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Molecular and morphological characterization of mealybugs (Hemiptera: Pseudococcidae) from Chilean vineyards

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Abstract

Mealybugs are major pests of grapevines worldwide. They cause economic losses by lowering the cosmetic value of fruits, reducing yields, transmitting viruses and resulting in the quarantine or rejection of produce in international trade. Knowledge of the species present in a vineyard is important for the adjustment of management strategies. We surveyed and accurately characterized the mealybugs infesting vineyards in one of the main production areas of Chile; 164 mealybugs were sampled from 26 vineyards in four regions of Chile and identified by DNA sequencing for two markers (cytochrome oxidase I and internal transcribed spacer 2) and morphological examination. *Pseudococcus viburni* (Signoret) was the most common species, followed by *Pseudococcus meridionalis* Prado and *Pseudococcus cribata* González. Molecular variability at the COI and ITS2 loci was observed in both *P. viburni* and *P. cribata*. A comparison of haplotypes of *P. viburni* worldwide provides support for a recent hypothesis that this species is native to South America, a finding with direct consequences for management. Neither *Pseudococcus longispinus* (Targioni & Tozzetti) nor *Planococcus ficus* Signoret were found.

Keywords: molecular identification, DNA sequencing, DNA barcoding, CO1, *Pseudococcus*, Hemiptera

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Introduction

Grape is one of the most economically important crops in Chile, with vineyards covering over 180,000 hectares in 2007, and about a third of production dedicated to table grapes (ODEPA, 2010). This crop is the principal fruit exported from Chile, accounting for about 42% of all fruit exports. Mealybugs (Hemiptera: Pseudococcidae) are the main phytosanitary problem confronting international sales of Chilean table grapes, because their presence in the produce requires quarantine restrictions in many markets (SAG, 2009–2010). For example, during the 2008–2009 season, mealybugs were responsible for 71.5% of all table grape rejections during inspections before export (SAG, 2009–2010). In addition, mealybugs may damage the vines directly and indirectly. Large populations may lower the vigor of the plant by feeding on phloem and may affect fruit quality by depositing honeydew on the fruit, on which sooty mold subsequently develops (Artigas, 1994; Geiger & Daane, 2001; Bentley *et al.*, 2008; Daane *et al.*, 2008a). Mealybugs also can cause long-term damage by transmitting viruses (Golino *et al.*, 1999;

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Millar *et al.*, 2002; Douglas & Krüger, 2008). The principal, recurrent problem in the management of mealybugs is the cryptic ecology of these species. They are small, feed in concealed areas and can be transported on plant material, workers and machinery, making them particularly successful invaders (Miller *et al.*, 2005). Mealybug biology, damage, current control techniques and the main pest species around the world have recently been reviewed (Daane *et al.*, in press).

Mealybugs constitute a very diverse group, with 2291 species belonging to 274 genera described worldwide (Ben-Dov *et al.*, 2010). The species are hard to tell apart because they are very similar morphologically and their taxonomic identification is based on keys dealing with various cuticular structures on adult females, viewed on slide-mounted specimens under a microscope. Furthermore, in some species, there may exist phenotypic variations between individuals, depending on the climatic conditions or the substrate on which they are growing. This can make identification impossible without considerable expertise (Cox, 1983; Gullan & Kosztarab, 1997; Charles *et al.*, 2000; Millar, 2002; Zaviezo *et al.*, 2010). These problems have led to the development and use of molecular tools for the correct identification of Pseudococcidae species (Beuning *et al.*, 1999; Downie & Gullan, 2004; Rung *et al.*, 2007; Demontis *et al.*, 2007; Cavalieri *et al.*, 2008; Saccaggi *et al.*, 2008; Hardy *et al.*, 2008; Malausa *et al.*, 2011; Correa *et al.*, 2011; Park *et al.*, 2011).

Despite the difficulties involved in differentiating between mealybug species, correct identification is essential when dealing with species considered as pests. It is important to know which species are present in the field to optimize the timing of insecticide applications, because different species living on the same host may have different biological characteristics (Geiger & Daane, 2001; Varela, 2006). Furthermore, the natural enemies of mealybugs tend to specialize on particular species; identification of the mealybugs present is, therefore, essential to the success of biological control programs (Chong & Oetting, 2007; Daane *et al.*, 2008b). In international trade, different markets identify different mealybug species as quarantine pests (Beuning *et al.*, 1999; González & Volosky, 2004; SAG, 2009–2010).

