Rapid Molecular Identification of Armored Scale Insects (Hemiptera: Diaspididae) on Mexican ‘Hass’ Avocado

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ABSTRACT ‘Hass’ avocado, Persea americana Mill., fruit imported into California from Mexico are infested with high levels of armored scale insects (Hemiptera: Diaspididae), constituting several species. The paucity and delicate nature of morphological characters traditionally used to diagnose armored scales often require careful preparation of slide-mounted specimens and expert knowledge of the group, for their accurate identification. Here, we present a simple, quick, and accurate means to identify armored scales on Mexican avocados, based on amplification of the internal transcribed spacer two of ribosomal DNA, by using the polymerase chain reaction (PCR). This region seems to show a high level of intraspecific conformity among scale specimens originating from different localities. A suite of species-specific reverse PCR primers are combined in a single reaction, with a universal forward primer, and produce a PCR product of a unique size, that after standard gel electrophoresis, allows the direct diagnosis of six diaspidid species: Abgrallaspis aguacatae Evans, Watson & Miller; Hemiberlesia lataniae (Signoret); Hemiberlesia sp. near latania; Hemiberlesia rapax (Comstock); Acutaspis albopicta (Cockerell); and Pinnaspis strachani (Cooley). Two additional species, Diaspis miranda (Cockerell) and Diaspis sp. near miranda, also are separated from the others by using this method and are subsequently diagnosed by secondary digestion of the PCR product with the restriction endonuclease smaI.

KEY WORDS armored scale insects, species-specific multiplex-polymerase chain reaction, avocado, internal transcriber spacer 2, Diaspididae

The armored scale insect Hemiberlesia lataniae (Signoret) (Hemiptera: Diaspididae), commonly known as latania scale, was once a significant problem on avocados, Persea americana Mill., in California. However, after the introduction of several parasitoid species in the 1940s to combat California red scale, Aonidiella aurantii (Maskell), on citrus (for review, see Compere 1961), the abundance of latania scale fell to very low levels, apparently as a fortuitous result of excellent coincidental biological control by one or more of these introduced parasitoids (Morse et al. 2009). Today, armored scale insects are no more than an occasional and very minor pest of avocados in California (Miller and Davidson 2005). However, worldwide, armored scale species are numerous and include some of the most damaging and refractory pests of perennial crops (Beardsley and Gonzalez 1975, Kosztarab 1990, Miller and Davidson 1990), perpetuating continued concern over the possible introduction of exotic armored scales, which based on their levels on imported avocado fruit, seem to be a significant problem in Mexico (Morse et al. 2009). Indeed, the most prominent of these (Morse et al. 2009), having been tentatively identified in initial reports as Hemiberlesia neodiffinis Miller & Davidson and then San Jose scale, Diaspidiotus perniciosus (Comstock) (California Avocado Commission 2007a,b), both of which are already present in the United States, was quickly reclassified as an undescribed exotic species with similarities to both. It has since been given the scientific name Abgrallaspis aguacatae Evans, Watson & Miller (Evans et al. 2009).

After the initial confusion over the identity of A. aguacatae, and heightened concern over the possible introduction of potentially damaging exotic pests, we instigated molecular characterization of the armored scale fauna present on avocado fruit imported into California from Mexico (see Morse et al. 2009). Ac-
accurately identifying an organism is also a critical step in assessing its potential for biological invasion. Species diagnoses can be problematic in taxa where definitive morphological characters are elusive or difficult to interpret. The identification of armored scales is hampered by their small size and by the need for high-quality slide-mounted specimens and expert knowledge of the family (e.g., Takagi 1969). Furthermore, diagnostic keys are based largely on delicate, and sometimes highly plastic (Stannard 1965), characters of the pygidial margin of adult females (Watson 2005, Evans et al. 2009). As such, traditional methods of identifying armored scales are time-consuming, require specialized knowledge, and are (to some extent) confined to identification of adult female specimens. The identification of males and early life stages is largely dependent on their association with, or proximity to, females.

