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An exceptional family: *Ophiocordyceps*-allied fungus dominates the microbiome of soft scale insects (Hemiptera: Sternorrhyncha: Coccidae)

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Abstract

Hemipteran insects of the suborder Sternorrhyncha are plant sap feeders, where each family is obligately associated with a specific bacterial endosymbiont that produces essential nutrients lacking in the sap. Coccidae (soft scale insects) is the only major sternorrhynchan family in which obligate symbiont(s) have not been identified. We studied the microbiota in seven species from this family from Israel, Spain and Cyprus, by high-throughput sequencing of ribosomal genes, and found that no specific bacterium was prevalent and abundant in all the tested species. In contrast, an *Ophiocordyceps*-allied fungus sp.—a lineage widely known as entomopathogenic—was highly prevalent. All individuals of all the tested species carried this fungus. Phylogenetic analyses showed that the *Ophiocordyceps*-allied fungus from the coccids is closely related to fungi described from other hemipterans, and they appear to be monophyletic, although the phylogenies of the *Ophiocordyceps*-allied fungi and their hosts do not appear to be congruent. Microscopic observations show that the fungal cells are lemon-shaped, are distributed throughout the host's body and are present in the eggs, suggesting vertical transmission. Taken together, the results suggest that the *Ophiocordyceps*-allied fungus may be a primary symbiont of Coccidae—a major evolutionary shift from bacteria to fungi in the Sternorrhyncha, and an important example of fungal evolutionary lifestyle switch.

KEYWORDS
evolutionary shift, primary symbiont, symbiosis

1 | INTRODUCTION

The phenomenon of endosymbiosis, microbial symbionts living within cells of a host, is widespread among arthropods. As not all symbionts are equally essential for the host, they are broadly classified into two categories: primary and secondary symbionts (Moran, McCutcheon, & Nakabachi, 2008). Secondary symbionts are generally regarded as noncrucial to the host's development and reproduction. Nonetheless, in recent years, copious effects of secondary symbionts are being revealed, for example conferring tolerance to pathogens and natural enemies, enhancing fecundity, causing reproductive manipulations and more, and it is now clear that they play important roles in their host's ecology and evolution (Douglas, 2015; McLean et al., 2016; Su, Zhou, & Zhang, 2013). Primary symbionts (PS) are essential for the hosts' development and reproduction, as they produce certain metabolites lacking in the hosts' diet. For example, in hemipteran insects of the suborder Sternorrhyncha, bacterial PS produce essential amino acids or carotenoids lacking in the phloem sap food source; PS of blood-sucking insects such as sucking lice, bed bugs and tsetse flies provide B vitamins (Baumann, 2005; Douglas, 2013; McLean et al., 2016).

In Sternorrhyncha, each family has established an obligate symbiosis with a specific bacterial lineage, and the phylogenies of both parties are congruent as a result of their codependence. For example: aphids (Aphididae) harbour the bacterial symbiont *Buchnera aphidicola*, whiteflies (Aleyrodidae) harbour the bacterium *Portiera aleyrodidarum*, psyllids (Psyllidae)—*Carsonella ruddii*, armoured scale insects (Diaspididae)—*Uzinura diaspidicola*, etc. (Baumann, 2005; Bennett & Moran, 2013; Douglas, 2016; Moran et al., 2008; Su et al., 2013). Despite the obligate nature of the association, replacement of a PS is not uncommon (Husnik & McCutcheon, 2016; Koga, Bennett, Cryan, & Moran, 2013; Sudakaran, Kost, & Kaltenpoth, 2017), including an example of several aphid species within the subfamily Cerataphidinae, in which the bacterium *B. aphidicola* has been replaced by a yeast-like symbiont as a PS (Fukatsu, Shigeyuki, Utako, Ishikawa, 1994; Vogel & Moran, 2013).

While bacterial symbionts of sap-sucking insects have been extensively studied in the last ~25 years, fungal symbionts have received less attention. Yeast-like micro-organisms inhabiting various insects have been known for a long time (see Buchner, 1965) and during the last two decades fungal symbionts, especially true yeasts (Ascomycota: Saccharomycotina), have been described from a wide range of insects, including hemipterans (Chen, Cheng, Kuan, & Hou, 1981; Fukatsu & Ishikawa, 1996; Gibson & Hunter, 2010; Hamby, Hernández, Boundy-Mills, & Zalom, 2012; Urbina, Frank, & Blackwell, 2013). True yeasts are usually located in fat body tissue and in the digestive tract (Chen et al., 1981; Gibson & Hunter, 2010; Hughes et al., 2011; Urbina et al., 2013). Known essential functions of fungal symbionts include synthesis of sterols (Noda & Koizumi, 2003) and nitrogen recycling (Sasaki, Kawamura, & Ishikawa, 1996), yet it is not clear if the associations between these fungi and their hosts are obligate (i.e., if both partners cannot survive without each other). Associations of insects with fungi are as old as 420 MY, yet there is currently little evidence of codiversification between host and symbiont lineages (Gibson & Hunter, 2010).

Soft scale insects (Hemiptera: Coccoidea: Coccidae) are soft-bodied insects that feed on phloem, typically of woody plants. The family Coccidae comprises about 1,140 species, placed in 171 genera that are grouped to 10 subfamilies (García Morales et al., 2016) (ScaleNet website-http://scalenet.info/), many of which are pests of agricultural crops worldwide (Ben-Dov, 1994). Coccids are one of the last major groups whose obligate primary symbionts are yet to be identified. As mentioned above, every plant sap feeding lineage carries obligate nutritional symbionts. It is therefore reasonable to hypothesize that coccids carry obligate symbionts as well. The literature on symbionts of soft scale insects is quite limited and suggests the presence of symbiotic fungi rather than bacteria. The first records of fungal symbionts in coccids go back to the second half of the 19th century, including some publications by Paul Buchner in the early 20th century (see summary in Tremblay, 1997). Brues and Glaser (1921) isolated fungal symbionts from *Pulvinaria innumerabilis* (Rathvon) and found that this symbiont produces proteolytic and lypolytic enzymes, suggesting a nutritional and/or metabolic role. A few microscopy studies found yeast-like symbionts (YLS) in the haemolymph and fat body of a few coccid species, as well as evidence of vertical transmission (Brues & Glaser, 1921; Steinhaus, 1955). Several YLS species were reported in the Mediterranean black scale, *Saissetia oleae* (Olivier), with different life stages differing in symbiont composition (Zacchi & Vaughan-Martini, 2002, 2003). A recent study found bacteria of the family Enterobacteriaceae in several coccid species, as well as in species belonging to other families in the superfamily Coccoidea (Rosenblueth, Sayavedra, Sámano-Sánchez, Roth, & Martínez-Romero, 2012). Despite these pieces of information, the identity of the PS of soft scale insects remains unknown. The goal of this study is, therefore, to characterize the microbiome (bacteria and fungi) of soft scale insects, in order to identify a candidate(s) for primary symbiont(s).

2 | MATERIALS AND METHODS

2.1 | Insects

Samples of seven species of soft scale insects were collected at different locations, host plants and dates (Table 1) and preserved in 96% ethanol for molecular analysis as described below. All species were identified to the genus or species level by Yair Ben-Dov. Two of the seven species were reared continuously outdoors: *Milviscutulus mangiferae* Green was maintained on a mango tree (*Mangifera*

TABLE 1 Details of soft scale insect species collected and used in the study

Subfamily	Species	Host plant	Country	Coordinates	Collection date
Ceroplastinae	*Ceroplastes* sp.	Unidentified	Spain	36°43′10.1″N 4°24′57.0″W	12-Feb-2016
	Ceroplastes floridensis	*Hedera helix*	Israel	32°42′45.7″N 35°06′25.2″E	23-Feb-2015
	Ceroplastes rusci	*Ficus carica*	Cyprus	34°54′16.5″N 33°37′23.0″E	14-Nov-2015
Coccinae	*Coccus hesperidum*	*Citrus sinensis*	Israel	32°36′44.8″N 35°05′58.8″E	2014
	Milviscutulus mangiferae	*Mangifera indica*	Israel	33°09′00.1″N 35°37′17.7″E	26-Feb-2015
	Parasaissetia nigra	*Ficus carica*	Israel	32°43′26″N 35°07′38″E	25-May-2015
	Saissetia sp.	*Ficus carica*	Cyprus	34°54′16.5″N 33°37′23.0″E	14-Nov-2015

indica L.), protected by an insect-proof net; a population of *Ceroplastes floridensis* Comstock was available right outside our laboratory, growing on common ivy (*Hedera helix* L.).

