ISSN: 0975-833X

Available online at http://www.journalcra.com

International Journal of Current Research
Vol. 8, Issue, 05, pp.31791-31800, May, 2016

INTERNATIONAL JOURNAL OF CURRENT RESEARCH

RESEARCH ARTICLE

FIRST STUDY ABOUT MORPHOLOGICAL AND MOLECULAR IDENTIFICATION OF *PSEUDOCOCCUS ELISAE* (HEMIPTERA: PSEUDOCOCCIDAE) IN COSTA RICA

[1]Melissa Palma-Jiménez and *,[2]Mónica Blanco-Meneses

[1]Master Program at the Universidad de Costa Rica
[2]Molecular Phytopathology Laboratory, CIPROC, Agronomy Department, Universidad de Costa Rica, San Pedro de Montes de Oca, San José, Costa Rica, P.O. BOX: 2060

ARTICLE INFO

Article History:
Received 27th February, 2016
Received in revised form
15th March, 2016
Accepted 24th April, 2016
Published online 31st May, 2016

Key words:

Central America,
Genetic variation,
Phylogeny,
Taxonomy.

ABSTRACT

The State Phytosanitary Service of the Ministry of Agriculture and Livestock (SFE, for its acronym in Spanish) declared national emergency in 2014 because of rising populations of mealybug *Pseudococcus elisae* (Hemiptera: Pseudococcidae) in banana plantations. Currently, there is a great concern due to the economic losses caused to farmers. To understand about the morphological and molecular relationship of these individuals, the present work was carried out with the aim of describing insects collected from 10 farms from the Atlantic region of Costa Rica and analyze three specific genes of mealybugs. The microscopy analysis was done in the Center for Research on Microscopic structures in 2012 and the molecular analysis was performed in the Molecular Phytophatology Laboratory ending in 2014, both at the University of Costa Rica. Morphological and molecular variations among mealybugs were identified; also discrepancies were noticed when comparing the results of the species among the three genes obtained from the study (18S ribosomal, E.F-1α and COXI), providing sequences that identified one of the species present in Costa Rica as *Pseudococcus elisae*, which was not previously described in the gene bank (NCBI).

Resumen:

El Servicio Fitosanitario del Estado del Ministerio de Agricultura y Ganadería (SFE) declaró emergencia fitosanitaria nacional para el año 2014 a causa del aumento de poblaciones de la cochinilla harinosa *Pseudococcus elisae* (Hemipters: Pseudococcidae) en el cultivo de banano. Actualmente existe una gran preocupación por las pérdidas económicas generadas a los productores. Para entender a cerca de la relación morfológica y molecular de estos individuos el presente trabajo se realizó con el objetivo de describir a los insectos colectados en 10 fincas de la región Atlántica de Costa Rica y se analizaron tres genes específicos de la cochinilla harinosa. La descripción morfológica se realizó en el CIEMic de la UCR en el año 2012 y el análisis molecular en el Laboratorio de Técnicas Moleculares aplicadas a la Fitoprotección del CIPROC para finalizar en el año 2014. Se identificó variación morfológica y molecular entre las cochinillas harinosas, también se observó discrepancias al comparar los resultados de las especies obtenidas entre los tres genes del estudio (18S ribosomal, F.E-1α y COXI), aportando además secuencias que identifican a una de las especies presente en Costa Rica como *Pseudococcus elisae*, la cual anteriormente no estaba descrita en el banco de genes (NCBI).

Citation: Melissa Palma-Jiménez and Mónica Blanco-Meneses, C. 2016. "First study about morphological and molecular identification of *Pseudococcus elisae* (Hemiptera: Pseudococcidae) in *Costa rica*", *International Journal of Current Research*, 8, (05), 31791-31800.

INTRODUCTION

In 1967 some specimens of *Pseudococcus elisae* were observed from United Fruit Company in Central America (Beardsley, 1986). These species feed by sucking the nitrogen content of the host plant by stylets located in their mouthparts; thereby, producing sugary waste, which is a good medium for the growth of sooty molds that are reproduced superficially in leaves and stems forming a black film (Ramos and Serna, 2004). Furthermore, the fact that it is a quarantine pest is enough to be rejected by the packing companies, which causes main economic losses to producers (Guillen *et al*., 2010; Rodríguez, 2013). The State Phytosanitary Service (SFE, for its acronym in Spanish) of the Ministry of Agriculture and Livestock, declared national phytosanitary emergency to the year 2014, because of increased populations of mealybug

Corresponding Author: **Mónica Blanco-Meneses,**
Molecular Phytopathology Laboratory, CIPROC, Agronomy Department, Universidad de Costa Rica

P. elisae which caused severe damage to banana crop. The affected area was estimated to 24,000 ha, of a total acreage corresponding to 41,200 ha (Rodríguez, 2013; SFE, 2015). According to numbers from the Central Agricultural Monitoring, prepared by the Business Intelligence area CentralAmericaData (2015) revealed that Central American banana exports decreased in 2015 compared to 2014. In the case of Costa Rica, the National Banana Corporation (CORBANA, for its acronym in Spanish) reported income from fruit exportation to $823 million by 2014 (CentralAmericaData, 2014), and for 2015, PROCOMER (2015) recorded from January to September (busiest months) a total cumulative of $588.8 million; for these months it meant 1.5% less than in the same period of 2014. Rodríguez (2013) mentions that factors such as pests and diseases are considered in this economical decrease; moreover, the emergency of mealybugs, has causing an increase of agrochemicals use in the crop area. The identification of these insects is routinely performed by phenotypic markers. It takes time to process the specimens before a correct classification can be made, besides most of the time an expert in the area needs to be consulted (Kondo *et al.*, 2008; Wu *et al.*, 2013). Sometimes the insect can have shown variation as a result of the environment, which makes the identification difficult (Miller *et al.,* 2014). This is particularly problematic because difficulties in identification may jeopardize the use of control and management methods specific to certain target species, which are currently favored over the use of broad-spectrum pesticides (Pacheco da Silva *et al.,* 2014). The analysis of genetic variation based on DNA fingerprinting techniques, has become an important method to understand the taxonomy and evolutionary studies in a variety of insects (Avise, 2004). Molecular analysis with sequencing technique is commonly used to differentiate populations of mealybugs (Downie and Gullan, 2004; Danforth *et al.,* 2005; Hardy, 2008; Malausa *et al.*, 2011). These methods can identify specimens regardless of the stage of development and geographical origin, and provide researchers with a powerful tool for fast, accurate and unambiguous identification of the different species of insects from agricultural fields (Cavalieri *et al.,* 2008). The information obtained can be used in the design and evaluation of new strategies for crop protection (Kazachkova *et al.,* 2007). The aim of this study was to conduct a characterization of the mealybug *Pseudococcus elisae* collected in banana plantations located in Costa Rica, by identifying morphological characters and using molecular analysis with three specific genes.

