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Genetic Diversity in Finger Millet Landraces Revealed by RAPD and SSR Markers

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Abstract

Genetic diversity assessment is the preliminary work for the development of variety and conservation of diversity. Finger millet is a very important crop in Nepal however, its genetic potential has not been fully utilized. Genetic diversity was assessed in forty landraces of finger millet using 9 RAPD and 5 SSR markers. These landraces were collected from Kaski and Dhading districts. None of single primers of these RAPD and SSR could separate all 40 landraces. The average number of bands were 6.33 and 7.8 per RAPD and SSR primers respectively. Mean polymorphism information content was of 0.314 for RAPD and 0.37 for SSR. Primer OPA-4 produced the highest number of bands and the lowest numbers of bands were produced by OPA-16. Among the SSR primers, SSR-06 produced the highest number of polymorphic bands and UGEP-53 produced the lowest bands. RAPD based dendrogram has generated four clusters and SSR based dendrogram has generated two clusters. In both dendrogram and principal component analyses, Purbeli landrace was found unique locating separately in the cluster and scatter plot. Nei's genetic distance produced by RAPD and SSR primers was similar that is 0.327 by RAPD and 0.296 by SSR markers. Genetic distance produced by SSR markers was higher than distance produced by RAPD marker. These landraces were from two districts and therefore have shown intermediate diversity. These molecular marker-based findings should would be more useful if we could link with agromorphological traits. Inclusion of large number of landraces collected from different areas are required to get higher level diversity in addition to associate genetic diversity with geographical sites. Groupings of these landraces could be useful for selecting landraces in breeding program as well as planning conservation program.

Keywords: Finger millet landraces, DNA fingerprint, Genetic diversity, DNA marker

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Introduction

Finger millet [*Eleusine coracana* (L.) Gaertn] is found mostly in warm temperate regions of the world from Africa to Asia and in Australia [1]. It is the primary food for millions in central Africa and Asia including southern parts of Nepal. The Consultative Group on International Agricultural Research (CGIAR) has estimated that 10% of the area under different millets is covered by finger millet [2]. Finger millet is the fourth important cereal crop grown in Nepal commonly known as *kodo* [3]. It is grown from Terai to High Hill of Nepal [4]. Finger millet is multipurpose cereal crop and its grains and flour are mostly used in preparation of many traditional products like *dhedo, roti, haluwa, paniroti, sada parautha, bharuwa parautha, khole, buniya, haluwa, sel*, pancake, pizza, doughnuts, namkins, chowmins, pastry momo, cheese balls, chocolates, birthday cake, biscuits, peanut cookies and alcoholic beverages like *jandh, rakshi, chhyang, tumba,* which have religious and cultural importance in many ethnic communities [5]. Along with this importance of finger millet, different landraces of finger millet are being grown across the country [6-7].

Knowledge on genetic diversity is important to manage and use of finger millet landraces and genotypes properly. Different molecular markers have been widely used in many plant species including finger millet for identification, genetic diversity analysis, phylogenetic analysis, population studies and genetic linkage mapping. DNA marker technology has been applied to a wide range of crop species including maize [8] *Panicum* millet [9], genus *Elusine* [10] and finger millet [11].

Das and Misra [12] reported the efficiency of RAPD (Random Amplified Polymorphic DNA) markers in

investigating genetic relationships at the molecular level, which is important for germplasm conservation and varietal identification. Muza et al. [13] reported a diversity of 26 germplasm lines of finger millet from Africa and India based on the southern blot hybridization patterns. Salimath et al. [10] reported that the molecular diversity of 20 finger millet accessions by using isozyme, RFLP (Restriction Fragment Length Polymorphism) and RAPD. Dida et al. [14] also developed a first genetic map of finger millet by using RFLP, AFLP (Amplified Fragment Length Polymorphism), EST (Expressed Sequence Tag) and SSR (Simple Sequence Repeat) markers. The map span was 721 cM on the A genome and 787 cM on the B genome and cover all 18 finger millet chromosomes. They developed a set of 82 SSR markers specific for finger millet by small-insert genomic libraries generated using methylation sensitive restriction enzymes and among them, 31 SSRs were mapped. Comparative analysis of this map with rice genetic map was a novel attempt that reported high level of conserved co-linearity between the finger millet and rice genomes [14].

Diversity at landrace and trait levels have been reported on Nepalese finger millet landraces [3,4,6,7,15–18]. This finger millet diversity has been poorly utilized in breeding and conservation [19] and only so far six varieties have been released for general cultivation [20]. Due to many factors diversity of finger millet is at risk of losing from the fields [4,20]. Study on genetic diversity helps to accelerate breeding and conservation works. Thus, the study was carried out to assess the genetic diversity and genetic relatedness of finger millet landraces collected from different eco-geological regions of Nepal, and to strengthen breeding and conservation of finger millet diversity.

Materials and Methods

Finger millet landraces

A total of 40 landraces of finger millet were collected from Local Initiatives for Biodiversity, Research and Development (LI-BIRD), Pokhara (**Table 1**). LI-BIRD (an NGO working in different parts of Nepal) has collected these landraces from two districts (Kaski and Dhading) of Nepal.