2.2 | High-throughput sequencing of ribosomal genes

Prior to DNA extraction, specimens were inspected for parasitoids under the microscope and only nonparasitized specimens were used. Individuals were surface-sterilized by submerging them for 3 min in 3% sodium hypochlorite solution and then rinsing three times with sterile double deionized water. Subsequently, DNA was extracted from pools of 9–10 female adults, with the exception of *P. nigra*, in which pools consisted of 2–4 females, due to their large size. DNA was extracted from whole insects using the Omega Bio-tek kit (Norcross, GA, USA; protocol for insect DNA), total DNA was eluted into 50 μl of PCR-grade water and stored at −20°C. Negative controls were added to each DNA extraction set.

The symbiotic bacterial and fungal communities of the seven soft scale insect species were assessed by high-throughput sequencing of small subunit (SSU) ribosomal RNA (rRNA) gene amplicons, using the Illumina Miseq platform. Briefly, the widely used primer set 341F/805R (with slight modifications, see Table 2), targeting the V3–V4 variable region of bacterial 16S rRNA, was used for amplification (Bartram, Lynch, Stearns, Moreno-Hagelsieb, & Neufeld, 2011; Kennedy, Hall, Lynch, Moreno-Hagelsieb, & Neufeld, 2014) (see also “Earth Microbiome Project” website- http://press.igsb.anl.gov/earthmicrobiome/protocols-and-standards/16s/). A two-stage PCR (or “targeted amplicon sequencing”) approach was performed to generate amplicon libraries, as described previously (Bybee et al., 2011; Green et al., 2015). In the first of the two-stage amplification procedure, the templates were amplified (28 cycles) using the primers 341F and 805R containing 5′ CS1 and CS2 linkers (Moonsamy et al., 2013). Similarly, the primers FF390 and FR1 (Table 2), also containing CS1 and CS2 linkers, respectively, were used for amplification of 18S rRNA genes of fungi (Vainio & Hantula, 2000). PCRs were performed in 10 μl reaction volumes using MyTaq HS 2X mastermix (Bioline, Taunton, MA, USA). Subsequently, a second PCR was established, with 1 μl of amplification product from the first stage used as input to the second reaction. The primers for the second stage amplifications were the AccessArray barcoding system primers (Fluidigm, South San Francisco, CA, USA), containing Illumina sequencing adapters, sample-specific barcodes (10 bases, with a minimum hamming distance of 3), and CS1 and CS2 linkers. The second stage PCRs were performed in 10 μl reaction volumes using MyTaq HS 2X mastermix (Bioline, Taunton, MA), and the PCR conditions were as follows: 5-min initial denaturation at 95°C, followed by eight cycles of: 95°C for 30 s, 60°C for 30 s, 72°C for 60 s. Final PCR products were purified and equalized using SequalPrep Normalization Plate Kit (Thermo Fisher Scientific), according to the manufacturer’s instructions. Samples were pooled in equimolar ratio and quantified using a Qubit 2.0 fluorometer. Sequencing was performed on an Illumina MiSeq sequencer using standard V3 chemistry with paired-end, 300 base reads. Fluidigm sequencing primers, targeting the CS1 and CS2 linker regions, were used to initiate sequencing. Demultiplexing of reads was performed on instrument. Library preparation and sequencing were performed at the DNA Services Facility at the University of Illinois at Chicago.

2.2.1 | OTU clustering using Usearch pipeline

Raw paired-end reads were first merged into single sequences using PEAR (http://sco.h-its.org/exelixis/web/software/pear/index.html). For each merged read pair, PEAR produces a joint quality score for the new assembled sequence. In the fungal data, forward reads from pairs that could not be merged were also retained (unpaired reverse sequences were excluded). In the bacterial data, only successfully merged reads were further utilized. Sequences containing traces of PhiX genome (NC_001422), and NCBI Univec sequences (http://www.ncbi.nlm.nih.gov/tools/vecscreen/univec/) were removed using FASTQ-SCREEN and BOWTIE2 tools (http://www.bioinformatics.babraham.ac.uk/projects/fastq_screen/; http://bowtie-bio.sourceforge.net/bowtie2/index.shtml). This allowed the removal of possible phiX spike-ins to the MiSeq run, and other potential contaminants. Most sequences included the complete primer sequences (>95% of the sequences). Sequences were further quality trimmed using Trimmomatic, ensuring >27 phred33 quality scores on a sliding window of 15 bp. The program FASTQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc) was used in order to visually validate high quality scores along the entire sequence length, and to ensure that most merged reads have the expected amplicon length. The sequences were further trimmed to a constant length (300 bp for the fungal data, and 400 bp for the bacterial data), by truncating their 3′ end. Reads shorter than the above cut-offs were excluded. The UPARSE program pipeline, part of the USEARCH program (usearch version v7.0.1090_i86linux32), was used for operational taxonomic unit (OTU) clustering, following the recommended pipeline (http://www.drive5.com/usearch/manual/uparse_pipeline.html). This program performs de novo OTU clustering, namely: the OTU clustering algorithm does not require matching the reads to any reference OTU databases. Specifically, this pipeline consists of the following steps: (i) reads de-replication; (ii) abundance sorting and singleton discarding, where a minimum of ten sequences was required per sequence; (iii) OTU clustering; and (iv) chimera filtering. This allowed obtaining absolute read counts per OTUs per sample. We further normalized counts by calculating the percentage of reads mapped to each OTU, and as a second measure: by converting to CPM values (Count Per Million), using the R module EdgeR default within-sample normalization for counts data (note that we have not performed any between-samples normalization steps). The resultant representative sequences of each OTU were compared to the NCBI NT database. Determination of taxonomy was performed using the weighted (80%) least common ancestor (LCA) algorithm in MEGAN6 community addition software (http://ab.inf.uni-tuebingen.de/software/megan6/). LCA parameters cut-off values included an *E* value <10e−100, minimum score of 200 and top per cent set to 10%. In order to

TABLE 2 Details of primers and PCRs performed in this study

	Target organism and gene	Primers' name	Primers' sequence (CS1 and CS2 linkers are underlined)	Product's length (bp)	Annealing temp./time	Source
High throughput sequencing	General bacteria 16S rRNA	341F 805R	ACACTGACGACATGGTTCTACACCTACGGGNGGCWGCAG TACGGTAGCAGAGACTTGGTCTGACTACHVGGGTATCTAATCC	390	57°C/30 s	Muyzer, de Waal, & Uitterlinden (1993) Klindworth et al. (2013)
	General fungi 18S rRNA	FF390 FR1	ACACTGACGACATGGTTCTACAAICCATTCAATCGGTAIT TACGGTAGCAGAGACTTGGTCTCGATAACGAACGAGACCT	390	55°C/45 s	Vainio and Hantula (2000)
Diagnostic screening PCR	*Ophiocordyceps* 18S rRNA	Ophio-F Ophio-R	CGTATTGCTTCGGCAGTA GGGCCTTCCGGCCAAGGGAGT	120	55°C/45 s	Present study
	Cladosporium 18S—ITS	Clad-F Clad-R	AGGCCCGCTTTGGCGGGTAGACCTTTCGGACAAGGAAAT	120	55°C/45 s	Present study
Phylogeny	*Ophiocordyceps* RPB1	CRPB1 RPB1c-r	CCWGGYTTYATCAAGAARGT CCNGCDATNTCRTTRTCCATRTA	790	55°C/60 s	Castlebury, Rossman, Sung, Hyten, & Spatafora (2004)
	Ophiocordyceps 18S	Ophio18S-1F Ophio18S-1R Ophio18S-3F	ATAGCGTATATTAAAGTTGTTGTGGT CCGTGTTGAGTCAAATTAAGCC GCTCGAATACATTAGCATGGAATAATGA	600 400	54°C/30 s	Present study
	Ophiocordyceps ITS	Ophio-F ITS4	CGTATTGCTTCGGCAGTA TCCTCCGCTTATTGATATGC	800	55°C/60 s	Present study Glass and Donaldson (1995)
	Coccidea COI	C1-1554F C1-2342R	CAGGAATAATAGGAACATCAATAA ATCAATGTCTAATCCGATAGTAAATA	700	52°C/50 s	Deng et al. (2012)

examine the similarity between different samples based on their OTU counts, sample level nMDS (nonmetric multidimensional scaling) clustering was conducted, based on Bray–Curtis dissimilarity distances, using the community ecology package VEGAN in R (R version 3.2.1, https://cran.r-project.org/web/packages/vegan/index.htm).

