

Molecular-assisted revision of red macroalgal diversity and distribution along the Western Antarctic Peninsula and South Shetland Islands

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Résumé – La flore marine de la zone Antarctique est supposée bien connue car relativement peu d'espèces y sont observées par rapport à d'autres régions du monde. En utilisant la taxonomie traditionnelle combinée aux outils moléculaires, nous avons étudié la diversité et biogéographie des communautés de macroalgues rouges le long de la côte Ouest de la Péninsule Antarctique (WAP) et des îles Shetlands du Sud (SShs) en considérant un gradient bathymétrique. Nous avons également comparé nos deux méthodes d'identification en terme de précision et de nombre de taxa identifiés. Nos résultats ont démontré de faibles et homogènes niveaux de diversité à l'échelle de la zone d'étude comme à échelle locale, avec au total environ 50 taxa identifiés. Nous avons également détecté trois complexes d'espèces cryptiques chez les genres *Callophyllis*, *Curdiea* et *Georgiella*, et amélioré l'identification de nos spécimens puisque 98% ont été identifiés jusqu'à l'espèce grâce aux outils moléculaires. Nos résultats ont aussi permis de révéler des différences significatives entre les assemblages de macroalgues rouges appartenant à trois sub-régions, correspondant à des latitudes différentes: SShs et partie Nord de la WAP (≈63°S), la partie centrale de la WAP (≈64°S) et la partie Centre-Sud de la WAP (≈67°S). Nos sub-régions ne correspondent pas à la séparation classique entre SShs/WAP proposée dans les études antérieures, et suggèrent d'une part, que le détroit de Bransfield n'est pas une barrière biogéographique forte pour les macroalgues rouge et d'autre part, que la dynamique actuelle et passée de la couverture de glace a pu jouer un rôle déterminant dans la distribution actuelle des assemblages de macroalgues rouges. Finalement, notre travail a permis de construire une banque de données génétiques associée à un herbier de collection de spécimens, et va permettre d'appuyer des travaux futurs pour mieux comprendre la diversité et distribution des communautés d'algues rouges dans la zone d'étude.

Barcoding ADN / Biogéographie / Espèces cryptiques / Inventaire de Biodiversité / Rhodophyta / Taxonomie intégrative

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Abstract – The Antarctic marine flora is well known as a flora composed of relatively few species in comparison with other marine realms. Using molecular taxonomy as complementary tool for traditional taxonomy, we studied red macroalgal diversity and biogeography along the Western Antarctic Peninsula (WAP) and South Shetlands Islands (SShs) coasts and across a bathymetric gradient; in addition, we compared both methods of identification in terms of accuracy and number of observed taxa. Our results show low diversity levels at the regional and local scales, with around 50 taxa registered in total. Molecular tools allowed us to detect putative cryptic species within the genera *Callophyllis*, *Curdiea* and *Georgiella*, and to identify 98% of our specimens at species level. Our results also allowed us to identify significant differences between red macroalgal assemblages of three distinct biogeographic sub-regions: SShs and Northern part of the WAP (at $\approx 63^\circ\text{S}$), Central part of the WAP (at $\approx 64^\circ\text{S}$) and Central-Southern part of the WAP (at $\approx 67^\circ\text{S}$). Our sub-regions do not correspond to the classical separation of SShs/WAP reported in previous studies and suggest that the Bransfield Strait is not a strong biogeographic barrier for red macroalgae. Since our three sub-regions correspond to three latitude levels, we propose that past and present-day dynamics of sea-ice disturbance may have shaped the observed differences in red algal communities. We believe that the construction of accessible genetic dataset associated with voucher specimens in the present work will benefit future studies of macroalgal diversity and distribution in the region.

Biodiversity survey / Biogeography / Cryptic species / DNA barcoding / Integrative taxonomy / Rhodophyta

INTRODUCTION

With more than 7000 species described to the day (Guiry & Guiry, 2018), the phylum Rhodophyta comprises the most diverse group of seaweeds, encompassing both macro- and microorganisms found in a wide diversity of habitats. Antarctic macroalgal diversity is considered to be low compared to temperate southern Pacific waters (one tenth of Australian algal diversity; Wiencke & Clayton, 2002), but is at the same time characterized by high level of endemism ($\approx 35\%$, Wiencke & Clayton, 2002; Ramírez, 2010; Wiencke *et al.*, 2014). These characteristics have been linked to the history of Antarctica. Indeed, the isolation of the Antarctic biota has begun with the sequential fragmentation of the Gondwana super-continent (130 Mya BP) and was completed with the opening of the Drake Passage and the separation of southern tip of South America and the Antarctic Peninsula about 40 Myr ago (Scher & Martin, 2006). Moreover, the strong intensification of the Antarctic Circumpolar Current (ACC) 25 Myr ago has also contributed to isolate Antarctic from sub-Antarctic waters, acting as an oceanographic barrier to dispersion for most of marine species (Sanches *et al.*, 2016). Since the Mesozoic, Antarctica has become increasingly cooler and, for several million years, Southern Ocean temperatures have been close to the freezing point for much of the year, and are considered as one of the most constant thermal regimes on earth (Barnes & Conlan, 2007). This relatively stable Antarctic environment and the strong isolation of Antarctica (but see Fraser *et al.*, 2018) are presumed to be the main reasons for the development of a unique biota, rich in endemic species well adapted to a cold-water system (Rogers, 2007).

The description of the Antarctic flora has begun 200 years ago with the expedition of Gaudichaud and Hooker (between 1817 and 1843, see Wiencke & Clayton, 2002) and new inventories have allowed to increase the number of recorded species during the last decade in the region (from 96 species in 1964 to about

130 species listed in Wulff *et al.*, 2009, and up to more 150 in Sanches *et al.*, 2016). In Antarctica, macroalgae are generally considered “well-known organisms” (Griffiths, 2010). Indeed, a long list of studies have deepened our understanding about the structure and the function of macroalgal communities, and have shown that significant differences exist between bioregions, habitats and ranges of depth (Zacher *et al.*, 2007; Oliveira *et al.*, 2009; Quartino *et al.*, 2013; Amsler *et al.*, 2014; Valdivia *et al.*, 2014; Amsler *et al.*, 2015; Griffiths & Waller, 2016; Sanches *et al.*, 2016; Marcías *et al.*, 2017). However, when compared to marine animals, and especially invertebrates, very few occurrence records are available along the coast of Antarctica for macroalgae (e.g. only 73 occurrence records in the Register of Antarctic Marine Species - RAMS - in 2010 while 29,727 occurrence records exist for molluscs alone, see De Broyer & Danis, 2011). It is clear that not all species have received the same attention and that major drawbacks in understanding patterns of diversity can arise in group of organisms characterized by simple morphology and for which errors in the taxonomic assignation of specimens have been detected, like macroalgae. Because of their fairly simple anatomy, rampant convergence, remarkable degrees of phenotypic plasticity in response to environmental factors, and incompletely understood life histories with alternation of heteromorphic generations, marine macroalgae are notoriously difficult to identify by means of traditional morphological characters (Saunders, 2005, 2008).

