ISSN 1479-2621

Plant Genetic Resources: Characterization and Utilization (2021) **19**(2); 122–132
doi:10.1017/S1479262121000162

Assessment of genetic variability amongst cultivated populations of Khasi mandarin (*Citrus reticulata* Blanco) detected by ISSR

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Received 13 May 2020, revised 25 February 2021; Accepted 4 March 2021 – First published online 8 April 2021

Abstract

Khasi mandarin (*Citrus reticulata* Blanco) is a commercial mandarin variety grown in northeast India and one of the 175 Indian food items included in the global first food atlas. The cultivated plantations of Khasi mandarin grown prominently in the lower Brahmaputra valley of Assam, northeast India, have been genetically eroded. The lack in the efforts for conservation of genetic variability in this mandarin variety prompted diversity analysis of Khasi mandarin germplasm across the region. Thus, the study aimed to investigate genetic diversity and partitioning of the genetic variations within and among 92 populations of Khasi mandarin collected from 10 cultivated sites in Kamrup and Kamrup (M) districts of Assam, India, using Inter-Simple Sequence Repeat (ISSR) markers. The amplification of genomic DNA with 17 ISSR primers yielded 216 scorable DNA amplicons of which 177 (81.94%) were polymorphic. The average polymorphism information content was 0.39 per primer. The total genetic diversity ($H_T = 0.28 \pm 0.03$) was close to the diversity within the population ($H_S = 0.20 \pm 0.01$). A high mean coefficient of gene differentiation ($G_{ST} = 0.29$) reflected a high level of gene flow ($N_m = 1.22$), indicating high genetic differentiation among the populations. Analysis of Molecular Variance (AMOVA) showed 78% of intra-population differentiation, 21% among the population and 1% among the districts. The obtained results indicate the existence of a high level of genetic diversity in the cultivated Khasi mandarin populations, indicating the need for preservation of each existing population to revive the dying out orchards in northeast India.

Keywords: AMOVA, gene flow, genetic diversity, Khasi mandarin, polymorphism

Introduction

The citrus genetic resources hold great economic importance for the Indian fruit industry (Hazarika, 2012). Khasi mandarin (*Citrus reticulata* Blanco) is a commercially grown mandarin variety in northeast India and plays a vital role in the management of *Citrus* germplasm in India (Hazarika, 2012; Singh *et al.*, 2016). It is suitable for the local conditions and has been growing over a long period (Ghosh, 1977). It is grown prominently in the lower Brahmaputra valley of Assam, which is one of the leading states of mandarin cultivation in northeast India (Singh *et al.*, 2016).

Most of the valuable germplasm of Khasi mandarin in this region is usually maintained in home gardens, backyard gardens and a handful of commercially established orchards by the farmers since very old times (Malik *et al.*, 2013). But in the present scenario, these non-commercial home gardens as well as the commercial cultivations of Khasi mandarin have been dying out (Deshmukh *et al.*, 2017). This is due to genetic erosion, cultivation of commercial cash crops, extensive shifting cultivation, changing climatic conditions and nutrient imbalance (Hazarika, 2012). Most of the orange orchards in the regions are propagated through seeds without incorporating any

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improved techniques like budded/grafted material (Patel *et al*., 2010). Besides, the plant material of seedling origin is susceptible to various soil-borne diseases and insect pests which lead to a decline of the orchards in the region (Deshmukh *et al*., 2017). On the other hand, rootstocks are known to increase the vigour, tree size, improve pest resistance and adapt to climatic and soil conditions (Hussain *et al*., 2017). Moreover, most of the orange orchards are located on the uncovered steep areas with no proper soil conservation measures, and as a result, heavy rains wash away the nutrient-rich soil leading to relatively less fertile surface soil. This leads to an inappropriate drop in productivity of the orchards (Singh *et al*., 2016; Bhandari *et al*., 2017). Thus, the decline of the orchards warrants special attention to conserve the genetic variability in Khasi mandarin, which is still lacking and has not been given much attention (Singh *et al*., 2016). Therefore, assessment of the genetic diversity within and among the cultivated populations of Khasi mandarin is essential to design a suitable genetic resource management programme as well as to support the farmers to continue cultivating them (Malik *et al*., 2012). In view of this, neutral molecular markers such as Inter-Simple Sequence repeat (ISSR) are highly reproducible and cost-effective which have been used in a wide range of applications in Citrus (Uzun *et al*., 2010; Sharafi *et al*., 2017).

Thus, the present study was designed to address the following objectives (i): to assess genetic diversity within and among the cultivated populations of Khasi mandarin collected from 10 sites covering Kamrup and Kamrup (M) districts, Assam, India using ISSR markers, (ii) to reveal the partitioning of the genetic variations among the districts, within, and among the cultivated populations of Khasi mandarin and (iii) to assess the efficiency of ISSR markers in evaluating genetic diversity in the cultivated populations of Khasi mandarin.

