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REGULAR ARTICLE

ASSESSING THE GENETIC RELATIONSHIP BETWEEN THREE SELECTED SPECIES OF *RAUVOLFIA* USING ISOENZYME PROFILES AND MORPHOLOGICAL CHARACTERS

A. Usha Raja Nanthini[2], M. Johnson[1*], F. Merlin Franco[1] and T. Renisheya Joy Jeba Malar

[1]*Department of Plant Biology and Biotechnology, St. Xavier's College, Palayamkottai, TN, South India*
[2]*Department of Biotechnology, Noorul Islam College of Arts and Science, Kumaracoil, TN, South India*

SUMMARY

Using Isoenzyme markers to study genetic diversity and relationship is the oldest and easiest method. In the current study, variation in Isozymic profiles as well as distinguishable morphological characters has been considered to reveal the genetic diversity and relationship existing between three species of *Rauvolfia* viz., *Rauvolfia serpentina* (L.) Benth. ex Kurz, *Rauvolfia micrantha* Hook. f. and *Rauvolfia tetraphylla* L. The enzymes selected for the study are Isoperoxidase, Isoesterasse, Acid Phosphatase, Alkaline Phosphatase and Poly phenol oxidase. The study shows that the species *R. serpentina* is distinct from *R. micrantha* and *R. tetraphylla* which show greater affinity towards each other.

Key words: Genetic diversity, Biochemical marker, Finger printing

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*Corresponding Author, Email: ptcjohnson@gmail.com; Tel.: +91-0462-4264316; Mob: + 91 9786 92 4334; Fax: + 91 462 2561 765

1. Introduction

Accurate identification, genetic diversity and evolutionary lineage of any taxon is assessed traditionally on the basis of morphological, anatomical and cytological characters such as plant height, reproductive features, habitat, habit and adaptation and chromosome number. Such characters may not often reflect the precise identification and genetic variance among the species and their ancestry. For instance, a study by Onus and Pickersgill [1] confirms that, morphological markers can fail to distinguish between homozygotes and heterozygotes, when there is dominance. To overcome such limitations of morphological, anatomical and cytological markers, modern molecular approaches are increasingly being adopted. One such marker is the Isoenzyme marker. Biochemical markers generated by isoenzymes can be used to make a distinction between morphologically indistinguishable species and varieties [2-4]. This quality renders Isoenzymes as valuable genetic markers in modern plant systematic studies and plant breeding programmes [5-9]. Using Isoenzyme markers to solve taxonomic and evolutionary puzzles is the oldest, easy and low-cost method [10-13]. Studies have shown that isoenzyme markers could be more reliable than RAPD markers [5, 14]. In addition, it permits to quantify the genetical homology and distance within and between species [15-19]. The existence and nonexistence of the plant isozymes can be revealed by the biochemical system of the cells. Isozymes often exhibit tissue or cell specificity [20] and each isozyme has a specific role in the metabolic pathway and functions in harmony with other enzymes within the organizational framework of cells. In the current study, Isozymic variation has been chosen to reveal the diversity existing at molecular level in *three* species of *Rauvolfia* viz., *Rauvolfia serpentina* (L.) Benth. ex Kurz, *Rauvolfia micrantha* Hook. f. *and Rauvolfia tetraphylla* L.

2. Materials and Methods

According to the APG-II classification, *Rauvolfia* belongs to the sub-family Rauvolfioideae of the family Apocynaceae [21]. The Genus consists of shrubs, bearing leaves in whorled phyllotaxy. The pentamerous flowers are found in corymbose or umbellate cymes. An annular or cup shaped disk is present. Fruits are drupaceous with a crustaceous pyrene [22-24]. All the three selected species of *Rauvolfia* (*Rauvolfia serpentina* (L.) Benth. ex Kurz, *Rauvolfia micrantha* Hook. f. and *Rauvolfia tetraphylla* L) were collected from Athmanilayam Nursey Gardens, Cheruvarakonam, Kerala, India. All this morphological characters were tabulated, similarity indices were obtained from the table and used for constructing a cladogram using the Software NTSYS (Numerical Taxonomy and Multivariate Analysis System) version 2.2 released by Applied Biostatistics Inc (Table – 1,2).

