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DNA BARCODING

Tissue-direct PCR, a rapid and extraction-free method for barcoding of ferns

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Abstract

Fern gametophytes and young sporophytes often provide too little material for DNA extraction and are particularly difficult to identify to genus. Here we developed an efficient procedure called 'Tissue-direct PCR', in which a slice of fern tissue is mixed with PCR reagents and primers, allowing certain genomic regions to be amplified directly in the thermal cycler. For these diminutive and featureless stages of ferns, Tissue-direct PCR combined with amplifying plant barcodes promises to make the identification of immature ferns easy and rapid. Tissue-direct PCR would also be very helpful for large-scale ecological studies surveying distribution and population structure.

Keywords: DNA barcoding, ferns, gametophytes, Tissue-direct PCR, young sporophytes

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Introduction

One of the most intriguing aspects of ferns (Moniliformopses, Viridiplantae) is the life cycle that alternates between free-living gametophyte and sporophyte phases. Nonetheless, studies on genetic composition, community structure and geographical distribution have been focuses mainly on sporophytes, despite the important role that gametophytes play in this regard. Gametophytes yield sporophytes, and therefore the insights drawn from sporophytes cannot necessarily reflect the organism as a whole, especially considering the high longevity of fern gametophytes in certain circumstances (Farrar 1967; Farrar *et al.* 2008, Li *et al.* in press).

The main obstacle to study gametophytes in the fields is that they are diminutive and lack enough diagnostic characteristics, making identification very difficult. An emerging idea to use one or a set of 'DNA barcodes' to identify species would seem to provide a promising solution to overcome this problem (Tautz *et al.* 2002; Herbert *et al.* 2003; Blaxter 2004; Valentini *et al.* 2008). Although DNA barcoding of plants has not been fully resolved (http://www.barcoding.si.edu/plant_working_group.html), using DNA-based identification, recent studies on ferns have successfully identified unknown gametophytes and demonstrated the power of DNA-based identification (Schneider & Schuettpelz 2006, Li *et al.* in press).

DNA extraction has always been the first step in the DNA barcoding process. However, in practice, DNA extraction from these small plant bodies of ferns (gametophytes or young sporophytes) is labour-intensive, time consuming, often results in low-yield and low-quality DNA, and sacrifices the entire specimen, which precludes further morphological studies and vouchering.

To make DNA barcoding more broadly applicable, we have developed a technique, 'Tissue-direct PCR', which skips the DNA extraction process and directly launches a PCR reaction with only minute amounts of fern tissue. Our method incorporates fewer procedures and is more suitable for high-throughput DNA barcoding processes. In this study, we present our detailed protocol for Tissue-direct PCR, demonstrate its broad range of applicability across fern taxa, and propose a comprehensive PCR combo strategy for future studies.

Materials and methods

Plant materials

All the fern gametophytes and young sporophytes (defined in this study as the first few leaves following sporophyte formation) used in this study were grown

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from spores, except those of *Lomariopsis spectabilis* (Kunze) Mett. which were collected from fields in Taiwan. All the mature sporophytes were field-collected and cultivated in the Dr Cecilia Koo Botanic Conservation Center (Pingtung, Taiwan).

Tissue-direct PCR

A tiny piece of fern tissue (roughly 1 mm^2) is sliced off using sterilized forceps and a needle, and put into a PCR tube with 5 μL ddH_2O on an iced rack. A concentration of 10 μL PCR mix is then added, including 10× buffer, 200 μM dNTP, 15 pmol of each primer, 1 U Taq polymerase (Pro Taq DNA polymerase, Protech, Taiwan) and ddH_2O to total reaction volume. Chloroplast *trnL-L-F* (*trnL* intron plus *trnL-F* IGS) region was used in all our tests. Primers used in this region were universal primer 'f' (Taberlet *et al*. 1991) and a newly designed primer on *trnL* intron for ferns (FernL 1Ir1 5′-GGYAATCCTGAGCCAAATC-3′, LY Kuo unpublished). To establish optimum conditions, we tested the success rate of Tissue-direct PCR under a null (a) and five treatment designs (b–f), including chemical and mechanical manipulations, using gametophytes, leaves of young sporophytes and leaves of mature sporophytes of *Acrostichum aureum* L. (Pteridaceae), *Aglaomorpha meyeniana* Schott. (Polypodiaceae) and *Anisocampium cumingianum* Presl. (Woodsiaceae). In chemical manipulations, DMSO and betaine were incorporated into the PCR mix because they have been shown to enhance PCR reactions (Frackman *et al*. 1998). Three different manipulations were made in our study: 1 M betaine (b), 5% DMSO (c) and 1 M betaine with 5% DMSO (d). In mechanical manipulations (e), PCR tubes with tissue and 5 μL ddH_2O were frozen in liquid nitrogen for 45 s and then sonicated for 45 s (Bransonic 52, Danbury, CT, USA). The purpose of the mechanical manipulations was to further break down tissues to release more cytoplasmic components. We also combined the chemical (d) and mechanical manipulations (e) as a mixed treatment (f). For each treatment (a–f) on each tissue and species, ten Tissue-direct PCR reactions were carried out to score success rates (percentage of successful amplifications).

