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ORIGINAL PAPER

Genetic diversity and biogeographical patterns of *Caulerpa prolifera* across the Mediterranean and Mediterranean/Atlantic transition zone

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Abstract Knowledge of spatial patterns of genetic differentiation between populations is key to understanding processes in evolutionary history of biological species. *Caulerpa* is a genus of marine green algae, which has attracted much public attention, mainly because of the impacts of invasive species in the Mediterranean. However, very little is known about the ecological and evolutionary history of the Mediterranean native *Caulerpa prolifera*, a species which is currently found at sites distributed worldwide. *C. prolifera* provides a good model to explore the patterns of genetic diversity at different scales across the Mediterranean and Atlantic area. This study aims to investigate the biogeographical patterns of diversity and differentiation of *C. prolifera* in the Mediterranean, with special focus on the Mediterranean/Atlantic transition zone. We used two nuclear (ITS rDNA and the hypervariable microsatellite locus CaPr_J2) and one chloroplast (*tuf*A) DNA markers on samples of *C. prolifera* from its entire range. Analyses of 51 sequences of the cpDNA *tuf*A of *C. prolifera,* 87 ITS2 sequences and genotypes of 788 ramets of *C. prolifera* for the locus CaPr_J2 revealed three different biogeographical areas: West Atlantic, East Atlantic and a larger area representing the Mediterranean, the Mediterranean/Atlantic transition zone and a Pacific site (Bali). It was found out that the Mediterranean/Atlantic transition zone is a biogeographical boundary for *C. prolifera*. A lack of connectivity was revealed between Atlantic and Mediterranean types, and identical sequences found in the Mediterranean and Indo-Pacific suggest either recent gene flow along the Red Sea connection or a possible ancient Indo-Pacific origin.

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Background

Spatial patterns of population genetic differentiation reflect the evolutionary history of biological species. The evolutionary processes that lead to population differentiation are determined by a variety of factors including species reproductive systems, population history, geographical distance and past geological/climatic events. One approach to identifying marine biogeographical discontinuities, and evaluating their importance as barriers to gene flow, is to determine population divergence for species with ranges expanding across candidate regions (Alberto et al. 2008). By combining geographical and evolutionary relationships, such phylogeographical studies have been powerful contributions to understanding population structure and evolution (Petit et al. 2005) and inferring the demographic and

historical processes that shaped evolution between populations within a species (Schaal et al. 1998; Avise 2000).

Caulerpa is a genus of marine green algae which has attracted much attention in the last two decades, mainly because two tropical *Caulerpa* species, *C. taxifolia* (M. Vahl) C. Agardh and *C. racemosa* (Forsskål) J. Agardh, have quickly spread into the Mediterranean, where they co-occur with indigenous *C prolifera* (Forsskål) J.V. Lamouroux, a species which is distributed worldwide. *C. prolifera* provides a good model to explore the patterns of genetic diversity at different scales across the Mediterranean and Atlantic area and to study the evolutionary history of Mediterranean marine species. However, while much research has focused on the Mediterranean invasive *Caulerpa* species (e.g., Durand et al. 2002; Meusnier et al. 2002; Varela-Álvarez et al. 2012; Zuljevic et al. 2012; Jongma et al. 2013), very little is known about the reproductive biology, dispersal history and biogeography of the native *C. prolifera*. The timing of appearance of this alga in the Mediterranean and its colonization history are unknown. There is also no information for the Atlantic area or any other distributional regions, neither it is known whether there is gene flow among *C. prolifera* from the Mediterranean and Atlantic.

Biogeographical studies using genetic markers on Mediterranean *Caulerpa* species have focused on invasive processes and revealed colonization histories with distinct sources of introduction of the invasive strains. Nuclear (ITS) and chloroplast (16S intron 2) sequences in *C. taxifolia* (Meusnier et al. 2002) have revealed it to be a complex of genetically and ecologically differentiated sibling genetic entities and confirmed the Australian origin of *C. taxifolia* in the Mediterranean. For *C. racemosa*, nuclear (ITS and 18S intron) loci differentiated distinct *C. racemosa* varieties in the Mediterranean into distinct taxonomic units and suggested that the invasive variety could be a recent hybrid from two different strains (Durand et al. 2002). Other molecular studies in *Caulerpa* species using allozymes (Benzie et al. 1997), chloroplast DNA RFLP (e.g., Lehman and Manhart 1997) and nuclear rDNA or chloroplast sequences (e.g., Jousson et al. 1998, 2000; Famà et al. 2000, 2002; Sauvage et al. 2013; Belton et al. 2013) have shown high intra-specific or even intra-individual differences in chloroplast DNA size and nuclear rDNA polymorphism. One hypervariable microsatellite marker (dinucleotide locus CaPr_J2) revealed high polymorphism in *C. prolifera* worldwide (Varela-Álvarez et al. 2011). Despite the availability of these markers, no studies have yet addressed the population genetic diversity of *C. prolifera* in the Mediterranean or other areas.

The Atlantic–Mediterranean transition is a controversial biogeographical boundary regarding gene flow magnitude and direction between Atlantic and Mediterranean biota. The opening of the Strait of Gibraltar at the end of the Messinian salinity crisis (about 6 million years ago) broke the land barrier between the Mediterranean and Atlantic water masses, allowing free migration across this area (Patarnello et al. 2007). However, genetic patterns for some marine species reveal a limitation to gene flow in this area preventing population admixture, suggesting that the Strait of Gibraltar represents a phylogeographical discontinuity. Strong differentiation between Atlantic and Mediterranean populations has been shown for many marine taxa (e.g., Duran et al. 2004; Baus et al. 2005; Alberto et al. 2008; Lowe et al. 2012). Two factors have been proposed to account for the maintenance of this biogeographical barrier in the area: The one-way surface current of Atlantic water flowing through the Strait of Gibraltar into the Mediterranean (Parilla and Kinder 1992; Bryden et al. 1994); the presence of a front forming a well-defined hydrogeographical boundary of surface waters between Almeria in southeast Spain and Oran in Algeria, the so-called Almeria–Oran oceanographic front (AOF) (Tintore et al. 1988). Analyses of population genetics of *Cymodocea nodosa* (Ucria) Ascherson, a marine angiosperm, revealed that the Almeria–Oran front rather than the Gibraltar Strait is the biogeographical limit between genetically distinct populations, (Alberto et al. 2008), and that any rare gene flow proceeded east to west along this region (Masucci et al. 2012) despite the leading water flow in the opposite direction, entering the Mediterranean. Although the green alga *C. prolifera* has a similar distribution to *C. nodosa* in both biogeographical areas across this transition zone, it extends much further into other warm regions, including the western Atlantic coasts, raising the question of a Mediterranean or Atlantic origin for the across the Mediterranean/Atlantic boundary.

