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1 **High genetic variability of *Alexandrium fundyense* (former *A. catenella*) directly**
2 **detected in environmental samples from the Southern Austral Ecosystem of Chile.**

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19 **Abstract**

20 Chilean waters are often affected by "*Alexandrium catenella*", one of the leading organisms
21 behind Harmful Algae Blooms (HABs). Studies of genetic variability for this species are
22 commonly carried out from cultured samples, an approach that may not accurately quantify
23 genetic variability of this organism in the water column. In this study, genetic variability of
24 "*A. catenella*" was determined by sequencing the rDNA region, in water samples from the
25 Canal Puyuhuapi (South Austral Ecosystem of Chile). "*A. catenella*" was detected in 8,8%
26 of samples analysed. All sequences obtained, as well as sequences originally reported as *A.*
27 *catenella* for Chile, were *A. fundyense* (Tamara complex group I), with three highly
28 frequent haplotypes (34%), and twenty new haplotypes. These haplotypes increase the
29 documented genetic variability from 2.8% to 3.14% in the study area. Through this new
30 method, genetic determination of *A. fundyense* (former *A. catenella*) can accurately be
31 monitored and ecological studies of this species can be implemented.

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33 **Key words:** *Alexandrium catenella, Alexandrium fundyense*, Molecular detection, Genetic
34 variability.

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40 **1. Introduction**

41 Dinoflagellates of the genus *Alexandrium* are the greatest cause of the harmful algal bloom
42 (HABs) syndrome known as Paralytic Shellfish Poisoning (PSP). In recent decades, HABs
43 caused by this genus have increased in frequency, severity, and geographic distribution
44 (Wang *et al.*, 2008), affecting marine ecosystems and the safety of seafood (Sellner et al.,
45 2003; Heisler et al., 2008). The genus *Alexandrium* has been separated into two major
46 species complexes based on morphological similarity and ribosomal sequence likeness: the
47 *minutum* group (*A. lusitanicum*, *A. angustitabulatum*, *A. andersonii* and *A. minutum*) and
48 the *Alexandrium tamarense* complex (originally grouped as "*Alexandrium tamarense"*, "*A.*
49 *catenella"* and "*A. fundyense"*), (Scholin et al., 1995; Wang et al., 2008). Recently based
50 on morphogenetic studies, a formal taxonomic revision of the *A. tamarense* complex
51 species has been proposed, identifying five genetic clades as different species: *A. fundyense*
52 (Group I); *A. mediterraneum* (Group II), *A. tamarense* (group III); *A. pacificum* (Group
53 IV); A. *australiense* (Group V) (Lily et al., 2007; Miranda et al., 2012; John et al., 2014;
54 Wang et al., 2014). Numerous strains of the *A. tamarense* complex have caused severe PSP
55 outbreaks in coastal regions around the world, with "*A. catenella"* and "*A. tamarense"*
56 being the species most frequently associated with such events (Hallegraef, 1993; Kamikawa
57 et al., 2007). Chile has been hit repeatedly by HABs linked to "*A. catenella"* since 1972 in
58 the Magallanes Region (Guzmán & Campodónico, 1975), and currently detected from the
59 Aysén Region to Chiloé Island (Mardones et al., 2010), causing a variety of negative
60 impacts that range from economic losses to human deaths. This discourages bivalve
61 shellfish aquaculture and fisheries activities in affected areas (Uribe et al., 2010). This
62 situation, has promoted the establishment of a monitoring program by government

63 authority, based on specific qualitative/quantitative monitoring of "*A. catenella"* in both the
64 water column and in sediments. These routine analyses are based on microscopic
65 observations of taxonomic features, including differences in the shape of plates, length to
66 width ratios and presence or absence of a ventral pore (Balech, 1985; John et al., 2014).
67 However, many authors concluded that this approach is inconclusive for species
68 identification, among other reasons, due to the presence of cells with intermediate
69 morphologies, providing erroneous information regarding the geographical distribution,
70 ecology and toxicity of this genus (Lilly, 2003; Gayoso and Fulco, 2006; John et al., 2014;
71 Wang et al., 2014). It is therefore necessary to complement the taxonomic approach with
72 molecular tools to ensure correct species identification (John et al., 2003; Lilly et al. 2007;
73 Touzet *et al.* 2007, Touzet *et al.* 2008).

74 Combinations of both molecular tools and bioinformatics analyses, mainly based on DNA
75 ribosomal sequences, have allowed inter- and intra-species identification, as well as
76 determination of genetic variability of dinoflagellates (Godhe et al., 2001; John et al., 2003;
77 Galluzzi et al., 2005; Lilly et al., 2007; Touzet et al., 2007; Touzet et al., 2008; Aguilera et
78 al., 2011; Richlen et al., 2012; Jhon et al., 2014). Genetic variability drives evolution and is
79 a characteristic of all species including harmful algae and is critical for their survival
80 (Rynearson & Armbrust 2005). In fact, many reports have shown genetic variability in
81 environmental samples of *"A. minutum"*, "*A. tamarense"*, "*A. catenella"*, and *A. fundyense*
82 (Chow et al., 2004; Penna et al., 2005; Alpermann et al., 2010; Aguilera et al., 2011;
83 Richlen et al., 2012). Generally, these studies involve the isolation of organisms from the
84 environment and the establishment of clonal cultures, however limits of *in vitro* culture and
85 loss of some strains while attempting to culture could in fact underestimate the genetic

86 variability of this species and lead to erroneous conclusions about this species in its natural
87 condition (Burkholder and Gilbert, 2006). For phylogenetic analyses to be of utility a
88 genetic marker of sufficient variability should be used. Recent work by Wang and
89 coauthors (2014) have suggested a revised taxonomy for the *A. tamarense* complex based
90 upon the ITS rDNA region of the genome, as other ribosomal markers show poor resolving
91 power for separating genetically distinct lineages.

