



A rapid method to score plastid haplotypes in red seaweeds and its use in determining parental inheritance of plastids in the red alga *Bostrychia* (Ceramiales)

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Abstract

A method for the rapid identification of plastid haplotypes in red algae is presented. This method is based on single-stranded conformation polymorphism (SSCP) to detect variation in DNA sequence of PCR-amplified plastid-encoded ribulose biphosphate carboxylase/oxygenase large and small subunit intergenic spacer. The method detects variation within a population of the mangrove red alga *Caloglossa leprieurii* from New South Wales, Australia. Three haplotypes (plastid variants) were easily scored in our samples, and a spatial variation of haplotypes within the intertidal is suggested. This method also reveals maternal inheritance of plastids in crosses between isolates of *Bostrychia radicans* and in multiple crosses between isolates of *B. moritziana*. Fluorescence microscopy shows that plastids are contained within the spermatia of these species and that bi- or paternal inheritance of plastids is possible. SSCP is a simple and rapid method for the detection of plastid variation (haplotypes) within populations of red algae that should also be applicable to brown seaweeds.

Introduction

Molecular methods for the study of evolution and population genetics are becoming increasingly prevalent in biology (Avice, 1994; Hillis et al., 1996), though their use in macroalgae is as yet limited. Population studies are important in community ecology research as aspects such as population variation, population subdivision, geneflow and recruitment can be addressed. The application of DNA-based molecular studies to the investigation of seaweeds has focused mostly on the use of DNA sequence to elucidate systematic and taxonomic problems. There are relatively few studies that have addressed issues below the species level and most of these have had a biogeographic focus (for example see Van Oppen et al., 1995; Van Oppen et al., 1996; Stache-Crain et al., 1997; Zuccarello & West, 1997; Kamiya et al., 1998). DNA

sequence methods have proved useful for phylogenetic reconstruction but they have the drawback that the large samples required for studies of diversity within populations cannot be screened quickly or cheaply.

Genetic studies on seaweeds that have determined variation at the population level, have relied mostly on protein electrophoresis (see Pearson & Murray, 1997; Benzie et al., 1997), although Van Oppen et al. (1995) were able to demonstrate the use of RAPD (randomly amplified polymorphic DNA) data in a study on the red alga *Phycodrys rubens*. Single-locus microsatellite markers have also been used successfully in a study of the population genetics of the red alga, *Gracilaria gracilis* (Wattier et al., 1997). Microsatellite methods are ideal for population genetic studies due to the high variability of microsatellite loci (i.e. multiple alleles). The development, however, of single-locus microsatellite markers can be expensive

and time consuming. These studies all point to the need for a generally applicable PCR-based method that can assess variation within populations.

DNA derived from organelles, and especially mitochondrial DNA (mtDNA), has proved to be extremely useful in population studies and phylogenetic reconstructions in animals (see Avise, 1994). The non-recombinant mode and rapid evolution of this genome often provides multiple haplotypes that can be ordered phylogenetically within a species and provide useful population genetic and demographic data. PCR primers for the highly variable control region of the mitochondrial genome are readily available (Hillis et al., 1996). Similar variable DNA regions are not known in the organelles of plants or algae with the usual method for scoring plastid haplotypes in plants being restriction fragment length polymorphism (RFLP) analyses (Avise, 1994; Hillis et al., 1996). This procedure, however, usually involves elaborate extraction procedures and protocols.

The red algae (Rhodophyta) and the brown algae (Phaeophyceae), have both the large subunit of the ribulose biphosphate carboxylase/oxygenase gene (*rbcL*) and the small subunit of the ribulose biphosphate carboxylase/oxygenase gene (*rbcS*) co-transcribed in the plastid (Kostrzewa et al., 1990; Fujiwara et al., 1993; Bhattacharya & Medlin, 1995), whereas the *rbcS* in green algae and land plants is encoded in the nucleus. The two plastid genes are separated by a short intergenic spacer region (Rubisco spacer). DNA sequence variations in this region have provided useful data for the reconstruction of algal inter- and intra-species phylogenies (Destombe & Douglas, 1991; Maggs et al., 1992; Bird et al., 1994; Goff et al., 1994; Stache-Crain et al., 1997; Zuccarello & West, 1997; Kamiya et al., 1998). In these studies, the intergenic region has been used to analyze isolates from different biogeographic locations, but in only one case did sequence data reveal variation at the population level (Zuccarello & West, 1997).

