

Short Communication

Internal Transcribed Spacer (ITS) as a Molecular Marker for Identification of *Dioscorea hispida* Dennst. (Dioscoreaceae)

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ABSTRACT

Dioscorea hispida is a source of food and also has important medicinal values. However, there are difficulties in identifying and recognizing *D. hispida* based on morphological characteristics. *D. hispida* collected from Besut District in the state of Terengganu, Malaysia, were subjected to PCR amplification with ITS1 and ITS2 (universal primers) but no amplified product was obtained. Therefore, a new set of primers was designed based on a specific signature sequence for *Dioscorea* spp. in the ITS region named as Dios_003 (5'-AAA CGG CTA CCA CAT CCA AG-3') and Dios_004 (5'-AGA CAA ATC GCT CCA CCA AC-3'). A specific band of 900 bp was detected for all accession numbers of *D. hispida*. Sequencing of the amplified ITS region was carried out and alignment of the sequences demonstrated that the ITS region amplified was identical in all the *D. hispida* accessions and was highly conserved among other *Dioscorea* spp. that were analyzed, i.e. *D. japonica*, *D. alata*, *D. elephantipes*, *D. communis*, *D. caucasia*, *D. sylvatica* and *D. polystachya*. Therefore, the new set of internal transcribed spacer (ITS) marker is an effective marker to identify *D. hispida*.

Keywords: *Dioscorea hispida*, Internal Transcribed Spacers (ITS), molecular marker

ABSTRAK

Dioscorea hispida adalah salah satu sumber makanan dan ia juga penting dalam nilai perubatan. Bagaimanapun, terdapat masalah dalam menentukan dan mengenalpasti *D. hispida* melalui ciri-ciri morfologi. *D. hispida* yang diperolehi daripada daerah Besut, Terengganu telah diampifikasikan menggunakan PCR dengan pencetus universal ITS1 dan ITS2 tetapi tiada ampifikasi yang diperolehi. Oleh yang

demikian, satu set pencetus baru telah direka berdasarkan jujukan tandatangan spesifik *D. hispida* iaitu Dios_003 (5'-AAA CGG CTA CCA CAT CCA AG-3') dan Dios_004 (5'-AGA CAA ATC GCT CCA CCA AC-3'). Satu jalur spesifik bersaiz 900 pb telah dikesan dalam kesemua aksesori *D. hispida*. Penjujukan kawasan ITS yang dikuatkan telah dilaksanakan dan penjajaran kesemua jujukan tersebut menunjukkan bahawa kawasan ITS yang dikuatkan adalah sama dalam kesemua aksesori *D. hispida* dan sangat terabadi antara *Dioscorea* spp. yang lain yang telah dianalisis, iaitu *D. japonica*, *D. alata*, *D. elephantipes*, *D. communis*, *D. caucasia*, *D. sylvatica* dan *D. polystachya*.

Kata kunci: *Dioscorea hispida*, Internal Transcribed Spacers (ITS), penanda molekul

INTRODUCTION

In Malaysia, *Dioscorea hispida* Dennst., known locally as *ubi gadung*, is a climber plant with the stem twinning to the left and arising from tuberous roots. The leaves are trifoliate and hairy with small prickles on the underside of the main vein. The male flower is large and branched while the female flower is unbranched. The tubers are covered with masses of fibrous roots and contain dioscorine which is extremely poisonous. *Dioscorea hispida* usually grows wild in Indonesia, and extends to Papua New Guinea, the Philippines and India including Malaysia (Suresh *et al.*, 2011). In this study, *D. hispida* accessions were collected from the district of Besut in the state of Terengganu, Peninsular Malaysia.