2.3 | Diagnostic screening of Ophiocordyceps sp. and Cladosporium sp

As will be described in the results section, two fungal lineages—an *Ophiocordyceps*-allied fungus (OAF) and a *Cladosporium* sp.—were abundant in the high-throughput sequencing, "marking" them as plausible primary symbiont candidates. Therefore, using newly designed genus-specific primers, we tested the infection frequency of these fungi in the coccid samples. Prior to DNA extraction, specimens were surface-sterilized as described above and then each female was ground individually (n = 30 for each species/population) in 50 μl of a buffer solution (10 mM Tris-Cl pH 8.2, 1 mM EDTA, 25 mM NaCl) containing 20 mg/ml proteinase K (Invitrogen, Grand Island, NY, USA). The lysates were incubated for 15 min in 65°C, then 10 min in 95°C and kept at −20°C until further use.

Ophiocordyceps-specific and *Cladosporium*-specific primers (Table 2), both targeting the 18S rRNA regions of these fungi, were designed based on the sequences obtained in the metaprofiling section and the following sequences from GENBANK database: JX968028 [*Ophiocordyceps sinensis* (Berk.)], AB968386 [*Ophiocordyceps arborescens* (S. Ban, T. Sakane & Nakagiri)], JN941692 [*Ophiocordyceps stylophora* (Berk. & Broome)], XR657242 (*Wallemia ichthyophaga* Johan-Olsen) and KJ443070 (*Cladosporium sphaerospermum* Penz). The primers' specificity was verified by testing them with 10 of our coccid DNA extractions, as well as DNA extracted from pure cultures of *Cladosporium* sp. and *Saccharomyces cerevisiae* (as a negative control), and sequencing the PCR-amplified products. After the primers' specificity was confirmed we screened 30 individual adult females from each coccid population for the presence of OAF and *Cladosporium*, adding positive and negative control samples in each set. The PCRs were performed in a final volume of 10 μl using the MyTaq™ Red Mix (Bioline). The PCR conditions for both primers were optimized and are specified in Table 2. PCR products were separated by electrophoresis in 1.5% agarose gels, stained with Gel Red and visualized under UV light.

2.3.1 | Phylogenetic analyses

To test whether the phylogenies of the coccid hosts and OAF (the fungus that has emerged as the main primary symbiont candidate, as will be detailed below, in the results section) are congruent, we sequenced fragments of OAF 18S ribosomal DNA (400–600 bp), OAF 18S-ITS (800–900 bp) and OAF RNA polymerase II largest subunit (RPB1) genes (730–740 bp), and a 700–800 bp fragment of the mitochondrial cytochrome oxidase I (COI) gene of the coccid hosts. The primers and PCR conditions are described in Table 2. Each of these gene fragments was amplified from at least three samples from each coccid species. Consensus sequences were constructed from the three (or more) replicates and were used for constructing the phylogenetic trees of the coccid hosts and *Ophiocordyceps*. Congruence between coccids' and OAF phylogenies was tested using TREEMAP 3 (Charleston, Robertson, & Sanderson, 2002). The OAF 18S and RPB1 sequences were also used for studying their phylogenetic relationships with other *Ophiocordyceps* species.

Nucleotide sequences related to the scale and fungal sequences were identified in genomic and nonredundant nucleotide sequence databases on GENBANK by BLASTN. Sequences were aligned using MAFFT (Katoh & Standley, 2013), and the best nucleotide substitution model was chosen by JMODELTEST 2.1.7 (Darriba, Taboada, Doallo, & Posada, 2012). The chosen model for RPB1 was TrN + I + G, for 18S-TrN + G, and for COI TIM2 + I + G. Maximum-likelihood phylograms were constructed using PHYML (Guindon et al., 2010). Topology searches included both NNI and SPR methods. Branch support was calculated using SH-like approximate likelihood ratio tests.

2.4 | Fluorescence in situ hybridization (FISH)

Nymphs and eggs of *P. nigra* and *M. mangiferae* were collected and processed following the method of Sakurai, Koga, Tsuchida, Meng, and Fukatsu (2005). Whole mount specimens were deposited directly into Carnoy's fixative (chloroform: ethanol: acetic acid, 6:3:1) and left overnight. After fixation, the samples were decolorized in 6% H_2O_2 (in ethanol) for 2 hr and then hybridized overnight in hybridization buffer (20 mM Tris-HCl [pH 8.0], 0.9 M NaCl, 0.01% sodium dodecyl sulphate, 30% formamide) containing 10 pmol of fluorescent probes/ml.

The probe Cy5-Ophio-R (5′-Cy5-GGGCCTTCCGGCCAAGG-GAGT-3′) was designed to specifically target OAF. Stained samples were whole mounted and viewed under an Olympus IX 81 (Japan) inverted laser scanning confocal microscope (Fluoview 500) equipped with a 543 nm helium-neon laser. CY5 was excited by 633 nm light, and the emission was collected through an IF 660 filter. Specificity of the detection was confirmed using no-probe controls.

In addition, observations with light microscopy were made on nymphs of *P. nigra*. The nymphs were placed in a drop of sterile water on a slide, gently squished gently between the slide and a cover glass and then observed using an Olympus BX-61 microscope.

3 | RESULTS

3.1 | High-throughput sequencing of ribosomal genes

3.1.1 | Bacteria

About 300 bacterial OTUs were identified in the coccid samples (after all the filtration steps, Appendix S1), but none of them was dominant in all the species (Table 3). For example, *Wolbachia* was highly dominant in *C. floridensis* but comprised only up to 2% of the total OTUs in the other coccid species. Similarly, *Rickettsia* was

TABLE 3 Relative abundance (mean ± *SE*) of the main bacterial OTUs, combined to the genus level, that were amplified from the coccid species

	Coccid species					
	First run (n = 323 OTUs)				**Second run (n = 277 OTUs)**	
Bacterial lineage	***M. mangiferae* (*n* = 3 pools)**	***C. floridensis*[a] (*n* = 5 pools)**	***C. hesperidum* (*n* = 2 pools)**	***P. nigra* (*n* = 3 pools)**	***Ceroplastes* sp. (*n* = 3 pools)**	***Saissetia* sp. (*n* = 4 pools)**
Wolbachia[b]	—	66.3 ± 14.3%	—	—	—	—
Rickettsia[b]	79.4 ± 5.5%	—	7.0 ± 6.6%	—	—	—
Weisiella[c]	—	—	22.9 ± 3.5%	—	—	—
Gluconobacter[d]	—	—	13.3 ± 8.4%	—	—	—
Acetobacter[d]	—	—	14.7 ± 13%	—	—	—
Staphylococcus[c]	—	—	—	13.2 ± 4.5%	—	12.2 ± 4.1%
Lactobacillus[c]	—	—	—	21.2 ± 21%	9.7 ± 16.4%	—
All others	20.6	33.7	49.1	65.6	90.3	87.8

Empty cells = the OTU represented (on average) less than 5% of the total OTUs in that coccid host. For the full list of OTUs and their relative abundances, refer to Appendix S2.

