*J. Phycol.* **42,** 1113–1124 (2006)

DOI: 10.1111/j.1529-8817.2006.0271.x

# A FORENSIC AND PHYLOGENETIC SURVEY OF *CAULERPA* SPECIES (CAULERPALES, CHLOROPHYTA) FROM THE FLORIDA COAST, LOCAL AQUARIUM SHOPS, AND E-COMMERCE: ESTABLISHING A PROACTIVE BASELINE FOR EARLY DETECTION[1]

*Wytze T. Stam*[2] *Jeanine L. Olsen*

Department of Marine Benthic Ecology and Evolution, Center for Ecological and Evolutionary Studies, Biological Centre, University of Groningen, PO Box 14, 9750 AA Haren, The Netherlands

*Susan Frisch Zaleski*

University of Southern California Sea Grant Program, 3616 Trousdale Parkway, Los Angeles, California 90089-0373, USA

*Steven N. Murray*

Department of Biological Science, California State University, Fullerton, PO Box 6850, Fullerton, California 92834-6850, USA

*Katherine R. Brown and Linda J. Walters*

Department of Biology, University of Central Florida, Orlando, Florida 32816, USA

**Baseline genotypes were established for 256 individuals of *Caulerpa* collected from 27 field locations in Florida (including the Keys), the Bahamas, US Virgin Islands, and Honduras, nearly doubling the number of available GenBank sequences. On the basis of sequences from the nuclear rDNA-ITS 1 + 2 and the chloroplast *tuf*A regions, the phylogeny of *Caulerpa* was reassessed and the presence of invasive strains was determined. Surveys in central Florida and southern California of >100 saltwater aquarium shops and 90 internet sites revealed that >50% sold *Caulerpa*. Of the 14 *Caulerpa* species encountered, *Caulerpa racemosa* was the most common, followed by *Caulerpa sertularioides*, *Caulerpa prolifera*, *Caulerpa mexicana*, and *Caulerpa serrulata*. None of the >180 field-collected individuals (representing 13 species) was the invasive strain of *Caulerpa taxifolia* or *C. racemosa*. With one exception (a sample of *C. racemosa* from a shop in southern California belonged to the invasive Clade III strain), no invasive strains were found in saltwater aquarium stores in Florida or on any of the internet sites. Although these results are encouraging, we recommend a ban on the sale of all *Caulerpa* species (including "live rock") because: morphological identification of *Caulerpa* species is unreliable (>12% misidentification rate) and invasive strains can only be identified by their aligned DNA sequences, and because the potential capacity for invasive behavior in other *Caulerpa* species is far from clear. The addition of the Florida region to the genetic data base for *Caulerpa* provides a valuable proactive resource for invasion biologists as well as researchers interested in the evolution and speciation of *Caulerpa*.**

***Key index words:*** **aquarium trade; *Caulerpa*; e-commerce; invasive species; ITS; marine conservation; phylogeny; *tuf*A**

***Abbreviations:*** **CTAB, cetyltrimethylammonium bromide; ITS, internally transcribed spacer; MCMC, Markov chain Monte Carlo analysis**

Coastal marine waters are among the most vulnerable and most heavily invaded habitats (Kolar and Lodge 2001, Grosholz 2002, Ruiz and Carlton 2003, Hochberg and Gotelli 2005). One of the best-documented accidental introductions of a marine organism was the release of *Caulerpa taxifolia* from the Monaco Oceanographic Museum into the Mediterranean Sea in 1984. When first discovered, the alga covered 1 $m^2$ (Meinesz and Hesse 1991). By 2000, it covered 131 $km^2$ of near-shore waters bordering six Mediterranean countries (Meinesz et al. 2001). That same year, professional divers discovered the alga at two locations in southern California: one in Agua Hedionda Lagoon near Carlsbad, San Diego County, and the other in Huntington Harbor, Orange County. Molecular forensics confirmed that both were the invasive Mediterranean "aquarium strain" (Jousson et al. 2000). Next, the alga showed up in Lake Conjola, Port Hacking, and Careel Bay, all in the Sydney, New South Wales area of Australia. Again, molecular identifications confirmed the invasive strain (Schaffelke et al. 2002).

*C. taxifolia* is not part of the California marine flora so its presence left no doubt that it was an introduction,

[1]Received 17 March 2006. Accepted 5 July 2006.
[2]Author for correspondence: e-mail w.t.stam@rug.nl.

and eradication efforts began almost immediately (Woodfield and Merkel 2004). In contrast, control efforts were delayed in Australia because *C. taxifolia* is part of the warm temperate to tropical Australian marine flora and initial discussions focused on the possibility of natural range expansions rather than on an introduction. Subsequent molecular studies over the past 5 years have confirmed that the invasive aquarium strain found in the Mediterranean, Southern California, and Southern Australia are of Australian origin (Moreton Bay area) and that spread has been human-mediated through the aquarium trade (Jousson et al. 2000, Meusnier et al. 2001, 2002, 2004, Wiedenmann et al. 2001, Walters et al. 2006).

At about the same time, an invasive strain of *Caulerpa racemosa* was also identified in the Mediterranean (Verlaque et al. 2000) and has now spread to the Canary Islands (Verlaque et al. 2004). This strain has been designated as *C. racemosa* var. *cylindracea* (Sonder) Verlaque, Huisman, and Boudouresque, based on an earlier combination, *C. racemosa* var. *laetevirens* f. *cylindracea* (Sonder) Weber-van Bosse, which is indigenous to southwest Australia. Once again, a human-mediated introduction is suspected, although the vector of the introduction is still unknown (Verlaque et al. 2003).

Additional species of *Caulerpa* are also showing signs of potentially invasive behavior, i.e. growing in dense and very large monospecific stands. For example, *Caulerpa scalpelliformis* (R. Brown ex Turner) C. Agardh was recorded in the Mediterranean (Verlaque and Fritayre 1994) and although native to southern Australia, extended its range northward along the eastern Australian coast (Davis et al. 1997). More recently, *Caulerpa brachypus*, a Caribbean native, was identified in Florida waters (Jacoby et al. 2004) and a Florida native, *Caulerpa verticillata*, has been reported in uncharacteristically large, monospecific stands (Raloff 2000). These observations, however, must be viewed with caution. In the process of introduction, a native species becomes non-native but not necessarily invasive (Nyberg and Wallentinus 2005). Here, we use "invasive" in the restricted sense for those strains that have been conclusively established as invasive, i.e. the Mediterranean Aquarium strain of *C. taxifolia* and the Australian strain of *C. racemosa* var. *cylindracea*. Use of the term "non-invasive" is misleading as this cannot be known. We prefer the term "native/wild-type."

Florida's long coast line, including the Florida Keys, is home to approximately14 species of *Caulerpa* among the ~20 species recognized in the greater Caribbean (Littler and Littler 2000, Guiry et al. 2005) and some 70 species worldwide (Silva 2002). Because Florida's surface water temperatures and climate are similar to previous invasion locations of *C. taxifolia* and *C. racemosa*, it is especially important to establish the best local knowledge possible as it provides the best insurance for early detection of non-native species and strains. No one knows whether the established invasive strains of *C. taxifolia* and *C. racemosa* are already present along the shores of Florida, in private/public aquaria, or available in shops and via internet retailers.

The aims of the present research were to: (1) establish baseline genetic identification of native *Caulerpa* species around the Florida coastline using nuclear-encoded rDNA-ITS and chloroplast encoded *tuf*A gene sequences; (2) establish genetic identification of *Caulerpa* species (including the possibility of invasive strains of *C. taxifolia* and *C. racemosa*) purchased from local aquarium shops in Florida and southern California, and from internet retailer and auction sites; and (3) reassess phylogenetic relationships among *Caulerpa* species using the >300 new sequences in addition to the approximately 300 existing in GenBank. With these results, we discuss the value of expanding the species sampling and biogeographic coverage beyond the currently recognized invasive forms. Our overall intent is to document the threat of the aquarium industry and e-commerce as vectors for dispersal of *Caulerpa* and the inadequacy of morphological identification—leading to the recommendation of a ban on the entire genus.

## MATERIALS AND METHODS

*Caulerpa samples.* A total of 256 samples representing 13 species of *Caulerpa* (supplemental Table 1) were collected from 27 field locations (Fig. 1) or purchased from >90 commercial sources. The CA-coded samples were purchased in 2000 and 2001 from aquarium shops in Orange County, San Diego County, and Los Angeles County, California (Zaleski and Murray 2006). The FL-coded samples were collected in 2003 and 2004 from: (1) field sites around the coast of Florida (including the Florida Keys), Honduras, the US Virgin Islands, and the Bahamas; (2) from central Florida aquarium shops; and (3) via e-commerce (Walters et al. 2006). Upon collection, the samples (typically 3–4 fronds from a single individual, including the stolon) were cleaned, patted dry with paper towels, and placed in heavyweight, zip lock plastic bags containing 20–30 g of silica gel crystals. A dried voucher specimen was also prepared for each sample according to standard herbarium procedures (available from L. J. Walters).