Materials and methods

Plant material and DNA extraction

Fresh leaf tissue of 92 individuals of Khasi mandarin was collected from plants that were 5–7 years old and stored at −80°C until DNA extraction. The samples were obtained from privately owned orange orchards and commercial citrus farms, with the permission of the owners, located in 10 sites (hereafter addressed as populations) in Kamrup and Kamrup (M) districts, Assam, India (Fig. 1). The collection sites were Boko (BO, 12 samples), Bamunigaon (BA, nine samples), Kahebama (K, 10 samples), Chayygaon (CH, 10 samples), Santipur (S, 10 samples), Dhupdhara (DH, five samples) covering Kamrup district and Panbari (P, 10 samples), Khetri (KH, 10 samples), Sonapur (SO, six samples) and Nortap (N, 10 samples) covering Kamrup (M) districts of Assam, India. The samples were collected from each site with due permission from the holders of each private orchard and the supervisor of each commercial citrus farms. The samples were taken for herbarium and authenticated at the Department of Botany, Gauhati University (accession no. 18168). Total genomic DNA was isolated from leaf tissue (2 g) using the cetyl-trimethyl-ammonium bromide method as described by Doyle and Doyle (1990). The DNA pellet was dissolved in 100 µl of Tris-EDTA buffer and stored at 4°C. DNA was determined qualitatively by running in 0.8% agarose gel buffered with 1X TAE and then quantified on NanoDrop1000 UV-spectrophotometer (Thermo Fisher Scientific Pvt. Ltd, USA).

PCR amplification

A total of 100 ISSR primers initially identified by the University of British Columbia, Vancouver, Canada, were manufactured from Eurofins Genomics, Bangalore, India. An initial set of 50 primers was screened for PCR amplification and 17 primers that produced clear and reproducible banding patterns with the sample DNA were chosen for final analysis. The PCR amplification protocol was optimized and was performed in a final volume of 25 µl containing 25 ng template DNA, 1X Taq buffer with 15 mM of magnesium chloride, 0.1 mM dNTPs, 0.3 µM primer and 0.5 U Taq DNA polymerase. The PCRs were carried out in a Veriti thermal cycler (Applied Biosystems, USA) under the profile: 94°C for 4 min followed by 36 cycles of 94°C for 30 s, 50–56°C for 45 s, 72°C for 2 min and with a final extension of 72°C for 7 min. The amplified products were resolved in 2% agarose gel in 1X TAE buffer for 3 h at 90 V and the amplicons were detected by staining with ethidium bromide (Sigma-Aldrich, Mumbai, India) and photographed using the UVitech gel documentation system (Bangalore Genei, Bangalore, India). Molecular weights of the PCR products were estimated by comparing them with 1 Kb and ØX/HaeIII digest DNA ladders.

Data analysis

Since ISSR markers show dominant inheritance, the loci are therefore considered to produce one amplifiable allele and the other null allele. Our study adopted a band-based approach; unambiguously scored as 1 and 0 for present and absent alleles, respectively (Bonin *et al*., 2007). Bands that resolved poorly on the gel were treated as missing ones. The efficiency of ISSR markers was evaluated by calculating the rare, shared and similar bands. Bands that amplified in more than 70% of the populations were treated as similar bands, those that amplified in 15–70% of the populations were treated as shared bands, while those that amplified

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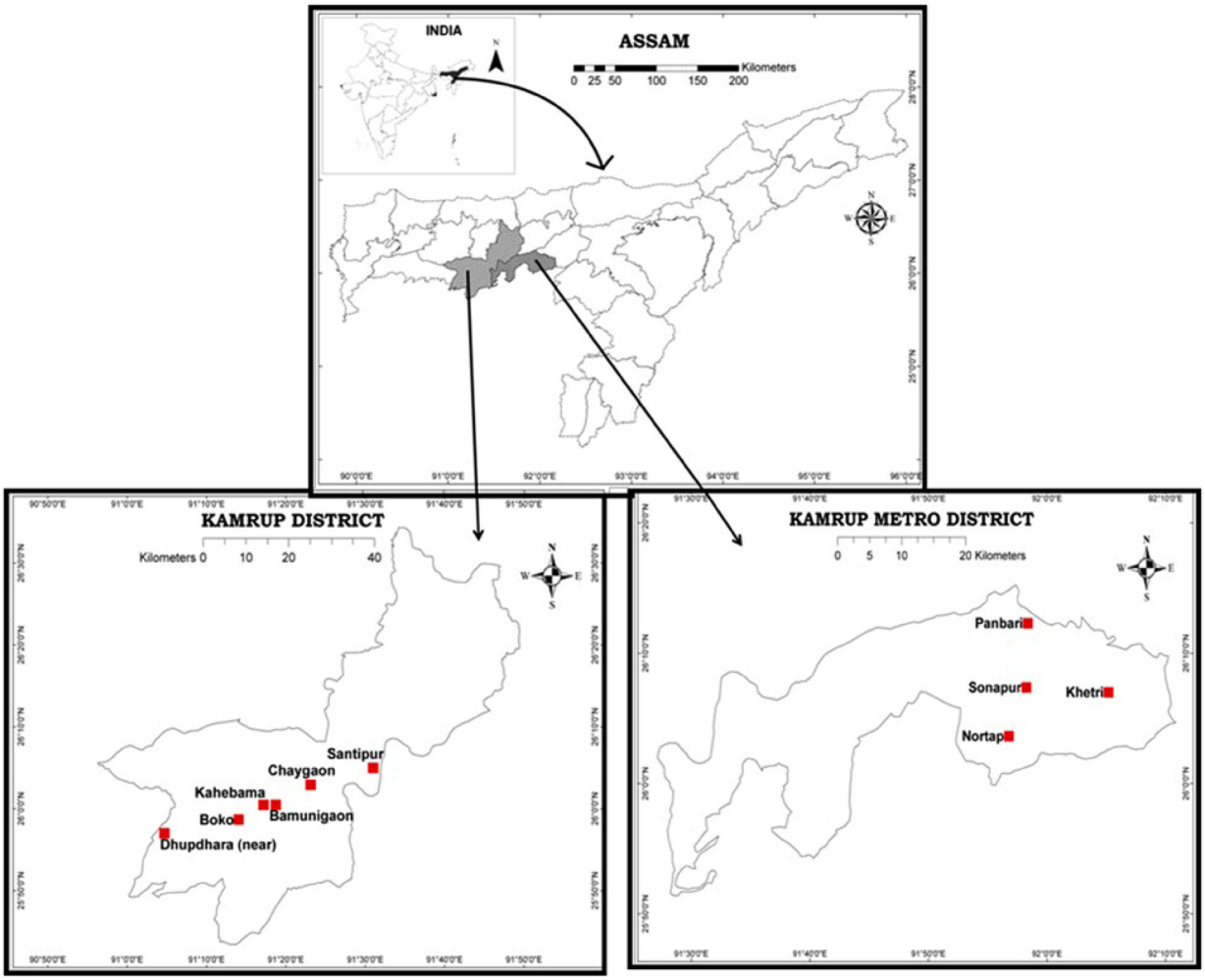

