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Morphological, Phytochemical and Molecular Characteristics of NSIC-Registered Varieties of Garlic (*Allium sativum* L.) in the Philippines

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ABSTRACT

Background: Garlic belongs to the largest monocot genus, *Allium*, comprising different species known for their use as spices and medicinal plants. It ranks second in volume and area of production among the *Allium* species, next to onoin. The Philippines is renowned for garlic varieties with a strong and pungent odour observed in the seven accessions registered at the National Seed Industry Council (NSIC), Department of Agriculture, Philippines. Morphological, phytochemical and molecular characterization of the different garlic varieties would provide baseline information important to the identification of the registered varieties, their conservation and improvement.

Methods: Physiologically mature and disease-free NSIC-registered garlic accessions were characterized and evaluated at the Institute of Crop Science, University of the Philippines Los Baños. The bulbs were separated from each other and planted in a homogenous field. Morphological characterization was done from the vegetative stage until bulb harvesting using an *Allium* spp. descriptor list. The mature bulbs of each accession were screened for phytochemicals and alliin content. SSR markers were used to fingerprint the eight garlic accessions.

Result: Qualitative characters showed low diversity (H' = 0.22), while quantitative characters had intermediate diversity (H'= 0.51). Identical phytochemicals, flavonoids and phenols, were detected across all the garlic accessions. Despite this, alliin content varied considerably with Bang-ar and Mindoro having the highest (37.70±0.70 mg/g) and lowest (17.22±0.68 mg/g) alliin contents, respectively. Only one SSR primer, ASA-24, showed polymorphic bands that can discriminate Batanes White and MMSU Gem from the other NSIC-registered garlic varieties.

Key words: Alliin content, Characterization, Diversity, SSR markers, Morphology.

INTRODUCTION

Garlic (*Allium sativum* L.) is a bulbous flowering plant belonging to the Amaryllidaceae family that is native to the Central Asian region extending to Northern Iran. It is cultivated throughout the world with Asia having 90% of the world's production, the bulk of which is coming from China with more than 20 MT of annual production in 2021 (FAO, 2022). Garlic is commonly used as a spice and ingredient in most Filipinos dishes (Lopez and Anit, 1994) and for traditional medicine in some indigenous communities to treat dog bites, phlegm and measles (Tantengco *et al*., 2018). Currently, the Philippines has seven NSIC-registered varieties of garlic, namely–Ilocos White, Ilocos Pink, Batanes Red, Mexican, Bang-ar, Ilocos Tan Bolters and MMSU Gem (BAFS, 2021). Despite the availability of these newly registered varieties, there is a dearth of information regarding their morphological, phytochemical and molecular characteristics.

Characterizing germplasm materials contributes to the efficient and effective use of genotypes for future crop improvement. It also allows the breeders to avoid duplication in collections (Dalir and Safarnejad, 2017). Morphological characterization is the first step in classifying and assessing plant genetic resources for it allows for identifying and selecting desirable traits (Malek *et al*., 2014). Likewise, phytochemical characterization is also an effective tool for differentiating among medicinal plant germplasm (Nsuala *et al*., 2017). Since garlic has vital use as food and medicine, the World Health Organization (WHO) emphasized the evaluation of the effectiveness of drugs found in the plant

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(Ayyanar *et al*., 2008). Several techniques have been adopted to detect the presence of chemical compounds in plants of which high-performance thin layer chromatography (HPTLC) method is commonly preferred (Akabari *et al*., 2015). HPTLC remains one of the most flexible, reliable and cost-efficient separation techniques for analyzing botanicals and herbal drugs (Attimarad, 2011). On the other hand, molecular markers are essential in breeding programs and germplasm conservation for determining phylogenetic relationships, identifying species and gene mapping (Amom *et al*., 2017). Farooq and Azam (2002) emphasized the role of DNA fingerprinting in germplasm management to ensure genetically diverse and uniform germplasm.

