Identification of mealybugs, soft scale insects and their predators in vineyards across the savannah agro-ecological region of Nigeria

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Abstract

Insect-vectored viruses are a major threat to grapevine production but there is a dearth of information on the occurrence and distribution of key grapevine pests in Nigeria. The recent detection of grapevine leafroll associated virus-1 (GLRaV-1), a known insect-vectored ampelovirus, in Nigeria elevates the importance of the identification of its potential vectors as a precursor to assessing the risk of grapevine leafroll disease spread. This study was conducted to determine the occurrence and diversity of potential vectors of grapevine viruses and their natural enemies in vineyards across the savannah agro-ecological region of Nigeria. Forty vineyard and nursery locations were surveyed during 2016 and 45 arthropod samples were collected. The samples were first morphologically identified, and DNA barcoding was conducted on a subset of 16 representative samples using universal primers specific to the Mitochondrial Cytochrome Oxidase subunit I (mtCOI) gene of most insects. The results indicated the presence of two species of scale insects (*Parasaissetia nigra* and *Saissetia coffeae*) and two mealybug species (*Maconellicoccus hirsutus* and *Ferrisia virgata*), some of which are potential grapevine virus vectors, in Nigerian vineyards. In addition, the natural enemies of these insect species were detected which includes three species of parasitoids (*Anagyrus kamali*, *Anagyrus pseudococci* and *Encarsia inaron*) and one predator (*Hyperaspidius mimus*). While the detection of mealybugs and scale insects underscore the risk of vector-mediated virus spread in Nigerian vineyards, the identification of their natural enemies indicates presence of natural biological control agents to facilitate an integrated management of economically important grapevine virus diseases in the country.

Keywords: Mealybugs; scale insects; parasitoids and predators; insect vectors; grapevine viruses.

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Introduction

Grapevine (*Vitis* sp.) belongs to the family *Vitaceae* (Zhang *et al* 2015). Grape is one of the world's most widely grown horticultural crops and its production is concentrated in relatively warm temperate climatic zones (Christian *et al* 2012). The predominant types of grapevine production are table and wine grapes, but a considerable proportion of the production is also devoted to other products such as raisins and juice (Reynolds, 2017). Grapevine is a relatively recently introduced crop in Nigeria and its production is concentrated in the savannah ecological zone of the country. Several table grape varieties are currently being grown successfully in Nigeria with the most common varieties being Anab-e-Shahi, Queen golden, Regina and Bangalore Blue (Okutu, 2004). The berries serve as sources of essential minerals, vitamins, and antioxidants for households whose diet is dominated by carbs. In addition, grape production in Nigeria could serve as a source of livelihood for the largely small acreage growers, thus contributing to poverty alleviation in rural communities. Based on conservative estimates, a fruit-bearing vine can provide about $60-$80 revenue to the grower per growing season. As a result, the viticulture industry is gradually expanding in Nigeria (Okutu, 2004). Over 70 graft-transmissible agents affect grapevine and at least 14 of these agents are vectored by arthopods (Basso *et al* 2017). These include the economically important grapevine leafroll-associated ampeloviruses and vitiviruses that are transmitted by several species of mealybugs and soft scale insects in a semi-persistent manner (Tsai *et al* 2010; Le Maguet *et al* 2012). In addition to their ability to spread viral diseases, mealybug and soft scale insects can also predispose the vines to sooty mold infection due to their sugar-rich honey dew excrements (Daane *et al* 2008; Ouvrard *et al* 2013). As such, species of both insects cause indirect damage to the vine through impairment of vine

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health and reduction of fruit quality. Therefore, vector management is an integral part of vineyard health management across all major viticultural regions of the world.

Arthropod pests in vineyards can be managed using insecticides and biological control agents such as insect predators and parasitoids (Ouvrard *et al* 2013) in an integrated manner. Several insect parasitoids and predators of mealybugs and soft scales insects have been identified in different viticultural regions of the world (Charles *et al* 2010; Daane *et al* 2008; Mahfoudhi and Dhouibi, 2009; Mani *et al* 2011; Walton and Pringle, 2004). The parasitoid oviposit within the adult insect body where it completes immature development and mummifies the host insect in the process (Daane *et al* 2008). The adult parasitoid emerges from the mummified body to later initiate a new generation. Because of significant consumption of prey during their development, arthropod predators contribute to significant death rates and control of mealybugs and scale insect pests in vineyards (Mani *et al* 2011). Therefore, a good measure of a healthy cropping system ecology is the presence of diverse natural enemies of economically important pests of the target crop.

