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METHODS FOR ACCURATE IDENTIFICATION OF CANEGRUBS

by

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SUMMARY

Canegrubs are the most important pests affecting Australian sugarcane. Implementation of appropriate and effective control measures is dependent on accurate identification of the species involved. This is because species differ in their life histories, susceptibility to insecticides and distributions. Methods for identification have been inadequate with no recent, serious attempt to survey morphological markers and to validate these genetically.

We determined that most of the morphological characters of larvae are fairly conservative. The exception is the raster pattern that allows separation of many of the species. Where interspecific differences in the raster patterns are subtle, we used head width, geographic distributions and soil-type preferences to separate species. Separation of species within the two groups *A. consanguineus/A. rugulosus* and *L. negatoria/L. frenchi/L. noxia* is difficult on raster patterns and impossible where species occur in the same area. Characters for separation of adults are reliable and, in most cases, are easy to observe. All identifications should be checked, as far as possible, by examining the male parameres, the shape of which is very diagnostic.

We developed these findings into multi-access, computer-based keys for identification of larvae and adults using the LucID program. The keys comprise a list of characters, taxa and a database of information on the characteristics of the taxa. The LucID Player is used to make identification with character states selected to match an unidentified specimen. The program queries the database to find all taxa that match the selected characteristics. Successive selection of characters narrows down the list of taxa until only one remains. Once an identification is made, the Player can be used to find information on the species; data on biology, distribution and life history of all species are included in our key.

We validated the specific status of the five morphologically similar species using DNA molecular markers based on the *cytochrome oxidase II* (*COII*) gene found on the mitochondrial DNA (mtDNA). The *COII* gene coalesces to monophyly for each of the species and this monophyly enabled us to develop a robust tool for recognising species boundaries. For example, *A. consanguineus* and *A. rugulosus* had been confused and they were synonymised with *A. mussoni*. We found that 19 base-pair differences distinguish *A. rugulosus* and *A. consanguineus* from one another. To identify morphologically similar species the *COII* gene can be initially amplified by polymerisation chain reaction (PCR). Species-level sequence differences were identified and used to create unique *COII* digestion profiles. The *Antitrogus* species markers were generated by *Mbo II* digestion of the *COII* PCR product, and the *Lepidiota* species markers by *Dra I* and *Stu I.*

As an adjunct to this project, we investigated the population genetics of *Antitrogus parvulus* (Childers canegrub) using the *COII* gene from mtDNA. We surveyed populations of *A. parvulus*, to understand its population dynamics and gene flow. We used base-pair differences to characterise haplotype diversity and divergence; investigate haplotype relationships to one another; and infer their phylogeographic structure. The gene flow of *A. parvulus* is largely phylogeographic and habitat fragmentation appears to be a major influence on the pattern of mtDNA phylogeography.

1.0 BACKGROUND

Amongst the 19 species of canegrubs important within the Australian sugar industry there is variability in lifecycles (1 and 2 years; spring, summer and autumn feeders), in where larvae live within the soil profile, and in susceptibility to insecticides. Before growers can implement integrated management strategies they must be certain of the identity of the target species.

Current methods for the identification of canegrubs are inadequate. The most recent comprehensive treatment was by Dodd in 1917. He provided descriptions of third-instar larvae and a key to species, but only four *Lepidiota/Dermolepida* species are fully named. Nineteen species in the genera *Lepidiota*, *Dermolepida*, *Antitrogus* and *Rhopaea* are known to be associated with sugarcane in Australia. These have been identified using the pattern of spines and hairs on the last ventral abdominal segment (the raster). However, the two complexes of *Antitrogus consanguineus/A. rugulosus* and *Lepidiota negatoria/L. frenchi/L. noxia* are impossible to separate on these characters. Other species can be separated, with varying reliability, on the basis of diagrams of rasters scattered through the literature. A modern, comprehensive treatment of the morphology of canegrubs and their adults is needed.

Advances in molecular methods for distinguishing species allows the rapid identification of morphologically similar immatures without the necessity for rearing through to the adult stage. By developing a reliable method for identifying morphologically similar species we can allow identification at the larval stage that would ensure that pest management measures can be more timely. Before reliable methods for species diagnosis can be devised, it is essential to assess the DNA diversity within each species. Mitochondrial DNA (mtDNA) is well suited for identifying morphologically similar species, as multiple copies are present in each cell and the mutation rate of mtDNA is high enough to provide numerous sequence differences between closely related species. mtDNA has been used to elucidate genetic variation and species limits in a variety of insect species complexes and the *cytochrome oxidase II* gene (*COII*) contains many species-level markers that allow DNA-diagnostic tools to provide a prompt and effective means of species identification. In this project, we used species-level base pair differences in *COII* to develop a PCR-RFLP protocol for species identification. Our aims were to provide an economical and robust technique that uses mtDNA to identify morphologically similar canegrubs and, in so doing, elucidate species relationships using mtDNA sequences.

2.0 OBJECTIVES

- Determine morphological characters for accurate identification of larvae and adults of all species of canegrubs.

- Validate the usefulness of morphological characters through DNA analysis.

