# On the identity and origin of the Mediterranean invasive *Caulerpa racemosa* (Caulerpales, Chlorophyta)

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Recent morphological and genetic studies on the *Caulerpa racemosa* (Forsskål) J. Agardh complex have demonstrated that three taxa occur in the Mediterranean Sea. One of them, the 'invasive variety', provisionally regarded as close to *C. racemosa* var. *occidentalis* J. Agardh, is currently spreading spectacularly throughout the Mediterranean. On the basis of new morphological and molecular studies (rDNA ITS1, 5.8S and ITS2 sequences), we confirm here that this invasion is the result of a recent introduction and we identify the invasive variety as *Caulerpa cylindracea* Sonder, endemic to south-west Australia, and currently known as *C. racemosa* var. *laetevirens* f. *cylindracea* (Sonder) Weber-van Bosse. *C. cylindracea* differs from the tropical north Australian *C. laetevirens* Montagne by its slender thallus, lack of large rhizoidal pillars, the slight inflation of the basal part of the upright axes immediately above the attachment to the stolon, by the range of morphological variations (branchlets clavate to cylindracea (Sonder) Verlaque, Huisman *et* Boudouresque is therefore proposed.

Key words: Australia, Caulerpa racemosa, introduced species, ITS1-ITS2, Mediterranean Sea, seaweed, taxonomy

# Introduction

Caulerpa racemosa (Forsskål) J. Agardh sensu lato (Caulerpales, Chlorophyta) is widely distributed in tropical to warm-temperate regions. In the Mediterranean Sea it was first collected in Sousse Harbour, Tunisia (Hamel, 1926), and subsequently reported from the Eastern Mediterranean Sea, but not noted to be a potential invader (e.g. Hamel, 1931; Mayhoub, 1976). It is regarded as a possible Red Sea migrant (i.e. Lessepsian species) (Por, 1978; Verlaque, 1994; Chisholm et al., 1995; Ribera & Boudouresque, 1995). At the beginning of the 1990s, the situation changed abruptly with the discovery in Libya (Nizamuddin, 1991) of a distichously branched form of C. racemosa unknown to the Mediterranean Sea. This taxon is currently undergoing a dramatic and continuous expansion throughout most of the Mediterranean Sea (see Verlaque et al., 2000). By late 2002, it occurred in at least 11 Mediterranean countries (Albania, Croatia, Cyprus, France, Greece, Italy, Libya, Malta, Spain, Tunisia and Turkey) and all the large islands (Balearic Islands, Corsica, Crete,

Correspondence to: M. Verlaque. Fax: 33 4 91 41 12 65. e-mail: verlaque@com.univ-mrs.fr Cyprus, Sardinia and Sicily). This invasion is, therefore, more widespread than that of the highly publicized *Caulerpa taxifolia* (Vahl) C. Agardh (Meinesz *et al.*, 2001).

Verlaque *et al.* (2000) suggested that three distinct taxa of the *C. racemosa* complex coexist in the Mediterranean: (i) an intermediate between *C. racemosa* var. *turbinata* (J. Agardh) Eubank and var. *uvifera* (C. Agardh) J. Agardh, termed here '*turbinata-uvifera*' (Verlaque *et al.*, 2000) (ii) *C. racemosa* var. *lamourouxii* (Turner) Weber-van Bosse f. *requienii* (Montagne) Weber-van Bosse, and (iii) the 'invasive variety' that has spread so quickly in the Mediterranean Sea. Verlaque *et al.* (2000) considered that the 'invasive variety' might be a recently introduced Atlantic or Indo-Pacific taxon close to *C. racemosa* var. *occidentalis* (J. Agardh) Børgesen and termed it 'aff. var. *occidentalis*'.

Famà *et al.* (2000) analysed DNA ITS sequences of *C. racemosa* from different localities both inside and outside the Mediterranean. They found: (i) intra-individual polymorphism of ITS1 (alignment of 167 positions including gaps) to be as high or higher than inter-individual polymorphism across all samples tested with no regional biogeographic structure or putative varietal structure even when ITS2 (alignment of 332 positions including gaps) was added: none of the six Mediterranean populations (five attributed to the 'invasive variety' and another to var. *lamourouxii*, i.e. the 'Mediterranean clade') could be distinguished from one another or from Western Australian and Gran Canarian populations. The presence of Western Australian samples, termed *C. racemosa* var. *laetevirens* f. *cylindracea* (Montagne) Weber-van Bosse, within the Mediterranean clade suggested that *C. racemosa* var. *occidentalis* (*sensu* Verlaque *et al.*, 2000) might be an introduction from western Australia but this hypothesis could be confirmed only by more extensive sampling.

Durand et al. (2002) continued the study of ITS sequences in the C. racemosa complex. Analyses of ITS1-ITS2 and an intron in the 18S gene supported the hypothesis that three distinct taxonomic units of C. racemosa occurred in the Mediterranean. Famà et al. (2000)'s ITS1 sequences of Western Australian and Gran Canarian samples were placed in the 'invasive variety' clade. The presence of sequences of C. racemosa from Rhodes Island (Greece), which was originally attributed to var. lamourouxii, in this clade suggested a possible misidentification (Durand et al., 2002). Durand et al. (2002) noted that Famà et al. (2000)'s suggestion of a possible introduction from Australia was interesting since Harvey (1858) described and illustrated a distichous variety, C. cylindracea Sonder var. macra Harvey, from Western Australia, which looks very similar to some Mediterranean isolates of the 'invasive variety'. With regard to C. racemosa with distichously arranged branchlets, another Australian taxon, C. racemosa var. corvnephora (Montagne) Weber-van Bosse, shows a likeness to C. racemosa var. laetevirens f. cylindracea.

