Identification of *Mgenia fuscovaria* (Stål) (Hemiptera: Cicadellidae), a Vector of Aster Yellows Disease on Grapevines in South Africa, and Differentiation from *Mgenia angusta* (Theron) by Nucleotide Sequences of the Mitochondrial Cytochrome Oxidase I (*cox1*) Gene

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Aster yellows phytoplasma (AY), only recently reported in South Africa and still limited in its distribution in the country, causes a serious disease of grapevine. A leafhopper, *Mgenia fuscovaria* (Stål)(Hemiptera: Cicadellidae), was shown to transmit AY to grapevine (*Vitis vinifera* L.) locally. A second *Mgenia* species, identified as *Mgenia angusta* (Theron), has also been found in vineyards in South Africa but has not been shown to transmit AY. *M. angusta* is morphologically similar to *M. fuscovaria*, but does differ regarding the size of the male sex organ and in the number of teeth of the blades making up the ovipositor. In this study, we determined the nucleotide sequence of the mitochondrial cytochrome oxidase I gene (*cox1*), commonly used in insect bar-coding, of a number of specimens of males, females and nymphs of both *M. fuscovaria* and *M. angusta*. No differences were observed between the *cox1* sequences of the male, female and nymph specimens putatively assigned to any specific species, but some nucleotide sequence differences were observed between specimens of *M. fuscovaria* and *M. angusta*. These differences, however, were insufficient to allow the development of PCR systems specific to each species.

INTRODUCTION

A worldwide trend of loss in "classical" (morphological description) insect taxonomy expertise, along with an overwhelming number of potential insect species, requires an alternative approach to insect taxonomic identification. DNA sequence data is a potential remedy for this taxonomy crisis. The sequencing of the 5′ end of the *cox1* gene has become the most widely used approach (www.barcodinglife.org), as the mitochondrial DNA is highly abundant in the cell and its amplification is reliable; and *cox1* is often variable from populations to higher taxonomic levels (Hebert *et al*., 2003). Hebert *et al*. (2003) proposed using a 650 base-pair region of the cytochrome c oxidase subunit I (cox-1) gene as the universal barcode sequence in animals to enable the identification of species. Sequence variation in this region has been found to be highly structured and partitioned between discrete clusters that correspond broadly to species-level entities (Papadoupolou *et al*., 2008).

During a recent study (Krüger *et al*., 2011), a leafhopper identified as *Mgenia fuscovaria* (Stål)(Hemiptera: Cicadellidae) was shown to transmit "*Candidatus* Phytoplasma asteris (16Sr1-B group)", also called aster yellows phytoplasma (AY) to grapevine (*Vitis vinifera* L.). AY is an organism only recently reported in South Africa (Engelbrecht *et al*., 2010) and causes a serious disease of grapevine. In South Africa, the disease is restricted to only some grapevine-growing regions of the Western Cape and is a quarantine organism, with various control measures being enforced and delineating surveys being done regularly to restrict the area affected by the disease. Very little is known of the local epidemiology of the phytoplasma, or of the biology of the vector. In order to conduct epidemiological studies and controlled transmission experiments in which nymphs are included, unequivocal identification of the species involved is required. This fact was reinforced by the discovery of a second *Mgenia* spe-

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cies, identified as *Mgenia angusta* (Theron) (Theron, 1984), in vineyards during this study. The size of the male sex organ and the number of teeth found on one of the blades making up the ovipositor (valvula 2) in the case of females morphologically differentiated *M. angusta* from *M. fuscovaria.* However, these differences will be difficult to utilise when studying large numbers of specimens, or specimens from sticky traps. In this study we have determined the nucleotide sequence of the mitochondrial cytochrome oxidase I gene (*cox1*), commonly used in insect bar-coding, of a number of specimens of both *M. fuscovaria* and *M. angusta*. The *cox1* sequences of males, females and nymphs of specimens putatively assigned to either *M. fuscovaria* or *M. angusta* were essentially identical within the species, with a number of minor, inconsistent single-nucleotide polymorphisms. However, a number of consistent nucleotide sequence differences were observed between sequences from *M. fuscovaria* and *M. angusta* specimens. Differences were insufficient to allow the development of standard PCR systems specific to each species. The *cox1* nucleotide sequences of both *M. fuscovaria* and *M. angusta* have been deposited with Genbank (accession numbers KP823217 and KP823218).

