

Analysis of Genetic Diversity in *Poeciloneuron pauciflorum* Bedd.—An Endemic Tree Species from the Western Ghats of India

Padmesh Pandaram Pillai^{1*}, Jayakumari Sanakan Sajan¹, Kuttapetty Manikantan Menon¹, Achuthan Sudarshanan Hemanthakumar¹, Alagramam Govindasamy Pandurangan¹, Peringatulli Narayanan Krishnan¹, Soorimuthu Seeni²

¹Tropical Botanic Garden and Research Institute, Thiruvananthapuram, India; ²Sathyabama University, Chennai, India.
Email: *padmeshpad@rediffmail.com, padmeshpillai@gmail.com

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ABSTRACT

Poeciloneuron pauciflorum is a narrow endemic having highly restricted distribution in the southern-western Ghats region of India. 18 accessions of *P. pauciflorum* collected from four different locations were analyzed for genetic variation using 20 random primers. Out of 141 amplicons generated, 130 of them were polymorphic (92.20% polymorphism). Contrary to the general concept of low genetic variation associated with endemic plant species, *P. pauciflorum* exhibits high genetic diversity as the similarity index value based on Nei & Li's similarity coefficient ranges from 0.36 to 0.95 with mean GS = 0.72 and Shannon's information measure (0.43). Cluster analysis showed grouping of all accessions from the State of Tamil Nadu into two major clusters with few outliers while those from the State of Kerala also clustered with them. Accessions from Kalliankadu forest segment harbors maximum diversity as indicated by various genetic diversity indices like *h*, *I*, *Ht*, *Hs*, *Gst*, *PL* and hence this site is recommended for in situ conservation of this narrow endemic. The main factors responsible for the high level of genetic diversity among accessions are probably related to the reproductive isolation and ecological breadth. The strong genetic variability among accessions indicates that the management for the conservation of the genetic diversity in *P. pauciflorum* should aim to preserve every accession. The present study assumes significance as it negotiates endemism and genetic variation in tree species, a global phenomenon having wide implications in species diversity and conservation.

Keywords: RAPD, Neoendemism, Xanthones

1. Introduction

Poeciloneuron pauciflorum Bedd.—(Clusiaceae) is a critically endangered tree species endemic to India having sparse distribution in the Southern Western Ghats of Kerala and Tamil Nadu States [1]. It is a medium sized tree growing up to 15 m tall and 2 m in girth, distributed in the evergreen forest on the hills, particularly along riverbanks or watercourse between 600 to 1500 MSL altitude. The species is seen in KMTR (Kalakadu Mudanthurai Tiger Reserve) forest segment of Tamil Nadu. Recently 12 new additional sites grouped under three major regions viz. Mahendragiri, Chambakuchi and Kallar comprising the Kanyakumari Wildlife Sanctuary of Tamil Nadu [2] and from Chemungi-Attayar forest region of Kerala [3] has been reported. The species yields economically important timber with the wood reddish in

colour, hard and heavy. The genus *Poeciloneuron* with the species *P. indicum* was initially considered as monotypic by Beddome [4] under the family Tenstromeiaceae and later in 1871 added *P. pauciflorum* as the second species. However, the genus with just two species finds itself in taxonomic reassessments based on morphological, wood anatomical and palynological data [5] as the extent of variation at the interspecific level is estimated to be high.

Like few other endemics of the Western Ghats, *P. pauciflorum* shows large amount of variation in plant morphology and is threatened by habitat loss. The family Clusiaceae is known for high amount of xanthones [6,7] and *P. pauciflorum* is no exception as it is a rich source of xanthones like (1,6-dihydroxy-7-methoxyxanthone and 1,6-dihydroxy-7-methoxyxanthone 6-*O*- β -d-glucoside) in addition to 12 others, such as 1,5-dihydroxy-, 1,5-dihy-

droxy-3-methoxy-, 1,7-dihydroxy-, 1-hydroxy-7-methoxy-, 2-methoxy-, 4-methoxy-, 1,4,5-trihydroxy-, 1,3,5-trihydroxy-, 1,3,6-trihydroxy-7-methoxy-, 1,3,7-trihydroxy-, 3-hydroxy-2-methoxyxanthone and (–)-epicatechin [8].

