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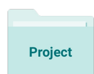

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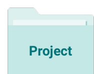

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RESEARCH NOTE

Genetic diversity analysis of *Tinospora cordifolia* germplasm collected from northwestern Himalayan region of India

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[Rana V., Thakur K., Sood R., Sharma V. and Sharma T. R. 2012 Genetic diversity analysis of *Tinospora cordifolia* germplasm collected from northwestern Himalayan region of India. *J. Genet*. **91**, 99–103]

Introduction

'Guduchi' (*Tinospora cordifolia* Willd Miers ex HooK. F. and Thoms) is a diploid ($2n = 22$), deciduous climbing shrub belonging to the family Menispermaceae (Anonymous 1976). It is distributed throughout the tropical and subtropical Indian subcontinent and China, ascending to an altitude of 300 m. *T. cordifolia* is mentioned in ancient Ayurvedic literature as a constituent of several formulations used for the treatment of general debility, dyspepsia, and urinary diseases (Ahmad *et al*. 2009). In the present study, an attempt was made to analyse morphophysiological and molecular polymorphism in *T. cordifolia* accessions collected from different parts of the northwestern Himalayan region of India.

Materials and methods

Twenty-seven accessions, collected from different ecological regions and altitudes in the northwestern Himalayan region of India ranging from 400 to 1300 m above mean sea level (table 1), were grown for morphophysiological characterization. Thirty-seven accessions (including 10 more accessions of unknown origin) were subjected to molecular diversity analysis using RAPD and ISSR markers.

The field experiment was laid out in a completely randomized block design (CRBD) with three replications. Each replication comprised of 50 plants per accession. The data on 15 randomly selected plants were recorded on various morphophysiological parameters viz. plant length (cm), stem diameter (mm), stomatal density (mm^2), trichomal density (mm^2), density of lenticels (per 10 cm), petiole length (cm) and plant biomass (g/plant) during months of July–August (2008 and 2009) when vegetative growth was maximum. The total carbohydrate content (CHO) was estimated after the plants attained the age of two years using a standard biochemical method (Sadasivam 1992). To ascertain the ploidy status of accessions, mitotic analysis was performed. For this 1–2 cm long root tips were collected and pretreated by cooling and then fixed in Carnoy's solution. The root tips were stored until microscopic observations according to Darlington and La Cour (1976).

Genomic DNA was extracted from fresh green leaves following CTAB method as described by Doyle and Doyle (1987) with some modifications for PCR analysis. Of the 144 RAPD primers (Operon Technologies, Alameda, USA) screened, 31 were selected based on their ability to produce robust amplification profiles. The PCR amplification reaction (25 μL) consisted of 20 ng/μL of DNA template, $10 \times$ PCR buffer, 100 μM of each dNTP, 0.2 μM RAPD primer, 25 mM $MgCl_2$ and 0.3 U of *Taq* polymerase (Banglore Genei, Bangalore, India). The PCR amplification was carried out in a Gene Amp PCR System 9700® (Applied Biosystems, Carlsbad, USA) with the following amplification parameters: initial denaturation at 94°C for 5 min, followed by 45 cycles of denaturation at 94°C for 1 min, primer annealing at 37°C for 1 min and amplification at 72°C for 2 min with a final extension at 72°C for 5 min. Amplified DNA was electrophoresed in a 1.4% agarose gel in $1\times$ TAE buffer by electrophoresis at 120 V for 2 h and visualized with ethidium bromide. As standard, 1-kb DNA ladder (Gene Ruler™) was used.

Of the 30 ISSR primers (15 to 23 nucleotides in length) screened, 15 were selected based on their ability to detect distinct amplicons across accessions. The PCR reaction mixture (25 μL) consisted of 20 ng/μL of DNA, 100 μM of each dNTP, $10\times$ PCR buffer, 25 mM $MgCl_2$, 0.4 μM ISSR primer and 0.3 U *Taq* polymerase (Banglore Genei,

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Keywords. genetic diversity; intersimple sequence repeat; ISSR; random amplified polymorphic DNA; RAPD; *Tinospora cordifolia*.

Table 1. Morphophysiological variability in 27 accessions of *T. cordifolia* under *ex situ* conditions.

