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COMPARATIVE PHYLOGEOGRAPHY OF TWO *AGAROPHYTON* SPECIES IN THE NEW ZEALAND ARCHIPELAGO[1]

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Abstract

Molecular studies have reported the co-existence of two species of *Agarophyton* in New Zealand: the newly described *A. transtasmanicum* with an apparently restricted distribution to some sites in the North Island, and the more wide-spread *A. chilense.* Here we compared the distribution, genetic diversity, and structure of both *Agarophyton* species throughout the archipelago using sequences of the nuclear Internal Transcribed Spacer 2 (ITS2) marker. *Agarophyton chilense'*s distribution was continuous and extensive along the North and South Islands, Stewart Island, and Chatham Island, and the genetic clusters were mostly concordant with boundaries between biogeographic regions. In contrast, specimens of *A. transtasmanicum* were collected in four sites broadly distributed in both the North and South Islands, with no clear spatial structure of the genetic diversity. Populations where the species co-occurred, tended to display similar levels in genetic diversity for the two species. Demographic inferences supported a post-glacial demographic expansion for two *A. chilense* genetic clusters, one present in the South Island and the eastern coast of the North Island, and the other present in northern South Island. A third genetic cluster located on the western coast of the North Island had a signature of long-term demographic stability. For *A. transtasmanicum*, the skyline plot also suggested a post-glacial demographic expansion. Last, we developed a new molecular tool to quickly and easily distinguish between the two *Agarophyton* species, which could be used to ease future fine-scale population studies, especially in areas where the two species co-exist.

Keywords: biogeographic and oceanographic barriers, demography, glacial-interglacial

cycles, Gracilariales, ITS2, parapatric genetic clusters, species co-occurrence, species diagnostic molecular tool.

Abbreviations: ITS2, Internal Transcribed Spacer 2; SSD, Sum squared deviations; BSP, Bayesian skyline plots; LGM, Last Glacial Maximum; AMOVA, Analysis of Molecular Variance; SAMOVA, Spatial Analysis of Molecular Variance; MCMC, Markov Chain Monte Carlo.

INTRODUCTION

A large proportion of marine algae in New Zealand are endemic (e.g., up to 40% of Rhodophyceae, Nelson 2012), which is considered to be a consequence of *in situ* radiations of ancient Gondwanan lineages after the separation of Zealandia from Australasia, and from taxa that colonized New Zealand after the Oligocene marine transgression, during which most of the archipelago was submerged (McDowall 2008, Neall and Trewick 2008, Sharma and Wheeler 2013, Wallis and Jorge 2018, McCulloch and Waters 2019). However, a number of marine species present in New Zealand also inhabit other regions of the southern hemisphere, such as Australia, sub-Antarctic islands, and South America (Waters 2008, Fraser et al. 2009b, Fraser et al. 2013, Guillemin et al. 2014). Phylogeographic studies have suggested that many of these disjoint distributions have been the result of west to east trans-oceanic dispersal at the end of the Pleistocene (Waters and Roy 2004, Waters et al. 2006, Waters 2008, Fraser et al. 2009b, Fraser et al. 2013, Guillemin et al. 2014).

The genus *Agarophyton* (Gracilariales) is one example of an algal taxon present across the South Pacific: *A. chilense* is distributed in New Zealand including Chatham Island, and South America, while *A. transtasmanicum* is present in mainland Australia, Tasmania, and New Zealand (Candia et al. 1999, Cohen et al. 2004, Guillemin et al. 2014, Preuss et al. 2020). The order Gracilariales is well represented in New Zealand, including eleven species from the genera *Agarophyton, Crassiphycus, Curdiea,* and *Melanthalia* (Gurgel et al. 2018, Neill & Nelson 2019, Preuss et al. 2020). Members of the Gracilariales possess an isomorphic haploid-diploid life cycle (Gurgel et al. 2018) and reproduction is either sexual or asexual (Santelices and Doty 1989). During sexual reproduction, spores (both haploid and diploid) develop into morphologically identical thalli attached by holdfasts to hard substrates (Kain and Destombe 1995). Asexual reproduction results from the survival and growth of thallus fragments separated from their holdfast. Such clonal propagation allows the species to form dense mats embedded in soft substrate, in protected bays or estuaries. The capacity to switch between sexual and asexual reproduction has contributed to the success of the invasive *A. vermiculophyllum* (Krueger-Hadfield et al. 2016) and to the extensive cultivation of *A. chilense,* in Chile (Santelices and Doty 1989, Buschmann et al. 2001). Their unattached thalli generally correspond to diploid tetrasporophytes growing vegetatively (Guillemin et al. 2008, Krueger-Hadfield et al. 2016). Various ecological traits further explain the success of these species, including a fast growth rate, low palatability in non-native habitats, and tolerance to a wide range of light and salinity conditions (Hammann et al. 2013, Hu and Lopez-Bautista 2014, Kim et al. 2016).

Two terete species of *Agarophyton* coexist in New Zealand: *A. chilense* and *A. transtasmanicum*. *Agarophyton chilense* grows in estuarine environments and along the open coast in sites characterized by moderate wave exposure (Nelson 1987, Nelson et al. 2015). Populations are usually found attached to cobbles, shells and sometimes on rocky substrates, but large agglutinations of entangled thalli have also been occasionally reported on soft substrates (e.g., mud or sand; Nelson 1987, Nelson et al. 2015). *Agarophyton transtasmanicum* shares the same habitat. Its floating thalli can be found in sandy/muddy substrates of highly protected bays, and only a few individuals are found growing on cobbles and rocks as the result of spore settlement (Wilcox et al. 2001, 2007, Nelson et al. 2015). In the absence of external diagnostic characters (Preuss et al. 2020), *A. transtasmanicum* was first identified from phylogenetic studies as a sibling lineage of *A. tenuistipitatum*, and genetically divergent from *A. chilense* (Candia et al. 1999, Cohen et al. 2004). *Agarophyton transtasmanicum* was reported among samples from Blockhouse Bay in New Zealand (near Auckland; Candia et al. 1999), mainland Australia and Tasmania (Byrne et al. 2002, Cohen et al. 2004, Preuss et al. 2020). The main phenotypic difference between *A. transtasmanicum* and *A. chilense* is related to the number of medullary layers and cortex-to-medulla transition (Preuss et al. 2020). A further difference between the two species was also demonstrated by the presence of gigartinine in the former species but not in the latter (Wilcox et al. 2001, 2007). Samples of *A. transtasmanicum* attached to rocky substrate were also observed in the southern west coast of North Island (e.g., the Whanganui and Manawatu River mouths), leading to the idea that the species' distribution range in New Zealand may still be underestimated (Preuss et al. 2020).

Gigartinine is an amino acid likely acting as a nitrogen reserve allowing sustained growth between nitrogen pulses (Laycock and Craigie 1977). Wilcox et al. (2007) proposed that the quick accumulation of nitrogen in form of gigartinine (in a few hours) by *A. transtasmanicum* could confer an ecological advantage over *A. chilense* in terms of nitrogen storage and ultimately of growth rate. Because the two species seem to occupy the same habitat, such an ecological advantage could lead to the replacement of *A. chilense* by *A. transtasmanicum*. The existence of such an ecological advantage is not supported by current field reports, since most of the known distribution *Agarophyton* along New Zealand's coasts is currently attributed to *A. chilense*. However, the lack of external features to distinguish the two species has likely biased these records (Preuss et al. 2020) and the actual distribution range of both species remains to be determined. Thus, the aims of this study were 1) to investigate the range of the distribution of *A. chilense* and *A. transtasmanicum* in the New Zealand archipelago based on genetic identification; 2) to compare patterns of genetic diversity, structure, and past demographic inferences of *A. chilense* and *A. transtasmanicum* to study co-existence of the two species from an evolutionary perspective; and 3) to develop an identification toolkit based on based on species-specific genetic markers to easily distinguish between these two species in future studies.

MATERIALS AND METHODS

Sampling

Because morphological distinction between the two species was not possible in the field, we performed a random sampling in two types of habitats: pebbles, seashells, or rocky substrate on which thalli could settle and attach to, versus soft substrates on which only floating vegetative fragments were present. A total of 559 individuals of *Agarophyton* were collected from 25 sites throughout New Zealand, including the North and South Islands, Stewart Island, and Chatham Island (Table 1).

Collections were made under a special permit (N°666) to NIWA from the New Zealand Ministry of Primary Industries. All samples were stored in plastic bags with silica gel to preserve the tissue before DNA extraction. Voucher specimens were deposited in the herbarium of the Museum of New Zealand Te Papa Tongarewa (WELT – Thiers 2019).