The present study was designed to test the hypothesis of a possible introduction from Australia, and to unravel the taxonomic status of the Mediterranean 'invasive variety' of *C. racemosa*. We carried out a comparative morphological and genetic study of the three Mediterranean putative *C. racemosa* varieties, new Australian collections of *C. cylindracea* Sonder, currently reported as *C. racemosa* var. *laetevirens* (Montagne) Weber-van Bosse f. *cylindracea* (Sonder) Weber-van Bosse (Silva *et al.*, 1996; Huisman, 2000), and other *C. racemosa* material from the Atlantic and the Indo-Pacific.

### Materials and methods

#### Specimen collection and herbarium studies

*Caulerpa racemosa* var. *laetevirens* f. *cylindracea* samples were collected by one of the authors of the present study (J.H.) from the type locality in Western Australia:

specimens H5527–5530, Carnac Island,  $(32^{\circ} 12' \text{ S}, 115^{\circ} 38' \text{ E})$ , near Rockingham, south of Perth, 12.xii.2001, 2 m deep, on limestone reef exposed to swell (Table 1).

The study was complemented by the following set of *C. racemosa* specimens (Table 1):

- C. racemosa var. turbinata-uvifera, specimen H5515, leg. Y. Valentin, Prainha, Brazil, 13.vii.2000, intertidal pools.
- C. racemosa var. turbinata-uvifera, specimen H7053, leg. L. Markovich, Saint-Barthélemy, Caribbean Islands, vii/2000, depth unknown.
- C. racemosa var. lamourouxii f. requienii, specimen H7054, leg. J.G. Harmelin and G. Bitar, Tripoli, Lebanon, viii/1999, depth unknown.
- C. racemosa var. peltata, specimen H5514, leg. P.
  Lopez Gonzales, Funchal, Madeira, 27.ix.2000, 4 m deep.

Tissues were fixed in 80% ethanol and preserved at  $-20^{\circ}$ C. The above material has been deposited in the Herbarium Verlaque, UMR-6540 DIMAR, COM, Marseilles, France.

We considered all the infraspecific taxa of *C. racemosa* from around the world to identify that most similar to the invasive one. Type localities and basionyms of the infraspecific taxa of *C. racemosa* cited are given in Table 2.

Voucher material was compared with the following specimens:

- Mediterranean and extra-Mediterranean specimens of *C. racemosa* cited in Verlaque *et al.* (2000).
- Caulerpa racemosa var. occidentalis (J. Agardh) Børgesen, Long Key, Florida, USA, 22.vi.2001, leg. P. Famà, specimen H6250, Herbarium Verlaque, COM, Marseilles.
- Caulerpa racemosa var. occidentalis, Martinique, viii.2001, leg. A. Ganteaume, specimen H6251, Herbarium Verlaque, COM, Marseilles.
- Caulerpa laetevirens Montagne, 1842, holotype, Toud Island, Torres Strait, Australia, specimen MA 10323, Herbarium Montagne, Laboratoire de Cryptogamie, Museum National d'Histoire Naturelle, Paris, France (PC).
- Caulerpa corynephora Montagne, 1842, holotype, Toud Island, Torres Strait, Australia, specimen MA 10282, Herbarium Montagne, Laboratoire de Cryptogamie, Museum National d'Histoire Naturelle, Paris, France (PC).

#### DNA extraction

Before genomic DNA extraction, tissue samples were dehydrated and frozen in liquid nitrogen. Total DNA was extracted using the Plant Tissue (Promega) genomic DNA purification kit. After O.D. measurement at 260/280 nm genomic DNA was resuspended in ddH<sub>2</sub>O at a final concentration of 1  $\mu$ g  $\mu$ l<sup>-1</sup>.

#### PCR amplification, RFLP and sequencing

PCR amplifications were performed using primers specific for *Caulerpa*, designed from an alignment of

all sequences of the nuclear ribosomal genes of Caulerpa species available in GenBank, a forward primer hybridizing in the 3' region of the 18S rDNA (5'-GCAATAACAGGTCTGTGATGC-3') sequence and a reverse primer located near the 5' extremity of the 28S rDNA (5'-TCCTCCGCTTATTGATATGCT-3'). The 1100 bp amplified region contains the 3' end of the 18S rDNA, including the intron (100 to 108 bases), the ITS1 (112 to 136 bases), 5.8S rDNA, ITS2 (281 to 315 bases), and the 5' end of the 28S rDNA. Genomic DNA was amplified by a DNA thermal cycler (Perkin Elmer Celtus). The 25  $\mu$ l PCR mix contained 10 ng of genomic DNA, 5  $\mu$ l of 10 × Taq DNA polymerase buffer, 8 µl of 1.25 mM dNTP mix (Pharmacia), 2.5 µl of each primer (20  $\mu M)$  and 1.25 U of Taq DNA polymerase (Promega). 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 for 35 cycles of amplification. Fragments were checked on 1.5% agarose gels and cleaned with Qiaquick kit (Wesburg). PCR products were then cloned into pGem-T Easy vector (Promega).

To evaluate intra-individual sequence polymorphism, DNA inserts were amplified from 10-16 positive clones for each sample, using the primer set described above, and digested with a series of restriction enzymes (Taq1, HaeIII, Sau3A and AluI). AluI was found to be the most informative and was chosen for the final RFLP analysis. Digestions were performed using 5  $\mu$ l of PCR product in a total volume of 10  $\mu$ l. Electrophoresis was performed using 2% agarose gels. For each distinct restriction pattern, one to three clones were selected and sequenced. Sequences were obtained for the combined ITS1, 5.8S, ITS2 region, and also for a Caulerpa-specific fast-evolving intron of the 18S rDNA using the dideoxy-nucleotide chain termination method (Sanger et al., 1977).

