Diversity in a widely distributed dioecious medicinal plant, *Tinospora cordifolia* (Willd.) Miers. ex. Hook F. and Thomas

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The nature and extent of diversity in *Tinospora cordifolia*, a dioecious climber, widely distributed in India, and one of the most important medicinal plants has remained underexplored. The present study reveals wide range of tinosporaside content (0.016–4.523 mg/g), berberine content (0.27–76.9 mg/g) and jaccard distances (0.0341–0.559). The neighbour joining tree, structure simulation and principal coordinate analysis resolved all the accessions into six sub-clusters, four of which were congruent in the analyses. Sub-cluster I uniquely included all male accessions, with above average leaf areas and below average tinosporaside contents with hairy and fibrous leaves. Further, analysis of molecular variance considering three populations showed that maximum variance (87%) was within the population. The result of this preliminary study revealed genetic diversity, population structure in *T. cordifolia*.

Keywords: AMOVA, berberine, ISSR, NJ tree, RAPD, *Tinospora*, tinosporaside.

MEDICINAL plants in the Indian system of medicine and health include several widely distributed taxa in India; yet, very few studies have reported on the nature and extent of diversity in these plants. Considering their usefulness, it is essential that detailed studies are undertaken to determine the nature and extent of genetic, phytochemical and morphological diversity so as to account for their wide and robust distribution, and also to enable their best utilization for the benefit of humankind. The plant of *Tinospora cordifolia* (Willd.) Miers. ex. Hook F. and Thomas (family Menispermaceae), is a large spreading, deciduous, succulent climber, widely distributed in almost the entire country[1] from the Himalayas to the southern part of peninsular India. The plant is also found in South East Asian countries such as Borneo, the Philippines, Malaysia, Indonesia, Thailand, Vietnam and China; as well as in North, West and South Africa. Its robust and wide distribution in the country has reportedly not been linked to any anthropogenic activities, despite its economic importance. *T. cordifolia* is an important medicinal plant commonly called 'Guduchi'/'Giloy' in Hindi and 'Amrita' in Sanskrit indicating immortality and signifies its uses for revitalization. In Ayurveda it is designated as a 'Rasayana', and is recommended to enhance general body resistance, promote longevity and as an antistress and adaptogen[1,2]. Almost all plant parts have medicinal properties. Leaves, stem, bark, fruits and even the whole plant have been used differently for treatment of a diverse range of disorders including allergies, diabetes, immune system, fever, gout, ulcer, skin inflammation, antidote to venoms, respiratory tract infections, diabetes, rheumatoid arthritis and even cancer[2–5]. Several reports on its chemical constituents, medicinal properties and validation of therapeutic claims have already been published[5–10]. The medicinal properties of the plant have been attributed to a variety of constituents, such as alkaloids, diterpenoids, lactones, glycosides, steroids, sequiterpenoids, phenolics, aliphatic compounds and polysaccharides that have been extracted from *T. cordifolia*. Of the various components, tinosporaside and berberine are reported as biomarkers for this plant and are dominant compounds[11,12]. Although a number of studies are available on various medicinal or therapeutic constituents isolated from the whole plant or its parts[11–16], the plant-to-plant variation in the phytochemical constituents of *T. cordifolia* has not been reported. An important question arises here on the extent of diversity and the dioecious nature of the plants – whether or not the diversity (morphological, chemical and molecular) is similar in both genders of the plants.

Considering the distribution and dioecious nature of the plants, it is expected that there will be extensive variation in genetic as well as phytochemical constituents in male and female plants. Thus, keeping in view the importance of *T. cordifolia* it is important to analyse genetic as well as phyto-chemical diversity in this plant. In this communication we report the extent of diversity in morphology, tinosporaside and berberine content and molecular marker based genetic diversity in a number of *T. cordifolia* plants.

