Incidence, diversity and evolution of *Rickettsia* and other endosymbionts that infect arthropods

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Abstract

The biology and evolution of many arthropod species cannot be understood without also considering their bacterial symbionts. In order to proliferate, many endosymbionts manipulate the reproduction of their hosts, and do so in a multitude of ways. The phenotypes that result from this bacterial manipulation have profound implications for the evolution of their hosts. These interactions will both influence, and be influenced by the abundance of the bacterial symbiont in arthropods.

One of the best-studied bacterial manipulations is male-killing, where endosymbionts kill their male hosts in order to distribute resources towards infected females (the only individuals who will transmit the infection). Ladybirds beetles are known to harbour male-killing symbionts, and so the incidence of bacteria was investigated in the whole of this family. It was found that endosymbionts infected over half of the host species, mainly at low prevalence, which indicates that symbiont incidence and diversity may be currently under-estimated. In addition, multiple symbionts were found in the same population, lending strength to the hypothesis that they are being maintained by balancing selection with host resistance genes.

The data was combined with a world-wide screen and other data from the literature, and used to estimate the distribution of across-species prevalences of the bacteria *Wolbachia*, *Rickettsia* and *Cardinium* in wild arthropod populations. A newly developed likelihood approach was used to find a best fit distribution, and properties of the distribution then used to predict how these symbionts manage to invade and spread through populations. The analysis revealed that the skew toward low prevalence infections may apply quite generally, suggesting that much of the diversity of endosymbionts will be missed from screens that test only a few individuals. In addition, the analysis highlighted differences in the incidence levels of different bacteria, and heterogeneity in prevalence distributions between clades of host species.

Contemporary patterns of endosymbiont abundance must also be understood within the long-term evolutionary context, best investigated with a phylogenetic approach. Comparison of *Rickettsia* and *Wolbachia* phylogenies with those of their hosts indicate that these symbionts frequently switch horizontally between related hosts.

Rickettsia have been less thoroughly investigated than *Wolbachia*, but these arthropod endosymbionts can also infect and cause serious diseases in humans and other mammals. In this study, 20 new strains of arthropod *Rickettsia* are identified and multiple genes sequenced to produce a robust phylogeny of the whole genus. *Rickettsia* are devised of two main clades, one of which primarily infects arthropods and the other infect a diverse range of protists, leeches, unidentified hosts from metagenomic samples, and some arthropods. Strategies such as male-killing and parthenogenesis induction appear to be recent innovations. Arthropod *Rickettsia* generally group basal to medically important strains, but some also cluster within the strains that infect vertebrates.

There is increasing evidence against the traditional view that intracellular symbionts are refractory to recombination. Recombination does occur in *Rickettsia* but seems to be uncommon. However, there is strong evidence of large scale horizontal gene transfer events. Numerous conjugation genes were also discovered which indicates that plasmids may be common throughout the whole genus.

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Contents

Chapter 1. Introduction

1.1 *Arthropod endosymbionts*

Bacteria and arthropods have evolved a bewildering array of symbiotic interactions, the most intimate being symbionts that reside within host cells. Although intracellular bacteria can be transmitted infectiously, many cannot survive outside of their host cell environment for long periods, and are therefore inherited by their host's offspring (Hertig and Wolbach 1924). Since the transmission of these bacteria depends on the survival and reproduction of their hosts, it has been suggested that they should evolve towards a benign or beneficial relationship with that host (Fine 1975; Lipsitch et al. 1996). However, facultative symbionts that manipulate their host's reproduction defy this prediction. These symbionts are inherited via the cytoplasm of their female host's eggs, but not by male gametes, because sperm do not contribute cytoplasm to the fertilised oocyte (Sears 1980). Therefore, if infectious transfer is uncommon, a symbiont in a male host is destined for extinction. This asymmetric mode of transmission creates four alternative routes that symbionts must adopt if they are to invade and spread within potential host populations: increase the fitness of infected females, increase the proportion of infected females, decrease the fitness of uninfected females or decrease the proportion of uninfected females (O'Neill et al. 1997). These strategies can be accomplished in a variety of ways, and the five main phenotypes are as follows.

1.1.1 Mutualists

Mutualistic endosymbionts are found in many arthropods, where they are thought to be an important driving force in allowing their hosts to explore and invade new ecological niches (Douglas 1994). Obligate mutualists are often found in hosts that have a nutritionally poor diet for the whole of their lifecycle, where they provision them with essential food, such as amino acids (Douglas 1998). These hosts include

those which feed on sap (Rouhbakhsh et al. 1996), cellulose (Breznak 1982), grain (VazquezArista et al. 1997) or blood (Douglas 1989). Since the host requires the symbiont for survival, they often have a relationship that can persist for millions of years (Moran et al. 1993; Chen et al. 1999; Casiraghi et al. 2001). However, after a long period of intracellular occupation, bacteria will inevitably lose genes that would have previously facilitated their free-living lifestyle (Blanc et al. 2007). In addition, repeated bottlenecks every transmission event serve to reduce their effective population size, and accumulate deleterious mutations in an irreversible way (Itoh et al. 2002). Such symbionts are sometimes considered to be "enslaved" by their hosts, which may result in an evolutionary dead end where other symbionts can take over nutrient provisioning (Koga et al. 2003; Gil et al. 2004). Endosymbionts may also be facultatively beneficial to their hosts. For example, there are a few known cases of symbiont-mediated protection from infectious elements or parasitic invertebrates (reviewed in Haine 2008). However, much less is known about these symbionts.

1.1.2 Cytoplasmic incompatibility

Inducing cytoplasmic incompatibility (CI) in their hosts is one way in which endosymbionts are known to decrease the fitness of uninfected females. This seems to be achieved by the endosymbiont modifying sperm to contain a toxin which is neutralised by infected oocytes, but not by uninfected oocytes (Werren 1997). In this case, the mating of an infected male with an uninfected female leads to eggs that are less likely to hatch (Yen and Barr 1971), while the fitness of infected females is independent of the male's infection status. In organisms with a haplodiploid sex determination system, cytoplasmic incompatibility can also cause a decrease in the proportion of uninfected females (Breeuwer and Werren 1995). If a diploid zygote is not infected with the symbiont, loss of the paternal chromosome can occur, converting the zygote from a diploid female into a haploid male (although in some systems, the result is zygote mortality – as is the case with classical CI).

1.1.3 Parthenogenesis induction

Endosymbionts that induce parthenogenesis in their hosts exemplify the strategy of increasing the proportion of infected females. By making all offspring female, the bacterium is ensured passage to all members of the next generation (Stouthamer 1997). Although in theory, this phenotype could manifest itself in diploid hosts, it has been observed only in haplodiploids, where the endosymbiont induces parthenogenesis by doubling of the chromosome (Weeks and Breeuwer 2001).

1.1.4 Feminisation

Another example of a phenotype that increases the proportion of infected females is host feminisation, whereby genetic males are converted to functional females through symbiont infection. This occurs in hosts that have a labile sex determination system, and is widespread is isopods and amphipods (less common in lepidoptera and hemiptera), where most species have very similar male and female sex chromosomes (Rigaud 1997), and females can easily be converted to males by the addition of just one hormone.

1.1.5 Male-killing

A final example of sex ratio distortion is a phenotype that raises the fitness of infected daughters through the outright killing of their male siblings. This phenotype is adaptive for the bacteria via a form of kin selection, and depends on a host ecology where the death of males provides a direct fitness benefit to their female relatives – who are likely to be infected by relatives of the male-killing bacterium (Hurst 1991). A necessary condition for invasion is thought to be a degree of antagonistic sibling interaction, such that the death of brothers partition resources towards infected female siblings or infected females avoid being predated (Hurst and Jiggins 2000). In addition, male death may cause a reduction in the rate of deleterious inbreeding, which consequently increases infected female reproductive success (Werren 1987). Male-killing will be one of the themes of this thesis, where I investigate the incidence and dynamics in field populations (Chapter 3), predictions about the

invasion of male-killers (Chapter 5), and the evolutionary origins of male-killing (Chapter 7).

1.2 *Incidence, diversity and phylogeny*

Having explored some of the phenotypes induced in their hosts, I now review the diversity of the bacterial symbionts themselves.

1.2.1 *Wolbachia*

The most common and best known arthropod endosymbiont is *Wolbachia*. *Wolbachia* is a member of the α-proteobacteria, most of which consist of intracellular bacteria (Amann et al. 1991). It is well established that the incidence of *Wolbachia* in wild arthropod populations is high. Random screens from field populations of many different species have found that approximately 20% of all arthropod individuals are infected (Werren et al. 1995b; Werren and Windsor 2000). However, since many *Wolbachia* are known infect host species at low prevalence (Jiggins et al. 2001), the true number of species infected is probably much higher; indeed, a recent study estimated that the proportion infected above 0.1% prevalence may be as high as 66% (Hilgenboecker et al. 2008). In addition, Wolbachia are found in most filarial nematodes, where they appear to be obligate mutualists (Bandi et al. 1998). Given that *Wolbachia* may be one of the most numerous bacteria on earth (Werren et al. 1995b), an overwhelming diversity of strain types exist, and have been assigned to eight supergroups (Lo et al. 2007). These supergroups show phylogenetic clustering by phenotype, and in particular, obligate mutualists and reproductive parasites show a strong tendency to group together, indicating that mutualism has evolved only once in *Wolbachia* (Fenn et al. 2006). These clades also show different levels of stability regarding the interactions with their hosts; for while many of the *Wolbachia* mutualisms seem to be ancient (Casiraghi et al. 2001), most of the facultative parasites (contained within the A and B supergroups), seem to have

undergone frequent host switching (Werren et al. 1995a). Mapping manipulative phenotypes such as cytoplasmic incompatibility and male-killing onto the *Wolbachia* phylogeny suggests that these traits are highly labile (Stouthamer et al. 1999). This and the observation that switching between phenotypes can be rapid (Hornett et al. 2008), suggests that these traits have a similar mechanistic basis. However, bacterial phenotype is also known to be influenced by host genotype (Sasaki et al. 2005), and another possibility is that these traits are easy to evolve, and that horizontal gene transfer may facilitate this evolution (Masui et al. 2000).

1.2.2.Diversity of parasites

While *Wolbachia* is the most highly studied bacterial arthropod endosymbiont, it is far from alone. Below, I review other endosymbionts that are known to manipulate their host's reproduction.

1.2.3 *Cardinium*

Another genus of bacteria with a diverse array of phenotypic manifestations is *Cardinium*, which is phylogenetically classified in the Bacteroidetes group of bacteria. To date, cytoplasmic incompatibility, feminisation and parthenogenesis induction have been described for *Cardinium* (Weeks et al. 2001; Zchori-Fein et al. 2001; Hunter et al. 2003). It is also known to be common in arthropod populations, although it is detected in less individuals than *Wolbachia* when low numbers of many arthropod species are tested (Weeks et al. 2003).

1.2.4 *Spiroplasma*

Spiroplasma are members of the mollicutes and get their name from their helical morphology (Gasparich et al. 2004). They are pathogens of arthropods, causing arthropod vectored plant diseases (Regassa and Gasparich 2006). Although assigned to the same genus, there are two very divergent types of *Spiroplasma*, both of which have been shown to induce male-killing in their hosts (Schulenburg et al. 2000). One

group is known to induce male-killing in several *Drosophila* species (Barile and Razin 1979; Montenegro et al. 2005). The other type of *Spiroplasma* (ixodetis), is non-helical, and is thought to be more widespread in arthropod populations (Duron et al. 2008). The number of known male-killing *Spiroplasma* in this group is growing (Hurst et al. 1999b; Jiggins et al. 2000b; Montenegro et al. 2005; Tinsley and Majerus 2006).

1.2.5 *Arsenophonus*

Bacteria in the genus *Arsenophonus* are classified to the γ-proteobacteria. One member of this genus is known to cause male-killing, in *Nasonia vitripennis*. Although *Arsenophonus* are inherited vertically, they may display a higher amount of horizontal transmission than other endosymbionts (Skinner 1985), and can be cultured outside of their hosts (Werren et al. 1986).

1.2.6 *Flavobacteria*

Flavobacteria species are also classified to the Bacteroidetes group of bacteria. In addition to causing male-killing in Coccinellidae beetles (Hurst et al. 1999a), bacteria in this genus are also known to be mutualists of cockroaches (Bandi et al. 1994).

1.2.7 *Rickettsia*

Rickettsia species are close relatives of *Wolbachia*. In common with *Wolbachia*, they induce male-killing and parthenogenesis in some hosts (Balayeva et al. 1995; Hagimori et al. 2006). However, they are also known to be arthropod vectored vertebrate pathogens (Azad and Beard 1998). *Rickettsia* will also be one of the themes in this thesis, where I investigate their diversity (Chapter 3, 4), life history evolution (Chapter 6), and molecular evolution (Chapter 6, 8).

1.3 The consequences of infection for host evolution

1.3.1 Genetic conflict

For the majority of animal species, an equal sex ratio is an evolutionary stable strategy (Fisher 1930). This is because deviations from a 50:50 ratio will result in the rarer sex having a higher reproductive success, and any individuals with a propensity to produce this sex, having a higher inclusive fitness. Therefore, this trait will spread, tending to restore equality. This suggests that bacterial endosymbionts that alter host sex ratio will tend to induce evolutionary change in their hosts. The resulting evolutionary dynamics resemble the intragenomic conflict arising whenever genes are inherited asymmetrically between the sexes (Hamilton 1967; Cosmides and Tooby 1981). Many striking features of organisms have been attributed to such intragenomic conflicts, including the presence of meiotic drive in plants, animals and fungi, genomic imprinting in mammals and cytoplasmic male-sterility in plants (Hurst et al. 1992). However, the consequences of conflicts between different species (intergenomic conflict) (Haig 1997), are no less profound, and so the ubiquity of endosymbiont parasites in arthropod populations, almost certainly has huge implications for the evolution of their hosts (Charlat et al. 2003). Some of these are outlined below.

1.3.2 Extinction

The dynamics of male-killers and feminisers suggest that after crossing a certain threshold, these parasites will spread to fixation in their hosts (O'Neill et al. 1997). Some male-killing bacteria are known to exist at extreme prevalence in butterfly hosts, and there have been reports of a complete lack of males in field populations (Charlat et al. 2007). In addition, in one population, this has resulted in complete sex role reversal, where females gather in leks in order to attract mates (Jiggins et al. 2000a). In addition, there have been reports of feminising *Wolbachia* at fixation in isopod populations (Bouchon et al. 1998). Clearly obligate sexual populations cannot

persist without males, therefore it follows that endosymbionts may have driven their host's populations extinct. Parthenogenesis-inducing bacteria may also cause extinction, by preventing the long-term benefits of sexual reproduction in responding to environmental change. For example, *Cardinium* in a haplodiploid mite has completely eradicated sex from the host population, so that all individuals exist in a haploid state (Weeks et al. 2001).

1.3.3 Speciation

There is also some evidence that endosymbionts might bring about speciation in their host populations. One way in which this can happen involves incompatibility between different *Wolbachia* strains (termed bidirectional incompatibility). This occurs when two populations of the same species are infected by different strains of *Wolbachia*, and low hatch rates occur when individuals infected with different strains mate (Yen and Barr 1973). Bidirectional cytoplasmic incompatibility therefore prevents gene flow between two infected populations, which can lead to full speciation. Cytoplasmic incompatibility (discussed above) can also cause population divergence under a range of theoretical conditions, even with appreciable levels of migration (Telschow et al. 2002). There is a some correlative empirical evidence from *Nasonia vitripennis* that *Wolbachia* induced incompatibility preceded other types of hybrid incompatibility early on in the speciation process (Bordenstein et al. 2001). But while speciation is widely considered to be theoretically plausible, there is little strong empirical evidence so far (Charlat et al. 2003).

1.3.3. Sex determination

Endosymbionts can cause irreversible changes to their host's sex determination system. In some populations of the woodlouse *Armadillium vulgare*, a complete loss of genetic females has occurred and instead the absence or presence of *Wolbachia* determines the sex of the individual (Rigaud 1997).

1.3.4. Effective population size

Although, phylogeny indicates that the symbionts that manipulate the reproduction of their hosts frequently switch to infect new host lineages over evolutionary time, in the short term, most endosymbionts are associated with their hosts long enough to be in linkage disequilibrium with their host's mitochondria (Jiggins 2003). While this provides a useful tool to measure the population dynamics of symbionts, it is also likely to affect the process of evolution of host mitochondria. One possible effect is a reduction in the effective population size of the mitochondria, although an increase is also possible under some conditions (Hurst and Jiggins 2005). Since there is an intense selection pressure to evolve resistance to the intracellular parasite, host strains that evolve resistance are likely to have a extremely high fitness and could sweep through a population while the mitochondria haplotype from this strain 'hitchhikes' with it. In addition, gene flow of haplotypes can become unidirectional, from infected to uninfected individuals due to inefficient transmission. In this case, if the symbiont is at equilibrium, this serves to reduce the effective population size of mitochondria (Johnstone and Hurst, 1996).

A similar assymetrical barrier can also arise for the host's nuclear genome. Theoretical models predict that any alleles that arise in infected females cannot spread through the population (Engelstadter and Hurst 2007). In essence, this makes the population dependent on genetic variation generated solely in the uninfected proportion of hosts.

1.4 *Insights from genomes*

While the effects of endosymbionts on hosts are therefore huge, the influence of the endosymbiotic lifestyle can also be detected in the genomes of the bacteria themselves. Indeed, the genomes of bacteria are much easier to study than their wider biology, due to their inability to be cultured outside of their hosts.

To date, around 16 genomes of obligate mutualists of arthropods have been sequenced (Moya et al. 2008). They range from symbionts that have been associated

with their hosts for millions of years (*Buchnera*), to bacteria that have only recently colonised their host's cells (*Sodalis*) (Moran et al. 1993; Weiss et al. 2006). The one overriding feature of these symbiont genomes is their reduced length, compared to those of free-living bacteria. Indeed, because of their lifestyle, they have lost all but the most essential genes for survival (Itoh et al. 2002). A second feature of these genomes is their much higher AT content (Sallstrom and Andersson 2005). In addition, these genomes often reveal the different nutritional supplements they are providing for their hosts (Moran and Wernegreen 2000).

Of the symbionts that manipulate their hosts, genome sequences are available for two bacteria that cause CI (*Wolbachia* of *Drosophila melanogaster* and of *Culex pipiens*), and one for a symbiont that causes a sex ratio distortion in its mite host (*Orientia tsutsugamushi*) (Wu et al. 2004; Cho et al. 2007; Klasson et al. 2008). These genomes are distinct from those of the obligate mutualists as they display a high degree of genomic plasticity, and have many transposases. 14% of the genome of *Wolbachia* from *D. melanogaster* consists of repetitive DNA and mobile elements, compared to 5.4 % of the genome of *Wolbachia* from *Brugia malayi* (which is mutualistic) (Wu et al. 2004; Foster et al. 2005); for *O. tsutsugamushi*, the figure is an astonishing 37.1%, which is on a par with the human genome (Cho et al. 2007). Although, there may be a selection pressure for evolutionary novelty, it appears that these endosymbionts are not effective at purging selfish genetic elements.

1.5 *Aims and outline of thesis*

The broad purpose of this thesis is to explore the incidence, diversity, genetics and wider biology of reproductive parasites, with the ultimate goal of understanding the role of these parasites in the evolution of their arthropod hosts. Chapters 3, 5 and 7 investigate a variety of bacteria, but a major focus throughout will be the genus *Rickettsia*, which has received much less attention than *Wolbachia*. Chapter 3 begins by demonstrating that the diversity of endosymbionts in arthropod populations has been underestimated, concentrating on a family of Coleoptera with an ecology

known to favour male-killing. Chapter 4 then increases the taxonomic scope, assessing the incidence of *Rickettsia* in worldwide screen. Chapter 5 undertakes a meta-analysis of screen data from *Rickettsia*, *Wolbachia* and *Cardinium*, using features of the distribution of prevalences across species to make inferences about host manipulation phenotypes. Chapter 6 investigates the evolution of *Rickettsia* with a phylogenetic approach, highlighting the different life history traits, and testing for recombination. Chapter 7 then explores the topic of host-switching in more detail, demonstrating that host jumps tend to occur between closely related species. Finally, Chapter 8 investigates the evolution of plasmids in *Rickettsia* strains, demonstrating extensive horizontal gene transfer.

Chapter 2. Methods

2.1 Primer sequences

Table 2.1 List of primer sequenced used in this thesis. Annealing temperature and extension time are given for the forward primers only.

