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Assessment of genetic variability of passion fruit using simple sequence repeat (SSR) markers

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ABSTRACT: Purple passion fruit (*Passiflora edulis Sims*) is the third most important fruit crop in Kenya that is produced for both local and export markets. In Uasin Gishu County, passion fruit had recently emerged as an important cash crop for the small-holder farmers. Understanding the structure and diversity of species is very important in plant breeding and in conservation of genetic resources related activities. This study was set out in 2017-2018 to determine the genetic diversity of purple passion fruits genotypes grown in Uasin Gishu County, Kenya using SSR markers. Among the 50 purple passion fruit accessions used in this study, the genetic distance coefficients among accessions ranged from 0.24 to 0.72, with an average of 0.48. The results of STRUCTURE analysis suggested that the 50 accessions could be grouped into five sub-populations. The clustering was based on the unweighted pair-group method of arithmetic averages (UPGMA) where accessions were divided into three major clusters. The UPGMA dendrogram revealed that accessions from identical or adjacent areas were generally, but not entirely, clustered into the same cluster. Comparison of the UPGMA dendrogram and the Bayesian STRUCTURE analysis showed general agreement between the population sub-divisions and the genetic relationships among accessions. Principal coordinate analysis (PCoA) with SSR markers revealed a similar grouping of accessions to the UPGMA dendrogram and STRUCTURE analysis. Analysis of molecular variance (AMOVA) indicated that 16% of the total was attributed to the diversity among sub-populations, while 84% was associated with differences within sub-populations. Overall, there was a considerable amount of genetic variability among passion fruit accessions grown in Uasin Gishu County of Kenya. The study represents the comprehensive investigation of the genetic diversity of passion fruit accessions which would be valuable for germplasm collection, genetic improvement, and efficient utilization.

Keywords: Accessions, genetic diversity, molecular markers, passion fruit, Uasin Gishu County.

INTRODUCTION

The purple passion fruit (*Passiflora edulis* Sims) belongs to the family Passifloraceae that comprises of 520 species distributed in tropical and subtropical regions of America, Asia and Africa (MacDougal and Feuillet, 2004). The passion fruit is an allogamous plant and produces edible fruit species; the purple (*P. edulis*) and the yellow passion fruit (*P. edulis* f. *flavicarpa*) (Martin and Nakasone, 1970) and sweet passion fruit (*Passiflora alata*) are also the most cropped species (Ulmer and MacDougal, 2004). Most *Passiflora* species are diploid, with 2*n* = 12, 18, or 20 chromosomes. The diploid (2*n* = 18) species are the most commercially important due to ease in genetic breeding to obtain interspecies hybrids (Yotoko et al., 2011). Most *Passiflora* species are cultivated as ornamentals, for their edible fruits, or medicinal properties and largely commercial value (Yockteng et al., 2011).

Passion fruit is the third most important commercial fruit crop in Kenya, produced for both local and export markets while the demands for consumption for fresh fruit and processed juice is on the rise (HCD, 2018). Passion fruit

is an important source of alkaloids, flavonoids and carotenoids, minerals and Vitamins A, C, and D that are beneficial to human health (Dhawan et al., 2004). The seeds contain essential fatty acids (55–66% linoleic acid, 18–20% oleic acid, and 10–14% palmitic acid), that can be used in the food and cosmetic industries (Malacrida and Jorge, 2012; da Silva et al., 2015). Passion fruit plants are known to contain compounds with anxiolytic, antihypertensive, sedative, and analgesic properties (Ngan and Conduit, 2011; Konta et al., 2014). In Kenya, the area under passion fruit production increased from 2,157 to 2,296 Ha in the year 2017 to 2018 (HCD, 2018; UNDPO, 2018). In the same year (2017-2018), it was reported that production and value of the crop plunged by 12,499 tons and Kshs 109 million mostly due to diseases such as woodiness virus, fusarium wilt and dieback disease.

