Deep genetic divergence between austral populations of the red alga *Gigartina skottsbergii* reveals a cryptic species endemic to the Antarctic continent

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Marie-Laure Guillemin

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1 Deep genetic divergence between austral populations of the red alga *Gigartina skottsbergii*
2 reveals a cryptic species endemic to the Antarctic continent.

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4 Emmanuelle Billard², Jeanette Reyes¹, Andres Mansilla³, Sylvain Faugeron^{4,5} & Marie-Laure
5 Guillemin^{1,5}.

6
7 ¹Instituto de Ciencias Ambientales y Evolutivas, Universidad Austral de Chile, Valdivia, Chile.

8 ²Univ Porto, CIBIO InBIO, Ctr Invest Biodiversidade & Recursos Genet, P-4485668 Vairao,
9 Portugal

10 ³Laboratorio de Macroalgas Antárticas y Subantárticas and Instituto de Ecología y
11 Biodiversidad (IEB), Universidad de Magallanes, Chile.

12 ⁴Centro de Conservación Marina and CeBiB, Facultad de Ciencias Biológicas, Pontificia
13 Universidad Católica de Chile, Santiago, Chile.

14 ⁵UMI 3614 Evolutionary Biology and Ecology of Algae, CNRS, Sorbonne Universités UPMC Univ.
15 Paris 06, Pontificia Universidad Católica de Chile, Universidad Austral de Chile

16
17 Email: marielaure.guillemin@gmail.com

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Abstract

The almost complete isolation of Antarctica after the intensification the Antarctic Circumpolar Current (ACC) during the middle-Miocene has been challenged by recent molecular data showing the existence of allelic exchange across the ACC. For organisms present on both sides of the ACC, two hypotheses have then been discussed to explain the origin of the Antarctic populations: 1) they correspond to recent immigrants from adjacent continents or 2) they have evolved in situ and have survived the dramatic effects of the last Quaternary glaciations in this region. The red algae *Gigartina skottsbergii* presents a disjoint distribution and is reported in both Antarctica and southern South America, a distribution pattern that largely exceeds its dispersal capacity. Mitochondrial sequences of the intergenic region *cox2-3* (n=233) and partial chloroplastic *RuBisCo* large subunit gene (n=26) sequences were obtained for individuals from the Chilean sub-Antarctic ecoregion and Antarctic Peninsula localities. The results strongly support the persistence of populations on each side of the Drake Passage during glacial periods and the existence of dispersal barrier due to the ACC. On both sides of the ACC, the last Quaternary glaciations have induced strong bottlenecks that were followed by rapid colonization events.

Keywords: Phylogeography, glacial refugia, seaweed, Antarctica, *rbcL*, *cox2-3*,

43 Introduction

44 The Southern Ocean is characterized by high levels of endemism of its fauna and flora
45 (Clarke and Crame 1989; Brandt et al 1999; Clarke and Johnston 2003; Wulff et al. 2009) that
46 has been related to the progressive isolation of the continent during the Mesozoic and the
47 reinforcement of the Antarctic Circumpolar Current (ACC) during the mid-Miocene (Lawver
48 and Gahagan 1998; Rogers et al. 2007; Poulin et al. 2014). Moreover, after the onset of
49 icehouse conditions in Antarctic, both the radiation of groups that have adapted to this
50 extreme environment and allopatric speciation driven by population fragmentation in
51 Antarctic refugia during glacial period seem to have contributed to the high Antarctic diversity
52 (Rogers et al. 2007; Thatje et al. 2008). Recent molecular data for several marine invertebrate
53 taxa, especially those with strong dispersal capabilities, have shown that divergence between
54 Antarctic and South American populations or sister species could be much more recent than
55 the physical separation of the continental landmasses and may rather have been driven by
56 more recent geographic and oceanographic changes like the evolution of the Scotia Arc and
57 the deepening of the Drake Passage (González-Wevar et al. 2012a; Poulin et al. 2014). The ACC
58 is generally considered to act as an impervious hydrographic barrier for most marine species
59 (Clarke et al. 2005; Thatje et al. 2005). Indeed, many studies have shown the absence of gene
60 flow between lineages across the ACC (Krabbe et al. 2010; Janosik et al. 2011; Stupnikova et al.
61 2013; Poulin et al. 2014; Weis et al. 2014). However, the permeability of this barrier has been
62 questioned by recent studies since low levels of exchanges across the ACC have been observed
63 for spider crabs (Clarke et al. 2005), ribbon worms (Mahon et al. 2010) and the sea star
64 *Odontaster meridionalis* (Janosik et al. 2011). These new evidences of the ability of species to
65 permeate the Polar Front have raised questions about the importance of historical land mass
66 connectivity versus more recent exchanges across the ACC in driving the distribution of the
67 Southern Ocean benthic biota (Thatje and Fuentes 2003; Tavares and De Melo 2004; Clarke et
68 al. 2005).

The persistence of high benthic marine diversity in the Antarctic continent is particularly puzzling when considering the major Quaternary climatic oscillations, which led to the formation of an ice-sheet reaching the limits of the continental plateau and likely eradicating life in shallow subtidal areas (Thatje et al. 2005). Many invertebrates are highly abundant and diverse along the Antarctic coasts (Clarke and Crame 1989; Clarke and Johnston 2003; Linse et al. 2006; Aronson et al. 2007; Rogers et al. 2007), suggesting that major climatic and oceanographic changes in the region did not impede their evolutionary success (Clarke and Crame 1989; Aronson et al. 2007). Several hypotheses have been proposed to explain the occurrence of such diversity despite major changes in habitat availability. The “deep-sea refugia” model proposes that species of the Antarctic shelf shifted their bathymetric range toward the deep sea during events of maximum ice cover, and later recolonized shelf areas following the deglaciation process (Kussakin 1973; Thatje et al. 2005; Allcock and Strugnell 2012). This hypothesis has been proposed for invertebrate species with wide eurybathic ranges, and has been confirmed by phylogeographic analyses (e.g. the crinoid *Promachocrinus kerguelensis*; Hemery et al. 2012). However, the model is not applicable to shallow benthic species, such as seaweeds and herbivores that feed on them, due to their dependence on light availability. Two alternative hypotheses, the “shelf in situ refugia” and the “island refugia” models, propose that some species might have survived in situ either because ice did not cover the entire shelf area at the same time, or alternatively because organisms sought refuge outside of the Antarctic continental shelf in more or less distantly surrounding islands (Thatje et al. 2005; Raupach et al. 2010; Diaz et al. 2012; González-Wevar et al. 2013).

In parallel, sub-Antarctic species have also experienced important changes in their respective distribution ranges due to Quaternary glacial cycles. During the Last Glacial Maximum (LGM), the southern tip of South America was covered by the Patagonian ice-sheet that extended approximately from Chiloé Island (42°S) to the Fuegian low lands (56°S) (Hulton et al. 2002), and this had various effects on species of southern Chile and Argentina

(Valdovinos et al. 2003; Aguirre et al. 2013). However, coastal ice-sheets were absent in the Cape Horn region and along the Scotia Arc (Hulton et al. 1994, 2002; Fraser et al. 2012); this likely offered glacial refugia for marine species. Contrasting postglacial recolonization pathways have been inferred from the genetic evidence of several Patagonian species (González-Wevar et al. 2012a). Similarly, several terrestrial species including amphibians, river fish, mammals and plants were restricted to glacial refugia or became locally extinct, whereas others persisted *in situ* (i.e. in the areas putatively covered by ice-sheets; Jakob et al. 2009; Vianna et al. 2011; Zemlak et al. 2011; Fraser et al. 2012). To date few studies have focused on marine Patagonian species and the existing results indicated diverse scenarios. These scenarios include potential post-glacial recolonization from distant Sub-Antarctic sources (e.g. *Durvillaea antarctica*, Fraser et al. 2010), from northern, unglaciated regions (e.g. *Mazzaella laminarioides*, Montecinos et al. 2012), or from local refugia in the southern sub-Antarctic region (Valdovinos et al. 2003). The potential occurrence of glacial refugia between Cape Horn and the South Sandwich archipelagos raises questions about the origin of both sub-Antarctic and Antarctic diversity.

With its present distribution embracing the southern coast (up to 40°S) of Chile and Argentina, sub Antarctic islands (Falkland Islands), Antarctic Islands (South Shetland, South Orkney Islands and South Georgia) and the Antarctic Peninsula, the red alga *Gigartina skottsbergii* is a suitable model to investigate the impact of major climatic changes on the subtidal flora in high southern latitudes. This species is highly patchy, with populations generally less than a square kilometer in size (Ramirez and Santelices 1991). It belongs to the order Gigartinales, which appears to have originated on Antarctic coasts when this continent was still attached to Australasia and South America (Hommersand et al. 1994). The northern limit of *G. skottsbergii*'s distribution is set by contrasting topological and oceanic characteristics including changes in the seawater surface temperature (i.e. the transition between cold waters to more temperate ones) (Ramirez and Santelices 1991). This

carraghenophyte alga is pseudo-perennial (Wiencke and Clayton 2002) and blades may reach up to 1–2m in diameter (Santelices 1988). *G. skottsbergii* is haploid-diploid and both phases of the isomorphic life cycle coexist in time and space (Piriz 1996; Avila et al. 1999; Westermeier et al. 1999). Propagation is achieved through sexual reproduction (Avila et al. 1999). Antarctic specimens are separated from South American plants by more than 2% *rbcL* base pair distance (Hommersand and Fredericq 2003), which is not uncommon among species within red algae (e.g. Gavio and Fredericq 2002). Furthermore, Antarctic and sub-Antarctic populations show physiological differences: while in Antarctica spores germinate at 0°C and juveniles grow only at temperatures below 5°C (Bischoff-Bäsmann and Wiencke 1996), spores from sub-Antarctic populations do not germinate at 0°C and can grow at up to 15°C (Buschmann et al. 1999) which might result from an adaptation to regional environmental conditions. These first genetic and physiological results suggest that there is some evolutionary divergence between *Gigartina* populations from Antarctica and South America. The objective of this study is to infer the evolutionary history of sub-Antarctic and Antarctic populations of the red alga *G. skottsbergii* (Setchell et Gardner) using mitochondrial and chloroplast markers Cox2-3 and *rbcL*. Two main processes were investigated: the divergence between Antarctic and South American populations, and the genetic consequences of last glacial cycle on both continents.

