>, [www.seasaroundus.org](http://www.seasaroundus.org), Figure 1a). The Irish EEZ contains continental shelf seas: the Celtic Sea and the Irish margin, including the Porcupine Bank. At the edge of the continental shelf the depth increases relatively rapidly from around 400 m to over 3 km. Much of the continental margin contains systems of canyons, clearly visible in the broad scale bathymetry. While the data widely available through biodiversity databases are not an exhaustive collection of all species records, the distribution of effort indicates that the pattern of sampling is very uneven (Figure 1b). Waters shallower than 250 m represent around one third of the total area of the Irish EEZ (Figure 1c), but 99.8 % of records are from these depths. Sampling in deeper waters is patchy, a pattern common to most deep sea regions of the world (Kelly et al 2010).

The uneven nature of sampling in deeper waters around Ireland precludes, for the time being, a systematic identification of diversity 'hotspots'. Three habitat features are, however, likely to contribute to areas of increased biodiversity. The continental shelf waters less than 200 m depth tend to have muddy or coarse sediment, however, in some areas rocky reefs exist. Extensive reefs were identified in INFOMAR ([www.infomar.ie](http://www.infomar.ie)) multibeam sonar surveys off the coast of Kerry. These sites, and similar areas, can have large densities of encrusting fauna, such as anemones, sponges, and soft corals. Further offshore, large areas of the continental margin slope are suitable habitat for the deep water corals *Lophelia pertusa*, *Madrepora oculata* and *Solenosmillia variabilis* (Davies and Guinotte 2011). These corals can form extensive reefs; although Howell et al (2011) point out that the environmental conditions suitable for reefs to form are narrower than the range of conditions where the coral species can grow. Deepwater reefs have been a focus for conservation and ongoing research to understand the extent to which the reefs support the wider ecosystem and the factors that cause reefs to build. Reefs can be damaged by deep water trawling and are subject to conservation measures including the creation of marine protected areas. A further habitat feature of the Irish EEZ is represented by the canyons of the continental margin. These areas can contain a variety of habitats, including cliffs and overhangs. Canyons are often considered to be sites of high biomass and biodiversity (De Leo et al. 2010).

*Marine Sampling and Collection*

Currently, to the authors' knowledge there are no formal guidelines on the sampling, collection and harvesting of marine specimens for medicinal purposes. As the marine environment becomes a greater source of medicines, in particular of a herbal type nature, formal guidelines similar to those of the World Health Organization's guidelines on good agricultural and collection practices (GACP) for medicinal plants (WHO Geneva, 2003) may be implemented for mariculture and aquaculture of medicinal bioactive-containing organisms. In order to sample in certain marine locations, such as Special Areas of Conservation, a permit or license is required from the authorities before any collection takes place. In Ireland, permits can be obtained from the National Parks and Wildlife Service (<http://www.npws.ie/>).

Within the Beaufort Marine Biodiscovery Programme, researchers at NUIG sample and collect the bulk of the marine specimens from the coast and deep-sea. All year round, samples of algae and marine invertebrates are collected and sent to the Marine Biodiscovery Laboratory at the Marine Institute to be made into extracts that are then bio-assayed both at the Marine Institute and more extensively by the Marine Biodiscovery Research partners.

Successful sampling and collection in the marine environment is fraught with accessibility difficulties that may not be experienced on land, simply due to the fact that the specimens are covered by water and not easy to reach. Sampling in the marine environment depends on a multitude of variables such as the timing due the tides, depths at which specimens may be found, weather conditions, sheltered or exposed locations and personnel safety as well as seasonality of specimens and patchiness in specimen distribution and abundance. These variables can very dramatically reduce the timeframe for sampling and consequently the amounts that can be collected for extractions and bioassays. Depending on the habitat of the marine specimens – specialised equipment may be required in order to collect the specimens such as boats and diving gear. For specimens found at depths greater than 50 metres submersibles (manned and/or remote operated vessels) are used requiring larger sea-going vessels and a trained crew to house and operate these vehicles.

