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NOTE

# Use of amplified fragment length polymorphism (AFLP) as a new tool to explore the invasive green alga *Caulerpa taxifolia* in Australia

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ABSTRACT: *Caulerpa taxifolia* is a high profile introduced alga in the Mediterranean Sea and California. It is native to tropical and subtropical regions of Australia. However, during 2000/01, 7 locations with abundant *C. taxifolia* were discovered in temperate waters around Sydney. To overcome limitations of resolution in previous studies using ITS (internal transcribed spacer) rDNA sequencing, we developed amplified fragment length polymorphism (AFLP) markers for *C. taxifolia*, using 4 primer combinations. With this technique we found a close relationship between isolates from Moreton Bay (native) and Lake Conjola (introduced). The Mediterranean samples, which previously could not be separated from the other isolates, formed a discrete cluster supported by high bootstrap values. AFLP had greatly improved resolution over other molecular markers, making it a promising technique for further studies on the population structure of *C. taxifolia* and the assignment of source populations.

KEY WORDS: AFLP · ITS · *Caulerpa taxifolia* · Introduced species · Source populations · Seaweed



Introduced marine species are of global concern because of their environmental and economic impacts on biodiversity, through habitat alteration and competition with native species. One of the best known introduced species is the green macroalga *Caulerpa taxifolia* (Vahl) C. Agardh (Ulvophyceae, Caulerpales). *C. taxifolia* is a robust, fast growing, popular ornamental plant in private, public and commercial aquaria. This species is assumed to have been introduced to the Mediterranean Sea in the 1980s and has since spread over 6000 ha ranging from the Balearic Islands to Croatia (Meinesz & Boudouresque 1996). The escape of a strain of this species from a public aquarium is believed to be the source of the Mediterranean introduction (Meinesz & Boudouresque 1996), and this strain is now known as the 'Aquarium-Mediterranean strain' (Jousson et al. 2000). In recognition of the risk of introduction of non-native *C. taxifolia*, Australia established an import ban on the species in1996. In 1999 the aquarium-Mediterranean strain was added to the US federal noxious weeds list (Meinesz 1999).

*Caulerpa taxifolia* is native to tropical and subtropical Australia with southern distribution limits recorded at Moreton Bay, southern Queensland (QLD) and Lord Howe Island (Lucas 1935, Cribb 1958, Lewis 1984). During 2000/01, *C. taxifolia* was discovered in California (Jousson et al. 2000, Kaiser 2000) and at 7 new Australian locations in the warm-temperate Sydney area (New South Wales, NSW). To receive Australian government funds for managing these incursions, it was essential to determine whether the new records were domestic translocations from native populations within Australia, or introductions from overseas.

Sequencing of the ITS (internal transcribed spacer) rDNA region has been the most commonly used method to identify source populations of *Caulerpa taxifolia*, allowing for comparison with a large number of published sequences (Jousson et al. 1998, Olsen et al. 1998, Meusnier et al. 2001). Jousson et al. (2000) identified *C. taxifolia* introduced to California as the aquarium-Mediterranean strain using ITS sequencing. Schaffelke et al. (2002) also used ITS sequencing to identify sources of 3 of the 7 new Australian *C. taxifolia* introductions, by comparison with isolates from the native range in northern Australia and published sequences. However, ITS sequences of isolates from an introduced location, Lake Conjola (NSW), and from the native Moreton Bay (QLD), were almost identical to sequences from Mediterranean and aquarium samples, and therefore a source region for the Lake Conjola introduction could not be assigned (Schaffelke et al. 2002).

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Other techniques used for identification of *Caulerpa taxifolia* to date include ssu (small subunit) sequencing of associated microflora (Meusnier et al. 2001), DNA digestion profile comparison (Wiedenmann et al. 2001) and allozyme analysis (Benzie et al. 2000). While these techniques remain useful for determining relationships on large biogeographic scales, they lack the resolution required for finer-scale analyses. For the invasive *C. taxifolia*, however, it has to be considered that failure to detect genetic differences may also be caused by isolates truly being originated from the same source populations.

Here, we report on the development and testing of a new molecular marker for *Caulerpa taxifolia*, Amplified fragment length polymorphism (AFLP). AFLP is a DNA fingerprinting technique based on selective PCR amplification of restriction fragments from a total digest of genomic DNA (Vos et al. 1995), and has been found to be an efficient method for studies of organisms in which insufficient variation is detected through sequence analysis.

**Materials and methods.** Specimens of *Caulerpa taxifolia* from Lake Conjola (NSW, introduced, n = 10), Moreton Bay (QLD, native, n = 10) and Villefranche-sur-Mer (France, introduced, n = 5), were collected and immediately dried in silica gel. Replicate samples were separated by at least 10 m and up to 15 km to ensure that different individuals were sampled. Voucher specimens are kept in the reference collection of the Centre for Research on Introduced Marine Pests (CRIMP) at the CSIRO Marine Laboratories in Perth, Australia.

