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Analysis of Genetic Variation in the Genus *Solanum* Using AFLP (Amplified Fragment Length Polymorphism) Markers

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ABSTRACT

Nine species of *Solanum* were investigated for genetic diversity studies using AFLP markers. Out of 15 primer combinations used, three gave good scorable polymorphic bands. An average of 89% polymorphism was detected among the species. Cluster analysis showed 2 major clusters, A and B. Cluster A was divided into 2 subgroups, one formed by *S. seaforthianum* and the other by *S. indicum* and *S. torvum.* Cluster B was again divided into 2 sub-clusters, 1 and 2. Maximum genetic distance was recorded between *S. erianthum* and *S. seaforthianum* (0.97) while least distance was found between *S. viarum* and *S. khasianum* (0.74) compared with all other species. The separation of *S. seaforthainum* and *S. caricaefolium* into distinct groups was very significant.

Keywords: diversity, Karnataka, molecular markers, Solanaceae
Abbreviations: AFLP, amplified fragment length polymorphism; **APS**, ammonium persulphate; **BPB**, bromophenol blue; **CTAB**, cetyl trimethyl ammonium bromide; **DNA**, deoxyribonucleic acid; **EDTA**, ethylene diamine tetraacetic acid; **GDE**, genetic diversity estimate; **JSC**, Jaccard's similarity co-efficient; **PAGE**, polyacrylamide gel electrophoresis; **PCR**, polymerase chain reaction; **SCAR**, sequence characterized amplified region; **SSR**, simple sequence repeat; **TBE**, Tris borate EDTA; **TE**, Tris EDTA; **TEMED**, tetra methyl ethylene diamine; **UPGMA**, unweighted pair group method arithmetic average

INTRODUCTION

The family Solanaceae consists of about 85 genera and 2800 species of prickly herbs, shrubs, climbers and small trees well represented all over the world and known to produce tropane alkaloids, derived from ornithine, pyridine and steroidal alkaloids (Wiart 2006). Among the different genera in this family, the genus *Solanum* is the biggest with approximately 1500 species distributed in varied climatic conditions ranging from tropical, subtropical to deciduous forests. Apart from the commonly cultivated species, there are certain plants which are medicinally useful and grown in kitchen gardens for fulfilling immediate medical needs (Parrotta 2001). The leaves of *S. nigrum* are rich in Vitamin C and are consumed by local populations as a vegetable (Jain and Robert 1991). The ripe fruits of *S. nigrum* are also rich in solasodine, known to possess anti-cancerous properties (Son *et al*. 2003). *S. erianthum* is employed for the treatment of vertigo and urinary troubles and is rich in solanidine and solasidine alkaloids present in the leaves and fruits up to a total concentration of 0.37 and 0.39%, respectively (Pullaiah 2002). The fruits of *S. indicum*, *S. surrattense*, and *S. khasianum* yield steroidal sapogenins, i.e., disogenin and solasodine sapogenins, administered for various ailments (Singh and Rajesh 2007). The polyphenols isolated from *S. torvum* potentially reduce oxidative stress in diabetes (Winthania *et al*. 2009). The polyphenols and anthocyanidin in various parts of *S. nigrum* have shown inhibitory effects on breast cancer cells and have proved to be a better remedy (Huang *et al*. 2010). A recent study on different extracts of fruit coat of *S. torvum* possessed antibacterial activity against human pathogenic strains (Sivapriya *et al*. 2011).

These species exhibit vast diversity in morphology and distribution. Amplified fragment length polymorphism (AFLP) is a powerful tool for estimating the genetic diversity within and among plant populations. The efficient use of this technique has been reported in analyzing the genetic diversity in a medicinal herb, *Tribulus terrestris* along with the other molecular tools (Maryam *et al*. 2008). Although there are many reports available on enumerating the genetic diversity in *Solanum*, emphasis has been given to the cultivated taxa. Kardolus *et al.* (1998) applied AFLP to analyze genetic diversity among potatoes and tomatoes, or for molecular characterization of wild potatoes (Lara-Cabrera and Spooner 2004). Variation among selected accessions of *S. melongena* has been reported by Karihaloo *et al.* (1995) using AFLP. The genetic diversity in southern African *S. retroflexum* and other related species was reported by Jacoby *et al.* (2003). Reports are also available on provenance studies in accessions of *S. nigrum* (Dehmer and Hammer 2004). Mkabwa *et al.* (2008) studied the genetic diversity of African hexaploid species *S. scabrum* using AFLP. Despite the existence of such reports, there is still a gap in the molecular characterization of wild and several other species of *Solanum*. Hence, the present study aims to characterize the genetic relationship among selected *Solanum* species.

