J Appl Phycol
DOI 10.1007/s10811-009-9466-1

Towards domestication of giant kelp (*Macrocystis pyrifera*) in Chile: selection of haploid parent genotypes, outbreeding, and heterosis

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Received: 19 December 2008 / Revised and accepted: 9 July 2009

Abstract *Macrocystis* is an important marine resource in Chile, with severe problems of over-exploitation. Our study describes genetic materials and techniques for a further improvement of laboratory-based mariculture. For a systematic hybridization program we have selected one pair (cultivar) of gametophytes with favorable somatic and reproductive characteristics from each of seven localities in southern Chile. Sporophytes from all 49 crosses were grown for 10 weeks to seedling size. We report here that sporophytes from sympatric parents (intra-cultivar matings) grow to different length, depending on the locality and, importantly, that sporophytes from several inter-cultivar crossings show superior growth, suggesting heterosis with symmetric or asymmetric reciprocity. The genetic materials and techniques described here, together with our newly developed standardized seedling production protocols now available, constitute a significant step towards domestication of *Macrocystis* in analogy to terrestrial agriculture.

Keywords Chile · Cultivar · Domestication · Heterosis · Hybridization · *Macrocystis* · Mariculture · Phaeophyta · Outbreeding

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Introduction

Macrocystis is a major component of many coastal habitats in cold-temperate climate zones of both hemispheres. Natural populations are an important resource for commercial production of alginate (Zemke-White and Ohno 1999). *Macrocystis* biomass is traditionally used in Chile as a raw product for alginic acid. Drift material or cut fronds are collected and traded in dried condition for processing. This situation has changed dramatically, since intense on-land mariculture of *Haliotis* spp. (abalone) had been introduced in Chile by the mid 1990s. These high-value mollusks have become an important economic resource. They are herbivores, and their preferred diet are the local brown kelps. Natural populations of *Macrocystis* and *Lessonia* from Chiloé in the South to Caldera in the North are now under significant harvesting pressure, and evidence for over-exploitation becomes increasingly visible (Vásquez 2008).

Thus, it is important to develop mariculture techniques for the production of additional kelp biomass along the coastline of Chile. In a previous study (Westermeier et al. 2006, 2007), we have described new laboratory methods, which can serve as a basis for a modern kelp mariculture industry. We introduced clonal gametophyte cultures as genetically defined stock material for the production of *Macrocystis* seedlings. Furthermore, aeration-culture of free-floating juvenile sporophytes facilitates and accelerates the production of seedlings for explantation on ropes in the sea. We showed that this technique can produce large batches (10^5 or more) of genetically identical seedlings from selected parent gametophytes in regular intervals at any time of the year.

The materials and methods now available for the manipulation of *Macrocystis* reproduction and cultivation fulfill all prerequisites necessary for crop improvement

Published online: 30 July 2009

programs as known for terrestrial plants, and it is now possible to explore this option in laboratory experiments. Our previous experiments for the development of *Macrocystis* and *Lessonia* seedling production were based on one single pair of parent gametophytes (Westermeier et al. 2006). However, we note substantial variation between local populations of *Macrocystis pyrifera*, and assume that this may be due to different genotypes. It is therefore important to improve the genetic basis for the production of *Macrocystis* seedlings for commercial use.

As a first approach, we expanded the geographical range of our breeding stock, now including seven localities in Southern Chile, each represented by one pair of male and female gametophytes. With this broader base we could compare the performance of genetically defined representatives of local populations under our standardized *Macrocystis* seedling production protocol. In addition, this approach offered the chance to explore the phenomenon of heterosis, which is an important principle in plant and animal breeding.

We report here the results of our hybridization studies using representatives of seven geographically separated *M. pyrifera* populations.

Materials and methods

Mature sporophylls of *Macrocystis pyrifera* (L.) C. Agardh were collected at seven sites in the 10th Region of Chile (Table 1). Spore suspensions and clonal gametophyte cultures were obtained as described by Westermeier et al. (2006). From each locality, ten female and ten male gametophytes were isolated and propagated by periodic fragmentation. Their somatic and reproductive performance (good vegetative growth and rich formation of eggs, spermatozoids, and zygotes under our laboratory maintenance and fertilization protocols) was used to select one favorable gametophyte pair from each locality. For the cross-breeding study to be described here, we define each of these parent gametophyte pairs as a cultivar of *M. pyrifera* and a representative of the gene-pool of its mother population. For convenience, we use the following coding for our seven gametophyte parent pairs: locality number supplemented with "f" standing for female and "m" for the respective male gametophyte (Table 1).

