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Genetic Diversity and Population Structure of a Pepper (*Capsicum* spp.) Collection Revealed by SSR Markers and Fruit Morphology

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ABSTRACT: Studies on genetic diversity, population structure, and cross-transferability have chosen the simple sequence repeat (SSR) marker as their preferred marker. This study examined the genetic diversity and population structure of 22 *Capsicum* spp. collection accessions (*C. annuum*, *C. chinense* and *C. frutescens*) using the recently developed 20 SSRs derived from the genus *Capsicum*. The fruit's phenotypic traits, such as shape, placenta type, apex, pericarp thickness, and colour at the immature and mature stages of development were investigated. In 22 accessions, 13 polymorphic SSRs were used for analysis. The total number of alleles (54) (ranging from 2 to 7, with an average of 4.15 per locus) as well as polymorphism information content values (0.34 to 0.86, with a mean of 0.56). Using SSRs, neighbour-joining and factorial analyses of 22 *Capsicum* accessions formed three major clusters, primarily based on their distinct types, and having no relationship with their geographical locations. Model-based STRUCTURE analysis also showed three genetically distinct populations (K = 3). *C. frutescens* accessions (chilies) were clustered together, and *C. annuum* accessions were separated into two clusters, indicating substantial genetic variation in the collection. Moreover, some admixture exists in all three clusters. The mature fruit colour, shape, and size vary between accessions. These thirteen SSR markers discriminate between the three *Capsicum* species in the collection. They may be implemented to conduct genetic management and marker-assisted breeding in *Capsicum* crops.

Keywords: Accession, Clustering, Conservation, Pepper (*C. annuum* L., *C. frutescens* L., *C. chinense* Jacq.), SSR

Introduction

Pepper (*Capsicum* spp.) is a globally consumed vegetable crop of the Solanaceae family, known for its nutritional composition. The pepper fruits are a rich source of bioactive substances [capsaicinoids (capsanthin, capsorubin)], carotenoids (β-carotene, β-cryptoxanthin, lutein, and zeaxanthin), phenolic (flavonoids), vitamins (C, E, and provitamin A), minerals, and essential oils (Antonio *et al*., 2018; Baenas *et al*., 2019; Hassan *et al*., 2019; Batiha *et al*., 2020; Villa-Rivera *et al*., 2020; González-López *et al.*, 2021). In addition to their nutritional benefits, the anti-bacterial, anti-inflammatory, anticancer, antimicrobial, and antioxidant properties of different *Capsicum* spp. have also been reported (Badia *et al*., 2017). It is believed that peppers originated in Western North America and South America, where they are more diverse. From these regions, they spread and diversified into other Southern and Central American areas (Pickersgill *et al*., 1991, 1997). The genus *Capsicum* consists of about 38 economically vital species, many of which are cultivated, namely *C. annuum*, *C. chinense*,

C. frutescens, *C. pubescens*, *C. baccatum* and *C. assamicum* (Pickersgill *et al*., 1997; Ramchiary *et al*., 2013; Di Dato *et al*., 2015), with more than 200 varieties (Bosland and Votava, 2012). More than 200 cultivars and landraces of these five species contributed to the global production of 4,255,050 tons in 2019 (FAO, 2021). The last three *Capsicum* species are not commercially available in Nigeria. The majority of cultivated *Capsicum* species are grown by farmers and are considered valuable economic crops. Due to its high nutritional value, phenolic compounds and pungency properties, it is widely consumed and a key part of indigenous diets in Nigeria (Adetula and Olakojo, 2006).

The three complexes of cultivated peppers can be distinguished by their capacity for cross-pollination: (i) The *C. annuum* complex, which includes *C. annuum*, *C. chinense*, *C. frutescens*, their wild relatives and *C. galapagoensis* Hunziker (Onus and Pickersgill, 2004). (ii) The *C. baccatum* complex, which includes *C. baccatum*, *C. praetermissum* Heiser et Smith, and *C. eximium* Hunziker. (iii) The more distantly related species, *C. baccatum* var. praetermissum (Heiser & P. G. Sm.) Hunz. Even though there are severe incompatibility barriers to hybridisation among these complexes, the development of viable hybrids, such as between *C. annuum* and *C. baccatum*, has been reported (Perry *et al*., 2007; Ince *et al*., 2010; Manzur *et al*., 2015). *Capsicum* species cultivated today are evidence of domestication (both conscious and unconscious selection activities) and an increase in co-evolutionary adaptation of plants to cultivation, human selection, and use in the various environments of *Capsicum* diversity (Gepts, 2010; González-Pérez *et al*., 2014). Furthermore, a high level of genetic variation within and between species has led to the emergence of many varieties and cultivars with superior fruits (Carvalho *et al*., 2014; Velázquez-Ventura *et al*., 2018).

Increasingly efficient genetic markers such as simple sequence repeat (SSR) markers (genomic and expressed sequence tags) have received increased interest from plant geneticists and breeders in exploring the genetic diversity analysis and population genetic structure of *Capsicum* species (Akyavuz *et al*., 2018; Xiao-zhen *et al*., 2019; Christov *et al*., 2021; Haq *et al*., 2022). Due to their advantages of being polymorphic, abundant, co-dominant, multi-allelic, cost-effective, simple, rapid genotyping, and significant transferability among genotypes, SSR markers have become increasingly popular as the most reliable method to detect genetic variation in crop plants in recent years. Previously published SSR markers (Minamiyama *et al*., 2006; Mimura *et al*., 2012) from some *Capsicum* germplasms have been applied for the assessment of genetic diversity in a collection of pepper genotypes. In addition, SSR loci potentially linked to resistance and pungency genes have been reported, allowing for the assessment of the genetic diversity analysis in the genus *Capsicum* (Di Dato *et al*., 2015; Adeyemo *et al*., 2017). Furthermore, some SSR markers have potential cross-transferability across species (Varshney et al., 2005). In a recent study, Moulin *et al*. (2022) examined the transferability of SSR markers created for *Capsicum annuum* by screening 203 F_2 populations that were the result of a cross between two *Capsicum baccatum* accessions. Out of 152 SSR markers, 62 were successfully transferred to *C. baccatum* species.

