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Dinucleotide microsatellite markers in the genus *Caulerpa*

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Abstract *Caulerpa* spp. are clonal green marine algae which often act as invasive species when growing outside their native biogeographical borders. Over the two past decades, *Caulerpa taxifolia* has spread along the Mediterranean coast, presently occurring at 70 sites and covering nearly 3,000 ha of subtidal area. New genetic markers (microsatellites) have been developed to assess clonal structure and genetic diversity of recently established populations of the invasive species *C. taxifolia* and *Caulerpa racemosa* in comparison with populations of the native *Caulerpa prolifera* in the Mediterranean. Our results show that nine polymorphic markers have been developed for *C. prolifera*, seven for *C. taxifolia*, and three for *C. racemosa*. Genetic diversity in *Caulerpa* was assessed in two geographical scales: one at a population scale where 40 thalli units were collected from *C. prolifera* in Cala d'Or, Mallorca, Spain, and another at a species scale, where 30 sample units were analyzed for *C. prolifera*, 24 for *C. taxifolia*, and 24 for *C. racemosa* from different sites in the Mediterranean, Atlantic, and Pacific Ocean. Number of alleles, expected heterozygosity, and marker amplification success are provided in each case.

Keywords *Caulerpa* · Clonal plant · Caulerpaceae · Microsatellite · Population genetics · Seaweed invasion

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Green algae of the genus *Caulerpa* (Bryopsidophyceae, coenocytic algae; Bessey 1907) have the capacity to propagate clonally and often show invasive behavior when introduced beyond their native ranges, particularly as competitors of seagrasses (e.g., de Villèle and Verlaque 1995; Williams 2007). Recently, the genus *Caulerpa* has been attracting considerable research attention in the Mediterranean Sea, where two tropical *Caulerpa* species, *Caulerpa taxifolia* (M. Vahl) C. Agardh and *Caulerpa racemosa* (Forsskål) J. Agardh, have spread into areas formerly occupied by seagrasses. In 1984, *C. taxifolia* was accidentally released into coastal waters of the Mediterranean Sea in Monaco and has spread along France and Italy, extending to the Spanish Balearic Islands, and covering nearly 3,000 ha of subtidal area (Ribera and Boudouresque 1995). This species has also reached the California coast in the USA (Jousson et al. 2000). How *Caulerpa* populations maintain themselves and spread, which may take place by clonal or/and sexual reproduction, is poorly understood (but

see Phillips 2009). New microsatellite markers are developed in our study and are tested for their potential cross-utilization among species to help understand the dynamics of propagation of *Caulerpa* species, and why their population dynamics and competitiveness varies between species and populations.

Herein, we present the development of polymorphic microsatellite loci for the genus *Caulerpa*, isolated from *Caulerpa prolifera* and *C. taxifolia*. Levels of polymorphism are investigated for *C. prolifera*, *C. racemosa*, and *C. taxifolia*, from the Mediterranean, Atlantic, and Pacific regions. In *C. prolifera*, polymorphism is investigated from a population in Cala d'Or, Mallorca, Spain.

Materials and methods

For construction of a microsatellite-enriched DNA library, fresh material was collected in Cala d'Or, Mallorca, Spain, from one individual of *C. prolifera* and one individual of *C. taxifolia*, with libraries constructed for each. DNA was purified by isolation of nuclei. Construction and screening of the genomic libraries are described in Varela-Álvarez et al. (2006), based on small modifications of protocols from Billote et al. (1999) and Kijas et al. (1994). A total of 18 sequences obtained from these libraries containing microsatellite repeats were used to design primers using PRIMER 3 (Rozen and Skaletsky 2000).

For polymorphism testing and scoring of microsatellites, sampled material was preserved in silica drying crystals, and DNA was extracted with the CTAB method (Doyle and Doyle 1990). DNA was digested with *Afa*I (RsaI) (Amersham Pharmacia Biotech), the same enzyme used for library construction, to facilitate polymerase chain reaction (PCR) amplification. Polymorphism was tested at two geographical scales. The first scale corresponded to three distant geographical areas: Mediterranean, Atlantic, and Pacific. The sample used consisted of the following: for *C. prolifera*, 30 sample units (thalli fragments) from Spain, Sicily, Cyprus, Gran Canaria, and Florida; for *C. taxifolia*, 24 sample units from Spain, Turkey, Florida, Polynesia, and Australia; and for *C. racemosa*, 24 sample units from Spain, Greece, Sicily, Cyprus, Florida, and Australia. The *C. racemosa* samples from Spain, Sicily, and Cyprus belong to *C. racemosa* var. *cylindracea*. The second sampling scale was only performed for *C. prolifera* and consisted of a sample of 40 thalli from a single population in Cala d'Or, Mallorca, Spain (collected in an 80×20-m area using random coordinates).