- Produce written and computer-based keys to enable extension staff and growers to accurately identify larvae and adults of canegrubs.

3.0 OUTCOMES

- Comprehensive morphological descriptions of larvae and adults of all species of Australian canegrubs.

- Written and computer-based keys for the accurate determination of larvae and adults of all species of Australian canegrubs.

- DNA-based validation of the specific status of *Antitrogus consanguineus*, *A. rugulosus*, *Lepidiota negatoria*, *L. frenchi* and *L. noxia.*

- DNA-based methods for identification of morphologically similar species of canegrubs (*Antitrogus consanguineus* and *A. rugulosus*; *Lepidiota negatoria*, *L. frenchi* and *L. noxia*).

- Use of DNA-based techniques to determine the population genetics of Childers canegrub (*Antitrogus parvulus*).

- Submission of a thesis for the degree of Doctor of Philosophy.

4.0 RESEARCH METHODOLOGY, RESULTS AND DISCUSSION

4.1 Morphology of larvae and adults

Full treatment of the methodology, results and discussion is given in manuscript presented as Appendix 1.

In the larvae, most of the morphological characters examined are fairly conservative. The exception is the raster pattern. The latter gives characters that allow separation of many of the species. Where interspecific differences in the raster patterns are subtle, we used head width, geographic distributions and soil-type preferences to separate species. Separation of species within the two groups *A. consanguineus/A. rugulosus* and *L. negatoria/L. frenchi/L. noxia* is difficult on raster patterns and impossible where species occur in the same area. Molecular methods for separating these species are given in the next section.

Characters for separation of adults are reliable and, in most cases, are easy to observe. All identifications should be checked, as far as possible, by examining the male parameres, the shape of which is very diagnostic.

4.2 Identification of larvae using molecular markers

Despite the morphological study, five species from two groups *Antitrogus rugulosus* and *A. consanguineus*, and *Lepidiota frenchi*, *L. negatoria* and *L. noxia* could not be confidently distinguished. For these species, adults are required for accurate morphological identification. However, rearing larvae to adults is time consuming (up to 2 years) and unreliable, as mortality can be high. The situation is not acceptable when pest management practices depend upon prompt identification.

We developed a molecular diagnostic tool for these species using molecular markers to distinguish the species from one another. We used DNA molecular markers to distinguish these species because these characters are most likely to be consistent over a species range and independent from environmental effects. We selected the *cytochrome oxidase II* (*COII*) gene found on the mtDNA as a diagnostic marker. Mitochondrial DNA is well suited for identifying morphologically similar species as individuals are usually homoplasmic with a single mtDNA sequence predominating in all tissues and is maternally inherited. mtDNA evolves rapidly at the sequence level and most differences between sequences reflect point mutations. Previous studies show that mtDNA can elucidate genetic variation and species limits in a variety of insect species complexes and the *COII* gene contains numerous species-level markers that show that DNA-diagnostic tools can provide a prompt and effective means of species identification.

Firstly, we assessed *COII* gene diversity within each species and confirmed their specific status. We showed that the *COII* gene coalesces to monophyly for each of the species and this monophyly enabled us to develop a robust tool for recognising species boundaries. For example, *A. consanguineus* and *A. rugulosus* had been confused and they were synonymised with *A. mussoni*. We found that 19 base pair differences distinguish *A. rugulosus* and *A. consanguineus* from one another and species monophyly corroborates the morphological and ecological data for *A. consanguineus* and *A. rugulosus*.

To identify morphologically similar species the *COII* gene can be initially amplified by polymerisation chain reaction (PCR). Species-level sequence differences were identified and used to create unique *COII* digestion profiles. The *Antitrogus* species markers were generated by *Mbo II* digestion of the *COII* PCR product, while the *Lepidiota* species markers were produced by *Dra I* and *Stu I*.

These techniques have been used to identify larvae in control trials, eg within the *Metarhizium* program.

Full details of the methodology, results and discussion are given in Appendix 2.

4.3 Identification keys

Classical dichotomous written keys to animals are often difficult for the novice to use. They are also impossible to use if a feature is missing. For example, the number of segments in the antennal clubs of adult canegrubs is an often-used character for their identification – if antennae are missing, it may be impossible to continue further in a classical key.

Multi-access keys do not suffer from the unanswerable-couplet problem because questions are not in any order – if a question can not be answered, the user simply goes onto another question that can be answered. Ultimately, a multi-access key eliminates all taxa except one that matches the specimen. Their structure makes them ideal for use on computers.

We used the LucID program (Centre for Pest Information Technology and Transfer 1999) to develop multi-access, computer-based keys for identification of larvae and adults of Australian canegrubs. The keys comprise a list of characters, taxa and a database of information on the characteristics of the taxa; the latter were taken from the conventional morphological descriptions written in 4.1. The LucID Player is used to make

identification with character states selected to match an unidentified specimen. The program queries the database to find all taxa that match the selected characteristics. Successive selection of characters narrows down the list of taxa until only one remains.

Once an identification is made, the Player can be used to find information on the species; data on biology, distribution and life history of all species are included in our key.