Generally, the morphological characteristics such as shape, texture, color, and odor are used in the identification of tuber plants (He *et al.*, 2011). However, these methods are difficult to apply accurately for discrimination and authentication because their morphological characteristics resemble each other especially when they are in a dried and sliced state (Lee *et al.*, 2008; He *et al.*, 2011). Nuclear ribosomal DNA (nrDNA) is tandemly organized with high copy numbers and each repeat unit consists of genes coding for the nuclear ribosomal small subunit (SSU), large subunit (LSU) and 5.8S nrDNA (Richard *et al.*, 2008). 5.8S nrDNA is located between the internal transcribed spacer ITS 1 and ITS 2 regions (He *et al.*, 2011; Baldwin *et al.*, 1995; Baldwin, 1992). The ITS regions are necessary in producing the mature rRNA molecules because they enable their own excision from the RNA transcript (Joseph *et al.*, 1999; Venema and Tollervey, 1999; Cote *et al.*, 2002; Tippery and Les, 2008).

The ITS region has been found to be an effective universal region for molecular identification of plants. For example, the ITS region has been used to infer the South African *Cucumis sagittatus* as the sister of *Cucumismelo* (Ghebretinsae *et al.*, 2007; Sebastiana *et al.*, 2010) and the differences in the nucleotide sequences of the ITS of *Scrophularia* varieties can be used to discriminate each plant (Lee *et al.*, 2010). In cultivated barley, the sequence data of the ITS region has also been used to assess genetic diversity (Sharma *et al.*, 2003). The nuclear ITS regions have been sequenced widely because of their relatively high variability and facility of amplification (Tippery and Les, 2008).

There has been controversy over the classification among *Dioscorea* spp. (Lawton, 1967; Miege *et al.*, 1982; Okonkwo, 1985; Degras, 1993; Sun *et al.*, 2012) which gave us the impetus for improvement on the identification of *D. hispida* using ITS as a molecular marker. The aims of this study were to classify and authenticate *D. hispida* accessions based on ITS as a molecular marker, as PCR and subsequent sequencing is believed to be more accurate and reliable for classifying and authenticating *D. hispida*. PCR with the ITS of the nuclear rDNA cistron as a primer might be a suitable marker for taxonomic classification over a wide range of levels. However, the serious interference from endophytes such as bacteria and fungi that live between living plant cells will result in low sequencing success of the ITS region (Sun *et al.*, 2012). Nevertheless, here we tried using the ITS as a molecular marker for the identification of *D. hispida*.

MATERIALS AND METHODS

Sample Collection

Dioscorea hispida samples were collected from various locations in the district of Besut, Terengganu (Table 1) and planted in the Nursery of the Faculty of Agriculture and Biotechnology, Universiti Sultan Zainal Abidin (UniSZA), Gong Badak Campus, Kuala Terengganu, Terengganu, Malaysia.

Primer Design

New primers for the ITS region were designed after the failure to amplify using known universal primers for ITS, 5'-GGA AGT AAA AGT CGT AAC AAC AAG G-3' (forward primer) and 5'-TCC TCC GCT TAT TGA TAT TGC-3' (reverse primer) which were obtained from the GenBank database. The nrDNA ITS sequence markers were developed by using the ITS of other *Dioscorea* spp. We designed the new primer set named Dios_003 (5'-AAA CGG CTA CCA CAT CCA AG-3') and Dios_004 (5'-AGA CAA ATC GCT CCA CCA AC-3') that were specific to *Dioscorea* spp. using software Primer3 Input (version 0.40).

DNA Extraction and Purification

Fresh leaves of *Dioscorea hispida* were collected from the Nursery of Faculty of Agriculture and Biotechnology, UniSZA and were extracted using Genomic DNA Mini Kit for Plants (Geneaid Biotech Ltd., United States) following the manufacturer's protocols. DNA quantity was assessed on 0.8% agarose gel following electrophoresis.

Polymerase Chain Reaction

Genomic DNA extracted from *D. hispida* accessions were subjected to polymerase chain reaction (PCR)-amplification in a 25 uL mixture which contained 0.2 mM dNTPs, 2.5 mM MgCl₂, 1 X buffer, 0.2 µM forward and reverse primers, 1.5 unit of *Taq* DNA polymerase and distilled water up to 25 uL per reaction with 30 ng of genomic DNA template. The reactions were started by an initial denaturation (94 °C) for 2 min, followed by denaturation (94 °C) for 1 min, annealing (65 °C) for 1 min and extension (72 °C) for 1 min for a total of 33 cycles and a final extension (72 °C) for 10 min. The samples were held at 10 °C. The products were analyzed using 1.5% agarose gel and stained with Midori Green to observe the band.