During the past two decades, molecular tools have been increasingly used to rapidly and accurately identify macroalgal specimens (Le Gall and Saunders, 2010) and the emerging field of molecular-assisted alpha taxonomy has allowed to revisit the phylogeny and species diversity in various orders and families (e.g. for red algae: Hommersand *et al.*, 2009; Sutherland *et al.*, 2011; Billard *et al.*, 2015; Guillemin *et al.*, 2016). Along the tip of South America and the Antarctic Peninsula, recent genetic studies have warranted the description of new genera and species of macroalgae (Hommersand *et al.*, 2009; Moniz *et al.*, 2012) and have pointed out the existence of various cryptic species in brown, red and green algae yet to be described (Young *et al.*, 2013; Mystikou *et al.*, 2014; Billard *et al.*, 2015; Guillemin *et al.*, 2016; Garrido-Benavent *et al.*, 2017; Pellizzari *et al.*, 2017). The DNA barcoding approach (Hebert *et al.*, 2003), based on the use of a standard genetic marker (*i.e.* generally the COI mitochondrial gene coding for subunit 1 of cytochrome oxidase, see Leliaert *et al.*, 2012 and reference therein), has been proposed as a simple tool to decipher species diversity and distribution in highly speciose groups. Even if the method presents well-known limitations (DeSalle *et al.*, 2005), it has been proved useful to detect cryptic diversity of algae (Saunders, 2005; Le Gall & Saunders, 2010) and has been used to revise spatial patterns of biodiversity in Atlantic kelp forests (Robuchon *et al.*, 2015). Despite the problems linked to their species delimitation and determination, Antarctic algae have not been cited as organisms where possible gaps in knowledge exist (see De Broyer & Danis, 2011), nonetheless, a comprehensive DNA library of life (Le Gall *et al.*, 2017) for those organisms is still missing. Moreover, it is clear that Antarctic benthic diversity has been seriously underestimated and that much fewer taxa have large circumpolar distributions or cross the Antarctic Circumpolar Current (ACC) than was previously postulated (see review in Clarke *et al.*, 2007 and Convey *et al.*, 2014) — macroalgae being no exception (Hommersand *et al.*, 2009; Billard *et al.*, 2015).

Within the Antarctic waters, the existence of various sub-regions / eco-regions was postulated and the authors generally have proposed a separation of the South Shetland Islands (SSIs) from the Western Antarctic Peninsula (WAP; Linse *et al.*, 2006; Spalding *et al.*, 2007; Terauds *et al.*, 2012) and even the existence of fine

scale differences between the Northern, Central and Southern part of the WAP (Linse *et al.*, 2006; Terauds *et al.*, 2012). These regions are characterized by different histories (e.g. different deglaciation ages: Ó Cofaigh *et al.*, 2014), marine current systems and environmental conditions such as temperature, salinity, light and substrate availability and period of sea ice coverage. At large geographic scales, recent studies have demonstrated that macroalgal species tend to form characteristic groups broadly following the delimitation of the proposed biogeographic regions (Griffiths & Waller, 2016; Sanches *et al.*, 2016; Pellizzari *et al.*, 2017). Of all the studies focused on macroalgae, only the one made by Pellizzari *et al.* (2017) has taken into account the existence of unnamed cryptic genetic lineages in some of the taxa under study. Species identification being an essential first step in ecological studies, it has been demonstrated that incorrect taxonomic classification of specimens can significantly bias any survey of biodiversity, producing an underestimation of both local and regional biodiversity (Bortolus, 2008). Indeed, in their recent survey of algal communities associated to distinct kelp forests in French Brittany, Robuchon *et al.* (2015) have shown that DNA barcoding significantly increases the alpha-diversity and generally allows to improve the taxonomic level of identification (*i.e.* higher number of samples resolved to the species level when using genetic data).

In the present study our aim was to contrast results of classical and molecular assisted taxonomy to investigate the variation of red macroalgal assemblages among Antarctic sub-regions. For the first time, a barcoding approach was consistently used to classify the whole red algal community in five areas located in the SSHs and along the Northern and Central part of the WAP at the lowest taxonomic level achievable. We expected the integrative classification method to increase species diversity when compared to the morphological classification (Robuchon *et al.*, 2015) since cryptic diversity could be taken into account (Saunders, 2005; Le Gall & Saunders, 2010; Young *et al.*, 2013; Guillemain *et al.*, 2016; Garrido-Benavent *et al.*, 2017). Finally we tested for differences in species assemblage between biogeographic sub-regions in the area under study.

MATERIALS AND METHODS

Sampling- Red macroalgae were sampled during the 2013 and 2014 Antarctic summer campaigns in five areas located in the SSHs (near the Chilean Capitán Arturo Prat base in Greenwich Island, and at Bahía Fildes in King George Island, hereafter referred to as PRAT and KGI, respectively) and along the Northern (near the Chilean O'Higgins Antarctic base, noted OHI) and Central part of the WAP (in Paradise Bay, near the Chilean Presidente Gabriel González Videla Antarctic base, noted GGV and in Marguerite Bay, noted MAR) (Fig. 1, Annex 1). The whole sampling region covered two distinct biogeographic sub-regions proposed by Linse *et al.* (2006): the SSHs and the WAP (see Fig. 1). Along the Central part of the WAP, GGV is located in the Gerlache Strait while MAR is located within the Bellingshausen Sea.

In each area, 4 to 7 sites separated by at most 80 km were sampled during our study (Annex 1). In PRAT, OHI, GGV and MAR, the sampling was done along transects (perpendicular to the shore, each site correspond to one transect), with three bathymetric zones defined for each transect (*i.e.* intertidal zone, subtidal zone from 0 to 15 m and subtidal zone from 15 to 30 m). An exhaustive research of