PCR conditions were carried out with initial heating at 94 °C for 5 min, followed by 35 cycles of 94 °C for 45 s, 58 °C for 45 s, 72 °C for 1 min and ended with 72 °C for 10 min. Amplification results were checked by electrophoresis on 1% (w/v) agarose gel, and if no bands were observed, double PCR (secondary PCR) was used. Double PCR was performed using the same thermal cycles with 1 μL of previous PCR products, 10× buffer, 200 μM dNTP, 15 pmol of each primer (10 mM), 1 U Taq polymerase (Pro Taq DNA polymerase, Protech) and ddH_2O for a 15 μL total reaction volume. Successful amplifications before and after double PCR were added together to score success rates for each individual treatment. To check if the amplified sequences were contaminated or not, we randomly selected successful amplifications and sequenced them for confirmation.

To assess the applicability of our method across fern lineages and on gametophytes with different growth forms, we further tested Tissue-direct PCR on the following taxa: *Angiopteris itoi* (Shieh) Kuo (Marattiaceae, multi layered gametophytes), *Adiantum flabellulatum* L. (Pteridaceae, cordate gametophytes), *Haplopteris anguste-elongata* (Hayata) Crane (Pteridaceae, ribbon-shaped gametophytes), *Belvisia mucronata* (Fee) Copel. (Polypodiaceae, cordate gametophytes), *Lomariopsis spectabili*s (Kunze) Mett. (Lomariopsidaceae, strap-shaped gametophytes), *Asplenium nidus* L. (Aspleniaceae, cordate gametophytes) and *Pentarhizidium orientalis* (Hook.) Hayata (Onocleaceae, cordate gametophytes). Both gametophyte and young sporophyte tissues were used. We employed four treatments in this study: a, d, e and f, as previously described. Three Tissue-direct reactions were repeated for each treatment on each species and tissue, and success rates were scored. The PCR reagents and conditions were the same as described above.

Results and disscussion

Optimization of Tissue-direct PCR

Table 1 shows the success rates for all treatments before and after employing double PCR. After double PCR, the total number of successful amplifications had increased greatly, suggesting the importance of double PCR. For the following discussion, treatment effects were referred to the success rates following double PCR.

In gametophytes, treatment designs had enhanced effects compared with the null treatment, except for *Acrostichum aureum*, in which both chemical and mechanical manipulations contributed negatively. For *Aglaomorpha meyeniana*, betaine improved the success rate more than DMSO (90% vs. 30%, $P < 0.05$ in Fisher's exact test), but it had the opposite effect for *Anisocampium cumingianum* (20% vs. 80%, $P < 0.05$ in Fisher's exact test). The mixed treatment design (f) appeared to increase the success rate the most in gametophytes. The average success rate was higher in young sporophytes than in gametophytes, which might be due to the presence of more cells within a 1-mm^2 slice from young sporophytes. Contrary to the situation in gametophytes, treatment designs in young sporophytes did not show any significant enhancement when compared with that of the null treatment.

Table 1 Tissue-direct PCR success rates following double PCR and before (numbers in bracket), shown as percentages, under different treatment designs to amplify *trnL-L-F*. Ten PCR reactions per species per tissue were carried out to score success rates

	Treatments					
Materials	a. Null	b. Betaine	c. DMSO	d. Betaine +DMSO	e. Liquid N +sonication	f. Mixed†
Gametophytes						
Acrostichum aureum	80 (70)	20 (0)	10 (10)	30 (0)	0 (0)	40 (0)
Aglaomorpha meyeniana	0 (0)	90* (0)	30 (10)	60* (10)	70* (60)	100* (100)
Anisocampium cumingianum	20 (0)	20 (0)	80* (10)	60 (20)	50 (0)	100* (0)
Average	33	43	40	50	40	80
Young sporophytes						
Acrostichum aureum	100 (100)	50 (0)	100 (100)	50 (40)	80 (80)	10 (0)
Aglaomorpha meyeniana	90 (80)	90 (0)	80 (80)	90 (50)	100 (100)	100 (100)
Anisocampium cumingianum	40 (0)	50 (0)	0 (0)	30 (0)	50 (0)	40 (0)
Average	77	63	60	57	77	50
Mature sporophytes						
Acrostichum aureum	10 (0)	40 (0)	10 (0)	0 (0)	10 (10)	0 (0)
Aglaomorpha meyeniana	50 (10)	70 (0)	70 (0)	80 (0)	40 (0)	0 (0)
Anisocampium cumingianum	80 (0)	30 (0)	50 (0)	30 (0)	0 (0)	40 (0)
Average	47	47	43	37	17	13

*Treatments b–f that showed significant improvement compared to null ($P < 0.05$, Fisher's exact test).
†Combined d and e.

