Molecular data suggest a hybrid origin for the invasive *Caulerpa racemosa* (Caulerpales, Chlorophyta) in the Mediterranean Sea

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Keywords:

Caulerpa racemosa;
concerted evolution;
hybrid;
ITS1–ITS2;
Mediterranean Sea;
molecular phylogeny;
polymorphism;
ribosomal DNA;
systematics;
18S intron.

Abstract

Morphological data has provided a basis for the hypothesis that three taxa belonging to the *Caulerpa racemosa* complex occur in the Mediterranean Sea: var. *turbinata–uvifera*, var. *lamourouxi*, and the 'invasive variety'. In order to test this hypothesis and to determine the origin of the 'invasive variety', the transcribed spacer ITS1–ITS2 and an 18S ribosomal DNA (rDNA) intron were analysed from 16 isolates of *Caulerpa racemosa*. The 'invasive variety' shows intraindividual polymorphism for both types of sequences. The ITS1–ITS2 data confirm that the three morphological varieties of *C. racemosa* from the Mediterranean Sea are distinct taxonomic units. The 18S intron data suggest that the new 'invasive variety' could be a recent hybrid between var. *turbinata–uvifera* and an unknown tropical strain. Incongruence between the phylogenetic tree computed from ITS1–ITS2 regions and the 18S intron indicates that homogenization processes of concerted evolution have run at different rates.

Introduction

Caulerpa racemosa (Forsskål) J. Agardh is a widely distributed pan-tropical to temperate-warm marine algal species (Caulerpales, Chlorophyta). It was first collected in the Mediterranean Sea in Sousse harbour, Tunisia (Hamel, 1926, 1930, 1931a; Djellouli *et al*., 1998). It has been regarded as an introduced species, possibly of Red Sea origin (Por, 1978; Verlaque, 1994; Ribera & Boudouresque, 1995), and has since been reported in the Levantine basin of the Eastern Mediterranean Sea, in Lebanon (Hamel, 1931b), Egypt (Aleem, 1950), Syria and Southern Turkey (Huvé, 1957; Mayhoub, 1976) and Israel (Rayss & Edelstein, 1960). Until recently, however, these populations remained confined and stationary, without any invasive tendency (Verlaque *et al*., 2000).

In the early 1990s, the situation abruptly changed and *C. racemosa* spread rapidly throughout the Mediterranean Sea, including the relatively cold waters of the northern part of the western basin: Libya (Nizamuddin, 1991), Sicily and Lampedusa Islands (Alongi *et al*., 1993; Famà *et al*., 2000), Greece (Panayotidis & Montesanto, 1994, 1998; Panayotidis & Zuljevic, 2001), Western Italy (Piazzi *et al*., 1994, 1997a,b, 2001; Bussotti *et al*., 1996; Gambi & Terlizzi, 1998; Piazzi & Cinelli, 1999; Famà *et al*., 2000), Sardinia (Cossu & Gazale, 1997), Western Turkey (Evirgen, 1997; Verlaque *et al*., 2000), Southern Italy (Buia *et al*., 1998; Famà *et al*., 2000), Cyprus (Argyrou *et al*., 1999), Balearic Islands (Verlaque *et al*., 2000) and France (Verlaque *et al*., 2000).

A comprehensive review of publications concerning *C. racemosa* in the Mediterranean Sea, together with morphological examination of fresh and preserved material, led Verlaque *et al*. (2000) to suggest that three distinct taxa of the *C. racemosa* complex coexist in the Mediterranean: (i) *C. racemosa* var. *turbinata* (J. Agardh) Eubank – var. *uvifera* (C. Agardh) J. Agardh, known since 1926 in Tunisia (Hamel, 1926) and since 1931 in the Levantine basin (Hamel, 1931b); (ii) *C. racemosa* var. *lamourouxii* (Turner) Weber-van Bosse f. *requienii* (Montagne) Weber-van Bosse, known since the 1950s in the Levantine basin (Huvé, 1957); and (iii) a so-called 'invasive variety', which has been spreading quickly

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since the early 1990s throughout most of the Mediterranean Sea (Balearic Islands, France, Western Italy, Sardinia, Sicily, Southern Italy, Lampedusa Island, continental Greece, Crete, Turkey, Cyprus, Libya and Tunisia).

According to Verlaque *et al*. (2000), this so-called invasive variety differs from the two others by its upright axes with clavate, more or less inflated branchlets, which are uncrowded and radially to distichously disposed. They suggested, on the basis of various criteria (e.g. taxonomy, kinetics of spread, tendency to proliferate; see Ribera & Boudouresque, 1995; Boudouresque, 1999), that this invasive variety could be a taxon recently introduced to the Mediterranean Sea.

To determine the origin of the invasion by *C. racemosa* in the Mediterranean Sea, it is essential to assess the taxonomic status of the so-called 'invasive variety'. In this study, we used molecular data from the variable regions of the nuclear ribosomal gene cluster to test the hypothesis of Verlaque *et al*. (2000), which was based upon morphological data.

Because of their relatively high evolution rate, the internal transcribed spacers (ITS1–ITS2) of ribosomal DNA (rDNA) are considered appropriate markers to distinguish between species within Ulvophyceae and to examine inter- and intraspecific relationships within the genus *Caulerpa* (Bakker *et al*., 1995; Leskinen & Pamilo, 1997; Pillmann *et al*., 1997). Recently, Famà *et al*. (2000) published a study of *C. racemosa* based only on ITS1 sequences. In the present study, we use combined ITS1–ITS2 sequences, and also a *Caulerpa*-specific fast-evolving intron of the 18S rDNA (see Olsen *et al*., 1998). Furthermore, the taxonomic and geographic sampling in the present study is wider-based than in Famà *et al*. (2000).

We amplified by polymerase chain reaction (PCR) a 1100-bp region spanning from the 3′ end of the 18S rDNA to the 5′ end of the 28S rDNA, including the 18S rDNA intron, and the ITS1 and ITS2 sequences. The PCR products were cloned, characterized with restriction enzymes and sequenced. We present phylogenetic analyses of data from ITS and the 18S intron.

