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Genetic diversity analysis of commercial Arabica coffee in Nepal using Molecular markers.

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Abstract
Coffee is an established plant for its flavor and has high commercial use. In Nepal, the popularity of coffee is increasing for its high economic value. However, its diversity and the status of its genetic mapping have not been studied in Nepal. In the present study, the genetic diversity of 28 coffee accessions was assessed by using twenty-four SSR markers with the aim of studying the variation of coffee in accord with the genetic markers from a molecular approach. With the use of DNA extraction and marker selection for its amplification using PCR tools, a total of 81 loci from SSR were identified. Of all SSR 63.22% showed for mean polymorphism. The mean polymorphic information content of SSR was 0.38, which showed low genetic diversity of SSR markers among *Coffea* genotypes. On the basis of the SSR marker, the unweighted pair group method with arithmetic mean (UPGMA) dendrogram constructed showed a similar group of distribution among 28 accessions, which was further supported by a principle coordinate analysis scatter plot. The phylogenetic relationships among the accessions were assessed by SSR marker, which also showed low diversity in coffee genotypes. Our study demonstrated the use of SSR markers in diversity analysis as the data were informative and highly reproducible for evaluating relationships among coffee cultivars in Nepal. The use of more markers systems and a high genotype pool would have been beneficial in accessing more accurately. Regardless, the information from the phylogenetic relationship study could be useful for breeding, varietal improvement, and for conservation programs.
Keywords: Coffee, DNA isolation, PCR, Molecular Markers, Dendogram, Scatter plot, Genetic diversity.

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Introduction
Coffee is regarded as one of the most desired drinks worldwide. The genus of coffee is *Coffea*, which belongs to the Rubiaceae family comprising almost 124 species after the addition of *Psilanthus* [1]. Africa, Asia, and Latin America are the primary tropical and subtropical locations where coffee is cultivated [1].

Mainly, three species of coffee are used in beverages, *Coffea arabica*, *C. canephora*, and *C. liberica*. Coffea arabica, one of the most well-liked varieties, is the one that typically attracts the most cultivation since it produces 70% of the world's coffee and has a superior flavor, a rich scent, and less caffeine [2]. *Coffea* is not an indigenous plant to Nepal, its seed was first brought and planted in Nepal in 1938 A.D. Coffee plantation covers more than 1000 ha of land with a total production of 250 mt of dry cherry; with its popularity, it has been one of the emerging and high-value cash generating commodities for hill farmers of Nepal [3][4]. Nepali coffee is renowned for its unique fragrance and taste, as it is grown at elevated altitudes (800-1600m), distinct from the typical coffee-producing regions found elsewhere in the world [5]. Any data on the first planted coffee variety and the diversity of *Coffea* species in the last 7 decades have not been officially recorded in Nepal.

Typically, traditional breeding is used to promote the genetic diversity of *arabica* by choosing and fusing elite genotypes from the population that are genetically varied. However, a significant degree of phenotypic similarity makes it difficult to choose genetically distinct parental lines based on morphological and agronomic traits. This is due to the low genetic diversity and high self-fertilization rate associated with arabica [6]. According to Lashermes, the genetic factors are more accurately tested by molecular markers [7]. The advancement of molecular markers has recently made it feasible to detect genetic diversity at the DNA level. Several DNA analysis techniques have been used to examine the genetic diversity of coffee. The methods differ in terms of consistency, expenses, sensitivity of the sequence, and technical requirements. The methods utilized in genome-wide scanning, such as RAPD (Random Amplified Polymorphic DNA), RFLP (Restriction fragment length polymorphism), and AFLP (Amplified fragment length polymorphism), do not require previous genetic sequence analysis for the design

of primers. SSRs (Simple Sequence Repeats) and SCARs (Sequence Characterized Amplified Regions), on the other hand, are more repetitive, based on sequence-specific primers, and have a restricted capacity to be passed between species. The development of simple sequence repeat (SSR) markers for *Coffea* has provided a key resource for investigating genetic diversity in the genus [8].

Future food security in agriculture and industry is largely dependent on plant genetic diversity, as claimed by Jump [9]. However, they noticed that natural population genetic diversity gets less attention. As is true for many crops, assessing the genetic diversity and resources within the genus *Coffea* is an important step in coffee breeding [8]. Considering that new coffee varieties are continuously produced through hybridization, it is important to determine the extent and sources of genetic variation both within and between new and existing coffee varieties [10]. Low genetic diversity was found in *C. arabica*, including wild and cultivated genotypes, according to earlier research on the characterization of *Coffea* germplasm, including analysis employing molecular markers [9,10,11,12].

