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Genetic drift in six cultivated populations of *Terminalia arjuna*

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ABSTRACT

Low genetic diversity among five cultivated populations of *Terminalia arjuna* was revealed using DNA fingerprints generated by ten commercially available random (RAPD) primers i.e. RPI01 to RPI10. Out of ten primers, eight primers generated total 79 bands with 87.34 percentage of polymorphism, while primer RPI02 and RPI08 did not show amplification. Applied all 8 primes having a good polymorphic informativeness among the populations (mean *PIC* = 0.355 ± 0.032 and *Ho* = 0.463 ± 0.018). Higher genetic variation, gene diversity (*H*), Shannon's Information index (*I*) and Percentage of polymorphic bands (*PPB*) among populations (*H* = 0.290 ± 0.162, *I* = 0.440 ± 0.221 and *PPB* = 87.34%) was observed compared to within populations (maximum *H* = 0.165 ± 0.212, I = 0.239 ± 0.304 and *PPB* = 39.24%, minimum *H* = 0.026 ± 0.104, *I* = 0.038 ± 0.150, *PPB* = 06.33%). Low average gene diversity (π = 0.075 ± 0.062) within population, higher pair-wise *F*st (ranged from 0.498 to 0.844) among the population and Analysis of Molecular Variance (*AMOVA*) shown adequate genetic variation among population (*Pv* = 75.57%) but serious low genetic variation within populations (*Pv* = 24.43, Φ_{ST} = 0.756). Among the populations of *T. arjuna* the higher relative differentiation (G_{ST} = 0.7843) with restricted gene flow G_{ST} (*Nm*) = 0.1375 was observed. Unbiased measures of genetic distance and phylogram revealed that all locations have their once genetic identity and they arranged in their respective clusters. Lowest distance was shown by accession collected from JNKVV and TFRI Jabalpur.

1. INTRODUCTION

In the last few decades, civilization and industrialization have been increased for the fulfilment of demand of rising population and which directly or indirectly effects on natural forest in the both developed and developing countries. Plantation forestry is most suitable and used approach for commercial production of forest wood as well as forest restoration [1, 2]. Enormous nurseries and clonal propagation laboratories are affianced in large scale production of plantlets and this genetically similar plant are beneficial for commercial forest wood production [3], but in other hand, this practice creates genetic drift in restoration of forest [4, 5]. Restoration of medicinal plants in the forest using nursery and clonal propagated plantlets creates genetic drift due to higher genetic similarity of mother and daughter plants. Medicinal plants play an important role in health carein developing countries, especially in remote areas and many loca healthcare systems were successfully used by local communities since ancient time [6, 7, 8]. A major healthcare system 'Ayurveda' having long, rich history in India and now most accepted worldwide, especially in developed countries because of its fewer side effects [9]. The popularity and demand of herbal produces adversely affected their viability in natural habits as a result of unsustainable exploitation of these wild sources has directed to many species being extinct, threatened or endangered [10, 11]. For the assessment of genetic viability of any species, estimation of genetic diversity, especially using molecular markers may play as an important tool. Many biochemical and molecular markers are routinely used for genetic diversity assessment, out of them random amplified polymorphic DNA analysis is most frequently used due to its simplicity and convenience [12, 13]. India has one of the richest natural resources and estimated to be around 25,000 effective plant-based formulations, used in folk medicine. It is estimated that there are over 7,800 medicinal drug-manufacturing units in India, which consume about 2,000 tonnes of herbs annually [9]. *T. arjuna* is one of the most important medicinal plants and used in Ayurveda from ancient time in India.

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T. arjuna (family *Combretaceae*) is a large deciduous 60-90 feet heighted and 2-2.5 m diameter at breast height tree found throughout India. This tree is usually an evergreen tree with new leaves appearing in the hot season (February to April) before leaf fall [14]. The thick white-to-pinkish-grey bark of *T. arjuna* has a wide spectrum of biological activity like anti-dysentric, antipyretic, astringent, cardiotonic, lithotriptic, anticoagulant, hypolipidemi, antimicrobial [15] and antiuremic [16] agent. Many active constituents include tannins, triterpenoidsaponins (arjunic acid, arjunolic acid, arjungenin, arjunglycosides), flavonoids (Argonne, arjunolone, luteolin), Gallic acid, ellagic acid, oligomericproanthocyanidins (OPCs), phytosterols, calcium, magnesium, zinc, and copper [17, 18, 19] was identified in *T. arjuna*. Wild and cultivated Fruit (Seed) and Bark (Stem) are used in Ayurveda, Folk, Homoeopathy, Siddha, Tibetan, Unani and Western system of medicine with estimated annual trade of 2000 – 5000 metric ton / annum (NMPB).