These xanthenes show various bioactivities like antimalarial [9], effective against anti-methicillin-resistant *Staphylococcus aureus* [10-12], tumor-promoting inhibition [13], selective cyclooxygenase-2 inhibition [14] and inhibitory effects on PAF-induced hypotension [15]. Besides, they are used for the treatment of mental disorder, infectious diseases and for exorcism activities by the Kani tribals of South India [16]. According to IUCN conservation assessment the plant species is red listed as endemic and critically endangered (*Poeciloneuron pauciflorum*. In: IUCN 2010. IUCN Red List of Threatened Species. Version 2010.4. <www.iucnredlist.org> as on 09 May 2011). Few fragmented populations distributed in a small geographical region entails an urgent reassessment of the threat category of this species for which we have employed RAPD as tool for estimation of genetic variation in samples collected from few locations across the region. RAPD as a cost effective DNA marker system has been successfully employed to detect varia-

tion at the inter-specific [17-19], intraspecific [20], intervarietal [21], intergeneric genome analysis [22] and at the cultivar levels [23].

The present study assumes significance as it negotiates endemism and genetic variation in tree species, a global phenomenon having wide implications in species diversity and conservation.

2. Materials and Methods

2.1. Collection of Plant Materials

Total of 18 accessions of *Poeciloneuron pauciflorum* were collected from parts of Tamil Nadu and Kerala. As the tree species is highly endemic, the distance between collection sites (inter-population) is approximately 20 km in Tamil Nadu and within each site (intra-population) accessions were collected at a distance of 0.2 km to 0.5 km. The distance between Kerala and Tamil Nadu forest segment is about 20 km. Populations of *P. pauciflorum* were located in four locations of Tamil Nadu and Kerala viz. Inchikuzhi, Veliyar and Kalliankadu in Kalakkadu-Mundanthurai Tiger Reserve (Tamil Nadu) and Attayar in Peppara Wild Life Sanctuary (Kerala) (**Figure 1**).