Over the past decade, the use of molecular techniques to identify species has increasingly been advocated in genera that lack definitive morphological characters. Typically, this has involved the use of the polymerase chain reaction (PCR) to amplify short stretches of DNA, which are then characterized by secondary experimental methods such as sequencing (e.g., Armstrong and Ball 2005) or digestion with restriction endonucleases (Stouthamer et al. 1999; Erlandson et al. 2003; Chiu et al. 2004; Rugman-Jones et al. 2006, 2009; Rung et al. 2009; Sumer et al. 2009). Indeed, many armored scale species, including those on Mexican Hass avocados, can be identified by sequencing a section of 28S rDNA (Morse and Normark 2006, Morse et al. 2009). However, such secondary methods require further investment of time and money. Thus, methods that allow direct interpretation of the product of the PCR reaction are favorable. In situations where a specimen is likely to be only one of a relatively few species, species-specific multiplex-PCR offers one solution. This technique involves the use of one PCR primer located in a region shared by several species (a universal primer) alongside several opposing primers, each of which lies in a region that is specific to only one of the species under consideration, and that when it amplifies, produces a PCR product of a size that is unique to that species. As a result, species can be identified directly after gel electrophoresis of the PCR product (Cornel et al. 1996, Fritz et al. 2004, Caripey et al. 2005, Hosseini et al. 2007, Saccaggi et al. 2008).

Here, we present a species-specific multiplex-PCR assay for the identification of the five most common described species of armored scale infesting Hass avocado fruit being imported into California from Mexico: A. aguacateae, H. lataniae, Acutaspis albopicta (Cockerell), Pinnaspis strachani (Cooley), and Diaspis miranda (Cockerell). We also include two currently undescribed species present on the Mexican fruit (referred to within as Hemiberlesia sp. near lataniae and Diaspis sp. near miranda) and an occasional species found on avocados in California, Hemiberlesia rapax (Comstock). This work seeks to provide a quick and accurate means to identify these diaspidid species, which can be applied by nonspecialist workers.

Materials and Methods

Scale Collection and DNA Extraction. Adult scales were collected from Mexican-grown Hass avocados, crossing into California by way of the CDFA Blythe Border Inspection Station, CA (for details of the collection process, see Morse et al. 2009). Specimens of both sexes (where possible) were collected directly into 95% ethanol and stored at ~20°C. In addition: specimens of H. rapax were obtained from avocado trees in Moro Bay, California; and further specimens of H. lataniae were obtained from avocado trees in Riverside County, CA; San Diego County, CA; and the province of Petorca, Chile. DNA was extracted from individual specimens by using the EDNA HiSpEx tissue kit (Saturn Biotech, Perth, Australia), following the manufacturer’s protocol. DNA isolation using this kit involves simple mixing of three proprietary solutions, no grinding of the specimen, and incubation at 95°C for only 30 min. Thus, it provides a quick and easy means of isolating DNA, while retaining a more-or-less intact specimen that can be retrieved and slide-mounted for morphological confirmation, if required. Each specimen was identified to species by matching its 28S rDNA sequence to that of a morphologically verified voucher specimen deposited previously in the California State Arthropod Collection, Plant Pest Diagnostics Center, Sacramento, CA (GenBank accession FJ040864–FJ040871) (for details and the location of other voucher specimens, see Morse et al. 2009, table 4). No further slide-mounted voucher specimens were prepared as part of the current study.

