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GENETIC DIVERSITY AMONG THE PHILIPPINE TRADITIONAL MAIZE (*ZEA MAYS* L.) POPULATIONS BASED ON SSR MARKERS

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SUMMARY

This paper reports the first genetic diversity analysis of Philippine traditional maize populations performed through a cost-effective DNA pooling strategy. The diversity among selected 100 traditional maize populations collected from Luzon, Visayas, and Mindanao was evaluated using twenty simple sequence repeats (SSR) markers at the Institute of Plant Breeding, University of the Philippines Los Baños, Laguna, Philippines. A total of 138 bands ranging from two to 12 bands per primer were detected. The average number of polymorphic alleles, polymorphism rate, effective multiplex ratio, marker index, resolving power, and expected heterozygosity are 6.283, 87.17%, 5.798, 4.104, 15.897, and 0.658, respectively. The polymorphism information content (PIC) varied between 0.141 to 0.848, with an average value of 0.620. A dendrogram was constructed with a dissimilarity coefficient ranging from 0.14 to 0.55 and a mean dissimilarity index of 0.425. Cluster analysis revealed 13 groups based on the result of Approximately Unbiased (AU) p-values from 10,000 bootstrap iterations. The cluster analysis enabled the classification of populations with ambiguous places of origin. Analysis of molecular variance (AMOVA) showed higher within-population diversity (70%) than among-population diversity (30%) with PhiPT (pairwise genetic differentiation metric) of 0.298 (P = 0.001). These results revealed the significant diversity of traditional maize populations in the Philippines and the power of SSR markers in diversity and cluster analyses despite the age of this marker technology. These findings will aid plant breeders in developing approaches towards knowledgeable and efficient execution of breeding programs using traditional maize populations.

Keywords: Traditional maize, Philippine germplasm, cluster analysis, SSR markers, AMOVA, bootstrapping

Key findings: The pooling strategy used in the study was found to be effective and can be replicated to provide cost-efficient germplasm characterization. The clustering pattern of Philippine traditional maize (*Zea mays* L.) populations, collected from Luzon, Visayas, and Mindanao, were found to be according to geographic location data. Breeders can potentially use the information as basis for generating heterotic groupings that may be agronomically validated, by choosing appropriate cross combinations from representative genotypes from each cluster. Furthermore, from the standpoint of germplasm management, this will contribute in creating a core germplasm collection.

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INTRODUCTION

Maize (*Zea mays* L.) exhibits great potential as a substitute staple crop next to rice. About one-third of Filipino farmers (1.8 million) depend on maize production as a source of income (Gerpacio *et al*., 2004). Philippine traditional corn varieties, also called farmer's varieties of traditional maize, such as, tiniguib, lagkitan, kalimpos, and kabagtik, have been reported to be characterized by distinctive attributes, while cultural minorities raise heirloom varieties (Altoveros and Borromeo, 2007). The Institute of Plant Breeding (IPB) is addressing the need to collect, characterize, and conserve traditional corn varieties from different locations across the country through the Corn Germplasm Utilization through Advance Research and Development (CGUARD) Program.

As in other parts of the world, maize production in the country faces constraints and challenges, such as, pests and diseases. Major pests and diseases of corn prevalent in the Philippines include the: Asiatic corn borer (ACB) caused by *Ostrinia furnacalis*, Fusarium ear rot by fungus *Fusarium verticillioides*, and the Philippine downy mildew (PDM) by the oomycete *Peronosclerospora philippinensis* (W. Weston) C.G. Shaw, which is considered as the most virulent downy mildew pathogens in maize (Payak, 1975). Several populations exhibiting resistance to pests and diseases, together with populations with high lysine and earliness, attest to a high breeding potential of the Philippine traditional maize varieties (Salazar *et al*., 2016). Thus, the Philippines' traditional maize offers great potential with a rich source of genetic material to address constraints in maize production.

The diversity of Philippine traditional maize populations has been studied in the past years using morphological characters. Reports on a moderate level of diversity using non-parametric morphological characters in the Philippine corn collection of the National Plant Genetic Resources Laboratory (NPGRL), traditional and farmers' corn from CGUARD, and additional NPGRL collections transpired (Siopongco *et al*., 1999; Bon *et al*., 2019; Baer *et al*., 2022). However, unambiguous estimation of genetic variation cannot be achieved based solely on morphological characters as these are often influenced by the environment, developmental stage-dependent, and limited in number (Chesnokov *et al.*, 2020; Adriansyah *et al*., 2021).

Genetic characterization of maize using molecular markers has been done in inbred lines and populations, lowland tropical, subtropical midaltitude highland inbred lines, tropical maize germplasm, and many others (Warburton *et al*., 2002; Xia *et al*., 2004; George *et al*., 2004; Xia *et al*., 2005; Qi-Lun *et al*., 2008; Mukri *et al.,* 2022). However, aside from the study conducted by Bautista *et al*. (2015), the genetic diversity of Philippine traditional maize populations has not been investigated thoroughly using molecular markers, which are more informative and indicative of unique allelic profiles (Warburton *et al*., 2002). Simple sequence repeat (SSR) markers are widely used in maize diversity analysis studies for their genome-wide distribution and known genomic location, high polymorphism rate, and reproducibility (Mushtaq *et al*., 2016; Bedoya *et al*., 2017; Adu *et al*., 2019).