Name	Target gene	Chapter	Sequence	Annealing temp	Extension time	Reference
BD1	*ITS region*	3	GTCGTAACAAGGT TTCCGTA	55°C	1 min	von der Schulenburg *et al.* 2001
4S	*ITS region*	3	TCTAGATGCGTTC GAAATGTCGATG			von der Schulenburg *et al.* 2001
WSP81F	*Wolbachia wsp*	3	TGGTCCAATAAGT GATGAAGAAAC	58°C	45 sec	Braig et al. 1998
WSP691R	*Wolbachia wsp*	3	AAAAATTAAACGC TACTCCA			Braig et al. 1998
RSSUF	*Rickettsia 16S*	3,4	CGGCTTTCAAAAC TACTAATCTA	58°C	1 min	von der Schulenburg *et al.* 2001
RSSUR	*Rickettsia 16S*	3, 4, 6	GAAAGCATCTCTG CGATCCG			von der Schulenburg *et al.* 2001
MGSO	*Spiroplasma 16S*	3	TGCACCATCTGTC ACTCTGTTAACCT C	57°C	30 sec	van Kuppeveld et al. 1992
HAIN1	*Spiroplasma 16S*	3	GCTCAACCCCTAA CCGCC			Hurst et al. 1998
FL1	*Flavobacteria 16S*	3	ATTGTTAAAGTTC	60°C	1 min	Hurst et al. 1997

Name	Target gene	Chapter	Sequence	Annealing temp	Extension time	Reference
			CGGCG			
FL2	*Flavobacteria 16S*	3	CTGTTTCCAGCTTA TTCGTAGTAC			Hurst et al. 1997
27F	*bacterial 16S*	6	AGAGTTTGATCCT GGCTCAG	58°C	1 min	Lane 1991
ATPAF2	*atpA*	6	ATCAAGCGTTGCA CAGATAG	52°C	1 min	this study
VITR	*atpA*	6	CGACTTACCGAAA TACCGAC			Vitorino *et al.* 2007
ATPA536R	*atpA*	6	GGAAGTGCCGTAA GTGAACC			this study
RCIT133F	*gltA*	6	GGTTTTATGTCTAC TGCTTCKTG	52°C	1 min	Davis *et al.* 1998
RCIT1197R	*gltA*	6	CATTTCTTTCCATT GTGCCATC			Davis *et al.* 1998
COXAF2	*coxA*	6	ACAGCCGTTGATA TGGCTA	55°C	1 min	this study
COXA1413R	*coxA*	6	CATATTCCAACCG GCAAAAG			this study
COXA322F	*coxA*	6	GGTGCTCCTGATA TGGCATT	55°C	1 min	this study
COXAR1	*coxA*	6	CATATTCCAGCCG GCAAAAG			this study
CI-J-2630	mitochondrial *COI*	7	CTTTCTATAGGAG CTGTATTTGC	55°C	1 min	Jiggins 2003
T12-N-3014	mitochondrial *COI*	7	CCAATGCACTAAT CTGCCATATTA			Simon et al. 1994

Name	Target gene	Chapter	Sequence	Annealing temp	Extension time	Reference
LCO1940LB	mitochondrial *COI*	7	GGTCAACAAATCATAAAGATATTGG	55°C	1 min	Folmer et al. 1994
CI-N-910I	mitochondrial *COI*	7	GCAATAATTATTGTAGCAGAGGTAAA			this study
CI-J-1718	mitochondrial *COI*	7	GGAGGATTTGGAAATTGATTAGTTCC	55°C	1 min	Simon et al. 1994
CI-N-856	mitochondrial *COI*	7	GTAAATATGTGATGAGCTCAAAC			this study
TRAD_PRF37F1	*TraD felis-type*	8	AAAAAGCAGTAGCCTTTGATCG	57°C	45 sec	Ogata et al. 2005
TRAD_PRF37R1	*TraD felis-type*	8	AGCTGCTGACCTTTACTTTTCC			Ogata et al. 2005
TRAD_F	*TraD felis-type*	8	AGTAACATTCCGTAAAGAATATG	TD[i] 58-48°C	1 min	this study
TRAD_R	*TraD felis-type*	8	GCGTCTTCAAAGCCTTCAGG			this study
TRAA_F	*TraA felis-type*	8	AGAGCTATGGGACGCTTTGC	TD 58-48°C	1 min	this study
TRAA_R	*TraA felis-type*	8	CCTTTCATCAGCGACAGCAT			this study
TRAAI_F	*TraA felis-type*	8	ATGGAACGGAGCAGAAGCAA	TD 58-48°C	1 min	this study
TRAAI_R	*TraA felis-type*	8	TCGCCATTCTCTAATCGCTC			this study
TRAA BELLII F	*TraA bellii-type*	8	TGGCACAGCAGAAAATATCG	TD 58-48°C	1 min	this study
TRAA BELLII R	*TraA bellii-type*	8	GAGATGGCTTTTGCCTT			this study

Name	Target gene	Chapter	Sequence	Annealing temp	Extension time	Reference
			GAA			
TRADTI BELLII F	*TraD bellii-type*	8	ATTTTTGCCGATTTTAGTCC	TD 58-48°C	1 min	this study
TRADTI BELLII R	*TraD bellii-type*	8	CGTAGATTGAAATTAGAACAA			this study
TRAD BELLII F	*TraD bellii-type*	8	TTAGTAATTGGGATGTTACC	TD 58-48°C	1 min	this study
TRAD BELLII R	*TraD bellii-type*	8	ACAGCACATAAATCAGCTTT			this study
TRAG BELLII F	*TraG bellii-type*	8	TGATCGCTTTGCTACTTATT	TD 58-48°C	1 min	this study
TRAG BELLII R	*TraG bellii-type*	8	CAATATTACTAATAGCAGCTTGGTC			this study
TRAH BELLII F	*TraH bellii-type*	8	TTAAGCCCTCTCTTTAGCGA	TD 58-48°C	1 min	this study
TRAH BELLII R	*TraH bellii-type*	8	CCTCAGAGGTAAGAAAAGCA			this study
TRAF BELLII F	*TraF bellii-type*	8	CCTTTAGTCTATTATAGTGCTG	TD 58-48°C	1 min	this study
TRAF BELLII R	*TraF bellii-type*	8	GCAGATAAATATGGCTTTCA			this study
TRAN BELLII F	*TraN bellii-type*	8	TTTGTTGTTGCATTATAGGC	TD 58-48°C	1 min	this study
TRAN BELLII R	*TraN bellii-type*	8	GCTATTAGTTTTGGCACAGG			this study
TRAU BELLII F	*TraU bellii-type*	8	TCAAAAGAGACAGCAACTGC	TD 58-48°C	1 min	this study

Name	Target gene	Chapter	Sequence	Annealing temp	Extension time	Reference
TRAU BELLII R	*TraU bellii-type*	8	GGAAGAGAAGGA TTATGTGG			this study
TRAW BELLII F	*TraW bellii-type*	8	TTTTAAGTAATTTA CCTTCCTGCCG	TD 58-48°C	1 min	this study
TRAW BELLII R	*TraW bellii-type*	8	GCTGGGGAAAAGC TTTAATATT			this study
TRAV BELLII F	*TraV bellii-type*	8	ATTATACCTTCCA AAGCATC	TD 58-48°C	1 min	this study
TRAV BELLII R	*TraV bellii-type*	8	CTTTAGCGTTGCT AATTGTA			this study
TRAB BELLII F	*TraB bellii-type*	8	TAAAAATTACCCG GATGTTG	TD 58-48°C	1 min	this study
TRAB BELLII R	*TraB bellii-type*	8	AGAGAGAATAAA GAAGCTGC			this study
TRAD2_F2	*TraD felis-type*	8	ATCGGCAATGATG CTAGGTG	TD 58-48°C	1 min	this study
TRAD2_F3	*TraD felis-type*	8	CGCAAAACCCAAG AAAGTCT	TD 58-48°C	1 min	this study
TRA10SP_F	*TraA felis-type*	8	TTGTTGGAGATAA CAGCCAGTTT	TD 58-48°C	1 min	this study
TRA10SP_F2	*TraA felis-type*	8	TGGACTATCTAGC TCACGAGGTC	TD 58-48°C	1 min	this study
TRAAICREAM_F	*TraA felis-type*	8	CTTTGCCGCGTGA AGTAAGT	TD 58-48°C	1 min	this study
TRAACREAM_R	*TraA felis-type*	8	ATCTCAATGGCTT CCTGCAT			this study
TRAAICRUFA2	*TraA felis-type*	8	CGAGGCATAGTAT	TD 58-	1 min	this study

Name	Target gene	Chapter	Sequence	Annealing temp	Extension time	Reference
4SP_F			GTGACGTT	48°C		
TRAACRUFA_R	*TraA felis-type*	8	ATCTCAATGGCTT CCTGCAT			this study
TRAD37CRUFA _R	*TraD felis-type*	8	GAGCATTATCAGG GTGCAAG			this study
TRAA24SP_R	*TraA felis-type*	8	TTAGCAGTGGCGG TAGAATG			this study
TRAD3724SP_R	*TraD felis-type*	8	TGAGCATTATCAG GGTGCAG			this study
TRAAIBU_R	*TraA felis-type*	8	GCGCAAATTCTAT TTCTGATGC			this study
TRAD37BU_R	*TraD felis-type*	8	CTTGCACCCTGAT AATGCTC			this study

[1] TD indicates a touch down PCR where 10 cycles decended in annealing temperature and the remaining cycles were performed at the lowest temperature

Chapter 3. The diversity and incidence of insect bacterial symbionts: what have previous studies missed?

3.1 *Introduction*

Symbiotic bacteria that are transmitted vertically from mother to offspring are common among arthropods. Some of these associations are essential for host survival and can persist for millions of years (Chen et al. 1999; Baldo et al. 2006). Other symbionts form shorter lived associations with their hosts, and may only infect a small proportion of host populations. Some of these are facultative mutualists, such as symbionts that make their hosts resistant to parasitoid wasps (Oliver et al. 2003). Others manipulate their host's reproduction in ways that enhance their transmission, such as distorting the host's sex ratio towards females, the sex that will transmit the bacteria on to the next generation. The discovery that symbionts in the genus *Wolbachia* infect about 17% of insects (Werren et al. 1995b), has simulated research into many aspects of symbiont biology, and prompted more surveys of symbiont diversity across various arthropod groups. These surveys not only replicated the original finding that *Wolbachia* is common, but also found that other symbionts such as *Cardinium* are widespread (Zchori-Fein and Perlman 2004), and that many *Wolbachia* infections only infect a small proportion of the host population (Jiggins et al. 2001; Tagami and Miura 2004).

Current knowledge of symbiont diversity may be unreliable. Previous surveys have generally examined small samples of individuals to screen many species for just a single bacterial taxon (Werren et al. 1995b; Weeks et al. 2003; Zchori-Fein and Perlman 2004). This will naturally detect highly prevalent infections, but means that we know little about symbionts that have a lower prevalence or whether a single host species is typically infected by one or many symbionts. Other studies have surveyed only a few host species, preventing assessment of interspecific diversity (Tsuchida et

al. 2002; Haynes et al. 2003). Screens have also tested primarily for *Wolbachia* or *Cardinium,* whereas the diversity of symbiotic associations is probably far greater. These studies have also tended to sample species where little is known about host ecology, making it difficult to draw conclusions about the factors that determine the distribution of symbionts. Despite being more time consuming, testing large samples of each host species for a range of bacterial symbionts will give us a more accurate picture of the diversity of symbionts both between and within species.

I investigated the diversity of bacterial symbionts in ladybird beetles (Coccinelidae). Ladybirds are particularly predisposed to male-killing bacteria as they lay their eggs in clutches and sibling cannibalism is common (Hurst and Jiggins 2000). Benefits of male-killing may include reduced sibling competition, inbreeding avoidance, evading cannibalism by brothers and opportunities to consume male eggs. Because these factors are determined by host ecology, male-killer distribution is thought to be driven by ecological parameters. Furthermore, ecological differences between ladybird species exist, which can be used to test hypotheses of male-killer invasion (Majerus and Hurst 1997).

In previous studies, male-killers in ladybirds have been identified by detecting a skewed sex ratio and then testing for the presence of the bacteria (Hurst et al. 1996). This has led to the discovery of male-killers from four different bacterial genera (*Wolbachia*, *Rickettsia*, *Spiroplasma* and Flavobacteria spp.) in ten species of ladybirds. Currently, *Wolbachia* are known to infect one species, *Rickettsia* and *Spiroplasma* infect three different species and Flavobacteria spp. infect five species of ladybird. The most extensively studied ladybird, *Adalia bipunctata*, has male-killers from three different families and despite gene flow between populations, exhibits bacterial heterogeneity across populations. However, the diversity of male-killers in ladybirds remains unclear. Only a few lines can be tested at a time as breeding is labour intensive, and so low prevalence male-killers will remain undetected. Furthermore, there is a large publication bias towards reporting only the positive results. In this study, I used polymerase chain reaction (PCR) to screen large numbers of 21 different species of wild-caught ladybird for the four bacterial genera

known to contain male-killers. As well as establishing where different symbionts occur in the family Coccinelidae, this study enabled us to detect between species patterns and within species patterns of symbiotic diversity.

3.2 *Materials and Methods*

3.2.1 Ladybirds

Twenty-one ladybird species were collected from the locations in Table 3.1 by beating vegetation whilst holding a collection tray underneath or sweeping vegetation with nets (thereby eliminating visual bias in collection rates). All species samples contained 20 females or more, providing a 90% chance of detecting infections at 12% prevalence or over; in most cases considerably larger sample sizes were used.

Sex was determined using morphology of the posterior abdominal tergite or the presence/absence of a sclerotised sipho seen with an underlighted microscope; criteria were verified by genital dissection. Sterile blades were used to remove an abdominal section for DNA extraction and the remainder preserved in ethanol.

3.2.2 DNA extraction and PCR

DNA was extracted using the Chelex method (Walsh et al. 1991) or using DNeasy columns for animal tissues (Qiagen, Valencia, CA). Samples extracted using columns were pooled with 5 ladybirds per column. The ribosomal DNA ITS region was amplified using BD1 and 4S (von der Schulenburg *et al.* 2001) from each sample to verify successful DNA extraction. Samples failing to yield a PCR product were discarded. Samples were then tested for *Wolbachia* presence using *wsp*81f and *wsp*691r ; *Rickettsia* using RSSUF and RSSUR; *Spiroplasma* using HaIn1 and MGSO; and Flavobacteria species using FL1 and FL2 (all primer sequences are given in chapter 2). Pooled samples that tested positive for any bacteria were then extracted separately to measure bacterial prevalence. Since accurate DNA extraction

for individual ladybirds in these samples cannot be confirmed, the bacterial prevalence estimated here is conservative.

3.3 *Results*

3.3.1 Symbiont diversity

I tested 2149 ladybirds from 21 different species for the presence of four bacterial genera that are known to cause male-killing in ladybirds (Table 3.1). Over half the species (11 of 21) were infected with at least one of the symbionts. *Rickettsia* were found in eight species, *Wolbachia* in six, and *Spiroplasma* in three species. No species were infected with Flavobacteria. The relative incidence of the four bacteria was significantly different to what has previously been found (χ^2= 10.3; *d.f.*= 3; p=0.016; previous work described in Introduction). On average, each host species was infected by 0.8 different symbionts.

Of the 11 infected species, six were infected by two different symbionts and, in all cases, both bacteria were found in a single population. These double infections fell in to two categories. In four of the ladybird species, the two different bacteria never infected the same individual. However, in *R. litura* and *C. rufa* both singly and doubly infected individuals were found. In *R. litura* infected individuals, there was an excess of double infections suggesting infection frequencies are not random in the population (χ^2=88.7; *d.f.*= 1; p<0.000).

3.3.2 Bacterial prevalence

The bacterial prevalence was very variable, ranging from 1% to 89%, with a median infection level of 5%. There are striking differences in the prevalence of symbionts in males and females (Table 3.1). Nine of the symbionts occurred in only females, compared to one which was only in males. Allowing a false discovery rate of 10% to correct for multiple tests (Benjamini and Hochberg 1995), of the 19 different infected populations, 10 had a significantly higher bacterial prevalence in females whereas one population had a higher prevalence in males (populations of the same species

with significantly different prevalence treated separately; uncorrected p-values shown in Table 3.1).

Table 3.1. Bacterial symbiont infection of ladybirds

Species name	Location[i]	Date (month/year)	Sample size	Sex Ratio[ii]	Sex ratio[ii] uninfected	Bacteria	Prevalence in females[iii]	Prevalence in males[iii]
***(a)* Infected**								
Coccinella 7-punctata	Dunwich and Edinburgh, UK	05/04, 09/04	115	0.47	0.49	*Wolbachia*	0.05 (0.01,0.14)	0.00*
Subcoccinella 24-punctata	Braintree, UK	07/04	220	0.51	0.46	*Rickettsia*	0.04 (<0.01,0.11)	0.15*(0.08,0.25)
Scymnus frontalis	UK and Germany	07/06	35	n/a		*Rickettsia*	0.24	0.10
Adalia 2-punctata	Edinburgh, UK	06/04	84	0.27***	0.35*	*Spiroplasma*	0.28 (0.17,0.41)	0.04*** (<0.01-0.22)
						Rickettsia	0.07 (0.02,0.16)	0.00*
	Queenstown, New Zealand	12/04	70	0.50	0.56	*Spiroplasma*	0.29 (0.15,0.46)	0.09* (0.02,0.23)
Anisosticta 19-punctata	Essex, UK	06/04	46	0.37	0.50	*Spiroplasma*	0.41 (0.24,0.61)	0.00***
	Ploen, Germany	05/04	123	0.46	0.46	Uninfected		
Halyzia sedecimguttata	Ploen, Germany	05/04	260	0.38***	0.39***	*Rickettsia*	0.01 (<0.01-0.05)	0.00
						Wolbachia	0.02 (<0.01-0.05)	0.00*
	Somerset, UK	07/04	24	0.50	0.50	Uninfected		

Adalia 10-punctata	Edinburgh, UK	05/04	112	0.52	0.52	*Rickettsia*	0.02 (<0.01,0.10)	0.00
	Piedmonte, Italy	04/05	46	0.41	0.44	*Rickettsia*	0.11 (0.02,0.29)	0.00*
Calvia quattuordecimguttata	Ploen, Germany	05/04	57	0.49	0.49	*Rickettsia*	0.03 (<0.01,0.18)	0.00
		10/04				*Wolbachia*	0.00	0.04 (<0.01,0.18)
Chilocorus bipustulatus	Verona, Italy	06/06	20	0.40	0.41	*Wolbachia*	0.08 (<0.01,0.38)	0.00
		05/04				*Spiroplasma*	0.08 (<0.01,0.38)	0.13 (<0.01,0.53)
	Hathersage, UK	08/05	15	0.40	0.40	Uninfected		
Rhyzobius (Rhizobius) litura	Ploen, Germany	05/04	70	0.37*	1.00**	*Rickettsia*	0.84 (0.70,0.93)	0.62** (0.41,0.80)
		04/04				*Wolbachia*	0.89 (0.75,0.96)	0.15*** (0.04,0.35)
	Thetford, UK	09/04	6	n/a		Uninfected		
Coccidula rufa	Ploen, Germany	07/04	49	0.35*	0.80	*Rickettsia*	0.59 (0.41,0.76)	0.41* (0.18,0.67)
						Wolbachia	0.78 (0.60,0.90)	0.18*** (0.04,0.43)
***(b)* Uninfected**								
Aphidecta obliterata	Edinburgh, UK	07/04-5	44	0.30**				
Exochomus quadripustulatus	Thetford, UK	05/04	95	0.63*				
Tytthaspis 16-punctata	Thetford, UK	07/05	53	0.55				
Propylea 14-punctata	various, UK	05/04	52	0.54				

Anatis ocellata	Edinburgh and Thetford, UK	08/04	65	0.31*
(from pupa)	Thetford, UK	07/04-5	111	0.45
Myzia oblongoguttata	Edinburgh, UK	07/05	85	0.49
Coccinella hieroglyphica	Balmoral, UK	08/04	83	0.55
Harmonia 4-punctata	Thetford, UK	07/04	33	0.30*
Coccinella miranda Wollaston	Tenerife, Spain	06/05	146	0.53
Myrrha octodecimguttata	Edinburgh, UK	05/04	30	0.37
	Murcia, Spain	05/05	67	0.27***

[i]Populations where symbiont type, sex ratio or infection level did not significantly differ were pooled together

[ii]Proportion of males, deviations from a 1:1 sex ratio were tested using an exact binomial goodness of fit test

[iii]Difference in prevalence between males and females was tested using a G-test, binomial confidence intervals are given against infected individuals only

*=p<0.05 **=p<0.01 ***=p<0.001

R. litura and *C. rufa* populations from Germany had much higher prevalence levels than other species, with nearly all individuals infected with *Rickettsia*, *Wolbachia*, or both bacteria. These populations were also unusual in containing large numbers of infected males (although the prevalence is still highest in females).

3.3.3 Population sex ratio

Of the 28 different populations of ladybirds, eight were female biased and one was significantly male biased. Are these sex ratios explained by the symbionts identified as killing males, or are there more male-killers yet to be identified? If the sex ratio is determined by male-killers, then the number of uninfected females will be the same as the number of uninfected males. If there is a large excess of uninfected females, this suggests that there may be other male-killers that are not detected by our assays. I tested whether the ratio of uninfected-males:uninfected-females differed from 1:1 (Table 3.1). Six of 29 populations still had a significant excess of uninfected females, and two had an excess of uninfected males (10% false discovery rate).

3.3.4 Ecology of male-killer invasion

Out of the 21 species of ladybird, 14 are aphidophagous, four feed primarily on other prey such as scale insects and adelgids, two are mycophagous and one is purely herbivorous. The incidence of male-killing in non-aphidophagous ladybirds did not differ from aphidophagous species (Fisher's exact test p=0.3972). However, in the one species of herbivorous ladybird (*Subcoccinella 24-punctata*), males were significantly more infected than females. This is not indicative of male-killing but the exclusion of male-killer incidence in this species did not alter the previous result (Fisher's exact test p=0.1827).

3.4 *Discussion*

I have demonstrated that *Rickettsia*, *Wolbachia*, and *Spiroplasma* bacteria are common among ladybirds. This is the first time that the incidence of *Spiroplasma, Rickettsia* or *Flavobacteria* in insects has been studied extensively, and these results suggest that some of these neglected groups of symbionts may be as common as *Wolbachia.* Many of the symbionts infect a small proportion of the population, and would have been missed by studies that examine only a few individuals of each species. It is therefore likely that both the taxonomic diversity of symbionts and the proportion of insect species infected by symbionts are far greater than previously suspected.

Symbiont diversity may actually be even greater than the data suggest. In the largest samples, I detected bacteria that infect less than 1% of individuals; these would have been missed in my smaller samples. Furthermore, I did not test for the presence of all known bacterial symbiont taxa. I also demonstrate that the bacterial prevalence was insufficient to explain the population sex ratio biases observed, suggesting that there may still be undiscovered diversity of sex ratio distorters. Whilst it is impossible to exclude the possibility that these unexplained female biases resulted from a bias in collection rates towards female beetles, the sampling methods used minimised the likelihood of this occurring.

Why have these symbionts spread through ladybird populations? In most cases, more females than males were infected, suggesting that they are sex ratio distorters. As all sex ratio distorters known in ladybirds are male-killers, it is likely that most of the symbionts are also male-killers. A surprising finding was that many of the bacteria also occur at a lower frequency in males. There have been few studies of whether males can survive male-killer infection in the wild. However, in *Drosophila*, infected males survive at high temperatures, probably because the density of bacteria has been reduced (Hurst *et al.* 2000). The widespread occurrence of infected males in the dataset could result from nuclear genes suppressing the male-killing phenotype, or from environmental effects.

Single populations commonly harboured more that one bacterial taxon. Theory predicts that such polymorphisms will be rare unless negative frequency dependent selection maintains them (Randerson *et al.* 2000). There is evidence that natural selection maintains multiple male-killers in the ladybird *A. bipunctata* (Jiggins and Tinsley 2005). The finding that such polymorphisms are common, lends strength to the hypothesis that bacterial symbionts may commonly be maintained in populations either by negative frequency-dependent selection or because different strains are favoured in different populations.

The relative frequency of the different symbiont taxa was significantly different from previous studies of male-killers in ladybirds, probably reflecting the different screening methods. Interestingly, in previous work Flavobacteria were the commonest male-killers, while in this study they were absent. This could be a consequence of different sampling strategies if Flavobacteria occur at a higher prevalence in fewer species, or in different ladybird species or geographical areas than the other bacteria.

There is an unusual pattern of bacterial infection in *R. litura* and *C. rufa*. These species have female biased population sex ratios, and their symbionts occur predominantly in females, suggesting they are sex ratio distorters. However, they are very atypical of male killers. First, as many as 60% of males are infected, suggesting that any sex ratio distortion is very inefficient. Second, many individuals are co-infected with both *Wolbachia* and *Rickettsia*, and two different male-killers have never been reported from a single individual in any other species. The cause of this pattern is a matter for speculation, but one hypothesis is that this may be evidence of partial suppression the male-killing phenotype. There is also an excess of ladybirds doubly infected with the two symbionts in *R. litura* possibly suggesting a selection pressure for this phenotype. Perhaps this pattern is due to the rate of male-killing being higher in doubly infected individuals.

