Industry allocated project number PPRI-11/17

AGRICULTURAL RESEARCH COUNCIL
PLANT PROTECTION RESEARCH INSTITUTE
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FINAL REPORT

Ref: PPRI-11/17

Identification of *Mgenia fuscovaria* and other vine associated leafhoppers by cytochrome oxidase 1 gene nucleotide sequence determination.

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FINAL REPORT
2013

Programme & Project Leader Information

	Research Organisation Programme leader	Project leader
Title, initials, surname	Prof. Gerhard Pietersen	Prof. Gerhard Pietersen
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Project Information

Research Organisation Project number	PPRI 11/17
Project title	Identification of *Mgenia fuscovaria* and other vine associated leafhoppers by cytochrome oxidase 1 gene nucleotide sequence determination.

Fruit kind(s)	Wine Grapes		
Start date (mm/yyyy)	01-04-2012	**End date** (mm/yyyy)	31-03-2013

Project keywords	*Mgenia fuscovaria*, leafhopper, Mitochondrial cytochrome oxidase 1 sequence,

Approved by Research Organisation Programme leader (tick box) ☐

THIS REPORT MUST INCLUDE INFORMATION FROM THE **ENTIRE** PROJECT

Executive Summary

Currently identification of the leafhopper *Mgenia fuscovaria*, a vector of aster yellows phytoplasma (AY) is done based on morphology, especially of the male genitalia, by a highly experienced taxonomist. Currently female leafhopper, nymphs, and deposited eggs cannot be identified by this means and controlled transmissions with nymphs confirmed as *M. fuscovaria* cannot be done. Also very limited numbers of individuals can be identified by the labour intensive morphological determination, and other than by Mike Stiller, the leafhopper taxonomist, cannot be done elsewhere. We have developed a technique to identify *M. fuscovaria* based on determining the sequence of the mitochondrial cytochrome oxidase I (mtCOI) gene that will allow Mike Stiller and researcher on related AY project to identify large numbers of individual males, females and nymphs, allowing transmission studies and biological studies to be conducted. The mtCOI sequence of *M. fuscovaria* was determined based on the sequence of five adult males. Two nymphs putatively identified as *M. fuscovaria* (as they were collected on the same occasion from the same site as the *M. fuscovaria* adults) were shown to have a sequence identical to that of the *M. fuscovaria* adult males. An additional *Mgenia* species, also found in grapevines from Robertson, was shown to have a mtCOI sequence differing from that of *M. fuscovaria* and was identified as *M. angusta.* The *M. angusta* mtCOI sequence is based on the near-identical sequences of nine males and four females. Phylogenetic trees, based on the mtCOI sequence of various leafhoppers show that the mtCOI sequence separates the two *Mgenia* species will be useful for identification of individuals.

Problem identification and objectives

Identification of the leafhopper *Mgenia fuscovaria*, a vector of aster yellows phytoplasma (AY) is currently done by a highly experienced taxonomist, Mr. M. Stiller (ARC-PPRI). Identification is based on insect morphology, especially of the male genitalia. Currently female leafhopper, nymphs, and deposited eggs cannot be identified by this means and controlled transmissions with nymphs confirmed as *M. fuscovaria* cannot be done. Furthermore only limited numbers of individuals can be identified by the labour intensive morphological determination, and other than by Mike Stiller, cannot be done elsewhere. In this project we proposed to develop a technique to identify the leafhopper based on the sequence of the mitochondrial cytochrome oxidase I (mtCOI) gene and to develop a method of *M. fuscovaria* identification that will allow Mike Stiller and other workers on AY to identify large numbers of individuals, females and nymphs allowing transmission studies and biological studies to be conducted. The technique will be utilised to identify specimens of *M. fuscovaria* and initially was also planned to use for the determination the distribution of this leafhopper in the wine grape production areas of South Africa, in order to gauge the potential of spread of AY. This latter aim however is similar to that of Dr. Andre' de Klerk and it was agreed amongst all parties that this would not be pursued in the current project. The leafhopper identification technique (which will be based on direct sequencing of the mtCOI gene) was also compared to other leafhopper species associated with vines, which, in this project were also first differentiated morphologically.