Information on patterns and levels of genetic diversity is valuable in crop breeding, such as, genetic variability analysis, identification of parental combinations, and introgression studies (Swarup *et al*., 2021). Development of new inbred lines, assignment of lines to heterotic groups, prediction of the degree of inheritance, variation and level of heterosis, and maintenance of the genetic base of breeding materials for genetic improvement, relies on the prior knowledge of genetic diversity in the germplasm (Fu, 2015; Gedil and Menkir, 2019).

This study investigated the genetic diversity of the first 100 Philippine traditional maize populations collected by the CGUARD Program. This intends to provide an overview of the genetic structure of this collection, as well as insights for Philippine corn research, particularly crop improvement and germplasm conservation and management.

MATERIALS AND METHODS

Plant material

The IPB Cereals Section of the University of the Philippines Los Baños (UPLB) provided a total of 100 Philippine traditional maize populations. The germplasm collection came from different geographical locations among the three Philippine island groups of Luzon, Visayas, and Mindanao through the CGUARD program (Supplementary Table 1). Selection of maize populations focused on kernel, aleurone, endosperm color, and seed texture. At most 35 seeds from each maize population were sown and maintained until the seedling stage at the Genetics Laboratory glasshouse at IPB, UPLB.

A total of 30 plants from each population were sampled for DNA extraction.

Extraction, quantification, and pooling of individual DNA samples

Three young, damage-free emerging light green leaves were collected from 30 individual maize plants per population 21 days after sowing. Genomic DNA extraction was conducted following the CIMMYT (2005) protocol, with modifications. Approximately 3 g of processed leaves were finely ground into a powder with liquid nitrogen using a mortar and pestle. DNA quality and quantity were determined by agarose gel electrophoresis carried out in 1% UltraPure agarose (Invitrogen Corp., Carlsbad, California, USA) in 1X Tris-Borate EDTA (TBE) running buffer at 100V for 40 min. The resulting gel was stained with 0.5 ug/mL ethidium bromide for 20 min in a shaker. The stained agarose gel was documented using the ENDURO^{TM} GDS gel documentation system (Labnet International, Inc., Edison, NJ, USA). The concentration of the DNA samples was estimated by manually comparing the samples' fluorescence intensities with that of the four known lambda (λ) DNA standards (Sigma-Aldrich Inc., St. Louis, Missouri, USA). The DNA bulk of each maize population was generated by pooling equal concentration (10 ng/uL) and amount of DNA working stock from each plant. One DNA-pooled sample consisted of 10 individual DNA samples from each population (Reyes-Valdés *et al.*, 2013).

Selection of SSR primers

SSR markers were selected according to the genomic location, with at least one marker for each chromosome, to provide a fair representation of the maize genome. Furthermore, the SSR markers that show distinct and polymorphic banding patterns during the screening of selected maize populations were utilized in the study. Thus, there is a non-uniform representation in terms of the number of SSR markers for each chromosome. SSR primers were obtained from the literature (Senior *et al*., 1998; Lu and Bernardo, 2001; Warburton *et al*., 2002; George *et al*., 2004) and were also selected based on resolving power, polymorphism, scorability, and ability to distinguish one population from another. The primer sequences were obtained from the Maize Genome Database (MaizeGDB, http://www.maizegdb.org) and were outsourced for synthesis to Invitrogen Corp. (Invitrogen Corp., Carlsbad, California, USA). A final set of 20 polymorphic SSR markers were used in the assay (Supplementary Table 2).

Amplification and detection conditions

Polymerase chain reaction (PCR) was carried out in a final volume of 10 uL using the SSR amplification conditions optimized by Canama and Hautea (2010). Each PCR reaction consisted of a 10ng genomic DNA template, 10X PCR buffer (10 mM Tris-Cl pH 8.3, 50 mM KCl), 50 mM $MgCL_2$ (Vivantis Technologies, Malaysia), 2.5 mM dNTPs (Amersham Pharmacia Biotech AB, Uppsala, Sweden), 2 uM/uL each of forward and reverse primers and 1U/total Taq polymerase (Vivantis Technologies, Malaysia). Amplifications were carried out in T100™ Thermal Cycler (Bio-rad Laboratories Inc., Hercules, CA, USA) with the amplification conditions performed as follows: initial denaturation at 94 °C for 5 min, followed by 34 cycles at 94 °C for 1 min denaturation, annealing at 60 °C for 1 min, with 72 °C for 2 min extension, and a final extension at 72 °C for 3 min.

PCR amplification products were resolved in 8% polyacrylamide gel using 1X TBE (90 mM Tris-borate, 2 mM EDTA) running buffer. Electrophoresis (C.B.S. Scientific Triple Wide Mini Vertical System^{TM}) was carried out for 90 min in 100 V. The polyacrylamide gel was stained with 0.5 ug mL^{-1} ethidium bromide for 15–20 min, and the bands were detected under UV light using the Enduro gel documentation system (Labnet International, Inc., Edison, New Jersey, USA). The amplified DNA fragments, with different patterns for each SSR marker, were scored manually and transformed into a binary matrix of presence (1) or absence (0) for each population within each pooled sample.

Genetic properties of markers

The number of alleles detected (A_o), number of polymorphic alleles observed per loci, and polymorphism rate (%) were recorded for each SSR marker. Polymorphism rate was obtained by dividing the number of polymorphic alleles observed over the number of bands observed multiplied by 100.

