Assessment of genetic and phenotypic diversity of the giant kelp, *Macrocystis pyrifera* , to support breeding programs

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1 Assessment of genetic and phenotypic diversity of the giant kelp, *Macrocystis pyrifera*, to
2 support breeding programs.

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20

21 Abstract

22 The accelerated development of seaweed aquaculture is stimulating research on the genetic
23 drivers of phenotypic diversity of the target species, in order to optimize breeding
24 strategies, to help determine the choice of source populations, and for the selection of traits
25 and varieties that fit with the environmental variability of the production site. This study
26 investigates the spatial variation of the genetic and phenotypic diversities in natural
27 populations of the giant kelp *Macrocystis pyrifera*, and evaluates the potential for
28 modifying agronomic traits through controlled breeding. Nine microsatellites and 12
29 morphological traits were used to describe the distribution of diversity present along the
30 Southeastern Pacific (SEP) Coast. We expected concordant patterns of spatial
31 discontinuities if the genetic background was driving morphological divergence across
32 habitats. Crossing experiments were made to assess the heritability of specific traits and
33 evaluate the performance of the F1 generation in the laboratory and in open sea cultivation
34 respectively. Our results revealed four genetic clusters along the latitudinal distribution of
35 *M. pyrifera* populations, tightly correlated with the existence of major environmental
36 discontinuities. These clusters also matched clusters of morphological diversity, suggesting
37 that both morphological and genetic diversities responded to the same environmental
38 drivers. In crossing experiments, no significant differences were detected between selfed
39 and outbred F1, in morphology, growth and chemical components, but a high variability
40 among all different crosses was observed, revealing a high degree of heritable phenotypic
41 variance. Although, the results suggest that the morphological variation of *Macrocystis*
42 along the SEP coast is strongly driven by the genetic background. Our controlled crosses

were also indicative of a high potential for using this genetic variability in breeding programs for sustainable aquaculture development.

Key words: breeding, genetic diversity, phenotypic diversity, microsatellites

1. Introduction

The use of seaweeds for food/feed, pharmaceuticals, textiles, cosmetics, and biofuels [1,2 3 4,5] and the continuously growing demand for raw material, is rapidly changing the way that we humans interact with this natural resource [6]. Encouraged by this increasing demand and the need to reduce the over-exploitation of natural resources, seaweed farming is expanding across several continents from East Asia to Europe, South America and East Africa [7]. Within aquaculture, the global production of seaweeds is 27.3 million tons (27%), and it has increased by 8% per year over the past decade [8].

Under this scenario, seaweed farming requires the urgent development of breeding programs to increase yield and optimize other relevant agronomic traits [9, 10]. There is a large amount of information on the development of macroalgal strains in red and brown algae [11,12,13]. However, the genetic science behind seaweed breeding and domestication is still in an initial phase, with little conceptual and empirical progress [14]. Several challenges related to the biological peculiarities of algae and their environment are yet to be faced. For instance, the marine environment is more complicated to manipulate than terrestrial environments, where water and nutrient supply, ploughing and other manipulations modify the physico-chemical properties of the soil, and avoid competitors,

predators and most pests and pathogens. Such manipulations are generally not possible in the sea, without significant logistical and infrastructure costs, which are sometimes accompanied by unwanted side effects [15]. Therefore, the increase in productivity must strongly rely on the modification of heritable traits. Yet, the genetic improvement of any agronomic trait must ensure the retention of adequate genetic variance in the targeted traits in order to ensure sufficient scope for adaptation to local environmental variation. Another major difference with land plants, where current breeding efforts are concentrated in already domesticated strains, is the use of wild variants for most seaweed aquaculture initiatives [16, 17]. Some of the cultivated algal species have never gone through a selective breeding process, based on genetic knowledge. Currently, no more than eight species are in the early stages of domestication [18]. One possible reason for this maybe the ease with which selected strains from wild populations can be cloned in order to establish a new seaweed farm. On the other hand, the complex life histories of algae, add additional conceptual and practical constraints to the implementation of breeding programs [18]. Indeed, trait correlations among life cycle stages may have negative consequences on overall production and/or breeding efforts [19]. For example, selecting for growth rate in the farmed red alga *Gracilaria chilensis* caused the dominance of heterozygous diploids that lost their capacity for sexual reproduction [20,21,22] and contributed to the critical loss of genetic diversity observed in this species [20]. Strong genetic diversity losses in cultivated populations can have serious consequences for the adaptability of these species and their susceptibility to pests and diseases [6,23,24,25,26,27].

