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EVALUATING THE GENETIC AFFINITY OF SELAGINELLA SPECIES USING DIFFERENT ISOZYMIC PROFILES

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ABSTRACT

The present study was intended to identify the genetic variability among the selected species of Selaginella viz., Selaginella intermedia (Bl.) Spring, Selaginella inaequalifolia (Hook. et Grev.) Spring, Selaginella involvens (Sw.) Spring & Selaginella tenera (Hook & Grev.) Spring), Selaginella wightii Hieron., Selaginella brachystachya (Hook. & Grev.) Spring & Selaginella repanda (Desv.) Spring, Selaginella radicata (Hook. & Grev.) Spring, Selaginella bryopteris (L.) Bak, Selaginella delicatula (Desv.) Alston with cones), Selaginella delicatula (Desv.) Alston without cones were collected from Western Ghats, South India using different isozymic profiles. PAGE was carried out using standard procedure of Sadasivam and Manickam (1991). The isoenzymes selected for the study includes, esterase, peroxidase, acid phosphatase, alkaline phosphatase and polyphenol oxidase. Multiple regions of activity with a total of thirty eight esterase bands, sixty six peroxidase bands, sixty three acid phosphatase bands, forty eight alkaline phosphatase bands and twelve polyphenol oxidase bands were observed in the isozymic system of studied Selaginella species. Unique banding profiles of esterase, peroxidase, acid phosphatase, alkaline phosphatase and polyphenol oxidase which observed in the studied Selaginella species were used to distinguish the selected Selaginella species and these isozymic profiles will act as biochemical fingerprint for the studied species in plant systematic studies.

KEYWORDS: Selaginella; Isozymic profiles; PAGE; Biochemical fingerprint; Plant systematic.

INTRODUCTION

Plants have always been the source of energy for survival and evolution of the animal kingdom, thus forming the base for every ecological pyramid. Over the last few decades, plant genomics has been studied extensively revolutionizing the area of research. Genetic diversity in germplasm collections is fundamental to broadening the genetic base of plant species. During the early period of research, classical strategies including comparative morphology, anatomy, physiology and embryology were employed in genetic analysis to determine inter and intra-species variability (Johnson et al., 2010). However, such data may not provide an accurate indication of genetic diversity because of environmental influences upon the expression of observed traits. Considering these difficulties, biochemical analysis has been applied to establish germplasm differences at various taxonomic levels (Nanthini et al., 2011).

Isozymes are good estimators for elucidating the possible mechanisms leading to the formation of genetic variability in plant populations. It is widely used in the studies of inter and intra-specific variation (Wang and Sheng, 2013). They also show lower levels of polymorphism in a population where the expression of various isozymes differs both temporally and spatially which can correctly identify several levels of taxa, accessions and individuals since the assumption of homology can be more accurate than for same genomic DNA markers (Johnson et al., 2012). Isozymes analysis has been used for over 60 years for various research purposes in biology viz., to delineate phylogenetic relationships, to estimate genetic variability, to study population genetics and developmental biology, to characterize the plant genetic resources management and plant breeding. Isozymes originate through amino acid alterations which cause changes in net charge or the spatial structure of the enzyme molecules which is identified by means of horizontal gel electrophoresis (Rodrigues et al., 1993). The strength of using isozymes is simplicity; it does not require DNA extraction or the availability of sequence information, primers or probes; they are quick and easy to use. Some plant species, however, can require considerable optimization of techniques for certain enzymes. Isozymes have been applied in many population genetic studies, including measurements of crossing rates, population structure and population divergence. Isozymes are particularly useful at the level of specific populations and closely related species and are therefore useful to study diversity in crops and their relatives (Smila et al., 2007). In the present study, Isozymic variation has been carried out to identify the genetic variability among the selected species of Selaginella collected from Western Ghats, South India.

MATERIALS AND METHODS

Totally, ten species of Selaginella were collected from various localities of Western Ghats, South India which includes Tamil Nadu viz., Kakachi Hills (Selaginella intermedia (Bl.) Spring, Selaginella inaequalifolia (Hook. et Grev.) Spring, Selaginella involvens (Sw.) Spring & Selaginella tenera (Hook & Grev.) Spring); Maruthuvalmalai (Selaginella wightii Hieron.); Shenbaganur (Selaginella brachystachya (Hook. & Grev.) Spring & Selaginella repanda (Desv.) Spring) and Kerala viz., Ponmudi (Selaginella radicata (Hook. & Grev.) Spring, Selaginella bryopteris (L.) Bak, Selaginella delicatula (Desv.) Alston with cones); Thenmalai (Selaginella delicatula (Desv.) Alston without cones).

Isozymic analysis

For isozymic analysis, young 2-3 month old plants from the natural habitat were collected in the wet paper and brought to the laboratory. After wash in tap water, the leaves were dissected out and the crude enzyme extracts were prepared by grinding in different buffers at varied pH based on enzymatic system (Table 1) as described by Sadasivam and Manickam (1991). The slurry was centrifuged at varied rpm at 4°C in a cooling centrifuge and the supernatant is stored at 4°C before use. The isolated enzymes then separated using PAGE. The enzymes were separated in the gel which is actively stained with the specific reaction mixture for the isozymes esterase, peroxidase, acid phosphatase, alkaline phosphatase and polyphenol oxidase as per Sadasivam and Manickam (1991).

Table 1: Isolation buffers based on enzyme system

Enzymes	Buffer with varied pH	rpm
Esterase	0.1M phosphate buffer (pH-7.0)	10,000 for 10 minutes
Peroxidase	1M sodium acetate buffer (pH-5.0)	10,000 for 10 minutes
Acid phosphatase	50 mM citrate buffer (pH-5.3)	10,000 for 10 minutes
Alkaline phosphatase	50 mM glycine buffer (pH-10.4)	10,000 for 10 minutes
Polyphenol oxidase	0.01 M phosphate buffer (pH-7.0) and 1% Tween 80	20,000 for 15 minutes

After electrophoresis, the gels were incubated in the staining solution for few minutes under dark condition till the clear bands appeared. The gels were fixed with 7% acetic acid solution for 30 min, washed with distilled water and photographed using the Vilber Loubermat gel documentation system. For the identification of similarity and variation among the selected Selaginella species, the isozymic profiles were converted into a "1" and "0" matrix, to indicate the presence or absence of the Rf values respectively. Genetic similarities (GS) were estimated (Nei and Li, 1979) and cladogram was constructed by UPGMA using NTSYSpc-2.0.