Hybridization is an important component in increasing the evolutionary potential of organisms (Rieseberg, 1997) and has been demonstrated in marine seaweeds (Scott & Hardy, 1994; Lewis & Neushul, 1995). Organelle DNA, mitochondrial and plastid, is thought to be inherited uniparentally in most organisms (Birky, 1995). Though plastid DNA is reported to be maternally inherited in most flowering plants (Corriveau & Coleman, 1988), its mode of transmission can vary and needs to be verified experimentally (Milligan, 1992). The pattern of organelle inheritance

in algae is less well understood. In the model green alga *Chlamydomonas*, plastid inheritance can vary (Sager, 1974) with certain species exhibiting predominantly uniparental plastid inheritance (mating-type plus strain in *C. reinhardtii*, Sears & Vanwinkle-Swift, 1994) while others have a predominantly biparental inheritance of plastids, (in *C. moewusii*, Lee & Lemieux, 1986).

The mode of plastid inheritance in the red algae has not been studied. Ultrastructural studies of male gametes (spermatia) are limited, and while some studies of spermatia reveal plastids, albeit with reduced or absent thylakoids (Kugrens & West, 1972; Cole & Sheath, 1980; Kugrens, 1980; Broadwater & Scott, 1983), others detail spermatia in which there are no plastids (Fetter & Neushul, 1981; Broadwater et al., 1991). The evidence, however, is ambiguous at best, as the presence of plastids in spermatia does no more than indicate the possibility of bi- or male plastid inheritance. As a result of this, only a DNA-based method, targeting plastid DNA, can be conclusive.

Methods for screening allelic variation in polymerase chain reaction amplified DNA, from the large sample sizes needed for studies of population biology, demography and phylogeny, are available (see Lessa & Applebaum, 1993). One of the simpler and less costly methods is single-stranded conformation polymorphism or SSCP. This method was first described by Orita et al. (1989). The method involves no special equipment beyond standard sequencing apparatus and has been improved and applied non-radioactively (Glavac & Dean, 1993; Hongyo et al., 1993). Although some of the latter techniques do involve special equipment (i.e. circulating temperature baths). The ability of the SSCP method to distinguish between mutant DNA fragments relies on the principles that the molecular conformation of single stranded DNA is nucleotide-sequence specific, that this conformation is altered by point substitutions, insertions and deletions, and that the altered conformation affects the mobility of the molecule to migrate through a polyacrylamide gel. This method has been reported to reveal up to 100% of the mutations in fragments of the 150–400 base pairs size.

In this study, we apply a rapid method of DNA extraction, Rubisco spacer PCR and SSCP techniques, to the study of genetic variation in the plastids of field collected material of *Caloglossa leprieurii* (Montagne) J. Agardh. Furthermore, this method was used to assess the inheritance of plastids in crosses within two different species of *Bostrychia* Montagne.

Materials and methods

Sample collection, preparation and DNA extraction

Caloglossa leprieurii was collected from Woollooware Bay, New South Wales (N.S.W.) on 10 June 1998. Pneumatophores of *Avicennia marina*, with attached algae, were collected from three 30 cm² quadrats 5 m apart perpendicular to the shoreline on an early morning low tide. Samples were placed in plastic bags and returned to the laboratory. Pneumatophores were washed in seawater and one plant of *C. leprieurii* was removed from each pneumatophore. The *C. leprieurii* used had approximately 4–5 blades each of 5–10 mm length. Due to the stoloniferous growth habit of this alga, individuals could not be recognized and only by sampling different pneumatophores could we be sure of collecting different individuals.

Bostrychia isolates [*Bostrychia moritziana* (Sonder ex Kützing) J. Agardh and *B. radicans* (Montagne) Montagne] were collected, isolated in unialgal culture and crossed as outlined previously in Zuccarello & West (1995).