MATERIALS AND METHODS

Sample collections

Female mealybugs were collected in 10 farms located along the Atlantic region of Costa Rica (Agrotubérculos, Aproveco, stol, CATIE, Corsega, Kopemaz, Laboratory of Biocontrol, Manu, Zent and Zorsales). The collection was carried out during visits to farms between 2010 and 2012 and the location of the sites was identified by geographic coordinates. Individuals were collected in 1.5 mL eppendorf tubes with 95% of ethanol. An average of 30 individuals per geographic region was collected

Place of study

The morphological analysis was performed at the Center for Research on Microscopic Structures (CIEMic, acronyms in Spanish) in 2012, and the molecular analysis was performed in the Molecular Phytophatology Laboratory for Research in Crop Protection (CIPROC, acronyms in Spanish) of Costa Rica, ending in 2014., both at the Universidad de Costa Rica, San Pedro, Montes de Oca.

Observation under the light microscope

Ten insects by locality were processed. The protocol described by Williams and Granara de Willink (1992) was used. To identify the translucent structures, the insects were examined with inverted light microscopy equipment, using increases 4x, 10x, 20x and 40x (Model IX51, Olympus Optical Co., Japan). The analyzed structures by light microscopy corresponded to the following: body shape, number of segments of the antenna, discoidal translucent pores around the eyes, mouthparts and stylets, description of posterior legs and presence of translucent pores, description of the circulus, ostioles, oral rim tubular ducts, anal lobe bar and cerarii.

Amplification of genomic DNA

The protocol by Murray and Thompson (1985) was used. One insect was used for each DNA extraction. The genomic DNA extracted was amplified by PCR and three pairs of primers were used (Table 2). For all PCR reactions in a 1x (ul) solution was used: 13.5 µL of H2O, 2.5 µL of buffer (10x), 2 µL of dNTPs (2 mM), 1.5 µL each for each pair primer (10µM), 0.3 µL of Dream Taq polymerase (5/µL) to 23 µL of master mix per eppendorf tube (all reagents Fermentas), and finally adding 2 µL of DNA (10 µg/mL). 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 2), 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. 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 50 ng/µL by the company 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 and by comparison of the chromatograms using the BioEdit program v7.0.5 (Hall, 2010). To determine the species according to the result of sequencing, the GenBank was used (NCBI, 2015). All sequences were aligned with the ClustalW program version 1.60 (Thompson *et al*., 1994). To corroborate the variation that might exist within populations of Costa Rica, sequencing was repeated on three different individuals. It allowed verifying the number of haplotypes within it.

Table 1. List of farms sampled: Location, population code, plant location, collection year and geographic coordinates

Location	Population Code	Plant location	Collection year	Geographic coordinates	
				Latitude	Longitude
Finca Agrotubérculos, Cahuita, Limón	*Agro*	Grass	2011	9º 40'00.00''N	82º 47'59.99''O
Finca Aproveco, Batán, Limón	*Apr*	Pseudostem	2012	10 º 05'04''N	83º 20' 28''O
Finca Bristol, Limón	*Bris*	Pseudostem	2012	10 º 01'44''N	83º 18'14''O
Finca CATIE, Turrialba	*CATIE*	Root	2010	9 º 53'23.06''N	83º 39'11.59''O
Corsega, San Carlos de Pacuarito, Limón	*Cor*	Pseudostem	2012	10 º 10'25''N	83º 46' 76''O
Finca Kopemaz, Matina, Limón	*Kope*	Pseudostem	2012	10 º02'54''N	83º 27' 78''O
Finca Lab Biocontrol, Turrialba	*LabBio*	Pseudostem	2010	9 º 53'23.06''N	83º 39'11.59''O
Finca Manu, Guápiles, Limón	*Manu*	Grass	2011	9 º 51'23.85''N	82º 58'36.45''O
Finca Zent, Matina, Limón	*Zent*	Pseudostem	2012	10 º 02'54''N	83º 27'78''O
Finca Zorsales, Carrandi, Limón	*Zor*	Pseudostem	2012	10 º 03'49''N	83º 16' 43''O

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

Gene	Primers	Primer sequence	PCR conditions	Amplicon average size (bp)	Primer source
18S	18S-2880 18S-B	CTGGTTGATCCTGCCAGTAG CCGCGGCTGCTGGCACCAGA	94°C, 4min; 30 ciclos de 94°C 1min, **67°C** 1min, 72°C 1min, 30s; 72°C 4min	630	(Malausa *et al.,* 2011) (Downie and Gullan, 2004)
EF-1α 5'	EF-1_M51.9 EF-1_rcM53-2	CACATYAACATTGTCGTSATYGG CTTGATGAAATCYCTGTGTCC	94°C, 4min; 30 ciclos de 94°C 1min, **62°C** 1min, 72°C 1min, 30s; 72°C 4min	439	(Downie and Gullan, 2004)
COXI	C1-J-2183 C1-N-2568	CAACATTTATTTTGATTTTTTGG GCWACWACRTAATAKGTATCATG	94°C, 4min; 30 ciclos de 94°C 1min, **45°C** 1min, 72°C 1min, 30s; 72°C 4min	385	(Malausa *et al*., 2011)

Table 3. GenBank information used for the phylogenetic trees construction: Species, host plant, origin country and GenBank accession number

Species	Host Plant	Origen Country	GenBank accession number		
			18S ribosomal	EF-1α	COXI
Dysmicoccus neobrevipes	*	China	JF965400.1	-	-
D. neobrevipes	*	USA	U20429.1	-	-
Mayetiola destructor	*	USA	KC177284.1	-	-
Pseudococcus longispinus	Citrus	South Africa	AY426038.1	-	-
P.longispinus	*Cycas* sp.	Mexico	HQ893787.1	-	-
P. jackbeardsleyi	*	Taiwan	KJ145237.1	-	-
P.viburni	*	South Africa	JQ651125.1	-	-
Eriococcus coccineus*	*	Australia	AY795536.1	-	-
D. brevipes	*	South Africa	-	AY427227.1	-
P. jackbeardsleyi	*	USA	-	EU188562.1	-
P.longispinus	*	USA	-	AY179487.1	-
P.longispinus	Citrus	South Africa	-	AY427262.1	-
P.maritimus	*Vitis vinifera*	South Africa	-	AY427217.1	-
Eriococcus coccineus*	*	USA	-	EU746893.1	-
D.neobrevipes	*	USA	-	-	EU267213.1
D.texensis	*	Brazil	-	-	KJ530628.1
Hypogeococcus pungens	*	Spain	-	-	JF714168.1
P.longispinus	*Sedirea japonica*	Japan	-	-	AB512118.1
P.longispinus	*	South Africa	-	-	DQ238222.1
P.longispinus	*	USA	-	-	AY179439.1
P.longispinus	*	Spain	-	-	JF714161.1
P.microadonium	*	France	-	-	GU134681.1
P. jackbeardsleyi	*	India	-	-	KC119455.1
Eriococcus azaleae*	*	USA:Oregon	-	-	KJ869284.1

* Not reported. ** Out group.

