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Stranded alone: The first reported Peruvian population of *Agarophyton chilensis* is a single-male's clone

Vanessa Robitzch, Natalia Arakaki, Stéphane Mauger, José Carlos Zapata Rojas, Marie-Laure Guillemain

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1 **Title:**

2 **Stranded alone: The first reported Peruvian population of *Agarophyton***
3 ***chilensis* is a single-male's clone**

4

5 **Authors:**

6 Vanessa Robitzsch¹, Natalia Arakaki², Stéphane Mauger³, José Carlos Zapata
7 Rojas⁴, and Marie-Laure Guillemin^{1,3*}

8

9 **Affiliations:**

10 ¹*Instituto de Ciencias Ambientales y Evolutivas, Facultad de Ciencias, Universidad*
11 *Austral de Chile, Casilla 567, Valdivia, Chile;* ²*Instituto del Mar del Perú, Banco de*
12 *Germoplasma de Organismos Acuáticos. Esquina Gamarra y General Valle s/n,*
13 *Chucuito, Callao, Perú;* ³*CNRS, Sorbonne Universités, UPMC University Paris VI, UMI*
14 *3614, Evolutionary Biology and Ecology of Algae, Station Biologique de Roscoff, CS*
15 *90074, Place G. Tessier, 296888 Roscoff, France;* ⁴*Asociación Las Brisas, Mirador*
16 *del Pacífico My. 142 – Lote 18, Ilo, Moquegua, Perú.*

17

18 ***Correspondence:** Marie-Laure Guillemin, Instituto de Ciencias Ambientales y
19 Evolutivas, Universidad Austral de Chile, Campus Isla Teja, Casilla 567, Valdivia,
20 Chile; **E-mail:** marielaure.guillemin@gmail.com

21

22 **Abstract:**

23 Gracilariales, a red macroalgal order, is considered one of the top ten most
24 invasive algal taxa. While *Agarophyton vermiculophyllum* is a well know example
25 of an algal invader worldwide, its congeneric species *A. chilensis* has mainly
26 remained within its original distribution range for thousands of years, and was
27 only engaged in a few dispersal journeys to arrive from New Zealand to Chile
28 over 19 000 years ago. Nowadays, *A. chilensis* is intensively farmed along the
29 Chilean coast and the establishment of new populations has mostly been due to
30 intentional/planned cultivation practices. No other source of range expansion
31 has ever been reported, since its first description. However, in 2014 it was
32 sighted for the first time at the docking of a small artisanal port in Peru, which
33 nowadays represents its northernmost population. We genetically assessed 60
34 specimens taken from Peru in 2015 and 2017, using two different types of
35 molecular markers (the ribosomal Internal Transcribed Spacer 2, ITS2 and six
36 nuclear microsatellites). Altogether, our results suggest the population in Perú to
37 be an unattached, vegetative, single male's clonal population. This population has
38 sustained itself successfully, reproducing asexually through fragmentation, for at
39 least four consecutive years and seems to have expanded in the area. The ITS2
40 sequences from these individuals corresponds all to one haplotype, the one
41 ubiquitous among populations from Chile, the eastern coast of New Zealand and
42 the Chatham Islands, not allowing to narrow down the potential area of origin of
43 the Peruvian population. Based on first the allelic frequencies of six
44 microsatellite loci estimated for 28 Chilean and five loci for seven New Zealander
45 populations and second the analyses of Bayesian clustering, we propose the
46 natural or unintentional anthropogenic introduction of a single male's thallus
47 from Chile to be the source of the Peruvian population.

48

49 **Keywords:** Agarophyte; Colonization; Microsatellite; Population genetic; Range
50 expansion; Red alga

51

52 **Introduction**

53 Algal colonizers have previously been reported to successfully expand their
54 original distribution range by spreading their reproductive propagules (spores
55 or zygotes) or thallus fragments, either through: oceanic current systems[1–3],
56 rafting on floating substrata [4–10], attached to ship hulls, in ballast waters, or as
57 a result of other anthropogenic activities such as aquaculture and the aquarium
58 trade[11,12]. It is thought that at least 346 algal taxa have successfully expanded
59 their original biogeographic range and formed new populations worldwide[12].
60 Amongst these, at least 17 non-buoyant seaweeds have colonized new
61 transoceanic territories across vast distances of open ocean[10,13]. Species of
62 the red macroalgal order Gracilariales are recognized among the top ten invasive
63 algal taxa and *Agarophyton vermiculophyllum* (previously known as *Gracilaria*
64 *vermiculophylla*[14]) is a well know example of an algal invader worldwide[11].
65 *Agarophyton chilensis*[15], despite its name, is originally from New Zealand and it
66 is considered most likely that recurrent colonizers naturally expanded the
67 species distribution range to the Chatham Islands and to the southern part of the
68 Pacific coasts of South America dating back at least 19 000 years ago[16,17].

