Molecular Characterization of Five Medicinally Important Species of *Typhonium* (Araceae) through Random Amplified Polymorphic DNA (RAPD)

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The interrelationship of five medicinally important species of *Typhonium* (Araceae) including *T. venosum*, which was previously placed under the genus *Sauromatum*, was inferred by analysis of random amplified polymorphic DNA (RAPD). DNA from pooled leaf samples was isolated and RAPD analysis was performed using 20 decamer oligonucleotide primers. Out of a total of 245 bands amplified, 12 were found to be monomorphic while 233 bands were polymorphic including 86 species-specific bands. The genetic similarities were analyzed from the dendrogram constructed by the pooled RAPD data using a similarity index. The dendrogram showed two distinct clades, one containing *T. roxburghii*, *T. trilobatum* and *T. venosum* and the other containing the remainder two species, *i.e. T. diversifolium* and *T. flagelliforme*. Both the clusters shared a common node approx. at 23.7% level of similarity. The maximum similarity of 31.2% was observed between *T. venosum* and *T. trilobatum*. In view of its close genetic similarity with other members of *Typhonium*, transfer of *Sauromatum venosum* to the genus *Typhonium* and merger of the two genera was supported.

Key words: Molecular Taxonomy, Genomic Relationship, RAPD, *Typhonium*

Introduction

The genus *Typhonium* (Araceae) is comprised of about 30 species (Mabberly, 1997) and is distributed throughout the tropical and subtropical regions of the world with greater concentration of species in Southeast Asia, Indo-Malaysia and Northeastern Australia. In India, it is represented by 16 species (Santapau and Henry, 1973). Among the species found in India, *T. trilobatum* (L.) Schott is the most common and grows wild in a wide range of habitats. Besides, *T. divaricatum* (L.) Decne, *T. flagelliforme* (Lodd.) Bl., *T. bulbiferum* Dalz., *T. venosum* (Dryand ex Ait.) Hett. & Boyce, and *T. roxburghii* Schott are the other species found in abundance in the country. In Orissa, the occurrence of three wild species namely, *T. trilobatum* (Haines, 1921–1925), *T. venosum* (syn. *Sauromatum venosum*) (Panda *et al*., 1995) and *T. flagelliforme* (Panda, 2002) has been reported.

Tuber and leaves of *T. trilobatum*, *T. flagelliforme*, and *T. venosum* are used by tribals as source of food and medicine. The tubers of many species are used as stimulant and for cure of piles, eaten with banana for stomach complaints, applied externally to the bite of venomous snakes and the same time given orally. The tribals also feed the raw tubers of *T. trilobatum* to the cattle against worm infection. It is also used as poultice in the treatment of tumors and haemorrhages. The rhizomes of *T. flagelliforme* have traditionally been used as an expectorant for coughs and as treatment for other pulmonary ailments. In the Philippines, the floral inflorescence is used to arrest bleeding and help in wound healing (Parry, 1980). This plant has been used extensively as one of the components of traditional herb for combating breast, lung, colon and liver cancer.

Being the source of food and medicine against a spectrum of ailments, the wild populations of the species have been severely eroded due to overexploitation. This necessitated conservation of the species in the wild and assessment of the genetic diversity of the genus *Typhonium* through molecular techniques. No report, however, is available on the molecular characterization and determination of genomic relationship among the species except that of Sriboonma *et al*. (1993).

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Molecular markers like RAPD, RFLP, ISSR and AFLP are stable, reliable and not influenced by the environment and thus being used nowadays for assessment of the genetic diversity and establishing genomic relationship among different plant taxa. The present work was undertaken to assess the genetic diversity among 5 medicinally important species of *Typhonium* and to understand the genomic relationship among them using RAPD marker.

Materials and Methods

Plant material

Five species of *Typhonium* (*T. diversifolium, T. flagelliforme, T. roxburghii, T. trilobatum* and *T. venosum*) were studied. Except for *T. diversifolium* and *T. venosum*, the other three species were collected from the premises of Regional Plant Resource Centre, Bhubaneswar, Orissa, India. *T. diversifolium* was collected from Darjeeling hills and *T. venosum* from Sanaghagra in Keonjhar district, Orissa, India. Leaves from ten different plants of the same species were collected at random and leaf samples of each species were pooled together and genomic DNA was isolated.