Allele frequency (f_{Ai}) was estimated by interpreting the allelic frequency as the ratio of a band. The computation was done by accounting for the pooling of individual samples per population using the formula described by Dubreuil *et al*. (1999):

Eq 1.

$$f_{A_i} = Q_{B_i}\left(\sum_{i=1}^{n} Q_{B_i}\right)^{-1}$$

where Q_{B1}, Q_{Bi},..., Q_{Bn} are the quantifications estimated for the bands B_1, B_i,..., B_n associated with the alleles A_1, A_i,..., A_n recorded across the three independently-pooled DNA samples. The estimated allele frequency within each locus was computed by the average of the allele frequencies observed among the DNA pooled samples. The approach was used in the genetic analysis of maize populations with pooled samples (Dubreuil *et al*., 1999; Rebourg *et al*., 2001). The allele frequencies calculated were subsequently used to obtain measures of diversity. The effective number of alleles (Ae) was measured using the formula:

Eq 2.

$$\mathrm{Ae} = \frac{1}{\sum_{i=1}^{n} p_i^2}$$

where p_i is the allele frequency of the i^{th} allele out of the total number of alleles at an SSR locus. The expected heterozygosity per locus (He), also known as the index of gene diversity, was calculated using the unbiased diversity index equation and the average expected heterozygosity for all loci (He) described by Nei (1973):

Eq 3.

$$\mathrm{He} = 1 - \sum_{i=1}^{n} p_i^2$$

and

Eq 4.

$$\mathrm{He\ (ave)} = \sum_{i=1}^{n} \frac{He}{L}$$

where p_i is the frequency of the i^{th} allele of n alleles at a locus and L is the total number of loci. Polymorphism information content (PIC) is the measure of discriminatory power of each SSR locus and serves as a measure of the allelic diversity within a locus (George *et al*., 2004). It measures the ability of an SSR marker to create polymorphism information within a pool of genotypes (Anderson *et al*., 1993; Perseguini *et al*., 2012). The formula by Botstein *et al.* (1980) was used to compute the PIC value of each SSR marker:

Eq 5.

$$\mathrm{PIC} = 1 - \sum_{i=1}^{n} p_i^2 - \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} 2p_i^2 p_j^2$$

where p_i and p_j are the average frequencies of the i^{th} and j^{th} allele obtained from a DNA pooled sample relative to the n total number of alleles at an SSR locus. PIC values are considered highly polymorphic when calculation results a >0.50 value; moderately polymorphic if 0.25 to 0.49, and poorly polymorphic if <0.25 (Botstein *et al*., 1980). The effective multiplex ratio (EMR) per marker is given by the formula: EMR = nβ, where n is the number of polymorphic loci while β is the proportion of polymorphic loci over the total number of alleles detected. Marker index (MI) is calculated by multiplying PIC with EMR (Powell *et al*., 1996). The resolving power (Rp) is calculated by the formula Σlb, where *lb* is 1-(2 x [0.5-p]), and p is the proportion of populations containing the band (Gilbert *et al*., 1999).

Cluster analysis and genetic differentiation

Jaccard dissimilarity matrix was calculated and used in agglomerative hierarchical clustering using the unweighted pair group method with arithmetic mean (UPGMA) method. The dendrogram was constructed using the *pvclust* function (Suzuki and Shimodaira, 2006) in R software (R Core Team, 2020). Multiscale bootstrap resampling with 10,000 iterations was generated to analyze the Approximately Unbiased P-value (AU), which determines the robustness of branches formed.

Principal Component Analysis (PCA) was performed in R software (R Core Team, 2020) using *ggfortify* R package (Tang *et al*., 2016). Analysis of molecular variance (AMOVA) among and within populations and the number of private alleles to analyze the distribution of the populations were done using the Genetic Analysis in Excel (GenAIEx) program, version 6.503 (Peakall and Smouse, 2012). The genetic differentiation estimate, PhiPT (proportion of variance among populations relative to total variance), which measures population variation relative to total variation, was calculated also using GenAlEx. The PhiPT

is an analog of Wright's fixation index (F_{ST}), which determines levels of differentiation according to a range of values, specifically very high for a value greater than 0.25, high for 0.15–0.25, moderate for 0.05–0.15, and low for a value below 0.05.

RESULTS

Robust SSR markers detect a high number of alleles

Twenty polymorphic SSR markers representing the 10 chromosomes of maize showed distinct banding patterns and revealed the genetic diversity of the 100 traditional maize populations. The average number of alleles detected (A_o) in each SSR marker across three DNA bulks, average number of polymorphic alleles per loci, polymorphism rate (%), and effective number of alleles (A_e) are presented in Table 1.

A total of 138 alleles were detected, with the number of alleles (A_o) ranging from two (phi088) to 12 (phi076 and phi128), having an average number of 6.9 alleles across 20 SSR loci. The number of polymorphic alleles per locus ranged from 1.0 (phi088) to 12 (phi076 and phi128). Forty percent (40%) of the SSR markers used in the study showed a 100% polymorphism rate with a total average of 87.17%. The average effective number of alleles was 3.781 ranging from 1.179 (phi088) to 7.341 (phi076). Mean expected heterozygosity is 0.658 ranging from 0.152 (phi088) to 0.863 (phi076). The minimum allele frequency obtained from the bulk samples ranged from 0.001 to 0.212, while the maximum allele frequency ranged from 0.185 to 0.917. The average PIC value of the 20 SSR markers used in the study is 0.620 at 80% (16 out of the 20) markers can be considered very informative (PIC > 0.5). Two SSR markers are moderately informative (PIC > 0.25) and two are less polymorphic. The marker observed to have the highest PIC value is phi076 (0.848), and the lowest is phi088 (0.141).