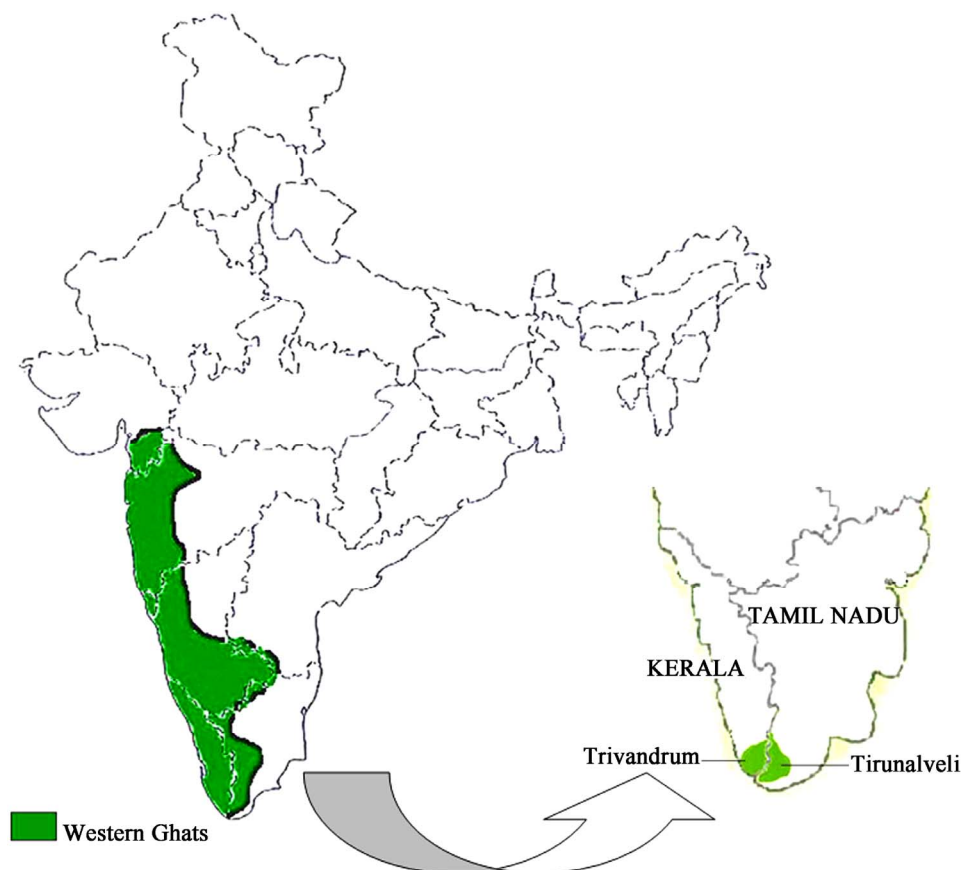


Figure 1. Map showing the Western Ghats and the distribution of *Poeciloneuron pauciflorum* (insight).

2.2. Genomic DNA Isolation and RAPD

Total genomic DNA from the young leaves was isolated following the modified Murray and Thompson [24] method using CTAB. After ethanol precipitation DNA was resuspended in 100 μ l of 1xTE buffer (pH 8.0). The DNA was quantified spectrophotometrically by taking the absorbance at 260 nm using Biophotometer (Eppendorf, Hamburg). RAPD assay was carried out in 25 μ l reaction mixture containing 0.5 μ l dNTPs (0.2 mM), 2.5 μ l 10X polymerase buffer (10 mM Tris-HCl, 1.5 mM MgCl₂, 50 mM KCl, 0.1% Triton X-100), 0.5 μ l Taq DNA polymerase (1.0 U/ μ l) (Finnzymes, Helsinki, Finland), 1 μ l primers (15 pmol) from Kit "C", Kit "D" and Kit "S" (Biogene, USA) and 50 ng of genomic DNA. The final volume was made up to 25 μ l by adding milli Q water. The amplification was performed in a thermal cycler (Eppendorf-ESP-S, Hamburg) with oil free operation. After the initial cycle of 2 min at 94°C, 2 min at 36°C and 2 min at 72°C, 38 cycles of 1 min at 94°C, 1 min at 36°C and 2 min at 72°C were performed. The last cycle was followed by 7 min extension at 72°C. Reaction mixture wherein template DNA replaced by distilled water was used as negative control. Amplified products were resolved in 1.4% agarose gel (1x TBE) followed by EtBr staining.

2.3. Genetic Data Analysis

Amplification with each random primer (45 primers) was repeated 3 times and those primers that produced reproducible and consistent bands (20 primers) were selected for data generation. Reproducible RAPD products were scored against the presence or absence of a fragment and denoted as "+" or "-", respectively. Dice coefficient of similarity defined as $2a/(2a + u)$, where "a" is the number of positive matches and "u" is the number of non matches was computed using the WINDIST software. The scored binary matrix was analyzed for the construction of phenogram and determination of confidence limits by bootstrap analysis using the WINBOOT software [26]. The genetic variation was analyzed for various parameters. The genotype and allelic frequency data were used to compute the genetic diversity indices *i.e.* observed number of alleles (na), expected number of alleles (ne), Shannon index of genetic diversity (I), Nei's gene diversity (h) at the population level, (Ht) heterozygosity at the polymorphic loci, average heterozygosity (Hs) and degree of genetic differentiation (Gst), using the statistical package *POPGENE* 1.3 [27]. The populations from which the samples taken for the present analysis were assumed to be in Hardy-Weinberg equilibrium implying that the population is at random mating. Based on the

above assumption, the bands were scored and estimation of heterozygosity (Ht) was done according to the formula: $Ht = 1 - \sum p_i^2$ where p_i is the frequency of the i^{th} allele in the population.

