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Entangled fates of holobiont genomes during invasion: nested bacterial and host diversities in *Caulerpa taxifolia*

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Abstract :

Successful prevention and mitigation of biological invasions requires retracing the initial steps of introduction, as well as understanding key elements enhancing the adaptability of invasive species. We studied the genetic diversity of the green alga *Caulerpa taxifolia* and its associated bacterial communities in several areas around the world. The striking congruence of α and ß diversity of the algal genome and endophytic communities reveals a tight association, supporting the holobiont concept as best describing the unit of spreading and invasion. Both genomic compartments support the hypotheses of a unique accidental introduction in the Mediterranean and of multiple invasion events in Southern Australia. In addition to helping with tracing the origin of invasion, bacterial communities exhibit metabolic functions that can potentially enhance adaptability and competitiveness of the consortium they form with their host. We thus hypothesize that low genetic diversities of both host and symbiont communities may contribute to the recent regression in the Mediterranean, in contrast with the persistence of highly diverse assemblages in southern Australia. This study supports the importance of scaling up from the host to the holobiont for a comprehensive understanding of invasions.

Introduction

Biological invasions are both a consequence and an active part of global change (McNeely 2006; Vitousek *et al.* 1997), and although the generalization of their negative impact is controversial, some introduced species have acute impacts on native communities. The underlying causes for

the invasive behavior of some species when introduced to new environments is a central question that has yet to be fully resolved (Sakai *et al.* 2001). In addition to some specific life-history traits prevalent in invasive species, such as clonality or selfing (Barrett 2015; Odom & Walters 2014), the genetic diversity of introduced pools (Roman & Darling 2007), as well as the alteration of biotic interactions, can influence the invasive behavior of numerous introduced species. Among the best known biotic interactions that may affect the potential of introduced species to become invasive are those related to the "enemy release hypothesis" (REH; Mitchell *et al.* 2006; Mitchell & Power 2003; Torchin *et al.* 2003; Torchin & Mitchell 2004). The ERH postulates that the absence of co-adapted predators, parasites or pathogens allows introduced species to avoid losses and allocate more resources to growth and reproduction in introduced ranges over ecological time scales, which may lead to the "evolution of improved competitive ability" over evolutionary time scales (EICA; Blossey & Notzold 1995).

Most empirical tests of invasive theories have focused on detrimental biotic interactions (mostly predation, parasitism and competition), whereas positive biotic interactions have received much less attention (but see Chisholm *et al.* 1996; Mitchell *et al.* 2006; Richardson *et al.* 2000). A poorly understood but likely very important biotic interaction is symbiosis with prokaryotes, despite reports suggesting that such microorganisms can play a determinant role (Klock *et al.* 2015; Mitchell *et al.* 2006; Richardson *et al.* 2000; Rodriguez-Echeverria 2010). Three main hypotheses have been proposed to describe the possible positive relationship between eukaryotic hosts and mutualists during an invasion (Klock *et al.* 2015). The Generalist Host Hypothesis proposes that invasive species may be relative generalist hosts (Parker 2001; Rodriguez-Echeverria *et al.* 2011), the Enhanced Mutualism Hypothesis that new mutualism is established

between introduced and native species (Richardson *et al.* 2000), and the Accompanying

Mutualist Hypothesis that invasive hosts are co-introduced with mutualistic symbionts

(Rodriguez-Echeverria *et al.* 2011).

Here, we test these hypotheses by simultaneously addressing the evolution of the genetic pool of

an invasive species and that of its associated bacterial flora, from its native and invasive ranges.

Our goal is to ascertain the most likely path and mechanism of introduction while screening for

possible interactions between host and associated bacteria that may help in understanding their

success as invasive species. A good candidate for exploring these relationships and their

potential influence on an invasive trajectory is the complex of *Caulerpa* species and their

associated bacterial fauna (Delbridge *et al.* 2004; Meusnier *et al.* 2001). The tropical green alga

C. taxifolia was first discovered in 1984 in the Mediterranean Sea (Meinesz & Hesse 1991),

where it spread to form dense meadows of large, cold-adapted, turbid-water thalli. These contrast

sharply with the isolated and patchy aggregations of warm-adapted, clear-water thalli with small

and delicate architecture observed in its native area of Queensland, in northeastern Australia

(Fama *et al.* 2002; Meusnier *et al.* 2002). The sudden Mediterranean spread resulted in important

detrimental effects including displacement of Mediterranean species from areas where *C.

t*axifolia* formed dense populations (BellanSantini *et al.* 1996; Boudouresque *et al.* 2009;

Francour *et al.* 1995; Williams 2007). This was the case for the seagrass *Posidonia oceanica, the*

foundation species of the most important (in terms of cover, biomass and biodiversity)

Mediterranean coastal ecosystem, which is experiencing severe declines (Marbà *et al.* 2014;

Marba & Duarte 2010). A distinct wave of invasion by *C. taxifolia* was recorded in the region of

New South Wales (NSW) in southeastern Australia in the early 2000s (Millar 2004; Schaffelke

et al. 2002), inducing similar pervasive effects on native species (Byers *et al.* 2010; but see Glasby 2013; Wright *et al.* 2007). More recently, an invasion was triggered in San Diego (California, USA), but it was successfully halted because it was detected early enough to be controlled (Anderson 2005).

