

Diversity of endosymbiotic bacteria in *Harmonia*
axyridis and *Halyzia sedecimguttata*

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Covid-19 impact statement

COVID-19 happened to me and my thesis. Since the outbreak of the pandemic, I have had a high fever that I have never had before. My health was affected and I had to be in isolation for two weeks. Covid-19 has had a significant impact on my opportunity to generate as much data as originally planned. In particular this was due to disruption in lab work and access to facilities. Ladybirds breeding takes months and requires daily attention to be fully successful. This was simply not possible, with ladybirds being kept on a maintenance diet of artificial food, which does not promote reproduction, for considerable periods of time. The timing of the first lockdown meant many months of preparation work had to be repeated and by then, time constraints meant the original scope was greatly reduced. The upshot was that the project developed a greater bioinformatics and literature-based focus. I have put a great deal of time and effort into learning these new skills, however this altered approach was also not without challenges, in particular the need to learn new bioinformatics skills remotely.

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Abstract

Ladybirds are economically important insects that can be used as beneficial control agents against pests such as aphids and powdery mildew management in the agricultural industry, due to their aphidophagous and mycophagous nature. With the emergence of resistance to insecticides in recent years, using beneficial agents may be the best choice for farmers, a situation that could increase the commercial demand for ladybirds. Ladybird species are also of interest to study because they are known to be infected with a range of bacterial endosymbiont species that alter the biology of their host. Sex-ratio distortion caused by male-killer endosymbionts is one such example of altered host biology, which might also prove to be a limiting factor for the rapid and commercial multiplication of ladybirds. However, there are also many examples of beneficial endosymbionts that provide useful services to their host. In this project, we tried to understand the diversity of bacterial community in two ladybird species, *Harmonia axyridis* and *Halysia sedecimguttata*, which are found widely in the UK but that differ in their ecologies. We used 16S rDNA sequences cloned and amplified from bacteria followed by comparison with known sequences on Genbank to characterise sequences of bacteria. Phylogenetic analysis was also used to identify other related microorganisms that might provide insights into the roles the bacteria might play in their ladybird hosts. Novel bacteria from 10 different classes, including those closely related to bacteria previously reported from invertebrate hosts, were identified. Of these 10 Classes, 7 were found in *H. axyridis* and 8 were found in *H. sedecimguttata*. Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, Bacilli and

Actinobacteria were identified in both ladybird species. Flavobacteriia and Terriglobia were only found in *H. axyridis* whereas Cytophagia, Chitinophagia and Hydrogenophilia were only found in *H. sedecimguttata*.

H. axyridis were commercially available ladybird before being banned due to the negative impact on the indigenous species. However, understanding the function of identified bacteria might help to engineer microbiome or endosymbionts in the native ladybirds in the future to make them a better pest regulator. In addition to this beneficial effect, it might also help us understand the interaction between microbes and invertebrate hosts and therefore tell us more about how populations of ladybirds are influenced by these microbes.

Chapter 1: General introduction

1.1 Morphology and classification of ladybirds

Majerus (1994) recounts the history and etymology of the term 'ladybird' to have been coined from the 7-spotted insect (*Coccinella septempunctata*) which was the most common species in Britain before proceeded by the Harlequin ladybird (*Harmonia axyridis*) (Hemsley and Holland, 2020). The red background of the insect's elytra represents a cloak of the Lady, Virgin Mary and the seven black spots symbolise her seven joys and seven sorrows as depicted in early paintings and sculptures. Hitherto, the term ladybird has been used to identify insects under the family Coccinellidae with tiny to moderate size (1-10 mm or 1/16 - 3/4 inch in length) with a circular or oval body as presented in Figure 1.1 (Roy *et al.*, 2013).



Figure 1.1 The 7-spot ladybird beetle (*Coccinella septempunctata*) (obtained from Wang *et al.*, 2013).

Ladybird beetles are characterised by the presence of short, clubbed antennae in the head and a wide pronotum located between the head and elytra (Figure 1.2). The pronotum has diverse decorations; however, it is not as brightly coloured as the elytra. The adults have short retractable legs under the body; the first posterior pair is attached at the prosternum while the other two pairs attach to the abdomen.

The feet (tarsi) are divided into four segments, however, only three are readily visible while the fourth segment is tiny (Roy *et al.*, 2013).

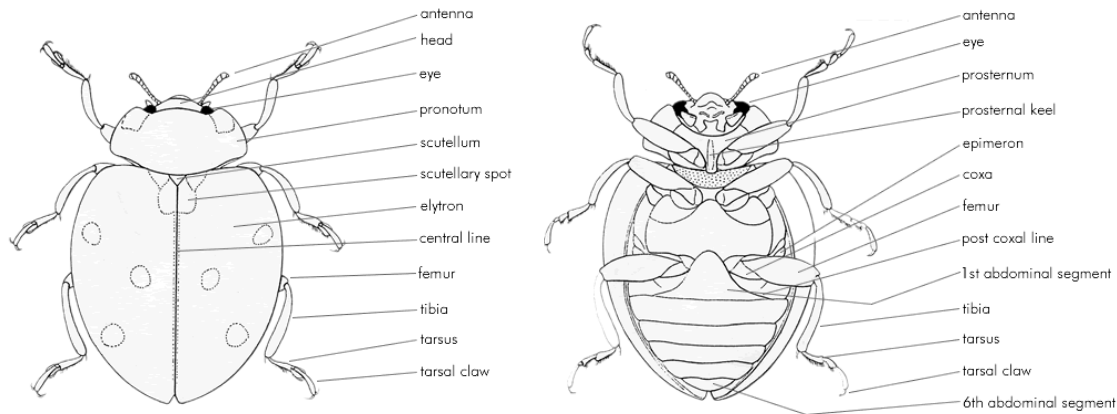


Figure 1.2 Morphology of a ladybird left (dorsal view) right (Ventral view) (adapted from UK ladybird webpage).

Overall, the ladybird beetles have brightly coloured and patterned elytra with black spot distributions. The elytra might be black spots on red (Figure 1.1) or vice-versa. In some cases, colours may be yellow and black, or brown with cream spots. However, there are exceptional cases where stripes run across the elytra instead of spotted patterns (Li *et al.*, 2014) (Figure 1.3). Elytral patterns demonstrate the diverse pigmentation polymorphism amongst insects. For instance, *Harmonia axyridis* is known to express over 200 elytral pigment patterns (Tan, 1946).

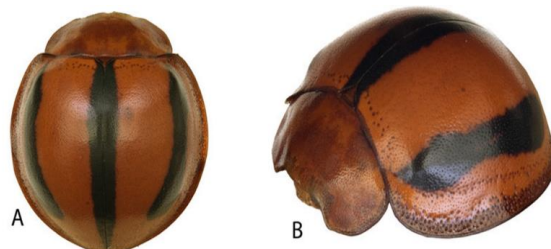


Figure 1.3 The Dorsal (A) and lateral (B) view of the striped elytra of the *Phrynocaria wallacii* (obtained from Li *et al.*, 2014).

Ladybirds belong to the domain Eukarya with their complete cells having a nucleus and other membrane-bound organelles (Staley, 2017). Notably, the classification of ladybird beetles has been consistently based on observable morphological features. These arthropods have jointed appendages and a chitin exoskeleton with a specific colour pattern that has been used, among other features, to stratify them into the various species. The taxonomic classification of ladybird as described by Lawrence and Newton, (1995) is shown below:

	Stages of classification	Taxonomic classification
1.	Domain:	Eukarya
2.	Kingdom:	Animalia
3.	Phylum:	Arthropoda
4.	Class:	Insecta
5.	Order:	Coleoptera
6.	Suborder:	Polyphaga
7.	Superfamily:	Cucujoidea
8.	Family:	Coccinellidae
9.	Common name	Lady beetle/ladybird

Such classification has confirmed the wide variability in terms of ecological habitat and morphological features among the ladybird beetles. Seago *et al.* (2011) stated that there are over 6000 species of identified ladybird beetles worldwide. However, the species distribution is based on environmental conditions (Liere *et al.*, 2019). Almost every garden has at least one species of ladybird and in some cases, up to five or more species are common (Roy *et al.*, 2013). A study conducted by Brown and Roy (2015) identified a competitive relationship between the invasive *H. axyridis* and the native species of ladybirds in the UK, which has resulted in a sharp decline in the populations of some species.

Approximately 46 species of the Coccinellidae subfamily have been identified in Britain as recorded by the UK Ladybird survey organisation (Roy and Brown, 2015). However, only 26 Coccinellid species have received researchers' attention and are readily recognisable as ladybirds by the UK ladybird survey (Table 1.1). Researchers in the field of entomology have suggested that there are several features that can be used to identify and characterise the Coccinellids into their respective species, without the involvement of molecular detection techniques. Some of these features are obvious such as number, size, colour and pattern of spots on the elytra and pronotum and abdominal colour variation (Rahaman and Aniszewski, 2015; Biranvand and Shakarami, 2015). However, given the extreme variation of colour-patterns within single species, such as *H. axyridis* (Tan and Li, 1932) and *Adalia bipunctata* (Lus, 1928, 1932), as well as similarity with morphs of other ladybird species, (cited as a factor in Müllerian mimicry rings, Brakefield, 1985), combining physical characteristics and molecular markers will allow greater accuracy and confidence in determining species identity. The species show diverse abdominal distinctions based on colour, shape, size of the pyramid, length and shape of the abdominal lines and how the abdominal lines join with the abdominal tip. A typical illustration based on abdominal examination is shown in Figure 1.4 as obtained from the experiment conducted by Rahaman and Aniszewski (2015), who describe detailed measurements of abdominal length, width, colour and shape and how these can be used to confirm species.

Sub-family	Species	Common name
Epilachninae	<i>Henosepilachna argus</i> (Geoffroy in Fourcroy)	Bryony ladybird
Epilachninae	<i>Subcoccinella 24-punctata</i> (Linnaeus)	24-spot ladybird
Coccinellinae	<i>Adalia 10-punctata</i> (Linnaeus)	10-spot ladybird
Coccinellinae	<i>Adalia 2-punctata</i> (Linnaeus)	2-spot ladybird
Coccinellinae	<i>Adonia variegata</i> (Goeze)	Adonis ladybird
Coccinellinae	<i>Anatis ocellata</i> (Linnaeus)	Eyed ladybird
Coccinellinae	<i>Anisosticta 19-punctata</i> (Linnaeus)	Water ladybird
Coccinellinae	<i>Aphidecta obliterate</i> (Linnaeus)	Larch ladybird
Coccinellinae	<i>Calvia 14-guttata</i> (Linnaeus)	Cream-spot ladybird
Coccinellinae	<i>Coccinella 11-punctata</i> (Linnaeus)	11-spot ladybird
Coccinellinae	<i>Coccinella 5-punctata</i> (Linnaeus)	5-spot ladybird
Coccinellinae	<i>Coccinella 7-punctata</i> (Linnaeus)	7-spot ladybird
Coccinellinae	<i>Coccinella hieroglyphica</i> (Linnaeus)	Hieroglyphic ladybird
Coccinellinae	<i>Coccinella magnifica</i> (Redtenbacher)	Scarce 7-spot ladybird
Coccinellinae	<i>Halyzia 16-guttata</i> (Linnaeus)	Orange ladybird
Coccinellinae	<i>Harmonia 4-punctata</i> (Pontoppidan)	Cream-streaked ladybird
Coccinellinae	<i>Harmonia axyridis</i> (Pallas)	Harlequin ladybird
Coccinellinae	<i>Hippodamia 13-punctata</i> (Linnaeus)	13-spot ladybird
Coccinellinae	<i>Myrrha 18-guttata</i> (Linnaeus)	18-spot ladybird
Coccinellinae	<i>Myzia oblongoguttata</i> (Linnaeus)	Striped ladybird
Coccinellinae	<i>Propylea 14-punctata</i> (Linnaeus)	14-spot ladybird
Coccinellinae	<i>Psyllobora 22-punctata</i> (Linnaeus)	22-spot ladybird
Coccinellinae	<i>Tytthaspis 16-punctata</i> (Linnaeus)	16-spot ladybird
Chilocorinae	<i>Chilocorus 2-pustulatus</i> (Linnaeus)	Heather ladybird
Chilocorinae	<i>Chilocorus renipustulatus</i> (Scriba)	Kidney-spot ladybird
Chilocorinae	<i>Exochomus 4-pustulatus</i> (Linnaeus)	Pine ladybird

Table 1.1 Twenty-six different species which are readily recognizable as ladybirds in the UK.

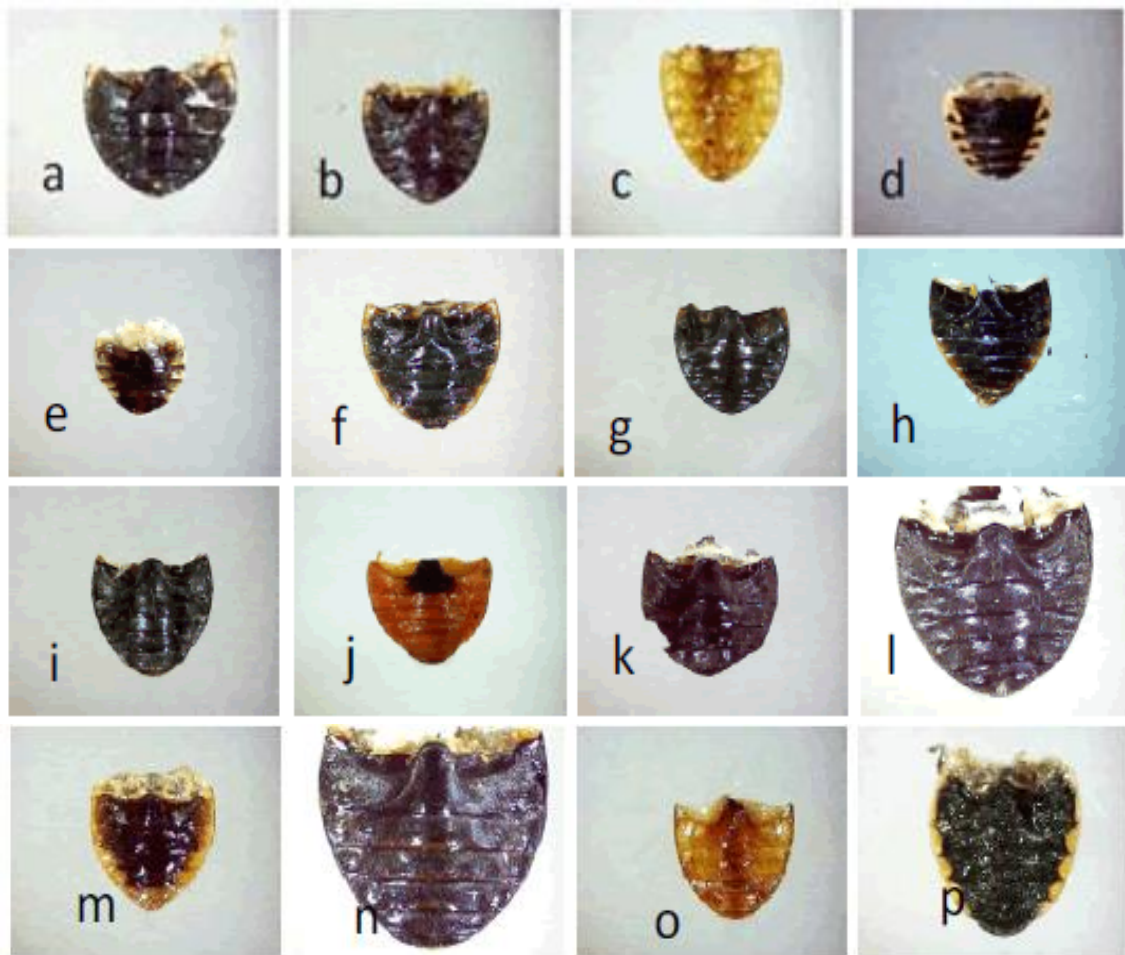


Figure 1.4 Examination of ladybird beetles abdomens and colour variation in 16 different species (obtained from Rahaman and Aniszewski, 2015). (a) *Coccinella trifasciata* (b) *Coccinella quinquepunctata* (c) *Halyzia sedecimguttata* (d) *Adalia bipunctata* (e) *Psyllobora vigintiduopunctata* (f) *Calvia quatuordecimguttata* (g) *Coccinella hieroglyphica* (h) *Adalia sexpustulata* (i) *Propylea quatuordecimpunctata* (j) *Chilocorus stigma* (k) *Coccinella undecimpunctata* (l) *Coccinella septempunctata* (m) *Adalia quadrimaculata* (n) *Anatis ocellata* (o) *Cryptolaemus montrouzieri* (p) *Hippodamia tredecimpunctata*.

1.2 Ladybird Lifecycle

The life span of many adult ladybird species is estimated to be one to two years. This depends on a variety of factors, particularly the weather relating to temperature and humidity, the availability of food source and predators and sexual activity (Nedved and Honek, 2012). The ladybird beetles undergo four major developmental stages for each complete cycle; the mature adults lay batches of between 10 and 50 eggs, which hatch into larvae then pupate before maturing to

adults (Majerus, 1994) (Figure 1.5). As such, these beetles are classified as holometabolous insects with a complete life cycle (Slipinski, 2013). Mating between the mature males and females usually takes place in spring or early summer, before oviposition of the yellow or orange eggs between June and July in the UK. The eggs hatch between 4 and 10 days after laying, each producing a bright coloured larva, which moults three times over 4-6 weeks to the pupal stage, which lasts about 15 days. During winter the adult ladybirds remain dormant in a stage referred to as “overwintering” (Awad *et al.*, 2013). As food becomes available again, they emerge and start to feed and mate. Most of the adults feed primarily on aphids and other plant-sap sucking insects until the end of summer. However, a few species, such as *Subcoccinella vigintiquatuorpunctata*, principally feed on the leaves of a variety of herbaceous plants (Majerus and Kearns, 1989); *Henosepilachna argus* feeds on white bryony and Cucurbitaceous plants, including melon or watermelon crops (Mora *et al.*, 2015) and *Halyzia sedecimguttata*, *Tytthaspis sedecimpunctata* and *Psyllobora vigintiduopunctata* all principally feed on powdery mildews (moulds of the family Erysiphaceae) (Sutherland and Parrella, 2009).

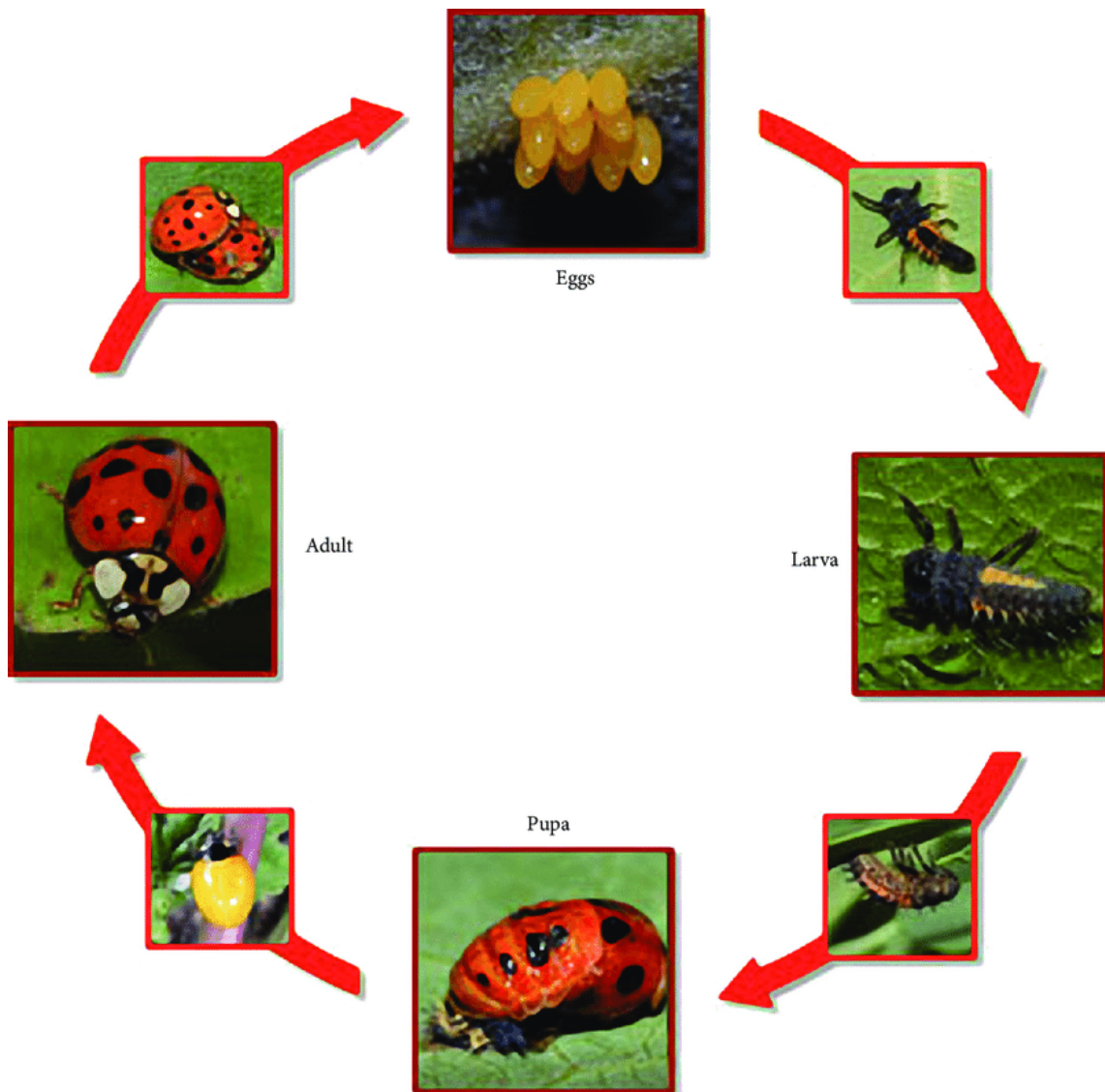


Figure 1.5 Life Cycle of a *Harmonia axyridis* ladybird (obtained from Wang *et al.*, 2013).

Normally, ladybirds complete one cycle per year, however, in some years, a few species such as the 2-spot and 14-spot ladybirds have a second generation. Moreover, *H. axyridis* ladybirds in Britain have been reported to have two

generations and occasionally a partial third generation in a year (Honek *et al.*, 2018).

Most of the species lay eggs that are oval with one end flattened and fastened to the plant or alternative suitable oviposition site. They deviate from light yellow to a deep orange hue. They are laid on the firm parts of plants, including stem, leaves and bark of trees, often nearby prey, or prey populations. Many species fix their eggs at one end, so they are found in an upright position, with exception of the pine ladybird which lays eggs on their sides. The number of eggs laid by females depends on some conditions, such as the type of food eaten by female adults and mating behaviour. For example, when the 2-spot ladybird was fed the black bean aphid, *Aphis fabae*, the total eggs laid was 738 (n=8) during her lifetime, averaging 9.3 daily, but the number was 1535 eggs (n=10), averaging 20.4 every day when the pea aphid, *Acyrtosiphon pisum*, was consumed (Hariri, 1966). The number of eggs produced, affected by the quantity of food eaten, has also been observed in the 11-spot ladybird (Ibrahim, 1955). The number of eggs laid in the 2 spot-ladybird has been shown to increase after each mating (Semyanov, 1970). Females mating with 10 different males had higher fecundity and egg hatch rates than females mating 10 times with the same male (Haddrill *et al.*, 2007), suggesting that higher promiscuity increases fecundity. The development time from egg to larva and then to pupa seems to be negatively correlated to an increase in ambient temperature. The data presented in Table 1.2 demonstrate a significant decrease in the time taken for egg hatching and transition from larva to pupa by the 7-spot ladybird, so that eggs will hatch in 5 days at 20°C, while at 30°C it is 1.9 days. However, if the

temperature goes above the upper limit of temperature tolerance (35°C), eggs may fail to hatch (Hodek, 1973).

Temperature (°C)	Egg (days)	Larva (days)	Pupa (days)
15	10.3	35.5	15.0
20	5.0	18.6	8.4
25.6	2.6	8.7	4.0
30	1.9	6.7	2.9
35	1.8	5.4	2.5

Table 1.2 Effect of temperature on the development of eggs to larva and pupa in days.

1.3 The microbial fauna in insects

Douglas (2015) refers to insects as multiorganismal entities due to the diverse range of microbial inhabitants that are known to inherently reside in the insect gut, haemocoel and even intracellularly. However, only a select group of microbial taxa are favored by the conditions of the insect gut, immunity and modes of transmission. Microbes colonise diverse niches within the insect body (Vallet-Gely *et al.*, 2008). Such microbial communities account for up to 10% of the insect dry mass. Insects have an intrinsic relationship with microbes, which has given the microbes a unique ability to colonise insect guts and with elusive features to evade immune response (Wernegreen, 2002). Besides, some microbes naturally find the insect gut to be a suitable environment for their successful colonisation (Dillon and Dillon, 2004). Such microbes colonise the insect cuticle, gut and cytoplasm of various cells (Douglas, 2015).

Whereas some microbes cause pathological manifestations, some provide significant benefits to their hosts in a symbiotic model (Ankrah and Douglas, 2018). Khaeso *et al.* (2018) described that such microbes influence a wide range of host traits and functions that range from detoxification of plant anti-herbivory

compounds or insecticides (van den Bosch and Welte, 2017) to modulation of the vector efficiency in pathogen transmission (Ricci *et al.*, 2012). Further, the gut bacteria in many insect species also contribute to B vitamin supplement, shielding from parasites and pathogens, regulation of immune responses and production of important semiochemicals that function in chemical defence behaviour to deter predators (Salem *et al.*, 2014). The pyrazines are a major group of these volatile compounds synthesised through the metabolic activity of bacterial symbionts and responsible for this characteristic deterrent (Schmidtberg *et al.*, 2019).

Zhang *et al.* (2019) identified the bacterial phyla within the insect gut to be dominated by Proteobacteria; Bacteroidetes; Firmicutes including *Lactobacillus* and *Bacillus* species; Clostridia; Actinomycetes; Spirochetes; Verrucomicrobia and Actinobacteria. Most insect species' microbiomes are comprised of all or most of these bacterial phyla. Chen *et al.* (2018) found that up to 70% of all insect species are inhabited by such bacteria leading to significant changes in insect reproduction, among other physiological functions. In addition to bacteria, the insects' guts also host protists, fungi and archaea (Engel and Moran, 2013).

There is a highly variant mode of interaction between insect physiology and invasive microbial activities. Vallet-Gely *et al.* (2008) described four major types of interaction: pathogenic, infectious, opportunistic and vectors, including passive vectors. A classical pathogenic mode of interaction is illustrated by the plague bacteria, *Yersinia pestis* and the flea (Gage and Kosoy, 2005). Because of *Y. pestis* colonies blocking blood flow to its gut, the flea is forced to ingest more blood from its rodent host and amidst repetitive regurgitations.

Vallet-Gely *et al.* (2008) also describe that insects acquire such microbes through ingestion, however, with highly dynamic consequences of interaction. For instance, the experimental investigations conducted by Nehme *et al.* (2007) confirmed that ingestion is the primary mode of bacterial acquisition of *Serratia marcescens* by *Drosophila* and the outcomes are unpredictable. A recent investigation conducted by Chambers *et al.* (2019) showed that infection of *Drosophila* by *Providencia rettgeri* negatively influences the ability of flies to resist starvation. In other investigations, Harada and Ishikawa (1997) showed that the ingestion of the *Erwinia aphidicola* significantly reduces the bodyweight of pea aphids. Nonetheless, *E. aphidicola* is a common gut endosymbiont of pea aphids (Gauthier *et al.*, 2015).

Molecular detection techniques have also confirmed the presence of diverse bacterial species, including *Bartonella* sp., *Rickettsia felis* and *Wolbachia pipientis* within the gut of arthropods of the Pulicidae family (*Ctenocephalides felis*) (Rolain *et al.*, 2003). Proteomic studies have also validated how the *Pseudomonas* sp. in *Paederus* beetles assists in protecting the insect from predation by synthesising a polyketide “pederin” substance (Piel, 2002). Another defensive trait is also enhanced by the *Hamiltonella defensa* bacteria inside their aphid host (Degnan and Moran, 2008). The experimental study conducted by Gerth *et al.* (2015) also identified the presence of *Wolbachia*, *Rickettsia*, *Arsenophonus* and *Cardinium* spp. among bees (Hymenoptera, Anthophila).

The colonisation of arthropod guts by microbial species is diverse and so is the nature of the association. Zug and Hammerstein (2012) stated that *Wolbachia*

bacterial species are extremely widespread in many orders of insects with the ability to modify the insects' reproductive physiologies. Aebi and Neumann (2011) indicate that *Wolbachia* or *Rickettsia* bacterial species are common among the honeybees (*Apis mellifera*) with the beneficial implication of reducing chances of fungal and viral infection in such insects. Other studies have also identified the presence of *Arsenophonus* spp (Yañez *et al.*, 2016).

The arthropods of the order Lepidoptera also host a significant variety of bacterial endosymbionts. The literature review conducted by Voirol *et al.* (2018) confirmed the presence of five dominant bacterial genera among moths and butterflies – *Wolbachia*, *Pseudomonas*, *Bacillus*, *Staphylococcus*, *Enterobacter* and *Enterococcus* (Duploux and Hornett, 2018). Voirol *et al.* (2018) suggested that *Wolbachia* and *Spiroplasma* can colonise and modulate the reproductive physiology of butterflies. Other studies have also identified *Wolbachia* within the ovaries and hemocytes of the woodlice species *Armadillidium vulgare* and *Porcellio dilatatus* (Le Clec'h *et al.*, 2013). Such relationships are dynamic and diverse and while some are mutually beneficial, others are pathogenically detrimental.

1.4 Symbiosis

The term symbiosis is derived from the Greek word *sumbiōsis*, which means 'living together' (De Bary, 1879). As such, symbiosis describes an association between two organisms of different species that live together in a close and beneficial physical relationship (Douglas, 2014). Symbiotic relationships exist in two forms depending on the location of the colonisation by the microorganism. Whereas in

ectosymbiosis, the partner remains on the body surface of the host, endosymbiosis delineates a case whereby one organism (endosymbiont) resides inside the body or cells of another organism (Van Arnam *et al.*, 2018). Endosymbiosis is a widely investigated phenomenon mainly by molecular biology researchers such as Rolain *et al.* (2003), whose experimental studies identified the presence and roles of various *Bartonella* spp., *Rickettsia felis* and *Wolbachia pipientis* inside cat fleas.

Endosymbiotic relationships exist among numerous species of organisms. Investigations on the *Oncocherca* worms have demonstrated the obligatory roles of the *Wolbachia* bacterial endosymbionts in mediating the microfilaria pathogenesis (Taylor *et al.*, 2000). Further, the experimental inquiries by Hoerauf *et al.* (2008) to determine the significance of the *Wolbachia* spp. found that use of the antibiotic doxycycline resulted in the death of 60% of the worms and hence they identified the obligatory association between worm and bacteria.

The symbiotic interrelationships exist in three forms; mutualism, commensalism and parasitism, regarding the impact that symbiont has on the host (Leung and Poulin, 2008). In mutualism, each organism benefits from the interaction, as typified in the interaction between the ungulates and their intestinal bacteria (Beckrich, 2015). As the ungulates derive benefit from the cellulase produced by the bacteria, which enables breakdown of cellulose; the bacteria derive benefit from the possession of a steady supply of nutrients issued by their host environment (Jami *et al.*, 2013). Mutualism is most common between the sap-feeding insects such as the aphids, hoppers, spittlebugs and cicadas, whereby the

endosymbionts provide the essential amino acids to enhance the host's nutrition (Wernegreen, 2012).

Contrary to mutualism, commensalism only awards benefits to one organism; however, the partner remains unharmed. The association is shared among the multicellular higher-order organisms. For instance, the orchid plants that grow on trunks and branches of other trees where orchids benefit by getting adequate sunlight and nutrition that flow on branches, while not harming the host tree in any way (Rasmussen and Rasmussen, 2018). Parasitism, on the other hand, is a relationship where only the parasite gets the benefits and the host is harmed. Hookworms and whipworms in the animal intestine are a classic example of parasitism (Little *et al.*, 2009).

1.5 Endosymbiotic bacteria in arthropods and their roles

Phylum Arthropoda encompasses organisms with segmented body parts, an exoskeleton with at least one jointed appendage and is the largest phylum in kingdom Animalia. These organisms host a wide range of intracellular endosymbiotic bacteria with diverse beneficial and harmful significance. Several researchers have investigated the types, density and roles of bacterial endosymbionts in such organisms and recent reviews have confirmed that the outcomes of such experiments have remained consistent over the years (Eleftherianos *et al.*, 2013). Among other microorganisms, bacteria form the largest and most diverse group of endosymbionts, colonising the arthropod body parts (Weinert *et al.*, 2015).

Endosymbiotic bacteria exist in two large groups; obligate and facultative strains. The obligate endosymbionts live in the cytosol of specialised host cells (bacteriocytes) and are vertically transmitted through reproduction. The microbes provide specific nourishments that are vital for the wellbeing and development of the hosts (Engel and Moran, 2013). One of the widely studied examples is the symbiosis between aphids and the bacterial symbiont, *Buchnera aphidicola*, which is thought to have originated approximately 200 million years ago (Baumann, 2005). As in other such associations, the *B. aphidicola* has a highly minimised genome; however, it retains essential genes that make it possible to provide nutrients (vital amino acids and vitamins) to its host (Shigenobu *et al.*, 2000). Some obligate endosymbionts also coexist in distinct bacteriocytes of sap-feeding insects with even more-minimised genomes; essentially, the supportive gene sets required by the host for encoding nutrient-provisioning biosynthetic pathways (McCutcheon and Moran, 2012).

Other insects such as the grain weevils may exploit immune mechanisms to control obligate symbionts by using antimicrobial peptides (AMPs) to prevent a bacteriocyte-associated symbiont from invading other host tissues (Login and Heddi, 2013). Few other cases have been demonstrated that AMPs produced by insect hosts target symbiotic bacterial populations (Mergaert, 2018). AMP expression at a controlled and balanced level plays a role in maintaining the homeostatic relationship and structuring the gut commensal microbial community in the fly *Drosophila* (Ryu *et al.*, 2008). Similarly, AMPs in the gut of honey bees are part of a system that determines the structure and composition of the gut

bacterial symbionts (Kwong *et al.*, 2017). It can also be proposed that the tsetse fly employs AMPs to manage its maternally inherited endosymbiont *Sodalis glossinidius* (Pontes *et al.*, 2011). Moreover, cysteine-rich peptides structurally related to AMPs are widely produced by the crypts (sections of the midgut) in the stinkbug *Riptortus pedestris* and are thought to be essential for regulating its *Burkholderia* crypt symbiont population (Futahashi *et al.*, 2011).

Facultative endosymbionts are not necessarily needed by the hosts and are often passed horizontally, vertically, or through the environment. The secondary endosymbionts have a facultative association with their host (Rave and Allen, 2003). A group of bacteria, including *Wolbachia pipientis*, *Spiroplasma* species and *Hamiltonella defensa* are mostly imparted vertically but occasionally go through horizontal transfer, leading to decoupled host-symbiont evolution. Unlike obligate endosymbionts, facultative endosymbionts harbour larger and more dynamic genomes (Werren *et al.*, 2008) and have mechanisms for actively occupying host tissues and for impacting on host biology in a way that drives the increased frequency of infected hosts in the host population. Endosymbiotic bacteria such as *Wolbachia* and *Spiroplasma* can also manipulate the reproductive properties of their insect host (O'Neill *et al.*, 1997). Such reproductive manipulation can occur via several routes, namely: parthenogenesis; male-killing; feminisation and cytoplasmic incompatibility. Parthenogenesis induction, in which female hosts produce eggs that develop and grow without sperm fertilisation, has been reported in the parasitoid wasps *Trichogramma deion*, *Trichogramma cordubensis* and *Muscidifurax uniraptor* (Stouthamer *et al.*, 1993). *Wolbachia* has been identified as

the causative factor of parthenogenesis in these hosts. When unfertilised eggs develop into females, the phenomenon is called thelytoky but if they develop into males, the phenomenon is called arrhenotoky. The production of both males and females by parthenogenesis is referred to deuterotoky. Male-killing, in which death of infected males occurs, usually early during development while infected females develop and grow, has been widely reported in invertebrates and particularly in ladybirds (Majerus and Hurst, 1997; Majerus and Majerus 2012). Male-killing has been associated with a diverse range of bacteria (Majerus and Majerus 2010b). Some bacteria have been associated with male-killing in more than one host species. For example, *Wolbachia* has been associated with the male-killing trait in the ladybird *Adalia bipunctata* and the butterfly *Acraea encedon* (Hurst *et al.*, 1999b). The functions of endosymbionts in ladybirds are discussed in more details in section 1.7.

Feminisation (Rousset *et al.*, 1992) is where genetically male hosts develop into functional females. For example, *Wolbachia* was the first agent responsible for feminization, identified in the terrestrial isopod *Armadillium vulgare*. *Cardinum* has also been identified as the bacterial feminiser in the spider mite *Brevipalpus phoemicis* (Weeks *et al.*, 2001).

Cytoplasmic incompatibility (CI) is one of the most common manipulations (Zheng *et al.*, 2011). In CI, mating between infected males and females that are either uninfected or infected with a different symbiont strain, either fails to produce viable offspring or produces very low numbers of viable offspring. *Wolbachia* was first

identified as the causative cytoplasmic incompatibility agent in *Culex* mosquitos (Yen and Barr 1973).

Different researchers have also described functions of facultative endosymbionts that create sex ratio biases, via male-killing, feminisation, cytoplasmic incompatibility and parthenogenesis induction (Werren *et al.*, 2008); host defense (Hedges *et al.*, 2008); host fitness (Hurst, 1991); host nutrition (Nováková *et al.*, 2009); protection from environmental stress (Oliver *et al.*, 2010); maximising forbearance to thermal stress (Montllor *et al.*, 2002); maximising opposition to parasitic wasps (Oliver *et al.*, 2003); causing host plant specialisation (Tsuchida *et al.*, 2004); conferring invasiveness (Feldhaar *et al.*, 2011) and host colouration (Tsuchida *et al.*, 2010).

In some associations, the endosymbiont *Wolbachia pipientis* protects the *Drosophila spp.* against some RNA viruses (Hedges *et al.*, 2008). It has also been found that *Spiroplasma* strains defend against nematode parasites in their host *Drosophila neotestacea* (Jaenike *et al.*, 2010). Infection of the mushroom-feeding woodland fly *D. neotestacea* with the parasitic nematode, *Howardula aoronymphium*, usually renders females completely sterile (Jaenike, 1995). However, the fertility of nematode parasitised females harbouring *Spiroplasma* symbionts is completely restored and nematodes become small, in poor health and unable to produce juveniles that cause the infection. Protection against nematodes is attributed to secreted, highly expressed, putative toxins called ribosome-inactivation proteins encoded by the *Spiroplasma* genome (Hamilton *et al.*, 2014). These are N-glycosidases that cleave the N-glycosidic bond between the rRNA

backbone and adenine by targeting a highly conserved adenine residue in the α -sarcin/ricin loop of the rRNA, exhibiting their toxic consequences through depurination of eukaryotic 28S ribosomal RNAs (Stirpe, 2004).

Researchers have provided an outline of the functions of different endosymbionts in insects (Clark *et al.*, 2010; Gibson and Hunter 2010). Recent investigations conducted by Kwong and Moran (2016) point out significant benefits to the honeybees by their gut microbiota including metabolic capabilities and defense against invading pathogens.

1.6 Transmission dynamics

Several researchers have also sought to understand the transmission mechanisms of symbiotic bacteria. Accordingly, diverse means of bacterial transmission, both vertical and horizontal, have been experimentally confirmed in different arthropod species. For instance, molecular biology experimental analysis was conducted by Yañez *et al.* (2016) to investigate the presence and transmission of the endosymbiotic genera *Arsenophonus*, *Wolbachia*, *Spiroplasma* and *Rickettsia* in honey bees (*Apis* spp.). *Arsenophonus* spp. were the only endosymbiotic bacteria found in queens of three different honey bees of the genus *Apis*. *Arsenophonus* strains were only found in the digestive tracts of the *A. mellifera* queens. The endosymbiont was not present in the ovaries and spermathecae of the queens or on the surface of sterilised eggs. These findings indicated that *Arsenophonus* species could not be transmitted vertically through the queen's reproductive organs in *A. mellifera* and consequently suggested the possibility of horizontal transmission, with *Arsenophonus* acquired from the

environment via contact with nest-mates, faeces or contaminated wax combs. Similarly, Chrostek *et al.* (2017) found that the *Rickettsia* and *Wolbachia* symbiont species' horizontal transfer among the leafhopper (*Euscelidius variegatus*) is often mediated by the plants upon which they feed. Li *et al.* (2017) also reported the horizontal transmission of *Rickettsia* spp among the whitefly species.

Vertical transmission is the most common mechanism for endosymbionts in insects. However, incongruence between the host and symbiont phylogenies suggests that many horizontal symbiont transfers have taken place during the course of evolution (Chrostek *et al.*, 2017). While some endosymbionts transmit horizontally, many are vertically transmitted from mother to offspring and have a variety of significant effects on their hosts (Herren *et al.*, 2013). For example, endosymbiotic *Spiroplasma* bacteria protect their hosts against parasites and therefore are attractive tools to control insect vector-borne diseases because it reduces the chances of insect host passing on the parasites to humans (Iturbe-Ormaechea *et al.*, 2011). Nonetheless, there are complex interactions between insect immunity and resident microbiota, which are beneficial to a range of host insects, including *Drosophila* spp., mosquito and aphid species (Douglas, 2014). The interaction is dynamic and diverse in a variety of insect species. Hence, this study focused on examining the types of endosymbiotic microorganisms found in insects and the research concentrated on ladybirds as the host insect.

1.7 Endosymbiotic bacteria in Ladybirds and their functions

Endosymbiosis is common in insects in which some form of mutualistic relationship between host and symbiont exists, whereas other endosymbiotic relationships

cause severe effects on the hosts' biological functions and are thus never mutually beneficial (Keeling and McCutcheon, 2017). The endosymbiosis in ladybirds is mainly heritable (Feldhaar and Gross, 2009). *Adalia bipunctata* has been popular to study host-parasite interactions for many researchers. *Adalia bipunctata* provides habitat to four different intracellular maternally acquired bacteria that kill male hosts during embryogenesis: one each of the genus *Rickettsia* (α -Proteobacteria) (Werren *et al.*, 1994), *Spiroplasma* (Mollicutes) (Hurst *et al.*, 1999a) and two distinct strains of *Wolbachia* (α -Proteobacteria) (Hurst *et al.*, 1999b). *Adalia bipunctata* has also been very useful to study sexually transmitted diseases (Hurst, 1995), the colour pattern which serves as a warning, also known as aposematism (Moore *et al.*, 1990), reproductive strategies (Bardoux *et al.*, 2003) and camouflage (Stevens *et al.*, 2008). According to Majerus and Majerus (2012), a combination of ecological and life-history factors such as the laying of eggs in clusters makes the ladybird susceptible to the male-killing phenomenon. There are several known male-killing systems in arthropod host species (Duron *et al.*, 2008). A summary of reported maternally inherited male-killing bacteria manipulating the reproductive biology of arthropods (Duron *et al.*, 2008) and Coccinellid hosts (Majerus and Majerus, 2012) are presented in Tables 1.3 and 1.4, respectively. These maternally inherited bacteria kill male embryos to enhance their spread through host populations. It is believed that the phenomenon also helps infected female hosts to gain indirect fitness benefits by reducing inbreeding and inbreeding depression, antagonistic interactions between siblings and competition for food or other resources (Hurst, 1991). The indirect fitness benefits

incurred by the host appear to be adequate, such that male-killing bacteria can even establish and persist in host populations when there is a direct fitness cost associated. It is widely known that male killers have indirect fitness benefits to female hosts due to resource reallocation when combined with sibling egg cannibalism (Majerus, 2001; Elnagdy *et al.*, 2011). However, there are also reports of direct benefits to hosts from harbouring male killers. The effect of male-killing bacterial infection on female fecundity, as measured by the number of eggs laid, survival and other life-history traits, including development time and body size, has been reported in ladybird host species infected with male killers (Elnagdy *et al.*, 2013). The number of eggs laid by adult females was significantly reduced or slightly but not significantly increased in infected ladybird females. The considerable decrease in the number of eggs laid by infected females was shown in *Adalia bipunctata* harbouring *Rickettsia*, *Spiroplasma* and *Wolbachia*, as well as in *Coccinula crotchii* harbouring *Flavobacteria*. Reduced numbers of eggs laid by infected females has also been previously reported in *Adalia bipunctata* harbouring *Rickettsia* (Hurst *et al.*, 1994). Male killer infected ladybirds have been reported to have reduced development time, increased survival, and body size (Elnagdy *et al.*, 2013). This has particularly been evidenced for the male-killing bacterial infection advantages for each of these three features demonstrated in specific infected strains. *Wolbachia*, *Spiroplasma* and *Rickettsia* increased survival in *Adalia bipunctata* adult females. A significant reduction of total development time and increased body size was found in *H. axyridis* adult females and adult female offspring infected with *Spiroplasma*, respectively. *H. axyridis* adult female offspring

infected with *Spiroplasma* also had significantly more ovarioles than uninfected female offspring, suggesting body size and ovariole number could indicate higher fecundity (Elnagdy *et al.*, 2013).

Moreover, the relationship between the diverse bacterial species and ladybird beetles plays significant roles in insect behavior and physiology, such as sibling egg cannibalism. Sibling egg cannibalism, an ephemeral phenomenon of feeding on dead male eggs in ladybirds, helps female siblings to survive until they find aphid prey (Majerus and Majerus, 1997). The association between male-killing bacteria and ladybirds is regarded to be an ultra-selfish behaviour, manipulating hosts' reproductive systems, leading to sex ratio distortion in ladybirds and survival and transfer of bacteria from generation to generation (Hurst, 1991; Majerus, 2003). Apart from male-killing, several resources have reported diverse roles of the endosymbiotic bacteria on their hosts (Table 1.3).

In addition to the male-killing bacteria, this study identifies the possibility of more endosymbionts in the ladybird species with other diverse, yet unidentified, roles. Hence, 16S rDNA sequencing was used as a tool (Weisburg, 1991) to investigate and understand bacterial diversity in ladybirds and to gain insights into their potential roles and impact on the host insect.

Organism	Phenotype	Host	Reference
<i>Rickettsia</i> sp. <i>α-proteobacteria</i>	Thelytokous parthenogenesis;	<i>Neochrysocharis Formosa</i> (Hymenoptera: Eulophidae)	Hagimori <i>et al.</i> (2006)
	Male-killing	<i>Brachys tessellatus</i> (Coleoptera: Buprestidae)	Lawson <i>et al.</i> (2001)
		<i>Adalia bipunctata</i> (Coleoptera: Coccinellidae)	Werren <i>et al.</i> (1994)
		<i>Adalia decempunctata</i> (Coleoptera: Coccinellidae)	von der Schulenburg <i>et al.</i> (2001)
<i>Wolbachia pipientis</i>	Cytoplasmic incompatibility	<i>Culex pipiens</i> (Diptera: Culicidae)	Yen and Barr (1973)
	Feminisation of genetic males	<i>Armadillidium vulgare</i> (Isopoda: Armadillidiidae)	Rousset <i>et al.</i> (1992)
	Thelytokous parthenogenesis	<i>Trichogramma</i> spp (Hymenoptera: Pteromelidae)	Rousset <i>et al.</i> (1992), Stouthamer <i>et al.</i> (1993)
	Male-killing	<i>Acrae encedon</i> (Lepidoptera: Nymphalidae)	Jiggins <i>et al.</i> (2000)
	Oogenesis	<i>Asobara tabida</i> (Hymenoptera: Braconidae)	Dedeine <i>et al.</i> (2001)
<i>γ-Proteobacteria</i> <i>Arsenophonus nasoniae</i>	Male-killing	<i>Nasonia vitripennis</i> (Hymenoptera: Pteromelidae)	Ghera <i>et al.</i> (1991)
<i>Cardinium hertigii</i> Bacteroidetes	Cytoplasmic incompatibility	<i>Encarsia pergandiella</i> (Hymenoptera: Aphelinidae)	Hunter <i>et al.</i> (2003)
		<i>Eotetranychus suginamensis</i> (Acari: Tetranychidae)	Gotoh <i>et al.</i> (2007)
	Feminisation of genetic males	<i>Brevipalpus</i> spp (Acari: Tenuipalpidae)	Weeks <i>et al.</i> (2001)
	Thelytokous parthenogenesis	<i>Encarsia</i> ssp (Hymenoptera: Aphelinidae)	Zchori-Fein <i>et al.</i> (2001) Zchori-Fein <i>et al.</i> (2004)
		<i>Aspidiotus nerri</i> (Hemiptera: Diaspididae)	Provencher <i>et al.</i> (2005)
<i>Flavobacterium</i> sp.	Male-killing	<i>Adonia variegata</i> (Coleoptera: Coccinellidae)	Hurst <i>et al.</i> (1999c)
		<i>Coleomegilla maculate</i> (Coleoptera: Coccinellidae)	Hurst <i>et al.</i> (1997a)
<i>Spiroplasma ixodetis</i>	Male-killing	<i>Adalia bipunctata</i> (Coleoptera: Coccinellidae)	Hurst <i>et al.</i> (1999a)
		<i>Anisosticta novemdecimpunctata</i> (Coleoptera: Coccinellidae)	Tinsley and Majerus (2006)
		<i>Harmonia axyridis</i> (Coleoptera: Coccinellidae)	Majerus <i>et al.</i> (1998)
		<i>Danaus chrisippus</i> (Lepidoptera: Nymphalidae)	Jiggins <i>et al.</i> (2000)
<i>S. poulsonii</i>	Male-killing	<i>Drosophila willistoni</i> group (Diptera: Drosophilidae)	Williamson <i>et al.</i> (1999)

Table 1.3 Bacteria with known manipulation of the reproductive biology of arthropod hosts.

