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Research Article

DNA FINGER PRINTING ANALYSIS OF EIGHT SPECIES OF *DENDROBIUM* FOUND IN WESTERN GHATS USING RAPD AND ISSR MARKERS

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ABSTRACT
The molecular marker is a useful tool for assessing genetic variation and resolving cultivars identities. The objective of the present study was to determine the variability of *eight species of Dendrobium*, (India) using Random Amplified Polymorphic Deoxyribonucleic acid (RAPD) and Inter Sample Sequence Repeat (ISSR) markers. Five decamer – primers and four ISSR markers could generate a total of 145 RAPD fragments and 133 ISSR fragments respectively (monomorphic and polymorphic). The size of amplified fragments was ranged from 300 to 1000 bp. The present work indicates that RAPD technology is an effective method to differentiate *Dendrobium* species.

Key Words: *Dendrobium, Random Amplified Polymorphic Deoxyribonucleic Acid, Inter Sample Sequence Repeat, Genetic Diversity, Polymorphism.*

INTRODUCTION
Dendrobium (epiphyte) is a huge genus of family Orchidaceae (orchids). It was established by Olof Swartz in 1799 and today contains about 1,200 species. Dendrobium herbs are used as traditional Chinese medicines. The genus occurs in diverse habitats throughout much of south, east and Southeast Asia, including the Philippines, Borneo, Australia, New Guinea, Solomon Islands and New Zealand. It is used for the treatment of alcoholic gastritis, also helpful for anti-pyretic, anti consumption and anti-diarrheal effects.

Dendrobium species used for DNA fingerprinting: Flickingeria nodosa, Dendrobium macrostachyum, Dendrobium crepidatum, Dendrobium herbaceum, Dendrobium aphyllum, Dendrobium heterocarpum, Dendrobium ovatum (L) kraenzlin, Dendrobium nutantiflorum, DNA based markers are effective and reliable tools for measuring genetic variability in plant species and studying evolutionary relationship. The evaluation based on RAPD profiles would be suitable for providing such information due to its high level of polymorphism of this technique. In recent years, DNA fingerprinting system based on RAPD analysis have been increasingly utilized for detecting genetic polymorphisms in several plant genera. The RAPD and ISSR technique has been applied to some research aspects, such as plant species identification (Zhang et al., 2001), diversity assessment (Jarret and Austin, 1994), detection of genetic variations in N fertilizer treated plants (Lo et al., 2004), detection of intraclonal variations (Villordon and LaBonte, 1995), and estimation of genetic diversity (Sagredo et al., 1998).

In the present investigation RAPD and ISSR were used to discriminate the Dendrobium species and genetic relationship was further analyzed according to the resultant RAPD and ISSR markers.

MATERIALS AND METHODS
Isolation of DNA
For the isolation from the plant sample, first of all 100mg of plant material was weighted, and was grinded in a chilled mortar and pestle. 900µl of cTAB (cetyl tri methyl ammonium bromide) Tris-HCl (2.5ml), EDTA (0.5ml), NaCl (2.04 gm), cTAB (0.5 gm) and β mercaptoethanol and PVP (0.25ml). The suspension was mixed well and incubated for 10 min at 60°C then it was transferred to refrigerator (4ºc)

for 5 mins. In this solution 900µl of ice cold chloroform-iso amyl alcohol (24:1). After centrifugation at 10,000rpm for 10 mins, the supernatant was transferred to another tube and 0.7 volume of ice cold propanol was added, mixed gently and then incubated for 15 min at -20°C. Mixture was centrifuged at 14,000rpm for 10 mins at 4°C then supernatant was discarded. The pelleted down DNA was then suspended in 500µl of 70% ethanol (spin at 5000rpm for 5 mins). Then supernatant was discarded and pellet was air dried and was dissolved in 200µl of 0.1X TE buffer.

Table 1: Types of Markers and Their Advantages and Disadvantages

Marker	Advantages	Disadvantages
RAPD	Results obtained quickly	Highly sensitive to laboratory Changes
	Fairly cheap	Low reproducibility within and between laboratories
	No sequence information Required	Cannot be used across populations nor across species
	Relatively small DNA quantities required	Often see multiple loci
	High genomic abundance	Dominant
	Good polymorphism	
	Can be automated	
ISSR	Highly polymorphic Robust in usage Can be automated	Usually dominant Species-specific

Then 1µl of RNase was added (for 30 mins at 37ºC) to remove RNA content. 1/10th volume of sodium acetate and 2.5 volume of absolute alcohol were added (for 20 mins at -20ºC). Then centrifuged at 14,000rpm for b10 mins at 4ºC, pellet was rinsed by adding 500µl of 705 ethanol and spinning at 5,000 rpm for 5 mins. Lastly the pellet was air dried and dissolved in 40µl of 1XTE buffer. The quality of genomic DNA was checked by using 0.8% agarose gel prepared in 1XTAE buffer (50 ml)r in presence of 5µl EtBr. The DNA samples were stored at -20°C until further analysis.