PCR amplifications were carried out in 10 μL reaction volume with approximately 5–10 ng of template DNA using the Qiagen Multiplex PCR Kit, where 2.0 μM of each

Table 1 Characterization of the ten microsatellite loci identified in *C. prolifera* and *C. taxifolia*

Primer name	Microsatellite repeat	Primers 5′–3′	Cloned allele size	Tm (°C)	Labeling	GenBank accession number
CaPr_M13	$(GA)_{21}$	F: AAGAGGTAGGACAGCGACCA R: GACCCACCCACCACTCAC	105	58	5′FAM	FJ969886
CaPr_J2	$(TC)_{17}$	F: GTAACCTAAACCATACCCCACCT R: TCAACCTCCCCTTGCGT	158	58	5′HEX	FJ969887
CaPr_M9	$(CT)_2T(CT)_8$	F: CTAGTCCTTTGCGAGAGC R: TCCATCTCCTGTTGTTGTT	150	58	5′FAM	FJ969888
CaPr_N12	$(GA)_6A_2(GA)_2A_2GA_2(AG)_3$	F: CGCGTGGGCTACCTTCT R: GGCTCCAATCTGGGGTG	154	58	5′HEX	FJ969889
CaPr_O17	$(AG)_{24}$	F: CTATGACCCCGGCTTTTGAT R: GTCTCAAAATTTCACCGTCTCA	221	58	5′FAM	FJ969890
CaPr_A1	$(GA)_{13}$	F: GTTAAACTGCAAGGGCATGG R: GATGTCAGTGTTGGCTGGTG	203	58	5′FAM	FJ969891
CaPr_N8	$A_{12}(TA)_5$	F: GCTCTGAACCACGGCTTATC R: CCTTCGGAGTCGATGAGAAC	162	52	5′HEX	FJ969892
CaPr_G4	$(CT)_6(TCA)_2(CT)_2$	F: TGCAGATTTCGCAGTTTGAG R: AAGATCCTGGGTAGAGCCAAA	156	55	5′HEX	FJ969893
CaPr_D4	$(CT)_{12}$	F: TTGGAATACCTTCGGGTGTC R: TCGGGAGAGAAATTGTCGTT	222	48	5′FAM	FJ969894
CaTa_A6	$(AG)_4G_2(AG)CG(AG)_5$	F: GTAAGAGTGGGAGTGAGAGT R: ACCTATCCATCTTGCTC	107	50	5′HEX	FJ969895

The name, motif, primer sequence, size of cloned allele, labeling, and GenBank accession number are given for each locus. The loci starting with CaPr derive from the *C. prolifera* library, and the loci starting with CaTa derive from the *C. taxifolia* library

Tm the optimal primer annealing temperature, *F* forward primer, *R* reverse primer

Table 2 Number of alleles, allele range, and unbiased expected heterozygosity for the three *Caulerpa* species that occur in the Mediterranean, assessed in mixed samples including individuals from the Mediterranean, Atlantic, and Pacific regions

	C. prolifera worldwide samples (Mediterranean–Atlantic)						*C. racemosa*[a] worldwide samples (Mediterranean, Atlantic, and Pacific)						*C. taxifolia* worldwide samples (Mediterranean, Atlantic, and Pacific)					
	Amp. success	*N*	No alleles	Range	He	Null alleles	Amp. success	*N*	No alleles	Range	He	Null alleles	Amp. success	*N*	No alleles	Range	He	Null alleles
CaPr_M13	90%	27	5	104–141	0.26	–	75%	18	Mp	–	–	–	79.1%	19	Mp	–	–	–
CaPr_J2	100%	30	6	150–164	0.74	√	41.6%	10	Mp	–	–	–	41.6%	10	Mp	–	–	–
CaPr_M9	83.3%	25	9	111–169	0.60	√	45.8%	11	6	93–152	0.56	–	66.6%	16	6	110–182	0.57	√
CaPr_N12	93.3%	28	5	118–184	0.64	–	45.8%	11	6	97–184	0.45	–	41.6%	10	6	100–184	0.77	√
CaPr_O17	53.3%	16	1	222	–	–	33.3%	8	3	208–222	0.22	–	58.3%	14	3	191–222	0.13	–
CaPr_A1	43.3%	13	2	201–203	0.14	–	0%	0	–	–	–	–	25%	6	1	201	–	–
CaPr_N8	56.6%	17	5	150–167	0.51	√	4.1%	1	1	153	–	–	45.8%	11	4	118–178	0.44	√
CaPr_G4	40.0%	12	5	90–156	0.45	–	25%	6	1	156	–	–	45.8%	11	3	108–204	0.16	–
CaPr_D4	60.0%	18	6	101–224	0.65	–	0%	0	–	–	–		62.5%	15	Mp	–	–	–
CaTa_A6	0%	0	–	–	–	–	0%	0	–	–	–		29.1%	7	7	91–196	0.82	–

