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A new species of *Pseudococcus* (Hemiptera: Pseudococcidae) belonging to the "*Pseudococcus maritimus*" complex from Chile: molecular and morphological description

MARGARITA CORREA[1,7], CARLOS AGUIRRE[2,7], JEAN-FRANÇOIS GERMAIN[3], PATRICIO HINRICHSEN[2], TANIA ZAVIEZO[1], THIBAUT MALAUSA[4] & ERNESTO PRADO[5,6]

[1]*Facultad de Agronomía e Ingeniería Forestal, Pontificia Universidad Católica de Chile, Casilla 306-22, Santiago, Chile. E-mail: macorre1@uc.cl, tzaviezo@uc.cl*

[2]*Instituto de Investigaciones Agropecuarias, La Platina, Casilla 439/3, Santiago, Chile. E-mail: phinrichsen@inia.cl, caguirre.d@gmail.com*

[3]*ANSES. Laboratoire de la santé des Végétaux, Campus International de Baillarguet, Montferrier-sur-Lez, France. E-mail: jean-francois.germain@anses.fr*

[4]*Institut National de la Recherche Agronomique, UMR 1301 INRA/UNSA/CNRS, Equipe BPI. 400 route des Chappes. BP 167. 06903 Sophia-Antipolis, France. E-mail: tmalausa@sophia.inra.fr*

[5]*EPAMIG. Lavras, Brazil. E-mail: epradoster@gmail.com*

[6]*Corresponding author*

[7]*Both authors contributed equally to this work*

Abstract

A new species of mealybug from Chile, *Pseudococcus meridionalis* Prado **sp. n.**, is described and illustrated based on the morphological and molecular characterization of adult females. This species belongs to the "*Pseudococcus maritimus*" complex and displays a wide host plant range, including Japanese pear, persimmon, pomegranate, pear and grape.

Key words: *Pseudococcus meridionalis* **sp. nov.**, Chile, mealybugs, phenotypic plasticity, cytochrome oxidase, internal transcribed spacer

Introduction

Approximately 25 mealybug species have been reported from Continental Chile (excluding Easter Island). Ten are indigenous and are mostly associated with native plants (Prado, 1991; Williams & Granara de Willink, 1992; Ben-Dov *et al*., 2010). The other species have a worldwide distribution and probably have been introduced. *Pseudococcus viburni* (Signoret), *P. calceolariae* (Maskell) and *P. longispinus* (Targioni Tozzetti) are the predominant species found on fresh fruits such as table grapes, apples, pears and plums exported from Chile, and there are no quarantine restrictions on their import to the many countries. However, the literature is contradictory regarding the presence of *P. maritimus* in Chile (González *et al*., 1973; Prado, 1991; Williams & Granara de Willink, 1992; Gimpel & Miller, 1996; González *et al*., 2001; Artigas, 1994, Gonzalez 2003a, González & Volosky 2005), and it is generally believed that earlier records are misidentifications of an undescribed *Pseudococcus* species. Indeed, unknown *Pseudococcus* species belonging to the "*Pseudococcus maritimus*" complex have been intercepted occasionally in phytosanitary export inspections, and have been referred to as *Pseudococcus* sp. 1 and *Pseudococcus* sp. 2 for many years (González, 2003a, 2003b).

Here we describe a species corresponding to *Pseudococcus* sp. 1 collected in Chile both morphologically and by means of molecular tools. Considering that morphological characterization is usually restricted to the adult female stage and because very closely related species may be morphologically indistinguishable (Rung *et al*., 2008; Pieterse *et al*., 2010), we have characterized this species using two molecular markers known to be efficient for fine-scale species identification (Malausa *et al*., 2011). We have also compared the DNA sequences of the new spe-

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cies, named *Pseudococcus meridionalis* Prado **sp. n.**, with other common *Pseudococcus* species. In addition, we compare the main morphological characters that distinguish the new species and other closely related species. We also determined that these morphological differences hold when populations are reared at different temperatures (Cox, 1983; Charles *et al.*, 2000).

Material and methods

Sampling sites. Samples were collected on cultivated and native plants in Chile, during two periods: (i) on cultivated and native plants during 2001-2003 seasons from Toconao in the north (23°11`23``S, 68°00`13``W) to Los Angeles in the south (37°27`48``S, 72°19`36``W), and (ii) on grapevines in 2009–2010 season from Buin (central Chile, just south of Santiago) (33°43`39``S, 70°42`36``W). Collected specimens were preserved in 75% or 95% ethanol. In addition, slide mounted specimens identified as *Pseudococcus* sp. 1, preserved at Servicio Agrícola y Ganadero (SAG, Chile) and at Instituto de Investigaciones Agropecuarias (INIA, Chile), were also examined.

Morphological examination. Ethanol preserved specimens from the 2001–2003 seasons were slide-mounted as follows. A small puncture was made in the thorax and individuals were macerated in 10% KOH for 24 hours at room temperature (or gently heated for 15 min). Afterwards they were rinsed three times in water, the body contents were removed with microtools and then passed through 75% and 95% ethanol for a few minutes before being placed in glycol ether (Cellosolve™, BioQuip Co., CA, USA) for approximately 10 minutes. Then they were stained with Double stain (BioQuip) for 10 to 30 min. Excessive staining was removed with 95% ethanol. The insects were subsequently placed in clove oil and finally mounted in Euparal (BioQuip). The samples collected from grapevines during the 2009–2010 season were prepared following Malausa *et al.* (2011).