In this study, the genetic diversity of the *Coffea* cultivars grown in Nepal is the main topic of investigation. The experiment and analysis are based on the simple objective of correctly identifying and validating the genotypes across Nepal as the status is unknown despite the long history of cultivation. Change in a gene is unlikely as coffee being, thus the use of markers to access current status is a stepping stone for further analysis. The use of SSR being co-dominant in nature was used for the diversity analysis, having distinct advantages which include cost-effectiveness, clear and unambiguous banding pattern, and high reproducibility.

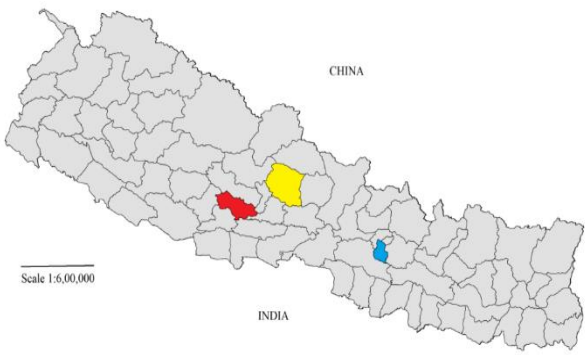


Figure 1. Germplasm collection of coffee genotypes. Red: Sampling Location in Gulmi [Nepal Coffee Research Centre]; Yellow: Coffee Development Board located in Pokhara Malepatan; Blue: Research Center (Nepal Agricultural Research Council, Khumaltar, Nepal).

Materials and methods

Plant Materials

We utilized a total of 28 different coffee varieties (**Table 1**), collected from Nepal Coffee Research Center, Gulmi, Nepal (Latitude: 27°56'17.85'' N to 27°5'44.87'' N; Longitude: 8° 25' 29.2''1 E to 83°25'30.20'' E), Coffee Development Center, Aapchaur-05 and Coffee Development Board, Pokhara, Malepatan, which were maintained ex-situ in Research Center (Nepal Agricultural Research Council, Khumaltar, Nepal).

Genomic DNA extraction

With few modifications, the Doyle and Doyle technique [15] was used to extract DNA from the materials. Using a mortar and pestle, one gram of leaf samples were obtained and crushed. A 700 µl extraction buffer containing 2% CTAB (w/v), Tris HCL pH 8.0 (0.1M), EDTA pH 8.0 (20mM), NaCl (1.4M), 2% PVP (w/v), and 1% β-mercaptoethanol was immediately added to the pulverized paste after it had been transferred to micro-centrifuge tubes. The tubes were centrifuged for 15 minutes at 15,000 rpm after being incubated at 65^0 C in a water bath for 1 hour. Carefully transferring the aqueous phase into new tubes, an equal amount of Chloroform:isoamyl alcohol (24:1) was added, and the mixture was well mixed by inversion for a few minutes. The mixture was centrifuged at 15,000 rpm for 15 min at 4^0C. After the phase separation, the supernatant was transferred into a new tube. To the supernatant, 0.2 ml sodium acetate was added in order to enhance the quality of DNA. An equal volume of isopropanol was added to each tube. The tubes were kept at -20^0C for 30 minutes and centrifuged at 15000 rpm for 7 minutes for precipitation. Then, the supernatant was removed, and the pellets were washed with 96% and 70% ethanol twice, respectively. The pellets were air-dried and resuspended in 1X TE buffer (Tris-HCl 10 mM, EDTA 1 mM, pH 8.0).

DNA quantification and purity

DNA quantification and quality were assessed by using Nanodrop (Quawell Q-5000). The purity of the DNA was assessed by the absorbance ($A_{260/280}$) ratio and running the DNA samples on 0.8% agarose comparing it with 1Kb DNA ladder (Thermo Fisher Scientific, Waltham, USA).

Table 1. Coffee sample varieties collected from the National Coffee Research Centre, Gulmi, Nepal. [G = green, Y = yellow, R = red]