In spite of the economic importance and diverse potential uses of passion fruit in Kenya, there has been scanty information on cultivated germplasm diversity. The majority of the passion fruit accessions in germplasm banks and other conservation programs have limited biological, genetic, and molecular characterizations, which partially is responsible for the limited passion fruit breeding programs and could have also resulted in the reduced productivity of existing programs (Cerqueira et al., 2015; Oluoch et al., 2018). Germplasm characterization is vital for understanding the genetic diversity and distinctness for effective conservation, management and efficient utilization in breeding programs. The knowledge of genetic background and variability of a particular plant species is an initial point for future breeding programs (Bertan et al., 2005). The genetic diversity of *Passiflora* species worldwide has been evaluated using morphological descriptors (Plotze et al., 2005; Santos et al., 2011; Ramaiya et al., 2014; do Carmo et al., 2017; Ocampo et al., 2017; Pérez and d'Eeckenbrugge, 2017), agronomic traits (Matheri et al., 2016; Galeano Mendoza et al., 2018) and physicochemical descriptors (da Silva et al., 2015; dos Reis et al., 2018).

Molecular characterization at the species level contributes to the acquisition of knowledge that can be valuable for the conservation and improvement of the diversity of *Passiflora*. The knowledge generated by genetic based characterization offers information for the future breeding efforts and other aspects of biochemical and ecological studies. Several studies have been reported on the genetic diversity of *Passiflora* species with random amplified polymorphic DNA (RAPD) (Vieira et al., 2019), amplified fragments length polymorphism markers (AFLP) (Ortiz et al., 2012), simple sequence repeat markers (SSR) (Oliveira et al., 2005; Pérez and d'Eeckenbrugge, 2017; Araya et al., 2017; Grisi, 2019; Vianna et al., 2019), inter-simple sequence repeats (ISSR) (dos Santos et al., 2011; Vianna et al., 2019) and sequence-related amplified polymorphism (SRAP) markers (Oluoch et al., 2018). Thus, the main objective of this study was to assess the genetic variability and decrypt the population structure of purple passion fruit accessions grown within in Uasin Gishu County, Kenya.

MATERIALS AND METHODS

Plant materials

A total of 50 passion fruit accessions were randomly collected from passion fruit farmers in six sub-counties (Ainabkoi, Soy, Moiben, Kapseret, Kesses and Turbo) with 8 passion fruit samples collected from each sub-county and 2 passion fruit from University of Eldoret (UoE) in Uasin Gishu County, Kenya. The passion fruits were sampled randomly from farmers between March and October 2017.

DNA extraction and PCR amplification

Approximately 0.2 to 0.25 g (15 to 18 seeds) from 50 accessions of passion fruit were used for DNA extraction using Dellaporta et al. (1983) method with minor modification. DNA quantity and quality were determined by 1% (w/v) agarose gel electrophoresis stained with ethidium bromide and compared with standard lambda DNA.

Twenty SSR markers developed for *P. edulis* (Oliveira et al., 2005) were used to assess diversity of passion fruit genotypes (Table 1). The SSRs were selected from a data base of SSRs applied in previous studies based on their high polymorphic content and broad coverage of the passion fruit genome (Oliveira et al., 2005) (Table 1).

The PCR reactions were performed in a Mastercycler (Eppendorf®) using a final volume of 20 µl Bioneer AccuPower® containing 4 µl pre-mix (1U Top DNA, 250 µM each dNTP, 10 mM Tris-HCl pH 9.0, 30 mM KCl, 1.5 mM $MgCl_2$, stabilizer and tracking dye), 0.5 ng/µl of each forward and reverse marker, 0.5 ng of DNA template, and 6 µl of double distilled water. The PCR cycles consisted of an initial denaturation at 95°C for 5 minutes, followed by 35 cycles at 95°C for 30 seconds, annealing temperature specific to the primer for 1 minute, extension at 72°C for 1 minute, and a final extension at 72°C for 5 minutes. The DNA fragments were separated on 2% agarose gel run at 100 volts for 3 hours using 1 M TAE buffer. The DNA fragments in gel were visualized by staining using 0.5 ug/mg ethidium bromide for 30 minutes and rinsed with distilled water for 20 minutes, visualized and photographed using ultraviolet (UV) transilluminator at 312 nm. Allele sizes were scored using a 100 base pair (bp) molecular size ladder.