Quantification of isolated DNA

Spectrophotometer method was used to quantify the purity of DNA sample. 10µl of DNA dissolved in 0.1XTAE buffer (also used as blank) volume made up to 2 ml. Absorbance was taken at 260 and 280nm.

Polymerase Chain Reaction (PCR)
Random Amplified Polymorphic Deoxyribonucleic acid (RAPD) markers:
Primers used
OP A 02-5'-TGCCGAGCTG-3'
OP A 03-5'-AGTCAGCCAC-3'
OP A 18-5'-AGGTGACCGT-3'
OP AB 09-5'-GGGCGACTAC-3'
OP AB 15-5'-CCTCCTTCTC-3'

PCR amplification reactions were performed with random decamer OP primers in a MJ research PTC 200 thermo cycler. For RAPD analysis, PCR reaction was performed in reaction mixture consisting 18.5µl sterile water, 1U Taq DNA polymerase(1µl), 10X PCR buffer(2.5µl), dNTP's (2.5mM each dNTP) (1µl), sample DNA(50-200mg)(1µl), and primer(1µl) with the following cycle repeated 39 times: pre denaturing at 94°C for 3 min, denaturing at 92°C for 1 min, annealing at 36°C for 2 min, elongation at 72°C for 2 min. Final elongation segment was held for 6 min at 72°C in thermal cycler PCR (thermo).Amplified DNA was analyzed by electrophoresis on Agarose gel for banding pattern.

Inter Sample Sequence Repeat (ISSR) Markers
Primer used:
A 807-5' AGAGAGAGAGAGAGAGT
B 811-5'GAGAGAGAGAGAGAGAC
C 815-5'CTCTCTCTCTCTCTCTG
D 818-CACACACACACACACAG

PCR amplification reactions of extracted DNA sample was carried out with ISSR primer in a MJ research PTC 200 thermo cycler . PCR reaction was performed in reaction mixture consisting 18.5µl sterile water, 1U Taq DNA polymerase(1µl), 10X PCR buffer(10mM Tris-HCL pH8.8, 500mM KCL, 15mM $MgCL_2$, 0.1% gelatin, 0.05% Tween 20 and 0.05% NP40)(2.5µl), dNTP's(2.5mM each dNTP)(1µl), sample DNA(50-200mg)(1µl), and primer(1µl) with the following cycle: pre denaturing at 94°C for 8 min, denaturing at 94°C for 30 sec, annealing at 50°C for 45 sec, , repeat step1 and 2 for 39 times, . Final elongation segment was held for 7 min at 72°C in thermal cycler PCR (thermo).Amplified DNA was analyzed by electrophoresis on Agarose gel for banding pattern.

RESULTS AND DISCUSSION

Genomic DNA isolation

Figure 1: DNA bands of isolated *Dendrobium*

In the present investigation**,** two different PCR-based approaches, RAPD and ISSR were carried out in order to determine the variability of *Dendrobium* species. The PCR reaction was repeated several times for each of the eight species to obtain highly reproducible bands. Results indicate that each species collected from different localities seemed similarity in RAPD and ISSR profiling by using different primers.

Table 2: Different Samples and their Absorbance Ratio and Quantification Value

Sample	Absorbance at 260nm	Absorbance at 280nm	Ratio	Quantification value ($Q = A_{260}*50*200$)
Flickingeria nodosa	0.020	0.017	1.17	200µg/ml
D. macrostachyum	0.048	0.033	1.45	480 µg/ml
D. crepidatum	0.031	0.016	1.93	310 µg/ml
D. herbaceum	0.030	0.017	1.76	300 µg/ml
D. aphyllum:	0.048	0.038	1.45	480 µg/ml
D.heterocarpum	0.013	0.007	1.85	130 µg/ml
D. ovatum	0.059	0.030	1.96	590 µg/ml
D.nutantiflorum	0.042	0.030	1.40	420 µg/ml

According to results *D. nodosa* was in pure form and *D. ovatum* contains max contamination.

