Frontiers in Health Informatics
ISSN-Online: 2676-7104
www.healthinformaticsjournal.com
2024; Vol 13: Issue 8
Open Access

VARIABILITY ASSESSMENT AMONG MONOTYPIC GENUS *TAMARINDUS* DISTRIBUTED IN ASSAM (INDIA) USING ISSR MARKERS

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Cite this paper as: Devanjal Bora, Biswajit Paul, Masram Pravin R., Manjula Yadav, Pradip Mohurle, Raj Kumar Pegu (2024). Variability assessment among monotypic genus *tamarindus* distributed in assam (india) using issr markers. *Frontiers in Health Informatics*, *13 (8) 6250-6263.*

ABSTRACT:

Tamarindus popularly known as Tamarind is a tropical or semi-tropical evergreen tree which can withstand drought for extensive period of time and hence, sustained as monotypic genus having various environmental and social issues, as human and animal food, a medicine and used for fuel as well. Several ISSR markers were used to study the genetic diversity and population structure of this important and monotypic medicinal plant collected from six different Agro-climatic zones of Assam within Northeast India as there is no information on the available genotypes of the species in the study area. Fresh and healthy leaf samples were collected from sites geographically located between 22°19' to 28°16' N latitude and 89°42' to 96°30' E longitude with elevation varying between 45 m to 1,960 m spread over an area of 78,438 km^2. Morphological characterizations of vegetal variants were done along with extraction of DNA with CTAB method followed by PCR technique. Dendrograms developed using the UPGMA and AMOVA was done with the help of GenAlEx for determination of genetic differentiation between six populations. Wide morphological variation for qualitative morphological traits among identified 94 *T. indica* genotypes across six different Agro-climatic zones of Assam is justified by Cophenetic Correlation Coefficient (CP) which is 0.8280; and Molecular variance (95% among populations and 5% within populations).

The analysis of individuals (variables) using polymorphic ISSR revealed high value of genetic diversity within Assam and clearly separate populations of southern Assam under Hills zone and Barak valley zone from Upper Brahmaputra valley and North Bank plain zone; and Lower Brahmaputra valley and Central Brahmaputra valley zones of Assam. This is the pioneer study of this kind in *T. indica* from this region of Northeastern India proximate to the eastern Himalaya mega biodiversity hotspot.

KEYWORDS: Agro-climatic zones, Assam, genotypic variability, ISSR markers, monotypic taxa, *Tamarindus*

INTRODUCTION:

The genus *Tamarindus* Tourn ex L. belonging to the family Fabaceae (subfamily Caesalpinioideae) is considered

monotypic as having only one species *T. indica* L. popularly known as Tamarind[1]. Plants (trees) are distributed through tropical or semi-tropical evergreen areas and reach a range of heights between 20 to 30 feet. Growth is very slow and can withstand drought for extensive period of time[2]. The plant is having 24 (2n) numbers of chromosomes[3] and capable of cross pollination within populations resulting in phenotypic variations[4]. This phenotypic variability enables Tamarind to adapt to variations in environment and boost the survival capacity and increase the chances of evolutionary changes like structural changes in flowers mainly the separation among the anthers and the stigma[5]. The fruit of the plant is multipurpose and has been used for edible purposes in many preparations including juices, jams or chewing gums and spices[6,7] and leaves are used as vegetables, as timber and fuel and fodder for animals[8,9,10] too in many parts of the world including India. In case of commercial production, India stood as world's largest producer of tamarind[11] and ranked 6th in the export market[12]. The fruit is a good source of iron, phosphorus, tartaric acid and calcium and an excellent source of secondary metabolites like riboflavin, thiamin, and niacin; but it contains only small amounts of vitamins A and C[13]. The physico-chemical and frictional characteristics was extensively studied for pharmaceutical applications[2]. Other than the medicinal use, the seed has significant use in the production of paper, jute, adhesive and preservatives in textile industry for printing and sizing purposes[14]. In many states of India, it is used in fortified wine production where almost all the parts of the plant (including seed, wood, leaves, bark, and roots) in dried form were used for flavoring and for taste[15]. It is also worth mentioning that the tree has a significant role in mitigating climate change as it is useful for the protection of fragile ecosystem affected by soil erosion which has been well studied in Africa[16].