MATERIALS AND METHODS

Samples

Putative *Mgenia fuscovaria* individuals were collected from various sites in the Western Cape. These were placed in 70% ethanol, and the collection site, date and other collection data were recorded. The samples were identified at the Agricultural Research Council – Plant Protection Research Institute (ARC-PPRI) using gross morphological traits along with a detailed analysis of the genitalia (Nault & Rodriguez, 1985). Morphogroups of putative *M. fuscovaria* adult females and nymphs were identified and individuals were stored separately in labelled tubes prior to DNA extraction, PCR and sequencing.

Identification by morphology

The morphology of, amongst other features, the male sex organ is used to distinguish species of leafhoppers. The last few segments of the abdomen of specimens were cut off and soaked in cold KOH until clear, and then examined under a stereo light microscope. The remainder of the specimen was stored for DNA extraction and PCR. The sex organ of males was examined for size and shape, while the number, shape and serrations of the blades making up the ovipositor of females were examined.

DNA extraction

DNA was extracted from the remains of the same insect individuals as had been identified morphologically and putatively classified as male, female or nymph members of *M. fuscovaria*. A number of individual specimens of each of the different classes was extracted to assess inter-specific variation of the *cox1* gene sequence.

Total DNA was extracted from individual insect specimens following a slightly modified version of the CTAB extraction method described by Doyle and Doyle (1990). The insect remains were macerated with a micro-pestle in micro-tubes containing 200 µl of 2% CTAB with freshly added 0.2% mercaptoethanol. Following this, a further 200 µl of 2% CTAB with 0.2% mercaptoethanol was added, and the mixture was incubated at 65°C for 30 min whilst shaking. An equal volume (400 µl) of chloroform was added and gently mixed in, after which the mixture was centrifuged for 10 min at 11 000 g. The supernatant was recovered and again treated with chloroform as above. The supernatant was then treated with an equal volume of isoamyl alcohol. Tubes were centrifuged at 12 000 g for 30 min at 10°C. The supernatant was discarded and 1 ml of 70% ethanol was added to the pellet and mixed, and the tubes were centrifuged at 12 000 g for 10 min at 4°C. The supernatant was discarded and the washing step was repeated if needed. After the final washing step, samples were left to air dry for 20 min. Pellets were re-suspended in 50 µl TE buffer and stored at -80°C.

PCR

Mitochondrial *cox1* genes were amplified using the primers described by Folmer *et al*. (1994) (LCO1490: 5'-ggtcaacaaatcataaagatattgg-3' and HC02198: 5'-taaacttcagggtgaccaaaaaatca-3'). Insect DNA extract (1 µl) was added to a final PCR reaction volume of 50 µl, consisting of 25 µl 2X Dream Taq Green PCR mastermix (ThermoScientific, Waltham, MA, USA), 5 µl of a 10 µM solution of each primer, and 15 µl molecular grade O (Sigma-Aldrich, St. Louis, MO, USA). A PCR cycling reaction was performed on a T100™ Thermal Cycler (Bio-Rad, CA, USA). Cycling conditions were set up as follows: initial denaturation at 94°C for 4 min, followed by 35 cycles of denaturation at 92°C for 60 s, annealing at 52°C for 60 s and elongation at 72°C for 90 s, with a final elongation step at 72°C for 10 min.

