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Genetic diversity of Robusta coffee (*Coffea canephora* Pierre Ex. A. Froehner) using EST-SSR markers

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Abstract: Research on genetic diversity demonstrated insights into variations that can be valuable for enhancing food security. Expressed sequence tag (EST)-simple sequence repeat (SSR) markers were employed to assess variations among thirty-nine robusta coffee accessions. In this study, the EST-SSR markers utilized identified a total of 15 alleles, averaging 3.0 alleles per locus. Primer CESR02 exhibited the highest polymorphic information content value at 0.59, the greatest genetic diversity value at 0.66, and the highest number of alleles (4). Primers CESR04 and CESR05 revealed the highest percentage polymorphism reaching 42.86. The phylogenetic dendrogram clustered the accessions into three main groups and six subgroups. Accessions 3, 26, and 30 were identified as the most genetically distinct. The most genetically related accessions represented 43.59%, while the most distinct accession recorded 2.56%. In the principal coordinate analysis (PCoA), the least genetically similar accessions constituted 20.51%, whereas 28.21% of the accessions demonstrated genetic similarity, representing the highest grouping. The findings of this study highlight the utility of EST-SSR markers in identifying and categorizing coffee accessions. This approach offers valuable insights into the genetic diversity among robusta coffee accessions, facilitating further efforts in their improvement and preservation.

Keywords: robusta coffee, germplasm, accessions, EST-SSR markers, genetic diversity.

Introduction

Coffee is an important horticultural crop, beloved non-alcoholic beverage consumed worldwide (Samoggia and Riedel, 2019). Owing to its substantial economic impact on developing nations in the past, coffee held the position of being the second most valuable commodity in the global market following oil (Cheserek et al., 2020). A proper understanding of the crop species, including the cultivated robusta coffee species, necessitates essential genomic knowledge (Xu et al., 2017). The presence of natural genetic variation in robusta coffee is advantageous for improvement initiatives just because it provides the foundation for genetic diversity, enabling the emergence of new species (Kumar et al., 2016). Evolution is the process through which new species develop from older ones. The study of genetic diversity has a significant impact on how a species evolves, enabling it to adapt to a new environment. Low levels of adaptability of crop species to new environments can result in a loss of genetic diversity in those species, rendering them more vulnerable to various challenges including extinction and pests and diseases attack (Arzani and Ashraf, 2016). The success of breeding initiatives relies on the genetic diversity present in *Coffea* spp. populations (Machado et al., 2017).

The understanding of genetic diversity in crop species has tremendously benefited from the numerous efforts invested in utilizing molecular markers to identify accessions that are genetically diverse (Rauf et al., 2010). Due to their independence from environmental factors, molecular markers have enabled the detection of genetic variation at the DNA level (Ebrahimi et al., 2017). Numerous molecular markers have been developed and employed over an extended period as a method for assessing the genetic diversity of crop species. These markers encompass random amplified polymorphic (RAPD), single-primer amplification reaction (SRAP), inter simple sequence repeats (ISSR), amplified fragment length polymorphism reaction (AFLP), and microsatellite (SSR) markers (Aboukhalid et al., 2017). Due to its broad content distribution, high polymorphism, neutrality, transferability, and repeatability, expressed sequence tag SSRs (EST-SSRs) markers are considered as one of the most attractive markers (Liu et al., 2019). EST–SSR markers, situated in the coding region of the genome, exhibit high transferability between species, codominant inheritance, and cost-effective (Mishra et al., 2011; Xu et al., 2013). In various crops, including coffee plants, gSSR and EST-SSR markers have been utilized for exploring genetic diversity, genotyping, and cultivar fingerprinting, among other applications (Zhu et al., 2013; Sousa et al., 2022). Under the Brazilian project, the development of an EST-SSR marker date gene bank for robusta coffee was made possible (Vieira et al., 2006). The majority of robusta coffee grown in Nigeria, particularly landraces, lacked sufficient information about their genetic diversity. This contributes to the genetic erosion of the majority of landraces/ cultivars that possess desirable traits and could be valuable in breeding programs. Therefore, the objective of this study was to employ EST-SSR markers to assess the extent of genetic variation cultivated among robusta coffee accessions.

