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Polymerase chain reaction–single strand conformation polymorphism analyses of nuclear and chloroplast DNA provide evidence for recombination, multiple introductions and nascent speciation in the *Caulerpa taxifolia* complex

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Abstract

Independent lines of evidence support an Australian origin for the Mediterranean populations of the tropical alga *Caulerpa taxifolia*. To complement previous biogeographical studies based on nuclear rDNA internal transcribed spacer (ITS), a new chloroplast marker was developed — the cp 16S rDNA intron-2. Sequence variability for both nuclear and chloroplast markers were assessed in 110 individuals using single strand conformation polymorphism. Comparison of intrapopulation genetic diversity between invasive Mediterranean and 'native' Australian populations revealed the occurrence of two divergent and widespread clades. The first clade grouped nontropical invasive populations with inshore-mainland populations from Australia, while the second clustered all offshore-island populations studied so far. Despite our finding of nine distinct nuclear and five distinct chloroplast profiles, a single nucleocytoplasmic combination was characteristic of the invasive populations and sexual reproduction was found to be very rare. *C. taxifolia* is clearly a complex of genetically and ecologically differentiated sibling species or subspecies.

Keywords: asexual reproduction, chloroplast 16S intron, invasive species, nuclear cytoplasmic disequilibrium, rDNA ITSs

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Introduction

Biotic invasions are recognized as a worldwide problem (Kolar & Lodge 2001). Over the past several decades, the number of invasive marine species has increased dramatically as a consequence of human mediated transport. This is the case for the tropical alga *Caulerpa taxifolia*. The alga was discovered in 1984 off the coast of Monaco in the Mediterranean (Meinesz & Hesse 1991), in 2000 near San Diego, California, USA (Jousson *et al*. 2000) and near Sydney, Australia and in 2001 (Wiedenmann *et al*. 2001). Using rDNA-internal transcribed spacer (ITS) sequences, Jousson *et al*. (1998) demonstrated that the alga proliferating in the western Mediterranean was accidentally introduced via one or several aquaria. Additional studies using different types of markers such as allozymes (Benzie *et al*. 2000), fingerprints (Wiedenmann *et al*. 2001), bacterial microflora associated with *C. taxifolia* (Meusnier *et al*. 2001), ITS sequences (Jousson *et al*. 2000; Meusnier *et al*. 2001; Famà *et al*. 2002; Schaffelke *et al*. 2002) and the presence/absence

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of an intron located in the *rbc*L gene of chloroplast DNA (Famà *et al*. 2002) have confirmed or are consistent with an original introduction from Eastern Australia.

More than 100 nuclear rDNA-ITS sequences from *C. taxifolia* and other *Caulerpa* species are available from GenBank. These sequences have proved valuable in sorting out interspecific phylogenies (Olsen *et al*. 1998) and identifying some biogeographical divisions at the intraspecific level (Meusnier *et al*. 2001; Schaffelke *et al*. 2002). Unfortunately, low sequence divergence combined with the peculiarities of concerted evolution (Dover 1989) affecting ITS sequences has also limited their utility at the intraspecific level. Intra-individual variability due to incomplete sequence homogenization has been observed in the ITS sequences of many plant (Sang *et al*. 1995; Buckler & Holtsford 1996; Campbell *et al*. 1997) and algal species (Serrão *et al*. 1999), including *C. taxifolia* (Jousson *et al*. 1998; Famà *et al*. 2002) and *C. racemosa* (Famà *et al*. 2000; Durand *et al*. 2002).

In contrast to nuclear DNA, chloroplast DNA is usually nonrecombinant because of uniparental inheritance (Reboud & Zeyl 1994; Birky 1995). The chloroplast genome evolves slowly in comparison with the nuclear genome in angiosperms (Wolfe *et al*. 1987). In several species of *Caulerpa*, Manhart (1995) described the occurrence of three introns located within the chloroplast (cp) 16S rDNA that are sufficiently variable for phylogeographical reconstruction.

In the present study we assessed genetic diversity in *C. taxifolia* using the second intron (intron-2) of the cp 16S rDNA and compared these new data with previous studies based on nuclear rDNA ITS. We also exploit the uniparental inheritance of the marker to evaluate the extent of sexual recombination. Strong nucleocytoplasmic linkage disequilibrium is expected for clonal species, whereas for sexually reproducing species, recombination regularly shuffles nuclear information among the different cytoplasms.