DNA extractions from samples were performed using the Chelex-100 (Biorad, Hercules, California) protocol of Goff & Moon (1993). Thalli, approximately 5–15 mm of an epiphyte-free axis were placed in 100–200 µl of a 10 mM Tris (pH 8.0) solution containing 5% ChelexTM-100 resin (BIORAD, Hercules, Ca, U.S.A.) in a 1.5 ml microfuge tube. The Chelex solution was shaken vigorously before each aliquot was removed to ensure proper proportions of the resin. Tissue was ground in the microfuge tube using KontesTM microfuge tube grinders until the solution became colored (cells disrupted), and only small fragments of tissue were visible. To remove exogenous DNA grinders were sterilized by placing them in 10% bleach for an hour or more, and washing repeatedly in autoclaved double-distilled water until all bleach odor was eliminated. The samples were kept on ice before and after grinding. After grinding, samples were placed in a floating rack in boiling water for 10 min and then immediately placed on ice. Samples were centrifuged for 10 min and then the supernatant was carefully removed and placed in new marked microfuge tubes. Care was taken not to remove the Chelex resin or the unground tissue.

PCR reactions

Two sets of Rubisco spacer primers were used. For the *Caloglossa leprieurii* amplification the primers were:

forward primer 5' -TATACTTCTACAGACACAGCTG A - 3' (rbcF1), reverse primer 5' -ATTTACACAGGA AACAGCTATGACATGTCAAATAATGGTAGTCCC CA - 3' (rbcR2-M2). The sequence complementary to the M13 reverse sequencing primer was added to the latter primer to aid in future DNA sequencing (Kamiya et al., 1998). For *Bostrychia* amplification the primers were: forward primer 5' -TGTGGACCTCTACAAACAGC - 3', and reverse primer 5' -CCCCATAGTTCCCAAT - 3' (Maggs et al., 1992).

PCR was performed using radioactively end-labelled forward primer. Final end-labelling conditions were: 100 pmol of primer, 1–2 µl of gamma-[³³P]ATP (0.27 10⁹ Bq ml⁻¹), 1X reaction buffer and 3 units of T4 polynucleotide kinase in a 10 µl volume, incubated at 37 °C for 20 min and heat-inactivated at 95 °C for 5 min. Amplification reactions were performed in 10 µl volumes using the following final concentrations: 1X PCR buffer (PromegaTM buffer A), 120 nmol dNTP's, 2 mM MgCl₂, 2.5 pmol of each primer (labelled forward, unlabelled reverse), 0.25% BSA (Sigma, Cat no. A-2153) and 0.5 U *Taq* polymerase (PromegaTM) and 1 µl Chelex-extracted template DNA. Amplifications were performed under a variety of conditions, the final optimal conditions were: an initial denaturation was carried out at 94 °C for 4 min, followed by 5 cycles of 93°/45°/72° for 1 min each, 20 cycles of 93°/55°/72° for 1 min each and a final step 72 °C for 5 min.

SSCP (single-strand conformation polymorphism) analysis.

SSCP gels were poured in a standard sequencing gel box (IBI-Kodak STS-45 sequencing apparatus, 350×420×0.4 mm dimensions), with 64-well shark-tooth combs.

For SSCP, 2 µl of PCR product was mixed with 10 µl denaturing solution (98% formamide, 10 mM NaOH, 0.025% bromophenol blue, 0.025% xylene cyanol), heated to 95 °C for 5 min and placed directly on ice. Two µl of this denatured DNA was loaded onto a non-denaturing acrylamide gel consisting of 0.5 X MDE acrylamide (FMC Bioproducts) in 0.6 X TBE and electrophoresis carried out at 30 W for 12 hours at 4 °C cold room. In addition, the surface of the gel was cooled continuously by a blowing fan. Other SSCP conditions used included gels consisting of 4.5% acrylamide [37.5:1 acrylamide: bisacrylamide], 1 X TBE) and electrophoresis, in 1 X TBE, at 50W for

approximately 5 h (4 °C). Although these latter conditions also produced resolvable haplotypes, the degree of separation (i.e. resolution) was reduced. After electrophoresis, gels were dried on Whatmann paper and exposed to X-ray film (Hyperfilm MP, Amersham) for 12–24 h.