Hypothesis and aims

The main objective of this work is to examine whether the Mediterranean/Atlantic transition zone currently forms a biogeographical separation between genetically distinct populations of *C. prolifera*, considered to be native in the Mediterranean. This objective is addressed using DNA evidence from two nuclear and one chloroplast loci. We also aimed to elucidate the direction of colonization across this boundary region, from a source in the Atlantic or in the Mediterranean. For this purpose, we used samples collected at the transition zone between the two seas but also we compared them with samples from other biogeographical areas, including East and Central Mediterranean, West Atlantic and Pacific. We aim to relate the genetic relationships with the geological history of the Mediterranean region in order to understand the evolutionary origin of Mediterranean *C. prolifera*, the *Caulerpa* native species in the Mediterranean, considered non-invasive.

Materials and methods

Model species

Caulerpa prolifera is a green coenocytic (multinucleate), alga that presents a single-generation life history in the Mediterranean, with individuals mainly in a diplophasic phase which is tetraploid, and *C. prolifera* also presents endopolyploidy up to 32Cx (Varela-Álvarez et al. 2012).

Sampling and DNA extraction

Samples of *C. prolifera* were obtained at 25 localities from the Mediterranean (Mainland Spain, Balearic Islands—Spain, France, Italy, Cyprus), Atlantic (Canary Islands—Spain, Portugal, Florida—USA) and Indo-Pacific coasts (provided by an aquarium supplier, Templo Aquatico in Lisbon). Sequences were retrieved from GenBank for *C. prolifera* from five additional locations: Martinique, Bali, Florida, Tunisia and Israel (Table 1; Figs. 1, 2) with the exception of one (DQ652388) containing ambiguities. For each locality, one or two individuals were sequenced (Table 1, see below specific loci). For detailed analysis of gene flow across the transition zone between the Atlantic and the Mediterranean, 13 locations were visited and between 29 and 78 samples were collected per meadow, reaching a total of 788 ramets sampled and analyzed (Table 2; map in Fig. 4).

After collection, samples were preserved in silica gel or dried at room temperature. DNA was extracted with the CTAB method (Doyle and Doyle 1990).

PCR amplification, sequencing and genotyping

One chloroplast and two nuclear markers were used in the samples collected. DNA targets for each of these two regions were difficult to amplify, so we used several primer pairs for each locus. The chloroplast locus is a partial region of cpDNA *tuf*A gene that was amplified by PCR following Famà et al. (2002) and also with two new specific sets of primers designed based on the *Caulerpa* sequences from GenBank. For ITS2, the more variable region, 170 bp, was amplified using primers from Meusnier et al. (2002): CaITS02-forward and CaITS01-reverse or using the universal primers ITS1 and ITS4 from White et al. (1990). (Primers are provided in Table S1 in supplementary material). For ITS2 amplicons, cloning was carried out using the pGEM T-Easy Vector system II and JM109 competent cells (Promega, Madison, USA). For each PCR product, up to eight clones were sent for sequencing to the CCMAR sequencing unit or to MACROGEN (Seoul, Korea).

For microsatellite amplification, we used conditions set in Varela-Álvarez et al. (2011). Because the objective of this study was not about intra-population genetic structure, but rather to study a biogeographical scale, we used only the microsatellite marker CaPr_J2, which has a high amplification rate (over 90 % for all the samples). The other markers amplified presenting null alleles and ambiguities in readings; therefore, they were not useful across these samples. Amplified fragments were separated using an ABI PRISM 3130xl (Applied Biosystems) automated capillary sequencer.

Sequence alignments and phylogenetic analyses

The resulting sequences (Table 1) were edited to eliminate ambiguities and aligned with Codon Code Aligner V.3.7.1 and MEGA 5.05 (Tamura et al. 2011). The final data set contained 52 sequences, of which 48 had a common region of 306 bp for *tuf*A (the shorter sequences of 240 bp (KF383329) was not included in any analyses or comparison), and 88 sequences of a common region of 151 bp for ITS2, including for both the outgroups.

The distribution of *tuf*A haplotypes and ITS profiles per location were plotted to visualize their geographical pattern (Figs. 1, 2). Phylogenetic relationships among them were performed: 1) statistical parsimony analyses on the *tuf*A and ITS2 cloned sequences and networks for each region with TCS 1.21 (Clement et al. 2000), with calculated maximum connection steps at 95 % level. For ITS2, networks were compared using indels in the alignment treated both as events and as missing data with the objective to find a phylogenetic pattern (Fig. 3). Maximum likelihood (ML) phylograms were constructed with the PhyML software (Guindon and Gascuel 2003) through the PHYLO-WIN graphical interphase (Galtier et al. 1996). Evolutionary models were selected using MODELTEST (Posada and Crandall 1998): HKY for *tuf*A and K80: Kimura 2-parameter for ITS2. For these last analyses, all indels were not considered; thus, the used ITS alignment was reduced to 132 positions. Bootstrap proportion values (50 %) were calculated for 1,000 bootstrap replicates. *C. taxifolia* was used as outgroup (accession numbers: DQ652360 and DQ652295).