Initial Sequencing of Internal Transcript Spacer (ITS) 2. The ITS2 of rDNA has proven very useful for identification purposes in several insect groups: particularly parasitoid Hymenoptera (for review, see Caripey et al. 2007) and mosquitoes (for review, see Collins and Paskewitz 1996, Walton et al. 1999). Intraspecific variation in this region is typically low, whereas differences between species, in both the size and sequence composition of ITS2, can be substantial (Hillis and Dixon 1991). Furthermore, the multicyclic nature of this region makes it relatively easy to amplify using standard PCR techniques. Initially, the entire internal transcribed spacer region of rDNA (ITS1, the interlying 5.8S, and ITS2) was amplified for the taxa A. albopicta, Hemiberlesia sp. near lataniae, D. miranda, and P. strachani, by using the PCR. Reactions were performed in 25-μl volumes containing 2 μl of DNA template (concentration not determined), 1× Thermopol PCR buffer (containing 2 mM MgSO4, New England Biolabs, Ipswich, MA), 200 μM each dNTP, 2 μl of bovine serum albumin (BSA) (NEB), 1 U Taq polymerase (New England Biolabs), and 0.2 μM each of the conserved primers ITS5 (5′-GGAAGTAAAAGTCTGAACACG-3′) and ITS4 (5′-TCCTCGGCTTATGATGC-3′) (White et al. 1990). Amplification was performed in a Mastercycler ep gradient S (Eppendorf North America, Inc., New York, NY) according to the following conditions: 3 min at 94°C, followed by 35 cycles of 30 sec at 94°C, 1 min at 55°C, and 1 min at 72°C, with a final extension of 7 min at 72°C.
An initial 3-min denaturing step at 95°C was followed by 35 cycles of 1 min at 94°C, 1 min at 54°C, and 1 min 30 s at 72°C; with a final extension of 3 min at 72°C. PCR products were visualized after electrophoresis on 1% agarose gels stained with ethidium bromide, cleaned using the Wizard PCR Prep DNA purification system (Promega, Madison, WI), and cloned into a plasmid vector (pGEM-T Easy Vector system, Promega) according to the manufacturer’s protocol. Insert-positive colonies were amplified using M13 PCR primers, and products were again visualized and cleaned before direct sequencing in both directions at the University of California, Riverside, Genomics Institute, Core Instrumentation Facility using an Applied Biosystems 3730 DNA analyzer (Applied Biosystems, Foster City, CA). Cloned products were sequenced manually in BioEdit version 7.0.5.3 (Hall 1999) and with a Big-Dye version 3.1 kit (Applied Biosystems, Foster City, CA). The sequences of three specimens of the remaining target species, by using the Wizard PCR Prep DNA purification system (Promega, Madison, WI), and cloned into a plasmid vector (pGEM-T Easy Vector system, Promega) according to the manufacturer’s protocol. Insert-positive colonies were amplified using M13 PCR primers, and products were again visualized and cleaned before direct sequencing in both directions at the University of California, Riverside, Genomics Institute, Core Instrumentation Facility using an Applied Biosystems 3730 DNA analyzer with a Big-Dye version 3.1 kit (Applied Biosystems, Foster City, CA). Cloned products were sequenced manually in BioEdit version 7.0.5.3 (Hall 1999) and deposited in GenBank (Benson et al. 2008) (accessions GQ284589–GQ284595). The sequences of three species (Hemiptera: Pseudococcidae) were retrieved from GenBank (accessions AF007263–AF007265) and used to delineate ITS2. From our initial sequences, the primer diaspid-ITS2-for (5’-ATGCCGATAGAGGCCTGCT-3’) was designed with the aid of Primer3 version 0.4.0 (Rozen and Skaletsky 2000) by using the following criteria: 1) they should complement the forward primer diaspid-ITS2-rev with ITS4 to gain good amplification, and cloned the PCR product before sequencing. All exceptions to this were the two Diaspis species, for which we substituted diaspid-ITS2-rev with ITS4 to gain good amplification, and cloned the PCR product before sequencing. All products were deposited in GenBank (accessions GQ284596–GQ284638).

