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Assessment of genetic diversity in Indian Perilla [*Perilla frutescens* (L.) Britton] landraces using STMS markers

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Inter-population diversity in 54 Indian *Perilla* landraces and 18 accessions of exotic origin was investigated using sequence tagged microsatallite (STMS) markers. The STMS markers clearly distinguished the Indian accessions from the exotic ones. Neighborhood-joining (NJ) clustering pattern revealed association of geographical diversity and genetic diversity for Indian *Perilla* germplasm. Population genetic parameters studied for 14 Indian *Perilla* populations from two distinct regions (North-eastern region and North-western Himalayas, Uttarakhand State) revealed greater diversity for accessions from the later region. Analysis of molecular variance, revealed that bulk of the variations existed between populations within groups, followed by variations within populations and between groups. The summary of group-wise F-statistics and gene flow revealed greater population differentiation for Uttarakhand populations as compared to accessions from North-eastern region of India.

Keywords: *Perilla frutescens*, genetic diversity, Indian Himalayas, molecular characterization, STMS markers

Introduction

Perilla frutescens (L.) Britton (Family: *Lamiaceae*) is an underutilized crop of Indian Himalayas with potential utility in agriculture. It is grown as a traditional crop in China, India, Japan, Korea, Thailand and other Asian countries. Two botanical varieties are recognized: var. *frutescens*—an oil crop and var. *crispa*—a medicinal or vegetable crop[1,2]. These are distinguishable on the basis of fragrance and the hardness of seeds[3]. The seeds of var. *frutescens* are larger and softer than those of var. *crispa*. Weedy plants of both types are common in East Asia[4]. *P. frutescens,* a tetraploid (2n=40) [5,6] is assumed to have main area of diversity in China because of its long history of cultivation[7,8,9] whereas the three wild species viz. *P. citriodora, P. hirtella and P. setoyensis* are diploid (2n=20) and are native to Japan[10].

There are many scientifically proven medicinal uses for *Perilla*. It has been used for centuries in oriental medicine as an antiasthmatic, antibacterial, antidote, antimicrobial, antipyretic, antiseptic, antispasmodic, antitussive, aromatic, carminative, diaphoretic, emollient, expectorant, pectoral, restorative, stomachic and tonic[2,3]. The seed oil is used for cooking, drying oil and as a fuel. The foliage is used as a pot herb, for medicine and is distilled to produce an essential oil for flavouring. The plants are grown as ornamentals. In addition to this, *Perilla* adds an antimicrobial agent to pickled foods.

Diversity and genetic relationships in East Asian *Perilla* have been widely studied[3,4,11-13], but limited information is available on Indian *Perilla* types. In India, the North-eastern region and parts of North-western Himalayas hold a great diversity of *P. frutescens* var. *frutescens*. However, in absence of its organized cultivation, it is being replaced by other cash crops, making it vulnerable to extinction. More than 200 Indian *Perilla* accessions collected from the North-eastern region and parts of Uttarakhand and maintained *ex situ* in the National Genebank at NBPGR, New Delhi, have yet to be systematically characterized. An understanding of the diversity present in landrace populations may help in identifying suitable sites for their *in situ* conservation, so that natural evolution continues and potential diversity is generated for use in crop improvement. Thus, the present study was undertaken to assess the level of diversity prevalent in Indian *Perilla* populations using STMS markers.

Materials and Methods

Plant Material and DNA Isolation

Seventy-two *Perilla* landraces representing Indian states viz., Arunachal Pradesh, Manipur, Meghalaya,

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Mizoram, Nagaland, Uttarakhand and exotic introductions from Australia, Bhutan, Japan, South Korea, Thailand and USA (Table 1) were selected for inter-population studies. Fresh young leaves from 5 plants (4 wks-old) were bulked for DNA extraction.