Typing assay at CaPr_J2 locus

For the dinucleotide locus CaPr_J2, alleles were scored in GeneMapper v.4.1 using the 500 ROXTM size standard (Applied Biosystems). Binning and allele rounding was also checked with TANDEM (Matschiner and Salzburger 2009). Genetic diversity was estimated using GENODIVE, which is suitable for polyploid data (Meirmans and Van Tienderen 2004) and calculates various different diversity and differentiation statistics, based into Nei's original work (Nei 1987). Summary statistics

Table 1 Sample localities, *tufA* haplotypes and ITS2 profiles and sequences codes used in this study: Area, Localities, Source, *N*: Number of individuals per sampling site, *tufA* Haplotypes vs. sequences accession numbers; ITS profiles vs. sequences accession numbers. Because some of the ITS2 rDNA sequences produced here are 161 bp (65 sequences), the shorter sequences (and also haplotypes and profiles found) are available as a mode of supplementary material. (Genbank does not archive sequences of less than 200 bp)

Area	Localities	Source	*N*	*Tuf*A haplotypes versus accession numbers	ITS2 profiles versus accession numbers and ITS2 sequences
Atlantic	Florida, USA	Stam et al. (2006)	21	**Hpl 1:** DQ652380, DQ652381, DQ652372, DQ652373, DQ652374, DQ652375, DQ652376, DQ652377, DQ652378, DQ652379, DQ652382, DQ652383, DQ652384, DQ652385, DQ652386, DQ652387, DQ652388, DQ652389, DQ652390, DQ652391, DQ652392	**M:** DQ652245, DQ652246, DQ652248 **D:** DQ652249 **C:** DQ652250 **E:** DQ652247
	Florida, USA	Famà et al. (2002)	1	**Hpl 1:** AJ417943	–
	Florida, USA	This study	2	**Hpl 1:** KF383331, KF383332	**A:** Seq 19, Seq 22, Seq 24 **B:** Seq 20 **F:** Seq 23 **G:** Seq 21
	Gando, Gran Canaria	This study	1	**Hpl 2:** KF383341	**N:** Seq 6, Seq 7
	Arinaga, Gran Canaria	This study	1	**Hpl 2:** KF383342	–
	El Poris, Tenerife	This study	1	**Hpl 2:** KF383340	**R:** Seq 1 **N:** Seq 2, Seq 3, Seq 4, Seq 5
	Martinique	Jousson et al. (1998)	1	–	**Z:** AJ228989 **H:** AJ228988
Transition zone	Fuseta, Algarve, Portugal	Cunha et al. (2013) and this study	1	**Hpl 3:** JX206458, JX206459, JX206460, JX206461, JX206462, JX206463, JX206464, JX206465	**J:** KJ944307 **K:** KJ944305 **M:** KJ944303, KJ944304, KJ944306, KJ944308, KJ944309
	Cadiz, Spain	This study		**Hpl 3:** KF383343	**I:** Seq 54 **T:** Seq 52 **L:** Seq 53, Seq 55, Seq 56, Seq 57, Seq 58
Mediterranean area	Alcaner, Spain	This study		**Hpl 3:** KF383344	**L:** Seq 45, Seq 46, Seq 47, Seq 48, Seq 49, Seq 50, Seq 51
	Campello, Spain	This study		**Hpl 3:** KF383345	**T:** Seq 59, Seq 60, Seq 61, Seq 62, Seq 63, Seq 64, Seq 65
	Formentera, Balearic Island	This study		**Hpl 3:** KF383333, KF383336	**L:** Seq 13, Seq 14, Seq 15, Seq 16, Seq 17, Seq 18
	St. Antonio, Ibiza, Balearic Island	This study		**Hpl 3:** KF383339	**L:** Seq 31, Seq 32, Seq 33, Seq 35, Seq 36, **S:** Seq 34
	CDOR, Mallorca, Balearic Island	This study		**Hpl 3:** KF383327, KF383328, KF383329, KF383330	**O:** KJ944301, KJ944302
	Addaia, Menorca, Balearic Island	This study		**Hpl 3:** KF383334	–
	Golfe Juan, France	Meusnier et al. (2001)		–	**Q:** AF259570 **Q:** AF259578

Table 1 continued

Area	Localities	Source	*N*	*Tuf*A haplotypes versus accession numbers	ITS2 profiles versus accession numbers and ITS2 sequences
	Aquarium Nausicaa, France	Meusnier et al. (2001)		–	**O:** AF259571
	Ischia, Italy			**Hpl 3:** FM956042	–
	Marzamemi, Sicily, Italy	This study	1	**Hpl 3:** KF383337	**T:** Seq 25, Seq 26, Seq 27
	Porto Palo, Sicily, Italy	This study	1	**Hpl 3:** KF383338	**L:** Seq 28 **T:** Seq 29, Seq 30
	Amathus, Cyprus	This study	1	**Hpl 3:** KF383335	**N:** Seq 8 **R:** Seq 9
	Paphos, Cyprus	This study	1		**L:** Seq 10, Seq 11, Seq 12
	Israel		1	**Hpl 3:** GU815499	**M:** JN662400
	Djerba, Tunisia	Meusnier et al. (2001)	2	–	**O:** AF460995
	Bali, Indonesia	Famà et al. (2002)	1	**Hpl 3:** AJ417942	–
	Indo-pacific (Aquarium)	This study	1	–	**U:** Seq 44 **T:** Seq 37, Seq 38, Seq 39, Seq 40, Seq 41, Seq 42, Seq 43

of genetic diversity for CaPr_J2 are given in Table 2. We analyzed the full data set, but we also performed a standardization using multiple random reduction [in excel: =INT(RAND()*N)] in each population to reach a common sample size of 29 individuals, the minimum sample size collected in a population.

Results

Analyses with the chloroplast DNA sequences

The 51 sequences analyzed of the cpDNA *tuf*A of *C. prolifera* from 44 samples from 19 localities (33 retrieved from GenBank, 18 produced in this study, Table 1) yielded three haplotypes (Fig. 1). These correspond to three different biogeographical areas: West Atlantic, East Atlantic and a larger area representing the Mediterranean including its nearest Atlantic sites at the Mediterranean/Atlantic transition zone, together with the only Pacific sequence, from Bali (Famà et al. 2002). The ancestral haplotype was the West Atlantic one (haplotype 1) with the two derived haplotypes equally distanced by one single mutation each. We also analyzed longer sequences, for regions where these were available, to look for more haplotypes. In Florida, regardless of the procedure of each sequence (sample from the field or aquarium supplier in the same area, available from Stam et al. 2006), in 648 aligned positions in 20 sequences from Florida, we found two haplotypes. The Mediterranean/Pacific sequences did not show any nucleotide difference in our alignment of 744 positions (five isolates).

