Genetic Assessment of Eggplant (*Solanum melongena* L.) and its Wild Crop Relatives in the Philippines through DNA Barcoding

Khris June L. Callano
Agriculture Department
Institute of Agriculture and Related Sciences
Davao del Sur State College

Previous eggplant diversity studies in the Philippines have utilized morphological and molecular markers to ascertain their genetic relationship. Since these studies were able to highlight only the relationship of the cultivated eggplant species, it is now becoming relevant to also look into the genetic relationship between the cultivated eggplant varieties and its wild crop relatives found in the country. In this study, DNA barcoding was done on eggplant and on its wild crop relatives to elucidate their genetic and taxonomic relationships. Only the phylogenetic tree constructed from *matK* gene sequences that successfully discriminated the studied *Solanum* species based on their taxonomic classification. Therefore, *matK* gene was perceived to be an efficient DNA barcode in discriminating *Solanum* species. Nonetheless, there was an observed close genetic affinity between *S. aethiopicum* and *S. melongena* in all generated phylogenetic trees with bootstrap values ranging from 52-88. These results are instrumental in elucidating the diversity of *Solanum* species.

Keywords: DNA barcoding, *nITS2, matK, rbcL, Solanum*

INTRODUCTION

The Solanaceae is a large plant family of over 3000 species including many important crops such as tomato, potato, eggplant, and pepper (Chase et al., 1993). It represents a group of dicotyledonous plants in Euasterid clade, which is divergent from the model plant Arabidopsis (Wu et al., 2015). Furthermore,

it is the third economically important plant family and ranks first in terms of vegetable crops (Dogonlar et al., 2002). Eggplant (*Solanum melongena* L.) is known as one of the most significant members of the Solanaceae family. According to Ishniiki et al. (1994a), eggplant may have originated in Asia, but most recent DNA sequencing studies suggested that it arose from Africa (Li et al., 2010; Weese & Bohs, 2010). This cultivated *Solanum* species is grown extensively as cash crop by most of the small-scale farmers in many countries, specifically in Asia. Together with China and India, Philippines is one of the top 10 eggplant-producing countries in the world. Economically, eggplant has been the leading vegetable crop in the country in terms of production volume and area planted since 2012 (PSA, 2019).

Despite the importance of the genus Solanum, phylogenetic relationships among the taxa are currently unclear (Sifau et al., 2014). Solanum taxonomy has been complicated largely due to the large number of species (Obute et al., 2005), overlapping ecogeographical distribution (Levin et al., 2005), morphological plasticity, similarity of genomes (Okoli, 1998) and existence of swarms of natural hybrids (Oyelana & Ugborogho, 2008). The inconsistencies and misconceptions generated by these factors had made past attempts to taxonomically resolve the complexities associated with the genus. In the advent of molecular biology, research attempts involving molecular analysis such as chloroplast DNA assessment (Sakata et al., 1991), isozyme marker study (Ishniiki et al., 1994b), restriction fragment length polymorphism (RFLP) markers (Doganlar et al., 2002), random amplified polymorphic DNA (RAPD) markers (Nunome et al., 2009), simple sequence repeats (SSR) markers (Nunome et al., 2009), amplified fragment length polymorphism (AFLP) markers (Mace et al., 1999) have been utilized for the characterization of eggplant materials from different regions of origin. So far, molecular analysis using DNA barcoding genes in eggplant and related species has not been exploited yet.

DNA barcoding is one of the advanced molecular marker-based methods that identify target plant species in a short duration. The main goal of DNA barcoding is to accurately identify nucleotide sequences of multiple plant species (Group et al., 2009). It is an efficient, quick, low-cost, and standard method for evaluation and identification of different plant species (Khan et al., 2015). Chloroplast gene regions like ribulose 1,5-biphosphate carboxylase/oxygenase large (rbcL) subunit, maturase K (matK) as well as nuclear internal transcribed spacer 2 (ITS2) have already been used as standard barcoding genes in almost

all flowering plant species (Hollingsworth et al., 2009; Chen et al., 2010; Jamil et al., 2014; Shinwara et al., 2014; Zahra et al., 2014; Khan et al., 2015).

Previously, morphological and molecular markers were used to study the genetic diversity of different eggplant varieties and its relatives in the Philippines. Due to climate change and selection pressure, the genetic constitution of these plant species has become prone to mutation. Thus, there is a need to constantly check their genetic make-up at the DNA level.

In this study, the efficiency of rbcL, matK and ITS2 genes in discriminating different *Solanum* species was reported. These genes were partially sequenced to assess the genetic relationship of eggplant and its wild relatives found in the Philippines. This assessment is instrumental in elaborating *Solanum* species' genetics, diversity, and taxonomy.