Workplan (materials and methods)

Samples
Putative *Mgenia fuscovaria* individuals were collected by Dr. Andre de Klerk and Prof. Kerstin Krüger from various sites in the Western Cape. These were placed in 70% ethanol and the collection site, date and other collection data recorded. The samples were submitted to Mr. Michael Stiller at the Agricultural Research Council-Plant Protection Research Institute (ARC-PPRI) for identification. Identification to species level was only possible with adult males as the male genitalia provide the species determining features. Morphogroups of putative *M. fuscovaria* adult females and nymphs were also identified and individuals separately stored in labelled tubes prior to DNA extraction, PCR and sequencing.

Identification by morphology
Morphology of the male sex organ is used to distinguish species of leafhoppers. The last few segments of the abdomen of specimens are cut off and soaked in cold KOH until clear and examined under a stereo light microscope. The remainder of the specimen is stored for DNA extraction and PCR.

DNA extraction
DNA was extracted from the remains of the same insect individuals as had been identified morphologically and putatively classified as male, female and nymphs members of *M. fuscovaria*. A number of replicates of individual specimens of the different classes were extracted to assess inter-specific variation of the mitochondrial cytochrome oxidase 1 gene (mtCOI) sequence.

Total DNA was extracted from individual insect specimens following a slightly modified version of CTAB extraction method described by Doyle and Doyle (1990). The insect remains were macerated in micro-tubes with a micro-pestle containing 200µl 2% CTAB with freshly added 0.2% mercapto-ethanol. Following this a further 200µl 2% CTAB/0.2% mercapto-ethanol was added and the mixture incubated at 65˚C for 30 min whilst shaking. An equal volume (400µl) of chloroform was added and gently mixed, after which it was centrifuged for 10 min at 11000g. Supernatant was recovered and again treated with chloroform as above. The supernatant was treated with an equal volume of iso-amyl alcohol. Tubes were then centrifuged at 12000g for 30 min at 10˚C. The supernatant was discarded and 1 ml of 70% ethanol was added to the pellet, mixed and tubes centrifuged at 12000g 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 20min. Pellets were re-suspended in 50ųl TE buffer and stored at -80˚C.

PCR
Mitochondrial cytochrome oxidase 1 genes were amplified using the primers described by Folmer et al., (1994) (LCO1490: 5'-ggtcaacaaatcataaagatattgg-3' and HC02198: 5'-taaacttcagggtgaccaaaaaatca-3'). Insect DNA extract (1µl) was added to a final PCR reaction volume of 50ųl consisting of 25µl 2X Dream Taq Green PCR mastermix (ThermoScientific, Waltham, MA, USA), 5 µl of a 10µM solution of each primer, and 15µl molecular grade H_2O (Sigma-Aldrich, St. Louis, MO, USA). PCR cycling reaction was performed on a T100™ Thermal Cycler (Bio-Rad, CA, USA). Cycling conditions were set up as follow; initial denaturation at 94˚C for 4 min, followed by 35 cycles of denaturation at 92˚C for 60s, annealing at 52˚C for 60s and elongation at 72˚C for 90s, with a final elongation step at 72˚C for 10 min.

Sequencing
To remove single stranded DNA from PCR products, 0.5µl of 10 U exonuclease (Fermentas, Maryland, USA) and 2µ of 2U FastAP® (Fermentas, Maryland, USA) was added to amplification products and reaction was carried out as per manufacturer's instructions. Purified amplicons were sequenced in both directions using the Folmer et al., 1994 primers in a Big Dye® Terminator v3.1 Cycle sequencing kit (Applied Biosystems, Foster City, USA) as per manufacturer's instructions. Sequences of amplicon products were determined using an ABI 3500xL automated sequencer (Applied Biosystems, Foster City, CA) at the core sequencing facility of the University of Pretoria, South Africa.

Analysis
The nucleotide sequence data was analysed and compare with leafhopper data available on Genbank and at the Barcode of Life database (August, 2012). CLC Main Workbench Ver 6 (CLC Bio, Aarhus N., Denmark) was used to do sequence assembly, trimming and alignments, as well as homology tree production.