The difference in feeding habits of the different ladybird species can inform us about the conditions for male-killer invasion. This is because aphid densities are ephemeral and a significant time may elapse between emergence from egg and finding a first meal. Therefore, you would expect to find more male-killers in aphidophagus ladybirds as the benefits of an egg meal are greater (Majerus and Hurst 1997). While other carnivorous (and some mycophagous) ladybirds will feed on aphids, their main form of prey (scale insects and adelgids) are not thought to go through such population crashes. And as eggs will also be laid in the immediate vicinity, the benefits of sibling cannibalism are significantly lowered. However, there was no significant difference in the incidence of male-killing between aphidophagous and non-aphidophagous species. It should be noted however, that the pattern of symbiont infection in the only ladybird species we tested that is purely herbivorous (and strictly non-aphidphagous) (*Subcoccinella 24-puctata*), suggests that the bacterium may not exhibit male-killing. In addition to this, female infection in the two other non-aphidophagous ladybirds (*Halyzia sedecimguttata* and *Chilocorus bipustulatus*), did not significantly differ from male infection. Therefore more experimental evidence will be needed to identify symbionts in these species as displaying the male-killing phenotype.

3.5 *Conclusion*

In conclusion, intensive sampling has uncovered widespread and extensive diversity of bacterial symbionts within one insect clade. These findings demonstrate that the methods employed in previous studies may be biasing the picture of symbiont diversity. Efforts, such as this study, to uncover infection diversity both between and within species may provide more information about what determines symbiont distribution and how they spread through populations.

Chapter 4. The incidence of *Rickettsia* in terrestrial arthropods

4.1 *Introduction*

Historically, endosymbiotic bacteria in arthropods have been detected because of the array of reproductive changes they inflict on their hosts, or because of the diseases they cause in their secondary hosts (as arthropod-vectored pathogens) (Hertig and Wolbach 1924; Yen and Barr 1973). More recently, the advent of PCR has made the detection of such bacteria much easier. Many studies test arthropod DNA for the presence of *Wolbachia* as it is common, and is currently known to produce more phenotypic changes in its host than any other bacteria (Stouthamer et al. 1999). There are also numerous screens of bacteria that cause vertebrate diseases such as *Rickettsia*, *Anaplasma*, *Erlichia*, and *Bartonella*, in blood feeding arthropods (mainly ticks) with the hope of establishing the risk of infection in certain areas (see (Parola and Didier 2001) for a review).

In addition to assessing their abundance, screening arthropods can also give clues as to which types of species are commonly infected. This in turn allows us to make inferences about the different phenotypes endosymbionts may be inflicting on their hosts. *Rickettsia* are unlike other disease-causing endosymbionts, because in addition to being transmitted horizontally, they are also transmitted maternally in many arthropod species. For this reason, *Rickettsia* also distort the sex ratio in some non-blood feeding insects in order to spread through insect populations. Many studies focussing on a single species of arthropod have uncovered new *Rickettsia* infections (Perlman et al. 2006) and currently, *Rickettsia* are known to infect members of the orders Coleoptera, Hemiptera, Hymenoptera, Acari, Siphonaptera, Psocoptera, Collembola and Diptera (Chapter 6).

A recent study showed that although *Wolbachia* infect arthropods at a higher prevalence, other bacteria, such as *Cardinium*, *Arsenophonus*, *Spiroplasma* (ixodes type) are also common (Duron et al. 2008b). This study also tested for the presence of *Rickettsia* but found them to be much rarer than the other bacteria. However, it is likely that Duron et al. (2008) underestimated the prevalence of *Rickettsia*, as the primers they used to amplify *Rickettsia* target only a subset of the diversity of strains (see Chapter 6). In the present study I tested DNA samples of 853 arthropod species from the classes *Arachnida*, *Entognatha*, *Malacostraca* and *Insecta* (Table 1) for the presence of *Rickettsia* using PCR to assess its incidence. These results are then used to make predictions about the phenotypic characteristics of *Rickettsia*-infected hosts.

4.3 *Methods*

4.3.1 Samples

Arthropods were collected from locations in Mexico, Panama, Chile, Ghana, South Africa, Papua New Guinea, USA, France, Spain, Kazakhstan, Russia and India. Samples were fixed in 95% ethanol and were kept at -20C. Most samples were identified to family level, but steps were taken to try to sample morphologically distinct arthropods and so most individuals should represent members of different species. Whole abdomens were dissected in sterile double-distilled deionised water on sterile Petri dishes. DNA was extracted using PureLink columns for animal tissues according to the manufacturer's protocol (Invitrogen, Carlsbad, CA).

4.3.2 Assay for Rickettsia

As a control to check the DNA extraction had been successful, each sample was tested using primers for the highly conserved region of eukaryotic 28S rDNA (Burke et al. 1993; Chapter 2). Any samples that failed to amplify a product were not included in the final screen. A portion of the final extract was then transferred to a 96 well plate to make the process of PCR amplification easier. Samples were then tested for the presence of *Rickettsia* using RSSUF and RSSUR (Chapter 2). A control that was previously shown to be positive for *Rickettsia* and a control that contained sterile

double-distilled deionised water were used in each PCR reaction. Any product observed that was not in the expected 300-400 bp range was discarded. If a sample tested positive for *Rickettsia*, then the original aliquot of DNA was tested to confirm the result and the PCR product was sequenced (Chapter 6).

4.4 *Results*

We tested a total of 853 arthropod species for the presence of *Rickettsia* (Table 4.1) of which nine species (1.1% (binomial confidence intervals 0.5%-2.0%) were found to be infected. These infections were found solely in five of the sixteen orders of the class Insecta; no positives were found in any of the other classes. Does infection frequency differ between insect orders? Of the species that tested positive, all are in the Hemipteroid assemblage or Endopterygota superorder (phylogenetically, these superorders are sister groups). Three species were Coleoptera (3/201), two species were Diptera (2/214), two species were Hemiptera (2/94), one species was Lepidoptera (1/26) and one species was Neuroptera (1/4). Infection levels did not significantly differ across orders (Fisher's exact test; p=0.087). The species were found in the following families; Meloidae, Elateridae, Curculionidae (Coleoptera), Bombyliidae (Diptera), Reduviidae, Cercopidae (Hemiptera), Noctuidae (Leidoptera) and Chrysopidae (Neuroptera).

Does infection frequency differ between locations? Of the nine infected species, three came from Panama (3/44), three came from Papua New Guinea (3/27), two came from Mexico (2/465) and one came from Ghana (1/76). No infections were found in temperate countries. However, the frequency of infection in tropical and temperate countries was not significantly different (Fisher's exact test; p=0.602). With the exception of the two infected species in Diptera, all of the other infections were found in different families of insects. The two species of Diptera were both from the Bombyliidae (bee fly) family. Therefore a portion of the host's mtDNA was sequenced to verify that they were different species (data not shown).

Table 4.1. Distribution of *Rickettsia* in arthropod species. *R* denotes how many species are infected with *Rickettsia*. Class and order of arthropods are given, as well as location collected from (for a full list of families see appendix). The individuals are all different species.

taxon	location	no. of species	*R*
Malacostraca			
Isopoda sp.	Chile	1	-
Arachnida			
Araneae sp.	Mexico	6	-
Holothyrida sp.	Mexico	1	-
Entognatha			
Collembola sp.	Chile	4	-
	South Africa	1	-
Insecta			
Blattodea sp.	Chile	2	-
	Mexico	2	-
	Ghana	1	-
Coleoptera sp.	Mexico	69	-
	Ghana	46	-
	India	29	-
	Panama	18	1
	Papua New Guinea	14	1
	South Africa	14	-
	New York, USA	6	-
	Chile	5	1
Dermaptera sp.	Ghana	1	-
	Chile	1	-
	unknown	1	-
Diptera sp.	Mexico	132	2
	New York, USA	40	-
	Ghana	11	-
	California, USA	8	-
	Michigan, USA	8	-
	South Africa	6	-
	Chile	6	-
	Panama	2	-
	Papua New Guinea	1	-
Hemiptera sp.	Mexico	32	
	Panama	20	1
	Ghana	10	1
	Chile	8	-
	Papua New Guinea	8	-
	India	7	-
	South Africa	5	-
	New York, USA	3	-
	unknown	1	-
Hymenoptera sp.	Mexico	201	-
	New York, USA	10	-
	India	7	-
	California, USA	3	-
	Ghana	3	-
	Florida, USA	3	-
	France	2	-
	Papua New Guinea	2	-
	unknown	2	-
	Spain	1	-
	South Africa	1	-
	Kazakhastan	1	-
	Russia	1	-
Lepidoptera sp.	Mexico	9	-
	New York, USA	7	-
	Ghana	3	-
	Chile	2	-
	Panama	2	-
	South Africa	1	-
	India	1	-
	Papua New Guinea	1	1
Mantodea sp.	South Africa	1	-
Neuroptera sp.	Mexico	4	1
Odonata sp.	Mexico	5	-
	Spain	3	-
Orthoptera sp.	Spain	2	-
	Mexico	3	-
	Ghana	1	-
	South Africa	1	-
	Panama	2	-
Psocoptera sp.	Chile	1	-
Siphonaptera sp.	Chile	1	-
Strepsiptera sp.	Chile	1	-
Thysanoptera sp.	South Africa	5	-
	Chile	2	-
	Mexico	1	-
	Papua New Guinea	1	-
Trichoptera sp.	South Africa	1	-

4.5 *Discussion*

This study indicates that *Rickettsia* are rare compared to other endosymbionts in arthropod populations. Current estimates are that *Wolbachia* infect 17-20% of arthropod individuals (Werren et al. 1995b; Werren and Windsor 2000), *Cardinium* 6-8% (Weeks et al. 2003; Zchori-Fein and Perlman 2004), *Arsenophonus* 5% and *Spiroplasma* (ixodes-type) 3% (Duron et al. 2008b). We detected *Rickettsia* in only 1% of individuals. In addition, approximately 100 individuals in this study were also assayed for the presence of *Wolbachia*, *Cardinium*, *Arsenophonus* and *Spiroplasma* (L. Weinert and J. Werren unpublished data), and all were found at higher prevalence (although, with the exception of *Wolbachia*, the end products were not sequenced and therefore could have resulted in false positives). While our findings therefore support the major conclusion of Duron et al. (2008), it is also true that the incidence of *Rickettsia* in the present study is much higher than that found by Duron et al. 2008 (1% compared with 0.04%), which is consistent with the fact that the primers used here target a much broader range of *Rickettsia*.

There are two reasons why results for *Rickettsia* might differ from those for other bacteria. First, there could genuinely be a lower incidence of *Rickettsia* across arthropod species. Although *Rickettsia* are known to infect all the major orders of arthropods, they may be restricted to species with a particular ecology or sex determination system, as the most intensively studied *Rickettsia* require specific conditions to allow them to invade a population. To be able to transmit horizontally through a vertebrate, they must infect blood-feeding species of arthropods. In addition, in order for the known sex ratio distortion traits to be adaptive, male-killing *Rickettsia* need to infect species with a permissive ecology, such as those with antagonistic sibling interactions (Hurst 1991). Parthenogenesis-inducing symbionts are currently only known to infect species with a haplodipoid sex determination system (Stouthamer 1997).

The second explanation, which is not mutually exclusive of the first, is that the low prevalence could reflect the difference in epidemiology of the different reproductive phenotypes. Male-killers are usually found at a low prevalence (Hurst and Jiggins 2000), whereas parthenogenesis induction and cytoplasmic incompatibility are often close to fixation in the species they infect (O'Neill et al. 1997). As mentioned in Chapter 3, low prevalence infections will be very difficult to detect in studies that screen single individuals of large species. Discriminating between these different hypotheses will form the basis of the Chapter 5.

A second aspect of the results reported here is that *Rickettsia* were detected in two orders not been previously known to harbour *Rickettsia* infections (Lepidoptera and Neuroptera). However, *Rickettsia* do not appear to cluster within different orders of hosts, making inferences about their phenotypic effects difficult. No *Rickettsia* were found in blood feeders although a small proportion of the Reduviidae family are known to blood feed and transmit disease (Uribe 1926), but a better level of identification would be required to see whether this is the case here. Of the different families, male-killing is thought to occur within Noctuidae and is thought to be particularly common in a subfamily of Curculionidae (Scolytinae) (Hurst 1991), which suggests that the *Rickettsia* infecting these individuals may be male-killers. None of the infected hosts have a haplodiploid sex determination system, suggesting that none of these *Rickettsia* induce parthenogenesis. Two members of Bombyliids were infected, which might indicate that they may be common in this family. However, they parasitise other insects as larva and nectar feed as adults, so there does not appear to be any particulars of their ecology as to why *Rickettsia* may be common. However, the phenotypic effects of most *Rickettsia* are still not known (Chapter 6).

The incidence of *Rickettsia* in ticks has a worldwide distribution, but different strains dominate in different localities (Korch 1994; Parola and Didier 2001). Here, I found no significant difference between the incidence of *Rickettsia* in temperate and tropical countries. This may genuinely indicate that arthropod *Rickettsia* have no tendency to cluster geographically but could also be explained by reduced statistical

power due to low samples sizes for infected individuals. However, the same finding is observed for *Wolbachia*, where temperate and tropical arthropod populations have similar incidences (Werren and Windsor 2000).

Chapter 5. The incidence and distribution of prevalence of *Wolbachia*, *Rickettsia* and *Cardinium* across arthropod species

5.1 *Introduction*

In order to understand the population dynamics of bacterial endosymbionts in arthropod populations, it is first necessary to investigate where symbionts are found (incidence) and the proportion of individuals infected (prevalence). These quantities are thought to be intimately related to the biology of the host species, particularly for endosymbionts that manipulate their host's reproduction. For example, incidence is thought to be determined largely by host ecology and genetics, and prevalence by reproductive phenotype. Accordingly, incidence and prevalence data can be used to test predictions relating to symbiont invasion and spread.

Here, I present a meta-analysis of existing data to estimate the distribution of prevalences of three bacterial endosymbionts across different arthropod species (Hilgenboecker et al. 2008) using a maximum likelihood approach. These distributions are then used to estimate the proportion of arthropod species infected, highlighting differences between different bacteria and host taxa, and to test and make predictions about endosymbiont mediated phenotype.

5.1.1 Predictors of prevalence and incidence

Table 5.1 lists the major factors thought to influence the incidence and prevalence of endosymbionts in arthropod populations. Male-killing is thought to spread in arthropod species when the death of male increases the survivorship of female siblings (Hurst 1991). Therefore, male-killers should be common in species with antagonistic sibling interactions. Observations of male-killer prevalence indicates

that they are generally found at low prevalence with only a few exceptions (Hurst and Jiggins 2000; Jiggins et al. 2000; Dyson and Hurst 2004).

In contrast, endosymbiont-induced parthenogenesis is determined by host genetics as it is currently only found in species where unfertilised individuals develop in to haploid males and fertilised individuals develop in to diploid females (haplodiploid) (Stouthamer 1997). This is probably due to the mechanism of endosymbiont parthenogenesis induction, which in all known cases, is due to the restoration of diploidy through gamete duplication (Stouthamer et al. 1999) (although, see Weeks and Breeuwer 2001).

Endosymbiont-induced feminisation on the other hand is restricted to hosts with a labile sex determination system, which is usually determined by the presence of a single hormone (Rigaud 1997). Although, *Wolbachia*-induced feminisation is known in a leafhopper and a butterfly, most cases are restricted to isopods (Bouchon et al. 1998; Kageyama et al. 2002; Negri et al. 2006).

Endosymbiont-induced cytoplasmic incompatibility is widespread throughout arthropods, and is known to be present among the orders Diptera, Coleoptera, Hemiptera, Hymenoptera, Orthoptera, Lepidoptera, Isopoda and the class Arachnida (Bourtzis et al. 2003). In addition, in order to invade an arthropod population, infection levels must reach a certain threshold, as there are no stable equilibria below a 50% prevalence (Turelli and Hoffmann 1991). Therefore, cytoplasmic incompatibility is thought to be the most common reproductive phenotype in arthropod populations.

Symbionts which are required for egg production (oogenesis) in arthropods are not thought to be mutualists, but parasites that have manipulated their hosts to be become dependent on them without bringing any further benefits (Pannebakker et al. 2007). It is not known how these symbionts invade their host population, but is thought to be uncommon, as currently, only three species of arthropods are known to be manipulated in this way (Dedeine et al. 2001; Perotti et al. 2006; Zchori-Fein et

al. 2006). Observation of prevalence in field populations of this type of manipulation indicates that this phenotype is at fixation in the species it infects (Dedeine et al. 2004).

Table 5.1. Determinants of incidence and prevalence according to phenotype

Reproductive phenotype	Bacteria[a]	Determinants of incidence	Ref	Typical prevalence	Ref
Male-killing	*Wolbachia*, *Rickettsia*	Ecology	(Hurst 1991)	Low	Reviewed in (Hurst and Jiggins 2000)
Parthenogenesis induction	*Wolbachia*, *Rickettsia*, *Cardinium*	Host genetics	(Stouthamer 1997)	Usually high	(Stouthamer et al. 2001)
Feminisation	*Wolbachia*, *Cardinium*	Host sex determination	(Rigaud 1997)	Low	(Bouchon et al. 1998)
Cytoplasmic Incompatibility	*Wolbachia*, *Cardinium*	Widespread: No known restrictions	(Bourtzis et al. 2003)	High.	Reviewed in (Hoffmann and Turellii 1997)
Required for oogenesis	*Wolbachia*, *Rickettsia*	Unknown	(Dedeine et al. 2001; Perotti et al. 2006; Zchori-Fein et al. 2006)	High	(Dedeine et al. 2004)
Obligate mutualism		Host ecology	(Douglas 1994)	High	(Douglas 1994)
Facultative mutualism	*Wolbachia*	Unknown	(Dedeine et al. 2003)	Variable	(Dedeine et al. 2003)

[a] Only *Wolbachia*, *Rickettsia* or *Cardinium* listed even though other bacteria are known

Finally, there are a few cases where *Wolbachia* strains appear to have a positive influence on their hosts, and the prevalence is variable (Dedeine et al. 2003). However, obligatory symbiosis, where the host requires the symbiont for survival, has not been demonstrated in *Wolbachia*, *Rickettsia*, or *Spiroplasma* and given that host phylogeny shows that these symbionts often move horizontally (Chapter 7), is not predicted to occur in these bacteria.

5.1.2 Studies of prevalence and incidence

There are a multitude of studies that have screened for endosymbionts in wild populations of arthropods. Most of these studies screen arthropods for bacterial DNA using primers that are specific to a particular bacterium, because universal primers may amplify up other kinds of bacteria (such as gut commensals). *Wolbachia* is the most widely screened bacterium although more data is accumulating for other species groups (Duron et al. 2008b). Accordingly, this study uses data from the three most widely sampled bacteria: *Wolbachia* (Werren et al. 1995; Werren and Windsor 2000), *Rickettsia* (Chapter 4) and *Cardinium* (Weeks et al. 2003; Zchori-Fein and Perlman 2004).

Most of the studies used here fall into two broad types. First, there are many studies that screen single individuals of many different species. These "single-individual" studies allow us to estimate the mean prevalence of endosymbiont infection across a species group, but give us no information on between-species variation in prevalence levels. A second group of studies screen multiple individuals of a single species, allowing us to estimate within-species prevalence. However, these "multi-individual" studies are more likely to be carried out on species already known to be infected, and so estimates of mean prevalence obtained from multi-individual screens may be upwardly biased (Hilgenboecker et al. 2008). In addition, a small number of recent studies combine both virtues, screening many individuals from a large number of species (Chapter 3; Mateos et al. 2006; Duron et al. 2008b). By combining data from all of these studies, we can estimate both mean prevalence levels, and between-species variation in prevalence of our three bacteria. These

estimates are then used to estimate bacterial strain richness, test theoretical predictions of symbiont invasion and also predict the phenotype that these symbionts are inflicting on their hosts.

5.2 *Methods*

5.2.1 Data collection

The data were collated from 50 studies that screened *Wolbachia* and/or *Rickettsia* and/or *Cardinium* from field collected arthropods representing over 43,000 individual arthropods screened. This data is summarised in Table 5.2. In addition to testing the data for biases (see below), many studies were excluded on *a priori* grounds. For example, I excluded studies that used 'long pcr' as this method has been previously shown to give anomalously high rates of *Wolbachia* infection (Jeyaprakash and Hoy 2000; Hoy et al. 2003; Meyer and Hoy 2008), and studies testing only a single population of one species, which are likely to be most biased. In addition, to lessen non-independence between the data, I included only a single population per species (or per genus or family when species-level identification was absent), retaining only the population (or species) with the most individuals sampled. (All results were also repeated with the complete data set, and were qualitatively unchanged). There will also be intrinsic biases due to differences in field collection methods and different approaches to screening DNA for bacteria (e.g. different primers). However, the large amount of data collected means that no one bias should dominate the signal.

Table 5.2. Summary of arthropod class and order with number of species and individuals tested[a].

		no. individuals tested (positive)		
taxa	no. of species	*Wolbachia*	*Rickettsia*	*Cardinium*
Arachnida				
Araneae	152	770 (115)	449	466 (54)
Ixodida	58	188	10026 (1375)	187
Mesostigmata	21	51 (6)	-	22
Opiliones	1	16	16	16
Oribatida	1	1 (1)	-	1 (1)
Prostigmata	66	442 (89)	-	14 (6)
Scorpiones	1	1	1	1
Crustacea				
Amphipoda	12	55	-	-
Decapoda	5	13	-	-
Isopoda	64	669 (123)	10	10
Entognatha				
Collembola	9	4 (1)	5	4
Insecta				
Astigmata	3	3	-	3
Blattaria	8	28	5	23
Coleoptera	425	3088 (122)	2385 (115)	44
Dermaptera	5	17	3	16
Diptera	571	3083 (600)	1688 (8)	1823
Ephemeroptera	2	2	-	-
Hemiptera	410	3039 (663)	1261 (38)	322 (3)
Holothyrida	1	-	1	-
Hymenoptera	686	4676 (2200)	1584 (6)	200 (7)
Isoptera	2	2	-	1
Lepidoptera	218	1571 (313)	38 (1)	9
Mantodea	4	11	9	9
Mecoptera	1	1	-	-
Neuroptera	7	3 (1)	4 (1)	3
Odonata	43	450 (6)	8	21
Orthoptera	38	233 (19)	55	75
Phasmida	2	2	-	2
Psocoptera	17	7	38 (2)	7
Siphonaptera	14	1011 (405)	344 (56)	-
Strepsiptera	3	2	1	2
Thysanoptera	20	36 (12)	9	7
Thysanura	1	1	-	-
Trichoptera	1	-	1	-
Myriapoda				
Chilipoda	1	1	-	-
Diplopoda	2	4	-	1

[a]Data collected from the following studies: (Werren et al. 1995; Breeuwer and Jacobs 1996; Bouchon et al. 1998; Hariri et al. 1998; West et al. 1998; Plantard et al. 1999; Rydkina et al. 1999; Cheng et al.

