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Ectocarpus

A model organism for the brown algae

Susana M. Coelho^{1,2}, Delphine Scornet^{1,2}, Sylvie Rousvoal^{1,2}, Nick Peters^{1,2}, Laurence Dartevelle^{1,2}, Akira F. Peters^{2,3}, J. Mark Cock^{1,2}

¹UPMC Université Paris 06, The Marine Plants and Biomolecules Laboratory, UMR 7139, Station Biologique de Roscoff, Place Georges Teissier, BP74, 29682 Roscoff Cedex, France. ²CNRS, UMR 7139, Laboratoire International Associé Dispersal and Adaptation in Marine Species, Station Biologique de Roscoff, Place Georges Teissier, BP74, 29682 Roscoff Cedex, France. ³Bezhin Rosko, 40 rue des pêcheurs, 29250 Santec, France.

ABSTRACT

The brown algae are an interesting group of organisms from several points of view. They are the dominant organisms in many coastal ecosystems, where they often form large, underwater forests. They also have an unusual evolutionary history, being members of the stramenopiles, which are very distantly related to well-studied animal and green plant models. As a consequence of this history, brown algae have evolved many novel features, for example in terms of their cell biology and metabolic pathways. They are also one of only a small number of eukaryotic groups to have independently evolved complex multicellularity. Despite these interesting features, the brown algae have remained a relatively poorly studied group. This situation has started to change over the last few years, however, with the emergence of the filamentous brown alga *Ectocarpus* as a model system that is amenable to the genomic and genetic approaches that have proved to be so powerful in more classical model organisms such as *Drosophila* or *Arabidopsis*.

BACKGROUND INFORMATION

Ectocarpus siliculosus is a small filamentous brown alga. Seaweeds of the genus *Ectocarpus* are found worldwide along temperate coastlines, where they grow on rocky substrates or epiphytically on other algae and seagrass. Research on *Ectocarpus siliculosus* has had a long history (Charrier et al. 2008), and this was one of the reasons that led to this species being selected as a genetic and genomic model organism for the brown algae about seven years ago (Peters et al. 2004). Other important arguments for selecting *Ectocarpus* included its small size, the fact that the entire life cycle can be completed relatively rapidly (3 months) in the laboratory (Müller et al. 1998), its high fertility and the ease with which genetic crosses can be carried out (Peters et al. 2004; Peters et al. 2008).

The brown algae are members of the stramenopiles (or heterokonts), together with organisms such as diatoms and oomycetes. The stramenopiles diverged from other major eukaryotic groups such as the opisthokonts (animals and fungi) and the archaeplastida (which includes land plants) over a billion years ago. One consequence of this unusual phylogenetic history is that brown algae exhibit many novel features, for example in terms of their metabolism and cell biology, making them prime targets for explorative research. The brown algae are also important because they are one of only a small number of eukaryotic groups that have evolved complex multicellularity (Cock et al. 2010a). However, another consequence of the large phylogenetic distance that separates stramenopiles from intensely-studied groups such as animals, fungi and green plants is that model organisms developed for these latter groups

are of limited relevance to brown algal biology. Given this context, the emergence of *Ectocarpus* as a model organism is expected to have a considerable impact on brown algal research.

SOURCES AND HUSBANDRY

Ectocarpus strain collections are maintained at the Culture Collection of Algae and Protozoa, (CCAP) Scottish Association for Marine Science, Oban, Scotland (<http://www.ccap.ac.uk/>), the Macroalgal Culture Collection at Kobe University (<http://www.research.kobe-u.ac.jp/rcis-ku-macc/>) and at the Station Biologique in Roscoff, France (<http://www3.sb-roscoff.fr/>). These three institutions currently hold, in triplicate, 328 *Ectocarpus* strains from a broad range of geographical locations and ecological niches. Sampling campaigns have been carried out recently around the coast of Britain, along the Channel coast in France, along the Pacific coasts of Peru and Chile, and in Korea, resulting in another collection of about 1500 strains, which is maintained at the Station Biologique in Roscoff. These strain collections are being exploited to study the biodiversity and ecology of *Ectocarpus* in the field and also as a source of genetic diversity for laboratory based studies.

In addition to the field-isolated strains, laboratory-based projects are also generating important biological material. For example, a segregating population was created for the construction of a genetic map (Heesch et al. 2010) and an ongoing TILLING (Targeting Induced Local Lesions in Genomes) project necessitates the maintenance of a large number of mutant lines. Altogether, more than 3000 genetically distinct, laboratory-generated strains are being maintained in Roscoff.

The *Ectocarpus* strain collection is organised as a centralized resource and a barcode system is being developed to identify and handle individual strains within the collection. Strains are maintained in duplicate as unialgal cultures free of eukaryotic contaminants in 5-10 mL of medium, at low light intensity ($1-3 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) and low temperature (5-15 °C), in growth chambers or incubators. The medium in these long-term storage cultures is renewed once a year. Data on geographical origin, morphology and other relevant features are maintained in a retrievable database that is being linked to the barcode storing system.

The culture collections have been of key importance for the establishment of *Ectocarpus* as a model organism. The collections are widely exploited, not only by members of the *Ectocarpus* Genome Consortium, but also by a broader community of scientists. The collections also serve as a basis for exchanges between laboratory- and field-based research programs.

RELATED SPECIES

The genus *Ectocarpus* currently contains three species, *E. siliculosus*, *E. fasciculatus* and *E. crouaniorum* (Peters et al. 2010b). However, there is accumulating evidence that these three taxa do not adequately describe the species diversity within the genus and additional species are likely to be defined in the future (Peters et al. 2010a). Phylogenetic analysis indicates that the Ectocarpales emerged relatively recently within the brown algae and that they are a sister group to the order Laminariales, which includes most of the large kelp species (Silberfeld et al. 2010). Brown algae belonging to other families within the Ectocarpales differ from *Ectocarpus* in terms of their physiology, cytology, life histories and ecology and may be suitable for comparative studies in the near future.