The available data, based on morphological identification, suggest that *Pseudococcus viburni* (Signoret) is the most abundant and widely distributed species in Chilean vineyards (Zaviezo, 2002; González & Volosky, 2004; Sazo *et al.*, 2008; Ripa & Luppichini, 2010; Daane *et al.*, in press). Other species also have been reported sporadically: *Pseudococcus longispinus* (Targioni & Tozzetti) and other new *Pseudococcus* species (Correa *et al.*, 2011; González, 2011). In addition, it has been suggested that *Planococcus ficus* Signoret may be present in Chilean vineyards, but this remains a matter of debate (González, 2011).

Here, we took profit from the recent development of molecular markers for mealybugs to characterize the taxa infesting Chilean vineyards, by coupling DNA and morphological analyses. We collected mealybugs from 26 vineyards in the main grape-producing areas of central Chile, DNA sequenced them at two loci (Cytochrome oxydase I and ITS2) and examined morphologically. As a secondary objective, we used the produced DNA data to test the hypothesis that *P. viburni* is native to South America (Daane *et al.*, 2008a; Charles, 2010). Indeed, this hypothesis has implications for pest management (e.g. choice of biocontrol agents) and the level of genetic diversity observed among individual DNA sequences is an indication of the native regions of taxa.

Materials and methods

Sample collection

We sampled mealybugs from 26 Chilean vineyards during the 2009–2010 and 2010–2011 seasons (table 1 and fig. 1). In each vineyard, we examined a large number of grapevine individuals, checking all parts of the plants, and collecting mealybugs at different stages of development, to ensure that we did not miss species with different phenological features and habitat preferences. Adult females and nymphs were stored at –20°C in 95% ethanol until laboratory analysis.

DNA extraction and PCR amplification

Genomic DNA was extracted with the DNeasy Tissue Kit (QIAGEN, Hilden, Germany), with the non-destructive protocol described by Malausa *et al.* (2011), to ensure that the specimen remained available for morphological examination. Polymerase chain reaction (PCR) was performed with the reagents and concentrations used by Malausa *et al.* (2011). The primers used for COI were COI-J-2183-F CAACATTTATTTTGATTTTTTGG and COI-N-2568-R GCWACWACRTAATAKGTATCATG from Gullan *et al.* (2003). For ITS2, the primers were: ITS2-M-F CTCGTGACCAAAAGAGTCCTG and ITS2-M-R TGCTTAAGTTCAGCGGGTAG, as described by Malausa *et al.* (2011).

PCR conditions were as follows: initial denaturation for 30 s at 98°C, followed by 35 cycles of denaturation for 10 s at 98°C, annealing for 15 s at temperatures of 48–60°C, elongation at 72°C for 15 s, and a final extension period for 5 min at 72°C. The quality of the PCR products was checked by electrophoresis in 2% agarose gels.

PCR products were sent to Genoscreen (Lille, France) for bidirectional sequencing. Consensus sequences were generated and checked with Seqscape v2.7 (Applied Biosystems, Foster City, CA, USA). Alignments were edited with Bioedit 7.01 (Hall, 1999). Sequences differing from the consensus sequences were considered to belong to a different haplotype. A median-joining haplotype network was built with the software NETWORK (Bandelt *et al.*, 1999) using our COI sequences and those available in GenBank for *P. viburni*. The sequences were from Europe (GU134686, found at >20 sites all over France and JF714166 found at one site in Spain), Brazil (GU134685, four sites from the region of Rio Grande do Sul), South Africa (FJ786966, number and location of sites unknown), USA (EU267207 and EU267206, number and location of sites unknown) and Iran (JF905460, number and location of sites unknown). The alignment used can be consulted in fig. S1 in the supplementary material.

Morphological examination

For each observed multilocus genotype (i.e. each combination of haplotypes for the two genetic markers), we morphologically examined at least one specimen (and up to 31). Specimens were prepared for slide-mounting as described by Malausa *et al.* (2011): (i) after making a small incision, they were heated in 10% KOH for 20 min; (ii) they remaining body contents were expelled, tapering the body with a micro spatula; (iii) the specimens were stained by incubation for 1 h in a saturated solution of fuchsine in a 1:1:1 mixture of distilled water, lactic acid and glycerol; (iv) then, the specimens were washed in glacial acetic acid for 1 h to stabilize the staining; (v) finally, the specimens were transferred to lavender oil for at

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Table 1. Mealybug populations sampled.

