Multiple guests in a single host: interactions across symbiotic and phytopathogenic bacteria in phloem-feeding vectors – a review

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41 15 **Running title:** Bacterial interactions in phloem phytopathogen vectors
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18   **Abstract**

19   Phloem-limited bacteria are a major threat for worldwide agriculture due to the heavy economic

20   losses caused to many high-value crops. These disease agents, namely phytoplasmas, spiroplasmas,

21   liberibacters and *Arsenophonus*-like bacteria, are transmitted from plant to plant by phloem-feeding

22   Hemiptera vectors. The associations established among pathogens and vectors often derive from co-

23   evolution, and hence could result in a complex network of interactions involving also the whole

24   microbial community harboured by the insect host. Interactions among bacteria may be beneficial,

25   competitive, or detrimental for the involved microorganisms, and can dramatically affect the insect

26   vector competence and consequently the spread of diseases. Interferences are observed among

27   different pathogen strains competing to invade the same vector specimen, causing selective

28   acquisition or transmission. Bacterial symbionts are another pivotal element for interactions existing

29   between vectors and phytopatogens, because of their central roles for insect life cycle. Some

30   symbionts, either obligate or facultative, were shown to have antagonistic effects on the colonization

31   by plant pathogens, by producing or stimulating the insect production of antimicrobial substances, or

32   competing for host infection. In other cases, evidences of mutual exclusion between symbiont and

33   pathogen suggested possible detrimental influence on phytopathogens displayed by symbiotic

34   bacteria; conversely examples of microbes enhancing pathogen load are available as well. Whether

35   and how bacterial exchanges occurring in vectors affect the relationships between insects, plants and

36   phytopathogens are still incompletely characterized issues, leaving room for many open questions

37   concerning the significance of some traits of these multitrophic interactions. However, such complex

38   interplays may have a serious impact on pathogen spread and control, having the potential to drive

39   new strategies for the containment of important diseases.

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Introduction

Phloem-limited bacterial phytopathogens, which are among the most devastating agricultural threats globally due to their wide host range and symptom severity, strictly rely on insect vectors to be spread from plant to plant. These pathogenic bacteria are walled Proteobacteria (α- and γ- subclades), and wall-less Mollicutes. The first group encompasses the α-proteobacterial 'Candidatus Liberibacter spp.', including important pathogens of citrus and vegetable crops (Haapalainen, 2014), and two Arsenophonus-related γ-Proteobacteria, namely 'Ca. Phlomobacter fragariae' and 'Ca. Arsenophonus phytopathogenicus' (Bressan, 2014). Plant pathogenic Mollicutes embrace the genera 'Ca. Phytoplasma' and Spiroplasma.

All vectors of plant pathogenic bacteria residing in the phloem are Hemiptera belonging to the suborders Auchenorrhyncha (with the families Cixiidae, Dictyopharidae and Flatidae in the Fulgoromorpha infraorder and Cicadellidae in the Cicadomorpha infraorder) and Sternorrhyncha (superfamily Psylloidaea). Vectors are able to ingest bacteria by feeding in the phloem with their piecing-sucking mouthparts. Liberibacters are transmitted by psyllids, and Arsenophonus-like bacteria are vectored by planthoppers in the family Cixiidae. On the other hand, phytoplasmas are transmitted by leafhoppers (family Cicadellidae), planthoppers (superfamily Fulgoroidea), and psyllids (superfamily Psylloidea); while spiroplasmas are vectored by leafhoppers only (Gasparich, 2010).

The interactions between plant pathogens and their vectors are not limited to a carrier-carried relation: different species or strains of a plant pathogen have divergent behaviours in different insect hosts. Moreover, phytopathogenic bacteria are included in a complex network of interactions occurring in vectors, being actual members of the multifaceted insect microbiomes, which have a significant influence on the biology of the hosts. Members of the Hemiptera, including all of the vectors of phloem-limited bacterial plant pathogens, rely on bacterial symbionts for supplying nutrients lacking in their unbalanced diet (Baumann, 2005). The nutritional provisioning operated by obligate

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68 symbionts has been a crucial condition for insect persistence and diversification on a limited food

69 niche such as plant phloem (Skidmore and Hansen, 2017), then affecting the host range of vectors.

70 High polyphagy deriving from mutualistic associations may in turn influence the chance of different

71 plants to be infected by a plant pathogen. Moreover, facultative symbionts are commonly found in

72 many vectors, showing protective functions, or being capable to manipulate the host's reproduction

73 (Zchori-Fein & Bourtzis, 2011). In addition, different species or strains of plant pathogens may be

74 hosted by the same individual vector (Table 1), possibly being transferred together to the host plant

75 (Bosco & D'Amelio, 2010). Such multipartite interactions most commonly result in microbial

76 synergies or interference, with potential implications for bacterial transmission as well (Bosco &

77 D'Amelio, 2010; Saldaña et al., 2017). This review summarizes the available knowledge concerning

78 microbial exchanges occurring in the vectors of phloem bacterial pathogens, with special regard to

79 the consequences on their transmission. Disease management could take advantage of these

80 interactions to develop microbe-based control strategies (Crotti et al., 2012) (Figure 1). Indeed,

81 despite their capability to easily adapt to, and grow in, different hosts such as plants and insects,

82 currently these phloem-restricted bacteria cannot be cultured or are difficultly cultivated in cell-free

83 media −with few exceptions such as spiroplasmas and a single liberibacter species− (Perilla-Herao

84 &Casteel, 2016), thus limiting experimentations aimed to identify new control strategies. Control is

85 generally based on the use of healthy plant propagation material, elimination of symptomatic plants,

86 and control of insect populations spreading the disease. Unravelling the interactions established

87 between phytopathogens and insect symbionts could offer an interesting tool to impair the

88 transmission of phloem-limited plant pathogens in a sustainable perspective.

89 **Phloem-limited bacterial plant pathogens**

90 Liberibacters

91 Transmitted by psyllids, '*Ca.* Liberibacter' pathogens include primarily obligate parasites of plants

92 and insects, responsible for several plant diseases, among which huanglongbing (HLB) in citrus trees

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93   and zebra chip (ZC) in potatoes are the most severe ones in terms of crop damage and economic

94   losses (Gottwald et al., 2007; Haapalainen, 2014). Three species of '*Ca*. Liberibacter' have been

95   indicated as the causal agents of citrus HLB, previously known as citrus greening, i.e. '*Ca*. L.