Sequencing

To remove single-stranded DNA from the PCR products, 0.5 µl of 10 U exonuclease (Fermentas, MD, USA) and 2 µl of 2U FastAP® (Fermentas, MD, USA) were added to the amplification products and the reaction was carried out as per the manufacturer's instructions. Purified amplicons were sequenced in both orientations using the individual Folmer *et al*. (1994) primers in a PCR reaction using Big Dye® Terminator v 3.1 cycle-sequencing kit (Applied Biosystems, Foster City, CA, USA) as per manufacturer's instructions. Sequences of amplicon products were determined using an ABI 3500xL automated sequencer (Applied Biosystems, Foster City, CA, USA) at the core sequencing facility of the University of Pretoria, South Africa.

Sequence analysis

Forward and reverse sequences were aligned and consensus sequences were obtained using CLC Main Workbench (CLC, Aarhus, Denmark). Sequences were subjected to multiple alignments with CLC workbench and maximum likelihood dendrograms were constructed in MEGA software version 6 (Tamura *et al*., 2011), with 1 000 replicates of bootstrap analysis.

RESULTS AND DISCUSSION

In total, 50 males, 35 females and a number of nymphs of the two species of *Mgenia* were examined and the individual

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remains were placed in individual labelled tubes for DNA extraction and PCR. The males of *M. fuscovaria* were shown to have a large, blade-like sex organ, while in *M. angusta* the sex organ is small and needle-like. Females were examined in a similar manner. At high magnification of the three pairs of blades that make up the ovipositor, the valvula 2 had a row of 10 to 12 teeth in *M. fuscovaria*, while *M. angusta*, also found in grapevines (A. de Klerk, unpublished data), had eight to nine teeth. No differences were found in the colour or shape of the body, or wing veins, amongst the individuals monitored. Nymphs could not be assigned to a species using morphological traits.

The sequence of *cox1* from representative *M. fuscovaria* male specimen 13-0076 was deposited in Genbank with accession number KP823217. The *cox1* sequence of putative *M. fuscovaria* nymphs (collected at the same locality where and on the occasion when the *M. fuscovaria* adults were collected) was identical to that of *M. fuscovaria* males. No *M. fuscovaria* females were sequenced.

The *cox1* nucleotide sequence of *M. angusta* was determined using nine male and four female specimens. The sequence of a representative *M. angusta* specimen (12-5157) was deposited with Genbank with accession number KP823218. The sequence could be used to differentiate *M. fuscovaria* and *M. angusta* individuals (Fig. 1).

Morphological differentiation of the two species of *Mgenia* was perfectly correlated with the sequence differences observed in all identified *M. fuscovaria* and all *M. angusta* specimens separating into two distinct clades, with no exceptions (Fig. 1). Small sequence differences amongst individual specimens of a given species were primarily due to ambiguous bases between the forward and reverse sequencing reactions, and were generally not present in any other individual.

CONCLUSIONS

The mitochondrial *cox1* gene sequence of *Mgenia fuscovaria*, as well as that of *Mgenia angusta*, a related grapevine-associated leafhopper, has been determined. These sequences differ sufficiently that species identification can be based on the sequence of the *cox1* sequence of the two species. While genes from *M. fuscovaria* females were

