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Morphological and molecular analyses of Leafhopper, *Amrasca biguttula* (Ishida) (Hemiptera: Cicadellidae) infesting eggplant (*Solanum melongena* L.) in Luzon Island, Philippines

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ABSTRACT

Eggplant (*Solanum melongena* L.) is the leading vegetable grown and consumed in the Philippines, and the eggplant leafhopper is one of its most important insect pests. Nine local populations of leafhopper infesting eggplants were collected from major eggplant growing areas in Luzon Island to determine if these populations are constituted singly or by different species and to assess the level of variability among them. Morphological analysis of the adult male abdominal and genital traits and partial mitochondrial *COI* gene sequence identified the eggplant leafhopper populations in Luzon Island as belonging to *Amrasca (=Sundapteryx) biguttula* (Ishida) (Hemiptera:Cicadelliae). Observed male specimens exhibited modifications in the pregenital abdominal tergites VII and VIII that were previously reported to not be found in other *Amrasca* species. Principal Component Analysis of four morphometric genital traits revealed high similarity among the leafhopper populations regardless of eggplant production areas. Partial *COI* sequence analysis idenfitied 11 sequence variants (haplotypes), with one predominant haplotype. Overall, the leafhopper populations from Luzon Island exhibited moderate haplotype diversity and low nucleotide diversity, indicating low genetic diversity. Topologies from a maximum likelihood tree indicate all eleven haplotypes cluster in a single clade with other *A. biguttula* infesting cotton and potato in India, but the Philippine population forms a separate subgroup although with weak bootstrap support. Further analysis with the Generalized Mixed Yule Coalescent (GMYC) method classified the different haplotypes into a single GMYC entity.

Introduction

The micro leafhopper genus *Amrasca*, within the sub-family Typhlocybinae (Hemiptera: Cicadellidae) includes a serious pest species, the cotton leafhopper, *Amrasca* (=*Sundapteryx*) *biguttula* (Ishida), that has a wide host range (Xu et al., 2017). Other names of this insect include the green jassid, Indian cotton jassid, and okra leafhopper (Bellis and Fletcher, 2005). This phloem-feeder harm plants by direct physical injury through depletion of plant nutrients during feeding. Heavy infestation of leafhopper can cause the leaves to appear yellowish or burned, crinkled and small, and the plant growth stunted resulting in yield loss of up to 50% (Kranthi et al., 2018). Unlike other leafhopper species, cotton leafhopper is not known to transmit any virus or mycoplasma diseases (Nielson, 1968).

Solanum melongena L. also known as eggplant, is the leading vegetable grown and consumed in the Philippines. Eggplant is one of the top 30 food items and one of the top 3 vegetables commonly consumed in the Philippines (FNRI, 2008). Total eggplant production in the Philippines has been increasing since 2000. In 2017, eggplant production reached 241,901 metric tons grown in 21,446 ha. It accounted for more than 30% of the total production volume of top vegetables in the country (Bureau of Agricultural Statistics, 2016). Eggplant production is very profitable and provides a ready source of cash income particularly for small, resource-poor farmers (Chupungco et al., 2014a).

A major constraint to eggplant production is the infestation by eggplant insect pests. The leafhopper is considered to be the second most serious insect pest of eggplant as it affects the occurrence and abundance of other major insect pest species such as eggplant borer (*Leucinodes orbonalis*), thrips (*Thrips palmi*) and whiteflies (*Bemisia tabaci*) (Navasero, 2015). As early as 1937, the insect was recorded in cotton and eggplant in the Philippines as *Empoasca flavescens* (Fabricius) (Woodworth, 1921,1922) and succeeding reports retained the

Abbreviations: COI, cytochrome oxidase I; DNA, deoxyribonucleic acid; LH, leafhopper; PCR, polymerase chain reaction

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name (Merino, 1936; Otanes and Butac, 1938, 1939). It was renamed by Capco (1957) and Baltazar (1968) as *Empoasca biguttula* (Matsumura), which was adopted by Gabriel (1975). The genus name was changed from *Empoasca* to *Amrasca* (Kapoor and Sori, 1972) and the latter is presently being adopted in the Philippines (Pascua and Pascua, 2002). The species occurring on eggplant was reported as single species based on morphological observations but no report has been published that clearly established the identity and diversity of leafhopper populations infesting eggplants in the Philippines. Given the increasing trend in eggplant production in the country and the potential damage from leafhopper, this study was conducted to provide information that will help in the effective management of this important insect pest in eggplant.

Over the years the identity of the Indian cotton leafhopper has created much confusion and many synonyms exist (Xu et al., 2017). The male genital structure is the most commonly used subject for insect species identification because it is highly specialized and modified (Sapin et al., 2013). More recently, a distinctive, previously unreported modifications of the male pregenital abdominal tergites (VII and VIII) has been used as identification key for *A. bigutulla* Ishida (Xu et al., 2017). The mitochondrial *cytochrome c oxidase I* (*COI*) gene has also been established as a core for a bio-identification or DNA barcoding system in animals (Hebert et al., 2003). *COI* sequences are also appropriate for assessing intraspecific variation due to its high degree of polymorphism (Hu et al., 2008). Recent works in India (Kranthi et al., 2018; Sagar et al., 2014) reported low genetic diversity for *A. biguttula* in cotton based on *COI* sequence analysis. *COI* sequences are also useful as a phylogenetic marker that may reflect evolutionary patterns within and among species (Galtier et al., 2009).

In this study, we evaluated nine populations of leafhopper infesting eggplants based on male adult pregenital abdominal and genital traits and mitochondrial *COI* gene sequence data to validate the single species identity and assess the genetic structure of leafhopper populations in major eggplant growing provinces in the Philippines. The key questions addressed by this study were: (a) whether leafhopper populations feeding on eggplant in major production areas in Luzon are constituted by *A. biguttula* Ishida alone or composed of different species and (b) whether these populations are genetically diverse or similar, based on morphological characters and *COI* sequence data.

Materials and methods

Collection and preservation

Leafhopper samples were collected from eggplant fields in major production areas in Luzon island (Table 1 **and Fig. S1)**. The provinces were selected based on the mean eggplant production area from 2006 to 2015 (Bureau of Agricultural Statistics, 2016) with different climate types. There are four recognized climate types in the Philippines which are based on rainfall PAGASA, 2015). I - Two pronounced seasons: dry from November to April and wet during the rest of the year; Type II - No dry season with a pronounced rainfall from November to January; Type III - Seasons are not very pronounced, relatively dry from November to April, and wet during the rest of the year; Type IV - Rainfall is more or less evenly distributed throughout the year. Insects were obtained using insect sweep nets, then stored in 70% ethanol. Samples for sequence analysis from different municipalities were kept individually in separate tubes and stored at −20 °C before DNA extraction.

Morphological analysis

Preparation of whole specimens. The leafhopper was carefully taken out from vials containing 70% ethanol using fine forceps and air-dried in paper towel. The side of the thoracic region was oriented horizontally along the long axis, glued on triangular point with a tiny drop of clear nail polish. The specimen was tilted slightly (45°) so that the dorsal surface would be entirely visible. The specimen was positioned with the abdomen flat against the card to help keep the head off the card surface. It is very important that the entire frontal surface of the head is visible. Whole specimens were viewed under a stereo zoom microscope (Nikon SMZ1500) under 40× magnification.

