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Endosymbiotic microorganisms of scale insects

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Abstract

The evolutionary and ecological success of insects is largely due to their associated bacteria and fungi that expand their metabolic capacities or allow them to resist stress or parasites. Some of these associations possibly originated hundreds of millions of years ago and have resulted in such interdependence that in some cases the insect and bacteria may not exist separately. This has also led to a significant reduction in the genome size of bacterial symbionts and to the maternal transfer of symbionts to progeny. The study of insect symbionts has recently gained great interest and some of the biological functions of symbionts within hosts have been identified. Scale insects or cochineals feed on the sap of plants, which is rich in carbon but poor in nitrogen and so they require symbionts to compensate for diet deficiencies. Some scale insects are devastating crop pests. In this article, we review the symbionts of some scale insects focusing on carmine- and wax cochineals, which have commercial, art and craft interest. In the cochineals studied we found diverse microbial communities that can synthesize amino acids, vitamins, fix nitrogen or recycle the waste products of nitrogen metabolism.

Key Words: cochineal, *Dactylopius*, nitrogen fixation, reduced-genomes, symbiotic fungi.

Microorganismos endosimbiontes de insectos escama

Resumen

Parte del éxito evolutivo y ecológico de los insectos se atribuye a las bacterias y hongos asociados a ellos que amplían sus capacidades metabólicas o les permiten resistir estrés o parasitosis. Las asociaciones posiblemente se originaron hace cientos de millones de años y el resultado es una interdependencia que, en algunos casos, insecto y bacteria no pueden existir separadamente, lo que ha llevado a una reducción significativa de los genomas de los simbiontes bacterianos y a la transferencia por vía materna de éstos a la progenie. Recientemente, el estudio de los simbiontes de insectos ha cobrado gran interés y se han identificado algunas de sus funciones biológicas dentro de los hospederos. Los insectos escama o cochinillas se alimentan de la savia de las plantas, por lo que requieren simbiontes para compensar las deficiencias de su dieta, rica en carbono pero pobre en compuestos nitrogenados. Algunas de las plagas más agresivas de los cultivos agrícolas son los insectos escama. En este artículo revisamos los simbiontes de la cochinilla del carmín y de la laca, de gran interés comercial y artesanal. En las cochinillas que se estudiaron encontramos diversas comunidades microbianas con la capacidad de sintetizar aminoácidos, vitaminas, fijar nitrógeno o reciclar los productos de desecho del metabolismo nitrogenado.

Palabras Clave: cochinilla, *Dactylopius*, fijación de nitrógeno, genomas reducidos, hongos simbióticos.

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Introduction

Insecta (insects) is the most successful class of animals on Earth. Seemingly, associated microorganisms have allowed insects to adapt to almost all ecosystems on the planet (Janson *et al*., 2008; Feldhaar, 2011). Insects with restricted diets, such as hematophagous insects that feed on blood (Rio *et al.*, 2016) or phytophagous insects that feed on sap, bark, leaves or seeds (Hansen & Moran, 2014) face nutritional deficiencies of nitrogen, vitamins or lipids. Bacteria or fungi can provide insects with nutrients or metabolites that are deficient in their diet (Douglas, 2015; Harris *et al.*, 2010; Hansen & Moran, 2014). Symbionts may inhabit inside insect cells and thus they have been designated "endosymbionts".

More than 50 years ago, Buchner (1965) published his microscopic observations in a compendium of insect-associated bacteria. In his book "Endosymbiosis of Animals with Plant Microorganisms", he described the morphology of bacteria that inhabit different tissues of phytophagous insects and he assumed that many insect bacteria have their origins in plants. Earlier microscopic studies of scale insect symbionts (or true cochineals) were performed by Walczuch in 1932 and Buchner included them in his 1965 review. Nowadays there is a large research interest in microorganisms associated with insects and several types of insect symbionts are known:

1) Primary or essential endosymbionts. They reside inside specialized cells that are called bacteriocytes in the case of insect cells containing bacteria (Baumann *et al.,* 2000), or mycetocytes when they harbor fungi. A transcriptional factor essential for bacteriocyte development was discovered in insects (Matsuura *et al.,* 2015) and this sets the basis to further understand the evolution of intracellular symbiosis in insects. Bacteriocytes are sometimes clustered in structures called bacteriomes (Fig. 1) that are found in distinct insect species (Baumann, 2005). Bacteriome location and numbers are variable. There are often two bacteriomes per insect, but there can be one to four. They are located in different regions of the insect. In the so-called "nutritional symbiosis", primary endosymbionts provide their hosts with nutrients that are deficient in the insect diet, such as essential amino acids and vitamins (Baumann, 2005; Moran *et al*., 2005).

2) Facultative or secondary endosymbionts. They are not essential for the survival of their host. They may also be found inside bacteriocytes. Some of them protect the insect from the attack of wasps, nematodes, fungi, bacteria and viruses (Xie *et al*., 2014; Jaenike *et al.*, 2010; Łukasik *et al.*, 2013; Chrostek *et al*., 2013). They can also help insects in the degradation of plant toxic compounds such as monoterpenes or alkaloids, allowing insects to colonize or parasite plants. Secondary endosymbionts can protect insects from high temperatures (Tsuchida *et al*., 2004; Burke *et al*., 2010). Others can manipulate the reproductive capacity of the host (Werren *et al*., 2008). It is known that