Table – 1: Similarity analysis of morphological characters of *Rauvolfia* spp.

Morphological character	*R. serpentina*	*R. micrantha*	*R. tetraphylla*
Shrubs	+	+	+
Leaves in whorls of three	+	+	-
Nerves 8-12 pairs	+	+	+
Flowers 0.5 mm across	+	+	-
Calyx with glands	+	-	-
Long corolla tube	+	-	-
Drupes 0.5 mm long	+	+	+
Drupes connate to the top	-	-	+

Table – 2: Similarity indices of morphological characters of *Rauvolfia* spp

Species	*R. serpentina*	*R. micrantha*	*R. tetraphylla*
R. serpentina	1.00000		
R. micrantha	0.83333	1.00000	
R. tetraphylla	0.54546	0.60000	1.00000

The enzymes selected for the study were Isoperoxidase, Isoesterasse, Acid Phosphatase, Alkaline Phosphatase and Poly phenol oxidase. For enzyme extraction, 500 to 1000 mg of freshly harvested young leaves were taken and homogenized with 3.5 ml of ice-cold homogenizing buffer in a pre-chilled pestle and mortar. For peroxidase, the young shoots were homogenized with 0.1M phosphate buffer (pH 7.0) and centrifuged at 12,000 rpm for 10min. For esterase, the young leaves were collected and ground with pre-chilled isolation buffer (0.1M phosphate buffer pH 9.2) and centrifuged at 12,000 rpm for 10 min. For acid and alkaline phosphatase the young leaves were harvested and homogenized in a mortar and pestle with citrate buffer and centrifuged at 20,000 rpm for 10 min. The supernatant was subjected to electrophoresis as Poly Acrylamide Gel Electrophoresis (PAGE) as per Sadasivam and Manickam [25]. Staining solutions for Isoperoxidase, Isoesterasse, Acid Phosphatase, Alkaline Phosphatase and Poly phenol oxidase were prepared as per Sadasivam and Manickam [25] for the detection of iso-enzymes. After the 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 gel documentation system manufactured by Biotech, Yercaud, India. Pairing affinity or similarity index was calculated by the method described by Sokal and Sneath [26]. Similarity indices of isoenzyme system were generated from the banding pattern thus obtained. Based on the isoenzyme banding profile the zymogram was constructed. As the last step, a combined similarity index of morphological characters

and isoenzyme system was generated from which, a cladogram was constructed.

3. Results

Alkaline Phosphatase (AKP)

In the Alkaline Phosphatase enzyme system, four regions (AKP 1 – 4) of activity with eight bands were obtained. In this enzyme system too, the selected three species failed to express the common banding profile. *R. serpentina* illustrated its presence only in AKP 2^1, AKP 2^3, AKP 3^1 and AKP 4^2 with MW-Rf 0.105, 0.152, 0.238 and 0.362 respectively. Bands of AKP 1^1(0.029), AKP 2^2 (0.124) and AKP 4^1 (0.305) were found only in *Rauvolfia micrantha.* AKP 4^3 with MW- RF 0.400 was jointly present in *R. micrantha* and *R. tetraphylla* (Table -3: Fig. 1 A and F).

Poly Phenol Oxidase (PPO)

Four regions of activity were observed for this enzyme system PPO 1, 2, 5 and 6. PPO 1^1 (0.026) and PPO 2^1 (0.198) were present commonly in the selected three species. PP0 5^1 (0.431) was restricted to *R. serpentina*, PPO 5^2 (0.474) was present only in *R. tetraphyla* and PPO 6^1 (0.560) was unique to *R. micrantha* (Table -3: Fig. 1 B and G). The poly phenol oxidase system distinguished the three species with unique presence and showed the similarity by the expression of common bands in the enzyme system.