Fig. 1. Map of Kamrup, Assam, India, showing geographic locations of the sampled populations.

in less than 15% of the population were treated as rare bands (Grativol *et al.*, 2011). Loci producing both monomorphic and polymorphic amplicons were analysed. Genetic variability was interpreted as the rate of polymorphism (%) and Polymorphism Information Content (PIC), a valuable marker that detects polymorphism within a population by considering both the number of alleles that are expressed and also the relative frequencies of those alleles (Smith *et al.*, 1997). The PIC was calculated from: $1-p^2-q^2$ (p = amplified band present; q = no band present). The ISSR Primer Index (SPI), which is indicative of the efficiency of the marker system, was then calculated from the PIC values by summing up the values of all the loci amplified by the given primer. The Probability Index (PI) that the two arbitrarily selected genotypes would show identical DNA amplification profiles using the same set of ISSR primer was calculated as $PI = (XD)^n$, where XD is the average genetic similarity index for all the pairwise comparisons and n represents the mean number of fragments obtained per genotype (Rajwade *et al.*, 2010).

Various genetic diversity parameters such as h (Nei's genetic diversity; Nei, 1973), I (Shannon's indices; Nei, 1987), H_S (average genetic diversity within the population), H_T (total genetic diversity among the population), G_{ST} (coefficient of genetic differentiation) and N_m (gene flow) was computed for each marker by a binary data matrix into POPGENE ver.1.31 (Yeh *et al.*, 2000). Other parameters calculated include N_a (observed number of alleles per locus), N_e (effective number of alleles per locus), H_e (Nei's genetic diversity, assuming Hardy–Weinberg equilibrium) and % P (percent polymorphism). To examine the geographic structure of genetic variation among the Khasi mandarin populations, correlations between genetic distance and geographic distance was performed using a Mantel test based on a pairwise matrix of Nei's (1978) unbiased genetic distances (computing 10,000 permutations; using XLSTAT 2017.2). The Pearson correlation coefficient was calculated at a 5% level of significance. Parameters such as Principal Coordinate Analysis (PCoA) and Analysis of Molecular Variance (AMOVA) were employed

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Table 1. Details of polymorphic ISSR markers used in the study

Serial No.	Primers	Sequence (5′–3′)	Band size range (bp)	Bands amplified[a]	Polymorphic bands[b]	Polymorphism (%) [c]	PIC[d]
1	UBC 806	$(AG)_9T$	800–180	10	9	90.0	0.49
2	UBC 807	$(AG)_9C$	800–260	9	9	100.0	0.49
3	UBC 808	$(AG)_9G$	600–190	12	6	50.0	0.48
4	UBC 809	$(GA)_9T$	1100–270	15	12	80.0	0.49
5	UBC 810	$(GA)_9C$	1000–280	12	11	91.6	0.49
6	UBC813	$(CT)_8A$	1300–280	10	4	40.0	0.39
7	UBC 825	$(AC)_8T$	1100–470	9	7	77.7	0.49
8	UBC 835	$(AG)_8Y$	1600–270	22	19	86.3	0.49
9	UBC 836	$(AG)_8YG$	700–90	12	10	83.3	0.49
10	UBC 840	$(GA)_8YT$	1200–90	12	8	66.6	0.49
11	UBC 842	$(GA)_8YG$	890–90	11	8	72.7	0.46
12	UBC 846	$(CA)_8RT$	1000–180	10	7	70.0	0.46
13	UBC 847	$(CA)_8RC$	1300–180	14	14	100.0	0.49
14	UBC 855	$(AC)_8YT$	1200–160	15	14	93.3	0.46
15	UBC 885	$BHB(GA)_7G$	1010–200	15	12	80.0	0.49
16	UBC 890	$VHV(GT)_8$	800–180	14	14	100.0	0.49
17	UBC 891	$HVHT(GT)_7G$	720–180	14	13	92.85	0.49