Table 1—Passport information of *Perilla* landraces for inter-/intra-population diversity

No.	Accession	Source	No.	Accession	Source
North-western Himalaya (Uttarakhand State)					
1.	IC281713	Chamoli	7.	IC538084	Champawat
2.	IC361361	Uttarkashi	8.	IC552395	Hardwar
3.	IC383469*	Pithoragarh	9.	Almora local*	Almora
4.	IC383391	Pithoragarh	10.	Bhowali local*	Nainital
5.	IC521293*	Pithoragarh	11.	Pithoragarh local*	Pithoragarh
6.	IC538007*	Nainital			
North-eastern region (State wise)					
12.	IC521289	Arunachal Pradesh	34.	IC526771*	Mizoram
13.	IC330441	Manipur	35.	IC549534	Mizoram
14.	Shillong local	Meghalaya	36.	IC552393	Mizoram
15.	IC335405*	Mizoram	37.	H 1796	Mizoram
16.	IC335408*	Mizoram	38.	H 2216	Mizoram
17.	IC369349	Mizoram	39.	IC419706	Nagaland
18.	IC374513	Mizoram	40.	IC423331*	Nagaland
19.	IC521283*	Mizoram	41.	IC516674	Nagaland
20.	IC521285	Mizoram	42.	IC521288	Nagaland
21.	IC521286	Mizoram	43.	IC521292*	Nagaland
22.	IC521290	Mizoram	44.	IC524473*	Nagaland
23.	IC521291	Mizoram	45.	IC524546	Nagaland
24.	IC526512	Mizoram	46.	IC524550	Nagaland
25.	IC526604	Mizoram	47.	IC524551	Nagaland
26.	IC526638	Mizoram	48.	IC524600	Nagaland
27.	IC526643	Mizoram	49.	IC524605	Nagaland
28.	IC526674	Mizoram	50.	IC526419	Nagaland
29.	IC526686	Mizoram	51.	IC526572	Nagaland
30.	IC526690	Mizoram	52.	IC419477	Nagaland
31.	IC526701*	Mizoram	53.	IC521284	Nagaland
32.	IC526719	Mizoram	54.	IC524622	Nagaland
33.	IC526755	Mizoram			
Accessions from exotic sources					
55.	EC216268	Australia	64.	EC592854	Thailand
56.	EC592848	Bhutan	65.	EC592842	USA
57.	EC592849	Bhutan	66.	EC592843	USA
58.	EC592850	Bhutan	67.	EC592844	USA
59.	EC592851	Bhutan	68.	EC592845	USA
60.	EC592853	Japan	69.	EC592846	USA
61.	EC592859	Japan	70.	EC592847	USA
62.	EC592855	South Korea	71.	EC592857	USA
63.	EC592856	South Korea	72.	EC592858	USA

*Accessions used for intra-population genetic diversity analysis

For intra-population studies, 10 plants each from 14 accessions were randomly selected for DNA isolation (Table 1). Genomic DNA was extracted using the CTAB method[14] and were diluted to a working concentration of 10 ngμL^{-1}.

STMS Analysis

Twenty-two microsatellite (STMS) primers[13,15] were used for genotyping the accessions (Table 2). Each reaction mixture (25 µL) contained 3.0 m*M* $MgCl_2$, 1U Taq DNA polymerase, 200 µ*M* each dNTP, 0.2µ*M* STMS primers and 30 ng genomic DNA in 10 m*M* Tris-HCl, 50 m*M* KCl, *p*H 8.3. The amplification regime included an initial denaturation step (95ºC/5 min), followed by 40 cycles of 94ºC/1 min, Ta/1 min and 72ºC/1 min, and a final extension step of 72ºC/8 min. The PCR products were mixed with 2.5 µL gel loading dye (6X dye: 0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol in water) and electrophoresed on a 3% (3 MetaPhor: 1 agarose) gel at 80V in 1X TAE buffer. A 100 bp DNA ladder was used as molecular size standard. The gels were stained with ethidium bromide and viewed under UV light. Patterns were scored for presence of each allele in an accession and Manhattan distances were calculated for this data. This matrix was subjected to neighborhood-joining analysis (NJ) to generate tree using average linkage procedure. All the computations were performed using NTSYS-pc v2.1 software[16] and resolving power (Rp) for the primers were calculated[17].