USES OF THE *ECTOCARPUS* MODEL SYSTEM

Research on *Ectocarpus* began in the 19th century with a description of species and investigation of their taxonomic positions (Dillwyn 1809). Subsequent studies were aimed at investigating the life cycle and the ultrastructure of the organism at different stages of the life cycle (Müller 1972). Additional work included identification of the sexual pheromone and its role in gamete recognition (Boland et al. 1995) and characterisation of the *Ectocarpus* virus EsV-1 (Delaroque et al. 2001). The following sections describe recent work carried out using *Ectocarpus* as a model organism. Additional emerging topics, not discussed here, include sex determination, gamete recognition and parthenogenesis.

The *Ectocarpus* life cycle

Ectocarpus has a haploid-diploid life cycle, involving alternation between two multicellular generations, the sporophyte and the gametophyte. Diploid sporophytes produce haploid meio-spores in unilocular sporangia. Following release the meio-spores germinate to give the haploid gametophyte generation. Gametophytes are dioecious, producing either male or female gametes, which fuse to produce the diploid zygotes that reinitiate the sporophyte generation. There are several possible variations on this basic life cycle; in particular gametes that do not find a gamete of the opposite sex with which they are able to fuse to form a zygote can develop parthenogenetically to produce sporophytes. It has long been something of a mystery as to how these partheno-sporophytes, which are derived from a haploid cell, are able to produce meio-spores (which are normally produced by a reductive meiotic division). Recent work has shown that partheno-sporophytes solve this problem in two different ways (Bothwell et al. 2010). Either there is an endoreduplication event very early during development resulting in a diploid individual, or the algae remain haploid and the meio-spores are produced via a non-reductive apomeiotic division in the developing unilocular sporangium.

Both generations of the *Ectocarpus* life cycle develop from single cells released into the medium by the parent(s). Moreover, the two generations exhibit clear morphological differences, and yet are sufficiently similar to allow the isolation of mutants that lead to the conversion of one generation into the other. These features, and the fact that life cycle generation is not determined by ploidy (see the section on haploid partheno-sporophytes above, for example), make *Ectocarpus* a particularly interesting model to study the genetic regulation of life cycle progression (Coelho et al. 2007). Interestingly, the two *Ectocarpus* generations exhibit radically different patterns of initial cell division, development starting with a symmetric division in the sporophyte, to produce a basal filament, but with an asymmetric division in the gametophyte, leading to the production of a rhizoid and an upright filament (Peters et al. 2008). This difference has facilitated screens for life cycle mutants. For example, the *immediate upright* (*imm*) mutant exhibits partial conversion of the sporophyte generation into the gametophyte generation (Peters et al. 2008). *imm* mutant sporophytes retain sporophyte reproductive characteristics, producing spores and not gametes, but they have several developmental features typical of the gametophyte including an asymmetric initial cell division that leads to the production of a rhizoid and an upright filament. More recently, a second mutant has been isolated which exhibits complete conversion of the sporophyte generation into a gametophyte (Coelho et al. 2011). This mutant has been named *ouroboros* after the symbol for the cycle of life. Current work is aimed at identifying the loci corresponding to the *imm* and *oro* mutations.

Morphogenesis

Analysis of the early development of the *Ectocarpus* sporophyte using a morphometric approach showed that the pattern of development is highly variable, both in terms of cell differentiation and branching of the filament (Le Bail et al. 2008). However, this variability appears to occur within a certain level of biological constraint, resulting in organisms with similar final architectures. This has allowed early development to be modelled using a stochastic automaton (Billoud et al. 2008). The variability in the pattern of early development in *Ectocarpus* contrasts with the highly ordered development of other model organisms such as *Fucus* or *Arabidopsis*. A recent study has shown that a mutation at the *ETOILE* locus results in a modified pattern of early development, mutant algae initiating branching more rapidly than wild type individuals (Le Bail et al. 2011).

Responses to biotic and abiotic stresses

Coastal ecosystems are relatively aggressive environments and *Ectocarpus* is not only prone to infection by a wide range of pathogens, including viruses, oomycetes, chytrids, hyphochytrids and parasites related to the Plasmodiophorea (Müller et al. 1998; Müller et al. 1999; Maier et al. 2000; Gachon et al. 2009), but is also subject to considerable abiotic stress due to continuous variations in temperature, salinity and light intensity. Microarray analysis has indicated that there is extensive reprogramming of the *Ectocarpus* transcriptome, even in response to relatively mild stresses, with transcripts for a large proportion of the expressed genes exhibiting changes in abundance (Dittami et al. 2009). Moreover, many of the genes that exhibit regulatory changes in response to stress are of unknown function suggesting that novel mechanisms of stress resistance remain to be discovered in this species.

Metabolism

Many brown algae are able to accumulate high concentrations of iodide from seawater. Seaweeds of the order Laminariales are thought to use apoplasmic iodine as an anti-oxidant, and the emission of iodine by this system may have a significant impact on atmospheric chemistry (Kupper et al. 2008). Analysis of the *Ectocarpus* genome identified not only enzymes that may be involved in this system but also a number of dehalogenases and haloalkane dehalogenases (Cock et al. 2010a). These latter enzymes may help *Ectocarpus* grow epiphytically on other brown algae by defending it against halogenated molecules produced by these hosts as defence molecules.

The carbon storage system of brown algae is unusual, involving the accumulation of reserves of mannitol and the β -1,3-glucan laminarin rather than α -1,4-glucans such as starch or glycogen. Recent analysis of the evolutionary origins of these storage systems (Michel et al. 2010b), using data from the *Ectocarpus* genome sequence, has brought into question the idea of a common origin for stramenopile and alveolate plastids, proposed under the "chromalveolate hypothesis" (Cavalier-Smith 1999). The study by Michel et al. (2010b) proposes that, whilst the alveolate lineage was probably able to acquire starch metabolism from its endosymbiont by modifying its existing glycogen pathway, the stramenopile lineage did not have this option because it had already lost the glycogen metabolic pathway. The existence of starch in one lineage but not the other therefore suggests two independent endosymbiotic events. The mannitol pathway was probably acquired later by the brown algal lineage via a horizontal transfer event from actinobacteria, along with another key metabolic pathway in brown algae, alginate biosynthesis (Michel et al. 2010a).

