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Research Article

Determination of Phytogenetic Relationships among Isoetes Species Using Random Primers

Yogendra K. TRIPATHI[1], Priyatansh GURHA[2], D. GHOSH[3], R.V. KUMAR[4], Ved PRAKASH[4]

[1]Department of Genetics, Barkatullah University, Bhopal - INDIA

[2]Department of Molecular Biology, University of Illinois, Illinois - USA

[3]Department of Genetics and Plant Breeding, Calcutta University, Kolkata - INDIA

[4]National Research Center for Agroforestry, Jhansi - INDIA

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Abstract: *Isoetes* L. is an ancient, primitive genus of lycopsids. The genus occupies a unique position in plant evolution as the closest relatives of tree lycopods. The morphological characters, namely leaf size, comb, and sporophyll size, are used diagnostically to help in the identification of the *Isoetes* species. Thus, species identification of *Isoetes* is always problematic and the knowledge on the genetic relationship of *Isoetes* species is also limited. Therefore, in order to find a suitable method for species identification and estimation of genetic relationship among different species of *Isoetes*, a Random Amplified Polymorphic DNA (RAPD) technique based on Polymerase Chain Reaction (PCR) was used for the described purpose. Out of 150 decamer primers tested, 58 produced good amplification products. A total of 4442 amplification products were scored and only 3479 bands (78.11%) were found to be polymorphic, with sizes ranging from 200 to 5500 bp. Unweighted pair group method using arithmetic average cluster analysis revealed a clear genetic difference among *Isoetes* species. The scientific data presented in this study suggest that RAPD-PCR could be a valuable tool for identification and estimation of genetic relationship among species of *Isoetes* L.

Key Words: *Isoetes* species, RAPD fingerprint, identification, genetic relationship

Introduction

Isoetes is a non-flowering lycopod inhabiting submerged and wet areas. All species of *Isoetes* are required enough light for luxuriant growth (Goswami, 1996). Lycopods are frequently mistaken as small aquatic grasses but they lack the hollow stems, nodes, and flat leaves of grasses. Leaves of *Isoetes* species are hollow and quill-like, and reveal cross like partitions. Each leaf is 1-2 cm wide and broadens to a swollen base where it attaches in clusters to a bulb-like, underground rhizome, which is a characteristic of all *Isoetes* (quillwort) species. This swollen base also contains male and female sporangia protected by a thin, transparent covering (velum), which is used diagnostically to help in the identification of the *Isoetes* species. However, an individual identification based on a morphological diversity record is now a more common practice. Therefore, with the help of morphological parameters, the identification of *Isoetes* species is difficult and morphological data cannot be used in the estimation of a genetic relationship (Stuber, 1989a, 1989b, 1989c).

To overcome these problems, the use of molecular markers is considered the best way for detecting genetic relationships and their identities among the species of *Isoetes*, since there is no effect of stage of development, environment, or management practices. Due to the advances in molecular biology techniques, large numbers of highly informative DNA markers have been developed for the species identification and for estimation of genetic relationships. In the last decade, the Random Amplified Polymorphic DNA (RAPD) technique based on Polymerase Chain Reaction (PCR) has been one of the most commonly used for the described purpose. The main objective of our study is to use RAPD fingerprinting for identification and estimation of genetic relationship among different species of *Isoetes*. Another important

E-mail: ygene2005@yahoo.co.in

aim of the study is to provide genetic data for cataloguing of all species of this genus.

Materials and Methods

Materials

Thirteen species (Table 1) of *Isoetes* species collected from 7 states of India, namely Madhya Pradesh, Uttar Pradesh, West Bengal, Maharashtra, Rajasthan, Andhra Pradesh, and Karnataka, were maintained in a greenhouse. The leaves of these 13 species of Isoetes were placed separately in an icebox. Freshly collected leaves of species of *Isoetes* were washed with distilled water and blot dried, and then cut into small pieces with a sterilised blade.