In all, 114 plants were collected from various phytogeographical zones of the country as stem cuttings, and maintained in the field at CSIR-NBRI garden under natural conditions. Eighty six accessions were selected for the present study which included 29 accessions from Uttar Pradesh, 25 from Madhya Pradesh, 19 from Jammu and Kashmir, 8 from Maharashtra, 3 from Kerala and 2 from Andhra Pradesh (Supplementary Table 1). Fifty Inter Simple Sequence Repeats (ISSR) primers and hundred Random Amplification of Polymorphic DNA (RAPD) primers were first tested. Ten of each primer (Table 1) that revealed good polymorphism and resolution were

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Table 1. The different primers, their sequences and annealing temperatures used for ISSR and RAPD methods

Marker type	Primer ID	Primer sequence (5′ → 3′)	Annealing temperature (°C)	Total	Bands polymorphic[a]
ISSR	807	AGAGAGAGAGAGAGAGT	50	12	7 (58)
	810	GAGAGAGAGAGAGAGAT	50	23	20 (87)
	811	GAGAGAGAGAGAGAGAC	50	18	13 (72)
	812	GAGAGAGAGAGAGAGAA	46	19	19 (100)
	813	CTCTCTCTCTCTCTCTT	45	23	22 (96)
	823	TCTCTCTCTCTCTCTCC	49	21	20 (95)
	835	AGAGAGAGAGAGAGAGYC	50	23	20 (87)
	840	GAGAGAGAGAGAGAGA(Y)C	46	15	14 (93)
	841	GAGAGAGAGAGAGAGAYC	50	18	11 (61)
	857	ACACACACACACACACC(Y)C	46[a]	29	29 (100)
	Total			201	175 (87)
RAPD	OPB-02	TGATCCCTGG	35	23	22 (96)
	OPF-05	CCGAATTCCC	35	21	20 (95)
	OPF-09	CCAAGCTTCC	35	15	6 (40)
	OPU-08	GGCGAAGGTT	35	16	16 (100)
	OPW-01	CTCAGTGTCC	35	16	11 (69)
	OPW-06	AGGCCCGATG	35	21	20 (95)
	OPW-09	GTGACCGAGT	35	18	15 (83)
	OPW-13	CACAGCGACA	35	19	11 (58)
	OPZ-04	AGGCTGTGCT	35	16	11 (69)
	OPAH-13	TGAGTCCGCA	35	18	14 (78)
	Total			183	146 (80)

[a]Numbers in parentheses are percentage of polymorphic bands.

selected for further analysis. The protocol for genomic DNA isolation, ISSR and RAPD profiling was followed as previously reported[17].

Dry stem powders were defeated with *n*-hexane and then extracted with methanol. The methanol extracts were dried and lyophilized. A solution of 10 mg/g of each extract was prepared and the analysis was carried out on a high-performance thin-layer chromatography (HPTLC) system (CAMAG-Switzerland), using known quantities of tinosporaside (1.0 mg/g) and berberine (0.1 mg/g) as standards[18].

The binary matrices were built attributing '1' to the presence and '0' to the absence of a band in both ISSR and RAPD profiles. The cumulative data matrix of both ISSR and RAPD were analysed for pairwise distance using the Jaccard coefficient using Free Tree software[19]. Further, neighbour joining-based tree with 1000 bootstrap values were generated and viewed using the Tree View program[20]. In order to estimate the number of subpopulations among the collected *T. cordifolia* germplasm, population structure analysis was carried out using model-based clustering method implemented in the program STRUCTURE[21] (ver. 2.3.4). The membership of each accession was tested for the range of genetic clusters from $K = 2$ to 10 with admixture model and without prior information on their origin. Three independent runs were assessed for each K and each run consisted of a 30,000 burn-in period and 100,000 iterations. The optimal value of K was determined by examination of the ΔK statistic and $L(K)$ using the program Structure Harvester[22]. A principal coordinate plot was made using the program GenAlex[23] (ver. 6.5), where the samples were considered in the same clusters as identified by STRUCTURE analysis. The number of observed alleles (Na), number of effective alleles (Ne), Shannon's information index (I) and molecular variance (AMOVA)[24] were calculated with GenAlex.

