

Molecular relationships within *Bostrychia tenuissima* (Rhodomelaceae, Rhodophyta)

Giuseppe C. Zuccarello,^{1*} John A. West,² Ulf Karsten³ and Robert J. King¹

¹School of Biological Science, University of New South Wales, Sydney, New South Wales 2052, Australia, ²School of Botany, University of Melbourne, Parkville, Victoria 3052, Australia and ³Alfred Wegener Institute for Polar and Marine Research, Am Handelshafen 12, D-27570 Bremerhaven, Germany.

SUMMARY

Bostrychia tenuissima King and Puttock is restricted to southern Australia and New Zealand. Previous studies in Australia have revealed two distinct patterns in the presence of osmotically active polyols. Southern populations only have D-sorbitol whereas northern populations have both D-sorbitol and D-dulcitol. These polyol patterns lead to speculation on the ecotypic differentiation of these two population types. Using single-stranded conformation polymorphism to rapidly score plastid haplotypes, plus DNA sequencing, a 100% congruence was found between polyol patterns and plastid haplotype. Analysis of 33 *B. tenuissima* isolates shows that the plastid relationships closely follow biogeographic regions, with south-eastern Australia (southern NSW and Victoria) and South Australia and Tasmania having one haplotype and central and northern NSW having two alternate haplotypes. There is an overlap at the haplotype boundary just south of Sydney, New South Wales.

Key words: biogeography, *Bostrychia tenuissima*, D-dulcitol, D-sorbitol, plastid haplotype, Rhodomelaceae, Rhodophyta, single stranded conformation polymorphism.

INTRODUCTION

Bostrychia tenuissima King et Puttock occurs on the eastern Australian coast south from 26°S and also in New Zealand, in mangroves, salt marshes and low energy, rocky, shore environments (King and Puttock 1989, 1994). It differs from all other *Bostrychia* species in that the axes are polysiphonous and ecorticate throughout and it possesses well developed peripherophtera (King and Puttock 1989).

Along with other algae that grow in mangrove habitats, *Bostrychia* is able to cope with a wide range of salinity through the production of compatible solutes, which include the polyols D-sorbitol and D-dulcitol (Karsten *et al.* 1990). For *B. tenuissima* in Australia, all isolates south of 34°S contained D-sorbitol, digeneaside

and an unknown compound, whereas isolates north of 34°S contained only D-sorbitol and D-dulcitol (Karsten *et al.* 1995). The polyol content reflected the salinity in the environment (i.e. increased polyols in high salinity treatments), which is consistent with these compounds playing a role in osmoacclimation. Karsten *et al.* (1995) also speculated that ecotypic differentiation occurred within the species, with southern isolates containing sorbitol only, possibly reflecting a reduced sensitivity of the sorbitol metabolic pathway to lower temperature.

In our continuing studies of the biogeography, evolution and physiology of mangrove algae we have developed a method to quickly score plastid haplotypes of multiple isolates. The intergenic spacer between the genes encoding the large and small subunits of ribulose-1-5-bisphosphate carboxylase/oxygenase (Rubisco spacer) enzyme has proved useful in understanding the phylogeny and biogeography of mangrove algae (Zuccarello and West 1997; Kamiya *et al.* 1998). Methods for screening DNA variation in polymerase chain reaction (PCR)-amplified DNA from large sample sizes are well known, although not yet applied in algal research (Lessa and Applebaum 1993). One of the simpler and less costly methods is single-stranded conformation polymorphism (SSCP), first described by Orita *et al.* (1989). 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. In *Bostrychia*, this method has been used to reveal all mutations of the Rubisco spacer studied so far and is able to distinguish single base pair changes (Zuccarello *et al.* 1999). In the present work, we investigate the genetic relationships of *B. tenuissima* isolates and relate these results to distribution of polyols.

*To whom correspondence should be addressed.

Email: <g.zuccarello@unsw.edu.au>

Communicating editor: H. Kawai.

Received 6 November 1998; accepted 16 January 1999.