Materials and Methods.

Sampling- Samples were collected by autonomous diving in the shallow subtidal zones and includes a total of 233 individuals of *G. skottsbergii*. Samples were extracted from 18 localities covering most of the distribution range of the species (Chilean and Antarctic coasts; Table 1). In order to avoid sampling genetically identical ramets we sampled fronds from distinct holdfasts. Individuals were sampled from the lower littoral down to the depth of 25m.

Each individual tissue sample was cut from a clean healthy frond and placed into a plastic bag filled with silica beds for rapid dehydration and preservation of DNA.

DNA extraction, PCR amplification and sequencing- Dried algal tissue was finely grounded using liquid nitrogen and DNA was extracted using the phenol–chloroform method described in Faugeron et al. (2001). The Cox2-3, an intergenic non-coding mitochondrial region located between the genes for cytochrome oxidase subunit 2 (COX2) and 3 (COX3) was amplified following Zuccarello et al. (1999). In total, 233 sequences of approximately 350 bp were obtained. Additionally, we amplified a 971 bp region of the chloroplastic gene *rbcl*, encoding the large subunit of the ribulose-1,5-bisphosphate carboxylase/oxygenase (RUBISCO) enzyme, using the primers F-*rbcl* and R-*rbcl* (Hommersand et al. 1994) and the PCR conditions described by Fredericq and Lopez-Bautista (2002). Sequences were obtained for a sub-sample of 26 individuals (Table 1) for the *rbcl* gene. All PCR reactions were performed in a Gene Amp PCR system 9700 (Applied Biosystems, Foster City, USA). The amplified samples from each individual were purified with the UltraClean™ kit (MO BIO Laboratories, Carlsbad, USA) and sequenced in both directions by Macrogen Inc. (Seoul, South Korea). Sequences were edited using Chromas v. 2.33 (McCarthy 1997) and alignments were obtained using the CLUSTAL function of Mega v 5 (Tamura et al. 2011). Sequences were deposited in Genbank with accession numbers KM261841 to KM261858 for the Cox2-3 region and accession numbers KM261859 to KM261862 for the *rbcl* region. Alignments of the sequences used for the Cox2-3 region and the *rbcl* for phylogenetic reconstructions are available in Online Resources 1 and 2, respectively.

For the *rbcl* and the coding part of the Cox2-3 sequences obtained, the McDonald-Kreitman test (<http://mkt.uab.es>, Egea et al. 2008) was performed to detect selection. The neutrality index (NI) was calculated as follows: $NI = (P_n/P_s)/(D_n/D_s)$, where P is the polymorphism within the population, D is the divergence or fixed difference between populations, n is for non synonymous and s is for synonymous mutations.

Phylogenetic reconstructions and estimation of divergence time – Phylogenetic

reconstructions for each marker dataset were performed with the Maximum Likelihood (ML) method using TreeFinder v March 2011 (Jobb et al. 2004) and a Bayesian inference (BI) using MrBayes v 3.1.2 (Huelsenbeck and Ronquist 2001). Outgroup species belonging to the genus *Sarcothalia* and *Iridaea* were chosen since they represent the closest known sister-taxa of *Gigartina skottsbergii* in the phylogenetic systematics of the Gigartinaceae (Hommersand et al. 1999). Outgroup sequences considered for the *rbcl* consisted of four species of *Sarcothalia* (*S. crispata*, SCU03085; *S. stiriata*, SSU03089; *S. livida*, SLU03087 and *S. circumcincta*, AF146219) and two sequences of *Iridaea cordata* from Antarctica (U02989 and GQ323780). For the *cox2-3*, two sequences of *S. crispata* (KM275591 and KM275592, both from Punta Estaquilla) and one sequence of *I. cordata* (KM275593 from Punta Hanna, South Shetland Islands) were used as outgroup. We also included in the analyses available *rbcl* sequences for *G. skottsbergii* (U03432, Ancud, Chile and AF146206, King George Island, South Shetland Islands; Hommersand et al. 1999). For both phylogenetic reconstruction methods, large indels within the non-coding intergenic region of the *Cox2-3* were treated as single mutation events.

ML analyses were performed using a mixed model taking into account the position of codons for the *rbcl* gene while only one model was used for the *cox2-3* intergenic region analysis. TreeFinder v March 2011 (Jobb et al. 2004) allows to choose between 32 substitution models for each partition of the data set. The best-fitted substitution models were selected using the Akaike Information Criterion implemented in the ModelTest package of the TreeFinder program (Posada and Crandall 1998; Jobb et al. 2004). The selected models for the *rbcl* data were TN+G for the first codon position, HKY+G for the second codon position and J3+G for the third codon position. For *cox2-3* the selected model was HKY+G. Using TreeFinder v March 2011, we performed a heuristic search in order to reconstruct the trees and node supports were assessed by non-parametric bootstrapping (1000 pseudo-replicates, Felsenstein 1985).

Bayesian inference was performed using the general criteria of the best fit model parameters defined for each dataset. Four independent analyses were run with four chains each (3 heated chains and one “cold” chain) for ten million generations. The settings were a heating parameter value of 0.2 and sampling every 1000 generations with randomly generated starting trees. The first 25% of sampled trees were discarded as “burn-in” to ensure convergence. The remaining trees were used to compute a consensus topology and posterior probability values. The split frequency (variance between the four independent runs) was below 0.0005, confirming that the posterior probability distribution was accurately sampled. Because the posterior probability bootstrap values were essentially identical in the independent runs starting from different, random topologies, we considered that the chains had converged.

Even though the lack of fossils impedes a precise calibration of molecular clocks in red algae, we used divergence rates already published for this group to estimate the historical divergence event between South American and Antarctic populations. A divergence rate of 0.109-0.127% per site per million years (Myr) has been proposed for *rbcL* (Kamiya et al. 2004). For *cox2-3*, a site mutation rate of 0.756-0.426% per Myr, based on the divergence of the red algae *Asparagopsis* spp. associated to the Panama isthmus closure, was proposed by Andreakis et al. (2007). Divergence time was estimated in BEAST v1.8 (Drummond et al. 2012) using the Yule model of tree prior, a gamma site heterogeneity model to allow variation among sites of the mutation rate, and a Log-normal relaxed clock with a uniform sampling within the range of published mutation rates. Four runs of ten millions MCMC iterations each were performed and the combined results were analyzed with Tracer v1.8 (Drummond et al. 2012). Effective sample size of the posterior distribution, the parameter of accuracy of the parameter estimation, was always superior to 300 in each individual run and in the combined analyses, indicating the MCMC appropriately converged to estimated values.

Genetic diversity - For *cox2-3*, we calculated five diversity indices for each sampled location and for the two phylogenetic lineages (i.e. *G. skottsbergii* from Chile and the Falkland Islands and *G. skottsbergii* from sub-Antarctic and Antarctic) using Arlequin v 3.5 (Excoffier and Lisher 2010): the number of haplotypes (nH); the number of private haplotypes (i.e. haplotypes found in a single population, nHpriv); the number of polymorphic sites (S); gene diversity (Hd, Nei 1987) and nucleotide diversity (π , Nei and Li 1979). For *rbcl*, only nH was calculated.

Network reconstruction and historical demography - Haplotype networks were reconstructed for *cox2-3* using the median-joining algorithm implemented in NETWORK v 4.510 (Bandelt et al. 1999). Moreover, for this molecular marker, three complementary approaches were used to infer the historical demography of *G. skottsbergii* from Chile and the Falkland Islands.

First, Tajima's D (Tajima 1989) and Fu's Fs (Fu 1997) statistics were calculated to detect significant past changes in population size. Significant departure from mutation-drift equilibrium was tested by 1000 bootstrap replicates in Arlequin (Excoffier and Lisher 2010). Under the assumption of neutrality, negative values characterize populations in expansion while positive values, associated with the loss of rare haplotypes, are considered as a signature of recent bottlenecks (Tajima 1989, Fu 1997).

Second, the observed distributions of pairwise differences were compared to estimated values under a model of sudden pure demographic expansion (Roger and Harpending 1992) using Arlequin (Excoffier and Lisher 2010). The model fit between the observed and estimated mismatch distributions was calculated through a generalized least squares approach, which was then tested with 1000 permutations. The date of growth/decline ($\tau=2\mu t$), measured in units of $1/2 \mu$ generations where t =time in years and μ =mutation rate per sequence per generation, was estimated using the demographic expansion parameters as

determined in the nonlinear least squares approach implemented in Arlequin (Excoffier and Lisher 2010).

Third, population growth rate and timing was estimated from coalescent simulations implemented in LAMARC 2.1.9 (Kuhner 2006). The maximum likelihood approach was applied using the Metropolis-coupled Markov chain Monte Carlo (MCMC) method with replication of chains and adaptive heating to achieve optimal sampling of the parameter space. The MCMC runs were performed three times with random seeds; each run used 10 initial chains with 500 samples and two long final chains with 10 000 samples. All initial chains and final chains were simulated using a sampling interval of 20 and a burn-in of 1000 samples. A tenfold evolutionary rate (4.26-7.56% per million years) was considered at population level, following the correction for time dependence of molecular rate proposed by Ho et al. (2011).

Results.

Four chlorotypes were detected for the chloroplast marker *rbcL*, with 23 polymorphic sites along the 971 base pair fragments sequenced (11 sequenced individuals, Table 1). For the *cox2-3* mitochondrial marker, 18 mitotypes were observed (233 sequenced individuals, Table 1). For this marker, 48 polymorphic sites were observed including two indels: one indel of 1bp characteristic of the mitotype C13 and one indel of 12bp for mitotypes C8 and C17. Sequence length of *cox2-3* sequences varied from 337 to 350bp.

