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Short Communication

Preliminary Evaluation of *rbcL*, *matK*, and SRAP Markers for the Molecular Characterization of Five Philippine *Allium sativum* varieties

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Abstract: *Allium sativum*, commonly known as "*bawang*", is one of the economically important crops in the Philippines mainly for spice and other uses including herbal medicine for hypertension and cancer. Several studies showing therapeutic properties of garlic often do not disclose the variety used while typical varietal identification rely on phenotypic or agronomic traits which may be inadequate to define distinct variants. This study aimed to evaluate the utility of chloroplast genes, *rbcL* and *matK*, as well as Sequence-Related Amplified Polymorphism (SRAP) to define and characterize five *A. sativum* varieties grown in the Philippines. Five garlic varieties (i.e., GNT4, GMX6, IP3A, GMR10 and GTB17) were collected from a demo farm in Batac, Ilocos Norte, Philippines. The bulbs were made to grow roots which were subsequently used for DNA extraction and PCR analysis. Results showed that PCR amplified sequences of *rbcL* and *matK* genes were able to identify *A. sativum* varieties with ample discriminatory power to differentiate closely related species. The identified SRAP markers were also able to show a degree of polymorphism among the five local varieties. The *rbcL* sequences were highly conserved, the *matK*-sequences were able to discriminate GTB17 while the SRAP using the primer combinations ME1-EM3 and ME3-EM3 discriminated all the five varieties. A summary of the concatenated molecular features of the five varieties was provided. Albeit preliminary, the study was able to show the potential of *matK and* SRAP in discriminating the local *A. sativum* varieties from each other and from other varieties of different origin.

Keywords: *Allium sativum*, molecular characterization, *rbcL*, *matK,* SRAP

1. INTRODUCTION

Allium sativum, commonly known as garlic or "*bawang*" in the Philippines, is an herbaceous monocot plant most distinguishable by the bulb at its leaf base. It belongs to the family Amaryllidaceae and is believed to have originated from Central Asia, although it is now widely used worldwide as a food additive or spice (Quisimbing, 1978; Fritsch & Friesen, 2002; Block, 2010; Agarwal, 1996). It has also been known as a natural treatment for various illnesses or diseases as it exhibits antimicrobial, antiseptic, antitoxic, antiviral effects and acts as depurative, diuretic, expectorant, etc. (Agarwal, 1996, Fufa, 2019). It contains chemically active compounds known for their therapeutic effects like flavonoids, saponins, essential oils, etc. and biologically active compounds which have antibiotic and fungicidal properties. In addition, it also has beneficial effects on the cardiovascular system and in cancer (Lanzotti, 2007; Banerjee & Maulik, 2002; Kumar *et al*., 2010; Li et al., 2018). Furthermore, *A. sativum* has also been found by some researches to have activities against insects, nematodes, rodents, and mollusks (Amonkar & Reeves, 1970; Singh & Singh, 2008; Nwanchukwu & Asawalam, 2014).

A. sativum, along with *A. cepa,* commonly known as onion, have varying characteristics such as size, color, flavor and more importantly, chemical components, making them important in traditional medicine, food additive, and in many other aspects (Boukeria *et al*., 2016). The Philippine Bureau of Plant Industry identified six *A. sativum* varieties that are produced in the Philippines. These are the Ilocos White, Tan Bolters, Batanes White, Batangas White, Ilocos Pink and Nueva Ecija Pink. In addition to these, the Mariano Marcos State University in Batac, Ilocos Norte also produces the Native, Mexican and Miracle varieties. Different garlic varieties have different chemical profiles that may be responsible for pest and disease resistance, as well as its medicinal properties. Moreno *et al.* (2016) determined the difference in chemical profile and cytotoxic activity of two varieties, Ilocos White and Native garlic.