RESULTS

Esterase assay

Multiple regions of activity with a total of thirty eight esterase bands were obtained in the esterase system of studied Selaginella species (Table 2). S. brachystachya displayed maximum number (5) of esterase bands and S. inaequalifolia expressed minimum number (2) of esterase bands. Esterase enzyme system of Selaginella showed eight active regions (Table 2). Region 1 depicted four active bands. EST 1^1 (0.01) was common to S. intermedia, S. involvens, S. repanda, S. radicata and S. bryopteris. EST 1^2 (0.03) showed its presence jointly in S. tenera, S. brachystachya, S. delicatula with and without cones. EST 1^3 (0.05) depicted its common existence in S. involvens, S. wightii and S. repanda. EST 1^4 (0.09) represented its occurrence in S. intermedia, S. bryopteris and S. delicatula without cones. Region 2 displayed three active bands of esterase enzymes. EST 2^1 (0.10) was observed in S. tenera, S. repanda and S. delicatula. EST 2^2 (0.14) established its mutual presence in S. repanda and S. radicata. EST 2^3 (0.16) was shared by S. involvens and S. wightii. Region 3 illustrated with three active esterase enzymes. EST 3^1 (0.20) was common in S. repanda and S. delicatula without cones. EST 3^2 (0.22), EST 3^3 (0.23) and EST 3^4 (0.25) were distinct to S. inaequalifolia, S. intermedia and S. delicatula with cones. EST 3^5 (0.27) established its mutual presence in S. tenera and S. radicata. Region 4 and 5 depicted with a single enzymatic band, EST 4^1 with MW-Rf value (0.34) was universal to S. radicata and EST 5^1 (0.49) showed its common presence in S. involvens, S. repanda and S. bryopteris. Region 6 expressed three active enzymatic bands. EST 6^1 (0.50), EST 6^2 (0.54) and EST 6^3 (0.58) exhibited its restricted character in S. brachystachya, S. involvens and S. repanda. Region 7 and 8 displayed with a single esterase band, EST 7^1 with MW-Rf (0.69) and EST 8^1 (0.76) were distinct to S. intermedia and S. wightii respectively.

Table 2: Esterase enzymatic profile of Selaginella species

MW Rf	R	Enzyme position	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11
0.01	1	EST 1^1	+		+				+	+	+		
0.03		EST 1^2				+		+				+	+
0.05		EST 1^3			+		+		+				
0.09		EST 1^4	+								+		+
0.10	2	EST 2^1				+		+				+	
0.14		EST 2^2						+		+			
0.16		EST 2^3		+			+						
0.20	3	EST 3^1						+					+
0.22		EST 3^2		+									
0.23		EST 3^3	+										
0.25		EST 3^4										+	
0.27		EST 3^5				+				+			
0.34	4	EST 4^1								+			
0.49	5	EST 5^1			+				+		+		
0.50	6	EST 6^1						+					
0.54		EST 6^2			+								
0.58		EST 6^3							+				
0.69	7	EST 7^1	+										
0.76	8	EST8^1					+						
Total			**4**	**2**	**4**	**3**	**3**	**5**	**4**	**4**	**3**	**3**	**3**

Note:R- Region, S1- S. intermedia, S2- S. inaequalifolia, S3- S. involvens, S4- S. tenera, S5- S. wightii, S6- S. brachystachya, S7- S. repanda, S8- S. radicata, S9- S. bryopteris, S10- S. delicatula with cones, S11- S. delicatula without cones.

Based on the esterase profile of the Selaginella species studied, the similarity indices were calculated and the cladogram was constructed (Fig. 1). The evolutionary tree which was constructed based on the esterase profile expressed two clusters (C_1 and C_2). The cluster (C_1) includes eight species of Selaginella viz., S. intermedia, S. involvens, S. repanda, S. bryopteris, S. radicata, S. tenera, S. brachystachya, S. delicatula with and without cones. The cluster (C_2) includes two Selaginella species, S. inaequalifolia and S. wightii. The cluster C_1 is again divided into two nodes (C_1N^1 and C_1N^2). The node C_1N^1 is branched into $C_1N^1B_1$ and $C_1N^1B_2$. The branch $C_1N^1B_1$ is again divided into two small branches ($C_1N^1B_1b_1$ and $C_1N^1B_1b_2$). The small branch $C_1N^1B_1b_1$ showed the unique presence of S. intermedia from the other studied species of Selaginella. The small branch $C_1N^1B_1b_2$ is promoted into two sub branches $C_1N^1B_1b_2A$ and $C_1N^1B_1b_2B$. The sub branch $C_1N^1B_1b_2A$ depicted the exclusive character of S. involvens whereas the sub branch $C_1N^1B_1b_2B$ explained the similarity between S. repanda and S. bryopteris. The branch $C_1N^1B_2$ showed the distinct nature of S. radicata from the other studied species of Selaginella. The node C_1N^2 is branched into two division $C_1N^2B_1$ and $C_1N^2B_2$. The branch $C_1N^2B_1$ represented the exclusive character of S. tenera. The branch $C_1N^2B_2$ is again divided into two small branches $C_1N^2B_2b_1$ and $C_1N^2B_2b_2$. The small branch $C_1N^2B_2b_1$ illustrated the similarity between S. delicatula with cones ($C_1N^2B_2b_1A$) and without cones ($C_1N^2B_2b_1B$). The small branch $C_1N^2B_2b_2$ explained the unique nature of S. brachystachya from the other studied Selaginella species. The cluster (C_2) is simply divided into two nodes C_2N^1 and C_2N^2 which showed the closeness between S. inaequalifolia and S. wightii.