Furthermore, with the availability of newly discovered SSR markers from *Capsicum* species, an investigation of their utility for the characterization of genetic diversity, population structure and cross-transferability is imperative. In recent years, the discovery of new SSR markers in *Capsicum* has been reported for determining the genetic relationships and levels of genetic variation among domesticated peppers in different breeding programs. For instance, Dubey *et al*. (2019) employed a comparative genomic approach to develop a set of 49 gene-based simple sequence repeat (SSR) markers linked to the genes involved in fruit development and ripening in *Capsicum*. According to this study, *C. annuum*, *C. frutescens*, and *C. Chinense* exhibit extensive MADS-RIN ortholog expression, which is consistent with non-climacteric ripening behaviour. In addition, the development of increasing cross-species mapping data has been widely employed in the identification of SSR in related species, which can be applied to detect interspecific sequence divergence of *Capsicum* species due to their high level of transferability (Carvalho *et al*., 2015). Additionally, Uncu (2018) generated a set of genome-anchored markers in *C. Chinense* for use in pepper introgression breeding to improve cultivated pepper germplasm if transferable within the *Capsicum* genus and for determining genetic diversity. More recently, Chhapekar *et al*. (2020) identified other genic SSR markers using transcriptome data in *C. chinense* and *C. frutescens*, which can be used in the breeding of *Capsicum* varieties with improved metabolites (pungency, carotenoids etc.) and agronomic traits. The utility of these newly developed SSR markers for evaluating in-depth genetic diversity and population structure has not been reported across species.

The morphological study will be useful for evaluating genetic diversity and cultivar/variety identification of genetic resources (Aiswarya *et al*., 2020; Atanasova *et al*., 2021). *Capsicum* germplasm for morphological traits are explored using conventional descriptors (Gepts, 2006; Upadhyaya *et al*., 2008; Brilhante *et al*., 2021). Fruit colour, size, pungency, and shape are essential fruit traits in *Capsicum* species (Moreira *et al*., 2018). The *Capsicum* species genetic diversity has been investigated using both phenotypic traits and SSR markers (Baral *et al*., 2004; Lima *et al*., 2017; Carvalho *et al*., 2017; Baba *et al*., 2016; Rabuma *et al*., 2020). There have been few studies on the fruit characteristics of the Nigerian species of *C. annuum*, *C. frutescens*, and *C. Chinense* species. On the other hand, *Capsicum* germplasm collections in Africa have the potential to broaden the genetic base of

cultivated peppers for breeding programs. To effectively conserve, manage, and improve the genetic diversity of the *Capsicum* germplasm collection, morphological variation in conjunction with SSR data is now imperative to grasp the extent of genetic diversity. To assess the genetic diversity and population structure of 22 pepper collections from different regions of Nigeria and two other African nations (Niger and Rwanda), we used a chosen set of recently developed SSR markers (Uncu, 2018; Dubey *et al*., 2019; Chhapekar *et al*., 2020). Likewise, we also found SSRs useful for understanding genetic relationships among *Capsicum* three cultivated species. We further used six fruit traits International Plant Genetic Resources Institute (IPGR) descriptors to characterize the morphological diversity of the collected *Capsicum* accessions to provide substantial information about the range of genetic variation in *Capsicum* species phenotypes. It is necessary to preserve *Capsicum* variability in Nigeria through germplasm collection and management. This is to prevent genetic diversity loss, considering climate change and rising global food demand.

Materials and methods

Collection of plant study materials, field evaluation and morphological trait assessments: In this study, between the 7th of December 2020 and the 6th of January 2021, we collected 22 accessions of *Capsicum* spp., including 18 local forms/landraces and varieties in the form of fruit, from small-scale farmers in eleven states in Nigeria. Additionally, *Capsicum* spp. seeds grown in Rwanda (2 accessions) and the Republic of Niger (2 accessions), which were purchased from local markets and department stores, were included as checks. These accessions were selected from many accessions throughout the states surveyed in Nigeria based on unique fruit shape morphology and commercial advantage. These accessions belong to *C. frutescens, C. annum* and *C. chinense*. Table 1 provides information on the origin of these accessions collected and used in this study. On January 27, 2021, ten seeds of each of the 22 accessions were sown in pots with a high-quality soil mixture in the experimental greenhouse for 42 days. The 22 accessions' seedlings were then grown in the field at the Department of Cell Biology and Genetics, University of Lagos, Akoka, Lagos. Fruit setting started on May 10, 2021, to assess fruit morphological traits. A total of 5 healthy seedlings were planted for each accession in rows at a spacing of 38 × 58 cm in one replication. Organic manure was applied 24 days after planting. After 42 days, a second manure was applied. When necessary, conventional procedures for soil preparation, manual weed control, and periodic watering are practised. Plants grew to maturity for measurement of morphological diversity. When the fruit background colour began to change from green to yellow in the field at the end of August 2021, qualitative analyses and records of fruit shape, fruit placental type, fruit apex, fruit epidermis thickness, fruit colour at immaturity, and fruit colour at maturity were performed. Fruits were longitudinally sectioned in halves. Traits were scored, and photo images were obtained from 19 accessions. Qualitative traits were analyzed descriptively. At the end of all the evaluations, three accessions did not fruit, which may be due to the photoperiod or day length variations and rainfall pattern in the south compared to the place of collection (northern region of Nigeria). All young leaf samples from five-week-old healthy plants in the greenhouse were collected and dried using silica gel in zip-lock plastic bags and stored at room temperature until DNA extraction and molecular analysis.

Table 1: List of *Capsicum* accessions used in the study

S/No	Code	Local names	Species	Origin
1	CF01	Mgbapka (Small pepper)	*C. frutescens*	Benue, Nigeria
2	CF02	Atawewe (Chilli pepper)	*C. frutescens*	Delta, Nigeria
3	CF03	Atawewe (Chilli pepper)	*C. frutescens*	Kogi, Nigeria
4	CF04	Atawewe (Chilli pepper)	*C. frutescens*	Kogi, Nigeria
5	CF05	Bambara kelekele Piment counte (Chilli pepper)	*C. frutescens*	Niamey, Niger
6	CF06	Dogodogo (Long pepper)	*C. frutescens*	Benue, Nigeria
7	CF07	Bawa (Long pepper)	*C. frutescens*	Kano, Nigeria
8	CF08	Shombo (Long pepper)	*C. frutescens*	Kogi, Nigeria
9	CF09	Piment de caine (Long pepper)	*C. frutescens*	Niamey, Niger
10	CA10	Atarodo	*C. annum*	Kano, Nigeria
11	CA11	Atarodo	*C. annum*	Ekiti, Nigeria
12	CA12	Atarodo	*C. annum*	Kogi, Nigeria
13	CA13	Atarodo	*C. annum*	Oyo, Nigeria
14	CA14	Tarumbu Jos atarodo	*C. annum*	Plateau, Nigeria
15	CA15	Atarodo	*C. annum*	Lagos, Nigeria