One of the main challenges that seaweed-breeding science is currently facing is the lack of general knowledge on the drivers of phenotypic diversity. While a large body of

literature has analysed phenotype responses to environmental variation, relatively little is known about genetic determinism of algal traits and their interactions with environmental determinants. Quantitative genetics approaches on algal models, such as QTL analysis [28,29], have recently emerged, and should provide valuable tools to assist breeding strategies in the near future . However, because most cultivated seaweeds are not yet domesticated, the production of new varieties must rely on an initial genetic pool collected from natural populations. Therefore, a critical initial stage in the establishment of a breeding program is the acquisition of solid knowledge concerning the natural variation in both the phenotypic and genotypic diversities [6]. Several fundamental questions can be tackled from such knowledge: 1) Can selection (either natural or artificial) modify traits of interest such as growth rate or shape (among many other traits)? By investigating signatures of evolutionary divergence between environments within the species range, it is possible to infer the evolvability of the species of interest under natural conditions, which is related to the capacity of different traits to accumulate additive genetic variation. The existence of such genetic diversity is essential to the success of trait improvement by selective breeding. 2) Can new varieties be cultivated anywhere or should landraces be established? Because aquaculture systems are deeply influenced by the natural environment, which cannot be easily modified or controlled, it is likely that selected strains or wild progenitors that evolved local adaptations will not be able to grow optimally in non-native environments. In this context, breeding strategies based on selection of local variants should maintain the genetic diversity necessary for optimal growth in the farm environment to secure the sustainability of the production. 3) Should breeding strategy be oriented towards hybrid vigor or “pure” (i.e. inbred) lines? The presence of inbreeding in natural populations may promote inbreeding depression. In this case, hybrid vigor is expected when crossing

different inbred lines. However, if local adaptation has taken place in natural populations, hybrids might break down optimal allelic combinations for specific environments. Also, depending on the level of local genetic diversity, and how representative of this diversity the collection of the initial progenitors was, a breeding program could suffer from high rates of inbreeding and loss of allelic variation if the relationships between the breeding candidates were not considered when making selection decisions. Therefore, efforts to develop diversified germplasms for experimental evaluation of inbreeding effects and local adaptation may complement studies of natural populations, as well as promoting backup conservation strategies [30].

This study aims to investigate the spatial structure of the genetic and phenotypic diversities of the giant kelp, *Macrocystis pyrifera* (L.) C. Agardh. This species is under a strong and increasing exploitation pressure, mainly for alginate production and as a source of feed for abalone [31]. Regulatory restrictions on kelp exploitation in many countries and the increasing demand for kelp biomass challenges the sustainable exploitation of natural populations, from which the large majority of the biomass is obtained. Biomass production through cultivation is an alternative that is being explored in several countries across its wide distribution range. In Chile, new legislation allows incentives for cultivation and repopulation of seaweeds, providing a positive environment for the installation of a kelp farming industry in the country. Pilot-production has demonstrated that 124 wet ton.ha⁻¹ of *M. pyrifera* can be achieved using wild individuals to seed ropes for suspended systems [32]. The development of *M. pyrifera* aquafarming is expected to emerge rapidly for several reasons: established procedures for cultivation in hatcheries [33] and open ocean [32] allow for the testing of the agronomic performance of a large array of genotypes and

pilot scale production; technology used to convert biomass to bioethanol implemented at the pilot scale [34,35]; and identification of novel components for food and pharmaceutical uses that add value to the biomass production [5,36,37].