S.N.	Code	Lab code	Variety	Sample Type	Source (location)	Year of Introduction	Young leaf color	Fruit color
1	C1	GCS81	Gulmi local	Seed	CDC, Gulmi	2071	Green	Red (G to R)
2	C2	GCS82	Chhetradeep	Seed	HRS, Malepatan, Pokhara	2071	Green	Red (G to R)
3	C3	GCS95	Selection-10	Plant	HRS, Malepatan, Pokhara	2071	Green	Maroon red (G to R)
4	C4	GCS98	Selection-12 (Cavery)	Seed	Thanapathi VDC, Thorga, Gulmi	2073	Green	Red (G to Y to R)
5	C5	GCS101	CRP-Yellow	Plant	HRS, Malepatan, Pokhara	2071	Green	Yellow (G to Y)
6	C6	GCS85	Catimor (Red)	Plant	HRS, Malepatan, Pokhara	2071	Green	Red (G to R)
7	C7	GCS93	Pacamara	Plant	HRS, Malepatan, Pokhara	2071	Green	Red (G to R)
8	C8	-	Tekisic	-	-	-	-	-
9	C9	GCS94	Pacas	Plant	HRS, Malepatan, Pokhara	2071	Green	Red (G to R)
10	C10	GCS105	Robusta Coffee	Seed	CDC, Aapchaur, Gulmi	2073	Green	-
11	C11	GCS86	Catuaii Amarelo (Brazillian)	Plant	HRS, Malepatan, Pokhara	2071	Green	Yellow (G to Y)
12	C12	GCS26	San Ramon	-	Indian	2057 B.S.	Light green	Dark red (G to R)
13	C13	-	Catisic	-	-	-	-	-
14	C14	GCS84	Bourbon Amarelo	Plant	HRS, Malepatan, Pokhara	2071	Green	Red (G to Y to R)
15	C15	GCS83	Mundo Novo (Brazillian)	Plant	HRS, Malepatan, Pokhara	2071	Green	Red (G to Y to R)
16	C16	GCS2	Caturra Amarelo	-	Winrock Int.	2059 B.S.	Light green	Yellow (G to Y)
17	C17	GCS99	Syangja Special	Plant	Highland coffee Nursery, Syangja	2071	Green	Red (G to R)
18	C18	-	Catuaii Vermelho	-	-	-	-	-
19	C19	GCS80	Argakhachi local	Seed	-	2072	Green	Red (G to R)
20	C20	GCS91	Kaski local	Seed	HRS, Malepatan, Pokhara	2071	Green	-
21	C21	GCS88	Indonesia	Plant	DCPA, Kaski	2071	Green	Maroon red (G to R)
22	C22	GCS79	Bourbon Vermelho	Plant	HRS, Malepatan, Pokhara	2071	Light green	Red (G to R)
23	C23	GCS87	Hawaii Kona	Plant	DCPA, Kaski	2071	-	-
24	C24	GCS106	Chandragiri	Seed	CCRI, India	2074	Bronze	-
25	C25	-	Sankhuwasabha local	-	Sankhuwasabha	-	-	-
26	C26	GCS92	Indotimtim	Plant	Puranchaur,Kaski	2072	Light bronze	Maroon red (G to R)
27	C27	GCS100	Bhandaridanda local	Seed	CRP, Selection	2075	Green	Maroon red (G to R)
28	C28	GCS96	Selection-09	Seed	CDC, Aapchaur, Gulmi	2073	Green	Maroon red (G to R)

*CRP = Coffee Research Project

Table 2. SSR primers used for PCR analysis for 28 Coffee accessions

Locus	Forward 5' - 3'	Reverse 5' - 3'	Published BP
Sat 207	CAATCTCTTTCCGATGCTCT	GAAGCCGTTTCAAGCC	82, 89, 93, 97
Sat 235	GCAAATCATGAAAATAGTTGGTG	TCGTTCTGTCATTAAATCGTCAA	167-750
Sat 244	GCATGTGCTTTTTGATGTCGT	CATACTAAGGAAATTATCTGACTGCT	178–306
SSR09	TTGGCTTTTGTCCCTCCTTCCTCTG	AGCCCATTTCCCTCTCATCATTTCAAG	124, 126, 130
AJ-250254	GGCTCGAGATATCTGTTTAG	TTTAATGGGCATAGGGTCC	132-166
AJ-250260	TGATGGACAGGAGTTGATGG	TGCCAATCTACCTACCCCTT	100-132
Sat 229	TTCTAAGTTGTTAAACGAGACGCTTA	TTCCTCCATGCCCATATTG	180-550
AJ-250257 (M29)	GACCATTACATTTCACACAC	GCATTTTGTTGCACACTGTA	120-150
AJ-250258 (M32)	AAC TCT CCA TTC CCG CAT TC	CTGGGTTTTCTGTGTTCTCG	99-131
AJ-250253 (M20)	CTTGTTTGAGTCTGTCGCTG	TTTCCCTCCCAATGTCTGTAG	157
MR-054	TGATGTGGAAGGCCATTG	GCCCCTATTATGACCCATGC	300
Sat_227	TGCTTGGTATCCTCACATTCA	ATCCAATGGAGTGTGTTGCT	132,146
Sat 254	ATGTTCTTCGCTTCGCTAAC	AAGTGTGGGAGTGTCTGCAT	200-220
Sat 41	AGTGTAACTTTAGTTCTTGC	ATTTAATGGGCATAGGGTC	200-650
CFGA 465	ACCCTTTACTACTTATTTACTCTC	ACATCCCCTTGCCATTTCTTC	157
AM 231573 (472)	AATCATGGGGACAGGACAAG	TCTGCTAGACTTGACATCTTTTGG	192
AJ 308764 (M764)	CTGGCATTAGAAAGCACCTTG	GCTTGGCTCACTGTAGGACTG	292, 318, 320
AJ 308767 (M767)	CAACACTATCTCTTGATTTTTCACT	CGTGCAAGTCACATACTTTACTAC	198
AJ 308746 (M746)	GGCCTTCATCTCAAAAACCT	TCTTCCAAACACACGGAGACT	100-200
AJ 308742(M742)	GGCTTCTTGGGTGTCTGTGT	CCATTGGCTTTGTATTTCTGG	110-200
AJ 308784 (M84)	TTGCTTGCTTGTTCTGTTAT	TGACACGAGAGTTAGAAATGA	110
AJ 308790 (M90)	TTTTCTGGGTTTTCTGTGTTCTC	TAACTCTCCATTCCCGCATT	126
AJ 308782 (M782)	AAAGGAAAATTGTTGGCTCTGA	TCCACATACATTTCCCAGCA	134
AJ 250256 (M27a)	AGGAGGGAGGTGTGGGTGAAG	AGGGGAGTGGATAAGAAGG	114