92 Therefore, the aim of the present work was to develop an effective molecular method to
93 detect "*A. catenella"* in natural conditions and avoid culture methods. Molecular data was
94 used to determine the genetic variability of this population, and was then compared with
95 sequences reported at different geographical sites from the Southern Austral Ecosystem of
96 Chile (SAE). This assay provides an alternative method for accurately monitoring and
97 performing ecological studies of this dinoflagellate throughout the world´s oceans.

98 2. Methods and materials

99 *2.1 Study area and sample collection*

100 Samples were obtained from the water column of the Puyuhuapi Channel (from
101 44º19'72º33'W to the North, to 44º57'S, 73º21'W to the South), at 24 water column stations
102 between November 2010 and September 2012 (Fig. 1). Water samples (0,5 L) were
103 obtained with Niskin bottles at depths of 0, 10, 25 and 50 m. The water was prefiltered at
104 100 µm, and then filtered through a cellulose ester filter with a diameter of 47 mm and pore
105 size of 0.22 µm with a pressure of less than 100 mm Hg. Filters were stored at -80°C until
106 processing.

107 *2.2. Total DNA extraction and determination of concentration and quality*

108 Total DNA extraction from filters was performed with 0.25 mL of extraction buffer (Tris-
109 HCl 20 mM pH 5.2, EDTA 50 mM, SDS at 10% (W/V), and Proteinase K (10 mg mL^{-1}),
110 followed by an incubation at 50°C for 10 min, and a subsequent freeze for 15 min. This
111 procedure was repeated three times. The lysis mixture was extracted with saturated phenol
112 (neutralized with Tris-HCl 0.5 M, pH 7.8 to 8.5), and chloroform-isoamyl alcohol (24:1).
113 Then, the nucleic acids were precipitated with 2 volumes of ethanol 95% (vol/vol) at -20°C
114 in presence of 0.1 volumes of sodium acetate 3M (pH 5.2). The mixture was incubated at -
115 20°C overnight, and centrifuged at 21,000g for 20 min. The pellet was washed twice with
116 cold ethanol 70% and air dried for 10 to 15 min. The pellet was resuspended in 100 µL
117 sterile water and stored at -80°C. DNA concentration and purity was estimated by
118 spectrophotometry in a Nanodrop 2000. The quality of the extracted DNA was evaluated by
119 electrophoresis (100V, 45 min) in a 1% agarose gel , stained with ethidium bromide (5 mg
120 mL^{-1}).

121 *2.3. Primer design, PCR amplification and specificity test.*

122 To design primers, sequences for "*Alexandrium catenella"* ITS1-5.8S-ITS2 ribosomal
123 region (~ 520 base pairs) obtained in both COPAS Sur Austral (personal database) and
124 NCBI database (using "*A. catenella* AND Chile" as search), were analysed. These
125 sequences combined with ITS sequences originating from representative genetic clades of
126 *Alexandrium tamarense* complex, were aligned with Vector NTI Advance ® software
127 version 10.3.0 (Invitrogen TM), and species-specific primers were designed in highly
128 conserved regions for the species of study, "*Alexandrium catenella"* . All samples were
129 analysed by polymerase chain reaction (PCR), with Taq 1X PCR buffer (Promega), 1 U
130 GoTaq® Flexi DNA polymerase (Promega), 3.0 mM $MgCl_2$, 0.4 mM of each dNTPs

131 (Promega), and 20 pmoles of each forward and reverse primer in a 25-µL reaction volume.
132 To determine the minimum amount of DNA to carry out PCR reactions, DNA isolated from
133 monoclonal culture cells of "*A. catenella"* strain PFB 37 (HM641243), was amplified. The
134 optimum DNA quantity amplified was 5 ng, with a range of 15-0.16 ng (data not shown).
135 The PCR program was: a preliminary denaturation step at 95ºC for 5 min; 5 cycles of pre-
136 amplification of 95ºC for 30 s, 50ºC for 30 s and 72ºC for 30 s; then, 35 cycles of 95ºC for
137 30 s, 55ºC for 30 s and 72ºC for 45 s; and a final extension of 72°C for 3 min. All the
138 reactions were performed in a Thermocycler T-personal Biometra®. The specificity of the
139 designed primers were evaluated using genomic DNA from vegetative cells or cysts of
140 *Alexandrium minutum*, in presence and absence of gDNA from "*A. catenella"* strain PFB
141 37 (HM641243). All PCR products were assessed by electrophoresis (100V, 45 min.) in
142 agarose gel at 1%, stained with ethidium bromide (5 mg mL^{-1}).