Materials and methods

Sample collection and DNA extraction

A total of 110 individuals (102 *C. taxifolia* and eight *C. prolifera*) was studied (Table 1). We sampled Mediterranean locations across the area occupied by *C. taxifolia*, from Croatia to the Balearic Islands. In Australia we collected samples from three populations: two located inshore along the Queensland coast at Kissing Point (near Townsville) and at Brisbane further south; and the third offshore on Kelso Reef, Great Barrier Reef. A single specimen was made available from the invasive population found north of San Diego, Calfornia, USA; one specimen from Bolinao, Philippines; and two specimens from Moorea, Tahiti. *C. prolifera* was chosen as outgroup. Protocols used for collection, storage and extraction of genomic DNA are described in Meusnier *et al*. (2001).

Single strand conformation polymorphism (SSCP)

Intrapopulation genetic variation for both nuclear and chloroplast markers was investigated using single strand conformation polymorphism (SSCP) (Kanazawa *et al*. 1986; Sunnucks *et al*. 2000). Differences of one or more nucleotides can be detected in fragments from 300 to 450 base pairs (bp) with nearly 90% accuracy. The method was applied to all 110 samples for both nuclear rDNA-ITS2 and cp 16S rDNA intron-2. The more variable part of the sequences was amplified. For the ITS2, forward and reverse primers were designed from all *Caulerpa* ITS sequences available in GenBank. For the cp intron-2, primers were designed after sequencing the complete intron-2 of the cp 16S rDNA (≈ 1 kb) in four *Caulerpa* from different species (two *C. taxifolia*, one *C. mexicana* and one *C. prolifera*); 170 bp of ITS2 were amplified using CaITS02-forward (5′-end of the ITS2: 5′AAGCAGTGGTTCAGTGAGCA3′) and CaITS01-reverse (3′-end of the ITS2, 5′GGCATTCAATCTCAGCGATT3′). 550 bp of the chloroplast intron were amplified using cp16S01-forward (5′GCCAAAGGTGTCCTGCTATA3′) and cp16S01-reverse (5′CATCAAGCCACCAAATAGCC3′) primers. Amplifications were performed on a Perkin-Elmer 9600 or 9700 thermocycler in 20-μL volumes (5 min at 94 °C, 30 cycles of 45 s at 94 °C, 45 s at 60 °C (for chloroplast intron-2) or 55 °C (for ITS 2), 1 min 30 s at 72 °C, followed by a cycle of 7 min at 72 °C). Eight μL of loading buffer (15% Ficoll, 0.25% Bromophenol Blue, 0.25% xylene cyanol), 5.3 μL of urea (5 M) and 19.7 μL TBE 1 × were added to the polymerase chain reaction (PCR) products. The solution was denatured during 5 min at 100 °C and snap-cooled on ice before loading.

The best electrophoresis conditions for ITS2 were: 15 μL denatured DNA on a 10% acrylamide gel (Bio-Rad) run in 0.5 × TBE at 200 V during 20 h at 4–6 °C; while for the chloroplast intron a 5% acrylamide was used. After electrophoresis, gels were silver-stained as described in Saumitou-Laprade *et al*. (1999). PCR-SSCP gels were highly reproducible under these conditions.

PCR amplification and sequencing

We verified that a given SSCP profile corresponded to a single sequence by sequencing at least two individuals for each different profile whenever possible. The sequencing covered the whole ITS region including ITS1 (513 bp long) and the SSCP portion of cp intron-2 (550 bp long) associated with each different ITS profile (Table 1).

ITS2 amplification conditions and sequencing were carried out as described in Meusnier *et al*. (2001). Cp intron amplifications were performed as above but we used an

Table 1 Sample collection used for SSCP and sequence analysis of internal transcribed spacer 2 of nuclear rDNA (ITS2); and intron-2 of cp rDNA16S gene. Sample size used for the SSCP is given in parentheses