The identification of economically important insect pest and their natural enemies can be achieved through morphological and/or molecular methods. Morphological identification typically relies on comparisons of the sample with type specimens or the use of taxonomic keys. Such taxonomic keys are often restricted to specific life stages of the insect and requires a trained expert for its interpretation. DNA-barcoding is an emerging molecular tool for identification and taxonomic resolution of both known and unknown species using a short DNA sequence or genetic marker in an insect DNA (Ashfaq and Herbert, 2016; Hebert *et al* 2003; Jalali *et al* 2015; Sinclair and Gresens, 2008). The maternally inherited mitochondrial cytochrome c oxidase subunit I *(COI)* gene is particularly useful for molecular identification due to the very high level of conservation of the protein-encoding genes (Folmer *et al* 1994; Kranzfelder *et al* 2017). Sequences derived from the gene-specific DNA amplicons can then be compared with corresponding sequences of known species in a barcode reference library (Wilson, 2012). The DNA barcoding technique is also versatile for the identification of an insect at any developmental stage (Webb *et al* 2012) and does not require specialized training in insect taxonomy to be accurately interpreted.

Recently, a survey to determine the repertoire of grapevine viruses and other graft-transmissible agents occurring in vineyards planted to introduced cultivars was conducted in Nigeria. This effort led to the identification of grapevine leafroll-associated virus 1 (GLRaV-1; genus *Ampelovirus*; family *Closteroviridae*) for the first time in Nigerian vineyards (Zongoma *et al* 2018). Since GLRaV-1 and several other grapevine-infecting viruses in the genus *Ampelovirus* are known to be vectored by mealybugs and soft scale insect species (Bertin *et al* 2010; Sforza *et al* 2003). The aim of the current study is to identify species of mealybugs, soft scale insects and their predators that are present in Nigerian vineyards using a combination of morphological and molecular assays. The results will provide science-based knowledge to aid the development of an integrated pest management strategy towards mitigating the potential impact of economically important grapevine viral diseases in the Nigerian grapevine industry, still at its infancy.

Materials and methods

Survey and sample collection

Surveys were conducted during 2016 raining season from June to July in the savannah agro-ecological zone of Nigeria. A total of 40 vineyard locations were visited across Bauchi (n = 3), Gombe (n = 4), Kaduna (n = 22), Katsina (n = 2), Plateau (n = 3) and Kano (n = 6) states. The 40 locations were identified through grower contacts. In each location, purposive sampling was used for vine selection. The sampled vines were inspected for the presence of mummified and non-mummified insects that resemble mealybugs, soft scale insects and their predators. Field-collected insect specimens were carefully excised using a paint brush and preserved in 70% (v/v) ethanol for later analysis. Infested cuttings were also sampled for morphological identification purposes.

Morphological identification

A total of 45 insect samples were collected during the survey from 22 of the 40 survey sites. The samples were morphologically identified at the Insect Museum, Department of Crop Protection, Ahmadu Bello University (ABU), Zaria, Nigeria, via comparisons of the field-samples with a reference collection at the museum. A subset of the insect-samples that were preserved in alcohol were shipped to the Texas A and M AgriLife Research and Extension, Weslaco, Texas, USA facility for molecular identification under a USDA-APHIS-PPQ permit.