In cross pollination, the absence of usual and alternative pollinators may lead to the evolution of self-pollination and this obviously will lead to reproductive isolation[17]. As *Tamarindus* also depend on different pollinators for reproduction in different geo-climatic zones; the pollinators shift as well as geographical variation will contribute to reproductive isolation leading to speciation and phenotypic variations as well as variations in chemical constituents; it is selected for study to access its genetic variability along six agro-climatic zones[18] throughout the state of Assam in India. The state is located between foot hills of Eastern Himalayas and earmarked by Patkai and Naga hill ranges from other sides. The knowledge of population structure and genetic variation is a prerequisite for conservation, sustainable utilization, and genetic improvements for any medicinal plant species, especially when it is a monotypic one. Due to high demands, medicinal plants are extensively exploited from their natural habitat posing a threat to their long-term survival and maintenance of sufficient genetic variability to cope with selection pressures exerted by ever-changing environmental factors[19]. The ability of a plant species to maintain sufficient genetic diversity depends on many factors including long-term evolutionary history, genetic drift, gene flow among the population, mode of reproduction, and mating system of the species[20]. Therefore, an estimate of genetic diversity and population structure is very much essential for designing conservation strategies and sustainable utilization of any medicinal plant[21]. Such efforts to understand the distribution, genetic diversity, and population structure of monotypic taxon *T. indica* are lacking. Although genetic diversity studies using DNA-based marker techniques are studied by several workers in Tamil Nadu[22,23,24]; in Karnataka[25]; and in South India[26] in the particular species, not a single such study was made in *T. indica* from Northeastern part of India. On the otherhand, in some other parts of India, the genetic diversity studies of the species based on morphometric,quantitative and qualitative characters have been done so far[27-30] which is also missing in the present study area.

However, several works on the Ethnobotany and ethnopharmacology of *T. indica* were done in this region where it was reported to be used in the management of jaundice[31] and its antioxidant and hepatoprotective effects were tested and found corroborative[32] along with its analgesic[33] and antiasthmatic properties[34]. Phytochemical studies of tamarind seeds were done and showed the presence of some bioactive compounds including presence of proteins, carbohydrates, and secondary metabolites like phenols, sapponins, tannins, and flavonoid etc.[35]. Even the fruit gum and pulp extract was successfully tested for preparation of mucoadhesive as a binder for tablets[36], to formulate herbal deodorant[37] and to develop Matrix tablets with tamarind seed mucilage[38]. Further, kinetic and equilibrium studies of tamarind fruit shell powder was also studied to test biosorption of heavy metals where the results were favouarable to removal of manganese[39] and zinc[40] from waste water to mitigate heavy metal pollution.

Descriptors of morphological attributes have been used as basic character in identification of plants, in breeding, commercialization, conservation of plant resources, cluster analysis for understanding of genetic similarities and dissimilarities[41,42] including components of flowers and fruits such as shape, color and general appearance[29]. As morphological descriptors have limitations in distinguishing hierarchical taxa[43], this study employed these descriptors for monotypic species. In respect to genotypic variation study, Inter Simple Sequence repeats (ISSRs) are more efficient among others and highlypolymorphic and reproducible which offers a rapid method of determination of diversity by combining AFLPs, SSRs, and RAPDs[44]. In the present work, we used several ISSR markers which were selected on the basis of prior screen before designing the present study methodology to study the genetic diversity and population structure of this

important and monotypic medicinal plant collected from six different Agro-climatic zones of Assam within Northeast India as there is no information on the available genotypes of the species in the study area which have limited the possibility of improvement of the accessions using old and conventional methods of collection for various R&D and industry.

MATERIAL AND METHODS:

Plant material & area of collection

Fresh and healthy samples free from disease and pest were collected from observed locations in surveys made during 2021-23 from six Agro-climatic zones of Assam (maximum 20 locations in each zone) geographically located between 22°19' to 28°16' N latitude and 89°42' to 96°30' E longitude spread over an area of 78,438 km^2 with elevation varying between 45 m to 1,960 m (Fig. 1). Information on the sample collection sites of genotypes of *T. indica* in the present study is provided in Table 1. Eleven major qualitative traits (vegetative and reproductive) were documented from the observed trees and from three to five randomly selected flower/fruit bearing branches of each genotype for documenting the observations. International Union of Plant Protection of New Vegetal Variants[45], and International Committee of Genetic Resources of Plant for the description of tropical plants[46,47,48] were followed for the morphological characterization of *T. indica* according to their descriptors. Fresh and young leaf samples were collected from wild, and then wrapped with cotton (wet) followed by putting in a airtight pouch (plastic) and kept in ice box to carry to the laboratory so that loss of moisture can be prevented. Labeled materials were kept in -20 degree for further study in the department of Botany, Assam Don Bosco University (ADBU) and voucher specimens (verified with Acc. 8859 of ASSAM at BSI Shillong) were kept in NEHAR, CARI Guwahati.

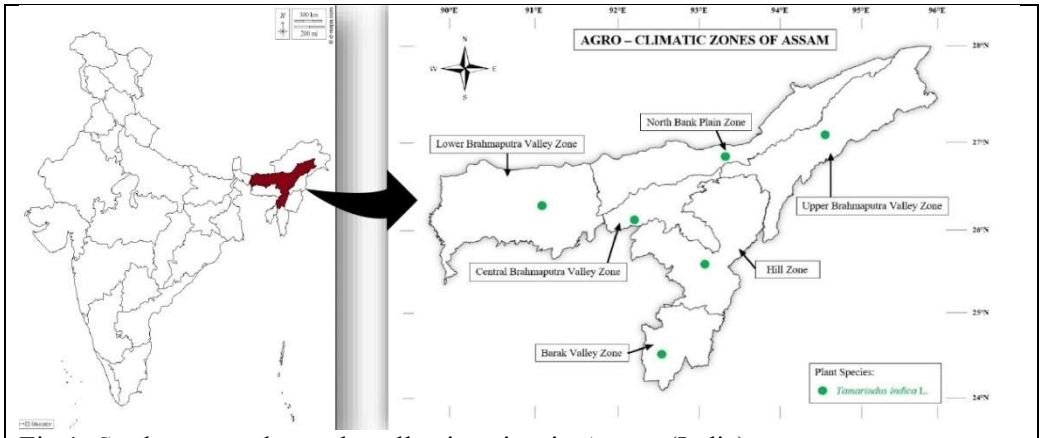


Fig 1: Study area and sample collection sites in Assam (India)