Results and discussion

The advantages of the Chelex/PCR/SSCP methodology over traditional methods of DNA extraction/PCR/Sequencing are multiple. Samples of both *Caloglossa* and *Bostrychia* from the field were easily selected and extracted, even from relatively small thalli (less than 1 cm in main axis length). This methodology is also very rapid, as large numbers of specimens (50) could be routinely collected, sorted and extracted in one half day. The ChelexTM extraction procedure is rapid and has been very successful in producing PCR amplifiable template DNA with various mangrove and non-mangrove red algae, predominantly within the order Ceramiales. PCR reactions can be performed the same day and SSCP gels run overnight. For PCR, it is also important to avoid too much tissue in the initial extraction as this can inhibit amplification. The addition of 0.25% BSA to the PCR mixture greatly increased the reliability of successful PCR amplifications from the field collected specimens which is probably due to the known effects of BSA in circumventing the inhibition effects of contaminants from environmental samples (Wintzingerde et al., 1997).

Gels made with MDE acrylamide provided the best resolution but are more expensive than normal acrylamide gels. Acrylamide gels (4.5% [37.5:1 acrylamide: bisacrylamide]) also gave satisfactory results, and occasionally, for other species, were able to resolve variation not apparent in the MDE gels. Both sets of gels were routinely used on our PCR amplifications. Room temperature gels, with and without 10% glycerol, did not resolve our particular products well, although not all combinations of concentrations (%) of acrylamide or cross-linker (bis-acrylamide) were tested (Glavac & Dean, 1993). It is also important to avoid heating of the gel by cooling with a fan and performing electrophoresis at low wattage.

Amplification products were between 250–350 base pairs in size, depending on the primer set used, which is an ideal size for SSCP analysis. Figure 1 shows a SSCP gel of three specimens of *Caloglossa*

Table 1. Haplotypes of parental strains and hybrid sporophytes of *B. radicans*. Culture numbers correspond to isolates from the J.A. West culture collection (for further details on these isolates see Zuccarello & West, 1997). Haplotype = arbitrary haplotype designation. Lane = gel lanes in Figure 3. n.a. = not applicable, i.e. parental strain. X = indicates cross between cultures, multiple crosses indicate different sporophytes derived from different released carpospores

Culture numbers			
Female	Male	Haplotype	Lane
3136VZ	n.a.	A	a
n.a.	3206MX	B	b
n.a.	3207MX	B	c
3136VZ X	3206MX	A	d
3136VZ X	3207MX	A	e
3207MX X	3136VZ	B	f
3017BZ	n.a.	C	g
n.a.	3043PE	D	h
3017BZ X	3043PE	C	i
3017BZ X	3043PE	C	j
3017BZ X	3043PE	C	k
3017BZ X	3043PE	C	l
3017BZ X	3043PE	C	m
3043PE X	3017BZ	D	n
3043PE X	3017BZ	D	o
3043PE X	3017BZ	D	p
3043PE X	3017BZ	D	q
3043PE X	3017BZ	D	r
3043PE X	3017BZ	D	s

leprieurii collected from a single transect in Woollooware Bay. Three haplotypes were easily resolved within this population. Not only was plastid variation uncovered by this method but a spatial variation in haplotype distribution is also indicated. Algal samples with three different haplotypes, in roughly equal proportions, were found in quadrat A, corresponding to the quadrat furthest from the low water mark. Two haplotypes are found in samples from quadrat B, though one haplotype is clearly dominant. In quadrat C, corresponding to the quadrat closest to the low water mark and only 10 m from quadrat A, only a single haplotype was observed from sampled algae. Though preliminary, this population and spatial variation of haplotypes shows that SSCP is able to detect haplotypes quickly and easily in the populations. These different haplotypes could correlate to genotypic/phenotypic adaptations (e.g. tol-

