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ULTRASTRUCTURAL AND MOLECULAR VARIANTS OF *PSEUDOCOCCUS ELISAE* (HEMIPTERA: PSEUDOCOCCIDAE) FROM NEOTROPICAL REGION

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A B S T R A C T

Is necessary to combine morphological and molecular techniques to establish appropriate taxonomic relationships in studies of mealybugs. These analyses allow understanding of their correct phylogenetic classification. The aim of the present investigation was to study *Pseudococcus elisae* (Borchsenius) (Hemiptera: Pseudococcidae) of banana crops (*Musa* spp.) from Neotropical regions using morphological and molecular techniques and analyzing their phylogenetic relationships. Morphological techniques such as light microscopy (LM) and scanning electron microscopy (SEM) were used, supported by molecular analyses of three genes: 18S, E.F-1α and COXI. The microscopy analysis was performed at the Center for Research on Microscopic Structures in 2012, and the molecular analysis was carried out at the Molecular Phytopathology Laboratory, ending in 2014, both at the University of Costa Rica. Using SEM, it was possible to identify ultrastructural variations in the bodies of the insects, the morphology of the dorsal margin cerarii and anal lobe cerarii, trilocular pores and the morphology of oral rim tubular ducts. The present investigation allowed recognition of ultrastructures and molecular variants of the mealybugs from Neotropical countries.

Keywords: 18S gene, E.F-1α gene, COXI gene, Epigenetic, Morphology, *Musa* spp.

INTRODUCTION

Mealybugs (Hemiptera: Pseudococcidae) are an extremely polyphagous group of insects (Abd-Rabou *et al.*, 2012). They comprise more than 2000 species and 290 genera (Hardy *et al.*, 2008). They feed on a wide variety of host plants worldwide (Miller *et al.*, 2007). Females are important agricultural pests and are mostly parasites that suck the phloem of the host plant (Kondo *et al.*, 2008). Two aspects of mealybug biology that make them ideal for the study of evolutionary biology and ecology grouping is the diversity of species and the phylogenetic history, which is not well known (Gullan and Cook, 2007; Hardy, 2013). Adult female mealybugs (*Pseudococcus* spp.) are characteristically elongated, oval, soft, and with distinct segmentation, measuring as much as 8–9 mm in length. They are wingless, and their mouthparts are thread-like (Mani and Shivaraju, 2016). Species of similar morphology of *Pseudocuccus* spp. are highly ubiquitous in the Neotropical area (Williams and Granara de Willink, 1992). According to Scalenet (2017), *Pseudococcus elisae* (Borchsenius) is a species associated with banana crops. It was previously identified in countries such as Costa Rica, Panama, Honduras, Guatemala, Colombia, and other tropical countries.

The high degree of morphological similarity between different taxa of mealybugs is a problem for their identification, as well as for studies of systematic biology or populations (Kondo *et al.*, 2008; Lin *et al.*, 2013). The processing and subsequent morphological study require time to analyze and identify the structures of the insect (Abd-Rabou *et al.*, 2012). Furthermore, the classification is performed in the adult stage that occurs when the mealybugs have their structures fully developed (Hardy *et al.*, 2008; Wu *et al.*, 2013). Only a few specialists in the world can identify mealybugs based on their morphology, and this is the main methodology for classification (Kondo *et al.*, 2008; Miller *et al.*, 2014; Pacheco da Silva *et al.*, 2014). This is a challenge due to the development of appropriate control strategies because even when keys

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are available, some related species are hard to identify at the species level (Malausa *et al.*, 2011).

According to Koteja and Azar (2008), some problems were encountered in the classification of mealybug families. Regarding the Putoidae family, the mealybugs have been categorized as neococcoids. Authors such as Kondo *et al.* (2008) have argued that this family belongs to the archaecoccoids, the other classification group of mealybugs. These authors have mentioned that the females of Putoidae are superficially more similar to those from the Pseudococcidae family (neococcoid) because most females of this family have trilocular pores, cerarii and dorsal ostioles. However, female adults of Putoidae differ in certain aspects from Pseudococcidae. For example, the dorsal trilocular pores (and especially cerarian ones) are larger than the ventral pores, and some of these have peculiar structures in some species and genera (Gavrilov-Zimin, 2015).

Hoffmann *et al.* (2005) mentioned that the morphological characteristics of mealybugs are often influenced by environmental factors. Additionally, Black *et al.* (2001) explained that the studies on genetic divergence and phenotypic aspects have been associated with factors such as geographic isolation, conditions of temperature, light, humidity and rain, which alter aspects of the biology and development of the insect. The application of a rapid and effective identification method to complement the morphological taxonomy of mealybugs and avoid environmental factors, such as molecular markers and DNA barcoding, would therefore be useful to assist with morphological taxonomy (Xu-Bo *et al.*, 2016).

Molecular markers are a particularly useful complement to morphological and ecological characterizations. So-called 'DNA barcoding' approaches can be used to assign voucher specimens with particular DNA sequences to morphologically characterize and identify taxa. Once this identification has been established, the molecular characterization can be used to obtain highly accurate results through tools based on species-specific PCR. This process avoids time-consuming and repetitive morphological examinations of the species (Abd-Rabou *et al.*, 2012; Hardy, 2013). To estimate the phylogeny of mealybugs, several studies of classification based on the identification of DNA sequences have been carried out, and they allowed a better understanding of their correct identification (Gullan and Cook, 2007; Hardy *et al.*, 2008; Rung *et al.*, 2008; Ashfaq *et al.*, 2011). Hardy (2013) suggested that after more than a decade of phylogenetic research based on DNA sequences, we still lack a robust phylogenetic hypothesis for evolutionary relationships among families of mealybugs, and the high degree of sequence divergence between the lineages identified has been a major obstacle in the estimation of a better phylogeny of this insect. Despite the growing number of molecular studies, morphological interpretations, and data based on a genetic hypothesis, there are still many challenges to solve, including phylogenetic relationships that are unresolved. In addition, each year, there are new species of mealybugs that are added to the list of agricultural pests (Gullan and Cook, 2007; Kondo *et al.*, 2008). The aim of the present investigation was to study the species *P. elisae* (Borchsenius), (Hemiptera: Pseudococcidae) from banana crops by morphological and molecular techniques as well as the phylogenetic relationships of three genes (*Musa* spp.) from the Neotropical region.

MATERIALS AND METHODS

Sample collections: Female mealybugs were collected from banana crops from the following Neotropical countries: Colombia, Guatemala, Honduras, Panama and the Dominican Republic. The collection was carried out during visits to farms between 2010 and 2011, and the location of the sites was identified by geographic coordinates. Individuals were collected in 1.5 mL Eppendorf tubes with 95% ethanol. An average of 30 individuals per geographical region were collected. For the source of the collected plant-level insects, we considered the pseudostem and the fruit (Table1).

Place of study: The morphological analysis was performed at the Center for Research on Microscopic Structures (CIEMic, acronyms in Spanish). The molecular analysis was carried out at the Molecular Phytopathology Laboratory for Research in Crop Protection (CIPROC, acronyms in Spanish) of Costa Rica, both at the University of Costa Rica, San Pedro, Montes de Oca.

Observation under a light microscope (LM): Ten insects per locality were processed. To identify the translucent structures, the insects were examined with inverted light microscopy equipment, using increases of 4x, 10x, 20x and 40x (Model IX51, Olympus Optical Co., Japan). The analyzed structures by light microscopy corresponded; body shape, number of segments of the antenna, discoidal translucent pores around the eyes, mouthparts and stylets, description of posterior legs and the presence of translucent pores, description of the circulus, ostioles, oral rim tubular ducts, anal lobe bar and cerarii.

Table 1. List of 5 countries sampled from the Neotropical region: geographical region, collection place, population code, plant location, collection year and geographical coordinates.

Geographical Region	Collection place		Plant location	Collection year	Geographical coordinates	
	Country	Location			Latitude	Longitude
Central America	Panama	Changuinola	Pseudostem	2011	9° 25'48.01"N	82° 31'12.02"O
	Guatemala	Escuintla	Pseudostem	2011	14° 17'48.29"N	90°47'19.41"O
	Honduras	La lima	Fruit	2010	14° 31'41.51"N	86°35'05.06"O
South America	Colombia	Urabá	Fruit	2010	7°49'24.82"N	76°39'03.34"O
Caribbean	Dominican Republic	Santiago	Fruit	2011	*	*

* Geographical coordinates unidentified.

Analysis with a Scanning Electron Microscope (SEM): Ten insects by locality were processed. The protocol by Palma-Jiménez and Blanco-Meneses (2015) was followed. The analyzed ultrastructures by SEM corresponded to the following: body morphology and dorsal ultrastructures of the insect, number of segments of the antenna, morphology of dorsal margin cerarii and anal lobe cerarii, trilocular pores and morphology of the oral rim tubular ducts.

