Assessment of Genetic Diversity of *Woodfordia fruticosa* (L.) Kurz in Himachal Pradesh using Isozyme Polymorphism

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Woodfordia fruticosa (L.) Kurz (Lythraceae) is a rare woody shrub, the dried flowers of which are used as an important drug to treat various human disorders. Genetic variation of thirty genotypes of *W. fruticosa* was investigated using nine enzyme systems. Isozyme analysis of eight enzymes revealed considerable and very useful polymorphism among thirty genotypes from ten sites of Himachal Pradesh. Allele frequency in the thirty genotypes ranged from 0.5 to 1.00 and at all polymorphic loci only two alleles could be identified. On the basis of dendrogram the thirty genotypes were separated into two clusters. Cluster I included genotypes from Mandi, Hamirpur and Bilaspur districts, whereas Cluster II included genotypes from Solan and Sirmour districts. Polymorphism in the selected genotypes was found to be 34.5 per cent.

Key Words: ***Woodfordia fruticosa*, Genetic markers, Isozymes, Polyacrylamide gel electrophoresis**

Introduction

Woodfordia fruticosa Kurz. of the family Lythraceae, commonly known as Fire flame bush, Shiranjitea, Dhai, Dhataki, Dawi, is a plant of tropical and sub-tropical region. The plant is found in majority of South-East Asian countries–Malaysia, Indonesia, Sri Lanka, China, Japan, Pakistan and Tropical Africa. The plant is reported to be present throughout India upto an altitude of 1500 m. It is a leafy shrub about 3.5 m high and it is attracting the attention of floriculturist due to its brilliant red flowers. The plant is a well known forest produce that has long been in regular demand amongst practitioners of traditional medicines in different South-East Asian countries. In India, it is a much used medicinal plant in Ayurvedic and Unani systems of medicines (Chopra *et al*., 1956). Although all parts of this plant possess valuable medicinal properties, there is a heavy demand for the flowers, both in domestic and international markets specialized in the preparation of herbal medicines. According to the Indian systems of medicine, this flower is pungent, acrid, cooling, toxic, alexiteric, uterine sedative, antihelmentic, and is useful in dysentery, leprosy, erysipelas, blood diseases, leucorrhea and menorrhagia. Many marketed drugs comprise flowers, fruits, leaves and buds mixed with pedicels and thinner twigs of the plant (Nadkarni, 1954; Chopra *et al*., 1956). The use of bark, leaves, fruits and flowers in tanning industry are well known in the Indian subcontinent (Chadha, 1976). The flower produces red dye that is being used throughout India for dyeing of fabrics or as an adjunct/mordant. Presently, genetic conservation of medicinal and aromatic plants has become of paramount importance because of ruthless extraction by upcoming biotechnology and pharmaceutical industry. For efficient conservation and management of medicinal plant diversity, the genetic composition of species collected from different phytogeographical regions of Himachal Pradesh need to be assessed. Therefore, it is important to study *W. fruticosa* at genetic and molecular levels for conservation and management of genetic diversity.

Keeping in view the importance of this medicinal plant species, it was considered appropriate to assess the genetic variability of the germplasm of *W. fruticosa* using molecular markers.

Molecular methods provide valuable tools for reliable and precise identification of plants at any stage of their growth and development. Isozymes offer the most reliable single gene markers (Arulsekar and Parfitt, 1986) and polymorphism at enzyme loci has been shown to be stable under a number of environmental conditions. Virtually any plant tissue can be analyzed for isozymic studies for cultivar identification (Torress, 1983). Moreover, results obtained with isozymes are easily repeatable and can be obtained without carrying out field experiments; this saves money, time and space. In the present study, 30 genotypes of *W. fruticosa* were analysed using isozyme analysis to estimate their genetic diversity.

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Materials and Methods

Thirty genotypes of *W. fruticosa* were selected from ten sites of five districts, *viz*., Mandi, Hamirpur, Bilaspur, Solan and Sirmour of Himachal Pradesh, which have been assigned code numbers as shown in Table 1. These sites were chosen on the basis of reported places of occurrence during the survey (Fig. 1).

Nine enzyme systems investigated were Peroxidase (PER), Acid phosphatase (ACP), Aspartate amino transferase (AAT), Esterase (EST), Catalase (CAT), Malate dehydrogenase (MDH), Alcohol dehydrogenase (ADH), Phosphorylase (PPH) and Alkaline phosphatase (ALP).