*DNA extraction.* DNA was extracted using a modification of the CTAB method of Olsen et al. (1998) excluding the final RNase incubation. Five to 10 mm of silica dried material was added to 800 μL preheated (60° C) 2% (w/v) CTAB buffer to which 2 μL β-mercaptoethanol was added. The mixture was incubated for 15 min at 60° C, followed by a prolonged incubation under gentle rotation for 45 min at room temperature. After two extractions with an equal volume of CIA (chloroform-isoamylalcohol 24:1 v/v), the DNA was precipitated with a 2/3 volume of cold isopropanol for 1 h at 4° C. The DNA was pelleted by centrifugation (30 min, 10,000*g*, 4° C), washed with 100 μL of cold 80% ethanol, air dried and dissolved in 100 μL 0.1 × TE (1 mM Tris, 0.1 mM EDTA, pH 8).

*PCR amplification.* Amplification of the ITS region and *tuf*A gene was performed by using the selected primer pair combinations (Table 1). For the ITS region, the JO4-JO6 pair was most commonly used. If amplification problems were encountered (i.e. multiple bands and/or low yield), the C1-JO6 pair or the combination JO4-Cau58R + Cau58F-JO6 pair was used for ITS. For the *tuf*A gene, the main primer combination used was the TufAF–TufAR pair developed by

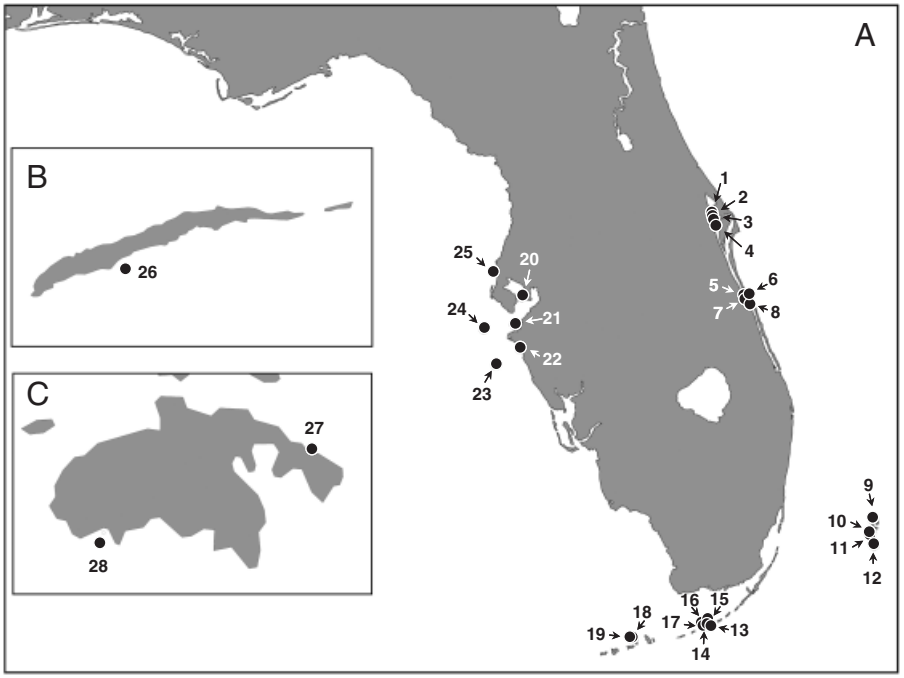



FIG. 1. Map with the field collection sites. Panel A. Florida and Bimini-Bahamas sites: 1, Merritt National Island Wildlife Refuge, Island Titusville; 2, Bennett Causeway Oceanside, Cocoa; 3, Indian River Lagoon, Grant; 4, Sebastian Inlet; 5, Ft. Pierce Inlet, Fort Pierce; 6, Little Jim Island, Fort Pierce; 7, South Jetty Park, Fort Pierce; 8, Wormrock site at Bathtub Reef, Stuart; 9, North Bimini; 10, North Turtle; 11, Turtle Rock; 12, Cat Key; 13, Goshen College Mangrove channel, Long Key; 14, Mangrove site at Goshen College Lab, Long Key; 15, Old Dan Key, Long Key; 16, Point North of Keys Marine Lab, Long Key; 17, Keys Marine Lab, Long Key; 18, Bahia Honda Beach, Bahia Honda Key; 19, Bahia Honda, Bahia Honda Key; 20, Picnic Park, Tampa Bay; 21, Skyway South, Tampa Bay; 22, Nokomis Beach, Nokomis; 23, Point of Rocks, Sarasota; 24, Larrys Ledge, St. Petersburg; 25, Tarpon Springs. Panel B. Honduras site: 26, Roatan Island. Panel C. St. John, US Virgin Islands sites: 27, West-facing Haulover Bay; 28, Fish Bay.

Famà et al. (2002). For low-yield amplifications, the TufAF–TufAR1 pair or the TufAF1–TufAR3 pair was used and for reamplifications the TufAF–TufAR2 pair was used.

A PCR reaction (final volume 25 μL) consisted of 1 × reaction buffer (Promega, Madison, WI, USA), 0.2 mM dNTP, 0.01% BSA, 1.5 or 2.5 mM $MgCl_2$ for ITS and 5.0 mM $MgCl_2$ for *tuf*A, 0.5 μM of each primer and 0.25 U $\mu L^{-1}$ Taq polymerase (Promega), and 1 μL template DNA. Amplifications were carried out in a GeneAmp PCR system 9700 (PE Applied Biosystems, Foster City, CA, USA) with the following profile: one cycle of 2 min at 94° C; 10 cycles of 15 s at 94° C, 1 min at 50° C and 2 min at 72° C; 30 cycles of 15 s at 94° C, 20 s at 50° C and 2 min at 72° C; and 1 cycle of 10 min at 72° C. PCR products were checked for correct length, purity, and yield on ethidium bromide-stained 1.5% TAE agarose gels. Good products were cleaned with ExoSap (USB, Corp., Cleveland, OH, USA) following the manufacturer's protocol and rechecked on an agarose gel.

In the case of low yields, the *tuf*A fragment was either reamplified or reaction products were pooled. The reamplification conditions were the same as described above but the $MgCl_2$ concentration was lowered to 1.5 mM. Pooled PCR products were cleaned with the GenElute PCR Clean-up Kit (Sigma-Aldrich CO., St. Louis, MO, USA) following the manufacturer's protocol. The pooled PCR products were eluted in 50 μL 1 mM Tris buffer with a 10× dilution of the provided elution buffer.

If multiple bands appeared on the diagnostic agarose gels, the band of appropriate length, as estimated from the annealing position of the applied primer pair (Table 1), was cut out from the gel and cleaned with the Wizard PCR Preps DNA Purification System (Promega) following the manufacturer's protocol. The fragment was eluted in 50 μL 0.1 × TE.

*Cloning of ITS fragments.* PCR amplified ITS1 + 2 fragments were ligated and transformed using the pGEM-T Easy Vector System, JM109 competent cells, and standard blue/white colony screening (IPTG, X-Gal/Ampicillin), all from Promega and following the manufacturer's instructions. The ratio of insert to vector was 3:1. A direct colony PCR was performed using the white colonies in which the initial amplification primers were used to verify the insert size. No DNA extraction is required. Colonies with the correct insert size were then grown overnight in 2× YT medium at 37° C on a shaker. Plasmid isolation was conducted using the Flexi-Prep Kit (Pharmacia, Roosendaal, The Netherlands). Plasmid yield was quantified using Image-Quant (ver. 4.2) software from Molecular Dynamics (MBT Benelux, Maarssen, The Netherlands). For each PCR product, up to 20 clones were sequenced, from which only non-identical sequences were used for further analysis.

*Sequencing.* Cycle sequencing was performed using approximately 75 ng of purified PCR product or 200 ng of double-stranded plasmid DNA template with the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems) using the original PCR primers for double-stranded sequencing. Fragment separation was carried out on an ABI 377 Automated Sequencer (PE Applied Biosystems).

*Sequence alignment and phylogenetic analyses.* Sequences were managed using Navigator and Factura Software (PE Applied Biosystems). The sequences from both strands were proofread.