DNA extraction and purification

Genomic DNA from young leaves of *Isoetes* species was isolated using CTAB (Cetyl trimethyl ammonium bromide) method (Dellaporta et al., 1983) with the following modification: 3.0 g of young leaf sample was ground to a very fine powder in liquid nitrogen using a mortar and pestle. The resulting powder was dispersed in a 50 ml sterile centrifuge tube containing 25 ml of pre-warmed (60 °C) DNA extraction buffer (100 mM Tris HCl, pH 8.0; 20 mM EDTA, pH 8.0; 1.4 M NaCl, 2% w/v CTAB, 0.2% 2-mercaptoethanol and 2% polyvinyl pyrollidone). This tube was incubated in a water bath at 65 °C for 45 min. After the incubation, 15 ml of 24:1 choloroform:isoamyl alcohol was added, mixed well with gentle inversions and centrifuged at 20,000 rpm for 20 min at room temperature. The upper aqueous layer was separated and an equal volume of pre-chilled Iso-propanol was added and gently mixed with quick inversion for DNA precipitation. The precipitated DNA was removed using wide bore pipette tips and transferred into Eppendorf tubes. The tubes were centrifuged at 10,000 rpm for 5 min. The resulting DNA pellets were washed 4 or 5 times in 70% ethanol, dried under vacuum, and dissolved in 500 µl of TE buffer (10 mM Tris HCl, 1 mM EDTA).

The RNA was removed by RNAse treatment at 37 °C for 1 h. For further purification, DNA solution was extracted once with an equal volume of phenol:choloroform:isoamyl alcohol (25:24:1; pH 8.0) followed by 2 extractions with chloroform:Isoamyl alcohol (24:1). The upper aqueous phase was precipitated by adding 2 volumes of chilled absolute alcohols, pelleted and dried in vacuum, and then dissolved in TE buffer. Quantification of DNA was accomplished by analysing the purified DNA on 1% agarose gels along side a diluted DNA marker as standard. The DNA was diluted in TE buffer to a concentration of 30-35 µl for use in PCR reactions.

PCR reactions

RAPD reactions were essentially performed as per Williams et al. protocols (Williams et al., 1990). However, some modifications were made to enhance the

Table 1. *Isoetes* species and their place of collection.

Serial no.	Accession no.	Species	Place
1.	G_1	*Isoetes reticulata*	Rajasthan
2.	G_2	*Isoetes dixitei*	Maharashtra
3.	G_3	*Isoetes rajashthansis*	Rajasthan
4.	G_4	*Isoetes tuberculata*	Rajasthan
5.	G_5	*Isoetes sampathkumarani Rao*	Andhra Pradesh
6.	G_6	*Isoetes pantii*	Madhya Pradesh
7.	G_7	*Isoetes coromedelina*	West Bengal
8.	G_8	*Isoetes unilocularsis*	Uttar Pradesh
9.	G_9	*Isoetes* sp. (but not yet described)	Tirupati (Andhra Pradesh)
10.	G_{10}	*Isoetes mirzapurensis*	Uttar Pradesh
11.	G_{11}	*Isoetes panchananii*	Maharashtra
12.	G_{12}	*Isoetes sahayandriensis mahabale*	Karnataka
13.	G_{13}	*Isoetes fuchsii*	Madhya Pradesh

reproducibility and consistency in the RAPD profile. RAPD amplification was performed in a 25 µl of reaction volume containing 1X PCR buffer (10 mM Tris HCl (pH 9.0), 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% gelatin) 200 µm each of dNTP (Sigma), 20 picomoles of random primers from Operon Technologies Inc., USA , 0.5 unit of taq DNA polymerase (Genetix) and 100 ng/reaction of DNA template. The amplification reaction was performed in a PCR machine (MJ Research Inc., USA, model PTC-1100) with a heated lid technology for 40 cycles. The PCR conditions were: an initial denaturation of genomic DNA at 93 °C for 4 min followed by 40 cycles of DNA template denaturation at 93 °C for 1 min, primer annealing at 35 °C for 1 min, DNA amplification at 72 °C for 1 min, and a final primer extension at 72 °C for 8 min.

The PCR products were separated on a 1.0% agarose gel containing ethidium bromide using 1X TAE buffer. The sizes of the amplified fragments were determined using size standards (100 bp DNA ladder plus or DNA ladder mix). The DNA fragments were visualised under a UV light and photographed using a gel documentation system. To test the reproducibility of the profile, the reaction was repeated at least twice.

Data analysis

Amplified and separated DNA fragments on agarose gels were scored for presence (1) or absence (0) of bands for each 13 species of *Isoetes* using 58 primers. Only the reproducible bands were considered and the faint or ambiguous bands that appeared unstable in the gel runs were ignored. In some cases, no band was detected, possibly due to the conditions of PCR products. The data were analysed using simqual route to generate Jaccard's similarity coefficient (Jaccard, 1908) using NTSYS_PC Version 2.0 (Exeter Software, NY, USA) program. These similarity coefficients were used to produce a dendrogram for which the unweighted pair group method using arithmetic average (UPGMA) algorithm and SAHN clustering were employed to depict the genetic relationship.

