

Full Length Research Paper

The groundnut leaf miner collected from South Africa is identified by mtDNA CO1 gene analysis as the Australian soybean moth (*Aproaerema simplixella*) (Walker) (Lepidoptera: Gelechiidae).

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Although the leaf miner attacking groundnut in Africa is widely reported as *Aproaerema modicella* (Deventer), a common groundnut (*Arachis hypogaea* L.) and soybean (*Glycine max* (L.) Merr.) pest in Indo-Asian countries, a proper taxonomic identification of the pest has not been done. A detection survey of the pest was conducted on groundnut, soybean and lucerne (*Medicago sativa* L.), the common host crops for *A. modicella*, at six widely separated sites in South Africa during the 2009-2010 growing season. Sixty specimens comprising 24 larvae, 24 pupae and 12 moths of GLM (54 from groundnut; six from soybean) were collected from the six survey sites, and their mitochondrial DNA (mtDNA) were sequenced and compared with those from the BOLD gene bank. Infestation by GLM was observed on groundnut and soybean, but not on lucerne. The mtDNA from all specimens of the pest, irrespective of whether they were from groundnut or soybean, matched 100% with the sequences in BOLD belonging to *Aproaerema simplixella* PS1, a strain also occurring in Australia, where it is a soybean pest. There was very little genetic diversity between and within the populations from the six sites, which suggested that the populations were maternally of the same origin.

Key words: *Arachis hypogaea* L., *Aproaerema modicella*, *Glycine max* (L.) Merr., lucerne, mitochondrial DNA.

INTRODUCTION

Groundnut (*Arachis hypogaea* L.) and soybean (*Glycine max* (L.) Merr.) are major legume crops grown for food and commercial purposes in most African countries. The production of these crops on the continent is seriously threatened by a leaf miner, widely reported as *Aproaerema modicella* (Deventer) (Lepidoptera: Gelechiidae) (Kenis and Cugala, 2006). First noticed as a serious pest on groundnut in 1998 in Uganda and described as groundnut leaf miner (GLM), the pest has raised considerable alarm and concern in the groundnut production industries of Malawi (Subrahmanyam et al.; 2000), Uganda (Page et al., 2000; Epieru, 2004), Mozambique

(Kenis and Cugala, 2006), Democratic Republic of Congo (Munyuli et al., 2003) and South Africa (Du Plessis, 2002). In South Africa, GLM is known to be present in all groundnut producing areas of the country (Du Plessis, 2003). However, the epidemic of GLM on groundnut in South Africa is sporadic and its severity appears to vary from place to place and from year to year, making it extremely difficult to predict. As a new pest, not much information is available on its ecology and ecophysiology that might help to predict its incidence and outbreaks.

The identity of the GLM in Africa, including South Africa, has generally been assumed to be *A. modicella* (Subrahmanyam et al., 2000; Page et al., 2000; Du Plessis, 2002; Munyuli et al., 2003; Epieru, 2004; Kenis and Cugala, 2006) although Shanower et al. (1993) has hinted that it might be a different species. Since no proper taxonomic identification has been done on this

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Table 1. Description of survey sites where GLM inspection and sample collection was done.

Province and inspection site	Climatic description			Plants inspected
	Mean annual rainfall (mm)	Summer temperature range (°C)	Winter temperature range (°C)	
Northern Cape Province				
Vaalharts (27° 95' 761" S; 24° 83' 991" E)	300 - 450	16 - 32	1 - 18	Groundnut, lucerne
North West Province				
Brits (25° 59' 135" S; 27° 76' 875" E)	300 - 700	22 - 34	15 - 20	Groundnut, soybean, lucerne
Potchefstroom (26° 73' 607" S; 27° 07' 553" E)	360 - 507	18 - 34	2 - 18	Groundnut, soybean
KwaZulu-Natal Province				
Manguzi (26° 95' 532" S; 32° 82' 356' 'E)	600 - 700	23 - 35	17 - 27	Groundnut
*Bhekabantu	-	-	-	Groundnut <i>Indigofera</i> spp.
Mpumalanga Province				
Nelspruit (25° 45' 452" S; 30° 97' 154" E)	500 - 780	24 - 30	17 - 24	Groundnut, lucerne

*Climatic data is not available because there is no weather station near the site.

new pest, the adoption of the name *A. modicella* was probably based on morphological characteristics of the larvae and adults and on crop damage symptoms that are similar to those of *A. modicella* in addition to strong prevalence of the pest on groundnut (Du Plessis, 2002, 2003; Kenis and Cugala, 2006). Van der Walt et al. (2008) examined the gonads of the female and male larvae of the GLM specimens collected in South Africa, and concluded that they were similar to those reported for *A. modicella* in Asia by Shanower et al. (1993), which reinforced the assumption that the pest was *A. modicella*.

