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Orginal Article

Genetic characterization and biochemical analysis in the populations of *P. corylifolia* L. from Western Maharashtra.

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Abstract

Psoralea corylifolia as one of the medicinal herbs is used in the treatment of psoriasis, leukoderma and in many medicinal formulations. The genetic profiles and secondary metabolite content of 9 populations comprising of 72 accessions of *P. corylifolia* collected from different locations in western Maharashtra have been analyzed. Out of 40 RAPD primers, 21 primers were found polymorphic which gave 226 polymorphic loci with 99.56% polymorphism. The PIC value on an average was 0.228 indicating better discriminating power of RAPD's in the present study. The cluster analysis and AMOVA showed more genetic variation within populations (76.47%) than between populations (23.53%). Methanolic extracts of dried seeds were analyzed for the total flavonoid and phenolic content while the acid hydrolysed ethanolic extracts were used for the estimation of isoflavonoids (daidzein and genistein) and coumarin (psoralen) content. Variation in the metabolite content was analyzed by one-way ANOVA and cluster analysis. An attempt has been made for the first to correlate the genetic and biochemical data in the populations of *P. corylifolia*.

Keywords: *AMOVA, daidzein, genistein, gene flow, RAPD.*

INTRODUCTION

Psoralea corylifolia L. [Indian bread root] commonly known as Babachi is reported as a rare and endangered, herbaceous medicinal plant distributed in the tropical and subtropical regions of the world [Kaufman et al. 1997]. The plant is well recognized in the Chinese and Indian folkloric medicine as a laxative, aphrodisiac, anthelminthic, diuretic and diaphoretic in febrile condition. The seeds have been widely recommended for curing asthma, leprosy, psoriasis, leukoderma and inflammatory skin diseases [Anonymous, 1988]. The ethanolic extracts of this plant have also been reported to act as inhibitors of DNA polymerase and topoisomerase [Sun et al. 1998]. The seed extracts have been reported to have antiplatelet [Tsai et al. 1996], antitumor, immunomodulatory properties [Latha et al. 2000] *in vitro* antimicrobial [Katsura et al. 2001] and antioxidant activities [Jiangning et al. 2005] have also been reported. The isoflavones daidzein and genistein have been reported to reduce the chances of breast and colon cancer. [Adlercreutz 1999]. *In vitro* production of these nutraceuticals through hairy roots culture has been reported. [Shinde et al. 2009]. No work has so far been reported regarding the genetic diversity studies between and within the populations of *P. corylifolia* and its correlation with the biochemical diversity. Genetic diversity studies will aid in devising pragmatic conservation strategies and in formulating core collections for plant species. The use of molecular markers is preferred over conventional morphological and biochemical markers for genetic diversity studies, since they are not influenced by environmental effects and the developmental stages of the

experimental material. Chemical diversity needs to be explored in relation to the efficient assessment of genetic diversity as it is an essential feature for conservation and utilization of resources.

The methods of multilocus analysis based on PCR with random primers are reliable for genetic diversity studies. Thus RAPD's were used for the diversity studies as they have number of advantages like examination of the genome completely, identification of polymorphism in number of loci and analysis of noncoding DNA sequences. An attempt is made to correlate the genetic data obtained from 40 RAPD markers along with the biochemical data. Such studies would help to establish the power of DNA fingerprinting and also explore metabolites for pharmacological studies and eventually for intellectual property rights. To the best of our knowledge this is the first report with respect to the studies on genetic diversity and its correlation with the biochemical diversity in *Psoralea corylifolia*.

MATERIAL & METHODS

Sample collection

9 populations comprising of 72 accessions of *P. corylifolia* were sampled from the agricultural fields and barren lands distributed in different regions of Maharashtra: Pune [P], Baramati [B], Malshiras [M], Kolhapur [Kol], Kopargoan [KO], Shirur [Sh], Shrigonda [Shri], Ahmednagar [N] and Nashik [Nas] [Table 1]. Each natural population was sampled as a bulk of mature seeds randomly collected from at least 10 plants that were separated from each other by 20-30 m. The set of seeds collected from every location is considered as an accession [Konate et al. 2007].