96   asiaticus' (CLas), '*Ca*. L. africanus' (CLaf), and '*Ca*. L. americanus' (CLam), the names of which

97   have been derived from the continents where these bacteria have been originally found and are mainly

98   distributed (Haapalainen, 2014). While CLaf is transmitted by the African citrus psyllid *Trioza*

99   *erytreae* Del Guercio (McClean & Oberholzer, 1965), CLas and CLam are mainly vectored by the

100 Asian citrus psyllid *Diaphorina citri* Kuwayama (Capoor et al, 1967; Teixeira et al., 2005). *D. citri*,

101 native to southeastern Asia, has been recently diffused in America probably in consequence of

102 international commerce (Halbert & Núñez, 2004; Bayles et al., 2017). Despite similar symptoms are

103 recorded after infection by each of the three HLB-causing species, CLas is the most destructive one,

104 inducing devastating epidemics in several countries (Haapalainen, 2014). On the other hand, ZC in

105 potatoes and other diseases in vegetable crops are caused by '*Ca*. L. solanacearum' (CLso), which

106 has been initially indicated with the name '*Ca*. L. psyllaurous' (Liefting et al., 2009). Geographically

107 distinct CLso haplotypes are known, whose differential distribution results in the association with

108 separate plant and insect host species. While in North America and Oceania this pathogen is vectored

109 by the potato/tomato psyllid *Bactericera cockerelli* Šulc, causing severe damage in potato and tomato

110 crops, in Europe -where it is transmitted by psyllids of the species *Trioza apicalis* Förster and

111 *Bactericera trigonica* Hodkinson- it is associated with diseases of the Apiaceae family plants, such

112 as carrot and celery.

113 In the last years, other liberibacter species have been identified, i.e. '*Ca*. L. europeaus' (CLeu) and

114 *Liberibacter crescens*, but differently from the aforementioned species these latter are not reported

115 as phytopathogens, rather showing an endophytic behaviour (Raddadi et al., 2011; Leonard et al.,

116 2012). Interestingly, *L. crescens*, found in mountain papaya in Puerto Rico, can be grown in axenic

117 cultures, making it an optimal candidate to study liberibacters' biology (Leonard et al., 2012; Fagen

118 et al., 2014a,b). On the other hand, CLeu, reported as an endophyte of pear, apple, blackthorn and

119 hawthorn, transmitted by *Cacopsylla* spp. (Raddadi et al., 2011; Camerota et al., 2012), has been

120 recently indicated as a pathogen in Scotch broom (*Cytisus scoparius*) in New Zealand (Thompson et

121 al., 2013). Recently, other two new candidate liberibacter species were recently reported: the '*Ca.*

122 Liberibacter caribbeanus' (CLca) detected in *Citrus sinensis* (L.) Osbeck and in the citrus psyllid *D.*

123 *citri* from Colombia (Keremane et al., 2015) and the '*Ca.* Liberibacter brunswickensis' (CLbr)

124 detected in the native Australian eggplant psyllid, *Acizzia solanicola* Kent & Taylor (Morris et al.,

125 2017). Neither these new species were associated with plant disease but a co-evolution with psyllids

126 as secondary symbionts is inferred (Morris et al., 2017).

127 *Arsenophonus*-like bacteria

128 *Arsenophonus* genus includes not only plant pathogens, but also insect parasites and symbionts

129 (Bressan, 2014). For instance, in a survey performed on 136 arthropod species it has been found that

130 *Arsenophonus* bacteria are associated with 5% of the tested hosts (Duron et al., 2008), where they

131 can establish complex interactions with beneficial or parasitic features (Wilkes et al., 2011).

132 Conversely, two species are cause of disease to strawberry and sugar beet plants (Danet et al., 2003;

133 Bressan et al., 2008). The first pathogenic agent was discovered at the end of last century in France

134 on strawberries affected by marginal chlorosis. Because at that time very little was known about this

135 genus, the pathogen was considered as a separate species that was named '*Ca.* Phlomobacter

136 fragariae' (Zreik et al., 1998); nonetheless the increase of sequence data availability led to propose it

137 to be an *Arsenophonus* (Bressan, 2014). The other plant pathogenic *Arsenophonus* is '*Ca.*

138 Arsenophonus phytopathogenicus' which infects sugar beet, causing a disease defined as "basses

139 richesses" syndrome, because diseased plants show decreased sugar content (Richard-Molard et al.,

140 1995). The insect vectors of pathogens in the *Arsenophonus* group are cixiids: '*Ca.* Phlomobacter

141 fragariae' is vectored by *Cixius wagneri* (China) (Danet et al., 2003), whereas '*Ca.* Arsenophonus

142 phytopathogenicus' is transmitted by *Pentastiridius leporinus* (L.) (Gatineau et al., 2002). These two

pathogens are phylogenetically distinct, and can differentially interact with plants and insects in different contexts. 'Ca. Arsenophonus phytopathogenicus' was observed in Italy to be related to a strawberry marginal chlorosis disease (Terlizzi et al., 2007); likewise it was detected in *C. wagneri*, which was able to inoculate it to sugar beet plants, whereas strawberries were not infected (Bressan et al., 2008). Moreover, the epidemiology of this group of diseases is complicated by the fact that they can be induced also by phytoplasmas transmitted by *Hyalesthes obsoletus* Signoret (Gatineau et al., 2002, Danet et al., 2003). Even being plant pathogens, there is evidence that many traits of *Arsenophonus*-like bacteria are characteristic of an insect symbiont lifestyle, such as reproductive tissue colonization and vertical transmission, absence of entomopathogenic activity, high infection rate and a life cycle prevalently related to insect hosts (Bressan, 2009b; 2014). Thus, these bacteria could easily initiate new associations with additional cixiid species. The complexity of their associations with insects and plants, jointly to cixiids' capability to easily adapt to new environments and host plants, could effectively explain the increasing appearance of emerging *Arsenophonus*-related diseases.