Table 1. Geographical location of selected ten sites and the sample codes of *W. fruticosa*

S. No.	Sites	Districts	Site code	Sample code	Altitude (m)
1.	Harabagh	Mandi	S_1	W_1; W_2; W_3	1100-1369
2.	Jogindernagar		S_2	W_4; W_5; W_6	
3.	Anu	Hamirpur	S_3	W_7; W_8; W_9	785
4.	Awahdevi		S_4	W_{10}; W_{11}; W_{12}	
5.	Ghumarwin	Bilaspur	S_5	W_{13}; W_{14}; W_{15}	350-670
6.	Bhararighat		S_6	W_{16} ;W_{17}; W_{18}	
7.	Nauni	Solan	S_7	W_{19}; W_{20}; W_{21}	1365-1400
8.	Kandaghat		S_8	W_{22}; W_{23}; W_{24}	
9.	Rajgarh	Sirmour	S_9	W_{25}; W_{26}; W_{27}	900-3994
10.	Dhanech		S_{10}	W_{28}; W_{29}; W_{30}	

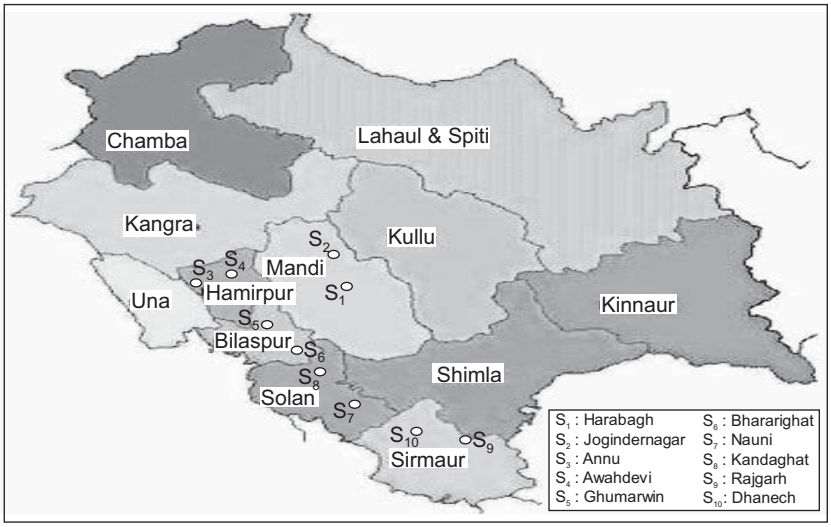


Fig. 1: Map showing ten selected sites of *Woodfordia fruticosa* in Himachal Pradesh

Sample Preparation

Fresh, young and green leaves were excised from 30 genotypes of *W. fruticosa* and then stored in liquid nitrogen till further use. Three hundred mg of leaf material was washed thoroughly and ground in prechilled pestle and mortar containing 1.5 ml Chase extraction buffer pH 7.5 (Chikkadevaiah and Nandinik, 2003). The homogenate was filtered through two folds of muslin cloth and then centrifuged at 20,000 rpm for 20 minutes at 4°C. The supernatant was taken and stored at –20°C till further study.

Electrophoresis

Electrophoresis was carried out for nine enzyme systems using miniVE electrophoretic and electrotransfer unit (Amersham Biosciences). The electrophoretic tank buffer was prechilled to 4°C to provide passive cooling. Two tray buffer systems were used for resolution of nine enzyme systems. First, tray buffer used was Lithium borate (lithium hydroxide 12 g, boric acid 118.9 g, distilled water 10.0 l and pH 8.3) as described by Scandalios (1969) and gel buffer used was Tris citrate (trizma base 62.0 g, citric acid 14.6 g, distilled water 10.0 l and pH 8.3) and this system was used for Peroxidase (PER), Esterase (EST), Aspartate amino transferase (AAT), Malate dehydrogenase (MDH) and Alcohol dehydrogenase (ADH). Second tray buffer used was Sodium borate (sodium hydroxide 20.0 g, boric acid 185.5 g, distilled water 10.0 l and pH 8.0) and gel buffer was Tris citrate (trizma base 121.1 g, citric acid 0.2 M, distilled water 10.0 l and pH 8.8) as described by Fowler and Morris (1977). This sytem was used for Acid phosphatase (ACP), Catalase (CAT), Alkaline phosphatase (ALP) and Phosphorylase (PPH).