Ecology

We have only limited knowledge about the ecology of *Ectocarpus* (Charrier et al. 2008) and there are many open questions in this domain. In ecophysiological experiments, different strains (which we now know belong to different species) survived temperatures of up to 23°C for arctic strains but up to 33°C for subtropical strains, and growth temperature optima were correlated with the conditions with which each strain was confronted in the field. When the responses to temperature of the two generations were different, the gametophyte was the less tolerant generation (Bolton 1983). The same tendency (large variability among different strains, and higher tolerance of the sporophyte) was found for salt tolerance (Thomas and Kirst 1991a, 1991b). The production of reproductive organs by sporophytes of Mediterranean strains of *Ectocarpus* was shown to be regulated by temperature, with meio-spore-containing unilocular sporangia being produced at lower temperatures and mito-spore-containing plurilocular sporangia being produced at higher temperature (Müller 1963). Recent phenological observations on *E. crouaniorum* from Brittany (Peters et al. 2010b) may provide an explanation for some of the differences between the generations; in this species the epilithic sporophytes are small and found all year round, often as microscopic forms, whereas the gametophytes are ephemeral, occurring only in spring. The sporophyte generation might therefore be expected to be adapted to cope with more extreme physical conditions. There are no phenological data for both generations of other species of *Ectocarpus*.

The different species of *Ectocarpus* may be found in different zones on the shore. In megatidal Brittany, *E. crouaniorum* occurs in the upper intertidal, whereas *E. siliculosus* and *E. fasciculatus* are found in the mid intertidal and the subtidal, respectively (Peters et al. 2010b). *Ectocarpus* is more often found epiphytic on marine macrophytes than epilithic but little has been published about the host ranges of the different species and their interactions with basiphytes. *E. fasciculatus* occurs regularly as an endophyte in the blades of *Laminaria digitata* (Russell 1983a, 1983b), but it is not known how it is able to overcome the host's defence. *Ectocarpus* is normally fixed to a substratum but it may be detached and can survive as a floating thallus. Morphological changes have been shown to occur when *Ectocarpus* grows without being attached to a substrate, possibly as a result of lost polarity (Russell 1967, 1967).

Thalli of *Ectocarpus* harbour a diverse biota of small, sessile or motile animals (e.g. crustaceans and nematodes), and protists (e.g. ciliates and benthic diatoms). The interactions with these organisms, some of which may feed on the *Ectocarpus* thallus or on released reproductive cells, has not been studied so far.

GENETICS, GENOMICS AND ASSOCIATED RESOURCES

Following the selection of *Ectocarpus* as a model, considerable effort was invested in the development of genomic and genetic tools for this organism. The most important of these was the assembly and analysis of the complete 214 Mbp genome sequence (Cock et al. 2010a). Additional tools that have been developed for *Ectocarpus* include RNA-seq data, a large EST collection, whole genome tiling arrays, deep sequencing of small RNAs, EST-based expression microarrays (Dittami et al. 2009), a genetic map (Heesch et al. 2010), stramenopile-adapted bioinformatic tools (Gschloessl et al. 2008) and proteomic (Ritter et al. 2010) and metabolomic methodologies. Genetic tools have also been developed including protocols for UV and chemical mutagenesis, phenotypic screening methods, genetic crosses, methods for handling large populations, a large number of genetic markers and defined strains for genetic mapping (Heesch et al. 2010).

Protocol 1

How to cultivate *Ectocarpus*

This protocol describes the standard procedure for growing *Ectocarpus* in the laboratory. Standard growth conditions are 13°C with a 12h:12h day:night cycle and 20 $\mu\text{mol photons.m}^{-2}.\text{s}^{-1}$ irradiance using daylight type fluorescent tubes (Philipps TL-D). The algae can be cultivated either in plastic Petri dishes or in 10 l bottles with bubbling if large amounts of biomass are required. The standard medium is Provasoli-enriched natural seawater (PES) but *Ectocarpus* can also be grown in artificial seawater allowing a more precise control over the culture conditions. The protocol starts with partheno-sporophyte (or sporophyte) material because this is the stage that is usually maintained as stock. All manipulations of cultures should be carried out in a clean environment, if possible under a laminar flow hood. Forceps should be dipped in ethanol and allowed to dry under the hood. Using a combination of this protocol and protocol 2 (Genetic crosses between *Ectocarpus* strains), the sexual life cycle can be completed in 3 to 4 months, depending on the strain.

MATERIALS

Reagents

Natural or artificial seawater <R>

Provasoli solution <R>

Equipment

140 mm diameter Petri dishes

60 mm diameter Petri dishes

Fine forceps

70% ethanol

METHOD

1. Inoculate autoclaved seawater supplemented with Provasoli solution with small pieces (1 mm) of (partheno-)sporophyte filaments. Culture should be at low density (just a few pieces of filament per Petri dish) to ensure that there are sufficient nutrients (cultures grown at high densities will not produce unilocular sporangia). The sporophyte filaments will stick to the bottom of the dish and in a few weeks they start to produce upright filaments. Change the medium regularly (once a fortnight for a 140 mm Petri dish culture).

Temperature induction of unilocular sporangia (10-13°C) has been reported for some strains (E. siliculosus sensu strictu, Müller 1963). However, this temperature shift may not induce unilocular sporangia in other Ectocarpus strains.

2. About 2 days after the uprights appear, plurilocular sporangia are produced. These contain mito-spores that allow asexual (clonal) reproduction of the sporophyte. About 1 week later, the unilocular sporangia that contain the meio-spores will appear. The meio-spores are the initial cells of the gametophyte generation.

3. Working under a sterile hood with a stereomicroscope, use a sterile Pasteur pipette to carefully dissect off one or two unilocular sporangia. Put a drop (30 μ l) of PES containing the unilocular sporangium on a coverslip that has been placed on a drop of medium in a Petri dish. Add 4-8 drops of PES around the sides of the Petri dish. This creates a moist chamber (Fig. 1) and prevents the drop containing the unilocular sporangium from drying up. A few hours after isolation, the unilocular sporangium should release its meio-spores into the medium. When this happens, discard the piece of filament bearing the empty unilocular sporangium using forceps or a Pasteur pipette under the stereomicroscope and fill the Petri dish with PES. Gametophytes should develop in about two weeks (100 to 200 per unilocular sporangium).