DNA extraction and PCR amplification

The seeds of *P. corylifolia* were scarified by immersing in pure sulphuric acid [Merck, India] for 60 mins to remove the pericarp and were then washed under running tap water till all the acid has been removed. After washing and imbibing them in water for 24 h, seeds were placed in pots containing soil and were grown in the Botanic garden of Department of Botany, University of Pune.

Leaf tissue, sampled population wise was used for DNA extraction. The DNA was extracted from 10 individuals of each locality. The genomic DNA was extracted using the protocol given by Doyle and Doyle [Doyle 1991]. The concentration of DNA was determined spectrophotometrically [Nanodrop] at 260nm and also by agarose gel electrophoresis.

PCR amplification

The polymerase chain reaction [PCR] conditions were finalized by varying the units of Taq polymerase, quantity of DNA, concentration of primers and the annealing temperature. 20µl PCR amplification mixtures contained 1X Taq buffer supplemented with 15mM of MgCl2 [Bangalore Genei, India], 2.5mM of dNTP's, 2.5U of the enzyme Taq DNA polymerase [Fermentas, USA], 12 pmoles of primers [Operon technologies, USA], 20ng of template DNA and sterile distilled water to make up the final volume. The amplification was performed in the thermal cycler [Corbett research, Australia] according to the following program: initial denaturation at95 ˚C for 3 min followed by 45 cycles of 15 sec at 950C [denaturation], 15s at 35˚C [annealing] and 3 mins at 72˚C [extension]. A final extension of 5 min at 72˚C ensured full extension of all amplified products. At the end of the PCR, amplification products were separated by electrophoresis on 1.5% agarose gel. It was performed in 1X TAE [Tris-Acetic acid-EDTA] running buffer at 50v for 4 h and then the gels were stained with ethidium bromide. The results were visualized under ultraviolet [UV] transilluminator and the image was captured on alpha imager. Molecular weights were estimated by using ΦX 174 DNA/BsuRI [Hae III] markers [Fermentas, USA].

Data analysis for RAPD markers

Amplified fragments were scored as discrete variables '1' for presence and '0' for absence of homologous bands to create a binary matrix of RAPD phenotypes. The PIC [polymorphic information content] value was calculated by applying the formula given by PIC= 1- Σ[Pij]2, where Pij is the frequency of the ith pattern revealed by the jth primer summed across all patterns revealed by the primers [Botstein et. al, 1980]. The data was also analyzed to obtain between and within population diversity using POPGENE program [Yeh et al, 1997] The Shannon information index, Nei's genetic diversity and percentage polymorphism were estimated at within population level. Nei's method of intra-population genetic diversity [Nei and Li, 1979], states that two random alleles in a population can be distinguished with the genetic

marker. The Shannon index was calculated as Ho= -∑pilog2 pi where pi is the frequency of given RAPD fragment [Lewinton, 1972]. The level of genetic diversity between populations was assessed by calculating [H_T] total genetic diversity; [G_{ST}] coefficient of gene differentiation; genetic diversity within a population [HS], genetic diversity among populations [D_{ST}], and the level of gene flow [Nm] for different groups of populations to understand the genetic diversity patterns in natural populations. Nei's GST is the proportion of genetic diversity that dwells among populations. The values for GST range from zero to one, with low values indicating little variation between populations [Culley et al. 2002]. The pair-wise Nei's genetic distance [Nei, 1978] between populations were used to generate a dendrogram [UPGMA] by using Tree View [version 1.6.6]. Components of variance partitioned within and among populations were also estimated using AMOVA. Input data files for the AMOVA v. 1.55 program [Excoffier, et al. 1992] were generated by AMOVAPREP [Miller 1998]. The number of permutations for significance testing was set at 1000. AMOVA variance components were used as estimates of the genetic diversity partitioning within and among populations.

Phytochemical analysis:

Preparation of plant extracts

0.1 g of the pulverized material [seeds] was extracted overnight with 5 ml of methanol. The extracts were filtered through Whatmann no. 1 paper and the filtrate was used for estimation of total phenolic and total flavonoid content. The extraction for the estimation of isoflavonoids and furanocoumarin was carried out by preparing the acid hydrolyzed extracts. [Nyugen et al. 1997]. 0.1g of the plant material was acid hydrolysed [1:1 water and sulphuric acid] followed by sonication for 15 mins and boiling in water bath for 60 mins. The extracts were kept overnight and then filtered through Whatmann no. 1 paper and the filtrate was used for the estimation of isoflavonoids and furanocoumarin.