Sequence published: [13,20,21,22,8]

PCR Reaction

A total of 24 primers were selected from a pool of markers (**Table 2**). The reactions were carried out in Mygene L series thermo cycler (LongGene Scientific Instrument Co. LTD. The reaction contains about 60-70 ng of template DNA, 2X master mix (Promega Corporation, USA), 0.5 µM of single Primer (Macrogene Inc., South Korea), with additional 25 mM MgCl2 (Himedia laboratories Pvt. Ltd, India), 0.2 mM dNTP Mix (Promega Corporation, USA), 0.2 U of T*aq* polymerase (Promega Corporation, USA), 1 mg/mL BSA. The thermocycler was programmed for an initial denaturation step of 4 min at 94 °C, followed by 35 cycles of 50s annealing according to primer Tm temperature, extension was performed at 72 °C for 80 sec, followed by a final extension at 72 °C for 7 min, with a temperature hold of 4 °C.

Visualization of PCR products

The PCR products were assessed by using 2.5% agarose gel electrophoresis (Cleaver Scientific, UK) in 1X TBE stained with ethidium bromide at 80V for 2 hr. Gel Documentation System (VWR®Genosmart 2, UK) was used to take pictures of the gel.

Data scoring and statistical analysis

The PCR-amplified bands were scored as present [1] or absent [0] based on size relative to marker (1Kb and 100 bp ladder, Thermo Fisher Scientific, Waltham, USA), according to the allotetraploid nature of *C. arabica*, even if it was a co-dominant marker, and scored to form a single data matrix. The total number of bands, polymorphic bands, and average number of bands per primer were calculated. Polymorphic information content (PIC) was calculated as PIC = $(1-\sum p_i^2)$, p_i is the frequency of the banding pattern [16]. The resolving

power of the marker is calculated as Rp = ΣIb [17], where I_b (band informativeness) takes the value of 1-(2*(0.5-p)) and p is the ratio of samples sharing the band. EMR was calculated as EMR = np *, where np is the total number of polymorphic bands and is the proportion of polymorphic bands to all bands. By multiplying PIC by EMR, the marker index (MI) was determined [18].

By utilizing the Jaccard similarity coefficient [19], the pairwise similarity matrix was created. Using NTSYS-pc (NTSYS-pc version 2.10 e) and the unweighted pair group technique with arithmetic averages (UPGMA) and a bootstrap value of 1000, a dendrogram representing the relationship between the genotypes was created and shown. Similar to this, scatter plots of the multidimensional distributions of coffee cultivars were created using the Principle component analysis (PCoA).

Results

The experiment was duplicated in all cultivars to get the optimum amplification results. Reliable amplification and reproducible pattern were seen for both the markers which were used further for data analysis.

Twenty-four SSR markers were initially screened against 28 coffee cultivars of which five SSR markers showed no polymorphic bands. The remaining nineteen SSR markers showed high polymorphism and produced a clear amplification pattern (as shown in **Figure 2**).

SSR polymorphism among cultivars

The SSR amplification allowed a total of 81 fragments amplified, 61 were polymorphic with the mean of 2.54 polymorphic fragments per primer (**Table 3**). The percentage of polymorphism ranged from 0 to 100%, with the mean of 63.22% polymorphism. Out of the nineteen primers with polymorphism only 6 primers showed either 80% or more polymorphism. The resolving power of SSR markers ranged from 0.57(AJ250260) to 8.64 (AJ250253), with the mean of 2.95 per primer. Similarly, the PIC of the twenty SSR primers ranged from 0.22 (AJ308764) to 0.65(AJ250258).