S/No	Code	Local names	Species	Origin
16	CA16	Green pepper	*C. annum*	Sokoto, Nigeria
17	CC17	Yellow pepper	*C. chinense*	Enugu, Nigeria
18	CC18	Yellow pepper	*C. chinense*	Kigali, Rwanda
19	CA19	Red bell pepper	*C. annum*	Edo, Nigeria
20	CA20	Red bell pepper	*C. annum*	Benue, Nigeria
21	CA21	Green bell pepper	*C. annum*	Kano, Nigeria
22	CA22	Green bell pepper	*C. annum*	Kigali, Rwanda

DNA isolation, primer selection and SSR genotyping: Silica-dried 12–20 leaves of each accession were lyophilized in a freeze-dryer (Bench Top), which were then ground into a fine powder. Genomic DNA isolation was performed according to the cetyltriethylammonium bromide (CTAB) extraction method described by Doyle and Doyle (1987). First, DNA was quantified using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) and DNA quality was assessed with 1% agarose gel electrophoresis in TBE 0.5X (Tris-borate-EDTA) buffer. Three different sets of SSR primer pairs (20) were chosen and tested for use in the study, comprising 8 gene-based SSR primer pairs developed previously from the genes involved in fruit development/ripening viz. *C. chinense, C. frutescens and C. annuum* (Dubey *et al*., 2019), 6 from genome wide based SSR markers developed previously from *Capsicum chinense* Jacq. with high potential for use in pepper introgression breeding (Uncu, 2018) and 6 from SSR primer pairs constructed from transcriptome profiling of potential industrial crops viz. *C. chinense* and *C. frutescens* (Chhapekar *et al*., 2020*)*. The forward and reverse primers were synthesized by Inqaba Biotec (South Africa). Primers are listed in Table 2. The first PCR optimization of selected three *Capsicum* accessions was used to identify the most effective SSR primer pairs. The melting temperature of the SSR primers as well as the PCR procedures as per Uncu (2018), Dubey *et al.* (2019), and Chhapekar *et al*. (2020) were followed. The PCR products were separated on 1.5% agarose gel, and 15 SSR markers that produced specific and clear PCR products were selected for analysis of genetic diversity and population structure of the collection of 22 *Capsicum* accessions. The PCR amplification profile was the same as described above. PCR amplifications were performed in a thermal cycler. The PCR DNA products (2.0 µL) were separated by electrophoresis using a 5% non-denatured polyacrylamide gel at 70W for 2 h. Ethidium bromide staining was used to visualize the SSR alleles. The 50-base pair (bp) DNA ladder was loaded along with the samples to estimate the size of the SSR alleles in the PCR products. Using a gel documentation system, UV illumination was used for visualization and photography. The distinct and clear alleles were scored as present (1) or absent (0).

Table 2: List of primers of SSR markers used for genotyping

Name	Forward primers (5′–3′)	Reverse primers (5′–3′)	Reference
SSR_CF-22* LOC107845460-up	GATACTTTACTGGATGGTTGCT	TGTTCTACACTCGTATTTGGG	Dubey *et al*., 2019
SSR_CF-30* LOC107847819-up	AGGGGTAGTAGTGGAAATTGTT	ACAGGTGAAGTAGAGGAAGATG	Dubey *et al*., 2019
SSR_CF-4* LOC107845304	AGTCTCTTCTTTGTGAGTGTTG	GACAGCTAACTAGACAGGTTTGT	Dubey *et al*., 2019
SSR_CF-43* LOC107866321-down	ATCTATGGAGTCATTTGGTGAG	GTTCTTGGGTCATACTTCTTTG	Dubey *et al*., 2019
SSR_CF-48* LOC107878054-down	TGGAGAGTTTAGTAGTTTCGTG	GAGTATGAAGATGAGCGTTAGA	Dubey *et al*., 2019
SSR_CF-7* LOC107855404	GTAGTCTCCATCTCCATACCTG	CGGGTGTAATCAACTCTCTTA	Dubey *et al*., 2019
SSR_CF-12* LOC107854549	GTGATACACCCTTATATGACCC	GTCGTACTTCTTGTCGTAGGTAT	Dubey *et al*., 2019
SSR_CF-13* LOC107854549	GATTCCCAGAAGTGTGAAAA	CTACTGTCATTGTTTGGTTGAC	Dubey *et al*., 2019
>MK84	TGGAATTGAAACGCAGCTAA	AATGCATGTTGCTGGGAAGT	Uncu, 2018
>MK190	GGGAAAGAGTGGCTTGCTC	TTCGGTAATCTTGTGCTGGA	Uncu, 2018
>MK310	CAGGCCCATCATTCAAATTC	ACTCTTTGTGGGGTTGATGG	Uncu, 2018
>MK487	ACCTCTTCAGCCAGTTGCAT	CACACTTGTGGGATTTCACG	Uncu, 2018
>MK544	TCCTCTCAAGTAAATGCCAAAG	AACAGGAAACGAAGGGAAAA	Uncu, 2018
>MK769	ACACATGCACATGGAGAGGA	CACATATCAATGCCCTAAACAG	Uncu, 2018
CFpSSR80	GACCTGATATTTCCCTCAGTC	CGAAATCTTTCTCTCATCGT	Chhapekar *et al.*, 2020
CFpSSR107	AGCTCGATGAGGATGAACTA	GAGGATTCGTTCTCTTGTGA	Chhapekar *et al.*, 2020

Name	Forward primers (5′–3′)	Reverse primers (5′–3′)	Reference
CFpSSR126	GAATGTGGTGGATGAATTG	CATCAAACTCCCATCAATCT	Chhapekar *et al.*, 2020
CFpSSR3	TTGAGGATGGCTACAGTAGAA	TGTATCCTTCTCAGCATTCAC	Chhapekar *et al.*, 2020
CFpSSR18	GAAATTATACCGAGCTTCACC	AAACCACTCTGCCTCTTTTAC	Chhapekar *et al.*, 2020
CFpSSR34	AATCTTGTGCCCAATGTAAG	CTTAGCATGAGCAACTCAAAG	Chhapekar *et al.*, 2020

The study of genetic diversity: The polymorphism information content (PIC) per locus, the observed number of alleles (N_A), gene diversity (expected heterozygosity, H_E) and minor allele frequency (MAF) to elucidate the characteristics of the polymorphic SSR markers in the collection were determined by PowerMarker version 3.25 software (Liu and Muse, 2005). H_E was calculated from allele frequencies using an unbiased formula of $1 - \sum pi^2$ ($1 \leq i \leq m$), where m is the number of alleles at the target locus and pi is the allele frequency of the ith allele at the target locus. H_O was calculated as the number of heterozygous individuals divided by the total number of individuals. The genetic similarity matrices between accessions were obtained with Jaccard's dissimilarity coefficient method in Darwin ver. 5.0.158 statistical (Perrier and Jacquemoud-Collet, 2006) software by Rohlf (1998). Agglomerative hierarchical clustering was applied using the unweighted pair-group method with arithmetic mean (UPGMA). Using Darwin software, Neighbour Joining (NJ) was obtained based on the distance matrix, and its robustness was tested using a 1000 replicate bootstrap analysis (Felsenstein, 1985). Also, to assess the genetic relationships of the investigated accessions, a factorial analysis of the genotypic data matrix was performed using Darwin software 22.