Accession	Altitude (m)	Location	Longitude	Latitude	Petiole length (cm)	Lenticel density/10 cm	Stomatal density (mm^2)	Trichome density (mm^2)	Stem diameter (mm)	Plant length (cm)	Biomass (g/plant)	CHO content (mg/g)
1	414	Kewardal	76°18′28″E	31°16′35″N	8.34	25.67	26.47	8.10	11.62	245.47	337.50	69.70
2	432	Panjoa	76°15′25″E	31°50′10″N	10.37	27.00	26.53	5.43	14.52	274.27	984.40	64.93
3	570	Bankhandi	76°12′19″E	31°55′48″N	10.32	17.00	28.27	10.83	11.93	362.93	376.70	68.98
4	574	Dhaliara	76°10′00″E	31°55′19″N	5.15	33.00	23.17	0	8.67	259.70	566.70	73.11
5	580	Dehra	76°10′03″E	31°55′17″N	9.32	28.67	31.83	6.67	13.22	311.90	697.70	71.68
6	585	Bhaleth	76°35′23″E	31°31′26″N	6.37	22.00	42.60	12.97	12.44	298.10	626.40	58.76
7	662	Mataur	76°15′25″E	32°05′16″N	7.27	27.33	24.43	8.87	14.02	352.73	716.70	77.21
8	666	Thural	76°31′39″E	31°55′34″N	8.57	76.67	40.33	6.00	14.67	469.73	4165.80	78.43
9	667	Kangra	76°15′25″E	32°05′15″N	9.97	28.67	31.50	6.43	14.67	391.60	2135.60	68.74
10	668	Dob	76°15′21″E	32°10′04″N	9.67	26.67	55.33	8.33	12.62	373.97	1816.70	66.41
11	783	Jiya	76°17′58″E	32°06′32″N	7.61	21.33	44.57	4.77	12.65	189.73	527.30	72.76
12	791	Ropri	76°32′45″E	31°59′17″N	8.87	28.00	33.90	3.43	11.36	238.13	856.70	76.84
13	792	Harityangal	76°37′22″E	31°31′31″N	6.67	27.67	47.43	9.93	16.59	517.83	6533.30	68.21
14	795	Ummer	76°32′45″E	31°59′16″N	9.27	33.00	14.53	4.20	13.67	291.80	931.20	70.19
15	815	Kalari	76°31′19″E	31°31′29″N	6.27	36.67	31.80	1.40	13.86	245.93	903.60	72.85
16	850	Hamirpur	76°34′22″E	31°36′25″N	7.27	32.67	24.83	5.20	13.80	302.23	846.50	66.69
17	900	Chander	76°38′48″E	31°12′38″N	7.57	31.67	42.27	6.90	11.92	265.13	498.70	66.19
18	960	Parour	76°23′48″E	32°12′38″N	6.00	35.00	32.09	29.07	10.05	279.77	485.20	72.98
19	960	Panchrukhi	76°33′50″E	32°01′55″N	7.80	16.00	49.87	6.67	12.30	251.40	452.20	72.90
20	973	Rangru	76°33′49″E	32°01′53″N	6.12	34.67	31.93	4.87	13.94	313.67	647.50	63.58
21	990	Lahla	76°33′46″E	32°04′32″N	6.26	19.67	31.17	5.77	11.43	343.63	598.70	71.58
22	1010	Daroh	76°28′38″E	31°45′32″N	7.14	32.67	31.07	9.83	11.82	330.50	697.30	67.68
23	1015	Bari	76°32′42″E	31°48′38″N	6.37	24.00	58.93	10.27	13.15	263.10	785.20	74.33
24	1030	Dehan	76°35′45″E	31°45′38″N	5.45	37.00	19.00	0	13.94	353.00	1146.70	68.66
25	1100	Yashwantnagar	77°12′43″E	30°53′29″N	9.20	33.00	21.80	10.83	13.94	309.80	635.40	76.18
26	1250	Kalaghat	77°10′36″E	30°52′15″N	7.20	26.67	42.53	5.13	15.60	413.40	2000.50	73.52
27	1280	Bindraban	76°27′08″E	32°06′10″N	5.20	26.33	43.43	8.80	13.45	285.27	613.70	71.27
Critical difference ($P = 0.05$)					1.25	2.91	3.28	1.28	0.97	30.97	119.730	0.75

Bangalore, India). PCR amplification was performed with initial denaturation at 94°C for 5 min followed by 39 cycles of denaturation at 94°C for 1 min, primer annealing at 57°C for 1 min, elongation at 72°C and a final extension at 72°C for 7 min. The amplified products were electrophoresed in 1 × TAE buffer at 100 V for 2–3 h in a 1.8% agarose gel using 1-kb DNA ladder as standard. The gels were stained with ethidium bromide (0.5 μg/mL) and the PCR products were visualized and photographed using the Gel-Documentation Unit (Bio-Rad, Hercules, USA).