Marine algae have long been known as a source of compounds with valuable biological properties and in some countries have been exploited for pharmaceutical purposes. Screening programs conducted in the last decades have revealed the exceptional biochemical diversity of these organisms, whose secondary metabolites cover an exceedingly wide range of biological activities (Smit, 2004; Tierney et al., 2010; Holdt and Kraan, 2011); to date, the occurrence of chemically mediated bioactivities has been documented for approximately 150 genera of benthic marine algae (Rindi et al., 2012). Ireland has a long tradition of seaweed usage (Guiry and Hession, 1998); seaweeds have been long used as human food and food supplements, as fertilizers in agriculture and horticulture, and as ingredients for cosmetics, bodycare and thalassotherapy products.

Most of the species used for applied purposes in Ireland are large-sized seaweeds distributed in the intertidal and shallow subtidal zones (i.e., wracks, brown algae of the genera *Fucus* and *Ascophyllum*; kelps, brown algae of the genera *Laminaria* and *Alaria*; dulse, the red alga *Palmaria palmata*). For the collection of these species, accessibility to the shore and tides are the only limitation. Timing and availability in general are not a problem; these algae typically occur in large populations, which are present on the shore for the whole year or most of it. Collection of seaweeds for biodiscovery or other biotechnological purposes, however, is subject to additional constraints. A large-scale biodiscovery program requires the collection of a large number of species, some of which may be rare or difficult to collect. Valuable bioactivities have been revealed in some species of algae with small size or filamentous structure, in which the biomass of individual specimens is limited.

In order to obtain reliable results, screening analyses for biological activities normally require large amounts of biomass; therefore, even when a species is found in the field, it may turn very time-consuming or impossible to obtain enough biomass for screening. The problem may be partially overcome by isolating in culture the species concerned and growing it in larger amounts in culturing devices. This, however, involves additional costs that may make the procedure unsustainable. Furthermore, type and amounts of secondary metabolites

depend in part on the environmental conditions in which the alga grows; so, the bioactivity responses of specimens grown in culture may not reflect the actual metabolism of the alga in the field.

Many species of benthic algae distributed in temperate seas are not present in the field for the whole annual cycle (or, if they are, their active growth and reproduction are restricted to certain seasons). This is a common case for many Irish brown and red seaweeds, which are best developed in spring and summer and disappear (or occur in reduced form) in autumn and early winter; for this reason, the sampling has to be limited to certain times of the year, in which these algae occur in the optimal form. This may be a substantial limitation for certain species. For example, brown algae of the genus *Cystoseira* are common in intertidal pools and in the subtidal zone around the Irish shores. In autumn and winter their thallus is limited to the holdfast and basal axes; new branches are generated in spring and the alga is fully developed in summer. Collection of these algae for biological screening can be successfully performed for no more than 1-2 months a year (generally May-June), in which the algae are actively growing. At this time of the year field specimens are in optimal conditions, well-developed and clean of contaminants; in subsequent months they will be covered by an assemblage of small- sized animal and algal epiphytes, which would be inevitably included in the extractions and contaminate the extraction products.

Another key aspect for successful sampling is the proximity of adequate facilities for processing and/or storage of the material collected. In general seaweeds to be used for biological screening are not immediately processed, but frozen and subsequently freeze-dried. The time between the collection and the freezing is a critical aspect and must be as short as possible; when seaweeds are kept in unfavourable conditions their secondary metabolites are quickly lost or degraded, making the material collected useless. In the Marine Biodiscovery program, particular attention has been paid to this aspect and every possible precaution has been taken in order to minimize the time interval between collection and freezing.

A very important aspect for the biological exploitation of marine

organisms is a correct identification of the specimens screened. A misidentification would result in an incorrect selection of the algae containing the targeted molecule of interest, with the likely consequence of a considerable waste of time and financial resources; incorrect identifications will also lead to misinterpretations of published studies, spreading confusion and misleading future work (Rindi et al., 2012). Identification of algae is a complex task that requires the expertise of skilled taxonomists and that involves several problematic aspects, which have become increasingly evident in recent years. Traditionally, species identification of algae has been based on morphological data. It is now appreciated, however, that this approach may not be the best for a correct characterization at species level.