Multiplex-PCR. Species-specific PCR primers were designed with the aid of Primer3 version 0.4.0 (Rozen and Skaletsky 2000) by using the following criteria: 1) they should complement the forward primer diaspid-ITS2-for; 2) at least one nucleotide at the 3’ end of the primer must be unique to one target species in the alignment; 3) complementarity between primers should be minimal; 4) they should work at similar annealing temperatures (can be manipulated by substituting nucleotides at the 5’ end); and 5) they should produce a PCR product of a unique size for each species that can be readily distinguished using standard agarose gel electrophoresis. The specificity of several primers for each taxon was tested individually and in multiplex-PCR against each species (unpublished data), identifying a set of primers which gave the best combination of the desired characteristics (Table 1). The optimized multiplex-PCR amplifies 2 µl of template DNA (concentration undetermined) from individual scales, in 25-µl reactions containing 1× Thermopol PCR buffer (New England Biolabs), 200 µM each dNTP, 2 µl of BSA (New England Biolabs), 0.2 µM each primer (Table 1), and 1 U of Taq polymerase (New England Biolabs). Amplification was performed in a Mastercycler ep gradient S (Eppendorf North America, Inc.) programmed for an initial denaturing at 95°C for 3 min; followed by 36 cycles of 94°C for 45 s, 61°C for 30 s, 72°C for 1 min; and a final extension of 3 min at 72°C. After amplification, 6 µl of each PCR product was visualized by electrophoresis on a 1.5% agarose gel stained with ethidium bromide.

The multiplex-PCR was tested on a panel of 100 adult armored scale specimens (including several males), collected from Mexican Hass avocados originating in several localities, which had previously been identified based on published 28S sequences (Morse et al. 2009). The utility of the multiplex-PCR to identify eggs and first-instar nymphs (crawlers) also was tested in those species for which we were able to collect these life stages (Table 1). Finally, the method was checked for the production of false positives with two further diaspidid species known to be present on Mexican Hass avocado fruit, Abgrallaspis perseae Davidson (which we intercepted on only two occasions, Morse et al. 2009) and Autacaspis tubercularis Newstead (specimens of which were sent to us having been intercepted on Mexican avocado entering California via the CDFA Needles Border Inspection Station, Needles, CA).

Table 1. Species-specific oligonucleotide (oligo) primers used in the multiplex-PCR and sizes of the resulting PCR products

<table>
<thead>
<tr>
<th>Species</th>
<th>Primer name</th>
<th>Oligo sequence&lt;sup&gt;a&lt;/sup&gt;</th>
<th>PCR product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abgrallaspis aguacaticae&lt;sup&gt;d&lt;/sup&gt;</td>
<td>A. agu R-2</td>
<td>5’-ATGCCGATAGAGGCCTGCT-3’</td>
<td>472</td>
</tr>
<tr>
<td>Acutaspis albopicta&lt;sup&gt;c&lt;/sup&gt;</td>
<td>A. acut R-1</td>
<td>5’-ATGCCGATAGAGGCCTGCT-3’</td>
<td>372</td>
</tr>
<tr>
<td>Diaspis mirandae&lt;sup&gt;d&lt;/sup&gt;</td>
<td>D. miranda R-1</td>
<td>5’-ATGCCGATAGAGGCCTGCT-3’</td>
<td>327</td>
</tr>
<tr>
<td>H. lataniae&lt;sup&gt;c&lt;/sup&gt;</td>
<td>H. lat R-3</td>
<td>5’-ATGCCGATAGAGGCCTGCT-3’</td>
<td>199</td>
</tr>
<tr>
<td>Hemiberlesia sp.</td>
<td>H. uralt R-1</td>
<td>5’-ATGCCGATAGAGGCCTGCT-3’</td>
<td>288</td>
</tr>
<tr>
<td>H. rapax</td>
<td>H. rap R-1</td>
<td>5’-ATGCCGATAGAGGCCTGCT-3’</td>
<td>508</td>
</tr>
<tr>
<td>P. strachani</td>
<td>P. stra R-3</td>
<td>5’-ACGATCTCGGCGCCCGTT-3’</td>
<td>521</td>
</tr>
</tbody>
</table>

<sup>a</sup> Underlined bases in the primer sequences are mismatched nucleotides, introduced to unify primer melting temperatures.

<sup>b</sup> Multiplex-PCR tested on crawlers.

<sup>c</sup> Multiplex-PCR tested on eggs.