MATERIALS AND METHODS

Collecting localities are given in Table 1. Methods of collection and culture are described in West and Zuccarello (1999). General methods for DNA extraction and SSCP analysis are given in Zuccarello *et al.* (1999), with only the amplification primers used and gel conditions being modified. The forward primer was 5'-TGTGGACCTCTACAAACAGC-3' (as in Maggs *et al.* 1992), the reverse primer was 5'-ATTTACACAGGAAA-CAGCTATGACATGTCAAATAATGGTAGTCCCA-3' (rbcR2-M2), with sequence complementary to the M13 reverse sequencing primer added at the 5' end to aid in future DNA sequencing (Kamiya *et al.* 1998). For SSCP gels, 2.5 µL radioactive-labelled PCR product was mixed with 10 µL denaturing solution (98% formamide, 10 mmol L⁻¹ NaOH, 0.025% bromophenol

blue, 0.025% xylene cyanol), heated to 95°C for 5 min and placed directly on ice. This denatured DNA (2.5 µL) was loaded on to a non-denaturing acrylamide gel consisting of 4.5% acrylamide (37.5:1 acrylamide:bisacrylamide), 1 × Tris-borate-EDTA (TBE) and electrophoresed in 1 × TBE at 50 W for 5–6 h in a 4°C room with a fan blowing directly on the plate surface. After electrophoresis, gels were dried on Whatman paper (Whatman, Maidstone, UK) and exposed to X-ray film (Hyperfilm MP; Amersham, Little Chalfont, Buckinghamshire, UK) for 12–24 h.

Sequencing

Samples of different haplotypes, plus multiple samples of the same haplotype, were sequenced. The reaction volume for these amplifications was 50 µL and the

Table 1. North to south distribution of *Bostrychia tenuissima* collecting sites in New South Wales (NSW), Victoria (VIC), South Australia (SA) and Tasmania (TAS)

State and collection no.	Locality name and latitude	Polyol	Haplotype	Sequenced
NSW				
3546	Brunswick Heads, 28°32'S	SD*	H3	X
3109	Gosford, 33°25'S	SD	H2	X
3110	Gosford, 33°25'S	SD*	H2	X
3111	Gosford, 33°25'S	SD	H2	
3114	Gosford, 33°25'S	SD	H2	
3576	Woolooware, 33°50'S	S*	H1	X
3308	Broughton Creek, 34°30'S	SD	H2	
3657	Sussex Inlet, 34°38'S	S*	H1	
3658	Sussex Inlet, 34°38'S	S*	H1	X
3672	Sussex Inlet, 34°38'S	S*	H1	X
3298	Moruya River, 35°30'S	S	H1	
3306	Nelligen, Batemans Bay, 35°44'S	S	H1	
3315	Tuross Lake, 35°47'S	S	H1	
3317	Narooma, 36°14'S	S	H1	
3319	Narooma, 36°14'S	S	H1	
3305	Bermagui River, 36°25'S	S	H1	
3663	Bermagui River, 36°25'S	S*	H1	
3321	Wapengo Lake, 36°35'S	S	H1	
3304	Merimbula, 36°52'S	S	n/a	
3322	Merimbula, 36°52'S	S*	H1	
3671	Merimbula, 36°52'S	S*	H1	X
VIC				
3814	Marlo, 37°47'S	S*	H1	X
3108	Williamstown, 37°52'S	S*	H1	
3581	Williamstown, 37°52'S	S*	H1	X
3612	Williamstown, 37°52'S	S*	H1	X
2747	Tooradin, 38°13'S	S	H1	X
3677	Phillip Island, 38°29'S	S*	H1	X
2853	Port Welshpool, 38°40'S	S	H1	
2933	Millers Landing, 39°05'S	S	H1	
SA				
2914	Torrens Island, 34°45'S	S*	H1	
2915	Torrens Island, 34°45'S	S	H1	
3330	Torrens Island, 34°45'S	S*	H1	
3895	Kangaroo Island, 35°45'S	S*	H1	
TAS				
3900	South George Town, 41°06'S	n/a	H1	X

S, D-sorbitol only pattern; SD, D-sorbitol/D-dulcitol pattern (from Karsten *et al.* 1995). *Data obtained in this study. H1, haplotype 1; H2, haplotype 2; H3, haplotype 3; n/a, not available. X, plastid rbcL/rbcS spacer region sequenced in these isolates