2. MATERIAL AND METHODS

Total 28 samples of *T. ajuna* were collected from Bhopal, Sagar, Gwalior, Chitrakoot and 2 places of Jabalpur (Madhya Pradesh) and conserved at MPCST Human Herbal Health Care Garden Bhopal (table 1).

2.1. DNA Isolation

Young leaves of *T. ajuna* were used for isolation of genomic DNA from HEPES Based Method [20, 21]. The yield of DNA was measured using Nano Drop UV-Spectrophotometer (ND-1000) at 260 nm using DNase RNase free water as a blank.

2.2. Amplified of DNA using RAPD markers

Ten commercially available RAPD primers (table 2) from Bangalore Genei, Bangalore were used in the amplification of 28 accessions of 6 populations. 25 ng Genomic DNA was amplification using Eppendorf gradient automatic thermal cycler. A PCR reaction of 50 µlwas prepared, adding 25 µl of 2× red dye PCR master mix (10 mMTris–HCl, 50 mMKCl, 1.5 mM MgCl2, 25 units/ml Taq DNA polymerase, 0.2 mMdNTPs, 5% glycerol, 0.08% NP-40 and 0.05% Tween-20), 2 µlprimer, 1 µl template DNA (25 ng/µl), 22 µl sterile DNase RNase Free water and PCR enhancement agents BSA (400 ng/µl). Condition of thermal cycler was set at initial denaturation of the DNA at 94 °C for 5 min, 08 cycles of 45 s at 94 °C, 1 min at 55 °C and 1.5 min at 72 °C; 30 cycles of 45 s at 94 °C, 1 min at 58 °C and 1 min at 72 °C and final extension at 72 °C at 10 min and a hold temperature at 4 °C. Control reactions without template DNA (negative control) and without the enzyme (positive control) were also run in the experiments. All the experiments were repeated thrice to ensure reproducibility. Amplified DNA fragments with a low range DNA ruler was separated by electrophoresis at 6–7 V/cm in TAE buffer for 3–4 h on 1.2% Agarose gel stained with Ethidium Bromide and photographed by Gel Documentation System (Alpha Innotech). Raw gel images were recorded and analysed through Alphaview software.

2.3. Statistical analysis

The molecular weight of all bands was calculated and binary matrix was prepared by scoring (0) as absence and (1) as the presence for all RAPD primers. Percentage of polymorphism was calculated according to the presence and absence of bands for all RAPD primers. For each RAPD markers, polymorphic information content (*PIC*) and heterozygosity (*H*) were calculated using online Software PICcalc (http://w3.georgikon.hu/pic/english/kodom.aspx) [22]. Similarity indices, distance matrix and phylogenetic tree were prepared using the PAST Software [23], using Jaccard's coefficient and UPGMA. Observed number of alleles (*Na*), Effective number of alleles (*Ne*) described by Welsh and McClelland [24], gene diversity (*H*) described by Nei [25], Shannon's information index (*I*) described by Lewontin [26], Relative differentiation (G_{ST}), estimate of gene flow from ($G_{ST}(Nm)$), total heterozygosity (*Ht*), within population heterozygosity (*Hs*), genetic identity and genetic distance within population described by McDermott and McDonald [27], Number of Polymorphic Bands (*NPB*) and Percentage of polymorphic bands (*PPB*) were calculated using Popgene version 1.31. Average genetic distances in the all populations was calculated and phylogenetic tree was analysed using MEGA5 software version 5 [28].

Average gene diversity (π), molecular diversity (θ_S and $\theta\pi$), matrix of pairwise F_{ST} (Genetic distances between populations), Average pairwise differences [29], Analysis of Molecular Variance (AMOVA), Sum of squares (SS), Variance components (*Vc*), Percentage of variation (*Pv*) and Fixation Index (Φ_{ST}) were calculated using software Arlequin version 3.5 [30].

3. RESULT

Isolated DNA from all 28 accessions of 6 populations of *T. arjuna* was amplified using ten plant RAPD primer (table 2) and observed under UV light after resolving on 1.5% agarose gel with low range molecular marker.