Materials and methods

Sample collection and genomic DNA extraction

Caulerpa racemosa samples were collected by scuba diving from the Mediterranean Sea, Red Sea and Pacific Ocean. *Caulerpa* isolates and collecting sites are listed in Table 1. Four distinct (remote) individuals were collected for isolate Nrs 2, 3 and 9 (*C. racemosa* var. *turbinata–uvifera*, var. *lamourouxii* f. *requienii* and 'invasive variety', respectively).

Tissues were fixed in 80% ethanol and preserved at −20 °C. Before genomic DNA extraction, tissue samples were dehydrated and frozen in liquid nitrogen. Total DNA was extracted using the genomic DNA purification kit from Promega (Madison, WI, USA). After optical density (OD) measurement at 260/280 nm genomic DNA was resuspended in ddH_2O at a final concentration of 1 $\mu g\ \mu L^{-1}$.

PCR amplification, restriction fragments length polymorphism (RFLP) and sequencing

Primers specific for *Caulerpa* were designed from an alignment of all sequences of the nuclear ribosomal genes cluster from *Caulerpa* species available in the GenBank database.

PCR amplifications were performed using a forward primer hybridizing in the 3′ region of the 18S rDNA sequence (5′-GCAATAACAGGTCTGTGATGC-3′) and a reverse primer located near the 5′ extremity of the 28S rDNA (5′-TCCTCCGCTTATTGATATGCT-3′). The amplified region contains the 3′ end of the 18S rDNA, including the intron (100–108 bases), the ITS1 (112–136 bases), 5.8S rDNA, ITS2 (281–315 bases) and the 5′ end of the 28S rDNA. Genomic DNA was amplified by a DNA thermal cycler (Celtus, Applied Biosystems, Foster City, CA, USA). The 50 μL PCR mix contained 10 ng of genomic DNA, 5 μL of 10X taq DNA polymerase buffer, 8 μL of 1.25 mM dNTP mix (Amersham Pharmacia Biotech, Piscataway, NJ, USA), 2.5 μL of each primer (20 μM) and 1.25 U of Taq DNA polymerase (Promega). Samples were amplified by 35 cycles of amplification. The procedure was denaturation at 94 °C for 1 min, primer annealing for 1 min at 57 °C and extension for 2 min at 72 °C. Fragments were checked on 1.5% agarose gels and cleaned with Qiaquick kit (Quiagen Inc., Valencia, CA, USA). PCR products were then cloned into pGem-T Easy vector (Promega).

To evaluate the sequence polymorphism, DNA inserts were amplified from 10 to 16 positive clones for each sample, using the primer set described above, and digested with a series of restriction enzymes (Taq1, HaeIII, Sau3A and AluI). Alu I was found to be the most informative and was chosen for the final RFLP analysis. Digestions were performed using 5 μL of PCR product in a total volume of 10 μL. Electrophoresis was performed using 2% agarose gels. For each distinct restriction pattern, one to three clones were selected and sequenced using the dideoxy-nucleotide chain termination method (Sanger *et al*., 1977).

Sequence alignment and phylogenetic analysis

Nucleotidic sequences were aligned automatically using the GeneWork program. The alignments were then optimized manually using the program SeeView (Galtier *et al*., 1996). The alignment is available from the authors upon request. The ITS1 + ITS2 alignment contains 435 positions, of which 91 are parsimony informative. The 18S intron alignment contains 177 positions, of which 41 are parsimony informative.

Table 1 Isolates of *Caulerpa racemosa* and *C. mexicana*: taxon, geographical origin, collectors and accession numbers. Mediterranean specimens of *C. racemosa* (Nrs 1–4 and 6–10) are named according to Verlaque *et al*. (2000). Specimens Nrs 5 and 11–16 are named from their morphological characters, according to the literature. Specimens 17–20 are named according to Olsen *et al*. (1998). Specimens LI, RH, GC, FU, PAN and WA are named according to Famà *et al*. (2000), without any change of these authors' code, for the sake of clarity. The 18S intron, ITS1 and ITS2 of rDNA sequences corresponding to the isolates were deposited in the GenBank database. New accession numbers associated with this study are given in bold.