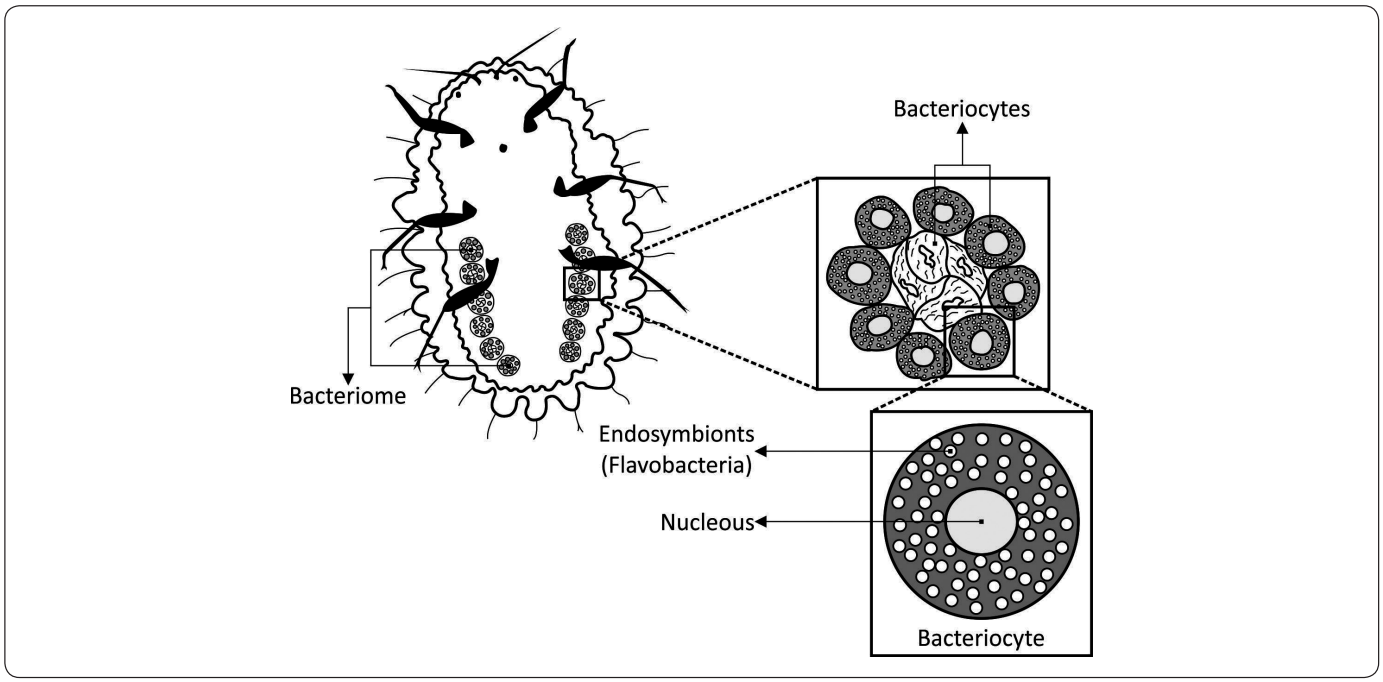

Figure 1. Diagram of a dorsal section of a scale insect (Monophlebidae and Coelostomidiidae families), in which the location of bacteriomes and endosymbionts are shown. Based on diagrams of Walczuch (1932) and FISH experiments of Matsuura *et al*., (2009), Dhami *et al*., (2012), and Rosas-Pérez *et al*., (2014).

secondary symbionts alter cellular functions of hosts, ranging from signal transduction to apoptosis (Bentley *et al*., 2007; Ikeya *et al*., 2009). When bacteria do not confer an increased fitness, they may be lost without any negative consequence for the insect (Moran *et al*., 2005).

3) Gut symbionts in crypts. They have been found only in some insects of the superfamily Pentatomomorpha, which includes bugs. They are often transmitted vertically by colonization of eggs, coprophagia or symbiotic capsules, but some species can be acquired from the environment by food as some of them are free-living. They also provide nutrients, degrade polymeric and toxic plant compounds and recycle nitrogen (Kikuchi *et al*., 2008).

4) Gut symbionts. They contribute to the breakdown of complex chemical compounds and toxins. Gut symbionts may be vertically transferred from mother to offspring by fecal contamination, coprophagy and trophallaxis that leads to the inoculation of the gut bacteria to the progeny (Salem *et al*., 2015 ; Abdul Rahman *et al.*, 2015). However, intestinal microbiota may be modified by changes in the diet. Except for termites, the number of species in insect microbiota is smaller than that of mammals. Diversity may vary in females and males and this is the case in monarch butterflies (Servín -Garcidueñas *et al*., 2014 and unpublished data). Using a functional approach, we have reported a meta-analysis of insect gut proteobacteria (Degli Esposti & Martínez-Romero, 2017).

In this review, we will discuss primary and secondary endosymbionts, leaving aside gut and crypt bacteria. In general, a single insect hosts two or more different species of endosymbionts in co-symbiosis. Inheritability and reduced genomes are the most outstanding features of endosymbionts. Most endosymbionts cannot be cultured on standard laboratory media and to acknowledge this, most endosymbionts are designated "*Candidatus*" before the name of the species. To simplify descriptions here, we will not use the "*Candidatus*" designation. Some endosymbionts share their evolutionary history with their hosts. Maternal transfer, reduced genomes and co-divergence will be reviewed in this article focusing on scale insect endosymbionts. In addition, we present the diversity of bacterial and fungal symbionts found in two models of scale insects studied in our laboratory.

Maternal transfer of bacterial symbionts

Vertical transfer refers to the transmission of endosymbionts from mothers to their offspring. It is a mechanism that ensures the permanence of these bacteria in insect populations. Endosymbionts are transmitted from the time the embryos are formed and when insects are born they already carry some bacteria (McFall-Ngai, 2002). By being transferred from one generation to another, bacteria can remain associated with insect species for millions of years. Of all endosymbionts studied so far, it is estimated that the oldest is *Sulcia muelleri* living for over 260 million years in a group of insects that includes cicadas, leafhoppers and planthoppers (Moran *et al*., 2005). *Sulcia muelleri* co-exists with different co-symbionts in different

Co-symbiont	Phylum	Genome size (kb)	Host (Family)	Reference
Nasuia deltocephalinicola	Betaproteobacteria *	112	*Macrosteles quadrilineatus* (Cicadellidae)	Bennett & Moran, 2013
Zinderia insecticola	Betaproteobacteria	208	*Clastoptera arizonana* (Clastopteridae)	McCutcheon & Moran, 2010
Vidania fulgoroideae **	Betaproteobacteria	ND	*Oliarus filicicola* (Cixiidae)	Bressan & Mulligan, 2013
Purcelliella pentastirinorum **	Gammaproteobacteria	ND	*Oliarus filicicola* (Cixiidae)	Bressan & Mulligan, 2013
Baumannia cicadellinicola	Gammaproteobacteria	686	*Homalodisca coagulata* (Cicadellidae)	Wu *et al*., 2006
Arsenophonus ***	Gammaproteobacteria	ND	*Macrosteles laevis* (Cicadellidae)	Kobialka *et al.,* 2016
Sodalis-like PSPU	Gammaproteobacteria	1,380	*Philaenus spumarius* (Aphrophoridae)	Koga & Moran, 2014
Hodgkinia cicadicola	Alfaproteobacteria	144	*Diceroprocta semicincta* (Cicadidae)	McCutcheon *et al.*, 2009

* All betaproteobacteria co-symbionts might have the same origin in Auchenorrhyncha (as suggested by Bennett & Moran, 2013).
** *Vidania* and *Purcelliella* can be both co-symbionts of *Sulcia* in the same insect.
*** *Arsenophonus* resides inside *Sulcia* cells. This insect also harbours a *Nasuia* co-symbiont
ND No data available.

Table I. Examples of co-symbionts (with phylum indicated) of *Sulcia muelleri*, the primary endosymbionts of Auchenorrhyncha.

insects (Table I) and a metabolic complementation between the different co-symbionts that inhabit an insect seems to exist (McCutcheon & Moran, 2010). This means that if the genes that encode the biosynthetic pathway of a metabolite do not exist in one of the symbionts, they are probably present in other symbiont bacteria of the same insect.

Some bacteria may have recently become endosymbionts acquired from the transfer of endosymbionts from another insect (horizontal transfer) or from the environment. When an insect acquires a new endosymbiont bacterium, the insect may gain new characteristics, such as the ability to feed on another plant (Moran, 2007). It has recently been described that endosymbionts such as *Wolbachia* can be transferred between insects through the plants on which the insects feed (Li *et al*., 2017; Sintupachee *et al*., 2006). Besides diet, endosymbionts can be exchanged by parasitoid wasps that transmit them when they sting different insects (Ahmed *et al*., 2015). In different species of aphids, males may transfer symbiotic bacteria to females during intercourse (Moran & Dunbar, 2006).

Endosymbiont reduced genomes and gene transfer to insect hosts

Since endosymbionts live inside insects and have been transferred vertically for millions of years, they display different characteristics than free-living bacteria. Endosymbionts have lost most of their genome, sometimes retaining even less than 10% of the original one (Bennett & Moran, 2013; Martínez-Cano *et al*., 2015). They no longer retain genes essential for free living. Genes preserved include those under selective pressure and those related to the synthesis of the nutrients that the insect requires. For this reason, endosymbionts of insects with similar diets have retained similar genes, in an example of convergent evolution.