Because of its sudden appearance, the GLM occurring in Africa is thought to be a recent invasion from the Indo-Asian continent (Kenis and Cugala, 2006) where *A. modicella* is native and seriously infests groundnut and soybean (Shanower et al., 1993). Whilst this is possible, the pest may have evolved and spread within Africa.

Whereas morphological studies have been the keystone of insect pest identification in the past, and continue to be in the present, modern molecular techniques offer complementary, faster and more precise options for species identification (Scheffer, 2000), and is especially useful in differentiating related species that share similar morphological characteristics. In addition, molecular techniques, e.g. DNA finger printing, especially those involving the mitochondrial DNA (mtDNA), are more reliable in pinpointing or tracing the geographical origin/links of pests and their paths of spread, (Scheffer, 2000; Simmons and Scheffer, 2004).

There were three objectives in the present study. The first was to build an alternative-host crop/plant list of the GLM occurring in South Africa. The second objective was to identify the species of the pest and the third objective

was to determine its inter and intra-population genetic diversity by analysing in both cases the mtDNA CO1 gene of specimens collected from widely separated sites.

MATERIALS AND METHODS

Detection survey and specimen collection sites

A detection survey involving three visits to the same sites was undertaken to determine the presence of GLM on groundnut and alternative host crops/plants in six locations (Table 1) in the North West, Northern Cape, Mpumalanga and KwaZulu-Natal provinces of South Africa.

The first visit was done between 11 and 16 January 2010, the second between 22 and 27 March 2010 and the last between 24 and 29 October 2010. In the North West province, the sites included the Agricultural Research Council research stations at Potchefstroom and Brits as well as farms surrounding the Brits research farm. In Northern Cape, the inspection site was the Vaalharts Research Station. In Mpumalanga, the inspection site was the Lowveld Agricultural Research Station near Nelspruit. In KwaZulu-Natal, the inspections were done at Bhekabantu and Manguzi in the northern part of the province.

The latter is unique from the other sites in that it is warm throughout the year and groundnut can thus be planted throughout the year. Being coastal, Manguzi is also expected to have higher humidity than the other sites.

Infestation recognition and specimen collection

The survey included visual inspection of old and young leaves of groundnut, soybean, lucerne (*Medicago sativa* L.) and any other known hosts of *A. modicella* for infestation by the pest, and the collection of GLM larvae and pupae for DNA analyses. During the survey, the presence of the pest and the damage symptoms on the crop were searched for. In the first survey visit, in addition to visual

Table 2. System used in the assigning of specimen identity in Figure 2.

Area	Specimens used for DNA analysis	Specimen identity
Manguzi	3 pupae	Man 1
	3 larvae	
	6 adults	
Bhekabantu	3 larvae	Man 2
	3 adults	
Vaalharts	9 pupae	Vaal, VD
	3 larvae	
Potchefstroom	3 pupae (soybean)	Pots*
	3 larvae (soybean)	
	3 pupae (groundnut)	Pot
	3 larvae (groundnut)	
Brits	3 pupae	Brits
	4 larvae	
	2 adults	
Nelspruit	6 larvae	Nel
	3 adults	

*All GLM specimens were collected from groundnut except at Potchefstroom where some specimens (Pots) were collected from soybean.

inspection of the groundnut plants and other host plants at each site, five specimens each of larvae and pupae were removed from infested groundnut (all survey sites) or soybean (Potchefstroom) plants and immediately placed in 10-ml polycarbonate vials containing absolute ethanol and closed with press-on plastic lids. The vials were taken to a laboratory and stored at minus 80°C in a cryogenic freezer until DNA sequencing. In addition, about 20 pupae per site were placed in a clear 250-ml plastic bottle that was perforated by a dissecting needle in many places to allow free air movement into and out of the bottle. The holes were small (≤ 2 mm in diameter) and not large enough to allow the GLM moths out of the bottles, which were closed with screw-on polycarbonate lids. The bottles containing the pupae were stored in a laboratory at room temperature until moths emerged. After visual inspection of the emerged moths, five of the moths from each site were placed in 10-ml polycarbonate vials containing absolute ethanol and the vials closed with press-on lids. As with vials containing larval and pupae specimens, the vials containing moths were stored at -80°C until DNA sequencing.