M. pyrifera is considered to be a highly plastic species [38,39], yet some morphological traits were considered to express a strong phylogenetic signal. Indeed, the spatial distribution of different morphotypes based on blade and holdfast shape along the coast was highly correlated with the presence of divergent clades of an ITS2-based phylogeny [40]. Using mitochondrial DNA, Macaya and Zuccarello [41] reported low genetic diversity across the South Eastern Pacific (SEP) but a concordance with the two major biogeographic discontinuities at 33°S, and 42°S, suggesting that environmental heterogeneity may be contributing to the distribution of the genetic diversity. Besides the relevance of this information, limited resolution of the molecular markers and the morphological survey restricts our understanding of the spatial patterns of phenotypic variation. The reduced genetic diversity and divergence among habitats or distant regions, and the high phenotypic plasticity were considered as strong arguments for a recent evolutionary history in the southern hemisphere where little or no adaptive divergence has occurred. Consequently, if natural selection had little or no impact on the species phenotypic diversity, it was considered that breeding and strain selection would be insufficient to modify traits and improve productivity under farming conditions. In this study, we challenged this view by developing a comparative study of genetic and morphological divergence across parts of the South American distribution range, with special emphasis on the region of Chiloé where environmental discontinuities are well known.

We quantified the genetic diversity and its spatial distribution in *M. pyrifera* across the SEP, and its association with morphological diversity. Secondly, we investigated the potential for modifying traits through controlled breeding by testing morphological, growth and chemical differentiation among crosses of *M. pyrifera* with different genetic backgrounds but cultivated in a common garden. Results are discussed in the context of seaweed domestication and sustainable production.

2. Materials and Methods

2.1 Morphological analyses

Adult sporophytes were collected at 16 locations along the Chilean coast (Table A1). At each site, between 20 and 30 mature individuals were collected along a transect of approximately 600 m by scuba diving, and transported immediately to the laboratory in boxes cooled with ice packs. Morphological analysis involved measuring the following characters of each thallus: number of stipes, total thallus length, total wet weight, number of blades, holdfast diameter and height. In addition, the following characters were measured for ten randomly selected blades per thallus: maximum blade length and width, blade angle with the stipe, maximum aerocyst length and width and substantiality (a measure of weight per projected blade area, expressed in g cm^{-2}). To assess the variation of the set of morphometric characters and the correlations between them, a Principal Component Analysis (PCA) was performed. The level of structuring of the total sample based on morphological traits was assessed by a K-means clustering analysis that performs an iterative alternating fitting process of assigned individuals to a number of specified

clusters ($K = 2, 3$ and 4) in order to maximize the morphological differentiation among groups. Finally, to evaluate the correspondence between morphology and genetic data, a discriminant analysis was performed assigning individuals to groups *a priori* defined from genetic clustering analyses, and the percentage of correct assignments was estimated as an indicator of the correspondence between morphological and genetic clustering (see below). All multivariate analyses were performed with JMP 10 (North Carolina, USA).

2.2 Genetic analyses

A 3x2 cm piece of blade tissue was excised from each individual for 13 of the 16 collected populations (Dalcague, Chaulinec and Meulin were not included, Table A1), washed with fresh water and immediately placed into a plastic bag with silica gel crystals for rapid dehydration. Total genomic DNA was isolated from finely ground tissue following [42]. Nine microsatellite loci were selected from [43]: Mp-BC-4N; Mp-BC-13; Mp-BC-25; Mpy-7; Mpy-9; Mpy-11; Mpy-14; Mpy-17 and Mpy-19. PCR reactions were carried out according to [43] with minor modifications in annealing temperatures. PCR products were analyzed on an ABI3130xl Genetic Analyzer (Applied Biosystems, Foster City, California, USA) using 500 LIZ internal standard. Raw allele sizes were scored with GENEMARKER v1.95 and assigned to specific alleles using FLEXIBIN [44].

