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Original article

Deciphering allelic variability and population structure in buckwheat: An analogy between the efficiency of ISSR and SSR markers

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ABSTRACT

Food and nutritional security continue to be the issues of concern in developing countries like ours. Exploring the reservoir of high potential unexplored genetic resources could address the world's food and nutritional insecurity. The availability of diverse data and the population structure of any crop germplasm is a valuable genetic resource for discovering genes that can help achieve food and nutritional stability. We used seven ISSR and seven SSR markers to investigate diversity among 63 buckwheat genotypes, including landraces from India's northwestern Himalayas. Various parameters such as percent polymorphism, PIC, resolving power, and marker index was used to evaluate the inequitable efficacy of these markers. We foundthat both marker systems are effective in detecting polymorphism in buckwheat germplasm. Seven ISSRs produced 55 polymorphic bands, while seven SSRs produced 32bands. When compared to ISSRs, SSRs had a greater average PIC value (0.43) than that of (0.36). ISSRs, on the other hand, had a resolving power of (4.38) compared to (1.42) for SSRs. The hierarchical cluster analysis dendrogram divided genotypes into three major clusters. We found that both marker systems were equally accurate in grouping buckwheat genotypes according to their geographical origins. Using 7 ISSR and 7 SSR markers, the model-based STRUCTURE analysis established a population with two sub-populations that correspond to species-based groupings. Within the population, there was a high level of genetic diversity. These results have consequences for both buckwheat breeding and conservation efforts.
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1. Introduction

Buckwheat (*Fagopyrum* sps) is an underutilized crop of the Himalayas that has tremendous potential as a nutraceutical and can serve as an alternative food crop (Singh et al., 2021). Though the plant does not belong to the Poaceae family, the edible component is a pseudocereal used as a cereal grain (Ahmad and Raj, 2012). While buckwheat is not considered a crop of paramount importance and holds an underutilized eminence in several parts of the world (Joshi et al., 2020), at the same timeits remarkable medicinal utility makes it an essentialpart of dietary intake at the local and regional levels (Ikeda, 2002). Owing to its short growth period, high adaptability, and capability to endure harsh environmental conditions, buckwheat thrives in the Himalayan region and utilizes the conditions optimally in comparison to other crops (Kumari and Chaudhary, 2020). Buckwheat is known as a traditional and valued crop in some production expenses, supporting local economies (Suzuki et al., 2020). Buckwheat is a nutrient-dense, gluten-free plant source with abundant health benefits owing to the higher presence of various bioactive components of buckwheat, such as flavonoids, polyphenols, polysaccharides,

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saponins, proteins, fatty acids, and trace elements (Gonçalves et al., 2016; He et al., 2019). Due to the presence of bioactive compounds, buckwheat has engrossed the attention of researchers owing to its healing and functional food properties. It has significantly proven to be anti-oxidative, cardio-protective, anti-cancer, hepato-protective, anti-hypertension, anti-tumor, anti-inflammatory, anti-diabetic, neuro-protection, cholesterol-lowering, and so on (Ge and Wang, 2020; Kwon et al., 2018). Owing to its medicinal and nutraceutical potential the genetic diversity of the buckwheat gene pool, which comprises conventional crop landraces, allows the production and augmentation of nutritionally dense varieties (Singh et al., 2020). Despite its incredible nutritional and nutraceutical potential, buckwheat production is on the decline dueto its low productivity, self-incompatibility, seed shattering and adhering seed coat. The appraisal of germplasm for required traits such as shatter resistance, loose hull, nutritional content, and so on will lead to the documentation of apposite genetic stocks for breeding programs (Rana et al., 2012) and will help in reinvigorating the cultivation of buckwheat. Although breeding of common buckwheat has been widespread in all buckwheat growing areas, tartary buckwheat is a speciality crop that is appreciated in less favorable environmental conditions. Therefore, special attention is given towards the optimization of nutritional quality parameters of buckwheat including optimization of high protein and amino acid composition. A strong understanding of the amount and extent of genetic variation within the buckwheat is important for a successful breeding program. An investigation into various characteristics like map associations, allele mining will form a strong base for determining novel traits that will help better understanding germplasm population structure and genetic diversity. Rapid advances in genomic technology will certainly improve genomic-based breeding for buckwheat quality in near future. In this study, we evaluated the efficacy of two different markers, Inter-simple sequence (ISSR) and Simple Sequence Repeat (SSR), in buckwheat genotype diversity analysis, using landraces from the north-western Himalayas of Jammu and Kashmir and Ladakh, India, and some were procured from NBPGR. Molecular markers like AFLP and RAPD had proven to be less efficient over SSR and ISSR markers such as dominant mode of inheritance and inference of homology from band co-migration (Goldstein, 1999). The SSR marker has high polymorphic information content (PIC) with highly conserved sequences. Due to their high reproducibility, multiallelic nature (Yu and Li,2007), they have been useful for integrating sequence-based physical maps in plant species, and have paved way for breeders and geneticists with an efficient tool to link phenotypic and genotypic variation. However, in the last decade, ISSR markers have been successfully utilized for diversity studies, which reveal many informative bands in a single amplification, therefore being one of the most frequently used markers in intra-specific diversity analysis (Sica et al., 2005; Yu and Li, 2007). Besides the repeatability of ISSR is better than RAPD because ISSR primers are longer and have higher annealing temperatures (Blair et al., 1999; Gilbert et al., 1999). The analysis of genotypes to explicate the genetic diversity and population structure has been elaborated comprehensively in the results and discussion. Moreover, the findings of this study will aid in the identification of parental lines for potential breeding programs, as well as the preservation of valuable genetic stock of this underutilized Himalayan wonder crop.