Meiospore germlings can be kept under very low light (2 μ mol photons.m⁻².s⁻¹) for up to 2 months. Under these conditions, they will not develop, and can be returned to standard growth conditions when necessary.

4. Isolate the gametophytes under the stereomicroscope. Be careful that they are not contaminated by sporophytes (a proportion of the meio-spores will develop as sporophytes, a phenomenon known as heteroblasty). Approximately ten gametophytes can be cultivated in PES in a 140 mm Petri dish. The gametophytes should become fertile in about 2 weeks, producing gametes in plurilocular gametangia.

5. Monitor the gametophytes under the stereomicroscope until they become mature (i.e. produce plurilocular gametangia). Remove most of the PES so that they are in a small drop and incubate in the dark at 13°C overnight. In the morning, add 1-2 ml of PES to the drop and place under strong light for ten minutes. This treatment induces synchronous gamete release. The release of the swimming zoids can be followed under the stereomicroscope.

Protocol 2

Genetic crosses between *Ectocarpus* strains

This protocol describes how to carry out crosses between different strains of *Ectocarpus*. By combining this protocol with Protocol 1 (How to cultivate *Ectocarpus*) it is possible to complete the *Ectocarpus* life cycle in about three or four months in the laboratory. Crossing gametophytes to obtain the sporophyte generation is the most technically challenging stage of this process because diploid sporophytes have to be distinguished from the haploid partheno-sporophytes that result from the parthenogenetic germination of unfused gametes. This requires careful monitoring of the progeny of the genetic cross until they have developed sufficiently to be transferred to a separate Petri dish. Genetic crosses allow several classical genetic methodologies to be applied in *Ectocarpus*, including allelic complementation tests, backcrosses, combination of different genetic mutations and outcrosses to create mapping populations.

MATERIALS

Reagents

Provosoli enriched seawater (PES) <R>

Microscope immersion oil (Zeiss)

Equipment

22 mm coverslips

Fine forceps

Pasteur pipettes

Vaseline

White plastic plumber's washers (1 mm thick, outer diameter 18 mm, inner diameter 12 mm)

60 mm Petri dishes

Steel needles

METHOD

1. Use Vaseline to stick a plastic washer to a microscope slide and put some more Vaseline along the top of the washer so that a coverslip can be stuck to the top of the ring. Keep the cultures at 10°C whilst preparing this material so that they do not release their gametes before the cross is set up.

2. Place three coverslips (22 mm) on a surface that will allow them to be picked up easily and spot a drop (about 10 μ l) of PES onto each coverslip.

3. Place small pieces of filament into each drop, two drops should just receive one of each of the parents, whilst the third will receive both parents. The two uniparental units are controls, to verify that both of the parent gametophytes release their gametes. Be careful not to cross contaminate between the three drops. Adjust the size of the drop to about 20 μ l by adding medium.

4. Gently but rapidly invert the coverslip and place it on the plastic ring. The bottom of the drop must not touch the microscope slide (Fig. 2a).

5. Incubate overnight at 13°C and then analyse under a microscope to determine whether zygotes have formed. Zygotes are identified by their larger size and confirmed (100x magnification may be required) by the presence of two stigmata (Fig. 2b). The stigmata will not be visible later (after 24-48h) so it is necessary to draw a sketch of the distribution pattern of the germlings. This sketch will allow the diploid sporophytes to be located and isolated later so that they can be transferred for culturing.

Zygotes with 3 or more stigmata can be formed as a result of polyspermy; this is rare but it does occur.

6. After about a week, invert the coverslip and place it in a Petri dish with PES and incubate at 13°C. Note that the microscope oil can be toxic (use Zeiss oil) so remove it from the slide with alcohol before placing the coverslip in culture. Remove the parthenogenically-derived germlings that surround the zygote-derived germlings with a fine-tipped steel needle (sharpened using abrasive paper and rinsed with

ethanol) so that the latter will be easier to isolate later. Under an inverted microscope, centre a zygote in the field using 10x or 40x, then work at 4x to remove parthenosporophytes with the needle. Monitor the configuration of the zygotes with respect to the drawing (individuals grow and some die).

7. When the zygote-derived germlings have grown sufficiently, they can be isolated and placed in culture. Transfer the growing sporophytes to 60 mm Petri dishes in PES by detaching with a steel needle then transferring using a Pasteur pipette.

Protocol 3

Isolation and regeneration of protoplasts from *Ectocarpus*

This protocol describes how to obtain isolated cells with no surrounding cell wall by enzymatic digestion of *Ectocarpus* filaments. The protoplasts are totipotent and regenerate to produce individual algae under appropriate culture conditions. The yield of protoplasts and their capacity to regenerate are highly dependent on the strain used, the stage of the life cycle and the culture conditions. The highest yields are obtained with young gametophyte filaments cultivated at low density. The naked, wall-less cells produced by this protocol can be used for several applications, including studies of cell wall regeneration, investigation of the role of the cell wall in determining cell fate and as a source of naked cells for the development of methods of introducing diverse molecules into the cell.

MATERIALS

Reagents

Provasoli-enriched seawater (PES) <R>

Antibiotic mix <R>

Chelation medium <R>

Digestion medium <R>

Cellulase (Sigma)

Alginate lyase (Boyen et al. 1990)

Equipment

Petri dishes

Pasteur pipettes

Orbital incubator

Forceps

40 µm cell strainers (BD Falcon)

METHOD

1. Weigh the algal material (app. 500 mg fresh weight) after removing any excess seawater
2. Place the filaments in a 90 mm plastic Petri dish and separate the filaments with forceps.