Preparation of male genitalia. The abdomen was carefully detached from the thoracic region with a pair of fine forceps and needle and placed in a evaporating dish with a small amount of 10% NaOH, boiled for 1 min, rinsed 1 to 2 times in distilled water, and stained with one or two drops of acid fuchsin solution for one minute. The washed genital portion was placed in a depression slide with 1 to 2 drops of glycerin. Photographs of the genitals were taken using a stereo zoom microscope (40x magnification) and measurements of different parts were taken and recorded. Afterwards, the specimens were kept in microvials containing glycerin.

Morphometric analysis. The following parts of adult male genitalia were measured (Fig. 1): the aedeagus (**ae**), length of anal tube (**at**), length of subgenital plate (**sp**), and length of ventral pygofer appendage (**vpa)** from a total of 433 male genitalia of leafhopper adults. The measurements were taken from 20 individual specimens per municipality. Data were analyzed by ANOVA at 5% significance level and by principal component analysis (PCA) based on a variance–covariance matrix using the Past 3.18 software (Hammer et al., 2001).

Species identification. The morphological terminology followed the diagnostic key for genera developed by Borror et al. (1971) and for the species as described by Xu et al. (2017).

DNA extraction, COI gene amplification and sequencing

Total DNA was extracted from leafhoppers using a protocol described by Muturi et al. (2013). The DNA was extracted from a whole-body sample of each leafhopper. The extracted DNA was stored in 10 μL Tris-EDTA buffer. The *COI* gene was amplified using universal primers, LCO1490 (F), 5′-GGTCAACAAATCATAAAGATATTGG-3′ and reverse primer HCO2198 (R), 5′-TAAACTTCAGGGTGACCAAAAAATCA-3′ (Folmer et al., 1994). A 20 μL polymerase chain reaction (PCR) was prepared composed of 1X PCR Buffer (Vivantis, Malaysia), 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 μM each of forward and reverse primers, and 1 U of Recombinant *Taq* polymerase (Vivantis, Malaysia). The PCR was run for about two hours in a G-Storm thermocycler (RainPhil, England) following the cycling program: 5 min. 95 °C, 35 × (30 s 95 °C, 45 s 55 °C, 1 min. 72 °C), 3 min. 72 °C (Chang et al., 2014). The amplicons were visualized in a 1.25% agarose gel electrophoresis stained with GelRed® (Biotium, USA), along with a 1 Kb plus DNA ladder (Invitrogen, USA) at 100 V for 35 mins. The PCR products were quantified from the electrophoretogram using Image Lab® (Bio-Rad, USA). The amplicons were sent to Macrogen Inc. (Seoul, South Korea) for purification and Sanger sequencing of the forward strand (single pass) with the PCR primers used as the sequencing primers.

COI gene sequence annotation and diversity analysis

The low-quality bases (≤Q25) at the ends of the received sequences were trimmed using the Sequencher® software (Gene Codes, USA). The trimmed sequences were queried to the NCBI non-redundant (*nr*) database using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). To identify the origin of the *COI* gene at the species level, the sequences were submitted to the Barcode of Life Data System (BOLD) (Ratnasingham and Hebert, 2007). The sequences were aligned using the MUSCLE algorithm in MEGA software with a reference *A. biguttula COI* gene sequence (ID: KJ867503.1) retrieved from NCBI and then manually proofread. The sequence diversity of the *COI* gene was

Table 1
Collection sites of eggplant leafhopper (LH) from Luzon, Philippines.

Region	Province	Municipality	Climate Type	Date of Collection	Latitude	Longitude	Elevation (m)
I	Ilocos Norte	Laoag	I	September 2017	18.19	120.66	76
		Batac			18.01	120.57	100
	Pangasinan	Villasis		April 2017	15.9	120.58	106
		Sta. Maria		July 2017	15.98	120.7	43
II	Isabela	Roxas	III	August 2017	17.1	121.61	127
		Aurora			17	121.7	89
		Cabatuan			16.93	121.64	114
	Cagayan	Sto. Niño		September 2017	17.9	121.59	61
		Alcala			17.94	121.64	59
		Solana			17.59	121.59	90
III	Tarlac	Gerona	I	February 2017	15.58	120.55	81
		Victoria			15.58	120.66	67
		Concepcion			15.3	120.75	48
IV-A	Laguna	Los Baños	I	April 2017	14.15	121.25	42
		San Pablo			14.2	121.32	138
		Victoria		August 2017	14.04	121.34	60
	Quezon	Dolores		July 2017	14.01	121.35	116
		Sariaya	III		13.84	121.47	38
		Candelaria			13.91	121.41	87
	Batangas	Balete	I	July 2016	14.03	121.1	39
		Lipa			13.99	121.2	341
		Batangas City		September 2016	13.69	121.07	50
V	Albay	Guinobatan	IV	May 2017	13.2	123.57	153
		Tobaco City	II		13.32	123.66	270
		Legaspi City	II		13.18	123.73	118

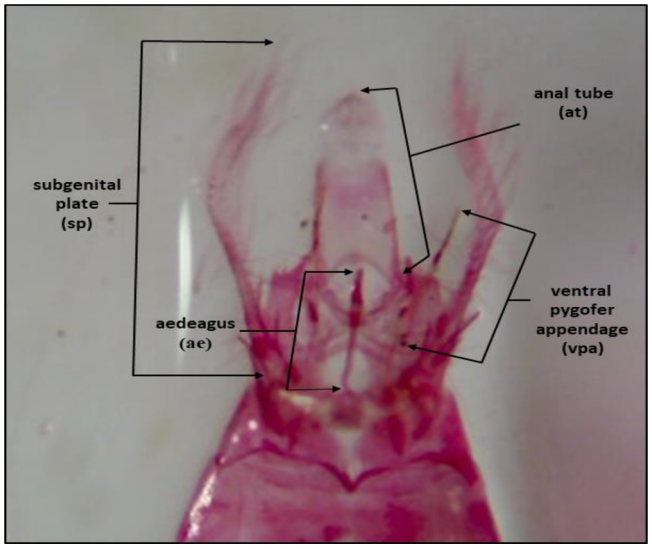

Fig. 1. Measured parts in the male genitalia of leafhopper.

calculated in terms of variable nucleotide sites, number of haplotypes, nucleotide diversity and haplotype diversity using DnaSP 6.10.04 (Librado and Rozas, 2009).

COI sequence divergence and population genetic analyses

To assess the *COI* sequence diversity, the number of variable nucleotide sites, number of haplotypes, nucleotide diversity and haplotype diversity were calculated using DnaSP 5.10 (Librado and Rozas, 2009). Sequences that had 100% nucleotide similarity were designated as a single haplotype. A minimum spanning haplotype network was also constructed to study the relationships among *A. biguttula* sequences using PopArt (Leigh and Bryant, 2015). Tajima's D test (Tajima, 1989) and Fu's Fs test (Fu, 1997) were used to study evolutionary neutrality or identify recent population expansions, calculated using DnaSP and considering only the number of segregating sites using coalescent simulation with 1000 replications for statistical support.