2. Materials and methods

2.1. Genotypes

Sixty-three genotypes of *Fagopyrum* species detailed in **supplementary Table 1** were used in this study. Part of the germplasm was collected from the traditional buckwheat growing areas of Jammu and Kashmir and Ladakh including Kargil, Leh, Machil, and Gurez, and 57 accessions were procured from National Bureau of Plant Genetic Resources (NBPGR), India through the Material Transfer Agreement protocol. The germplasm was grown in germination trays kept at green house for 3 weeks and after three weeks shoot of plants were kept at −80 °C till further use.

2.2. DNA extraction

Genomic DNA from young buckwheat shoots was extracted by CTAB method with minor modifications (Doyle and Doyle, 1987). Nanodrop tested the quantity as well as the consistency of the DNA (mySPEC, Wilmington, USA). Furthermore, the isolated genomic DNA was run on a 1% agarose gel to visually confirm its quality. Lastly, the prime quality DNA was diluted to a concentration of 25 ng/μL for further use.

2.3. Molecular analysis

2.3.1. ISSR genotyping

The present diversity and population study were carried out using 7 ISSR primers selected from available literature (Zong et al., 2008) as shown in **supplementary Table 2,** among 63 buckwheat genotypes. Universal Gradient Thermal Cycler with 96 wells (Applied biosystems), a 5 μL reaction mixture including 0.5 μL of template DNA (25n g/μL), 3.5 μL of Kappa PCR reaction mixture from Sigma Aldrich (KM 1002 including Taq polymerase) and 0.8 μL primer (10 pm/μL), 0.2 μL of sterilized Millipore water was amplified. Initial denaturation was kept at 5 min at 94 °C, followed by 45 cycles of 94 °C, 30 s; 52 °C, 1 min; 72 °C, 2 min and a final extension of 10 min at 72 °C. Two percent (2%) agarose gel were utilized for the parting of final products using a100 bp ladder (Promega, USA), standard molecular weight marker for ISSRs. Under UV light the gel was assessed visually and recognized with a gel documentation system (Genius, Syngene NG/1151 Cambridge UK).

2.3.2. SSR genotyping

For evaluating polymorphism among 63 buckwheat genotypes, 7 SSR primers were chosen from the literature (Iwata et al., 2005; Song et al., 2018). In **supplementary Table 2,** the particulars of SSRs are specified**.** In a 96 well Universal Gradient Thermal Cycler (Applied Biosystems) PCR amplification was performed for a 5 μL reaction mixture. 0.4 μL forward and reverse (10 pmol/μL), 0.2 μL Millipore water, 3.5 μL Sigma Aldrich Kappa reaction mixture (KM 1002 containing Taq polymerase), and 0.5 μL template DNA (25 ng/μL) were used in the reaction. Initial denaturation was kept 5 min at 94 °C, followed by 35 cycles of 94 °C, 1 min; 50–55 °C, 1.30 min; 72 °C, 2 min and a final extension of 10 min at 72 °C. The amplified products were visualized on a 3% agarose gel under UV gel documentation system (Genius, Syngene NG/1151 Cambridge UK). Among the 63 genotypes amplified in ISSR and SSR using binary scoring according to the 100 bp molecular weight marker (Promega, USA) the gel records were manually scored and scanned forthe clear and repeatable alleles.