Amplification of Genomic DNA: The protocol by Murray and Thompson (1985) was used. One insect was used for each DNA extraction. The extracted genomic DNA was amplified by PCR. Initially, five pairs of primers were used to observe which ones had a polymorphism of interest in a sub-sample of ribosomal, nuclear, and mitochondrial DNA of the mealybugs. At the end of testing, three pairs of these primers were selected. For all PCR reactions, a 1x (µL) solution was used: 13.5 µL of H_2O, 2.5 µL of buffer (10x), 2 µL of dNTPs (2 mM), 1.5 µL each for each pair primer (10 µM), and 0.3 µL of Dream Taq polymerase (5/µL) to 23 µL of master mix per Eppendorf tube. All reagents were from Fermentas, and 2 µL of DNA (10 µg/mL) was finally added. The amplification reaction was performed using the following thermal profile: an initial predenaturation at 94°C for 4 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing for 1 min at the temperature specified in each primer pair (Table 1), and chain elongation at 72°C for 1 min and 30 s, followed by a final extension at 72°C for 4 min. The reactions and cycling conditions were carried out in an automated thermocycler Eppendorf Mastercycler pro (Table 2). The PCR product was separated on an agarose gel (agar + 0.5X TBE buffer). The PCR product was digested with Exonuclease I (ExoI) from Fermentas. Sequencing was performed on the purified PCR product at a concentration of 50ng/µL by Macrogen, Inc. (South Korea).

Sequence Alignment and Phylogenetic Analysis: Sequences in both directions were obtained. The quality of the sequences was confirmed in a bidirectional alignment comparing the chromatograms with the BioEdit program v7.0.5 (Hall, 1999). To identify the species according to the results of sequencing, GenBank was used (NCBI, 2017). All sequences were aligned through ClustalW program version 1.60 (Thompson *et al.*, 1994). For the phylogenetic analysis, the sequences of the species previously reported from GenBank were included for all three genes studied. The individual origin was verified according to the host plant and the country (Table 3). The analysis of phylogenetic trees was performed using the program Molecular Evolutionary Genetic Analysis (MEGA) version 5.0 (Tamura *et al.*, 2007). The evolutionary history was inferred using the maximum likelihood (ML) method based on the Tamura-Nei model. The percentage of trees in which the associated taxa clustered together is shown next to the branches (random parameter of 2000 replications was used). The trees for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood approach, then selecting the topology with the superior log likelihood value.

RESULTS

Morphological analysis by LM: By LM, it was possible to identify the species *P. elisae* according to the following characteristics: elongated oval body, seventeen cerarii pairs, and each cerarius with two enlarged conical setae and auxiliary setae, except on the head cerarius, with three conical setae. Three stylets were identified. The antennae had eight segments (Figure 1. A).

Table 2. Primer information used for PCR amplification: 18S ribosomal region, nuclear elongation factor 1α (E.F-1α) and mitochondrial cytochrome c oxidase subunit I (COXI).

Gene	Primers	Primer sequence	PCR Conditions	Amplicon size (bp)	Primer source
18S	18S-2880	CTGGTTGATCCTGCCAGTAG	94°C, 4min; 30 Cycles of 94°C 1min, 67°C	630	(Malausa *et al.,* 2011)
	18S-B	CCGCGGCTGCTGGCACCAGA	1min 72°C 1min, 30s; 72°C 4min		(Downie and Gullan, 2004)
E.F-1α 5'	E.F-1α_M51.9 E.F-1α _rcM53-2	CACATYAACATTGTCGTSATYGG	94°C, 4min; 30 Cycles of 94°C 1min, 62°C	439	(Downie and Gullan, 2004)
		CTTGATGAAATCYCTGTGTCC	1min, 72°C 1min, 30s; 72°C 4min		
COXI	C1-J-2183	CAACATTTATTTTGATTTTTTGG	94°C, 4min; 30 Cycles of 94°C 1min, 45°C	385	(Malausa *et al.,* 2011)
	C1-N-2568	GCWACWACRTAATAKGTATCATG	1min, 72°C 1min, 30s; 72°C 4min		

Table 3. GenBank information used for the phylogenetic trees construction: species, host plant, origin country and Genbank accession number, according to the gene analyzed.

Species	Host plant	Origin country	GenBank		
			18S ribosomal	E.F-1α	COXI
Pseudococcus elisae	*Musa* spp.	Costa Rica	KP402189.1	-	-
P. elisae	*Musa* spp.	Costa Rica	KX639737	-	-
P. jackbeardsleyi	*Musa* spp.	Costa Rica	KT956119.1	-	-
Dysmicoccus neobrevipes	*	USA	U20429.1	-	-
*D. brevipes***	*	Taiwan	EU307273.1	-	-
P. elisae	*Musa* spp.	Costa Rica	-	KP402191.1	-
P. jackbeardsleyi	*Musa* spp.	Costa Rica	-	KT956120.1	-
D. brevipes	*Musa* spp.	Costa Rica	-	KP402192.1	-
*D. bovinsis***	*	USA	-	AY427285.1	-
P. elisae	*Musa* spp.	Costa Rica	-	-	KP402194.1
P. elisae	*Musa* spp.	Costa Rica	-	-	KP402195.1
P. jackbeardsleyi	*Musa* spp.	Costa Rica	-	-	KT956121.1
*D. bovinsis***	*	China	-	-	KP692714.1

There were translucent pores in the methacoxas on the femur and tibia (Figure 1. B). Seven pores around the eye were observed (Figure 1. C). There was an absence of anal lobe bar in the anal lobe cerarii, two conical setae on the anal lobe cerarii, and sclerotized lobe cerarii. Multilocular pores were observed ventrally (Figure 1. D).

Morphological analysis by SEM: Body: An oval-shaped body was found in all mealybugs (Figure 2 A and B). Mealybugs from Guatemala were characterized by a prominent mouthpart structure and antennae and thicker legs, compared to the rest of the individuals studied. Eight antennal segments were counted; the last was thicker than the above.