[a] One of the five pools was substantially different from the other four. If omitted, *Wolbachia*'s percentage would be 80 ± 1.9%.

[b] α-proteobacteria: Rickettsiales.

[c] Firmicutes: Bacilli.

[d] α-proteobacteria: Rhodospirillales.

highly dominant in *M. mangiferae*, whereas in the other coccid species was rare or absent. The relative abundances of all other OTUs were very low. Notably, some of the OTUs were identified as *Buchnera* (the primary symbiont of aphids), *Tremblaya* and *Moranella* (the primary symbionts of mealybugs) and *Uzinura* (the primary symbiont of armoured scales). However, these OTUs were present in very low copy numbers or totally absent in some of the pooled samples (except for one sample of *Saissetia* sp., from Cyprus, in which *Tremblaya* comprised 15% of the total OTUs). Clustering data are presented in the Appendices S2 and S3.

3.1.2 | Fungi

Seventy-four and eighty fungal OTUs were mapped (after all the filtering steps) in the 1st and 2nd runs, respectively. The vast majority of the OTUs (92% and 99%, respectively) belonged to the phylum Ascomycota, most of which were from the class Sordariomycetes. A single OTU was highly abundant and accounted for 30%–90% of the fungal OTUs in all the coccid species, except for *Saissetia* sp. from Cyprus (Table 4). In the MEGAN analysis, the identity of this OTU was resolved with confidence level of >80% only to the order level (Hypocreales). In NCBI BLASTN and in the Fungal Barcoding Database (http://www.fungalbarcoding.org), the sequence of this OTU was >99% identical to various species of *Ophiocordyceps* (Ascomycota: Sordariomycetes: Hypocreales: Ophiocordycipitaceae) as well as *Hirsutella rhossiliensis*, which is the teleomorph of *Ophiocordyceps* (Ban, Sakane, & Nakagiri, 2015). As mentioned in the Materials and Methods, we also sequenced a 730–740 bp fragment of the RPB1 gene. While the 18S data were inconclusive about the identity of this OTU beyond the order level, the RPB1 gene sequences clearly

TABLE 4 Relative abundance (mean ± *SE*) of the main fungal OTUs amplified from the coccid species

	Coccid species						
	First run (n = 74 OTUs)			**Second run (n = 80 OTUs)**			
Fungal lineage	***M. mangiferae* (*n* = 3 pools)**	***C. floridensis* (*n* = 5 pools)**	***P. nigra* (*n* = 4 pools)**	***Saissetia* sp. (*n* = 4 pools)**	***Ceroplastes* sp. (*n* = 3 pools)**	***C. rusci* (*n* = 4 pools)**	***C. hesperidum* (*n* = 4 pools)**
Ophiocordyceps-allied fungus	33 ± 4%	50 ± 13%[a]	64 ± 14%[b]	—	89 ± 6%	72.5 ± 6%	73 ± 18%
Saccharomyces cerevisiae	33 ± 5%	—	19 ± 16%	—	—	—	—
Cladosporium sp.	—	26 ± 6.5%	—	10 ± 5%	6 ± 4%	21 ± 6%	—
Alternaria alternata	23 ± 2.5%	—	—	—	—	—	—
Wallemia spp.	—	10 ± 9%	—	—	—	—	—
Metarhizium anisopliae	—	—	—	80% ± 5%	—	—	21 ± 18%
All others (%)	11	14	17	10	5	6.5	6

Empty cells = the OTU represented (on average) less than 5% of the total OTUs in that coccid host. For the full list of OTUs and their relative abundances, refer to Appendix S2.

[a] One of the five pools was substantially different from the other four. If omitted, *Ophiocordyceps arborescens* percentage is 59 ± 10%.

[b] One of the four pools was substantially different from the other three. If omitted, *Ophiocordyceps arborescens* percentage is 78 ± 4.7%.

supported placement of these fungal symbionts within Ophiocordycipitaceae, based on MEGAN LCA analysis. Moreover, in the phylogenetic analyses (see section below), our sequences clustered with strong support in a clade of various *Ophiocordyceps* spp. Hence, here we conservatively refer to this OTU as *Ophiocordyceps*-allied fungus (OAF). In the mango soft scale, OAF and the yeast *Saccharomyces cerevisiae* codominated the fungal community (33% each, on average), followed by *Alternaria* (23%) (Table 4). The fungal community of *Saissetia* sp. (from Cyprus) differed from the other coccids, as it was dominated by *Metarhizium anisopliae* (Hypocreales: Clavicipitaceae) instead of OAF. *Metarhizium* represented about 21% of the fungal OTUs in *C. hesperidum* but only up to 1.5% in the rest of the coccids. A *Cladosporium* sp. (Capnodiales: Cladosporiaceae) was present in variable proportions in our samples, from <1% in *M. mangiferae* and *C. hesperidum* to 26% in *C. floridensis*. Lastly, a fungus of the genus *Wallemia* (Basidiomycota: Wallemiomycetes: Wallemiales) comprised 10% of the fungal community in *C. floridensis*, 4% in *P. nigra* but only 0%–1% in the other coccids. For the full list of OTUs and their relative abundances, refer to Appendix S1. Clustering data are presented in Appendices S4 and S5.

3.2 | Screening of OAF and Cladosporium sp

OAF was detected in all the individuals screened in the seven coccid species included in our study. *Cladosporium*, in contrast, was detected in varying proportions: 100% infection in *C. floridensis*, *C. hesperidum* and *P. nigra*, 75% infection in *C. rusci*, 62% infection in *M. mangifera*, 16% infection in *Saissetia* sp. (Cyprus) and 8% infection in the samples of *Ceroplastes* sp. from Spain.

3.3 | Phylogenetic analyses of OAF and their coccid hosts

We constructed phylograms using two nuclear genetic markers: RNA polymerase II subunit (RPB1) and 18S ribosomal DNA (18S rDNA). Each marker places the coccids' symbionts as close relatives of members of the genus *Ophiocordyceps* with strong support (Figures 1 and 2). 18S rRNA sequence places them within one of two previously recovered clades of yeast-like symbionts of leafhoppers (Nishino, Tanahashi, Lin, Koga, & Fukatsu, 2016), planthoppers (Noda, Nakashima, & Koizumi, 1995) and one species of aphid (Fukatsu & Ishikawa, 1996; marked with asterisks in Figure 2). Interestingly, OAF from our samples appear to also be closely related to *O. coccidiicola* and *O. cochlidiicola*, previously reported as coccid and moth parasites, respectively (Nikoh & Fukatsu, 2000).

We also constructed a phylogram of soft scale mitochondrial COI gene (Figure 3). Relationships among scale taxa within the genus *Ceroplastes* and related genera are well resolved at this marker and are not congruent with the relationships shown in the fungal phylogenies, although the latter are considerably less resolved. A symbiont phylogram constructed from concatenated RPB1 and 18S sequence alignments is shown alongside the host phylogram in Figure 3 to facilitate comparison. While some host and symbiont clades show similar relationships, for example *Saissetia* sp. and *Parasaissetia nigra*, others are clearly incongruent, for example *C. floridensis* and *M. mangiferae* (TREEMAP congruence analyses: $p = .98$ for the 18S vs. COI, $p = .18$ for RPB1 vs. COI). The alignments used to construct the phylogenies are attached in the Appendix S6.

3.4 | Fluorescence in situ hybridization (FISH)

Microscopic observations in *P. nigra* and *M. mangifera* using an OAF-specific probe revealed lemon-shaped cells, about 10 μm long and 5 μm wide, scattered in many parts of the body, either in the haemolymph and/or in the fat body. OAF was clearly visible also in eggs (Figure 4). Lemon-shaped cells were clearly observed also in the light-microscope, with a darker area visible in the centre. Many of them were multiplying by budding (Figure 5).

