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MOLECULAR IDENTIFICATION AND PRELIMINARY GENETIC DIVERSITY ANALYSIS OF TOBACCO STEMBORER, *Scrobipalpa aptatella* (Walker) (LEPIDOPTERA: GELECHIIDAE) FROM NORTHERN MINDANAO USING *CYTOCHROME C OXIDASE I* [1]

Aaron V. Ogot, Romnick A. Latina, and Barbara L. Caoili[2]

[1]Part of the BS Agricultural Biotechnology (major in Crop Protection-Entomology) thesis of the senior author, under the supervision of the co-authors. Institute of Weed Science, Entomology, and Plant Pathology, College of Agriculture and Food Science, University of the Philippines Los Baños, Laguna. 4031. Presented during the 49th Anniversary and Annual Scientific Convention of the Pest Management Council of the Philippines, Inc. May 10-12, 2017, Boracay Island, Malay, Aklan.

[2]Institute of Weed Science, Entomology, and Plant Pathology, College of Agriculture and Food Science, University of the Philippines Los Baños, College. 4031 Laguna, Philippines; Corresponding author: BLC, e-mail: blcaoili@up.edu.ph

ABSTRACT

***Scrobipalpa aptatella* (Walker) or tobacco stemborer (TSB) is a reported pest of tobacco worldwide. Its damage is characterized by stem tunnels created by larval feeding that consequently result to stem galling and wilting as the infestation progresses. Proper identification of TSB is crucial in establishing effective control measures to prevent serious infestation. DNA barcoding was used to establish the identity of TSB infesting tobacco plantations. Using *cytochrome c oxidase I* (*COI*) gene as the molecular marker, TSB samples collected from Claveria, Misamis Oriental and Bukidnon revealed 99.0% nucleotide similarity to *S. aptatella* (Walker) *COI* sequence (GenBank Acc. No. KF388766). Multiple alignment of the *COI* sequences showed single nucleotide polymorphisms within and between the collected populations. Haplotype and nucleotide diversity analyses resulted to values of 0.2584 and 0.00074, respectively, that revealed five haplotypes. These haplotypes showed distinct separation in the phylogenetic tree constructed. Evidence of TSB population expansion was also observed based on Tajima's *D* and Fu's *Fs* tests of neutrality. The occurrence of *S. aptatella* in these production areas calls for the development of control measures against TSB infestation.**

Key words: *cytochrome c oxidase subunit I* (*COI*), DNA barcoding, haplotypes, *Nicotiana tabacum, Scrobipalpa aptatella*

INTRODUCTION

Tobacco (*Nicotiana tabacum* L.) is cultivated in over a hundred countries covering almost four million hectares of agricultural land. In the Philippines, 27

provinces grow tobacco with nearly 90% of production concentrated in the provinces of Ilocos Sur, Ilocos Norte, La Union, Pangasinan, and Isabela. The local tobacco industry contributes 0.4% to the total employment in the agricultural sector (NTA, 2015). Most tobacco plantations are devoted to cigarette production which accounts for 99.0% total sales value (Casorla et al., 2012).

Like any agricultural crop, tobacco also suffers damages from a variety of pest and diseases. One identified insect pest of tobacco is *Scrobipalpa aptatella* (Walker) [=*S. heliopa* (Lower)], commonly known as the tobacco stemborer (TSB) (Gabriel, 2000). This stemborer species is also reported to infest eggplant (*Solanum melongena* L.) (Kisha, 1985). The larvae of *S. aptatella* mainly inflict damage in the succulent stems, which results to stem galling (Jagadish, 1979). They also feed on the leaf midrib and bud. As the infestation advances, other symptoms such as wilting of leaves and sucker formation are observed (De Faveri & Malipatil, 2007). The TSB attacks all stages of the plant but prefers the seedling and transplant stages. During these stages, high infestations can totally wipe out plantations. Hence, early detection of the presence of the borer is needed to prevent further infestation.

The success of early detection and monitoring of TSB depends on proper identification of the insect pest. However, since the destructive stage of the insect is the larva, accurate identification of the pest is difficult. Traditional identification of TSB relies heavily on distinguishing morphological characters of adults. This technique, although very useful, is limited to expertise of taxonomists and state of samples identified (Chan et al., 2014). Damage on the host plants as a basis of identification of the insect pest is also an unreliable method because the nature of damage of TSB infestation resembles that of another lepidopterous pest of tobacco, the potato moth, *Phthorimaea operculella* (Zeller) (De Faveri & Malipatil, 2007).