Phylogenetic analyses

The analysis of genetic and phylogenetic relationships of *COI* sequences was done using MEGA 7.0.18 (Kumar et al., 2016). The sequence similarities between populations and haplotypes were studied by calculating the genetic distance using the Tamura 3-parameter model (T92) (Tamura, 1992). Maximum Likelihood analyses were performed using the Tamura 3-parameter model with invariant sites (T92 + I), which was determined to be the best substitution model based on Bayesian Information Criterion (BIC) in MEGA 7. Various *COI* sequences of *A. biguttula* from GenBank and representative sequences of cotton leafhopper from India (Kranthi et al., 2018) were included in the analyses for comparison. Some sequences from GenBank were omitted from the analyses (MH570182.1, MH570181.1, and MH570179.1) as these had short sequences. Sequences from *Empoasca vitis* and *Nephotettix virescens* were included as outgroups. Clustering probabilities were statistically tested by the bootstrap method with 1000 replicates.

Species delimitation

The General Mixed Yule-Coalescent (GMYC) analysis was also performed to identify any possible subspecies from the different haplotypes. The GMYC model is a method for species delimitation based on phylogenetic data derived from a single locus (Fujisawa and Barraclough, 2013; Pons et al., 2006). It has become a popular approach for species delimitation as it does not require previous species information, allowing it to be useful for studying organisms with unclear taxonomical classification (Talavera et al., 2013). The GMYC characterizes the transition between intra-specific and interspecific events and is considered to be more robust and less subjective than traditional barcoding analysis methods (da Souza et al., 2015).

Ultrameric trees were first obtained using BEAST v2.5 (Bouckaert et al., 2014) using a constant clock. We used the Yule prior as it is the simplest model by convention (Fujisawa and Barraclough, 2013). Sequence alignment was partitioned into each of the three codon

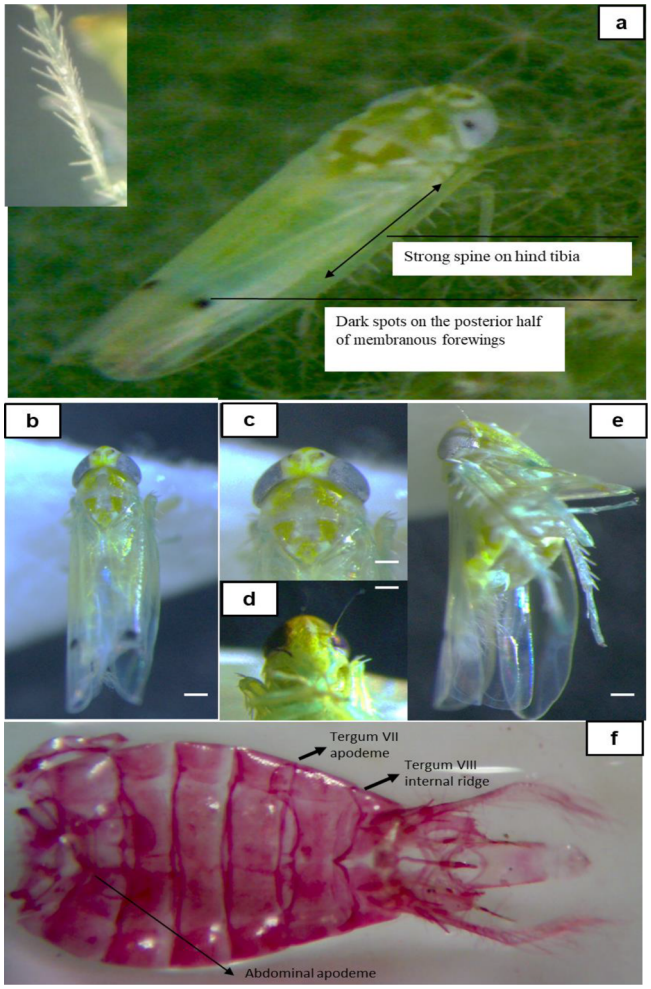

Fig. 2. Morphology of the adult male leafhopper, *Amrasca biguttula* (Ishida) in eggplant.; a) view of the strong rows of spurs and spines on the hind tibia b) adult, dorsal view; c) head and thorax, dorsal view; d) face and head, ventral view; e) adult, right lateral view; f) ventral view of male abdomen of *A. biguttula* (45×). Scale bars = 0.2 mm.

positions. Nucleotide substitution models were determined by jModelTest (Posada, 2008) based on Bayesian Information Criterion (BIC). The best models were GTR for the first and second codon positions and GTR + I for the third codon position partitions. The length of the MCMC chain was 10 000 000 with sampling every 1000. TreeAnnotator v2.5.0 (Rambaut and Drummond, 2018) was used to summarize the posterior distribution with 10% burn-in. GMYC clustering was performed using the SPLITS package (Ezard et al., 2009) implemented in R software (version 3.5.0, https://r-project.org).

Results and discussion

Leafhopper species in eggplant is A. bigutulla Ishida

Representative specimens of the collected populations are presented in Fig. 2**a–f.** Adult eggplant leafhopper is about 2.5 to 3.0 mm long, yellowish-to-yellowish-green in body color; forewings are shiny, membranous, with conspicuous dark spot toward the tip of the forewing (Fig. 2**a and b**). Adults have intricate markings on dorsal pronotum and vertex of the head **(**Fig. 2**c)**. The hind tibiae of the legs are equipped with rows of numerous strong setae and spurs (Fig. 2**a and e)**. Forewing coloration varies from yellowish-green to yellow during summer (Fig. 2**a**).

The details of male abdomens from the sampled specimens are shown in Fig. 2**f**. The eggplant leafhopper specimen showed well developed bulb-like abdominal apodemes and the long, slender subgenital plates possessing macrosetae restricted to basal half with distal half possessing numerous, conspicuous, long and fine setae, typical characteristics of the genus *Amrasca.* The eggplant leafhopper specimen possess a pair of large lateral apodemes in tergum VII that extend anterad into segment VI while tergum VIII has a pair of arched internal ridges. This distinctive, previously unreported modifications of the male pregenital abdominal tergites (VII and VIII) has been used as identification key for *A. bigutulla* Ishida and delimits this species from the other *Amrasca* species (Xu et al., 2017). **Fig. S2** shows the representative specimen from the different collection sites.

Taken together, the results above indicate that the eggplant leafhopper in the Philippines is the same species as the cotton leafhopper, *A. bigutulla* Ishida.

High similarity in eggplant leafhopper populations based on adult male genitalia

Results of morphometric analysis are presented in Tables 2 and 3 and Figs. 3 and 4. The length of aedeagus **(ae)** from the nine leafhopper populations ranges from 12.01 to 29.18 μm **(**Table 2). Anal tube **(at)** length measures 17.09–33.20 μm. The length of subgenital plate **(sp)** shows a wide variation with measurement ranging from 10.96 to 68.82 μm. Longest sp (68.82 μm) was recorded in Candelaria, Quezon while the shortest (10.96 μm) was observed in Tabaco, Albay. The length of ventral pygofer appendage **(vpa)** among the nine leafhopper populations ranges from 16.18 to 69.07 μm. The shortest and longest vpa was recorded among the Quezon leafhopper population. ANOVA test results detected no significant variation in the four genital traits

Table 2
Mean, standard deviation, and range of genital trait measurements of leafhopper samples in major eggplant growing provinces in Luzon Island, Philippines.

