

SHORT COMMUNICATION

# Markers derived from amplified fragment length polymorphism gels for plant ecology and evolution studies

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## Abstract

We describe the types of polymerase chain reaction (PCR) markers that we have isolated using amplified fragment length polymorphisms (AFLP) in closely related taxa from diverse plant genera. With these markers, both inter- and intraspecific differences have been identified. The characterization of the nucleotide sequences and fragment length polymorphisms of such AFLP-derived PCR markers is promising for investigating the ecology and evolution of closely related plant taxa.

*Keywords:* amplified fragment length polymorphisms (AFLP), direct repeats, plant molecular markers, sequence-characterized amplified region (SCAR)

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## Introduction

The realization that the Quaternary has been a time of intense speciation and rapidly changing plant distributions globally (e.g. Comes & Kadereit 1998; Winkworth *et al.* 1999) has highlighted the need to identify fast-evolving regions of plant genomes for studying ecological and evolutionary processes. Restriction endonuclease-based methods such as restriction fragment length polymorphisms (RFLP) (Crawford *et al.* 1993; Mason-Gamer *et al.* 1995) and amplified fragment length polymorphisms (AFLP) (Mueller & Wolfenbarger 1999) provide one means for locating polymorphic regions (Lou & Boutry 1995; Lockhart & McLenachan 1997). Although AFLP is an extremely reliable method (Jones *et al.* 1998), our experience is that the effectiveness of AFLP is reduced when DNA quality is poor, presumably because of interfering plant metabolites and/or DNA degradation. This has limited our use of herbarium tissue and some material collected from natural populations.

However, we have taken advantage of the sensitivity of AFLP to locate polymorphic regions using a relatively small number of closely related species and/or intraspecific taxa for which high-quality DNA template can be obtained.

Sequence-characterized amplified region (SCAR) markers (e.g. Melotto *et al.* 1996; Lockhart & McLenachan 1997; Schupp *et al.* 1999; Shan *et al.* 1999) were derived from AFLP profiles and used to rapidly analyse collections from natural populations and herbarium accessions. In this communication, we report the different types of SCAR markers that we have isolated using AFLP from diverse genera in the New Zealand flora.

## Materials and methods

AFLP profiles were obtained for duplicate accessions (three to seven taxa) of the New Zealand plant genera: *Myrsine* (Myrsinaceae), *Nothofagus* (Nothofagaceae), *Rhopalostylis* (Palmae), *Phormium* (Phormiaceae) and *Myosotis* (Boraginaceae). AFLP reactions were performed as described in the Gibco BRL AFLP Analysis System I manual, with the modifications of Lockhart & McLenachan (1997). Electrophoresis and visualization of AFLP profiles in the present work used denaturing 5% polyacrylamide (PAA) gels and silver staining (Vos *et al.* 1995; Promega Corporation 1998). Previously, 4% SEPARIDE had also been used to successfully isolate an AFLP-derived chloroplast marker for direct sequencing studies in alpine *Ranunculus* (Lockhart & McLenachan 1997; Lockhart *et al.*, in press).

Selective AFLP primers were either AT rich or GC rich and these were arbitrarily chosen. Those which gave useful

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**Table 1** Overview of results at different stages in attempts to derive markers

Selective primer combinations used that gave AFLP profiles with polymorphic bands	No. of bands cut	No. of bands amplified	No. of bands sequenced	GenBank locus match	No. of useful PCR markers	Primers used to amplify polymorphic regions	No. of taxa investigated to date
<i>Myrsine</i>							
<i>MseI</i> -CTA/ <i>EcoRI</i> -AAA, AAT, ATA, ATT	17	12	10	none	1	(MdAf9F, MdAf9R)	25
<i>MseI</i> -CAG/ <i>EcoRI</i> -AAA, AAT, ACG, AGG	5	4	4	none	1	(MaAf5F, MaAf5R)	50
<i>Nothofagus</i>							
<i>MseI</i> -CTA/ <i>EcoRI</i> -ACG, AAT	13	9	9	none	1	(NmAf2F, NmAf2R)	230
<i>Phormium</i>							
<i>MseI</i> -CTG/ <i>EcoRI</i> -AGG, ACA	11	8	8	emb   AJ2906 64.1	1	(PtAf7oF, PtAf7oR PtAf7iF, PtAf7iR)	61 61
<i>Rhopalostylis</i>							
<i>MseI</i> -CAT/ <i>EcoRI</i> -AAT, ATT, AAA	5	5	4	none	1	(RbAf3F, RbAf3R)	15
<i>MseI</i> -CTG/ <i>EcoRI</i> -AGG, AGC, ACG, ACC	9	9	5	none			
<i>MseI</i> -CTC/ <i>EcoRI</i> -AGG, AGC, ACG, ACC	7	7	3	none			
<i>Myosotis</i>							
<i>MseI</i> -CTG/ <i>EcoRI</i> -AGG	1	1	1	none			
<i>MseI</i> -CTC/ <i>EcoRI</i> -ACC	2	2	2	none			
<i>MseI</i> -CAC/ <i>EcoRI</i> -ACG	1	1	1	none	1	(MyGC5F, MyGC5R)	22
<i>MseI</i> -CTA/ <i>EcoRI</i> -AAT	3	3	3	none			
<i>MseI</i> -CAT/ <i>EcoRI</i> -ATA	1	1	1	none			
<i>MseI</i> -CAT/ <i>EcoRI</i> -AAT	1	1	1	none	1	(MyAT3F, MyAT3R)	21

