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Research Article

GENETIC DIVERSITY ASSESSMENT OF ACID LIME (*Citrus aurantifolia*, Swingle) LANDRACES OF EASTERN NEPAL USING RAPD MARKERS

NN Munankarmi[1,] *, RL Shrestha[2], N Rana[3], JKC Shrestha[3], S Shrestha[3], R Koirala[2,3] and S Shrestha[3]

[1]Central Department of Biotechnology, Tribhuvan University (T.U.), Kirtipur, Kathmandu, Nepal
[2]Agricultural Research Station (ARS), Pakhribas, Dhankuta, Nepal
[3]Nepal Academy of Science and Technology (NAST), Khumaltar, Lalitpur, Nepal

*Corresponding author email: nabin2045@gmail.com

Abstract

Acid lime (*Citrus aurantifolia* Swingle) is an important commercial fruit crop, cultivated from terai to high hill landscapes of Nepal. However, production and productivity is very low due to various reasons including infestations by various diseases and pests, lack of diseases and pests resistant and high yielding varieties. In this context, determination of genetic variation at molecular level is fundamental to citrus breeders for the development of elite cultivars with desirable traits. In the present study, Random Amplified Polymorphic DNA (RAPD) marker technique has been employed to assess genetic diversity in 60 acid lime landraces representing different agro-ecological zones of eastern Nepal. Nine selected arbitrary primers generated 79 RAPD fragments of which 75 were polymorphic (94.94%). Phenogram was constructed by NTSYS-PC ver. 2.21i using UPGMA cluster analysis based on Jaccard's similarity coefficient to deduce overall genetic diversity and relationships of the acidlime genotypes under study. Sixty acid lime landraces formed seven clusters and similarity value ranged from 38% to 98% with an average of 72%. Genetic variation at different agro-ecological zones was assessed using Popgene ver. 1.32 and found 47% to 69.6% polymorphism. Shannon's index and Nei's gene diversity showed highest level of acid lime diversity in Terai zone (PPB, 69.62%; H, 0.213; I, 0.325) followed by mid-hill zone (PPB, 67.09%; H, 0.208; I, 0.317). The results obtained will be useful to citrus breeders for elite cultivar development. The RAPD-PCR technique is found to be the rapid and effective tool for genetic diversity assessment in acid lime landraces of Nepal.

Key words: Lime; Citrus; molecular marker; Polymerase Chain Reaction; PCR

Introduction

Acid lime (*Citrus aurantifolia* Swingle), member species of family Rutaceae is commonly known as 'Kagati' in Nepali. It is a rich source of vitamin "C" which is used as juice, pickles and salad preparations. Besides, it also has medicinal properties and used for the prevention of various diseases such as bones and joints, piles, dysentery, cold, influenza, constipation and scurvy (Dhillon and Randhawa, 1993). It is an important commercial fruit crop that ranks third after mandarin and sweet orange in terms of area coverage and cultivated in 60 out of 75 districts of terai to high hill landscapes of Nepal (NCRP, 2012).

Production and productivity of acid lime in Nepal is low at 8.4 ton per ha (MoAC, 2011), as compared to other countries like Argentina with 19 ton per ha and India with 12.2 ton per ha (FAO, 2006). This might be due to various reasons including lack of high yielding varieties, low quality planting materials, lack of use of disease resistant rootstocks, prevalence of various bacterial, fungal and viral diseases, lack of use of advanced crop management practices etc. Development of elite cultivars of acid lime with desirable qualitative and quantitative traits can be achieved via conventional and non-conventional breeding, protoplast fusion, genetic engineering, molecular marker assisted breeding and mutational breeding (Dominquez *et al.*, 2002; Viloria and Grosser, 2005; Rauf *et al.*, 2013).

Cultivation range of acid lime in Nepal is 800 m asl to 1400 m asl in the mid hills stretching from east to west, but potentiality of cultivation range could be much wider from 125 m asl to 1800 m asl. The normal production period is limited between September and December (Dhakal and Bhattarai, 2002). High level of variation in fruit quality, seasonality in flowering, harvesting time, productivity and disease resistance among acid lime accessions of different Agro ecological zones have been reported (Sapkota, 2006).

Maximum utilization of any germplasm for breeding can be achieved by understanding the level of genetic diversity it contains (Vinu *et al.*, 2013). Genetic diversity estimates are also important to understand its adaptive potential in different environments (Lowe *et al.*, 2004). Evaluation of genetic divergence and relatedness among breeding materials has significant implications for crop improvements. And knowledge on genetic diversity in acid lime accessions could help breeders and geneticists to

understand the structure of germplasm and to predict which combination would produce best offspring and facilitate in widening up the genetic basis of breeding material for selection (Singh, 2005).

Genetic diversity within and among different populations or different agro-ecological regions can be assessed using morphological, biochemical and molecular approaches (Chawla, 2005; Vinu *et al.*, 2013). Assessment of genetic diversity using morphological traits is not promising as such traits are influenced by environmental factors and management practices (Reddy *et al.*, 2002). Use of biochemical markers such as isozymes and seed proteins has been restricted due to limited availability of polymorphic markers for genetic analyses (Shrestha, 2001). In this context, various Polymerase Chain Reaction (PCR) - based molecular marker tools such as Simple Sequence Repeats (SSRs), Amplified Fragment Length Polymorphism (AFLP), Random Amplified Polymorphic DNA (RAPD) and Inter Simple Sequence Repeats (ISSR) have emerged as powerful tools for screening biodiversity. These techniques have been widely used to study the genetic diversity, taxonomy, cultivar identification (Fang *et al.*, 1997; Filho *et al.*, 1998; Novelli *et al.*, 2000) and the construction of genetic linkage maps (Kijas *et al.*, 1997; Sanker and Moore, 2001) in various Citrus spp. Of these markers, RAPD markers (Wiliams *et al.*, 1990) that result from the PCR amplification of genomic DNA fragments using short oligonucleotides (usually 10-mers) of arbitrary sequence as primers have been widely used for diversity analyses as they are simple to use, cost effective and amplify multiple DNA loci through PCR (Wiliams *et al.*, 1990; Abkenar and Isshiki, 2003; Baig *et al.*, 2009). Other advantages of RAPD include requirement of very small amounts of genomic DNA, elimination of blotting and radio-active detection steps (Cipriani *et al.*, 1996). For these reasons many fruit tree crops have been successfully fingerprinted using RAPD markers, e.g. grape (Huseyin and Sabitagaoglu, 2008), strawberry (Sugimoto *et al.*, 2005), olive (Sanz-Cortes *et al.*, 2001) and pineapple (Sripaoraya *et al.*, 2001). However, despite its limitations such as sensitivity to reaction conditions, problems with repeatability, and amplification of non-homologous sequences, it has been successfully used for the assessment of genetic diversity in plants (Maria *et al.*, 2008). In citrus species, RAPD markers have been used for various purposes such as genetic diversity analysis (Abkenar and Isshiki, 2003; Mariniello *et al.*, 2004; Campos *et al.*, 2005; Novelli *et al.*, 2006; Shaaban *et al.*, 2006; Shahsavar *et al.*, 2007; Hvarleva *et al.*, 2008), and phylogenetic analysis (Nicolosi *et al.*, 2000).