The morphological terminology follows that of Williams and Granara de Willink (1992) and Gimpel and Miller (1996). The cerarii were numbered from 1 to 17 starting from the anal lobe pair (number 1). Measurements and numbers for the holotype are given as an average or by a value from the left side – right side (i.e. 4–5, indicating 4 on the left side and 5 on the right side), followed by the range for 20 paratypes in parentheses. Enlargements on illustrations are not proportional. The studied specimens were collected from three host plants and from two regions separated by ca. 450 km (north and central region of Chile).

The specimens are deposited at the Museo Nacional de Historia Natural (MNNC-Chile, Santiago, Chile), United States National Museum of Natural History (USNM, Washington, DC, USA), Muséum National d'Histoire Naturelle (MNHN, Paris, France) and ANSES Montpellier, Campus International de Baillarguet, Montferrier-sur-Lez, France.

Phenotypic plasticity assay. The ovisac of an adult female of *P. meridionalis* and of *P. viburni* were isolated and each divided in three boxes, and reared on sprouted potatoes under laboratory conditions (25°C, 60%RH) until egg hatching. Then, each box was transferred to climatic chambers regulated at different temperatures: 17°C, 24°C and 28°C at approximately 60%RH, each with a photoperiod of 12 hours, until they had completed their development. The resultant adult females were slide mounted and the number of the following were counted: (i) abdominal dorsal oral rim tubular ducts (OR), (ii) oral collar ducts (OC) between cerarii 10 and 11 and (iii) oral collar ducts on the head. Data were analyzed using two-way ANOVA, with species and temperatures as independent factors, and the means were separated using Tukey ($\alpha = 0.05$). Because *P. maritimus* is unknown from Chilean territory and could not be imported into Chile for quarantine (and ethical) reasons, phenotypic plasticity and crossing experiments between *P. meridionalis* and *P. maritimus* were not performed.

DNA extraction, amplification and sequencing. For samples collected between 2001 and 2003, two specimens, one collected on *Pyrus pyrifolia* (Santiago, Chile) and one on *Annona cherimola* Mill. (La Serena, Chile) were subjected to DNA sequencing. DNA was extracted following a four steps procedure: (i) incubation for 30 min at 60°C in 2x CTAB (2% CTAB, 100 mM Tris, 20 mM EDTA, 1.4 M NaCl and 1/100 volume of 2-mercaptoethanol); (ii) addition of 1 volume of chloroform-isoamilic alcohol (24:1) and centrifugation at 10,000 rpm for 5 min; (iii) addition of 1/10 volume of 5 M NaCl and 2.5 volumes of ethanol followed by centrifugation at 10,000 rpm for 5 min; and (iv) washing the pellet with 70% ethanol, resuspended with 50 uL of TE buffer (10 mM Tris pH 8.0, 1 mM EDTA) and incubated with RNAse A (10 mg/mL) at 37 ºC for 30 min. The DNA quality was checked after electrophoresis in a 0.8% agarose gel, and then a portion of the ribosomal RNA transcription unit corresponding to ITS1-5.8S-ITS2 was amplified with the universal primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS26

(5'-ATATGCTTAAGTTCAGCGGGT-3') (White *et al*., 1990). The PCR was made in a total volume of 20 µL, with 0.2 mM of each dNTP, 1.5 mM of $MgCl_2$, 12.5 pmol of each primer, 2 µL of 10x PCR buffer (100 mM Tris, 500 mM KCl) and 3 µL of a 1/100 dilution of genomic DNA. The PCR conditions were as follows: initial denaturation at 94°C for 3 min, followed by 30 cycles of (i) denaturation at 94°C for 3 min, (ii) annealing at 57.5°C for 45 s, (iii) elongation at 72°C for 1.5 min and a final extension at 75°C for 5 min. The PCR product were visualized on ethidium bromide-stained 1% agarosegel. The DNA was recovered by cutting the band of the gel, dissolving the agarose and adsorbing the DNA on a silica resin. The DNA was suspended in ultrapure water (30 µL) and cloned using the pGEM®-T Easy cloning kit (Promega, WI, USA) following the manufacturer recommendations. Colonies were selected by PCR and a miniprep (Sambrook *et al*., 1989) was made with two clones per individual that were sequenced in both directions using the universal primers M13F or M13R (pGEM®-T Easy cloning kit) and a primer at 5.8S (5'-GATGAAGACCGCAGCTAAC-3'). The sequencing was performed by Macrogen, Inc. (Seoul, Korea). The voucher is stored at the Museo Nacional de Historia Natural (MNNC, Santiago, Chile) under the code MNHN6808. As no sequence of internal transcribed spacer 2 (ITS2) was available for *Pseudococcus maritimus*, a sample collected in California, USA, was sequenced following the same protocol described above.

