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Morphological and molecular identification of *Dysmicoccus brevipes* (Hemiptera: Pseudococcidae) in Costa Rica

Melissa Palma-Jiménez and Mónica Blanco-Meneses

Abstract
The aim of the present study was to identify the species of mealybug from Rebusca Farm in Sarapiquí, Costa Rica. Light microscopy and molecular analysis were done. The microscopy analysis was performed at the Center for Research on Microscopic Structures, and the molecular analysis was carried out at the Molecular Phytopathology Laboratory, at the University of Costa Rica. After analyze the insects, was possible to associate it to the species *Dysmicoccus brevipes* Cockerell, due to: the morphology of the body, mouthparts, antennae segments, presence of translucent pores, description of hind legs, ostioles, circulus and cerarii. In the molecular data, BLAST hits from GenBank revealed similarities of between 92-100% to the species *D. brevipes* to the studied genes (18S, E.F-1α and COXI). Until the date, this species had been associated to pineapple crop. This study evidence the risk of the pest to be disseminated and to search other hosts for food.

Keywords: Central America, *Musa* sp., taxonomy, nuclear gene, ribosomal gene, mitochondrial gene

1. Introduction
Mealybugs are small, soft-bodies insects that are covered with a layer of fine mealy wax, which often extends laterally to form a series of short filaments [1]. They belong to the family Pseudococcidae, one of about 20 families in the scale insect superfamily Coccoidea. Three of the largest genus of mealybugs: *Pseudococcus* Westwood; *Trionymus* Westwoodia and *Dysmicoccus* Williams, belong here [2].
Mealybug *Dysmicoccus* sp. is considered a major threat to agricultural production in general [3]. This affects the aesthetic value of the plant, to causing wilting, product of transmission of pathogens and/or toxins during feeding. It could also become a serious problem due to its mode of dispersal and the prevailing ecological conditions [4].
According to Carter (1932) [5], the mealybug species *Dysmicoccus brevipes* Cockerell, previously referred as *Pseudococcus brevipes*, is originated from tropical America, and it has spread to all zoogeographical regions, mainly in the tropics and subtropics [6]. It is probably one of the most common mealybugs in Central and South America [7].
There are a number of species in the *Dysmicoccus* genus, but *D. brevipes* is the most similar to *D. neobrevipes* Beardsley, native to tropical America and has a pantropical distribution, with a small number of records from subtropical or Mediterranean localities [8]. It is known to have been introduced to China, Japan, Sri Lanka and Lithuania [9]. Beardsley (1992) [10] collected some samples in Hawaii and therefore, it was able to define them as two separate species, a pink form (*D. brevipes*) and a grey form (*D. neobrevipes*).
The distribution of these mealybugs in tropical countries has been studied. In Costa Rica this kind of mealybug is present in a wide range of host plants, including: pineapple, coffee, sugar cane, rice, palms, plantain and banana [1].
Particularly, in banana plantations, *Dysmicoccus* sp. has been reported in several producing countries. Kondo *et al*. (2008) [11] identified *Dysmicoccus* (*neobrevipes, brevipes* and *texensis* Tinsley, in plantations of Colombia. Meanwhile Blanco *et al.* (2002) [12] identified *Dysmicoccus* (*alazon* Williams; *bispinosus* Beardsley and *brevipes*) in Cuba. Williams and Matile-Ferrero [13] reported *Dysmicoccus* (*brevipes* and *grassii* Leonardi) in banana plantations in Ethiopia. Navasero [14] reports in the Philippines *D. neobrevipes* and *D. brevipes* Beardsley. Fernández *et al.* (2001) [15] identified *D. grassi* in Las Palmas, Canary Islands. In Mindanao, Philippines identified the species *D. neobrevipes* [16].

In Sri Lanka comercial banana plantations were found to be infested and small-scale home gardens to the mealybug *D. neobrevipes* [4]. Sirisena *et al*. (2013) [17], mention that *D. neobrevipes* is a recent accidental introduction Sri Lanka and its infestation level on banana is ranked as high.

