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INTRA–SPECIFIC GENETIC DIVERSITY IN WATER YAM (*Dioscorea alata* L.) ACCESSIONS COLLECTED FROM MYANMAR, PAPUA NEW GUINEA AND JAPAN

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ABSTRACT

Understanding germplasm diversity is valuable in improving underutilized water yam (*Dioscorea alata* L.) diversity. In this study, we investigated intra-specific genetic diversity based on morphological traits, SSR polymorphisms and ploidy levels in water yam accessions collected from Myanmar, Papua New Guinea (PNG) and Japan. The results of agro-morphological analysis showed that the distance between leaf lobes, petiole color, and wrinkles on the tuber surface strongly contributed to grouping. Furthermore, SSR polymorphisms were observed in all 12 microsatellite loci analyzed, revealing a mean polymorphic information content of 0.63, with total of 58 alleles detected. Analysis of molecular variance indicated 98% variation within populations and 2% among populations. Meanwhile, ploidy analysis revealed three ploidy levels: 29 diploids ($2n = 40$), 8 triploids ($2n = 60$) and 4 tetraploids ($2n = 80$). Notably, only diploids were observed among accessions from Japan. In all three levels of analysis, no association with geographical origin was observed. Moreover, intra-specific diversity from each origin was relatively close, except during ploidy analysis. These findings suggest, for the first time, that accessions from Japan are as diverse as those from Myanmar and PNG.

Key words: flow cytometry, morphological traits, ploidy, SSR marker, water yam

INTRODUCTION

Yams (*Dioscorea* spp.) are a multi-species tuber crop distributed in Africa, Asia, Oceania, and South America. Water yam is the most widely distributed species because of its agronomic flexibility and productive potential. However, despite the importance of water yam in the diets of millions of people, as well as its contribution to national economies, the continuous accumulation of deleterious somatic mutations and viral infections have led to the clonal degeneration of many genotypes, leading to significant genetic erosion (Baco et al. 2007). Meanwhile, in Africa, water yam is less popular than White yam, while in Asia, it is less popular than Jinenjo (*D. japonica* T.) and Chinese yam (*D. polystachya* T.) varieties. These problems combined with farmers' expectations highlight the need for development of improved varieties that combine higher and more sustainable yields with more marketable attributes. The current problem facing researchers is that traditional cultivars have not been adequately characterized and, as a result, breeding and selection of water yam cultivars with improved characteristics do not exist (Otoo et al. 2009). In order to develop new elite

water yam genotypes showing improved agronomical traits as well as resistance to pests and diseases, plant breeders require a wide range of diversity.

Several reports have documented the genetic diversity of water yam accessions (Obidiegwu et al. 2009, Siqueira et al. 2012, Girma et al. 2017); however, most have concentrated on accessions from West Africa, particularly those from the International Institute of Tropical Agriculture (IITA). Water yam accessions from Myanmar, which is believed to be the center of origin, and Papua New Guinea (PNG), considered the secondary center of diversity, are therefore extremely important (Lebot 2009). Furthermore, water yam accessions from Japan, which has a long history of commercial cultivation of this species as well as being geographically isolated from other yam growing regions, is also likely to play a major role in improving the diversity of this species. However, to the best of our knowledge, little is known about the genetic diversity of water yam accessions from these three regions.

The genebank collections of Tokyo University of Agriculture (TUA) contain water yam germplasm collected mainly from Myanmar, Japan and PNG. Understanding the genetic diversity of these collections will therefore provide much-needed baseline data for efficient breeding programs. Thus, the objective of this study was to elucidate the genetic diversity of water yam accessions collected from Myanmar, Japan and PNG. To do so, 12 agro-morphological trait measurements were examined to determine the variability of selected accessions under field conditions as an important tool and preliminary requirement of cultivar classification. Analysis of Simple Sequence Repeat (SSR) polymorphisms was also carried out. SSRs are the preferred molecular marker system for diversity analyses, since they provide a range of useful features suitable for cultivar identification and genetic diversity assessment. Finally, ploidy analysis was also conducted. Based on the results, future utilization of the genetic diversity in water yam accessions from these three regions was discussed.