Population Studies

The most informative STMS primers (Table 2) were further used to study intra-population diversity of 14 accessions (Table 1). Frequency of an allele in each population/group was calculated. The accessions x allele frequency matrix for all the loci were used to calculate the genetic diversity parameters using POPGENE version 1.32[18]. In order to estimate population substructure AMOVA was carried out using Arlequin version 3.0[19].

Results

Inter-population Diversity

All the 22 STMS primers used for inter-population diversity were polymorphic. The representative gel photograph of the 72 accession is shown in Fig. 1. Resolving power ranged from 0.17 (GBPFM 63) to 4.53 (GBPFM 157). The NJ tree based on Manhattan distances (Fig. 2) of *Perilla* populations depicted two distinct clusters. The cluster I comprised of the indigenous accessions whereas the cluster II represented

Table 2—21 STMS primers used for diversity analysis

Primer ID	Primer sequences (5'- 3')	Repeat motif	Allele size (bp)	T_a (°C)
KWPE1	F caaaagccttacaactttga R agcgtttgtatttcatggac	$(GAA)_{18}$	198	55
KWPE5	F atctccaagcttatgaatgc R ctggtagtgagcctgttcat	$[(ATG)_6(GA)_6]$	195	55
KWPE19*	F caacccttcacgatcactat R acatttaagagagagagcaag	$(ACG)_7$	215	55
KWPE25	F acatttaagagagagagcaag R acgaacgggcttcaatctt	$[(GT)_8(GA)_{14}]$	212	55
KWPE26	F gaggcaatgctggtacttc R gaacgggcttcaatcttc	$[(AG)_6(AG)_7(GA)_{13}]$	243	55
KWPE29	F aagacaaggaggaagatgc R taggtgttcgctctcctgtg	$(GAA)_5$	164	55
KWPE32	F agaacaacattgtagctcgg R acgaccaaccagtagatgat	$(CCT)_4$	225	55
KWPE39	F agaacaacattgtagctcgg R gacgaaccagcaaacgac	$(CCT)_4$	298	55
KWPE48	F caccccatcttttggat R agcaggatggtggtggtc	$(GA)_9$	215	55
KWPE51	F ccatacctggaacaaacatt R gaccctagcttctctccatt	$A(N)_9$	184	55
KWPE53	F actcaccagaagagaagaaga R gccactgacctgttaatatctg	$(CT)_{16}$	187	55
KWPE57	F atcacatctctctctttctgga R ccagtcactccatcatctcta	$(CT)_{16}$	156	55
KWPE58	F agagagttacctgcgattttc R cttcaatattcggccatctt	$(TG)_9$-$(AG)_{12}$	162	55
GBPFM63	F gattggaagtccaaatccct R tgcccgcaaattatacctaa	$(TC)_{21}$-$(CA)_{12}$	309-323	56
GBPFM70	F ccctccaaatcaatattcca R tagctgccatacgaacatga	$(ATTTG)_3$, $(AC)_5$	234-258	54
GBPFM75	F catagttcatggcttccacc R cctgagcacagaaacagatca	$(CT)_{12}$	164-172	52
GBPFM91	F ccactcaaatccgcttctaa R aatgttggttgcgtttcatt	$(AG)_9$	234-274	54
GBPFM111*	F atcatggatgaatcgcactt R ccattctccaaatgttactctattt	$(ACACA)_8$	175-205	56
GBPFM155	F tttgtgacaatacgcaccac R ccaattgctcaatgctctct	$(GAA)_{10}$	276-312	60
GBPFM157	F aaagagctgatggacgtgag R aggtgctactgtgtcaaggc	$(ACC)_5$, $(ACA)_4$, $(CAA)_7$	196-217	56
GBPFM203*	F gttttgttgcagctcgattt R tgggtttggaaagtattgatg	$(GA)_5TAA(AG)_{26}$	176-234	54
GBPFM204	F tcgaaaaattgcagatcacc R ttgtcttttgcctcttttgc	$(AG)_{17}$	143-189	54