#### Sequence alignment and phylogenetic analysis

Nucleotide sequences were aligned automatically using the Mac Vector program. A set of published and unpublished sequences of *C. racemosa* (Famà *et al.*, 2000; Yeh, 2000; Durand *et al.*, 2002; Kooistra, 2002) was included in analyses (Table 1). The alignments were then optimized manually using the program ClustalW (Thompson *et al.*, 1994). The alignment is available from the authors upon request.

Trees were computed using the distance matrix neighbour-joining method (NJ) (Saitou & Nei, 1987), maximum parsimony (MP) and maximum likelihood (ML) in Paup 4.0b10 (Swofford, 2000). Gaps are distributed proportionally. 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 for multiple hits, and for unequal transition and transversion rates using Kimura's two-parameter model (Kimura, 1980). In parsimony analyses, gaps were treated as missing data. We performed heuristic searches with the branch swapping option, using the TBR algorithm. The statistical robustness of nodes was ascertained by bootstrapping (Felsenstein, 1992) with 1000 iterations for NJ and MP and 100

## **Results and discussion**

# Morphological data

A comparison of liquid-preserved specimens of the C. racemosa 'invasive variety' from the Mediterranean and C. racemosa var. laetevirens f. cylindracea from Carnac Island, Western Australia, showed no morphological differences. In both, the slender thallus is fixed to the substratum by means of thin rhizoids, 1-10(-20) mm long and 0.3-0.8(-1.0)mm in diameter that are closely arranged along the stolon (Figs 1 and 6). The stolon is 0.7-2.0 mm in diameter and bears simple or occasionally branched upright axes, 1-11 cm (up to 19 cm in the Mediterranean Sea) high and 3-10 mm across. The basal part of upright axes is slightly inflated immediately above the attachment to the stolon (Figs 4 and 9: arrows). Upright axes bear uncrowded vesiculate branchlets that are radially or distichously arranged (sometimes both on the same thallus) on a cylindrical rachis (Figs 2, 3, 7 and 8). The branchlets are clavate, (1.5-)2-5(-7)mm long and 1-2 mm (up to 3 mm in the Mediterranean) in greatest diameter shortly below the rounded apices, and upwardly directed. This description is in good agreement with the protologue of C. cylindracea Sonder (Sonder, 1845) and the description and illustrations of C. cylindracea Sonder and C. cylindracea var. macra Harvey (Harvey, 1858) (Figs 5A-C).

Sonder (1845) described C. cylindracea for material collected from Western Australia by J.A.L. Preiss. The exact locality is not known due to Sonder's practise of referring all of the Preiss collections to 'Novae Hollandiae Occidentale', i.e. Western Australia. It is known, however, that Preiss collected from several locations in the southwest (Marchant, 1990). We failed to obtain the holotype of C. cylindracea Sonder, but our Australian specimens were collected from the type locality and are in good agreement with the illustration of an authentic specimen, i.e. a specimen identified by Sonder (Kützing, 1857: tab. 15, fig. III, as *Chauvinia 'cylindrica'* annoted: 'Sonder, Herb.') (Fig. 10). The Mediterranean specimens also agree with this illustration.

Herbarium specimens of *C. racemosa* var. *occidentalis* (J. Agardh) Børgesen from the tropical West Atlantic (Figs 11 and 12; Table 3) and older descriptions and illustrations (Agardh, 1873; Reinke, 1900: Fig. 57, as *C. racemosa* var. *chemnitzia*; Børgesen, 1907: Figs 28 and 29) were distinguishable from *C. racemosa* var. *laetevirens* f. *cylindracea* 