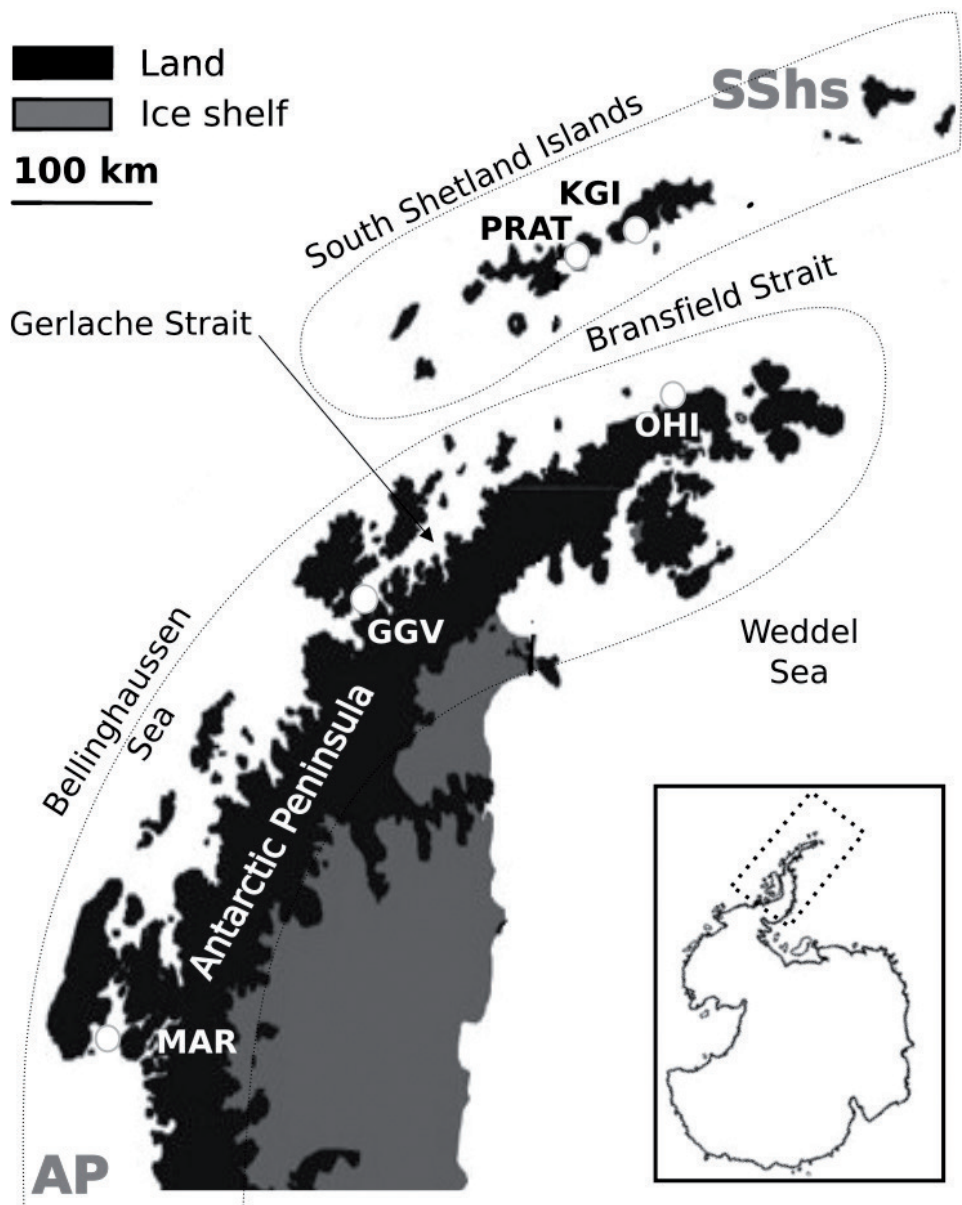


Fig. 1. Map of the study region including the five sampling areas: PRAT = Greenwich Island, KGI = King George Island, OHI = O'Higgins Antarctic base, GGV = Paradise Bay and MAR = Marguerite Bay. The study region covered the two distinct biogeographic regions proposed by Linse et al. (2006); the South Shetlands Islands (SShs) and the Antarctic Peninsula (AP).

different morphotypes was completed during a 30 min sampling effort in each bathymetric zone for each site. Specimens for which we were able to observe noticeable variations in thallus shape, color or thickness and elasticity were

considered as distinct morphotypes and at least one specimen of each morphotype was sampled. In KGI, a non-exhaustive research of morphotypes, without controlling the sampling time effort, was conducted in seven sites along a bathymetric gradient going from the intertidal zone to a 40 m depth. Intertidal samplings were conducted during diurnal low tide hours; subtidal samplings were done by means of SCUBA diving.

All specimens were pressed as vouchers after removing a small portion of the thallus that was stored in silica gel for subsequent DNA analysis (see Annex 1). Voucher specimens are housed in the herbarium of the Universidad Austral de Chile and available from the corresponding author on request (*i.e.* voucher code correspond to "Specimen ID" in Annex 1). In this study, the specimens of the Corallinaceae were not included since the sampling was highly biased to collecting in the intertidal zone only in most of our sites.

Identification process of red algal specimens- A total of 882 specimens of red macroalgae were collected over the sampling region (Annex 1). Two methods were used during the process of identification and taxonomic assignation of specimens. First, we used floristic keys, species list and field guides available for the region (Wiencke & Clayton, 2002; Hommersand *et al.*, 2009; Ramírez, 2010) to identify red macroalgae to the lowest possible taxonomic level on the basis of morphological criteria alone [see Annex 1, "Species (Identification based on morphological characters)"]. Second, two genetic markers were used to provide a complementary species identification based on molecular criteria: a partial sequence of the mitochondrial Cytochrome c Oxidase I gene (5P-COI) and a partial sequence of the plastid gene *rbcL*, encoding the large subunit of the ribulose-1,5-bisphosphate. Dry tissues were grinded by hand in liquid nitrogen. All samples belonging to the genus *Georgiella*, *Gigartina*, *Iridaea*, *Picconiella* and *Trematocarpus* were extracted using extraction protocol from Saunders (1993) modified by Faugeron *et al.* (2001). For all the other samples, DNA extraction was performed using extraction kit E.Z.N.A.[®] Poly-Gel DNA Extraction (Omega Bio-Tek Inc., Norcross, USA). For all specimens, the amplification of 5P-COI was performed using the primers developed by Saunders (2005) (GaZF1: TCAACAAATCATAAAGATATTGG and GaZR1: ACTTCTGGATGTCCAAAAAYCA). *rbcL* amplification was performed only for a subsample of the red algae including: 1) all specimens that did not amplify for the 5P-COI and 2) up to 23 specimens for each group of samples assigned to the same species with the 5P-COI (more details available below). *rbcL* amplification was performed using the primers developed by Hommersand *et al.* (1994) (F-*rbcL*: TTGCATAYGATATTGATYTATTTGAA and R-*rbcL*: RAGCTGTTTKTAAAGGWCCACAA). For both gene fragments the same PCR mix was used. Amplifications were performed in a final volume of 30 µL that included: 0.12 µL of Kapa Taq (5 U/µL), 3 µL of Kappa Taq buffer B (10x) (Kapa Biosystems); 0.6 µL of MgCl₂ (25 mM); 0.6 µL dNTPs (2.5 µM) and 1.5 µL of each primer (10 µM). Depending on the species and marker under study, 0.2 to 0.6 µg/µL BSA (100X) (New England Biolabs, MA, USA) was added in each PCR mix. For both markers, cycling conditions consisted of an initial denaturing step of 5 min at 94°C, followed by 5 cycles (94°C for 30 s, Tm1°C for 45 s, and 72°C for 45 s; Tm1 = 40°C for 5P-COI and 45°C for *rbcL*), 25 additional cycles (94°C for 30 s, Tm2°C for 30 s, and 72°C for 30 s; Tm2 = 54°C for 5P-COI and 56°C for *rbcL*) and a final elongation step of 7 min at 72°C. PCR products were purified using commercial kit E.Z.N.A.[®] DNAProbe Purification (Omega Bio-Tek Inc., Norcross, USA) and sequenced with primers used for amplification at the AUSTRAL-omics Core-Facilities (Valdivia, Chile). Please note that both the forward and reverse

primers were used for the 5P-COI sequencing while only the F-*rbcL* primer was used for the *rbcL* sequencing.

Sequences were edited using Chromas v.2.33 (McCarthy, 1997), and aligned using MEGA v.5 (Tamura *et al.*, 2011). For the 5P-COI, uncorrected p-distances were calculated in Mega v 5 (Tamura *et al.*, 2011) and sequences separated by p-distances higher than 1% were considered as potentially belonging to distinct species (Robuchon *et al.*, 2015). Molecular species identification was performed using the basic local alignment search tool (BLAST) from NCBI (Altschul *et al.*, 1990) and comparing the sequences obtained in this study with those already deposited in GenBank. Before phylogenetic tree reconstructions, GenBank sequences presenting more than 95% of similitude from the ones obtained in our study were added to the 5P-COI and the *rbcL* datasets (*i.e.* 19 and 42 sequences for the 5P-COI and the *rbcL*, respectively). For both markers, and in order to limit the complexity of the trees for some highly diverse genera, only the first six most similar species present in GenBank were added to our data sets. Tree reconstructions were conducted separately for the COI-5P and the *rbcL* markers using a neighbour-joining (NJ) algorithm and the Juke-Cantor model in MEGA v.5 (Tamura *et al.*, 2011).