Nr	Taxon	Origin	Collector	Accession no.
1	*C. racemosa* var. *turbinata-uvifera*	Bizerte, Tunisia	A. Djellouli	**AJ297632***
2	*C. racemosa* var. *turbinata-uvifera*	Salakta, Tunisia	N. Ben Maiz	**AJ297633***
3	*C. racemosa* var. *lamourouxii* f. *requienii*	Cyprus	M. Verlaque	**AJ297634***
4	*C. racemosa* var. *lamourouxii* f. *requienii*	Lebanon	J.G. Harmelin and H. Zibrowius	**AJ297635***
5	*C. racemosa* var. *lamourouxii*	Ishigaki, Japan	C.F. Boudouresque and A. Meinesz	**AJ297636***
6	*C. racemosa* 'invasive variety'	Samos, Greece	J. Laborel	**AJ297637* clone a** **AL297638* clone b**
7	*C. racemosa* 'invasive variety'	Saronikos, Greece	P. Panayotidis	**AJ297639* clone a** **AJ297640* clone b**
8	*C. racemosa* 'invasive variety'	Cyprus	M. Verlaque	**AJ297641* clone a** **AJ297642* clone b** **AJ297643* clone c**
9	*C. racemosa* 'invasive variety'	Marseille, France	T. Pérez	**AJ297644* clone a** **AJ297645* clone b**
10	*C. racemosa* 'invasive variety'	Marseille, France	S. Ruitton	AJ228997† AJ228998† AJ228999† Jousson *et al*. (1998)
11	*C. racemosa* var. *clavifera*	Red Sea	A. Meinesz	**AJ297646***
12	*C. racemosa* var. *clavifera*	Indian Ocean	C. Marschall	**AJ297647***
13	*C. racemosa* var. *peltata*	Ishigaki, Japan	C.F. Bou-dou-resque and A. Meinesz	**AJ297648***
14	*C. racemosa* var. *macrophysa*	Ishigaki, Japan	C.F. Boudou-resque and A. Meinesz	**AJ297646* clone a** **AJ297650* clone b**
15	*C. racemosa* var. *racemosa*	Heron I., Queensland, Australia	M. Manuel	**AJ297651***
16	C. racemosa var. racemosa	Balicasag I., Philippines	A. Gómez and M.A. Ribera	**AJ297652* clone a** **AJ297653* clone b**
17	*C. mexicana*	Florida	J.R.M. Chisholm	AJ007818* Olsen *et al*. (1998)
18	*C. mexicana*	Israel	F. Weinberger	AJ007815* Olsen *et al*. (1998)
19	*C. mexicana*	Panama	W.H.C.F. Kooistra	AJ007817* Olsen *et al*. (1998)
20	C. mexicana	Canary Islands	Y.S.D.M. De Jong and W.F. Prud'homme van Reine	AJ007816* Olsen *et al*. (1998)
LI	*C. racemosa* var. *occidentalis*§	Livorno, Italy	L. Piazzi	AF256088‡, isolate LI1, clone A AF256093‡, isolate LI1, clone F AF256096‡, isolate LI1, clone I AF256099‡, isolate LI1, clone L AF256104‡, isolate LI1, clone Q Famà *et al*. (2000)
RH	*C. racemosa* var. *lamourouxii*	Rhodes, Greece	M. van Rijssel	AF256140‡, isolate RH1, clone A AF256141‡, isolate RH1, clone B AF256142‡, isolate RH1, clone C AF256143‡, isolate RH1, clone D Famà *et al*. (2000)
GC	*C. racemosa* 'var. not available'	Gran Canaria, Canary Islands, Spain	H.J. van de Strate	AF256144‡, isolate GCa1, clone A AF256147‡, isolate GCs1, clone C AF256148‡, isolate GCl1, clone A Famà *et al*. (2000)
FU	*C. racemosa* 'var. not available'	Fuerteventura, Canary Islands, Spain	H.J. van de Strate	AF256151‡, isolate FU1, clone B Famà *et al*. (2000)
PAN	*C. racemosa* 'var. not available'	Panama	W.H.C.F. Kooistra	AF256154‡, isolate PAN Famà *et al*. (2000)

Table 1 (Continued)

Nr	Taxon	Origin	Collector	Accession no.
WA	*C. racemosa* var. *laetivirens* f. *cylindracea*	Perth, Western Australia	M. Polifrone and S. Williams	AF256155‡, isolate WA1, clone A AF256156‡, isolate WA1, clone B AF256157‡, isolate WA1, clone C AF256158‡, isolate WA1, clone D AF256159‡, isolate WA1, clone E AF256160‡, isolate WA2, clone A AF256161‡, isolate WA2, clone B Famà *et al*. (2000)

*ITS1, ITS2 and intron.
†ITS1 and ITS2 only.
‡ITS1 only.
§This taxon was only considered similar, and not definitely assigned, to var. *occidentalis*, by Verlaque *et al*. (2000).

Trees were computed using the distance matrix Neighbour-Joining (NJ) method (Saitou & Nei, 1987) in the PHYLO-WIN package (Galtier *et al*., 1996), and Maximum Parsimony (MP) in PAUP 3.1 (Swofford, 1991). In distance analyses we used the option 'pairwise gap removal' which does not exclude *a priori* positions with gaps in distance computation. Distances were corrected using Kimura's two-parameter model (Kimura, 1980). In parsimony analyses, gaps were treated as missing data. We performed heuristic searches, using the TBR algorithm. The statistical robustness of nodes was ascertained by bootstrapping (Felsenstein, 1992) with 500 iterations. Four published sequences (AJ007815, AJ007816, AJ007817 and AJ007818) of *Caulerpa* mexicana Sonder ex Kützing (Olsen *et al*., 1998) were used as outgroup taxa (Table 1). Our ITS1 and ITS2 sequences were aligned together with a number of sequences published by Jousson *et al*. (1998) (Table 1). In addition, our ITS1 sequences were compared with some of the sequences published by Famà *et al*. (2000) (Table 1).

Results

RFLP analysis was performed to check for possible inter-individual polymorphism within populations. We found intraindividual polymorphism for all isolates of the Mediterranean 'invasive variety' of *C. racemosa* (Nrs 6–10), and for two extra-Mediterranean isolates (Nr 14 from Japan and Nr 16 from Philippines), with either two or three distinct restriction patterns for each individual (result not shown). In each case, one of the patterns is representative of 80–90% of clones from a given individual. In contrast, we found no differences between individuals within a given population (Nrs 2, 3 and 9).

Sequence data are consistent with the results of RFLP analysis. Polymorphic individuals are polymorphic for both ITS sequences and for the 18S intron. Hereafter, for isolates in which intraindividual polymorphism has been detected, the sequence representing the majority of the clones has been named 'a' and the other sequences 'b' and 'c'.

The trees obtained using NJ and MP methods yielded the same topologies.

The result of a combined analysis of ITS1 and ITS2 sequences, including three sequences (AJ228997, AJ228998 and AJ228999) of *C. racemosa* (invasive variety) from Marseille (Jousson *et al*., 1998), is shown in Fig. 1. In the tree, all isolates referred to the Mediterranean 'invasive variety' of *C. racemosa* (Nrs 6–10), including a, b and c sequences, fall within the same clade (clade 1 in Fig. 1) which is supported by a high bootstrap value (Fig. 1). Within this clade, the sequences do not cluster according to geographical origin, nor do polymorphic sequences from a given individual make monophyletic units. Instead, the tree shows four subclades, within the 'invasive variety' clade, each supported by a high bootstrap value. Polymorphic sequences from a given individual generally fall within distinct subclades, and a given subclade contains distinct geographical origins. Polymorphic sequences observed in some extra-Mediterranean isolates (Nr 14 from Japan and Nr 16 from Philippines) do not cluster with sequences from the 'invasive variety'.

The two remaining *C. racemosa* varieties from the Mediterranean Sea, var. *turbinata–uvifera* and var. *lamourouxii* f. *requienii*, constitute two distinct clades (clades 2 and 4, respectively, in Fig. 1), each one being supported by 100% of bootstraps. These two clades are not closely related to each other or to the invasive clade. Thus, from this analysis, each of the three morphological varieties of *C. racemosa* in the Mediterranean Sea, including the 'invasive variety', appears as a distinct taxonomic unit.