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**Table 1.** Isolates of *Caulerpa racemosa* and *C. mexicana*: taxon, geographical origin, collectors and accession numbers. Mediterranean specimens of *C. racemosa* (Nrs 1–2, 5–7 and 9–12) are named according to Durand *et al.* (2002). Specimens Nrs 3–4, 8, 13–16, 23–25 and 27 were identified by their morphological characters, according to the literature. For the published sequences, the specimens are named according to the authors. Specimens LI, RH, GC, FU, PAN and WA are named according to Famà *et al.* (2000), without any change of these author's code, for the sake of clarity. In agreement with the present study, the isolate of *C. racemosa* var. *laetevirens* f. *cylindracea* is labelled 'var. *cylindracea*'. The 18S intron, ITS1 and ITS2 rDNA sequences corresponding to the isolates were deposited in the GenBank database. Accession numbers without reference are from Durand *et al.* (2002). 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. turbinata-uvifera	Prainha, Brazil	Y. Valentin	AY334305
4	C. racemosa var. turbinata-uvifera	Saint-Barthélemy, Caribbean Islands	L. Markovich	AY334304
5	C. racemosa var. lamourouxii	Famagusta, Cyprus	M. Verlaque	AJ297634
6	C. racemosa var. lamourouxii	Selaata, Lebanon	J.G. Harmelin and H. Zibrowius	AJ297635
7	C. racemosa var. lamourouxii	Tripoli, Lebanon	J.G. Harmelin and G. Bi- tar	AY334303
8	C. racemosa var. lamourouxii	Ishigaki, Japan	C.F. Boudouresque and A. Meinesz	AJ297636
9	C. racemosa 'invasive variety'	Samos, Greece	J. Laborel	AJ297637 clone a AJ297638 clone b
10	C. racemosa 'invasive variety'	Saronikos, Greece	P. Panavotidis	AJ297639 clone a
10		Sarolinios, Creece	1 · · · unuj o uuis	AJ297640 clone b
11	C. racemosa 'invasive variety'	Famagusta, Cyprus	M. Verlaque	AJ297641 clone a
				AJ297642 clone b
				AJ297643 clone c
12	C. racemosa 'invasive variety'	Marseilles, France	T. Pérez	AJ297644 clone a
				AJ297645 clone b
13	C. racemosa var. clavifera	Red Sea	A. Meinesz	AJ297646
14	C. racemosa var. clavifera	Mayotte Island, Indian Ocean	C. Marschall	AJ297647
15	C. racemosa var. peltata	Ishigaki, Japan	C.F. Boudouresque and A. Meinesz	AJ297648
16	C. racemosa var. peltata	Madeira, Atlantic	P. Lopez Gonzales	AY334302 clone a AY334301 clone b
17	C. racemosa var. peltata	Green Island, Taiwan	W.J. Yeh	AY205288 (Yeh, 2000, unpublished)
18	C. racemosa var. peltata	Dali, Taiwan	W.J. Yeh	AY205289 (Yeh, 2000, unpublished)
19	C. racemosa var. laetevirens	Fengchueisha, Taiwan	W.J. Yeh	AY205290 (Yeh, 2000, unpublished)
20	C. racemosa var. laetevirens	Kinting, Taiwan	W.J. Yeh	AY205291 (Yeh, 2000, unpublished)
21	C. racemosa var. macrophysa	Green Island, Taiwan	W.J. Yeh	AY206420 (Yeh, 2000, unpublished)
22	C. racemosa var. macrophysa	Nanwan, Taiwan	W.J. Yeh	AY206421 (Yeh, 2000, unpublished)
23	C. racemosa var. macrophysa	Ishigaki, Japan	C.F. Boudouresque and A. Meinesz	AJ297649 clone a AJ297650 clone b
24	C. racemosa var. racemosa	Heron Island, Australia	M. Manuel	AJ297651
25	C. racemosa var. racemosa	Balicasag Island, Philip- pines	A. Gómez and M.A. Ribera	AJ297652 clone a
				AJ297653 clone b
26	C. racemosa "var. not available"	Panama, Atlantic	W.H.C.F. Kooistra	AF479702 (Kooistra, 2002)
27	C. racemosa var. cylindracea	Carnac Island, Western Australia	J. M. Huisman	AY173116 clone a AY173117 clone b
				AY173118 clone c
28	C. mexicana	Israel	F. Weinberger	AJ007815 <sup><i>a</i></sup> (Olsen <i>et al.</i> , 1998)
29	C. mexicana	Canary Islands, Spain	Y.S.D.M. De Jong and W.F. Prud'homme van Reine	AJ007816 <sup>a</sup> (Olsen <i>et al.</i> , 1998)
30	C. mexicana	Panama	W.H.C.F. Kooistra	AJ007817 <sup>a</sup> Olsen et al. (1998)
31	C. mexicana	Florida	J.R.M. Chisholm	AJ007818 <sup>a</sup> (Olsen et al., 1998)
LI	C. racemosa var occidentalis <sup>e</sup>	Livorno, Italy	L. Piazzi	AF256088 <sup>b</sup> , isolate L11, clone A AF256093 <sup>b</sup> , isolate L11, clone F AF256096 <sup>b</sup> , isolate L11, clone I AF256099 <sup>b</sup> , isolate L11, clone L AF256104 <sup>b</sup> isolate L11, clone

Q (Famà et al., 2000)

#### Table 1. (continued)

Nr	Taxon	Origin	Collector	Accession no.
RH	C. racemosa var. lamourouxii	Rhodes, Greece	M. van Rijssel	AF256140 <sup>b</sup> , isolate RH1, clone A AF256141 <sup>b</sup> , isolate RH1, clone B AF256142 <sup>b</sup> , isolate RH1, clone C AF256143 <sup>b</sup> , isolate RH1, clone D (Famà <i>et al.</i> , 2000)
GC	C. racemosa "var. not available"	Gran Canaria, Canary Islands, Spain	H.J. van de Strate	AF256144 <sup>b</sup> , isolate GCa1, clone A AF256147 <sup>b</sup> , isolate GCs1, clone C AF256148 <sup>b</sup> , isolate GCl1, clone A (Famà <i>et al.</i> , 2000)
FU	C. racemosa "var. not available"	Fuerteventura, Canary Islands, Spain	H.J. van de Strate	AF256151 <sup><math>b</math></sup> , isolate FU1, clone B (Famà <i>et al.</i> , 2000)
PAN	C. racemosa "var. not available"	Panama	W.H.C.F. Kooistra	AF256154 <sup>b</sup> , isolate PAN (Famà <i>et al.,</i> 2000)
WA	C. racemosa var. laetevirens f. cylindracea	Perth, Western Australia	M. Polifrone and S. Williams	AF256155 <sup>b</sup> , isolate WA1, clone A AF256156 <sup>b</sup> , isolate WA1, clone B AF256157 <sup>b</sup> , isolate WA1, clone C AF256158 <sup>b</sup> , isolate WA1, clone D AF256159 <sup>b</sup> , isolate WA1, clone E AF256160 <sup>b</sup> , isolate WA2, clone A AF256161 <sup>b</sup> , isolate WA2, clone B (Famà <i>et al.</i> , 2000)

<sup>a</sup>ITS1, ITS2 and intron. <sup>b</sup>ITS1 only. <sup>c</sup>This taxon was only considered similar, and not definitely assigned, to var. *occidentalis*, by Verlaque *et al.* (2000).