4 | DISCUSSION

In this study, we combined molecular, phylogenetic and microscopic approaches to provide the first detailed characterization of the microbial symbiotic complex associated with soft scale insects. Taken together, our results suggest that the primary symbiont of soft scale insects may be a fungus of the genus *Ophiocordyceps* (or at least closely related to this genus, OAF). This hypothesis is supported by the following evidence: (i) A primary symbiont would be common to the vast majority, if not all, species of the family, and would be the most dominant member of the microbial symbiotic community, for example *Buchnera aphidicola* in aphids (Jousselin et al., 2016; Lavy, Sher, Malik, & Chiel, 2015). Here, no specific bacterium was ubiquitous in all the tested coccid species in the high-throughput sequencing (Table 3), whereas OAF was prevalent in all the species and was the most dominant fungus (i.e., highest relative abundance) in five of the seven coccid species tested, constituting 50%–90% of all the fungal OTUs (Table 4). The fact that the coccid species included in this study were collected at different locations, times and host plants provide further support. (ii) Another characteristic of a primary symbiont is its presence in all individuals of a given taxon. Indeed, using specific primers, we found that all individuals of all the tested species carry OAF including in *Saissetia* sp. (collected in Cyprus), where a *Metharizium anisopliae* predominated the microbial community (Table 4). The mould fungus *Cladosporium*, which was also found in all the species in the high-throughput sequencing, was much less abundant (1%–26% of the total OTUs) than OAF, and was not detected in all individuals. (ii) By means of microscopy we found that OAF is present in the eggs, strongly suggesting vertical transmission. Obligate symbionts of Sternorrhyncha are strictly vertically transmitted, with the process starting in some cases at early stages of oogenesis or embryogenesis (Douglas, 2015). Nonetheless, this in itself is not proof for being an obligate primary symbiont, as secondary facultative symbionts are also vertically transmitted, as well as some parasites (Salem, Onchuru, Bauer, & Kaltenpoth, 2015).

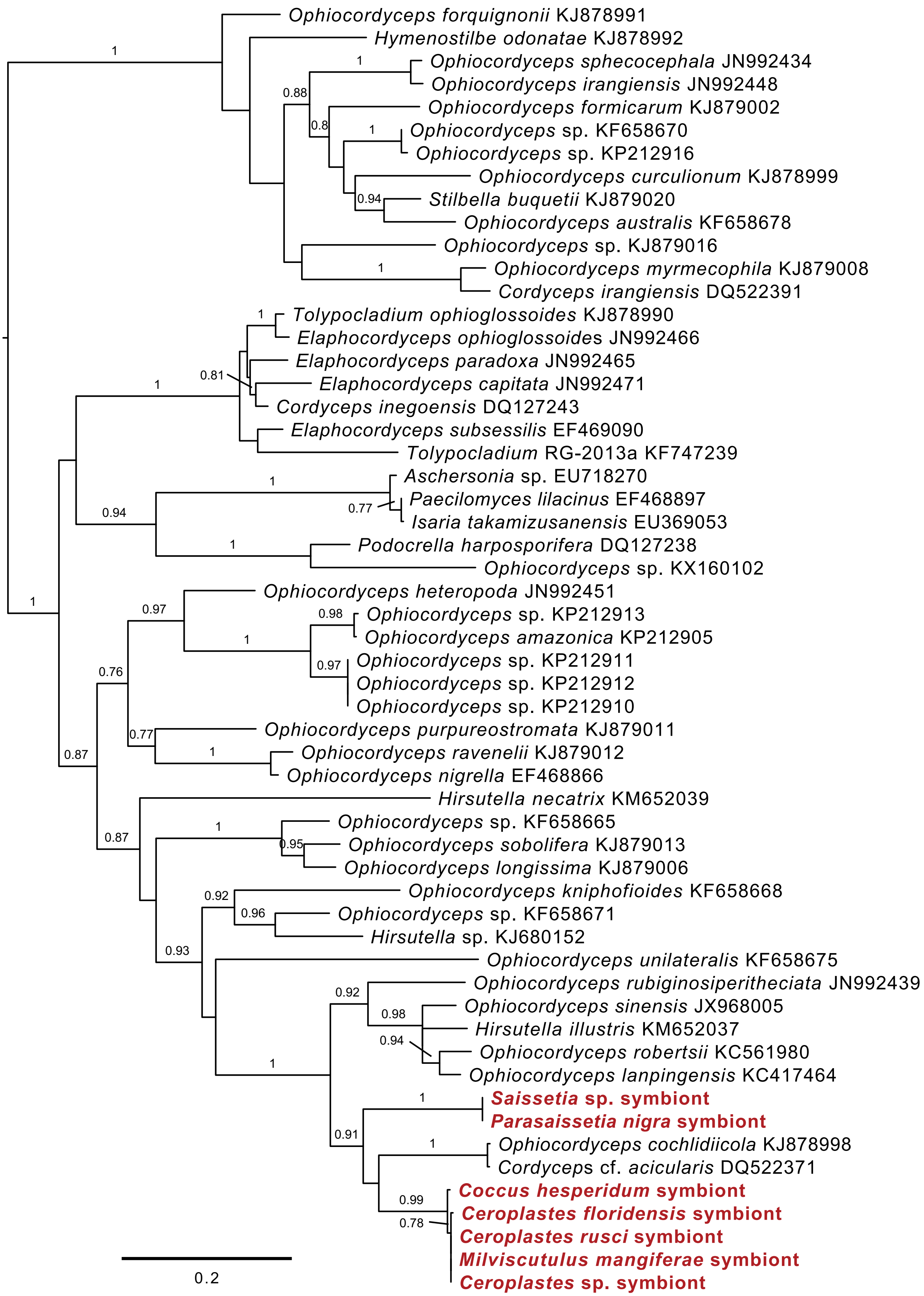
Ophiocordyceps forquignonii KJ878991
Hymenostilbe odonatae KJ878992
Ophiocordyceps sphecocephala JN992434
Ophiocordyceps irangiensis JN992448
Ophiocordyceps formicarum KJ879002
Ophiocordyceps sp. KF658670
Ophiocordyceps sp. KP212916
Ophiocordyceps curculionum KJ878999
Stilbella buquetii KJ879020
Ophiocordyceps australis KF658678
Ophiocordyceps sp. KJ879016
Ophiocordyceps myrmecophila KJ879008
Cordyceps irangiensis DQ522391
Tolypocladium ophioglossoides KJ878990
Elaphocordyceps ophioglossoides JN992466
Elaphocordyceps paradoxa JN992465
Elaphocordyceps capitata JN992471
Cordyceps inegoensis DQ127243
Elaphocordyceps subsessilis EF469090
Tolypocladium RG-2013a KF747239
Aschersonia sp. EU718270
Paecilomyces lilacinus EF468897
Isaria takamizusanensis EU369053
Podocrella harposporifera DQ127238
Ophiocordyceps sp. KX160102
Ophiocordyceps heteropoda JN992451
Ophiocordyceps sp. KP212913
Ophiocordyceps amazonica KP212905
Ophiocordyceps sp. KP212911
Ophiocordyceps sp. KP212912
Ophiocordyceps sp. KP212910
Ophiocordyceps purpureostromata KJ879011
Ophiocordyceps ravenelii KJ879012
Ophiocordyceps nigrella EF468866
Hirsutella necatrix KM652039
Ophiocordyceps sp. KF658665
Ophiocordyceps sobolifera KJ879013
Ophiocordyceps longissima KJ879006
Ophiocordyceps kniphofioides KF658668
Ophiocordyceps sp. KF658671
Hirsutella sp. KJ680152
Ophiocordyceps unilateralis KF658675
Ophiocordyceps rubiginosiperitheciata JN992439
Ophiocordyceps sinensis JX968005
Hirsutella illustris KM652037
Ophiocordyceps robertsii KC561980
Ophiocordyceps lanpingensis KC417464
Saissetia sp. symbiont
Parasaissetia nigra symbiont
Ophiocordyceps cochlidiicola KJ878998
Cordyceps cf. acicularis DQ522371
Coccus hesperidum symbiont
Ceroplastes floridensis symbiont
Ceroplastes rusci symbiont
Milviscutulus mangiferae symbiont
Ceroplastes sp. symbiont
1
1
0.88
0.8
1
0.94
1
1
0.81
1
1
0.77
0.94
1
0.97
0.98
1
0.97
0.76
0.77
1
0.87
1
0.87
1
0.95
0.92
0.96
0.93
0.92
0.98
0.94
1
1
0.91
1
0.99
0.78
0.2