The Consortium of Barcode of Life (CBOL) has recommended *rbcL* for its universality, and *matK* for its discriminatory power (CBOL, 2009), and these have been used for the identification and phylogenetic analysis of *Allium* species (Ipek et al., 2014; Lee et al., 2017, Abugalieva et al., 2017; Zarei et al., 2020). Sequence-Related Amplified Polymorphism (SRAP) markers have also been demonstrated to reveal 69.1% polymorphic loci in 40 garlic germplasms from China (Chen, et al., 2013). With these, the study aimed to aimed to evaluate the utility of chloroplast genes, *rbcL* and *matK*, as well as Sequence-Related Amplified Polymorphism (SRAP) to define and characterize five *A. sativum* varieties grown in the Philippines.

2. METHODOLOGY

2.1. Acquisition of Samples

Garlic varieties that were readily available at the time of sampling were collected from the demo farm of Mariano Marcos State University in Batac, Ilocos Norte, Philippines (Table 1). Bulb specimens for each variety were vacuum sealed until used for germination of the cloves. Further experimentation was conducted at the Department of Biochemistry and Molecular Biology (DBMB), University of the Philippines – Manila.

Table 1. Local garlic varieties collected from Batac, Ilocos Norte, Philippines that was used in this study

Sample Code	Identification	Locality	Genbank Accession No.
GNT4	Native	Batac, Ilocos Norte, Philippines	MN239994
GMX6	Mexican		MN239995
IP3A	Ilocos Pink		MN239996
GMR10	Miracle		MN239997
GTB17	Tan Bolters		MN239998

2.2 DNA Extraction

One garlic bulb specimen for each variety was used for DNA extraction. Garlic cloves were pealed and half-submerged in water using floaters to allow roots to grow. About 1 cm of the root tips were cut and used for DNA extraction. DNA extraction was performed following the manufacturer's protocol for i-genomic Plant DNA Extraction Mini Kits (iNTRON Biotechnology, South Korea). Briefly, 50 mg of the root tips were homogenized in a 1.5 mL microcentrifuge tube using a micropestle. Binding, washing and elution of DNA was performed in a spin column. To verify the success of extraction, samples were subjected to 1.2% agarose gel electrophoresis with 0.5X TAE buffer and 1µL GelRed and visualized through a gel documentation system (Bio-rad, USA). Garlic root DNA isolates were then stored in -20°C freezer until used for PCR amplification.

2.3 PCR Amplification, Agarose Gel Electrophoresis, DNA Sequencing, and Sequence-Related Amplified Polymorphism (SRAP) Analysis

Three markers (*rbcL*, *matK* and SRAP) were used in PCR amplification using the Bio-rad T-100 Thermocycler (USA). Genomic DNA was amplified using Promega (USA), and Invitrogen Platinum *Taq* Polymerase for *rbcL/matK* and SRAP, respectively. PCR reaction mixture and thermal cycle profile were set up according to the manufacturer's protocol. Primer sequences and thermal profile is shown in Table 2. The 30 µl PCR reaction mixture of the markers, *rbcL* and *matK* are as follows: 22.7 µL of nuclease-free water, 3 µL of 10X PCR buffer, 1.2 µL of 50 mM $MgCl_2$, 0.6 µL of 10 mM deoxynucleotide triphosphates (dNTP), 0.6 µL of 10 mM forward and reverse primers, 0.3 µL of *Taq* DNA polymerase (5 U/ µL), and 1 µL of the DNA template (20 ng).

Table 2. List of primer pairs used to amplify the *rbcL, matK* and SRAP region. Primer combinations for SRAP were: ME1-EM3 and ME3-EM3; Thermal profile for each marker was also presented.

Marker	Primer name	Primer Sequence (5'-3')	Thermal Profile
rbcL	*rbcL*a-F	5'-ATGTCACCACAAACAGAGACTAAAGC-3'	94^0C for 4 minutes, 94^0C for 30 seconds, 55^0C for 30 seconds, 72^0C for 1 minute, 35 cycles, and 72^0C for 10 minutes.
	*rbcL*a-R	5'-GTAAAATCAAGTCCACCRCG-3'	
matK	*matK*-xf	5'-TAATTTACGATCAATTCATTC-3'	94^0C for 1 minute, 94^0C for 30 seconds, 54^0C for 20 seconds, 72^0C for 50 seconds, 35 cycles and 72^0C for 5 minutes.
	matK-MALP	5'-ACAAGAAAGTCGAAGTAT-3'	
SRAP	ME1 ME3 EM3	5'-TGA GTC CAA ACC GGA TA-3' 5'-TGA GTC CAA ACC GGA AT-3' 5'-GAC TGC GTA CGA ATT GAC-3'	94^0C for 5 minutes, 94^0C for 1 minute, 35^0C for 1 minute, and 72^0C for 1 minute, 5 cycles; 94^0C for 1 minute, 50^0C for 1 minute, 72^0C for 1 minute, 35 cycles, and 72^0C for 5 minutes.