Table 1 Collection dates and habitats of the seven cultivars used for our hybridization study

Locality/ cultivar	Habitat	Gametophyte clones		Collection date
		Female	Male	
1	Interior Sea	1f	1m	Apr 2003
2	Interior Sea	2f	2m	Oct 2003
3	Interior Sea	3f	3m	Nov 2002
4	Interior Sea	4f	4m	Oct 2003
5	Open Pacific	5f	5m	Jan 1997
6	Open Pacific	6f	6m	Aug 1999
7	Interior Sea	7f	7m	Feb 2003

The geographic range extended from 40° 37′S (locality 6) to 43° 07′S (locality 7). Since some of our genotype stocks are of commercial significance, we refer to location numbers instead of coordinates. For each location/cultivar the female clone is marked with "f" and the male with "m"

Each one of these 14 gametophyte clones was mated with all gametophytes of opposite sex. The resulting 49 crosses were carried out under standardized conditions as described by Westermeier et al. (2006). Gametogenesis was induced by mixing male and female gametophyte fragments, followed by exposure to increased irradiation with white fluorescent light at +10°C in sealable household polyethylene bags. Sporophytes produced by intra-cultivar crossings were defined as controls. After 3 weeks, juvenile sporophytes were introduced into 1-L gas-washing bottles with aeration and magnetic stirring, as described by Westermeier et al. (2006). Sporophyte cultures were successively expanded to 2-, 5-, and 10-L bottles, and then transferred to Plexiglass cylinders of 20 and 50 L volume. Culture media were exchanged in weekly intervals. As a final indoor step, sporophyte batches at a length of ca 30 mm were transferred to 800-L greenhouse tanks with running natural sea-water.

During our experiments, 30 sporophytes were selected randomly at weekly intervals to measure average thallus length. The data obtained after 10 weeks were subjected to statistical analysis. Data sets from different crossing products were compared by the Mann–Whitney rank sum test, using SigmaPlot for Windows (version 9.0, Systat Software Inc.). After 10 weeks, tank culture of sporeling batches from selected crosses was continued for various intervals until transplantation to the open sea.

Results

Figure 1 shows thallus length increase for some representative sporophyte batches of our crossing program. Considerable differences between individual crosses became apparent. While the fastest growers reached 9 cm length within 10 weeks (7f×6m), others needed up to 20 weeks to grow to a comparable size (6f×5m), or did not exceed 4 to 6 cm at all under our indoor culture conditions (5f×6m). Furthermore, in advanced stages of some crosses, apical erosion occurred and caused a reduction in blade length (6f×7m). Therefore, in order to obtain a standard character

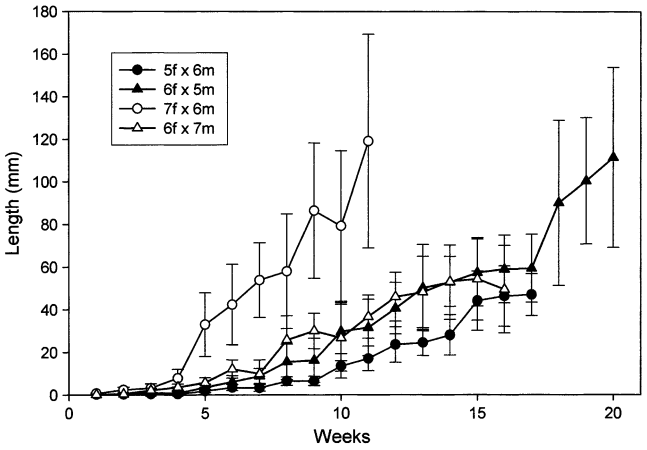

Fig. 1 Weekly thallus length measurements (n=30) for four representative hybridization products, illustrating growth from initiation of sexualization (time axis zero) up to 10 weeks, and diverging development thereafter. *Vertical bars* indicate standard deviations

applicable to all crosses, we measured the thallus length after 10 weeks of growth. Table 2 summarizes these data for all our 49 crossing combinations.