Determination of secondary metabolite content

Estimation of total phenolics content

The total phenolics content was estimated by modifying the method given by Singleton and Rossi, 1965. The estimation of total phenolics was carried out by introducing 0.1ml of the sample into 2.5ml of folin-ciocalteau reagent and 2ml of Na2CO3 followed by incubation for 30 mins. The absorbance was recorded at 765nm. The total phenolics content was expressed as tannic acid equivalent.

Extraction and estimation of total flavonoid content

The total flavonoid contain was estimated by the method given by Chang et al. 2002. The total flavonoid content was estimated by introducing 0.1 ml of the sample into 1.5ml of 95% methanol, 0.1ml of 10% aluminum chloride [Merck, India] and 0.1ml of 1M potassium acetate [Himedia, India] which was followed by the incubation of 30 mins. The absorbance was recorded at 415nm and the total flavonoid content was expressed as quercetin equivalent.

Extraction and estimation of Isoflavonoids

Isoflavonoids daidzein and genistein and furanocoumarin psoralen content were estimated spectrophotometrically [Nanodrop] at 250nm, 260nm and 240nm respectively. The total content of isoflavonoids was expressed as daidzein and genistein equivalent while the furanocoumarin content was expressed as psoralen equivalent.

Data analysis for secondary metabolite content:

The % dry weight data was ranged between the minimum and the maximum value. The midpoint of these values was considered as the centre of the hypothetical scale and the values which were more than the intermediate value were considered as present [1] for a particular metabolite while the values which were lesser than the intermediate value were considered as absent [0]. The binary matrix of the biochemical data was used to calculate the pair wise similarity for all analyzed populations using Biodiversity professional 2 software. The biochemical data was also analyzed statistically by performing one-way ANOVA using SPSS software [version 9].

RESULTS & DISCUSSION

Genetic diversity

Out of 40 RAPD primers, 22 primers showed significant polymorphism, clear and polymorphic RAPD profiles are shown [Figure 1]. The RAPD profile yielded a total of 225 polymorphic loci with 99.56% polymorphism. PIC values provide an estimate of the prejudiced power of the marker by taking into account not only the no. of alleles at the locus but also the relative frequencies of the alleles. The PIC values calculated ranged from [0.122-0.337] with an average of 0.228 [maximum value for a dominant marker is 0.5] showing better discriminating power of RAPD's in the investigated populations.

The results for within population genetic diversity were computed taking into consideration percentage polymorphic loci, Shannon's diversity index and Nei's genetic diversity [Table 2]. The percentage polymorphic loci was found to be higher in population from Shirur [57.96%] and Shrigonda [56.19%] while the percentage was found to be lower in populations from Pune [16.37%]. The percent polymorphic loci for the remaining populations [P2- P7] ranged from 40%-50%. Percent polymorphic loci visually display certain degree of polymorphism with simple calculations but it is only an estimated value in measurement of genetic diversity. Thus there is an uncertainty in calculating the degree of evenness in the band frequency and so other parameters like Shannon's diversity index and Nei's genetic diversity were also considered in assessing the genetic diversity [Qain and Ge, 2001]. Shannon's diversity index is based on the phenotype frequency of the bands while Nei's diversity is based on the Hardy-Weinberg's assumption and so they are more reliable. The results show that the Shannon's diversity index was higher in populations from Shrigonda [0.2660] and Shirur [0.2486] while the values were low for populations from Pune [0.0821]. Nei's genetic diversity was found to be higher in populations from Shrigonda [0.1742] and Shirur [0.1585] while it was lowest in populations from Pune [0.0535].

The total genetic diversity between populations of *P. corylifolia* was found to be 0.1507 and that within the populations was 0.1193.