Results and Discussion

A total of 150 primers (10-mer) tested for their capacity to differentiate among the 13 species of *Isoetes*. The best 58 primers that detected polymorphism among species of *Isoetes* and gave reproducible banding patterns were chosen. The 58-decamer primers produced total of 4442 fragments in sizes ranging from 200 to 5500 bp. Out of the 4442 amplification products, 3479 bands (78.11%) were polymorphic (Table 2). The average number of bands per primer was 76.59 (ranging from 65 to 96 bands/primer, and the average number of polymorphic bands was 59.98 (ranging from 59 to 98 bands/primer). OPA-01 (5'-CAGGCCCTTC-3'), OPO-01 (5'-GGCACGTAAG-3'), OPM-06 (5'-CTGGGCAACT-3'), OPN-01 (5'-CTCACGTTGG-3'), and OPM-09 (5'-GTCTTGCGGA-3') exhibited the highest level of variability and the percentage of polymorphic bands was 100% (Table 2). Finally, these 5 primers were selected for cluster analysis. The generated dendogram UPGMA based on the distance matrix showed a more detailed genetic relationship among the species of *Isoetes* (Figure). Thirteen species of *Isoetes* showed 5 clusters. The 1st one had G1, G4, G8, G7, G5, G10, and G13 whereas the 2nd one had only one species, namely G12. G6 and G9 occupied the 3rd group of clusters, while only G3 species were placed in the 4th cluster. The last 5th cluster had only G11 species. A dendogram generated between species matrix showed 2 main clusters (Figure). G1, G4, G8, and G7 occupied in the first 2 subclusters of the 1st cluster, while G2, G10, G5, G13 occupied in the 3rd and the 4th subclusters of the 1st cluster. At first, G12 species stood alone, and then followed the 2nd cluster, which had G6 and G9 species. G3 and G11 species appeared at the lower end of the dendrogram.

The genetic variability of the plants results from interaction of mutation, selection, random genetic drift, and differential migration. Mutation pressure and selection procedure are the major factors changing the level of genetic equilibrium. Geographically ecological and reproductive isolation had a material effect on the level of the genetic diversity of the species (Tripathi et al., 2002). Molecular techniques provide an alternative approaches for evaluating the genetic relationship in shrubs. Since they are not subjected to any environmental effects and are independent of the development stages of plants, these methods were used to identify the species (Ma et al., 2004). The development of molecular techniques was selected in alternative DNA based procedures for detecting polymorphism (Tripathi et al., 2005). The RAPD technique reveals an extensive amount of variation leading to species identification (Swoboda and Bhalla, 1997). Because of the conserved morphology among *Isoetes* species, species identification and estimation of

Table 2. Oligonucleotide primers selected for amplification and polymorphism.

Serial no.	Primers	Sequence	Scored bands	Polymorphic bands	Polymorphism rate (%)
1	OPA-01	5'-CAGGCCCTTC-3'	78	78	100.00
2	OPA-13	5'-CAGCACCCAC-3'	76	50	65.79
3	OPA-14	5'-CAAACGTCGG-3'	76	63	82.89
4	OPN-01	5'-CTCACGTTGG-3'	76	76	100.00
5	OPN-02	5'-ACCAGGGGCA-3'	79	66	83.54
6	OPN-03	5'-GGTACTCCCC-3'	69	43	62.23
7	OPN-04	5'-GACCGACCCA-3'	71	45	63.38
8	OPN-05	5'-ACTGAACGCC-3'	74	61	82.43
9	OPN-06	5'-GAGACGCACA-3'	78	52	66.67
10	OPN-07	5'-CAGCCCAGAG-3'	82	69	84.15
11	OPN-08	5'-ACCTCAGCTC-3'	79	53	67.09
12	OPN-15	5'-CAGCGACTGT-3'	91	78	85.71
13	OPN-20	5'-GGTGCTCCGT-3'	75	36	48.00
14	OPF-01	5'-ACGGATCCTG-3'	85	72	84.71
15	OPF-05	5'-CCGAATTCCC-3'	81	55	67.90
16	OPF-08	5'-GGGATATCGG-3'	65	39	60.00
17	OPF-09	5'-CCAAGCTTCC-3'	92	79	85.87
18	OPF-10	5'-GGAAGCTTGG-3'	74	35	47.30
19	OPF-13	5'-GGCTGCAGAA-3'	74	48	64.86
20	OPF-15	5'-CCAGTACTCC-3'	66	52	78.79
21	OPO-01	5'-GGCACGTAAG-3'	95	95	100.00
22	OPO-02	5'-ACGTAGCGTC-3'	71	58	81.69
23	OPC-01	5'-TTCGAGCCAG-3'	82	56	68.29
24	OPC-02	5'-GTGAGGCGTC-3'	73	60	82.19
25	OPC-03	5'-GGGGGTCTTT-3'	72	59	81.94
26	OPC-04	5'-CCGCATCTAC-3'	75	62	81.67
27	OPC-05	5'-GATGACCGCC-3'	77	64	83.12
28	OPC-06	5'-GAACGGACTC-3'	81	68	83.95
29	OPC-07	5'-GTCCCGACGA-3'	80	54	67.50
30	OPC-08	5'-TGGACCGGTG-3'	75	62	82.67
31	OPC-09	5'-CTCACCGTCC-3'	72	59	81.94
32	OPC-10	5'-TGTCTGGGTG-3'	77	51	66.23
33	OPM-01	5'-GTTGGTGGCT-3'	79	53	67.09
34	OPM-02	5'-ACAACGCCTC-3'	66	53	80.30
35	OPM-03	5'-GGGGGATGAG-3'	70	57	81.43
36	OPM-04	5'-GGCGGTTGTC-3'	89	76	85.39
37	OPM-05	5'-GGGAACGTGT-3'	69	56	81.16
38	OPM-06	5'-CTGGGCAACT-3'	79	79	100.00
39	OPM-07	5'-CCGTGACTCA-3'	67	41	61.12