The pattern of genetic relationship between the genotypes was assessed by UPGMA method of cluster analysis using Jaccard coefficient and the dendogram representing the genetic relationship is presented in Figure 2. The dendogram based on SSR marker data grouped the 28 cultivars into four major clusters. Chandagiri taking the first major cluster. Two Sub-clusters made up the Second main cluster containing Tekisic, Selection-10, Selection-12 and CRP-Yellow on the first sub-cluster, whereas Sankhuwasabha Local, Indorimrim, Selection-9 and Bhandaridada Local were present on Second sub-cluster. Robusta coffee taking the third major cluster. Four minor clusters were formed from the fourth major cluster, and the first sub-minor cluster included Catimor, Chettradeep, Pacamara, and Pacas, whereas the second sub-minor cluster contained, Mundonovo, Catuaii Vermelho, Hawaii Kona, Arghakhachi Local and third minor sub-cluster contained Catuaii Amarelo, Sanaramon, Bourbon Amarelo, Bourbon Vermelho, Caturra Amarelo, Catisic, Syanja Special, Kaski Local, Indonesia, Gulmi local.

The scatter plot distributed the accessions across two coordinates (**Figure** 2), which showed significant level of diversity among them forming 2 major clusters showing its similarity from each other which further accords the dendogram. Chettradeep, Catimor, Pacas, Catuaii Amarelo, Sanaramon, Bourbon Amarelo, Bourbon Vermelho, Caturra Amarllo, Catisic, Syanja Special, Kaski Local, Indonesia, Gulmi local, Mundonovo, Catuaii Vermelho, Hawaii Kona, Arghakhachi Local, Pacamara formed the first cluster. And second cluster of Tekisic, Selection-10, Selection-12, CRP-Yellow, Sankhuwasabha Red, Indorimrim, Selection -9, and Bhandaridada Local.

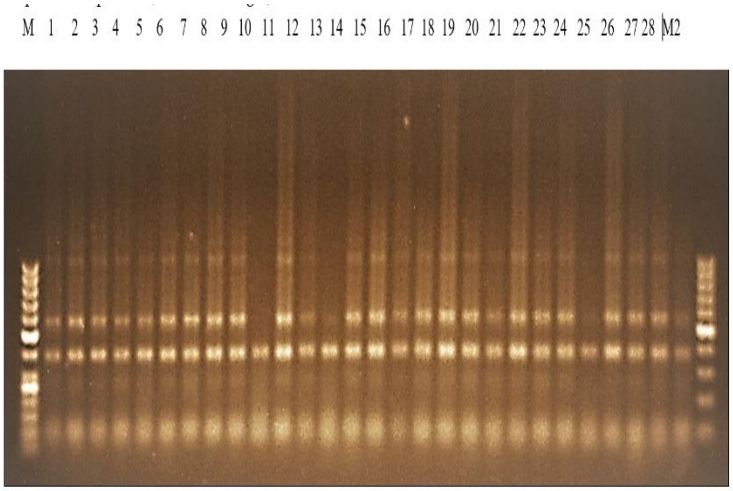


Figure 2. M1 and M2 represent 100bp ladder. PCR amplification product on marker M782 across 28 genotype. The coded names for the cultivars:[1: Gulmi Local; 2: Chhetradeep; 3: Selection-10; 4: Selection-12; 5: CRP-Yellow; 6 Catimor; 7: Pacamara; 8: Tekisic; 9: Pacas; 10:Robusta Coffee; 11:Catuai Amarelo; 12: San ramon;13: Catisic; 14: Bourbon Amarelo; 15:Mundo novo; 16: Caturra Amarelo; 17:Syanja Special; 18: Catuaii Vermelho; 19: Argakhachi local; 20: Kaski local; 21:Indonesia; 22: Bourbon Vermelho; 23: Hawaii Kona; 24: Chandagiri; 25: Sankhuwasabha local; 26: Indotimtim; 27: Bhandaridada local; 28:Selection-9].

Table 3. Detail on Genetic Information generated by SSR markers in coffee 28 coffee cultivars.

