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Simple Sequence Repeat Marker Analysis of Genetic Diversity among Progeny of a Biparental Mapping Population of Sweetpotato

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***Abstract*. Genetic diversity is critical in sweetpotato improvement as it is the source of genes for desired genetic gains. Knowledge of the level of genetic diversity in a segregating family contributes to our understanding of the genetic diversity present in crosses and helps breeders to make selections for population improvement and cultivar release. Simple sequence repeat (SSR) markers have become widely used markers for diversity and linkage analysis in plants. In this study, we screened 405 sweetpotato SSR markers for polymorphism on the parents and progeny of a biparental cross of New Kawogo × Beauregard cultivars. Thereafter, we used the informative markers to analyze the diversity in this population. A total of 250 markers were polymorphic on the parents and selected progeny; of these, 133 were informative and used for diversity analysis. The polymorphic information content (PIC) values of the 133 markers ranged from 0.1 to 0.9 with an average of 0.7, an indication of high level of informativeness. The pairwise genetic distances among the progeny and parents ranged from 0.2 to 0.9, and they were grouped into five main clusters. The 133 SSR primers were informative and are recommended for use in sweetpotato diversity and linkage analysis.**

Sweetpotato [*Ipomoea batatas* (L.) Lam] is a vegetatively propagated, highly heterozygous and outcrossing dicotyledonous crop (Woolfe, 1992). The outcrossing nature of sweetpotato has led to the development of several landraces due to chance seedlings and the propagation of spontaneous mutations in secondary centers of diversity such as East Africa (Villordon et al., 2006; Yada et al., 2010a). Over 80% of global sweetpotato production occurs in the developing world, particularly in Africa and Asia (FAOSTAT, 2012). The importance of sweetpotato as a food, feed, nutrition, and income-security crop is widely recognized (FAOSTAT, 2012). Its ability to grow under marginal conditions and its high nutritional value (orange-fleshed types) appeals to resource-poor sweetpotato farmers (Karyeija et al., 1998).

Sweetpotato breeding has relied on the ability of breeders to identify parental genotypes with desirable traits and combine these traits through hybridization schemes such as the polycross and controlled cross nurseries (Grüneberg et al., 2009). It has been hypothesized that, crosses of genetically diverse parents result in high levels of heterosis in sweetpotato and this approach can be employed to enhance genetic gains. Studies to evaluate this hypothesis are ongoing at the International Potato Center (CIP), Lima, Peru (Grüneberg et al., 2009). However, such anticipated genetic gains could be limited by the high level of self- and cross-incompatibilities in some of the diverse parental genotypes (Gurmu et al., 2013; Martin, 1965).

The application of molecular approaches will be critical for exploiting heterosis for sweetpotato improvement; genetic markers will be required for analysis of diversity among parental genotypes and selection of diverse parents for use in population improvement basing on their genotypic and phenotypic profiles.

Because of self-incompatibility and severe inbreeding depression in sweetpotato, heterotic groups are currently based on the long-term geographic adaptations of breeding lines such as the African, Asian, and South and North American heterotic groups (Grüneberg et al., 2009). Molecular markers will enable identification of potential heterotic gene pools within populations of breeding programs.

Molecular markers for use in analysis of heterotic groups in polyploid crops such as sweetpotato should have the ability to detect multiple, codominantly inherited alleles, have uniform genomic distribution, be easily generated and scored, and have the ability to easily differentiate closely related genotypes such as an F_1 progeny (Buteler et al., 2002). Simple sequence repeat markers are currently the most suitable markers for paternity analysis and identification of heterotic gene pools in sweetpotato as they occur throughout the genome and are codominantly inherited and relatively easy to score (Jarret and Bowen, 1994). Sweetpotato SSR markers have been used for paternity (Buteler et al., 2002) and diversity analyses (Karuri et al., 2010; Koussao et al., 2014; Veasey et al., 2008; Yada et al., 2010b; Zhang et al., 2000).