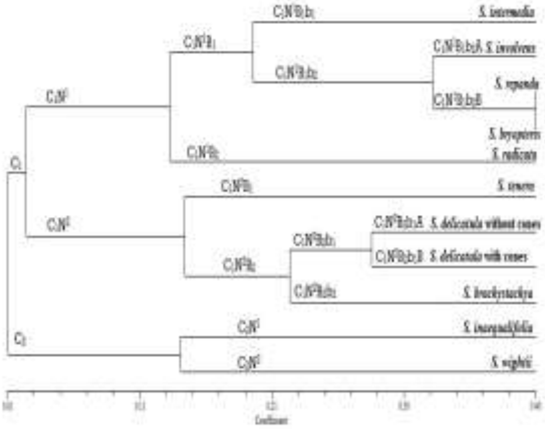

Fig. 1: Cladogram of Selaginella species based on esterase profile

Peroxidase assay

Peroxidase enzyme system of Selaginella produced sixty six peroxidase bands with ten active regions (Table 3). S. brachystachya displayed highest number (11) of peroxidase bands and S. inaequalifolia expressed lowest number (2) of peroxidase bands (Table 3). Region 1 illustrated with six active bands. PRX 1^1 (0.01) was shared by S. wightii and S. bryopteris. PRX 1^2 (0.02), PRX 1^3 (0.04) and PRX 1^4 (0.05) were distinct to S. brachystachya, S. involvens and S. inaequalifolia respectively. PRX 1^5 (0.08) represented its presence in S.

delicatula with and without cones. PRX 1^6 (0.09) showed its common presence in S. intermedia, S. brachystachya and S. radicata. Region 2 depicted six active enzymatic bands. PRX 2^1 (0.11), PRX 2^4 (0.16) and PRX 2^6 (0.19) exhibited its restricted character in S. repanda, S. involvens and S. radicata respectively. PRX 2^2 (0.12) was shared by S. bryopteris and S. delicatula. PRX 2^3 (0.15) was showed its common existence in S. brachystachya and S. delicatula without cones. PRX 2^5 (0.18) represented its mutual presence in S. repanda and S. bryopteris. Region 3 illustrated with five active enzymatic bands. PRX 3^1 (0.21) and PRX 3^5 (0.28) exhibited its restricted character in S. tenera and S. brachystachya. PRX 3^2 (0.22) was demonstrated its common occurence in S. intermedia, S. delicatula with and without cones. PRX 3^3 (0.23) was showed it's jointly presence in S. involvens, S. brachystachya and S. bryopteris. PRX 3^4 (0.26) established its mutual presence in S. tenera and S. radicata. Region 4 expressed four peroxidase bands. PRX 4^1, PRX 4^3 and PRX 4^4 with MW-Rf values 0.32, 0.36 and 0.38 were represented its unique existence in S. repanda, S. radicata and S. tenera respectively. PRX 4^2 (0.35) showed its common presence in S. wightii, S. brachystachya and S. bryopteris. Region 5 illustrated with three enzymatic bands. PRX 5^1 (0.42) and PRX 5^3 (0.47) were expressed their unique presence only in S. involvens. PRX 5^2 (0.46) established its mutual presence in S. tenera and S. wightii. Region 6 showed four active peroxidase bands. PRX 6^1, PRX 6^2, PRX 6^3 and PRX 6^4 with MW-Rf values 0.52, 0.53, 0.56 and 0.59 were displayed its unique presence in S. brachystachya, S. radicata, S. wightii and S. involvens respectively. Similarly, region 7 displayed four peroxidase bands. PRX 7^1 (0.60) and PRX 7^3 (0.66) exhibited its restricted expression in S. bryopteris and S. wightii. PRX 7^2 (0.64) was observed in S. radicata, S. delicatula with and without cones. PRX 7^4 (0.69) was shared by S. involvens and S. tenera. Similar to region 6 and 7, region 8 also observed with four enzymatic bands. PRX 8^1, PRX 8^3 and PRX 8^4 with MW-Rf values 0.70, 0.76 and 0.78 were depicted its unique character in S. brachystachya, S. intermedia and S. repanda correspondingly. PRX 8^2 (0.74) was commonly shared by S. brachystachya and S. radicata. Region 9 expressed with a single enzymatic band PRX 9^1 (0.88) was restricted to S. delicatula without cones. Region 10 produced four active enzymatic bands. PRX 10^1 (0.91) and PRX 10^3 (0.95) were distinct to S. tenera and S. radicata respectively. PRX 10^2 showed it's jointly distribution in S. brachystachya and S. bryopteris. PRX 10^4 (0.97) was dispersed commonly in six species of Selaginella viz., S. inaequalifolia, S. involvens, S. wightii, S. brachystachya, S. repanda and S. bryopteris.

Table 3: Peroxidase profile of Selaginella species

MW Rf	R	Enzyme position	S1	S2	S3	S4	S5	S6	S7	S8	S9	S 10	S 11
0.01	1	PRX 1^1					+				+		
0.02		PRX 1^2						+					
0.04		PRX 1^3			+								
0.05		PRX 1^4		+									
0.08		PRX 1^5										+	+
0.09		PRX 1^6	+					+		+			
0.11	2	PRX 2^1							+				
0.12		PRX 2^2									+	+	
0.15		PRX 2^3						+					+
0.16		PRX 2^4			+								
0.18		PRX 2^5							+		+		
0.19		PRX 2^6								+			
0.21	3	PRX 3^1				+							
0.22		PRX 3^2	+									+	+
0.23		PRX 3^3			+			+			+		
0.26		PRX 3^4				+				+			
0.28		PRX 3^5						+					
0.32	4	PRX 4^1							+				
0.35		PRX 4^2					+	+			+		
0.36		PRX 4^3								+			
0.38		PRX 4^4				+							
0.42	5	PRX 5^1			+								
0.46		PRX 5^2				+	+						
0.47		PRX 5^3			+								
0.52	6	PRX 6^1						+					
0.53		PRX 6^2								+			
0.56		PRX 6^3					+						
0.59		PRX 6^4			+								

0.60	7	PRX 7^1									+		
0.64		PRX 7^2								+		+	+
0.66		PRX 7^3					+						
0.69		PRX 7^4			+	+							
0.70	8	PRX 8^1						+					
0.74		PRX 8^2						+		+			
0.76		PRX 8^3	+										
0.78		PRX 8^4							+				
0.88	9	PRX 9^1											+
0.91	10	PRX 10^1				+							
0.92		PRX 10^2						+			+		
0.95		PRX 10^3								+			
0.97		PRX 10^4		+	+		+	+	+		+		
Total			3	2	8	6	6	11	5	8	8	4	5

Note: R- Region, S1- S. intermedia, S2- S. inaequalifolia, S3- S. involvens, S4- S. tenera, S5- S. wightii, S6- S. brachystachya, S7- S. repanda, S8- S. radicata, S9- S. bryopteris, S10- S. delicatula with cones, S11- S. delicatula without cones.