In this study, our aims are (i) comparing bacterial α-diversity in invaded versus native ranges and their change during the course of invasion; (ii) assessing the strength of the relationship between the algae and the bacterial OTUs that characterize its endophytic communities; (iii) using the information from host genetics and bacterial community profiles to deliver insights regarding the putative number of introduction events and their origin; and iv) examining the functions of the most relevant bacterial lineages compared to habitat modifications associated with invasion by *C. taxifolia*, to inform hypotheses on how endophytic bacteria might have influenced the switch of *C. taxifolia* from an introduced to an invasive alga. To achieve these goals, we estimated the host and bacterial diversity of *C. taxifolia* from the native Queensland and the introduced NSW and Mediterranean regions (Fig. 1) to compare the genetic diversity of both compartments in native versus invasive populations.

Materials and Methods

Sampling and DNA extraction

We focused on three areas: the native Queensland (QLD) and the invaded coasts of the Mediterranean (MED) and New South Wales (NSW). In invasive areas, samples were collected (Fig. 1) in 2009 in the Mediterranean (Tunis and Villefranche, MED) during Northern Hemisphere summer (July to August), and in 2010 in Australia (New South Wales, NSW: Port

Jackson, Lake Conjola, Narrawallee, Burrill Lake) during the Southern Hemisphere summer (January-February). In the Northern part of its native area (Queensland, QLD), *Caulerpa taxifolia* was collected in April 2010 along the beach of Kissing Point, a beach near Townsville, here referred to as Townsville.

For host population genetics, a total of 236 sampling units (SUs, composed of several fronds linked together by the same stolon and sampled at least 1–2 m apart from each other) were collected from 8 localities in Australia and from 3 in the Mediterranean (Fig. 1; Table 1), with a sampling density of approximately 30 sampling units (SUs) within approximately 1–2 km^2. Additionally, historical samples that were silica-dried, and thus could only be used for host population genetics, were added: one sample from the Oceanographic Museum of Monaco, two from the San Diego 'aborted' invasion (Anderson 2005), and one from a native area of the Pacific (Tahiti), together with 19 sampling units from the native area of Brisbane (QLD), four samples collected in 1999 in Kelso Reef (QLD) and in Croatia (MED), all from previous studies (Jousson *et al.* 1998; Meusnier *et al.* 2001; Meusnier *et al.* 2004).

Samples for microsatellite genotyping were preserved either frozen (-20°C), in silica gel, or in CTAB (cetyltrimethyl ammonium bromide) buffer until extraction in the laboratory. Total genomic DNA was extracted using the CTAB method (Doyle & Doyle 1987).

For bacterial communities, a total of 3 to 6 disinfected SUs yielding a threshold concentration above 50 ng μL^{-1} were amplified and sequenced per location. For each location, a sample of sediment and 3 non-disinfected SUs were also analyzed as a control for endophytes *versus* whole communities. These were picked from a collection of sample of 15 fresh SUs taken from from

each locality (Table S2, Supplementary material). Those 15 SUs were subdivided into two subsets: one with the DNA extracted immediately from one portion (disinfection control) and one after a previously reported disinfection pre-treatment (Aires *et al.* 2012). Sediment sampled in the same locations wasfrozen (-80°C) until DNA extraction. Bacterial DNA extraction was performed using the FastDNA® SPIN Kit for Soil (MP Biomedicals, LLC).

Population genetics analysis

Eight microsatellite loci specifically developed for *C. taxifolia* were amplified from 195 SUs (Arnaud-Haond *et al.* 2013), and fragments were separated on an ABI 3130 XL automatic sequencer (Applied Biosystems, Foster City, CA, USA).

Due to the partial clonality of *Caulerpa taxifolia*, multi-locus genotypes (MLGs) of each sampling unit were used to infer their membership in clonal lineages based on their probability of multiple occurrence through independent sexual events (Arnaud-Haond *et al.* 2005; Arnaud-Haond *et al.* 2007). Genotypic diversity was assessed (see Table 1) using the main indices summarized by Arnaud-Haond *et al.* (2007) and computed in GenClone (Arnaud-Haond & Belkhir 2007). Genetic differentiation was then estimated through F_{ST} estimates (Belkhir *et al.* 1996-2001) and network analysis based on allele sharing distance as previously proposed for clonal organisms (Bowcock *et al.* 1994; Kivelä *et al.* 2015; Moalic *et al.* 2011; Rozenfeld *et al.* 2008) and detailed in the Supplementary material.