Polyacrylamide gel electrophoresis was carried out to separate various isozymes due to its high resolving power, transparency and chemical inertness. Anionic system (Davis, 1964) was used for different enzyme systems. The gels were cast on the modules along with combs, which were removed after solidification and then these modules were placed in tank filled with tray buffer. The gentle inward pressure to both the tabs and locks into the lower notch were made to make contact between the gels and tray buffer. Thirty enzyme extracts were loaded in each

well with the help of a micropipette alongwith a drop of tracking dye (0.2 per cent bromophenol blue) in one well to work out the relative mobility (RM) values. Electrophoresis was carried at a constant current of 25 mA and 250 V till the marker dye reached the other end.

The bands of the enzyme activity were revealed by immersing the gels in specific histochemical stains. Staining recepies for ADH and AAT used were as described by Shaw and Prasad (1970). Staining of MDH was done according to Shaw and Koen (1968) while the methods of Scandalios (1969) were employed for EST and ACP. Staining procedures of Graham *et al.* (1964) were employed for PER. Staining of CAT was done according to Conkle *et al.* (1982) and methods of Tanksley and Orton (1983) were employed for PPH and ALP.

The genotypes were determined from the banding patterns obtained for each isozyme. In enzyme systems with multiple loci, the locus with greatest anodal migration was designated as I. Similarly, the fastest isozyme at a locus was represented by 'a' being controlled by allele 'A' (Mowrey *et al.*, 1990). The numbering of the additional loci and alleles within an enzyme system progressed sequentially in cathodal direction. The genotypes on the basis of isozymes were compared for each locus to assess genetic variability.

Different genetic parametes were obtained by analysing the data. The allele frequency was calculated as the proportion of a particular allele present at a given locus. Other genetic parameters studied to differentiate the genotypes included the proportion of polymorphic loci (Nei, 1975), the polymorphic index (Allard *et al.*, 1978), the average and effective number of alleles per locus (Crow and Kimura, 1970) and the observed (h_o) and expected heterozygosity (h_e), according to Nei (1975). The observed and expected heterozygote frequencies were compared using F-statistics (Wright, 1965). The inter-relationships of genotypes could be found on the basis of the following parameters.

Similarity index was calculated according to Hunter and Kannenberg (1971) based on presence or absence of bands. The identical bands in the particular enzyme system were rated as zero. If the two individuals differed for the presence or absence of a band within the same locus, these were given a rating of one. Thus, a diversity index comprised of cumulative ratings obtained in all the enzyme systems for each pair of the genotypes. Dendrogram was constructed from genetic distance values by the unweighted pair group arithmetic average (UPGMA) method and NTSYS-pc software version 2.0 to analyse the patterns of genotype relatedness.

Results and Discussion

In the present investigation, eight enzyme systems out of the nine were found to be polymorphic in nature. Only three (AAT, PPH, MDH) were found to produce informative markers, which were able to differentiate amongst the ten sites, whereas the alkaline phosphatase enzyme system showed monomorphic banding pattern. The qualitative description of these three enzymes is depicted in the form of zymograms.