For the phylogenetic analysis, sequences were included from species previously reported by GenBank for all three genes studied, by renowned authors in the field, as: Gullan *et al.* (2003), Downie and Gullan (2004), Hardy and Gullan (2008), among others; and the individuals origin was verified with respect to the host plant and the country (Table 3). The analysis of phylogenetic trees was performed using the program MEGA version 4.0 (Molecular Evolutionary Genetic Analysis) (Tamura *et al.,* 2007). The random parameter of 2000 replications was used to search for phylogenetics trees and UPGMA method for the three genes presented the best groupings between the species analyzed.

RESULTS

Morphological characterization

The species results corresponded to those described by the key of Gimpel and Miller (1996), which is used by the staff of SFE of Costa Rica and the key to Miller *et al.* (2007) of USDA (United States Department of Agriculture) (Figs. 1 and 2).

Body Shape: Elongated oval (2.9 x 1.5 mm) (Fig. 1 A) with 17 pairs of cerarii were quantified (Fig. 1 A).

Mouthparts: The presence of three stylets was observed (Fig 1. B).

Cerarii: Each cerarius with two enlarged conical setae and auxiliary setae (Fig. 1. A), except on the cerarius 12 (C12) and on the head, with three conical setae (C15-C17) (Fig. 1. C).

Oral rim tubular ducts: Were observed in the region of the head of the insect, adjacent to the head cerarii (C17), between each antenna (Fig 1. C.), these ducts were absent on the dorsum of segment VII of the abdomen (Fig. 2).

Circulus: A circulus divided by a line between segments III and IV, which presented a diamond-shaped contour (Fig. 1 D).

Discoidal pores at the eye edge: Seven to nine pores around the eye were presented (Fig 1.E).

Antennae: Eight segments were presented (Fig 1. F.).

Translucent pores in metacoxas: Located in two sections of the metacoxas: femur and tibia. Absent on trochanter or coxa (Fig 1. G).

Ostioles: The typical structure of the ostioles was observed, which has lip-shaped, with trilocular pores inside (Fig. 2).

Anal lobe bar: Was absent in the anal lobe cerarii (Fig. 2).

Anal lobe cerarii: With two conical setae on sclerotized area (Fig. 2).

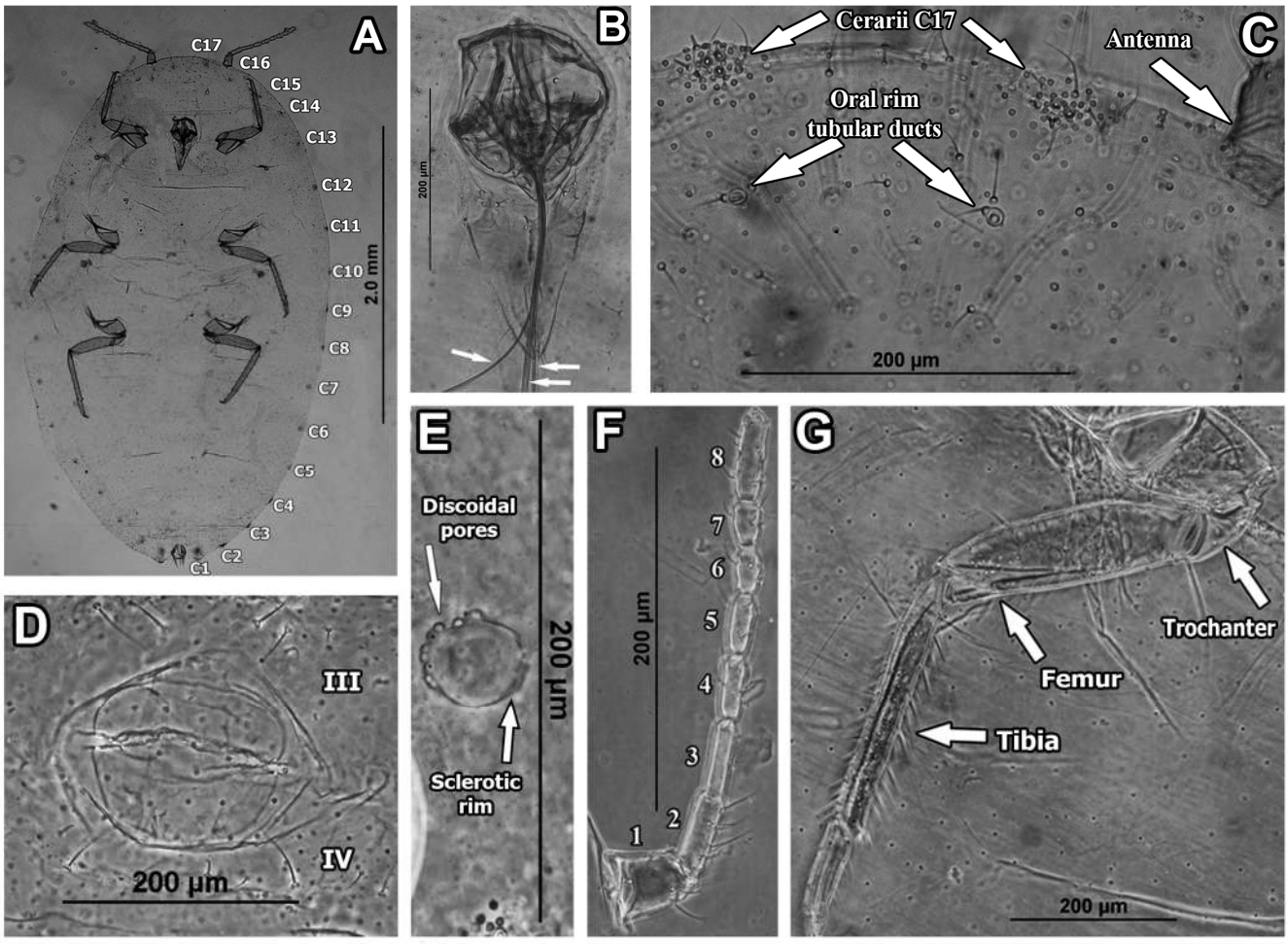

Fig. 1. Morphological characters analyzed to identify the *Pseudococcus elisae* mealybug from banana crop from farms in the Atlantic and Central area of Costa Rica by light microscopy. Mealybug collected during years 2010-2012. A. Body of the insect shaped elongated oval with 17 pairs of cerarii. B. Mouthparts with three stylets. C. Cerarii C17 associated to oral rim tubular ducts. D. Circulus. E. Eye surrounded by discoidal pores and sclerotic rim. F. Antennae with eight segments. G. Presence of translucent pores on femur and tibia of the metacoxa, translucent pores not on trochanter