Acid lime is a cross-pollinated crop with wide sexual compatibility between *Citrus* and related genera. Besides, high frequencies of bud mutation, a high level of genetic erosion and narrow genetic base have also been reported in acid lime (Scora, 1988). Furthermore, low quality planting materials and poor orchard management practices are also contributing factors for low quality fruits and production (NCRP, 2012; Shrestha *et al.*, 2012a). A survey conducted in 14 major cities of Nepal showed that 94.5% (1875.0 tons) of lime sold from Kalimati market (one of the wholesale markets at Kathmandu) and 68% of the lime sold in rest 13 cities were imported from India (Dhakal and Bhattarai, 2002). In this context, development of elite acid lime cultivars with desirable traits such as disease resistance, nematode resistance, high yield, juice content etc., holds great promise. Therefore, study of genetic diversity of acid lime landraces of Nepal at molecular level is one of the fundamental tasks to be performed for this purpose.

Genetic diversity assessment of acid lime landraces of Indian origin has been carried out recently using RAPD markers (Kumar *et al.*, 2013). Prior to this study, SSR based genetic diversity analysis was carried out using same acid lime samples of eastern Nepal (Shrestha *et al.*, 2012a). Selection of elite acid lime genotypes based on phenotypic attributes and physicochemical properties have also been carried out using same samples used in this study (Shrestha *et al.*, 2012b). In the present study, an attempt has been made to evaluate the genetic diversity of existing acid lime landraces at different agro ecological zones of Eastern Nepal using dominant marker system, the RAPD.

Materials and Methods

Sample collection and DNA isolation

A total of 60 young expanding healthy leaf samples (6 - 8 weeks old) were collected for DNA extraction from the farmer's orchards of eastern Nepal (Fig. 1) and dried immediately in silica gel in a air tight plastic container and brought to Molecular Biotechnology Laboratory, Nepal Academy of Science and Technology (NAST), Khumaltar for DNA extraction and subsequent molecular analysis. Leaf samples were collected randomly, from the selected trees of three agro-ecological domains representing Terai, Mid-hills and High-hills (Table 1)

Leaf tissues (100 mg) were ground to a fine powder in liquid nitrogen. The total genomic DNA was extracted by following manufacturer's instruction of DNeasy Plant DNA extraction mini-kit (QIAGEN, www//qiagen.com). The extracted DNA (200 µl) was stored at –20˚C until use. The quantity and quality of DNA were determined by spectrophotometer (Bio-photometer, Eppendorf, Germany).

Table 1 Altitudinal range, accessions number and locality details of sample collection sites of acid lime landraces

Above 1200 m asl			600-1200 m asl			Less than 600 m asl		
Acc. No	**Altitude**	**VDC-Ward no**	**Acc. no**	**Altitude**	**VDC-Ward no**	**Acc. No**	**Altitude**	**VDC-Ward no**
LT-1	1605	Okhre-8	LD-49	1185	Bodhe-1	LM-43	135	Sunpur-2
LT-17	1750	Fachmara-7	LKv-60	1285	Balara-1	LM-44	135	Sunpur-2
LT-18	1710	Fachmara-9	LKm-61	1285	Balara-1	LD-45	135	Sunpur-2
LT-15	1655	Fachmara-9	LKr-62	1285	Balara-1	LD-58	135	Sunpur-2
LD-50	1638	Rajarani-9	LD-48	1181	Bodhe-1	LS-34	128	Narsing-2
LT-8	1505	Okhre-8	LD-25	1180	Balara-1	LS-35	128	Narsing-4
LT-22	1505	Sudap-1	LD-26	1175	Balara-1	LS-36	128	Narsing-4
LT-9	1500	Okhre-5	LD-27	1175	Balara-1	LS-37	128	Narsing-4
LT-21	1485	Fachamara-1	LD-28	1175	Balara-1	LS-38	128	Narsing-4
LT-20	1410	Fachamara-8	LD-29	1175	Balara-1	LS-39	128	Narsing-4
LT-16	1405	Fachamara-7	LD-30	1175	Balara-1	LS-40	128	Narsing-4
LT-19	1350	Fachamara-7	LD-59	1175	Balara-1	LS-41	128	Narsing-4
LT-13	1315	Fachamara-7	LT-4	1155	Okhre-1	LS-42	128	Narsing-4
LT-12	1310	Fachamara-7	LT-5	1155	Okhre-3	LS-56	128	Narsing-4
LT-14	1308	Fachamara-7	LT-6	1150	Okhre-3	LS-57	128	Narsing-4
LT-23	1308	Sudap-7	LD-31	1150	Dhnk -3	LM-51	125	Pathari-2
LT-3	1305	Okhre-8	LT-7	1145	Okhre-2	LM-52	125	Pathari-2
LD-24	1290	Balehara-8	LT-10	1135	Okhre-3			
LT-2	1285	Okhre-1	LT-11	1130	Okhre-3	LM-54	125	Pathari-2
LD-46	1278	Bodhe-2	LD-32	1130	Balhra-3	LM-55	125	Pathari-2
			LD-33	1130	Balhra-1	-	-	-

Note: LT = Lime Terhathum, LD = Lime Dhankuta, LM = Lime Morang, LS = Lime Sunsari, LKm = Lime Madrasi, LKr = Lime Rampur, LKv = Lime Bana-rasi, VDC = Village Development Committee, m = meter, asl = above sea level.