Specimens were ground using mortar and pestle. Tissue was crushed in 700 µl CTAB extraction buffer (50 mM Tris, pH 8.0, 0.7 M NaCl, 10 mM EDTA, 1% hexadecyltrimethylammonium bromide, 0.1% 2-mercaptoehanol) using a pellet pestle mixer (Crown Scientific), prior to incubation at 65°C for 1 h with 100 µg $ml^{-1}$ Proteinase K. DNA was extracted using phenol and chloroform, precipitated in ethanol and eluted in 100 to 200 µl of Milli-Q water (Millipore). Extractions were checked on agarose gels and concentration was quantified using the GeneQuant pro RNA/DNA fluorometer (Amersham Pharmacia Biotech). Samples were equilibrated to 25 ng of DNA in 1 µl water.

AFLP fingerprints were generated using a modified protocol from Vos et al. (1995). Approximately 50 ng of DNA were digested with 6 U *Eco*RI and 5 U *Mse*I (New England Biolabs), 100 mM DTT (Amersham Pharmacia Biotech), 1 mg $ml^{-1}$ BSA (New England Biolabs), 100 mM spermidine (Sigma) and 1 mg $ml^{-1}$ RNase A (Amersham Pharmacia Biotech) in a total volume of 40 µl. Digests were incubated at 37°C for 6 h, with enzymatic activity terminated by incubation at 70°C for 15 s followed by cooling on ice.

An adapter ligation solution containing 1 U T4 DNA Ligase (Amersham Pharmacia Biotech), 10X Ligation Buffer (Amersham Pharmacia Biotech), 10 mM ATP (Amersham Pharmacia Biotech), 50 pmol $µl^{-1}$ *Eco*RI adapter, 250 pmol $µl^{-1}$ *Mse*I adapter and Milli-Q water to the volume of 10 µl was added to restriction digests and incubated at 37°C for 3.5 h. After incubation samples were diluted 1:10 with TE Buffer (10 mM Tris-HCl and 0.1 mM EDTA) (pH 8.0).

Five µl of each restriction ligation was then added to a preselective PCR mix containing 10X PCR buffer (Fisher Biotech), 25 mM $MgCl_2$ (Fisher Biotech), 10 mM each dNTPs (Fisher Biotech), 10 µM *Eco*RI (GACTGCGTACCAATTC)+A primer (*Eco*R1+A; Genset), 10 µM *Mse*I (GATGAGTCCTGAGTAA)+C primer (*Mse*I+C; Genset), 2 U *Taq* DNA polymerase (Fisher Biotech) and Milli-Q water to the volume of 20 µl. The PCR was performed in a Perkin Elmer 9700 thermal cycler for 20 cycles of: 94°C for 30 s, 56°C for 60 s and 72°C for 60 s. Preamplification PCR products were diluted 1:10 with TE Buffer.

Four primer combinations were used in selective PCR amplifications. These included 5′ Fam labelled *Eco*RI+AAC and 5′ Fam labelled *Eco*RI+AG each combined with *Mse*I+CTT and *Mse*I+CTA. 2.5 µl of each diluted preamplification sample was added to a selective PCR mix containing 10X PCR buffer, 25 mM $MgCl_2$, 10 mM each dNTPs, 10 µM *Eco*RI and *Mse*I primer (Genset), 0.5 U *Taq* DNA polymerase and Milli-Q water to the volume of 20 µl. The touchdown PCR commenced with a cycle of 94°C for 30 s, 65°C for 60 s and 72°C for 60 s. In subsequent cycles, the annealing temperature was reduced by 0.7°C per cycle until 56°C was reached, followed by 23 cycles at 56°C.

Selective PCR products were electrophoresed on a denaturing polyacrylamide-gel run on an ABI Prism 377 automated sequencer. Selective amplifications from the 4 primer combinations for all samples were replicated 3 times to test for reproducibility of results.

Fragment mobility was estimated by concurrent electrophoresing of a size standard (TAMRA 500, Automated Biosystems, Perkin Elmer) with each sample run. Digital profiles were visualised using ABI PRISM GeneScan 3.1 (PE Applied Biosystems) software. Fragments between 100 and 400 bp were scored using ABI PRISM Genotyper Version 1.1.1 (Perkin Elmer). Peaks were not scored if their height was less than 32% of the highest peak (in the sample), or if they were preceded by higher scored peaks within 1.6 bp or followed by higher scored peaks within 3 bp. Samples with fragments that were non-repeatable in replicate runs were excluded from the analyses.