Experimental evolution studies performed in the laboratory have shown that an initial massive loss of DNA blocks occurs rapidly in bacteria (Nilsson *et al*., 2005). Comparison between the genomes of endosymbionts and those of free-living bacteria suggests that after deletions have occurred there is a proliferation of repetitive elements that causes chromosomal rearrangements. Later on, there is a slow accumulation of deleterious mutations. Non-functional genes are then lost (Moran, 2003; Manzano-Marín & Latorre, 2016). The presence of pseudogenes in the genomes of some endosymbionts suggests that their genome is still in a reduction process.

Most endosymbionts have A + T rich genomes (Table II). They also have high rates of non-synonymous nucleotide substitutions in protein-encoding genes. Endosymbionts live in restricted environments that prevent recombination with other bacteria. The strict vertical transmission has an effect on the population structure since there is a bottleneck with a limited number of bacteria passing to the next generation. Successive bottlenecks during evolution reduce bacterial diversity and increase their mutation fixation rate (Moran, 2007).

In the evolutionary history of insects, it seems that endosymbiont replacements have occurred several times (Moran *et al*., 2005; Gruwell *et al.*, 2010; Toju *et al.*, 2013; Sudakaran *et al.*, 2017) and this was shown experimentally with a secondary endosymbiont taking the place of a primary endosymbiont (Koga *et al.*, 2003). The elimination of endosymbionts is also evident when bacterial genes of endosymbionts no longer present are found in the genomes of insects. It is presumed that these genes were transferred laterally by past endosymbionts that are no longer currently living in the insects, but have left a trace of their existence. Some insect genes of bacterial origin are transcribed in the bacteriome and may be required for the endosymbiotic relationship (Husnik *et al.*, 2013; Sloan *et al.*, 2014.). Lateral gene transfer from bacteria to the insect has been studied in four species of the suborder Sternorrhyncha (aphids, psyllids, whiteflies and mealybugs) (Nikoh & Nakabachi, 2009; Husnik *et al.*, 2013; Sloan *et al.*, 2014). Not all the transferred genes are the same in each species but they encode similar functions, such as peptidoglycan and amino acid biosynthesis (Sloan *et al*., 2014). Large genome fragments of *Wolbachia* have been found transferred to the chromosomes of mosquitoes (*Aedes aegypti*), vinegar fruit fly (*Drosophila ananassae*), beetles (*Callosobruchus chinensis*) and several species of parasitoid wasps of the genus *Nasonia*. Some of these genes have been found to be functional (Kondo *et al*., 2002; Dunning Hotopp *et al*., 2007; Nikoh *et al.,* 2008; Klasson *et al*., 2009; Choi *et al.*, 2015).

Extreme examples of reduced genomes are found in *Nasuia* and *Vidania* endosymbionts with genomes of 112 and 119 kb, respectively (Bennett *et al*., 2016). Endosymbionts with extreme genome reduction may need the insect contribution of essential enzymes or proteins as occurs in mitochondria. In some cases, several of these proteins are encoded by genes that were transferred from endosymbionts to the insect (Sloan *et al*., 2014). Remarkably, it was shown that a protein encoded by a gene of bacterial origin in an aphid, is transported to the endosymbiont (Nakabachi *et al*., 2014).

Shared evolutionary histories among symbionts and hosts

If endosymbiotic bacteria and their hosts remain associated for a long evolutionarily time, they can co-speciate or co-diverge together. In those cases, there is a phylogenetic congruence that can be traced back to a single infection event (Baumann *et al*., 2000).

Occasionally endosymbionts have evolutionary histories different from the hosts. This is more frequent in secondary co-symbionts due to endosymbiont losses, new acquisitions of environmental bacteria or lateral transfers from one insect species to another. Furthermore, it has been observed that secondary

Endosymbiont (Class)	Host (FAMILY)	Genome size (pb)	%GC	Number of pseudogenes	Function	Location	Reference
Walczuchella monophlebidarum (Flavobacteria) [P]	*Llaveia axin axin* (MONOPHLEBIDAE)	309,299	32.6	27	Provides amino acids	Bacteriome & ovaries	Rosas-Pérez *et. al*. (2014)
Sodalis TME1 (Gammaproteobacteria) [S]		3,400,000	55.6	ND	Provides amino acids & recycles uric acid	Bacteriome & ovaries	Rosas-Pérez *et. al*. (2017)
Tremblaya phenacola (Betaproteobacteria) [P]	*Phenacoccus avenae* (PSEUDOCOCCIDAE)	170,756	42.2	178	Provides amino acids & vitamins	Bacteriome	Husnik & McCutcheon (2016)
Tremblaya princeps (Betaproteobacteria) [P]	*Maconellicoccus hirsitus* (PSEUDOCOCCIDAE)	138,415	61.8	136	Provides amino acids & vitamins	Bacteriome	Husnik & McCutcheon (2016)
Doolittlea endobia (Gammaproteobacteria) [S]		834,723	44.2	564	Provides amino acids & vitamins	*Tremblaya* cytoplasm	Husnik & McCutcheon (2016)
Tremblaya princeps (Betaproteobacteria) [P]	*Ferrisia virgata* (PSEUDOCOCCIDAE)	141,620	58.3	132	Provides amino acids & vitamins	Bacteriome	Husnik & McCutcheon (2016)
Gullanella endobia (Gammaproteobacteria) [S]		938,041	28.9	461	Provides amino acids & vitamins	*Tremblaya* cytoplasm	Husnik & McCutcheon (2016)
Tremblaya princeps (Betaproteobacteria) [P]	*Planococcus citri* (PSEUDOCOCCIDAE)	138,927	58.8	125	Provides amino acids & vitamins	Bacteriome	Husnik & McCutcheon (2016)
Moranella endobia (Gammaproteobacteria) [S]		538,294	43.5	419	Provides amino acids & vitamins	*Tremblaya* cytoplasm	Husnik & McCutcheon (2016)
Tremblaya princeps (Betaproteobacteria) [P]	*Pseudococcus longispinus* (PSEUDOCOCCIDAE)	144,042	58.9	134	Provides amino acids & vitamins	Bacteriome	Husnik & McCutcheon (2016)
2 *Sodalis*-allied (Gammaproteobacteria) [S]		8,190,816	53.9	ND	Provides amino acids & vitamins	*Tremblaya* cytoplasm	Husnik & McCutcheon (2016)
Tremblaya princeps (Betaproteobacteria) [P]	*Tryonimus perrisii* (PSEUDOCOCCIDAE)	143,340	57.8	116	Provides amino acids & vitamins	Bacteriome	Husnik & McCutcheon (2016)
Hoaglandella endobia (Gammaproteobacteria) [S]		628,221	42.8	510	Provides amino acids & vitamins	*Tremblaya* cytoplasm	Husnik & McCutcheon (2016)
Tremblaya princeps (Betaproteobacteria) [P]	*Paracoccus marginatus* (PSEUDOCOCCIDAE)	140,306	58.2	124	Provides amino acids & vitamins	Bacteriome	Husnik & McCutcheon (2016)
Hoaglandella endobia (Gammaproteobacteria) [S]		352,837	30.6	273	Provides amino acids & vitamins	*Tremblaya* cytoplasm	Husnik & McCutcheon (2016)

Table II. Bacterial endosymbionts in scale insects.