Figure 1 shows the Maximum Likelihood (ML) phylogenetic trees constructed using the two molecular markers. For both markers, tree topologies based on Bayesian and ML analyses were largely congruent and shared comparable support values for major nodes (Figure 1). Regardless of the marker used, tree topologies were broadly similar among phylogenetic reconstruction methods and clearly showed that all *G. skottsbergii* sequences obtained in this study form a monophyletic group, clearly split into two well supported lineages (Figure 1,

support values >89%) that are strongly divergent from the outgroup species. The first lineage is composed of all individuals from Chile and the Falkland Islands while the second is composed of individuals from the Antarctic Peninsula, the South Shetland Islands and the South Orkney Islands (Figure 1). The available *rbcL* sequence AF146206 in GenBank for *G. skottsbergii* previously sampled in King George Island in the South Shetland Islands (Hommersand et al. 1999) corresponds exactly to the chlorotype R4 present in our Antarctic lineage. The uncorrected p-distance, measured using only 665bp, between the *rbcL* sequence U03432 and the closest sequence in our data set (i.e. chlorotype R1, present only in Ancud, Chile, see Table 1) is of 0.3%. This is congruent with the location where the specimen corresponding to the U03432 sequence was collected, on Chiloé Island within the bay of Ancud (Hommersand et al. 1999). P distances between sequences from the Antarctic and South American *G. skottsbergii* lineages were of 9.2 ± 1.5 % for *cox2-3* and of 2.1 ± 0.5 % for *rbcL*. When performed between the Antarctic and South American *G. skottsbergii* lineages, no significant departure from neutrality was detected using the McDonald-Kreitman test for the *rbcL* and the coding part of the *Cox2-3* sequences analyzed (NI= 0.969, p = 0,980; NI= 2.191, p= 0.536, for the *rbcL* and the *Cox2-3*, respectively).

Divergence between the Antarctic and South American *G. skottsbergii* lineages was estimated at 9.4 Myr (95% CI: 3.2-16.4Myr) based on *cox2-3* data and at 14.9 Myr (95% CI: 2.6-35.9Myr) based on *rbcL* data.

For both markers, no genetic diversity was observed in the Antarctic lineage of *G. skottsbergii*. Only one haplotype was detected for the *cox2-3* region and for the *rbcL* gene, the mitotype C18 and the chlorotype R4, respectively (Table 1). The Antarctic lineage is spread over more than 1600 km of coast, from Marguerite Bay (67°S) to the South Orkney Islands (60°S) (Table 1, Figure 2C). Within the South American lineage of *G. skottsbergii* the genetic diversity in *cox2-3* was generally low with the number of mitotypes per sampling site (nH)

being lower than 3 and the gene diversity (H_d) as lower than 0.3 in 6 of the 11 studied populations (Table 1). The highest genetic diversity was observed in the population of PAG, BCH and FAL ($n_H = 5$ and $H_d > 0.5$ in all three populations, Table 1). These three populations were also the ones with the highest number of private haplotypes, with three private haplotypes in PAG and FAL and two in BCH (Table 1). For the *rbcl* gene, only three chlorotypes were observed within the South American lineage of *G. skottsbergii*. Only one chlorotype was found in each single population and the only private chlorotype (R1) was observed in the ANC population (Table 1). The chlorotype R3 was shared between BOR in Tierra del Fuego and FAL located in the Falkland Islands (Table 1).

The *cox2-3* mitotype network revealed the presence of two main haplogroups, one for South America and one for Antarctica, which are separated by 31 bp (Figure 2A), a result fully congruent with the phylogenetic reconstructions (Figure 1). Within the South American haplogroup, characterized by a typical star-like topology, pairs of mitotypes were separated by 1 to 5 bp, except for the mitotypes C17 and C8 that were differentiated by a unique 12bp indel. This haplogroup is also characterized by one frequent and widespread mitotype (C1, 81% of the samples, Table 1) and several less frequent mitotypes (Figure 2A). Low frequency mitotypes were predominantly restricted to a single or few nearby local populations. For example, the mitotype C3 was observed in BLO and PAG, these being two populations located in the Moraleda Channel. Additionally, mitotype C7 was observed in BCH, BOR and TOR which are located in the southern part of the Magellanic region and Tierra del Fuego (Figure 2B). One mitotype, C13, was also shared between BCH in the southern part of the Magellanic region and FAL located in the Falkland Islands (Figure 2B). As expected, the star-like topology is coupled with a unimodal mismatch distribution (Figure 3A), and the values of Tajima's D and Fu's F_s statistics were both negative and significant ($D = -2.01$, $p = 0.001$; $F_s = -1.26$, $p = 0.0001$) a result congruent with a sudden demographic population expansion model. Population size changes depicted from the mismatch distribution was $\tau = 0.73$ (90% confidence interval of 0.00

to 2.23). Assuming the start of exponential demographic expansion when population size was 1% of present-day's estimate, it was estimated to initiate 20 000 – 36000 years before present. A large positive exponential growth rate, $g = 3004$ (2458-4666 95% confidence interval), was also detected. It is possible that demographic expansion led to a large increase in N_e (approximately 170 000 to 300 000-fold increase, Fig. 3B), which may have started during or just before the LGM, depending on the combination of growth and mutation rates considered within their respective confidence limits.

Discussion

The patterns of genetic structure for *G. skottsbergii* seem to confirm the absence of gene flow between Antarctic and South American populations. Regardless of the marker analyzed, phylogenetic reconstructions using mtDNA and cpDNA sequences showed strong congruence and clear support for two distinct lineages consisting of populations from South America and the Falklands on the one hand and populations from the Antarctic Peninsula, Shetlands and Orkney Islands on the other hand. The absence of shared haplotypes between the two regions may reflect the isolation created by the Antarctic Circumpolar Current between the Antarctic Peninsula and the South American continent. Divergences among lineages of *G. skottsbergii* for the Cox2-3 (9.2 ± 1.5 %) and for the *rbcL* (2.1 ± 0.5 %) are within the range reported for interspecific distances between sister species in Rhodophyta (ranging from 2.55% to 4.70% and from 0.77% to 5.08% for the Cox2-3 and the *rbcL*, respectively; McIvor et al. 2001; Destombe et al. 2010; Hernández-Kantún et al. 2014). The large range estimated, of 2.6 to 35.9 My depending on the genetic marker and the mutation rate considered (with central tendency around 9 to 15My), for divergence time between the two lineages seems to predate Pleistocene glaciations. This time of divergence is however more recent than the separation of the Antarctic continent from the South American continent (approximately 24-40 Myr ago),

and seems to include the period of intensification of the ACC circulation 11-12 Myr ago (Dalziel et al. 2013). These climatic and oceanographic changes have been shown to be major drivers in the isolation of marine Antarctic fauna. For example, González-Wevar et al. (2010) have shown that the diversification within a genus of mollusk (*Nacella*) took place long after the separation of the continents. For these marine mollusks, the appearance of the most genetically distant clades (Kerguelen, Antarctic, and South America) took place between 9 and 5 Myr. These results were further supported by a recent study of comparative phylogeography of different invertebrate taxa showing a shared Antarctic and South American distribution (González-Wevar et al. 2012a; Poulin et al. 2014). In these studies, divergence times between Antarctic and South American lineages ranged from 1.0 to 13.6 Myr, largely overlapping the estimates for *G. skottsbergii*. Interestingly, divergence estimates in the mitochondrial markers for *G. skottsbergii* (9.2%) fall within the 7-11% range for shallow subtidal and intertidal invertebrates (González-Wevar et al. 2010, 2012a; Poulin et al. 2014), corroborating the occurrence of a shared evolutionary history despite imprecisions in the respective mutation rates. These authors proposed that the connection between Antarctic and South American populations could have been maintained by a stepping stone process along the archipelago of the Scotia Arc. Indeed, geological evidence has recently been reported of a now-submerged volcanic arc in the Central Scotia Sea that existed during the early Miocene (Dalziel et al. 2013). This archipelago was located closer to the Antarctic Peninsula and the Cape Horn region than the South Sandwich Islands are today, and may thus have provided a corridor for genetic connectivity across the Drake Passage until approximately 10-11 Myr ago (Poulin et al. 2014). Our study further shows that *G. skottsbergii*, a seaweed characterized by a very restricted dispersal capacity of its spores, may also have maintained a certain level of connectivity (even after the mid-Miocene) between the continents of Antarctica and South America through the volcanic arc of islands linking both sides of the Scotia Sea. This connection could have been

maintained until the beginning of Pliocene's glaciations, as in the case of brooding invertebrates, through rafting of adults (Nikula et al. 2010; Haye et al. 2012; Poulin et al. 2014).

The Antarctic and South American lineages of *G. skottsbergii* exhibit different patterns of genetic diversity. While a strong demographic expansion was inferred in the sub-Antarctic region, a total absence of genetic diversity was observed in the Antarctic lineage. The presence of a single haplotype over more than 1600 km from Marguerite Bay to the South Orkney Islands is intriguing. Even if this lack of diversity limits our capacity to test for different demographic scenarios in Antarctica, it suggests that a very strong demographic bottleneck occurred during glacial contraction, followed by a sudden and recent recolonization process that did not allow for new mutations. Similarly, the overall low genetic diversity and the presence of the same common haplotype C1 in every population from the Chilean coast to the Falkland Islands seems to support the hypothesis of persistence in a single glacial refugium followed by a massive demographic expansion over 2500 km. Such a pattern of genetic homogeneity over a broad geographical range has usually been related to high dispersal potential (Bortolotto et al. 2011; González-Wevar et al. 2012b). The scenario of rapid recolonization leading to the presence of only one haplotype over thousands of kilometers of coast is, however, difficult to envision for *G. skottsbergii*. Indeed, this alga is a non-buoyant species, and spore dispersal is considered to be very limited (Ramirez and Santelices 1991). Nevertheless, signatures of long distance dispersal have been observed in other apparently non-dispersive algal species like *Adenocystis utricularis* or *Bostrychia intricata* and have been explained by the organisms' potential ability to raft on floating substrates (Fraser et al. 2010, 2013). In contrast with our results, in a previous study using RAPDs nuclear markers (Faugeron et al. 2004), significant genetic differentiation among South American populations was observed and has been related to the poor dispersal capacity of for *G. skottsbergii*. Differences in mutation rates and/or level of drift effects (effective population size of uniparentally inherited loci is only one-fourth that of nuclear loci) may account for the differences between

level of genetic structure obtained with RAPDs (Faugeron et al. 2004) and cytoplasmic sequences (our study). Also, during spatial expansion, gene surfing effects may contribute to the reduction of diversity in cytoplasmic markers in the recolonized region (Excoffier and Ray 2008). Indeed, large-scale spread of mitochondrial genetic variants has been observed during recolonization process in seaweeds (Fraser et al. 2010). The ACC is a strong west to east current that connects the Antarctic Peninsula and the South Shetland Islands to the Antarctic Islands located in the South Scotia Ridge. Particle movement modeling and particle tracking has shown that passive drifters travel northeastwards across the Scotia Sea, connecting the Antarctic Peninsula and the South Shetland Islands to South Orkney Islands and South Georgia (Thorpe et al. 2004). Movement of drifting seaweed along the ACC could be connected to west-east spatial expansion pattern. On the other hand, the postglacial colonization of the Peninsula could have been promoted by stepwise spatial expansion through spore propagation. Such spatial expansion could have been facilitated by strong coastal currents, as the Antarctic Peninsula Coastal Current (APCC; Moffat et al. 2008), a southward current that forms during the ice-free seasons and extends to Alexander Island. However, we lack knowledge on the actual mechanisms of dispersal in *G. skottbergii* to appropriately determine the role of dispersal during post-glacial population dynamics on the present day genetic diversity.