Data generated by Genotyper software were analysed using the software package Peakmatcher 6.0 (GNU General Public License, Version 2). Peak-

matcher converts text-based Genotyper data into a presence/absence (1/0) table by identifying optimal markers based upon repeatability, with at least 2 replications of a sample required. Genotyper data from 3 replications for all samples were analysed using Peakmatcher. Markers that were present in all samples (monomorphic) were not considered informative and were removed from the data set.

Distance matrices (Nei's genetic distance) derived from presence/absence of AFLP fragments were analysed using UPGMA (unweighted pair group method with arithmetic means) clustering algorithms with the software package PHYLIP (phylogeny inference package) Version 3.57c (Felsenstein 1995, available at: http://evolution.genetics.washington.edu/phylip.html). The consensus tree was calculated using both majority rule and strict consensus methods and was drawn using the software package TreeView (Page 1996). Branches were tested using bootstrap re-sampling for 2000 replicates.

**Results and discussion.** AFLP allows simultaneous screening of different DNA regions distributed randomly throughout the genome (Mueller & Wolfenbarger 1999). Large numbers of replicable markers may be generated that can be used in high-resolution genotyping of fingerprinting quality (Mueller & Wolfenbarger 1999). Peakmatcher produced 13 (*Eco*RI+AAC/*Mse*I+CTT), 8 (*Eco*RI+AAC/*Mse*I+CTA), 24 (*Eco*RI+AG/*Mse*I+CTT) and 19 (*Eco*RI+AG/*Mse*I+CTA) polymorphic DNA markers. The phylogenetic analysis produced a tree with 2 distinct clades, supported by bootstrap values above 80% (Fig. 1).

*Caulerpa taxifolia* isolates from Lake Conjola and Moreton Bay clustered in 1 clade, with isolates from the Mediterranean comprising a second clade (Fig. 1). This is the first indication that the incursion to Lake Conjola is not related to the Aquarium-Mediterranean strain but likely to be a domestic translocation from Moreton Bay, or other populations in Australia with this genotype. However, a more detailed investigation using a larger number of locations and isolates is required to elucidate the population structure of *C. taxifolia* in Australia.

In previous studies, close relationships have been found between Australian and Mediterranean isolates of *Caulerpa taxifolia* (Benzie et al. 2000, Wiedenmann et al. 2001), with Australia proposed as the possible origin for the Mediterranean *C. taxifolia* populations (Jousson et al. 2000, Meusnier et al. 2001). However, our preliminary AFLP results show Mediterranean samples to be a clade well-separated from the Australian Moreton-Bay/Lake-Conjola clade (Fig. 1). Schaffelke et al. (2002) showed earlier, using ITS sequencing, that a number of other native Australian isolates are clearly distinct from Mediterranean *C. taxifolia*.

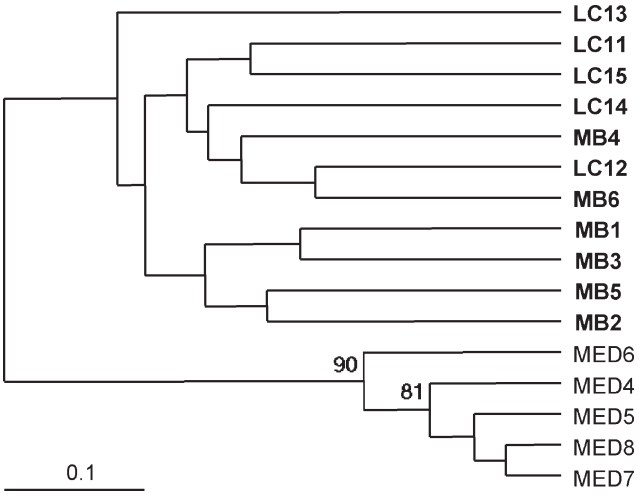


Fig. 1. *Caulerpa taxifolia*. Consensus tree after UPGMA (unweighted pair group method with arithmetic means) analyses of distance matrices derived from presence/absence of AFLP fragments. Numbers are bootstrap values above 80% (2000 replicates). Scale bar represents genetic distance between isolates or groups of isolates. Terminal forklengths are not informative. LC = Lake Conjola, New South Wales (introduced); MB = Moreton Bay, Queensland (native); MED = Villefranche-sur-Mer, France (introduced)

The hypothesis of an Australian origin of the Mediterranean *C. taxifolia* may be further progressed using AFLPs in a more comprehensive study.

Using AFLP allowed us to answer a specific question concerning a *Caulerpa taxifolia* incursion in Australia, which previously could not be resolved using ITS sequencing. External timeframes for decisions on incursion response options dictated that only a limited number of isolates could be analysed.

In conclusion, AFLP has the potential to be a highly useful marker for more detailed studies of the population structure of this invasive species. Future development of the technique will include further testing of the repeatability and of a greater number of diagnostic markers from different selective primer combinations on a larger sample size.

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