Analyses with nuclear rDNA ITS2 region

Samples from 37 individuals from 18 worldwide localities (Fig. 2; Table 1) resulted in 87 ITS2 sequences that yielded an alignment of 151 common positions. These represented 21 distinct sequences (hereafter called profiles for clarity, to make it clear when we refer to chloroplast sequences = haplotypes, or nuclear sequences = profiles, the latter potentially recombinant). We found eight profiles in the Mediterranean Sea and 16 in the Atlantic, with only four shared profiles found mainly in the East Atlantic (with exception of profile M, Fig. 2). The profiles found in the Canary Islands (East Atlantic) were also found in the Mediterranean, but several of these were only shared with the more distant eastern Mediterranean region. The profiles found in the Atlantic sites located near the transition zone with the Mediterranean were mostly Mediterranean for Cadiz, but all Atlantic for the Algarve (Fuseta). Of the two profiles found in the Pacific, one (profile T) was dominant in two locations in the Mediterranean (Sicily and Alicante). Private profiles were common in the West Atlantic,

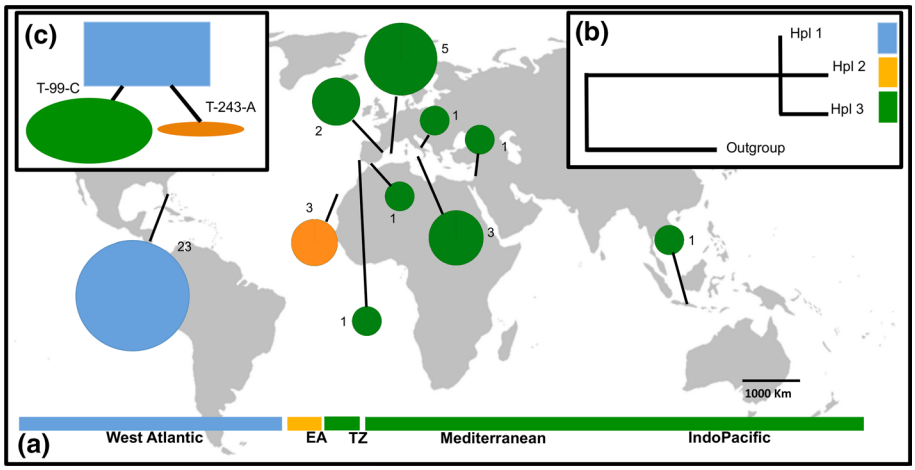

Fig. 1 Geographical distribution of *C. prolifera* chloroplast DNA sequences. Geographical ranges and phylogenetic relationships between *C. prolifera* isolates based on cpDNA *tuf*A sequences. *a* *Colored dots* on the map represent haplotypes at sampling localities (see Table 1 for detailed locality information). The size of the pie charts is proportional to the number of individuals sequenced (*number in brackets*). *b* Maximum likelihood phylogram based on the three haplotypes obtained in 48 sequences of 306 nucleotides of cpDNA. *c* Minimum-spanning network of cpDNA haplotypes using molecular variance parsimony, where the *square* represents the ancestral haplotype. *Blue* haplotype (West Atlantic) includes 23 sequences, *Green* haplotype has also 22 sequences (Mediterranean and Indo-Pacific), and *Orange* haplotype (East Atlantic) includes three distinct sequences. *Branches* represent a single nucleotide change (marked along the branch)

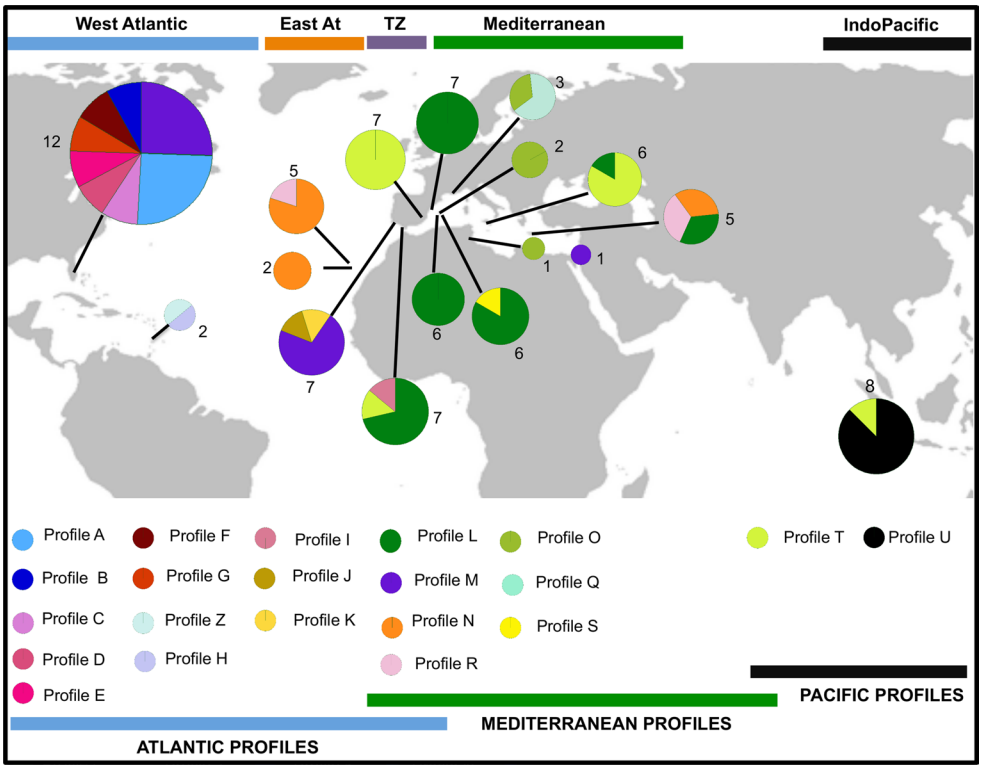

Fig. 2 *Caulerpa prolifera* rDNA ITS2 profile distribution pie charts along the full biogeographical range. The size of the pie charts is proportional to the number of sequences available (*number in brackets*), one or two individuals sampled in each location. The *colors* in the pie charts represent one of the 21 profiles found. The *color key* for the haplotypes found is below the map. *Profiles A–N* found in the Atlantic, *Profiles L–S* found in the Mediterranean and *Profiles T–U* found in the Indo-Pacific area. Shared profiles among areas: Mediterranean/Atlantic: *L–N*; Mediterranean/Pacific: *T*