The development of molecular systematics that has taken place in the last 20 years has substantially reshaped species concepts in all algal groups, showing numerous cases in which morphological features are inadequate to identify algal species. Several studies have revealed cryptic diversity in many macroalgae, including genera and species occurring in Ireland. At the same time, it is well known that the morphology of many marine macroalgae is affected by strong phenotypic plasticity and may vary in relation to environmental factors such as temperature, salinity, wave intensity and interactions; this is a particularly serious problem with morphologically simple macroalgae, in which environmentally driven variation may easily lead to misidentifications. For these reasons, DNA barcoding is a practice which is rapidly gaining great popularity, and it is now accepted that for some genera of marine algae molecular data are a mandatory requirement for a reliable identification. Problems related to taxonomic identification need to be considered carefully in any bioactivity investigation, and two basic procedures should be adopted: (1) voucher specimens should be deposited in reliable repositories (such as public herbaria or museums); and, (2) DNA sequences should be produced for samples with valuable properties and deposited in public repositories (such as GenBank) so that they are publically available.

Many of the above criteria are also relevant to the collection of marine

sponges for the biodiscovery programme (e.g. accessibility, size, effect of environmental conditions, time between collection and freezing, correct identification). Most specimens are collected currently by SCUBA in depths of <50 m. Some specimens yielding potential hits were collected on the lower shore at low spring tides. Many of the intertidal species, however, are very small and unless focus is placed on biotechnological or synthetic approaches for development of bioactives the development of novel chemicals from these species is not feasible. Sampling protocols developed by Australian Institute of Marine Science (AIMS) have been adapted for use by the Biodiscovery Programme in Ireland, e.g. taking careful note of field characteristics and the appearance of the sponge *insitu*, and ensuring that we take only a proportion of the population in a sustainable manner. Careful attention is also paid to correct preservation of sampled material. For marine sponges subsamples are held in ethanol, DNA extraction buffer (6-8M GmCl), and the rest is placed at -80 oC for subsequent freeze-drying.

It is thus critical that the marine specimens are correctly identified, to ensure that the same species can be resampled, and so vouchers are kept for taxonomic identification and molecular systematics. Generally, the information taken along with the sample will be recorded for later electronic data entry such as date of collection, personnel who took the sample, taxonomist who identified the species, global position co-ordinates of the sampling site, habitat and the level of epiphytes found on the specimen.

In 2010 and 2011, research cruises targeted two deep canyons off the Irish continental shelf to sample marine specimens at depths up to 3000 m using the national deep-sea research vessel, the R.V. Celtic Explorer. The key to sampling at such depths is a remotely operated vehicle (ROV). Conventional sampling with box cores is problematic in canyons due to the mixed seafloor composition (the core will not sample if the seafloor is too rocky or sloped) and the risk of fouling the core on the uneven topography. The ROV is equipped with the latest underwater camera equipment including a HDTV camera and recording system which enables the capture of high definition footage and real-time targeting of specimens observed at the sea floor. Specimens are retrieved using a combination

of a suction hose and/or a robotic arm, both of which require the skills of an experienced ROV pilot, particularly if there is any current. The expeditions were aimed at retrieving target marine specimens for drug discovery and yielded deep-sea specimens such as sponges, echinoderms, tunicates, ascidians and also sediment samples from which microbes were isolated.

#### *Marine Biodiscovery Laboratory*

The Marine Biodiscovery Laboratory is based at the Marine Institute, Oranmore Co. Galway and was founded in 2009. This laboratory centralises the samples taken by the NUIG researchers along the intertidal zones and the deep-sea. Once they have been identified by the taxonomists, the specimens are archived for use by all the Beaufort researchers. At the biodiscovery laboratory, all specimens are logged-in to the Marine Biodiscovery Database, given unique identity numbers, freeze-dried and solvent extracted. Both freeze-dried material and extracts may be requested by the Beaufort researchers who then perform extensive bioassays aimed at detecting natural products active against human diseases or look at the potential to yield biomaterials for use in medical devices. It is intended that this become a national resource and an international one at a later stage.

There are many different extraction strategies aimed at the isolation of marine natural products (Houssen and Jaspars 2006, Ebada et al 2008). The strategy developed within the Marine Biodiscovery Laboratory is based on four constraints: personnel number, chemistry, method robustness (i.e. all marine specimens are treated in the same way initially) and cost. Standardised methods have been developed for freeze-drying, aqueous washing, organic solvent extraction and storage of extracts and freeze-dried materials. All samples are initially freeze-dried either from a fresh harvest or from a frozen (minimum -20°C) collection using a three day method in a large seven tray freeze-dryer. All freeze-dried specimens are vacuum packed and stored in the dark at -20°C until required. The specimens are kept as intact as possible and undergo a five minute aqueous wash at 4°C (ice bath, deionised water and gentle stirring) to rid the specimen of external sea salts. The specimen is separated from the wash by decanting. Separately the specimen and aqueous wash are freeze-dried. Note the timing between wash and freeze-drying is kept to a minimum. The freeze-dried