2.4. Data analysis

2.4.1. Diversity analysis

For each genotype, the summary produced by each marker was valued (1) for the occurrence of a band and (0) when the band was absent, for the purpose to determine genotype credentials, variation and diversity analysis of buckwheat genotypes, the efficacy of these two marker systems was evaluated with the help of key factors such as number of alleles, number of assay units, polymor-

phic band, and so on independently and in combination (Zargar et al., 2016). The phylogenetic tree was built with DARwin software using a pair-wise distance matrix obtained by constructing a dissimilarity matrix using a common allele index (Perrier & Jacquemoud-Collet, 2006). Using the obtained dissimilarity index, an unweighted neighbor joining tree was created (Saitou & Nei, 1987). The genetic distance between accessions was calculated using the NEI coefficient and a bootstrap resampling (1000) approach across markers and individuals (Nei, 1972). In addition, DARwin software was used to perform Principal Coordinate Analysis (PCoA) on the combined data (Perrier & Jacquemoud-Collet, 2006).

2.4.2. Population structure

The population structure analysis was done by STRUCTURE software version 2.3.4 (Pritchard et al., 2000), it was used to study the genetic structure and number of clusters in the data set. From 1 to 10, the number of supposed populations (K) was set. Burn-in and MCMC were set to 50,000 for each run, and iterations were set at 5. The optimal K value for the population was determined using STRUCTURE HARVESTER (Earl, 2012), an online software. Individual genotypes were assigned to groups using the greatest likelihood run. Genotypes were considered as "distinct" based on obtained probability value of 80 percent, while genotypes with a probability value of less than 80 percent were considered as "admixture", i.e., these genotypes appear to have a mixed lineage from parents from diverse geographical origins or gene pools. The anticipated heterozygosity (gene diversity) and population differentiation (Fst) between individuals in a subpopulation was calculated by STRUCTURE program. To study the partitioning of genetic variance among the populations, we conducted an analysis of molecular variance (AMOVA) by using the program GenAlEx 6.3 (Peakall and Smouse, 2006).

3. Results

3.1. Allele diversity in Fagopyrum using two different marker systems

The combined marker approaches (ISSR and SSR) were exceedingly proficient in distinguishing the 63 genotypes. The findings are summarized in (Tables 1 and 2). Because of the large number of polymorphic loci that can be obtained, ISSR and SSR markers are anticipated to provide more specific genetic information than RAPD and AFLP markers (Dow and Ashley, 1996; Goto et al., 2006). In this investigation, the 7 ISSR and 7 SSR primers amplified 55 and 32 polymorphic bands, respectively. ISSR had an average of 7.85 polymorphic bands per assay unit, whereas SSR had an average of 4.57 (Table 2). The effectiveness of a given marker is established by the stability between the level of polymorphism that can be distinguished and its capability to identify several polymorphisms (Powell et al., 1996). The marker index, on the other hand, is a property of a marker that reflects its discriminatory potential, and it was determined for all of the markers. For ISSR a higher marker index value was observed. The Polymorphic Information Content (PIC value) of a primer is a critical property that determines its ability to distinguish between different individuals. ISSR markers had an average PIC of 0.36, while SSR markers had an average PIC of 0.43 (Table 1). In the ISSR assay (Table 1), primer U-834 (0.42) had the highest PIC, while primer GB-FE-035 (0.46) had the highest PIC in the SSR (Table 1). For each primer, the further efficacy of a marker, which reflects the primer's discriminatory proficiency to differentiate genotypes or individuals, was calculated. ISSR had an average resolving power of 4.38, while SSR had a resolving power of 1.42. Primer U-834 had the highest resolving power of 10.9 among ISSR markers, while primer Fem 1303 had the best resolving power of 2.28 among SSR markers.