The present study evaluated the occurrence of morphological, phytochemical and molecular diversity in a large number of accessions of *T. cordifolia*. The morphological characters of all the aerial vegetative parts, viz. leaves, stem and bark were evaluated for all 86 accessions. Several morphological variations were noticed in the leaves; however, no significant variations were observed in the morphology of stems and barks. On the basis of screening, primers that resulted in distinct well separated bands in case of all the tested DNAs (ten each of ISSR and RAPD primers) were selected for further analysis of the full set of 86 DNAs and *Cissampelos pareira* L. was taken as out-group taxon.

The results of the morphological and HPTLC analysis revealed considerable diversity in leaf shape, size and base structure (Figure 1). The leaf laminae also exhibited two types of textures glabrous or non-glabrous and hairy. However, a majority of plants had only glabrous leaves and only a small set of male accessions collected from

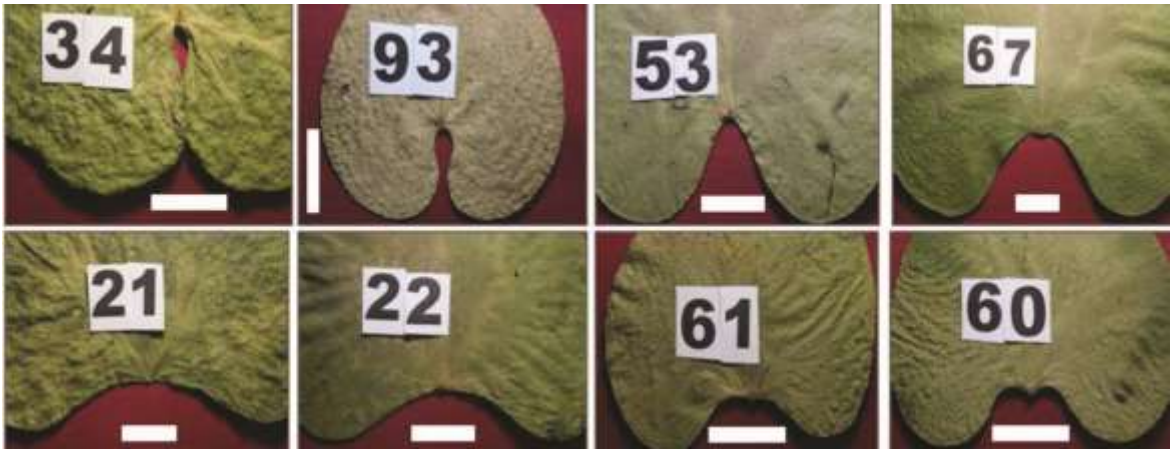

Figure 1. The range of variations in leaf lamina at the point of its attachment to the petiole. The large numbers refer to the accessions as in Table 1. The solid white bar in each image represents a length of 2 cm.

Table 2. The average value of leaf morphology traits, tinosporaside and bererine content depicted according to NJ clusterwise and also gender wise. Overall range and average value of these traits across the accessions are also given

NJ tree cluster	No. of accessions	Gender (M, F)[a]	Tinosporaside content ± SD (mg/g)	Berberine content ± SD (mg/g)	Leaf area ± SD (cm^2)	Lamina to petiole ratio ± SD
I	6	M	0.171 ± 0.15	16.425 ± 8.24	139.266 ± 31.46	0.918 ± 0.15
	0	F	–	–	–	–
II a	4	M	0.084 ± 0.05	8.22 ± 7.40	107.6 ± 19.04	1.335 ± 0.17
	5	F	0.198 ± 0.16	6.895 ± 4.13	102.59 ± 19.78	1.628 ± 0.65
IIb	2	M	0.111 ± 0.07	40.785 ± 36.13	73.175 ± 12.58	1.427 ± 0.25
	10	F	0.158 ± 0.19	12.641 ± 10.94	84.337 ± 17.10	1.216 ± 0.17
IIc	8	M	0.256 ± 0.16	24.801 ± 19.06	99.431 ± 19.30	1.079 ± 0.18
	12	F	0.418 ± 0.52	20.058 ± 14.95	103.97 ± 21.76	1.193 ± 0.22
IId	9	M	2.111 ± 1.72	27.395 ± 18.82	90.994 ± 22.64	1.319 ± 0.31
	10	F	1.188 ± 0.82	31.412 ± 12.86	97.34 ± 24.98	1.161 ± 0.17
IIe	4	M	0.976 ± 0.39	43.54 ± 6.22	103.767 ± 12.07	1.232 ± 0.08
	9	F	0.847 ± 0.25	27.088 ± 3.81	112.741 ± 7.75	1.367 ± 0.05
	Overall range	M	0.036–4.523	0.27–76.9	47.8–199.3	0.763–1.743
	Overall range	F	0.016–2.387	2.82–51.96	54.1–204	0.897–2.791
	Overall mean ± SD	M	0.618 ± 0.80	26.861 ± 13.66	102.372 ± 21.82	1.218 ± 0.18
	Overall mean ± SD	F	0.561 ± 0.44	19.618 ± 10.07	100.195 ± 10.45	1.313 ± 0.19