Binary matrices were built by combining RAPD and ISSR data, attributing '1' to the presence and '0' to the absence of a band. The Jaccard genetic similarity matrix was used to build an unweighted pair-group method with arithmetic means (UPGMA) dendrogram using NTSYS-pc v2.0 (Rohlf 1993). WINBOOT software (Yap and Nelson 1996) was used for the assessment of robustness of the dendrogram typology and the estimation of robustness of cluster analysis.

Results and discussions

Accessions were variable for all morphophysiological traits including petiole length, density of lenticels, stomatal and trichomal density, stem diameter, plant length, plant biomass and total carbohydrates. The accessions grown in common environmental and soil conditions showed distinct variation, which suggested existence of genetic variation among different genotypes. Some key examples include mean petiole length which was 7.61 cm with a range of 5.15–10.37 cm. Density of lenticels varied from 16–76.67 per 10 cm with a mean of 29.95 cm, while stomatal density varied from 14.53–58.93 mm^2 with a mean of 34.50 mm^2. Significant variation was also recorded for morphometric traits. Mean stem diameter was 13.03 mm, with a range of 8.67–16.59 mm. A fivefold difference in plant biomass was observed with a mean of 1169.77 g per plant. Plant length varied from 189.73–517.83 cm with a mean of 316.10 cm. Similarly, the accessions differed significantly for total carbohydrate content. Total carbohydrate content ranged from 58.76–78.43 mg/g with a mean of 70.53 mg/g in the accessions evaluated. Cytological analysis showed that all accessions were diploid ($2n = 2x = 22$).

The genetic similarity matrix generated using pooled RAPD and ISSR data ranged from 0.33 to 0.79 with an average of 0.56. The results of the consensus tree indicated that the dendrogram (figure 1) separated accessions into three main clusters at 33% variation. Cluster A was the largest cluster and consisted of 27 accessions that formed four subclusters (49.5% similarity). Subclusters I, II, III and IV consisted of 2, 14, 8 and 2 accessions, respectively. The subcluster I showed 56% genetic similarity, whereas subcluster II, III and IV showed a genetic similarity index of 57.0, 59.0 and 57.4 per cent. The accession 33 did not cluster with any group. Cluster B consisted of seven accessions showing 48.0% similarity. Three accessions, namely 15, 18 and 36 formed cluster C at 49% similarity level being the most divergent. The bootstrap support was high in case of combined RAPD-ISSR data used indicating its robustness.

There are limited reports of genetic diversity analysis using molecular markers in genus *Tinospora* (Ahmad *et al*. 2006; Rout 2006). There are also seminal reports on genetic variability studies in *T. cordifolia*, which is necessary to initiate a breeding programme (Shinde and Dhalwal 2010). The genetic diversity values deduced through molecular markers indicates the degree of heterogeneity in the accessions. However, clustering of majority of accessions in one group reflected the narrow genetic structure of *Tinospora* populations in the region. The patterns of genetic structure in plant populations are mainly related to the species life history and breeding system, and knowledge of these patterns is necessary for the management, use and conservation of biological diversity (Zanella *et al*. 2011). *T. cordifolia* is diocious in nature and is primarily propagated through vegetative means. This condition precludes genetic recombination and reshuffling of genes that is manifested in the form of genetic variation. The species is used in almost every household in the Himalayan region of India for the cure of various ailments including that of cattle. This practice encourages natural way of *in situ* conservation of this species. The perpetuation of genetically similar genotypes across large geographical region is a possibility, which is evident from the study. The levels of genetic diversity and the pattern of the population's structure may be related to the low dispersal of seeds, clonal reproduction, and the population's colonization history.

No correspondence was observed between plant biomass and CHO content and genetic clusters developed on the basis of DNA marker data. The accessions 15, 18 and 36 (figure 1) have been placed distantly from rest of the accessions, suggesting high levels of diversity present compared to other accessions or they may be genetically highly heterogeneous in nature. In all, neither the pattern of genetic diversity corresponded with the elevation or place of collection, nor did it always match with the plant biomass and CHO content. Similar observations have been made earlier in case of Cymbopogon (Kumar *et al*. 2009). In general, the chemical elites superior for CHO content were different and did not match with the morphological elites. However, accessions 8 and 23 (both in cluster A) were superior for both morphological and CHO content, hence can be genetically and commercially exploited.