MATERIALS AND METHODS

Materials

Nine *Solanum* species were included in the present study (**Table 1**). They were identified with the help of a regional flora (Saldanha and Nicolson 1976). Fresh, young leaves (3–4 g) from each species were collected from the Botanical Garden, University of Agricultural Sciences, Karnataka, India.

Methods

Total genomic deoxyribonucleic acid (DNA) was isolated by a cetyl tri-methyl ammonium bromide (CTAB) method (Doyle and

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Research Note

Table 1 List of species of *Solanum* selected for AFLP analysis.

Botanical name	Synonym	Codes
S. seaforthianum	-	*S. sea*
S. torvum	-	*S. tor*
S. indicum	-	*S. ind*
S. americanum	*S. nigrum*	*S. ame*
S. erianthum	*S. verbascifolium*	*S. eria*
S. caricaefolium	-	*S. car*
S. khasianum	-	*S. kha*
S. surrattense	*S. xanthocarpum*	*S. sur*
S. viarum	-	*S. via*

Doyle 1990). Isolated DNA was purified and the concentration of DNA in the samples was determined with agarose (Titan Biotech, Bhiwadi, Rajasthan, India) gel electrophoresis using λ-DNA (Bangalore Genei, Peenya, Bangalore, India) as the standard. DNA samples were dissolved in 10:1 Tris EDTA (TE) and stored at -20°C until AFLP analysis. A standard procedure for AFLP was followed as per Vos *et al.* (1995) with minor modifications. In the selective amplification step, the first PCR cycle was reduced to 11 cycles and the second PCR program was enhanced by an additional PCR cycle. Fifteen enzyme combinations of *Eco*RI/*MSe*I and *Pst*I/*Mse*I (New England Biolabs, Labmate, Bengalore) were used for the double digestion of template DNA. The *Eco*RI/*MSe*I adapters and *Pst*I/*Mse*I adapters were ligated to the ends of restriction fragments.

Pre-selective amplification

Before selective polymerase chain reaction (PCR), a pre-amplification step was carried out to amplify the DNA fragments. The pre-selective primers have a single base overhang which selects for fragments having an extra base downstream of the restriction site. The single base is either C (Cytosine) or A (Adenine). The previous research on the genus *Solanum* showed good results with these two bases, hence these bases were retained for our study (Klaus *et al*. 2004). The amplification of these primers was accomplished with the following PCR cycle. The PCR programme consisted of 20 cycles of 30 sec at 94°C for DNA denaturation, 60 sec at 56°C for DNA annealing and 60 sec at 72°C for DNA extension followed by constant 10°C until use.

Selective amplification

The pre-amplified product was diluted for selective PCR using 15 primer combinations (**Table 2**). The PCR programme was performed for 11 cycles with the following cycle profile: A) 30 sec denaturation at 94°C; B) 30 sec annealing; C) 1 min extension at 72°C. The annealing temperature in the first cycle was 65°C which was subsequently reduced in each cycle by 0.7°C for the next 10 cycles. In the next step, there were 24 cycles of 30 sec at 94°C, 60 sec at 56°C and 60 sec at 72°C followed by constant 10°C until use. All amplification reactions were performed in a thermocycler (Primus 96, Peqlab, Germany).