Materials and Methods

Plant materials

The study encompassed thirty-nine accessions of robusta coffee which comprises of accessions obtained from coffee germplasm of Cocoa Research Institute of Nigeria and accessions (landraces) sourced from farmers. A list of the coffee accessions, their corresponding codes, locations and coordinates of the collection areas are provided in Table 1. The collected accessions were planted and maintained in the field at Cocoa Research Institute of Nigeria from 2017 to 2018. Cocoa Research Institute of Nigeria is located at latitude 7° 12' 55" N and 3° 51' 45" E, at an altitude of 122 m above sea level, with an average annual rainfall ranging between 1200-1500 mm. During the dry season, the plants were irrigated using watering cans. Five EST-SSR markers were used for this study. About 500 g of fresh, young, disease-free leaf samples were collected from the coffee plants in the field. These samples were immediately placed into the cooler box containing ice blocks before undergoing lyophilization. Genomic DNA was extracted from young lyophilized leaf samples of the thirty nine robusta coffee accessions. The extraction process

involved grinding the samples, following the Sodium Dodecyl Sulphate (SDS) method (Dellaporta et al., 1983). The quantity and quality of the extracted DNA were checked by running of the individual accession in a mixture of 5 µL of DNA and 0.5 µg/mLof ethidium bromide in 1 % agarose gel electrophoresis. The electrophoresis was conducted for 30 min at 100 voltage. The quantity and purity of DNA were confirmed using a NanoDrop spectrophotometer.

Table 1. List of Robusta coffee: accessions number, code, sources and coordinate of collection.

Accession no.	Code	Source	GPS DD coordinate		
			Latitude (0)	Longitude (0)	Altitude (m)
T1	M36	Germplasm	7.20354	3.86183	422.25
T2	SG2	Germplasm	7.21880	3.86473	464.83
T3	IYA2	Landrace	7.79267	5.80625	1595.50
T4	ORA1	Landrace	7.86340	5.74112	1746.89
T5	EJU2	Landrace	8.05854	5.75192	1418.88
T6	DAC	Landrace	7.86157	6.07288	1407.06
T7	OMU1	Landrace	7.77586	5.77326	1643.60
T8	C105	Germplasm	7.20353	3.86472	422.25
T9	KB3	Landrace	7.82385	6.07772	1385.77
T10	ZN5	Germplasm	7.20660	3.86193	463.25
T11	OLU1	Landrace	7.49692	5.63078	1143.70
T12	OMU2	Landrace	7.77584	5.77324	1643.60
T13	OMA	Landrace	6.92361	3.44661	268.50
T14	OLU2	Landrace	7.49692	5.63078	1143.70
T15	ATK	Landrace	6.92245	3.44086	163.63
T16	T24	Germplasm	7.20353	3.86182	422.25
T17	ORA2	Landrace	7.86340	5.74112	1746.89
T18	AJA 1	Ajassor	5.87446	8.80908	452.21
T19	AJA 2	Ajassor	5.87444	8.81817	452.21
T20	OKU	Okundi	5.96118	8.77028	348.13
T21	E130	Germplasm	7.20353	3.86182	422.25
T22	C111	Germplasm	7.20354	3.86181	422.25
T23	EJU1	Landrace	8.05854	5.75192	1418.88
T24	IFE1	Landrace	7.89475	5.77761	1623.89
T25	C36	Germplasm	7.20357	3.86762	422.25
T26	D57	Germplasm	7.20359	3.86683	422.25
T27	OWE2	Landrace	7.19928	5.02489	1097.97
T28	IYA3	Iyamoye	7.79308	5.80627	1590.45
T29	OWE1	Landrace	7.19847	5.02451	848.81
T30	IYA1	Iyamoye	7.79306	5.80631	1595.50
T31	IBE	Landrace	5.56055	7.62828	165.56
T32	A81	Germplasm	7.20376	3.86572	422.25
T33	IFE2	Landrace	7.77611	5.77307	1641.23
T34	SEK	Landrace	7.48864	5.63687	1076.68
T35	IYA4	Iyamoye	7.79305	5.80631	1698.00
T36	C96	Germplasm	7.20445	3.87281	433.25
T37	SG1	Germplasm	7.21891	3.87571	465.81
T38	F63	Germplasm	7.21992	3.87632	432.35
T39	EJU3	Landrace	8.05854	5.75192	1418.21