MATERIALS AND METHODS

Plant materials

A total of 43 accessions (Table 1) from Myanmar (19 accessions), PNG (12 accessions) and Japan (12 accessions) were used for agro-morphological and SSR analyses. Forty-one of the 43 accessions were used for ploidy analysis because two of the accessions were not suitable for ploidy analysis. This study was conducted from 2016 to 2017. Two seed tuber per accession, weighing approximately 100g each was individually planted in plastic pots filled with mixture of subsoil and 250g of cow-dung compost applied to each plant as base fertilizer and grown in a greenhouse of TUA, Miyako Subtropical Training and Research Farm, Okinawa, Japan (N 24°70’, E 125°28’). Plants were stalked and stems trained, and irrigated. First sowing was carried out in April 2016 and repeated in April 2017 using the same method.

Analysis of intra-specific genetic diversity in agro-morphological traits

The following agro-morphological traits were characterized after harvest in December 2016: tuber shape, roots on the tuber surface, the presence/absence of cracks on the tuber, the tendency of tubers to branch, and the presence of wrinkles on the tuber. In addition, the following morphological traits of upper parts were characterized during the growing season of 2017: petiole color, distance between lobes, position of the widest part of the leaf, and leaf shape. The characters used and methods of data recording were carried out according to the general descriptors for yam (*Dioscorea* spp.) (IPGRI/IITA 1997). Agro-morphological data were recorded either directly from the measurements using a 1-9 scale or a binary recording (1 = present and 0 = absent). Correspondence analysis was applied to all 12 traits (BellCurve for Excel, SSRI Co., Ltd.). As a diversity index, the Shannon–Weaver diversity index (*SH*) was subsequently calculated as $SH = -\Sigma^{n} p_1 \log_2 p_1$, where n and p_1

represent the number of classes of the trait and the proportion of accessions in the ith class of the trait, respectively (Shannon and Weaver 1948).

Table 1. Geographic origin of the water yam accessions used.

Accession number	Geographic origin
A-9	Papua New Guinea
A-10	Papua New Guinea
A-17	Japan
A-18	Japan
A-21	Japan
A-23	Papua New Guinea
A-24	Papua New Guinea
A-26	Papua New Guinea
A-27	Papua New Guinea
A-28	Papua New Guinea
A-33	Papua New Guinea
A-37	Myanmar
A-39	Myanmar
A-43	Papua New Guinea
A-45	Japan
A-46	Papua New Guinea
A-48	Papua New Guinea
A-50	Japan
A-53	Myanmar
A-54	Myanmar
A-57	Myanmar
A-61	Japan
A-70	Japan
A-73	Myanmar
A-74	Myanmar
A-76	Myanmar
A-78	Myanmar
A-80	Myanmar
A-86	Myanmar
A-87	Myanmar
A-88	Myanmar
A-98	Japan
A-99	Japan
A-100	Myanmar
A-102	Myanmar
A-104	Myanmar
A-108	Japan
A-131	Japan
A-132	Japan
A-133	Japan
A-135	Myanmar
A-136	Myanmar
A-137	Myanmar

