First record of *Caulerpa cylindracea* (Caulerpaceae, Chlorophyta) in Andalusia (Southern Spain)

María Altamirano[1*], Nikos Andreakis[2], Virginia Souza-Egipsy[1], Marianela Zanolla[1] & Julio De la Rosa[3]

[1]Dpto. Biología Vegetal (Botánica), Facultad de Ciencias, Campus de Teatinos s/n, Universidad de Málaga, E-29071 Málaga, Spain
[2]Australian Institute of Marine Science, Townsville, Qld, Australia
[3]Dpto. Botánica, Facultad de Ciencias, Universidad de Granada, C/Severo Ochoa s/n, E-18003 Granada, Spain
altamirano@uma.es; n.andreakis@aims.gov.au; virginia.souza@ica.csic.es; marianela@uma.es; jdlarosa@ugr.es

Abstract

Altamirano, M., Andreakis, N., Souza-Egipsy, V., Zanolla, M. & De la Rosa, J. 2014. First record of *Caulerpa cylindracea* (Caulerpaceae, Chlorophyta) in Andalusia (Southern Spain). *Anales Jard. Bot. Madrid* 71(2): e007

Three different species of *Caulerpa* (Caulerpaceae, Chlorophyta) co-occur in the Mediterranean Sea: two of them are found at the central-eastern basin and are typically considered non aggressive components of the Lessepsian flora [(*C. chemnitzia* (Esper) J.V. Lamouroux and *C. racemosa* var. *lamourouxii* (Turner) Weber-van Bosse f. *requienii* (Montagne) Weber van Bosse)]; a third taxon, *C. cylindracea* Sonder has aggressively expanded its range since its first observation in 1990, and it is nowadays reported from nearly all the Mediterranean countries. We report a population of *C. cylindracea* from Almería (Andalusia, Southern Iberian Peninsula) at −30 m depth as to be the westernmost record of the invasive variety on the Mediterranean European coast. Therefore, we made use of morphological description and molecular phylogenetics to provide a complete identification of this invasive seaweed in Southern Spain. Our findings are discussed in light of the composition of the receptor communities, such as maërl bed, edges of *Posidonia oceanica* (Linnaeus) Delile, and their ecology. Our results confirmed the suggested directionality of the invasive pathway to be westward to the Strait of Gibraltar, mainly supported by sea currents and vectors of anthropogenic origin.

Keywords: ITS sequence, maërl, Mediterranean Sea, *Caulerpa cylindracea*, phylogenetic analysis.

Resumen

Altamirano, M., Andreakis, N., Souza-Egipsy, V., Zanolla, M. & De la Rosa, J. 2014. Primera cita de *Caulerpa cylindracea* (Caulerpaceae, Chlorophyta) en Andalucía (Sur de España). *Anales Jard. Bot. Madrid* 71(2): e007

En el Mar Mediterráneo se encuentran tres especies diferentes de *Caulerpa* (Caulerpaceae, Chlorophyta): dos de ellas se localizan en la cuenca centro oriental y se consideran componentes no agresivos de la flora Lessepsiana (*C. chemnitzia* (Esper) J.V. Lamouroux and *C. racemosa* var. *lamourouxii* (Turner) Weber-van Bosse f. *requienii* (Montagne) Weber van Bosse); un tercer taxon, *C. cylindracea* Sonder ha expandido de manera agresiva su rango de distribución desde su primera observación en 1990, y actualmente se registra en casi todos los países de la cuenca mediterránea. En este trabajo se informa sobre una población de *C. cylindracea* en Almería (Andalucía, Sur de España), a −30 m de profundidad, que representaría la cita más occidental de la variedad invasora en las costas mediterráneas europeas. Se realiza una completa identificación de esta alga invasora en el Sur de España mediante una descripción morfológica y filogenia molecular. Los datos obtenidos se discuten en relación a la composición de las comunidades receptoras, tales como fondos de maërl y bordes de *Posidonia oceanica* (Linnaeus) Delile, y su ecología. Los resultados confirman la direccionalidad de la ruta invasora de la especie hacia el oeste en las proximidades del Estrecho de Gibraltar, principalmente debido a corrientes marinas y vectores de origen antropogénico.

Palabras clave: análisis filogenético, *Caulerpa cylindracea*, maërl, Mar Mediterráneo, secuencia ITS.

INTRODUCTION

Populations of an invasive *Caulerpa* taxon have been systematically reported from nearly all Mediterranean countries since the early nineties. (Nizamuddin, 1991; Klein &Verlaque, 2008, Rivera-Ingraham & al., 2010), genetically related to populations of southwestern Australia (Verlaque & al., 2003). Formerly this taxon was identified as *C. racemosa* var. *occidentalis* (J. Agardh) Børgesen (Verlaque & al., 2000), to be renamed few years later as *C. racemosa* var. *cylindracea* (Sonder) Verlaque, Huisman & Boudouresque, i.e. the 'invasive variety' (Verlaque & al., 2003). Two other varieties of *C. racemosa* were accepted to co-occur with the invasive one in the Mediterranean Sea, *C. racemosa* var. *turbinata* (J. Agardh) Eubank and *C. racemosa* var. *lamourouxii* (Turner) Weber-van Bosse f. *requienii* (Montagne) Weber-van Bosse), both confined at the central-eastern Mediterranean Sea and considered as typical seaweed components of the Lessepsian flora, with a non-aggressive character (Verlaque & al., 2000). All these varieties were accepted on the basis of morphological identification and molecular phylogenetic analyses (Famà & al., 2000; Durand & al., 2002; Verlaque & al., 2000, 2003).

However, recent molecular studies of the *Caulerpa racemosa-peltata* complex have revealed the existence of at least eleven distinct species-level entities in the complex (Sauvage & al., 2013; Belton & al., 2014), together with a confirmed high phenotypic plasticity of certain lineages/species that morphologically partial overlap with some others (Belton & al., 2014). The latter revision of this complex drove to reinstate earlier-described species for subspecies, varieties and forms of *C. racemosa* and *C. peltata*, among them the invasive variety of *C. racemosa*, for which the earlier name *C. cylindracea* Sonder was reinstated (Belton & al., 2014).