Table 2.	Basionym ar	id type	locality of	the infraspecific	taxa of	`C. racemosa	cited
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Taxon	Basionym	Type locality
var. <i>clavifera</i>	Fucus clavifer Turner, 1807-1808: 126-127, pl. 57	Red Sea
var. corynephora	C. corynephora Montagne, 1842:14	Toud Island (Warrior Islet), Torres Strait, Australia
var. cylindracea	C. cylindracea Sonder, 1845:50	Western Australia
var. laetevirens	C. laetevirens Montagne, 1842:13	Toud Island (Warrior Islet), Torres Strait, Australia
var. <i>lamourouxii</i>	Fucus lamourouxii Turner, 1811-1819:79-80, pl. 229	Red Sea
F. laxa	C. laxa Greville, $1853:3$ , pl. II: upper figs $1-2$	Eastern India
F. macra	C. cylindracea var. macra Harvey, 1858: pl. XXX, fig. 2	King George's Sound, Western Australia
var. macrophysa	Chauvinia macrophysa Kützing, 1857: 6, pl. 15: fig. II	Central America, Eastern Pacific
var. occidentalis	C. chemnitzia var. occidentalis J. Agardh, 1873: 37	Upper Gulf of Mexico to Recife, Brazil
var. <i>peltata</i>	C. peltata J.V. Lamouroux, 1809:332	Antilles, West Indies
var. racemosa	Fucus racemosus Forsskål, 1775: 191	Suez, Egypt, Red Sea
F. requienii	Herpochaeta requienii Montagne, 1856: 454	Red Sea
var. turbinata	C. clavifera var. turbinata J. Agardh, 1837: 173	Sinai Peninsula, near Tor, Egypt, Red Sea
var. <i>uvifera</i>	<i>Fucus uvifer</i> Turner, <i>nom. illeg.</i> , 1811–1819:81–82, pl. 230	Red Sea

by the larger size, stout thallus, stolons up to 3-5 mm in diameter, vigorous rhizoidal pillars, upright axes not inflated immediately above the attachment to the stolon, and clavate, spaced lowest branchlets, the more distal branchlets becoming progressively more crowded and swollen at the apex with the uppermost, outwardly bent surface of the swollen part flattened or convex. As a result, *C. racemosa* var. *occidentalis* is closer to var. *turbinata* than to var. *laetevirens* f. *cylindracea*.

The holotype of *C. laetevirens* (Figs 13 and 14; Tables 2 and 3) differs from *C. racemosa* var. *laetevirens* f. *cylindracea* in having a stout thallus,

rhizoidal pillars, upright axes not slightly inflated immediately above the attachment to the stolon and crowded branchlets (see also Taylor, 1950; Coppejans & Prud'homme van Reine, 1992, as *C. racemosa* ecad *laetevirens*).

The holotype of *C. corynephora* Montagne, from the same type locality as *C. laetevirens* (Fig. 15; Tables 2 and 3), differs from *C. racemosa* var. *laetevirens* f. *cylindracea* in having a stout thallus, larger size, spaced rhizoidal pillars and upright axes not slightly inflated immediately above the attachment to the stolon and bearing a series of subopposite branchlets (Prud'homme van Reine *et al.*,



**Figs 1–9.** *Caulerpa racemosa* (Forsskål) J. Agardh. Figs 1–4. Mediterranean specimens of 'invasive variety'. Fig. 1. Habit of plants (Herbarium specimens, H2794, Cyprus, arrow: rhizoids). Fig. 2. Detail of distichous arrangement of branchlets (Herbarium specimen, H5526, France). Fig. 3. Detail of radial arrangement of branchlets (Herbarium specimen, H2794, Cyprus). Fig. 4. Detail of attachment of upright axes to the stolon (arrow) (France, Marseilles). Fig. 5. Illustrations of *C. cylindracea* Sonder (Harvey, 1858: pl. XXX). A. *C. cylindracea* var. *cylindracea*. B. *C. cylindracea* var. *macra* Harvey. C. Branchlets of different ages. Figs 6–9. *C. racemosa* var. *laetevirens* (Montagne) Weber-van Bosse f. *cylindracea* (Sonder) Weber-van Bosse [Herbarium (Figs 6 and 7) and alcohol preserved specimens (Figs 8 and 9), H5527, Carnac Island, Western Australia]. Fig. 6. Habit of plant (arrow: rhizoids). Fig. 7. Detail of distichous arrangement of branchlets. Fig. 8. Detail of radial arrangement of upright axes to the stolon (arrow). (Scale bars: Figs 1, 5A–B and 6 = 1 cm; Fig. 5C: no scale available; Figs 2–4 and 7–9 = 5 mm).

1996, as *C. racemosa* ecad *corynephora*). Weber-van Bosse (1898: 365-366) suggested the possibility of var. *corynephora* being a reduced form of *C. racemosa* var. *laetevirens*, possibly paralleling the relationship of *C. cylindracea* var. *macra* Harvey to *C. cylindracea* Sonder, since imbricated radial and distichous branchlets may occur on the same individual.

Finally, *Caulerpa laxa* Greville (Table 2) is currently included as a form of *C. racemosa* var.



**Figs 10–12.** *Caulerpa* species. Fig 10. Illustration of *Caulerpa cylindracea* Sonder based on an authentic specimen of Sonder (Kützing, 1857: tab. 15, fig. III, as *Chauvinia 'cylindrica'* Sonder) (arrow: rhizoids). Figs 11 and 12. *Caulerpa racemosa* var. *occidentalis* (J. Agardh) Børgesen from Martinique (herbarium specimen H6251) and Florida (herbarium specimen H6250), respectively (arrow: rhizoidal pillars). (Scale bars: Figs 10-12 = 1 cm).