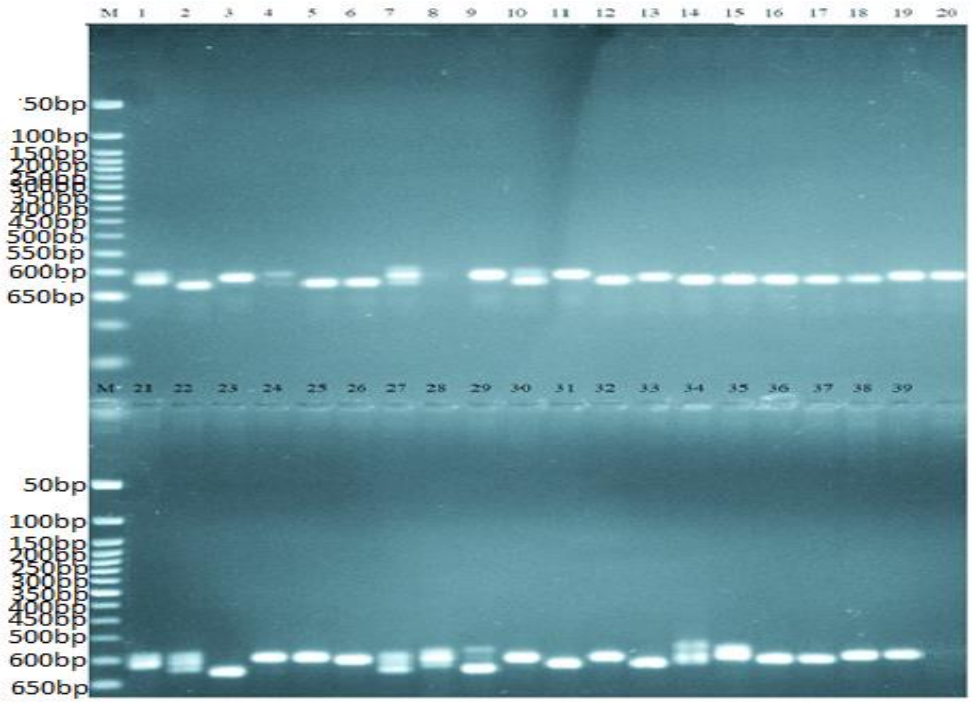

Figure 1. Image of DNA bands of the thirty-nine coffee accessions on CESR02 EST-SSR markers.

EST-SSR markers and PCR amplification

The DNA extracted from different samples were appropriately coded. PCR conditions for all the EST-SSR primers pairs were optimized using template DNA. The PCR reactions were carried out in a total volume of 20 µL, comprising 1.0 µL template DNA, 0.2 µL of each primer, 10 µL Mix (*Taq* polymerase, dNTPs, and PCR buffer), and 8.8 µL of sterile distilled water. PCR amplification was carried out with an initial denaturation step at temperature of 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 57°C for 30 s, extension at 72°C for 20 s, and a final extension step for 10 min at 72°C. The PCR products were separated on a 2.0% agarose gel, stained with ethidium bromide and the gels were visualized under ultraviolet (UV) light. A Polaroid camera was used to photograph the gels through a UV gel documentation system. Primers were obtained from the published article by Hendre and Aggarwal (2014). The list and details of EST-SSR primers are provided in Table 2.

Data Analysis

DNA bands corresponding to different alleles were identified through gel electrophoresis These observed DNA bands were then converted into a binary matrix where the presence of an allelic variation was scored as 1 and the absence of an allelic variation was scored as 0 (Hildebrand et al., 1992; He et al., 2021). Polymorphism among thirty-nine accessions of robusta coffee was evaluated using EST-SSR markers. To estimate genetic diversity the following parameters were determined; number of alleles, gene diversity, polymorphic information content (PIC) and percentage polymorphism at each EST-SSR locus. Power marker version 3.25 was employed for these analyses. (Liu and Muse, 2005). The resulting similarity matrix was used for plotting a phylogenetic dendrogram to illustrate the relationship among the accessions in cluster form using Power marker version 3.25 (Liu and Muse, 2005).