Descriptive statistics for population genetic diversity, including number of alleles (N_{all}), observed (H_o) and expected (H_e) heterozygosities, estimators of inbreeding (F_{IS}) and pairwise population differentiation (F_{ST}) were calculated using GENETIX v 4.05.2 [45].

Isolation by distance was evaluated by a Mantel test with 5000 permutations performed in GENETIX. The identification of genetic clusters was made using the Bayesian clustering

approach implemented in STRUCTURE [46]. The analysis considered possible admixture and correlated allele frequencies among populations as optional settings. The MCMC chain discarded the first 50000 iterations as burn-in, and kept only the subsequent 100000 iterations. The analysis was performed 10 times for each of the k-clusters (k = 1 to 12), and all these runs were integrated using STRUCTURE HARVESTER (available at <http://taylor0.biology.ucla.edu/structureHarvester/>). The uppermost likely number of cluster was defined following Evanno's criteria Δk [47].

2.3 Germplasm collection and crossing experiment

Fertile sporophytes were collected from the sampled sporophytes at the 16 locations (Table A1). The germplasm collection was prepared following [30]. From the collection, three male and three female gametophytes from Puchilco (PUH) and Pargua (PAR) (PAR1♀, PAR20♀ and PUH6♀; PAR1♂, PAR20♂ and PUH6♂), were selected for their different genetic background (see Section 3.2.). These gametophytes were transferred from germplasm to new culture conditions to promote vegetative growth, following [48,49]. Once sufficient gametophyte biomass was obtained, sexual fertility was induced following [49]. Both inbred and outbred crosses were performed. After 4-6 weeks, juvenile sporophytes were observed. F₁ sporophyte individuals (n= 30) of each cross were weighed, and morphological characters were measured. With the initial and final length and weight, the specific growth rate (SGR) was calculated as $SGR = [(\ln x_2 - \ln x_1)/(t_2 - t_1) * 100]$, where x_1 and x_2 are the measured trait at the beginning (t_1) and end (t_2) of the period.

After 15-20 weeks of cultivation under controlled conditions, between 100-300 individuals of each cross were transplanted to a 21-hectare outdoor floating cultivation system in southern Chile (Quenac; see [32]). The sporophytes were attached to ropes, three individuals per meter, at 4 m depth and monitored for 4-7 months. Each month, three randomly selected individuals of each cross were collected to determine SGR. Also, the number of blades per sporophyte were counted. A fragment of blade tissue of each of these samples was cut, washed and dried in silica gel crystals for genetic analysis. When sporophytes reached maturity, indicated by the presence of sori, the individuals were removed and the reproductive tissue brought to laboratory to collect isolated female and male gametophytes for the germplasm collection. The rest of each of three individuals per cross was completely dried at 60°C for 24-48 h, milled and mixed to ensure homogenization for chemical analysis.

2.4 Chemical characterization

Carbohydrates (alginate, mannitol and glucans) were determined by first completing a 2-step enzymatic depolymerization. The first 24 h process used cellulases and alginate-lyase to extract mannitol, convert all glucans to glucose and to solubilize all alginate. Glucans and mannitol were determined via HPLC/IR. The second 24 h process used an oligoalginate-lyase to break all oligo-alginates into monomers. Ammonia was added in solution which spontaneously converts 4-deoxy-L-erythro-5-hexoseulose urinate (DEHU), an alginate monomer to 5-hydroxypyridine-2-carboxylic acid (5-HPA) which could be detected and quantified on HPLC/UV (for detailed protocols see [35]).

Fucoxanthin was quantified by HPLC, following [50]. Phloroglucinol was quantified using Folin-Ciocalteu method [51] and Total Phenolic Content (TPC) was determined by the Folin-Ciocalteu method using Gallic acid as standard [52,53,54]. Finally, amino acids were quantified via acid digestion and derivatization followed by HPLC/UV detection [55,56].