The location of both Antarctic and South American glacial refugia is difficult to infer from the results presented here. The extreme genetic homogeneity within the *G. skottsbergii* Antarctic clade is in disagreement with the hypothesis that genetic structure and global genetic diversity in Antarctica may have been promoted by the fragmentation and isolation of micro-refugia where marine species were able to survive during repeated ice advances and retreats (Thatje et al. 2005; Allcock and Strugnell 2012). The possible presence of micro-refugia has been particularly highlighted in the archipelagoes located near the West Antarctic Peninsula and the South Shetland Island (See Fraser et al. 2012 for review). Indeed, new studies using molecular

data have shown that the South Shetland Islands is the most speciose region for endemic Southern Ocean octopuses of the genus *Pareledone* (Allcock et al. 2011). Even though the data herein lack the explicit molecular diversity to test adequately for the number and location of glacial refugees in this region, the survival of *G. skottsbergii* during the LGM seems more likely in the northernmost latitude (South Shetland and South Orkney Islands). Indeed, whereas *G. skottsbergii* is found in dense beds within these northern archipelagos, less than ten specimens could be collected in Paradise Bay and only one specimen was sampled in Marguerite Bay (Note that the same sampling effort was applied in Paradise Bay, Marguerite Bay, O'Higgings and Punta Prat; Table 1; M-L Guillemin, personal communication). It is understood that an increased sampling effort could better elucidate the southern limits of *G. skottsbergii*, but it seems that in Marguerite Bay the alga has reached a biotic or abiotic limit to its distribution.

The absence of genetic structure for the mitochondrial and chloroplast markers in southern South America also limits our ability to infer the number and location of glacial refugia. Because of their longstanding demographic stability, populations from glacial refugia are expected to present higher levels of genetic diversity than those populations that have formed following postglacial expansions (Provan and Bennett 2008). In addition, long-term isolation in distant refugia often leads to genetic differentiation due to mutation accumulation and genetic drift (Hewitt 2000). Along the Chilean coast, recolonization pathways have differed between organisms in terms of the number and origin of sources. A recent recolonization from a single distant/exterior source has been proposed to explain the high genetic homogeneity of populations of the red alga *Mazaella laminarioides* (Montecinos et al. 2012) and the brown alga *Durvillaea antarctica* that exist south of 42°S (Fraser et al. 2010). In *M. laminarioides*, a single refugium has been proposed, located in the North of the Island of Chiloe and the mainland coast north of the 41°S (Montecinos et al. 2012). In *D. antarctica*, broad sampling across the southern hemisphere has shown that all individuals present off the Patagonian coast of Chile share the same haplotype (Fraser et al. 2010) and the authors have indicated

that this species likely recolonized the region from a refugium in New Zealand sub-Antarctic archipelagoes. Yet others have highlighted the role that the Southern Fjords and Channels Region played as a refuge for several animal species, like marine mollusks (Valdovinos et al. 2003; González-Wevar et al. 2010) and the river otter (Vianna et al. 2011). The existence of multiple refugia, scattered among the Southern Fjords and Channels, has been proposed to explain the high levels of endemism and the high diversity of mollusks in this region (Valdovinos et al. 2003). In *G. skottsbergii*, the overall low genetic diversity and the presence of the same common haplotype, C1, in every population from the Chilean coast to the Drake Passage and the Falkland Islands seem to support the hypothesis of a single glacial refugium. Strong currents (i.e. the Cape Horn Current and the Malvinas-Falkland Current) connect the southern coast of Chile from 42°S to Cape Horn and the Falkland Islands. These currents have been shown to provide high connectivity among all inhabiting regions for the genus *Nacella* (González-Wevar et al. 2012b). In this genus, the existence of asymmetric gene flow, from West to East, was shown to be related to the prevailing circulation patterns in this region (González-Wevar et al. 2012b). Due to these strong directional currents, the hypothesis of a postglacial recolonization of the Chilean coast by *G. skottsbergii* from a refuge located in the Falkland Islands is most unlikely. Again, more polymorphic markers should help identifying regions with higher / lower genetic diversity as putative locations of glacial refugia / recolonized areas, respectively, for *G. skottsbergii*.

Conclusions

In accordance with previous results of Hommersand et al. (2009) the data presented here clearly show two divergent and respectively monophyletic clades of *G. skottsbergii* that may correspond to two cryptic species. *G. skottsbergii* (Type locality: Slogget Bay, Fuegia - Silva 1996; <http://www.algaebase.org>) is distributed in the Southern and sub-Antarctic coast of

Chile and the Falkland Islands. The Antarctic species, still to be formally described and named, occurs in the Antarctic Peninsula, the South Shetland Islands and the South Orkney Islands. Despite drastic reductions in population size, as revealed by strong signals of bottlenecks, both species persisted and maintained their separation on either side of the ACC even during the glacial period. The divergence time between the two cryptic species, estimated as 9.4 to 14.9 Myr (comparable with other invertebrate species, but with a large confidence interval of 2.6-35.9Myr), indicates that algae with limited dispersal capabilities were able to cross the Scotia Sea after separation of the continents, potentially via a stepping stone process through the volcanic arc of islands. Our work also sheds light on the possible importance of dispersal by rafting in the recolonization process of a species for which natural propagation is mainly achieved via sexual reproduction (Avila et al. 1999; Westermeier et al. 2012). Future prospects on glacial refugia for Antarctic and sub-Antarctic seaweeds shall also contribute to better understand the dispersal mechanisms during demographic expansion in these regions characterized by complex topography of coastal shelf and coastal currents.

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Figure 1: Maximum likelihood rooted trees for the *cox2-3* and the *rbcl* haplotypes of *Gigartina skottsbergii*. Maximum likelihood bootstrap values are indicated above each node and Bayesian posterior probabilities are noted between brackets. Only high support values are shown (>75 for bootstraps and >0.95 for posterior values, respectively; - correspond to branches not observed in the Bayesian inference reconstruction).

Figure 2: Haplotype networks of *Gigartina skottsbergii* and their geographic distribution based on *cox2-3*. The haplotype networks are presented in (A) while the pie charts showing the geographical distribution of haplotypes of the South American haplogroup are shown in (B) and the pie charts showing the geographical distribution of haplotypes of the Antarctic haplogroup are shown in (C). In the networks, each circle represents a haplotype and its size is proportional to the frequency in which the haplotype was encountered (correspondence between circle sizes and numbers of individuals is indicated in the box A). The black square in-between C7, C9 and C14 represents a hypothetical un-sampled haplotype. For haplotypes separated by more than one mutational step, the number of steps is indicated in bp. Pie charts' color-code corresponds to the one used in haplotype networks. Abbreviations for population codes are as in Table 1. Numbers of sequenced individuals are given between brackets.

Figure 3: Mismatch distributions (A) and population growth rate estimates (B) of the South American haplogroup of *Gigartina skottsbergii* for the *cox2-3* marker. Growth rate and timing of population dynamic changes were estimated from coalescent simulations implemented in LAMARC 2.1.9 (Kuhner 2006). The observed distribution of the number of pair base differences between sequences is indicated by the grey bars while the expected distribution under a model of sudden demographic expansion is represented by a black line. Effective population size (i.e. N_e) fluctuations throughout time are represented in the LAMARC graph. The dotted lines represent the 95% confidence intervals of the estimated growth rate, whereas the grey

533 shaded area corresponds to the average growth rate combined with the range of mutation
534 rates proposed by Andreakis et al (2007) corrected by a tenfold evolutionary rate as suggested
535 at population level for time dependence of molecular rate by Ho et al. (2011).

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1 Deep genetic divergence between austral populations of the red alga *Gigartina skottsbergii*
2 reveals a cryptic species endemic to the Antarctic continent.

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4 Emmanuelle Billard², Jeanette Reyes¹, Andres Mansilla³, Sylvain Faugeron^{4,5} & Marie-Laure
5 Guillemin^{1,5}.

6
7 ¹Instituto de Ciencias Ambientales y Evolutivas, Universidad Austral de Chile, Valdivia, Chile.
8 ²Univ Porto, CIBIO InBIO, Ctr Invest Biodiversidade & Recursos Genet, P-4485668 Vairao,
9 Portugal

10 ³Laboratorio de Macroalgas Antárticas y Subantárticas and Instituto de Ecología y
11 Biodiversidad (IEB), Universidad de Magallanes, Chile.

12 ⁴Centro de Conservación Marina and CeBiB, Facultad de Ciencias Biológicas, Pontificia
13 Universidad Católica de Chile, Santiago, Chile.

14 ⁵UMI 3614 Evolutionary Biology and Ecology of Algae, CNRS, Sorbonne Universités UPMC Univ.
15 Paris 06, Pontificia Universidad Católica de Chile, Universidad Austral de Chile

16
17 Email: marielaure.guillemin@gmail.com

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Abstract

The almost complete isolation of Antarctica after the intensification the Antarctic Circumpolar Current (ACC) during the middle-Miocene has been challenged by recent molecular data showing the existence of [allelic](#) exchange across the ACC. For organisms present on both sides of the ACC, two hypotheses have then been discussed to explain the origin of the Antarctic populations: 1) they correspond to recent [immigrants](#) from [adjacent](#) continents or 2) they have evolved in situ and have survived the dramatic effects of the last Quaternary glaciations in this region. The red algae *Gigartina skottsbergii* presents a disjoint distribution and is reported in both Antarctica and southern South America, a distribution pattern that largely exceeds its dispersal capacity. Mitochondrial sequences of the intergenic region cox2-3 (n=233) and partial chloroplastic RuBisCo large subunit gene (n=26) sequences were obtained for individuals from the Chilean sub-Antarctic ecoregion and Antarctic Peninsula localities. The results strongly support the persistence of populations on each side of the Drake Passage during glacial periods and the existence of dispersal barrier due to the ACC. On both sides of the ACC, the last Quaternary glaciations have induced strong bottlenecks that were followed by rapid colonization events.