Jabalpur (Tc56, Tc62, Tc65, Tc67, Tc68 and Tc70) had hairy leaf laminae (Supplementary Table 2). Morphological variations were observed only in the stem surface and the colour of the bark. Some plants had smooth stems with greyish bark at maturity while others had a slightly rough surface with light grey or whitish bark (data not shown).

HPTLC analysis revealed that tinosporaside content ranged from 0.016 to 4.523 mg with Tc89 exhibiting the least and Tc21 exhibiting the highest amount of tinosporaside. Similarly, the berberine content was found to vary in the range 0.27 mg/g (least amount in Tc86)–76.9 mg/g (highest amount in Tc93) (Supplementary Table 2). The average value of tinosporaside content across all male and female accessions was found to be 0.618 ± 0.80 and 0.561 ± 0.44 mg/g respectively (Table 2). Similarly, the average value of berberine content across all male and female accessions was found to be 26.86 ± 13.66 and 19.618 ± 10.07 mg/g respectively (Table 2).

In order to best resolve the affinities of accessions to each other, the ISSR and RAPD data were considered together. Here, a total of 384 bands were scored, of which 346 bands were polymorphic (~84% polymorphism). Least (0.0341) and maximum (0.559) pairwise distances were found in the accession ranges Tc107–Tc108 and Tc63–Tc70 respectively. The distance data were used for computing a neighbour joining (NJ) tree after 1000 replicate bootstrap (Figure 2 *a*). The out-group taxon is well