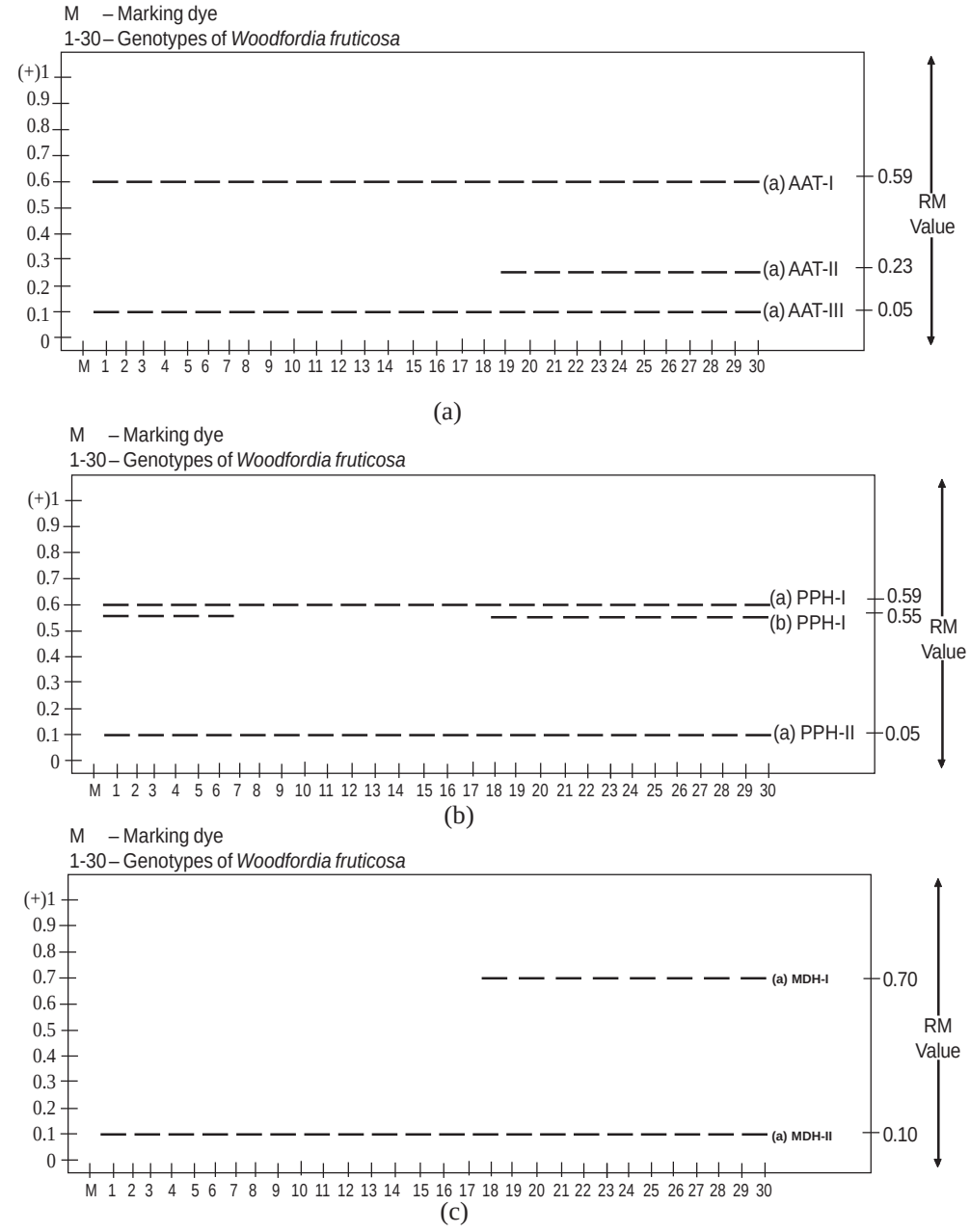
Isozymic Banding Patterns

Isozyme phenotypes of different enzyme systems have been described as under:

Aspartate amino transferase (EC2.6.1.2): Isozymic pattern of aspartate amino transferase with thirty genotypes produced three zones of enzyme activity (Fig. 2a). The most anodal zone locus AAT I displayed single band AAT Ia at RM value of 0.59. In the second zone locus AAT II showed a single band AAT IIa at RM value of 0.23 and was polymorphic in nature. It was present in genotypes from Nauni and Kandaghat of Solan district and Dhanech and Rajgarh sites of Sirmour district. The third zone locus AAT IIIa was found to be monomorphic and was present in all the thirty genotypes at the RM value of 0.05.

Phosphorylase (EC 2.4.1.1):- Two zones of enzyme activity were detected in phosphorylase enzyme system (Fig. 2b). The most anodal zone displayed a locus PPH I with two bands. PPH Ia at the RM value of 0.59 was present in all the thirty genotypes whereas PPH Ib was found to be polymorphic in nature and was present in the genotypes from Harabagh and Jogindernagar sites of Mandi district, Nauni and Kandaghat sites of Solan district and Rajgarh and Dhanech sites of Sirmour district. This band was present at the RM value of 0.55. The second zone locus PPH II showed single band PPH IIa at the RM value of 0.05 in all the thirty genotypes.