<u>Phytoplasmas</u>

Phytoplasmas are known to be responsible for diseases in over a thousand of economically important crops globally distributed (Marcone, 2014): typical symptoms include yellowing, witches' broom, virescence, phyllody, bolting, reddening of leaves and stems, decline and stunting of plants (Hogenhout et al., 2008). To date, all known phytoplasmas are reported to be pathogenic for at least one plant, even though asymptomatic hosts may be recruited. Phytoplasma taxonomy has been hampered by their recalcitrance to be cultured in vitro; therefore these bacteria are partially classified in the provisional genus 'Ca. Phytoplasma' based on sequence analysis; up to now 42 'Ca. Phytoplasma' species have been reported (Zhao & Davis, 2016). A more exhaustive categorization defines phylogenetic clusters (16SrI-XXXIII groups, each one divided in many subgroups) according to 16S rRNA gene sequence (Lee et al., 1993, 1998b; Zhao & Davis, 2016).

168 Since most phytoplasmas are capable to cause symptoms to a number of plants belonging to different

169 families, such phytopathogens are regarded as some of the most troubling disease agents in these

170 areas. Moreover, some phytoplasmas are successfully transmitted by polyphagous vectors, furtherly

171 incrementing their chance to infect a huge number of plants. For example, Aster Yellows

172 phytoplasmas (16SrI) are vectored by many polyphagous leafhoppers to several plants (Weintraub &

173 Beanland, 2006), including different flowers, vegetables, or grapevine. The broad range of wild and

174 cultivated plants that are affected by these pathogens can be explained by the polyphagy recorded for

175 most of vectors, along with the great diversity of phytoplasma subclades within this group

176 (Hogenhout et al., 2008).

177 Considering vector-phytoplasma interplays, many specific interactions are acknowledged between

178 different phytoplasma phylogenetic groups and distinct taxa of vectors. As an example, only

179 leafhoppers in the family Cicadellidae have been reported to transmit phytoplasmas of the 16SrI

180 group (Alma et al., 2015). On the other hand, many phytoplasmas are indistinctively vectored by

181 distant insects. For instance, phytoplasmas of the phylogenetic groups 16SrV and 16SrXII may be

182 vectored by members of either Fulgoromorpha and Cicadomorpha, and 16SX phytoplasma can be

183 transmitted both by Auchenorrhyncha and Sternorrhyncha (Alma et al., 2015). However, a single

184 family with major vector importance can be generally recognized even for pathogens transmitted by

185 distinct taxa: in the case of 16SrV phytoplasmas, most of vectors belong to Cicadellidae, 16SrXII

186 phytoplasmas are mainly transmitted by cixiids, and the major vectors 16SrX phytoplasmas are

187 members of Psyllidae.

188 Spiroplasmas

189 Spiroplasmas are regarded as an extremely harmful group for global agriculture, even though only

190 few species have been accounted as phytopathogens, i.e. *Spiroplasma citri* in citrus, *S. kunkelii* in

191 maize and *S. phoeniceum* in aster (Gasparich, 2010). All plant pathogenic spiroplasmas are

192 phylogenetically related, being included in the same taxonomic lineage, namely the Citri clade

(Garsparich, 2010). Despite spiroplasmas and phytoplasmas establish similar pathogenic relationships with host plants, inducing analogous symptoms, major biological differences are evident between these genera. Distinctions include the bacterial shape, as spiroplasmas are characterized by the helical morphology and phytoplasmas are pleomorphic, and cultivation suitability, as spiroplasmas can be cultured in nutrient-rich media while phytoplasmas are recalcitrant to cultivation (Gasparich, 2010).

S. citri is mainly related to heavy economic losses to citrus productions; however this pathogen, as well as its vectors, may be found on many different host plants. Namely, *S. citri* is the agent of citrus stubborn, brittle root disease of horseradish, sesame yellowing, and carrot purple leaf (Zarei et al., 2017); it is transmitted by the leafhoppers *Circulifer haematoceps* (Mulsant & Rey) in the Mediterranean basin and *Circulifer tenellus* (Baker) in North America (Renaudin, 2006). The main areas affected by *S. citri*-related diseases are the Mediterranean countries of Europe, North Africa, and western Asia, as well as the Nearctic region, whereas the pathogen is absent in South America.

S. kunkelii is an important pathogen of maize crops, even though its distribution is restricted to the Americas. Its natural vector is the cicadellid *Dalbulus maidis* (Delong & Wolcott), which is a specialist of the genus *Zea* present in the Nearctic and Neotropical areas. *D. maidis* is co-evolved with maize, where it can be among the most prevalent leafhoppers (Palomera et al., 2012).

The third plant pathogenic spiroplasma species is *S. phoenicium*, which was retrieved from periwinkle plants affected by yellows in Syria. This pathogen is experimentally transmitted by the leafhopper *Macrosteles fascifrons* (Stål); however, at present no information is available concerning the natural vectors of *S. phoeniceum* in the infested area (Saillard et al., 1987).

Bacterial phytopathogen-vector relations

In the vectors, the phloem-restricted pathogens are transmitted in a persistent manner: once ingested by trophic activity on infected plants, bacterial cells multiply in the insect midgut, cross the

217 epithelium, replicate in the hemolymph and, ultimately, infect the salivary glands to be further

218 injected in the new host plant (Figure 1; Gasparich, 2010; Bressan, 2014; Haapalainen, 2014). This

219 process implies complex interplays, spanning from beneficial to adverse. A benign role was suggested

220 for CLas in *D. citri* (Duan et al., 2009; Mann et al., 2011), although an increased susceptibility to

221 selected insecticides was observed in infected psyllids, resulting in fitness decrement (Mann et al.,

222 2011). Similarly, a negative density-dependent effect of CLso infection on the fecundity of *B.*

223 *cockerelli* was reported by (Nachappa et al., 2014), whereas no significant detrimental effects on the

224 biology of infected individuals occurs according to Thinakaran et al (2015). Effects of vector

225 manipulation by a phytopathogen have been observed also at the hemolymph level, as in CLas-

226 infected *D. citri* showing changes in proteins related to energy metabolism, immunity, and lipid

227 transport (Kruse et al., 2018). Differential effects have been reported for insect-phytoplasma

228 associations: for example, shorter survival and a lower egg production were observed in individuals

229 of *Scaphoideus titanus* Ball infected by 16SrV phytoplasmas (Bressan et al., 2005a), whereas a

230 positive influence have been recorded for 16SrI phytoplasmas in *Macrosteles quadrilineatus* DeLong

231 & Caldwell (Beanland et al., 2000).