with nine unique to the Florida region and two to the Caribbean. In contrast, only two private profiles were found in the Mediterranean. The Mediterranean is dominated by profile L, and it is also present in one location in the East Atlantic (Cadiz). In the samples/populations where only one profile has been found, we reanalyzed the data set with longer ITS sequences and there was still no variation: for example, for, Alcaner 161 bp—8 sequences, Formentera 161 bp—6 sequences, Alicante 161 bp—7 sequences, Cala dor 520 bp—2 sequences, Gando 161 bp—2 sequences.

Either phylogenetic analysis based on ITS2 regions or haplotypes network was highly unresolved and showed a

Table 2 Genetic Diversity for CaPr_J2 locus in populations of *C. prolifera* in the Mediterranean/Atlantic transition zone. CADI: Cadiz, Spain; ELPO: El Poris, Tenerife, Canaries, GAND: Gando, Gran Canaria, Canaries, FLORI: Florida Keys, USA; TABA: Tabarca, Spain; LOPA: Lo Pagan, Murcia, Spain; LESC: Les Cases de Alcaner, Cataluña, Spain; LAMA: La Mata, Alicante, Spain, FORM: Estany des Peix, Formentera, Balearic Islands; ADDA: Addaia, Menorca, Balearic Islands; AMER: Amerador, Alicante, Spain; CDOR: Cala Dor, Balearic Island, Spain; IBIZ: Ibiza, Balearic Island, Spain. GPS COORD: GPS coordinates. N: a minimum common sample size standardized to $N = 29$ per population. Num: Number of alleles found. Eff_num: Effective number of alleles, Ho: Observed heterozygosity, Hs: Heterozygosity within populations, PG: genets found (minimum estimate, based on a single locus), Gis: Inbreeding coefficient (* = $p < 0.05$). Num/RAMET: Alleles per ramet encountered in each population and its frequency. For example in Cadiz, 28 ramets had 2 alleles and 1 ramet had 1 allele. PR: Proportion of homozygote/heterozygote ramets for each population

Code	GPS Coord	N/N	Num/Num	EN/EN	Ho/Ho	Hs/Hs	PG/PG	Gis/Gis	Num/RAMET frequency for 1, 2, 3, 4	PR: proportion ramets
CADI	36°46.94′N	29/29	4/4	2.14/2.14	1.00/1.00	0.54/0.54	3/3	−0.84*/−0.84*	2, 3 (0:28:1:0)	At least 3 % heterozygotes
	6°25.10′W									At most 97 % fixed heterozygotes
ELPO	28°09.52N	67/29	1/1	1.00/1.00	0/0	0/0	1/1	–/–	1 (67:0:0:0)	100 % fixed heterozygotes
	16°25.57W									–
GAND	27°56.379′N	66/29	10/8	1.93/1.85	0.22/0.20	0.49/0.48	9/5	0.53/0.57	1, 2, 3 (51:5:10:0)	At least 22.73 % heterozygotes
	15°22.066′W									At most 77.27 % heterozygotes
FLORI	23°43.157N	62/29	5/4	3.02/2.89	0/0	0.68/0.67	5/4	1/1	1, 2 (61:1:0:0)	At least 1.62 % heterozygotes
	80°08.888W									At most 98.38 % heterozygotes
TABA	38°09.97N	77/29	2/2	1.48/1.31	0.01/0	0.33/0.24	3/2	0.96/1	1, 2 (76:1:0:0)	At least 1.41 % heterozygotes
	0°28.66W									At most 98.59 % heterozygotes
LOPA	37°48.84N	72/29	2/2	1.90/1.89	0/0	0.48/0.48	2/2	1.00/1	1 (72:0:0:0)	100 % fixed heterozygotes
	0°47.326W									–
LESC	40°33.250N	76/29	3/1	1.13/1	0.03/0	0.12/0	4/1	0.67/–	1, 2 (72:4:0:0)	At least 2.71 % heterozygotes
	0°31.965E									At most 97.29 % heterozygotes
LAMA	38°01.750N	73/29	2/2	1.79/1.67	0.2/0.10	0.44/0.41	3/3	0.54/0.75	1, 2 (58:15:0:0)	At least 20.55 % heterozygotes
	0°39.032W									At most 79.45 % heterozygotes
FORM	38°43.626N	39/29	2/2	2.00/1.99	0.89/0.89	0.50/0.50	3/3	−0.77*/−0.76*	1, 2 (4:35:0:0)	At most 89.74 % fixed heterozygotes
	001°25.025E									At least 10.25 % heterozygotes
ADDA	40°00.368′N	78/29	1/1	1.00/1	0/0	0/0	1/1	–/–	1 (78:0:0:0)	100 % fixed heterozygotes
	4°12.024′E									–
AMER	38°26.056′N	75/29	2/2	1.58/1.61	0.30/0.34	0.37/0.39	3/2	0.17/0.11	1, 2 (52:23:0:0)	At least 30.66 % heterozygotes
	0°22.355′W									At most 69.33 % heterozygotes
CDOR	39°22.164N	39/29	2/2	1.21/1.29	0/0	0.18/0.23	2/2	1/1	1, 2 (37:2:0:0)	At least 5.13 % heterozygotes
	3°13.887E									At most 94.87 % heterozygotes

Table 2 continued

Code	GPS Coord	N/N	Num/Num	EN/EN	Ho/Ho	Hs/Hs	PG/PG	Gis/Gis	Num/RAMET frequency for 1, 2, 3, 4	PR: proportion ramets
IBIZ	38°58.649N	35/29	13/11	5.02/4.92	0.60/0.62	0.81/0.81	15/13	0.26/0.23	1, 2, 3, 4 (14:9:8:4)	At least 60 % heterozygotes
	1°17.036E									At most 40 % heterozygotes