143 *2.4. Cloning and purification of plasmids*

144 The PCR product corresponding to the expected amplification size was cut from the
145 agarose gel, and purified using the Gel extraction kit E.Z.N.A. (Omega Biotek) according
146 to the manufacturer´s instructions. Purified DNA was ligated into the pGEMT-Easy vector
147 and transformed to competent *Escherichia coli* JM109 cells using the thermal shock
148 method. Positive white colonies were selected from X-gal/amp agar plates and grown in
149 LB/amp liquid medium. Plasmids were purified using the Plasmid Miniprep kit II E.Z.N.A.
150 (Omega Biotek), following the manufacturer´s instructions. All positives clones were
151 checked by PCR amplification.

152 *2.5. Sequencing and phylogenetic analysis*

153 Sequencing of the purified plasmids containing the cloned fragment of ITS1-5.8S-ITS2
154 from *A. catenella* ribosomal DNA was performed by MACROGEN (Korea) using BigDye
155 Terminator Cycle Sequencing Kit (PE Applied Biosystems, Foster City, CA). To verify that
156 the sequenced clones corresponded to the study species, preliminary analysis of the
157 sequences obtained was performed using the BLAST program. In order to compare
158 sequences of ITS1-5.8S-ITS2 rDNA, aligned haplotype diversity (Hd) was calculated using
159 DNA Sequence Polymorphism software version 5.10.01 (Librado and Rozas, 2009). For
160 phylogenetic analyses, representative ITS1-5.8S-ITS2 rDNA sequences of "*A. catenella"*
161 from the Southern Austral Ecosystem of Chile and ITS sequences originating from
162 representative genetic clades of the Tamarense complex available in GenBank were used
163 (Additional Table 1), and defined as **CM**: this study; **AA**: sequences described in Aguilera
164 *et al.* 2011; and **VV**: sequences described in Varela *et al.* 2012. The ITS1-5.8S-ITS2 rDNA
165 sequence from *A. minutum* strain AM 18S was used as the out-group. Sequences were
166 aligned and manually edited using MEGA 6 Software (Tamura *et al.* 2013). jModeltest
167 version 2.1 (Darriba *et al.* 2012) was used to determine the best substitution model and
168 associated parameters for the phylogenetic analysis. The model selected for ITS analysis
169 based on the Akaike Information Criterion (AICc) was HKY+G with base frequencies of
170 A: 0.247, C: 0.151, G: 0.244, T: 0.358; base substitution rates of AC: 0.029, AG: 0.149,
171 AT: 0.070, CG: 0.047, CT: 0.219, GT: 0.070; proportion of invariable sites I= 0; and
172 gamma distribution shape parameter= 0.84. Phylogenetic analyses were performed using
173 the maximum likelihood method with 1000 bootstraps replicates with MEGA 6 Software.

174 *2.6 Environmental map of A. fundyense (former A. catenella) haplotypes*

175 In order to show the spatial distribution of the most representative haplotypes of *A. fundyense,* described in the Southern Austral Ecosystem of Chile, an environmental map was created.

178

179 3. Results

180 *3.1 Primer design, PCR amplification and specificity test for A. fundyense (former A. catenella).*

182 Using the primers Alex-F (5'-CACTAACCTGCTAATGATATTGTGG-3') and Alex-R (5'-AGTCTTCAGCTTGTCTCAGTTGT-3') designed in this work, the PCR reaction was only positive in samples containing "*A. catenella"* DNA (Fig. 2). The samples containing gDNA from vegetative cells or cyst of *A. minutum* did not show amplification or unspecific products, demonstrating the specificity of the primers.

187 *3.2 Amplification of A. fundyense (former A. catenella) from environmental samples*

188 Positive amplification of *A. fundyense* (former *A. catenella*) was detected in 19 samples analysed (8.8%), between 10 m and 50 m in the water column. All samples of 0 m strata showed no amplification, suggesting no detectable *A. fundyense* DNA. The amplicons of ~500 bp obtained provided a total of thirty one (31) sequences, which were stored in the GenBank database under the following accessions: KC544998- KC545026; KF924270 - KF924271. All sequences obtained were analysed using the BLAST program, with a 99% and 100% identity with the ITS1-5.8S-ITS2 regions of ribosomal DNA of the *A. tamarense* complex group I (*A. fundyense*) strains isolated from southern Chile.

196 *3.3 Sequencing and bioinformatics analysis*

197 For sequence analysis, we used representative ITS1-5.8S-ITS2 ribosomal DNA sequences
198 from the *A. tamarense* complex (included former *A. catenella*) from the Southern Austral
199 Ecosystem of Chile available in Genbank. The alignment of obtained sequences revealed an
200 average genetic diversity of 3.14%. From all sequences, 33 haplotypes were found (Table
201 1), with a total haplotype diversity (Hd) of 0.9633 and nucleotide diversity (π) of 0.03145.
202 The group sequences obtained in this study (CM) displayed high genetic diversity, with 23
203 haplotypes, and values of Hd and π of 0.9742 and 0.03480, respectively; three highly
204 frequent haplotypes, representing 34% of total haplotypes (A= 14%; B= 12%; C= 8%),
205 were found from all sequences (CM; AA and VV). Another three haplotypes (from D to F;
206 in total= 12%) were found but at lower frequencies, only dispersed in CM and VV
207 sequences. The other twenty-seven haplotypes found were unique and distributed
208 throughout all sequences (in total= 32.7%). When considering only the sequences obtained
209 from this study, twenty-new haplotypes sampled directly from the water column were
210 determined, representing a 154% increase in haplotypes described for this area.

