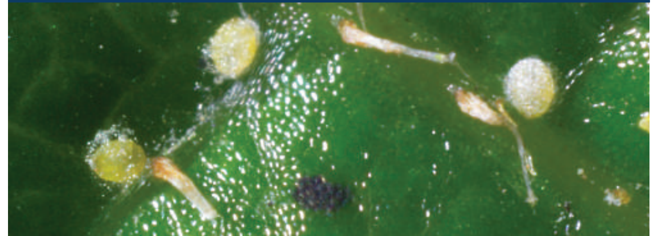

Scale on kiwifruit leaf. Photograph by Tim Holmes of Plant & Food Research Ltd

Identification of scale insects on kiwifruit in New Zealand

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Introduction

Many insects are cryptic with respect to their species identity, especially to the untrained eye. Furthermore, some life-stages of insects, such as eggs and immature stages, are particularly difficult to distinguish using morphology. There are many reasons why it might be necessary to identify an insect to species level. One reason might be to guide decision making for the type(s) of insect control measures to use if a species is resistant to a particular class of insecticide or if a biological control agent is species-specific. In addition, some insect species are regarded as quarantine pests in various markets and knowledge of an insect's identity can guide decisions on where produce from a particular field or orchard can be distributed, or it can facilitate quarantine pest inspection / identification at border control. Finally, some species of insect are able to vector human pathogens whilst cryptic sibling species may not; for example anopheline mosquitoes and malaria.

Typically for these types of assessments, testing needs to be conducted with high levels of accuracy whilst maintaining high rates of throughput.

Insect species identification using *prep*GEM® Insect

Three species of armoured scale insect are found on kiwifruit vines (*Actinidia* spp.) in New Zealand: latania scale, *Hemiberlesia lataniae* (Signoret); greedy scale, *Hemiberlesia rapax* (Comstock); and oleander scale, *Aspidiotus nerii* (Bouché). When found on fruit, some of these species are quarantine pests in key export markets. Distinguishing among the three scale species currently requires microscopic examination of the morphological characters of slide-mounted mature specimens. The immature stages of greedy and latania scale, however, cannot be distinguished morphologically. A molecular diagnostic test for all the life stages of these three armored scale insect pests would assist in the allocation of the kiwifruit crop to appropriate markets.

Here we describe the development of a species diagnostic test that employs rapid DNA extraction using *prep*GEM® Insect (ZyGEM NZ Ltd, Hamilton, New Zealand) followed by multiplex PCR utilising sequence variation in the cytochrome oxidase (COI and COII) genes to distinguish among the three species. The method allows hundreds of samples to be processed in a day and has provided a detailed picture of the distribution and abundance of these pests across green and gold kiwifruit in orchards throughout New Zealand (Edwards et al. 2008).

Materials and methods

Scale insects were collected directly from kiwifruit leaf and fruit material into 96-well 50 µl PCR plates to reduce handling. Adult scale insects were removed from under their protective cap using an alcohol and flame-sterilised needle before transfer, whilst immature stages were transferred directly into plates. Each plate contained 92 insects with four wells reserved for positive (three species of scale insect) and negative (one water) controls. Complete plates were sealed and stored at -20°C prior to the DNA extraction.

96-well PCR plates containing scale insects were centrifuged at 1084 x *g* for 2 minutes to ensure that the insects were in the extraction mixture.

Nucleic acids were extracted using the *prep*GEM Insect DNA extraction kit. Proteinase digestion was conducted as described by the manufacturer except that the volume of buffer was reduced to 40 µl. The *prep*GEM digestion was carried out on a PCR machine at 75°C for 15 minutes followed by 97°C for 5 minutes, for 1 cycle. Plates were then stored at 4°C until required. Prior to PCR, the extracted DNA was diluted 1:9 with 5% DMSO in ddH$_2$O. The PCR master mix contained 20 mM Tris-Cl (pH 8.4), 50 mM KCl, 1.5 mM MgCl$_2$, 0.2 mM dNTP, 0.2 µmol of each the four primers, 2.5 units of *Taq* DNA polymerase (Invitrogen, Carlsbad, CA, USA), with 5.0 µl of diluted template and a total reaction volume of 50 µl.