3.2. Genetic relationship among Fagopyrum genotypes

Depending on the area of collection and variances among *Fagopyrum* species (*F. tartaricum and F. esculentum*) in the germplasm used in this study, all of the dendrograms revealed an alike configuration of linkage among a maximum of the genotypes (Fig. 1**A-C**). Cluster analysis was used independently and in conjunction with ISSR and SSR data sets to determine the genetic connection between buckwheat genotypes. For ISSR-based diversity analyses, the dissimilarity coefficient varied from 0.11 to 0.85 (Fig. 1**A**), however for SSR it valued from 0 to 1 (Fig. 1**B**). The majority of the genotypes from Kargil, as well as a few from Leh, were clustered together. The majority of the genotypes acquired from Machil, on the other hand, were gathered together. Genotypes no. 18 (BWM-18) and 16 (BWZ-16) demonstrate dissimilarity, with a value equal to 1, whereas genotypes no. 50 (BWM-50) and 56 (BWM-59) are substantially similar, with a value equal to 0 for SSR primers. The dendrogram acquired with SSR markers is sym-

Table 1
Various parameters revealing the discriminatory power of primer.

S. no	Primer	NB	NPB	NMB	NUB	PPB	PIC	MI	Rp
1	U-808	3	3	0	0	100	0.35	0.003	2.06
2	U-818	11	11	0	0	100	0.37	0.004	6.54
3	U-815	5	5	0	0	100	0.37	0.003	1.58
4	U-842	2	2	0	0	100	0.35	0.004	0.85
5	U-834	15	15	0	0	100	0.42	0.004	10.9
6	U-MO5	10	10	0	0	100	0.35	0.005	3.77
7	U-840	10	9	0	0	90	0.36	0.004	5.01
AVG		8	7.85	0	0	98.5	0.36	0.003	4.38
1	Fem1322	5	5	0	0	100	0.44	0.001	1.55
2.	Fem 1303	5	5	0	0	100	0.42	0.001	2.28
3.	Fem 1528	4	4	0	1	100	0.44	0.001	1.14
4.	GB-FE-014	4	4	0	0	100	0.41	0.001	1.14
5.	Fem 1407	5	5	0	0	100	0.44	0.001	1.68
6.	GB-FE-035	5	5	0	0	100	0.46	0.000	1.04
7.	GB-FE-012	4	4	0	0	100	0.44	0.001	1.17
Avg		4.57	4.57	0	0	100	0.43	0.0008	1.42

NB: number of bands, NPB: number of polymorphic bands, NMB: number of monomorphic bands, NUB: number of unique bands, PPB:percentage of polymorphic bands, PIC: polymorphism information content, MI: marker index, Rp: resolving power.

bolized in (Fig. 1**B**) and genotypes classified into three main clusters displayed similar results. The similar genotypes out of 63 in the case of ISSR primers were genotype no. 9 (BWM-9) and 11 (BWM-11) as similarity value equal to (0.1) while BWM-M2 and BWM-S (59,63) are dissimilar as their value tallies to 0.8. The dendrogram created using ISSR markers **(**Fig. 1**A)** separated genotypes into three main clusters and yielded comparable results. The dendrogram created from the collective ISSR and SSR data sets yielded results that were almost identical as shown in **(**Fig. 1**C)** and values ranging from 0.14 to 0.92. PCoA was used to look at the structural patterns of the 63 buckwheat accessions. The germplasm with the highest populations displayed a very distinct PCoA analysis, with the population I concentrated primarily in quadrants 2 and 3, and population II primarily in quadrants 1 and 4 as shown in Fig. 2.

Table 2
Levels of polymorphism and comparison of the discriminatory power of ISSR and SSR.