[a]Total bands = 216.
[b]Total polymorphic bands = 177.
[c]Average % polymorphism = 81.94%.
[d]Polymorphism information content.

to determine the overall distribution of diversity within and among populations using GenALEx ver.6.4 (Peakall and Smouse, 2006). A comparison of genetic distance between the populations was implemented using the distance matrix based on Nei's genetic distance (Nei, 1972). UPGMA (unweighted pair group method with arithmetic mean) denoted as a dendrogram was built using the TFPGA (Tools for Population Genetic Analyses) software based on Nei's genetic distances after bootstrapping the data 1000 times (Miller, 1997). A similarity matrix within the population was constructed with Jaccard's coefficients using the WINDIST package of WINBOOT (Yap and Nelson, 1996).

Results

Polymorphism and genetic diversity

The genomic DNA amplification with 17 selected ISSR primers used in the analysis of 92 individuals from 10 populations of Khasi mandarin and one out-group of Rough lemon yielded 216 clear and reproducible bands with a size range of 90–1600 bp of which 81.94% (177) were polymorphic and the remaining 18.05% (39) were monomorphic. The total number of bands per primer ranged from nine ($(AG)_9C$ and $(AC)_8T$) to 22 ($(AG)_8Y$) and the percentage of polymorphic bands per primer ranged from 100 to 40%. The PIC value for ISSR markers for Khasi mandarin was 0.49 with an average of 0.4 per primer (Table 1). The SPI ranged from a low value of 0.78 ($(CT)_8A$) to a high value of 8.15 ($(AG)_8Y$). The PI values ranged from 0.06 ($(GA)_9T$) to 0.27 ($(AG)_9C$) when calculated for individual primer (Table 2). The number of rare bands (NRB) ranged from 0 ($(CT)_8A$) to 47.61% ($(AG)_8Y$), while the number of shared bands (NSB) ranged from 14.60% ($(AC)_8T$) to 69.62% ($(CA)_8RC$). The percentage of polymorphic ISSR bands per population ranged from 51.3 to 65.7%, and the genetic diversity values ranged between 0.06 and 0.19 (Table 3). Of the 10 populations of Khasi mandarin, the highest level of polymorphism (65.7%) was observed for the Bamunigaon population; while Dhupdhara showed the lowest levels (51.3%). A significant but negative correlation was found between genetic distance and geographic distance (rAB = −0.94, $P = 0.001$, online Supplementary Figs. S1 and S2), at a 5% level of significance. The h and I among all the populations were 0.28 (±0.18) and 0.43 (±0.25), respectively. The H_T was 0.28, while H_S was 0.20. The study detected a G_{ST} value of 0.29 and N_m value of 1.22 (online Supplementary Table S1).

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Table 2. Characteristics of polymorphic ISSR markers used in the study

ISSR primer	ISSR Primer Index (SPI)	Probability Index (PI)	TNB[a]	NPB[b] (%)	NRB[c] (%)	NSB[d] (%)	NSiB[e] (%)
UBC 806	3.22	0.17	714	621 (86.97)	49(6.86)	426 (59.66)	239 (33.47)
UBC 807	3.83	0.27	530	530 (100)	186 (35.09)	344 (64.90)	102 (19.24)
UBC 808	2.48	0.12	873	315 (36.08)	110 (12.60)	205 (23.48)	558 (63.91)
UBC 809	4.33	0.06	1108	753 (67.96)	168 (15.16)	509 (45.93)	431 (38.89)
UBC 810	3.46	0.26	547	454 (82.99)	220 (40.21)	234 (42.77)	93 (17.00)
UBC 813	0.78	0.11	885	327 (36.94)	0	157 (17.74)	728 (82.25)
UBC 825	2.00	0.22	623	437 (70.14)	162 (26.00)	91 (14.60)	370 (59.39)
UBC 835	8.15	0.08	1027	748 (72.83)	489 (47.61)	259 (25.21)	441 (42.94)
UBC 836	3.47	0.15	775	589 (76.00)	139 (17.93)	450 (58.06)	186 (24.00)
UBC 840	3.28	0.13	822	450 (54.74)	110 (13.38)	340 (41.36)	372 (45.25)
UBC 842	2.34	0.12	866	587 (67.78)	58 (6.69)	529 (61.08)	279 (32.21)
UBC 846	1.75	0.19	677	398 (58.78)	68 (10.04)	330 (48.74)	279 (41.21)
UBC 847	5.85	0.15	767	767 (100)	233 (30.37)	534 (69.62)	68 (8.86)
UBC 855	4.45	0.16	752	659 (87.63)	190 (25.26)	469 (62.36)	93 (12.36)
UBC 885	4.57	0.08	1017	738 (72.56)	157 (15.43)	277 (27.23)	583 (57.32)
UBC 890	3.59	0.08	992	992 (100)	162 (16.33)	554 (55.84)	276 (27.82)
UBC 891	4.76	0.13	832	739 (88.82)	121 (14.54)	418 (50.24)	253 (30.40)