For the DNA extraction of samples collected in 2009, we followed the protocol described in Malausa *et al*. (2011) using the DNeasy Tissue Kit (QIAGEN, Hilden, Germany) and keeping the samples intact for further morphological examination. Those samples were amplified at two DNA regions: ITS2 and Cytochrome oxidase I (COI, positions 2183–2568). PCR were performed using Phusion High-Fidelity DNA polymerase 530L (FINNZYMES, Espoo, Finland) in 23 µl of mix plus 2 µl of diluted DNA. Reagent concentrations were: 1x Phusion HF buffer, 0.01 U/ µl Phusion enzyme, 200 µM dNTPs and 0.5 µM of each primer. For the ITS2 region, the primers designed in Malausa *et al*. (2011) were used: ITS2-M-F 5'-CTCGTGACCAAAGAGTCCTG-3' and ITS2-M-R 5'-TGCTTAAGTTCAGCGGGTAG-3'. For the COI region, the primers described in Gullan *et al*. (2003) were used: C1-J-2183-F 5'-CAACATTTATTTTGATTTTTTGG-3' and C1-N-2568-R 5'-GCWACWACRTAATAKGTATCATG-3'. PCR conditions were: initial denaturation at 98°C for 30 s, followed by 35 cycles of (i) denaturation at 98°C for 10 s, (ii) annealing for 15 s at 56°C for COI and 60°C for ITS2, (iii) elongation at 72°C for 15 s and by a final extension period of 5 min at 72°C. PCR products were checked by electrophoresis in a 2% agarose gels. Positive PCR products were sent to Genoscreen (Lille, France) for bidirectional sequencing. Consensus sequences were generated and checked with Seqscape v2.7 (ABI). Alignments were manually edited with Bioedit 7.01 (Hall, 1999). The voucher specimen is stored in the collection of ANSES Montpellier, Campus International de Baillarguet, Montferrier-sur-Lez, France, as slide #1002243.

DNA alignments and molecular characterization. In order to compare the sequences obtained from *P. meridionalis*, an alignment for each marker (ITS2 and COI) was generated by ClustalW using closely related *Pseudococcus* species, and the divergence distances were calculated. For the ITS2 region the sequences were: *Pseudococcus viburni* (Signoret) (AF006820.1), *Pseudococcus longispinus* (Targioni Tozzetti) (AF007264.1), *Pseudococcus calceolariae* (Maskell) (AF007263.1), *Pseudococcus maritimus* (Erhorn) (JF758861), *Pseudococcus meridionalis* Prado (JF780514), *Planococcus citri* (Risso) (FJ430145.1) and *Planococcus ficus* (Signoret) (GU134677.1). For the COI region the sequences were: *P. viburni* (GU134686.1), *P. longispinus* (AY179439.1), *P. calceolariae* (GU134684.1), *P. maritimus* (Daane *et al.*, submitted), *P. meridionalis* (JF780513), *Pl. citri* (GU134692.1) and *Pl. ficus* (GU134693.1).

Results

Molecular characterization. For the two samples from the 2001–2003 collections, we obtained a sequence of 1,355 bp corresponding to the region ITS1-5.8S-ITS2. For the sample from the 2009 collection, one COI sequence of 363 bp and one ITS2 sequence of 618 bp were obtained. The sequences for ITS1-5.8S-ITS2 (2001–2003), ITS2 and COI (2009) are deposited in GenBank (accession numbers JF776370, JF780514 and JF780513). In the case of *P. maritimus*, we obtained an ITS1-5.8S-ITS2 sequence of 965 bp (GenBank accession number JF758861).

The ITS2 sequences from individuals collected in 2001–2003 and 2009 were identical. For this sequence, the divergence distances between *P. meridionalis* and other species were high (Figure 1): 11.9% (*P. maritimus)*, 33.2% (*P. viburni*), 56.0% (*P. longispinus*), 58.8% (*P. calceolariae*), 48.0% (*Planococcus citri and Pl. ficus)*. For the cytochrome oxydase I gene, the comparisons were limited to a 181 bp region (Figure 2), because it was the only region