D. neobrevipes and *D. brevipes* could be confused each other because of the similar external morphology. Characteristics such as: discoidal pores near the eyes, ventral multilocular pores restricted to segments VI, VII and VIII, translucent pores on hind femur and tibia, and oral rim tubular ducts absence, are shared [1].

In *D. neobrevipes* the ventral sclerotization of the anal lobes is elongate, whereas in *D. brevipes* these areas are quadrate. Beardsley (1992) [10] also noted that *D. brevipes* has several long setae on either side of the mid-dorsal axis of the VIII abdominal segment, which ranged as 45-80 µm long, whereas in *D. neobrevipes* the longest setae in this region are about 15 µm long, no longer than the other dorsal setae [9].

The authors Yan-Biao *et al*. (2014) [18] explain that the ventral sclerotization of the anal lobes and the length of the setae on the dorsum of the abdomen, are the primary characteristics used to differentiate *D. neobrevipes* of *D. brevipes*. However, it is not always easy to identify. Kondo *et al*. (2008) [11] mention the importance of scale insect specialists.

The correct identification and classification of these insects, is the first step in developing control methods [19]. That is why the use of DNA markers has become the most common strategy to measure differences between species of the same genus and between populations. Among the molecular markers used in the study of insects, it was mentioned the ribosomal, nuclear and mitochondrial genes [20]. It is important to have information on the regional fauna in order to compare and differentiate the presence or absence of species of mealybugs [21].

Relationships among molecular lineages in species of *D. brevipes* reveal their own biogeographic patterns. Comparing molecular sequences of *D. brevipes*, it is suggested that these species inside *Dysmicoccus* genus may have a complex, which includes cryptic lineages [18].

The aim of present research was to study the morphology and perform a molecular analysis of the mealybug *Dysmicoccus brevipes* of banana crop (*Musa* sp.) from Rebusca farm in Sarapiquí, Costa Rica, Central America.

2. Materials and Methods

2.1 Sample collection

Female mealybugs from *Musa* sp. ground in Rebusca farm, Sarapiquí, Heredia province close to the Caribbean region from Costa Rica, Central America were collected in 2010. Twenty mealybugs in 1.5 mL Eppendorf tube with 95% ethanol were collected. The coordinates were: latitude 10° 29'00.00"N and longitude 84°01'00.00"O. 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 Phytopathology Laboratory at the Center for Research in Crop Protection CIPROC (acronyms in spanish) of Costa Rica, ending in 2014, both at the University of Costa Rica, San Pedro, Montes de Oca.

2.2 Observation under the light microscope

Ten insects were processed. The protocol described by Williams and Granara de Willink (1992) [1] was used.

To identify the translucent structures, the insects were examined with light microscopy equipment, using increases 4x, 10x, 20x and 40x and photographed with the inverted microscope (model IX51, Olympus Optical Co., Japan).

The analyzed structures by light microscopy corresponded to the following: body shape, number of segments of the antenna, translucent pores around the eyes, mouthparts and stylets, description of metacoxas (posterior legs) and presence of translucent pores, description of the circulus, ostioles, oral rim tubular ducts, anal lobe and cerarii.

2.3 Amplification of genomic DNA

The protocol by Murray and Thompson (1985) [22] was used. One insect was used for each DNA extraction. The genomic DNA extracted was amplified by PCR. Initially, five pairs of primers were used to observe which ones had polymorphism of interest in a sub-sample of DNA ribosomal, nuclear, and mitochondrial of mealybugs. At the end of testing, three pairs of these primers were selected (Table 1).

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

Gene	Primers	Primer sequence	PCR conditions	Amplicon 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	673	[20, 23]
E.F-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	374	[23]
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	370	[20]

For all PCR reactions a 1x (ul) solution it 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).

2.4 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 [24]. To determine the species according to the result of sequencing, the GenBank previous reports were used [25]. All sequences were aligned with the ClustalW program version 1.60 [26].