*Primers used for intra-population diversity
T_a is the annealing temperature

mainly exotic populations. At the sub-cluster level, a fair association between geographical diversity and genetic diversity was revealed, particularly for indigenous populations in cluster I. The bootstrap value for the two major groups was 100%, indicating a strong statistical support for major clusters.

Population Parameters of *Perilla* Landraces

The allele frequency per locus generated by STMS primers among populations (intra-population diversity) showed polymorphism at all the four loci viz. KWPE 19a, KWPE 19b, GBPFM 111 and GBPFM 203. GBPFM 111 locus was the most variant

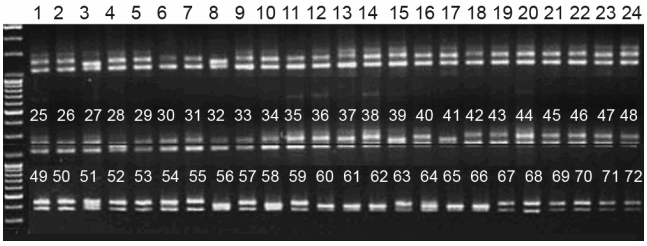

Fig. 1—Gel photograph of 72 *Perilla frutescens* var. *frutescens* accessions obtained with primer KWPE 19.

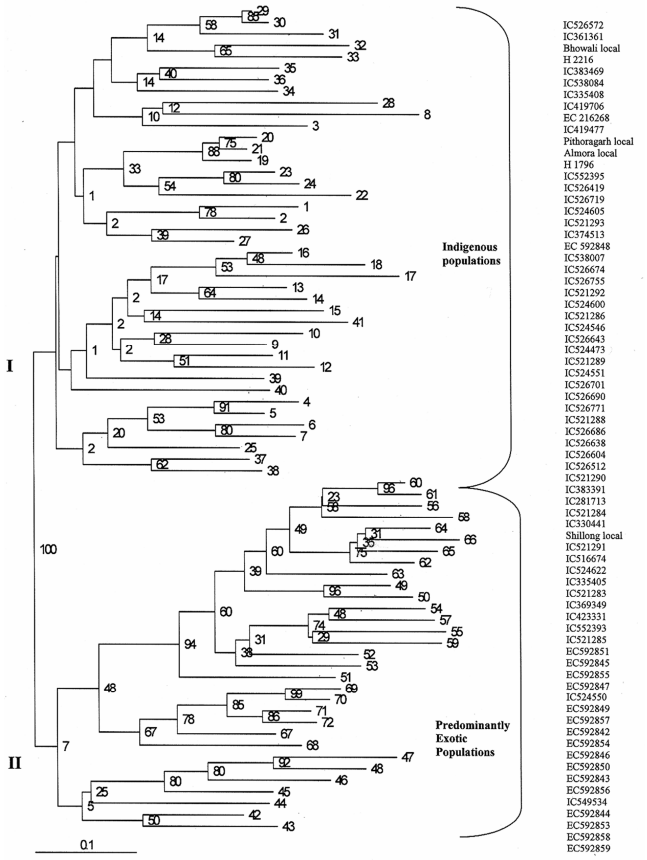

Fig. 2—Neighborhood-joining (NJ) tree based on Manhattan distances in 72 *Perilla* landraces obtained by analysis of STMS polymorphism. 98 PCR products obtained with 22 STMS primers pairs. The number at branch points is per cent bootstrap value based upon analysis of 1000 bootstrap samples. The values at the tip of branches indicate he different samples whose accession numbers are indicated in the figure.

with five alleles followed by four, three and two alleles at GBPFM 203, KWPE 19b and KWPE 19a, respectively. Three alleles were found to have frequency of lower than 0.05 and were rare alleles.