Molecular identification

Based on results of the morphological identification, DNA barcoding was performed on a subset of 16 representative samples from eight fields. DNA was extracted from individual insects according to the Dellaporta *et al* (1983) extraction protocol. The DNA extracts were quantified on a NanoDrop 2000 series spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) and stored at -20°C until use. Following quality analysis, the insect DNA samples were subjected to molecular barcoding using universal primers C1-J-2195-F and L2-N-3014-R that are capable of amplifying ~700 bp DNA fragment specific to the mitochondrial cytochrome oxidase subunit I (mtCOI) gene of several arthropods (Frohlich *et al*., 1999). The specificities of the DNA amplicons were confirmed by cloning of the fragments into the pJET1.2/blunt vector using the CloneJET PCR Cloning Kit (Thermo Fisher Scientific) as prescribed by the manufacturer. Two to three plasmids with PCR-verified correct size inserts per cloned-

DNA amplicon were isolated from recombinant chemically competent *Escherichia coli* cells using the GenElute Plasmid Miniprep Kit (Sigma-Aldrich, St. Louis, MO). The plasmids were sequenced in both directions with a pair of vector-specific primers by the Sanger method in a commercial facility (ELIM BIOPHARM, Hayward, CA, USA).

Bioinformatic analysis

The raw sequences were trimmed to remove cloning vector-specific sequences. Sequences derived from each insect sample were compared among themselves, following which the CAP contig assembly program of the BioEdit software (Hall, 1999) was used to derive a consensus sequence from sequence haplotypes (99-100% nucleotide [nt] identities) present in each insect sample. Divergent sequences, i.e. sequences of recombinant plasmids from the same insect sample that shared <99% nt identities with one another, were analyzed as independent sequences. Each of the sequence haplotypes was subjected to BLASTN analysis (Altschul *et al* 1990). The MUSCLE alignment program (http://www.ebi.ac.uk/Tools/msa/muscle/) was used to generate multiple sequence alignments for the derived sequences and to determine the sequence identity matrix. The alignment file was also used for phylogenetic analysis with the neighbour-joining algorithm of the molecular evolutionary genetics analysis (MEGA) software version 7.0 (Kumar *et al* 2016).

Results

Morphological identification

The morphological identification of the 45 insect specimens led to the identification of 60% (27/45) of the samples as mealybugs, 33.3% (15/45) as soft scale insects, 4.4% (2/45) as lady beetles and 2.2% (1/45) as a wasp. The most prevalent of the identified insects were mealybugs with occurrence in 81.8% (18/22) of surveyed-sites, followed by soft scale insects in 50% (11/22) of sampled sites. The lady beetle and wasp samples were each found only in one sampled site. The results showed greater prevalence of mealybugs and soft scale insects, both potential vectors of GLRaVs and vitiviruses, across vineyards in Nigeria. Based on comparisons with the museum specimens, the mealybug samples were identified as *Maconellicoccus* sp. and *Ferrisia* sp. (Hemiptera: Pseudococcidae), the scale insect samples as *Parasaissetia* sp. and *Saissetia* sp. (Hemiptera: Coccidae), the lady beetle as *Hyperaspis* sp. (Coleoptera: Coccinelidae) and the wasp as *Prospaltella* sp. (Hymenoptera: Aphelinidae) (Table I). Even though some of the mealybug samples were observed to be mummified suggesting the presence of parasitoid in these samples, it was impossible to morphologically identity these immature natural enemies.

Molecular detection

The sequences derived from the DNA barcoding analysis have been deposited in the Genbank under the accession numbers MK090638-53. The BLASTN analysis of sequences derived from the 16 representative samples confirmed the presence of species of mealybugs and soft scale insects in Nigerian vineyards (Table I). The mealybugs were identified as *Maconellicoccus hirsutus* Green (pink hibiscus mealybug) (GenBank accession nos. MK090638-45) and Ferrisia virgata (Cockerell) (stripped mealybug) (GenBank accession no. MK090646) (Table 1). The soft scale insects were identified as *Parasaissetia nigra* (Nietner) (GenBank accession no. MK090647) and *Saissetia coffeae* (Walker) (GenBank accession nos. MK090648-49) (Table 1). In addition, BLASTN analysis of sequences derived from the mummified bodies of *M. hirsutus* samples collected from two locations in Bauchi and Gombe states revealed the presence of two parasitoids, *Anagyrus pseudococci* (Girault) and *Anagyrus kamali* Moursi (Hymenoptera: Encyrtidae) (GenBank accession nos. MK090650 and MK090651, respectively). The BLASTN analysis of sequences derived from the wasp and the ladybird beetle samples linked them to be specific to *Encarsia inaron* (Walker, 1939) (Hymenoptera: Aphelinidae) (GenBank accession no. MK090652), and *Hyperaspidius mimus* Casey (Coleoptera: Coccinellidae) (GenBank accession no. MK090653), respectively.