DNA extraction, PCR amplification, sequencing, and alignment

Genomic DNA was isolated from pulverized dry tissue using the Chelex method following Cohen et al. (2004). The Internal Transcribed Spacer 2 (ITS2) was amplified using the primers CD12F (5´-TACAAATTGGACTTTGGCATTCTGGG-3´; Wattier et al. 1997) and AB28 (5´-GGGATCCATATGCTTAAGTTCAGCGGGT-3´; Goff and Moon 1993). Amplification reactions (30 µL) contained 1x reaction buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.3 µM of each primer, 1 U Taq DNA polymerase (Thermo Fisher Scientific, Inc.), and 3 µL (ca. 50nM) of template DNA. The PCR cycles were performed as described by Cohen et al. (2004). PCR products were purified using the UltraClean™ DNA Purification kit (MO BIO Laboratories, Carlsbad, CA, USA) and sequenced, using both PCR primers, with an ABI Automatic Sequencer at the AUSTRAL-OMICS core-facility

(Universidad Austral de Chile, Chile). Sequences were edited using Chromas v.2.33 (McCarthy 1997) and the sequences' alignments were made with AliView (Larsson 2014). Sequences of low frequency ribotypes were independently read twice, and any difficulty identifying variable sites led to sample re-sequencing. Within each species, indels were manually recoded as substitutions, considering one mutational event for each indel or group of consecutive indels.

Ninety-three sequences of *A. chilense* published by Guillemin et al. (2014) were added to our ITS2 data set. In total, 652 ITS2 sequences obtained from 27 sites were available for phylogeographic analyses (Table 1). Twenty of these sites were on hard substrates with settled individuals, while seven sites were soft substrates colonized by floating thalli (Table 1). Species assignment was achieved by comparing ITS2 sequences obtained in this study with the ones already published in Cohen et al. (2004).

Phylogeographic structure and population genetic diversity

For each species, genealogical relationships between ITS2 ribotypes were reconstructed using Median-Joining implemented in NETWORK v5.0.1.1 (Bandelt et al. 1999). Population genetic structure was examined by calculating pairwise Φ_{ST} between populations within each species; and significance of the Φ_{ST} values was estimated using 1,000 permutations (Excoffier et al. 1992). For each *Agarophyton* species independently, Analysis of Molecular Variance (AMOVA) was used to test for the partition of genetic variance among sites and among bioregions. The bioregions were defined using the Coastal Biogeographic Regions' Classification map provided by the Ministry for the Environment

of New Zealand (http://www.mfe.govt.nz/more/science-and-data/classification-systems/marine-classification-systems). Additionally, the existence of differentiated genetic clusters in *A. chilense* was evaluated by Spatial Analysis of Molecular Variance implemented in SAMOVA 2.0 (Dupanloup et al. 2002). This analysis allows the identification of groups of spatially contiguous populations by maximizing the among-group component (F_{CT}) of the overall genetic variance. SAMOVA was performed using 1,000 simulated annealing procedures for *K*-values ranging from 2 to 10 groups. Configurations containing groups with a single population were not considered. An AMOVA was then used to estimate the partition of genetic variance among sites and among genetic clusters. Tests for isolation by distance within species were performed using Mantel tests. All analyses were performed in ARLEQUIN v3.5 (Excoffier and Lischer 2010).

The genetic diversity of *A. chilense* and *A. transtasmanicum* was evaluated using five indices, calculated within each sampling site, within each genetic cluster, and within each species. The number of ribotypes (nR), gene diversity (H), nucleotide diversity (π), number of private ribotypes (Rpriv), and number of polymorphic sites (S) were calculated in DNASP 4.10.3 (Rozas et al. 2003). The number of ribotypes corrected for differential sample size (nRrar) was estimated using the rarefaction method developed in CONTRIB 1.02 (Petit et al. 1998). A rarefaction size of eight was used for estimating nRrar at each site in both species. The rarefaction size corresponds to the lowest number of sequences available in any sampled locality: GOLD in *A. chilense* and OHOP in *A. transtasmanicum* (Table 1). In *A. transtasmanicum*, the locality HINA was excluded from the rarefaction analysis due to its small sample size (N = 3, Table 1). Mann-Whitney U-tests were

performed to test for significant differences of genetic diversity (indices nRrar, H, and π were used) between the *Agarophyton* species in R Studio v1.2.1335 (R Core Team, 2019).

Demographic history

Tajima's D (Tajima 1989) and Fu's F (Fu 1997) tests were used to assess significant excess of rare alleles, performing 10,000 bootstrap replicates in ARLEQUIN v3.5 (Excoffier and Lischer 2010). Observed mismatch distributions, the distribution of the numbers of pairwise differences among all ribotypes, were fitted to a model of demographic growth coupled with spatial expansion (Ray et al. 2003) and its statistical significance was tested with the sum of squared deviations (SSD) statistic after 10,000 bootstraps. Bayesian skyline plots (BSP) were constructed using BEAST v2.5.1 (Drummond et al. 2012). The optimal nucleotide substitution model for ITS2 was estimated using MODELGENERATOR (Keane et al. 2006). Based on the Bayesian Information Criterion (BIC), the substitution models selected were F81 for *A. chilense* and F81 + I + G for *A. transtasmanicum*. Bayesian skyline plots were constructed with 50 million iterations. Posterior distributions of parameters were approximated by Markov Chain Monte Carlo (MCMC) sampling, with samples drawn every 10,000 iterations after a discarded burn in of the first 5 million iterations. A log-normal, relaxed molecular clock was used, with a mutation rate 10 times higher (Ho et al. 2011) than the substitution rate of 0.71% - 0.83% per million years estimated for ITS1 (Zuccarello and West 2002) to test for historical changes of effective population size.

Development of a molecular tool to identify cryptic Agarophyton *species in New Zealand*

A partial sequence of the mitochondrial cytochrome c oxidase subunit I gene (5P-COI) was obtained for a subset of *A. chilense* and *A. transtasmanicum* specimens. Seventeen specimens of *A. chilense* from New Zealand (sample sites: AHUR, BRIG, CASS, CHAT, HINA, MOMO, MOUT, OHOP, OKAR, PARE, OTAG, WAIN, STEW, WITI, SHEL, and RAGL; population abbreviations as in Table 1) and five specimens from Chile (sample sites: FCAL, FLEN, FTUB, NDIC, and NTUB; see Table 1 from Guillemin et al. 2008 for more information about sampling sites) were sequenced. For *A. transtasmanicum*, 10 specimens were sequenced: four from the North Eastern region (OHIW and OHOP), four from the North and South Cook Strait (WANG and MOIN), and two from the Southern South Island (CALA) (two specimens sequenced per site; population abbreviations and bioregions as in Table 1). The PCR conditions and primers (i.e., GaZF1 and GaZR1) described by Saunders (2005) were used for all individuals of both species.

Two consensus sequences, one for each species, were generated using GENEIOUS (http://www.geneious.com) to design new specific primers. First, a primer pair was designed in the highly conserved domain of the 5P-COI in order to amplify a positive control of the PCR in both species. These primers were Agarophyton_COI_F (5'-CACCTACTTCTACAATTGCTGATG -3') and Agarophyton_COI_R (5'-ATGGTAATGCCTGTWATGATTGG -3'). Second, two species-specific forward primers were designed in the 5P-COI region where a high number of fixed polymorphic sites discriminated between the two species: Agarophyton_chilense_COI_F (5'-

CTAAAACAGGAACAGCTAATAGTAG -3') and Agarophyton_transtasmanicum_COI_F (5'- AACAGGGACAGCTAATAACAATAG -3'). The predicted sizes of the PCR products were 160 base pairs (bp) for Agarophyton_COI_F / Agarophyton_COI_R, 403 bp for Agarophyton_chilense_COI_F / Agarophyton_COI_R, and 399 bp for Agarophyton_transtasmanicum_COI_F / Agarophyton_COI_R.

In order to test the primers' ability to distinguish between the two *Agarophyton* species, two multiplex PCRs were performed for each sample. PCRs were carried out in a final volume of 10 µl containing 1X buffer (Go Taq Flexi, Promega; Madison, USA), 0.5 µM of each internal control primer (Agarophyton_COI_F and Agarophyton_COI_R), 0.5 µM of one of the two species-specific primers (Agarophyton_chilense_COI_F or Agarophyton_transtasmanicum_COI_F), 1.5 mM $MgCl_2$, 0.15 mM dNTPs, 0.35 U Go Taq Flexi polymerase (Promega; Madison, USA), and ~5 ng of DNA template for each reaction. DNA amplifications were performed using an initial denaturation phase at 94°C for 5 min, followed by 40 cycles with a 30 s denaturation at 95°C, a 1 min annealing at 56°C, and a 1 min extension at 72°C, with a final extension at 72°C for 10 min. Amplified fragments were visualized under UV light after electrophoresis on 2% agarose gels stained with GelRed™ (Biotium, Fremont, USA).

RESULTS

A total of 652 ITS2 sequences, with an alignment length between 479 bp - 483 bp and 494 bp - 497 bp, were obtained for *A. chilense* and *A. transtasmanicum*, respectively. The alignment of the newly obtained sequences with those already published in Cohen et

al. (2004) confirmed the presence of the two species. In total, 556 samples were classified as *A. chilense* and 96 samples as *A. transtasmanicum* (Table 1). Among the *Agarophyton* collected in New Zealand, 72.5% were collected attached to hard substrates (rocks, cobbles, shells) by a holdfast, while 27.5% were collected unattached, embedded in muddy substrate (Table 1).