Microbial community analysis

Amplification of the 16Sr V3-V4 DNA region of amplicons was performed using barcoded fusion primers (V3 forward: 16Sar, V4 reverse: 16Sbr) with Roche-454 A Titanium sequencing adapters with a unique 8 bp key-tagged universal primers (Wang & Qian 2009). Amplicons were analyzed using tagged pyrosequencing (GS FLX Titanium, 454-Life Sciences-Roche technology®).

The data analyses are detailed in the Supplementary material. The analyses were performed using the QIIME v. 1.6.0 pipeline (Caporaso *et al.* 2010) to filter datasets, remove chimeras, perform clustering (at the 97% identity level), and infer taxonomy using BLAST with an 80% confidence threshold and a minimum e-value threshold of 10-3 (default value). α-Diversity was estimated both for each SU and within each location by clustering replicates using Shannon's diversity index, Chao 1 richness and number of unique OTUs estimates to estimate the coverage of the community reached via the sequencing effort performed. Estimates were standardized (for the disinfected samples) to the smallest number of sequences obtained in the sample delivering the lowest yield. β-Diversity was assessed using a weighted UniFrac algorithm, taking into account both the phylogenetic distance and the frequency of phylotypes among samples. The differentiation and clustering of communities were illustrated through Principal coordinate analysis (PCoA) plots and network analysis, and their significance was tested using ANOSIM based on Bray Curtis dissimilarity.

Congruence between host and bacterial community patterns

The relationship between the levels of the α-diversity of the host (clonal and genetic diversities through R, Â and H_{nb}) and bacterial (endophytic communities, through the standardized number of estimated OTUs) compartments. As for β-diversity, a Mantel test was performed to test for the relationship between patterns of divergence among locations for both compartments (Fst versus Bray-Curtis values).

Results

Population genetics of the host

A total of 54 distinct multi-locus genotypes (MLGs; Table 1) were identified with 8 microsatellites, with few sampling units (SUs) sharing identical MLGs in the native range of Queensland (particularly Townsville and Kelso Reef, with slightly more identical MLGs observed in Brisbane), whereas clonal dominance was clear in the invaded ranges. Several distinct dominant MLGs were discriminated in NSW. In contrast, the Mediterranean sites were characterized by a core of nearly identical MLGs differing at only one locus (GE3) (Table 1, Fig. 2). The MLG characterizing the aquarium strain was retrieved in 2010 in Villefranche and Tunis (circled in red in Fig. 2), while the slightly different and most common MLG sampled in those two locations corresponds to the one isolated during the successfully aborted invasion in California (Fig. 2).

Both clonal diversity (G, R) and evenness (Simpson D*, Pareto β), as well as genetic diversity (heterozygosity and allelic richness), were highest in the native area of Queensland and lowest in the Mediterranean, with a wide range of intermediate values in NSW (Table 1, Table S1, Fig. 2).

The network of MLGs analyzed at the percolation threshold (Dpe=1.25) showed a high and significant value of clustering (<C>=0.8), illustrating the significant regional grouping of MLGs (Fig. 2, Queensland, NSW and the Mediterranean; Table S6), with two distant clusters corresponding to North and South locations in Queensland and the four samples from Kelso Reef appearing as intermediate among those.

The unique core cluster of Mediterranean MLGs appeared closer to one MLG from Brisbane than to those from the northern part of the native range (Kelso Reef and Townsville). Finally, a large cluster grouped all samples from the invaded range of New South Wales closer to samples from Townsville and Kelso Reef than to those from Brisbane, except for the two MLGs detected in Port Jackson that belonged to the Townsville cluster. In contrast, the large South Australian cluster formed a cohesive and distinct assemblage of closely related MLGs with a core MLG found in all three locations (Lake Conjola, Burrill Lake and Narrawallee), although some unique and divergent MLGs were also detected in Narrawallee.

Endophytic microbial communities

α-Diversity analysis

The α-diversity (standardized for the smallest number of sequences) for the endophytic community ranged widely, from 29 in a SU from the Mediterranean to 694 in an SU from NSW, and higher values were usually observed for non-disinfected samples (i.e., endophytic and epiphytic strains, Table S2). When pooling replicates to obtain estimates of diversity per location, the communities with highest diversity were found in Queensland (Townsville) and NSW (Narrawallee; Table S3, Supplementary material).

ß-Diversity

A clear and significant differentiation of disinfected communities versus non-disinfected and sediment communities was supported by ANOSIM (p<0.001) and illustrated by the PCoA projection (Fig. 33), with a lower but significant differentiation between the pool of non-disinfected SUs and that of the sediment (p<0.01, Table S4). The differentiation between disinfected communities and the cluster of non-disinfected and sediment communities was also reflected in differences in frequencies of the OTUs at the class and order levels (Fig. S1, Fig. S2).