Based on the peroxidase profile of studied Selaginella species, the similarity indices were calculated and the cladogram was constructed (Fig. 2). The phylogentic tree was constructed based on the peroxidase profile of studied Selaginella species expressed two clusters, C_1 and C_2. The cluster C_1 displayed the similarity and variation between three species of Selaginella viz., S. intermedia, S. radicata, S. delicatula with and without cones. The cluster C_2 represented the closeness and divergence among seven species of Selaginella viz., S. inaequalifolia, S. repanda, S. involvens, S. wightii, S. bryopteris, S. brachystachya and S. tenera. The cluster C_1 is divided into two nodes, C_1N^1 and C_1N^2. The node C_1N^1 is branched into $C_1N^1B_1$ and $C_1N^1B_2$ which explained the similarity between S. intermedia and S. radicata. Similarly, the node C_1N^2 is branched into $C_1N^2B_1$ and $C_1N^2B_2$ that established the similarity between S. delicatula with and without cones. The cluster C_2 is divided into two nodes C_2N^1 and C_2N^2. The node C_2N^1 is branched into $C_2N^1B_1$ and $C_2N^1B_2$. The branch $C_2N^1B_1$ is again branched into two small branches $C_2N^1B_1b_1$ and $C_2N^1B_1b_2$. Both small branches viz., $C_2N^1B_1b_1$ and $C_2N^1B_1b_2$ represented the similarity between S. inaequalifolia and S. repanda. The branch $C_2N^1B_2$ is further divided into two small branches, $C_2N^1B_2b_1$ and $C_2N^1B_2b_2$. The small branch $C_2N^1B_2b_1$ showed the unique expression of S. involvens. The small branch $C_2N^1B_2b_2$ is again sub branched into $C_2N^1B_2b_2A$ and $C_2N^1B_2b_2B$. $C_2N^1B_2b_2A$ determined the closeness between S. wightii and S. bryopteris. $C_2N^1B_2b_2B$ expressed the distinct nature of S. brachystachya. The node C_2N^2 simply depicted the exclusive character of S. tenera from the other studied species of Selaginella.

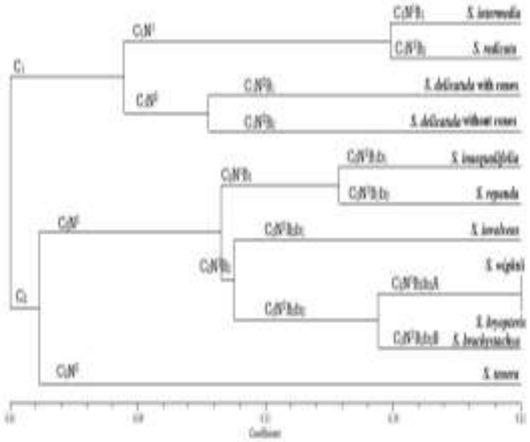

Fig. 2: Cladogram of Selaginella species based on peroxidase profile

Acid phosphatase assay

A total of sixty three bands with ten regions were observed in the acid phosphatase enzyme system (Table 4). Region 1 displayed with four active bands. S. intermedia showed high number (12) of acid phosphatase enzyme bands. S. involvens and S. brachystachya expressed low number (1) of enzymatic bands (Table 4). ACP 1^1 (0.01) established its mutual presence in S. intermedia and S. tenera. ACP 1^2 (0.03) was common to S. radicata, S. bryopteris and S. delicatula with cones. ACP 1^3 (0.04) was shared by S. intermedia and S. inaequalifolia. ACP 1^4 (0.07) showed its common presence in S. involvens, S. wightii, S. brachystachya, S. bryopteris and S. delicatula without cones. Region 2 depicted five active acid phosphatase bands. ACP 2^1 (0.11) was distributed jointly in S. tenera and S. radicata. Similarly, ACP 2^2 (0.12) was mutually shared by S. bryopteris and S. delicatula. ACP 2^3 (0.14) showed its presence only in S. wightii. ACP 2^4 (0.15) displayed its universal distribution in S. intermedia, S. inaequalifolia, S. radicata and S. delicatula without cones. ACP 2^5 (0.19) was widespread in S. intermedia, S. tenera and S. bryopteris. Region 3 expressed four active enzymatic bands. ACP 3^1, ACP 3^2, ACP 3^3 and ACP 3^4 with MW-Rf values 0.20, 0.25, 0.26 and 0.28 were distinct to S. delicatula with and without cones, S. intermedia and S. bryopteris correspondingly. Region 4 showed five active acid phosphatase bands. ACP 4^1 (0.30), ACP 4^2 (0.31), ACP 4^3 (0.36) and ACP 4^5 (0.39) were exhibited its restricted expression in S. inaequalifolia, S. radicata, S. delicatula without cones and S. delicatula with cones respectively. ACP 4^4 (0.38) was shared by S. intermedia and S. bryopteris. Region 5 displayed four active enzymatic bands. ACP $5^1$and ACP 5^4 with MW-Rf values 0.44 and 0.49 were established their unique presence in S. wightii. ACP 5^2 (0.46) was commonly observed in S. intermedia, S. inaequalifolia and S. delicatula with cones. ACP 5^3 (0.47) displayed its distinct presence in S. repanda. Region 6 represented two active enzymatic bands. ACP 6^1 (0.55) and ACP 6^2 (0.57) were established its unique existence in S. intermedia and S. delicatula with cones respectively. Region 7 described five acid phosphatase bands. ACP 7^1, ACP 7^3, ACP 7^4 and ACP 7^5 with respective MW-Rf values 0.61, 0.65, 0.68 and 0.69 were exhibited its restricted expression in S. radicata, S. inaequalifolia, S. wightii and S. intermedia correspondingly. ACP 7^2 (0.63) was uniformly distributed in S. intermedia and S. bryopteris. Region 8 delivered four active acid phosphatase bands. ACP 8^1 (0.71) established its mutual presence in S. wightii and S. repanda. ACP 8^2 (0.74), ACP 8^3 (0.76) and ACP 8^4 (0.77) were displayed their distinct presence in S. intermedia, S. delicatula with cones and S. inaequalifolia correspondingly. Region 9 showed a single enzymatic band, ACP 9^1 with MW-Rf value 0.88 was observed its presence jointly in S. inaequalifolia, S. radicata and S. delicatula without cones. Region 10 illustrated three active bands of acid phosphatase. ACP 10^1 (0.90) was shared by S. intermedia and S. wightii. Similarly, ACP 10^2 (0.96) established its mutual existence in S. repanda and S. delicatula with cones. ACP 10^3 (0.98) was observed in S. tenera, S. delicatula with and without cones.