Species	Reference(s)	Causative agent	Reference(s)
<i>Adalia bipunctata</i>	Lus (1947), Hurst <i>et al.</i> (1992)	<i>Rickettsia</i> ; <i>Spiroplasma</i> sp.; <i>Wolbachia</i> spp.	Werren <i>et al.</i> (1994), Hurst <i>et al.</i> (1999a,b)
<i>Hippodamia quinquesignata</i>	Shull (1948)	Not identified	
<i>Harmonia axyridis</i>	Matsuka <i>et al.</i> (1975), Hu (1979), Majerus <i>et al.</i> (1998)	<i>Spiroplasma</i> sp.	Majerus <i>et al.</i> (1999)
<i>Cheilomenes sexmaculata</i> (<i>Menochilus sexmaculatus</i>)	Nijima and Nakajima (1981), Majerus and Majerus (2010a)	γ -proteobacterium	Majerus and Majerus (2010a)
<i>Coleomegilla maculate</i>	Hurst <i>et al.</i> (1996)	Flavobacteria	Hurst <i>et al.</i> (1997b)
<i>Adonia variegata</i>	Hurst <i>et al.</i> (1999c)	Flavobacteria	Hurst <i>et al.</i> (1999c)
<i>Coccinula sinensis</i>	Majerus and Majerus (2000)	Flavobacteria	Majerus (2001)
<i>Adalia decempunctata</i>	von der Schulenburg <i>et al.</i> (2001)	<i>Rickettsia</i>	von der Schulenburg <i>et al.</i> (2001)
<i>Anisosticta novemdecimpunctata</i>	Tinsley and Majerus (2006)	<i>Spiroplasma</i> sp.	Tinsley and Majerus (2006)
<i>Propylea japonica</i>	Majerus and Majerus (2010b)	<i>Rickettsia</i>	Majerus and Majerus (2010b)

Table 1.4 Summary of reports of maternally inherited female-biased sex ratios and identification of causative male-killing bacteria in Coccinellids.

1.8 16S Ribosomal DNA as a tool for understanding bacterial communities in insects

Morphological identification of bacteria, as done through culture and microscopy, has been the ultimate approach over recent decades. However, such an approach fails in the accurate identification of unculturable intracellular bacteria, such as the *Spiroplasma* spp. (Hamsher *et al.*, 2013). Culture-based techniques are more complicated and time-consuming for slow-growing and fastidious organisms and the interpretations of the results are often subjective to the researcher's judgment (Stager and Davis, 1992). Reports have shown that numerous bacterial isolates remained unidentifiable even after the application of all available phenotypic tests, based on the extensive guidance flow charts developed by reference laboratories such as the Center for Disease Control and prevention in the United States (CDC) and the collection de l'Institut Pasteur and BioMerieux laboratories in France

(Weisburg *et al.*, 1991). Such inefficiencies thus prompt the need for molecular-based techniques for genetic screening.

As such, the 16S ribosomal RNA (rRNA) gene detection, using molecular biology techniques becomes the gold standard for any bacterial identification (Weisburg *et al.*, 1991). Janda and Abbott (2007) report that the 16S rRNA genetic marker is commonly and widely used to study bacterial phylogeny due to three reasons: its presence in all bacterial species, its conserved nature and its large size for easy bioinformatics analysis. Similarly, Weisburg *et al.* (1991) indicate that the 16S rRNA is widely used for phylogenetic studies and determining evolutionary relationships among bacteria, archaeobacteria and eukaryotic organisms. In prokaryotes, the 16S rRNA genes are crucial and present, with at least one copy in a genome (Acinas *et al.*, 2004).

The universality of the 16S rRNA genes makes them an ideal target for phylogenetic studies and taxonomic classification (Woese *et al.*, 1990). The gene has a sequence of about 1,500 bp existing within the bacterial genome (Patel, 2001). The gene products of the rRNA genes can fold into a complex, stable, secondary structure, comprising of stems and loops (Noller *et al.*, 1985). While almost all bacterial species have conserved sequences of some of the circuits which are involved in essential functions, other features remain widely variant and specific between classes (Jonasson *et al.*, 2002). Hence, the presence of species-specific variable regions in 16S rDNA (Drancourt *et al.*, 2000) can be utilised as a suitable molecular approach for investigating bacterial phylogeny (Woese *et al.*, 1990).

The innovation of the Polymerase Chain Reaction (PCR) technique in 1985 has enabled mapping of the variant regions, V1-V9, of the 16S rRNA genes (rDNA) and hence is applied for species identification and subsequent phylogenetic studies (Lane *et al.*, 1985).

Primers of 16S rDNA between 15 and 20 nucleotide base pairs have been designed from the conserved parts that flank a target region used for the bacterial phylogenetic analysis. The first sets of primers were constructed from the conserved elements of 16S rDNA arrangement from variant species and were named according to their positioning on *E. coli* 16S rDNA (Lane *et al.*, 1985); this has become the protocol for subsequent primer design. For example, primer E685 corresponds to the eubacteria P4 region (Kuijper *et al.*, 2003) and primer A344 targets the archaeal H339 region (Cannone *et al.*, 2002).

With the advancement of the sequencing technique, the number of 16S rDNA sequences is increasing in the database in recent decades which has helped researchers to design more primers for bacterial studies, using tools like those in the ARB (from Latin *arbor*, tree) project (Ludwig *et al.*, 2004). Moreover, phylum-specific primers were designed in the late 90s (Nübel *et al.*, 1997). Nucleotide polymorphisms have been identified even in the conserved regions with more 16S rDNA sequence data available in the Ribosomal Database Project (RDP) (Cole *et al.*, 2009). Consequently, the originally widely used primers may not be suitable for a small group of bacteria, as noticed in recent studies (Huws *et al.*, 2007). Sequencing and the subsequent analysis have thus been used to study the genetic relationships among organisms of different species of the genus *Xanthomonas* and

the species *Stenotrophomonas maltophilia* (Hauben *et al.*, 1997). In this study, 16S rDNA sequencing followed by Basic Local Alignment Search Tool (BLAST) (Altschul *et al.*, 1990) has been used to identify endosymbiotic bacterial diversity present in ladybirds.

1.9 Ladybirds as biological control or beneficial invertebrates

Ladybird beetles are economically and ecologically significant insects. Researchers have confirmed that ladybirds are economically important insects that can be used as beneficial control agents against aphids, powdery mildew and management of other invertebrate pests in the agricultural sector, due to their aphidophagous, carnivorous and mycophagous nature (Sutherland and Parrella, 2009; Roy *et al.*, 2013). However, some ladybird species are herbivorous and have different mouthparts from those in carnivorous species, which helps them in scraping the layers of plant tissues (Roy *et al.*, 2013).

Aphids are the primary source of nutrients for both the larvae and adult members of the aphidophagous ladybirds. Roy *et al.* (2013) presented an account of how such ladybirds control the aphid population and outlined that females deposit their eggs near the aphid population for the progeny to locate in the vicinity. The female ladybirds do the same for the coccid prey too.

Female ladybirds do not lay eggs in patches where they detect larval tracks left by members of the same ladybird species, by responding to the oviposition-detering pheromone (ODP) present in the tracks (Ruzicka, 1997; Frechette *et al.*, 2004). Previous studies have shown that the seven-spot ladybird beetle *Coccinella septempunctata* and the two-spot ladybird beetle *Adalia bipunctata* were deterred

from oviposition in patches of prey contaminated by the ODP where conspecific larvae were already present (Doubilia *et al.*, 1998). The ODP is released from the anal disk on the tenth abdominal segment of larvae and produced by one individual of a ladybird species to cause a specific interaction with another individual of the same species (Laubertie *et al.*, 2006). Hemptinne *et al.* (2001) attributed the ODP effect to its composition consisting of a mixture of long-chain hydrocarbons, mainly alkanes of which n-pentacosane is the principal constituent. The hydrophobic cuticle of plants allows these molecules to spread readily, leaving a strong cue. Furthermore, they are not easily oxidised, resulting in a large and long-lasting signal (Mishra and Omkar, 2006). The response to ODP by avoiding aphid colonies that have previously been exploited and competition for oviposition sites is crucial for gravid female ladybirds because it would protect their offspring from conspecific larvae and reduce the risk of competition and cannibalism which is a major threat to egg survival in ladybirds (Osawa, 1989, Doubilia *et al.*, 1998).

Prey detection is a survival trait in many insect species, and ladybirds apply dynamic prey detection techniques, which directly facilitate location and hence capture their prey. Nakamuta (1984) has shown that ladybirds can detect their prey in the short-range only, using visual cues, whereas Sloggett *et al.* (2011) reported the significance of chemical cues for long-range prey detection. Moreover, Obata (1986) and Ninkovic *et al.* (2001) suggested that the chemicals released after aphid damage of plants, act as cues and attractions for *H. axyridis* and 7-spot ladybirds rather than chemicals produced by aphids themselves.

Some coccinellids rely on conidia and hyphae of powdery mildews as their primary source of food, especially all members of Halysziini (e.g. the orange ladybird, *Halyszia sedecimguttata*) (Sutherland and Parrella, 2009). Another tribe of Coccinellidae, the Tythaspidini had been identified as facultative mycophages, which feed on fungus and other plant pests too (Turian, 1969). Such ladybird insects are thus significant in the management of the fungal populations in farms. Notably, researchers have tested the significance of such insects in the control of farm pests (Tabata *et al.*, 2011).

The multicoloured Asian ladybird *H. axyridis* was first applied to agricultural industries in the USA as a biological control agent of aphids and coccids at the beginning of the 20th century (Gordon, 1985). Brown *et al.* (2011a) indicated that *H. axyridis* were introduced as biological control agents in 13 European, two North American, two South American, two Asian and two African countries. This coccinellid is characterised as large, voracious and able to withstand or recover quickly from difficult conditions which has made it a choice for use as a biological control agent of pests on crops (Brown *et al.*, 2011b). As such, *H. axyridis* ladybird beetles have been widely used to control pests on diverse plants as shown in Table 1.5.

Crops	References
Pecan	Tedders and Schaefer (1994)
Red pine	McClure (1987)
Apple	Brown and Miller (1998)
Citrus fruit	Michaud (2002)
Soybean	Costamagna and Landis (2006)
Sweet corn	Musser and Shelton (2003)
Alfalfa	Buntin and Bouton (1997)
Cotton	Wells <i>et al.</i> (2001)
Tobacco	Wells and McPherson (1999)
Winter wheat	Colunga-Garcia and Gage (1998)

Table 1.5 Use of *H. axyridis* as a biological control on different crops.

Hence, the Harlequin ladybird *H. axyridis* can and has been widely used as a beneficial agent for various pest management in different crops. It can help to conserve biodiversity while also being a more environmentally friendly alternative to chemical pesticides to control pests (Davies and Britton, 2015). However, it has successfully become an invasive species in many regions where it was introduced as a biocontrol agent (and indeed some where it was not deliberately introduced) and is considered to pose a threat to native ladybird populations (Camacho-Cervantes *et al.*, 2017). Native ladybird species have become at risk of being suppressed or outcompeted after Harlequin ladybird invasion. Previous research has demonstrated that many formerly widespread native ladybird species have seen a decline in populations over the last 3 decades in several countries in North and South America and Europe. This coincided with increasing population of two invasive alien species including *C. septempunctata* and *H. axyridis* globally (Roy *et al.*, 2012; Haelewaters *et al.*, 2022). Similarly, it has been indicated that native coccinellid beetles (*Coccinella transversoguttata richardsoni*, *Coccinella novemnotata* and *Adalia bipunctata*) have declined in North America in association with the establishment of invasive species of ladybirds (Hesler and Beckendorf, 2021). Previous studies have suggested that the Harlequin ladybirds compete with native aphidophagous species for food resources and could be reducing native ladybird diversity in those locations where it has been present (Roy *et al.*, 2012; Brown *et al.*, 2011b). It has been shown, for example, that native ladybird beetles could be harmed as parasitic microsporidia spores that can be carried by the Harlequin ladybird, while not causing any harm to the carrier, are fatal to the native

ladybird *Coccinella septempunctata* (Vilcinskas *et al.*, 2013). In addition to outcompeting native species for food resources *H. axyridis* can predate on native ladybird species, in particular eggs and larvae (Pell *et al.*, 2008). This is referred to as intraguild predation which could be a cause of the decline of native species (Roy *et al.*, 2012). The Harlequin ladybird seems to be well adapted to consuming other ladybird species and tolerates diverse prey defensive chemicals in its diet (Sloggett *et al.*, 2011). *H. axyridis* resist to the alkaloids of intraguild ladybird prey, making them harmless by metabolising them within the body. The success of *H. axyridis* as an intraguild predator may be partially dependent on this tolerance of intraguild prey defensive chemistry and its own strong chemical defences, represented in alkaloids that possibly provide protection against predators and render it less vulnerable to natural enemies. For example, it has been shown that larval *Coccinella septempunctata* intraguild predators are less likely to consume *H. axyridis* eggs with higher harmonine (Kajita *et al.* 2010). The endosymbionts harboured by ladybird species may play a role in detoxification of defensive chemicals in intraguild prey as predicted for herbivorous insects that rely on symbiotic microbes to detoxify plant defence compounds (Wei *et al.*, 2022).

The Harlequin ladybird as an invader is perceived to negatively affect not only native species but also human health and well-being (Koch *et al.*, 2006). This insect can become a household pest when it overwinters inside homes and other structures within its invasive area. It can become a nuisance to humans because of massive accumulations to seek shelter from winter in the autumn in attics, wall voids and other inaccessible places in houses (Koch and Galvan 2008). Airborne

volatiles emitted from dead *H. axyridis* aggregating in these sheltered places can cause allergic reactions in humans (Nakazawa *et al.* 2007). The inadvertent harvesting of grapes contaminated with defensive chemicals from *H. axyridis* can occur. These chemicals have a bitter, unpleasant taste, leading to *H. axyridis* being considered a pest of fruit crops as they 'taint' wine in this way during production (Pickering *et al.* 2004).

In contrast to *Harmonia axyridis*, the orange ladybird *Halyzia sedecimguttata* has not been utilised as a commercial biological agent against mildew (its primary diet). However, research done by Tabata *et al.* (2011) suggested that mycophagous ladybird beetles that feed on powdery mildew might be useful in integrated disease management to protect crops against a wide range of mildew. Plants infected by powdery mildew emit olfactory cues that are significantly different than those of uninfected plants. These characteristic olfactory cues are volatiles compounds associated with the infection of plants by powdery mildew, including 3-octanol and its analogues 1-octen-3-ol and 3-octanone. In particular, one individual compound, 1-octen-3-ol, which is the most abundant of the characteristic fungal compounds identified, significantly attracts mycophagous beetles. Mycophagous ladybirds preferentially respond to the odours of infected plants when foraging for powdery mildew (Tabata *et al.*, 2011).

Yun *et al.* (2014) suggested the presence of diverse gut microbial diversity based on the host diet. This study would predict distinct endosymbiotic bacterial species in the carnivorous *H. axyridis* and the mycophagous *Halyzia sedecimguttata* species given their different diets. This in turn suggests the ladybird hosts may be

impacted in different ways by their respective endosymbionts or bacterial communities. Therefore, it is crucial to understand the factors impacting on the population and physiological competencies of these ladybird species, and to understand the significance of the bacterial communities of *H. axyridis* and *H. sedecimgutta* ladybirds. This in turn may provide insight into the role these ladybirds can play in the control of the farm pests.

Moreover, applying the use of carnivorous and mycophagous ladybird species as beneficial agents may be the best choice for farmers to control various herbivorous pests and fungal diseases due to the harmful effects resulting from the use of insecticides and fungicides in recent years. Yet, there is an evident gap in the identification of the physiologically significant endosymbionts and their parasite control mechanisms among such ladybird insects. Hence, this research study seeks to identify different bacteria in *Harmonia axyridis* and *Halyzia sedecimguttata*. This in turn will allow the future development of a more complete understanding of the life-history of different ladybird species, and provide opportunity to understand the functions of the bacteria and how they might enable ladybirds to better be harnessed for beneficial applications in the control of 'plant pests'.

1.10 Ladybird biodiversity and invasion

The Harlequin ladybird migrated from Asia over a decade ago and has now almost overrun the endogenous seven spot and orange ladybirds in the UK. This has caused some concern with conservationists who consider all species essential to an important environmental cycle.

Factors that contribute to global biodiversity being at risk of decline include changes in land use and climate, nitrogen deposition and invasive species (Valtonen *et al.*, 2017). In general, as land use alters, changes such as habitat loss favour a few species that are already widespread, while harming many others. The predicted outcomes involve a reduction in population sizes and a reduction in species richness, both locally and regionally. Community compositions become similar across sites, known as biotic homogenisation (Mckinney and Lockwood, 1999). Much work on global biodiversity loss has been carried out to understand diversity patterns at national and regional scales (Sala *et al.*, 2000). The replacement of native species by newly introduced and established, non-native types, is, along with biotic homogenisation, integral to this, as illustrated by many previous examples in our Earth history (Olden *et al.*, 2018). One of the five principle drivers for loss of biodiversity, as mentioned by the Conference of the Parties in Nagoya 2010, is Invasive Alien Species (IAS), which have a direct and ecological influence on other types through several mechanisms (Egoh *et al.*, 2020). There are many different examples, specifically, predators of invertebrate species that directly impact native species either by hunting, competition for land and food, or introduction of pathogens which were not known before in native habitats, as well as lowering their immunity and mating systems (Roy *et al.*, 2012). These invader

properties can be partially accounted for by the idea that newly established invaders are less overwhelmed by natural enemies in comparison with native species. As a result, the invaders accumulate in that area and hence this is responsible for the fast spread of these individuals of invasive species (Haelewaters *et al.*, 2017).

Accordingly, worldwide biodiversity is highly threatened by an increased invasion of several species (Roy *et al.*, 2019). The increasing prevalence of non-native, invasive species results in an annual estimated cost of 70 billion US\$ to the global economy (Bradshaw *et al.*, 2016). This cost is used on a range of corrective measures such as pest control, damage repair, clean up education, vector control, medical research and restoration programmes. Invasion biology scientists work on identifying the sources and the path over which IAS spread, in order to control such species. They are also trying to understand the factors responsible for the tendency of some species to become invasive when others do not. The increasingly widespread use of genetic and phylogenetic tools can infer invasion routes and estimate the population diversity (Garnas *et al.*, 2016). The extensive application of these methods has led to growing evidence that patterns in biogeography, introduction history, subsequent movement and spread of many IAS are more complicated than originally thought (Garnas *et al.*, 2016). Together

these factors can well define the evolutionary process and the history of the invasive alien species.

Due to the ability of *H. axyridis* to effectively outcompete indigenous aphidophagous insects in a variety of new environments, it has been considered as a model species in invasion biology (Roy *et al.*, 2016). Despite its Asian origin, it has spread extensively throughout the world in Europe, North and South America and in a few locations in Africa. The North American populations started to become invasive in the 1980s. *H. axyridis* was intentionally released and introduced multiple times for biological control use in the USA during the twentieth century (Gordon, 1985). When it colonises new habitats in different parts of the world in Europe, Africa, North and South America where it has invaded, it directly affects native insect species and even other common types of ladybirds (Grez *et al.*, 2016). As an advantage, *H. axyridis* is a strong predator of pest insects associated with different crop fields and agricultural systems (Riddick, 2017).

Invasive populations may be exposed to a severe bottleneck following migration from their native origin and introduction into a new range (Dlugosch and Parker, 2008). Such a bottleneck affects their adaptability, due to a loss in genetic diversity, resulting from the limitation on the number of different genotypes introduced from the source population (Auger-Rozenberg *et al.*, 2012). Some invaders may tolerate

low genetic diversity well and survive despite founder effects (Roman and Darling 2007). This may be due to phenotypic plasticity, which is represented in the ability of an individual organism to modify its phenotypic trait or activity (for example, metabolism) in response to environmental stimuli or inputs (Garland and Kelly, 2006). However, multiple introductions can help invasive species to restore or even increase their genetic diversity and hence their adaptive abilities, limiting the impact/severity of bottlenecks (Dlugosch and Parker, 2008).

The results of a previous study conducted by Javal *et al.* (2019) suggest the possibility that invasive species may be introduced numerous times from their native area. These introductions were probably human-mediated, which may have occurred via increased international trading, reforestation activities or other anthropogenic factors such as tourism. These introductions are likely to have been followed in some cases by a secondary dispersal within the invaded ranges, leading to increased population size. This emphasises the significance of increasing the number of introduction events in invasion success and shows that an invasive population may act as a source for a new infestation. This is termed the bridgehead effect (Lombaert *et al.*, 2010), through which IAS can spread across broad geographic ranges. This approach was applied to the Harlequin ladybird *Harmonia axyridis* that was introduced as a biological control agent

without becoming established for several years. The invasion history worldwide of this species followed a bridgehead scenario in which an invasive population in eastern North America serves as the source of the colonists that invaded remote new continents including Europe, South America and Africa. In fact, multiple introductions occur commonly in many invasive insect species (Garnas *et al.*, 2016).

Role of host-associated symbionts in the rapid growth and spread of invaders can be explained by many hypotheses, including the Enemy Release Hypothesis, which suggests that invasive species are less affected by natural enemies such as pathogens and parasites than native species (Keane and Crawley 2002). Invasive species may lose symbiotic microbes and/or acquire symbionts unique to their introduced range (Lester *et al.*, 2017). In addition, moving to a new range may mean invasive species are no longer affected by microbial pathogens or other pests or predators. The release or escape from such enemies in a new geographical location would provide a competitive advantage for introduced species over native species and hence they may become prevalent (Torchin *et al.*, 2003). For example, it has been reported that microbial pathogens of red imported fire ants, *Solenopsis invicta*, are less prevalent during invasion than in their native zone in South America (Yang *et al.*, 2010). In addition to the key role of

endosymbionts in invasion processes, the absence of beneficial microbial symbionts may inhibit the invasive species from being successfully introduced into a new habitat during invasion (Lester *et al.*, 2017).

In the invaded areas of North America, the diversity of the bacterial community of *H. axyridis* reduced when compared to the native areas of Asia (Li *et al.*, 2022).

This indicated that the microbiota composition of *H. axyridis* has been restructured in the invaded North American ranges in comparison with the native Asian ranges.

The composition and distribution of reproductive endosymbiotic bacteria *Wolbachia*, *Rickettsia* and *Spiroplasma* has been investigated in native and invasive ranges of *H. axyridis* (Goryacheva *et al.*, 2015; Goryacheva *et al.*, 2017).

The proportion of infected individuals with *Wolbachia* and *Rickettsia* in native and invasive populations of *H. axyridis* was about 3–5 %. Regardless of their reproductive effects, this suggested that these bacteria have no effect on the establishment of invasive populations of *H. axyridis*. *Spiroplasma* was exclusively detected in native *H. axyridis* populations (Goryacheva *et al.*, 2017). The proportion of *Spiroplasma*-infected individuals in native populations of *H. axyridis* was approximately 8 %. The lack of *Spiroplasma* in invasive populations of *H. axyridis* indicated that this endosymbiont does not contribute to the invasion success of *H. axyridis*.

A second hypothesis that has been used to explain the success of invasive species is the Novel Weapons Hypothesis, which states that invasive species may carry biological weapons such as pathogens and parasites that support their competitive capability into a new habitat (Fassbinder-Orth *et al.*, 2013). These pathogens and parasites are harmless to the invasive host but can infect and kill native competitors giving the invader a selective advantage (Vilcinskas, 2015). For example, it has been demonstrated that the invasive ladybirds, *Harmonia axyridis*, benefit from their ability to carry abundant microsporidia, which are fungi-related obligate parasites that are not harmful to the Harlequin ladybird host but lethal to indigenous ladybird species such as *Coccinella septempunctata* (Vilcinskas *et al.*, 2013).

The importance of microbial symbionts in promoting insect invasions has been reviewed in Lu *et al.* (2016). For instance, it was shown that the presence of *Rickettsia* in the notorious sweet potato whitefly, *Bemisia tabaci*, an invasive agricultural pest in the USA, increases the performance, the survival rate and the production of female-biased offspring (Himler *et al.*, 2011). In contrast, interaction with symbiotic microbes can negatively influence the life history traits of their hosts such as dispersal (Goodacre *et al.*, 2009). For example, it was demonstrated that the infection of the spider *Erigone atra* by a particular maternally inherited

endosymbiont, *Rickettsia*, negatively affects long-distance movement through aerial dispersal which is termed 'ballooning'.

1.11 Aim of the study

The aim of this study is to identify and understand the diversity of bacterial communities in the ladybirds for beneficial applications in the control of 'plant pests'. Accordingly, the study seeks to meet the following research objectives:

1. To identify the different bacterial microbiota in *H. axyridis* and *Halyzia sedecimguttata* using 16S rDNA sequences. The objective of this identification is to test the initial hypothesis that the microbial community harboured by these two ladybird species will differ owing to their different diet and ecological habitat which in turn play a role in transmitting the bacteria horizontally from the environment.
2. To determine the phylogenetic relationship among the microbial species in the two ladybird species using 16S rDNA sequences and compare these with known invertebrate microbiota.
3. To identify the mode of transmission of bacteria in the ladybird species *H. axyridis* by investigating infection in different life stages (eggs, larvae, pupae, adult males and females) to establish whether they are transmitted vertically from mother to offspring or horizontally through the environment.
4. To identify the bacteria present in different segments of the ladybird's body to investigate their distribution in different parts of ladybird bodies to try to begin to understand any function or effect they may have on the host.

Chapter 2: Prevalence and importance of bacteria in

Harmonia axyridis and *Halysia sedecimguttata*

2.1 Introduction

Beneficial insects such as predators or parasitoids can eradicate populations of herbivorous insect pests in crop fields, providing a vital ecosystem service that saves billions of dollars every year (Losey and Vaughan, 2006). Recently, identification of the factors influencing the abundance of naturally occurring predators and parasitoids of crop pests, pests and pest control effectiveness in crop fields has been carried out, with the goal of designing and managing agricultural systems to make this service as comprehensive and effective as possible (Gurr *et al.*, 2017). These factors include providing predators and parasitoids with food such as nectar and pollen or alternative prey or host species or a mild microclimate such as shelter that keep them alive during harsh weather conditions. The potential outcomes of this study were pest population suppression in agricultural systems, crop yield enhancement and intensification of agricultural production. Improved pest management could lead to positive results that include increasing crop production and reducing the use of chemical pesticides in agricultural habitats (Haan *et al.*, 2020).

Generally, insect populations harbour a collection of obligatory and facultative symbionts, including mutualists, parasites and commensals, some of which can have significant fitness implications (Engel and Moran, 2013). In many cases, these microbial symbionts provide benefits such as enhanced nutritional availability (Douglas *et al.*, 2001) or protection against parasitoids and pathogens (Oliver *et al.*, 2003; Scarborough *et al.*, 2005).

The successful ecological development of insects is because of their accompanying bacteria or fungi from millions of years ago, which enhance their metabolic capabilities or enable them to deal with several stress conditions or parasites (Moran, 2007). Recently, there has been a large interest in the study of insect symbionts and certain biological functions performed by symbionts have been recognised (Rosenblueth *et al.*, 2017). Insects that have restricted diets, for example those specialising in feeding on vertebrate blood, phloem and xylem sap are sometimes found to be deficient in sterols, essential amino acids and vitamins such as thiamine, pyridoxine and folate (Rio *et al.*, 2016). Fungal and bacterial symbionts such as *Wigglesworthia*, *Wolbachia*, *Rhodococcus*, *Coxiella* and *Rickettsia* can supply their insect host with deficient nutrients by the retention of these nutrients biosynthesis genes (Douglas, 2015). These symbionts living either in or on several kinds of hosts, can help them to cope with stressful environments

and protect them from pathogens or predators, by boosting hosts' immune systems. Besides, they can be involved in cell-specific processes, such as the acquisition of multiple sets of genes (Beinart, 2019). The resistance of hosts to insecticide can also be affected by endosymbionts such as *Wolbachia* and *Rickettsia* (Liu and Guo, 2019). In general, the susceptibility of hosts to chemical pesticides increases when infected with these symbiotic bacteria, but resistance also increases in other cases, depending on insect host species, symbiont species and insecticide substances. For example, it was shown that the infection of whiteflies, *B. tabaci*, with *Wolbachia* increases their susceptibility to insecticides including thiamethoxam, imidacloprid and pyriproxyfen compared with those individuals free of the bacteria (Ghanim and Kontsedalov, 2009; Kontsedalov *et al.*, 2008). In contrast, it has been found that the resistance of whiteflies to other insecticides such as acetamiprid and spiromesifen increases in the presence of *Wolbachia* (Ghanim and Kontsedalov, 2009). Variation in symbiotic population density may induce gradual alterations in insecticide resistance or susceptibility of insects on different host plants (Li *et al.*, 2018). For example, it has been found that the buprofezin-resistant strain of the small brown planthopper, *Laodelphax striatellus*, harbours more *Wolbachia* than the susceptible strain. Similarly, it has been observed that the thiamethoxam-resistant whitefly population houses a

higher density of *Rickettsia* compared with the susceptible population (Pan *et al.*, 2013). The fitness costs of *Wolbachia* and *Rickettsia* to their hosts might cause the higher susceptibility of their hosts to insecticides (Liu and Guo, 2019). It has been shown that the increased susceptibility of pea aphids infected with *Serratia* to insecticides is likely to be related to the negative effects on their fitness, such as decreased fecundity and body weight, induced by this symbiotic bacterium (Skaljac *et al.*, 2018). Although degrading and detoxifying of insecticides have not been demonstrated in *Wolbachia* and *Rickettsia*, other symbionts can degrade or detoxify toxic compounds conferring insecticide resistance to their hosts (Shen and Dowd, 1991). For example, *Burkholderia*, a gut-bacterial symbiont found in soil, provides resistance to fenitrothion, an organophosphorus insecticide, in the bean bug, *Riptortus pedestris* (Kikuchi *et al.*, 2012). The mechanisms by which such degradation or detoxification happen are currently unknown. Accordingly, population density of endosymbionts and their effect on fitness of hosts, metabolism, immune system and gene expression may be the determinants of resistance levels (Liu and Guo, 2019).

The identified insect endosymbionts are varied in terms of their phylogenetic placement, effect on host fitness, host tissue tropism and their dependency on the host rather than their abundance. Symbionts may belong to the Proteobacteria

while others belong to Actinobacteria, Chlamydiae, Bacteroidetes and Firmicutes, providing essential amino acids or fixing nitrogen (Lu *et al.*, 2016). They could reside on the surface of their hosts, extracellularly in the gut, or intracellularly in cells of various tissues, including the gut, and blood (Douglas, 2000). Symbiotic associations could be facultative in which symbionts are culturable with hosts that have relatively low fitness without symbiont presence. Obligate associations are where both the symbiont and the host cannot survive without each other. The effect of symbionts on their hosts could be parasitic, mutualistic or commensalist. Another classification could be regarding their biological aspects, the association of facultative symbionts could vary depending on the host physicochemical factors or the external environment (Rosenblueth *et al.*, 2017). *Wolbachia*, *Rickettsia* and *Cardinium* are endosymbiotic bacteria typically present in a variety of arthropod species including insects (Sazama *et al.*, 2019). Over half of insect species harbour *Wolbachia* and it is perhaps one of the most plentiful endosymbionts on the planet (Landmann, 2019). The global incidence frequency of *Wolbachia* has been estimated to be 52% for aquatic insect species versus 60% for terrestrial insects (Sazama *et al.*, 2017), while 24% and 13% of all insect species are estimated to carry *Rickettsia* and *Cardinium* infections, respectively (Weinert *et al.*, 2015).

There are two transmission models for symbionts to expand their distribution. The main mechanism that facilitates the spread of symbionts within a population is via vertical transfer from the mother to her offspring. Horizontal transmission, from one individual to another within a population is occasionally observed between species (Shaikevich and Romanov, 2022). Horizontal transmission of symbiotic bacteria can occur through various routes, such as the environment (Kikuchi *et al.*, 2007), host plant sap (Caspi-Fluger *et al.*, 2012), mating (Moran and Dunbar, 2006), predator-prey interactions (Tolley *et al.*, 2019), parasitoids of insects (Ahmed *et al.*, 2015) or exchange of hemolymph by physical contact (Shaikevich and Romanov, 2022).

Co-infection with different strains of the same symbiont within the host commonly occurs and could lead to decreasing bacterial density and symbiont loss (McLean *et al.*, 2018). In aphids as an example, it has been found that infections with multiple strains of the symbiont *Hamiltonella* result in lower *Hamiltonella* concentration compared to single infections and loss of one of the bacterial strains in a few events of double infections.

In the history of ladybirds, scientists, researchers and experts in the ecological field have always seen ladybirds as a worldwide tool for biological control achievement. Much work on the potential of *Harmonia axyridis* has been carried out, however,

there are still some critical issues that restrict their use for biological control. Not only has their acceptance been limited, but also their release has been banned outside their native range (van Lenteren *et al.*, 2008). *H. axyridis* has a key role in biodiversity changes of other native insects mainly as a predator or as a food competitor (Rondoni *et al.*, 2017). Also, they have colonised in large numbers, which may be an inconvenience for other species or human life (Roy and Brown, 2015). It has now been demonstrated that *H. axyridis* exhibits a mixture of immunological, behavioural, chemical and microbiological protection systems against their enemies, that usually directly kill or decrease their fertility, thus, preserving *H. axyridis*' strong invasive abilities (Verheggen *et al.*, 2017; Ceryngier *et al.*, 2018). These anti-predator/defensive mechanisms are effective in deterring predators and restricting most parasitoids. However, somehow they are not effective against some birds and a few invertebrates such as *Phalacrotophora* parasitoid flies, that evade the ladybird immune response and some spiders that can easily consume both *H. axyridis* and *C. septempunctata* are not deterred or affected by chemical defences of these ladybirds (Ceryngier *et al.*, 2018).

Insects such as carnivorous and mycophagous ladybird species used as beneficial agents may be the best choice for farmers to control various herbivorous pests and fungal diseases due to the emergence of resistance to insecticides and

fungicides in recent years. However, the release of *H. axyridis* outside its native range is banned. When using a biological control agent that invades and outcompetes native species, it might be the best solution instead of banning its release, to employ symbionts that can reduce or get rid of the negative consequences resulting from its release. Another solution that might be of benefit is to recruit symbionts that allow a native species to be a suitable biocontrol agent and keep it active. So, in this situation understanding symbionts may help us to control the invasive species or boost the native species to design better biocontrol programmes. Knowledge of symbionts will provide a more complete understanding of potential novel biocontrol agents.

Ladybirds also have a high diversity in diet, including three different habits: predatory by nearly all species; phytophagous and mycetophagous for example by *Halyzia sedecimguttata* which feeds on fungi. These habits are generally associated with a high level of adaptation in different environments (Escalona *et al.*, 2017).

It has been found that multiple species of bacteria may infect *H. axyridis* across 15 different phyla (Dudek *et al.*, 2017). The prevalence of different species within these phyla varied from presence in a single individual to presence in all individuals tested. The presence and functions of different bacteria has not been investigated

in different populations. The ladybird *H. axyridis* can serve as a good example for investigating the role of endosymbiotic bacteria on the establishment of insect invasive populations, based on two key factors. First, the hypothesis that invasive species may acquire new microbial symbionts in their invaded areas (Lester *et al.*, 2017). Second, that multiple founder individuals may come from a variety of native insect populations and result in the formation of insect populations in their new habitat that integrate a variety of microbial communities from numerous sources (Lu *et al.*, 2016). This leads to the possibility of invasive insect populations harbouring more microbial diversity than their indigenous populations. In order to understand the make-up and complexity of microbial symbionts within a particular ladybird host population, multiple individuals from multiple native populations need to be investigated. Once the symbionts present in different populations have been compared it would be possible to determine the validity of these two hypotheses. The ladybird *H. axyridis* is a generalist, as are many other species of ladybird. In contrast some species are specialists, feeding on specific prey. It is interesting to consider whether the bacterial communities associated with these different life histories are similar or different. In particular, are the same bacteria found associated with both generalists and specialists, and does the lifestyle affect the numbers of different bacterial genera found in such associations. To investigate

this, two species were selected for this study, the generalist *H. axyridis* and the specialist *H. sedecimguttata*.

H. sedecimguttata, are ladybird beetles characterised with sixteen creamy-white, large spots found on the elytra (eight spots on each elytron), with oval body shape and bright orange elytral colour (Rahaman and Aniszewski, 2016). They prefer cool environments and are usually seen on deciduous or coniferous trees, especially on sycamore and ash (Roy *et al.*, 2013). They are considered an obligatory, mycophagous species and feed primarily on mildews that form and develop on the leaves of these trees (Sutherland and Parrella, 2009). However, it has been recorded that when mildews have not grown in abundance on the leaves of deciduous plants, they may sometimes eat honeydew or small aphids (Roy *et al.*, 2013). Feeding on these alternative types of food occasionally enables them to survive during what would otherwise be periods of starvation. The abundance and distribution of orange ladybirds have increased in Britain and Belgium in the late 20th and early 21st centuries (Roy *et al.*, 2012). The prevalence of their principal food (i.e., mildew) might have increased as a result of high temperatures. However, this mycophagous species has shown declines in abundance after the arrival of *H. axyridis* in Britain. The changes in the abundance of *H. sedecimguttata* could be attributed to the negative effects of *H. axyridis* on the diversity of *H.*

sedecimguttata through predation and competition (Brown *et al.*, 2011a). In contrast, the abundance of *H. sedecimguttata* evaluated between the 1970s and 2010s, before and after the arrival of *H. axyridis* in the Czech Republic, did not vary (Honek *et al.*, 2016).

16S rDNA sequences were investigated using PCR, cloning and sequencing for both species (see 2.3.2, 2.3.3 and 2.3.4). These investigations and previous work by Dudek *et al.* (2017) and Schmidtberg *et al.* (2019) suggested that the range of bacteria may be far greater than could be efficiently identified by the PCR and cloning approach, hence additional Next Generation metagenomic sequencing (see 2.3.8) was used to investigate the bacteria present in four *H. axyridis* individuals in more detail than would be practical via the PCR and cloning approach.

2.2 Objectives

The aim of this part of the study is to identify and understand the diversity of the bacterial communities in the ladybird species *Harmonia axyridis* and *Halyzia sedecimguttata* using 16S rDNA sequences, generated from cloning and PCR and metagenomic approaches. The objective of this identification is to test the initial hypothesis that the microbial community harboured by these two ladybird species will differ, owing to their different diet and ecological habitat, which in turn play a role in transmitting the bacteria horizontally from the environment.

2.3 Materials and Methods

2.3.1 Collection of ladybirds

The ladybird samples were provided by Tamsin Majerus or collected myself, in both cases from wild populations at University of Nottingham Park in Nottingham, the UK and brought to the laboratory. The sex was not determined, and all samples were adults. The total number of samples is 10 of each ladybird species. Two *Harmonia axyridis* and two *Halyzia sedecimguttata* ladybirds were collected in 2016 and another 8 of each in 2017 and 2018. They were identified by eye and captured using collecting techniques as described in Majerus *et al.* (1989). They were kept in the fridge until used for DNA extractions.

2.3.2 DNA extraction

Genomic DNA was extracted from the above-mentioned whole ladybirds. Ladybirds were immersed in 70% ethanol, firstly to remove any contamination from surrounding bacteria via surface sterilisation and secondly to preserve the sample from any further degradation. Note bacteria within the ladybird gut associated with prey items may still be present (see Chapter 5). Ladybirds were then placed in 1.5 ml Eppendorf tubes, frozen in liquid nitrogen and ground up using a sterile, autoclaved blue tip. The quick-Start Protocol for Qiagen DNeasy® Blood & Tissue Kit (cat. nos. 69504 and 69506) was used for DNA extraction. 180 µl of pre-warmed lysis buffer ATL was added to the sample. The samples were ground again to make sure they were as finely crushed as possible. 20 µl of proteinase K solution was added to each sample and mixed well by flicking the tube without using a vortex. The samples were then incubated at 56°C for 3 hours or overnight in a

Biometra Thermoblock. After completion of incubation, 4 µl of RNase A was added and tubes vortexed gently for 10 seconds to ensure contents mixed well. 200 µl of lysis buffer AL and 200 µl of 100% ethanol were added and gently vortexed again for 10 seconds. The tubes were then placed back into the heated block to incubate at 56°C for a further 10 minutes. Then the samples were pipetted out into labelled mini-spin columns with collection tubes and centrifuged (Eppendorf centrifuge 5418) at 8,000 rpm for 1 minute. The filtrate was discarded and the mini-spin column was placed into a newly labelled collection tube. 500 µl of AW1 buffer was added to the spin column and centrifuged at 8,000 rpm for 1 minute. The filtrate was discarded and the mini-spin column was placed into a newly labelled collection tube. 500 µl of AW2 buffer was added to the spin column and centrifuged at approx. 14,000 rpm for 3 minutes. Once again, the filtrate was discarded and the spin column was transferred into a newly labelled 1.5 ml Eppendorf tube. 100 µl of buffer AE was added to the column directly without touching the mesh and it was left to stand for 2-5 minutes at room temperature. It was centrifuged at 8,000 rpm for 1 minute to get the first solution of DNA. The spin column was transferred into another newly labelled 1.5 ml Eppendorf tube and the steps from the addition of 100 µl buffer AE to centrifugation were repeated to produce a second solution of DNA. The first solution was not combined with the second solution. It was not necessary to use the second solution because insufficient DNA was detected in the second solution.

The method above was subsequently improved by transferring the frozen ladybirds to homogenisation tubes where three steel beads were placed in each tube. The

homogenisation tubes were run into Precellys 24 Tissue Homogenizer at 7,200 rpm for 10 seconds three times. 100 ul of dH₂O was used for elution instead of buffer AE. This modified method was used for later experiments described in Chapters three and four because it is quicker, easier, safer and consistent.

2.3.3 PCR amplification of 16S rDNA gene and purification of PCR products

Each DNA sample was analysed for bacterial presence through a PCR reaction using general bacterial 16S rDNA primers (Weisberg *et al.*, 1991) synthesised and supplied by SIGMA. The sequences of these primers and other related information are as shown in Table 2.1. 1µl dH₂O was used as a no-DNA control in PCR. The components of the PCR reaction mixture are given in Table 2.2. A thermocycler (FTC152D, UK) PCR machine was used for PCR with conditions consisting of 1 cycle of initial denaturation at 94°C for 2 minutes, 35 cycles of denaturation at 94°C (15 s), annealing at 55°C (30 s), extension at 72°C (3 min) and 1 cycle of final extension at 72°C (20 min) as shown in Table 2.3. All the PCR and sequencing reactions were performed in MJ Research Dyad Dual 96-well Thermal Cycler unless otherwise stated.

Name of primer	Primer sequence	Primer target	Expected product size (bp)	Reference
Forward primer (27f)	5'-GAGAGTTTGATCCTGGCTCAG-3'	16S ribosomal DNA (rDNA) gene	1500	Weisburg <i>et al.</i> , 1991
Reverse primer (1495r)	5' -CTACGGCTACCTTGTACGA-3'			

Table 2.1 Forward and reverse primer details used for the PCR reaction.

Reagent	Volume	Final concentration
Reddymix	5 µl	1.5 units Taq DNA polymerase, 10 mM Tris-HCL, 50 mM KCl, 1.5 mM MgCl ₂ , 0.001% gelatin, 0.2 mM dNTP, stabilisers
Forward primer (27f)	0.5 µl	0.5 µM
Reverse primer (1495r)	0.5 µl	0.5 µM
dH ₂ O	3 µl	
DNA samples extracted from <i>Harmonia axyridis</i> or <i>Halyzia sedecimguttata</i> ladybird	1 µl	
Final volume	10 µl	

Table 2.2 PCR reaction mixture.

Steps	Temperature	Time	Number of cycles
Initial denaturation	94°C	2 min	1
Denaturation	94°C	15 s	35
Annealing	55°C	30 s	35
Extension	72°C	3 min	35
Final Extension	72°C	20 min	1

Table 2.3 PCR conditions.

The PCR products were electrophoresed in 1% agarose gel containing 200 µg/ml of ethidium bromide and then visualised using UV light in Gel doc XR system, Bio-Rad. The PCR products from ladybird DNA samples showing clear bands on the gel for 16S rDNA gene at the expected length were sent for sequencing by the company Macrogen, initially as a 16S rDNA PCR product and then followed by individually selected and purified clones, and the closest matching sequences on GenBank were identified using BLAST online (Altschul *et al.*, 1990).

2.3.4 Cloning of 16S rDNA into TOPO® vector

The purification of target sequences of bacteria was done by a cloning technique as mentioned in Inoue *et al.* (1990) using the TOPO® vector. The TOPO® cloning reactions (3 µl) were set up as indicated in Table 2.4. The cloning reaction mixture was mixed gently by flicking the tube and incubated at room temperature (22–23 °C) for 5 minutes. The reaction was placed on ice. The NEB 5-alpha competent *E.*

coli cells stored at -70°C were thawed by holding the tube in the palm of the hand. Once the cells were thawed, they were transferred into the tube in the ice bath and incubated in ice for 10 minutes. A chilled, sterile pipette tip was used to transfer 100 µl of the competent cells to chilled, small falcon tubes and the cells stored on ice. 1.5 - 2 µl of the cloning reaction was added to each Falcon tube. For the control of transformation, 1.5 µl PUC19 (plasmid control) was added. The tubes were gently swirled several times to mix the contents well. The tubes were stored on ice for 30 minutes. The tubes were transferred to a rack placed in a preheated 42°C circulating water bath. The tubes were stored in the rack for exactly 30 seconds without shaking the tubes and were rapidly transferred to an ice bath and allowed to cool down for 5 minutes. 800 - 950 µl of SOC medium (2% (w/v) Tryptone, 0.5% (w/v) Yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂ and 20 mM Glucose) was added to each tube and then the tubes were transferred to a shaking incubator set at 37°C to incubate the cultures for 45 - 60 minutes. After incubation, 100 µl of transformed competent cells were transferred onto agar LB medium containing 40 mg/ml X-gal and 100 µg/ml of ampicillin. The plates were stored at room temperature until the liquid had been absorbed then inverted and incubated at 37°C for 12-16 hours to observe the transformed colonies.

Reagent	Volume	Concentration
Fresh PCR product	1.0 µl	1 µg/µl
Salt solution	0.5 µl	1.2 M NaCl, 0.06 M MgCl ₂
Water	1.0 µl	NA
TOPO® vector	0.5 µl	10ng/µl
Final volume	3.0 µl	

Table 2.4 TOPO® cloning reaction mixture.

2.3.5 Blue-white screening

The selection of transformed colonies was done using blue-white screening. This selection technique is one of the most common ones used to detect transformed colonies with recombinant DNA. The recombinant bacterial colonies stay white as β -galactosidase is not produced because of the insertion of the DNA fragment of interest into the *lacZ* operon. Whereas non-recombinant bacteria produce functional β -galactosidase which hydrolysed 5-bromo-4-chloro-3-indolyl- β -D-galactoside (X-gal) in the agar plate into 5-bromo-4-chloro-indoxyl, which spontaneously dimerises to produce an insoluble blue pigment called 5,5'-dibromo-4,4'-dichloro-indigo (Zamenhof and Villarejo, 1972). Based on this principle, 10 white colonies from each plate were picked separately with sterile tips and mixed in 10 sterile Eppendorf tubes containing 100 μ l sterile dH₂O and vortexed for 10 seconds. Once the colonies were properly mixed in dH₂O, 1 μ l of white colony solution from each tube was mixed with M13 forward and reverse primers and other reagents (Table 2.5) separately, for PCR to check the presence of insert bands. The PCR conditions used were initial denaturation at 95°C for 10 min, 28 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 3 min and a final extension at 72°C for 5 min. The PCR products were run in a 1% agarose gel for band visualisation as described in 2.3.3 above. A zero DNA PCR was included in each set of reactions.

Reagent	Volume	Final concentration
Reddymix	15 µl	1x
Forward primer (M13) 5'-CGCCAGGGTTTTCCCAGTCACGAC-3'	1.5 µl	0.5 mM
Reverse primer (M13) 5'-CACACAGGAAACAGCTATGACCAT-3'	1.5 µl	0.5mM
dH ₂ O	11 µl	
White colonies aliquot of <i>Harmonia axyridis</i> or <i>Halyzia sedecimguttata</i> ladybird	1 µl	
Final volume	30 µl	

Table 2.5 PCR to check the presence of insert bands using M13 primers.

2.3.6 Identification of bacterial community

PCR products from white colonies from *Harmonia axyridis* and *Halyzia sedecimguttata* ladybird samples, which gave a clear band of the expected size for the insert on the gel, were sent for sequencing. In total 47 colonies were selected for *Harmonia axyridis* from 8 different individuals. In detail 7 white colonies from sample number 1, 15 white colonies from sample number 2, 4 white colonies from sample number 3, one white colony from sample number 4, 6 white colonies each from sample number 5 and sample number 10, 3 white colonies from sample number 6 and 5 white colonies from sample number 7 were selected for *Harmonia axyridis*. Similarly, 21 white colonies were selected for *Halyzia sedecimguttata* from 4 individuals. In detail 7 white colonies from sample number 1, 5 white colonies from sample number 2, 8 white colonies from sample number 8 and one white colony from sample number 9 were also selected for *Halyzia sedecimguttata*. Sequencing was carried out by the company Macrogen and the sequences were used in BLAST (Altschul *et al.*, 1990) as query sequences to identify similar sequences from the library or database of sequences available, which are deposited at the National Institutes of Health (NIH), USA. The aim of this project is to understand the diversity of bacterial community in ladybirds. Since the

isolated 16S rDNA sequences were from uncharacterised or unidentified bacteria, sequencing followed by BLAST was used to identify bacterial community.

2.3.7 Phylogenetic analysis of 16S rRNA gene sequences

Phylogenetic relationships were evaluated using the sequences which were obtained in this study, alongside the 16S rRNA bacterial sequences available in GenBank. Phylogenetic analysis was carried out using programmes available on the website Phylogeny.fr (Dereeper *et al.*, 2008). Sequences were manually cleaned and then converted to FASTA file format. The sequences were aligned by MUSCLE (Edgar, 2004) and curated by Gblocks (Castresana, 2000) using default settings for both programmes. The phylogenetic trees were constructed by PhyML selecting GTR (na) as a substitution model and the number of substitution rate categories set at 8. The newick format output from PhyML was opened in FigTree v1.4.4 which was used for visualisation of the tree. Options within FigTree were selected to change the font size and line length to improve the resolution of the trees. The Bootstrapping procedure was used for statistical tests for branch support and the number of bootstraps was set at default 100.