69 In late February 2014, the presence of *A. chilensis* at the Grau Port in Morro
70 Sama, Tacna, Peru, was reported for the first time. First specimens were

71 collected in May of the same year and were morphologically and genetically
72 (based on the ribulose 1,5-bisphosphate carboxylase/oxygenase gene, *rbcl*,
73 plastid) coherently identified[18]. *Agarophyton chilensis* is a red alga commonly
74 found and cultivated along the shores of Chile. Here, it is found from Raul Marin,
75 in the South (43°S), all the way up to the Bahia Mejillones (23°S) in northern
76 Chile[15,19,20]. At its currently northernmost distribution, in Coquimbo (29°S)
77 and Caldera (27°S, an introduced population likely originating from Coquimbo),
78 *A. chilensis* is intensively farmed and harvested for the production of agar[15,19].
79 All northern populations, north of Coquimbo, at 29°S, are populations introduced
80 by fishermen for cultivation purposes and were not part of the historical natural
81 distribution range of the species[15,21]. Some of these northern farms have
82 already disappeared probably due to poor management choices (Wilkomirsky,
83 personal communication). Among these farms, La Herradura Bay, Coquimbo
84 (29°S), remains the oldest reported and established population, dating back to at
85 least the 1950s. In Peru, *A. chilensis* is not farmed, which indicates that the new
86 Peruvian population was not intentionally introduced, and it has been
87 hypothesized to have rather come from a nearby farm in northern Chile.
88 However, since the *rbcl* genetic marker studied in the Morro Sama locality is not
89 variable at the species level (i.e., same sequence across the entire Pacific
90 range[16]) for *A. chilensis*, the origin of the new Peruvian population remained
91 unsolved[18]. Two scenarios could have led to the colonization of the Peruvian
92 coasts: (1) a direct transoceanic colonization from New Zealand to Peru, or (2) a
93 natural or anthropogenic range expansion from Chilean populations to the
94 North.

95 *Agarophyton chilensis*, has a complex reproductive life cycle with an alternation
96 between haploid (male and female gametophytes) and diploid
97 (tetrasporophytes) individuals. The species can reproduce sexually through the
98 production of spores or asexually by fragmentation of its thallus. The latter
99 resulting in individuals unattached to the substrata (lacking attachment discs)
100 and forming populations of thalli drifting over, or embedded in sandy/muddy
101 seabeds, which are commonly found in estuaries and bays[19]. Farmers also take
102 advantage of fragmentation to seed their farms with cuttings of thalli and grow
103 new algae[22]. In this process of induced asexual reproduction in farms, a loss in
104 fertility of the farmed unattached population (which also mainly comprises
105 vegetative tetrasporophytes) has further been recorded[19]. In contrast, sexually
106 reproducing natural populations are composed of individuals growing anchored
107 to pebbles, shells or the rocky substratum by attachment discs[19]. Most
108 individuals in natural populations are fertile, and the populations typically
109 comprise a mixture of males, females, and tetrasporophytes[19]. In the case of
110 Peru, the first collected individuals were reported to be infertile and growing
111 buried in sand[18], and thus, so far, rather resembling a farmed Chilean
112 population. Yet, there is little knowledge on the demographics of the Peruvian
113 population since an assessment of population dynamics and genetics is still
114 needed.

115 This study seeks to comprehensively assess the putative origin as well as the
116 phase and sex ratio of the Peruvian population of *A. chilensis*, combining
117 knowledge on the biology of the species, metadata of the site, and phenotypic
118 and genotypic data. Using population genetics including a large genetic data base

119 of the ribotype of the Internal Transcribed Spacer 2 (ITS2) and microsatellite
120 nuclear DNA markers of *A. chilensis* specimens collected along its entire
121 distribution range (seven locations from New Zealand, including Chatham
122 Islands, and 28 in Chile[16,19]), we verify whether or not the newly found
123 Peruvian population is representative of another case of transoceanic
124 colonization from New Zealand or rather a range expansion from the Chilean
125 coast. Additionally, Peruvian samples were taken in 2015 and 2017 to assess
126 potential temporally recent genetic and demographic changes within the
127 population of Morro Sama.

128

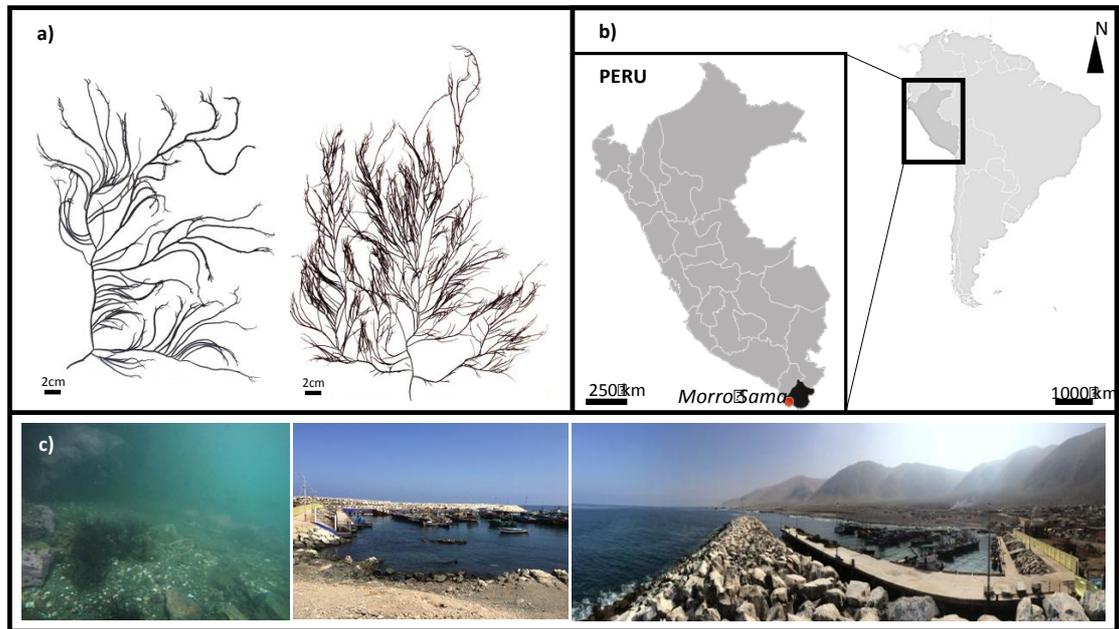
129 **Materials and Methods**

130 *Sampling location, direct thalli examinations, and DNA extraction*

131 The 12th of September of 2015, 30 samples of *Agarophyton chilensis* were
132 collected under the structure of a small dock for artisanal fishers' boats[18], at
133 the Grau Port, in Morro Sama, Tacna, Peru (17° 59' 39.7" S and 70° 52' 59.1" W;
134 Fig. 1). Another 30 specimens were sampled on the 31st of August, 2017. During
135 both samplings, a brief field evaluation of the *A. chilensis* population was carried
136 out and basic environmental parameters were measured using a field
137 thermometer (for temperature in °C), a WTW Multiparameter (Multi 3630, for
138 pH), and an Atago Master Refractometer (for salinity).

