Development of a Multiplex PCR for Identification of Vineyard Mealybugs

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ABSTRACT A simple molecular tool was developed and tested to identify seven mealybug species found in North American vineyards: *Pseudococcus maritimus* Ehrhorn, *Pseudococcus viburni* (Signoret), *Pseudococcus longispinus* (Targioni-Tozzeti), *Pseudococcus calceolariae* (Maskell), *Planococcus ficus* (Signoret), *Planococcus citri* (Risso), and *Ferrisia gilli* Gullan. The developed multiplex PCR is based on the mitochondrial cytochrome c oxidase subunit one gene. In tests, this single-step multiplex PCR correctly identified 95 of 95 mealybug samples, representing all seven species and collected from diverse geographic regions. To test the sensitivity, single specimen samples with different *Pl. ficus* developmental stages (egg to adult female and adult male) were processed PCR and the resulting output provided consistent positive identification. To test the utility of this protocol for adult males caught in sex baited pheromone traps, *Pl. ficus* adult males were placed in pheromone traps, aged at a constant temperature of 26 ± 2°C, and processed with the multiplex each day thereafter for 8 d. Results showed consistent positive identification for up to 6 d (range, 6–8 d). Results are discussed with respect to the usefulness of this molecular tool for the identification of mealybugs in pest management programs and biosecurity of invasive mealybugs.

KEY WORDS multiplex PCR, pest identification, vineyards, *Planococcus*, *Pseudococcus*

Over the past decade there has been increasing concern over mealybug infestations in vineyards in Europe (Sforza et al. 2003), New Zealand (Charles et al. 2010); North America (Daane et al. 2008); South Africa (Walton and Pringle 2004); South America (Ripa and Luppichini 2010); and elsewhere. Mealybug species commonly found in vineyards can feed on the vine's root, trunk, canes, leaves, or fruit clusters and, as the mealybug feeds, it eliminates carbohydrate-rich honeydew that can accumulate on the leaves and fruit clusters and serve as a substrate for sooty mold fungi (Charles 1982, Ben-Dov 1995). In most of the world's wine grape regions, however, it is not the damage from mealybug feeding or contamination of fruit clusters that are of most concern, but the transmission of viruses, particularly a complex of species collectively known as grapevine leafroll-associated viruses (GLRaV) (de Borbon et al. 2004, Charles et al. 2009, Bertin et al. 2010, Tsai et al. 2010).

Currently, several mealybug species are known GLRaV vectors, including *Pseudococcus maritimus* Ehrhorn, *Pseudococcus viburni* (Signoret), *Pseudococcus longispinus* (Targioni Tozzeti), *Pseudococcus calceolariae* (Maskell), *Pseudococcus comstocki* (Kuwana), *Planococcus ficus* (Signoret), *Planococcus citri* (Risso), *Heliococcus bohemicus* Sulc, and *Phenacoccus aceris* (Signoret) (Cabaleiro and Segura 1997, Engelbrecht and Kasdorf 1990, Golino et al. 2002, Sforza et al. 2003, Tsai et al. 2008). Of these, *Ps. maritimus*, *Ps. viburni*, *Ps. longispinus*, *Pl. ficus*, and *Pl. citri* can be found in North American vineyards (Daane et al. 2008), along with the newly described *Ferrisia gilli* Gullan (Gullan et al. 2003). In addition, *Ps. calceolariae* is an important GLRaV vector in New Zealand (Bell et al. 2009, Charles et al. 2009); whereas *Ps. calceolariae* is found in North America (McKenzie 1967), it is rarely found in vineyards. These species have different geographic ranges (Ben-Dov 1995, ScaleNet 2011), in part because of biological constraints such as temperature tolerances (Gutierrez et al. 2008) but also because of geographic isolation among species and populations throughout the world's grape-growing regions. For example, in most of North America, *Ps. maritimus* is the primary mealybug pest in vineyards (Geiger and Daane 2001, Grasswitz and James 2008), but in some parts of California and Mexico, the invasive *Pl. ficus* has become the more important species (Castillo et al.

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2005, Daane et al. 2008) and is not found elsewhere in North America.

Preventing the spread of these mealybug species is an important quarantine issue, and early identification of new invasions is a key part of such a program. However, the rapid and accurate identification of mealybug species is not easily accomplished. Taxonomic separation can be difficult, particularly for the nymphal stages that are primarily involved with dispersal (Beuning et al. 1999, Gullan 2000, Hardy et al. 2008). For example, many of the early North American records of mealybugs on grapes (*Vitis* spp.), pears (*Pyrus* spp.), and apples (*Malus domestica* Borkh.) were described as *Ps. maritimus*, and yet, of the 1,000 slides labeled as *Ps. maritimus* at the United States Museum of Natural History, there were at least 10 different species (Miller et al. 1984). It was particularly difficult to separate the *Ps. maritimus* from *Ps. viburni* until the needed taxonomic descriptions of these closely related species were provided (Miller et al. 1984, Gimpel and Miller 1996). Separation between *Pl. ficus* and *Pl. citri* provides similar difficulties (Rotundo and Tremblay 1982), and their taxonomic identification is only through careful slide preparation to discern slight differences in the distribution and presence of multilocular pores and tubular ducts on the adult females (Williams and Granara de Willink 1992).