Previous to analyses of species diversity and composition, two datasets were generated for Antarctic red algae: a “morphological” dataset based only on species identification using the morphological criteria and an “integrative” dataset that combines information given by the morphological and molecular criteria. In order to generate the integrative dataset, morphological identification was contrasted for each specimen for which genetic data were available, with the results of a species identification based on molecular criteria. When a complete concordance was found between both methodologies of species identification, the species name given in the morphological identification was kept in the “integrative” dataset. When a discordance was observed between the two methods and sequences were available in NCBI that allow to name our specimens, the name given by molecular species identification tools (*i.e.* 5P-COI and/or *rbcL*) were kept in the “integrative” dataset. For specimens for which a discordance was observed between the two methods but the information present in NCBI was not sufficient to name our samples, the name of the genus followed by sp.1 (2, 3, etc.) was given to the genetic entities. In this last case, for all except four specimens, the name of the genus obtained after comparing our two molecular markers and the NCBI database were concordant between the 5P-COI and the *rbcL* sequences. Four specimens were identified as *Cryptonemia* in the morphological dataset, as *Grateloupia* when using the 5P-COI marker and as *Cryptonemia* when using the *rbcL* marker. *Grateloupia* and *Cryptonemia* are closely related genera and we chose to classify these specimens as species of *Cryptonemia* (Annex 1).

Specimens for which no sequences were available were named in the “integrative” dataset depending on the situation: 1) if a complete congruence between morphological and molecular identification was observed for all specimens of a morphological species for which sequences were obtained, species name given by the morphological identification were kept in the “integrative” dataset for all specimens (*i.e.* with and without molecular data); 2) if we observed a discordance between morphological and molecular identification for at least one specimen sequenced within a morphological species, non sequenced specimens were given the name corresponding to the ones identified by molecular criteria and sampled at the same site and at the same depth; 3) if, for a species defined using the morphological criteria no sequences were obtained at all, all specimens kept the name given by the morphological identification in the “integrative” dataset.

Data analyses- To characterize pattern of red seaweed diversity, presence / absence of species was recorded for each site within the five areas under study (*i.e.* KGI, PRAT, OHI, GGV and MAR). For each site, information available for the three bathymetric zones was pooled together and analyzed jointly. Within each sampled area, absolute values of species richness were determined using both the “morphological” and the “integrative” datasets. Moreover, for the whole sampled region, we constructed species accumulation curves for both the “morphological” and the “integrative” datasets. Accumulation curves and standard errors were calculated using the statistical software R version 3.01 (R Core Team, 2014) via the *specaccum* function from *vegan* package (Oksanen *et al.*, 2014) and combining data from increasing numbers of sites in random order repeated 1000 times. On the basis of the presence / absence data, Jaccard index was used to construct a matrix of similarity between our areas, for both the “morphological” and the “integrative” datasets, using PRIMER 6 (Clarke & Warwick, 2001).

Compositional changes in species assemblages across sampled areas were analyzed by nonmetric multidimensional scaling (nMDS) ordination of distance matrices calculated using Jaccard’s index. All sites were used for the analysis with a total sampling effort over the whole sampling region of 15, 38 and 41 in the intertidal, shallow subtidal (0-15 m) and deep subtidal (15-30 m) zone, respectively. A stress value was also calculated for the ordination in order to judge the quality of the plot. Stress values <0.1 generally indicate good representation of the similarity matrix and a low potential of misinterpretation whereas caution should be applied when interpreting the nMDS results when stress values >0.2. Finally an analysis of similarities (ANOSIM) was used to identify statistically significant differences between species assemblages among sampling areas, considering data from all bathymetric zones pooled together, and to test for biogeographic delimitations based on the results of the nMDS analysis (see Results for more details).

RESULTS

A total of 882 specimens of the phylum Rhodophyta, 511 sequences of 5P-COI (632 bp) and 98 sequences of *rbcL* (958 bp) were obtained (Annex 1, cf. doi/10.7872/crya/v39.iss4.2018.Suppl.Mat.). For 362 of the 882 specimens (*i.e.* 41%) no sequences are available. All GenBank numbers are listed in Annex 1.

Comparison between the two methods of species identification- Fifty-one taxa in total, ranging from order to species taxonomic ranks, were detected using classical taxonomy, *i.e.* based on morphological characters only (Annex 1). The use of the integrative method allowed us to identify forty-nine taxa (Annex 1). These 49 taxa were classified as part of 11 orders and 17 families (Fig. 2, Fig. 3, Annex 1). We were unable to define the family level for a group of 8 samples for which no sequences were obtained, keeping the order level identification [*i.e.* Gigartinales (Kallymeniaceae or Palmariaceae); Annex 1]. Among the 868 algae that were determined at least at the genus level using classical taxonomy, the genus name was changed for only 16% (*i.e.* 143) of them in our integrative dataset (Annex 1). More than half (*i.e.* 76, 53%) of these 143 specimens were Bangiaceae (namely, specimens determined morphologically as *Porphyra* changed to *Pyropia* and *Wildemanian* after using molecular tools, Annex 1), a family in which genera are notoriously difficult to recognize using classical taxonomy (see for reference Sutherland *et al.* 2011).

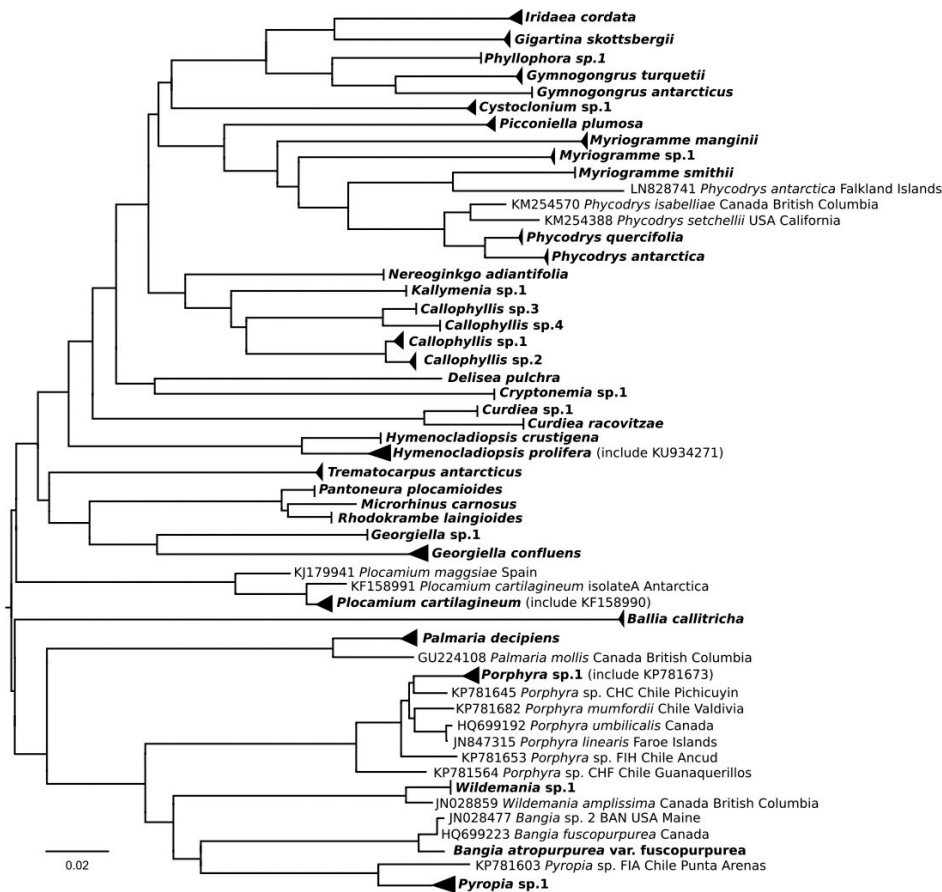


Fig. 2. Unrooted neighbour-joining (NJ) phylogenetic tree based on the COI sequences (632 bp) of Antarctic red algae. Sequences downloaded from public database are indicated by their GenBank number while specimens collected during our surveys are noted in bold. Species names, as given in the “Integrative dataset”, are noted next to collapsed branches.