Maximum likelihood phylogenetic inferences clustered the bacterial genera, *Burkholderia contaminans* MN826151.1 and *Delftia tsuruhatensis* MT374262.1, identified in two *Harmonia axyridis* sequences generated from two DNA samples, Uncultured bacterium KX507403.1, identified in 12 DNA samples, *Flavobacterium alkalisola* MN310902.1, identified in 6 DNA samples and *Myroides profundus* KF730743.1, identified also in 9 DNA samples. These were used to create the

preliminary phylogenetic tree of 16S rRNA sequences generated from DNA samples extracted from *Harmonia axyridis*.

Novosphingobium clariflavum MN629069.1, identified in two *Halyzia sedecimguttata* samples, *Staphylococcus warneri* KC213938.1 and *Streptococcus mitis* JN713499.1 identified in two sequences from one DNA sample, were used to create the preliminary phylogenetic tree of 16S rRNA sequences generated from DNA samples extracted from *Halyzia sedecimguttata*.

Burkholderia contaminans MN826151.1, *Delftia tsuruhatensis* MT374262.1, *Novosphingobium* sp. MK863544.1, *Staphylococcus warneri* KC213938.1, *Streptococcus mitis* JN713499.1, Uncultured bacterium KX507403.1, *Flavobacterium alkalisola* MN310902.1 and *Myroides profundus* KF730743.1 were used to construct the preliminary phylogenetic tree of 16S rRNA sequences generated from both *Harmonia axyridis* and *Halyzia sedecimguttata* sequences.

Finally, additional trees were created incorporating 16S rRNA sequences from a wider range of genera representing approximately 10 different BLAST hits in each case, plus an outgroup, to illustrate the broader phylogenetic placement of the bacteria identified from the ladybird sample. The *Cedrus deodara* chloroplast or the archaeon *Methanococcus voltae* sequence was selected as an outgroup as they are phylogenetically distant from the other genera represented in the trees, as appropriate.

2.3.8 Metagenomic analysis of *Harmonia axyridis* symbionts

Investigation of the microbiome of four *Harmonia axyridis* individuals was carried out. Two male and two female *H. axyridis* were frozen at -80°C and then placed in

DNA/RNA Shield™ before shipping via Cambridge Bioscience to the US company Zymo Research Corporation. Zymo carried out DNA purification, 16S library preparation, Illumina MiSeq sequencing and bioinformatic analysis following the methods outlined below:

The samples were processed and analyzed with the ZymoBIOMICS® Targeted Sequencing Service (Zymo Research, Irvine, CA).

DNA Extraction: One of three different DNA extraction kits was used depending on the sample type and sample volume. In most cases, the ZymoBIOMICS®-96 MagBead DNA Kit (Zymo Research, Irvine, CA) was used to extract DNA from the four adult ladybirds, using an automated platform.

Targeted Library Preparation: Bacterial 16S ribosomal RNA gene targeted sequencing was performed using the Quick-16S™ NGS Library Prep Kit (Zymo Research, Irvine, CA). In most cases, the bacterial 16S primers amplified the V3-V4 region of the 16S rRNA gene. These primers have been custom-designed by Zymo Research to provide the best coverage of the 16S gene while maintaining high sensitivity.

The sequencing library was prepared using an innovative library preparation process in which PCR reactions were performed in real-time PCR machines to control cycles and therefore limit PCR chimera formation. The final PCR products were quantified with qPCR fluorescence readings and pooled together based on equal molarity. The final pooled library was cleaned with the Select-a-Size DNA

Clean & Concentrator™ (Zymo Research, Irvine, CA), then quantified with TapeStation® (Agilent Technologies, Santa Clara, CA) and Qubit® (Thermo Fisher Scientific, Waltham, WA).

Control Samples: The ZymoBIOMICS® Microbial Community Standard (Zymo Research, Irvine, CA) was used as a positive control for each DNA extraction, if performed. The ZymoBIOMICS® Microbial Community DNA Standard (Zymo Research, Irvine, CA) was used as a positive control for each targeted library preparation. Negative controls (i.e. blank extraction control, blank library preparation control) were included to assess the level of bioburden carried by the wet-lab process.

Sequencing: The final library was sequenced on Illumina® MiSeq™ with a v3 reagent kit (600 cycles). The sequencing was performed with 10% PhiX spike-in.

Bioinformatics Analysis: Unique amplicon sequences variants were inferred from raw reads using the DADA2 pipeline (Callahan *et al*, 2016). Potential sequencing errors and chimeric sequences were also removed with the Dada2 pipeline. Chimeric sequences were also removed with the DADA2 pipeline. Taxonomy assignment was performed using Uclust from Qiime v.1.9.1 with the Zymo Research Database, a 16S database that is internally designed and curated, as reference. Composition visualization, alpha-diversity, and beta-diversity analyses were performed with Qiime v.1.9.1 (Caporaso *et al.*, 2010).

Absolute Abundance Quantification: A quantitative real-time PCR was set up with a standard curve. The standard curve was made with plasmid DNA containing one

copy of the 16S gene and one copy of the fungal ITS2 region prepared in 10-fold serial dilutions. The primers used were the same as those used in Targeted Library Preparation. The equation generated by the plasmid DNA standard curve was used to calculate the number of gene copies in the reaction for each sample. The PCR input volume was used to calculate the number of gene copies per microliter in each DNA sample. The resulting values are shown in the gene_copies column of the absolute abundance results table.

The number of genome copies per microliter DNA sample (genome_copies) was calculated by dividing the gene copy number by an assumed number of gene copies per genome. The value used for 16S copies per genome is 4. The value used for ITS copies per genome is 200. The amount of DNA per microliter DNA sample (DNA_ng) was calculated using an assumed genome size of 4.64×10^6 bp, the genome size of *Escherichia coli*, for 16S samples, or an assumed genome size of 1.20×10^7 bp, the genome size of *Saccharomyces cerevisiae*, for ITS samples. This calculation is shown below:

Calculated Total DNA = Calculated Total Genome Copies $\times$ Assumed Genome Size (4.64×10^6 bp) $\times$ Average Molecular Weight of a DNA bp (660 g/mole/bp) $\div$ Avogadros Number (6.022×10^{23} /mole)

2.4 Results

In this study a total of 20 ladybirds (10 *Harmonia axyridis* and 10 *Halyzia sedecimguttata*) were analysed for their bacterial diversity using 16S rDNA, following methods outlined in section 2.3.2. Total genomic DNA was extracted from all the samples and a clear band of high molecular weight DNA was visible in 1% agarose gel using Lambda DNA/HindIII Marker as shown in Figure 2.1. There was no noticeable difference in the quality of genomic DNA as a result of the modification in the extraction method described in 2.3.2. All DNA samples were assessed for the presence of bacterial DNA through PCR using 16S rDNA primers as described in Table 2.1. The presence of the bands shown in Figure 2.2 along with the 100 bp DNA ladder confirms the presence of bacterial 16S rDNA in extracted genomic DNA samples. The PCR products at this stage were potentially a mixture of different bacterial 16S rDNA, an example sequence trace is displayed in Appendix Figure 1. As described in section 2.3.4, in order to isolate the different 16S rDNA sequences, the PCR products were cloned using the TOPO® vector, thus ensuring that only a single 16S rDNA sequence was present in each clone. The transformed colonies were identified through the white-blue screening method, as described in section 2.3.5.

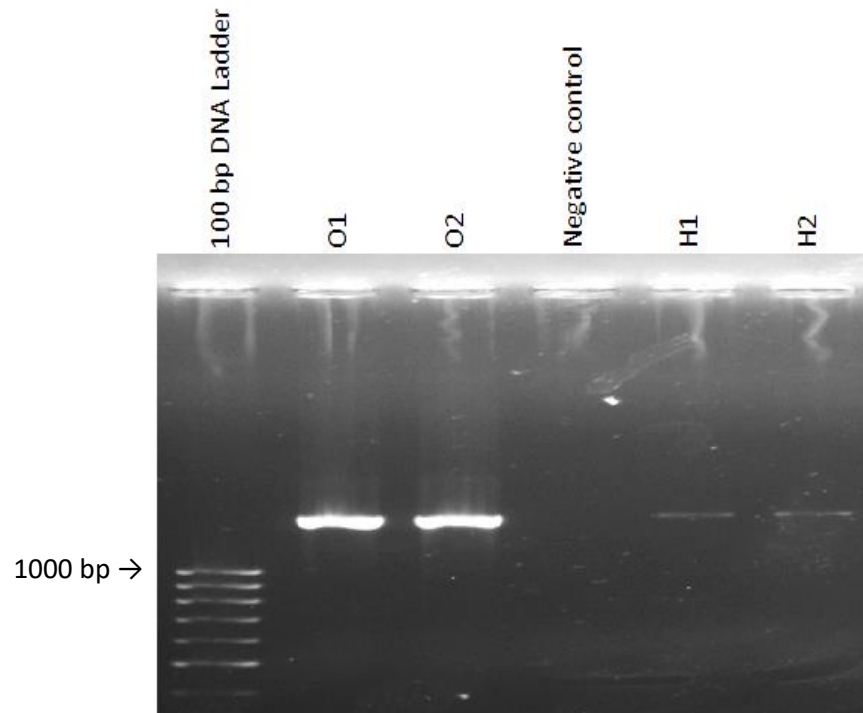


Figure 2.1 Genomic DNA extracted from Ladybirds. Lane1 & 12 - λ DNA/HindIII Marker; Lane2 to Lane 6 - Genomic DNA extracted from sample No. 3 to sample No. 7 (H3 – H7) of *Harmonia axyridis*; Lane 7 to Lane 11 - Genomic DNA extracted from sample No. 3 to sample No. 7 (O3 – O7) of *Halysia sedecimguttata*. [1](#) [6](#)

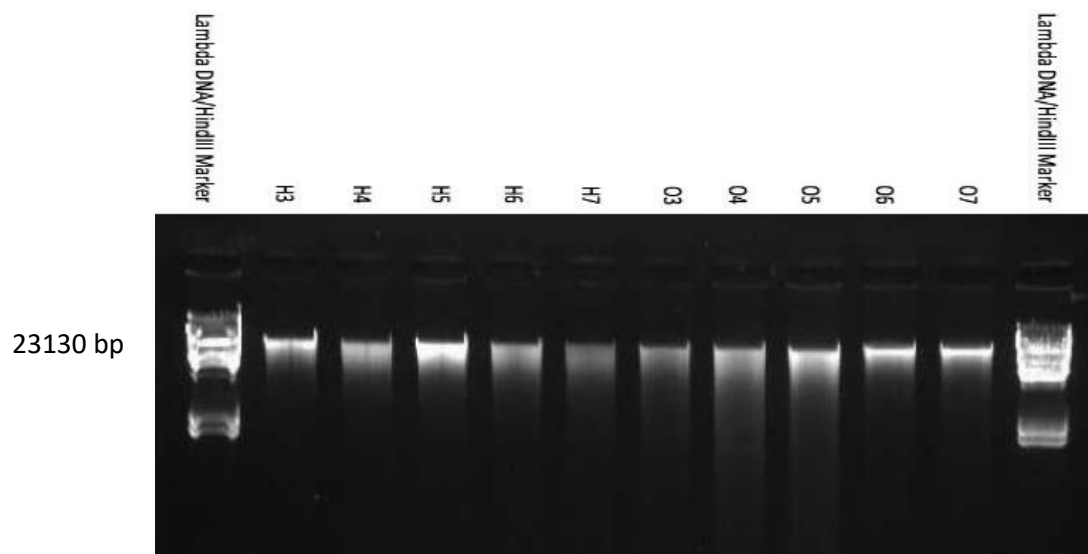


Figure 2.2 PCR products of 16S rDNA primers. Lane1 - 100 bp DNA Ladder; Lane2 & Lane 3 - amplified 16S rDNA from *Halysia sedecimguttata* (O1 & O2); Lane4- Negative control; Lane5 & Lane 6 - amplified 16S rDNA from *Harmonia axyridis* (H1 & H2). [7](#)

Cloning was successfully completed for a total of eight samples from *Harmonia axyridis* individuals and four samples from *Halyzia sedecimguttata* individuals. Transformed white bacterial colonies were selected and M13 forward and reverse primers (as shown in Table 2.5) were used to PCR out pure 16S rDNA from the TOPO® vector insert site. The PCR products that gave a clear band were submitted for sequencing to the company MacroGen. The quality of sequences was checked again to ensure they were not mixed, as illustrated in Appendix Figure 2. This ‘clean’ sequence trace indicates the success of the cloning approach for purifying amplified 16S rDNA from mixed PCR products. Finally, the resulting sequences were identified using BLAST (Altschul *et al.*, 1990) as described in section 2.3.6, and details of the closest sequence matches are presented in Tables 2.6 and 2.7. As indicated in Table 2.6, *Harmonia axyridis* and *Halyzia sedecimguttata* showed the presence of Proteobacteria, Actinobacteria, Bacteroidetes, Firmicutes along with unidentified bacteria. Further, Appendix Table 1 demonstrates greater taxonomic resolution obtained after a comparative analysis of submitted sequences with the existing sequences available in BLAST databases. In the case of *Harmonia axyridis*, fourteen confirmed bacterial genera i.e *Burkholderia*; *Delftia*; *Janthinobacterium*; *Rhizobium*; *Salinibacterium*; *Auriemonas*; *Flavobacterium*; *Novosphingobium*; *Acinetobacter*; *Granulicatella*; *Rodentibacter*; *Lactococcus*; *Myroides*; *Stenotrophomonas* were found. In addition, the BLAST hits included samples identified as ‘uncultured bacterium’. This name refers to the fact that the bacterium in question was not cultured but was simply isolated from an environmental or host species sample. In comparison, in *Halyzia sedecimguttata*

bacterial species belong to 11 genera including *Duganella*; *Rhizobium*; *Novosphingobium*; *Klenkia*; *Staphylococcus*; *Sphingomonas*; *Janthinobacterium*; *Methylobacterium*; *Alkanindiges*; *Streptococcus* and *Acinetobacter*. Preliminary (not shown) and additional phylogenetic trees were created as described in 2.3.7. The 'unidentified' or 'uncultured' bacteria were omitted from the phylogenetic analysis presented in the additional trees (Figures 2.7 and 2.8), due to the lack of taxonomic detail provided in the database but were included in Figures 2.3 to 2.6 to infer their taxonomy.

The phylogenetic analysis demonstrates clear separation of the samples included in the trees Figure 2.3 to 2.6, by class, allowing the class of bacteria identified in this study and that of previously described uncultured/unidentified bacteria to be determined. The lower taxonomic levels cannot be unambiguously determined for all samples hence are not given.

The four individual ladybirds investigated contained between 9 and 217 different bacterial species. 217 different bacteria were identified (Appendix Table 2) and in addition for one sample (female 1) 38.6% of reads were assigned as 'other'. These are reads that have not been recognisably matched to previously identified taxa. These 'other' reads are excluded from further analysis. The stringent process for sample preparation and amplification ensures that amplification is controlled for any contamination, via use of zero DNA controls and sequences trimmed, checked for chimera and unique sequences identified. The vast majority of species identified in the metagenomics analysis are present in very small proportions of the individual ladybird's microbiome, with most making-up less than 1%, on

average, of the total. Of those that amount to more than 1% of an individual's reads, approximately 10% on average of the reads come from 'other'. It should be noted that in many cases the vast majority of these highly represented species make up a large proportion of the reads from one individual only. Figures 2.9 and 2.10 show the breakdown of different 16S reads by individual. Figure 2.9 shows the breakdown with respect to Class and Figure 2.10 at species level. Table 1 in the Appendix gives the full taxonomic details of the metagenomics results. For example, the reads classed as 'other' make up over 38.6% of the reads from Female 1 and between 0.03 and 2.2% of reads from the other three individuals. Individual bacterial species that are highly represented include Cyanobacteria, *Pinus wallichiana*; the Firmicute *Lactococcus lactis*; three Proteobacteria, *Serratia grimesii-liquefaciens-proteamaculans*, *Serratia marcescens* and *Serratia marcescens-nematodiphila*; Actinobacteria, *Rhodococcus rhodochrous-ruber* and Proteobacteria, *Pinus sp46807* and *Stenotrophomonas maltophilia*.

Type of bacteria identified		Number of <i>H. axyridis</i> host in which the bacteria are found	Number of <i>H. sedecimguttata</i> host in which the bacteria are found
Phylum	Class		
Proteobacteria	Alphaproteobacteria	7	7
Proteobacteria	Betaproteobacteria	8	2
Proteobacteria	Gammaproteobacteria	2	3
Firmicutes	Bacilli	18	5
Actinobacteria	Actinobacteria	1	1
Acidobacteria	Terriglobia	1	-
Bacteroidetes	Flavobacteriia	10	-
Bacteroidetes	Cytophagia	-	1
Bacteroidetes	Chitinophagia	-	1
Pseudomonadota	Hydrogenophilia	-	1

Table 2.6 Summary of bacteria identified in ladybird samples via cloning experiments as described in section 2.3.4. Phylogenetic trees confirmed the class as shown in Figures 2.3 to 2.6.

Accession of uncultured bacterium identified	Phylum	Class
HM811652.1	Proteobacteria	Betaproteobacteria
MH934454.1	Proteobacteria	Betaproteobacteria
JQ191643.1	Proteobacteria	Betaproteobacteria
MH717852.1	Proteobacteria	Alphaproteobacteria
KX507403.1	Firmicutes	Bacilli
JN023276.1	Acidobacteria	Terriglobia
LC409580.1	Proteobacteria	Betaproteobacteria
JQ386884.2	Proteobacteria	Alphaproteobacteria
KF091179.1	Proteobacteria	Betaproteobacteria
LC410755.1	Proteobacteria	Betaproteobacteria
GQ080953.1	Proteobacteria	Betaproteobacteria
DQ532133.1	Proteobacteria	Alphaproteobacteria
EF157084.1	Firmicutes	Bacilli
KC541035.1	Proteobacteria	Gammaproteobacteria
GU361873.1	Firmicutes	Bacilli
GU201968.1	Bacteroidetes	Flavobacteriia
DQ800532.1	Firmicutes	Bacilli
JX457156.1	Bacteroidetes	Flavobacteriia
KM221316.1	Proteobacteria	Gammaproteobacteria
GQ443093.1	Bacteroidetes	Flavobacteriia
EU137419.1	Proteobacteria	Betaproteobacteria
HM264061.1	Proteobacteria	Betaproteobacteria
AB636931.1	Proteobacteria	Alphaproteobacteria
HM299709.1	Proteobacteria	Alphaproteobacteria
KF188985.1	Proteobacteria	Betaproteobacteria
KF843005.1	Firmicutes	Bacilli
KF494748.1	Firmicutes	Bacilli
JX255231.1	Bacteroidetes	Chitinophagia
KJ123785.1	Proteobacteria	Alphaproteobacteria
KU514522.1	Proteobacteria	Betaproteobacteria
EU536499.1	Proteobacteria	Alphaproteobacteria
AB294312.1	Proteobacteria	Alphaproteobacteria
FM873225.1	Proteobacteria	Alphaproteobacteria
KX507758.1	Proteobacteria	Gammaproteobacteria
OL952481.1	Proteobacteria	Alphaproteobacteria
JQ460737.1	Firmicutes	Bacilli
AB494281.1	Proteobacteria	Alphaproteobacteria
KU514497.1	Proteobacteria	Betaproteobacteria
MW686473.1	Pseudomonadota	Hydrogenophilia

Table 2.7 Taxonomy of uncultured and unidentified bacteria that were identified in BLAST search results. These bacteria are shown in Figures 2.3 to 2.6 and classes confirmed with the clustering in the trees.

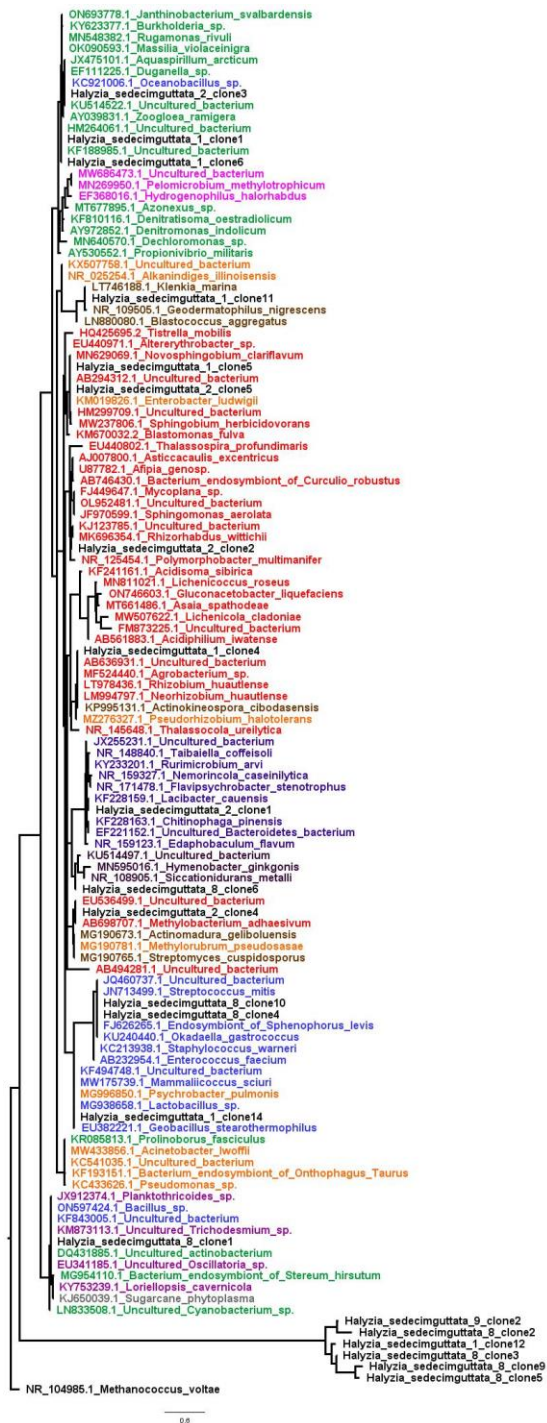


Figure 2.3 Phylogenetic analysis of 16S rRNA bacterial gene in *Halysia sedecimguttata* individuals. Branch length indicates the phylogenetic distance. BLAST sequences are highlighted in different colours. Each colour represents a bacterial class: green (Betaproteobacteria); light blue: (Bacilli); pink (Hydrogenophilia); orange (Gammaproteobacteria); brown (Actinobacteria); red (Alphaproteobacteria); dark blue (Chitinophagia); dark purple (Cytophagia); light purple (Cyanophyceae) and gray (Mollicutes). See Figs 2.3a and 2.3b for larger font size of species details.

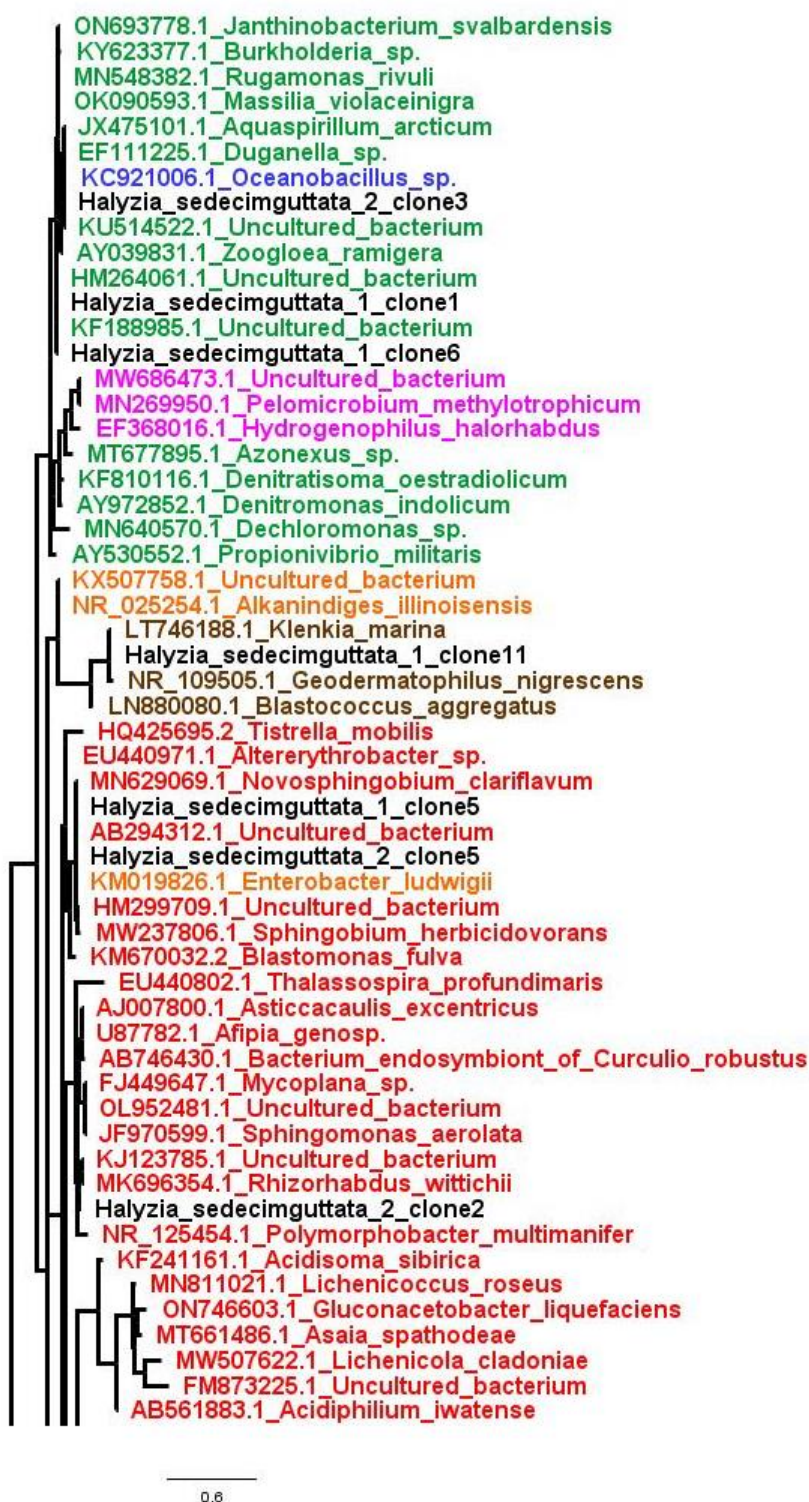


Figure 2.3a Phylogenetic analysis of 16S rRNA bacterial gene in *Halyzia sedecimguttata* individuals. Branch length indicates the phylogenetic distance. Top half of Fig 2.3

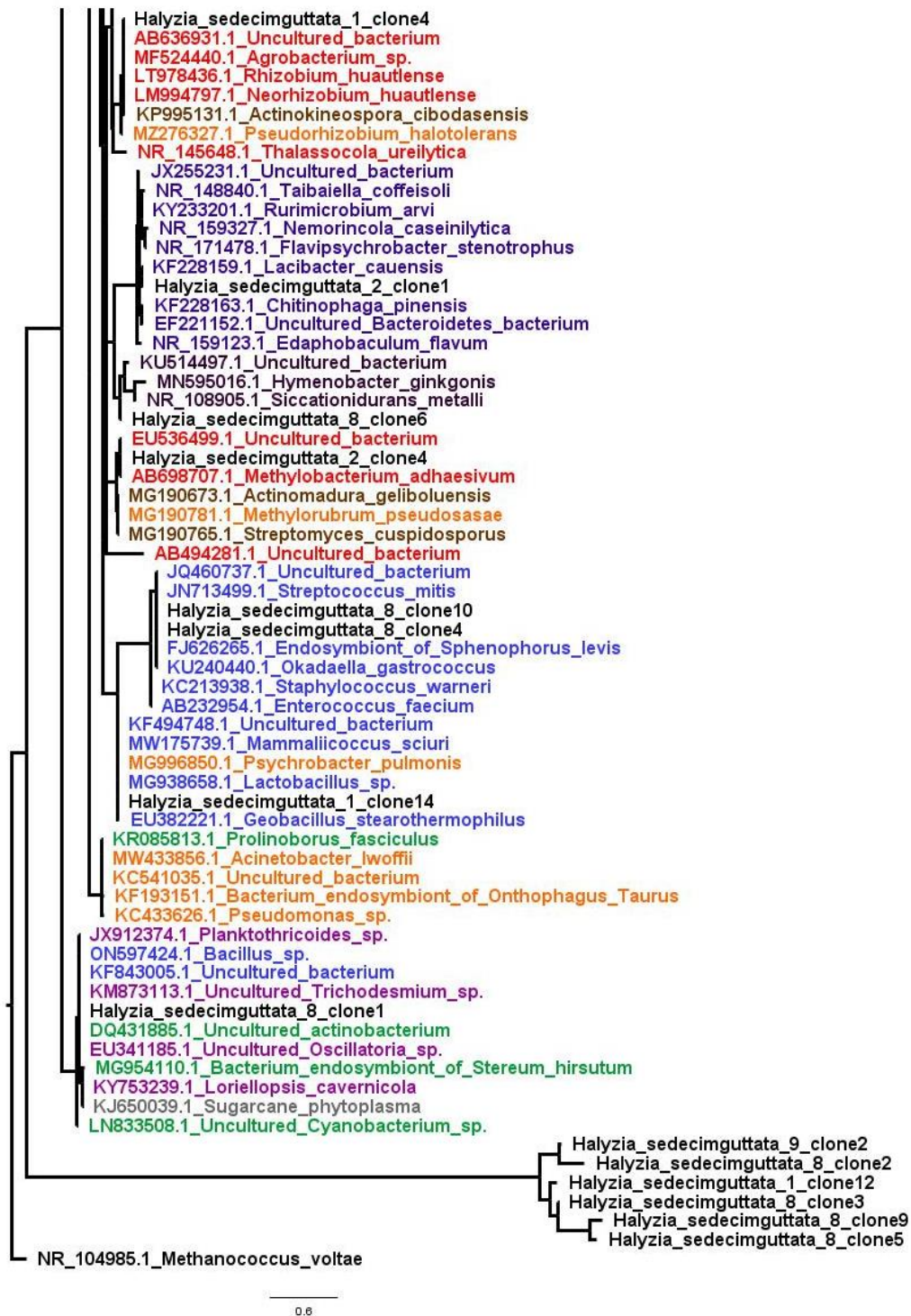


Figure 2.3b Phylogenetic analysis of 16S rRNA bacterial gene in *Halyzia sedecimguttata* individuals. Branch length indicates the phylogenetic distance. Bottom half of Fig 2.3.

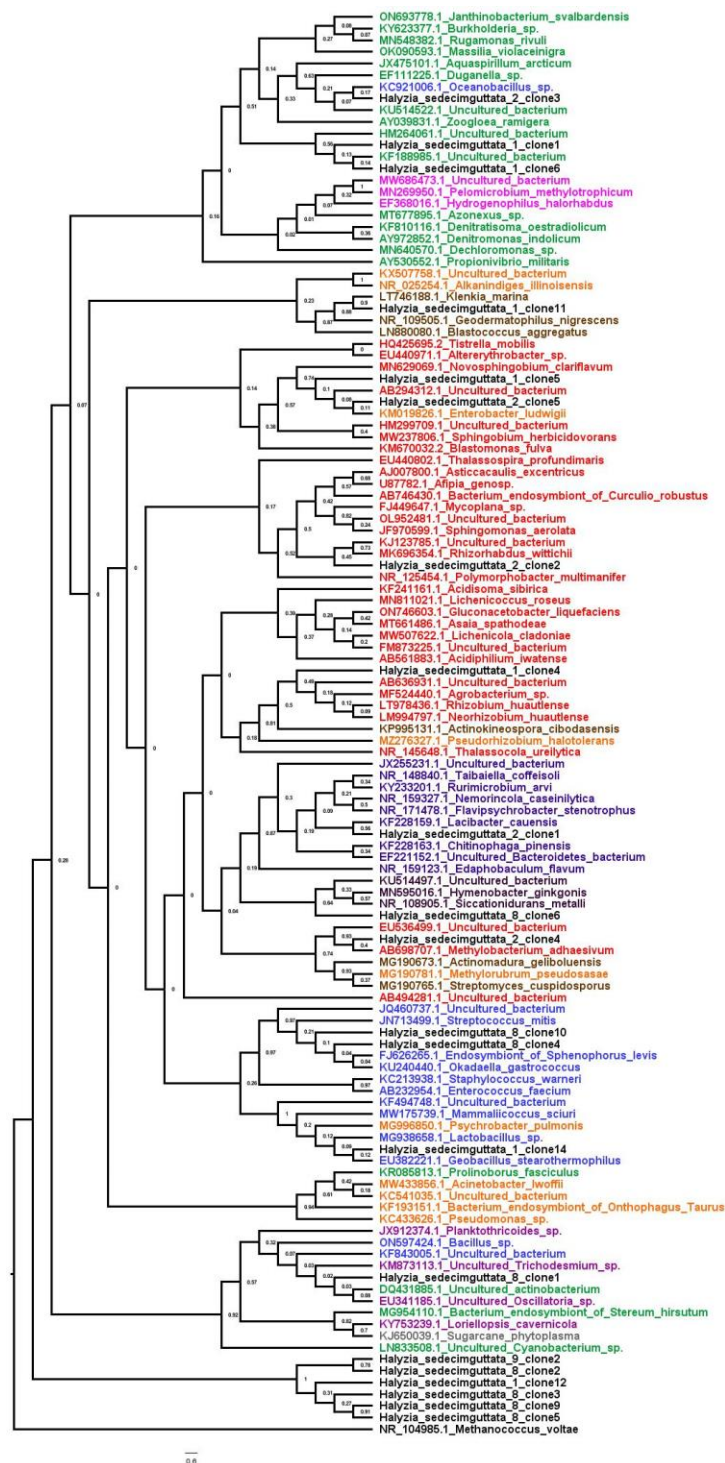


Figure 2.4 Phylogenetic analysis of 16S rRNA bacterial gene in *Halysia sedecimguttata* individuals with bootstraps. Numbers indicate bootstrap values with 100 replicates. BLAST sequences are highlighted in different colours. Each colour represents a bacterial class: green (Betaproteobacteria); light blue: (Bacilli); pink (Hydrogenophila); orange (Gammaproteobacteria); brown (Actinobacteria); red (Alphaproteobacteria); dark blue (Chitinophagia); dark purple (Cytophagia); light purple (Cyanophyceae) and gray (Mollicutes). See Figs 2.4a and 2.4b for larger font size of species details.

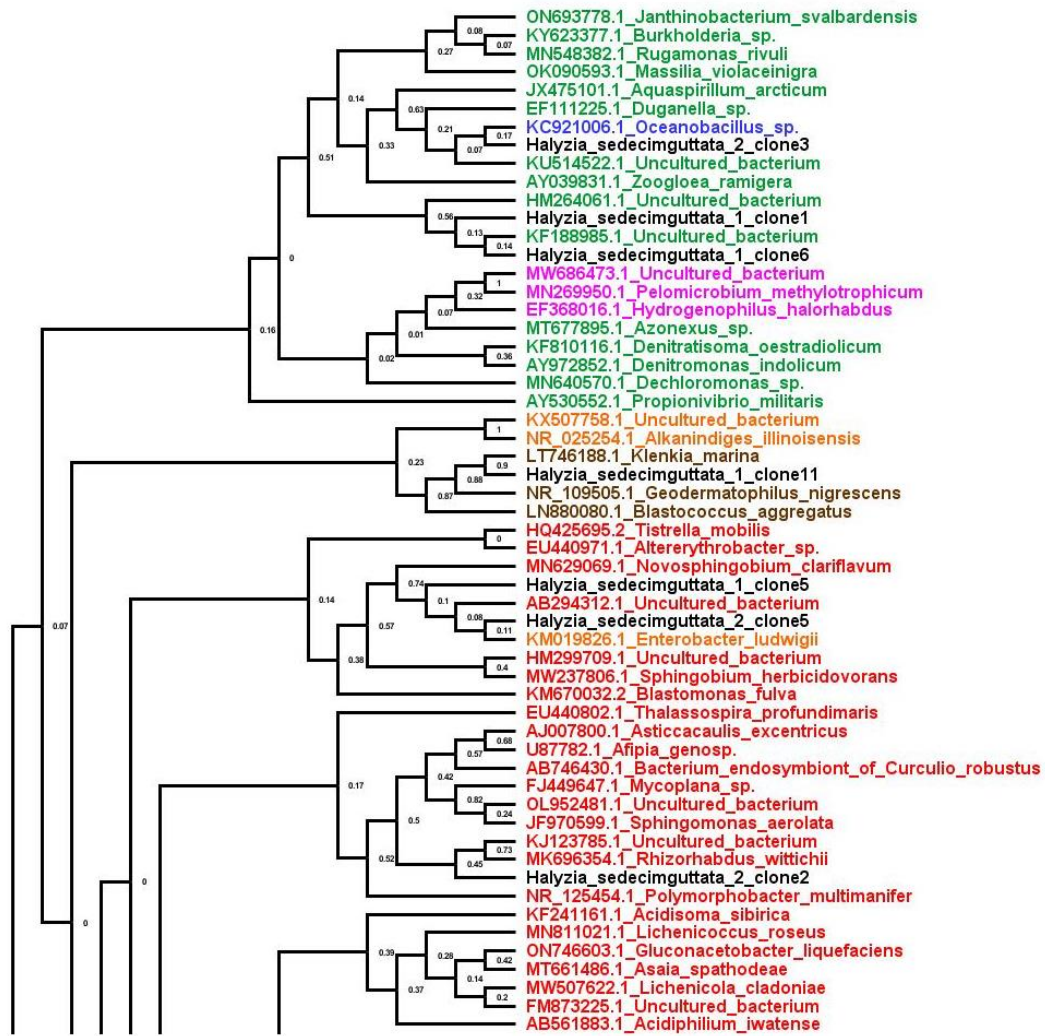


Figure 2.4a Phylogenetic analysis of 16S rRNA bacterial gene in *Halyzia sedecimguttata* individuals with bootstraps. Numbers indicate bootstrap values with 100 replicates. Top half of Fig 2.4.

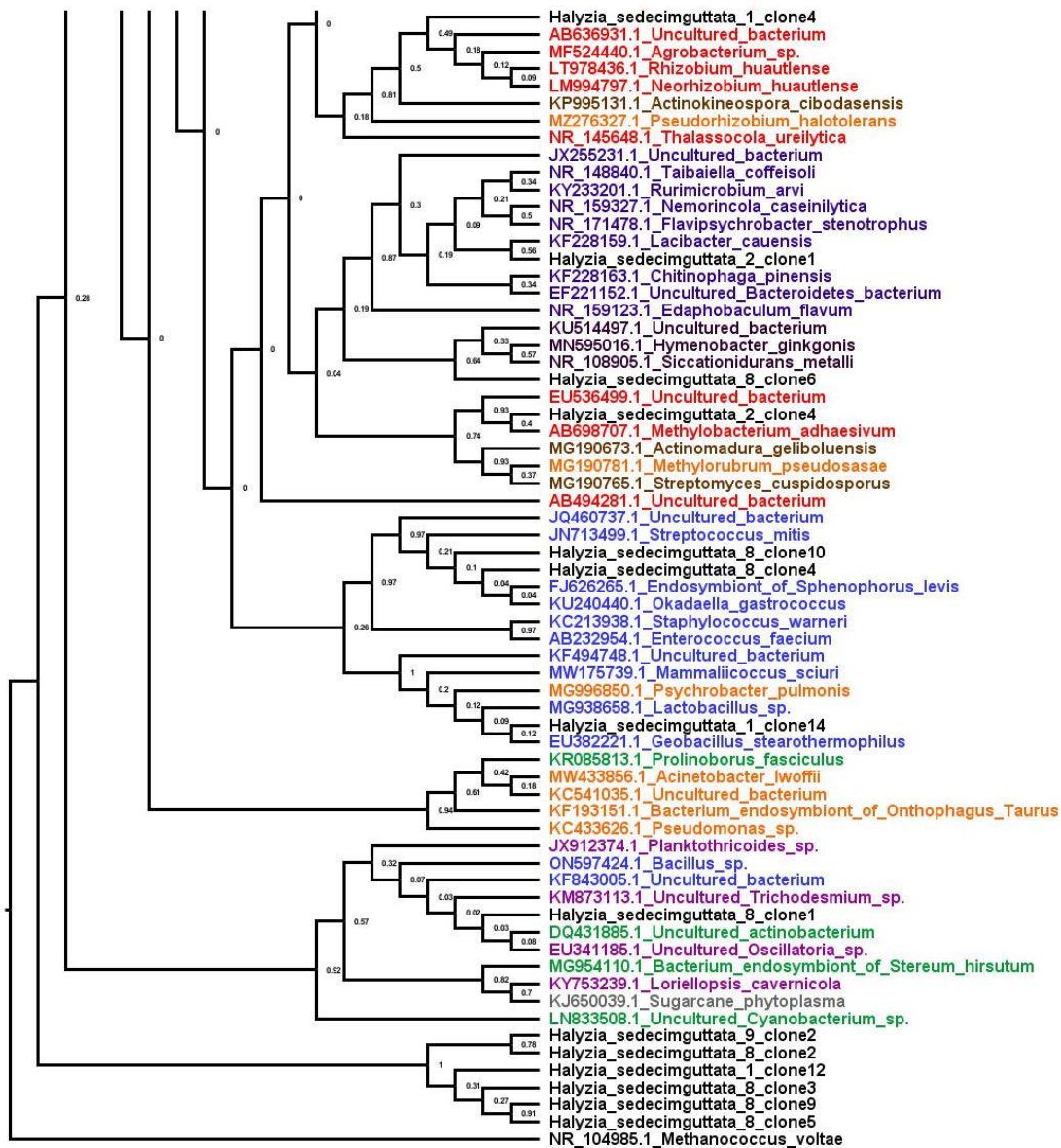


Figure 2.4b Phylogenetic analysis of 16S rRNA bacterial gene in *Halyzia sedecimguttata* individuals. Branch length indicates the phylogenetic distance. Bottom half of Fig 2.3.

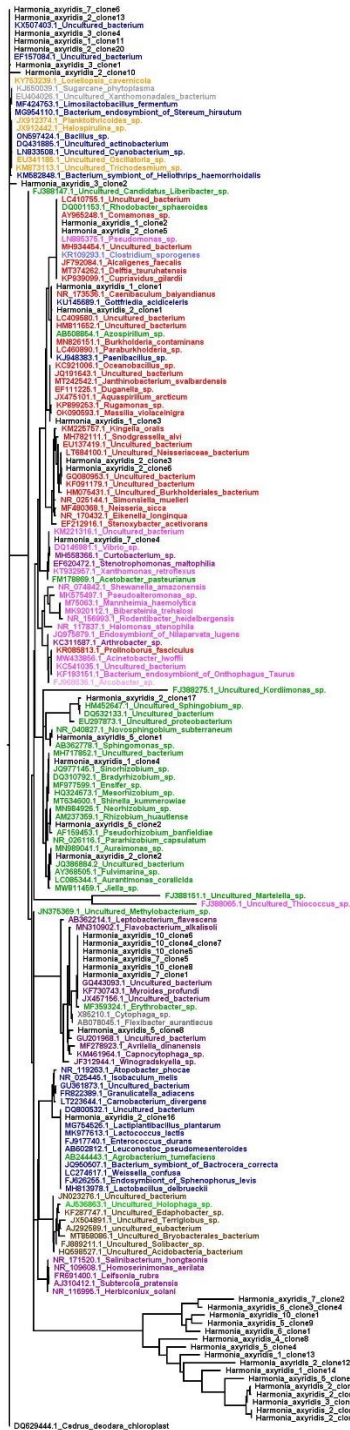
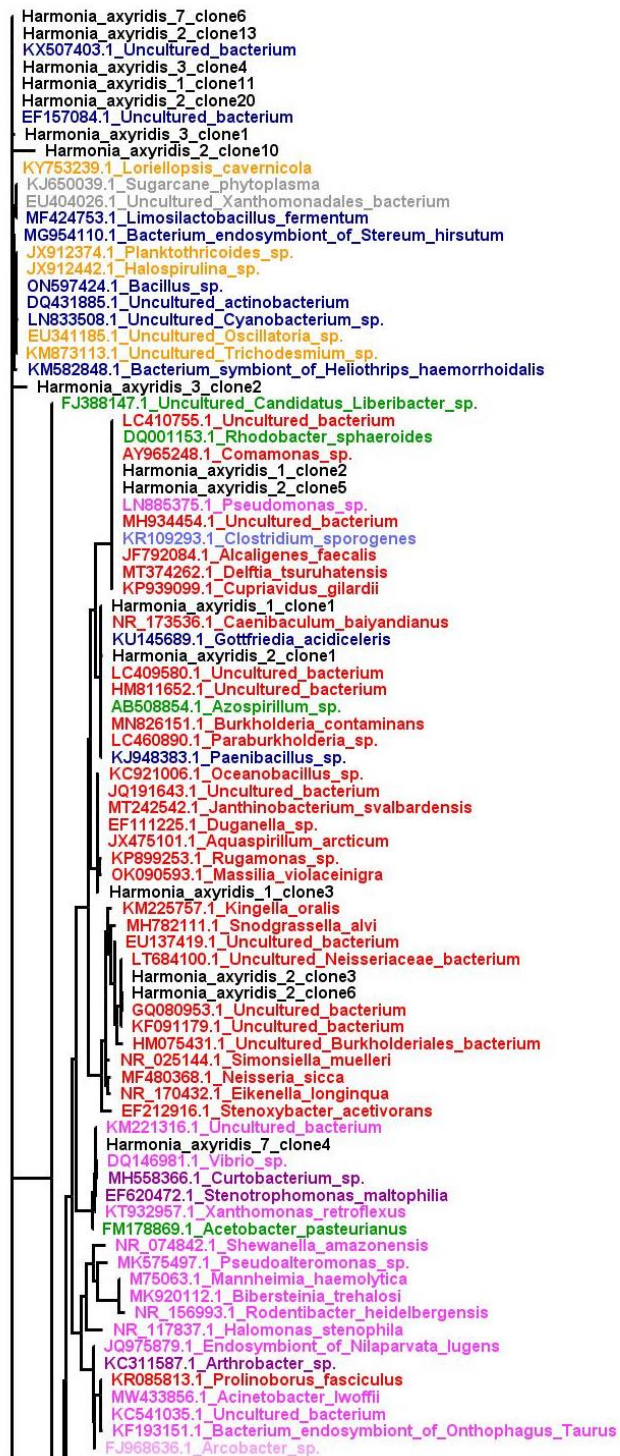


Figure 2.5 Phylogenetic analysis of 16S rRNA bacterial gene in *Harmonia axyridis* individuals. Branch length indicates the phylogenetic distance. BLAST sequences are highlighted in different colours. Each colour represents a bacterial class: dark blue (Bacilli); orange (Cyanophyceae); light gray (Mollicutes); dark green (Alphaproteobacteria); red (Betaproteobacteria); pink (Gammaproteobacteria); light blue (Clostridium); light purple (Actinobacteria); light pink (Epsilonproteobacteria); dark blue (Flavobacteriia); dark gray (Cytophagia); light green (Holophagae); brown (Terriglobia). See Figs 2.5a and 2.5b for larger font size of species details.



0.3

Figure 2.5a Phylogenetic analysis of 16S rRNA bacterial gene in *Harmonia axyridis* individuals with bootstraps. Numbers indicate bootstrap values with 100 replicates. Top half of Fig 2.5.

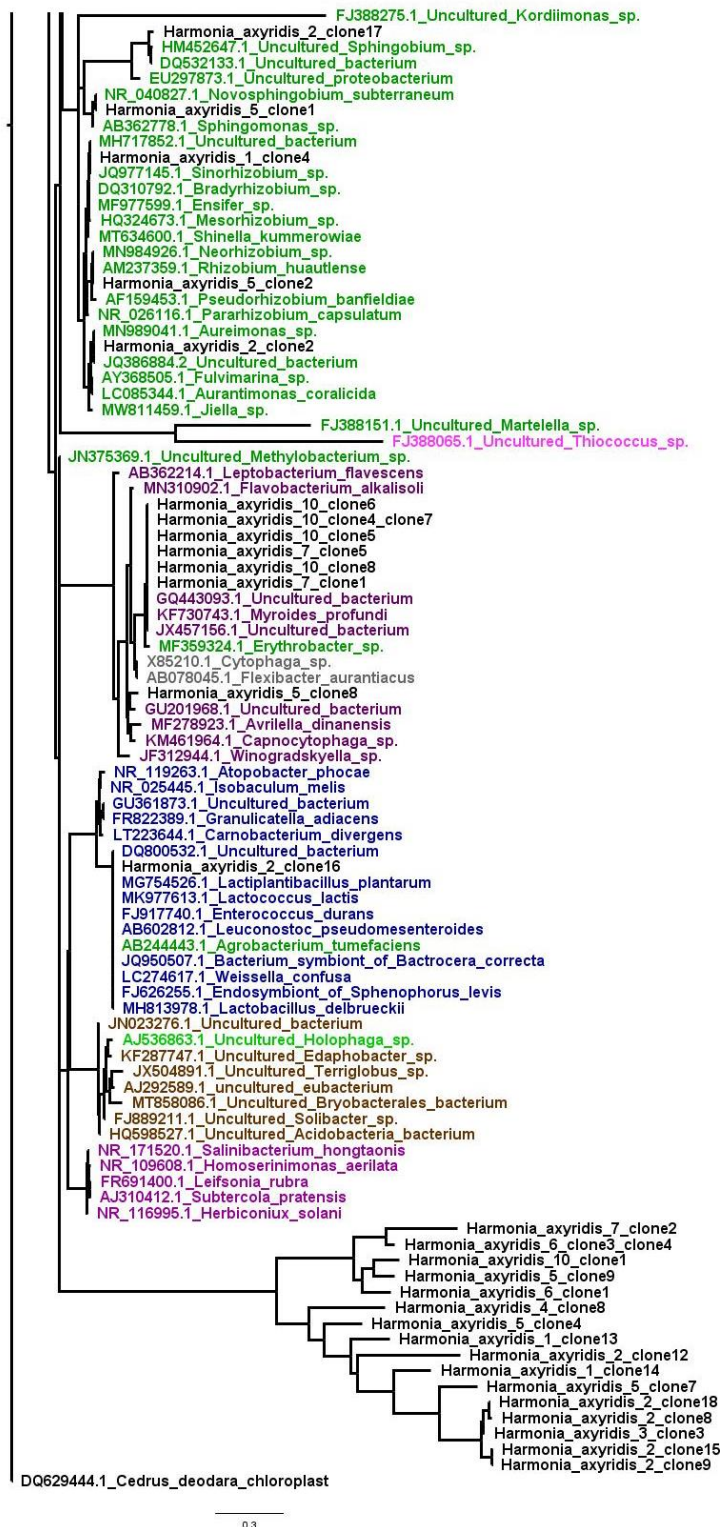


Figure 2.5b Phylogenetic analysis of 16S rRNA bacterial gene in *Harmonia axyridis* individuals with bootstraps. Numbers indicate bootstrap values with 100 replicates. Bottom half of Fig 2.5.