Population structure analysis: The population structure was analysed using the STRUCTURE software package (Pritchard *et al*. 2000). Using the Bayesian clustering model method, the SSR data were analysed to define the number of clusters and assign individuals to K (putative number of populations). Genetic structure was simulated using K = 1–5, with an admixture model for ancestry with correlated allele frequencies (Falush *et al*., 2003). Each run involved 1,000,000 Markov Chain Monte Carlo simulations, after a burn-in period of 1,000,000 iterations. Ten independent runs were performed for each value of the K. The most likely value of K (the number of inferred ancestral populations) was determined by comparing mean values and the variability of log-likelihoods in each K, and the rate of change in the log-likelihoods between adjacent K values, and ΔK was used to estimate the optimum K value (Evanno *et al*., 2005). Of the five independent runs, the one delivering the highest likelihood value was used to assign the entries to the indicated sub-population clusters (Pritchard *et al*., 2000).

Results

Morphological variability in fruit traits: Fruits from 19 accessions were available and matured during the research. We examined 19 accessions since 3 did not bear fruit. Among the six qualitative traits studied, based on IPGRI descriptors, a variation was detected in the collection of 19 *Capsicum* spp. (Figures 1 and 2). The various traits evaluated in the collection are presented in Table 3. The mature fruits of the 22 accessions were different in colour. Light red to dark red (different shades) were the fruit colours most often seen in the accessions (72.22%), followed by green (11.11%), yellow-orange (11.11%), and yellow-red (5.55%). The most varied fruit morphology was found in *C. frutescens* accessions, particularly in fruit size, shape, and length. The fruit shapes showed were block-shaped, almost round, campanulate, triangular, and elongated in the collection (Figure 1).

Genetic diversity revealed by SSR markers: We optimized 20 primers for PCR amplification efficiency using three randomly selected accessions. Of the three sets of markers evaluated, there was no amplification for SSR_CF-48*, SSR_CF-7*, SSR_CF-12*, SSR_CF-13* and CFpSSR3. Fifteen (15) primers (75%) successfully produced clear and specific PCR products of the expected size in these three accessions on 2% agarose gel electrophoresis. Only 15 SSR primers were applied to analyse all 22 *Capsicum* accessions in the study. Finally, 14 primer loci that showed clear amplicons were used to evaluate polymorphism in 22 accessions and produced the expected size range (bp) across all *Capsicum* accessions. As shown in Table 4, genetic characteristics, and allele size (bp) (approximate) within the collection are listed. One SSR was removed from the study because it showed poor amplicon resolution and missing alleles. In all, 13 primers produced polymorphic amplicons and detected 54 alleles (Table 4). Among the 13 SSR markers, the number of alleles (Na) ranged from 2 to 7 (>MK190 and CFpSSR107), with an average of 4.15 alleles per marker (Table 4). The polymorphism information content (PIC) values ranged from 0.34 (>MK310) to 0.86 (CFpSSR107), with an average value of

0.56 and the expected (H_E) varied from 0.38 to 0.87, with a mean of 0.62. Minor allele frequency at the SSR level spanned from 0.18 to 0.77 with an average value of 0.51. Figure 3 a representative image of the gel electrophoresis pattern obtained with SSR markers.

Pairwise genetic dissimilarity: A genetic dissimilarity matrix was created (Table 5) to understand the extent of genetic divergence among the 22 accessions. In this study, the dissimilarity coefficients ranged from 0.13 to 0.93, with a mean of 0.68. In a pairwise comparison, the maximum dissimilarity was obtained between CA05 and CC18, with an index of 0.93, while the minimum dissimilarity was obtained between CF03 and C04, with an index of 0.13.

Analysis of genetic relationships of Capsicum spp. accessions using distance-based model analysis: The NJ tree created by the UPGMA-based cluster analysis of the 22 *Capsicum* accessions from different country origins revealed three major clusters, which are indicated by different coloured lines in Figure 4. *C. frutescens* formed cluster I which contains 8 accessions, apart from one *C. frutescens* (CF09) which clustered with 5 accessions of *C. annuum* forming cluster II. In contrast, cluster III had 8, mainly *C. annuum* accessions; 6, and 2 *C. chinense* accessions. When a factorial analysis was used to examine the relationship between the 22 accessions, three distinct clusters were formed (Figure 5). The result of the factorial analysis showed a clear separation between *C. frutescens* and *C. annuum* (Figure 5). Factorial analysis revealed that accessions *2 C. chinense accession*s (CC17 and CC18) clustered with the *C. annuum* accessions.

Population genetic structure of Capsicum spp.: The Bayesian clustering analysis showed that the Δ*K* identified a maximum at *K* = 3, which indicates that the collection was divided into three genetically distinct clusters (I, II, and III in Figure 6a). Cluster I included 7 *C. frutescens accessions*, predominantly the 5 chilli types (from Nigeria and the Niger Republic) and 2 *C. frutescens* spp (long pepper). Cluster II consisted of eight accessions (6 *C. annum* and 2 *C. chinense*). Of the 7 accessions belonging to Cluster III, 5 were from *C. annum* and 2 from *C. frutescens*. Notably, all these accessions were clustered per species irrespective of where they originated from. It is important to note that clusters II and III captured *C. annum* accessions. Out of 22 accessions, 8 had no significant admixture, 8 had only a slight admixture level from nearly all subgroups, and 6 belonged to a particular group (Figure 6b). The proportions of the colour bars are admixtures in each accession. Thus, a level of introgression from the three species was seen within each of the three genetic groups. The long pepper from Kogi state (CF08) is grouped with 6 others in Cluster III and has the highest admixture. The STRUCTURE plot for K = 3 is presented in Figure 5a. The population structure analysis revealed a clustering pattern consistent with the NJ and factorial analysis.