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aqueous wash is placed in a weighed aluminium envelope, re-weighed, labelled, placed in a numbered box and kept in the dark  $-20^{\circ}\text{C}$  in a freezer room. Where possible, slightly more than 5 g of the freeze-dried specimen is taken and milled using a planetary ball mill. Milling takes place in stainless steel jars that have been pre-cooled. Generally a fine powder is obtained when milling at 450 rpm. Milling is quite rapid, for example, algae take approximately 2 minutes, ascidians take approximately 5 minutes whereas some sponges may take 10 minutes. Every 5 minutes the milling jars are cooled to avoid excessive heating of the sample. Some material may be lost during the milling process (adhering to the jar wall and stainless steel balls). Currently 5 g of material is firstly extracted in dichloromethane over a four hour period in a rotary vapour unit under a slight vacuum and in a nitrogen enriched atmosphere (system is flushed 3 times with nitrogen). After four hours have passed, the marine specimen is separated from the dichloromethane using a Büchner funnel setup. The dichloromethane extract is then evaporated under nitrogen and the marine specimen once more extracted over four hours but this time in methanol. Again, the specimen is separated from the extract solvent using the Büchner funnel setup. The methanol extract is combined with the dichloromethane one and evaporated to dryness under nitrogen. The dry extract amounts for both are recorded. These combined extracts are then labelled and stored at  $4^{\circ}\text{C}$  if they are to be used within a week or at  $-80^{\circ}\text{C}$  if not. Generally, one third of the specimen by weight can be extracted in this manner; the dichloromethane extraction yielding smaller quantities and the methanol extraction yielding the larger amount). Work is in progress to repeatably aliquot out 20 mg quantities into vials prior to evaporation and to store the dry extracts in vials such that only one vial is used at a time to minimise the number of freeze-thaw cycles suffered by the entire extract.

Once bioactivity has been indicated, the extract is fractionated at first using planar chromatography and the fractions are sent for bioassaying. In order to isolate a large amount of the bioactive, the planar chromatography method is transferred from reverse phase planar chromatography to column chromatography (flash and high pressure liquid chromatography) and the fractions bioassayed again to ensure bioactivity remains. Generally, some more chromatography

method development takes place in order to isolate out sufficient quantities for chemical structure identification and to continue with the bioassays.

One of the main bioassays, due to its rapidity, within the Marine Biodiscovery Laboratory is based on bacterial bioluminescence and a thin layer chromatography (TLC) bioautography technique (Kreiss et al 2010, Möhrle et al 2007). Using this approach, the presence of gram negative anti-bacterial bioactive compounds is indicated along with their positions on the TLC plate. We have noted that anti-bacterial compounds lacking a UV chromophore have been detected using this technique. The extracts are spotted on a TLC plate in duplicate, with the second spot being twice the concentration of the first. The TLC plate is developed using a 9:1 methanol:deionised water solvent mixture, dried and then dipped into a *Vibrio fischeri* suspension (it is important that the culture is luminescent). Next the TLC plate is viewed under a luminescence detector (See Figure 2). Generally, a lack of luminescence indicates the presence of an anti-bacterial compound. The technique works very well and in equipment terms, it is quite an inexpensive bioassay, requiring only a thin layer chromatography plate, a TLC development tank, a shaker incubator to culture *Vibrio fischeri* and a luminescence detector that allows the TLC plate to be viewed without disturbing the silica surface. The presence of anti-bacterial compounds is known within 5 minutes and, as such, it is very rapid. Immediately after the bioassay, the anti-bacterial compounds present are cut-out of the plate, recovered and concentrated and the mass spectrum obtained. The mass spectrum is compared to those of known marine bioactives found in AntiMarin (Professor John Blunt, University of Canterbury, NZ) and other databases such as PubChem (National Center for Biotechnology Information, NCBI). Another TLC-bioautography bioassay used in the Marine Biodiscovery Laboratory is based on the plant fungus *Cladoporium cucmerinum*. This assay is more time-consuming, taking 2-3 days. In addition, to the TLC bioautography technique, disc diffusion bioassays are also conducted. The Laboratory has plans to introduce one or two additional preliminary bioassays. However, the more complex and extensive bioassays will be conducted by the partner institutions such as quorum sensing, anti-biofilm/fouling, and anti-cancer bioassays.

