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Molecular Identification of Mealybugs (*Hemiptera*: *Pseudococcidae*) on Cultivated, Ornamental and Wild Host Plants in Swaziland

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Authors' contributions

This work was carried out in collaboration between both authors. Author YA designed the study, performed the statistical analysis, wrote the protocol and wrote the manuscript. Author NM conducted the survey and collected mealybug specimens from cultivated, ornamental and wild host plants. Both authors read and approved the final manuscript.

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ABSTRACT

Mealybugs are considered as the most important insect pests of fruits, vegetables and cotton in Swaziland. However, lack of sound identification and information on the mealybug species attacking these crops in the country represents a major barrier to establish satisfactory pest management strategies. In this study, a DNA barcoding approach was used to identify mealybug species attacking wild hosts, ornamentals and cultivated crops in the country. Molecular identification using fragment of mitochondrial cytochrome oxidase sub-unit I revealed the presence of six mealybug species belonging to four genera. A significant (0.00-0.68%) within species similarity and between species sequence divergence (6.90-16.80%) was observed. Of the species identified *Phenacoccus madeirensis* and *Phenacoccus solenopsis*, were the dominant and were highly polyphagus. *Phenacoccus solenopsis,* was recorded from 12 host plants belonging to six families in three regions, whereas, *P. madeirensis* was recovered from five wild host plants growing in Middleveld

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regions. Other mealybug species collected were *Saccharicoccus sacchari, Planococcus citri, Paracoccus burnerae* and *Phenacoccus solani.* The study has validated the efficacy of sequence diversity in the COI gene for identifying mealybugs. This is the first DNA-based characterization of mealybugs from Swaziland and the findings will help in decision making while considering biological control programs.

Keywords: Cytochrome oxidase; mealybugs; molecular identification; Swaziland.

1. INTRODUCTION

Expansion of agricultural ecosystems coupled with rise of globalization and worldwide trade drives the spread and establishment of many organisms beyond their native ranges [1]. The risk and rate of alien insect pest invasion in Africa has dramatically increased over the past decades and invasive pests continued to disperse in the continent due to movement of infested plant material between countries [2,3]. The spread of introduced mealybug pests such as the cotton mealybug, *Phenacoccus solenopsis* Tinsley [4], the cassava mealybug, *Phenacoccus manihoti* Matile-Ferrero [5] and others [6] are examples of this phenomenon. Nevertheless native and introduced mealybugs in natural and agricultural ecosystems of African countries such as Swaziland have received little attention despite their economic importance in crop production. Consequently, many indigenous and/or introduced invasive mealybugs remain undetected and lack of knowledge on the species diversity and host range of these organisms represents a major barrier to the establishment of adequate pest management strategies.

Accurate identification of species is fundamental to both basic and applied research. Success in classical biological control programs depend critically on accuracy of species discrimination and identification. However, morphology-based identification of many mealybug species is challenging. Mealybugs are morphologically very similar and their identification to species level using standard taxonomic characteristics is cumbersome, difficult, time-consuming and even impossible for immature stages and males, or for adults of very closely related species [7,8]. Contradictory reports have been published about the taxonomic status of closely related mealybug species [9,10]. Moreover, taxonomic keys often exist only for adult females [11,12] which makes assigning species names to specimens often impossible in studies where immature stages make up a large proportion of the sample. Years of experience also may be required before investigators can effectively use the taxonomic keys that are available.

Uses of molecular tools to discriminate insect populations, and insects' adaptation to various stresses are wider in applications [13,14]. In such a way, DNA barcoding based approaches were proved to resolve problems related with morphological identification of mealybugs [15] and can provide valuable information for investigating mealybug associations and interactions with natural enemies [16,17]. DNA-barcoding is generally considered to be reliable, cost-effective and easy molecular identification tool with a wide applicability across animal taxa [18]. As such it could be very useful to routinely identify difficult taxa of economic importance. This statement is particularly valid for mealybug families that comprise large numbers of notorious pest species, whose identification often requires highly specialised taxonomic skills [19,15]. However, the use of DNA barcoding databases is of a considerable advantage only when these databases are large enough to cover the range of intra- and interspecific genetic diversity observed in the field. Unfortunately, this is far from the case in the Pseudococcidae, where a large percentage of the DNA barcodes generated to date are new and absent from international databases [20,21]. In this study, mealybugs infesting crops, ornamentals and wild host plants in the Lubombo, Highveld, Middleveld and Lowveld regions of Swaziland were surveyed and subjected for molecular characterization based on the methods described in Chatzidimitriou et al. [15]. A marker located in the region of the cytochrome oxidase subunit I (COI) mitochondrial gene was used in identification of mealybug species [21,22]. The study also reports cultivated and wild host plants of the identified mealybug species in Swaziland.