DNA analyses

DNA extraction

From the specimens collected at the six survey sites, a total of 60 specimens (9 to 12 specimens per site) comprising pupae, larvae and adults (Table 2) were used for DNA analyses. All the specimens processed for DNA analyses were from groundnut except for three larvae and three pupae that were collected from soybean at Potchefstroom. The specimens were identified in relation to the area from which they were collected as shown in Table 2. The DNA was extracted from the specimens following the method of McPherson et al. (1991). The specimens were added

individually to 500 μ l Buffer PL1 of the NucleoSpin PlantII kit (Macherey-Nagel) and 2 μ l of 10 mg/ml proteinase K (Sigma-Aldrich), homogenised using the TissueLyser (Qiagen) and incubated overnight at 60°C. The samples were then centrifuged at 6.0 relative centrifugal force for 20 min. The rest of the protocol was performed on a robotic platform (Genesis RMP200 (Tecan)). A total of 400 μ l supernatant was mixed with 450 μ l binding buffer PC and transferred to a silica membrane plate. The mixture was pulled through the membrane by a vacuum system. The bound DNA was washed to remove proteins and salts with 400 μ l buffer PW1 and twice with 700 μ l buffer PW2. The bound DNA was eluted twice with 100 μ l volumes of elution buffer preheated to 70°C.

DNA amplification and sequencing

DNA amplification by PCR was performed with the primers Ron and Nancy. The PCR conditions were as follows: 1x KAPA Robust Ready Mix (KAPA Biotech), 1x Enhancer A, 0.4 μ M of each primer and 20 ng DNA. The PCR was performed in a verity PCR-cycler (Applied Biosystems) with the following conditions: 95°C for 5 min followed by 40 cycles of 95°C at 30 s, 55°C at 60 s and 72°C for 90 s and a final extension of 72°C for 10 min. Post-PCR purification was done using the NucleoFast Purification System (Separations). Sequencing was performed with each primer and BigDye Terminator V1.3 (Applied Biosystems) followed by electrophoresis on the 3730xl DNA Analyser (Applied Biosystems). Sequences were analysed using the Sequencing Analysis Version 5.3.1 software (Applied Biosystems).

Editing of DNA sequences

DNA sequences were manually edited (for base calling errors) pruned

and aligned by ClustalW using the BioEdit Sequence Alignment Editor (Hall, 1999) to create consensus sequences which were saved in the fasta format in MEGA5 (Hall, 1999).

Determining evolutionary relationships

The evolutionary history was inferred using the Neighbour-Joining method (Saitou and Nei, 1987) with bootstrap analysis based on 1000 replicates (Felsenstein, 1985). A phylogenetic tree was constructed based on the neighbour joining method. The evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980) and are in the units of the number of base substitutions per site. The rate variation among sites was modelled with a gamma distribution (shape parameter = 1). The analysis involved 60 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 363 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura et al., 2011).

Additionally, all consensus sequences were entered in BOLD to positively identify species. All specimens were identified to be from the same species, except one sample, which was identified to be from a different species, and was therefore used as an outgroup in the analysis. Additionally, the sequences were also exposed to Multiple Sequence Alignment by ClustalW (<http://www.genome.jp/tools/clustalw/>) to verify level of similarity between samples.

RESULTS

Host plants

All the groundnut crops inspected at the six survey sites were infested by GLM, and so were the soybean crops inspected at Vaalharts, Potchefstroom and Brits. In contrast, GLM infestation was absent from all lucerne crops inspected at Nelspruit, Brits and Vaalharts. At Vaalharts, this was despite volunteer lucerne plants growing on the edges of groundnut fields infested by GLM. At Bhekabantu only two GLM larvae were observed on a plant of an *Indigofera* species, even though there were a number of plants of the same species within 5 m of a groundnut crop heavily infested with GLM.