Analysis of genetic diversity based on SSR polymorphisms

Leaf samples were collected from individual plants of each accession grown in the greenhouse of TUA in September 2016. Collected leaves were then lyophilized and kept under dry cool conditions until use. Genomic DNA was extracted from young leaves using a modified cetyl trimethylammonium bromide (CTAB) procedure, as described by Mignouna et al. (2003), and quantification was carried out using a BioSpec-nano spectrophotometer (Shimadzu, Japan) at 260 nm. Extracts were diluted in sterilized distilled water to obtain a DNA concentration of 20ng/µL. A total of 12 SSR primer pairs selected from the report by Tamiru *et al*., (2015) were then used to classify the accessions. Polymerase chain reaction (PCR) was conducted in a 96-well plate using GenAmp PCR system 9700 (Applied Biosystems, USA) in a total volume of 10 µL containing 1 × ExTaq Buffer (TaKaRa, Japan), 200 µM of dNTPs (deoxynucleotide triphosphates), 1 µM of each forward and reverse primer, 0.5 U of Takara ExTaq HS, and approximately 20 ng of template DNA. The amplification conditions were 3 min initial denaturation at 94°C, followed by 35 cycles of denaturation at 94°C for 30 s, annealng at 55°C for 30 s, and extension at 72°C for 1 min, with a final extension at 72°C for 10 min. The resulting products were electrophoresed on 8% polyacrylamide gel (mono: bis 29: 1) in 1 × TBE (tris-borate-ethylenediaminetetraacetic acid) buffer at 110 V for 80 min. Gels were stained with ethidium bromide solution, and photographs were taken using the gel documentation analysis system Atto Printgraph 2M (Atto Corp., Japan). A 20-bp DNA ladder was used as a reference to estimate the size of specific DNA bands in the gel images of PCR-amplified products using CS Analyzer 4 software version 2.2.4 (ATTO Corp., Japan).

Genetic diversity parameters such as the number of alleles (NA) and expected heterozygosity (He) were computed using GenAlex software version 6.50 software (Peakall and Smouse, 2012). Furthermore, to determine the discriminatory power of the microsatellite loci, the polymorphic information content (PIC) of each locus was computed using PIC = 1−∑Pi2, where Pi2 refers to the sum of the *i*th allelic frequency of each microsatellite locus for the genotypes. Principal Coordinates Analysis (PCoA) was performed to reveal the structure of the genetic diversity within each population using the same software. The genetic distance between accessions based on allele size in base pairs was subsequently determined from the SSR assays and PCoA was conducted based on the genetic distance using GeneAlEx 6.5. To assess the distribution of gene diversity and estimate the components of variance in each population, analysis of Molecular Variance (AMOVA) was also performed.

Analysis of intra-specific genetic varation in ploidy level

Ploidy variation was determined in 41 of the 43 genotypes (18 from Myanmar, 11 from PNG and 12 from Japan) using a Partec ploidy Analyser (Sysmex Partec, Germany). Young leaves were collected from individual plants of each accession grown in the greenhouse of TUA. Rice (*Oryza sativa* L.) was used as an inter-reference. The chromosome number of rice (2x =40) was determined by microscopic observation. Ploidy levels were determined following the procedure of Babil et al. (2010). Leaf blade samples of water yam (approximately 5 mm^2) and rice were chopped with a sharp razor blade to homogenize the tissues and release nuclei in a Petri dish containing 0.4 mL nuclei extraction buffer (CyStain® UV Precise P reagent kit). The homogenate was mixed and filtered through a nylon filter (50-µm pore size) into a plastic tube then the cell suspension was incubated for 1-2 min at room temperature before staining the extracted nuclei with 1.6 mL staining buffer (CyStain® UV Precise P reagent kit). After staining for five minutes at room temperature (approximately 25°C), flow cytometry was conducted at a rate of 5-20 nuclei/s. The DNA index (DI = mean channel number of the sample/mean channel of the reference standard) of each accession was then calculated based on the relative amount of DNA in nuclei at the G_1 stage compared with rice. Flow cytometry was repeated two or three times with different leaf samples to confirm the DI of each accession. Ploidy levels of the accessions were then determined by comparing the DI of the accessions with that of the diploid accession "A-50", which has a chromosome number of 2*n* = 40 (Babil et al. 2010).

RESULTS AND DISCUSSION

Intra-specific genetic diversity in agro-morphological traits

The SH index of each trait (Table 2) ranged from 0.78 (position of the widest part of the leaf) to 1.38 (tendency of the tubers to branch). Of the 12 agro-morphological traits analyzed, high variability was observed in six: the tendency of the tubers to branch, leaf shape, tuber shape, petiole color, the presence of wrinkles on the tuber surface, and the presence of roots on the tuber surface. Of the 43 accessions, 67% were not branched, 21% were slightly branched, 5% were branched, and 7% were highly branched. A low branching tendency is one of the most important agronomic traits when considering the mechanization of harvesting. The high proportion of accessions showing no branching in the genetic resource of TUA is therefore significant. The average SH for the Myanmar, PNG, and Japan accessions was 1.17, 0.86, and 0.99, respectively. According to our observations in phenotypic analysis, the Myanmar accessions are the most diverse followed by Japan.