Principal coordinate analysis (PCoA) was constructed with the use of PAST Software version 3.26b (Hammer, 2001). The allelic data was used to calculate the polymorphic information content (PIC) value based on the formula described by Anderson et al. (1993)

$$\text{PIC} = 1 - \sum_{i=1}^{k} (P_i)^2$$

Where k is the total number of alleles detected for an EST-SSR marker, and Pi represents the frequency of the i^{th} allele.

The percentage of polymorphism loci (P) was calculated by dividing the number of polymorphic bands by the total number of amplified bands for each primer.

$P = \frac{\text{Np}}{\text{Nt}}$ x 100

Where Np is the number of polymorphic alleles and Nt is the total number of alleles (polymorphic and monomorphic alleles).

Table 2. EST-SSR Identification no, primers sequences, temperature and product sizes.

S/N	Markers ID	Primer sequence	Tm (C)	Product sizes (bp)
1	CCESSRO1	TGGTAGCACTGTCGGAAGCATAT GACCCATCTAACTTGCTGCATTTT	58	450
2	CCESSRO2	AGGGGCTGGTTATTTTTTGGG GGGGGTAAATACGGGAAAGCAGA	58	650
3	CCESSRO3	GCAGCAACAATCACTTCCACAGC TGCTGTTGTAACTGCGGGATTTG	60	650
4	CCESSRO4	TTCTGGCCGATTGATTGTGAT GCGACAAGGCTGACAAACTACTAC	58	600
5	CCESSRO5	GGCGCTAGAGTTGGTTGTTTGC CAGGCATTGGAACCAGCGAAC	60	550

Table 3. EST-SSR primers and allelic information of 39 Robusta coffee accessions.

Primer ID	Number of allele	Sample size	Polymorphic bands	Total bands scored	Allelic diversity	Gene diversity	PIC	Percentage polymorphic
CESRO1	2.00	39	2	6	0.05	0.50	0.37	33.33
CESRO2	4.00	39	4	11	0.10	0.66	0.59	36.36
CESRO3	3.00	39	1	7	0.02	0.56	0.49	14.28
CESRO4	3.00	39	3	7	0.07	0.56	0.47	42.86
CESRO5	3.00	39	6	14	0.15	0.53	0.43	42.86
Mean	3.0	39	3.2	9	0.08	0.56	0.47	33.94

Results

EST-SSR analysis

A cumulative of 15 alleles was identified across the 39 robusta coffee accessions at the five EST-SSR loci, as illustrated in Table 3. The allele count per primer varied between 2 to 4, with an average of 3.0 alleles at each locus. The polymorphism, as indicated by EST-SSR loci, demonstrated the Polymorphic Information Content (PIC) ranging from 0.37 (CESR01) to 0.59 (CESR02), averaging at 0.47. The lowest PIC value of 0.37 was observed in CESRO1 primer with 2 alleles and the highest PIC value of 0.59 was detected in CESRO2 with 4 alleles. The allelic diversity values ranged from 0.02 to 0.15 with an average of 0.08 diverse alleles per locus. Also, the highest gene diversity was revealed in loci CESR02 (0.66), and the lowest gene diversity was recorded in loci CESR01 (0.50) with an average value of 0.56 per locus. The five EST-SSR primers amplified a total of 45 scorable bands. The primer CESR05 had the highest scorable bands of 14, while primer CESR01 had the lowest scorable bands of 6. The total number of polymorphic bands was 16 with primer CESR05 indicating the highest number of bands (6), while primer CESR03 recorded the least number of bands (1). The highest percentage polymorphism of 42.86% was dictated in primer CESR04 and CESR05 and the lowest percentage polymorphism of 14.28% was revealed by primer CESR03. The percentage polymorphism ranged from 14.28% to 42.86% with average of 33.94%. Figure 1 shows the image of the amplified DNA of the thirty-nine robusta coffee accessions on the locus.

Genetic relationship clustering

The Jaccard's (Jaccard, 1908) similarity coefficients were used based on the genetic matrix data to construct the phylogenetic dendrogram which grouped the accessions based on similarity and dissimilarity into three main clusters and six sub-clusters with genetic distance values ranging from 0.33 to 1.00 respectively. The phylogenetic dendrogram which shows the genetic relationship is represented in (Figure 2). Accessions 3, 26, and 30 were the most genetically distinct among all the accessions. 43.59% of the total accessions were genetically related, while only 2.56% of the accessions exhibited high genetic distance.