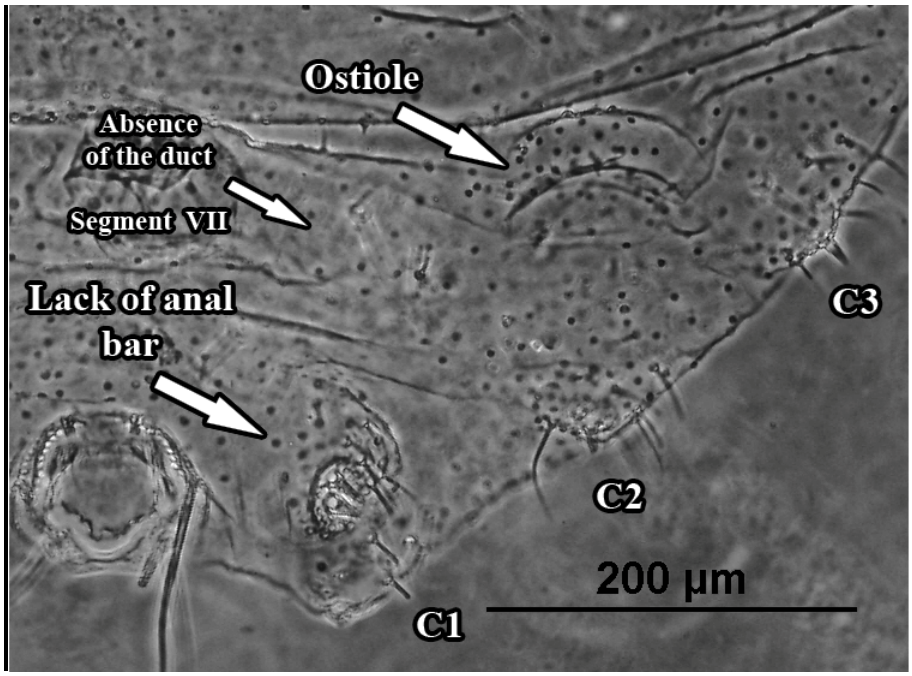

Fig. 2. Posterior area of abdomen of *Pseudococcus elisae,* mealybug from banana crop from farms of the Atlantic and Central region of Costa Rica by light microscopy. Mealybug collected during years 2010-2012. Lack of anal bar in the anal lobe cerarii (C1). Oral rim tubular duct absent on segment VII next to cerarius

Molecular analysis

Using 18S ribosomal gene and the elongation factor 1α gene (EF-1α), a total of 26 mealybugs were sequenced. With the mitochondrial COXI gene a total of 10 mealybugs were sequenced. The 18S ribosomal gene produced an average amplicon of 639 base pair (bp), the EF-1α gene an average amplicon of 359 bp and for the COXI gene an amplicon of 411 bp. According to the results reported by Blast tool of NCBI, it was identified that the 18S ribosomal gene is not representative for the description of these mealybugs. Most of the hit results from this genomic region corresponded to very high identity percentages of 99-100% to mealybugs who belong to different genus. As an example genus as: *Dysmicoccus, Pseudococcus, Oracella, Paracoccus, Vryburgia, Trionymus, Paradoxococcus, Cataenococcus* [= *Paraputo*], *Erium, Planococcus* and *Sphaerococcus* between other (data not shown), this percentage is only two to four different bases within the sequence. This clearly shows the lack of specific genetic polymorphisms within the 18S genomic region. For all locations, the mealybugs of the studied farms with highest hits of similarity to the top five species from GenBank correspond to *Pseudococus viburni* and *Dysmicoccus neobrevipes*. For these reasons, the data generated by the 18S ribosomal region of the genome were not taken as transcendental in the development of this research. Moreover, the EF-1α nuclear gene shows a very low similarity hits in GenBank to the population studied. The highest percentages of similarity correspond to values between 80-90%, which represents a number close to the 40 bases that are polymorphic between isolates reported and those belonging to this study, which confirms the low specificity in the search with species reported previously. On the COXI genomic region for the mitochondrial gene, a pattern of low identity between the populations studied and reported in GenBank was observed. Most isolates have between 90-96% of identity, which represents between 13-36 polymorphisms that differentiate the population of Costa Rica from individuals from other places (unpublished data). The highest identity corresponds to 99% of *P. nr. microadonidum* respect to mealybugs from *CATIE* and *Manu*. Analyzing the morphological characteristics and relating them with the genetic characteristics, it was possible to determine the most common sequences of mealybugs banana in Costa Rica. These data were reported to GenBank and sequences were deposited under accession numbers: KP402189, KP402191, KP402193, KP402194, KP402195 and KP402197.

Phylogenetic trees analysis

For the 18S ribosomal gene (Fig. 3), the closest species to the hits found in GenBank were used, which corresponded to *D. neobrevipes* from China (JF965400.1) and USA (U20429.1) and *P. viburni* from South Africa (JQ651125.1). These two species formed the largest monophyletic group, although morphologically have different structures in relation to the population studied. It can be seen that the only remaining accessions out of this majority group are individuals from *Manu1, CATIE1* and *CATIE3*. Interrelated and from a clade are the taxa: *CATIE1* and *3*, which were grouped together with *P. longispinus*, reported by GenBank from Mexico (HQ893787.1) and South Africa (AY426038.1), sharing a bootstrap value of 96% similarity. It is noteworthy that the species *P. elisae* was not registered previously in GenBank for the 18S gene. Some of the accessions from the same geographic location (*CATIE, Manu* and *Agro*) have two haplotypes for this genetic region. The out group used corresponded to *Eriococcus coccineus* (AY795536.1). In the Figure 4 the phylogenetic tree corresponding to the elongation factor 1α gene can be seen.