Caulerpa cylindracea seems to show a westward directionality of expansion in the Mediterranean, since the first report of the species in Libya in 1990 (Nizamuddin, 1991; Klein & Verlaque, 2008; Rivera-Ingraham & al., 2010). For this fast expansion potential and the vast impact on local biota (e.g. on maërl beds, edges of *Posidonia oceanica*, photophilic algae communities), homogenizing the sea bottom independently of species richness of the recipient marine

* Corresponding author.

communities (revised by Klein & Verlaque, 2008), the taxon is considered to be one amongst the ten most invasive seaweed species in the Mediterranean Sea (Boudouresque &Verlaque, 2002; EEA, 2007).

Until recently, taxonomical confusion occurred among taxonomical entities of *Caulerpa* found in the Mediterranean sea, specially with those assigned to the *C. racemosa-peltata* complex, with isolates commonly identified as *C. racemosa sensu lato* (Rivera-Ingraham & al., 2010). This was due to similarities in gross morphology, conspicuous phenotypic plasticity or evidences from ecological performance and expansive behaviour of local populations. Nowadays, identification of these taxa can be achieved by means of fixed morphological features and genetic evidence (Famà & al., 2000; Durand & al., 2002; Verlaque & al., 2000, 2003; Nuber & al., 2007; Sauvage & al., 2013; Belton & al., 2014).

Both morphological and molecular analyses are necessary to discriminate among *Caulerpa* taxonomical units, especially to precisely identify the invasive one *C. cylindracea*. The large number of the nuclear rDNA ITS1-5.8S-ITS2 region sequences obtained from specimens collected worldwide can be used to distinguish among *Caulerpa* taxa (Famà & al., 2000; Durand & al., 2002; Verlaque & al., 2000, 2003; Nuber & al., 2007; Sauvage & al., 2013; Belton & al., 2014). Yet, to identify the "invasive taxon", a combination of morphological identikit and molecular data has been applied in solely 5 amongst the 13 Mediterranean countries in which *C. racemosa sensu lato* has been reported so far (France, Italy, Greece, Croatia and Cyprus) (reviewed in Klein & Verlaque, 2008), since the first report from Libya in 1990 (Nizamuddin, 1991) (see Fig. 1 for an updated distribution map).

In this study we make use of both morphological differences and molecular evidences to assess the identity and origins of *C. cylindracea*, collected for first time in Andalusia region (Southern Spain), corresponding up till now to the westernmost identified population of the European Mediterranean coasts. Our results are discussed following the course of the invasive process and the present distribution of the remaining *Caulerpa* species and varieties encountered in the central and eastern parts of the basin.

MATERIAL AND METHODS

Specimen's preliminary identification & collection

An introduced population of *Caulerpa cylindracea* was localized by SCUBA diving at −30 m of depth at Villaricos coast (Almería, SE Iberian Peninsula, 37°17′17″N 1°41′04″O, Fig. 1 ∗) in October 2009; several thalli were collected by M. Altamirano and J. de la Rosa from four areas within the population separated at least 5 m from one another. Selected samples were either preserved in 4% formalin in seawater for morphological identification or carefully cleaned from epiphytes and desiccated in silica gel for genetic analysis. Herbarium sheets were prepared and deposited in the Herbarium of the University of Málaga (MGCPhyc 5087).

Morphological identification

The following morphological characters from different parts of the thalli (Fig. 2) were analyzed: height, width and attachment to stolons of the fronds; height, diameter, shape and arrangement of the ramuli; diameter of the stolons; length, width, spacing and morphology of the rhizoids.

DNA extraction, PCR amplification, cloning & sequencing

Total DNA was extracted following procedure described in Andreakis & al. (2004); quantity and quality of DNA was determined with a ND-1000 spectrophotometer (Nanodrop,
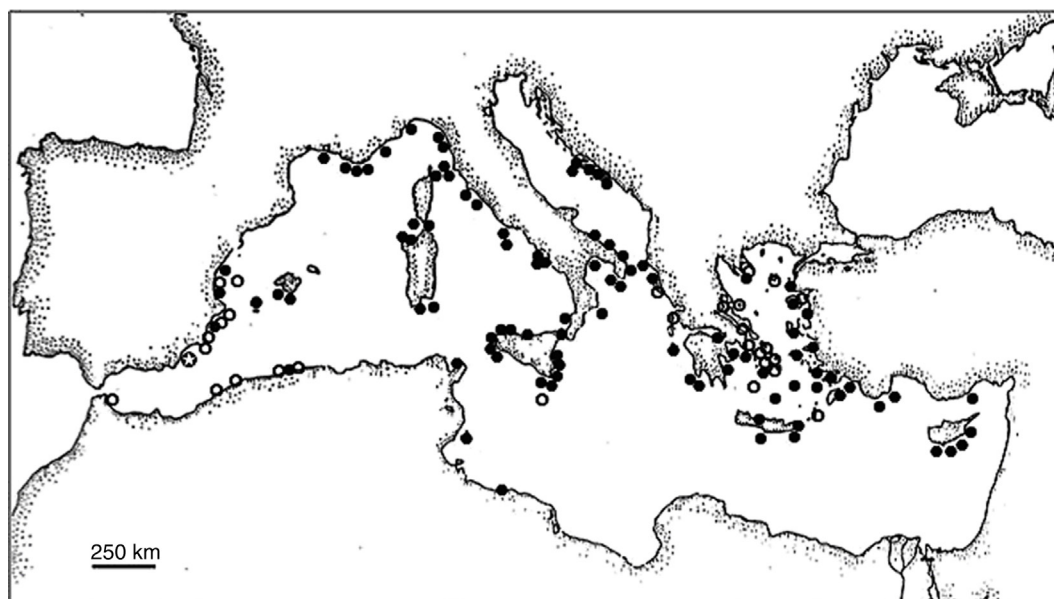