Crop damage symptoms

The symptoms of damage found on the groundnut leaves mirrored those described for GLM in Mozambique and elsewhere (Kenis and Cugala, 2006; Iavanya, 2009). The symptoms varied with time of season and growth stage of the crop (Figure 1). Early in the growth season, the mines are not large and the larvae produce small necrotic areas mostly in the middle of the leaflets (Figure 1A and B) or a slight folding at the end of a leaflet. Leaf folding and webbing (Figure 1B) may be less visible compared to the mid and late season symptoms. In late growth stages of the groundnut crop, the affected leaves are severely necrotic and distorted (Figure 1C). In severely affected plants, almost all leaflets are affected/infested (Figure 1D)

or there is complete defoliation (Figure 1E).

Pest description and morphology

The moths, when newly emerged from the pupae, have light-grey coloured wings. As they age, they turn dark grey or brownish and mottled; with dark brown forewings, and pale brown hind wings covered with scales and whitish towards the lower part (Figure 1F). The moth is about 4 to 5 mm long. Larvae were pale green when they were collected small and dark-green when collected bigger, with a shiny black head capsule. Larvae became cream coloured towards pupation. The moths lived for about 6 to 9 days.

Species identification by mtDNA (CO1)

Based on comparisons with published sequences from the BOLD genebank, one sample was identified as possibly *Helicoverpa armigera* (Lepidoptera: Noctuidae) (99.3% match), but the remaining samples (59) were identified as *Aproaerema simplexella* PS1 (Walker) (Lepidoptera: Gelechiidae) (100% match). In addition, the topmost 15 matches after *A. simplexella* PS1 among the sequences available in the BOLD genebank (Table 3) included 11 *A. simplexella* (93.53 to 98.08% match), one *Aproaerema lerauti* (Lepidoptera: Gelechiidae) (93.53% match), two *Aproaerema isoscelixantha* (Lepidoptera: Gelechiidae) (92.81 to 93.05% match) and one *Aproaerema captivella* (Lepidoptera: Gelechiidae) (92.33% match). There was very little genetic diversity within and between the specimens from the six surveyed sites (Figure 2).

DISCUSSION

It has generally been assumed that the GLM occurring on groundnut in Africa has its origins in Asia, with all reports from the African continent assuming the name *A. modicella* (Deventer) for the pest (Kenis and Cugala, 2006; Du Plessis, 2002, 2003). Contrary to this assumption, irrespective of the place or crop (groundnut or soybean) from which the specimens were taken, the mtDNA CO1 sequences of the GLM specimens examined in this study matched 100% with those of *A. simplexella* PS1 (previously *Stomopteryx subsecivella* (Ziller)) (Bailey, 2007). This particular strain of *A. simplexella* is thought to be native to Australia where it is reported to be a pest for soybean (Common, 1990; Bailey, 2007). The evidence obtained from DNA analysis in the present study therefore suggests that the GLM in South Africa may have links with Australia. This is further supported by the fact that all GLM specimens taken from the six widely separated sites in South Africa were identified as *A. simplexella* PS1, with *A. modicella* not