Pop.	Region	Site	GPS	Collection date
1	O'Higgins	Chimbarongo	34°43′4.19″S/71°2′32.00″W	8/04/2010
2	O'Higgins	Chimbarongo	34°43′39.76″S/71°2′17.56″W	8/04/2010
3	O'Higgins	Chimbarongo	34°43′16.63″S/71°2′7.84″W	8/04/2010
4	O'Higgins	Chimbarongo	34°44′2.12″S/71°2′25.18″W	8/04/2010
5	O'Higgins	Chimbarongo	34°43′22.28″S/71°2′48.61″W	30/04/2010
6	O'Higgins	Chépica	34°41′32.77″S/71°9′46.65″W	10/06/2010
7	O'Higgins	Nancagua	34°36′24.74″S/71°7′32.64″W	9/04/2010
10	O'Higgins	Santa Cruz	34°40′42.46″S/71°23′7.92″W	12/04/2010
11	O'Higgins	Nancagua	34°40′54.34″S/71°12′52.75″W	6/04/2010
12	O'Higgins	Nancagua	34°40′47.54″S/71°12′25.92″W	11/06/2010
13	O'Higgins	Nancagua	34°40′51.50″S/71°13′43.91″W	6/04/2010
14	O'Higgins	Nancagua	34°40′55.84″S/71°13′24.77″W	6/04/2010
19	O'Higgins	Placilla	34°36′40.67″S/71°4′16.54″W	10/06/2010
22	O'Higgins	Placilla	34°38′04.07″S/71°07′29.71″W	30/12/2010
23	O'Higgins	Placilla	34°37′27.05″S/71°07′29.52″W	30/12/2010
24	O'Higgins	Placilla	34°37′10.43″S/71°07′00.33″W	30/12/2010
20	O'Higgins	Nancagua	34°41′5.59″S/71°14′22.69″W	30/04/2010
21	O'Higgins	Nancagua	34°40′30.74″S/71°12′27.76″W	29/04/2010
25	Valparaíso	Los Andes	32°50′46.67″S/70°38′47.91″W	16/02/2011
8	Valparaíso	Casablanca	33°21′41.89″S/71°19′0.31″W	9/04/2010
9	Valparaíso	Casablanca	33°21′5.76″S/71°20′53.45″W	9/04/2010
15	Maule	Molina	35°3′46.79″S/71°19′10.84″W	18/04/2010
16	Maule	Molina	35°4′18.54″S/71°18′47.55″W	18/04/2010
17	Metropolitana	Buin	33°43′44.61″S/70°42′37.12″W	10/06/2010
18	Metropolitana	Buin	33°44′8.20″S/70°42′41.46″W	10/06/2010
26	Metropolitana	Pirque	33°40′26.48″S/70°35′12.09″W	20/03/2011

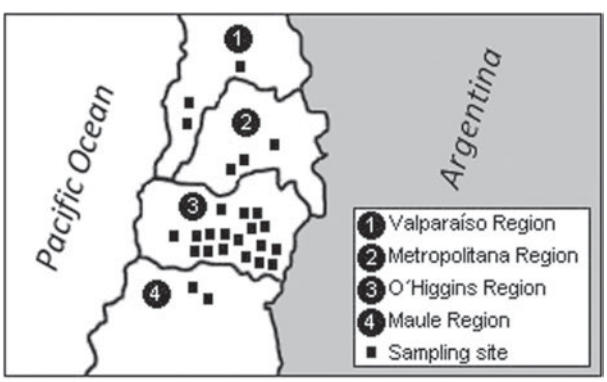

Fig. 1. Location of sampling sites in Chile.

least 1 h, placed in a drop of Canada balsam on a slide and covered with a coverslip.

The slide was then labeled and observed immediately under a microscope. Identification was based on the taxonomic keys of Williams & Granara de Willink (1992), Gimpel & Miller (1996) and Williams (2004). The voucher specimens are deposited in the Laboratoire de la Santé des Végétaux, ANSES, Campus International de Baillarguet, Montferrier-sur-Lez, France.