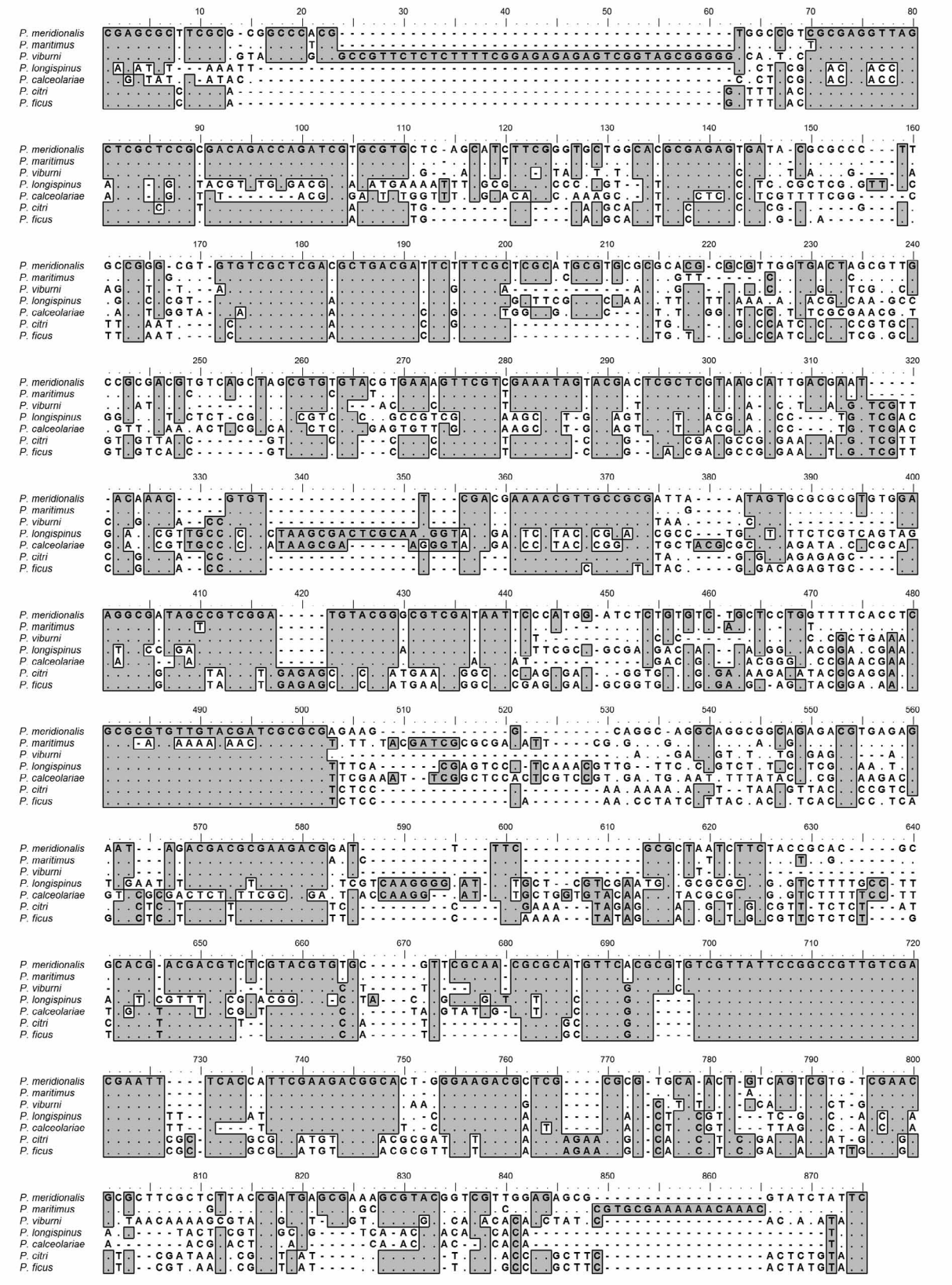

FIGURE 1. Sequence alignment of the Internal Transcribed Spacer 2 (ITS2) region of *Pseudococcus meridionalis*, *Pseudococcus maritimus*, *Pseudococcus viburni*, *Pseudococcus longispinus*, *Pseudococcus calceolariae*, *Planococcus citri* and *Planococcus ficus* (JF776370 and JF780514, JF758861, AF006820.1, AF007264.1, AF007263.1, FJ430145.1 and GU134677.1, respectively). The sequence of *P. meridionalis* is used as reference in the alignment. Nucleotide sequences of the other species are displayed only at sites where differences were observed.