211 A phylogenetic tree was constructed using the sequences obtained in this study, the
212 sequences from AA and VV, ITS sequences originating from representative genetic clades
213 of the *Tamarense* complex, and *A. minutum* as an outgroup. This tree showed two main
214 clades: A basal clade including sequences from *A. tamarense* complex group II – V, and a
215 superior clade containing only sequences belonging to *A. tamarense* complex group I (*A.*
216 *fundyense*). All sequences obtained in this study as well sequences originally reported as
217 "*A. catenella"* to date for Chile, belonged to this clade. Sub-clades show a frequent
218 haplotype B and another 7 haplotypes distributed only in the Aysén Region, and included a

219 unique divergent sequence (KC545015). The other sub-clades show frequent haplotypes A
220 and C, and a further 20 haplotypes distributed throughout all areas. This clade also includes
221 4 divergent sequences KC545006; KC545011; KC545020; KC545024, found only in this
222 study.

223

224 *3.4 Environmental map of Alexandrium fundyense (former A. catenella)*

225 An environmental map to visualize the geographic distribution of the most frequent
226 haplotypes in the southern austral zone can be seen in Figure 4. Haplotypes A and C are
227 distributed across the Southern Austral Ecosystem of Chile, covering the regions of Los
228 Lagos, Aysén and Magallanes. Haplotype B was found exclusively in the region of Aysén,
229 with a wide distribution across the study area (Puyuhuapi Channel).

230 **4. Discussion**

231 Harmful Algal Blooms (HABs) in Chile, particularly in the regions of Los Lagos, Aysén
232 and Magallanes, have been linked to cases of Paralytic Shellfish Poisoning (PSP), a toxic
233 complex that has caused serious damage to the economy, environment and human health
234 (Buschmann, 2005). "*Alexandrium catenella"*, has been identified as the main species
235 responsible for the production of PSP in the south of Chile (Guzman *et al.* 1975, Lembeye
236 *et al.* 1998, Mardones *et al.* 2010), where the emergence, persistence, and toxicity of their
237 blooms are the result of the interaction of several ambient factors, such as salinity and
238 temperature (Guisande *et al.* 2002, Laabir *et al.* 2011, Lim *et al.* 2011, Anderson *et al.*
239 2012). This situation has led to extensive efforts to develop approaches that contribute to

240 toxic algae monitoring. Genetic variability is a characteristic property among harmful algae
241 and is critical to the survival and evolution of the species (Rynearson & Armbrust 2005),
242 enabling the maintenance of a variety of genotypes, which can generate toxic events
243 according to environmental conditions. Therefore, the correct determination of this
244 parameter is fundamental to understanding the physiology and ecology of these organisms.
245 In this study, we present a molecular method for detecting and determining the original
246 genetic variability of *Alexandrium fundyense* (former *A. catenella)* directly from water
247 samples, avoiding traditional culture methods. Bioinformatics analyses demonstrate that all
248 positive samples detected from the environmental samples were identified by sequence data
249 as *A. fundyense* (Group I) with a similarity between 99-100%. This confirms species-
250 specific detection using the proposed primers. Additionally, sequences analysed were
251 grouped with a North American Group I (NA / Group I) of "tamarense complex" (Scholin
252 *et al.* 1995, John *et al.* 2003 and Lilly *et al.* 2007), actually *A. tamarense* complex group I
253 (*A. fundyense*), clade that includes genotypes with a wide biogeographical distribution, in
254 agreement with other studies of isolates of *A. fundyense* (formerly *A. catenella*) described
255 from this area (Aguilera *et al.* 2011, Jedlicki *et al.* 2012, Varela *et al.* 2012).

256 Molecular detection of *A. fundyense* (formerly *A. catenella)* in the water column was
257 limited to depths between 10 and 50 m. The highest number of detections was observed at a
258 depth of 10 m, which corresponds mainly to vegetative cells, which were associated with
259 depths from 3 m to 25 m (Martin *et al.* 2005, Townsend *et al.* 2005). Moreover, we
260 reported a high rate of molecular detection at 50 m, even higher than at 25 m, from all
261 samples from all campaigns (data not shown). These detections could be cysts of *A.*
262 *fundyense*, which have been associated with depths between 30 m and seafloor sediments

263 (Kirn *et al.* 2005, Ishikawa *et al.* 2014). We failed to detect this organism in surface waters,
264 probably due to high susceptibility to UV radiation reported for this microalgae (Martinez
265 *et al.* 2000), and the specific environmental factors of the study area. The Puyuhuapi
266 Channel presents strong temperature and salinity gradients, forming a thermocline and
267 halocline that separate the surface layer (0-10 m depth) from the deep layer (10-50 m deep)
268 (Silva *et al.* 1997, Silva *et al.* 2002).

269 The presence of *A. fundyense* (formerly *A. catenella*) can be modulated by various
270 environmental factors, particularly temperature and salinity (Anderson *et al.* 1990,
271 Guisande *et al.* 2002, Laabir *et al.* 2011, Lim *et al.* 2011). Based on temperature profiles
272 obtained from the campaigns, the presence of *A. fundyense* was associated with
273 temperatures between 9 and 13°C (with the highest abundance at 10°C), coinciding with
274 Balech (1995), who proposed 12°C as the optimum temperature for the development of this
275 dinoflagellate. In salinity profiles, the presence was linked to values between 28 and 33 psi,
276 coinciding with the optimum range reported for this organism (Siu *et al.* 1997, Aguilera
277 2012, Ishikawa *et al.* 2014).