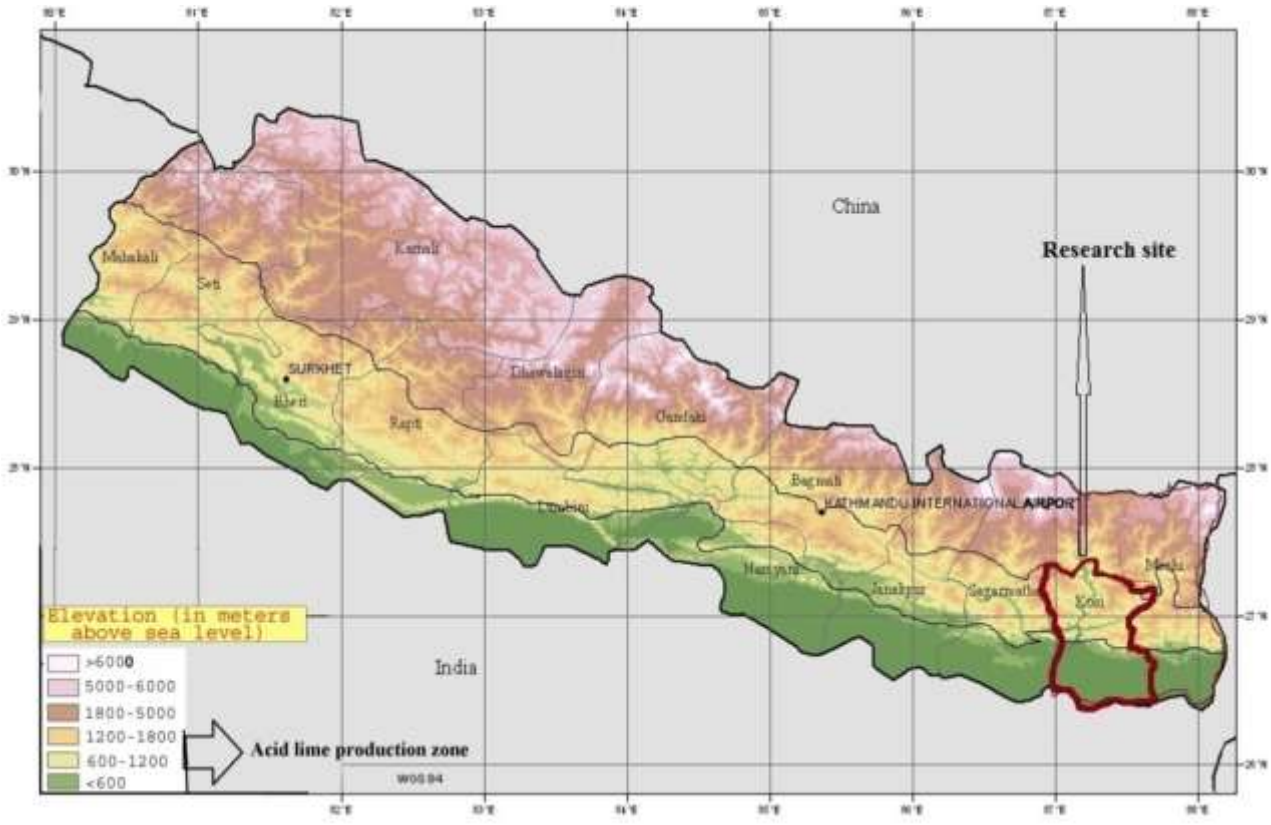

Fig. 1: Map of Nepal showing sample collection sites

RAPD-PCR amplification and primer screening

RAPD-PCR reaction conditions were optimized by varying concentration of different PCR parameters such as template DNA, $MgCl_2$ and primer. RAPD cycling condition described by Edwards (1998) was used for the optimization and subsequent RAPD profiling experiments. The PCR

program consisted of initial denaturation at 95˚C for 2 min, 45 cycles of 95˚C for 20 sec, followed by annealing at 37˚C for 1 min; extension at 72˚C for 1 min and final elongation at 72˚C for 10 minute. Using optimized RAPD-PCR reaction conditions, 40 arbitrary UBC primers (Vancouver, Canada) were screened using one genomic DNA sample of acid lime. Of these 40 primers, nine primers that produced multiple, scorable polymorphic and reproducible bands were finally selected for RAPD profiling involving all acid lime landraces under study. PCR amplification was performed in 25µL reaction volume in Thermal cycler (Bioer Technology Co. Ltd., China Version 2001.1.0) containing 0.1 mM dNTPs, 3 mM $MgCl_2$, 2.5 µl of 10× Taq buffer [100 mM Tris-HCl, pH 8.8 at 25˚C, 500 mM KCl 0.8% (v/v), Nonidet P40], 2.0 U Taq DNA polymerase (Fermentas, Life sciences; 5 U/µl), 0.4 pmol of each primer (Eurofins Genomic Test Pvt. Ltd., Banglor, India) and 25 ng of template DNA.

PCR products were analyzed on 1.5% (w/v) agarose gel after running in 1X TAE Buffer at 100 V for 45 minutes (9.0 V/cm) and Ethidium Bromide staining (0.5µg/ml) (Sambrook and Russell, 2001) for visualization and documentation using Gel doc system (Syngene, UK). The molecular size of PCR products was estimated by comparing the position of bands with 100 bp plus DNA ladder (Gene Ruler TM, Fermentas, Life Sciences).