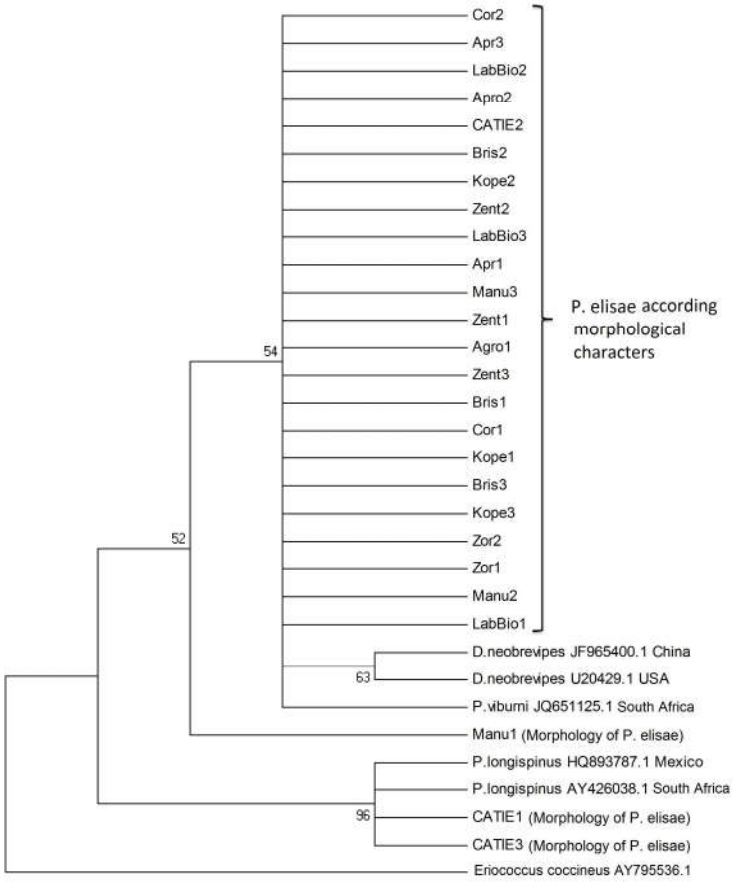

Fig. 3. UPGMA phylogenetic tree calculated from the number of differences between 18S ribosomal haplotypes. Bootstrap values (2000 replications) are displayed for each of 10 different locations of the study and GenBank accessions. *Eriococcus coccineus* (AY795536.1) was used as out group

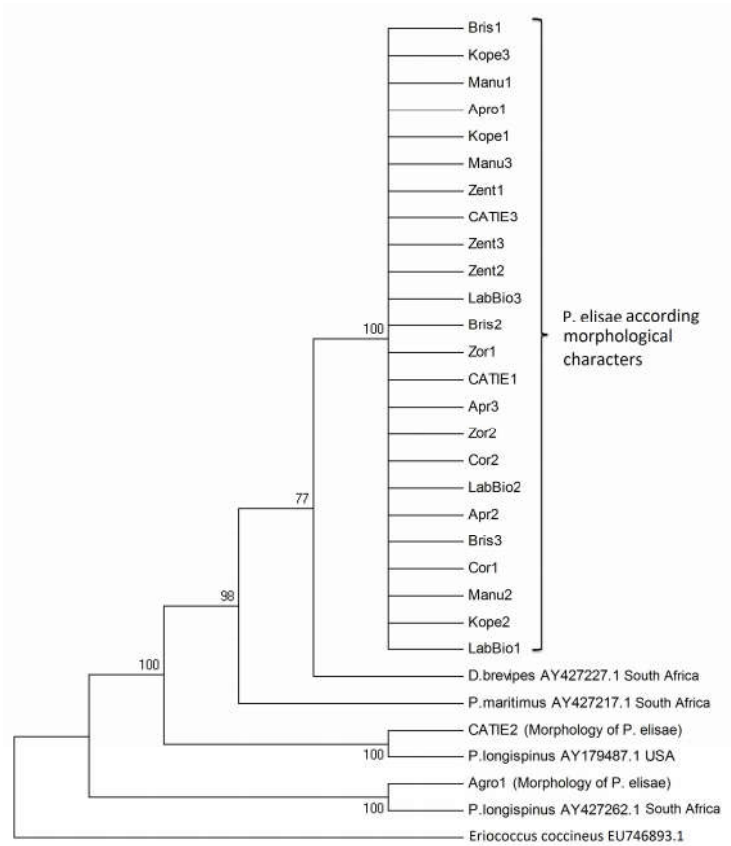

Fig. 4. UPGMA phylogenetic tree calculated from the number of differences between elongation factor 1α gene (EF-1α) haplotypes. Bootstrap values (2000 replications) are displayed for each of 10 different locations of the study and GenBank accessions. *Eriococcus coccineus* (EU746893.1) was used as outgroup

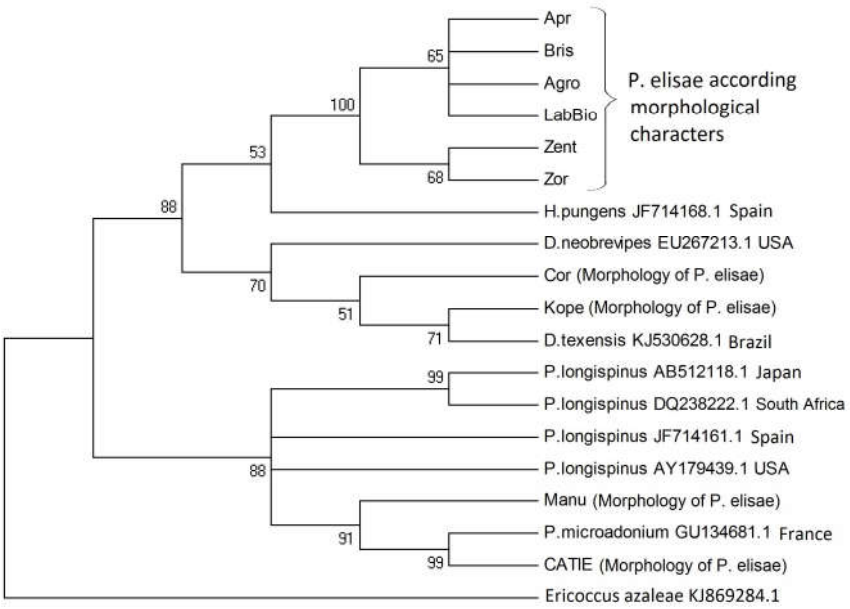

Fig. 5. UPGMA phylogenetic tree calculated from the number of differences between mitochondrial cytochrome oxidase subunit I gene (COXI) haplotypes. Bootstrap values (2000 replications) are displayed for each of 10 different locations of the study and GenBank accessions. *Eriococcus azaleae* (KJ869284.1) was used as outgroup