278 Phylogenetic analysis show a high genetic diversity in the ITS sequences analysed in this
279 study. The average genetic diversity calculated was 3.14%, a much larger value than that
280 reported among isolates from this same study region (2.8%) (Aguilera *et al.* 2011), from the
281 austral region of Chile (1.14) (Varela *et al.* 2012), from the Mediterranean Sea (0%) and
282 from the Japanese Pacific (0.05%) (Penna *et al*. 2005). These high values may be explained
283 by the high number of samples analysed, and the high sensitivity of the proposed molecular
284 technique. However, consistent with our results, a sustained increase in the genetic
285 diversity values obtained in isolates of *A. fundyense* from this area has been observed.

286 Older established populations present large genetic differences in the nuclear rDNA
287 (Collins *et al.* 2009), generating new haplotypes over a short period of time. A phylogenetic
288 tree that included sequences between 1994-2012, showed that three haplotypes were most
289 common in all areas (haplotypes A, B and C). These haplotypes have persisted over time
290 (2004-2012), suggesting an event of population expansion. According to this, generally
291 high genetic diversity has been observed within populations (Hd: 0.9633), especially the
292 CM sequences (Hd: 0.9742), and high nucleotide diversity between haplotypes (π=
293 0.03145), which demonstrated high divergence in terms of population differentiation, and
294 suggests a possible demographic expansion event. Many factors may contribute to this
295 dispersion, among which we highlight well-boats (given the high levels of aquaculture
296 activity in these regions), currents, natural movement of this species, and the cyst state of
297 this dinoflagellate, which is highly resistant to environmentally adverse conditions
298 (Lembeye 2003).

299 This study provides an important contribution to current knowledge on the genetic diversity
300 of *A. fundyense* (former *A. catenella*) in the Southern Austral Ecosystem of Chile. The
301 genetic information obtained directly from environmental samples offers a more realistic
302 ecological vision of this dinoflagellate, considering the dynamic oceanographic conditions
303 described for the south of Chile, (Sievers 2008, Quiroga *et al.* 2009, Diaz *et al.* 2011,
304 Daneri *et al.* 2012). Finally, this proposed method can serve as a complementary tool to
305 current monitoring programs of HABs caused by *A. fundyense* (formerly *A. catenella*) and
306 can contribute to the understanding of the molecular ecology of this dinoflagellate.

307 **5. Conclusions**

308 In this study, specie-specific PCR detection assay for "*A. catenella"* were used to study the
309 genetic diversity of this specie directly from water samples of the Southern Austral
310 ecosystem. Bioinformatics rDNA analysis showed that previous *A. catenella* corresponds to
311 *A. fundyense* (Tamara complex group I), with three highly frequent haplotypes, and new
312 haplotypes that increase the genetic diversity (from 2.8% to 3.14%) of *A. fundyense*
313 (formerly *A. catenella*) described in the study area. This PCR assay contributes in
314 understanding the distribution and dynamics of *A. fundyense* (formerly *A. catenella*), and
315 shows significant advantages for studying the genetic diversity of this species at a global
316 scale.
317

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522 **Table 1. Samples, sample size and haplotype distributions of *Alexandrium fundynese***
523 **(former *A. catenella*). CM: this study; AA: sequences described in Aguilera *et al.* 2011;**
524 **and VV: sequences described in Varela *et al.* 2012**.

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Samples	Sample Size	Number of Haplotypes	Haplotype Diversity (Hd)	Nucleotide Diversity (π)
AA	7	5	0.9048	0.03161
VV	12	10	0.9697	0.01139
CM	31	23	0.9742	0.03480
Total	50	33	0.9633	0.03145

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538 **Table A1. DNA Sequences used in this study for phylogenetic analyses. Strain**
539 **assignment, origin, date of collection, and accession number in GenBank.**