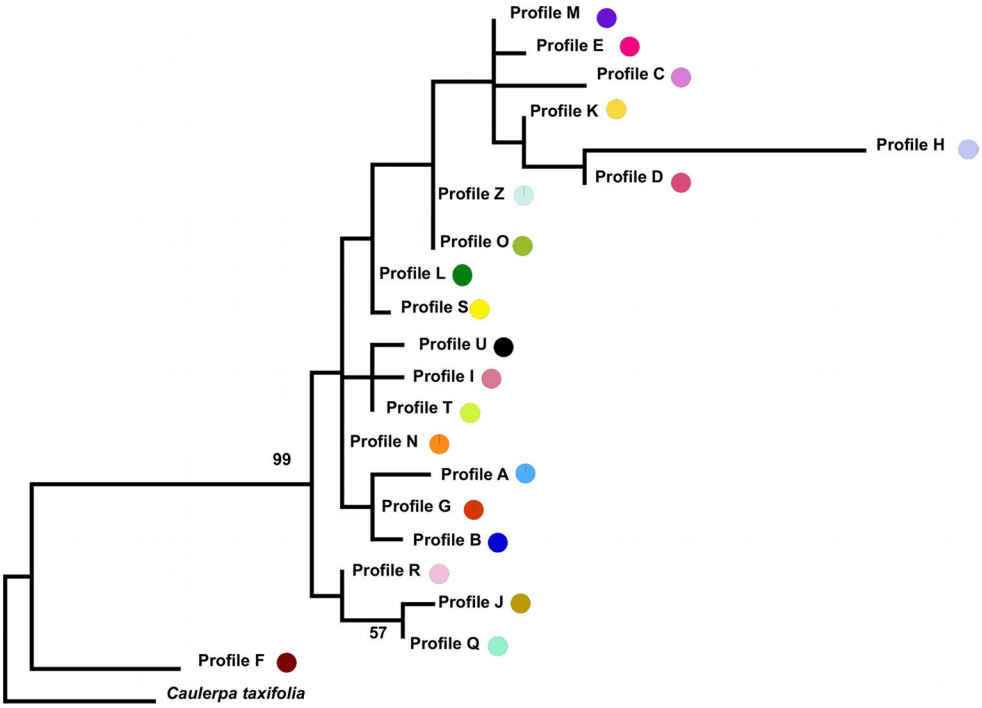

Fig. 3 Molecular phylogenetic analyses by maximum likelihood method for ITS2 region of *C. prolifera* from its full geographical range: Atlantic, Mediterranean and Pacific areas. The evolutionary history was inferred by using the maximum likelihood method based on the Kimura 2-parameter model (Kimura 1980). The bootstrap consensus tree inferred from 1,000 replicates. *Branches* corresponding to partitions reproduced in <50 % bootstrap replicates are collapsed. The analysis involved 22 nucleotide sequences: 21 ITS2 profiles found in *C. prolifera* and the outgroup: *C. taxifolia*. Codon positions included were 1st + 2nd + 3rd + noncoding. All positions containing gaps and missing data were eliminated. There were a total of 132 positions in the final data set

similar lack of geographical structuring (ML phylograms shown in Fig. 3 and haplotype network in Fig. S1 in supplementary material). Regardless of the lack of phylogeographical signal for any analyses, several interesting patterns are noteworthy. The western Atlantic contains a most ancestral profile (F), highly divergent from all others in the same region and in all other regions. Profile U characteristic of the Pacific area is very closely related to two other, from the Pacific/Mediterranean (T, with only one mutation step) and the transition zone in Cadiz (I, with two mutation steps).

Analyses with dinucleotide locus CaPr_J2

A total of 788 ramets of *C. prolifera* from four Atlantic and nine Mediterranean locations were genotyped for the microsatellite loci CaPr_J2. The total number of alleles varied from 1 to 13 between populations, with a total number of 21 alleles and 33 genotypes (Table 2). Genetic diversity measured as effective number of alleles varied from 1 to 5.02 (average 1.60). Ramets showed from 1 to 4 alleles per sample, and the proportion of possible homozygote ramets was much higher than heterozygotes for all the populations, with the exception of Cadiz where 97 % of the ramets were a possible heterozygote sample (Table 2). Ho was inferior to Hs for all the populations with the exception of Formentera, but Gis values (inbreeding coefficient) were significantly different from zero only for two populations possibly due to low power having only one marker used. Ibiza showed an unusual pattern of genetic diversity in all the parameters estimated. After standardizing genetic variability statistics among samples to 29 ramets per population (the minimum common number of individuals sampled in Cadiz), parameters did not vary greatly relative to the full data set.

The allele frequencies at the microsatellite locus CaPr_J2 clearly distinguish Atlantic locations versus "Mediterranean" (Fig. 4) and also revealed different allele frequencies