Molecular tools provide an additional method to separate mealybug species and aid in their classification (Hardy et al. 2008, Malausa et al. 2011). Polymerase chain reaction (PCR) was first used to identify closely related mealybugs by Beuning et al. (1999) in New Zealand for three of the vineyard mealybug species (*Ps. viburni*, *Ps. calceolariae*, and *Ps. longispinus*). Demontis et al. (2007) and Cavalieri et al. (2008) later described PCR-based separation of *Pl. ficus* and *Pl. citri*, which is invaluable for these two taxonomically similar species in regions where they overlap. Cavalieri et al. (2008) used restriction fragment length polymorphism (RFLP) analysis of the mitochondrial gene cytochrome coxidase subunit I (COI) to distinguish populations of *Pl. ficus* and *Pl. citri*, which allowed the identification of two species using only one PCR reaction but added a post-PCR step to the protocol. Multiplex PCR offers another rapid identification process for multiple species, where a number of species-specific primers are used in a single PCR reaction. This process was successfully used by Demontis et al. (2007) in Italy to separate *Pl. ficus* and *Pl. citri*, and by Saccaggi et al. (2008) in South Africa to separate *Pl. ficus*, *Pl. citri*, and *Ps. longispinus*. Here, we sought to build upon these studies by developing a multiplex marker that could be used for the North American mealybug species that are either known vectors of GLRaVs or that are known to be isolated to a few geographic regions and considered to be invasive to other vineyard regions. We also tested the sensitivity of the multiplex PCR primers against individual mealybugs, different mealybug stages (egg to adult) and adult male mealybugs captured and aged in pheromone traps.

Materials and Methods

Mealybug Collection and DNA Extraction. The multiplex PCR integrity was tested using seven targeted mealybug species, collected from vineyards in Europe, Middle East, North America, South Africa, and South America (Table 1). The vineyard mealybug species used were *Ps. maritimus*, *Ps. viburni*, *Ps. longispinus*, *Ps. calceolariae*, *Pl. ficus*, *Pl. citri*, and *F. gilli*. In addition, nontarget specimens of *Ps. comstocki* (Kuwana), *Phenacoccus gossypii* Townsend & Cockerell, and *Phenacoccus solenopsis* Tinsley were tested as negative controls. The multiplex PCR intraspecific integrity also was tested with samples of the same species collected from different regions (Table 1). The initial taxonomic identification of specimens collected in North America were made by Kent Daane, Vaughn Walton, or Gillian Watson (Plant Pest Diagnostics Branch, CA Department of Food and Agriculture); specimens collected by René Sforza in Eurasia were identified by Dr. Jean-François Germain (Laboratoires Nationaux de la Protection des Végétaux, Montpellier, France); specimens from Argentina were supplied by Dr. Jose Luis Miano (Instituto Nacional de Tecnología Agropecuaria, Mendoza, sArgentina) and identified by taxonomists in that organization; specimens collected by Tania Zaviezo in Chile were from an insectary colony; and specimens from Egypt were identified by Dr. Majid Fallahzadeh (Department of Entomology, Islamic Azad University, Jahrom Branch, Fars, Iran). The vineyard mealybug species listed, as well as nontarget mealybug species, collected from the field or supplied from an insectary colony were immediately placed in 95% ethanol, until they could be stored at −20°C. Voucher specimens are either material in 95% ethanol and stored at −20°C (placed at the University of California, Berkeley, Quarantine collection), slide mounted (in Balsam), or deposited as GeneBank sequences (Table 1).

Genetic Data for COI. For each multiplex PCR sample, one large (third-instar or adult female) mealybug was tested per sample per location, unless stated otherwise. For each sample, genomic DNA was extracted with the DNeasy tissue kit (Qiagen, Inc., Valencia, CA).

Two representative sequences consisting of 588 bp from the mitochondrial COI gene were gathered from GenBank for *Ps. viburni* (EU267206-EU267207); *Ps. longispinus* (EU267194, DQ238222); *Pl. ficus* (DQ238220, EU250573); *Pl. citri* (EU267197, EU267198); and *F. gilli* (EU267202, EU267203). At the time of inquiry (September 2009), GenBank COI sequences were not available for *Ps. maritimus* and *Ps. calceolariae*. For these species, data were obtained from a sample of *Ps. maritimus* from Fresno County, CA, and from a sample of *Ps. calceolariae* from Santiago, Chile (Table 1), using PCR with primers Pat 5′ TCC-AAT-GCA-CTA-ATC-CAT-ATT-A three 'and Jerry 5' CAA-CAT-TTA-TTT-TGA-TTT TTT-GG 3′ (Simon et al. 1994). Amplification was performed in a Biometra T- personal thermal cycler (Biometra Göettingen, Germany). An initial denaturing step at 95°C for 5 min was followed by 33 cycles of 30 s at 94°C, 30 s at

Table 1. Mealybug collections used to assess the multiplex PCR with listings of mealybug species, location of the sampled population (Country, State or Region/Province, County or City), the species-specific results of the PCR band, the number of sites sampled for each region, and the type of voucher material stored

