# Polymerase Chain Reaction-Restriction Fragment Length Polymorphism Method to Distinguish Three Mealybug Groups Within the *Planococcus citri–P. minor* Species Complex (Hemiptera: Coccoidea: Pseudococcidae)

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**ABSTRACT** The mealybug species *Planococcus citri* (Risso) and *Planococcus minor* (Maskell) (Hemiptera: Coccoidea: Pseudococcidae) have special significance to U.S. quarantine and U.S. agriculture. Commonly intercepted at U.S. ports-of-entry, they are difficult to identify based on morphological characters. This study presents a molecular method for distinguishing *P. citri*, *P. minor*, and a genetically distinct group that is morphologically identical to *P. citri*, from Hawaii. This method uses polymerase chain reaction (PCR) followed by restriction fragment polymorphism analysis (RFLP) using the restriction enzymes BspH1, BsmH1, and HpH1. The resulting band patterns can be visualized in a 2% agarose gel and are sufficient to differentiate between the three entities mentioned above. PCR-RFLP diagnostics can be used for all life stages and is cheaper and faster than DNA sequencing.

**KEY WORDS** *Planococcus citri*, *Planococcus minor*, diagnostics, PCR, restriction fragment length polymorphism

Mealybugs in the genus *Planococcus* encompass several serious pests; for example *Planococcus ficus* (Signoret) on grapes (Dalla Montá et al. 2002; Walton and Pringle 2004) and *Planococcus lilacinus* (Cockerell) on several hosts such as cacao and other tropical fruits (Ben-Dov 1994, Peña et al. 2002). In an analysis of invasive species of mealybugs, Miller et al. (2002) list 12 additional species of *Planococcus* that have been characterized as pests, including *Planococcus citri* (Risso) and *Planococcus minor* (Maskell). These morphologically similar species have special significance to U.S. quarantine and U.S. agriculture. *P. citri* is abundant and a serious polyphagous pest in the United States, attacking citrus, ornamentals, tropical fruit, house plants, and other agricultural crops (Ben-Dov 1994, Ebeling 1959, Franco et al. 2001, Dunkelblum et al. 2002). *P. minor* is also polyphagous and occurs in all zoogeographic regions of the world except the Palearctic (Ben-Dov 1994, Ben-Dov et al. 2006, Williams 2004). *P. minor* is not currently known to be present in the United States, but it is frequently intercepted from >30 importing countries at U.S. ports-of-entry (Miller et al. 2002) on a wide variety of hosts, including coffee (*Coffea* L.), yam (*Dioscorea* L.), banana (*Musa* L.), cacao (*Theobroma cacao* L.), *Heliconia* L., *Plumeria* L., *Psidium* L., and *Punica* L. The introduction and establishment of *P. minor* within the United States is considered to represent a serious threat to U.S. agriculture and is a high priority for exclusion by the U.S. Department of Agriculture's Animal and Plant Health Inspection Service/Plant Protection and Quarantine Program.

Separating *P. citri* from *P. minor* by using only morphological characters has been difficult (Cox and Wetton 1988). The standard method, applicable only to adult females, uses six characters that are scored using a point system, often referred to as the "Cox score" (Cox 1989). A score of 35 or less means that the unidentified specimen is *P. minor;* a score >35 indicates that the specimen belongs to *P. citri.* In addition to its inapplicability to immature or male specimens, environmental conditions such as temperature and humidity can affect the characters used to calculate the Cox score (Cox 1981, 1983); therefore, we believe that it is necessary to find an additional method to aid species identification.

Our previous work (Rung et al. 2008) was directed at finding molecular markers to evaluate species limits of *P. citri* and *P. minor.* Phylogenetic analysis using sequences of the mitochondrial cytochrome oxidase-1 (COI) gene and the nuclear protein-coding gene elongation factor 1-$\alpha$ (EF1-$\alpha$) revealed three distinct clades within the *P.citri–P.minor* species complex: the "*P. citri*" and the "*P. minor*" clades, corresponding to the two named species and encompassing specimens from various locations around the world; and the "Hawaiian clade," which includes specimens morphologically indistinguishable from *P. citri* and occurring only