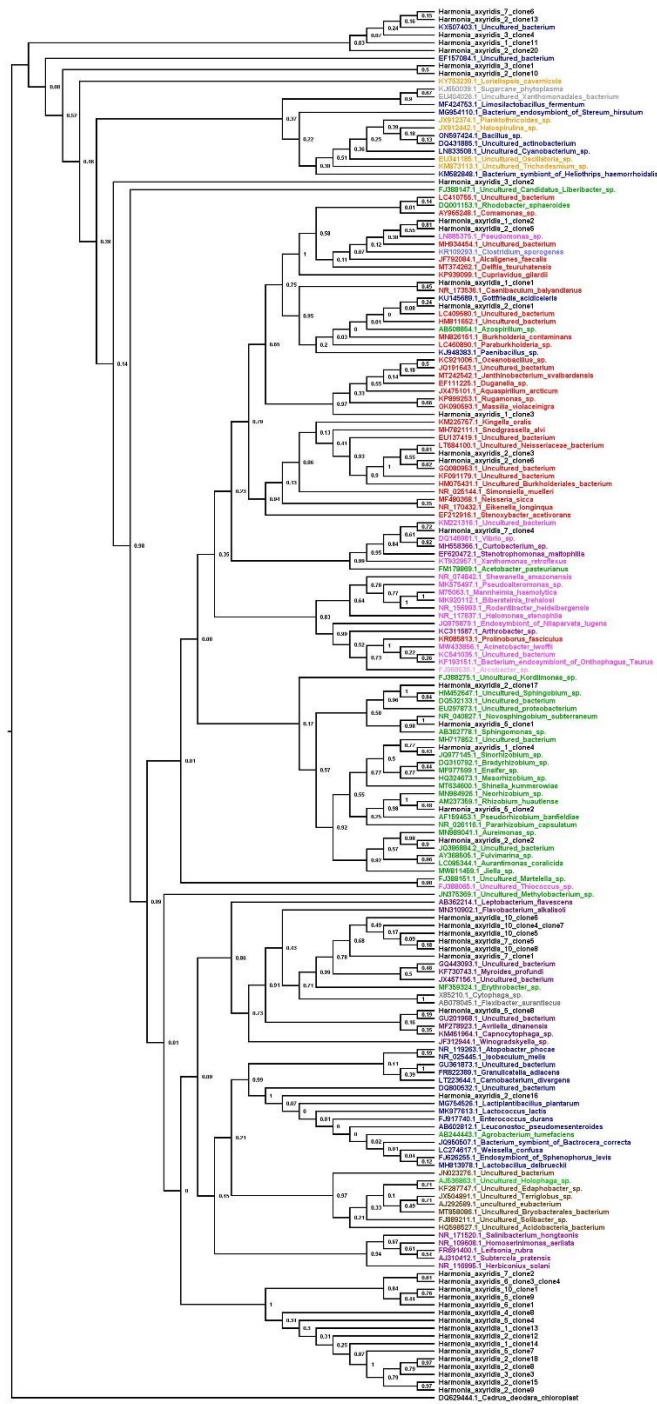


Figure 2.6 Phylogenetic analysis of 16S rRNA bacterial gene in *Harmonia axyridis* individuals with bootstraps. Numbers indicate bootstrap values with 100 replicates. BLAST sequences are highlighted in different colours. Each colour represents a bacterial class: dark blue (Bacilli); orange (Cyanophyceae); light gray (Mollicutes); dark green (Alphaproteobacteria); red (Betaproteobacteria); pink (Gammaproteobacteria); light blue (Clostridium); light purple (Actinobacteria); light pink (Epsilonproteobacteria); dark blue (Flavobacteriia); dark gray (Cytophagia); light green (Holophagae); brown (Terriglobia). See Figs 2.6a and 2.6b for larger font size of species details.

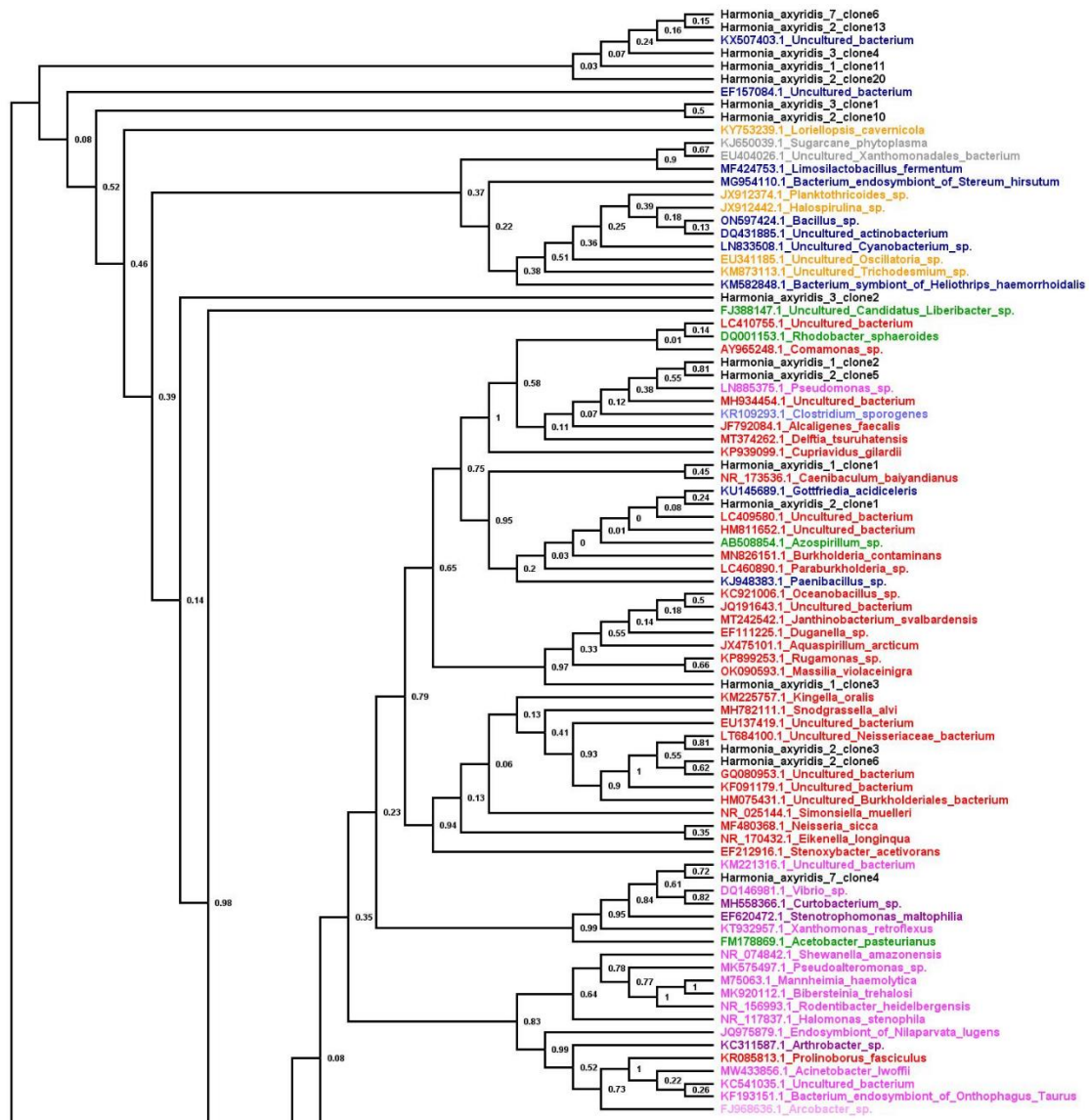


Figure 2.6a Phylogenetic analysis of 16S rRNA bacterial gene in *Harmonia axyridis* individuals with bootstraps. Numbers indicate bootstrap values with 100 replicates. Top half of Fig 2.6. 12

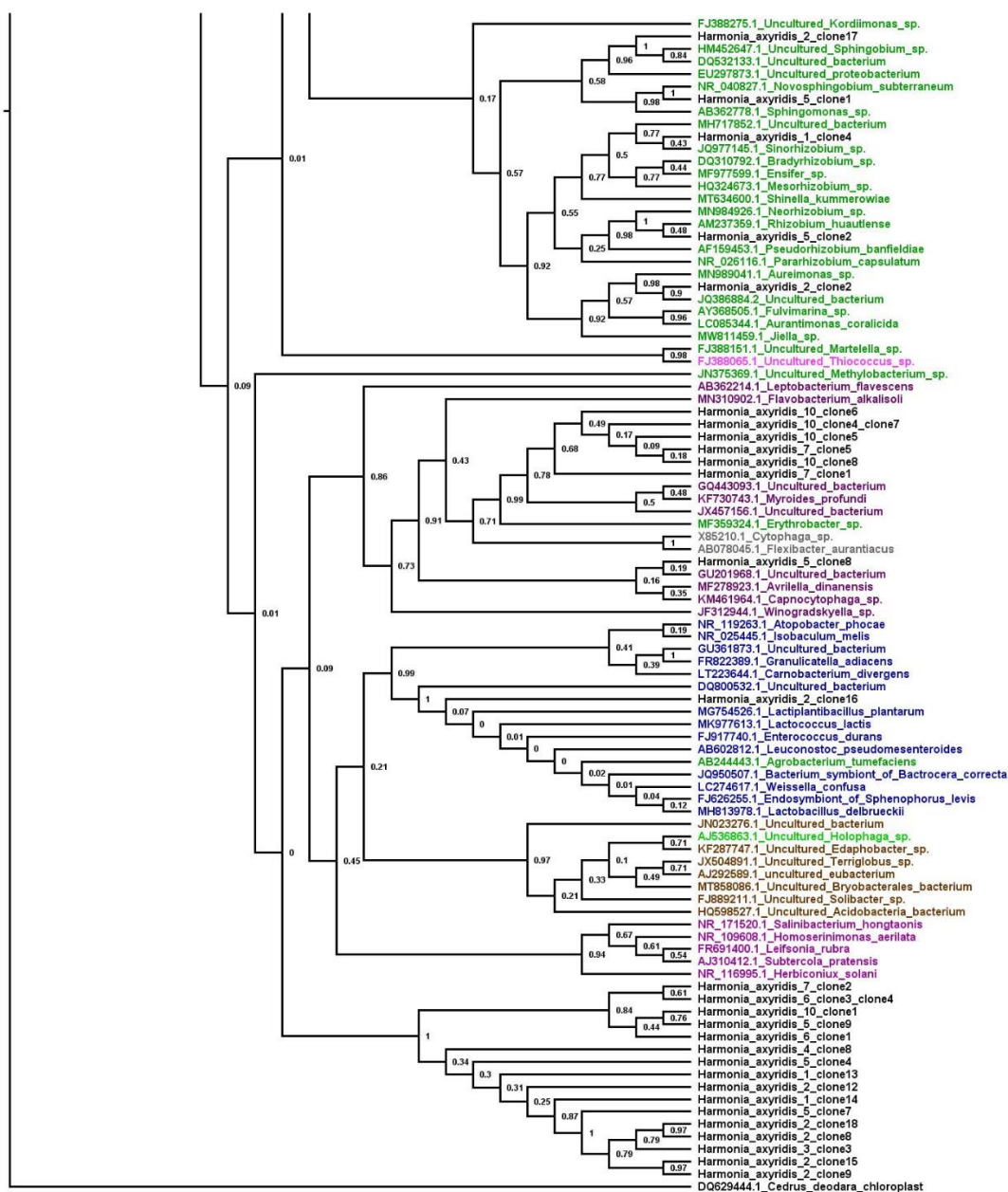


Figure 2.6b Phylogenetic analysis of 16S rRNA bacterial gene in *Harmonia axyridis* individuals with bootstraps. Numbers indicate bootstrap values with 100 replicates. Bottom half of Fig 2.6.

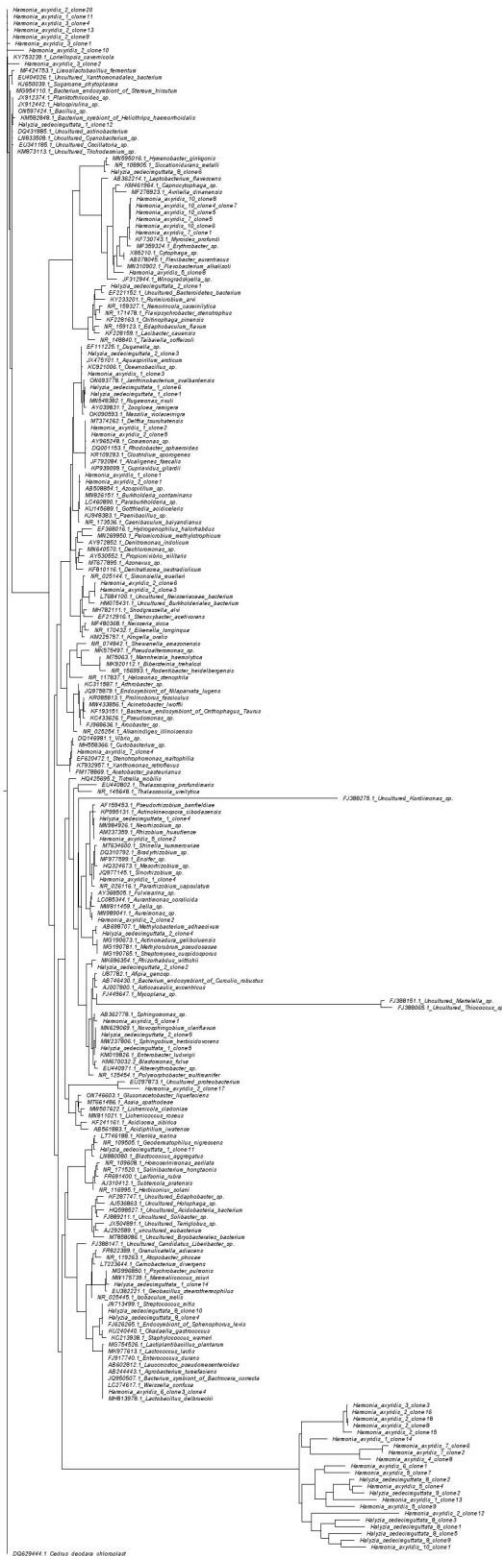


Figure 2.7 Phylogenetic analysis of 16S rRNA bacterial gene in *Harmonia axyridis* and *Halysia sedecimguttata* individuals. Branch length indicates the phylogenetic distance. See Figs 2.7a and 2.7b for larger font size of species details. 13

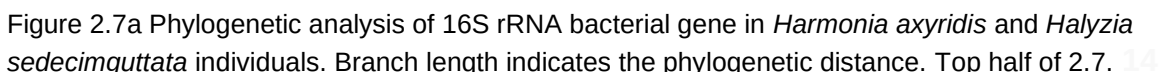




Figure 2.7b Phylogenetic analysis of 16S rRNA bacterial gene in *Harmonia axyridis* and *Halyzia sedecimguttata* individuals. Branch length indicates the phylogenetic distance. Bottom half of 2.7.

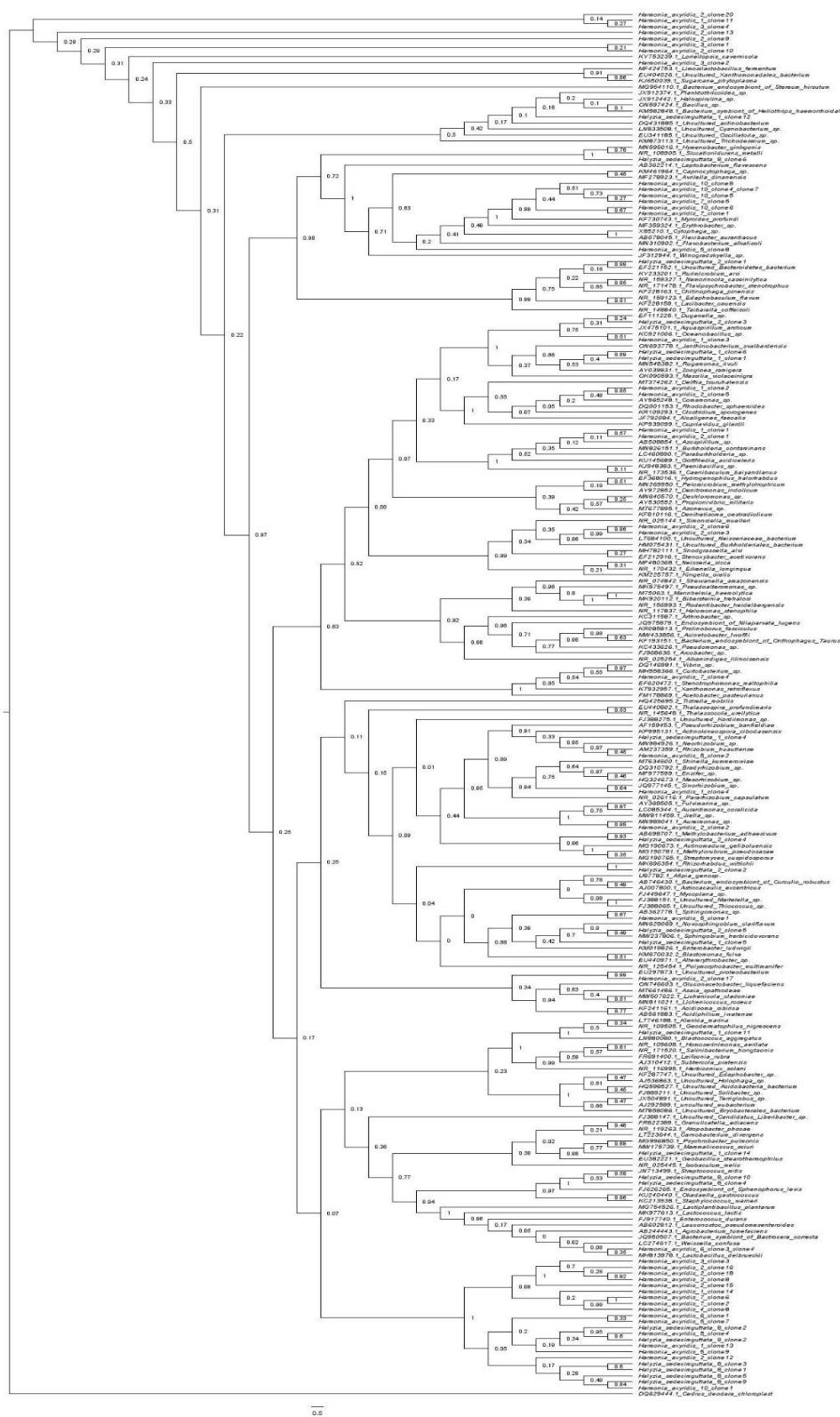


Figure 2.8 Phylogenetic analysis of 16S rRNA bacterial gene in *Harmonia axyridis* and *Halysia sedecimguttata* individuals with bootstraps. Numbers indicate bootstrap values with 100 replicates. See Figs 2.8a and 2.8b for larger font size of species details.

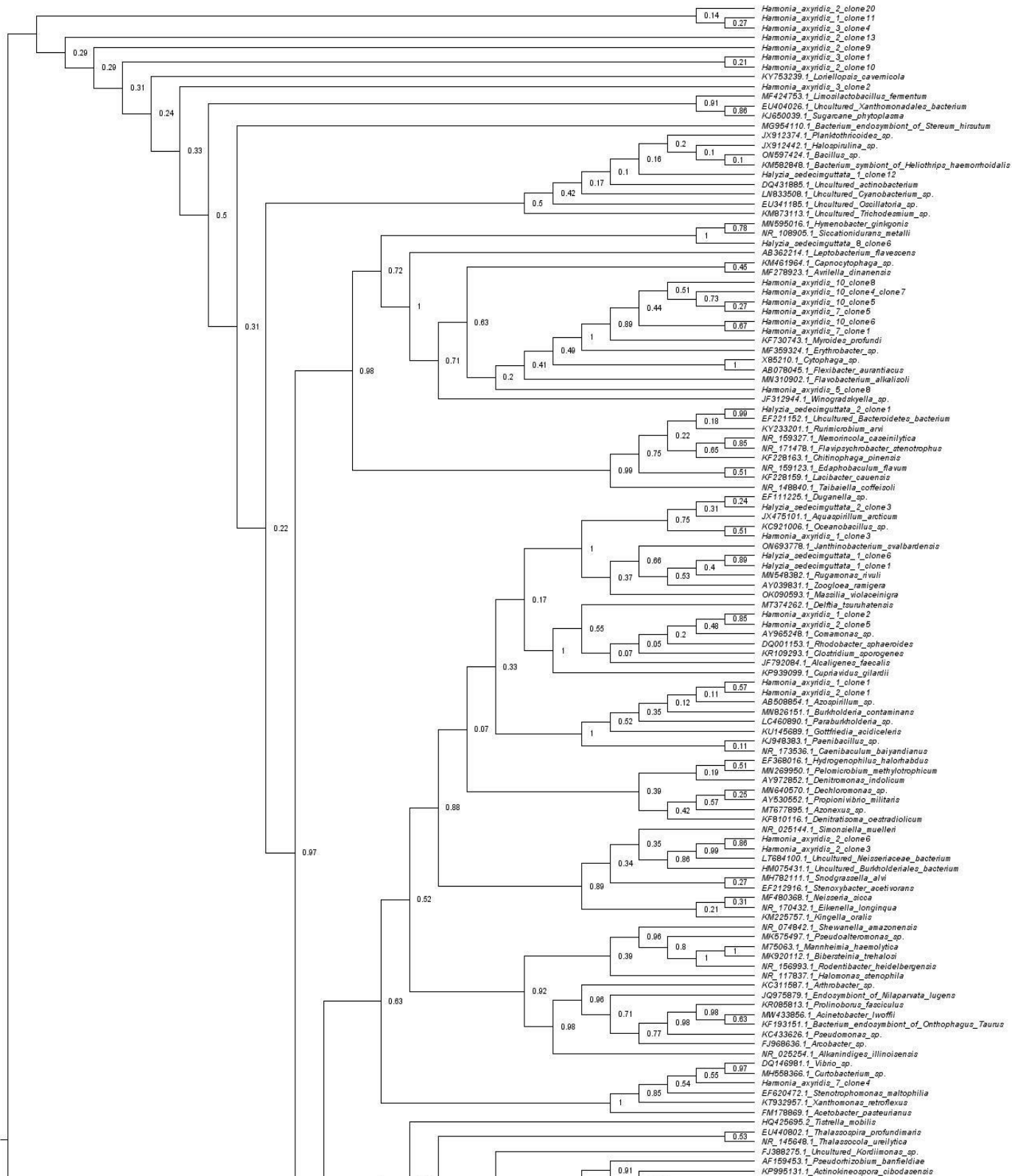


Figure 2.8a Phylogenetic analysis of 16S rRNA bacterial gene in *Harmonia axyridis* and *Halyzia sedecimguttata* individuals with bootstraps. Numbers indicate bootstrap values with 100 replicates. Top half of Fig 2.8. 17

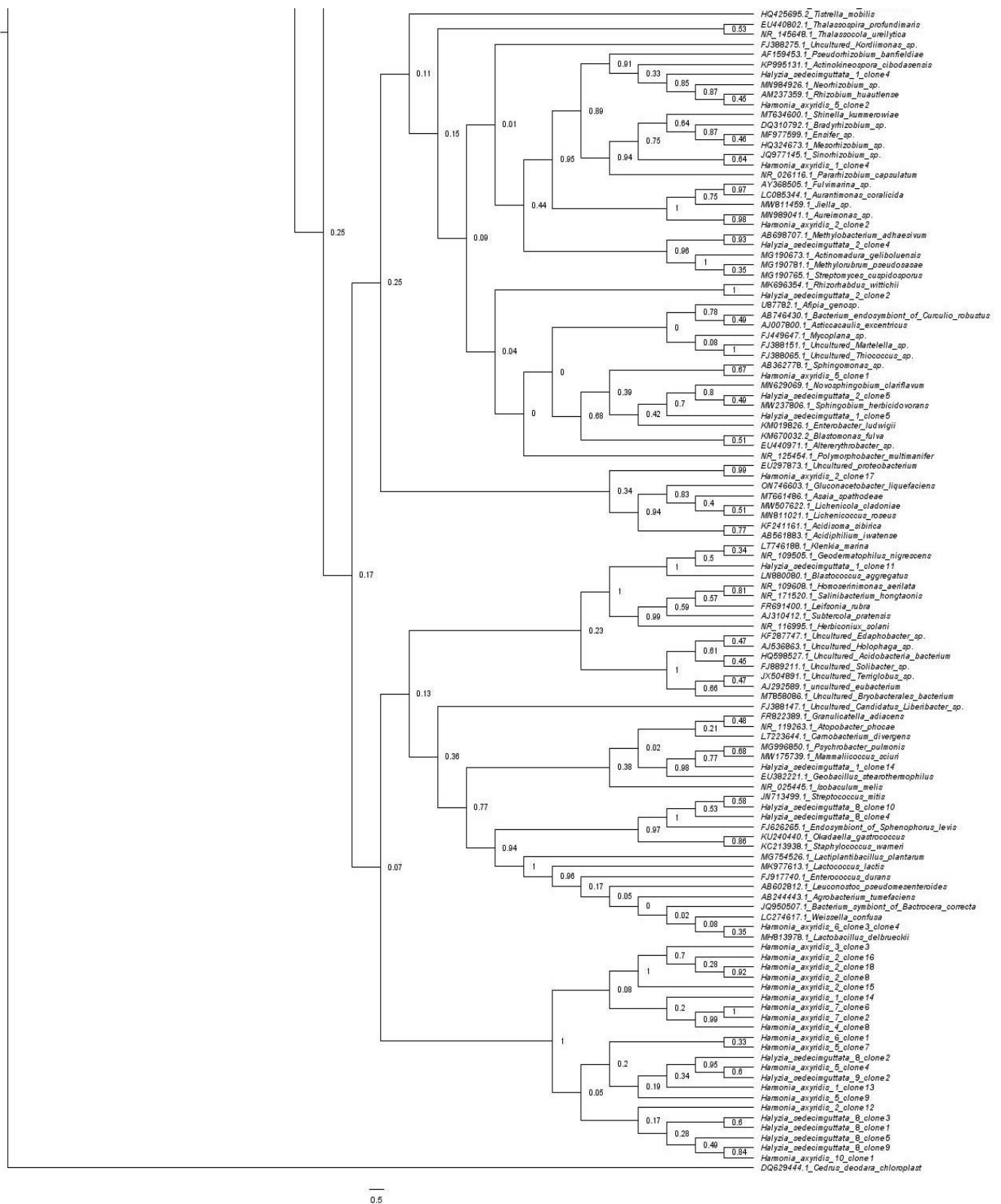


Figure 2.8b Phylogenetic analysis of 16S rRNA bacterial gene in *Harmonia axyridis* and *Halyzia sedecimguttata* individuals with bootstraps. Numbers indicate bootstrap values with 100 replicates. Bottom half of Fig 2.8. 18

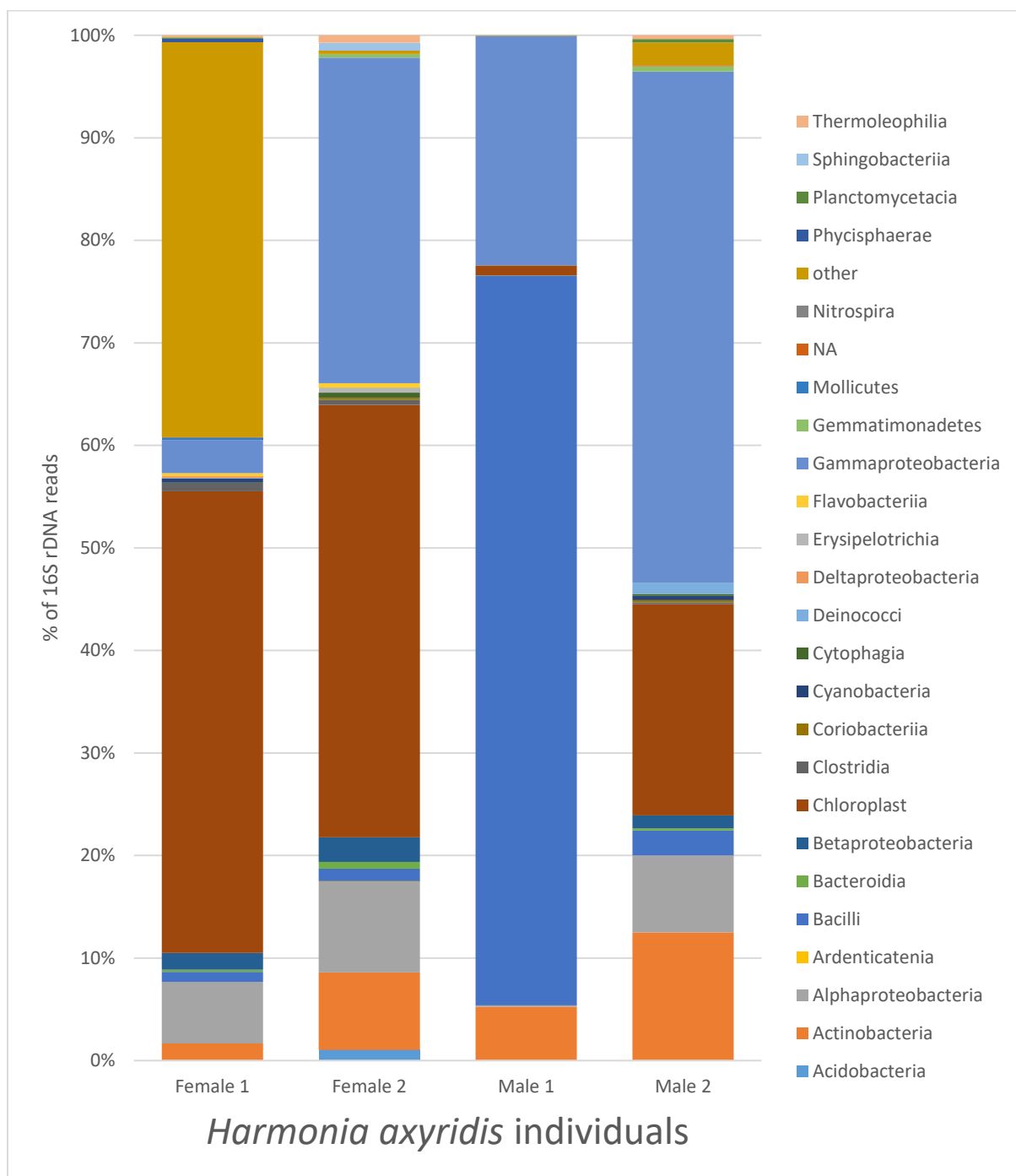


Figure 2.9 Stacked column graph illustrating the make-up of 16S rDNA sequences from four *Harmonia axyridis* individuals, two female, two male with respect to Class. See Appendix Table 4 for details of full taxonomy of bacteria. 19

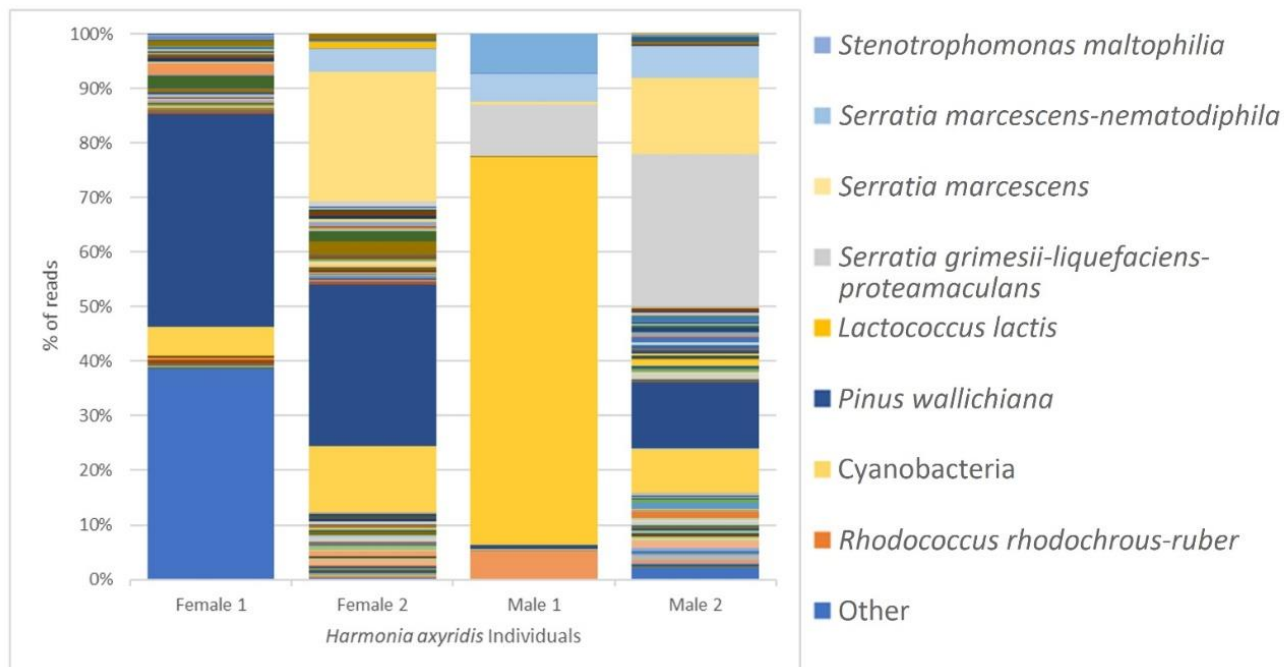


Figure 2.10 Stacked column graph illustrating the make-up of 16S rDNA sequences from four *Harmonia axyridis* individuals, two female, two male at species level. The key covered the most significant species identified, those present as >5% of the reads. See Appendix Table 4 for details of full taxonomy of bacteria. 20

2.5 Discussion

The current study investigated the diversity of the bacterial community in 8 *Harmonia axyridis* and 4 *Halyzia sedecimguttata* samples. The 16S rRNA gene sequences are closely aligned with sequences collected from GenBank. The phylogenetic trees were constructed using the closely related query sequences available in GenBank and the newly obtained sequences to validate the presence of the bacteria within the studied host species. Additional 16S sequences were included in the phylogenetic analysis in order to assess the phylogenetic positions of the sequences generated in this study. Phylogenetic analysis supported the matches to the *Harmonia axyridis* 16S sequences listed in Appendix Table 1. It also supported a close relationship with *Burkholderia* sp.; *Delftia* sp.; *Paraburkholderia* sp.; *Paenibacillus* sp.; *Cupriavidus gilardii*; *Comamonas* sp.; *Pseudomonas* sp.; *Rhodobacter sphaeroides*; *Clostridium sporogenes*; *Alcaligenes faecalis*; *Snodgrassella alvi*; *Simonsiella muelleri*; *Eikenella longinqua*; *Limosilactobacillus fermentum*; *Halospirulina* sp.; *Flavobacterium* sp.; *Enterococcus durans*; *Leuconostoc pseudomesenteroides*; *Agrobacterium tumefaciens*; *Weissella confuse*; *Lactococcus lactis*; *Lactobacillus delbrueckii*; *Myroides* sp.; *Erythrobacter* sp.; *Cytophaga* sp. and *Lactiplantibacillus plantarum*. Within these two phylogenetic trees (Figure 2.7 and Figure 2.8), different monophyletic groupings gathered respective bacterial species, the presence of the following phyla was shown Proteobacteria, Actinobacteria, Bacteroidetes and Firmicutes along with unidentified bacteria in *Harmonia axyridis* and *Halyzia sedecimguttata* ladybird species, similar outcomes were reported by (Dudek et al.,

2017). These bacteria have been identified using several isolation methods and are generally found to be associated with many types of insects, in particular with arthropods. These phyla have commonly shared environments such as soil (Kaluzhnaya *et al.*, 2012). BLAST analysis revealed the presence of several bacterial species in both *Harmonia axyridis* and *Halyzia sedecimguttata*. In the case of *Harmonia axyridis*, bacterial genera such as *Burkholderia* (2 individuals); *Delftia* (2 individuals); *Janthinobacterium* (1 individual); *Rhizobium* (2 individuals); *Salinibacterium* (1 individual); *Auriemonas* (1 individual); *Flavobacterium* (4 individuals); *Novosphingobium* (1 individual); *Acinetobacter* (1 individual); *Granulicatella* (1 individual); *Rodentibacter* (1 individual); *Lactococcus* (1 individual); *Myroides* (4 individuals) and *Stenotrophomonas* (1 individual) were identified. The first five identified bacterial species are gram-negative bacteria and found in soil and plant leaf surfaces (Lai *et al.*, 2011). These results corroborate a recent study where *Burkholderia*, *Delftia*, *Lactococcus* and *Rhizobium* species were identified in invasive ladybird *Harmonia axyridis* (Dudek *et al.*, 2017). While *Lactococcus*, *Flavobacterium*, *Acinetobacter*, *Delftia* and *Stenotrophomonas* were also identified by (Schmidtberg *et al.*, 2019). In comparison, in *Halyzia sedecimguttata* bacterial species belong to *Duganella* (2 individuals); *Rhizobium* (1 individual); *Novosphingobium* (2 individuals); *Klenkia* (1 individual); *Staphylococcus* (3 individuals); *Sphingomonas* (3 individuals); *Janthinobacterium* (2 individuals); *Methylobacterium* (1 individual); *Alkanindiges* (1 individual); *Streptococcus* (3 individuals) and *Acinetobacter* genera (2 individuals). *Duganella*, *Rhizobium*, *Novosphingobium*, *Bacterioidetes*, *Sphingomonas*, *Janthinobacterium*

and *Methylobacterium* species all are gram-negative bacteria found in soil and leaf surfaces (Lai *et al.*, 2011). This is in line with the majority of insect species which are reported to contain gut colonising species, such as Proteobacteria, Bacteroidetes and Firmicutes, that include *Lactobacillus*; *Bacillus*; Clostridia; Actinomycetes; Spirochetes; Verrucomicrobia and Actinobacteria (Zhang *et al.*, 2018).

Flavobacterium species induce male-killing in *Coleomegilla maculate* (Hurst *et al.*, 1997a); *Adonia variegata* (Hurst *et al.*, 1999c); *Coccinula sinensis* (Majerus and Majerus, 2000) and *Coccinula crotchii* (Elnagdy *et al.*, 2014). The bean bug, *Riptortus*, is an important agricultural pest, which can make beneficial symbiosis with *Burkholderia* from soil (Jung and Lee, 2019). *Burkholderia* horizontally transmits via the environment to every generation in the *Riptortus-Burkholderia* gut symbiosis system and *Burkholderia* symbiosis is not essential to hosts. However, it has been reported that non-infected *Riptortus* shows retarded body morphology and development and lower fecundity (Kikuchi and Fukatsu, 2014). Thus, *Burkholderia* plays a beneficial role in this symbiotic relationship but specific details of benefits from bacteria to the host remain to be determined, *Burkholderia* symbionts deficient of O-antigen are found to show a reduction in ability to colonise *Riptortus*, suggesting that O-antigen biosynthesis genes are critical to establish the symbiotic association but not required to maintain a proper symbiont population (Kim *et al.*, 2017). The *Riptortus-Burkholderia* gut symbionts may induce bacterial cell envelope changes to thus, increase *Burkholderia* symbionts' susceptibility to host immunity factors (Kim *et al.*, 2016). Otherwise, some strains of *Burkholderia*

help the host bean bug to develop resistance to insecticides and hence the host survives in the sprayed crop field (Itoh *et al.*, 2018).

Rhizobium association is environmentally acquired and not vertically transmitted as *Rhizobium* species are abundant in the soil of insect habitat (Kikuchi *et al.*, 2007). *Rhizobium* forms several nitrogen-fixing species symbioses associated with the roots of plants (González *et al.*, 2019). Other species such as *Janthinobacterium*, *Granulicatella*, *Rodentibacter* were not reported in the tested ladybirds previously and the present study was the first to identify their presence in *Harmonia axyridis*. Montagna *et al.* (2015) demonstrated the abundant presence of *Janthinobacterium* (Oxalobacteriaceae) in the phytophagous beetles *Cryptocephalus aquitanus* and *Cryptocephalus marginellus*. No previous reports were obtained about the symbiotic association between *Aureimonas*-beetle or insect species and this outcome of the present study requires further research. *Methylobacterium* is a Gram-negative bacterium, which colonises in plants and provides beneficial effects for plant growth and health (Lai *et al.*, 2020). Further, *Methylobacterium* sp. was reported to be one of the dominant genera at the pupal stage of *Dendroctonus armandi* (white pine beetle) and is hypothesised to influence insect development (Hu *et al.*, 2013). Bacterioidetes (*Cardinium hertigii*) have been reported to be the cause of cytoplasmic incompatibility in *Encarsia pergendiella* (Hunter *et al.*, 2003), feminisation of genetic males in *Brevipalpus* spp. (Weeks *et al.*, 2001) and thelytokous parthenogenesis in *Encarsi* spp. (Zchori-Fein *et al.*, 2004) and *Aspidiotus nerri* (Provencher *et al.*, 2005). The presence of *Methylobacteria* sp. in *Halyzia sedecimguttata* has not been reported in previous

studies, however, its existence was reported in *Harmonia axyridis* (Dudek *et al.*, 2017).

In general, the diversity of the bacterial community varies with the environmental habitat, diet, developmental stage and phylogeny of the hosts. The species of ladybirds used in this project vary in their diet. *Harmonia axyridis* is described as carnivorous (Roy *et al.*, 2019) whereas the orange ladybird *Halyzia sedecimguttata* is mycophagous (Sutherland and Parrella, 2009). Due to the horizontal transmission nature of the identified bacteria, it could be possible that they are linked with the feeding habits of ladybirds or the food chain, as Harlequin ladybirds primarily feed on aphids which suck the juice out of leaves and stems of plants which might have been inhabited by above-mentioned bacteria. Once ladybirds feed on aphids, bacterial symbionts may transmit horizontally to their guts. This speculation is based on the presence of the same male killing *Spiroplasma* in both insects (Romanov *et al.*, 2020). Without specific knowledge of the recent diet of the ladybird used in this study, it is not possible to confirm horizontal transmission from aphids or other component of the ladybird diet. In addition, The BLAST results are ambiguous for several samples suggesting that the PCR primers used were not fully specific for *Acinetobacter*. Hence, while the approach used in this project is useful, the primers need redesigning before it can be used for the selection and identification of bacteria in ladybird hosts.

Based upon the results of this study, the bacterial communities associated with both *Harmonia axyridis* and *Halyzia sedecimguttata* are composed mostly of bacteria, many of which may be found in their environment. These include unusual

and/or undescribed bacteria which may be critical for their survival, may be beneficial, detrimental or may have no quantifiable effect on the hosts. The shared occurrence of the same bacterial species in both ladybirds is perhaps due to the sharing of the same habitat, alternatively it may indicate that these bacteria serve a similar function in both hosts. Many species of bacteria are linked to the agricultural environment. In conclusion, our results demonstrate that different ladybird species are infected by diverse bacterial communities. Further work is clearly needed to identify any potential functions of these bacteria or impacts on the host ladybirds.

Chapter 3: Prevalence of *Acinetobacter*-like bacteria in *Harmonia axyridis* and *Halyzia sedecimguttata*

3.1 Introduction

A significant number of bacterial species are known to be arthropod endosymbionts (Boucias and Pendland, 2012). However, there is still little information on the insect-related microbial community in many insect host groups. Data on the bacterial community associated with ladybirds are limited (Roy *et al.*, 2011). Most previous research has only worked on a small portion of the bacterial metagenome and concentrated on certain bacterial species, in particular male-killing bacteria, such as *Spiroplasma* spp. (Majerus *et al.*, 1999; Nakamura *et al.*, 2005), *Rickettsia* spp. (Werren *et al.*, 1994) and *Wolbachia* spp. (Hurst *et al.*, 1999b). However, it has been reported in a recent study that the most prevalent bacterial phyla present in *Harmonia axyridis* are Actinobacteria, Proteobacteria, Firmicutes, Bacteroidetes, Thermotogae and Cyanobacteria (Dudek *et al.*, 2017). *Acinetobacter* species are gram-negative, Gammaproteobacteria, widely distributed in the environment, such as in soil and water, as free-living organisms (Bergogne-Berezin and Towner, 1996). The occurrence of *Acinetobacter* has been reported within a variety of insect species (Minard *et al.*, 2013). Different species of *Acinetobacter* have been identified in natural populations of the tsetse fly *Glossina palpalis* (Geiger *et al.*, 2009), *Culex quinquefasciatus* (Pidiyar *et al.*, 2004), *Aedes albopictus* (Zouache *et al.*, 2011), *Leptinotarsa decemlineata* (Muratoglu *et al.*, 2011) and *Tribolium castaneum* (Wang *et al.*, 2020). The *Acinetobacter* species isolated from *G. palpalis* were phylogenetically closely

related to the species *Acinetobacter baumannii* and *Acinetobacter junii*. These species were also identified as the closest phylogenetic relatives of *Ae. albopictus* isolates along with *Acinetobacter johnsonii*, *Acinetobacter schindlerii*, *Acinetobacter calcoaceticus* and *Acinetobacter lwoffii*. The most closely related strain based on genetic similarity at the 16S rRNA gene is *Acinetobacter vivianii* in the case of the *Acinetobacter* strain isolated from the flour beetle *Tribolium castaneum*. Unfortunately, it was not able to find specifically 16S *Acinetobacter* sequences from these insect species, so direct phylogenetic comparison has not been possible.

Only in *Stomoxys calcitrans* has the role of *Acinetobacter* been shown in insect biology, as these bacteria are essential for the fly larvae to develop completely (Lysyk et al., 1999). It is unclear what *Acinetobacter* associated with *G. palpalis* contribute to, but it has been suggested that they may produce *in situ* trypanolytic molecules that destroy trypanosomes (Geiger et al., 2009). The possibility of *Acinetobacter* species in enhancing blood meal digestion and nectar assimilation for their mosquito host *Ae. albopictus* has also been proposed because these bacterial isolates can metabolise components of both blood and plant sap (Minard et al., 2013). Moreover, some species of this bacterial genus can degrade a wide range of contaminants including biphenyl, phenol, benzoate, crude oil and acetonitrile and remove phosphate or heavy metals (Abdel-El-Haleem, 2003). This suggests that mosquitoes associated with *Acinetobacter* may have a greater ability to colonise and adapt to human-altered environments because *Ae. albopictus* can invade suburban areas and inhabit contaminated areas (Minard et al., 2013). In

addition, the isolated strain of *Acinetobacter* spp. from *T. castaneum* is likely involved in the biodegradation of polystyrene as this bacterium can degrade polystyrene and thus it can be used in petroleum-based plastic degradation studies and improving remediation methods (Wang *et al.*, 2020).

Results, as summarised in Appendix Table 1, identified bacteria with *Acinetobacter lwoffii* as the closest match (99% similarity) in both *H. axyridis* and *H. sedecimguttata*. Four individual ladybirds, two of each species, investigated contained *Acinetobacter* species. It is possible, therefore, that these bacteria may have a conserved function not only across different ladybird species, but potentially more broadly across other beetles and even broader insect groups.

Bacterial symbionts harboured by insect hosts can transmit vertically from parent to offspring or/and transmit horizontally from the environment (Wierz *et al.*, 2021). It is interesting to note that Schmidtberg *et al.* (2019) reported *Acinetobacter* that were identified in invasive *H. axyridis* from Germany as pyrazine producers. However, it is also possible that a repeat analysis of the sequence data might provide different results, due to the continued addition of new data, meaning what was the most similar sequence at the time of the study has been replaced by something more closely related, added more recently and hence providing a more accurate phylogenetic placement.

The acquisition and transmission of *Acinetobacter* species in insect species where it has been identified are still unknown. However, determining where it is localised in the insect body may indicate how it is acquired and transmitted (Minard *et al.*, 2013). It has been reported that *Acinetobacter* species inhabit the gut of several

insect species such as *Cx. quinquefasciatus*, *G. palpalis*, *Ae. Albopictus* and *T. castaneum* (Pidiyar *et al.*, 2004; Geiger *et al.*, 2009; Zouache *et al.*, 2009; Wang *et al.*, 2020). This could indicate the possibility of horizontal transmission of *Acinetobacter* strains through the environment (Zouache *et al.*, 2011). For example, these bacteria may be transmitted to mosquitoes when feeding on nectar where they are extremely abundant (Muturi *et al.*, 2018). However, the potential of vertical inheritance through generations is not ruled out (Minard *et al.*, 2013).

3.2 Objectives

To survey the prevalence of *Acinetobacter*-like bacteria in additional *Harmonia axyridis* and *Halyzia sedecimguttata* individuals, as assessed through sequencing of PCR amplicons obtained using primers targeting the *Acinetobacter* 16S rRNA gene. *Acinetobacter* has been identified in four out of twelve individual ladybirds, two of each species in this study and in *H. axyridis* samples from Germany (Schmidtberg *et al.*, 2019), and might be common in the two ladybird species. Screening of adult ladybirds and their offspring was used to investigate whether the bacteria transmit vertically from mother to offspring or transmit horizontally from the environment.