2. MATERIALS AND METHODS

2.1 Survey Sites and Survey Methods

Field surveys were conducted from February to March of 2016 and January to April 2017 in crop fields, gardens, roadsides and grasslands bordering crop fields in all the four regions (Highveld, Middleveld, Lowveld and Lubombo) of

Swaziland. The surveys were aimed at collection and identification of mealybug pest and non-pest species in various localities of the country. A total of 63 localities in the four regions of Swaziland were visited to evaluate the distribution and host range of the mealybugs in the country. In all the surveys, geographic coordinates of localities visited were recorded using a GARMIN 12X portable Geographic Positioning System (GPS). At each locality natural habitats and crop fields were monitored for mealybug infestations. Infested host plants were identified in *situ*, and the parts of the plant attacked were recorded. Specimens of mealybugs were collected and placed singly into small glass vials containing absolute alcohol. The vials were sealed and labelled. The labels contained all relevant information about the samples collected.

2.2 Molecular Studies

Representative mealybug specimens of the plants infested in the 63 localities of the four regions included in the surveys were selected for molecular analysis. Tissue samples of the representative mealybug specimens collected in the surveys were sent to Inqaba Biotechnological Industries in Pretoria, South Africa for DNA extraction, Polymerase Chain Reaction (PCR) amplification, and sequencing. Primer pairs C1J2195 TTGATTYTTTGGTCATCCAGAAGT and TL2N3014 TCCAATGCACTAATCTGCCATATTA [23] were used to amplify 840 bp of the mitochondrial COI gene.

DNA sequence chromatograms from Inqaba Biotech were edited and assembled using the Staden package [24]. Individual sequences were then blasted on Barcode of Life Data Systems (BOLD, http://www.boldsystems.org) for species level identification and those that could not be identified using the BOLD system were submitted to the GenBank (http://www.ncbi.nlm.nih.gov). The query sequences were then aligned with accessions from the GenBank of morphologically identified adult female mealybugs that showed closer similarity [15].

All data sets were aligned using ClustalX [25] and manually corrected and trimmed by deleting the flanking regions using BioEdit 5.0.9 sequence alignment editor [26] to achieve a final data set of 32 sequences (21 quirey sequences and 11 Barcode sequences from GenBank) with a 592bp. Basic sequence statistics were calculated using DnaSP [27]. Pairwise distances were calculated using the Kimura-2-parameter model (K2P) [28] and a phylogenetic tree was reconstructed using the neighbour-joining method [29]. Clade support was estimated using 10,000 bootstrap replicates using MEGA 7 [30].

3. RESULTS

3.1 Mealybug Identification

The BOLD Identification System (IDS) returned with species-level identification for only few of the mealybug (Hemiptera: Pseudococcidae) specimens submitted. Five of the 21 sequences submitted to the BOLD system have been matched to *Phenacoccus madeirensis* Green (GenBank accession number JQ085561) sequence with 100% similarity. The remaining sequences were compared with sequences in the GenBank using MegaBlast. Based on the results of the blast in the systems, they were identified as *Phenacoccus solenopsis* Tinsley*, Planococcus citri* (Risso), *Phenacoccus solani* Ferris, *Paracoccus burnerae* (Brain) and *Saccharicoccus sacchari* (Cockerell) (Table 1).

Further validation of the results were done using pairwise genetic divergence and phylogenetic analyses. The optimal Neighbor-Joining tree with the sum of branch length = 0.37765815 is shown in Fig. 1. Six clades each representing a species are apparent (Fig. 1). Uncorrected pairwise sequence distances within clades (species) ranged from 0 to 0.68% (Table 2). Twelve of the twenty one individuals from Swaziland are included in the first clade together with the positively identified sequences of *Phenacoccus solenopsis* from the GenBank.