Table 4: Genetic characteristics of thirteen SSR markers used for assessment of genetic diversity in 22 *Capsicum* accessions

		Size Range			
SSR loci	**Na**	**(bp)**	**MAF**	**He**	**PIC**
SSR_CF-22* LOC107845460-up	6	390-400	0.27	0.83	0.81
SSR_CF-30* LOC107847819-up	4	120-130	0.59	0.55	0.47
SSR_CF-4* LOC107845304	3	400-420	0.41	0.69	0.63
SSR_CF-43* LOC107866321-down	4	270-280	0.59	0.55	0.47
>MK84	3	310-325	0.68	0.46	0.39
>MK190	2	320-325	0.64	0.49	0.41
>MK310	3	220-225	0.77	0.38	0.34
>MK487	5	120-140	0.41	0.69	0.63
>MK769	3	180-190	0.45	0.67	0.60
CFpSSR80	5	140-175	0.5	0.69	0.65
CFpSSR107	7	180-225	0.18	0.87	0.86
CFpSSR18	4	155-165	0.55	0.57	0.48
CFpSSR34	5	850-870	0.64	0.56	0.53
Mean	4.15		0.51	0.62	0.56

Na: number of alleles, He: gene diversity,
MAF: Minor allele frequency, PIC: polymorphic information content.

Table 3: The 6 qualitative traits of 19 *Capsicum* accessions under experimental field conditions

Traits	Characters	No of accessions			
		Total	***C. frutescens***	***C. annum***	***C. chinense***
Fruit shape					
	Elongated	6	6	-	-
	Campanulate	2	-	2	-
	Triangular	2	1	-	1
	Almost round	5	-	4	1
	Block shaped	4	-	4	-
Placenta type					
	Axile	12	-	10	2
	Marginal	7	7	-	-
Fruit apex					
	Acute	7	7	-	-
	Tapered	1	-	1	-
	Depressed	2	-	1	1
	Flat	1	-	1	-
	Truncate	8	-	7	1
Fruit wall (pericarp thickness					
	Light	4		4	-
	Intermediate	10	5	4	1
	Thick	5	2	2	1
Fruit colour at immature stage					
	Light green	6	1	3	2
	Green	9	5	4	-
	Dark green	4	1	3	-
Fruit colour at mature stage					
	Light red	5	2	3	-
	Red	5	2	3	-
	Dark red	4	2	2	-
	Green	2	-	2	-
	Yellow-red	1	1	-	-
	Yellow-orange	2	-	-	2

Table 5: Pairwise dissimilarity of 22 *Capsicum* accessions estimated the Jaccard index

	CF01	CF02	CF03	CF04	CA05	CF06	CF07	CF08	CF09	CA10	CA11	CA12	CA13	CA14	CA15	CA16	CC17	CC18	CA19	CA20	CA21
CF02	0.28																				
CF03	0.52	0.53																			
CF04	0.59	0.60	0.13																		
CA05	0.57	0.64	0.50	0.57																	
CF06	0.61	0.55	0.55	0.62	0.71																
CF07	0.76	0.78	0.67	0.60	0.70	0.62															
CF08	0.72	0.68	0.74	0.79	0.85	0.50	0.84														
CF09	0.68	0.64	0.70	0.75	0.67	0.59	0.70	0.59													
CA10	0.74	0.76	0.76	0.81	0.78	0.81	0.76	0.67	0.57												
CA11	0.68	0.69	0.74	0.79	0.67	0.79	0.79	0.65	0.50	0.46											
CA12	0.84	0.86	0.86	0.90	0.70	0.79	0.78	0.74	0.54	0.62	0.44										
CA13	0.79	0.77	0.86	0.81	0.83	0.68	0.77	0.68	0.45	0.60	0.54	0.52									
CA14	0.79	0.81	0.81	0.86	0.79	0.78	0.77	0.73	0.58	0.48	0.54	0.68	0.56								
CA15	0.81	0.84	0.74	0.79	0.89	0.70	0.84	0.64	0.65	0.48	0.60	0.69	0.63	0.57							
CA16	0.81	0.84	0.84	0.79	0.89	0.80	0.74	0.75	0.65	0.55	0.54	0.69	0.50	0.50	0.33						
CC17	0.87	0.89	0.89	0.85	0.90	0.86	0.81	0.81	0.73	0.58	0.77	0.80	0.65	0.54	0.61	0.48					
CC18	0.86	0.88	0.79	0.74	0.93	0.75	0.79	0.75	0.76	0.67	0.75	0.79	0.63	0.73	0.33	0.42	0.55				
CA19	0.79	0.76	0.85	0.89	0.86	0.67	0.81	0.61	0.63	0.69	0.77	0.80	0.75	0.70	0.67	0.67	0.52	0.67			
CA20	0.71	0.67	0.78	0.83	0.85	0.62	0.83	0.62	0.50	0.71	0.69	0.78	0.67	0.67	0.55	0.55	0.52	0.62	0.28		
CA21	0.77	0.74	0.74	0.79	0.89	0.57	0.84	0.57	0.65	0.72	0.75	0.83	0.73	0.73	0.42	0.64	0.67	0.50	0.48	0.29	
CA22	0.77	0.74	0.84	0.79	0.89	0.70	0.79	0.70	0.59	0.77	0.75	0.83	0.63	0.73	0.64	0.50	0.40	0.57	0.40	0.19	0.42

Figure 1: Morphological diversity of nineteen *Capsicum* accessions showing contrasting phenotypes for fruit shape, size colour at early stage and intermediate stage

Figure 2: Longitudinal section of some *Capsicum* species showing placental types: marginal (top) and axile (below) and pericarp thickness