Mealybug species	Sample location	Samples positive/tested[a]	Voucher specimens[b]
Pseudococcus maritimus	USA: CA: Fresno County	3/3	EtOH, JN112800
Pseudococcus maritimus	USA: CA: Napa County	1/1	EtOH, SM
Pseudococcus maritimus	USA: CA: San Joaquin County	5/5	EtOH
Pseudococcus maritimus	USA: CA: Riverside County	1/1	EtOH, SM
Pseudococcus maritimus	USA: WA: Benton County	4/4	EtOH
Pseudococcus maritimus	USA: OR: Jackson County	3/3	EtOH
Pseudococcus maritimus	USA: NC: Buncombe County	1/1	EtOH, SM
Pseudococcus maritimus	USA: NE: Custer County	1/1	-
	Subtotal	18	
Pseudococcus viburni	USA: CA: Santa Cruz County	1/1	EtOH
Pseudococcus viburni	USA: CA: San Luis Obispo County	9/9	EtOH, SM
Pseudococcus viburni	Chile: Santiago: Santiago	5/5	EtOH, SM, JN112803
	Subtotal	15	
Pseudococcus longispinus	USA: CA: San Luis Obispo County	3/3	EtOH, SM
Pseudococcus longispinus	USA: CA: Fresno County	1/1	EtOH, SM
Pseudococcus longispinus	Chile: Cachapoal: Requnoa	1/1	JN112804
Pseudococcus longispinus	Chile: Santiago: Santiago	1/1	EtOH, SM
Pseudococcus longispinus	Australia: South Australia: Adelaide	2/2	EtOH, SM
	Subtotal	8	
Pseudococcus calceolariae	Chile: Santiago: Santiago	5/5	EtOH, JN112801
	Subtotal	5	
Planococcus ficus	USA: CA: San Joaquin County	4/4	EtOH, JN120845
Planococcus ficus	USA: CA: Fresno County	4/4	EtOH, SM
Planococcus ficus	USA: CA: Stanislaus County	2/2	EtOH
Planococcus ficus	USA: CA: Napa County	1/1	EtOH, SM
Planococcus ficus	USA: CA: Kern County	3/3	EtOH
Planococcus ficus	USA: CA: San Luis Obispo County	3/3	EtOH, SM, JN120846
Planococcus ficus	Mexico: Sonora: Hermosillo	4/4	EtOH, SM
Planococcus ficus	Argentina: Mendoza Province	3/3	EtOH, JN120844
Planococcus ficus	Italy: Sicily: Trapani	1/1	-
Planococcus ficus	Italy: Sardinia: Sassari	3/3	EtOH. SM
Planococcus ficus	Italy: Apulia: Bari	1/1	EtOH
Planococcus ficus	Portugal: Lisbon: Lisboa	3/3	EtOH
Planococcus ficus	Spain: Catalonia: Barcelona	3/3	EtOH, JN120846
Planococcus ficus	Greece: Crete: Heraklion	3/3	EtOH
Planococcus ficus	Greece: Crete: Chania	1/1	EtOH
Planococcus ficus	Israel: Keshet: Golan	1/1	JN120847
	Subtotal	40	
Planococcus citri	USA: CA: San Luis Obispo County	3/3	EtOH, JN120843
	Subtotal	3	
Ferrisia gilli	USA: CA: El Dorado County	5/5	EtOH, SM
Ferrisia gilli	USA: CA: Fresno County	1/1	EtOH
	Subtotal	6	
	Total target mealybugs	95	
Phenacoccus solenopsis	USA: CA: Sacramento County	0/3	EtOH, JN112802
Pseudococcus comstocki	USA: CA: Kern County	0/3	EtOH
Phenacoccus gossypii	Pakistan: Balochistan	0/3	EtOH
	Total nontarget mealybugs	9	

[a] Each sample represents a separate site, although different sites were often from within one region (e.g., different vineyards in Fresno County) and some samples were provided through insectary colonies that were initiated with field collected material from that location.

[b] Voucher material deposited as samples in 95% alcohol (EtOH), slide-mounts (SM) of adult mealybugs, DNA sequences deposited in GenBank, or both (GenBank accession number provided).

47°C, and 1 min 30 s at 72°C; with a final extension of 10 min at 72°C. All reactions used *Taq*PCR Master Mix Kit (Qiagen) with a $MgCl_2$ concentration of 4 mM and 0.25 μM of each primer. For each reaction, 1 μl of genomic DNA was used for a total reaction volume of 12.5 μl. PCR products were visualized after electrophoresis on a 1.2% agarose gels, stained with ethidium bromide, and cleaned using QIAquick PCR Purification Kit (Qiagen). Purified PCR was submitted to the University of California, Berkeley DNA sequencing facility for direct sequencing of both strands using the ABI Big Dye V3.1 terminator sequencing reaction kit (Perkin-Elmer/ABI, Weiterstadt, Germany) on an ABI 3707xl DNA Analyzer (Perkin-Elmer) with POP seven and a 50-cm array. DNA sequences were aligned manually in SeqMan two version 5.07 (DNASTAR, Madison, WI).

Multiplex-PCR. COI sequences from tested mealybugs were aligned in MEGA version 4.0.2 (Tamura et al. 2007) and species-specific forward PCR primers (Table 2) were visually searched from within sequence areas with a high number of mismatched bases between species. Primer design followed a combination of criteria from Rugman-Jones et al. (2009) and Saccaggi et al. (2008) whereby: 1) complementary between primers is minimal, 2) the designed primers had at least one but no more than three G or C bases

Table 2. Species specific oligonucleotide primers used in the multiplex PCR and the sizes of the PCR fragments

Insect species (abbreviated name)	Forward primer	Oligo sequence	Estimated PCR product size (bp)
Pseudococcus calceolariae	PCa	5′-TGCAACAATAATTATTGCCATC-3′	650
Pseudococcus longispinus	PL	5′-CCATTTATCTTTGATCCACAG-3′	600
Planococcus ficus	PF	5′-CTTTGTTGTAGCTCACTTTCAC-3′	450
Pseudococcus maritimus	PM	5′-CTGATTTCCTTTATTAATTAATTCAAC-3′	400
Planococcus citri	PC	5′-TAATCTATTTTTATCTATCAATTTAACC-3′	350
Pseudococcus viburni	PV	5′-ATATTTCTTCTATTGGTTCATTC-3′	250
Ferrisia gilli	FG	5′-GAATCATTAATTTCTAAACGTTTACTAA-3′	150
Universal reverse primer	MB-R	5′-CAATGCATATTATTCTGCCATATTA-3′	

to promote specific binding at the 3′ end of the primer, 3) the primers should work at similar annealing temperatures, 4) species primers should have three or more base pair differences with all other species, and 5) designed primers should produce different sized PCR fragments that can be visualized with gel electrophoresis. Species specificity of primers was confirmed using Basic Local Alignment Search Tool on GenBank.

