Genetic Crosses between *Ectocarpus* Strains

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Protocol

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This article describes a procedure for conducting crosses between different strains of *Ectocarpus*. 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

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous material used in this protocol.

RECIPES: Please see the end of this article for recipes indicated by <R>. Additional recipes can be found online at http://cshprotocols.cshlp.org/site/recipes.

Reagents

Ectocarpus cultures (see **How to Cultivate *Ectocarpus*** [Coelho et al. 2012a])

Ethanol (70%)

Provasoli solution <R>

Provasoli-enriched seawater (PES) <R>

Vaseline

Equipment

Abrasive paper

Coverslips (round; 22-mm diameter)

Fine forceps, dipped in ethanol and allowed to dry under the hood

Microscope, upright and inverted

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Microscope immersion oil (Zeiss)

Microscope slides

Pasteur pipettes

Petri dishes (60-mm)

Steel needles

White plastic washers (1 mm thick; 18 mm outer diameter; 12 mm inner diameter) (obtained from purveyors of plumbing supplies)

METHOD

1. Using Vaseline, stick a plastic washer on a microscope slide and put some more Vaseline around the top of the washer so that a coverslip can be stuck to the top of the ring. Keep the *Ectocarpus* cultures at 10°C while assembling this apparatus so that they do not release their gametes before the cross is set up.
2. Place three coverslips (22-mm diameter) on a surface that will allow them to be picked up easily and spot a drop (~10 µL) of PES onto each coverslip.
3. Place small pieces of *Ectocarpus* filament into each drop of PES. Place one of each of the parents into the first two drops of PES, and place both parents into the third drop. The two uniparental samples are controls, which allow verification that both of the parent gametophytes release their gametes. Be careful not to cross-contaminate the three drops. Adjust the size of the drops to ~20 µL by adding more PES.
4. Gently, but rapidly, invert each coverslip and place it on a plastic ring. Do not allow the bottom of the drop to touch the microscope slide (Fig. 1A). Incubate overnight at 13°C.
5. Analyze under an upright microscope to determine whether zygotes have formed. Identify zygotes by their size (they are larger than gametes) and confirm by the presence of two stigmata

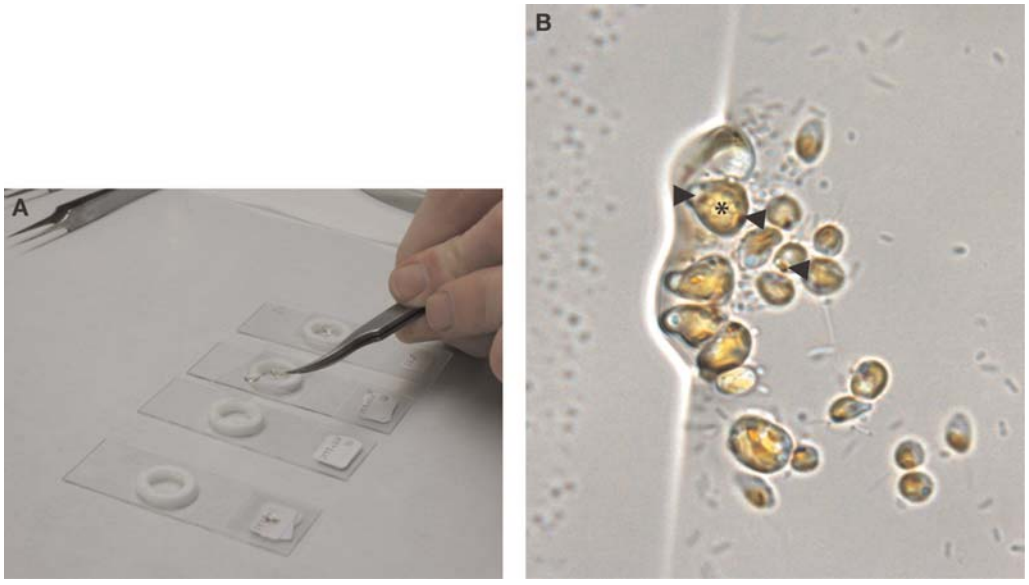

FIGURE 1. (*A*) Genetic crosses are performed in hanging drops of PES that contain two compatible *Ectocarpus* strains. Male and female gametes are released from the fertile gametophyte filaments and syngamy occurs within the drop. Zygotes stick preferentially to the edges of the drop. The hanging drop procedure allows the genetic cross to be monitored by observation under an upright microscope. (*B*) Zygotes and gametes in a hanging drop. Zygotes (asterisk) are identified by the presence of two stigmata (arrowheads).