With these limitations in morphological and damage-based species identification, molecular-based approaches such as DNA barcoding is highly suggested to reinforce these traditional methods. DNA barcoding is a technique developed for rapid but accurate identification of species with the use of short, standardized DNA sequence (Hebert & Gregory, 2005). The technique was developed to aid and minimize the limitations of the morphology-based identification of species in cases where the organism has a divergent morphology (Chan et al., 2014). The most common DNA barcode is the *cytochrome c oxidase subunit I* (*COI/COXI*), which is located in the mitogenome. Mitochondrial genes, being maternally inherited, do not undergo recombination, thus exhibiting faster rate of evolution (Brown et al., 1979). This made *COI* one of the most commonly used molecular markers for species delimitation not only for insects but also other animals (Hebert et al., 2003a). Aside from molecular barcoding, *COI* can also be used in genetic diversity studies. Multiple studies on insect diversity based on the variations of one of the regions of *COI*, the Folmer region, include the Casuarina Moth, *Lymantria xylina* Swinhoe (Wang et al., 2018), *Aedes albopictus* (Skuse) (Fang et al., 2018), *Culex pipiens* L. (Simonato et al., 2016), and diamondback moth, *Plutella xylostella* (L.) (Juric et al., 2017). Since TSB is under the family Gelechiidae which are microlepidopterans, its identification based on morphological characters is confounded by its minute size and cannot easily be done without proper expertise and tools. In the case of cryptic species

screening, Huemer et al. (2014) established the use of DNA barcoding as a screening tool for cryptic diversity analysis of the genus *Caryocolum* (Lepidoptera: Gelechiidae). Their results revealed the presence of eight species within this genus with an intraspecific divergence greater than 3.5% in the DNA barcode marker.

To date, there are no available studies on the molecular identification and genetic diversity of TSB in the Philippines. Since accurate identification as well as the knowledge on the genetic diversity of insect pest species are vital for the development of proper management and control strategies, this study was designed to establish the identity of the TSB species infesting tobacco fields in northern Mindanao, specifically Misamis Oriental and Bukidnon, based on *COI* gene, and to perform preliminary diversity analysis.

MATERIALS AND METHODS

Field collection of tobacco stem borer

Lepidopterous stemborer species were collected from tobacco plantations in Brgy. Panampawan, Claveria, Misamis Oriental province; and Brgy. Salimbalan, Baungon; and Brgy. Lindaban, Manolo Fortich, in Bukidnon province. Infested stems of tobacco plant were collected and dissected for larval sampling. The TSB larvae were segregated depending on their life stage.

Amplification of *COI* of the tobacco stem borer

Total genomic DNA extraction was done following the standard protocol of the Animal and Fungi DNA Preparation Kit™ (Jena Biosciences Gmbh, Jena, Germany, www.jenabioscience.com) with slight modifications. The total genomic DNA samples were quantified and quality-checked using BioDrop 125 (Biodrop Ltd., www.biodrop.co.uk) spectrophotometer. The *cytochrome c oxidase subunit I* (*COI*) gene amplification was done with the use of the following set of primers; LepF1 5'- ATTCAACCAATCATAAAGATATTGG-3' and LepR1 5'- TAAACTTCTGGATGTCC AAAAAATCA-3', which targets the Folmer region of the *COI* gene, a widely used marker for DNA barcoding of animals, including insects, although the LepF1 and LepR1 primer sequences have been modified for specific use on lepidopterans (Hajibabaei et al., 2006). Also, for the molecular identification of the TSB, the available barcodes in databases such as GenBank and BOLD (Barcoding of Life Database) are the *COI* Folmer region. Polymerase chain reaction was carried out using a 25 µL reaction mix containing 12.5 µL of 2X Taq master mix (Vivantis, Malaysia), 10 pmol each of the primers, 2.5 mM $MgCl_2$, and 100 ng DNA template. The thermal profile used was based on the study of Hebert et al. (2013) with the following conditions: initial denaturation for one min at 94°C, five cycles at 94°C for 40 sec, 45°C for 40 sec, 72°C for one min followed by 35 cycles at 94°C for 40 sec, 51°C for 40 sec, 72°C for one min and final elongation at 72°C for five min. The amplified *COI* gene fragments were resolved in 1.0% agarose gel. The molecular size of the amplicons were estimated using VC 100bp Plus DNA ladder (Vivantis, Malaysia).