Most AFLP selective PCR primers that gave discrete profiles identified polymorphisms in different plant groups. Not all, but most gel cuts gave DNA that could be re-amplified with pre-amplification primers. Most fragments that could be re-amplified were cloned and sequenced in the pGEM®-T easy vector (Promega) using M13 forward and reverse sequencing primers. Not all clones made have been investigated. Larger cloned fragments, (those between 350 bp and 500 bp in size) were investigated. Primer pairs (20-25mers), and in a few cases nested primers, were designed and used to amplify products from genomic DNAs. Some of these products (~50%) showed no variability between accessions when run on PAA gels or when sequenced. Primer sequences for phylogenetically informative PCR markers shown in the table are available from p.j.lockhart@massey.ac.nz.

banding patterns are shown in Table 1. Polymorphic bands were cut from wet gels and eluted in sterile water. Most of these bands could be reamplified (see Table 1) using *MseI* and *EcoRI* preamplification primers (GIBCO BRL AFLP Analysis System I) freshly diluted from frozen stocks. Amplification products were purified using Qiaquick polymerase chain reaction (PCR) purification kits (Qiagen) and ligated into pGEM<sup>®</sup>-T easy vector (Promega) before cell transformation. The nucleotide sequences of cloned fragments were determined using the ABI377 sequencing protocol (Perkin Elmer). Primers were designed at the 5' and 3' ends of fragments to amplify by PCR the intervening regions from the genomic DNAs in other intraspecific or closely related taxa. This was done using a standard PCR protocol (Lockhart & McLenachan 1997).

Products were electrophoresed on agarose and/or PAA gels. When single bands were present, the DNA sequence of two to six accessions was determined using an ABI377 sequencing protocol to check for sequence variation. In some cases, where different alleles were suspected of being present, markers were characterized using dye-terminated single-base sequencing reactions. The fragment lengths of these products were visualized on a Li-cor 4200 DNA sequencer.

## Results and Discussion

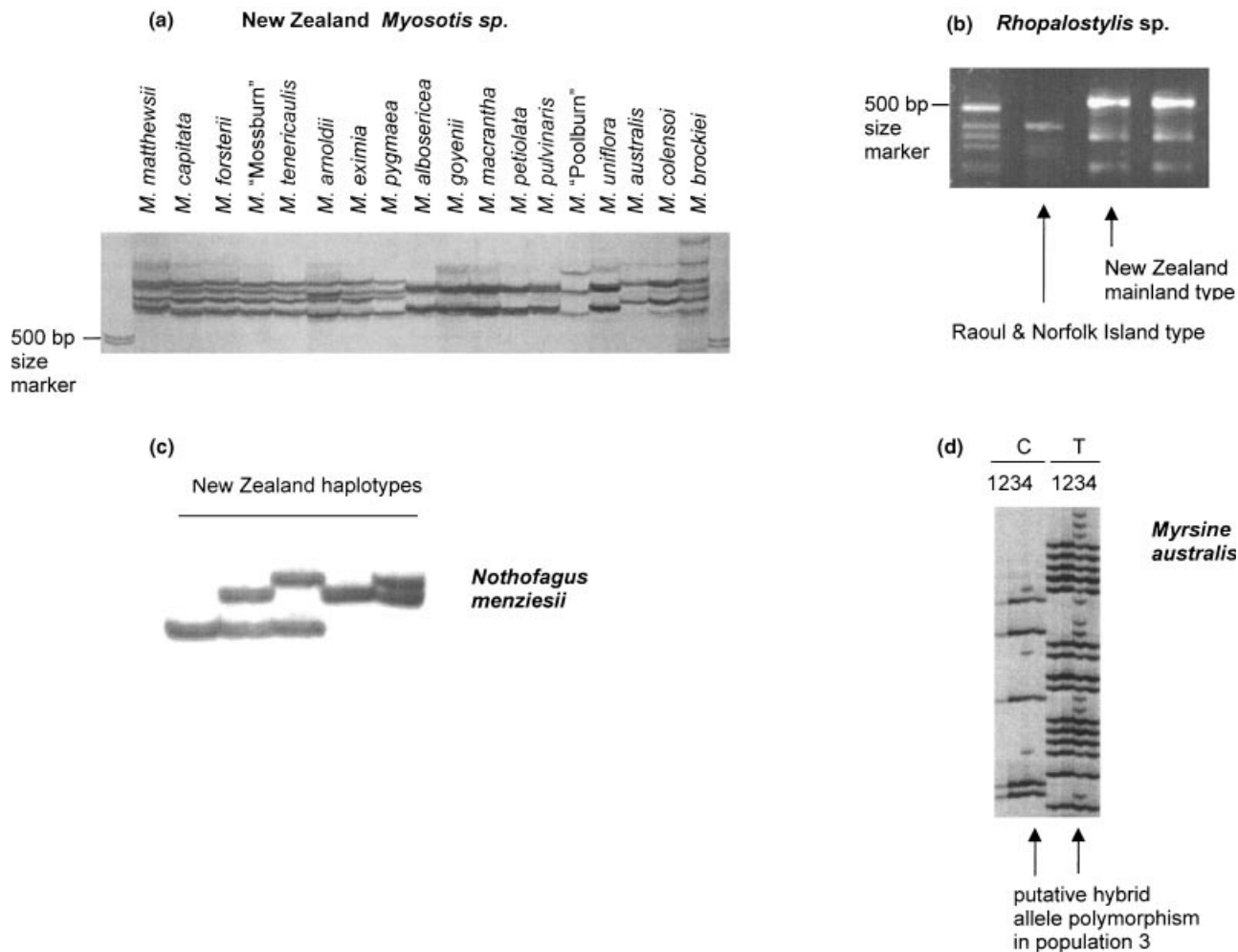
Table 1 provides an overview of our results at different stages in our attempts to find PCR markers for different plant groups. Table 2 and Fig. 1 show the variability observed with these markers in closely related taxa and/or populations studied to date. Variation has been detected in respect of the size, sequence and number of alleles amplified by the AFLP-derived primer pairs. All markers showed greater phylogenetic variation among New Zealand taxa than did sequences for the nuclear internal transcribed spacer region.