The first two principal coordinates in the scatter plot (Fig. 1) obtained based on correspondence analyses explained 23.24% and 20.99% of the total variation, respectively. Traits that strongly contributed to grouping at the first axis were distance between lobes, the point of tuber branching, petiole color and the position of the widest part. Additionally, traits that contributed to the grouping at the second axis were the presence of wrinkles on the tuber surface, petiole color, presence/absence of cracks on the tubers, roots on the tubers, and tuber shape. In other words, these traits could be used as key morphological traits in evaluating water yam genetic resources. No grouping based was observed in terms of geographical origin in the scatter plot obtained with the first two principal coordinates (Fig. 1), suggesting a common origin and/or exchange of accessions in the past. Similarly, Anokye et al. (2014) evaluated 107 morphological characters to assess 49 accessions of water yam from Ghana, and revealed that characters that contributed towards differentiation of the accessions were tuber skin and flesh color, leaf margin color, leaf shape, petiole wing color, spine shape on the stem, and branching of stems aboveground. According to their report, morphological descriptors also grouped the accessions into distinct clusters independent of the place of collection.

Table 2. Shannon diversity indexes of the agro-morphological traits.

Trait	Shannon Diversity Index			
	Myanmar	**PNG***	**Japan**	**Average**
Tuber shape	1.50	0.91	1.25	1.22
Tendency to branch	1.73	0.92	1.48	1.38
Point where tuber branches	1.03	0.80	0.87	0.90
Roots on tuber surface	1.00	0.88	0.91	0.93
roots on tuber surface	1.09	0.85	0.93	0.96
Location of roots on the tuber	0.92	0.70	1.00	0.87
Wrinkles on tuber surface	1.12	0.88	0.91	0.97
Absence/presence of cracks	0.93	0.79	0.82	0.85
Leaf shape	1.42	1.10	1.19	1.24
Distance between lobes	0.92	0.77	0.79	0.83
Position of the widest part of leaf	0.88	0.74	0.71	0.78
Petiole color	1.45	1.03	1.05	1.18
Average	1.17	0.86	0.99	1.01

*****Papua New Guinea

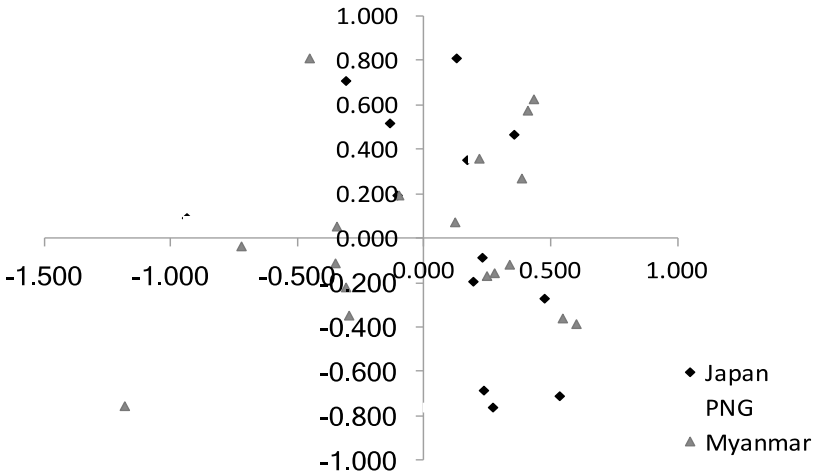

Fig. 1. Scatter plot based on PCoA of agro-morphological traits of water yam accessions from Myanmar, Papua New Guinea (PNG), and Japan

SSR polymorphisms in the accessions

A total of 58 alleles were amplified using 12 microsatellite loci in 43 accessions, with the number of alleles per locus varying from three to seven (Table 3). SSR markers YM53 and YM33 recorded the highest number of alleles (7 alleles each). *He* at each locus ranged from 0.52 to 0.80, with an average of 0.68 (Table 3). The mean PIC for all SSR markers used was 0.63, ranging from 0.44 (YM44) to 0.77 (YM53) (Table 3). Markers with more alleles but a lower allele frequency had a larger PIC as found in YM53 (0.77) followed by YM24 (0.75).