Most mealybugs from Costa Rica farms formed a clade with a bootstrap or genetic identity of 100%. This clade was joined to a separate node with 77% of similarity that relates to the species *D. brevipes* from South Africa (AY427227.1); however the variation at morphological characters is very different in relation to the population studied. On different clades is: *P. maritimus* from South Africa (AY427217.1), a grouping of *CATIE2* with *P. longispinus* from USA (AY179487.1) and another clade of *Agro1* with the species *P. longispinus* from South Africa (AY427262.1). Likewise, morphological differences confirming that individuals of the other countries do not correspond to these species observed in Costa Rica. For this gene it was observed that the accessions having different haplotypes are the *CATIE* and *Agro*, both with 2 haplotypes. The external group used for this gene corresponded to *Eriococcus coccineus* species (EU746893.1). In the phylogenetic tree for mitochondrial gene COXI, it was determined that six locations from Costa Rica were grouped forming a monophyletic group with 100% bootstrap, and have the closest relationship with *H. pungens* of Spain (JF714168.1) (Fig. 5). The *Cor* location was associated with a 51% bootstrap regard to previous locations and it has a 70% bootstrap similarity related to the species *D. neobrevipes* (EU267213.1) from USA. The *Kope* location was associated with a 71% bootstrap of similarity to the species *D. texensis* from Brazil (KJ530628.1). Other two major clades divide the mealybugs from *Manu* and *CATIE* of the rest of the population from Costa Rica. The *CATIE* location was related to the species *P. microadonium* from France (GU134681.1) with a 99% bootstrap of similarity, this at the same time shared relationship with *Manu* and was associated with a 91% bootstrap of similarity, this at the same time with 88% of similarity with *P. longispinus* from different geographic regions: Japan (AB512118.1), South Africa (DQ238222.1), USA (AY179439.1) and Spain (JF714161.1). All are in separate clades that support the great genetic diversity among the accessions. The variation at morphological characters in relation to the population studied is relevant. *Eriococcus azaleae* (KJ869284.1) was used as outgroup.

DISCUSSION

When comparing the results of the morphological characters for specimens identified as *P. elisae* regarding molecular data of the study, there is some inconsistency, mainly determined between the three genes and the information that has been released in GenBank (NCBI, 2015). Before this study *P. elisae* species had not been reported for any of the genetic regions used in this study. Gimpel & Miller (1996) provided a key, illustrations and discussion of *P. elisae* in their publication on the "*Pseudococcus maritimus* Complex". Upon knowledge of this complex, Correa *et al.* (2011) comment that mealybug species of the *Pseudococcu*s genus, that are found in phytosanitary investigations and are not known, have been referred to as *Pseudococcus* sp. According to several authors (Downie and Gullan, 2004; Hardy, 2008; Malausa *et al*., 2010; Beltra *et al*., 2012), the Pseudococcidae family is characterized by a few unique and distinctive morphological characters for classification, these vary in number and position. This is why sometimes there have been difficulties in establishing classifications and phylogenies consistent. However, the species of Costa Rica farms are grouped together, indicating a diversity characteristic of the area and this is reinforced by the analysis of results when comparing the three phylogenetic groups (Figs. 3, 4 and 5). Phylogenetic clustering of 18S ribosomal gene demonstrates a high percentage of similarity and genetic conservation, slow development and lack of resolution of relations between families of insects, mentioned by several authors for that gene (Downie and Gullan, 2004; Kondo *et al.,* 2008; Ashfaq *et al.,* 2011) (Fig. 3). Different results were observed when comparing the 18S ribosomal gene with FE-1α gene, which showed a larger pattern of intraspecific

polymorphisms (data not shown). It is probably that the nuclear gene to be linked to sexual reproduction and have a role in the process of protein translation is affected by heterozygosity in addition to possible changes in the translation process and thus can explain the presence of more of these polymorphisms (Downie and Gullan, 2004; Djernaes and Damgaard, 2006). Authors like Downie and Gullan (2004) explained the possible presence of an intron for all taxa, corresponding to paralogous copies (homologous sequences separated by a duplication event) for the EF-1α gene, which has also been very divergent (25%) in the study of mealybugs and according to Danforth and Lin (2004) can cause problems in the phylogenetic analysis, avoiding proper clustering of the sequences of the study. Even Danforth *et al.* (2005), mention that problems can arise from multiple paralogous copies. In the case of mitochondrial gene, it is inherited through the maternal line, therefore without genetic recombination with the father, potential paralogous genes problems and heterozygosity are avoided (Lin and Danforth, 2004). Despite this, Malausa *et al.* (2011), indicate that there are limitations in the study of molecular markers of mitochondrial type, especially for species of *Pseudococcus*. Danforth and Lin (2004) and Kondo *et al.* (2008), explain that this genomic region has sites that are evolving at a very fast rate and tend to saturate more quickly, resulting in a small but significant variation in DNA mitochondrial sequences between species. This variation leads to the formation of homoplasy levels, creating erroneous interpretations, because of false similarities, for the reason that results of phylogenetic analysis evolutionarily seem closer than they really are (Lin and Danforth, 2004; Rung *et al.,* 2008). Molecular characteristics using multiple genes, as described in this research, have identified genetic variations. Downie and Gullan (2004) explain that the molecular data and morphological characters presented discordance by factors such as homoplasy. It is; therefore, likely some mealybugs classified as a particular species according to some morphological characters, have been reported mistakenly by another name, as in the case of the mealybug *P. elisae*, which is not recorded in the GenBank and is the major pest reported in banana plantation. This species is characterized by a body type like elongated oval. It has 17 pairs of cerarii, the presents in the head (frontal cerarii o C17) show three conical setae and the anal lobe cerarii two conical setae. It has two oral rim tubular ducts in the head region between two antennae, adjacent to C17; these are also present in the tergal region, are usually recognized until 13 of these ducts and are absent in segment VII of sternal abdomen. This last characteristic separated *P. elisae* from *P. jackbeardsleyi*. The mouthpart shows three stylets. Along the posterior border of the eyes are numerous discoidal pores, about nine are present. In the antennae, eight segments are accounted. The posterior pair of legs have translucent pores usually restricted to the femur and tibia. It has a circulus between segments III and IV. This mealybug lacks the anal bar in the anal lobe. It also has two pairs of ostioles with trilocular pores inside (Williams and Granara de Willink, 1992; Gimpel andMiller, 1996; Miller *et al*., 2007). Species obtained in the molecular results as *P. viburni* and *P. maritimus*, have been confused with each other. Among the most important characteristics, *P. viburni* has one to three discoidal pores associated with the eye area, while *P. maritimus* has at least one and not more than three of these pores, both species lack a sclerotic rim in the eye area; these differences contrast with *P. elisae*, in which seven to nine dicoidal pores circling the eyes were seen in this study. In addition, *P. viburni* has oral rim tubular ducts between the antennae and the frontal coxa, while *P. maritimus* has an oral rim tubular duct between 15 and 16 cerarii (Gimpel and Miller, 1996; Miller *et al.,* 2007). Another species resulted from molecular data is *P. longispinus*. Among the main characteristics, there is the absence of discoidal pores in the eye area. The penultimate cerarii and the anal lobe cerarii have an obvious sclerotic base. There have been identified abundant oral rim tubular ducts adjacent to the cerarii of the margin of the body. This characteristic is shared with *P. microadonidum*, species also obtained in the molecular data (Miller *et al.,* 2007). These features are absent in the mealybugs described morphologically as *P. elisae*. Regarding the most representative characteristics of the genus and species *D. neobrevipes* and *D. brevipes*, species obtained in the molecular results, they are having a robust body, the oral rim tubular ducts structures are absent, also are reported two to three discoidal pores around the eyes and in the case of *D. neobrevipes*, it presents the penultimate cerarii of the anal lobe with more than two conical setae (Williams and Granara de Willink, 1992; Miller *et al*., 2007). In the case of *D. texensis*, it lacks of discoidal pores around the eyes, has an elongated and sclerotic area in the annals lobes and is characterized by robust legs (Williams and Granara de Willink, 1992). These features are not present in the morphological analysis of this research.