Table 4: Acid phosphatase profile of Selaginella species

MW Rf	R	Enzyme position	S1	S2	S3	S4	S5	S6	S7	S8	S9	S 10	S 11
0.01	1	ACP 1^1	+			+							
0.03		ACP 1^2								+	+	+	
0.04		ACP 1^3	+	+									
0.07		ACP 1^4			+		+	+			+		+
0.11	2	ACP 2^1				+				+			
0.12		ACP 2^2									+	+	
0.14		ACP 2^3					+						
0.15		ACP 2^4	+	+						+			+
0.19		ACP 2^5	+			+					+		
0.20	3	ACP 3^1										+	
0.25		ACP 3^2											+
0.26		ACP 3^3	+										
0.28		ACP 3^4									+		
0.30	4	ACP 4^1		+									
0.31		ACP 4^2								+			
0.36		ACP 4^3											+
0.38		ACP 4^4	+								+		
0.39		ACP 4^5										+	
0.44	5	ACP 5^1					+						
0.46		ACP 5^2	+	+								+	
0.47		ACP 5^3							+				
0.49		ACP 5^4					+						
0.55	6	ACP 6^1	+										
0.57		ACP 6^2										+	
0.61	7	ACP 7^1								+			
0.63		ACP 7^2	+								+		
0.65		ACP 7^3		+									
0.68		ACP 7^4					+						
0.69		ACP 7^5	+										
0.71	8	ACP 8^1					+		+				
0.74		ACP 8^2	+										
0.76		ACP 8^3										+	
0.77		ACP 8^4		+									
0.88	9	ACP 9^1		+						+			+
0.90	10	ACP 10^1	+				+						
0.96		ACP 10^2							+			+	
0.98		ACP 10^3				+						+	+
Total			12	7	1	4	7	1	3	6	7	9	6

Note: R- Region, S1- S. intermedia, S2- S. inaequalifolia, S3- S. involvens, S4- S. tenera, S5- S. wightii, S6- S. brachystachya, S7- S. repanda, S8- S. radicata, S9- S. bryopteris, S10- S. delicatula with cones, S11- S. delicatula without cones.

Based on the acid phosphatase profile of studied Selaginella species, the similarity indices were calculated and the cladogram was constructed (Fig. 3). The evolutionary tree showed two clusters, C_1 and C_2. The cluster C_1 displayed the similarity and variation among five species of Selaginella viz., S. intermedia, S. inaequalifolia, S. radicata, S. tenera, S. delicatula with and without cones. Similarly, the cluster C_2 represented the closeness and divergence among five species of Selaginella viz., S. involvens, S. brachystachya, S. bryopteris, S. wightii and S. repanda. The cluster C_1 is divided into two nodes, C_1N^1 and C_1N^2. The node C_1N^1 is branched into $C_1N^1B_1$ and $C_1N^1B_2$. The node C_1N^2 displayed the unique character of S. delicatula with cones. The branch $C_1N^1B_1$ is further divided into $C_1N^1B_1b_1$ and $C_1N^1B_1b_2$. The branch $C_1N^1B_2$ represented the exclusive nature of S. tenera. $C_1N^1B_1b_1$ presented the similarity between S. intermedia ($C_1N^1B_1b_1A$) and S. inaequalifolia ($C_1N^1B_1b_1B$). $C_1N^1B_1b_2$ demonstrated the closeness between S. radicata ($C_1N^1B_1b_2A$) and S. delicatula without cones ($C_1N^1B_1b_2B$). The cluster C_2 is divided into two nodes, C_2N^1 and C_2N^2. The node C_2N^1 is branched into $C_2N^1B_1$ and $C_2N^1B_2$. The branch $C_2N^1B_1$ explained the similarity between S. involvens and S. brachystachya. The branch $C_2N^1B_2$ exposed the distinct occurrence of S. bryopteris. The node C_2N^2 is branched into $C_2N^2B_1$ and $C_2N^2B_2$ which depicted the similarity between S. wightii and S. repanda.