**Table 3.** A morphological comparison between specimens of *Caulerpa racemosa* var. *cylindracea*, var. *occidentalis*, var. *laetevirens* and var. *corynephora* 

	var. cyl	indracea	var. occidentalis		var. <i>laetevirens</i>	var. corynephora	
	Carnac Island, Australia H5527-5530	Marseilles, France H2736-2743	Long Key, Florida H6250	Martinique H6251	Toud Island, Aus- tralia Holotype	Toud Island, Aus- tralia Holotype	
Rhizoids							
Morphology	Thin rhizoids	Thin rhizoids	Rhizoidal pillars	Rhizoidal pillars	Rhizoidal pillars	Rhizoidal pillars	
Length, mm	1 - 20	2 - 20	20 - 60	20 - 30	25 - 40	Sectioned	
Diameter, mm	0.3 - 0.5	0.3 - 0.5	1.5 - 2.0	1.0 - 2.0	1.5 - 2.0	1.5 - 2.0	
Stolons							
Diameter, mm	0.5 - 1.0	0.5 - 1.0	4.0 - 5.0	3.0 - 4.0	2.0 - 3.0	3.0 - 4.0	
Upright axes							
Height, mm	24 - 110	25 - 78	25 - 140	140 - 150	55 - 105	90 - 100	
Attachment to the stolon	Slightly inflated	Slightly inflated	Cylindrical	Cylindrical	Cylindrical	Cylindrical,	
Branchlets							
Arrangement	Uncrowded; radial to distichous	Uncrowded; radial to distichous	Uncrowded to crowded; radial	Uncrowded to crowded; radial	Crowded; radial	Uncrowded; distichous, sub-opposite	
Shape	Clavate	Clavate	Clavate to clavate flattened	Clavate to clavate flattened	Cylindrical to cla- vate	Cylindrical to clavate	
Length, mm	4-6	4-5	4-8	6-8	4-9	4-9	
Diameter, mm	1.0-2.0	1.0-2.0	1.5-3.0	1.5-2.5	1.0-2.0	1.5-2.5	

*laetevirens* [*C. racemosa* var. *laetevirens* f. *laxa* (Greville) Weber-van Bosse], which grows in localities strongly exposed to swell (Weber-van Bosse, 1898; Silva *et al.*, 1996).

# Sequence and RFLP analyses

As we previously showed in the 'invasive variety' (Durand *et al.*, 2002), RFLP analysis revealed intra-individual polymorphism in the isolate of *C*.

*racemosa* var. *laetevirens* f. *cylindracea*, three distinct restriction patterns. The sequence representing the majority of the clones has been named 'a' and the other sequences 'b' and 'c'.

Sequence data are consistent with the results of RFLP analysis. Polymorphic individuals were polymorphic for both ITS sequences and for the 18S intron.

The ITS1, 5.8S and ITS2 alignment consisted of 704 positions, of which 247 were parsimony



**Figs 13–15.** Types of *Caulerpa* species, photos by courtesy of the Muséum National d'Histoire Naturelle, Herbier Cryptogamique. Figs 13–14. Holotype of *Caulerpa laetevirens* Montagne (Montagne, 1842) (MA 10323, Herbarium Montagne, Herbier Cryptogamique, PC). Fig. 14. Detail of stolon and rhizoidal pillars (arrow). Fig. 15. Holotype of *Caulerpa corynephora* Montagne (Montagne, 1842) (MA 10282, Herbarium Montagne, Herbier Cryptogamique, PC). (Scale bars: Figs 13–15 = 1 cm).

informative. Neighbour-joining, maximum parsimony and maximum likelihood analyses (Fig. 16) showed no significant differences. The two previously identified *C. racemosa* varieties from the Mediterranean Sea, var. *lamourouxii* f. *requienii* and var. *turbinata-uvifera*, fall into two distinct clades (clades 1 and 3, respectively), both with 100% bootstrap support. Australian *C. racemosa* var. *laetevirens* f. *cylindracea* sequences (Nrs 27a-c) fall within the 'invasive variety' clade (clade 4), with high bootstrap support. The other extra-Mediterranean isolates of *C. racemosa* fall into the robust clades 2, 3 and 5 (Fig. 16).

A comparison of our ITS1 sequences with some of the sequences published by Famà *et al.* (2000) is shown in Figure 17. As in our previous study (Durand *et al.*, 2002), the tree, constructed from ITS1 alone (164 positions, of which 88 were parsimony informative), shows a significantly lower degree of resolution than the tree based on ITS1-ITS2. There is no resolution of the topology within clade 2, comprising all isolates referred to the Mediterranean 'invasive variety' of *C. racemosa*, our sequences of *C. racemosa* var. *laetevirens* f. *cylindracea* as well as Famà *et al*'s sequences of





0.1

**Fig. 17.** Neighbour-joining tree showing a comparison of our ITS1 sequences of *Caulerpa racemosa* isolates with published sequences, with *Caulerpa mexicana* as the outgroup. The maximum parsimony (MP) and maximum likelihood trees for the same dataset show the same topology. Numerals at internal nodes are the NJ/MP bootstrap values (1000 and 1000 replicates, respectively). Bootstraps < 70% are not indicated. Sequences from Famà *et al.* (2000) are labelled in bold; morphological determination and geographical origin are indicated in Table 1. *C. racemosa* var. *laetevirens* f. *cylindracea* is labelled '*cylindracea*'.