No incongruence was observed between the results obtained for the COI and the *rbcL* for the 89 samples sequenced for both markers (Fig. 2, Fig. 3, Annex 1) (cf. doi/10.7872/crya/v39.iss4.2018.Suppl.Mat.). In various cases, the molecular dataset led to lumping entities recognized as different taxa using morphological criteria. For example, all specimens identified in morphological dataset as *Plocamium aff. hookeri*, *Plocamium aff. secundatum*, *Plocamium* sp. and *Plocamium cartilagineum* (Linnaeus) P.S.Dixon corresponded to a unique genetic entity determined both by COI and *rbcL* gene (Fig. 2, Fig. 3, Annex 1). Thus, all specimens were identified as *Plocamium cartilagineum* in the integrative dataset. In the same way, the COI sequences of two specimens recognized morphologically as *Gracilaria pulvinata* Skottsberg and *Rhodymenia subantarctica* R.W.Ricker were lumped within *Gymnogongrus* specimens under the name *Gymnogongrus antarcticus* Skottsberg (Annex 1). The only specimen recognized morphologically as *Trematocarpus dichotomus* Kützing was also lumped with the rest of the *Trematocarpus antarcticus*

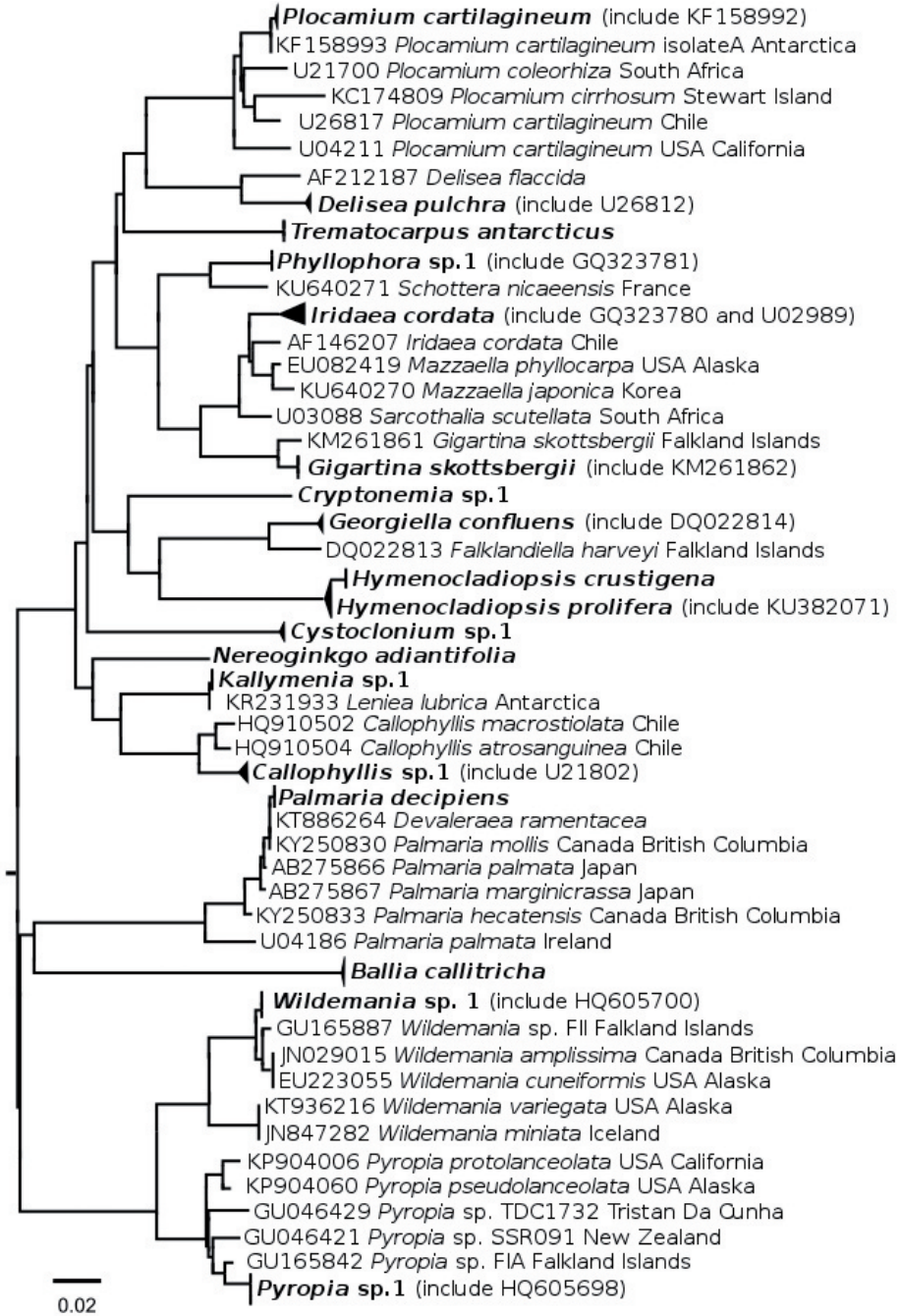


Fig. 3. Unrooted neighbour-joining (NJ) phylogenetic tree based on the *rbcL* sequences (958 bp) of Antarctic red algae. Sequences downloaded from public database are indicated by their GenBank number while specimens collected during our surveys are noted in bold. Species names, as given in the "Integrative dataset", are noted next to collapsed branches.

(Hariot) Fredericq & R.L.Moe specimens after sequencing the COI gene (Annex 1, cf. doi/10.7872/crya/v39.iss4.2018.Suppl.Mat.). On the other hand, we detected putative cryptic species in three genera: *Curdiea*, *Georgiella* and *Callophyllis*. In *Curdiea* (between *Curdiea racovitzae* Hariot and *Curdiea* sp.1) and *Callophyllis* (between *Callophyllis* sp.1, sp. 2 and between *Callophyllis* sp. 3 and sp. 4) the divergence between putative cryptic species were low, just above our 1% threshold. Five specimens of *Georgiella* showed a COI sequence divergence of 6% when compared to the rest of the specimens of *Georgiella confluens* (Reinsch) Kylin studied and were renamed *Georgiella* sp.1. Interestingly, specimens of *Curdiea* sp.1 were only found in the SSHs and the northern part of the WAP (*i.e.* KGI and OHI), whereas specimens of *Curdiea racovitzae* were found over the whole sampling region (Fig. 1, Annex 1).

Geographic distribution

Both accumulation curves for sampling effort including all sites at all depths for the “Morphological dataset” and the “Integrative dataset” were highly similar (Fig. 4). They both reached an asymptote point around 50 taxa in our study region. Species richness expressed in absolute values of the number of taxa displayed the same pattern between the “Morphological dataset” and the “Integrative dataset” (Fig. 5). The species-specific richness varied between 22 taxa in GGV to 28 taxa in PRAT (estimation computed using the “Integrative dataset”, Fig. 5). For the nMDS and ANOSIM analyses, both the “Morphological dataset” and the “Integrative dataset” gave very similar results and only the “Integrative dataset” results are presented here. The existence of three distinct groups was suggested by a separation of the three northern areas of sampling (*i.e.* KGI and PRAT in the SSHs and OHI in the northern part of the WAP), the Gerlache Strait area (GGV) and the Bellingshausen Sea (MAR) (Fig. 6).