Fig. 1. Updated geographical distribution of *C. racemosa var. cylindracea* in the Mediterranean Sea. Black dots denote invaded locations cited in Klein & Verlaque, 2008. White dots indicate recently occupied geographical locations (Sciberras & Schembri, 2007, Ruíz & al., 2007; Klein & Verlaque, 2008; Guillén & al., 2010; Bouiadjra & al., 2010; Rivera-Ingraham & al., 2010; Tsiamis & al., 2010; Bentaallah & Kerfouf, 2013). Circled star indicates the new reported population in Almería (Andalusia, Southern Spain).
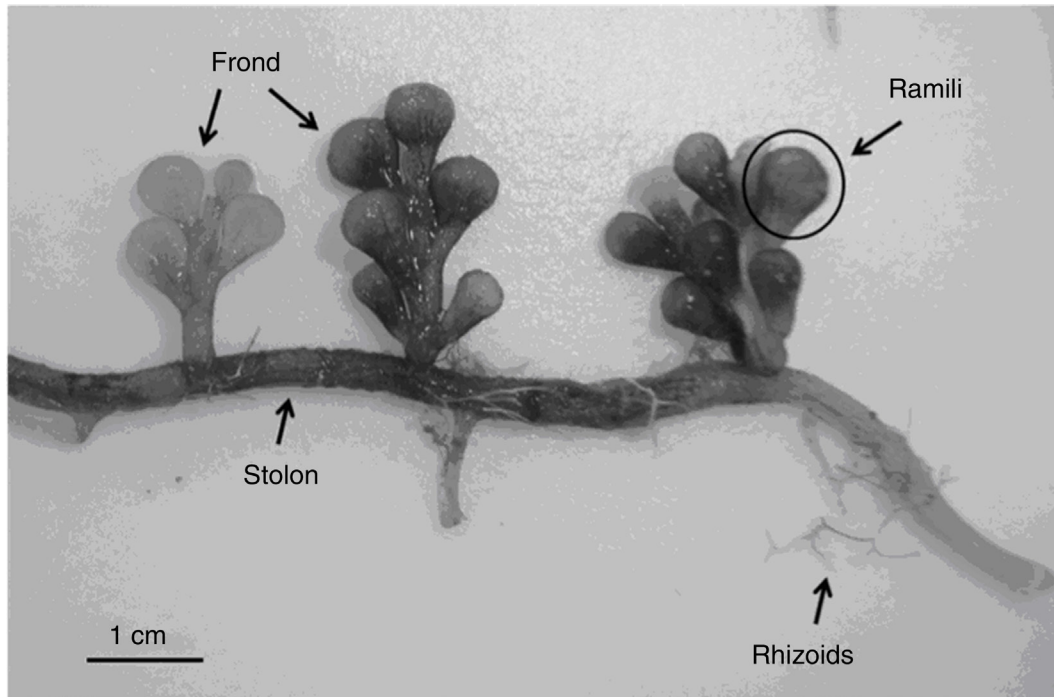
Fig. 2. Habit of *Caulerpa cylindracea* in Almería (Andalusia, Southern Spain) at −30 m depth in October 2009 (MGC Phyc 5087).

Wilmington, Detroit, USA). The ITS1-5.8S-ITS2 rDNA locus was PCR amplified using PCR primers and conditions reported in Durand & al. (2002). PCR amplifications were performed in a DNA thermal cycler 2720 (Applied Biosystems) in 50 µl PCR mix contained 10 ng of genomic DNA, 1 µl of 5% BSA (GE Healthcare, Buckinghamshire, UK), 5 µl of 10X Taq DNA polymerase buffer $MgCl_2$ – free, 5 µl of 25 mM $MgCl_2$, 1 µl of 10 mM dNTP mix (Roche, Mannheim, Germany), 5 µl of each primer (10 µM) and 2U of Taq DNA polymerase (Roche, Mannheim, Germany). PCR products were checked on a 0.8% UltraPure Agarose gel (Invitrogen, Carlsbard, California, USA), and purified with NucleoSpin Extract II kit (Macherey-Nagel, Düren, Germany). Purified PCR products were cloned into TOPO TA Cloning vector (Invitrogen, Paisley, UK) and 4 to 8 positive clones for each sample were sequenced to evaluate levels of intra-individual variation. Sequencing reactions were performed in both directions using universal primers M13F and M13R and a BigDye v3.1 Sequencing kit followed by capillary electrophoresis on a DNA analyzer 3730XL (Applied Biosystems).

Sequence alignment and phylogenetic analyses

Electropherograms were assembled in Sequencher 4.9 (Gene Codes). More than 100 nuclear rDNA-ITS sequences from *Caulerpa* species are available in GenBank. Amongst these, four sequences of *Caulerpa mexicana* Sonder ex Kützing (AJ007815-18; Olsen & al., 1998) were selected as outgroups in all phylogenetic reconstructions and were aligned against 27 ITS1-5.8S-ITS2 sequences produced in this study and 40 publically available sequences to create a final dataset of 71 sequences (see Table 1 for sequence accession numbers). Alignments were optimized in MUSCLE (http://www.ebi.ac.uk/Tools/msa/muscle/), trimmed in BIOEDIT version 7.0.9.0 (Hall, 1999) and refined by eye. Prior to phylogeny reconstruction, the best fitting model of evolution was estimated for the dataset as implemented in jModeltest 2 (Darriba & al., 2012). Model-constrained ML phylogenies were inferred in PAUP* 4.0b10 (Windows version; Swofford 2002) under heuristic search option, ten random sequence additions to find the highest likelihood tree and gaps treated as missing data. Bootstrap support for individual clades was calculated on 100 replicates using the same options and constraints as used in the tree-inferences. Bayesian phylogenies and posterior probabilities of individual clades were calculated using a variant of the Markov chain Monte Carlo algorithm implemented in MrBayes v3.1.2 (Huelsenbeck & Ronquist, 2001). The GTR substitution model was used in BI since the jModeltest output could not be implemented in MrBayes. In this case model parameters were treated as unknown variables with uniform default priors and were estimated as part of the analysis. Two runs of four Markov chains were run for 1,000,000 generations using random starting trees and the same model employed in branch length estimates, with trees saved every 100 generations. We assumed the two runs have reached the stationary distribution when the value for the standard deviation of split frequencies achieved values below 0.01. All trees prior to stationarity and convergence were discarded, and a consensus tree was calculated from the remaining trees. To assess the influence of the indels on the distance estimation, ML and BI computations were repeated in PAUP* and MrBayes with gaps coded using the simple method described by Simmons and Ochoterena (2000) implemented in FastGap v1.2 (Borchsenius, 2009). For the same reason, ML exploratory phylogenies were inferred in MEGA v6.06 under the GTR model and the