139 Both collections were evaluated under a stereoscope in search for reproductive
140 structures as well as attachment discs. For further genetic analyses, a fragment

141 of each of the 60 samples was kept in dry silica gel prior DNA extraction
142 following the protocol in Cohen et al. (2004)[17].



143

144 **Fig. 1: Description of the Peruvian population of *Agarophyton chilensis*,**
145 **Morro Sama, Tacna, Peru.** Panel (a) are photographs of two samples taken in
146 September 2015, by N. Arakaki. Panel (b) indicates the exact sampling location of
147 Puerto Grau, in Morro Sama, Tacna Region, Perú. Panel (c) are three
148 photographs: The *A. chilensis* population *in situ* (underwater); the shoreline and
149 access road to the Port; and the structure of the docking site (from left to right).

150

151 *Genetic Data acquisition*

152 The ploidy level and sex of the vegetative Peruvian individuals were determined
153 using the genetic sex markers published by Guillemin et al. (2012)[23]. Ploidy
154 level given by genetic sex markers was cross-checked with microsatellite

155 genotypes (i.e., haploid gametophytes should not present more than one allele
156 for each microsatellite locus).

157 Microsatellite data were generated according to Guillemin et al. (2005; with the
158 following codes for each of the six microsatellite loci: Grc-8B2, Grc-7F12, Grc-
159 2B2, Grc-6C7, Grc-7D3, and Grc-AC/CT23)[24] on an ABI 3100 sequencing
160 platform (Applied Biosystem, Foster City, CA). Genotypes from Morro Sama were
161 added to previously published genetic data sets composed of a total of 2112
162 individuals from seven locations in New Zealand (including the Chatham Islands)
163 and 28 locations in Chile (for more details on the sample locations and site
164 descriptions of published samples see Guillemin et al. 2008, 2014[16,19], and the
165 suppl. Table S1). All 182 individuals from New Zealand were diploids and
166 individuals from Chile were composed of 1394 diploids and 536 haploids. Allele
167 sizes among the different data sets were calibrated by including individuals of
168 known genotypes from Chile in each separate fragment analysis run. A total of
169 six microsatellite loci were scored in South America and two localities in New
170 Zealand (Scorching Bay (P-SCB) and Pautahanui Inlet (P-PIW) in the south of the
171 northern island; locality codes, as in Guillemin et al. (2008, 2014) [16,19]; suppl.
172 Table 1). Locus Grc-AC/CT23[24], was missing for the rest of the New Zealand
173 samples, for which only five microsatellites were scored. Please note that the
174 DRYAD data set for microsatellites genotypes used in Guillemin et al. (2014)[16]
175 is available at <http://hdl.handle.net/10255/dryad.73932>.

176 Additionally, the Internal Transcribed Spacer 2 (ITS2, operon of nuclear
177 ribosomal genes) was sequenced for a subset of the Peruvian samples collected
178 in 2015. The ITS2 PCR reactions and sequencing followed the protocol described

179 in Cohen et al. (2004)[17] and were performed on an ABI 3100 Sequencer
180 (Applied Biosystem, Foster City, CA). Sequences were edited using
181 CHROMAS[25] and multiple sequence alignments were constructed with
182 BIOEDIT[26].

183

184 *Microsatellites and ITS data analyses*

185 First, allelic frequencies and probabilities to generate the Peruvian genotypes
186 under the null hypothesis of free recombination [27] were estimated in various
187 populations and regions. Allele frequencies were estimated using GenoDive
188 v.2.0b27[28], for each microsatellite locus, for every single sampling location in
189 Chile and New Zealand (i.e., 35 locations in total), or among regions (after
190 grouping locations following Guillemin et al. 2008, 2014)[16,19]. Regions in New
191 Zealand were assigned as in Guillemin et al. (2014)[16]: West New Zealand, East
192 New Zealand, and Chatham Islands (suppl. Table 1). Eight regions were defined
193 in Chile (i.e., Caldera, Coquimbo, Concepción, Valdivia, Seno Reloncavi, Channel
194 of Chacao, Chiloé Island, and Continental Chiloé; Guillemin et al. 2008[19], suppl.
195 Table 1). Within each location or region, the likelihood of producing a Peruvian
196 genotype was estimated using the product of the frequency of each allele
197 characterizing the genotype (i.e., two per locus for diploid genotypes, one per
198 locus for haploid genotypes). Since only five microsatellites were available for
199 most populations from New Zealand (including Chatham Islands) these
200 calculations were done (1) using five microsatellite loci among samples from all
201 available locations (in South America and New Zealand), and (2) using six
202 microsatellite loci among samples from South America.

203 Second, using allele frequencies estimated above, genic differentiation between
204 the population of Morro Sama and all the other populations sampled was
205 calculated using the Fisher's exact probability test and 1,000 permutations, in
206 GENEPOP v.4.2[29,30]. Genic differentiation was estimated using the five
207 microsatellite loci available in all populations.

