Project title

Molecular systematics of the siphonous green algae

Summary of project

The siphonous green algae are very common along tropical and temperate Australian shorelines. This project will investigate how many species of this group there are in Australia, how they can be identified using DNA sequences and morphological characters, and how the different species are related to one another in an evolutionary context. The gained knowledge will benefit Australia because they form the baseline for identification of introduced species that could potentially harm marine ecosystems. It will also provide new insights in the diversity of limestone-boring algae, which play a key role in the health of the Great Barrier Reef.

Project aim

This project will integrate molecular and morphological data to establish a solid taxonomic and phylogenetic framework for the Australian siphonous green seaweeds (order Bryopsidales). The project has three major goals:

1. Establish a reliable higher-level phylogenetic hypothesis of the Bryopsidales
2. Assess species boundaries in Australian species belonging to four important families (Codiaceae, Halimedaceae, Rhipiliaceae, Udoteaceae) and establish reliable species-level phylogenies for these families
3. Document the species diversity of *Ostreobium* in Australian waters

Background information

The siphonous green algae form a morphologically diverse group of seaweeds. They are readily distinguished from other green algae by their ability to form large, differentiated thalli comprised of a single, giant tubular cell called siphon [1]. The morphology of these algae ranges from simple, branched thalli to more complex organizations with interwoven siphons, differentiated thalli and diverse morpho-ecological specializations. They form an important component of the marine flora in Australia's tropical and warm-temperate marine environments. They comprise roughly 500 recognized species [2] of which approximately half are Australian [3].

1. Higher-level phylogenetic relationships of Bryopsidales

Only a few studies have addressed the higher-level phylogenetic relationships of the Bryopsidales [1,4,5]. While these studies have drastically improved our understanding of their evolutionary history, they have not led to a solid higher-level phylogeny because of limited gene and taxon sampling. The following three issues have not been resolved:

(1) *Early diversification of major lineages*: The divergence between the two suborders and the early-branching *Ostreobium* lineage has not been established with confidence because only a single *Ostreobium* specimen was sequenced in previous studies, introducing a very long branch in an unstable position [1].

(2) *Radiation of the core Halimedineae*: The families Udoteaceae, Halimedaceae, Pseudocodiaceae, Rhipiliaceae and Caulerpaceae have diverged rapidly and none of the previous studies has been able to establish their relationships with confidence.

(3) *Position of the Pseudobryopsis-Trichosolen group*: Despite their ecological importance as major bloom formers [6], the phylogenetic position of these genera has not been confidently established.

2. Species-level systematics of four important families

It has been shown that purely morphology-based species boundaries are poorly defined in the Bryopsidales [e.g. 7,8] and recent improved taxonomies have first delimited species-level clusters in DNA data and subsequently used statistical analysis to identify those morphological correlates of species boundaries that can be used as diagnostic characters [9,10]. Little work has focused on the molecular assessment of species boundaries in Australian siphonous algae. While outstanding new results have recently been produced for the Caulerpaceae in Fred Gurgel's group (Univ Adelaide), other families have been neglected. The Codiaceae, Rhipiliaceae, Udoteaceae and Halimedaceae are diverse in Australia (34, 15, 24 and 35 species, respectively [3]) and of significant importance in Australian coastal ecosystems. Regional morpho-taxonomic monographs are available for all groups [11-13] and form the necessary framework for morphological identification.

3. Biodiversity of *Ostreobium*

The species *Ostreobium quekettii* has a very peculiar biology [14]. While all other Bryopsidales are textbook seaweeds, this species has a reduced morphology and lives inside carbonate substrates. It is best known living inside coral skeletons, boring its way through by chemical dissolution of $CaCO_3$. Although only one species of *Ostreobium* was previously believed to live in coral skeletons, a recent environmental sequencing study shows that at least 12 lineages exist [15]. Considering that the study was limited to two species of coral in a very restricted geographical area, it would seem safe to assume that the newly discovered biodiversity is just the tip of the iceberg and a vast *Ostreobium* diversity may be found if such studies were to be carried out on a larger scale.

Project justification

The taxonomic issues outlined above limit understanding of the biodiversity of siphonous green algae, their important roles in tropical and warm-temperate ecosystems and decisions related to conservation and ecological management. Here, new collections are introduced and new tools applied to extend our knowledge of the systematics of the group both at higher levels and at the species level.

The PI has a large collection of > 5,000 Bryopsidales samples of which 748 are Australian, and another ca. 2,000 Australian samples will be collected during the field work proposed here. The PI is an experienced siphonous green algal taxonomist and has experience with the entire range of techniques that will be applied. The PhD students involved in the project have a background corresponding to their respective contributions. The team has a working knowledge of all the techniques, including reliably working PCR primers and protocols, high-throughput sequencing and bioinformatics. PhD student Marcelino has already carried out a pilot project to trial environmental sequencing methods for *Ostreobium* biodiversity assessment. All primers and PCR protocols work, and the samples are presently in the queue at a sequencing facility.