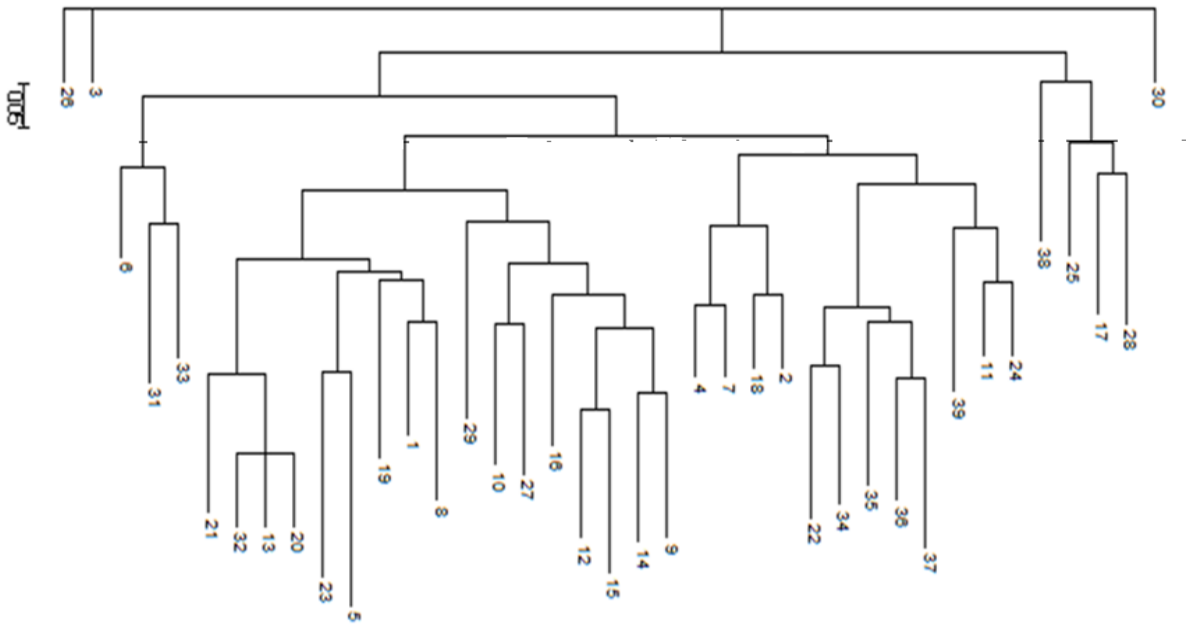

Figure 2. Phylogenetic dendrogram illustrating genetic relationship among the thirty-nine accessions of robusta coffee.

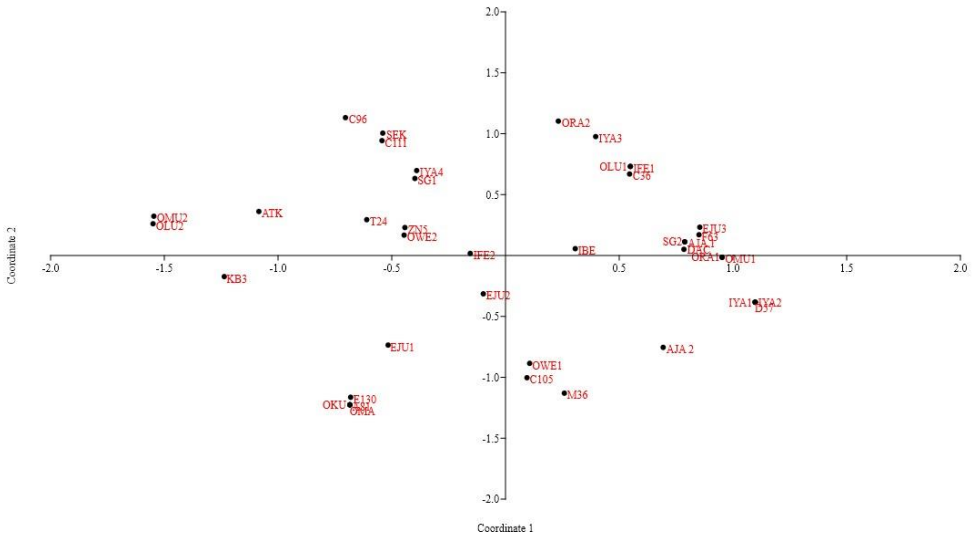

Figure 3. Principal coordinates analysis (PCoA) revealing the relationship among thirty nine Robusta coffee accessions based on EST-SSR markers