[a]Total no. of bands.
[b]No. of polymorphic bands.
[c]No. of rare bands.
[d]No. of shared bands.
[e]No. of similar bands.

Partitioning of genetic diversity

The total genetic variation was partitioned into variation among districts (regions), among populations and within populations ($P = 0.001$). The AMOVA analysis revealed that of the total genetic diversity, 1% was attributable among the regions (Kamrup and Kamrup (M)), 21% was attributable to the among-population differentiation and the rest 78% to the intra-population diversity. The analysis showed that more genetic variation within the populations exists in the Kamrup region in comparison to the Kamrup (M) region (online Supplementary Table S2). The PCoA analysis indicated that first and second PCs accounted for 15.70 and 6.26%, of the total variation (Fig. 2). Quadrant (Q) I and QIV grouped samples from Chayygaon and Nortap and samples from Boko, Panbari, and a single sample from Kahebama, while QII grouped samples from Santipur, Bamunigaon and Dhupdhara. Similarly, QIII grouped Khetri and Sonapur samples into a moderately distinct cluster. The PCoA pattern was also confirmed by the UPGMA dendrogram except for Dhupdhara, which was grouped with Santipur and Bamunigaonin in QII in the 2D plot, which was distinctly separated in the dendrogram. The dendrogram grouped Khetri and Sonapur populations in one cluster (I), Dhupdhara formed the root, while the rest of the seven populations were grouped in a major cluster (II), which was split into two sub-clusters (IIa and IIb); Chayygaon and Nortap; Boko and Panbari populations formed cluster IIa, while Kahebama, Santipur and Bamunigaon populations were grouped into cluster IIb (Fig. 3).

Discussion

The ISSR markers are efficient in estimating genetic variability at the species level in a wide range of plant species (Govindaraj *et al.*, 2015; Nilkanta *et al.*, 2017; Singh *et al.*, 2017; Yan *et al.*, 2019). The maximum NRB produced by $(AG)_8Y$ (489 across 92 accessions) indicates the effectiveness of the prevalence of AG repeats in the recognition of a particular accession, especially in the studied populations of Khasi mandarin from Kamrup and Kamrup (M) districts, India. While the shared and similar bands detected resemblance among the populations from different geographic locations (Tatikonda *et al.*, 2009). A similar study was reported by Bhagwat *et al.* (2014) using ISSR markers in *Symplocos laurina* Wall., where the NRB ranged from 30

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Table 3. Polymorphism parameters estimated using ISSR marker data

Population names	No. of accessions collected	H_e[a] (SD)[b]	%P[c]
Boko	12	0.19 (0.10)	64.35
Bamunigaon	09	0.17 (0.15)	65.74
Kahebama	10	0.17 (0.13)	62.50
Santipur	10	0.10 (0.10)	61.11
Chayygaon	10	0.17 (0.17)	60.18
Dhupdhara	05	0.15 (0.13)	51.38
Panbari	10	0.14 (0.15)	57.40
Khetri	10	0.17 (0.09)	61.57
Sonapur	06	0.06 (0.13)	56.94
Nortap	10	0.19 (0.10)	61.11
Average		0.28 (0.18)	60.22

[a]Nei's genetic diversity (assuming Hardy–Weinberg equilibrium).
[b]Standard deviation.
[c]Percent polymorphism.