Genetic differentiation

Nei's genetic differentiation Gst is defined as the proportion of genetic diversity that resides among populations. The value for Gst was 0.2083 [values for Gst ranges from 0-1] indicating little variation among populations. The estimation of gene flow depends on the no. of migrants per generation, higher the value of gene flow lower is the genetic differentiation among the populations. The level of gene flow [Nm] among the populations of *P. corylifolia* was found to be 1.90 [>1] which indicate that there was a certain frequency of gene flow. AMOVA analysis also proved less variation among populations than within populations of *P. corylifolia*. The results show about 23.53% variation among populations and 76.47% variation within populations. The above results indicate that less genetic differentiation among populations than within populations of *P. corylifolia*.

Cluster analysis

The Nei's genetic distance [D] and genetic identity [I] between populations ranged from 0.0185 to 0.0786 and 0.924 to 0.9816 respectively. The UPGMA dendrogram was constructed based on the matrix of Nei's genetic distances which grouped the 9 populations of *P. corylifolia* into two clusters [Fig 2]. The first cluster comprises of populations from barren lands Shirur [pop8] and Shrigonda [pop9]. The second cluster comprises of the remaining populations collected from the irrigated regions [Malshiras, Kolhapur, Kopargaon, Nagar, Baramati and Nashik] with the populations from Pune which were collected from the residential areas. The clustering pattern significantly indicates that all the populations from barren lands are grouped away from the populations of irrigated regions. The clustering pattern revealed that there is certain degree of genetic differentiation among populations of *P. corylifolia*. Thus the analyses of the genetic diversity by RAPD markers using different approaches [cluster analysis, Shannon's diversity measures, and Nei's diversity measures] revealed similar interpretations of the genetic structure of the populations of *P. corylifolia*.

Phytochemical analysis

The populations under study were analyzed for the presence of isoflavonoids [daidzein and genistein], coumarin [psoralen], total flavonoid and total phenolic content spectrophotometrically. Data analysis was carried by performing One-way ANOVA using SPSS software [version 9] and cluster analysis was performed using Biodiversity professional version 2. ANOVA was carried out by performing LSD and Duncan's test [Table 4].

The statistical analysis shows that the content of daidzein was found to be higher in populations from Shrigonda [6.58±1.01] and Shirur [6.51± 1.70] while the content of genistein was found to be higher in the populations from Kolhapur [6.60±1.03], Shrigonda [5.82±0.65] and Shirur [5.54±1.25] while the content of isoflavonoids daidzein and genistein was found to be lowest in the populations from Baramati [1.90±0.41] and [2.03±0.50] respectively. The coumarin content ranged from [3.33±0.33] in populations from Shrigonda to [1.86±0.25] in the populations from Baramati. The total flavonoid content was found to be higher in the populations from Shirur [6.01±0.38] while it lowest in the populations from Baramati [2.22±0.65]. The phenolic content was found to be higher in the

populations from Shrigonda [8.05±0.77] and Pune [8.00±0.62] while it was found to be lowest in the populations from Nashik [5.63±0.94] and Baramati [5.83±2.37]. Thus the results of the phytochemical analysis shows that all the analyzed content was found to be significantly higher in the populations from Shirur and Shrigonda while it was found to be lowest in the populations from Baramati and Nagar. The cluster analysis showed the formation of two major clusters on the basis of the biochemical data. The first cluster comprises of the two clusters of which the first cluster comprises of the populations from Pune, Shrigonda and Nashik while the second cluster consists of populations from Malshiras, Kopargaon and Kolhapur. The second major cluster comprises of the populations from Baramati, Nagar along with Shirur [Fig 3].

Genetic diversity is the sum of the genetic information carried by the organism and it plays an influential role in the species and community diversity and is the basis of population survival, development and evolution [Barrett and Kidwell, 1998]. Genetic diversity includes the distribution pattern of genetic variation [i.e. the genetic structure of the populations as well as the genetic variation][Hamrick and Loveless, 1989]. Thus evaluation of genetic diversity is significant for us to understand the adaptability of species, distribution of genetic resources and origin of species.

In the present study 22 primers yielded 226 polymorphic loci with 99.56% polymorphism indicating that there is a high level of genetic diversity among populations of *P. corylifolia*. Though *P. corylifolia* is a self-pollinated plant, chances of cross-pollination cannot be denied. RAPD being dominant marker in case of chances of cross-pollination the existing locus couldn't represent its homozygosis. Shannon's diversity index can thus overcome this defect as it estimates the genetic diversity by considering the occurrence and frequency of amplified product as the phenotype frequency of the locus. Also Nei's diversity which is another parameter considered in the estimation of genetic diversity reflects the evenness and richness of an allele in a population. The value obtained is proportional to the loci variation [Wang, 1996]. The present study indicate that the Shannon's diversity index is 0.2694 while the Nei's diversity index is 0.1565 showing significant genetic diversity in the populations of *P. corylifolia*.