FIGURE 1 A maximum-likelihood phylogram constructed from RNA polymerase II subunit (RPB1) sequences of fungi. The symbiotic fungal taxa amplified from our samples are shown in red, bolded text. Branches are labelled with SH-like support values >0.75. Branch tips are labelled with NCBI accession numbers and taxonomic names [Colour figure can be viewed at wileyonlinelibrary.com]

Zacchi and Vaughan-Martini (2002) isolated seven species of fungi from the coccid *Saissetia oleae* (from Italy). Five of these seven species were found also in our deep sequencing, but in very low, even negligible, copy numbers and the other two were absent in our samples. Likewise, members of the Enterobacteriaceae, which Rosenblueth et al. (2012) previously found in coccids, were amplified in negligible copy numbers in our study. These discrepancies are possibly due to the use of different probes in each study. The *Wolbachia* and *Rickettsia* that were found in high relative abundance in *C. floridensis* and *M. mangiferae*, respectively, are likely secondary symbionts of these species, as they often are in other sternorrhynchan families (e.g., Chiel et al., 2007; Singh, Kumar, Thomas, Ramamurthy, & Rajagopal, 2013; Zytynska & Weisser, 2016). Among the many bacterial OTUs that were amplified from our samples, there were also sequences of *Uzinura diaspidicola*, *Buchnera aphidicola*, *Tremblya princeps* and *Moranella endobia* (Appendix S1). The presence of these bacteria is surprising as they are PS of other sternorrhynchan insects with strict fidelity to their hosts. Nonetheless, it should be noted that: (i) with the exception of one *Saissetia* sample, these OTUs were amplified in negligible numbers (and totally absent in some of the samples); (ii) attempts to amplify *Tremblaya* with specific primers yielded negative results. It is therefore likely that these are artefacts.

Typically, due to the mutual dependence, primary symbionts codiversify with their hosts, resulting in congruent phylogenies of both parties. However, in our study, the phylogenies of OAF and their coccid hosts were incongruent. Host and obligate inherited symbiont phylogenies may be incongruent due to insufficient signal or inappropriate evolutionary models of nucleotide substitution (Clark, Moran, Baumann, & Wernegreen, 2000). Another possible explanation is that primary symbionts have been acquired independently, such the timescale in which congruence is being examined is inappropriate. For example, Husnik and McCutcheon (2016) recently showed that mealybugs have repeatedly replaced their obligate symbionts with new ones, by independently acquiring bacteria allied with *Sodalis*. Interestingly,

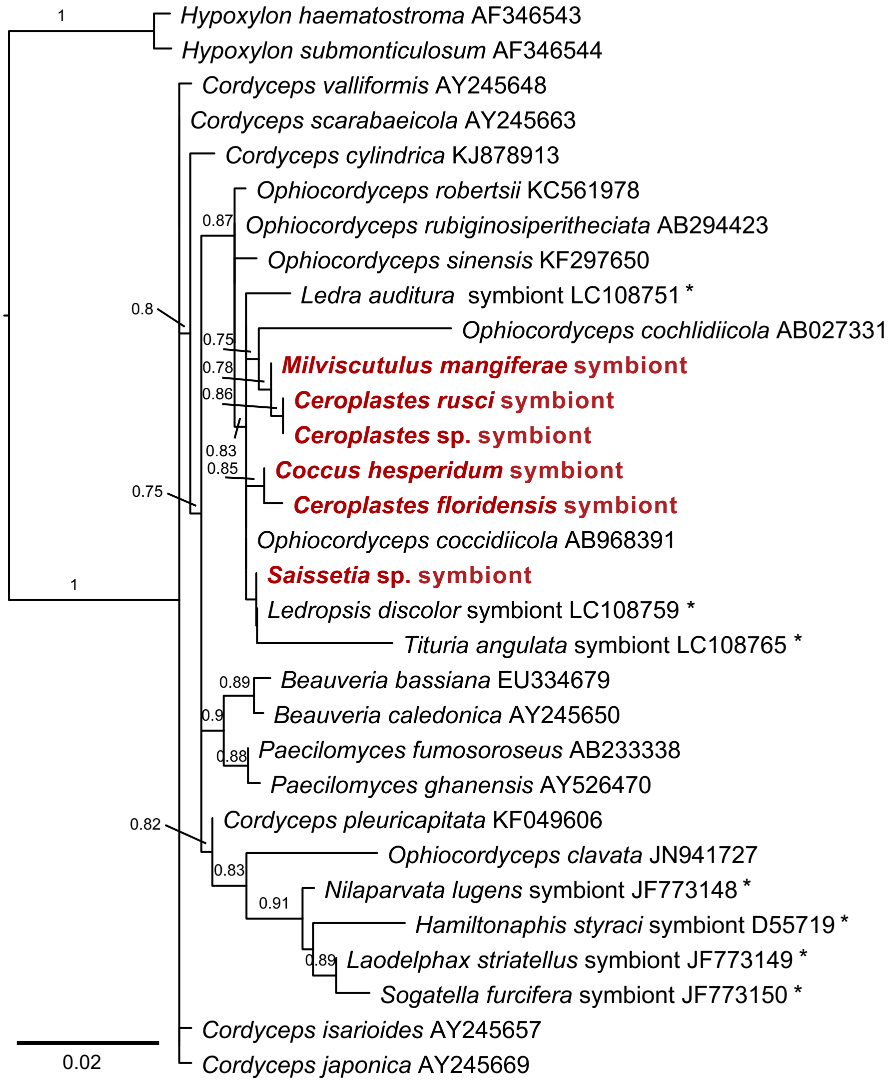

FIGURE 2 A maximum-likelihood phylogram constructed from 18S ribosomal DNA sequences of fungi. The symbiotic fungal taxa reported here are shown in red, bolded text. Previously described yeast-like symbionts are marked with an asterisk. Branches are labeled with SH-like support values >0.75. Branch tips are labelled with NCBI accession numbers and taxonomic names. The phylogram is rooted with members of the Pyrenomycete genus, *Hypoxylon* [Colour figure can be viewed at wileyonlinelibrary.com]

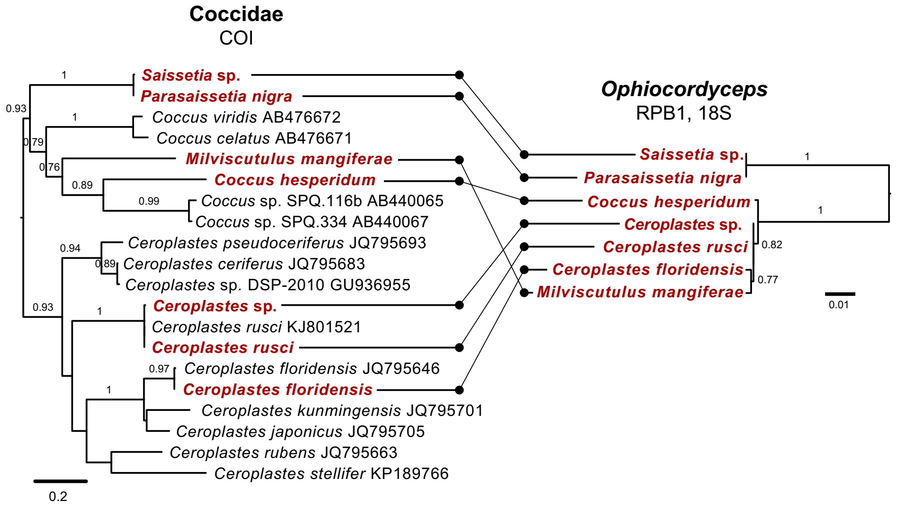

FIGURE 3 A maximum-likelihood phylogram constructed from COI sequences of soft scale insects shown opposite a phylogram of their *Ophiocordyceps* symbionts, constructed from two concatenated symbiont genes: RPB1 and 18S-ITS rDNA. Solid lines connect the tips of hosts to their respective symbionts. Hosts and symbionts reported here are shown in red, bolded text. Branches are labeled with SH-like support values >0.75. Branch tips are labelled with NCBI accession numbers and taxonomic names [Colour figure can be viewed at wileyonlinelibrary.com]

Ophiocordyceps symbionts in ledrine planthoppers also appear to have been acquired independently (Nishino et al., 2016). One additional option is that OAF has been acquired by one or a few coccid species and then horizontally transferred to other lineages.