Significance/benefits

Study of Australia's biodiversity and its evolutionary history aligns within the National Research Priority of *An Environmentally Sustainable Australia*. It fits with the Priority Goal *Sustainable use of Australia's Biodiversity*, because taxonomy underpins many other disciplines. This project aligns to several ABRS Research Priorities as outlined above. The significance of the project is further augmented by the ecological importance of the taxa:

(1) The calcified Bryopsidales form massive meadows on the Great Barrier Reef where they accrete carbonate at > 1m per thousand years [16]. The taxonomic framework that will be established here underpins future ecophysiological investigations of how these calcifying organisms and the GBR in general will respond to ocean acidification.

(2) *Ostreobium* also plays a key role in the coral reef ecosystem [14]. It facilitates coral survival by transferring a portion of its photosynthate to the coral during bleaching events [17]. It also has a key function in bioerosion. *Ostreobium* is the main agent of carbonate dissolution in coral skeletons and contributes greatly to reef bioerosion. As such, *Ostreobium* plays a major yet understudied role in coral reef health and is key to understanding how reefs will develop (or dissolve) under environmental change. It is likely that different species of *Ostreobium* will respond differently, but this has been ignored in experiments because it has always been assumed that there was only one species. The project presented here will document the presumably vast species diversity of *Ostreobium* and establish culture strains of the different species for future experimental research. In a parallel project starting in the PI's lab, a draft genome of *Ostreobium* will be sequenced.

(3) Several bryopsidalean taxa are vigorous invasive species that are known to have profoundly affected the ecology and native biota in areas of introduction (e.g., *Codium fragile*, *Caulerpa taxifolia* and *Caulerpa racemosa* var. *cylindracea*). Given that seaweed species are more reliably identified with DNA sequences than based on morphology for reasons listed above, it is important to establish reference DNA datasets of native species, so that when species are introduced, they can be unambiguously identified as being new.

Outputs

Results will be published in international and Australian refereed journals, and papers will be presented at appropriate international and Australian conferences. Outputs will include:

- At least one paper presenting a higher-level phylogenetic tree of the Bryopsidales based on chloroplast genomes and nuclear ribosomal cistron sequences.
- At least 3 papers on the species-level systematics of Australian representatives of the families Halimedaceae, Udoteaceae, Rhipiliaceae and Codiaceae.
- At least 3 papers on the biodiversity and molecular phylogeny of *Ostreobium*.
- A public reference set of DNA sequences for Australian Bryopsidales as a basis for molecular identifications.
- A public database of images of representative specimens of the Australian species of the Halimedaceae, Udoteaceae, Rhipiliaceae and Codiaceae.

Outcomes

- Phylogenetic information for Bryopsidales, with a global scope but emphasizing the Australian diversity.
- Revised classifications of Australian members of the Halimedaceae, Rhipiliaceae, Udoteaceae and Codiaceae derived from multi-gene DNA sequence datasets
- Insights in the biodiversity of the limestone-boring genus *Ostreobium* in Australia, which will also be framed in a global framework and a genomic context through parallel work on the biodiversity from other regions and a project aimed at sequencing a draft genome.
- Training of three PhD students and at least two honours students in algal systematics, including field procedures, morphological and molecular identification, and molecular phylogenetics.
- Development of molecular tools (procedures and reference DNA sequences) for molecular identification of native and introduced Bryopsidales.

Contributions to the ABRS

- Voucher specimens from field collections will be deposited in Australian herbaria and be available through Australia's Virtual Herbarium.
- DNA sequence data will be made publicly available through Genbank.
- An open-source online repository illustrating representative specimens of the Australian species of Bryopsidales will be produced, further increasing the visibility of Australian biodiversity and of the ABRS.

Method

Sampling: Field work will be carried out on Lord Howe Island, Ningaloo Reef, Heron Island and in Victoria. Samples of the focal taxa will be collected by snorkeling and SCUBA diving. Small, young, cleaned pieces of thallus will be preserved in silica gel and RNA later for later molecular analysis and the remainder of the specimen will be preserved in ethanol for anatomical investigation and/or pressed, depending on the size of the specimen. We will target a wide range of corals and other limestone substrates to obtain environmental samples for *Ostreobium* biodiversity assessments. A very small piece will be chipped off, surface-cleaned, cut into slices with a Dremel tool and preserved in RNA later. The subsamples taken for DNA analysis (silica-gel, RNA later) will be frozen in the field, transported back to the lab and stored in frozen condition to reduce DNA damage.