MATERIALS AND METHODS

Sample Collection

Leaf samples of seven *Solanum* species, namely, *S. aethiopicum* (Fig. 1a), *S. capsicoides* (Fig. 1b), *S. diphyllum* (Fig. 1c), *S. mammosum* (Fig. 1d), *S. melongena* (Fig. 1e), *S. nigrum* (Fig. f) and *S. torvum* (Fig. 1g) were collected from the experimental area at Cordillera St., Umali Subd., Brgy., Batong Malake, Los Baños, Laguna. Young leaves of these *Solanum* species were collected in three replicates for genomic DNA isolation.

Figure 1. Representative photos of (a) ***Solanum aethiopicum*** L., (b) ***S. capsicoides*** Allioni, (c) ***S. diphyllum*** L., (d) ***S. mammosum*** L., (e) ***S. melongena*** L., (f) ***S. nigrum*** L. and (g) ***S. torvum*** Sw

Genomic DNA Isolation

Fresh and young leaves were selected and wiped with 70% ethanol before they were ground in 700µl CTAB buffer pre-heated to 60oC. Genomic DNA isolation of the seven Solanum species was carried out using the modified Milligan method (Herbert et al., 2003). The homogenized mixture was transferred into a sterile microcentrifuge tube and was incubated in a water bath at 60°C for 45 minutes. A mixture of 24:1 chloroform:isoamyl alcohol was added to the mixture and was centrifuged at 5,000rcf for 12 min. The aqueous phase was then transferred to another sterile microcentrifuge tube to which 2µL of RNase, with a final concentration of 0.1mg/mL added. After this, the chloroform:isoamyl step was repeated. The aqueous phase was transferred to another microcentrifuge tube with the same volume of the isopropanol and was incubated at 20°C for 20 minutes.

After incubation, the mixture was centrifuged at 5,000 rcf for 5 minutes. The pellet was washed with 700µL of 70% ethanol and was allowed to sit in the wash buffer for 20 minutes before centrifugation at 3,000rcf for 10 minutes. The pellet was dried for four (4) minutes and was resuspended at 50 µL 1x TE in small increments (10-100 µL), depending on the size of the pellet.

The quality of the extracted DNA was determined using gel electrophoresis and Epok Biotek Spectrophotometer. Isolated plant genomic DNA from the samples was stored at 40°C until further use.

Gene Amplification

Barcoding genes, rbcL, matK and nITS2 were amplified using gene-specific primers. Polymerase Chain Reaction (PCR) with three technical replicates was done using the genomic DNA isolated from the fresh leaves of seven Solanum species.

rbcL. A set of primer, rbcl_F (5'-ATGTCACCACAAACAGAGACTAAAGC-3') and rbcL_R (5'-GTAAAATCAAGTCCACCRCG-3') (Kress & Erickson, 2007) was used to amplify rbcL gene from the Solanum samples. A total volume of 20 µL of PCR reaction mixture was prepared: 11.8µL sterile nanopure water, 2µL of 1x PCR buffer, 1µL of 2.5mM MgCl2, 1.6 µL of 0.8mM dNTP mix (GeneAll), 0.6 µL of 0.3 µM of each primer, 0.4 µL of Taq pol (Invitrogen),

and 2 µL of template DNA. The PCR amplification was done using BIO RAD thermal cycler with the following cycling profile: initial denaturation at 94°C for 4 min, followed by 5 cycles of 94°C for 30 s, 55°C for 1 min and 72°C for 1min, then 35 cycles of 94°C for 30 s, 55°C for 1min and 72°C for 1 min, and a final elongation step at 72°C for 10 min.

matK. A set of primer, matK_F (5'-CGTACAGTACTTTTGTGTTTACGAG-3') and matK_R (5'-ACCCAGTCCATCTGGAAATCTTGGTTCC-3') (Fazenkas et al., 2008) was used to amplify matK gene from the *Solanum* samples. A total volume of 20µL of PCR reaction mixture was prepared: 11.8µL sterile nanopure water, 2 µL of 1x PCR buffer, 1µlLof 2.5mM MgCl2, 1.6 µL of 0.8mM dNTP mix (GeneAll), 0.6 µL of 0.3 µM of each primer, 0.4 µL Taq pol (Invitrogen), and 2 µL of template DNA. The PCR amplification was done using BIO RAD thermal cycler with the following cycling profile: initial denaturation at 94°C for 3 min, followed by 5 cycles of 95°C for 30s, 52°C for 20s and 72°C for 1min, then 35 cycles of 94°C for 30 s, 55°C for 1min and 72°C for 1 min, and a final elongation step at 72°C for 10 min.

nITS2. A set of primer, nITS2_F (5'- ATGCGATACTTGGTGTGAAT -3') and nITS2_R (5'- GACGCTTCTCCAGACTACAAT -3') (Ajmal Ali et al., 2015), were used to amplify matK gene from the *Solanum* samples. A total volume of 20µL of PCR reaction mixture was prepared: 11.8µL sterile nanopure water, 2µL of 1x PCR buffer, 1µL of 2.5mM MgCl2, 1.6 µL of 0.8mM dNTP mix (GeneAll), 0.6µl of 0.3µM of each primer, 0.4µL of Taq pol (Invitrogen), and 2µL of template DNA. The PCR amplification was done using BIO RAD thermal cycler with the following cycling profile: initial denaturation at 94°C for 3 min, followed by 5 cycles of 95°C for 30 s, 56°C for 30s and 72°C for 1min, then 35 cycles of 94°C for 30 s, 55°C for 1min and 72°C for 1 min, and a final elongation step at 72°C for 10 min.