A common feature of most of the polymorphic regions that we have identified is the presence of small direct repeats. Such repeats have also been observed elsewhere in fragments derived from fingerprint profiles. In some cases the genomic regions showed elevated substitution rates (Lou & Boutry 1995; Lockhart *et al.*, in press). In *Rhopalostylis* a single primer pair amplified multiple fragments (Fig. 1b), one of which contained both inverted and direct repeats. In *Phormium*, no repeats were observed; however, the variable region in the derived AFLP marker showed a GenBank match (Expectation value =  $3e^{-07}$ ) to a truncated retrotransposon.

Recently, in genome mapping studies, the use of SCAR markers has been cautioned against since primers may amplify homologous regions (of identical or near identical size) from different chromosomes (Shan *et al.* 1999). However, such markers can be useful for molecular ecology, since they allow characterization of genomic complexity. Phylogenetic analyses of an earlier derived chloroplast

**Table 2** Observations on derived markers which appear phylogenetically useful in New Zealand taxa

	<i>Myosotis</i>	<i>Rhopalostylis</i>	<i>Nothofagus</i>	<i>Myrsine</i>	<i>Phormium</i>
Marker	MyAT3F	RbAf3	NaAf2	MaAf5	PtAf
Initial observation on agarose and/or PAA gels	multiple products of different sizes (Fig. 1a)	multiple products of different sizes (Fig. 1b)	single or doublet products (Fig. 1c)	single or doublet products	single band only observed in closely related taxa
Suspected complexity of PCR product(s)	co-amplification of multilocus alleles	co-amplification of multilocus alleles	co-amplification of single locus alleles	co-amplification of single locus alleles	unique allele type
Observed variation in taxa investigated to date	unique and shared haplotypes in 22 closely related species	two distinct haplotypes separating sub-species	five distinct haplotypes in 230 accessions of <i>N. menziesii</i>	putative hybrid alleles in population 3 visualised on Li-cor (Fig. 1d)	direct sequencing identifies eight haplotypes in 61 accessions



**Fig. 1** (a) Silver-stained 5% PAA gel showing haplotypes in 18 different species of *Myosotis* which are genetically very similar (Winkworth *et al.* 1999) (b) ethidium bromide-stained 1% agarose gel showing banding patterns for New Zealand species of *Rhopalostylis*; (c) silver-stained 5% PAA gel showing banding patterns in individuals from different New Zealand populations of *Nothofagus menziesii*; (d) Li-cor single-base sequencing gel (ddCTP and ddTTP reactions) showing banding patterns in individuals from different New Zealand populations of *Myrsine australis*.

SCAR marker (Lockhart *et al.*, in press) and those being investigated in our present ongoing studies (unpublished observations), demonstrate that AFLP markers can be informative for understanding the evolutionary histories of populations and species.

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This research forms part of molecular systematic studies investigating the origins and distributions of endemic New Zealand flora. K. Stöckler is a PhD student studying New Zealand forest flora including *Nothofagus* and *Myrsine*. R. Winkworth is a PhD student studying alpine genera including *Myosotis*. K. McBreen is a postdoctoral researcher studying *Phormium*. P. McLenachan is a molecular biologist with interests in genome evolution. S. Zauner is a PhD student studying comparative genome evolution. P. Lockhart is a lecturer in Bioinformatics and Advanced New Zealand Botany. His research interests include sequence evolution and the development of phylogenetic methodologies.

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