*Marine Biodiscovery Data Management System*

The Marine Biodiscovery Database is aimed at managing data generated by the National Marine Biodiscovery Programme and linking the data collected by the project's stakeholders to existing biodiversity, genetic and chemical resources.

The database model implemented for the tracking of samples is partially based on the database schema used by the AIMS system – an in-house system designed to support the Australian Biodiscovery programme.

The Marine Biodiscovery data management system provides the means to visualize all steps of a sample's biochemical workflow (sequential extracts, fractions, bioassays), the taxonomic identification history of a sample and its 'chain of custody'. This allows the team to keep track of biological material that is transferred among Marine Biodiscovery partners and outsourced to external organisations for bioassay screening, etc.

The database currently contains records on 118 species and 86 extracts. The database system includes tools aimed at merging data collected during the Marine Biodiscovery Project with other chemical, genetic, and climatological information available in external, third party databases and online web servers. For instance, geo-referenced information on species distribution harvested from the Global Biodiversity Information Facility ([www.gbif.org](http://www.gbif.org)) is correlated with environmental factors from bathymetry, slope and seabed classification data available at the Marine Institute (INFOMAR project) to assist in making strategic decisions about where to target sampling effort.

The Biodiscovery data management system also links information from specimens collected during the project to Nuclear Magnetic Resonance spectra and Mass Spectrometry information available in the AntiMarin and AntiBase (Professor Hartmut Laatsch, University of Göttingen, DE) chemical databases in order to facilitate structure determination of the natural compounds extracted and thus speed up the drug discovery process.

***Summary***

The Beaufort Biodiscovery programme is at the midpoint of a funding cycle

intended to establish a national capacity in marine biodiscovery. The programme seeks to broaden the range of species screened from Irish waters, using taxonomic expertise to achieve this. This has already been shown to produce results, in that initial screens have identified activities in species where similar activities had not previously been reported. Wider aspects to the Beaufort programme, not covered in this review, include microbial metagenomic screening of extracts from sponges and from extreme environments, culture of marine organisms to study glues and the cell differentiation processes (including stem cell activity) and the use of marine-origin materials in biomaterials for medical applications. Research in the second half of the programme will inevitably include a focus on sustaining the research through further sampling and screening, greater characterization of hits and the development of strategic research and exploitation partnerships.

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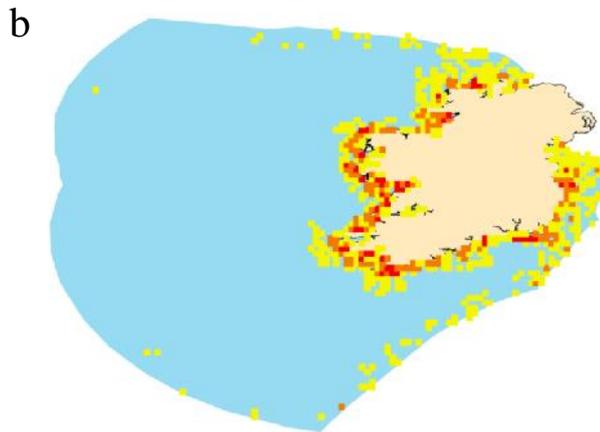
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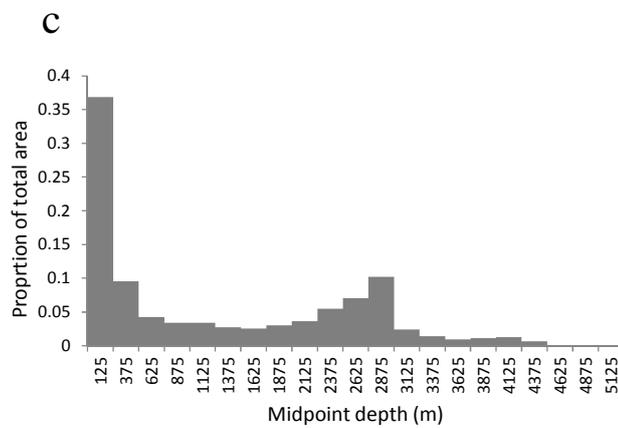
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**Species richness**