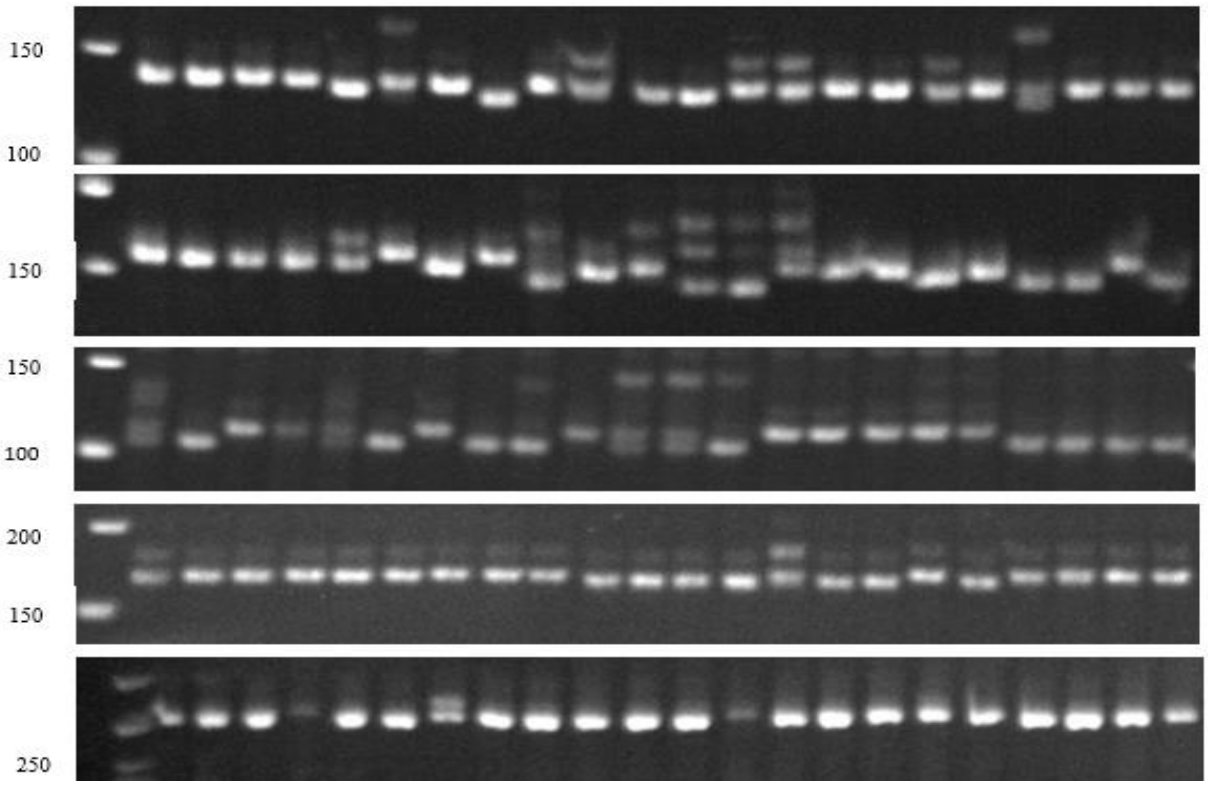

Figure 3: Representative gel images showing allelic variations as revealed by polymorphic SSR loci (CFpSSR80, CFpSSR107, MK487, MK84 and SSR_CF-30) in the 22 *Capsicum* collection (Lanes 22–3), First lane: 50 bp DNA ladder,

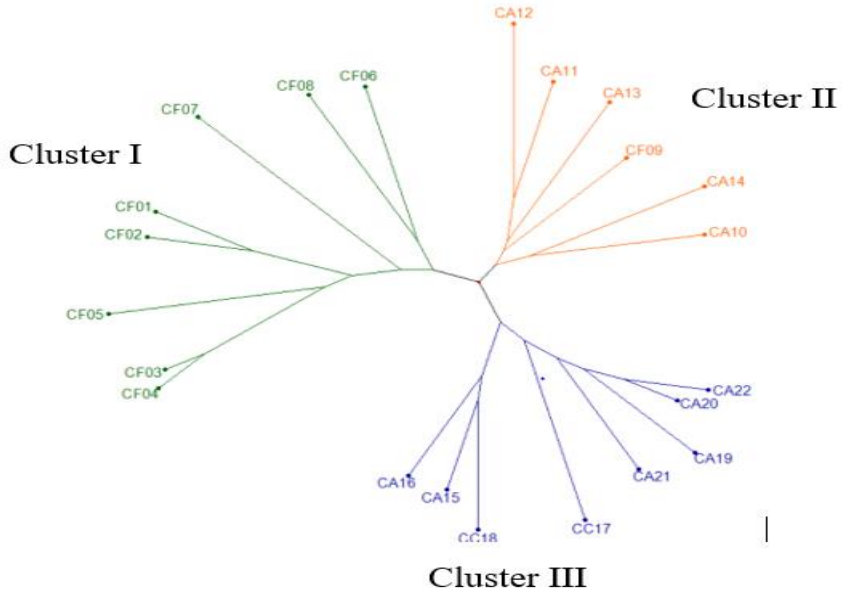

Figure 4: Neighbour-joining (NJ) dendrogram based on genetic distance, among 22 accessions of the three *Capsicum* species. For accession species ID, see Table 1

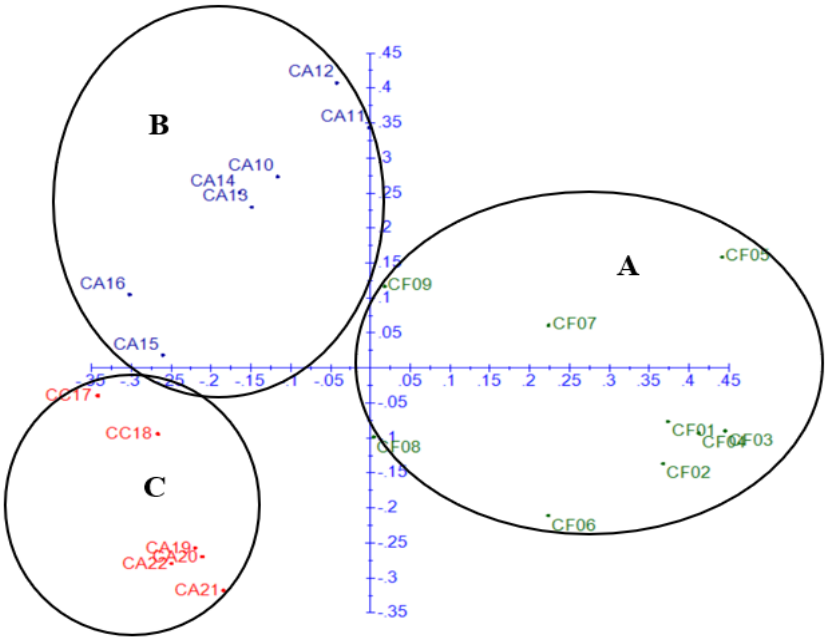

Figure 5: Factorial plot of SSR markers showing three groups in 22 accessions of the three *Capsicum* species. (A, B and C represent three groups)