To corroborate the variation that might exist within populations from Costa Rica, sequencing was repeated on three different individuals, except the mitochondrial gene, and compared with the highest matches in GenBank. The result data were reported to GenBank and sequences were deposited under accession numbers: KP402186, KP402187 y KP402192.

For the phylogenetic analysis, sequences were included from species previously reported by GenBank for all three genes studied, such as: *Dysmicoccus brevipes*, *D. neobrevipes*, *D. texensis*, *Pseudococcus viburni* Signoret; and outgroups were: *Planococcus minor* Maskell, and *Phenacoccus baccharidis* Williams. The individual origin was verified according to the host plant and the countries: USA, Brazil, China, South Africa, India and France, which correspond to the only reports so far with better relationship according to the sequences of the study (Table 2). The analysis of phylogenetic trees was performed using the program MEGA version 7.0 (Molecular Evolutionary Genetic Analysis) [27]. The evolutionary history was inferred by using the Maximum Likelihood method (ML) based on the Tamura-Nei model. The percentage of the trees, in which the associated taxa clustered together, is shown next to the branches (random parameter of 2000 replications). 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, and then selecting the topology with superior log likelihood value. All positions containing gaps and missing data were eliminated.

Table 2: Mealybugs used for the phylogenetic trees construction: Species, host plant, origin country and GenBank accession number.

Species	Host Plant	Origin Country	GenBank accession number		
			18S ribosomal	E.F-1α	COXI
Dysmicoccus brevipes	*	USA	AY426037	-	-
D. brevipes	*	China	JF965399	-	-
D. brevipes	*	Bolivia	AY426046.1	-	-
D. brevipes	*Ananas comosus*	Malaysia	KU891790.1	-	-
Dysmicoccus neobrevipes	*	China	JF965400.1	-	-
D. neobrevipes	*	USA	U20429.1	-	-
Pseudococcus elisae	*Musa* sp.	Costa Rica	KX639737.1	-	-
P. jackbeardsleyi	*Musa* sp.	Costa Rica	KT956119.1	-	-
*Planococcus minor***	*	China	JF965404.1	-	-
Dysmicoccus sp.	*Lechea sessiliflora*	USA	-	AY427240.1	-
D. brevipes	*	USA: Hawaii	-	AY427227.1	-
D. neobrevipes	*	USA: Hawaii		AY427208.1	
Pseudococcus elisae	*Musa* sp.	Costa Rica	-	KP402191.1	-
P. jackbeardsleyi	*Musa* sp.	Costa Rica	-	KT956120.1	-
*Planococcus minor***	*	Trinidad and Tobago	-	EU250499.1	-
D.brevipes	*	USA	-	-	EU267214.1
D.brevipes	*	Japan	-	-	LC121502.1
D.neobrevipes	*	USA	-	-	EU267213.1
D.neobrevipes	*	Japan	-	-	LC121499.1
D.neobrevipes	*	China	-	-	KJ187532.1
*Phenacoccus baccharidis***	*	Brazil	-	-	KJ530632.1

Species no reported (*). Outgroup (**).

3. Results

3.1 Morphological characterization

These results correspond to the anatomy of the studied mealybugs described through light microscopy technique.

Body: Shape of the body corresponds to a width oval type (2.2 x 2 mm) (Fig. 1). Body setae have the same size.

Oral rim tubular ducts: Absents. There are exemplified by the head region of mealybug, which is where these structures are observed in other genus with similar characteristics as *Pseudococcus* (Fig. 1B).

Mouthparts: Three stylets were observed (Fig. 1 C).

Translucent pores in metacoxas: Located on two sections of the metacoxas: femur and tibia with numerous translucent pores (Fig. 1. D).

Antennae: Eight segments were observed (Fig. 1 E).

Discoidal pores at the edge of the eye: It has three pores in the eye edge associated with a sclerotic rim (Fig. 1 F).

Circulus: Divided by a line between segments III and IV (Fig. 1 G).