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**Table 1. List of specimens tested in this study and their corresponding haplotypes in Rung et al. (2008), Cox scores, and collection data**

| Species | Label as in Rung et al (2008) | Corresponding haplotype in Rung et al (2008) | Collection data |
|---|---|---|---|
| C | MB59 | COI.H1 | Brazil. Espírito Santo, Vitória, 10 Dec. 2004, M. Culik |
| C | Dmps27 | COI.H1 | U.S. HI, Pali, Oahu, 25 April 2005, J. Yalemar, from *Pipturus* sp. |
| C | MB35.9 | COI.H2 | U.S. CA, San Luiz Obispo, 7 June 2006, C. Darling, from *Radermachera sinica* (Hance) |
| C | MB10 | COI.H2 | U.S. CA, Riverside, 18 May 2005, M. Lanthi |
| C | MB43 | COI.H9 | U.S. FL (no further info) |
| C | MB6 | COI.H12 | Israel. 5 June 2005, from *Rumex acetosa* L. |
| C | MB60 | COI.H13 | Brazil. Espírito Santo, Linhares, 16 Dec. 2006 |
| C | MB44.4 | COI.H15 | U.S. HI (no further info) |
| C | MB47 | COI.H16 | U.S. FL, Citrus Co., July 2006, G. Hodges, from *Clerodendrum paniculatum* L. |
| C | MB56 | C01.H19 | South Africa. Stellenbosh, 2006 |
| M | MB12 | COI.H3 | Australia. Queensland, 21 Aug. 2005, from *Morinda citrifolia* L. |
| M | MB58 | COI.H3 | Indonesia. 26 June 2006, from "ornamental plants". |
| M | MB38 | COI.H4 | Trinidad and Tobago. Centeno, 10 July 2006, A. Francis |
| M | MB14 | COI.H5 | Thailand. Thani Province, 17 June 2004, P.S. Cranston, from "Ranbutan fruit" |
| M | MB45.1 | COI.H7 | American Samoa. Mepusage, Tutuila, 4 July 2006, M. Schaedick, from *C. paniculatum* L. |
| M | MB1 | COI.H7 | Viet Nam, from *Nephelium lappaceum* L. (no further info). |
| M | MB50 | COI.H8 | Trinidad and Tobago. St. Augustine, UWI campus, 28 July 2006, A. Francis, from cocoa. |
| M | MB65 | COI.H10 | Brazil. Espírito Santo, Linhares, 21 Feb. 2005, D. Martins, from Biden pilosa roots |
| M | MB34 | COI.H17 | Ecuador. 25 April 2006, C. Gaona |
| H | MB44.2 | COI.H6 | U.S. HI (no further info) |
| H | MB44.3 | COI.H6 | U.S. HI (no further info) |

Cox scores given within parentheses after the specimen label were calculated from other specimens present in the same infestation as the voucher specimen. Abbreviations: C, *P. citri;* M, *P. minor;* H, Hawaiian clade; NS, no score.

in Hawaii. Although for the most part, COI and EF1-$\alpha$ recovered the same clades, placement of a few specimens was inconsistent across the two genes. Whether the inconsistency was due to hybridization or ancestral polymorphism was not determined.

Even though DNA sequence data can generally be used to distinguish these species, it is not suitable for routine identifications because it is time-consuming and relatively expensive. The purpose of this contribution is to present a diagnostic method that can be used to rapidly differentiate between *P. citri* and *P. minor.* Even though the "Hawaiian clade" does not correspond to a named species, we also provide a means to diagnose individuals of this clade, in case it becomes a concern in the future. Our method combines PCR with restriction fragment length polymorphism (RFLP) analysis and can be performed in a single day. However, as noted previously (Rung et al. 2008), maximum accuracy of COI-based identifications will be achieved when used in concert with morphological and geographical data.

## Materials and Methods

As described in Rung et al. (2008), adult females of *Planococcus* obtained from different hosts in various locations around the world were collected and preserved in 95–100% ethanol (Table 1). In the laboratory, specimens were transferred to vials containing 100% ethanol and stored in a −80°C freezer until DNA extraction. DNA extraction followed the protocol described in Rung et al. (2008).

To develop and evaluate the PCR-RFLP diagnostic method, samples were selected to include different haplotypes of *P. citri*, *P. minor*, and the Hawaiian clade, previously sequenced by Rung et al. (2008). Primers CJ-J-2183 (alias Jerry) (5′-CAACATTTATTTTGATTTTTTGGN) and 3014-R2 (5′-AATGTATGATTTAAATTAGGTG) were used to amplify 840 bp (size calculated from sequences) of the COI. PCR was performed with TaKaRa Ex *Taq* following the manufacturer's protocol with 4 $\mu$l of DNA in a total of 50 $\mu$l of solution. The conditions for PCR using a Stratagene Robocycler (Stratagene, La Jolla, CA) were as follows: 94°C for 4 min followed by 30 to 50 cycles of 94°C for 1 min, 48–56°C for 1 min, and 72°C for 1min 30 s with a final extension at 72°C for 4 min. PCR products were purified using the QIAquick PCR purification kits (QIAGEN, Valencia, CA) and eluted in 40 $\mu$l of pure water.