The mean allelic frequencies at GBPFM 111 were 0.02, 0.36, 0.33, 0.09 and 0.13 for allele A, B, C, D and E respectively. IC335408, IC521283 and IC524473 were most diverse at this locus for distribution of alleles B and C. At GBPFM 203, IC 335405 and IC538007 were diverse with respect to alleles C and D; A and B with the allele frequencies of 0.45 and 0.35; 0.30 and 0.60, respectively. KWPE 19b was variable with respect to all the three alleles A, B and C with mean allele frequencies of 0.23, 0.69 and 0.07, respectively. Allele A was found common in most of the populations at this locus. IC 335408 from Mizoram was identified as most diverse with equal frequency distribution of alleles A and B within population, whereas IC 538007 and IC 423331 showed uneven frequency of alleles A and B. KWPE 19a was invariant as the infrequent allele B was present in only two (Pithoragarh local, IC 335405) out of 14 populations. A few populations, viz. IC521293, IC383469, Bhowali local and Almora local, were invariant as one allele was fixed with frequency P=1 (Table 3). Allele A at KWPE 19a and allele B at GBPFM 203 were found at relatively high frequencies of 0.79 and 0.80 (Data not shown).

Maximum values for Shannon information index were obtained in IC335405 followed by IC538007, IC335408 and H2216. No intra-population variation was recorded for IC521293, IC383469, Bhowali local and Almora local (Table 3). The expected heterozygosity was low (mean value = 0.11) except for accessions IC335405 (0.28) and 0.24 for IC538007 and IC335408. The diversity parameters for *Perilla* populations showed greater diversity in populations from North-eastern region with more number of effective alleles, greater Shannon information index and greater expected heterozygosity as compared to populations from Uttarakhand. However, two accessions of Uttarakhand region (Pithoragarh local & IC538007) showed greater values for these indices.

Analysis of molecular variance (AMOVA) revealed maximum variation among populations within groups (76.83%) followed by within populations (13.52%) and among groups (9.65%) (Table 4). The F-statistics and gene flow for all loci identified by STMS primers revealed that Uttarakhand populations have relatively low level of heterozygosity consequently resulting in greater population differentiation. The mean gene flow value for all loci was relatively more in North-eastern populations. (Table 5). Population-wise NJ tree (Fig. 3) based upon Nei's genetic distances[20], showed the presence of one major cluster comprising all the accessions except IC335405 (Mizoram), which was found to be the most distinct among all the 14 populations.

Table 3—Diversity parameters in *Perilla* landraces

Population	Sample size	% of polymorphic loci	Na	Ne	I	Ho	He
IC521293	10	0	1.00 ± 0.00	1.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
IC383469	10	0	1.00 ± 0.00	1.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Bhowali local	10	0	1.00 ± 0.00	1.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Pithoragarh local	10	25	1.50 ± 0.43	1.21 ± 0.43	0.20 ± 0.40	0.05 ± 0.10	0.12 ± 0.24
IC538007	10	75	2.00 ± 0.82	1.40 ± 0.52	0.39 ± 0.37	0.00 ± 0.00	0.24 ± 0.24
Almora local	10	0	1.00 ± 0.00	1.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
IC423331	10	25	1.25 ± 0.50	1.05 ± 0.11	0.08 ± 0.16	0.00 ± 0.00	0.05 ± 0.09
IC521292	10	25	1.25 ± 0.50	1.09 ± 0.17	0.10 ± 0.21	0.02 ± 0.05	0.07 ± 0.13
IC335405	10	75	2.25 ± 0.96	1.58 ± 0.79	0.47± 0.44	0.20 ± 0.33	0.28 ± 0.28
IC335408	10	50	1.50 ± 0.58	1.43 ± 0.51	0.33 ± 0.38	0.00 ± 0.00	0.24 ± 0.28
H2216	10	50	1.50 ± 0.58	1.20 ± 0.28	0.22 ± 0.27	0.02 ± 0.05	0.15 ± 0.19
IC521283	10	25	1.25 ± 0.50	1.18 ± 0.36	0.15 ± 0.30	0.00 ± 0.00	0.11 ± 0.22
IC526701	10	25	1.25 ± 0.50	1.15 ± 0.30	0.14 ± 0.28	0.12 ± 0.25	0.10 ± 0.20
IC524473	10	25	1.25 ± 0.50	1.18 ± 0.36	0.15 ± 0.30	0.00 ± 0.00	0.11 ± 0.22
Mean		28.57	1.36 ± 0.42	1.18 ± 0.27	0.16 ± 0.22	0.03 ± 0.06	0.11 ± 0.15