$((CT)_8RG)$ to 135 $((GGAT)_4)$, the number of similar bands ranged from 0 $((TC)_8G)$ to 857 $((TG)_8RC)$ and NSB ranged from 343 $((CA)_8T)$ to 726 $((CT)_8RG)$. Since ISSRs are usually dominant markers, the PIC score for any locus can range from 0 to 0.5 (Singh *et al.*, 2013). The high average PIC value observed with ISSR markers in the present study indicates a significant level of genetic diversity among the mandarin populations considered in the study. The PIC score is a measure of variability at a particular locus. The more the PIC value for a locus, the higher is the chance that polymorphism will exist between two randomly selected genotypes at that locus. A similar average PIC value (0.47) was identified in *Trichosanthes dioica* using ISSR markers (Kumar and Agarwal, 2019). A moderate average PIC value for ISSRs was found in other Citrus species (Sharafi *et al.*, 2017; Uchoi *et al.*, 2017). While Meena *et al.* (2019) identified a low average PIC value of 0.23 in *Ephedra foliata* using ISSR markers. Moreover, the highest SPI value obtained in the present study using $(AG)_8Y$ primer indicates the importance of AG repeats for detecting polymorphism in Khasi mandarin populations. The SPI indicates the information content of the ISSR markers that can be obtained per analysis. The higher the SPI value, the more is the usefulness of the marker (Lamare and Rao, 2015). A high SPI value of 5.68 $((CT)_8RA)$ was observed in *Musa acuminate* Colla using ISSR markers (Lamare and Rao, 2015). Besides, a high SPI value of 8.91 (CACCACCACGC) was identified in *T. dioica* using ISSR markers (Kumar and Agarwal, 2019). Further, the low PI value observed in the present study indicates that the ISSR primers are efficient in distinguishing different accessions. The PI value indicates the ability of a set of primers to uniquely distinguish two genotypes (Rajwade *et al.*, 2010). Thus, the high level of overall polymorphism, PIC and SPI identified in the present study using ISSR markers proved informative for analysing diversity in the Khasi mandarin populations and this finding is in line with the studies by Sharafi *et al.* (2016) and Singh *et al.* (2016). The level of polymorphism percentage found in the present study is comparable with the previous findings carried out in *C. reticulata* Blanco ($P = 95.62\%$, Pal *et al.*, 2013), wild Citrus spp. ($P = 89.4\%$, Shahsavar *et al.*, 2007), *Citrus indica* ($P = 87\%$, Kumar *et al.*, 2010), a few commercially popular citrus species ($P = 100\%$, Marak and Laskar, 2010) and other citrus cultivars using ISSR ($P = 88.2\%$, Sharafi *et al.*, 2016). The level of polymorphism in the present study is also comparable with an important tree species, that is, *T. dioica* Roxb. ($P = 95.96\%$, Kumar and Agarwal, 2019) cultivated in Assam–Bengal regions of India and a commercial fruit crop species, that is, *Prunus armeniaca* L. ($P = 94.84\%$, Li *et al.*, 2013) cultivated in central Asia. The high level of genetic polymorphism in the present study might be due to genetic factors because the orchards bearing the Khasi mandarin trees planted by farmers across the region consist of citrus clones that are grown from seeds of diverse sources. Most of the existing ones may contain trees that are either of zygotic or nucellar origin besides being zygotic twins (Mondal and Saha, 2014).

The AMOVA analysis indicates that a greater part of the variation resides within populations in both Kamrup and Kamrup (M) regions. The high intra-population differentiation indicates that the basis of the population from which the existing populations originated might have been genetically diverse. Genetic drift, population isolation and breeding methods have been cited to explain a high level of intra-population variation (Hogbin and Peakall, 1999). Besides, traits such as longevity, pollination, high fertilization and out-crossing rates along with the life history of citrus species may have promoted the maintenance of high intra-population genetic variation (Yu *et al.*, 2018; Meena *et al.*, 2019). A similar pattern of high intra-population genetic variation has been established in woody species belonging to families such as Pinaceae, Fagaceae and Myrtaceae (Hamrick and Godt, 1996), as well as in other perennial woody species such as *S. laurina* Wall., *Melocanna baccifera* (Roxb.), *Zizipbus mauritiana* (Lamk.) and *E. foliata* (Deshpande *et al.*, 2001; Nilkanta *et al.*, 2017; Singh *et al.*, 2017; Meena *et al.*, 2019). These inferences were also supported by the PCoA ordination, where the populations illustrated distinct clustering and few were exclusively grouped in the ordination space and were overlapping to some extent. The PCoA revealed that mandarin accessions were scattered, indicating their possibility of being more divergent at the molecular level. Moreover, previous studies also reported molecular

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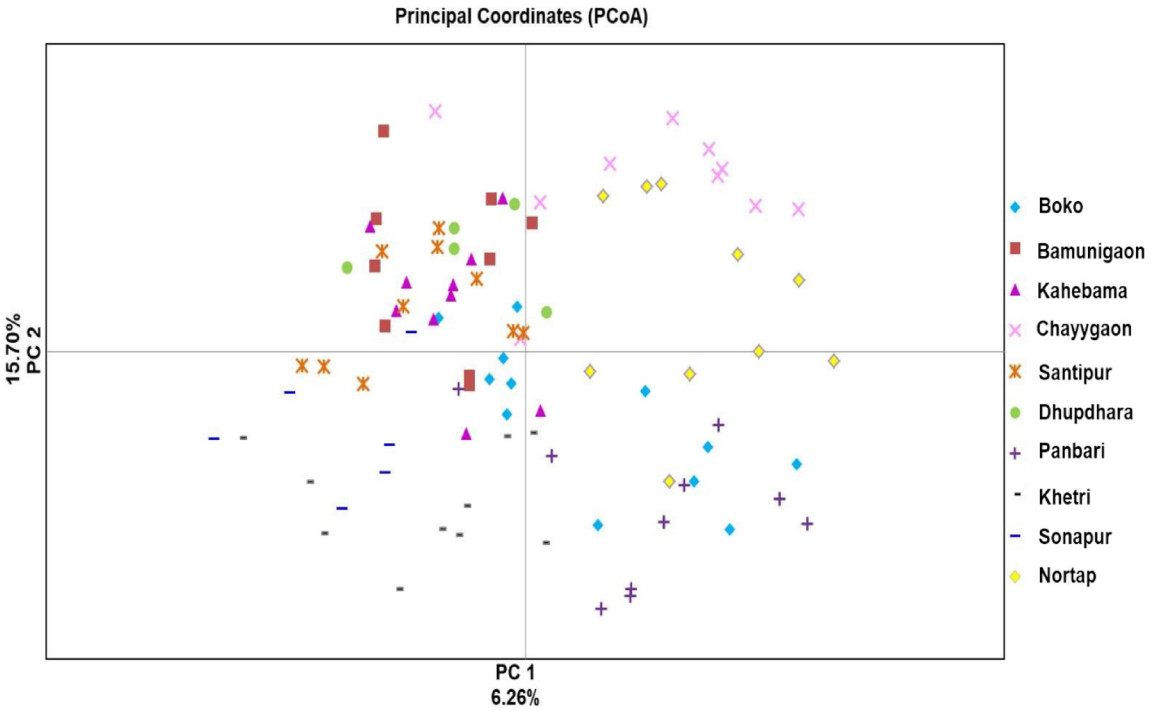