TABLE 1. Primer combinations used for PCR amplification of the nuclear rDNA internal transcribed spacer 1 and 2 (ITS1 + 2) and chloroplast elongation factor TU gene (*tuf*A).

| | Forward primer | | | Reverse primer | | |
|---|---|---|---|---|---|---|
| Target region | Code | Sequence | Annealing position | Code | Sequence | Annealing position |
| ITS1 + 2 | JO4 | 5′-GGAAGGAGAAGTCGTAACAAGG-3′ | 3′ end 18S rDNA | JO6 | 5′-ATATGCTTAAGTTCAGCGGGT-3′ | 5′end 26 rDNA |
| ITS1 + 2 | C1 | 5′-GTACACACCGCCCGTCGCTCC-3′ | 3′ end 18S rDNA | JO6 | 5′-ATATGCTTAAGTTCAGCGGGT-3′ | 5′end 26 rDNA |
| ITS1 | JO4 | 5′-GGAAGGAGAAGTCGTAACAAGG-3′ | 3′ end 18S rDNA | Cau58R | 5′-GGACGACAATGCATTCGG-3′ | 5′end 26 rDNA |
| ITS2 | Cau58F | 5′-CGAATTGCAACATTTCGTG-3′ | 5.8S rDNA | JO6 | 5′-ATATGCTTAAGTTCAGCGGGT-3′ | 5.8S rDNA |
| *tuf*A | TufAF | 5′-TGAAACAGAAMAWCGTCATTATGC-3′ | 210 in *tuf*A gene | TufAR | 5′-CCTTCNCGAATMGCRAAWCGC-3′ | 1184 in *tuf*A gene |
| *tuf*A | TufAF | 5′-TGAAACAGAAMAWCGTCATTATGC-3′ | 210 in *tuf*A gene | TufAR1 | 5′-CCATAGGAATTGGACTATCA-3′ | 1096 in *tuf*A gene |
| *tuf*A | TufAF1 | 5′-GAGCAGCTCAAATGGATGGT-3′ | 284 in *tuf*A gene | TufAR2 | 5′-AAACTTGGGCTTGAAAACGA-3′ | 955 in *tuf*A gene |
| *tuf*A | TufAF | 5′-GAGCAGCTCAAATGGATGGT-3′ | 284 in *tuf*A gene | TufAR3 | 5′-CAATTTTCACTCGATCTCCT-3′ | 1123 in *tuf*A gene |

All primers were designed in the Olsen/Stam laboratory, except TufAF and TufAR, which were designed by Famà et al. (2002).

Sequences were initially identified by a BLAST (Altschul et al. 1997) search of GenBank and subsequently added to the reference alignments of related sequences using BioEdit 6.0.7 (Hall 1999). For the *tuf*A gene, an overall *Caulerpa* reference alignment was generated that consists of all the *tuf*A sequences determined by Famà et al. (2002, GenBank accession numbers AJ417928–AJ417973), and Senerpont Domis et al. (2003, GenBank accession numbers AJ512411–AJ512426, AJ412466, and AJ41267). For the ITS sequences, separate reference alignments were made for *C. taxifolia* and *C. racemosa*. For *C. racemosa,* three additional subalignments were required because of the polyphyletic nature of this species (Famà et al. 2002).

Alignments were subjected to a phylogenetic analysis using both Bayesian (MrBayes 2.01, Ronquist and Huelsenbeck 2003) and parsimony (PAUP 4.0b10, Swofford 2003) frameworks. Optimal models for sequence evolution for the Bayesian analyses were determined with ModelTest 3.7 (Posada and Crandall 1998) using hierarchical likelihood ratio tests (hLRTs). For the complete *tuf*A, the best model was the GTR + I + G, and for the ITS regions it was the HKY + G model. The MCMC searches were run for $5 \times 10^6$ generations; burn-in was determined by visually plotting the likelihood versus generation. PAUP was used for further handling of the trees obtained from each Bayesian analysis. After removal of the trees associated with the burn-in, a consensus tree was constructed for the *tuf*A analysis (Fig. 1) and an unrooted phylogram for both ITS analyses (Figs. 3 and 4). Parsimony analyses were evaluated by bootstrap analysis (1000 replications). For the parsimony analyses of the *tuf*A alignment, identical sequences were removed (i.e. represented by only one single sequence) in order to avoid endless rearrangements during the heuristic search. The subsequent bootstrap analysis was run with "maxtrees" set at 100.

## RESULTS

*Identification and classification*. Detailed information on the final identity of the *Caulerpa* samples can be found in supplemental Table 2 along with their GenBank accession numbers. The complete *tuf*A alignment (820 bp) and the ITS alignments for *C. taxifolia* (590 bp) and *C. racemosa* Clade III (661 bp) are also provided in GenBank.

Phylogenetic trees based on Bayesian and parsimony inferences had identical topologies. Posterior probabilities and bootstrap values were high to very high in the *tuf*A tree (Fig. 2). Almost all sequences unambiguously joined one of the species clades present in the reference tree. Only samples FL018, FL101, and FL145 clustered together in a well-supported new clade (see below), and the position of sample FL108, tentatively a *C. racemosa* clade I, remains ambiguous.

Posterior probabilities and bootstrap values were also high for the subset ITS trees for *C. taxifolia* (Fig. 3) and *C. racemosa* Clade III (Fig. 4). This would not be the case if all *Caulerpa* species were analyzed together because of intra-individual polymorphisms associated with the multi-copy nature of ITS, which promotes long branches and reduced resolution. Previous surveys of *C. taxifolia* and *C. racemosa* have extensively investigated ITS polymorphism (Jousson et al. 1998, 2000, Famà et al. 2000, Durand et al. 2002, Schaffelke et al. 2002, Verlaque et al. 2003). In our survey, we encountered 36 cases of ITS polymorphism in directly

TABLE 2. Summary of error types in identification

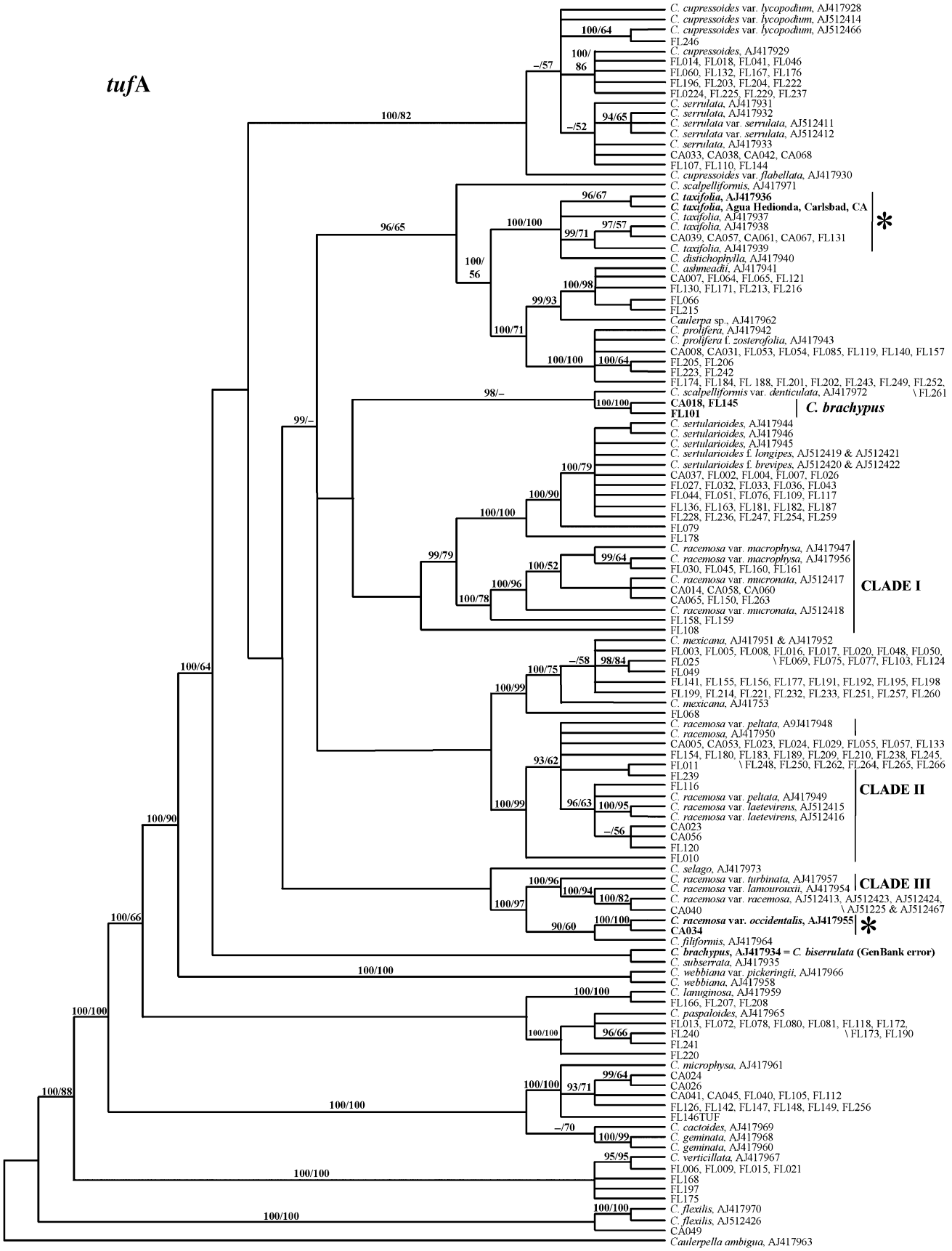
| | *N* | % | Comments |
|---|---|---|---|
| Total samples processed | 258 | | |
| Samples identified | 242 | **93.8** | |
| Samples unidentified | 16 | 6.2 | Due to contamination from other organisms:<br>**ITS** from a red alga, ciliate, sponge, plasmophoride, copepod, ascomycete, coelenterate, diatom, hydrozoan, and stramenopile (14 samples)<br>***tuf*A** from the diatom *Skeletonema costatum* (Greville) Cleve, GenBank AF545615 (1 sample) |
| Samples identified with ITS only | 38 | 15.7 | |
| Samples identified with *tuf*A only | 154 | 63.7 | |
| Samples identified with both | 50 | 20.7 | |
| Samples with polymorphic ITS | 34 | 14.0 | Most frequent in *Caulerpa mexicana* and *Caulerpa racemosa* |
| Disparities in initial vs. final identification | 38 | **15.7** | |
| Morphological misidentification | 30 | 12.4 | Grape forms:<br>*C. racemosa* (including putative varieties) and *Caulerpa microphysa* (13 times)<br>Feather forms:<br>*Caulerpa ashmeadii* but actually *Caulerpa taxifolia* (1)<br>*Caulerpa sertularioides* but actually *C ashmeadii* (4)<br>*C. mexicana* but actually *C. ashmeadii* (1)<br>*C. mexicana* but actually *Caulerpa verticillata* (1)<br>Serrated/curly forms:<br>*Caulerpa serrulata* but actually *Caulerpa cupressoides* (1)<br>*C. cupressoides* but actually *Caulerpa lanuginosa* (2)<br>*C. cupressoides* but actually *Caulerpa paspaloides* (2)<br>Blade forms:<br>*Caulerpa prolifera* but actually *Caulerpa brachypus* (1)<br>Other:<br>*Caulerpa webbiana* but actually *Caulerpa flexilis* (1)<br>*C. sertularioides* but actually *C. racemosa* (1)<br>*C. sertularioides* but actually *C. paspaploides* (1)<br>*C. racemosa* but actually *C. mexicana* (1) |
| Taxonomic uncertainty from the outset | 2 | 0.8 | *Caulerpa macrophysa*, *C. racemosa* v. *macrophysa* (Guiry et al. 2005) |
| Molecular misidentification | 2 | 0.8 | |
| Clerical mistake | 1 | 0.4 | |
| GenBank errors | 5 | 2.0 | See text |