2000; Werren and Windsor 2000; Jiggins et al. 2001; Van Borm et al. 2001; Rokas et al. 2002; Shoemaker et al. 2002; Tsuchida et al. 2002; Gorham et al. 2003; Gotoh et al. 2003; Haynes et al. 2003; Kikuchi and Fukatsu 2003; Kittayapong et al. 2003; Nirgianaki et al. 2003; Parola et al. 2003; Rolain et al. 2003; Thipaksorn et al. 2003; Weeks et al. 2003; Blair et al. 2004; Hartelt et al. 2004; Rasgon and Scott 2004; Tagami and Miura 2004; Zchori-Fein and Perlman 2004; Kyei-Poku et al. 2005; Reeves et al. 2005; Goodacre et al. 2006; Kim et al. 2006; Loftis et al. 2006; Mateos et al. 2006; Oteo et al. 2006; Prakash and Puttaraju 2006; Reeves et al. 2006; Enigl and Schausberger 2007; Nijhof et al. 2007; Duron et al. 2008b; Duron et al. 2008a; Hornok et al. 2008; Mura et al. 2008; Sarih et al. 2008 A. Aebi and G. Stone unpublished; Chapter 3; Chapter 4)

5.2.2 Statistical methods

To analyse the data, a refinement of the method of Hilgenboecker et al. 2008 was used. The approach assumes that sampling of individuals within each species is random. If this is true, in a sample of n individuals from a species with prevalence q ($0 \leq q \leq 1$), the number infected will follow a binomial distribution with parameters n and q. The approach allows both sample size and prevalence to vary from species to species, but assumes that the distribution of prevalences across all species can be described by a Beta distribution. The Beta distribution is quite flexible, and depending on the values of its two parameters, can be unimodal with a single peak anywhere on the range [0-1], uniform across that range, or bimodal with peaks at 0 and 1.

The Beta distribution was parameterised in terms of the mean prevalence across species, denoted $\mu=E[q]$, and a parameter denoted ρ that describes the correlation in infection probability among conspecifics, or, equivalently, the increase in the sample variance attributable to between-species differences in prevalence: $Var[q] = \mu(1-\mu)\rho$. Given these assumptions, the likelihood of obtaining the screen data is a Beta-binomial distribution, with parameters μ, and ρ (see Appendix 2).

The method then consists of the following three stages. (1) The data from each of the bacterial screens (both single-individual and multi-individual) is used to obtain Maximum Likelihood estimates of the parameters μ and ρ; (2) A second

model is fit in which μ and ρ apply solely to the multi-individual studies, and a third parameter, μ_S, models the mean infection prevalence in the single-individual studies. If the multi-individual studies represent a reasonably unbiased sample of species, then the estimates of μ and μ_S should be close in value, and the three-parameter model should not yield a substantially improved fit. To formally compare the fit of the two models, a Likelihood-Ratio-Test (comparing twice the difference in log likelihoods to a Chi-squared distribution with one degree of freedom) is used. If the LRT is significant, then I must conclude that the multi-individual studies represent a biased subset of species. Otherwise, the two-parameter Beta-distribution with ML parameter estimates can be used as an acceptable description of the distribution of prevalences across the species group. (3) If the distribution has been accepted as a reasonable fit, I can then estimate the fraction of all species that are infected with the different bacteria at a prevalence of c or above, a quantity denoted x_c (see Appendix 2). Confidence intervals for μ, ρ or x_c can be determined from the curvature of the likelihood surface, i.e., by finding fixed values of the chosen parameter that decrease the maximised log likelihood by 2 units.

The method above is based very closely on the approach of Hilgenboecker et al. (2008), but there are three important differences. First, in stage (1), all available data is used to estimate the parameters of interest, while Hilgenboecker et al. (2008) used only multi-individual screens. Second, the procedures differ in stage (2), where Hilgenboecker et al.'s approach implicitly treats the same Beta distribution as applying simultaneously to infected species only, and to all species. Finally, in stage (3), my method generates confidence intervals for x_c, and for the other parameters.

5.3 *Results*

5.3.1 How common are endosymbionts in arthropods?

Of the three different bacteria tested, *Wolbachia* was the only bacterium where fitting a single distribution to multi-individual studies and single-individual studies (data from 1898 species, of which 923 are multi-individual screens) did not have a significantly worse likelihood than fitting separate mean prevalence parameters to the two types of study (χ^2=1.136; *d.f.*=1; *p*= 0.2866). This implies that the multi-individual studies generally did not over-represent the prevalence of *Wolbachia* in arthropod populations. Combining all the data, the maximum likelihood distribution estimated that ~40% of arthropod species harbour *Wolbachia* at or above a 1% prevalence (Figure 5.1).

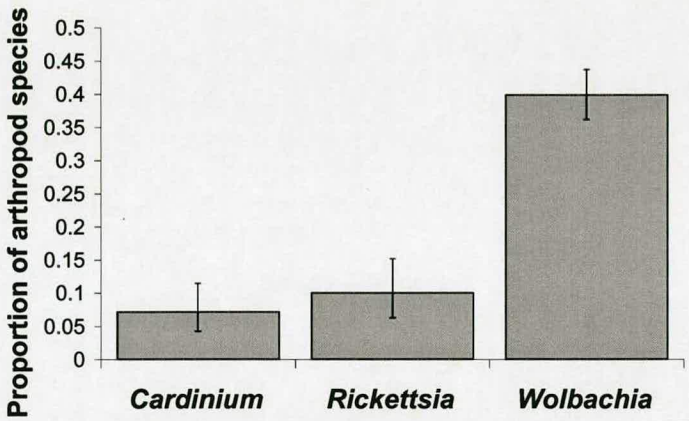


Figure 5.1. The proportion of arthropod species infected above a 1% prevalence ($x_{0.01}$) for each of the different bacteria. Confidence intervals (calculation described in the method) for $x_{0.01}$ are represented as error bars.

In contrast to *Wolbachia*, for *Rickettsia* and *Cardinium*, fitting different mean prevalence parameters to the multi-individual and single-individual studies significantly improved the fit of the model (*Rickettsia* χ^2=1.136; *d.f.*=1; *p*=0.0376; *Cardinium* χ^2=1.136; *d.f.*=1; *p*=0.0019). The multi-individual studies of *Rickettsia* indicated a higher mean prevalence than the whole dataset, implying that the multi-

individual *Rickettsia* studies were biased towards reporting populations previously known to be infected. We hypothesised that this was due to an over-representation of hard ticks (Ixodida), in the multi-individual studies. Ixodida have a much higher rate of horizontal transmission than other taxa, and also a particular medical and economic importance. When the order Ixodida was removed from the dataset, the parameters fit to multi-individual and single-individual studies did not differ significantly (χ^2=0.421; *d.f.*=1; p= 0.5166). The estimated distribution for *Rickettsia* prevalences with Ixodida removed is shown in Figure 5.2, and was used to estimate that the proportion of arthropod species infected above a 1% prevalence was ~10% (Figure 5.1).

The multi-individual studies that tested for *Cardinium* on the other hand, seemed to under-represent the number of infections as the mean prevalence of the multi-individual studies was significantly lower than the mean prevalence of the single-individual studies. This again seemed to reflect the taxon sampling in the different types of studies, as *Cardinium* are absent in *Diptera*, and rare in *Hymenoptera* and *Hemiptera*, and many multi-individual studies concentrated on these groups (Table 5.1). Combining all of the data, the proportion of arthropod species infected with *Cardinium* above 1% prevalence was estimated to be ~7% (Figure 5.1) although this may well be an underestimate as the single individual studies, which gave a higher mean prevalence are thought to be relatively unbiased.

5.3.2 Bacterial distribution of prevalence

The Maximum Likelihood Beta distribution for *Wolbachia* and *Cardinium* was bimodal with a high frequency of low (or zero) and high prevalence infections (Figure 5.2). In contrast, Figure 5.2 shows that the Beta distribution for *Rickettsia* indicates a high frequency of purely low prevalence infections. The probability distributions all have a positive skew, probably reflecting the large proportion of species completely free from infection. In order to test whether the three distributions were significantly different from each other, pairwise comparisons were made for the different bacteria. These comparisons were formally tested using an extension of the

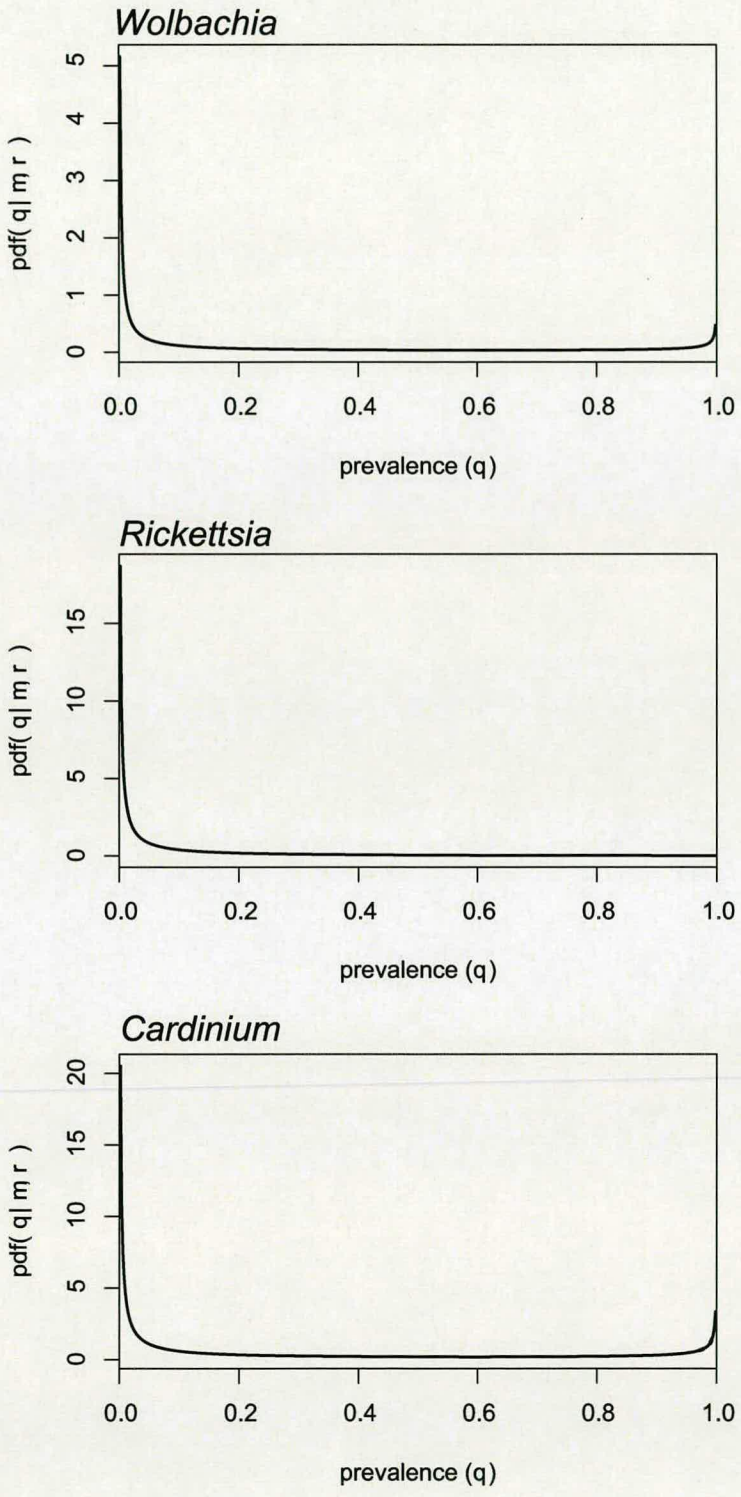


Figure 5.2 Maximum Likelihood distribution of prevalence across different species of arthropod for each of the different bacteria. The probability density function is plotted again prevalence. On the *y*-axis, m=mu and r=rho.

previous method, where the fit of a single distribution applied to both bacteria (the two parameter model) was compared to the fit of a model in which each bacterium was assigned its own distribution (four parameter model). A likelihood ratio test was carried out and the *p* value, which was then Bonferroni corrected for multiple tests (corrected values are given in the text).

For the comparison between *Wolbachia* and *Rickettsia*, the distributions were significantly different (χ^2=265.35; *d.f.*=2; p<0.0001), and this appeared to be due to differences in both mean prevalence (μ) and the variance parameter (ρ) which determines the shape of the distribution for a given mean prevalence. The same applied to *Rickettsia* and *Cardinium* (χ^2=13.269; *d.f.*=2; *p*=0.0039).

In contrast, for *Wolbachia* and *Cardinium*, the distributions again differed significantly (χ^2=153.072; *d.f.*=2; p<0.0001) but ρ had a similar maximum likelihood value for both bacteria. To test whether the shape of the distribution for these two bacteria did differ significantly, a new three parameter model was used where the mean prevalence was allowed to differ but ρ had the same value. When the likelihood of this model was compared to the four parameter model the difference was not significant (χ^2=0.052; *d.f.*=1; *p*=0.8220).

Therefore, the overall distributions of the three bacteria were each found to be significantly different (Figure 5.2), but for *Wolbachia* and *Cardinium* the distributions had a similar shape despite their different mean prevalences (Figure 5.1).

5.3.3 Heterogeneity in incidence and prevalence across orders

To assess whether there is heterogeneity in the distribution of prevalences across different orders of arthropods, we tested a model where one beta distribution was fit to the prevalences in one particular order, and another to the prevalences in the rest of the data. The fit of this four-parameter model was then compared to a single distribution fit to all orders. Since our estimates for *Cardinium* may be unreliable (due to the significant difference between the single- and multi-individual studies),

we did not conduct any further tests on this bacterium. In addition, only orders containing 50 or more sampled species were analysed.

For *Rickettsia*, we first tested our hypothesis that the prevalences for the order Ixodida would differ significantly from other orders. This hypothesis was supported strongly (χ^2=30.882; *d.f.*=2; $p<10^{-6}$). When Ixodida were removed, the remaining four orders represented by more than 50 species, had distributions that did not differ significantly from the pattern of the entire *Rickettsia* dataset after Bonferroni correction (*Diptera* χ^2=6.603; *d.f.*=1; p= 0.1472; *Coleoptera* χ^2=8.334; *d.f.*=1; p=0.06120; *Hemiptera* χ^2=5.295; *d.f.*=1; p=0.2833; *Hymenoptera* χ^2=5.210; *d.f.*=1; p=0.2955). The separate distributions for these four orders are shown in Figure 5.3.

For *Wolbachia*, unlike *Rickettsia*, four out of eight most widely sampled infected orders (*Araneae*, *Diptera*, *Coleoptera* and *Hymenoptera*) had distributions that differed significantly from that of the complete dataset, even after Bonferroni correction. The distribution of all eight orders is shown in Figure 5.4.

Although the order *Araneae* showed a significantly different distribution from the entire *Wolbachia* dataset (χ^2=21.041 *d.f.*=2 p=0.0002), the mean prevalence was very similar, indicating that the important difference was that this order does not adopt the bimodal shape that most of the *Wolbachia*-infected orders exhibit. As *Araneae* does not have a concentration of high prevalence infections, but still has a similar mean prevalence, this suggests that it has a higher frequency of intermediate infections. This can be seen by the shallower decline of the distribution curve in Figure 5.4. The order *Prostigmata* shows a similar pattern with the same proportion of species infected above the 0.01 threshold, but this did not show a deviation from the entire dataset (χ^2=4.647 *d.f.*=2 p=0.7833).

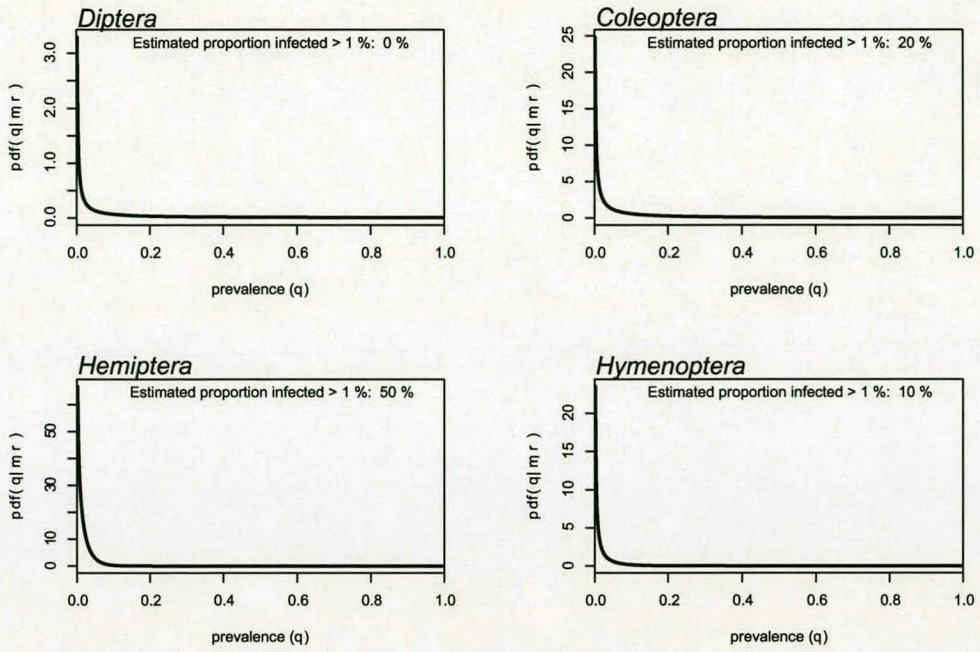


Figure 5.3. Maximum Likelihood distribution of prevalence of *Rickettsia* over four different orders (only the orders represented by more than 50 species are pictured here).

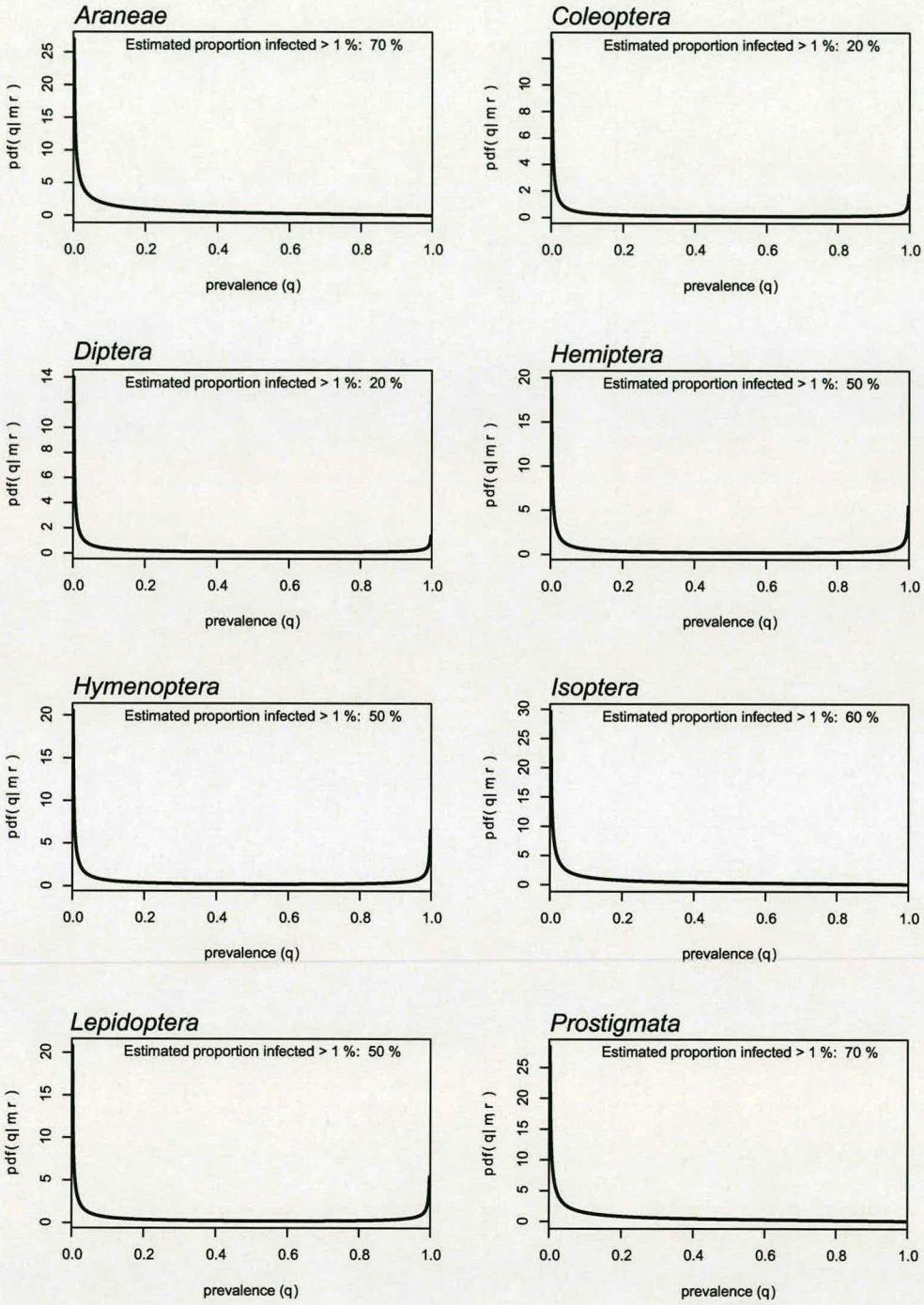


Figure 5.4. Maximum Likelihood distribution of prevalence of *Wolbachia* over eight different orders (only the orders represented by more than 50 species are pictured here).

The four parameter model also had a significantly higher likelihood for the orders *Diptera* (χ^2=37.232 *d.f.*=2 p<0.0001) and *Coleoptera* (χ^2=19.873 *d.f.*=2 p=0.0004), and in both cases they had a lower mean prevalence and a much steeper decline in the shape of the distribution curve in Figure 5.4 (note the different scale of the y-axis compared to the other orders). In contrast, a significantly higher mean prevalence was observed for *Hymenoptera* (χ^2=26.970 *d.f.*=2 p<0.0001).

5.3.4 Are symbionts that infect species with an ecology that supports male-killing found at lower prevalence?

It is known that male-killers tend to be found at lower prevalence than other endosymbionts. The Lepidopteran genus *Acraea* and the Coleopteran family Coccinellidae are known to cannibalise their dead siblings, and this is thought to promote male-killer infection (because infected females can thereby gain a fitness advantage). We therefore hypothesised that *Wolbachia* symbionts would have a different distribution in these taxa, with a lower mean prevalence compared to the rest of the data. By fitting a separate distribution to the 71 species placed in either *Acraea* or Coccinellidae we found that this hypothesis was supported (χ^2=6.347; *d.f.*=2; p<0.042); μ for *Acraea* and Coccinellidae = 0.125, μ for all other species = 0.174. The maximum likelihood distribution for these suspected male-killers is shown in Figure 5.5. While the difference in mean prevalences accords with predictions, the bimodality of the distribution is surprising. However, closer investigation showed that the high prevalence peak was attributable to a single data point, where *Acraea encedana* has an extreme level of infection. This is very atypical of a male-killer (although one other case is known Charlat et al. 2005). It seems likely that there has been a detection bias here, due to the near absence of males in the high-prevalence populations.