The multiplex universal reverse primer MB-R (Table 2), is a version of Pat (Simon et al. 1994), developed for Pseudococcidae by using available mitochondrial t-RNA leucine (UUR) data from GenBank. Genetic data (GenBank accession numbers in parentheses) for *Pl. citri* (AF483206), *Maconellicoccus hirsutus* (Green) (AF483207), *Melanococcus albizziae* (Maskell) (AF483205), and *Dysmicoccus brevipes* (Cockerell) (AF483204) were aligned in MEGA version 4.0.2 (Tamura et al. 2007); the reverse primer was visually chosen using base conservation as criteria. The optimized multiplex-PCR amplified 1 μl of template DNA (unknown concentration) from mealybugs. All reactions used the QIAGEN Multiplex PCR Kit (Qiagen) with a final $MgCl_2$ concentration of 3 mM and 0.20 μM of each primer. Amplification was performed in a Biometra T- personal thermal cycler (Biometra, Göettingen, Germany) with an initial activation of HotStarTaq DNA Polymerase (Qiagen) at 95°C for 15 min, followed by 30 cycles of 30 s at 94°C, 90 s at 53°C, and 90 s at 72°C, and then a final extension of 10 min at 72°C. After amplification, 4 μl of each PCR product was visualized by electrophoresis on a 2% agarose gel stained with ethidium bromide.

The utility of the multiplex-PCR was tested on mealybug species from different locations (Table 1) and checked for the production of false positives using the three nontarget samples of Pseudococcidae, as well as a no mealybug (blank) control. For each sample, a single adult mealybug was used. The entire identification protocol (including DNA extraction, PCR, and electrophoresis) was completed in 4–5 h.

Size and Number of Mealybugs. Mealybugs range in size from <0.5 mm for the egg and crawler (unsettled first-instar) to >5 mm for the adult female, with more than a 1,000-fold increase in body weight (K. M. Daane, unpublished data). It is the smaller eggs, first instars, and adult males that are more difficult to identify and have a greater need for PCR identification tools. Different stages of *Pl. ficus* were used to determine if mealybug size, as representative of the amount of DNA available per sample, would impact the PCR results. A single egg; first, second, and third instar; an adult female; and an adult male mealybug were each tested separately using the multiplex PCR, as described previously. As with all trials, a DNA sizing ladder (Invitrogen Corp., Carlsbad, CA) suitable for sizing double-stranded DNA from 50 to 800 bp was used. For reference on agarose gels, the 350 bp band is brighter than the other bands in the ladder. In addition, a DNA mass ladder (Invitrogen Corp., Carlsbad, CA) was used to estimate mass of DNA samples providing bands 200, 120, 80, 40, 20, and 10 ng. In these trials we kept the same elution volume regardless of the insect size (e.g., egg versus adult female). Five samples of each mealybug stage were analyzed.

Time (Degradation) Trails for Adult Male Captures. Regulatory pheromone trapping for *Pl. ficus*, *Ps. maritimus*, and *Ps. viburni* is being conducted by state and university personnel in California, Oregon, Washington, and Idaho, primarily to monitor for the invasive *Pl. ficus*, but also to determine the presence of GLRaV vectors in vineyard regions. For these studies, the fast and accurate identification of aged adult male mealybugs caught in pheromone traps is important. Adult males caught in sex pheromone baited traps are not always collected immediately after trap capture, with pheromone traps typically checked every 7–14 d. Therefore, time degradation of samples of adult males caught in pheromone traps may impact the utility of the multiplex PCR. Differently aged adult males of *Pl. ficus* were tested to estimate how long after trap capture the multiplex PCR would provide consistent results. Samples of *Pl. ficus* adult male mealybugs were collected from a colony at the University of California, Kearney Agricultural Center. The adult males were placed in the stickum of a Pherocon Delta IIID sticky trap (Suterra, Bend, OR), which is a kind of trap used with a sex pheromone lure to monitor male mealybug flights (Walton et al. 2004). The male mealybugs, embedded in the trap's stickum, were held at room temperature (26 ± 2°C) until analysis with the multiplex PCR. To assess the time period allowed for successful multiplex identification of pheromone trap collected males, genomic DNA extractions were performed on samples containing a single adult male mealybug and samples containing three adult males, at 24-h intervals until the multiplex PCR regularly failed to amplify the *Pl. ficus* specific band. Five samples were analyzed for

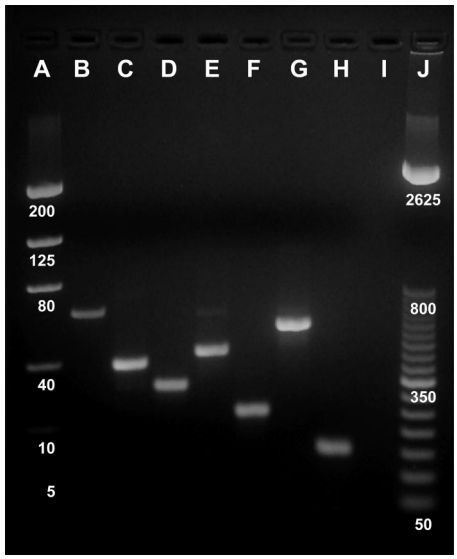

Fig. 1. Sample gel from multiplex PCR testing different mealybug species showing (A) DNA mass ladder (in nanograms), (B) *Ps. calceolariae*, (C) *Ps. maritimus*, (D) *Pl. citri*, (E) *Pl. ficus*, (F) *Ps. viburni*, (G) *Ps. longispinus*, (H) *F. gilli*, (I) negative water control, and (J) DNA sizing ladder (in base pairs).

