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Successful DNA amplification from *Acacia* (Leguminosae) and other refractory Australian plants and fungi using a nested/semi-nested PCR protocol

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Abstract

Despite the promise of the polymerase chain reaction (PCR) as a method for rapid generation of informative phylogenetic characters, it has proven difficult or impossible to amplify the commonly used internal transcribed spacer (ITS) 1+2 regions using standard approaches for several groups of Australian plants and fungi. This paper describes a two step nested or semi-nested PCR protocol that was utilised to provide the first consistent amplification of the ITS region from *Acacia* Mill. (Mimosoideae: Leguminosae). Subsequently these methods have proven to provide reliable amplification of ITS from previously intractable Australian members of the Rutaceae and Cortinariaceae. In addition to use with fresh material, this method has also proven effective with herbarium material up to 26 years old. It is hoped these methods may facilitate use of the ITS region from a broader range of plants and fungi, including herbarium material.

Introduction

In recent years the use of PCR for obtaining DNA sequences for systematics has expanded greatly. Of the DNA regions sequenced, the internal transcribed spacer (ITS) region of 18S-26S nuclear ribosomal DNA has proven to be particularly useful for plant (Baldwin *et al.* 1995) and fungal (Edel 1998) systematics. The relatively small size of ITS and its high copy number facilitates its amplification and sequencing (Baldwin *et al.* 1995), and rapid concerted evolution of the nuclear ribosomal DNA multigene family acts to promote intragenomic uniformity of repeats (Arnheim *et al*. 1980; Appels & Dvorak 1982; Arnheim 1983; Hillis *et al*. 1991), decreasing the likelihood of paralogy confounding phylogenetic reconstructions based on ITS sequences. Ease of alignment, and sufficient sequence variation between closely related species, has also contributed to the use of ITS for systematics. The popularity of ITS has resulted in more than 45,000 records being submitted to the GenBank DNA sequence database, which makes comparison of sequences with other taxa, worldwide, extremely easy.

The many advantages of the ITS region has seen it used for examining relationships between several Australian plant groups, e.g. Leguminosae: Papilionoideae (Crisp *et al.* 2000), *Hovea* R.Br. (Leguminosae, Thompson *et al.* 2001), *Beaufortia* R.Br. suballiance (Myrtaceae, Ladiges *et al*. 1999; Brown *et al*. 2001) and *Eucalyptus* L'Her. (Myrtaceae, Steane *et al*. 1999; Udovicic & Ladiges, 2000). Likewise, when it was necessary to sequence a region of nuclear DNA for acacias, ITS was deemed to be the ideal candidate. Initially, amplification of DNA from *Acacia* Mill. was attempted using primers that had proven successful for another legume genus, *Lupinus* L. (ITS18, ITS26, S3, S4, S5 and S6; Käss & Wink 1997). PCR reactions with these primers, in a variety of combinations, and using standard protocols either amplified nothing or multiple fragments. When single bands (from samples that had multiple fragments amplified) were isolated from agarose gel and sequenced, the sequences invariably matched fungal DNA sequences in GenBank. Consequently it was necessary to use primers more specific for higher plants in an attempt to avoid amplifying these fungal sequences by PCR.

Angiosperm-specific primers, 17SE and 26SE (Sun *et al*. 1994), were tried. These

primers also resulted in multiple PCR fragments and preferential amplification of unwanted fungal DNA or chloroplast ribosomal DNA. If higher annealing temperatures (above 62˚C) were used, no DNA was amplified. When the 17SE and 26SE primer sequences were compared to the partial 18S and 26S sequences available for *Acacia* in GenBank - from *A. fimbriata* A.Cunn. ex G.Don (Martin & Dowd 1993) - it was found that there were four bp differences between the 18S sequence and the 17SE primer at the 5' end.

J. Miller (CSIRO Canberra) cloned the ITS region for several *Acacia* taxa and designed a new forward primer in the 18S (AcF). This primer was found to amplify the ITS region in combination with 26SE. High stringency PCR conditions (annealing temperatures above 60˚C and hotstart PCR) were required to produce a single amplicon. Difficulties were experienced in obtaining a high yield of PCR product, especially when higher annealing temperatures were used. To overcome this problem a two step, nested or semi-nested PCR strategy was utilised, which incorporated a PCR additive (Q-solution, QIAGEN) to change the melting properties of the double stranded template DNA. The nested PCR approach was originally developed for detection of hepatitis C viral sequences in blood samples (Garson *et al*. 1990), and because of its sensitivity for amplification of low-copy number templates it is frequently used for characterisation and identification of micro-organisms. Here, the ultra-sensitivity of nested PCR is used to assist in amplification of the ITS region of Australian acacias, Rutaceae and fungi.