232 The molecular mechanisms regulating plant pathogens retention, multiplication and spread in the

233 body of some species, and not in others, are still poorly understood. The biological adaptation of

234 vectors to harbour plant pathogens suggest a co-evolution between insects and bacteria; however,

235 these interactions have polyphyletic traits, indicating multiple independent evolution events

236 (Orlovskis et al., 2015). The evolution of pathogen transmission shares some traits with insect

237 symbiosis, as most of plant pathogens are phylogenetically related to many symbiotic bacteria of

238 Hemiptera, and similarly to endosymbionts they have reduced genomes, reflecting the adaptation to

239 obligate associations (Bendix & Lewis, 2018). Indeed, a major consequence of a host-dependant life

240 style is an extreme gene loss, due to the lack of a selection process capable to maintain superfluous

241 genes in the rich environment provided by the insect body (Latorre & Manzano-Marín, 2017). In most

242 cases, the associations between plant pathogens and their vectors are believed to be originated from

243 bacterial internalization and successful survival in insects feeding transiently in infected plants (plant-

244 first model). Conversely, some phytopathogens, especially those in the Enterobacteriaceae family,

245 may have been initially insect commensals (i.e. non-harmful associates) that have evolved as plant

246 pathogens following repeated inoculations in the phloem by their insect hosts (insect-first model)

247 (Bové & Garnier, 2002; Nadarasah & Stavrinides, 2011).

248 Traits affecting vector suitability and specificity are thought to be related to difference in insect

249 physiology, immunity, and behaviour, as well as to their geographical and seasonal distribution

250 (Perilla-Hernao & Casteel, 2016). For instance, divergent plant host-dependant feeding behaviours

251 have been suggested to play an important role in differential transmission competence observed in

252 the leafhopper phytoplasma vectors *Euscelidius variegatus* (Kirschbaum) and *Empoasca decipiens*

253 Paoli (Galetto et al., 2011). Moreover, the vector immune system may limit pathogen invasion. In *D.*

254 *citri*, CLas acquisition by adult specimens was proven to be significantly less efficient than by

255 nymphs due to differential immune responses, like melanization and apoptosis of gut cells (Kruse et

256 al., 2017). Similarly, immune response may be the cause of limited phytoplasma cell number found

257 in non-transmitting individuals of different vector species after experimental exposure to the

258 pathogens (Galetto et al., 2009). A crucial phase of the transmission process is the protein interaction

259 between pathogen cells and those of the host, regulating pathogen crossing of gut and salivary glands

260 epithelia. The main strategy for bacterial internalization reported for plant pathogenic agents is endo-

261 exocytosis (Kwon et al., 1999; Hogenhout et al., 2008; Cicero et al., 2016), mediated by different

262 membrane proteins (Labroussaa et al., 2010, 2011; Béven et al., 2012; Duret et al., 2014; Konnerth

263 et al., 2016; Arricau-Bouvery et al., 2018). The absence of specific adhesion machinery to host cells

264 seriously weakens the vector competence (Weintraub & Beanland, 2006). For example, *S. citri* strains

265 lacking adhesion-related proteins are not transmissible by insects (Kruse et al., 2017).

266 The transmission of a plant pathogen by vectors is affected also by the fact that different species or

267 strains of a plant pathogen have divergent behaviours in different insect hosts. This is especially

268 observed for those phytopathogens that most probably derive from insect symbionts, such as

269 *Arsenophonus* bacteria and spiroplasmas. Both the genera *Arsenophonus* and *Spiroplasma* encompass

270 inter- and intracellular symbiotic bacteria displaying a diversity of roles, from mutualism to

271 reproductive manipulation, or may even be entomopathogenic (Gasparich, 2010, Bressan, 2014). In

272 '*Ca*. A. phytopathogenicus' and '*Ca*. P. fragariae', it has been shown that the exploitation of plants

273 resulted from independent evolutionary events from a common endosymbiotic ancestor (Bressan,

274 2014). This evidence, along with the observation of typical symbiotic traits in insects, like high

275 prevalence and maternal transmission, suggests their transition from endosymbiotic to plant

276 pathogenic life style (Bressan, 2014). Besides, some species belonging to other phytopathogen groups

277 could actually derive from insect commensals. For example, phylogenetic studies demonstrated a

278 match between the affinity level of liberibacter species restricted to different continents and the

279 geographical distribution of psyllid hosts. This supported the hypothesis of a co-evolution between

280 CLbr, behaving as an insect secondary symbiont, and its host *A. solanicola* (Morris et al., 2017). On

281 the other hand, co-evolved associations involving a plant pathogen and an insect vector may lead to

282 mitigate possible harmful effects exhibited on the host fitness (Purcell, 1982). The growing number

283 of observed transitions from insect endosymbiosis to plant pathogeny and vice versa is certainly

284 indicative of the possibility that new bacterial species, currently believed to be horizontally

285 transmitted insect commensals or mutualists, will become emerging plant pathogens in the future.

286 The study of phytopathogen-vector interactions has a remarkable pertinence from a disease

287 containment perspective, because differential molecular targets for control could be derived from

288 distinct associations involving co-evolution, mutualism or insect injury. For example, the

289 enhancement of insect immunity could be a specific control objective in case of pathogen-vector

290 interactions where the bacterium is definitely recognized and attacked by immune cells due to non-

291 beneficial interchange (Weiss & Aksoy, 2011). In contrast, some phytopathogens that are anciently

292 related to and co-evolved with their insect hosts are able to escape the immune response. For example,

293 *S. citri* has been reported to evade phagocytosis and limit phenoloxidase activity in its vector *C.*

294 *haematoceps* (Eliautout et al., 2016). In those cases, control approaches based on immune

295 augmentation may be insufficient.