In total, 58 COI sequences representing 19 different haplotypes sampled from localities in 16 countries were analyzed using the program Sequencher (Gene Codes, Ann Arbor, MI). A complete list of haplotypes sequenced and their localities can be found in Rung et al. (2008). Restriction enzymes that could be used to distinguish the three clades were identified. Sequences from *P. minor* (comprising seven haplotypes sampled from localities in nine countries) were found to have a unique recognition site for BspH1 that is absent in the other two clades (Fig. 1A). The single haplotype from the "Hawaiian clade" (from the same locality in the Hawaii) was found to have a unique recognition site for BsmA1 that is absent in *P. minor* and *P. citri* (Fig. 1C). A unique recognition site for

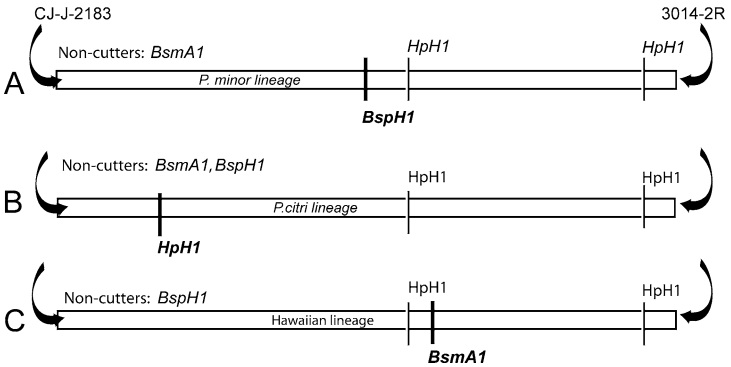


**Fig. 1.** Schematic diagram of the mitochondrial cytochrome oxidase fragment used in this study. Approximate location of restriction sites HpH1, BspH1, and BsmA1, when present, are indicated.

*HpH1* was found in sequences from *P. citri* (including 12 haplotypes sampled from localities in nine countries), but this enzyme also had two additional recognition sites common to all three clades (Fig. 1). Unless the reaction fails, digests resulting from this enzyme always give "positive results" (see Results) as two or three cuts will be made per sample.

Restriction digests of PCR samples of known identity were performed using the restriction enzymes BspH1, HpH1, and BsmA1 (New England Biolabs, Ipswich, MA) following the manufacturers' protocol, with 7 $\mu$l of DNA template in a total of 20 $\mu$l of solution. Digests were performed in all of the known haplotypes of *P. minor* (nine specimens, see Table 1) and the Hawaiian clade (two specimens), and in 66% of the known *P. citri* haplotypes (eight haplotypes, 10 specimens). Each sample was digested two separate times, with BspH1 and HpH1. Because the incubation temperature of BspH1 and HpH1 is different than the incubation temperature of BsmA1, incubation with this enzyme was performed separately. The resulting fragments were visualized in 2% agarose gel along with a 100-bp DNA ladder (Invitrogen, Carlsbad, CA) to ascertain the size of the resulting fragments.

## Results and Discussion

Throughout this section, the exact size of bands resulting from various digests, as calculated from sequences of known identity, is given between brackets. BspH1 digests of eight known *P. citri* haplotypes, and of the only known haplotype of the "Hawaiian clade" (Table 1) all resulted in a single band of the original size (840 bp), confirming the absence of BspH1 recognition site for these sequences (Fig. 2). BspH1 digests of seven known *P. minor* haplotypes (Table 1) all resulted in a strong band between the 400–500-bp ladder marks. This band corresponds to two approximately equal-sized fragments (410 and 430 bp, respectively) resulting from the digest (Fig. 1A).

Digests performed on samples of *P. citri* and *P. minor* by using BsmA1 all resulted in a single band of the original size, confirming that these sequences do not have a recognition site for this enzyme. BsmA1 digests of samples of the Hawaiian clade, however, resulted in two bands near the 500- and 300-bp marks, respectively (510 and 330 bp; data not shown).