3. Results

3.1. RAPD Polymorphism

The 20 random primers used for the estimation of intraspecific variation in 18 samples of *P. pauciflorum* (Tables 1 and 2) provide interesting insights into the prevailing

Table 1. List of *Poeciloneuron pauciflorum* samples with details of collection site and location coordinate.

SI No.	Sample ID	Location	Latitude/Longitude
1	Pp 1	Near Veliyar	8°36.651"N 77°15.564"E
2	Pp 2	Near Veliyar	8°36.682"N 77°15.531"E
3	Pp 3	Near Veliyar	8°36.642"N 77°15.574"E
4	Pp 4	Near Veliyar	8°36.694"N 77°15.521"E
5	Pp 5	Near Veliyar	8°36.738"N 77°15.794"E
6	Pp 6	Near Veliyar	8°36.721"N 77°15.564"E
7	Pp 7	Near Veliyar	8°36.788"N 77°15.720"E
8	Pp 8	Kalliankadu	8°37.482"N 77°16.432"E
9	Pp 9	Kalliankadu	8°37.456"N 77°16.414"E
10	Pp 10	Kalliankadu	8°37.489"N 77°16.112"E
11	Pp 11	Kalliankadu	8°37.542"N 77°16.342"E
12	Pp 12	Inchikuzhi	8°37.484"N 77°18.094"E
13	Pp 13	Inchikuzhi	8°37.454"N 77°18.014"E
14	Pp 14	Inchikuzhi	8°37.521"N 77°18.014"E
15	Pp 15	Inchikuzhi	8°37.621"N 77°18.421"E
16	Pp 16	Inchikuzhi	8°37.458"N 77°18.261"E
17	Pp 17	Attayar	8°41.117"N 77°11.369"E
18	Pp 18	Attayar	8°41.186"N 77°11.314"E

Table 2. List of primers and their sequence used for RAPD analysis of *Poeciloneuron pauciflorum*.

Sl No.	Primers	Primer sequence 5'→3'	No. of bands	No. of polymorphic bands
1	S61	TTCGAGCCAG	11	11
2	S62	GTGAGGCGTC	6	4
3	S63	GGGGTCTTT	9	9
4	S64	CCGCATCTAC	7	7
5	S65	GATGACCGCC	5	3
6	S66	GAACGACTC	9	8
7	S68	TGGACCGTG	5	5
8	S69	CTCACCGTG	8	6
9	S70	TGTCTGGGTG	6	6
10	S71	AAAGCTGCGG	9	7
11	C73	AAGCCTCGTC	8	8
12	C74	TGCGTGCTTG	9	8
13	C75	GACGGATCAG	9	9
14	C76	CACACTCCAG	6	5
15	S-80	ACTTCGCCAC	6	6
16	D-08	GTGTGCCCA	6	6
17	D-10	GGTCTACACC	5	5
18	D-13	GGGGTGACGA	5	5
19	D-17	TTCCACGG	6	6
20	D-18	GAGAGCCAAC	6	6
Total No. of bands			141	130
Mean per primer			7.05	6.50

genetic variability in this narrow endemic tree. Out of 141 products generated, 130 were polymorphic (92.20%). On