The uncorrected pairwise distance between the sequences in this clade ranged from 0.00 to 0.17%. In the second clade are included two sequences from this survey and a positively identified sequence of *P. solani.* The with a within-clade sequence divergence in the second clade range from 0.00 to 0.26%. Clade three has a two sequences: one individual from sugarcane in Lowveld of Swaziland and a *Saccharicoccus sacchari* sequence downloaded from the GenBank. The two individuals in this clade share a haplotype with a within-clade sequence divergence of 0.00%; (Table 2). The fourth clade is comprised of a *Paracoccus burnerae* sequence from the GenBank a mealybug sequence from x-mass cactus in the Middleveld. This clade also has a within-clade sequence divergence of 0.00% (Table 2). The fifth clade

Table 1. Mealybug specimens collected in four ecologically unique regions of Swaziland: site codes, host plant, location, co-ordinates and identification.

Code	Host plant	Family	Region	Location	Co-ordinates	Final identification
MHV009	*Solanum mauritianum* (W)	Solanaceae	Highveld	Ngonini	S25°80, E31°04	*Phenacoccus solenopsis* Tinsley
MHV012	*Chromoleana odorata* (W)	Asteraceae	Highveld	Buhleni	S25°92', E31°53'	*Phenacoccus solenopsis* Tinsley
MHV017	*Bougainvillea sp*. (O)	Nytaginaceae	Highveld	Ngololweni	S27°09', E31°42'	*Phenacoccus solenopsis* Tinsley
MHV003	*Abutilon theophrasti* (W)	Malvaceae	Highveld	Ngonini	S25°76', E31°42'	*Phenacoccus solani* Ferris
MHV005	*Bidens pilosa* (W)	Asteraceae	Highveld	Ngonini	S25°79', E31°41'	*Phenacoccus solani* Ferris
MLV024	*Abutilon theophrasti* (W)	Malvaceae	Lowveld	Bhalekane	S26°07', E31°55'	*Phenacoccus solenopsis* Tinsley
MLV025	*Solanum ptychanthum* (W)	Solanaceae	Lowveld	Bhalekane	S26°07', E31°55'	*Phenacoccus solenopsis* Tinsley
MLV034	*Helianthus annus* (C)	Asteraceae	Lowveld	Big Bend	S26°51', E31°56'	*Phenacoccus solenopsis* Tinsley
MLV036	*Gossypium herbaceum* (C)	Malvaceae	Lowveld	Big Bend	S26°51', E31°56'	*Phenacoccus solenopsis* Tinsley
MLV037	*Parthenium hysterophorus*(W)	Asteraceae	Lowveld	Big Bend	S26°51', E31°56'	*Phenacoccus solenopsis* Tinsley
MLV028	*Saccharum officinale* (C)	Poaceae	Lowveld	Vuvulane	S26°13', E31°91'	*Saccharicoccus sacchari* Ckll
MLB004	*Zea mays* (C)	Poaceae	Lubombo	Shewula	S26°06', E32°01'	*Phenacoccus solenopsis* Tinsley
MLB006	*Curcurbita pepo* (C)	Cucurbitaceae	Lubombo	Shewula	S26°06', E32°01'	*Phenacoccus solenopsis* Tinsley
MLB008	*Sida* sp. (W)	Malvaceae	Lubombo	Shewula	S26°06', E32°01'	*Phenacoccus solenopsis* Tinsley
MMV009	*Asparagus aethiopicus* (O)	Asparagaceae	Middleveld	Luyengo	S26°57', E31°17'	*Phenacoccus madeirensis* Green
MMV013	*Impatiens* sp. (O)	Balsaminaceae	Middleveld	Luyengo	S26°57', E31°17'	*Phenacoccus madeirensis* Green
MMV014	*Amaranthus graecizans* (W)	Amaranthaceae	Middleveld	Luyengo	S26°57', E31°17'	*Phenacoccus madeirensis* Green
MMV012	*Peperomai obtusifolia L*. (O)	Piperaceae	Middleveld	Luyengo	S26°57', E31°17'	*Phenacoccus madeirensis* Green
MMV008	*Gerbera* sp. (O)	Asteraceae	Middleveld	Luyengo	S26°57', E31°17'	*Phenacoccus madeirensis* Green
MMV001	*Mangifera indica* (C)	Anacardiaceae	Middleveld	Luyengo	S26°58', E31°16'	*Planococcus citri* (Risso)
MMV011	*Sohlumbergera* sp. Lam (O)	Cactaceae	Middleveld	Luyengo	S26°57', E31°17'	*Paracoccus burnerae* (Risso)