RAPD profiling and data analysis

All nine primers selected from primer screening experiment were used for RAPD profiling of all 60 acid lime landraces under study. RAPD profiles generated by each of the nine primers were used to generate a binary data matrix with '0''1' coding, where the presence of the band corresponded to value 1 and the absence to value 0. Amplification failure was scored as "9", which was designated in the analysis procedure as an indicator of missing data (Transue *et al.*, 1994). The binary data matrix created was analysed using MS- Excel 2007 for the estimation of the banding characteristics namely: 1) Total number of bands (TNB), 2) number of polymorphic bands (NPB), 3) Percent Polymorphism (PP), 4) Polymorphic Information Content (PIC), and 5) Resolving Power (RP) for each primer used, which are defined by, PP = NPB/TNB generated by each primer.

$$PIC = 1 - \sum_{j=1}^{n} (P_{ij})^2$$

Where, Pij is the frequency of the ith pattern revealed by the jth primer summed across all patterns revealed by the primers, where P is the proportion of accessions containing the band. RP was calculated as (Prevost and Wilkinson, 1999).

We used statistical software NTSYS-PC version 1.7 (Rohlf, 2009) to deduce genetic similarity and relationships among acid lime accessions collected from different agro-ecological zones and to construct the phenogram. Similarity indices were calculated using SIMQUAL (Similarity for Qualitative data) computational algorithm. Based on similarity matrices, Sequential, Agglomerative, Hierarchial and Nested (SAHN) clustering was performed using UPGMA algorithm (Sneath and Sokal, 1973). Estimates of similarity was computed on the basis of Jaccard's coefficient (Jaccard, 1908).

S_{ij} = a/a+b+c

Where,

S_{ij} = the similarity between two individuals, i and j;

a = the number of bands present in both i and j;

b = the number of bands present in i and absent in j;

c = the number of bands present in j and absent in i, and

d = the number of bands absent in both i and j.

Genetic Relationships among the Acidlime accessions were also studied using a Principal Coordinate Analysis (PCoA) using (MVSP) Multivariate statistical package version 3.2 (Kovach, 2007). Genetic diversity assessment of Acidlime landraces in different agro-ecological zones was determined by computing Shannon's Information Index (I) and Nei's gene diversity (H) (Yeh *et al.*, 1997).

Results

Estimation of genetic polymorphism in acid lime accessions using RAPD primers

Out of 40 UBC RAPD primers, 26 primers gave amplification products with acid lime genomic DNA. However, only nine primers amplified multiple polymorphic scorable bands and hence selected for RAPD profiling involving all 60 acid lime landraces. The RAPD gel picture amplified by primer UBC 16 is shown in Fig. 2. A total of 79 loci were amplified by nine primers across 60 acid lime accessions, of which 75 were polymorphic and 4 were monomorphic. The average number of bands per locus was 8.8, where highest number (11) of amplified bands was observed for primer UBC 11 and lowest (7) for primer UBC 74. The maximum number of polymorphic bands (11) was amplified by the primer UBC 16 and minimum (7) by primers UBC 18, UBC 74 and UBC 6. The percentage polymorphism ranged from 87.5% to 100% with an average value of 94.94%. The amplicon size ranged from 250 bp to 2500 bp. The PIC value ranged from 0.78 for primer UBC 66 to 0.88 for primer UBC 16 with an average value of 0.83. Similarly, the Resolving Power (R_P) of RAPD primers ranged from 7.4 (UBC 66) to 15.63 (UBC 16) with an average of 10.0 (Table 2).