Agarose Gel Electrophoresis

PCR products were resolved in a 1 % agarose gel that was prepared in 22mL of 0.5x TAE buffer with 0.6 µL GelRed. Gel electrophoresis was run on Mupid One System at 50 volts for ~30min. The gel image was obtained using BIO-RAD gel doc. The size of the amplified PCR products was determined using 1kb+ ladder.

Gene Sequencing

The PCR products were sent to 1st BASE DNA Sequencing Services (Seri Kembangan, Selangor, Malaysia) for Sanger sequencing. The sequences were generated as electrophoretogram and text files from the company's official website.

Sequence Analyses

Sequences generated from 1st BASE DNA Sequencing Services were analyzed via Staden Package 2.0 entailing PreGap4 and Gap4 to allow selection of sequences with non-overlapping as well as high amplitude base sequence peaks. This software can trim and edit long run of sequences to come up with a consensus sequence for individual samples. The generated consensus sequences were then subjected to Basic Local Alignment Search Tool (BLAST) under the nucleotide BLAST (BLASTN) algorithm (Altschul et al., 1990). This proess was executed to determine the sequences' homology to the available sequences in the National Center for Biotechnology Information (NCBI) database.

Phylogenetic Analyses

Phylogenetic and molecular analyses were executed through MEGA version 7 by Tamura et al. (2013). The rbcL, matK and nITS2 genes of Coffea arabica were incorporated in the analyses to serve as an outgroup species. Eventually, the distance and test of neutrality were computed and bootstrap consensus tree was constructed.

RESULTS AND DISCUSSION

Genomic DNA (gDNA) isolation was done on the leaves of the seven *Solanum* species as a preliminary step for its molecular characterization. Over the years, several researchers (Doganlar et al., 2002; Behera et al., 2006; Frary et al., 2007; Demir et al., 2010; Ge et al., 2011; Caguiat and Hautea, 2014; Acquardo et al., 2017) have also successfully utilized leaf samples from Solanum species for their molecular studies.

PCR amplification of the *Solanum* samples' rbcL, matK and nITS2 genes from seven species was done. Based on Kress and Erickson (2007), Fazenkas et al.

(2008), and Ajmal Ali et al. (2015), the expected band sizes of rbcL (Fig. 2.1a) matK (Fig. 2.1b) and nITS2 (Fig. 2.1c) are 554 bp, 870 bp and 500 bp, respectively. These band sizes were observed in all seven *Solanum* species. Despite the fact that chloroplast genome can be used as an excellent marker to establish phylogenetic relationships between and within plant genera and families (Shinwari et al., 1994; Hurst & Jiggins, 2005; Gao et al., 2008; Dong et., 2012; Khan et al., 2013), there are still very few published studies (Sifau et al., 2014; Jamil et al., 2014) regarding the utilization of these genes in analyzing the genetic and taxonomic structures of eggplant and its wild relatives. So far, eggplant has not benefited from the enormous potential of DNA barcodes to explore genetic diversity by detecting nucleotide differences between cultivars and species like other *Solanum* species such as tomato (Wang et al., 2015), potato (Enyedi & Pell, 1992; Barthet & Hilu, 2007; Spooner, 2009) and pepper (Jarret, 2008; Parvathy et al., 2014; Parvathy et al., 2018). In general, eggplant and its wild relatives have been less often used in molecular genetics research because eggplant is produced less and consumed widely compared to tomato and potato (Fukuoka et al., 2012).

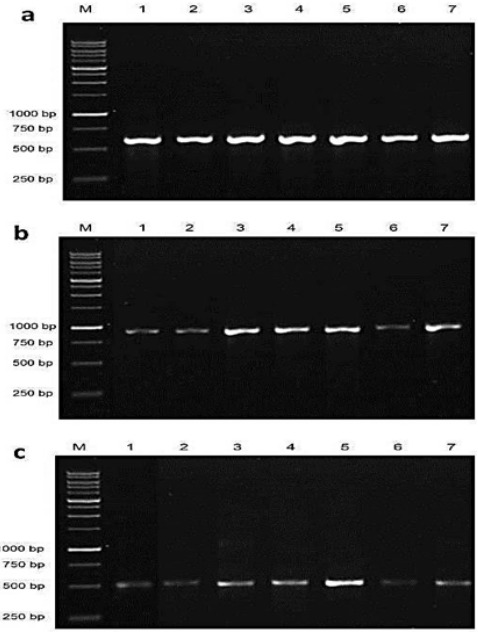

Figure 2.1. Representative electrophoretic gel of PCR products from (a) rbcL (b) matK (c) nITS2 genes in *Solanum* species. M-1kb+ DNA ladder (Invitrogen); lane 1 (*S. aethiopicum L.*); lane 2 (*S. capsicoides L.*); lane 3 (*S. diphyllum Allioni*); lane 4 (*S. mammosum L.*); lane 5 (*S. melongena L.*); lane 6 (*S. nigrum L.*); lane 7 (*S. torvum Sw.*)