Malate Dehydrogenase (EC 1.1.1.37):- Malate dehydrogenase enzyme system displayed a banding pattern with two zones of enzyme activity (Fig. 2c). The locus MDH I showed a single band MDH Ia at the RM value of 0.70 in the genotypes from Nauni and Kandaghat sites of Solan district and Rajgarh and Dhanech sites of Simour district. The second zone locus MDH II showed single band MDH IIa at the RM value of 0.10 in all the thirty genotypes and thus was monomorphic in nature.



1-3 - Harabagh 4-6 - Jogindernagar 7-9 - Anu 10-12 - Awahdevi 16-18 - Bhararighat
19-21 - Nauni 22-24 - Kandaghat 25-27 - Rajgarh 13-15 - Ghumarwin 28-30 - Dhanech

Fig. 2: Isozymic patterns of (a) Aspartate amino transferase, (b) Phosphorylase, and (c) Malate dehydrogenase

Genetic interpretation studies revealed that a total of 24 isozyme loci were scored, coding for nine enzyme systems in thirty genotypes of *W. fruticosa*. The number of loci coding for individual enzyme ranged from 1 to 4 and a total of 24 alleles were identified in the present investigation (Table 2). Allele frequency varied between 0.5 to 1.0, respectively (Nei, 1975) depending upon whether a locus displayed heterozygous or homozygous conditions. Various monomorphic loci identified were PER I, AAT I, AAT II, AAT III, ACP I, ACP II, ACP III, CAT I, CAT II, CAT III, EST III, ADH II, ADH III, ALP I, ALP II, ALP III, PPH II, MDH I, MDH II. The polymorphic loci identified were PER IIA, PER IIb, PPH Ia, PPH Ib, EST Ia, EST Ib, EST IIa, EST IIb, ADH Ia

and ADH IIb. A locus was considered polymorphic if the frequency of the most common allele was not more than 0.99 (Nei, 1975). The proportion of polymorphic loci varied from 0.83 for Mandi district, 0.20 for genotypes from Hamirpur, Bilaspur and Solan districts and 0.187 for genotypes from Sirmour district. In the present study, the average number of alleles per locus varied from 0.95 for genotypes from Mandi district upto 1.00 for genotypes from Sirmour district. Expected heterozygosity ranged from 0.0416 to 0.062. F-Statistics showed negative values, depicting more heterozygosity than the observed values (Table 2). Goncharenko *et al*. (1994) also obtained negative values of Wright Fixation showing excess of heterozygosity between *Populus sylvestris* populations. However, such observations obtained in the present study may be biased due to very small sample size. Nei (1975) observed that in order to reduce the sampling error of heterozygosity a large number of loci rather than a large number of individuals per locus must be examined and to study allele frequency distribution also, large number of individuals must be examined.

Genetic Relation

Isozyme polymorphism data was analysed considering all the bands obtained from nine enzymes. According to the dendrogram thirty genotypes were divided into two major clusters (Fig. 3). The upper cluster demarcated the genotypes into two sub clusters. The first one consisted of genotypes from Mandi district, whereas cluster included genotypes from Hamirpur and Bilaspur districts. The lower bifurcation divided the genotypes of Solan and Sirmour into two sub-sub clusters. In the present investigation, total number of isozyme bands obtained were 29 and the RM value ranges from 0.03- 0.95 for

Table 2. Genetic variation parameters among thirty genotypes of *W. fruticosa*

Parameters	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Total no. of loci	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24
Total no. of alleles	23	23	23	23	23	23	21	21	21	21	21	21	21	21	21
Proportion of polymorphic loci	0.083	0.083	0.083	0.083	0.083	0.083	0.020	0.020	0.020	0.020	0.020	0.020	0.020	0.020	0.020
Polymorphic index	0.0416	0.0416	0.0416	0.0416	0.0416	0.0416	0.020	0.020	0.020	0.020	0.020	0.020	0.020	0.020	0.020
Average no. of alleles per locus	0.95	0.95	0.95	0.95	0.95	0.95	0.87	0.87	0.87	0.87	0.87	0.87	0.87	0.87	0.87
Effective no. of alleles per locus	0.043	0.043	0.043	0.043	0.043	0.043	0.42	0.42	0.42	0.42	0.42	0.42	0.42	0.42	0.42
Mean observed heterozygosity (h_0)	0.083	0.083	0.083	0.083	0.083	0.083	0.020	0.020	0.020	0.020	0.020	0.020	0.020	0.020	0.020
Expected heterozygosity (h_e)	0.0416	0.0416	0.0416	0.0416	0.0416	0.0416	0.020	0.020	0.020	0.020	0.020	0.020	0.020	0.020	0.020
F-statistics	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1

Table 2. Contd.