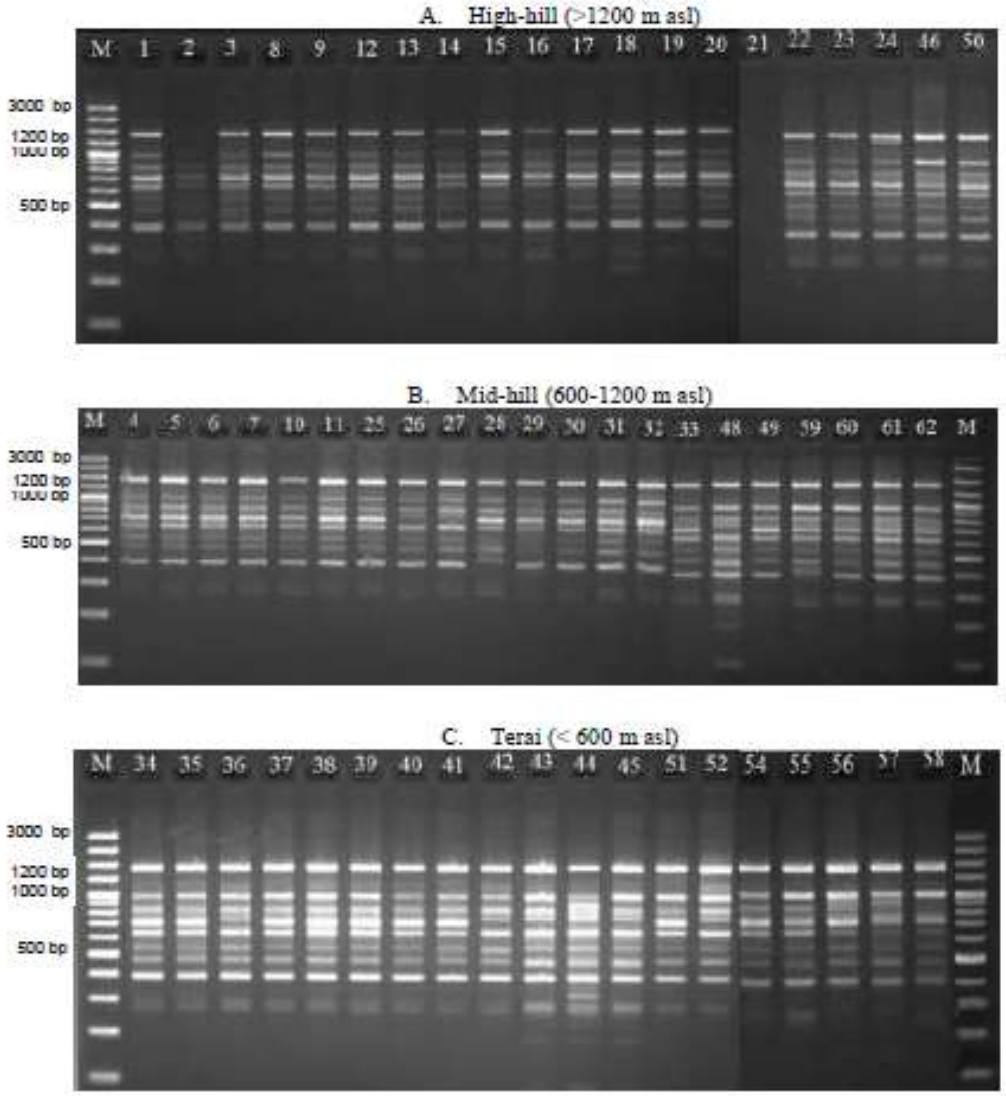

Fig. 2. ISSR profile generated for 60 acid lime landraces by primer UBC 16. Lanes marked 1-62 represents acid lime samples 1-62 from various ago-ecological zones; Lanes marked M are 100bp plus molecular weight marker. A] represents High-hill accessions, B] represents Mid-hill accessions and C] represents Terai accessions.

Table 2. RAPD Primers and their sequences, Total Number of Bands (TNB), Number of Polymorphic Bands (NPB), Percentage Polymorphism (PP), Amplicon size range, Polymorphic Information Content (PIC), and Resolving Power (R_P) values generated by nine primers using DNA of 60 acid lime accessions.

Primer Code	Primer Sequence (5' - 3')	TNB	NPB	Polymorphisms (%)	Amplicon size range (bp)	PIC	R_p
UBC 4	CCTGGGCTGG	10	9	90	320-2000	0.87	14.8
UBC 6	CCTGGGCCTA	8	7	87.5	250-1200	0.85	11.6
UBC 16	GGTGGCGGGA	11	11	100	300-1180	**0.88**	**15.63**
UBC 18	GGGCCGTTTA	8	7	87.5	300-1200	0.84	10.16
UBC 43	AAAAACCGGG	10	9	90	350-1800	0.83	8.7
UBC 51	CTACCCGTGC	9	9	100	400-2500	0.83	7.4
UBC 66	GAGGGCGTGA	8	8	100	500-2000	**0.78**	**7.4**
UBC 74	GAGCACCRGA	7	7	100	400-1800	0.77	7.67
UBC 85	GTGCTCGTGC	8	8	100	300-1800	0.83	6.7
Total		**79**	**75**		**Average**	**0.83**	**10.00**

Estimation of overall genetic diversity of acid lime accessions using similarity coefficients and PCoA

The binary data derived from amplified bands of RAPD markers were used to create similarity matrix to estimate genetic similarity among acid lime accessions. Based on Jaccard's similarity coefficient level (Nei and Li, 1979), genetic similarity among acid lime landraces ranged from 38% to 100% with an average of 72%. A maximum similarity of 100% was observed between accessions LS-39 and LS-40 while minimum similarity of 38% was observed between LS-56 and LT-16 accessions.

Total accessions were separated into two major clusters (I and II) and five (III, IV, V, VI and VII) minor clusters. Majority of accessions were grouped into cluster I followed by cluster II separated at 0.717 similarity coefficient level. There were narrow genetic distance among cluster groups I, II and VII (73.1%, 72% and 71.7% respectively), where as cluster groups III, IV, and VI were observed to have wider diversity (67.1%, 61.8%, 60% respectively). The cluster group VII has been observed higher level of dissimilarity (0.54%) than the other groups (Fig 3).

A PCoA (Principal Co-ordinate analysis) based on the Euclidean distance matrix revealed that the first axis comprised of Eigen value of 158.5 and percentage of variance of 19.209% whereas second axis comprised of Eigen value of 99.197 and percentage of variance of 12.022% with a cumulative variance of 31.232% (Table 5). Plots of the first two coordinates were used to generate a PCoA graph (Fig. 4).

Genetic diversity estimation of acid lime in different agro-ecological domains

Genetic diversity of acid lime landraces from different agro-ecological zones were assessed on the basis of Percentage of Polymorphic Band (PPB), Nei's Gene Diversity (H) and Shannon's Information Index (I) using Popgene ver. 1.32. All the diversity indices were highest in terai accessions (PPB, 69.62%; H, 0.213; I, 0.325) followed by mid-hill and high-hill (Table 4).

Table 3. Two major and four minor clusters along with their accessions.

S.N	Clusters	Accessions
1	I	LT-1, LT-15, LT-3, LT-13, LT-18, LT-17, LT-20, LT-14, LD-24, LT-8, LD-31, LD-27, LD-30, LT-4, LD-25, LT-6, LT-5, LT-11, LT-7, LT-10, LT-9, LT-12, LD-32, LT-21, LT-22, LT-23, LD-28, LD-29, LD-33, LS-36, LD-26
2	II	LD-46, LS-41, LS-37, LS-38, LS-39, LS-40, LS-34, LS-35, LKm-61, LKr-62, LM-51, LD-50, LD-48, LS-42, LM-44, LM-43, LD-45, LKv-60, LM-54, LM-55, LD-49, LM-52
3	III	LT-19
4	IV	LT-16
5	V	LD-59, LS-57
6	VI	LT-2
7	VII	LS-56, LD-58

Table 4 Genetic variation of acid lime landraces at different agro-ecological zones.