The disinfected SUs from Australia and the Mediterranean form two differentiated clusters that are closer to each other than to 'environmental' samples (Fig. 3a). Mediterranean endophytic communities segregated significantly (p<0.05, Supplementary Table S5) from all Australian ones (Figs. 2b, 3a and 3b). Two groups of Australian SUs emerged, corresponding to localities from the Australian invaded and native ranges, except for the SUs sampled in Port Jackson (NSW), which clustered together on PCoA with those from Townsville (QLD) in the native area (Figs. 2b and 3b). Except for this case, and for Burrill Lake, for which the lack of replication did not allow statistical analysis, all samples showed statistically significant differentiation among regions (QLD, NSW, Med, p<0.05, R=0.86), while no heterogeneity among localities was detected within each invaded region (Med, NSW without Port Jackson; p>0.05; Fig. 3b; Table S5).

Bacterial community characterization

From the phylum Proteobacteria, the most represented are Alpha-, Beta- and Gammaproteobacteria. The former and the latter are common to all regions and treatments, while OTUs belonging to the class Betaproteobacteria are characteristic of disinfected samples

(endophytic communities; Fig. S1). Burkholderiales is the most widely represented order of this class, and it is highly represented in the Mediterranean Sea, Townsville and Port Jackson and absent in other samples from NSW (Fig. S5). A mirror image was observed for Rhizobiales, all disinfected SUs being shared throughout the Australian NSW invasive range except those from Port Jackson, which clustered overall with the SU from the native range of Townsville (Fig. 2b, Fig. 3b). For Gammaproteobacteria, specifically the order Pseudomonadales, sequences from SUs sampled in the native areas, the Mediterranean, and Port Jackson often shared OTUs absent from the cluster samples from NSW, which in turn exhibited a wide diversity of characteristic OTUs (Fig. S4).

The networks of haplotypes (Fig. 4) showed either a complete admixture of haplotypes independently of their geographical origin (for the most common OTUs within Burkholderiales, assigned by BLAST to *Alicycliphilus denitrificans*), or geographic clustering of the most common OTUs (within Pseudomonadales, assigned to *Acinetobacter* sp). In addition to being rare in NSW (except Port Jackson), "*Acinetobacter*-like" OTUs from NSW were also very distinct : only the SUs from Port Jackson one SU from Narrawallee share haplotypes in common with the Mediterranean and Queensland areas. Interestingly, this SU from Narrawallee which bacterial communities departs from the most ones characterized in NSW is also the SU having a genome most distant from all other NSW SUs (Fig. 3b).

Congruence between host and Bacterial community patterns

This study shows a striking congruence between the levels of diversities (both *α* and *ß*) of the genomic and bacterial compartments of the native and invaded ranges (Table 1, Fig. 2). A significant relationship was detected between *α*-diversities (Figure S6) of the host genome (as

estimated through heterozygosity: $r^2=0.63$ & $p=0.03$, and standardized allelic richness $r^2=0.61$ & $p=0.04$) and the α-diversity of endophytic communities (the standardized number of estimated OTUs), whereas no relationship was detected with clonal diversity R (as estimated through R: $r^2=0.13$, $p=0.43$). Similarly, the Mantel test performed to test for the relationship between patterns of β-diversity among location for the population genetics of the algae (Fst values) and for their endophytic communities (Bray-Curtis distance) showed a significant relationship ($Z=21.7$, $p=0.04$), and the clusters appearing below the percolation thresholds for both the host and the bacterial networks showed a high congruence (Table S6). Together with the specific nature of endophytic communities compared to environmental controls (Fig. 3), this reveals a concordant signature of the invasion process on both the host and endophyte genomes.

Discussion

Difference in invasion scenarios were described and explained by the interaction of migration with other ecological and/or evolutionary forces (in particular life history traits and mating system) during the invasion process (Facon *et al.* 2006; Puth & Post 2005; Rius *et al.* 2014).This study illustrates two contrasted scenario of invasion for the same species, supporting a unique introduction of a pre-adapted genotype reproducing clonally in the Mediterranean sea *versus* multiple introductions of different genotypes in NSW. The magnitude and distribution of the diversity of both *Caulerpa taxifolia* and the bacterial community in two distinct invaded areas provide remarkably coherent and relevant information suggesting that the holobiont concept may allow to better understand the interaction of migration with ecological and evolutionary forces during this process.

Aquarium origin and clonal spread in the Mediterranean versus multiple introductions in New South Wales

