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Parasitoids (Hymenoptera) of Mealybug Pests (Hemiptera: Pseudococcidae) from Southern Brazil: Molecular and Morphological Characterization

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Abstract

Parasitoids of three mealybug pests (Hemiptera: Pseudococcidae), *Planococcus ficus* (Signoret), *Pseudococcus sociabilis* Hambleton, and *Pseudococcus viburni* (Signoret) have been identified for the first time in Brazil. Mealybugs were collected in fruit-growing areas along southern Brazil during 2013–2016. An integrative approach, combining morphological and molecular methods, was used to identify the Brazilian parasitoids to the species level. Fifteen species were recorded, including 14 primary parasitoids belonging to Encyrtidae and Platygastridae and a single secondary parasitoid species belonging to Signiphoridae. The encyrtid parasitoids *Acerophagus flavidulus* (Brèthes), *Anagyrus calyxtoi* Noyes and *Zaplatycerus* sp., and the signiphorid secondary parasitoid *Chartocerus axillaris* De Santis are reported for the first time in Brazil.

Keywords Integrative taxonomy · Encyrtidae · biological control agents · Pseudococcidae · DNA barcoding

Introduction

Mealybugs (Hemiptera: Coccomorpha: Pseudococcidae) are important pests worldwide, infesting fruit plants such as apples, persimmon, strawberry, and grapevines (Charles et al. 2015; Pacheco da Silva et al. 2017). Nymphs and adult females damage crops by secreting honeydew, which facilitates the development of sooty mold, and by transmission of toxins and viruses, which may eventually kill the plant (Golino et al. 2002; Franco et al. 2009; Daane et al. 2012). The invasive mealybug *Pseudococcus viburni* (Signoret), known as the obscure mealybug, is a common species damaging fruits in temperate regions (Ciampolini et al. 2002; Daane et al. 2008; Dapoto et al. 2011; Mudavanhu et al. 2011; Correa et al. 2012), and is considered the most common species in fruit crops in southern Brazil (Pacheco da Silva et al. 2017). The vine mealybug *Planococcus ficus* (Signoret) shows high infestation rates in vineyards and fig plants (Daane et al. 2012), and it has recently expanded across Serra Gaúcha, the most important wine production region of Brazil (Pacheco da Silva et al. 2016). Finally, the Hambleton mealybug *Pseudococcus sociabilis* Hambleton has been reported on persimmon fruits from the same area (Pacheco da Silva et al. 2017). Mealybug control commonly relies on pesticides, but those are inefficient in the long-term because of the cryptic habits and presence of hydrophobic waxes on the body surface of mealybugs (Franco et al. 2009). In this context, biological control agents (BCAs), such as encyrtid parasitoid wasps and ladybird beetles (Coleoptera: Coccinellidae), especially the mealybug destroyer *Cryptolaemus montrouzieri* Mulsant, play an important role in regulating pest insect

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populations, and represent the best approach for mealybug suppression (Daane et al. 2012).

Parasitoid wasps are able to attack different insect orders (Heraty et al. 2013), and can be used on augmentative biological control programs. Chalcidoidea (Hymenoptera) is the most diverse group of parasitoids, comprising 25 families and about 22,500 described species (Heraty et al. 2013; Noyes 2019). This group has been successfully used as BCAs in agricultural landscapes (Noyes and Hayat 1994; Heraty 2009). For example, African populations of the cassava mealybug *Phenacoccus manihoti* Matile-Ferrero were controlled through the use of *Anagyrus lopezi* (De Santis) (Encyrtidae) from South America (Neuenschwander et al. 1988; Herren and Neuenschwander 1991) and the Rhodes grass mealybug *Antonina graminis* (Maskell) is kept under control after the release of the parasitoid *Neodusmetia sangwani* (Subba Rao) (Encyrtidae) in 1970 in Brazil (Batista Filho et al. 2017). Similarly, *Acerophagus flavidulus* (Brèthes) and *Leptomastix epona* (Walker) have been introduced from South America to California to control the obscure mealybug (Daane et al. 2008), while *Coccidoxenoides perminutus* Girault and *Anagyrus pseudococci* (Girault) were introduced against the vine mealybug in California (Sime and Daane 2014).