Endosymbiont (Class)	Host (FAMILY)	Genome size (pb)	%GC	Number of pseudogenes	Function	Localization	Reference
Dactylopiibacterium carminicum (Betaproteobacteria) [P]	*Dactylopius coccus* (DACTYLOPIIDAE)	3,589,384	62.7	ND	Fixes N & recycles uric acid	Ovaries	Vera-Ponce de León *et al.* (2017)
Wolbachia bourtzisii *w*DacA (Alphaproteobacteria) [S]		1,170,000	35.1	ND	Provides riboflavin & heme groups	Ovaries	Ramírez-Puebla *et al.* (2016)
Wolbachia pipientis *w*DacB (Alphaproteobacteria) [S]		1,498,000	34.1	ND	Provides riboflavin & heme groups	Ovaries	Ramírez-Puebla *et al.* (2016)
Uzinura diaspidicola (Flavobacteria) [P]	*Diaspis echinocacti* (DIASPIDIDAE)	263,431	30.2	7	Provides amino acids	Bacteriocytes	Sabree *et. al.* (2013)
Sulcia muelleri str. GWSS (Flavobacteria) [P]	**Homalodisca vitripennis* (CICADELLIDAE)	245,530	22.4	ND	Provides amino acids	Bacteriome	Husnik & McCutcheon (2016)
Baumannia cicadellinicola (Gammaproteobacteria) [S]		686,192	33.2	9	Provides vitamins	Bacteriome	Wu *et al*. (2006)
Buchnera aphidicola str. APS (Gammaproteobacteria) [P]	**Acyrtosiphon pisum* (APHIDIDAE)	640,681	22.5	13	Provides riboflavin	Bacteriome	Shigenobu *et al*. (2000)
Carsonella rudii (Gammaproteobacteria) [P]	**Pachypsylla venusta* (PSYLLIDAE)	159,662	16.6	ND	Provides amino acids	Bacteriome	Tamames *et al*. (2007)
Blochmannia floridanus (Gammaproteobacteria) [P]	**Camponotus floridanus* (FORMICIDAE)	705,557	27.4	6	Provides amino acids, participates in urea detoxification & N recycling	Gut & ovaries	Gil *et al*. (2003)

ND, no data available; [P], primary endosymbiont; [S], secondary endosymbiont; *, non-scale insect.

Table II. Bacterial endosymbionts in scale insects (*continued*).

endosymbionts have a higher nucleotide substitution rate than primary symbionts (Bennett *et al*., 2014). Repeated sequences are found in co-symbionts such as *Sodalis* (enterobacteria) (Siguier *et al*., 2014).

It is considered that mitochondria evolved from an ancestral alphaproteobacterium more than a billion years ago and underwent a process of genome reduction (Gray, 1992; Gray, 1999; Martin, 2017) that might have been similar to the evolutionary process followed by insect endosymbionts (Sloan *et al.*, 2014; Dunning Hotopp, 2011; McCutcheon, 2016). Mitochondria have very small genomes, in many cases rich in A + T such as in Insecta and Nematoda (Arunkumar & Nagaraju, 2006). Mitochondria have co-speciated with their hosts and they have transferred lots of genes to their hosts that encode proteins that are necessary for the mitochondria to carry out their metabolic functions (McCutcheon, 2016).

Fungi symbionts in insects

Although bacteria-insect associations are usually the most studied, about eight orders of insects are known to host fungi. Arthropods and fungi have coexisted for around 200 million years and their earliest relationship dates from the Jurassic (Vega & Blackwell, 2005). Their interactions include all types of symbiosis, such as the obligatory mutualism of cicadas (Chen *et al*., 1981), commensalism of the Attini tribe of ants where fungi are their main food source, and parasitism (Hughes *et al*., 2016). Fungal symbionts can inhabit cavities within the insect such as the micangio in bark beetles (Jones *et al*., 1999; Klepzig & Six, 2004; Ganter, 2006), or in mycetocytes that are found in the rice pest *Nilaparvata lugens* or in *Drosophila melanogaster* (Cheng & Hou, 2001; Ebbert *et al*., 2003). Fungi can also inhabit the lumen of the digestive system, the Malpighian tubules, gonads and even venom-producing glands in *Comperia merceti* wasp (Gibson & Hunter, 2010; Rivera *et al*., 2009; Ricci

et al., 2011; Vera-Ponce de León *et al*., 2016). Insects acquire fungi from other insects or by mycofagia when the insects feed directly from mycelia or yeasts as several species of *Drosophila* do (Gibson & Hunter, 2010; Becher *et al*, 2012). Fungi, like endosymbiont bacteria, can be transferred from the mother to her offspring such as the yeast-like symbionts in the case of *N. lugens* (Cheng & Hou, 2001) and *Wickerhamomyces anomalus* yeast in *Anopheles stephensi* mosquitoes (Ricci *et al*., 2011). The elimination of fungi causes a fitness decrease in their hosts (Sasaki *et al*., 1996; Menezes *et al*., 2015). The effects of fungi on host fitness have been demonstrated in cicadas (Sasaki *et al*., 1996) and in some beetles (Anobiidae and escolitins) (Ayres *et al*., 2000; Nasir & Noda, 2003). Fungi can supply insects with nitrogen metabolites and essential lipids lacking in their diet. They also participate in the degradation of high-density biological polymers, uric acid recycling, biotransformation of toxic chemicals in the environment, and pheromone production (D'Ettorre *et al*., 2002; Nasir & Noda, 2003; Vega & Blackwell, 2005; Vera-Ponce de León *et al*., 2016).

Bacterial endosymbionts of scale insects

Scale insects belong to the superfamily Coccoidea (Hemiptera: Sternorrhyncha) and include many plant pest species that cause enormous economic losses in agriculture (Miller *et al.,* 2005). They all secrete a protective wax cover that gives the insect a cotton or waxy powder appearance (Fig. 2). Most of them feed on the plant's phloem and are known as "cochineals". There are about 7,800 species. Most adult females remain sessile once they introduce their stylet into the host plant. The stylet is a specialized mouth organ to suck sap. Some species of scale insects damage plants by transmitting diseases and by excretion of honeydew, a sticky waste substance composed of sugars and minerals that promotes fungal growth. Honeydew is a source of food for birds, mammals and other insects, especially for several species of ants (Kondo *et al*., 2009).

Symbiotic bacteria that provide scale insects with nitrogen metabolites, vitamins and co-factors have been described (Table II and Figure 3). Endosymbionts cannot be cultured in media in the laboratory because their highly reduced genomes lack many genes needed for independent living. Thus, their study relies on metagenomic analyses. Symbiont genome sequences deduced from metagenomes allows the inference of their metabolic capabilities and their role in symbiosis (e.g. Shigenobu *et al*., 2000; Sabree *et al*., 2013; Rosas-Pérez *et al*., 2014).