Therefore, characterizing the different garlic varieties using these tools would provide baseline information important to the conservation and improvement of the crop. Thus, this study aimed to characterize the NSIC-registered garlic varieties along with two landraces for their morphological characteristics and phytochemical properties and to develop DNA fingerprints.

MATERIALS AND METHODS

Germplasm acquisition

Physiologically mature and well-developed bulbs from average-sized to big cloves of the six NSIC-registered varieties and two additional local accessions were obtained from the Department of Agriculture-Ilocos Integrated Agricultural Research Center (DA-ILIARC) in the Philippines. The planting materials were protected from diseases and mechanical damage for good growth and development. Bulbs were prepared first by splitting the clove from each other with the thick protective scale intact.

Crop establishment

The experiment was conducted from February 2020 to June 2021 at the Crop Breeding and Genetic Resources, Institute of Crop Science, College of Agriculture and Food Science (CAFS), University of the Philippines, Los Baños (UPLB). Plots measuring 1.5 × 0.3 m were prepared following two passes of plough and harrow at seven-day intervals. The different garlic varieties were assigned randomly to each plot containing 24 hills with a planting distance of 15 × 15 cm. Cloves were sown upright up to 2/3 of the length at 3 cm planting depth. Plastic tunnels were installed to protect the plants from excessive rain. Weeding was done twice a week or as the need arose either manually for those within the bed, or using a grass cutter when between plots. Irrigation was applied 1-2 days before planting to ensure sufficient moisture until 70-85 days after planting. Mature bulbs were harvested once 75% of the leaves have turned yellow or approximately 100-120 days after planting.

Morphological characterization and evaluation

The eight garlic accessions were characterized using the *Allium* spp. descriptor lists developed by the International Plant Genetic Resources Institute, European Cooperative Programme for Crop Genetic Resources Networks (ECPGR) and Asian Vegetable Research and Development Center (IPGRI, 2001). Morphological characterization focused only on the vegetative stage using only the bulb and leaf descriptors.

Phenotypic diversity was estimated using the standardized Shannon Weaver's diversity index (H') and following the diversity index criteria (H'≥0.67= high; 0.34 ≤ H2≤0.66 = intermediate; 0.01≤H′≤0.33= low) of Eticha *et al*., (2006). On the other hand, qualitative data were scored using presence (+) and absence (-). A dendrogram was generated based on the unweighted pair group method of the arithmetic mean (UPGMA) computed through Gower's distance using XLSTAT software.

Phytochemical screening and alliin content determination

For each accession, 1.0 gram of fresh cloves was ground in 10 ml methanol and water solution having a ratio of 8:2, which was then filtered and subjected to further analysis. The derivation of reagents was performed with 0.6 g of ninhydrin dissolved in 190 ml isopropanol and 10 ml of acetic acid. Chromatographic conditions used HPTLC silica gel 60 F254 (Merck) plates at 10 × 10 cm or 20 × 10 cm. Plates were pre-washed with methanol and then dried at 120°C for 20 minutes. The mobile phases used n-butanol, water, acetic acid and formic acid with a ratio of (28:8:9:2). Sample applications were done using the standard, 3 uL and 5 uL of test solution, applied in 8 mm bands with 2 mm as minimum part and 8 mm from the lower edge of the plate. The developing solvent was done at 10 × 10 cm or 20 × 20 cm Twin through the chamber. The unsaturated 5 mL developing solvent was done by developing a distance of 60 mm from the lower edge of the plate. After drying for 5 minutes, the plates were immersed in ninhydrin reagent for 1 second, then heated at 120°C until the band's colors were stable for 2-5 minutes. Examination of the results was done under white light. One-way Analysis of Variance (ANOVA) followed by post hoc analysis (Tukey's highly significance difference test) was used to determine significant differences in alliin content.

The different *Allium* accessions were screened for the presence of alkaloids, phenols, terpenoids, saponins and tannins using alkali, acetate, Salkowski, froth and $FeCl_3$ tests, respectively. The presence of specific phytochemicals was indicated by color changes (intense yellow for flavonoids, greenish-gray for tannins), formation of precipitates (white for phenols), interface (reddish-brown for terpenoids) and foam (saponins) (Mamta and Jyoti, 2012).