3.1. Genetic diversity, according to primers

Percentage of polymorphism was calculated according to the presence and absence of bands for use primers. Out of ten primers, eight primers generated total 79 bands with 9.875 ± 2.10 meanbands per primer while primer RPI02 and RPI08 did not show amplification. Out of 79 bands total 69 polymorphic bands (87.34%) were observed with average 8.625 ± 2.330 polymorphic bands per primer. Maximum percentage of polymorphic bands (100.00) were observed in RPI-07 and minimum 66.67 was observed in RPI-06 primer. Among the populations, mean of heterozygosity (*Ho*) observed was 0.463 ± 0.018 with 0.355 ± 0.032 of mean polymorphic information content (*PIC)* (table 3).

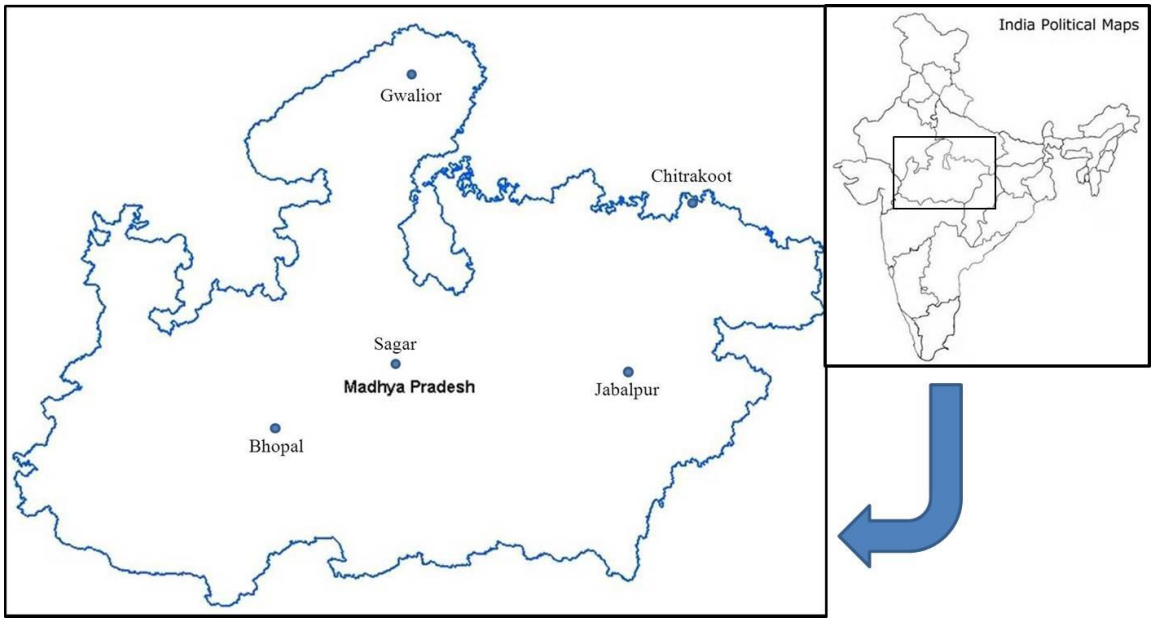

Fig. 1: Location of Sample collection.

Table 1: *T. ajuna*from different locations.

SL	Location of Collection	Sample Code	Population	No of Sample
1	Tropical Forest Research Institute, Jabalpur	JTTA-01 to 05	JTTA	5
2	Gwalior	GTA-01 to 05	GTA	5
3	Jawaharlal Agricultural University, Jabalpur	JJTA-01 to 05	JJTA	5
4	Chitrakoot	CTA-01 to 03	CTA	3
5	Bhopal	BTA-01 to 04	BTA	4
6	Sagar	STA-01 to 06	STA	6
			Total	**28**

Table 2: RAPD Primers for the molecular diversity analysis.

SL	Name of Primer		Accession No.
1.	RPI – 01	:	AM 765819
2.	RPI – 02	:	AM750044
3.	RPI – 03	:	AM 773310
4.	RPI – 04	:	AM 773769
5.	RPI – 05	:	AM 773770
6.	RPI – 06	:	AM 773771
7.	RPI – 07	:	AM 773312
8.	RPI – 08	:	AM 773773
9.	RPI – 09	:	AM 773315
10.	RPI - 10	:	AM 750045

Table 3: Genetic diversity, according to primers.