Restriction digests with HpH1 exhibited a different pattern, as sequences from all clades are cut (a "pos-

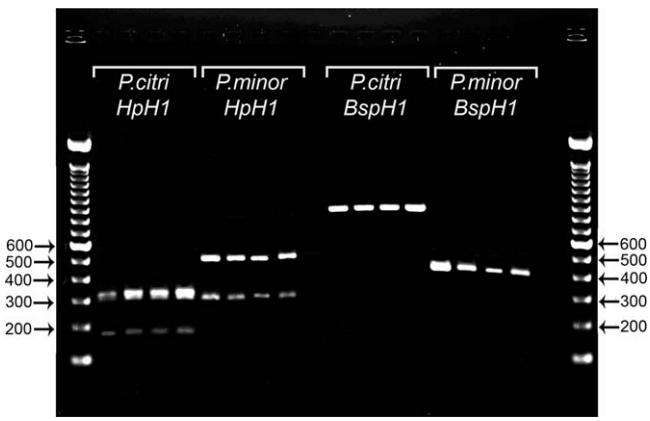


**Fig. 2.** Digital photograph of a 2% agarose gel with representative samples of *P. citri* and *P. minor* after restriction enzyme digests of the mitochondrial cytochrome oxidase fragments by using HpH1 and BspH1.

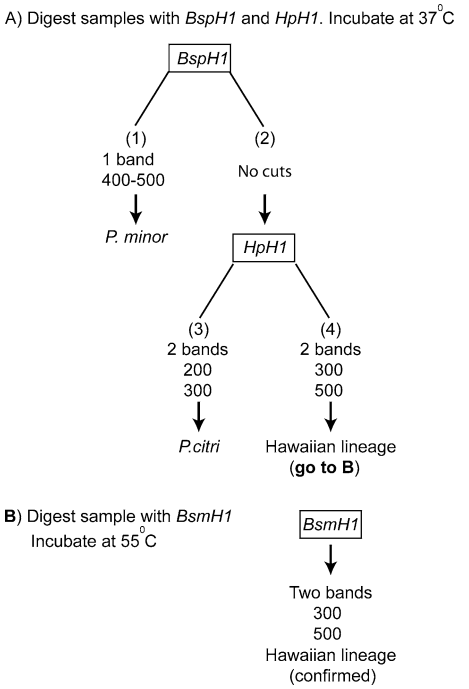


**Fig. 3.** Diagrammatic representation of the identification process used in this work. Numbers represent the approximate location of the bands as given by the ladder.

itive result") (Figs. 2 and 3). Digests of samples of *P. minor* and the Hawaiian clade resulted in two bands: a strong band near the 500-bp mark (510 bp) and a weaker band a little above the 300-bp mark (320 bp; also see Fig. 2). HpH1 digests of *P. citri* resulted in a strong band ≈300 bp and a weaker of ≈180 bp (Fig. 2). The strong band ≈300 bp corresponds to two approximately equal-sized fragments (300 and 320 bp, respectively) that result from an internal cut (Fig. 1B). The additional fragment that results from *HpH1* digest of these sequences, with 40 bp, is not visible on the gel.

Because restriction digest using HpH1 positively distinguishes *P. minor* from *P. citri*, it might seem as though this single digest would be sufficient for identifying these species. It must be kept in mind, however, that this test will not distinguish the "Hawaiian clade" from *P. minor*, a species with which the "Hawaiian clade" seems to be neither genetically nor morphologically unified (Rung et al. 2008). Additionally, U.S. quarantine currently restricts entry of *P. minor*, but not of the "Hawaiian clade," so a first test to positively identify *P. minor* is of high priority. For this purpose, we recommend a series of restriction digests as diagrammed in Fig. 3.

First, the restriction enzyme *BspH1* can be used to separate *P. minor* from the other two clades. A digest with this enzyme resulting in a strong band near the 500-bp ladder mark means that the sample belongs to *P. minor* (Fig. 3A, 1), whereas the absence of a cut is an indication that the sample belongs to either *P. citri* or the "Hawaiian clade" (Fig. 3A, 2) or that the reaction failed for some reason.

To distinguish *P. citri* from the "Hawaiian clade," at least one additional digest is required. Using HpH1, digests of both *P. citri* and the "Hawaiian clade" result in two readily observable bands (although *P. citri* is actually cut three times; see above) that differ in size and placement in the gel. Samples from *P. citri* will show the presence of a strong band near the 300-bp mark, and a weaker band near the 200-bp mark in an HpH1 digest (Fig. 3A, 3). Samples from the "Hawaiian clade" show the presence of a strong band near the 500-bp mark and a weaker band near the 300-bp mark (Fig. 3A, 4). Because samples of *P. minor* from failed BspH1 reactions also seem to belong to the "Hawaiian clade," a further digest with BsmA1 is desirable to positively confirm the identification (Fig. 3B).