Population	Mean ± SD (range) (μm)				*n*
	ae	at	sp	vpa	
Albay	22.27 ± 2.54 (17.39–27.02)	26.77 ± 2.21 (21.26–32.82)	15.60 ± 1.50 (10.96–19.07)	57.03 ± 3.51 (48.77–65.66)	53
Batangas	19.90 ± 3.44 (13.41–25.4)	24.35 ± 3.07 (17.84–29.01)	15.83 ± 1.85 (11.64–18.97)	57.24 ± 4.83 (40.56–63.2)	20
Cagayan	21.71 ± 2.29 (17.18–28.02)	26.39 ± 1.55 (22.83–29.31)	15.49 ± 1.65 (12.67–18.91)	56.77 ± 3.50 (47.92–65.49)	60
Ilocos Norte	22.19 ± 2.11 (14.82–25.63)	27.50 ± 2.19 (22.00–31.80)	16.35 ± 1.49 (13.67–18.97)	56.50 ± 3.17 (51.13–66.88)	40
Isabela	21.16 ± 2.64 (15.77–26.49)	26.25 ± 2.89 (17.09–32.81)	17.36 ± 2.45 (13.42–29.11)	58.05 ± 4.16 (50.66–67.85)	40
Laguna	22.05 ± 2.69 (14.99–28.33)	26.93 ± 1.91 (22.08–31.40)	16.43 ± 1.62 (12.46–19.46)	58.13 ± 4.41 (45.77–68.97)	60
Pangasinan	23.28 ± 2.29 (17.82–29.01)	27.89 ± 2.12 (22.65–32.40)	16.27 ± 1.31 (13.44–20.18)	58.22 ± 3.90 (50.74–68.08)	60
Quezon	22.66 ± 3.45 (12.01–29.18)	27.34 ± 2.49 (17.21–33.20)	19.01 ± 10.15 (13.00–68.82)	58.96 ± 10.40 (16.18–69.07)	60
Tarlac	24.05 ± 2.62 (16.80–28.88)	28.07 ± 1.79 (22.33–31.68)	16.78 ± 1.50 (13.34–20.82)	59.86 ± 3.67 (52.03–69.39)	40
Range	12.01 – 29.18	17.09 – 33.20	10.96 – 68.82	16.18 – 69.39	

Where: ae - length of aedeagus, at- length of anal tube, sp - length of subgenital plate, vpa – length of ventral pygofer appendage vpa, *n* – number of samples. Values in parentheses are the minimum and maximum measurements.

Table 3
Principal components and Eigenvalues of parameters measured in the genital morphological diversity of leafhopper.

Principal Component	Eigenvalue	% variance
1	35.4823	59.821
2	12.8063	21.591
3	6.70006	11.296
4	4.32576	7.2929

among the leafhopper populations (*P value* < 0.05) (Table S1).

Principal component analysis showed that PCA 1 and PCA 2 accounted for a combined 81.41% of the variation of the populations (Table 3). The loading plots of each component (Fig. 3) shows that the lengths of the ventral pygofer appendage (vpa) and subgenital plate (sp) have the greatest contributions to the variations explained by components 1 and 2, respectively. Plotting the two components (Fig. 4) reveals that most of the data points clustered together, which indicates high similarity among the leafhopper populations except for the Quezon (QT, QC and QS) and Batangas-Magapi (BM) populations which were observed to be the natural outlying populations. Although, there were more individuals collected in Quezon (60) compared to Batangas (20), the data suggest wider variability among the Quezon population. Generally, the lack of differentiation in the scatter plot indicates that the morphometric characters in leafhopper are not affected by the geographic location and that the populations examined belong to the same species. The graph also shows variability in subgenital plate (component 2) between populations as suggested by the wide spread of data points along the y-axis.

Diversity in eggplant leafhopper populations based on COI sequence analysis

Haplotype and nucleotide diversity. Of the 72 samples sequenced, 68 had clean reads and were used for further analysis. The trimmed clean sequences queried against the BLAST-nr database were all identified to be *A. biguttula* mitochondrial *cytochrome oxidase I* gene. The *COI* leafhopper sequence data showed a nucleotide composition of high A + T content (44.0% + 25.9%) than G + C content (14.3% + 15.8%), which is expected from the AT-rich nature of an insect mitochondrial genome (Periasamy et al., 2015; Shashank et al., 2015). Multiple sequence alignment found no insertions, deletions, and stop codons which is consistent with amplified functional *COI* genes (Shashank et al., 2015).

Haplotypes. Haplotype analysis of polymorphic sites in the DNA revealed 11 variable sites, which are comprised of 7 singleton and 4 parsimony informative sites. A total of eleven (11) sequence variants (haplotypes) were detected (Table 4). Sequence data for the obtained haplotypes have been deposited in GenBank (Accession numbers: MK391406 – MK391416).

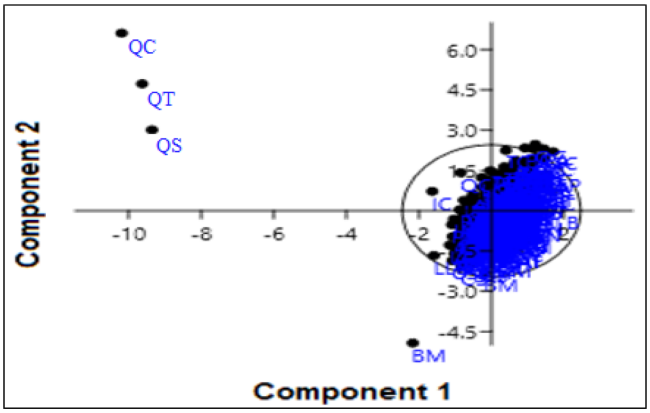

Fig. 4. Principal component analysis plot of genital traits among the leafhopper populations in Luzon (Ilocos Norte: Batac = INBT, Laoag = INL; Pangasinan: Villasis = PV, Sta. Maria = PSM, PA = Asingan; Isabela: Roxas = IR, Cabatuan = IC; Cagayan: Solana = CS, Alcala = CA, Sto. Niño = CSN; Tarlac: Concepcion = TC, Gerona = TG,; Laguna: Los Baños = LLB, Victoria = LV, San Pablo = LSP; Quezon: Candelaria = QC, Tiaong = QT, Sariaya = QS; Batangas: Magapi = BM; Albay: Guinobatan = AG, Tobaco = AT, Legazpi = AL).

Table 4
Number and overall frequency of leafhopper haplotypes from partial COI sequences from various provinces in Luzon Island, Philippines. *n* indicates the number of individuals genotyped.

Region	Province	Haplotype Frequency											*n*
		1*	2	3	4	5	6	7	8	9	10	11	
I	Ilocos Norte	6		1							2		9
	Pangasinan	7							1				8
II	Isabela	3				1						1	5
	Cagayan	7			1				1				9
III	Tarlac	4							2				6
IV-A	Laguna	7	1							1			9
	Quezon	5								1			6
	Batangas	1						1					2
V	Albay	6				1	1						8
Overall frequency		46	1	1	1	2	1	1	4	2	2	1	62

*Widespread haplotype.