In this analysis, extra-Mediterranean isolates of *C. racemosa* fall within two distinct robust clades (clades 3 and 5 in Fig. 1). Interestingly, isolates do not cluster according to morphology, or strictly according to geographical origin. *Caulerpa racemosa* var. *racemosa* from Queensland (Australia) and Philippines constitute clade 3

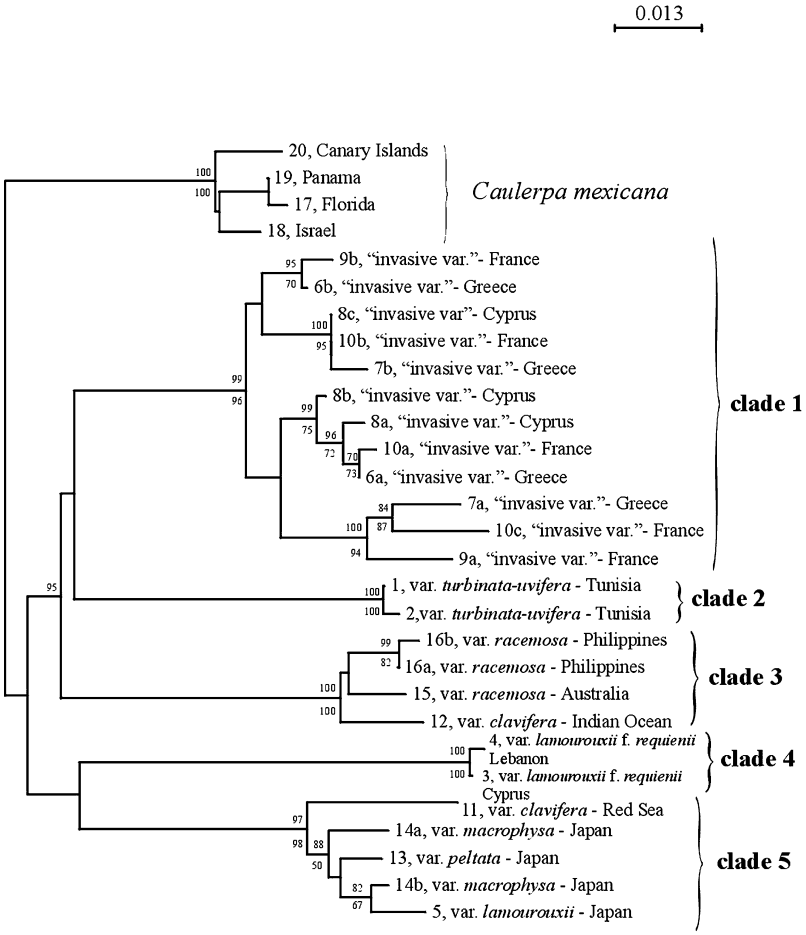

Fig. 1 Molecular phylogenetic tree based on a comparison of ITS1–ITS2 sequences from a range of *Caulerpa racemosa* isolates. This tree was obtained using the Neighbour-Joining (NJ) method with the *C. mexicana* lineage as the outgroup. Results of 500 NJ bootstrap replicates are shown above the branches. The Maximum-Parsimony (MP) tree for the same dataset shows the same topology. There are 18 most-parsimonious trees of 351 steps. The result of a bootstrap (MP) analysis is noted below the branches. Bootstraps <50% are not indicated.

with *C. racemosa* var. *clavifera* from the Indian Ocean. Clade 5, comprising isolates from Japan and the Red Sea, includes algae that classical taxonomic classification refers to as var. *lamourouxii*, var. *peltata*, var. *macrophysa*, and var. *clavifera*. Finally, note that isolates referred to as var. *lamourouxii* from the Mediterranean Sea and Japan fall within two different clades (respectively 4 and 5). Apart from the grouping of clades 1–2–3 (95% bootstraps), the relationships between the five clades are essentially unresolved in this analysis, probably because of substitutional saturation.

A comparison of our ITS1 sequences with some of the sequences published by Famà *et al.* (2000) is shown in Fig. 2. This tree, constructed from ITS1 alone, shows a significantly lower degree of resolution (especially with the MP method) than the tree based on ITS1 + ITS2 (Fig. 1). In this analysis of ITS1, as in the ITS1–ITS2 tree, all isolates referred to the Mediterranean 'invasive variety' of *C. racemosa* (Nrs 6–10), as well as sequences LI1 A, F, I, L and Q ('var. *occidentalis*', i.e. the invasive variety) from Famà *et al.* (2000) fall within a clade (clade 1 in Fig. 2) supported by 63% (NJ) of bootstraps (bootstrap value <50% with MP). It is of interest to note that in addition, several isolates from Famà *et al.* (2000) (WA1 A–E, WA2 A–B, GCa1 A, GCs1 A, GCl1 A–B) from Perth (Western Australia) and Gran Canaria (Canary I.) are spread throughout this clade. There is no resolution of the topology within this clade. Our sequences corresponding to the two other morphological varieties from Mediterranean Sea, var. *turbinata–uvifera* and var. *lamourouxii* f. *requienii*, still constitute two distinct clades with this more reduced dataset (clades 2 and 4 in Fig. 2) supported, respectively, by 94% (NJ) to 60% (MP) and 100% (NJ and MP) of bootstraps, but in contrast, sequences referred to *C. racemosa* var. *lamourouxii* from Rhodes Island by Famà *et al.* (2000) (RH1) fall within clade 1 with sequences from the 'invasive variety'.

As in the ITS1–ITS2 tree (Fig. 1), *C. racemosa* var. *racemosa* from Queensland (Australia) and Philippines constitute clade 3 with *C. racemosa* var. *clavifera* from the Indian Ocean. Isolates from Japan and the Red Sea constitute clade 5.