The following conclusions can be drawn from these results:

1. Considerable differences exist between cultivars. Within the same cultivation program, intra-cultivar matings showed differences in thallus length by a factor of four between the most potent (1f×1m) and the weakest (7f×7m) parent pair. As shown in Table 3, most differences between thallus lengths were statistically significant ($p \leq 0.01$).
2. Sporophyte batches of many inter-cultivar crosses grow better than their parent cultivars (Table 2; Fig. 2).
3. Surprisingly, our best-growing hybrids were the offspring of the rather poor parent cultivars 6 and 7. It is also noteworthy that the male, but not the female of our best local cultivar 1, generated superior offspring with additional partners (2f×1m and 4f×1m).

Table 3 Statistical comparison (by the Mann–Whitney rank sum test) for the growth performance (10-week thallus length) of the intra-cultivar crossings (controls): ++p<0.001; +$p \leq$0.01; −p>0.05

Cultivars	2	3	4	5	6	7
1	++	++	++	++	++	++
2		+	++	++	++	++
3			+	++	++	++
4				+	+	++
5					−	+
6						+

4. In late indoor stages of our crossing program, i.e., between weeks 10 and 20 of growth, additional differences between sporophyte batches appeared. Poor development or complete lack of stipe and haptera (Fig. 3), apical frond erosion, termination of growth, or complete breakdown occurred in some crosses, making them unsuitable candidates for mariculture. Such cross-specific differences may escalate under field conditions.
5. Due to differences in fecundity, total numbers of sporelings reaching the tank stage in our crossing experiments varied between 10^3 and 5×10^4. The highest proliferation potential was seen in our inter-cultivar cross 6f×5m with 5×10^4 sporelings exhibiting good growth and reaching 29.6 mm at 10 weeks (Table 2). This indicates that the negative effects mentioned above are not caused by excessive sporeling density, but are more likely attributable to genetic deficiencies in specific crosses.
6. In order to examine the significance of our laboratory growth experiments, 30 seedlings from selected crosses were explanted side by side on ropes within a commercial mariculture unit in the sea. As illustrated by the following examples, these preliminary results confirm that the genotype-specific growth potentials revealed by our laboratory protocol continue in the sea

Table 2 Thallus lengths, reached after 10 weeks of growth

	1m	2m	3m	4m	5m	6m	7m
1 f	*24.8±5.3*	19.4±5.2	7.8±4.2	8.1±4.4	16.6±6.4	**41.7**±20.4	4.7±4.2
2 f	**53.3**±5.2	*14.8±3.4*	6.3±1.4	12.2±3.1	15.6±7.7	11.8±3.6	**27.8**±5.9
3 f	5.0±1.7	6.9±1.5	*12.6±2.0*	6.5±2.1	21.4±4.8	**50.7**±24.8	6.5±1.2
4 f	**40.6**±9.9	5.2±2.9	12.1±2.2	*10.1±3.1*	17.0±4.3	**26.4**±5.4	10.9±2.6
5 f	3.9±1.3	7.0±2.5	10.6±4.8	2.7±1.4	*8.2±2.8*	13.5±5.6	12.7±4.8
6 f	19.5±9.9	13.0±2.7	**40.6**±19.0	14.8±5.1	**29.6**±13.6	*7.9±2.6*	**26.7**±15.7
7 f	22.1±11.4	**29.1**±5.9	6.1±2.0	18.7±7.2	**25.9**±6.3	**79.2**±35.4	*6.1±2.1*

The figures in each box give the average thallus length in millimeter obtained from 30 randomly selected sporophytes and the corresponding standard deviation. The diagonal (italicized, upper left to lower right) contains all intra-cultivar matings (controls) arranged according to their growth performance. All other boxes contain outcrossing products and their reciprocal versions. Values rendered in bold show average thallus length >25 mm