208 Third, Bayesian clustering assuming genetic admixture and correlated allele
209 frequencies was performed in STRUCTURE v.2.3.4[31,32] with a burn-in of
210 50,000 and a run-length of 100,000 iterations, making 10 runs for each K from 1
211 to 15 and without any prior population assignment, to evaluate with which
212 individuals of the 35 total sampling locations, the Peruvian samples were more
213 likely to cluster with. In order to achieve this, two different datasets were
214 consecutively run: (1) using all sampling sites and all available microsatellite
215 markers (six loci among South American samples and two sites in New Zealand,
216 and five loci for the remaining sites in New Zealand and Chatham Island, with the
217 sixth locus coded as missing data), (2) using only those sampling sites with
218 which the Peruvian samples clustered within the first data set and all six
219 available microsatellite markers. For STRUCTURE analyses, haploids were
220 artificially diploidized as homozygotes at every locus. The most likely number of
221 K was determined using the Evanno method implemented in STRUCTURE
222 HARVESTER v.0.6.94[33].

223 To assess ribotype diversity present in Peru, the ITS2 sequences obtained were
224 compared to the sequences available from Chile and New Zealand (108
225 sequences from Chile and 93 from New Zealand, Guillemin et al. 2014[16]) using
226 MEGA7[34].

227

228 **Results**

229 *Characteristics of the Morro Sama Agarophyton chilensis population*

230 In 2015 the Peruvian *A. chilensis* population covered only an area of about 18 m
231 in diameter. During the field evaluation in 2017, the site was described as
232 sheltered, without currents, turbid with 10 m visibility, depths ranging from 1-4
233 m, sea surface temperature of 16-17°C, pH of 7.65, 34.7 salinity, and a total *A.*
234 *chilensis* population of about 100 m x 25 m consisting of isolated patches
235 separated by approximately 1 meter between patches. The substratum was
236 described as mostly muddy-sandy, with a couple of rocks and some boulders.
237 The evaluation of the population further reported estimates of a 1:9 ratio of fixed
238 to buried or entangled (loose) specimens or fragments.

239 None of the specimens collected in 2015 or 2017 showed presence of
240 reproductive structures and they were categorized as vegetative samples. No
241 fixation discs were observed, suggesting that the entire population was
242 composed of loose thalli.

243 Among all 60 samples, the sex marker only amplified PCR products
244 representative to the male marker[23]. The results of the sex markers'
245 amplifications were coherent with the single, unique genotype obtained in Peru
246 (see below; with no more than one allele at each microsatellite locus).

247 From Peru, 29 individuals collected in 2015 and 28 in 2017 were successfully
248 genotyped at all six microsatellite loci. All 57 Peruvian samples corresponded to
249 one single genotype: 357bp for locus Grc-8B2, 192bp for locus Grc-7F12, 318bp

250 for locus Grc-2B2; 201bp for locus Grc-6C7; 297bp for locus Grc-7D3 and 220bp
251 for locus Grc-AC/CT23 (the genotype is given as allele lengths of each of the six
252 microsatellite loci, in base-pairs (bp) following Guillemin et al. 2005)[24]. All
253 allele lengths found in Peru had already been recorded in Chile[19,24]
254 (Guillemin et al. 2005, 2008). Further, we found one single ribotype among all
255 Peruvian sequenced ITS2 fragments (480 bp) generated for 22 samples collected
256 in 2015 (corresponding to “r1”, after Guillemin et al. 2014[16]).

257 Altogether, our results suggest the population in Peru to be an unattached,
258 vegetative, single male’s clonal population.

259

260 *Possible origins of Morro Sama’s male clone*

261 The ribotype r1 corresponds to the one ubiquitous among Chilean populations
262 and to the most common ribotype on the eastern coast of New Zealand and the
263 Chatham Islands[16]. Therefore, based on this genetic marker it is only possible
264 to discard the western coast of New Zealand as possible origin for the Peruvian
265 population.