3.3 Methods

3.3.1 DNA extraction

Genomic DNA was extracted from 11 *Harmonia axyridis* individuals and 10 *Halyzia sedecimguttata* individuals as well as 7 pairs of *Harmonia axyridis* (adults and their offspring that emerged and became adults) according to the method described in Chapter 2.

3.3.2 Primer design

Cloned sequences from bacterial symbionts of both *Harmonia axyridis* and *Halyzia sedecimguttata* which were identified as closely related to *Acinetobacter* species (see Chapter 2) were aligned and compared with 16 published *Acinetobacter* sequences on Genbank. These sequences belong to *Acinetobacter lani*, *Acinetobacter schindleri*, *Acinetobacter albensis*, *Acinetobacter lwoffii*, *Acinetobacter haemolyticus*, *Acinetobacter harbinensis*, *Acinetobacter movanagherensis*, *Acinetobacter bohemicus*, *Acinetobacter kyonggiensis*, *Acinetobacter graminis*, *Acinetobacter oryzae*, *Acinetobacter bouveti*, *Acinetobacter johnsonii*, *Acinetobacter gandensis* and *Acinetobacter baumannii*. An *in silico* PCR was carried out to check for specificity. This identified two other genera in addition to *Acinetobacter*, *Moraxella* and *Psychrobacter* both also Gammaproteobacteria and no other species that would be amplified. The alignment was used to design primers that were used to amplify these sequences. The set of primers were *Acinetobacter lwoffii*-like forward (TGTGTAGCCCTGGTCGTAAG) and *Acinetobacter lwoffii*-like reverse (TCCAGGTGTAGCGGTGAAAT) were designed using the programme Primer 3

(Rozen and Skaletsky, 2000). The melting temperature of the primers was set at 59 °C. The GC content of the primers was 55% and the PCR product length was approximately 500 bp.

3.3.3 DNA amplification and alignment

Each DNA sample was tested by polymerase chain reaction (PCR) using the above-mentioned designed primers through a modified PCR reaction. The components of the PCR reaction mixture are as shown in Table 3.1. The 3 µl of DNA was replaced with 3 µl of dH₂O as a no-DNA control representing the negative control, in a total volume of 30 µl. All primers were at 10µM. A thermocycler (FTC152D, UK) PCR machine was used for PCR with conditions consisting of 1 cycle of initial denaturation at 94°C for 2 minutes, 35 cycles of denaturation at 94°C (15 s), annealing at 58°C (30 s), extension at 72°C (1 min) and 1 cycle of final extension at 72°C (10 min). *Harmonia axyridis* and *Halyzia sedecimguttata* individuals were tested for the presence of *Acinetobacter* species using the 16S rDNA PCR reaction with specifically designed primers as described. For *Harmonia axyridis* ladybirds, DNA samples were extracted from different life stages. This included both adults and their offspring (as described in 3.3.1).

Reagent	Volume	Final concentration
Reddymix	15 µl	1.5 units Taq DNA polymerase, 10 mM Tris-HCL, 50 mM KCl, 1.5 mM MgCl ₂ , 0.001% gelatin, 0.2 mM dNTP, stabilisers
Forward primer (27f)	1.5 µl	0.5 µM
Reverse primer (1495r)	1.5 µl	0.5 µM
dH ₂ O	9 µl	
DNA samples extracted from <i>Harmonia axyridis</i> or <i>Halyzia sedecimguttata</i> ladybird	3 µl	
Final volume	15 µl	

Table 3.1 PCR reaction mixture.

3.3.4 DNA gel Electrophoresis

The PCR products were electrophoresed in 1% agarose gel containing ethidium bromide to assess product quality. The samples were run in a gel tank with TAE buffer for 30 minutes at 100V and then visualised using UV light on a Nugenius GelDoc.

3.3.5 Sample selection and analysis method

The PCR products from ladybirds that generated clear bands of the expected size, on the gel (35 samples, 10 from *Harmonia axyridis*, 11 from *Halyzia sedecimguttata* and 14 from different life stages including 7 female and their corresponding offspring) were sent for Sanger sequencing at the company MacroGen and the closest matching sequences on GenBank were identified using the BLAST online search tool (Altschul *et al.*, 1990). Biological sequence alignment editor BioEdit (Hall, 1999) software was used to align the sequences for the phylogenetic analysis. The aligned sequences were saved in FASTA format and phylogenetic relationships were reconstructed using the maximum likelihood method that was implemented using a systematic computational algorithm of web-based phylogeny.fr which is a systematic search algorithm that ensures finding the best solution if one exists (Dereeper *et al.*, 2008). The method was integrated with various software programmes including MUSCLE (Edgar, 2004), which was used for multiple alignments; Gblocks (Castresana, 2000), which was implemented for curation to remove misaligned positions and divergent regions i.e. remove alignment noise; and PhyML (Guindon *et al.*, 2010, Anisimova and Gascuel, 2006), which was used to construct the phylogenetic tree as described in section 2.3.7.

Figtree v1.4.4 was used for visualisation of the tree. The Bootstrapping procedure was used for statistical tests for branch support and the number of bootstraps was set at default 100. The unambiguous, clean sequences were used for creating a phylogenetic tree using different *Acinetobacter* species sequences and the identified BLAST hits sequences that closely related to the amplified sequences available in GenBank. The *Acinetobacter* species sequences included were those that had been identified through BLAST as having high similarity to the newly generated sequences in this study. The other *Acinetobacter* species sequences had been isolated from *A. albopictus* in previous research (Minard *et al.*, 2013). The sequences used in the tree were expanded to include a broader range of genera than simply the top BLAST hits. This was done using the boxes available in NCBI to limit the search to exclude, for example, 'uncultured bacteria'.

3.4 Results

The presence of the bands illustrated in Figure 3.1 along with a 1 kb DNA ladder may indicate the presence of an amplifiable target in the extracted genomic DNA samples. Whilst this does not confirm the presence of *Acinetobacter*, as the primers may amplify more than one bacterial 16S sequence (see section 2.4), sequencing can be used to identify the bacteria present. A subset of these PCR products was sent for sequencing. The resulting sequences had two outcomes, either one clear sequence was detected (9 out of 35 samples), or the sequence trace showed evidence of multiple sequences with mixed traces (10 out of 35 samples). The mixed traces were excluded from further analysis. In addition, a number of samples totalling 16 out of 35 samples failed to produce sequence data. Again, these were excluded from further analysis.

Where unambiguous, 'clean' sequences were obtained, these were compared with each other. The resulting comparisons showed that a number of different sequence variants could be identified. Some of these were identical to each other, being detected in more than one individual and from the two species studied, e.g. *Harmonia axyridis* sample 8 and *Halyzia sedecimguttata* sample 8 were identical when aligning to each other.

Sequences were subsequently compared with those available on GenBank using BLAST and results are represented in Table 3.1. As is apparent from these BLAST comparisons, many of the ladybird sequences are found to be mostly similar to the same set of sequences from GenBank, e.g. the BLAST analysis shows that the sequence shared between *Harmonia axyridis* sample 8 and *Halyzia*

sedecimguttata sample 8 has a similar hit to the sequence obtained from *Harmonia axyridis* sample 3. Similarly, the BLAST search for the sequences obtained from *Halyzia sedecimguttata* sample 3, *Halyzia sedecimguttata* sample 5 and *Harmonia axyridis* sample 5 also showed similar hits with different accession numbers.

The BLAST results suggested that one mother-offspring pair both produced a 16S sequence that matched *Acinetobacter baumannii*. This applies to BLAST hits of the sequences obtained from the amplification of DNA sample number 3 extracted from *Harmonia axyridis* stage 1 (mother) and stage 4 (offspring) as shown in Table 3.2. Whilst this does not demonstrate vertical inheritance, it indicates the possibility, although acquisition from a common environmental source is also possible. The phylogenetic tree shown in Figure 3.2 indicates that *Harmonia axyridis* and *Halyzia sedecimguttata* bacterial clone sequences are clustering with the *Acinetobacter* sequences that identified as the BLAST hits for the clone sequences. This indicates that *Harmonia axyridis* and *Halyzia sedecimguttata* contain a closely related type of microbe that resembles *Acinetobacter* species. Out of the samples tested, 8 ladybird samples included positive BLAST hits for *Acinetobacter*. Of these only 3 were unambiguously the closest match, others also had equivalent matches for other species of bacteria.

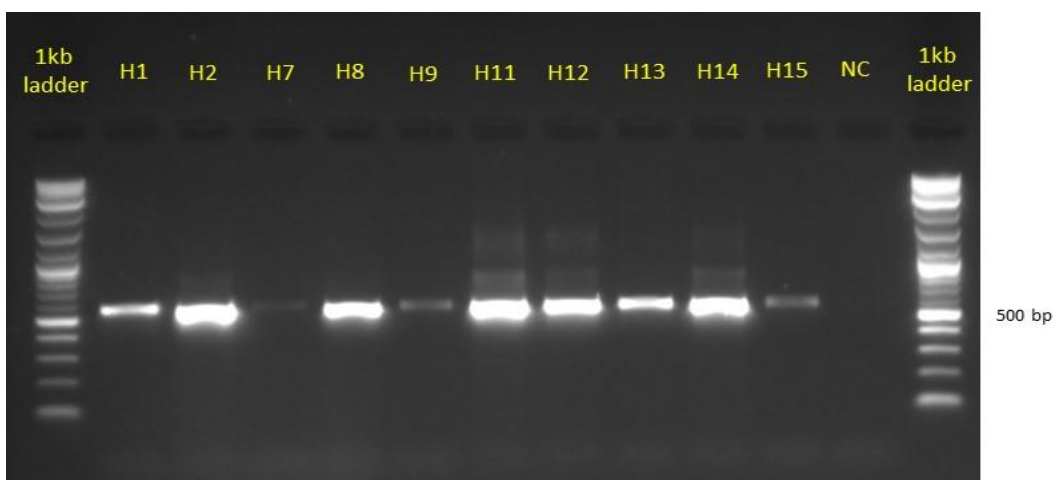


Figure 3.1 PCR products from 10 *Harmonia axyridis* individuals when amplified using 16S rRNA primers designed from *Acinetobacter* sequences. H1 indicates PCR product for *Acinetobacter*-like species gene amplified from *Harmonia axyridis* sample 1 and so on; NC: negative control; bp: base pair. 21

sample identity	PCR +/-	Sequence trace	Closest match	Percentage identity
<i>Harmonia axyridis</i> 1	+	Mixed	NA	
<i>Harmonia axyridis</i> 2	+	One sequence detected	<i>Rahnella</i> sp. MT605339.1	100%
			<i>Ewingella americana</i> MN826581.1	100%
			<i>Rouxiella chamberiensis</i> MT505141.1	100%
			<i>Serratia</i> sp. MN491073.1	100%
			Enterobacteriaceae bacterium MK248151.1	100%
			<i>Hafnia</i> sp. MF151923.1	100%
			Yersiniaceae bacterium MT596546.1	100%
			<i>Obesumbacterium proteus</i> MT081721.1	99%
			<i>Burkholderia</i> sp. KY681834.1	99%
			<i>Aranicola</i> sp. AM491468.1	99%
<i>Harmonia axyridis</i> 5	+	One sequence detected	<i>Acinetobacter johnsonii</i> MN826586.1	99%
			<i>Acinetobacter baumannii</i> MN435357.1	99%
			<i>Acinetobacter gandensis</i> OK569846.1	99%
			<i>Acinetobacter bouvetii</i> OK147821.1	99%

			<i>Acinetobacter oryzae</i> OK136220.1	99%
			<i>Acinetobacter haemolyticus</i> MW199127.1	99%
			<i>Acinetobacter lwoffii</i> OK178910.1	99%
			<i>Stenotrophomonas</i> sp. MH883926.1	99%
			<i>Bacillus siamensis</i> MG008667.1	99%
			<i>Paenibacillus graminis</i> JX401447.1	99%
<i>Harmonia axyridis</i> 7	+	NA		
<i>Harmonia axyridis</i> 8	+	One sequence detected	<i>Serratia marcescens</i> MT645673.1	100%
			<i>Staphylococcus aureus</i> MH603395.1	100%
			<i>Pseudomonas</i> sp. MH259878.1	100%
			<i>Acinetobacter baumannii</i> MH071347.1	100%
			<i>Pantoea agglomerans</i> KR822262.1	100%
			<i>Enterobacter cloacae</i> KJ850207.1	100%
			<i>Raoultella planticola</i> MZ203710.1	100%
			<i>Klebsiella</i> sp. MZ203699.1	100%
			<i>Bacillus subtilis</i> HQ123474.1	100%
			<i>Ralstonia</i> sp. MK058439.1	99%
<i>Harmonia axyridis</i> 9	+	Mixed	Uncultured <i>Acinetobacter</i> HQ891176.1	
<i>Harmonia axyridis</i> 11	+	Mixed	Uncultured bacterium KF095394.1	
<i>Harmonia axyridis</i> 12	+	NA		
<i>Harmonia axyridis</i> 13	+	NA		
<i>Harmonia axyridis</i> 14	+	NA		
<i>Harmonia axyridis</i> 15	+	Mixed	<i>Myroides marinus</i> MN220571.1	
<i>Halyzia sedecimguttata</i> 1	+	NA		
<i>Halyzia sedecimguttata</i> 2	+	One sequence detected	<i>Pantoea eucrina</i> MN889276.1	97%
			<i>Erwinia</i> sp. MG681219.1	97%
			<i>Enterobacter</i> sp. AY914094.1	96%
			<i>Siccibacter colletis</i> MN833502.1	96%
			<i>Pseudomonas</i> sp. MH769299.1	96%

			<i>Bacillus</i> sp. MH769293.1	96%
			<i>Franconibacter helveticus</i> OK103794.1	96%
			<i>Streptomyces pilosus</i> ON015754.1	96%
			<i>Providencia rustigianii</i> KJ638995.1	96%
			<i>Escherichia</i> sp. JX471112.1	96%
<i>Halazia sedecimguttata</i> 3	+	One sequence detected	<i>Acinetobacter albensis</i> MT378378.1	92%
			<i>Acinetobacter lanii</i> MK990276.1	92%
			<i>Acinetobacter lwoffii</i> MH125161.1	92%
			<i>Acinetobacter kyonggiensis</i> LC508807.1	92%
			<i>Acinetobacter harbinensis</i> MK402066.1	92%
			<i>Acinetobacter bohemicus</i> MK402065.1	92%
			<i>Acinetobacter schindleri</i> KY110864.1	92%
			<i>Acinetobacter haemolyticus</i> KX588554.1	92%
			<i>Acinetobacter movanagherensis</i> NR_145841.1	92%
			<i>Acinetobacter guillouiae</i> MT154257.1	94%
<i>Halazia sedecimguttata</i> 4	+	NA		
<i>Halazia sedecimguttata</i> 5	+	Mixed	<i>Acinetobacter lwoffii</i> MF966262.1	
<i>Halazia sedecimguttata</i> 7	+	NA		
<i>Halazia sedecimguttata</i> 8	+	One sequence detected	<i>Serratia marcescens</i> MT645673.1	100%
			<i>Staphylococcus aureus</i> MH603395.1	100%
			<i>Pseudomonas</i> sp. MH259878.1	100%
			<i>Acinetobacter baumannii</i> MH071347.1	100%
			<i>Pantoea agglomerans</i> KR822262.1	100%
			<i>Enterobacter cloacae</i> KJ850207.1	100%
			<i>Raoultella planticola</i> MZ203710.1	100%
			<i>Klebsiella</i> sp. MZ203699.1	100%
			<i>Bacillus subtilis</i> HQ123474.1	100%

			<i>Ralstonia</i> sp. MK058439.1	99%
<i>Halyzia sedecimguttata</i> 11	+	NA		
<i>Halyzia sedecimguttata</i> 13	+	Mixed	Uncultured bacterium JF236894.1	
<i>Halyzia sedecimguttata</i> 14	+	One sequence detected	<i>Serratia symbiotica</i> KF751204.1	96%
			<i>Gilliamella apicola</i> KF600148.1	95%
			<i>Arsenophonus symbiont</i> EU727113.1	98%
			<i>Frischella</i> sp. MW538483.1	95%
			<i>Cronobacter</i> sp. HQ407281.1	94%
			<i>Orbus sasakiae</i> NR_109477.1	93%
			<i>Enterobacter cloacae</i> MN594805.1	94%
			<i>Klebsiella quasivariicola</i> MN082107.1	92%
			<i>Atlantibacter subterranea</i> MN134591.1	92%
			<i>Pectobacterium aroidearum</i> MK450566.1	92%
<i>Harmonia axyridis</i> 1 stage 1	+	Mixed	Uncultured bacterium JF154913.1	
<i>Harmonia axyridis</i> 1 stage 4	+	Mixed	Uncultured bacterium HQ804871.1	
<i>Harmonia axyridis</i> 3 stage 1	+	One sequence detected	<i>Serratia marcescens</i> MH361384.1	98%
			<i>Staphylococcus aureus</i> MH603395.1	98%
			<i>Pseudomonas</i> sp. MH259878.1	98%
			<i>Acinetobacter baumannii</i> MH071347.1	98%
			<i>Pantoea agglomerans</i> KR822262.1	98%
			<i>Enterobacter cloacae</i> KJ850207.1	98%
			<i>Raoultella planticola</i> MZ203710.1	98%
			<i>Klebsiella</i> sp. MZ203699.1	98%
			<i>Bacillus subtilis</i> HQ123474.1	98%
			<i>Ralstonia</i> sp. MK058439.1	97%

<i>Harmonia axyridis</i> 3 stage 4	+	One sequence detected	<i>Escherichia coli</i> OM996123.1	98%
			<i>Shigella flexneri</i> MT624787.1	98%
			<i>Pseudescherichia vulneris</i> MT186163.1	98%
			<i>Citrobacter koseri</i> MT149900.1	98%
			<i>Acinetobacter baumannii</i> MH071349.1	98%
			<i>Cronobacter sakazakii</i> KY524291.1	98%
			<i>Limosilactobacillus fermentum</i> MF108372.1	98%
			<i>Lactococcus lactis</i> MF098001.1	98%
			<i>Photorhabdus luminescens</i> MF429509.1	98%
			<i>Leuconostoc lactis</i> MF429083.1	98%
<i>Harmonia axyridis</i> 5 stage 1	+	mixed	Uncultured bacterium EU778496.1	
<i>Harmonia axyridis</i> 5 stage 4	+	mixed	Uncultured bacterium JN697819.1	
<i>Harmonia axyridis</i> 6 stage 1	+	NA		
<i>Harmonia axyridis</i> 6 stage 4	+	NA		
<i>Harmonia axyridis</i> 7 stage 1	+	NA		
<i>Harmonia axyridis</i> 7 stage 4	+	NA		
<i>Harmonia axyridis</i> 8 stage 1	+	NA		
<i>Harmonia axyridis</i> 8 stage 4	+	NA		
<i>Harmonia axyridis</i> 9 stage 1	+	NA		
<i>Harmonia axyridis</i> 9 stage 4	+	NA		

Table 3.2 BLAST hits of the sequences obtained from the amplification of DNA samples extracted from *Harmonia axyridis* and *Halyzia sedecimguttata*. Different adult samples are indicated by a number. Stage 1 indicates mother. Stage 4 indicates offspring. 'Mixed' sequence traces were excluded from further analysis. NA indicates that no sequence was obtained from the sample. DNA samples number 3, 4, 6 and 10 extracted from *Harmonia axyridis*, number 6, 9, 10 and 12 extracted from *Halyzia sedecimguttata* and stage 1 and 4 from samples number 2 and 4 extracted from *Harmonia axyridis* failed to produce PCR products and were not tested.

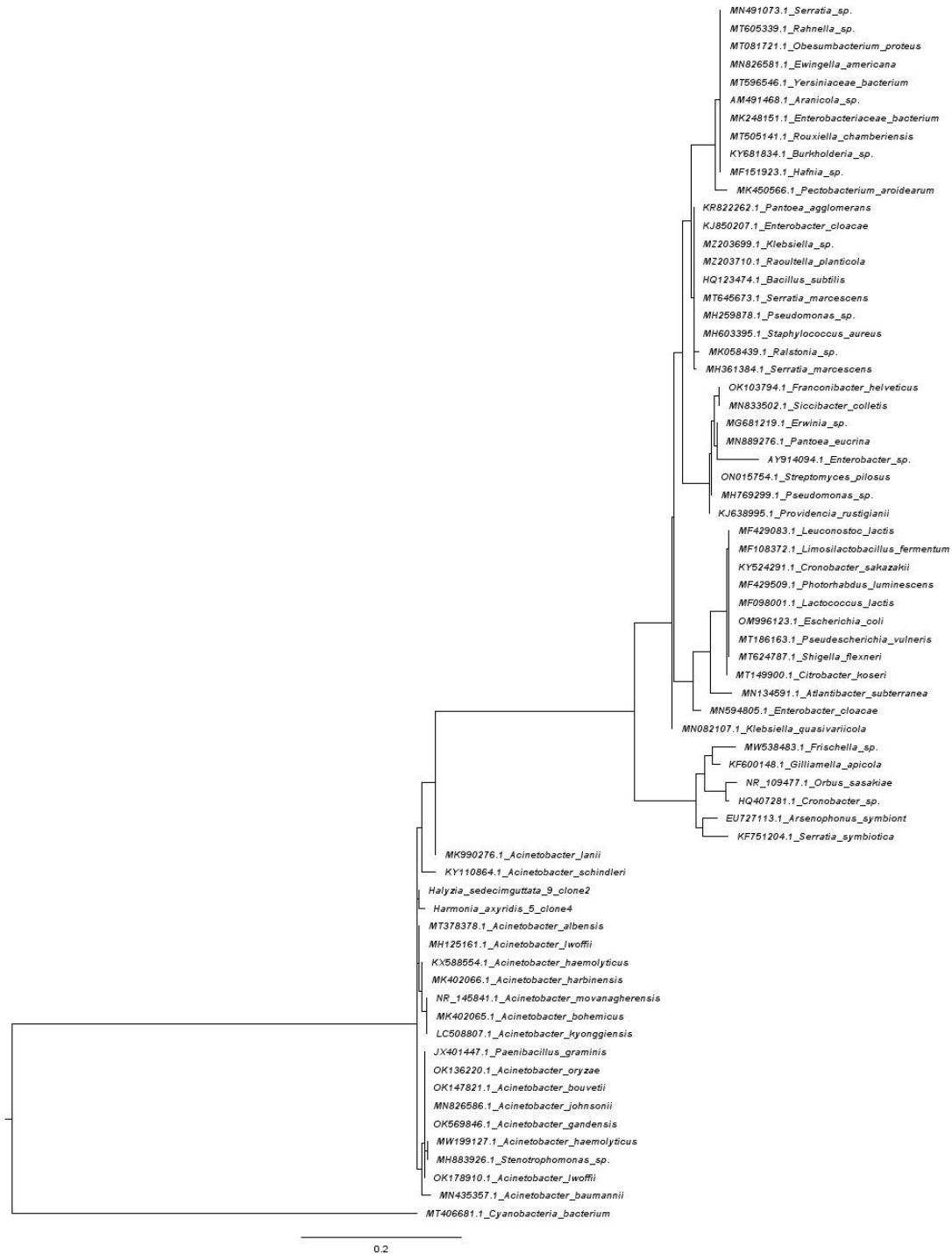


Figure 3.2 Phylogeny of *Acinetobacter* strains based on 16S rRNA bacterial gene sequences found in *Harmonia axyridis* sample number 5 and *Halyzia sedecimguttata* sample number 9. Branch length indicates the phylogenetic distance. Figures 2.3 to 2.8 showed phylogenetic analysis of further clones.

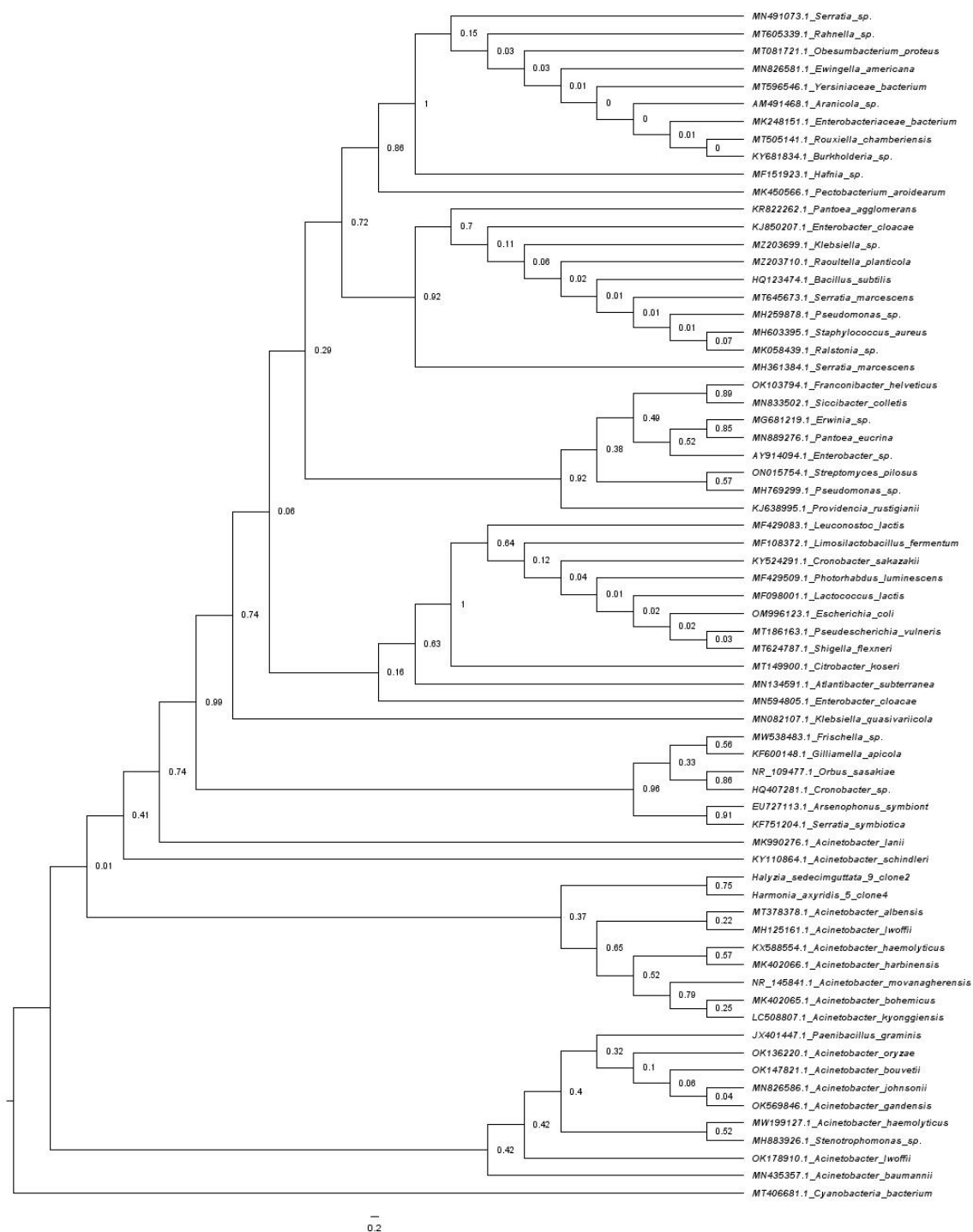


Figure 3.3 Phylogeny of *Acinetobacter* strains based on 16S rRNA bacterial gene sequences found in *Harmonia axyridis* sample number 5 and *Halyzia sedecimguttata* sample number 9 with bootstraps. Numbers indicate bootstrap values with 100 replicates. Figures 2.3 to 2.8 showed phylogenetic analysis of further clones. 23

3.5 Discussion

In the present study, the presence of *Acinetobacter*-like sequences was investigated in the ladybirds *Harmonia axyridis* and *Halyzia sedecimguttata*, using primers specifically designed to target these bacterial strains. However, the primers were not as specific to the *Acinetobacter* 16S sequence as intended. This limits the conclusions that can be drawn from this part of the study. There were clearly some samples that contained *Acinetobacter*, however further investigation with a more specific primer set is required to draw any more general conclusions. In addition, given the small sample size and single location, clearly a larger sample and comparison with populations in different locations would be needed to confirm the wider frequency. It is noted that it is possible that these bacteria are present in more individuals and that the PCR may have failed to detect it in those individuals that were apparently negative, potentially due to low concentration in the DNA samples in question. This is likely to be relevant to the larval stages, where all but one of the samples failed to produce any sequence data.

It is noted that individuals of both species in this study appeared in some cases to have more than one strain or different bacterial species coexisting within them, as evidenced through the multiple sequence traces retrieved from the PCR products from a single individual. The presence of mixed sequence traces indicates that the primers were able to amplify more than one target. This may be due to a lack of specificity in the primer design, however, despite this it could also be possible that there was more than one *Acinetobacter*-like bacterial strain or species present in the samples tested. Further BLAST analysis revealed the presence of

Acinetobacter species in both *Harmonia axyridis* and *Halyzia sedecimguttata* DNA preparations.

The phylogenetic analysis described in Chapter 2 shows a clade of sequences, at the bottom of the trees shown in Figures 2.7 and 2.8. This clade includes *Harmonia axyridis* 5 clone 4 and *Halyzia sedecimguttata* 9 clone 2 which are shown in Figures 3.2 and 3.3. Specific BLAST analysis confirmed these two sequences are 99% identical to each other (964/967 identical bases). Further, the two sequences both showed a high identity to *Acinetobacter lwoffii* (908/910 identities, for the *Harmonia axyridis* clone and 996/998 identities, for the *Halyzia sedecimguttata* clone). This strongly suggest that the *Acinetobacter* identified in this experiment is a strain of *Acinetobacter lwoffii*. It is noted that in Figures 2.3 to 2.8 the clades containing the clone sequences that appear to be distinct from the clade containing *Acinetobacter*. This is likely to be an inaccuracy caused by the shorter clone sequences in comparison to the *Acinetobacter lwoffii* sequence (1498 base pairs). Different *Acinetobacter* species have previously been reported from *H. axyridis* (Dudek *et al.*, 2017; Schmidtberg *et al.*, 2019). It is possible that the mixed sequence traces observed in some samples therefore represented either further *Acinetobacter* species, or variants of the species previously identified in *H. axyridis*. Several *Acinetobacter* species have previously been identified in natural populations of other insects, including the tsetse fly *Glossina* spp. (Geiger *et al.*, 2009) and *Aedes albopictus* (Zouache *et al.*, 2011), suggesting the possibility that they may have a similar relationship with many host species.

The high prevalence of a range of *Acinetobacter* or *Acinetobacter*-like species found in the two ladybird species studied here indicates that this type of bacterium may be common in both hosts. These two ladybird species have different ecologies with a different diet, thus the diet itself seems an unlikely source of the bacteria in each case.

The function of the phenotype of these bacteria in terms of whether they are parasitic, or beneficial to their host, however, remains uncertain. *Acinetobacter* are gram-negative bacteria, in many cases existing as free-living saprophytes commonly found in soil, in water, or even on vertebrate skins (Doughari *et al.*, 2011). Previous studies have also shown that members of *Acinetobacter* can cause opportunistic human infections (Doughari *et al.*, 2011). The role played by *Acinetobacter* in insects has only been elucidated in a small number of cases, for example in the fly *Stomoxys calcitrans*, which requires these bacteria to ensure the complete development of the fly larvae (Lysyk *et al.*, 1999). Some *Acinetobacter* species isolated from the mosquito *Aedes albopictus* have already been shown to be effective pollutant degraders (Lal and Khanna, 1996) and have a potential role in improving nectar assimilation (Minard *et al.*, 2013). In anthropogenic structures, such as wind turbines, ladybirds often hibernate (Dudek *et al.*, 2015) during which their overwintering survival is high. *Acinetobacter* might potentially be associated with traits such as over-wintering, ladybirds moving around wind turbines on contaminated soil by bacteria, or could be involved in the metabolism of the plant sap components, which occur within their sap-sucking prey. The bacteria in these cases are thus likely to be transmitted horizontally and

acquired from the environment. This does not, however, preclude the possibility that the bacteria may be transmitted vertically by other mechanisms, including potentially maternal transmission (Kikuchi, 2009).

To get a better understanding of the prevalence of *Acinetobacter* in ladybirds, examining a greater number of samples and diversity of sequences should be considered. This would allow a greater understanding of the generality of the interaction between *Acinetobacter* and ladybirds more broadly and be reflected positively to facilitate the possibility of isolating more *Acinetobacter* sequences from a greater range of populations and ladybird species. Further, it may start to provide some insight into the hitherto unknown function of these seemingly prevalent bacteria in ladybird hosts.

In conclusion, this study demonstrated the occurrence of *Acinetobacter* within the *Halysia sedecimguttata* and *Harmonia axyridis* ladybirds samples from Nottingham and has begun to characterise this microbial diversity. Further study is required to understand the functions of these identified *Acinetobacter* species in their ladybird hosts.

Chapter 4: Prevalence of *Cedrus* chloroplast-like or Cyanobacteria in different life stages and body parts of *Harmonia axyridis*

4.1 Introduction

Although little is known about the complex microbial community associated with the Harlequin ladybird *Harmonia axyridis*, a subset of bacterial species are known to be symbionts of this insect (Dudek *et al.*, 2017). This lack of knowledge is attributed to the fact that most of the total bacterial diversity cannot be grown in the laboratory; for example, only about 14% of all microorganisms in the sponge *Haliclona* sp. can be cultured (Sipkema *et al.*, 2011). As a result, it is not possible to exploit traditional culture-based methods to identify a wide range of insect endosymbionts. Molecular tools have provided evidence for the existence of bacteria that cannot be cultured. Obtaining DNA sequence information from samples via PCR amplification followed by cloning or direct sequencing enabled phylogenetically relevant markers, such as 16S rRNA gene sequences, to be characterised (Amann *et al.*, 1995). A subset of host-associated bacteria tends to be obligate, intracellular symbionts that have not been cultured outside of the host or cultured via cell lines as a result of this relationship. Examples of symbiotic bacteria of insects that have never been grown using traditional culturing methods, include the most common examples of bacteria closely associated with their hosts with important relevance, as in cases of aphids and *B. aphidicola* acting as obligate endosymbionts (Clark *et al.*, 2010). The most common and well recognised obligate endosymbionts are mitochondria and chloroplasts that have developed

alongside plants and animals over many millions of years of evolution. Sequencing of 16S rRNA genes extracted from genomic DNA can not only characterise several yet uncultured symbionts from insects but also almost all the microorganisms present in samples. Uncultured bacteria that do not grow on laboratory media play important roles in the cycling of carbon, nitrogen and other elements, as well as synthesising novel natural products affecting the ecosystem and the organisms associated with it (Stewart, 2012).

Although most of the previous research focussed on symbionts in ladybirds was devoted to male-killing bacterial species, such as *Spiroplasma* spp. (Majerus *et al.*, 1999), *Rickettsia* spp. (Werren *et al.*, 1994) and *Wolbachia* spp. (Hurst *et al.*, 1999b), more recently, studies analysing the larger bacterial community associated with *Harmonia axyridis* reported the presence of bacteria of the genera *Serratia*, *Lactococcus*, *Corynebacterium*, *Delftia*, *Pseudomonas*, *Stenotrophomonas*, *Acinetobacter*, *Providencia*, *Methyobacterium*, *Flavobacterium*, *Comamonas*, *Enterobacter*, *Enterococcus* and several unclassified bacteria (Schmidtberg *et al.*, 2019). Members of these bacterial genera identified in *Harmonia axyridis* such as *Lactococcus lactis* (Heon and Lee 1991), *Staphylococcus sciuri* (Robacker, 2007), *Serratia* (Dalmaga and Vrioni, 2011), *Pseudomonas* (Cheng and Reineccius, 1991), *Acinetobacter*, *Stenotrophomonas* and those belonging to the family Enterobacteriaceae (Gao *et al.*, 2016) are reportedly able to produce volatile alkylpyrazines. Pyrazines are found in a variety of insect orders (Moore *et al.*, 1990). Beetles belonging to the predator family Coccinellidae, which includes the two-spot ladybird *Adalia*

bipunctata, the seven-spot ladybird *Coccinella septempunctata*, the convergent ladybird *Hippodamia convergens* and the Harlequin ladybird *Harmonia axyridis* use pyrazine as allomones or pheromones that serve as deterrents or attractants in many interactions (Verheggen *et al.*, 2017). The hypothesis has been put forward that there is a potential role of the symbionts in pyrazine production in *Harmonia axyridis* (Dudek *et al.*, 2017; Schmidtberg *et al.*, 2019). This hypothesis is supported by the abundance of bacteria, especially those of the genera *Lactobacillus* and *Serratia*, which are known to produce pyrazine (Schmidtberg *et al.*, 2019).

Other potential functions of the bacteria associated with ladybirds arise from further investigation of the bacteria identified in other hosts, as described in Chapter 3. One interesting finding to emerge from previous studies investigating the microbiota of *H. axyridis* (Dudek *et al.*, 2017; Schmidtberg *et al.*, 2019 and findings reported in Chapters 2 and 3) is that there are a group of bacteria present that appear to be related to chloroplasts, plastids and/or Cyanobacteria. Some of the *H. axyridis* samples investigated in this study produced 16S rDNA PCR products with closest sequence matches that included *Cedrus deodara* chloroplast-like sequence, various crop plant plastid sequences e.g. *Oryza rufipogon* (rice), *Triticum aestivum* (wheat), *Brachypodium distachyon* (grass) and Cyanobacteria. Similar bacteria have been identified in the metagenomics analysis carried out in this study. These bacteria are phylogenetically very distant from *Acinetobacter*, investigated in Chapter 3, which suggests that they may potentially have a different function. Essentially, these bacteria are interesting because at first glance it seems

that they may simply be a gut contaminant. This might be due to the ladybird eating aphids which have themselves fed on a plant and hence which contain chloroplasts. These chloroplasts then pass into the ladybird's gut and some chloroplast DNA is present with ladybird and other bacterial DNA following the DNA extraction process. However, Li *et al.* (2022) also detected Cyanobacteria which may be related to the *Cedrus* chloroplast-like bacteria identified in this study, suggesting that these might actually be ladybird symbionts not simply gut contaminants. Li *et al.* (2022) used a metagenomic approach and hence the location of the Cyanobacteria they identified within the *Harmonia axyridis* was unknown. By investigating different body parts, the current study may be able to distinguish between gut contaminants and genuine symbionts.

Cyanobacteria live in various environments, even harsh environments including bare rocks, lava flows, volcanic islands, caves, benthic waters rich in hydrogen sulfide and mineral springs (Krivosheina, 2008). They are characterised by their ability of fixing atmospheric nitrogen and tolerance of darkness. They are also rich in proteins, amino acids, polysaccharides, vitamins, and microelements making them a food source for invertebrate species such as insects. However, the poisonous characteristic of cyanobacteria severely limits the number of organisms that feed on them. Invertebrates can consume cyanobacteria without being affected when mixed with detritus and other algae because they do not appear to be toxic (Krivosheina, 2008).

Investigating the presence of these chloroplast-like bacteria in different life stages as per the investigation of *Acinetobacter* described in Chapter 3, will provide further

information about the type of transmission involved for these chloroplast-like bacteria. In addition, investigation of the location within the host's body may provide greater insight into both the mode of transmission of the bacteria, their relationship with the ladybird host and their potential function. The null hypothesis is that these bacteria play no role in the ladybird hosts, but instead are part of the gut contents, potentially passing from some component of the diet or environment that contains chloroplasts. A more interesting hypothesis is that these bacteria represent bacterial relatives of the cyanobacteria that are thought to have been the ancestors of chloroplasts (Sato, 2021). If the null hypothesis is correct, it is predicted that there will be no evidence of vertical transmission or presence in body parts such as legs and wings, but relatives of Cyanobacteria may be present in the abdomen.

4.2 Objectives

The prevalence of *Cedrus* chloroplast-like or Cyanobacteria in different life stages and different body parts of *Harmonia axyridis*, viz. adults, eggs, larvae, offspring, head, abdomen, wings and legs, as assessed through sequencing of PCR amplicons, were surveyed using primers targeting the *Cedrus* chloroplast-like 16S rRNA gene. Further, by identifying the likely location(s) and transmission route of these bacteria, this investigation sought to gain further insight into any possible roles they may have associated with the ladybird host. Given the widespread occurrence of Cyanobacteria (Krivosheina 2008), primers were designed to target the *Cedrus* chloroplast-like bacterial 16S rRNA gene found in the cloning experiment described in Chapter 2.

4.3 Methods

4.3.1 Ladybird artificial diet

The artificial ladybird food was composed of a variety of carefully weighed ingredients that have been developed by our group over years of breeding ladybirds as described by Majerus and Kearns (1989). This consists of 18 g of desiccated liver (OXOID, LP0026) ground with 30 g of sucrose (Fisher Chemical, 1862754) in a pestle and mortar following this 12 g of yeast (Sigma Aldrich, 73145-500G-F) was added to the mortar and the mixture was ground further to thoroughly mix all components. In a fresh 500 ml bottle, 4 g of agar (Sigma Aldrich, A7002) was dissolved in 300 ml of dH₂O by heating in a microwave oven for 4 minutes at maximum setting. The agar was allowed to cool so the bottle could be handled with bare hands after which the ground mixture from the mortar was transferred to the cooled agar. 20 ml treacle (Tate & Lyle Black Treacle Syrup) was added into the agar mixture. The final ingredient was 5X multivitamin tablets (Multivitamins *by Sainsbury's*). Using the pestle and mortar these were crushed and the powder was poured into the agar bottle mixture and thoroughly stirred. Once powdered tablets were thoroughly dispersed throughout the mixture this was then rapidly poured into Petri dishes so that the base of the dish was evenly covered with the molten food mix. This was allowed to set and then stored in the freezer until required. One plate was thawed out at room temperature and used to feed the ladybirds, keeping it in a fridge until completely used. The artificial food was prepared by slicing it, in the Petri dish, making small blocks about 4mm square. 2 to 5 blocks were picked from

the Petri dish using forceps and placed in the ladybird individual's and pair's dishes respectively.

4.3.2 Breeding and Feeding ladybirds

Ladybird breeding aims to generate sufficient individuals of different life stages that DNA can be extracted from these, as well as from the different body parts of individual ladybirds.

In order to investigate potential vertical transmission of bacteria, it was necessary to breed and rear the ladybirds and their offspring. To overcome the lack of breeding facilities, a new housing facility needed to be created.

The housing for ladybirds is a novel method developed by our group, using small



Figure 4.1 VITOPOD HEATED PROPAGATOR with electronic thermostat. 24

plant propagators with an electronic thermostat as shown in Figure 4.1. All ladybirds were reared in these propagators during their stay in the laboratory.

Adults of *H. axyridis* were reared in groups of 10 without determining sex, in approximately 10 Petri dishes per propagator, after they had been collected. Pieces of paper were cut, folded and placed in these dishes as oviposition sites.

They were fed daily with a combination of the artificial ladybird food made in agar, *Acyrtosiphon pisum* aphids and *Ephestia khueniella* eggs (Agralan Ltd, UK068). The *Ephestia khueniella* eggs used for feeding ladybirds were investigated by a student in the lab. Their project identified *Wolbachia* as the predominant bacteria present in the *Ephestia khueniella* samples tested. The student used generalised 16S primers (27f and 1495r). All 16S sequences generated were clear with no evidence of mixed sequences and gave a 100% match to *Wolbachia* (Majerus pers. comm.). When available, aphids were collected from sycamore trees and stored in the fridge then fed to the ladybirds during their feeding regime. The propagator was set at 23°C (Laugier *et al.*, 2013), to maintain humidity a small piece of rolled-up tissue was soaked in dH₂O and placed with the ladybirds inside the Petri dish. The electronic thermostat and a lamp were connected to an analogue timer mains plug which was set to remain switched on for 16 hours and switched off for 8 hours (16:8, Daylight: Night ratio). Once mating was observed the pairs would be transferred to a fresh separate Petri dish. Once eggs were observed, first the parents would be removed to a separate dish and then for DNA extraction some eggs were transferred to an Eppendorf and frozen at -70°C. The remaining eggs were kept in the Petri dish until the larvae emerged. To avoid sibling cannibalism the larvae were also separated as individuals into 60 mm diameter small Petri dishes. For DNA extraction 5-8 larvae were collected from each of 6 matriline, transferred to Eppendorf tubes and stored at -70°C. The remainder were fed artificial ladybird food made in agar until pupation and

emergence of adults. Newly emerged adults were also directly transferred to Eppendorf tubes, without feeding and stored at -70°C until used for DNA extractions.

4.3.3 Ladybird dissection

Female and male ladybirds were placed in corked glass tubes and immersed in 100% ethanol as sterilisation and euthanising methods to prepare the samples for dissection. The samples were lifted from the corked glass tubes with forceps,



Figure 4.2 *Harmonia axyridis* separated into five tissue segments: (A) elytra, (B) wings, (C) head, (D) abdomen, (E) legs. 25

washed in sterile dH₂O, transferred to Petri dishes and dissected under a dissection microscope. The samples were cut into five parts as in Figure 4.2: head, abdomen, elytra, wings and legs. All body parts were cut off using sterile dissecting scissors.

Each body part was picked from the Petri dish using forceps and stored in an Eppendorf tube. All body parts were stored separately and processed within that same tube i.e. the legs from one individual were put into one tube and processed in that tube. Similarly, the head was placed in a separate tube as were each of the other types of tissues. Samples were temporarily stored at -70°C until used for DNA extraction.

4.3.4 DNA extraction

DNA samples extracted from *Harmonia axyridis* adults, eggs, larvae and their adult offspring as well as different body parts including head, abdomen, elytra and legs were tested for the presence of *Cedrus* chloroplast-like species using the 16S rDNA PCR reaction with specifically designed primers as described in section 4.3.5. Genomic DNA was extracted from the different body parts according to the method described in Chapter 2, with a small number of differences as outlined below. Egg samples were not transferred to homogenisation tubes but kept in Eppendorf tubes. Due to the soft nature of ladybird eggs, it was not necessary to freeze them in liquid nitrogen or use the homogeniser as the eggs are easily crushed with a microcentrifuge pestle in their respective Eppendorf tubes. There was also no need to use liquid nitrogen for the four different body parts as they were stored at -70°C and DNA extraction commenced immediately on removal from the freezer.

4.3.5 Primer design

Cloned sequences of DNA extracted from the four different life stages (eggs, larvae, pupae and adults) and different body parts (head, abdomen, elytra and

legs) (Figure 4.2) of *Harmonia axyridis*, which were identified as closely related to *Cedrus deodara* chloroplast-like (see Chapter 2), were aligned and compared with the published *Cedrus deodara* chloroplast-like sequence on GenBank. An *in silico* PCR was carried out to confirm specificity. The alignment was used to design primers that were used to amplify these sequences. The set of primers were chloroplast-like forward (CTTTGTCCCGGCCATTGTAG) and chloroplast-like reverse (GGTAGGGGCAGAGGGAATTT), and were designed using the programme Primer 3 (Rozen and Skaletsky, 2000) and the 16S rDNA sequences from 6 different clones. The melting temperature of the primers was set at 58°C. The GC content of the primers was 55% and the PCR product length was approximately 600 bp. This process generated the primers sequences that were used.

4.3.6 DNA amplification and alignment

Each DNA sample was tested by polymerase chain reaction (PCR) using the above-mentioned designed primers through a modified PCR reaction. The components of the PCR reaction mixture are as shown in Table 4.1. The 3 µl of DNA was replaced with 3 µl of dH₂O as a no-DNA control representing the negative control, in a total volume of 30 µl. All primers were at 10µM. A thermocycler (FTC152D, UK) PCR machine was used for PCR with conditions consisting of 1 cycle of initial denaturation at 94°C for 2 minutes, 35 cycles of denaturation at 94°C (15 s), annealing at 58°C (30 s), extension at 72°C (1 min) and 1 cycle of final extension at 72°C (10 min).

Reagent	Volume	Final concentration
Reddymix	15 µl	1.5 units Taq DNA polymerase, 10 mM Tris-HCL, 50 mM KCl, 1.5 mM MgCl ₂ , 0.001% gelatin, 0.2 mM dNTP, stabilisers
Forward primer (27f)	1.5 µl	0.5 µM
Reverse primer (1495r)	1.5 µl	0.5 µM
dH ₂ O	9 µl	
DNA samples extracted from <i>Harmonia axyridis</i> or <i>Halyzia sedecimguttata</i> ladybird	3 µl	
Final volume	15 µl	

Table 4.1 PCR reaction mixture.

4.3.7 DNA gel Electrophoresis

The PCR products were electrophoresed in 1% agarose gel containing ethidium bromide to assess product quality. The samples were run in a gel tank with TAE buffer for 30 minutes at 100V and then visualised using UV light on a Nugenius GelDoc.

4.3.8 Sample selection and analysis method

The PCR products from ladybirds that generated clear bands on the agarose gel (70 samples, 48 from different life stages and 22 from different body parts) were sent for Sanger sequencing at the company Macrogen and the closest matching sequences on GenBank were identified using the BLAST online search tool (Altschul *et al.*, 1990). Biological sequence alignment editor BioEdit (Hall, 1999) software was used to align the sequences for the phylogenetic analysis. The aligned sequences were saved in FASTA format and phylogenetic relationships were reconstructed using the maximum likelihood method that was implemented using a systematic computational algorithm of web-based phylogeny.fr which is a systematic search algorithm that ensures finding the best solution if one exists (Dereeper *et al.*, 2008). The method was integrated with various software

programmes including MUSCLE (Edgar, 2004), which was used for multiple alignments; Gblocks (Castresana, 2000), which was implemented for curation to remove misaligned positions and divergent regions i.e. remove alignment noise; and PhyML (Guindon *et al.*, 2010, Anisimova and Gascuel, 2006), which was used to construct the phylogenetic tree as described in section 2.3.7. Figtree v1.4.4 was used for visualisation of the tree. The Bootstrapping procedure was used for statistical tests for branch support and the number of bootstraps was set at default 100. The unambiguous, clean sequences were used for creating a phylogenetic tree using different uncultured bacterium sequences and a *Cedrus deodara* chloroplast-like sequence available in GenBank. The uncultured bacterium sequences included were those that had been identified through BLAST as having high similarity to the newly generated sequences in this study. The *Cedrus deodara* chloroplast-like sequence was also shown to be similar and was included for this reason. The other uncultured bacterium sequences had been isolated from environments such as the Pacific Ocean or Prairie dogs and Rock squirrels occurring in the foothills of the Rocky Mountains in Boulder County, Colorado in previous research (Apprill *et al.*, 2011, Jones *et al.*, 2008).