Agarophyton chilense had a wide geographical distribution and was observed in all bioregions but was absent from three sampling sites, namely: Whanganui (WANG, attached thalli), in North Cook Strait, Ohiwa (OHIW, unattached thalli) in North Eastern, and Moutere Inlet (MOIN, unattached thalli) in South Cook Strait. The species was present attached to hard substrates in sites located nearby OHIW and MOIN, in Ohope (OHOP) and Moutere Inlet (MOUT), respectively (Table 1). The species was also encountered attached on rocky/shell substrates in 19 sites, and unattached, embedded in mud in five sites (Table 1). *Agarophyton transtasmanicum* was found in four regions, attached to hard substrates in three sites and unattached on soft substrate in another three sites. Attached individuals represented 78% of our sampling in *A. chilense* and 57% in *A. transtasmanicum* (Table 1). These proportions differed significantly from expected frequencies under random sampling in each substrate type (over the whole study, 72.5% of samples were expected to be attached to hard substrates given the number of samples and the number of sites with soft or hard substrate available; $X^2 = 7.58$ for *A. chilense*; and $X^2 = 44.35$ for *A. transtasmanicum*; critical value $X^2_{(0.05,1)} = 3.84$). The species co-occurred in three sites. In Ohope (OHOP) and Hinahina (HINA), individuals of both species were sampled attached to rocky/shell substrates, while in Catlins Lake (CALA) the individuals of both species

were sampled unattached, forming dense mats in muddy substrates. In the South Cook Strait, *A. chilense* was found on hard substrate in Moutere Inlet (MOUT) while *A. transtasmanicum* thalli were floating in MOIN, the two sites being only a few meters apart.

Intraspecific genetic diversity and structure

Among the 556 individuals of *A. chilense* sampled throughout New Zealand, 45 ITS2 ribotypes were detected (Fig. 1, see also Table S1). A total of 42 polymorphic sites were observed in the 483 bp fragment, including four indels of 1 bp or 2 bp in length (Table 1; Fig. 1; GenBank accession numbers MN145944-MN145972, see Table S2). Ribotype r01 was the most frequent in New Zealand (60%, 333 of the 556 *A. chilense* ITS2 sequences) and the only one shared with Chilean populations (Guillemin et al. 2014). Among the 96 individuals of *A. transtasmanicum* sampled, ten ITS2 ribotypes were detected (Fig. 2, see also Table S3). In the case of *A. transtasmanicum*, a total of four polymorphic sites were observed in the 496 bp fragment, including two indels of 1bp (Table 1; Fig. 2; GenBank accession numbers MN145973-MN145982, see Table S2). At the species level, ribotype richness after rarefaction (nRrar), gene diversity (H), and nucleotide diversity (π) were all slightly higher in *A. chilense* than in *A. transtasmanicum,* but differences were not statistically significant (nRrar= 13.63 and nRrar= 9.00, U= 34, p= 0.771; H= 0.614 ± 0.022 and H= 0.504 ± 0.058, U= 33, p= 0.480; π= $7.06\ 10^{-3} \pm 0.36\ 10^{-3}$ and π= $2.40\ 10^{-3} \pm 0.30\ 10^{-3}$, U= 24, p= 0.330; values are given for *A. chilense* and *A. transtasmanicum*, respectively, Table 1).

Within *A. chilense,* spatial clustering (SAMOVA; Table S4) revealed the existence of three genetically distinct groups of populations distributed in parapatry: one genetic cluster on the west coast of the North Island (the NW genetic cluster), another restricted to the Cook Strait (the Cook genetic cluster), and a third one distributed mainly along the eastern coasts of both North and South Islands, as well as the west coast of the South Island and Chatham Island (hereafter called the Eastern genetic cluster). Supporting the SAMOVA, the three genetic clusters were also recognized in the ribotype network (Fig. 1). The ribotype network obtained for *A. chilense* was reticulated (i.e., with unresolved mutational pathways), especially between ribotypes belonging to the NW genetic cluster (RAGL and SHEL, Fig. 1), showing evidence of homoplasy. However, except for ribotype r14 (sampled from GOLD, Guillemin et al. 2014), all ribotypes close to each other in the network were part of the same genetic cluster. No ribotypes were shared among clusters, suggesting a clear geographic pattern of genetic divergence. The network also revealed an unambiguous differentiation of the Eastern genetic cluster (E genetic cluster), with all ribotypes belonging to the E genetic cluster separated by a minimum of four mutational steps from the ones belonging to the Cook and NW genetic clusters. The central and most frequent ribotype of the E genetic cluster (r01) was present in all 19 localities where this cluster was observed. The Cook and NW genetic cluster were not completely divergent, with ribotype r14 (sampled from the Cook Strait) being connected to ribotype r27 (sampled from the northernmost sampling site on the west coast of the North Island).

Among the 45 ITS2 ribotypes retrieved in *A. chilense*, 37 (82%) were private (i.e., present in only one population; 34 of which were unique ribotypes; Table S1) and distributed in a

total of 13 populations. After rarefaction, gene and nucleotide diversities were the highest in the two populations of the west coast of the North Island (SHEL and RAGL) and in one Chatham Island population (CHIS) (Table 1). In general, the genetic diversity was lower in the South Island than in the North Island, except for the north eastern populations WITI and OHOP, in which a single ribotype was found (Table 1). All the other monomorphic populations were located in the South Island (Table 1, Fig. 1).

The ribotype network obtained for *A. transtasmanicum* was reticulated, with up to six mutational steps depending on the selected pathways (Fig. 2). The ribotypes r01 and r05 were the most common, representing 69% and 17% of the samples sequenced, respectively (Fig. 2). The most frequent ribotype, r01, was present in all localities sampled in the Cook Strait and the North Island (WANG, OHIW, OHOP and MOIN), but was not found in the southern region of the South Island (CALA and HINA, Fig. 2). Private ribotypes were observed in all localities except OHOP (North Eastern region; Table 1; Table S2). The highest genetic diversity in *A. transtasmanicum* was observed in MOIN, located in the South Cook Strait region. No significant differences in nRrar were observed between North and South Islands (Table 1).

In *A. chilense*, the AMOVA showed that the genetic variance was principally explained by variation among genetic clusters (%var= 87.08, $p < 0.001$, Table 2); while variation among populations within genetic clusters was much lower, but still significant (%var= 6.58, $p < 0.001$; Table 2). Similarly, biogeographical regions were significantly differentiated (%var= 67.61, $p < 0.001$, Table 3) and the variation among populations within bioregion was still significant even though lower (%var= 20.69, $p < 0.001$; Table 3). In *A.*

transtasmanicum, the AMOVA showed that most genetic variance was explained by the variance among populations within a bioregion (%var= 45.85, p< 0.001), while the genetic variance among bioregions was non-significant (%var= 15.19, p= 0.46, Table 4).

No significant isolation by distance was detected in both *Agarophyton* species (*A. chilense* and *A. transtasmanicum*; r^2= -0.049, p= 0.65 and r^2 = 0.544, p=0.07, respectively).

Historical demography

For *A. chilense*, all three genetic clusters showed a unimodal mismatch distribution fitting the spatial population expansion model (Fig. 3A; hypothesis of spatial expansion could not be rejected, SSD values ranging from 0.0003 to 0.0453, p> 0.05, Table 5). This was further supported by the negative values obtained for the Fu's Fs and Tajima's D tests (Fs= -5.92, p< 0.01; D= -0.76, p= 0.22 for the NW genetic cluster; Fs= -7.72, p< 0.001 and D= -2.43, p< 0.001 for the Cook genetic; Fs= -3.40, p< 0.001 and D= -2.20, p< 0.001 for the E genetic cluster; Table 5). The BSP analysis for the E genetic cluster showed a demographic growth that started at the end of the Last Glacial Maximum (LGM, around 18,000-20,000 years ago, Fig. 3B), whereas no evidence of clear demographic change emerged for the Cook and NW genetic cluster.

For *A. transtasmanicum*, the mismatch distribution was bimodal (Fig. 3A). Non-significant values were obtained for the Fu's F_S and Tajima's D tests (Table 5), and the BSP analysis showed a demographic growth that also started at the end of the LGM (Fig. 3B).

Confidence intervals were large in all BSP analyses, damping most trends in estimated population size (Fig. 3B). Indeed, shifts in the BSP barely reached the 95%

credibility interval even in *A. chilense* E genetic cluster and *A. transtasmanicum*. These results should be interpreted with caution since a slight increase in population size in a BSP could be an artifact of random sampling by the Markov Chain Monte Carlo (MCMC) during gene genealogy reconstruction and not necessarily the result of historical demographic size changes (Ho and Shapiro 2011).

Agarophyton *species diagnostic molecular tool*

5P-COI sequences for 22 specimens of *A. chilense* and nine specimens of *A. transtasmanicum* revealed 51 fixed differences between the two *Agarophyton* species (GenBank accession numbers MN145983-MN146013). No variability was detected among the *A. transtasmanicum* individuals, while two polymorphic sites and three haplotypes were detected in *A. chilense*. Amplifications of the COI using the two sets of multiplex PCR primers provided a direct identification of the two *Agarophyton* species with 100% accuracy (Fig. 4). Whatever the primer combination used (Agarophyton_COI_F / Agarophyton_COI_R / Agarophyton_chilense_COI_F or Agarophyton_COI_F / Agarophyton_COI_R / Agarophyton_transtasmanicum_COI_F), the internal control amplification products (of 160bp) were always positive (Fig. 4).