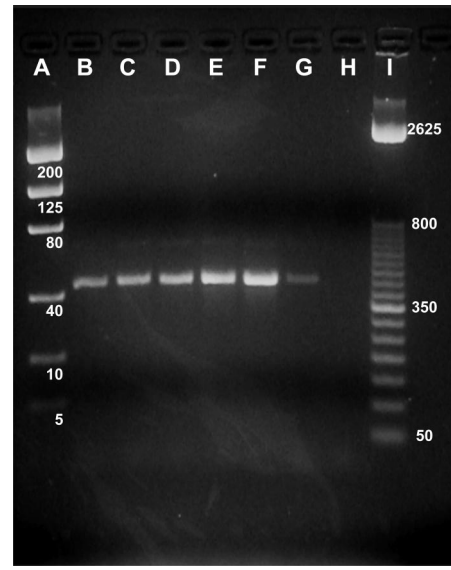

Fig. 2. Sample gel from multiplex PCR testing different *Pl. ficus* stages showing (A) DNA mass ladder (in nanograms), (B) adult male (C) first-instar nymph, (D) second-instar nymph, (E) third-instar nymph, (F) adult female, (G) egg, (H) negative water control, and (I) DNA sizing ladder (in base pairs).

each time period and sample size (one or three male mealybugs).

Results

Sequence of *Ps. maritimus* and *Ps. calceolariae*. The primer pair of Pat and Jerry successfully PCR amplified and direct sequenced 700 bp of the COI gene for *Ps. maritimus* and *Ps. calceolariae.* Sequence data for these mealybug species were used for the multiplex design and also submitted to GenBank for *Ps. maritimus* and *Ps. calceolariae* (Table 1).

Multiplex. Amplification of the COI by using multiplex PCR primers yielded species-specific fragments (between 150 and 650 bp) that provided the direct diagnosis of the seven targeted mealybug species (Fig. 1). The reliability and reproducibility of the multiplex assay was demonstrated by running a panel of mealybugs from a broad geographic distribution, with the field biology, or taxonomically determined species matching the PCR output. The method accurately identified all samples (100% accuracy) of *Ps. maritimus*, *Ps. viburni*, *Ps. longispinus*, *Ps. calceolariae*, *Pl. ficus*, *Pl. citri* and *F. gilli* (Table 1). The nontarget mealybugs (*Ps. comstocki*, *Ph. gossypii*, and *Ph. solenopsis*) failed to generate any PCR signal and, therefore, did not yield a false positive PCR band.

Size and Number of Mealybugs. Multiplex PCR reliably produced distinct PCR bands for *Pl. ficus* adult males and all immature female stages (Fig. 2). Evident band masses for individual crawlers (40–60 ng) and adult males (≈10 ng) were weaker than the band mass for the adult female mealybug (150–200 ng).

Time (Degradation) Trials for Adult Male Captures. In repeated trials, multiplex PCR identification of trap collected *Pl. ficus* males provided consistently accurate positive bands for males aged up to 6 d. There was degradation of the band strength, as measured by the mass ladder, and PCR bands were not always visible after 6 d of time in pheromone traps, with the longest clearly recognizable positive bands at 8 d (Fig. 3).

Discussion

We developed a multiplex PCR to identify key mealybug species found in North American vineyards. The multiplex PCR was developed from 588 bp of the COI gene region by using a combination of sequences deposited in GenBank and sequencing produced from our samples of *Ps. maritimus* and *Ps. calceolariae.* Sequences were aligned and species primers were chosen using sequence differences to produce seven forward primers paired with one universal reverse primer that diagnosed mealybugs through different size PCR products. The utility of these primers to discriminate against the targeted mealybug species was evaluated by collecting samples from a broad geographic range, and confirming their identification through multiplex PCR. Results indicate that the developed multiplex PCR accurately identified the targeted mealybugs, which represented samples derived from a wide geographic range. Identification of mealybugs through classical taxonomic keys often require a high level of expertise and can be time consuming when, in some cases, immature stages must be reared to the adult stage for proper identification (Gullan 2000, Hardy et

Fig. 3. Sample from multiplex PCR testing adult male mealybugs aged for different number of days in the stickum of a pheromone trap and a negative water control are shown by the horizontal label, the left-side vertical label is the DNA mass ladder (in nanograms), and the right-side vertical label is the DNA sizing ladder (in base pairs). In this sample, adult males aged at 7 d are quite discernable, whereas there is only a weak and questionable band at 8 d.

al. 2008). The developed molecular tool will help screen mealybugs in this and other circumstances. The primary use for the multiplex PCR may be related to biosecurity, particularly in those countries, states, provinces, or regions where regulations are in place to halt the spread of invasive pests. Such a molecular approach was developed for use in New Zealand to identify *Pseudococcus* species found on apple (Beuning et al. 1999).

Previous molecular tools were developed successfully for *Pl. ficus*, *Pl. citri*, and *Ps. longispinus* (Demontis et al. 2007, Rung et al. 2008, Saccaggi et al. 2008). However, *Ps. maritimus* was not included in these earlier studies and this species is the primary mealybug found in North American vineyards. In addition, *Ps. viburni* has been reported in North American vineyards and pome fruit orchards and is the key vineyard pest in many regions, such as South America (Ben-Dov 1995). *Pseudococcus viburni* and *Ps. maritimus* are commonly misidentified (Miller et al. 1984) and their inclusion in the current multiplex will be of benefit in North America and elsewhere. Also included were *Pl. citri*, *Pl. calceolariae*, and *F. gilli*, which are all found in North America, particularly in California, and their inclusion completed the development of a molecular diagnostic tool for mealybugs collected in vineyards that are either known vectors of GLRaVs (Cid et al. 2010, Tsai et al. 2010) or that are known to be isolated to a few geographic regions and considered to be invasive to other vineyard regions. Therefore, primers were redesigned to account for the seven mealybug species tested, which created more pressure on each primer to show specificity for one species. In addition, the redesigned primers provided better annealing with the DNA template and reduced chemical properties of hairpin formations that can eventually hinder PCR reactions. The developed multiplex adds to other molecular identification tools for mealybugs found in citrus (Pieterse et al. 2010); pears (Rung et al. 2009, Park et al. 2010); and grapes (Demontis et al. 2007, Rung et al. 2008, Saccaggi et al. 2008, Bertin et al. 2010).