Presently, one major obstacle to the use of SSR markers for sweetpotato improvement is the limited development of core sweetpotato genomic tools. Currently, there are a total of 23,406 expressed sequence tag (EST) sequences of *I. batatas* deposited at the National Center for Biotechnology Information (NCBI, 2014) database. Sweetpotato genomics is in the early stages with slightly over 600 published EST-based SSR markers (Buteler et al., 1999; Hu et al., 2004b; Schafleitner et al., 2010; Wang et al., 2011). Most of these markers have not been used for genetic studies and so have limited information on their use for sweetpotato improvement. The lack of a reference genome sequence for sweetpotato or its closest progenitor has also made it difficult to mine sweetpotato single-nucleotide polymorphism (SNP) markers for use in sweetpotato genomic improvement.

In our quest to identify SSR markers for genetic improvement of key traits in sweetpotato, we developed a trait-mapping population from a biparental cross of diverse cultivars, New Kawogo and Beauregard. We expected that this population had the

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potential to exhibit high levels of genetic diversity and heterosis for storage root yield, dry matter, starch and β-carotene content as the parents were from diverse gene pools. The objectives of the study were to assess the level of polymorphism of the SSR markers and assess the genetic diversity among the progeny and parents of this putatively diverse cross so as to identify useful SSR markers for future diversity and linkage studies in sweetpotato.

Materials and Methods

Plant material. The population used in this study consisted of 287 F_1 progeny from a biparental cross between 'New Kawogo' (NK) and 'Beauregard' (B). The cross was made in 2010 at the National Crops Resources Research Institute (NaCRRI), Namulonge in Uganda. 'New Kawogo' (female) is a sweetpotato weevil and sweetpotato virus disease (SPVD) resistant, high dry matter content, and white-fleshed released Ugandan landrace cultivar (Mwanga et al., 2001; Stevenson et al., 2009). 'Beauregard' (male) is a weevil and SPVD susceptible, low dry matter content, and orange-fleshed (high β-carotene content) popular U.S. cultivar (Rolston et al., 1987).

Genomic DNA extraction. Genomic DNA was extracted from young leaves of each progeny at the NaCRRI Biosciences Laboratory using a modified C-TAB method (Doyle and Doyle, 1990). A piece of leaf (ca. 100 g) of each progeny was put in labeled 2-mL microfuge plastic tubes. A total of 800 μL of 2× CTAB buffer (2 mL mercapto ethanol:700 mL CTAB 2×) was added to submerge the leaf samples. An autoclaved porcelain bead was put inside each microfuge tube. The samples were crushed using the FastPrep-24 instrument (FastPrep system, Solon, OH). After incubation at 45 °C for 20 min, 800 μL of chloroform:isoamyl alcohol (24:1) was added to the homogenates. The tubes were inverted several times and then spun at 14,000 rpm for 10 min in a micro centrifuge. The aqueous phases were transferred to new sterile tubes, 50 μL of 10× CTAB was added to samples and vortexed gently. Thereafter, 800 μL of chloroform:isoamyl alcohol (24:1) was added to each sample, and spun at 14,000 rpm for 5 min. The aqueous phases were transferred in to new sterile tubes to which, 600 μL of isopropanol was added and stored over night at –20 °C.

The samples were then spun down at 14,000 rpm for 20 min. The pellets were recovered and washed in 500 μL of 70% ethanol and then spun at 14,000 rpm for 15 min. The supernatant was discarded. The pellets were again washed in 500 μL of 95% ethanol and centrifuged at 14,000 rpm for 15 min. The supernatant was discarded. The pellets were then dried using a Speed Vac Concentrator (Thermo Fisher Scientific, Waltham, MA) for 10 min. The dried pellets were finally suspended in 100 μL of TE buffer.

SSR genotyping. The DNA concentrations of the genomic DNA samples from NaCRRI were measured at North Carolina State University, Raleigh, NC, using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE).