DISCUSSION

The present study reveals important differences between *Agarophyton chilense* and *A. transtasmanicum* in terms of species distribution and genetic structure, while genetic diversity and habitats colonized (rocky and muddy) by the two *Agarophyton* species were

fairly similar. Indeed, *A. chilense*'s distribution was continuous and extensive, including the North and South Islands, Stewart Island, and Chatham Island, and the species exhibited genetic discontinuities concordant with biogeographic transitions. The distribution of *A. transtasmanicum* was more restricted and patchier, but the species was found in both the North and South Islands. Both *Agarophyton* species were found attached to hard substrate and as drifting thalli in soft-bottom habitats and had comparable genetic diversity at the population level. Species distribution patterns did not provide evidence of any clear ecological differences between the two species. We discuss our results in the light of the evolutionary history of *A. transtasmanicum* in New Zealand. Then we examine the potential outcome linked to the co-occurrence of the two species, and finally, we discuss the evolutionary history and biogeography of *A. chilense* in the archipelago.

Ancient presence of Agarophyton transtasmanicum *in New Zealand*

The early observations of *A. transtasmanicum,* limited to inlets located near Auckland city (in both the Manukau Harbour, corresponding to the Western North Island bioregion and the Waitemata Harbour, Orakei Basin, corresponding to the North Eastern bioregion), supported the hypothesis of a recent introduction of the species linked to anthropogenic activities (Wilcox et al. 2001, 2007, Nelson et al. 2015). Seaweeds transported by human activities usually show discontinuous distributions, with populations generally restricted to harbors or highly disturbed environments in the region of introduction (Trowbridge 1995, Smith et al. 2004). For example, unattached populations of the red alga *Chondria harveyana* are restricted to Porirua Harbour in the North Island, New

Zealand, where it was introduced during the 19th century by marine transport from Tasmania (Adams 1983). In the same way, *Gracilaria salicornia*, introduced in Honolulu Harbour and Kaneohe Bay, Hawaii, in the 1970s, is still present with a distribution restricted to both bays three decades later (Smith et al. 2004). The extensive geographic survey of *Agarophyton* populations provided in the present study revealed a still patchy distribution, but with the presence of the species in two new bioregions (South Cook Strait and Southern). The present study also confirms the presence of *A. transtasmanicum* in the North Cook Strait (Whanganui and Manawatu River mouths, Preuss et al. 2020). *Agarophyton transtasmanicum*'s distribution range includes the sites of CALA and HINA located at the mouth of the Catlins River, a sparsely populated area with no present day passenger or freight boat services, suggesting limited contemporary impact of maritime transport as a possible source of colonizers. The Catlins region was settled by European immigrants in the 1840s and became a sawmilling hub for about 70 years from 1865 and on, with coastal vessels being the primary means of transport until the late 1800s, and a number of vessels transporting timber to Dunedin and Christchurch from small ports that are no longer operating (Fortrose, Waikawa). Moreover, recently established populations of Gracilariales generally form vegetative mats embedded in soft substrates, whereas long-established populations tend to be sexually reproductive populations attached to hard substrates by holdfasts as the result of spore settlement (Nettleton et al. 2013, Krueger-Hadfield et al. 2016). For example, 32 of the 35 sites where the invasive *A. vermiculophyllum* was collected in its recently introduced range (Europe and North America) were composed exclusively of unattached thalli embedded in soft substrate

(Krueger-Hadfield et al. 2016). This was not the case in *A. transtasmanicum* for which half the sampled populations of the present study were attached to solid substrates, suggesting a regular occurrence of sexual reproduction in New Zealand. Samples from Manukau Harbour collected by Wilcox et al. (2001) were also reported as attached to cobble substrates. Taken together, these results are not in agreement with the hypothesis of a recent anthropogenic introduction of the species into the archipelago.

A low genetic diversity and absence of spatial genetic structure in a newly colonized region have also been reported as common characteristics of introduced seaweeds when compared to the region of origin (Kim et al. 2010, Le Cam et al. 2019). Such a genetic pattern is generally attributed to founder effects, linked to the arrival of a few individuals and the rapid expansion in the introduced range (Pérez et al. 2006, Kim et al. 2010, Lejeusne et al. 2011). The present study only focused on New Zealand's *A. transtasmanicum* populations, and no comparison with other regions where the species is present (Australia including Tasmania) was performed. However, when compared to the highly abundant and widely distributed *A. chilense* in the archipelago, no significant difference in genetic diversity was observed at the species or population level (Table 1). Indeed, no significant differences between *A. chilense* and *A. transtasmanicum* were detected when comparing within-population ribotype or nucleotide diversity, nor between richness after rarefaction. In three of the four populations where both species coexist, *A. transtasmanicum* presented a higher genetic diversity than *A. chilense* (for both H and π) and one unique ribotype was observed in each species in the fourth population. Additionally, no clear differences in genetic diversity have been detected between Australia

and New Zealand (but those results were only based on 13 ITS1 sequences in total; Preuss et al. 2020). All results obtained to date suggest that *A. transtasmanicum* has a much longer demographic history in the New Zealand archipelago than expected for a recently introduced species. As previously suggested for other marine species (Neall and Trewick 2008, Waters 2008), we postulate that *A. transtasmanicum* could have colonized New Zealand from Australia at the end of the last glacial era as a result of trans-oceanic dispersal following main marine currents (flowing from west to east in the region). However, the species' patchy distribution and the existence of numerous ribotypes in the southern South Island may suggest a more complex history of *A. transtasmanicum* in the archipelago. Whether the species was present before the LGM or introduced more recently (but without a founder effect genetic signature) remains to be determined. A better coverage of the species' distribution range (along the coasts of Australia and Tasmania as well as within New Zealand) is needed to infer the species' history more precisely.

Co-occurrence of Agarophyton chilense *and* A. transtasmanicum *in New Zealand*

The co-occurrence of *A. chilense* and *A. transtasmanicum* in a single habitat brings an interesting perspective to the possible evolutionary origin of these species. First, co-existence of competing species within a specific ecological niche is generally considered a transient state, along a path that leads to the exclusion of one species (Olden et al. 2004, Gallardo et al. 2016). This competitive exclusion can be rapid when one species has a clear ecological advantage over the other. A potential advantage of *A. transtasmanicum* over *A. chilense* could come from the production of gigartinine in the former but not the latter, as

proposed by Wilcox and collaborators (2001, 2007). However, *A. transtasmanicum* was restricted to a few and widely separated sites in the archipelago, while *A. chilense* was present throughout the range of sampled populations. Moreover, *A. transtasmanicum* never clearly dominated in regions where it co-existed with *A. chilense* (i.e., 54% of *A. transtasmanicum* in CALA, 30% in HINA and OHOP). Therefore, *A. transtasmanicum* does not seem to be an overwhelmingly successful competitor in New Zealand.

It has been proposed that ecologically fairly similar species might actually coexist in the long term as a result of differences in micro-niches (MacDougall et al. 2009). Overlapping distribution ranges between seaweed species generally involve a complete spatial segregation at the microscale (Peters et al. 2015, Muangmai et al. 2016, Montecinos et al. 2017). Subtle differences in temperature, wave exposure, or tidal height between the micro habitats of sister species have been associated with distribution range overlaps in algae (e.g., in red algae: Muangmai et al. 2015b, Muangmai et al. 2016; and in brown algae: Fraser et al. 2009a, Tellier et al. 2009). The red alga *Bostrychia intricata* shows spatial segregation related to wave exposure and tidal height between three cryptic lineages (N2, N4 and N5) around Moa Point in North Cook Strait, New Zealand (Muangmai et al. 2016), that was associated with subtle physiological differences previously found in these cryptic species in experimental studies (Muangmai et al. 2015b). Observations of the two species in the same ecological niche may suggest that allopatric speciation occurred in distant regions but in similar environments, in which case the current coexistence would result from secondary contact. Testing such hypotheses would require a global survey of the evolutionary history of *Agarophyton,* and the experimental assessment of the level of

reproductive isolation and niche differentiation between the four species constituting the genus (Gurgel et al. 2018).

Phylogeographic structure of Agarophyton chilense *in New Zealand*

Three genetic clusters, distributed in parapatry, were detected in *A. chilense* and phylogeographic discontinuities were almost completely concordant with the described boundaries between bioregions (Ayers and Waters 2005, Ross et al. 2009, Shears et al. 2008). The main boundaries between genetic clusters observed in *A. chilense* in the present study included Cape Farewell, at the northern tip of the South Island (separating the Cook and the E genetic clusters) and Cape Reinga, at the northern tip of the North Island (separating the NW and the E genetic clusters). In New Zealand, a south/north and west/east genetic structure has commonly been reported for marine organisms and matches the presence of biogeographic boundaries and oceanographic barriers to gene flow (e.g., in marine invertebrates: Ross et al. 2009, Gardner et al. 2010; and in seaweeds: Muangmai et al. 2014, 2015a, Zuccarello and Martin 2016). The most prominent genetic break corresponds to the divergence among populations located around 42°S, at the north of the South Island near Cape Farewell (Ayers and Waters 2005, Goldstien et al. 2006, Ross et al. 2009). The origin of this break has been associated with the historical of sea-level changes which closed the Cook Strait region during glacial periods (most recently 15-24,000 years ago; Lewis et al. 1994), and may be maintained by a sharp present-day thermal gradient acting as an environmental barrier to gene flow (Ayers and Waters 2005, Goldstien et al. 2006). Several studies have also observed a west/east genetic divergence at the northern tip

of the North Island (Jones et al. 2008, Ross et al. 2009, Veale and Lavery 2012), where the strong southward oceanic splits into the eastern and western arms of the Auckland Current, which may strongly limit gene flow across this region. Moreover, marine intrusion and tectonic events during the upper Miocene and Pliocene have affected the topography of the shoreline at the northern tip of the North Island (Stevens and Hogg 2004) and have also been proposed as the cause of the west/east genetic break at Cape Reinga (Cooper and Millener 1993, Waters and Craw 2006).