Materials and Methods

Material of *Acacia*, Rutaceae (*Phebalium* Vent. and allied genera) and fungi (Cortinariaceae: *Cortinarius* (Pers.) Gray and *Dermocybe* (Fr.:Fr.) Wünsche) was sampled from the field, living collections or herbarium collections. Fresh material was stored at 4˚C until isolation of DNA was performed within a week of collection. Alternatively, material collected in the field was dried rapidly in silica gel and stored until convenient to isolate (Chase & Hillis 1991). Where possible herbarium material was chosen from collections under five years of age, although some collections sampled were up to 26 years old.

Genomic DNA was isolated using a CTAB protocol (Udovicic *et al.* 1995), followed by purification with Qiagen Tip 20 columns or by using Prep-a-Gene (Bio Rad). Herbarium samples were preferentially isolated using commercial kits, QIAGEN DNeasy mini kits or NucleoSpin kits (Macherey-Nagel, Düren, Germany), because of the small quantity of starting material needed and the consistent quality of resulting DNA.

The polymerase chain reaction (Mullis & Faloona 1987; Saiki *et al*. 1988) was used to amplify the ITS 1 + 2 region using a two step process (Fig. 1). Total volume of all DNA amplifications was 50 µl. First round reactions contained 0.2 mM dNTPs, 3 mM $MgCl_2$, 10 pmol each primer (Table 1), 1.25 units HotStar Taq DNA polymerase (Qiagen), 30–100 ng of template DNA and 10 µl Q-solution (Qiagen).

Thermal cycling was performed on an Eppendorf Mastercycler gradient thermal cycler with one hold at 95˚C for 15 min preceding 30 cycles of 94˚C for 30 s, 64˚C for 30 s, 72˚C for 20 s, and followed by one hold at 72˚C for 5 min.

In second round reactions a 0.5–1 µl aliquot of PCR product from the first round reaction was used as the template. Alternatively, template DNA was obtained from agarose gels of first round reactions by "stabbing" the target band with a 10 µl pipette tip, followed by agitation in 20 µl of ultrapure water, 10 µl of which was used in the second round PCR. Apart from primers for nested or semi-nested PCR (Table 1), reaction components and conditions were unchanged from the first round. Products of amplifications were visualised by agarose gel electrophoresis and stained with ethidium bromide.

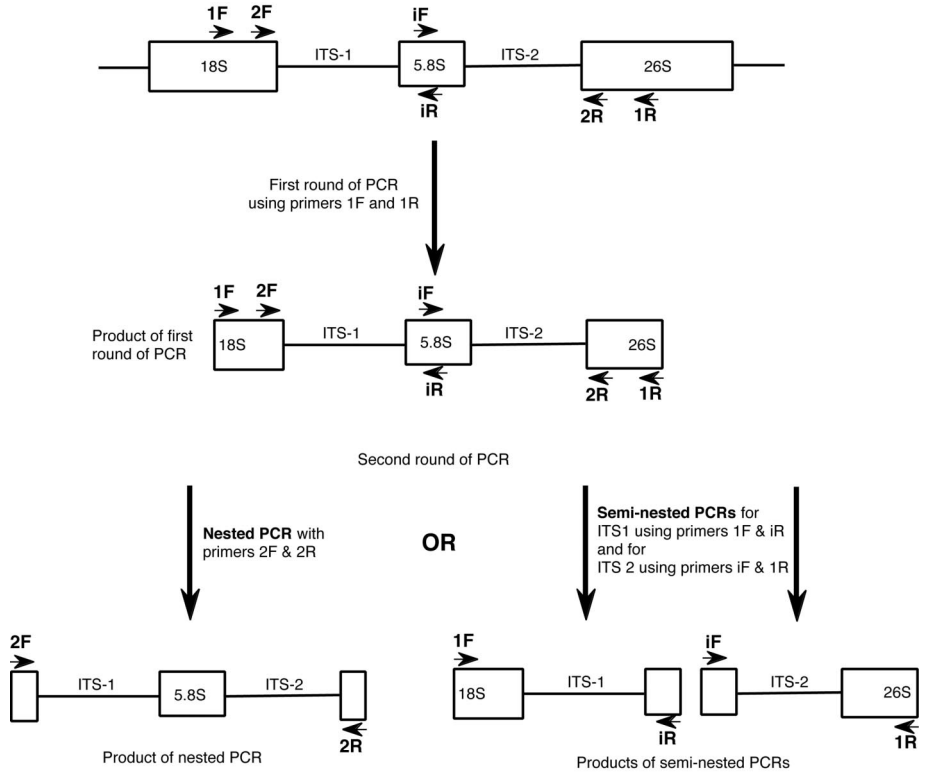

Figure 1. Diagram of nested and semi-nested PCRs, including location of primers. 1F and 1R denote primers used for the first round of PCR, 2F and 2R are primers used for nested PCR, and iF and iR are internal primers used for semi-nested PCR. See Table 1 for primers used for different taxa. Note that DNA regions and relative positions of primers are not to scale.