Electrophoresis

Following selective amplification, reaction products were mixed with 20 µl of loading buffer (98% formamide; 10 mM ethylene diamine tetra acetic acid (EDTA) pH 8.0, 0.1% bromophenol blue (BPB) and xylene cyanole). Each sample (4 µl) was loaded onto a 6% polyacrylamide gel electrophoresis (PAGE). The gel matrix was prepared using 40% acrylamide, 7 M urea and 10X tris borate EDTA (TBE) buffer. To 35 ml of gel solution 256 µl of 10% ammonium persulphate (APS) and 22 µl of tetra methyl ethylene diamine (TEMED) were added and gels were cast in a gel apparatus. 1X TBE was used as running buffer. Electrophoresis was performed at constant power (1500 V) for 2 hrs. After electrophoresis gels were fixed in 10% acetic acid for 30 min and washed with milliQ water for 2 min (3 washes) and kept in silver stain (1 g $AgNO_3$: 1.5 ml 40% H_2CO) for 30 min. The gel was washed in milliQ water for 10 sec only and kept in developing solution (3% solution of $NaCO_3$; 1.5 ml 40% H_2CO: 150 µl $NaSO_3$) until the bands developed. The gel was then fixed in 10% acetic acid for 5 min and washed in milliQ water to remove acid and to prevent gel cracking.

Table 2 Primers with three nucleotide extension used for re-amplification.

Primers (5′-3′)
Eco+ACG: GACTGCGTACCAATTCACG
Eco+ACT: GACTGCGTACCAATTCACT
Eco+TAC: GACTGCGTACCAATTCTAC
Eco+AAC: GACTGCGTACCAATTCAAC
Eco+AGG: GACTGCGTACCAATTCAGG
Eco+ACC: GACTGCGTACCAATTCACC
Mse+CAG: GATGAGTCCTGAGTAACAG
Mse+CAT: GATGAGTCCTGAGTAACAT
Mse+CTT: GATGAGTCCTGAGTAACTT
Mse+CAA: GATGAGTCCTGAGTAACAA
Mse+CTG: GATGAGTCCTGAGTAACTG
Mse+CTA: GATGAGTCCTGAGTAACTA
Pst+ GC: GACTGCGTACATGCAGGC
Pst+CA: GACTGCGTACATGCAGCA
Pst+CG: GACTGCGTACATGCAGCG

Data analysis

For the diversity analysis, bands were scored as present (1) or absent (0) to form a raw data matrix. A square symmetric similarity matrix was then obtained using Jaccard's Similarity Coefficient (JSC; Dudley 1993):

$$JSC = [a/ (n-d)]$$

where a is the number of fragments in common between two species; n is the total number of fragments scored, and d is the number of fragments absent in both species.

From the raw data matrix Genetic Diversity Estimates (GDEs) were then calculated as 1- JSC (Jaccard 1908); for cluster analysis, Unweighted Pair Group Method Arithmetic Averages (UPGMA) (Sneath and Sokal 1973) was used.

RESULTS AND DISCUSSION

Nine *Solanum* species were subjected to AFLP analysis using 15 primer combinations of *Eco*RI, *Mse*I and *Pst*I out of which only three (P-CA; M-CTG, P-CG; M-CTA and P-CA; M-CTA) gave good scorable bands with 92% polymorphism. The number of bands and profiles varied significantly between primers. In total, 152 amplified fragments (**Table 3, Fig. 1**) were detected by silver staining. The total number of molecular markers specific to each species varied from 17 to 32. Highest polymorphism (92%) was detected in P-CA; M-CTG and the least (86.95%) was obtained by P-CG; M-CTA. On average, 89% polymorphism was recorded.

Genetic relationship between *Solanum* species

The similarity matrix was generated using JSC, which was converted to GDEs (**Table 4**), and which in turn was used for UPGMA cluster analysis. The cluster analysis (**Fig. 2**) showed 2 major clusters, A and B. Cluster A was subdivided into 2 groups, one formed by *S. seaforthianum* and the other

Table 3 Polymorphism in *Solanum* species related to the three primer combinations.

Primer combination	No. of bands produced	No. of polymorphic bands	Polymorphism (%)
P-CA/ M-CTG	50	46	92
P-CG/M-CTA	46	40	86.95
P-CA/M-CTA	56	50	89.25
TOTAL	152	136	89.4

Table 4 Mean (s.d) of the AFLP – based pair wise genetic diversity estimates (GDEs) between nine species of *Solanum.*

