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Development of a doubled haploid mapping population of *Pyropia yezoensis* and measurement of the economic characters of blade

Version 2

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ABSTRACT

Pyropia yezoensis is one of the most valuable and widely cultivated seaweeds across the world. However, there is limited data regarding its genetic background underlying complex economic traits. The molecular genetic linkage map of *P. yezoensis* was not constructed. The most probable cause was the lack of mapping population, which was the basic of linkage analysis. In the present study, a red-type and fast-growing pigmentation mutant Py-HT and a wild-type strain Py-LS who had enough variation for traits of interest at both DNA and phenotypic level were crossed under control. The heterozygote (heterozygous conchocelis) was identified among conchocelis colonies which were developed from single zygotospore released from zygotosporangia of fertilized Py-HT blade, based on the result that 91.9% F1 gametophytic blades developed from the conchospores of heterozygous conchocelis were linearly sectored with 2-4 color sectors of parental colors. Afterwards, 57 chimeric blades with four color sectors were screened from thousands of F1 blades and 228 color sectors were separated according the boundaries of color sectors for single culture. A single zygotospore released from zygotosporangia of one sector through selfing was selected and then developed into homozygous conchocelis, which was declared a double haploid strain. Finally, a mapping population with 148 strains obtained from 37 of the 57 four color sectored blades was developed, which was used for the construction of genetic linkage maps and analysis of quantitative trait loci of *P. yezoensis* blades.

EXTERNAL LINK

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PROTOCOL STATUS

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Preparation of conchocelis

1 Stock culture of free-living conchocelis of *Pyropia yezoensis* wild-type strain Py-LS and red-type pigmentation mutant Py-HT were maintained in the laboratory, at **19 °C** under 10 μmol photons m^{-2} s^{-12} (10:14 LD) provided by cool-white, 40-W fluorescent lamps, in an **250 ml** SEBC bottle with **200 ml** culture medium, which was usually renewed 50% every 10~12 month.

The culture medium was natural seawater collected from the East China Sea off Qushan Island enriched with MES medium (**[M]10 Volume Percent**). The formula of MES medium was shown in Table 1 and Table 2.

Table 1. Formula of MES medium

sodium nitrate (NaNO3)	2.8 g
sodium glycerophosphate	0.4 g
MP II solution	200 ml
Tris (hydroxymethyl) methyl aminomethane	4.0 g
distilled water	700 ml

3 M HCl was used to adjust pH to 7.6 and the final volume was 1000ml.

Table 2. Formula of MP II solution

EDTA·2Na	3.0 g

H3BO3	2.5 g
MnCl2·4H2O	0.35 g
FeCl3·6H2O (62.5 mg/mL)	2 ml
ZnCl2 (12.5 mg/mL)	2 ml
CoCl2·6H2O (5 mg/ml)	2 ml
Fe·Citrate (30 mg/mL)	2 ml
distilled water	1000 ml

Pyropia yezoensis has a biphasic life cycle that alternates between macroscopic, foliose, gametophytic blades and microscopic, sporophytic filaments (referred to as the conchocelis phase). The gametophytic blade, which develops from a conchospore produced by mature conchocelis, is haploid, monoecious and consists of membranous monostromatic blade that is anchored by a small rhizoidal holdfast. The conchocelis, which develops from a carpospore produced by the mature gametophytic blade, is a shell-boring form in nature. When the conchocelis grows in either seawater or liquid culture medium, it is called freeliving conchocelis.

Collection of conchospores.

2 A small amount of conchocelis (about **10 µg**) of each strain was sampled from stock culture and incubated in a 90 mm Petri dish at **23 °C** under 20 µmol photons m^{-2} s^{-1} (10:14 LD) to induce the formation of conchosporangia.

When conchosporangia appeared, the conchocelis was transferred into a **250 ml** Erlenmeyer flask containing **200 ml** culture medium and cultured with aeration in an incubator at **19 °C** under 40 µmol photons m^{-2} s^{-1} (10:14 LD).

Vinylon monofilaments (about 3 cm long) were placed in the flask for attachment of conchospores. Then, the monofilaments with attached conchospores were transferred to a new flask and cultured under the same condition to obtain conchospore germlings (gametophytic blades).

The general cultural condition is a **250 ml** Erlenmeyer flask containing **200 ml** culture medium and cultured with aeration in an incubator at **19 °C** under 40 µmol photons m^{-2} s^{-1} (10:14 LD). The culture medium was renewed 50% every 5 days.

Cross of blades

3 When gametophytic blades of each strain grew up to 1-2 cm long, they were detached from vinylon monofilaments and cultured individually (one blade cultured in one flask).

When spermatangia appeared in the blade, but before spermatia release, the upper peripheral portions of one blade of Py-HT strain and one blade of Py-LS strain were cut out and co-cultured until carposporangia appeared.