Agarose gel electrophoresis was performed to verify the presence of amplified bands using 1.2% concentration with 0.5X TAE Buffer and 1 µL of gel red, and run at 100V for 30 minutes. The gel containing the successfully amplified products was visualized using gel documentation system (Bio-Rad, USA) (Figures 1a and 2a). PCR amplicons were sent to Macrogen, South Korea for bidirectional sequencing.

SRAP was performed using twenty-five primer combinations to determine the polymorphic sites of each garlic variety. The 10 µl PCR reaction mixture of the SRAP marker are as follows: 3.6 µL of nuclease-free water, 5 µL of Invitrogen Master Mix, 0.2 µL of 10 mM forward and reverse primers, and 1 µL of DNA template (20 ng). Agarose gel electrophoresis was performed to verify the presence of amplified polymorphic bands using 2.2% concentration with 0.5X TAE Buffer and 1 µL of gel red and was subjected to electrophoresis at 75V for 45 minutes. Visualization was done using gel documentation system (Bio-Rad, USA).

2.4 Sequence Analysis for *rbcL* and *matK*

Sequence assembly and alignment was done using Geneious Prime 2020 (http://www.geneious.com/) and MEGA X software (Kumar, Stecher, Li, Knyaz, and Tamura 2018), respectively. BLASTn analysis (Altschul, et al., 1990) was generated to determine the sequence homology to *Allium* sequences deposited at NCBI GenBank. Sequences generated were trimmed at both the 5' and 3' ends to obtain a consensus sequence with high quality base calls. *Allium schoenoprasum* (chives), *Allium ampeloprasum* (wild leek) and *Allium cepa* (onion) were used as outgroup taxa to evaluate the discriminatory power of these two markers. Maximum-Likelihood trees were generated from the resulting sequences using models determined by the *Find Best DNA/Protein Models (ML)* algorithm of MEGA X.

2.5 SRAP Analysis

Presence (1) or absence (0) of distinct bands in the gel image were noted and compared among the samples used in this study as polymorphic markers (Li & Quiros, 2001).

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3. RESULTS

3.1 PCR Amplification of *rbcL* and *matK* genes

Amplicons of the *rbcL* gene were generated from the four local *A. sativum* varieties analyzed (Fig. 1). Approximately 600 bp amplicon was generated for GMX6, IP3A, GMR10 and GTB17. GNT4 sequence was obtained from a previous amplification experiment (Olivar *et al*, unpublished). The five *rbcL* sequences were deposited in GenBank: GNT4 (MN239994), GMX6 (MN239995), IP3A (MN239996), GMR10 (MN239997) and GTB17 (MN239998) (Table 1).

Sequences of the *rbcL* gene of the five local *A. sativum* varieties were compared. Upon alignment and trimming,

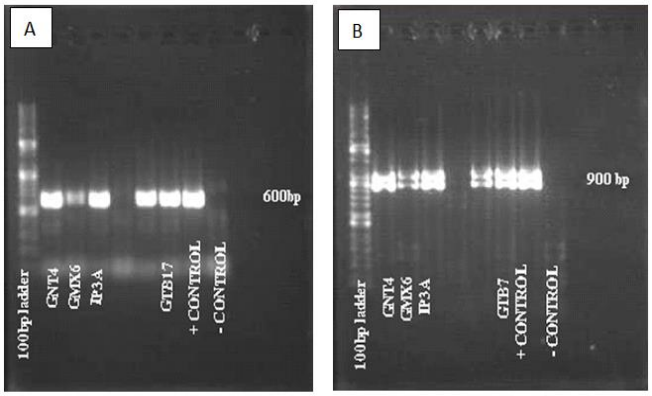


Figure 1. Gel profile showing PCR amplicons using (A) *rbcL* and (b) *matK* marker resolved in 1.2% agarose gel. A 100 bp Plus DNA ladder was used as molecular marker.