The genetic structure of the population refers to the distribution pattern of genetic variation which is the combined effect of mutation, gene flow, natural selection and genetic drift. It also reflects the genetic differentiation within and between the populations [Liu and Zhao, 1999]. The co-efficient of genetic differentiation [Gst], the most commonly used index expressed by genetic variation between populations accounts for the proportion in the total variation among populations. The study indicates that the total diversity was found to be 0.1507, within population genetic diversity was 0.1193 while the co-efficient of genetic differentiation was found to be 0.2083. The AMOVA analysis showed 76.47% variation within populations and 23.53% variation among populations. The studies indicate that about 76.47% variation was obtained within populations, pointing out that though *P. corylifolia* is a self-pollinated plant some chances of cross-pollination occurs.

Gene flow, the genetic counterpart of dispersal is an important content for study of genetic structure. In plants gene flow is transferred by migrants of pollen, seeds, spores and other carriers. The gene flow by pollen spread has significant impact on the distribution of genetic variation both between and within populations. If gene flow Nm>1 then it shows that there is less genetic variation among the populations of *P. corylifolia*. The study showed that the gene flow among populations of *P. corylifolia* was 1.90 showing less genetic differentiation among populations of *P. corylifolia*. This could be the result that the populations were isolated in different patches collected from different regions. Thus gene flow was high because of the pollination by insect or seed dispersal within a small patch which led to less genetic variation.

Nei's genetic distance and genetic identities reflects the degree of hereditary differences among the populations of *P. corylifolia*. The present work shows 0.0185 to 0.0786 and 0.924 to 0.9816 values for Nei's genetic distance and genetic identities respectively. The cluster analysis for the genetic marker shows that the populations of *P. corylifolia* are grouped into two major clusters. The first cluster shows the populations from Shirur and Shrigonda grouped together while the second group consists of remaining 7 populations. The group I consists of all the populations of *P. corylifolia* from the barren lands while the group II comprises of the populations of *P. corylifolia* from irrigated land along with the populations from residential area [Pune]. The within populations diversity was found to be lowest in

samples from residential areas as compared to barren lands and farm lands due to restricted gene flow and small population size. The results show that the separation of the population of *P. corylifolia* is based on the landing pattern.

The correlation of the biochemical and genetic data in the populations of *P. corylifolia* highlighted that both the analysis are in accordance with each other. Except the populations from the barren lands [Shirur and Shrigonda] did not show any separate grouping but they were grouped within the two major cluster. This difference in the biochemical analysis could be due to the difference in the climatic conditions, pH of the soil and irrigation facilities which would have led to variation in the secondary metabolite content.

Very few studies regarding correlation of genetic and biochemical diversity in medicinal plants belonging to different families have been reported. Smelcerovic et al. [2006] reported the correlation of the secondary metabolites content with RAPD data among six Hypericum species from Siberia. The correlation between the distribution of withanolides and the genetic diversity by AFLP markers between the different accessions of Withania somnifera was reported by Dhar et al. [2006]. Kumar et al. [2009] reported poor correlation between the molecular and chemical diversity in Cymbopogon species by analyzing the molecular diversity by using SSR markers. Sharma et al. [2009] reported significant level of variation in the andrographolide content with RAPD data among the genotypes of Andrographis paniculata collected from various regions of Chhattisgarh. Analysis of Actonoium species by RAPD and biochemical ways showed well defined groups [Fico et al. 2003]. The patterns of relatedness observed in biochemical analysis appear to correspond with the genetic profiles generated by RAPD's of *P. corylifolia*, suggesting that there may be a genetic basis for the chemical profiles observed.