3. Add 15 ml of chelation medium and incubate in the dark at 13°C for 20 min.
4. Replace the chelation medium with 15 ml of digestion medium and incubate in the dark at 13°C with gentle agitation for up to 6 hours.
5. Check the material regularly under a microscope. When the cell wall has been digested (Fig. 3), pass through a 40 µm filter to remove the undigested filament material.
6. Wash the protoplasts twice by centrifugation at 50-100 g for 15 min at 4°C and resuspend in regeneration medium. Count the number of protoplasts obtained using a Neubauer cell.
7. To obtain regeneration of the protoplasts, cultivate at very low density in regeneration medium in the dark at 13°C. After two days, start very slowly to reduce the osmolarity by adding drops of PES supplemented with antibiotics. After a further day, transfer the regenerating protoplasts to low light, and continue to reduce the osmolarity by adding drops of PES with antibiotic until complete replacement of the regeneration medium. Finally, after a further day, protoplasts (that at this point have regenerated their cell wall) can be transferred to 20 µmol photons.m⁻².s⁻¹, i.e. the standard culture conditions described in protocol 1.

Protocol 4

Extraction of high quality genomic DNA from *Ectocarpus*

For some applications, such as genome sequencing (Cock et al. 2010b) and high-throughput genotyping with multiple markers (Heesch et al. 2010), it is necessary to use high quality genomic DNA. This protocol describes how to obtain several micrograms of high quality, caesium chloride purified DNA from one gram of *Ectocarpus* filaments. We also recommend using DNA of this quality for quantitative RT-PCR control reactions. Simpler, more rapid, kit-based methods are however preferable for experiments that involve the treatment of large numbers of individuals, such as genotyping large populations with a small number of markers or PCR screening of large populations.

MATERIALS

Reagents

Liquid nitrogen

2% CTAB extraction buffer <R>

Proteinase K

Chloroform:isoamylalcohol (24:1, CIA)

Ethanol (100%, 80%)

12 M LiCl <R>

β-mercaptoethanol

Isopropanol

Tris-EDTA (TE, pH8) <R>
Phenol (for DNA)
3 M sodium acetate (NaAc, pH 5.2) <R>
CsCl
Hoechst N° 33258 bis-benzimide
TE saturated butanol <R>
Isopropanol
Distilled water

Equipment

Mortar and pestle
5 teflon tubes
Wheaton grinder (1 ml)
2 ml Eppendorf tubes
55°C water bath
Pipettes
Filter tips
Centrifuge
Ultracentrifuge
Weighing balance
Polyallomer bell-top tube
Vacuum centrifuge
Minicentrifuge
Beckman heater (for ultracentrifuge tube sealing)
Parafilm
26G needle
2 ml syringe with 18G needle
Aluminium foil
UV illuminator
Filter tips
Pipettes

METHOD

1. Grind about 1 g of frozen filaments in a mortar and pestle under liquid nitrogen for about 15 min.
2. Transfer the powder to a 40 ml teflon tube and add 2 ml of 2% CTAB buffer. Mix well, but gently.
3. Further grind 1 ml aliquots in a Wheaton grinder (5 min per aliquot) and transfer the aliquots to a new teflon tube using a 1 ml filter tip. After all the aliquots have been ground, rinse the Wheaton several times with 1 ml aliquots of CTAB buffer, recover these rinses in the teflon tube and make the total volume up to 15 ml with CTAB buffer. Mix vigorously for 10 min.

4. Add 25 units of Proteinase K. Mix gently and incubate at 55°C for 2 hours.
5. Add 1 volume of chloroform / isoamylalcohol, (24:1) mix gently and centrifuge at 10,000 rpm (12,000 g) in a JA20 rotor at 12°C for 20 min. Transfer the upper phase into a new teflon tube and measure the volume.
6. Add 0.3 volumes of 100% ethanol drop by drop whilst stirring gently. Mix well and add 1 volume of chloroform / isoamylalcohol.
This step removes polysaccharides. The ethanol must be added gradually to prevent precipitation of the DNA.
7. Centrifuge at 12,000 rpm (10,000 g) in a JA20 rotor at 12°C for 20 min, transfer the upper phase to a new teflon tube and measure the volume.
8. Add 0.25 vol. of 12 M lithium chloride and mix it vigorously. Add 1% β-mercaptoethanol and mix well. Incubate at -20°C overnight.
This step precipitates RNA.
9. Centrifuge at 10,000 rpm (12,000 g), in a JA20 rotor at 4°C for 30 min, transfer the supernatant to a new teflon tube and measure the volume.
A pellet of RNA should be visible on the side of the tube. The RNA can be recovered by adding 500 μl of water and stored at -20°C.
10. Add 0.8 vol. of 100% isopropanol and incubate at -20°C for at least 2 hours (ideally overnight). Centrifuge at 10,000 rpm (12,000 g) in a JA20 rotor at 4°C for 30 min, discard the supernatant and retain the pellet.
11. Add 500 μl of TE buffer pH 8 or water and dissolve the pellet gently. Transfer the DNA solution to a 2 ml tube and measure the volume. Add 0.5 volumes of phenol pH 8.0 and 0.5 volumes of chloroform / isoamylalcohol and mix vigorously. Centrifuge at 13,000 rpm (17,950 g), 12°C, for 10 min, transfer the supernatant into a new 2 ml tube and measure the volume.
12. Repeat the phenol / chloroform / isoamylalcohol extraction.
13. Add 1 volume of chloroform / isoamylalcohol and mix gently. Centrifuge at 13,000 rpm (17,950 g), 12°C, for 10 min, transfer the supernatant into a new 2 ml tube and measure the volume.
14. Add 2.5 volumes of 100% ethanol and a tenth volume 3M sodium acetate pH5.2. The DNA should precipitate as a white fluffy material. Incubate at -20°C overnight, centrifuge at 13,000 rpm (17,950 g), 4°C, for 30 min and discard the supernatant. Wash the pellet with 200 μl of 80% ethanol. Centrifuge at 13,000 rpm (17,950 g), 4°C, for 15 min and discard the supernatant. Dry the pellet and dissolve in 200 to 500 μl of Tris-EDTA buffer (pH 7.5).

15. Weigh out 1.818 g of caesium chloride into an Eppendorf tube and add 1 mL of TE pH 8. Add at least 20 µg of DNA in TE pH 8 buffer solution and adjust the volume to 1.5 ml. Add 7.4 µl Hoechst N° 33258 bis-benzimide, mix well and check that the caesium chloride is fully dissolved (if not add more TE pH 8 buffer).