Peroxidase (PRX)

Four Regions of activity (PRX 1 – 3 and 5) were observed in this enzyme system. The first (0.027) and last (0.432) bands were restricted to *Rauvolfia serpentina* (PRX 1^1 and PRX 5^1). Second band MW-RF 0. 189 (PRX 2^1) was shared by two selected species *Rauvolfia micrantha* and *Rauvolfia tetraphylla.* PRX 3^1 (0.216) was jointly present in *Rauvolfia serpentina* and *Rauvolfia micrantha* (Table - 3: Fig. 1 C and H).

Acid Phosphatase (ACP)

Multiple regions of activity were obtained for this enzyme system ACP 1 to 7. *R. serpentina* showed its unique banding profile in region ACP 2^2, 4^3, 6^1 and 7^1 with 0.185, 0.361, 0.546 and 0.611 MW-RF values. *R. micrantha* demonstrated solely in regions ACP 3^1 (0.277) and ACP 4^2 (0.333). *R. tetraphylla* failed to show its unique presence in this enzyme system. The regions ACP 1^1 and ACP 5^1 were observed in *R. serpentina and R. micrantha.* Region ACP 1^2 (0.055) and ACP 2^1 (0.166) were jointly present in *R. micrantha* and *R. tetraphylla* (Table – 3: Fig. 1 D and I). Acid Phosphatase system failed to express the common banding profiles between the selected three *Rauvolfia* species.

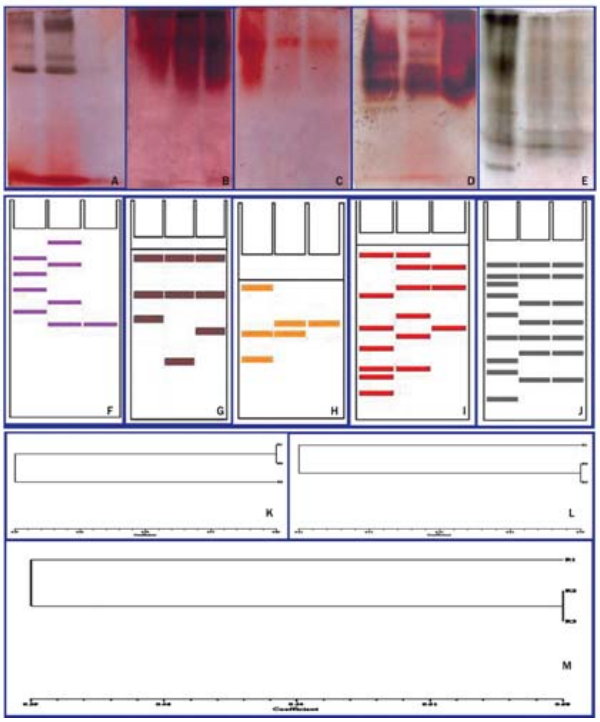

Fig. 1. Analysis of Genetic Relationship between Three Selected Species of *Rauvolfia.*

A – Alkaline Phosphatase banding pattern of *Rauvolfia* spp., B – Poly Phenol Oxidase banding pattern of *Rauvolfia* spp., C – Isoperoxidase banding pattern of *Rauvolfia* spp., D – Acid Phosphatase banding pattern of *Rauvolfia* spp., E – Isoesterase banding pattern of *Rauvolfia* spp., F – Zymogram of Alkaline Phosphatase of *Rauvolfia* spp., G - Zymogram of Poly Phenol Oxidase of *Rauvolfia* spp., H - Zymogram of isoperoxidase of *Rauvolfia* spp., I - Zymogram of Acid Phosphatase of *Rauvolfia* spp., J - Zymogram of Isoesterase *Rauvolfia* spp., K - Cladogram of selected Morphological characters of *Rauvolfia* spp., L - Isoenzymes based cladogram of *Rauvolfia* spp., M - Cladogram based on the morphological characters and isoenzyme systems of *Rauvolfia* spp.