Agro-ecological zone	Sample Size	Number of polymorphic bands	PPB (%)	H	I
High-hill	20	59.49	47.000	0.178	0.272
Mid-hill	21	53.00	67.090	0.208	0.317
Terai	19	55.00	69.620	0.213	0.325
Average		55.83	63.820	0.197	0.300
Species level (Multiagro-ecological zone)	60	75.00	94.940	0.231	0.369

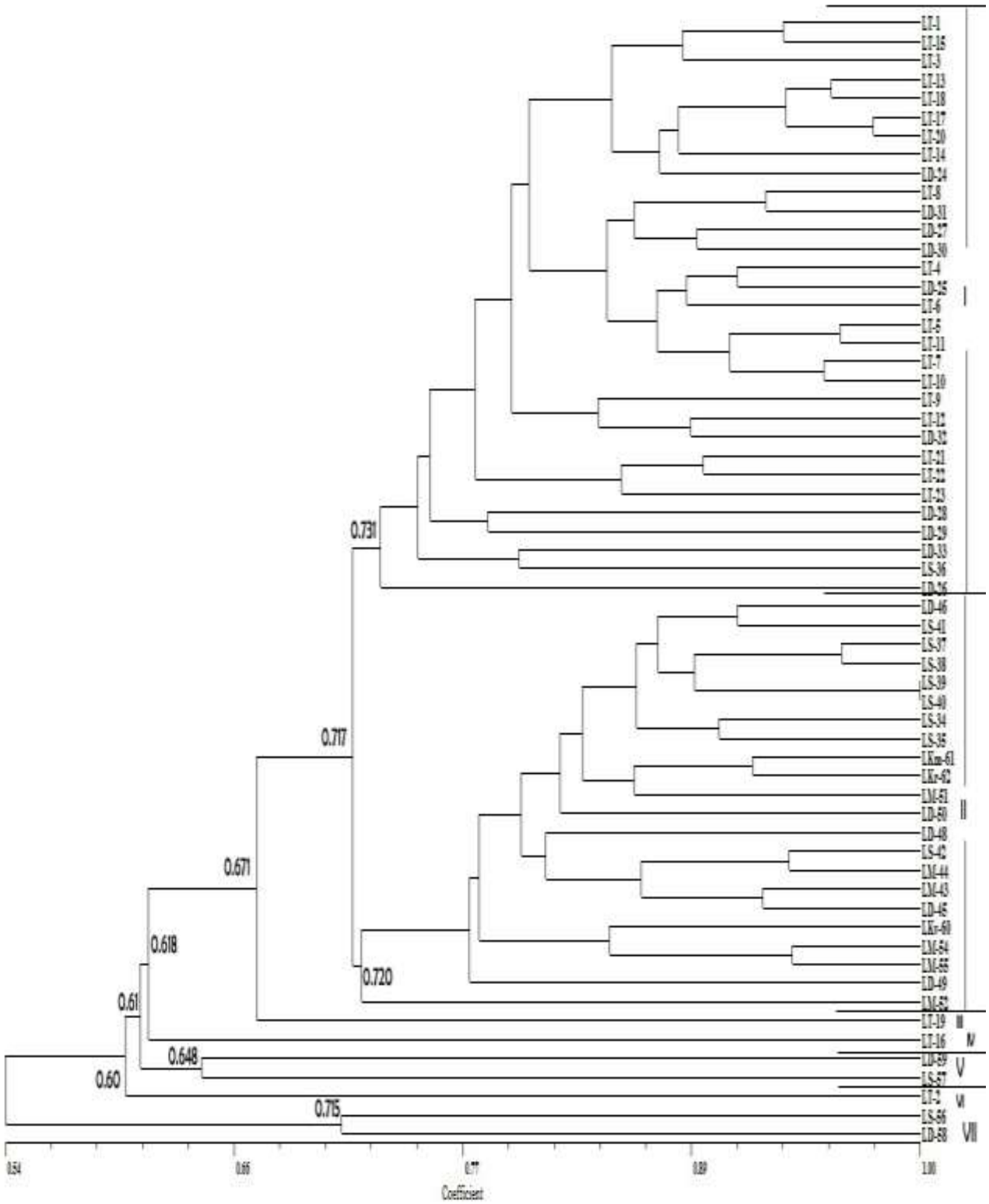

Fig. 3: UPGMA phenogram derived from similarity matrix of Jaccard's coefficient, demonstrating the genetic relationships among 60 acid lime landraces, based on binary data matrix created for 79 RAPD loci generated by nine primers (refer to table 1 for sample details).

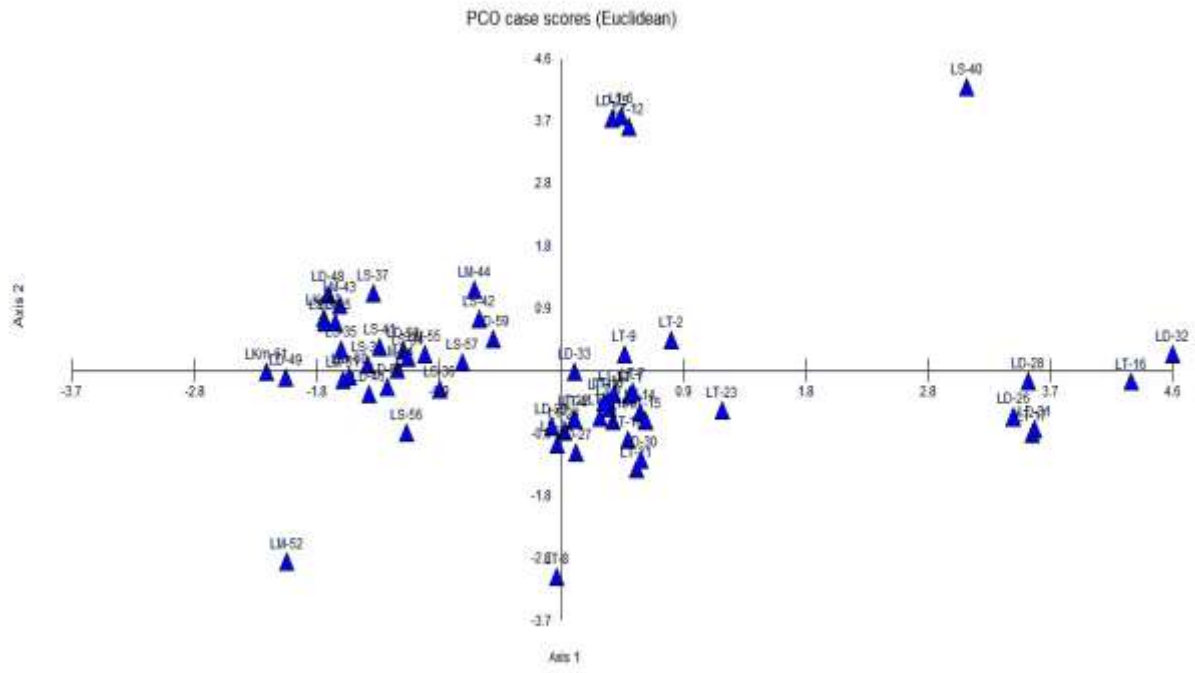

Fig. 4: Principal co-ordinates analysis (PCoA) of 60 accessions of Acid lime using MVSP 3.21

Table 5 Eigen values and the percentage for the co-ordinates of PCoA.