One haplotype (Hap 1) was found to be widespread or common in all regions sampled in Luzon. Island. Other haplotypes were found to be unique to certain regions. The widespread haplotype occupies the central position in the constructed network, and all other haplotypes are differentiated by one or two nucleotides (Fig. 5). The nucleotide diversity in the overall Luzon population was $\pi = 0.0008$, while haplotype diversity was Hd = 0.442 (Table 5). This indicates that different

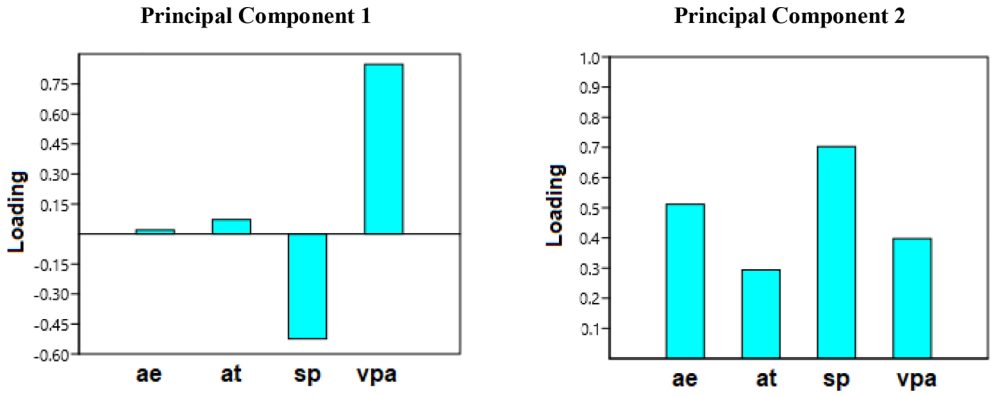

Fig. 3. Loading plots of genital morphometric traits in leafhopper where ae = aedeagus; at = anal tube; sp = subgenital plate; vpa = ventral pygofer appendage.

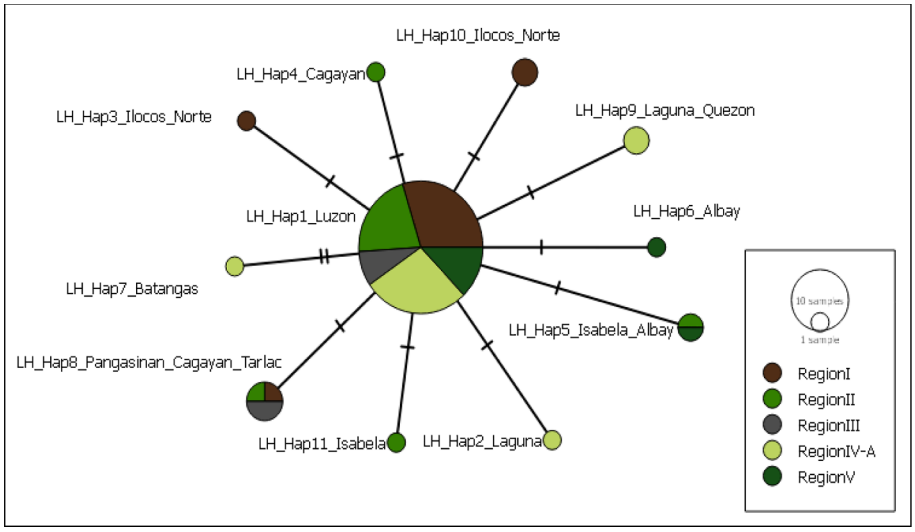

Fig. 5. Minimum Spanning Network illustrating evolutionary relationship of *A. biguttula* haplotypes. Each circle represents a single haplotype. Area of circles is proportional to the frequency of observed haplotypes.

Table 5
Number of samples studied (n), number of segregating sites (S), nucleotide diversity (π), number of haplotypes (h), haplotype diversity (Hd), Tajima's D and Fu's F_S tests for *A. biguttula* populations studied in the Philippines.

Region	Province	*n*	S	π	h	Hd	*Tajima's D**	*Fu's Fs*[†]
I	Ilocos Norte Pangasinan	17	3	0.0007	4	0.399	−1.4	−1.43
II	Isabela Cagayan	14	4	0.0009	5	0.505	**−1.8***	**−2.45**[†]
III	Tarlac	6	1	0.0009	2	0.533	0.85	1.02
IV-A	Laguna Quezon Batangas	17	4	0.0009	4	0.419	−1.57	−1.8
V	Albay	8	2	0.0008	3	0.464	−1.31	−1.5
Luzon	–	62	11	0.0008	11	0.442	**−2.16***	**−3.05**[†]

*Significant at $p < 0.05$.
[†] The statistic should be considered as significant at the 5% level if the *P* value is below 0.02.

haplotypes or sequence variants occur occasionally in the different populations. However, the extent of the variation is limited, as the haplotypes differ by only one or two point mutations.

Geographical structure. Tajima's D test indicated a significant, negative value for the overall Luzon population, and Fu's F_S statistic was also negative (Table 5). For analysis by each region in the Philippines, all regions had non-significant negative results for evolutionary neutrality tests, except for Region II (Isabela and Cagayan). Negative significant values for these tests, as observed in the overall Luzon population and the population in Region II (Isabela and Cagayan) suggest an excess of new mutations due to selective sweeps or population growth (Alonso and Armour, 2001). Significant negative values in the neutrality tests may also be indicative of positive selection. The latter may be more likely due to the low observed nucleotide diversity. This may be because of indiscriminate pesticide use (Arora, 2009; Murugesan and Kavitha, 2010) which may act as a strong selective pressure against leafhopper, possibly reducing genetic variation. However, it is important to note that the relatively low sample sizes for each region obtained in this study may have resulted in non-significant values and could possibly not be enough to assess evolutionary neutrality in other regions.

The presence of similar haplotypes in different and geographically distant provinces (such as the presence of Hap 5 in Isabela and Albay which are separated by around 800 km), may indicate similar selective pressure especially considering that all of the samples were collected from eggplant fields. The presence of unique haplotypes in certain regions may be due to numerous factors such as different cropping systems, pesticide usage and agro-climatic conditions in the sampled regions. In addition, multiple cropping systems within the same host range of the insect may also exist, which can provide populations a continuous source of food, and may limit their movement to other locations. This may be true for leafhoppers sampled from eggplant, which is often planted by small-scale farmers in backyard gardens (Chupungco et al., 2014b), and is planted alongside other vegetables such as okra. The combination of these factors may give rise to unique haplotypes. Nonetheless, similarity of selective pressures (such as pesticide usage) and same climates in the different regions sampled in the Philippines may also limit the extent of variability observed.

Genetic divergence. Genetic distance based on *COI* sequences between populations, ranged from 0.0004 to 0.0022 (Table 6), indicating a high degree of genetic similarity for populations in Luzon Island,

Table 6
Mean Tamura 3-parameter (T92) genetic distances between populations of *A. biguttula* in Luzon, Philippines.