Table – 3: MW- Rf Values and Banding Profile of *Rauvolfia* Species

MW-RF	Band Positions	*R. serpentina*	*R. micrantha*	*R. tetraphylla*
POLY PHENOL OXIDASE				
0.026	PPO 1^1	+	+	+
0.198	PPO 2^1	+	+	+
0.431	PPO 5^1	+	-	-
0.474	PPO 5^2	-	-	+
0.560	PPO 6^1	-	+	-
PEROXIDASE				
0.027	PRX 1^1	+	-	-
0.189	PRX 2^1	-	+	+
0.216	PRX 3^1	+	+	-
0.432	PRX 5^1	+	-	-
ACID PHOSPHATASE				
0.018	ACP 1^1	+	+	-
0.055	ACP 1^2	-	+	+
0.166	ACP 2^1	-	+	+
0.185	ACP 2^2	+	-	-
0.277	ACP 3^1	-	+	-
0.305	ACP 4^1	+	-	+
0.333	ACP 4^2	-	+	-
0.361	ACP 4^3	+	-	-
0.462	ACP 5^1	+	+	-
0.546	ACP 6^1	+	-	-
0.611	ACP 7^1	+	-	-
ESTERASE				
0.048	EST 1^1	+	+	+
0.17	EST 2^1	+	+	+
0.284	EST 3^1	+	-	-
0.414	EST 5^1	+	-	-
0.455	EST 5^2	-	+	+
0.496	EST 5^3	+	-	-
0.512	EST 6^1	-	+	+
0.577	EST 6^2	+	+	+
0.675	EST 7^1	-	+	+
0.691	EST 7^2	+	-	-
0.731	EST 8^1	+	-	-
0.748	EST 8^2	-	+	+
0.869	EST 9^1	+	-	-
ALKALINE PHOSPHATASE				
0.029	AKP 1^1	-	+	-
0.105	AKP 2^1	+	-	-
0.124	AKP 2^2	-	+	-
0.152	AKP 2^3	+	-	-
0.238	AKP 3^1	+	-	-
0.305	AKP 4^1	-	+	-
0.362	AKP 4^2	+	-	-
0.400	AKP 4^3	-	+	+

Esterase (EST)

In the Esterase enzyme system, eight regions (EST 1-3, 5-9) of activity were obtained. EST 1^1(0.048), 1^2 (0.170) and 6^2 (0.577) were commonly shared by selected three species. EST 3^1(0.284), 5^1(0.414), 7^2(0.691), 8^1(0.731) and 9^1 (0.869) were observed only in *R. serpentina*. EST 5^2(0.455), 6^1(0.512), 7^1 (0.675) and 8^2(0.748) were restricted to *R. micrantha* and *R. tetraphhlya* (Table -3: Fig. 1 E and J).

A similarity index was generated based on the morphological features of the three species, with emphasis on the floral characters. The results are given in Table – 1, based on which, a cladogram was generated

(Fig. 1 K). Highest percentage (0.833333) of similarity is observed between *R. serpentina and R. micrantha* while the highest percentage (0.4546) of variation is seen between *R. serpentina* and *R. tetraphylla* (Table-2). The cladogram of morphological character shows two clusters, of which cluster (C2) includes only one species- *R. tetraphylla, that* showed highest percentage of divergence with the other two species. The Cluster 1 (C1) showed two nodes viz., C_1N_1 - *R. serpentina* and C_1N_2 - *R. micrantha* (Fig. 1 K).