The choice of rbcL+matK as the core barcodes for this study was based on the straightforward recovery of rbcL region and the discriminatory power of the matk gene as well. The gene *matK* is one of the most rapidly evolving coding sections of the plastid genome (Herbert et al., 2003; Lahaye et al., 2008; Ford et al., 2009), and is perhaps considered as closest plant analogue to the CO1 animal barcode (Hollingsworth et al., 2011). Unfortunately, matK is hard to amplify (Hilu & Liang, 1997; CBOL, 2009). In contrast, the barcode region of rbcL is easy to amplify, sequence and align in most land plants (Kress et al., 2005). It also provides useful backbone to the barcode dataset despite having only modest discriminatory power (Liu et al., 2010; Pang et al., 2010; Nock et al., 2011). Despite their disadvantages, no other two-marker or multi-marker plastid barcode gave appreciably greater species resolution than rbcL+matK combination (CBOL, 2009; Hollingsworth et al., 2011). With the present study, the plant samples were only represented by a single accession per species. According to Hollingsworth et al. (2011), in cases such as this, the use of more barcoding genes will naturally increase the discrimination success among the studied individuals. Hence, internal transcribed spacer of the large subunit of ribosomal DNA (nITS) was added in the existing standard barcode combination (i.e. rbcL+matK) to increase the phylogenetic informative sites (Chen et al., 2010; Gao et al., 2010; Yao et al., 2010) of the studied *Solanum* species. Furthermore, barcode combination like this can provide genetic trees that are based on the phylogentically relevant information contained in many loci in the whole genome (Hampl et al., 2001).

All generated sequences from rbcL, *matK* and nITS2 genes from the seven *Solanum* samples were compared against the databank in the NCBI database through a BLAST search. The *matK* (Table 1) and nITS2 (Table 2) genes partial sequences were identified to be 99% homologous to the maturase K (matK) and internal transcribed spacer 2 (nITS2) genes of *S. aethiopicum, S. capsicoides, S. diphyllum, S. mammosum, S. melongena, S. nigrum and S. torvum*. Whereas, BLAST analysis in rbcL gene partial sequences (Table 3) were able to identify *S. aethiopicum, S. capsicoides, S. diphyllum, S. mammosum, S. nigrum* as *S. macrocarpon, S. indicum, S. pseudocapsicum, S. anguivi* and *S. americanum,* respectively. It was only the rbcL partial sequences of *S. melongena* and *S. torvum* that were found to be 99% identical to the 1, 5-bisphosphate carboxylase genes of their respective species in NCBI. Indeed, rbcL gene sequences are only efficient in identifying plant species up to the genus level. In 2012, Bafeel et al., reported that rbcL gene sequences could identify 92% of the samples to the genus level

and only 17% on the species level in the DNA barcoding studies of arid plants. In addition, several studies (Kress & Erickson, 2007; Fazenkas et al., 2008; Lahaye et al., 2008) stated that the variation in the rbcL region was insufficient to discriminate a certain group of plant samples up to species level.

To further discriminate and analyze the genetic relationship of the *Solanum* samples used in the present study, bootstrap consensus trees were constructed using maximum parsimony method for rbcl, *matk*, nITS2 and combination of rbcL+matK+nITS2 partially sequenced genes. The sequences of Coffea arabica were used as an outgroup sample in the phylogenetic tree since it belongs to family Rubiceae which is one of the most related families to Solanaceae (Wu & Tanksley, 2010). For a more in-depth analysis, the discrimination was based on the subgenus level of the said samples. The genus *Solanum* contains economically important plants such as tomato, potato, and eggplant that comprise more than 3500 species in six subgenera such as Archaesolanum, Bassovia, Leptostemonum, Minon, Potatoe, and *Solanum* (Lester & Hasan, 1991). Specifically, eggplant and its wild relatives are included in four of these subgenera: subgenera Leptostemonum, Solanum, Potatoe, and Archaesolanum.

Figure 2 shows the bootstrap consensus tree which was generated using the rbcL sequences of the seven *Solanum* species. Apparently, the clustering failed to completely group the *Solanum* species that belonged to the same subgenus. *S. nigrum* and *S. diphyllum*, both classified under subgenus *Solanum*, separated and joined cluster A and formed an independent cluster (B), respectively, whereas cluster A was able to group *S. aethiopicum, S. melongena, S. mammosum*, and *S. torvum* according to their respective subgenus (Leptostemonum). With a bootstrap value of 52, *S. aethiopicum* and *S. melongena* joined together in a subcluster under cluster A, indicating close genetic affinity between the two species. Although *S. nigrum* and *S. torvum* clustered together with bootstrap value of 51, such grouping can be quite unreliable since both species were from different subgenera (*Solanum* and *Leptostemonum*, respectively). These results imply that rbcL gene of about 554bp (Kress & Erickson, 2007) has less discriminating power if used with Solanum species. According to Chase et al. (2005), the entire rbcL gene sequence (~1430bp) of some plant species must be sequenced to, at least, obtain enough and relatively reliable species discrimination.