Where,
Na =Observed number of alleles; Ne = Effective number of alleles; I = Shannon's information index; Ho = Observed heterozygosity; He = Expected heterozygosity

Table 4—Analysis of molecular variance (AMOVA) for *Perilla* landraces from two regions

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation
Among groups	1	11.863	0.07930 Va	9.65
Among populations within group	12	77.108	0.63146 Vb	76.83
Within population	126	14.000	0.11111 Vc	13.52
Total	139	102.971	0.82186	

Fixation Indices- FSC:0. 85037, FST:0.86481, FCT:0.09648

Table 5—F-statistics and gene flow for all loci (mean values)

Region (group)	Sample size	F_{IS}	F_{IT}	F_{ST}	Nm
North-western Himalaya (Group I)	60	0.853	0.985	0.895	0.029
North-eastern region (Group II)	80	0.643	0.879	0.661	0.128

Where,
F_{IS} = inbreeding coefficient of an individual (I) relative to subpopulation; F_{IT} = inbreeding coefficient of an individual (I) relative to the total populations (T); F_{ST} = effect of subpopulations (S) compared to the total populations (T); Nm = Gene flow estimated from $F_{ST} = 0.25 (1 - F_{ST}) / F_{ST}$)

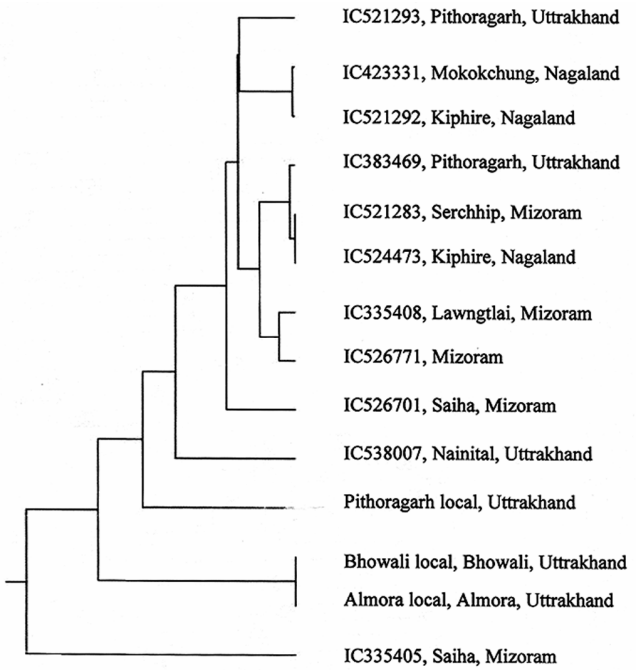

Fig. 3—Neighborhood-joining (NJ) tree of 14 *Perilla* populations based upon STMS polymorphism data. The relative lengths are indicated by scale. The NJ tree was obtained by analysis of 10 individual plants per sample and is based on allele frequencies. Map of India showing collection sites of the *Perilla* germplasm.