an average, the primers generated 7.05 amplicons and 6.50 polymorphism per primer. The number of products generated by these arbitrary 10-mer primers was found to range from 5 to 11 with primer S61 giving the maximum (11) and primer S65, D10 and D13 giving the minimum (5) number of amplicons. While majority of the primers produced 100% polymorphism, there was not a single primer that resulted in complete monomorphism. The similarity matrix developed using the WINDIST software showed similarity index ranging from 0.36 to 0.95 with mean value of 0.72 (Table 3). Nei's gene diversity at population level (h), Shannon index (I), expected number of alleles (ne) were calculated to estimate genetic variation. The accessions included in this study showed relatively high level of genetic diversity, i.e. h = 0.3 and I = 0.43. The mean genetic diversity based on Nei's statistics [28] also supports the other data. The mean value of heterozygosity (Ht) observed in the various accessions of *P. pauciflorum* was found to be 0.45. The mean value of average heterozygosity value was 0.30. The heterozygosity values and degree of genetic differentiation (Gst) is shown in Tables 4(a)-(b). The other diversity measures as indicated in Tables 4(a)-(b) also revealed more diversity at the inter and intra population levels. The gene flow (Nm) among all accessions is 0.98, calculated on the assumption that the accession under study follows the inland model [29] which predicts a simple relationship between the numbers of migrants an accession receives per generation.

$F_{st} = 1/4 (Nm+1)$ from which Nm was derived as $Nm = (1 - F_{st})/4F_{st}$. The Gst value obtained from the POPGENE analysis was substituted for Fst and derived the rate of gene flow [30].

Table 3. Similarity matrix of accessions of *Poeciloneuron pauciflorum* analyzed using dice's coefficient.

1.00																			
0.91	1.00																		
0.89	0.94	1.00																	
0.87	0.92	0.92	1.00																
0.91	0.93	0.96	0.90	1.00															
0.90	0.89	0.93	0.89	0.95	1.00														
0.80	0.81	0.83	0.78	0.81	0.77	1.00													
0.92	0.86	0.85	0.83	0.88	0.88	0.76	1.00												
0.55	0.55	0.53	0.58	0.54	0.55	0.54	0.55	1.00											
0.83	0.79	0.80	0.79	0.82	0.81	0.71	0.85	0.55	1.00										
0.82	0.83	0.80	0.77	0.82	0.79	0.78	0.84	0.56	0.82	1.00									
0.70	0.70	0.69	0.66	0.71	0.71	0.61	0.72	0.40	0.69	0.72	1.00								
0.68	0.68	0.68	0.65	0.70	0.68	0.63	0.70	0.38	0.67	0.72	0.94	1.00							
0.62	0.63	0.63	0.58	0.64	0.62	0.60	0.62	0.36	0.62	0.63	0.80	0.79	1.00						
0.65	0.68	0.68	0.65	0.70	0.69	0.62	0.68	0.41	0.67	0.67	0.90	0.88	0.84	1.00					
0.53	0.56	0.58	0.56	0.59	0.55	0.46	0.56	0.46	0.55	0.57	0.72	0.71	0.61	0.75	1.00				
0.65	0.65	0.68	0.63	0.68	0.66	0.60	0.63	0.44	0.63	0.62	0.76	0.75	0.65	0.72	0.78	1.00			
0.62	0.65	0.65	0.63	0.65	0.63	0.58	0.59	0.45	0.63	0.61	0.72	0.70	0.67	0.70	0.71	0.85	1.00		
P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12	P13	P14	P15	P16	P17	P18		

Table 4. (a) Mean genetic Diversity of 18 accessions of *Poeciloneuron pauciflorum* based on Nei's (1987) statistics; (b) Multi-population analysis showing mean value of Genetic diversity indices.

(a)								
Mean	Ht	Hs	Gst	Nm	na	ne	h	I
	0.45	0.29	0.33	0.98	1.73	1.54	0.30	0.43
(b)								
Population	h	I	Nm	Gst	Ht	Hs	PL	% PL
Veliyar	0.33	0.49	3.13	0.14	0.33	0.28	18	90.00
Kalliankadu	0.43	0.61	1.23	0.29	0.43	0.30	19	95.00
Inchikuzhi	0.34	0.50	1.91	0.21	0.34	0.27	18	90.00
Attayar	0.26	0.39	2.2	0.04	0.26	0.25	15	75.00

Ht-heterozygosity at the polymorphic loci, Hs-average heterozygosity, Gst-degree of genetic differentiation, na-observed number of alleles, ne-expected number of alleles, h-Nei's gene diversity at population level, I-Shannon index of genetic diversity, PL-Polymorphic Loci, %PL-percentage Polymorphic Loci.