The comparison of the Mediterranean and NSW invasion cases provides contrasting patterns that strongly suggest signatures of unique versus multiple introductions, respectively. The hypothesis of a unique event of release of *Caulerpa taxifolia* from the Monaco aquarium into the Mediterranean was first published by Meinesz & Boudouresque 1996. This hypothesis was confirmed by phylogeographic studies through Internal Transcribed Spacer (ITS) sequencing (Jousson *et al.* 1998; Meinesz & Boudouresque 1996). The microsatellites used here (Arnaud-Haond *et al.* 2013) allowed to increase the resolution power. The high polymorphism revealed in the native area allow the interpretation of the low diversity of MLGs sampled in the Mediterranean Sea (as well as in historical samples of the aborted invasion of California) as a likely outcome of the restricted diversity available in an aquarium acting as the source for the invasion. The perfect match in the identity of the MLG characterized from the aquarium and those in San Diego and some Mediterranean SUs confirms the origin of the Mediterranean and Californian strains as a single aquarium strain spread by commercial trade. Additionally, the genetic proximity of these invasive SUs with the ones sampled in Brisbane (Fig. 2) also supports an origin of this commercial aquarium strain closer to the populations of the southern part of the native range, as previously suggested on the basis of morphological and genetic singularities (Benzie *et al.* 2000; Fama *et al.* 2002; Meusnier *et al.* 2001; Meusnier *et al.* 2002; Meusnier *et al.* 2004; Schaffelke *et al.* 2002). Slightly different MLGs were also observed in the Mediterranean, suggesting that in addition to clonal growth, either somatic mutations or sexual recombination have occurred. The genetic difference affected a single locus and only one allele motif at a time, while heterozygosity was maintained in three other loci, favoring somatic

mutations in the populations established in the Mediterranean, consistent with the lack of observation of female gametes and sexual reproduction in the Mediterranean (Zuljevic & Antolic 2000).

In contrast, much higher diversities were observed at both the host and endophytic bacterial compartment levels in the invaded lagoons of NSW (Table 1), where the origin and mechanism of the more recent introduction had thus far remained an open question due to the low power of ITS (Schaffelke *et al.* 2002). A first attempt to use Amplified Fragment Length Polymorphism (Murphy & Schaffelke 2003) allowed preliminary inferences as to the origin of the samples from Port Jackson and Lake Conjola (NSW). Here, the joint profile of higher diversity and the dichotomy in the distribution of host genomes, similar to that of bacterial communities, clearly support multiple origins of the populations of NSW. Microsatellite data, strengthened by the remarkably analogous pattern observed for associated bacterial communities, support an origin of the Port Jackson population close to Townsville, as previously suspected on the basis of ITS analysis (Schaffelke *et al.* 2002), whereas the other algae from NSW show distinct signatures . These cluster further apart, at equivalent distances from samples collected in the Northern part of the native range and from Mediterranean specimens. However, the population from Narrawallee exhibits a slightly distinct pattern of higher genetic diversity, suggesting the possibility of distinct introductions in this inlet. Additional analyses of bacterial communities in the southern part of the native range would likely bring relevant information to narrow down the screening of the approximate origin of Southern NSW *C. taxifolia*, as the relatedness of the Port Jackson population to that in Townsville is supported by both microsatellite and bacterial data.

This inference supports the hypothesis of several distinct events and likely vectors of introductions in NSW possibly via ship or aquaculture transfer, the two main vectors of marine introductions (Ruiz *et al.* 2000; Williams 2007). The heavy traffic of recreational boats in the area may favor the transport of algae in lakes and inlets (Schaffelke *et al.* 2002), and although ballast water does not seem to be a major vector of introduction for green algae (Williams & Smith 2007), it may be efficient over short distances and may be considered as a plausible vector in the case of Port Jackson in particular. Finally, aquaculture transfer of shellfish is also a major source of invasion (Ruiz *et al.* 2000) that should not be discarded, given that many shellfish are commercialized along the Eastern coast of Australia, some of them, including *Saccostrea glomerata*, which is distributed from Queensland to New South Wales, having been subject to extensive translocation practices across hundreds of kilometers (Banks *et al.* 2006).

Is the bacterial community part of the invasion process?

Importantly, the footprint of the origin of introduced populations as well as the signature of the introduction events having affected the effective population size of the algae appear on the levels of diversity revealed both for the host genome and the endophytic bacterial communities. The epiphytic bacterial communities, clustering with sediment samples, seem to mainly deliver information on environmental conditions and bacteria acquired from the environment. In contrast, the distribution of endophytic communities exhibits a remarkable correspondence with that of genetic polymorphism of *C. taxifolia*. This high level of coherence (Table 1, Fig. 2) suggests that a significant part of the endophytic community is possibly submitted to some form of vertical transmission. The clonal mode of *C. taxifolia* has the peculiarity that vertical transmission can be ensured during the events of clonal spread and fragmentation. Even if the

mechanism of transmission of bacteria during gamete release and sexual reproduction remains unknown, the similar heterogeneity and differentiation of host and bacterial communities in the sister lagoons and inlets of NSW supports a co-introduction of part of the endophytic bacterial communities. Such coherence between the distributions of host and bacterial lineages, show that endophytic communities thus have the potential to play an active role in the successful establishment and spread of *C. taxifolia* in invaded areas.