Table 1. PCR primers used for different taxa.

Taxa	1st Round Primers		2nd Round Primers		Internal Primers	
	1F	**1R**	**2F**	**2R**	**iF**	**iR**
Acacia	AcF[1]	26SE[2]	S3[3]	S4[3]	S6[3]	S5[3]
Rutaceae	ITS18[3]	ITS26[3]	S3[3]	S4[3]	S6[3]	S5[3]
Fungi	ITS1[4]	ITS4-B[5]	ITS1[4]	ITS4[4]	ITS3[4]	ITS2[4]

Notes: Origins of primers as denoted by superscript numerals: 1= J. Miller, CSIRO, Canberra; 2= Sun *et al*. (1994); 3= Käss and Wink (1997); 4= White *et al.* (1990); 5= Gardes and Bruns (1993).

Results and Discussion

For *Acacia*, the first round of amplification resulted in a DNA fragment of approximately 750–760 bp. This product was generally only present in low quantities and was difficult or impossible to visualise. The second round of nested PCR usually amplified a single fragment of DNA approximately 650 bp long (Fig. 2). If the second round of amplification resulted in a low yield of product (Fig. 2, lane 2), multiple fragments (Fig. 2, lane

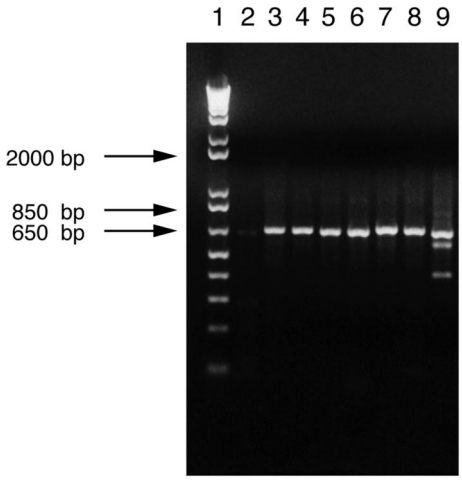

Figure 2. Second round of nested PCR amplification of *Acacia* DNA using primers S3 and S4. Aliquots of 5 µl taken from 50 µl PCR reactions, electrophoresed in a 1.5% agarose gel, stained with ethidium bromide and visualised with UV light.
1: 1kb Plus molecular weight ladder; 2: *Acacia verticillata* (L'Her.) Willd., note low yield of product; 3: *A. elata* Benth.; 4: *A. latisepala* Pedley; 5: *A. translucens* Cunn. ex Hook.; 6: *A. tumida* F.Muell. ex Benth.; 7: *A. ampliceps* Maslin; 8: *A. colei* Maslin & Thompson; 9: *A. platycarpa* F.Muell., note the multiple amplification products.

9), or was unsuccessful, semi-nested PCR was then used. Semi-nested PCR amplified fragments of approximately 400 bp for ITS 1 and 450 bp for ITS 2 (Fig. 3). Subsequent use of this protocol for Rutaceae and fungal DNA yielded comparable results to *Acacia*.

Primer selection was found to be of particular importance for amplification of ITS. Use of non-specific primers resulted in little or no amplification of the target template or preferential amplification of non-target templates. However, problems with amplification were not always due to non-specificity of primers; for example, in this study a new forward primer was synthesised to target the 3' end of the 18S rDNA gene of *Acacia.* Although being a perfect match to a published sequence for *A. fimbriata*, the *Acacia*-specific primer failed to amplify the ITS region. Such problems may be due to secondary structure in the target area of the primer. Alternatively, the species of *Acacia* trialed may have had mismatches at the priming site, although, this is unlikely given the conservative nature of the 18S rDNA sequence. In the case of Rutaceae, primers used for legumes (Käss & Wink 1997) worked satisfactorily for nested/semi-nested PCR. For fungi, primers ITS1 and ITS4-B were used for the first round of amplification. These were determined to give the best amplification of DNA from *Dermocybe* and *Cortinarius* after trialing the commonly used universal primers, ITS1 and ITS4 (White *et al*. 1990) and fungal (ITS1-F) and basidiomycete (ITS4-B) specific primers (Gardes & Bruns 1993) in all combinations.