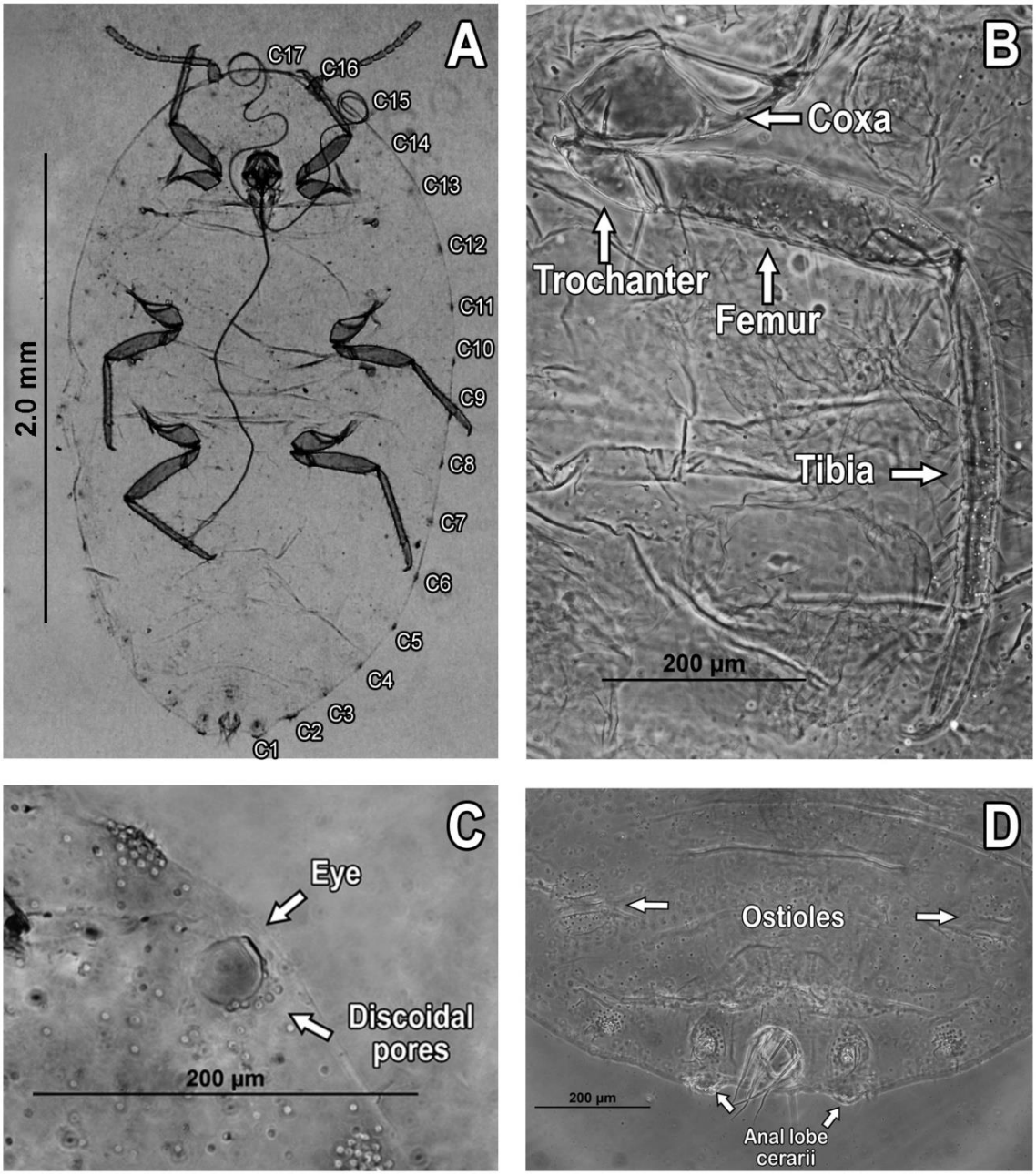

Figure 1. Translucent structures by light microscopy of *Pseudococcus elisae* mealybugs from all samples collected from the Neotropical region in banana crops during 2010 and 2011. A. Elongated oval body (Panama), 17 cerarii, eight antennae segments and three stylets. B. Translucent pores in the methacoxas on the femur and tibia, not considered on the coxa and trochanter (Dominican Republic). C. Five pores around the eye (Honduras). D. Absence of the anal bar (Guatemala).

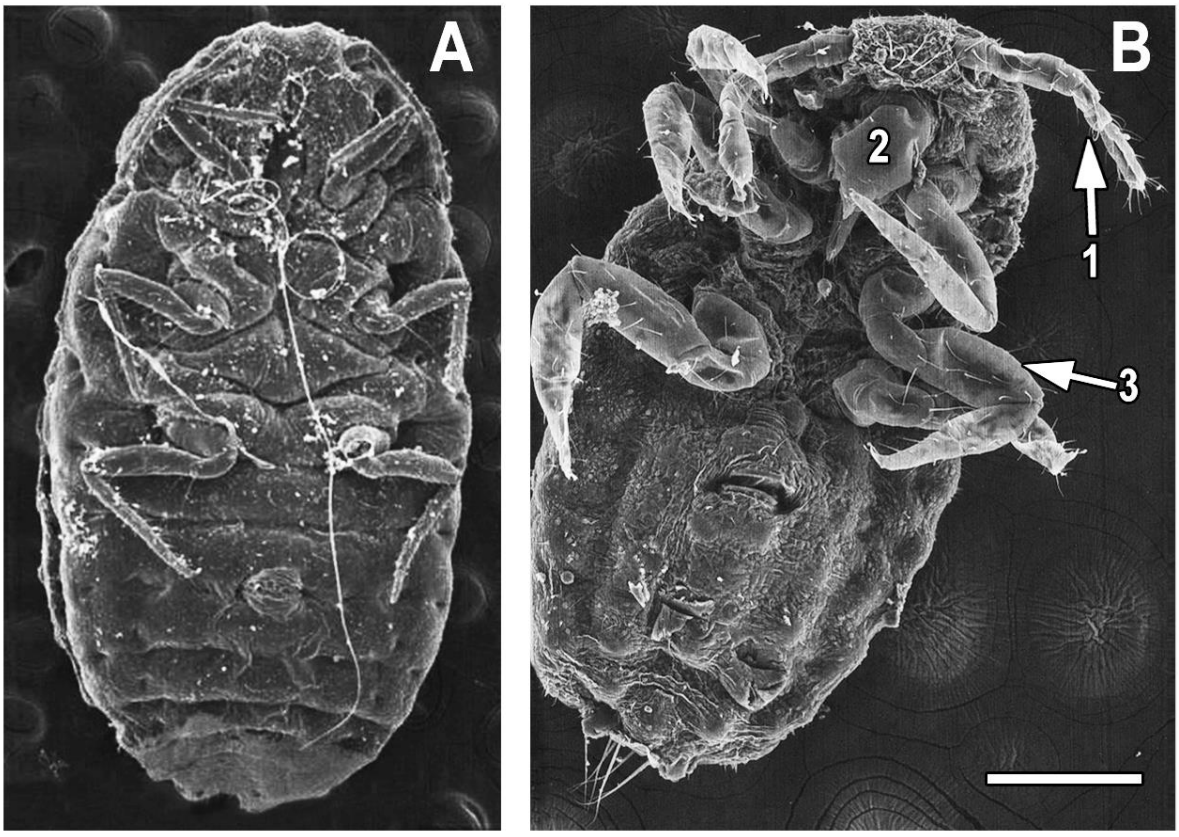

Figure 2. Ultrastructural morphology of the mealybug body in *Pseudococcus elisae* by SEM. Samples collected from the Neotropical region in banana crops during 2010 and 2011. A. Oval shaped body (Panama). *B. Antenna* (1), labium (2) and thicker legs (3) (Guatemala). Scale bar = 0,14 mm (A); 43,95 µm (B).

Twenty-two openings on the dorsal area of the mealybug body from Colombia were observed. Additionally, 8 openings on the frontal area of the body were quantified (Figure 3 A and B). Wax structures in the output of these openings were identified (Figure 3. C), specifically the cylindrical type (Figure 3. D); these could be the result of clusters of wax secreted by the ultrastructures; data not found in the literature.

Three sections of mealybugs: head, thorax (prothorax, mesothorax and metathorax) and abdomen were analyzed to identify the ultrastructural morphology of the dorsal body. Those sections were clearly divided and seen from the ventral area of the body (Figure 4).

Two pairs of ostioles, a pair in the anterior region (head) and another pair in the region of dorsal abdomen, were observed (Figure 4. A). A projection of dermal tissue in the dorsal area of mealybugs from Guatemala was identified. This ultrastructure was seen by SEM and has not been reported in the literature yet, possibly because the dorsal area of the insect has been less described, and this technique has not been widely used in the study of this insect (Figure 4. B).

Cerarii: An aggregation of two or more conical setae, surrounded by trilocular pores with or without lanceolates setaes were observed. Those were located in the dorsal margin of the body of the mealybugs. Conical setae are used as support when the wax produced is secreted by the trilocular pores (Cox 1987, Miller *et al* 2007). Seventeen pairs of cerarii for all specimens from both right and left sides, organized from the cephalic tagma (head) to the lower region in the anal lobes (lower area), were visualized (Figure 5. A-C).

In the dorsal margin of the cerarii, a pattern of two and three central conical setae with a variation in the number of setae flogged around were observed. In the case of the head cerarii, four conical setae next to the antenna were identified. In mealybugs from Colombia, elevations of tissue with spinules, as the base for the central conical setae on the cerarii, are presented (Figure 6. A).

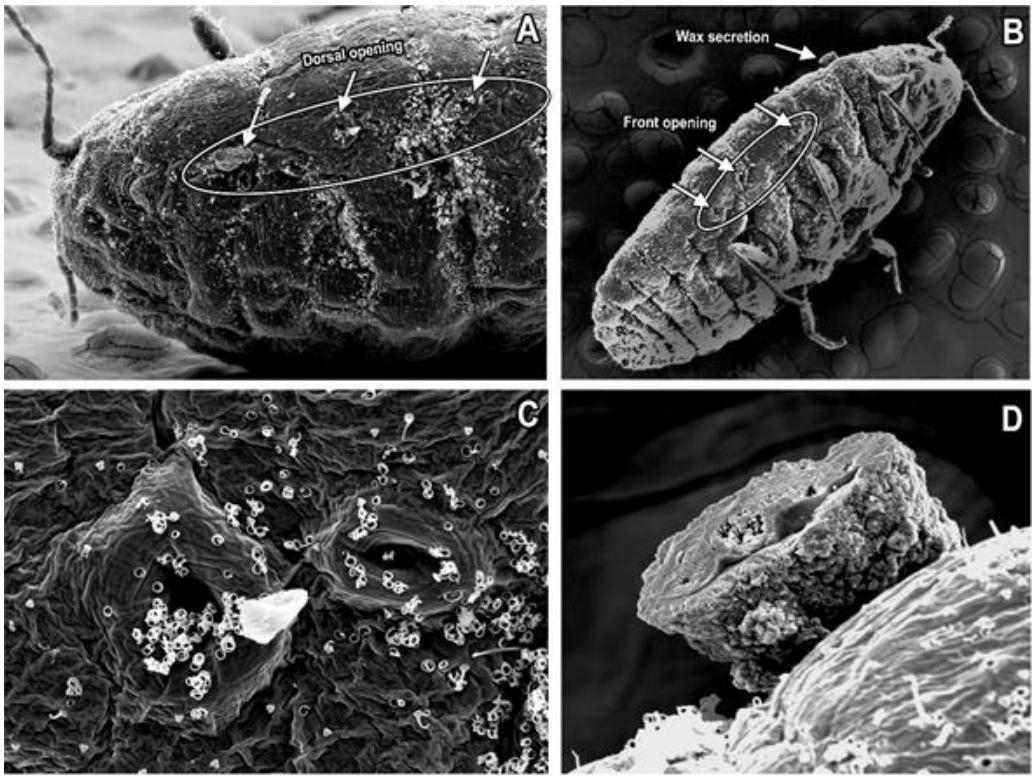

Figure 3. Ultrastructural morphology of the body of *Pseudococcus elisae* by SEM collected from Colombia in the banana crop during 2011. A. Dorsal opening. B. Frontal opening. C. Exemplification of the openings. D. Incrustation over a dorsal opening. Scale bar = 59 µm (A); 9,43 µm (B); 0,13 mm (C); 9,43 µm (D).