	S. seaforthianum	***S. torvum***	***S. indicum***	***S. americanum***	***S. erianthum***	***S. caricaefolium***	***S. khasianum***	***S. surrattense***	***S. viarum***
S. seaforthianum	-								
S. torvum	0.87	-							
S. indicum	0.85	0.85	-						
S. americanum	0.93	0.91	0.81	-					
S. erianthum	0.97	0.92	0.91	0.78	-				
S. caricaefolium	0.95	0.91	0.92	0.88	0.8	-			
S. khasianum	0.96	0.91	0.93	0.94	0.9	0.82	-		
S. surrattense	0.95	0.93	0.96	0.93	0.9	0.85	0.76	-	
S. viarum	0.95	0.96	0.88	0.93	0.9	0.85	0.74	0.77	-

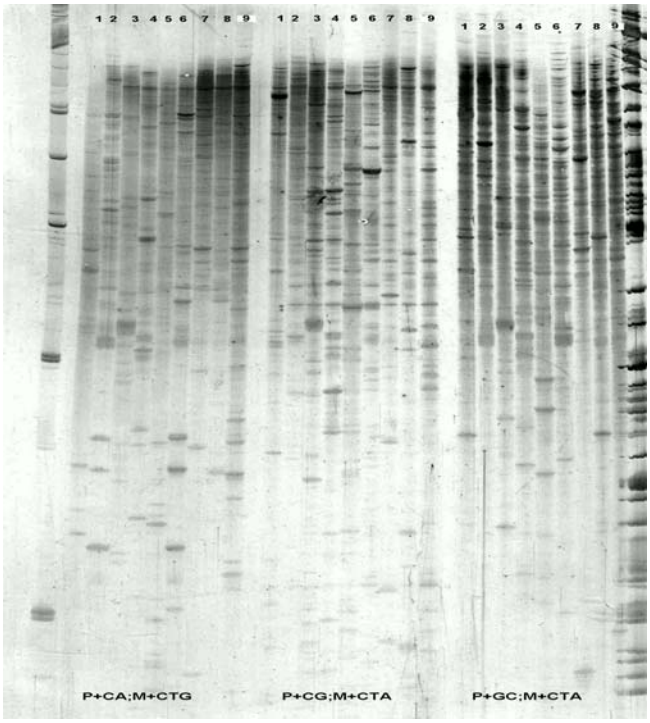

Fig. 1 AFLP analysis with different primer combinations in the genus *Solanum*. Lanes: 1, *S. seaforthianum*; 2, *S. torvum*; 3, *S. indicum*; 4, *S. nigrum*; 5, *S. erianthum*; 6, *S. caricaefolium*; 7, *S. khasianum*; 8, *S. surrattense*; 9, *S. viarum*.

S. indicum and *S. torvum.* Cluster B was again divided into 2 sub-clusters, 1 and 2. Sub-cluster 1 was divided into Groups A and B while sub-cluster 2 was formed by *S. erianthum* and *S. nigrum*. In Group A there were subgroups 1 and 2, the former formed by *S. surrattense* and the latter by *S. viarum* and *S. khasianum*. Group B was formed by *S. caricaefolium* alone. The genetic diversity estimated among the species is shown in **Table 4**. *S. erianthum* and *S. seaforthianum* were separated by maximum genetic distance (0.97) while least distance was found between *S. viarum* and *S. khasianum* (0.74) compared with all other species. The species selected for this study share several growth habits ranging from herbs, shrubs to climbers. Although the clustering pattern does not depict much about the morphology of individual species, the separation of *S. seaforthianum* into a distinct group is very significant. It is the only climber, while the others are either herbs or shrubs. Another important observation noticed in our study was the grouping of *S. caricaefolium* into a separate cluster. This is the only introduced or exotic member and has a different gene pool from other members of the group. The grouping of *S. indicum* and *S. torvum* is also significant as both are prickly shrubs.