Results

Molecular characterization

We obtained 164 individual sequences for each marker. Six haplotypes were identified for COI, and seven for ITS2, resulting in 12 multilocus genotypes (table 2). The sequences obtained in this work are available from GenBank under accession numbers JN983129-JN983139. Multilocus genotypes #A–E consisted of sequences similar or very similar to sequences already available for *P. viburni* (Malausa *et al.*, 2011; Beltrà *et al.*, 2012). Multilocus genotype F consisted of COI and ITS2 sequences absolutely identical to those in the description of the species *Pseudococcus meridionalis* Prado: JF780513 for COI and JF780514 for ITS2 (Correa *et al.*, 2011). Multilocus genotypes #G–L did not correspond to any sequences deposited in international databases.

Considering that all the haplotypes were very similar to the published sequences assigned to *P. viburni*, we found that the most common and widely distributed were haplotype #1 for COI and haplotype #1 for ITS2 (multilocus genotype #A). Only multilocus genotype #A was found in the Valparaiso region, whereas four other multilocus genotypes in addition to multilocus genotype #A were observed in the O'Higgins region (table 3).

The multilocus genotype corresponding to the recently described species *P. meridionalis* was found only in the Metropolitana region, whereas multilocus genotypes #G–L, which could not be assigned to any species on the basis of molecular data, were found at three sampling sites in the O'Higgins region.

When we compared the Chilean COI haplotypes with other available haplotypes (fig. 2), the Chilean *P. viburni* haplotype #1 (H1) was also found in France, Spain and South Africa, whereas Chilean haplotypes #2 and #3 (H2 and H3) were present only in Chile. Several haplotypes absent from Chile were found in other countries: Brazil, U.S and Iran, (H4, H5 and H6, respectively, in fig. 2).

Morphological characterization

The molecular results were confirmed by the examination of slide-mounted specimens. Multilocus genotypes #A–E were

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Table 2. Multilocus genotypes for the various species found: *P. viburni* (COI: 1–3; ITS2: 1, 2), *P. meridionalis* (COI: 4; ITS2: 3) and *P. cribata* (COI: 5, 6; ITS2: 4–7), with the identification code of the slide-mounted specimens.

Multilocus genotype	COI haplotype	ITS2 haplotype	Slide-mounted specimen #	Morphological identification
A	COI-1 JN983135	ITS2-1 JN983131	1002270, 1002269, 1101177, 1002260, 1002259, 1002268, 1002229, 1002228, 1002267, 1002266, 1002265, 1002227, 1002258, 1002264, 1002271, 1002263, 1002257, 1002262, 1002256, 1002261, 1002255, 1002230, 1002275, 1002274, 1002254, 1002253, 1002273, 1002272, 1101179, 1101181, 1101185	*P. viburni*
B	COI-1 JN983135	ITS2-2 JN983133	1002252	*P. viburni*
C	COI-2 JN983136	ITS2-1 JN983131	1002233, 1002234, 1101182	*P. viburni*
D	COI-2 JN983136	ITS-2 JN983133	1002232, 1002231, 1101180, 1101183, 1101184	*P. viburni*
E	COI-3 JN983137	ITS2-1 JN983131	1101178, 1002226	*P. viburni*
F	COI-4 JF780513	ITS2-3 JF780514	1002243, 1002242, 1002241, 1002240, 1002239, 1002238, 1002237, 1002236, 1002235	*P. meridionalis*
G	COI-5 JN983138	ITS2-4 JN983130	1101187, 1101188, 1101197, 1101198	*P. cribata*
H	COI-5 JN983138	ITS2-5 JN983132	1101190, 1101195	*P. cribata*
I	COI-5 JN983138	ITS2-6 JN983134	1101186, 1101189, 1101191, 1101193, 1101194	*P. cribata*
J	COI-5 JN983138	ITS2-7 JN983129	1101199	*P. cribata*
K	COI-6 JN983139	ITS2-5 JN983132	1101196	*P. cribata*
L	COI-6 JN983139	ITS2-6 JN983134	1101192	*P. cribata*

assigned to *Pseudococcus viburni*. All the character states useful for the diagnosis of *P. viburni* (Gimpel & Miller, 1996) were present in the specimens of this species: oral-rim tubular ducts (OR), usually absent in the submedial row from segment III–VII; with a medial row and a lateral row of OR on each side, 13 (10–18) OR on the dorsum of segments I–VIII; dorsal OR absent on the submargin between cerarii 15 and 16; 2 (1–3) discoid pores close to each eye; numerous translucent pores on hind tibia and femur; 10 (8–16) oral collar tubular ducts (OC) in clusters on the mesad of cerarius 12 and 1 (0–2) OC associated with cerarii 10 and 11.