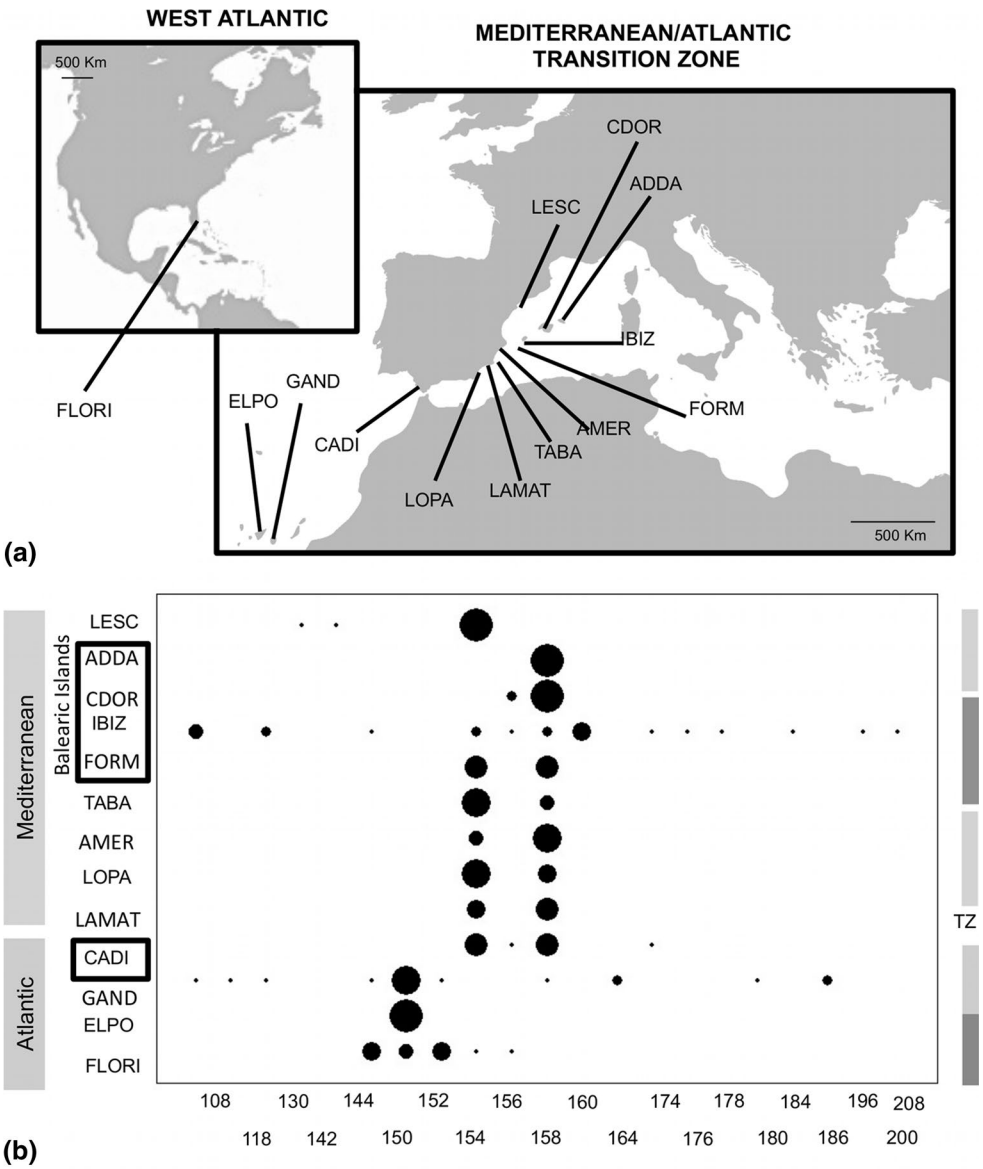

Fig. 4 Allele frequencies of locus CaPr_J2 in the Mediterranean/Atlantic transition zone and Florida. **a** Location of the 13 meadows sampled along the transition zone between the East Atlantic and Mediterranean areas and Florida Keys genotyped with CaPr_J2 locus. Site codes are given in Table 1. **b** Microsatellite allele frequencies from locus CaPr_J2. Each *circle* represents one allele, and the diameter of the circle is function of the allele frequency

between East Atlantic and West Atlantic. Additionally, for the transition zone, allele frequencies were related to the Mediterranean and not the Atlantic.

Discussion

Phylogeographical patterns detected

Three main biogeographical areas have been found in this study within the global distribution of *C. prolifera*: West Atlantic, East Atlantic and Mediterranean/Pacific revealed by sequences of cpDNA and nuclear DNA as well as by allele frequencies at one nuclear microsatellite locus. These three regions contained distinct cpDNA haplotypes and several unique, high-frequency diagnostic alleles, indicating the absence of recent connectivity. The Atlantic and Mediterranean types showed some admixture in the Atlantic transition zone (Portugal, southwest Spain). The higher genetic diversity and the presence of the most ancestral sequences suggest that the West Atlantic populations are older than the East Atlantic and Mediterranean populations.

Despite the occurrence of high-frequency dominant profiles in each region, admixture of some ITS2 profiles between Mediterranean and East Atlantic suggests some gene flow, although this could be a consequence of recent human introductions, as *C. prolifera* is often used in aquaria. However, the other markers used in our study show isolation between these regions, except for the particularly distinct case of Ibiza in the Balearic Islands, with

unique allele frequencies. ITS have shown very little intra- and inter-individual variation in Mediterranean *C. taxifolia* (e.g., Jousson et al. 1998; Famà et al. 2000; Meusnier et al. 2001). For Mediterranean *C. racemosa* (Famà et al. 2002), ITS showed high intra-individual variation and also the origins of colonization in the Mediterranean (Verlaque et al. 2003). Our results for *C. prolifera* ITS2 mainly show much higher diversity in the Atlantic, and particularly in the western Atlantic, than in the Mediterranean. This is somewhat contradictory to the general idea that this species is native from the Mediterranean and may suggest an early colonization. In the Indo-Pacific area, we found two ITS sequences differing by only 1 bp, one of them was also found in two Mediterranean locations, corroborating the results found with cpDNA, in suggesting that these Pacific and Mediterranean populations have a recent common origin.

Within the Mediterranean populations, the single microsatellite locus here used is not sufficient for full clonal resolution but allows a relative comparison of the minimum number of clones detected per sample, standardized to a common sample size, bearing in mind that this is likely to yield an underestimate of the real number of clones represented but can be interpreted as a comparative clonality index. The standardized minimum number of genets in Florida was the highest (3 vs. 1–2) in comparison with any of the other localities, with exception of one site, Ibiza which was highly influenced by distinct dominant alleles. Both populations in the Canary Islands showed high frequency of the same single allele, suggesting colonization from a single source. In addition, all sampled ramets from the Canary Islands had a cpDNA haplotype distinct from all other sampled sites, which might have originated from a colonization source not present in our samples. Yet, the finding of high number of alleles (microsatellite) at one site (even if only one of them presents a high frequency) does not support a recent founder effect but rather an ancient presence in the area.