Primers	Number of total Alleles	Band Size range	Number of Bands	No of polymorphic Alleles	Polymorphism Percentage	Resolving Power (RP)	Polymorphic Information Content (PIC)	Effective multiplex Ratio (EMR)	Marker Index (MI)
Sat 207	5	100-500	1-5	3	60.00	3	0.43	1.8	0.77
Sat 235	2	200-250	1-2	1	50.00	0.93	0.48	0.5	0.24
Sat 244	3	285-600	1-3	3	100.00	3.43	0.53	3	1.59
SSR09	1	150	1	0	0.00	0	0	0	0
AJ-250254	4	190-700	1-4	3	75.00	2.64	0.55	2.25	1.24
AJ-250260	2	350-900	1-2	1	50.00	0.57	0.63	0.5	0.32
Sat 229	2	110-180	1-2	1	50.00	1	0.51	0.5	0.26
AJ-250257 (M29)	7	110-800	1-7	6	85.71	7.14	0.51	5.14	2.62
AJ-250258 (M32)	5	100-1000	1-5	5	100.00	5.86	0.65	5	3.25
Sat 41	5	150-800	1-5	4	80.00	3.57	0.61	3.2	1.95
AJ-250253 (M20)	6	180-1000	1-6	6	100.00	8.64	0.47	6	2.82
MR-054	5	150-800	1-5	5	100.00	7.36	0.44	5	2.2
Sat_227	1	200	1	0	0.00	0	0	0	0
Sat 254	4	200-750	1-4	3	75.00	2.5	0.55	2.25	1.24
CFGA 465	1	200	1	0	0.00	0	0	0	0
AM 231573 (472)	3	300-800	1-3	2	66.67	3.07	0.26	1.33	0.35
AJ 308764 (M764)	3	190-550	1-3	2	66.67	3.29	0.22	1.33	0.29
AJ 308767 (M767)	3	170-600	1-3	2	66.67	2.5	0.4	1.33	0.53
AJ 308746 (M746)	3	400-1000	1-3	2	66.67	2.5	0.42	1.33	0.56
AJ 308742 (M742)	4	290-1000	1-4	3	75.00	4.93	0.24	2.25	0.54
AJ 308784 (M84)	3	150-390	1-3	3	100.00	4.29	0.47	3	1.41
AJ 308790 (M90)	1	100	1	0	0.00	0	0	0	0
AJ 308782 (M782)	4	170-800	1-4	3	75.00	3.57	0.45	2.25	1.01
AJ 250256 (M27a)	4	150-500	1-4	3	75.00	4.57	0.32	2.25	0.72
TOTAL	**81**			**61**		**75.36**	**9.14**	**50.21**	**23.91**
MEAN	**3.375**			**2.541**	**63.22**	**3.14**	**0.380**	**2.092**	**0.996**

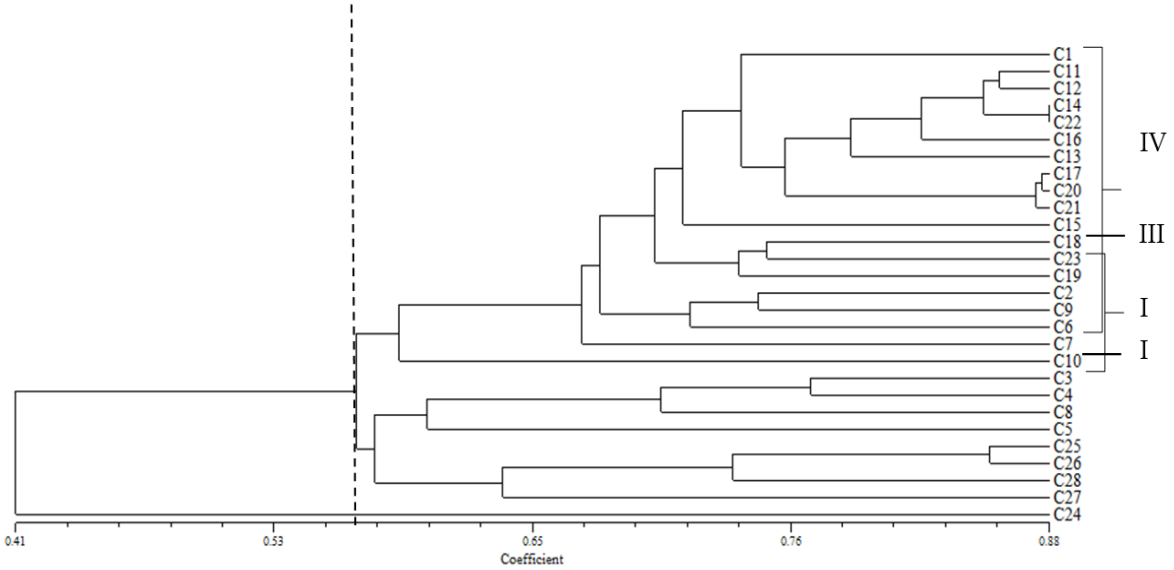
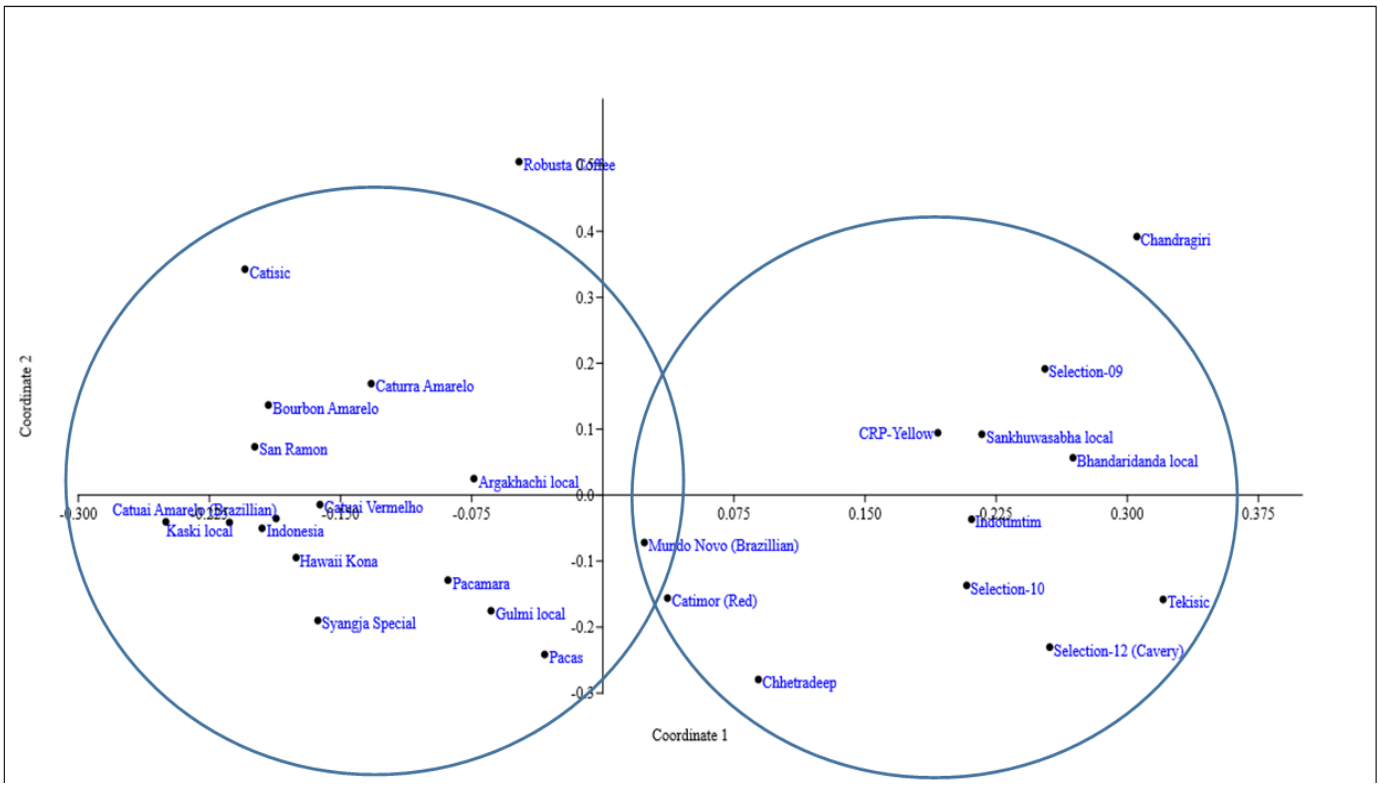