Despite their importance as BCAs and high diversity, Chalcidoidea is still less studied than other insect groups, especially if compared to groups with larger sized insects (Noyes 2000). Out of 17 Chalcidoidea families recorded in Brazil, the most speciose are Chalcididae (247), Eulophidae (202), and Pteromalidae (155), followed by Encyrtidae with 140 species (Noyes 2019). Morphological discrimination among chalcidoid wasps relies mostly on external female characters, but identification keys are still lacking for many taxa. DNA barcoding has become increasingly popular for parasitoid identification (Rugman-Jones et al. 2011; Fernández-Triana et al. 2014; Beltrà et al. 2015; Correa et al. 2016), particularly when complexes of cryptic species have been reported (see Triapitsyn et al. 2007). Few molecular studies have been performed on the Encyrtidae though, which leaves them particularly underrepresented in public databases.

Efficient selection of biological control agents depends on appropriate knowledge about parasitoid presence on each mealybug species. In this study, parasitoid wasps infesting *Ps. sociabilis*, *Ps. viburni*, and *Pl. ficus* populations in Rio Grande do Sul (Brazil) were characterized based on morphology and DNA markers.

Material and methods

Sampling

Mealybug specimens were collected from 22 sampling sites on fruit production areas across Rio Grande do Sul State in Brazil (covering Encruzilhada do Sul and the so-called Serra Gaúcha region, including Antônio Prado, Bento Gonçalves, Caxias do Sul, Farroupilha, Monte Belo, and São Valentin do Sul), between 2013 and 2016 (Table 1). Commercial crops included apple *Malus domestica* Borkh (Rosaceae), persimmon *Diospyros kaki* L. (Ebenaceae), strawberry *Fragaria x ananassa* Duchesne (Rosaceae), and grapes *Vitis labrusca* L. and *Vitis vinifera* L. (Vitaceae). Mealybug-infested fruits and leaves were collected into plastic boxes and taken to the Laboratory of Entomology of Embrapa Uva e Vinho. Mealybugs were examined for parasitism using a stereo microscope, and mummified mealybugs were kept in gel capsules until the emergence of adult wasps. The remaining specimens were reared in plastic containers closed with voile tissue, provided with potato sprouts *Solanum tuberosum* L. for food, and held at 25 ± 1°C, relative humidity of 70 ± 10%, and photoperiod of 14 h. These mealybugs were periodically checked for signs of parasitism. Capsules were checked daily for parasitoid emergence, and adults were placed in Eppendorf tubes with 95% ethanol and stored at − 20°C.

Molecular characterization and phylogenetic analyses

DNA was extracted at Laboratório de Biologia Molecular from Embrapa Uva e Vinho, through a non-destructive method, using the prepGEM Insect DNA extraction kit (ZyGEM, Lane Hamilton, New Zealand) and following the manufacturer's recommendations. Primary vouchers were kept in ethyl alcohol 70% for later morphological inspection. Polymerase chain reactions (PCRs) were completed using QIAGEN PCR Mastermix (Hilden, Germany), composed by 23 μL of reaction mix (water, Tmix and primers) and 2 μL of diluted DNA (1–20 ng). Primers used for PCR amplification were 5′ – GAGAGTTMAASAGTACGTGAAAC – 3′ and 5′ – TCGG -ARGGAACCAGCTACTA – 3′ for the 28S-D2 gene region and 5′ – GGTCAACAAA-TCATAAAGATATTGG 3′ and 5′ – TAAACTTCAGGGTGACCAAAAAATCA – 3′ for COI (Folmer et al. 1994). Annealing temperature was 58°C for 28S-D2 and 50°C for COI. After 15 min of polymerase activation at 95°C, a total of 35 (28S-D2) or 40 (COI) cycles were completed with 30 s at 95°C for denaturation, 90 s at 58°C (28S-D2) or 50°C (COI) for hybridization, and 60 s at 72°C for elongation; a final 10 min extension step at 72°C was included. PCR products were run through electrophoresis and sent for bidirectional sequencing to Beckman Coulter Genomics (Danvers, USA). Consensus sequences were constructed using Seqscape v.27 (Applied Biosystems, Foster City, CA, USA) and then blasted against the Genbank nucleotide database by using MEGABLAST (http://www.ncbi.nlm.gov/BLAST). New sequences are deposited in Genbank under accession numbers presented in Table 1.