The presence of endosymbionts within bacteriocytes has been detected in ten families of scale insects by culture independent methods (von Dohlen *et al*., 2001; Zchori-Fein *et al*., 2005; Gruwell *et al*., 2007; Matsuura *et al*., 2009; Gruwell *et al.,*

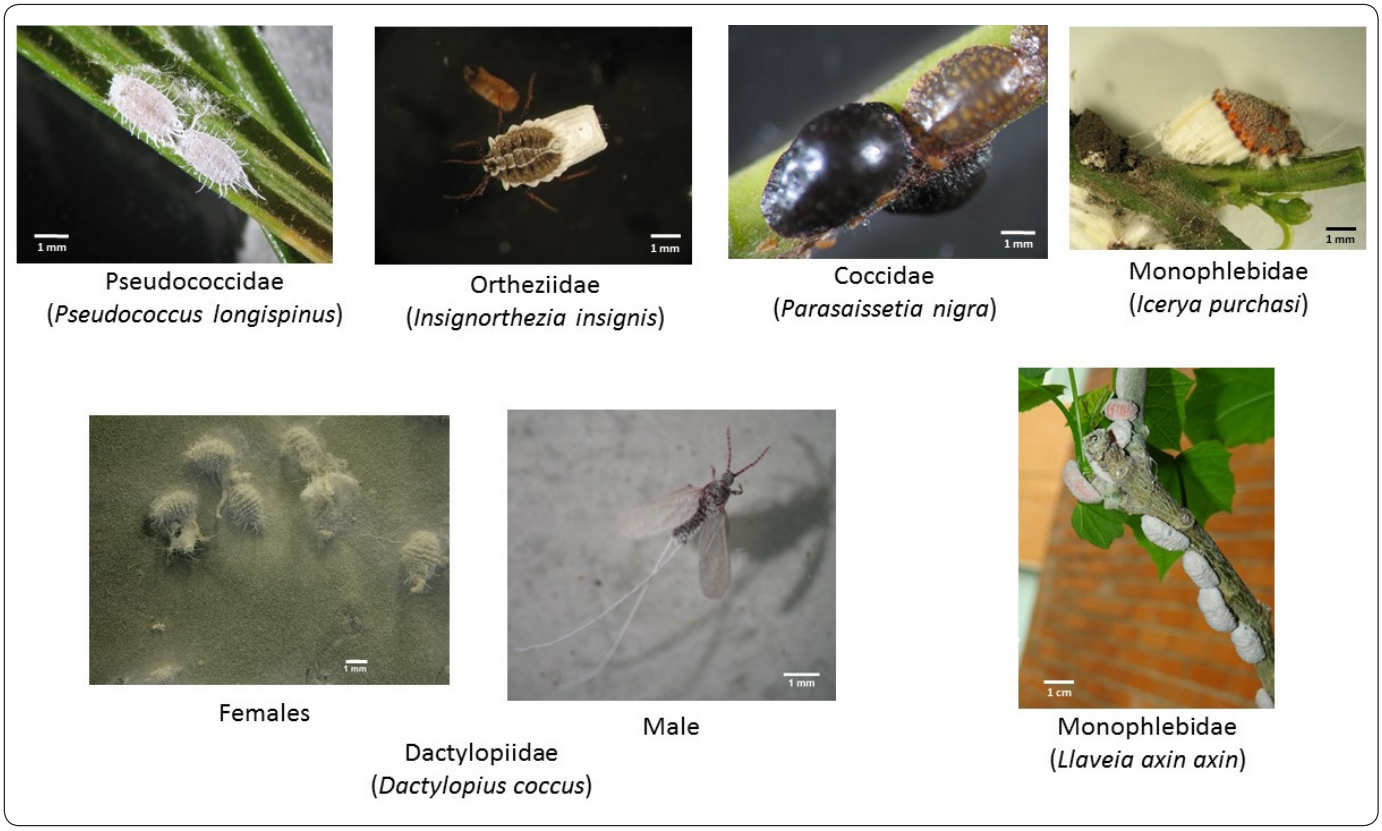

Figure 2. Photographs of some scale insect species mentioned in the review. Archive images from our group.

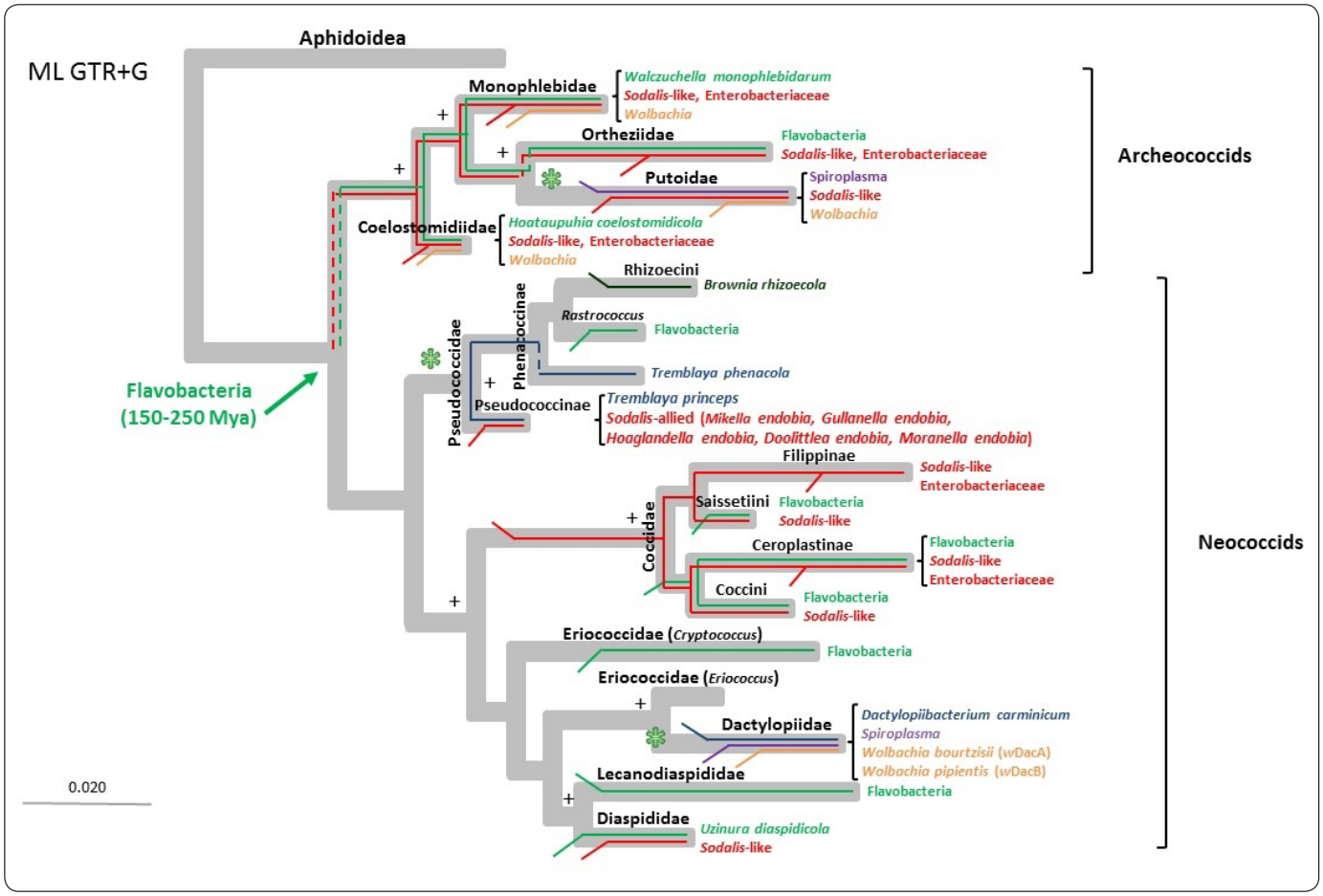

Figure 3. Phylogenetic reconstruction of 18S rRNA genes (570 bp) of scale insects and their relationship to bacterial endosymbionts with outgroup from Aphidoidea superfamily. One representative from each family or clade of scale insects was used. Different colors indicate the taxonomic identity of the endosymbionts: Flavobacteria, green; Alphaproteobacteria, yellow; Betaproteobacteria, blue; Gammaproteobacteria, red; Spiroplasma, purple. Dotted branches show endosymbiont losses. Branches from outside correspond to endosymbionts acquired by horizontal transfer. Asterisk shows the likely loss of Flavobacteria and replacement by other endosymbionts. All Flavobacteria except *Brownia rhizoecola* are phylogenetically related. The + sign at the nodes indicates a bootstrap value > 50%. Not all endosymbionts are present in all species from the same family. Diagram based on von Dohlen *et al.*, 2001; Zchori-Fein *et al.*, 2005; Gruwell *et al.*, 2007; Matsuura *et al.*, 2009; Gruwell *et al.*, 2010; Rosenblueth *et al.*, 2012; Dhami *et al.*, 2013; Husnik & McCutcheon, 2016; Vera-Ponce de León *et al.*, 2017.