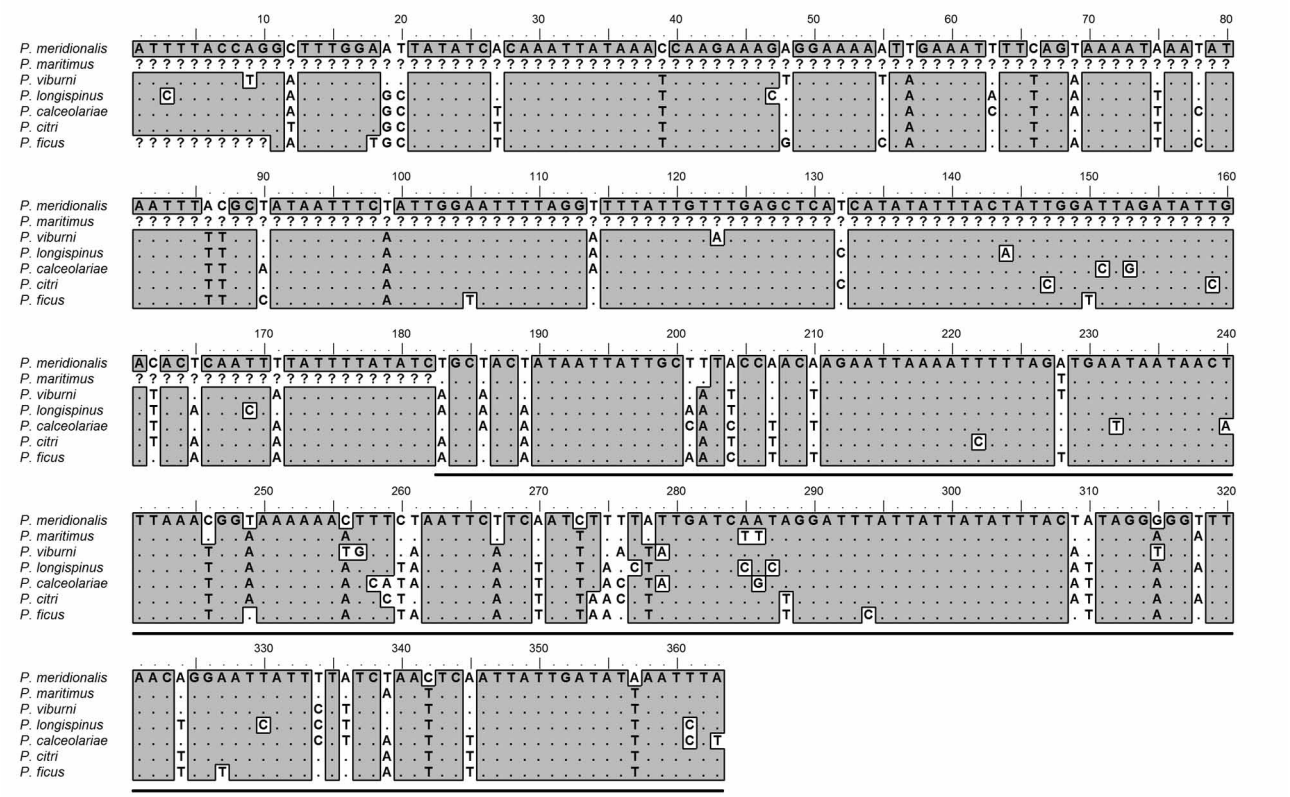

FIGURE 2. Sequence alignment of the cytochrome oxidase subunit I (COI) region of *Pseudococcus meridionalis* (JF780513), *Pseudococcus maritimus* (Daane *et al.*, submitted), *Pseudococcus viburni* (GU134686.1), *Pseudococcus calceolariae* (GU134684.1), *Pseudococcus longispinus* (AY179439.1), *Planococcus ficus* (GU134693.1) and *Planococcus citri* (GU134692.1). The sequence of *P. meridionalis* is used as reference in the alignment. Nucleotide sequences of the other species are displayed only at sites where differences were observed. The region at which the divergence distances were estimated is underlined.

sequenced for all the three species *P. viburni*, *P. maritimus* and *P. meridionalis* (Malausa *et al*., 2011; Daane *et al.*, submitted). Divergence distances at COI between *P. meridionalis* and other species were also high: 6.1% (*P. maritimus*), 12.2% (*P. viburni*), 19.4% (*P. calceolariae*), 16.6% (*P. longispinus*), 15.5% (*Pl. citri* and *Pl. ficus*).

The extent of divergence between *P. meridionalis* and the closely related species *P. maritimus* and *P. viburni* is high when compared to that observed at the intra-specific level in Pseudococcidae. Previous studies at the same DNA regions documented intra-specific divergence distances ranging from 0.3% (within *P. longipisnus* and *P. viburni*) to 0.8% (within *Pl. citri*) at COI and from 0.1% (within *P. comstocki* (Kuwana)) to 0.9% (within *P. viburni*) for ITS2. In addition, inter-specific divergences between species of the same genus have been shown to range from 1.9% (*Pl. citri* vs *Pl. minor* (Maskell)) to 6.9% (*Pl. citri* vs *Pl. ficus*) at COI, and from 0.6% (*Pl. citri* and *Pl. minor*) to around 60% (*P. viburni vs P. calceolariae*) at ITS2 (Pieterse *et al*., 2010; Malausa *et al.*, 2011; Malausa et al., unpublished data). Hence, it is here considered that the divergence distances found between *P. meridionalis* and both *P. maritimus* and *P. viburni* correspond to inter-specific divergences.

Morphological distinction between *P. meridionalis* and closely related species. The species described here is close to *P. maritimus* and *P. viburni* and its characters overlap with several characters of both species. However, a set of characters make it possible to unambiguously identify *P. meridionalis* (Table 1). No phenotypic plasticity related to temperature was observed on the characters used for morphological characterization, with *P. meridionalis* and *P. viburni* shown as distinct groups (Fig. 3). The two-way ANOVA on the three chosen characters showed a significant effect of species ($p<0.0001$ for all). There was also a significant effect of temperature and of species x temperature interaction for the dorsal abdominal oral rim tubular ducts (OR) and for the oral collar tubular ducts (OC) between cerarii 10 and 11 ($p<0.0001$). For *P. viburni*, the characters were similar at every tested temperature, but for *P. meridionalis* more dorsal abdominal OR were found at 25°C and fewer OC between cerarii 10 and 11 were found at 18ºC. Nonetheless, the ranges of the two species never overlapped.

TABLE 1. Comparison between *P. meridionali*s and two closely related *Pseudococcus* species. Means are given and the extreme values that were observed are shown in parentheses.

Character	*P. meridionalis*	*P. maritimus* (*)	*P. viburni* (*)
Dorsal OR between cerarii 15 and 16	without	with	without
Ventral OC on each side of head	7 (6–16)	9 (3–25)	2 (0–6)
Ventral OC between cerarii 10 and 11	5 (3–17)	14 (6–20)	1 (0–2)
Dorsal OR on abdomen	29 (2–44)	27 (19–35)	13 (10–18)
Dorsal OR near cerarii 2 and 7	present	present	absent
Longest anal-lobe seta	80 (78–120)	151 (136–68)	125 (109–136)
Longest interantennal seta	98 (66–98)	124 (105–149)	102 (73–134)

(*) After Gimpel and Miller (1996).