The speed, accuracy, reliability, and prevalence of molecular techniques indicate their value as a standard tool for monitoring agricultural pests and assisting in research (Armstrong and Ball 2005). Testing can be relatively rapid and multiple samples can be screened at the same time. This test can aid in the screening of vine produce for infestation, taxonomic identification, and assist in research on the biology and ecology of vine-associated mealybugs. Two aspects of the developed multiplex PCR that would determine its utility are its ability to discern first-instar mealybugs collected from the vine, as well as adult males caught in pheromone traps. Using *Pl. ficus*, the multiplex PCR provided a repeatable evident band for individual eggs and first instars (40–60 ng) and adult males (10 ng), whereas the band mass for the adult female mealybug was clearly brighter (150–200 ng). In this study, we used the same elution volume for all samples, regardless of the insect size (e.g., egg versus adult). For greater band mass, a smaller elute volume could be used and we note that band mass should not be used to indicate mealybug stage.

It is the largely unidentifiable smaller mealybug stages that present the greater use for the multiplex PCR. Mealybug sex pheromones developed for mealybugs are largely species specific (e.g., Millar et al. 2002, Figadère et al. 2007, Zou and Millar 2009, El-Sayed et al. 2010); however, for quarantine issues verification of trapped mealybugs is necessary, and some sex-pheromone baited traps have collected nontarget adult male mealybugs (Bentley et al. 2008). The sensitivity of the multiplex PCR to identify adult males collected in pheromone traps was shown, with reliable identification of *Pl. ficus* aged for 6 d in pheromone traps. After this time, the genomic DNA extraction failed to give a reliable DNA template, likely because of degradation. The trial was conducted with male mealybugs aged at room temperature and it is expected that ambient conditions may either accentuate or reduce the amount of time samples are viable for identification. For this reason, when possible the male mealy-

bugs should be removed from traps, placed preserved in 95% EtOH, and stored at −20°C (or lower) as soon as possible. When traps are checked every 7–14 d, these results also suggest a high probability of false negative PCR outcome.

False negative and false positive outputs are a potential problem for identification employing the mitochondrial COI locus through multiplex PCR. False negatives may occur when the specimen has not been properly preserved or when high intraspecific variation exists in a species causing a primer to fail. Data from other studies show intraspecific variation of the COI is low and usually between 1 and 2% (Avise 2000), supporting development of species-specific primers. In Pseudococcidae, COI data for *Pl. citri* and *Planococcus minor* (Maskell) gathered from every continent, excluding Antarctica, showed the average intraspecific pairwise distance to be 0.3% and 0.2%, respectively (Rung et al. 2008). Although this information does not provide estimates of genetic divergence among the other species of mealybugs included in this study, it suggests sequence divergences within Pseudococcidae species are low enough to support multiplex identification studies employing the COI gene.

False positives would arise if sequences did not show enough interspecific variation in mealybugs to permit a putative species-specific primer to fail by annealing with other closely related species. This is not a perceived problem as a large survey of COI pairwise distances for congeneric insect pairs from within the orders Coleoptera, Diptera, and Hymenoptera typically average 8–16% (Hebert et al. 2003), providing many suitable locations for species-specific primer design. Of the sequences used in this study, the highest pairwise distance was 15.2% between *Ps. maritimus* and *Ps. calceolariae* and the lowest was 7% between *Pl. citri* and *Pl. ficus*, allowing ample sequence divergence for robust primer design. Regardless, to assess for false positives during multiplex PCR, we included three nontarget mealybugs *Ps. comstocki*, *Ph. Gossypii*, and *Ph. solenopsis* in our testing of species primers and found them to not be cross reactive. In addition to the false positive results, lower sequence divergence in closely related species is not a problem because of the sensitivity of a well developed PCR reaction. In our species primers, we permitted as few as three polymorphic sites in a primer, and this allowed the successful discrimination of species as in other multiplex studies with Pseudococcidae (Demontis et al. 2007, Saccaggi 2008). As new data are generated, a redesign of the multiplex key may be required to account for the diversity. To verify a negative result, the protocols for sequencing the COI provided in this study should clear up any ambiguity.

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References Cited

Armstrong, K. F., and S. L. Ball. 2005. DNA barcodes for biosecurity: invasive species identification. Philos. Trans. R. Soc. Lond., Ser. B: Biol. Sci. 360: 1813–1823.

Avise, J. C. 2000. Phylogeography: the history and formation of species, Harvard University Press, Cambridge, MA.

Bell, V. A., R.G.E. Bonfiglioli, J.T.S. Walker, P. L. Lo, J. F. Mackay, and S. E. McGregor. 2009. Grapevine leafroll associated virus 3 persistence in *Vitis vinifera* remnant roots. J. Plant Pathol. 91: 527–533.

Ben-Dov, Y. 1995. A systematic cataloque of the mealybugs of the world (Insecta: Homoptera: Coccoidea: Pseudococcidae, and Putoidae) with data on geographical distribution, host plants, biology, and economic importance, Intercept Ltd., Hampshire, United Kingdom.

Bentley, W. J., L. G. Varela, F. G. Zalom, R. J. Smith, A. H. Purcell, P. A. Phillips, D. R. Haviland, K. M. Daane, and M. C. Battany. 2008. Grape: pest management guidelines. University of California IPM Pest Management Guidelines: Grapes, Insects and Mites. Publ. 3448.