Indexes with their abbreviation		ISSR	SSR
Number of assay units	U	7	7
Number of polymorphic bands	np	55	32
Number of monomorphic bands	nnp	0	0
Average number of polymorphic band /assay	np/U	7.85	4.57
Number of Loci	L	56	32
Number of loci/assay unit	nu	8	4.57
Average number of alleles per locus	nav	8	4.57
Fraction of polymorphic loci	β	2	1
Effective multiplex ratio	E	4.29	0.79
Expected heterozygosity	He	0.462	0.284

3.3. Population structure and relationship among 63 genotypes

Further STRUCTURE analysis was performed to see how many populations could be produced from 63 genotypes using seven ISSR and seven SSR markers. We were able to establish two populations with some genetic mixing in this experiment. Likewise, the two buckwheat species (*Fagopyrum esculentum* and *Fagopyrum tartaricum*) have been divided based on microsatellite markers (Bashir et al., 2021). Using all 63 genotypes, the STRUCTURE simulations were run with K ranging from 1 to 10, with 5 runs for each K. The population subgroups at K were equal to 2 in this study. As indicated in Fig. 3, population I have 25 genotypes (39.6%), population II contains 36 genotypes (57.1%), and the two admixture genotypes, BWM-29 and BWM-46 also shown in **Supplementary Table 1**. Furthermore, as found by PCoA and NJ clustering studies, population configuration investigation established the consortium of genotypes. The anticipated heterozygosity, which governs the likelihood that two randomly designated individuals, will be dissimilar (heterozygous) at a particular locus, fluctuated from 0.224 to 0.242 in the first subpopulation, with an average of 0.233. The population differentiation measurements (Fst) varied from 0.349 (in the 2nd subpopulation) to 0.455 (in the 1st subpopulation), with an average of 0.402 to summarize the genetic differentiations among groups and on the three separate clusters analogously allocated close to various populations **(**Table 3 **and** Table 4**)**. This shows the buckwheat species are highly diverse and the markers employed are sufficient to carry out the study. The molecular variance within the population was found to be higher (70%), while among the population it was 30% as shown in Fig. 4 as per AMOVA analysis.