A total of 405 published EST-SSR markers (Buteler et al., 1999; Hu et al., 2004b; Schafleitner et al., 2010; Wang et al., 2011) were used in this study. The primers were redesigned by addition of an M13 tail universal primer sequence (TGTAAAACGACGGCCAGT) to the 5′ end of the forward primer sequence and synthesized by Eurofins Genomics (Huntsville, AL). The complementary M13 sequences were fluorescently labeled with VIC (green), 6FAM (blue), NED (yellow), and PET (red) tags from Applied Biosystems (Foster City, CA) for automated detection of the polymerase chain reaction (PCR)-amplified products.

The PCR for DNA amplification was performed in a 10 μL reaction volume. The reaction consisted of 3.0 μL (20–40 ng/μL) DNA template, 1.0 μL 10× PCR buffer, 1.0 μL of 15 mM $MgCl_2$, 0.8 μL of 10 mM DNTPs mix, 0.2 μL forward primer (1.0 μM), 1.0 μL reverse primer (1.0 μM), 0.5 μL M13 primer (1.0 μM), 0.1 μL Taq polymerase (50 U/μL) and 2.4 μL PCR water. The PCR conditions were as follows: one hold at 94.0 °C for 4 min, followed by 15 cycles of 94.0 °C denaturation for 30 s, 55.0 °C annealing for 30 s, and 72.0 °C extension for 1 min, plus 25 cycles of 94.0 °C for 30 s, 50.0 °C annealing for 30 s, and 72.0 °C extension for 1 min, ending with two holds at 72.0 °C for 7 min, and at 4.0 °C for infinite time.

The PCR amplifications were performed using an Eppendorf Mastercycler (Eppendorf AG, Hamburg, Germany). We amplified each

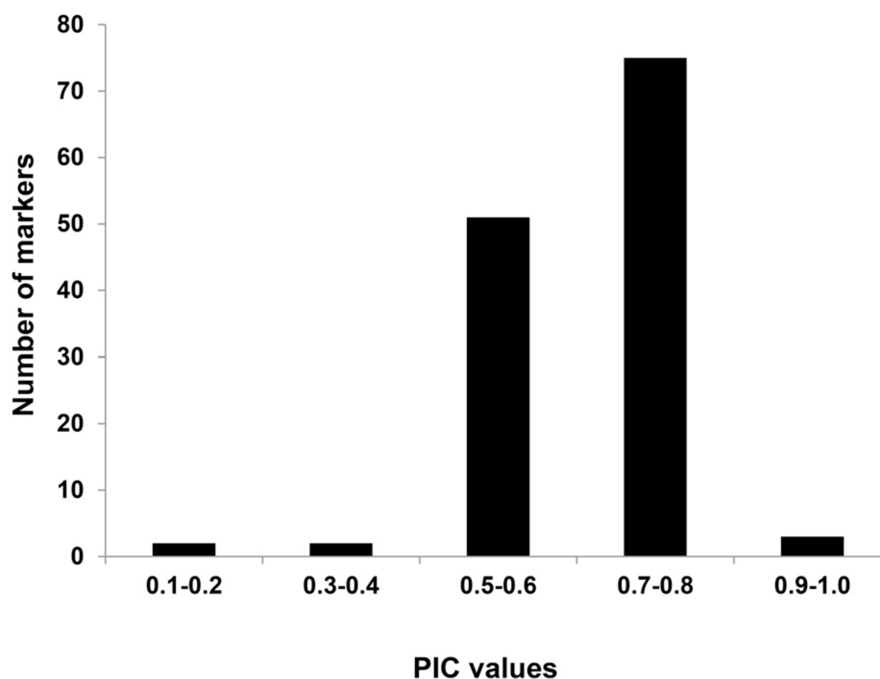

Fig. 1. Distribution of polymorphic information content (PIC) values of 133 sweetpotato simple sequence repeat (SSR) markers.