Populations are generally clustered in congruence to geographical location

The dissimilarity coefficient ranged from 0.14 to 0.55 with a mean of 0.425. At 10,000 bootstrap iterations, robust population groupings were inferred as the majority of clusters have >95 AU bootstrap values. The analysis gave a minimum bootstrap threshold value of 77%, considered to provide good branch support. A bootstrap threshold value of 70% and above corresponds to a 95% probability that the clade is real (Hillis and Bull, 1993). Thirteen clusters could be confidently distinguished (Figure 1). At 0.425 dissimilarity coefficient and an AU value of 98, Cluster 8 presents the largest and the most diverse group composed of 37 populations from Luzon, Visayas, and Mindanao.

Four populations — APN 138 (Bukidnon), APN 020 (Unknown origin), APN 128 (Bukidnon), and APN 077 (Isabela) — did not cluster in any group and were designated as independent groups, namely, Cluster 1, Cluster 2, Cluster 9 and Cluster 11, respectively. The cluster dendrogram revealed that APN 138 is the collection's most distinct population.

Majority of the populations coming from Luzon were grouped in Cluster 13 at a 0.470 dissimilarity coefficient. The populations in this cluster came from Northern Luzon including the provinces of Abra (Cordillera Administrative Region), Cagayan (Region 2 - Cagayan Valley), Isabela (Region 2 - Cagayan Valley) and Marinduque (Region 4B - MIMAROPA). The populations from Abra present a relatively narrow genetic base clustering at a 0.365 dissimilarity coefficient. The rest of Luzon population was grouped in the highly diverse Cluster 8 (the largest cluster in terms of population number), along with populations from Visayas and Mindanao. The Luzon populations in this cluster mostly came from the Southwestern Tagalog (MIMAROPA) and CALABARZON Luzon provinces: Palawan (Region 4B - MIMAROPA), Romblon (Region 4B - MIMAROPA), Laguna (Region 4A - CALABARZON) and Quezon (Region 4A - CALABARZON). Maize populations, APN 107 and APN 108 both collected in Marinduque (Region 4B - MIMAROPA), are separated into Cluster 13 and Cluster 8, respectively. In addition, APN 120 from Pangasinan (Region 1 - Northern Luzon) grouped with APN 119 from Iloilo (Region 6 - Western Visayas) in Cluster 12, while APN 077 from Isabela (Region 2 - Northeastern Luzon) is the lone population comprising Cluster 11.

Populations collected from Visayas were grouped across Clusters 6, 7, 8, and 11. Visayan populations from Cebu (Region 7 - Central Visayas), Negros Occidental (Region 6 - Western Visayas), and Negros Oriental (Region 7 - Central Visayas) were found in Cluster 6 along with the populations from Mindanao and unknown sources. These populations all have white endosperm color and flint seed texture. Seven populations from Visayas were grouped

Table 1. Characterization of 20 SSR loci of 100 Philippine traditional maize.

Primer No.	Primer Name	Allele Frequency (f_{Ai})		Number of Alleles Detected (Ao)	Effective Number of Alleles (Ae)	No. of Poly-morphic Alleles	Poly-morphism Rate (%)	Effective Multiplex Ratio (EMR)	Marker Index (MI)	Resolving Power (Pp)	Expected Hetero-zygosity (He)	Polymorphic Information Content (PIC)
		Minimum	Maximum									
1	umc1479	0.190	0.355	4.00	3.756	3.00	75	2.250	1.543	16.920	0.7338	0.6856
2	umc1711	0.212	0.304	4.00	3.759	4.00	100	4.000	2.783	18.000	0.7434	0.6957
3	bngl176	0.003	0.319	10.00	4.714	9.00	90	8.100	6.151	18.660	0.7878	0.7594
4	phi090	0.038	0.873	3.00	1.303	2.00	67	1.333	0.276	6.880	0.2182	0.2067
5	phi088	0.083	0.917	2.00	1.179	1.00	50	0.500	0.070	6.520	0.1522	0.1406
6	phi096	0.001	0.275	8.00	4.684	6.67	83	5.556	3.938	21.200	0.7538	0.7088
7	phi076	0.010	0.185	12.00	7.341	12.00	100	12.000	10.175	26.780	0.8628	0.8479
8	umc1153	0.009	0.330	9.00	5.226	9.00	100	9.000	7.071	17.460	0.8086	0.7857
9	phi128	0.001	0.187	12.00	6.981	12.00	100	12.000	10.082	27.800	0.8568	0.8402
10	phi081	0.004	0.359	10.00	4.168	9.00	90	8.100	5.871	16.740	0.7602	0.7248
11	phi112	0.005	0.500	8.00	3.137	8.00	100	8.000	5.152	11.620	0.6812	0.644
12	umc1728	0.008	0.189	7.00	5.878	7.00	100	7.000	5.643	29.300	0.8299	0.8062
13	umc1287	0.001	0.322	7.00	4.538	7.00	100	7.000	5.219	18.120	0.7797	0.7456
14	umc1121	0.078	0.728	4.00	1.799	3.00	75	2.250	0.938	8.240	0.4445	0.4171
15	phi108411	0.002	0.607	5.00	2.193	4.00	80	3.200	1.529	9.880	0.5441	0.4778
16	umc2341	0.005	0.491	5.00	2.693	5.00	100	5.000	2.797	11.020	0.6287	0.5593
17	phi035	0.011	0.499	5.00	2.182	4.00	80	3.200	1.400	11.920	0.5418	0.4374
18	phi032	0.122	0.386	4.00	3.401	3.00	75	2.250	1.468	15.540	0.7058	0.6525
19	phi084	0.008	0.370	11.00	4.470	10.00	91	9.091	6.802	16.020	0.7764	0.7482
20	phi050	0.006	0.644	8.00	2.219	7.00	88	6.125	3.181	9.320	0.5495	0.5193
	AVERAGE	0.040	0.442	6.900	3.781	6.283	87.170	5.798	4.104	15.897	0.658	0.620
	Total			138.00								
	Min Value	0.001	0.185	2.000	1.179	1.000	50.000	0.500	0.070	6.520	0.152	0.141
	Max Value	0.212	0.917	12.000	7.341	12.000	100.000	12.000	10.175	29.300	0.863	0.848