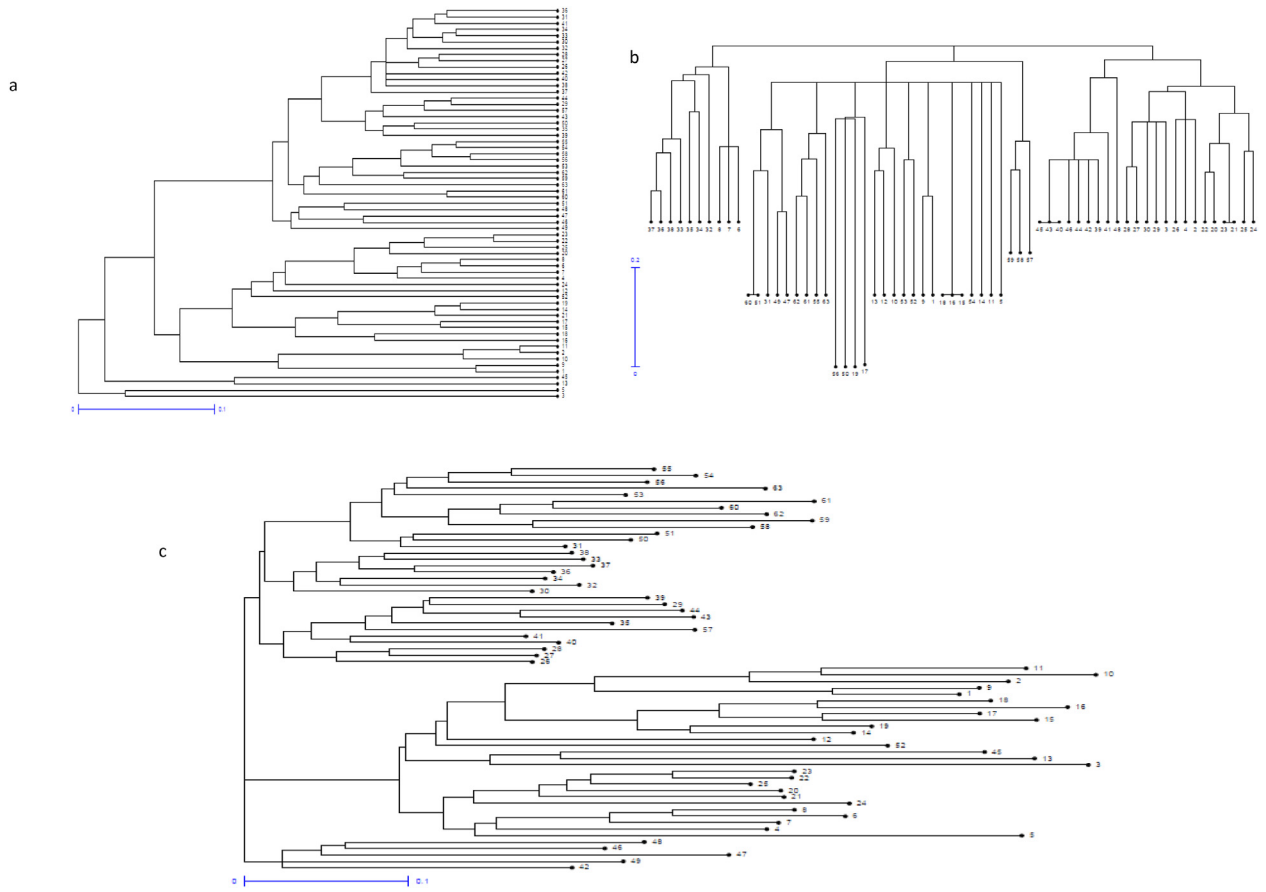

Fig. 1. A-C: A. Cluster tree based on7 ISSR markers B. 7 SSR and C. combine ISSR and SSR among 63 genotypes of Buckwheat. DARwin software was used for a pair-wise distance matrix and Nj method for tree construction.

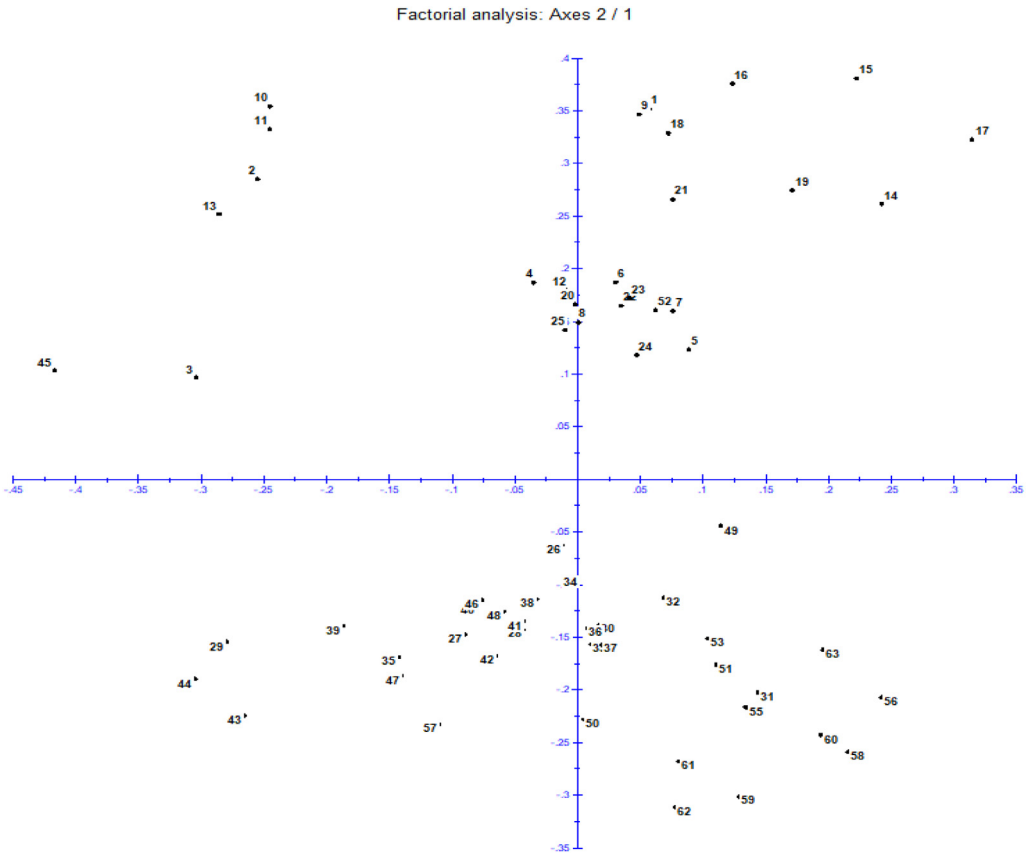

Fig. 2. CoA analysis-based results of 63 buckwheat genotypes using 7 ISSR and 7 SSR primers.