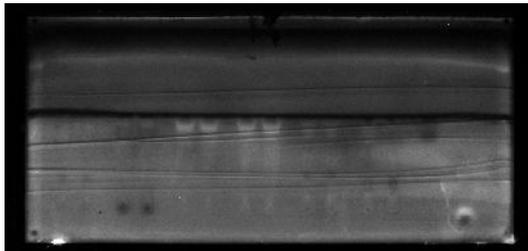
- 1 - 75
- 75 - 199
- 199 - 525



**Figure 1** Distribution of depths and sample records from the waters around Ireland. a) Bathymetry of the waters around Ireland with exclusive economic zone area highlighted. b) Species records from the OBIS databases ([www.iobis.org/](http://www.iobis.org/)). The number of species is summarized for areas of 0.1 latitude by 0.1 degree longitude with no symbol in areas where lacking records in the databases. c) Proportion of the EEZ in different depth categories. EEZ data from VLIZ (2011) with bathymetry from the ETOPO1 database (Amante and Eakins 2009).



a)



b)

**Figure 2** Thin Layer Chromatography Bioautography assay.

a) Image of 366 nm exposure of thin layer chromatography of marine specimens, order of spot application from left to right on the TLC plate: a red algae, a starfish, a sea squirt, another specimen of the same species sea squirt, a sponge, a different species sponge and a positive control for *Vibrio fischeri*, b) same TLC plate as in a) above and now the plate has been coated in luminescent bacteria and observed over 5 minutes using a luminescence detector. Note the areas indicating the presence of anti-bacterial compounds show up as dark spots. Note each extract applied on the horizontal (see X-axis); each extract separates into its components following elution, observe the vertical tracks (see Y-axis).

## **F2) Protocol for culturing and maintaining Strains of Algae of small dimensions**

### *Establishing cultures*

Unialgal cultures of algae are established either from vegetative fragments or from released reproductive bodies (zygotes or spores). For algae with apical growth cut extremities to guaranty minimum contamination with other organisms using the tip of a sterilized Pasteur pipette. Always start cultures with little material and watch closely for a few weeks, checking the material daily. If signs of contamination become evident, cut new piece and start again. Cyanobacteria and diatoms are among the most common contaminants occurring in cultures of marine algae. Their development may be suppressed adding to the culture vessels germanium dioxide (for the diatoms) and/or penicillin salts (for the cyanobacteria), respectively.

For marine cultures, in our lab, we use Von Stosch enriched seawater medium (VS5) modified following Guiry and Cunningham (1984) and for freshwater cultures Jaworski's medium (JM, both liquid and agarized; Tompkins et al. 1995). There are other media that work just as well.

### *Culture conditions*

Most of the strains maintained in ISRG are kept at 10 °C and 15°C, 20–40  $\mu\text{mol photons m}^{-2} \text{ s}^{-2}$ , at 14:10 h light:dark in Sanyo MLR-351 culture chambers (Sanyo Electric Co., Osaka, Japan) using plastic dishes containing approximately 30 ml of medium (Bibby Sterilin, Stone, U.K.) or glass culture flask. For cultures of small dimensions, once the material has been cleaned and isolated into unialgal cultures, test tubes containing 15-40 ml of medium can be used.

### *Maintaining cultures*

It is recommendable to maintain small amounts of algal biomass, in order to keep it actively growing. The material may be multiplied by separating and transferring a part of the culture to another culture vessel with new medium every 10–15 days, in some cases a month. This allows keeping more material, minimizing the risk of losing a culture due to possible problems with contaminations or medium.



### **F3) Abstracts of Presentations during this Project**

#### ***Beaufort Meeting, Belfast, 2009***

##### ***Exploring and unknown world: Diversity and Taxonomy of Algal Epibionts on Sponges of Irish Shores***

Mónica B.J. Moniz, Fabio Rindi, Carsten Wolff, Kelly Stephens and Grace P. McCormack

In general very little information is available about the diversity of algae growing as epibionts on sponges, and most studies are restricted to the southern hemisphere. We are currently analysing the epibiontic algal flora of the sponges of Irish shores, with the purpose of enumerating its species diversity, examining possible associations between species of algae and species of sponges, and selecting algal epibionts suitable as source of pharmaceuticals. So far, we have recorded 72 algal species, of which 24% have been reported to produce molecules with medical and pharmaceutical properties. Most algal specimens growing on sponges were of small size, and some species could be identified only after isolation in culture. A number of species are currently maintained in culture; among them some have been selected as promising candidates for production of bioactive compounds. For these species it is planned to scale up the production in order to obtain sufficient amounts for bioactivity screening.