Table 1. Geographical location, accession numbers and references of *Caulerpa* isolates used in this study. For the publically available sequences employed in the phylogeny we used the nomenclature deposited with each of the sequence records in NCBI by the Authors listed in the last column

No	Taxon	Origin	Accesión no. and reference
1	*C. cylindracea*	Villaricos, Almería, Spain	Cr1 B1*
2	*C. cylindracea*	Villaricos, Almería, Spain	Cr1 E1*
3	*C. cylindracea*	Villaricos, Almería, Spain	Cr1 F1*
4	*C. cylindracea*	Villaricos, Almería, Spain	Cr1 H1*
5	*C. cylindracea*	Villaricos, Almería, Spain	Cr9 A3*
6	*C. cylindracea*	Villaricos, Almería, Spain	Cr9 B3*
7	*C. cylindracea*	Villaricos, Almería, Spain	Cr9 C3*
8	*C. cylindracea*	Villaricos, Almería, Spain	Cr9 F3*
9	*C. cylindracea*	Villaricos, Almería, Spain	Cr1 A1*
10	*C. cylindracea*	Villaricos, Almería, Spain	Cr1 C1*
11	*C. cylindracea*	Villaricos, Almería, Spain	Cr1 D1*
12	*C. cylindracea*	Villaricos, Almería, Spain	Cr25 G5*
13	*C. cylindracea*	Villaricos, Almería, Spain	Cr33 A7*
14	*C. cylindracea*	Villaricos, Almería, Spain	Cr1 G1*
15	*C. cylindracea*	Villaricos, Almería, Spain	Cr9 D3*
16	*C. cylindracea*	Villaricos, Almería, Spain	Cr9 G3*
17	*C. cylindracea*	Villaricos, Almería, Spain	Cr9 H3*
18	*C. cylindracea*	Villaricos, Almería, Spain	Cr25 A5*
19	*C. cylindracea*	Villaricos, Almería, Spain	Cr25 F5*
20	*C. cylindracea*	Villaricos, Almería, Spain	Cr25 H5*
21	*C. cylindracea*	Villaricos, Almería, Spain	Cr33 B7*
22	*C. cylindracea*	Villaricos, Almería, Spain	Cr33 C7*
23	*C. cylindracea*	Villaricos, Almería, Spain	Cr33 D7*
24	*C. cylindracea*	Villaricos, Almería, Spain	Cr33 E7*
25	*C. cylindracea*	Villaricos, Almería, Spain	Cr33 F7*
26	*C. cylindracea*	Villaricos, Almería, Spain	Cr33 G7*
27	*C. cylindracea*	Villaricos, Almería, Spain	Cr33 H7*
28	*C. racemosa* var. *cylindracea*	Samos, Greece	AJ297637 clone a, AJ297638 clone b (Durand & al., 2002)
29	*C. racemosa* var. *cylindracea*	Saronikos, Greece	AJ297639 clone a, AJ297640 clone b (Durand & al., 2002)
30	*C. racemosa* var. *cylindracea*	Famagusta, Cyprus	AJ297641 clone a, AJ297642 clone b, AJ297643 clone c (Durand & al., 2002)
31	*C. racemosa* var. *cylindracea*	Marseilles, France	AJ297644 clone a, AJ297645 clone b (Durand & al., 2002)
32	*C. racemosa* var. *cylindracea*	Carnac Island, Australia	AY173116 clone a, AY173117 clone b, AY173118 clone c (Verlaque & al., 2003)
33	*C. racemosa* var. *cylindracea*	Canary Islands, Spain	AY173119 clone a, AY173120 clone b (Verlaque & al., 2004)
34	*C. racemosa* var. *cylindracea*	Peljesac, Croatia	DQ778924 (Nuber & al., 2007)
35	*C. racemosa* var. *cylindracea*	Peljesac, Croatia	DQ778925 (Nuber & al., 2007)
36	*C. racemosa* var. *cylindracea*	Mljet, Croatia	DQ778926 clone a (Nuber & al., 2007)
37	*C. racemosa* var. *cylindracea*	Mljet, Croatia	DQ778935 clone b (Nuber & al., 2007)
38	*C. racemosa* var. *cylindracea*	Mljet, Croatia	DQ778936 (Nuber & al., 2007)
39	*C. racemosa* var. *cylindracea*	Mljet, Croatia	DQ778937 (Nuber & al., 2007)
40	*C. racemosa* var. *turbinata-uvifera*	Bizerte, Tunisia	AJ297632 (Durand & al., 2002)
41	*C. racemosa* var. *turbinata-uvifera*	Salakta, Tunisia	AJ297633 (Durand & al., 2002)
42	*C. racemosa* var. *turbinata-uvifera*	Prainha, Brazil	AY334305 (Verlaque & al., 2003)
43	*C. racemosa* var. *turbinata-uvifera*	Saint-Barthelemy, Caribbean Islands	AY334304 (Verlaque & al., 2003)
44	*C. racemosa* var. *lamourouxii* f. *requienii*	Famagusta, Cyprus	AJ297634 (Durand & al., 2002)
45	*C. racemosa* var. *lamourouxii* f. *requienii*	Selaata, Lebanon	AJ297635 (Durand & al., 2002)
46	*C. racemosa* var. *lamourouxii*	Ishigaki, Japan	AJ297636 (Durand & al., 2002)
47	*C. racemosa* var. *lamourouxii*	Tripoli, Lebanon	AY334303 (Verlaque & al., 2003)