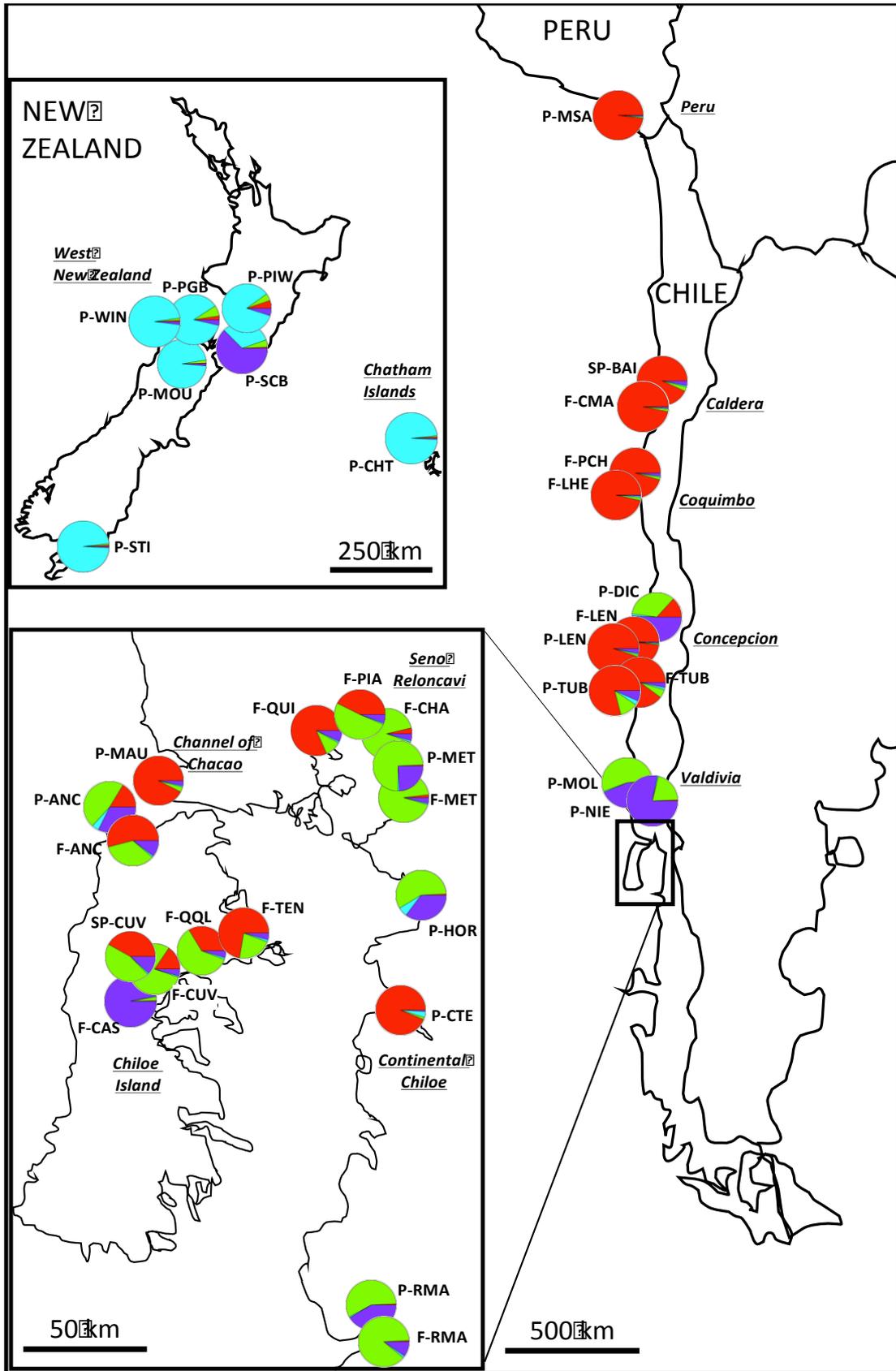
266 For the data set of five microsatellites, the likelihood to produce the Peruvian
267 genotype was 0.00 in all populations and regions of New Zealand, 0.03 in the
268 region of Coquimbo (north Chile), and 0.23 in the region of the Channel of
269 Chacao (south Chile; see suppl. Table S1 for further details). For the complete
270 data set of six microsatellites, the likelihood to produce de Peruvian genotype
271 was the highest in the region of the Channel of Chacao followed by the region of
272 Coquimbo (0.14 and 0.02, respectively; suppl. Table S1). Among the 28 Chilean

273 sampling locations (no grouping into regions), the most likely to produce the
274 Peruvian genotype was the location of Chaitén (in the continental Chiloé region,
275 $p = 0.10$), followed by the location of Maullín (in the Channel of Chacao region, p
276 $= 0.05$). Amongst the northern locations, the most likely source of the Peruvian
277 genotype was Playa Changas (in the region of Coquimbo, $p = 0.01$). Please note
278 that our values of likelihood to produce the Peruvian genotype within a Chilean
279 population are comparable to ones already estimated for *A. chilensis* in Chile
280 using the same molecular markers. Indeed, the probability of a given multilocus
281 genotype to be observed in the N samples genotyped from its own sampling site
282 as a consequence of different events of sexual reproduction was estimated to be
283 in between $3.3E-06$ and 0.125 for diploid samples and between $4.5E-04$ and
284 0.586 for haploid samples within our Chilean data set (see Supplementary Online
285 Material from Guillemin et al. 2008[19]).

286 Genic differentiation assessed between the Peruvian population and the rest of
287 the populations sampled were in all case highly significant (all $p < 0.0001$)

288 Using the Evanno method in the Bayesian clustering analysis of STRUCTURE,
289 $K=2$ and $K=4$ were suggested as the most likely total number of genetic groups in
290 our data set, differing slightly from the results in Guillemin et al. (2014)[16]
291 where $K=5$ was estimated for the whole *A. chilensis* distribution area. Results of
292 $K=2$ can be found in Figure S1 and clustering results for $K=4$ are given for each
293 population in Figure 2. For $K = 2$, the Peruvian genetic cluster (P-MSA, in red,
294 Figure S1) also included 12 Chilean populations (SP-BAI, F-CMA, F-PCH, F-LHE,
295 P-LEN, F-LEN, P-TUB, F-TUB, P-MAU, F-QUI, P-CTE, F-TEN). For $K = 4$, one group
296 was clustering all sites from New Zealand and Chatham Islands, except P-SCB

297 (light blue in Figure 2). P-SCB shows a mixture of light blue and purple genetic
298 group. Both light green and purple genetic groups were mostly present in
299 populations located in Southern Chile (from the region of Valdivia to the region
300 of Chiloé Continental) and in P-DIC (region of Concepción). The Peruvian genetic
301 cluster (P-MSA, in red, Fig. 2) also included another 12 Chilean populations with
302 over 70% assignment to that cluster (i.e.: SP-BAI, F-CMA, F-PCH, F-LHE, F-LEN, P-
303 LEN, F-TUB, P-TUB, F-QUI, P-MAU, P-CTE, F-TEN). Among these 13 South
304 American populations (i.e., P-MSA and the 12 populations listed above), another
305 STRUCTURE analysis was performed to narrow down which populations were
306 closest to the Peruvian genotype. The Evanno method suggested 4 genetic
307 clusters among these 13 populations. Some Chilean populations highly clustered
308 with P-MSA with: 77% of the population of Chaitén (P-CTE), 38% of the
309 population of Maullín (P-MAU), and 31% of the population of Playa Changa (F-
310 PCH) assigned to the Peruvian cluster. All other populations were rather
311 assigned to one of the other 3 genetic clusters.