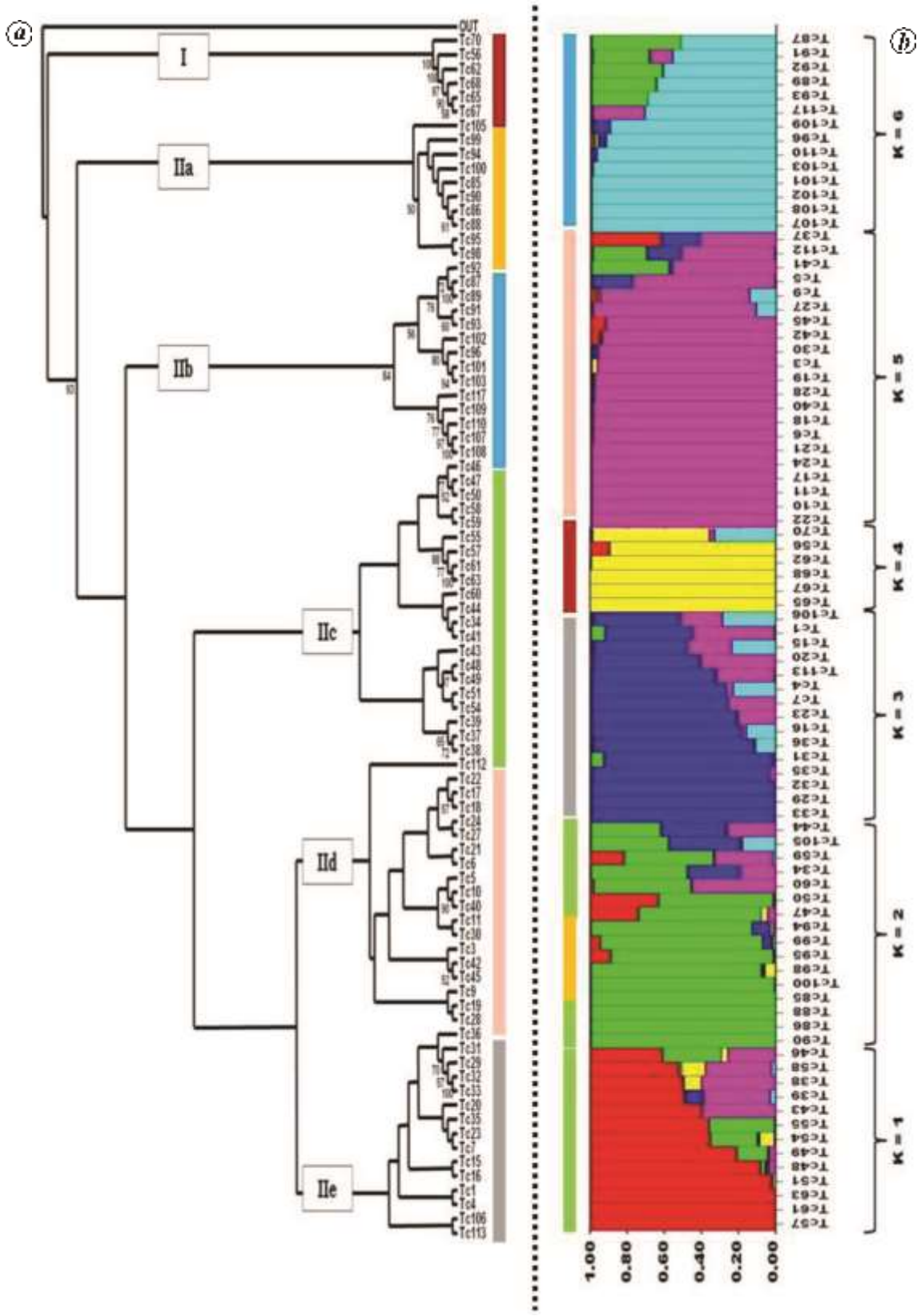

Figure 2. ***a***, The NJ-tree based on Jaccard's coefficient derived from ISSR and RAPD data with 1000 bootstrap value. ***b***, The bar plot derived from STRUCTURE analysis with $K = 6$ (maximum ΔK at $K = 6$). The cluster distribution of the accessions is colour coded with six colours to further depict admixtures, if any. Additional colour bars corresponding to the total clusters in both NJ tree as well as the K plot have been provided such that the colour coding enables easy identification of the clusters congruent in both plots.

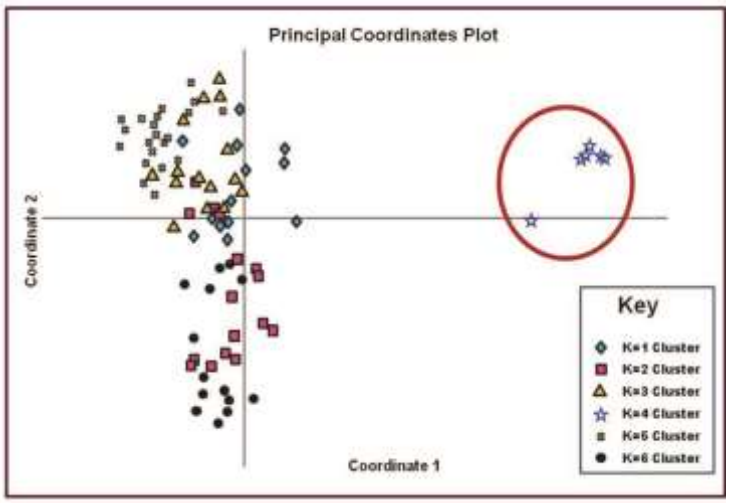

Figure 3. Principal coordinates were plotted from the genetic distance data of the combined ISSR and RAPD bands. The groups are identified by different symbols and for each accession in the group the same symbol is used to illustrate distribution or aggregation of the accessions. The group of accessions within the circle are the same as those of cluster I (of Figure 2 *a*) or $K = 4$ cluster (Figure 2 *b*).