Table 1. Landraces of finger millet collected from different regions for this study

SN	Landrace	Site and district
1	Setothulo	Kaski
2	Kalo ghudo-2	Pumdibhumdi-6, Kaski
3	Bachuwa-1	Kaski
4	Thulo kalo-1	Kaski
5	Mangsire-2(k)	Kaski
6	Chimte	Dhading
7	Thulo kalo-2	Kaski
8	Kholse	Kaski
9	Setosano	Kaski
10	Kalo ghudo-3	Kaskikot-5, Kaski
11	Setojhyapa	Kaskikot-2, Kaski
12	Tori pane	Kaskikot-2, Kaski
13	Seto	Kaskikot-2, Kaski
14	Mangsire-1	Dhikur pokhara-2, Kaski
15	Kalo ghude-1	Dhikur Pokhara-2, Kaski
16	Mangsire-1	Chimkeswori-3, Kaski
17	Katike-2	Chimkeswori-2, Kaski
18	Kaile	Chimkeswori-3, Kaski
19	Khukur kane-2	Chapkot-6, Kaski
20	Dalle	Chapkot-9, Kaski
21	Khukur kane-1	Chapkot-9, Kaski
22	Chamare	Dhading
23	Champate	Dhading
24	Setokodo	Dhading
25	Mangsire-2(d)	Dhading
26	Katike-1	Dhading
27	Jhyape seto-1	Kaski
28	Pumdeli	Kaski
29	Dalle kodo-1	Kaski
30	Purbeli	Kaski
31	Seto usro-2	Kaski
32	Kalo ghude-1	Kaski
33	Dhudekodo	Kaski
34	Usrokodo	Kaski
35	Dalle kodo-2	Kaski
36	Gairegaule	Kaski
37	Kalo ghude-2	Kaski
38	Mangsire	Kaski
39	Raikare	Kaski
40	Setobhachuwa	Kaski

k: Kaski district , d: Dhading district

Isolation of genomic DNA

The DNA was extracted from the leaf using the modified CTAB (Cetyl Trimethylammonium Bromide) method [21]. Young leaves (2 g) were collected from the 8 days old plants. The leaves were then cut into small pieces with scissors, lyophilized in liquid nitrogen and stored at -40°C. The lyophilized leaves were grounded to a fine powder

using a pestle and mortar and transferred to a 2 ml centrifuge tube. About 600 µl of warm CTAB buffer (65°C) was added and the contents were mixed well on a rotating shaker and incubated for 1 h at 65°C in a water bath with occasional mixing. The tubes were taken out, cooled to room temperature; and 600 µl of chloroform-isoamyl alcohol (24:1) was added and mixed gently by inverting. The tubes were centrifuged at 12000 rpm for 12 min. The aqueous phase was transferred to a new 1.5 ml tube (approx. 400 µl), to which an equal volume of chloroform-isoamyl alcohol was added and mixed gently for 5-6 times. The solution was then centrifuged at 12000 rpm for 2 min. The aqueous phase (approx. 300 µl) was then transferred to 1.5 ml centrifuge tube. On the same tube 600 µl of chilled isopropanol was added for the precipitation of DNA. The precipitated DNA was carefully taken out into new tube with the help of a pipette. The DNA was washed with 70% ethanol, and dried for more than 1 hour and finally dissolved in 50 µl of the 1x TE buffer (1 mM EDTA, 10 mM Tris-HCl, pH 8.0) and stored at -20°C. DNA concentration was estimated by Q5000 UV vis Spectrophotometer (Quawell). Working template DNA solution was prepared with the concentration of 50 ng/ µl using 1x TE buffer.

RAPD primers and amplification

RAPD analysis was performed by using 10-mer primer from Operon Technologies (Alameda, CA, USA). Nine arbitrary RAPD primers were tested for amplification (**Table 2**). DNA amplification was performed in 20 µl reaction volume containing 1.5 mM $MgCl_2$, 10 mM dNTPs, 0.8 picomole of primer, 1.5 U Taq DNA polymerase and 50 ng template DNA. PCR amplification was performed in a Thermo cycler (MULTIGENE OPTIMAX, Labnet International, Inc.) with the following cycling conditions: denaturation at 94°C for 1 min, annealing at 37°C for 1 min and elongation at 72°C for 2 min for 40 cycles after an initial denaturation for 5 min at 94°C. After finishing of amplification 12 µl aliquots of amplification products were loaded in a 1% (w/v) agarose gel (Bioneer) for electrophoresis in 1X TAE buffer (Bioneer). Gels were stained with Ethidium bromide (0.5 µg/ml for 30 min) and visualized under exposure of UV light within gel doc system (UVDI, Major Science). There was standard 1 kb ladder (Promega) for comparing band size.

Table 2. RAPD and SSR primers used in this study

SN	Primer	Sequence	Repeat motifs	Tm (°C)	Ref
A. 10-mer RAPD primer					
1	OPA 03	AGTCAGCCAC		38.6	[22]
2	OPA-16	AGCCAGCGAA		38.6	[23]
3	OPA 08	GTGACGTAGG		38.6	[23]
4	OPA-04	AATCGGGCTG		42.7	[22]
5	OPA 13	CAGCACCCAC		42.7	[24]
6	OPC-06	GAACGGACTC		38.6	[25]
7	OPC-14	TGCGTGCTTG		48.6	[26]
8	OPB-01	GTTTCGCTCC		46.8	[27]
9	OPA-10	GTGATCGCAG		42.7	[28]
B. SSR primer					
1	SSR-06	F: GCCTCGAGCAT CATCATCAG R: CAACCTGCACT TGCCTGG		55	[29]
2	SSR-08	F: TTCCCTGTTA AGAGAGAAATC R: TGTATTTGGTG AAAGCAAC		55	[29]
3	UGEP-10	F: AAACGCGATGA ATTTTAAGCTC R: CTATGTCGTGT CCCATGTCG	(GA)19	60	[30]
4	UGEP-53	F: TGCCACAACT GTCAACAAAAG R: CCTCGATGGCC ATTATCAAG	(AG)26	60	[30]
5	UGEP-1	F: TTCAGTGGTGA CGGAAGTTCT R: GGCTCCATGA AGAGCTTGAC	(TC)11	60	[31]