Figure 3 shows a tree computed from an alignment of sequences of the *Caulerpa*-specific variable region in the 18S rDNA. This intron sequence is inserted between position 94 and 194–202 of the 18S sequence and has a length of 100–108 nucleotides. It has been found in the

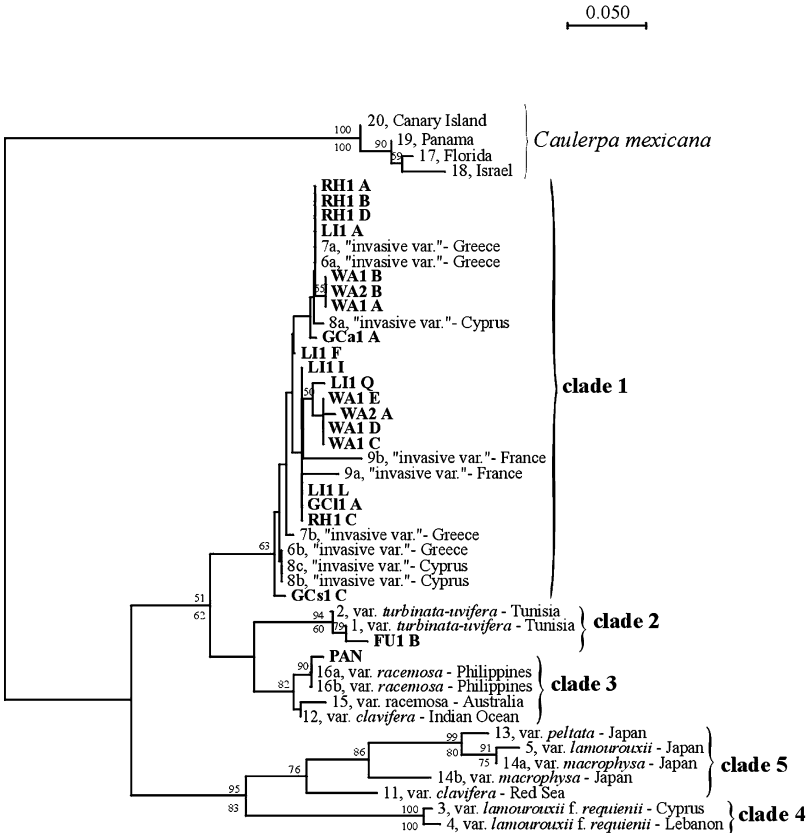

Fig. 2 Molecular phylogenetic tree showing a comparison of our ITS1 sequences of *Caulerpa racemosa* isolates with published sequences (Famà *et al.*, 2000). Sequences from Famà *et al.* (2000) are labelled in bold; morphological determination and geographical origin are indicated in Table 1. This tree was obtained using the NJ method with the *C. mexicana* lineage as the outgroup. Results of 500 NJ bootstrap replicates are shown above the branches. Maximum-Parsimony analysis of the dataset gives numerous (>20 000) minimal trees (182 steps). The result of a bootstrap (MP) analysis is noted below the branches. Bootstraps <50% are not indicated.

same position for all *C. racemosa* isolates and is known to occur in *C. mexicana*, *C. taxifolia* and *C. prolifera* (Olsen *et al.*, 1998).

In our study, the intron tree shows a major inconsistency with the tree derived from ITS sequences, in that clade 1 (invasive variety) is split into two distinct unrelated clades, named here 1A and 1B. All other major clades are largely consistent with our ITS1–ITS2 tree and have been numbered in the same way for clarity.

Among sequences from the Mediterranean 'invasive variety', distinct sequences from polymorphic isolate Nrs 7–9 belong to both 1A and 1B clades. Clade 1 A (Nrs 7a, 8b, 8c, 9b) is the sister-group of Mediterranean *C. racemosa* var. *turbinata–uvifera* (clade 2) with high statistical support (98%). In contrast, clade 1B (Nrs 6a, 6b, 7b, 8a, 9a) is the sister-group of clade 3 of extra-Mediterranean isolates (*C. racemosa* from Philippine, Queensland–Australia, and Indian Ocean; with 86% of bootstraps); one of the sequences of *C. racemosa* var. *racemosa* from Philippine (Nr 16a) falls within clade 1B.

Other minor inconsistencies between the tree derived from the 18S intron and the tree derived from ITS1–ITS2 include the positioning of sequence Nr 14b (var. *macrophysa*, Japan), which has evolved significantly faster than all other sequences and branches in a basal position, probably as a result of the well-known 'long branch-attraction' artifact (Felsenstein, 1978), and the position of Nr 13 (var. *peltata*, Japan) in clade 3, expected to fall within clade 5 according to the ITS analysis.

Discussion

Three distinct taxonomic units of *Caulerpa racemosa* in the Mediterranean Sea

The question has remained open until now as to whether the Mediterranean specimens identified morphologically as *C. racemosa* var. *turbinata–uvifera*, var. *lamourouxii* f. *requienii* and 'invasive variety' illustrate the capacity of a single taxon to change, or belong to three distinct taxa.

In favour of the second hypothesis, it has been pointed out that no transitional forms between these three varieties have been observed in the Mediterranean Sea (Verlaque *et al.*, 2000). Further evidence is provided by the results of regional surveys, transplantations, and culture studies on the morphological polymorphism of *C. racemosa*. Under low light, the most important generalized response is a change in the symmetry of the upright axes from radial to bilateral and sometimes to unilateral, accompanied by fewer branchlets. However, depending

on the material chosen, this change comes with two different and independent patterns of morphological branchlet variation: the algae produce new axes with distichous or unilateral branchlets that are trumpet-like with an obconical apex or shield-like with a flat apex (peltata-like) (Tandy, 1934; Peterson, 1972; Enomoto & Ohba, 1987; Ohba *et al*., 1992), or clavate to cylindrical with a rounded apex (Nasr, 1947; Peterson, 1972; Calvert, 1976). These two variations seem to be genotype-dependent and could characterize distinct taxonomic groups (Verlaque *et al*., 2000). The range of morphological variations in populations from the Mediterranean Sea suggests that *C. racemosa* var. *turbinata–uvifera* belongs to the first group and *C. racemosa* var. *lamourouxii* f. *requienii* to the second group (Verlaque *et al*., 2000). No culture studies have been done on the 'invasive variety'.