3.2. Cluster Analysis

The samples of *Poeciloneuron* clustered broadly under two major groups with sub-clusters within each group. All the samples except one from Veliyar grouped and constituted cluster I at a moderate confidence interval limit of 78.2% while samples from Inchikuzhi formed cluster II along with the two samples of Attayar at 92.7% confidence limit (Figure 2). The grouping of Inchikuzhi and Attayar samples showed high robustness as they were supported at 92.7% and 94.6% confidence interval limits, respectively. All the four samples from Kalliankadu were found to form outliers. Three samples were placed characteristically between the two clusters and one outside the clusters (P-9).

4. Discussion

It is well known that the nature and distribution of genetic variability within and among natural populations of species constitutes its genetic structure which in turn is affected by edaphic/demographic factors [31,32] and evolutionary processes [33]. The characteristic genetic structure of a population reflects the interactions of various factors like long-term evolutionary history of the species (shifts in the distribution, habitat distribution, habitat fragmentation and population isolation), genetic drift, mating system, gene flow and selection [34]. However, attributes like the progenitor, probability of common origin, kin structure and inbreeding within populations all have significant effects on genetic differentiation among populations [35].

Above all, the extent of geographical distribution of the species is yet another single major factor that determines its variability [36-39]. In general, it is accepted

that long term conservation strategies for plant species demands better understanding of the ecological and genetic variables prevailing in that particular niche over a period of time. Therefore, genetic variability analysis has significant implication in designing conservation strategies for species which are endemic and endangered in status.

Genetic Variation in Narrow Endemics

The RAPD data generated out of 20 random primers for *P. pauciflorum* collected from forest segments that span across two southern most States of India provided interesting insights into the existing diversity in this highly endemic tree species. The coefficient of genetic similarity ranging from 0.36 to 0.95 with mean value of 0.72 suggests high variability in the species despite its endemic status. The grouping of samples apparently reflects characteristic fragmentation of the population with all samples from Tamil Nadu side of the Western Ghats forming two independent clusters at the two ends of the phenogram with few samples forming outliers in between them. Those samples from the Kerala side of the Western Ghats though less in number tend to form grouping with the samples from Inchikuzhi. Multi-population analysis to determine intra and inter population diversity shows that most of the diversity indices (Ht, Hs, Gst, Nm, h, I, PL) are high for samples from Kalliankadu followed by Inchikuzhi, Veliyar and Attayar. It is generally accepted that endemics tend to have lower levels of genetic variation than their wide spread congeners [40], a condition often attributed to reproductive isolation and geographical fragmentation. Endemics are generally considered to be inbreeders as their progenitors as founders were assumed to have benefited from self compatibility. It is

presumed that at a later stage of divergence they became reproductively isolated from the parent population and resorted to inbreeding as the only possible mode of reproduction. There are many schools of thought that endorse low levels of genetic variation in endemic plant species based largely on geographical distribution pattern and reproductive isolation. In contrast to our expectation of low genetic diversity, *P. pauciflorum* shows high genetic diversity and the mean genetic diversity values. Nei's statistics also support this level of genetic diversity. High genetic differentiation ($G_{st} = 0.33$) and low gene flow ($N_m = 0.98$) indicates the possible threat to the taxa in spite of its present diverse nature showing strong tendency to genetic drift and inbreeding depression unless measures are taken for introducing sufficient number of migrants into the population. Despite its endemic status and restricted geographical range *P. pauciflorum* shows

high level of genetic diversity and the two major genetic consequences of small population size for long periods of time are high levels of genetic drift and inbreeding [41, 42]. Inbreeding species maintain relatively more of their genetic diversity among populations rather than within populations than do out crossers [43]. Some of the factors such as recent speciation from a more wide-spread species, recent changes in distribution or habitat, breeding system, somatic mutations, multiple founder events, tropical forest fragmentation leading to decreased gene flow, increased inbreeding producing a high differentiation among remnant populations, invoked to explain high genetic diversity of the narrow endemic species like, *Delphinium viridescens* [44], *Symplocos laurina*, *Eurya nitada* [45], *Primula interjacens* [46], *Abeliophyllum distichum* [47], and *Antirhea aromatica* [48] may also explain the observed endemism of *P. pauciflorum*.