Table 1.
BLASTN result of rbcL conserved consequences in the seven Solanum species

SPECIES	BLASTN BEST MATCH	QUERY COVER	E-VALUE	PERCENT IDENTITY (%)
S. aethiopicum	*S. macrocarpon* ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit-like (rbcL) gene, partial sequence	100%	0.0	99.66%
S. capsicoides	*S. indicum* ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit-like (rbcL) gene, partial sequence	99%	0.0	99.33%
S. diphyllum	*S. pseudocapsicum* ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit-like (rbcL) gene, partial sequence	98%	0.0	99.98%
S. mammosum	*S. anguivi* ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit-like (rbcL) gene, partial sequence	99%	0.0	99.17%
S. melongena	*S. melongena* ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit-like (rbcL) gene, partial sequence	99%	0.0	99.17%
S. nigrum	*S. americanum* ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit-like (rbcL) gene, partial sequence	99%	0.0	99.49%
S. torvum	*S. torvum* ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit-like (rbcL) gene, partial sequence	100%	0.0	99.33%

Table 2.
BLASTN result of matK conserved consequences in the seven Solanum species

SPECIES	BLASTN BEST MATCH	QUERY COVER	E-VALUE	PERCENT IDENTITY (%)
S. aethiopicum	S. aethiopicum tRNA-Lys (trnK) gene, partial sequence; and maturase K (matK) gene	100%	0.0	99.87%
S. capsicoides	S. capsicoides tRNA-Lys (trnK) gene, partial sequence; and maturase K (matK) gene	99%	0.0	99.75%
S. diphyllum	S. diphyllum tRNA-Lys (trnK) gene, partial sequence; and maturase K (matK) gene	99%	0.0	99.03%
S. mammosum	S. mammosum tRNA-Lys (trnK) gene, partial sequence; and maturase K (matK) gene	99%	0.0	99.88%
S. melongena	S. melongena tRNA-Lys (trnK) gene, partial sequence; and maturase K (matK) gene	98%	0.0	99.88%
S. nigrum	S. nigrum tRNA-Lys (trnK) gene, partial sequence; and maturase K (matK) gene	99%	0.0	99.75%
S. torvum	S. torvum tRNA-Lys (trnK) gene, partial sequence; and maturase K (matK) gene	99%	0.0	99.05%

Table 3.
BLASTN result of nITS2 conserved consequences in the seven Solanum species

SPECIES	BLASTN BEST MATCH	QUERY COVER	E-VALUE	PERCENT IDENTITY (%)
S. aethiopicum	*S. aethiopicum* 5.8s ribosomal RNA gene, partial sequence; internal transcribed spacer 2, partial sequence	99%	0.0	99.87%
S. capsicoides	*S. capsicoides* 5.8s ribosomal RNA gene, partial sequence; internal transcribed spacer 2, partial sequence	99%	0.0	99.75%
S. diphyllum	*S. diphyllum* 5.8s ribosomal RNA gene, partial sequence; internal transcribed spacer 2, partial sequence	98%	0.0	99.03%
S. mammosum	*S. mammosum* 5.8s ribosomal RNA gene, partial sequence; internal transcribed spacer 2, partial sequence	99%	0.0	99.88%
S. melongena	*S. melongena* 5.8s ribosomal RNA gene, partial sequence; internal transcribed spacer 2, partial sequence	99%	0.0	99.88%
S. nigrum	*S. nigrum* 5.8s ribosomal RNA gene, partial sequence; internal transcribed spacer 2, partial sequence	98%	0.0	99.75%
S. torvum	*S. torvum* 5.8s ribosomal RNA gene, partial sequence; internal transcribed spacer 2, partial sequence	98%	0.0	99.05%

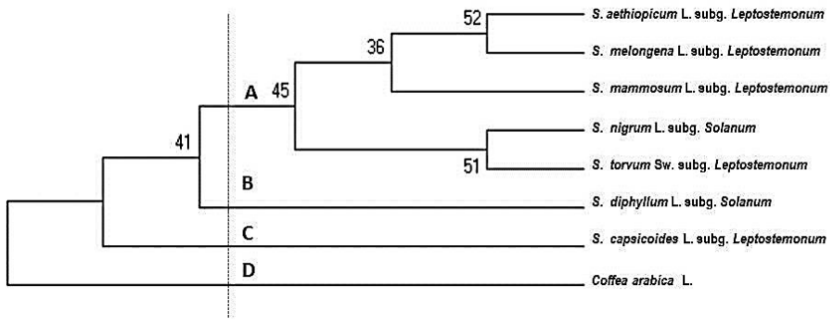

Figure 2. The bootstrap consensus tree based on partial rbcL gene sequences of *Solanum aethiopicum, S. capsicoides, S. diphyllum, S. mammosum, S. melongena, S. nigrum,* and *S. torvum*