16. Transfer the solution to a 2 ml Polyallomer bell-top tube and weigh the tube. Adjust the weight with TE pH 8 buffer so that pairs of tube differ in weight by less than 5 mg. Place the small metal cap on the tube and heat seal.

17. Spin the sample in an ultracentrifuge at 90,000 rpm (650,000 g), 20 °C, for at least 24 hours.

Note that larger preparations in 13.5 ml tubes should be centrifuged for at least 40 hours.

18. Remove the tubes from the rotor and visualise the DNA on a UV table. There should be two bands, the upper band corresponding to organelle DNA and the lower to nuclear DNA.

19. Make a small hole in the top of the tube with a 26 G needle, then puncture the tube with a 18 G needle attached to a 2 ml syringe just underneath the desired DNA band. Carefully aspirate the DNA and transfer it to a 2 ml Eppendorf tube.

20. Add 1 volume of TE-saturated butanol. Mix well, and centrifuge 2 min at 13,000 rpm (17,950 g) to allow the two phases to separate and discard the upper phase (which is the butanol with the extracted Hoechst dye). Repeat this extraction 2-3 times and then check the DNA solution on a UV transilluminator. The solution will fluoresce blue if Hoechst is still present, if this is the case carry out 2 or 3 more butanol extractions.

21. Measure the volume of the DNA solution, add 2.5 volumes of 100% ethanol and precipitate the DNA overnight at -20 °C. Centrifuge at 13,000 rpm (17,950 g), 4 °C, for 30 min and discard the supernatant. Wash the pellet with 50 µl of 80% ethanol and centrifuge at 13,000 rpm (17,950 g), 4 °C, for 5 min. Discard the supernatant and dry the pellet to remove any ethanol. Dissolve the pellet in 50 µl of Tris-EDTA buffer (pH 8.0). Check the quantity and quality of the DNA using a Nanodrop machine and by running an aliquot on a 0.8% agarose gel.

22. To check the stability and the digestibility of the DNA dilute about 100 ng of DNA to 30 µl with an appropriate restriction digestion buffer and incubate samples either with or without added restriction enzyme (we usually use *XhoI* or *SalI*) at 37°C overnight. Check the two samples on a 0.8% agarose gel. The undigested sample should produce a band of about 25 kbp whereas the digested sample should have a smeared appearance.

Protocol 5

Immunostaining of *Ectocarpus* cells

This protocol has been optimised for the detection of tubulin but could be used with any suitable antibody. *Ectocarpus* has small but relatively transparent cells and the uniseriate filaments can be grown directly attached to the surface of the coverslips. These features make them particularly suitable for high resolution imaging approaches, both *in vivo* or after fixation.

All incubations described below are carried out on a platform shaker at room temperature. Use high quality microscope slides (e.g. Fisher 12-550-42), to avoid imperfections in the glass that can be a problem for confocal laserscan microscopy analysis.

MATERIALS

Reagents

PHEM <R>

mPBS <R>

NaBH₄ <R>

Solution C <R>

Solution C plus enzymes <R>

Blocking solution <R>

Clearing solution (BABB) <R>

Equipment

Weighing balance

Microscope coverslips and slides

METHOD

1. Dip slides with attached *Ectocarpus* material rapidly into liquid nitrogen (immerse for less than one second) and immediately immerse in PHEM solution in a fresh 35x10 Petri dish for 1h (or overnight at 4°C)
2. Remove the PHEM, rinse three times with mPBS (5 min each time) and immerse in 5% Triton-X-100 in mPBS. Incubate overnight at room temperature.
3. Rinse three times with mPBS and then immerse in 100 mM NaBH₄. Incubate for 4 h or until the solution stops bubbling (indicating that the reducing reaction is complete).
4. Rinse three times with mPBS. Immerse in solution C, then in solution C plus enzymes. Incubate for 1h.
5. Rinse three times with mPBS. Immerse in the blocking solution and incubate overnight at room temperature.
6. Rinse three times with mPBS. Add the primary antibody and incubate for 4h or overnight. We carry out the incubation in a 140 mm Petri dish lined on the bottom with parafilm. Pipette the primary antibody

onto the parafilm then place the slide on top. Place the Petri dish in a larger Petri dish with ~½ inch of wet paper towels on the bottom. Cover the pyrex dish with plastic film (Saran wrap) and put into a light-proof cardboard box. This keeps the chamber humid so that the filaments do not dry out.

7. Rinse three times with mPBS and add the secondary antibody. Incubate for 4h or overnight as described above. Exclusion of light is particularly important for the secondary antibodies.

8. If you want to carry out a double labelling, label with the first pair of antibodies (monoclonal primary, then secondary) as above, then block with blocking solution overnight before labelling with the second pair of antibodies (polyclonal primary, then secondary).

9. Rinse three times with mPBS and rinse three times with 100% methanol at -20°C. Note that methanol dries very quickly, do not let the cells dry out. Mount in clearing solution (BABB) by pipetting about 20 µl onto the microscope slide, then rapidly place a coverslip on the slide (touch the edge of the slide on a paper towel to remove excess methanol). Wait for about 20 seconds then paint around the perimeter of the coverslip with nail polish (e.g. Sally Hensen's clear "Hard as Nails"). Wait for an hour and then repeat the sealing with the nail polish to eliminate small holes that could cause the material to dry out.

Recipes

ANTIBIOTIC MIX

<u>Reagent</u>	<u>Quantity (for 100 ml)</u>
Penicillin G	1 g
Streptomycin	0.5 g
Chloramphenicol	0.1 g

Dissolve the penicillin G and streptomycin in 90 ml of water, then add the chloramphenicol dissolved in 10 ml of 100% ethanol. Filter through a 0.22 µm membrane and store 5 ml aliquots at -20°C. Add 5 ml of the antibiotic mix to 1 liter of natural or artificial seawater.