Genetic diversity analysis

The SSR marker alleles were scored based on the band sizes for all the passion fruit accessions. The PowerMarker software package (Liu and Muse, 2005) was used to

Table 1. Description of the 20 SSR markers used to characterize the 50 passion fruit accessions

Marker	Forward	Reverse	Annealing Temperature (°C)	Range Size (bp)
PE01	CAGGATAGCAGCAGCAATGA	AGCCAAATGTCAAACTGAAC	54	160-200
PE03	GCAGCGAGGGAAGAAAAA	TGAGACATCGTGCGTGAA	60	140-160
PE04	ATGCTTTTGGAAATCCGTTT	TGCTCATGCAAAGTCACTGG	54	200-250
PE07	TGCTCATTGATGGTGCTTG	TCGTCTCTTCTCCTCCTTCA	60	100-150
PE08	TCTAATGAGCGGAGGAAAGC	CCGGATACCCACGCATTA	54	280-300
PE09	GGAAATCCGAAAACTGGTTG	GGGCCTTTATCCATGTTTGA	56	250-300
PE11	GCATAAGTTGTCGGTCTTGG	CCTCGAACCTCTATCATCCA	60	280-320
PE12	CGTAATATTGTTTGGGCACT	ATCATGGGCGAACTCATTT	60	120-150
PE18	CCGTGAACCAACCATTTCTC	CCGTGAACCAACCATTTCTC	60	180-250
PE19	TTAACAGGACTTAGCACTTGA	CTCATCCTTCTTCCATCTTTG	52	220-280
PE23	CAATCCCTTGACCCATAGA	CGTCCATCCTTCTCCTTT	56	150-200
PE24	TCAAACTGAACTCGTAAAGG	GTGCTGGGAGACTGATGTT	56	230-250
PE37	CAAAAGGATAGGCCTGATGTC	TGCTTGGTCATCCACTGAAG	60	240-260
PE38	GATCGGTCCTCGGTTAGAC	AGTCACACAGCATGAGAAATC	56	220-280
PE42	GTCACTTCATTCTTCCTTTCC	TTAGCCCACTCAAACACAA	56	280-300
PE59	GAACACTTCGCATGGCTAGA	TTCCGAATCAAACCGTAACT	56	240-280
PE66	CCATAGTCCCAACAAGCATC	GCTGTGGACCCTAACTCAGTC	60	240-260
PE75	CACAATCGGTGGGAAAGATA	GTAGTTTTGGGCAGTTTGC	60	140-150
PE88	CTTCAGGGTCACACACATT	GTTCATCCTTTAGTGGGCT	60	260-290
PE90	TCAGGAAGATTGCATGTTAGT	CTGGGTTTTGTTTATGTTGC	60	200-250

calculate the following summary statistics; percentage of polymorphic loci, mean number of alleles per polymorphic locus, observed and expected heterozygosities (HO and HE, respectively), and polymorphic information content (PIC). Analysis of molecular variance (AMOVA) was used to calculate the genetic variance within and among populations using the ARLEQUIN 3.01 software (Excoffier et al., 1992).

The genetic structure analysis was done based on Bayesian model (Hubisz et al., 2009) as implemented in the STRUCTURE program version 2.3.4. The number runs for K values ranging from 1 to 10 were executed with a burn-in length of 100,000 tailed by 1,000,000 Monte Carlo Markov Chain (MCMC) interactions using admixture model. The number of subpopulations was determined using the Delta *K* (Δ*K*) *ad hoc* method proposed by (Evanno et al., 2005) and implemented in the online tool Structure Harvester (Earl and VonHoldt, 2012) to estimate the most likely *K* in each set of passion fruit accessions.

Phylogenetic trees were produced using genotyping data with 20 SSR markers using the hierarchical clustering method based on the dissimilarity matrix calculated with Manhattan index, as implemented in the DARwin software (version 6.0.9). Principal coordinate analyses were also performed using DARwin 6.0.9 software (Perrier and Jacquemoud-Collet, 2006).

RESULTS

The 20 SSRs markers showed that the 50 passion fruit accessions studied generated a total number of 207 alleles with a range of 6 to 17 alleles per marker with an average of 10 alleles. All the 20 SSR markers gave polymorphic bands, with marker PE 38 (Table 2) giving the highest allele number and heterozygosity of 17 and 0.89 respectively. Marker PE 37 gave the highest allele frequency of 0.89 while PE 90 marker gave the highest polymorphic information content of 0.89. The average values of allele frequency and heterozygosity were 0.54 and 0.50, respectively (Table 2).

Genetic diversity analysis was executed to explore the genetic variations among and within groups of passion fruit accessions (Table 3). AMOVA revealed that the diversity among populations and within individuals of passion fruit accessions were 16 and 84%, respectively (Table 3).