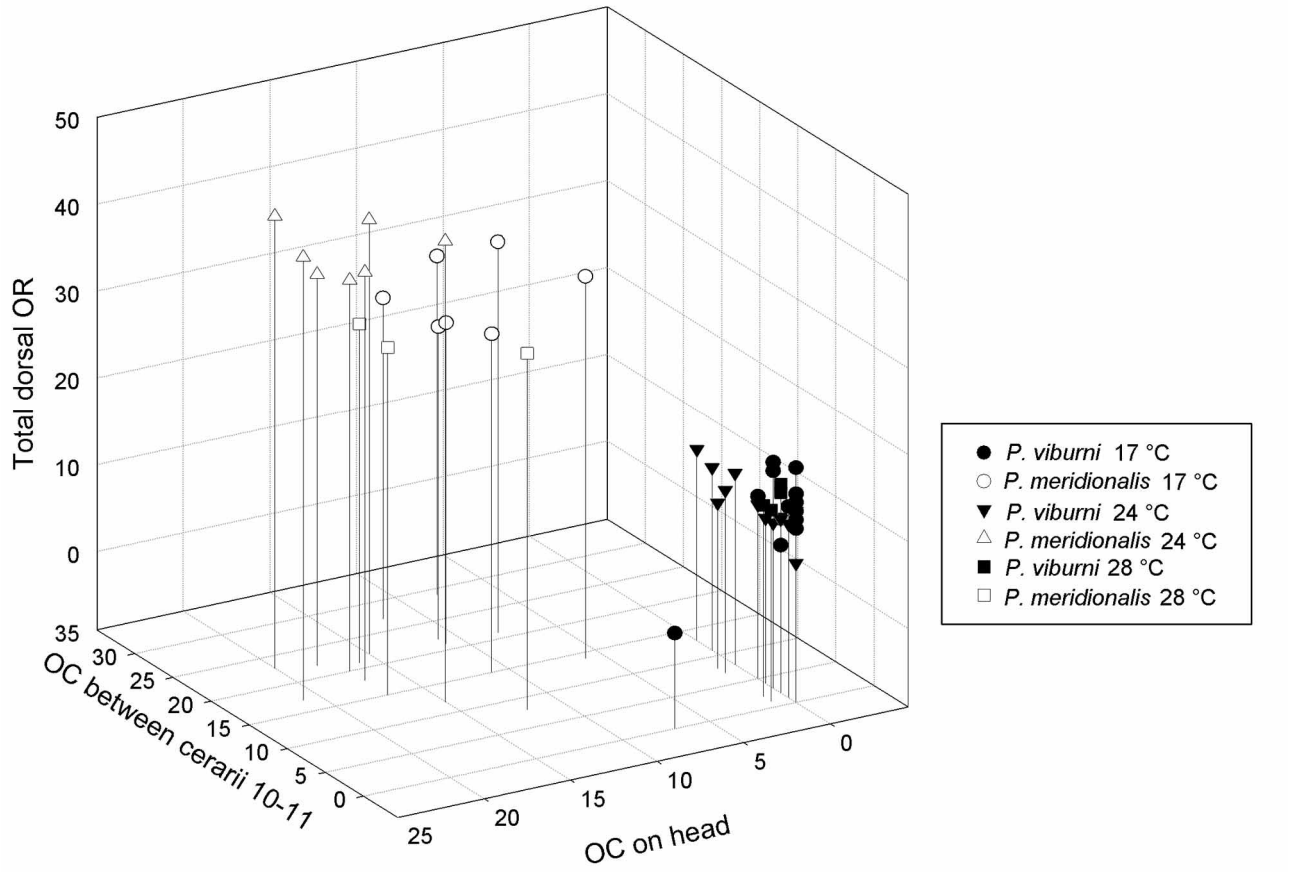

FIGURE 3. Differences on some important morphological characters of *P. viburni* and *P. meridionalis* reared at different temperatures: 17°C (N=15 for *P. viburni*, N=7 for *P. meridionalis*), 24°C (N=15 for *P. viburni*, N=7 for *P. meridionalis*), 28°C (N=6 for *P. viburni*, N=3 for *P. meridionalis*).

***Pseudococcus meridionalis* Prado sp. n.**
(Fig. 4)

Diagnosis. *P. meridionalis* belongs to the *Pseudococcus maritimus* complex, characterized by the presence of discoidal pores near the eyes (Gimpel & Miller, 1996) and is very similar to *P. maritimus* and *P. viburni*. One of the most important diagnosis characteristics of *P. maritimus*, the presence of a marginal OR between cerarii 15 and 16, is absent from *P. meridionalis*. Other important characteristics of *P. meridionalis* include OC present in ventromar-

ginal areas of thorax, translucent pores present on hind tibia and femur, and discoidal pores near eyes not located on sclerotized rim.