	1	2	3	4	5	6	7	8	9
1. Ilocos Norte									
2. Cagayan	0.0012								
3. Isabela	0.0015	0.0010							
4. Pangasinan	0.0010	0.0005	0.0008						
5. Tarlac	0.0014	0.0008	0.0012	0.0006					
6. Laguna	0.0012	0.0007	0.0010	0.0005	0.0009				
7. Quezon	0.0011	0.0006	0.0009	0.0004	0.0008	0.0006			
8. Albay	0.0012	0.0008	0.0010	0.0005	0.0010	0.0008	0.0007		
9. Batangas	0.0025	0.0020	0.0023	0.0018	0.0022	0.0020	0.0019	0.0021	

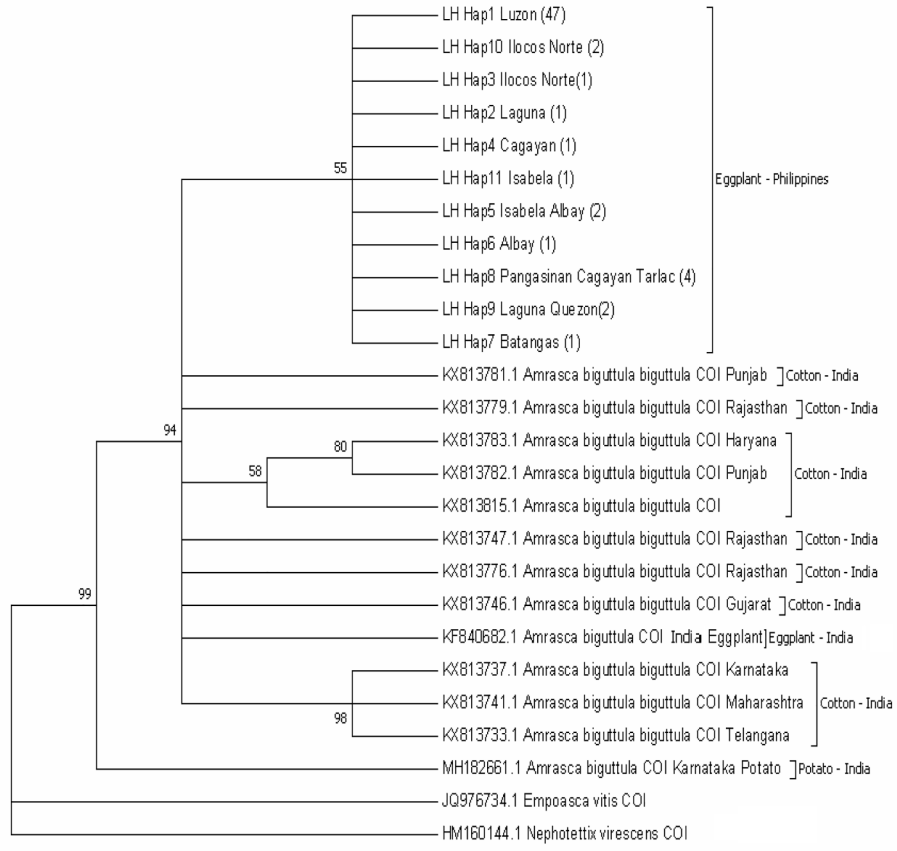

Fig. 6. Maximum Likelihood (ML) tree based on partial *COI* sequences of *A. biguttula* haplotypes from the Philippines (Hap 1 – 11), with other *A. biguttula biguttula* sequences from India and other insect genera used as the outgroups. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches.

Philippines. Observed intraspecific genetic variation of *COI* sequence within and among different geographical locations falls within the limit of the 2% genetic distance for insect species boundary (deWaard et al. 2011; Fu et al. 2014). The results are in concurrence and comparable with similar studies on cotton leafhoppers, *Nilaparvata lugens* and *Sogatella furcifera* (Hemiptera: Delphacidae) (Kranthi et al, 2018). Observed genetic similarities of *COI* between population groups, in combination with the morphometric analysis, suggests that only *A. biguttula* is infesting eggplant in Luzon, Philippines.

Phylogenetic analysis

Results of the phylogenetic (Maximum Likelihood, ML) and General Mixed Yule-Coalescent (GYMC) analyses are presented in Figs. 6 and 7, respectively. Posterior probabilities of the GMYC tree are indicated in **Fig. S2**. It should be noted that *A. biguttula biguttula* and *A. bigutulla* are all synomyms of the correct scientific name of the Indian cotton leafhopper, *Amrasca* (Sundapteryx) *biguttula* (Ishida) (Xu et al., 2017).

Both ML and GMYC trees show that *COI* sequences from Philippine eggplant leafhopper populations (*A. biguttulla*) group together. GMYC analysis further classified Philippine haplotypes into a single entity/species as likelihood for the formation of a subspecies within the Philippine haplotypes was not supported (**Fig. S3**), due to the low observed genetic diversity of the studied populations. This result also indicates that eggplant leafhopper populations in Luzon Island are not genetically isolated by distance because Luzon island is relatively small and distances are not large when compared to the north and south Indian cotton leafhopper populations, which were isolated by distance (Kranthi et al., 2018).

The results of the ML and GMYC analyses in this study also showed that the *COI* sequences from Philippine eggplant leafhopper populations grouped separately from *COI* sequences from leafhoppers infesting cotton (*A. biguttula biguttula*) and eggplant (*A. biguttula*) from India, but with weak bootstrap support (55%) (Fig. 6). The weak dissociation and separation of the Philippine populations from Indian populations in the ML and GMYC analyses, indicates that *A. biguttula* in the Philippines has close phylogenetic relationships with cotton and eggplant *A. biguttula* from India, and probably the most likely origin of the Philippine eggplant leafhopper population. However, it should be noted that *COI* sequences of *A. biguttula* from nearby Asian countries are not available for comparison to accurately determine the origin of leafhopper infesting eggplants in the Philippines.

The results of the ML and GMYC analyses in this study also showed that *A. biguttula* sampled from potato and cotton in the same district of India (Karnataka) did not group together in the phylogenetic analysis (Figs. 6 and 7). In contrast, the leafhopper sampled from eggplant in India grouped with select haplotypes sampled from cotton. This may be due to a difference in agroecological zones since potato and cotton require different climatic regimes while eggplant and cotton are generally lowland crops and planted in similar areas. These results suggest that leafhopper populations in India are not merely isolated by distance (Kranthi et al., 2018) but maybe isolated due to different agro-climatic conditions. This also begs assessment of the morphological characteristics of *A. biguttula* from potato in India.

The *A. biguttula* subgroup of the Philippine populations represent eggplant-growing provinces in Luzon spanning around 900 km with varying agroecological zones in which a single widespread haplotype was observed and no substantial differences between haplotypes. Although the sampled populations were far apart and had different geographic features and climate types, all populations were collected from eggplant which could be a limiting factor in the observed genetic diversity of *A. biguttula*. It will be critical to sample populations of leafhopper feeding on other crops and/or populations from other islands of the Philippines to determine if this will hold true and to determine if the different Philippine populations of *A. biguttula* are isolated by distance and agro-climatic conditions.