The advantage of the nested PCR approach has been the ability to use a high annealing temperature, allowing high stringency PCR for avoidance of non-target amplification. Subsequent sequencing revealed a high G + C content (>70%) in the ITS region of *Acacia*, compared to a G + C content of 50–60 % in the fungal contaminants. This could explain the preferential amplification of contaminants at standard annealing temperatures, because at these temperatures unmelted G + C regions can prevent primer binding (Borman *et al.* 2000). Use of high annealing temperatures reduced the incidence of non-

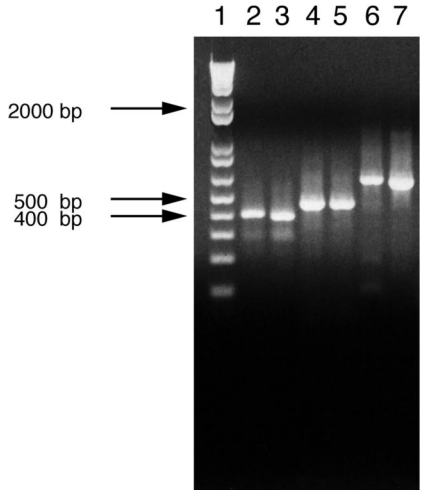

Figure 3. A comparison of PCR products resulting from semi-nested and nested amplification of *Acacia* DNA. Aliquots of 5 µl taken from 50 µl PCR reactions, electrophoresed in a 1.5% agarose gel, stained with ethidium bromide and visualised with UV light. Lane1: 1kb Plus molecular weight ladder; Lanes 2 and 3 show semi-nested PCR of ITS 1 for *A. mearnsii* De Wild. and *A. paradoxa* DC., respectively; Lanes 4 and 5 show semi-nested PCR of ITS 2 for *A. mearnsii* and *A. paradoxa*, respectively; Lanes 6 and 7 show nested PCR of ITS 1+2 for *A. mearnsii* and *A. paradoxa*, respectively.

specific amplification, and while resulting in a low yield in the first round of amplification because of the unstable nature of the primer and template hybrid (Varadaraj & Skinner 1994), the second round of amplification served to greatly increase the yield of desired product. Despite the high annealing temperature used for PCR, for *Acacia* a small proportion (approximately 10%) of second round amplifications resulted in non-target templates still being amplified (Fig. 2, lane 9). In these cases it was possible to amplify the *Acacia* ITS region using semi-nested PCR in the second round of amplification.

For *Acacia*, as described in materials in methods, aliquots of the PCR product from the first round of amplification served as the template for the second round of amplification, and this method was also used for fungal (*Dermocybe* and *Cortinarius*) DNA. Occasionally in Rutaceae this approach resulted in a background of smeared DNA, in addition to the expected product, when visualised on an agarose gel. This problem may be caused by nonspecific amplification in the original PCR (Hengen 1995), but this was unlikely in this case due to the high stringency conditions used. Another common reason for the occurrence of DNA smears is the use of far too much DNA template for the second round of amplification, but as found by Hengen (1995) dilution of the primary PCR product up to 10 000-fold may not resolve the smear into a discrete band after the second round of amplification. Alternatively, a greatly reduced amount of template for the second round PCR can be obtained by band-stab PCR, in which a needle or toothpick is used to "stab" the appropriate DNA band on a gel, and subsequently the needle or toothpick is dipped into the second round PCR reaction mix (Bjourson & Cooper 1992; Kadokami & Lewis 1994). This method achieves the aim of a greatly reduced amount of template for the second round PCR and isolates the desired fragment from the smeared DNA background. It is also quicker than cutting the desired band out of the gel and isolating it. Instead of toothpicks or needles, pipette tips were used to stab the band, and then, rather than mixing this directly into the PCR reaction, it was agitated in a separate tube con-

taining 20 µl of water. This allowed storage of this template DNA in a freezer for later use, and enabled multiple second round reactions to be carried out from the one sample.

A useful feature of nested PCR is the ability to amplify the ITS region from low concentrations of genomic DNA. As found in this study, this is particularly relevant when using DNA isolated from small amounts of material, such as herbarium specimens, which in addition, typically yield small quantities of degraded genomic DNA (Jansen *et al*. 1999). Previously, nested PCR has been used with some success for amplification of the *rbc*L region from DNA isolated from herbarium material (Savolainen *et al.* 1995). The use of herbarium material allows ready access to many taxa, including rare or even extinct species (Jansen *et al.* 1999). These specimens are already vouchered and have often been identified by specialists, thus increasing the reliability and repeatability of molecular systematic research. The use of herbarium material is time and cost effective, reducing the need to re-collect specimens (Wood *et al*. 1999). The nested PCR methodology described here may be an effective tool for application in systematic studies using plant and fungal material preserved in herbaria.

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