sequenced PCR products involving samples from several different species (supplemental Table 2). Seven cases involved *C. taxifolia* or *C. racemosa*. In two of these cases, BLAST searches of these directly sequenced PCR products suggested membership of the *C. racemosa* invasive Clade III (CA034 and FL185). In both cases, PCR products were cloned and sequenced. FL185 was confirmed as a Clade II "native/wild type," whereas CA034 clustered in the invasive Clade III.

A new finding is the position of *C. brachypus* (CA018, FL101, FL145) as a probable sister-group to *C. scalpelliformis*. Previous analyses have placed it in a more basal position within the *Caulerpa* tree (Fig. 2). In fact, the *tuf*A sequence stored under accession number AJ417934 is not *C. brachypus*. Reexamination of the original specimen used by Famà et al. (2002) revealed that their sample is a *Caulerpa biserrulata* Sonder (W. F. Prud'homme van Reine and L. N. de Senerpont Domis, personal communication). Samples CA018, FL101, and FL145 were correctly identified *C. brachypus*, and formed a distinct clade in the *tuf*A tree, which was completely separated from the *C. brachypus*/*Caulerpa subserrata* Okamura clade in the reference tree (Fig. 2). This GenBank mistake is relevant for the state of Florida because of the anomalous behavior of *C. brachypus* in its waters (Jacoby et al. 2004).

Caulerpa *around the Florida coast and environs*. More than 180 individuals, representing 13 species of *Caulerpa,* were sequenced from 27 locations (see supplemental Table 1). These sequences represent a first inventory of the native genotypes and thus serve as a baseline against which future, suspect samples can be compared (see supplemental Table 2). No field-collected samples of *C. taxifolia* or *C. racemosa* belonged to any established invasive strains. It has been suggested that *C. brachypus* may also be exhibiting invasive properties in Florida waters (Jacoby et al. 2004). At present, we cannot comment further as we found no *C. brachypus* in the field sampling.

Caulerpa *availability in saltwater aquarium shops*. In Florida, Walters et al. (2006) surveyed 47 aquarium shops in the Tampa, Orlando, and Daytona Beach areas in 2003–2005. *Caulerpa* was sold in 53% of the shops. *C. racemosa, Caulerpa sertularioides, Caulerpa prolifera*, and *Caulerpa mexicana* were the most common among the nine *Caulerpa* species available. Of the 29 samples we sequenced from this source, none


tufA
C. cupressoides var. lycopodium, AJ417928
C. cupressoides var. lycopodium, AJ512414
C. cupressoides var. lycopodium, AJ512466
FL246
C. cupressoides, AJ417929
FL014, FL018, FL041, FL046
FL060, FL132, FL167, FL176
FL196, FL203, FL204, FL222
FL0224, FL225, FL229, FL237
C. serrulata, AJ417931
C. serrulata, AJ417932
C. serrulata var. serrulata, AJ512411
C. serrulata var. serrulata, AJ512412
C. serrulata, AJ417933
CA033, CA038, CA042, CA068
FL107, FL110, FL144
C. cupressoides var. flabellata, AJ417930
C. scalpelliformis, AJ417971
C. taxifolia, AJ417936
C. taxifolia, Agua Hedionda, Carlsbad, CA
C. taxifolia, AJ417937
C. taxifolia, AJ417938
CA039, CA057, CA061, CA067, FL131
C. taxifolia, AJ417939
C. distichophylla, AJ417940
C. ashmeadii, AJ417941
CA007, FL064, FL065, FL121
FL130, FL171, FL213, FL216
FL066
FL215
Caulerpa sp., AJ417962
C. prolifera, AJ417942
C. prolifera f. zosterofolia, AJ417943
CA008, CA031, FL053, FL054, FL085, FL119, FL140, FL157
FL205, FL206
FL223, FL242
FL174, FL184, FL 188, FL201, FL202, FL243, FL249, FL252,
C. scalpelliformis var. denticulata, AJ417972 \ FL261
CA018, FL145
FL101
C. brachypus
C. sertularioides, AJ417944
C. sertularioides, AJ417946
C. sertularioides, AJ417945
C. sertularioides f. longipes, AJ512419 & AJ512421
C. sertularioides f. brevipes, AJ512420 & AJ512422
CA037, FL002, FL004, FL007, FL026
FL027, FL032, FL033, FL036, FL043
FL044, FL051, FL076, FL109, FL117
FL136, FL163, FL181, FL182, FL187
FL228, FL236, FL247, FL254, FL259
FL079
FL178
C. racemosa var. macrophysa, AJ417947
C. racemosa var. macrophysa, AJ417956
FL030, FL045, FL160, FL161
C. racemosa var. mucronata, AJ512417
CA014, CA058, CA060
CA065, FL150, FL263
C. racemosa var. mucronata, AJ512418
FL158, FL159
FL108
CLADE I
C. mexicana, AJ417951 & AJ417952
FL003, FL005, FL008, FL016, FL017, FL020, FL048, FL050,
FL025 \ FL069, FL075, FL077, FL103, FL124
FL049
FL141, FL155, FL156, FL177, FL191, FL192, FL195, FL198
FL199, FL214, FL221, FL232, FL233, FL251, FL257, FL260
C. mexicana, AJ41753
FL068
C. racemosa var. peltata, A9J417948
C. racemosa, AJ417950
CA005, CA053, FL023, FL024, FL029, FL055, FL057, FL133
FL154, FL180, FL183, FL189, FL209, FL210, FL238, FL245,
FL011 \ FL248, FL250, FL262, FL264, FL265, FL266
FL239
FL116
C. racemosa var. peltata, AJ417949
C. racemosa var. laetevirens, AJ512415
C. racemosa var. laetevirens, AJ512416
CA023
CA056
FL120
FL010
CLADE II
C. selago, AJ417973
C. racemosa var. turbinata, AJ417957
C. racemosa var. lamourouxii, AJ417954
C. racemosa var. racemosa, AJ512413, AJ512423, AJ512424,
CA040 \ AJ51225 & AJ512467
C. racemosa var. occidentalis, AJ417955
CA034
CLADE III
C. filiformis, AJ417964
C. brachypus, AJ417934 = C. biserrulata (GenBank error)
C. subserrata, AJ417935
C. webbiana var. pickeringii, AJ417966
C. webbiana, AJ417958
C. lamuginosa, AJ417959
FL166, FL207, FL208
C. paspaloides, AJ417965
FL013, FL072, FL078, FL080, FL081, FL118, FL172,
FL240 \ FL173, FL190
FL241
FL220
C. microphysa, AJ417961
CA024
CA026
CA041, CA045, FL040, FL105, FL112
FL126, FL142, FL147, FL148, FL149, FL256
FL146TUF
C. cactoides, AJ417969
C. geminata, AJ417968
C. geminata, AJ417960
C. verticillata, AJ417967
FL006, FL009, FL015, FL021
FL168
FL197
FL175
C. flexilis, AJ417970
C. flexilis, AJ512426
CA049
Caulerpella ambigua, AJ417963