Genomic DNA isolation

DNA was isolated from freshly collected young leaves by the CTAB method as described by Doyle and Doyle (1990). RNA was extracted with RNaseA (Quiagen) treatment: @ 60 μg for 1 ml of crude DNA solution at 37 °C followed by two washings with phenol/chloroform/iso-amyl-alcohol (25:24:1 v/v/v) and subsequently two washings with chloroform/iso-amyl-alcohol (24:1 v/v). After centrifugation, the upper aqueous phase was separated, 1/10 volume of 3 M sodium acetate (pH 4.8) was added and DNA precipitated with 2.5 volume of pre-chilled absolute ethanol. The extracted DNA was dried and then dissolved in 10 mM Tris-HCl [tris(hydroxy methyl) amino methane]/1 mM EDTA (ethylene diamine tetra acetic acid disodium salt) ($T_{10}E_1$ buffer, pH 8). Quantification was made by running the dissolved DNA in 0.8% agarose gel along side uncut λ DNA of known concentration. The DNA was diluted to 25 ng/μl for RAPD.

RAPD analysis

For RAPD analysis, PCR amplification of 25 ng of genomic DNA was carried out using 25 standard decamer oligonucleotide primers out of which 20 primers [OPA02, OPA03, OPA04, OPA10, OPA16, OPA18, OPD02, OPD03, OPD05, OPD07, OPD08, OPD10, OPD18, OPD20, OPN02, OPN04, OPN05, OPN06, OPN08 and OPN16 (Operon Technologies, Alameda, USA)] produced satisfactory amplification. The RAPD analysis was performed by the methods of Williams *et al.* (1990). Each amplification reaction mixture of 25 μl contained 20 ng of template DNA, 2.5 μl of 10X assay buffer (100 mM Tris-HCl, pH 8.3, 0.5 M KCl and 0.01% gelatin), 1.5 mM $MgCl_2$, 200 μM each of dNTPs, 20 ng of primer and 0.5 U Taq DNA polymerase (Bangalore Genei Pvt. Ltd., Bangalore, India). The amplification was carried out in a thermal cycler (Perkin Elmer, 9600). The first cycle consisted of denaturation of template DNA at 94 °C for 5 min, primer annealing at 37 °C for 1 min and primer extension at 72 °C for 2 min. In the subsequent 42 cycles, the period of denaturation was reduced to 1 min while the primer annealing and primer extension time were maintained as in the case of the first cycle. The last cycle consisted of only primer extension at 72 °C for 7 min. The PCR products were separated on a 1.5% agarose gel containing ethidium bromide solution (0.5 μg/ml of gel solution). The size of the amplicons was determined using standards (100 bp ladder plus, MBI Fermentas, Graiciuno, Vilnius, Lithuania). The DNA fragments were observed under UV light and photographed.

Data analysis

The presence/absence of bands in RAPD analysis was recorded in binary (0, 1) form. All the bands (polymorphic and monomorphic) were taken into account for calculation of similarity with a view to avoid over-/underestimation of the distance (Gherardi *et al*., 1998). Jaccard's coefficient of similarity (Jaccard, 1908) was measured and a dendrogram based on similarity coefficients generated by the un-weighted pair group method using arithmetic averages (UPGMA) (Sneath and Sokal, 1973) and SHAN clustering. The statistical analysis was done using the computer package NTSYS-PC (Rohlf, 1997). Resolving power (Rp) of the RAPD primer was calculated according to Prevost and Wilkinson (1999): $Rp = \Sigma IB$, where IB

(band informativeness) = 1 – [2 × (0.5 – *P*)], *P* being the proportion of the 5 species containing the band.