Table 3. Analysis of molecular variance for 73 accessions from three populations

Source	Degree of freedom	Sum of squares	Variance	% variance
Among population	2	354.900	5.731	13%
Within population	70	2798.963	39.985	87%
Total	72	3153.863	45.716	100%

$F_{ST} = 0.125$; $P < 0.001$.

separated in the NJ tree from the rest of the *T. cordifolia* accessions, which in turn were grouped into six clusters. In order to examine whether leaf morphological traits, phytochemical traits and molecular markers have any congruence whatsoever, a table was generated where the accessions were listed in the order of their occurrence in the NJ tree. This table was then populated with the values for morphological and phytochemical traits (Supplementary Table 2) to determine any congruence among different accessions.

The observation that the combined ISSR and RAPD data resolved an NJ tree with all accessions divided in six broad clusters, suggests that these (clusters) represent many genetic lineages. However, by itself, the NJ tree does not reveal genetic admixtures, if any, among the accessions. In order to assess this, a simulation was carried out using model-based clustering implemented in STRUCTURE program, where the data were analysed for membership of each accession among the possible clusters. This analysis of simulation revealed that six subpopulations best resolved the data in terms of least deviations in probabilities of the estimates and the highest delta K values (Supplementary Figure 1 *a* and *b*). The NJ-based clustering and model based sub-population were plotted together to compare accession composition (Supplementary Figure 1 *a* and *b*). The coloured bars representing the total clusters in the NJ tree as well as the structure plot show that as many as four clusters of accessions are congruent by both methods and only two clusters show significant admixtures. The admixtures of accessions are also clearly indicated by principal coordinate analysis (Figure 3), where only one group of accessions ($K = 4$ cluster, Figure 3) is clearly separated from all others. Significantly it is the same cluster, which also separates out from all others in the combined NJ tree (Figure 2 *a*) and the STRUCTURE simulation plot (Figure 2 *b*).

Further, to test the significance of the genetic structure, AMOVA analysis was done by taking accessions from three states, i.e. UP (Pop1), MP (Pop2) and J&K (Pop3) as these contributed maximum number of accessions (~85%). It was noticed that 87% of the total genetic variation was within the population and 13% was among the population (Table 3). The average pairwise Φ_{PT} (similar to F_{ST}) was 0.125 and supported the strong population structure among the collected accessions. The mean of Nei genetic diversity (He), effective number of alleles (Ne) and Shannon's information index (I) were estimated as 0.198 ± 0.06, 1.333 ± 0.011 and 0.30 ± 0.008 respectively (Table 4). The accessions from MP had higher values for these genetic parameters when compared to accessions from UP and J&K.

Table 4. Different genetic diversity estimates for three populations of *T. cordifolia* based on ISSR and RAPD data

Population	Na	Ne	I	He
Pop1	1.435 ± 0.043	1.326 ± 0.019	0.295 ± 0.014	0.194 ± 0.010
Pop2	1.526 ± 0.039	1.346 ± 0.018	0.318 ± 0.014	0.208 ± 0.010
Pop3	1.335 ± 0.044	1.326 ± 0.019	0.289 ± 0.014	0.192 ± 0.010
Mean	1.432 ± 0.024	1.333 ± 0.011	0.301 ± 0.008	0.198 ± 0.006

Na, Number of different alleles; Ne, Effective number of alleles; He, Nei's (1973) gene diversity; I, Shannon's information index.