SSR primer and amplification

Eight pairs of SSR primers were tested for amplification and among them only five primers were found good for DNA profiling (**Table 2**) based on readable and reproducibly of the bands. SSR amplification was performed in 20 µl reaction volume containing 50ng genomic DNA, 10 µl of 2X Green GoTaq® Reaction Buffer (pH 8.5), 400µM dATP, 400µM dGTP, 400µM dCTP, 400µM dTTP and 3 mM $MgCl_2$ (Promega) and 10 picomole of each forward and reverse primer. These components were gently mixed and centrifuged prior to adding 2 drops of mineral oil. The amplification was performed in a Thermo cycler (MULTIGENE OPTIMAX, Labnet International, Inc.). The cycling conditions were 1 cycle of 95°C for 5 min followed by 30 cycles of 95°C for 60 sec, 55°C for 1 min 30 sec, 72°C for 2 min, and finally 1 cycle of 72°C for 10 min.

After finishing amplification 12 µl aliquots of amplification products was loaded in a 1% (w/v) agarose gel (Bioneer) for electrophoresis in 1X TAE buffer (Bioneer). After completion of electrophoresis, gels were stained with Ethidium bromide (0.5 µg/ml for 30 min) and visualized under exposure of UV light within gel doc system (UVDI, Major Science). There was standard 100 bp ladder (Promega) for comparing size of the band.

Gel image and molecular data analysis

Gels were processed and adjusted brightness and contrast in MS Picture Manager before scoring. Ladder were labelled and gel band scoring scale (**Figure 1**) was developed in MS PowerPoint. Based on this gel scoring scale, all bands of both RAPD and SSR profiles were scored for the presence (1) or absence (0) and developed separate binary matrix. Molecular weights of bands were estimated using GelAnalyzer 19.1. Number of amplified bands, polymorphism information content (PIC) and genetic distance were estimated. PIC was estimated following the method of Roldan-Ruiz et al. [32] for RAPD scores and of Smith et al. [33] for SSR scores. GenAlEx 6.5 software was used to estimate these parameters. Cluster and principal component analyses were applied separately for RAPD and SSR profiles. Additional cluster analysis was done combining both RAPD and SSR data.

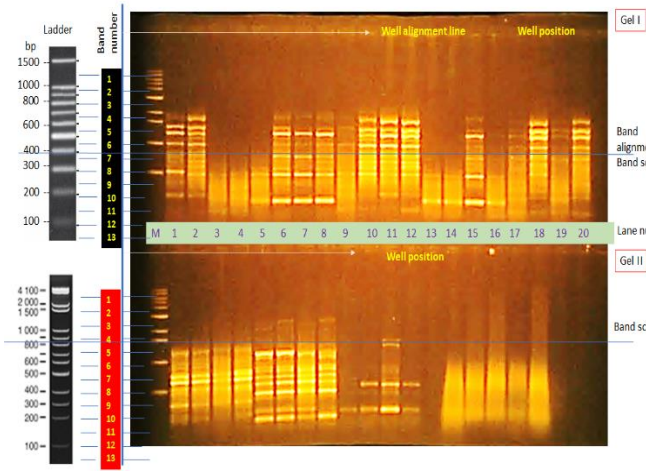

Figure 1. Gel band scoring scale used in MS PowerPoint for gel image analysis one by one

To determine robustness of the dendrogram, the data were bootstrapped with 1000 replications. Mantel test [34] was applied for estimating correlation between SSR and RAPD coefficients matrices. Data were processed in MS Excel and analyzed using GenAlEx 6.5 (for estimating PIC and genetic distance) and NTSYSpc 2.0 (for cluster and principal component analysis).

Results

RAPD and SSR profiles have generated different bands for each of 40 finger millet landraces. As an example, profiles of 40 landraces based on OPA-10 RAPD primer and UGEP-10 SSR primer are given in **Figure 2** and **Figure 3** respectively. None of single primers of these RAPD and SSR could separate all 40 landraces. SSR profiles indicated that some loci are heterogenous, producing 4 distinct bands. Finger millet is tetraploid self-pollinated crops, therefore, majority of loci have two bands showing homozygosity for that loci.