408 base pairs were obtained for length consistency in performing the phylogenetic analysis. The BLASTn analysis for the *rbcL* region generated a 99-100% identity match with *Allium sativum* sequences deposited at NCBI GenBank. A 99-100% sequence identity without other similarity to other species is an indicator of positive identification (Hofstetter et al., 2019).

Phylogenetic analysis was conducted on the five local *A. sativum* varieties used in this study, along with seven *A. sativum* reference sequences and three closely related *Allium* species as outgroup (Fig.2). Analysis was done through construction of the maximum-likelihood tree using the Jukes-Cantor model. All five *A. sativum* sequences examined in this study clustered to a single branch with the reference *A. sativum* sequences MG226586, HQ690458, KU499886 and HF572830.

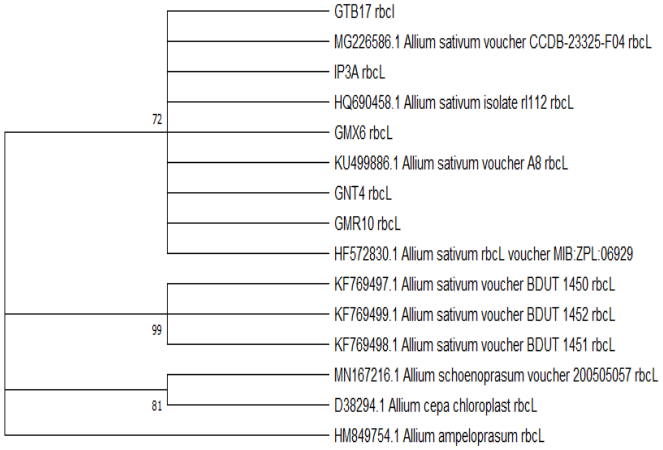


Figure 2. Maximum Likelihood tree of aligned *rbcL* sequences generated using the Jukes-Cantor model. The bootstrap consensus tree was inferred from 1000 replicates and condensed to >70% bootstrap values. There was a total of 408 positions in the final dataset. The tree was rooted to the outgroup *A. ampeloprasum*.

The *rbcL* region was not able to distinguish the five varieties from one another but were discriminated from three other *A. sativum* sequences KF769497,

KF769498 and KF769499, as well as from closely related *Allium* species *A. schoenoprasum*, *A. cepa* and *A. ampeloprasum.* A total of 54 variable sites were observed to be responsible for the branching of these three from the rest of the *A. sativum* sequences (Fig 5, A).

A similar approach was used for the analysis of the *matK* gene. Sequences were obtained from the *matK* region of five *A. sativum* variants (Figure 3). Upon alignment and trimming, 653 base pairs were obtained for length consistency in performing the phylogenetic analysis. The BLASTn analysis for *matK* region generated a 98.63-100% identity match with the *A. sativum* sequences deposited at NCBI GenBank.

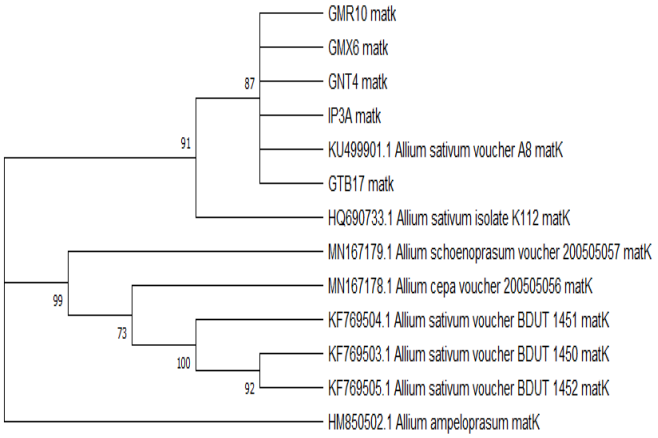


Figure 3. Maximum Likelihood tree of aligned *mat*K sequences generated using the Tamura 3-Parameter model (T92). The bootstrap consensus tree was inferred from 1000 replicates and condensed to >70% bootstrap values. There was a total of 653 positions in the final dataset. The tree was rooted to the outgroup *A. ampeloprasum*.