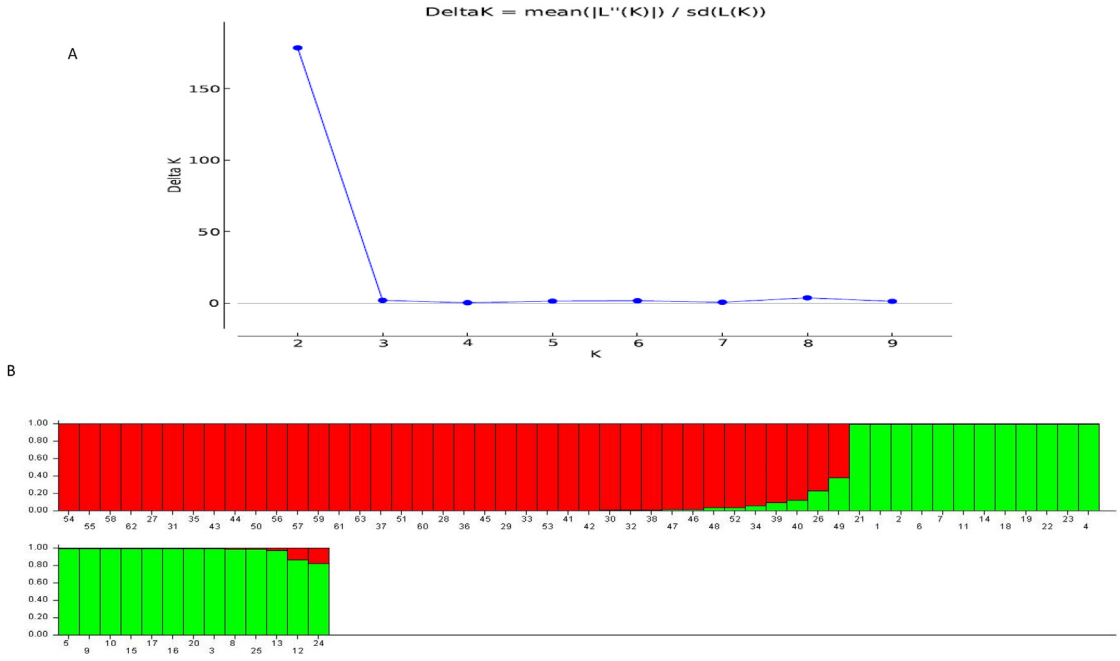

Fig. 3. A-B: a Graphical representation of the optimal number of groups in the program STRUCTURE inferred using structure harvester. b STRUCTURE plot of membership coefficients for all the accessions of buckwheat in the study sample sorted in the same order and classified according to successive selected preset K values ranging from 1 to 10. For K = 2 the groups are identified.

Table 3
Heterozygosity and Fst value calculated for 2 buckwheat sub-populations.

Sub-population(K)	Expected heterozygosity	Fst value
1	0.224	0.455
2	0.242	0.349
Average	0.233	0.402

Table 4
Genetic differentiation based on Fst values between two buckwheat sub- populations identified by population structure analysis.

	Pop A	Pop B
Pop A	–	
Pop B	0.1441	–

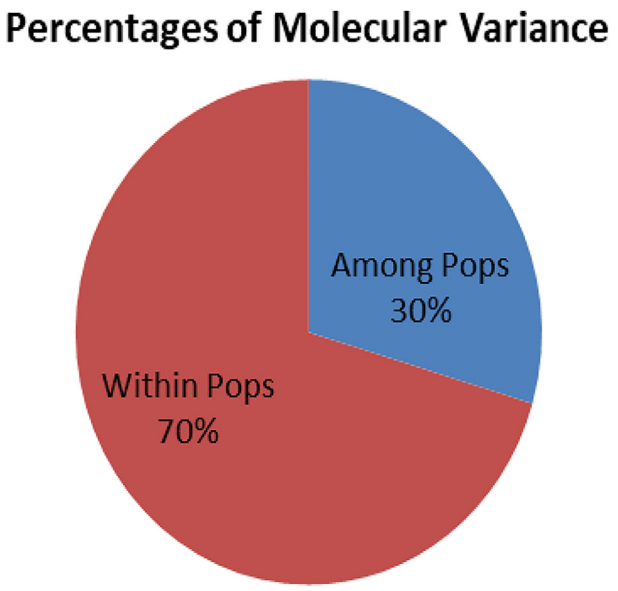

Fig. 4. AMOVA showing variation within and among 63 buckwheat genotypes.