4.4 Results

The presence of the bands illustrated in Figure 4.3 along with a 1 kb DNA ladder indicated the likely presence of *Cedrus* chloroplast-like species in extracted genomic DNA samples. The PCR products amplified from the DNA samples that tested positive were sent for sequencing. The resulting sequences had two outcomes, either one clear sequence was detected (32 out of 51 samples), or the sequence trace showed evidence of multiple sequences with mixed traces (16 out of 51 samples). In addition, few samples (3 out of 51 samples) failed to produce sequence data. These along with the mixed traces ones were excluded from further analysis. As discussed in Chapter 3, these may be indicative of lack of specificity of the primers and may indicate more than one bacterial strain or species present.

Where unambiguous, 'clean' sequences were obtained, these were compared with each other. The resulting comparisons showed that a number of different sequence variants could be identified. Some of these were identical to each other, being detected in more than one sample and from different life stages and body parts. For example, all different life stage samples from lineage 15 and lineage 23, except the larval and the egg stages respectively, were identical when aligning to each other.

Sequences were subsequently compared with those available on GenBank using BLAST and results are represented in Tables 4.2 and 4.3. As is apparent from these BLAST comparisons, many of the ladybird sequences are found to be most similar to particular sequences from GenBank, e.g. the BLAST analysis shows that

the majority of the sequences are closely related to uncultured bacterium accession number [LC349021.1](#). Other hits include uncultured bacterium accession number [KX022163.1](#) and uncultured bacterium accession number [HM243988.1](#). The uncultured bacterium accession number [KX022163.1](#) is the BLAST search closest match for two larval sequences from lineage 14 and 17, one offspring sequence from lineage 15, 3 head sequences from samples 2, 6 and 7, 2 abdomen sequences from samples 1 and 7 and one elytron sequence from sample 1, but the uncultured bacterium accession number [HM243988.1](#) from the BLAST search was the closest match for only one egg sequence from lineage 21. No significant similarity was found for two offspring sequences from lineage 15 and 21; one egg sequence from lineage 17; 4 head sequences from samples 2, 3, 5 and 7; 3 elytra sequences from samples 3, 6 and 9; 2 abdomen sequences from samples 3 and 9 and one leg sequence from sample 6.

The phylogenetic tree shown in Figure 4.4 indicates that male and female *Harmonia axyridis* i.e. both sexes can carry more than one type of bacteria that amplified with the designed primer. Although not all *Harmonia axyridis* eggs appear to be infected, *Harmonia axyridis* larvae and offspring may have more than one type of bacteria that amplified with the designed primer. These bacteria may be present in every life stage of *Harmonia axyridis* ladybird or at least often present. The phylogenetic tree shown in Figure 4.4 indicates that the sequences amplified from *Harmonia axyridis* body parts are distinct from the other uncultured bacteria sequences. Additionally, *Harmonia axyridis* body parts can also carry more than one type of bacteria that amplified with the designed primer.

The full results are available in Appendix Tables 2 and 3. Tables 4.2 and 4.3 contains summary taxonomic details of the sequences amplified from the different life stages and body parts of *H. axyridis*. It is noted that the sequences have identical identity to bacteria that are highly divergent in their taxonomy, which means that taxonomic assignment is difficult to impossible. For this reason Tables 4.1 and 4.2 contain only Phylum and Class designations which were confirmed by phylogenetic analysis presented in Figures 2.3 to 2.6.

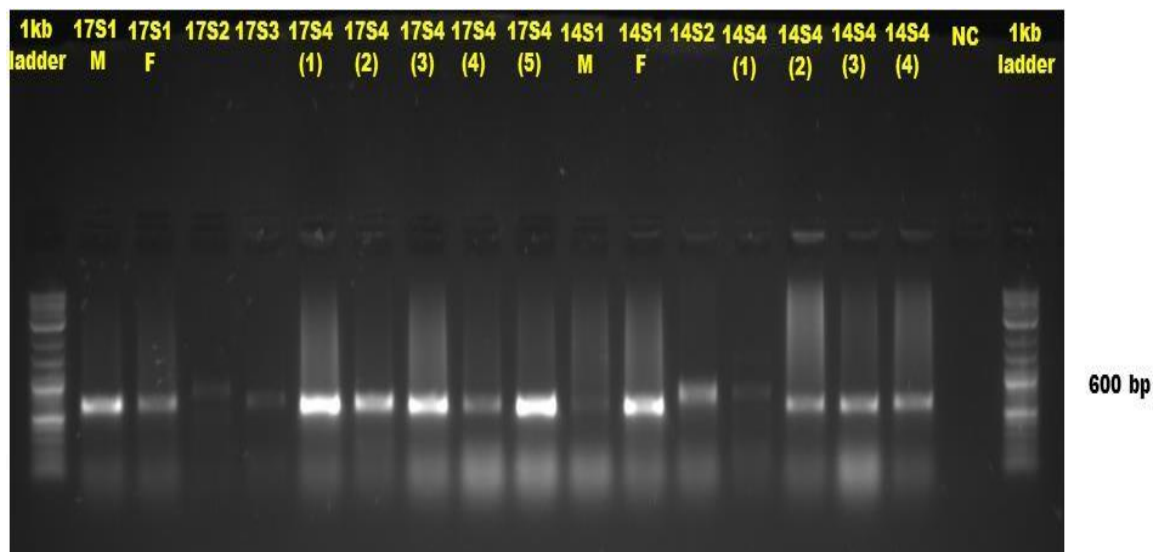


Figure 4.3 PCR products from *Harmonia axyridis* different life stages lineage 14 and 17 when amplified using 16S rRNA primers designed from *Cedrus deodara* chloroplast-like sequence. The numbers from (1) to (5) indicate the sample number; S1: parental stage; M: male; F: female; S2: eggs; S3: larvae; S4: offspring; NC: negative control; bp: base pair.

Closest match	Phylum	Class	Number of samples where the bacteria are identified
<i>Limosilactobacillus fermentum</i>	Firmicutes	Bacilli	32
Sugarcane phytoplasma	Mycoplasmata	Mollicutes	32
Uncultured Xanthomonadales bacterium	Mycoplasmata	Mollicutes	30
Bacterium endosymbiont of <i>Stereum hirsutum</i>	Firmicutes	Bacilli	32
Uncultured Cyanobacterium sp.	Firmicutes	Bacilli	32
Uncultured Trichodesmium sp.	Cyanobacteria	Cyanophyceae	31
Uncultured <i>Oscillatoria</i> sp.	Cyanobacteria	Cyanophyceae	32
Uncultured actinobacterium	Firmicutes	Bacilli	31
<i>Bacillus</i> sp.	Firmicutes	Bacilli	27
<i>Loriellopsis cavernicola</i>	Cyanobacteria	Cyanophyceae	24

Table 4.2 Summary of taxonomy of closest BLAST hits of the sequences obtained from the amplification of DNA samples extracted from *Harmonia axyridis* adults, eggs, larva and offspring.

Closest match	Phylum	Class	Number of samples where the bacteria are identified
<i>Limosilactobacillus fermentum</i>	Firmicutes	Bacilli	5
Sugarcane phytoplasma	Mycoplasmata	Mollicutes	7
Uncultured Xanthomonadales bacterium	Mycoplasmata	Mollicutes	6
Bacterium endosymbiont of <i>Stereum hirsutum</i>	Firmicutes	Bacilli	8
Uncultured Cyanobacterium sp.	Firmicutes	Bacilli	8
Uncultured Trichodesmium sp.	Cyanobacteria	Cyanophyceae	7
Uncultured <i>Oscillatoria</i> sp.	Cyanobacteria	Cyanophyceae	8
Uncultured actinobacterium	Firmicutes	Bacilli	8
<i>Bacillus</i> sp.	Firmicutes	Bacilli	8
<i>Loriellopsis cavernicola</i>	Cyanobacteria	Cyanophyceae	5

Table 4.3 Summary of taxonomy of closest BLAST hits of the sequences obtained from the amplification of DNA samples extracted from *Harmonia axyridis* head, abdomen, elytra and legs.

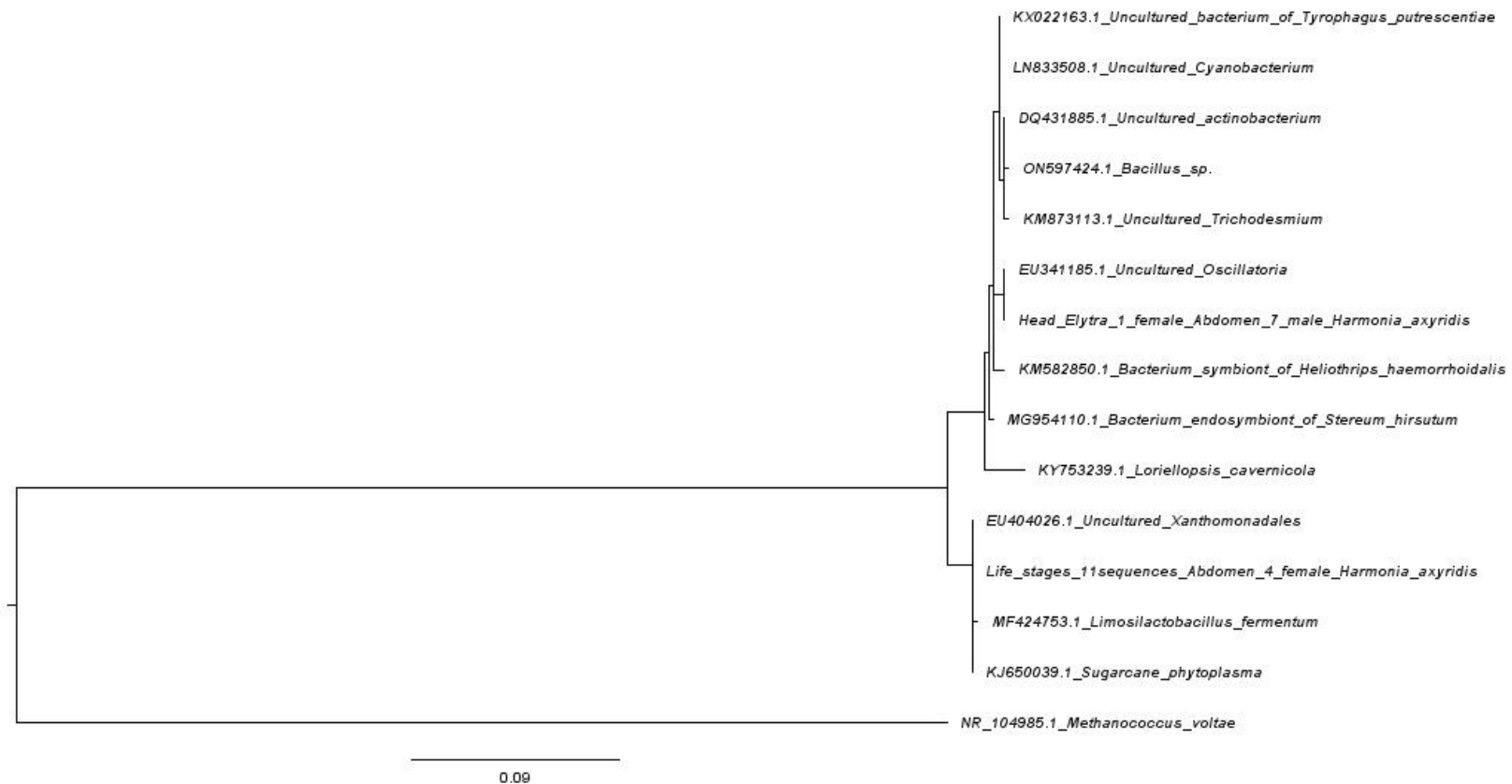


Figure 4.4 Phylogeny of unidentified bacterial strains based on 16S rRNA bacterial gene found in different life stages and body sections. Life stages include adults, eggs, larvae and offspring and body sections include head, abdomen, elytra and legs. Branch length indicates the phylogenetic distance. 27

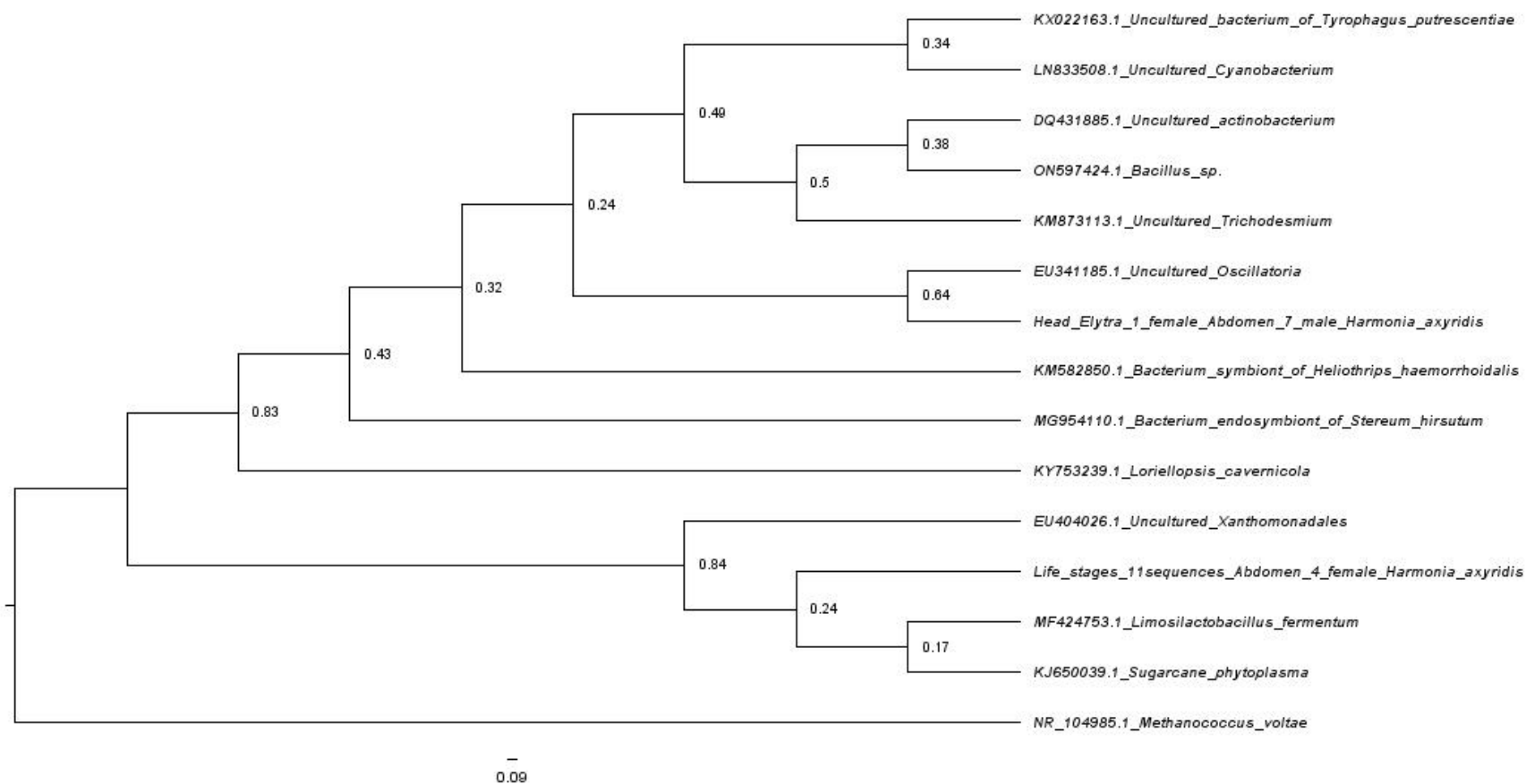


Figure 4.5 Phylogeny of unidentified bacterial strains based on 16S rRNA bacterial gene found in different life stages and body sections. Life stages include adults, eggs, larvae and offspring and body sections include head, abdomen, elytra and legs. Numbers indicate bootstrap values with 100 replicates. 28

4.5 Discussion

This study provides an estimate of the prevalence of *Cedrus* chloroplast-like bacteria in different life stages and body parts of the Harlequin ladybird, *Harmonia axyridis*. The findings revealed that both males and females harbour one or more *Cedrus* chloroplast-like bacteria that amplified using the *Cedrus* chloroplast-like primers. Although not all eggs were infected, one or more *Cedrus* chloroplast-like bacterial strain was identified in larval stages, emerging adults and different body segments. In both different life stages and body parts, BLAST showed the existence of a particular *Cedrus* chloroplast-like bacterial species isolated from the shared environment which may indicate horizontal transmission but does not rule out vertical transmission as it was found in parents and offspring. It is noted that offspring may inherit bacteria vertically, directly from their mother, but as they live in the same environment, could hence acquire the same bacteria horizontally from their mother. No information indicates that this bacterium was previously isolated from ladybirds or insects. A specific *Cedrus* chloroplast-like bacterium may be present in more than one different insect. For instance, analysis of the 16S rRNA gene sequence identified a *Cedrus* chloroplast-like bacterium of the host *Drosophila paulistorium* in wild *Cx. quinquefasciatus* mosquitoes indicating that bacterial inhabitants could be common for different insect species (Pidiyar *et al.*, 2004).

The phylogenetic analysis indicates the 16S sequence generated from *H. axyridis* symbiont samples clusters with Cyanobacteria from a variety of sources and/or of different genera and species. Also, worth noting is that when BLAST searches

were originally carried out, the closest matches included *Cedrus deodara* chloroplast-like sequences. A repeat of the BLAST search more recently found 16S sequences as represented in the phylogenetic analysis, which potentially indicates that the initial chloroplast hits whilst obviously similar, were the top hits because at the time GenBank lacked more closely related sequences.

Most insect species harbour at least one symbiotic bacterium that is specific to that species (Amann *et al.*, 1995). The number of bacterial species that have been characterised number not much more than 7000, representing a small fraction of the total bacterial diversity that can be grown in the laboratory (Achtman and Wanger, 2008). The number of described bacteria is influenced by the time and effort required to identify novel isolates as well as not being able to culture many of them (Rosselló-Mora and Amann, 2001). However, in recent years, two related approaches have been employed and contributed towards broadening the range of bacteria that can be cultivated through bringing the environment into the laboratory as well as growing close to other environmental bacteria, which has allowed the cultivation of many new species (Stewart, 2012). It would be possible to find out about the functions played by these microbes in the environment, ecology, host health and product production if a solution could be found of overcoming the difficulty of growing them *in vitro*.

Chapter 5: General discussion

5.1 Review of aims and main findings

The main aims of the study were to identify the different bacterial microbiota in two ladybird species *Harmonia axyridis* and *Halyzia sedecimguttata* using 16S rDNA sequences and to place bacteria found phylogenetically. Secondary aims were to identify where bacteria were located within the host and to provide information about transmission. The findings provide an overview of the different bacteria present in samples of the ladybird species *H. axyridis* and *H. sedecimguttata* collected in Nottingham, U.K.. In addition, it investigated the *Cedrus* chloroplast-like species present in different life stages and body parts of the Harlequin ladybird *H. axyridis* from the same population. As such it provides a comparison to two previous studies investigating the microbiota of *H. axyridis* from two different populations, namely invasive wild populations from Gołańcz in the Wielkopolska region of Poland (Dudek *et al.*, 2017) and in and around Giessen, Germany (Schmidtberg *et al.*, 2019).

Overall, results indicate that the bacterial communities identified in *H. axyridis* and *H. sedecimguttata* are composed mostly of symbiotic bacteria widely distributed in their environment along with infection by some unusual and undescribed bacteria. Proteobacteria; Actinobacteria; Bacteroidetes and Firmicutes along with unidentified bacteria were the dominant phyla of bacteria identified via 16S cloning, PCR and sequencing in both *H. axyridis* and *H. sedecimguttata*. In addition to these, Cyanobacteria and uncharacterised 'other' phyla, were also dominant in the metagenomic sequencing of *H. axyridis*. Members of these phyla are often

identified as dominant bacteria associated with arthropods and are commonly found in the environment, such as in soil (Capri *et al.*, 2011). It is unclear if the variation in the identified bacteria was due to dietary differences as *H. axyridis* are carnivorous insects while *H. sedecimguttata* are mycophagous insects (Roy *et al.*, 2019; Sutherland and Parrella, 2009). The components of different diets may have been colonised by, or contain, different types of bacteria that may be transmitted horizontally. The similarity in the bacterial species identified in both ladybird species may be attributed to the fact that these two species inhabit the same environment. As well as many symbiont species in common between the two ladybird hosts, there are several examples of bacteria previously identified in other beetles. It is possible that some at least of the differences in identified bacteria are simply due to the different approaches used to study the two ladybird species. In particular, as the metagenomic approach was not used in *H. sedecimguttata* the results are limited in comparison to *H. axyridis*. The bacterial community composition identified here shows overlap with other findings in other studies (Dudek *et al.*, 2017; Schmidtberg *et al.*, 2019), although notably the study of Schmidtberg *et al.* (2019), which focuses on gut bacteria and the possible effects of different diet on the bacteria present, did not identify Cyanobacteria. Although the results are limited, the metagenomics investigation of four individual *H. axyridis* demonstrated a wide variety in terms of number of different symbiont phylum per individual, in particular Male 1 contained predominantly Firmicutes, Proteobacteria and Actinobacteria. Whereas Male 2 contained 7 main phyla and multiple different species within the majority of these phyla (Figure 2.11).

Cyanobacteria are a challenging group of organisms, which are only beginning to be effectively and usefully classified (Walter *et al.*, 2017), with morphological and ecological characteristics not necessarily being reflected in phylogenetic analyses. An earlier study proposed that there should be 170 genera of Cyanobacteria based on 16S rRNA sequences only (Kozlov *et al.*, 2016). Next-Generation-Sequencing approaches are beginning to be used to address this issue (Walter *et al.*, 2017 for a review). The exact relationship between Cyanobacteria and their 'host' is not fully understood. However, Cyanobacteria are amongst the majority of organisms that cannot (currently) be cultured, meaning reliance on accurate molecular taxonomy is crucial in understanding relationships and functions. Given these limitations in classification, it seems unwise to speculate on the function of the Cyanobacteria identified in the investigations presented here. For the purpose of this discussion, the word 'symbiont' will be used despite these reservations.

One group of additional matches came from plastids. Intriguingly, apicoplasts, so-called because they are reduced plastids found in apicomplexan parasites, such as *Plasmodium* and *Toxoplasma*, are thought to have evolved from red algal endosymbionts (McFadden and Yeh, 2017). At some point in their evolution the Apicomplexa lost their photosynthetic ability, but retained some of the plastid functions. They cause diseases in a wide range of organisms including invertebrates (Lim and McFadden, 2010). These were only found in 13 sequences of BLAST hits and hence it is not possible to determine whether they represent genuine endosymbionts, or simply environmental or dietary components present in the sample tested. Further, as with the chloroplast-like bacteria, whilst potentially

interesting it is not possible to deduce any possible role they may have in ladybird hosts. Whilst less important or prevalent, these bacteria are potentially interesting and hence mentioned here for completeness.

This study estimated the prevalence of *Acinetobacter* in *H. axyridis* and *H. sedecimguttata*. Whilst PCR indicated the presence of the *Acinetobacter*-like bacteria in almost all individuals tested, as discussed in Chapter 3, these results may not be reliable as they could reflect false positive PCR products. A previous study demonstrated that the *Acinetobacter* genus was identified in *H. axyridis* (Schmidtberg *et al.*, 2019). *Acinetobacter* are gram-negative bacteria that are mostly free-living saprophytes inhabiting the environment such as soil, water, or even on vertebrate skins (Doughari *et al.*, 2011) and reported to produce pyrazines (Gao *et al.*, 2016). Pyrazines are present in the reflex bleeds of *H. axyridis* with a potential role in antipredatory defense behaviour. *Acinetobacter* are likely to be transmitted horizontally and acquired from the environment where *H. axyridis* hibernate. It is also possible that they can be transmitted vertically (Kikuchi, 2009). The occurrence of previously unidentified bacteria was confirmed in *H. axyridis* different life stages and body parts. Previous research described unidentified bacteria found in *H. axyridis* (Schmidtberg *et al.*, 2019). The existence of a particular unidentified bacterial species isolated from the environment may indicate the horizontal transmission of microbes from the environment without excluding the possibility of vertical transmission from mother to offspring.

Increasingly Next Generation Sequencing is providing insights into not just the identity of bacterial symbionts, but also detailed information about their genomes,

the biosynthetic pathways that are present and those that are missing. This in turn provides insights into the factors that may be necessary for their survival, growth and reproduction, but which need to be provided by their host, or the culture medium, if they are to be grown in culture. This can only be achieved if the bacteria are characterised by growing in culture which will require indepth understanding of the enviromental and growth conditions nessesary for their cultivation.

5.2 The contribution of the study to the insect microbiome

Some of the bacterial genera identified in *H. axyridis* including *Burkholderia*, *Delftia*, *Rhizobium*, *Flavobacterium*, *Acinetobacter*, *Lactococcus*, *Serratia*, *Rhodococcus* and *Stenotrophomonas* overlap with those of invasive *H. axyridis* from Poland or Germany (Dudek *et al.*, 2017; Schmidtberg *et al.*, 2019). In contrast, the microbiome of invasive *H. axyridis* in this study consists of other bacterial genera belong to *Janthinobacterium*, *Salinibacterium*, *Aureimonas*, *Novosphingobium*, *Granulicatella*, *Capnocytophaga*, *Rodentibacter*, *Myroides* and unidentified bacteria which were not identified in samples of *H. axyridis* from Poland or Germany. Some symbiotic bacteria including *Aureimonas*, *Capnocytophaga* and *Rodentibacter* were also not previously identified in other insect species. However, the other bacterial symbionts were found to be associated with insect hosts. In particular, the bacterial symbionts *Salinibacterium* and *Mycoplana* were detected in aphid samples (Grigorescu *et al.*, 2018; Wu *et al.*, 2018), the symbiotic bacteria *Novosphingobium* was found in association with spiders (Zhang *et al.*, 2018), the bacterial symbionts *Granulicatella* and *Streptococcus* were isolated from mosquitoes (Segata *et al.*, 2016; Omoke *et al.*,

2021) and the microbial symbiont *Myroides*, was identified in the migratory locust (Tan *et al.*, 2023).

This study is the first research on the bacterial community associated with the orange ladybird *H. sedecimguttata*. Results indicate that the microbiome of *H. sedecimgutta* encompasses *Janthinobacterium*, *Rhizobium*, *Novosphingobium*, *Acinetobacter*, *Duganella*, *Klenkia*, *Staphylococcus*, *Methylobacterium*, *Alkanindiges*, *Mycoplana*, *Streptococcus*, *Hymenobacter*, *Pelomicrobium* and unidentified bacteria. This study of a second ladybird species allows a comparison between species. Obviously two species are not sufficient to draw a general conclusion about ladybird microbiomes. However, it is notable that many of the bacterial genera identified have been found in both *H. axyridis* and *H. sedecimguttata*. Although the bacterial symbionts *Klenkia* and *Pelomicrobium* were not previously detected in insects, the other symbiotic microbes were shown to be associated with other species of insect. Specifically, the bacterial symbionts *Janthinobacterium*, *Duganella* and *Hymenobacter* were identified in the cereal leaf beetle (Wielkopolan *et al.*, 2021) and the symbiont *Alkanindiges* was determined in the carpenter ant (Koto *et al.*, 2020). The symbiotic bacteria *Staphylococcus* and *Methylobacterium* were identified in samples of invasive *H. axyridis* from Germany (Schmidtberg *et al.*, 2019) and in *H. sedecimguttata* in this study, however, not in *H. axyridis* from Nottingham. This serves to illustrate the important point that populations of any species are likely to vary in the microbiome and hence a broader understanding of ladybird microbiomes in general, and of more populations as well as different species is required.

5.3 The relevance of the findings to *H. axyridis* invasions and biological control

If we can understand how microbiota influence the survival, adaptation and reproduction of their ladybird hosts, it is possible that ways of manipulating the microbiota could also be used to successfully control the hosts. For example, bacteria that limit reproduction are being used to control the spread of other pests such as mosquitos carrying Dengue fever (O'Neill *et al.* 2019). Potentially preventing the ladybirds from reproducing, or reducing their viable offspring numbers, might be sufficient to prevent or limit invasion, with a greatly reduced risk that they will survive, reproduce and spread. Similarly, there is potential to harness the ladybird microbiota in ensuring that ladybirds can work as efficient and useful biocontrol agents, without having a negative impact on other local ladybird species.

5.4 The function of the identified bacteria

Some of the bacteria that have been identified in the two ladybird species play critical roles in their insect hosts, while the role played by others has not yet been recognised so far. There are bacteria that were previously identified in ladybirds, and the role they play was hypothesised. These include *Lactococcus*, *Staphylococcus*, *Serratia*, *Pseudomonas*, *Acinetobacter* and *Stenotrophomonas* and those belonging to the family Enterobacteriaceae (Schmidtberg *et al.*, 2019). The ladybird hosts use pyrazine produced by any of these bacterial symbionts as deterrents or attractants in many ladybirds interactions (Verheggen *et al.*, 2017). Flavobacteria was identified as the caustive agent of male-killing endosymbionts in ladybird host species (Hurst *et al.*, 1997a; Majerus, 2001). The functions of other

bacterial symbionts have been identified in other insect hosts. The insecticide resistance, growth rate and body size of the bean bug host are enhanced by *Burkholderia* symbionts (Kikuchi and Fukatsu, 2014). *Streptococcus* symbiotic bacteria can degrade pyrethroid insecticides such as permethrin and may also contribute to insecticide resistance in their mosquitoes hosts (Omoke *et al.*, 2021). D-amino acid amidase enzyme that is produced by *Delftia* sp. may increase the survival rate of their whitefly host (Xie *et al.*, 2012). *Janthinobacterium* symbionts assist their herbivorous insect host to absorb the nutrient phytate by producing the enzyme phytase that degrades phytate which is the dominant class of organic phosphorus compounds synthesised by plants (Zhang *et al.*, 2011). Rhizobacteria harboured by termite hosts synthesise the vitamins and amino acids required for the development of insect host by fixing dinitrogen into ammonia (Paniagua *et al.*, 2018). *Bacillus* symbionts detoxify plant defence chemicals in bark beetle hosts (García-Fraile, 2018). Along with other bacterial symbionts isolated from bark beetle hosts including *Stenotrophomonas maltophilia* and those belonging to the genera *Pseudomonas*, *Serratia*, *Methylobacterium*, *Paenibacillus* and *Sphingomonas*, they provide nutrition to their beetle host by degrading cellulose. *Novosphingobium* symbionts increase the survival and fitness of their invasive beetle hosts by degrading the phenolic defensive chemical, naringenin, in pines (Cheng *et al.*, 2018). It was suggested that *Asaia* symbionts may play a role in vitamins or cofactors biosynthesis utilised by their mosquito species hosts (Crotti *et al.*, 2010). In summary, many of the symbionts identified play roles in insect hosts, with a small number having previously identified roles specifically in

ladybirds. For many of the bacteria identified by the metagenomic approach in particular, any roles they may have either in insects in general or more specifically in ladybirds are yet to be identified.

5.5 The limitations of the study

The nature of the interaction between the ladybird hosts and the bacteria is unclear from the findings. Whilst the investigations of the different body parts and different life stages aimed to distinguish between bacteria that were true endosymbionts, living within the host, and 'contaminants', these findings were inconclusive. The bacteria identified could potentially simply be part of the gut contents, and hence simply passing through the host, as opposed to bacteria that might be genuine gut endosymbionts, playing a role in nutrient absorption or toxin breakdown for example. Similarly, bacteria found in the mother, her eggs, resultant larvae and new adults could be vertically transmitted symbionts. Absence of positive PCR results in many samples meant that it was not possible to confirm vertical transmission. These limitations were in part due to the non-specificity of the PCR primers and in part due to the small numbers of samples tested and even smaller numbers that produced informative results.

5.6 Priorities for future research

Moving forwards, future research should ideally focus on identifying further details about the potential roles of bacterial identified. There are several useful approaches that could be taken. Pre-treatment of ladybird samples to ensure that gut contents do not confuse findings is an important step. Rearing ladybirds and then extracting DNA from newly emerged adults, ahead of any feeding would

ensure only vertically transmitted bacteria are present in the samples. An alternative approach for samples from the wild could involve starving adults ahead of DNA extraction, or feeding them on artificial diets that are also tested for bacteria. Designing specific primers that amplify a single bacterial Genus (if not species), would also help in ensuring that positive results are true positives and hence reflect the bacteria present.

In terms of investigating functions of particular bacteria, experiments designed to manipulate the presence/absence of particular bacteria may prove useful. Treatment with particular antibiotics may allow the production of ladybirds free of specific bacteria. Where potential roles have been identified in other insects, ideally relatively closely related host species, such as ladybirds or other beetles, experiments could be designed to see if eliminating the bacteria has an impact of the phenotype in terms of the potential function/role. A complementary experiment might involve culturing a particular bacterial species and 'infecting' ladybird hosts with it, either via microinjection at the pupal stage, or via diet for species that can survive passage through the gut and potentially act as gut endosymbionts.

A final priority is to investigate further the generality of the findings by investigating different host ladybird species, larger sample sizes and populations of hosts from different locations and environments. The metagenomics results suggested that individual ladybirds may vary in the number of different bacteria present within their bodies. Previous work on male-killers in ladybirds straightforwardly identified the male-killers as the predominant bacteria present in the maternal line, suggesting that these bacteria are able to outcompete other bacteria (Majerus and Majerus

2012; Majerus and Majerus 2010a; Majerus and Majerus 2010b; Majerus *et al.*, 1999; Majerus *et al.*, 1998). Investigation of interactions between different bacteria and identification of combinations that predominate might provide greater insight into the roles of these microbiota. Investigation of different hosts, including those with different diets and life histories may provide greater understanding of the functions and variety of ladybird symbionts.

5.7 Conclusion

Overall the findings in this thesis have developed the insights into the variety of bacterial species found within two ladybird host species. Results have confirmed some earlier studies, using different populations of *H. axyridis*, identifying the same genera of bacteria, and further, suggesting that the presence of several bacterial classes is common to populations from different countries, albeit in the small sample sizes tested. Further, the study was the first to investigate the bacteria present in *Halysia sedecimgutta*, and in doing so identified some bacteria in common with the findings from *H. axyridis*. It is possible that some of the bacteria identified only in *H. sedecimgutta* and not *H. axyridis* play roles which are specific to the former host and relate to its fungal diet, or other aspect of its life history, distinct from that of the generalist *H. axyridis*.

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Appendix

Clone identity	Most closely related organism (Where Known)	Accession	Percentage identity	Percentage coverage
H1-1	Uncultured bacterium	HM811652.1	100%	100%
	<i>Burkholderia contaminans</i>	MN826151.1	99%	100%
	<i>Burkholderia cepacia</i>	MT355406.1	99%	100%
	<i>Burkholderia lata</i>	MN988625.1	99%	100%
	<i>Burkholderia cenocepacia</i>	MK615919.1	99%	100%
	<i>Burkholderia paludis</i>	MK503788.1	99%	100%
	<i>Paraburkholderia</i> sp.	LC460890.1	99%	100%
	<i>Burkholderia metallica</i>	OL851724.1	99%	100%
	<i>Paenibacillus</i> sp.	KJ948383.1	99%	100%
	<i>Gottfriedia acidiceris</i>	KU145689.1	99%	100%
	<i>Caenibaculum baiyandianus</i>	NR_173536.1	99%	100%
H1-2	<i>Delftia tsuruhatensis</i>	MT374262.1	100%	100%
	<i>Delftia lacustris</i>	MT269010.1	100%	100%
	Uncultured bacterium	MH934454.1	100%	100%
	<i>Delftia acidovorans</i>	MZ203805.1	100%	100%
	<i>Cupriavidus gilardii</i>	KP939099.1	99%	100%
	<i>Comamonas</i> sp.	AY965248.1	99%	100%
	<i>Pseudomonas</i> sp.	LN885375.1	99%	100%
	<i>Rhodobacter sphaeroides</i>	DQ001153.1	99%	100%
	<i>Clostridium sporogenes</i>	KR109293.1	99%	100%
	<i>Alcaligenes faecalis</i>	JF792084.1	99%	100%
H1-3	<i>Janthinobacterium svalbardensis</i>	MT242542.1	99%	100%
	<i>Janthinobacterium lividum</i>	MG825082.1	99%	100%
	Uncultured bacterium	JQ191643.1	99%	100%
	<i>Janthinobacterium tractae</i>	ON237933.1	99%	100%
	<i>Aquaspirillum arcticum</i>	JX475101.1	99%	100%
	<i>Duganella</i> sp.	EF111225.1	99%	100%
	<i>Oceanobacillus</i> sp.	KC921006.1	99%	97%
	<i>Massilia violaceinigra</i>	OK090593.1	97%	99%
	<i>Rugamonas</i> sp.	KP899253.1	98%	98%
	<i>Massilia brevitalea</i>	MH667847.1	97%	99%
H1-4	<i>Rhizobium herbae</i>	MK611738.1	99%	100%
	Uncultured bacterium	MH717852.1	99%	100%
	<i>Rhizobium giardinii</i>	KR014110.1	99%	100%
	<i>Sinorhizobium</i> sp.	JQ977145.1	99%	100%
	<i>Ensifer</i> sp.	MF977599.1	99%	100%
	<i>Bradyrhizobium</i> sp.	DQ310792.1	99%	100%
	<i>Shinella kummerowiae</i>	MT634600.1	98%	100%
	<i>Pararhizobium capsulatum</i>	NR_026116.1	98%	100%
	<i>Pararhizobium polonicum</i>	LN845890.1	98%	97%
	<i>Mesorhizobium</i> sp.	HQ324673.1	97%	100%
H1-11	<i>Cedrus deodara chloroplast</i>	DQ629444.1	99%	94%
	Uncultured bacterium	KX507403.1	99%	93%
	<i>Loriellopsis cavernicola</i>	KY753239.1	98%	90%
	Uncultured <i>Trichodesmium</i> sp.	KM873113.1	97%	93%
	<i>Bacillus</i> sp.	ON597424.1	97%	93%
	Uncultured actinobacterium	DQ431885.1	97%	93%
	Uncultured <i>Cyanobacterium</i> sp.	LN833508.1	97%	93%

	Sugarcane phytoplasma	KJ650039.1	96%	93%
	Uncultured <i>Oscillatoria</i> sp.	EU341185.1	96%	93%
	Bacterium endosymbiont of <i>Stereum hirsutum</i>	MG954110.1	97%	87%
	<i>Planktothricoides</i> sp.	JX912374.1	96%	87%
H1-13	<i>Salinibacterium hongtaonis</i>	NR_171520.1	97%	94%
	<i>Salinibacterium xinjiangense</i>	KF923458.1	96%	94%
	<i>Leifsonia rubra</i>	FR691400.1	96%	94%
	<i>Salinibacterium amurskyense</i>	KF306368.1	96%	93%
	<i>Herbiconiux solani</i>	NR_116995.1	96%	94%
	<i>Herbiconiux ginsengi</i>	NR_043879.1	96%	93%
	<i>Subtercola pratensis</i>	AJ310412.1	96%	94%
	<i>Leifsonia poae</i>	DQ232613.2	96%	94%
	<i>Homoserinimonas aerilata</i>	NR_109608.1	96%	93%
	<i>Leifsonia flava</i>	KY033218.2	96%	94%
	Uncultured bacterium	JN023276.1	98%	94%
H1-14	Uncultured Acidobacteria	HQ598527.1	98%	93%
	Uncultured actinobacterium	AB874556.1	96%	94%
	Uncultured <i>Solibacter</i> sp.	FJ889211.1	96%	94%
	Uncultured <i>Edaphobacter</i> sp.	KF287747.1	92%	93%
	Uncultured eubacterium	AJ292589.1	91%	94%
	Uncultured Bryobacterales	MT858086.1	91%	93%
	Uncultured proteobacterium	EF018823.1	94%	82%
	Uncultured <i>Holophaga</i> sp.	AJ536863.1	93%	83%
	Uncultured <i>Terriglobus</i> sp.	JX504891.1	91%	86%
	Uncultured bacterium	LC409580.1	99%	100%
H2-1	<i>Burkholderia contaminans</i>	MN826151.1	99%	100%
	<i>Burkholderia cepacia</i>	MT355406.1	99%	100%
	<i>Burkholderia lata</i>	MN988625.1	99%	100%
	<i>Burkholderia paludis</i>	MK932026.1	99%	100%
	<i>Burkholderia cenocepacia</i>	MK615919.1	99%	100%
	<i>Paraburkholderia</i> sp.	LC460890.1	99%	100%
	<i>Burkholderia metallica</i>	OL851724.1	99%	100%
	<i>Paenibacillus</i> sp.	KJ948383.1	99%	100%
	<i>Bacillus</i> sp.	KJ948345.1	99%	100%
	<i>Azospirillum</i> sp.	AB508854.1	99%	100%
	<i>Aureimonas</i> sp.	MN989041.1	99%	100%
H2-2	Uncultured bacterium	JQ386884.2	99%	100%
	<i>Aureimonas altamirensis</i>	MF525345.1	98%	100%
	<i>Aurantimonas coralicida</i>	LC085344.1	98%	100%
	<i>Aureimonas glaciei</i>	OL912976.1	98%	100%
	<i>Fulvimarina</i> sp.	AY368505.1	98%	100%
	<i>Aurantimonas marina</i>	MT941030.1	97%	100%
	<i>Aurantimonas endophytica</i>	NR_152083.1	96%	100%
	<i>Jiella</i> sp.	MW811459.1	96%	100%
	<i>Aurantimonas aggregata</i>	NR_159240.1	95%	100%
	<i>Aurantimonas litoralis</i>	MW793439.1	95%	100%
	Uncultured <i>Neisseriaceae</i> bacterium	LT684100.1	100%	100%
H2-3	Uncultured bacterium	KF091179.1	100%	100%
	Uncultured Burkholderiales bacterium	HM075431.1	91%	100%
	<i>Snodgrassella alvi</i>	MH782111.1	90%	99%
	<i>Simonsiella muelleri</i>	NR_025144.1	90%	98%
	<i>Eikenella longinqua</i>	NR_170432.1	90%	98%
	<i>Delftia tsuruhatensis</i>	MT374262.1	99%	100%

	<i>Delftia lacustris</i>	MT269010.1	99%	100%
	Uncultured bacterium	LC410755.1	99%	100%
	<i>Delftia acidovorans</i>	MZ203805.1	99%	100%
	<i>Cupriavidus gilardii</i>	KP939099.1	99%	100%
	<i>Comamonas</i> sp.	AY965248.1	99%	100%
	<i>Pseudomonas</i> sp.	LN885375.1	99%	99%
	<i>Rhodobacter sphaeroides</i>	DQ001153.1	99%	100%
	<i>Clostridium sporogenes</i>	KR109293.1	99%	100%
	<i>Alcaligenes faecalis</i>	JF792084.1	99%	100%
H2-6	Uncultured bacterium	GQ080953.1	100%	100%
	Uncultured Neisseriaceae bacterium	LT684100.1	99%	100%
	Uncultured Burkholderiales bacterium	HM075432.1	91%	100%
	<i>Simonsiella muelleri</i>	NR_025144.1	89%	100%
	<i>Eikenella longinqua</i>	NR_170432.1	89%	98%
	<i>Snodgrassella alvi</i>	MH782111.1	89%	100%
H2-8	<i>Cedrus deodara</i> chloroplast	DQ629444.1	99%	93%
	Uncultured bacterium	KX507403.1	99%	92%
	<i>Loriellopsis cavernicola</i>	KY753239.1	99%	88%
	Uncultured <i>Oscillatoria</i> sp.	EU341185.1	97%	93%
	<i>Bacillus</i> sp.	ON597424.1	96%	93%
	Uncultured <i>Cyanobacterium</i> sp.	LN833508.1	96%	93%
	Uncultured <i>Trichodesmium</i> sp.	KM873113.1	96%	92%
	Bacterium endosymbiont of <i>Stereum hirsutum</i>	MG954110.1	97%	86%
	Sugarcane phytoplasma	KJ650039.1	94%	92%
	Uncultured Xanthomonadales	EU404026.1	95%	83%
	<i>Limosilactobacillus fermentum</i>	MF424753.1	95%	83%
H2-9	<i>Cedrus deodara</i> chloroplast	DQ629444.1	99%	93%
	Uncultured bacterium	KX507403.1	99%	92%
	Uncultured <i>Trichodesmium</i> sp.	KM873113.1	98%	92%
	<i>Bacillus</i> sp.	ON597424.1	98%	92%
	<i>Loriellopsis cavernicola</i>	KY753239.1	99%	89%
	Uncultured <i>Cyanobacterium</i> sp.	LN833508.1	97%	92%
	Sugarcane phytoplasma	KJ650039.1	97%	92%
	Uncultured <i>Oscillatoria</i> sp.	EU341185.1	97%	92%
	<i>Halospirulina</i> sp.	JX912442.1	96%	92%
	<i>Planktothricoides</i> sp.	JX912374.1	96%	90%
H2-10	<i>Cedrus deodara</i> chloroplast	DQ629444.1	93%	93%
	Uncultured bacterium	KX507403.1	93%	92%
	Uncultured <i>Trichodesmium</i> sp.	KM873113.1	91%	92%
	<i>Bacillus</i> sp.	ON597424.1	91%	93%
	<i>Loriellopsis cavernicola</i>	KY753239.1	92%	89%
	Uncultured actinobacterium	DQ431885.1	91%	92%
	Uncultured <i>Cyanobacterium</i> sp.	LN833508.1	91%	92%
	Sugarcane phytoplasma	KJ650039.1	91%	92%
	<i>Halospirulina</i> sp.	JX912442.1	90%	92%
	<i>Planktothricoides</i> sp.	JX912374.1	90%	91%
H2-12	Uncultured bacterium	DQ532133.1	98%	93%
	Uncultured <i>Martelella</i> sp.	FJ388151.1	92%	42%
	Uncultured <i>Kordiimonas</i> sp.	FJ388275.1	92%	42%
	Uncultured <i>Thiococcus</i> sp.	FJ388065.1	91%	42%
	Uncultured proteobacterium	EU297873.1	87%	48%
	Uncultured Candidatus <i>Liberibacter</i> sp.	FJ388147.1	92%	40%
	Uncultured <i>Sphingobium</i> sp.	HM452647.1	93%	36%

	Uncultured <i>Aurantimonas</i> sp.	JN375331.1	86%	39%
	Uncultured <i>Methylobacterium</i> sp.	JN375369.1	86%	39%
H2-13	<i>Cedrus deodara</i> chloroplast	DQ629444.1	99%	93%
	Uncultured bacterium	KX507403.1	99%	93%
	<i>Loriellopsis cavernicola</i>	KY753239.1	98%	90%
	Uncultured <i>Trichodesmium</i> sp.	KM873113.1	97%	93%
	<i>Bacillus</i> sp.	ON597424.1	97%	93%
	Uncultured actinobacterium	DQ431885.1	97%	93%
	Uncultured <i>Cyanobacterium</i> sp.	LN833508.1	97%	93%
	Sugarcane phytoplasma	KJ650039.1	96%	93%
	Uncultured <i>Oscillatoria</i> sp.	EU341185.1	97%	93%
	<i>Planktothricoides</i> sp.	JX912374.1	96%	91%
H2-15	<i>Cedrus deodara</i> chloroplast	DQ629444.1	99%	94%
	Uncultured bacterium	KX507403.1	99%	94%
	<i>Loriellopsis cavernicola</i>	KY753239.1	99%	91%
	Uncultured <i>Oscillatoria</i> sp.	EU341185.1	97%	94%
	<i>Bacillus</i> sp.	ON597424.1	97%	94%
	Uncultured <i>Trichodesmium</i> sp.	KM873113.1	97%	94%
	Uncultured <i>Cyanobacterium</i> sp.	LN833508.1	97%	94%
	Bacterium endosymbiont of <i>Stereum hirsutum</i>	MG954110.1	97%	89%
	Sugarcane phytoplasma	KJ650039.1	95%	94%
	Uncultured actinobacterium	DQ431885.1	97%	85%
	Uncultured Xanthomonadales	EU404026.1	96%	87%
H2-16	<i>Limosilactobacillus fermentum</i>	MF424753.1	96%	86%
	<i>Cedrus deodara</i> chloroplast	DQ629444.1	99%	94%
	Uncultured bacterium	KX507403.1	99%	94%
	<i>Loriellopsis cavernicola</i>	KY753239.1	99%	91%
	Uncultured <i>Oscillatoria</i> sp.	EU341185.1	97%	94%
	<i>Bacillus</i> sp.	ON597424.1	97%	94%
	Uncultured <i>Trichodesmium</i> sp.	KM873113.1	97%	94%
	Uncultured <i>Cyanobacterium</i> sp.	LN833508.1	96%	94%
	Bacterium endosymbiont of <i>Stereum hirsutum</i>	MG954110.1	97%	89%
	Sugarcane phytoplasma	KJ650039.1	95%	94%
	Uncultured actinobacterium	DQ431885.1	97%	85%
H2-17	<i>Limosilactobacillus fermentum</i>	MF424753.1	96%	86%
	Uncultured bacterium	DQ532133.1	95%	94%
	Uncultured <i>Sphingobium</i> sp.	HM452647.1	96%	50%
H2-18	Uncultured proteobacterium	EU297873.1	88%	60%
	<i>Cedrus deodara</i> chloroplast	DQ629444.1	99%	94%
	Uncultured bacterium	KX507403.1	99%	93%
	<i>Loriellopsis cavernicola</i>	KY753239.1	99%	90%
	Uncultured <i>Oscillatoria</i> sp.	EU341185.1	97%	94%
	<i>Bacillus</i> sp.	ON597424.1	97%	94%
	Uncultured <i>Cyanobacterium</i> sp.	LN833508.1	96%	94%
	Uncultured <i>Trichodesmium</i> sp.	KM873113.1	96%	93%
	Bacterium endosymbiont of <i>Stereum hirsutum</i>	MG954110.1	97%	87%
	Sugarcane phytoplasma	KJ650039.1	94%	93%
	Uncultured actinobacterium	DQ431885.1	96%	83%
H2-20	Uncultured Xanthomonadales	EU404026.1	95%	85%
	<i>Cedrus deodara</i> chloroplast	DQ629444.1	99%	94%
	Uncultured bacterium	KX507403.1	99%	94%