2.5 Statistical analysis of strain selection

The growth rates and chemical concentrations of the nine strains were compared with a 1-way ANOVA after assurance of normality and homoscedasticity. If significant differences were detected, *a posteriori* Tukey test was performed to identify the source of variation. Growth rates based on the number of blades per individual at the hatchery stage were compared with a Kruskal-Wallis Test. Statistical analysis was performed in JMP 10.0.0.

3. Results

3.1 Microsatellite population structure

The nine microsatellite loci revealed 287 alleles in 373 genotyped individuals (5 – 72 per locus; Table 1). The average number of alleles per population varied between 5.2 and 13.4 in Palqui and Pargua, respectively (Table 1). Heterozygosity for all populations, except Algarrobo, exceeds 0.5 with maximum values of 0.71 in Pargua. There was significant heterozygote deficiency ($F_{IS} < 0.01$) in all populations but Los Choros and Puchilco, with significant values ranging from 0.048 in Pucatrihue to 0.250 in Antofagasta (Table 1).

3.2 Patterns of genetic structure

The STRUCTURE analysis indicated the existence of four distinct genetic clusters (Fig. 1): Cluster 1 dominated by the Antofagasta population in northern Chile; cluster 2 comprised Punta Choros and Algarrobo, both in northern-central Chile; cluster 3 included southern Pacific and canal Chacao, from Chome to Pargua (FCO and PAR) populations, and cluster 4 included populations of the interior sea of Chiloé. Most the individuals had a high probability of belonging to their clusters, however a few (e.g. one to three individuals per cluster) showed an admixture with other clusters (Fig. 1). When assignment of individuals was restricted to only two clusters, the main genetic discontinuity separated northern (i.e. Algarrobo, Punta Choros and Antofagasta) from all southern populations. With three clusters, a new discontinuity appeared separating Chiloé populations of the interior sea from those of the wave exposed coast. Only Pargua, in the Chacao channel (separating Chiloé island from the continent) considered a protected coast site, was assigned to the open coast genetic cluster. Finally, the Antofagasta population appeared as unresolved, with mixed assignments of most its individuals. When defining a fourth cluster, Antofagasta appeared as a highly-differentiated population from all the other populations (Fig. 1). In summary, the analysis illustrated a clear pattern of spatial genetic differentiation within *M. pyrifera* populations along the SEP coast, with strong genetic discontinuities. No signature of isolation by distance was detected (Mantel test: $R^2 = 0,024$; $p = 0,124$).

3.3 Morphological variability

Three groups were identified in the PCA (Fig. 2). One was composed of individuals belonging to the northern populations only (Antofagasta, Los Choros and Algarrobo). The

second included individuals collected on the exposed coast, south of the first group, and the Chacao channel (Chome, Mehuín, Pucatrihue, Faro Corona, Pargua) and one population from interior sea of Chiloé (Metri). The third group included the remaining individuals from the interior sea of Chiloé (Puchilco, Quenac, Palqui, Dalcahue, Chaulec, Mehuín and Queilen).

The differences between the groups were explained by length, weight, disc diameter and number of blades, all of which had higher values in the interior sea of Chiloé. Sporophytes from the exposed coast had a distribution differentiated mainly by the number of stipes and blade width, with southern individuals having more stipes and thinner blades, closer in character to the *pyrifera* morphotype than northern individuals which were closer to the *integrifolia* morphotype.

K-means clustering revealed strong differentiation between individuals from the interior sea of Chiloé, and the rest of the southern and northern populations when $K = 2$ (Fig. 3). For $K = 3$, two northern populations (Punta Choros and Algarrobo) were differentiated from the rest of the populations while Quenac (from interior Chiloé) formed a single population-cluster (Fig. 3). Finally, for $K = 4$, the clustering pattern was similar to the genetic clustering (Fig. 1), except that Quenac was still isolated in a different cluster and Antofagasta was not differentiated from the other populations on the exposed shores south of 33°S. Sporophytes found at Quenac had a particular morphology, with a pronounced conical holdfast, short but significantly wider laminae and longer aerocysts, which was distinct from other individuals of the interior sea.