Table 1. *(continued)*

No	Taxon	Origin	Accesión no. and reference
48	*C. racemosa* var. *clavifera*	Red Sea	AJ297646 (Durand & al., 2002)
49	*C. racemosa* var. *clavifera*	Mayotte Island, France	AJ297647 (Durand & al., 2002)
50	*C. racemosa* var. *peltata*	Ishigaki, Japan	AJ297648 (Durand & al., 2002)
51	*C. racemosa* var. *laetevirens*	Fengchueisha, Taiwan	AY205290 (Yeh & Chen, 2004)
52	*C. racemosa* var. *laetevirens*	Kenting, Taiwan	AY205291 (Yeh & Chen, 2004)
53	*C. racemosa* var. *macrophysa*	Green Island, Taiwan	AY206420 (Yeh & Chen, 2004)
54	*C. racemosa* var. *macrophysa*	Nanwan, Taiwan	AY206421 (Yeh & Chen, 2004)
55	*C. racemosa* var. *macrophysa*	Ishigaki, Japan	AJ297649 clone a, AJ297650 clone b (Durand & al., 2002)
56	*C. racemosa* var. *racemosa*	Heron Island, Australia	AJ297651 (Durand & al., 2002)
57	*C. racemosa* var. *racemosa*	Balicasag Island, Philippines	AJ297652 clone a, AJ297653 clone b (Durand & al., 2002)
58	*C. mexicana*	Canary Islands, Spain	AJ007816 (Olsen & al., 1998)
59	*C. mexicana*	Florida, USA	AJ007818 (Olsen & al., 1998)
60	*C. mexicana*	Israel	AJ007815 (Olsen & al., 1998)
61	*C. mexicana*	Panamá	AJ007817 (Olsen & al., 1998)

*New accession numbers.

"complete deletion" or "the use all sites" options for gaps/ missing data treatment activated (Tamura & al., 2011). To evaluate the partition of individual sequence polymorphisms, genealogical relationships among ITS1-5.8S-ITS2 copies within individuals were calculated using the Median Joining algorithm (ε=0, equally weighted characters) implemented in the software Network v4.5.1.6 (http://www. fluxus-technology.com). The method identifies groups of closely related sequences and uses "median vectors" to connect sequences into a tree or network. Median vectors can be interpreted biologically as extinct individuals or haplotypes that have not been sampled yet (Bandelt & al., 1999).

RESULTS

Morphological identification

Plants found in Villaricos, exhibited morphological features (Table 2) that fit the description given by several authors for the invasive species *C. cylindracea* from different populations in the Mediterranean Sea (Verlaque & al., 2000; Verlaque & al., 2003; Klein & Verlaque, 2008; Cebrián & Ballesteros, 2009; Guillén & al., 2010). Thalli exhibited creeping stolons up to 2.2 mm diameter where thin rhizoids and rhizoidal pillars outgrow for attachment to the substratum (Fig. 2). Erect fronds are up to 2 cm high, bearing uncrowded clavate ramuli radially and distichously distributed counted in low number (<8 per frond). Fronds were slightly inflated above the attachment to the stolon.

Phylogenetic reconstruction

The ITS1-5.8S-ITS2 alignment comprised 71 sequences (62 unique types) varying from 584 bp (DQ778935, var *cylindracea*, Croatia) to 1082 bp in length (AY205290, var *laetevirens*, Taiwan). Final alignments were characterized by length variation and consisted of 1200 characters of which

275 were parsimony informative. The best-fitting model of evolution given the data was the TVM+I+G (nucleotide frequencies: A=0.2426, C=0.2072, G=0.2584, T=0.2919; proportion of invariable sites I=0.329; gamma distribution with shape parameter α=0.52) as suggested by jModeltest 2 (Darriba & al., 2012). ML analysis constrained with optimal model and parameters resulted in a single ML tree (-lnL=1217.3337; Fig. 3); BI showed nearly identical topologies; in addition, the different gap treatments applied in the analyses revealed no significant influence of the indels in distance estimates (data not shown). Phylogenetic reconstructions reproduced five highly supported, distinct clades (as in Nuber & al., 2007). Sequences of *C. cylindracea* from

Table 2. Morphological characteristics of *Caulerpa cylindracea* from Almería (Andalusia, Southern Spain) at −30 m depth in October 2009

|	n	media±SD	range
Fronds			
Height (mm)	39	12.4±3.2	7-20
Width (mm)	39	6.7±0.9	5-8
Attachment to stolons		cylindrical	
Ramuli			
Height (mm)	52	4.3±0.6	6-3
Diameter (mm)	52	2.2±0.4	1.5-3.5
Shape		clavate	
Arrangement		uncrowded, radial distichously	
Stolons			
Diameter (mm)	20	1.7±0.2	1.4-2.2
Rhizoids			
Length (mm)	38	14.9±5.1	7-23
Width (mm)	25	1.1±0.3	0.8-1.9
Spacing (mm)	47	11.7±4.1	5-20
Morphology		rhizoidal pillars thin rhizoids	
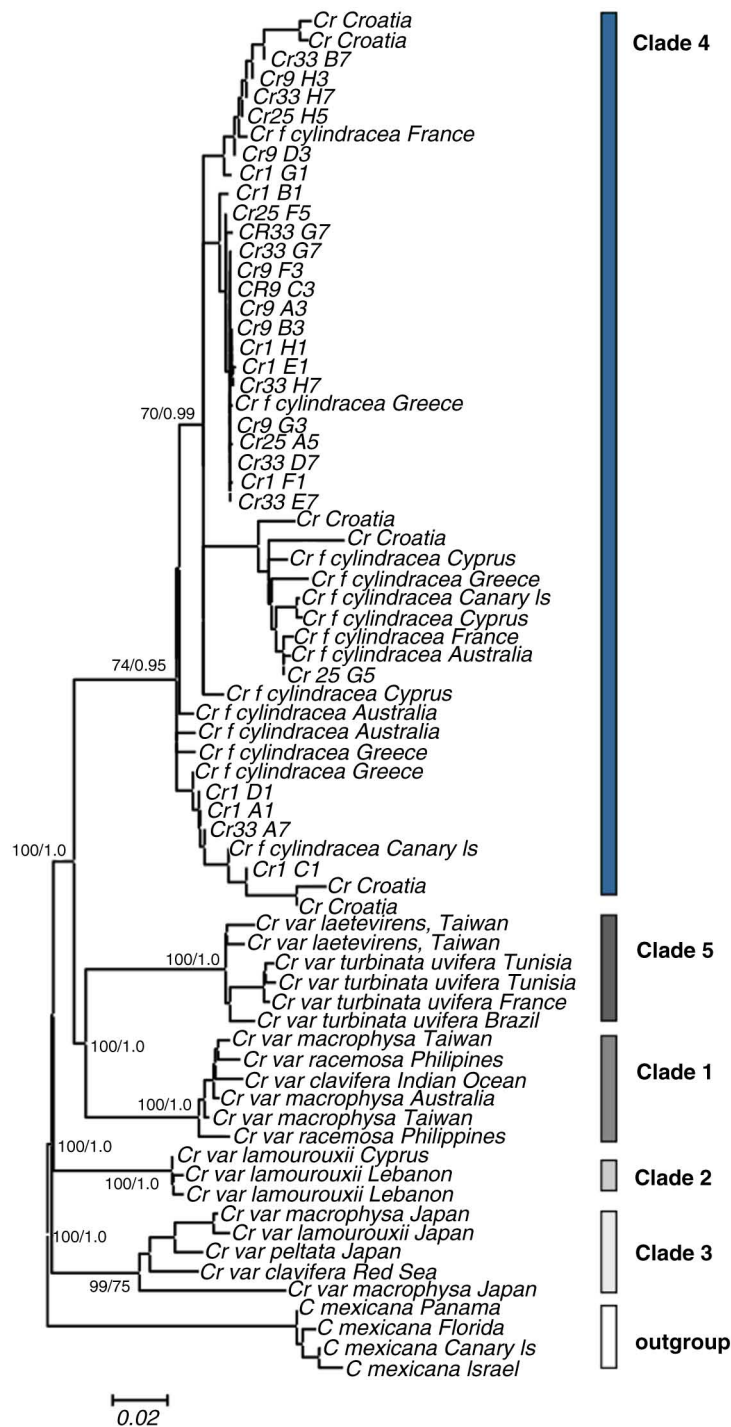
Fig. 3. Maximum likelihood phylogeny of *Caulerpa* taxa reconstructed from ITS1-5.8S-ITS2 sequences. *C. mexicana* Sonder ex Kützing is used as outgroup. Numbers on nodes indicate bootstrap support and Bayesian posteriors respectively.