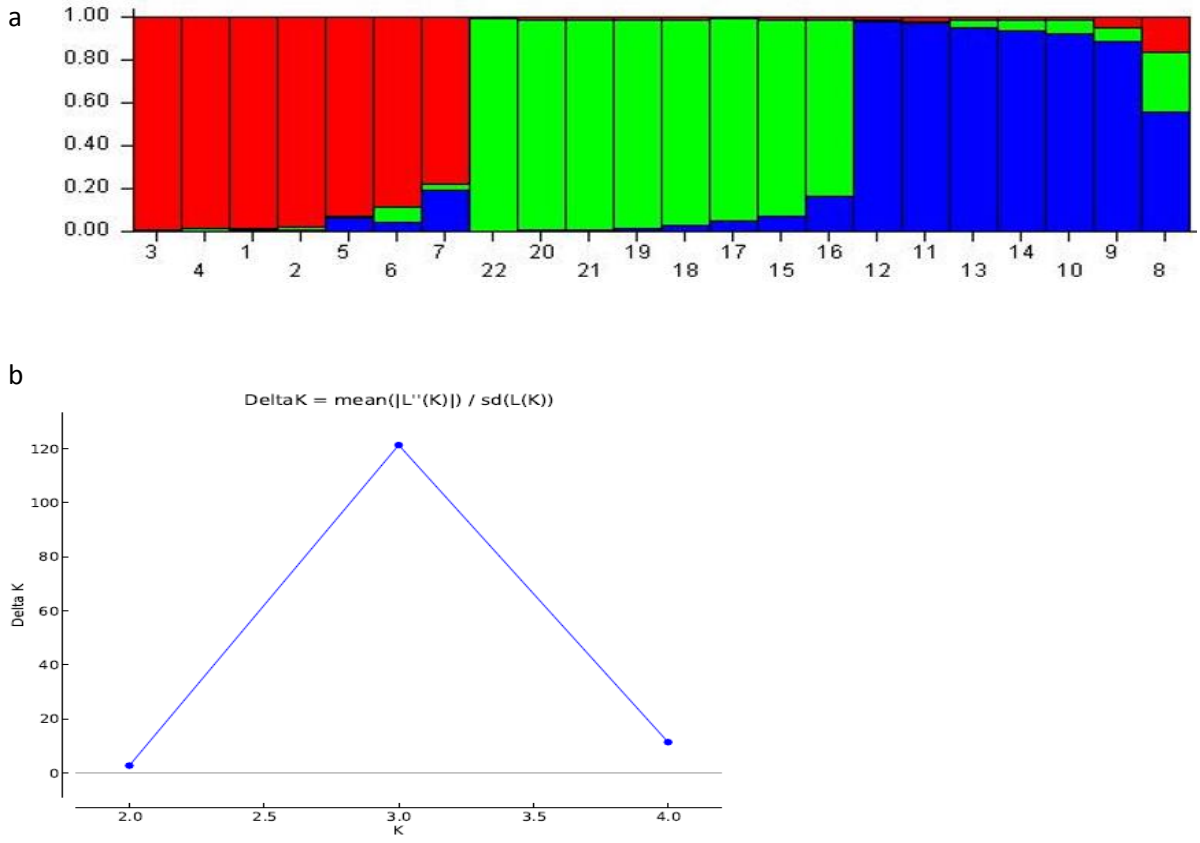

Figure. 6: (a) The population structure obtained with the STRUCTURE software at K=3, 22 accessions of *Capsicum* (1–22 corresponds to Table 1) grouped into three major groups and (b) the estimated value delta K (ΔK) plot based on the allelic data of 13 SSR loci

Discussion

For a crop like *Capsicum*, genetic structure diversity and morphological data have become crucial steps in modern breeding programs, management and conservation strategies. A collection of cultivated *Capsicum* spp. can be used to identify potential genotypes with high diversity (heterozygosity), favorable alleles, and phenotypically desirable traits for successful and long-term pepper introgression breeding (Uncu *et al*., 2018). The next-generation sequencing (NGS) and batch-design primers in the genus *Capsicum* have contributed to the discovery of many SSR markers. In plant genomes, SSR markers are abundant, and they are most often used in genetic diversity analyses (Varshney *et al*., 2005). Additionally, genetic variation can be assessed through morphological traits based on descriptors (Bianchi *et al*., 2016).

This study investigated 22 *Capsicum* species with SSR markers and fruit descriptors. An understanding of the genetic diversity of pepper accessions was conducted using phenotyping, cluster, factorial, and model-based population analyses. Both size and colour at the fruit's immature and mature stages differ significantly between accessions; similar results among Brazilian accessions have been reported (Bianchi *et al*., 2020). Due to variations in the carotenoid composition and content in the pericarp, pepper (*Capsicum* spp.) fruit has a variety of colours, including green, yellow, orange, brown, and red (Borovsky and Paran, 2008). Ripening is generally associated with the degradation of chlorophyll as well as the carotenoid accumulation in mature fruits as they progress from physiological immaturity to maturity (Kim *et al*., 2010). Only two ripening stages are likely to occur in fruits. Comparatively, three ripening stages can be seen in this study's four mature pepper fruit accessions. Red-colour peppers are due to the accumulation of capsanthin and capsorubin (Paran and Van der Knaap, 2007). Ripening has a significant impact on fruit quality and shelf life. Among the accessions in the collection, some have thick fruit walls or pericarps, suggesting significant genetic variations among the accessions. The fruit of the CF09 accession from Niamey, Republic of Niger, had a unique shape, size, and pericarp thickness. A thicker fruit wall is a crucial factor in fruit quality because it may increase resistance to pathogens and parasites during field growth and post-harvest (Rêgo *et al*., 2011). A study on the inheritance of pericarp thickness showed that thin pericarps are dominant and have significant additive variance components (Ben-Chaim and Paran, 2000). In the present investigation, two types of placental dissection were observed in *Capsicum* species. In this study, the accessions have remarkable morphologically diverse traits such as fruit shape, apex shape, and placental type, showing the extent of the phenotypic diversity of the collection. Studies on the *Capsicum* plant have shown that the characterization of variability for fruit-related characteristics is considered necessary for selecting promising accessions for developing cultivars with improved quality-related traits such as color, shape, and health-promoting carotenoids (Pickersgill, 1991; Carvalho *et al*., 2014; Baba *et al*., 2016; Cardoso *et al*., 2018). This study also showed that some accessions' fruit morphology was not maximal, and that some accessions' fruiting occurred later than others or not at all. This shows that *Capsicum* plant species are distributed throughout many geographic areas and appear to have a high potential for adaptation to a wide range of environmental habits and conditions, particularly in Nigeria and other African countries. The morphology of the pepper fruit in the southern part of Nigeria may be influenced by environmental factors in this study, including heavy rainfall, sunlight, temperature, humidity, soil composition, and cultivation methods. As a result of the environmental factors in the south, accessions collected from northern Nigeria, where peppers are widely grown, suffered growth challenges. Furthermore, plant traits may be controlled by a blend of genetic factors and phenotypic plasticity (Klingenberg, 2019). Morphological traits are marginally useful, precise genetic relationship in a group of peppers is assessed using molecular markers for the study of genetic diversity and population structure.