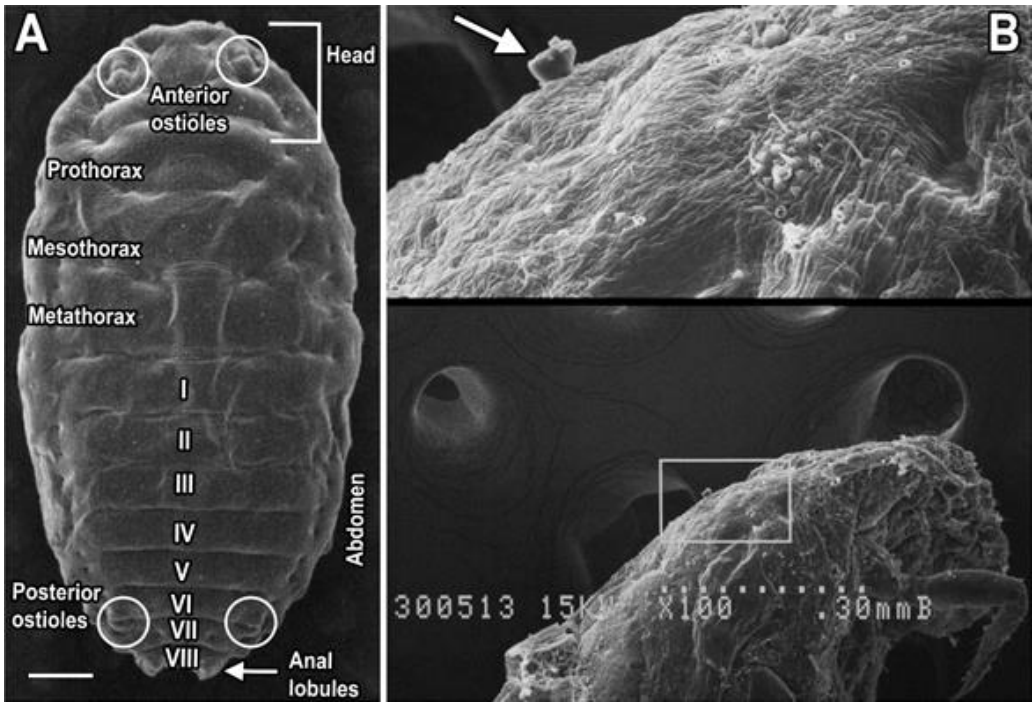

Figure 4. Ultrastructural morphology of the dorsal area in *Pseudococcus elisae* by SEM from all samples collected from the Neotropical region in the banana crop during 2010 and 2011. A. Regular dorsal ultrastructure (Honduras). B. Projection in the dorsal area of the thorax of mealybugs from Guatemala. Scale bar = 0,15 mm (A); 0,30 mm (B).

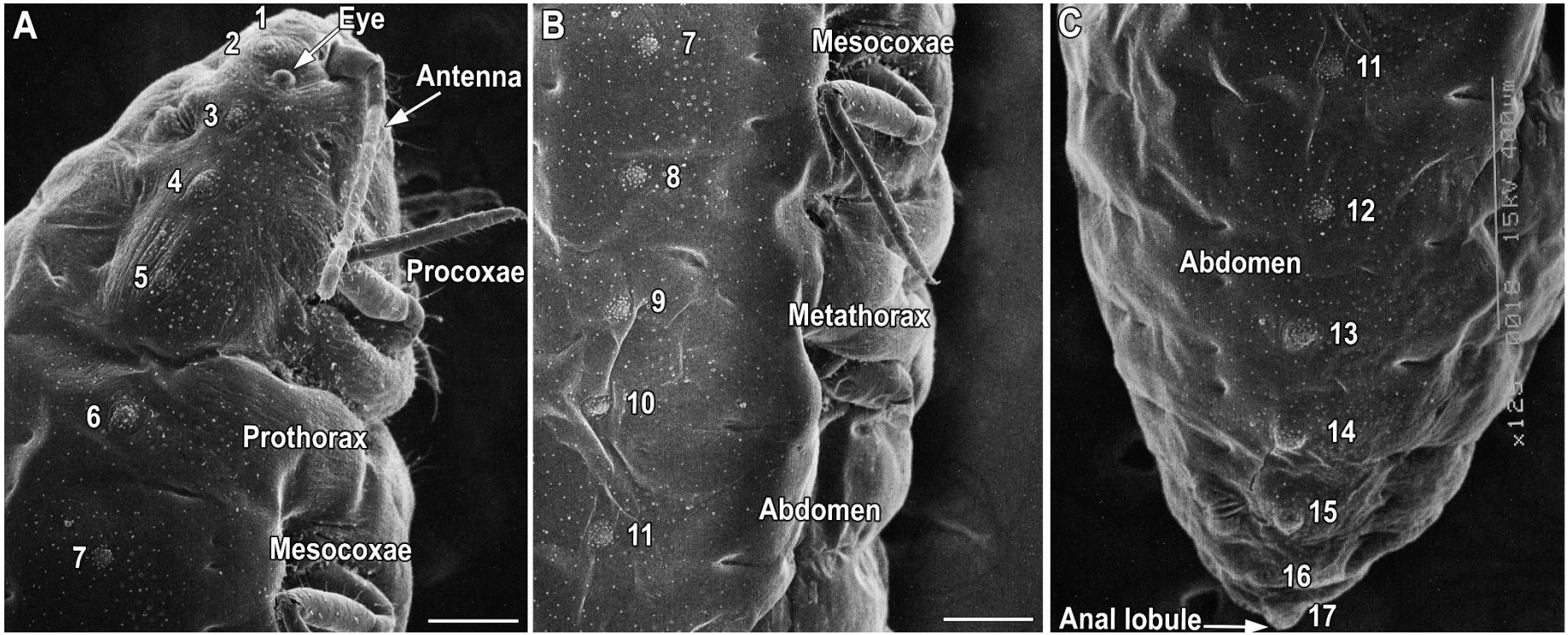

Figure 5. Ultrastructural morphology and distribution of cerarii of the body margin in *Pseudococcus elisae* (head, thorax and abdomen) by SEM from all samples collected from the Neotropical region in the banana crop during 2010 and 2011 (shown here from Panama samples A-C). A. Dorsal margin of the head and prothorax section. B. Dorsal margin of the thorax. C. Dorsal abdomen cerarii, back ostiole and anal lobe. Scale bar = 53,33 µm (A); 50 µm (B); 400 µm (C).