The success of *C. taxifolia* as an invader has been attributed to a number of traits, such as its capacity for vegetative proliferation and fragmentation (Ceccherelli & Cinelli 1999; Smith & Walters 1999), a strong resistance to desiccation (Schaffelke & Deane 2005) and low palatability to local predators (Gollan & Wright 2006) in line with the ERH, all likely to improve the competitiveness of *C. taxifolia*. Additionally, its role as an engineer species inducing sharp habitat modifications both in the Mediterranean (Chisholm & Moulin 2003; Holmer *et al.* 2009) and in Southern Australia (Byers *et al.* 2010; Gallucci *et al.* 2012) likely plays an important role in the displacement of native species. In the Mediterranean, the capacity to grow on anoxic sediment rich in organic matter, hydrogen sulfide and inorganic phosphorous (Chisholm *et al.* 1996), and to induce in turn changes in the sediment, may be related to the function of rhizoids as 'root-like' systems associated with specific bacterial lineages that induce enhanced nutrient fixation (Chisholm *et al.* 1996; Chisholm & Moulin 2003; Holmer *et al.* 2009). This physiological peculiarity may also explain the release of organic matter stimulating sulfate reducers, which yields sulfides highly toxic to their seagrass competitors (Holmer *et al.* 2009).

Data here are limited to partial 16S characterization, which, together with the lack of *in situ* labeling or experimental cross-validation, only allows indirect inferences and preliminary hypotheses on the metabolism of OTUs exhibiting the best BLAST alignment. As a preliminary step, we compared the bacterial functions of the closest relative presenting the highest BLAST scores with some known sediment particularities in presence of *Caulerpa sp.* in the Mediterranean, including anoxia, phosphate precipitation and discharge in waste water (Chisholm *et al.* 1997), as well as enhanced rates of nitrogen fixation and high sulfide invasion (Chisholm & Moulin 2003; Holmer *et al.* 2009). Chisholm *et al.* (1996) suggested that endocellular bacteria could allow the rhizoids of *C. taxifolia* to function as roots, providing it with the ability to take up organic and inorganic nutrients from sediments and potentially facilitating the removal of toxic sulfide while enhancing N_2 fixation. Our screening of endophytic communities revealed a large prevalence of OTUs assigned to bacteria classically known as N_2-fixing.

The two most prevalent and ubiquitous OTUs in epiphytic communities in the three regions belong to the Burkholderiales and Pseudomonadales. Their closest cultured relatives according to BLAST being respectively, *Alicycliphilus denitrificans* (type strain K601) a nitrate-reducing betaproteobacterium able to grow in anaerobic conditions (Dullius *et al.* 2011; Mechichi *et al.* 2003), and *Acinetobacter* sp., encompassing bacteria promoting plant growth via different mechanisms, including nitrogen fixation. The latter showed a clustering of lineages partly in line with that of their host, with haplotypes shared between the native area and the Mediterranean but also with Port Jackson (Fig. 4), whereas a secondary and smaller cluster characterize most NSW samples. Such tightly associated OTUs may be useful tracers of the origin of invasion, provided

an improved sampling of the native area. Interestingly, the "*Alicycliphilus denitrificans*-like" OTU is the sister OTU of the one identified on *Caulerpa racemosa*, that showed a segregation of haplotypes according to their host variety (Aires *et al.* 2013). Despite strict segregation of these OTU haplotypes at the species and variety levels, no differentiation was observed here among geographic regions, possibly due to larger effective population size than *Acetinobacter sp.* or to a different mode of transmission. Together with their absence in control samples, these results suggest a tight association of host with endophytic lineages whose putative function presents a remarkable analogy with the characterized upheaval in sediments in the invaded areas (Chisholm *et al.* 1996; Chisholm & Moulin 2003; Holmer *et al.* 2009), causing the displacement of many native species (Ceccherelli & Cinelli 1997; Garcias-Bonet *et al.* 2008).

The holobiont concept to better understand the process of invasion

The results reveal a tight association between the eukaryotic genome and the prokaryotic compartments, suggesting the heritability of part of the bacterial community and confirming its potential as a marker of the origin and path of dispersal (Meusnier *et al.* 2001; Nieberding & Olivieri 2007). A consequence of this association, together with the putative function of bacteria, is their possible role in the "species"-based mechanisms (such as ERH and EICA, mentioned above) of invasion. This would support the Accompanying Hypothesis (Klock *et al.* 2015; Rodriguez-Echeverria 2010) to hold true for a broad part of the endophytic community. These results are also in line with recent synthesis underlining the ecological influence of bacteria on marine organisms (Wahl *et al.* 2012). Our results suggest that these species-based mechanisms must be reconsidered in terms of invasive "meta-organisms" or "holobionts" rather than single

species because functions performed by endophytic bacteria may have facilitated the establishment and spread of *Caulerpa taxifolia* in the areas where it was introduced.