Strain	Morpho-species designation	Origin	Sample Date	Accession number
CM	*"A. fundyense"*	Canal Puyuhuapi, Aysén, Chile	2012	KF924270 – KF924271
CM	*"A. fundyense"*	Canal Puyuhuapi, Aysén, Chile	2010	KC544998 - KC545026
PFB36	*"A. catenella"*	Isla San Pedro, Los Lagos, Chile	2009	HM641244
PFB37	*"A. catenella"*	Isla Quemada, Aysén, Chile	2009	HM641243
PFB41	*"A. catenella"*	Isla Luz, Aysén, Chile	2009	HM641242
PFB38	*"A. catenella"*	Isla Cailin, Los Lagos, Chile	2009	HM641245
PFB39	*"A. catenella"*	Bahía Asasao, Los Lagos, Chile	2009	HM641240
PFB42	*"A. catenella"*	Isla Jorge, Aysén,Chile	2009	HM641241
PFB45	*"A. catenella"*	Bahía Aysén, Aysén, Chile	2009	HM641246
PE02	*"A. catenella"*	Puerto Edén, Magallanes, Chile	2009	HQ997913
CB01	*"A. catenella"*	Canal Beagle, Magallanes, Chile	2009	HQ997911
PE01	*"A. catenella"*	Puerto Edén, Magallanes, Chile	2009	HQ997910
CB02	*"A. catenella"*	Bahía Low, Aysén, Chile	2009	HQ997919
IO01	*"A. catenella"*	Canal Beagle, Magallanes, Chile	2009	HQ997915
Q09	*"A. catenella"*	Quellón, Los Lagos, Chile	2009	HQ997916
Q10	*"A. catenella"*	Quellón, Los Lagos, Chile	2009	HQ997918
IO02	*"A. catenella"*	Bahía Low, Aysén, Chile	2009	HQ997914
Q05	*"A. catenella"*	Quellón, Los Lagos, Chile	2004	HQ997912
SD01	*"A. catenella"*	Santo Domingo, Aysén, Chile	2004	HQ997921
Q07	*"A. catenella"*	Quellón, Los Lagos, Chile	2004	HQ997920
ACC07	*"A. catenella"*	Canal Costa, Aysén, Chile	1994	HQ997917
CCMP	*A. tamarense* complex group I	Gulf of Maine , USA	2012	KF646464- KF646487
CCMP	*A. tamarense* complex group I	Sequim Bay,WA, USA	2012	KF646488-KF646501
ACC01	*A. tamarense* complex group I	Canal Costa, Aysén, Chile	1994	KF646308-KF646309
ACQH01	*A. tamarense* complex group I	Qt. Msr. Harbor, WA,USA	2002	KF646361

AL10	*A. tamarense* complex group I	Monterey Bay, CA, USA	-	KF646366
AL52	*A. tamarense* complex group I	Pacifica Pier, CA, USA	-	KF646374
SZNB21	*A. tamarense* complex group IIA	Gulf of Naples, Italy	-	KF646509
SZNB8	*A. tamarense* complex group IIA	Gulf of Naples, Italy	-	KF646510
CCAP1119-20	*A. tamarense* complex group III	Loch Maddy, England	-	KF646461- KF646462
CCAP1119-29	*A. tamarense* complex group III	Loch Maddy, England	-	KF646463
ATIR3	*A. tamarense* complex group III	Cork Harbor, Ireland	-	KF646424
ATSW01-1	*A. tamarense* complex group III	Essvik, Sweden	-	KF646458
SP3B8-3	*A. tamarense* complex group III	La Coruna Bay, Spain	-	KF646507
ACHK-NT	*A. tamarense* complex group IV	Hong Kong, China	-	KF646334
OF878-C5	*A. tamarense* complex group IV	Ofunato Bay, Japan	-	KF646505
ACHK	*A. tamarense* complex group IV	Hong Kong, China	-	KF646326
ACJP03	*A. tamarense* complex group IV	Kashima , Japan	-	KF646351
ATP	*A. tamarense* complex group IV	Antarctica	-	KF646440
ACDH01	*A. tamarense* complex group IV	Yangyz River, China	-	KF646331
ACPP01	*A. tamarense* complex group IV	Port Phillip Bay, Australia	-	KF646356
ATCIO1	*A. tamarense* complex group IV	South China Sea, China	-	KF646392
ATDH02	*A. tamarense* complex group IV	Yangyz River, China	-	KF646408
ATMJ01	*A. tamarense* complex group IV	Minjiang Estuary, China	-	KF646425
G. Crux	*A. tamarense* complex group IV	Singapore	-	KF646503
ATBB01	*A. tamarense* complex group V	Australia	-	KF908802
ATBB01	*A. tamarense* complex group V	Australia	-	KF908817
AM	*A. Minutum*	Taiwan, China	2011	JF906998

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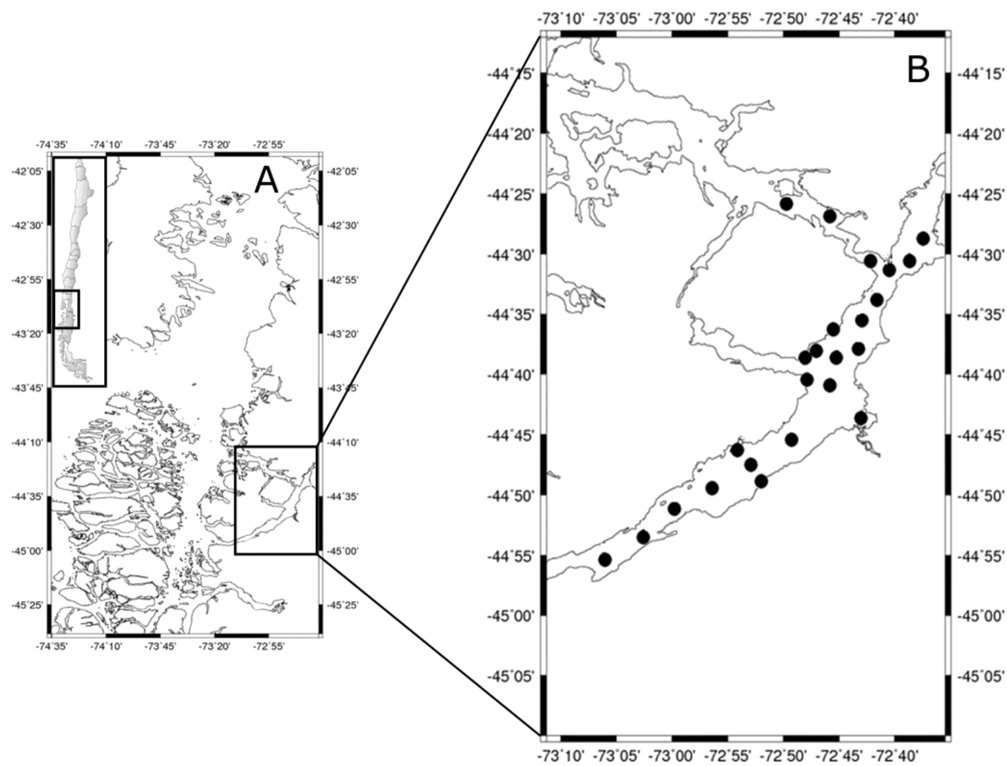