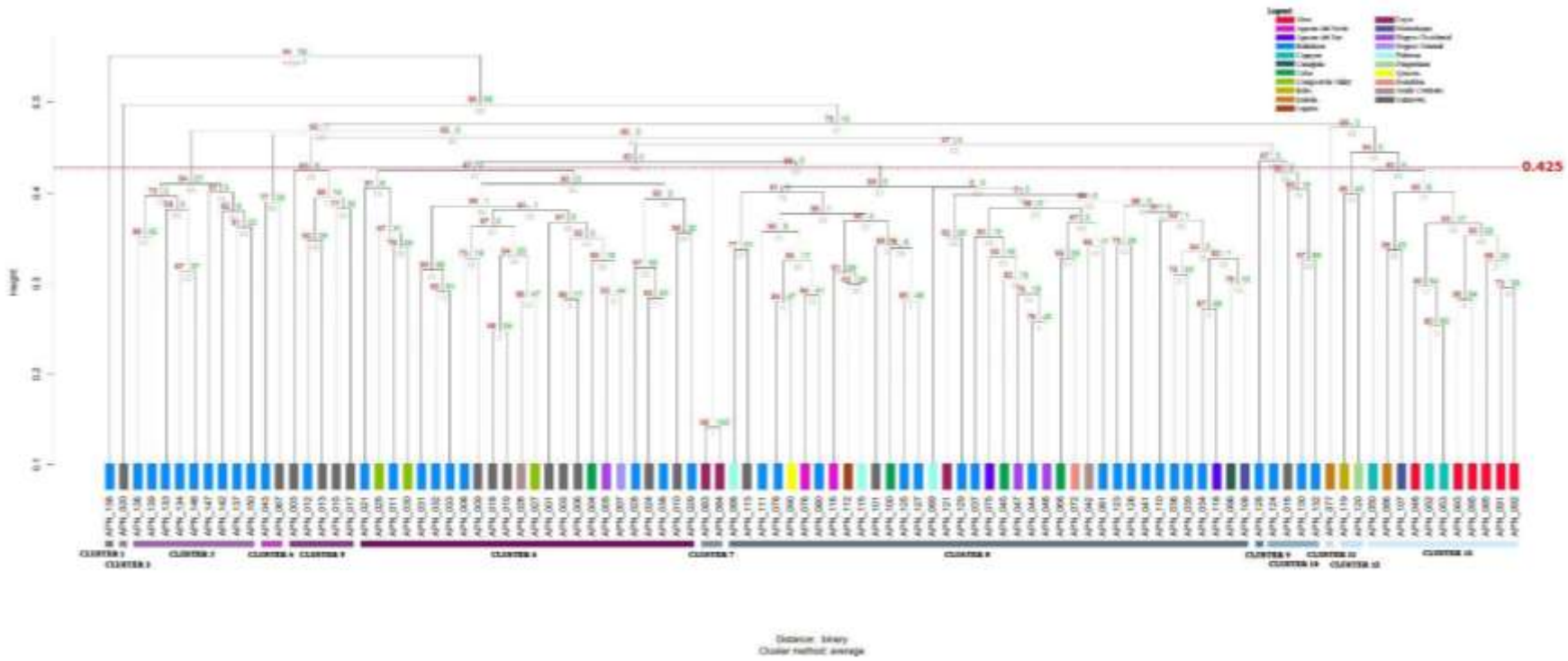

Figure 1. Dendrogram derived using the combined analysis of 20 SSR markers of 100 traditional maize populations using multiscale bootstrap re-sampling with 10,000 bootstrap iterations. The corresponding designations and phenotypic characteristics of each population are summarized in Table 1. The values are presented in the figure as percentages, with AU values (red) representing approximately unbiased p-value, BP values (green) indicating a bootstrap probability (bootstrap value = 10,000), and #edge (gray) representing the number of subclusters (98 total clades). The broken red line indicates the 0.425 dissimilarity coefficient wherein 13 Clusters were derived.

in Cluster 8 from the provinces of Cebu (Region 7 - Central Visayas), Leyte (Region 8 - Eastern Visayas), Negros Occidental (Region 6 - Western Visayas), and Camiguin (Region 10 - Northern Mindanao). Morphologically, these populations have red and white kernel color, red and colorless aleurone, white endosperm, and flint seed texture. The maize populations, APN 083 and APN 084, both collected from Leyte, showed the highest genetic similarity, which comprised Cluster 7. Although both populations show differences in morphological data, such that APN 083 have a white kernel and colorless aleurone, while APN 084 have red color for both characters, they are genetically highly similar.