2010; Rosenblueth *et al*., 2012; Dhami *et al*., 2013; Husnik & McCutcheon, 2016) (Figure 3). It is possible to locate these bacteria within bacteriocytes by fluorescent *in situ* hybridization (FISH) (Fig. 1; Kono *et al*., 2008; Matsuura *et al*., 2009; Dhami *et al*., 2012; Rosas-Pérez *et al*., 2014). Enterobacteriaceae (gammaproteobacteria), especially those close to the genus *Sodalis* (*Sodalis*-like) and Flavobacteria (Bacteroidetes), have been found in six families, namely Monophlebidae, Ortheziidae, Coelostomidiidae, Coccidae, Lecanodiaspididae and Diaspididae of the superfamily Coccoidea of scale insects (Table II; Figure 3). There is evidence that flavobacteria have been associated with scale insects since they appeared on Earth, about 150-250 million years ago. *Sodalis* has been reported in phylogenetically distant insects like the orders Coleoptera (such as weevils), Diptera (such as tsetse fly), Phthiraptera (like lice), and Hemiptera (like bugs, psyllids and spittlebugs) (Dale & Maudlin, 1999; Lefèvre *et al.*, 2004; Fukatsu *et al.*, 2007; Koga & Moran, 2014; Hosokawa *et al*., 2015; Morrow *et al.*, 2017).

Apparently, scale insects of the Diaspididae family (Gruwell *et al*., 2007), and several families of the Arqueococcids (Figure 3; Rosenblueth *et al*., 2012; Dhami *et al*., 2013) co-speciated with their flavobacterial endosymbionts. In contrast, a high number of host changes have been reported in the enterobacterial co-symbiont. It has even been shown that several individual insects bear two different species of enterobacteria (Rosenblueth *et al*., 2012). It seems that several insects that lost their flavobacteria and enterobacteria, acquired back a

flavobacterium by horizontal transfer from other scale insects, as is the case of several Neococcids. Flavobacteria have been replaced by other endosymbionts in at least three events, for example in species of the Putoidae family (by *Spiroplasma* and *Wolbachia*), Pseudococcidae (by *Tremblaya*) and Dactylopiidae (by *Dactylopiibacterium*, *Spiroplasma* and *Wolbachia*). The Phenacoccinae subfamily of the Pseudococcidae family, after acquiring *Tremblaya,* apparently lost it and got back a *Flavobacterium*. Two clades of Phenacoccinae acquired Flavobacterium, one of them from other scale insects and the other (*Brownia rhizoecola*) from outside scale insects. *Brownia* shows a greater identity with flavobacteria from unrelated insects such as those of leafhoppers and of cockroaches (*Sulcia muelleri* or *Blattabacterium* spp.) than with flavobacteria of scale insects (Gruwell *et al*., 2010; Rosenblueth *et al*, 2012.) (Fig. 3).

The endosymbiont bacteria of the Pseudococcidae family have been extensively studied since 1992 (Munson *et al*., 1992; Thao *et al*., 2002). They represent an exceptional case in endosymbiosis. Inside the cytoplasm of *Tremblaya princeps* (Betaproteobacteria) inhabits an enterobacterium close to the genus *Sodalis* (Husnik & McCutcheon, 2016).

Bacteria of the genus *Wolbachia* are secondary endosymbionts of over 40% of all arthropods and also of nematodes of the Onchocercidae family (Darby *et al*., 2012). In arthropods, some wolbachias manipulate the reproduction of their host, either by parthenogenesis, male feminization, male killing or cytoplasmic incompatibility (Werren *et al*., 2008). Some wolbachias benefit their hosts by providing them with protection against viral infections (Teixeira *et al*., 2008; Chrostek *et al*., 2013), or metabolites as riboflavin (Moriyama *et al*., 2015) or heme (Brownlie *et al*., 2009). *Wolbachia* have also been found in several species of scale insects (Figure 3) but it is not yet known whether they have an effect on the reproduction of their hosts.

Our research group has worked with three species of scale insects, *Llaveia axin axin* (Monophlebidae), *Dactylopius opuntiae*, and *Dactylopius coccus* (Dactylopiidae) (Figure 3). We described the bacterial (Ramírez-Puebla *et al*., 2010, 2016; Rosas-Pérez *et al*., 2014, 2017) and fungal (Vera-Ponce de León *et al*., 2016) communities associated with these two Mexican scale insect species. To illustrate the interactions of scale insects with their bacterial symbionts, we review the biology of these scale insects and the possible effects of their microbiota.

Llaveia axin axin (WAX COCHINEAL) AND BACTERIAL SYMBIONTS

Mesoamerican natives called the cochineal *Llaveia axin axin* "niij" (Fig. 2). Its annual life cycle presents hemimetabolism (gradual changes of egg, nymph and adult, without passing through the pupa stage). Females go through three stages of growth until they reach the adult state. They spend their entire life feeding from their host plant and at the end of their cycle they lay eggs in soil and then die. Under proper environmental conditions, the eggs hatch, nymphs emerge and disperse by moving or being blown by the wind. When they find an adequate plant, they settle on it. Like all scale insects, *L. axin axin* is exclusively phytophagous, feeding on the sap of plants such as *Acacia cochliacantha*, *Acaciella angustissima*, *Jatropha curcas* and *Spondias* sp. (Rincón-Rosales & Gutiérrez-Miceli, 2008). This hemipteran is of economic value in southern Mexico and since pre-Hispanic times a wax extracted from it is used to produce a lacquer to coat handicrafts (Williams & MacVean, 1995). It is worth mentioning that the population of niij has declined in recent years, mainly due to overexploitation, deforestation and the burning of the forests they inhabit (Rincón-Rosales & Gutiérrez-Miceli, 2008; Rosas-Pérez *et al*., 2014). The biology of this insect is little known. We studied its microbiota with metagenomic approaches.

Two endosymbiotic bacteria, a flavobacterium and an enterobacterium were found in the abdominal bacteriomes in *L. axin axin* (Fig. 2). DNA and RNA obtained from the bacteriomes were sequenced. Metagenomic and metatranscriptomic analyses of *L. axin axin* showed that both bacteria provide essential amino acids that the insects do not get from their diet. The flavobacterium, now named *Walczuchella monophlebidarum*, has a very small genome (309 kb) and its genetic repertoire is very similar to that of other phylogenetically distant flavobacteria, indicating a convergence in the evolution of different flavobacterial endosymbionts (Rosas-Pérez *et al*., 2014). Due to its reduced genetic repertoire, *Walczuchell*a metabolically depends on *Sodalis* TME1 and their host.

The enterobacterium co-symbiont of *L. axin axin*, now named *Sodalis* TME1 (Rosas-Pérez *et al*., 2017), has a 3.41 Mb genome with high functional diversity. Its large number of mobile elements is indicative of an ongoing genomic reduction process. *Sodalis*, as other enterobacteria that are co-symbionts of flavobacteria, is considered a newly acquired endosymbiont (Michalik *et al*., 2014). *Sodalis* TME1 has genes that may participate in the suppression of defense mechanisms in the insect. *Sodalis* encodes a Type 3 secretion system (T3SS) and enzymes for polyamine synthesis, which have been recognized as modulators of the defense system of insects (Dale *et al*., 2001; Jelsbak *et al*., 2012).