Table 1 Parasitoids of mealybugs found in Rio Grande do Sul–Brazil. Parasitoid identity, number of specimens found (*N*), host mealybug, host plant substrate, location, collection code, and accession number.

Parasitoid identity	*N*	Host mealybug	Host plant	Location (city)	Collection code	Accession number	
						28S-D2	COI
Acerophagus flavidulus	28	*Ps. viburni*	Apple	Caxias do Sul	15160	MW465441	---
Anagyrus calyxtoi	2	*Ps. viburni*	Persimmon	Farroupilha	15177	MW465445	---
Anagyrus calyxtoi	1	*Ps. viburni*	Apple	Caxias do Sul	15164	---	MW463922
Anagyrus vladimiri	78	*Pl. ficus*	Grapes	Pinto Bandeira	22611	---	---
Anagyrus sp. 1	28	*Ps. viburni*	Strawberry	Farroupilha	15075	MW465438	---
Anagyrus sp. 1	12	*Ps. viburni*	Grapes	São Valentin do Sul	15192	---	MW463920
Anagyrus sp. 2	1	*Ps. viburni*	Apple	Caxias do Sul	15166	MW465439	---
Anagyrus near *quilmes*	20	*Ps. viburni*	Strawberry	Farroupilha	15076	MW465437	MW463921
Allotropa sp. 1	16	*Ps. viburni*	Strawberry	Farroupilha	22608	MW465435	MW463918
Allotropa sp. 2	8	*Ps. viburni*	Strawberry	Farroupilha	22632	---	---
Blepyrus clavicornis	166	*Ps. viburni*	Strawberry	Farroupilha	22575	MW465436	MW463919
Blepyrus schwarzi	2	*Ps. sociabilis*	Persimmon	Farroupilha	15176	MW465443	MW463923
Blepyrus sp.	4	*Ps. viburni*	Grapes	Encruzilhada do Sul	22591	MW465447	MW463926
Chartocerus axillaris	2	*Ps. viburni*	Strawberry	Farroupilha	15142	MW465440	---
Clausenia nr. *purpurea*	1	*Pseudococcus* sp.*	Persimmon	Farroupilha	22630	MW465448	MW463924
Coccidoxenoides perminutus	12	*Pl. ficus*	Grapes	Pinto Bandeira	22566	MW465444	---
Zaplatycerus sp.	26	*Ps. viburni*	Grapes	Encruzilhada do Sul	22571	MW465446	MW463925

**Pseudococcus* sp. corresponds to mummies that were collected directly in the field so the mealybug species could not be determined.

Sequence alignments were built for each locus (28S-D2 and COI) using the ClustalW algorithm in Bioedit v.7.02 (Hall 1999). Single gene trees were reconstructed directly from the alignments using a maximum likelihood (ML) approach as implemented in PhyML v3 (Guindon et al. 2010). A general time-reversible (GTR) model with discrete gamma-distribution with four rate categories (+G) plus invariant positions (+I) was used, estimating the gamma parameter and the fraction of invariant positions from the alignment. Branch support was computed using 500 bootstrap replicates. Genetic divergence was calculated in MEGA7 (Kumar et al. 2016) using 28S data and the Tamura-Nei model (Tamura and Nei 1993). All positions containing gaps and missing data were eliminated.

Morphological identification

Morphological identification of vouchers was carried out by the second author at the CEPAVE (La Plata, Argentina). Specimens were mounted using conventional techniques and, when necessary for more detailed study, slide-mounted in Canada balsam following Noyes (1990). Identification was based on taxa-specific keys (De Santis 1964; Gibson et al. 1997; Noyes 1980, 2000; Triapitsyn et al. 2014) and direct comparison with reference material. Voucher specimens are deposited in the collection of *División Entomología* of *Museo de La Plata* (see Table 1).