Keywords: Phylogeography, glacial refugia, seaweed, Antarctica, *rbcL*, cox2-3,

43 Introduction

44 The Southern Ocean is characterized by high levels of endemism of its fauna and flora
45 (Clarke and Crame 1989; Brandt et al 1999; Clarke and Johnston 2003; Wulff et al. 2009) that
46 has been related to the progressive isolation of the continent during the Mesozoic and the
47 reinforcement of the Antarctic Circumpolar Current (ACC) during the mid-Miocene (Lawver
48 and Gahagan 1998; Rogers et al. 2007; Poulin et al. 2014). Moreover, after the onset of
49 icehouse conditions in Antarctic, both the radiation of groups that have adapted to this
50 extreme environment and allopatric speciation driven by population fragmentation in
51 Antarctic refugia during glacial period seem to have contributed to the high Antarctic diversity
52 (Rogers et al. 2007; Thatje et al. 2008). Recent molecular data for several marine invertebrate
53 taxa, especially those with [strong dispersal](#) capabilities, have shown that divergence between
54 Antarctic and South American populations or sister species could be much more recent than
55 the physical separation of the continental landmasses and may rather have been driven by
56 more recent geographic and oceanographic changes like the evolution of the Scotia Arc and
57 the deepening of the Drake Passage (González-Wevar et al. 2012a; Poulin et al. 2014). The ACC
58 is generally considered to act as an impervious hydrographic barrier for most marine species
59 (Clarke et al. 2005; Thatje et al. 2005). Indeed, many studies have shown the absence of gene
60 flow between lineages across the ACC (Krabbe et al. 2010; Janosik et al. 2011; Stupnikova et al.
61 2013; Poulin et al. 2014; Weis et al. 2014). However, the permeability of this barrier has been
62 questioned by recent studies since low levels of exchanges across the ACC have been observed
63 for spider crabs (Clarke et al. 2005), ribbon worms (Mahon et al. 2010) and the sea star
64 *Odontaster meridionalis* (Janosik et al. 2011). These new evidences of the ability of species to
65 permeate the Polar Front have raised [d](#) questions about the importance of historical land mass
66 connectivity versus more recent exchanges across the ACC in driving the distribution of the
67 Southern Ocean benthic biota (Thatje and Fuentes 2003; Tavares and De Melo 2004; Clarke et
68 al. 2005).

The persistence of high benthic marine diversity in the Antarctic continent is particularly puzzling when considering the major Quaternary climatic oscillations, which led to the formation of an ice-sheet reaching the limits of the continental plateau and likely eradicating life in shallow subtidal areas (Thatje et al. 2005). Many invertebrates are highly abundant and diverse along the Antarctic coasts (Clarke and Crame 1989; Clarke and Johnston 2003; Linse et al. 2006; Aronson et al. 2007; Rogers et al. 2007), suggesting that major climatic and oceanographic changes in the region did not impede their evolutionary success (Clarke and Crame 1989; Aronson et al. 2007). Several hypotheses have been proposed to explain the occurrence of such diversity despite major changes in habitat availability. The “deep-sea refugia” model proposes that species of the Antarctic shelf shifted their bathymetric range toward the deep sea during events of maximum ice cover, and later recolonized shelf areas following the deglaciation process (Kussakin 1973; Thatje et al. 2005; Allcock and Strugnell 2012). This hypothesis has been proposed for invertebrate species with wide eurybathic ranges, and has been confirmed by phylogeographic analyses (e.g. the crinoid *Promachocrinus kerguelensis*; Hemery et al. 2012). However, the model is not applicable to shallow benthic species, such as seaweeds and herbivores that feed on them, due to their dependence on light availability. Two alternative hypotheses, the “shelf in situ refugia” and the “island refugia” models, propose that some species might have survived in situ either because ice did not cover the entire shelf area at the same time, or alternatively because organisms sought refuge outside of the Antarctic continental shelf in more or less distantly surrounding islands (Thatje et al. 2005; Raupach et al. 2010; Diaz et al. 2012; González-Wevar et al. 2013).

In parallel, sub-Antarctic species have also experienced important changes in their respective distribution ranges due to Quaternary glacial cycles. During the Last Glacial Maximum (LGM), the southern tip of South America was covered by the Patagonian ice-sheet that extended approximately from Chiloé Island (42°S) to the Fuegian low lands (56°S) (Hulton et al. 2002), and this had various effects on species of southern Chile and Argentina

(Valdovinos et al. 2003; Aguirre et al. 2013). However, coastal ice-sheets were absent in the Cape Horn region and along the Scotia Arc (Hulton et al. 1994, 2002; Fraser et al. 2012); this likely offered glacial refugia for marine species. Contrasting postglacial recolonization pathways have been inferred from the genetic evidence of several Patagonian species (González-Wevar et al. 2012a). Similarly, several terrestrial species including amphibians, river fish, mammals and plants were restricted to glacial refugia or became locally extinct, whereas others persisted *in situ* (i.e. in the areas putatively covered by ice-sheets; Jakob et al. 2009; Vianna et al. 2011; Zemlak et al. 2011; Fraser et al. 2012). To date few studies have focused on marine Patagonian species and the existing results indicated diverse scenarios. These scenarios include potential post-glacial recolonization from distant Sub-Antarctic sources (e.g. *Durvillaea antarctica*, Fraser et al. 2010), from northern, unglaciated regions (e.g. *Mazzaella laminarioides*, Montecinos et al. 2012), or from local refugia in the southern sub-Antarctic region (Valdovinos et al. 2003). The potential occurrence of glacial refugia between Cape Horn and the South Sandwich archipelagos raises questions about the origin of both sub-Antarctic and Antarctic diversity.

With its present distribution embracing the southern coast (up to 40°S) of Chile and Argentina, sub Antarctic islands (Falkland Islands), Antarctic Islands (South Shetland, South Orkney Islands and South Georgia) and the Antarctic Peninsula, the red alga *Gigartina skottsbergii* is a suitable model to investigate the impact of major climatic changes on the subtidal flora in high southern latitudes. This species is highly patchy, with populations generally less than a square kilometer in size (Ramirez and Santelices 1991). It belongs to the order Gigartinales, which appears to have originated on Antarctic coasts when this continent was still attached to Australasia and South America (Hommersand et al. 1994). The northern limit of *G. skottsbergii*'s distribution is set [by contrasting topological and oceanic characteristics including changes in the seawater surface temperature \(i.e. the transition between cold waters to more temperate ones\)](#) (Ramirez and Santelices 1991). This

carraghenophyte alga is pseudo-perennial (Wiencke and Clayton 2002) and blades may reach up to 1–2m in diameter (Santelices 1988). *G. skottsbergii* is haploid-diploid and both phases of the isomorphic life cycle coexist in time and space (Piriz 1996; Avila et al. 1999; Westermeier et al. 1999). Propagation is achieved through sexual reproduction (Avila et al. 1999). Antarctic specimens are separated from South American plants by more than 2% *rbcL* base pair distance (Hommersand and Fredericq 2003), which is not uncommon among species within red algae (e.g. Gavio and Fredericq 2002). Furthermore, Antarctic and sub-Antarctic populations show physiological differences: while in Antarctica spores germinate at 0°C and juveniles grow only at temperatures below 5°C (Bischoff-Bäsmann and Wiencke 1996), spores from sub-Antarctic populations do not germinate at 0°C and can grow at up to 15°C (Buschmann et al. 1999) which might result from an adaptation to regional environmental conditions. These first genetic and physiological results suggest that there is some evolutionary divergence between *Gigartina* populations from Antarctica and South America. The objective of this study is to infer the evolutionary history of sub-Antarctic and Antarctic populations of the red alga *G. skottsbergii* (Setchell et Gardner) using mitochondrial and chloroplast markers Cox2-3 and *rbcL*. Two main processes were investigated: the divergence between Antarctic and South American populations, and the genetic consequences of last glacial cycle on both continents.

Materials and Methods.

Sampling- Samples were collected by autonomous diving in the shallow subtidal zones and includes a total of 233 individuals of *G. skottsbergii*. Samples were extracted from 18 localities covering most of the distribution range of the species (Chilean and Antarctic coasts; Table 1). [In order to avoid sampling genetically identical ramets we sampled fronds from distinct holdfasts. Individuals were sampled from the lower littoral down to the depth of 25m.](#)

Each individual tissue sample was cut from a clean healthy frond and placed into a plastic bag filled with silica beds for rapid dehydration and preservation of DNA.

DNA extraction, PCR amplification and sequencing- Dried algal tissue was finely grounded using liquid nitrogen and DNA was extracted using the phenol–chloroform method described in Faugeron et al. (2001). The Cox2-3, an intergenic non-coding mitochondrial region located between the genes for cytochrome oxidase subunit 2 (COX2) and 3 (COX3) was amplified following Zuccarello et al. (1999). In total, 233 sequences of approximately 350 bp were obtained. Additionally, we amplified a 971 bp region of the chloroplastic gene *rbcl*, encoding the large subunit of the ribulose-1,5-bisphosphate carboxylase/oxygenase (RUBISCO) enzyme, using the primers F-*rbcl* and R-*rbcl* (Hommersand et al. 1994) and the PCR conditions described by Fredericq and Lopez-Bautista (2002). Sequences were obtained for a sub-sample of 26 individuals (Table 1) for the *rbcl* gene. All PCR reactions were performed in a Gene Amp PCR system 9700 (Applied Biosystems, Foster City, USA). The amplified samples from each individual were purified with the UltraClean™ kit (MO BIO Laboratories, Carlsbad, USA) and sequenced in both directions by Macrogen Inc. (Seoul, South Korea). Sequences were edited using Chromas v. 2.33 (McCarthy 1997) and alignments were obtained using the CLUSTAL function of Mega v 5 (Tamura et al. 2011). Sequences were deposited in Genbank with accession numbers KM261841 to KM261858 for the Cox2-3 region and accession numbers KM261859 to KM261862 for the *rbcl* region. Alignments of the sequences used for the Cox2-3 region and the *rbcl* for phylogenetic reconstructions are available in Online Resources 1 and 2, respectively.