The dendrogram based on UPGMA cluster analysis constructed from the 20 SSR markers in DARwin software package among the passion fruit accessions were grouped into four sub-groups (Figure 1) as revealed by population structure in Figures 3 and 4.

The Bayesian clustering method with admixed model indicated that the 50 passion fruit accessions were clustered into five genetic groups (*K* = 5) (Figure 3 and 4). Figure 3 and 4 shows the estimated population structure based on Delta *K* (*ΔK*) when it reaches its maximum value following the *ad-hoc* method and subpopulation clusters (K) that are represented by different colors, respectively.

A further molecular analysis using SSR data in Figure 5 displays the scatter plot that splits the passion fruit accession into defined clusters by the first two coordinates.

Table 2. Summary of statistical analysis of genetic diversity of the 50 passion fruit accessions based on 20 SSR markers used in this study.

Marker	Allele Frequency	Allele Number	Gene Diversity	Heterozygosity	PIC
PE01	0.86	12	0.26	0.72	0.55
PE03	0.49	9	0.63	0.13	0.56
PE04	0.48	7	0.67	0.51	0.61
PE07	0.42	9	0.48	0.76	0.64
PE08	0.45	10	0.66	0.52	0.54
PE09	0.69	12	0.06	0.55	0.56
PE11	0.22	8	0.06	0.45	0.67
PE12	0.39	13	0.01	0.60	0.81
PE18	0.45	11	0.47	0.34	0.57
PE19	0.58	6	0.50	0.23	0.85
PE23	0.42	9	0.68	0.56	0.61
PE24	0.73	10	0.42	0.61	0.48
PE37	0.89	8	0.08	0.40	0.60
PE38	0.24	17	0.76	0.89	0.73
PE42	0.34	14	0.87	0.68	0.79
PE59	0.63	11	0.48	0.67	0.74
PE66	0.53	10	0.52	0.20	0.66
PE75	0.50	16	0.68	0.46	0.65
PE88	0.82	6	0.06	0.24	0.58
PE90	0.67	9	0.09	0.50	0.89
Mean	0.54	10	0.42	0.50	0.65

Table 3. Analysis of molecular variance (AMOVA).

Sources of variations	Degrees of freedom	Sum of squares	Variance components	Percentage (%) of variation
Among Populations	5	877.8	102	16
Within Individuals	45	6945.1	985	84
Total	49	256832.8	1184	100

The principal co-ordinate analysis resembled more strongly the dendrogram obtained with SSR data analysis (Figure 2). The first and second principal components comprised 48.6 and 16.2% respectively that accounted for 54.8% of the total variation (Figure 5).

DISCUSSION

The SSRs studies showed that there was significant genetic variability among the 50 passion fruit accessions studied generating a total number of 207 alleles with a range of 6 to 17 alleles per marker. This could be owed to the fact that microsatellites are often valuable for merely pointing out differences in directly related germplasm sources (Peakall et al., 1998). Furthermore, differences in laboratory procedures might have led to the diverse results reported by diverse studies mainly associated with the origin and sources of passion fruit genotypes studied (Di et al., 2006), as well as the marker type applied and the appropriate platform for resolution of amplified products (Tyagi et al., 2014). Several studies in passion fruit have previously reported similar results (Ortiz et al., 2012; Cerqueira-Silva et al., 2015; Silva et al., 2016; Vianna et al., 2019; Grisi et al., 2019).

The polymorphic information content (PIC = 0.65 average) of most loci permit that the evaluated markers are highly informative (higher than 0.50) according to Botstein et al. (1980) criteria. The observed high PIC in the present study could be due to the fact that passion fruit accessions were selected from individual farmers who might have obtained seed or seedlings from diverse sources. Generally, passion fruit farmers raise their own seedlings, either from seeds collected in their neighborhoods or from fresh fruits purchased from the market (Ocampo et al., 2017). Higher PIC has also been associated with the distribution and equilibrium of the population´s allelic frequencies (Missio et al., 2010), that allows to affirm that