Table 2. Percentage uncorrected sequence divergence within and between mealybug species

	Phenacoccus solenopsis	***Phenacoccus solani***	***Saccharicoccus sacchari***	***Planococcus citri***	***Paracoccus burnerae***	***Phenacoccus madeirensis***
Phenacoccus solenopsis	0.00-0.17					
Phenacoccus solani	6.90-8.59	0.00-0.26				
Saccharicoccus sacchari	12.67-12.87	14.29-14-70	0.00			
Planococcus citri	11.87-12.07	12.47-13.68	10.31-10.69	0.68		
Paracoccus burnerae	13.07-15.77	12.27-12.48	11.29	8.77-8.96	0.00	
Phenacoccus madeirensis	15.77-15.98	14.91-15-32	15.78	15.36-15.80	16.80	0.00

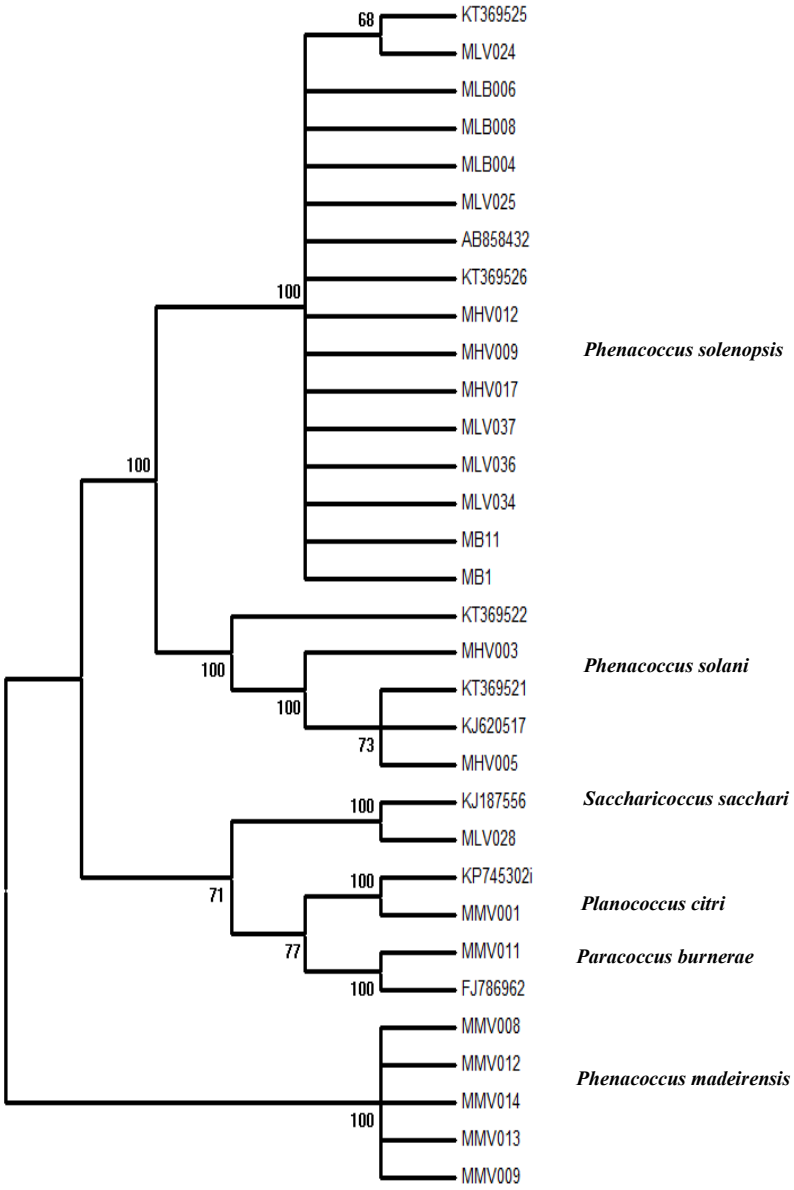

Fig. 1. NJ tree showing the relationships among the six mealybug species. Bootstrap support is indicated on the branches. Specimen codes for samples from Swaziland are as indicated in Table 1. KT369525, AB858432, KT369526, KT369522, KT369521, KJ620517, KJ187556, KP745302 and FJ78962 are GenBank accession numbers of sequences used for identification and analyses of genetic distances.

included a sequence from this survey and its closest match downloaded from the GenBank. The two sequences had shown a sequence divergence of 0.68% (Table 2). Five sequences of individuals collected from wild host plants in the Middleveld shared a haplotype and were clustered in the sixth clade (Table 1).

3.2 Mealybug Distribution, Host Plant Range and Species Composition

Mealybug species varied in their distribution with only *P. solenopsis* found in three the four regions of Swaziland (Table 1). This invasive species was recovered from five cultivated and seven wild host plants (Table 1).

Table 3. Species composition of mealybugs recorded from cultivated and wild host plants in the four regions of Swaziland.

Region	No. of sites with mealybug infestation	Species composition (%)					
		P. solenopsis	***P. solani***	***S. sacchari***	***P. burnerae***	***P. citri***	***P. madeirensis***
Highveld	13	69.2	23.1	7.7	-	-	-
Middleveld	14	21.4	-	-	7.1	14.3	57.1
Lowveld	28	96.4	-	3.6	-	-	-
Lubombo	8	100	-	-	-	-	-
Total	63	74.6	4.8	3.2	1.6	3.2	12.7