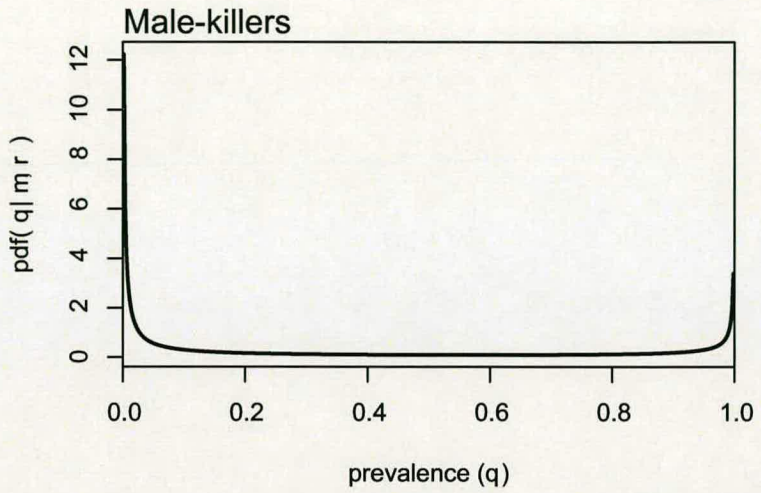


Figure 5.5. Maximum Likelihood distribution of prevalence of *Wolbachia* in the Lepidopteran genus *Acraea* and the Coleopteran family Coccinellidae. The ecology of these species is thought to make them particularly prone to male-killing.

5.4 *Discussion*

Arthropod endosymbionts have been known to be common, since estimated that ~20% of field-sampled arthropod individuals are infected by *Wolbachia* alone (Werren et al. 1995). By estimating the distribution of prevalence across different arthropod species, I have estimated that *Wolbachia* infect around 40% of species at a frequency of 1% or higher, and that *Rickettsia* and *Cardinium* infect around 10% of species. (The *Wolbachia* estimate is compatible with the earlier estimate of Hilgenboecker et al. 2008, despite the much larger data set used here.) Given that other strains of symbionts are known to be common in arthropods, it is therefore likely that one or more facultative endosymbionts infect at least half of all arthropod species (taking in to account multiple infections within a species). Incidence levels as

high as these are likely to have profound effects on host evolution. For example, as vertically inherited symbionts are in linkage disequilibrium with host mitochondria, they are likely to alter mitochondrial diversity in non-neutral ways (Hurst and Jiggins 2005). Theory predicts that a reduction in mitochondrial effective population size will actually be more pronounced in species with a lower prevalence (Johnstone and Hurst 1996) and could therefore explain why there is a large deviation from neutrality in mitochondria across invertebrate lineages (Bazin et al. 2006).

In addition to the generally high incidence levels (Figure 5.1), the approach presented here also highlights the differences and similarities in the distributions of prevalence among the three endosymbionts (Figure 5.2). *Wolbachia* are already known to infect more randomly-sampled arthropod individuals than any other known symbiont (Duron et al. 2008b), but it was hitherto unknown whether this implied that more species were infected or whether a similar number of species were infected at generally higher prevalence – both of which would give the same pattern when screening large numbers of single individuals of different species.

Furthermore, it has been shown how differences in the distribution of prevalence across arthropod species can help us predict how symbionts are spreading through populations. This is because the within-species prevalence is predicted to differ among phenotypes. For example, a bimodal distribution of prevalence is consistent with a bacterium causing a range of phenotypes that select for high prevalence (cytoplasmic incompatibility, parthenogenesis induction, being required for oogenesis) and low prevalence (male-killing, feminization). Such a bimodal distribution was found for *Wolbachia* (Figure 5.2), and *Wolbachia* are indeed known to induce all these phenotypic alterations in their hosts (Stouthamer et al. 1999; Dedeine et al. 2001). Interestingly, despite its lower mean prevalence and incidence level, the shape of the distribution for *Cardinium* (Figure 5.2) did not differ significantly from that of *Wolbachia*, suggesting that *Cardinium* and *Wolbachia* might be inducing similar phenotypes in their hosts. (However it should be noted that the *Cardinium* data set was the smallest, and showed the strongest signs of taxonomic bias in the multi-individual studies).

In contrast to the bimodality of the other distributions, the *Rickettsia* distribution was positively skewed (Figure 5.2) indicting that the vast majority of *Rickettsia* infect their host species at a low prevalence. Although, *Rickettsia* are known to induce parthenogenesis and are required for oocyte production, the absence of high prevalent infections suggests these phenotypes are rare. In addition, they are almost certainly not inducing cytoplasmic incompatibility. This suggests that the majority of these strains are probably male-killers, or alternatively may be facultatively beneficial in some environments.

In addition to heterogeneity between bacteria, this study has also demonstrated significant heterogeneity of the distribution of prevalences between host orders, particularly for *Wolbachia* infection (Figure 5.4). Again, these can be used to make inferences, or test hypotheses about bacterially-induced phenotypes. The highest numbers of species infected were in the orders Aranae and Prostigmata with approximately 70% infected. Spiders (Araneae) are known to be a particular hotspot for endosymbiont infection, although the reasons are unknown (Goodacre et al. 2006; Duron et al. 2008a). The positive skew of the distribution indicates that most of these infections are at low prevalence within species, suggesting that they may be sex ratio distorters or facultative mutualists. A similar shape was also observed for mites despite the observation that haplodiploidy has arise at least twice in Prostigmata (Wrensch and Ebbert 1993), which indicates that parthenogenesis inducing *Wolbachia* are probably rare. A high number of species were also infected within Isopoda (~60%) Hemiptera, Hymenoptera, and Lepidoptera (~50%) (Figure 5.4). Isopoda seem particularly predisposed to feminising *Wolbachia*, which might explain the high incidence observed (Bouchon et al. 1998). Endosymbionts might also be able to invade Hymenoptera species because many species are haplodiploid, and so their sex determination systems are more easily manipulated. It is currently unclear why Hemiptera and Lepidoptera might harbour higher numbers of endosymbionts. However, these represent two particularly speciose orders, and simulation experiments predict that groups with a higher tempo of radiation will be

more predisposed to infection when the genetic distance of the donor and recipient hosts is a major factor in establishing an infection (Engelstadter and Hurst 2006).

While these inferences are speculative, I also tested and found support for an existing hypothesis – that the genus *Acraea* and the family Coccinellidae would show a significantly different distribution of *Wolbachia* prevalence due to an ecology that supports male-killing. This gives us a slightly increased confidence in our ability to make inferences from the distribution's shape.

However, it should be noted that there are some important limitations to the approach used above. The most important is probably the assumption that the distribution of prevalences can be satisfactorily modelled by a Beta distribution. The assumptions that each data point represents a random population sample, and is statistically independent from every other data point may also be questionable (for example, if cospeciation between host and parasite were common). The robustness of my analysis to deviations from these and other assumptions will be investigated in future. But if found to be reasonably robust, the approach introduced here could be used to test a wide variety of theoretical hypotheses, for example, that cytoplasmic incompatibility should be less common in female than in male heterogametic taxa (Hurst et al. 2002), or that endosymbionts should appear at higher incidence in speciose orders (Engelstadter and Hurst 2006).

Chapter 6. Evolution and diversity of *Rickettsia* bacteria

6.1 *Introduction*

Rickettsia bacteria are intracellular symbionts of eukaryotes. The genus is classified in the family Rickettsiaceae within the alpha-proteobacteria, and is closely related to the genera *Erlichia* and *Wolbachia* (Hotopp *et al.* 2006; Williams *et al.* 2007). *Rickettsia* are most noted for causing human diseases, including Rocky Mountain spotted fever and epidemic typhus, which has been a major source of mortality at times in human history (Gross 1996). However, all known vertebrate-associated *Rickettsia* are vectored by arthropods as part of their life-cycle, and many other *Rickettsia* are found exclusively in arthropods with no known secondary host (for convenience, we will refer to the former as "vertebrate *Rickettsia*" and the latter as "arthropod *Rickettsia*"). In recent years, arthropod *Rickettsia* have been discovered in a diverse range of hosts, suggesting that they are more common than had been suspected (Werren et al. 1994; Chen et al. 1996; Davis et al. 1998; Fukatsu and Shimada 1999; Van der Schulenburg et al. 2000; Lawson et al. 2001; von der Schulenburg et al. 2001; Sakurai et al. 2005; Gottlieb et al. 2006; Hagimori et al. 2006; Perotti et al. 2006; Zchori-Fein et al. 2006). Nevertheless, research effort has tended to concentrate on the medically important vertebrate *Rickettsia*, or on the more common arthropod endosymbionts, such as *Wolbachia* and *Cardinium*, and so we know little about the biology of arthropod *Rickettsia*. Even less is known about the closely related bacteria that have been recently discovered in organisms such as leeches and protists, and in metagenomic studies sequencing all DNA in an environmental sample (Hine *et al.* 2002; Kikuchi *et al.* 2002; Dykova *et al.* 2003; Vannini *et al.* 2005; Gihring *et al.* 2006; Lu *et al.* 2006; Fraune and Bosch 2007; Percent *et al.* 2008; Rintala *et al.* 2008). This neglect is unfortunate, because comparing the vertebrate pathogens with related species can help to elucidate the

mechanisms of pathogenicity, transmission and virulence (Maurelli 2007; Rohmer *et al.* 2007). However, this requires a robust phylogeny for the genus.

Historically, *Rickettsia* were classified into three major groups based on serological characteristics, namely the 'typhus group', 'spotted fever group' and 'scrub typhus group', although subsequent DNA sequencing led to the latter being reassigned to the related genus *Orientia* (Tamura *et al.* 1995). The relationship of species within the remaining two groups of *Rickettsia* has been the subject of intensive study over the last decade as progressively more informative genes have been sequenced (Roux *et al.* 1997; Andersson *et al.* 1999; Roux and Raoult 2000; Sekeyova *et al.* 2001) culminating in a multi-genic approach (Vitorino *et al.* 2007). As a result it has been suggested that the spotted fever group consists of two sister clades, one of which is now designated 'transitional' (Gillespie *et al.* 2007) (although see Fournier *et al.* 2008). A fourth so-called "ancestral" clade, including *Rickettsia bellii* and *Rickettsia canadensis*, is thought to be basal to the other groups and is largely non-pathogenic to vertebrates. However, the position of *R. canadensis* remains uncertain (Vitorino *et al.* 2007).

While many studies have helped to clarify the relationships between the vertebrate *Rickettsia*, only one recent study has explored the relationship of the well classified groups to the newly discovered arthropod *Rickettsia* (Perlman *et al.* 2006). The authors found that most arthropod *Rickettsia* are basal to the vertebrate *Rickettsia* and that the *Rickettsia* associated with leeches, protists and freshwater environments fell into two phylogenetic groups, distinct from the arthropod and vertebrate groups. The only known exceptions are a small number of arthropod *Rickettsia* that fell within the group otherwise infecting leeches (Campbell *et al.* 2004; Perlman *et al.* 2006; Perotti *et al.* 2006). However, Perlman *et al.* (2006) were only able to provide little statistically significant support for relationships among the arthropod *Rickettsia*. This is almost certainly because the study relied on partial sequences of *16S* rDNA, which is extremely slowly evolving, and so lacking in phylogenetic resolution. Improving this situation is challenging because amplifying other genes in basal strains has proven problematic, perhaps because the genes in

question may either be missing or too divergent for PCR amplification using existing primers. Also, resolving some deep nodes in the *Rickettsia* species tree continues to be a problem. The reasons for this are unclear but could be exacerbated by long-branch attraction. One of the best ways to minimise this effect is to sample for more taxa and add them to the tree in the hope of breaking up (thereby shortening) the long branches.

Here, to explore the diversity of arthropod *Rickettsia*, I screened 4454 arthropods to uncover new *Rickettsia* strains and sequenced four genes from five known and 20 new bacterial strains. I used the recently published *Orientia tsutsugamushi* genome (Cho *et al.* 2007) to design PCR primers allowing amplification of rapidly evolving genes from strains that lie between the genera *Rickettsia* and *Orientia*. To include other hosts, I also searched published metagenomic databases for *Rickettsia* sequences. With this data, I have been able to produce the first well-resolved phylogeny of the entire genus *Rickettsia*, showing how the vertebrate *Rickettsia* relate to the other taxa. This phylogeny has allowed identification and nomenclature of additional novel groups. Furthermore, I was able to compare host associations among these groups, identify major life history transitions, and investigate the extent of recombination within the genus.

6.2 *Methods*

6.2.1 Bacterial strains

I obtained most of the *Rickettsia* strains I sequenced from three PCR screens of insects collected in the wild (Table 6.1). These used primers that amplify the *16S rDNA* of *Rickettsia* (von der Schulenburg *et al.* 2001), and are therefore thought to target a broad range of *Rickettsia*. The first screen tested 2149 ladybirds from 21 different species collected from the UK, Germany, Spain and New Zealand for the presence of *Rickettsia* (Chapter 3). I sequenced a *Rickettsia* from a single individual from each of the eight species shown to be infected. The second screen tested 1458

Table 6.1. *Rickettsia* strains sequenced

***Rickettsia* obtained from:**	**Host Order**	**Host species**
this study:		
Chapter 4	*Lepidoptera*	*Noctuid* sp. (moth)
	Neuroptera	*Chrysopidae* sp. (lacewing)
	Coleoptera	*Elaterid* sp. (beetle)
	Coleoptera	*Curculionid* sp. (weevil)
	Diptera	*Bombylid sp.* (bee fly)
	Diptera	*Bombylid sp.* (bee fly)
	Hemiptera	*Reduviidae* sp. (assassin bug)
	Coleoptera	*Meloidae* sp. (blister beetle)
	Hemiptera	*Cercopidae* sp. (spittlebug)
Chapter 3	*Coleoptera*	*Subcoccinella_24punctata* (24 spot ladybird)
	Coleoptera	*Halyzia 16guttata* (orange ladybird)
	Coleoptera	*Calvia 14guttata* (cream spot ladybird)
	Coleoptera	*Coccidula rufa* (ladybird)
	Coleoptera	*Rhyzobius litura* (ladybird)
	Coleoptera	*Scymnus frontalis* (ladybird)
	Coleoptera	*Adalia bipunctata* (2 spot ladybird) edinburgh
	Coleoptera	*Adalia decempuntata* (10 spot ladybird)
Gall wasp screen	*Hymenoptera*	*Pediobius rotundatus*
	Hymenoptera	*Aulogymnus balani/skianeuros*
	Hymenoptera	*Aulogymnus trilineatus*
previous studies:		
Jiggins and Tinsley, 2005	*Coleoptera*	*Adalia bipunctata* (2 spot ladybird) moscow
	Coleoptera	*Adalia bipunctata* (2 spot ladybird) cambridge
	Coleoptera	*Adalia bipunctata* (2 spot ladybird) ribe
Chen *et al.*, 1996	*Hemiptera*	*Acyrthosiphon pisum* (pea aphid)
Lawson *et al.*, 2001	*Coleoptera*	*Brachys tessellatus* (buprestid beetle)

individuals of Hymenoptera associated with galls induced by oak gall wasps (Hymenoptera: Cynipidae, Cynipini; Stone *et al.* 2002), comprising nine species of oak gall wasps, 26 species of associated chalcid parasitoids, and ten species of oak gall wasp inquilines (Hymenoptera: Cynipidae, Synergini) (A. Aebi and G. Stone, unpublished data). I sequenced a *Rickettsia* from single individuals from three of the

four species that were infected. The third study screened 847 individuals, each of which was a different species of arthropod from the classes *Arachnida*, *Entognatha*, *Malacostraca* and *Insecta*. The individuals from *Arachinida* comprised six of the order *Araneae* and one *Holothyrida*. The five *Entognatha* were all *Collembola* and the individual from *Malacostraca* was from the order *Isopoda*. The individuals from the *Insecta* comprised 240 of the order *Hymenoptera*, 218 *Diptera*, 206 *Coleoptera*, 86 *Hemiptera*, 28 *Lepidoptera*, nine *Orthoptera*, nine *Thysanoptera*, eight *Odonata*, eight *Heteroptera*, five *Homoptera*, five *Blattodea*, four *Neuroptera*, three *Dermaptera*, and one individual each of *Mantodea*, *Pscoptera*, *Siphonaptera*, *Strepsiptera*, and *Trichoptera* (Chapter 4). The insects were collected from worldwide locations. All nine *Rickettsia* isolates from this screen were sequenced. I also included a *Rickettsia* from the pea aphid *Acyrthosiphon pisum* (Sakurai *et al.* 2005), a male-killing *Rickettsia* from the buprestid beetle *Brachys tessellatus* (Lawson *et al.* 2001) and three *Rickettsia* strains from the ladybird beetle *Adalia bipunctata*, each of which has been shown to be genetically distinct (Schulenburg *et al.* 2001; Jiggins and Tinsley 2005).

6.2.2 PCR and sequencing

To obtain estimates of phylogeny from different portions of the genome, I sequenced four different genes, which are at least 200 kbps apart in the *R. bellii* genome. Of the genes used in a previous study to produce a multi-gene vertebrate *Rickettsia* phylogeny (Vitorino *et al.* 2007), I sequenced *16S rDNA* and *atpA* (encodes for ATP synthase F1 alpha subunit), which are the only ones that have homologues conserved enough to produce alignments in *Orientia tsutsugamushi*. I also targeted the *coxA* gene (encodes for subunit I of cytochrome C oxidase) as it is used in *Wolbachia* multilocus strain type analysis (Baldo *et al.* 2006) and is found in *Orientia* and all *Rickettsia* genomes except for *Rickettsia typhi*. I also used the *gltA* gene (encodes for citrate synthase), which is commonly sequenced from *Rickettsia* strains (Roux *et al.* 1997) and, although it is absent from the *Orientia tsutsugamushi* genome, it is conserved throughout all other Rickettsiales (Cho *et al.* 2007). This provides four genes for the multi-gene analysis. All primer sequences are given in Chapter 2. The PCR products were incubated at 37C for 40 minutes with shrimp alkaline

phosphatase (Promega, Southampton, UK) to digest unincorporated dNTPs and exonuclease I (NEB, Hertfordshire, UK) to digest the PCR primers. They were then sequenced using Big Dye technology (Applied Biosystems, CA) in both directions using the PCR primers and run on a 3730 capillary sequencer (Applied Biosystems, CA).

6.2.3 Phylogenetic analysis

Nucleotide sequences were edited and assembled using Sequencher 4.1 (GeneCodes, MI), and aligned using the ClustalW application within Bioedit v.7.0.1. All sequences within alignments were checked to ensure they encoded functional proteins (with the exception of the *16S* gene). The model of sequence evolution used for each gene was selected by only including parameters that significantly improved the fit of the model to our data. These parameters were identified by comparing alternative models using hierarchical likelihood ratio tests in the program Modeltest v.3.7 (Posada and Crandall 1998). The evolutionary models used were as follows: *16S* - HKY+G, *gltA* - K81uf+I+G, *coxA* - GTR+G and *atpA* - GTR+G.

Phylogenetic hypotheses were inferred using maximum likelihood in PAUP v.4.b10 and using the Bayesian MC^3 approach implemented in MrBayes v3.1 (Huelsenbeck and Ronquist 2001). I combined the data with published sequences from all the known non-vertebrate *Rickettsia* strains, and all the *Rickettsia* from the ancestral, typhus and transitional groups, as well as *Rickettsia helvetica*, *Rickettsia montanaensis*, *Rickettsia massiliae*, *Rickettsia japonica*, *Rickettsia conorii*, *Rickettsia peacockii* and *Rickettsia rickettsii* from the spotted fever group (Figure 6.1a). I also included *O. tsutsugamushi* as an outgroup (I checked that this species is a genuine outgroup by reconstructing a 16S rDNA tree rooted with *Wolbachia pipientis*; data not shown). All accession numbers are given in Table S1. Maximum parsimony trees were created using the tree-bisection reconnection branch swapping method, and these were then used both to estimate model parameters and as a starting tree for the maximum likelihood analysis. The maximum likelihood trees were then found using the nearest-neighbour-interchanges branch swapping method. The robustness of the tree topologies was assessed by repeating the analysis using 1000 bootstrapped

datasets. The GTR+I+G model of evolution was used for the concatenated dataset of the three genes.

The Bayesian analysis incorporated four Markov chains (three heated and one cold chain), consisting of 1,000,000 generations with sampling every 100 generations. Two simultaneous runs with different random start trees were performed, and the first 25% of samples were discarded as burn-in. For the Bayesian analysis including missing data, the data were partitioned for the four different genes and assigned the appropriate evolutionary model (given above), then unlinked so that the parameters were estimated separately and allowed to have a different evolutionary rate. The MCMC analysis was then run for 6,000,000 generations, after which the standard deviation of split frequencies (a measure of the similarity of the two independent trees in the run) fell below a proposed threshold for model convergence of 0.01 (Huelsenbeck and Ronquist 2001). For the phylogeny that contains missing data, I only used the Bayesian approach, as missing data adds exponential complexity to a maximum likelihood approach that maximises over the entire 'parameter landscape', but only increases complexity linearly with Bayesian techniques as nuisance parameters are marginalised out.

Split networks for each of the four genes were constructed using the neighbour-net method in SplitsTree4 (Bryant and Moulton 2004; Huson and Bryant 2006). Networks represent multiple trees simultaneously, and they can therefore identify conflicting signals in the data. These may arise from either genetic exchange between bacterial strains, or from systematic error in the underlying model of evolution. The neighbour-net method computes a matrix of distances (much like the neighbour joining method) and produces a network with weights assigned to each split that are proportional to the number of sites that support the split. I used non-parametric bootstrapping to identify splits supported with >95% confidence, and only included these statistically significant splits in the network (otherwise representing the data as a bifurcating tree) (Huson and Bryant 2006).

6.2.4 Phylogenetic tests

I tested whether there were significant topological differences between the maximum likelihood trees of the four genes and a tree produced from the concatenated sequences of all four genes using the Shimodaira-Hasegawa test (Shimodaira and Hasegawa 1999). The test statistic for a given gene is generated by comparing the maximised likelihood score for that gene with topology unconstrained, to the likelihood obtained when topology was fixed at the maximum likelihood topology obtained from the concatenated dataset. The null distribution of the test statistic for a gene is generated from 1000 nonparametric bootstrapped datasets, although to reduce the computational burden, nuisance parameters were fixed at values estimated from the original dataset (RELL method). This test was applied to each of the genes with the *Rickettsia* strain from *C. rufa* removed for reason of recombination (see below).

I tested for recombination between *Rickettsia* strains in two ways. First, I used the maximum χ^2 test (Maynard Smith 1992) implemented in RDP v3b22 (Martin *et al.* 2005). This test takes all possible triplets of sequences, removes any gaps, and makes an alignment of just the polymorphic sites. A window is then slid along this alignment in single nucleotide steps. At each position a χ^2 statistic is calculated as a measure of the likelihood that recombination has occurred between these sequences. The size of the window was set at approximately 3/4 the numbers of polymorphic sites present for each triplet. To correct for the large number of multiple tests performed, we obtained an analysis-wide significance threshold of χ^2 by repeating the analysis on 1000 datasets that were simulated without recombination (simulations performed using SEQGEN (Rambaut and Grassly 1997)). The maximum χ^2 test of recombination is one of the most powerful tests of recombination (Posada 2002) but it can occasionally falsely infer the presence of recombination under some conditions, such as in regions that contain mutational hot-spots (Bruen *et al.* 2006). Therefore I also used the pairwise homoplasy index (PHI) test of recombination (Bruen *et al.* 2006) implemented in SplitsTree4. The test exploits the fact that when recombination has occurred, sites that are physically close in the sequence should yield compatible phylogenies more often than distant sites. The phi statistic (Φ_w) quantifies the degree of congruence between parsimonious trees at

closely-linked sites up to 100bp (w=100). A p-value can then be obtained by comparing this statistic to a distribution of values obtained when the position of sites along the sequence is determined at random. To speed computation, this null distribution can be approximated by a normal distribution, whose mean and variance are calculated analytically from the data.