	<i>Loriellopsis cavernicola</i>	KY753239.1	99%	92%
	Uncultured <i>Trichodesmium</i> sp.	KM873113.1	97%	94%
	<i>Bacillus</i> sp.	ON597424.1	97%	94%
	Uncultured <i>Cyanobacterium</i> sp.	LN833508.1	97%	94%
	Uncultured <i>Oscillatoria</i> sp.	EU341185.1	97%	94%
	Sugarcane phytoplasma	KJ650039.1	97%	94%
	Bacterium endosymbiont of <i>Stereum hirsutum</i>	MG954110.1	97%	88%
	Uncultured Xanthomonadales	EU404026.1	96%	88%
H3-1	<i>Cedrus deodara</i> chloroplast	DQ629444.1	98%	90%
	Uncultured bacterium	KX507403.1	98%	90%
	Uncultured <i>Trichodesmium</i> sp.	KM873113.1	96%	90%
	<i>Bacillus</i> sp.	ON597424.1	96%	90%
	<i>Loriellopsis cavernicola</i>	KY753239.1	97%	85%
	Uncultured <i>Cyanobacterium</i> sp.	LN833508.1	96%	90%
	Uncultured <i>Oscillatoria</i> sp.	EU341185.1	95%	89%
	Sugarcane phytoplasma	KJ650039.1	95%	90%
	Uncultured <i>Acinetobacter</i>	HG917238.1	95%	90%
	<i>Halospirulina</i> sp.	JX912442.1	94%	90%
H3-2	<i>Cedrus deodara</i> chloroplast	DQ629444.1	98%	71%
	Uncultured bacterium	KX507403.1	98%	71%
	Uncultured <i>Cyanobacterium</i>	KJ574140.1	97%	71%
	<i>Loriellopsis cavernicola</i>	KY753239.1	97%	71%
	Bacterium endosymbiont of <i>Stereum hirsutum</i>	MG954110.1	96%	71%
	<i>Bacillus</i> sp.	ON597424.1	96%	71%
	<i>Halospirulina</i> sp.	JX912442.1	96%	71%
	<i>Planktothricoides</i> sp.	JX912374.1	96%	71%
	Sugarcane phytoplasma	KJ650039.1	96%	71%
	Bacterium symbiont of <i>Heliothrips haemorrhoidalis</i>	KM582848.1	95%	71%
H3-3	Uncultured bacterium	EF157084.1	98%	71%
	<i>Cedrus deodara</i> chloroplast	DQ629444.1	98%	71%
	Uncultured <i>Cyanobacterium</i>	KJ574140.1	97%	71%
	<i>Loriellopsis cavernicola</i>	KY753239.1	97%	71%
	Bacterium endosymbiont of <i>Stereum hirsutum</i>	MG954110.1	96%	71%
	<i>Bacillus</i> sp.	ON597424.1	96%	71%
	<i>Halospirulina</i> sp.	JX912442.1	96%	71%
	<i>Planktothricoides</i> sp.	JX912374.1	96%	71%
	Sugarcane phytoplasma	KJ650039.1	95%	71%
	Bacterium symbiont of <i>Heliothrips haemorrhoidalis</i>	KM582848.1	95%	71%
H3-4	<i>Cedrus deodara</i> chloroplast	DQ629444.1	99%	94%
	Uncultured bacterium	KX507403.1	99%	94%
	<i>Loriellopsis cavernicola</i>	KY753239.1	99%	91%
	Uncultured <i>Trichodesmium</i> sp.	KM873113.1	98%	94%
	<i>Bacillus</i> sp.	ON597424.1	97%	94%
	Uncultured actinobacterium	DQ431885.1	97%	94%
	Uncultured <i>Cyanobacterium</i> sp.	LN833508.1	97%	94%
	Sugarcane phytoplasma	KJ650039.1	97%	94%
	Uncultured <i>Oscillatoria</i> sp.	EU341185.1	97%	93%
	Bacterium endosymbiont of <i>Stereum hirsutum</i>	MG954110.1	98%	87%

H4-8	<i>Flavobacterium lacunae</i>	MW020255.1	92%	92%
	<i>Flavobacterium columnare</i>	AB015481.1	92%	93%
	<i>Flavobacterium arcticum</i>	KU529277.1	91%	94%
	<i>Flavobacterium rivuli</i>	NR_115084.1	91%	94%
	<i>Flexibacter aurantiacus</i>	AB078045.1	91%	94%
	<i>Flavobacterium enshiense</i>	NR_118321.1	91%	93%
	<i>Myroides</i> sp.	KF611893.1	91%	94%
	<i>Winogradskyella</i> sp.	JF312944.1	91%	94%
	<i>Flavobacterium lutivivi</i>	NR_148774.1	91%	94%
	<i>Leptobacterium flavescens</i>	AB362214.1	91%	92%
H5-1	<i>Novosphingobium subterraneum</i>	NR_040827.1	99%	94%
	<i>Novosphingobium aromaticivorans</i>	KF381499.1	98%	94%
	<i>Novosphingobium percolationis</i>	MZ707565.1	97%	94%
	<i>Novosphingobium pokkali</i>	NR_149820.1	97%	94%
	<i>Sphingomonas</i> sp.	AB362778.1	97%	94%
	<i>Novosphingobium taihuense</i>	MK138638.1	97%	93%
	<i>Novosphingobium hassiacum</i>	NR_028962.1	97%	94%
	<i>Novosphingobium huizhouense</i>	MZ707566.1	97%	94%
	<i>Novosphingobium ginsenosidimutans</i>	NR_133800.1	97%	92%
	<i>Novosphingobium capsulatum</i>	M59296.1	96%	94%
H5-2	<i>Rhizobium huautlense</i>	AM237359.1	99%	92%
	<i>Rhizobium rosettiformans</i>	KP721486.1	97%	94%
	<i>Neorhizobium</i> sp.	MN984926.1	99%	89%
	<i>Rhizobium panacihumi</i>	OM971214.1	97%	94%
	<i>Pseudorhizobium banfieldiae</i>	AF159453.1	97%	93%
	<i>Rhizobium rhizolycopersici</i>	MT703880.1	97%	94%
	<i>Sinorhizobium</i> sp.	MN606155.1	97%	94%
	<i>Rhizobium gallicum</i>	FR728933.1	97%	92%
	<i>Agrobacterium deltaense</i>	MN638807.1	97%	94%
	<i>Agrobacterium rhizogenes</i>	MZ298110.1	97%	94%
H5-4	Uncultured bacterium	KC541035.1	99%	94%
	<i>Acinetobacter lwoffii</i>	MW433856.1	99%	93%
	<i>Prolinoborus fasciculus</i>	KR085813.1	99%	94%
	Bacterium endosymbiont of <i>Onthophagus Taurus</i>	KF193151.1	98%	92%
	<i>Bacillus methylotrophicus</i>	KF818634.1	99%	89%
	<i>Pseudomonas</i> sp.	KC433626.1	98%	91%
	<i>Arcobacter</i> sp.	FJ968636.1	97%	92%
	Endosymbiont of <i>Nilaparvata lugens</i>	JQ975879.1	97%	93%
	<i>Bacillus weihenstephanensis</i>	JF935054.1	98%	89%
	<i>Arthrobacter</i> sp.	KC311587.1	97%	92%
H5-7	Uncultured bacterium	GU361873.1	99%	94%
	<i>Granulicatella adiacens</i>	FR822389.1	99%	94%
	<i>Granulicatella elegans</i>	JN801174.1	99%	91%
	<i>Carnobacterium divergens</i>	LT223644.1	97%	94%
	<i>Enterococcus italicus</i>	MT994627.1	97%	94%
	<i>Atopobacter phocae</i>	NR_119263.1	97%	93%
	<i>Isobaculum melis</i>	NR_025445.1	96%	94%
	<i>Enterococcus camelliae</i>	NR_044121.1	96%	94%
	<i>Enterococcus avium</i>	MK182809.1	96%	94%
	<i>Carnobacterium mobile</i>	LT223645.1	96%	94%
	<i>Enterococcus viikkiensis</i>	NR_117976.1	96%	94%
	Uncultured bacterium	GU201968.1	90%	94%
H5-8	<i>Capnocytophaga</i> sp.	KM461964.1	90%	94%

	<i>Myroides phaeus</i>	NR 117475.1	90%	94%
	<i>Flavobacterium columnare</i>	KC912660.1	89%	94%
	<i>Flavobacterium arcticum</i>	KU529277.1	89%	94%
	<i>Flavobacterium ichthyis</i>	MH729075.1	89%	93%
	<i>Flavobacterium alkalisola</i>	MN310902.1	89%	94%
	<i>Flavobacterium kingsejongi</i>	NR 159896.1	89%	94%
	<i>Myroides fluvii</i>	MK129421.1	89%	93%
	<i>Avrillella dinanensis</i>	MF278923.1	89%	94%
H5-9	<i>Rodentibacter heidelbergensis</i>	NR 156993.1	82%	94%
	<i>Bibersteinia trehalosi</i>	MK920112.1	82%	92%
	<i>Pseudoalteromonas</i> sp.	MK575497.1	82%	93%
	<i>Mannheimia haemolytica</i>	M75063.1	82%	92%
	<i>Vibrio natriegens</i>	ON003474.1	83%	87%
	<i>Halomonas stenophila</i>	NR 117837.1	82%	93%
	<i>Halomonas denitrificans</i>	EU541350.1	82%	94%
	<i>Halomonas pacifica</i>	KJ575078.1	82%	94%
	<i>Rodentibacter pneumotropicus</i>	MK287673.1	82%	89%
	<i>Shewanella amazonensis</i>	NR 074842.1	81%	94%
H6-1	<i>Lactococcus lactis</i>	DQ340068.1	99%	94%
	<i>Lactococcus cremoris</i>	NR 116443.1	99%	94%
	<i>Enterococcus durans</i>	FJ917740.1	99%	93%
	<i>Leuconostoc pseudomesenteroides</i>	AB602812.1	99%	92%
	<i>Agrobacterium tumefaciens</i>	AB244443.1	99%	92%
	<i>Lactobacillus acidophilus</i>	MG543795.1	99%	92%
	bacterium symbiont of <i>Bactrocera correcta</i>	JQ950507.1	99%	92%
	<i>Lactobacillus delbrueckii</i>	KJ526924.1	99%	93%
	Endosymbiont of <i>Sphenophorus levis</i>	FJ626255.1	99%	92%
	<i>Weissella confusa</i>	LC274617.1	99%	91%
H6-3	<i>Lactococcus lactis</i>	MK977613.1	99%	94%
	Uncultured bacterium	DQ800532.1	99%	94%
	bacterium symbiont of <i>Bactrocera correcta</i>	JQ950507.1	99%	92%
	<i>Enterococcus durans</i>	FJ917740.1	99%	92%
	<i>Leuconostoc pseudomesenteroides</i>	AB602812.1	99%	92%
	<i>Agrobacterium tumefaciens</i>	AB244443.1	99%	92%
	<i>Lactococcus cremoris</i>	NR 116443.1	99%	94%
	<i>Weissella confusa</i>	LC274617.1	99%	90%
	<i>Lactobacillus delbrueckii</i>	MH813978.1	99%	90%
	<i>Lactiplantibacillus plantarum</i>	MG754526.1	99%	88%
H6-4	<i>Limosilactobacillus fermentum</i>	MF355330.1	99%	88%
	<i>Lactococcus lactis</i>	MK977613.1	99%	94%
	Uncultured bacterium	DQ800532.1	99%	94%
	bacterium symbiont of <i>Bactrocera correcta</i>	JQ950507.1	99%	92%
	<i>Enterococcus durans</i>	FJ917740.1	99%	92%
	<i>Leuconostoc pseudomesenteroides</i>	AB602812.1	99%	92%
	<i>Agrobacterium tumefaciens</i>	AB244443.1	99%	92%
	<i>Lactococcus cremoris</i>	NR 116443.1	99%	94%
	<i>Weissella confusa</i>	LC274617.1	99%	90%
	<i>Lactobacillus delbrueckii</i>	MH813978.1	99%	90%
H7-1	<i>Lactiplantibacillus plantarum</i>	MG754526.1	99%	88%
	<i>Limosilactobacillus fermentum</i>	MF355330.1	99%	88%
	<i>Myroides profundus</i>	KF730743.1	99%	94%

	<i>Myroides odoratimimus</i>	EU373415.1	99%	94%
	Uncultured bacterium	JX457156.1	98%	94%
	<i>Myroides gitamensis</i>	NR_125560.1	98%	92%
	<i>Myroides phaeus</i>	NR_117475.1	95%	94%
	<i>Myroides marinus</i>	MN216276.1	96%	89%
	<i>Erythrobacter</i> sp.	MF359324.1	94%	88%
	<i>Flavobacterium ardleyense</i>	NR_158093.1	91%	94%
	<i>Cytophaga</i> sp.	X85210.1	91%	93%
	<i>Flavobacterium columnare</i>	KR080246.1	91%	94%
H7-2	<i>Myroides odoratimimus</i>	EU373415.1	99%	93%
	<i>Myroides profundus</i>	KF730743.1	99%	93%
	<i>Myroides gitamensis</i>	NR_125560.1	98%	91%
	<i>Myroides marinus</i>	KT260532.1	97%	91%
	<i>Myroides pelagicus</i>	NR_114110.1	97%	91%
	<i>Myroides phaeus</i>	NR_117475.1	96%	92%
	<i>Myroides albus</i>	MK734183.1	97%	89%
	<i>Erythrobacter</i> sp.	MF359324.1	97%	87%
	<i>Flavobacterium niveum</i>	NR_171470.1	93%	94%
H7-4	<i>Flavobacterium alkaliphilum</i>	MN310902.1	93%	93%
	Uncultured bacterium	KM221316.1	99%	94%
	<i>Stenotrophomonas maltophilia</i>	EF620472.1	99%	94%
	<i>Stenotrophomonas indicatrix</i>	MZ823476.1	98%	94%
	<i>Pseudomonas nitroreducens</i>	KJ002756.1	98%	94%
	<i>Vibrio</i> sp.	DQ146981.1	98%	94%
	<i>Xanthomonas retroflexus</i>	KT932957.1	98%	94%
	<i>Pseudomonas fluorescens</i>	KY495220.1	98%	93%
	<i>Curtobacterium</i> sp.	MH558366.1	98%	93%
H7-5	<i>Stenotrophomonas geniculata</i>	KJ147086.1	98%	94%
	<i>Acetobacter pasteurianus</i>	FM178869.1	97%	94%
	<i>Myroides odoratimimus</i>	EU373415.1	99%	94%
	Uncultured bacterium	JX457156.1	99%	94%
	<i>Myroides profundus</i>	KF730743.1	99%	94%
	<i>Myroides gitamensis</i>	NR_125560.1	99%	92%
	<i>Myroides phaeus</i>	KU682197.1	96%	92%
	<i>Myroides marinus</i>	MN216276.1	96%	89%
	<i>Erythrobacter</i> sp.	MF359324.1	94%	88%
H7-6	<i>Flavobacterium tibetense</i>	MF511809.1	91%	94%
	<i>Cytophaga</i> sp.	X85210.1	91%	93%
	<i>Flavobacterium alkaliphilum</i>	MN310902.1	91%	94%
	<i>Myroides profundus</i>	KF730743.1	99%	94%
	<i>Myroides odoratimimus</i>	EU373415.1	98%	94%
	<i>Myroides gitamensis</i>	NR_125560.1	99%	92%
	Uncultured bacterium	GQ443093.1	98%	93%
	<i>Myroides marinus</i>	KT260532.1	97%	91%
	<i>Myroides phaeus</i>	NR_117475.1	90%	94%
H10-1	<i>Myroides pelagicus</i>	NR_114110.1	97%	92%
	<i>Myroides injeensis</i>	NR_134774.1	97%	91%
	<i>Erythrobacter</i> sp.	MF359324.1	97%	88%
	<i>Flavobacterium gelidilacus</i>	EU000234.1	94%	94%
	Uncultured bacterium	EU137419.1	97%	93%
	<i>Snodgrassella alvi</i>	OU943324.1	95%	94%
	<i>Kingella oralis</i>	KM225757.1	94%	94%
	<i>Kingella negevensis</i>	NR_157664.1	94%	94%

	<i>Kingella kingae</i>	AY551998.1	94%	93%
	<i>Stenoxymbacter acetivorans</i>	EF212916.1	94%	94%
	<i>Neisseria sicca</i>	MF480368.1	93%	94%
	<i>Neisseria perflava</i>	NR_117694.1	93%	94%
	<i>Neisseria meningitidis</i>	FJ932762.1	93%	94%
	<i>Neisseria subflava</i>	MF480374.1	93%	94%
H10-4	<i>Myroides odoratimimus</i>	EU373415.1	99%	94%
	Uncultured bacterium	JX457156.1	99%	94%
	<i>Myroides profundus</i>	KF730743.1	99%	94%
	<i>Myroides gitamensis</i>	NR_125560.1	99%	92%
	<i>Myroides phaeus</i>	NR_117475.1	96%	94%
	<i>Erythrobacter</i> sp.	MF359324.1	94%	88%
	<i>Flavobacterium tibetense</i>	MF511809.1	91%	94%
	<i>Flavobacterium ardleyense</i>	NR_158093.1	91%	94%
	<i>Cytophaga</i> sp.	X85210.1	91%	93%
	<i>Flavobacterium alkaliphilum</i>	MN310902.1	91%	94%
H10-5	<i>Myroides odoratimimus</i>	EU373415.1	99%	94%
	Uncultured bacterium	JX457156.1	99%	94%
	<i>Myroides profundus</i>	KF730743.1	99%	94%
	<i>Myroides gitamensis</i>	NR_125560.1	99%	92%
	<i>Myroides phaeus</i>	NR_117475.1	96%	94%
	<i>Myroides marinus</i>	MN216276.1	96%	89%
	<i>Erythrobacter</i> sp.	MF359324.1	94%	88%
	<i>Flavobacterium tibetense</i>	MF511809.1	91%	94%
	<i>Flavobacterium ardleyense</i>	NR_158093.1	91%	94%
	<i>Flavobacterium alkaliphilum</i>	MN310902.1	91%	94%
H10-6	<i>Myroides profundus</i>	KF730743.1	99%	94%
	<i>Myroides odoratimimus</i>	EU373415.1	99%	94%
	Uncultured bacterium	JX457156.1	98%	94%
	<i>Myroides gitamensis</i>	NR_125560.1	98%	92%
	<i>Myroides phaeus</i>	NR_117475.1	95%	94%
	<i>Myroides marinus</i>	MN216276.1	97%	89%
	<i>Erythrobacter</i> sp.	MF359324.1	94%	88%
	<i>Flavobacterium ardleyense</i>	NR_158093.1	91%	94%
	<i>Cytophaga</i> sp.	X85210.1	91%	93%
	<i>Flavobacterium columnare</i>	KR080246.1	91%	94%
H10-7	<i>Myroides odoratimimus</i>	EU373415.1	99%	94%
	Uncultured bacterium	JX457156.1	99%	94%
	<i>Myroides profundus</i>	KF730743.1	99%	94%
	<i>Myroides gitamensis</i>	NR_125560.1	99%	92%
	<i>Myroides phaeus</i>	NR_117475.1	96%	94%
	<i>Erythrobacter</i> sp.	MF359324.1	94%	88%
	<i>Flavobacterium tibetense</i>	MF511809.1	91%	94%
	<i>Flavobacterium ardleyense</i>	NR_158093.1	91%	94%
	<i>Cytophaga</i> sp.	X85210.1	91%	93%
	<i>Flavobacterium alkaliphilum</i>	MN310902.1	91%	94%
H10-8	<i>Myroides profundus</i>	KF730743.1	99%	94%
	<i>Myroides odoratimimus</i>	EU373415.1	99%	94%
	Uncultured bacterium	JX457156.1	98%	94%
	<i>Myroides gitamensis</i>	NR_125560.1	99%	92%
	<i>Myroides phaeus</i>	NR_117475.1	95%	94%
	<i>Myroides marinus</i>	MN216276.1	96%	89%
	<i>Erythrobacter</i> sp.	MF359324.1	94%	88%

	<i>Flavobacterium ardleyense</i>	NR_158093.1	91%	94%
	<i>Cytophaga</i> sp.	X85210.1	91%	93%
	<i>Flavobacterium tibetense</i>	MF511809.1	91%	94%
O1-1	Uncultured bacterium	HM264061.1	99%	100%
	<i>Duganella zoogloeoides</i>	AB495150.1	99%	100%
	<i>Duganella phyllosphaerae</i>	MT760182.1	99%	100%
	<i>Burkholderia</i> sp.	KY623377.1	99%	100%
	<i>Zoogloea ramigera</i>	AY039831.1	98%	100%
	<i>Rugamonas rivuli</i>	MN548382.1	98%	100%
	<i>Rugamonas rubra</i>	MK071274.1	98%	100%
	<i>Janthinobacterium svalbardensis</i>	ON693778.1	97%	99%
	<i>Rugamonas aquatica</i>	MK968139.2	97%	100%
	<i>Massilia</i> sp.	KY302128.1	97%	100%
O1-4	<i>Rhizobium huautlense</i>	LT978436.1	99%	100%
	<i>Agrobacterium</i> sp.	MF524440.1	99%	100%
	Uncultured bacterium	AB636931.1	99%	100%
	<i>Neorhizobium huautlense</i>	LM994797.1	99%	100%
	<i>Rhizobium panacihumi</i>	OM971566.1	98%	99%
	<i>Actinokineospora cibodasensis</i>	KP995131.1	98%	99%
	<i>Rhizobium smilacinae</i>	NR_148270.1	98%	99%
	<i>Pseudorhizobium halotolerans</i>	MZ276327.1	98%	99%
	<i>Pseudorhizobium flavum</i>	NR_133843.1	98%	99%
	<i>Pseudorhizobium endolithicum</i>	MK542974.1	97%	99%
O1-5	<i>Novosphingobium clariflavum</i>	MN629069.1	100%	100%
	<i>Novosphingobium resinovorum</i>	MN629068.1	99%	100%
	Uncultured bacterium	HM299709.1	99%	100%
	<i>Sphingomonas</i> sp.	EU827494.1	99%	100%
	<i>Sphingobium herbicidovorans</i>	MW237806.1	99%	100%
	<i>Novosphingobium naphthalenivorans</i>	MT299725.1	99%	99%
	<i>Enterobacter ludwigii</i>	KM019826.1	99%	99%
	<i>Altererythrobacter</i> sp.	EU440971.1	97%	99%
	<i>Blastomonas fulva</i>	KM670032.2	97%	99%
	<i>Blastomonas aquatica</i>	KJ528316.1	97%	99%
O1-6	<i>Duganella zoogloeoides</i>	ON614201.1	100%	100%
	Uncultured bacterium	KF188985.1	99%	100%
	<i>Burkholderia</i> sp.	KX928272.1	98%	100%
	<i>Rugamonas rivuli</i>	MN548382.1	98%	100%
	<i>Duganella phyllosphaerae</i>	MN421180.1	98%	100%
	<i>Zoogloea ramigera</i>	AY039831.1	98%	100%
	<i>Rugamonas rubra</i>	MK071274.1	98%	100%
	<i>Rugamonas aquatica</i>	MK968139.2	98%	100%
	<i>Janthinobacterium svalbardensis</i>	ON693778.1	97%	100%
O1-11	<i>Klenkia marina</i>	LT746188.1	98%	95%
	<i>Klenkia brasiliensis</i>	NR_126197.1	98%	93%
	<i>Klenkia terrae</i>	NR_109441.1	99%	88%
	<i>Klenkia taihuensis</i>	NR_109679.1	97%	91%
	<i>Klenkia soli</i>	NR_109440.1	98%	88%
	<i>Geodermatophilus nigrescens</i>	NR_109505.1	95%	94%
	<i>Geodermatophilus obscurus</i>	L40620.1	95%	93%
	<i>Geodermatophilus chilensis</i>	KX943330.2	96%	93%
	<i>Blastococcus aggregatus</i>	LN880080.1	95%	94%
	<i>Geodermatophilus tzadiensis</i>	HE654549.1	95%	93%
	<i>Geodermatophilus amargosae</i>	NR_126209.1	95%	93%

O1-12	Uncultured bacterium	KF843005.1	98%	94%
	<i>Bacillus</i> sp.	ON597424.1	98%	94%
	Uncultured <i>Trichodesmium</i> sp.	KM873113.1	98%	94%
	Uncultured actinobacterium	DQ431885.1	98%	94%
	Uncultured Cyanobacterium sp.	LN833508.1	98%	94%
	Uncultured <i>Oscillatoria</i> sp.	EU341185.1	98%	93%
	Sugarcane phytoplasma	KJ650039.1	96%	94%
	<i>Planktothricoides</i> sp.	JX912374.1	98%	86%
	Bacterium endosymbiont of <i>Stereum hirsutum</i>	MG954110.1	98%	87%
	<i>Loriellopsis cavernicola</i>	KY753239.1	96%	91%
O1-14	<i>Staphylococcus warneri</i>	MN512291.1	98%	94%
	<i>Staphylococcus pasteurii</i>	MG255966.1	98%	94%
	Uncultured bacterium	KF494748.1	98%	94%
	<i>Staphylococcus devriesei</i>	OL818273.1	98%	94%
	<i>Streptococcus</i> sp.	KF733696.1	98%	92%
	<i>Mammaliicoccus sciuri</i>	MW175739.1	98%	92%
	<i>Geobacillus stearothermophilus</i>	EU382221.1	97%	94%
	<i>Lactobacillus</i> sp.	MG938658.1	97%	91%
	<i>Psychrobacter pulmonis</i>	MG996850.1	98%	89%
	<i>Enterococcus faecium</i>	MF429322.1	98%	88%
O2-1	Uncultured Bacteroidetes bacterium	EF221152.1	95%	100%
	Uncultured bacterium	JX255231.1	95%	100%
	<i>Chitinophaga pinensis</i>	KF228163.1	91%	100%
	<i>Flavipsychrobacter stenotrophus</i>	NR_171478.1	90%	100%
	<i>Nemorincola caseinilytica</i>	NR_159327.1	90%	100%
	<i>Lacibacter cauensis</i>	KF228159.1	89%	100%
	<i>Edaphobaculum flavum</i>	NR_159123.1	89%	100%
	<i>Rurimicrobium arvi</i>	KY233201.1	93%	81%
	<i>Taibaiella coffeisolii</i>	NR_148840.1	88%	97%
	<i>Chitinophaga polysaccharea</i>	NR_125662.1	87%	99%
	<i>Chitinophaga oryzae</i>	KJ734873.1	87%	99%
O2-2	Uncultured bacterium	KJ123785.1	99%	100%
	<i>Staphylococcus</i> sp.	LN876462.1	99%	100%
	<i>Sphingomonas segetis</i>	NR_175421.1	98%	100%
	<i>Rhizorhabdus wittichii</i>	MK696354.1	98%	99%
	<i>Sphingomonas agri</i>	NR_159163.1	98%	99%
	<i>Sphingomonas sediminicola</i>	KY228985.1	98%	99%
	<i>Sphingomonas jaspsi</i>	KX982825.1	98%	99%
	<i>Sphingomonas lutea</i>	NR_153746.1	98%	99%
	<i>Sphingomonas ginkgonis</i>	MG843843.1	97%	99%
	<i>Sphingomonas palustris</i>	NR_157774.1	97%	99%
O2-3	Uncultured bacterium	KU514522.1	99%	100%
	<i>Janthinobacterium lividum</i>	EU275366.1	99%	100%
	<i>Janthinobacterium aquaticum</i>	NR_170539.1	99%	100%
	<i>Aquaspirillum arcticum</i>	JX475101.1	99%	100%
	<i>Duganella</i> sp.	EF111225.1	99%	100%
	<i>Janthinobacterium svalbardensis</i>	ON693761.1	99%	100%
	<i>Oceanobacillus</i> sp.	KC921006.1	99%	96%
	<i>Massilia plicata</i>	JX475102.1	97%	100%
	<i>Massilia violaceinigra</i>	OK090593.1	98%	97%
	<i>Psychrobacter pulmonis</i>	KY783398.1	97%	99%
O2-4	Uncultured bacterium	EU536499.1	99%	100%
	<i>Methylobacterium adhaesivum</i>	AB698707.1	98%	100%

	<i>Methylobacterium gossypicola</i>	MW237792.1	98%	100%
	<i>Methylobacterium goesingense</i>	MF796690.1	98%	100%
	<i>Sphingomonas</i> sp.	KJ016142.1	98%	100%
	<i>Methylobacterium extorquens</i>	LT594351.1	96%	99%
	<i>Methylobacterium pseudosasaiae</i>	MG190781.1	96%	100%
	<i>Streptomyces cuspidosporus</i>	MG190765.1	96%	100%
	<i>Actinomadura geliboluensis</i>	MG190673.1	96%	100%
	<i>Methylobacterium aminovorans</i>	MW564213.1	96%	100%
O2-5	<i>Novosphingobium clariflavum</i>	MN629069.1	99%	100%
	Uncultured bacterium	AB294312.1	99%	100%
	<i>Novosphingobium resinovorum</i>	MN629068.1	99%	100%
	<i>Sphingomonas</i> sp.	MF289199.1	99%	100%
	<i>Sphingobium herbicidovorans</i>	MW237806.1	99%	100%
	<i>Novosphingobium naphthalenivorans</i>	MT299725.1	99%	100%
	<i>Novosphingobium barchaimii</i>	MN421331.1	99%	100%
	<i>Novosphingobium clariflavum</i>	LC618512.1	99%	100%
	<i>Enterobacter ludwigii</i>	KM019826.1	98%	99%
	<i>Sphingomonas bosoensis</i>	AB275605.1	98%	99%
O8-1	Uncultured bacterium	FM873225.1	96%	94%
	<i>Lichenicoccus roseus</i>	MN811021.1	93%	93%
	<i>Lichenicola cladoniae</i>	MW507622.1	93%	91%
	<i>Gluconacetobacter liquefaciens</i>	ON746603.1	92%	94%
	<i>Gluconacetobacter sacchari</i>	LC108744.1	92%	94%
	<i>Asaia spathodeae</i>	MT661486.1	92%	94%
	<i>Asaia siamensis</i>	MT661483.1	92%	94%
	<i>Acidisoma sibirica</i>	KF241161.1	92%	93%
	<i>Asaia platycodi</i>	JF514557.1	92%	93%
	<i>Acidiphilium iwataense</i>	AB561883.1	92%	94%
O8-2	Uncultured bacterium	KX507758.1	98%	93%
	<i>Alkanindiges illinoisensis</i>	NR_025254.1	97%	94%
	<i>Acinetobacter wuhouensis</i>	MK511823.1	95%	94%
	<i>Acinetobacter guillouiae</i>	OK083429.1	95%	94%
	<i>Alkanindiges hongkongensis</i>	NR_115179.1	97%	87%
	<i>Acinetobacter lwoffii</i>	KJ531441.1	95%	93%
	<i>Acinetobacter bereziniae</i>	NR_117625.1	94%	94%
	<i>Acinetobacter chinensis</i>	NR_165666.1	94%	94%
	<i>Acinetobacter sichuanensis</i>	NR_171416.1	94%	94%
	<i>Acinetobacter junii</i>	MH021658.1	94%	94%
O8-3	Uncultured bacterium	OL952481.1	98%	95%
	<i>Mycoplana</i> sp.	FJ449647.1	98%	94%
	<i>Sphingomonas aerolata</i>	JF970599.1	98%	94%
	<i>Sphingomonas faeni</i>	FR682703.1	98%	94%
	<i>Pseudomonas</i> sp.	KR856235.1	97%	91%
	<i>Bacillus velezensis</i>	KR708865.1	97%	89%
	Bacterium endosymbiont of <i>Curculio robustus</i>	AB746430.1	95%	91%
	<i>Afipia genosp</i>	U87782.1	94%	94%
	<i>Asticcacaulis excentricus</i>	AJ007800.1	94%	92%
	<i>Polymorphobacter multimanifer</i>	NR_125454.1	93%	94%
O8-4	Uncultured bacterium	JQ460737.1	99%	94%
	<i>Streptococcus mitis</i>	JN713499.1	99%	94%
	<i>Streptococcus oralis</i>	NR_117719.1	98%	94%
	<i>Streptococcus dentisani</i>	HG315102.1	98%	94%
	<i>Streptococcus pneumoniae</i>	OM368578.1	98%	94%

	<i>Streptococcus downii</i>	MF443455.1	98%	92%
	<i>Endosymbiont of Sphenophorus levis</i>	FJ626265.1	98%	87%
	<i>Okadaella gastrococcus</i>	KU240440.1	94%	94%
	<i>Enterococcus faecium</i>	AB232954.1	94%	94%
	<i>Staphylococcus warneri</i>	KC213938.1	94%	92%
O8-5	Uncultured bacterium	AB494281.1	93%	92%
	<i>Thalassospira profundimaris</i>	EU440802.1	83%	92%
	<i>Tistrella mobilis</i>	HQ425695.2	83%	93%
	<i>Thalassospira xiamenensis</i>	KY457421.1	83%	92%
	<i>Thalassospira marina</i>	NR_164633.1	83%	92%
	<i>Thalassospira australica</i>	NR_146708.2	83%	92%
	<i>Thalassocola ureilytica</i>	NR_145648.1	83%	93%
	<i>Tistrella bauzanensis</i>	NR_117256.1	83%	93%
	<i>Thalassospira indica</i>	NR_153721.1	83%	92%
	<i>Thalassospira permensis</i>	KP645211.1	83%	92%
O8-6	Uncultured bacterium	KU514497.1	97%	94%
	<i>Hymenobacter ginkgonis</i>	MN595016.1	97%	92%
	<i>Hymenobacter bucti</i>	KX098333.1	95%	94%
	<i>Hymenobacter rivuli</i>	NR_163628.1	95%	92%
	<i>Hymenobacter polaris</i>	MN784633.1	95%	92%
	<i>Hymenobacter setariae</i>	MH549147.2	95%	94%
	<i>Hymenobacter baengnokdamensis</i>	MK752551.1	95%	92%
	<i>Siccationidurans metalli</i>	NR_108905.1	94%	92%
	<i>Hymenobacter marinus</i>	NR_149279.1	95%	91%
	<i>Hymenobacter pomorum</i>	NR_165754.1	94%	93%
O8-9	<i>Pelomicrobium methylotrophicum</i>	MN269950.1	99%	94%
	Uncultured bacterium	MW686473.1	99%	93%
	<i>Azonexus</i> sp.	MT677895.1	90%	94%
	<i>Dechloromonas</i> sp.	MN640570.1	90%	93%
	<i>Hydrogenophilus halorhabdus</i>	EF368016.1	90%	93%
	<i>Propionivibrio militaris</i>	AY530552.1	90%	94%
	<i>Propionivibrio dicarboxylicus</i>	NR_026477.1	90%	94%
	<i>Denitromonas indolicum</i>	AY972852.1	90%	93%
	<i>Denitromonas aromaticus</i>	AB049763.1	90%	93%
	<i>Denitratisoma oestradiolicum</i>	KF810116.1	90%	93%
O8-10	Uncultured bacterium	JQ460737.1	99%	94%
	<i>Streptococcus mitis</i>	JN713499.1	99%	94%
	<i>Streptococcus oralis</i>	NR_117719.1	99%	94%
	<i>Streptococcus dentisani</i>	HG315102.1	98%	94%
	<i>Streptococcus pneumoniae</i>	OM368578.1	98%	94%
	<i>Streptococcus downii</i>	NR_175455.1	99%	92%
	<i>Endosymbiont of Sphenophorus levis</i>	FJ626265.1	99%	87%
	<i>Okadaella gastrococcus</i>	KU240440.1	94%	94%
	<i>Enterococcus faecium</i>	AB232954.1	94%	94%
	<i>Staphylococcus warneri</i>	KC213938.1	95%	92%
O9-2	Uncultured bacterium	KC541035.1	99%	94%
	<i>Acinetobacter lwoffii</i>	MW433856.1	99%	93%
	<i>Prolinoborus fasciculus</i>	KR085813.1	99%	94%
	Bacterium endosymbiont of <i>Onthophagus Taurus</i>	KF193151.1	98%	93%
	<i>Bacillus methylotrophicus</i>	KF818634.1	99%	90%
	<i>Pseudomonas</i> sp.	KC433626.1	98%	92%
	<i>Bacillus weihenstephanensis</i>	JF935054.1	98%	90%

Table 1 Species of bacteria identified based on sequence matches with sequences in the BLAST bacterial database. H indicates *Harmonia axyridis* and O indicates *Halysia sedecimguttata* followed by the sample number and clone identity. Organism written in green are identified as a closest match within 2 or more individuals from the ladybird species and those written in orange are identified as a closest match in at least one individual of the two ladybird species.

Lineage	stage	PCR +/-	Sequence trace	Closest match	Per. Ident
14	Male	+	Mixed	Uncultured bacterium LC349021.1	94%
14	Female	+	Mixed	Uncultured bacterium LC349021.1	96%
14	Eggs	+	one sequence detected	<i>Limosilactobacillus fermentum</i> MF424753.1	96%
				Uncultured bacterium KX603597.1	96%
				Sugarcane phytoplasma KJ650039.1	96%
				Uncultured Xanthomonadales bacterium EU404026.1	96%
				endosymbiont of <i>Stereum hirsutum</i> MG954110.1	93%
				symbiont of <i>Heliothrips haemorrhoidalis</i> KM582850.1	93%
				Uncultured Cyanobacterium LN833508.1	93%
				Uncultured <i>Trichodesmium</i> KM873113.1	93%
				Uncultured <i>Oscillatoria</i> sp. EU341185.1	93%
				Uncultured actinobacterium DQ431885.1	93%
				<i>Bacillus</i> sp. ON597424.1	93%
				<i>Loriellopsis cavernicola</i> KY753239.1	92%
14	Larvae	+	one sequence detected	Uncultured bacterium	99%

				KX022163.1	
				Uncultured Cyanobacterium LN833508.1	99%
				Uncultured actinobacterium DQ431885.1	98%
				endosymbiont of <i>Stereum hirsutum</i> MG954110.1	98%
				symbiont of <i>Heliothrips haemorrhoidalis</i> KM582850.1	98%
				Uncultured <i>Trichodesmium</i> KM873113.1	98%
				<i>Bacillus</i> sp. ON597424.1	98%
				Uncultured <i>Oscillatoria</i> sp. EU341185.1	98%
				<i>Loriellopsis cavernicola</i> KY753239.1	97%
				Sugarcane phytoplasma KJ650039.1	95%
				<i>Limosilactobacillus fermentum</i> MF424753.1	95%
14	Offspring 1	+	one sequence detected	Sugarcane phytoplasma KJ650039.1	91%
				Uncultured bacterium LC349021.1	91%
				Uncultured Xanthomonadales bacterium EU404026.1	91%
				<i>Limosilactobacillus fermentum</i> MF424753.1	91%
				symbiont of <i>Heliothrips haemorrhoidalis</i> KM582850.1	89%
				endosymbiont of <i>Stereum hirsutum</i> MG954110.1	88%
				Uncultured Cyanobacterium LN833508.1	88%

				Uncultured <i>Trichodesmium</i> KM873113.1	88%
				Uncultured <i>Oscillatoria</i> sp. EU341185.1	88%
				Uncultured actinobacterium DQ431885.1	88%
				<i>Bacillus</i> sp. ON597424.1	88%
				<i>Loriellopsis cavernicola</i> KY753239.1	88%
14	Offspring 2	+	one sequence detected	Sugarcane phytoplasma KJ650039.1	99%
				Uncultured bacterium LC349021.1	99%
				Uncultured Xanthomonadales bacterium EU404026.1	99%
				<i>Limosilactobacillus fermentum</i> MF424753.1	98%
				symbiont of <i>Heliothrips haemorrhoidalis</i> KM582850.1	96%
				Uncultured Cyanobacterium LN833508.1	95%
				Uncultured <i>Oscillatoria</i> sp. EU341185.1	95%
				endosymbiont of <i>Stereum hirsutum</i> MG954110.1	95%
				Uncultured <i>Trichodesmium</i> KM873113.1	95%
				Uncultured actinobacterium DQ431885.1	95%
				<i>Bacillus</i> sp. ON597424.1	95%
				<i>Loriellopsis cavernicola</i> KY753239.1	94%
14	Offspring 3	+	one sequence detected	Uncultured bacterium LC349021.1	98%

				Sugarcane phytoplasma KJ650039.1	98%
				Uncultured Xanthomonadales bacterium EU404026.1	98%
				<i>Limosilactobacillus fermentum</i> MF424753.1	98%
				endosymbiont of <i>Stereum hirsutum</i> MG954110.1	95%
				symbiont of <i>Heliothrips haemorrhoidalis</i> KM582850.1	95%
				Uncultured Cyanobacterium LN833508.1	95%
				Uncultured <i>Trichodesmium</i> KM873113.1	95%
				Uncultured <i>Oscillatoria</i> sp. EU341185.1	95%
				<i>Bacillus</i> sp. ON597424.1	95%
				<i>Loriellopsis cavernicola</i> KY753239.1	94%
14	Offspring 4	+	Mixed	Uncultured bacterium LC349021.1	94%
14	Offspring 5	+	one sequence detected	Sugarcane phytoplasma KJ650039.1	97%
				Uncultured bacterium LC349021.1	97%
				Uncultured Xanthomonadales bacterium EU404026.1	97%
				<i>Limosilactobacillus fermentum</i> MF424753.1	96%
				endosymbiont of <i>Stereum hirsutum</i> MG954110.1	93%
				symbiont of <i>Heliothrips haemorrhoidalis</i> KM582850.1	93%

				Uncultured Cyanobacterium LN833508.1	93%
				Uncultured <i>Trichodesmium</i> KM873113.1	93%
				Uncultured <i>Oscillatoria</i> sp. EU341185.1	93%
				Uncultured actinobacterium DQ431885.1	93%
				<i>Loriellopsis cavernicola</i> KY753239.1	92%
15	Male	+	one sequence detected	Uncultured bacterium LC349021.1	96%
				<i>Limosilactobacillus fermentum</i> MF424753.1	96%
				Sugarcane phytoplasma KJ650039.1	96%
				Uncultured Xanthomonadales bacterium EU404026.1	96%
				endosymbiont of <i>Stereum hirsutum</i> MG954110.1	93%
				symbiont of <i>Heliothrips haemorrhoidalis</i> KM582850.1	93%
				Uncultured Cyanobacterium LN833508.1	93%
				Uncultured <i>Trichodesmium</i> KM873113.1	93%
				Uncultured <i>Oscillatoria</i> sp. EU341185.1	93%
				Uncultured actinobacterium DQ431885.1	93%
				<i>Bacillus</i> sp. ON597424.1	93%
				<i>Loriellopsis cavernicola</i> KY753239.1	92%
15	Female	+	one sequence detected	Uncultured bacterium LC349021.1	96%

				<i>Limosilactobacillus fermentum</i> MF424753.1	96%
				Sugarcane phytoplasma KJ650039.1	96%
				Uncultured Xanthomonadales bacterium EU404026.1	96%
				endosymbiont of <i>Stereum hirsutum</i> MG954110.1	93%
				symbiont of <i>Heliothrips haemorrhoidalis</i> KM582850.1	93%
				Uncultured Cyanobacterium LN833508.1	93%
				Uncultured <i>Trichodesmium</i> KM873113.1	93%
				Uncultured <i>Oscillatoria</i> sp. EU341185.1	93%
				Uncultured actinobacterium DQ431885.1	93%
				<i>Bacillus</i> sp. ON597424.1	93%
				<i>Loriellopsis cavernicola</i> KY753239.1	92%
15	Eggs	+	one sequence detected	Uncultured bacterium LC349021.1	96%
				<i>Limosilactobacillus fermentum</i> MF424753.1	96%
				Sugarcane phytoplasma KJ650039.1	96%
				Uncultured Xanthomonadales bacterium EU404026.1	96%
				endosymbiont of <i>Stereum hirsutum</i> MG954110.1	93%
				symbiont of <i>Heliothrips haemorrhoidalis</i> KM582850.1	93%

				Uncultured Cyanobacterium LN833508.1	93%
				Uncultured <i>Trichodesmium</i> KM873113.1	93%
				Uncultured <i>Oscillatoria</i> sp. EU341185.1	93%
				Uncultured actinobacterium DQ431885.1	93%
				<i>Bacillus</i> sp. ON597424.1	93%
				<i>Loriellopsis cavernicola</i> KY753239.1	92%
15	Larvae	+	mixed	Uncultured bacterium LC349021.1	89%
15	Offspring 1	+	Mixed	Uncultured bacterium LC349021.1	97%
15	Offspring 2	+	one sequence detected	Uncultured bacterium LC349021.1	96%
				<i>Limosilactobacillus fermentum</i> MF424753.1	96%
				Sugarcane phytoplasma KJ650039.1	96%
				Uncultured Xanthomonadales bacterium EU404026.1	96%
				endosymbiont of <i>Stereum hirsutum</i> MG954110.1	93%
				symbiont of <i>Heliothrips haemorrhoidalis</i> KM582850.1	93%
				Uncultured Cyanobacterium LN833508.1	93%
				Uncultured <i>Trichodesmium</i> KM873113.1	93%
				Uncultured <i>Oscillatoria</i> sp. EU341185.1	93%

				Uncultured actinobacterium DQ431885.1	93%
				<i>Bacillus</i> sp. ON597424.1	93%
				<i>Loriellopsis cavernicola</i> KY753239.1	92%
15	Offspring 3	+	Mixed	Uncultured bacterium KX022163.1	81%
15	Offspring 4	+	Mixed	No significant similarity found	
17	Male	+	one sequence detected	Uncultured bacterium LC349021.1	99%
				Sugarcane phytoplasma KJ650039.1	99%
				Uncultured Xanthomonadales bacterium EU404026.1	99%
				<i>Limosilactobacillus fermentum</i> MF424753.1	99%
				endosymbiont of <i>Stereum hirsutum</i> MG954110.1	96%
				symbiont of <i>Heliothrips haemorrhoidalis</i> KM582850.1	96%
				Uncultured Cyanobacterium LN833508.1	96%
				Uncultured <i>Trichodesmium</i> KM873113.1	96%
				Uncultured <i>Oscillatoria</i> sp. EU341185.1	96%
				Uncultured actinobacterium DQ431885.1	96%
				<i>Loriellopsis cavernicola</i> KY753239.1	95%
17	Female	+	Mixed	Uncultured bacterium LC349021.1	96%
17	Eggs	+	Mixed	No significant similarity found	
17	Larvae	+		Uncultured bacterium	100%

			one sequence detected but short	KX022163.1	
				Uncultured Cyanobacterium LN833508.1	100%
				<i>Bacillus</i> sp. ON597424.1	100%
				Uncultured actinobacterium DQ431885.1	100%
				Uncultured <i>Oscillatoria</i> sp. EU341185.1	100%
				symbiont of <i>Heliothrips haemorrhoidalis</i> KM582850.1	99%
				endosymbiont of <i>Stereum hirsutum</i> MG954110.1	99%
				Sugarcane phytoplasma KJ650039.1	98%
				<i>Limosilactobacillus fermentum</i> MF424753.1	97%
				<i>Loriellopsis cavernicola</i> KY753239.1	97%
17	Offspring 1	+	one sequence detected	Sugarcane phytoplasma KJ650039.1	94%
				Uncultured bacterium LC349021.1	94%
				Uncultured Xanthomonadales bacterium EU404026.1	94%
				<i>Limosilactobacillus fermentum</i> MF424753.1	94%
				endosymbiont of <i>Stereum hirsutum</i> MG954110.1	91%
				symbiont of <i>Heliothrips haemorrhoidalis</i> KM582850.1	91%
				Uncultured Cyanobacterium LN833508.1	91%
				Uncultured <i>Trichodesmium</i> KM873113.1	91%

				Uncultured <i>Oscillatoria</i> sp. EU341185.1	91%
				Uncultured actinobacterium DQ431885.1	91%
				<i>Bacillus</i> sp. ON597424.1	91%
				<i>Loriellopsis cavernicola</i> KY753239.1	90%
17	Offspring 2	+	one sequence detected	Sugarcane phytoplasma KJ650039.1	98%
				Uncultured bacterium LC349021.1	98%
				Uncultured Xanthomonadales bacterium EU404026.1	98%
				<i>Limosilactobacillus fermentum</i> MF424753.1	98%
				endosymbiont of <i>Stereum hirsutum</i> MG954110.1	95%
				Uncultured Cyanobacterium LN833508.1	95%
				Uncultured <i>Trichodesmium</i> KM873113.1	95%
				Uncultured <i>Oscillatoria</i> sp. EU341185.1	95%
				Uncultured actinobacterium DQ431885.1	95%
				symbiont of <i>Heliothrips haemorrhoidalis</i> KM582850.1	95%
				<i>Bacillus</i> sp. ON597424.1	95%
				<i>Loriellopsis cavernicola</i> KY753239.1	94%
17	Offspring 3	+	one sequence detected	<i>Limosilactobacillus fermentum</i> MF424753.1	96%
				Sugarcane phytoplasma KJ650039.1	96%

				Uncultured bacterium LC349021.1	96%
				Uncultured Xanthomonadales bacterium EU404026.1	96%
				endosymbiont of <i>Stereum hirsutum</i> MG954110.1	93%
				symbiont of <i>Heliothrips haemorrhoidalis</i> KM582850.1	93%
				Uncultured Cyanobacterium LN833508.1	93%
				Uncultured <i>Trichodesmium</i> KM873113.1	93%
				Uncultured <i>Oscillatoria</i> sp. EU341185.1	93%
				Uncultured actinobacterium DQ431885.1	93%
				<i>Bacillus</i> sp. ON597424.1	93%
				<i>Loriellopsis cavernicola</i> KY753239.1	92%
17	Offspring 4	+	Mixed	Uncultured bacterium LC349021.1	96%
17	Offspring 5	+	one sequence detected	Uncultured bacterium LC349021.1	96%
				<i>Limosilactobacillus fermentum</i> MF424753.1	96%
				Sugarcane phytoplasma KJ650039.1	96%
				Uncultured Xanthomonadales bacterium EU404026.1	96%
				endosymbiont of <i>Stereum hirsutum</i> MG954110.1	93%
				symbiont of <i>Heliothrips haemorrhoidalis</i> KM582850.1	93%