'var. *occidentalis*' (i.e. the invasive variety), several isolates from Western Australia (i.e. *C. racemosa* var. *laetevirens* f. *cylindracea*), Gran Canaria (var. not available), and Greece (referred to '*C. racemosa* var. *lamourouxii*'). Our sequences corresponding to the two other morphological varieties from Mediterranean Sea, var. *turbinata-uvifera* and var. *lamourouxii* f. *requienii*, still fall into two distinct clades (clades 1 and 5) (Fig. 17).

The 18S intron alignment contained 233 positions, of which 60 were parsimony informative. As in our previous study (Durand *et al.*, 2002), the analyses show a major inconsistency with the ITS tree. Australian *C. racemosa* var. *laetevirens* f. *cylindracea* and invasive variety sequences are split into two distinct unrelated clades, clades 2 and 4 (Fig. 18). The sequence Nr 27a of the *C. racemosa* var. *laetevirens* f. *cylindracea* falls into clade 2, whereas sequences 27b and 27c are in clade 4. The other results have been already discussed in Durand *et al.* (2002).

## The species introduction hypothesis

Morphological comparisons of *Caulerpa racemosa* taxa from the Mediterranean, Australia, and other areas have demonstrated that the Mediterranean 'invasive variety' is in good agreement with *C. racemosa* var. *laetevirens* f. *cylindracea* from southwestern Australia. The trees based on ITS1, 5.8S and ITS2 (247 informative positions) give better phylogenetic information on the *C. racemosa* complex than those constructed from ITS1 alone (88 informative positions). ITS analyses place *C. racemosa* var. *laetevirens* f. *cylindracea* with the Mediterranean 'invasive variety' samples, suggesting that they are conspecific. The invading strain may have come from Western Australia.

#### The hybridization event hypothesis

Durand *et al.* (2002) suggested the possibility that the 'invasive variety' could be the result of a recent hybridization between an undetermined introduced taxon of *C. racemosa* with a Mediterranean population of var. *turbinata-uvifera*. The identical genetic characteristics of the Mediterranean and Australian populations disprove this hypothesis: in the event of hybridization, this would necessarily have taken place prior to the introduction into the Mediterranean Sea.

# *Taxonomic affinities of* Caulerpa racemosa *var*. laetevirens *f*. cylindracea

Weber-van Bosse (1898), who did not consider *C.* racemosa var. occidentalis, included *C. cylindracea* Sonder and all other taxa with cylindrical branchlets, i.e. *C. laetevirens* and *C. laxa* Greville, under





*C. racemosa* var. *laetevirens* (Montagne) Webervan Bosse. However, *C. racemosa* var. *laetevirens* f. *cylindracea* is readily distinguished from all other varieties, including var. *occidentalis*, var. *laetevirens*, var. *laxa* and var. *corynephora*, by the thin

rhizoids, the basal part of the upright axes being slightly inflated above the attachment to the stolon, and the uncrowded branchlets that are radially or distichously disposed and clavate to cylindrical but never trumpet-like or shield-like.

Ohba & Enomoto (1987), Enomoto & Ohba (1987) and Ohba et al. (1992), using cross-gradient cultures, have shown that C. racemosa var. laetevirens (with many densely imbricate and cylindrical branchlets) and C. peltata (with few and shield-like branchlets) were two morphological expressions of the same entity. In the tree based on ITS, 5.8S and ITS2, C. racemosa var. laetevirens and var. peltata from Taiwan did fall within the same distinct robust clade as var. turbinata-uvifera, which can bear trumpet-like or shield-like branchlets (Fig. 16, clade 3). However, C. racemosa var. peltata from Japan (and two other Japanese isolates referred to var. lamourouxii and var. macrophysa) falls within another distinct robust clade, that is not closely related (Fig. 16, clade 5). So, resolution of the taxonomic status of C. racemosa var. peltata and var. laetevirens requires further studies before following Silva et al.'s (1996) conclusion that C. racemosa var. peltata should be treated as a separate species (C. peltata) with C. laetevirens as a synonym.

In conclusion, our genetic and morphological data provide undisputed evidence that *C. racemosa* var. *laetevirens* f. *cylindracea* and var. *laetevirens* (including var. *peltata*), which are allopatric in Australia (Fig. 19), are two distinct taxa. We

propose the following new combination: *Caulerpa* racemosa (Forsskål) J. Agardh var. cylindracea (Sonder) Verlaque, Huisman et Boudouresque, stat. nov.

Basionym: *Caulerpa cylindracea* Sonder (1845) *Botanische Zeitung*, 3: 50. Type locality: Western Australia.

Homotypic synonym: *Caulerpa racemosa* (Forsskål) J. Agardh var. *laetevirens* (Montagne) Weber-van Bosse f. *cylindracea* (Sonder) Webervan Bosse (Weber-van Bosse, 1898: 366–368, pl. XXXIII, figs 17, 19 and 20).

Heterotypic synonym: *Caulerpa cylindracea* Sonder var. *macra* Harvey (Harvey, 1858: pl. XXX). Harvey (1858) described this taxon on the basis of its distichous habit only. Since radial and distichous branchlets may occur on a same individual, we consider this entity to be a superfluous synonym of Sonder's taxon.

# *The vector of introduction into the Mediterranean Sea*

Attempts to determine the vector of a marine species' introduction can be only speculative. In the present case, there is little evidence in favour of the Lessepsian migration hypothesis (see Verlaque *et* 



**Fig. 19.** Map of the distribution of *Caulerpa racemosa* var. *laetevirens* (including var. *peltata*) and var. *cylindracea* in Australia ("?": south-east limit unknown) (from Lucas, 1936; Tolbert & Osmond, 1976; Saenger, 1979; Womersley, 1984; Huisman & Walker, 1990; Huisman *et al.*, 1991; Wynne & Thinh, 1996; Huisman, 1997, 2000; Phillips, 1997).