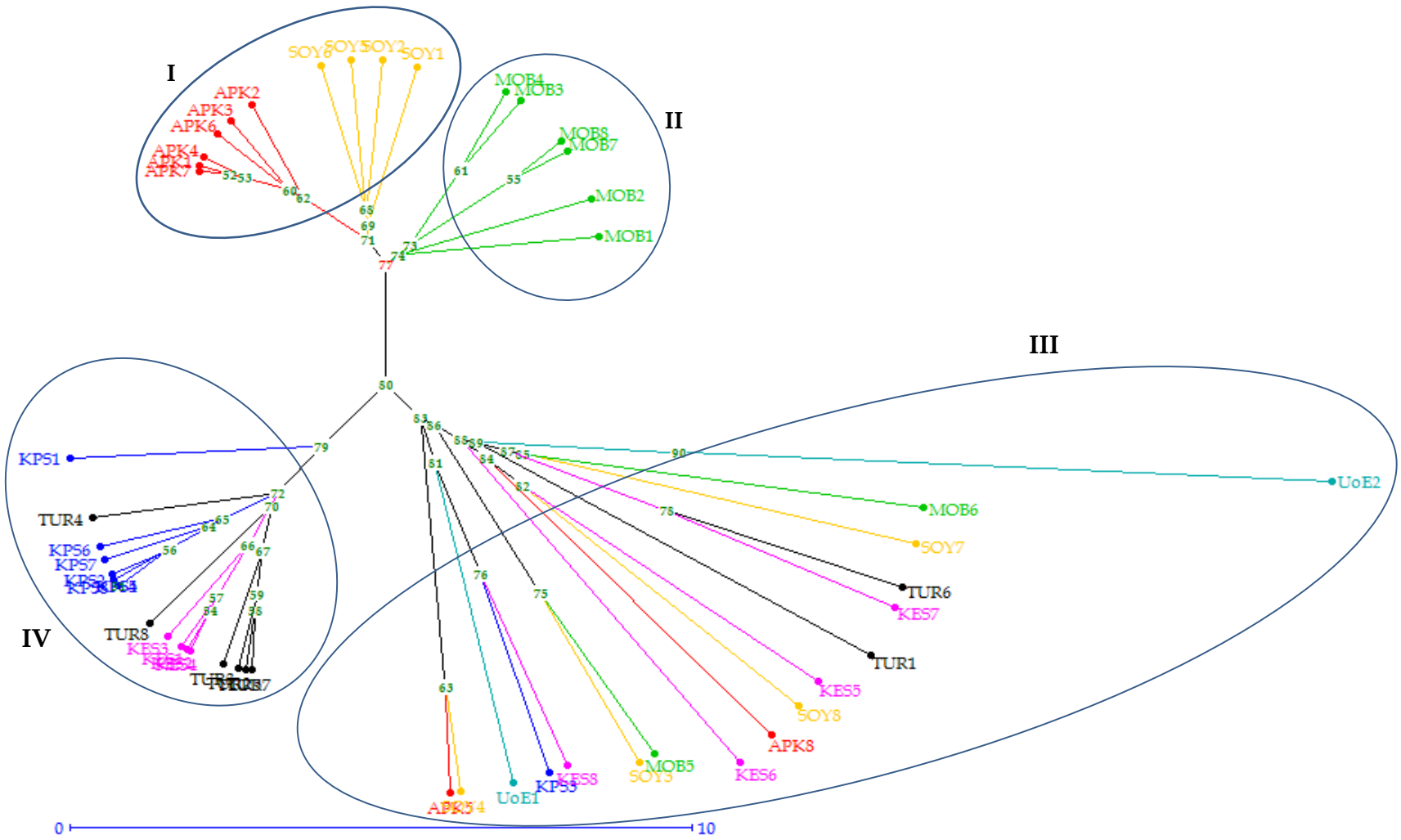

Figure 1. Unrooted tree using Unweighted pair-group method of arithmetic averages (UPGMA) illustrating the genetic relationships among the 50 passion fruit accessions based on the 20 SSR markers**.**