Figure 2.1(Above). UPGMA dendogram showing genetic similarity among 28 cultivars based on SSR markers forming four main clusters. The coded names for the cultivars:[C1:Gulmi Local; C2: Chhetradeep; C3: Selection-10; C4 Selection-12; C5: CRP-Yellow; C6 Catimor; C7: Pacamara; C8: Tekisic; C9: Pacas; C10:Robusta Coffee; C11:Catuai Amarelo; C12: San ramon;C13: Catisic; C14: Bourbon Amarelo; C15:Mundo novo; C16: Caturra Amarelo; C17:Syanja Special; C18: Catuaii Vermelho; C19: Argakhachi local; C20:Kaski local; C21:Indonesia; C22: Bourbon Vermelho; C23:Hawaii Kona; C24: Chandagiri; C25: Sankhuwasabha local; C26:Indotimtim; C27: Bhandaridada local; C28:Selection-9].

Figure 2.2(Below). PCoA analysis on the basis of SSR marker showing two main clusters from the analysis with Robusta and Chandragiri not being in any cluster. Chettradeep, Catimor, Pacas, Catuaii Amarelo, Sanaramon, Bourbon Amarelo, Bourbon Vermelho, Caturra Amarllo, Catisic, Syanja Special, Kaski Local, Indonesia, Gulmi local, Mundonovo, Catuaii Vermelho, Hawaii Kona, Arghakhachi Local, Pacamara formed the first cluster. And second cluster of Tekisic, Selection-10, Selection-12, CRP-Yellow, Sankhuwasabha Red, Indorimrim, Selection -9, and Bhandaridada Local.