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553 **Figure 1.** (A) Study area in southern Chile. (B) Portion of Puyuhuapi Channel showing

554 sampled stations (filled circle) for molecular detection of *Alexandrium fundyense* (formerly

555 *A. catenella*).

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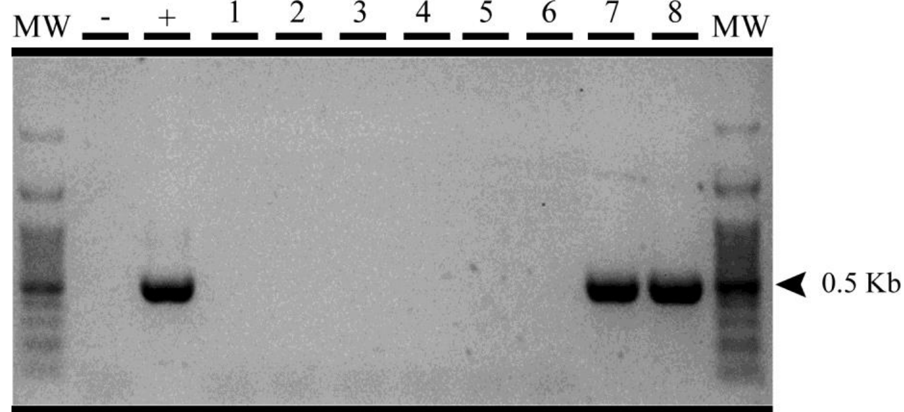

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565 **Figure 2.** Primer specificity test for the amplification of ribosomal DNA region of
566 *Alexandrium fundyense* (formerly *A. catenella*). **Lanes 1-5**: gDNA from vegetative cells of
567 *Alexandrium minutum*; **Lane 6**: gDNA from cyst of *Alexandrium minutum*; **Lane 7**: gDNA
568 from vegetative cells of *Alexandrium fundyense (*former *A. catenella)*; **Lane 8**: mix of
569 gDNA *Alexandrium minutum* and *Alexandrium fundyense* (formerly *A. catenella*); **-** :
570 negative control (PCR water); + : positive control (plasmid with *A. catenella* sequence)
571 **MW**: Molecular weight marker (100bp ladder).

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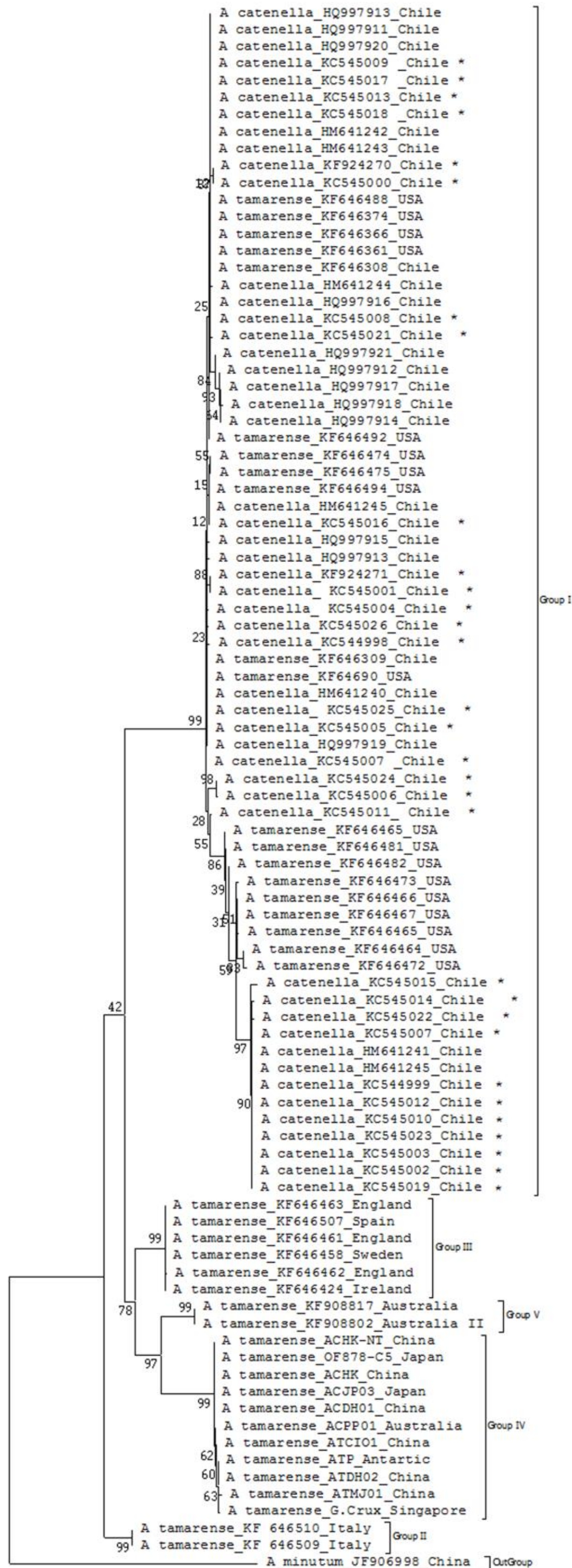