Table 1: Information on the sample collection sites of genotypes of *T. indica* in the present study

Agro-climatic zones	Code	Accession	Collection site	GPS coordinates
Upper Brahmaputra valley zone (Z1)	T1	T1-1	Bokajan, Golaghat	26.569095, 93.736911
		T1-2	Golaghat, Golaghat	26.500084, 93.960675
		T1-3	Titabar, Jorhat	26.585091, 94.205275
		T1-4	Majuli, Majuli	26.928372, 94.171899
		T1-5	Teok, Jorhat	26.812478, 94.426710
		T1-6	Demow, Sivasagar	27.111779, 94.737340
		T1-7	Lakwa, Sivasagar	27.006505, 94.860152
		T1-8	Sonari, Charaideo	27.019857, 95.021820
		T1-9	Longpotia, Sivasagar	27.061623, 95.096110
		T1-10	Dibrugarh, Dibrugarh	27.457113, 94.903715
		T1-11	Tengakhat, Dibrugarh	27.370000, 95.168538
		T1-12	Namrup, Dibrugarh	27.172673, 95.348313
		T1-13	Digboi, Tinsukia	27.383132, 95.630724
		T1-14	Margherita, Tinsukia	27.275839, 95.676419
		T1-15	Kakopathar, Tinsukia	27.631541, 95.677078
North Bank plain zone (Z2)	T2	T2-1	Tangla, Udalguri	26.651454, 91.906543
		T2-2	Kabirali, Darrang	26.642401, 92.033897

		T2-3	Khoirabari, Udalguri	26.599193, 91.826780
		T2-4	Kalaigaon, Udalguri	26.582805, 91.973182
		T2-5	Hugrajuli, Sonitpur	26.807740, 92.392276
		T2-6	Chamuagaon, Udalguri	26.713285, 92.343329
		T2-7	Gadhaijhar, Darrang	26.569910, 92.207397
		T2-8	Dhekiajuli, Sonitpur	26.693134, 92.496879
		T2-9	Behali, Biswanath	26.840797, 93.376240
		T2-10	Biswanath Chariali, Biswanath	26.714584, 93.141972
		T2-11	Monabari, Biswanath	26.772079, 93.236229
		T2-12	Gohpur, Sonitpur	26.871975, 93.604941
		T2-13	North Lakhimpur, Lakhimpur	27.226929, 94.110129
		T2-14	Gogamukh, Dhemaji	27.424467, 94.325601
		T2-15	Dhakuakhana, Lakhimpur	27.300184, 94.464441
		T2-16	Silapathar, Dhemaji	27.589694, 94.731308
		T2-17	Jonai, Dhemaji	27.829563, 95.240100
Lower Brahmaputra valley zone (Z3)	T3	T3-1	Barkajuli, Baksa	26.671570, 91.530093
		T3-2	Khamari Gaon, Baksa	26.672249, 91.402244
		T3-3	Panbari, Chirang	26.619044, 90.832368
		T3-4	Ballomguri, Chirang	26.638005, 90.645242
		T3-5	Borkukuria, Kamrup	26.442650, 91.585877
		T3-6	Malipara, Barpeta	26.279699, 91.188972
		T3-7	Jania, Barpeta	26.331782, 90.918859
		T3-8	Tapatari, Bongaigaon	26.319531, 90.694557
		T3-9	Lalkura, Dhubri	26.236876, 90.385572
		T3-10	Garbhanga, Kamrup (Metro)	26.035585, 91.699171
		T3-11	Dakuapara, Kamrup	25.973848, 91.245041
		T3-12	Sildubi, Goalpara	25.953242, 90.537282
Barak valley zone (Z4)	T4	T4-1	Hailakandi, Hailakandi	24.449214, 92.555544
		T4-2	Patharkandi, Karimganj	24.600151, 92.325134
		T4-3	Dulbacherra, Karimganj	24.480257, 92.430972
		T4-4	Bidyanagar, Karimganj	24.517735, 92.462552
		T4-5	Lalpani, Cachar	24.517093, 92.726449
		T4-6	Katlicherra, Hailakandi	24.462409, 92.553252
		T4-7	Algapur, Hailakandi	24.863678, 92.688499
		T4-8	Badarpur, Karimganj	24.861399, 92.560241
		T4-9	Silchar, Cachar	24.831555, 92.779041
		T4-10	Lakhipur, Cachar	24.784030, 93.019753
		T4-11	Udarbond, Cachar	24.881381, 92.874065
		T4-12	Kachipur, Cachar	24.832657, 92.838019
		T4-13	Boalipar, Hailakandi	24.723856, 92.588316
		T4-14	Kachudaram, Cachar	24.679707, 92.944637
		T4-15	Silcoorie, Cachar	24.720295, 92.762752
		T4-16	Binnakandi, Cachar	24.717710, 92.982439
		T4-17	Kajidahar, Cachar	24.708234, 92.832963
Central Brahmaputra valley zone (Z5)	T5	T5-1	Amsing, Kamrup (Metro)	26.141135, 91.896437
		T5-2	Chandrapur, Kamrup (Metro)	26.207023, 91.898437
		T5-3	Mayang, Morigaon	26.242427, 92.024611
		T5-4	Sonapur, Kamrup (Metro)	26.030042, 91.919847
		T5-5	Jagiroad, Morigaon	26.144005, 92.183211
		T5-6	Morigaon, Morigaon	26.232668, 92.325272
		T5-7	Pabitara, Morigaon	26.209749, 92.067431