With the exception of one lineage of aphids (Gibson & Hunter, 2010; Hongoh & Ishikawa, 2000; Vogel & Moran, 2013), each family in the large suborder Sternorrhyncha relies on a bacterial primary symbiont(s) to supplement its dietary needs, including closely related families within the diverse superfamily Coccoidea (Baumann, 2005; Dhami, Turner, Deines, Beggs, & Taylor, 2012; Douglas, 2015; Michalik, Szklarzewicz, Kalandyk-Kołodziejczyk, Jankowska, & Michalik, 2016; Moran et al., 2008; Rosas-Pérez, Rosenblueth, Rincón-Rosales, Mora, & Martínez-Romero, 2014). Hence, Coccidae is the only family so far in the Sternorrhyncha (or at least the two major subfamilies studied here: Ceroplastinae and Coccinae) which seems to be dominated by fungal rather than bacterial symbionts. It will be useful to screen other coccid subfamilies to determine how widespread the symbiosis with OAF is.

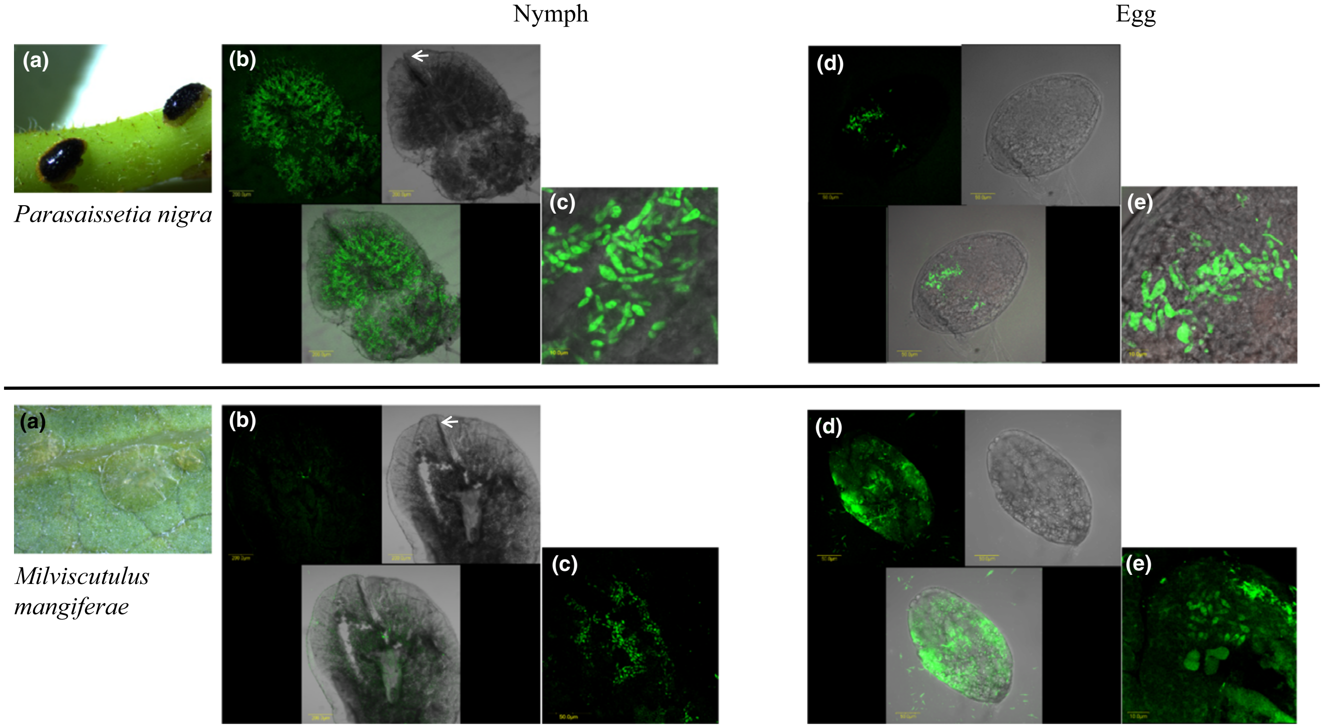

FIGURE 4 FISH staining of *Ophiocordyceps*-allied fungus in a nymph and an egg of *Parasaissetia nigra* (upper panel) and *Milviscutulus mangiferae* (lower panel). In both panels, from left to right: (a) a picture of *P. nigra* nymphs (upper panel) and *M. mangiferae* (lower panel); (b) nymph: upper left picture—*Ophiocordyceps* fluorescence channel, upper right picture—bright field channel, lower picture—merge of the two channels. Scale bar = 200 μm; (c) zoom in on (b) to obtain high magnification of *Ophiocordyceps* cells in the nymph's body. Scale bar = 10 μm; (d) egg: upper left picture—*Ophiocordyceps* fluorescence channel, upper right picture—bright field channel, lower picture—merge of the two channels. Scale bar = 50 μm; (e) zoom in on (d) to obtain high magnification of *Ophiocordyceps* cells in the egg. Scale bar = 10 μm. White arrows point to the anal clefts [Colour figure can be viewed at wileyonlinelibrary.com]

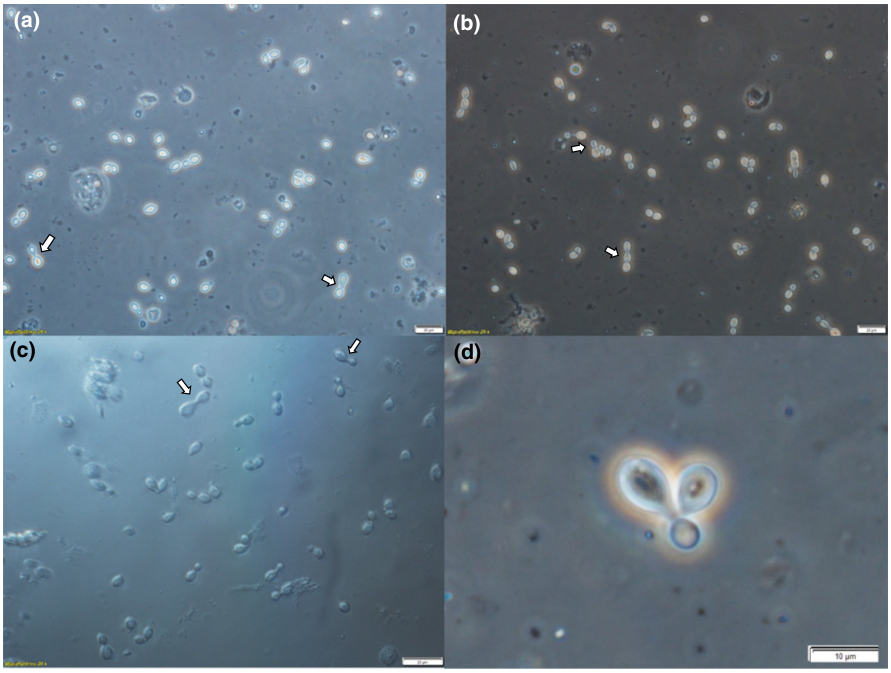

FIGURE 5 Fungal cells in a nymph of *Parasaissetia nigra*, as seen in light microscopy. (a-c) view with magnification of ×200, scale bar = 20 µm. Arrows point to budding fungal cells; (d) Zoom in (magnification ×500, scale bar = 10 µm) on fungal cells to show the darker area in the cells' center [Colour figure can be viewed at wileyonlinelibrary.com]