Multiple pathogen infections and competition

297 The interaction among pathogens, plants and vectors can be extremely complex. Mixed infections by

298 different bacterial pathogens can quite commonly be observed in the phloem of the same plant. The

299 simultaneous occurrence of multiple pathogens in the same plant is rather frequent in herbaceous

300 plants and trees belonging to many families; either related and phylogenetically distant pathogenic

301 agents may co-exist (Križanac et al., 2010; Nicolaisen et al., 2011; Arratia-Castro et al., 2016; Satta

302 et al., 2016; Swisher et al., 2018). Moreover, a single insect can feed on several plants, or even

303 different plant species, during its life cycle, possibly being exposed to mixed pathogen infections. As

304 a consequence, insect vectors may acquire many pathogen species or strains during the same feeding

305 event, or by feeding sequentially on host plants infected by different bacteria (Križanac et al., 2010;

306 Raddadi et al., 2011; Swisher et al., 2018) (Table 1). However, in some cases, the co-occurrence of

307 multiple pathogens in the same insect's body is inhibited by interferential interactions such as

308 selective acquisition or transmission of a single microbe (Bosco & D'Amelio, 2010). For example,

309 in the leafhopper *Dalbulus maidis* (Delong & Wolcott), which is the natural vector of maize bushy

310 stunt phytoplasma (MBSP) and corn stunt spiroplasma (CSS), competition for transmission was

311 reported after co-occurrence during a long-term latency period (de Oliveira et al., 2007). This

312 competition resulted in suppression of prolonged transmission of MBSP after acquisition of CSS, as

313 the latter is thought to have faster rates of multiplication and spread, hence being more competitive

314 during the latency period required for successful transmission. Similar results were obtained with the

315 cicadellid *M. quadrilineatus*, vector of several strains of Aster Yellows Phytoplasma. Leafhoppers

316 exposed to sequential acquisition of different phytoplasma strains most frequently transmitted the

317 first provided isolate exclusively (Freitag, 1967). These evidences suggest competitive colonization

318 of the insect's body, where the first strain starting multiplication and reaching the salivary glands is

319 more competitive and hence preferentially transmitted (Bosco & D'Amelio, 2010). The same

320 competitive colonization process was proposed for *Osbornellus horvathi* Matsumura, since '*Ca*. P.

321 asteris' and '*Ca*. P. phoenicium' double-infected adult leafhoppers were able to transmit the former,

322 but not the latter, to different plants in experimental conditions (Rizza et al., 2016). Considering

323 *Arsenophonus*-related plant pathogens, no specific transmission trial from double-infected sources

324 has been reported yet; however there are evidences that separated populations of *Cixius wagneri*

325 (China), the only known vector of both pathogens, exclusively transmit '*Ca*. A. phytopathogenicus'

326 or '*Ca*. P. fragariae' but do not carry the two bacteria together (Bressan et al., 2008). Many factors

327 must be taken into account to explain exclusive pathogen acquisition by *C. wagneri*, including vector

328 ecology and population dynamics, which could lead to limited chance for the same individual to be

329 exposed to both pathogens; however the competition between '*Ca*. A. phytopathogenicus' and '*Ca*.

330 P. fragariae' for insect colonization cannot be ruled out.

331 The competition between two bacterial pathogens in the vectors has been better dissected by Rashidi

332 et al. (2014), by using the leafhopper *E. variegatus* and two unrelated phytoplasmas, namely

333 Chrysantheum Yellows phytoplasma (CYP) and Flavescence Dorée Phytoplasma (FDP),

334 experimentally transmitted to broad bean plants. The authors found that insects sequentially exposed

335 to acquisition of CYP and FDP showed unilateral interference, with the suppression of FDP

336 transmission regardless of the feeding order. On the other hand, the acquisition of each pathogen was

337 not affected by the presence of the other one, suggesting no competition at the earlier infection stages.

338 The barrier where competition takes place was rather identified in salivary glands, which were more

339 rapidly invaded by CYP due to its capability to multiply faster than FDP, even though the latter

340 bloomed to higher concentrations. The higher speed in reaching salivary glands displayed by CYP

was suggested to be related to: i) long co-evolution with the insect host and consequent mitigated

immune response, and ii) broad phytoplasma host range supporting the evolution of traits that

promote acceptability by a broad vector range (Rashidi et al., 2014). Transcriptomic analysis of

infected leafhoppers with single phytoplasma strains demonstrated the activation of insect immune

response (by activation of Kazal type 1 serine protease inhibitor and melanisation pathway) after

infection by FDP, which reduces the host fitness and is then perceived as a potential pathogen (Galetto

et al., 2018). Instead, the most competitive CYP increased energy metabolism, providing molecular

confirmation for different competition levels.

The knowledge on competition between co-occurring pathogen strains in the same host, although

being still limited, could support the study of pathogen transmission. Indeed, the observation and

characterization of competition events may contribute to unravel meaningful details of the processes

determining insect invasion and spread of phytopathogens, possibly identifying weaknesses of single

associations and revealing new control targets. Moreover, competitive transmission of different plant

pathogens may seriously alter disease epidemiology in the field.

Symbiont-pathogen interactions

The Auchenorrhyncha and Sternorrhyncha, including the vectors of plant pathogenic bacteria,

harbour both obligate and facultative endosymbionts which play important roles in supplying

nutrients and providing the host with other fitness benefits (Baumann, 2005; Morrow et al., 2017).

The main obligate (primary) symbiont are 'Ca. Sulcia muelleri' in Auchenorrhyncha, and 'Ca.