Results of RAPD

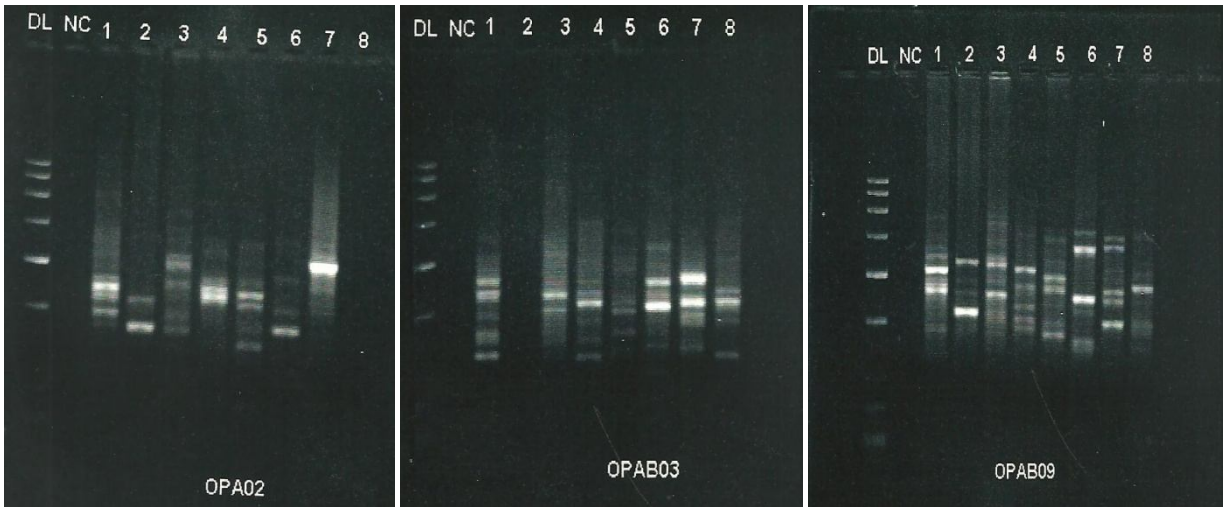

Figure 2: Showing Monomorphic Bands in region of 300-500bp in sample 1,2,3,5 and 6; 1,4,5,6,7 and 8 for primer opa02, opab03,opab09 respectively and primer opa02, opab03,opab09 showing other monomorphic bands in region of 500-1000bp in sample 1,2,4,5 and 6; 1,3,5,7 and 8; 1,2,3,4,6 and 7 respectively. some polymorphic bands observed in sample 5and7; 4, 5and7; 2, 6, 7 and 8 respectively. (dl- dna ladder, nc- negative control)

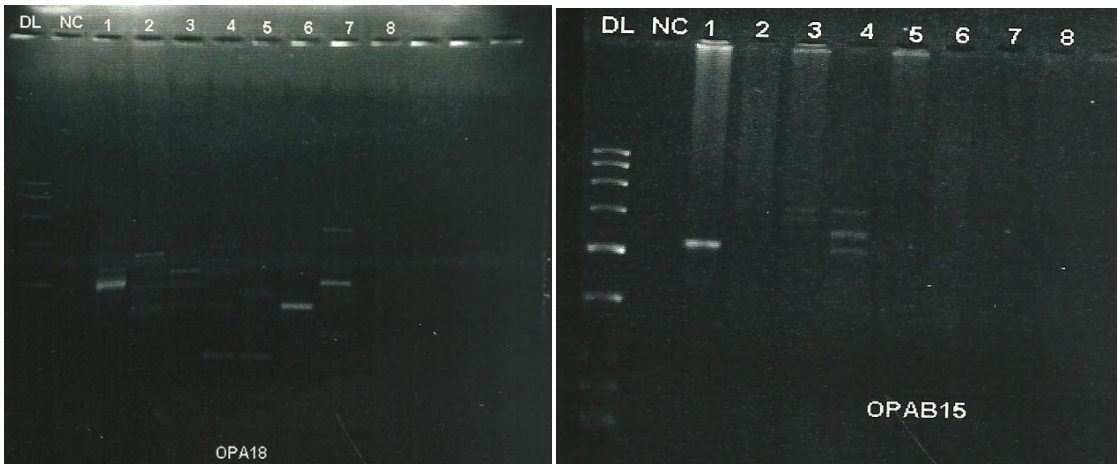

Figure 3: Showing Bands With Primer Primer Opa18, Opab15 Which Cannot Be Used As Differential Markers Because They Show Very Less Amplification In All Samples.