				Uncultured Cyanobacterium LN833508.1	93%
				Uncultured <i>Trichodesmium</i> KM873113.1	93%
				Uncultured <i>Oscillatoria</i> sp. EU341185.1	93%
				Uncultured actinobacterium DQ431885.1	93%
				<i>Bacillus</i> sp. ON597424.1	93%
				<i>Loriellopsis cavernicola</i> KY753239.1	92%
19	Male	+	Mixed	Uncultured bacterium LC349021.1	93%
19	Female	+	one sequence detected	Uncultured bacterium LC349021.1	96%
				<i>Limosilactobacillus fermentum</i> MF424753.1	96%
				Sugarcane phytoplasma KJ650039.1	96%
				Uncultured Xanthomonadales bacterium EU404026.1	96%
				endosymbiont of <i>Stereum hirsutum</i> MG954110.1	93%
				symbiont of <i>Heliothrips haemorrhoidalis</i> KM582850.1	93%
				Uncultured Cyanobacterium LN833508.1	93%
				Uncultured <i>Trichodesmium</i> KM873113.1	93%
				Uncultured <i>Oscillatoria</i> sp. EU341185.1	93%
				Uncultured actinobacterium DQ431885.1	93%
				<i>Bacillus</i> sp. ON597424.1	93%

				<i>Loriellopsis cavernicola</i> KY753239.1	92%
19	Eggs	+	one sequence detected	Sugarcane phytoplasma KJ650039.1	99%
				Uncultured bacterium LC349021.1	99%
				Uncultured Xanthomonadales bacterium EU404026.1	99%
				<i>Limosilactobacillus fermentum</i> MF424753.1	99%
				endosymbiont of <i>Stereum hirsutum</i> MG954110.1	96%
				symbiont of <i>Heliothrips haemorrhoidalis</i> KM582850.1	96%
				Uncultured Cyanobacterium LN833508.1	96%
				Uncultured <i>Trichodesmium</i> KM873113.1	96%
				Uncultured <i>Oscillatoria</i> sp. EU341185.1	96%
				Uncultured actinobacterium DQ431885.1	96%
				Bacillus sp. ON597424.1	96%
				<i>Loriellopsis cavernicola</i> KY753239.1	95%
19	Larvae	+	one sequence detected but short	Sugarcane phytoplasma KJ650039.1	100%
				Uncultured bacterium LC349021.1	100%
				Uncultured Xanthomonadales bacterium EU404026.1	100%
				<i>Limosilactobacillus fermentum</i> MF424753.1	99%

				Uncultured <i>Trichodesmium</i> KM873113.1	98%
				Uncultured Cyanobacterium LN833508.1	98%
				<i>Bacillus</i> sp. ON597424.1	98%
				Uncultured actinobacterium DQ431885.1	98%
				endosymbiont of <i>Stereum hirsutum</i> MG954110.1	97%
				symbiont of <i>Heliothrips haemorrhoidalis</i> KM582850.1	97%
				Uncultured <i>Oscillatoria</i> sp. EU341185.1	97%
				<i>Loriellopsis cavernicola</i> KY753239.1	96%
19	Offspring	+	one sequence detected	Sugarcane phytoplasma KJ650039.1	98%
				Uncultured bacterium LC349021.1	98%
				Uncultured Xanthomonadales bacterium EU404026.1	98%
				<i>Limosilactobacillus fermentum</i> MF424753.1	98%
				endosymbiont of <i>Stereum hirsutum</i> MG954110.1	96%
				symbiont of <i>Heliothrips haemorrhoidalis</i> KM582850.1	96%
				Uncultured Cyanobacterium LN833508.1	96%
				Uncultured <i>Trichodesmium</i> KM873113.1	96%
				Uncultured <i>Oscillatoria</i> sp. EU341185.1	95%

				Uncultured actinobacterium DQ431885.1	95%
				<i>Bacillus</i> sp. ON597424.1	95%
21	Male	+	one sequence detected	Uncultured bacterium LC349021.1	96%
				<i>Limosilactobacillus fermentum</i> MF424753.1	96%
				Sugarcane phytoplasma KJ650039.1	96%
				Uncultured Xanthomonadales bacterium EU404026.1	96%
				endosymbiont of <i>Stereum hirsutum</i> MG954110.1	93%
				symbiont of <i>Heliothrips haemorrhoidalis</i> KM582850.1	93%
				Uncultured Cyanobacterium LN833508.1	93%
				Uncultured <i>Trichodesmium</i> KM873113.1	93%
				Uncultured <i>Oscillatoria</i> sp. EU341185.1	93%
				Uncultured actinobacterium DQ431885.1	93%
				<i>Bacillus</i> sp. ON597424.1	93%
				<i>Loriellopsis cavernicola</i> KY753239.1	92%
21	Female	+	one sequence detected	Sugarcane phytoplasma KJ650039.1	90%
				Uncultured bacterium LC349021.1	90%
				Uncultured Xanthomonadales bacterium EU404026.1	90%
				<i>Limosilactobacillus fermentum</i>	90%

				MF424753.1	
				symbiont of <i>Heliothrips haemorrhoidalis</i> KM582850.1	88%
				endosymbiont of <i>Stereum hirsutum</i> MG954110.1	88%
				Uncultured Cyanobacterium LN833508.1	88%
				Uncultured <i>Trichodesmium</i> KM873113.1	88%
				Uncultured <i>Oscillatoria</i> sp. EU341185.1	87%
				Uncultured actinobacterium DQ431885.1	87%
				<i>Bacillus</i> sp. ON597424.1	87%
21	Eggs	+	one sequence detected	Uncultured bacterium HM243988.1	99%
				Uncultured actinobacterium DQ431885.1	98%
				Uncultured Cyanobacterium LN833508.1	98%
				Uncultured <i>Trichodesmium</i> KM873113.1	98%
				<i>Bacillus</i> sp. ON597424.1	98%
				Uncultured <i>Oscillatoria</i> sp. EU341185.1	97%
				endosymbiont of <i>Stereum hirsutum</i> MG954110.1	97%
				symbiont of <i>Heliothrips haemorrhoidalis</i> KM582850.1	97%
				Sugarcane phytoplasma KJ650039.1	95%
				Uncultured Xanthomonadales bacterium EU404026.1	95%
				<i>Limosilactobacillus fermentum</i>	95%

				MF424753.1	
21	Larvae	+	Mixed	Uncultured bacterium LC349021.1	97%
21	Offspring 1	+	Mixed	No significant similarity found	
21	Offspring 2	+	one sequence detected	<i>Limosilactobacillus fermentum</i> MF424753.1	96%
				Sugarcane phytoplasma KJ650039.1	96%
				Uncultured bacterium LC349021.1	96%
				Uncultured Xanthomonadales bacterium EU404026.1	96%
				endosymbiont of <i>Stereum hirsutum</i> MG954110.1	93%
				symbiont of <i>Heliothrips haemorrhoidalis</i> KM582850.1	93%
				Uncultured Cyanobacterium LN833508.1	93%
				Uncultured <i>Trichodesmium</i> KM873113.1	93%
				Uncultured <i>Oscillatoria</i> sp. EU341185.1	93%
				Uncultured actinobacterium DQ431885.1	93%
21	Offspring 3	+	one sequence detected	Sugarcane phytoplasma KJ650039.1	99%
				Uncultured bacterium LC349021.1	99%
				Uncultured Xanthomonadales bacterium EU404026.1	99%
				<i>Limosilactobacillus fermentum</i> MF424753.1	98%
				endosymbiont of <i>Stereum hirsutum</i> MG954110.1	96%

				symbiont of <i>Heliothrips haemorrhoidalis</i> KM582850.1	96%
				Uncultured Cyanobacterium LN833508.1	96%
				Uncultured <i>Trichodesmium</i> KM873113.1	96%
				Uncultured <i>Oscillatoria</i> sp. EU341185.1	96%
				Uncultured actinobacterium DQ431885.1	96%
21	Offspring 4	+	Mixed	Uncultured bacterium LC349021.1	93%
21	Offspring 5	-	NA		
23	Female	+	one sequence detected	Uncultured bacterium LC349021.1	96%
				<i>Limosilactobacillus fermentum</i> MF424753.1	96%
				Sugarcane phytoplasma KJ650039.1	96%
				Uncultured Xanthomonadales bacterium EU404026.1	96%
				endosymbiont of <i>Stereum hirsutum</i> MG954110.1	93%
				symbiont of <i>Heliothrips haemorrhoidalis</i> KM582850.1	93%
				Uncultured Cyanobacterium LN833508.1	93%
				Uncultured <i>Trichodesmium</i> KM873113.1	93%
				Uncultured <i>Oscillatoria</i> sp. EU341185.1	93%
				Uncultured actinobacterium DQ431885.1	93%
				<i>Bacillus</i> sp. ON597424.1	93%

				<i>Loriellopsis cavernicola</i> KY753239.1	92%
23	Eggs	+	Mixed	Possible contamination and would be ignored	
23	Larvae	+	one sequence detected	Uncultured bacterium LC349021.1	96%
				<i>Limosilactobacillus fermentum</i> MF424753.1	96%
				Sugarcane phytoplasma KJ650039.1	96%
				Uncultured Xanthomonadales bacterium EU404026.1	96%
				endosymbiont of <i>Stereum hirsutum</i> MG954110.1	93%
				symbiont of <i>Heliothrips haemorrhoidalis</i> KM582850.1	93%
				Uncultured Cyanobacterium LN833508.1	93%
				Uncultured <i>Trichodesmium</i> KM873113.1	93%
				Uncultured <i>Oscillatoria</i> sp. EU341185.1	93%
				Uncultured actinobacterium DQ431885.1	93%
				<i>Bacillus</i> sp. ON597424.1	93%
				<i>Loriellopsis cavernicola</i> KY753239.1	92%
23	4 th instar larva -1	+	one sequence detected	Sugarcane phytoplasma KJ650039.1	97%
				Uncultured bacterium LC349021.1	97%
				Uncultured Xanthomonadales bacterium EU404026.1	97%
				<i>Limosilactobacillus fermentum</i>	97%

				MF424753.1	
				endosymbiont of <i>Stereum hirsutum</i> MG954110.1	94%
				symbiont of <i>Heliothrips haemorrhoidalis</i> KM582850.1	94%
				Uncultured Cyanobacterium LN833508.1	94%
				Uncultured <i>Trichodesmium</i> KM873113.1	94%
				Uncultured <i>Oscillatoria</i> sp. EU341185.1	94%
				Uncultured actinobacterium DQ431885.1	94%
				<i>Bacillus</i> sp. ON597424.1	93%
23	4 th instar larva -2	+	one sequence detected	Sugarcane phytoplasma KJ650039.1	99%
				Uncultured bacterium LC349021.1	99%
				Uncultured Xanthomonadales bacterium EU404026.1	99%
				<i>Limosilactobacillus fermentum</i> MF424753.1	99%
				endosymbiont of <i>Stereum hirsutum</i> MG954110.1	96%
				symbiont of <i>Heliothrips haemorrhoidalis</i> KM582850.1	96%
				Uncultured Cyanobacterium LN833508.1	96%
				Uncultured <i>Trichodesmium</i> KM873113.1	96%
				Uncultured <i>Oscillatoria</i> sp. EU341185.1	96%
				Uncultured actinobacterium DQ431885.1	96%
				<i>Bacillus</i> sp.	96%

				ON597424.1	
23	4th instar larva -3	+	one sequence detected	<i>Limosilactobacillus fermentum</i> MF424753.1	96%
				Sugarcane phytoplasma KJ650039.1	96%
				Uncultured bacterium LC349021.1	96%
				Uncultured Xanthomonadales bacterium EU404026.1	96%
				endosymbiont of <i>Stereum hirsutum</i> MG954110.1	93%
				symbiont of <i>Heliothrips haemorrhoidalis</i> KM582850.1	93%
				Uncultured Cyanobacterium LN833508.1	93%
				Uncultured <i>Trichodesmium</i> KM873113.1	93%
				Uncultured <i>Oscillatoria</i> sp. EU341185.1	93%
				Uncultured actinobacterium DQ431885.1	93%
23	Offspring 1	-	NA		
23	Offspring 2	+	one sequence detected	Uncultured bacterium LC349021.1	96%
				<i>Limosilactobacillus fermentum</i> MF424753.1	96%
				Sugarcane phytoplasma KJ650039.1	96%
				Uncultured Xanthomonadales bacterium EU404026.1	96%
				endosymbiont of <i>Stereum hirsutum</i> MG954110.1	93%
				symbiont of <i>Heliothrips haemorrhoidalis</i> KM582850.1	93%

				Uncultured Cyanobacterium LN833508.1	93%
				Uncultured <i>Trichodesmium</i> KM873113.1	93%
				Uncultured <i>Oscillatoria</i> sp. EU341185.1	93%
				Uncultured actinobacterium DQ431885.1	93%
				<i>Bacillus</i> sp. ON597424.1	93%
				<i>Loriellopsis cavernicola</i> KY753239.1	92%
23	Offspring 3	-	NA		
23	Offspring 4	+	Mixed	Uncultured bacterium LC349021.1	99%
23	Offspring 5	+	one sequence detected	Uncultured bacterium LC349021.1	96%
				<i>Limosilactobacillus fermentum</i> MF424753.1	96%
				Sugarcane phytoplasma KJ650039.1	96%
				Uncultured Xanthomonadales bacterium EU404026.1	96%
				endosymbiont of <i>Stereum hirsutum</i> MG954110.1	93%
				symbiont of <i>Heliothrips haemorrhoidalis</i> KM582850.1	93%
				Uncultured Cyanobacterium LN833508.1	93%
				Uncultured <i>Trichodesmium</i> KM873113.1	93%
				Uncultured <i>Oscillatoria</i> sp. EU341185.1	93%
				Uncultured actinobacterium DQ431885.1	93%
				<i>Bacillus</i> sp. ON597424.1	93%

				<i>Loriellopsis cavernicola</i> KY753239.1	92%
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Table 2 BLAST hits of the sequences obtained from the amplification of DNA samples extracted from *Harmonia axyridis* adults, eggs, larvae and offspring. NA indicates that no sequencing was carried out, due to the lack of PCR product for the sample.

Induvial no.	Body section	PCR +/-	Sequence trace	Closest match	Per. Ident
1	Head	+	one sequence detected	Uncultured bacterium KX022163.1	98%
				Uncultured <i>Oscillatoria</i> sp. EU341185.1	98%
				Uncultured Cyanobacterium LN833508.1	98%
				Uncultured actinobacterium DQ431885.1	98%
				<i>Bacillus</i> sp. ON597424.1	98%
				Uncultured <i>Trichodesmium</i> KM873113.1	98%
				endosymbiont of <i>Stereum hirsutum</i> MG954110.1	98%
				symbiont of <i>Heliothrips haemorrhoidalis</i> KM582850.1	98%
				<i>Loriellopsis cavernicola</i> KY753239.1	96%
				Sugarcane phytoplasma KJ650039.1	96%
1	Abdomen	+	Mixed	Uncultured bacterium KX022163.1	96%
				Uncultured bacterium KX022163.1	98%
				Uncultured <i>Oscillatoria</i> sp. EU341185.1	98%
				Uncultured Cyanobacterium LN833508.1	98%
				<i>Bacillus</i> sp. ON597424.1	98%
				Uncultured actinobacterium DQ431885.1	98%
				Uncultured bacterium KX022163.1	98%
				Uncultured <i>Oscillatoria</i> sp. EU341185.1	98%
				Uncultured Cyanobacterium LN833508.1	98%
				<i>Bacillus</i> sp. ON597424.1	98%
1	Elytra	+	one sequence detected	Uncultured bacterium KX022163.1	98%
				Uncultured <i>Oscillatoria</i> sp. EU341185.1	98%
				Uncultured Cyanobacterium LN833508.1	98%
				<i>Bacillus</i> sp. ON597424.1	98%
				Uncultured actinobacterium DQ431885.1	98%

				endosymbiont of <i>Stereum hirsutum</i> MG954110.1	98%
				symbiont of <i>Heliothrips haemorrhoidalis</i> KM582850.1	98%
				Uncultured <i>Trichodesmium</i> KM873113.1	98%
				<i>Loriellopsis cavernicola</i> KY753239.1	96%
				Sugarcane phytoplasma KJ650039.1	96%
				Uncultured Xanthomonadales bacterium EU404026.1	96%
1	Legs	+	Mixed	Uncultured bacterium LC349021.1	78%
2	Head	+	Mixed	No significant similarity found	
2	Abdomen	+	Mixed	Uncultured bacterium KX022163.1	86%
2	Elytra	-	NA		
2	Legs	-	NA		
3	Head	+	Mixed	No significant similarity found	
3	Abdomen	+	Mixed	No significant similarity found	
3	Elytra	+	Mixed	No significant similarity found	
3	Legs	-	NA		
4	Head	-	NA		
4	Abdomen	+	one sequence detected	<i>Limosilactobacillus fermentum</i> MF424753.1	96%
				Sugarcane phytoplasma KJ650039.1	96%
				Uncultured bacterium LC349021.1	96%
				Uncultured Xanthomonadales bacterium EU404026.1	96%
				endosymbiont of <i>Stereum hirsutum</i> MG954110.1	93%

				symbiont of <i>Heliothrips haemorrhoidalis</i> KM582850.1	93%
				Uncultured Cyanobacterium LN833508.1	93%
				Uncultured <i>Trichodesmium</i> KM873113.1	93%
				Uncultured <i>Oscillatoria</i> sp. EU341185.1	93%
				Uncultured actinobacterium DQ431885.1	93%
				<i>Bacillus</i> sp. ON597424.1	93%
4	Elytra	-	NA		
4	Legs	-	NA		
5	Head	+	Mixed	No significant similarity found	
5	Abdomen	-	NA		
5	Elytra	+	Mixed	Uncultured bacterium LC349021.1	91%
5	Legs	-	NA		
6	Head	+	One sequence detected but short	Uncultured bacterium KX022163.1	99%
				Uncultured Cyanobacterium LN833508.1	99%
				<i>Bacillus</i> sp. ON597424.1	99%
				Uncultured actinobacterium DQ431885.1	99%
				symbiont of <i>Heliothrips haemorrhoidalis</i> KM582850.1	98%
				Uncultured <i>Trichodesmium</i> KM873113.1	98%
				Uncultured <i>Oscillatoria</i> sp. EU341185.1	98%
				endosymbiont of <i>Stereum hirsutum</i> MG954110.1	98%
				<i>Limosilactobacillus fermentum</i>	97%

				MF424753.1	
				<i>Loriellopsis cavernicola</i> KY753239.1	97%
				Sugarcane phytoplasma KJ650039.1	97%
6	Abdomen	+	One short sequence detected	Uncultured Cyanobacterium LN833508.1	99%
				<i>Bacillus</i> sp. ON597424.1	99%
				Uncultured bacterium LC349152.1	99%
				Uncultured actinobacterium DQ431885.1	99%
				symbiont of <i>Heliothrips haemorrhoidalis</i> KM582850.1	98%
				Uncultured <i>Trichodesmium</i> KM873113.1	98%
				Uncultured <i>Oscillatoria</i> sp. EU341185.1	98%
				endosymbiont of <i>Stereum hirsutum</i> MG954110.1	98%
				<i>Limosilactobacillus fermentum</i> MF424753.1	97%
				<i>Loriellopsis cavernicola</i> KY753239.1	97%
6	Elytra	+	One sequence detected but short	No significant similarity found	
6	Legs	+	Mixed	No significant similarity found	
7	Head	+	Mixed	No significant similarity found	
7	Abdomen	+	One sequence detected	Uncultured <i>Oscillatoria</i> sp. EU341185.1	98%
				Uncultured bacterium KX022163.1	98%

				Uncultured Cyanobacterium LN833508.1	98%
				Uncultured actinobacterium DQ431885.1	98%
				<i>Bacillus</i> sp. ON597424.1	98%
				Uncultured <i>Trichodesmium</i> KM873113.1	98%
				endosymbiont of <i>Stereum hirsutum</i> MG954110.1	98%
				symbiont of <i>Heliothrips haemorrhoidalis</i> KM582850.1	98%
				<i>Loriellopsis cavernicola</i> KY753239.1	96%
				Sugarcane phytoplasma KJ650039.1	96%
				Uncultured Xanthomonadales bacterium EU404026.1	96%
7	Elytra	+	One sequence detected	Sugarcane phytoplasma KJ650039.1	100%
				Uncultured bacterium LC349021.1	100%
				Uncultured Xanthomonadales bacterium EU404026.1	100%
				<i>Limosilactobacillus fermentum</i> MF424753.1	99%
				endosymbiont of <i>Stereum hirsutum</i> MG954110.1	97%
				symbiont of <i>Heliothrips haemorrhoidalis</i> KM582850.1	97%
				Uncultured Cyanobacterium LN833508.1	97%
				Uncultured <i>Trichodesmium</i> KM873113.1	97%

				Uncultured <i>Oscillatoria</i> sp. EU341185.1	97%
				Uncultured actinobacterium DQ431885.1	97%
				<i>Bacillus</i> sp. ON597424.1	97%
7	Legs	-	NA		
8	Head	-	NA		
8	Abdomen	+	One sequence detected	Sugarcane phytoplasma KJ650039.1	99%
				Uncultured bacterium LC349021.1	99%
				Uncultured Xanthomonadales bacterium EU404026.1	99%
				<i>Limosilactobacillus fermentum</i> MF424753.1	99%
				endosymbiont of <i>Stereum hirsutum</i> MG954110.1	96%
				symbiont of <i>Heliothrips haemorrhoidalis</i> KM582850.1	96%
				Uncultured Cyanobacterium LN833508.1	96%
				Uncultured <i>Trichodesmium</i> KM873113.1	96%
				Uncultured <i>Oscillatoria</i> sp. EU341185.1	96%
				Uncultured actinobacterium DQ431885.1	96%
				<i>Bacillus</i> sp. ON597424.1	96%
8	Elytra	-	NA		
8	Legs	-	NA		
9	Head	-	NA		
9	Abdomen	+	Mixed	No significant similarity found	
9	Elytra	+	Mixed	No significant similarity found	
9	Legs	-	NA		
10	Head	-	NA		

10	Abdomen	-	NA		
10	Elytra	-	NA		
10	Legs	-	NA		

Table 3 BLAST hits of the sequences obtained from the amplification of DNA samples extracted from *Harmonia axyridis* head, abdomen, elytra and legs. NA indicates that no sequencing was carried out, due to the lack of PCR product for the sample.

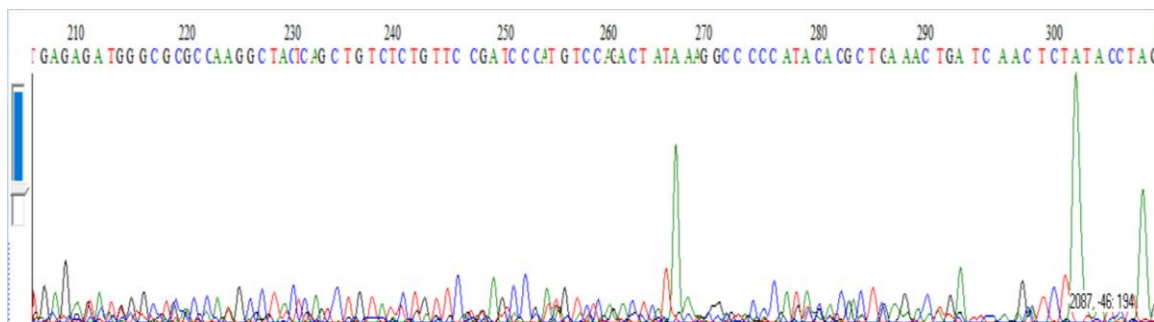


Figure 1 Mixed sequences trace showing mixed 16S rDNA amplified from *Harmonia axyridis* sample 1.²⁹

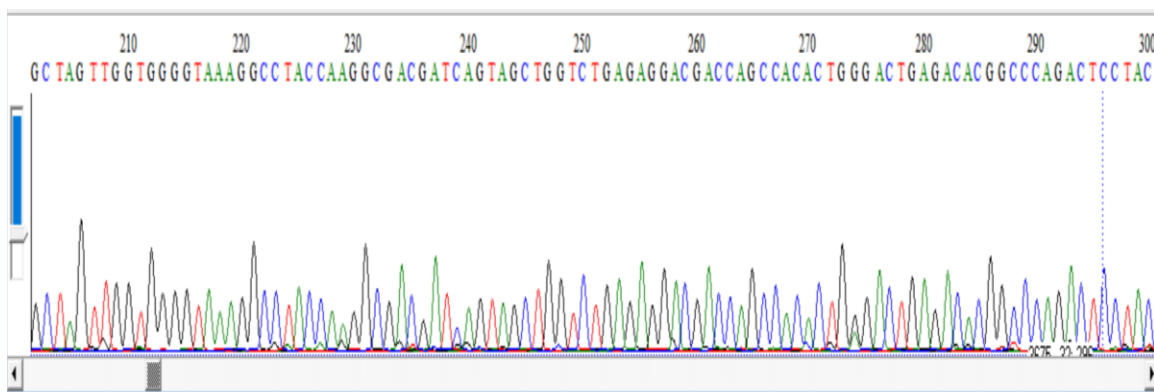


Figure 2 Clean sequences trace showing cloned 16S rDNA amplified from *Harmonia axyridis* sample 1.³⁰

Mean %	kingdom	phylum	class	order	family	genus	species
10.3116	other	other	other	other	other	other	other
0.0115	Archaea	Thaumarchaeota	NA	NA	NA	NA	sp1562
0.0623	Bacteria	Acidobacteria	Acidobacteria	NA	NA	NA	sp3336
0.0605	Bacteria	Acidobacteria	Acidobacteria	NA	NA	NA	sp3502
0.0627	Bacteria	Acidobacteria	Acidobacteria	NA	NA	NA	sp3506
0.0349	Bacteria	Acidobacteria	Acidobacteria	NA	NA	NA	sp3596
0.0362	Bacteria	Acidobacteria	Acidobacteria	NA	NA	bacterium	enrichment
0.0512	Bacteria	Actinobacteria	Actinobacteria	Bifidobacteriales	Bifidobacteriaceae	Bifidobacterium	longum
0.0334	Bacteria	Actinobacteria	Actinobacteria	Corynebacteriales	Corynebacteriaceae	Corynebacterium	flavescens
0.0508	Bacteria	Actinobacteria	Actinobacteria	Corynebacteriales	Corynebacteriaceae	Corynebacterium	freneyi-xerosis
0.0769	Bacteria	Actinobacteria	Actinobacteria	Corynebacteriales	Corynebacteriaceae	Corynebacterium	nuruki
0.0428	Bacteria	Actinobacteria	Actinobacteria	Corynebacteriales	Mycobacteriaceae	Mycobacterium	pallens
0.0444	Bacteria	Actinobacteria	Actinobacteria	Corynebacteriales	Nocardiaceae	Rhodococcus	equi-hoagii
1.4525	Bacteria	Actinobacteria	Actinobacteria	Corynebacteriales	Nocardiaceae	Rhodococcus	rhodochrous-ruber
0.2246	Bacteria	Actinobacteria	Actinobacteria	Frankiales	Geodermatophilaceae	Blastococcus	aggregatus
0.0380	Bacteria	Actinobacteria	Actinobacteria	Frankiales	Geodermatophilaceae	Blastococcus	endophyticus
0.0215	Bacteria	Actinobacteria	Actinobacteria	Frankiales	Geodermatophilaceae	NA	sp6085
0.0384	Bacteria	Actinobacteria	Actinobacteria	Frankiales	NA	NA	sp6166
0.0857	Bacteria	Actinobacteria	Actinobacteria	Frankiales	Nakamurellaceae	Nakamurella	flavida
0.0477	Bacteria	Actinobacteria	Actinobacteria	Frankiales	Sporichthyaceae	NA	sp6154
0.0572	Bacteria	Actinobacteria	Actinobacteria	Kineosporiales	Kineosporiaceae	Kineococcus	glutinatus
0.0101	Bacteria	Actinobacteria	Actinobacteria	Micrococcales	Brevibacteriaceae	Brevibacterium	linens
0.0901	Bacteria	Actinobacteria	Actinobacteria	Micrococcales	Cellulomonadaceae	Cellulomonas	chitinilytica-composti
0.0511	Bacteria	Actinobacteria	Actinobacteria	Micrococcales	Dermacoccaceae	Dermacoccus	nishinomiyaensis
0.1208	Bacteria	Actinobacteria	Actinobacteria	Micrococcales	Intrasporangiaceae	Aquipuribacter	hungaricus

0.5731	Bacteria	Actinobacteria	Actinobacteria	Micrococcales	Intrasporangiaceae	Knoellia	aerolata
0.0618	Bacteria	Actinobacteria	Actinobacteria	Micrococcales	Intrasporangiaceae	Knoellia	aerolata-sinensis-subterranea
0.0508	Bacteria	Actinobacteria	Actinobacteria	Micrococcales	Intrasporangiaceae	Knoellia	flava
0.0433	Bacteria	Actinobacteria	Actinobacteria	Micrococcales	Intrasporangiaceae	Lapillicoccus	sp6479
0.1829	Bacteria	Actinobacteria	Actinobacteria	Micrococcales	Intrasporangiaceae	NA	NA
0.0210	Bacteria	Actinobacteria	Actinobacteria	Micrococcales	Intrasporangiaceae	Ornithinimicrobium	murale-sp6491
0.1231	Bacteria	Actinobacteria	Actinobacteria	Micrococcales	Intrasporangiaceae	Ornithinimicrobium	sp6490-sp6492
0.0517	Bacteria	Actinobacteria	Actinobacteria	Micrococcales	Intrasporangiaceae	Tetrasphaera	sp6526
0.0355	Bacteria	Actinobacteria	Actinobacteria	Micrococcales	Microbacteriaceae	Leifsonia	NA
0.0192	Bacteria	Actinobacteria	Actinobacteria	Micrococcales	Microbacteriaceae	Microbacterium	saccharophilum
0.0371	Bacteria	Actinobacteria	Actinobacteria	Micrococcales	Micrococcaceae	Arthrobacter	citreus
0.0535	Bacteria	Actinobacteria	Actinobacteria	Micrococcales	Micrococcaceae	Arthrobacter-Pseudarthrobacter	NA
0.2407	Bacteria	Actinobacteria	Actinobacteria	Micrococcales	Micrococcaceae	Kocuria	polaris
0.0186	Bacteria	Actinobacteria	Actinobacteria	Micromonosporales	Micromonosporaceae	Asanoa	iriomotensis-ishikariensis-siamensis
0.0371	Bacteria	Actinobacteria	Actinobacteria	Micromonosporales	Micromonosporaceae	Catellatospora	sp7406
0.0481	Bacteria	Actinobacteria	Actinobacteria	Micromonosporales	Micromonosporaceae	Micromonospora	sp7444
0.2471	Bacteria	Actinobacteria	Actinobacteria	Propionibacteriales	Nocardiodaceae	Marmoricola	scoriae
0.0650	Bacteria	Actinobacteria	Actinobacteria	Propionibacteriales	Nocardiodaceae	Marmoricola	sp7575
0.0361	Bacteria	Actinobacteria	Actinobacteria	Propionibacteriales	Nocardiodaceae	NA	sp7702
0.2148	Bacteria	Actinobacteria	Actinobacteria	Propionibacteriales	Nocardiodaceae	Nocardioides	alpinus

0.0329	Bacteria	Actinobacteria	Actinobacteria	Propionibacteriales	Nocardioidaceae	Nocardioides	islandensis
0.0056	Bacteria	Actinobacteria	Actinobacteria	Propionibacteriales	Nocardioidaceae	Nocardioides	lentus-pyridinolyticus
0.0984	Bacteria	Actinobacteria	Actinobacteria	Propionibacteriales	Nocardioidaceae	Nocardioides	mesophilus
0.0210	Bacteria	Actinobacteria	Actinobacteria	Propionibacteriales	Nocardioidaceae	Nocardioides	oleivorans-sp7690
0.0614	Bacteria	Actinobacteria	Actinobacteria	Propionibacteriales	Nocardioidaceae	Nocardioides	sp7654
0.2808	Bacteria	Actinobacteria	Actinobacteria	Propionibacteriales	Nocardioidaceae	Nocardioides	sp7658
0.0604	Bacteria	Actinobacteria	Actinobacteria	Propionibacteriales	Nocardioidaceae	Nocardioides	sp7670
0.1465	Bacteria	Actinobacteria	Actinobacteria	Propionibacteriales	Nocardioidaceae	Nocardioides	sp7692
0.0435	Bacteria	Actinobacteria	Actinobacteria	Propionibacteriales	Propionibacteriaceae	Aestuariimicrobium-Naumannella	halotolerans-sp7705
0.0059	Bacteria	Actinobacteria	Actinobacteria	Propionibacteriales	Propionibacteriaceae	Friedmanniella	flava-lacustris
0.2777	Bacteria	Actinobacteria	Actinobacteria	Propionibacteriales	Propionibacteriaceae	Friedmanniella	lacustris
0.1135	Bacteria	Actinobacteria	Actinobacteria	Propionibacteriales	Propionibacteriaceae	Friedmanniella	lucida
0.0206	Bacteria	Actinobacteria	Actinobacteria	Propionibacteriales	Propionibacteriaceae	Friedmanniella	lucida-okinawensis
0.2878	Bacteria	Actinobacteria	Actinobacteria	Propionibacteriales	Propionibacteriaceae	Friedmanniella	okinawensis
0.1414	Bacteria	Actinobacteria	Actinobacteria	Propionibacteriales	Propionibacteriaceae	Microlunatus	aurantiacus

0.0152	Bacteria	Actinobacteria	Actinobacteria	Propionibacteriales	Propionibacteriaceae	Micrococcus	ginsengisoli-sp7740
0.2227	Bacteria	Actinobacteria	Actinobacteria	Propionibacteriales	Propionibacteriaceae	Propionibacterium	acnes
0.0264	Bacteria	Actinobacteria	Actinobacteria	Propionibacteriales	Propionibacteriaceae	Propionisphaera	sp7800
0.0683	Bacteria	Actinobacteria	Actinobacteria	Propionibacteriales	Propionibacteriaceae	Propionisphaera	sp7802
0.0146	Bacteria	Actinobacteria	Actinobacteria	Pseudonocardiales	Pseudonocardaceae	Actinomyces	sp7854
0.1093	Bacteria	Actinobacteria	Actinobacteria	Pseudonocardiales	Pseudonocardaceae	Pseudonocardia	sp8091
0.0990	Bacteria	Actinobacteria	Coriobacteriia	Coriobacteriales	Coriobacteriaceae	Collinsella	aerofaciens
0.0299	Bacteria	Actinobacteria	Thermoleophilia	Gaiellales	Gaiellaceae	Gaiella	sp10792
0.0230	Bacteria	Actinobacteria	Thermoleophilia	Gaiellales	Gaiellaceae	Gaiella	sp10800-sp10801
0.0133	Bacteria	Actinobacteria	Thermoleophilia	Gaiellales	NA	NA	sp10950
0.0421	Bacteria	Actinobacteria	Thermoleophilia	Solirubrobacterales	NA	NA	sp11029
0.0848	Bacteria	Actinobacteria	Thermoleophilia	Solirubrobacterales	NA	NA	sp11059
0.0526	Bacteria	Actinobacteria	Thermoleophilia	Solirubrobacterales	Patulibacteraceae	Patulibacter	minatonensis
0.0852	Bacteria	Actinobacteria	Thermoleophilia	Solirubrobacterales	Solirubrobacteraceae	Solirubrobacter	sp11110
0.0054	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	NA	NA	sp12571
0.0188	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	NA	NA	sp12572-sp12578-sp12693
0.0442	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	NA	NA	sp12589
0.0412	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	NA	NA	sp12763
0.0256	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	NA	NA	sp12804
0.0224	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Porphyromonas	sp13299

0.0451	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	Prevotella	copri-sp13942
0.0526	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	Prevotella	loescheii
0.0243	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	Alistipes	sp14336-sp14354
0.0453	Bacteria	Bacteroidetes	Cytophagia	Cytophagales	Cytophagaceae	Hymenobacter	arizonensis
0.1210	Bacteria	Bacteroidetes	Cytophagia	Cytophagales	Cytophagaceae	Hymenobacter	roseosalivarius
0.0451	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Capnocytophaga	sp16515
0.1140	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	sp16891
0.0368	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Sediminibacter-Subsaxibacter	furfurosus-sp17593
0.0676	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Chitinophagaceae	Flavisolibacter	sp18182
0.0424	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Chitinophagaceae	Flavisolibacter	sp18204
0.0654	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Chitinophagaceae	NA	sp18498
0.0110	Bacteria	Chloroflexi	Ardenticatenia	Ardenticatenales	NA	NA	sp20888
0.0531	Bacteria	Cyanobacteria	Chloroplast	Myrmecia israelensis	Myrmecia israelensis	Myrmecia	israelensis
6.4074	Bacteria	Cyanobacteria	Chloroplast	NA	NA	NA	NA
0.0389	Bacteria	Cyanobacteria	Chloroplast	NA	NA	NA	sp22867
0.0147	Bacteria	Cyanobacteria	Chloroplast	Orthotrichum rogeri	Orthotrichum rogeri	Orthotrichum	rogeri
20.3319	Bacteria	Cyanobacteria	Chloroplast	Pinus wallichiana	Pinus wallichiana	Pinus	wallichiana
0.2474	Bacteria	Cyanobacteria	Chloroplast	Platycladus orientalis	Platycladus orientalis	Platycladus	orientalis
0.1032	Bacteria	Cyanobacteria	Chloroplast	Rubus hybrid cultivar	Rubus hybrid cultivar	Fragaria-Rubus	hybrid-x
0.0901	Bacteria	Cyanobacteria	Cyanobacteria	NA	FamilyII	Chroococcidiopsis	sp23063-sp23575
0.0416	Bacteria	Cyanobacteria	Cyanobacteria	NA	FamilyII	Chroococcidiopsis	sp23085
0.0640	Bacteria	Cyanobacteria	Cyanobacteria	NA	NA	NA	sp23640

0.0212	Bacteria	Deinococcus-Thermus	Deinococci	Deinococcales	Deinococcaceae	Deinococcus	antarcticus
0.0380	Bacteria	Deinococcus-Thermus	Deinococci	Deinococcales	Deinococcaceae	Deinococcus	papagonensis
0.1835	Bacteria	Deinococcus-Thermus	Deinococci	Deinococcales	Deinococcaceae	Deinococcus	sp24005
0.0590	Bacteria	Deinococcus-Thermus	Deinococci	Deinococcales	Trueperaceae	Truepera	radiovictrix
0.0368	Bacteria	Firmicutes	Bacilli	Bacillales	Bacillaceae	Bacillus	NA
0.0439	Bacteria	Firmicutes	Bacilli	Bacillales	Bacillaceae	Bacillus	psychrosaccharolyticus-simplex
0.0442	Bacteria	Firmicutes	Bacilli	Bacillales	Paenibacillaceae	Cohnella	arctica-lupini
0.0883	Bacteria	Firmicutes	Bacilli	Bacillales	Paenibacillaceae	Cohnella	suwonensis
0.0480	Bacteria	Firmicutes	Bacilli	Bacillales	Paenibacillaceae	Paenibacillus	durus-sabinae-sophorae
0.0312	Bacteria	Firmicutes	Bacilli	Bacillales	Planococcaceae	Lysinibacillus	contaminans
0.0576	Bacteria	Firmicutes	Bacilli	Bacillales	Planococcaceae	Sporosarcina	psychrophila
0.0723	Bacteria	Firmicutes	Bacilli	Bacillales	Staphylococcaceae	Staphylococcus	capitis-caprae-epidermidis
0.0577	Bacteria	Firmicutes	Bacilli	Bacillales	Staphylococcaceae	Staphylococcus	epidermidis-hominis
0.0269	Bacteria	Firmicutes	Bacilli	Bacillales	Staphylococcaceae	Staphylococcus	lentus
0.0339	Bacteria	Firmicutes	Bacilli	Bacillales	Staphylococcaceae	Staphylococcus	saprophyticus-xylosus
0.0386	Bacteria	Firmicutes	Bacilli	Lactobacillales	Carnobacteriaceae	Atopostipes	suicloacalis
0.0773	Bacteria	Firmicutes	Bacilli	Lactobacillales	Carnobacteriaceae	Carnobacterium	inhibens-jeotgali-viridans
0.0498	Bacteria	Firmicutes	Bacilli	Lactobacillales	Carnobacteriaceae	NA	sp28233
0.0968	Bacteria	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	NA
0.0121	Bacteria	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	intestinalis
0.0173	Bacteria	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	johnsonii

18.0758	Bacteria	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Lactococcus	lactis
0.0349	Bacteria	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Streptococcus	mitis-oralis-sanguinis
0.0543	Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	Clostridium	bowmanii-estertheticum
0.0173	Bacteria	Firmicutes	Clostridia	Clostridiales	Family XVII	Thermaerobacter	composti
0.0534	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Lachnoclostridium	sp32442
0.0071	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	NA	sp32255
0.0174	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	NA	sp32622
0.0270	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	NA	sp32668
0.0173	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	NA	sp32764
0.0464	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	NA	sp33366
0.0160	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	NA	sp33760
0.0394	Bacteria	Firmicutes	Clostridia	Clostridiales	NA	NA	sp30555
0.0308	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	NA	sp34838
0.0115	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	NA	sp34863
0.0134	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	NA	sp34879
0.0403	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	NA	sp35507
0.1175	Bacteria	Firmicutes	Erysipelotrichia	Erysipelotrichales	Erysipelotrichaceae	Turicibacter	sanguinis
0.0325	Bacteria	Gemmatimonadetes	Gemmatimonadetes	Gemmatimonadales	Gemmatimonadaceae	Gemmatimonas	sp37828
0.0848	Bacteria	Gemmatimonadetes	Gemmatimonadetes	Gemmatimonadales	Gemmatimonadaceae	Gemmatimonas	sp37864
0.0457	Bacteria	Gemmatimonadetes	Gemmatimonadetes	Gemmatimonadales	Gemmatimonadaceae	NA	sp37886
0.0316	Bacteria	Gemmatimonadetes	Gemmatimonadetes	Gemmatimonadales	Gemmatimonadaceae	NA	sp38019
0.0165	Bacteria	Gemmatimonadetes	Gemmatimonadetes	NA	NA	NA	sp37610
0.0186	Bacteria	Nitrospirae	Nitrospira	Nitrospirales	Nitrospiraceae	Nitrospira	sp39753
0.0680	Bacteria	Planctomycetes	Phycisphaerae	NA	NA	NA	sp41171

0.0810	Bacteria	Planctomycetes	Planctomycetacia	Planctomycetales	Planctomycetaceae	Gemmata	sp41490
0.0199	Bacteria	Planctomycetes	Planctomycetacia	Planctomycetales	Planctomycetaceae	Gemmata	sp41528
0.0104	Bacteria	Planctomycetes	Planctomycetacia	Planctomycetales	Planctomycetaceae	NA	sp42199
0.1803	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Methylobacteriaceae	Methylobacterium	adhaesivum
0.0197	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Methylobacteriaceae	Methylobacterium	komagatae-sp43876
0.0325	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Methylobacteriaceae	Methylobacterium	oxalidis
0.0142	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	NA	NA	sp44617
0.2090	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	Agrobacterium-Rhizobium	radiobacter-tumefaciens
0.0587	Bacteria	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Amaricoccus	kaplicensis
0.0389	Bacteria	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Paracoccus-Paracoccus	huijuniae-sp44862
0.0941	Bacteria	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Paracoccus	carotinifaciens-marcusii
0.0439	Bacteria	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Rubellimicrobium	mesophilum
0.1391	Bacteria	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Rubellimicrobium	roseum
0.0274	Bacteria	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Rubellimicrobium	sp45098
0.0233	Bacteria	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Rubellimicrobium	sp45105
0.2342	Bacteria	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Acetobacteraceae	Acidiphilium	sp45502
0.0732	Bacteria	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Acetobacteraceae	Acidiphilium	sp45514

0.1359	Bacteria	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Acetobacteraceae	Craurococcus	sp45575-sp45582
0.0375	Bacteria	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Acetobacteraceae	Roseomonas	vinacea
0.0640	Bacteria	Proteobacteria	Alphaproteobacteria	Rhodospirillales	NA	*Alysiosphaera	sp46509
0.0645	Bacteria	Proteobacteria	Alphaproteobacteria	Rhodospirillales	NA	NA	sp45944
0.2695	Bacteria	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Rhodospirillaceae	Azospirillum-Skermanella	sp45983-sp46213
0.0804	Bacteria	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Rhodospirillaceae	Defluviicoccus	sp46056
0.0848	Bacteria	Proteobacteria	Alphaproteobacteria	Rickettsiales	Mitochondria	Arundo-Oryza meyeriana	NA
0.8205	Bacteria	Proteobacteria	Alphaproteobacteria	Rickettsiales	Mitochondria	NA	NA
0.0113	Bacteria	Proteobacteria	Alphaproteobacteria	Rickettsiales	Mitochondria	NA	sp46943
1.1113	Bacteria	Proteobacteria	Alphaproteobacteria	Rickettsiales	Mitochondria	Pinus	sp46807
0.0468	Bacteria	Proteobacteria	Alphaproteobacteria	Rickettsiales	Mitochondria	Rubus hybrid cultivar	hybrid
0.5257	Bacteria	Proteobacteria	Alphaproteobacteria	Rickettsiales	Mitochondria	Sanguinaria	canadensis
0.1038	Bacteria	Proteobacteria	Alphaproteobacteria	Rickettsiales	Mitochondria	Tetraphis-Tetraphis pellucida	pellucida
0.0329	Bacteria	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Erythrobacteraceae	Erythrobacter	marinus
0.0935	Bacteria	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Erythrobacteraceae	Erythrobacter	sp47537-sp47561-sp47565

0.0879	Bacteria	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Erythrobacteraceae	NA	sp47561
0.0878	Bacteria	Proteobacteria	Alphaproteobacteria	Sphingomonadales	NA	NA	sp48028
0.1034	Bacteria	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Sphingobium	yanoikuyae
0.0544	Bacteria	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Sphingomonas	astaxanthinifaciens-humi
0.0178	Bacteria	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Sphingomonas	gei-xinjiangensis
0.1880	Bacteria	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Sphingomonas	kyeonggiensis-leidy
0.0389	Bacteria	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Sphingomonas	sp47831
0.2010	Bacteria	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Sphingomonas	sp47868
0.0567	Bacteria	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Sphingomonas	sp47872
0.0467	Bacteria	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Sphingomonas	wittichii
0.0649	Bacteria	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Sphingopyxis	contaminans
0.0297	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Alcaligenaceae	Parasutterella	excrementihominis
0.0713	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Burkholderiaceae	Burkholderia-Burkholderia-Paraburkholderia	cenocepacia-cepacia-vietnamiensis
0.3249	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Burkholderiaceae	Burkholderia-Paraburkholderia	bryophila
0.4076	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Burkholderiaceae	Burkholderia-Paraburkholderia	insulsa

0.0454	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Burkholderiaceae	Cupriavidus	basilensis-numazuensis
0.1170	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Burkholderiaceae	Lautropia	mirabilis
0.0091	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Burkholderiaceae	Lautropia	sp48667
0.0724	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Curvibacter	delicatus
0.0115	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Hydrogenophaga	bisanensis
0.0088	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Polaromonas	jejuensis
0.0632	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Variovorax	ginsengisoli-paradoxus
0.0229	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	Noviherbaspirillum	sp49444
0.1177	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	Noviherbaspirillum	sp49445
0.0261	Bacteria	Proteobacteria	Betaproteobacteria	Neisseriales	Neisseriaceae	Neisseria	flavescens
0.0084	Bacteria	Proteobacteria	Betaproteobacteria	Nitrosomonadales	Nitrosomonadaceae	NA	sp50229
0.0156	Bacteria	Proteobacteria	Deltaproteobacteria	Bdellovibrionales	Bacteriovoracaceae	Bacteriovorax	sp50758
0.0091	Bacteria	Proteobacteria	Deltaproteobacteria	Desulfovibrionales	Desulfovibrionaceae	Desulfovibrio	sp52711
0.0093	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	*Hamiltonella	defensa
0.0182	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Citrobacter-Escherichia-Shigella	braakii-flexneri-freundii

0.0108	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Enterobacter	cloacae-ludwigii-sp58653
0.0610	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Escherichia-Shigella	coli
0.0139	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Pantoea	vagans
9.5780	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Serratia	grimesii-liquefaciens-proteamaculans
9.5855	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Serratia	marcescens
3.7093	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Serratia	marcescens-nematodiphila
0.0693	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Serratia	sp59859
0.0689	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Acinetobacter	bouvetii-johnsonii
0.0329	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Acinetobacter	johnsonii
0.0610	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Alkanindiges	sp62596
0.2814	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Enhydrobacter-Moraxella	aerosaccus-osloensis
0.0614	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	NA
0.0624	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	aeruginosa
0.0233	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	azotoformans-fluorescens-synxantha

0.0435	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	baetica-fluorescens
0.0950	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	benzenivorans-putida
0.5831	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	costantinii-fluorescens-poae
0.1601	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	fluorescens-protegens
0.0784	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	poae
0.3197	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	putida
0.0160	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	stutzeri
0.0286	Bacteria	Proteobacteria	Gammaproteobacteria	Xanthomonadales	NA	Acidibacter	sp65535
0.0234	Bacteria	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Nevskiaceae	Nevskia	solis
1.8127	Bacteria	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Stenotrophomonas	maltophilia
0.0256	Bacteria	Saccharibacteria	NA	NA	NA	NA	sp66110
0.0425	Bacteria	Tenericutes	Mollicutes	Entomoplasmatales	Spiroplasmataceae	Spiroplasma	secondary

Table 4: Metagenomic analysis of results from 16S rDNA sequencing from four *Harmonia axyridis* adult ladybirds (two male, two female). Mean % indicates the proportion of reads averaged over the four individual ladybirds' results, for each taxon identified. *Table 6*