Discriminant analysis of the morphological data using genetic clustering as *a priori* grouping revealed a high congruence of the spatial distribution of the morphological and genetic variability (Figure 4). The main difference was the population from Metri that was

assigned to the same group as the northernmost population from Antofagasta. The results were consistent and revealed only 5.0%, 12.6% and 17.4% of misclassified individuals in K= 2, 3 and 4, respectively (Table 2). Independent of the level of structure, the correct assignment always exceeded 80%, revealing a strong correspondence between genetic and morphological data.

In a second discriminant analysis (Table 3), individuals were assigned based on environmental groups defined as the three biogeographical units recognized on the SEP coast [57]: Peruvian province (18.4° - 29°S), intermediate area (30° - 41°S) and Magellan province (42° - 56°S). The percentage of correct assignment decreased mainly for the northern genetic cluster (63%), with 31.5% and 5.6% incorrectly assigned to Pacific/Canal Chacao cluster and Chiloé cluster, respectively.

3.4 Crossing experiments

One female and one male gametophyte from each of three sporophytes with different genetic backgrounds, were selected from the established germplasm, based on contrasting morphological characteristics of the wild parental sporophytes, all were of the *pyrifer* morphotype (Table 4). PAR1 and PAR20 belong to the Southern Pacific-Chacao channel genetic cluster, and PUH6 to the Chiloé genetic cluster, all located in the interior sea (e.g. same habitat but different genetic clusters).

Strong and significant differences between crosses were observed in growth rate using weight (g), length (cm) and number of blades per plant. Under hatchery conditions (Fig. 5), Bal 1 and Bal13 had the worst performance, whereas in the open sea culture (Fig. 6) they resumed their growth, and Bal 5 had the lowest growth rates under natural conditions. No

significant differences were observed between the inbred and outbred crosses, neither in hatchery nor in open sea culture. Furthermore, holdfast morphology developed differently between crosses, with some extreme variability, i.e. from a well-developed structure (e.g. Bal 1, Fig. 7) to no holdfast (e.g. Bal 3, Fig. 7). In open culture, morphological differences were also observed between crosses (Fig. 8), but mainly in terms of total length and weight.

3.5 Chemical characterization

Chemical analyses were performed for all crosses, except Bal 3 that did not survive the culture conditions in open water. Carbohydrates, bioactive molecules and aminoacids exhibited strong variability and significant differences (Table A2) between crosses. These differences were on several occasions striking: alginate yield was over 3 times higher for Bal 14 than Bal 1, and mannitol was 7.7 times higher for Bal 14 than for Bal 1 (Figure 9A). This same situation was observed for two of the 3 bioactive compounds measured (phenols and phloroglucinol), Bal 14 had values more than 4 times higher for both compounds than Bal 9 (Figure 9B). In the case of aminoacids, 7 out of 16 showed significant differences between the crosses (Table A2). Six of the total number of aminoacids showed differences that did not vary significantly, but the other 11 aminoacids showed significant variation and Arginine and Leucine showed variations up to 2.5 times.

4. Discussion

Our analysis based on microsatellites markers and morphological data provides clear evidence of spatial structure within the distribution range of *M. pyrifera* along the