The Philippine maize populations coming from Mindanao make up the most representative sample in the study. The populations collected from Bukidnon (Region 10 - Northern Mindanao) are a diversified collection that can still be divided into five unique groups, with two populations—Clusters 1 and 9 populations—considered a distinct group. Cluster 3 with an AU value of 94, comprises nine populations solely from Bukidnon. Cluster 4, Cluster 5, and Cluster 10 were all Bukidnon populations grouped with populations of unknown sources. In Cluster 6, Bukidnon populations were grouped among other Mindanao populations coming from Compostela Valley (Region 11 - Davao) and South Cotabato (Region 12 - SOCCSKSARGEN). Representative Mindanao populations, collected from Agusan del Norte (Region 13 - Caraga), Agusan del Sur (Region 13 - Caraga) and South Cotabato (Region 12 - SOCCSKSARGEN), also grouped along with those collected from Bukidnon in the highly diverse Cluster 8.

Principal component analysis (PCA; Figure 2) showed a pattern comparable to that observed from cluster analysis. The first principal component explained 8.84% of the total marker variation. The second principal component accounted for 6.23% of the variation. Most populations from Luzon are clustered separately from the populations of those collected from Mindanao and Visayas. On the other hand, maize populations from the Visayas clustered with both Mindanao and Luzon populations. Mindanao populations formed several clusters and is the island where the most distant population was identified.

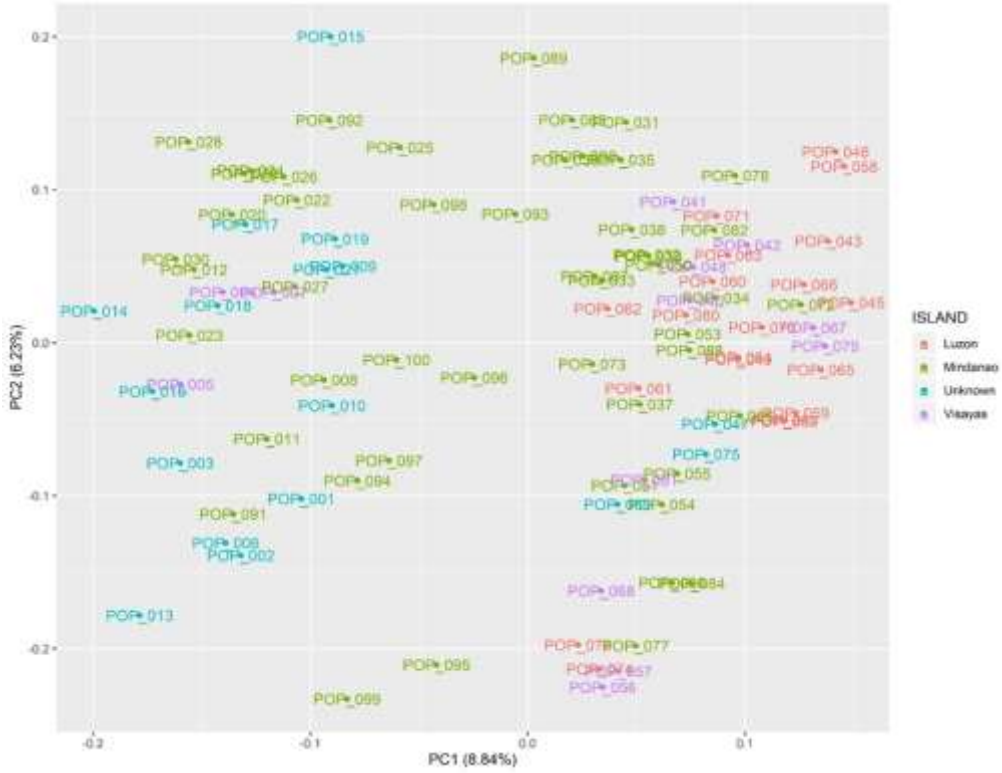

Figure 2. Scatterplot of the first and second principal component scores of 100 Philippine traditional maize population based on 20 SSR markers.

SSR profiles reveal a high level of genetic differentiation

Table 2 presents the results of the analysis of molecular variance (AMOVA) based on PhiPT values. Of the total genetic variations, 30% indicated a variation among populations, while 70% variation within populations. Four (4) private bands were determined among the populations. Two were found in APN 119 and one in both, APN 007 and APN 041. Nonetheless, a high variation level was detected among the traditional maize populations used in the study (PhiPT = 0.298, $P < 0.001$).

Table 2. Analysis of molecular variance based on 20 SSR markers for 100 populations of Philippine traditional maize (*Zea mays* L.).

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Sum of Squares	Variance Components	Variation (%)	Genetic Differentiation	P (rand > = data)
Among populations	99	246.437	24.873	4.647	30%	PhiPT = 0.298	0.001
Within populations	200	2186.667	10.933	10.933	70%		
Total	299	4649.103		15.580	100%		

Note: P (rand >= data), the probability for PhiPT is based on standard permutation across the full data set.

DISCUSSION

Effectiveness of pooling strategy in genetic diversity assessment

Calculation of allelic frequency for the DNA pooled sampling method was successfully demonstrated in restriction fragment length polymorphism (RFLP) analysis and has since been established for its efficiency in the analysis of maize populations (Rebourg *et al*., 1999). The pooling strategy for determining allele frequencies in this study followed said method and was similar to several reports (Prasanna, 2012; Reyes-Valdés *et al*., 2013). The approach was used to evaluate, at most, 30 individual DNA samples for each population divided into three DNA pooled samples (Reyes-Valdés, 2013). Having at least 15 individual representatives in each population adequately assesses genetic diversity (Prasanna, 2012). The pooling strategy ensured quality amplification while saving time and resources, and allowed for better coverage on the estimation of allele frequency (f_{Ai}) per maize population. The SSR markers used in this study provided a fair representation of the 10 chromosomes of maize to depict the genetic diversity in each specific locus. The robust amplification patterns, which resulted in the determination of a wide range of allele frequencies in the populations investigated, can be ascribed to better resolving power and handling of dilution attributed to the use of three DNA pools consisting of 10 samples each (Reyes-Valdés *et al.,* 2013). Moreover, reports from using SSR markers on inbred lines, such as studies on residual heterozygosity, SSR loci mutations, or duplications that resulted in amplification of similar sequences located in different regions in the genome (Legesse *et al*., 2007), gave an insight into the genomic and genetic structure within individuals and populations. These factors affecting the variations within individuals and populations must also play a significant role during an assessment at a population level.