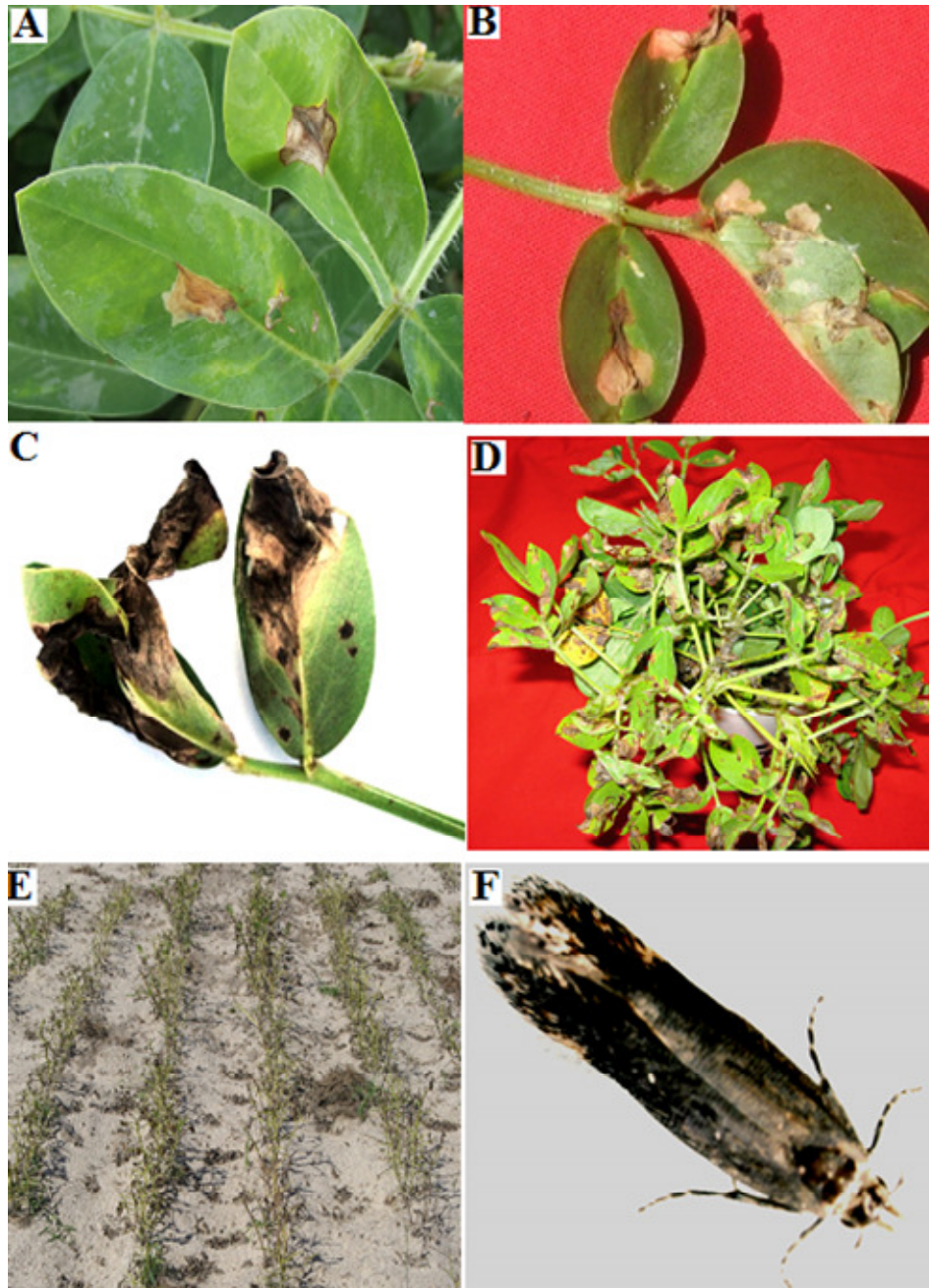


Figure 1. Pictogram showing (i) symptoms of GLM infestation in groundnut; early season leaf symptoms (A, B), late season symptoms (C), whole plant symptoms (D) and crop defoliation (E), and (ii) the adult groundnut leaf miner moth (F); the necrotic bubble/blotches in the middle of leaflets is shown in (A), the folding and webbing of leaflet is shown in (B) and the extensive necrosis of leaflets is shown in C.

listed in the most closely related species (Table 3). This infers that all infestations of GLM in Africa may be caused by the former, and not the latter species. Based on morphological characteristics, Shanower et al. (1993) suggested that the species found in Africa may be different from that found in India or Indonesia, describing the GLM in India as *Anacampsis nerteria* (Meyr.) (Meyrick, 1906), the one in Africa as *Stomopteryx*

subsecivella and another in India-Indonesia as *A. modicella* (Deventer). It is thus clear that a large degree of uncertainty has always existed as to the correct classification of GLM in Africa. No attempt has however been made to discriminate between the species genetically.

Previous to our DNA analysis, *A. simplexella* PS1 was known to be present only in Australia (Common, 1990;

Table 3. The 16 topmost matches of mtDNA of groundnut leaf miner specimens collected from South Africa with sequences from BOLD Genbank.