Bertin, S., V. Cavalieri, C. Graziano, and D. Bosco. 2010. Survey of mealybug (Hemiptera: Pseudococcidae) vectors of *Ampelovirus* and *Vitivirus* in vineyards of northwestern Italy. Phytoparasitica 38: 401–409.

Beuning, L. L., P. Murphy, E. Wu, T. A. Batchelor, and B.A.M. Morris. 1999. Molecular-based approach to the differentiation of mealybug (Hemiptera: Pseudococcidae) species. J. Econ. Entomol. 92: 463–472.

Cabaleiro, C., and A. Segura. 1997. Field transmission of grapevine leafroll associated virus 3 (GLRaV-3) by the mealybug *Planococcus citri.* Plant Dis. 81: 283–287.

Castillo, A.A.F., H. G. Hernández, and K. M. Daane. 2005. Los pijo harinosos de la vid, vol. No 9. ISBN No. 968-558-64-2, Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias. Libro Técnico, Mexico.

Cavalieri, V., G. Mazzeo, G. T. Garzia, E. Buonocore, and A. Russo. 2008. Identification of *Planococcus ficus* and *Planococcus citri* (Hemiptera: Pseudococcidae) by PCR-RFLP of COI gene. Zootaxa 1816: 65–68.

Charles, J. G. 1982. Economic damage and preliminary economic thresholds for mealybugs (*Pseudococcus longispinus* T.-T.) in Auckland vineyards. N.Z. J. Agric. Res. 25: 415–420.

Charles, J. G., K. J. Froud, R. van den Brink, and D. J. Allan. 2009. Mealybugs and the spread of grapevine leafroll-associated virus 3 (GLRaV-3) in a New Zealand vineyard. Australas. Plant Pathol. 38: 576–583.

Charles, J. G., V. A. Bell, P. L. Lo, L. M. Cole, and A. Chhagan. 2010. Mealybugs (Hemiptera: Pseudococcidae) and

their natural enemies in New Zealand vineyards from 1993–2009. N.Z. Entomol. 33: 84–91.

Cid, M., S. Pereira, C. Cabaleiro, and A. Segura. 2010. Citrus mealybug (Hemiptera: Pseudococcidae) movement and population dynamics in an arbor-trained vineyard. J. Econ. Entomol. 103: 619–630.

Daane, K. M., M. L. Cooper, S. V. Triapitsyn, V. M. Walton, G. Y. Yokota, D. R. Haviland, W. J. Bentley, K. E. Godfrey, and L. R. Wunderlich. 2008. Vineyard managers and researchers seek sustainable solutions for mealybugs, a changing pest complex. Calif. Agric. 62: 167–176.

de Borbon, C. M., O. Gracia, and G.S.G. Talquenca. 2004. Mealybugs and grapevine leafroll-associated virus 3 in vineyards of Mendoza, Argentina. Am. J. Enol. Vitic. 55: 283–285.

Demontis, M. A., S. Ortu, A. Cocco, A. Lentini, and Q. Migheli. 2007. Diagnostic markers for *Planococcus ficus* (Signoret) and *Planococcus citri* (Risso) by random amplification of polymorphic DNA-polymerase chain reaction and species-specific mitochondrial DNA primers. J. Appl. Entomol. 131: 59–64.

El-Sayed, A. M., C. R. Unelius, A. Twidle, V. Mitchell, L. A. Manning, L. Cole, D. M. Suckling, M. F. Flores, T. Zaviezo, and J. Bergmann. 2010. Chrysanthemyl 2-acetoxy-3-methylbutanoate: the sex pheromone of the citrophilous mealybug, *Pseudococcus calceolariae.* Tetrahedron Lett. 51: 1075–1078.

Engelbrecht, D. J., and G.G.F. Kasdorf. 1990. Transmission of grapevine leafroll disease and associated closteroviruses by the vine mealybug, *Planococcus ficus.* Phytophylactica 22: 341–346.

Figadère, B. A., J. S. McElfresh, D. Borchardt, K. M. Daane, W. Bentley, and J. G. Millar. 2007. Trans-alpha-necrodyl isobutyrate, the sex pheromone of the grape mealybug, *Pseudococcus maritimus.* Tetrahedron Lett. 48: 8434–8437.

Geiger, C. A., and K. M. Daane. 2001. Seasonal movement and distribution of the grape mealybug (Homoptera: Pseudococcidae): developing a sampling program for San Joaquin Valley vineyards. J. Econ. Entomol. 94: 291–301.

Gimpel, W. F., Jr., and D. R. Miller. 1996. Systematic analysis of the mealybugs in the *Pseudococcus maritimus* complex (Homoptera: Pseudococcidae). International Contributions on Entomology 2: 1–163.

Golino, D. A., S. T. Sim, R. Gill, and A. Rowhani. 2002. California mealybugs can spread grapevine leafroll disease. Calif. Agric. 56: 196–201.

Grasswitz, T. R., and D. G. James. 2008. Movement of grape mealybug, *Pseudococcus maritimus,* on and between host plants. Entomol. Exp. Appl. 129: 268–275.

Gullan, P. J. 2000. Identification of the immature instars of mealybugs (Hemiptera: Pseudococcidae) found on citrus in Australia. Aust. J. Entomol. 39: 160–166.

Gullan, P. J., D. A. Downie, and S. A. Steffan. 2003. A new pest species of the mealybug genus *Ferrisia* Fullaway (Hemiptera: Pseudococcidae) from the United States. Ann. Entomol. Soc. Am. 96: 723–737.

Gutierrez, A. P., K. M. Daane, L. Ponti, V. M. Walton, and C. K. Ellis. 2008. Prospective evaluation of the biological control of vine mealybug: refuge effects and climate. J. Appl. Ecol. 45: 524–536.