***COI* gene nucleotide sequence analysis**

The amplified DNA samples were sent to Apical Scientific Sdn. Bhd. (Taman Serdang Persadana, 43300 Seri Lembangan, Selangor, Malaysia) through AsiaGel Corporation (Quezon City, Philippines) for sequencing. The nucleotide sequences were processed and analyzed using BioEdit (Ibis Biosciences, Carlsbad, CA, USA). Phylogenetic analysis using maximum likelihood based on Tamura-Nei substitution model with 1000 bootstraps was carried out in MEGA 7 (Kumar et al., 2016) while genetic diversity analysis based on the number of polymorphic sites (NPS), number of haplotypes (Nh), haplotypic diversity (h), nucleotide diversity index (π), and Tajima's *D* and Fu's *Fs* neutrality analysis, was estimated using DnaSP 5.1 (Rozas et al., 2017).

RESULTS AND DISCUSSION

Molecular identification of tobacco stem borer using *COI*

The mitochondrial gene, *COI*, has been favored for taxonomical studies due to its high rate of evolution and has been used as a DNA barcode to resolve species identification issues. *COI* has been used as a genetic marker in a wide range of animal phyla. Hebert et al. (2003b) established *COI* profiles of eight orders of the class Insecta including representative species from the order Lepidoptera. In 2013, Hebert et al. conducted a feasibility study on creating a DNA barcode library of Australian lepidopteran fauna, wherein a total of 41,650 specimen were processed that included the TSB, *S. aptatella*. Factors affecting the recovery of the DNA barcode such as age, body size, and collector were assessed in the study because these factors are crucial for the quality and quantity of the barcode gene that can be obtained from specimen found in collections.

Using different developmental stages, such as adults, pupae, and late instar larvae, the partial sequence of the *COI* region was successfully amplified from the three populations of the TSB collected from northern Mindanao, namely: Brgy. Salimbalan, Baungon, Bukidnon, BB, (GenBank Acc. No. MH286496 and MH286499), Brgy. Panampawan, Claveria, Misamis Oriental, CLM, (GenBank Acc. No. MH286497, MH286500-502), and Brgy. Lindaban, Manolo Fortich, Bukidnon, MFB, (GenBank Acc. No. MH286498). The generated amplicons were approximately 650 base pairs (bp) (Figure 1), which is in agreement with the reported lepidopteran *COI* barcode region by Hebert et al. (2013). The partial nucleotide sequences had 99.0% similarity to *S. aptatella* (GenBank KF388766) based on BLASTn (nucleotide BLAST) results (Table 1).

A maximum likelihood tree based on Tamura-Nei model was constructed for the three populations of *S. aptatella*, *S. leucocephala* (Lower) (Genbank KF390714), *Australiopalpa tristis* Povolný (Genbank KF387945) and *Sarotorna myrrhina* Turner (Genbank KF389078), with the two latter species as outgroups (Figure 2). The identity of the collected individuals was confirmed with the formation of a monophyletic clade for *S. aptatella* with a high bootstrap support of 99%. Within the *S. aptatella* clade, five subclades were observed.