Eigen Values		
	Axis 1 (Co-ordinate 1)	**Axis 2 (Co-ordinate 2)**
Eigenvalues	158.500	99.197
Percentage	19.209	12.022
Cum. Percentage	19.209	31.232

Discussion

RAPD for the assessment of genetic diversity in Acid lime

Assessment of genetic diversity is essential for the characterization, conservation and utilization of genetic resources. It is used for selection and monitoring of genetic resources and contribute to predict the potentiality of the genotypes for breeding purposes (Chakravarthi and Naravaneni, 2006). PCR based molecular techniques are more precise over the morphological and biochemical markers for genetic diversity assessment (Chawla, 2005; Weising *et al.*, 2005). RAPD, an arbitrarily primed PCR-based marker technique is simple, cost effective and powerful tool for the analysis of plant genome. Although it is criticized for its low reproducibility, it is usually overcome by optimizing PCR reaction parameters and maintaining stringent conditions (Vaishali *et al.*, 2008). It has therefore been used extensively in assessing genetic diversity and relationships in wide variety of plant species (Transue *et al.*, 1994; Sugawara *et al.*, 2002; Sugimoto *et al.*, 2005; Maria *et al.*, 2008; Vaishali *et al.*, 2008). In present study also, optimized RAPD-PCR reaction and cycling conditions were used for RAPD profiling and genetic diversity assessment of Acidlime landraces.

Experiments with acid lime landraces have demonstrated the potential of RAPD markers as a rapid, reproducible and useful method for assessing genetic diversity among acid lime landraces. Out of 40 RAPD primers screened, only nine primers gave remarkable polymorphic bands. Average polymorphism revealed by nine primers for all 60 acid lime accessions was 94.94% (Table 4), which indicated high level of genetic diversity among landraces from different agro-ecological domains. This indicates the heterogeneity of the genotypes being used from Terai to high hill landscapes of eastern Nepal. It has been held that commercial citrus species including limes and lemons are complex hybrids of true citrus species i. e. mandarin, pummelo and citron (Uzun and Yesiloglu, 2012). Also, many varieties of the commercial citrus species arose via somatic mutations. The number of DNA fragments amplified ranged from 7 to 11 which is quite consistent with number reported by other authors such as 3 to 15 (Nicolosi *et al.*, 2000) and 5 to 14 (Baig *et al.*, 2009). In this investigation, PIC value ranged from 0.78 (primer UBC 66) to 0.88 (primer UBC 16) with an average of 0.80. The value of PIC between 0 and 1 estimates the degree of polymorphism of the marker (Arya *et al.*, 2011). PIC value of less than 0.25 indicates low polymorphism, a value between 0.25 and 0.50 indicate average polymorphism and a value higher than 0.5 indicates a highly polymorphic locus (Botstein *et al.*, 1980). It has been reported that primers with comparably higher PIC value are more useful than others for distinguishing accessions (Teklewood and Becker, 2006). Therefore, all primers selected for this investigation are highly informative primers for the assessment of the

genetic diversity of acid lime landraces collected from different agro-ecological zones of eastern Nepal as PIC values of all primers are above 0.77. In contrast, PIC value of SSR based study of same accessions (Shrestha *et al.*, 2012a), were shown to be comparatively low at 0.18-0.75 with an average of 0.50. This might be due to the difference in inherent properties of these two marker systems. SSR being specifically primed PCR of codominant inheritance and deals with specific loci of organism's genome, while RAPD is an arbitrarily primed PCR of dominant inheritance and searches the genome more widely (Ellegren, 2004; Chawla, 2005; Shrestha *et al.*, 2005; Wang *et al.*, 2009; Shrestha, 2014). Resolving power (Rp) is an index developed to compare the value of different primers in terms of the informative bands obtained in a given set of germplasm and has been found to correlate strongly with genotype diagnosis and so has potential for a number of applications (Prevost and Wilkinson, 1999). The primer resolving power (R_P) provide quantitative data allowing direct comparisons between primers (Sokal, 1979) and primer with high R_P value have a greater capacity to separate different accessions (Prevost and Wilkinson, 1999). In this investigation, the primer UBC 16 that has the highest PIC (0.88) and R_P (15.63) values, is the most suitable primer to differentiate different Acidlime accessions of this study.

Morphological traits have been frequently used for the determination of relationship among plants and its varieties (Ortiz *et al.*, 1998). Unfortunately, morphological markers do not often reflect genetic relationships because of their interaction with the environment epistasis and largely unknown genetic control of the traits (Smith and Smith, 1998). Based on phenotypic diversity, four landraces (two from high-hills, LT-17 and LT-23 and each from mid-hills, LD-49 and terai, LM-44) were found superior and selected for conservation, breeding and variety development purpose (Shrestha *et al.*, 2012b). In present investigation, the first two are clustered in cluster I while remaining two is in cluster II. Based on Jaccard's coefficient, they are almost similar i.e. LT-17 and LT-23 (86.4%), LT-17 and LM-44 (70%), LT-17 and LD-49 (75.5%), LT-23 and LM-44 (64.7%), LM-23 and LD-49 (64.8%) and LM-44 and LD-49 (66.7%). Molecular marker may provide information on the history and biology of cultivars, but not necessary to reflect what may be observed in phenotypic traits (Avise, 2004). There are many controlling genes spread throughout the genome for the development of quantitative traits like fruit weight, juice content, total soluble solid etc. (Martin and Herrmann, 1998). Among the three agro-ecological zones, high genetic diversity was observed in Terai landraces than Mid-hills and High-hills. This may be due to the planting materials carried by the farmers from different hill districts with migration and introduction from neighboring country in Terai agro-zone. On the other hand, low level of genetic variability were observed in mid hill and high hill as in this zone most of the acid lime trees were established in natural conditions.