Robustness of SSR markers chosen for molecular characterization

The number of alleles detected in this study is comparatively higher than in several studies which utilized SSR markers in analyzing maize populations. It is higher than the reported 104 total alleles with an average of 5.2 per locus in 165 accessions of landrace and inbred Chinese waxy maize assayed with the same number of SSR markers (Zheng *et al*., 2013). The number of alleles detected in this study is also higher on a per-marker basis in a bulk analysis of 124 maize landraces (Qi-Lun *et al*., 2008). It is comparable to the results of SSR analysis of 98 Turkish landraces in which 28 markers detected 172 alleles with an average of 6.21 alleles per locus (Cömertpay *et al*., 2012). Meanwhile, maize populations from Algerian Sahara registered a higher number of alleles at 197, with a mean of 10.9 alleles per locus (Aci *et al*., 2018). The effective number of alleles is also higher than a study on the Creole maize population in Puerto Libertador, Córdoba, which reported an average of 3.365 (Pérez *et al*., 2018).

Genetic diversity analysis is better measured using the PIC value than the number

of alleles alone since it also considers the relative frequencies per allele (Laborda *et al*., 2005). The average PIC value computed in the study is comparable with Algerian landraces from the Sahara (Aci *et al*., 2018). This is also higher than reported on Swiss landraces (Freitag *et al*., 2012) and US maize inbreds (Senior *et al*., 1998). Moreover, 85% of the markers used in the study are highly informative and indicative of the robustness of the selected SSR markers used to evaluate the genetic diversity of Philippine traditional maize populations.

SSRs are abundant, highly polymorphic, randomly, and widely distributed in the genome. It is highly informative due to its co-dominant nature, such that heterozygous individuals are distinguished from homozygous ones (George *et al*., 2004). Through assessment per SSR locus, diversity patterns of the Philippine traditional maize populations revealed a large amount of variation in each SSR locus evaluated and suggested a broad genetic base of the selected populations.

Population genetic diversity of Philippine traditional maize populations

The Philippines, as an archipelago, allows for a degree of isolation among the maize populations. Acquiring differences while being exposed to different environmental conditions, a large diversity is expected among populations. The genetic diversity of species that come from diverse and distinct geographical regions is likely to be higher than that of geographically localized species (Hamrick and Godt, 1989). This paper describes a pioneer study on the genetic characterization of the traditional maize population in the Philippines.

The distribution of 100 maize traditional populations in the study is as follows: 19 from Luzon, 12 from the Visayas, 52 from Mindanao, and 17 populations cannot be classified as to their origin. The majority of the maize populations from Mindanao came from Bukidnon province (40 populations). These populations reflect the initial populations collected by the CGUARD program and this study is the preliminary genetic analysis of the collection.

The observed variation in visible seed morphological characters hints at the level of diversity of the populations. Kernel color, aleurone color, endosperm color, and texture exhibited varying characteristics. Despite evident phenotypic characteristics, DNA markers reveal that clustering of the populations is not formed based on similarities of seed characters alone. The majority of maize populations showed a tendency to group based on their geographical location. Luzon populations are divided separately into four different groups. Visayas populations are distributed into four clusters as well. Mindanao populations, regardless of having the most number of representative sample populations, were formed into five groups and two very distantly unique clusters suggesting that these populations are genetically broad. Meanwhile, maize populations from unknown sources tend to group with Visayas and Mindanao accessions that indicate most of these populations are possibly collected within these islands.

The populations obtained from Abra were observed to have a narrow genetic basis, with most of these clustering into a single group. These suggest that maize populations from Abra may be unique and have a particular molecular characteristic that distinguishes them from accessions of other regions. It is also interesting to note that populations coming from the northern part of Luzon are clustered separately from populations collected in the southern part of Luzon. The clustering of maize populations from Northern Luzon, such as, those from Cagayan and Isabela, alongside Abra in Cluster 13, further suggests that Northern Luzon populations are more distinct from rest of the Philippine maize populations in the collection. Furthermore, populations from the southern portion of Luzon clustered under the most diversified group, Cluster 8, suggesting that these populations might comprise a larger genetic base, yet have discrete genetic similarities with accessions from Visayas and Mindanao. The northern part of Luzon is more isolated and distant from the rest of the Philippines. The two representative populations from Marinduque are grouped separately in Clusters 8 and 12, indicating that each population share similarities of populations collected from northern and southern Luzon. The fact that Marinduque is an island province may have contributed to this occurrence. Thus, clustering pattern among these populations suggests that geographical location plays an important role in their variances. Results from the past survey suggested that Region 2, or Cagayan Valley, is classified as upland plain, while Region 4A, or Southern Tagalog Region, is classified as rainfed lowlands (Gerpacio *et al*., 2004). These agro-ecological classifications are based on the shape of the landscape and topography of the areas. Moreover, APN 052 and APN 053, are

the most similar populations from Luzon, clustering only at a 0.255 dissimilarity coefficient, or with 75% genetic similarity. These populations were both collected from the province of Cagayan.