Table 3. Summary of the diversity parameters generated using SSR primers.

Primer ID	Number of alleles (Na)	Expected heterozygosity (He)	PIC*
YM02	5	0.70	0.65
YM03	4	0.58	0.53
YM09	5	0.71	0.66
YM12	3	0.58	0.52
YM21	5	0.69	0.64
YM24	6	0.78	0.75
YM33	7	0.79	0.76
YM37	5	0.69	0.64
YM44	3	0.52	0.44
YM53	7	0.80	0.77
YM55	4	0.59	0.54
YM75	4	0.70	0.64
Total/mean	58.00	0.68	0.63

***** Polymorphic information content

The usefulness of molecular markers can be measured based on their PIC value. Girma et al. (2015) recorded an average PIC of 0.43 when screening 121 water yam accessions showing aerial tuber production and six without aerial tuber maintained at the Genebank of IITA, Nigeria, using eight SSR markers. In his results, PIC ranged from between 0.33 and 0.50, lower than those obtained in the present study. Moreover, Otoo et al. (2015) revealed a high PIC value when investigating 49 water yam accessions from Ghana and Nigeria using 14 SSR markers. The average PIC value for all markers in Otoo's research was 0.91. These findings suggest a very high PIC compared with this study. Similar to our results, Obidiegwu et al. (2009 assessed 89 accessions of water yam collected from Benin, Congo, Côte d'Ivoire, Equatorial Guinea, Gabon, Ghana, Nigeria, Sierra Leone, and Togo using 13 SSR markers, revealing an average PIC value of 0.65 (range: 0.30 to 0.82). Overall, the results suggest that the SSR marker sets used in the present study, especially YM 53 and YM24, are highly efficient and could be used to analyze genetic diversity in water yam genetic resources. Moreover, the Na, He, and PIC (Table 4) values observed in each region were similar. That is, the PIC of the Myanmar accessions was 0.64, of the PNG accessions was 0.63 and of the Japan accessions was 0.61 (average: 0.63). This suggests that the water yam accessions from Japan are equally diverse as those from Myanmar and PNG, regions considered the center of diversity.

Table 4. Summary of SSR polymorphism observed in water yam accession from Myanmar, Papua New Guinea and Japan

Population	Number of alleles (Na)	Expected heteroygosity (He)	PIC*
Myanmar	5.00	0.69	0.64
PNG**	4.75	0.67	0.63
Japan	4.67	0.67	0.61
Total/mean	14.42	0.68	0.63

* Polymorphic information content, ** Papua New Guinea

The AMOVA results (Table 5) revealed that the highest percentage of variation (98%) was within populations of water yam, while the gene diversity among populations was low (2%). This may be attributed to the diverse geographical origin of the accessions, generating variability within populations. Our AMOVA analyses agree with those of Mengesha et al. (2013), who revealed a low but significant mean fixation index (FST) for yellow yam in Ethiopia, suggesting that the majority of microsatellite diversity was within rather than between populations. Moreover, similar to our findings, Siqueira et al. (2012) revealed a weak population structure among sampling regions, with most of the genetic diversity concentrated within regions (95.9%; 4.1% of the variance conserved among regions).

Table 5. Summary of the AMOVA results

Source	df	SS	Estimated variance	% Total
Among populations	2	30.29	0.25	2%
Within populations	40	462.61	11.57	98%
Total	42	492.91	11.82	100%

PCoA of the molecular data showed that the first two axes of the resulting scatter plot (Fig. 2) accounted for 11.93% and 20.64% of the total variation, respectively. Moreover, members of the same geographic origin were not assigned to the same group (Fig. 2) and there was no distinct grouping of accessions by population. These observations perhaps imply that geographical location did not play a

major role in differentiation of this species, as observed in almost every quadrant (I, II, III, and IV). This interpretation is in agreement with Malapa et al. (2005), who evaluated 83 water yam accessions from Asia, Africa, and Melanesian using amplified fragment length polymorphism (AFLP) markers and found no distinction between country cultivars. Thus, the non-distinction between country cultivars shown in the clustering pattern strengthens the inference that the clones must have been widely distributed, in agreement with previous studies by Egesi et al. (2006) and Tamiru et al. (2015) using AFLP markers.