SL	Primer	Total bands	*NPB*	*PPB*	*H* value	*PIC* value
1	RPI01	10	9	90.00%	0.476	0.362
2	RPI02	0	-	-	--	--
3	RPI03	10	9	90.00%	0.480	0.365
4	RPI04	10	9	90.00%	0.417	0.330
5	RPI05	13	11	84.62%	0.497	0.374
6	RPI06	9	6	66.67%	0.475	0.362
7	RPI07	12	12	100.00%	0.470	0.360
8	RPI08	0	-	-	--	--
9	RPI09	9	8	88.89%	0.408	0.325
10	RPI10	6	5	83.34%	0.480	0.365
	Total	**79**	**69**	**87.34%**		
Mean ± SD		**9.875 ± 2.100**	**8.625 ± 2.330**		**0.463 ± 0.018**	**0.355 ± 0.032**

Table 4: Genic Variation Statistics for All Loci Nei (1987).

Population	Sample Size	*Na*	*Ne*	*H*	*I*	*NPB*	*PPB*
Within Population							
JTTA	5	1.063 ± 0.245	1.047 ± 0.190	0.026 ± 0.104	0.038 ± 0.150	5	06.33
GTA	5	1.089 ± 0.286	1.076 ± 0.250	0.041 ± 0.132	0.057 ± 0.186	7	08.86
JJTA	5	1.114 ± 0.320	1.077 ± 0.228	0.045 ± 0.128	0.066 ± 0.187	9	11.39
CTA	3	1.101 ± 0.304	1.081 ± 0.243	0.045 ± 0.135	0.065 ± 0.193	8	10.13
BTA	4	1.076 ± 0.267	1.056 ± 0.202	0.032 ± 0.112	0.046 ± 0.163	6	07.59
STA	6	1.392 ± 0.491	1.295 ± 0.389	0.165 ± 0.212	0.239 ± 0.304	31	39.24
Among populations							
	28	1.873 ± 0.335	1.478 ± 0.3203	0.290 ± 0.162	0.440 ± 0.221	69	87.34

Na = Observed number of alleles
Ne = Effective number of alleles [Kimura and Crow (1964)]
H = Nei's (1973) gene diversity
I = Shannon's Information index [Lewontin (1972)]

Table 5: Nei's Analysis of Gene Diversity in Subdivided Populations.

	Sample	*Ht*	*Hs*	*Gst*	*Nm**
Among population	28	0.273 ± 0.028	0.059 ± 0.005	0.7843	0.1375

* Nm = estimate of gene flow from Gst
Nm = 0.5(1 - Gst)/Gst;

Table 6: Average gene diversity (π) and molecular diversity (θ_S and $\theta\pi$).

SL	Populations	Molecular Diversity		Average gene diversity (π)
		θ_S	$\theta\pi$	
	JTTA	2.400	2.600	0.033
	GTA	3.360	4.000	0.051
	JJTA	4.320	4.400	0.056
	CTA	5.333	5.333	0.068
	BTA	3.273	3.333	0.042
	STA	13.577	15.667	0.198
	Mean ± SD	5.377 ± 4.14	5.889 ± 4.88	0.075 ± 0.062

3.2. Genetic Variation

For the estimation of genetic variation in the populations, Observed number of alleles (*Na*), Effective number of alleles (*Ne*), gene diversity (*H*), Shannon's Information index (*I*), Number of Polymorphic Bands (*NPB*) and Percentage of polymorphic bands (*PPB*) were calculated. According to this parameter within population STA maximum genetic variation (*Na*= 1.392 ± 0.491, *Ne = 1.295 ± 0.389*, *H* = 0.165 ± 0.212, *I* = 0.239 ± 0.304, *NPB* = 31% and *PPB*= 39.24%), was observed compared to other population and population JTT As how minimum genetic variation (*Na* = 1.063 ± 0.245, *Ne* = 1.047 ± 0.190, *H*= 0.026 ± 0.104, *I*= 0.038 ± 0.150, *NPB*= 5% and PPB= 06.33%) (table 4). Compare to within population, among the populations higher genetic variations (*Na* = 1.873 ± 0.335, *Ne*=1.478 ± 0.320, *H*= 0.290 ± 0.162, *I*= 0.440 ± 0.221, *Ht*=0.273 ± 0.028 and *Hs*= 0.059 ± 0.005, NPB = 69 and PPB= 87.34% was observed (table 4, and 5).