Maize populations representing the Visayas islands have a broader genetic base as they are found to cluster within both Luzon and Mindanao populations. The location of Visayas islands being between Luzon and Mindanao may suggest a correlation. Mindanao accessions are mostly representatives from Bukidnon, one of the major maize-producing regions in the Philippines (Delos-Santos *et al*., 2019). Maize populations from Bukidnon are distributed among the five large clusters that Mindanao represents. The most similar maize populations from Bukidnon are APN 032 and APN 033 clustering at a 0.290 dissimilarity coefficient.

Despite their morphological differences, the most genetically similar accessions among the maize populations are APN 083 and APN 084 (both obtained from Leyte, Eastern Visayas), indicative of a high similarity between the two accessions, which may have diverged from a single population of origin and cultivated separately. Therefore, at a 0.140 dissimilarity coefficient, a large morphological difference can still be identified, giving an overview of the intensity of observable diversity among the selected populations.

Unique maize populations, APN 138, APN 020, APN 128, and APN 077, which did not cluster with other populations, could be considered as separate heterotic groups that can be used for breeding purposes. Among the populations in the study, APN 138 (a lone member of Cluster 1) from Bukidnon is the most distant population sharing a 0.550 dissimilarity coefficient against the rest of the populations utilized in this study. Heterosis among the 13 clusters could be agronomically confirmed by intercrossing representative genotypes from each cluster.

Populations with no recorded place of collection that clustered in groups 2, 4, 5, 6, and 9, most likely came from the Mindanao region, specifically in Bukidnon, based on the dendrogram. These accessions include APN 020, APN 067, APN 003, APN 013, APN 015, APN 017, APN 009, APN 018, APN 019, and APN 016. In addition, APN 024 and APN 010 in group 6 clustered within another Bukidnon, Mindanao sub-cluster at a 0.39 dissimilarity coefficient, signifies that this might have also been collected from the same region. Moreover, APN 001, APN 002, and APN 006 in Cluster 6, as well, could have been collected from the Visayas region since they form a sub-cluster with populations collected from Visayas at about 0.37 dissimilarity coefficient. Similarly, in Cluster 8, APN 113 clustered at 0.37 dissimilarity coefficient with APN 088 collected from Palawan, Luzon, which suggests a common collection origin. Furthermore, APN 101 clustered at a 0.34 dissimilarity coefficient with APN 100 (Cebu), APN 125 (Bukidnon), and APN 127 (Bukidnon) could have also been collected from Mindanao.

Based on the AMOVA results, a large genetic variation in maize populations appears to exist in the Philippines. The acquired high PhiPT value (30%) indicates a high genetic variation level among populations. Bracco and colleagues (2009) obtained comparable values in maize landraces from indigenous settlements in Northern Argentina analyzed with SSR markers. Their results showed that 34% of the variation was among populations. In contrast, Qi-Lun and colleagues (2008) reported lower genetic differentiation among populations in maize landraces in China with SSR markers. Their study revealed that 13% of the total variation is found among landraces, while 87% is within landraces. The level of genetic differentiation among populations in the Philippine traditional maize analyzed in this study is considered relatively high, especially for an introduced crop. A low number of private bands found across the populations suggests free gene flow between populations, indicating that seed exchange may have been performed among regions, implying a low level of genetic divergence. Sharing of seeds could be through human-mediated plant materials exchange. However, the existence of a limited number of private alleles in Mindanao (Bukidnon) and Visayas (Negros Oriental and Iloilo), which are among the major maize-producing regions, suggests that these populations are beginning to diverge. It is also possible that the founder effect or genetic drift has played a role in the modern diversity structure of Philippine maize. The traditional maize population in the Philippines is but a fraction of the very diverse maize around the globe. Over time, this fraction of maize changes allele frequencies due to random sampling error resulting in the loss of some alleles and fixation of other alleles.

Conservation efforts of the traditional maize population have been started in the Philippines to harness its potential in breeding programs. Genetic diversity analysis is essential for the better management of these collections. Thus, germplasm conservation

strategies must narrow duplications while harnessing as much allele diversity as possible. Moreover, the Philippine traditional maize population can be a source of new alleles for breeding programs geared toward abiotic stress adaptation, including the emergence of new biotic stresses.

This study provides a detailed overview of the genetic base in Philippine traditional maize populations. The information can be used as a guide, along with other relevant data on disease, pest, and agronomic characteristics, in choosing the parental lines for a suitable cross combination specific for breeding maize appropriate in the Philippine setting. The genetic information on highly similar accessions collected from the same region can also be a basis for efficiently managing the germplasm collection of the traditional maize population of the Philippines. Further characterization of other maize populations in locations not covered in this study is an area for future research.

CONCLUSIONS

The study successfully established the genetic diversity among 100 Philippine traditional maize populations selected using the molecular data from 20 SSR DNA markers of pooled DNA analysis. The pooling strategy provides a cost-effective characterization of the germplasm. The overview of the allelic similarity and difference relationships among the populations was also elucidated. The significant levels of variation found among the Philippine maize populations shall be explored through breeding programs using conventional and new breeding approaches. Plant breeders can use the information to aid them in selecting parents for various breeding purposes, such as, hybrid breeding, forming heterotic groups, genome mapping, and quantitative trait loci identification for important biotic and abiotic traits among others. This study's findings provide only an overview of the diverse genetic background found in traditional Philippine maize populations. Further studies of maize populations distributed in the Philippines not covered this study, can be considered using the set of SSR markers established.

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