For the *rbcl* and the coding part of the Cox2-3 sequences obtained, the McDonald-Kreitman test (<http://mkt.uab.es>, Egea et al. 2008) was performed to detect selection. The neutrality index (NI) was calculated as follows: $NI = (P_n/P_s)/(D_n/D_s)$, where P is the polymorphism within the population, D is the divergence or fixed difference between populations, n is for non synonymous and s is for synonymous mutations.

Phylogenetic reconstructions and estimation of divergence time – Phylogenetic

reconstructions for each marker dataset were performed with the Maximum Likelihood (ML) method using TreeFinder v March 2011 (Jobb et al. 2004) and a Bayesian inference (BI) using MrBayes v 3.1.2 (Huelsenbeck and Ronquist 2001). Outgroup species belonging to the genus *Sarcothalia* and *Iridaea* were chosen since they represent the closest known sister-taxa of *Gigartina skottsbergii* in the phylogenetic systematics of the Gigartinaceae (Hommersand et al. 1999). Outgroup sequences considered for the *rbcl* consisted of four species of *Sarcothalia* (*S. crispata*, SCU03085; *S. stiriata*, SSU03089; *S. livida*, SLU03087 and *S. circumcincta*, AF146219) and two sequences of *Iridaea cordata* from Antarctica (U02989 and GQ323780). For the *cox2-3*, two sequences of *S. crispata* (KM275591 and KM275592, both from Punta Estaquilla) and one sequence of *I. cordata* (KM275593 from Punta Hanna, South Shetland Islands) were used as outgroup. We also included in the analyses available *rbcl* sequences for *G. skottsbergii* (U03432, Ancud, Chile and AF146206, King George Island, South Shetland Islands; Hommersand et al. 1999). For both phylogenetic reconstruction methods, large indels within the non-coding intergenic region of the *Cox2-3* were treated as single mutation events.

ML analyses were performed using a mixed model taking into account the position of codons for the *rbcl* gene while only one model was used for the *cox2-3* intergenic region analysis. TreeFinder v March 2011 (Jobb et al. 2004) allows to choose between 32 substitution models for each partition of the data set. The best-fitted substitution models were selected using the Akaike Information Criterion implemented in the ModelTest package of the TreeFinder program (Posada and Crandall 1998; Jobb et al. 2004). The selected models for the *rbcl* data were TN+G for the first codon position, HKY+G for the second codon position and J3+G for the third codon position. For *cox2-3* the selected model was HKY+G. Using TreeFinder v March 2011, we performed a heuristic search in order to reconstruct the trees and node supports were assessed by non-parametric bootstrapping (1000 pseudo-replicates, Felsenstein 1985).

Bayesian inference was performed using the general criteria of the best fit model parameters defined for each dataset. Four independent analyses were run with four chains each (3 heated chains and one “cold” chain) for ten million generations. The settings were a heating parameter value of 0.2 and sampling every 1000 generations with randomly generated starting trees. The first 25% of sampled trees were discarded as “burn-in” to ensure convergence. The remaining trees were used to compute a consensus topology and posterior probability values. The split frequency (variance between the four independent runs) was below 0.0005, confirming that the posterior probability distribution was accurately sampled. Because the posterior probability bootstrap values were essentially identical in the independent runs starting from different, random topologies, we considered that the chains had converged.

Even though the lack of fossils impedes a precise calibration of molecular clocks in red algae, we used divergence rates already published for this group to estimate the historical divergence event between South American and Antarctic populations. A divergence rate of 0.109-0.127% per [site per](#) million years (Myr) has been proposed for *rbcL* (Kamiya et al. 2004). For *cox2-3*, a site mutation rate of 0.756-0.426% per Myr, based on the divergence of the red algae *Asparagopsis* spp. associated to the Panama isthmus closure, was proposed by Andreakis et al. (2007). Divergence time was estimated in BEAST v1.8 (Drummond et al. 2012) using the Yule model of tree prior, a gamma site heterogeneity model to allow variation among sites of the mutation rate, and a Log-normal relaxed clock with a uniform sampling within the range of published mutation rates. Four runs of ten millions MCMC iterations each were performed and the combined results were analyzed with Tracer v1.8 (Drummond et al. 2012). Effective sample size of the posterior distribution, the parameter of accuracy of the parameter estimation, was always superior to 300 in each individual run and in the combined analyses, indicating the MCMC appropriately converged to estimated values.

Genetic diversity - For *cox2-3*, we calculated five diversity indices for each sampled location and for the two phylogenetic lineages (i.e. *G. skottsbergii* from Chile and the Falkland Islands and *G. skottsbergii* from sub-Antarctic and Antarctic) using Arlequin v 3.5 (Excoffier and Lisher 2010): the number of haplotypes (nH); the number of private haplotypes (i.e. haplotypes found in a single population, nHpriv); the number of polymorphic sites (S); gene diversity (Hd, Nei 1987) and nucleotide diversity (π , Nei and Li 1979). For *rbcl*, only nH was calculated.

Network reconstruction and historical demography - Haplotype networks were reconstructed for *cox2-3* using the median-joining algorithm implemented in NETWORK v 4.510 (Bandelt et al. 1999). Moreover, for this molecular marker, three complementary approaches were used to infer the historical demography of *G. skottsbergii* from Chile and the Falkland Islands.

First, Tajima's D (Tajima 1989) and Fu's Fs (Fu 1997) statistics were calculated to detect significant past changes in population size. Significant departure from mutation-drift equilibrium was tested by 1000 bootstrap replicates in Arlequin (Excoffier and Lisher 2010). Under the assumption of neutrality, negative values characterize populations in expansion while positive values, associated with the loss of rare haplotypes, are considered as a signature of recent bottlenecks (Tajima 1989, Fu 1997).

Second, the observed distributions of pairwise differences were compared to estimated values under a model of sudden pure demographic expansion (Roger and Harpending 1992) using Arlequin (Excoffier and Lisher 2010). The model fit between the observed and estimated mismatch distributions was calculated through a generalized least squares approach, which was then tested with 1000 permutations. The date of growth/decline ($\tau=2\mu t$), measured in units of $1/2 \mu$ generations where t =time in years and μ =mutation rate per sequence per generation, was estimated using the demographic expansion parameters as

determined in the nonlinear least squares approach implemented in Arlequin (Excoffier and Lisher 2010).

Third, population growth rate and timing was estimated from coalescent simulations implemented in LAMARC 2.1.9 (Kuhner 2006). The maximum likelihood approach was applied using the Metropolis-coupled Markov chain Monte Carlo (MCMC) method with replication of chains and adaptive heating to achieve optimal sampling of the parameter space. The MCMC runs were performed three times with random seeds; each run used 10 initial chains with 500 samples and two long final chains with 10 000 samples. All initial chains and final chains were simulated using a sampling interval of 20 and a burn-in of 1000 samples. A tenfold evolutionary rate (4.26-7.56% per million years) was considered at population level, following the correction for time dependence of molecular rate proposed by Ho et al. (2011).

Results.

Four chlorotypes were detected for the chloroplast marker *rbcL*, with 23 polymorphic sites along the 971 base pair fragments sequenced (11 sequenced individuals, Table 1). For the *cox2-3* mitochondrial marker, 18 mitotypes were observed (233 sequenced individuals, Table 1). For this marker, 48 polymorphic sites were observed including two indels: one indel of 1bp characteristic of the mitotype C13 and one indel of 12bp for mitotypes C8 and C17. Sequence length of *cox2-3* sequences varied from 337 to 350bp.

Figure 1 shows the Maximum Likelihood (ML) phylogenetic trees constructed using the two molecular markers. For both markers, tree topologies based on Bayesian and ML analyses were largely congruent and shared comparable support values for major nodes (Figure 1). Regardless of the marker used, tree topologies were broadly similar among phylogenetic reconstruction methods and clearly showed that all *G. skottsbergii* sequences obtained in this study form a monophyletic group, clearly split into two well supported lineages (Figure 1,

support values >89%) that are strongly divergent from the outgroup species. The first lineage is composed of all individuals from Chile and the Falkland Islands while the second is composed of individuals from the Antarctic Peninsula, the South Shetland Islands and the South Orkney Islands (Figure 1). The available *rbcL* sequence AF146206 in GenBank for *G. skottsbergii* previously sampled in King George Island in the South Shetland Islands (Hommersand et al. 1999) corresponds exactly to the chlorotype R4 present in our Antarctic lineage. The uncorrected p-distance, measured using only 665bp, between the *rbcL* sequence U03432 and the closest sequence in our data set (i.e. chlorotype R1, present only in Ancud, Chile, see Table 1) is of 0.3%. This is congruent with the location where the specimen corresponding to the U03432 sequence was collected, on Chiloé Island within the bay of Ancud (Hommersand et al. 1999). P distances between sequences from the Antarctic and South American *G. skottsbergii* lineages were of 9.2 ± 1.5 % for *cox2-3* and of 2.1 ± 0.5 % for *rbcL*. When performed between the Antarctic and South American *G. skottsbergii* lineages, no significant departure from neutrality was detected using the McDonald-Kreitman test for the *rbcL* and the coding part of the *Cox2-3* sequences analyzed (NI= 0.969, p = 0,980; NI= 2.191, p= 0.536, for the *rbcL* and the *Cox2-3*, respectively).

Divergence between the Antarctic and South American *G. skottsbergii* lineages was estimated at 9.4 Myr (95% CI: 3.2-16.4Myr) based on *cox2-3* data and at 14.9 Myr (95% CI: 2.6-35.9Myr) based on *rbcL* data.