Parameters	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
Total no. of loci	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24
Total no. of alleles	21	21	21	21	21	21	21	21	21	24	24	24	24	24	24
Proportion of polymorphic loci	0.020	0.020	0.020	0.020	0.020	0.020	0.020	0.020	0.020	0.187	0.187	0.187	0.187	0.187	0.187
Polymorphic index	0.020	0.020	0.020	0.020	0.020	0.020	0.020	0.020	0.020	0.062	0.062	0.062	0.062	0.062	0.062
Average no. of alleles per locus	0.87	0.87	0.87	0.87	0.87	0.87	0.87	0.87	0.87	1.00	1.00	1.00	1.00	1.00	1.00
Effective no. of alleles per locus	0.42	0.42	0.42	0.42	0.42	0.42	0.42	0.42	0.42	0.044	0.044	0.044	0.044	0.044	0.044
Mean observed heterozygosity (h_0)	0.020	0.020	0.020	0.020	0.020	0.020	0.020	0.020	0.020	0.187	0.187	0.187	0.187	0.187	0.187
Expected heterozygosity (h_e)	0.020	0.020	0.020	0.020	0.020	0.020	0.020	0.020	0.020	0.062	0.062	0.062	0.062	0.062	0.062
F- statistics	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1

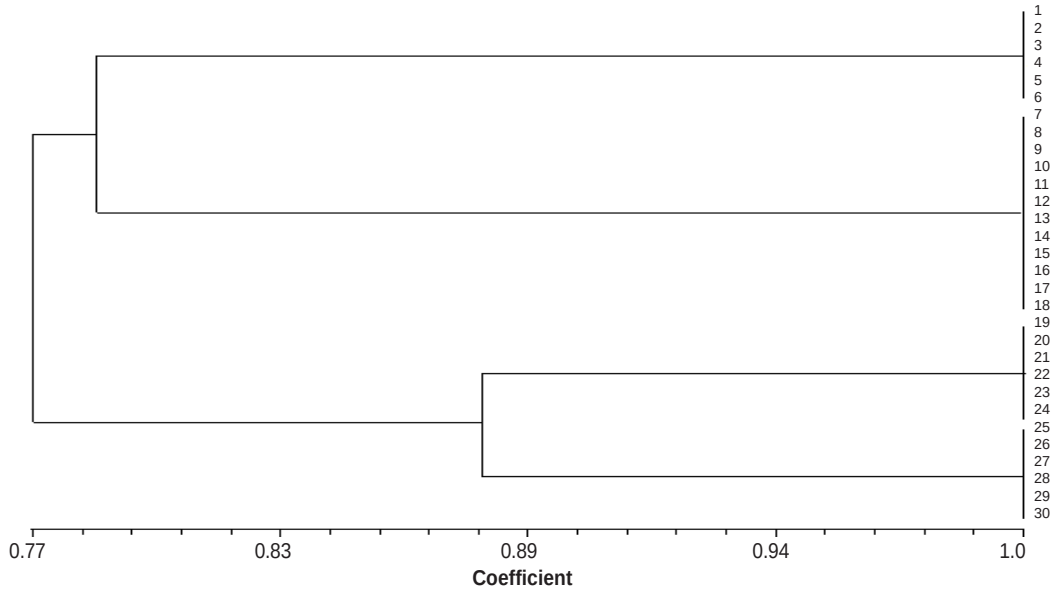


Fig. 3: Dendrogram showing genetic relatedness among 30 genotypes of *Woodfordia fruticosa*

thirty genotypes (Table 3). Only in five loci of the total twenty four studied show polymorphism that was not sufficient to characterize the individual genotypes.

Table 3. Summary of isozyme analysis obtained from thirty genotypes of *W. fruticosa*

Total number of enzyme studied	9
Total number of isozyme bands	29
Polymorphic isozyme bands	10
RM value range	0.03-0.95
Percent polymorphism	34.48 %

Genetic analysis based on isozyme analysis of thirty genotypes from the ten sites of Himachal Pradesh indicates low genetic variability of *W. fruticosa* germplasm. To the best of our knowledge this is the first report of isozyme polymorphism in *W. fruticosa*.

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