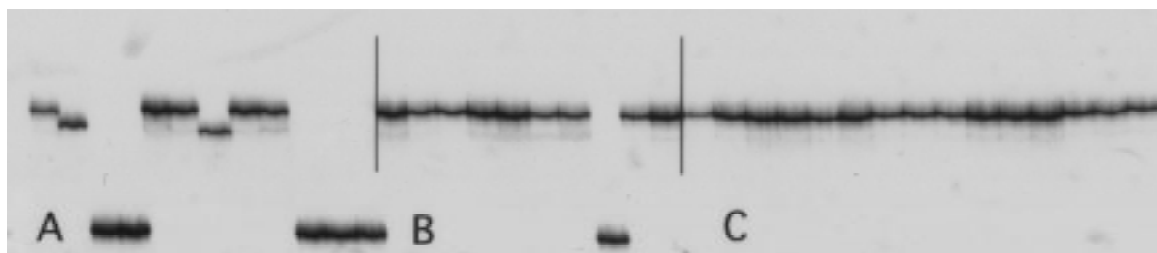


Figure 1. SSCP analysis of amplified Rubisco spacer region from *Caloglossa leprieurii* collected from Woollooware Bay, NSW. Lines indicate separation between samples from the 3 different quadrats (A, B, C).

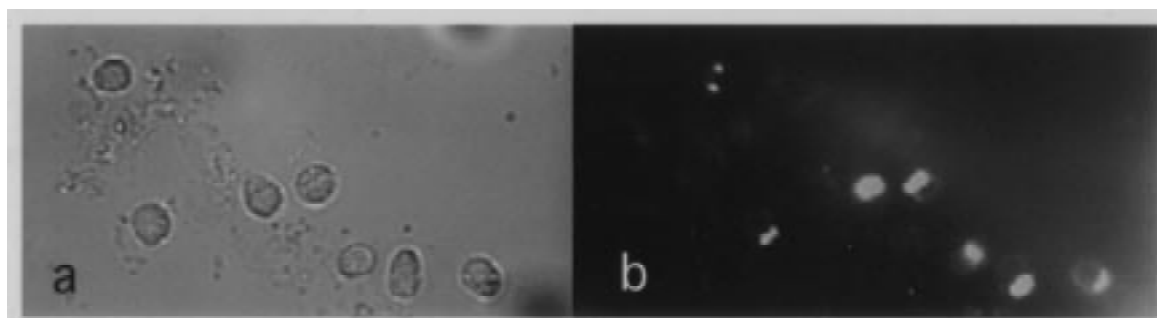


Figure 2. Micrograph of released spermatia from *Bostrychia moritziana* (live specimen). a= Bright field image. b= same image seen under fluorescence. Autofluorescent plastids are visible.

erance to desiccation) or may suggest very limited dispersal/recruitment of thalli in the field. Work is currently in progress to answer these questions.

Previous studies have shown that isolates of *Bostrychia radicans* from different locations around the world are able to hybridize (Zuccarello & West, 1995, 1997). DNA sequence data from these isolates has also revealed sequence variation in the Rubisco spacer. For example, isolate 3017 from Brazil differed from isolate 3043 from Peru by a single base pair substitution. Fluorescence microscopy of the released spermatia of *Bostrychia moritziana* clearly shows that plastids are found within the spermatia (Figure 2). Spermatia autofluorescence was identical in color to vegetative cell autofluorescence. Interestingly, only ca. 70% of the observed spermatia contained autofluorescent plastids which varied considerably in size. Fixation and DAPI staining of the spermatia, to visualize plastid genome DNA was unsuccessful, possibly due to plastid autofluorescence and small size of the plastid genome. The presence of autofluorescent bodies does not definitely prove the presence of plastids, but as they are a similar color to vegetative plastids this interpretation appears to be reasonable. Nor can we be sure that they contain plastid DNA, but if they do they allow for the possibility that the spermatia once

fused to the egg (trichogyne) could transfer a plastid to the future zygote cytoplasm. Plastid presence, based on endogenous autofluorescence in spermatia, has been demonstrated likewise in *B. radicans* and *Caloglossa leprieurii*. These data are consistent with ultrastructural data that indicate plastids within many red algal spermatia (Kugrens & West, 1972; Cole & Sheath, 1980; Kugrens, 1980; Broadwater & Scott, 1983). Only molecular data using a plastid marker will confirm the mode of inheritance of this organellar genome.