4. Discussion

Allele diversity among 63 genotypes of *Fagopyrum* was determined by ISSR and SSR markers which correspond to the study conducted by (Bashir et al.,2021), they employed SSR markers to access genetic diversity in buckwheat germplasm, which helped in the identification of highly polymorphic SSRs such GB-FE-035 in *Fagopyrum esculentum* and Fem 1322 in *Fagopyrum tartaricum*. Fem-1322 has the highest Polymorphic Information Content (PIC) of 0.93, compared to 0.56 for primer GB-FE-035 similar to our results. The greatest PIC value was also observed in primer U-834 (0.94) in the study reported by (Zong et al., 2008). In another study, genetic diversity was analyzed in 79 Tartary buckwheat species and the percentage (92.6%) of polymorphic fragments amplified in bulked DNA analysis, which is identical to our study in the case of ISSR which is equal to 98.5 percent. The difference between the PIC values may be due to the different genotypes and sample size as reported by (Ahmad and Raj, 2012) while estimating the genetic diversity in 82 walnut cultivars, the investigation reported PIC value of (0.39) in SSR than that of (0.250) in ISSR. Similarly among 51 common bean genotypes (Zargar et al., 2016) also witnessed a higher PIC value in SSR (0.300) in comparison to RAPDs (0.243). The discrepancy in PIC values between the two systems could be attributed to the different genotypes and number of markers employed in this investigation. A recent study done by (Shukla et al., 2018) observed a higher resolving power in ISSR (9.94) therefore, efficient in detecting polymorphism and discrimination among 31 buckwheat accessions. Similar results were obtained in sugarcane genotypes. In this case, also higher resolving power in ISSR (3.8) compared to SSR(0.1) was found in 81 sugarcane genotypes (Ranya and Ahmed, 2018).

The topology of the marker systems' respective dendrograms exhibited a high degree of resemblance although there were minor discrepancies in the placement of various genotypes. The disparity in resolution between two marker systems may be explained by the fact that the two marker approaches targeted different parts of the genome. ISSR markers were found all over the genome, revealing the genome's diversity, while SSR markers only amplified the functional regions of the open reading frame (ORF). The current situation of vast genetic diversity among populations may have resulted from the free exchange of germplasm among the study's various areas.

Our analysis suggests that two separate genetic populations of *Fagopyrum* were distributed in different regions of northwestern Himalayas with some genetic mixing via STRUCTURE analysis and Fst values. The results obtained from the investigation are similar to the analysis carried on 51 common bean genotypes with high Fst score (0.330) as well (Zargar et al., 2016). Similar results were obtained by in estimation of genetic structure of 12 *P. harmala* genotypes by using ISSR, RFLP and SSR markers and obtained high Fst value (0.486) (Ranya and Ahmed, 2018). However, the higher molecular variance within the population might be attributed due to the self-incompatibility nature of *F. esculentum* (Matsui and Yasui, 2020). It was also discovered that cross-pollinating species have more genetic variation within their populations than self-mating species, whereas self-mating species have a fewer genetic variation (Duminil et al., 2007; Nybom, 2004). As evidenced by NJ-based research, the admixtures found in two distinct populations indicate subgrouping of genotypes. It's possible that the subgrouping is due to species structuring or adaptation in various geographical areas. Among all of these mechanisms, the geographic range can play a significant role in the maintenance of genetic variation (Maki, 2003; Meloni et al., 2006).

5. Conclusion

ISSR and SSR marker approaches are considered to be effective in deciphering polymorphism information in buckwheat. As a result, they are more useful in identifying buckwheat genotypes. In terms of genotype grouping, both marker systems are accurate. This is extremely important for the management and protection of germplasm from various geographical locations. Furthermore, the results of the population structure study will be opportune in conducting association mapping in buckwheat for a variety of economically significant traits. This will be used as a starting point for breeding programs. Moreover, all of the findings from this study will be useful in deciding ISSRs and SSRs are conducive for future research and characterization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.sjbs.2021.07.061.

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