Fig. 2. Principal Coordinate Analysis of Khasi mandarin populations.

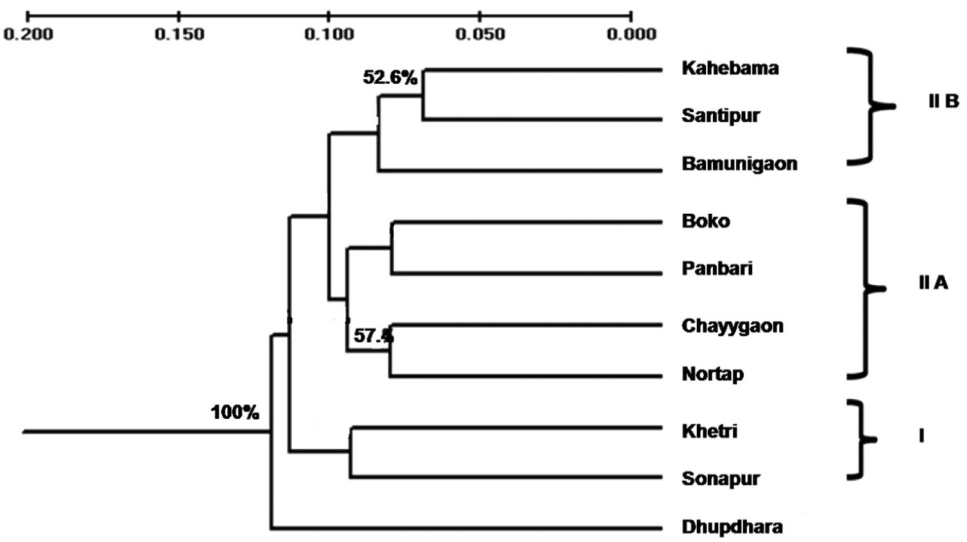

Fig. 3. UPGMA cluster analysis of Khasi mandarin populations collected from 10 locations in Kamrup and Kamrup (M), Assam using the distance matrix generated using Nei's (1972) algorithm. The percent values refer to bootstrap values.

divergence in the mandarin group (Campos *et al.*, 2005). Genetic differentiation among the populations and high intra-population diversity indicates that the management programme for the conservation of genetic variability in Khasi mandarin should aim to preserve every present population.

According to the results of cluster analysis based on genetic similarity, geographical affinity was shown between the populations. The result showed a good correspondence among the PCoA, UPGMA clustering and that clusters were related to the geographic distance between populations except for populations from Nortap and Boko, which were genetically similar to populations from Chayygaon and Panbari grouping together despite the distance separating the two districts, suggesting wider genetic variability of these two populations. For instance, Nortap

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and Chayygaon; Boko and Panbari populations clustering in the UPGMA tree as group II were located in the right-side (QI and QIV) of the PCoA diagram in two well-separated clusters. While the left quadrants (QII and QIII) in the PCoA diagram contained the remaining populations, which formed cluster II b and I in the UPGMA tree. This result, however, is not surprising considering that most mandarin trees are seedlings of either zygotic or nucellar origin. The ability of ISSR markers to separate closely related populations corroborates the findings by ColettaFilho *et al.* (2000) and Alansi *et al.* (2016). This similarity may be due to the reason that trees in each district might have been grown from seedlings originating from the same nursery or collection centre authorized to supply citrus seedlings, while some accessions might have been grown from unknown seedling sources. The difference in genetic makeup among populations from different locations could be attributed to evolutionary forces for favourably allowing seedling trees with genomes better suited to particular locations to develop through juvenility till reproductive maturity.