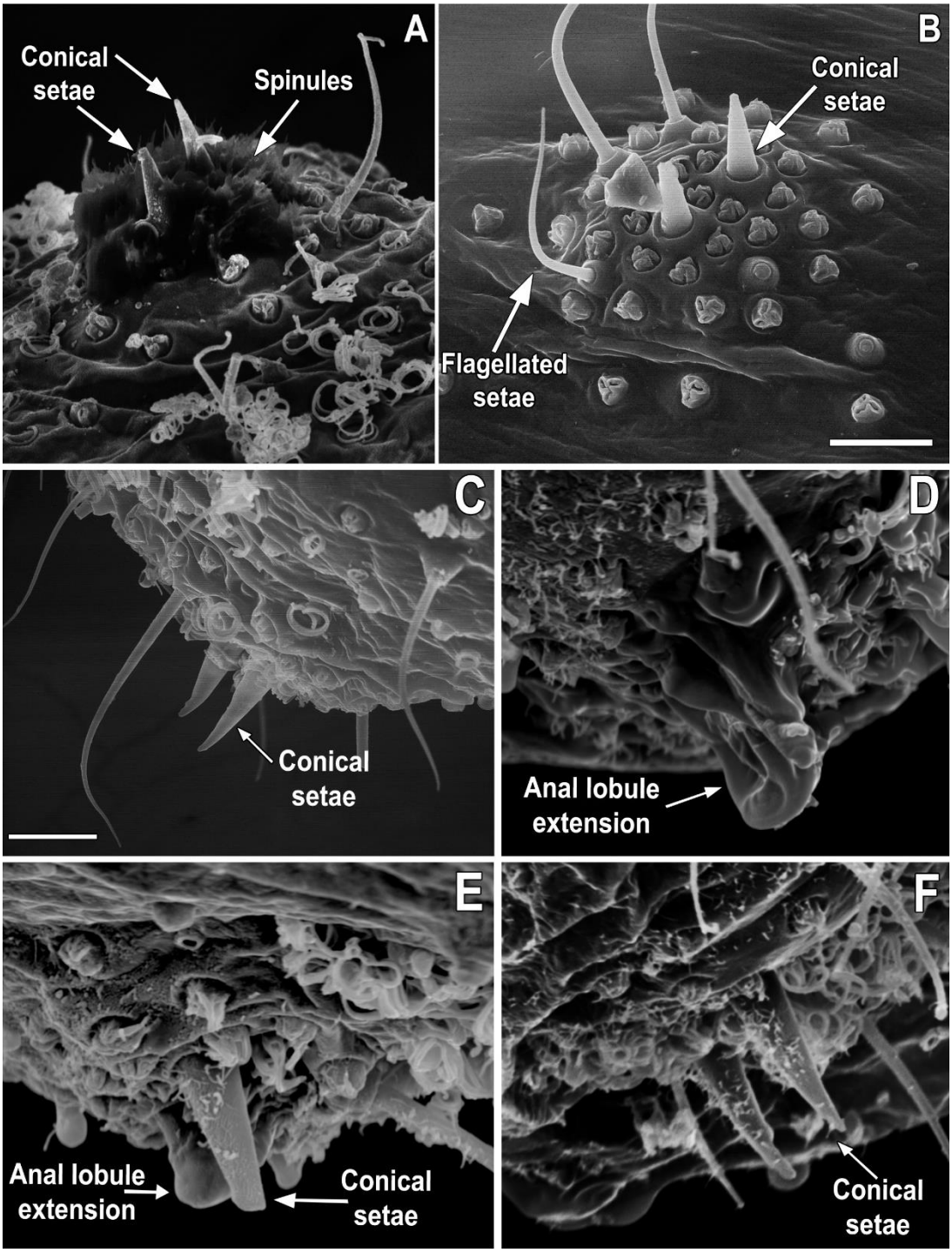

Figure 6. Ultrastructural morphology of the cerarii in the dorsal margin of the *Pseudococcus elisae* body by SEM. Samples collected from the Neotropical region in the banana crop during 2010 and 2011. A. Spinules on the margin cerarius of mealybugs from Colombia. B. Flat base on the margin cerarius of mealybugs (Guatemala). C. Two conical setae present in the anal lobe cerarii (Panama). D-F. Cerarii development sequence in Dominican Republic: D. Presence of a dermal extension on the cerarii. E. Second cerarii with conical setae, adjacent to a dermal extension of the anal lobe. F. Third cerarii with two conical setae and without the dermal extension on the lobe. Scale bar = 2,4 µm (A); 3,17 µm (B), 3,08 µm (C); 1,78 µm (D); 2,67 µm (E); 5,62 µm (F).

For the rest of the mealybugs, a pattern of central conical setae, surrounded by lanceolates setae and a generous amount of trilocular pores were observed on the dorsal margin cerarii (Figure 6. B). Two central conical setae surrounded by trilocular pores on the anal lobe cerarii, a characteristic pattern for most geographical regions, were observed (Figure 6. C). The ultrastructure morphology of the anal lobe cerarius of mealybugs from the Dominican Republic was very different compared to the rest of the mealybugs that were studied. The anal lobes do not show setae at all; rather, an extension of dermal tissue in the anal lobe was presented (Figure 6. D). In the following cerarius (second cerarius), a lobe extension was identified, and well-defined conical setae were observed (Figure 6. E), but were in the third cerarii of the two tapered setae (Figure 6. F). It is likely that this shape represents a transition of setae development; however, there are no reports on cerarii developing an anal lobe with an extension and the conical setae simultaneously.

Trilocular pores: This kind of pore was the most abundant type, and it was present in all mealybugs secreting wax (Ramos and Serna, 2004). These were identified as scattered on the body, in the dorsal area, in the ventral area, and in the margin as part of the cerarii but were specifically absent in the lines of ventral abdominal segments. Different basal pore morphology was presented according to the body location. Those found in the ventral area had a circular base (Figure 7. A); those observed in the dorsal area were bordered by a rim of dermis tissue (Figure 7. B); and those found close to the cerarii did not have this edge (Figure 11. C). In addition, these pores were characterized by a sclerotized loculu, an ultrastructure where the wax was secreted (Figure 7. A and B).

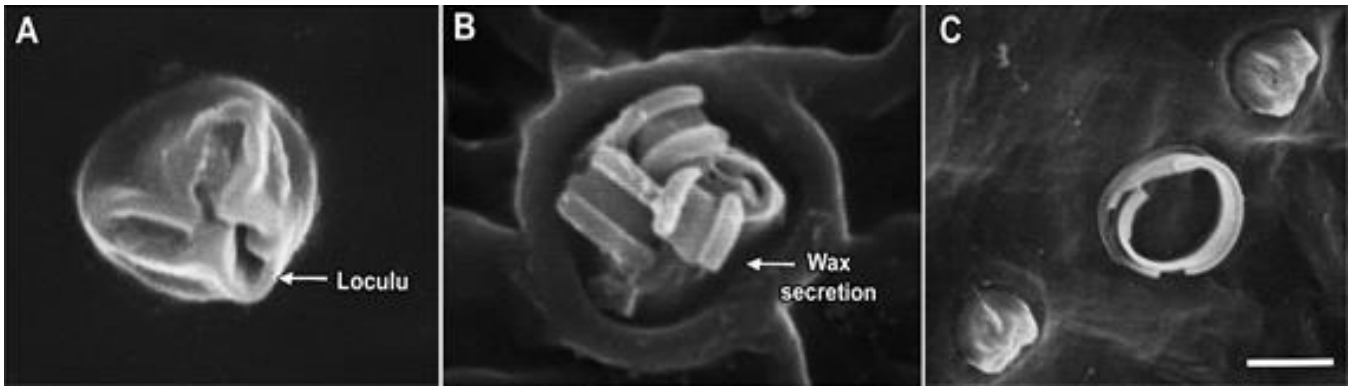

Figure 7. Ultrastructural morphology of the trilocular pores of *Pseudococcus elisae* by SEM. Samples collected from the Neotropical region in the banana crop during 2010 and 2011. A. Ventral trilocular pore on a circular base (Panama). B. Dorsal simple trilocular pore (Panama). C. Trilocular pore from the lateral area of the body, surrounded by the dermis (Panama). Scale bar = 0,30 µm (A); 1,03 µm (B); 0,32 µm (C).

Oral rim tubular duct: An unusual and varied form of oral rim tubular duct morphology was identified by SEM. In mealybug from Guatemala, two types of oral rim tubular duct were observed: a circular simple border duct and a lateral projections duct (Figure 8. A). For the Dominican Republic, simple circular tubular ducts and circular tubular ducts with lateral projections were observed, with both rims serrated on the outer area (Figure 8. B). Mealybugs from Colombia and Panama, showing some ultrastructure of the ducts, turned out to have circular types with a simple oral rim (Figure 8. C). Some mealybugs from the Dominican Republic had ducts with narrow rims (Figure 8. D). In Colombia and Honduras, thicker tubular ducts were identified compared to the rest of the previous accessions (Figure 8. E).

The main results for the distinctive translucent structure with discoidal pores around the eye (translucid pores in posterior legs and anal lobe cerarii are the same for all specimens) are summarized in Table 4. Additionally, the particular ultrastructure of the body, cerarii morphology and oral rim tubular duct morphology identified in the mealybugs from Neotropical countries, from light microscopy and scanning electron microscopy, are shown.