Even with its innate limitation in discriminating the *Solanum* species, rbcL gene in the present study and even in green algae (Manhart, 1994), palm trees (Hahn, 2002), grasses (Gamache and Sun, 2015), mangoes (Fitmawati & Juliantari, 2017), and subtropical tuberous herbaceous plants (Gao et al., 2018) was the easiest to amplify and sequence. This particular characteristic made this barcoding gene as the benchmark locus in phylogenetic investigations (Vijayan & Tsou, 2010).

Among the phylogenetic trees produced from the partially sequenced rbcL, *matK* and nITS2 genes of the Solanum samples, it was only the bootstrap consensus tree constructed from *matK* gene sequences (Fig. 3) that successfully discriminated the seven *Solanum* species based on their taxonomic classification. With a bootstrap value of 78, *S. aethiopicum, S. melongena, S. torvum* and *S. capsicoides*, and *S. mammosum* joined together in one cluster (A), forming a group of *Solanum* species of the same subgenus (Leptostemonum). On the other hand, *S. diphyllum* (cluster B) and *S. nigrum* (cluster C) formed separate and independent clusters despite the fact that these two species belong to the same subgenus (*Solanum*). Consistently, *S. aethiopicum* and *S. melongena* showed close genetic affinity but with a relatively high bootstrap value (69) as compared to rbcL (52). The following findings clearly show that *matK* is an invaluable gene in plant systematics because of its high discriminating power. Basically, the 1500bp matK gene is nested in the group II intron between the 5' and 3' exons of trnK in the large single copy region of the chloroplast genome of most green plants (Sugita et., 1985; Steane, 2005; Daniell et al., 2006). Muller et al. (2006) reported that in a phylogenetic analysis of a data set composed of *matK*, rbcL and trnT-F sequences from basal angiosperms, it was *matK* gene sequences that contributed more parsimoniously informative characters over the highly conserved chloroplast gene, rbcL. Sequence information from this chloroplast gene alone has generated phylogenies as robust as those constructed from data sets comprised of 2-11 other genes combined (Hilu et al., 2003). In addition, the molecular information generated from matK has been used to resolve phylogenetic relationships from shallow to deep taxonomic levels (Johnson & Soltis, 1994; Hayashi & Kawano, 2000; Hilu et al., 2003; Cameron, 2005; Turuspekov et al., 2017). However, some researchers (Kores et al., 2000; Kugita et al., 2003; Hidalgo et al., 2004; Jankowiak et al., 2004; Spies & Spies, 2018) also reported that *matK* may not be functional in some

plant species due to its rapid rate of substitution, along with the rare presence of frameshift indels and a few cases of premature codons.

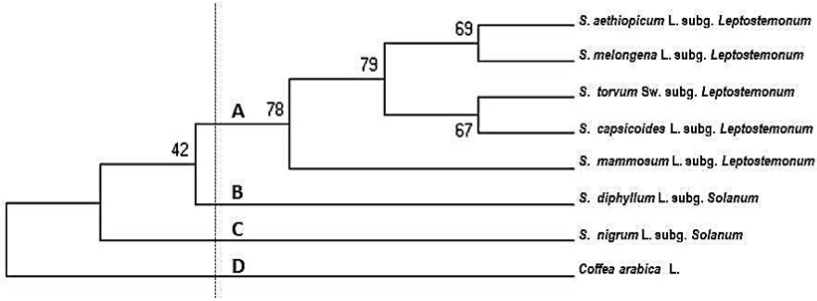

Figure 3. The bootstrap consensus tree based on partial matK gene sequences of *Solanum aethiopicum*, *S. capsicoides*, *S. diphyllum*, *S. mammosum*, *S. melongena*, *S. nigrum* and *S. torvum*

The bootstrap consensus tree generated from the nITS2 gene sequences of the seven *Solanum* species is presented in Figure 4. Unfortunately, nITS2 gene sequences were not able to completely discriminate the seven Solanum species according to their subgenera. *S. aethiopicum*, *S. melongena*, and *S. torvum* grouped together according to their subgenus in cluster A, with even a very high bootrstrap value of 98. However, cluster B unfortunately included *S. mammosum* subgenus Leptostemonum to the group of species (i.e. S. diphyllum and S. nigrum) that belong to subgenus *Solanum*. As observed from the previous phylogenetic trees, *S. aethipicum*, and *S. melongena* were consistently held together but in a much higher bootstrap value (88), relatively higher than what were detected in rbcL (52) and *matK* (69). Aside from rbcL and *matK*, DNA barcoding also relies on nuclear phylogenetic markers such as internally transcribed spacer (ITS) found in the assemblage of the nuclear ribosomal DNA (nrDNA) (Wiseman, 2003). In this study, nITS2 region was used because it is believed to have high discriminatory power (Baldwin, 1992; Buckler & Holtsford, 1996; Clevinger & Panero, 2000; Iwen et al., 2002; Wissemann, 2003, Yao et al., 2010; Gu et al., 2013; Feng et al., 2016; Moorhouse-Gann et al., 2018) and amplification success rate (Torres et al., 1990; Baldwin et al., 1995; Ferguson et al., 1999; Edrogan & Mehlenbacher, 2000; Young & Coleman, 2004; Chen et al., 2010; Cheng et al., 2016; Liu et., 2019). The discriminatory power of such region was not