The results of the ANOSIM analysis support this finding, with significant differences encountered between the three northern areas, and the two southern areas while no significant differences were detected between KGI and PRAT, KGI and OHI and OHI and PRAT (Table 1). The results of the ANOSIM analysis also detected a significant difference between red algal assemblages of GGV and MAR (*i.e.* the two southern sites located along the WAP, Table 1, Fig. 6).

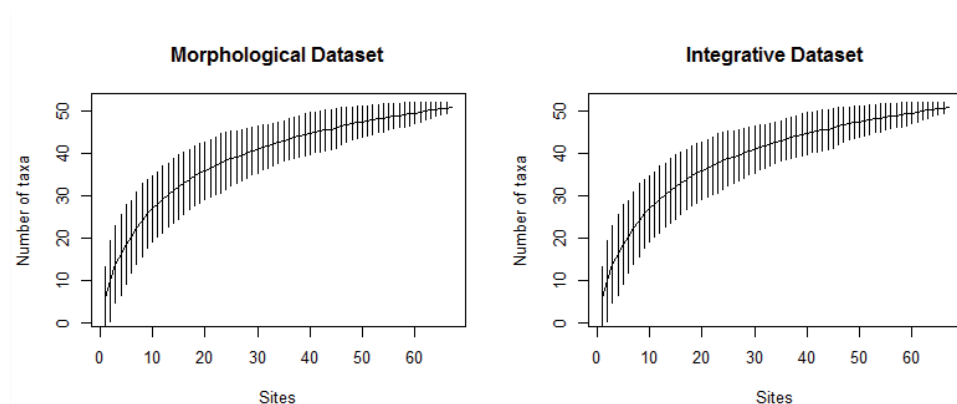


Fig. 4. Taxa accumulation curves obtained from identification based only on morphological characters (“Morphological dataset”) and from integrative identification including molecular markers (“Integrative dataset”) for red macroalgae communities sampled in our study region.

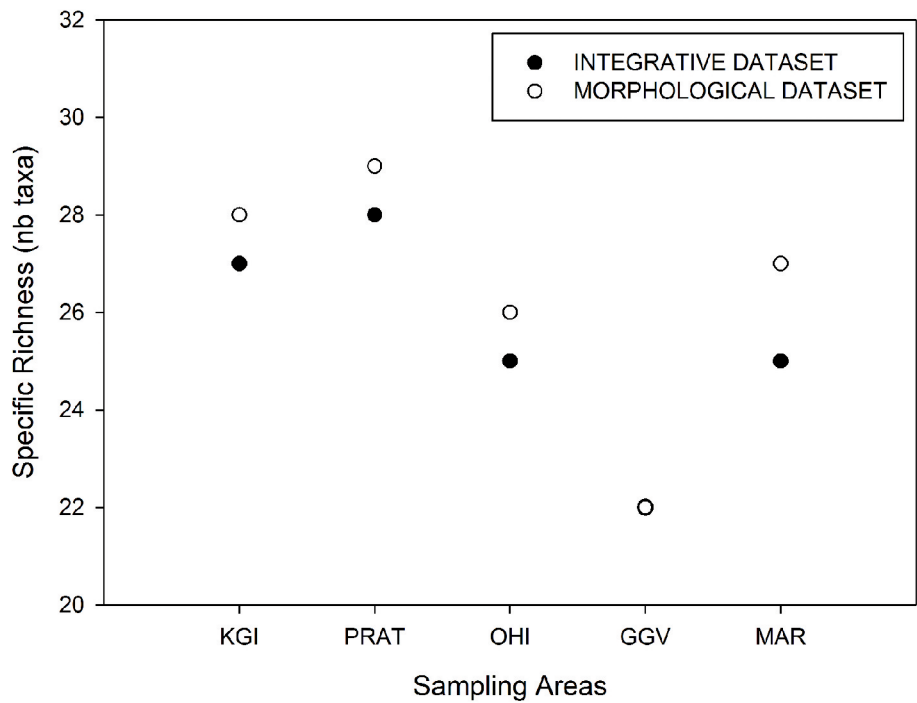


Fig. 5. Absolute values of specific richness calculated for each sampling area using the “Morphological dataset” and the ‘Integrative dataset”. For each site, information available for the three bathymetric zones was pooled together and analysed jointly.

Table 1. Results of the pairwise ANOSIM test. Matrix was calculated using Jaccard’s index and the “Integrative dataset”. Significant values are indicated with a star. Geographic areas: PRAT = Greenwich Island, KGI = King George Island, OHI = O’Higgins Antarctic base, GGV = Paradise Bay and MAR = Marguerite Bay.

Results of pairwise tests between geographic areas, all bathymetric zones pooled together		
Global R Statistic = 0.253		
Groups	R Statistic	p-value
KGI, MAR	0.390	0.001*
KGI, GGV	0.273	0.011*
KGI, OHI	0.007	0.467
KGI, PRAT	0.067	0.212
MAR, GGV	0.514	0.001*
MAR, OHI	0.405	0.005*
MAR, PRAT	0.443	0.001*
GGV, OHI	0.256	0.01*
GGV, PRAT	0.172	0.025
OHI, PRAT	0.151	0.096

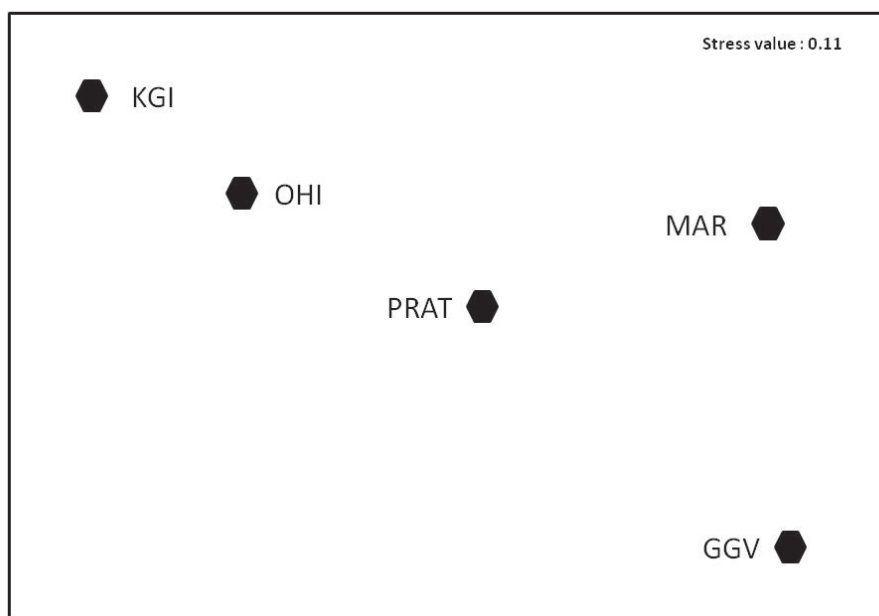


Fig. 6.

DISCUSSION

Our work is the first study where integrative taxonomy, coupling information from morphological characters and molecular tools, has been consistently used to study red macroalgal diversity along the coasts of the South Shetland Islands and the Northern and Central part of the Western Antarctic Peninsula. Our qualitative sampling allowed us to register around 50 taxa over the whole sampled region, being consistent with previously registered number of species in these areas (Wiencke & Clayton, 2002; Pellizarri *et al.*, 2017). Our complementary identification method (*i.e.* “Integrative Dataset”), based on both morphology and molecular markers, confirmed that the region is characterized by a low specific diversity level; nevertheless, it also allowed us to detect putative cryptic species within three genera: *Curdiea*, *Georgiella* and *Callophyllis*. *Georgiella* had been reported previously as a monotypic genus restricted to Antarctic waters (including some islands of the Scotia Arc, Wiencke *et al.*, 2014). Finally, our results allowed us to identify significant differences between red algal assemblages of three distinct geographic sub-regions around the South Shetland Islands and the Western part of the Antarctic Peninsula, following a latitudinal gradient: SShs and Northern part of the WAP (*i.e.* all areas located on both side of the Bransfield Strait: KGI, PRAT and OHI, 63°S), Central part of the WAP (GGV, 64°S) and Center-Southern part of the WAP (MAR, 67°S).