Phylogenetic analysis was conducted on five local *A. sativum* varieties used in this study, along with five reference sequences and three closely related *Allium* species as outgroup. Analysis was done through construction of the maximum-likelihood tree using the Tamura-3-parameter model (T92. A total of 45 variable sites were observed in the *matK* sequence alignment of ten *A. sativum* species (Fig 5, B). All five *A. sativum* sequences examined in this study clustered with the reference sequence KU499901. Meanwhile, the other reference sequences, HQ690733, KF769503, KF769504 and KF769505 formed different branches. The outgroup sequences were also successfully discriminated from the *A. sativum* samples.

3.2 SRAP analysis

After visualization, out of twenty-five primer pairs only two yielded polymorphic bands, consequently, ME1-EM3 and ME3-EM3 primer pairs were chosen. Bands resolved after gel electrophoresis using SRAP markers were evaluated (Figure 4). Polymorphic bands at

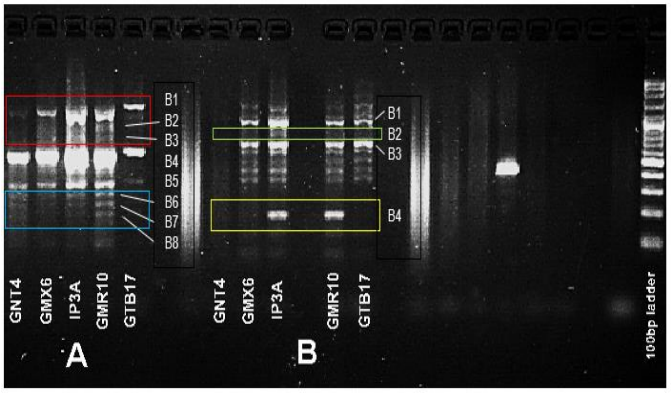


Figure 4. Gel profile showing PCR amplicons from SRAP primers combinations (A) ME1-EM3 and (B) ME3-EM3 resolved in 2.2% agarose gel. Polymorphic bands at around 1200, 1000, 800 bp (red), 300,250,200 bp (blue), 900 (green), and 200 bp (yellow) were highlighted. A 100 bp Plus ladder was used as molecular marker.

around 1200, 1000, 800 bp (i.e., enclosed in red box), and 300,250,200 bp (i.e., enclosed in blue box) for ME1-EM3 were generated, and 900 (i.e., enclosed in green box), and 200 bp (i.e., enclosed in yellow box) for ME3-EM3 were generated. A distinct polymorphic band at around 200 bp was present in the IP3A and GMR10 varieties while a loss of the 1,200 bp band was noted in GNT4. IP3A and GMR10 had the greatest number of bands generated for both primers, GNT4 had the least, followed by GMX6 and GTB17. Interestingly, GNT4 did not produce bands for ME3-EM3. Either this was due to failed amplification or absence of the target polymorphic region, is subject to further investigation.

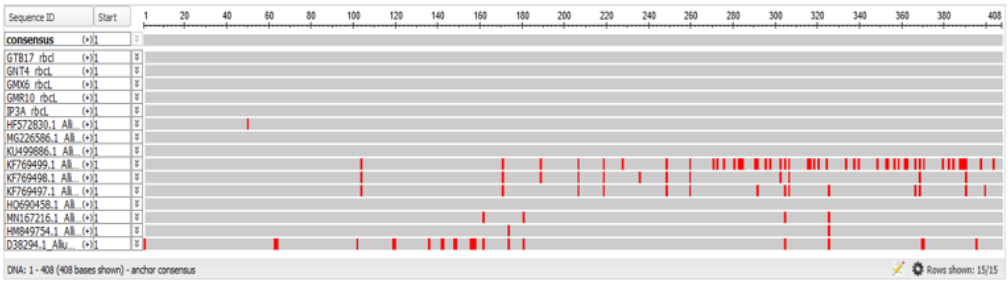
3.3 Sequence alignment and summary of concatenated molecular profiles