Results

Morphological identification

Fifteen parasitoid species, belonging to the families Encyrtidae, Platygastridae, and Signiphoridae, were found parasitizing the three mealybugs studied (Table 1). Eleven species were associated with *Ps. viburni*, including the encyrtids *Acerophagus flavidulus* (Brèthes), *Anagyrus calyxtoi* Noyes, *Anagyrus* sp. 1, *Anagyrus* sp. 2, *An.* near *quilmes*, *Blepyrus clavicornis* (Compere), *Blepyrus* sp., and *Zaplatycerus* sp.; one signiphorid hyperparasitoid (*Chartocerus axillaris* De Santis); and two platygastrids (*Allotropa* sp. 1 and *Allotropa* sp. 2). Only one encyrtid, *Blepyrus schwarzi* (Howard), was found parasitizing *Ps. sociabilis*, while two encyrtids were found associated with *Pl. ficus* namely, *Anagyrus vladimiri* Triapitsyn and *Coccidoxenoides perminutus* Girault. *Clausenia* sp. was collected from an unidentified *Pseudococcus* specimen. It should be pointed out that the species *Ac. flavidulus*, *An. calyxtoi*, and *Ch. axillaris* and the genus *Zaplatycerus* Timberlake are reported for the first time from Brazil.

Inconsistencies between morphological and molecular identification have been observed in the genera *Blepyrus* Howard and *Anagyrus* Howard. *Blepyrus* specimens from Encruzilhada do Sul vineyards most likely represent an undescribed cryptic species, showing high molecular divergences for both COI (22.36%) and 28S (8.81%) with

B. clavicornis from Serra Gaúcha Region; however, no morphological differences were observed between them. *Anagyrus quilmes* Triapitsyn, Logarzo & Aguirre sensu stricto and *An.* near *quilmes* present differences in wing setation and color of head and antennal segments, but they show high molecular similarity (see below).

Molecular characterization and phylogenetic analyses

A total of 379 sequences were obtained from 216 parasitoids. Among all the parasitoids collected, fragments of 719 to 931 base pairs for 28S-D2 were successfully amplified for 15 different haplotypes corresponding to 15 taxa. For COI, fragments of 520 to 659 base pairs were obtained, corresponding to 22 haplotypes from 12 different taxa. Nine haplotypes were observed for *Anagyrus* sp. 1, whereas two haplotypes were detected for *Allotropa* sp. 1, and *Zaplatycerus* sp., with divergences of 0.30% (2/656) and 2.57% (15/582) between haplotypes, respectively. Distance-based phylogenetic trees are shown in Fig. 1. Sequence divergences for the 28S gene ranged from 0.003 to 0.193 within Encyrtidae, from 0.451 to 0.493 between Encyrtidae and Platygastridae, and from 0.129 to 0.163 between Encyrtidae and Signiphoridae (Table 2). Blast hits resulted in some *Anagyrus* 28S sequences (*An. calyxtoi*, *Anagyrus* sp. 1, and *Anagyrus* sp. 2) showing more than 97% similarity with *An. pseudococci* (Girault) from GenBank (AY599315.1). *Acerophagus flavidulus* and *Co. perminutus* 28S sequences blasted with more than 98% similarity to *Ac. flavidulus* (KU499433.1) and *Co. perminutus* (KY211082.1), respectively. The 100% similarity for 28S (*An. quilmes*: MG731478-88) and 94% similarity for COI sequences (*An. quilmes:* MG731507-17) show a close relationship between our *An.* near *quilmes* and *An. quilmes* from Argentina. *Anagyrus vladimiri* COI sequences blasted with more than 99% similarity with *An.* near *pseudococci* (KU499515.1), a cryptic species recently described as *An. vladimiri* (Andreason et al. 2019). Lower similarity values (below 96%) were observed between other species and GenBank sequences.