Species	Geographical region and origin	Locality	Code	SSCP profiles		No. of sequences		GenBank accession nos	
				ITS2	intron-2	ITS2	intron-2	ITS2	intron-2
Caulerpa taxifolia	Mediterranean Basin (48)	Stari Grad Bay, Croatia (12)	Med	A	I	2†	1‡	AF259587 AF259586	AF461000
		Sicily, Italy (2)		A	I	2*	1‡	AJ228963 AJ007819	AF460998
		Elba, Italy (1)		A	I	1*	1‡	AJ007820	
		Capo Mele, Italy (5)		A	I	1†	1‡	AF259588	
		Menton, France (8)		A	I		1‡		AF460996
		St Raphaël, France (8)		A	I				
		Toulon, France (6)		A	I				
		Balearic Islands (6)		A	I	9†**	1‡	AF259569 AF259577 AF259590 AF259580– AF259585	AF460999
	Queensland coast (40)	Brisbane, Australia (19)	Aus-Bri	A	I	2‡	2‡		AF461001
		Kissing Point, Australia (21)§	Aus-KP	B (9)	I	1‡	1‡	AF401317	AF461002
				C (6)	I	1‡	1‡	AF401318	
				D (3)	I	2‡ + 1*	2‡	AF401319 AJ007823	
				E (1)	I	1‡	1‡	AF401320	
				F (1)	II	1‡	1‡	AF460994	AF461004
				G (1)	II	1‡	1‡	AF401321	
	Great Barrier Reef (9)	Kelso Reef, Australia (9)	Aus-KR	H (8)	III	3‡	2‡		AF461005
				I (1)	III	1‡	1‡	AF401316	
	Malaysia (1)	Bolinao, Philippines (1)	Phil	J	V	1†	1‡	AF259589	AF461007
	Polynesia (2)	Tahiti (2)	Tah	H	IV	1† + 1‡	2‡	AF259579	AF461006
	California (1)	Carlsbad, USA (1)	Carl	A	I	1‡	1‡		AF461003
	Monaco Aquarium (1)	Monaco (1)	AqMon	A	I	1*	1‡	AJ007822	AF460997
Caulerpa prolifera	Nausicaa Aquarium (1)	Boulogne, France (1)	AqNau	α	1	1‡	1‡	AF259571	AF461008
	Mediterranean Basin (7)	Golfe Juan, France (5)	Med-G	β	1	2‡	2‡	AF259578 AF259570	AF461009
	Djerba, Tunisia (2)		Med-Tun	γ	1	2‡	2‡	AF460995	AF461010

*Sequences from Olsen *et al*. (1998).
†Sequences from Meusnier *et al*. (2001).
‡This study.
§The individual designated as 'Townsville' is actually from Kissing Point but is not the same specimen. The designation 'Townsville' is retained here because that specimen was cited as 'Townsville' in Olsen *et al*. (1998).
**DNA was cloned before sequencing, cf. Meusnier *et al*. (2001).

M13-tailed cp16S01-reverse primer (Steffens *et al*. 1993; Roy *et al*. 1996) to sequence the PCR products directly. PCR reactions were purified using a Qiagen®kit (QIAquick™, Qiagen, Valencia, CA, USA). The fragment was sequenced in both directions using universal M13-reverse primer (IRD 700, fluorescence-labelled) on a 4200-L automated DNA sequencer (LiCor).

Sequence alignment and phylogenetic analyses

The ITS and intron-2 sequences were managed and aligned using BIOEDIT version 4.7.8 (Hall 1999). Phylogenetic analyses were performed using PAUP version 4.0b 3 (Swofford 1999). Models for sequence evolution were determined using MODELTEST version 3 (Posada & Crandall 1998). Although both maximum parsimony (MP) and maximum likelihood (ML) methods were used, ML cannot be applied to gaps, which are relevant in the present data analysis. MP analyses utilized a branch and bound search with tree bisection-reconnection and branch swapping. Gaps were coded as 'new state' or as 'missing data'. When ML was applied to the data sets, the HKY + G model (Hasegawa *et al*. 1985) of sequence evolution was used. Bootstrap values for all analyses were obtained from 10 000 replicates. Phylogenetic trees were displayed using TREEVIEW version 1 (Page 1996).