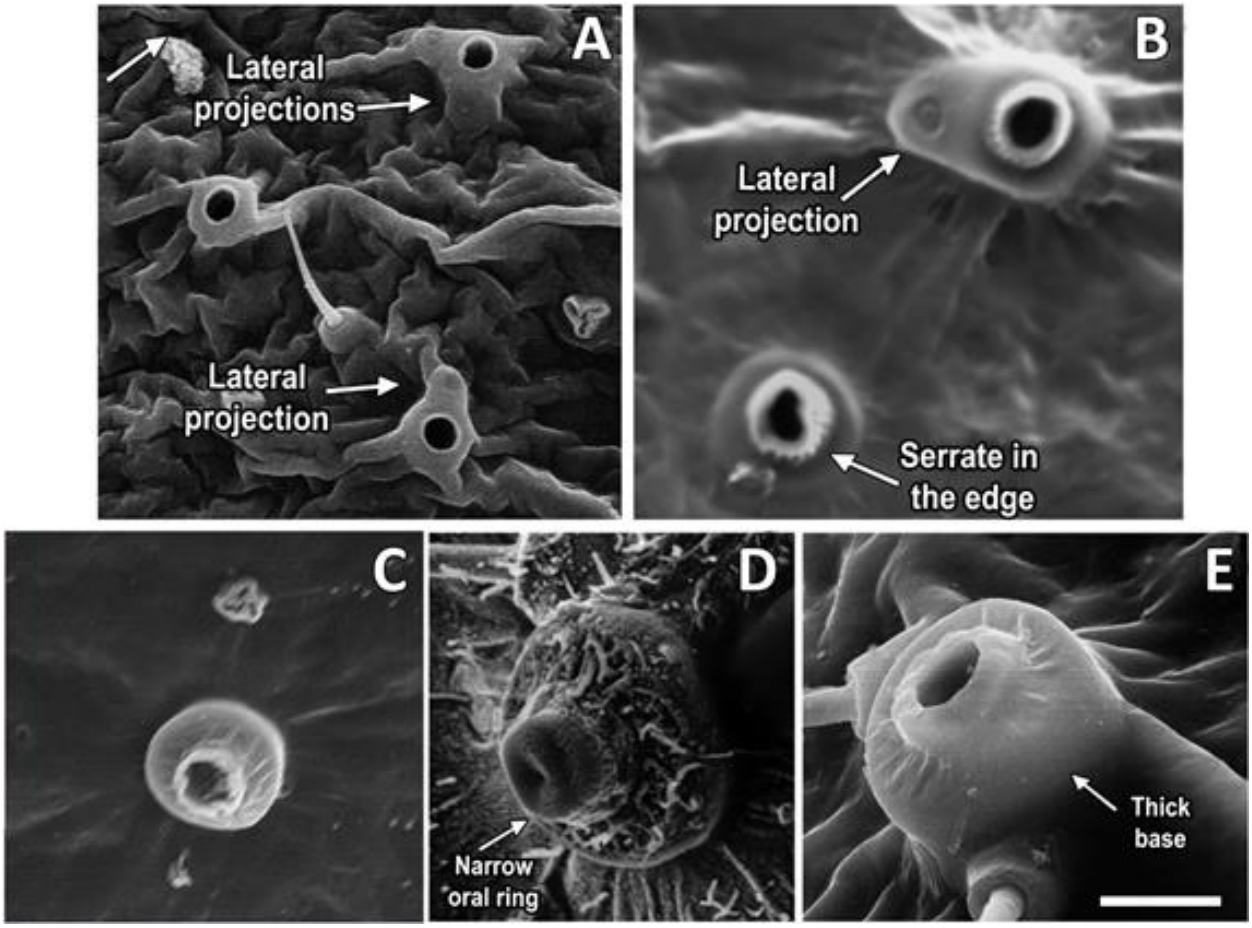

Figure 8. Ultrastructural morphology of the oral rim tubular ducts of *Pseudococcus elisae* by SEM. Samples collected from the Neotropical region in a banana crop during 2010 and 2011. A. Simple oral rim tubular duct from (Panama). B. Circular and lateral projection ducts from Guatemala. C. Serrated outside oral rim from Dominican Republic. D. Narrow oral rim from the Dominican Republic. E. Tick ducts base (Honduras). Scale bar = 0,61 µm (A); 2,06 µm (B); 0,46 µm (C); 0,83 µm (D); 0,71 µm (E).

Table 4. Comparative table of distinctive translucid structures and ultrastructures identified in the mealybugs from Neotropical countries by light microscopy and scanning electron microscopy.

Countries from Neotropical Region	Discoidal pores around the eye*	Body**	Cerarii morphology** (17 cerarii all)	Oral rim tubular duct morphology**
Panama	6	Regular dorsal and frontal area.	Flat base. Two conical setae in anal lobe.	Simple oral rim tubular duct
Guatemala	4	Thicker ultrastructure.		Lateral projection.
Honduras	5	Regular dorsal and frontal area.		Tick ducts
Colombia	6	Openings on the dorsal and frontal area.	Spinules on the margin.	Simple oral rim tubular duct
Dominican Republic	6	Regular dorsal and frontal area.	Dermal extension in anal lobe.	Serrate and narrow outside oral rim

*Light Microscopy; ** Scanning Electron Microscopy.

Molecular Analysis

Sequences details: The DNA sequencing results from the 18S ribosomal gene, the E.F-1α nuclear gene and the COXI gene were studied. Our identifications were confirmed by the matches between our sequences and the GenBank database. Sequencing results showed a total of 641 base pairs (bp) analyzed for the 18S ribosomal gene, 391 bp for E.F-1α gene and 429 bp for the COXI mitochondrial gene. These were incorporated into the GenBank database. These corresponded to the followings accessions: KX639737-KX639740 and KP402190. According to the results reported from the Blast tool (NCBI, 2017), some sequences matched different species from the same geographical region after comparing the analyzed genes to each other. In the 18S ribosomal gene, the hits had high identity percentages (99-100%) for mealybugs, such as *P. elisae, Pseudococcus jackbeardsleyi* (Gimpel and Miller) and *Dysmiccus neobrevipes* (Beardsley). The percentages showed a lack of polymorphisms within the genetic region. The geographical region of the Dominican Republic was less related to the other mealybugs. Nuclear gene E.F-1α showed low identity percentages (89-92%). According to GenBank reports, the most similar species were *P. elisae, P. jackbeardsleyi,* and *D. brevipes.* The mitochondrial gene COXI showed a pattern of low identity between the population studied and GenBank reports, and most isolates had an identity between 90% and 96%. The reported species were *P. elisae* and *P. jackbeardsleyi.*

Phylogenetic trees analysis: Figures 9, 10 and 11 show the results of phylogenetic trees obtained through the MEGA program (version 4.0). Here, we studied the genomic regions for 18S ribosomal, E.F-1α and COXI genes. For each phylogenetic tree, the closest species to the hits found in GenBank were used (Table 3). 18S ribosomal gene. A monophyletic group including Panama, Colombia, Honduras and Guatemala was related to *P. jackbeardsleyi* (KT956119.1) and *P. elisae* (KX639737). It had a 67% bootstrap value. *D. neobrevipes* (U20429.1) from the USA was related to all last species with an 85% bootstrap value. The species *P. elisae* (KP402189.1) from Costa Rica showed a relationship of 100% to the last species. The Dominican Republic was not associated. *Dysmicoccus brevipes* (Cockerell) (EU307273.1) was used as the outgroup (Figure 9).

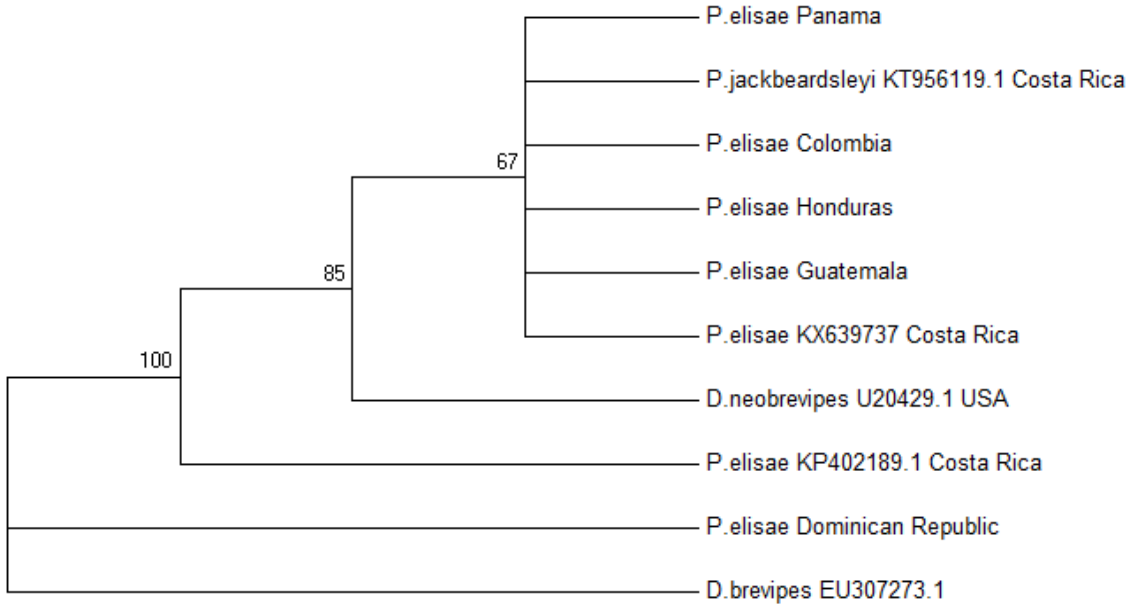

Figure 9. Maximum likelihood phylogenetic tree calculated from the number of differences between the 18S ribosomal haplotypes. Bootstrap values (2000 replications) are displayed for each of the different locations of the study and GenBank accessions. *Dysmicoccus brevipes* (EU307273.1) was used as the outgroup.