Carsonella ruddii' in psyllids. Moreover, *Sulcia* requires complementary (co-primary) symbiotic

bacteria to integrate its nutrient supply to the insect (McCutcheon & Moran, 2010). Similarly, psyllids

harbour secondary symbionts, such as *Sodalis* or *Arsenophonus* bacteria, with nutritional roles

(Morrow et al., 2017). In addition, the function of some symbionts of hemipterans vectors is still

unrecognized. For example, many bacteria generally known as reproductive manipulators, such as

Wolbachia, *Cardinium*, *Rickettsia* and *Arsenophonus*, have been found in several vector species;

366 however their role has not been characterized yet (Marzorati et al., 2006; Gonella et al., 2011; Jing et

367 al., 2014; Morrow et al., 2017; Iasur-Kruh et al., 2017). Moreover, some insect beneficial

368 microorganisms (e.g. *Rickettsia* and *Cardinium*), capable to colonize the salivary glands, may be

369 transferred from insect to plant and vice versa, possibly establishing endophytic relationships as well

370 (Caspi-Fluger & Zchori-Fein, 2010; Gonella et al., 2015; Iasur-Kruh et al., 2017). Despite the

371 emerging recognized need to study microbial communities affiliated to non-model insects

372 (Prosdocimi et al., 2015), which recently led to a growing number of evidences of co-existence of

373 plant pathogens and other microbes in the insect vectors, few studies directly investigated their

374 interactions (Table 2). Symbiont-pathogen exchanges were firstly studied in psyllids, and more

375 specifically in the CLas vector *D. citri*. This psyllid harbours three main endosymbionts: a species of

376 *Wolbachia*, the γ-Proteobacterium '*Ca.* Carsonella ruddii', an endosymbiont which may provide

377 nutritional benefits to its host (Thao et al., 2000), and '*Ca.* Profftella armatura', a β-Proteobacterium

378 with defensive function (Nakabachi et al. 2013). Fagen et al. (2012) firstly observed a negative

379 correlation between CLas infection rate with the relative abundance, within the microbial community,

380 of *Profftella*. Based on its genome sequence, *Profftella* was predicted to produce defensive toxins,

381 i.e. diaphorin and diaphorin-related polyketides. CLas-infected [CLas(+)] insects were found to have

382 dramatically elevated levels of two proteins involved in polyketide biosynthesis. In contrast, the

383 protein responsible for initiating diaphorin biosynthesis is down-regulated in CLas(+) *D. citri*

384 (Ramsey et al., 2015). Moreover, Ramsey et al. (2015) observed that the ratio between levels of

385 diaphorin and the related polyketide is significantly increased in CLas (+) compared to CLas

386 uninfected [CLas(-)] *D. citri*, suggesting changes in *Profftella* polyketide metabolism in response to

387 the presence of the pathogen or in direct or indirect response to changes induced by the pathogen in

388 infected plants. The up-regulation of the polyketide synthase (PKS) gene expression in CLas(+) *D.*

389 *citri* may be a specific response of *Profftella* to the presence of CLas, as part of an infection response

390 that may be mediated by *D. citri* (Ramsey et al., 2015). Such an interactive response may involve

391 *Carsonella* as well, which could provide the host with essential amino acids required for polyketide

392 production (Ramsey et al., 2015).

393 Besides psyllid-liberibacter interactions, further evidences of antagonistic relationships between

394 symbiotic bacteria and plant pathogens are reported for some Auchenorrhyncha vectors of

395 phytoplasmas. A bacterium in the Xanthomonadaceae, provisionally named *Dyella*-like bacterium

396 (DLB) (Iasur-Kruh et al., 2017), was isolated from the planthopper *H. obsoletus*, and showed anti-

397 phytoplasmal activity in inoculated plants (Iasur-Kruh et al., 2018). Indeed, despite being isolated

398 from an insect source, DLB showed endophytic traits: it was consistently found in the wild bush *Vitex*

399 *agnus-castus* L., and it was able to long-term colonize the phloem of different plant species, including

400 many hosts of phytoplasmas and liberibacters (Lidor et al., 2018). Once established in grapevines

401 infected by phytoplasmas, DLB reduced disease symptoms (Iasur-Kruh et al., 2018). Based on DLB

402 genome analysis, the authors suggested that such a drop of symptoms is related to inhibition of

403 pathogens, rather than competition or production of substances stimulating plant growth or defense

404 (Lahav et al., 2016; Iasur-Kruh et al., 2018). Moreover, DLB was demonstrated to inhibit the growth

405 of the cultivable model Mollicute *Spiroplasma melliferum* (Iasur Kruh et al., 2017).

406 Acetic acid bacteria in the genus *Asaia* are widespread in insects, including leafhoppers transmitting

407 phytoplasmas, and they were proposed to interact with insect vectors, possibly altering their spread

408 (Crotti et al., 2009). Strains with different phenotypes previously isolated from mosquitoes were

409 orally supplied to the experimental vector of FDP *E. variegatus*, which was successfully colonized.

410 One *Asaia* strain producing an air-liquid interface biofilm, after establishing in *E. variegatus,* reduced

411 its acquisition of FDP from broad beans in experimental conditions (Gonella et al., 2018). These

412 authors suggested that the strain of *Asaia* could affect the capability of the phytoplasma to cross the

413 gut epithelia for reaching salivary glands, even though the mechanisms regulating this interference

414 remain to be elucidated. However, such an alteration was imperfect and, when the pathogen

415 succeeded in colonizing the insect, transmission rates to broad beans were similar to those recorded

416 for control leafhoppers unexposed to *Asaia* (Gonella et al., 2018).

417 Additional interplays between symbiotic bacteria and plant pathogens have been suggested by

418 multiple prevalence studies, as in some cases positive correlation or mutual exclusion could be

419 detected between symbiotic and phytopathogenic bacteria. For example, the obligate symbiont

420 *Nasuia*, largely widespread in the family Cicadellidae, is present in most of leafhopper species

421 transmitting phytoplasmas, while non-vector species were shown to lack it (Wangkeeree et al., 2012).

422 It has been suggested that *Nasuia* could be required for successful transmission. Likewise, in the

423 planthopper FDP vector, *Dictyophara europaea* L., a negative correlation between infections by

424 phytoplasma and *Wolbachia* was reported, suggesting that the *Wolbachia* strain infecting *D. europaea*

425 displays antagonistic activities against the pathogen, or alternatively competes for insect colonization

426 (Krstić et al., 2018). On the other hand, in *D. citri* an increase in the ubiquitous *Wolbachia* titre was

427 reported with CLas infection (Fagen et al., 2012), indicating a more complicated interplay mechanism

428 with strain-specific variability. Direct interaction has been documented between *Wolbachia* and

429 CLas, as the first suppress the holing lytic promoter in a CLas-infecting phage in *D. citri* (Jain et al.,

430 2017).