Anal lobe bar: It was absent (Fig. 1. H).

Ostioles: The typical structure of the ostioles was observed which is lip-shaped and has triloculars pores inside (Fig. 1 H).

Cerarii: 17 pairs of cerarii were quantified. Was noted a pattern of flagellated setae surrounded the conical setae. The presence of a mild sclerotic base in the annals lobes cerarii was presented. Furthermore, it was observed that the base of anal lobe cerarii provided two conical setae (Fig. 1 H) and the anteriorly on the abdomen four conical setae were observed with a circular base (Fig. 1 I).

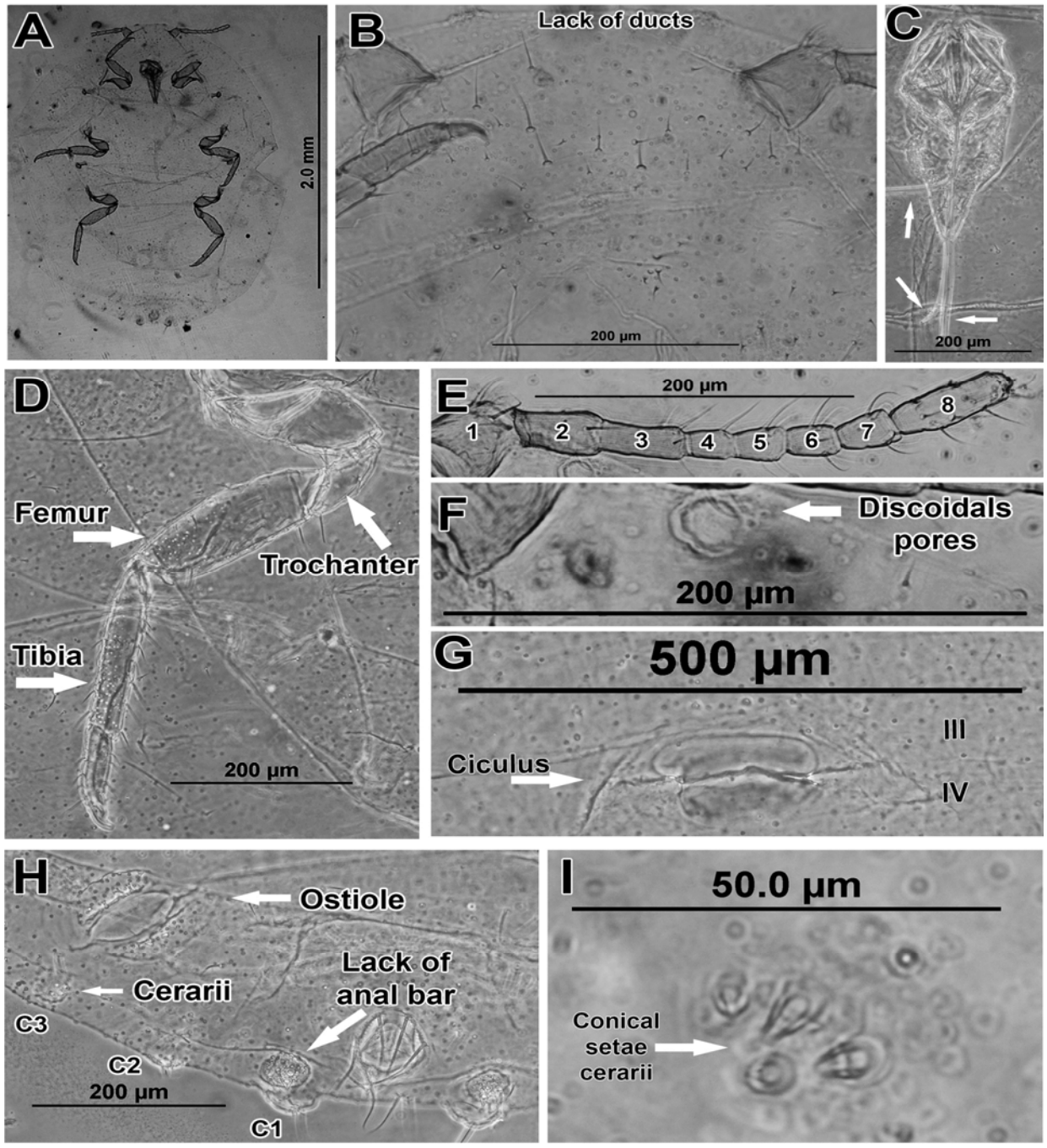

Fig 1: Morphological characters analyzed to identify the *Dysmicoccus brevipes* mealybug from banana crop from Rebusca farm in Costa Rica by light microscopy. Mealybug collected in 2010. **A.** Body of the insect: width oval type with 17 pairs of cerarii. **B.** Head region. **C.** Mouthparts with three stylets. **D.** Presence of translucent pores on femur and tibia of the metacoxa, translucent pores not on trochanter. **E.** Antennae with eight segments. **F.** Three translucent pores in the eye edge associated with a sclerotic rim. **G.** Dividing line circulus in the segments III and IV. **H.** Posterior area of abdomen which indicates the ostiole, cerarii C1-C3 and absence of anal bar (C1). **I.** Cerarii C2 and C3 with four conical setae.

3.2 Molecular description

All specimens were morphologically identified as *Dysmicoccus brevipes*. We obtained BLAST hits from GenBank sequences; to 18S gene revealed similarities of 100% to *D. brevipes* and 99% with *D. neobrevipes*; to E.F-1α gene the hits with similarities of 95% were to *D. brevipes* and 93% with *D. neobrevipes*; for COXI hits with similarities of 95% were to *D. neobrevipes* and 92% to *D. brevipes*.

On a maximum likelihood phylogenetic tree, the genomic region 18S ribosomal showed a clade with the taxa *D. brevipes* CR 1, 2 and 3 which were linked with the following species: *D. brevipes* (AY426037.1) from USA, Bolivia (AY426046.1), China (JF965399.1) and Malaysia (KU891790.1) to a bootstrap of 89%. Other clade was formed by the most similar species related to *D. brevipes* in our study: *Pseudococcus elisae* Borchsenius from Costa Rica (KX639737.1), *D. neobrevipes* from USA (U20429.1) and China (JF965400.1), and *Pseudococcus jackbeardsleyi* from Costa Rica (KT956119.1); which were grouped sharing a bootstrap of 60%. *Planococcus minor* (JF965404.1) was used as outgroup (Fig. 2).