In conclusion, the results presented here support the use of the Accompanying Mutualist Hypothesis to describe, at the scale of the holobiont, the mutualistic relationship between *Caulerpa taxifolia* and its endophytic bacterial community during the course of invasion. The spread of the incidentally escaped aquarium strain of the green algae in the Mediterranean was initially presented as an archetype of the genetic paradox of invasions (Frankham 2005; Roman & Darling 2007). The present comprehensive analysis of diversity patterns based on the recent development of hyper-variable markers for the host genome and the unlocking of massive metagenomic characterization of the endophytic bacterial communities made it a triple paradox at the holobiont scale: genotypic (with a single clone), genetic (this clonal lineage logically resulting in limited allelic diversity), and endophytic (extremely reduced diversity of bacterial communities compared to the native area). The paradox, however, may well be lost, or it may not last more than several decades at least in the case of *C. taxifolia*: since 2004, the species once called "killer algae" has vanished from several localities (Ivesa *et al.* 2006; Meinesz *et al.* 2010; Montefalcone *et al.* 2015; Tejada & Sureda 2013), and the surface covered has regressed by approximately 80%. This contrasts with the present state in NSW, where the more variable introduced populations currently show no sign of decline, approximately 15 years after their introduction. Among the main speculations that can be proposed to explain the Mediterranean regression, the poor resistance to temperature variations (particularly to cold winters), the genetic degeneration of the clonally spread host and the emergence of an antagonistic bacterial or virus strain may have affected the holobiont. Insufficient phenotypic plasticity, genetic degeneration

and susceptibility to changes in abiotic and biotic environments are indeed more likely in a drastically impoverished genetic lineage, considering the genetic background of both the host and the symbionts. This regression is relatively recent and may be temporary. If the reason underlying the failure of the invasion is associated with the low diversity in distinct compartments, there may be greater impacts of a newly introduced variety recently discovered in Turkey and Italy (Jongma *et al.* 2013; Turan *et al.* 2011), *C. taxifolia* var. *distichophylla*, which is morphologically similar and extremely close genetically to the large male clone present in the Mediterranean for the past three decades, and the newly introduced variety is more diverse (Jongma *et al.* 2013). Moreover, if interbreeding occurs, a very possible event considering their morphological and genetic proximity (Jongma *et al.* 2013), this variety may also provide a genetic and/or bacterial rescue to the initially isolated holobiont clone.

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Data Accessibility

Author Contributions

SAH, MV and ES designed the study, SAH and TA performed the sampling, SAH, TA, RC and

SJL produced the data, SAH and TA analyzed data, all authors contributed to their interpretation,

SAH and TA wrote with CMD, MV and ES wrote and edited the manuscript

Tables
Table 1. Details of the samples collected, with the number of sampling units (N), year of collection, approximate depth, approximate area sampled, number of distinct MLGs discriminated (G) standardized for the lowest number of samples (13) in locations where more than 10 sampling units were collected, standardized (Â) allelic richness, and number of estimated OTUs (Sp) also standardized for the lowest number of sequences obtained among samples.

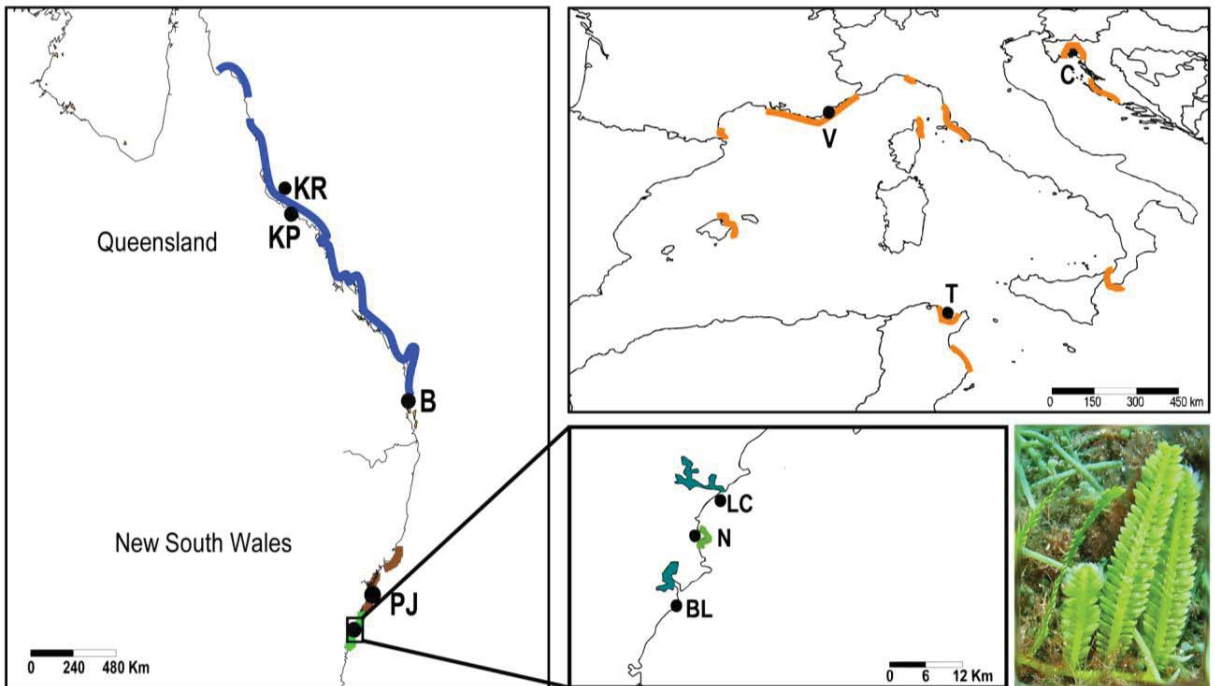| Region	Location	N	Year	App. depth	App. area	Ĝ	R	A	Â	Hnb	Sp
NE Australia	All	44	-	-		11.95	0.76	45	33.6	0.68	1425
	Kelso Reef	2	1999	-	-	-	-	16	-	-	-
	Kissing Point	23	2010	0-2	1	12.71	0.95	25	22.45	0.43	661
	Brisbane	19	1999	-	-	8.56	0.56	16	14.86	0.23	-
SE Australia	All	100	2010	-		4.33	0.00	38	24.28		1960
	Port Jackson	30	2010	2-6	1	1.68	0.02	17	14.12	0.13	270
	Lake Conjola	30	2010	0-2	1	1	0	14	14	0.38	625
	Narrawallee	17	2010	0-2	1	4.31	0.25	24	22.25	0.46	696
	Burrill Lake	32	2010	0-2	1	4.95	0.10	18	16.65	0.40	501
Mediterranean	All	38	-	-	-	4.03	0.11	12	12.90	0.27	321
	Aquarium	2	-	-	-	-	-	11	-	-	-
	Villefranche	13	2009	11-16	1-2	4	0.25	12	13	0.27	94
	Croatia	1	1999	-	-	-	-	12	-	-	-
	Tunis	22	2009	?	1-1.5	3.15	0.14	12	12.90	0.27	120
Pacific	San Diego	2	2000	-	-	-	-	14	-	-	-
	Tahiti	2	1999	-	-	-	-	17	-	-	-