satisfactorily expressed in the phylogenetic analysis of the seven *Solanum* species. More likely, the use of the entire ITS region in barcoding *Solanum* species can eventually provide better sequences, since barcoding genes are also species-specific. According to Hollingsworth et al., (2011), some plant species require the entire ITS region to obtain quality and readable sequences. Despite its inefficiency in discriminating the seven *Solanum* species, nITS2 gene was witnessed to have high amplification rate like the rbcL gene.

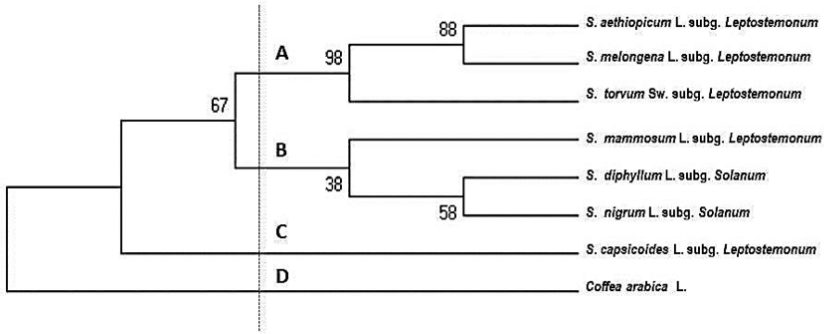

Figure 4. The bootstrap consensus tree based on partial nITS2 gene sequences of *Solanum aethiopicum, S. capsicoides, S. diphyllum, S. mammosum, S. melongena, S. nigrum* and *S. torvum*

A bootstrap consensus tree was made based on combined partially sequenced rbcL, matK and nITS2 genes (Fig. 5) to thoroughly analyze the genetic relationship of the seven *Solanum* species. The constructed phylogenetic tree was not able to completely discriminate the seven Solanum species based on their respective subgenera. *S. capsicoides* formed a distinct cluster (C) that separated itself from other species under subgenus Leptostemonum, whereas, *S. mammosum*, which is under subgenus Leptostemonum, joined S. diphyllum and S. nigrum in cluster B, both under the subgenus Solanum. Despite the observed disparity in cluster B, the taxonomic classification of *S. diphyllum* and *S. nigrum* was confirmed through their distinct subclustering at bootstrap value of 70. It should be noted as well that *S. torvum* grouped with *S. aethiopicum* and *S. melongena* in cluster A with a very high bootstrap value of 93, a concrete indication of genetic relatedness. This observation was also seen by Sifau et al. (2014) when have studied the partitioning and distribution of random amplified polymorphic

DNA (RAPD) variation among eggplant species in Southwest Nigeria. The observed close genetic affinity between *S. aethiopicum* and *S. melongena* in this combined sequence analysis conformed to the results from rbcL, matK and ITS2 genes' phylogenetic trees. In recent years, diversity researches on eggplant and its related species through morphological assessment (Fawzi & Habeeb, 2016); molecular analysis using amplified fragment length polymorphism (AFLP) (Meyer et al., 2005); restriction fragment length polymorphism (RFLP) (Sakata et al., 1991); inter-simple sequence repeats (ISSRs) (Isshiki et al., 2008); single nucleotide polymorphism (SNP) (Acquardo et al., 2017); barcoding genes (Weese & Bohs, 2010; Sifau et al., 2014); and even morpho-molecular investigations (Caguiat & Hautea, 2014) have consistently reported the close genetic relationship of *S. aethiopicum* and *S. melongena*. Taxonomically, *S. melongena* subg. Leptostemonum is placed into S. sect Melongena (Mill.) Dunal, while *S. aethiopicum* subg. Leptostemonum belongs to S. sect. Oliganthes (Dunal) Bitter (Daunay et al., 1998). Although these Solanum species differ from the sections they belong to, S. aethiopicum is one of the wild Solanum species considered as genetically related to *S. melongena*. However, Whalen (1984) emphasized that *S. aethiopicum* was not directly involved in the cultivated eggplant's evolution when he scrutinized the conspectus of species groups in Solanum subgenus Leptostemonum.