The possibility of a temperature-dependent morphological variation can also be excluded on the basis of the overlapping temperature range of the Mediterranean localities where var. *turbinata–uvifera*, var. *lamourouxii* f. *requienii* and the 'invasive variety' occur (Verlaque *et al*., 2000).

Our analysis of ITS1–ITS2 sequences (Fig. 1) is congruent with the hypothesis drawn from morphological data (Table 2). The existence of three distinct taxonomic units of *C. racemosa* in the Mediterranean Sea is well supported not only by the high bootstrap values at nodes, but also by the tree morphology, with long internal and short-terminal branches.

The fact that the Famà *et al*. (2000) isolate of *C. racemosa* var. *lamourouxii* from Rhodes Island (RH1, Fig. 2) falls within clade 1 (invasive variety) suggests a possible misidentification, rather than contradicting the preceding conclusion. Indeed, some seasonal forms of the invasive variety exhibit very few branchlets (unpublished data) and can be confused with *C. racemosa* var. *lamourouxii* f. *requienii*, always totally deprived of branchlets. In addition, a form of *C. racemosa* var. *lamourouxii* never reported from the Mediterranean (f. *lamourouxii*) can also exhibit very few branchlets (Weber-van Bosse, 1898). It is worth noting that Fig. 2 of Famà *et al*. (2000) does not illustrate the studied isolate from Rhodes I. (Mediterranean) but is a copy of a figure published by Huvé (1957; from Weber-van Bosse, 1898); the specimens illustrated are of extra-Mediterranean origin, and belong to both f. *lamourouxii* and f. *requienii*. Thus we consider that Famà *et al*. (2000) did not distinguish molecularly between the three distinct taxonomic units of Mediterranean *C. racemosa* because they sampled only the 'invasive variety'.

The phylogenetic relationships between the three above mentioned taxa (var. *turbinata–uvifera*, var. *lamourouxii* f. *requienii* and 'invasive variety'), and their relationships with extra-Mediterranean *C. racemosa* taxa, are not satisfyingly resolved in our tree (Fig. 1), apart from the grouping of clade 1 (invasive), clade 3 (Philippine, Queensland–Australia, Indian Ocean) and clade 2 (Mediterranean *turbinata–uvifera*) in the NJ tree. Better resolution would require the use of a different, slower evolving molecular marker.

Intraindividual polymorphism in sequences of the ribosomal gene cluster

The individual copies of tandemly repeated genes such as rDNA evolve coordinately within species. This phenomenon has been called concerted evolution and is thought to be caused by sequence-homogenizing mechanisms, such as gene conversion and/or unequal crossing-over between individual copies of a gene family. As these processes act between the arrays on homologous and nonhomologous chromosomes, the whole family of repeats would be expected to undergo homogenization in a given interbreeding population (Schlötterer & Tautz, 1994).

The polymorphisms in internal transcribed spacers (ITS1 and ITS2) and in the 18S intron, which occur in all isolates of the Mediterranean 'invasive variety' (Nrs 6–10), a Japanese isolate (Nr 14) and a Philippine isolate (Nr 16) of *C. racemosa*, thus appear unusual, even if polymorphism in rDNA has already been reported, especially in fungi (Sanders, 1999) and plants (e.g. Buckler *et al*., 1997; Campbell *et al*., 1997). Such polymorphism (up to six distinct sequences) has already been noted in *C. racemosa* by Famà *et al*. (2000).

In plants, some of the divergent rDNA ITS or IGS sequences have been considered as pseudogenes based on high substitution rates and low predicted secondary structure stability. However in our dataset, paralogues found in the same individual of *C. racemosa* 'invasive variety' do not show significantly unequal substitution rates (compare the lengths of terminal branches between sequences labelled a, b and c in Fig. 1), hence it is improbable that some of them would be pseudogenes. Another explanation has been proposed for rDNA polymorphism, in the case of fungi of the genera *Glomus* and *Scutellospora* (Sanders, 1999). According to this author, the existence of distinct sequences of ribosomal genes implies that recombination does not occur, because mechanisms that keep the sequences of rDNA copies the same operate most frequently during recombination events. To accommodate the co-occurrence of nuclei with distinct rDNA sequences, Sanders (1999) advocates the absence of sexual reproduction, and the existence of interindividual hyphal anastomosis in these coenocytic fungi. Like these fungi, Caulerpales are coenocytes, i.e. all nuclei are enclosed within one cell wall, so that an individual can be considered, in genetic terms, as a population of discrete nuclei. This explanation, however, is unlikely in *C. racemosa*. Indeed, sexual reproduction does exist in this species, and it has been definitely observed in the Mediterranean 'invasive variety' (Panayotidis & Zuljevic, 2001). Furthermore, inter-individual