Principal coordinate analysis

Principal coordinate analysis (PCoA) is a factorial analysis used to identify the distance and relatedness among the coffee accessions. To understand the genetic diversity among accessions of robusta coffee, the principal coordinate analysis (PCoA) (Figure 3) was conducted. Based on their genetic similarities, the 39 accessions were divided into four main groups by the PCoA. Two of the groups had 11 accessions clustered together, representing 28.21% of the total accessions. The remaining two groups had 8 and 9 accessions grouped, which accounted for 20.51% and 23.08% respectively. This section may be divided by subheadings. It should provide a concise and precise description of the experimental results, their interpretation, as well as the experimental conclusions that can be drawn. All figures and tables should be cited in the main text (such as Figure 1 and Table 1) and numbered in the order they appear in the text.

Discussion

The EST-SSR markers provide useful information on the genetic diversity of coffee with the use of EST-SSR markers, as depicted in Figure 1. The primers CESR02 indicated a high polymorphic information content value of 0.59 as well as a high genetic diversity of 0.66 (Table 2). findings of the current study align withwith those of Missio et al. (2011) who similarly reported high polymorphism in robusta coffee. Prakash et al. (2005) noted elevated genetic diversity in the India robusta coffee gene pool when compared with the coffee originated from Africa. This observation was made using SSR and AFLP markers. The notable level of polymorphism observed may be attributed to the allogamous nature induced by self-incompatibility of the robusta coffee species (Depecker et al., 2023). These findings corroborate the wide genetic diversity observed by Sousa et al. (2022) who worked on *C. canaphora* using SSR markers. They justified the observed genetic diversity by attributing it to the receptivity of pollen grains from other plants, contributing to heterozygosity.

Among the primers, primer CESR02 stands out as more informative, as evidenced by its the highest PIC value and a greater number of alleles which are indicators used to measure the polymorphism of a marker (Guo and Elston, 1999). This implies that primer CESR02 demonstrated a heightened capacity to discern the presence of genetic diversity

among the studied coffee accessions. The mean value of allele number in this study was 3.0 different from the mean allele numbers of 10.5 reported by Poncet et al. (2006), with the use of 25 EST-SSR markers. As per Poncet et al. (2006) varied mean numbers of alleles have been documented in *Coffea* spp. According to Tshilenge et al. (2009), a high level of polymorphism was observed among robusta coffee accessions using RAPD markers. These results indicated that the EST-SSR markers used in this study revealed some levels of genomic DNA diversity in robusta coffee. Zhai et al. (2013) reported a low level of polymorphism in radish (*Rephanu sativus* L.) species with the use of EST-SSR markers. These findings demonstrate that the PIC value in a population depends highly on the genetic constitution assessed and the genotypes used (Tasma and Arumsari, 2013). Thus the observed PIC value becomes crucial (Missio et al., 2011). The genetic relationship among the 39 robusta coffee accessions was assessed through the phylogenetic dendrogram and the principal coordinate analysis (PCoA) which clustered the accessions into different groups. The clustering of landraces alongside with germplasm accessions suggests their genetic similarity, providing guidance to avoid inadvertent duplication during selection. Moreover, a significant proportion of the accessions clustered in the phylogenetic dendrogram exhibited similar groupings in the principal coordinate analysis (PCoA). Nonetheless, within the PCoA clustering, there were instances of overlapping accessions, suggesting the potential duplication of these entries. This overlap offers valuable insights into identifying duplicate accessions and serves as a guide to prevent the inadvertent of the same accession for the breeding program.