References

Doyle, J. J., and Doyle, J. L., 1990. Isolation of Plant DNA from fresh tissue. Focus 12:13-15

Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R: DNA primers for amplification of mitochondrial cytochrome C oxidase subunit I from diverse metazoan invertebrates. Mol Mar Biol Biotechnol 1994, 3:294-299

Results and discussion

Fifty males and 35 females of the two species of *Mgenia* were examined and the individual remains placed in individual labelled tubes for DNA extraction and PCR. The males of *M. fuscovaria* was shown to have a large and blade-like sex organ, while in the other two species of *Mgenia* (*M. angusta* and *M. capeneri*) it is generally known to be 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, we found that one of these parts has a row of teeth that which number 10-12 teeth in *M. fuscovaria.* A second *Mgenia* species, *M. angusta,* also found in grapevine during this study (de Klerk, *unpublished data*) had 8-9 teeth. No differences in the colour or shape of the body, or wing veins, between the two were found.

The sequence of mtCOI from *M. fuscovaria* (Appendix 1) was determined using the consensus of five male individuals. The mtCOI sequence of putative *M. fuscovaria* nymphs (collected at the same local and occasion where *M. fuscovaria* adults were collected) was identical to that of *M. fuscovaria* males. Thus far useful sequences to *M. fuscovaria* females have not been generated.

The mtCOI nucleotide sequence of *M. angusta* (appendix 1) was determined using nine male and 4 female specimens. The sequence could be used to differentiate *M. fuscovaria* and *M. angusta* individuals (Figure 1)