Sequence analysis

The results of the BLASTN analysis of the derived-sequences, further verified using neighbour-joining phylogenetic tree, showed the mealybug and soft scale insect sequences clustering into clades specific to *M. hirsutus*, *F. virgata*, *P. nigra* and *S. coffeae*, along with global sequences of the respective insect species (Figure 1). In pairwise comparisons showed in Table 2, the *M. hirsutus* derived sequences shared 99.8-100% nt identities among themselves and the same range of nt identities with corresponding global sequences of this mealybug species, while the *F. virgata* sequence shared 98.3-100% nt identities with corresponding global sequences of this mealybug species. For the *P. nigra* sequence, the shared nt identities was 99.1-99.7% with corresponding global sequences of this scale insect species, while the *S. coffeae* sequences derived in this study shared 93.9% nt identities among themselves and 81.6-84.3% nt identities with corresponding global sequences of this scale insect species. Sequences specific to *Anagyrus kamali* and *Anagyrus pseudococci* shared 84.6-100% nt identities among themselves and 84.5-99.6% nt identities with corresponding global sequences of several species in the genus *Anagyrus*. The sequence specific to *Encarsia inaron* shared 90.1-92.2% nt identities with corresponding global sequences of several species in the genus *Encarsia*. And the sequences specific to *Hyperaspidius mimus* shared 99.8-100% nt identities among themselves and 90.1-90.9% nt identities with corresponding global sequences of species in the genus *Hyperaspidius*.

Table 1. Molecular and morphological identification of insect samples collected from vineyards in savannah agroecological zone of Nigeria.

Location	Coordinate	Arthropod[a]	Molecular ID[b]	Morphological ID[c]
Bauchi	10º17.350′N, 009º51.373′E	Wasp[1]	*Encarsia inaron*	*Prospaltella sp*
Bauchi	10º17.350′N, 009º51.373′E	Mealybug[1]	*Maconellicoccus hirsutus*	*Maconellicoccus sp*
Bauchi	10º17.350′N, 009º51.373′E	Lady Beetle[1]	*Hyperaspidius mimus*	*Hyperaspis sp*
Bauchi	10º17.350′N, 009º51.373′E	Parasitoid[1]	*Anagyrus nr. Pseudococci*	*Maconellicoccus* sp. mummy
Bauchi	10º17.350′N, 009º51.373′E	Scale insect[1]	*Parasassetia nigra*	*Parasassetia sp.*
Bauchi	10º17.350′N, 009º51.373′E	Mealybug[1]	*Maconellicoccus hirsutus*	*Maconellicoccus sp*
Bauchi	10º17.350′N, 009º51.37′E	Mealybug[1]	*Maconellicoccus hirsutus*	*Maconellicoccus sp*
Kaduna	11º11′36.558′N, 7º37′9.414′E	Mealybug[2]	*Maconellicoccus hirsutus*	*Maconellicoccus sp*
Bauchi	10º15′42.348′N, 9º56′28.674′E	Mealybug[3]	*Maconellicoccus hirsutus*	*Maconellicoccus sp*
Kano	11º57′17.928′N, 8º33′44.574′E	Mealybug[4]	*Maconellicoccus hirsutus*	*Maconellicoccus sp*
Gombe	10º15′29.06′N, 11º9′45.186′E	Parasitoid[5]	*Anagyrus kamali* Moursi	*Maconellicoccus* sp. mummy
Kaduna	11º10′2.508′N, 7º38′40.914′E	Mealybug[6]	*Maconellicoccus hirsutus*	*Maconellicoccus sp*
Kano	11º58′59.46′N, 8º32′30.744′E	Mealybug[7]	*Ferrisia virgata*	*Ferrisia sp*
Kano	11º57′17.928′N, 8º33′44.574′E	Mealybug[4]	*Maconellicoccus hirsutus*	*Maconellicoccus sp*
Kaduna	10º43′5.238′N, 7º35′6.282′E	Lady Beetle[8]	*Hyperaspidius mimus*	*Hyperaspis sp*
Kaduna	10º43′5.238′N, 7º35′6.282′E	Scale insect[8]	*Saissetia coffeae*	*Saissetia sp*

[a]Arthropod with the same number superscript were sampled from the same field.
[b]Molecular identification was based on amplification, cloning and sequencing of partial mitochondrial cytochrome c oxidase subunit I (mtCOI) gene sequences.
[c]Morphological identification was based on comparison of samples with reference specimens in the collection of the Insect Museum, Department of Crop Protection, Ahmadu Bello University, Zaria, Nigeria.