<sup>d</sup> Multiplex-PCR tested on eggs and first-instar nymphs (crawlers) also was tested in those species for which we were able to collect these life stages (Table 1). Finally, the method was checked for the production of false positives with two further diaspidid species known to be present on Mexican Hass avocado fruit, Abgrallaspis perseae Davidson (which we intercepted on only two occasions, Morse et al. 2009) and Autacaspis tubercularis Newstead (specimens of which were sent to us having been intercepted on Mexican avocado entering California via the CDFA Needles Border Inspection Station, Needles, CA).
The method accurately identified 20 specimens previously identified by the sequence of their 28s rDNA (Morse et al. 2009). The multiplex-PCR also produced distinct products of the correct size for each of the eight desired species. The exceptions, Diaspis sp. near miranda, Diaspis sp. near miranda, produced inconsistent and poor amplification products most likely due to a single nucleotide mismatch at the 3’-terminal end of the reverse primer (see GenBank accessions GQ284631–GQ284638). However, sequences were obtained for these two species using the primer ITS4 in place of diaspid-ITS2-rev.

Interspecific variation in the size of the ITS2 (which ranged from 576 bp to 716 bp in H. rapax and A. aguacatae, respectively) was not sufficient to separate the eight species. However, the rDNA sequences of the ITS2 were highly conserved within species, and sufficiently different between species, to allow the design of a set of diagnostic multiplex-PCR primers (Table 1). Amplification of ITS2 by using these primers in a multiplex-PCR, yielded species-specific fragments (between 194 bp and 588 bp), allowing the direct diagnosis of six of the eight desired species and the discrimination from the others of the two remaining Diaspis species (Fig. 1). These latter species can subsequently be diagnosed by digesting the PCR product for 2 h with 10 U of the restriction endonuclease smal (New England Biolabs), which cuts Diaspis sp. near miranda but not Diaspis sp. near miranda (data not shown). The reproducibility of the ITS2 multiplex-PCR was demonstrated using a panel of 100 adult female and male specimens, collected from avocados originating in several geographic localities, and previously identified by the sequence of their 28s rDNA (Morse et al. 2009). The method accurately identified 20 specimens as A. aguacatae, 20 as H. lataniae, 25 as H. sp. near lataniae, six as H. rapax, 15 as A. albopicta, seven as D. miranda, six as Diaspis sp. near miranda, and a single specimen as P. strachani. The multiplex-PCR also produced distinct products of the correct size for each of twelve samples of individual scale eggs and first instar crawlers (see Table 1 for species). Finally, neither Abgrallaspis perseae nor Aulacaspis tubercularis yielded “false positive” bands.

Results

The primers diapid-ITS2-for and diapid-ITS2-rev amplified and allowed direct sequencing of the ITS2 of six of the eight desired species. The exceptions, D. miranda and Diaspis sp. near miranda, produced inconsistent and poor amplification products most likely due to a single nucleotide mismatch at the 3’-terminal end of the reverse primer (see GenBank accessions GQ284631–GQ284638). However, sequences were obtained for these two species using the primer ITS4 in place of diaspid-ITS2-rev.

Discussion

Traditional morphology-based methods for the identification of armored scales often require specialist knowledge of the group and careful, time-consuming preparation of slide-mounted specimens. Furthermore, most existing morphological keys rely heavily on characters of the adult female, such that males and immature life stages can often be only tentatively identified by their association with female specimens. Identification methods based on DNA sequences may offer a quicker, more widely applicable, and more cost-efficient alternative. The multiplex-PCR developed here incorporates a universal forward primer and seven reverse primers in a single reaction. After subsequent standard gel electrophoresis, six species (Abgrallaspis aguacatae, H. lataniae, Hemiberlesia sp. near lataniae, H. rapax, Acutaspis albopicta, and P. strachani) and a further species pair (D. miranda and Diaspis sp. near miranda) can clearly be separated based on the size of the resulting product of the PCR reaction. The two Diaspis species can then be separated based on whether or not the product of the multiplex-PCR is cut by the restriction endonuclease smal.