Results

ITS2 SSCP profiles

ITS2 showed variability at the intra- and interpopulation levels for both *C. prolifera* and *C. taxifolia*. We determined three SSCP profiles for *C. prolifera* (α, β and γ, Fig. 1a), and 10 profiles for *C. taxifolia* (A–J, Fig. 1a). In both species, SSCP profiles consisted of three to six major bands (Fig. 1a). These multibanding patterns can be explained by the several folding conformations that each DNA molecule can take under SSCP conditions (Desmarais *et al*. 1995). Weaker bands can also be indicative of incomplete homogenization of the multiple rDNA copies through concerted evolution. Profile F is of special interest, because it showed a minimum of nine major bands, which corresponded to a combination of profiles E and G. This suggests that both ITS types are equally frequent in this individual.

For *C. taxifolia*, no polymorphism was observed among the Mediterranean samples (Fig. 2a). In contrast, substantial polymorphism was found among Australian samples. Intrapopulation polymorphism was marked, except in the nontropical population from Brisbane. Regardless of their geographical origin, nontropical populations were all characterized by the same unique profile (profile A, Fig. 2a).

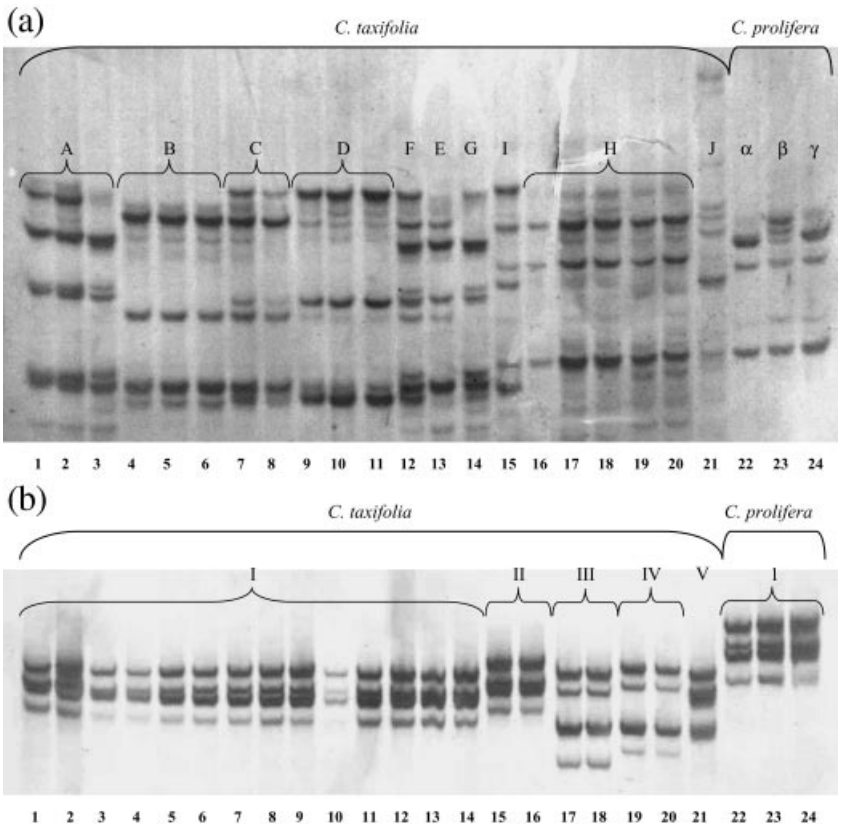

Fig. 1 SSCP gel for *C. taxifolia* and *C. prolifera*. (a) SSCP profiles of ITS2: 10 profiles (labelled A–J) for *C. taxifolia* (lanes 1–21) and three profiles (labelled α, β and γ) for *C. prolifera* (lanes 22–24). For *C. taxifolia*: lane 1, Monaco Aquarium; lane 2, Carlsbad; lane 3, Brisbane; lanes 4–10, Kissing Point; lane 11, Townsville (also Kissing Point but reported as Townsville in Olsen *et al*. 1998); lanes 12–14, Kissing Point; lanes 15–18, Kelso Reef; lanes 19–20, Tahiti; lane 21, Philippines. For *C. prolifera*: lane 22, Nausicaa Aquarium; lane 23, Golfe Juan; lane 24, Djerba. (b) SSCP profiles of chloroplast intron-2: five profiles (labelled I–V) for *C. taxifolia* (lanes 1–21) and one (labelled 1) for *C. prolifera* (lanes 22–24). For *C. taxifolia*: lanes 1–2, Balearic Islands; lane 3, Menton; lane 4, Monaco Aquarium; lane 5, Carlsbad; lane 6, Croatia; lanes 7–8, Brisbane; lanes 9–12, Kissing Point; lane 13, Townsville (also Kissing Point but reported as Townsville in Olsen *et al*. 1998); lanes 14–16, Kissing Point; lanes 17–18, Kelso Reef; lanes 19–20, Tahiti; lane 21, Philippines. Within *C. prolifera*: lane 22, Nausicaa Aquarium; lane 23, Golfe Juan; lane 24, Djerba.