ARTIFICIAL SEA WATER

<u>Reagent</u>	<u>Quantity (for 1 litre)</u>	<u>Final concentration</u>
NaCl	26.29 g	450 mM
KCl	0.74 g	10 mM
CaCl ₂	0.99 g	9 mM
MgCl ₂ (6H ₂ O)	6.09 g	30 mM
MgSO ₄ (7H ₂ O)	3.94 g	16 mM

Adjust to pH 7.8, autoclave and store at 4°C.

BLOCKING SOLUTION

<u>Reagent</u>	<u>Quantity (for 100 ml)</u>
mPBS	100 ml
non-fat dry milk	2.5 g

Prepare fresh just prior to use.

2% CTAB EXTRACTION BUFFER

<u>Reagent</u>	<u>Quantity (for 30 ml)</u>
100 mM TrisHCl (pH 6)	3 ml of 1M stock
1.5 M NaCl	9 ml of 5M stock
2% CTAB	3.75 ml of 16% stock
50 mM EDTA (pH 7)	6 ml of 250 mM stock
50 mM DTT	0.231g

CLEARING SOLUTION (BABB)

<u>Reagent</u>	<u>Quantity (for 30 ml)</u>
benzyl benzoate	20 ml
benzyl alcohol	10 ml

16% (w/v) CTAB

<u>Reagent</u>	<u>Quantity (for 100 ml)</u>	<u>Final concentration</u>
CTAB stock (hexadecyltrimethylammonium bromide)	16 g	16%

Dissolve the CTAB by incubating at 42 °C overnight.

CHELATION MEDIUM

<u>Reagent</u>	<u>Quantity (for 1 litre)</u>	<u>Final concentration</u>
NaCl (MW= 58.44g/mole)	40.9 g	700 mM
MgCl ₂ -6H ₂ O (MW= 203.3g/mole)	6.1 g	30 mM
MgSO ₄ -7H ₂ O (MW= 246.48g/mole)	7.39 g	30 mM
KCl (MW= 74.55g/mole)	1.49 g	20 mM
EGTA (MW= 380.35g/mole)	7.6 g	20 mM

Adjust to pH 5.5 using HCl; autoclave or filter through 0.2 µm and store at 4°C.

DIGESTION MEDIUM

<u>Reagent</u>	<u>Quantity (for 1 litre)</u>	<u>Final concentration</u>
NaCl (MW= 58.44g/mole)	23.4 g/L	400 mM
MgCl ₂ -6H ₂ O (MW= 203.3g/mole)	26.4 g/L	130 mM
MgSO ₄ -7H ₂ O (MW= 246.48g/mole)	5.4 g/L	22 mM
KCl (MW= 74.55g/mole)	11.9 g/L	160 mM
CaCl ₂ (MW= 147.02g/mole)	0.3 g/L	2 mM
MES (MW= 195.2g/mole))	1.95 g	10 mM

Adjust to pH 6.5, filter through a 0.2 µm filter and store at 4°C.

Just before use, add 1% w/v cellulase and 3U/ml alginate lyase.

0.5 M EDTA pH 8

<u>Reagent</u>	<u>Quantity (for 1 litre)</u>	<u>Final concentration</u>
EDTA	186.12 g	0.5 M

Dissolve the EDTA in 100 ml of water by adjusting the pH to 8.0 with NaOH. Autoclave.

12 M LiCl

<u>Reagent</u>	<u>Quantity (for 200 ml)</u>	<u>Final concentration</u>
LiCl (MW= 42.39 g/mol)	101.736 g	12 M

Take care during the dissolving step as this is very exothermic.

Filter the solution at 0.45 µm and autoclave.

mPBS

<u>Reagent</u>	<u>Quantity (for 3.2 litres)</u>	<u>Final concentration</u>
NaCl (MW=58.44)	25.6 g	136 mM
KCL (MW=74.55)	0.64 g or 8.64 ml of 1 M stock	2.7 mM
KH ₂ PO ₄ (MW=136.09)	0.7408 g	1.7 mM
Na ₂ HPO ₄ (MW=141.96)	3.648 g	8.03 mM
glycerol (MW=92.10)(density=1.261g/cm ³)	160 ml	685 mM
sodium azide (MW=65.02)	3.2 g	15.4 mM
BSA (MW=67,000)	3.2 g	0.015 mM

The pH should be 7 or slightly above. Adjust the volume to 3.2 l with distilled water.

This solution is stable and can be used for several weeks.

3 M NaAc

<u>Reagent</u>	<u>Quantity (for 200 ml)</u>	<u>Final concentration</u>
NaAc (MW= 82.03 g/ml)	49.218 g	3 M

Dissolve the sodium acetate in 120 ml of water and adjust pH to 5.2 with glacial acetic acid. Autoclave.

NaBH₄

<u>Reagent</u>	<u>Quantity (for 100 ml)</u>	<u>Final concentration</u>
mPBS	100 ml	
NaBH ₄	0.3784 g	100 mM

5 M NaCl

<u>Reagent</u>	<u>Quantity (for 200 ml)</u>	<u>Final concentration</u>
NaCl (MW= 58.442 g/mol)	116.88 g	5 M

Dissolve the NaCl in 120 ml of water, adjust the total volume to 200 ml and autoclave.

PHEM

<u>Reagent</u>	<u>Quantity (for 200 ml)</u>	<u>Final concentration</u>
PIPES	3.68 g	60 mM
Hepes	1.19 g	21 mM
EGTA	0.76 g	10 mM
1M MgCl ₂	400 µl	2 mM
NaCl	8 g	685 mM

Adjust the pH to 7.5 with KOH

Adjust the volume to 179 ml volume with distilled water, then add 1 ml of 25% glutaraldehyde and 20 ml of 32% paraformaldehyde

This solution works best if it is made fresh just prior to use.