Almeria clustered in clade 4 with all Mediterranean and Australian isolates. Clade 4 has been previously identified as the clade of *C. racemosa* var. *cylindracea* (Sonder) Verlaque, Huisman & Boudouresque (Verlaque & al., 2003), i.e. the 'invasive variety' (Fig. 3).

Genealogical relationships amongst ITS1-5.8S-ITS2 sequence copies from the 4 different specimens are depicted in Fig. 4. Among the 27 sequences analyzed (Table 1), the same haplotype was recovered from specimens CR9, CR25, CR33. Further, two more haplotypes were additionally shared by specimens CR9 and CR33. two and three . The remaining 15 copies were present only once and separated one to 37 substitution steps from one another. No clear patterns of variation were encountered (i.e. copies of the same individual clustering together) indicating high levels of intra-individual variation and intermingled genealogies.
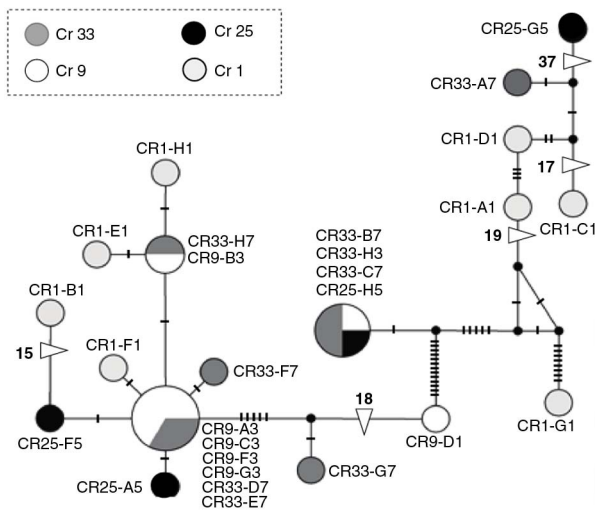
Fig. 4. Median-joining genealogical network reconstruction based on ITS1-5.8S-ITS2 copies cloned from individuals Cr33, Cr25, Cr9 and Cr1. Numbers on circles denote specimen names reported in Table 1; circles represent distinct ITS1-5.8S-ITS2 copies; circle size is proportional to the frequency of that copy; triangles indicate large substitutions denoted by the number; bars across lines connecting clone sequences indicate bp substitutions.

Recipient community characteristics

Caulerpa cylindracea was located at Villaricos (Southern Spain) at the circalittoral zone at −30 m depth forming extended (aprox. 100 m^2) tight but not dense webs on different types of communities. Seawater temperature was 24 °C at the time of collection. At this place, the recipient communities to the invasion of *C. cylindracea* were maërl beds formed by species as *Phymatolithon calcareum* (Pallas) Adey & McKibbin, *Lithothamnion coralloides* (P.L. Crouan & H.M. Crouan) P.L. Crouan & H.M. Crouan, *Lithophyllum racemus* (Lamarck) Foslie and *Tricleocarpa fragilis* (L.) Huisman & Townsend, *Posidonia oceanica* meadow at its edges, and photophilic community dominated at the time of collection by *Dictyopteris polypodioides* (A.P. De Candolle) J.V. Lamouroux. Gametophytes and tetrasporophytes of another invasive species, *Asparagopsis taxiformis* (Delile) Trevisan (Bonnemaisoniales, Rhodophyta), were also present in these communities.

DISCUSSION

Morphological identification coupled with molecular phylogenetic analysis provides neat evidence that isolates of *Caulerpa* collected from Almería (Andalusia, Southern Spain) correspond to the invasive species *C. cylindracea*. The isolates found in Almería represent the most westerly population of this taxon in the Mediterranean European coast and are genetically related to the group of genotypes encountered in the central and eastern Mediterranean Sea. Population was encountered in the same depth as the populations reported from the east Spanish coasts corresponding to circalittoral sites (Ruíz & al., 2007; Cebrián & Ballesteros, 2009; Guillén & al., 2010). Collected thalli exhibited similar morphological and ecological characteristics with short fronds and not dense arranged mats. This study represents

the first complete identification of *C. cylindracea* in the Alboran Sea (Western Mediterranean), confirming the previously suggested directionality of the invasive pathway to be towards west, to the Strait of Gibraltar.