*al.*, 2000). This conclusion is strengthened by the fact that *C. racemosa* var. *cylindracea* is a south Indian Ocean species that has a relatively restricted distribution along southwestern shores of Western Australia (Fig. 19) and has never been reported from the Red Sea or the north of the Indian Ocean.

Within the Mediterranean, the occurrence of recently established populations of *C. racemosa* var. *cylindracea* close to harbours provides compelling evidence in favour of its secondary dispersal via shipping (ballast water, anchors, fishing nets or other structures). As far as the primary introduction into the Mediterranean Sea is concerned, the chances of survival of a putative inoculum, under such conditions (transit of several weeks from Western Australia to the Mediterranean), seem low. An intermediate scenario could be a step-bystep transit (from harbour to harbour), but there is no report of such a recent spread of *C. racemosa* var. *cylindracea* at any site in the Indian Ocean or Red Sea.

Another possible vector is the aquarium trade, such as occurred for the introduced *C. taxifolia* (Jousson *et al.*, 1998), and indeed *C. racemosa sensu lato* is one of the most widely used *Caulerpa* species in aquaria along with *C. taxifolia* and *C. sertularioides* (S. Gmelin) Howe. Unlike *C. taxifolia*, *C. racemosa* var. *cylindracea* is not commonly used as a decorative plant in marine aquaria, but in the Perth region of Western Australia at least it has been observed in display aquaria in several aquarium supply shops. Consequently, an accidental escape from a Mediterranean aquarium cannot be excluded.

Finally, we know of at least one example of the intentional introduction of *C. taxifolia* (January 1992, Saint-Cyr-les-Lecques, France; Meinesz, 1999: 82–88). The almost simultaneous and unexpected introduction of two different species of *Caulerpa*, both native to Australia, *C. taxifolia* from the south-east (Meusnier *et al.*, 2001; Famà *et al.*, 2002) and *C. racemosa* var. *cylindracea* from the south-west region, into the Mediterranean Sea (including the relatively cold waters of the north western basin) may indicate the possibility of an intentional act.

# *The probability of spread of the introduced* C. racemosa *var*. cylindracea

In Australia, the distribution of *C. racemosa* var. *cylindracea* is from tropical Western Australia around the southwest coast to Hopetoun  $(33.9^{\circ} \text{ S}, 120.1^{\circ} \text{ E})$ . The southern-most latitude is  $35^{\circ} \text{ S}$  (Albany region) (Womersley, 1984). The exact northern and eastern limits are unknown, due to the paucity of published records from the tropical coast of Western Australia (see Huisman *et al.* 1998), but it has not been reported from Shark Bay

or the Houtman Abrolhos Islands (where var. *laetevirens* and var. *corynephora* occur), nor from South Australia and New South Wales, in the east (Lucas, 1936; Womersley, 1984; Huisman *et al.*, 1991; Huisman, 1997) (Fig. 19).

*Caulerpa racemosa* var. *cylindracea* is common near Perth and at Rottnest Island (Huisman & Walker, 1990; Huisman, 2000). It grows epilithically, generally in high light conditions (i.e. on the upper surfaces of reefs), down to a depth of about 6 m. In the intertidal region, it is an opportunistic species and grows during winter on reef flats covered with sand during the summer months (Womersley, 1984; Carruthers *et al.*, 1993). It does not usually form dense monospecific stands but grows intermixed with other algae. At Albany (King Georges Sound), populations are represented by the rather depauperate form known as var. *macra* Harvey (Harvey, 1858; Womersley, 1956).

Rottnest Island, which is part of a chain of reefs, is subject to winter warming by the Leeuwin Current, but is also influenced by cooler currents (Huisman & Walker, 1990). *C. racemosa* var. *cylindracea* occurs in areas where the surface water temperatures range from about 14°C or 16°C in winter to 22.5°C in summer (Jeffrey, 1981; Womersley, 1984; Pearce *et al.*, 1999). Salinities of southwest Australia waters range from 35.27 to 37.00 PSU (Chubb & Barker, 2002). In culture, *C. racemosa* var. *cylindracea* grows under a wide range of salinities (10 – 40 PSU) and light conditions (20 – 140 µmol photons m<sup>-2</sup> s<sup>-1</sup>), showing best growth at 30 and 40 PSU (Carruthers *et al.*, 1993).

The ecological requirements of C. racemosa var. cylindracea and the similarity of the marine environment inhabited by the Australian populations to that of the Mediterranean go some way to explaining the amazing success of the introduction since the first Mediterranean report of the alga from Libya (Nizamuddin, 1991). C. racemosa var. *cylindracea* appears to be one of the most invasive introduced species ever recorded in the Mediterranean Sea. It grows in the subtidal zone from 1 m down to 60 m depth, on all types of hard and soft substrata and in different communities, with the only exception being unstable sandy substrata (De Biasi et al., 1999; Piazzi et al., 2001a; Tolay et al., 2001a,b; it survives winter sea temperatures down to 13-17°C (10.5°C at Marseilles; Verlaque et al., 2000).

Consequently, *C. racemosa* var. *cylindracea* seems destined for great success in the Mediterranean, and, only a few years after its introduction, the first significant signs of a negative impact on some communities and human activities have been reported (Balata *et al.*, 2000; Cecere *et al.*, 2000; Ceccherelli *et al.*, 2001; Piazzi *et al.*, 2001*b*; Magri *et al.*, 2001).

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