The present study revealed that 13 of the 20 SSRs used in this study were highly polymorphic, as demonstrated by allele richness, gene diversity, and PIC, underscoring the markers' utility. Five out of 20 tested markers showed no amplification, which may be considered non-transferable, while others revealed interspecific length polymorphisms. Five markers used in this study, namely CFpSSR80, CFpSSR34, >MK487, SSR_CF-22*, LOC107845460-up and CFpSSR107, showed a high potential for allelic variation with alleles ranging from five to seven in the collection, which is higher than the number of alleles per locus in Dubey (2019). These discrepant findings may be attributed to different genetic backgrounds used in the investigations. Furthermore, the high number of loci with heterozygous alleles suggests that they could be useful for future breeding programs of *Capsicum* spp. across the collection of *Capsicum* species evaluated, six SSR markers designed from *Capsicum chinense* were cross-species transferable, indicating a high level of flanking sequence conservation within the primer binding regions (Varshney *et al*., 2005). The mean number of alleles (4.15) among accessions is a sign of a considerable level of genetic diversity in the collection and is higher than in other studies (Rai *et al*., 2013; Dutta *et al*., 2023). However, this value is lower in another study with a mean of 6 alleles (Buso *et al*., 2016). The average PIC value in this study is 0.56, which is comparable to an earlier study reported in *Capsicum* (Chhapekar *et al*., 2020) but higher than the value reported in Guzma´n *et al*. (2019). Moreover, earlier research using 9 SSR markers obtained a mean PIC of 0.62 (Adeyemo *et al*., 2017). According to Botstein *et al*. (1980), a molecular marker is highly polymorphic and useful when it has a PIC value greater than 0.5. The PIC measures

the informativeness of a molecular marker for diversity studies. Thus, the efficient markers (13) from the present study would be suitable for future genetic analyses, including QTL association and population genetic studies of *Capsicum*. Additional SSR marker polymorphism measures are gene diversity, and minor allele frequency which show significant genetic diversity among all accessions. Overall, the present study found a moderate genetic variation within *Capsicum* accessions, which could be attributed to low breeding among the cultivated accessions with farmers. Accession CA05 had a high dissimilarity distance to other accessions. This shows that the short pepper variety from the Niger Republic is rare and could be a valuable resource for pepper improvement research. Additionally, the *Capsicum* grown in Nigeria is distinguished from that grown in other African countries like the Niger Republic in this study. Population size, mutation, genetic drift, inbreeding, gene flow, and selection are combinations of factors that affect the level of genetic diversity between and within populations (Leroy *et al*., 2018). The results of the NJ clustering, scatter plot of factorial, and STRUCTURE analyses produced similar results among the 22 accessions, revealing three distinct genetic clusters. In this study, the three analyses revealed that the two yellow pepper (*C. chinense*) varieties, CC17 and CC18, from different parts of the African continent (West and East Africa), clustered together. Furthermore, the bell pepper (*C. annuum*) accessions from Rwanda and Nigeria grouped in the same cluster irrespective of the colours, suggesting genetic relatedness and shared morphology. The genetic distance between C. *chinense* and *C. annuum* was found to be relatively close. The clustering patterns of accessions presented in this study reflect species types and were not necessarily related to the accession origin of the collection. This corroborates the earlier findings of Baba *et al*. (2016) and Moreira *et al*. (2018) studies that investigated the genetic relationships of *Capsicum* accessions based on AFLP marker analysis.

In this study, a cluster included predominantly *C. frutescens* and on the other hand, *C. chinense* accessions clustered with some *C. annuum* accessions, while the third cluster had the rest of the *C. annuum* accessions. This finding corresponds to Lee *et al*. (2016), who reported a moderately close relationship between *C. annuum* and *C. chinense*. *C. chinense* and *C. frutescens*, on the other hand, formed a distinct cluster in another study (Pereira-Dias *et al*., 2019). The present results show that there is a close relationship between *C. annuum* and *C. chinense*. This might be due to their high morphological similarity and close genetic relatedness; *C. annuum, C. chinense*, and *C. frutescens* have traditionally been regarded as "complex". Each of these three species, however, has at least one distinguishing feature that allows identification. Domesticated *C. annuum* var. annuum and its wild ancestor (*C. annuum* var. glabriusculum), for example, show distinct characteristics. Furthermore, considerable admixed accessions were found in the structure analysis. The observed admixtures might result from genomic hybridization events including recombination involving introgression between varieties with different fruit phenotype diversity, during early stages of domestication processes over time, natural recombination, and substantial gene flow (Zhang *et al*., 2016). According to earlier results, Guzma´n *et al*. (2020) and Rai *et al*. (2013) assessed the genetic diversity of eleven *Capsicum* spp. (42 genotypes) and five *Capsicum* spp. (48 genotypes), respectively, and reported admixture patterns like those observed in our present results.

The successful exploitation of germplasm depends on the extensive characterization of the existing population for the exact selection of suitable genotypes (Guerra *et al*., 1999; Khodadabi *et al*., 2011). The current work's thorough fruit characterization of the *Capsicum* collection adds to our understanding of the genus' genetic variability. Furthermore, to improve *Capsicum* cultivars in the face of climate change, breeders need to assess the variability in available germplasm to identify unique alleles for specific traits (Pereira-Dias *et al*., 2019). The study reveals genetic variability among cultivated peppers thereby helping the selection of different traits such as high yield, fruit colour, fruit size and shape, as well as complex traits such as disease and pest resistance. Moreover, the results suggested that these markers might be useful for identifying *Capsicum* germplasm.

Conclusions

We presented the distinctiveness of fruit characteristics in a pepper collection. Also, previously developed SSR markers produced molecular data used for genetic diversity and population structure analyses in the collection. The fruit traits of the *Capsicum* showed high variation in shape and colour. These characteristics can be used in *Capsicum* as markers for choosing desirable parents for hybridization. We also supplied information on 13 SSR markers' effectiveness as tools for identifying genetic variation in germplasm. They would therefore have considerable potential to effectively support several marker-assisted selections to maximize and broaden genetic diversity in the *Capsicum* genus in breeding programmes. There was a moderate amount of genetic variability. These results can support future breeding work for further phenotypic and molecular analyses of a large collection in Africa. However, it has been difficult to improve major native *Capsicum* cultivars. The establishment of breeding programmes in Nigeria is highly critical. Furthermore, given the world's changing

climate, Nigeria's genetic resources for *Capsicum* species should be managed and conserved in germplasm banks. Thus, preserving pepper's variability will enhance adaptable varieties.

Authors' Contributions:

Conceptualization: O.A.A., O.K.A., D.O.O.; methodology: O.A.B.., M.O.A., T.R.O., Field work: P.L.O., S.Y.O., C.D.N. E.O.G; Laboratory work: T.O.P., M.O.A. O.A.A. Statistical analysis, O.A.A., O.A.B., O.K.A., D.O.O; writing original draft preparation: E.O.G., T.O.P., K.T.A.; writing review and editing: O.A.A., C.D.N., D.O.O., M.L.A.; supervision: O.A.A.; project administration: O.K.A., D.O.O.; Photography: O.K.A., M.O.A. All authors read and approved the manuscript.

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