Even though the use of BspH1 is sufficient to separate *P. minor* from the other two clades, and BsmA1 is sufficient to separate the Hawaiian clade from the two named species, the highest degree of accuracy can only be achieved when restriction digests of BspH1 and HpH1 are performed simultaneously, followed by BspH1 (to confirm identification of the Hawaiian clade, whenever necessary). Because many factors can cause a restriction digest to fail, a negative PCR-RFLP result from a single restriction enzyme should not be the decisive factor to assign species identity. For example, if only a BspH1 digest is performed on a sample and the result is a band of the original size, one should not immediately conclude that the sequence does not belong to *P. minor*. Other factors could account for this negative result, such as poor reaction conditions. Using the two additional restriction digests (Fig. 3) on this sample will confirm, to the greatest degree possible, that the specimen does not belong to *P. minor*.

One possible pitfall of PCR-RFLP–based identifications is that the target restriction site could be absent from yet un-sampled haplotypes. This would cause PCR-RFLP testing of the new haplotypes to fail to correctly assign the specimens to species. This problem may be most likely to arise for specimens belonging to the Hawaiian clade, for which only one haplotype has been identified. Another potential pitfall could be the presence of a given restriction site in a mealybug species not included in this study. The inadvertent inclusion of specimens of such a species in the PCR-RFLP protocol could result in incorrect species identifications. Therefore, until COI sequence data are available from other *Planococcus* species, we cannot predict whether our PCR protocol would amplify other species. We suggest that only specimens conforming to the morphological descriptions of *P. citri* and *P. minor* be evaluated using our PCR-RFLP protocol. Because of the potential complications explained above, PCR-RFLP diagnostics should be applied with caution. We advise that morphological identification and DNA sequencing be performed for specimens that come from localities not previously sampled by us, or for specimens that reveal a PCR-RFLP band pattern different from that described in this work. Whenever possible, a nondestructive DNA extraction, such as the extraction described in Materials and Methods, should be performed because it allows a voucher to be kept for future morphological inspection. This

method can be applied to identify immatures, but for the reasons explained above, DNA sequencing of a few samples first is advisable for unsampled localities.

The results of our previous work (Rung et al. 2008) showed that COI, in combination with morphological and geographical data, can be used to accurately identify the *P. minor* clade and can be used to identify the *P. citri* clade and the Hawaiian clade in most cases. Additionally, we concluded that, even though adult females of *P. minor* can almost always be identified accurately with the values used in the Cox score alone, the same is not true for *P. citri* individuals. In our analysis, all specimens that clustered with the *P. minor* clade had been previously identified as *P. minor* based on their morphology. In contrast, of the 31 specimens found to belong to *P. citri*, ≈12% had borderline Cox scores (35) and ≈12% had scores compatible with their identification as *P. minor* (Rung et al. 2008). When a *P. citri* individual is misidentified as *P. minor* (a "false positive" identification) because it has a low Cox score, shipments containing agricultural products may be unnecessarily detained or excluded from entering the country, causing unnecessary economic losses. In situations like these, our method is expected to be extremely helpful.

Because results from COI and EF1-$\alpha$ conflict in the placement of a few specimens, we cannot be sure that this gene will always give an accurate identification. It is not known whether *P. minor* and *P. citri* hybridize in natural conditions. If they do, mitochondrion introgression could potentially occur, resulting in individuals that have the nuclear genome of *P. minor* and the mitochondrion of *P. citri*, or vice versa, resulting in inaccurate identifications. In the case of greatest interest to U.S. quarantine, that of essentially *P. minor* samples carrying a *P. citri* mitochondrion, the samples would not be identified as *P. minor*. A very small proportion of specimens in our study (one specimen of 19) fell in this category: a specimen from Brazil that was identified as *P. citri* based on morphology and COI sequence data clustered with *P. minor* in the EF1-$\alpha$ data set. Until another nuclear gene is sequenced for these species, and conflicting information is resolved, results from COI should be interpreted with caution.

When performed as described here, all tasks included in this diagnostics may take up to 24 h to obtain results. One way to shorten this time is by grinding the specimen before adding proteinase K, in which case the overnight incubation with proteinase K can be substituted with 10-min incubation at 70°C. The disadvantage of this method is that the voucher is destroyed.

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