The expression profiles of bacterial and insect genes in the bacteriome and in the ovary showed that *Sodalis* TME1 may secrete an effector, possibly injected by T3SS, to achieve the colonization of the bacteriome. While *Sodalis* actively recycles the insect's waste nitrogen, the insect activates genes for transporters of different types of nutrients and produces an antimicrobial peptide (drosomycin-like) perhaps to keep the density of endosymbionts under control or to facilitate the metabolic exchange between symbiotic partners through membrane permeabilization (Mergaert *et al*., 2017).

In the ovary, *Walczuchella* seems to activate a response to oxidative stress, which the insect may use as a defense mechanism. In the ovary, the insect dramatically increases the production of energy for the development of eggs and activates genes encoding lysozyme and peptidoglycan binding proteins, maybe to control bacterial growth.

Fungi associated with the wax cochineal *L. axin axin*

A 500 bp fragments of fungal 18S rRNA and 26S rRNA ribosomal genes were recovered from three metagenomes from the wax cochineal, two from bacteriomes, and a third from a fully macerated insect (Vera-Ponce de León *et al*., submitted). These sequences allowed the identification of three phyla, Basidiomycota, Chitridiomycota and Ascomycota (Vera-Ponce de León, submitted). In the meta-assemblies of the metagenomes, genes that encode for triglyceride synthesis, purine metabolism and for uricase enzymes of *Aspergillus* sp. were found. This suggests that fungi associated with *L. axin axin* may produce lipids or provide nitrogen by recycling molecules such as uric acid and its derivatives, as has also been observed in beetles, grasshoppers and in the carmine cochineal (Sasaki *et al*., 1996; Nasir & Noda, 2003; Vera-Ponce de León *et al*., 2016). Genes encoding enzymes like phenol-monooxygenase, involved in the detoxification of xenobiotic compounds, were found, supporting the hypothesis that fungi may help the insect degrade the chemicals produced by the plants for their protection against insects. For example, *Jatropha curcas*, one plant on which *L. axin axin* insects feed, produces phorbol esters in high concentrations and various host plants of the niij produce tannins. It was observed that fungi found in the micangio of *Dendroctonus* beetles are capable of biotransforming toxic terpenoids and turning them into pine pheromones that attract insects of the same species (Brand *et al*., 1976).

Insects were collected for several years and using a culture-dependent approach, three fungi were isolated in culture. Phylogenetic analysis of 26S rRNA genes identified the three isolates within the genus *Aspergillus* spp. (Vera-Ponce de León *et al*., submitted). *Aspergillus* sequences were also found in the metagenome analyses and clearly, a larger diversity was shown by a culture-independent approach than by a culture-dependent approach. The former revealed several unknown fungi.

Dactylopius coccus (carmine cochineal)

D. coccus is known as the carmine cochineal (Fig. 2), and it is of great economic importance because carminic acid (carmine or red E120) is obtained from these insects. Carminic acid is an anthraquinone glycoside that is industrially used as a red dye for food, textiles, and cosmetics (Deveoglu *et al*., 2011). The genus *Dactylopius* comprises 11 species: *Dactylopius ceylonicus* (Green), *D. confusus* (Cockerell), *D. opuntiae* (Cockerell), *D. bassi*, *D. coccus* (Costa), *D. tomentosus* (Lamarck), *D. austrinus* (De Lotto), *D. confertus* (De Lotto), *D. gracilipilus* (Van Dam & May), *D. salmianus* (De Lotto), *D. zimmermanni* (De Lotto) (Williams & Ben-Dov, 2015). The first six species are found in Mexico and *D. coccus* is the only one that has been domesticated and depends on human intervention to complete its reproductive cycle. Due to the high concentration and quality of its pigment, *D. coccus* has been cultivated since pre-Columbian times (Rodríguez *et al*., 2005). Individuals have a size of 1-6 mm in length. Like other scale insects, their life cycle consists of a hemimetabolous metamorphosis that lasts approximately 110 days. This species exhibits sexual dimorphism. Males are winged and smaller than females (Pérez-Guerra & Kosztarab, 1992). All individuals, males and females, are covered by a cottony secretion wax that protects them against predators or from the environment (Chávez-Moreno *et al*., 2009). These insects feed on the sap of *Opuntiae* and *Nopalea* cacti (Cactaceae: Opuntioidae) (Chávez-Moreno *et al*., 2009). The sap is composed of water (85-95%), carbohydrates (3-7%) and fiber (1-2%); however, it is poor in protein (0.5-1%) and lipids (0-0.2%) (Stintzing & Carle, 2005).

Bacteria associated with *D. coccus* and their role in insect metabolism

The diversity of bacteria associated with *Dactylopius* cochineals has been well studied (Pankewitz *et al*., 2007; Ramírez-Puebla *et al*., 2010, 2015, 2016) not only in the domesticated species *D. coccus* but also in wild species. In *D. coccus* we found *Wolbachia*, *Spiroplasma*, *Dactylopiibacterium carminicum* (a betaproteobacterium), and diverse fungi (Ramírez-Puebla *et al*., 2010, 2015, 2016; Vera Ponce de León, 2016).

Two *Wolbachia* strains were found associated with *D. coccus* that were classified as *Wolbachia bourtzisii w*DacA and *Wolbachia pipientis w*DacB after a general proposal of species names in *Wolbachia* based on phylogenomic analyses (Ramírez-Puebla *et al*., 2015, 2016). The sizes of the genomes of *Wolbachia* (Ramírez- Puebla *et al*., 2016) are similar to those of other wolbachias and the *Spiroplasma* genome is also within the size range of its group genomes (1 Mb). All genes required for the synthesis of riboflavin and ubiquinone (vitamin Q) were found in the genomes of *Wolbachia*, suggesting that it provides these vitamins to its host. One of the *Wolbachia* species, the most abundant, may cause cytoplasmic incompatibility that affects the sex ratio in the progeny.

A bacterial phylogroup (END-1, now called *Dactylopiibacterium carminicum*) was the only bacterial species found in *D. coccus* (Ramírez-Puebla *et al*., 2010). *Dactylopiibacterium* is related to *Uliginosibacterium gangwonense.* From the *D. coccus* metagenomes we were able to reconstruct the entire genome of *Dactylopiibacterium*. Its characteristics are different to all betaproteobacterial genomes previously described as endosymbionts associated with Hemiptera and other scale insects (Table I; Table II). The size of the *Dactylopiibacterium* genome (3.6 Mb) is similar to those of free-living bacteria. This suggests that it is a recent symbiont that has not undergone

a long process of genome reduction characteristic of other endosymbionts. Furthermore, the functional annotation of this genome has shown that the whole genetic machinery for biological nitrogen fixation (BNF) is present (Vera-Ponce de León *et al*., 2017). Acetylene reduction assays and analysis of *nif* gene expression of *Dactylopiibacterium* showed that BNF occurs in whole *D. coccus* insects, ovaries and intestines. There is good evidence that the betaproteobacterium *Dactylopiibacterium* fixes nitrogen in the cochineal and we suppose it alleviates the nitrogen deficiencies in the sap that the cochineal ingests.