Discussions

The marker technique could locate polymorphic loci across the germplasm, however, the level of polymorphism that was found is modest when compared to similar analyses of plants [20]. The highest level of polymorphism is observed in *Coffea arabica* and *Coffea canephora*, which are the most extensively cultivated and frequently utilized in breeding programs. These findings align with previous research on genetic diversity within commercially grown *Coffea* cultivars. This high level of genetic variation in these two species underscores their significance in ongoing breeding efforts and Coffee production [21,20], which reports low diversity of *Coffea*. This might be due to the narrow genetic basis of the cultivated coffee, as few *C. arabica* were introduced for breeding programs from Ethiopian origin worldwide. SSR markers offer a variety of other uses besides genetic diversity analysis, such as serving as markers for desirable qualities. It was discovered that Sat207 and Sat235 are closely related to the locus Ck-1, which contains a key gene that confers resistance to the coffee berry disease (CBD), among the markers used in the current study. Both CLR and CBD are fungal diseases that harm the coffee plant, with CLR being brought on by the fungus *Hemileia vastatrix* and CBD being caused by *Colletotrichum kahawae*. Both of these diseases are exceedingly common and a major factor in the worldwide collapse of the coffee industry [23]. In our study, the two markers used Sat207 and Sat235 showed good responses on CLR and CBD. The presence of the gene was marked by the bands which can be used for linkage analysis of SH3 and Ck-1 in Nepali coffee genotypes.

Within this research, the mean genetic value for 24 SSR markers was lower than previously reported values for commercial arabica varieties. Also, one prominent observation was seen for Robusta coffee positioning it separately on the scatter plot which strengthened the data as Robusta belongs to species *C. canephora,* and the major species in our study was *C. arabica.* However, differences in the species level the arabica coffee arose from a cross between two coffee species: *Coffea canephora* and *Coffea eugenioides* [24]. The utilization of SSR markers in coffee genotype analysis has revealed a high level of genetic diversity within and among coffee populations [25]. The presence of multiple alleles at SSR loci indicates that coffee genotypes are genetically heterogeneous, reflecting the complex evolutionary history and diverse origins of coffee species. However, the result showed the positioning of genotypes having a small difference from each other can indicate low diversity among the species. Using six SSR markers, 15 commercial varieties were found to have less genetic diversity and polymorphism than wild coffee accessions [14]. 34 SSR markers were used to investigate 12 Colombian-grown arabica coffee varietals, and the results showed a low level of genetic variation with a mean of 2 alleles and 0.22 PIC per primer. [21]. Similar findings with a very low level of genetic diversity were seen in another study utilizing 26 Brazilian commercial cultivars of arabica coffee inbred lines and 23 SSR markers (mean of 2.87 alleles and 0.33 PIC per primer) [26]. With a mean of 2.8 alleles and 32% polymorphism, minimal genetic variation was found in 55 commercial arabica coffee varietals purchased from France using 32 SSR markers [27]. Similar results were obtained from the Geleta research, where 12 SSR markers were used to find a mean of 2.0 alleles and 42% polymorphism in eight Nicaraguan commercial arabica coffee varietals [23]. Yemeni arabica coffee cultivars showed a low level of genetic variation using 58 SSR markers, with a mean of 2.5 alleles and 0.32 PIC per primer [23]. Researchers [26] contend, however, that some commercial arabica coffee cultivars have a substantial level of genetic variability. A possible explanation for these variations may be attributed to the limited sample size and the diversity among coffee genotypes. Most researchers have investigated a few number of genotypes generated from the two species ("Typica" and "Bourbon") and the Hybrido de Timor [21], a naturally occurring interspecific hybrid of *C. arabica* and *C. canephora*, is the most widely used hybrid in coffee line breeding. A common ground for breeding coffee lines can reduce the diversity among the genotypes cultivated. The limited genetic diversity and low polymorphism of Typica and Bourbon have also been reported in certain research [14,20]. This may be related to *C. arabica*'s predominant autogamy, which promotes genetic diversity reduction, and the several cycles of selection and backcrossing that occurred during the creation of better varieties [21].

The coffee varieties that were the focus of our investigation were also developed from a number of collections of accessions to coffee germplasm that were acquired from farmers. The disparities in the results of the aforementioned research were mostly due to variations in the genetic bases of the material sources under investigation, sample size, the marker systems used, and the number and kind of nucleotide motifs selected for the SSR primers [21]. The low diversity of coffee in our study can be explained by the low variation

pool of arabica coffee for breeding as most of the varieties have the same parent and the gene pool is narrowed. The low diversity of coffee in Nepal can also be explained by the use of the same parent in different geographic places giving out the same or similar gene attributes. The coffee genotypes collected from different sources may have the same gene attributes but with different names according to their geographical status. This could explain the hypothesis of low diversity data. The scatter plot from our study also shows the similarity of scattering of accessions on the SSR marker system used hence pointing towards the low diversity among arabica coffee. Since no past research has been done on accessing and identifying coffee status in Nepal, the validation of the diversity is in a grey space. Regardless, the current study acts as a base for further research on identifying genotypes using more advanced molecular tools to validate.

Conclusion

This study demonstrated the use of SSR markers for accessing the diversity of coffee accessions in Nepal. SSR marker favored for further diversity analysis. Developing suitable coffee breeding and conservation techniques might benefit from knowing the extent and distribution of genetic diversity within the cultivars. Further assessment of CBD using marker-trait analysis can be done to improve the cultivars found in Nepal.

Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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