To date key transitions in the order Rickettsiales, I calibrated a 16S rDNA phylogeny of the order using the substitution rate of this gene estimated for the endosymbiont *Buchnera* (Moran *et al.* 1993). This tree was reconstructed with a molecular clock enforced. I checked that enforcing a clock did not significantly reduce the likelihood of the tree by comparing the likelihoods of a tree with and without a clock enforced using a likelihood ratio test.

6.3 *Results*

6.3.1 Strains identified and genes sequenced

The screens identified 20 novel strains of arthropod *Rickettsia* from six orders of insects, and these are listed in Table 6.1. These strains were combined with five previously described arthropod *Rickettsia* (listed at the bottom of Table 6.1) to give 25 strains in total. I successfully sequenced all four of the chosen genes from 18 of these strains, and one or more genes from the remaining seven.

6.3.2 *Rickettsia* Phylogeny

To obtain a phylogeny of the genus *Rickettsia*, I combined a concatenated alignment of the four genes I sequenced, with data from other *Rickettsia* strains available from Genbank (accession number available in Table S6.1 in Appendix 3). For most of the previously described arthropod *Rickettsia*, only *16S* rDNA sequence is available, and so I allowed for missing data in the alignment where a gene had not been sequenced. Missing data should not decrease phylogenetic resolution for taxa with complete data, and is likely to be a problem for other taxa only when the number of characters is very low (Wiens 2006).

Figure 6.1a shows that the concatenated alignment with missing data gave a well-resolved tree with strong support for most nodes. Nevertheless, it is important to determine whether there are conflicting signals between the individual genes. Therefore, I used SH tests to compare our concatenated topology to the maximum likelihood trees inferred from each of the four genes (Table 6.2). Only the *16S* gene tree topology was marginally significantly different (although this is no longer significant when controlling for multiple tests by Bonferroni correcting the *p* values).

Table 6.2. Comparison of the tree topologies obtained from the four genes against the topology of the concatenated dataset using four SH tests. Each dataset was forced to adopt the topology from the concatenated dataset and the log likelihood of this tree was compared to the log likelihood of the unconstrained tree. The taxa used in this analysis are shown in Figure 1b.

	Likelihood of tree topology			
Dataset	unconstrained	concatenated	-2Δ*l*	*p*
16S	1486.10	1502.03	31.85	0.045
AtpA	2129.98	2140.90	21.85	0.161
CoxA	3484.47	3490.98	13.02	0.201
GltA	3931.44	3942.56	22.24	0.069

It is also important to investigate the influence of missing data on the phylogeny. Therefore, I constructed a second tree that included only taxa with complete sequences for the three genes *atpA*, *coxA* and *gltA* (excluding *16S* due to its marginally significant SH test). This 'complete data' tree is shown in Figure 6.1b. Overall, the topologies of the two trees are very similar (Figure 6.1a and 6.1b), but most nodes had higher support in the tree with complete sequences. In particular, there is strong bootstrap support for the group largely composed of ladybird symbionts in the complete data tree (Figure 1b) but not on the missing data tree (Figure 1a). An exception is the placement of *R. canadensis,* which is uncertain in the complete data tree but is well supported on the missing data tree (probably

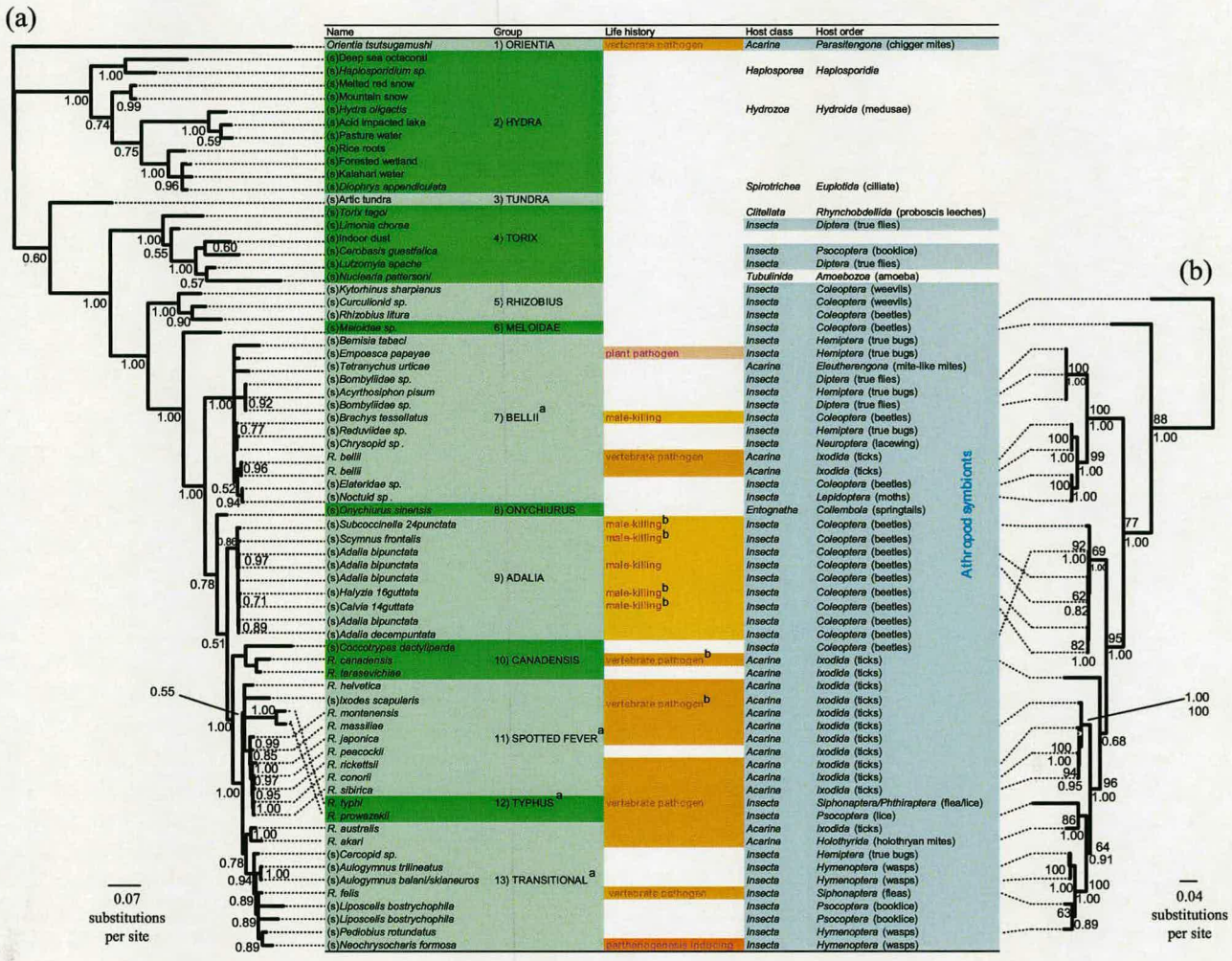


Figure 6.1. Phylogeny of *Rickettsia*. The name of the host prefixed by (s) is given where the bacterium does not have a species name, as well as names for each *Rickettsia* group, life history and host order. (a) Bayesian phylogeny using concatenated sequences of *atpA*, *coxA*, *gltA*, *16S*. Posterior support for each node is shown. (b) Maximum likelihood phylogeny based on complete sequences of *atpA*, *coxA* and *gltA*. Bootstrap support is given as a percentage above the node, and posterior support from a Bayesian tree is given as a decimal below the node.

[a] Previously characterised groups.

[b] Only circumstantial evidence connects the trait to the strain.

because the missing data tree includes two closely related taxa; Figure 6.1a). The composition of the transitional group and the placement of *Rickettsia prowazekii* also differ in the two trees. *Rickettsia* within the typhus group (*R. prowazekii* and *Rickettsia typhi*) are striking in that they reside on longer branches than other *Rickettsia* in the trees. This is indicative of rate heterogeneity, which can cause a long branch attraction artefact where the taxa will appear in an incorrect place. In the missing data tree the transitional group is monophyletic, while in the complete data tree *R. prowazekii* groups with *Rickettsia akari* (Figures 6.1a and 6.1b). However, constraining *R. akari* and the transitional group to be monophyletic in the complete data tree only causes a marginally significant drop in the likelihood (SH test; χ^2=20.003 p=0.066).

Together, these phylogenetic analyses reveal five distinct and well-supported major clades of *Rickettsia* (Figure 6.1), one (designated the hydra group) containing protist-associated *Rickettsia* and a number with unknown host associations from sequences amplified from environmental samples, a second clade (torix) containing *Rickettsia* from amoeba, leeches and arthropods, a third (rhizobius) contains three beetle *Rickettsia*, a fourth (melloidae) containing a single beetle *Rickettsia*, a fifth (bellii) containing arthropod *Rickettsia* and a sixth clade of diverse bacteria containing both arthropod and vertebrate *Rickettsia*. This final clade can be further subdivided into the following groups: onychiurus, adalia, canadensis, spotted fever group, typhus group and transitional group, although bootstrap support for some of these groupings is less strong (all groups are also summarized in Figure 6.2).

6.3.3 Host Shifts

By mapping host species onto our phylogeny, we are able to make inferences about patterns of host-switching in the genus. It is clear from Figure 6.1 that *Rickettsia* bacteria have an extremely diverse host range, occurring in arthropods, vertebrates, plants, amoebae, cilliates, annelids and hydrozoa, and that there have been numerous shifts between these hosts. The earliest shift splits the genus into two major divisions; the hydra and torix groups and all other arthropod *Rickettsia*. As mentioned, the hydra group are symbionts of protists and undetermined hosts.

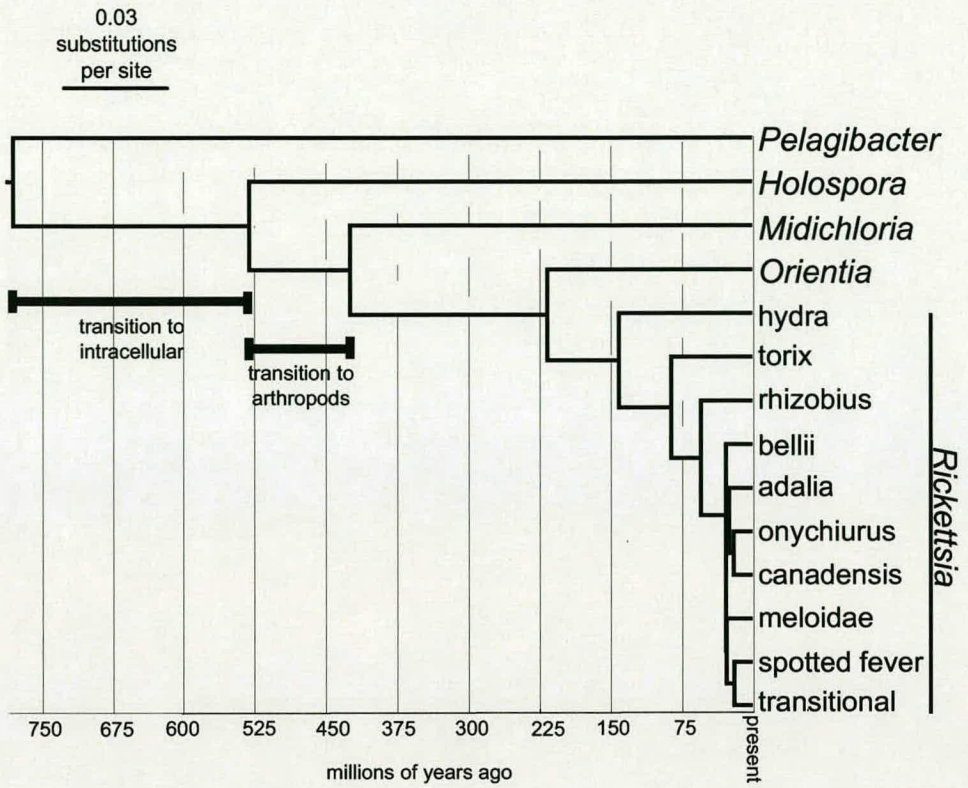


Figure 6.2. Relationships and approximate dates of divergence of the major clades within the order Rickettsiales. The 16S rDNA phylogeny was reconstructed using one member of each of the groups shown with a molecular clock enforced (enforcing the clock did not reduce the likelihood of the tree: likelihood ratio test $-2\Delta ln$ =13.84, d.f.=12 p=0.311).

Although one member of this group was found in the marine ciliate *Diophrys* from brackish water (Vannini *et al.* 2005), and another from a deep sea octacoral, all others are from freshwater environments or damp terrestrial environments. In general it appears that marine *Rickettsia* are rare. Indeed, from over 13 billion open reading frames compiled from marine metagenomic datasets (Seshadri *et al.* 2007) I detected no homologues of greater than 91% identity to the *16S* gene of hydra group *Rickettsia*. The next split in the tree separates all the remaining *Rickettsia* from the torix group (Figure 6.1) which contains symbionts of leeches (phylum Annelida), an amoeba (Perlman *et al.* 2006) and arthropods (a sandfly, a cranefly, a biting midge,

and a booklouse). In the torix group, as with the hydra group, the vast majority of the hosts are aquatic (the sole exception being the booklouse).

The remainder of the arthropod *Rickettsia*, including all strains sequenced in the present study, form a monophyletic group (Figure 6.1). Parsimony suggests that the ancestral state of this clade is to infect arthropods, with one or more lineages subsequently evolving to also infect vertebrates. In addition, there have been multiple transitions between blood feeding and non blood feeding insects. Perlman *et al.* (2006) demonstrated that forcing *R. bellii* to group with other blood feeders gives a significantly worse tree. SH tests of the phylogeny in this study showed that forcing *R. canadensis* and *R. felis* to group with other blood feeders similarly gives a significantly worse fit (SH tests on all groups: $p< 0.001$).

These results therefore show clearly that there have been numerous host shifts, sometimes between taxonomically distant hosts. However, it is equally clear that related *Rickettsia* tend to share related hosts. Multiple different strains were detected within ladybird beetles, ticks, lice, parasitic wasps and bee-flies, and in all cases, two or more of these strains cluster together. Nevertheless, this pattern cannot be explained by ancestral infection followed by co-speciation of parasite and host. Comparisons of ladybird beetle and *Rickettsia* phylogenies indicate at least one case of horizontal transfer between related hosts (Chapter 7). Closed, well-studied systems such as oak gall wasp communities are ideal to study horizontal symbiont transmission (Schonrogge and Crawley 2000; Rokas *et al.* 2002). Unfortunately only four parasitoid individuals from the oak gall wasp screen were infected, not allowing me to test the influence of host relatedness, host interaction frequency and geographic isolation on the frequency of horizontal transfer events.

In addition to clustering according to host type, Figure 6.1 also demonstrates phylogenetic clustering by ecology (although it is often difficult to separate these effects). For example, the two major groups of vertebrate *Rickettsia*, the spotted fever or typhus groups, consist solely of vertebrate *Rickettsia*, containing no arthropod *Rickettsia*. However, the transitional group differs from this pattern containing both

vertebrate *Rickettsia* and *Rickettsia* infecting non-blood feeding arthropods (Figure 1). A second ecological adaptation to increase transmission is to skew the sex ratio of the host towards females, which are the sex that most efficiently transmits the infection to offspring for vertically transmitted *Rickettsia*. Some of these *Rickettsia* are known or suspected to kill male hosts early in their development, and there appears to be two separate origins of this adaptation on the tree (once within a buprestid beetle in the bellii group and once within ladybirds in the adalia group). There are 11 strains of *Rickettsia* that infect ladybird beetles, and nine of these cluster in a single monophyletic group. The ones that cluster elsewhere are probably not male-killers (male ladybird beetles are also heavily infected – Chapter 3). A third possible source of ecological clustering relates to herbivorous hosts. Such clustering may reflect ecology in two possible ways. Firstly, many symbionts are known to supplement their hosts with amino acids that are rare in phloem sap (although a mutualistic role for *Rickettsia* has never been demonstrated). Secondly, *Rickettsia* may be transmitted horizontally through plants (one case is already known). It has previously been asserted that the bellii group consists mainly of herbivorous arthropod symbionts (Perlman *et al.* 2006). Four *Rickettsia* in this group are indeed known to infect sap sucking arthropods (a whitefly, a leaf hopper, an aphid and a red spider mite), and three of these group separately from the other members of the bellii group (Figure 6.1). However, I have uncovered a large number of predatory insect hosts in this group, and sap sucking insects in other groups (a spittlebug symbiont is in the transitional group). Therefore, the view that members of the bellii group are mainly associated with herbivorous arthropods is not supported by these new data.

6.3.4 Recombination

Recombination events complicate the inference of species trees, and so it is important to investigate the extent of recombination in the *Rickettsia* genus. I found one clear instance of recent recombination between different *Rickettsia* groups (this taxon was excluded from the analyses above). In the phylogenetic trees of the four individual genes (Figure S6.1), the symbiont of the ladybird *Coccidula rufa* (s*C. rufa*) appears in the transitional group on the *16S* and *gltA* trees, and in the bellii group on the *atpA* and *coxA* trees. An alignment of the polymorphic sites and a

hybridisation network indicates that s*C. rufa* is a chimera of sequences from these two groups (Figure 6.3).

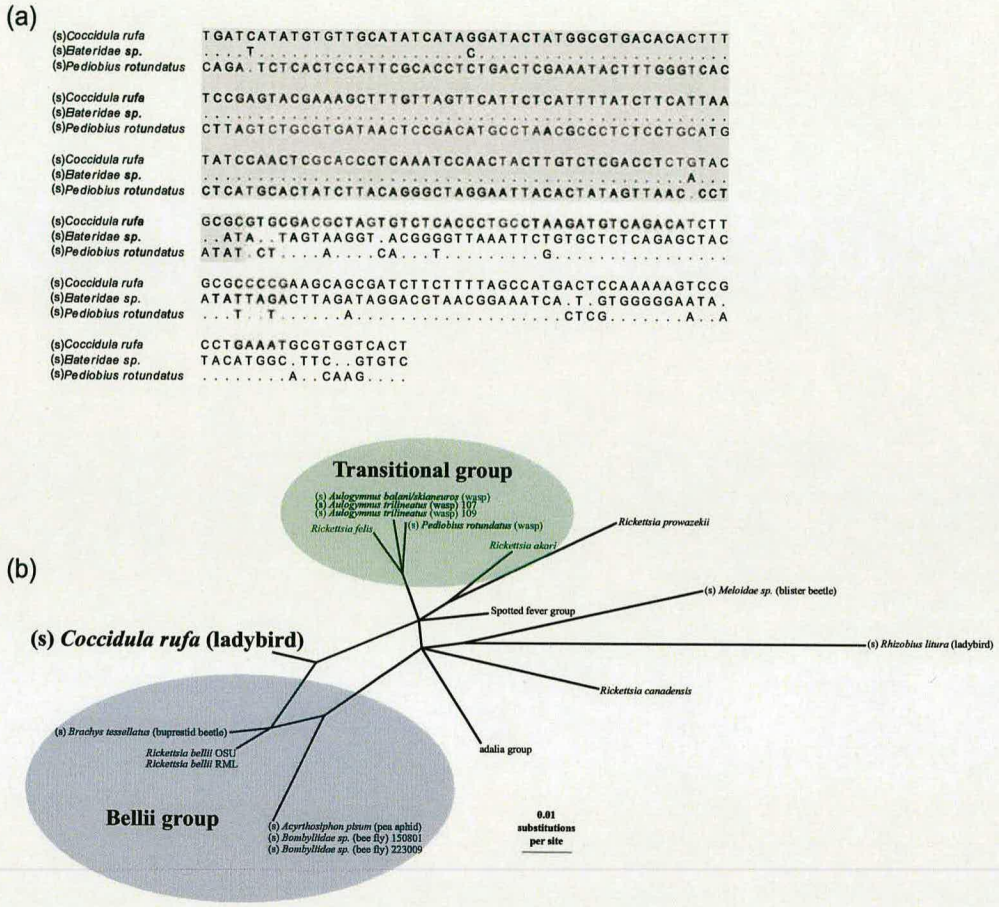


Figure 6.3. Sequence alignment and hybridisation network showing the symbiont of *Coccidula rufa* to be a recombinant. (a) Alignment of concatenated genes *atpA*, *coxA*, *gltA*, *16S* showing just polymorphic sites. Nucleotides that are identical to the *C. rufa* sequence are shown as a dot. The (s)*C. rufa* sequence of *atpA* and *coxA* (shaded) are most similar to (s)*Elaterid* sp. in the bellii group, while the *gltA* and *16S* sequences (unshaded), are most similar to (s)*Pediobius rotundus* in the transitional group. (b) A hybridisation network of the concatenated sequences of *atpA*, *coxA*, *gltA* and *16S*. A neighbour-net split network was generated and splits were then filtered by weight to include only the (*s*)*C.* rufa split. A hybridisation network was then performed on the split network, to provide an explicit example of descent from the two different groups.

To verify that the recombination pattern for s*C. rufa* was not the result of contamination, this result was confirmed by sequencing three strains from different individuals of *C. rufa*. This appears to be the only case of recombination between the four genes because when s*C. rufa* is excluded from analyses, there is little evidence of topological differences between the datasets (see SH tests above).

I did, however, detect some evidence of recombination events within two of the four genes. The maximum χ^2 test and phi test identified multiple recombination breakpoints in the *gltA* and *coxA* genes. In *coxA*, the breakpoint pattern indicted that there had been some recombination between an ancestor of the *Adalia* group and of the *Rhizobius* group (maximum χ^2 test χ^2=42.79; p<0.001; phi test p<0.001). In *gltA*, there was evidence of recombination between *R. akari* of the transitional group and the *Adalia* group (maximum χ^2 test χ^2=46.78; p<0.001; phi test p=0.021). In contrast, no recombination was detected within the *16S* and *atpA* genes (*16S* maximum χ^2 test χ^2=8.92;p=0.783; phi test p=0.960; *atpA* maximum χ^2 test χ^2=12.13; p=0.57; phi test p=0.759).

Split networks were constructed for each of the four genes to identify possible sources of conflicting signal and recombination in the data (Figure S6.2). This method has an advantage over tree-based methods as posterior support and bootstrap values measure robustness solely with respect to sampling error (as opposed to systematic bias), and with large sample size robustness will generally be high as noise in the data is filtered out. The split network constructed for the *16S* gene was tree-like (containing no significant splits). In contrast the other three genes showed a small amount of phylogenetic conflict, with statistical support for two different trees. In all cases, one of these trees corresponded to that shown in Figure 6.1, suggesting that this tree accurately reflects the evolutionary history of most of the genome. The discrepancies were as follows. The *atpA* split network showed additional support for a tree where *R. prowazekii* is basal to the other vertebrate groups. This pattern is consistent with a tree based on protein alignments of the ten *Rickettsia* genomes (Gillespie *et al.* 2008). The *coxA* split network supported a closer relationship between *R. litura* symbiont and the *Adalia* group, which is consistent

with the recombination pattern for this gene. The *gltA* split network also supported this same relationship although this was not reflected in the recombination breakpoint pattern (Figure S6.2).