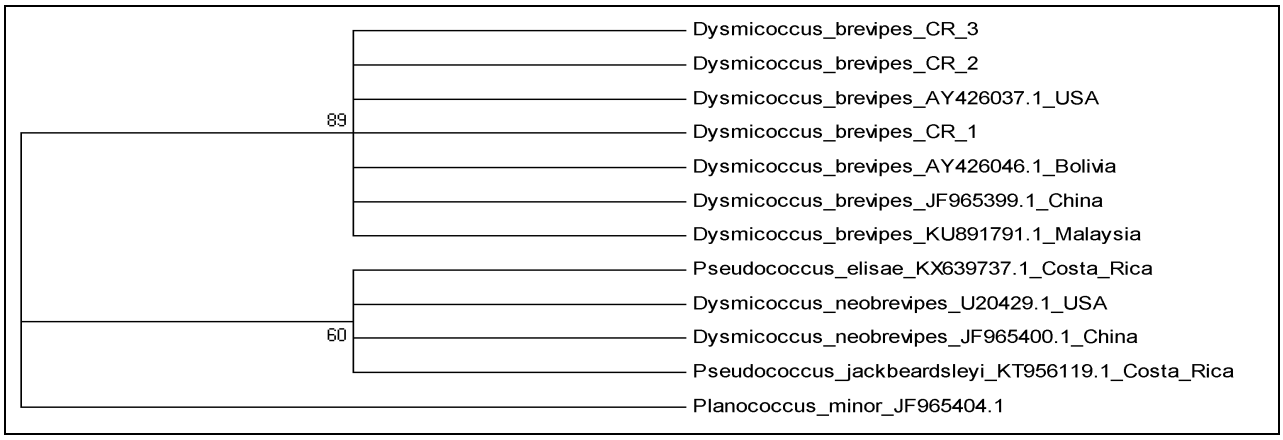

Fig 2: Molecular Phylogenetic analysis by Maximum Likelihood method, calculated from the number of differences between genomic region 18S ribosomal. The percentage of the tree in which the associated taxa clustered together is shown next to the branches (Bootstrap values of 2000 replications). *Planococcus minor* (JF965404.1) was used as outgroup.

Fig. 3 showed the maximum likelihood phylogenetic tree to the genetic region of Elongation Factor-1α. Clade of 61% bootstrap was showed. It had a groping formed by *D. brevipes* CR 1, which shared a relationship of 96% bootstrap to the species *P. elisae* (KP402191.1) and *P. jackbeardsleyi* (KT956120.1), both from Costa Rica. *Dysmicoccus* sp. from USA (AY427240.1) and *D. brevipes* CR 2 was part of the 61% bootstrap clade. A relationship of 98% to *D. brevipes* CR 3 and *D. brevipes* from USA (AY427227.1) was showed. *D. neobrevipes* (AY427208.1) from USA did not have relationship in the phylogenetic tree. *Planococcus minor* (EU250499) was used as outgroup.

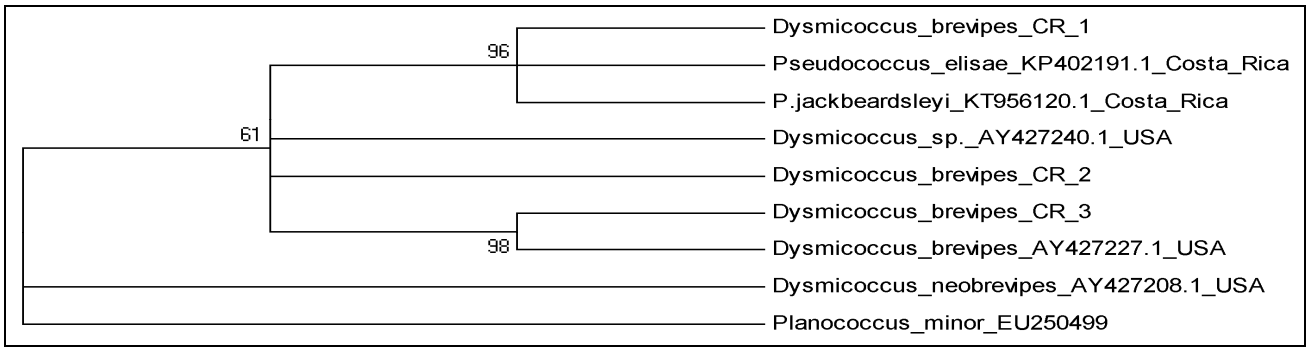

Fig 3: Molecular Phylogenetic analysis by Maximum Likelihood method, calculated from the number of differences between genomic region Elongation Factor-1α. The percentage of the tree in which the associated taxa clustered together is shown next to the branches (Bootstrap values of 2000 replications). *Planococcus minor* (EU250499) was used as outgroup.

The maximum likelihood phylogenetic tree for mitochondrial genomic region COXI determined a bootstrap of 58% to the species *D. brevipes* CR, related to *D. neobrevipes* from USA (EU267214.1), Japan (LC121499.1) and China (KJ187532.1). These species shared a relationship of 99% to *D. brevipes* from Japan (LC121502.1). *Phenacoccus baccharidis* (KJ530632.1) was used as outgroup (Fig. 4).