Phylogenetic tree estimates were congruent for both 28S and COI (Fig. 1). Encyrtidae is monophyletic in both cases, and the genera *Zaplatycerus*, *Aenasius*, and *Blepyrus* group together with high bootstrap support (> 85%). A second clade, including *Leptomastidea* spp. and *Anagyrus* spp., was also found with high bootstrap support in both cases. The relative position of other genera such as *Clausenia* Ishii, *Coccidoxenoides* Girault or *Acerophagus* Smith and *Metaphycus* Mercet within the family remains unresolved. Furthermore, our *Anagyrus* sp. 2 sequences cluster together with sequences from *An. calyxtoi*, while *An. quilmes* from Genbank cluster with *An.* near *quilmes*.

Parasitoid-host relationship

Parasitism rate was low in all sampling sites except in the late-season vineyard, showing up to 70% of adult *Pl. ficus* females parasitized by *An. vladimiri* (Table 1). In the strawberry field, besides the great diversity of species parasitism reached only 2.15% of total *Ps. viburni* collected. The parasitism rates were not measured for the other crops and localities. Encyrtidae showed the greatest number of species recorded (11 species) and was the most abundant family (about 90%). *Anagyrus* was

Table 2 Estimates of evolutionary divergence between 28S-D2 sequences

		1	2	3	4	5	6	7	8	9	10	11	12	13	14
1.	*Acerophagus flavidulus*														1.
2.	*Anagyrus* sp. 1	0.189													2.
3.	*Anagyrus* near *quilmes*	0.152	0.036												3.
4.	*Anagyrus calyxtoi*	0.155	0.044	0.009											4.
5.	*Anagyrus* sp. 2	0.159	0.047	0.012	0.003										5.
6.	*Blepyrus clavicornis*	0.145	0.126	0.100	0.096	0.100									6.
7.	*Blepyrus schwarzi*	0.147	0.123	0.101	0.097	0.101	0.057								7.
8.	*Blepyrus* sp.	0.143	0.122	0.099	0.096	0.099	0.052	0.005							8.
9.	*Clausenia* nr. *purpurea*	0.156	0.107	0.082	0.084	0.087	0.082	0.092	0.087						9.
10.	*Coccidoxenoides perminutus*	0.193	0.133	0.107	0.103	0.103	0.119	0.125	0.129	0.111					10.
11.	*Zaplatycerus* sp.	0.135	0.107	0.085	0.085	0.089	0.055	0.063	0.058	0.065	0.093				11.
12.	*Chartocerus axillaris*	0.163	0.152	0.135	0.133	0.129	0.144	0.148	0.146	0.137	0.162	0.129			12.
13.	*Allotropa* sp. 1	0.481	0.468	0.456	0.465	0.468	0.451	0.468	0.462	0.489	0.493	0.477	0.485		13.
14.	*Allotropa* sp. 2	0.482	0.468	0.456	0.465	0.468	0.451	0.468	0.462	0.489	0.493	0.477	0.484	0.003	14.

The number of base substitutions per site from between sequences is shown. Analyses were conducted using the Tamura-Nei model (Tamura and Nei 1993). The analysis involved 19 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 667 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura et al. 2013).