Multilocus genotype #F corresponded to the morphological description of *Pseudococcus meridionalis* Prado. This species has several features in common with *P. viburni*: dorsal OR absent on the submargin between cerarii 15 and 16; 2 (1–3) discoid pores close to each eye; 9 (7–13) OC in clusters on the mesad of cerarius 12 and numerous translucent pores on hind tibia and femur. However, this species was characterized by three morphological characteristics not associated with any species of the '*Pseudococcus maritimus* complex' (Gimpel & Miller, 1996). The most obvious of these character states was the many OR on the abdomen, in transverse rows, with up to 9 OR per row, and 38 (34–43) OR on dorsum segments I–VIII. There were also 19 (13–23) OR on dorsal cephalothoracic segments, with a transverse row at the cerarius 12 level. Finally, there were 9 (6–13) OC clustered between cerarii 10 and 11.

The specimens displaying multilocus genotypes #G–L (which did not contain previously documented DNA sequences) were morphologically similar to *Pseudococcus cribata* González. These specimens had the following features: a dorsal OR between cerarii 15 and 16; presence of 1 to 2 OR close to the frontal cerarii and cerarii 8 and 10, which were not very marked or absent; a mean of 38 OR on the abdomen. On the venter, no discoid pores were found close to the eyes, and multilocular pores were present around the vulva.

The species most closely related to *P. cribata*, based on morphologically characterization, is *Pseudococcus calceolariae* (Maskell). *Pseudococcus cribata* differed from *P. calceolariae* by the slight or even absent cerarii 8 and 10; the presence of 1 to 2 dorsal OR close to cerarius 17; the higher density of trilocular pores on anal cerarii than in *P. calceolariae* and the presence of at least 10 OR between the anterior spiracle and cerarius 12.

Discussion

Pseudococcus viburni was the most common mealybug found in this survey of Chilean vineyards, consistent with previous reports based on morphological taxonomy (Zaviezo, 2002; González, 2003a,b; Ripa & Luppichini, 2010). The second species found was *P. meridionalis* Prado (Correa *et al*., 2011). This species had also been called *Pseudococcus sp.1* (González, 2003a) and recently described as *Pseudococcus rubigena* González (González, 2011). Nevertheless, to our knowledge, *Pseudococcus meridionalis* is the valid name for this species. In our study, *P. meridionalis* was much less frequent than *P. viburni*, but nonetheless with high densities in a few vineyards of the Metropolitana region, confirming its status as a pest of grapes. The third species found would correspond morphologically to *P. cribata* (González, 2011), and the DNA

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Table 3. Geographic distribution and abundance of multilocus genotypes for the different species found: *P. viburni* (1–5), *P. meridionalis* (6) and *P. cribata* (7–12).

Population		Multilocus genotype no.											
N°	Region	1	2	3	4	5	6	7	8	9	10	11	12
1	O'Higgins	4	1										
2	O'Higgins	5											
3	O'Higgins	5											
4	O'Higgins	4											
5	O'Higgins	5											
6	O'Higgins	9				1							
7	O'Higgins	10											
10	O'Higgins	8	1										
11	O'Higgins	3				1							
12	O'Higgins	5											
13	O'Higgins	4											
14	O'Higgins	5											
19	O'Higgins	10											
20	O'Higgins	5											
21	O'Higgins	3		4	5								
22	O'Higgins							2	2	3			1
23	O'Higgins								1	4			
24	O'Higgins							2	1	1	1	1	
25	Valparaíso	6											
8	Valparaíso	10											
9	Valparaíso	9											
15	Maule	4											
16	Maule	4											
17	Metropolitana						5						
18	Metropolitana						4						
26	Metropolitana						4						