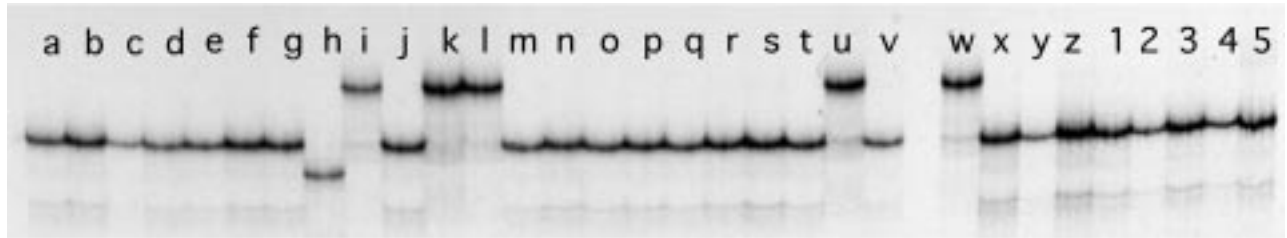


Fig. 1. Single-stranded conformation polymorphism gel of plastid *rbcL/rbcS* spacer region for most samples of *Bostrychia tenuissima*. (a) isolate 3576, (b) 3581, (c) 3612, (d) 3658, (e) 3672, (f) 3671, (g) 3677, (h) 3697, (i) 3109, (j) 2915, (k) 3111, (l) 3114, (m) 3305, (n) 3306, (o) 3321, (p) 3658, (q) 3892, (r) 2914, (s) 2933, (t) 3108, (u) 3110, (v) 3298, (w) 3308, (x) 3315, (y) 3317, (z) 3319. (1) 3322, (2) 3330, (3) 3657, (4) 3663, (5) 3671. Haplotype 1 includes a, b, c, d, e, f, g, j, m, n, o, p, q, r, s, t, v, x, y, z, 1, 2, 3, 4, 5; haplotype 2 includes i, k, l, u, w; haplotype 3 is h.

H1	CTTTGTCGAAACTCCAACAGCTAATGTTTAAAGTAAAAAATCTAATATGAATTTATACATT
H2G.GT...TGC...A.....A.A...
H3	T.....A.....C...A.CG..TC.TAC...A.....G.T...
H1	TAAAAAAGTTTGTAGTTTGTAGTT--AATTTTTTTTATACAAA-----AATCTATTA----T
H2	...C.CTT...G.T.....ACA.....CA.....T...TGTA...A...C...AAAGC
H3--C.....A.GTAGT....AAC--.T..CTATAC.G.AA.A.CCATAA
H1	TAT--AGATTAATTATTATAAGTATAAGGAGTATATAATAGTGAGACTAACTCAAGGAAC
H2	...TA.A.A...A.-...C.T.....A..C.....A.....
H3	G.AAT.....C.....C.....C.....T...A.....
H1	TTTTTCTTTTTTACCTGACTTAACTGAAGAACAATAAAAAAGCAAATTAATTACGCAAT
H2C.....C..T.....T.....G.....T..
H3C..C...T....C.....C.....A.....C..T.....
H1	AGAACAAGACTGGGCAATTAATATTGAATATACAGAGGATCCGCATCCACGCAACAAC
H2	.TCT...A.T.....T.....A.....A..T.GT.
H3	...A.TA.A.TT.....G.T.....A..C..T.....A.....T.

Fig. 2. DNA sequence of the plastid *rbcL/rbcS* spacer region of the three haplotypes. H, haplotype; (...) Nucleotide is the same as in the first row; (-) alignment gap.

final concentration for all reactions was: 1 × PCR buffer (Promega buffer A; Promega, Madison, WI, USA), 120 nm deoxynucleoside triphosphate, 2 mmol L⁻¹ MgCl₂, 5 pmol of each primer, 0.25–0.10% bovine serum albumin (Sigma A-2153; Sigma Chemical Co., St Louis, MO, USA) and 1 U *Taq* polymerase (Promega™). Chelex-extracted DNA (2 µL) was added to 48 µL of the PCR cocktail. Automated sequencing was performed on an ABI Prism 377 DNA Sequencer™ (Perkin Elmer, Norwalk, CT, USA) after cycle sequencing of the purified PCR product with dye-labeled dideoxynucleotides (Perkin Elmer). Cycle sequencing was performed on a P-E 2400 Thermal Cycler (Perkin Elmer) and samples were cleaned up by the ethanol precipitation method following the manufacturer's recommendations.

Evolutionary distance values were calculated between aligned haplotype sequences, using the Kimura 2-parameter model, transition/transversion ratio of 1:1, in the PHYLIP 3.5 package (Felsenstein 1993). Polyol analysis followed the methods of Karsten *et al.* (1991).

RESULTS

Single-stranded conformation polymorphism analyses revealed three distinct haplotypes (Fig. 1, Table 1).

DNA sequencing of identical haplotypes showed no sequence variation. Non-identical haplotypes differed in sequence and in length (Fig. 2). Haplotype 1 was 286 b.p., H2 was 298 b.p. and H3 was 296 b.p. in length. Evolutionary distances were: 0.1872 between H1 and H2; 0.2358 between H1 and H3 and 0.3232 between H2 and H3. Polyol analysis of isolates showed a 100% correlation between polyol type and plastid haplotype (Table 1; i.e. haplotype 1 with D-sorbitol only and haplotypes 2 and 3 with D-sorbitol and D-dulcitol).