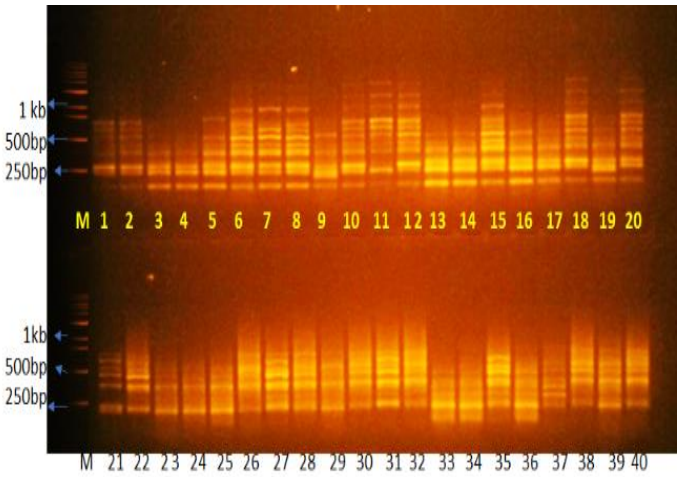

Figure 2. RAPD profiles of 40 finger millet landraces amplified by OPA-10 primer. M, 1kb ladder and lane number represent the sequence number of Table 1

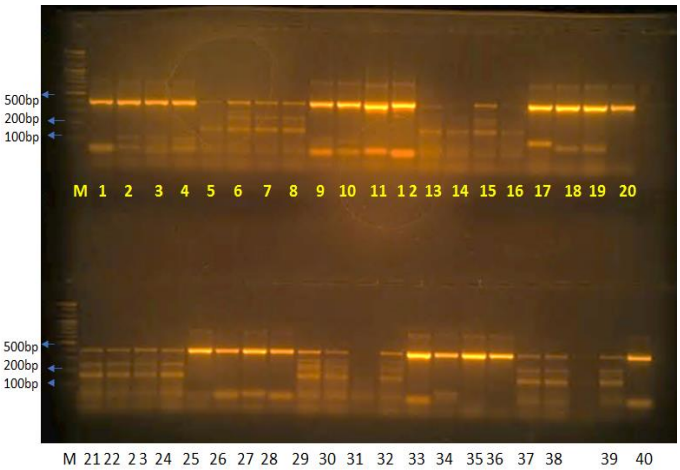

Figure 3. SSR profiles of 40 finger millet landraces amplified by UGEP-10 primer. M, 100 bp ladder and lane number represent the sequence number of **Table 1**

Genetic diversity based on RAPD markers

Nine RAPD primers generated 57 different bands fragments, with average of 6.33 bands per primer and 73.68% polymorphic bands. Primer OPA-4 produced the highest number of bands and the lowest numbers of bands were produced by OPA-16 (**Table 3**). The polymorphic information contents range from 0.15 in primer OPA-13 to 0.45 in OPA-08. Mean PIC value was 0.314±0.174. Primer OPA-04 amplified the maximum number of bands. OPC-14 produced 8 bands, OPA-03, OPA-08 and OPA-10 each produced 7 bands, OPB-01 showed 6 bands, OPA-13 and OPC-06 each showed 5 bands and OPA-16 produced the lowest numbers of bands (3).

Table 3. RAPD and SSR primers with number of amplified bands, polymorphism information content (PIC) and standard deviation

SN	RAPD marker	Bands amplified	Polymorphism information content (PIC)	SD
RAPD marker				
1	OPA-03	7	0.27	0.23
2	OPA-16	3	0.28	0.20
3	OPA-08	7	0.45	0.08
4	OPA-04	9	0.40	0.15
5	OPA-13	5	0.15	0.18
6	OPC-06	5	0.27	0.21
7	OPC-14	8	0.33	0.20
8	OPB-01	6	0.42	0.05
9	OPA-10	7	0.26	0.23
	Mean	6.33	0.314	0.174
SSR marker				
1	SSR-06	15	0.32	0.18
2	SSR-08	8	0.34	0.09
3	UGEP-53	8	0.49	0.00
4	UGEP-10	1	0.35	0.16
5	UGEP-1	7	0.37	0.16
	Mean	7.8	0.37	0.12

The phylogenetic analysis based on UPGMA method separated 40 finger millet landraces into four major groups (**Figure 4**). The Nei's genetic distance obtained by the RAPD markers were ranged from 0.027 in between Dalle kodo-2 and Seto bhachuwa to 0.853 in between Dale kalo-2 and Purbeli. First group consisted of twenty-two late flowering landraces, and second, third and fourth group consisted of seven, eight and three landraces respectively according to mid to late flowering. Second and third groups were mid maturing landrace and forth as the early maturating landraces. Scatter plot of these landraces was well distributed among the principal coordinates with four major groups (**Figure 5**). Variance accounted by principal components I, II and III were 33.28%, 21.71% and 15.67% respectively. Kukurkane-2 and Purbeli landraces have scattered separately.

Genetic diversity based on SSR markers

Five SSR primers generated 39 different bands fragments, with average of 7.8 bands per primer and 87.18% polymorphic bands. Primer SSR-06 produced the highest number of polymorphic bands and UGEP-53 produces the lowest bands during amplification. The polymorphic information contents range from 0.32 in primer SSR-06 to 0.49 in UGEP-53 with mean PIC value of 0.37± 0.123. SSR-6 showed the maximum number of bands (15) followed by SSR-08 (8), UGEP-10 (8), UGEP-07 (7) and UGEP-01 (1).

The phylogenetic analysis based on UPGMA grouping separated 40 finger millet landraces into two major groups (**Figure 6**). First group consisted of twenty-four landraces and second group comprised of sixteen landraces. The UPGMA grouping had further made two sub groups from each main group. These landraces were not grouped according to their collection district.