Among the distinctive features of the *H. pungens*, species of mealybug obtained from molecular data, it has a round body shape, three circuli in the sternal abdomen and absence of trilocular pores (Miller *et al.*, 2007). Features absents of mealybugs in this study. Based on our analysis, the identified populations are fairly stable morphologically, but genetically there is probably greater selection pressure that caused genes variation. In addition, we observed little variation between in individuals from the pseudostem of banana plants (Table 1). Moreover, mealybugs from the grass that are in the vicinity of plantations often have the same genetic sequences as those found in different parts of the plant, this according to the 18S and EF-1α genes. This might suggest that these species are easily adapted to both habitat and weeds, which could serve as an alternate host of the insect.

While *P. elisae* has been associated as a pest of banana crop (SFE, 2015), this species is known for presenting few plants as hosts (Gimpel and Miller, 1996; Ben-Dov, 2015). In Espirito Santo, Brazil; Culik *et al*. (2006) collected a specimen of *P. elisae* Borchsenius in the inflorescence of coffee crop (*C. canephora*) and an additional sample of this species in a weed in another unidentified collection site. Gratereaux (2009) explained that mealybugs of the genus *Dysmicoccus* sp. are polyphagous, and those in pineapple plantations survive on crop stubble or alternate hosts around of the crop and weed plants that may be favor the presence of the pest and allow the continuity of their reproductive cycle. In conclusion, the results of this study suggest that different groups of molecular markers studied (ribosomal nuclear and mitochondrial) could be used for better understanding of genetics population studies along with the identification of morphological characters of mealybugs.

Acknowledgements

We are grateful to César Guillén, entomologist of CORBANA, for providing the mealybugs collection used in this study. We thank Carlos Sanabria from SFE, by the corroboration of morphological characterization. We also thank all CIEMic staff, especially to Alcides Sánchez for his guidance during the project execution and the Molecular Phytophatology Laboratory for the equipment availability. We also want to thank Gregory Evans, by the document review. This research, , was supported by the Vice-Rectorate of Research at the Universidad de Costa Rica by funding the project code: 813-B1-273 and scholarship code: 810-B2-232.

Conflict of Interest

We declare do not have conflict of interest.

REFERENCES

Ashfaq, M., Ara, J., Noor, A.R., Hebert, P.D.N. and S. Mansoor. 2011 Molecular phylogenetic analysis of a scale insect (*Drosicha mangiferae*; Hemiptera: Monophlebidae) infesting mango orchards in Pakistan. *European Journal of Entomology,* 108: 553–559.

Avise, J.C. 2004. Molecular markers, natural history and evolution, 2nd ed. by Sinauer Associates, Sunderland, Massachusetts. pp. 684.

Beardsley, J.W. 1986. Taxonomic notes on *Pseudococcus elisae* Borchsenius, a mealybug new to the Hawaiian fauna (Homoptera: Pseudococcidae). *Proceedings of the Hawaiian Entomological Society*, 26: 31-34.

Beltrà, A., Soto, A. and Malausa, T. 2012. Molecular and morphological characterization of Pseudococcidae surveyed on crops and ornamental plants in Spain. *Bulletin of Entomology Research,* 102: 165-72.

Ben-Dov, Y. 2005. ScaleNet, *Pseudococcidae elisae* catalogue. (http://www.sel.barc.usda.gov/catalogs/pseudoco/Pseudococcuselisae.htm) (accessed on 1 August 2015).

Cavalieri, V., Mazzeo, G., Tropea, G.G., Buonocore, E. and Russo, A. 2008. Identification of *Planococcus ficus* and *Planococcus citri* (Hemiptera: Pseudococcidae) by PCR-RFLP of COXI gene. *Zootaxa*. 1816: 65–68.

Correa, M., Aguirre, C., Germain, J.F., Hinrichsen, P., Zaviezo, T., Malausa, T. and E. Prado. 2011. A new species of *Pseudococcus* (Hemiptera: Pseudococcidae) belonging to the "*Pseudococcus maritimus*" complex from Chile: molecular and morphological description. *Zootaxa*. 2926: 46–54.

CentralAmericaData. 2014. Información de mercados en Centroamérica: Banano costarricense pierde terreno. [in Costa Rica] (http://www.centralamericadata.com/es/article/home/Banano_costarricense_pierde_terreno) (accessed on 7 January 2016).

CentralAmericaData. 2015. Información de mercados en Centroamérica: Monitoreo agrícola regional. [in Costa Rica] (http://www.centralamericadata.com/es/article/home/Monitoreo_agrcola_regional_Exportacin_de_banano) (accessed on 7 January 2016).

Culik, M.P., Dos Santos Martins, D. and Gullan, P.J. 2006. First records of two mealybug species in Brazil and new potential pests of papaya and coffee. *Journal of Insect Science*, 6: 1-6.

Danforth, B.N., Lin, C.P. and Fang, J. 2005. How do insect nuclear ribosomal genes compare to protein-coding genes in phylogenetic utility and nucleotide substitution patterns?. *Systematic Entomology*, 30: 549–562.

Djernaes, M. and J. Damgaard, J., 2006. Exon-intron structure, paralogy and sequenced región of elongation factor-1 alphain hexapoda. *Arthropod Systematics and phylogeny,* 64(1): 45-52.