The distribution of the genetic diversity also reflects past demographic dynamics in *A. chilense*. For the E genetic cluster, demographic inferences were congruent among the ribotype network, neutrality tests, mismatch distribution, and BSP analysis, and supported a scenario of postglacial expansion. The populations AHUR, PARE, PAUA, and SCOR, all located in the North Island, had the highest levels of genetic diversity within the E genetic cluster, suggesting that glacial refugia might have been located in this region. However, a single ribotype was observed in the northernmost populations OHOP and WITI, limiting our capacity to draw clear conclusions. Demographic inferences were less congruent for the NW and the Cook genetic clusters. The ribotype network of the Cook genetic cluster had a star-like shape, suggesting a recent demographic expansion, which was further supported by the neutrality tests and a mismatch distribution fitting a geographic expansion model. However, the BSP analysis did not support any significant demographic change. This latter result may be attributed to the reduced number of sampled individuals and variability of the genetic marker (ITS2), which limit the statistical power of the Bayesian inference (Ho and Shapiro 2011, Grant et al. 2012, Grant 2015). It is possible that a geographically restricted

demographic expansion (maybe after the LGM), from a local resilient population established on the western side of the Cook Strait, explains the distribution range and genetic diversity encountered in the Cook genetic cluster. The network structure of the NW genetic cluster is reticulated and rather suggested demographic stability. However, Tajima's and Fu's tests detected an excess of low frequency ribotypes (expected under rapid demographic expansion, but only the Fu test was statistically significant), and the BSP analysis suggested a mild demographic growth in the past 100,000 years, although with poor statistical confidence. These discrepancies are more difficult to explain. However, the shape of the genealogy evidenced in the ribotype network suggested that the various unique ribotypes observed in the region were not the product of recent mutations from an ancestral or founder ribotype. The higher genetic diversity (nRrar, H, and π) observed in SHEL and RAGL compared to other more southerly and easterly populations further suggested that the NW population may have been demographically resilient during the LGM.

The LGM is considered one of the most important Quaternary events that has shaped the geographical distribution and genetic diversity patterns of terrestrial and marine species (Hewitt 2000, 2004, Maggs et al. 2008, Marko et al. 2010). In marine organisms, including seaweeds, a reduction of genetic diversity from lower to higher latitudes has commonly been observed, matching theoretical expectations from recolonization events among deglaciated areas at the end of the LGM (Australia: Fraser et al. 2009c; New Zealand: Macaya and Zuccarello 2010, Buchanan and Zuccarello 2012, Muangmai et al. 2015a; Chile: Montecinos et al. 2012, Guillemin et al. 2016). The same pattern was observed in *A. chilense*, reinforcing the hypothesis of a strong impact of the glacial-

interglacial cycles on the dynamics of South Island marine populations. The statistical limitations of using a single genetic marker, impeded clear conclusions about the locations of glacial refugia, recolonization routes, and the timing of demographic changes and range shifts. Further studies, using more polymorphic (e.g., microsatellites markers; Assis et al. 2016) or numerous (e.g., Single Nucleotide Polymorphisms, SNPs; Vendrami et al. 2019) molecular markers will be needed to better infer the evolutionary history of *A. chilense* in New Zealand.

CONCLUSIONS

This study revealed the coexistence of two *Agarophyton* species in several widely separated locations in New Zealand. The species had similar genetic diversity at the local scale and occupied hard and soft bottom habitats. *Agarophyton chilense* presented an extensive distribution, with genetic discontinuities in agreement with the biogeographic structure of New Zealand's coasts and the historical changes associated with glacial-interglacial cycles. Ribotype r01, the most commonly sampled in Chile (Guillemin et al. 2014), dominated in all New Zealand localities where the E genetic cluster was present (this included the east coasts of both North and South Islands, as well as the western coast of the South Island and Chatham Island). While the results obtained in the present study corroborated the hypothesized colonization of South America from New Zealand (Guillemin et al. 2014) it provides little additional precision about the origin of the founders and the oceanic processes associated with the transoceanic transport of the algae. Contrastingly, the distribution of genetic diversity in *A. transtasmanicum* did not match any

clear biogeographic or historical feature, and it is difficult to draw conclusions about the evolutionary history of the species in New Zealand. The coexistence of the two species however raises several interesting questions about competitive exclusion, niche differentiation, and the effectiveness of the reproductive barriers discussed above. Lastly, the new molecular tool developed here allows quick and easy identification of the two *Agarophyton* species. It represents a useful tool for future monitoring of eventual changes in the species' distributions and in the execution of common-garden or transplant experiments needed to address open questions.

DATA SHARING AND DATA ACCESSIBILITY: The authors confirm that all data underlying the findings are fully available without restriction. Sequences are available in GenBank (accession numbers: MN145944 to MN145972 and MN145973 to MN145982).

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Table 1: Position of sampling sites and indices of genetic diversity calculated for the nuclear marker ITS2 in *Agarophyton chilense* and *A. transtasmanicum* throughout the New Zealand archipelago. For each site, the abbreviation (code), the population type (attached or unattached) and the geographic coordinates are indicated. N: total number of sequences with the number of sequences added from Guillemin et al. (2014) in brackets; nR: number of ribotypes; nRrar: number of ribotypes corrected for unequal sample size after rarefaction method; H: gene diversity; π: nucleotide diversity; S: number of polymorphic sites; Rpriv: number of private ribotypes; -: not estimated (in N < 8). [a]Standard deviations are in brackets. Bioregions (bold) were defined using the Coastal Biogeographic Regions Classification map provided in http://www.mfe.govt.nz/more/science-and-data/classification-systems/marine-classification-systems.

			Agarophyton chilense						*Agarophyton transtasmanicum*					
Site (code)	Population type	Position	N	nR/nRrar	H [a]	π (.10^{-3}) [a]	S	Rpriv	N	nR/nRrar	H [a]	π (.10^{-3}) [a]	S	Rpriv
Western North Island														
Shelly Beach (SHEL)	Attached	36° 34 'S, 174° 22' E	26	7/3.49	0.822 (0.037)	3.24 (0.000)	9	3						

Raglan (RAGL)	Attached	37° 48' S, 174° 53' E	24	11/4.52	0.877 (0.045)	5.00 (1.04)	11	7						
North Cook Strait														
Whanganui (WANG)	Attached	39° 56' S, 174° 59' E							30	2/0.27	0.067 (0.061)	0.13 (0.12)	1	1
Paremata (PARE)	Attached	41° 06' S, 174° 52' E	34 (14)	2/0.94	0.401 (0.073)	0.83 (0.15)	1	0						
Pauatahanui (PAUA)	Unattached	41° 05’ S, 174° 54’ E	28	5/1.75	0.508 (0.096)	0.14 (0.36)	5	3						
Scorching Bay (SCOR)	Attached	41° 18’ S, 174° 48’ E	15 (15)	3/1.51	0.514 (0.116)	1.14 (0.30)	2	2						
Eastern North Island														
Ahuriri (AHUR)	Attached	39° 28' S, 176° 53' E	18	4/1.86	0.575 (0.101)	2.08 (0.39)	3	3						
North Eastern														

Whitianga (WITI)	Attached	36° 50' S, 175° 41' E	14	1/0.00	0	0	0	0						
Ohiwa (OHIW)	Unattached	37° 59' S, 177°11' E							29	2/0.48	0.133 (0.081)	0.27 (0.17)	1	2
Ohope (OHOP)	Attached	37° 58' S, 177° 04' E	19	1/0.00	0	0	0	0	8	1/0.00	0	0	0	0
West Coast South Island														
Okari River (OKAR)	Attached	41° 48' S, 171° 27' E	19	1/0.00	0	0	0	0						
South Cook Strait														
Whanganui Inlet (WAIN)	Attached	40° 35’ S, 172° 37’ E	23 (11)	4/1.04	0.249 (0.116)	0.54 (0.27)	3	3						
Golden Bay (GOLD)	Attached	40° 48’ S, 172° 48’ E	8 (8)	2/1.00	0.250 (0.180)	2.07 (1.49)	4	1						
Moutere Inlet (MOUT)	Attached	41° 08' S, 173° 00' E	49 (11)	5/0.50	0.158 (0.070)	0.93 (0.65)	11	3						