Scatter plots of these landraces have been found well distributed among the principal coordinate that also separated two major groups (**Figure 7**) which corresponds with cluster analysis (**Figure 6**). This first principal component accounted for 47.02%, second components accounted for 15.53% and third one had explained 14.67% of total variation. Purbeli and Mangsir-2(d) were scattered separately.

Comparison between RAPD and SSR primers

Nine RAPD markers produced 57 bands with 6.33 number of bands per primers and five SSR markers produced 39 bands with average number of 7.8 bands per primer (**Table 4**). Nei's genetic distance produced by RAPD and SSR primers was similar that is 0.327 by RAPD markers and 0.296 by SSR markers. Genetic distance produced by SSR markers was higher than distance produced by RAPD marker. Mantel test indicated the lower correlation (r=0.30) between RAPD and SSR base coefficient matrices.

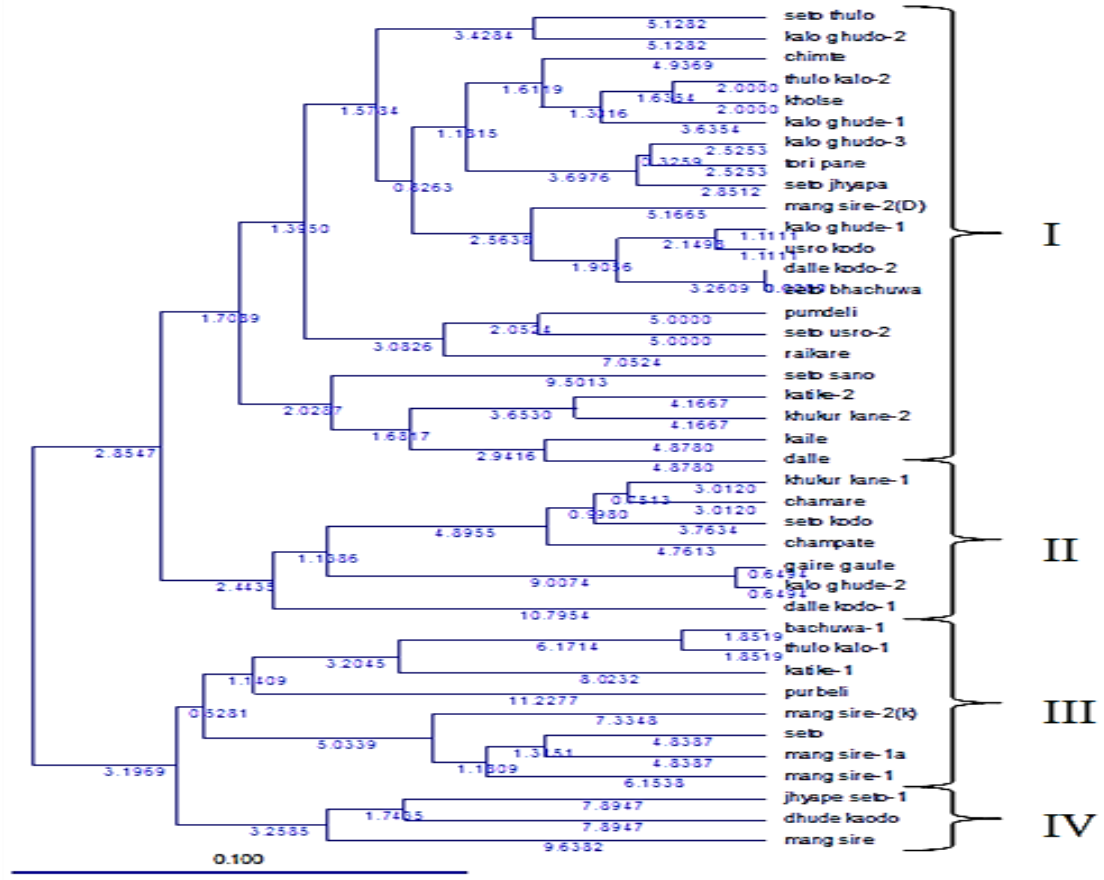

Figure 4. Dendrogram of 40 finger millet landraces based on Nei's genetic distance of 9 RAPD markers using UPGMA methods with bootstrap of 1000 replications