(100× magnification may be required; Fig. 1B). Because the stigmata will not be visible later (after 24–48 h), 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 three or more stigmata can be formed as a result of polyspermy; this is rare, but it does occur.

6. After ~1 wk, invert the coverslip, place it in a Petri dish with PES, and incubate at 13°C. Note that the microscope oil can be toxic, so remove it from the slide with alcohol before placing the coverslip in culture. Using a fine-tipped steel needle (sharpened with abrasive paper and rinsed with ethanol), remove the parthenogenically derived germlings that surround the zygote-derived germlings, so that the latter will be easier to isolate later. On an inverted microscope, center a zygote in the field **using 10× or 40× objectives, then work at 4×** 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. Using a steel needle, first detach the growing sporophytes, and then using a Pasteur pipette, transfer them to 60-mm Petri dishes containing PES.

RELATED INFORMATION

Combining this protocol for genetic crosses between *Ectocarpus* strains with the procedure described in **How to Cultivate *Ectocarpus*** (Coelho et al. 2012a), it is possible to complete the life cycle of the organism in 3–4 mo in the laboratory. For further details regarding the biology of *Ectocarpus* and the development of genomic and genetic tools for this organism, see ***Ectocarpus*: A Model Organism for the Brown Algae** (Coelho et al. 2012b).

RECIPES

Provasoli-Enriched Seawater (PES)

Reagent	Quantity (for 1 L)
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 using a 5-µm mesh. Aliquot into Nalgene bottles (in glass bottles a precipitate can form), autoclave, and store at 13°C. Autoclave the filtered seawater and the Provasoli solution separately to avoid precipitation. This recipe is based on Starr and Zeikus (1993); we use half-strength PES (i.e., 10 mL of Provasoli solution per 1 L of autoclaved seawater).

Provasoli Solution

Solution 1 (10×)

Reagent	Quantity (for 1 L)	Final concentration
H_3BO_3 (MW = 61.83)	1.9 g	30.7 mM
$FeCl_3$ (MW = 162.21)	0.05 g	0.3 mM
$MnSO_4 \cdot H_2O$ (MW = 169.02)	0.273 g	1.6 mM
$ZnSO_4 \cdot 7H_2O$ (MW = 287.54)	0.0367 g	0.127 mM
$CoSO_4 \cdot 7H_2O$ (MW = 281.1)	0.008 g	28 µM
EDTA (0.5 M, pH 8)	11.4 mL	5.7 mM

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Solution 2 (10×)

Reagent	Quantity (for 500 mL)
Vitamin B12 (cyanocobalamine)	3.35 mg
Thiamine hydrochloride (vitamin B1) (MW = 337.27)	165 mg
Biotin $C_{10}H_{16}N_2O_3S$ (MW = 244.31)	1.65 mg
TRIS (Trisma base) $C_4H_{11}NO_3$ (MW = 121.14)	166.5 g

Solution 3 (10×)

Reagent	Quantity (for 1 L)	Final concentration
$(NH_4)_2Fe(SO_4)_2{\cdot}6H_2O$ (MW = 392.14)	1.17 g	3 mM
EDTA (0.5 M, pH 8)	6.8 mL	3.4 mM

Solution 4 (10×)

Reagent	Quantity (for 1 L)	Final concentration
$NaNO_3$ (MW = 84.99)	23 g	270 mM

Solution 5 (10×)

Reagent	Quantity (for 1 L)	Final concentration
$C_3H_7Na_2O_6P{\cdot}5H_2O$ "glycerophosphate" (MW = 216.04)	3.33 g	15.4 mM

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

For 1 L of Provasoli solution, add 100 mL of each of Solutions 1, 3, 4, and 5 plus 10 mL of Solution 2 to MilliQ water (the 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 L with MilliQ water. Aliquot into small glass bottles (20, 50, 100, or 200 mL), autoclave, and store at 4°C.

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