Molecular characterization

DNA extraction and quality check

Mature, healthy and well-developed leaf samples were collected and stored at -20°C before processing. DNA was extracted following the modified protocol of Doyle and Doyle (1987) and quantified using Epoch Microplate Spectrophotometer (Biotek, USA). The quality was evaluated

by running the samples in agarose gel electrophoresis. Bands were visualized by staining the agarose gels with GelRed™ (Biotium, USA) and viewed using the UVIdoc Documentation System.

PCR Amplification using SSR markers

DNA sequences were amplified using the three microsatellite primer pairs (Table 1) through polymerase chain reaction (PCR). The final volume of the PCR mixture was 20 µl containing 1X PCR buffer, 1 U/µl Taq Polymerase, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 µM each of forward and reverse primers and 520 ng/µl of DNA. PCR reaction began with an initial denaturation at 94°C for 3 minutes and was followed by 35 cycles of denaturation at 94°C for 30 seconds, primer annealing at each annealing temperature (Table 1) for 45 seconds and elongation at 72°C for 1 minute. This concluded with a final elongation step at 72°C for 10 minutes.

Molecular marker scoring and analyses of banding patterns

The PCR product was resolved on 1.4% and 1.7% agarose gel by running at 100 V for 30 minutes. A 50-bp DNA ladder was used as a molecular size standard. The gels were stained with GelRed and subsequently photographed using a gel photo-documentation system bands were scored as present (1) and absent (0) for each SSR marker. DNA fingerprints were visualized through the elucidation of their banding patterns. Polymorphism information content (PIC) value was calculated for each primer following the method of Botstein *et al*. (1980).

RESULTS AND DISCUSSION

Morphological characteristics

Qualitative descriptors for both clove and bulb had low diversity, H'= 0.22 while quantitative descriptors had intermediate diversity, H'= 0.51 (Fig 1; Table 2). For clove descriptors, both qualitative and quantitative traits showed intermediate diversity, H'= 0.38, except for the number of cloves per bulb, having low diversity (0.23). On the other hand, no diversity was found in all of the qualitative bulb descriptors but bulb skin color with intermediate diversity (0.34) while both the quantitative descriptors, bulb height and weight, exhibited intermediate diversity (0.65) (Fig 1; Table 3). Interestingly, only the intensity of anthocyanin coloration at the pseudostem base registered high diversity, H'= 0.82, among all the qualitative descriptors while only the number of cloves per bulb recorded low diversity for

Table 1: Characteristics of the three microsatellite loci amplified in garlic accessions (Cunha *et al*., 2012).

Primer code	Forward and reverse primer sequence (5′-3′)	Repeat motif	Ta (°C)	Allele size (bp)	GenBank accession no.
Asa08	F: TGATTGAAACGAATCCCACA R: GGGGGTTACCTGAACCTGTTA	(GT)8	56	209-257	JN084088
Asa10	F: TTGTTGTTCTGCCATTTT R: GATCTAAGCCGAGAGAAA	(AC)7	48	225-239	JN084089
Asa24	F: TTGTTGTGCCGAGTTCCATA R: CAGCAATTTACCAAAGCCAAG	(GT)4(GT)3(GT)5	48	149-161	JN084096

Table 2: Qualitative morphological trait differences among garlic accessions (n=8).