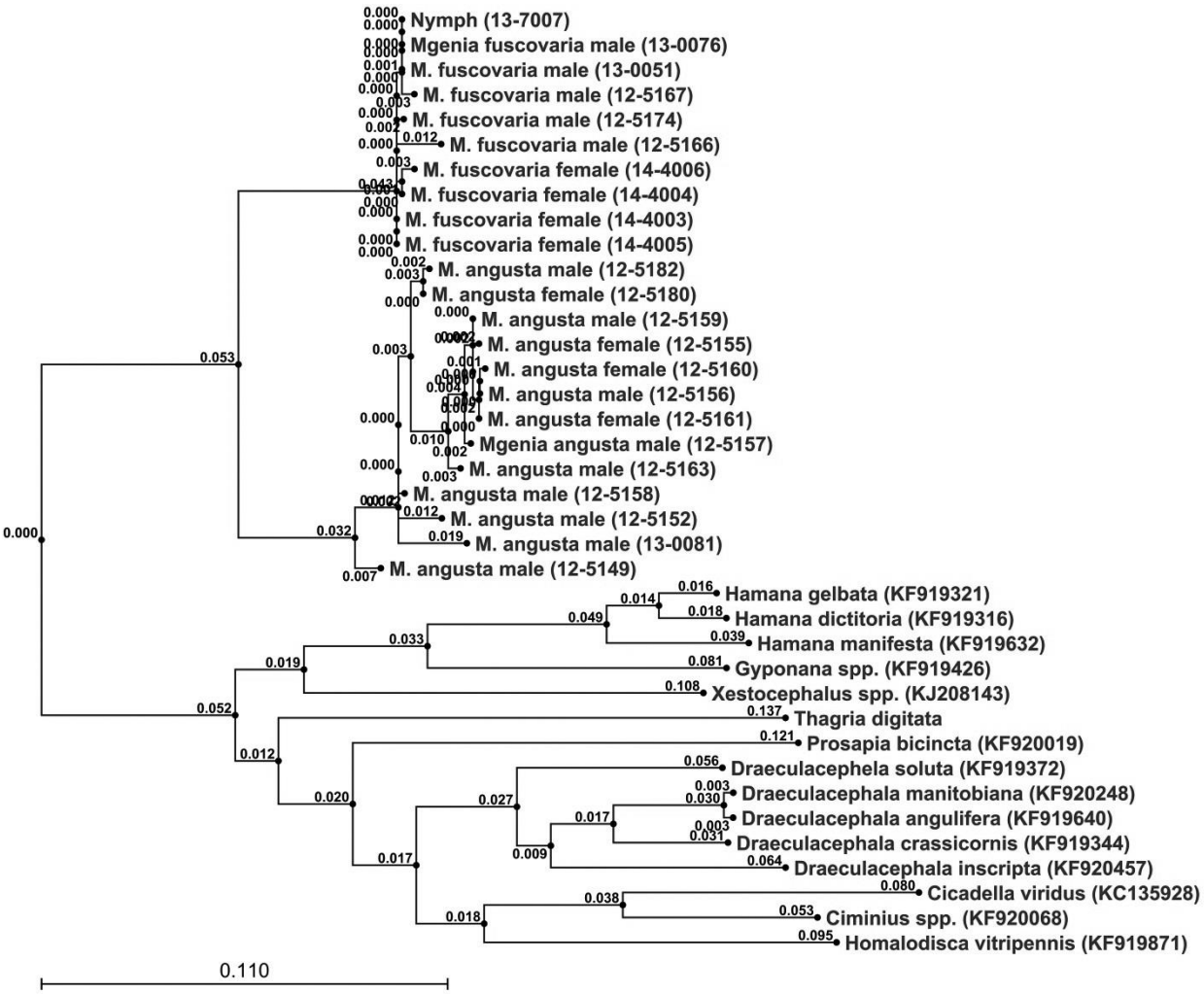

FIGURE 1

Maximum likelihood phylogenetic tree of the mitochondrial cytochrome oxidase subunit 1 gene (*cox1*) of *Mgenia fuscovaria*, *M. angusta* and the closest leafhopper sequences currently available on Genbank (as determined by BLAST analysis). Tree produced by UPGMA program, Jukes Cantor substitution model.

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not sequenced, identity within *M. fuscovaria* males and nymphs, and *M. angusta* males and females, suggest that the sequences generated can be used to identify *M. fuscovaria* and *M. angusta* males, females and nymphs. Differences in sequence were insufficient to develop species-specific PCRs. However, we have achieved the objective of using sequence data to help identify specimens of both species, especially nymphs, for further AY insect-transmission studies.

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