Table 2 Voucher information and GenBank accession numbers of *trnL-L-F* amplified and sequenced from genomic DNA

Species	Voucher	Accession no.
Acrostichum aureum	KBCC* K017715	FJ807658
Aglaomorpha meyeniana	KBCC K016952	FJ807657
Anisocampium cumingianum	KBCC K014215	FJ807659

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Lower success rates in mature sporophytes might be due to higher amounts of secondary metabolites present in the leaf tissue of mature plants. The majority of successful amplifications were obtained after double PCR. This might be attributed to the dilution of secondary metabolite concentration during double PCR. No enhancement was shown for mechanical manipulations (e), which break cells more severely and presumably release more secondary metabolites. Tissue-direct PCR is less necessary for mature sporophytes, where there is little problem with species identification and DNA extraction. On the other hand, in gametophytes and young sporophytes where these problems are acute, Tissue-direct PCR worked well.

To check if the sequences amplified were derived from the correct species, we randomly selected them for sequencing. All the resulting sequences matched the *trnL-L-F* sequences amplified from the same species' total genomic DNA extracts (Table 2) .

Applicability of Tissue-direct PCR in wide range of fern lineages

We assessed the applicability of Tissue-direct PCR on gametophytes and young sporophytes for seven other species, representing major fern lineages and with a range of different gametophyte growth forms (cordate, ribbon-shaped, strap-shaped and multilayered). Tissue-direct PCR successfully amplified *trnL-L-F* from gametophytes and young sporophytes from every species except for the gametophytes of *Asplenium nidus*, indicating a wide taxonomic range of applicability (Table 3). In addition, the success of Tissue-direct PCR was not limited to only single-layered cordate gametophytes, but also included other growth forms, such as multilayered and ribbon-like ones (Table 3).

Tissue-direct PCR combo

Compared with a previously published technique on DNA amplification from filmy fern gametophytes (Ji *et al.* 1994), we not only skipped the crude DNA extraction step, but also demonstrated that various treatment designs can improve the amplification success rate. Moreover, in this study, Tissue-direct PCR worked successfully across diverse fern species and tissues, including gametophytes, young sporophytes, mature sporophytes (Table 1) and even spores (data not shown).

However, there was no single best treatment for all situations (Table 1). As a result, we propose a 'Tissue-direct

Table 3 Applicability of Tissue-direct PCR on various fern taxa. Four treatments were employed in this study, each with three PCR replicates per species per tissue, a: null, d: betaine+DMSO, e: liquid nitrogen+sonication and f: combining d and e, as described in materials and methods

	Gametophyte					Young sporophyte				
	a	d	e	f	Average (%)	a	d	e	f	Average (%)
Angiopteris itoi	+++	++	+++	++	83	+++	+++	+++	+++	100
Adiantum flabellulatum			+	+	17	++	++	++	+++	75
Haplopteris anguste-elongata	+++	++	+	+++	75	+	++	+	+++	58
Belvisia mucronata	+++	+	+++	+++	83	+	+++	+++	++	75
Lomariopsis spectabilis		++	+	+++	50	+++	+++	+++	+	83
Asplenium nidus					0	+++	+++	+++	+++	100
Pentarhizidium orientalis	+++	+++	+++	+++	100	+++	+++	+++	+++	100

The number of '+'denote the number of successful amplifications

PCR combo' for future application in species identification of juvenile ferns. To ensure the highest success rate from different species, Tissue-direct PCR combo incorporates two amplification conditions: null (a) and mixed treatments (f), as described above, and double PCR is always recommended.

Future perspectives

Tissue-direct PCR, as presented in this study, increases the efficiency of sequence amplification from fern gametophytes and young sporophytes, with minimal time and labour requirements. Moreover, as only a minute amount of tissue is required, it also reduces the possibility of contamination due to crude DNA extraction, especially in situations where there is interwoven or overlapping growth between fern gametophytes and bryophytes in natural habitats. Therefore, in combination with DNA barcoding, Tissue-direct PCR provides a rapid and high-throughput method for identifying those featureless ferns in their earliest developmental stages, and furthermore, it also enables the exploration of gametophyte genetic composition and diversity in nature. We believe that Tissue-direct PCR can help to facilitate future attempts to tackle fundamental questions in fern biology, by making the gametophyte phase more accessible to study.

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