The pair wise similarity matrix was generated from the binary data using the Jaccard's coefficient of similarity which showed the genetic similarity coefficient ranging from 0.38 to 1.00 with an average of 0.72. RAPD based similarity is found to be comparatively higher than reported using SSR markers (0.43-0.53) for same acid lime samples (Shrestha *et al.*, 2012a). The highest genetic similarity (100%) was found between the accessions of sunsari i.e. LS-39 and LS-40 and highest genetic distance (38% similarity) was observed between LS-56 and LT-16. Accessions grouped in clusters III, IV, V, VI and VII have higher level of genetic distance. As RAPD being multilocus marker and searches the genome more widely than SSR, it has given different clustering pattern. However, clustering of some of the accessions such as LD-59, LS-57, and LS-56 away from the rest of the accessions in SSR-based phenogram is congruent with RAPD-based phenogram also. In addition to cluster analysis, PCoA (Principal Co-ordinate Analysis) was carried out to determine the genetic diversity of acid lime landraces and showed similar results with that of the phenogram. Genotypes in different clusters of the phenogram may harbor diverse genetically attributed traits (both qualitative and quantitative), that should be identified and utilized further for the development of elite cultivars through breeding.

Use of RAPD data in Acid lime breeding programs

Improvement and selection of good quality traits are important steps in the variety development program. Breeding of good quality traits requires selection of parents with a wider genetic diversity (Pangali *et al.*, 1997) For this, sufficient knowledge about genetic diversity in the gene pool is required to adopt the efficient and valuable breeding approach. In the present investigation, the value of Shannon's information index and Nei's gene diversity were found to be 0.325 and 0.213 respectively in terai agro-ecological zone which shows higher level of diversity among the accessions studied. This indicates diverse gene pool in Terai in comparison to mid and high hills, which might be due to Terai landscapes being more accessible for the movement of germplasm in country as well as from neighboring country India.

Production of any crop is related to a number of activities including agronomic practices, diseases and pest's management, use of improved varieties, use of various root stocks etc. (Machado *et al.*, 2011). A number of sanitary problems that challenges the citriculture are those of biotic and abiotic limiting factors. Major biotic constraint is susceptibility to many diverse pathogens and insects including virus, viroids, fungi, nematodes and bacteria resulting into the manifestation of various diseases such as Citrus Greening Disease (CGD) or Haunglongbing (HLB) disease, bacterial canker, Alternaria brown spot, Dagger

nematode, Tristeza, Crinkly leaf, Cachexia, Exocortis etc. (Deng *et al.*, 2000; Rauf *et al.*, 2013; Harper *et al.*, 2014). Such susceptibility causes huge losses to citrus industry, one of the most important fruit crop industries in the world. Infestation by various disease epidemics can ruin any citrus industry when there is narrow genetic base among the cultivated accessions (Machado *et al.*, 2011). Therefore, assessment of genetic diversity using molecular markers is one of the most fundamental tasks to be performed in order to understand genetic structure of available gene pool for further utilization and conservation.

Breeding for resistance to important diseases has been one of the top priorities in citrus cultivar improvement program. Use of various disease resistant rootstocks such as *Poncirus trifoliata*, is a traditional practice being utilized by citrus industries around the globe to save quality scions from various diseases (bacterial and viral) and pests. *Poncirus trifoliata* is resistant against Citrus Tristeza Virus (CTV), which is the causal agent of one of the most important citrus viral diseases. In this connection, citrus tristeza virus (CTV) resistance gene (Ctv) and major gene responsible for the citrus nematode resistance (*Tyr*1) have been identified from *Poncirus trifoliata*, which can be effectively utilized for the development of resistant cultivar development either via genetic engineering or via marker assisted breeding strategies (Deng *et al.*, 2000). In our case, many of the accessions have been grafted on *Poncirus trifoliata* (Lkv-60, Lkm-61, Lkr-62, LD-26, LD-27, LD-28, LD-29, LD-30, LS-36, LS-37, LS-38, LS-39, LS-40, LS-41, LS-42, LS-56, LS-57, LM-51, LM-52, LM-54 and LM-55) while others are of seed origin.

Conclusion

Acid lime is highly demanded fruit crop of Nepal. However, Nepal is not self-sufficient in Acid lime production and large volume has to be imported from India to fulfill the market demand. Although, geo-climatic condition of Nepal is highly suitable for acid lime cultivation, its production per hectare is comparatively very low in comparison to other countries. The low production may be attributed to a number of reasons including lack of high yielding varieties, diseases and pests' infestations, poor agronomic practices and so on.

Many diseases and pests of citrus can be associated both with scions and rootstocks. Therefore, establishment of healthy citrus industry is challenging as a number of factors need to be considered. Citrus breeders need to consider not only the enhanced yield but in the meantime should also take care of fruit quality, flavor, taste as well as disease and pest resistance of both scions and the rootstocks. In this context, various biotechnological tools such as molecular markers and genetic engineering could be promising for the enhancement of various qualitative as well as quantitative traits of commercial cultivars of acid lime.

Molecular markers such as RAPDs and SSRs have got wide application in genetic diversity assessment of various agronomic crops. Many qualitative and quantitative agronomic traits such as fruit size, high juice content, disease and insect resistance etc. have genetic basis of inheritance and can be enhanced by the use of molecular markers and marker assisted selection (MAS) technique. From the present study, genetic diversity of acid lime landraces of eastern Nepal has been assessed using RAPD marker technique. Genetic diversity assessment is fundamental task for plant breeders. Based on genetic diversity estimation of this study, acid lime breeding program can be expanded aiming at development of elite cultivars. Finally, acid lime genotypes considered in this study are the valuable genetic materials of Nepal for long term conservation and utilization for the development of elite cultivars.

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