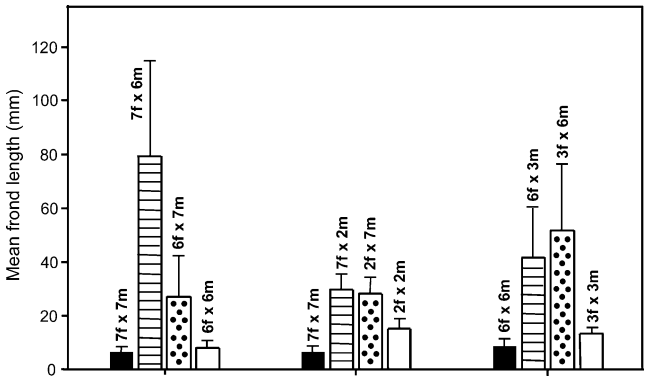

Fig. 2 Three cases of heterosis in outbreeding experiments with different cultivars of *M. pyrifera*. *Left and right columns* in each group represent thallus lengths of 10-week-old sporophytes resulting from intra-cultivar matings (controls). The *two center columns* in each group show thallus lengths of reciprocal inter-cultivar hybrids of the same age. Note heterosis with equal (*center group*), moderately (*right*), or strongly asymmetric reciprocity (*left group*). In each group, values of the inter-cultivar hybrids (*center columns*) were significantly different ($p<0.001$) from those of both intra-cultivar parental matings (*outer columns*). *Vertical bars* indicate standard deviations

under mariculture conditions: (a) our weak intra-cultivar cross 5f×5m (Table 2) reached a frond length of 1.01±1.3 m and a biomass of 1.19±0.2 kg per frond after 4 months in the sea. (b) In the same time span, our inter-cultivar cross 6f×5m grew to 3.12±0.8 m and 3.05±0.4 kg biomass. Similarly, the values for the inter-cultivar cross 6f×3m after 4 months of growth in the sea were 5.19±1.5 m frond length and 4.63±0.9 kg biomass per individual. (c) After 6 months in the sea our best hybrid produced 12 kg fresh weight per individual, in contrast to 200–300 g per individual reached by poor genotypes (Westermeier, unpublished data).

Discussion

Our experimental data reveal substantial differences between *Macrocystis* genotypes, and confirm the occurrence of heterosis in a marine crop system. The dominating phase of kelps is a diploid sporophyte like in higher plants. In contrast to higher plants, however, the obligatory sexual life cycle of kelps involves an independent free-living haploid gametophytic phase. Dispersal is effected by motile zoospores, which according to Graham (2003) are estimated to settle within a 10-m distance from the mother thallus. Consequently, natural kelp populations show a high degree of self-fertilization, which results in inbreeding-depression, and is considered to be responsible for periodic kelp bed fluctuations. Raimondi et al. (2004) showed for Californian *M. pyrifera* that outbreeding caused significant positive effects (heterosis) in offspring produced by geographically distant parents.

Since these experiments, as well as traditional seeding of mariculture ropes with natural spore suspensions (Gutierrez et al. 2006), make use of natural spore batches that contain many millions of genetically different meiospores, they have only limited value to reveal heterosis. Instead, it will be compulsory to use genetically defined clonal stock gametophytes to make use of bastard luxuriance in kelp breeding.

Heterosis, the superiority of hybrids over their genetically homogeneous parents is a major principle of plant and animal breeding. Due to the dominant diploid state combined with obligatory sexual reproduction and dioecism, the occurrence of heterosis in kelps is not surprising, and a first case has been reported for *Undaria* by Hara and Akiyama (1985). Heterosis breeding strategies are presently applied in *Laminaria* mariculture in China, using intraspecific matings between *Laminaria japonica* cultivars (Li et al. 2008) and reciprocal crosses between cultivars of *L. japonica* and *L. longissima* (Li et al. 2007; Zhang et al. 2007).