Three major biogeographic sub-regions across South Shetland Islands and West Antarctic Peninsula

In our study, significant differences between red macroalgal assemblages were detected between three geographic sub-regions: the South Shetland Islands and

the Northern West coast of the Antarctic Peninsula (*i.e.* KGI, PRAT and OHI); the Gerlache Strait (*i.e.* GGV) and Marguerite Bay located within the Bellingshausen Sea (*i.e.* MAR). Some widespread species, as *Curdiea racovitzae*, *Georgiella confluens*, *Gigartina skottsbergii* Setchell & N.L.Gardner, *Iridaea cordata* (Turner) Bory de Saint-Vincent, *Myriogramme manginii* (Gain) Skottsberg, *Palmaria decipiens* (Reinsch) R.W.Ricker, *Plocamium cartilagineum* and *Trematocarpus antarcticus* were common to the three identified sub-regions. Nevertheless, some species were found to be characteristic of each of the different geographic sub-regions: bladed Bangiales (both *Porphyra* sp.1 and *Pyropia* sp.1), *Cystoclonium* sp.1 and *Phycodrys antarctica* Skottsberg were recorded only in the South Shetland Islands and the Northern West coast of the Antarctic Peninsula; *Delisea pulchra* (Greville) Montagne and *Kallymenia* sp.1 were recorded only in the Gerlache Strait and *Hymenocladopsis crustigena* R.L.Moe and *Notophycus fimbriatus* R.L.Moe were recorded only in Marguerite Bay. *Callophyllis* sp.4, *Cryptonemia* sp.1 and *Myriogramme smithii* (J.D.Hooker & Harvey) Kylin were limited to the two most southern areas of GGV and MAR. Species distribution uncovered in our study remains subject to sampling limitations and some species could have been overlooked during our 2013 and 2014 field surveys. This could especially be the case within the two bio-regions of the Gerlache Strait and the Bellingshausen Sea, each of them only being represented by one sampling area and a reduced sampling effort (~15 days in 2014). For example, both *Hymenocladopsis crustigena* and *Notophycus fimbriatus*, recorded only in the Bellingshausen Sea in our study (*i.e.*, MAR) where first described along the coastlines of Anvers Island and Argentine Island, respectively (Hommersand *et al.*, 2009). Anvers and Argentine Islands are located within the Gerlache Strait at less than 100 km of GGV. The absence of these two species from our GGV samples could be more related to local oceanic and climatic conditions in our sampling area (GGV is a much more sheltered area than the coasts of Anvers and Argentine Islands) than to a clear separation of the Central part of the WAP and Center-Southern part of the WAP in distinct geographic sub-regions. Even if significant, separations between bioregions have then to be taken with caution and new sampling localities in the Gerlache Strait and the Bellingshausen Sea will be needed to confirm our results.

Our three detected biogeographic sub-regions do not correspond to the ones proposed by Linse *et al.* (2006) or to the marine ecoregions proposed by Spalding *et al.* (2007). In these works the South Shetlands Islands were distinguished from the Center/North of the Western Antarctic Peninsula, considered as a single region. Moreover, the environmental domains and expert-defined bioregions described by Terauds *et al.* (2012) separate the Center/North of the Western Antarctic Peninsula in three distinct latitudinal zones: North-east Antarctic Peninsula (where OHI is located, 63°S), North Antarctic Peninsula (where GGV is located, 64°S) and South Antarctic Peninsula (where MAR is located, 67°S). For red macroalgae we did not detect the proposed separation between the North-east Antarctic Peninsula (OHI) and the South Shetlands Islands (KGI and PRAT) (Terauds *et al.*, 2012), suggesting that the Bransfield Strait is not a strong biogeographic barrier for these organisms. In the same way, the previous work of (Griffiths & Waller, 2016) reported a high overlap in terms of intertidal species composition (27% of species) between King George Island and the Antarctic Peninsula (considering both invertebrates and macroalgae).

Potential processes underpinning the biogeography of Antarctic macroalgae

At large scale, macroalgal community structure has been deemed to be primarily determined by both historical factors and the present dynamics of currents and sea-ice disturbance (Spalding *et al.*, 2007; Pellizzari *et al.*, 2017). Historical effects of Antarctic species range contraction in refugia during glacial periods and subsequent allopatric divergence and speciation could have led to regional differences in species community structure. Indeed, cryptic species and/or genetic lineages have been detected in a wide array of marine animals (see Allcock & Strugnell, 2012 for review) and, for most species complex, allopatric speciation while in isolation in small refugia along the Antarctic shelf has been hypothesized to be at the origin of this unsuspected diversity. It is possible that the slight, but significant, differences in red algal community structure detected in our study between the three distinct latitudinal zones could be linked to the historical glacial legacy of the region. However, within the red algal samples under study, we detected only a low amount of cryptic species and the pattern encountered for community structure was not mirrored by specific richness. Indeed, no clear gradient of taxa diversity was observed, with 28 taxa recovered in KGI, our northernmost sampling point, and 27 taxa recovered in MAR, our southernmost sampling point (considering species richness calculated with our “Integrative dataset”). Moreover, no genetic structure was observed among our three detected biogeographic sub-regions for six common species of red algae sampled from KGI to MAR (*i.e.* *Curdiea racovitzae*, *Iridaea cordata*, *Georgiella confluens*, *Gigartina skottsbergii*, *Palmaria decipiens* and *Plocamium cartilagineum*; Guillemin *et al.*, 2018). This result has been related with the drastic effect of genetic bottleneck during glaciation and subsequent expansion while rafting on the strong currents flowing along the coast of the South Shetlands Islands and the Western Antarctic Peninsula (Guillemin *et al.*, 2018). Indeed, Griffiths & Waller (2016) already reported the importance of rafting in explaining the existence of wide-ranging macroalgae shaping in great extent the biogeography of Antarctic and Sub-Antarctic intertidal communities in the Southern Ocean. In the same way, fronds drifting on the cyclonic circulation inside the Bransfield Strait (Savidge *et al.*, 2009) could easily lead to recurring connectivity between the North-east Antarctic Peninsula (OHI) and the South Shetlands Islands (KGI and PRAT). At last, since our three sub-regions correspond to three latitude levels, the dynamic of sea-ice disturbance in the present day could also partly shape the observed differences in red algal communities (Clarke *et al.* 2007, Terauds *et al.*, 2012). Indeed, the distribution of some species seems limited to the northern most areas of our sampling region, as for example *Porphyra* sp.1 and *Pyropia* sp.1 encountered only in the high intertidal zone of the three milder areas sampled.

Species richness has been described to decline with increasing latitude along the Antarctic Peninsula (Moe & Delaca, 1976), a pattern commonly explained by an increasing effect of ice scour and encasement at high latitudes (Waller, 2008). Indeed, these results seem well supported by the study of diversity of macroalgal communities following a glacial retreat in King George Island (Quartino *et al.*, 2013), where species diversity correlated positively with time elapsed since substrate availability for recolonization. In our study no clear gradient of taxa diversity was observed, even if slightly less taxa can be observed in the southern part of our study region (*i.e.* GGV and MAR) than in the SSHs (*i.e.* PRAT and KGI). The low species richness in OHI may be explained by specific and local limitations (e.g. substrate availability, oceanic conditions increasing time of ice encasement or probability of ice scouring) blurring possible observation of an existing latitudinal gradient.