SEP coast, with strong discontinuities in the distribution of both the genetic and the phenotypic diversities. Four major clusters were identified, which coincide with the geographic distribution of the populations (North, Central, South Pacific/Chacao channel, and Interior Sea of Chiloé). the lack of isolation by distance further supports the idea that, at the spatial scales considered in this study (from tens to hundreds of kilometers, and up to 2,600 km in total), the genetic diversity is structured into major clusters representing mainly regional groupings separated by discontinuities in the genetic identity of individuals. Some of these discontinuities are co-located with environmental breaks. For instance, a sharp discontinuity along the Chacao channel, in between the Island of Chiloé and the continent (35 km long and 4-6 km wide) separates the interior sea from the open coast, environments that differ in terms of wave exposure, salinity variation, water stratification and nutrient abundance. A second major discontinuity separates populations south of 33°S, characterized by strong but intermittent upwelling regimes, from populations north of 30°S dominated by weaker but more persistent over time upwelling [58]. These discontinuities correspond to previously described biogeographic boundaries (i.e. 30-33°S and 40-42°S) [57, 59] and are strongly associated with the phylogeographic discontinuities of a large number of invertebrates (see [60] and references therein) and seaweeds [61], which on occasions leads to speciation [62]. Habitat heterogeneity plays an important role in kelp divergence by favoring adaptation to particular environmental conditions, as shown for the *Lessonia* species complex [62, 63, 64, 65]. Phylogeographic analyses of *M. pyrifera* across the southern hemisphere have also revealed genetic discontinuities associated with these environmental frontiers [41] suggesting that the distribution of genetic diversity is strongly driven by the distribution of different habitats. The northernmost cluster, represented by a single sampled population (Antofagasta), does not appear to be isolated by

any known environmental discontinuity. Analysis of other seaweed species along the SEP coast have indicated genetic discontinuities that do not coincide with biogeographic boundaries (e.g. *Mazzaella laminarioides* [66]), but with large interruptions in suitable habitat (e.g. long sandy beaches). Even though there is no such interruption of the rocky shore between Choros and Antofagasta, there is a total absence of *M. pyrifera* along a large section of coastline running approximately 600 km, south of Antofagasta [67]. Such an interrupted distribution might be the cause of the significant differentiation of the Antofagasta population, as gene flow seems to occur over relatively short distances. Indeed, dispersal of this species is dominated by spore dispersal at scales of a few meters, leading to high inbreeding within and strong differentiation among populations [68]. It is possible that the Algarrobo-Choros cluster, located within the 30-33°S biogeographic transition between the Peruvian Province and the Intermediate Area [57], is poorly connected to the northern cluster because of both restrictions in dispersal due to the distances between populations and local adaptations caused by habitat divergence.

The strong concordance between morphological and genetic clustering further suggests environmental conditions are driving the evolutionary divergence between regions. Phenotypic plasticity has often been considered as an explanation for the diversity of phenotypic traits found along the coast. The morphological characters of the sporophytes considered here include those that used to be diagnostic for the distinction between *M. integrifolia* and *M. pyrifera*: holdfast shape, blade and aerocysts size. The observations of the *integrifolia*- morphotype along wave exposed rocky shores, and the *pyrifera* morphotype in the Interior Sea of Chiloé were considered plastic responses to the exposure to wave action (or the absence of it) [39]. There is, however, evidence of genetic control of some traits, as demonstrated by the differential growth of juveniles of *Macrocystis* under

variable nutrient concentrations within common garden experiments [69]. Evidence of a phylogenetic signature of the morphological divergence between *pyrifer* and *integrifolia* types suggest that *M. pyrifer* is experiencing an incipient evolutionary divergence between the two morphotypes along southern hemisphere coastlines that can potentially be explained by the different environments that they inhabit [40]. However, gene flow is still occurring between both groups as indicated by laboratory results [70], which are consistent with our results for a few admixed individuals in each cluster. Here, not only did we observe spatial clusters for morphological data, but when combining data sets in a discriminant analysis, the existence of these morphological clusters was well explained by their association with the genetic clusters. In other words, the species seems to be experiencing evolutionary divergence between different habitats. This reinforces the idea that the phenotypic diversity observed in *M. pyrifer* is an evolutionary response to environmental heterogeneity rather than pure phenotypic plasticity. The results of our limited crossing experiments, with as few as 9 male/female combinations, strongly reinforce this hypothesis. First, considerable variation was observed for all the analyzed traits, including shape, size, growth rate and chemical composition. Second, this limited sampling of natural diversity provided, after a single generation, evidence of strongly heritable variation, as each progeny was highly homogeneous in the common garden experiments (both in tanks and out-door), but very different from any other progeny. For instance, the observation of variation in the holdfast morphology, ranging from normal *pyrifer* type, to reduced structures, to total absence of a holdfast, is experimental proof of the strong genetic determinism of holdfast shape. Parental sporophytes all came from sheltered habitats, where selection for holdfast size may be weak and could allow individuals with small sized holdfasts to survive. Therefore, these populations may have