PROVASOLI SOLUTION

<u>Solution 1 (10x)</u>	<u>Quantity (for 1 litre)</u>	<u>Final concentration</u>
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H ₃ BO ₃ (MW=61.83)	1.9 g	30.7 mM
FeCl ₃ (MW=162.21)	0.05 g	0.3 mM
MnSO ₄ (H ₂ O) (MW=169.02)	0.273 g	1.6 mM
ZnSO ₄ (7 H ₂ O) (MW=287.54)	0.0367 g	0.127 mM
CoSO ₄ (7 H ₂ O) (MW=281.1)	0.008 g	28 µM
0.5 M EDTA pH8 (MW=292.24)	11.4 ml	5.7 mM

<u>Solution 2 (10x)</u>	<u>Quantity (for 500 ml)</u>
Vitamin B12 (cyanocobalamine)	3.35 mg
Thiamine hydrochloride (vitamin B1) (MW=337.27)	165 mg
Biotin C ₁₀ H ₁₆ N ₂ O ₃ S (MW=244.31)	1.65 mg
TRIS = Trisma base C ₄ H ₁₁ NO ₃ (MW=121.14)	166.5 g

<u>Solution 3 (10x)</u>	<u>Quantity (for 1 litre)</u>	<u>Final concentration</u>
(NH ₄) ₂ Fe(SO ₄) ₂ (6H ₂ O) (MW=392.14)	1.17 g	3 mM
0.5 M EDTA pH8	6.8 ml	3.4 mM

<u>Solution 4 (10x)</u>	<u>Quantity (for 1 litre)</u>	<u>Final concentration</u>
NaNO ₃ (MW=84.99)	23 g	270 mM

<u>Solution 5 (10x)</u>	<u>Quantity (for 1 litre)</u>	<u>Final concentration</u>
C ₃ H ₇ Na ₂ O ₆ P(5H ₂ O) "glycerophosphate" (MW=216.04)	3.33 g	15.4 mM

Prepare each stock solution separately, autoclave and store at 4°C in a glass bottle. Use a dark bottle for solution 2.

For 1 litre of Provasoli solution add 100 ml each of solution 1, 3, 4 and 5 plus 10 ml of solution 2 to milliQ water (starting pH should be between 9.6 and 9.8).

Adjust to pH 7.8 with concentrated HCl (37%) and adjust the volume to 1 litre with milliQ water.

Aliquot into small glass bottles (20, 50, 100 or 200 ml), autoclave and store at 4°C.

PROVASOLI ENRICHED SEAWATER (PES) (Starr and Zeikus 1993)

<u>Reagent</u>	<u>Quantity (for 1 litre)</u>
Natural seawater	1 l
Provasoli solution	20 ml

If possible seawater should be collected by boat at some distance from the coast. Filter the seawater at 5 µm. Aliquot into Nalgene™ bottles (in glass bottles a precipitate can form), autoclave and store at 13°C. We use half-strength PES, i.e. 10 ml of Provasoli solution is added to 1l of autoclaved seawater.

The filtered seawater and the Provasoli solution are autoclaved separately in order to avoid precipitation.

REGENERATION MEDIUM

<u>Reagent</u>	<u>Quantity (for 1 litre)</u>	<u>Final concentration</u>
MgCl ₂ -6H ₂ O (MW=203.3g/mole)	30.5 g	150 mM
KCl (MW=74.55g/mole)	7.5 g	100 mM
NaHCO ₃ (MW=84.01g/mole)	0.34 g	4 mM
KNO ₃ (MW=101.11g/mole)	0.202 g	2 mM
NaH ₂ PO ₄ (MW=119.98g/mole)	0.012 g	100µM

Adjust the volume with natural seawater.

SOLUTION C

<u>Reagent</u>	<u>Quantity (for 1 litre)</u>	<u>Final concentration</u>
NaCl (MW=58.44)	5.8 g	100 mM
MgCl ₂ (or 20 ml of 1 M stock)	4.1 g	20 mM
KCl (MW=74.55)	0.15g	2 mM
BSA (MW=67,000)	2 g	29.8 µM
MES (MW=195.2)	2.13 g	0.01 mM
sorbitol (MW=182.17)	154.85 g	850 mM
EGTA (MW=292.24)	0.38 g	1 mM

Adjust the pH to 5.8 with TRIS base, then adjust the volume to 1 l with distilled water. Store 50ml aliquots at -20°C

SOLUTION C PLUS ENZYMES

<u>Reagent</u>	<u>Quantity (for 25 ml)</u>
Solution C	25 ml
CELF	175 mg
hemicellulase	1 g

Add the enzymes just prior to use.

TE SATURATED BUTANOL

<u>Reagent</u>	<u>Quantity (for 200 ml)</u>	<u>Final concentration</u>
Butanol	100 ml	50%

Tris-EDTA buffer (pH 8) 100 ml 50%

The saturated butanol is the upper phase.

TRIS-EDTA

<u>Reagent</u>	<u>Quantity (for 1 l)</u>	<u>Final concentration</u>
Tris-HCl (pH 7.5) MW=121.14g/mole	1.21g	10 mM
EDTA-4Na-2H ₂ O MW=416.2g/mole	0.42g	1 mM

Adjust to pH 8 and autoclave.

1 M TRIS-HCl

<u>Reagent</u>	<u>Quantity (for 200 ml)</u>	<u>Final concentration</u>
Trizma base (MW= 121.1 g/mol)	24.22 g	1 M

Dissolve the Trizma base in 100 ml of water, adjust to pH 7.5 with concentrated HCl, then adjust the volume to 200 ml and autoclave.

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WWW RESOURCES

<http://bioinformatics.psb.ugent.be/webtools/bogas/> *Ectocarpus* genome access. The *Ectocarpus* genome sequence can be browsed at this site.

FIGURE LEGENDS

Figure 1 – “Moist chamber” used for the isolation of unilocular sporangia. After dissection, the unilocular sporangia are transferred to a small drop on a coverslip placed inside a Petri dish. Four to eight drops of PES are placed around the edge of the Petri dish to keep the chamber moist.

Figure 2a – Genetic crosses are performed in hanging drops containing two compatible *Ectocarpus* strains. Male and female gametes are released from the fertile gametophyte filaments and syngamy occurs within the drop. Zygotes stick preferentially to the edges of the drop. The hanging drop procedure allows the genetic cross to be monitored by observation under an upright microscope.

Figure 2b – Zygotes and gametes in a hanging drop. Zygotes (asterisk) are identified by the presence of two stigmata (arrow heads).

Figure 3 - Protoplasts of *Ectocarpus* after incubation in digestion medium for 5 hours.

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