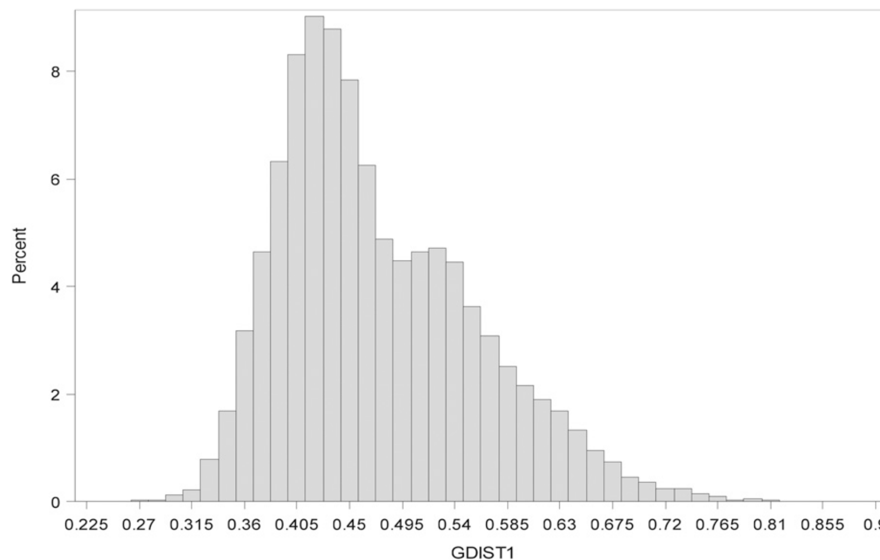

Fig. 2. Pairwise genetic distance distribution (GDIST1) among the progeny and parents of the 'New Kawogo' (NK) × 'Beauregard' (B) sweetpotato mapping population.

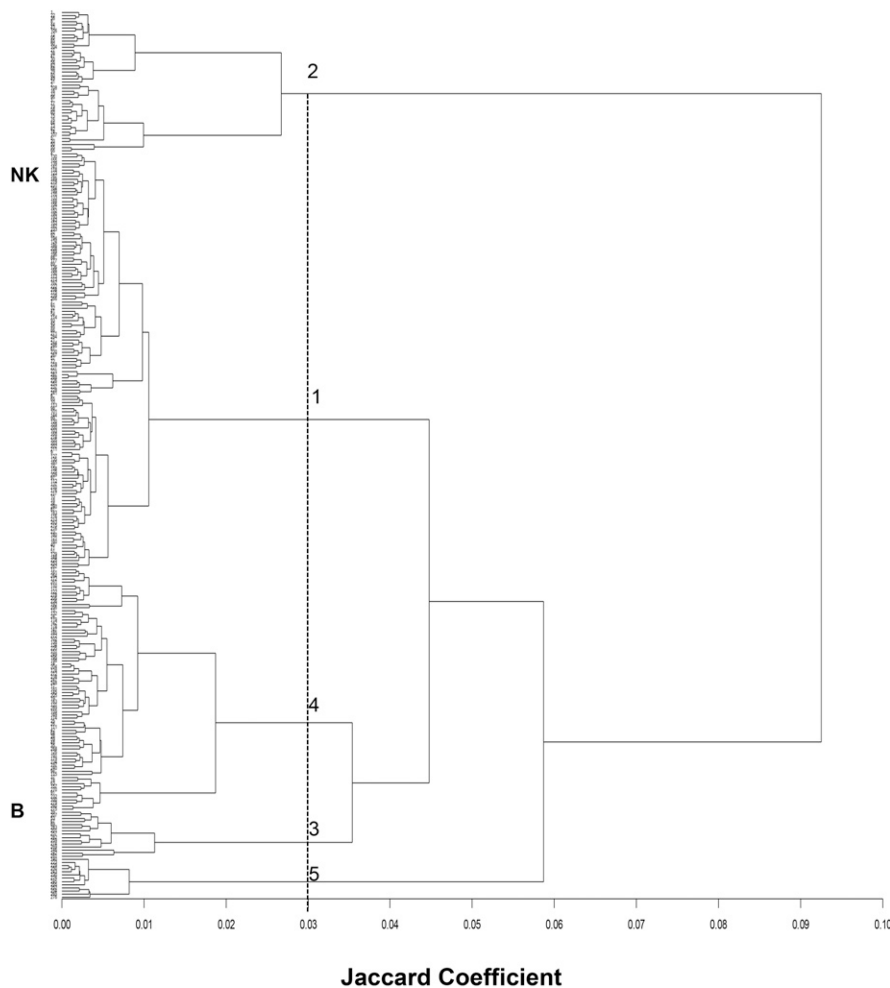

Fig. 3. Dendrogram of genetic relationships among the progeny and parents of the ‘New Kawogo’ (NK) × ‘Beauregard’ (B) sweetpotato mapping population, numbers 1, 2, 3, 4, and 5 represent the clusters.