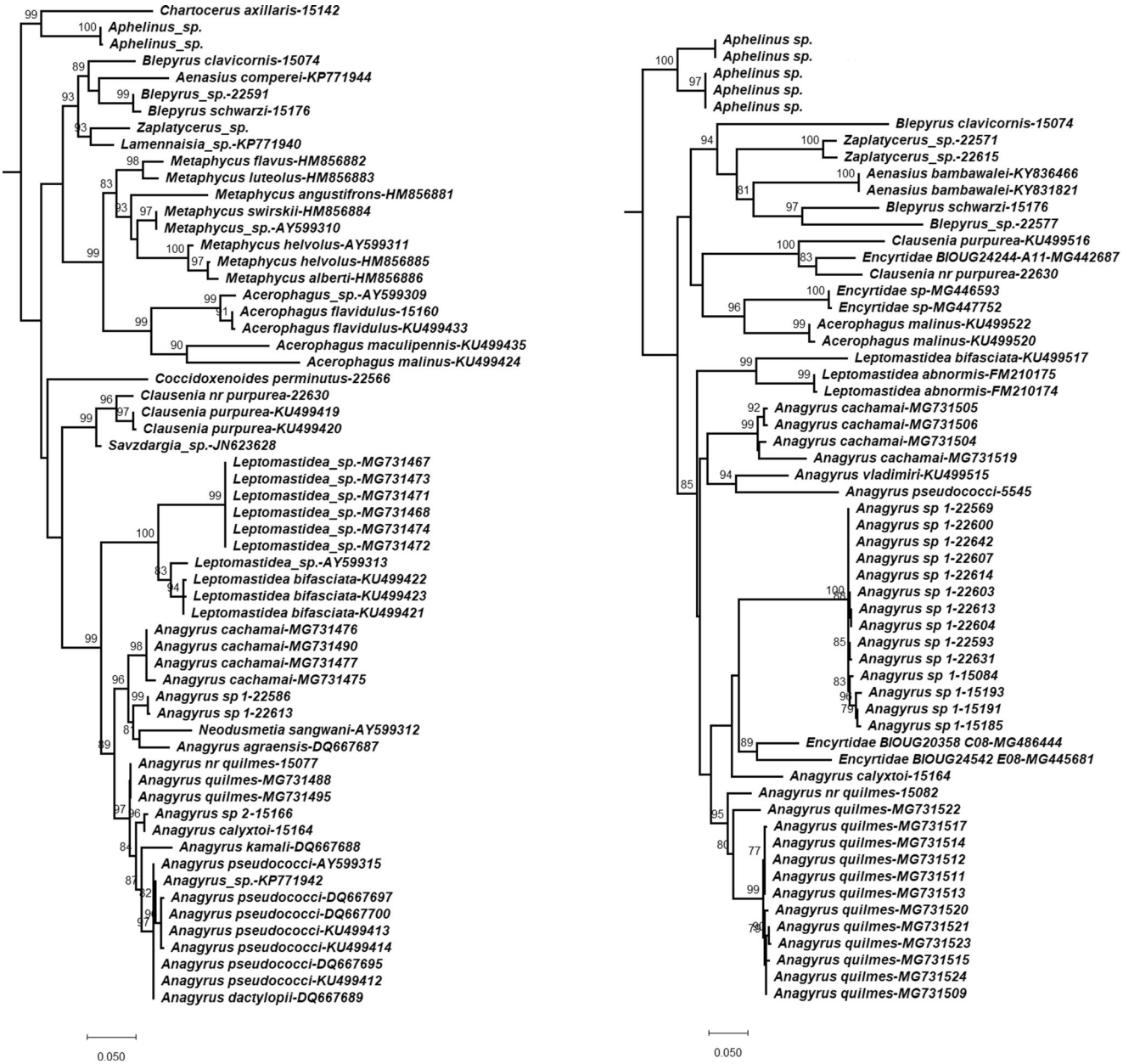

Fig. 1 Maximum-likelihood tree for the 28S (left) and COI (right) datasets. Bootstrap values (500 replicates) are displayed. Rooted by *Aphelinus* sp. and *Chartocerus axillaris*.

the most diverse genus within the family, with five species being represented (Table 1).

Acerophagus flavidulus was the main parasitoid species found in one apple orchard infested by *Ps. viburni* and was not observed in other fruit crops. In persimmon orchards, three parasitoid species were found sporadically in two locations, one infested with *Ps. sociabilis* and other with *Ps. viburni.* As many as seven species of parasitoids were observed in an organic *Ps. viburni*–infested strawberry farm, with *Blepyrus clavicornis* accounting for 70.8% of the total mealybugs parasitized and being the main primary parasitoid, followed by *Anagyrus* spp. representing 20.5% and *Allotropa* spp. with 8.7% of the parasitism. Parasitoids were also found in three vineyards, the two infested with *Ps. viburni* included *Anagyrus* sp. 1, *Blepyrus* sp., and *Zaplatycerus* sp., whereas the vineyard infested with *Pl. ficus* included *An. vladimiri* and *Co. perminutus*.