The key has been tested with a small number of BSES staff. Further testing is required before a completed version can be released to the industry. Before release, negotiation with the copyright owners is required to negotiate a user license for LucID.

A copy of version 1.0 of the program, called grubID, is attached as Appendix 4.

4.4 Other uses for outputs

To understand the biology of invading species and to design control strategies, a genetic approach to geographic structure is necessary. Knowledge of a pest's population genetic structure may enable a canegrower to make more informed pest management decisions to prevent or delay secondary pest problems and ensure crop protection is most effective. Consequently, questions regarding invasions, such as: the origin of colonists; whether invasions involve multiple colonisation events; the effectiveness of local (property-scale) control measures and the spread of a current infestation or of resistance alleles, should be addressed.

Sequence variation in mitochondrial DNA (mtDNA) reflects adult female vagility due to the maternal, non-recombining nature of mtDNA inheritance and its rapid evolution at the sequence level. In part 4.2 we showed that the localisation of related mtDNA alleles in some species reflects maternal gene flow. Species mobility and habitat fragmentation appear to exert important influences on the pattern of mtDNA phylogeography, and in turn, the population genetics of a pest, so we would predict that as females of *Antitrogus parvulus* (Childers canegrub) are non-volant then populations should exhibit genetic differentiation amongst nearby geographic locales.

As an adjunct to this project, we investigated the population genetics of *A. parvulus* using the *COII* gene from mtDNA. We surveyed populations of *A. parvulus*, to understand its population dynamics and gene flow. We used base-pair differences to characterise haplotype diversity and divergence; investigate haplotype relationships to one another; and infer their phylogeographic structure.

Full details of the methods and materials, results and discussion of the results are given in Appendix 3.

Female mating behaviour appears to be a major factor contributing to the phylogeographic variation of *A. parvulus*. Adults lie active in their pupal cells until released by rain and mate shortly afterwards and the females re-enter the soil from close to their emergence site and lay eggs a few days later. Our data underpin this behaviour whereby most haplotypes from the same region and population group together. This mating behaviour indicates that Childers beetles may not have significant rates of immigration and emigration between discrete patches of suitable habitat and is likely to be a significant factor leading to the phylogeographic variation of mtDNA.

The gene flow of *A. parvulus* is largely phylogeographic and habitat fragmentation appears to be a major influence on the pattern of mtDNA phylogeography. However, the population structure of *A. parvulus* is not solely determined by habitat discontinuity as little divergence is apparent between populations from South Kolan and Gin Gin and substantial divergence between populations from Childers+Cordabla and Horton is apparent. For South Kolan and Gin Gin populations genetic interchange may be following a 'stepping stone' model using nearby favourable soil patches to find a suitable mate. Between the Childers and Horton populations is a ridge that rises to 128 m above sea level. This ridge is broken by a small creek and continues to the north to end in a knoll-like feature called 'Bare End' (about 105 m above sea level). This feature could be confounding female dispersal and prevent genetic interchange between Horton and Childers+Cordabla populations.

5.0 RECOMMENDATIONS

- grubID should be developed further and released to the industry to provide a technique for the accurate identification of canegrubs.

- Techniques for the identification of canegrubs using molecular markers should be used in all trials potentially involving some of the five morphologically similar species. BSES Indooroopilly has the equipment and expertise to do these identifications and has already done some.

- Consideration should be given to the uses to which molecular markers can be put; the adjunct study to this project demonstrates that they can be used to answer fundamental questions regarding the gene flow in pest species. These studies would be important in understanding the development and spread of insecticide resistance.

6.0 PUBLICATIONS

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Miller L J and Allsopp P G. 2000. Identification of Australian canegrubs (Coleoptera: Scarabaeidae: Melolonthini). *Invertebrate Taxonomy* in press.

7.0 REFERENCES

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8.0 ACKNOWLEDGMENTS

David Yeates (Department of Zoology and Entomology, University of Queensland) provided important supervision of this project at the University of Queensland; his input and advice in many aspects of the study have been invaluable. Glenn Graham (Centre for Identification and Diagnostics, University of Queensland) likewise contributed his experience to the molecular parts of the study. Craig Moritz (Department of Zoology and Entomology, University of Queensland) had important input into the study of the population genetics of Childers canegrubs. BSES staff helped in the collection of material.

9.0 APPENDICES

Appendix 1 - Manuscript *Identification of Australian Canegrubs (Coleoptera: Scarabaeidae: Melolonthini)* by L J Miller and P G Allsopp.

Appendix 2 - Manuscript *Identification of morphologically similar canegrubs (Coleoptera: Scarabaeidae: Melolonthini) using a molecular diagnostic technique* by Liza J Miller, Peter G Allsopp, Glenn C Graham and David K Yeates.

Appendix 3 – Chapter from L J Miller's PhD thesis *Population genetics of* Antitrogus parvulus *Britton (Coleoptera: Scarabaeidae: Melolonthini), a common pest of sugarcane in south east Queensland.*

Appendix 4 – grubID version 1.0 (attached CD).