In the present study, the values of H_T and H_S suggest the existence of high genetic variation at the species level than at the population level, although they do not show any distinct morphological variation (data not shown). This is consistent with the findings of Meena *et al.* (2019) where similar estimates of high genetic diversity were obtained in *E. foliata* ($H_T = 0.23$ and 0.25; $H_S = 0.16$ and 0.23). In general, a backcross of the hybrids with the varieties of *C. reticulata* Blanco makes the mandarin group phenotypically and genotypically most diverse (Pal *et al.*, 2013). Moreover, mandarins were found to be the most polymorphic among the ancestral species (Barkley *et al.*, 2006; Oueslati *et al.*, 2017). The genetic variation detected in Khasi mandarin using ISSR markers corroborates with the hypothesis that populations produced from out-crossing species can generate higher genetic diversity as compared to inbreeding ones (Verma and Rana, 2011).

Further, the high level of total genetic diversity observed among the populations of Khasi mandarin in the study might be attributed to the effect of a broader gene pool and later, introduction to plantations from different gene pools might have established different alleles across the populations. Besides, this could be due to free genetic exchange within the mandarin group and closely related species such as *Citrus sinensis* Osbeck (sweet orange) and *Citrus paradisi* Macfad. to form cultivars of hybrid origin, respectively. Moreover, Khasi mandarin has a long cultivation period of over 20 years and produces a large number of seeds per year that grow rapidly following the same season after dehiscence, implying that the abundant seed set might contribute to accumulating mutations and harbour a high amount of diversity among the populations of Khasi mandarin (Bernet *et al.*, 2010). This inference is supported by the values of G_{ST} observed in the study that indicates high genetic differentiation among the populations of Khasi mandarin. The G_{ST} value larger than 0.25 is a threshold beyond which considerable population differentiation occurs (Han *et al.*, 2007). The G_{ST} value in the present study is comparatively higher than the estimate found in *Citrus aurantifolia* cultivar ($G_{ST} = 0.13$), an important commercial fruit crop (Munankarmi *et al.*, 2018). Moreover, some other angiosperms such as *Z. mauritiana* (Lamk.) (Singh *et al.*, 2017) and *Styrax obassia* Sieb. Et Zucc. (Wang *et al.*, 2015) also have revealed higher genetic differentiation. However, the value of genetic differentiation in the present study is higher than the average coefficients of long-lived perennial ($G_{ST} = 0.19$) and out-crossing ($G_{ST} = 0.23$) species (Nybom, 2004; Meena *et al.*, 2019). Mean G_{ST} values of 0.59, 0.19 and 0.23 were assembled for selfing, mixed mating and out-crossing plants, respectively, based on the estimates of genetic diversity obtained with RAPD markers (Nybom and Bartish, 2000). Based on these estimates, the amount and pattern of genetic variation in Khasi mandarin are more comparable to out-crossing species than to selfing. These inferences also reflect genetically distinct populations in the region differing concurrently in terms of allelic frequencies and sizes, implying that new mutations may be contributing to the high genetic diversity found in the cultivated Khasi mandarin populations. Based on differentiation among populations, the present study showed significantly high gene flow. Population differentiation and effective population size correspond to three different groups of N_m values: high (≥1.000), moderate (0.25–0.99) and low (0.00–0.24) (Slatkin, 1985). The N_m value >1.0 indicates a significant movement of genetic material across populations through farmers in the region satisfying the lowest number of migrants per generation necessary to avoid genetic drift (Slatkin, 1987). Thus, the value of gene flow that explains the inter-population genetic differentiation and the high intra-population differentiation indicates potentially superior fitness of the population (Hamrick and Godt, 1996). Pollen dispersal, which is usually the primary means of gene flow, depends on the geographic distribution of the diverse compatible species available in a particular area (Pons *et al.*, 2011). The genetic diversity within the cultivated populations of Khasi mandarin suggests that since some of the cultivated populations are not distantly situated from wild habitats, thereby movement of pollens from wild to cultivated populations through an aerial distance of nearly 100–150 km might result in allele combinations which would not otherwise occur geographically (Miller and Gross, 2011). This may have led to increased mixing and gene flow among geographically isolated populations. In the present study, the balance between genetics and environment in determining variation is interpreted in terms of populations that differed genetically, originating from the same district which can be exemplified further by floristic and pollen study.

https://doi.org/10.1017/S1479262121000162 Published online by Cambridge University Press

Thus, there exists a high genetic diversity among the cultivated Khasi mandarin populations in the lower Brahmaputra valley of Assam, northeast India, suggesting that these orchards are effective reservoirs of plant genetic resources and can play a vital role in the conservation of mandarin orange germplasm. The present study highlights the utility of the ISSR marker system in the characterization of the Khasi mandarin populations based on polymorphism level. The present study may act as a baseline for sustainable utilization, breeding and conservation of this valuable germplasm. The information generated from this study will add to the existing knowledge on mandarin orange genetic resources and will be helpful to protect the existing diversity of these rapidly declining mandarin orange resources in northeast India.

Supplementary material

The supplementary material for this article can be found at https://doi.org/10.1017/S1479262121000162.

Acknowledgements

The authors acknowledge Padma Rabha and other orchard owners for allowing access to leaf samples used in the study. The authors thank Dr Akan Das and Priya Boro for help in sample collection. The authors also thank Krishna Das for help in the preparation of the map.

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