For both markers, no genetic diversity was observed in the Antarctic lineage of *G. skottsbergii*. Only one haplotype was detected for the *cox2-3* region and for the *rbcL* gene, the mitotype C18 and the chlorotype R4, respectively (Table 1). The Antarctic lineage is spread over more than 1600 km of coast, from Marguerite Bay (67°S) to the South Orkney Islands (60°S) (Table 1, Figure 2C). Within the South American lineage of *G. skottsbergii* the genetic diversity in *cox2-3* was generally low with the number of mitotypes per sampling site (nH)

being lower than 3 and the gene diversity (H_d) as lower than 0.3 in 6 of the 11 studied populations (Table 1). The highest genetic diversity was observed in the population of PAG, BCH and FAL ($n_H = 5$ and $H_d > 0.5$ in all three populations, Table 1). These three populations were also the ones with the highest number of private haplotypes, with three private haplotypes in PAG and FAL and two in BCH (Table 1). For the *rbcl* gene, only three chlorotypes were observed within the South American lineage of *G. skottsbergii*. Only one chlorotype was found in each single population and the only private chlorotype (R1) was observed in the ANC population (Table 1). The chlorotype R3 was shared between BOR in Tierra del Fuego and FAL located in the Falkland Islands (Table 1).

The *cox2-3* mitotype network revealed the presence of two main haplogroups, one for South America and one for Antarctica, which are separated by 31 bp (Figure 2A), a result fully congruent with the phylogenetic reconstructions (Figure 1). Within the South American haplogroup, characterized by a typical star-like topology, pairs of mitotypes were separated by 1 to 5 bp, except for the mitotypes C17 and C8 that were differentiated by a unique 12bp indel. This haplogroup is also characterized by one frequent and widespread mitotype (C1, 81% of the samples, Table 1) and several less frequent mitotypes (Figure 2A). Low frequency mitotypes were predominantly restricted to a single or few nearby local populations. For example, the mitotype C3 was observed in BLO and PAG, these being two populations located in the Moraleda Channel. Additionally, mitotype C7 was observed in BCH, BOR and TOR which are located in the southern part of the Magellanic region and Tierra del Fuego (Figure 2B). One mitotype, C13, was also shared between BCH in the southern part of the Magellanic region and FAL located in the Falkland Islands (Figure 2B). As expected, the star-like topology is coupled with a unimodal mismatch distribution (Figure 3A), and the values of Tajima's D and Fu's F_s statistics were both negative and significant ($D = -2.01$, $p = 0.001$; $F_s = -1.26$, $p = 0.0001$) a result congruent with a sudden demographic population expansion model. Population size changes depicted from the mismatch distribution was $\tau = 0.73$ (90% confidence interval of 0.00

to 2.23). Assuming the start of exponential demographic expansion when population size was 1% of present-day's estimate, it was estimated to initiate 20 000 – 36000 years before present. A large positive exponential growth rate, $g = 3004$ (2458-4666 95% confidence interval), was also detected. It is possible that demographic expansion led to a large increase in N_e (approximately 170 000 to 300 000-fold increase, Fig. 3B), which may have started during or just before the LGM, depending on the combination of growth and mutation rates considered within their respective confidence limits.

Discussion

The patterns of genetic structure for *G. skottsbergii* [seem to](#) confirm the absence of gene flow between Antarctic and South American populations. Regardless of the marker analyzed, phylogenetic reconstructions using mtDNA and cpDNA sequences showed strong congruence and clear support for two distinct lineages consisting of populations from South America and the Falklands on the one hand and populations from the Antarctic Peninsula, Shetlands and Orkney Islands on the other hand. The absence of shared haplotypes between the two regions may reflect the isolation created by the Antarctic Circumpolar Current between the Antarctic Peninsula and the South American continent. Divergences among lineages of *G. skottsbergii* for the Cox2-3 (9.2 ± 1.5 %) and for the *rbcL* (2.1 ± 0.5 %) are within the range reported for interspecific distances between sister species in Rhodophyta (ranging from 2.55% to 4.70% and from 0.77% to 5.08% for the Cox2-3 and the *rbcL*, respectively; McIvor et al. 2001; Destombe et al. 2010; Hernández-Kantún et al. 2014). The large range estimated, of 2.6 to 35.9 My depending on the genetic marker and the mutation rate considered (with central tendency around 9 to 15My), for divergence time between the two lineages seems to predate Pleistocene glaciations. This time of divergence is however more recent than the separation of the Antarctic continent from the South American continent (approximately 24-40 Myr ago),

and seems to include the period of intensification of the ACC circulation 11-12 Myr ago (Dalziel et al. 2013). These climatic and oceanographic changes have been shown to be major drivers in the isolation of marine Antarctic fauna. For example, González-Wevar et al. (2010) have shown that the diversification within a genus of mollusk (*Nacella*) took place long after the separation of the continents. For these marine mollusks, the appearance of the most genetically distant clades (Kerguelen, Antarctic, and South America) took place between 9 and 5 Myr. These results were further supported by a recent study of comparative phylogeography of different invertebrate taxa showing a shared Antarctic and South American distribution (González-Wevar et al. 2012a; Poulin et al. 2014). In these studies, divergence times between Antarctic and South American lineages ranged from 1.0 to 13.6 Myr, largely overlapping the estimates for *G. skottsbergii*. Interestingly, divergence estimates in the mitochondrial markers for *G. skottsbergii* (9.2%) fall within the 7-11% range for shallow subtidal and intertidal invertebrates (González-Wevar et al. 2010, 2012a; Poulin et al. 2014), corroborating the occurrence of a shared evolutionary history despite imprecisions in the respective mutation rates. These authors proposed that the connection between Antarctic and South American populations could have been maintained by a stepping stone process along the archipelago of the Scotia Arc. Indeed, geological evidence has recently been reported of a now-submerged volcanic arc in the Central Scotia Sea that existed during the early Miocene (Dalziel et al. 2013). This archipelago was located closer to the Antarctic Peninsula and the Cape Horn region than the South Sandwich Islands are today, and may thus have provided a corridor for genetic connectivity across the Drake Passage until approximately 10-11 Myr ago (Poulin et al. 2014). Our study further shows that *G. skottsbergii*, a seaweed characterized by a very restricted dispersal capacity of its spores, may also have maintained a certain level of connectivity (even after the mid-Miocene) between the continents of Antarctica and South America through the volcanic arc of islands linking both sides of the Scotia Sea. This connection could have been

maintained until the beginning of Pliocene's glaciations, as in the case of brooding invertebrates, through rafting of adults (Nikula et al. 2010; Haye et al. 2012; Poulin et al. 2014).

The Antarctic and South American lineages of *G. skottsbergii* exhibit different patterns of genetic diversity. While a strong demographic expansion was inferred in the sub-Antarctic region, a total absence of genetic diversity was observed in the Antarctic lineage. The presence of a single haplotype over more than 1600 km from Marguerite Bay to the South Orkney Islands is intriguing. Even if this lack of diversity limits our capacity to test for different demographic scenarios in Antarctica, it suggests that a very strong demographic bottleneck occurred during glacial contraction, followed by a sudden and recent recolonization process that did not allow for new mutations. Similarly, the overall low genetic diversity and the presence of the same common haplotype C1 in every population from the Chilean coast to the Falkland Islands seems to support the hypothesis of persistence in a single glacial refugium followed by a massive demographic expansion over 2500 km. Such a pattern of genetic homogeneity over a broad geographical range has usually been related to high dispersal potential (Bortolotto et al. 2011; González-Wevar et al. 2012b). The scenario of rapid recolonization leading to the presence of only one haplotype over thousands of kilometers of coast is, however, difficult to envision for *G. skottsbergii*. Indeed, this alga is a non-buoyant species, and spore dispersal is considered to be very limited (Ramirez and Santelices 1991). Nevertheless, signatures of long distance dispersal have been observed in other apparently non-dispersive algal species like *Adenocystis utricularis* or *Bostrychia intricata* and have been explained by the organisms' potential ability to raft on floating substrates (Fraser et al. 2010, 2013). In contrast with our results, in a previous study using RAPDs nuclear markers (Faugeron et al. 2004), significant genetic differentiation among South American populations was observed and has been related to the poor dispersal capacity of for *G. skottsbergii*. Differences in mutation rates and/or level of drift effects (effective population size of uniparentally inherited loci is only one-fourth that of nuclear loci) may account for the differences between

level of genetic structure obtained with RAPDs (Faugeron et al. 2004) and cytoplasmic sequences (our study). Also, during spatial expansion, gene surfing effects may contribute to the reduction of diversity in cytoplasmic markers in the recolonized region (Excoffier and Ray 2008). Indeed, large-scale spread of mitochondrial genetic variants has been observed during recolonization process in seaweeds (Fraser et al. 2010). The ACC is a strong west to east current that connects the Antarctic Peninsula and the South Shetland Islands to the Antarctic Islands located in the South Scotia Ridge. Particle movement modeling and particle tracking has shown that passive drifters travel northeastwards across the Scotia Sea, connecting the Antarctic Peninsula and the South Shetland Islands to South Orkney Islands and South Georgia (Thorpe et al. 2004). Movement of drifting seaweed along the ACC could be connected to west-east spatial expansion pattern. On the other hand, the postglacial colonization of the Peninsula could have been promoted by stepwise spatial expansion through spore propagation. Such spatial expansion could have been facilitated by strong coastal currents, as the Antarctic Peninsula Coastal Current (APCC; Moffat et al. 2008), a southward current that forms during the ice-free seasons and extends to Alexander Island. However, we lack knowledge on the actual mechanisms of dispersal in *G. skottbergii* to appropriately determine the role of dispersal during post-glacial population dynamics on the present day genetic diversity.

The location of both Antarctic and South American glacial refugia is difficult to infer from the results presented here. The extreme genetic homogeneity within the *G. skottsbergii* Antarctic clade is in disagreement with the hypothesis that genetic structure and global genetic diversity in Antarctica may have been promoted by the fragmentation and isolation of micro-refugia where marine species were able to survive during repeated ice advances and retreats (Thatje et al. 2005; Allcock and Strugnell 2012). The possible presence of micro-refugia has been particularly highlighted in the archipelagoes located near the West Antarctic Peninsula and the South Shetland Island (See Fraser et al. 2012 for review). Indeed, new studies using molecular

data have shown that the South Shetland Islands is the most speciose region for endemic Southern Ocean octopuses of the genus *Pareledone* (Allcock et al. 2011). Even though the data herein lack the explicit molecular diversity to test adequately for the number and location of glacial refugees in this region, the survival of *G. skottsbergii* during the LGM seems more likely in the northernmost latitude (South Shetland and South Orkney Islands). Indeed, whereas *G. skottsbergii* is found in dense beds within these northern archipelagos, less than ten specimens could be collected in Paradise Bay and only one specimen was sampled in Marguerite Bay (Note that the same sampling effort was applied in Paradise Bay, Marguerite Bay, O'Higgings and Punta Prat; Table 1; M-L Guillemin, personal communication). It is understood that an increased sampling effort could better elucidate the southern limits of *G. skottsbergii*, but it seems that in Marguerite Bay the alga has reached a biotic or abiotic limit to its distribution.