Hardy, N. B., P. J. Gullan, and C. J. Hodgson. 2008. A classification of mealybugs (Hemiptera: Pseudococcidae) based on integrated molecular and morphological data. Syst. Entomol. 33: 51–71.

Hebert, P.D.N., S. Ratnasingham, and J. R. de Waard. 2003. Barcoding animal life: cytochrome c oxidase subunit 1 divergences among closely related species. Philos. Trans. R. Soc. Lond., Ser. B: Biol. Sci. 270: S96–S99.

Malausa, T., A. Fenis, S. Warot, J.-F. Germain, N. Ris, E. Prado, M. Botton, F. Vanlerberghe-Masutti, R. Sforza, C. Cruaud, A. Couloux, and P. Kreiter. 2011. DNA markers to disentangle complexes of cryptic taxa in mealybugs (Hemiptera: Pseudococcidae). J. Appl. Entomol. 135: 142–155.

McKenzie, H. L. 1967. Mealybugs of California with taxonomy, biology and control of North American species. University of California Press, Berkeley and Los Angeles, CA.

Millar, J. G., K. M. Daane, J. S. McElfresh, J. A. Moreira, R. Malakar-Kuenen, M. Guillen, and W. J. Bentley. 2002. Development and optimization of methods for using sex pheromone for monitoring the mealybug *Planococcus ficus* (Homoptera: Pseudococcidae) in California vineyards. J. Econ. Entomol. 95: 706–714.

Miller, D. R., R. T. Gill, and D. J. Williams. 1984. Taxonomic analysis of *Pseudococcus affinis* (Maskell), a senior synonym of *Pseudococcus obscurus* Essig, and a comparison with *Pseudococcus maritimus* (Ehrhorn) (Homoptera: Coccoidea: Pseudococcidae). Proc. Entomol. Soc. Wash. 86: 703–713.

Park, D.-S., Y. J. Leem, K.-W. Hahn, S.-J. Suh, K.-J. Hong, and H.-W. Oh. 2010. Molecular identification of mealybugs (Hemiptera: Pseudococcidae) found on Korean pears. J. Econ. Entomol. 103: 25–33.

Pieterse, W., D. L. Muller, and B. J. van Vuuren. 2010. A molecular identification approach for five species of mealybug (Hemiptera: Pseudococcidae) on citrus fruit exported from South Africa. Afr. Entomol. 18: 23–28.

Ripa, R. S., and P. B. Luppichini. 2010. Management of vineyard pests (in Spanish), Instituto Investigaciones Agropecuarias, Miniserio de Agricultura, La Cruz, Chile.

Rotundo, G., and E. Tremblay. 1982. Hybridization and sex-pheromone responses in two closely related mealybug species (Homoptera: Pseudococcidae). Syst. Entomol. 7: 475–478.

Rugman-Jones, P. F., J. G. Morse, and R. Stouthamer. 2009. Rapid molecular identification of armored scale insects (Hemiptera: Diaspididae) on Mexican 'Hass' avocado. J. Econ. Entomol. 102: 1948–1953.

Rung, A., S. J. Scheffer, G. Evans, and D. Miller. 2008. Molecular identification of two closely related species of mealybugs of the genus *Planococcus* (Homoptera: Pseudococcidae). Ann. Entomol. Soc. Am. 101: 525–532.

Rung, A., D. R. Miller, and S. J. Scheffer. 2009. Polymerase chain reaction-restriction fragment length polymorphism method to distinguish three mealybug groups within the *Planococcus citri - P. minor* species complex (Hemiptera: Coccoidea: Pseudococcidae). J. Econ. Entomol. 102: 8–12.

Saccaggi, D. L., K. Kruger, and G. Pietersen. 2008. A multiplex PCR assay for the simultaneous identification of three mealybug species (Hemiptera: Pseudococcidae). Bull. Entomol. Res. 98: 27–33.

ScaleNet. 2011. (http://www.sel.barc.usda.gov/scalenet/scalenet.htm).

Sforza, R., E. Boudon-Padieu, and C. Greif. 2003. New mealybug species vectoring grapevine leafroll-associated viruses-1 and-3 (GLRaV-1 and-3). Eur. J. Plant Pathol. 109: 975–981.

Simon, C., F. Frati, A. Beckenbach, B. Crespi, H. Liu, and P. Flook. 1994. Evolution, weighting, and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. Ann. Entomol. Soc. Am. 87: 651–701.

Tamura, K., J. Dudley, M. Nei, and S. Kumar. 2007. MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol. Biol. Evol. 24: 1596–1599.

Tsai, C., A. Rowhani, D. A. Golino, K. M. Daane, and R.P.P. Almeida. 2010. Mealybug transmission of grapevine leafroll viruses: an analysis of virus-vector specificity. Phytopathology 100: 830–834.

Tsai, C. W., J. Chau, L. Fernandez, D. Bosco, K. M. Daane, and R.P.P. Almeida. 2008. Transmission of grapevine leafroll-associated virus 3 by the vine mealybug (*Planococcus ficus*). Phytopathology 98: 1093–1098.

Walton, V. M., and K. L. Pringle. 2004. A survey of mealybugs and associated natural enemies in vineyards in the Western Cape Province, South Africa. South Afr. J. Enol. Vitic. 25: 23–25.

Walton, V. M., K. M. Daane, and K. L. Pringle. 2004. Monitoring *Planococcus ficus* in South African vineyards with sex pheromone-baited traps. Crop Prot. 23: 1089–1096.

Williams, D. J., and M. C. Granara de Willink. 1992. Mealybugs of Central and South America, CAB International, Wallingford, United Kingdom.

Zou, Y., and J. G. Millar. 2009. Synthesis of the pheromone of the longtailed mealybug, a sterically congested, irregular monoterpenoid. J. Org. Chem. 74: 7207–7209.

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