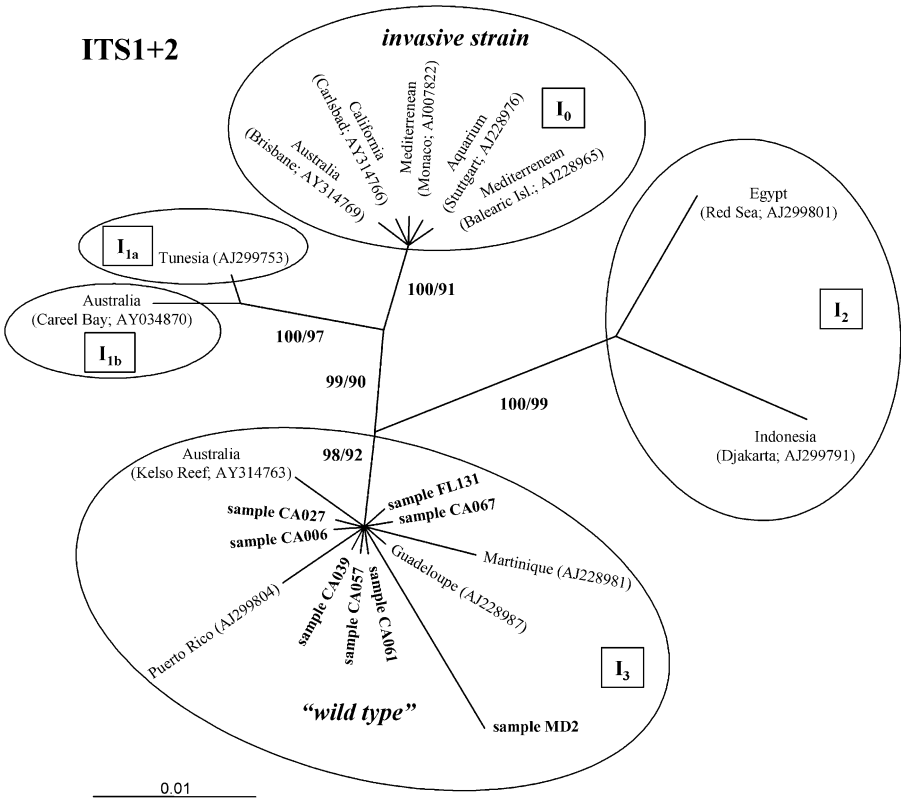



FIG. 3. Bayesian phylogenetic tree based on ITS1 sequences from *Caulerpa taxifolia*. The analysis was run under the HKY + G model of sequence evolution for $5 \times 10^6$ generations with a burn-in of $1 \times 10^6$ generations. Reference sequences are indicated by their sample location and GenBank accession number (Jousson et al. 1998, 2000, Olsen et al. 1998, Schaffelke et al. 2002, Meusnier et al. 2004). Other samples are indicated by their code (see Tables 1 and 3). For numbers along branches, see legend to Figure 2. Boxed symbols refer to ITS indelotypes (Meusnier et al. 2004), type $I_3$ being characteristic for the "native/wild type" and type $I_0$ for the invasive strain. Scale bar, expected changes per site.

were *C. taxifolia* and all 17 *C. racemosa* samples were "native/wild type."

In California, Zaleski and Murray (2006) surveyed >50 saltwater aquarium shops in Orange, San Diego, and Los Angeles counties between 2000 and 2001. *Caulerpa* was sold in 52% of the shops. *C. racemosa, Caulerpa serrulata,* and *C. taxifolia* were the most common among the 14 *Caulerpa* species available. Of the 68 samples, we sequenced from these older purchases (before California legislation), the six *C. taxifolia* samples all belonged to the "native/wild type." Among the 19 *C. racemosa* samples, three (CA034, CA035, and CA040) belonged to Clade III (Fig. 4) but only one (CA034) can be considered an invasive type being a member of the *C. racemosa* v. *cylindracea* subclade.

Caulerpa *availability via e-commerce.* The availability of *Caulerpa* species through e-commerce is enormous as documented by Walters et al. (2006) in their 2003–2005 survey of 90 internet sites through the US and UK. Twelve species were available, with *C. racemosa* (35%), *C. sertularioides* (20%), *C. prolifera* (15%–20%), *C. mexicana* (10%), and *C. serrulata* (10%) being the most common. *C. taxifolia* was encountered only once. Of the 53 samples we sequenced from these e-commerce sources, none of the *C. racemosa* or *C. taxifolia* samples belonged to the established invasive strains of these species.

*Detecting source populations.* Biogeographic affiliations of the >120 aquarium shop and e-commerce samples remain inconclusive in most cases because of either inadequate geographic sampling in the data base of sequences or insufficient resolution provided by the markers. For example, *C. sertularioides* differs by <1% in ITS1 between Pacific and Caribbean sources but only four accessions are available. In the case of *C. racemosa,* the situation is further complicated by the fact that the taxon is polyphyletic with three clades, none of which correspond to regional geographic locations. Of the 53 purchased samples, 50 belonged to Clades I and II ("native/wild types") and three belonged to Clade III (which includes the subclade with the known invasive strain) (Fig. 2). Within Clade III (Fig. 4), CA034 belongs to the invasive subclade, whereas CA040 and CA035 do not. In short, identifying a sample as belonging to Clade III is still insufficient to determine whether or not it is

FIG. 2. Bayesian consensus tree based on *tuf*A sequences. The analysis was run under the GTR + I + G model of sequence evolution for $5 \times 10^6$ generations with a burn-in of $1 \times 10^6$ generations. Species names and GenBank accession numbers are given for the reference sequences (Famà et al. 2002, Senerpont Domis et al 2003). Other samples are indicated by their code (see supplemental Tables 1 and 2). Numbers above branches are Bayesian posterior probabilities (left number, $\geq$ 90%) and bootstrap values (right number; $\geq$ 50%). The tree is rooted with *Caulerpella ambigua* (Famà et al. 2002). Clades I–III refer to paraphyly of *Caulerpa racemosa*. *, clades with documented invasive strains.