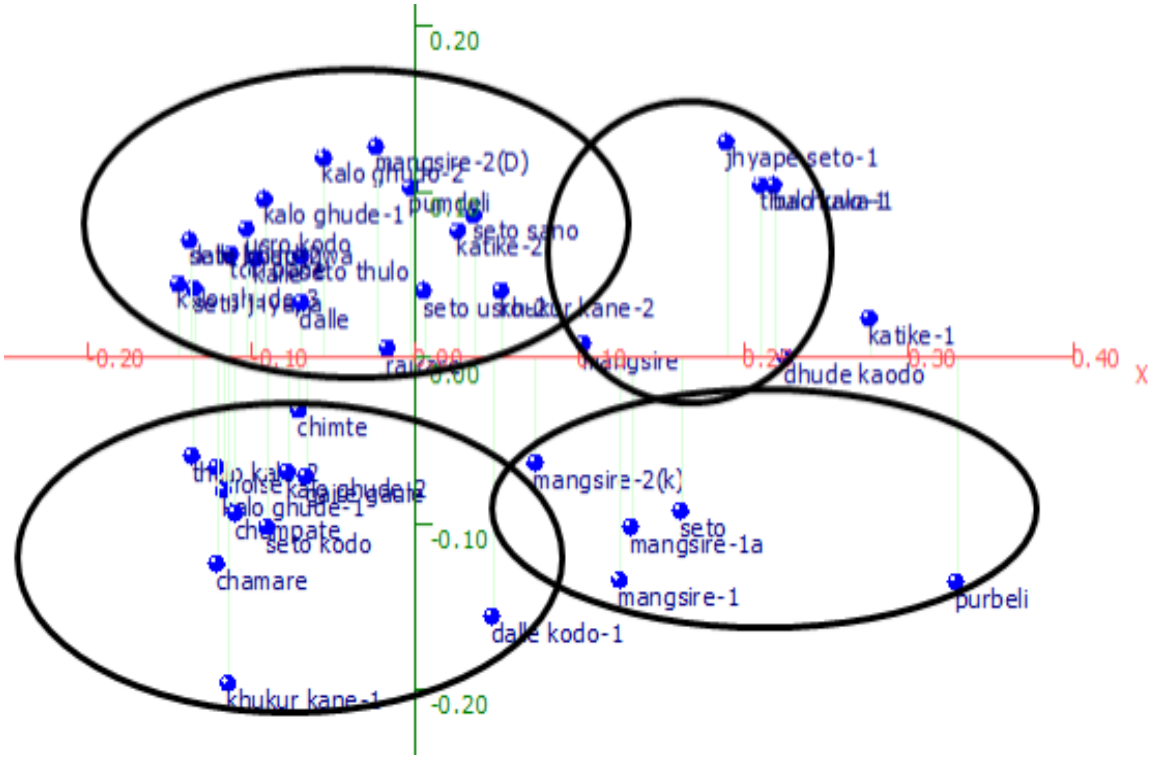

Figure 5. Scatter plot of 40 finger millet landraces based on 9 RAPD markers

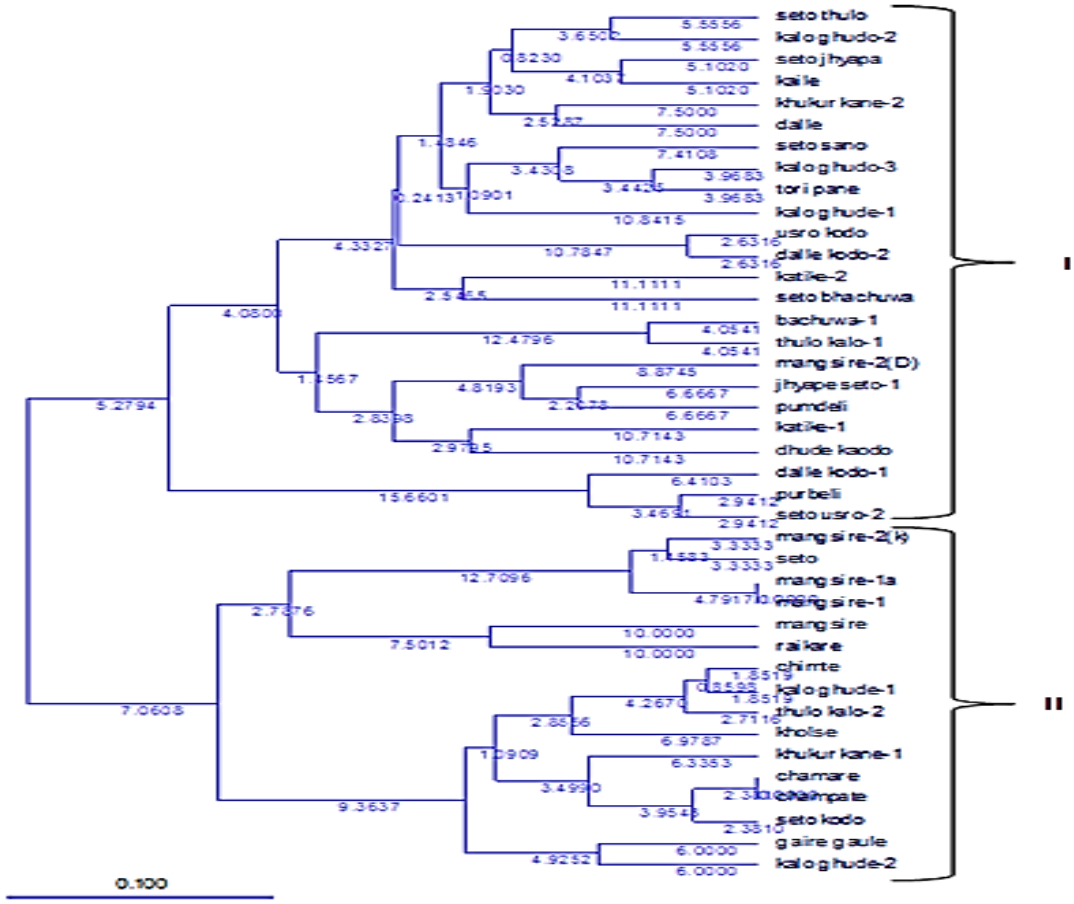

Figure 6. Dendrogram of 40 finger millet landraces using UPGMA methods with bootstrap of 1000 replications based on Nei's genetic distance estimated from five SSR markers