Our results with sexual crosses of clonal gametophytes of *M. pyrifera* from different localities along a 250-km stretch of South Chilean coastline correspond well with the observations in a natural kelp bed (Raimondi et al. 2004):

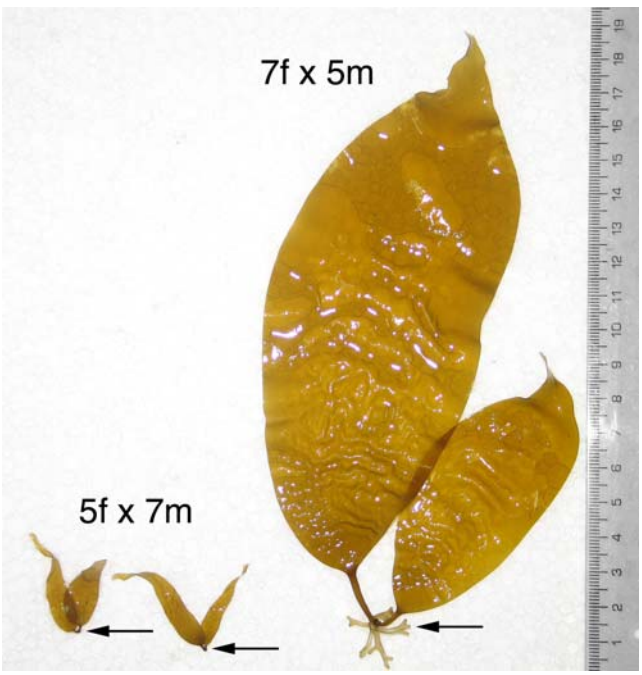

Fig. 3 Advanced *M. pyrifera* seedling sporophytes from a reciprocal inter-cultivar hybridization experiment after 17 weeks of indoor and tank cultivation. Note the remarkable degree of asymmetry of the crosses. The female parent (*7f*) of the luxuriant sporophyte represents the weakest local genotype in our study. Also visible: rich development or complete lack of stipe and haptera (*arrows*)

intra-cultivar matings of our seven local parents produce sporophytes with statistically different growth potential at relatively low "inbreed-degeneration" levels (Tables 2 and 3). The superiority of some of our inter-cultivar hybrids (Table 2, Figs. 2 and 3) compares well with crossing products of commercial *L. japonica* clones described by Li et al. (2008). Furthermore, our observations include cases of hybrid depression and reciprocal asymmetry (Table 2; Fig. 3).

The work reported here deals with growth potential of intra- and inter-cultivar crossings mainly under indoor conditions. We are aware of the fact that many important features for mariculture requirements (e.g., growth performance at different seasons and different geographic locations, stipe and haptera formation, blade thickness, frond-splitting and erosion, susceptibility to epiphytism, etc.) become crucial after the indoor stage in tank culture, and even more after explantation to the sea. Such late effects and differences between various crosses must be evaluated in follow-up full-scale mariculture pilot projects presently under way.

Unlike terrestric crops, which have been cultivated for 10,000 years, marine algae are still largely used without the breeding techniques commonly used in higher plants. It now appears possible that this condition can be changed due to advanced laboratory techniques combined with traditional plant breeding methods. One important prerequisite for heterosis breeding is the establishment of genetically homogeneous parent lines. In higher plants, the creation of pure-breed lines requires elaborate and time-consuming inbreeding programs or complicated alternative techniques. In contrast, the life history of kelps facilitates the creation of pure-breed parent lines almost instantly: male and female kelp gametophytes are free-living, and can be propagated as haploid clones in unlimited manner. Sporophytes represent the desired biomass. Seedlings can be produced in genetically defined fashion at any time and any desired numbers by mixing and sexualizing selected male and female gametophytes.

Additional advantages of gametophyte stock clones are stability and fertility over decades, as confirmed for *Lessonia trabeculata* (Westermeier et al. 2006) and *M. pyrifera* (Table 1, this study). Stability of kelp gametophyte clones is further improved by establishment of axenic cultures on agar plates. Such culture units are conveniently maintained as stock material with minimum labor input (Müller et al. 2008). Furthermore, cryo-preservation (Quansheng et al. 2008) will offer an additional element for long-term stability of stock material.

In summary, the present status of laboratory management for *M. pyrifera* seedling production in Chile is sufficiently advanced to be converted into large-scale production by cooperation schemes with established mariculture facilities.

Acknowledgments This work has been supported by Fondef Grant DO4I 1288 (CONICYT – CHILE).

We thank L. Muñoz, A. Ruiz, P. Murua and C.Atero for their support in laboratory activities. For valuable comments on earlier versions of this manuscript we thank Prof. R. Knippers, Konstanz University. The constructive advice of two anonymous reviewers is gratefully acknowledged.

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