CONCLUSION

An attempt has been made to correlate the genetic and biochemical diversity among populations of *P. corylifolia*. RAPD and chemical analysis showed similarity in the grouping pattern with slight difference in the clustering. Effective seed dispersal by humans, animals and wind could have contributed to the gene flow between the populations and genetic differentiation among the populations of *P. corylifolia* from different regions of Maharashtra. The chemical variation in the medicinal plants is not only the result of genetic variability, but it also depends on the number of other factors. Thus better understanding of the biochemical and genetic variations in the populations of *P. corylifolia* will help in selection and conservation of elite genotypes for further analysis.

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Table 1. Location of populations of *P. corylifolia* from different regions of Maharashtra.

DNA extraction and PCR amplification:

Population	Regions	Latitude (N)	Longitude (E)	Land type
P1	Pune	18°31'	73°45'	Residential
P2	Nashik	20°01'	73°47'	Irrigated fields
P3	Baramati	18°11'	74°32'	Irrigated fields
P4	Kopargaon	19°55'	74°25'	Irrigated fields
P5	Nagar	19°05'	74°44'	Irrigated fields
P6	Malshiras	17°52'	74°54'	Irrigated fields
P7	Kolhapur	16°41'	74°13'	Irrigated fields
P8	Shirur	18°49'	74°22'	Barren land
P9	Shrigonda	18°49'	74°41'	Barren land

Table 2 Population genetic studies within populations of *Psoralea corylifolia* collected from different regions of Maharashtra

Population	Locality	% polymorphic loci	Effective no. of alleles	Nei's gene diversity	Shannon information index
P1	Pune	16.37	1.087	0.0535	0.0821
P2	Nashik	28.32	1.150	0.0934	0.1432
P3	Baramati	46.02	1.158	0.1605	0.1745
P4	Kopargaon	47.79	1.200	0.1249	0.1977
P5	Nagar	46.02	1.214	0.1341	0.2093
P6	Malshiras	45.58	1.171	0.1136	0.1838
P7	Kolhapur	50.88	1.175	0.1149	0.1884
P8	Shirur	57.96	1.256	0.1585	0.2485
P9	Shrigonda	56.19	1.294	0.1742	0.2660

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Table 3 Results of AMOVA analysis of 9 populations of *P. corylifolia*.

Source of variation	df	Sum of squares	Mean squares	Variance components	Total variance (%)	P-Value
Among populations	8	511.58	63.94	5.72	23.53	<0.001
Within populations	63	1172.51	18.61	18.611	76.47	<0.001

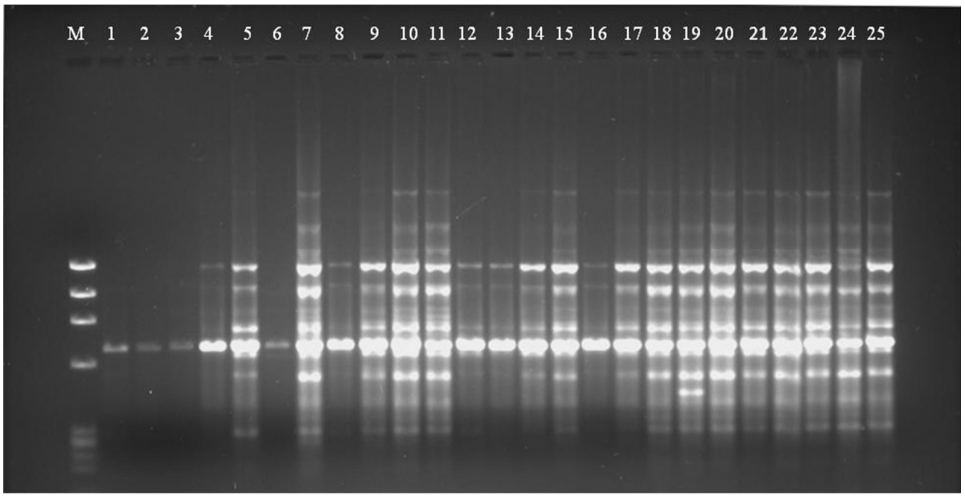

Fig1: RAPD profile obtained by primer OPA-20. Lane 1: Molecular marker Φ X , Lane 2: M5, Lane 3: M9, Lane 4: M10, Lane 5: M11, Lane 6: Kol 2, Lane 7: Kol7, Lane 8: Kol 9, Lane 9: Kol 10, Lane 10: Sh 1, Lane 11: Sh2, Lane 12: Sh3, Lane 13: Sh 4, Lane 14: Sh 5, Lane 15: Sh 6, Lane 16: Sh 7, Lane 17: Sh 8, Lane 18: Sh 9, Lane19: Sh 10, Lane 20: Shr 1, Lane 21: Shr 2, Lane 22: Shr 3, Lane 23: Shr 4, Lane 24: Shr5, Lane 25: Shr6.