		T5-8	Khetri, Kamrup (Metro)	26.102092, 92.081303
		T5-9	Bhuragaon, Morigaon	26.392282, 92.248680
		T5-10	Bangaldhara, Morigaon	26.180658, 92.381095
		T5-11	Sonari Gaon, Nagaon	26.451714, 92.471873
		T5-12	BhumuraGuri, Morigaon	26.404477, 92.557986
		T5-13	Nagaon, Nagaon	26.339700, 92.693324
		T5-14	Roha, Nagaon	26.217418, 92.530543
		T5-15	Hojai, Hojai	25.992555, 92.846449
		T5-16	Lanka, Hojai	25.912852, 92.960884
Hills zone (Z6)	T6	T6-1	Baithalangso, West Karbi Anglong	25.923502, 92.439286
		T6-2	Hamren, West Karbi Anglong	25.836416, 92.572702
		T6-3	Panimur, Dima Hasao	25.708637, 92.822054
		T6-4	Umrangso, Dima Hasao	25.505044, 92.738467
		T6-5	Howraghat, Karbi Anglong	26.514172, 93.429855
		T6-6	Diphu, Karbi Anglong	25.839180, 93.439920
		T6-7	Barlangfer, Karbi Anglong	25.676623, 93.294729
		T6-8	Langting, Dima Hasao	25.496723, 93.115590
		T6-9	Dijao, Dima Hasao	25.497837, 93.170361
		T6-10	Wadrengdisa, Dima Hasao	25.493968, 93.197305
		T6-11	Lungdingkro, Dima Hasao	25.467989, 93.165874
		T6-12	Semkhor, Dima Hasao	25.259336, 93.303514
		T6-13	Maibang, Dima Hasao	25.298530, 93.136185
		T6-14	Purana Hajong, Dima Hasao	25.339851, 93.215915
		T6-15	MupaNobdi, Dima Hasao	25.312542, 93.214183
		T6-16	Haflong, Dima Hasao	25.167503, 93.015450
		T6-17	Mahur, Dima Hasao	25.178598, 93.117776

DNA Extraction

Quantity of DNA extracted from varied samples may vary depending upon the content of common metabolites like polyphenols and polysaccharides etc. As there is no any universal DNA protocol for the targeted species, a powerful detergent, cetyltrimethylammonium bromide (CTAB) which captures lipids from cell membranes and facilitate nuclear content to release is selected for the purpose of DNA extraction[49]. Resulted pellets obtained after centrifugation was dissolved in TE buffer for further quality test.

Quality Checking of DNA

The 0.8% agarose gel was used to study the results of DNA extraction and 2% agarose gel was used to view the products of PCR. The gel then was put on standard electrophoresis followed by adding 1x TBE on both sides till it sank. Then, 5 µl of DNA was mixed with dye 1 µl loading included in the gel cautiously. Electrophoresis was carried out at 100V for 30 minutes. Finally for visualization of DNA bands, the gel was kept on the Bio step UV trans-illuminator for analyzing and checking the DNA quality[50].

PCR Analysis with ISSR

PCR analysis was conducted by mixing 2 µl Primer ISSR (total twenty one Primers were scanned) with the reagents (12,5 µl My Taq Red Mix), 9 µl ddH_2O, and 1.5 µl DNA Template making a total volume of 25µl. PCR Rotor-Gene Q machine was used for amplification. This study used twenty ISSR primers for screening of suitable primers with concentrations of each primer being 0.4 µM. Initial denaturation was done at 95°C lasted for five minutes which was followed by 35 cycles (denaturation at 95°C for 30 seconds, annealing at 45°C to 60°C (depending on the primer) for 30 seconds, extension at 72°C for 30 seconds) and final extension at 72°C for 10 minutes. To ensure the stability of amplification, three replications of all PCR reactions were carried out. For negative controls, all substrates with a mix without adding DNA were amplified. The twenty one ISSR primers selected[51-55] for the screening are in Table 2 where seven finally selected Primers (with * in list) were screened out for further analysis. The amplification products were separated in size on DNA electrophoresis (Mupid-next submarine electrophoresis) system with 0.8% agarose gel in 1x TBE buffer at 100V lasted for 30 minutes at a standard room temperature. Photography of gel was done under the Bio step UV Light Trans-illuminator where gel was coloured with ethidium bromide for easy view. The PCR results were analyzed by

using a scoring system.