Results of ISSR

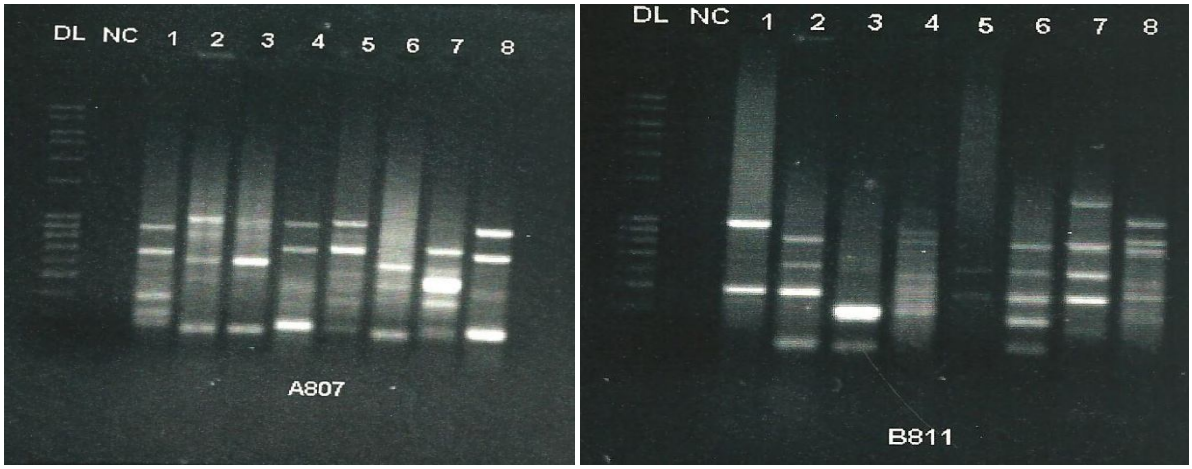

Figure 4: Showing All Samples with primer a807 which show monomorphic bands in region of 200-300 and in region of 600-700 monomorphic bands in sample 2, 3 and 6. Around 15 polymorphic bands were observed. Primer b811 shows monomorphic bands in sample 2, 3 and 6 in 200-300bp and sample 1, 2,5,6,7 and 8 in 500-600bp region so both can be used as differential markers.

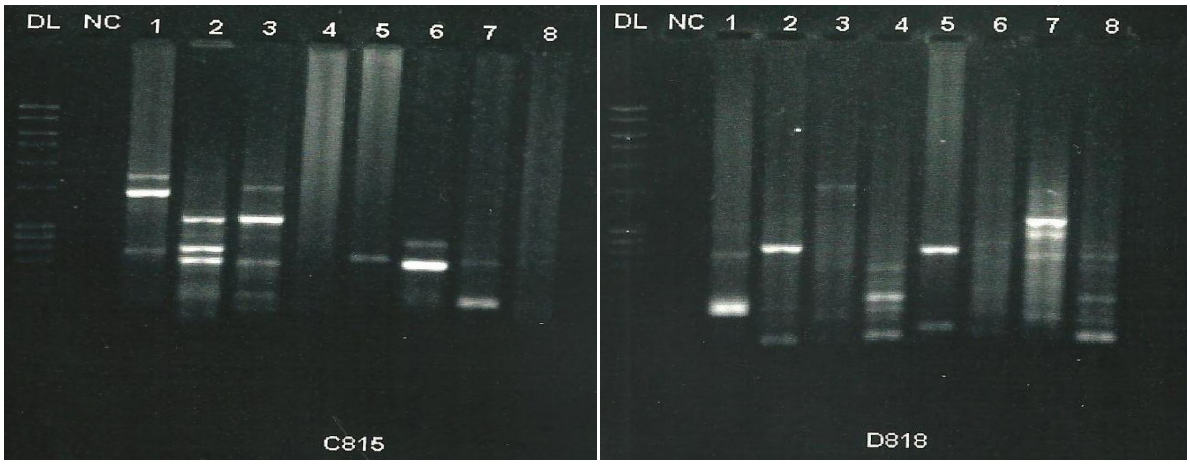

Figure 5: Showing Primer C815 And D818 Which Show Very Less Amplification So Cannot Be Used As Differential Markers.

A total of 10 and 9 primers were used in RAPD and ISSR respectively. Only 5(RAPD) and 4(ISSR) were able to produce reproducible bands in all the eight *Dendrobium* species. Total primers produced 145 RPD fragments and 133 ISSR fragments and both monomorphic and polymorphic bands were observed.
The present work indicates that RAPD technology is an effective method to differentiate *Dendrobium* species. The genetic variation observed by this method in this report may also demonstrate the genetic relationships among the eight *Dendrobium* species. The RAPD and ISSR technique appliedfor present investigation is very highly sensitive and reproducible and as little as 10-20 ng DNA template is sufficient for analysis.

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