Table 2. (continued)

Serial no.	Primers	Sequence	Scored bands	Polymorphic bands	Polymorphism rate (%)
40	OPM-08	5'-TCTGTTCCCC-3'	96	70	72.92
41	OPM-09	5'-GTCTTGCGGA-3'	65	65	100.00
42	OPM-10	5'-TCTGGCGCAC-3'	73	60	82.19
43	OPE-01	5'-CCCAAGGTCC-3'	81	68	83.95
44	OPE-02	5'-GGTGCGGGAA-3'	90	77	85.56
45	OPE-03	5'-CCAGATGCAC-3'	65	52	80.00
46	OPE-04	5'-GTGACATGCC-3'	69	56	81.16
47	OPE-05	5'-TCAGGGAGGT-3'	76	50	65.79
48	OPE-06	5'-AAGACCCCTC-3'	68	55	80.88
49	OPE-07	5'-AGATGCAGCC-3'	73	60	82.19
50	OPE-08	5'-TCACCACGGT-3'	67	54	80.59
51	OPE-09	5'-CTTCACCCGA-3'	79	66	83.54
52	OPE-10	5'-CACCAGGTGA-3'	70	57	81.43
53	OPE-13	5'-CCCGATTCGG-3'	76	63	82.89
54	OPE-15	5'-ACGCACAACC-3'	71	58	81.69
55	OPE-16	5'-GGTGACTGTG-3'	89	63	70.79
56	OPE-17	5'-CTACTGCCGT-3'	83	57	68.67
57	OPE-20	5'-AACGGTGACC-3'	85	72	84.71
58	OPE-11	5'-GAGTCTCAGG-3'	76	63	82.89
	Total		4,442	3,479	
	Average		76.59	59.98	78.16
	Range		65-96	36-95	47.30-100.00

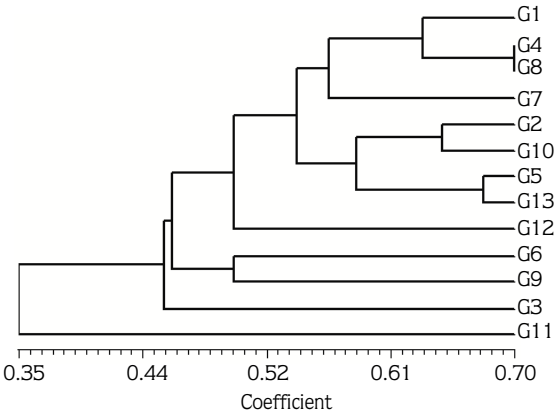

Figure. UPGMA clustering of *Isoetes* species based on RAPD polymorphisms.

genetic relationships have been problematic. Therefore, there is a need to use molecular markers for solving these problems. Various researchers also used a number of molecular markers for revealing unknown or extinct lineages within *Isoetes* (Hoot et al., 2004). They suggest that, with the help of molecular markers, it is possible to catalogue the *Isoetes* species for future conservation of this endangered species. RAPD markers have been proved to be useful by providing a bunch of simply inherited genetic markers for genome mapping (Bradshaw et al., 1995) or to discriminate among closely related taxa. Our work indicated that RAPD markers could be used for estimation of genetic relationships, which ultimately help in the identification of *Isoetes* species.

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