DISCUSSION

Single-stranded conformation polymorphism scored three haplotypes and sequencing revealed sequence and length differences between different haplotypes and no variation between isolates of the same haplotype. This is consistent with other results we have obtained on the Rubisco spacer with other species of *Bostrychia* (Zuccarello *et al.* 1999 and unpubl. data) and is consistent with data on other PCR products in other systems (e.g. Glavac and Dean 1993) where 100% sensitivity can be achieved. The level of sequence variation between these haplotypes is more than that found within biogeographically diverse and

reproductively isolated samples of *Caloglossa leprieurii* (Montagne) Martens (Delesseriaceae, Rhodophyta) (Kamiya *et al.* 1998), or within biogeographically separated isolates of *Bostrychia radicans* (Montagne) Montagne (Zuccarello and West 1997). The phylogenetic relationships are difficult to establish with these data as no confidently alignable Rubisco spacer sequence from other species is available, although evolutionary distance values would indicate that haplotype 3 is the most genetically divergent haplotype and that it is related to its more geographically distant position (northern New South Wales coast, 28°32'S).

The haplotypes correlate exactly with populations defined on the basis of polyol content. Haplotype 1 consists of isolates that produce D-sorbitol only, digeneaside and an unknown compound (Karsten *et al.* 1995). All of the isolates from that study were scored, except for isolate 3304 in which a satisfactory DNA extraction was not possible. Haplotype 1 isolates were found in Tasmania, South Australia, Victoria and southern New South Wales. Haplotype 2 isolates containing D-sorbitol and D-dulcitol occur primarily north of 34°S. Haplotype 3 also contains D-sorbitol and D-dulcitol and is the most northern sample at 28°S. One haplotype 1 isolate (3576) was collected at Woolloomare, within Botany Bay (33°50'S), a location considerably north of a population of haplotype 2 (Broughton Creek, 34°30'S, isolate 3308) containing D-sorbitol and D-dulcitol. In earlier data the populations now designated H1 and H2 showed no crossover in distribution. Karsten *et al.* (1995) speculated that the polyol patterns might be correlated to ecotypic differentiation, with the D-dulcitol metabolic pathways more sensitive to lower temperatures and, therefore, missing in southern populations. The new data make this interpretation unlikely. The polyol patterns correlate more accurately with the phylogenetic history of the plants (i.e. plastid haplotypes). If plastid DNA sequence data represent accurate evolutionary relationships, then a polyol pattern may be associated with a particular haplotype. Dispersal of haplotypes along the NSW coast could have then led to present distributions. This hypothesis rests on the assumption that all haplotype 1 isolates will contain only D-sorbitol, whereas all haplotype 2 and 3 isolates have D-sorbitol and D-dulcitol and, further, that the plastid haplotypes reflect true genetic and phylogenetic relationships. Our current hypothesis also predicts that if present distribution of haplotypes and associated polyols is due to dispersal and not grand-scale ecological factors, it is potentially possible for different haplotypes and polyol patterns to coexist. Different haplotypes have been shown to coexist within a few centimeters in another mangrove alga, *Caloglossa leprieurii* (Zuccarello *et al.* 1999).

Reproductive isolation may have also occurred during this genetic differentiation, as reproductive isolation seems to be associated with very little plastid

sequence divergence (Zuccarello and West 1997; Kamiya *et al.* 1998) and this area is being actively investigated.

Our present results show that there is a 100% correlation between polyol patterns and plastid haplotypes, indicating a strong genetic component to differences in osmotically active sugar alcohols in *Bostrychia tenuissima*. The SSCP method is easily adaptable to scoring large sample sizes of field-collected material (more than 50 samples can be processed in a few days) and this research will be actively pursued. This work on haplotype and polyol patterns will continue to determine if plants with different osmolytes and haplotypes co-occur, especially in populations between Woolloomare Bay and Broughton Creek in central NSW.

ACKNOWLEDGEMENTS

We thank Rosario Braga for providing the samples collected in Gosford, NSW and Doug McBride for those obtained in Tasmania. This study was supported in part by a Vice-Chancellor's post-doctoral fellowship to GCZ and Australian Research Council small grants to GCZ and RJK (SG1B003963125) and to JAW (SG 0933526–1994, SG19812824–1998). This is publication no. 1518 of the Alfred Wegener Institute for Polar and Marine Research, Bremerhaven. We also thank Oliver Nixdorf, University of Bremen, Germany, for the polyol analysis.

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