3.3. Relative differentiation and gene flow

Among the populations of *T. arjuna* the Relative differentiation (G_{ST} = 0.4033) and estimate of gene flow from G_{ST} (*Nm*) = 0.7396 was observed (table 5).

3.4. Gene diversity and pairwise difference

Mean of average gene diversity (π) and molecular diversity (θ_S and $\theta\pi$) was 0.075 ± 0.062, 5.377 ± 4.14 and 5.889 ± 4.88 respectively, with maximum π = 0.198, θ_S = 13.577 and $\theta\pi$ = 15.667 in population STA and minimum π = 0.033, θ_S = 2.4 and $\theta\pi$ = 2.6 in population JTTA was observed (table 6). Maximum F_{ST} of 0.844 was observed in between a population of BTA and GTA, while minimum 0.498 was observed in between population BTA and CTA (table 7, figure 2) with significance (p =0.0500) at 110 permutations. The maximum average number of pair wise differences between populations STA and JTTA and minimums between populations JTTA and JJTA, while maximum corrected average pair wise difference (Nei's) between populations STA and JTTA and minimums between populations CTA and BTA was observed. The maximum average number of pair wise differences within population STA and minimum within population JTTA was observed (table 8, figure 3).

3.5. Analysis of Molecular Variants (AMOVA)

Within populations and among populations, 75.57 and 24.43 Percentage of variation (*Pv*) respectively, with Fixation Index (Φ_{ST}) 0.756 was calculated with significance level p=0.001 at 1023 permutations (table 9).

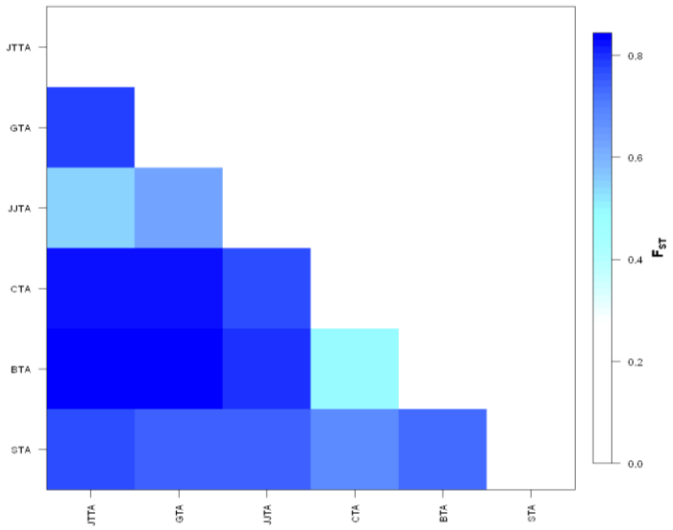

Fig. 2: Matrix of pair-wise Fst.

Table 7: Population pairwise F_{ST}.

	JTTA	GTA	JJTA	CTA	BTA	STA
JTTA	--					
GTA	0.786*	--				
JJTA	0.544*	0.621*	-			
CTA	0.826*	0.823*	0.770*	-		
BTA	0.841*	0.844*	0.792*	0.498*	-	
STA	0.771*	0.748*	0.743*	0.684*	0.735*	-

Number of permutations: 110.
*Significance Level (p) =0.0500.

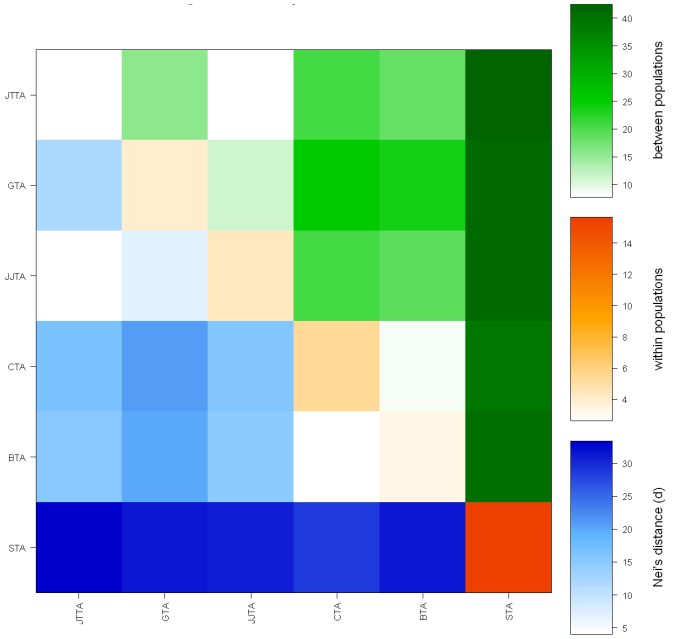

Fig. 3: Avarage number of pairwise distance.