of the DNA samples using each SSR marker independently, but pooled PCR products of 2–3 reactions for genotyping by capillary electrophoresis using an automated ABI3730 Sequencer (Applied Biosystems). The allele data were then analyzed using GeneMarker 2.2.0 (SoftGenetics, State College, PA). The allele scores were converted to binary data, i.e., 1 (allele present) and 0 (allele absent).

Data analysis. The SSR marker allele frequencies were computed as the sum of a particular allele at a given locus divided by the number of individuals in the mapping population. These frequencies were used to determine the level of informativeness of each SSR marker using the PIC estimates (Weir, 1996) as

$$PIC_l = 1 - \sum_{i=1}^{n} P_i^2$$

where p_i is the frequency of the ith allele among a total of lth alleles within a locus.

Pairwise Jaccard genetic distances among the progeny and parents were calculated to generate a triangular matrix. This matrix was subjected to cluster analysis and multidimensional scaling for graphical display of genetic relationships. The dendrogram was constructed using the unweighted pair group method of arithmetic averages (UPGMA) algorithm. All the analyses were conducted using the Numerical Taxonomy and Multivariate Analysis Systems (NTSYS-pc) version 2.2 (Rohlf, 2005) and SAS 9.4 (SAS Institute Inc., 2013).

The storage root yield of progeny and parents was evaluated in Uganda for two seasons at three sites: NaCRRI, Ngetta Zonal Agricultural Research and Development Institute (NgeZARDI), and National Semi-Arid Resources Research Institute (NaSARRI) in 2012 and 2013. The first season’s trials were planted in June 2012 and harvested in Nov. 2012 while the second season’s trials were planted in Nov. 2012 and harvested in May 2013. Storage root yield ($t \cdot ha^{-1}$) of the progeny (F_1) and high parent (Hp) heterosis were determined. High parent heterosis indicates that the hybrid progeny performs significantly better than the high parent for the trait. The high parent is the higher yielding of the two parents. High parent heterosis (HpH) for storage root yield was calculated as $HpH = (F_1 - Hp)/Hp$ (Hochholdinger and Hoecker, 2007), and was used to analyze the relationship between high parent heterosis and genetic diversity in the biparental cross.

Results

Upon screening of the 405 SSR markers for polymorphism on the parents and 10 randomly selected progeny, a total of 250 markers were polymorphic, while 113 were monomorphic. The remaining markers did not amplify the test DNA samples even after several adjustments of the PCR conditions and concentrations of the PCR reactants. The DNA of the 287 progeny and the parents were amplified using the 250 polymorphic markers. However, out of the 250 markers used, 76 markers gave ambiguous and difficult to score electropherogram peaks with allele profiles differing by one base pair. As a result, they were not included in the final analysis as they could have led to erroneous results.

Of the remaining 174 potentially useful SSR markers, 41 markers had very few alleles, with a total number of fragments of less than 10 per marker for all the genotypes. The markers characterized to have high levels of rare alleles (ca. less than 10 fragments per marker) were excluded as those were not to account significantly for variability in this population. For the final diversity analysis and subsequent analyses, we selected 133 SSR markers based on their polymorphic information content and allele composition.

The PIC values for the 133 markers used in the analysis ranged from 0.1 to 0.9 (Fig. 1) with an average PIC value of 0.7. Primers IbE34 and IBS107 had the lowest and highest PIC values, respectively. The majority of the SSR markers had PIC values >0.5 and so were informative. The number of alleles per marker for the primers ranged from 2 to 6. Of the primers used, 25 had two alleles per marker, 41 had three alleles per marker, 30 had four alleles per marker, 20 had five alleles per marker, and 17 had six alleles per marker.