Discussion

The molecular characterization of *Perilla* landraces using STMS markers indicated enough polymorphism to differentiate the intra- and inter-population diversity. The STMS primers used in the present study were species specific and have already been successfully used for genetic diversity studies of *Perilla* populations from Japan and Korea[13,15]. The indigenous and exotic accessions formed distinct groups. Though, at the major cluster level there was no strong association between geographical origin and genetic diversity, yet at sub-cluster level this association was better revealed

particularly for indigenous accessions as majority of them grouped in cluster I. Some of the Uttarakhand accessions were clearly discriminated in distinct sub-clusters. Clustering pattern, therefore, provides the opportunity to select diverse accessions from distinct microclimatic niches representing specific morphological adaptations. Low level of heterozygosity observed is expected in *Perilla* as it is an autogamous plant with limited out crossing (0.148-0.5%)[13].

Studies on Population Genetic Parameters

All the four loci, GBPFM 111, GBPFM 203, KWPE 19b and KWPE 19a, were variable with respect to the alleles. Although a few populations viz. IC521293, IC383469, Bhowali local and Almora local, were invariant as one allele was fixed with frequency P=1. Such common and localized alleles occurring in only one or few habitats may be biologically specialized alleles that enhance adaptation only in certain habitats[21]. These are often the alleles of most interest to breeders, because breeders are concerned with improving performance in the specialized habitats of their own eco-geographical regions.

F-statistics (Table 5) revealed greater population differentiation in *Perilla* populations from Uttarakhand compared to populations from North-eastern region. On an average 89.5% of variation among populations for Uttarakhand accessions and 66.08% variation among populations for North-eastern region was noticed, which is indicative of greater overall population differentiation among the *Perilla* landraces. Greater F_{IS}, F_{IT} and F_{ST} values for *Perilla* populations from Uttarakhand compared to North-eastern region revealed greater inbreeding and population differentiation among populations from the former region. The F-statistics measures the correlation between genes drawn at different levels of a (hierarchically) subdivided population, which is influenced by several evolutionary forces, such as mutation and migration, but it was originally designed to measure how far populations had gone in the process of fixation owing to genetic drift[22]. The populations from North-eastern region were characterized by greater genetic diversity as compared to Uttarakhand populations. This may be due to the fact that populations from North-eastern region showed more number of effective alleles, greater Shannon's information index and expected hererozygosity.

The NJ tree of 14 *Perilla* populations revealed closeness among the accessions from North-eastern region and Uttarakhand except IC335405. This relatedness of Uttarakhand populations with North-eastern populations probably indicates the migration of populations from one area to another. The differentiation of IC335405 (Mizoram) is expected because it showed distinct and maximum values for observed and effective number of alleles, Shannon's information index and expected hererozygosity (Table 3) as compared to other populations. Diversity studies using molecular markers have assisted in developing *in situ* conservation strategies and the effectiveness of DNA markers for providing a scientific basis for long-term strategies for managing crop genetic diversity on-farm have been reported[23,24].

Understanding the population genetic structure of *Perilla* landraces will be helpful for *in situ* (on-farm) management of these landraces. Farmers' options regarding the size and relative placement of their fields impact significantly on local crop diversity[25]. As there is no organized cultivation of *Perilla* and majority of the populations are weedy races grown in farmers' backyards, they suffer from genetic drift owing to smaller populations. The occasional crossing between cultivated and weedy types leads to setting up of differentiation-hybridization cycle and release of more potential variability[25].

The centre of diversity of *Perilla* is still obscure[9] and the genus *Perilla* contains only one tetraploid species, *P. frutescens*, which is traditionally cultivated in Asia. Though the main area of diversity of *P. frutescens* is assumed to be China because of its long history of cultivation[3,7,8], the other areas where the tetraploid *P. frutescens* is widely distributed or presently cultivated including India are also important for the utilization and conservation of genetic resources because of high expected genetic diversity in these areas.

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