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574 **Figure 3.** Consensus maximum likelihood phylogenetic tree based on ITS sequences
575 (ITS1-5.8S-ITS2) of *Alexandrium fundyense* (formerly *A. catenella*) obtained in this study
576 (represented with *). Sequences of different *Alexandrium fundyense* (formerly *A. catenella*)
577 species/strains obtained in Chile, and sequences of different groups of the *Alexandrium*
578 *tamarense* complex, are also included. Total taxa n= XXX, with a final data set of XXXX
579 nucleotide positions. Numbers at the nodes represent bootstrap values (1000 replicates).
580 The tree was rooted using *Alexandrium minutum* strain AM as the outgroup
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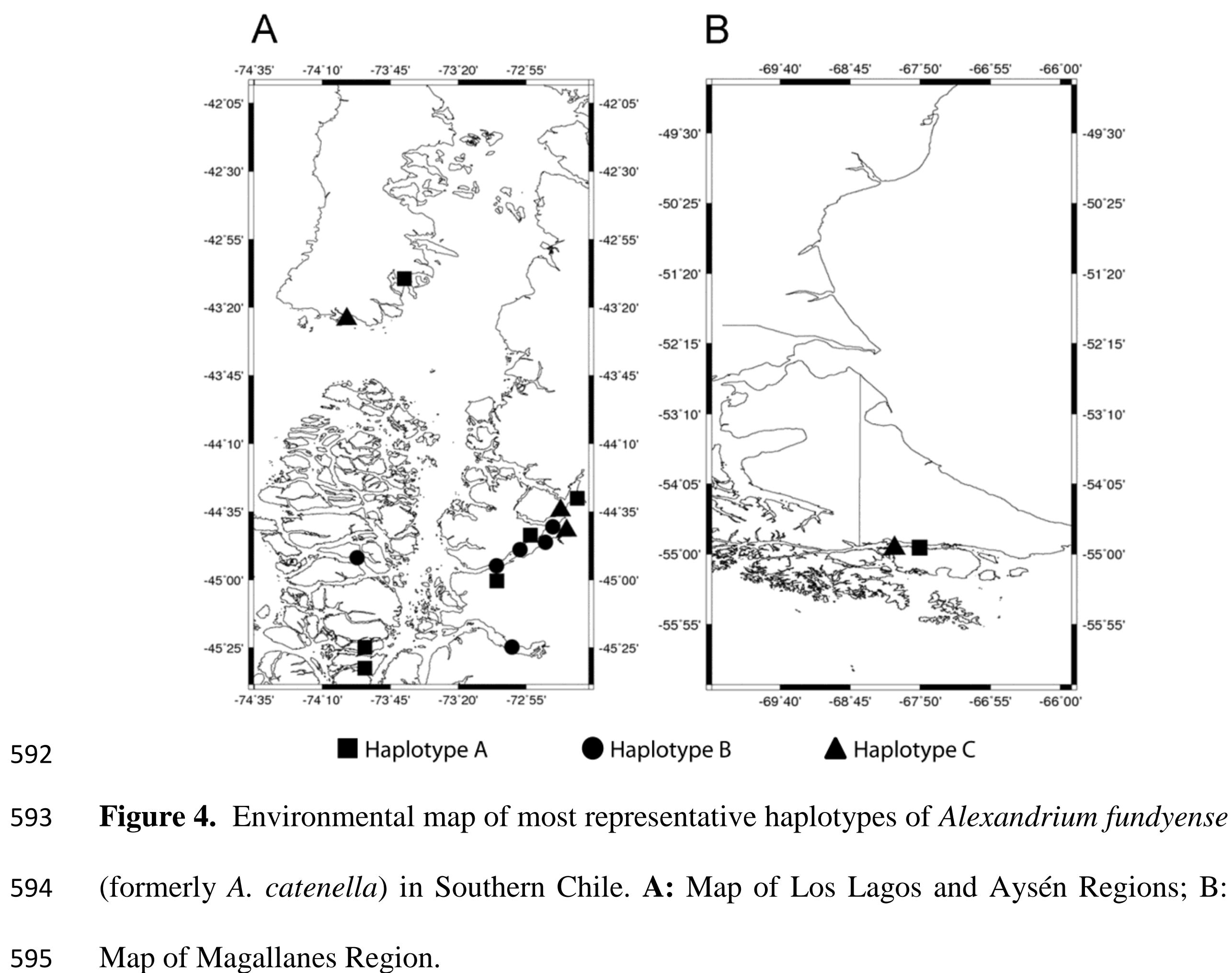

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593 **Figure 4.** Environmental map of most representative haplotypes of *Alexandrium fundyense*

594 (formerly *A. catenella*) in Southern Chile. **A:** Map of Los Lagos and Aysén Regions; B:

595 Map of Magallanes Region.