retained some genetic variance for holdfast shape. This might not be expected in wave-exposed populations where the drag forces eliminate individuals weakly attached to the substratum, and therefore tend to eliminate genetic variance for holdfast shape (i.e. purifying selection). These results may explain the phylogenetic signal of holdfast morphology previously described [40]. Such a hypothesis could be further tested by analyzing the variance of holdfast phenotypes in the progeny of sporophytes living in protected *versus* wave-exposed habitats. To conclude, holdfast shape may acquire some characteristics from the influence of the environment during early development [39], but the genetic background of the different progeny is the main driver of variability in these common garden experiments. Similar conclusions can be drawn for the chemical composition, which also strongly suggests that sporophyte physiology is under genotypic control [69], although seasonal variation is also known [32].

Heritable variation of phenotypic diversity is one of the fundamental predictions of Darwin's theory of evolution under natural selection. Altogether, our results strongly suggest that the diversity of phenotypic traits is under the strong influence of natural selection. Besides a recent evolutionary history of the species in the southern hemisphere [41], the amount and distribution of this heritable variation is likely the result of evolutionary divergence between the different habitats. Therefore, the usually recognized phenotypic plasticity of the giant kelp, as an explanation of its broad distribution, should be reconsidered and local adaptation should be experimentally tested among habitats.

The introduction of new varieties for seaweed cultivation is posing a number of biological challenges. For example, over reliance on genetically uniform breeds that, often have unstable performance and get discarded from the production lines after only a few

years [71]. This genetic homogeneity also increases vulnerability to environmental stress and pests, because of intensification [72]. However, these varieties have fixed certain economically important traits, questioning the influence of phenotypic plasticity in Laminariales. Many traits related to economic production and quality are quantitatively inherited, and determined by the combined interaction between genetic and environmental factors. Therefore, understanding the relationship between the genotype and the environment and their role in shaping phenotypes will accelerate our capacity to selectively breed and improve the agronomic performance of cultivated strains. Recent advances in QTL analyses of seaweed traits [73] offer an alternative approach for demonstrating the role of the genetic background, and allow for a move towards the development of tools to assist selective breeding. Additionally, by suggesting that some processes of local adaptation are occurring in giant kelp populations along the SEP coast, our results should be relevant to the development of cultivars that fit into local/regional environments. Indeed, *M. pyrifera* as well as most other kelps that are being incipiently cultivated are still wild species that evolved genetic combinations that optimize the fitness of different genotypes in their local environment. Therefore, initial steps of selective breeding should assess unwanted consequences of breaking these optimal combinations and take into consideration the nature of the genetic resources and natural variation available in wild seaweed stocks, in order to achieve sustainable improvement of the agronomic performances of the cultivars in their native environment [18]. In this context, wild-type genetic diversity needs to be tested under farming conditions and preserved and stored in germplasms [30] for subsequent breeding experiments.

In the current context of an increasing demand for seaweed biomass not only for hydrocolloids industry, but also for a much larger range of high value molecules for

different industries, understanding and preserving the natural genetic diversity of the breed-stock is a pre-requisite for developing efficient breeding strategies that will increase production through farming. Genotype and phenotype diversities within wild populations offer a large panel of interesting traits for the industry. We should also take advantage of the evolutionary history of the species, which has promoted genetic combinations optimized for the different habitats a species can occupy naturally. In this context, the high heritable variance for phenotypic diversity revealed by *M. pyrifera* represents a natural heritage, potentially highly valuable to the success of future breeding programs.

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Author Contributions

C.C., S.F., and A.H.B. planned and designed the research. C.C. carried out the sampling and the laboratory work. C.C. and S.F. performed the analyses. C.C., S.F., and A.H.B. wrote the manuscript.

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