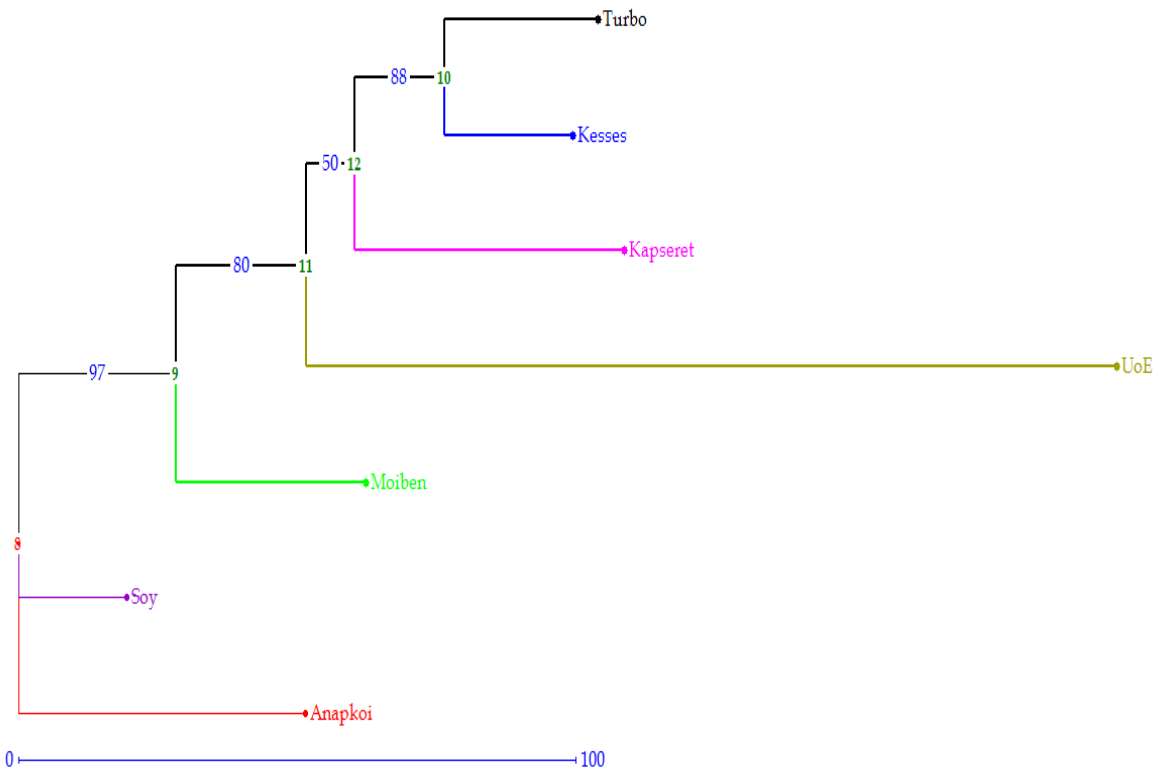

Figure 2. Unrooted tree using unweighted pair-group method of arithmetic averages (UPGMA) illustrating the genetic relationship among locations of passion fruit accessions based on the 20 SSR markers.

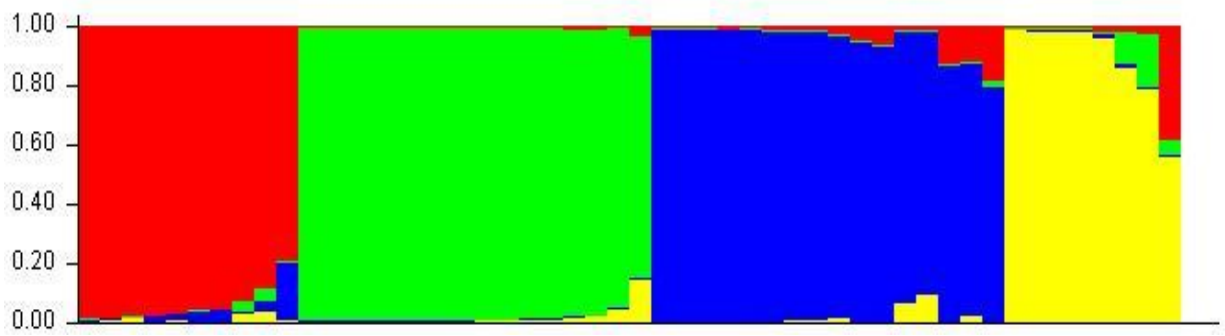

Figure 3. Population structure of the 50 passion fruit accessions obtained with the structure program based on 20 SSR markers for K = 4. Each colour represents a subpopulation and the length of the coloured segment shows the estimated membership proportion of each sample to designed group.

the selected markers are consistent and effective to detect genetic variability in passion fruit genotypes (Pérez and d'Eeckenbrugge, 2017). Similar to the results of the study, high PIC values regarding passion fruits have been reported by other authors such as Pérez and d'Eeckenbrugge (2017) with an average value of 0.744 and Grisi et al. (2019) with a mean value of 0.59.