Table 8: Distance method: Pairwise difference.

	JTTA	GTA	JJTA	CTA	BTA	STA
JTTA	2.600	15.400	7.680	20.400	18.400	42.567
GTA	12.100	4.000	11.080	25.267	23.700	41.233
JJTA	4.180	6.880	4.400	20.533	18.900	41.100
CTA	16.433	20.600	15.667	5.333	8.333	38.944
BTA	15.433	20.033	15.033	4.000	3.333	40.667
STA	33.433	31.400	31.067	28.444	31.167	15.667

Above diagonal: Average number of pairwise differences between populations.
Diagonal elements: Average number of pairwise differences within population.
Below diagonal: Corrected average pairwise difference.

Table 9: Analysis of Molecular Variance (ANOVA).

Source of variation	D*f*	Sum of squares (*SS*)	Variance components (*Vc*)	Percentage of variation (*Pv*)	Fixation Index
Among populations	5	248.929	10.054	75.57	Φ_{ST}: 0.756*
Within populations	22	71.500	03.250	24.43	
Total	27	320.429	13.304		*P**= <0.001

*Significant (1023 permutations)

Table 10: Unbiased Measures of Genetic distance.

	JTTA	GTA	JJTA	CTA	BTA	STA
JTTA	--					
GTA	0.179	--				
JJTA	**0.062**	0.103	--			
CTA	0.256	0.335	0.248	--		
BTA	0.232	0.315	0.230	0.065	--	
STA	**0.660**	0.616	0.610	0.552	0.605	--

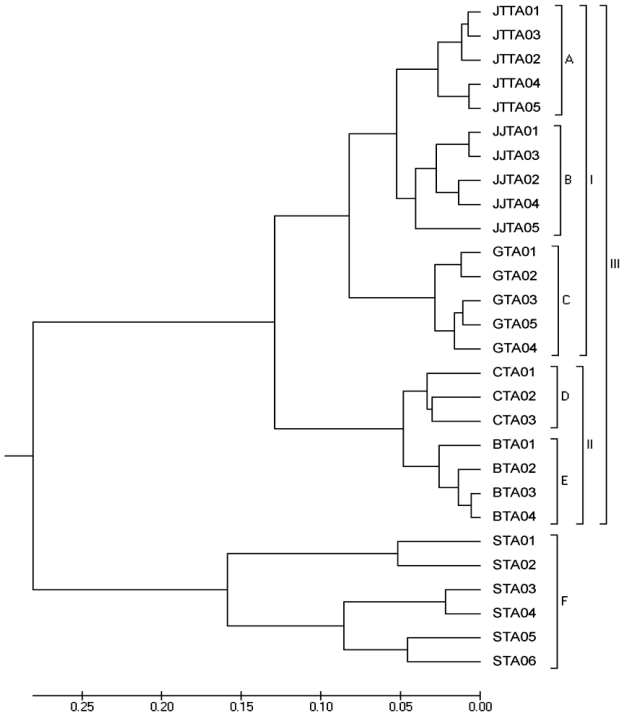

Fig. 4: Dendrogram of 28 accessions Based Nei's (1978) Genetic distance Method UPGMA

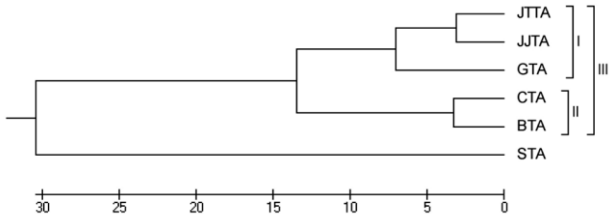

Fig. 5: Dendrogram of 6 populations Based Nei's (1978) Genetic distance Method UPGMA.