The absence of genetic structure for the mitochondrial and chloroplast markers in southern South America also limits our ability to infer the number and location of glacial refugia. Because of their longstanding demographic stability, populations from glacial refugia are expected to present higher levels of genetic diversity than those populations that have formed following postglacial expansions (Provan and Bennett 2008). In addition, long-term isolation in distant refugia often leads to genetic differentiation due to mutation accumulation and genetic drift (Hewitt 2000). Along the Chilean coast, recolonization pathways have differed between organisms in terms of the number and origin of sources. A recent recolonization from a single distant/exterior source has been proposed to explain the high genetic homogeneity of populations of the red alga *Mazaella laminarioides* (Montecinos et al. 2012) and the brown alga *Durvillaea antarctica* that exist south of 42°S (Fraser et al. 2010). In *M. laminarioides*, a single refugium has been proposed, located in the North of the Island of Chiloe and the mainland coast north of the 41°S (Montecinos et al. 2012). In *D. antarctica*, broad sampling across the southern hemisphere has shown that all individuals present off the Patagonian coast of Chile share the same haplotype (Fraser et al. 2010) and the authors have indicated

that this species likely recolonized the region from a refugium in New Zealand sub-Antarctic archipelagoes. Yet others have highlighted the role that the Southern Fjords and Channels Region played as a refuge for several animal species, like marine mollusks (Valdovinos et al. 2003; González-Wevar et al. 2010) and the river otter (Vianna et al. 2011). The existence of multiple refugia, scattered among the Southern Fjords and Channels, has been proposed to explain the high levels of endemism and the high diversity of mollusks in this region (Valdovinos et al. 2003). In *G. skottsbergii*, the overall low genetic diversity and the presence of the same common haplotype, C1, in every population from the Chilean coast to the Drake Passage and the Falkland Islands seem to support the hypothesis of a single glacial refugium. Strong currents (i.e. the Cape Horn Current and the Malvinas-Falkland Current) connect the southern coast of Chile from 42°S to Cape Horn and the Falkland Islands. These currents have been shown to provide high connectivity among all inhabiting regions for the genus *Nacella* (González-Wevar et al. 2012b). In this genus, the existence of asymmetric gene flow, from West to East, was shown to be related to the prevailing circulation patterns in this region (González-Wevar et al. 2012b). Due to these strong directional currents, the hypothesis of a postglacial recolonization of the Chilean coast by *G. skottsbergii* from a refuge located in the Falkland Islands is most unlikely. Again, more polymorphic markers should help identifying regions with higher / lower genetic diversity as putative locations of glacial refugia / recolonized areas, respectively, for *G. skottsbergii*.

Conclusions

In accordance with previous results of Hommersand et al. (2009) the data presented here clearly show two divergent and respectively monophyletic clades of *G. skottsbergii* that may correspond to two cryptic species. *G. skottsbergii* (Type locality: Slogget Bay, Fuegia - Silva 1996; <http://www.algaebase.org>) is distributed in the Southern and sub-Antarctic coast of

Chile and the Falkland Islands. The Antarctic species, still to be formally described and named, occurs in the Antarctic Peninsula, the South Shetland Islands and the South Orkney Islands. Despite drastic reductions in population size, as revealed by strong signals of bottlenecks, both species persisted and maintained their separation on either side of the ACC even during the glacial period. The divergence time between the two cryptic species, estimated as 9.4 to 14.9 Myr (comparable with other invertebrate species, but with a large confidence interval of 2.6-35.9Myr), indicates that algae with limited dispersal capabilities were able to cross the Scotia Sea after separation of the continents, potentially via a stepping stone process through the volcanic arc of islands. Our work also sheds light on the possible importance of dispersal by rafting in the recolonization process of a species for which natural propagation is mainly achieved via sexual reproduction (Avila et al. 1999; Westermeier et al. 2012). Future prospects on glacial refugia for Antarctic and sub-Antarctic seaweeds shall also contribute to better understand the dispersal mechanisms during demographic expansion in these regions characterized by complex topography of coastal shelf and coastal currents.

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Figure 1: Maximum likelihood rooted trees for the *cox2-3* and the *rbcl* haplotypes of *Gigartina skottsbergii*. Maximum likelihood bootstrap values are indicated above each node and Bayesian posterior probabilities are noted between brackets. Only high support values are shown (>75 for bootstraps and >0.95 for posterior values, respectively; - correspond to branches not observed in the Bayesian inference reconstruction).

Figure 2: Haplotype networks of *Gigartina skottsbergii* and their geographic distribution based on *cox2-3*. The haplotype networks are presented in (A) while the pie charts showing the geographical distribution of haplotypes of the South American haplogroup are shown in (B) and the pie charts showing the geographical distribution of haplotypes of the Antarctic haplogroup are shown in (C). In the networks, each circle represents a haplotype and its size is proportional to the frequency in which the haplotype was encountered (correspondence between circle sizes and numbers of individuals is indicated in the box A). The black square in-between C7, C9 and C14 represents a hypothetical un-sampled haplotype. For haplotypes separated by more than one mutational step, the number of steps is indicated in bp. Pie charts' color-code corresponds to the one used in haplotype networks. Abbreviations for population codes are as in Table 1. Numbers of sequenced individuals are given between brackets.

Figure 3: Mismatch distributions (A) and population growth rate estimates (B) of the South American haplogroup of *Gigartina skottsbergii* for the *cox2-3* marker. Growth rate and timing of population dynamic changes were estimated from coalescent simulations implemented in LAMARC 2.1.9 (Kuhner 2006). The observed distribution of the number of pair base differences between sequences is indicated by the grey bars while the expected distribution under a model of sudden demographic expansion is represented by a black line. Effective population size (i.e. N_e) fluctuations throughout time are represented in the LAMARC graph. The dotted lines represent the 95% confidence intervals of the estimated growth rate, whereas the grey

533 shaded area corresponds to the average growth rate combined with the range of mutation
534 rates proposed by Andreakis et al (2007) corrected by a tenfold evolutionary rate as suggested
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Cox23

rbcl

Figure
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Table1: Sampling sites of *G. skottsbergii* and associated genetic diversity for two molecular markers (cox23: mitochondrial; rbcL: chloroplast). For each site, the abbreviation (code) and the geographic coordinates are indicated. For more information on molecular diversity indices see foot note references.

Sampling site	Code	Coordinates	Cox23							rbcL		
			N	nH	Hd	π (.10-2)	S	nHpriv	Hap.	N	nH	Hap.
Chile												
Ancud	ANC	41°51'S/73°47'W	18	1	0	0	0	0	C1(18)	2	1	R1(2)
Calbuco	CAL	41°48'S/73°13'W	17	3	0.18	0.44	13	1	C1(15), C8(1), C10(1)			
Bahia Low	BLO	43°47'S/73°58'W	17	3	0.23	0.10	3	1	C1(15), C3(1), C11(1)	1	1	R2(1)
Puerto Aguirre	PAG	45°10'S/73°32'W	19	5	0.68	0.31	4	3	C1(10), C2(4) , C3(3), C4(1) , C5(1)			
Bahia Chilota	BCH	53°20'S/70°43'W	16	5	0.53	0.30	5	2	C1(11), C7(1), C12(2) , C13(1), C14(1)	2	1	R2(2)
Bahia Inútil	BIN	53°10'S/72°55'W	17	3	1.72	0.49	13	1	C1(13), C8(1), C9(3)	2	1	R2(2)
Isla clarence	ICL	54°03'S/71°58'W	16	1	0	0	0	0	C1(16)	1	1	R2(1)
Isla London	ILO	54°57'S/72°20'W	13	1	0	0	0	0	C1(13)	1	1	R2(1)
Bahía Orange	BOR	55°31'S/68°08'W	11	2	0.18	0.05	1	0	C1(10), C7(1)	1	1	R3(1)
Puerto Toro	TOR	55°06'S/67°06'W	5	3	0.80	0.29	2	1	C1(2), C6(1) , C7(2)			
Falkland Islands												
Falkland	FAL	51°37'S/57°45'W	17	5	0.51	0.59	15	3	C1(12), C13(1), C15(2) , C16(1) , C17(1)	5	1	R3(5)
South Orkney Islands												
Orkney	ORK	60°44'S/45°37'W	5	1	0	0	0	0	C18(5)			
South Shetland Islands												
Punta Hanna	HAN	62°39'S/60°38'W	14	1	0	0	0	0	C18(14)	1	1	R4(1)
Punta Prat	PRA	62°28'S/59°40'W	29	1	0	0	0	0	C18(29)	2	1	R4(2)
Isla Aitcho	AIT	62°25'S/59°44'W	9	1	0	0	0	0	C18(9)	1	1	R4(1)
Antarctic Peninsula												
O'Higgins	OHI	63°18'S/57°53'W	9	1	0	0	0	0	C18(9)	4	1	R4(4)
Paradise Bay	PAR	64°50'S/62°52'W	3	1	0	0	0	0	C18(3)	2	1	R4(2)
Marguerite Bay	MAR	67°45'S/68°52'W	1	1	0	0	0	0	C18(1)	1	1	R4(1)
Chile and Falkland Antarctic Peninsula and Islands			166	17	0.34	1.05	24			15	3	
			70	1	0	0	0			11	1	

Molecular diversity indices are as: N: number of sequences; nH: number of haplotypes; Hd: gene diversity; π : nucleotide diversity; H_{priv}: number of private haplotypes; S: number of polymorphic sites; Hap.: list of haplotypes present in each population. In the haplotype list the name of the haplotype is directly followed by the number of sampled individuals presenting the haplotype between parenthesis and private haplotype are noted in bold characters.

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