314 **Fig. 2: Genetic clustering of *Agarophyton chilensis* using six microsatellite**
315 **loci.** Sampling sites are coded with P and F (for natural vs. farmed populations,
316 respectively) and a three-letter code for the population's name (following
317 Guillemin et al. 2008, 2014, and as in suppl. Table S1). The assigned regions are
318 given in underlined bold letters (see also suppl. Table S1). Pie charts indicate the
319 proportion of the population's individuals belonging to a total of four (K=4)
320 possible genetic clusters as assigned by STRUCTURE v.2.3.4[31,32]. Each of these
321 K-clusters is coded by a different color.

322

323 **Discussion**

324 This study infers the putative origin of the newly found Peruvian
325 population of *Agarophyton chilensis*, and describes the phase and sex ratios of
326 this population using genetic data available for specimens across the species'
327 entire, currently known distribution[16,18,19]. In brief, the Peruvian population
328 was found to comprise clones of one single vegetative male of *A. chilensis*, which
329 seemingly has been reproducing asexually through fragmentation of its thallus,
330 forming a small, patchy population. This population has sustained itself
331 successfully for at least four consecutive years and appears to have grown in
332 area (2014[18]; and from this study for 2015 and 2017). While the ITS2 nuclear
333 DNA marker could not discard New Zealand as a possible transoceanic source,
334 based on allele frequencies of microsatellite markers, our study strongly
335 suggests Chile as possible source of the Peruvian male, whereas all New Zealand
336 locations were discarded. Limited dispersion of Gracilariales spores[19] suggests
337 that the Peruvian population rather originated from a single stranded fragment

338 of the thallus of a male specimen than from one haploid spore. Further, all the
339 samples analyzed during both sampling periods were vegetative indicating that
340 the population has not been reproducing sexually in recent years. The reasons
341 for this remain undetermined but may be due to the recent (and singular) arrival
342 of the species to Peru or the lack of favorable environmental conditions.
343 Similarly, in a study on *A. vermiculophyllum*, newly arrived thalli seemed to
344 rather reproduce vegetatively through fragmentation, forming a vegetative
345 unattached population[35].

346 To date, invasion and expansion of geographic ranges among *Agarophyton*
347 have been proposed to rely on the arrival of one or a few floating
348 tetrasporophytes, which increase the initial population density and algal
349 biomass via vegetative reproduction through fragmentation, after which the
350 production of a few reproductive structures by these long-standing unattached
351 populations may lead to the production of haploid spores and the fixation of
352 male and female gametophytes on nearby hard substrata, finally recovering the
353 sexual life cycle of the species[16,19,35,36]. In the case of the Morro Sama
354 population, even if the male clone developed functional reproductive structures
355 (i.e., male gametangia), due to the absence of close-by reproductive females, the
356 sexual life cycle cannot be restored. Indeed, in Gracilariales, the male gametes
357 are short-lived (<6h)[37] and fecundation occurs at a few meters at most[38,39].
358 The nearest known locality where *A. chilensis* females have been reported is
359 located in the region of Caldera, almost 1000 km away from Morro Sama
360 (Guillemin et al. 2008). Additionally, most surveyed farms and/or sandy
361 unattached populations have been described to be highly dominated by

362 vegetative tetrasporophytes (in *A. chilensis*[16,19] and in *A. vermiculophyllum*
363 [36,40]). In Chile, only one *A. chilensis* farm mostly contained female
364 gametophytes, the rest being only or mostly tetrasporophytes[19], with
365 commonly complete absence of male gametophytes. Ecological differences
366 between phases may be one explanation for the dominance of vegetative
367 tetrasporophytes in unattached populations[41–43]. For instance, in *A. chilensis*,
368 vegetative tetrasporophytes have enhanced growth when compared to
369 gametophytes, with the male's growth rate being slowest in this species[41,42].
370 Altogether, Morro Sama is a unique case among unattached, recently described
371 *Agarophyton* populations, and the first asexually reproducing population
372 dominated by male thalli ever reported.

373 *Agarophyton chilensis* is negatively buoyant, however, fragments of thalli could
374 either be transported (1) entangled on marine vessels[44,45], (2) on other
375 positively buoyant rafts like tree trunks or other large floating
376 seaweeds[10,46,47], (3) carried by birds (e.g., in *A. vermiculophyllum* by either
377 migrating birds[48], or as nesting material of local birds,[49]), or (4) attached to
378 other imported marine goods such as oysters, shells, or clams (e.g., in *A.*
379 *vermiculophyllum*[50–52]). In terms of geographic distance and likelihood of
380 drift through natural currents or anthropogenically induced transport, northern
381 Chilean populations could be regarded as the more likely source for the arrival of
382 male propagules in southern Peru. Morro Sama is located directly on the path of
383 the Humboldt Current that flows north from 42°S (i.e., North of the Chiloé
384 Island)[9] and is found only 600 km away from Bahia Mejillones, the northern-
385 most point where *A. chilensis* was transported to be cultivated in Chile. The farm