PCR Primers

AnDIP2	5'-CACATTCAAATATTGAAAACAACTTTA-3'
HlDIP2	5'-TTTATAATATTAGAAACTGATAATAACCT-3'
HrDIP3	5'-AACAAAATTATTTTACAAAATCAAAT-3'
c2-N-3662	5'-CCACAAATTTCTGAACATTGACC-3'

The primers AnDIP2, HlDIP2 and HrDIP3 recognise distinct species-specific regions within the *COI* or *COII* genes of the three species of scale insect, whilst c2-N-3662 recognises a common region in the *COI* gene for all three species.

The cycling conditions were 2 min at 94°C, 35 cycles of 40 sec at 94°C, 40 sec at 52°C and 1 min at 72ºC, and an extension of 10 min at 72°C. Reaction volumes for PCR were 50 µl. 8 µl of each of the resulting 96 PCR products were run on a 1.5% agarose gel made with SYBR® Safe gel stain (Invitrogen) in 1x TAE buffer.

Species were scored by product size:

- 206 bp = latania scale,
- 437 bp = greedy scale
- 633 bp = oleander scale

Results and discussion

The *prep*GEM method was able to extract sufficient DNA as a template for PCR. This in itself is impressive given the size of scale, as their immature stages are less than 1 mm in length. Furthermore, the method does not require any physical manipulation of the sample, such as grinding or sonication, potentially enabling the process to be automated using any standard liquid-handling robotic system.

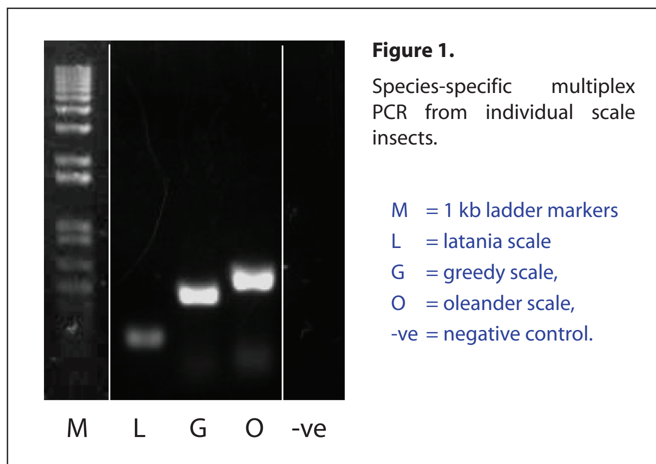

Figure 1.

Species-specific multiplex PCR from individual scale insects.

M = 1 kb ladder markers
L = latania scale
G = greedy scale,
O = oleander scale,
-ve = negative control.

Up to three species of scale insect could be diagnosed in a single multiplex PCR (Figure 1). The best results were obtained using a dilution step after the proteinase digestion and an annealing temperature of 52°C in the multiplex PCR. The dilution step was necessary to enable the highest possible rate of successful PCRs and amount of product, presumably by diluting inhibitors of *Taq* DNA polymerase present in the insects.

We then went on to conduct the test on a large collection of adult and immature scale insects sampled from different plant tissues and locations around New Zealand and were able to identify a total of 3,418 scale insects to species level using the *prep*GEM Insect and diagnostic multiplex PCR method: 1,904 (56%) were identified as latania scale, 1,473 (43%) as greedy scale, and 41 (1%) as oleander scale (Edwards et al. 2008). The method is rapid and the species can easily be scored based on PCR product size.

Overall the use of *prep*GEM Insect in conjunction with multiplex PCR offers the advantages of being rapid, easily scalable to high throughput automation and enables the species-typing of life stages of insect that are not easily differentiated morphologically, such as eggs and immature scale insects.

Conclusions

The *prep*GEM Insect method provides a very simple and rapid way of producing template DNA for PCR from small insects such as the immature stages of scale insects, many of which are difficult to distinguish morphologically.

In conjunction with multiplex PCR, *prep*GEM Insect provides a rapid means of species identification .

The use of *prep*GEM for DNA extraction of small insects lends itself for use in conjunction with high throughput automation systems.

Reference:

Edwards R., Carraher C., Poulton J., Sandanayaka S., Todd J. H., Dobson S., Mauchline N., Hill G., McKenna C., Newcomb R. D. (2008) DNA diagnostics of three armored scale species on kiwifruit in New Zealand. *Journal of Economic Entomology* **101**: 1944-1949.

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