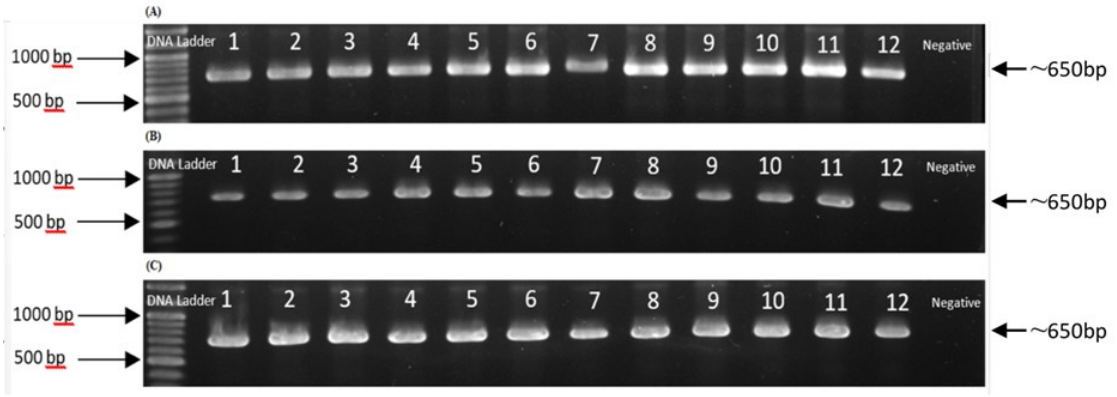

Figure 1. Molecular weight estimation of the amplicons of *cytochrome c oxidase I* (*COI*) gene region of *Scrobipalpa aptatella* from (A) Claveria, Misamis Oriental, (B) Manolo Fortich, Bukidnon, and (C) Baungon, Bukidnon. The *COI* gene fragments were amplified using the primer pair, LepF1 and LepR1 (Hajibabei et al., 2006) and resolved using 1% agarose gel.

Table 1. BLAST Results yielding >99% nucleotide identity with *Scrobipalpa aptatella,* GenBank Acc. No. KF388766, for the molecular identification of tobacco stemborers collected in northern Mindanao using *cytochrome c oxidase I* nucleotide sequences.

Sample Code	Collection Site	Coordinates & Elevation	Nucleotide Identity (%)
BB 1, 3-14	Brgy. Salimbalan, Baungon, Bukidnon	N-8°16.056' E-124°42.161' 610 m	99.85
BB 2, 15			99.39
CLM 1, 2, 4-10, 13-14	Brgy. Panampawan, Claveria, Misamis Oriental	N-14°09.951' E-121°14.388' 858 m	99.85
CLM 3, 15			99.70
CLM 11			99.70
CLM 12			99.54
MFB 1-15	Brgy. Lindaban, Manolo Fortich, Bukidnon	N-08°16.771' E-124°50.773' 772 m	99.85

The largest subclade is composed of samples from the three collection sites. All samples from Brgy. Lindaban, Manolo Fortich, Bukidnon are grouped within this clade. The absence of variation within this population can be due to its relatively new establishment, compared to the other two populations. The CLM population which was collected from Brgy. Panampawan, Claveria, Misamis Oriental, and the first plantation reported to be infested with TSB, were distributed in four of the five subclades, while another subclade formed from BB samples. The CLM subclades have little deviation in its sequences from the first clade (Table 2), indicating variations of *COI* gene sequences within this population. The largest deviation from the major clade can be observed from the sequences of individuals BB2 and BB15 from Baungon, Bukidnon. The