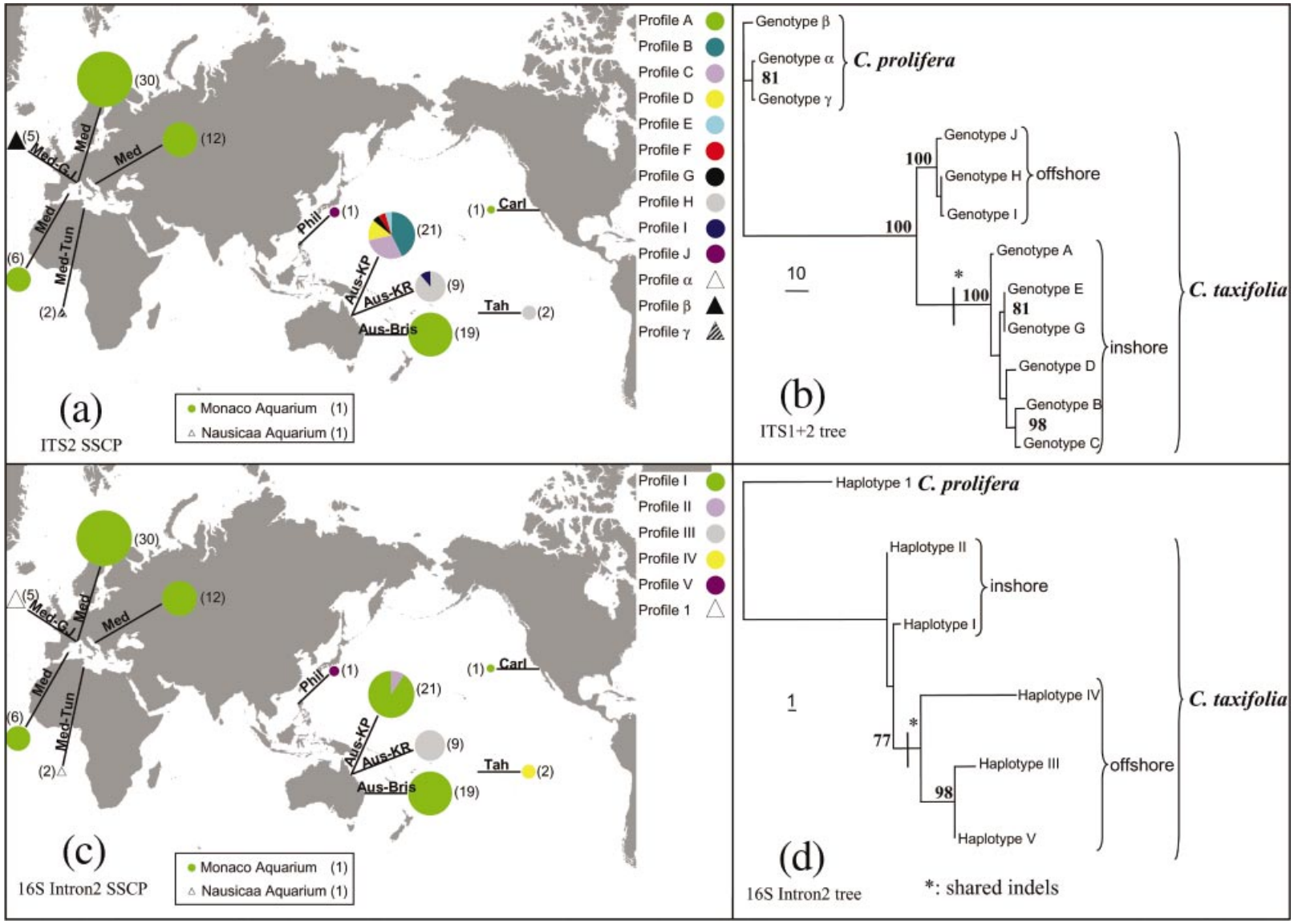

Fig. 2 Biogeographical distribution of ITS2 and 16S-cp intron-2 profiles and their phylogenetic relationships (for locality code see Table 1). (a) ITS2 profiles revealed by SSCP in *Caulerpa taxifolia* (circles) and *Caulerpa prolifera* (triangles). The number of individuals analysed is shown in parentheses. (b) One of the two most parsimonious trees obtained by intraspecific phylogenetic analysis based on complete ITS1 + two sequences of each genotype. Length = 17, consitancy index = 0.940, retention index = 0.947, gap = 'new state' and bootstrap values (= 50%) are above branches. Scale bare = 10 steps. (c) Intron-2 profiles revealed by SSCP in *C. taxifolia* (circles) and *C. prolifera* (triangles). The number of individuals analysed is shown in parentheses. (d) Single most parsimonious tree obtained by intraspecific phylogenetic analysis based on 550 bp intron-2 sequences of each genotype. Length = 82, consistency index = 0.963, retention index = 0.900, gap = 'new state' and bootstrap values (= 50%) are above branches.

Despite the multibanding patterns shown in Fig. 1a, each ITS2 SSCP profile corresponded to a single dominant sequence with the exception of Profile F. This demonstrates that the SSCP method was able to discriminate perfectly between different sequences, i.e. nuclear genotypes, among populations. The smallest observed difference between genotypes was two nucleotides (between profiles H and I observed at Kelso Reef). The sequence of profile F is a combination of sequences from genotypes E and G. Consequently, we did not use this profile for phylogeographical analysis. However, it does reveal the existence of an intraindividual polymorphism due to hybridization or sexual recombination (see below).

Phylogenetic analysis of full-length ITS

Results of the MP analysis of the ITS sequences (gaps coded as 'new state') are given in Fig. 2b, which shows one of the two most-parsimonious trees. Three well-defined clades are supported by 100% bootstrap values. Within the group of *C. taxifolia*, two clades could be identified clearly. The first clade (referred to as offshore-island) covers the three genotypes (H, I and J) from Kelso Reef (GBR, Australia) and the other tropical specimens from Tahiti and the Philippines. All sequences from this clade shared the same three insertions (data not shown). The second clade (referred to as inshore-mainland) grouped genotype A, characteristic of the invasive and 'nontropical' populations, with the five other genotypes found at Kissing Point (B, C, D, E, G). Within this clade, genotypes B and C and genotypes E and G consistently grouped together.