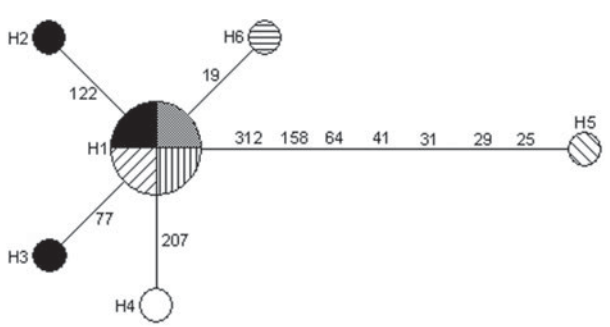

Fig. 2. Median-Joining COI haplotype network for *P. viburni*. Numbers indicate the location of the mutation within the sequence. For more details of the alignment, refer to fig. S1 in the supplementary material (▥, Spain; ■, Chile; ▨, South Africa; ▤, France; □, Brazil; ▧, California, USA; ▤, Iran).

sequences obtained did not match any sequence already present in an international database or publication. However, this taxon, characterized by two haplotypes at COI and four at ITS2, was found at three sites in the O'Higgins region and may be, therefore, also considered a pest of grapes. On the other hand, *P. longispinus* and *Pl. ficus* were not found at the sites studied, although they have been mentioned as grape pests in Chile (González & Volosky, 2004). The rarity of *Pl. ficus* in Chilean vineyards remains surprising, given that most grape-producing regions of the world, including France, the United States, South Africa, Argentina and Uruguay (Daane *et al.*, in press), are infested with this species. Indeed, the occurrence of *Pl. ficus* in Chile is a matter of debate (González, 2011). *Pseudococcus longispinus* has previously been collected in grapes in Chile, where it is known to be commonly associated with grapes (González & Volosky, 2004; Ripa & Luppichini, 2010; González, 2011). Therefore, the absence of *P. longispinus* from our two-year-long survey suggests that this species is not common on grapes in the regions sampled.

One remarkable result in this survey was the haplotype diversity and distribution for COI and ITS2 in *P. viburni* and *P. cribata*. Three COI haplotypes and two ITS2 haplotypes were found by us for *P. viburni* in Chile, and a different haplotype had been previously found in Brazil (Malausa *et al.*, 2011). This contrasts with the situation found for *P. viburni* in Europe, where, despite the large number of populations sampled and the diversity of hosts sampled, only one haplotype has been found (Malausa *et al.*, 2011; Beltrà *et al.*, 2012). The European haplotype corresponds to the most common haplotype found in Chile. Also, one haplotype with high divergence from the Chilean ones has been found for *P. viburni* in California (Genbank accession EU267206), which may correspond to another strain or sibling species, or sequence ambiguities. These findings support the hypothesis of a neotropical origin of *P. viburni* (Daane *et al.*, 2008a; Charles, 2010) because the level of genetic diversity seems to be higher in this biogeographic region than elsewhere. However, a more thorough sampling should be carried out in other regions of the world in order to better support this hypothesis.

For *P. cribata*, which was collected only in a few close sites (populations 22, 23 and 24), the samples displayed considerable DNA variation (two COI haplotypes and four ITS2 haplotypes). This suggests that this species may also be neotropical in origin, or at least is not a recent invader,

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although this conclusion remains speculative. On the other hand, for *P. meridionalis*, only one haplotype was found at each marker. In previous similar studies (Malausa *et al*., 2011; Abd-Rabou *et al*., 2012; Beltrà *et al*., 2012), a clear difference was found between native species, which had several haplotypes for the COI and ITS2 loci, and recent invaders, which systematically presented a single haplotype for each marker. If this pattern holds true in Chile, then *P. meridionalis* is probably not native to this country, because no variation at either of the loci was found in this species, despite repeated sampling from different host plants (Correa *et al*., 2011; this study). If confirmed, these patterns may be of use in the development of biological control strategies, because the native region of a species is generally considered the most suitable place to look for natural enemies (Moore, 1988).

This survey identified *P. viburni*, *P. meridionalis* and *P. cribata* as pests of grape in Chile's main grape production area. The genetic variability of *P. viburni* and *P. cribata*, at the two molecular markers used, suggest that they are either native or long-established in this biogeographic region. In contrast, no genetic variability was found in *P. meridionalis*, suggesting that this species may have been introduced recently into Chile.

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Supplementary material

The online figure can be viewed at http://journals.cambridge.org/ber.

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