The pairwise genetic distances among the progeny and parents ranged from 0.22 to 0.90 with an average of 0.43 (Fig. 2). The pairwise genetic distance distribution was slightly skewed toward the left though two main groups were observed. Since this was a biparental cross, a low range of pairwise genetic distances would typically be expected.

The progeny and parents were grouped into five major clusters with the number of genotypes per cluster ranging from 13 (cluster 3) to 132 (cluster 1) (Fig. 3). ‘New Kawogo’ was grouped in cluster 1 that had the largest number of genotypes; whereas, ‘Beauregard’ was grouped in cluster 4, which had the second largest number of genotypes. A total of 73 progeny were grouped in different clusters from the clusters in which the parents were grouped.

A similar pattern in the relationships among the progeny and parents was observed in the multidimensional scaling plot (Fig. 4). The genotypes were grouped into four main clusters with most of the genotypes grouped in one main cluster. However, some genotypes did not fall in any of the four major groups. This showed that these genotypes were genetically different from the rest of the sibs in this family.

Fifty-one of the progeny exhibited high parent heterosis for storage root yield. The magnitude of heterosis exhibited by progeny ranged from 0.01 to 0.61. Progeny NKB216 exhibited the highest amount of heterosis in this population. Pairwise genetic distances

Fig. 4. Plot of the first and second dimension of the multidimensional scaling analysis of genetic relationships among the progeny and parents of the 'New Kawogo' (NK) × 'Beauregard' (B) sweetpotato mapping population.

were not correlated with the magnitude of high parent heterosis exhibited by progeny in this population. From the pairwise genetic distances, NKB216 was not the most distantly related to 'New Kawogo', the higher parent. The most distantly related progeny to the higher parent was NKB5, but had a low heterosis estimate of 0.18.

Discussion

SSR markers are currently the most useful molecular markers for genetic studies in sweetpotato. However, most of the EST-SSR markers developed (Buteler et al., 1999; Hu et al., 2004b; Schafleitner et al., 2010; Wang et al., 2011) and used for this study had not been tested on other populations. The 99 cDNA SSR primers that were published by Wang et al. (2010) were not used in this study.

The low levels of SSR marker polymorphism observed in our study was similar to the levels of polymorphism reported at the development of these EST-SSR markers. For instance, out of the 120 EST-SSR primers designed, 75 were reported to be polymorphic (Hu et al., 2004b). Likewise, only nine out of 63 designed EST-SSR primers were observed to be polymorphic (Buteler et al., 1999). Schafleitner et al. (2010) identified 1661 microsatellite sequences from the mining of sweetpotato ESTs from which, they designed 233 primers but found only 195 to be polymorphic. Recently, 816 EST-SSR primers were designed and only 195 exhibited polymorphism between parental cultivars, E Shu 3 Hao and Guang 2k-30 of a mapping population (Wang et al., 2011).

A polymorphic information content value of less than 0.25 is considered as low, and between 0.25 and 0.5 as medium, while that greater than 0.5 is considered as high (Xie et al., 2010). Most of the 133 SSR markers used in our study had high PIC and so were useful for diversity and linkage analyses. However, close to 50% of all the SSR markers initially screened were not polymorphic. This overall low polymorphism of EST-SSR markers may be due to the intense selection against variation in the conserved EST-SSR regions of the genome (Scott et al., 2000). The recalcitrance in polymorphisms of EST-SSR markers in sweetpotato could be solved by developing markers from within the intron regions of candidate genes that show more polymorphism instead of ESTs as more sweetpotato genomics tools are developed. Sweetpotato SSR markers developed from introns of candidate carotenoid genes of *Ipomoea* and other species showed high levels of polymorphism and across species transferability (Arizio et al., 2014).

Since there is no reference genome sequence of hexaploid sweetpotato or its close diploid progenitor, ESTs will still remain a vital source of SSR markers. EST-SSR markers still have the advantage of being transferrable across species (Mian et al., 2005; Zhang et al., 2014). Hu et al. (2004a) reported that SSR markers developed from ESTs of *I. trifida* could be 100%, 83.3%, 75%, and 66.7% transferable in *I. batatas*, *I. tiliacea*, *I. triloba*, and *I. lacunose*, respectively. As a result, EST-SSR markers are amenable for use in trait mapping and functional genomic analysis and diversity analysis across species.