T. cordifolia remained relatively less studied for the nature and extent of diversity. Only a few studies of genetic diversity revealed low level of genetic diversity[25–28]. All the foregoing studies were carried out with limited number of accessions, collected mostly from local or small regional areas and do not reflect a comprehensive account of the actual diversity as may be extant in *T. cordifolia* plants in their native state. The present study is a more comprehensive analysis of diversity based on 86 accessions. Considering the dioecious nature of the plant and thereby obligate cross pollination, extensive genetic diversity is expected. At the morphological level a considerable range of variations in leaf area, shape, size and leaf base, leaf surface traits, stem traits, bark colour and texture were observed. The morphological traits do not seem to have any specific gender association, since no congruence between any of the traits and the gender of the plants was observed.

The NJ tree based on ISSR and RAPD enabled us to identify two broad clusters in the accessions (Figure 2 *a*). The six accessions of cluster I have above average leaf areas, uniquely hairy and fibrous leaf textures, with below average or very low tinosporaside content. However, leaves of all other accessions were glabrous (Supplementary Table 2). No congruence was observed between the sub-clustering of the accessions and their geographical provenance. The leaf morphology traits, tinosporaside and berberine contents and the data of molecular markers revealed a considerably wide range of diversity in the accessions. The comparative data of all clusters showed that the accessions in cluster I collected from Jabalpur, were male accessions and exhibited above average values of leaf area and have large, broad lamina that also exhibit uniquely the fibrous and hairy leaf surfaces (Table 2 and Supplementary Table 2). Unlike the above, all other accessions were glabrous and exhibited very low (below average) quantities of tinosporaside (Table 2). These results contrast the observed morphological variation in samples not congruent with the molecular marker data[27]. This non-congruence of variation could be due to smaller sample size, as well as, a narrower range of collection of the accessions used in the earlier study[27]. The results of molecular data have also been correlated with agronomic traits in the case of mungbean[29] and morphological and fruit traits in papaya[30]. Similarly in the case of betelvine (*Piper betle* L.), which is also a dioecious liana, cumulative SPAR profile analysis clearly separated the accessions on the basis of gender[31,32].

The identification of sub-clusters of the accessions based on molecular marker data suggested that the accessions used in this study may have a considerable range of diversity. The model-based clustering also clearly resolved the entire data as best fit for a simulation of six different sub-populations (Figure 2 *b* and Supplementary Figure 1 *a* and *b*). The six sub-populations showed considerable congruence with the six sub-clusters of the NJ tree (Figure 2 *a* and *b*). It is noteworthy that four of six sub-populations were congruent in both the NJ tree and the K plot (Figure 2 *a* and *b*), while only two sub-populations in the K plot (Figure 2 *b*) showed admixtures. Additional support for possible genetic admixture of accessions was also demonstrated through principal coordinate analysis (Figure 3) with the sub-population ($K = 4$) clearly separated in the PCoA plot. This group of accessions is clearly different from the other accessions in molecular, morphological as well as phytochemical profiles. Such a distinct group of accessions can constitute a potentially useful group of accessions for further selection as well as downstream processing for metabolite production. Further, the genetic structures of the accessions determined through AMOVA considering three populations from three states which showed 87% variability within population and the F_{ST} value (0.125; $P < 0.001$) (Table 3) support population structure. The accessions collected from MP (population 2, Table 4) had comparatively higher value of Na, Ne, He and I indicating that these accessions are comparatively more diverse with potential alleles. Unlike the case of betelvine, the *T. cordifolia* accessions could not be resolved into gender specific groups (male and female separately) which is in agreement with an earlier finding by Rana *et al.*[27]. Thus, in *T. cordifolia* there could be different molecular mechanisms for gender distinction relative to other dioecious taxa including betelvine.

The present study has revealed a wide range of diversity among the *T. cordifolia* accessions collected from different phyto-geographical zones of the country. Tinosporaside and berberine contents vary widely from accession

to accession. This information may be useful for researchers and pharmaceutical industries for quality control and best utilization of this natural resource. The usefulness of the study is in documenting the potential congruence of some of the molecular data, phytochemical constituents and leaf morphology. The study thus addresses the important question on the wide and robust distribution of the plants. It appears that the extant wide range of genetic diversity could also account for the wide distribution.

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