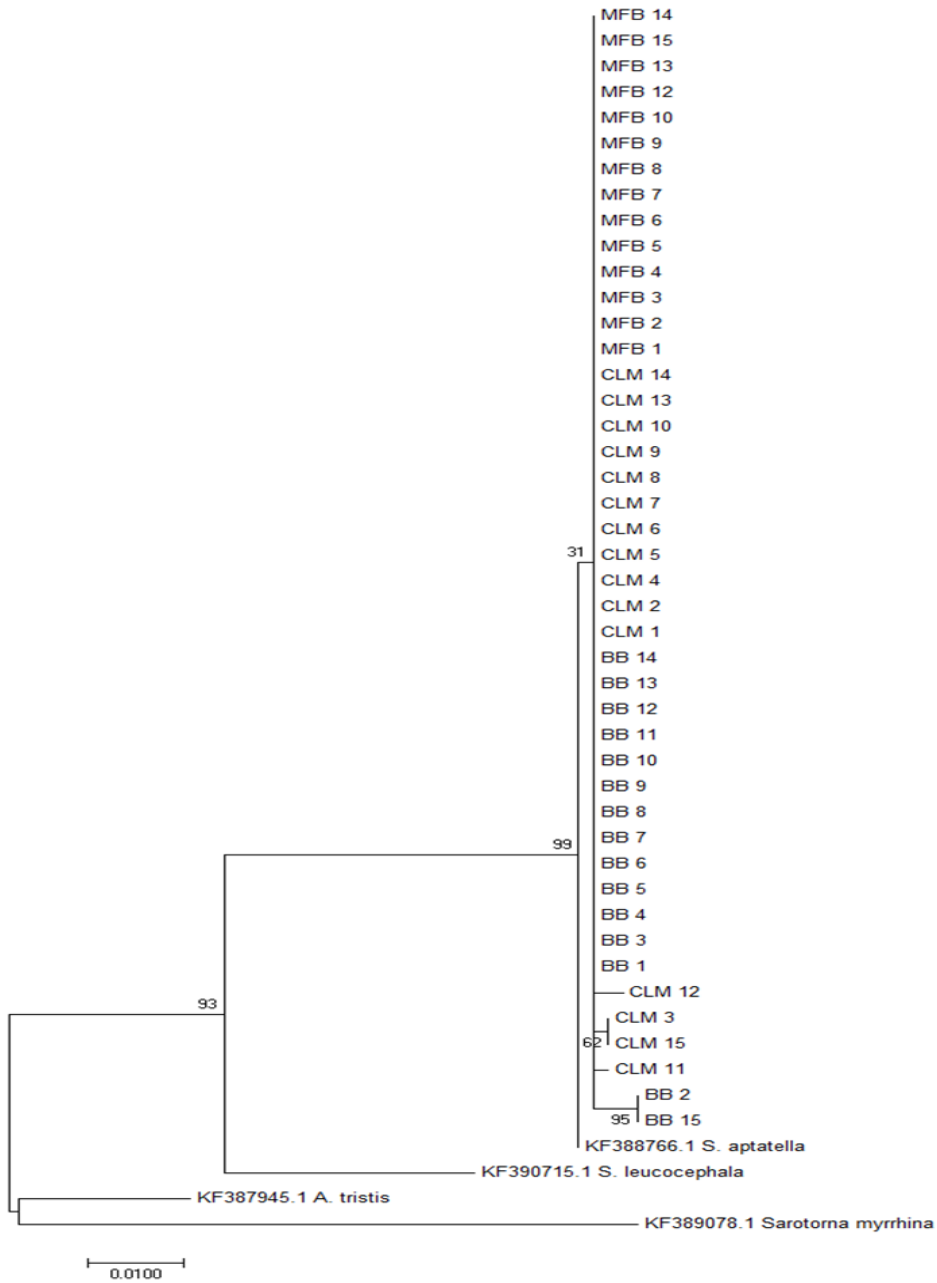

Figure 2. Molecular phylogenetic analysis based on *cytochrome c oxidase I* (*COI*) gene of populations of *Scrobipalpa aptatella* from Brgy. Panampawan, Claveria, Misamis Oriental (CLM), Brgy. Lindaban Manolo Fortich Bukidnon (MFB) and Brgy. Salimbalan, Baungon, Bukidnon (BB) using maximum likelihood method based on Tamura-Nei substitution model. Bootstrap support are indicated at nodes. The scale bar represents 0.01 substitutions/state change per position. *S. leucocephala* KF390714, *Australiopalpa tristis* KF387945, and *Sarotorna myrrhina* KF389078 were included as outgroups.

Table 2. Summary of nucleotide polymorphisms and amino acid change among haplotypes of TSB collected in Mindanao. *Scrobipalpa aptatella* KF388766 as reference sequence.

Haplotype	Representative Samples	Nucleotide position							
		205	211	271	343	361	373	616	646
KF388766 *S. aptatella*		C	A	C	T	T	A	T	C
Haplotype 1	MFB						G		
Haplotype 2	BB 2 & 15			G		C	G		T
Haplotype 3	CLM 3 & 15	T					G		
Haplotype 4	CLM 11				C		G		
Haplotype 5	CLM 12		G				G	C	
Amino acid change		-	-	-	-	-	-	-	-

CLM and BB individuals that have variations can be observed to form different subclades, which is probably due to the independent adaptation and population growth of each population. In a similar study using *COI* gene to identify species under the genus *Spodoptera* from different geographical locations, the maximum likelihood tree also revealed intraspecific variations of species from different geographic locations (Ramamurthy et al., 2015). Based on the same phylogenetic analysis, haplotypes were also observed on *Helicoverpa armigera* (Hübner) collected from five countries (Behere et al., 2007). In the same study, genetic diversity analysis also showed low level intraspecific variations among the different populations.