When gaps were excluded from the analysis, MP and ML gave the same tree topology (data not shown) but bootstrap values were low. Inclusion of the gaps, coded as 'new state' (only possible in the MP analyses) produced the high bootstrap support shown in Fig. 2.

SSCP profiles of cp intron-2

The cp intron showed variability at both the intra- and interpopulation levels. There were six SSCP profiles (Fig. 1b), five for *C. taxifolia* (marked I–V) and one for *C. prolifera* (profile 1). SSCP profiles always consisted of four bands with no weaker bands (Fig. 1b).

For *C. taxifolia* the offshore-island samples were all characterized by a unique chloroplast profile specific to their origins (profile II for Kelso-Reef, profile IV for Tahiti and profile V for Philippines, Fig. 2c). In contrast, the same profile was observed in the different inshore-mainland locations along with the invasive samples (profile I, Fig. 2c). Specifically, some individuals from Kissing Point (i.e. northern tropical mainland location) share the identical chloroplast profile with individuals collected in Brisbane (i.e. more southern, temperate mainland location). This profile I was the unique profile observed within Brisbane and invasive-type populations.

Each SSCP profile was found to correspond to a single sequence. Again, this confirms the discriminatory power of the SSCP method for distinguishing, in this case, cytoplasmic haplotypes. The smallest difference between haplotypes (two nucleotides) was found between haplotypes originating from the same population (haplotypes I and II from Kissing Point, Figs 1 and 2c).

Phylogenetic analysis of cp intron-2

Results from the MP analyses of the sequences (gaps coded as 'new state') of the cp intron-2 resulted in a single tree (Fig. 2d). Within *C. taxifolia*, one clade can be clearly identified (77% bootstrap). This clade includes the three haplotypes (III, IV, V) from offshore-island populations (Tahiti, Philippines and Kelso Reef). These haplotypes shared the same 4-bp deletion (poly A in position 471, data not shown). Although haplotypes I and II were very similar in their sequences, the number of informative sites was too low to resolve this node and they were not grouped within a clade. However, these two haplotypes are clearly divergent from the offshore-island clade (Fig. 2d).