Results

A total of 245 numbers of loci were amplified in the RAPD analysis using 20 decamer oligonucleotide primers. Out of these, 12 bands were monomorphic while 233 bands were polymorphic including 86 species-specific bands (Table I). The RAPD banding pattern of the five species of *Typhonium* is represented in Fig. 1. The maximum of 114 loci was amplified in case of *T. trilobatum* and the minimum (73) in case of *T. roxburghii*. The highest number of 27 unique bands was detected in *T. diversifolium* and 10, the lowest, for *T. roxburghii*. In *T. trilobatum*, the highest number of 102 polymorphic bands was generated and the least (61) for *T. roxburghii* (Table II). The amplicons were amplified in the range of 100 to 3000 base pairs. The maximum (19) and minimum (7) number of bands were amplified with the primer OPD18 and OPA16, respectively (Table I). Among the primers used, OPD18 generated maximum polymorphic bands but the highest number of unique bands was noted with the primer

Table I. Details of RAPD analysis in five species of *Typhonium*.

Primer	Nucleotide sequence	Total bands	Polymorphic bands	Monomorphic bands	Unique bands	Resolving power
OPA02	5′TGCCGAGCTG3	12	11	1	3	12.8
OPA03	5′AGTCAGCCAC3′	8	8	0	3	6.4
OPA04	5′AATCGGGCTG3′	11	11	0	3	10.8
OPA10	5′GTGATCGCAG3	10	9	1	2	9.6
OPA16	5′AGCCAGCGAA3′	7	7	0	1	7.2
OPA18	5′AGGTGACCGT3′	10	10	0	4	8
OPD02	5′GGACCCAACC3′	13	13	0	2	13.2
OPD03	5′GTCGCCGTCA3′	14	13	1	4	13.2
OPD05	5′TGAGCGGACA3′	11	11	0	3	10.4
OPD07	5′TTGGCACGGG3′	11	11	0	5	6.8
OPD08	5′GTGTGCCCCA3′	10	10	0	3	7.6
OPD10	5′GTGTGCCCCA3′	11	10	1	3	8.4
OPD18	5′GAGAGCCAAC3′	19	19	0	5	14.4
OPD20	5′ACCCGGTCAC3′	15	15	0	7	11.2
OPN02	5′ACCAGGGGCA3′	12	12	0	5	8
OPN04	5′GACCGACCCA3′	12	12	0	6	10.8
OPN05	5′ACTGAACGCC3′	17	15	2	8	12.8
OPN06	5′GAGACGCACA3	12	9	3	7	9.2
OPN08	5′ACCTCAGCTC3′	15	15	0	7	10.8
OPN16	5′AAGCGACCTG3	15	12	3	5	12
Total		245	233	12	86	

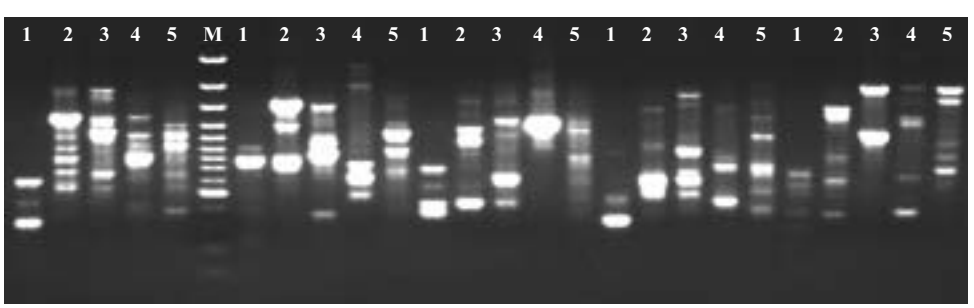

Fig. 1. RAPD banding pattern of five species of *Typhonium* as revealed through primer OPD20, OPN02, OPN04, OPN06, OPN08. The lanes represent: 1, *T. roxburghii*; 2, *T. trilobatum*; 3, *T. flagelliforme*; 4, *T. diversifolium*; 5, *T. venosum*.

Table II. Banding pattern in five species of *Typhonium*.