Table 2: List of Primers with sequence and respective annealing temperature

Sl. No.	Primer code	Primer Sequence	Annealing temperature
1*	ISSR-UBC-807	5' AGAGAGAGAGAGAGAGT 3'	50.0° C
2*	ISSR-UBC-807	5' AGAGAGAGAGAGAGAGT 3'	47.0° C
3*	ISSR-UBC-809	5' AGAGAGAGAGAGAGAGG 3'	47° C
4*	ISSR-UBC-812	5′ GAGAGAGAGAGAGAGAA 3′	50.0° C
5	ISSR-UBC-817	5' CACACACACACACACAA 3'	51.7° C
6*	ISSR-UBC-818	5′ CA CACACACACACA CAG 3′	47° C
7	ISSR-UBC-825	5' ACACACACACACACACT 3'	45.0° C
8	ISSR-UBC-826	5' ACACACACACACACACC 3'	55.0° C
9	ISSR-UBC-827	5' ACACACACACACACACG 3'	56.5° C
10	ISSR-UBC-835	5' AGAGAGAGAGAGAGAGYC 3'	56.9° C
11	ISSR-UBC-836	5' AGAGAGAGAGAGAGAGYA 3'	56.9° C
12	ISSR-UBC-841	5' GAGAGAGAGAGAGAGAYC 3'	58.0° C
13	ISSR-UBC-842	5' GAGAGAGAGAGAGAGAYG 3'	59.8° C
14	ISSR-UBC-855	5' ACACACACACACACACYT 3'	55.9° C
15	ISSR-UBC-856	5' ACACACACACACACACC 3'	56.9° C
16*	ISSR-HB-12	5' CACCACCACGC 3'	50.0° C
17*	ISSR-Primer-20	5' GAGAGAGAGAGAGAGAGAA 3'	47.0° C
18	ISSR-1	5' ACACACACACACACACTT 3'	51.0° C
19	ISSR-4	5' GAGAGAGAGAGAGAGAT 3'	50.0° C
20	ISSR-10	5' CTCCTCCTCCTCCTC CTC 3'	50.0° C
21	ISSR-11	5' AGGAGGAGGAGGAGGCC 3'	50.0° C

Data Analysis

For statistical analysis, scoring bands was done as per requirement of the statistical software. The scored band data was tabulated using excel and subjected to statistical analysis using DendroUPGMA available at http://genomes.urv.es/UPGMA/, for developing dendrograms, using the UPGMA (Unweighted Pair Group Method with Arithmetic Mean) algorithm mostly used in ecology and systematics[56] and in Numerical taxonomy[57]. Amplified bands were scored '1' if present, and '0' if absent regardless of the intensity of bands. Ambiguous bands were considered missing data and scored '-1' for use in software purpose. The output data is a dendrogram (original Greek term *dendron* meaning "tree" and *gramma* meaning "drawing") often used to illustrate the arrangement and organization of diverse and different clusters represented by U- shaped lines produced by hierarchical clustering where these lines connect data points in a hierarchical tree. However, dendrogram must not be similar to a phylogenetic tree as it does not bear any evolutionary information. For input data, numbers of set of variables are placed in rows and the number of variables is placed in columns to determine a similarity or a distance matrix. The Jaccard coefficient has been used to compare between sets of variables where Similarity Matrix and Distance matrix which are represented through Dendrogram in Newick format. Jaccard similarity coefficient

which is also known as Tanimoto coefficient is often used to measure the similarity between two sets of input binary data and the value is defined ranging between '0' and '1'as the size of the intersection divided by the size of the union of the sample sets. In Addition to this, the Cophenetic Correlation Coefficient (CP) is also measured to test how faithfully a dendrogram preserves the pairwise distances between the original unmodeled data points. It gives a value between '0' and '1', where '1' represents a perfect match. This coefficient is only calculated when the number of rows is lower with respect to sample size and population ratio.

For determination of genetic differentiation between six populations, (phiPT) was done that allows intra-individual variation to be suppressed and is therefore considered ideal for comparing codominant and binary data, with 999 permutations[58]. Determination of population differentiation at molecular level was performed by analysis of molecular variance (AMOVA) among the populations and within every population of the geographical region using GenAlEx - Genetic Analysis in Excel[59,60] available at http://biology.anu.edu.au/GenAlEx. Fine scale genetic structure was investigated using spatial autocorrelation analysis using GenAlEx 6.5[61] involving 999 permutations. Principal Coordinate Analysis (PCoA) was also done which is a multifaceted and multivariate technique allowing one to determine and plot the major patterns where a multivariate data set (having multiple loci and multiple samples) is involved. Though the mathematics involved in this process is complex, but PCoA is a process representable in graphical format by which the major axes of variation are located within a multidimensional data set easily understandable by the user. The technique followed by GenAlEx is based on an algorithm forwarded by Orloci, 1978[62].