Qualitative traits	Common trait	Unique trait	H'	Diversity
Intensity of anthocyanin coloration at pseudostem base	Weak	Strong	0.82	High
Bulb skin color	White	Light violet	0.34	Intermediate
Compactness of clove	Compact	Medium	0.51	Intermediate
Clove scale color	Cream	Brown	0.34	Intermediate
Distribution of cloves	Radial	Non-radial	0.34	Intermediate
Clove flesh color	White	Yellowish	0.34	Intermediate
Bulb structure type	Regular multi-fan groups		0	No diversity
Shape of mature garlic bulb	Heart-shaped, basal plate retracted		0	No diversity
Bulb base shape	Rounded		0	No diversity
Bulb shape in cross-section	Circular		0	No diversity
Shape of the compound bulb in horizontal section	Circular		0	No diversity
Pseudostem length	Short		0	No diversity
Average			0.22	Low

quantitative descriptors. The low diversity observed for qualitative traits can be attributed to geographical relatedness as discussed previously in Egea *et al*. (2017) and Kumar *et al*. (2018) where accessions collected from neighboring locations tend to cluster together. Comparably, Trani *et al*. (2005) observed high genetic diversity for quantitative traits among garlic germplasm in Sao Paolo, Brazil. This is, however, contrary to the findings of Panthee *et al*. (2006) on the discriminating value of qualitative characters at the varietal level, as with the case of garlic accessions from greater Asia and Southeast Asia having highly similar quantitative traits for clove and bulb (Hirata *et al*., 2016). This only confirms that morphological traits alone do not always readily reflect genetic variations among germplasm collections.

Cluster analysis using both qualitative and quantitative traits revealed three clusters (Fig 2). Mindoro White can be delineated by its light violet clove skin colour. Batanes White, Bang-ar and Ilocos Pink showed similar morphological characters having long bulb height and low intensity of anthocyanin coloration at pseudostem base. On the other hand, the rest of the accessions have relatively smaller bulbs exhibited in its bulb height and weight.

Alliin content determination

The eight garlic accessions were found to contain significantly different alliin contents (Table 4). Among these, Bang-ar and Mindoro White showed the highest, 37.70±0.70 mg/g and lowest, 17.22±0.68 mg/g, alliin contents, respectively. The observed higher alliin content in the varieties grown in areas under Type I climate (with pronounced wet and dry seasons), compared with Mindoro, cultivated within Type 3 climate (without pronounced wet and dry seasons) corroborates the findings of Singh and Hiremath (2013) that alliin content varies among garlic varieties grown under differing climatic conditions. In addition, Huchette *et al*. (2005) confirmed that along with genotype influence, environmental conditions affect alliin accumulation in garlic. According to Rahman (2007), alliin is the main sulfur compound in raw and powdered garlic, averaging ~8 mg/g alliin per clove, with the highest attainable

Table 3: Quantitative morphological trait differences among garlic accessions (n=8).

Quantitative traits	Highest	Lowest	H'	Diversity
Height of bulb (cm)	8.27	2.94	0.65	Intermediate
Weight of bulb (g)	12.58	4.96	0.65	Intermediate
Width/diameter of pseudostem (cm)	6.32	4.42	0.49	Intermediate
Number of cloves per bulb	20	11	0.23	Low
Weight of clove (g)	10.9	4.3	0.53	Intermediate
Average			0.51	Intermediate