Genetic diversity analysis of *S. aptatella* populations

Diversity analysis of the 44 *S. aptatella COI* gene sequences generated from TSB samples taken from three tobacco plantation sites in northern Mindanao also revealed five haplotypes similar to what the phylogenetic tree has presented. These haplotypes are labelled as Hap 1, Hap 2, Hap 3, Hap 4, and Hap 5 (Table 3). Hap 1 has the most number of individuals and represents 86.36% of the collected TSBs. Each of Hap 2 and Hap 3 represents 4.55% of the samples collected, while Hap 4 and Hap 5 are singleton haplotypes. The population collected in Brgy. Lindaban, Manolo Fortich, Bukidnon belongs only to one haplotype, Hap 1. The other three haplotypes consist of only individuals collected from Brgy. Panampawan, Claveria, Misamis Oriental. Low haplotype diversity (h) and nucleotide diversity index (π) values (Table 4) for each population and as a combined or single Mindanao population denote that the differences between haplotypes within the populations are just single nucleotide polymorphisms and are manifestation of slow demographic expansion. Although there were a lot of polymorphic sites when haplotypes were compared, most of these differences are indeed just single nucleotide variations. Multiple nucleotide sequence alignment from each population revealed distinct single nucleotide polymorphisms brought about by transitions and transversions (Table 2). In the samples from Claveria, Misamis Oriental, four substitutions were observed and classified as transition point mutation, while in those from Baungon, Bukidnon, three substitutions were observed with two transitions and a transversion. Meanwhile, the alignment of *COI* gene from individuals collected from Manolo Fortich, Bukidnon

Table 3. Distribution of *COI* haplotypes of tobacco stemborer *Scrobipalpa aptatella* derived from 44 nucleotide sequences from Brgy. Salimbalan, Baungon, Bukidnon (BB), Brgy, Panampawan, Claveria, Misamis Oriental (CLM,) and Brgy. Lindaban, Manolo Fortich, Bukidnon (MFB).

Haplotype	No. of samples	Collection Site/Samples
Hap 1	38	BB_1, BB_3, BB_4, BB_5, BB_6, BB_7, BB_8, BB_9, BB_10, BB_11, BB_12, BB_13, BB_14, CLM_1, CLM_2, CLM_4, CLM_5, CLM_6, CLM_7, CLM_8, CLM_9, CLM_10, CLM_13, CLM_14, MFB_1, MFB_2, MFB_3, MFB_4, MFB_5, MFB_6, MFB_7, MFB_8, MFB_9, MFB_10, MFB_12, MFB_13, MFB_14, MFB_15
Hap 2	2	BB_2 and BB_15
Hap 3	2	CLM_3 and CLM_15
Hap 4	1	CLM_11
Hap 5	1	CLM_12

Table 4. Sample size (N), number of polymorphic site (NPS), number of haplotypes (Nh), haplotypic diversity (h), and nucleotide diversity index (π) of *Scrobipalpa aptatella* used in the study.

Site	N	NPS	Nh	h	π
Brgy. Panampawan, Claveria, Misamis Oriental	15	4	4	Hd: 0.4667	0.00097
Brgy. Lindaban, Manolo Fortich, Bukidnon	14	0	1	Hd: 0.0000	0.00000
Brgy. Salimbalan, Baungon, Bukidnon	15	3	2	Hd: 0.2476	0.00109
Combined populations	44	7	5	Hd: 0.2548	0.00074

revealed no polymorphisms. The absence of polymorphism is probably due to the relatively young population of the TSB in the area since the tobacco plantation was just recently established. When each *COI* haplotype sequences was translated, all point mutations were shown to be just silent synonymous substitutions. The alignment of amino acid sequence of each haplotype revealed no observable difference from the amino acid sequence of *S. aptatella* (Accession No. AGS95458) in the GenBank. With the knowledge that the plantations in Mindanao are relatively new, management strategies are needed because, in general, populations of *S. aptatella* from Claveria, Misamis Oriental, and Bukidnon are already expanding although very slowly.