The Mediterranean/Atlantic transition zone

Our results show that the Atlantic sites at the transition to the Mediterranean, Cadiz (Spain) and Fuseta (Portugal) are similar to the Mediterranean rather than other Atlantic sites, indicating an introduction of ramets from the Mediterranean to the Atlantic in this area. However, it is interesting to note that ITS2 profiles for Cadiz and Fuseta differed. Although the dominant ITS sequences for Cadiz were also found in other Mediterranean sites, Fuseta had unique ones but also shared profiles with the West Atlantic (Florida). These results suggest that this might be an admixed contact zone between Atlantic and Mediterranean genetic types, as reported for the seagrass *C. nodosa* (Alberto et al. 2008). Here, we hypothesize that recombination occurred between individuals with different chloroplasts and homogenization caused the loss of one type of ITS (Quijada et al. 1997; Fuertes Aguilar et al. 1999). This "admixture process" already has been described in other *Caulerpa*, even for *Caulerpa* species with a low rate of sexual reproduction (Jousson et al. 1998; Famà et al. 2002; Schaffelke et al. 2002). The Fuseta area will be more subjected to share profiles among isolates from Mediterranean Sea and Atlantic Ocean, and this is why profile L only appears in the Cadiz isolates.

Hypotheses about the evolutionary origin of *Caulerpa prolifera* in the Mediterranean Sea

We describe below the two hypothetical scenarios for the origin of *C. prolifera* in the Mediterranean Sea, and how congruent these are with our data:

Hypothesis 1 Atlantic origin of Mediterranean populations.

The present-day biota in the Mediterranean Sea are largely the result of colonization, mostly from the Atlantic Ocean (Almada et al. 2001; Domingues et al. 2005) and to a minor extent from the Red Sea since the opening of the Suez Canal. As most of the biota in the Mediterranean Sea, our results showing higher diversity and ancestral types in the Atlantic indicate that *C. prolifera* colonized the Mediterranean from the Atlantic. This could have occurred after the Messinian salinity crisis (6 million years ago) or more recently. The reduced genetic diversity in the colonized area compared to the source population can be interpreted as a genetic signature of a relatively recent colonization.

Our data also allow to infer other recent events that have affected *C. prolifera* at the Atlantic–Mediterranean transition zone. Expansion from the Mediterranean Sea into the East Atlantic along southern Iberia is suggested by cpDNA sequences from Cadiz (Spain) and Fuseta (Portugal) being equal to the Mediterranean, yet the distinct ITS2 types found there contradict this hypothesis suggesting the opposite colonization direction. An alternative hypothesis would thus be a contact zone showing some degree of admixture with either Atlantic or Mediterranean alleles becoming fixed at different loci, as was found for the seagrass *C. nodosa* along this region (Alberto et al. 2008).

The particularly high genetic diversity identified in Ibiza can be a consequence of persistence of ancient diversity. However, since Ibiza contains unique genetic types not found elsewhere in the Mediterranean areas here sampled, it is more in agreement with a scenario of admixture by multiple colonizations from distinct differentiated sources. Such an effect causing high genetic diversity is likely to be found in areas subjected to boat traffic from multiple

sources as found for an invasive seaweed in New Zealand (Voisin et al. 2005).

This hypothetical scenario 1 does not explain why the cpDNA *tuf*A haplotypes and ITS2 profiles from the Indo-Pacific are similar to the Mediterranean, and not to the Atlantic ones.

Hypotheses 2 Indo-Pacific origin of Mediterranean populations.

There are two facts that give us clues about possible connectivity (and a possible recent colonization in one of these directions) between Mediterranean areas and Indo-Pacific areas. The samples from the Indo-Pacific area (collected in an aquarium) shared equal or close related profiles for ITS with the Mediterranean samples, and the Bali samples share equal sequences for *tuf*A. *C. prolifera* has been recorded in several locations in the Indo-Pacific area in algaebase (see Guiry and Guiry 2014); however, we cannot ascertain the exact location of this specific sample from the Indo-Pacific area collected in the Aquarium.

More ancient colonization events and an Indo-Pacific origin in the Mediterranean are also possible. During the Messinian high salinity crisis, most of the preexisting Indo-Pacific biota went through extinction, with few exceptions of taxa of Miocene origin that had survived in shallow water refuges, as, for example, killifishes (Hrbek and Meyer 2003). Another classical Tethyan relic, a Messinian survivor, is the dominant and characteristic Mediterranean seagrass *Posidonia oceanica* (Aguirre et al. 2006), which occupies a habitat similar to that of *C. prolifera*, and it has also been hypothesized for *Halophila stipulacea* (Forsskål) Ascherson (Pérès 1967). Hence, the Mediterranean *C. prolifera* could descend from survivors of the Messinian salinity crisis. In this case, we could hypothesize that this presumed native *Caulerpa* species in the Mediterranean could also have dispersed from an Indo-Pacific ancestral population. However, the lack of further data on the current genetic structure of *C. prolifera* in the Indo-Pacific precludes further inferences about this question until more samples should become available.

This second hypothesis becomes stronger considering that many tropical species have been colonizing the Mediterranean during the last decades, mostly from an Indo-Pacific origin (Galil 2009). Besides anthropogenic introductions, this may also be the result of climatic change (sea water temperature increase) and the opening to the Indo-Pacific area through the Suez Canal that allowed natural dispersal through. A historical review shows that tropical biota survived in the Mediterranean till the end of the Pliocene Climate Optimum and that in the present we may be witnessing a recolonization of the Mediterranean by Tethyan descendants, rather than an invasion by alien species (Por 2009).

Conclusions

Our data based on three distinct loci answered the major question of this study that the Mediterranean/Atlantic transition zone is a biogeographical boundary for *C. prolifera,* and there are three main biogeographical areas within the global distribution of *C. prolifera*: West Atlantic, East Atlantic and Mediterranean/Pacific. Populations in the Canary Islands showed high frequency of the same single allele and a cpDNA haplotype distinct from all other sampled sites suggesting colonization from a single source. The western Atlantic area contained both higher genetic diversity and the most ancestral sequence types, supporting the hypothesis that this region could have been the species geographical origin. Identical sequences found in the Mediterranean and Indo-Pacific suggest recent gene flow along the Red Sea connection, since an alternative hypothesis of a possible ancient Indo-Pacific origin would lead to expectation of higher current divergence levels, not supported by these data.

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