#### ***Ectocarpus 2010, A decade of algal genomics, 2010, Ghent.***

##### ***Understanding the Phylogeny of the green algal order Prasiolales (Trebouxiophyceae, Chlorophyta)***

Mónica B. J. Moniz, Fabio Rindi

Green algae of the order Prasiolales are one of the few algal groups with marine,

freshwater and terrestrial representatives. They are particularly interesting since they are widespread in colder regions and marine species living in the two hemispheres are geographically disjunct. Previous investigations have shown a separation of species from different habitats but not with different morphologies, especially among the marine *Prasiola* species. We analysed the phylogeny of this group using the chloroplastic genes *rbcL* and *psaB* and the nuclear marker ITS. Species from non-European regions such as China, Antarctica, Australia, New Zealand and Mexico were added to previous data sets to better understand the biogeography of this order. The *rbcL* gene tree shows the separation of seven clades. The marine clades of *Prasiola stipitata*/*P. meridionalis* and *Rosenvingiella radicans* and *R. polyrhiza* show very good support and seem to be stable clades. The marine clade of *Prasiola* has representatives of a wide geographic range, including representatives from Atlantic and Pacific oceans. It seems that what has been called *Rosenvingiella polyrhiza* in the southern hemisphere is not the same as its namesake inhabiting in the northern hemisphere. Also, what has been named *Prasiola calophylla* in Antarctica comes in a very distinct clade from true *P. calophylla*. The order *Prasiolales* appears to have diversified quickly and conquered very different environments in a short period of time. Information on molecular markers with a rapid evolution rate will aid in resolving the phylogeny of this order and understanding which habitats are more primitive

***Beaufort Meeting 2010, Cork***

***New insights into the taxonomy and systematics of selected Irish seaweeds***

Mónica B.J. Moniz and Fabio Rindi

It has been recognized that in certain habitats algal-sponge interactions have a great ecological significance but only a few studies have analysed in detail the diversity of the algal assemblages associated with sponges. We examined the composition and temporal variation of the algal assemblage associated with a population of *Haliclona indistincta* (Demospongiae, Haplosclerida) at a sheltered

site on the western Irish shore (Corranroo, Galway Bay) for a complete annual cycle (September 2009-August 2010). Sixty seven species of macroalgal epibionts were recorded. Overall, the algal assemblage was most diverse and best developed in the period late winter-early spring 2010. Some species were isolated in culture and cultivated in batch to be examined for production of bioactives; positive results were obtained for some species. Phylogenetic work on the green algal order Prasiolales and the red algal order Peyssonneliales based on sequences of several molecular markers has led to important insights into the phylogeny and taxonomy of these groups. For the Prasiolales, sequences of the chloroplast genes *rbcL* and *psaB* have clarified phylogenetic relationships in this group and shed light into its biogeography. Phylogenetic trees from these genes are in agreement and show that some species occurring in Ireland, namely *Prasiola crispa* and *Prasiola calophylla*, require a taxonomic reassessment. The genus *Peyssonnelia* has been reinvestigated using sequences of the chloroplast *rbcL* and *psbA* genes and the barcoding mitochondrial gene *cox1*. The results so far indicate a high level of intraspecific diversity and suggest that a reassessment of species boundaries will be necessary for some species (*P. squamaria*, *P. inamoena*, *P. stoechas*).

***Young Systematics Forum, 2010, London***

***Understanding the Phylogeny of the green algal order Prasiolales  
(Trebouxiophyceae, Chlorophyta)***

MónicaB.J. Moniz

The Prasiolales are one of the few algal groups with marine, freshwater and terrestrial representatives and are easily identifiable by the observation of their stellate axial chloroplast with a central pyrenoid. These algae are widespread in colder regions and marine species living in the two hemispheres are geographically disjunct. I am analyzing the phylogeny of the more speciose genera *Prasiola* and *Rosenvingiella* using the chloroplast genes *rbcL* and *psaB* and adding, in comparison to previous studies,

taxa from non-European regions such as China, Antarctica, Australia, New Zealand and Mexico to better understand the biogeography of this order. Phylogenetic trees from these genes are in agreement and show that some species within this order need reevaluation, namely *Prasiola crisper* and *Prasiola calophylla*. Studies like this bring us closer to understand how this group, which apparently has diversified quickly, conquered very different environments and understand which was the primitive habitat.