Moutere Inlet (MOIN)	Unattached	41° 08' S, 173° 00' E							13	5/2.97	0.705 (0.122)	3.77 (0.63)	4	2
Momorangi Bay (MOMO)	Attached	41° 16' S, 173° 56' E	20	2/0.40	0.100 (0.088)	0.21 (0.18)	1	1						
Fiordland														
Fiordland (CASS)	Attached	45° 55' S, 166° 54' E	17	2/0.47	0.118 (0.101)	0.24 (0.21)	1	1						
Riverton (RIVE)	Attached	46° 21' S, 168° 00' E	20	2/0.40	0.100 (0.088)	0.21 (0.18)	1	1						
Southern														
Stewart Island (STEW)	Attached	46° 54’ S, 168° 08’ E	35 (17)	2/0.23	0.057 (0.053)	0.12 (0.11)	1	1						
Catlins Lake (CALA)	Unattached	46° 28'S, 169° 38' E	11	1/0.00	0	0	0	0	13	3/1.84	0.590 (0.122)	2.84 (0.53)	3	1
Hinahina (HINA)	Attached	46° 28' S, 169° 40' E	26	4/0.92	0.222 (0.106)	0.48 (0.24)	3	3	3	3/-	1.000 (0.272)	2.690 (0.90)	2	2

Otago Harbour (OTAG)	Attached	45° 50' S, 170° 39' E	19	1/0.00	0	0	0	0						
Hoopers Inlet (HOOP)	Unattached	45° 51' S, 170° 39' E	27	1/0.00	0	0	0	0						
East Coast South Island														
South Brighton (BRIG)	Attached	43° 31' S, 172° 43' E	18 (5)	2/0.44	0.111 (0.096)	0.23 (0.20)	1	0						
Ferrymead (FERR)	Unattached	43° 33' S, 172° 41' E	25	1/0.00	0	0	0	0						
Chatham Islands														
Chatham Island (CHIS)	Attached	43° 46' S, 176° 33' E	28 (12)	7/2.42	0.680 (0.059)	2.63 (0.064)	8	5						
Chatham Island (CHAT)	Unattached	43° 46' S, 176° 33' E	33	2/0.97	0.436 (0.066)	0.90 (0.14)	1	0						
NW genetic cluster			50	14	0.850 (0.075)	4.11 (0.64)	12	10						

Cook genetic cluster			80	9	0.191 (0.355)	0.93 (0.45)	15	7						
E genetic cluster			426	23	0.376 (0.029)	.099 (0.10)	23	20						
Total			**556**	**45/13.63**	**0.614 (0.022)**	**7.06 (0.36)**	**42**	**-**	**96**	**10/9.00**	**0.504 (0.058)**	**2.40 (0.30)**	**4**	**-**

Table 2: Analysis of molecular variance (AMOVA) of *Agarophyton chilense* for the ITS2 marker with hierarchical partitioning of variance among genetic clusters and among sites within genetic cluster. Genetic clusters were defined according to the SAMOVA results (NW genetic cluster= SHEL and RAGL; Cook genetic cluster = WAIN, MOUT, and GOLD; and E genetic cluster= WITI, OHOP, AHUR, PARE, PAUA, SCOR, MOMO, BRIG, FERR, OTAG, HOOP, CALA, HINA, STEW, RIVE, CASS, OKAR, CHAT, and CHIS, see Figure 1); d.f.: degree of freedom.

Source of variation	d.f.	% variation	Fst	p-value
Among genetic clusters	2	87.08	0.87	<0.001
Among sites within genetic cluster	21	6.58	0.51	<0.001
Residual	532	6.34	-	-
Total	555	-	-	-

Table 3: Analysis of molecular variance (AMOVA) of *Agarophyton chilense* for the ITS2 marker with hierarchical partitioning of variance among bioregions and among sites within bioregion. Bioregions were defined using the Coastal Biogeographic Regions Classification map provided in http://www.mfe.govt.nz/more/science-and-data/classification-systems/marine-classification-systems (Western North Island= SHEL and RAGL; North Eastern= WITI and OHOP; North Cook Strait= PARE, PAUA, and SCOR; South Cook Strait= WAIN, MOUT, GOLD, and MOMO; Fiordland= CASS and RIVE; Southern= STEW, HINA, CALA, HOOP, and OTAG; East Coast South Island= FERR and BRIG; and Chatham Island= CHAT and CHIS, see Table 1); d.f.: degree of freedom. Localities of OKAR in West Coast South Island and AHUR in Eastern North Island were excluded from the analysis since only one site was sampled within each of these two bioregions (Table 1).

Source of variation	d.f.	% variation	Fst	p-value
Among bioregions	7	67.61	0.68	<0.001
Among sites within bioregions	14	20.69	0.64	<0.001
Residual	497	11.70	-	-
Total	518	-	-	-

Table 4: Analysis of molecular variance (AMOVA) of *Agarophyton transtasmanicum* for the ITS2 marker with hierarchical partitioning of variance among bioregions, among sites within bioregion and within sampling site. Bioregions were defined using the Coastal Biogeographic Regions Classification map provided in http://www.mfe.govt.nz/more/science-and-data/classification-systems/marine-classification-systems. Localities from the Cook Strait were clumped in a region named North/South Cook Strait. Regions were: North Eastern= OHIW and OHOP; North/South Cook Strait= WANG and MOIN; and Southern= HINA and CALA (see Table 1). d.f.: degree of freedom.

Source of variation	d.f.	% variation	Fst	p-value
Among bioregions	2	15.19	0.15	0.46
Among sites within bioregions	1	45.85	0.54	<0.001
Residual	90	38.96	-	-
Total	95	-	-	-

Table 5: Test of historical demographic change based on ITS2 sequences of *Agarophyton chilense* and *A. transtasmanicum*. Each genetic cluster of *A. chilense* (defined according to the SAMOVA results) was treated independently. Significance of Tajima´s D and Fu´s F_S tests and the goodness-of-fit for SSD were determined using 1000 bootstrap replicates in the software ARLEQUIN v 3.11 (Excoffier and Lisher 2010); § p-value > 0.05 means the null hypothesis of sudden expansion cannot be rejected; SSD: sum of squared deviations.

	A. chilense- NW genetic cluster	*A. chilense*- Cook genetic cluster	*A. chilense*- E genetic cluster	*A. transtasmanicum*
Tajima´s D (p-value)	-0.76 (0.22)	-2.43 (<0.001)	-2.20 (<0.001)	0.94 (0.83)
Fu´s Fs (p-value)	-5.92 (<0.01)	-7.72 (<0.001)	-3.40 (<0.001)	-3.37 (0.07)
SSD (p-value) §	0.0453 (0.20)	0.0011 (0.23)	0.0003 (0.10)	0.0208 (0.55)

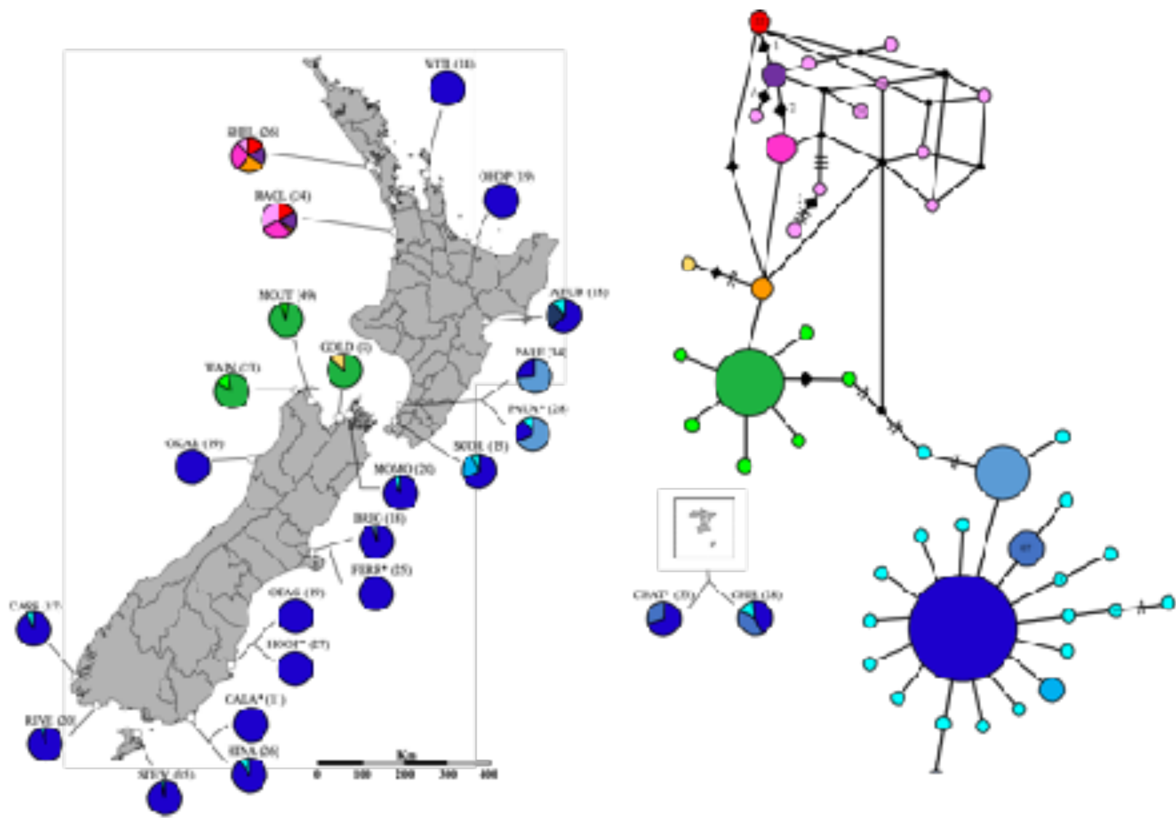

Figure 1: ITS2 ribotype distribution and ITS2 network including 556 sequences of *Agarophyton chilense.* Length of ITS2 sequences vary between 479 base pairs (bp) and 483 bp. Pie charts represent ribotype frequency in each sampling site, sequence number in each site is noted between parenthesis, colors correspond to the ones presented in the ITS2 network. In the network, circle sizes are proportional to the ribotype frequency and connection lengths correspond to mutation steps. For ribotypes separated by more than one mutational step, black bars represent the number of steps. Black diamond: indel, for each indel the number of bp is noted above. Black circle: hypothetical un-sampled ribotype. Rare and private ribotypes, in each genetic cluster, are represented by the same color for clarity. Ribotype r14, substantiating the ambiguous differentiation between Cook and NW genetic

clusters is colored in yellow. Ribotype number as in Table S1 and S2. *: population of floating thalli; population abbreviations as in Table 1. Genetic clusters as defined by the SAMOVA are: NW genetic cluster= SHEL and RAGL; Cook genetic cluster= WAIN, MOUT, and GOLD; and E genetic cluster= WITI, OHOP, AHUR, PARE, PAUA, SCOR, MOMO, BRIG, FERR, OTAG, HOOP, CALA, HINA, STEW, RIVE, CASS, OKAR, CHAT, and CHIS.