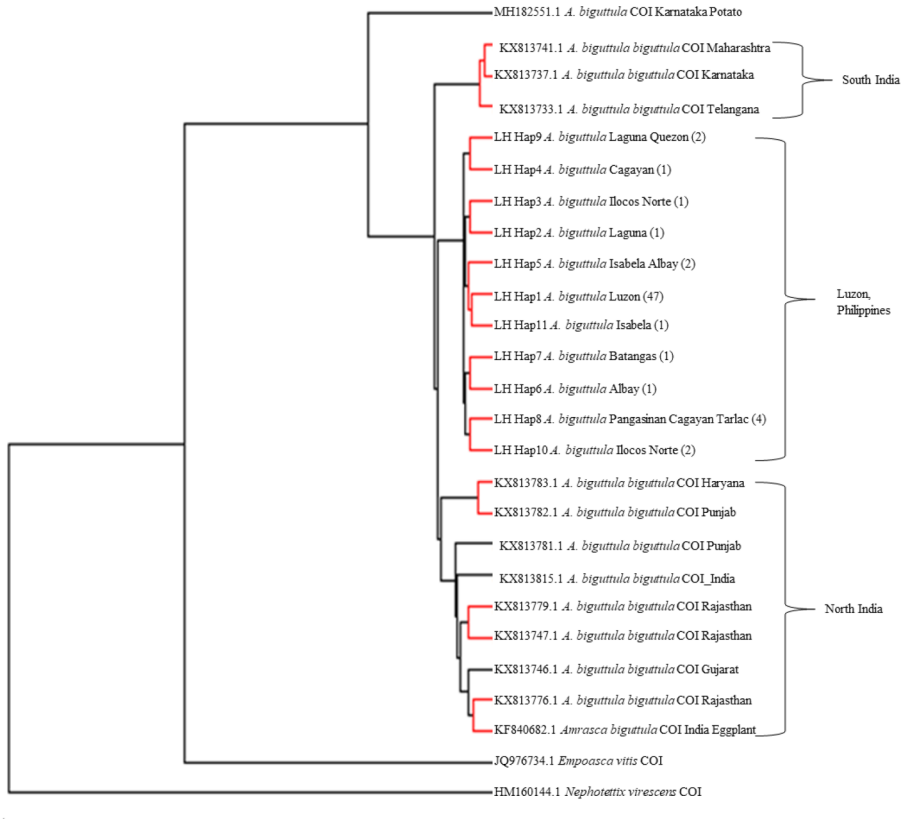

Fig. 7. Generalized Mixed Yule Coalescent species delimitation tree showing the different GMYC entities based on partial *COI* gene sequence. Numbers in parenthesis indicate number of haplotypes observed.

Conclusion and recommendations

Insights on pest population structure and genetic distribution are important to design and implement successful IPM strategies. From the present study, we observed that the populations of leafhoppers found in eggplant from major growing provinces in Luzon, Philippines consist of one species, *Amrasca (Sundapteryx) biguttula* (Ishida) (Syn = *A. biguttula* (Ishida)). Leafhopper populations were also found to be genetically similar, suggesting that a single management strategy for IPM can be implemented in Luzon. However, populations from the two other major regions in the Philippines, Visayas and Mindanao, were not accounted for in this study. Given the insular nature of the Philippines and how leafhopper populations are likely to be isolated by distance and agro-climatic condition, it is possible that there will be a greater degree of variation upon observation of the other Philippine eggplant production regions. Considering also the polyphagous nature of the leafhopper, populations feeding on other crops such as okra or cotton should be sampled and compared with available data in order to obtain a more accurate view of the genetic and morphological distribution of this pest.

Disclaimer

The contents and views are the responsibility of the authors and do not necessarily reflect the views of the funding agencies and their respective governments.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aspen.2019.12.009.

References

Alonso, S., Armour, J.A.L., 2001. A highly variable segment of human subterminal 16p reveals a history of population growth for modern humans outside Africa. PNAS 98, 864–869.

Arora, S., 2009. Analysis of insecticides in okra and brinjal from IPM and non-IPM fields. Environ. Monit. Assess. 151 (1–4), 311–315.

Baltazar, C.R., 1968. Supplementary host list and checklist of Philippine plant pests. Philipp. J. Sci. 97, 177–228.