Purification of PCR Products

All PCR products were purified before DNA sequencing using GeneJet™ PCR Purification Kit (Fermentas, United States) according to the manufacturer's instruction. The purified PCR products were then sent to a commercial company (1st Base Laboratory, Malaysia) for automated DNA sequencing.

DNA Sequence Analysis

Raw DNA sequence data was edited using Chromas and Bioedit software (Hall, 1999). Sequences were compared with GenBank database using Basic Local Alignment Search Tool (BLAST) at the National Center for Biotechnology Information (NCBI) site (<http://www.ncbi.nih.gov>).

Table 1. *Dioscorea hispida* samples used in this study.

Accession no.	Sampling Site	Bunch	District	Location			
DH 003	Kg Denger	Bunch A	Besut	N 05 ⁰ 29.337'	46.030'	E	102 ⁰
DH 005	Kg Denger	Bunch B	Besut	N 05 ⁰ 29.337'	46.030'	E	102 ⁰
DH 009	Kg Pasir Akar	-	Besut	N 05 ⁰ 29.933'	39.299'	E	102 ⁰
DH 012	Kg Seberang Batu 13	Bunch A	Besut	N 05 ⁰ 29.907'	34.017'	E	102 ⁰
DH 015	Kg Seberang Batu 13	Bunch B	Besut	N 05 ⁰ 29.907'	34.017'	E	102 ⁰
DH 018	Kg Keruak	-	Besut	N 05 ⁰ 29.422'	28.552'	E	102 ⁰
DH 024	Kg La	-	Besut	N 05 ⁰ 29.789'	31.660'	E	102 ⁰

Note: Kg stands for *Kampung* or village. Each bunch was sampled from different plants

RESULTS AND DISCUSSION

The ITS region was successfully amplified using the specifically designed ITS primers as shown in Figure 1. The results showed that the Dios_003/ Dios_004 primer pair amplified a 900 bp DNA product which was then purified and sequenced.

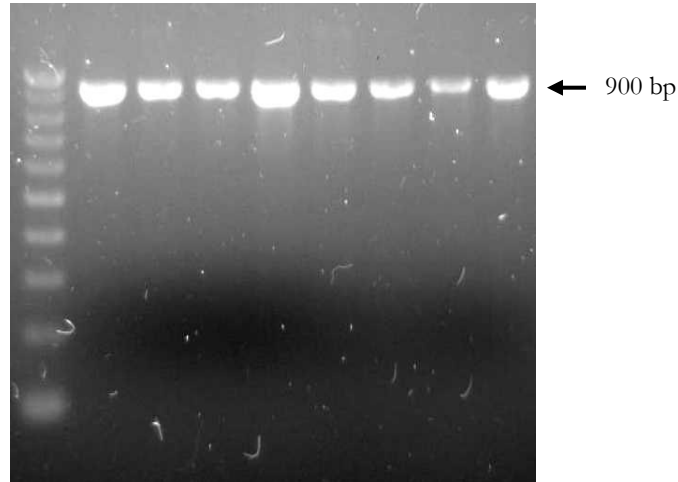


Fig. 1. PCR amplification of extracted DNA from *Dioscorea hispida* accessions using the Dios_003/Dios_004 primers. From left: 100 bp ladder, DH 3, DH 5, DH9, DH 12, DH 15, DH 18, DH 22 and DH 24.