Analysis of Molecular Variance (AMOVA) revealed high proportion (84%) of genetic diversity within populations than among subpopulation, showing the existence of low genetic differentiation among subpopulations. Similar results have been previously reported on Kenyan passion fruits using SSR markers with the genetic variance among being 57% while 43% within populations (Matheri et al., 2016). In another study, Oluoch et al. (2018) revealed that 52% of the variation was owed to be among population variance, while 48% was due to within-population variance using SRAP markers. The high genetic diversity within populations could be due to collecting germplasm from adjacent regions and close kinship in the passion fruit accessions.

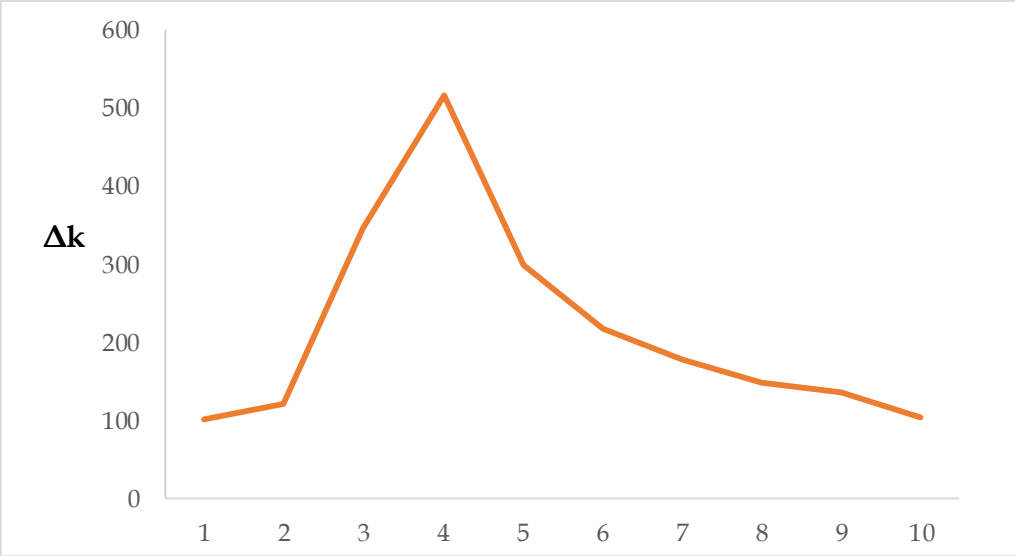

Figure 4. Structure estimation of the number of subgroups for the K values ranging from 1 to 10, by delta K (ΔK) = 4 values of the 50 passion fruit accessions.

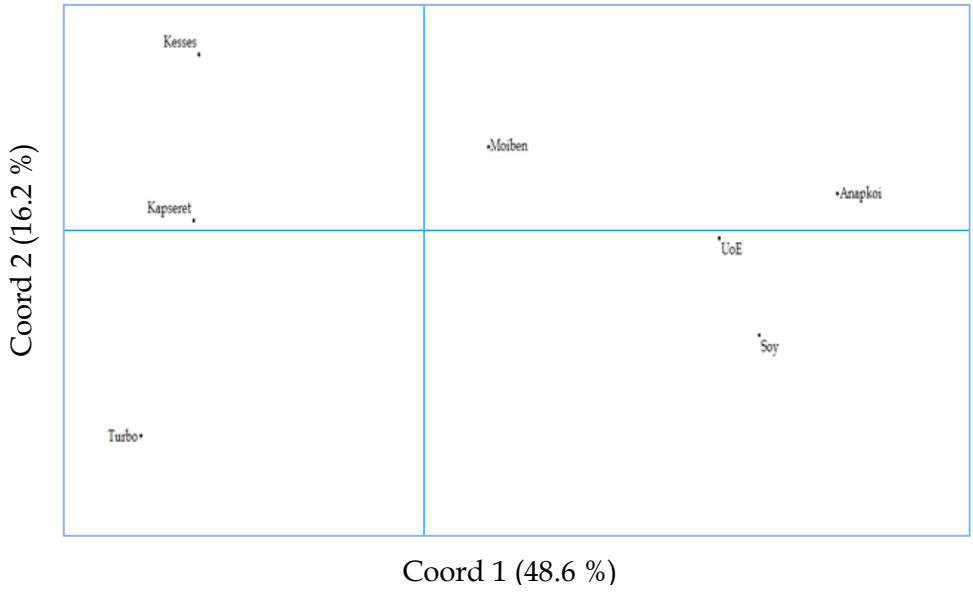

Figure 5. A scatter plot of passion fruit collecting locations based on first and second components of principal coordinate analysis using data from 20 SSR markers.