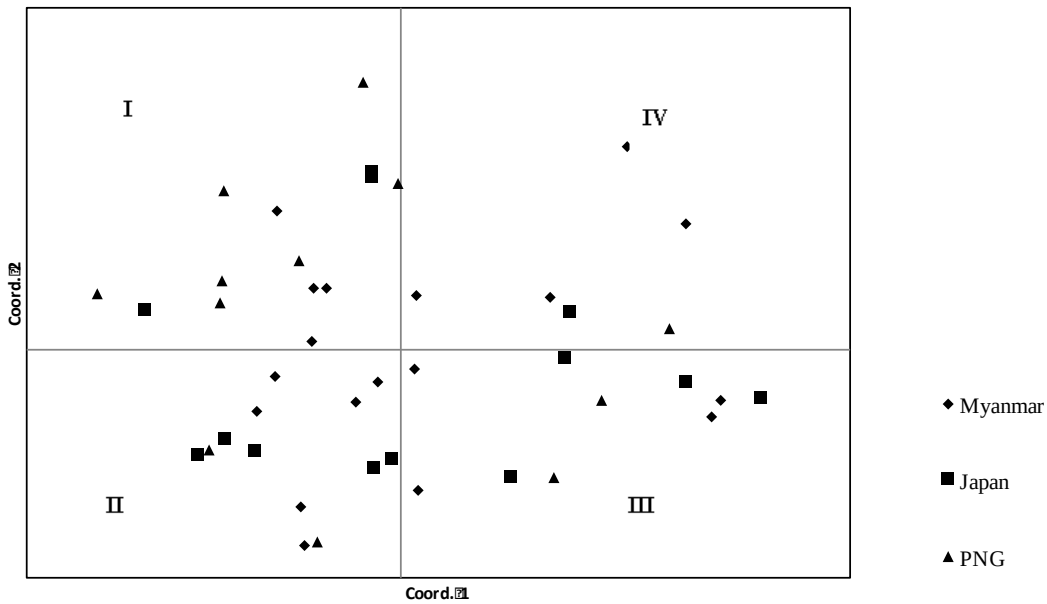

Fig. 2. Scatter plot based on PCoA of SSR polymorphisms observed in water yam accessions from Myanmar, Papua New Guinea (PNG), and Japan.

Intra-specific genetic diversity in ploidy level

The present study also investigated intra-specific ploidy variation. The DI of the standard diploid accession "A-50", which has a chromosome number of $2n = 40$, was confirmed as 1.6. Using this standard, the accessions were divided into three groups based on DI values. Values of the first group ranged from 1.4 to 1.9, indicating a ploidy level identical to that of "A-50"; of the second group ranged from 2.0 and 3.1, suggesting a triploid ($2n = 60$) ploidy level; and of the third group ranged from 3.2 and 3.5, suggesting a tetraploid ($2n = 80$) ploidy level. The position of G_2 peaks of each water yam accession was also considered while deciding the DI range of each ploidy group. Of the 41 accessions examined, 29 (70.7%) were diploid, 8 (19.5%) were triploid and 4 (9.8%) were tetraploid as shown in Table 6. Interestingly, all three ploidy levels were observed in accessions from Myanmar and PNG, while only diploid variation was observed in accessions from Japan.