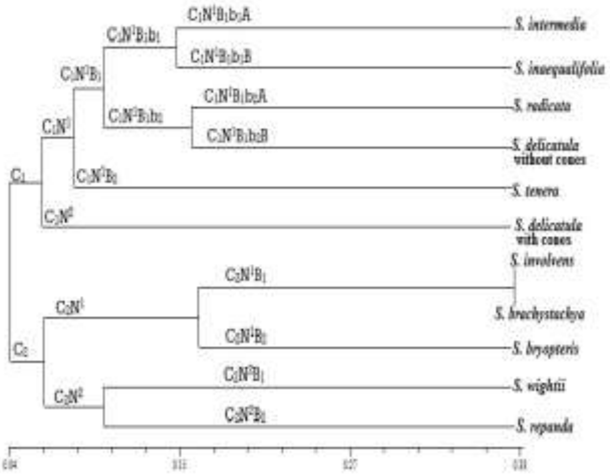

Fig. 3: Cladogram of Selaginella species based on acid phosphatase profile

Alkaline phosphatase assay

In the alkaline phosphatase enzyme system, ten regions of activity with forty eight bands were obtained (Table 5). S. bryopteris displayed maximum number (10) of alkaline phosphatase bands expression and S. radicata represented with a single enzymatic band. Region 1 illustrated with four enzymatic bands (Table 5). ALP 1^1 (0.01) was restricted to S. bryopteris. ALP 1^2 (0.02) showed its common presence in S. involvens, S. tenera and S. wightii. ALP 1^3 with MW-Rf value 0.08 was shared by S. brachystachya and S. radicata. ALP 1^4 (0.09) was common to S. bryopteris, S. delicatula with and without cones. Region 2 exposed five alkaline phosphatase bands. ALP 2^1 (0.11) was uniformly distributed in S. involvens and S. tenera. ALP 2^2 (0.12) was shared by S. inaequalifolia and S. wightii. ALP 2^3 (0.14) was observed commonly in S. intermedia, S. inaequalifolia, S. tenera and S. repanda. ALP 2^4 (0.16) was distinct to S. brachystachya. ALP 2^5 (0.18) was mutually shared by S. involvens and S. bryopteris. Region 3 represented three active alkaline phosphatase bands. ALP 3^1 (0.22) was restricted to S. brachystachya. ALP 3^2 and ALP 3^3 with MW-Rf values 0.23 and 0.28 were expressed only in S. tenera. Region 4 displayed four active enzymatic bands. ALP 4^1 (0.30) was shared by S. intermedia and S. tenera. ALP 4^2 (0.33), ALP 4^3 (0.37) and ALP 4^4 (0.38) were showed their unique presence in S. involvens, S. bryopteris and S. tenera respectively. Region 5 displayed three enzymatic bands. ALP 5^1, ALP 5^2 and ALP 5^3 with MW-Rf values 0.46, 0.48 and 0.49 were restricted to S. involvens, S. bryopteris and S. brachystachya correspondingly. Region 6 observed with a single enzymatic band, ALP 6^1 (0.58) was distinct to S. brachystachya. Region 7 illustrated with three alkaline phosphatase bands. ALP 7^1 (0.60) and ALP 7^3 (0.64) were showed its unique existence in S. delicatula and S. brachystachya respectively. ALP 7^2 (0.61) was uniformly distributed in S. involvens and S. bryopteris. Region 8 depicted three alkaline phosphatase bands. ALP 8^1, ALP 8^2 and ALP 8^3 with MW-Rf values 0.75, 0.77 and 0.79 were expressed its exclusive character in S. bryopteris, S. wightii and S. involvens respectively. Region 9 delivered three enzymatic bands. ALP 9^1 (0.81) and ALP 9^2 (0.83) were restricted to S. bryopteris and S. delicatula with cones. ALP 9^3 (0.85) was shared by S. involvens and S. bryopteris. Region10 showed two alkaline phosphatase bands. ALP 10^1 (0.90) established its unique occurrence in S. intermedia. ALP 10^2 (0.92) was observed in S. involvens, S. wightii, S. repanda, S. bryopteris and S. delicatula without cones.

Table 5: Alkaline phosphatase profile of Selaginella species

MW Rf	R	Enzyme position	S1	S2	S3	S4	S5	S6	S7	S8	S9	S 10	S 11
0.01	1	ALP 1^1									+		
0.02		ALP 1^2			+	+	+						
0.08		ALP 1^3						+		+			
0.09		ALP 1^4									+	+	+
0.11	2	ALP 2^1			+	+							
0.12		ALP 2^2		+			+						
0.14		ALP 2^3	+	+		+			+				
0.16		ALP 2^4						+					
0.18		ALP 2^5			+						+		
0.22	3	ALP 3^1						+					
0.23		ALP 3^2				+							
0.28		ALP 3^4				+							
0.30	4	ALP 4^1	+			+							
0.33		ALP 4^2			+								
0.37		ALP 4^3									+		
0.38		ALP 4^4				+							
0.46	5	ALP 5^1			+								
0.48		ALP 5^2									+		
0.49		ALP 5^3						+					
0.58	6	ALP 6^1						+					
0.60	7	ALP 7^1										+	
0.61		ALP 7^2			+						+		
0.64		ALP 7^3						+					
0.75	8	ALP 8^1									+		
0.77		ALP 8^2					+						
0.79		ALP 8^3			+								
0.81	9	ALP 9^1									+		
0.83		ALP 9^2										+	
0.85		ALP 9^3			+						+		
0.90	10	ALP 10^1	+										
0.92		ALP 10^2			+		+		+		+		+
Total			**3**	**2**	**9**	**7**	**4**	**6**	**2**	**1**	**10**	**3**	**2**

Note: R- Region, S1- S. intermedia, S2- S. inaequalifolia, S3- S. involvens, S4- S. tenera, S5- S. wightii, S6- S. brachystachya, S7- S. repanda, S8- S. radicata, S9- S. bryopteris, S10- S. delicatula with cones, S11- S. delicatula without cones.

Based on the alkaline phosphatase profile of studied Selaginella species, the similarity indices were calculated and the cladogram was constructed (Fig. 4). The evolutionary tree displayed two clusters, C_1 and C_2. The cluster C_1 is divided into two nodes, C_1N^1 and C_1N^2. The node C_1N^1 is branched into $C_1N^1B_1$ and $C_1N^1B_2$. The branch $C_1N^1B_1$ is further divided into $C_1N^1B_1b_1$ and $C_1N^1B_1b_2$. $C_1N^1B_1b_1$ represented the exclusive nature of S. intermedia from the other studied species of Selaginella. $C_1N^1B_1b_2$ explained the similarity between S. inaequalifolia and S. repanda. The branch $C_1N^1B_2$ exposed the distinct occurrence of S. tenera. The node C_1N^2 is branched into $C_1N^2B_1$ and $C_1N^2B_2$. The branch $C_1N^2B_1$ is again branched into $C_1N^2B_1b_1$ and $C_1N^2B_1b_2$. $C_1N^2B_1b_1$ presented the similarity between S. involvens ($C_1N^2B_1b_1A$) and S. bryopteris ($C_1N^2B_1b_1B$). The branch $C_1N^2B_1b_2$ displayed the unique presence of S. wightii. The branch $C_1N^2B_2$ demonstrated the closeness between S. delicatula with cones ($C_1N^2B_2b_1$) and without cones ($C_1N^2B_2b_2B$). The cluster C_2 is divided into two nodes C_2N^1 and C_2N^2 which displayed the closeness between S. brachystachya and S. radicata.