Phylum	Class	Order	Family	Genus	Species	Specimen similarity (%)
Arthropod	Insecta	Lepidoptera	Gelechiidae	<i>Aproaerema</i>	<i>Simplixella</i> PS1	100
Arthropod	Insecta	Lepidoptera	Gelechiidae	<i>Aproaerema</i>	<i>Simplixella</i>	98.08
Arthropod	Insecta	Lepidoptera	Gelechiidae	<i>Aproaerema</i>	<i>Simplixella</i>	97.84
Arthropod	Insecta	Lepidoptera	Gelechiidae	<i>Aproaerema</i>	<i>Simplixella</i>	97.84
Arthropod	Insecta	Lepidoptera	Gelechiidae	<i>Aproaerema</i>	<i>Simplixella</i>	97.84
Arthropod	Insecta	Lepidoptera	Gelechiidae	<i>Aproaerema</i>	<i>Simplixella</i>	97.84
Arthropod	Insecta	Lepidoptera	Gelechiidae	<i>Aproaerema</i>	<i>Simplixella</i>	97.84
Arthropod	Insecta	Lepidoptera	Gelechiidae	<i>Aproaerema</i>	<i>Simplixella</i>	97.84
Arthropod	Insecta	Lepidoptera	Gelechiidae	<i>Aproaerema</i>	<i>Simplixella</i>	97.84
Arthropod	Insecta	Lepidoptera	Gelechiidae	<i>Aproaerema</i>	<i>Simplixella</i>	97.79
Arthropod	Insecta	Lepidoptera	Gelechiidae	<i>Aproaerema</i>	<i>Simplixella</i>	97.73
Arthropod	Insecta	Lepidoptera	Gelechiidae	<i>Aproaerema</i>	<i>Simplixella</i>	97.36
Arthropod	Insecta	Lepidoptera	Gelechiidae	<i>Aproaerema</i>	<i>Simplixella</i>	93.53
Arthropod	Insecta	Lepidoptera	Gelechiidae	<i>Aproaerema</i>	<i>Lerauti</i>	93.53
Arthropod	Insecta	Lepidoptera	Gelechiidae	<i>Aproaerema</i>	<i>Isoscelixantha</i>	93.05
Arthropod	Insecta	Lepidoptera	Gelechiidae	<i>Aproaerema</i>	<i>Isoscelixantha</i>	92.81
Arthropod	Insecta	Lepidoptera	Gelechiidae	<i>Aproaerema</i>	<i>Captivella</i>	92.33

Bailey, 2007). Now, given the presence of this strain of *A. simplexella* in Africa, it is at present difficult to tell which between Africa and Australia is the native continent of the pest. However, the sudden visibility of the pest in Africa points to the possibility that it is a recent invasion to Africa, and this appears to be confirmed by lack of intra- and inter-population diversity in the mtDNA CO1 gene among the specimens collected in the present study (Figure 2).

The distribution range of *A. simplexella* PS1 in Australia covers almost all of the country (Common, 1990; Bailey, 2007). Even though groundnut is a major crop in Australia, *A. simplexella* PS1 has not been reported to attack groundnut in Australia. In that country, *A. simplexella* PS1 is generally regarded as a minor pest for soybean, and is commonly known there as a soybean moth (Common, 1990; Bailey, 2007). This suggests that the pest has stronger preference for soybean than for groundnut in Australia. In contrast, although it has been noted to infest soybean in South Africa (in this study), the pest has so far not been reported to be serious on that crop in South Africa or elsewhere on the African continent. In South Africa, this is despite that soybean (311,000 ha) production far exceeds that of groundnut (150,000 ha) (<http://www.thecropsite.com>). It is therefore surprising that unlike in Australia, the pest has caused severe problems with groundnut rather than soybean in South Africa and in the rest of Africa. Also, whilst lucerne is expected to be one of its alternative hosts (Du Plessis, 2003), our study suggests that it may not be a preferred host as it could not be found on that crop at Vaalharts, Brits and Nelspruit, despite the presence of the pest on groundnut crops nearby. Nonetheless, lucerne and other host plants may play an important role in maintaining a

small population in-between seasons when the groundnut crop is not present.

Conclusion

Mitochondrial DNA CO1 analysis identified GLM in South Africa as *A. simplexella* PS1 (100% match on the BOLD system), thought to be native to Australia, which suggested that Australia may be the origin of the pest. It is most likely that GLM being reported on groundnut in other parts of Africa is *A. simplexella* PS1.

The phylogenetic tree of the specimens of *A. simplexella* PS1 obtained from the six widely separated sites in South Africa indicated that there was very little genetic diversity between and within the populations suggesting that the pest might be from the same origin and could be a recent introduction to South Africa.

Given that the sequences of GLM in South Africa matched those of *A. simplexella* PS1 and the damage symptoms of the pest on groundnut are similar to those of *A. modicella* found in Asia, there is need to determine if the two species are indeed genetically different. This has a bearing on the development and use of groundnut lines resistant to GLM in countries where it is a problem. For the purpose of formulating strategies of managing the pest, there is also a need to determine the host range of the pest as well as its in-between season survival tactics in Africa.