The sequence alignment for *rbcL* and *matK* sequences are summarized in Figure 5 together with the results of the SRAP. As can be noted, from the 408-nt *rbcL* sequences, no polymorphism is observed among the local varieties studied and are thus, highly conserved, although two of the closest relatives reported in Genbank showed differences in nucleotide numbers 50, 174, and 327, For the 653 nt *matK*-sequence, GTB17 was discriminated at 5 nucleotide positions (i.e., 4, 76, 85, 126 and 445). While the rest of the variants have similar *matK*-sequences, they differ from the closest relatives reported in Genbank in nucleotide numbers 245, 289, 509 and 535. Meanwhile, the SRAP discriminates all the five varieties based on the 8 bands generated from ME1-EM3 primers and 4 bands from ME3-EM3 primer pairs.

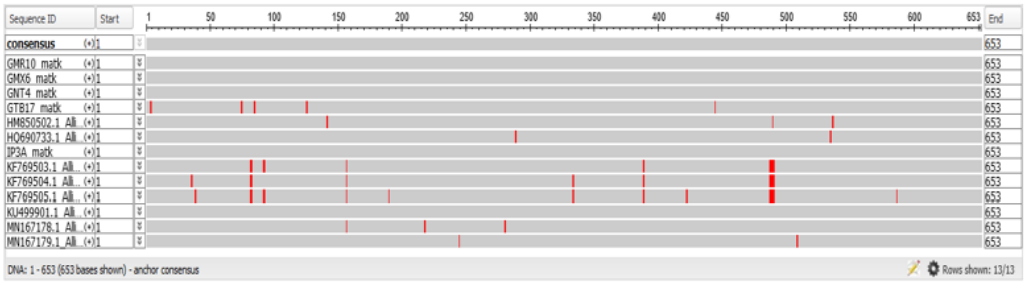
4. DISCUSSION

Species under the *Allium* genus can be distinguished from each other based on their morphological characteristics (Khosa, 2014). However, within species variation may prove to be difficult to distinguish based on morphological traits alone. Differentiation among *A. sativum* varieties is primarily based on average plant height, average bulb size and bulb color as described in

A.



B.



C.

Garlic varieties studied	*rbcl* sequence (1-408 nt)			*matK* sequence (1-653 nt)									SRAP											
	Nucleotide position number			Nucleotide position number									Band number for ME1-EM3								Band number for ME3-EM3			
	50	174	327	4	76	85	126	245	289	445	509	535	1	2	3	4	5	6	7	8	1	2	3	4
GMR10	A	A	T	A	A	T	A	G	G	A	A	A	1	1	1	1	1	1	1	1	1	0	1	0
GMX6	A	A	T	A	A	T	A	G	G	A	A	A	1	0	0	1	1	0	0	0	1	0	1	1
GNT4	A	A	T	A	A	T	A	G	G	A	A	A	0	0	0	1	1	1	1	1	F	F	F	F
IP3A	A	A	T	A	A	T	A	G	G	A	A	A	1	1	1	1	1	1	1	1	1	1	1	1
GTB17	A	A	T	C	C	G	C	G	G	C	A	A	1	0	0	1	1	0	0	0	1	0	1	0
HF572830.1 (GB)	C	A	T	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
HM849754.1 (GB)	A	G	C	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
HO690733.1 (GB)	N	N	N	A	A	T	A	G	A	A	A	G	N	N	N	N	N	N	N	N	N	N	N	N
MN167179.1 (GB)	N	N	N	A	A	T	A	A	G	A	T	A	N	N	N	N	N	N	N	N	N	N	N	N