***59th British Phycological Society Winter Meeting, Cardiff, 2011***

***Understanding the biogeographic distribution and the species concept of species *Prasiola crisper* (Trebouxiophyceae, Chlorophyta)***

Mónica B. J. Moniz, Fabio Rindi.

*Prasiola crisper* is the type species for green algal genus *Prasiola*. It is a terrestrial green alga widespread in cold regions, often found associated with bird colonies or sites subjected to persistent mammalian urination and/or in city streets with high humidity conditions. It is one of the most common terrestrial algae in Antarctica but it is also present in the northern hemisphere. Using two chloroplast genes (*rbcL* and *PsaB*), we show that *Prasiola crisper* includes at least two cryptic taxa: the *Prasiola crisper* present in the northern hemisphere and Antarctica, which represents the genuine species (type locality: Isle of Skye, Scotland) and another taxon found so far on King George Island and in Antarctica. Morphological examination based field and cultured material revealed no clear differences between these genotypes. We conclude that genetic diversity within this species has been underestimated

*5<sup>th</sup> European Phycological Congress, 2011, Rhodes*

*Understanding sponges' algal communities*

Mónica B .J. Moniz, Fabio Rindi, Kelly Stephens, Carsten Wolff and Grace McCormack

Although it has been recognized that certain algal-sponge interactions have great ecological significance, to date only a few studies have analyzed in detail the diversity of the algal assemblages associated with sponges. Most of the limited information available has been produced for marine tropical species and freshwater species; to date, data concerning marine temperate species are almost non-existent. The diversity of algae associated with sponges of Irish shores has been investigated by a combination of microscopical investigations and metagenomic studies (last generation 454 sequencing). We summarize here the current information available on the diversity and ecology of algal epibionts living on sponges, presenting the results of an investigation of the algal community associated with a population of *Haliclona indistincta* (Demospongiae, Haplosclerida) at a sheltered site on the western Irish shore (Corranroo, Galway Bay). The epibionts were collected from 3 samples comprised of 10 individuals of *H. indistincta* each month for a complete annual cycle (October 2009-September 2010) and identified morphologically. In the course of the study 66 species of macroalgae were recorded. Overall, the algal assemblage showed significant seasonal modifications and was most diverse and best developed in the period late winter-early spring 2010. Most algal species did not appear to be specifically associated with *H. indistincta* and were also observed on abiotic substrata at the sampling site. Libraries obtained by 454 sequencing identification of the algal community indicate an unexpectedly high diversity. The relationships between the algal community distributed in the inner and surrounding water and the algal community associated with the sponge are examined based on the implications of the metagenomic results.

*CAREX Conference on Life in Extreme Environments, 2011, Dublin*

*Describing Prasiolales diversity in Antarctica*

Mónica B.J. Moniz, Fabio Rindi, Michael D. Guiry

The Prasiolales are one of the few algal groups with marine, freshwater and terrestrial representatives. These algae are widespread in polar and cold-temperate regions. Specimens of *Prasiola* were collected at different sites from Antarctica and compared with European, American and Australasian specimens using the chloroplast genes *rbcL* and *psaB*. Phylogenetic trees from these genes were in agreement and showed that algae previously identified as *Prasiola crista* and *Prasiola calophylla* require re-evaluation. In Antarctica both *P. crista* and *P. crista* var. *antarctica* occur and we propose the status of an independent species, *P. antarctica*, for the latter. In addition, there is another species present which is morphologically similar to *P. calophylla* but genetically different and we also propose that this is a new species. True *P. calophylla* has not yet been identified in Antarctica. These algae have adapted to nitrogen rich environments such as penguin rookeries. They can also survive in dry conditions for long periods of time and possess physiological mechanisms for tolerance of high levels of UV radiation. The dry form is easily dispersed by wind or by birds. For these reasons, *Prasiola* species are extremophiles worthy of further investigation