Ophiocordyceps—the largest genus of hypocrealean fungi—comprises many species that parasitize insects (Pontoppidan, Himaman, Hywel-Jones, Boomsma, & Hughes, 2009; Salgado-Neto, Valmorbida, Guedes & Blume, 2015; Sasaki, Miyamoto, Yamamoto, Tamai, & Yajima, 2012; Wang & Yao, 2011). The family Ophiocordycipitaceae, together with two more entomopathogenic families—Clavicipitaceae and Cordycipitaceae—collectively represent the most diverse clade of arthropod pathogens in Hypocreales, with a relatively ancient origin (Araújo & Hughes, 2016; Sung et al., 2007). Kobayasi and Shimizu (1978) have reported the finding of pathogenic *Cordyceps/Hirsutella* spp. in two coccid hosts and termed them *C. coccidiicola* and *H. coccidiicola*, respectively (currently unified to a single species, *O. coccidiicola*; Mycobank # 311794 [http://www.mycobank.org/]). However, apart from this publication there is hardly any information about *O. coccidiicola*. It was used in several phylogenetic studies (Ban et al., 2015; Nikoh & Fukatsu, 2000; Sung et al., 2007; Tian et al., 2010), but it is not clear what is the source of material in these studies (i.e., hosts' identity and whether it was collected from fruiting bodies that grew out from their coccid hosts) and how abundant is this pathogenic fungus in nature. The 18S rRNA phylogeny (Figure 2) clearly shows that *O. coccidiicola* is closely related to the OAF amplified in our study. It should be noted that all the samples of soft scale insects we collected looked healthy and we never found any samples showing disease symptoms or fruiting bodies, including the two species which we reared continuously, *C. floridensis* and *M. mangifera*. Further research is required to know if *O. coccidiicola* and the OAF found in our study represent the same species, or two closely related species. To the best of our knowledge, there are only three other reports of fungi from this clade (Ophiocordycipitaceae—Clavicipitaceae—Cordycipitaceae) that have become mutualists, or at least nonpathogenic symbionts: *Ophiocordyceps* in some leafhoppers (Hemiptera: Auchenorrhyncha: Cicadellidae) (Nishino et al., 2016) and YLS (of the Clavicipitaceae) in three planthopper species (Hemiptera: Auchenorrhyncha: Delphacidae) as well as in a tribe of aphids (Gibson & Hunter, 2010; Hongoh & Ishikawa, 2000; Suh, Noda, & Blackwell, 2001). Interestingly, the closest relatives of the aphid's *C. brasiliensis* YLS were found to be fungi of the genera *Cordyceps* and *Metarhizium*, of the families Cordycipitaceae and Clavicipitaceae, respectively (Vogel & Moran, 2013). In our study, *Metarhizium anispoliae* was the dominant fungal symbiont in the *Saissetia* sp. population from Cyprus, thereby being markedly different from the other tested coccid hosts, from Israel and Spain. While the reason for this difference is not clear and should be further studied, it appears that lineages of these hypocrealean fungi have undergone a shift from insect pathogens to mutualists (or nonpathogens, or at least sublethal pathogens). Other fungi that were found in our study—*Cladosporium*, *Alternaria* and *Wallemia*—were also reported from a mealybug (Sternorrhyncha: Pseudococcidae, a sister taxon to Coccidae) (Iasur-Kruh et al., 2015). In the light of our results, we postulate that these fungi are facultatively or opportunistically associated with soft scales. *Alternaria alternata* is a well-known plant pathogen; thus, it may have been acquired by *M. mangiferae* via feeding on an infected host plant. *Cladosporium* and *Wallemia* are ubiquitous mould fungi that may have been acquired by some of the coccids from the environment.

How has the lineage of OAF shifted from a parasitic to a nonparasitic lifestyle? At this point we can only speculate, similarly to Nishino et al. (2016), that at some point in the evolutionary history an ancestral parasitic *Ophiocordyceps* infected an ancestral soft scale insect (Sung, Poinar, & Spatafora, 2008), lost its virulence and/or horizontal transmission ability, got trapped and became domesticated and vertically transmitted. With time, the parasite may have turned into an obligate mutualist possibly replacing a bacterial PS (Sachs, Essenberg, & Turcotte, 2011). However, it should be underlined that currently we do not know if OAF is a mutualist or merely a nonpathogenic symbiont.

Through microscopy, we identified OAF cells scattered throughout many parts of the host's body, either in the haemolymph or in fat body tissues, resembling the recent report of Nishino et al. (2016). Unlike this scattered distribution, bacterial PS in other sternorrhynchan families (aphids, whiteflies etc.) are typically localized inside specific cells—bacteriocytes—that are aggregated into organ-like bacteriomes (Douglas, 2015). Yet, this characteristic neither supports nor refutes the possible role of OAF as a PS. The YLSs of the brown planthopper are found in abdominal fat body cells (Chen et al., 1981; Cheng & Hou, 2001), which resembles the distribution pattern we see in our coccid samples, although at this point we cannot determine unambiguously whether OAF is intra- or extracellular. In both nymphs and eggs OAF exhibits a single-celled, yeast-like anamorph growth stage, very similar to the fungi found in other species of Coccidae (Steinhaus, 1955; Tremblay, 1997). It looks like OAF stopped producing stromata, remaining in an anamorph-like state, sacrificing its filamentous morphology, undergoing evolutionary convergence that has left it resembling Saccharomycetes, resembling (again) the YLS of planthoppers (Cheng & Hou, 2001). The presence of OAF in the eggs (Figure 4) strongly suggests vertical transmission of this symbiont from mother to offspring. The common transmission mechanism of PS in other sternorrhynchan families is trans-ovarial, but in coccids the mechanism may be different due to: (i) identity—OAF is a fungus, not a bacterium; (ii) distribution—bacterial PS in other sternorrhynchan insects are intracellular and are aggregated in bacteriomes, whereas OAF may be extracellular and scattered throughout most of the host's body. Thus, further research is needed to elucidate the exact transmission mechanism of OAF in coccids.

How the OAF symbiont affects its soft scale insects is currently unknown. The closely related YLS from a planthopper synthesizes sterols and amino acids and also recycles nitrogen (Douglas, 2016; Fan et al., 2015; Vera-Ponce de León, Sanchez-Flores, Rosenblueth, & Martínez-Romero, 2016). The YLS of the aphid *Cerataphis brasiliensis* seems to retain a broader metabolic repertoire, including synthesis of essential and nonessential amino acids, lipids and chitin, and nitrogen metabolism (Vogel & Moran, 2013). Genome sequencing of the coccid symbiont may shed more light on its ecological role. Additionally, in order to test whether OAF is indeed an obligate symbiont, attempts should be made to eliminate it from the host, in order to see if aposymbiotic coccids can survive, develop and reproduce.

To summarize, the work reported here sheds a light on the microbial community of soft scale insects. The coccid-OAF symbiosis incorporates two fascinating evolutionary lifestyle switches: a large family of insects that, unlike its relatives, is dominated by a fungal rather than a bacterial symbiont; a fungal symbiont that evolved from a lineage of insect pathogens.

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DATA ACCESSIBILITY

The following sequences were deposited to NCBI GenBank database: coccids' COI- KY924793 to KY924799; OAF RPB1- KY924800 to KY924806; OAF 18S-ITS- KY924807 to KY924813; OAF 18S-KY924814 to KY924819. The raw data of the high-throughput sequencing were submitted to SRA database of NCBI under project no. PRJNA392317, accession numbers SRR5786053 to SRR5786099.

AUTHOR CONTRIBUTIONS

P.G.P. designed and performed the research, analysed data and wrote the manuscript; M.J.B. performed the research, wrote the manuscript and analysed data; M.L. analysed data; A.M. analysed data; Y.B.D. identified the insects; N.M.D. performed the research; S.J.P. designed the research and analysed the results; L.I.K. designed the research; E.C. designed the research, analysed the data and wrote the manuscript. All authors reviewed the manuscript.

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