3.6. Distance Matrix and phylogenetic

Populations STA and JTTA showed maximum genetic distance (*D*=0.660) and population JJTA and JTTA showed minimum genetic distance (*D*=0.062). 0.338 Average genetic distances were calculated using MEGA5. When dendrogram (Phylogenetic tree) was prepared using a binary matrix of 28 accessions, six clusters, i.e. cluster A to cluster F were generated, which represent the accessions of their respective population (figure). Population JJTA and JTTA showed lowest genetic distance followed by population GTA and prepared a major cluster I. Population CTA and BTA create a cluster II, which is similar to cluster I and prepare a mega cluster called cluster III. Population STA was shown similarity with mega cluster III (table 10, figure 4 and 5).

4. DISCUSSION

Medicinal plants are the source of a large number of essential drugs in Western medicine and are the basis of herbal medicine, which is not only the primary source of health care for most of the world's population living in developing countries but also enjoys growing popularity in developed countries. The increased demand for botanical products is met with an expanding industry and accompanied by calls for assurance of quality, efficacy and safety. Plants used as drugs, dietary supplements and herbal medicines are identified at the species level [31].

The genetic variability of a population is usually measured by the average hetrozygosity per locus, while the gene differences between two populations may be measured by the genetic distance recently proposed by Nei [32, 33]. In this study, a total of 79 alleles was scored by 10 RAPD primers in 26 accessions, out of which, 69 alleles (87.34%) were polymorphic in nature. Barker [3] suggested that loci with at least four alleles should be suitable for studying the genetic diversity. The alleles more than the recommended number observed in this study supports the suitability of markers [34]. Therefore, it revealed that the used primers had sufficient potency for population studies and this is the first report, which accesses the genetic diversity among and within populations of *T. arjuna*. The occurrence of low genetic diversity at the population level was also reported in many plant species [35, 36, 37, 38, 39, 40, 41].

The *PIC* value of each RAPD primers was determined by both the number of alleles and their frequency distribution within a population and was used to assess their information level (high *PIC*> 0.5, moderate 0.5 >*PIC*> 0.25 and low *PIC*< 0.25) [42, 43]. Therefore, in this study, mean polymorphic information content (*PIC*=0.355 ± 0.032) of primers, showed moderate level of polymorphic information among six populations of used markers and suggested that these markers were equally effective in determining polymorphisms. The *PIC* value has been used for evaluating genetic variation in many studies using RAPD markers and other molecular markers [39, 44, 45, 46, 47, 48]

Genetic variation was estimated by various parameters; out of them the effective number of alleles is lesser than the observed number of all the lociin all calculations. Effective number of alleles and observed number of alleles among the population was found higher than within population, indicating more allelic polymorphism among the population, compared to within the population. Shannon's information index, which measures the level of diversity, further supports the suitability of markers, and occurrence of genetic polymorphism [49]. Observed and expected heterozygosity defines the probability that a given individual randomly selected from a population will be heterozygous at a given locus and the observed heterozygosities were lower than the expected [34]. This information index and heterozygosity also support the more diversity among the population, compared to within populations. The unbiased expected gene diversity (Nei's) ranged from 0.14 (OarJMP29) to 0.92 (ILSTS059) with the overall mean 0.73 ± 0.19. The F-estimates were applied to all the 25 studied loci to determine the extent of inbreeding in the population.

Gene flow is a collective term that includes all the mechanism resulting in the movement of genes from one population to another [50]. Higher relative differentiation in within groups was observed in comparing to the relative differentiation. On the other hand, gene flow was higher among the accessions in respect to the gene flow of within the groups. Assessment of gene flow from one population to another is an important statically parameter for study of genetic diversity. This parameter for enlightening diversity in many medicinal plants was successfully applied [51, 52, 53, 40].

The significance of the covariance components was calculated with the different possible levels of genetic structure (among groups, among populations within groups and within populations). Within populations and among populations, 75.57 and 24.43 Percentage of variation (*Pv*) respectively, with Fixation Index (Φ_{ST}) 0.756 was calculated with significance level p=0.001 at 1023 permutations. The significance of the covariance components with the different possible levels of genetic structure, including among groups, among populations within groups and within populations was extensively used for the study of molecular variance [51, 54].

5. CONCLUSION

Sustainable genetic diversity is an important issue for forest restoration more willingly than low genetic diversity is suitable for commercial forestry for their economic importance and value. Viable diversity upholds in platelets generated through clonal propagation and horticulture is slightly difficult and this may be created genetic drift.

6. ACKNOWLEDGMENT

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Conflict of Interests: There are no conflicts of interest.

7. DATA ARCHIVING STATEMENT

Genotypes of the individuals analyzed are available from the corresponding author after request.

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