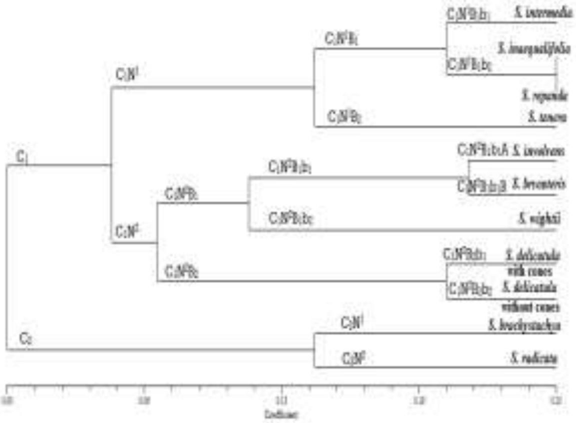

Fig. 4: Cladogram of Selaginella species based on alkaline phosphatase profile

Polyphenol oxidase assay

Two regions of activity with twelve polyphenol oxidase bands were observed in the polyphenol oxidase enzyme system of studied Selaginella species (Table 6). Region 1 showed three active enzymatic bands. PPO 1^1 (0.01) was restricted to S. bryopteris. PPO 1^2 (0.02) was distributed in S. radicata, S. delicatula with and without cones. PPO 1^3 (0.05) was observed in S. intermedia, S. inaequalifolia, S. involvens, S. tenera, S. wightii, S. brachystachya and S. repanda. Region 2 failed to show the active polyphenol oxidase enzymes. Region 3 observed with a single enzymatic band, PPO 3^1 (0.29) was distinct to S. brachystachya.

Table 6: Polyphenol oxidase profile of Selaginella species

MW Rf	R	Enzyme position	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11
0.01	1	PPO 1^1									+		
0.02		PPO 1^2								+		+	+
0.05		PPO 1^3	+	+	+	+	+	+	+				
0.29	3	PPO 3^1						+					
Total			**1**	**1**	**1**	**1**	**1**	**2**	**1**	**1**	**1**	**1**	**1**

Note: R- Region, S1- S. intermedia, S2- S. inaequalifolia, S3- S. involvens, S4- S. tenera, S5- S. wightii, S6- S. brachystachya, S7- S. repanda, S8- S. radicata, S9- S. bryopteris, S10- S. delicatula with cones, S11- S. delicatula without cones.

The similarity indices were calculated and phylogenetic tree was constructed based on the polyphenol oxidase profile of studied Selaginella species (Fig. 5). In the cladogram, four clades were displayed viz., C_1, C_2, C_3 and C_4. The clade C_1 is divided into two nodes, C_1N^1 and C_1N^2. The node C_1N^1 is branched into $C_1N^1B_1$ and $C_1N^1B_2$. The branch $C_1N^1B_1$ explained the similarities among the four species of Selaginella viz., S. intermedia, S. inaequalifolia, S. involvens and S. tenera. The branch $C_1N^1B_2$ showed the closeness between S. wightii and S. repanda. The clade C_2 expressed the distinct nature of S. radicata. The clade C_3 represented the exclusive character of S. bryopteris. The clade C_4 illustrated the similarity between S. delicatula with and without cones.

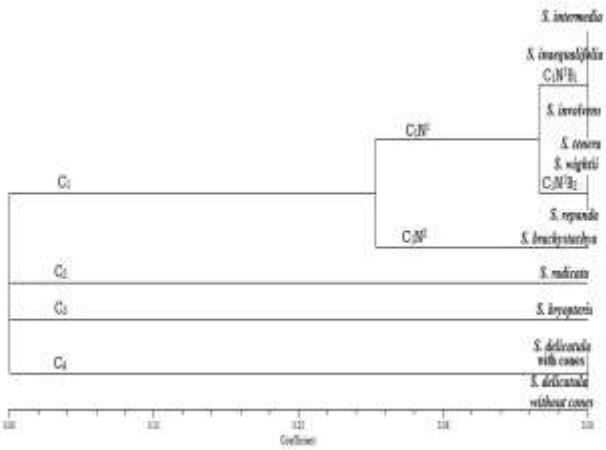

Fig. 5: Cladogram of Selaginella species based on polyphenol oxidase profile

Amalgamated isozymic profile

Based on the five enzymatic profiles viz., peroxidase, esterase, acid phosphatase, alkaline phosphatase and polyphenol oxidase of studied Selaginella species, the similarity indices were calculated and the amalgamated cladogram was constructed (Table 7). The evolutionary tree represents two main clusters C_1 and C_2. The cluster C_1 is diverged into two nodes C_1N^1 and C_1N^2. The node C_1N^1 is branched into $C_1N^1B_1$ and $C_1N^1B_2$ where C_1N_2 showed the unique existence of S. bryopteris. $C_1N^1B_1$ is again branched into $C_1N^1B_1b_1$ and $C_1N^1B_1b_2$. $C_1N^1B_1b_1$ displayed the similarity between two sub branches denoted by $C_1N^1B_1b_1A$ (S. intermedia) and $C_1N^1B_1b_1B$ (S. inaequalifolia). The branch $C_1N^1B_1b_2$ showed two sub branches $C_1N^1B_1b_2A$ and $C_1N^1B_1b_2B$. $C_1N^1B_1b_2A$ expressed the similarity between S. involvens and S. repanda. $C_1N^1B_1b_2B$ displayed the individuality of S. wightii. $C_1N^1B_2$ displayed the similarity between S. tenera ($C_1N^1B_2b_1$) and S. brachystachya ($C_1N^1B_2b_2$). The cluster C_2 is divided into two nodes C_2N^1 and C_2N^2. The node C_2N^1 showed the distinct character of S. radicata. C_2N^2 displayed the similarity between S. delicatula with cones and S. delicatula without cones (Fig. 6).