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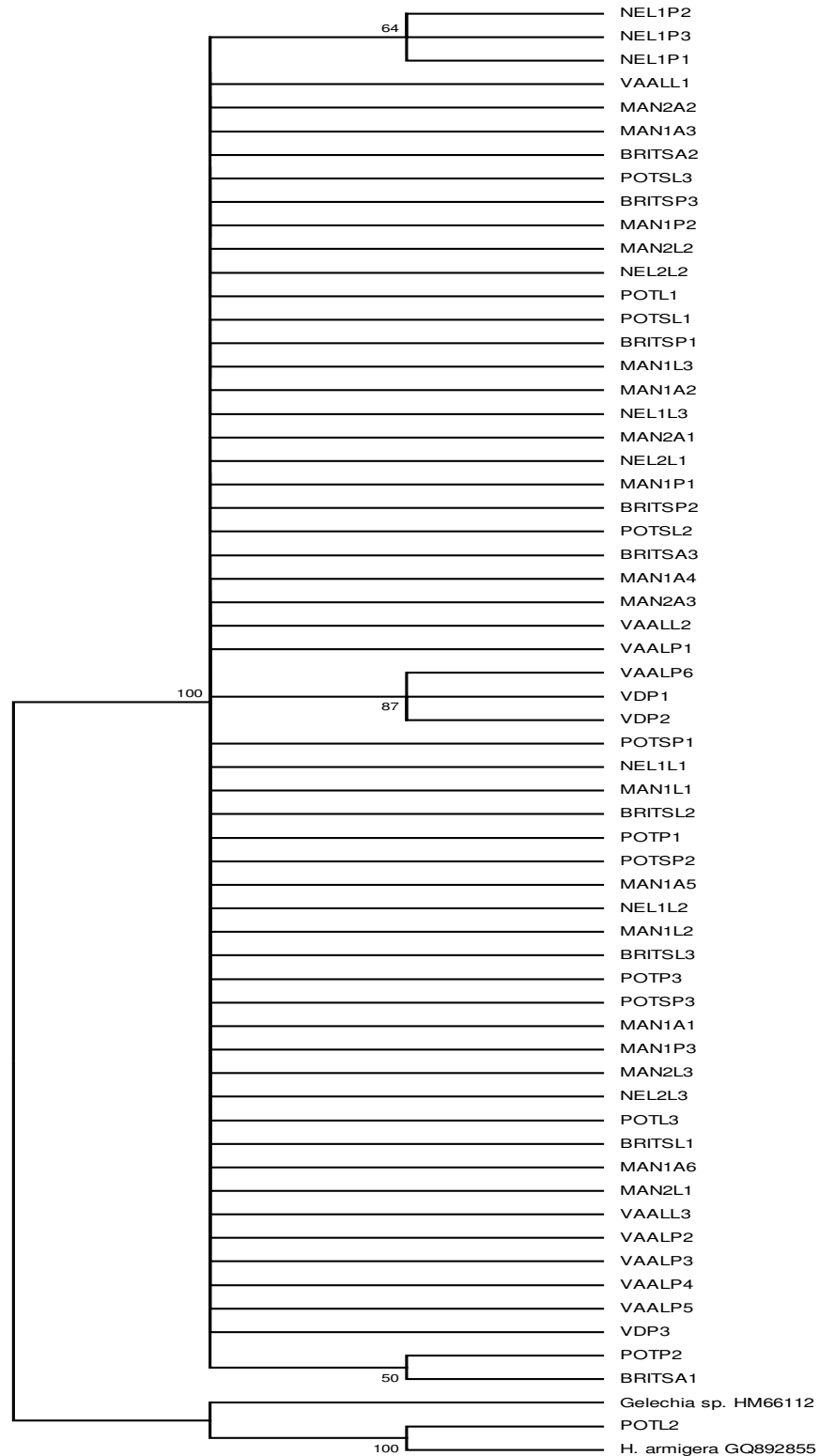


Figure 2. Phylogenetic relationships based on mtDNA COI regions of groundnut leaf miner identified as *Aproaerema simplexella* PSI specimens collected from six survey sites. The names on taxa positions reflect the sampling areas (Vaal and VD denote Vaaharts; Brits, Pot, Nel, Man 1 and Man 2 denote Brits, Potchefstroom, Nelspruit, Manguzi and Bhekabantu respectively) and whether the specimen was larva (L), pupa (P) or adult (A). Numbers at the nodes represent bootstrap proportions (50% or more; 1000 replicates). Numbers after species names indicate Genebank accession numbers.

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