Neutrality test for *COI* gene evolution

This part of the study excluded the population from Brgy. Lindaban, Manolo Fortich, Bukidnon because there were no observed polymorphisms. Thus, only the populations from Claveria, Misamis Oriental, and Baungon, Bukidnon, were subjected to the neutrality tests. These tests are done not just to reject the neutral evolution hypothesis but also to provide strong summary statistics to interpret DNA sequence polymorphisms in population studies and to

somehow explain demographic expansion of some species using some molecular markers (Tajima, 1989; Simonsen et al., 1995; Dogan & Dogan, 2016). Based on Tajima's *D* test, the *COI* sequences from each population showed non-significant deviation of the *D* value from 0, which follows the neutrality mutation hypothesis. Similarly, for the Fu's *F*s test, separate analysis of the two populations showed strong inclination to neutrality hypothesis (Table 5). The combined *COI* sequences from both populations, on the other hand, implied non-neutral evolution. Both tests generated negative values or very low statistics indicative of excess of *COI* alleles at low frequencies as a result of recent population expansion. The tobacco plantation in Claveria, Misamis Oriental, was one of the first tobacco plantations in Mindanao and was established in 2013 while the plantation in Manolo Fortich, Bukidnon, was just established in 2016. The infestation of TSB was also first reported in that area in 2014 but the first outbreak was in 2015 (Mr. Lupo Villanueva, Personal Communication, November 13, 2016). The Baungon, Bukidnon, and Manolo Fortich, Bukidnon TSB populations were just recently reported. Although, its presence in the Philippines was reported as early as in 1957, no record of outbreak has been hitherto reported. TSB is reported to have an alternate host, eggplant (*Solanum melongena* L.), and this alternate host was observed and reported to be planted in other barangays in Claveria, Misamis Oriental. The outbreak in Northern Mindanao may be due to the presence of TSB in the area but in low numbers and on its alternate host. With the establishment of tobacco plantations, TSB infested its main host and the populations started to expand. TSB was also observed in neighboring countries such as Indonesia and Malaysia, but the introduction of TSB from these countries had been ruled out since planting materials used by PMFTC, Inc. are all sourced from Brazil in which TSB is not reported. With the current knowledge of the plantations, Tajima's *D* and Fu's *F*s test suggest that the TSB populations in Claveria, Misamis Oriental and Baungon, Bukidnon, contained excess of rare haplotypes due to the expanding population.

Tobacco production remains to be a steadfast agricultural industry in the Philippines. With the recent reports of possible TSB resurgence, an established identification protocol is needed for the development of control measures against this insect pest. DNA barcoding, which is a very straightforward, molecular-based protocol using *COI* as used in this pioneering local study, offers an effective way of identification. The technique accurately confirmed the insect pest infesting tobacco plantations in Mindanao as *S. aptatella*. Through genetic diversity analysis and neutrality test, which are all based on *COI* sequence

Table 5. Tajima's *D* and Fu's *Fs* neutrality analysis of *Scrobipalpa aptatella cytochrome c oxidase I* (*COI*) gene.

Site	Tajima's *D*		Fu's *Fs*	
	D	Significance	*Fs*	p-value
Brgy. Panampawan, Claveria, Misamis Oriental	-1.51811	P > 0.10^{ns}	-1.287	0.076
Brgy. Salimbalan, Baungon, Bukidnon	-0.57961	P > 0.10^{ns}	1.960	0.822
Combined populations	-1.88522	P > 0.10*	-2.165	0.050

polymorphic information, presence of variation among the individuals of the population proved the existence of haplotypes and suggested the recent population expansion phenomenon particularly in the Misamis Oriental and Bukidnon populations. The low value of haplotype and nucleotide diversity indicates that although the population is expanding, such is happening at a slow rate. The knowledge on the structure and movement of TSB populations, at their young stage, offers an opportune time for devising integrated approaches to control and manage these pests, and prevent further expansion. The data on and identity of the sex pheromone of female TSB are already available (Baker et al., 1985). Hence, pheromone traps infused with the pesticides that tested to be effective (Sreedhar, 2014) could be recommended as a management strategy. With right timing of application, these pheromone traps could lower the population density of adult males, thus lowering the reproduction of the species. Moreover, strict monitoring of the movement of tobacco planting materials and other possible TSB dispersal agents should be employed.

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