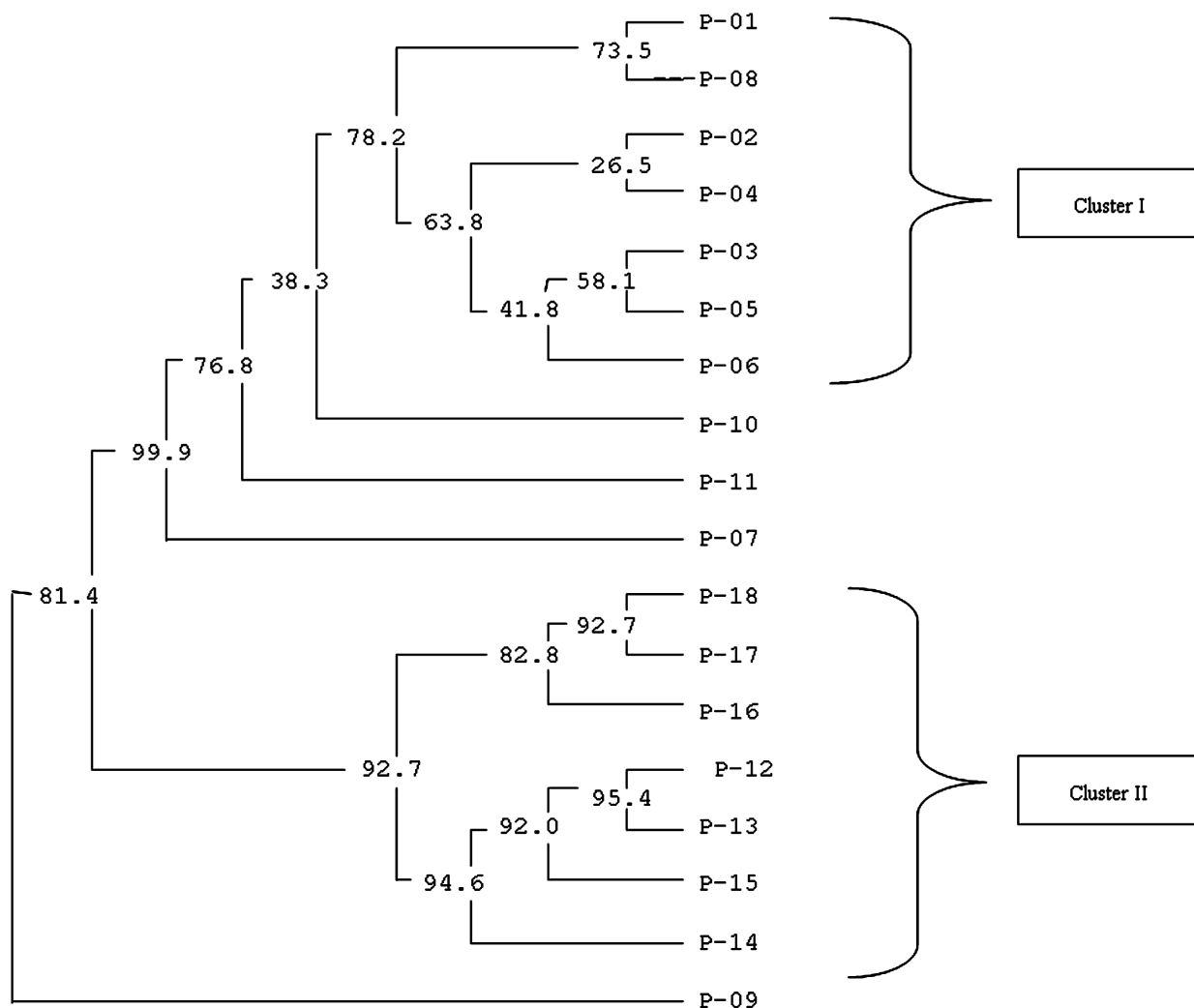


Figure 2. Phenogram based on UPGMA analysis of *Poeciloneuron pauciflorum* accessions. Numbers at the fork indicate bootstrap values.