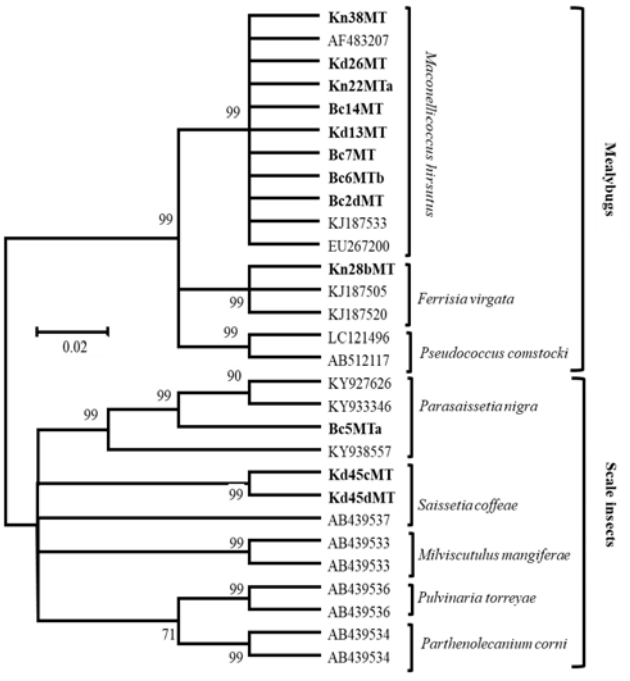

Figure 1. Neighbour-joining phylogenetic tree showing the diversity of mealybug and soft scale insect species detected in Nigerian vineyards during a survey conducted in the savannah agro-ecological zone of the country. Bootstrap supports for tree branches (1,000 replicates are shown at the internodes). Sequences derived in this study are shown in bold fonts, while those from GenBank are in regular fonts.

Table 2. The range of pairwise nucleotide sequence identities within and between insect species identified in this study and corresponding global sequences in GenBank.
Insect species Nucleotide identity levels

Insect species	Nucleotide identity levels	
	This study	GenBank
Maconellicoccus hirsutus	99.8-100	99.8-100
Ferrisia virgata	100	98.3-99.8
Parasassetia nigra	100	99.1-99.7
Saissetia coffeae	93.9	81.6-84.3
Anagyrus sp.	84.6-100	84.5-99.6
Encarsia sp.	100	90.1-92.2
Hyperaspidius sp.	99.8-100	90.1-90.9

Discussion

Viruses are economically important graft-transmissible agents of grapevine and major concerns in vineyards due to the negative impacts of their associated diseases (Alabi *et al* 2016). While the inadvertent introduction or movement of infected cuttings for propagation by humans is the primary means of long distance spread of GLRaV-1 and other grapevine viruses, phloem-feeding insects such as species of mealybugs (Pseudococcidae) and soft scale insects (Coccidae) play a pivotal role in short distance (vine-to-vine) and long distance (between vineyards) spread of the introduced viruses (Herrbach *et al* 2017). In addition, these insects are also capable of causing direct economic losses as pests of the grapevine when their infestation levels within vineyards become high (Daane *et al* 2008). An accurate identification of pests and potential

virus vectors within vineyards is the first step towards the implementation of appropriate measures for their control. The survey confirmed the occurrence of *M. hirsutus, F. virgata*, *P. nigra* and *S. coffeae* in Nigerian vineyards (Table 1) for the first time. Whereas none of these detected insect species is a known vector of GLRaV-1, a virus recently detected in Nigerian vineyards (Zongoma *et al* 2018), however, *P. nigra* and *S. coffeae* have been documented as vectors for GLRaV-3 (Herrbach *et al* 2017). It is plausible to assume that the mechanism of acquisition and transmission of ampeloviruses by mealybugs and scale insect species would be similar hence the presence of the identified potential vectors in GLRaV-1 infected fields poses a risk of vector-mediated virus spread. Therefore, it is important for farmers to consider implementing control measures for these pests as part of their vineyard management practices.