In other insects, it has been observed that bacterial symbionts associated with gut, protists or fungal gardens, are able to fix nitrogen and provide it to the host (Ulyshen, 2015; Pinto-Tomás *et al*., 2009; Morales-Jiménez *et al*., 2013). In ant fungus gardens, *Klebsiella variicola* strains are found. These bacteria are not insect-specialized as they thrive in crop plants and hospital patients (Rosenblueth *et al*., 2004; Martínez *et al*., 2004).

Furthermore, sequence analysis of clones of bacterial ribosomal genes (16S rRNA) from amplicons obtained by PCR of total DNA extracted from various species of *Dactylopius*, revealed alphaproteobacteria close to *Sphingomonas*, *Mesorhizobium*, and *Hepatincola porcellionum*. Twelve ribo-clones of *D. confusus* were related to *Acinetobacter* (gammaproteobacteria) and betaproteobacteria such as *Herbaspirillum* and *Massilia* were also reported (Ramírez-Puebla *et al*., 2010). Several of these bacteria had been previously reported as plant endophytes (Rosenblueth & Martínez-Romero, 2006) and may colonize the cochineal gut.

Fungi in cochineal carmine and their role in recycling nitrogen

The study of the fungal communities of *D. coccus*, *D. confusus*, and *D. opuntiae* by a culture-dependent approach revealed a total of 14 different fungal phylogroups associated with three *Dactylopius* species (Fig. 4). Although there is not a clear pattern between insect species and their fungal phylum groups, we observed that two yeast species *Rhodotorula mucilaginosa* and *Cryptoccocus saitoi* were present in females of all three species and in all locations sampled (Fig. 4). *Penicillium* fungi were detected in males of three *Dactylopius* species (Vera-Ponce de León *et al*., 2016). FISH analysis with probes targeting fungal ribosomal genes revealed the presence of *C. saitoi* in Malpighian tubules and embryos of *D. coccus* and *D. opuntiae*. Fungal ribosomal genes of Ascomycota, Basidiomycota, Chitridiomycota and Glomeromycota classes were found by metagenomic analyzes of hemolymph, ovaries, and guts of *D. coccus* females. Many sequences did not match to any previously reported sequence showing that there are novel fungi well adapted to live inside the carmine cochineals. Fungal genes involved in the bioconversion of uric acid (UA)

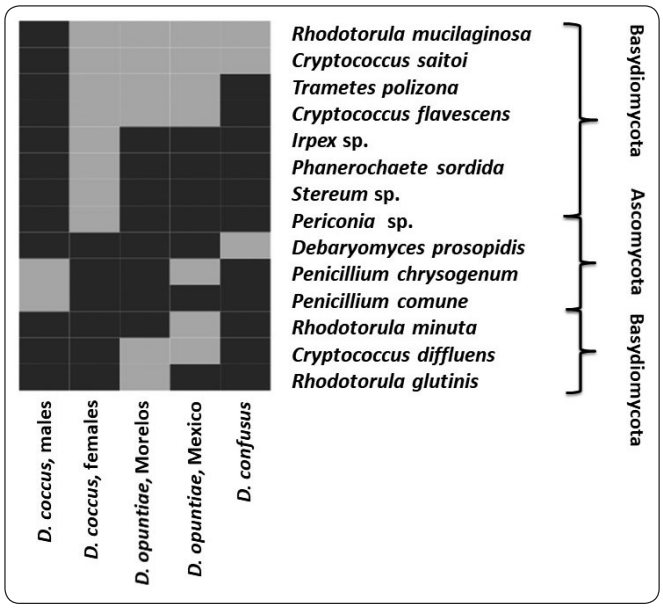

Figure 4. Heat map indicating the presence (light gray) and absence (dark gray) of different species of fungi found with a culture-dependent approach by sequencing ITS of three *Dactylopius* species (Vera-Ponce de León *et al.*, 2016).

to urea were identified in the metagenome. It is known that UA is the major waste metabolite from the nitrogen cycle in terrestrial insects. It has been estimated that this compound constitutes about 80% of the product of nitrogen catabolism in these organisms (Pant, 1988). In *D. coccus*, concentrations between 4 and 34 ng UA/μg^{-1} tissue, and uricase activity were detected in intestines and eggs. Importantly, no insect uricase gene was found in the metagenomes (Vera-Ponce de León *et al*., 2016). It was observed that most fungal phylogroups associated with *Dactylopius* use UA as their sole nitrogen source (Vera-Ponce de León *et al*., 2016). In tissues of *D. opuntiae* treated with antifungals to remove fungi, it was observed that UA concentration increased and uricase activity decreased. Furthermore, a significant decrease in the size and weight of treated insects was observed in comparison to control insects (Vera-Ponce de León *et al*., 2016). These results suggest that symbiotic fungi play an important role in nitrogen supply in *Dactylopius*. Such is the case in *Nilaparvata* leafhoppers where yeasts bio-convert UA to amino acids that can be used by their host.

Conclusions

It is common for many different insects to harbor various co-symbionts (Table I). This also happens in scale insects. There is a consensus that Flavobacteria are among the oldest symbionts, with largely reduced genomes, that provide essential amino acids to the host; but apparently, they frequently have to be metabolically complemented by other bacteria (Wu *et al*., 2006). Co-symbionts may confer some other additional benefits to the

hosts besides helping to provide the ten essential amino acids we metazoans need. This may explain the co-occurrence of Flavobacteria with enterobacteria in many insects including the wax cochineal. However, these symbionts are not found in the carmine cochineal, in which there is a more diverse community. A comparative summary of some of the characteristic of the carmine and wax cochineal symbiosis is presented in Table III. In the carmine cochineal, we studied in particular *Dactylopiibacterium* that fixes nitrogen to compensate for the carbon-nitrogen imbalance found in cactus sap on which the insect feeds. *Dactylopiibacterium* may provide essential amino acids to the carmine cochineal as it has complete pathways for the biosynthesis of amino acids. Among the cochineals that we studied, we also detected differences in the composition of their associated fungi (Vera-Ponce de León *et al*., 2016). Despite the taxonomic differences, there are common functions in fungi from *Nilaparvata*, *Dactylopius*, and the wax cochineal *L. axin-axin* to recycle uric acid.

This review shows the enormous plasticity of insect microbiota in different hosts and conditions. This is outstandingly illustrated in the case of *Nilaparvata lugens* which is the most destructive rice pest that originally could not attack resistant rice plants that lacked asparagine needed by the insect (Shigematsu *et al.,* 1982), but the insects overcame the resistance when carrying yeast-like symbionts that provided amino acids (Ferrater *et al*., 2013).

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Insect host	Symbionts		Phylum	Possible role in symbiosis
Dactylopius coccus (carmine cochineal)	Bacteria	*Dactylopiibacterium carminicum*	Proteobacteria (β)	Biological nitrogen fixation
		*Wolbachia burtzisii w*DacA *Wolbachia pipientis w*DacB	Proteobacteria (α)	Riboflavin and hemo synthesis
	Fungi	*Cryptococcus saitoi Rhodotorula mucilaginosa*	Basidiomycota	Uric acid recycling
		Penicillium sp.	Ascomycota	
Llaveia axin axin (wax cochineal)	Bacteria	*Walczuchella monophlebidarum*	Bacteroidetes (Flavobacteria)	Essential amino acids synthesis
		Sodalis TME1	Proteobacteria (γ) (Enterobacteriaceae)	Metabolic complementation of *Walczuchella*, uric acid recycling
	Fungi	*Aspergillus* sp.	Ascomycota	Lipid synthesis, uric acid recycling

Table III. Symbiotic relationships, observed in carmin and wax cochineal.

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