Figure 5. Summary of the alignment profiles of the garlic varieties and their concatenated molecular features. A- *rbcL* alignment of 408 nt, B- *matK* alignment of 653 nt, and C- summary of concatenated molecular features. Legends: F-Failed amplification, N-No data, GB-Genbank. As highlighted in yellow in panel C, *rbcL* sequences are highly conserved, *matK*-sequences discriminates GTB17. SRAP discriminates all the five varieties.
URLA:
https://www.ncbi.nlm.nih.gov/projects/msaviewer/?coloring=diff&consensus=true&key=kSIL-40gUvn-Dhz-3R8qAHqrUx0MbAJpDm8meTJ9IFOxYCo2nwznWjP1Bty4hA_5XuED9R3RRtQBzhXDE8Uf3i33EPk8xRY,GKuCcgSp23B3h5V3VJajifMi2pKF44vmh-Cv9rvyqdw476O5GYMVQJ2lqKIAw8G-kKbNstOWiJPPiduE3YLRmeOw3r7ygtg&columns=d:120,b:50,x:17,aln.
URLB:
https://www.ncbi.nlm.nih.gov/projects/msaviewer/?coloring=diff&consensus=true&key=ohE4yL4TYcrNPS_N7iwZM0mYU0AMMQI0DjImJDIgIA6xPSpp9VM1rCP6FoRb90-KHpJDhl2iBqdBvVWwU7ZfrW2EUIp8tlY,VOfOPkjllzw7y9k7GNrvxb9umKTH1cnQxdbtwPnE6-p62eGNPbdH-2A3VSB3XxEiQDodLgMKWA8fFQsYDR4BBTMsDiliHgg&columns=d:120,b:50,x:17,aln,e:50

the Philippine Department of Agriculture Garlic Production Guide (Department of Agriculture, 2012). Cultivars that are actually the same but with different names typically arise from this kind of phenotypic/agronomic traits classification (Egea et al., 2017).

In this study both the *rbcL* and *matK* DNA regions were shown to be highly conserved in *A. sativum*, making them suitable barcoding markers for the molecular identification and discrimination of *A. sativum* from other closely related *Allium* species.

Unlike *rbcL*, *matK* is highly variable and have been used to differentiate closely related species (Dong et al., 2012). The *matK* sequences obtained have correctly identified all the *A. sativum* samples at 98-100% match.
All five varieties showed sequence homology with the reference sequence KU499901 which presumably originated from Egypt based on its GenBank entry. However, the other, reference sequences HQ690733 (China), KF769503, KF769504 and KF769505 (India) showed clear distinction from the rest. This result suggests that the matK DNA marker exhibited both inter- and intra-species discriminatory power, albeit with some degree of resolution. Although unable to discriminate among varieties, this study has shown the existence of *A. sativum* species that have polymorphic *rbcL* and *matK* regions. In particular, garlic sequences deposited in GenBank that originated from China and India were distinct from the local varieties in the Philippines. Intraspecific variation in *matK* has also been reported in other species (Hori et al., 2006; Spies and Spies, 2018).

Sequence-Related Amplified Polymorphism (SRAP) markers are DNA-based dominant markers with primers designed to target open reading frames. (Li and Quiros, 2001). Two primer sets were evaluated to generate distinct banding patterns. Two polymorphic bands (900 bp and 200 bp) were observed from the ME3-EM3 primer set that was able to differentiate the varieties IP3 and GMR10 from the other three varieties. Meanwhile,

the ME1-EM3 primer was able to discriminate GNT4. This preliminary result on the use of SRAP markers show its potential to discriminate among varieties within *A. sativum* species, which may be used to augment the discriminatory power of *matK*.

5. CONCLUSION AND RECOMMENDATION

In this study, the utility of DNA markers to identify *A. sativum* species were shown. The *rbcL* and *matK* DNA regions was utilized as molecular markers for the species identification of *Allium sativum*. The *rbcL* sequences were highly conserved, the *matK*-sequences discriminated GTB17 while the SRAP using the primer combinations ME1-EM3 and ME3-EM3 discriminated all the five local garlic varieties. Albeit preliminary, the data suggest that the *matK* sequences and the SRAP showed potential as markers for augmenting the current phenotypic classification of *A. sativum*, and for discriminating varieties of the same *A. sativum* from different origins. An increase in sample size with more varieties are needed to validate these findings.

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