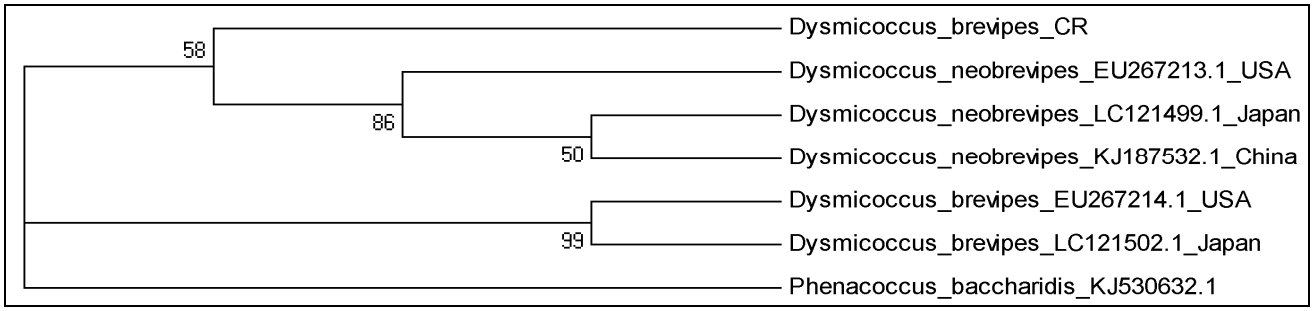

Fig 4: Molecular Phylogenetic analysis by Maximum Likelihood method, calculated from the number of differences between genomic region mitochondrial (COIX). The percentage of the tree in which the associated taxa clustered together is shown next to the branches (Bootstrap values of 2000 replications). *Phenacoccus baccharidis* (KJ530632.1) was used as outgroup.

4. Discussion

The morphological analysis allowed identifies *Dysmicoccus brevipes* as specimen of the study. A close relationship was established according to the description of the species *D. neobrevipes* in the literature [28]. Comparing the molecular data registered on GenBank (2016) [25], different bootstrap values were showed in the three phylogenetic trees of the studied genes. The phylogeny associated the species *D.*

brevipes from Costa Rica to the specie *D. brevipes* from others geographic regions and less similarity showed to be associated to the species *D. neobrevipes* from other countries. Only mitochondrial gene (COXI) showed species of *D. brevipes* not directly related with the species of study.

The 18S ribosomal gene showed 99-100% similarity regarding to the greatest hits from species reported in GenBank. The most related species were: *D. brevipes, D. neobrevipes*, *P. elisae*, *P. jackbeardsleyi, P. viburni, Pseudococcus baliteu*, among others. This proves the low level of gene specificity.

The percentage of Elongation Factor 1α gene indicated a big difference at the genetic level between 89-95% identities to the species reported in GenBank. This percentage represents of 18-39 different basis between the species of the study and those found by Blast tool. Two different haplotypes were identified with variations in the sequence from nucleotide 10 to nucleotide 370 of an average amplicon of 374 bp, indicating that there is variation between the specimens on a single farm of the study. One of the associations was linked to the species *P. elisae* and *P. jackbeardsleyi,* and the other association was matched to species of *D. brevipes*. Both shared the same phylogenetic group with a bootstrap of 61%.

The results obtained from the COXI gene showed values of identity between 91-96% (up to 16 different bases) regarding to the species associated in the GenBank. Within the highest hits, the species *D. brevipes* was not reported as it was presented in the other two genes (ribosomal and nuclear). In this case, the species with higher percentages of identity were *D. neobrevipes* and less associated *D. brevipes.*

The DNA information exposed in the phylogenetic trees, suggested no variation among specimens from Costa Rica in 18S gene, but a little variation in EF-1a and also in the COI data was showed. The results do not mean high phylogenetic differentiation among Costa Rican samples. However, compared to other countries, it suggests a population with characteristics inherent from this geographical area according to the difference at the genetic identity.