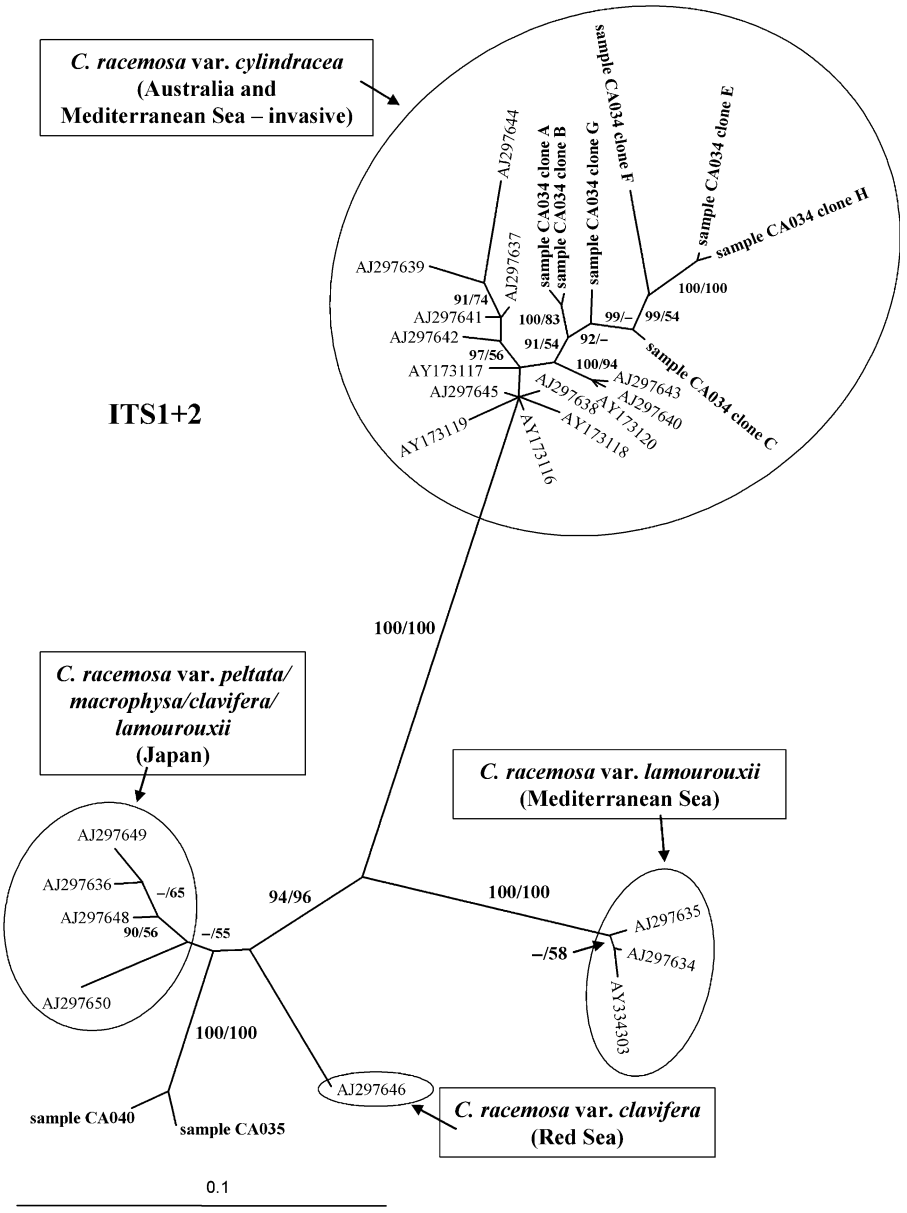



FIG. 4. Bayesian phylogenetic tree based on ITS1 sequences from *Caulerpa racemosa* clade III (Fig. 2). The analysis was run under the HKY + G model of sequence evolution for $5 \times 10^6$ generations with a burn-in of $1 \times 10^6$ generations. Reference sequences are indicated by their GenBank accession number (Verlaque et al. 2003). For numbers along branches, see the legend to Figure 2. For sample CA034, all seven cloned sequences (clones A–C and E–H) are included. Scale bar, expected changes per site.

the known invasive strain. By analogy, this is also true for *C. taxifolia*, in which additional information is needed from indel patterns of the ITS1 (Meusnier et al. 2004).

Of the ~20 species recognized in the Caribbean, almost all are also found in the Pacific Ocean. In our surveys, we did not find any exclusively Pacific Ocean species, of which there are ~30. This does not rule out the possibility, however, that a cosmopolitan species detected by us could have originally been obtained from the Pacific Ocean.

*Misidentification rates and other mistakes.* Error types and rates are summarized in Table 2. Sequencing was successful in 93.8% of the 258 samples; the remaining 6.2% (16 samples) were contaminated with other organisms for one or both sequences. The amplification of ITS is particularly prone to picking up contaminants because the primers are universal. In 50% of the cases where ITS contaminations were found, the *tuf*A gene could not be amplified, which indicates insufficient algal DNA in the extraction.

Misidentification occurred in 38 of the remaining 241 samples (15.7%), of which 12.4% were due to morphological misidentification. We found this surprisingly high given that all of us have considerable professional experience with *Caulerpa* identification. The most common mix-up involved *C. racemosa* with other "grapelike" morphologies associated with varieties of *C. racemosa* v. *macrophysa* and *Caulerpa . microphysa*. Confusion among "featherlike" morphologies involved *Caulerpa ashmeadii*, *C. sertularioides*, *C. mexicana*, and *C. taxifolia* (e.g. FL131 originally identified as

*C. ashmeadii,* which proved to be *C. taxifolia*) also occurred. In contrast, <1% (two cases involving CA005 and FL209) of the misidentifications were due to sequencing mistakes (lack of agreement between ITS and *tuf*A). GenBank errors were discovered in four cases (as discussed above for *C. brachypus*) but also involving accession numbers AJ228984–AJ228987, which are not *C. taxifolia,* but *Caulerpa cupressoides*. Sample FL046 was unambiguously identified as *C. cupressoides* based on morphology, *tuf*A, and ITS (Fig. 2 and Supplemental Table 2). The ITS sequence of this sample aligned with the above GenBank accessions. These sequences were obtained by cloning ITS fragments from one sample collected in Japanese waters (Jousson et al. 1998). The odd position in the phylogenetic reconstruction presented by Jousson et al. (1998) can now be explained. The Japanese sample was misidentified—it is *C. cupressoides* not *C. taxifolia*. Finally, clerical mistakes were minor, occurring in only one case.

## DISCUSSION

*The interactive value of phylogeny.* The *tuf*A tree (Fig. 2) represents the most comprehensive phylogeny for *Caulerpa* currently available, having more than quadrupled the prior number of *tuf*A sequences from 64 to 268. This has had the effect of stabilizing clades and identifying and/or confirming polyphyletic taxa (e.g. *C. cupressoides, C. serrulata, C. scalpelliformis*, and *C. racemosa*). We have also shown, for the first time, that *C. brachypus* is the probable sister-group to one of the clades of *C. scalpelliformis* (*C. scalpelliformis* var. *denticulata* (Dacaisne) Weber-van Bosse from the Mediterranean Sea), rather than to *C. subserrata*. The 21 species in the tree represent about 30% of described species with the skew toward Caribbean/Atlantic Ocean representatives. A goal of baseline molecular surveys is to increase worldwide and regional sampling to the point that both geographic and invasive identification can be performed almost automatically. At present, we are still very far away from achieving this DNA-barcoding objective for *Caulerpa*.

The subanalysis of ITS1 + 2 for *C. taxifolia* (Fig. 3) and *C. racemosa* (Fig. 4) involving Florida field samples confirmed that the recognized invasive forms of *C. taxifolia* and *C. racemosa* were not present. This, however, was not the case for an aquarium shop purchase of *C. racemosa* (sample CA034) made in 2001. Because ITS has the power to identify geographic populations, any species displaying potentially invasive behavior should definitely be investigated with these sequences.

Being able to distinguish genotypes is critical to both detection and to understanding the potential underlying causes for observed changes in local abundance that may or may not be connected to an introduction. For example, a number of researchers have reported abnormally high abundances of indigenous *Caulerpa* species in their local area (e.g. *C. verticillata* [Raloff 2000], *C. cupressoides* [J. L. Olsen, unpublished observations in Pago Bay, Guam, USA]), leading to concerns that these species might be becoming invasive. In these cases (at least so far), there has been no way of knowing whether the increased abundances were due to a foreign genotype, thus lending support to the possibility of competitive superiority, or the result of proximal ecological changes, such as eutrophication, competition, or predation affecting the indigenous genotypes.

Genetic assessment of local populations can also uncover evidence for clonality, lower diversity, and/or inter-population hybridization, which are often associated with introductions. The latter has been shown in many higher plant studies (Ellstrand and Schierenbeck 2000) and at least provisionally in *C. taxifolia* (Meusnier et al. 2004). Understanding how invasiveness evolves will require large amounts of data of this type (Grosholz 2002, Lee 2002).

In terms of practical considerations, DNA sequencing of the *tuf*A and/or ITS1 + 2 is easy and cost-effective. The DNA extraction and PCR amplification can be performed in most labs, while the sequencing itself can be commercially outsourced at between US$3 and 5 per sequence. Access to data management and analysis of sequences is all public domain.

Whereas *tuf*A provides easy identification of the species clades for the entire genus, ITS1 + 2 is still required for identification of intraspecific invasive forms within specific clades, which necessitates a cloning step. Moreover, the peculiarities of concerted evolution affecting ITS sequence homogenization (see references in Materials and Methods) make it necessary to assess intraspecific variation in new accessions. We caution that identification of the Mediterranean invasive strain of *C. taxifolia* by PCR ITS-length variation alone is insufficient because some "native/wild-type" strains of *C. taxifolia* also have deletions, but these deletions differ from those described for *C. taxifolia* (Meusnier et al. 2004). Although an intermediate screening step using single-strand conformation polymorphisms (SSCP; allowing detection of sequence difference among fragments of the same length or nearly the same length) might be considered, in terms of time, equipment, and expertise, sequencing remains the easiest and most valuable tool for management and research communities.

*Why the entire genus needs to be banned.* At the US Federal level, the *C. taxifolia*-Mediterranean clone (now "aquarium strain") was added to the USDA's APHIS-Noxious Species List in 1999. The State of California imposed stricter regulations by banning nine species of *Caulerpa* (*C. taxifolia, C. mexicana, C. sertularioides, C. ashmeadii, C. floridana* W. R. Taylor, *C. cupressoides, C. racemosa, C. scalpelliformis*, and *C. verticillata*) in 2001 (Anonymous 2005). The city of San Diego, CA, subsequently banned all species in the genus. After nearly 5 years, the Aquatic Nuisance

Species (ANS) Task Force released a National Management Plan for the genus *Caulerpa* in the Fall of 2005. The plan focuses on supporting *Caulerpa* research and outreach, and the USDA APHIS is considering whether to enhance regulation of the entire genus.