External appearance. *P. meridionalis* shows four long caudal tails; the length of the wax tail of cerarius 2 reaches less than half the length of the wax tail of the anal cerarius. Body color is dark gray. A compact ovisac is produced containing orange color eggs.

Mounted material. (data in brackets for paratypes) Adult female. Holotype elongated oval, length 2.09 mm (1.6–3.1), width 1.19 mm (0.80–1.75). Anal lobes well developed. Longest anal lobe seta 80 mm (78.2–119.5), approximately twice greatest width of anal ring (n = 23). Antennae each 480–468 µm long, with eight segments. Average lengths of each segment as follows: I 65.0 mm (53.7–85.4); II 62.2 mm (51.2–78.0); III 61.0 mm (51.2–75.0); IV 45.0 mm (31.7–56.1); V 55.0 mm (46.3–63.4); VI 40.2 mm (36.6–51.2); VII 47.5 mm (41.5–51.2); VIII 105.0 mm (95.1–109.8). Legs well developed; hind trochanter + femur 345–350 mm (300–400) ; hind tibia + tarsus 405–390 mm (345–460); ratio of length of hind tibia + tarsus to hind trochanter + femur 1.17–1.11:1 (1.01–1.22:1). Ratio of length of hind tibia to tarsus 2.5–2.5:1 (2.0–2.9:1). Translucent pores: 40–39 (16–60) present on hind femur and 48–45 (26–52) on hind tibia; absent from hind coxa and trochanter. Hind tibia with 22–21 (21–38) setae. Longest seta on hind trochanter 97.6–107.3 (80–131) mm long. Labium 150 mm long (117–183). Circulus 1.6 times as wide as long, 162 mm wide (158–176). Longest anal-ring seta 125 mm long (117–173).
Cerarii numbering 17 pairs. Each anal lobe cerarius lightly sclerotized, with three setae of different sizes, long, medium and short. Cerarii 1–11 each mainly with 2 conical setae per cerarius (rarely 3 in ce2 and 6); ce12 with 3 (rarely 2); ce13 and 14 with 2; ce15 with 3; ce16 with 4 (3 or 4); and ce17 with 3 (sometimes 4). All cerarii with auxiliary setae, more numerous in anal cerarius with 5 (4–7), ce2 with 4 (3–7) and ce17 with 4 (2–5).

Dorsal surface with flagellate setae of mostly one size, only somewhat longer on segment VIII; with 5 dorsomedial setae on segment VIII (3–9), longest of 22 mm long (14.6–24.4). Multilocular pores absent. Trilocular pores scattered throughout. Each oral rim tubular duct (OR) usually with 0 to 3 associated discoidal pores and 0 or 1 seta**,** present on submargin close to ce2 to 9 and 11, between ce13 and 14, and close to ce17 (ce17 rarely with 2 OR on paratypes); absent near ce10 on holotype but sometimes present on paratypes; absent near ce12, 15 and 16. In addition, abdomen with 6 OR located in the medial line (3–9) and 9 in the submedial lines (9–21); also present on thorax as submarginal (as mentioned above in association with cerarii), 2 in the medial line (1–6) and 9 in submedial line (3–13). Few dorsal OC, located in margin**.** Discoidal pores of one size scattered over dorsum approximately same size as on venter. Discoidal pores present near eyes 1 or 2 (0–4).

Ventral surface with normal flagellate setae, longest on abdominal segment VII each 57 mm long (43–63); 3 cisvulvar setae on each side (2–3), 40 mm long (30–43). Longest interantennal seta 98 mm long (66–98). Multilocular pores present on abdominal segments IV–VIII but not extending laterally to margin; pores restricted to an anterior band on segment VII and around vulva but present as both an anterior and a posterior band on VI and V but more dispersed on the V anterior band; on IV they are dispersed; absent from head and thorax on holotype but occasionally present on paratypes. Trilocular pores scattered throughout the body. One size OC in the holotype, some paratypes show 2 sizes with the second type being somewhat longer and narrower, located in the medial and submedial part of abdomen; associated with multilocular pores on posterior band of abdominal segments IV–VII, also present submarginally on head and thorax; numerous between ce1 and 7, much less frequent between ce8 and 11; then with 5–7 (3–17) OC between ce10 and 11; 4–9 (7–27) close to ce12; 9–12 (7–13) near ce13; 7–8 (6–28) between ce14 and 15; none near ce16; 7–8 (6–16) on each side of head. OR each with 1 (0–2) discoidal pore and 1(0–1) setae associated with rim; with 1 submarginal between ce8 and 9, also 1 submarginal duct at level of cerarii ce10 and 12 (2–3), and 1 submedial between ce9 and 10; thus a total of 4 OR between anterior spiracle and abdominal segment 2 on each side of abdomen on holotype but varying from 2 to 9 on paratypes. Posterior spiracle greatest length 68.3–70.7 mm long (60.9–80.5).