The observed wide genetic distance range in this biparental cross is attributed to the diverse gene pools from which 'New Kawogo' and 'Beauregard' were drawn. Grüneberg et al. (2009) hypothesized that crosses of parents of wide gene pools could result in high levels of diversity in the progeny and potential heterosis.

The number of progeny exhibiting high parent heterosis for storage root yield in our study was 51 (18%) though the magnitude of high parent heterosis was low in most of the progeny. There was no direct relationship between pairwise genetic distances of the progeny and the higher parent and the level of high parent heterosis exhibited by the progeny in this study. On the basis of the pairwise genetic distances, we expected the most distantly related progeny from 'New Kawogo', the higher parent for storage root yield to have exhibited the highest amount of heterosis, but this was not the case. This could be attributed to the random reassortment of multiple alleles in the diverse cross. From this study we suggest that a combination of several diverse genotypes in a breeding scheme could result in a critical number of progeny exhibiting heterosis for storage root yield and other traits to enhance breeding.

Studies in India showed that sweetpotato breeding families generated from more diverse parents exhibited higher levels of progeny diversity and heterosis than families generated from closely related parents (Chaurasiya et al., 2013). In these studies, dominance gene action was observed in these high heterosis families (Chaurasiya et al., 2013). Hybrids between diverse parents were reported to exhibit high levels of heterosis in allohexaploid wheat (Birchler et al., 2003). To date, there is no concrete explanation of the molecular basis of heterosis (Chen, 2013).

Both cluster and multidimensional scaling results indicated that some progeny were genetically different from the parents in this population. This was expected because of the random reassortment of alleles in the diverse biparental cross. Studies on the diversity analysis of families of sweetpotato breeding populations at molecular level have not been reported to the best of our knowledge. However, the robustness of SSR markers in sweetpotato germplasm diversity analysis has been demonstrated in collections in Brazil (Veasey et al., 2008), Kenya (Karuri et al., 2010), Uganda (Yada et al., 2010b), and Burkina Faso (Koussao et al., 2014). SSR markers were also used to analyze diversity among cultivated sweetpotato and its progenitors in

the recent studies to disentangle the origins of cultivated sweetpotato (Roullier et al., 2013). This study proposed multiple origins of cultivated sweetpotato from at least two autopolyploidization events from a single progenitor that *I. batatas* shared with *I. trifida* (Roullier et al., 2013).

In the future, breeding programs could genotype the compatible parental genotypes with SSR markers to select the diverse parents for constituting new populations for selection. This could result in populations exhibiting substantial amounts of heterosis for key traits. Breeders could also select the best performing progeny (clones) for various agronomic traits, genotype them with SSR markers to select the potential diverse and superior parents for the next cycle of population improvement. This could minimize the chances of recombining closely related parents and enhance genetic gains in sweetpotato breeding as core genomic tools are being developed.

In conclusion, out of the 405 SSR markers designed for use in this study, only 133 (33%) were finally used in all the subsequent analysis. This was due to low SSR marker polymorphism levels in this population, amplification of markers that gave ambiguous allele profiles and presence of rare alleles in some markers. It is worth noting that, the 133 SSRs used had high levels of polymorphism and effectively revealed the level of genetic diversity in this biparental mapping population. These markers can be useful for tagging agronomic traits using logistic regression and quantitative trait loci analysis. SSRs are abundant in plant genomes, easily transferable across species and across laboratories, codominantly inherited, and amenable for automation making them useful for genetic studies in hexaploid sweetpotato.

We also recommend that more sweetpotato SSR markers be developed in future for enhancing genetic studies in sweetpotato. Ultimately, the sweetpotato genomics and breeding community needs to develop a reference genome sequence for mining SNPs and developing SSR markers for maximum exploitation of genomics-assisted sweetpotato improvement.

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