6.4 *Discussion*

I have identified a large number of new strains of *Rickettsia*, including several new groups, and shown that arthropod *Rickettsia* are both common and diverse. I have also constructed the largest and most robust phylogenetic analysis of the genus to date. Importantly, I used a multiple locus approach, as using of single genes to build species phylogenies can seriously confound the true relationship between strains, especially with loci that are prone to recombination (Baldo and Werren 2007).

6.4.1 The evolutionary history of *Rickettsia*

It is useful to view these results in the context of the evolution of the whole order Rickettsiales. To do this, I have used a molecular clock to date the divergence of different groups, and this is shown in Figure 6.2. The common ancestor was presumably free-living, as the earliest diverging genus of the order is *Pelagibacter* (which account for 26% of the bacterial rDNA sequences from sea water (Rappe *et al.* 2002)). About 525-775 million years ago there was a transition to living within cells, followed by a split into endosymbionts of protists (*Holospora*) (Amann *et al.* 1991; Horn *et al.* 1999) and a clade that primarily infects arthropods. The most parsimonious interpretation of the tree is therefore that the transition to infecting arthropods occurred approximately 425-525 million years ago in this lineage (Figure 3), which is can be compared to the first appearance of most metazoan phyla in the Cambrian geological boundary (542-543 million years ago).

The genus *Rickettsia* is approximately 150 million years old (Figure 6.2). Parsimony would suggest that the common ancestor of *Rickettsia* infected arthropods, and that species in the hydra and torix groups then switched to infect other eukaryotes such as protists, leeches and numerous unidentified hosts (many of

which may be protists) (Figures 6.1 and 6.2). However, care should be taken with this interpretation, as symbionts of arthropods are more thoroughly sampled than those of other animals. In addition, two patterns call into question the interpretation that the ancestral state was arthropod infection. First, the genome sequence of *R. bellii* includes many genes that are more related to other amoeba symbionts than to other *Rickettsia* (Ogata *et al.* 2006). This is compatible with an ancestor of *R. bellii* infecting amoeba and exchanging genes with other amoebal symbionts. Second, of the arthropod hosts within the torix group (three *Diptera* and a louse), all of the Dipteran hosts have larval stages that feed on aquatic microbiota, with the other hosts within the group also being aquatic. Although host switching could occur in either direction, transmission from protist to arthropod is more intuitive given that the related genus *NeoRickettsia* is transmitted between hosts through ingestion (Gibson *et al.* 2005). Further sampling of other eukaryotic hosts may resolve the question of the ancestral state.

Regardless of this, I have shown that the remaining clade of *Rickettsia* (i.e. those not in the hydra or torix groups), all have associations with arthropods; either as the only known host or in conjunction with a vertebrate or plant host (Figure 6.1). The rhizobius and meloidae groups, which all infect beetles, diverged from the other taxa early in the evolution of this clade. There was then a rapid radiation about 50 million years ago that led to most of the strains we know of. This includes the bellii group, which is probably the largest group of arthropod *Rickettsia* as it contains all but three strains from the worldwide sample. This sample includes both a diverse array of arthropods (it rarely includes the same host genus twice), and it will tend to pick up high prevalence infections (only a single specimen of each host species was tested).

These results show clearly that switching between arthropod hosts has been a common feature of *Rickettsia* evolution. Within the genus, closely related bacteria sometimes infect different host phyla and classes (Figure 6.1), but the genus arose long after the major arthropod orders diverged (Gaunt and Miles 2002) (Figure 6.2). However, the host phylogeny is not entirely unrelated to the bacterial phylogeny, and

there are many cases of related *Rickettsia* strains infecting related hosts. In the case of many mutualistic symbionts, the bacterial phylogeny precisely mirrors the host phylogeny, indicating that the bacteria and host have co-speciated (Moran *et al.* 1993). However, this is not the case in the *Rickettsia.* Even in the adalia group, where a group of related bacteria all infect related hosts, the host and bacterial phylogenies are different. Therefore, *Rickettsia* symbioses are short-lived on an evolutionary scale, which is consistent with most of these infections being parasitic.

The analysis has also allowed me to reconstruct the changes in the ecology of the genus. *Rickettsia* are almost entirely restricted to terrestrial and freshwater habitats (Figure 6.1). Within the genus, there have been three major transitions in life history; becoming sex ratio distorters, arthropod vectored vertebrate pathogens and, in one case, an arthropod vectored plant pathogen. Based on current data, infecting plants and parthenogenesis induction in the arthropod host has arisen only once, and male-killing twice. Until the effect of *R. bellii* on vertebrates in the field has been properly defined, we cannot say for sure how many times vertebrate pathenogenesis has evolved (Fournier et al. 2008).

6.4.2 Recombination

The recent discovery of plasmids in the genus *Rickettsia* opens up the possibility that horizontal gene transfer may be common between strains (Ogata *et al.* 2005; Baldridge *et al.* 2007; Blanc *et al.* 2007; Baldridge *et al.* 2008). Furthermore, there have been reports of recombination between *Rickettsia* strains (Amiri *et al.* 2003; Jiggins 2006). This has implications for the evolution of *Rickettsia,* as beneficial genes can sweep through different genetic backgrounds and bacterial species, which could have important implications for the spread of genes altering bacterial pathogenicity. Recombination can also complicate the inference of relationships between strains, as recombination violates the assumption that a strain has one evolutionary history.

It is clear from the data that these different genes have very similar phylogenetic histories and recombination must therefore be infrequent (although it is

possible that the exchange of plasmids may be common – Chapter 8). However, we detected one clear-cut case of recombination between different groups of *Rickettsia*. In the symbiont of the ladybird beetle *Coccidula rufa* (Figure 6.3) the sequences of *atpA* and *coxA* place (s)*C. rufa* within the bellii group, whereas *gltA* and *16S* place it within the transitional group (Figure S6.1). In the *Rickettsia felis* genome (from the transitional group), the gene sequences of *atpA* and *coxA* are approximately 670kb apart. If this represents one recombination event and the genes are syntenic with the *R. felis* genome, it will have included approximately 45% of the genome. The biggest known recombination event in *Rickettsia*, which occurred in *Rickettsia massiliae*, is a 54 kb segment containing many genes that facilitate conjugal DNA transfer. Intriguingly, although *R. massiliae* is in the spotted fever group, this region of DNA was also thought to originate from the bellii group (Blanc *et al.* 2007). As well as this, Gillespie *et al.* (2007) found that many of the genes on the *Rickettsia felis* plasmid have a closer relationship to the bellii group. This evidence suggests that conjugation with the bellii group *Rickettsia* may have an important role in the evolution of the groups containing vertebrate pathogens.

I also detected recombination within the *coxA* and *gltA* genes. This is particularly surprising given that the individual gene topologies did not seem to conflict in any way (Table 6.2). This can only be explained if the recombination event is ancient, and indeed the breakpoint patterns affected all members in particular groups suggesting the events predated the divergence of the different groups. Even though recombination machinery has been detected in *Rickettsia* genomes (Andersson *et al.* 1998), this is the first evidence that housekeeping genes recombine, and could have implications for the inference of relationships, since housekeeping genes (in particular *gltA* in *Rickettsia*) are often used to build phylogenies. Therefore recombination should be investigated more fully, especially when using single genes to build phylogenies. These ancient recombination events involve the adalia group and the rhizobius group, as well as the transitional group. This would seem to indicate that recombination is not unique to the bellii and vertebrate groups, and may be widespread throughout all arthropod *Rickettsia* and possibly the other basal

groups. However, the recombination signal is different to the above cases, as it is intragenic and over a small area.

6.4.3 Transmission and population dynamics

It is clear from the data that *Rickettsia* are common and diverse bacteria. However, the basic biology of most of these strains is entirely unknown and it is therefore unclear how these have spread through populations. As *Rickettsia* are primarily intracellular, they cannot survive for long in the external environment (but see (Rasgon et al. 2006) for cell-free persistence of related *Wolbachia*). For this reason, they are most readily maintained by either vertical transmission (mother to offspring) in their arthropod hosts or, in the case of blood-sucking arthropods, by horizontal transmission through an infected vertebrate (one case is also known of transmission through a plant (Davis *et al.* 1998)). Because infectious transmission between arthropod hosts is thought to be rare, the general view is that exclusively arthropod *Rickettsia* are maintained within a host species primarily by transovarial transmission, and therefore must enhance the fitness of infected females (Werren 2005). Some *Rickettsia* raise infected female fitness in an indirect way by manipulating host reproduction towards infected daughters at the expense of sons, either by killing male offspring as embryos (male-killing) or by inducing parthenogenesis (Hurst *et al.* 1996; Hagimori *et al.* 2006). The closely related bacterium *Orientia tsutsugamushi* also causes a female biased sex ratio in its mite host (Takahashi *et al.* 1997). Theoretically, arthropod *Rickettsia* could also be maintained by directly providing a fitness benefit to infected females as shown for other bacterial groups (Montllor *et al.* 2002; Koga *et al.* 2003; Oliver *et al.* 2003; Ferrari *et al.* 2004; Chiel *et al.* 2007), e.g. by providing essential nutrients or protection from other infective agents. In most cases where the arthropod relationship has been studied in depth, *Rickettsia* are pathogenic (Azad and Beard 1998; Schulenburg *et al.* 2001; Sakurai *et al.* 2005; Kontsedalov *et al.* 2008) or have no observable effect (Azad *et al.* 1992; Wedincamp and Foil 2002), making a mutualistic role for *Rickettsia* in those hosts unlikely.

For those *Rickettsia* that are vertebrate pathogens but vectored by arthropods, the effects of the bacteria on their arthropod hosts are generally poorly understood (Azad and Beard 1998). *Rickettsia prowazekii* is clearly pathogenic to infected lice, and transmission through humans is essential to the maintenance of the bacteria in arthropod populations. In every other case, human infections are accidental, but transmission through other vertebrates may allow the bacteria to persist in populations. Many of the bacteria that can infect vertebrates are also transmitted vertically by the arthropod host (Azad *et al.* 1992). In these cases, even very occasional horizontal transmission through the vertebrate host can enhance the maintenance of bacteria in arthropod populations.

My data also has implications for transmission. I have shown that *Rickettsia felis* (transitional group), *R. canadensis* (canadensis group) and *R. bellii* (bellii group) are more closely related to *Rickettsia* in non-blood feeding hosts than to those found in other blood feeding hosts. Therefore, are these strains even transmitted horizontally? As far as I am aware, even in cases where the bacteria can infect vertebrates (as is the case with *R. felis*), there has been no recorded instance of transmission back to arthropods (i.e. ectoparasites can not pick up the infection from vertebrates). Therefore, while there are multiple origins of infecting blood-feeding arthropods, the ability to be transmitted from vertebrates back into the arthropod host may have arisen once only, and subsequently been lost in the transitional group after the divergence of *R. akari* and *australis*.

We still do not have a complete understanding of how *Rickettsia* are maintained within host populations or how they move horizontally between host species. A better understanding of these dynamic processes can be achieved by detailed studies of representatives from the different groups described here.

6.5 *Conclusion*

I have identified twenty new arthropod *Rickettsia* and described the major transitions and life-history strategies throughout the phylogeny. This raises many questions

about how these bacteria are maintained and spread throughout populations of arthropods and vertebrates. *Rickettsia* are known to distort the sex ratio of their hosts by male-killing and inducing parthenogenesis, and are also horizontally transmitted through vertebrates and plants. However, these phenotypes are probably not manifest in the majority of strains discovered and so there may be other ways in which *Rickettsia* are maintained in host populations. For example, there seem to be intriguing links to host oogenesis in some strains and a possible case of a beneficial effect in the torix group (Kikuchi and Fukatsu 2005; Zchori-Fein *et al.* 2006). Exploring the biology of these new strains is essential if we are to learn more about the genus.

Chapter 7. The evolutionary origins of *Wolbachia* and *Rickettsia* infection in ladybird beetles (Coccinellidae)

7.1 *Introduction*

Arthropod endosymbionts should have phylogenies that exactly mirror the phylogenies of their hosts, if their only mode of transmission is vertical. Good examples of this are the ancient associations of some mutualists with their hosts, where there is speciation of the symbiont upon host speciation (co-speciation) (Chen et al. 1999; Clark et al. 2000; Lo et al. 2003). However, facultative symbionts, such as those that manipulate their host's reproductive system, often have phylogenies that do not reflect those of their hosts (Werren et al. 1995a; Zhou et al. 1998). Although these symbionts may be maternally inherited, these infections are transient over evolutionary time, which implies that, in order to persist, these endosymbionts must switch to infect different host lineages.

Once an infection is established, there are four trajectories that a symbiont can follow. First, the infection could be lost, leading to lineage sorting and causing a mixture of infected and uninfected populations. Second, the symbiont may diverge into distinct bacterial strains that are maintained within a single host species, leading to one host having two distinct strains. Third, the symbiont might co-speciate with its host, in which case, both phylogenies will be congruent. Finally, the symbiont may switch to infect a new host, in which case the symbiont phylogeny should be decoupled from host phylogeny. However, host tracking (a phenomenon of switching to related hosts because of shared physiology/ecology) may lead to a pattern wrongly indicative of co-speciation.

Patterns of host switching have been particularly well studied in the bacterium *Wolbachia* (Jiggins et al. 2002; Baldo et al. 2008) but also recently in *Cardinium* (Weeks et al. 2003) and *Rickettsia* (Chapter 6). A general pattern is

closely related symbionts tending to cluster among closely related hosts, although closely related symbionts can sometimes infect very distantly related hosts. Most studies to date show that the degree of switching between closely related hosts is sufficiently high enough to decouple host phylogeny from bacterial phylogeny. In addition to clustering by host relatedness, a few studies have attempted to test whether there is strain clustering in hosts with a shared ecology, with mixed results (Haine and Cook 2005; Sintupachee et al. 2006).

However, while studies have attempted to characterise patterns of horizontal transfer within a species (Ballard 2004; Viljakainen et al. 2008) or within a single genus (Michel-Salzat et al. 2001; Jiggins et al. 2002; Baldo et al. 2008), no studies have attempted to understand patterns of host switching between more distantly related hosts. There is plenty of experimental evidence to suggest that success of trans-infection (where a symbiont is injected in to a novel host species) declines with increasing genetic distance between recipient and donor, but it is not known how this affects transmission dynamics in wild populations (Moret et al. 2001; Jaenike et al. 2007; Tinsley and Majerus 2007). In addition, all the literature has been focused on *Wolbachia*, as a larger number of informative genes are available to discriminate between strains. However, understanding patterns of transmission in different symbionts will enable us to understand more about the observed heterogeneity in incidence and prevalence.

In this study, I investigate the evolutionary relationships of *Wolbachia* and *Rickettsia* that infect species of the same family Coccinellidae. Ladybirds are an ideal system to investigate routes of transmission, as their ecology makes them predisposed to male-killing symbionts. Data on the incidence and prevalence of ladybird symbionts is also available, allowing us to test hypotheses about how the phenotypic effects of a symbiont affects patterns of host switching (Chapter 3). In addition, it has been shown experimentally in ladybirds that the success of infection, the expression of the male-killing phenotype and transmission efficiency negatively correlates with host genetic distance (Tinsley and Majerus 2007).

7.2 *Methods*

7.2.1 Data collection

Bacterial strains:

I obtained most of the strains from a study screening large numbers of ladybird species for the presence of symbionts (Chapter 3). From this study, the *wsp* gene was sequenced in six strains of *Wolbachia* and the *atpA*, *gltA*, *16S*, and *coxA* genes were sequenced in eight strains of *Rickettsia* (Chapter 6). A further six *wsp Wolbachia* strains were used from Genbank (Hurst et al. 1999; Jeyaprakash and Hoy 2000), giving a total of 12 strains from ladybirds. Also, three strains of *Rickettsia* shown to be genetically distinct were included (Schulenburg et al. 2001), giving a total of 11 strains of *Rickettsia* from ladybirds. The non-ladybird strains of *Wolbachia* used in permutation and phylogenetic analyses were all the known male-killing strains of *Wolbachia,* as well as randomly selected strains (the *wsp* sequence from 50 strains were selected from the ~1500 strains in Genbank, using the sample function in R statistical package). Of the 50 randomly selected strains, those with partial sequence and an excess of N nucleotides were discarded, as were multiple strains from the same host species or from *Wolbachia* super groups other than A or B, leaving a total of 19 strains.

Host strains:

Ladybird species were collected and their DNA was extracted as described in Chapter 3. Primers used were CI-J-2630 with T12-N-3012 (Simon et al. 1994), and LCO1940LB (Folmer et al. 1994) with CI-N-910i, and CI-J-1718 (Simon et al. 1994) with CI-N-856 (all primer sequences given in chapter 2). In addition, mitochondrial sequences were also obtained from Genbank (accession numbers are given on phylogenetic trees). Nucleotide sequences were edited and assembled using Sequencher 4.1 (GeneCodes, MI).

7.2.2 Phylogenetic analysis

All the sequences were aligned using the ClustalW application within Bioedit v.7.0.1 and were checked to ensure they encoded functional proteins. The hypervariable regions of the alignment were removed as they are difficult to align. A model of sequence evolution was obtained for each gene using hierarchical likelihood ratio tests in the program Modeltest v.3.7. The general time reversible model with a proportion of invariable sites (GTR + I) was used for *Wolbachia* and *Rickettsia* phylogenies, and the Tamura-Nei model with a proportion of invariant sites (TrN+I) was used for the ladybird phylogeny. Phylogenetic trees were produced using the Bayesian MC^3 approach implemented in MrBayes v3.1 (Huelsenbeck and Ronquist 2001), consisting of 4,000,000 generations with sampling every 100 generations. Two simultaneous runs with different random start trees were performed, and the first 25% of samples were discarded as burn-in. Shimodara-Hasegawa (SH) tests were carried out in the same manner as described in Chapter 6.

7.2.3 Permutation tests

All permutation tests were completed in the R statistical package. To reduce the effect of potential recombination with each of the genes, pairwise distances were calculated for all data, as well as distances along a tree (patristic distances). The test statistics quoted correspond to pairwise distances unless otherwise stated (patristic distances yielded similar results). Moran's autocorrelation test requires that the trait being tested be weighted according to their similarities. Usually a straightforward inverse of the genetic distance is used but as some trait values in this study are identical (i.e. genetic distance between some bacteria), a matrix of weights was calculated on the following criteria. A genetic distance of zero corresponded to a weight of 1, and the maximum genetic difference corresponded to a weight of 0. The weight was then calculated from the maximum genetic distance (d_{max}) and the genetic distance between the i^{th} pair of taxa (d_i): $W=(d_{max} - d_i)/ d_{max}$.

7.3 *Results*

7.3.1 *Wolbachia* and *Rickettsia* do not cluster together by host

Figure 7.1 shows that there appears to be no clear pattern of infection of either the distribution of *Wolbachia* or *Rickettsia* in ladybird hosts across the ladybird host phylogeny. However, even though most of the hosts that appear in the tree have previously been tested for the presence of *Wolbachia* and *Rickettsia* (Chapter 3) the unequal sampling effort of host species could mean that "uninfected" species may simply be those that have been poorly sampled. I tested whether *Wolbachia* and *Rickettsia* were clustered together on the host tree by calculating an index of clustering, *c*. This was calculated from the average pairwise distance between hosts infected by *Rickettsia* (*r*), the average pairwise distance between hosts infected by *Wolbachia (w*), and the average pairwise distance between all infected hosts (*a*) using the equation $c=(r+w)/2a$. The value of *c* under the null hypothesis that symbionts are randomly distributed across hosts is one. A null distribution of *c* was generated by permuting bacterial genes across the host phylogeny and recalculating *c* 100,000 times. There was no significant relationship between genetic distance of ladybird hosts and symbiont genus (c=1.0179 p=0.3128), suggesting that related ladybirds are not more or less likely to share the same genus of bacteria than distantly related ladybirds.

7.3.2 Multiple invasions of symbionts into ladybird hosts

Based on the phylogeny of *wsp* sequences (Figure 7.2), *Wolbachia* that infect ladybird hosts fell in to at least four distinct clades with high posterior support. A fifth clade may also be evident, even though the placement of the wsp strain from *Calvia quattuordecimguttata* has little support, as an SH test showed that forcing it to group with the *wsp* strain from *Coleomegilla maculata* marginally reduced the likelihood of the tree ($-2\Delta ln$=40.274 $d.f.$=37 p=0.070).There are also three independent clades of *Rickettsia* from ladybird hosts based on a phylogeny that includes all known *Rickettsia* from arthropod hosts (Figure 6.1).

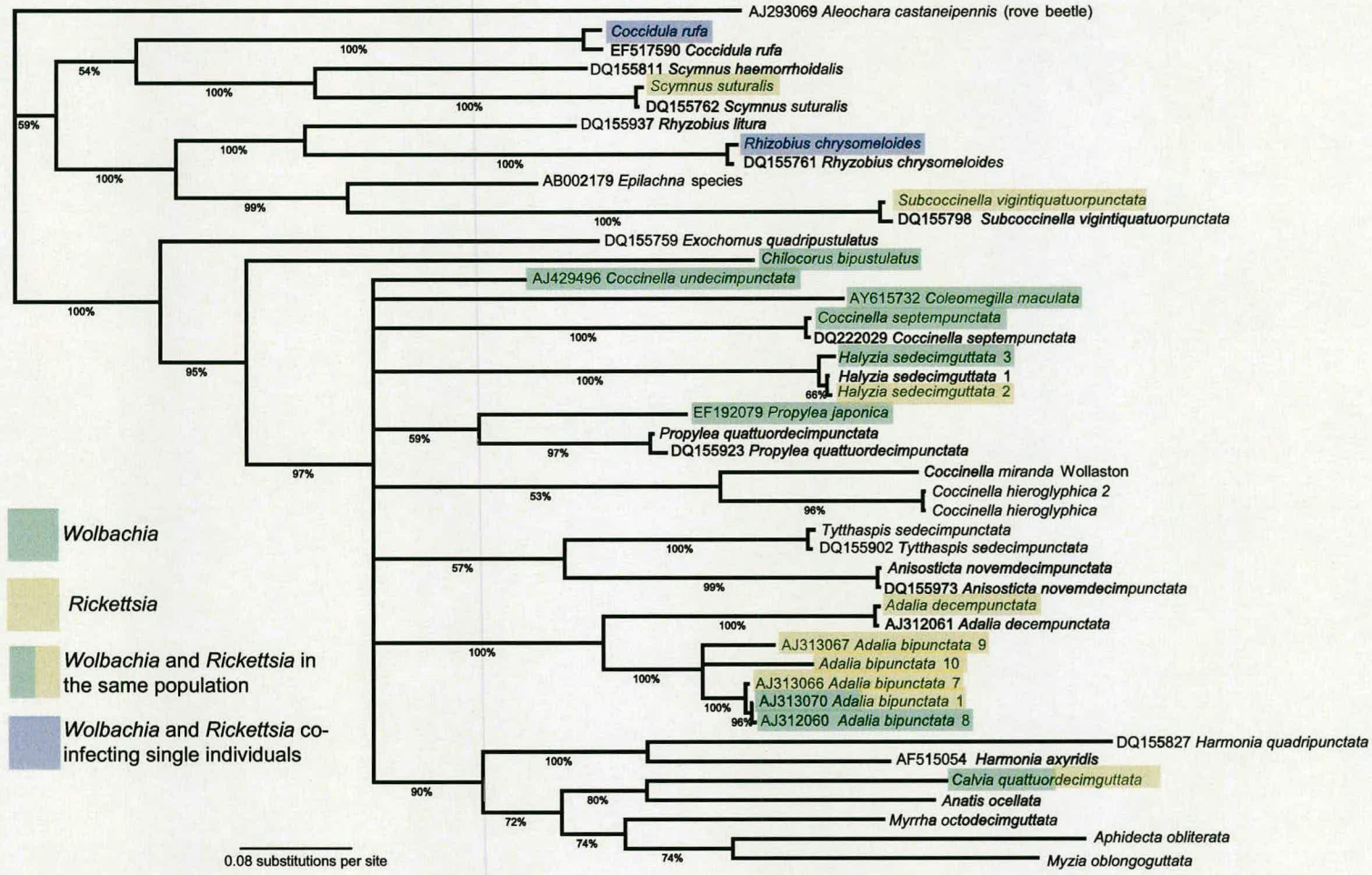


Figure 7.1 Bayesian phylogeny of ladybird beetles. Posterior probability values are given along the corresponding branch and the scale bar represents the length of branches. The key represents infection by different bacteria in ladybird populations.