Type material. HOLOTYPE: adult female on one slide, Chile, La Serena, 10.V.2002, on pomegranate (*Punica granatum*), Col. M. Zambrano (depositary Museo Nacional de Historia Natural, Chile, MNNC). **PARATYPES**: Four adult females on four slides, same data as holotype; 29 specimens on 24 slides, Chile, Calera de Tango, Santiago, 18.III.2002 and 30.IV.2002, on *Pyrus pyrifolia*; 12 specimens on 10 slides, Chile, Huelquén, Santiago, 17.IV.2002, on *Pyrus pyrifolia*; one specimen on one slide, Chile, San Bernardo, SAG fruit inspection, on *Pyrus communis*; eight specimens on eight slides, Chile, Buin, March 2010, on *Vitis vinifera*. Paratypes deposited in MNNC,USNM; MNHN and ANSES.

Non-type material. The specimens identified previously as *Pseudococcus* sp.1 preserved at Servicio Agrícola y Ganadero (SAG, Chile), and at Instituto de Investigaciones Agropecuarias (INIA, Chile) were also identified as *P. meridionalis.*

Hosts and distribution. This species has been collected in Chile from Antofagasta Region (23°11`23``S, 68°00`13``W) to Los Angeles (37°27`48``S, 72°19`36``W) on blueberries, nectarine, peach, pear, Japanese pear, persimmon, plum, cherimoya, pomegranate and grapevine.

Etymology. The species name "meridionalis" refers to the location of this species at the southern part of South America, Chile.

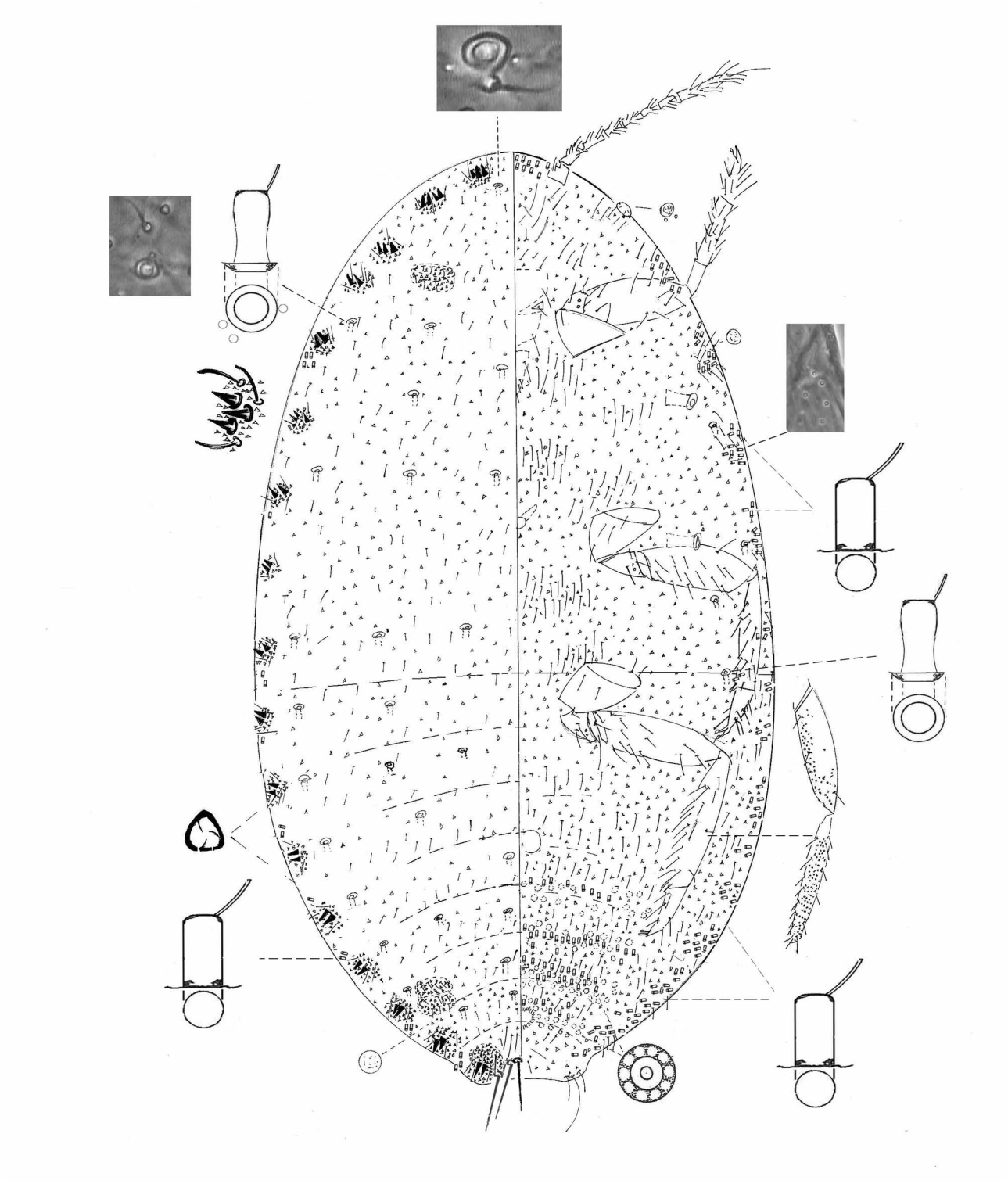

FIGURE 4. *Pseudococcus meridionalis* Prado **sp. n.** Holotype adult female, Chile.

Acknowledgments

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