Figure Captions

Figure 1. Main sampling locations in Queensland (Blue; **KP-** Kissing Point/Townsville, **KR-** Kelso Reef, **B-** Brisbane), NSW (Green and brown, **PJ-** Port Jackson, **LC-** Lake Conjola, **N-** Narrawallee, **BL-** Burrill Lake) and the Mediterranean (Orange, **C-** Croatia, **V-** Villefranche, **T-** Tunis). Color codes are standardized with figures illustrating the alpha and beta diversities of samples based on microsatellites for algae and metabarcoding for bacterial communities. The picture on the lower right shows a typical Mediterranean specimen of *Caulerpa taxifolia*, and some intermingled stolons of the also-invasive sister species *C. racemosa* can be seen in the background (credits Sophie Fallour).

Figure 2. (a) Network of multi-locus genotypes (MLGs) of *Caulerpa taxifolia* based on Allele Sharing Distance (percolation threshold D_{pe}=1.25 non-shared alleles per loci). Each dot represents a MLG, and links are tuned from light grey to black at decreasing distances among MLGs. The size of nodes is standardized except for MLGs encountered more than 5 times in the dataset (for New South Wales (NSW), 60 replicates were shared among BL, N, LC, and PJ; 30 replicates were collected from the Mediterranean, with 25 identical MLGs, including the samples from the aborted Californian invasion). (b) Network of endophytic microbial communities based on Bray Curtis Distance (percolation threshold, D_{pe}=0.84). For both networks, blue dots represent genes from the native Queensland (QLD) collected in Kelso Reef (turquoise), Townsville (dark blue) and Brisbane (light blue). Brown and green dots represent genes the invasive NSW (Brown for Port Jackson; light green for Narrawallee; dark green for both Lake Conjola and Burrill Lake, which share many MLGs). For the MLG network, orange dots in the Mediterranean encompassed an admixture of marginally distinct MLGs (1 single locus) mostly

common, including the Aquarium Strain, circled in red. (c) Levels of clonal, genetic and bacterial diversity in QLD (blue), NSW (green) and the Mediterranean (orange). The number of OTUs is divided by 100 to allow it to be projected on the same axis as allelic richness.

Figure 3. Principal Component Analysis of bacterial communities. The panels illustrate the segregation for (a) the entire sample of microbial assemblages and (b) endophytic (disinfected) SUs only.

Figure 4 Haplotype network of the OTU assigned through BLAST to (a) *Alicycliphilus denitrificans* (Betaproteobacteria) and (b) *Acinetobacter* sp. (Gammaproteobacteria). Each dot represents a haplotype or a set of very closely related haplotypes merged by the star contraction algorithm. The length of links is approximately proportional to the nucleotidic distance among haplotypes but was slightly modified between the two dominant haplotypes (distinct by only one mutation) to improve visualization. The color code is identical to the sampling and network figures, with blue for Queensland, green and brown for NSW (brown for Port Jackson, light green for Narrawallee, and dark green for both Lake Conjola and Burill Lake), and orange for the Mediterranean.

Lorem ipsum

a)

b)

- Queensland
- Tahiti
- New South Wales
- Mediterranean Sea
- Aquarium strain

c)