Conclusion

The presence of genetic variability in crops is crucial, as it creates a chance for the breeders to develop new varieties. Among the EST-SSR markers, primer CESR02 proved to be the most informative regarding the genetic diversity of robusta coffee studied. This is attributed to its capability to produce a substantial number of alleles, coupled with the highest PIC value. The identification of genetic variation in this study, suggests that the coffee accessions have the potential to serve as valuable genetic material for breeding programs. The data acquired through the application of EST-SSR markers, would aid in evaluating the genetic variation among coffee accessions. The congruence observed in clustering of the accessions through both phylogenetic dendrogram and PCoA suggests that these two methods can be utilized to assess the genetic relatedness of the robusta coffee accessions. This is because they complement each other effectively. The outcomes of this research will simplify the management, conservation, and identification of landrace accessions that exhibit genetic relatedness to those in the germplasm. It is therefore recommended that to incorporate genetically distinct accessions into coffee germplasm to increase the variation with coffee populations.

Supplementary Materials

No supplementary material is available for this article.

Author contributions

Conceptualization, M.B.N. and AC.O.: methodology, M.B.N.; A.C.O; BOA; OPA; L.S.F.: software, M.B.N.: validation, M.B.N.; A.C.O.; and L.S.F,: formal analysis, M.B.N.: investigation, M.B.N.: resources, M.B.N.; and CRIN.: data curation, M.B.N.; A.C.O.; and L.S.F.: writing—original draft preparation, M.B.N.: writing— review and editing, M.B.N.; A.C.O.; and L.S.F.: visualization, M.B.N.: supervision, A.C.O.; B.O.A.; O.P.A.; and CRIN.: project administration, M.B.N.; A.C.O.; and CRIN.: funding acquisition, M.B.N.: All authors have read and agreed to the published.

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Conflict of interest statement

The authors declare no conflict of interest.

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تنوع ژنتیکی قهوه روبوستا (*Coffea canephora Pierre* Ex. A. Froehner) با استفاده از نشانگرهای EST-SSR

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چکیده: تحقیقات در مورد تنوع ژنتیکی، بینش‌هایی را در مورد تغییراتی که می‌تواند برای افزایش امنیت غذایی ارزشمند باشد نشان می‌دهد. نشانگرهای مبتنی بر توالی بیان‌شده (EST) و توالی تکرار ساده (SSR) برای ارزیابی تغییرات در بین ۳۹ نمونه قهوه روبوستا استفاده شد. در این مطالعه، نشانگرهای EST-SSR مورد استفاده، در مجموع ۱۵ آلل را شناسایی کردند که به طور متوسط ۳ آلل در هر مکان است. آغازگر CESR02 بالاترین مقدار محتوای اطلاعات چندشکلی را با ۰/۵۹، بیشترین مقدار تنوع ژنتیکی را با ۰/۶۶ و بیشترین تعداد آلل (۴) را نشان داد. آغازگرهای CESR04 و CESR05 بیشترین درصد چندشکلی را نشان دادند که به۴۲/۸۶ رسید. دندروگرام فیلوژنتیک، نمونه‌ها را به سه گروه اصلی و شش زیر گروه تقسیم نمود. ژنوتیپ‌های ۳، ۲۶ و ۳۰ به عنوان متمایزترین از نظر ژنتیکی شناسایی شدند. بیشترین قرابت بین ژنوتیپ‌های مورد بررسی ۴۳/۵۹ درصد بوده، در حالی که کمترین قرابت ۲/۵۶ درصد مشاهده شد. در تجزیه به مؤلفه اصلی (PCoA)، ژنوتیپ‌های با قرابت ژنتیکی کم و زیاد، به ترتیب ۲۰/۵۱ درصد و ۲۸/۲۱ درصد از نمونه‌ها را تشکیل داده که مبین قدرت بالای تفکیک آنالیز در گروه‌بندی است. در این تحقیق، کاربرد نشانگرهای EST-SSR در شناسایی و طبقه‌بندی ترکیبات قهوه مورد بررسی قرار گرفت. یافته‌های این تحقیق بینش ارزشمندی در مورد تنوع ژنتیکی میان قهوه‌های روبوستا ارائه می‌دهد و تلاش‌های بیشتر در بهبود و حفظ آنها را تسهیل می‌کند.

کلمات کلیدی: قهوه روبوستا، ژرم‌پلاسم، اکسیشن، نشانگرهای EST-SSR، تنوع ژنتیکی.

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