At this moment the westernmost populations of *C. cylindracea* in the world are located in Canary Islands (Western Atlantic Ocean), where the species has been observed since the late 1990s, and whose origin was confirmed to be from Mediterranean populations (Verlaque & al., 2004). The nearest known population from Canary Islands is in Ceuta (North coast of Africa), where the species was observed for first time in 2007 (Rivera-Ingraham & al., 2010). For these western populations, long distance anthropogenic mediated transport should be suspected, as the nearest populations by the time of the first observations, were very far away. However, for the new reported population of *C. cylindracea* in Almería (Southern Spain), a short-distance transport from near populations in Murcia province (Ruíz & al., 2007) might be accepted, mainly due to shipping and diving activities at the zone. Up to now, no new populations have been reported in Southern Spain, but this could be due to the severe limit that depth over 30 m poses in locating new introductions.

Two amongst the three *Caulerpa* taxa encountered in the Mediterranean Sea (Verlaque, 2000, 2003), namely, *C. chemnitzia* (Esper) J.V. Lamouroux (formerly *C. racemosa* var. *turbinata* (J. Agardh) Eubank) and *C. racemosa* var. *lamourouxii* f. *requienii* occur only in the central and eastern part of the basin. In comparison, the third taxon, *C. cylindracea*, following initial observation in 1990 (Nizamuddin, 1991), expanded its distribution range in the last 20 years by sea currents and vectors of anthropogenic origin (Klein & Verlaque, 2008). Several eco-physiological characteristics may account for the distinctive distribution patterns encountered between the invasive and the non-invasive *Caulerpa* species in the Mediterranean Sea. For instance, it has been stated that the non invasive taxa do not occur in locations where winter isotherms drop off below 15 °C (Verlaque & al., 2000). The invasive species on the other hand, has been recorded even below 10°C during colder winters (Verlaque & al., 2000). Furthermore, differences in ecological behavior (i.e. bathymetric distribution, type of substratum and light adaptation) are remarkable between invasive and non-invasive taxa thus reflecting differences in colonization capabilities and survival rates of newly introduced propagules.

The invasive species has been reported from shallow waters down to 60 m depth, from sciaphilic to photophilic conditions and on both soft and hard substrata, being able of competing against a wider range of communities (Klein & Verlaque, 2008). Taken together, these features represent an advantage in challenging local communities when compared with the other two non-invasive forms. However, differences in minimum lethal temperature boundaries among varieties, may explain their current geographical distribution patterns in the Mediterranean Sea: *C. chemnitzia* and *C. racemosa* var. *lamourouxii* are confined to the central and eastern part of the basin; the invasive species occurs also in colder waters. This behavior is additionally suggested by the relative growth rates of the invasive taxon during the winter period, which remains positive down to 10 °C in laboratory conditions (Flagella & al., 2008). The present report and that for

Ceuta (Rivera-Ingraham & al., 2010) validate geographical distribution niche models, developed for this invasive species based on surface temperature distribution occurring in the Mediterranean and European coastlines (Verbruggen & al., 2009).

High intra-individual levels of sequence polymorphisms are typically encountered in all isolates belonging to the Mediterranean *C. cylindracea* and this is believed to be the result of incomplete sequence homogenization by concerted evolution, differences in ploidy levels and multinucleate coenocytic thalli, all responsible of promoting intra-individual polymorphisms in ITS1-5.8S-ITS2 copies (Famà & al., 2000; Kapraun, 2005). Given our data, we cannot clarify on the contribution of sexual reproduction *versus* clonal propagation to the extreme levels of ITS sequence variation observed at the intra-individual level. It is highly probable however that both, the introduction of positively selected genetic variants and the elevated population density encountered locally, contribute to the recent establishment and remarkable expansion of *C. cylindracea* population on the Mediterranean Spanish coast.

ACKNOWLEDGEMENTS

REFERENCES

Andreakis, N., Procaccini, G. & Kooistra, W.H.C.F. 2004. *Asparagopsis taxiformis* and *Asparagopsis armata* (Bonnemaisoniales, Rhodophyta): genetic and morphological identification of Mediterranean populations. *European Journal of Phycology* 39: 273-283. http://dx.doi.org/10.1080/0967026042000236436

Bandelt, H.J., Forster, P. & Röhl, A. 1999. Median-joining networks for inferring intraspecific phylogenies. *Molecular Biology and Evolution* 16: 37-48.

Belton G.S., Prud'homme van Reine W.F., Huisman J.M., Draisma S.G.A. & Gurgel C.F.D. 2014. Resolving phenotypic plasticity and species designation in the morphologically challenging *Caulerpa racemosa–peltata* complex (Chlorophyta, Caulerpaceae). Journal of Phycology. http://dx.doi.org/10.1111/jpy.12132

Bentaallah M. El A. & Kerfouf A. 2013. Prolifération de l'algue *Caulerpa racemosa* dans les écosystèmes littoraux de l'Algérie: état des lieux et des connaissances. *Physio-Géo - Géographie Physique et Environnement*, volume VII, supplément: 61-68.

Borchsenius F. 2009. FastGap 1.2. Department of Biosciences, Aarhus University, Denmark. Published online at http://www.aubot.dk/FastGap_home.htm

Boudouresque, C.F. & Verlaque, M. 2002. Biological pollution in the Mediterranean Sea: invasive *versus* introduced macrophytes. *Marine Pollution Bulletin* 44: 32-38.

Bouiadjra, B.B., Taleb, M.Z., Marouf, A., Benkada, M.Y. & Riadi, H. 2010. First record of the invasive alga *Caulerpa racemosa* (Caulerpales, Chlorophyta) in the Gulf of Arzew (western Algeria). *Aquatic Invasions* 5: 97-101.

Cebrián, E. & Ballesteros, E. 2009. Temporal and spatial variability in shallow- and deep-water populations of the invasive *Caulerpa racemosa* var. *cylindracea* in the Western Mediterranean. *Estuarine, Coastal and Shelf Science* 83: 469-474.