To test for the parental mode of plastid inheritance in the red alga *Bostrychia radicans*, crosses were performed between isolates of *B. radicans* from which different plastid haplotypes had already been demonstrated (Table 1, Figure 3) (Zuccarello & West, 1997). SSCP analysis showed maternal inheritance of the PCR-amplified plastid gene in reciprocal crosses. Furthermore the analysis of 5 different hybrid sporophytes derived from different carpospores was consistent, in all cases, with maternal inheritance. In the limited samples analyzed here, it does not appear that the nuclear genotype (different isolate) or plastid genotype (haplotype) in any way affects the plastid inheritance mode, as is seen in other organelle inheritance patterns in plants and animals (Birky, 1995). 'Leakage',

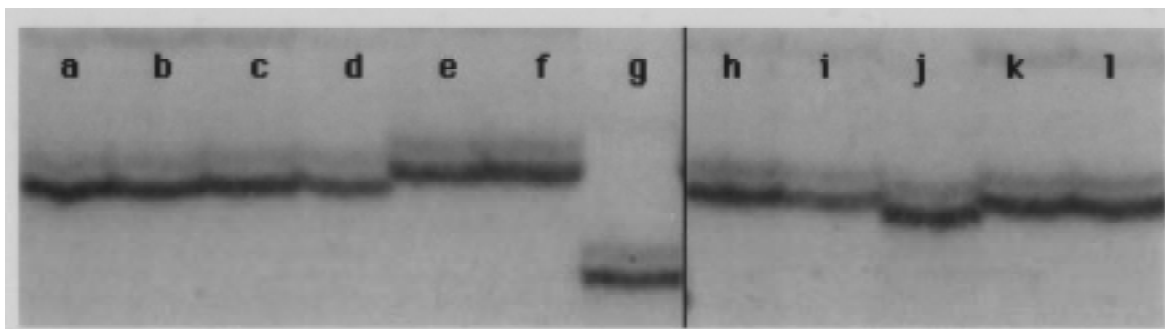


Figure 3. SSCP of amplified Rubisco spacer region of parental isolates and hybrid F₁ tetrasporophytes of *Bostrychia radicans*. For lane designation see Table 1.

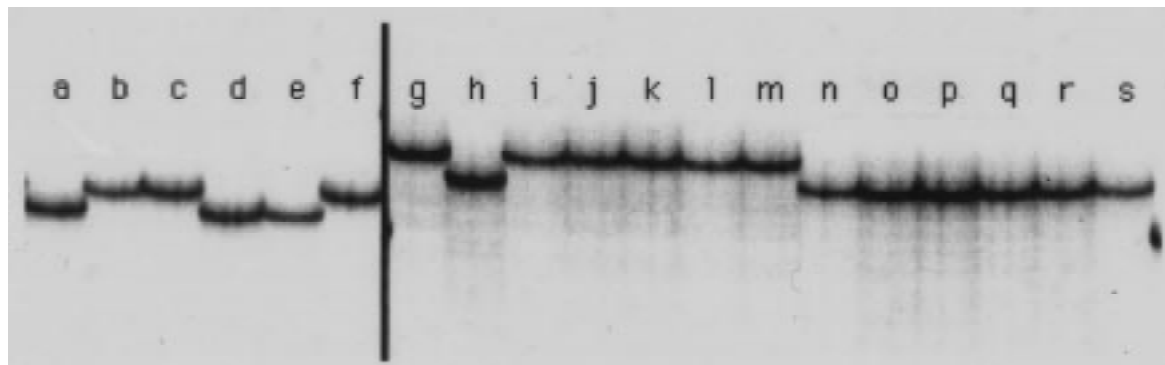


Figure 4. SSCP of amplified Rubisco spacer region of parental isolates and hybrid F₁ tetrasporophytes of *Bostrychia moritziana*. For lane designation see Table 2.