Nevertheless, variation in level of ice disturbance could still explain the strong difference between the intertidal and the subtidal communities in our study (Mystikou *et al.*, 2014; Valdivia *et al.*, 2014; Marcias *et al.*, 2017). Indeed, at least 2.5 more taxa were encountered in the subtidal zone than in the intertidal zone, whatever the area under study, and this ratio was the most extreme in MAR (Bahia Margarita) where the subtidal zone was eight times more diverse than the intertidal zone (Annex 1). Only *Palmaria decipiens* and *Iridaea cordata* were commonly encountered in the intertidal zone of the five areas under study, two species that were reported to be able to survive to extreme environmental conditions and were even recorded in the Ross Sea (Wiencke & Clayton, 2002) and the continental coasts of East Antarctica (Wiencke *et al.* 2014).

Integrative identification as a tool to study Antarctic macroalgal diversity and biogeography

Morphological delimitation of macroalgal species commonly faces problems linked with a lack or plasticity of characters and morphological stasis (Leliaert *et al.*, 2014). This has led to taxon misidentification, incorrect taxon delineation and a global underestimation of floral diversity, a phenomenon also affecting our understanding of Antarctic macroalgal community structure (Moniz *et al.*, 2012; Young *et al.*, 2013; Pellizzari *et al.*, 2017). In our study, some molecular data contrasted with morphological data, leading to 1) the detection of uncovered cryptic diversity and 2) of the lumping under the same integrative name of various morphologically different (polymorphic) taxa, showing very little to no genetic divergence. In previous studies, acquisition of molecular data sets has led to both lumping (González-Wevar *et al.*, 2010) and splitting (see review in Kaiser *et al.*, 2013) morphologically recognized Antarctic taxa. However, simple approximation based on unique divergence threshold adopted to delimit taxa, as in our study (*i.e.*, sequences considered as characteristic of distinct species when separated by more than 1% of divergence for the 5P-COI), only represents a first step in the estimation of red macroalgal diversity in Antarctica. This relatively rapid and low-cost method is usually used for large-scale barcode-based identification whereas combined species delineation approaches, based on sequences from multiple independent genes, are recommended for reliably identifying species (e.g. Montecinos *et al.*, 2017). Complementary studies will be needed to confirm the existence of cryptic species in the genera *Curdiea*, *Georgiella* and *Callophyllis*.

Most studies where cryptic species were detected show that cosmopolitan species are much less common than previously reported within Antarctic communities (Kaiser *et al.*, 2013; see also De Wever *et al.*, 2009 for an example of microchlorophyte cryptic species). These observations have raised questions about our capacity to clearly measure species diversity and detect fine-scale biogeographic divisions in taxa where classical taxonomy is problematic (Kaiser *et al.*, 2013). However, contrasting with Robuchon *et al.* (2015), the use of integrative taxonomy in our study did not lead to an increase in alpha-diversity in Antarctic red algae. Molecular data mostly led to lumping entities previously recognized as different taxa using morphological criteria, ultimately reducing the number of taxa in the “Integrative dataset” when compared to the “Morphological dataset” (51 and 49 taxa, respectively). Interestingly, in the case of the genus *Plocamium*, all samples were lumped under the name *Plocamium cartilagineum*, supporting the doubts raised by Hommersand and collaborators (2009) about the validity of the species *Plocamium hookeri* and *Plocamium secundatum*. Even if the complementary use of molecular data and

classical taxonomy in our work had had little impact on results obtained about diversity and structure of communities it had, nonetheless, improved level of species identification (98% of our specimens in the “Integrative dataset” were determined at species level, while only 89% of them had been determined at species level in the “Morphological dataset”).

The relative low availability of sequences allowing comparisons of Antarctic macroalgae within databanks, still prevented us going further than the genus on specimen name attribution in some cases. We believe that the construction of accessible genetic dataset associated with voucher specimens in the present work will benefit future studies of macroalgal diversity and distribution in the region. The 49 taxa of red macroalgae detected in the “Integrative dataset”, with more than 20 taxa per area, are consistent with the number of species previously listed for the region. A total of 75 species of red macroalgae has been listed in Wiencke & Clayton (2002) for the whole Antarctic waters while only 21 has been reported on King George Island (Oliveira *et al.*, 2009), 27 for the Marguerite Bay region (Mystikou *et al.*, 2014) and 46 along the coasts of the South Shetland archipelago (*i.e.* excluding Corallinaceae, Pellizzari *et al.*, 2017). Nevertheless, it is worth to note that our work represents only an incomplete view of the Antarctic red macroalgal diversity since molecular data has been obtained for only 59% of our collected specimens and some families have been particularly difficult to sequence (*i.e.* Delesseriaceae, Kallymeniaceae; less than 55% of specimens with molecular data; Annex 1, cf. doi/10.7872/crya/v39.iss4.2018.Suppl.Mat.). For example, we did not detect the divergent genetic group named “*P. cartilagineum* isolate A” reported in the study of Young *et al.* (2013) even with molecular data obtained for 65 of our 88 samples of *Plocamium*.

CONCLUSION

Our work is the first attempt to document red seaweeds diversity along the Antarctic Peninsula and the South Shetlands Islands coasts using integrative taxonomy. It highlighted the importance of using complementary tools to lead biodiversity surveys even in areas considered as well known, as cryptic diversity and morphology-based assignation errors can be underlined. On the contrary to our expectations, molecular tools did not increase alpha-diversity, but allowed us to be more accurate when identifying species. Errors in naming species can have strong implications when studying biogeographic patterns or species role in ecological processes (Bortolus, 2008) and integrative taxonomy can clearly offers better possibilities to understand structure and dynamics of marine benthic ecosystems. We believe that the collection of COI and *rbcL* sequences associated to voucher specimens of red macroalgae sampled in our study region (*i.e.* from the SSHs down to the center-southern of the WAP) can be used as a platform to generate new phylogenetic, ecological and biogeographical studies in Antarctica and the Southern Ocean. Indeed, Antarctic macroalgae are extremely stenothermic organisms for which rapid climatic changes in the WAP could affect resilience and distribution and more work will be needed to clarify their actual diversity and distribution and test for potential changes depending on future scenarios (Wiencke *et al.*, 2014). Human activities could also add to threats linked to global climatic changes. Economic interests in sub-Antarctic and Antarctic marine communities have grew recently and,

as red macroalgae offers great economic prospects for molecules extraction, they could be particularly at risk.

Acknowledgments. This research was supported by the Instituto Antártico Chileno (INACH) T_16-11 and RG_15-16 projects and sampling in King George Island was funded by the Project Anillo ART1101 from Comisión Nacional de Investigación Científica y Tecnológica. Additional support came from the International Research Network “Diversity, Evolution and Biotechnology of Marine Algae” (GDRI N° 0803) and from the Centro FONDAP IDEAL no. 15150003. While writing NV was supported by FONDECYT grants no. 1161699 and 1181300. The authors thank P. Brunning, I. Garrido, J. L. Kappes, T. Heran, Y. Henriquez, and L. Vallejos for their help in the field. The authors would also like to thank the Chilean Navy (especially the captain and crew of the ships Almirante Oscar Viel and Lautaro), the staff from the Chilean Army in the O’Higgins base and the Air Force of Chile (FACH) for the logistic support of our fieldwork in sub-Antarctica and Antarctica.

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