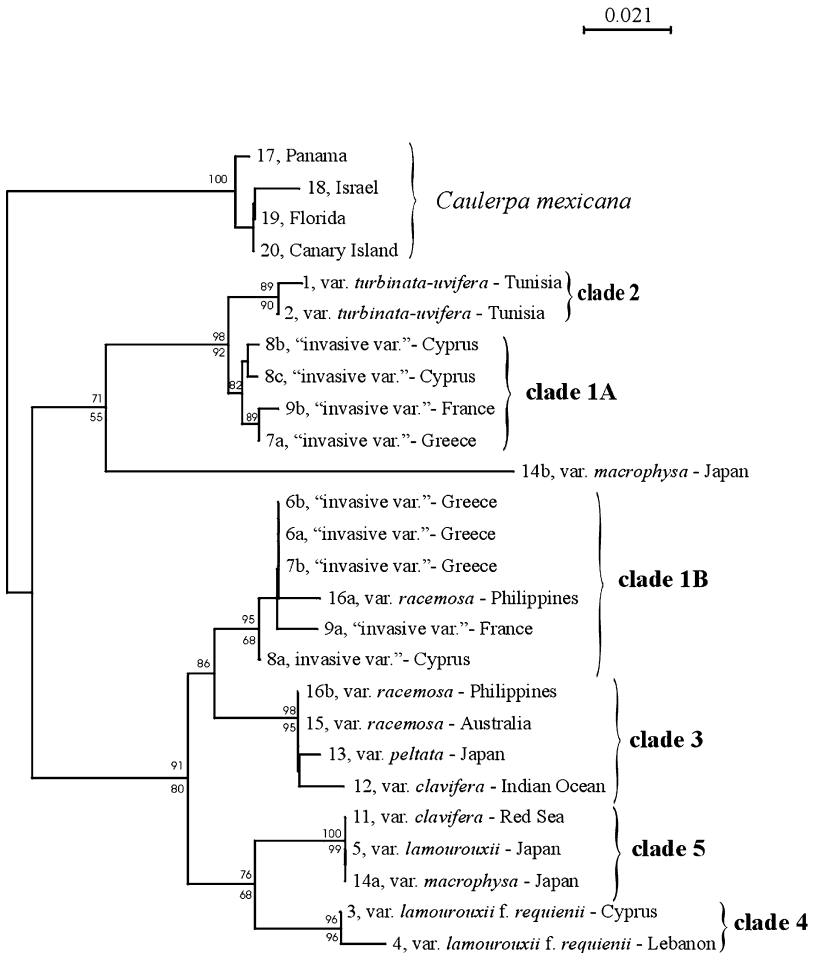

Fig. 3 Molecular phylogenetic tree based on a comparison of 18S intron sequences from a range of *Caulerpa racemosa* isolates. This tree was obtained using NJ method with the *C. mexicana* lineage as the outgroup. Results of 500 NJ bootstrap replicates are shown above the branches. The MP tree for the same dataset shows the same topology. There are four most-parsimonious trees of 113 steps. The result of a bootstrap (MP) analysis is noted below the branches. Bootstraps <50% are not indicated.

hyphal anastomosis with exchange of nuclei has not been observed in the genus *Caulerpa*.

Furthermore, in *C. racemosa* only a portion of the studied group show polymorphism (the Mediterranean 'invasive variety', an isolate from Philippines, and another from Japan). The lack of sequence polymor phism in the remaining taxa can be viewed as evidence for concerted evolution in *C. racemosa*. As a consequence, an alternative explanation for polymorphism would be that the 'invasive variety' was produced by a recent hybridization event between distinct taxa, resulting in a genome in which sequences of distinct origins coexisted. Since this putative hybridization, concerted evolution may have begun, leading to partial but still incomplete homogenization of the sequences. Intra-individual polymorphism of ITS sequences has already been related to putative hybridization events in plants (e.g. Sang *et al.*, 1995; Campbell *et al.*, 1997).

Consistently, the so-called 'invasive variety' was observed for the first time in 1991 (Nizamuddin, 1991), and its spread throughout the Mediterranean Sea has been extremely rapid. The tree derived from ITS sequences also suggests that distinct (and geographically dispersed) isolates of the 'invasive variety' have diverged only very recently. Indeed within clade 1 (Fig. 1), each subclade contains polymorphisms from distinct individuals and geographical origins, and sequences from a given individual generally fall within different subclades, suggesting that the intraindividual polymorphisms of ribosomal gene clusters observed in Mediterranean invasive *C. racemosa* have been inherited from a common ancestor.

Hybrid origin of the Mediterranean 'invasive variety' suggested by the analysis of the 18S intron

The hypothesis of a recent hybridization event, followed by rapid spreading, is further supported by the analysis of sequences of the 18S intron. The most striking feature of the tree derived from sequences of the 18S rDNA intron (Fig. 3) is the splitting of sequences from the Mediterranean invasive variety into two nonsister-group clades (1A and 1B). It could be argued that such a topology, incongruent with the tree derived from ITS sequences, is

Table 2 Correspondence between clades (from ITS1 to ITS2 tree; see Fig. 1), geographical origin, morphological criteria and possible taxonomic treatment. For isolate numbers, see Table 1.

Isolate Nr	Geographical origin	Morphological criteria	Subspecific taxa	Species	Clade Nr
15, 16ab	Philippines and Queensland (Australia)	Crowded, subspherical branchlets. Small rhizoidal pillars	var. *racemosa*	*C. racemosa*	3
1, 2	Mediterranean	Crowded, subspherical to complanate branchlets. Small rhizoidal pillars	var. *turbinata-uvifera*		2
6ab, 7ab, 8abc, 9ab, 10abc	Mediterranean	Loose, subspherical, radial to distichous branchlets. Small rhizoidal pillars	'Invasive variety'		1
3, 4	Mediterranean	No branchlets. Spaced large rhizoidal pillars	var. *lamourouxii* f. *requienii*		4
11	Red Sea	Loose, spherical branchlets	var. *clavifera*		5
12	Indian Ocean	Loose, spherical branchlets	var. *clavifera*		3
13	Japan	Loose, peltate branchlets	var. *peltata*		5
14ab	Japan	Loose, spherical branchlets	var. *macrophysa*		5
5	Japan	No branchlets	var. *lamourouxii*		5
17, 19	Florida, Panama			C. *mexicana*	
20, 18	Canary I., Israel				

artifactual and results from a lack of phylogenetic information in the intron sequences. We feel that this is unlikely, for several reasons. First, the evolutionary rate of the intron sequences is similar to that of ITS sequences. More important, apart from clade 1, all major clades that are well supported in the ITS tree (Fig. 1) are also retrieved in the intron tree, with only minor inconsistencies. The overall morphology of the intron tree shows a good resolution of *C. racemosa* taxonomic units, with rather long internal branches (leading to the various taxa), and short-terminal branches (within each clade).

One subgroup of 'invasive variety' sequences (clade 1 A in Fig. 3) is found with high confidence to be the sister-group of the *turbinata–uvifera* clade (clade 2 in Fig. 3). The latter taxon has been recorded since the 1920s in the Mediterranean. The sister-group relationship is supported by 98% of bootstraps and a long common branch. The other subgroup of 'invasive variety' sequences (clade 1B in Fig. 3) is related to clade 3 of *C. racemosa* from the Philippines, Queensland (Australia) and Indian Ocean with 86% of bootstraps.