431 The studies regarding synergies and interferences between symbiotic agents and plant pathogens offer

432 significant cues for disease treatment; moreover, further work is still required to describe new

433 interactive associations. Future work concerning such interplays should be aimed not only to identify

434 direct anti-pathogen activity expressed by symbionts, but also to alter the mutualistic exchange

435 recorded among vectors, symbionts and phytopathogens, and to influence insect ecology (e.g. by

436 driving plant choice and governing interactions with stresses).

437

Conclusions and open issues

439 The interactive roles of phytopathogenic and symbiotic bacteria in insects certainly represent an

440 emerging topic for researchers focusing on the transmission process of disease agents. A multi-actor

441 picture, involving insects, plants, and microbes, is resulting as the condition where the transmission

442   of plant pathogens arises. Consequently, the bacterial interactions occurring in insects affect the life

443   cycle of the host as well. First, considering the reported evolutive bilateral transition of the role of

444   many disease agents in their vectors from symbiotic to phytopathogenic life style, the effects of these

445   bacteria are a key issue for the study of insect-microbe relationships; however they are still mostly

446   unknown. Such effects may also result in the uneven competitive behaviours described for both

447   closely and distantly related pathogens. Many questions arise from this hypothesis. How is insect

448   immunity involved in differential growth rates of plant pathogens? What are the traits of vector-

449   pathogen interaction originating possible diversity in host responses? Are these bacteria at different

450   steps of transition from symbiont to pathogen or vice versa (e.g. the most competitive pathogens

451   supply the host with fitness advantages)? Most of these questions were addressed by Galetto et al.

452   (2018) using the *E. variegatus*-CYP-FDP model, but more work is needed to expand the analysis of

453   competitiveness conditions to different pathogens and vectors. Moreover, it is still unclear whether

454   non-competitive or beneficial interactions take place among pathogens in insects where multiple

455   infections are observed. Finally, how the plants are implicated in these interactions? Many examples

456   are available on the effects displayed by phytopathogens on the plant processes in favour of insects,

457   such as the promotion of insect attraction to infected hosts, allowing the pathogen spreading

458   (Orlovskis et al., 2015). However, whether pathogens that are capable to modulate their attractiveness

459   could display enhanced competitiveness against horizontally transmitted microbes (including other

460   phytopathogens) is poorly understood. Deep surveys of molecular and cellular machineries of insect-

461   phytopathogen-host plant relations could provide the answers to these issues.

462   Additional open questions involve the role of bacterial symbionts in plant pathogen competition and

463   spread. Only few examples of interactions between symbionts and pathogens have been described, in

464   spite of the high number of symbiotic bacteria depicted in most of vectors: direct evidences of an

465   interference with the transmission process in the insect or with symptom development in the plant

466   have been provided only for phytoplasmas (Gonella et al., 2018; Iasur-Kruh et al., 2018).

467 Furthermore, the mechanisms regulating beneficial or hostile exchanges have been only rarely

468 elucidated, and some bacterial pathogens were shown to exhibit mutualistic effects on their vectors,

469 while other caused fitness costs (Hogenhout et al., 2008; Tamborindeguy et al., 2017). An open field

470 for future research is the awareness of whether harmful or beneficial roles are in some way the result

471 of interactions with bacterial symbionts co-inhabiting the same host. A similar evidence of indirect

472 effect on the insect fitness as a consequence of symbiont suppression was observed in virus-

473 transmitting aphids. In the soybean aphid *Aphis glycines* Matsumura, a drop in the concentration of

474 the endosymbiotic *Buchnera* was observed in insects exposed to the beetle-transmitted bean pod

475 mottle virus, resulting in reduced aphid fecundity (Cassone et al., 2015).

476 Finally, a still unexplored field for vectors of phloem-limited pathogen is the manipulation of

477 symbiotic microbes to drive their interaction with plant pathogens toward antagonistic activities, by

478 means of paratransgenesis. A similar approach was proposed for example for a xylem-restricted

479 pathogenic agent, i.e. the *Xylella fastidiosa* strain causing Pierce disease to grapevine. A bacterium

480 reported as an insect symbiont and an endophyte, *Alcaligenes xylodoxidans denitrificans*, was

481 proposed as a candidate agent to be genetically transformed to display anti-*Xylella* molecules (Bextine

482 et al., 2004).

483 Along with being of certain interest to elucidate biological mechanisms regulating insect-bacteria

484 relationships, the gain of knowledge concerning microbial interactions occurring in insect vectors

485 have important implications for disease epidemiology and control. From the epidemiological point of

486 view, the competition among plant pathogens alters the rates of transmission by vectors, and possibly

487 influences their fitness as well, with a final impact of the spread of diseases on different plants. From

488 the point of view of disease control, the study of microbial interactions in the vectors could provide

489 valuable tools to manage crop infections by altering vector competence via symbiotic control

490 approaches (Alma et al., 2010). Possible strategies include the identification of detrimental effects

491   played by symbionts on plant pathogens in the insect, or the selection of new molecular targets to

492   interrupt beneficial interplays among bacteria.

493

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Table legends

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Table 1 Multiple bacterial infections in the vectors of phloem-limited pathogens. Only reports

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showing mixed infections in the same host individual, involving distinct plant disease agents or

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symbiotic bacteria with phytopathogens, are listed.

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Table 2 Symbiont-pathogen interactions reported in the vectors of phloem-limited plant pathogenic

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bacteria.

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Figure legends

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Figure 1 Insect symbionts could be useful for controlling the transmission of phloem-limited plant

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pathogens. Phloem-restricted plant pathogens are indicated as red, purple or violet dots, while

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microbial symbionts are depicted with other different colours. Microbe movements are indicated with

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red arrows. Symbiont-mediated control mechanisms of pathogen transmission are listed on the right

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and corresponding numbers are depicted in gut (in green circles), hemolymph (in orange circles) and

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salivary glands (inset, in blue circles).