Table 7: Similarity indices of Selaginella species using amalgamated isozymic profile

Taxon	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11
S1	1.00										
S2	0.17	1.00									
S3	0.12	0.12	1.00								
S4	0.16	0.12	0.13	1.00							
S5	0.11	0.18	0.19	0.10	1.00						
S6	0.08	0.08	0.18	0.13	0.11	1.00					
S7	0.16	0.17	0.20	0.08	0.19	0.07	1.00				

S8	0.05	0.03	0.02	0.05	0.00	0.07	0.03	1.00			
S9	0.09	0.02	0.14	0.02	0.07	0.06	0.12	0.05	1.00		
S10	0.04	0.01	0.00	0.05	0.00	0.05	0.02	0.11	0.04	1.00	
S11	0.04	0.03	0.04	0.04	0.04	0.09	0.05	0.15	0.08	0.21	1.00

Note: S1- S. intermedia, S2- S. inaequalifolia, S3- S. involvens, S4- S. tenera, S5- S. wightii, S6- S. brachystachya, S7- S. repanda, S8- S. radicata, S9- S. bryopteris, S10- S. delicatula with cones, S11- S. delicatula without cones.

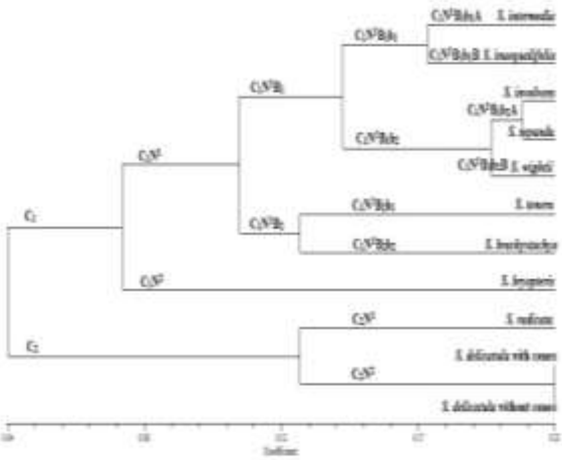

Fig. 6: Amalgamated cladogram of Selaginella species based on isozymic profiles

DISCUSSION

Assessment of genetic variability within and between species or populations for purpose of conservation and to seek useful genotypes can be achieved by employing a variety of techniques. Among these, use of molecular markers finds greater utility due to its ability to reveal polymorphism at the genetic level (Rea et al., 2004). They have become increasingly popular in plant research since they can potentially provide a much higher number of markers. Among the different types of markers available, biochemical and molecular markers have become exceedingly popular for estimation of genetic changes within and between the species (Johnson et al., 2012). Variations in isozyme expression of tissues reflect changes in metabolic activities during growth, development and differentiation of plant by means of biochemical indices. Characterization of purified enzyme is important to reveal physiological functions and action of particular enzymes. Changes in isozyme action during development of a tissue can be detected by pattern shifts on PAGE profile. Isozyme markers have been useful in determining genetic relationships among closely related species and cultivars. Isozyme electrophoresis is chosen for its relative simplicity because it provides direct visualization of gene products and can provide a unique fingerprint for each genetically distinct clone (Barta et al., 2003; Mukhlesur et al., 2004; Angelov and Ivanova, 2012).

Irudayaraj and Johnson (2011) identified the phylogenetic relationships among Sphaerostephanos arbuscula, S. unitus and S. subtruncatus using isoperoxidase analysis. Johnson et al. (2012) exposed inter-specific variation on three species of tree fern Cyathea by means of isoperoxidase analysis. Johnson et al. (2012) assessed the genetic variation between different populations of Thelypteris ciliata collected from different localities on Tirunelveli hills, using isoperoxidase profiling. Similar to the previous observations in the present study also isozymic analysis viz., esterase, peroxidase, acid phosphatase, alkaline phosphatase and polyphenol oxidase were carried out to know inter-specific relationship among the selected ten species of Selaginella. The evolutionary tree of studied Selaginella species based on the esterase profile showed the distinct nature of S. intermedia, S. involvens, S. radicata, S. tenera and S. brachystachya. These will be an additional supportive evident to distinguish the morphologically unique species of Selaginella viz., S. intermedia, S. involvens, S. radicata, S. tenera and S. brachystachya. In peroxidase system of Selaginella, twenty five distinct bands were observed. The phylogenetic tree based on the peroxidase profile of studied Selaginella species explained the uniqueness of S. involvens, S. brachystachya and S. tenera. Isozymes such as esterase and peroxidase have been utilized to assess the genetic similarity and differences at the various taxonomic levels (Nanthini et al., 2011). Similarly in the present study also, these isozymes are used as fingerprint for the identification of Selaginella species. They revealed the variation at species level.

Acid phosphatase system of studied Selaginella species was observed with a total of sixty three enzymatic bands with twenty one classified bands in the acid phosphatase profile. The cladistic tree based on the acid phosphatase profile displayed the restricted character of S. tenera, S. delicatula with cones and S. bryopteris. In addition, the acid phosphatase system revealed the developmental stage variation in S. delicatula with and without cones. In morphotaxonomy, the cones of fern allies play an important role to distinguish the fern allies at species level. In the present study, the result of the acid phospahstase profiles provided an alternative for the identification of S. delicatula without cones. In the alkaline phosphatase enzyme system of studied Selaginella species, twenty exceptional bands were obtained. The evolutionary tree displayed the distinctive character of S. intermedia, S. tenera and S. wightii. Polyphenol oxidase enzyme system of studied Selaginella species was observed with twelve active bands and showed only two distinct enzymatic bands. The phylogenetic tree of Selaginella species displayed the uniqueness of S. brachystachya, S. radicata and S. bryopteris based on polyphenol oxidase profile. The amalgamated cladogram of isozymic profiles displayed the distinct character of S. wightii, S. bryopteris and S. radicata. Unique banding profiles of esterase, peroxidase, acid phosphatase, alkaline phosphatase and polyphenol oxidase were observed in the studied Selaginella species which were used to distinguish the selected Selaginella species and these profiles will act as biochemical fingerprint for the studied species in plant systematic studies.

CONCLUSION

The result obtained in the present study explained that the PAGE analysis can provide an easy, low cost and quick way for the identification of Selaginella species and also have a better knowledge of the genetic affinity of germplasm.

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