Results and Discussion:

The morphological characterization of qualitative traits among individuals of all the six populations (collectively observed data) distributed in six Agro-climatic zone of the study area is enumerated in Table 3 and the results were observed critically.

Table 3: Record of collectively observed morphological variation for qualitative traits among the genotypes of *T. indica* in six Agro-climatic zones of Assam

Z	GenTy	N-LF	D-CP	D-LF	C-LF	A-LF	S-PD	S-SD	C-SD	C-FL	C-PL	F-BH
Zones	Genotypes	Nature of Life form	Density of canopy	Density of leaf	Colour of Leaf	Arrangement of leaves	Shape of fruit or Pod	Shape of Seed	Colour of seed	Colour of flower	Colour of Pulp	Fruit Bearing habit
Z1	T1-1 to T1-15	Erect	Medium	Medium	Green	Alternate	Curved	Rhomboid (regular)	Brown	Yellow, veins red	Dark brown	Regular
Z2	T2-1 to T2-17	Erect	Medium	Medium	Green	Alternate	Curved	Rhomboid (irregular)	Brown	Yellow, veins red	Dark brown	Regular
Z3	T3-1 to T3-12	Erect-Bushy	Spars	Medium-Dense	Light green	Alternate	Curved (short)	Nearly round	Dark brown	Yellow, veins red	Brown	Irregular
Z4	T4-1 to T4-17	Bushy	Dense	Dense	Dark green	Alternate	Curved (long)	Flattened	Reddish brown	Yellow, veins red	Brown	Regular
Z5	T5-1 to T5-16	Erect-Bushy	Spars	Dense	Light green	Alternate	Curved (short)	Irregularly round (small)	Dark brown	Yellow, veins red	Brown	Irregular

Z6	T6-1 to T6-17	Bushy	Dense	Medium-Dense	Dark green	Alternate	Straight – curve (long)	Flattened	Reddish brown	Pale Yellow, veins reddish	Brown	Regular

The observations of targeted *T. indica* genotypes pointed to a significant amount of variation and disparity in all the eleven morphological traits. Three different types of nature of life forms (tree) were recorded, most genotypes are bushy (36 per cent), followed by erect forms (34 per cent), and forms in between erect to bushy (30 per cent) with varying canopy density of dense (36 per cent), medium (34 per cent) and spars (30 per cent). Though all the arrangement of leaves are alternate, density of leaves in a twig varies between dense (35 per cent), medium (34 per cent) and medium to dense (31 per cent). The leaf colour of most of the genotypes are dark green (36 per cent) followed by green (34 per cent) and light green (30 per cent). In case of shape of fruit or pod, notably curved (77 per cent) and straight to curve (17 per cent) characters were observed; but in case of seed, three characters were observed as flattened (34 per cent), rhomboid (32 per cent including regular and irregular shapes) and nearly round or irregularly round (28 per cent). Similarly, in case of colour of seed, three characters were observed as reddish (34 per cent), brown (32 per cent) and dark brown (28 per cent including some with more blackish in colour). Colour of pulp and flowers were also observed where flower colour varies between yellow with red veins (77 per cent) and flower colour pale yellow with reddish veins (17 per cent); and in case of pulp, brown (62 per cent) and dark brown (32 per cent). Over 66 percent of the fruits showed the regular and about 28 percent irregular or uneven type of fruit bearing habit. The observation in eleven characters clearly gives us 2 to 3 set of data in each character which corresponds to statistical analysis of genetic data described below. These morphological traits used in this study could be considered as important phenotypic marker for further evaluation of other monotypic species.

The present study revealed ample variation for eleven qualitative morphological characteristics among identified 94 *T. indica* genotypes across six different Agro-climatic zones of Assam. The variation is mainly influenced by the factors such as different agricultural practices and land-use types where several environmental and growing climatic factors influence possible gene flow or genetic drift of gene for a particular trait. The spontaneous and impulsive mutation perhaps occurred in a particular character over the years (may be expressed in one geographical area among many) may be counted among the reasons for variation in a particular morphological trait. Among various factors, besides changes in the climatic condition, availability or unavailability of nutrients, presence of human and animal activities in the natural habitat and growing abiotic and biotic stress may also cause the variability in morphological traits within a species in general and especially in a monotypic species as it has limitations in variability. Understanding the diversity at phenotypic traits will lead to developing principles to conserve the diversity and identifying the productive types which could help researcher and breeder to select the desirable genotypes for further improvement.

The seven primers used to screen ISSR diversity of T. indica populations generated 94 polymorphic amplification fragments across the whole sample collected from six agro-climatic zones (Fig. 2). Results of AMOVA among the six populations and within every population of the samples in study area is represented through similarity matrix (Table 4) and distance matrix (Table 5) using the Jaccard coefficient and represented through Dendrogram in Newick format (Fig. 3). To test the faithfulness of the dendrogram, the Cophenetic Correlation Coefficient (CP) is also measured and found 0.828085975727652 which is a significant result in terms of data prejudice. As the CP value is significantly lower within the range of 0 to 1; this indicates less similarity between the samples tested.