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TABLES

Vector taxonomic position	Vector family	Vector species	Phytopathogen multiple infection	Symbiont - phytopathogen multiple infection	Reference
Auchenorrhyncha - Fulgoromorpha	Cixiidae	*Hyalesthes obsoletus* Signoret		'*Ca.* Sulcia muelleri' + *Wolbachia* + '*Ca.* Vidania fulgoroidaeae' + '*Ca.* Purcelliella pentastirinorum' + 16SrXII phytoplasma	Gonella et al., 2011
		Pentastiridius leporinus L.		'*Ca.* Sulcia muelleri' + '*Ca.* Purcelliella pentastirinorum' + *Wolbachia* + '*Ca.* Arsenophonus phytopathogenicus'	Bressan et al., 2009a
Auchenorrhyncha-Cicadomorpha	Cicadellidae	*Amplicephalus curtulus* Linnavuori & DeLong	Phytoplasmas, groups: 16SrI + 16SrXII		Longone et al., 2011
		Amplicephalus funzaensis Linnavuori	Phytoplasmas, groups: 16SrI + 16SrVII		Perilla-Henao et al., 2016
		Circulifer tenellus (Baker)	16SrVI phytoplasma + *S. citri*		Lee et al., 1998a Swisher et al., 2018
		Euscelidius variegatus Kirshbaum	Phytoplasmas, groups: 16SrI + 16SrV	bacterium of *E. variegatus* (BEV) + 16SrI phytoplasma *Asaia* + 16SrV phytoplasma	Rashidi et al., 2014 Galetto et al., 2009 Gonella et al., 2018
		*Euscelis incisus (*Kirschbaum)	Phytoplasmas, groups: 16SrI + 16SrIII + '*Ca.* Phytoplasma pruni'		Orságová et al., 2011
		Euscelis lineolatus Brulle	Phytoplasmas, groups: 16SrII+16SrXII		Landi et al., 2013
		Exitianus atratus Linnavuori	Phytoplasmas, groups: 16SrI + 16SrVII		Perilla-Henao et al., 2016
		Graminella nigrifrons (Forbes)	Phytoplasmas, groups: 16SrI + 16SrVII 16SrI + 16SrX		Arocha-Rosete et al., 2011
		Macrosteles sexnotatus (Fallén)		'*Ca.* Sulcia muelleri' + *Nasuia* + 16SrI phytoplasma	Ishii et al., 2013
		Macrosteles striifrons Anufriev		'*Ca.* Sulcia muelleri' + '*Ca.* Nasuia deltocephalinicola' + 16SrI phytoplasma	Ishii et al., 2013
		Matsumuratettix hiroglyphicus (Matsumura)		Bacterium associated with *M. hiroglyphicus* (BAMH) (*Nasuia*) + '*Ca.* Sulcia muelleri' + 16SrXI phytoplasma	Wangkeeree et al., 2012
		Osbornellus horvathi Matsumura	'*Ca.* Phytoplasma asteris' + '*Ca.* Phytoplasma phoenicium'		Rizza et al., 2016

		Paratanus exitiosus (Beamer)	Phytoplasmas, groups: 16SrII + 16SrVII + 16SrXII		Longone et al., 2011
		Recilia dorsalis Motschulsky		BAMH + *'Ca.* Sulcia muelleri' +16SrXI phytoplasma	Wangkeeree et al., 2012
		Recilia sp. nr. *vetus*		BAMH + *'Ca.* Sulcia muelleri' +16SrXI phytoplasma	Wangkeeree et al., 2012
		Scaphoideus titanus Ball		*'Ca.* Cardinium hertigii' + 16SrV phytoplasma	Marzorati et al., 2006
Sternorrhyncha	Psyllidae	*Cacopsylla chinensis* (Yang & Li)	Phytoplasmas, groups: 16SrII + 16SrX		Liu et al., 2011
		*Cacopsylla melanoneura (*Förster*)*		CLeu + *'Ca.* Phytoplasma mali'	Camerota et al., 2012
		Cacopsylla pyri L.	Phytoplasmas, groups: 16SrI + 16SrXII 16SrX + 16SrXII 16SrI + 16SrX	CLeu + *'Ca.* Carsonella ruddii' + *Arsenophonus* + *Ralstonia* + *'Ca.* Phytoplasma pyri'	Križanac et al., 2010 Raddadi et al., 2011 Camerota et al., 2012
		Cacopsylla pyricola Förster		*Arsenophonus* + *'Ca.* Phytoplasma pyri'	Cooper et al., 2017
		Diaphorina citri Kuwayama		*'Ca.* Carsonella ruddii' + *'Ca.* Profftella aramtura' + *Wolbachia* + CLas	Kruse et al., 2017 Ramsey et al., 2017
				Ralstonia + CLas	Kolora et al., 2015
	Triozidae	*Bactericera cockerelli* (Sulc)		*Erwinia* sp. + *Wolbachia* + *Staphylococcus* sp. + *Enterococcus* sp. + CLso	Kolora et al., 2015
		Bactericera trigonica Hodkinson	CLso + phytoplasmas, (group 16SrVI+ 16SrI)		Swisher et al., 2018

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Entomologia Experimentalis et Applicata

Table 2 Symbiont-pathogen interactions reported in the vectors of phloem-limited plant pathogenic bacteria.

Insect	Phytopathogen	Symbiont	Interaction	Reference
Diaphorina citri	CLas	'Ca. Profftella armatura'	Upregulation of genes involved in biosynthesis of diaphorin polyketide.	Ramsey et al., 2015
		Wolbachia	Positive correlation	Fagen et al., 2012
Hyalethes obsoletus	16SrXII phytoplasma	*Dyella*-like bacterium (DLB)	Reduction of phytoplasma-related symptoms in grapevine	Iasur-Kruh et al., 2018
Euscelidius variegatus	16SrV phytoplasma	*Asaia* sp.	Reduced phytoplasma acquisition in Asaia-infected individuals	Gonella et al., 2018
Matsumuratettix hiroglyphicus *Recilia dorsalis* *Recilia* sp. nr. vetus	phytoplasmas	Bacterium associated with *M. hiroglyphicus* (BAMH) (*Nasuia*)	BAHM suggested to be required for successful phytoplasma transmission	Wangkeeree et al., 2012
Dyctiophara europaea	16SrV phytoplasma	*Wolbachia*	Mutual exclusion	Krstić et al., 2018

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