Bellis, G, Fletcher, M., 2005. Indian cotton leafhopper (Amrasca biguttula biguttula). PaDIL Species Fact Sheet.
Borror, D.J., DeLong, D.M., Triplehorn, C., 1971. An introduction to the study of insects, third ed. Ohio State University, USA, Holt, Rinehard and Winston, pp. 852.
Bouckaert, R., Heled, J., Kühnert, D., Vaughan, T., Wu, C.H., Xie, D., et al., 2014. BEAST 2: a software platform for Bayesian evolutionary analysis. PLoS Comput. Biol. 10, 1–6. https://doi.org/10.1371/journal.pcbi.1003537.
Bureau of Agricultural Statistics., 2016. Philippine Statistics Authority - Bureau of Agricultural Statistics.
Capco, S., 1957. A list of plant pests of the Philippines with special reference to field crops, fruit trees and vegetables. Philipp. J. Agric. 1, 7–80.
Chang, J.-C., Ponnath, D.W., Ramasamy, S., 2014. Phylogeographical structure in mitochondrial DNA of eggplant fruit and shoot borer, *Leucinodes orbonalis* Guenée (Lepidoptera: Crambidae) in South and Southeast Asia. Mitochondrial DNA 27, 198–204. https://doi.org/10.3109/19401736.2014.880890.
Chupungco, A.R., Elazegui, D.D., Nguyen, M.R., de los Santos, S.G.G., 2014. Supply Chain of the Eggplant Industry in Selected Areas in the Philippines. In: Gerpacio, R.V., Aquino, A.P., (eds.). Socioecon. Impacts Bt Eggplant Ex-ante Case Stud. Philipp., Ithaca, New York, USA and Los Banos, Laguna, Philippines: International Service for the Acquisition of Agri-biotech Application (ISAAA) and SEAMEO SOutheast Asian Regional Center for Graduate Study and Research in Agriculture (SEARCA), p. 6.
Chupungco, A.R., Elazegui, D.D., Nguyen, M.R., de los Santos, S.G.G., 2014. Supply Chain of the Eggplant Industry in Selected Areas in the Philippines. In: Gerpacio, R.V., Aquino, A.P., (eds.). Socioecon. Impacts Bt Eggplant Ex-ante Case Stud. Philipp., Ithaca, New York, USA and Los Banos, Laguna, Philippines: International Service for the Acquisition of Agri-biotech Application (ISAAA) and SEAMEO SOutheast Asian Regional Center for Graduate Study and Research in Agriculture (SEARCA), p. 14.
Souza, C. da S de, Oliveira, C., Pereira, L.H.G., 2015. Knodus moenkhausii (Characiformes: Characidae): one fish species, three hydrographic basins - a natural or anthropogenic phenomenon? DNA Barcodes;3:129–38. DOI:10.1515/dna-2015-0016.
deWaard, J.R., Hebert, P.D.N., Humble, L.M., 2011. A comprehensive DNA barcode library for the looper moths (Lepidoptera: Geometridae) of British Columbia, Canada. PLoS One 6, e18290.
Ezard, T., Fujisawa, T., Barraclough, T.G., 2009. SPLITS: SPecies' Limits by Threshold. Statistics.
FNRI., 2008. Philippine Nutrition: Facts and Figures. Food and Nutrition Research Institute, Department of Science and Technology, Bicutan, Taguig, Metro Manila.
Folmer, O., Black, M., Hoeh, W., Lutz, R., Vrijenhoek, R., 1994. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Mol. Mar. Biol. Biotechnol. 3, 294–299. https://doi.org/10.1371/journal.pone.0013102.
Fu, Y.X., 1997. Statistical tests of neutrality of mutations against population growth, hitchhiking and background selection. Genetics 147, 915–925 doi:genetics.org//147/2/915.
Fu, J.Y., Han, B.Y., Xiao, Q., 2014. Mitochondrial coi and 16sRNA evidence for a single species hypothesis of E. vitis, J. formosana and E. onukii in East Asia. PLoS One 9, e115259.
Fujisawa, T., Barraclough, T.G., 2013. Delimiting species using single-locus data and the generalized mixed yule coalescent approach: a revised method and evaluation on simulated data sets. Syst. Biol. 62, 707–724. https://doi.org/10.1093/sysbio/syt033.
Gabriel, B.P.,1975. Insects and mites injurious to Philippine crop plants. Department of Entomology. U. P. at Los Baños, College, Laguna, Philippines.
Galtier, N., Nabholz, B., Glémin, S., Hurst, G.D.D., 2009. Mitochondrial DNA as a marker of molecular diversity: a reappraisal. Mol. Ecol. 18, 4541–4550. https://doi.org/10.1111/j.1365-294X.2009.04380.x.
Hammer, Ø., Harper, D.A., Ryan, P.D., 2001. PAST: paleontological statistics software package for education and data analysis. Palaeontol. Electronica 4 (1), 9.
Hebert, P.D.N., Cywinska, A., Ball, S.L., deWaard, J.R., 2003. Biological identifications through DNA barcodes. Proc. Biol. Sci. 270, 313–321. https://doi.org/10.1098/rspb.2002.2218.
Hu, J., Zhang, J.L., Nardi, F., Zhang, R.J., 2008. Population genetic structure of the melon fly, Bactrocera cucurbitae (Diptera: Tephritidae), from China and Southeast Asia. Genetica 134, 319–324. https://doi.org/10.1007/s10709-007-9239-1.
Kapoor, V., Sori, A., 1972. The nomenclature status of cotton jassids, *Amrasca devastans* (Distant). Entomol. Record. 84, 2396.
Kranthi, S., Ghodke, A.B., Puttuswamy, R.K., Mandle, M., Nandanwar, R., Satija, U., et al., 2018. Mitochondria COI-based genetic diversity of the cotton leafhopper *Amrasca biguttula* biguttula (Ishida) populations from India. Mitochondrial DNA Part A DNA Mapping, Seq. Anal. 29, 228–235. https://doi.org/10.1080/24701394.2016.1275595.
Kumar, S., Stecher, G., Tamura, K., 2016. MEGA7: molecular evolutionary genetics analysis Version 7.0 for bigger datasets. Mol. Biol. Evol. 33, 1870–1874. https://doi.org/10.1093/molbev/msw054.
Leigh, J.W., Bryant, D., 2015. POPART: full-feature software for haplotype network construction. Methods Ecol. Evol. 6, 1110–1116. https://doi.org/10.1111/2041-210X.12410.
Librado, P., Rozas, J., 2009. DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. Bioinformatics 25, 1451–1452. https://doi.org/10.1093/bioinformatics/btp187.
Merino, G., 1936. The Philippine Cicadellidae (Homoptera). Philipp. J. Sci. 61, 307–400.
Murugesan, N., Kavitha, A., 2010. Host plant resistance in cotton accessions to the leafhopper, *Amrasca devastans* (Distant). J. Biopesticides 3 (3), 526.
Muturi, S.M., Karanja, L.S., Wachira, F.N., Wambulwa, M.C., 2013. Ten (10) M Ammonium acetate is an efficient molecular concentration for the extraction of genomic DNA from small insects used for rolling circle amplification;12:5790–8. DOI:10.5897/AJB2013.12848.
Navasero, M.V., 2015. Insect pests of eggplant. Pages 154-183 in Pests and Diseases of Economically Important Crops in the Philippines. In: de la Cueva, F.M., Pascual, C.B., Bajet, C.M., Dalisay, T.U. (eds.). Pest Management Council of the Philippines, Inc. c/o Crop Protection Cluster, University of the Philippines Los Banos, College, Laguna.
Nielson, M.W., 1968. The leafhopper vectors of phytopathogenic viruses (Homoptera, Cicadellidae): Taxonomy, biology and virus transmission. Tech. Bull. No. 1382. Agricultural Research Service, US Department of Agriculture, Washington, DC, USA.
Otanes, F., Butac, F., 1938. A preliminary study on insect pests of cotton in the Philippines with suggestion for their control. Philippine J. Agric. 10 (4), 341–371.
Otanes, F., Butac, F., 1939. Cotton insect pests in the Philippines. Philipp. J. Agric. 10 (4), 341–372.
Pascua, L.T., Pascua, M.E., 2002. Cotton leafhopper in the Philippines: a review. Philipp. J. Sci. 131, 69–74.
Periasamy, M., Schafleitner, R., Muthukalingan, K., Ramasamy, S., 2015. Phylogeographical structure in mitochondrial DNA of legume pod borer (Maruca vitrata) population in tropical Asia and sub-Saharan Africa. PLoS One 10, 1–24. https://doi.org/10.1371/journal.pone.0124057.
Pons, J., Barraclough, T.G., Gomez-Zurita, J., Cardoso, A., Duran, D.P., Hazell, S., et al., 2006. Sequence-based species delimitation for the DNA taxonomy of undescribed insects. Syst. Biol. 55, 595–609. https://doi.org/10.1080/10635150600852011.
Posada, D., 2008. jModelTest: phylogenetic model averaging. Mol. Biol. Evol. 25, 1253–1256. https://doi.org/10.1093/molbev/msn083.
Rambaut, A., Drummond, A.J., 2018. TreeAnnotator v2.5.0.
Ratnasingham, S., Hebert, P.D.N., 2007. BOLD: the barcode of life data system: Barcoding. Mol. Ecol. Notes 7, 355–364. https://doi.org/10.1111/j.1471-8286.2007.01678.x.
Sagar, D., Balikai, R.A., Bhat, R.S., 2014. Molecular and Taxonomic Diversity of Cotton Leafhopper, Amrasca biguttula biguttula (Ishida) in Major Cotton Growing Districts of Karnataka. Vegetos- An Int. J. Plant Res. 27, 225. https://doi.org/10.5958/2229-4473.2014.00036.6.
Sapin, G.D., Caoili, B.L., Recuenco-Adorada, J.D., 2013. Morphometrics of Four Genital Traits in Some Philippine Populations of the Asian Corn Borer, *Ostrinia furnacalis* (Guenée) (Lepidoptera: Crambidae). Philipp. Ent. 27, 134–150.
Shashank, P.R., Ojha, R., Venkatesan, T., Jalali, S.K., Bhanu, K.R.M., 2015. Molecular characterization of brinjal shoot and fruit borer, *Leucinodes* orbonalis (Guenée) (Lepidoptera: Crambidae) based on mitochondrial marker cytochrome oxidase I and their phylogenetic relationship. Indian J. Exp. Biol. 53, 51–55.
Tajima, F., 1989. Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. Genetics123:585–95. DOI:PMC1203831.
Talavera, G., Dincă, V., Vila, R., 2013. Factors affecting species delimitations with the GMYC model: insights from a butterfly survey. Methods Ecol. Evol. 4, 1101–1110. https://doi.org/10.1111/2041-210X.12107.
Tamura, K., 1992. Estimation of the number of nucleotide substitutions when there are strong transition-transversion and G + C-content biases. Mol. Biol. Evol. 9, 678–687. https://doi.org/10.1093/oxfordjournals.molbev.a040752.
Woodworth, H., 1921. A host index of insects injurious to Philippine crops. I. Philipp. Agric. 10, 22.
Woodworth, H., 1922. A host index of insects injurious to Philippine crops. II. Philipp. Agric. 10, 328.
Xu, Y.E., Wang, Y., Dietrich, C.H., Fletcher, M.J., Qin, D., 2017. Review of Chinese species of the leafhopper genus Amrasca Ghauri (Hemiptera, Cicadellidae, Typhlocybinae), with description of a new species, species checklist and notes on the identity of the Indian cotton leafhopper;4353:360–370.