ITS sequences including the 5.8S gene were determined for *Dioscorea hispida* (sequences not yet submitted to GenBank). The size of the ITS region which spanned ITS 1-5.8S-ITS 2 ranged from 400 to >1000 bp (Li *et al.*, 2011). In this study, all *D. hispida* accessions (eight samples) revealed an ITS region length of 900 bp. There was no difference in nucleotide sequences among the different accessions of *D. hispida*, suggesting that there is no intraspecific variation in the nrDNA ITS region of *D. hispida* that were sampled from the district of Besut. This is in agreement with the findings of Li *et al.* (2011) who reported that the ITS region is not suitable for the discrimination of the same species but very efficient in the discrimination of different species due to the low intraspecific variation (<0.5%) and high interspecific variation (16%). These sequences were compared with the ITS region of other *Dioscorea* spp. in the GenBank database using BLAST. The results obtained were summarized in Table 2 and indicated that the *Dioscorea* spp. has 99% similarities compared with *D. hispida*.

From the results in Table 2, the ITS region of *D. japonica* was shown to be very similar with *D. hispida* because it contained only one base substitution at nt

263. The divergence between *D. hispida* and other *Dioscorea* spp. were very low because of the base substitutions that occurred occasionally, meaning that there indeed existed the core structure or conserved region (Schultz *et al.*, 2012).

Dioscorea hispida revealed the unique nucleotide at position 263 where the base is Guanine (G) instead of Adenine (A), compared with *D. japonica*, *D. alata*, *D. elephantipes*, *D. communis*, *D. caucasia*, *D. sylvatica* and *D. polystachya*. The unique nucleotide indicated that the species was *D. hispida*. However, further research should be done in order to compare *D. hispida* with other *Dioscorea* spp. such as *D. esculenta*, *D. tripidata* and *D. rotundata*; to find out if this G at position 263 of the ITS region is indeed specific for *D. hispida* and could be used as a basis for species identification.

As a conclusion, primers for the molecular marker ITS were successfully designed and amplified in all accessions of *D. hispida* that were collected in Besut, Terengganu. *D. hispida* (that were studied in this research), *D. japonica*, *D. alata*, *D. elephantipes*, *D. communis*, *D. caucasia*, *D. sylvatica* and *D. polystachya* were found to be very closely related based on the sequences of the ITS region with 99% similarities. *D. hispida* appeared to have a unique nucleotide at position 263 of the ITS region where the base was Guanine instead of Adenine for the other *Dioscorea* spp.

Further studies using the maturase K (*matK*) gene should be carried out as *matK* has been recently used as a DNA barcode for the identification of *Dioscorea* due to greater nucleotide variation (Sun *et al.*, 2012). Future assessments need to take into account the ability for species discrimination as well as the cost of experiments. Other molecular studies of *D. hispida* may be necessary prior to the availability of the full genome sequence of this species.

Table 2. The nucleotide differences of *Dioscorea* spp. compared to *Dioscorea hispida* according to ITS region.

No.	<i>Dioscorea</i> spp.	Identities (bp)	Location of nucleotide changes	Nucleotide changes	GenBank accession no.	References
1	<i>D. japonica</i>	792/793	nt 263	A → G	EU 817835.1	Gao <i>et al.</i> , 2008
2	<i>D. alata</i>	787/793	nt 126 nt 159 nt 263 nt 297 nt 307 nt 726	C → T T → C A → G C → T G → A T → C	EU 817831.1	Gao <i>et al.</i> , 2008
3	<i>D. elephantipes</i>	791/793	nt 39 nt 263	A → T A → G	FJ 215767.1	Merckx <i>et al.</i> , 2008
4	<i>D. communis</i>	790/793	nt 236 nt 263 nt 265	T → C A → G T → C	EU 186223.1	Merckx <i>et al.</i> , 2008
5	<i>D. caucasia</i>	789/793	nt 263 nt 356 nt 357 nt 478	A → G G → A C → T A → C	FJ 215769.1	Merckx <i>et al.</i> , 2008
6	<i>D. sylvatica</i>	789/793	nt 39 nt 263 nt 356 nt 357	A → T A → G G → A C → T	FJ 215768.1	Merckx <i>et al.</i> , 2008
7	<i>D. polystachya</i>	788/793	nt 263 nt 286 nt 311 nt 493 nt 766	A → G T → C C → T C → T T → C	EU 817838.1	Gao <i>et al.</i> , 2008

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