On the basis of the present and companion studies (Walters et al. 2006, Zaleski and Murray 2006), we recommend that the entire genus be banned including the sale of "live rock." We base this recommendation on the following considerations: first, morphological identification of *Caulerpa* is unreliable and prone to significant error rates (Olsen et al. 1998, Verlaque et al. 2003) that cannot be overcome by better training or closer scrutiny. Virtually every *Caulerpa* paper published over the past decade has bemoaned the ongoing problems of morphological identification that are due to morphological plasticity and the presence of multiple intermediate forms that are not environmentally stable (Senerpont Domis et al. 2003). In addition, the recognition of sub-specific varieties and forms (e.g. within *C. racemosa*) further exacerbates identification efforts by artificially elevating these infraspecific taxa to the species level in the minds of non-experts. The 15.7% error rate documented in the present study is certainly an underestimation of what could be expected from state and federal agencies that rarely employ experts on marine seaweeds. Morphological misidentifications from aquarium and internet sites were as high as 90%, reflecting both ignorance and intentional mislabeling of completely different species as a marketing strategy (e.g. the red alga *Botryocladia occidentalis* [Boergesen] Kylin as "red grape *Caulerpa*") (Walters et al. 2006). Second, *Caulerpa* is a common component of "live rocks," which are pieces of coral rubble containing the propagules of many organisms. Retailers have no way of knowing whether or not they are stocking *Caulerpa* in this form. *C. racemosa, C. sertularioides, C. mexicana*, and *C. verticillata* were identified from "live rock" samples by Walters et al. (2006) along with 53 other marine species. *Caulerpa* was encountered in 17% of the cases after 1-month aquarium culture. Third, and perhaps most crucially, it is not clear what the capacity for invasive behavior is. The invasive capacity of *C. taxifolia* and *C. racemosa* are well documented, but what about the other species of *Caulerpa*? On the one hand, invasion success is a function of intrinsic factors such as clonal reproduction (Ceccherelli and Cinelli 1999, Smith and Walters 1999), facultative heterotrophy (Williams 1984, Chisholm et al. 1996), and rapid growth rates—all of which are characteristic of *Caulerpa*, as well as extrinsic factors associated with the recipient habitat including eutrophication, lack of competition, and possible cross-fertilization with local indigenous populations/species. Reproductive barriers are probably weak in *Caulerpa* such that contact between normally isolated biogeographic populations may lead to hybridization and introgression with unknown fitness effects (Meusnier et al. 2002, 2004). Furthermore, the fact that *Caulerpa* species "mass" spawn (Clifton and Clifton 1999) with only very slight temporal differences means that new contacts of normally separated regional populations, with different spawning regimes, might disrupt this delicate barrier. It is already known that impacts associated with climate change operate through the life history of a species so that viability as well as timing of reproduction may be adversely affected. For example, such effects have been documented in northward range extensions of barnacles (Herbert et al. 2003).

On the other hand, invasion success is also a function of propagule pressure, i.e. the frequency and dosage of repetitive introductions into a non-native area (Levine 2000, Kolar and Lodge 2001, Lockwood et al. 2005). The view until recently has been that introductions of *Caulerpa* have been point events involving one or a few escapes of the genetically identified invasive aquarium strains from Public Aquaria (Mediterranean case) or home hobbyists who purchased the "aquarium strain" of *C. taxifolia* (southern California case) (Jousson et al. 1998, Wiedenmann et al. 2001, Meusnier et al. 2002, Verlaque et al. 2003, 2004). It is now recognized that e-commerce sites may be the most important vectors of dispersal—even more than aquarium shops (Walters et al. 2006, Zaleski and Murray 2006). In our survey of >100 shops and 90 internet sites, more than half carried *Caulerpa* species, thus drastically increasing the risk of escape and/or intentional introduction in this form of propagule pressure. Aquarium dumping is well documented in Florida waters, with some 16 non-native fish species identified (Semmens et al. 2004). Hobbyists using *Caulerpa* as an ornament in their aquaria are confronted with the need for constant thinning, given the alga's rapid growth rate. This, in turn, provides added opportunities for hobbyists to sell their extra stock on online auctions or to dispose of the *Caulerpa* in nearby waters. The latter case is the probable cause of the California invasions in both Aqua Hedionda and Huntington Harbor (Woodfield and Merkel 2004).

With the above three points in mind, the "presumption of innocence" principle applied to *Caulerpa* species that have not yet shown invasive behaviors puts a heavy burden on stakeholders as the "polluter pays" principle is not in place and virtually impossible to enforce (Perrings et al. 2005). Given that risk assessments are always judgment calls, it seems to us that a bit of the "precautionary principle" is just good common sense in the case of *Caulerpa*, where there is well-established evidence for invasiveness. This is a doubly important consideration for the state of Florida because of its extensive coastline and large number of coastal residents.

The fact that *C. taxifolia* was rarely encountered via e-commerce and never in the Florida aquarium shops that we surveyed is somewhat heartening and suggests that the laws and public awareness are working to some extent. Unfortunately, the same cannot be said for *C. racemosa*, which is the most widely available species from all sources. Zaleski and Walters currently

have funding to develop a public outreach campaign to inform citizens more generally about the vulnerability of Florida's waters to marine algal invasions, and to enlist cooperation from aquarium hobbyists and dive clubs in keeping an invasive species "watch".

Grosholz (2002) identified two major obstacles to understanding invasion biology: uncertainty about which species are native and which are introduced; and the spatial and temporal scales of genetic changes in coastal and regional populations. The present study has taken the first step in identifying populations of *Caulerpa* around the Florida coasts. Understanding the interplay between gene flow and selection in a metapopulation context remains a challenge.

In conclusion, our survey of field, aquarium shop, and e-commerce samples of *Caulerpa* has doubled the available data base. It has also established a baseline of local genotypes in Florida waters and placed them in a phylogenetic framework for the entire genus. Finally, our survey highlights the need for a full ban on the genus in conjunction with the unreliability of morphological identification and wide availability of *Caulerpa* species in aquarium shops and via internet commerce.

We thank S. A. Boele-Bos, J. H. Veldsink for technical assistance and the students J. de Bie, D. Beugeldsijk, B. Hogerdijk, T. Janzen, L. Lammerts, L. Moria, M. Poortvliet, M. Smelter, S. Smith, B. Westerhof, P. Wolfs, E. van der Zee from the undergraduate course Marine Biology 2, University of Groningen. In Florida and the Caribbean, K. Beach, C. Glardon, M. Black, A. Simpson, S. Reed, I. Kuffner, Sea World, and the UCF Dive Club assisted with collections. We thank L. McCauley at the University of Central Florida for technical assistance with the GIS mapping program. This research was supported by the National Fish and Wildlife Foundation/Pinellas County Environmental Fund, the National Sea Grant Aquatic Nuisance Species Research Program (project number R/C E-49), the Tampa Bay Estuary Program Mini-Grant, the Florida Department of Agriculture, the University of Central Florida, the University of Tampa, Florida Institute of Oceanography through cruise time aboard the *R/V Bellows*, the National Sea Grant College Program of the US Department of Commerce's National Oceanic and Atmospheric Administration under NOAA (grant #NA66RG0477) administered by University of Southern California Sea Grant (project number R/CZ-63PD), and the California Sea Grant College Program (grant #NA16RG2256).

Anonymous. 2005. California Laws & Regulations http://www.invasivespeciesinfo.gov/laws/state/ca.shtml

Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W. & Lipman, D. J. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* 25:3389–402.

Ceccherelli, B. & Cinelli, F. 1999. The role of vegetative fragmentation in dispersal of the invasive alga *Caulerpa taxifolia* in the Mediterranean. *Mar. Ecol. Prog. Ser.* 182:299–303.

Chisholm, J. R. M, Dauga, C., Ageron, E., Grimont, P. A. D. & Jaubert, J. M. 1996. "Roots" in mixotrophic algae. *Nature* 381:382.

Clifton, K. E. & Clifton, L. M. 1999. The phenology of sexual reproduction by green algae (Bryopsidales) on Caribbean coral reefs. *J. Phycol.* 35:24–34.

Davis, A. R., Roberts, D. E. & Cummins, S. P. 1997. Rapid invasion of a sponge-dominated deep-reef by *Caulerpa scalpelliformis* (Chlorophyta) in Botany Bay, New South Wales. *Austral. J. Ecol.* 22:146–50.

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. *J. Evol. Biol.* 15:122–33.

Ellstrand, N. C. & Schierenbeck, K. A. 2000. Hybridization as a stimulus for the evolution of invasiveness in plants. *Proc. Natl. Acad. Sci. USA* 97:7043–50.

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). *Eur. J. Phycol.* 35:349–56.

Famà, P., Kooistra, W. C. H. F. & Zucarello, G. 2002. Molecular phylogeny of the genus *Caulerpa* (Caulerpales, Chlorophyta) inferred from chloroplast *tuf*A gene. *J. Phycol.* 38:1040–50.

Grosholz, E. (2002). Ecological and evolutionary consequences of coastal invasions. *TREE* 17:22–7.

Guiry, M. D., Rindi, F. & Guiry, G. M. 2005. *Algae Base Version 4.1*. World-wide electronic publication, National University of Ireland, Galway, http://www.algaebase.org/

Hall, T. A. 1999. Bioedit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp. Ser.* 41:95–8.

Herbert, R. J. H., Hawkins, S. J., Sheader, M. & Southward, A. J. 2003. Range extension and reproduction of the barnacle *Balanus perforatus* in the eastern English Channel. *J. Mar. Biol. Assoc. UK* 83:73–82.