Individual fertilized blade was then transferred to a **500 ml** flask with **450 ml** culture medium and cultured until carposporangia mature. In the present study, only fertilized blade of Py-HT strain was selected for releasing carpospores.

The blade with mature carposporangia was dried in the shade at **19 °C** and then immersed in a 90 mm Petri dish with **50 ml** culture medium for **01:00:00** to induce the release of carpospores. After that, the blade was subcultured in the flask and used for releasing carpospores at the next several days.

The carpospores from Py-HT blade grew into conchocelis colonies in 90 mm Petri dishes at 19 °C under 20 µmol photons m^{-2} s^{-1} (14:10 LD). When the color of conchocelis colonies became distinguishable microscopically, individual colonies with the color of wild-type were picked out by means of a finely drawn Pasteur pipette and transferred to Petri dishes or test tubes for culture.

During the culture of carpospores and conchocelis colonies, the culture medium can only be renewed after the colonies became macroscopic.

It is reported in our previous work [doi: 10.1046/j.1440-1835.2000.00203.x.] that the mutations associated with color phenotypes are recessive, because all of the heterozygous conchocelis resembled the wild type color when they were crossed with the wild type (wt). Therefore, the color of heterozygote of Py-HT × Py-LS was wild type color.

Determination of heterozygote

4 After approximately 3 months in culture, when wild type color conchocelis clumps grew into approx. 10 mm in diameter, they were cultured at 23 °C under 30 µmol photons m^{-2} s^{-1} (10:14 LD) for induction of conchosporangia, respectively.

go to step #2

After approximately 3 weeks in culture, F_1 gametophytic blades developed from conchospores were detached from the monofilaments with a surgical blade. Color phenotypes and blade types of the F_1 blades were examined microscopically. When more than 90% F_1 blades of one conchocelis were 2-4 color-sectored blades, the conchocelis was identified as the heterozygote.

For *Pyropia yezoensis*, the blades are monoecious and could be self-fertilized, the heterozygote could only be identified through F_1 blades if they are mainly color-sectored, which depended on tissue culture techniques.

Construction of a doubled haploid population

5 Only four-color sectored mosaic blades were screened from the F_1 blades.

Every four-color sectored blade was then cut into four color-sectors along the boundaries of adjacent color-sectors and every color-sector was subsequently cultured individually.

A DH strain was obtained when one of the carpospores was released from a self-fertilized color-sector and developed into a single conchocelis.

Collection of carpospores and culture of conchocelis was according to go to step #3 .

In our previous work [DOI: 10.11964/jfc.20161210641], a mapping population of 148 DH strains was established from 37 four-color sectored mosaic blades.

In Pyropia yezoensis, the blades are monoecious and could be self-fertilized. Therefore, the DH population can be established by self-fertilization because spermatia (male gamete) and carpogonium (female gamete) always occur diffusely on a single sector that is developed from one of the tetrad cells after mitosis. Thus, the gametes formed on a single color-sector are genetically identical. We considered that self-fertilization of a color-sector was a procedure of chromosome doubling of gamete.

Measurement of the economic characters of blade

6 go to step #2

All the conchospores of 148 strains were collected individually and attached to Vinylon monofilaments.

Gametophytic blades of each strain developed from conchospores were cultured in a 300 ml flask with 250 ml culture medium at 19 °C under 28-30 μmol photons m^{-2} s^{-1} (14:10 LD). The medium was renewed 50% every 10 days.

Blades were detached from monofilaments after 30 days in culture. Then, 100 intact blades with holdfasts and tips were screened randomly and transferred to a 600 ml flask with 500 ml culture medium (half old and half new medium) under 38-40 μmol photons m^{-2} s^{-1}.

After 5 days in culture, 50 intact blades (35-day old) were screened randomly and half medium was renewed.

After 5 days in culture, 30 intact blades (40-day old) were screened randomly and transferred to a 1200 ml flask with 1000 ml culture medium (half old and half new medium). The medium was renewed 50% every 5 days.

The length, width and fresh weight of the 40-day old blades (L40, W40 and FW40) were measured. The blade length was measured from the holdfast to the blade tip. The blade width was measured at the widest part of the blade. The fresh weight of blade was measured after the surface water of the blade was sucked up with a paper towel.

After 10 days in culture, the length, width and fresh weight of the 50-day old blades (L50, W50 and FW50) were measured.

The specific growth rate of blade length, width and fresh weight between 40th and 50th day (LGR, WGR and FWGR) were calculated based on a formula. Take LGR for example,

$$\mathrm{LGR}\ (\%) = \frac{\ln(\mathrm{L50}) - \ln(\mathrm{L40})}{\mathrm{n}} \times 100$$

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where ln was the natural logarithm, L50 and L40 were the length of blade at 40th and 50th day, respectively, and n was the interval of 10 days.