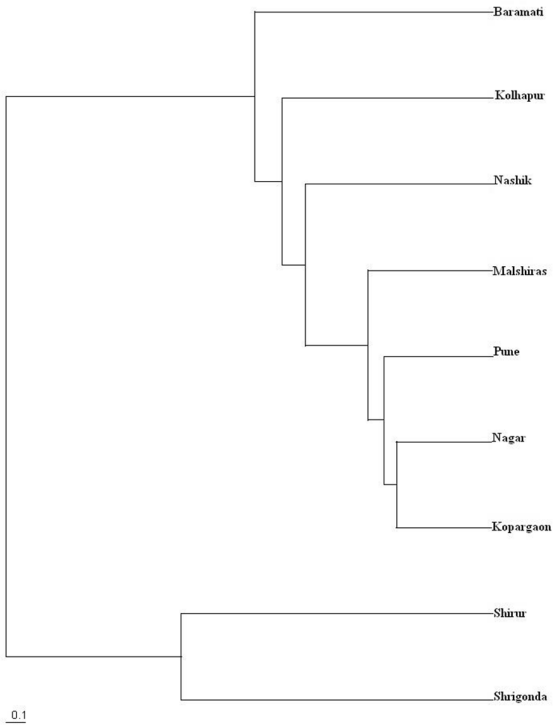

Fig2. Cluster analysis among 9 populations of *Psoralea corylifolia* based on Nei's genetic distances.

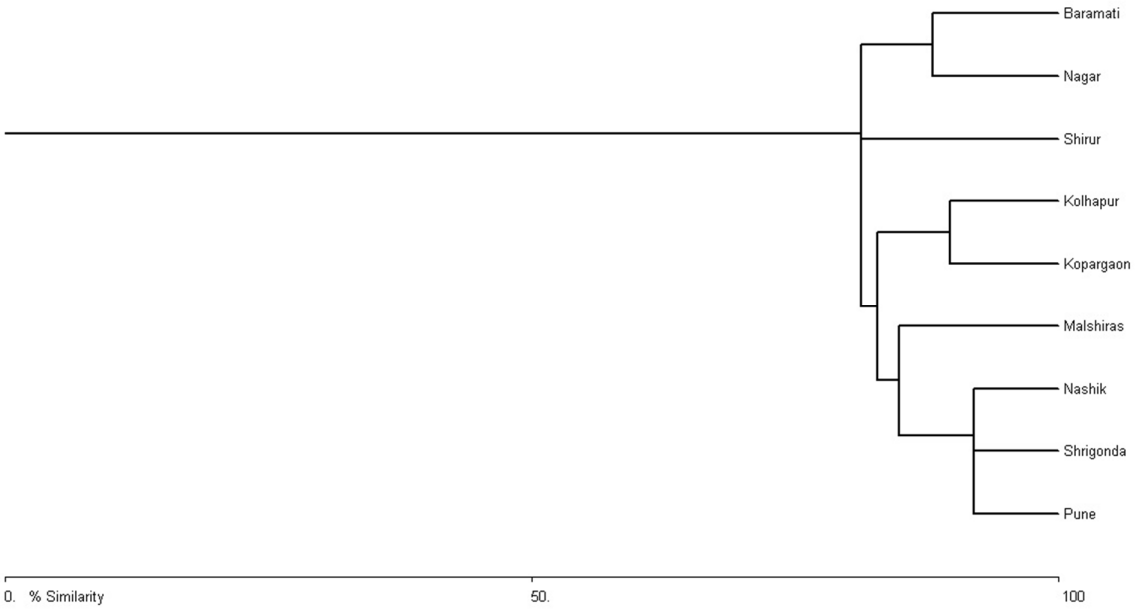

Fig 3.Hierarchical cluster analysis based on biochemical data in populations of *P. corylifolia*.