Amp. success amplification success, *N* number of sample units where amplification was successful, *Mp* multiple peaks, *He* expected heterozygosity

[a] The *C. racemosa* samples from Spain, Sicily, and Cyprus, 19 samples in total belong to *Caulerpa racemosa* var. *cylindracea*. The name of the variety of the other five *Caulerpa* samples (from Greece, Florida, and South Australia) is not available

Table 3 Characterization of diversity in a *C. prolifera* meadow from Cala d'Or, Mallorca, Spain, based on seven microsatellites and a sample size of 40

	C. prolifera Cala d'Or meadow, Mallorca, Spain					
	Amp. success	*N*	No alleles	He	Range	Null alleles
CaPr_J2	100%	40	2	0.27	160–162	√
CaPr_M9	40%	16	5	0.70	104–170	–
CaPr_N12	67.5%	27	5	0.52	129–183	–
CaPr_O17	75%	30	2	0.05	222–254	–
CaPr_N8	70%	28	4	0.31	110–160	√
CaPr_G4	45%	18	6	0.71	94–167	√
CaPr_D4	80%	32	8	0.48	106–224	–

N sample size amplified, *No* number of alleles, *He* expected heterozygosity

primer (one of each primer pair 5′ labeled with FAM or HEX), 0.5× Q solution, and 1× PCR Qiagen Master Mix were used. The PCR program, following the Qiagen kit instructions, was 95°C for 15 min; followed by 95°C for 30 s, *Tm* temperature (Table 1) for 30 s, and 72°C for 60 s, for 40 cycles; and a final extension at 72°C for 5 min. For each PCR reaction, we used a positive control (the cloned microsatellite isolated in the library) and a negative control. For genotyping, a mixture of 1 μL of PCR product, 0.15 μL of genescan 500 Liz size standard (Applied Biosystems), and 8.5 μL of Hi-Di Formamide (Applied Biosystems), denatured at 95°C for 5 min, was electrophoresed on a 3730xl DNA Analyser (Applied Biosystems). The genotypes were analyzed with GeneMapper 4.0 (Applied Biosystems). Expected heterozygosities (non-biased) were estimated using GENETIX 4.02 (Belkhir et al. 2005) and are given for samples collected from a range of distant sites just as representing diversity levels for the loci, under the assumption that individuals are diploid (see below). The presence of null alleles was tested with MICROCHECKER (Van Oosterhout et al. 2004) under the assumption that all individuals sampled are diploid.

Results and discussion

Specific primers were designed for 18 sequences containing dinucleotide repeats from the libraries previously developed by Varela-Álvarez et al. (2006). Ten loci were found to be polymorphic in at least one species. The name, motif, primer sequence, size of cloned allele, and GenBank accession number are given for each locus in Table 1. Number of alleles, allele sizes, expected heterozygosities, and amplification success were different for each marker in each species. The species *C. prolifera* had the highest amplification success for the three Mediterranean *Caulerpa* spp. (Table 2). The levels of heterozygosity represent the diversity of the markers under the assumption that all individuals are diploid.

For *C. prolifera*, eight polymorphic markers were found with two to nine alleles (Table 2). For *C. racemosa*, three polymorphic markers were found with three to six alleles (Table 2). For *C. taxifolia*, seven polymorphic markers were found with three to seven alleles (Table 2). Null alleles were detected for three loci in *C. prolifera* and three in *C. taxifolia* (Table 2). No large allele dropout was detected among the data for any marker. Three loci (CaPr_M13, CaPr_J2, and CaPr_D4) showed multiple bands when tested in *C. racemosa* and *C. taxifolia*. The flanking regions of these markers seem to be conserved in the three species but may have been duplicated in *C. racemosa* and *C. taxifolia*, thus producing the multiple banding patterns observed. Table 3 shows variability of seven microsatellite loci in *C. prolifera* in a sample (n=40 units) of a meadow in Cala d'Or, Mallorca, Spain. Number of alleles per locus ranged from 2 to 8, with an average of 4.4 alleles per locus. Expected heterozygosity was calculated after removal of clonal replicates, and the values ranged from 0.05 to 0.71. Null alleles were present in three loci: CaPr_J2, CaPr_N8, and CaPr_G4. CaPr_M13 and CaPr_A1 did not amplify in this population.

In this study, we were able to isolate ten new microsatellite markers for the *Caulerpa* genus, of which nine were polymorphic in *C. prolifera*, seven in *C. taxifolia*, and three in *C. racemosa*. Amplification success was low in general, a result that might be expected if these taxa represent complexes of cryptic species, as has been shown in *C. racemosa* (Famà et al. 2002) and hypothesized for *C. taxifolia* (Meusnier et al. 2002).

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