Species	Total no. of bands	Polymorphic bands	Monomorphic bands	Unique bands
T. roxburghii	73	61	12	10
T. trilobatum	114	102	12	18
T. flagelliforme	109	97	12	19
T. diversifolium	100	88	12	27
T. venosum	113	101	12	12

OPN05. Maximum similarity was seen among the species with the primer OPA02. While the highest number of unique loci got amplified in OPN05, the lowest numbers of species-specific loci were found in the primer OPA16 *i.e.* 1 in each case. Among the RAPD primers used, the resolving power ranged from 6.4 for OPA03 to 14.4 for OPD18. *T. venosum* showed maximum similarity (31.2%) with *T. trilobatum* and the latter with *T. roxburghii* at 28.10% (Table III). Similarly, *T. diversifolium* and *T. flagelliforme* were close to each other exhibiting 28.57% similarity. The constructed dendrogram (Fig. 2) showed two distinct clades, one containing *T. roxburghii*, *T. trilobatum* and *T. venosum* and the other containing the remainder two species, *i.e. T. diversifolium* and *T. flagelliforme*. Both the clusters shared a common node at approx. 23.7% level. In the first clade, of the three species, *T. trilobatum* formed a cluster with *T. venosum* having 31.2% similarity and together shared a common node with *T. roxburghii* at 25.9% level of similarity. In the second cluster, *T. flagelliforme* and *T. diversifolium* had a similarity index of approx. 26%.

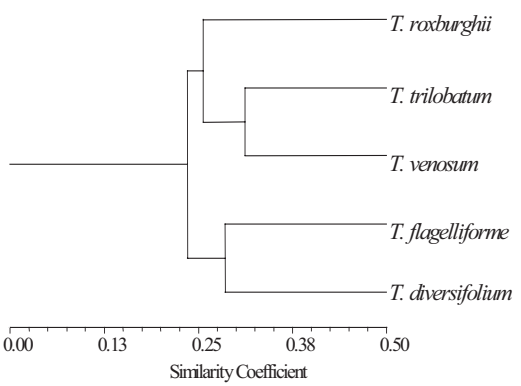

Fig. 2. Dendrogram as revealed from RAPD data through SHAN clustering.

Discussion

The study was limited to RAPD analysis of only five important species of *Typhonium* namely, *T. roxburghii, T. trilobatum, T. venosum, T. flagelliforme* and *T. diversifolium*. Most of these species are important as medicinal and food plants in India and elsewhere and due to over-exploitation, their wild populations are declining rapidly. This necessitates assessment of the genetic diversity of the genus through molecular characterization and evaluation of their medicinal properties.

Hooker (1894) recognized *Sauromatum* and *Typhonium* as two separate genera under the tribe Arineae distinguishable on the basis of emergence of leaves either after or before flowering. Of the remaining 4 species of the genus *Typhonium*, based on the characters of the limb of the spathe, he placed *T. trilobatum* and *T. roxburghii* under one group along with others and *T. diversifolium* and *T. flagelliforme* in two separate groups. The close similarity between *T. trilobatum* and *T. roxburghii* has also been established by RAPD analysis in the present study as they had a similarity of more than 28%.

Table III. Similarity values between different species of *Typhonium*.

	T. roxburghii	*T. trilobatum*	*T. flagelliforme*	*T. diversifolium*	*T. venosum*
T. roxburghii	100				
T. trilobatum	28.10	100			
T. flagelliforme	24.39	27.78	100		
T. diversifolium	19.33	19.31	28.57	100	
T. venosum	23.20	31.21	28.87	21.99	100

As remarked by Sriboonma *et al.* (1993), the infra-generic classification of *Typhonium* has not yet been established and as a result, the placement of species in one group or the other has been based on personal judgments. Engler (1920) recognized two sections under the genus, namely, section *Heterostalis* Engl. characterized by the clavate sterile flowers and section *Eutyphonium* Engl. without clavate sterile flowers. He placed T. *flagelliforme* under the former section and *T. trilobatum* under the latter. The data obtained in the present study also supports this view. However, inclusion of both the species under one group based on shoot morphology (Murata, 1990) could not be established from data obtained by RAPD analysis.

T. venosum, earlier included under *Sauromatum* and known as *S. venosum*, was quite closer to *T. trilobatum* showing a similarity coefficient of about 30% and formed a single clade along with *T. roxburghii*. Unlike other two species, *T. venosum* even did not form a separate node by itself to be treated as a genetically very distant species so to say a separate genus. Therefore, the taxonomic merger of the genus *Sauromatum* with *Typhonium*, as has been done by Hetterscheid and Boyce (2000), appears justified.

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