Goal 1: DNA will be extracted from culture strains or meticulously cleaned thallus pieces using a modified CTAB protocol. Following quality control, the extracted DNA will be sent out for high-throughput sequencing, multiplexing 5 samples per lanes with barcode adapters. Sequencing will be done with Illumina GAIIx technology, resulting in paired-end 2x150bp reads. Reads will be assembled with a standard pipeline that includes several programs (Velvet/Oases, SOAPdenovo, Trans-ABySS). Prior experience suggests that the sequencing coverage for 5 samples per lane exceeds 100x for the plastid genome, yielding contigs covering large pieces of the plastid genome as well as the complete nuclear ribosomal cistron and substantial pieces of the mitochondrial genome. Open reading frames will be extracted and annotated using reciprocal BLAST searches and phylogenetic sorting methods. Plastid genes and the ribosomal cistron will be aligned and subjected to phylogenetic analysis using appropriate partitioned models.

Goal 2: Three markers (partial *tuf*A, 28S nrDNA and EF1α) will be amplified from ca. 1,000 CTAB-extracted samples using established protocols. Species boundaries will be inferred from the data using different techniques, including GMYC (individual loci) and BP&P (multi-locus) [18,19]. For ca. 150 samples representative of different species, an additional 3 markers (*rbc*L, *psb*A, 18S nrDNA) will be sequenced to increase the length of the alignment from which the species-level phylogenies will be inferred.

Goal 3: The ca. 480 limestone samples to be targeted for *Ostreobium* biodiversity will be homogenized in a bead-beater and extracted with a CTAB method. We have recently established protocols to amplify pieces of *tuf*A, *rbc*L and UPA of ca. 370 bp length. Based on the primers from these protocols, we will construct fusion primers that include barcode tags and bridge adapters. The amplicons resulting from PCR with these primers can be directly pooled and will be sequenced on the Illumina MiSeq (2 x 250 bp) to obtain complete coverage of each individual sequence. To (molecularly) identify species in the resulting DNA sequences, all data will be pooled and subjected to species delimitation algorithms (e.g. GMYC [18]). With this knowledge available, the biodiversity and species composition of each sample can then be determined.

Personnel and Facilities

PI Heroen Verbruggen is a productive early-career researcher with 11 years of experience in the application of molecular and morphological data to the systematics and evolutionary biology of green seaweeds. Until March 2012, he was a postdoctoral fellow at Ghent University (Belgium). The School of Botany has attracted him to Melbourne, where he has started his own team as an ARC Future Fellow. Verbruggen will coordinate the project, participate in some of the field work, supervise and train the students, and participate substantially in producing the outputs.

Three PhD students will be involved in the project. They will all participate in those parts of the field work most relevant for their project. PhD student Joana Costa and a second PhD student (to be appointed) will be jointly responsible for growing algal strains and carrying out DNA sequencing for the higher-level and species-level systematics aspects. The unnamed PhD student will primarily

focus on taxonomic results while Ms Costa will use the produced phylogenies in her project about the evolutionary dynamics of ecological niches. PhD student Vanessa Marcelino will carry out sequencing of the environmental samples containing *Ostreobium*.

Two Honours students (to be appointed) will work with the PhD students and the PI on sub-projects. They will receive field and lab training in taxonomic techniques.

The molecular work will largely be done through the School of Botany at the University of Melbourne. The department has all equipment required to carry out the proposed research. Sequencing runs (Sanger and Illumina) will be outsourced to a specialized service provider (to be determined). The School of Botany provides a superb intellectual environment for the research students. They will be part of the Plant Systematics group (PIs Ladiges, Drinnan, Bayly & Verbruggen) in which many students are working on similar projects in other taxa.

References

[1] Verbruggen et al. 2009 *Mol. Phylogen. Evol.* 50: 642; **[2]** www.algaebase.org; **[3]** Schils 2012 PhycoBase; **[4]** Woolcott 2000 *Phycologia* 39: 471; **[5]** Lam & Zechman 2006 J. Phycol 42: 669; **[6]** Pauly et al. 2011 *Mar. Biol.* 158: 2239; **[7]** Fama et al. 2002 *J. Phycol.* 38: 1040; **[8]** Kooistra et al. 2002 *Mol. Phylogen. Evol.* 24: 121; **[9]** Verbruggen et al. 2005 *J. Phycol.* 41: 606; **[10]** Verbruggen et al. 2006 *Eur. J. Phycol.* 41: 337; **[11]** Womersley 1984 The marine benthic flora of southern Australia, Part 1; **[12]** Millar & Kraft 2001 Phycologia 40: 21; **[13]** Kraft 2007 Algae of Australia, Green algae; **[14]** Verbruggen & Tribollet 2011 *Curr. Biol.* 21: R876; **[15]** Gutner-Hoch & Fine 2011 Coral Reefs 30: 643; **[16]** Drew 1993 Spums J. 23: 93; **[17]** Fine & Loya 2002 *Proceedings B* 269: 1205; **[18]** Monaghan et al. 2009 Syst. Biol. 58: 298; **[19]** Yang & Rannala 2010 PNAS 107: 9264.