Hochberg, M. E. & Gotelli, N. J. 2005. An invasions special issue. *Trends Ecol. Evol.* 20:211.

Jacoby, C., Lapointe, B. & Creswell, L. 2004. *Are Native and Non-indigenous Seaweeds Overgrowing Florida's East Coast Reefs*? Florida Sea Grant College Program SGEF-156.

Jousson, O., Pawlowski, J., Zaninetti, L., Meinesz, A. & Boudouresque, C. F. 1998. Molecular evidence for the aquarium origin of the green alga *Caulerpa taxifolia* introduced to the Mediterranean Sea. *Mar. Ecol. Prog. Ser.* 172:275–80.

Jousson, O., Pawlowski, J., Zaninetti, L., Zechman, F. W., Dini, F., Di Giuseppe, G., Woodfield, R., Millar, A. & Meinesz, A. 2000. Invasive alga reaches California. *Nature* 408:157–8.

Kolar, D. S. & Lodge, D. M. 2001. Progress in invasion biology: predicting invaders. *Trends Ecol. Evol.* 16:199–204.

Lee, E. C. 2002. Evolutionary genetics of invasive species. *Trends Ecol. Evol.* 17:386–91.

Levine, J. M. 2000. Species diversity and biological invasions: relating local process to community pattern. *Science* 288:852–4.

Littler, D. S. & Littler, M. M. 2000. *Caribbean Reef Plants*. OffShore Graphics Inc., Washington, DC, 542 pp.

Lockwood, J. L., Cassey, P. & Blackburn, T. 2005. The role of propagule pressure in explaining species invasions. *Trends Ecol. Evol.* 20:223–8.

Meinesz, A. & Hesse, B. 1991. Introduction et invasion de l'algue tropicale *Caulerpa taxifolia* en Méditerranée Nord-occidentale. *Oceanol. Acta* 14:415–26.

Meinesz, A., Belsher, T., Thibault, T., Antolic, B., Ben Mustapha, B., Chiavarini, C., Cinelli, F., Cottalorda, J., Djellouli, A., El Abed, A., Orestano, C., Grau, A., Ivesa, L., Jaklin, A., Langar, H., Masutti-Pascual, E., Peirano, A., Tunesi, L., De Vaugelas, J., Zavodnik, N. & Zuljevic, A. 2001. The introduced green alga *Caulerpa taxifolia* continues to spread in the Mediterranean. *Biol. Invas.* 3:201–10.

Meusnier, I., Olsen, J. L., Stam, W. T., Destombe, C. & Valero, M. 2001. Phylogenetic analyses of *Caulerpa taxifolia* (Chlorophyta) and of its associated bacterial microflora provide clues to the origin of the Mediterranean introduction. *Mol. Ecol.* 10: 931–46.

Meusnier, I., Valero, M., Destombe, C., Godé, C., Desmarais, E., Bonhomme, F., Stam, W. T. & Olsen, J. L. 2002. Polymerase chain reaction—single strand polymorphism analyses of nuclear and chloroplast DNA provide evidence for recombination, multiple introductions and nascent speciation in the *Caulerpa taxifolia* complex. *Mol. Ecol.* 11:2317–25.

Meusnier, I., Valero, M., Destombe, C., Olsen, J. L. & Stam, W. T. 2004. Invasive algal species splits: successive deletions in the rDNA ITS1 suggests nascent speciation in *Caulerpa taxifolia*. *Eur. J. Phycol.* 39:83–92.

Nyberg, C. D. & Wallentinus, I. 2005. Can species traits be used to predict marine macroalgal introductions? *Biol. Invas.* 7:265–79.

Olsen, J. L., Valero, M., Meusnier, I., Boele-Bos, S. A. & Stam, W. T. 1998. Mediterranean *Caulerpa taxifolia* and *C. Mexicana* (Chlorophyta) are not conspecific. *J. Phycol.* 34:850–6.

Perrings, C., Dehnen-Schmutz, K., Touza, J. & Williamson, M. 2005. How to manage biological invasions under globalization. *Trends Ecol. Evol.* 20:212–5.

Posada, D. & Crandall, K. A. 1998. Modeltest: testing the model of DNA substitution. *Bioinformatics* 14:817–8.

Raloff, J. 2000. Algal bloom is smothering Florida coral. *Sci. News* 157:373.

Ronquist, F. R. & Huelsenbeck, J. P. 2003. Mrbayes 3.1: bayesian phylogenetic inference under mixed models. *Bioinformatics* 19:1572–4.

Ruiz, G. M. & Carlton, J. T. 2003. *Invasive Species: Vectors and Management Practices*. Island Press, Washington, DC, 536 pp.

Schaffelke, B., Murphy, N. & Uthicke, S. 2002. Using genetic techniques to investigate the sources of the invasive alga *Caulerpa taxifolia* in three new locations in Australia. *Mar. Poll. Bull.* 44:204–10.

Semmens, B. X., Buhle, E. R., Salomon, A. K. & Pattengill-Semmens, C. V. 2004. A hotspot on non-native marine fishes: evidence for the aquarium trade as an invasion pathway. *Mar. Ecol. Prog. Ser.* 266:239–44.

Senerpont Domis, L. N. de, Famà, P., Bartlett, A. J., Prud'homme van Reine, W. F., Espinosa, C. A. & Trono, G. C. Jr. 2003. Defining taxon boundaries in members of the morphologically and genetically plastic genus *Caulerpa* (Caulerpales, Chlorophyta). *J. Phycol.* 39:1019–37.

Silva, P. C. 2002. Overview of the Genus *Caulerpa*. *Proceedings of the International Caulerpa Conference*, San Diego, CA, p. 11.

Smith, C. M. & Walters, L. J. 1999. Fragmentation as a strategy for *Caulerpa* species: fates of fragments and implications for management of an invasive weed. *Mar. Ecol.* 20:307–19.

Swofford, D. L. 2003. *PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods). Version 4*. Sinauer Associates, Sunderland, MA.

Verlaque, M., Afonso-Carrillo, J., Candelaria Gil-Rodríguez, M., Durand, C., Boudouresque, C. & Le Parco, Y. 2004. Blitzkrieg in a marine invasion: *Caulerpa racemosa* var. *cylindracea* (Bryopsidales, Chlorophyta) reaches the Canary Islands (north-east Atlantic). *Biol. Invas.* 6:269–81.

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

Verlaque, M., Durand, C., Huisman, J. M., Boudouresque, C. F. & Parco, Y. 2003. On the identity and origin of the Mediterranean invasive *Caulerpa racemosa* (Caulerpales, Chlorophyta). *Eur. J. Phycol.* 38:325–39.

Verlaque, M. & Fritayre, P. 1994. Modifications des communautés algales méditerranéennes en présence de l'algue envahissante *Caulerpa taxifolia* (Vahl) C. Agardh. *Oceanol. Acta* 17:659–72.

Walters, L. J., Brown, K. R., Stam, W. T. & Olsen, J. L. 2006. E-Commerce and *Caulerpa*, unregulated dispersal of invasive species. *Front. Ecol. Environ.* 75–9.

Wiedenmann, J., Baumstark, A., Pillen, T. L., Meinesz, A. & Vogel, W. 2001. DANN fingerprints of *Caulerpa taxifolia* provide evidence for the introduction of an aquarium strain into the Mediterranean Sea and its close relationship to an Australian population. *Mar. Biol.* 138:229–34.

Williams, S. L. 1984. Uptake of sediment ammonium and translocation in a marine green macroalga *Caulerpa cupressoides*. *Limnol. Oceanogr.* 62:766–71.

Woodfield, R. & Merkel, K. 2004. *Eradication and Surveillance of Caulerpa Taxifolia within the Agua Hedionda Lagoon, Carlsbad, California*. Third year status report. Merkel Associated, Inc, San Diego, 12 pp.

Zaleski, S. M. & Murray, S. N. 2006. Taxonomic diversity and geographic distribution of aquarium-traded species of *Caulerpa* (Chlorophyta, Caulerpaceae) in southern California, USA. *Mar. Ecol. Prog. Ser.* 314:97–108.

## SUPPLEMENTARY MATERIAL

The following supplementary material is available for this article online at www.blackwellsynergy.com

**Table S1.** *Caulerpa* sample list. Those coded CA were purchased from aquarium shops in California (see Zaleski and Murray 2006). Those coded FL include those collected from field sites in Florida, Honduras, US Virgin Islands and the Bahamas; in aquarium shops in Florida, and via e-commerce (see Walters et al. 2006). All CA samples were collected by S. M. Z. and all FL samples by K. R. B. and L. J. W., unless stated otherwise.

**Table S2.** Molecular analyses and identifications. Comparisons between initial morphological identification and subsequent identification based on DNA sequences of the nuclear rDNA Internal Transcribed Spacer 1 and 2 (ITS1 + 2) and chloroplast elongation factor TU gene (*tuf*A).