386 of Bahia Mejillones seems to have disappeared nowadays due to poor
387 management choices (Wilkomirsky, personal communication) but Morro Sama is
388 still located only 980 km north of the region of Caldera (27°S) and 1,170 km
389 north of the region of Coquimbo (29°S), the oldest established farm in northern
390 Chile[15]. However, at its northern distribution in Chile, *A. chilensis* populations
391 are farms mostly or even entirely composed of vegetative tetrasporophytes[19]
392 and our genetic investigation did not refer to a northern population as the most
393 likely source of the Peruvian male. Nonetheless, even if barely reproductive,
394 farmed tetrasporophytes along Chile's northern coasts are still able to produce
395 haploid gametophytes via sexual reproduction every once in a while and these
396 spores can attach and grow on pebbles and shells scattered close to the farms
397 (few fixed males recorded up to 27°S, SP-BAI, region of Caldera[19]). Thus, it is
398 still possible that the male thallus fragment from Morro Sama originated from a
399 rare male specimen in northern Chile in the Coquimbo or the Caldera region. Our
400 genetic data, however, suggest the region of the Channel of Chacao to be the most
401 likely source of the Peruvian genotype (see our results from STRUCTURE;
402 Channel of Chacao is located more than 2,600km south of Morro Sama). In the
403 region of the Channel of Chacao and around the Island of Chiloé, large natural
404 populations of *A. chilensis* are established at the opening of estuaries and rivers
405 growing from spores and fixed on small pebbles, shells, or in rock pools.
406 *Agarophyton* thalli seem highly resistant and resilient to physiological stress and
407 are able to survive long trips while drifting[16,40,50]. Supporting this idea,
408 ecophysiological studies have demonstrated that *Agarophyton* thalli have the
409 ability to withstand a wide range of physico-chemical conditions (in *A.*
410 *chilensis*[53]; in *A. vermiculophyllum*[48,54]). Furthermore, it has been shown

411 that rapid evolution can occur during range expansion by sieving advantageous
412 genotypes during transport, which for example allows for successful
413 establishment in new environments[55]. In *A. vermiculophyllum*, adaptation to
414 new environmental conditions during colonization has been proposed due to
415 higher tolerance to extreme heat, cold, or low-salinity[56] observed in non-
416 native populations compared to native ones. Hence, the mere distance between
417 Channel of Chacao and Morro Sama, and their contrasting latitudinal position
418 (i.e., the two locations are separated by 25°) does not represent a sufficient
419 impediment as to reject South Chile as a possible source of the Peruvian clone.
420 Indeed, *A. chilensis* has already undertaken and survived the much longer
421 journey from New Zealand to Chile and was able to colonize most of Chile's
422 coast[16] and long-distance dispersal has been suggested as an important factor
423 for the distribution of marine macrophytes in the southern Pacific Ocean[13].

424 Regardless of the exact Chilean source population, natural colonization by rafting
425 thalli was possibly fostered by the strong northward-flowing Humboldt
426 Current[9,10]. Yet introduction linked to anthropogenic activities is not to be
427 discarded, especially since the traffic of boats from Morro Sama has been
428 reported in the northern parts of Chile[57–59]; and the Peruvian population of *A.*
429 *chilensis* is actually found right at the landing pier for artisanal fisheries
430 (Desembarcadero de Pesca Artesanal, DPA) of the Grau Port (Sama District,
431 Tacna Province), a very small port built in 1995, with the capacity of mooring up
432 to 15 artisanal fishing boats, which daily provide a wide variety of marine
433 products.

434 Despite the high numbers of samples and locations included in this study, the
435 source of the Peruvian male thallus still remains elusive, especially because of
436 the anthropogenic farming activities in Chile[19]. Indeed, fresh thalli are
437 commonly exchanged between fishermen's and southern populations were used
438 to seed the artificially introduced farms in the North[19], potentially blurring
439 accurate genetic signature for single geographic sites/regions. *Agarophyton*
440 *chilensis*, because of its economic interest, is relatively well known in Chile where
441 most of the natural and farmed populations have been mapped. However, some
442 recognized populations (unattached in the seawater lagoon of Puerto Saavedra,
443 region of Araucanía, 38°S; attached on rocks at Punta Curaumilla or buried in
444 sand in Quintero, region of Valparaíso, 33°S and 32°S, respectively) have not yet
445 been genotyped. It could be interesting to add these populations to the Chilean
446 data set in order to better infer the origin of the Moro Sama population.
447 Potentially, the utilization of next generation sequencing techniques and the
448 acquirement of much higher numbers of genetic markers, such as SNPs, for
449 population genetics may give a better resolution of the colonization pathways
450 and sources of *Agarophyton* populations. Until then, the Peruvian *A. chilensis*
451 population does not seem to be an ecological threat nor is the species
452 categorized as invasive but it will be interesting to keep on monitoring the
453 further development and proliferation of this isolated and unique clonal male.

454

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673

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680

681 **Author Contributions Statement**

682 V.R. did data analysis, prepared the figure and table; V.R. and M-L.G. wrote the
683 main manuscript text; S.M. generated molecular data; J.C.Z.R and N.A. collected
684 and analyzed samples as well as site descriptive information. All authors
685 critically reviewed and approved the manuscript's final version prior
686 submission.

687

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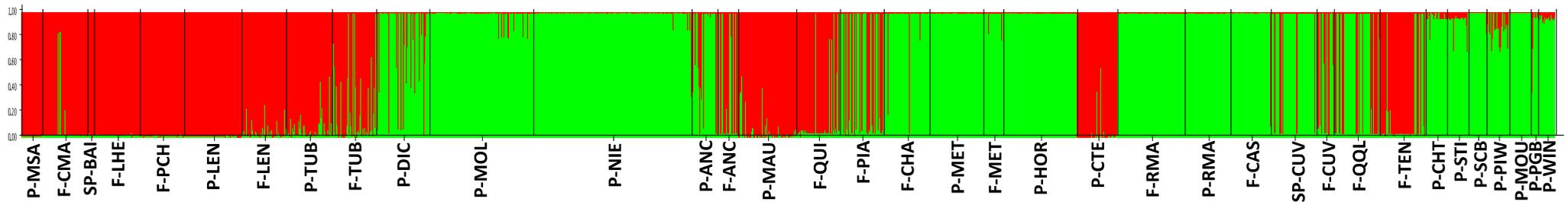
700 **Supplementary Information**