A. 3738FJ	ATTGGATCTATGGAAAAGATATAACGTTTAACTATACTTCTACAGATACAGCAGACTTTGT
B. 3209SA
C. 3275SAT.....
A. 3738FJ	AGAAACTCCAACAGCTAATGTTTAAATAAAAAATTTTCTTCACTAGTATTTTTTAAATTAT
B. 3209SA
C. 3275SA
A. 3738FJ	TTGTATGTATACAAAATAATTTAAATCTTTTATATACAGATCAATTCTCATAAGGAGTGAA
B. 3209SA
C. 3275SA
A. 3738FJ	TAGTGAGACTAACACAAGGAACCTTTTTCCTTTTACCAGACTTAACTGATGAACAAATCA
B. 3209SAC.....
C. 3275SAC.....
A. 3738FJ	AAAAACAAATTGACTACGCAATTTCTCAAAATTTGGGCAGTAAATATTGAATATACAGAAG
B. 3209SA
C. 3275SA
A. 3738FJ	ATCCGCATCCTAGAAATAGCT
B. 3209SA
C. 3275SA

Figure 5. DNA sequence of the Rubisco spacer from *Bostrychia moritziana* isolates. See Table 2. Haplotype A= A.3738FJ. Haplotype B= B.3209SA. Haplotype C= C.3275SA.

Table 2. Haplotypes of parental strains and hybrid sporophytes of *B. moritziana*. Culture numbers correspond to isolates from the J.A. West culture collection. Haplotype = arbitrary haplotype designation. Lane = gel lanes in Figure 4. n.a. = not applicable, i.e. parental strain. X = indicates cross between two isolates

Culture numbers			
Female	Male	Haplotype	Lane
3738FJ X	3197SA	A	a
3738FJ X	3209SA	A	b
3738FJ X	3275SA	A	c
3738FJ	n.a.	A	d
n.a.	3197SA	B	e
n.a.	3209SA	B	f
n.a.	3275SA	C	g
3197SA X	3738FJ	B	h
3235SA X	3738FJ	B	i
n.a.	3738FJ	A	j
3197SA	n.a.	B	k
3235SA	n.a.	B	l

the usual term for the alternate type of organelle inheritance (usually paternal) pattern, is rare in most organisms (Avisé, 1994), and was not detected in the limited number of samples analyzed in this study.

Our studies on the biogeography and reproductive patterns of the mangrove alga *Bostrychia moritziana* have led to the screening using SSCP and sequencing of the Rubisco spacer in many isolates, and to crosses between different isolates. In this species no haplotype variation could be found within the western Pacific (Australia, New Zealand, Fiji-unpublished data). Crosses between isolates that did show Rubisco spacer variation are shown in Table 2 and Figure 4. These crosses again show solely maternal inheritance of plastids. Sequencing of these haplotypes also shows that the SSCP method was able to distinguish variation of just a single base pair difference (Figure 5). Haplotype A differs from haplotype B by a single base change, from an adenosine to a cytidine in position 218, while haplotype A differs from haplotype C by 2 base changes, a cytidine to a thymidine in position 31 and an adenosine to a cytidine in position 218. SSCP screening appears to be extremely sensitive in detecting extremely low levels of sequence variation. This is confirmed by complete sequencing of identical haplotypes which, as yet, have not revealed a single case

of DNA sequence variation not observed by SSCP (unpublished data).

The application of SSCP is currently the best approach available for the rapid screening of plastid variation in the seaweeds. We believe this method should also be applicable to the Rubisco spacer region of brown algae through the use of different primer combinations (Stache-Cairns et al., 1997). In the initial use of this methodology it is recommended that concurrent sequence data be gathered to confirm the SSCP results. A drawback of this method for population genetic studies may be the limited degree of plastid DNA sequence variation. The plastid genome is believed to mutate more infrequently at the DNA sequence level than the mitochondrial genome (Avisé, 1994). Data from this study support this contention, within a single population of *Caloglossa leprieurii* and *Bostrychia radicans* only a few haplotypes have been revealed (Zuccarello & West, 1997, this study and unpublished data).

This is the first study to conclusively show maternal inheritance of plastids in marine red algae. We are still ignorant to the fate of mitochondria in crosses between red algae, although studies in this area are currently in progress. Future work in our laboratory utilizing SSCP is aimed at demonstrating the utility of this region and these methods in describing genetic variation, demography and systematics of algal populations.

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