The inconsistency observed between molecular diversity and morphological diversity cannot be easily explained. However, the lack of perfect congruence between morphological and molecular data suggests that the morphological system may be useful for the morphotypes management but is not appropriate to study the genetic structure of the *T. cordifolia*. For example, accessions 15 and 18 are divergent as per RAPD as well as ISSR grouping while they are quite similar to other genotypes based on

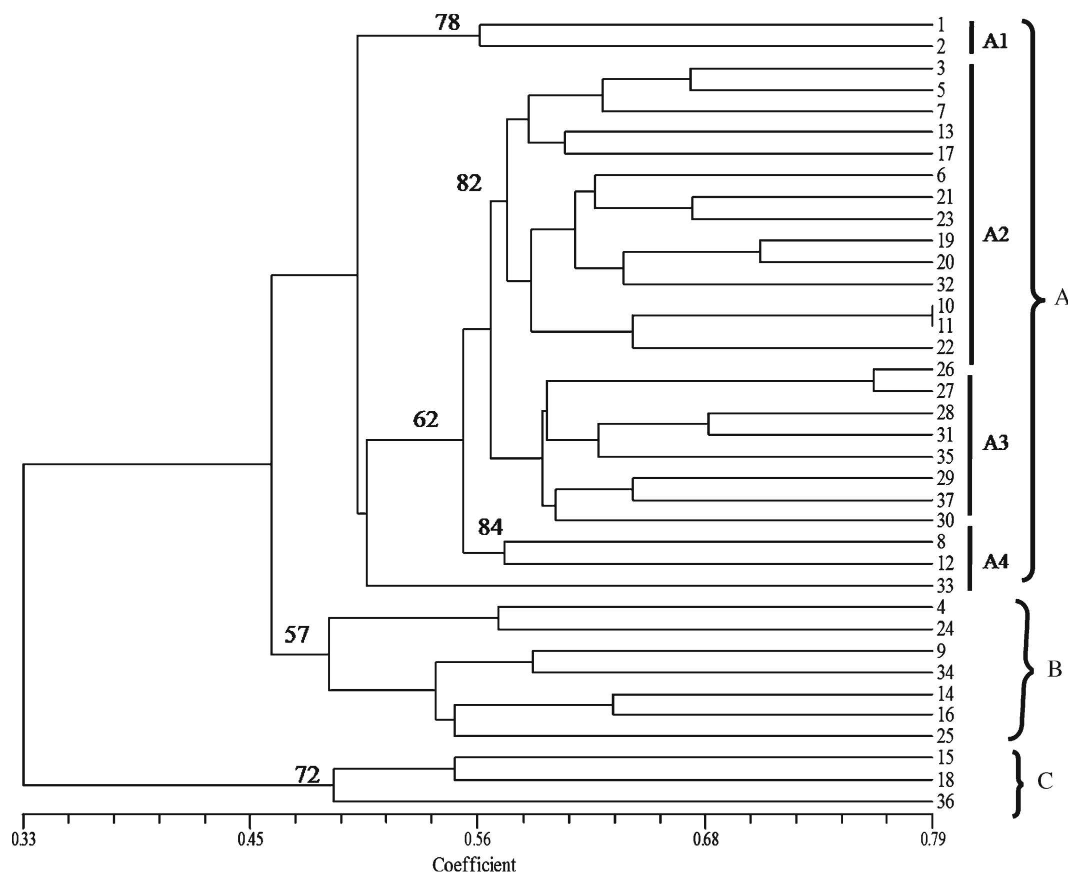

Figure 1. Dendrogram of 37 *Tinospora* accessions based on RAPD and ISSR data. The number of branches represents bootstrap values generated by 1000 replicates using the Winboot program. Serial numbers and geographical location of the accessions correspond to table 1.

morphological similarity. Similar morphotypes can be the result of different genotypes under a set of genotype × environment interactions and vice versa (Singh *et al*. 2011). The investigation is based on limited number of markers, and hence further analysis with more number of functional makers particularly from genes involving biosynthetic pathway of carbohydrate content and genes governing morphological characters is required that can give better picture of genetic relationship among the accessions. The study also suggests that evaluation and characterization of germplasm through both DNA as well as morphological markers validate the existence of genetic diversity among *Tinospora* populations of northwestern Himalayan region, however, there is need to formulate effective collection and conservation strategies.

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