When gaps were excluded from the analysis both MP and ML grouped haplotype IV with haplotypes I and II. Haplotype IV was found in the Tahiti sample and exhibited a unique 12-bp insertion at position 265 (data not shown).

Linkage disequilibrium between ITS 2 and cp intron-2

Because the same individuals were analysed for both the nuclear and cytoplasmic markers, we were able to investigate linkage disequilibrium between genomic compartments (Table 2). A unique chloroplast haplotype has been found for all *C. prolifera*. Within *C. taxifolia*, nuclear genotypes from Mediterranean and Australian inshore-mainland populations (A–G) were all associated with either cytoplasmic haplotypes I or II. In contrast, nuclear genotypes from the offshore-island clade (H to J) were associated with cytoplasmic haplotypes III, IV and V. The only exception to this strong nucleocytoplasmic disequilibrium was detected for the nuclear genotype H which was found in two different haplotypes (III and IV) in Kelso Reef and Tahiti samples. In the Kissing Point population, profile F proved to be a combination of two nuclear genotypes (E and G) and was associated with haplotype II.

Discussion

Separate analyses of ITS and cp intron-2 (Fig. 2b,d) yielded congruent topologies with respect to the split between inshore-mainland and offshore-island populations. The inshore-mainland group includes the invasive form of

Table 2 Association between chloroplast and nuclear SSCP profiles in *Caulerpa taxifolia* and in *Caulerpa prolifera*. Profiles for *C. prolifera* are shown as the outgroup in italics. The number of samples analysed within each locality and for each nucleocytoplasmic combination is indicated in parentheses. Abbreviations as in Table 1

	Intron II 16S (chloroplast)					
	I	II	III	IV	V	*1*
ITS2 (nuclear)						
A	AqMon (1) Carl (1) Med (30) Aus-Bri (19)					
B	Aus-KP (9)					
C	Aus-KP (6)					
D	Aus-KP (3)					
E	Aus-KP (1)					
F		Aus-KP (1)				
G		Aus-KP (1)				
H			Aus-KR (8)	Tah (2)		
I			Aus-KR (1)			
J					Phil (1)	
α						*AqNaus (1)*
β						*Med-GJ (5)*
γ						*Med-Tun (2)*

C. taxifolia and individuals from two coastal populations located at Kissing Point/Townsville and Brisbane. Note that the clade includes specimens from both temperate and tropical latitudes based on co-occurrence of the same haplotype (haplotype I, Fig. 2). Two conclusions can be drawn from these topologies. First, Australia is definitely confirmed as the origin of the Mediterranean and other invasive strains as hypothesized in a number of recent studies (Meusnier *et al*. 2001; Wiedenmann *et al*. 2001; Famà *et al*. 2002; Schaffelke *et al*. 2002). Second, differences between inshore-mainland vs. offshore-island populations suggest the possibility of strong divergence related to different habitat and/or environmental conditions, in particular temperature and turbidity.

Concerning the first point, introduced populations are often characterized by a reduced genetic variability due to founder effects and/or clonal vegetative reproduction (Abbott 1992; Lodge 1993; Kolar & Lodge 2001). In our study, no SSCP polymorphism was found within the invasive 'Med' strains nor within the mainland Australian population from Brisbane located in more temperate waters. These populations showed a single nucleocytoplasmic combination (A/I, Table 2). In sharp contrast, every other population studied was polymorphic for at least one of the marker loci. Similarly, Benzie *et al*. (2000) found a single genotype in the invasive Mediterranean populations and in a population from the Brisbane area, and low but significant polymorphism for at least one of the six allozyme loci surveyed in tropical Australian populations.

Our combined nucleocytoplasmic analyses further allow us to hypothesize that at least two consecutive founder events have occurred that involve the invasive strain. First, the Brisbane samples have most probably come from the Northern Queensland populations and second, the invasive Mediterranean strains are themselves derived from the Brisbane populations. How the tropical-northern populations found their way to a more temperate-southern location is unknown though human transport is suspected. In any case, adaptation to cold-water tolerance has occurred.