Accordingly, narrow endemics are mostly referred as products of speciation that differ in their origin and status of rarity only in certain aspects that in turn are controlled by such factors as geographic area, ecological breadth, reproductive isolation and most importantly the amount of genetic variation inherent in the taxa [49]. In fact, the first three factors defines the genetic structure of the taxa to a considerable extent that perpetuation and establishment relies mostly on the extent of genetic variation.

The niche variation hypothesis which refers to species adaptation to narrow ecological conditions also explains the significant role of heterozygosity in genetic diversity and endemism and thereby the genesis of narrow endemics. Thereby other major factor besides selection pressure that determine heterozygosity is effective population size which in turn could be the product of (a) smaller total population size compared to those species having cosmopolitan distribution (b) genetic bottle neck at the time of origin of the species which in due course with rapid speciation may produce a certain low level of genetic variation, a condition that could be transitory (c) inbreeding/selfing may reduce effective population size and hence heterozygosity and (d) population fragmentation due to geographical or reproductive isolation. We assume that some or all of these factors must have contributed to the peculiar distribution and current status of endemism seen in *P. pauciflorum*. The populations from which the samples taken for the present analysis were assumed to be in Hardy-Weinberg equilibrium implying that the population is at random mating. Though direct measure of heterozygosity was not calculated in the present study the indirect measures reflected through various diversity indices clearly demonstrates high variability in the taxa that is not otherwise expected in this category of narrow endemics in general and in a taxon like *P. pauciflorum* in particular where the natural mode of reproduction is through selfing as indicated by the floral structure (bi-sexual flowers, stigma acute wherein both stigma and anther are placed in the same level suggestive of inbreeding). Therefore the general concept of associating low genetic variation in natural populations of plant species with endemism entails further extrapolation to negotiate the prevalence of unseemingly high diversity in this species. However such situations of associating endemism with high genetic diversity has been earlier reported in vascular plants like rare ferns *Adenophorus periens* [50], endemic *Agave victoriae-reginae* [51], narrow endemic tree species *Antirhea aromatica*, *Antirrhinum charidemi* and *A. valentinum* [52], narrow endemic conifer *Cupressus macrocarpa*, which has highly restricted distribution shows fairly good variability. All seven species of the Californian endemic grass genus

Orcuttia that have as much genetic variability as any other widely distributed members of Graminae [53]. Among these, *Antirhea aromatica* has exceptionally high genetic diversity and variability, despite its low population density [48]. The phenomenon is likely to be associated with the reproductive system as in certain members of the family Rubiaceae, there is pre-zygotic incompatibility crossing system which reduces inbreeding and thereby loss of genetic diversity. We also propose similar or associated mechanism underlying the reproductive biology of *P. pauciflorum* that reduces the possibility of selfing and maintains the observed levels of genetic diversity in this narrow endemic species.

In conclusion our results show that this narrow endemic tree species has generated a systematic process of genetic isolation yet capable of maintaining genetically viable populations in few patches of the Western Ghats region. Further the data is suggestive that *in situ* conservation may be adopted for those populations in Kallankadu as they are shown to harbor maximum amount of variability possibly present in the species. We also recommend an *ex situ* conservation strategy through conventional or non-conventional approach involving simple micropropagation to increase the number of plantlets and thereby delist the species from its current endemic status.

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Abbreviations: RAPD: Random Amplified Polymorphic DNA; GS: Genetic Similarity Coefficient.