Such a distribution of polymorphic sequences of 18S intron from the Mediterranean 'invasive variety' strongly suggests a hybrid origin for this form of *C. racemosa*. An alternative explanation would be that coexistence of distinct intron sequences results from horizontal transfer, but this hypothesis seems highly improbable given that the position of the intron within the 18S rDNA sequence is constant in the whole dataset.

According to the hybridization hypothesis, the parent taxa seem to be *C. racemosa* var. *turbinata–uvifera*, a taxon classically known from the Mediterranean Sea but often hypothesized to be the result of an ancient introduction (Por, 1978), and an undetermined tropical taxon (belonging to clade 3).

In the tree shown in Fig. 2 (analysis of ITS1 including sequences from Famà *et al.*, 2000), sequences from *C. racemosa* var. *laetevirens* f. *cylindracea* from Western Australia fall within clade 1, with sequences of Mediterranean 'invasive variety'. This is an interesting result because Harvey (1858, plate 30, Fig. 2) illustrates as *C. cylindracea* var. *macra* a form of *C. racemosa* var. *laetivirens*, collected in Western Australia, which looks closely similar to the Mediterranean isolates of the 'invasive variety' Further studies are required to assess whether this taxon constitutes the possible second parent of the 'invasive variety'. The undetermined material from Gran Canaria (in Famà *et al.*, 2000; see Fig. 2: GC) could either belong to the same entity, or to the 'invasive variety'.

Three possible scenarios may account for the hybridization event and the occurrence of the 'invasive variety' in the Mediterranean: (i) the coexistence of the two parent taxa in an unknown region, and their hybridization followed by the introduction of this hybrid to the Mediterranean Sea; (ii) the hybridization of the two parent taxa, not naturally coexistent, via cultivation by aquariologists, and then the accidental introduction of this hybrid into the Mediterranean Sea and (iii) the recent introduction of the undetermined tropical parent taxon into the Mediterranean Sea (so far not discovered) and its subsequent hybridization with the Mediterranean taxon *turbinata–uvifera*.

The hybridization hypothesis, via heterosis, could account for the remarkable spread of the 'invasive variety' throughout the Mediterranean sea.

Incongruence between ITS1 + ITS2 tree and 18S intron tree

If the tree derived from the intron (Fig. 3) is interpreted as indicative of a hybridization event, why does the ITS tree not show the same topology? We already stated that the occurrence of ribosomal gene cluster polymorphism by itself is consistent with a recent hybridization event followed by partial homogenization of sequences as a result of concerted evolution.

The nuclear ribosomal genes are organized into one or more arrays containing hundreds to thousands of tandemly arranged repeats. Each repeat contains a transcribed region comprising 18S rDNA, the internal transcribed spacer 1 (ITS1), 5.8S rDNA, ITS2, and 28S rDNA. An intergenic, untranscribed spacer region (IGS) occurs between two units. The two internal transcribed spacer sequences (ITS1 and ITS2) are cleaved from precursor (45S) transcripts during formation of the mature rRNAs.

A contrasting pattern of homogenization has already been observed between IGS and ITS spacer regions in *Drosophila melanogaster* (Polanco *et al*., 1998). Those authors found no single course of evolution for the rDNA: different regions follow different homogenization trajectories depending on the locations of reciprocal exchanges along the rDNA unit. Our results suggest that a similar difference in rate of concerted evolution exists in *Caulerpa racemosa*, between the variable region of the 18S rDNA considered as an intron, and the internal transcribed spacers. The latter sequences would homogenize more rapidly, explaining why a single clade of sequences from the invasive variety is obtained in the tree derived from the ITS sequences.

Hillis *et al*. (1990) reported that concerted evolution of rDNA in parthenogenetic lizards of hybrid origin is strongly biased toward one of two parental sequences, and that the concept of a 'permanent hybrid' genome does not apply to repeated DNA sequences, which continue to evolve in concerted fashion. Similarly, the hybrid origin of the taxon is no longer detectable in the ITS sequences of the Mediterranean 'invasive variety' of *C. racemosa*. But in contrast to the case of parthenogenetic lizards, the sequences from the hybrid are not significantly closer to one parent than to the other. Note however, that in the NJ ITS tree (Fig. 1), clade 1 (the supposed hybrid invasive variety) and clades 2 and 3 (the two parents according to the intron tree) make a monophyletic unit with 95% of bootstraps.

Events of this type may not be uncommon in the *C. racemosa* complex, as illustrated by the independent polymorphism in isolates from Philippines (Nr 16a and b) and Japan (Nr 14a and b). In the case of *C. racemosa* from Phillipines (Nr 16), clones 16a and 16b fall within clade 3 in the ITS tree, but fall, respectively, within clades 1B and 3 in the intron tree, which possibly also reflects a hybrid origin.

Finally, that some individuals have more than two distinct sequences (three for isolate Nrs 8 and 10), together with the four distinct subclades in the invasive clade (clade 1) in the tree from ITS, may reflect a more complex history, with several rounds of hybridization, or alternatively may simply reflect the pattern of homogenization of the two parental sequences.

Conclusion

Hybridization processes have been largely documented as being important in geographical speciation and expansion of ground plants (e.g. Potts & Reid, 1988; Abbott, 1992; Arnold, 1992; Milne *et al*., 1999). Examples of hybridization are less numerous for marine plants and algae (Mathieson *et al*., 1981; Athanasiadis, 1996; Benzie *et al*., 1997; Yoon & Boo, 1999). A hybridization process could explain the origin of *C. racemosa* 'invasive variety', which has been spreading rapidly in the Mediterranean Sea for 10 years. Of note is that this taxon spreads not only in warm to temperate water, but also in relatively cold water (down to 10 °C), which is unusual for *Caulerpa* species (Verlaque *et al*., 2000). Such property could be because of heterosis.

Acknowledgments

The authors are indebted to the collectors who kindly provided them with material (see Table 1), to Michael Paul for improving the English text and thank specially Carole Borchiellini, Gary Buckhart and Mike Mitchell for insightful discussion and critical reading of the manuscript. This work was supported by a Fifth Framework Program of the European Union (Algal Introductions in European Seas).

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Received 31 August 2001; accepted 5 October 2001