The distribution of *P. citri* was restricted to the Middleveld region of the country and was recovered only from mango. *Saccharicoccus sacchari* was recovered from sugarcane in the Lowveld region. *Phenacoccus madeirensis* was common in wild host plants growing in the Middleveld region. This species was recorded from five wild host plants belonging to three families. *Paracoccus burnerae* was recorded from *Sohlumbergera* spp. Lam. in the Middleveld, whereas *P. solani* was recorded from *Abutilon theophrasti* and *Bidens pilosa* in the Highveld region (Table 1). This is the first record of the mealybugs and their host plants in Swaziland. The presence of mealybugs in the country is long known but no research on their identification and host range was conducted before this study.

Mealybug species composition varied between sites (Table 3). *Phenacoccus solenopsis* was the predominant mealybug species in three of the four regions surveyed (Table 3), followed by *P. madeirensis* and P. *solani*. *Paracoccus burnerae, S. sacchari* and *P. citri* constituted only 1.6, 3.2 and 3.2% of the mealybugs species recovered, respectively.

4. DISCUSSION

Comparisons of the COI gene sequences from local mealybug populations collected during the survey with the available sequence data of other mealybug species in GenBank and BOLD systems revealed the presence of six mealybug species belonging to four genera. However, only five of the 21 sequences submitted to the BOLD system were identified to a species level with 100% similarity as *P. madeirensis*. The DNA barcode libraries that are available on the Barcode of Life Data Systems did not properly represent the taxonomic diversity of the remaining five Pseudococcidae species collected in the survey. Hence, the nucleotide data of the remaining 16 sequences in this study were identified using Megablast in the GenBank and Kimura's two parameter (K2P) genetic distance [28] as it is the most commonly used and widely accepted distance metric in DNA barcoding. Incomplete taxon coverage is reported to result in false positives (producing an erroneous positive identification] and false negatives (erroneously discarding a query) and may affect the liability of insect DNA barcoding [31]. Limitation of incomplete taxon coverage in the BOLD systems libraries in this study was resolved through the use of sequences of morphologically identified specimens in the GenBank.