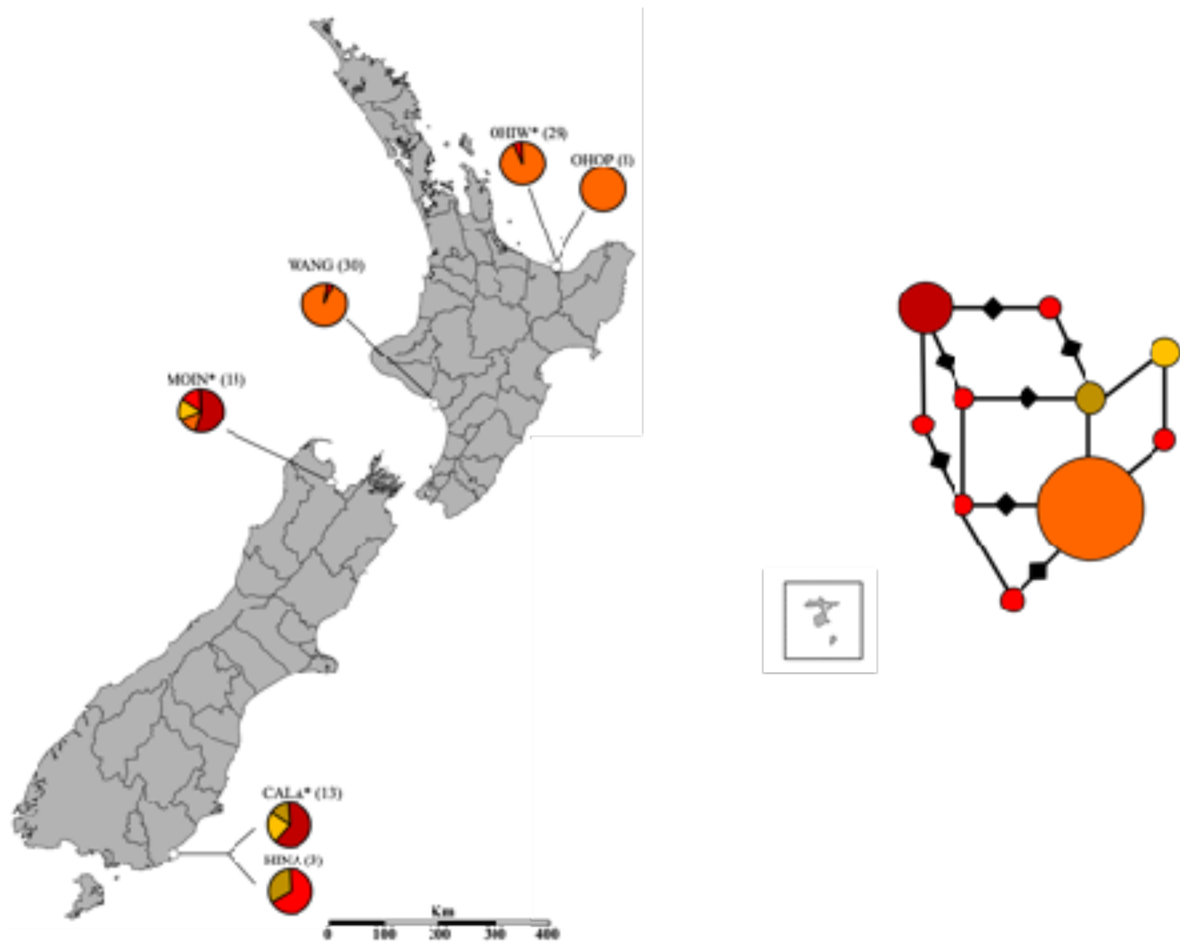

Figure 2: ITS2 ribotype distribution and ITS2 network including 96 sequences of *Agarophyton transtasmanicum*. Length of ITS2 sequences vary between 494 base pairs (bp) and 497 bp. Pie charts represent ribotype frequency in each sampling site, sequence number in each site is noted between parenthesis, colors correspond to the ones presented in the ITS2 network. In the network, circle sizes are proportional to the ribotype frequency and connection lengths correspond to mutation steps. For ribotypes separated by more than one mutational step, black bars represent the number of steps. Black diamond: indel, for each indel the bp is noted above. Rare and private ribotypes are represented by the same color

for clarity. Ribotype number as in Table S2 and S3. *: population of floating thalli; population abbreviations as in Table 1.

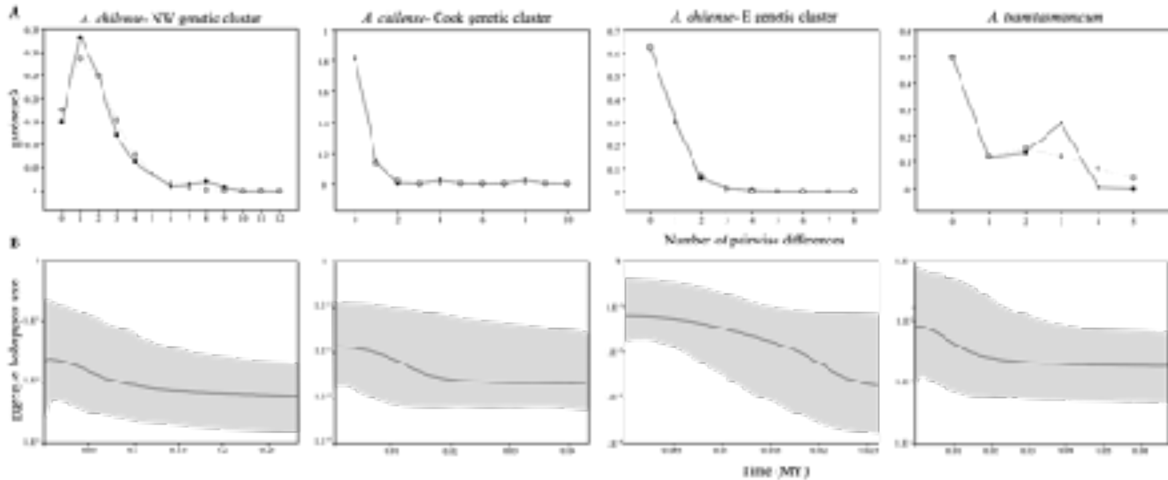

Figure 3: Historical demography of *Agarophyton chilense* and *A. transtasmanicum* for ITS2 sequences. A) Observed mismatch distribution, represented by black circles, and expected mismatch distribution under a spatial expansion model represented by white circles. B) Changes in effective population size estimated by Bayesian Skyline plots. Solid lines are the median posterior effective population size through time; gray area indicates the 95% confidence interval for each estimate. Each genetic cluster of *Agarophyton chilense*, defined according to the SAMOVA results, was treated independently.

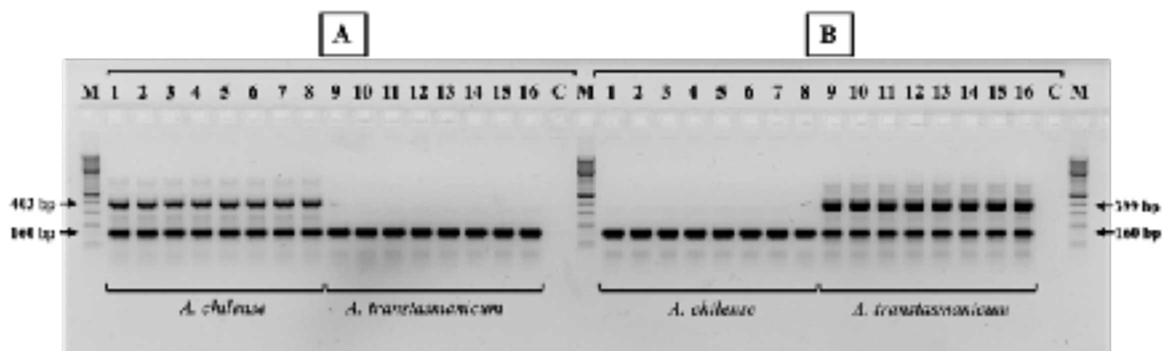

Figure 4: Agarose gel showing results of the two multiplex polymerase chain reaction (PCR) for the diagnostic tool developed in this study, allowing the identification of the two cryptic *Agarophyton* species in New Zealand. A) PCR reactions using the Agarophyton_COI_F / Agarophyton_COI_R / Agarophyton_chilense_COI_F primer combination; B) PCR reactions using the Agarophyton_COI_F / Agarophyton_COI_R / Agarophyton_*trantasmanicum*_COI_F primer combination. Samples of *A. chilense* correspond to lanes 1-8 (1: AHUR, 2: CHAT, 3: OKAR, 4: OTAG, 5: WAIN, 6: MOUT, 7: SHEL, 8: RAGL). Samples of *A. transtasmanicum* correspond to lanes 9-16 (9 and 10: MOIN, 11 and 12: CALA, 13 and 14: OHIW, 15: OHOP, 16: WANG). Population abbreviations are as in Table 1. C: negative control. M: 100bp molecular weight marker.