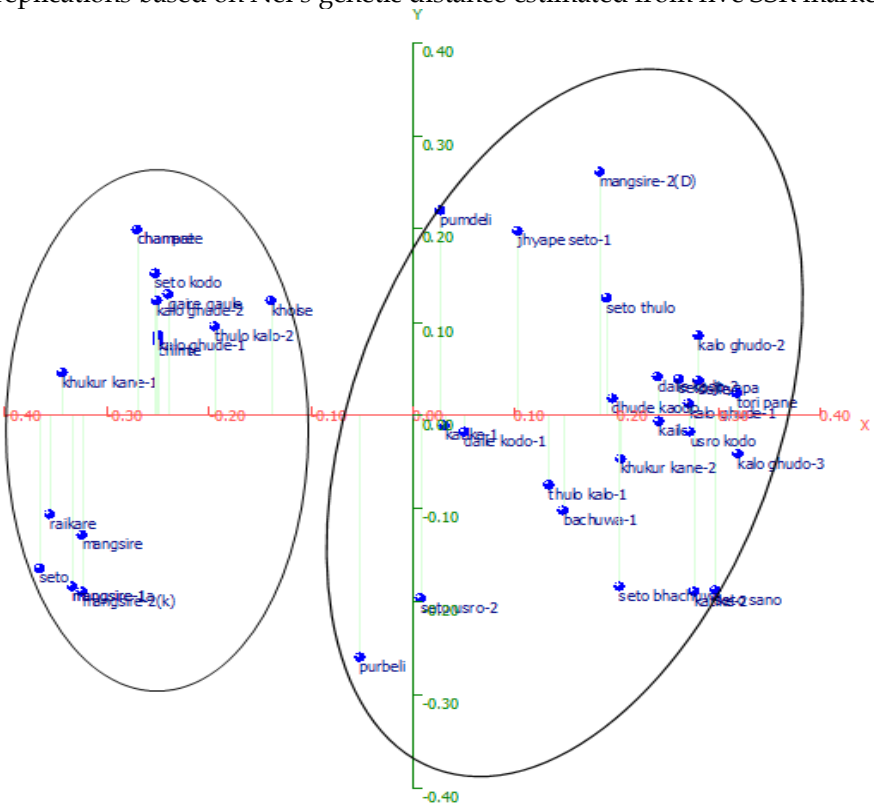

Figure 7. Scatter plot of 40 finger millet landraces using 5 SSR markers

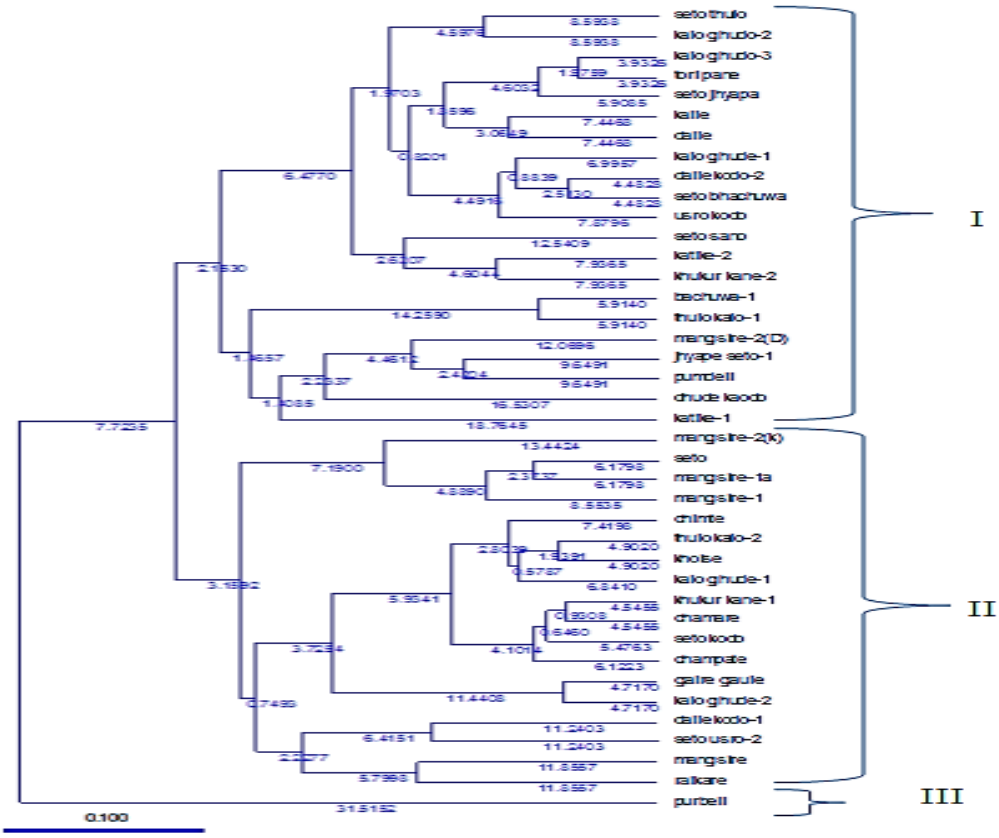

Figure 8. Dendrogram showing 40 finger millet landraces based on Nei's genetic distance calculated from 9 RAPD and 5 SSR markers profile using UPGMA methods with bootstrap of 1000 replications.