The dendrogram generated from the molecular analysis revealed four cluster groups of passion fruit accessions with group III and IV generating the largest and diverse accessions compared to group I and II. The clustering of accessions in different groups is probably considered as a result of exchange of planting material between the areas and/or due to hybridization. In most instances, these clusters, revealed the majority of accessions that were geographically close were generally clustered into the same cluster except some accessions. Passion fruit accessions collected from Anapkoi (group II) were found in the same cluster while accessions collected from Moiben and Soy (group I) were found in the same cluster. Some passion fruit accessions collected from all the regions in Uasin Gishu County were found in cluster III forming a separate group. The genetic dissimilarity and similarity observed between and within the various clusters of passion fruits could possibly be due to collecting germplasm from adjacent regions with close kinship or resemblance and differences in ancestry. Genetic variability

within a population of a species is affected by a number of factors comprising the seed dispersal, gene flow, natural selection, geographic range, and the center of diversity (Hamrick and Godt, 1989). Marker selection is critical to the accuracy of passion fruit cultivar identification since it affects the results depending on marker(s) combinations employed. Liao and Guo (2014) reported that a cultivar can be in a cluster with a few cultivars in a dendrogram, and perhaps cluster with other cultivars in another dendrogram based on diverse primer combinations. The Mantel test also displayed slight correlation between genetic and geographic distances which probably could be due to outcrossing, or artificial transfer of accessions from one region to another.

The cluster analysis was executed to deduce the genetic structure, estimate the lineage and presence of possible populations of the sampled individuals. The microsatellites studied using the Bayesian clustering approach performed by the program STRUCTURE generated four clusters $\Delta k = 4$ from the 50 passion fruits accessions with considerable admixture among purple passion fruits accessions of different geographic regions and probably mixed ancestry from parents belonging to different gene pools. The 50 passion fruit accessions were less defined according to its geographic regions possibly due to the self-incompatibility of passion fruit and its outcrossing pollination. Similar results were also reported by Pérez and d'Eeckenbrugge (2017) studying 51 yellow passion fruit accessions of different geographic origins. Admixture is considered as a consequence of exchange of plant material between the areas and/or hybridization.

The results of the three analyses performed (UPGMA cluster, Bayesian model-based method, and PCoA) agreed with the existence of clusters or subpopulations. In spite of minor differences, the results were largely consistent. The results of the analyses show that the accessions analyzed are reasonably genetically heterogeneous and indicate that there is small correspondence with geographic distribution of accessions. Similarly, other studies have reported reasonably genetically heterogeneous for cultivated accessions of *P. edulis* f. *flavicarpa* (Pérez and d'Eeckenbrugge, 2017), *P. ligularis* (Bernal-Parra et al., 2014) and *Carica papaya* (Matos et al., 2013). In contrast, Ortiz et al. (2012) reported a high genetic homogeneity in cultivated material of purple passion fruit (*P. edulis* f. *edulis*) with microsatellite markers and AFLP in Colombia. Nonetheless, inadequate selection plan may not have been developed in Uasin Gishu County, Kenya for the purple passion fruit as farmers inconsistently select the finest fruits in each harvest, without considering cross pollination (Ocampo et al., 2017). Passion fruit growers normally performs phenotypic or mass selection when establishing or renewing new orchards in function of their surveillances, or of a phenotype enforced by local or international markets (Ocampo et al., 2017). Purple passion fruit producers generate their own seedlings, either from seeds collected in their neighborhood or from fresh fruits purchased at the market (Ocampo et al., 2017) where they graft the yellow passion fruit (root stock) and purple passion fruit (scion) (pers. obs.). Generally, the genus *Passiflora* have a very extensive genetic variability both within the genus and within the most cultivated species (Ocampo et al., 2017).

Conclusion

The findings of this study demonstrate the presence of considerable amount of genetic variability among passion fruit accessions grown in Uasin Gishu County, Kenya. The study represents the most comprehensive investigation of the genetic diversity and population structure of passion fruit accessions in Uasin Gishu County. This could offer an opportunity for laying foundation for further research, and provides valuable information for germplasm collection, genetic improvement, and efficient utilization.

CONFLICT OF INTERESTS

The authors declare that they have no conflict of interest.

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