Effective chemical control measures are available for the management of mealybugs and scale insects in vineyards. However, contact and systemic pesticides may be cost prohibitive especially for small scale farmers, in addition to the potential detrimental effects of synthetic chemicals on the environments and their non-target effects on beneficial insects. To address the problems associated with the use of synthetic pesticides, biological control agents such as insect predators and parasitoids are being incorporated into integrated pest management programs. Indeed, the presence of biocontrol agents in a field is an indicator of sustainability. In this vein, the detection of three parasitoids, *Anagyrus kamali*, *Anagyrus pseudococci*, and *Encarsia inaron* in few of the surveyed vineyards is a welcome development. Previous studies by Noyes and Hayat (1995) have documented members of the family Encyrtidae, to which the genus *Anagyrus* belong, as natural enemies of mealybugs. Roltsch *et al* (2006) credited *Anagyrus kamali*, one of two parasitoids detected in this study, with the reduction of *M. hirsutus* densities to non-economic levels throughout southern California, USA. Similarly, Daane *et al* (2008) reported that parasitoids such as *Acerophagus* sp., *Chrysoplatycerus* sp. and *Anagyrus pseudococci* can parasitize *Ferrisia gilli* Gullan, a documented mealybug vector or GLRaV-3 (Wistrom *et al* 2016). The ladybird beetle, *Hyperaspidius mimus*, detected in this study is also a known polyphagous insect predator along with several members of the family Coccinellidae that often serve as predators of mealybugs and soft scale insects (Giorgi *et al* 2009; Daane *et al* 2012). Of interest is the identification of a species in the genus *Encarsia*, known parasitoids of whiteflies (Heinz and Parrella, 1994) in the mummified body of a *M. hirsutus* suggesting that the predatory range of these wasp species could be wider than currently reported. Overall, the results point to the presence of a diverse population of beneficial natural enemies of mealybugs and soft scale insects in Nigerian vineyards that could be exploited for control of these potential virus vectors.

In this study, the successful identification of the mealybugs, soft scale insects and their potential predators and parasitoids was achieved via a complementary use of morphological and molecular tools and techniques. Whereas morphological identification of field-collected insect specimens is commonly used globally for species identification, its limitations lie in the need for taxonomic expertise, sampling of different life stages of the insect, intactness of the sampled insects, availability of robust identification keys, among other factors. These limitations could be overcome with DNA barcoding because universal primers that target conserved genes of insects could be employed on any or parts of the specimen regardless of the insect life stage that was sampled. An additional benefit of the DNA barcoding approach used in this study is that the target gene fragment is specific to the insects and their parasitoids.

The sequence information obtained from the cloned gene fragment could then be compared with reference sequences in public databases for specific identification of the insect sample and its parasitoid (if present). Thus, the use of molecular techniques has enabled a better resolution of several insect taxa since its advent (Ashfaq *et al* 2011; Daane *et al* 2018) as applicable for the identification of insect samples collected during our vineyard surveys (Table I) with a high degree of confidence. The primary challenge associated with the DNA barcoding approach lies in resource availability, but this can be overcome through research collaborations as exemplified in this study.

In conclusion, our results point to the presence of pests and potential vectors of economically important grapevine viruses in Nigeria. It also provided an indication of presence of diverse population of beneficial natural enemies of mealybugs and soft scale insects under field conditions in Nigerian vineyards that could be exploited for control of the insect pests. This, to the best of our knowledge is the first report on diversity of arthropods in Nigerian vineyards and the first documentation of three parasitoids and a predator in a vineyard ecosystem in Nigeria. Future studies will determine the epidemiological roles of the detected mealybug and soft scale insect species and the relative parasitism abilities of the parasitoids and predator found in Nigerian Savanna vineyards

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