Table 4. Comparison between the RAPD and SSR markers in 40 finger millet landraces

Parameter	RAPD marker	SSR marker
Number of primers used	9	5
Number of bands obtained	57	39
Polymorphic bands, n	42	35
Monomorphic bands, n	15	4
Mean number of bands per primer	6.33	7.80
Mean genetic distance	0.32	0.29
Highest genetic distance	0.80	0.93
Lowest genetic distance	0.027	0.04
Correlation coefficient between two matrices	0.30	

Cluster analysis based on both RAPD and SSR markers

Correlation between RAPD and SSR marker based coefficients was low therefore, cluster analysis was done combining both RAPD and SSR profiles. Three clusters were observed (**Figure 8**). There were 21 landraces in cluster I and 18 landraces in cluster II. Purbeli landrace make a separate cluster indicating unique landrace compare to other landraces. Kalo ghudo-3 and Tori pane landraces were very closely related.

Discussion

RAPD and SSR markers are being commonly used for diversity assessments. Majority of the findings of genetic diversity have not been greatly utilized particularly in Nepal for further breeding and conservation works. It is very common that diversity is generally revealed by different DNA markers. We have used finger millet landraces from just few farmers of two districts. These landraces therefore have shown intermediate diversity. Landraces generally possesses intra level diversity but such diversity could not generally be estimated through one sample per landrace, therefore, population level study might be more appropriate for revealing intra landrace diversity. All markers have sown polymorphism producing different band sizes. These findings are similar to other many studies.

Das et al. [24] found that amplification generated by OPA-13 was of 12 bands with the size range from 300 to 3000 base pairs. OPA-4 produced the highest

number of bands (8-9), and OPA-13 was 100% polymorphic. The genetic similarity and group analysis based on similarity coefficient indicated two major groups, first major group had one genotype and a second major group contained 29 genotypes. Babu et al. [22] found the maximum number of bands in OPA-04. Babu et al. [22] reported the diversity of 32 finger millet genotypes using 50 RAPD markers and reported a total 529 loci of which 479 loci (91%) were polymorphic and informative to differentiate the accessions. Sharma et al. [35] used the OPA-13 for the variability study of blast pathogen in rice and finger millet.

Panwer et al. [29] found 10 amplified bands with 60% polymorphism by SSR-06 and 15 amplified bands with 40% polymorphism by SSR-08 in finger millet with PIC value of 0.523 and 0.511 respectively. Arya et al. [30] found the similar result while using UPEG-10 and UPEG-53 SSR markers for assessing genetic diversity and population structure in Indian and African finger millet where UPEG-10 produced two bands and UPEG-53 produced five bands with PIC value of 0.2392 and 0.6681 respectively. Nethra et al. [31] used 35 SSR primers to find degree of genetic diversity in finger millet in which UGEP-1 produced 2 bands with PIC value of 0.16, UGEP-10 amplified 3 allelic bands with PIC of 0.53 and UGEP-53 produced 3 allelic bands with PIC value of 0.51. Dida et al. [36] reported the population structure of 79 finger millet accessions with 45 SSR markers and identified significant difference of plant architecture and yield among Asian and African sub-population.

Salimath et al. [10] experimented with three different DNA marker techniques, viz., RFLP (8 probe-3-enzyme combination), RAPD (18 primers) and ISSR (6 primers) to analyze the diversity of 22 accessions belonging to 5 species of Eleusine. The results revealed 14, 10, and 26% polymorphisms in 17 accessions of E. coracana from Africa and Asia. They suggested that the ISSR marker was good as compared to RFLP and RAPD in terms of the quantity and quality of data output

Different types of markers are considered for generating reliable diversity assessment. Two types of markers in this study have shown low correlation. This may be due to the difference on number of primers used in each marker type. Moulin et al. [37] reported the 0.55 correlation value between RAPD and ISSR marker based coefficient matrices. Additionally scoring SSR codominant markers as presence and absence scale might have some role on revealing true genetic diversity and low correlation among two coefficient matrices.

Breeding and conservation work in Nepal need to strengthen in order to develop climate resilient high yielding varieties. This crop is poorly studied though many farmers are growing over diverse climate and soil conditions. Economic trait based study might have immediate application therefore, further studies are necessary to assess the important traits linked markers through covering large number of landraces collecting from wide areas. This will also help to relate the genetic diversity in association with farming land, and ultimately help to guide conservation works. Highly polymorphic markers and distantly related landraces in this study could be of used for breeders, conservationists and landrace owners. Finger prints of these landraces will be helpful for identification of landraces and developing ownership certificates for particular landrace.

Conclusion

Finger millet landraces collected from different areas have shown genetic diversity that can be valuable resources for variety development and diversity management. Estimated genetic distance were ranged from 0.027 to 0.853 with RAPD markers and from 0.04 to 0.93 with SSR markers. Multivariate analysis generated different groups of these 40 landraces. These landraces were only from two districts, and therefore further analysis need to carry out covering wider areas so that any association between diversity and geographical areas could be estimated.

Author's Contribution

BKJ conceptualized the research proposal, monitored lab work and wrote paper. DJ finalized the proposal, reviewed papers, and performed the lab works, scoring and data analysis. SKG supervised the research activities and supported on data analysis and interpretation. All authors read and approved the final manuscript.

Competing Interests

No competing interests were disclosed.

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Ethical Approval and Consent
Not applicable

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