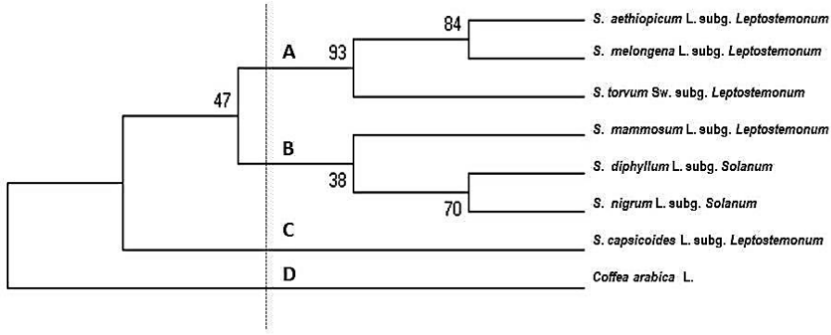

Figure 5. The bootstrap consensus tree based on combined partial rbcL, *matK* and nITS2 genesequences of *Solanum aethiopicum, S. capsicoides, S. diphyllum, S. mammosum, S. melongena, S. nigrum* and *S. torvum*

Solanum subgenus Leptostemonum is a large group within *Solanum*, comprising almost one third of the genus (Nee, 1999). This particular group has been recognized by different botanists and taxonomists (Symon, 1981; Whalen, 1984), with rank and circumscription varying with the taxonomic treatment. One of the main features that distinguish this subgenus among others is the presence of sharp epidermal prickles on stems and leaves; thus, species that belong to it are commonly called "spiny Solanums." Out of the seven *Solanum* species studied, *S. aethiopicum, S. melongena,* and *S. torvum* were phenotypically witnessed to bear such characteristics. This might be one of the reasons for their close genetic affinity as far as the barcoding genes are concerned. Through the years, *S. aethiopicum* and *S. torvum* have been inter-crossed with *S. melongena* to eventually produce interspecific hybrids with improved agronomic traits. The interspecific hybrids generated from *S. aethiopicum* x *S. melongena* (Ano et al. 1991; Daunay et al. 1998; Prohens et al., 2012) as well as *S. torvum* x *S. melongena* (Blesto et al., 1998; Kumchai et al., 2013; Kouassi et al., 2016; Plazas et al., 2016) were impartially fertile or even completely sterile. Thus, it is deemed necessary to identify more candidate wild and related species that are compatible to produce fertile eggplant hybrids.

S. aethiopicum and *S. melongena* and other related *Solanum* species are thought to have been developed from the wild ancestor, S. insanum (Swarup, 1995; Prohens et al., 2003; Barchi et al., 2010; Boyaci et al., 2015). Evidently, the taxonomy and evolution of the Old World members of the subgenus Leptostemonum has been problematic because of Solanum species' complexity (Knapp et al., 2013). Morphological similarity between Old and New World species led some authors (Dunal, 1852; Whallen, 1984; Weese & Bohs, 2010; Stern et al., 2011; Vorontsova & Knapp, 2012) to postulate multiple evolutionary concepts in eggplant. But, the evolution of the cultivated eggplant, S. melongena, was eventually attributed to mutation (Swarup, 1995), hybridization (Daunay et al., 1998; Collonnier et al., 2001; Singh & Kumar, 2007) and human selection (Weese & Bohs, 2010).

CONCLUSION AND RECOMMENDATION

The rbcL, matK, and nITS2 barcoding genes can be amplified in the DNA samples from *S. aethiopicum, S. capsicoides, S. diphyllum, S. mammosum,*

S. melongena, S. nigrum, and *S. torvum* using the published protocols with minor modifications.

BLAST analysis showed that the matK and nITS2 genes partial sequences were 99% homologous to the maturase K (matK) and internal transcribed spacer 2 (nITS2) genes of *S. aethiopicum, S. capsicoides, S. diphyllum, S. mammosum, S. melongena, S. nigrum*, and *S. torvum* in the NCBI database, whereas the rbcL gene partial sequences of the seven Solanum samples were 99% identical to rbcL gene sequences of other Solanum wild relative species. This is because rbcL gene sequences were only efficient in identifying plant species up to genus level.

Among the phylogenetic trees produced from the partially sequenced rbcL, matK, and nITS2 genes of the Solanum samples, only the phylogenetic tree constructed from matK gene sequences partially discriminated the seven Solanum species based on their taxonomic classification. Therefore, matK gene was perceived to be an effective DNA barcode in discriminating Solanum species.

Nonetheless, there was an observed close genetic affinity between *S. aethiopicum* and *S. melongena* in all of the generated phylogenetic trees with bootstrap value ranging from 52-88. This finding conforms to several genetic diversity studies in Solanum species using morphological and molecular markers. Therefore, it is strongly believed that *S. aethiopicum* and *S. melongena* are closely related to each other.

It is recommended to include Solanum verbascifolium, a wild Solanum species which is also reported to be present in the country, in future DNA barcoding studies to make the genetic assessment more comprehensive.

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