Downie, D.A. and Gullan, P.J. 2004. Phylogenetic analysis of mealybugs (Hemiptera: Cocoidea: Pseudococcidae) based on DNA sequences from three nuclear genes, and a review of the higher classification. *Systematic Entomology,* 29: 238–259.

Gimpel, W.F. and Miller, D.R. 1996. Entomology International: Systematic analysis of the mealybugs in the *Pseudococcus maritimus* complex (Homoptera: Pseudococcidae). vol. 2. by Associated Publishers, Gainesville, Florida, USA, pp. 1-164.

Gullan, P.J., Downie, D.A. and Steffan, S.A. 2003. A new pest species of the mealybug genus Ferrisia Fullaway (Hemiptera: Pseudococcidae) from the USA. *Annals of the Entomological Society of America*, 96: 723-737.

Gratereaux, B.W.V. 2009. Potencial del uso de hongos entomopatógenos para el control de cochinilla (*Dysmicoccus brevipes*) en producción orgánica de piña (*Ananas comosus*). M.S. thesis CATIE, Turrialba, Costa Rica. pp. 62-68.

Hall, T.A. 2010. BioEdit, version 7.2.5th ed. for Windows 95/98/NT. Carlsbad, California.

Hardy, N.B., Gullan, P.J. and Hodgson, C.J. 2008. A subfamily-level classification of mealybugs (Hemiptera: Pseudococcidae) based on integrated molecular and morphological data. *Systematic Entomology*, 33: 51-71.

Kazachkova, N., Maijer, J. and Ekbom, B. 2007. Genetic diversity in pollen beteles (*Meligethes aeneus*) in Sweden: role of spatial, temporal and insecticide factors. *Agricultural and forest Entomology,* 9: 259-269.

Kondo, T., Gullan, P.J. and Williams, D.J. 2008. Coccidology. The study of scale insects (Hemiptera: Sternorrhyncha: Coccoidea). *Revista Corpoica – Ciencia y Tecnología Agropecuaria*, 9: 55-61.

Lin, C.P. and Danforth, B.N. 2004. How do insect nuclear and mitochondrial gene substitution patterns differ?. *Molecular Phylogenetics and Evolution*, 30: 686–702.

Malausa, T., Fenis, A., Warot, S., Germain, J.F., Ris, N., Prado, E., Botton, M., Vanlerberghe-Masutti, F., Sforza, R., Cruaud, C., Couloux, A. and Kreiter, P. 2011. DNA markers to disentangle complexes of cryptic taxa in mealybugs (Hemiptera: Pseudococcidae). *Journal of Applied Entomology,* 135: 142-155.

Miller, D.R., Rung, A. and Parikh, G. 2014. Scale Insects, edition 2, a tool for the identification of potential pest scales at U.S.A. ports-of-entry (Hemiptera, Sternorrhyncha, Coccoidea*). ZooKeys,* 431: 61–78.

Miller, D.R., Rung, A., Venable, G.L. and Gill, R.J. 2007. ScaleNet. Scale Insects: Identification tools, images, and diagnostic information for species of quarantine

significance, Systematic Entomology Laboratory, USDA-ARS. [in USA] (http://www.sel.barc.usda.gov/scalekeys/ScaleInsectsHome/ScaleInsectsHome.html) (accessed on 7 October 2007).

Murray, M.G., and Thompson, W.F.. 1985. Rapid isolation of high molecular weight plant DNA. Nucleic Acids *Research,* 8: 4321-4325.

(NCBI) National Center for Biotechnology Information. 2015. Rockville Pike, Bethesda, USA. [in USA] (http://www.ncbi.nlm.nih.gov) (accessed on 1 July 2015).

Pacheco da Silva, V.C., Bertin, A., Blin, A., Germain, J.F., Bernardi, D., Rignol, G., Botton, M. and Malausa, T. 2014. Molecular and Morphological Identification of Mealybug Species (Hemiptera: Pseudococcidae) in Brazilian Vineyards. *PLoS ONE* 9: e103267.

(PROCOMER) Promotora del Comercio Exterior Costa Rica. 2015. Prevén aumento de consumo de banano en Polonia. [in Costa Rica] (http://www.procomer.com/es/noticias#prevn-aumento-de-consumo-de-banano-en-polonia) (accessed on 7 January 2016).

Ramos, P.A. and Serna, C.F. 2004. Coccoidea de Colombia, con énfasis en las cochinillas harinosas (Hemiptera: Pseudococcidae) [Coccoidea of Colombia, with an emphasis on mealybugs (Hemiptera: Pseudococcidae)]. *Revista Facultad Nacional de Agronomía de Medellín,* 57: 2383-2412.

Rodríguez, V.A. 2013. El financiero: Costa Rica declara emergencia fitosanitaria por plagas que afectan al banano. [in Costa Rica] (http://www.elfinancierocr.com/economia-y-politica/Costa-Rica-emergencia-fitosanitaria-afectan_0_425357478.html) (accessed on 10 December 2013).

Rung, A., Scheffer, S.J., Evans, G. and Miller, D. 2008. Molecular Identification of Two Closely Related Species of Mealybugs of the Genus *Planococcus* (Homoptera: Pseudococcidae). *Annals of the Entomological Society of America,* 101: 525-532.

Sanger, F., Nicklen, S. and Coulson, A.R. 1997. DNA sequencing with chain-terminating inhibitors. *Proceedings of the national academy of sciences of U.S.A*., 74: 5463-5467.

Servicio Fitosanitario del Estado (SFE). 2015. Guía Técnica para el monitoreo de *Pseudococcus elisae* y *Diaspis boisdulli* en *Musa* AAA. Departamento de Operaciones Regionales, Ministerio de Agricultura y Ganadería, Costa Rica [in Costa Rica]. (https://www.sfe.go.cr/intranet/documentos/planes_de_accion/Guia_Tecnica_Cochinilla_en_banano.pdf) (accessed on 1 October 2015).

Tamura, K., Dudley, J., Nei, M. and Kumar, S. 2007. MEGA, version 4.0th ed. for Windows. Center for Evolutionary Functional Genomics Arizona,

Thompson, J.D., Higgins, D.G. and Gibson, T.J.. 1994. Clustal W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. *Nucleic Acids Research,* 22: 4673-4680.

Williams, D.J. and M. Granara de Willink. 1992. Mealybugs of Central and South America. *CAB International, Wallingford*, UK, pp. 635.

Wu, S.A., Nan, N., Gullan, P. and Deng, J. 2013. The taxonomy of the Japanese oak red scale insect, *Kuwania quercus* (Kuwana) (Hemiptera: Coccoidea: Kuwaniidae), with a generic diagnosis, a key to species and description of a new species from California. *Zootaxa,* 3630: 291–307.

****