Water yam is thought to display the greatest intra-specific diversity among all species of *Dioscorea*. Studies on intra-specific ploidy variation in water yam have demonstrated that the most widely recognized forms are diploids, followed by triploids, while tetraploids are uncommon, tending to exist in diversified centers in Asia and the South Pacific (Abraham and Nair 1991, Arnau et al. 2009, Lebot 2009). The present authors adopt the basic chromosome number (x =20), as proposed by Arnau et al. (2009). However, in the past, ploidy analyses were carried out in accordance to the basic chromosome number (x = 10). For example, ploidy analyses of water yam accessions mostly from

West African countries, Chad, and Puerto Rico revealed that 84.9% were hexaploid (2*n* = 60) and 15.1% were tetraploid (2n = 40) (Egesi et al. 2002). In addition, Gamiette et al. (1999) reported three ploidy levels, tetraploid (2*n* = 40), hexaploid (2*n* = 60), and octoploid (2*n* = 80), in accessions from West Indies, with few clones from India, Nigeria, New Caledonia, and the Ivory Coast. The presence of tetraploid (2n = 40), as identified in their reports was similar to the present report.

Table 6. Summary of the observed ploidy variation

Origin	Diploid (2*n* = 40)	Triploid (2*n* = 60)	Tetraploid (2*n* = 80)
Myanmar	10 (24.4%)	6 (14.6%)	2 (4.9%)
PNG*	6 (14.6%)	2 (4.9%)	2 (4.9%)
Japan	13 (31.7%)	0	0
Total	29 (70.7%)	8 (19.5%)	4 (9.8%)

*Papua New Guinea

In this study, genetic variability at the ploidy level was high in accessions from Myanmar and PNG, with all three levels of ploidy observed. This high level of ploidy variation is in accordance with a previous report suggesting that Myanmar is the primary center of diversity and PNG the secondary center of diversity for water yam (Coursey 1967). In contrast, no triploids or tetraploids were observed among the 12 accessions from Japan, possibly because triploid and tetraploid maturity takes longer and growth is slower than that of diploids (Singh 2016). In line with this, accessions from Japan appear to be adapted to the short growing season. However, triploid variants tend to be more vigorous in terms of vegetative growth and tuber production than diploid and tetraploid variants (Malapa et al. 2005, Lebot 2009).

Association between ploidy level and geographic origin was not observed, as in Babil et al. (2010). Overall, the most significant finding in the present study was the ploidy information from Asia pacific accessions of water yam, especially those from the centers of diversity. Identification of ploidy level is a preliminary step towards the establishment of water yam breeding programs in the Asia Pacific. It provides information on the variation in ploidy within water yam species in a region for which such data is scarce, with the exception of Babil et al. (2010) who examined accessions from Myanmar. Polyploidy is fundamental for advanced breeding programs, and is therefore strongly related to the utilization of genetic resources of water yam. Triploids and tetraploid accessions were closely clustered in the scatter plots based both on phenotypic traits as well as SSR polymorphisms. Phenotypically, leaves of triploid accessions were observed to be larger than that of tetraploid cultivars. Moreover, a relationship was also observed between morphological traits, SSR polymorphisms and ploidy among accessions. This is sometimes displayed in terms of phenotypic features, especially in the leaves and tubers (Babil et al. 2010, Girma et al. 2015).

The results of the present study also illustrate the existence of genetic polymorphisms in the water yam germplasm. Various factors can influence genetic diversity, notably mutation, population gene flow and sexual recombination. The high genetic diversity observed in this study could be the result of an accumulation of mutations, which can create variation within a species or population. In addition, spontaneous hybridization must have contributed to the ancestry of certain accessions, although the selection of somatic mutants was likely the main source of variability used by farmers in plant improvement practices. Vegetatively propagated crops usually maintain a high level of heterozygosity, especially in dioecious plants such as yam. This genetic variation offers significant potential for genetic improvements because it implies a high level of genetic variance upon which selection can be made (Obidiegwu et al. 2009, Otoo et al. 2015).

CONCLUSION

The most significant finding in this study was the genetic diversity information from Myanmar and PNG, centers of diversity for which information was previously scarce. In addition, this is the first report to analyze Japanese accessions in detail. In all three diversity investigations, no major association with geographic origin was observed. The findings obtained are therefore highly valuable in terms of breeding and conservation of genetic resources in water yam. Moreover, the high genetic diversity in Japanese water yam clones was observed for the first time, highlighting their potential utilization in water yam improvements.

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