Darriba, D., Taboada, G.L., Doallo, R. & Posada, D. (2012) jModelTest 2: more models, new heuristics and parallel computing. Nature methods 9, 772-772. http://dx.doi.org/10.1038/nmeth.2109

Durand, C., Manuel, M., Boudouresque, C.F., Meinesz, A., Verlaque, M. & Le Parco, Y. 2002. Molecular data suggest a hybrid origin for the invasive *Caulerpa racemosa* (Caulerpales, Chlorophyta) in the Mediterranean Sea. *Journal of Evolutionary Biology* 15: 122-133.

European Environmental Agency. 2007. Halting the loss of biodiversity by 2010: proposal for a first set of indicators to monitor progress in Europe EEA. Technical report No 11/2007

Famà, P., Olsen, J.L., Stam, W.T. & Procaccini, G. 2000. High levels of intra- and inter-individual polymorphism in the rDNA ITS1 of *Caulerpa racemosa* (Chlorophyta). *European Journal of Phycology* 35: 349-356.

Flagella, M.M., Lorenti, M. & Buia, M.C. 2008. Temperature response in a shallow-water Mediterranean population of *Caulerpa racemosa* var. *cylindracea* (Caulerpales, Chlorophyta), and a possible strategy of season anticipation. *Botanica Marina* 51: 278-284.

Guillén, J., Jiménez, S., Martinez, J., Triviño, A., Múgica, Y., Argilés, J. & Bueno, M. 2010. Expansion of the invasive algae *Caulerpa racemosa* var. *cylindracea* (Sonder) Verlaque, Huisman & Boudouresque, 2003 on the region of Valencia seabed. *Thalassas* 26: 135-149.

Hall, T. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* 41: 95-98.

Huelsenbeck, J.P. & Ronquist, F. 2001. MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics* 17: 754-755.

Kapraun, D.F. 2005. Nuclear DNA content estimates in multicellular green, red and brown algae: phylogenetic considerations. *Annals of Botany* 95: 7-44.

Klein, J. & Verlaque, M. 2008. The *Caulerpa racemosa* invasion: A critical review. *Marine Pollution Bulletin* 56: 205-225.

Nizamuddin, M. 1991. *The Green Marine Algae of Libya*. Elga Publishers, Bern, Switzerland.

Nuber, N., Gornik, O., Lauc, G., Bauer, N., Zuljevic, A., Papes, D. & Zoldos, V. 2007. Genetic evidence for the identity of *Caulerpa racemosa* (Forsskål) J. Agardh (Caulerpales, Chlorophyta) in the Adriatic Sea. *European Journal of Phycology* 42: 113-120.

Olsen, J.L., Valero, M., Meusnier, I., Boele-Bos, S. & Stam, W.T. 1998. Mediterranean *Caulerpa taxifolia* and *C. mexicana* (Chlorophyta) are not conspecific. *Journal of Phycology* 34: 850-856.

Rivera-Ingraham, G.A., García-Gómez, J.C. & Espinosa, F. 2010. Presence of *Caulerpa racemosa* (Forsskal) J. Agardh in Ceuta (Northern Africa, Gibraltar Area). *Biological Invasions* 12: 1465-1466.

Ruíz, J.M., Ramos, A. & García, R. 2007. *Informe sobre la presencia del alga tropical invasora* Caulerpa racemosa *en el litoral murciano en 2006*. Instituto Español de Oceanografía, Biblioteca del Centro Oceanográfico de Murcia, Spain.

Sauvage T., Payri C., Draisma S.G.A., Prud'homme van Reine W.F., Verbruggen H., Belton G.S., Gurgel C.F.D., Gabriel D., Sherwood A.R. & Fredericq S. 2013. Molecular diversity of the *Caulerpa racemosa-peltata* complex (Caulerpaceae, Caulerpales) in New Caledonia, with new Australasian records for the variety *cylindracea*. Phycologia 52: 6-13.

Sciberras M. & Schembri P.J. 2007. A critical review of records of alien marine species from the Maltese Islands and surrounding waters (Central Mediterranean). *Mediterranean Marine Science*, 8(1): 41-66. http://dx.doi.org/10.12681/mms.162

Simmons M.P. & Ochoterena, H. 2000. Gaps as characters in sequence-based phylogenetic analyses. Systematic Biology 49, 369-381.

Swofford, D.L. 2002. *PAUP. Phylogenetic Analysis Using Parsimony (*and Other Methods)*. Version 4. Sinauer Associates, Sunderland, Massachusetts.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. & Kumar, S. (2011) MEGA5: Molecular Evolutionary Genetics Analysis Using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. Molecular Biology and Evolution 28, 2731-2739. http://dx.doi.org/10.1093/molbev/msr121

Tsiamis K., Montesanto B., Panayotidis P., Katsaros C. & Verlaque M., 2010. Updated records and range expansion of alien marine macrophytes in Greece (2009). *Mediterranean Marine Science*, 11: 61-79.

Verbruggen, H., Pauly, K., Tyberghein, L., Mineur, F. & Maggs, C. (2009). Niche models of invasive seaweed species: predicting spread and areas at risk of suffering blooms, In: Mees, J. & al. (ed.) (2009). *VLIZ Young Scientists' Day, Brugge, Belgium 6 March 2009: book of abstracts. VLIZ Special Publication,* 41: pp. 18.

Verlaque, M., Boudouresque, C.F., Meinesz, A. & Gravez, V. 2000. The *Caulerpa racemosa* complex (Caulerpales, Ulvophyceae) in the Mediterranean Sea. *Botanica Marina* 43: 49-68.

Verlaque, M., Durand, C., Huisman, J.M., Boudouresque, C.F. & Le Parco, Y. 2003. On the identity and origin of the Mediterranean invasive *Caulerpa racemosa* (Caulerpales, Chlorophyta). *European Journal of Phycology* 38: 325-339.

Verlaque, M., Alfonso-Carrillo, J., Gil-Rodríguez, C., Durand, C., Boudouresque, C.F. & Le Parco, Y. 2004. Blitzkrieg in a marine invasion: *Caulerpa racemosa* var. *cylindracea* (Bryopsidales, Chlorophyta) reaches the Canary Islands (north-east Atlantic). *Biological Invasions* 6: 269-281.

Yeh, W. & Chen, G.Y. 2004. Nuclear rDNA and internal transcriber spacer sequences clarify *Caulerpa racemosa* vars. from other *Caulerpa*. *Aquatic Botany* 80: 193-207.