Table S1: Geographical distribution of ITS2 ribotypes in *Agarophyton chilense.* Number of individuals is given for each ribotype in each sampling site (Code, abbreviation as in Table 1).

Genetic clusters	Code	r01	r07	r08	r09	r10	r11	r12	r13	r14	r15	r16	r17	r18	r19	r20	r21	r22	r23	r24	r25	r26	r27	r28	r29
NW	SHEL																	4	1	1	5	1	7	7	
	RAGL																	4			4		1	7	2
Cook	WAIN								20								1								
	GOLD								7	1															
	MOUT								46		1	1													
E	PARE	9											25												
	PAUA	6											19												
	SCOR	10												1	4										
	AHUR	11																							
	WITI	14																							
	OHOP	19																							
	OKAR	19																							
	MOMO	19																							
	CASS	16																							
	RIVE	19																							
	STEW	34														1									
	CALA	11																							
	HINA	23	1																						
	OTAG	19																							

	HOOP	27																							
	BRIG	17																							
	FERR	25																							
	CHIS	12	11	1	1	1	1	1																	
	CHAT	23	10																						
	TOTAL	**333**	**21**	**1**	**1**	**1**	**1**	**1**	**73**	**1**	**1**	**1**	**45**	**1**	**4**	**1**	**1**	**8**	**1**	**1**	**9**	**1**	**8**	**14**	**2**

Continued - Table S1

Genetic clusters	Code	r30	r31	r32	r33	r34	r35	r36	r37	r38	r39	r40	r41	r42	r43	r44	r45	r46	r47	r48	r49	r50	**TOTAL**
NW	SHEL																						**26**
	RAGL	1	1	1	1	1	1																**24**
Cook	WAIN													1	1								**23**
	GOLD																						**8**
	MOUT															1							**49**
	PARE																						**34**
	PAUA							1	1	1													**28**
	SCOR																						**15**
	AHUR										5	1	1										**18**
	WITI																						**14**

	OHOP																						**19**
	OKAR																						**19**
	MOMO																1						**20**
E	CASS																	1					**17**
	RIVE																		1				**20**
	STEW																						**35**
	CALA																						**11**
	HINA																			1	1	1	**26**
	OTAG																						**19**
	HOOP																						**27**
	BRIG																						**18**
	FERR																						**25**
	CHIS																						**28**
	CHAT																						**33**
	TOTAL	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**5**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**556**

Note that ribotypes 02, 03, 04, 05, and 06 are not listed in Table S1. These ribotypes where only encountered in *Agarophyton chilense* in Chile and have been reported in Guillemin et al. (2014).

Table S2: Ribotypes and GenBank access numbers of *Agarophyton chilense* and *A. transtasmanicum*.

Species	Ribotype	Genbank access N°	Sequence deposited in Genbank	Source
A. chilense	r01	HQ998639	CH-LEN-LEF102	Guillemin et al. 2014
	r07	HQ998748	NZ-CHT-CHAT15	Guillemin et al. 2014
	r08	HQ998749	NZ-CHT-CHAT2	Guillemin et al. 2014
	r09	HQ998750	NZ-CHT-CHAT20	Guillemin et al. 2014
	r10	HQ998753	NZ-CHT-CHAT14	Guillemin et al. 2014
	r11	HQ998754	NZ-CHT-CHAT3	Guillemin et al. 2014
	r12	HQ998755	NZ-CHT-CHAT16	Guillemin et al. 2014
	r13	HQ998759	NZ-PGB-GB4	Guillemin et al. 2014
	r14	HQ998764	NZ-PGB-GB3	Guillemin et al. 2014
	r15	HQ998772	NZ-MOU-MOUT16	Guillemin et al. 2014
	r16	HQ998777	NZ-MOU-MOUT3	Guillemin et al. 2014
	r17	HQ998779	NZ-PIW-PIW12	Guillemin et al. 2014
	r18	HQ998792	NZ-SCB-SB10	Guillemin et al. 2014

r19	HQ998796	NZ-SCB-SB14	Guillemin et al. 2014
r20	HQ998816	NZ-STI-STIS2	Guillemin et al. 2014
r21	HQ998824	NZ-WIN-WIN10	Guillemin et al. 2014
r22	MN145944	SHEL_01	This study
r23	MN145945	SHEL_05	This study
r24	MN145946	SHEL_06	This study
r25	MN145947	SHEL_07	This study
r26	MN145948	SHEL_16	This study
r27	MN145949	SHEL_20	This study
r28	MN145950	SHEL_22	This study
r29	MN145951	RAGL_01	This study
r30	MN145952	RAGL_04	This study
r31	MN145953	RAGL_03	This study
r32	MN145954	RAGL_10	This study
r33	MN145955	RAGL_15	This study
r34	MN145956	RAGL_13	This study
r35	MN145957	RAGL_33	This study
r36	MN145958	PAUA_02	This study
r37	MN145959	PAUA_19	This study
r38	MN145960	PAUA_26	This study
r39	MN145961	AHUR_02	This study
r40	MN145962	AHUR_04	This study
r41	MN145963	AHUR_08	This study
r42	MN145964	WAIN_03	This study
r43	MN145965	WAIN_08	This study

	r44	MN145966	MOUT_30	This study
	r45	MN145967	MOMO_18	This study
	r46	MN145968	CASS_40	This study
	r47	MN145969	RIVE_14	This study
	r48	MN145970	HINA_25	This study
	r49	MN145971	HINA_31	This study
	r50	MN145972	HINA_39	This study
A. transtasmanicum	r1	MN145973	OHIW_01	This study
	r2	MN145974	OHIW_15	This study
	r3	MN145975	WANG_35	This study
	r4	MN145975	MOIN_04	This study
	r5	MN145977	MOIN_07	This study
	r6	MN145978	MOIN_31	This study
	r7	MN145979	MOIN_39	This study
	r8	MN145980	CALA_01	This study
	r9	MN145981	HINA_11	This study
	r10	MN145982	HINA_18	This study

Note that ribotypes 02, 03, 04, 05, and 06 are not listed in Table S1. These ribotypes where only encountered in *Agarophyton chilense* in Chile and have been reported in Guillemin et al. (2014).

Table S3: Geographical distribution of ITS2 ribotypes in *Agarophyton transtasmanicum*. Number of individuals is given for each ribotype in each sampling site (Code, abbreviation as in Table 1).

Code	r01	r02	r03	r04	r05	r06	r07	r08	r09	r10	**TOTAL**
OHIW	27	2									**29**
WANG	29		1								**30**
MOIN	2			1	7	2	1				**13**
CALA					8	3		2			**13**
OHOP	8										**8**
HINA								1	1	1	**3**
TOTAL	**66**	**2**	**1**	**1**	**15**	**5**	**1**	**3**	**1**	**1**	**96**

Table S4: Spatial analysis of molecular variance (SAMOVA) of *Agarophyton chilense* for the ITS2 marker. K, number of groups. F_{SC}, proportion of total genetic variance due to differences between populations within each group. F_{CT}, proportion of total genetic variance due to the differences between groups. Population abbreviations as in Table 1. * $p < 0.001$. K for which Fct was maximized is indicated in bold.

Composition of groups (*K*)	F_{CT}	F_{SC}
K = 2 (SHEL, RAGL, WAIN, GOLD, MOUT) (PARE, PAUA, SCOR, AHUR, WITI, OHOP, OKAR, MOMO, CASS, RIVE, STEW, CALA, HINA, OTAG, HOOP, BRIG, FERR, CHIS, CHAT)	0.882	0.497
***K* = 3** (SHEL, RAGL) (WAIN, GOLD, MOUT) (PARE, PAUA, SCOR, AHUR, WITI, OHOP, OKAR, MOMO, CASS, RIVE, STEW, CALA, HINA, OTAG. HOOP, BRIG, FERR, CHIS, CHAT)	**0.919**	**0.259**
K = 4 (SHEL, RAGL) (WAIN, GOLD, MOUT) (PARE, PAUA) (SCOR, AHUR, WITI, OHOP, OKAR, MOMO, CASS, RIVE, STEW, CALA, HINA, OTAG, HOOP, BRIG, FERR, CHIS, CHAT)	0.914	0.084