Mitochondrial lineages of mealybug *Dysmicoccus* sp. from China revealed previously unknown biogeographic patterns [29]. The researchers found different haplotypes, one in Mainland China, two on the island of Hainan, while that mealybugs collected in the provinces of Yunnan, Guangxi and Fujian shared the same haplotype, suggesting a limited genetic variation of the mealybugs of these other locations. The authors explain that *Dysmicoccus* sp. could be a species complex, which includes at least two cryptic lineages, sister species or may be some complex taxa. Beardsley (1965) [30] featured a complex of mealybugs of *Dysmicoccus* with similar morphological characteristics within closely related species: *D. neobrevipes, D. brevipes* and *D. texensis.*

Species of *Pseudococcus* and *Dysmicoccus*, shared high phylogenetic relationship. This is explicable invoking homoplasy in this divergent sequence [23]. That explain why we had strong relationship of *D. brevipes* from Costa Rica to species *P. elisae* and *P. jackbeardsleyi*. Downie and Gullan (2004) [23] explain that molecular characters of mealybugs are affected by homoplasy, and none of the single gene analyzed could to a set of strong supported relationships in mealybugs phylogeny. Also Palma-Jiménez and Blanco-Meneses (2016) [31] mention that homoplasy create erroneous interpretations in the phylogenetic analysis.

The geographical environment is an important factor that involves the clime of the region; also the host plant is determinant to understand the effect in the molecular relationship as the found in the molecular result. The 18S and Elongation Factor genes associated *D. brevipes* CR to *P. elisae* and *P. jackbeardsleyi,* both from Costa Rica and from *Musa* sp host.

According to Williams and Granara de Willink (1992) [1], one of the most important morphological differences is the absence of oral rim tubular ducts in *Dysmicoccus* sp. Also, Granara de Willink (2009) [32] explains that the morphological differences between species of this genus, as: *D. neobrevipes* and *D. brevipes* are minimal and in some cases may be variations of the same species. Also mention that the mealybug *D. neobrevipes* could be confused with the species *D. brevipes* for features such as: 17 pairs of cerarii, anal lobe with two conical setae, two pair of ostiloles, discoidal pores at the edge of the eyes, multilocular pores limited to the segments VI, VII and VIII of the frontal area, translucent pores in the femur and tibia of the metacoxae, and absence of oral rim tubular ducts. However, *D. neobrevipes* can be distinguished by the absence of longer dorsal setae on the segments VII and VIII and the same way because of the presence of a big circulus. In *D. brevipes* the circulus is subquadrate [6]. Both species have four to seven conical setae on the anal lobe cerarii, *D. neobrevipes* has a sclerotized area of each anal lobe cerarii [33], meanwhile *D. brevipes* has not sclerotized area [1]. Here we saw a mild sclerotized area.

Granara de Willink (2009) [32] mention that the solution to these minor differences, aside from the DNA studies, is that other studies should be done, such as the biological studies to consider the magnitude of the variations. Biao *et al.* (2012) [29], explain that the potential impact for expansion of the mealybug in different agricultural populations worldwide depends on the taxonomy and the biology that shows the different haplotypes, which will require much additional research.

5. Conclusion

In the present research, the study of morphological data determined that the species most closely related correspond to *D. brevipes*. The study supported by molecular data, allowed understanding high degree of genetic variation, identified a population with molecular characteristics inherent from this geographical area. As Downie and Gullan (2004) [23] mention, it is essential to sample many more species of the large and economically important genera, such as *Dysmicoccus*, to test for generic monophyly and species synonymy.

Until the date, the identification of *D. brevipes* in Costa Rica has been associated to pineapple crop [34]. Therefore, this study provided insight into the ability of dissemination of this polyphagous pest, present in Costa Rica in a different uncommon crop. Therefore, it is likely for the effect of the environment this bug tends to search other hosts for food and propagation as part of its biology. So far, the report of *D. brevipes* in *Musa* sp. has been in countries as: Colombia [35], Cuba [12], Ethiopia [13] and Philippines [14].

The phylogenetic relationships found in mealybug are always open to questions. It should be important to considerate relationships based on adult male morphology, even the geography and plant host also as some author mention [36].

6. Acknowledgments

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