The isoenzyme profile emphasized pairing affinity or similarity index analysis of *Rauvolfia* species is shown in Table – 4 from which a cladogram was constructed (Fig. 1 L). Highest percentage (0.6809) of similarity is observed between *R. micrantha* and *R. tetraphylla* while the highest percentage (0.6539) of variation *is observed between R. serpentina* and *R. tetraphylla*. Similar to the cladogram based on morphological character, the isozyme profile based cladogram also showed two clusters but cluster (C1) includes only one species - *R. serpentina that* showed highest percentage of divergence with the other two species. The Cluster 2 (C2) showed two nodes viz., C_2N_1 - *R. micrantha* and C_2N_2 – *R. tetraphylla* (Fig. 1 L). This contradicts the results of morphology based cladogram.

Table – 4: Similarity indices of isoenzyme systems of *Rauvolfia* spp.

Species	*R. serpentina*	*R. micrantha*	*R. tetraphylla*
R. serpentina	1.000000		
R. micrantha	0.333333	1.000000	
R. tetraphylla	0.292683	0.702703	1.000000

A combined cladogram of morphological characters and isoprofile systems was done by amalgamating the morphological characters and isoenzymes profiles obtained (Table – 5). This hybrid system reveals the similarity and variation between the selected three species (Table – 5; Fig. 1 M). The cladogram shows two clusters. Cluster (C1) includes only one species- *R. serpentina that* shows the highest percentage of divergence with the other two species while Cluster 2 (C2) shows two nodes viz., C_2N_1 - *R. micrantha* and C_2N_2 – *R. tetraphylla* (Fig. 1 M). The results reflect the outcome of the isoenzyme based cladogram and contradicts the results of morphology based cladogram.

Table – 5: Combined similarity indices of morphological characters and isoenzyme systems of *Rauvolfia* spp

Species	*R. serpentina*	*R. micrantha*	*R. tetraphylla*
R. serpentina	1.0000		
R. micrantha	0.4333	1.0000	
R. tetraphylla	0.3461	0.6809	1.0000

4. Discussion

The similarity and variation between *Rauvolfia serpentina*, *Rauvolfia micrantha* and *Rauvolfia tetraphylla* are reported with respect to the enzymes esterase, peroxidase, acid phosphatase, alkaline phosphatase and polyphenol oxidase, (Fig. 1 A to E). Since 1930, electrophoresis, along with the zymogram technique has been the tool of choice for studies of heritable variation by geneticists, systematists and population biologists. The present study shows that the three selected species are easily separable isozymically, besides revealing the affinities. In electrophoresis, each zone is occupied by a particular isozyme in the form of band and is representative of the appearance of a particular gene locus coding for that isozyme. In certain enzyme system, more than one distinct band could be resolved in a particular zone. These bands could represent allelic isozymes, coded by different alleles of the same gene at locus and thus occupy that particular zone on the gel [5]. In the present study also the similar kind of banding profiles are observed in all enzyme systems indicating the presence of multiple alleles.

Isozymes such as esterase and peroxidase have been utilized to assess the genetic similarity and differences at the various taxonomic levels [1, 7, 8, 27-30]. Similarly in the present study also, these isozymes are used as biochemical marker for the systematic study of *Rauvolfia* species. Unique banding profiles of esterase, peroxidase, acid phosphatase, alkaline phospahatase and polyphenol oxidase were observed in *Rauvolfia serpentina*, *Rauvolfia micrantha* and *Rauvolfia tetraphylla,* which represent the fingerprint of that particular species. Such finger printing is useful in differentiating the species and act as biochemical markers for these species in plant systematic studies. From the study, it is understandable that the species *R. serpentina* is distinct from *R. micrantha* and *R. tetraphylla* which show greater affinity towards each other. This assumes significance as *R. serpentina* and *R. micrantha* are native to India where as *R. tetraphylla* is an introduced and cultivated species. Though *R. micrantha* and *R. tetraphylla* originated in two different continents (Asia and America, respectively), they are more related to each other whereas *R. serpentina* appears to be less related. The use of both the distinguishable morphological characters as well as isoenzyme profiles provides a hybridized picture of the relationship between the three selected species of *Rauvolfia*.

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