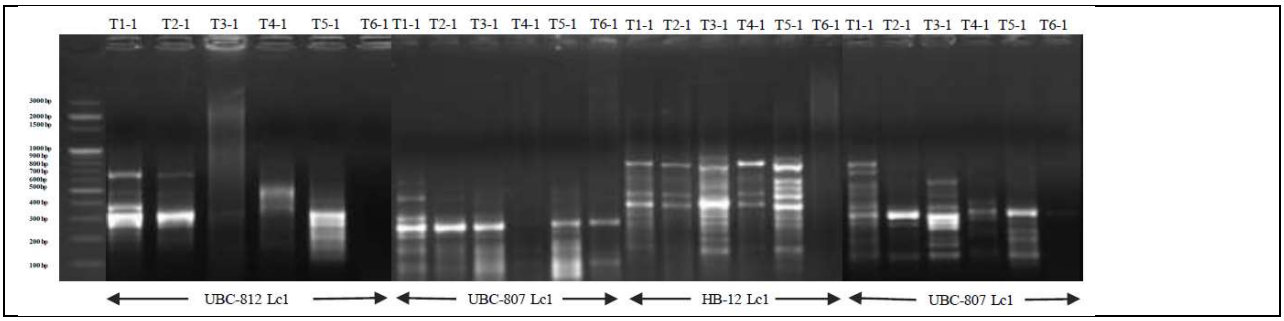
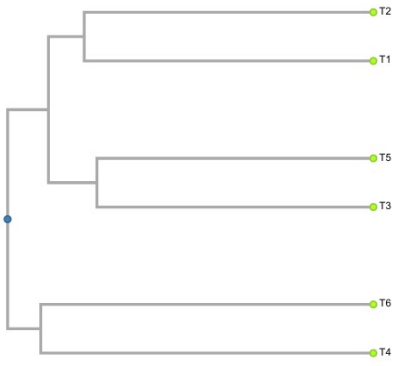


Fig. 2: ISSR profiles obtained after amplification of 6 populations of *T. indica* from six agro-climatic zones



: North Bank plain zone

: Upper Brahmaputra valley zone

: Central Brahmaputra valley zone

: Lower Brahmaputra valley zone

: Hills zone

: Barak valley zone

NB: (T1:0.340, T2:0.340):0.042, (T3:0.325, T5:0.325):0.057):0.048, (T4:0.391, T6:0.391):0.039)

Fig. 3: Output Dendrogram in Newick Format

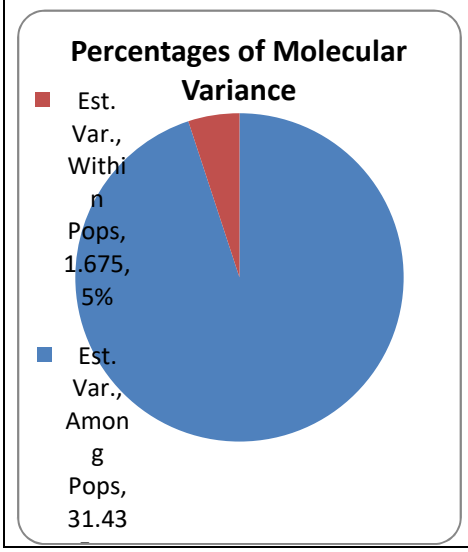
Table 4: Similarity Matrixbased on Jaccard coefficient

	T-1	T-2	T-3	T-4	T-5	T-6
T-1	1	0.321	0.240	0.171	0.214	0.082
T-2		1	0.284	0.260	0.211	0.122
T-3			1	0.197	0.350	0.088
T-4				1	0.133	0.219
T-5					1	0.079
T-6						1

Table 5: Distance Matrix based on Jaccard coefficient

	T-1	T-2	T-3	T-4	T-5	T-6
T-1	0	0.679	0.760	0.829	0.786	0.918
T-2		0	0.716	0.740	0.789	0.878
T-3			0	0.803	0.650	0.912
T-4				0	0.867	0.781
T-5					0	0.921
T-6						0

The Analysis of Molecular Variance (AMOVA) based on PhiPT values (Fig. 4) indicated that most of the genetic diversity occurred among populations (95%) while the variability within populations calculated as 5% only, to the observed genetic diversity. This implies that though the individuals of the same agro-climatic zone have similar attributes, these get differentiated in different agro-climatic conditions involving various factors. These factors may be influenced by human interference at various level and climatic variations over the period of time including the variations of pollinators.



Source	df	SS	MS	Est. Var.	%
Among Pops	5	2464.348	492.870	31.435	95%
Within Pops	88	147.439	1.675	1.675	5%
Total	93	2611.787		33.111	100%

Pop	T1	T2	T3	T4	T5	T6
n	15	17	12	17	16	17
SSWP	32.267	24.824	19.500	32.000	19.438	19.412

Stat	Value	P(rand >= data)
PhiPT	0.949	0.001

PhiPT max	0.975
Phi'PT	0.974

Fig. 4: Summary of AMOVA Analysis

Principal Coordinate Analysis (PCoA) results depicted in graphical format in Fig. 5 also corresponds the dendrogram prepared through UPGMA. The results clearly placed the individuals in three groups of clades where individuals of Hills zone and Barak valley are closely associated and distantly placed from other two classes.