F.E-1α gene. In this genomic region, mealybugs from Colombia, the Dominican Republic, Guatemala, Panama and Honduras were grouped with the species *P. jackbeardsleyi* (KT956120.1) and *P. elisae* (KP402191.1), which were both from Costa Rica. The grouped clade had a 100% bootstrap value. *D. brevipes* (KP402192.1) was a

species that was less related. *Dysmicoccus boninsis* (Kuwana) (AY427285.1) was used as the outgroup (Figure 10). COXI mitochondrial gene. Five countries formed a clade including Honduras, Colombia, Panama Dominican Republic and Guatemala; this was related to *P. jackbeardsley* (KT956121.1) and *P. elisae* (KP402194.1) from Costa Rica. It had a 65% bootstrap value. *P. elisae* (KP402195.1) did not have a direct relationship to the last group. *D. boninsis* (KP692714.1) was used as the outgroup (Figure 10).

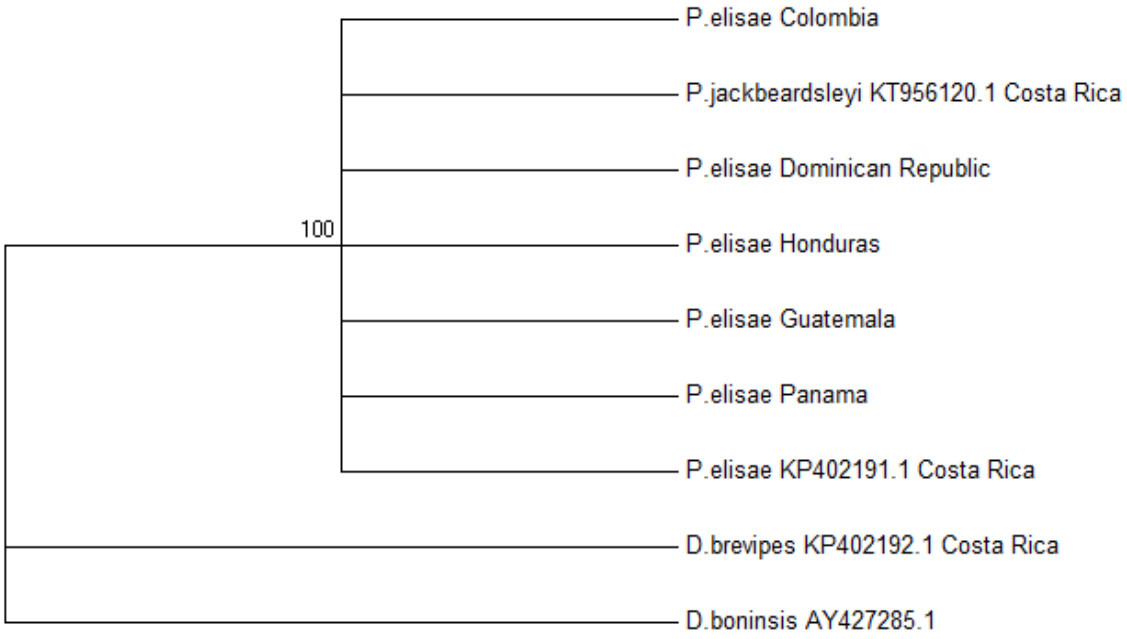

Figure 10. Maximum likelihood phylogenetic tree calculated from the number of differences between the Elongation Factor-1α (F.E-1α) haplotypes. Bootstrap values (2000 replications) are displayed for each of the different locations of the study and GenBank accessions. *Dymicoccus bovinsis* (AY427285.1) was used as the outgroup.

COXI mitochondrial gene. Five countries formed a clade including Honduras, Colombia, Panama Dominican Republic and Guatemala; this was related to *P. jackbeardsley* (KT956121.1) and *P. elisae* (KP402194.1) from Costa Rica. It had a 65% bootstrap value. *P. elisae* (KP402195.1) did not have a direct relationship to the last group. *D. boninsis* (KP692714.1) was used as the outgroup (Figure 11).

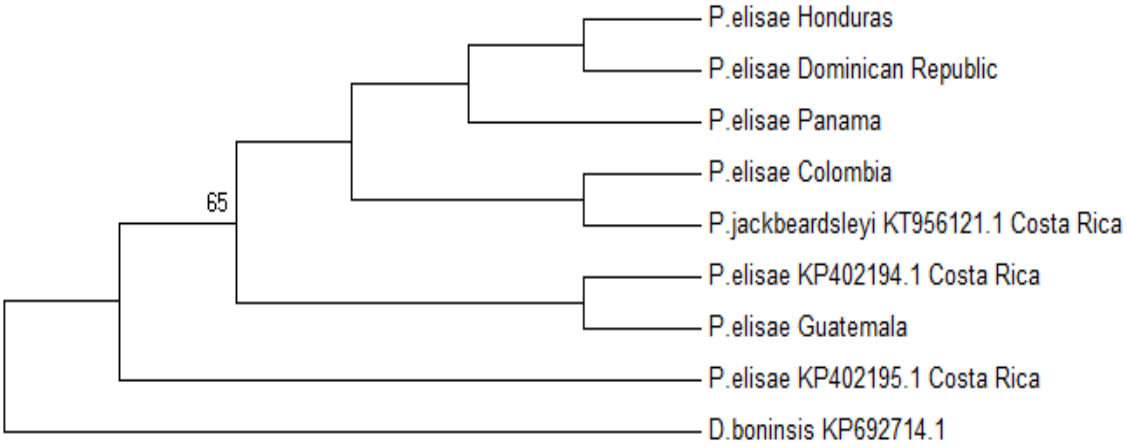

Figure 11. Maximum likelihood phylogenetic tree calculated from the number of differences between the mitochondrial cytochrome oxidase I (COXI) haplotypes. Bootstrap values (2000 replications) are displayed for each of the different locations of the study and GenBank accessions. *Dysmicoccus bovinsis* (KP692714.1) was used as the outgroup.

DISCUSSION

Morphology: Body: An oval-shaped body was a frequent body morphology in species of the present study, but there was variation in size. This variation might be the result of two factors: the natural growth of the insect or the environmental effect. Physiological aspects in mealybug development causes the body to extend during egg production after reaching maturity, and some species are much smaller than others. The sizes of mealybugs have been associated with species rather than genera (Cox, 1987).

Antenna: Seven and 8 segments in the antenna were identified. According to Ramos and Serna (2004), it is normal to see 6 to 9 segments in the antenna in the Pseudococcidae family; the latter is slightly thicker and longer than the penultimate segment. As Gullan (2000) indicated, female mealybugs have four stages of development, and in the third stage, the female mealybug can be easily recognized by the segment number on the antennae. The 7 segments in the antenna are associated with species of the genus *Ripersia* (Radicicola Morrison) or with an immature state in which the mealybug is not fully developed (Ferris, 1978). In some kinds of mealybugs, 6 to 8 segments of the antenna have been identified in *Dysmicoccus* (Granara de Willink, 2009), while eight-segment antennas have been identified in the mealybugs of different genera such as the *Plotococcus* (Miller and Denno) (Miller, 2005), *Eucaliptococcus* (Quin, 1988), *Phenacoccus* (Cockerell) (Williams *et al.*, 2011), *Paracoccus* (Ferris), *Planococcus* (Ferris) and *Pseudococcus* (Williams and Granara de Willink, 1992).