701

702 **Suppl. Table S1: Details on the sampled populations (Natural or Spontaneous vs. Farms (P, SP, or F, respectively)) and probabilities P to**
 703 **generate the Peruvian genotype among each population based on allele frequency analyses of microsatellite data (six vs. five microsatellites**
 704 **(MSATS)). Probabilities to generate the Peruvian genotype among each region are given in bold between brackets. N is the Number of**
 705 **genotyped individuals.**

Country	Region	Name	Population	Geoposition	N	P [6 MSATS]	P [5 MSATS]
Perú	Perú	<i>Morro Sama</i>	P-MSA	17° 59' S, 70° 52' W	29	1.00 (1.00)	1.00 (1.00)
Chile	Caldera	<i>Bahía Inglesa</i>	SP-BAI	27° 09' S, 70° 53' W	9	0.003 (0.00)	0.003 (0.00)
		<i>Cultivos Marinos</i>	F-CMA	27° 04' S, 70° 50' W	63	0.000	0.000
	Coquimbo	<i>Playa Changa</i>	F-PCH	29° 57' S, 71° 20' W	62	0.011 (0.002)	0.016 (0.003)
		<i>La Herradura</i>	F-LHE	29° 58' S, 71° 21' W	64	0.000	0.000
	Concepción	<i>Dichato</i>	P-DIC	36° 32' S, 72° 56' W	74	0.000 (0.002)	0.000 (0.002)
		<i>Lenga</i>	P-LEN	36° 45' S, 73° 11' W	80	0.002	0.002
			F-LEN	36° 45' S, 73° 11' W	62	0.000	0.000
	Valdivia	<i>Tubul</i>	P-TUB	37° 15' S, 73° 26' W	64	0.007	0.008
			F-TUB	37° 15' S, 73° 26' W	62	0.002	0.002
		<i>Los Molinos</i>	P-MOL	39° 50' S, 73° 23' W	145	0.000 (0.000)	0.000 (0.000)
	Channel of Chacao	<i>Niebla</i>	P-NIE	39° 52' S, 73° 23' W	221	0.000	0.000
		<i>Mauñín</i>	P-MAU	41° 37' S, 73° 35' W	81	0.053 (0.014)	0.064 (0.023)
		<i>Ancud</i>	P-ANC	41° 52' S, 73° 48' W	36	0.000	0.000
	F-ANC		41° 52' S, 73° 48' W	29	0.004	0.007	
	Seno Reloncavi	<i>Quillape</i>	F-QUI	41° 32' S, 72° 44' W	61	0.013 (0.000)	0.017 (0.000)
<i>Piedra Azul</i>		F-PIA	41° 30' S, 72° 47' W	61	0.000	0.000	
<i>Chaica</i>		F-CHA	41° 38' S, 72° 39' W	64	0.000	0.000	
<i>Metri</i>		P-MET	41° 36' S, 72° 42' W	75	0.000	0.000	
	F-MET	41° 36' S, 72° 42' W	28	0.000	0.000		

Chiloé Island	<i>Tenaún</i>	F-TEN	42° 20' S, 73° 23' W	64	0.009 (0.000)	0.020 (0.001)	
	<i>Quiquel</i>	F-QQL	42° 21' S, 73° 35' W	64	0.001	0.001	
	<i>Curaco de Veléz</i>	SP-CUV	42° 26' S, 73° 36' W	24	0.001	0.002	
		F-CUV	42° 26' S, 73° 36' W	64	0.000	0.000	
	<i>Castro</i>	F-CAS	42° 30' S, 73° 47' W	56	0.000	0.000	
Continental Chiloé	<i>Hornopiren</i>	P-HOR	41° 58' S, 72° 28' W	103	0.000 (0.000)	0.000 (0.000)	
	<i>Chaitén</i>	P-CTE	42° 55' S, 72° 42' W	56	0.102	0.104	
	<i>Raul Marín</i>	P-RMA	43° 46' S, 72° 57' W	94	0.000	0.000	
		F-RMA	43° 46' S, 72° 57' W	64	0.000	0.000	
New Zealand	Chatham Islands	<i>Chatham Islands</i>	P-CHT	43° 52' S, 176° 33' W	30	— (—)	0.000 (0.000)
	East New Zealand	<i>Stewart Islands</i>	P-STI	46° 54' S, 168° 07' E	30	— (0.000)	0.000 (0.000)
		<i>Pautahanui Inlet</i>	P-PIW	41° 06' S, 174° 49' E	32	0.000	0.000
		<i>Scorching Bay</i>	P-SCB	41° 18' S, 174° 48' E	25	0.000	0.000
	West New Zealand	<i>Moutere Inlet</i>	P-MOU	40° 59' S, 173° 01' E	30	— (—)	0.000 (0.000)
		<i>Whanganui Inlet</i>	P-WIN	40° 34' S, 172° 32' E	25	—	0.000
		<i>Golden Bay</i>	P-PGB	40° 48' S, 172° 48' E	10	—	0.000



Suppl. Figure S1: STRUCTURE bar plot from 36 collection sites of *Agarophyton chilensis* (complete trans-Pacific distribution) based on five polymorphic microsatellite markers. Posterior probability of assignment of *A. chilensis* samples to one of two ($K = 2$) genotype clusters are shown in the bar plot by the colors green and red, generated using a Bayesian clustering analysis. Black lines divide the collection sites, which are coded as in Suppl. Table S1.