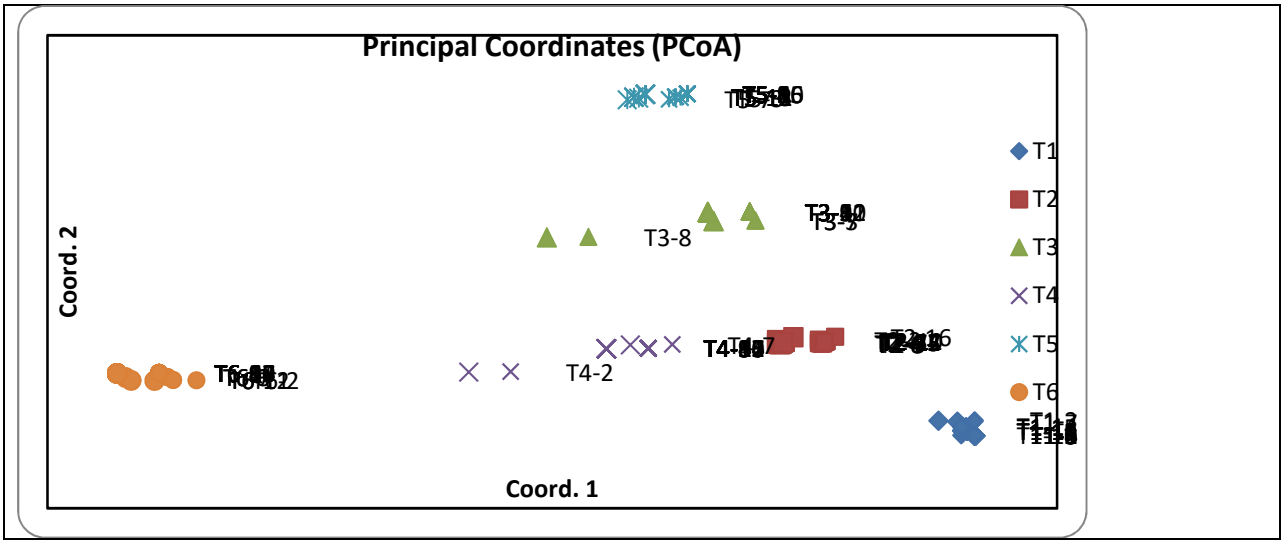


Fig. 5: Summary of Principal Coordinate Analysis

Though the morphological variations are highly linked with various climatic factors like land use types, high rainfall, domestication, biotic and abiotic stress etc. which influence the vegetative as well as the reproductive growth of genotypes;[37] and eventually reflects adaptive evolution or plasticity in phenotypic expression[63]. However, the present study reports genotypic variation too across agro-climatic zones. The variation in morphological traits suggests that there are extensive prospects and opportunities for phenotypic selection of the species for greater quantitative as well as qualitative upgrading in future productivity. These variations observed could be attributed to the different genetic, environmental and climatic influences as well as growing conditions of the tree species because all the agro-climatic zones are considerably different. Both the morphological and genetic diversity among the monotypic species could potentially form the foundation for future breeding programs, thereby contributing considerably towards medicinal and nutritional benefits, and income generation, in particular among the indigenous folk of Assam.

CONCLUSION:

The analysis of individuals (variables) among six different Agro-climatic populations of *T. indica* using polymorphic ISSR revealed high value of genetic diversity within different populations of Assam and clearly separate populations of southern Assam under Hills zone and Barak valley zone from Upper Brahmaputra valley and North Bank plain zone; and Lower Brahmaputra valley and Central Brahmaputra valley zones of Assam.The phenotypic and genotypic variation observed in different agro-climatic zones corresponds with the fact that there is variation of all climatic factors including pollinators who are sole responsible for continuation of life in general and in particular to this monotypic species. This is the pioneer study of this kind in *T. indica* from this region of Northeastern India proximate to the eastern Himalaya mega biodiversity hotspot. Six primerpairs with different strength (with varying conditions) were recommended that are useful for genotyping of a Monotypic tree species of a region, though there are no major morphological differentiation observed and no any lower taxa at sub-species and/or variety level of this taxon is established till now.

Source of support:

The authors declare that there is no source of financial support for the study, however, logistic support received from ADBU and CARI Guwahati to carry out the research work.

Acknowledgments:

Authors are grateful to Director of Research, Departmental Research Committee, ADBU for continuous encouragement and Director General, CCRAS for providing necessary support. Authors are also thankful to Dr. R. Bharali, Dept. of Molecular Biology and Biotechnology, Cotton University, Assam for his support in statistical analysis and fellow Researchers at CARI Guwahati, Kapil, Bimisa, Bikash, and Rohit for their support during field collection, laboratory experimentation and in preparation of the manuscript.

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