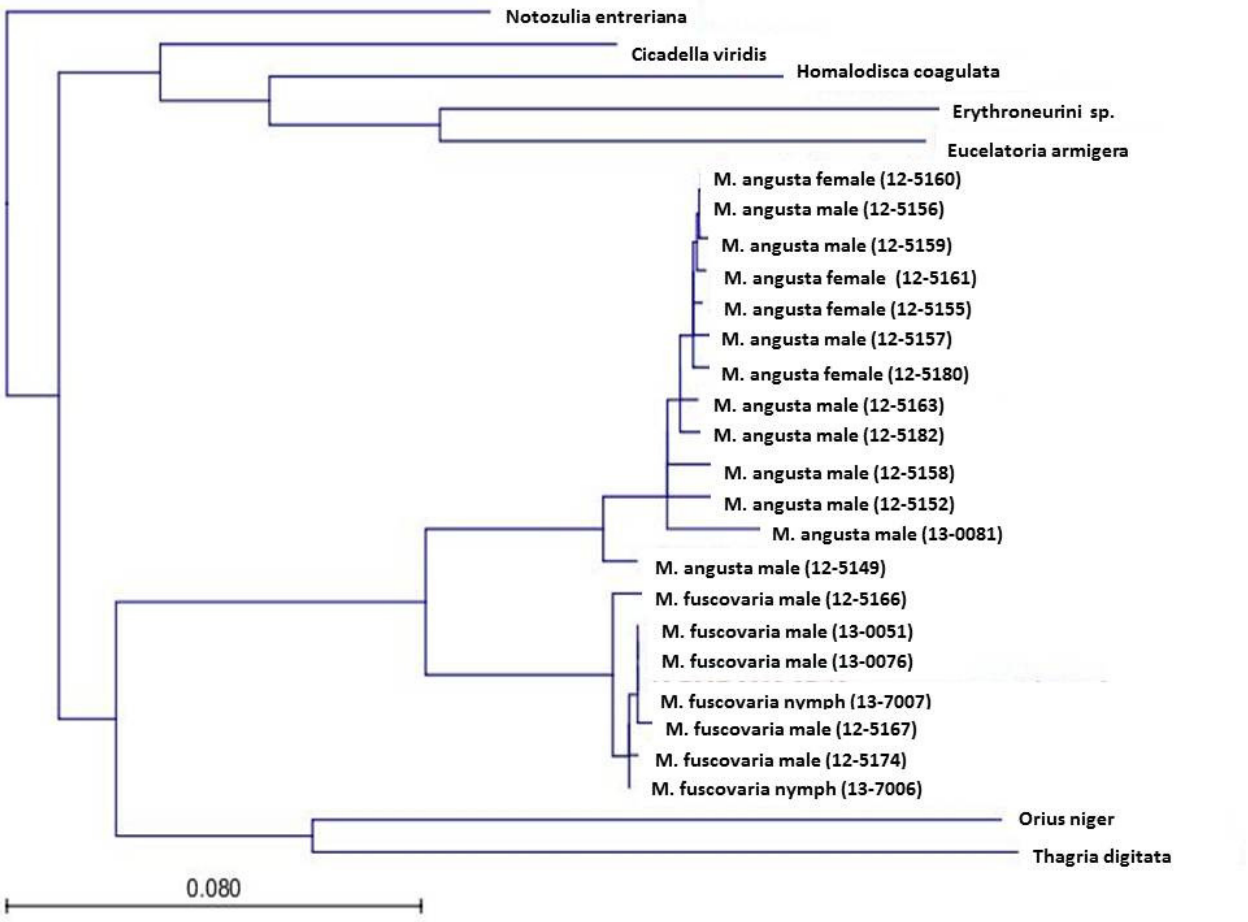

Figure 1: Maximum likelihood phylogenetic tree of the mitochondrial cytochrome oxidase subunit 1 gene (mtCOI) of *Mgenia fuscovaria, M. angusta* and the closest leafhopper sequences currently available on Genbank (as determined by BLAST analysis. Tree produced by UPGMA program, Jukes Cantor substitution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

Complete the following table

Milestone	Target Date	Extension Date	Date Completed	Achievement
1. Establish and optimise mtCOI PCR	October, 2012		October, 2012	Done, PCR works well and appears specific to mtCOI of insects, as published.
2. Collect putative *M. fuscovaria* from various sites in areas in which it has been found (Vredendal, Robertson and Wamakersrivier).	June, 2012.		March, 2013.	Obtain Mgenia specimens from both Dr. A de Klrek and Prof. K. Kruger, from Robertson and from Vredendal areas.
3. Identify *M. fuscovaria* adult male specimens by conventional morphological descriptions. Sort females and nymphs into morphogroups	June 2012		March, 2013	50 males, and 35 females of *Mgenia* , and a number of unidentified nymphs were examined for identification by morphology.
4. Determine sequence of putative *M. fuscovaria* females and nymphs and confirm relationship with male sequence	December, 2012		April, 2013	The sequence of mtCOI from *M. fuscovaria* was determined using the consensus of five male individuals. The mtCOI sequence of putative *M. fuscovaria* nymphs (collected at the same local and occasion where *M. fuscovaria* adults were collected) was identical to that of *M. fuscovaria* males. Thus far useful sequences to *M. fuscovaria* females have not been generated.
5. Determine sequence of other vine-associated leafhoppers.	April, 2013		April, 2013	The mtCOI nucleotide sequence of *M. angusta* was determined using nine male and 4 female specimens. The sequence could be used to differentiate *M. fuscovaria* and *M. angusta* individuals.
6. Analyse all the sequence data.	April, 2013		April, 2013	Partially completed. mtCOI sequences to *M. fuscovaria* and *M. angusta* males, females and nymphs must be deposited in Genbank
5. Journal publication/s – final milestone	July, 2013		July, 2013	Still to be finalised.

Accumulated outputs

Establishment of the Insect universal barcoding PCR system directed at amplification of the mitochondrial cytochrome oxidase I gene (mt COI) (2012)
Determine the Mgenia fuscovaria mtCOI gene nucleotide sequence (2012)
Determine the Mgenia angusta mtCOI gene nucleotide sequence (2012)

Conclusions

The mitochondrial cytochrome oxidase 1 gene sequence of *Mgenia fuscovaria* has been determined, as well as that of *Mgenia angusta*, a related grapevine associated leafhopper. These sequences differ sufficiently that species identification can be based on the sequence of the mtCOI sequence of the two species. While sequences from *M. fuscovaria* females and *M. angusta* nymphs are still lacking, identity within *M. fuscovaria* males and nymphs, and *M. angusta* males and females suggested that the sequences generated can be used to identify *M. fuscovaria* and *M. angusta* males, females and nymphs.