Cerarii: These are structures from the Pseudococcidae family. The number of cerarii in this family varied from sixteen to eighteen pairs, located on the margins of the insect body; seventeen pairs of cerarii with a common panther were found (Williams and Granara de Willink, 1992; Ramos, 2004; Hodges and Hodges, 2006; Miller *et al.*, 2007) and identified in the present research. According to different authors, in various genera of mealybugs, in banana crops, seventeen cerarii in each dorsal margin of the body were quantified, including species of the genus *Pseudococcus* and *Dysmicoccus* (Kondo *et al.*, 2008), *Paraputo* (before *Cataneococcus*) (Williams and Matile-Ferrero, 1999), *Planococcus* (Williams and Matile-Ferrero, 1999; González *et al.*, 2002), *Phenacoccus* (Mohammad and Moharum, 2012) and *Paracoccus* (Miller *et al.*, 2007; Muturi *et al.*, 2013). Zhang *et al.* (2012) and Sirisena *et al.* (2015) explained that the number of conical setae commonly found in each cerarius of the Pseudococcidae family is two, and they are accompanied by a group of trilocular pores and flagellated auxiliary setae. However, this could change depending on the species. For this reason, there are from three to four conical setae (Williams and Granara de Willink, 1992). Mealybugs analyzed by SEM showed differences in the cerarii ultrastructure. For example, mealybugs from Colombia showed spinules over these structures. The closest mealybug found in the literature with this characteristic was *Phenacoccus gossypiphilus* (Townsend and Cockerell), which presents, as described by the author, a cerarii consisting of spiny setae (Abbas *et al.,* 2009). The margin cerarii morphology was different from the anal lobe cerarii, just as Ferris (1978) indicated. Usually, anal lobes cerarii are more sclerotized, and their setae are longer than the remaining cerarii of the body. Rarely, thick setae could be truncated at the apex or born in a sclerotic process, as observed in the mealybugs from the Dominican Republic in the present investigation by SEM. Gullan (2000) explains that mealybugs in an adult stage can be distinguished from stages of nymphs because of the present conical, lanceolate and robust setae in the anal lobe cerarii as well as in the penultimate cerarii; however, the author does not mention a truncated development seta. Opposite to Gullan (2000), Nanda and Ghose (1989) explained that mealybug *Rastrococcus iceryoides* nymphs (Green) are easily recognizable by a distinctive truncated development of their setae in the cerarii.

Trilocular pores: The trilocular pores are features of the Pseudococcidae family, and their distribution varies depending on the genus. According to Zhang *et al.* (2012), each trilocular pore consists of three structural loculi, arranged in secreted helical strands of wax on the edge of the pore. This occurs because of the outlet pressure that appears when the wax is produced. This first coating on the body is called "soft wax" or "wet wax". Kumar *et al.* (1997) found trilocular pores on the ventral and dorsal body surface in all stages of the female in the *Maconellicoccus hirsutus* mealybug (Green). Zhang *et al.* (2012) identified the presence of these pores in the dorsal surface of the nymphs in the third instar of *Pseudococcus fraxinus* (Frêne) as being very numerous. Images of this work by SEM showed the presence of these pores throughout the body of mealybugs (dorsal

and ventral), which are especially abundant in the ventral area and they secrete wax helically. Oral rim tubular duct: Species of the *Pseudococcus* genus have large ducts with a very distinctive edge (Williams and Granada de Willink, 1992). According to Chandler and Watson (1990), the oral rim duct tubular structure could be sclerotic and in some cases varies considerably in the rim structural detail. This classification depends on the species. In addition, Cox (1987) mentioned that these ducts are similar in the neck, but they have a raised edge of integument around the opening, which is a morphological variation. He also explained that these ducts can be totally simple in shape, present or absent in the dorsal and ventral surface, and are commonly found individually. However, the morphology of these structures is not associated with a particular species.

In the present study, it was possible to identify ultrastructural differences analyzed by SEM as different from those observed by LM. Among these ultrastructures were body morphology and dorsal ultrastructures of the insect, morphology of the dorsal margin cerarii and anal lobe cerarii, and morphology of the oral rim tubular ducts. The State Phytosanitary Service of the Ministry of Agriculture and Livestock from Costa Rica was informed of an increase in the mealybug population of the species *P. elisae*. This occurred as a consequence of climate change, specifically in the Caribbean region of the country, the main banana area (Rodríguez, 2013). These alterations, caused by the changes in climate conditions and food requirements, could be reflected on the morphological appearance of mealybugs, as identified in the ultrastructures of this study.

Molecular: According to results from different studies, the phylogenetic relationships between species of mealybugs are still misunderstood (Downie and Gullan 2004; Hardy *et al.*, 2008). Pacheco da Silva *et al.* (2014) explained that it is not possible to ensure that molecular procedures in mealybugs are reliable for a population that has not been sampled already. On the other hand, some of the identified mealybugs have no concordance with the classification related to morphological characteristics through molecular analysis. Moreover, there are reports that have identified some variants between the phenotype and genotype of the mealybug from tropical regions (Palma-Jiménez and Blanco-Meneses, 2016). Hardy *et al.* (2008) explained that the species of the Pseudococcidae family have not been easily understood because of their molecular variation patterns. Abd-Rabou *et al.* (2012) determined the existence of a "cryptic complex" within the Pseudococcidae family. The authors analyzed three DNA markers (28S-D2, COXI and ITS2) and found genetic variation between populations of the same species of mealybugs, justifying further investigation of the possible occurrence of complexes of cryptic taxa.

Despite certain differences in the phylogenetic associations, the relationships between mealybug populations suggest that this is a complex taxon, and the alterations in the genetic patterns in the populations are influenced by geographical factors. In a study of both molecular and morphological data, molecular variability in some so-called 'species' was identified. The authors found that the 'species' *Planococcus ficus* (Signoret) is composed of at least two divergent clusters, consisting on the one hand of European and Turkish populations and on the other hand of Egyptian, Israeli and Californian populations. Furthermore, the authors found genetic variability in the genes ITS2 and LCO of *Planococcus citri* (Risso) from the Egyptian and French populations, but they did not find a common haplotype in any of the populations. They mentioned that these differences may be explained by simple geographic population differentiation (Abd-Rabou *et al.,* 2012).

Xu-Bo *et al.* (2016) explained the case of *Formicococcus* sp. (Hemiptera: Pseudococcidae) mealybugs, which showed two haplotypes in the COXI gene, but the sequences of the 28S gene remained unchanged. Downie and Gullan (2004) explained how the nuclear fragment was conserved as well and lacks sufficient variation to resolve the close relationship between related species. Tan *et al.* (2009) and Xu-Bo *et al.* (2016) mentioned the existence of divergent patterns between two markers and explained the influence of cryptic speciation in mealybugs. According to Abd-Rabou *et al.* (2012), the analysis to understand the correct phylogenetic association of the COXI gene in these mealybugs remains challenging. This is because of the few conserved sites inside the mitochondrial sequence. Wu *et al.* (2015) determined that most of the *Pseudococcus solenopsis* (Tinsley) mealybugs in Southern China, obtained from the same provinces, displayed slight differences regarding the COXI sequence. The authors suggested that since *P. solenopsis* invaded China, this species may have spread from one place to another, which resulted in variations.

In the present study, we observed differences regarding the COXI sequence in *P. elisae*, and a high presence of A and T nucleotides was identified. Rattanawannee and Chongrattanameteekul (2016) mentioned that all PCR products of COXI from all collection localities were high in A+T content. Malausa *et al.* (2011) explained the presence of polymorphisms as high levels of homoplasy and point mutations in COXI. Downie and Gullan (2004) mentioned a high mutation rate and high rate of evolution of this gene as being 3 times greater than the nuclear genome (Rattanawannee and Chongrattanameteekul, 2016). However, the COXI gene has been used to study intraspecific variations of insect pests, such as mealybugs (Rosas-García *et al.,* 2010).

It is important to consider not only the use of one type of DNA as a genetic marker in molecular ecology and phylogeographic studies because there is a tendency toward misinterpretation and this may yield an incomplete evolutionary history of the target species (Ballard and Rand, 2005). The combination of mitochondrial and nuclear DNA genes could allow researchers to answer more complex evolutionary questions about target organisms (Rattanawannee and Chongrattanameteekul, 2016). For this reason, several authors have mentioned the importance of sampling more than one gene (Downie and Gullan, 2004; Hardy *et al.*, 2008; Malausa *et al.*, 2011).

According to Downie and Gullan (2004), given the variation established in evolutionary rates between genes, in regions within genes and more specifically at nucleotides forming codons as well as the different properties of recombination and gene flow that affect the sequence variation within and between taxa, it should be no surprise to observe results from different regions of the genome that are often inconsistent.

CONCLUSION

By the use of the LM technique, we successfully validated the identification of *P. elisae*. The implementation of SEM allowed determination of the ultrastructural variants of the body, dorsal margin cerarii and anal lobe cerarii, trilocular pores and morphology of the oral rim tubular ducts. Our study was one of the first studies to identify variants using this technique to describe this mealybug from the Neotropical region in banana crops (*Musa* spp.), and our results were reinforced by molecular analysis (genes 18S, E.F-1α and COXI). This investigation brought forth questions about the taxonomic status of some populations of mealybugs sampled from different geographical regions, due to the morphology and molecular variability of *P. elisae*. We obtained distinctive patterns that were influenced by the environmental conditions of Neotropical countries. The mealybugs identified through molecular analysis did not show concordance with the ultrastructural characteristics, because these have not been previously mentioned in the literature as taxonomic characters. The relationships among mealybug populations suggested that these are complex taxa, and the alterations in the genetics (because of different levels of evolution) and phenotypic patterns in the populations are influenced by geographical factors. To explain the influence of many factors, future studies may consider increasing taxon sampling and the informative number of genetic markers.

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