Multiplex-PCR can be an extremely efficient identification tool if a specimen is expected to be one of only a handful of species (Gariepy et al. 2008). The eight species incorporated in this study have all been intercepted on Hass avocado fruit being imported into California from Mexico (Morse et al. 2009). One of the difficulties encountered in designing a multiplex PCR method is finding “room” for all the “target” species. Although the ITS2 sequence displays significant interspecific variation, with each species that is added to the multiplex, the design of a species-specific primer that has a similar annealing temperature to the others,
and produces a PCR product of a distinguishably unique size becomes more difficult. Furthermore, as the number of primers is increased, so is the probability of spurious bands being formed as a result of mispriming, which may in turn lead to false positive identifications (Gariepy et al. 2007, Hosseini et al. 2007). Such limitations imposed on us the need to adopt a secondary method (restriction digestion) to discriminate between D. miranda and Diaspis sp. near miranda. Furthermore, we were unable to include two additional species, Abgrallaspis perseae and Aulacaspis tubercularis, which have also been intercepted (in very low numbers) on Mexican Hass avocado entering California. However, using our multiplex-PCR with these species did not result in the production of false positives, effectively identifying them as something other than the eight included species. Actually, within the armored scale insect fauna found on Mexican avocados, Aulacaspis tubercularis is morphologically distinct and easy to diagnose (G. Watson, personal communication), but in the routine application of our method, specimens that do not produce a PCR product (or a product of an unexpected size) could subsequently be identified by sequencing of the 28S gene (Morse and Normark 2006, Morse et al. 2009). In addition, because the EDNA HiSpEx tissue kit we advocate for the DNA extractions is nondestructive, specimens may still be identified based on their morphology after extraction.

In contrast to traditional morphology-based identification keys for armored scale insects, multiplex-PCR is a quick and simple method which can be used by workers with basic laboratory skills. Our entire multiplex PCR protocol, from DNA extraction through to the completion of electrophoresis, can be performed in \(\approx 5\) h. If required, restriction digestion of the multiplex PCR product (and further electrophoresis) adds another 3 h. Furthermore, our method is particularly useful for identifying males and immature stages that are often not considered in morphological keys. We estimate that using our method, a single laboratory technician could readily diagnose 100 live or recently deceased specimens (regardless of sex or life stage) in a normal working day. As such, our multiplex-PCR represents a valuable tool for monitoring the armored scale fauna entering California on Mexican Hass avocados. Furthermore, with concern rising over the possible accidental introduction of a potentially damaging exotic armored scale, our multiplex PCR could provide an efficient means of monitoring resident/native armored scale populations in California and other avocado producing regions around the world. That said, one drawback of multiplex-PCR is that it is largely context-specific. Our method has been designed to work within the constraints of a known set of species and a known degree of inter- and intraspecific genetic variation. The application of the method to species and/or geographic areas outside the constraints of our study may be unwise without first surveying and sequencing the resident armored scale fauna. The ITS2 of specimens of those species included here, but from populations outside the geographic range of our study, may differ sufficiently in just the right places to prevent annealing of the “correct” primer and/or facilitate annealing of an “incorrect” primer, resulting in the production of spurious PCR products and the potential for false positive identifications. Similarly, we are unable to predict how our multiplex PCR would “respond” to the presence of additional species. Two species, known to occur on Mexican avocado but not detected on Hass fruit entering California (Morse et al. 2009) that have not been included in our study are Hemiberlesia diffinis (Newstead) and H. neodiffinis (Miller and Davidson 1998). A more complete list of armored scale pests that have been recorded as feeding on avocado worldwide is provided by Evans et al. (2009).

Accurate identification to species is a fundamental, but sometimes problematic, step in any study that addresses the basic biology, (potential) economic impact, or methods available for controlling armored scale insect pests (Burger and Ulenberg 1990). Provided live, or very recently deceased, specimens are available at the time of collection, the DNA-based method presented here allows quick and accurate identification of eight species of armored scale, resident on Mexican Hass avocado, regardless of size, life stage, or sex of the specimen.

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References Cited


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