Technology development, products and patents

None

Suggestions for technology transfer

Submission of sequence of *Mgenia fuscovaria* and *M. angusta* to Genbank. Training of Mr. Stiller in doing PCR and sequencing.

Human resources development/training

Student level (BSc, MSc, PhD, Post doc)	Cost to Project
1. Gert Pietersen (BSc)	R10000
2. Inge Pietersen (BSc)	R1000

Publications (popular, press releases, semi-scientific, scientific)
None

Presentations/papers delivered
None

Total cost summary of the project

TOTAL COST IN REAL TERMS	COST	CFPA	DFTS	Deciduous	SATI	Winetech	THRIP	OTHER	TOTAL
YEAR 1						R80000		R24000	R104000
YEAR 2									
YEAR 3									
YEAR 4									
YEAR 5									
TOTAL									**R104000**

Appendix 1:

mtCOI sequence of *Mgenia angusta* male (Representative individual 12-5157)

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        1 ACTAATTCGT ATTGAACTTA GACAACCAGG ATCATTTTTA ATAAACGATC AATTATATAA
       61 CGTTTTAGTT ACTATACACG CATTTATTAT AATTTTCTTC ATAGTTATAC CAATCATAAT
      121 TGGTGGATTT GGAAATTGAT TAATTCCAAT AATGATTGGA GCACCCGATA TAGCATTTCC
      181 ACGAATAAAC AATATAAGAT TTTGATTATT ACCCCCATCA ATTATTATAT TATTATCAAG
      241 ATCAATAGTT GAAACAGGAT CAGGTACAGG ATGAACAATT TACCCGCCTT TATCTTCCAA
      301 TATTGCACAC TCAAGAATAA GAGTTGATTT AACAATCTTC TCCCTTCATA TAGCAGGGAT
      361 CTCTTCAATT TTAGGTGCAA TTAATTTCAT TACAACAATT ATTAATATAC GAATAATAGG
      421 AATAAGATAT GATCGAGCTC CATTATTTGT ATGATCAATT CTAATTACAG CAATNCTATT
      481 AATAATTTCA TTACCAGTAT TAGCTGGTGC AATTACAATA TTATTAACAG ATCGAAACTT
      541 AAATACATCA TTTTTTGACC CATCAGGTGG GGGNGATCCA ATTTTATATC AACATTTATT
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mtCOI sequence of *Mgenia fuscovaria* male (Representative individual 13-0076)

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        1 ATTAATTCGT ATTGAATTAA GACAACCAGG ATCATTTTTA ATAAACGATC AATTATATAA
       61 TGTATTAGTA ACTATACATG CATTTATTAT AATTTTTTTT ATAGTTATAC CAATCATAAT
      121 TGGTGGATTT GGAAATTGAT TAATTCCAAT AATAATTGGA GCACTGATAT AGCATTTCCA
      181 CGAATAAATA ACATAAGATT TTGATTACTT CCTCCATCAA TTATCCTATT ATTATCAAGA
      241 TCAATAATTG AAACAGGGTC AGGAACAGGA TGAACAATTT ACCCCCCATT ATCATCAAAT
      301 ATTGCACATT CAAGAATAAG AGTTGATTTA ACAATTTTCT CTCTTCATAT GGCAGGGATC
      361 TCTTCTATTT TAGGTGCAAT TAACTTTATT ACAACAATTA TCAATATACG AATAATAGGA
      421 ATAAGTTATG ACCGAACTCC ATTATTTGTT TGATCAATTT TAATTACAGC AATCCTATTA
      481 ATAATTTCAT TACCAGTATT GGCTGGTGCA ATTACTATAT TATTAACAGA TCGAAACTTA
      541 AAATACATCA TTTTTTGACC CATCAGGTGG TGGTGATCCA ATTTTATTTC AGCACTTATT
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