Recently deposited sequences of morphologically identified individuals of *P. solenopsis* and *P. solani* in the GenBank [15] made accurate identification of specimens of these species collected in this survey possible. The species *P. solani* and *P. solenopsis* are morphologically similar in appearance [15] that it is difficult to tell apart. These species were considered to be morphological variants of a single species (*P. solenopsis)* [8] until Chatzidimitriou et al. [15] delimited them into separate species using DNA barcoding. The interspecific sequence distance between the *P. solani* and *P. solenopsis* individuals (6.90-8.59%) in this study satisfies the 10 times the average intraspecific variation within the species (0.00-0.17%) that Hebert et al. [32] proposed as a screen for new species. Similarly, COI sequences of morphologically identified individuals of *P. citri,* and *P. burnerae* deposited by Pieterse et al. [33] in the GenBank were ultimate for solid identification of these species collected from Swaziland. The degree of separation between the GenBank sequence and individual sequence of *P. citri* from Swaziland (0. 68%) is far lower than the genetic distance between *P. citri* and its congeneric-species

P. ficus (4.3-6.1%) reported by Mansour et al. [19].

Results of this study revealed that cytochrome *c* oxidase subunit I (COI) gene could play a pivotal role in characterization and differentiation of morphologically similar mealybug species [34, 20]. However, queries with a large genetic distance with their best DNA barcode match should be treated cautiously to avoid misidentification. Comparing them with sequences of morphologically identified individuals is one of the options. Unfortunately, a large percentage of the DNA barcodes of Pseudococcidae generated to date are new and are therefore absent from international databases [20,21]. There is a need to improve the availability of Barcode sequences for these taxa and studies like this contribute to the development of a DNA barcoding system for the identification of mealybugs.

Phenacoccus solenopsis was the most common mealybug found in this survey. This mealybug has been described as a serious invasive pest in Pakistan and India [8], Sri Lanka [35] and China [36,22]. In Africa, it was first reported from Hibiscus in the southern Guinea Savanna of Nigeria [37] but its dispersion currently extends as far as Benin, Cameroon, Mali and Senegal [4]. However, the pest has never been reported from southern Africa. Therefore, the establishment of this invasive mealybug species in Swaziland is of special concern, as this can serve as source populations for invasion into the rest of southern Africa. This mealybug was recovered from twelve host plants belonging to seven families, consistent with its polyphagus nature [38]

Phenacoccus madeirensis is the second common species that was recorded from three ornamental and one wild host plant species confirming its status as a pest of ornamentals in Swaziland. This mealybug is a widespread pest on ornamental plants [39] and has been recorded on much of the African mainland [40]. It is a very polyphagous mealybug, attacking 152 plant species in 46 families [41]. The number of host species recorded in this survey is likely to increase. The other mealybug species recorded from ornamental plants in this study is the citrus mealybug, *Planococcus citri* [42] and Harsimran et al. [43] cited it as a pest of citrus in Africa [44]. Further study on the distribution and host plant range of this mealybug should shed some light on its economic importance for the country.

Phenacoccus solani was recorded from *Bidens pilosa* and *Abutilon theophrasti* in the Highveld region of Swaziland. This mealybug was reported from *B. pilosa* in the vineyards of the neighbouring South Africa [45] and is known to be pest of pepper and tarragon [46] in Europe. The oleander mealybug, *Paracoccus burnerae,* was recorded from mango in the Middleveld region of the country. This species is common in Africa known to occur from Kenya to South Africa. It has been recorded on 35 plant species, including important food and beverage crops such as potatoes, olives and coffee (www.sel.barc.usda.gov/scalenet/scalenet.htm). In South Africa, *P. burnerae* is known to attack citrus [47], hence it is likely to attack fruit crops in Swaziland and pose a threat to citrus production in country. Consistent with previous reports [48], the pink sugarcane mealybug, *Saccharicoccus sacchari* was recorded only from commercially grown sugarcane in the Lowveld region.

5. CONCLUSION

There is high diversity of mealybugs in crops, ornamentals and wild host plant species in Swaziland. The number of hosts affected by mealybugs and the mealybug species diversity in Swaziland is likely to be far more comprehensive. Using the COI sequences from this study, anyone with direct or indirect access to a DNA sequencer will be able to identify, to a high degree of certainty, any mealybug life stage or tissue fragment. However, identification using molecular techniques should be done with great caution. Proper identification of pest mealybugs is fundamental for genetic research on the organisms and, consequently, translational work to improve management of the identified mealybug pests.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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