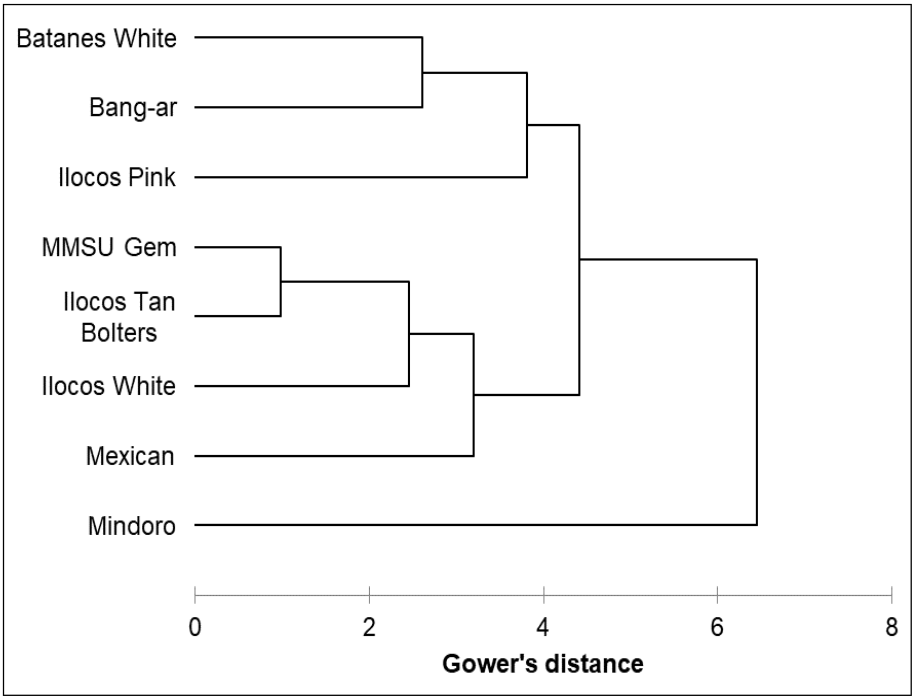


Fig 1: Dendrogram of eight garlic accessions based on morphological characteristics.

Table 4: HPTLC quantification of alliin content of garlic accessions.

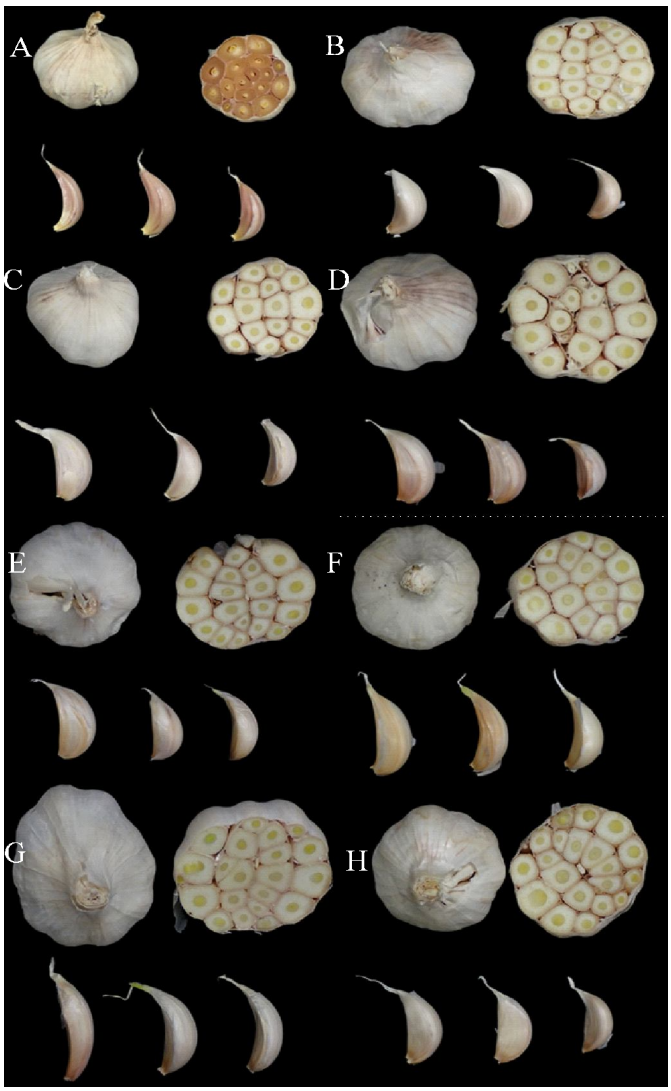
Accessions	Alliin content (mg/g)*
Mindoro	17.22±0.68^{f}
Ilocos white	28.71±0.62^{bc}
Batanes white	32.28±0.76^{b}
Ilocos pink	27.95±1.10^{cd}
Mexican	22.58±1.30^{e}
Bang-ar	37.70±0.70^{a}
Ilocos Tan Bolters	24.35±0.31^{de}
MMSU Gem	30.35±1.43^{bc}

*Accessions with the same letter are not significantly different (α=0.05).

content of 20-25 mg/g in powder form. The alliin content of Philippine garlic varieties is comparable with those of the Indian garlics and higher than those from China (Siddiqui *et al*., 2016) and the different ecophysiological groups in Iran (Akbarpour *et al*., 2021) and Argentina (Gonzalez *et al*., 2009). Kim *et al*. (1994) reported that the degree of pungency is positively correlated with alliin content.