The demonstration of very strong linkage disequilibrium, with an almost 1:1 correspondence between nuclear and cytoplasmic genotypes in *C. taxifolia* (Table 2), indicates predominant asexual or clonal reproduction. Further indirect evidence for strong asexuality comes from an earlier allozyme study of several species of *Caulerpa*, including *C. taxifolia* in which Benzie *et al*. (1997) found significant heterozygote excesses in the Kissing Point and Great Barrier Reef populations they examined.

Two important new findings in our study are evidence for within- and between-population recombination events. In the tropical population from Kissing Point the ITS profile F is a combination of the G and E profiles. The combination of ITSs in this individual shows that hybridization is probably relatively recent, because ITSs have not yet been homogenized (Van Houten *et al*. 1993; Wendel *et al*. 1995; Roelofs *et al*. 1997). The two parental types, G and E, differ not only in their nuclear genotype but also in their chloroplast haplotypes (Table 2). The nuclear genotype E is linked to the more frequent haplotype I while genotype G is linked with haplotype II. We hypothesize that the individual bearing the F 'profile' resulted from a recent crossing between parents G and E, the G parent being the mother as F is linked to the same cytoplasm as G.

Our results also indicate an older recombination event between haplotypes now found in different geographical

populations, i.e, Tahiti and Kelso Reef, Australia. Nuclear genotype H has chloroplast haplotypes III and IV. Here we can hypothesize that recombination occurred between individuals with different chloroplasts, and that later, homogenization caused the loss of one type of ITS (Fuertes Aguilar *et al*. 1999; Quijada *et al*. 1997). Taken together, these results are consistent with previous studies (Jousson *et al*. 1998; Famà *et al*. 2002; Schaffelke *et al*. 2002) and indicate that sexual reproduction, although rare and apparently absent in the invasive strain, does occur in *C. taxifolia* as a stochastic event. It is possible, as suggested by Schaffelke *et al*. (2002), that individuals' fertility depends on the environmental conditions. In particular, Zuljevic & Antolic (2000) observed that cold-water temperature inhibits sexual reproduction in the invasive Mediterranean strain.

As to the second point regarding ecotypic differentiation, we hypothesize that differences between inshore-mainland and offshore-island populations are due to habitat-linked environmental variation. For example, the northern tropical strains (regardless of whether or not they are inshore-mainland or offshore-island) are small (< 10 cm tall) and delicate, whereas the more southern, cold-water strains (which include Brisbane and the invasive forms of *C. taxifolia*) are large (25–50 cm or more) and robust, with thick stolons, wide fronds and large pinnules (Benzie *et al*. 2000; Schaffelke *et al*. 2002). Gigantism is also associated with the invasive Mediterranean populations (Meinesz & Hesse 1991). As revealed in the present study, inshore-mainland and offshore-island genotypes can be separated geographically, but they can also be found in relatively close proximity without any apparent gene flow detectable. Thus, habitat and not geographical distance appears to play a role in the observed differentiation. The same geographical pattern of genetic divergence between inshore-mainland and offshore-island habitats has also been found by Schaffelke *et al*. (2002). Using ITS sequencing, they have shown that all the samples collected in six different sites located on the Great Barrier Reef were grouped together with other tropical reef populations coming from the Caribbean and the Red Sea in what they called a 'Reef Clade'. This is equivalent to our 'offshore-island' clade.

Given the strong correlation between clades based on independent molecular data sets, the presence of distinct morphologies and habitat differences, a simple explanation of phenotypic plasticity for *C. taxifolia* can be ruled out. Strong ecotypic differentiation and possible incipient speciation are more likely but extremely difficult to prove unequivocally. Field experiments involving reciprocal transplants will not be allowed in any country and laboratory experiments involving temperature tolerance are technically very difficult.

In conclusion, tracking invasions of *C. taxifolia* and establishing biogeographical source populations have proved difficult because of low variability of available markers and the need to screen hundreds of samples. Our results suggest that *C. taxifolia* is actually a complex of species or subspecies. At least two sibling species or speciating ecotypes have been identified that were not recognized previously until the invasive form emerged in the Mediterranean. Researchers undertaking regional-scale phylogeographical studies will thus have to remain vigilant in their initial assumptions about species autonomy prior to investigating questions related to gene flow and interpopulational connectivity.

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