Discussion

Biological control programs against *Pseudococcus* species generally rely on New World encyrtids such as *Acerophagus* Smith and *Anagyrus*. Our results suggest that further attention should be paid to poorly studied genera, such as *Blepyrus* and

Zaplatycerus Timberlake, which have not been used very often as BCAs (Noyes 2000). The encyrtids *Ac. flavidulus*, *Anagyrus* spp., and *B. clavicornis* were the most common parasitoids infesting *Ps. viburni* populations in the Serra Gaúcha region. Our analyses yield new evidence on mealybug parasitoids compared with previous surveys. Daane et al. (2008) observed four encyrtid species on *Ps. viburni* populations (*Ac. flavidulus*, *An. pseudococci*, *Leptomastix dactylopii* Howard, and *Leptomastidea abnormis* (Girault)), while five encyrtids were recorded in South Africa (*Anagyrus* sp., *Acerophagus* sp., *Ac. maculipennis* (Mercet), *Pseudectroma* sp., and *Tetracnemoidea* sp.) (Wakgari and Giliomee 2004) and no parasitoids were observed in New Zealand (Charles et al. 2010). In this study, 11 parasitoids were found associated with *Ps. viburni*, which provides further support for the hypothesis of a South American origin of this species (Charles 2011; Correa et al. 2015). The new parasitoids found here for *Ps. viburni* and *Ps. sociabilis* are most likely native South American species with strong host specificity, which makes them ideal candidates as biological control agents.

Other parasitoids reported here were only found sporadically. Species from *Clausenia* have been used in the biological control of *Ps. comstocki* (Kuwana) (Guerrieri and Pellizzari 2009), but only one female *Clausenia* specimen was observed in this study. Similarly, platygastrid parasitoids (e.g., *Allotropa* spp.) have been previously used in mealybug management (Roltsch et al. 2006; Quaglietti et al. 2017), but they were only found in low numbers here. The same happened with the signiphorid hyperparasitoid *Ch. axillaris*, which was rarely collected in this survey.

The parasitoids *An. vladimiri* and *Co. perminutus* are known to be important biological control agents against the vine mealybug (*Pl. ficus*), an exotic species recently observed damaging vineyards in Serra Gaúcha region (Pacheco da Silva et al. 2016). They were still observed in the last year of our survey, showing high levels of parasitism during the late season (March and April, 2016). *Coccidoxenoides perminutus* had been recently recorded in Brazil parasitizing *Pl. citri* (Risso) and could have easily infested *Pl. ficus* (Fernandes et al. 2016). Our observation also agrees with the fact that *Co. perminutus* infests *Pl. ficus* populations in Africa, where it is probably an endemic species (Walton et al. 2004; Mahfoudhi and Dhouibi 2009).

Occasional outbreaks of *Ps. sociabilis* and *Ps. viburni* in Southern Brazil are probably due to common pesticides killing off the local parasitoid community, and alternative strategies to combat mealybugs are needed to improve fruit production. Our results show that the most suitable natural enemies against the obscure mealybug are *Ac. flavidulus*, *Anagyrus* species, and *B. clavicornis*, and that the establishment of *An. vladimiri* and *Co. perminutus* as biological control agents (BCAs) should contribute to reduce vine mealybug outbreaks. Encyrtid parasitoids are key BCAs around the world and their morphological and molecular characterization will be an important step towards efficient management of mealybug pest populations across South America, not just Brazil.

The integrative taxonomy approach used here revealed the presence of several cryptic species parasitizing mealybugs. Females of *Anagyrus* sp. 1 are morphologically close to *An. quilmes* but clearly distinct at the molecular level, whereas another species (i.e., *An.* near *quilmes*) showed significant molecular similarity despite being morphologically distinct from *An. quilmes*. *Anagyrus* is a highly diverse genus with a complex taxonomic history, in which other cryptic species have already been identified (Andreason et al. 2019; Triapitsyn et al. 2018). Our investigation confirmed the presence of new parasitoid species infesting *Ps. viburni* in Brazil, but many more potential biological control agents might remain undescribed.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflicts of interest.

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