Phytochemical screening

Detection of phytochemicals revealed that only flavonoids and phenols are present in all the garlic varieties studied (Table 5). Similarly, Arify *et al*. (2018) confirmed the presence of phenols and flavonoids and the absence of tannins in garlic accessions from India. Strati *et al*. (2018) regarded



(A. Mindoro; B. Ilocos White; C. Batanes White; D. Ilocos Pink; E. Mexican; F. Bang-ar; G. Ilocos Tan Bolters; H. MMSU Gem).

Fig 2: Bulb structure of eight garlic genotypes.

Table 5: Qualitative phytochemical analysis of eight garlic accessions.

Accessions/variety	Flavonoids	Phenols	Terpenoids	Saponin	Tannins
Mindoro	+	+	-	-	-
Ilocos white	+	+	-	-	-
Batanes white	+	+	-	-	-
Ilocos pink	+	+	-	-	-
Mexican	+	+	-	-	-
Bang-ar	+	+	-	-	-
Ilocos tan bolters	+	+	-	-	-
MMSU gem	+	+	-	-	-

(+) denotes the observable presence of the compound.

(-) denotes compound may be absent or present in undetectable amounts.

PRIMER	ALLELE SIZE (bp)	LOCI	ACCESSION							
			Mindoro	Ilocos White	Batanes White	Ilocos Pink	Mexican	Bang-ar	Ilocos Tanbolters	MMSU Gem
ASA08	250	A1								
ASA10	450	B1								
	350	B2								
	250	B3								
	150	B4								
	100	B5								
ASA24	275	C1								
	250	C2								
	225	C3								
	200	C4								

Fig 3: Banding patterns of garlic varieties, including other *Allium* spp. accessions using SSR markers.

Allium species, especially garlic and leek, to be generally rich in phenols and flavonoids. Phenols are considered responsible for the pungency of garlic, while flavonoids influence the stability of *Allium* spp. when cooked and stored (Lanzotti, 2006).

Molecular analysis-SSR markers

Three primers were able to amplify five DNA fragments, three monomorphic and the other polymorphic. ASA-08 primer generated only one monomorphic band at 250 bp while ASA-10 produced two at 250 and 150 bp. On the other hand, two polymorphic bands were detected at 250 and 225 bp in the ASA-24 primer amplification profile (Fig 3). This runs contrary to the results obtained by Kumar *et al*. (2018) where ASA-24 primer failed to amplify any DNA fragment and Anwar *et al*. (2020) where ASA-10 primer was found to be highly polymorphic. Furthermore, it is only in Batanes White that ASA-24 primer was not able to generate any DNA fragment at all amplicon sizes while MMSU Gem is the only accession that has one polymorphic band amplified by the same primer. Thus, only one primer, ASA-24, can be used to discriminate Batanes White and MMSU-Gem among the NSIC-registered varieties and landraces of garlic in the Philippines and could be used to delineate the registered varieties.

CONCLUSION AND RECOMMENDATIONS

We were able to successfully characterize the six NSIC-registered varieties and two additional local accessions from the Philippines. Minimal differences were observed in the morphology among the different garlic accessions; however, distinctive differences were observed in their alliin contents and genotypic characteristics. The primer, ASA-24, showed polymorphic bands that can discriminate Batanes White and MMSU Gem from the other garlic accessions. The established characteristics could serve as an additional resource in identifying registered garlic varieties in the Philippines.

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Conflict of interest

All authors declared that there is no conflict of interest.

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