The first report on the application of AFLP in *Solanum* taxonomy was by Kardolus *et al.* (1998), who proved the efficiency and reliability of this technique in generating biosystematics from 19 taxa of section *Petota* (potato) and three taxa of section *Lycopersicum* (tomato), indicating that this technique is useful up to the species level. The studies by Klaus *et al.* (2004) on germplasm accessions from 44 genotypes from five species of *Solanum* confirmed 5 major clusters exhibiting higher infra-specific variation despite close geographic origins. Jacoby *et al.* (2003) reported 62% polymorphism among 14 different genotypes of *S. retroflexum* which were clearly separated into similar groups based both on morphology and AFLP marker analysis. Lara-Cabrera and Spooner (2004) also reported successful application of AFLP in cladistic and phenetic analysis of the section *petota*. AFLP analysis by Furini and Wunder (2004) could efficiently assign species name for eight out of nine accessions that were not previously classified. Our findings are in agreement with an earlier study (Solis *et al*. 2007) which reported 90.04% polymorphism in Chilean native potato germplasm using AFLP markers Straadt and Rasmussen (2008) also reported species-specific AFLP markers for *S. phureja* and *S. tuberosum* in their study on introgression of DNA of *S. phureja* into *S. tuberosum* with 17 AFLP primers. Investigations have also shown AFLP to be a powerful co-dominant marker in studying the genetic diversity in 58 eggplant accessions (Yi *et al*. 2009). Further, microstaellite markers have also been identified to distinguish closely related accessions of the *S. nigrum* complex arising from similar pedigrees (Van Biljan *et al*. 2010). A recent study has evaluated the application of diagnostic molecular markers for the selection of low bruising potato varieties (Urbany *et al*. 2011).

AFLP is also effective in depicting the genetic divergence and phylogenetic analysis of species other than *Solanum* also. AFLP markers were useful for identifying specific hybrids, marker-assisted selection and genetic resource management in seven species of *Jatropha* (Sudheer *et al*. 2008). Similarly, Negi *et al.* (2006) reported 80% polymorphism in 25 genotypes of *Withania somnifera*.

Nine *Solanum* species were characterized by AFLP analysis. Further studies are needed to assess intra-specific variation and to develop character-specific markers.

CONCLUSION

AFLP is a very useful tool in depicting the genetic diversity among the different species of *Solanum*. The results have clearly shown the differences in the genotypic make up of the species. The more number of bands obtained by the AFLP gels had the limitation of identifying unique bands in the species under the study. Further, other molecular markers such as simple sequence repeats (SSR) or sequence characterized amplified region (SCAR) markers might be used to assess the uniqueness of each species.

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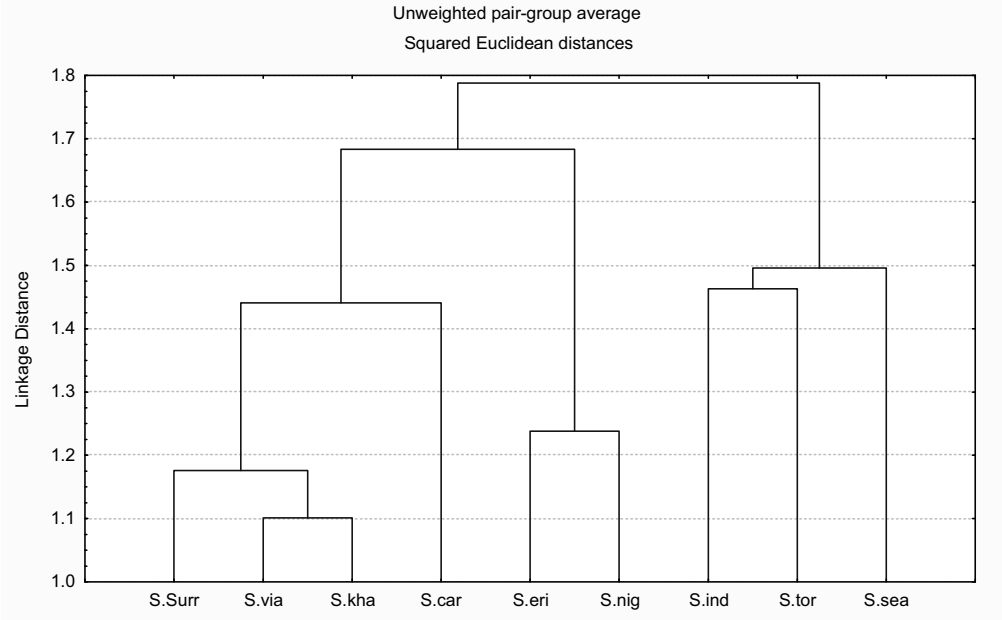

Fig. 2 UPGMA cluster analysis of AFLP data generated by three primer combinations for nine species of *Solanum* depicting patterns of genetic diversity. Scale depicts genetic diversity estimates.

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