7.3.3 Clustering by relatedness but not by ecology

In Figure 7.2, many of the ladybird symbionts cluster together in monophyletic groups. To assess the whether this was statistically significant, I tested whether the *Wolbachia* strains found in ladybird hosts are more closely related to each other than they are to the *Wolbachia* that infect other arthropods. To do this I compared the average pairwise distance between ladybird *Wolbachia* and the average pairwise distance between all the *Wolbachia* in the sample (selection described in methods). A null distribution was estimated by permuting whether or not the host was a ladybird, over the bacterial tree, and recalculating the difference in the distances 100,000 times. The pairwise distances between ladybird/ladybird hosts were significantly closer compared to pairwise distances of ladybird/other randomly selected hosts (average pairwise difference between ladybirds=0.1870 p=0.0343, one tailed test). This suggests that, although there have been several horizontal transmission events between ladybirds and other hosts, there is still clustering of the ladybird *Wolbachia*. It is clear from Figure 6.1, that this is also the case for *Rickettsia*, as ladybirds are the only members of the entire 'adalia group'.

I also investigated whether this clustering was due to ecology. All the previously described symbionts of ladybirds are male-killers and many of the new strains described in chapter 3 show sex biases, indicating that they are also sex ratio distorters. It is possible that bacteria will have switched between hosts because of a related ecology that supports a male-killing phenotype. To test this hypothesis, I collected sequences from all known male-killing *Wolbachia* from other host families. It is expected that if the distribution is driven by ecology, ladybird symbionts will be more related to these male-killers than other randomly selected symbionts. However, the average pairwise distance between ladybird symbionts and other male-killers (distance=0.2902) was larger than the average pairwise distance between ladybird symbionts and non-male-killing *Wolbachia* (distance=0.2606). This is clear to see on the *Wolbachia* phylogeny, where there is no tendency for the ladybird symbionts to cluster with other male-killers (Figure 4.2). Unfortunately, there is only one instance

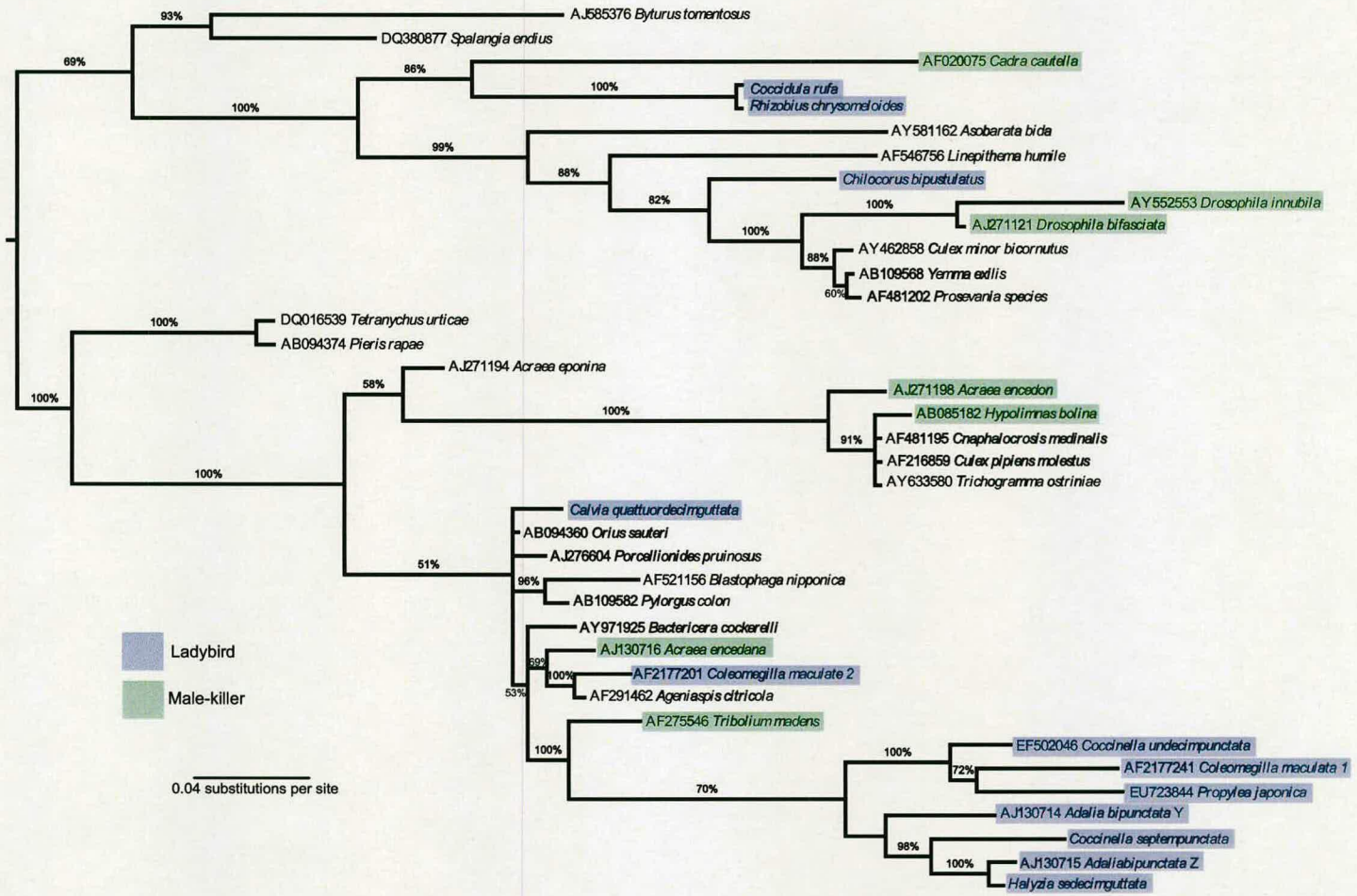


Figure 7.2. Bayesian phylogeny of *Wolbachia* bacteria. Bacterial names correspond to the species name of the host they infect. Posterior probabilities are given on the corresponding branches and scale bar represents length of branches. The key represents whether i) the host a ladybird or ii) whether the bacteria kills male hosts.

of male-killing in *Rickettsia* from a host other than a ladybird, which makes this hypothesis difficult to test, but none of the ladybird symbionts group with this strain (Figure 6.1).

7.3.4 Transmission between ladybird species

To investigate routes of horizontal transmission, I tested whether symbiont strains tended to transmit between closely related ladybird hosts more than distantly related ones. This was done by correlating pairwise distances of bacterial sequences with their host's pairwise distances. To generate the null distribution, bacterial strains were permuted over ladybird hosts 100,000 times and the correlation was re-calculated for each permutation. A significant positive correlation was found between *Wolbachia* and their hosts (pearsons correlation coefficient r=0.5511; one-tailed permutation test: p=0.0049) suggesting that closely related symbionts have primarily switched between related hosts. This same pattern was marginally significant for *Rickettsia* (r^2=0.3605; one-tailed permutation test: p=0.0687).

In this analysis, I included multiple strains of the same host species. These strains were all genetically distinct and in most cases were not monophyletic in the tree (for example *Adalia bipunctata* strain X and Y in Figure 7.2). However, this test may be confounded if there is natural polymorphic variation within bacterial strains from a single invasion of a particular host. Therefore, I repeated the analysis including only strains from different host species. The results were similar, although the positive correlation with *Wolbachia* and their hosts was highly significant (r^2=0.6475; one-tailed permutation test: p=0.0005), and the positive correlation between *Rickettsia* and their hosts became clearly non-significant (r^2=0.1920; one-tailed permutation test: p=0.2812).

If bacterial strains from distantly related ladybirds occur in independent clades, then this is likely to have a great influence on the results of these analyses. For example, *Coccidula rufa* and *Rhizobius chrysomeloides* are more closely related to each other than other ladybirds possessing *Wolbachia*, and indeed, their

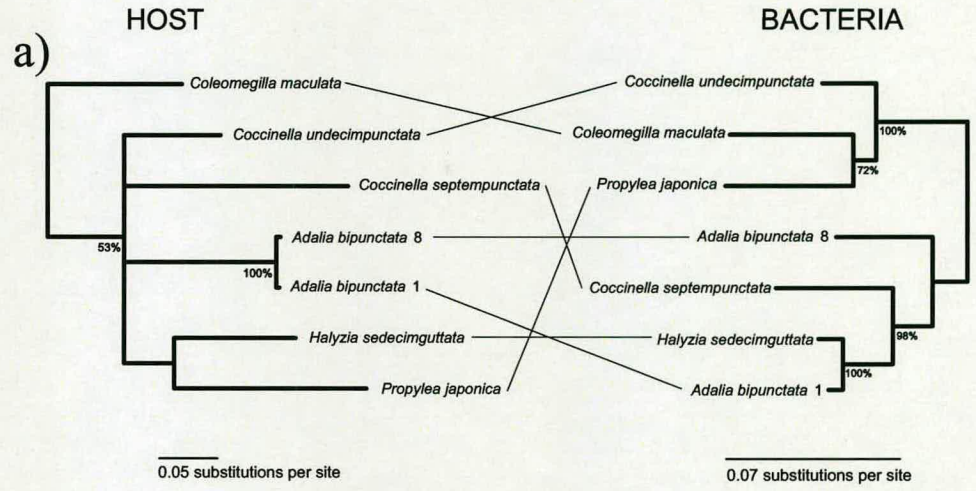
Wolbachia strains are identical and distinct from the other ladybird *Wolbachia* strains (Figure 7.2). To investigate whether more closely related bacterial strains preferentially transmit to closely related ladybirds (either by cospeciation or switching), I repeated the correlation tests on individual clades of bacteria that are exclusively ladybird symbionts (although there is only one clade in both the *Wolbachia* phylogeny (Figure 7.2) and the *Rickettsia* phylogeny (Figure 6.1) that contain more than three strains). Within the *Wolbachia* clade, bacterial pairwise difference was not correlated with host distances within this individual clade (r^2=0.2321; one-tailed permutation test: p=0.1170). Figure 7.3a shows a tanglegram comparing this *Wolbachia* clade with the host phylogeny, and there seems to be a high degree of incongruence between the two phylogenies. In particular, *Adalia bipunctata* strain 1 forms a monophyletic group with *Halyzia sedecimguttata* and *Coccinella septempunctata* to the exclusion of a strain from the same host species (*A. bipunctata* strain 8) with high posterior support.

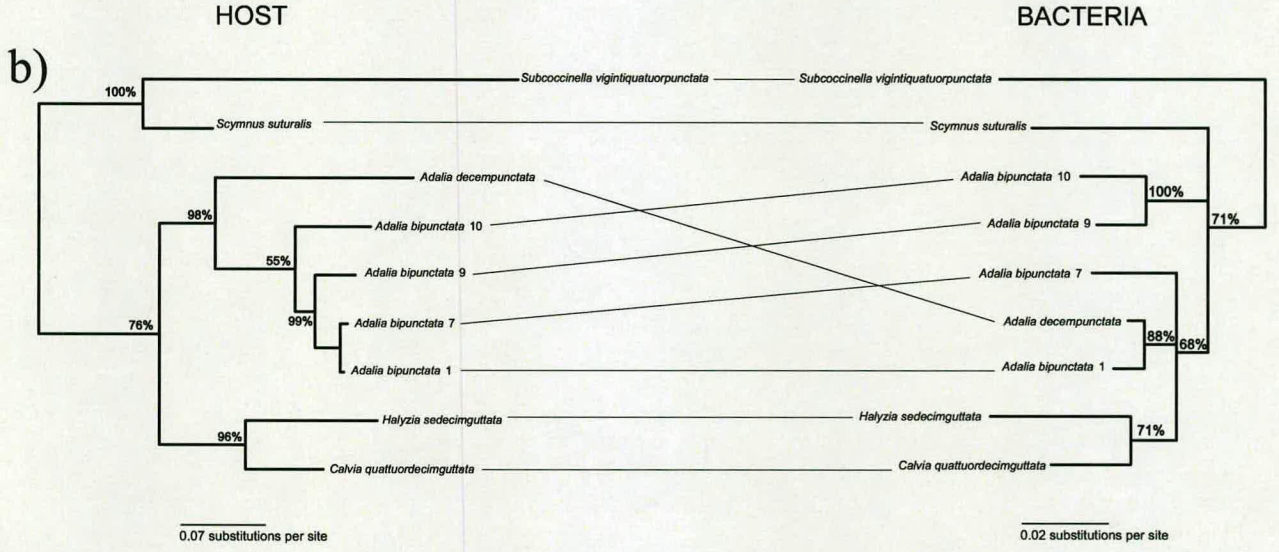
Conversely, there was a significant positive correlation between *Rickettsia* and their hosts when just the 'adalia group' symbionts are considered (r^2=0.7248; one-tailed permutation test: p=0.0346), indicating that either the bacteria has undergone co-speciation with their hosts or there is switching to similarly related hosts (host tracking). This pattern is also reflected in the tanglegram of this clade (Figure 7.3b). However, it appears that there may have been horizontal transmission between the *Adalia bipunctata* strains and the *Adalia decempunctata* strain, as forcing all the *Adalia bipunctata* symbionts to be monophyletic marginally reduces the likelihood of the phylogeny (SH test; -2Δln=12.780 *d.f.*=8 p=0.075).

7.3.5 Phenotypic similarities between hosts

Since bacterial genotype and/or host genotype has been shown to have an effect on the reproductive phenotype that is manifest in the host (Sasaki et al. 2002; Jaenike et al. 2007), I investigated to what extent this happened in my data. Within host species sampling of sex ratio, female prevalence and male prevalence has been collected for some of the bacterial strains described here (chapter 3), and so I

Figure7.3 Tanglegrams of ladybird host phylogeny with a) *Wolbachia* and b) *Rickettsia*. Posterior probabilities are given on the corresponding branch and the scale bars represent length of branches. Connecting lines are drawn to connect bacteria strains to the host species they infect (or mitochondrial haplotypes within the host species *Adalia bipunctata*).





used Moran's test for spatial autocorrelation to test whether there was a relationship between these factors and both bacterial and host pairwise distances. The null distribution of this statistic was obtained by permuting the trait measurement over bacterial taxa in the tree 100,000 times. First, I assessed whether the relatedness of host had any effect on the host population sex ratio, but did not find a significant effect for hosts infected with *Wolbachia* (Moran's test for autocorrelation I=0.3686 p=0.1812) or hosts infected with *Rickettsia* (I=0.1670 p=0.6199 respectively). Second, I tested whether related bacteria tended to infect hosts with similar sex ratios. Again, there was no significant relationship between the host sex ratio and either *Wolbachia* (I=0.4482 p=0.1832) or *Rickettsia* bacterial distances (I=0.0534 p=0.8907).

It is also interesting to note that closely related *Rickettsia* tended to occur at similar prevalence in their host populations. For example, a higher prevalence of *Rickettsia* in male ladybirds is reported in *C. rufa*, *R. chrysomeloides*, *Scymnus suturalis*, and *Subcoccinella vigintiquatuorpunctata*, whereas a lower prevalence is recorded in the other hosts, whose *Rickettsia* form a monophyletic group (Figure 7.1). This pattern was statistically significant (I=0.5487 p<0.0000). The same pattern is significant for female prevalence also (I=0.4710 p=0.00067). Conversely, closely related *Wolbachia* do not tend to occur at similar prevalence. Bacterial distance and the prevalence of the bacterium in females were not correlated (Moran's test for autocorrelation I=0.3844 p=0.25841). The same pattern was also observed for male prevalence (I=0.3375 p=0.34081).

The analysis was then repeated using host rather than bacterial distances. In contrast to the results using bacterial distances, closely related hosts had very similar prevalence of *Wolbachia* and, independently, *Rickettsia.* The prevalence of *Wolbachia* in females and host distance was highly significantly correlated (I=0.5535 p=0.00592), and marginally so for male prevalence (I=0.5026 p=0.05522). Again, the prevalence of *Rickettsia* was similar in closely related female hosts (I=0.2750 p=0.08772) and also male hosts (Moran's test for autocorrelation; I=0.3620 p=0.07401), although only marginally so in both cases.

7.4 *Discussion*

In this study, I explored the routes of horizontal transfer of bacterial symbionts between hosts that are members of the same family (Coccinellidae). It is clear from the data that host relatedness has an important effect on switching between hosts. In Figure 7.2 and Figure 6.1, closely related symbionts sometimes share very distantly related hosts. For example, *Rickettsia* has been known to switch between hosts that span different kingdoms (Animala-Plantae and Protista-Animala; Figure 6.1), but this is very rare. There is clearly preferential host switching between closely related hosts, and this pattern is evident at all taxonomic levels. In this study, I show that switching between different orders of arthropods hosts does occur but that switching between related family members is more common. I also find that within a family, switching between hosts happens more frequently between close relatives. Finally, this study and others have shown that many individual species harbour closely related strains of bacteria associated with different mitotypes (Jiggins 2003; Hiroki et al. 2004; Baldo et al. 2007), and that switching between hosts of the same genera and species sometimes happens frequently enough to obscure the phylogenetic signal created by maternal inheritance (Haine et al. 2005; Baldo et al. 2008). This has important implications for the incidence of symbionts observed across different hosts, as the distribution of related strains within host species will partly result from chance horizontal host switches. This will result in the host taxa that speciate fastest or with a high population density having the highest incidence of infection (Engelstadter and Hurst 2006).

There appeared to be no association between *Wolbachia* relatedness and host relatedness within an individual clade (Figure 7.3a), but when all taxa were included, host relatedness and bacterial relatedness are positively correlated. This indicates that there is a large amount of horizontal transfer between related hosts, but that the success of infection is partly determined by host relatedness. In contrast to *Wolbachia*, *Rickettsia* strains did cluster by host relatedness within a clade, (Figure

7.3b) (although there is probably one instance of a symbiont switching to a related genus). This pattern must be partially explained by the relatedness of four distinct strains that naturally infect *A. bipunctata*, and therefore may be accountable by the bacterial strains accumulating polymorphism after one invasion. Indeed, it has been shown that these strains may be causing an ancient polymorphism in their associated mitochondrial genealogies (Jiggins and Tinsley 2005), and there is more evidence to suggest that this association is older than the association of *Wolbachia* with *A. bipunctata* (Schulenburg et al. 2002).

The reasons for preferential host switching between relatives could result from shared physiology, shared vectors such as parasites or predators, or from a shared ecology. There is good evidence to suggest that a shared physiology between related insect hosts is important for facultative endosymbionts such as *Wolbachia*. Trans-infection experiments indicates that it is relatively easy to establish a successful infection when the donor is of the same species or a related species (Boyle et al. 1993; Grenier et al. 1998; Sasaki and Ishikawa 2000), but notoriously difficult in distantly related individuals (Dobson et al. 2002; Kang et al. 2003). This means that the observation that there is preferential switching on all taxonomical levels could be caused by an increased successful transfer rate in more native rather than naïve hosts (Tinsley and Majerus 2007). There is also evidence to suggest that the infection can be picked up and passed on in parasitoids (Heath et al. 1999; Huigens et al. 2000; Huigens et al. 2004) and mites (Jaenike et al. 2007). This can also result in related hosts having similar strains when they share vectors, although this will also result in distantly related hosts having similar strains. More evidence is needed to assess the importance of this factor in the wild. Finally, there are many examples of trans-infection between hosts where the reproductive phenotype is maintained (Boyle et al. 1993; Sasaki and Ishikawa 2000; Poinsot and Mercot 2001; Tinsley and Majerus 2007) or altered (Sasaki et al. 2002; Sakamoto et al. 2005; Sasaki et al. 2005), even sometimes in distantly related hosts (Moret et al. 2001). This means that organisms with a shared ecology that would facilitate an invasion of the same reproductive phenotype could be more likely to harbour related strains, regardless of the taxonomic distance between them. For instance, organisms that cannibalise dead

male siblings may be more predisposed to male-killing, and therefore a male-killer from an unrelated species could easily pass across. However, in this study, sex ratio distorting ladybird symbionts do not cluster with other male-killers either in *Wolbachia* or *Rickettsia*. It is possible that the apparent clustering of reproductive phenotypes on the bacterial phylogeny is simply a result of selection favouring the same phenotype in related hosts, and not due to constraints resulting in the conservation of phenotypes. These results suggest that it may be far easier to evolve a male-killing phenotype rather than adapt to a naïve host with an ecology which would support a male-killer.

Since similar reproductive phenotypes are manifest in related hosts, it can therefore be hypothesised that other phenotypic properties may be similar. There is weak evidence suggesting that prevalence occurs at similar levels in related ladybird hosts. This may imply that some aspect of host physiology, such as host resistance, may control bacterial prevalence levels in populations. However, it should also be noted that prevalence will be affected by the presence of other male killers in the same population (Randerson et al. 2000), which was impossible to control for. Closely related *Rickettsia* also exhibited similar prevalence levels in their hosts, although this is not surprising given that there is a correlation between host genotype and bacterial genotype in *Rickettsia*. Further experimental evidence will be needed to establish causal affects between phenotype and genotype.

7.5 *Conclusions*

I have investigated both the extent and routes of bacterial host switching in ladybirds, and demonstrated that preferential switching between related hosts occurs at all levels. This seems to be driven primarily by physiology, or shared vectors. but does not seem to be because hosts share an ecology that supports male-killing (at least in ladybirds). Related hosts also exhibit other bacterial phenotypic characteristics in common. Taken together, these results have important implications for the

heterogeneity observed in the incidence of bacterial symbionts across arthropod hosts, and impacts on the origin of male-killing in endosymbionts.

Chapter 8. Discovery and evolution of conjugation genes in arthropod *Rickettsia*

8.1 *Introduction*

Rickettsia genomes are smaller than their free-living counterparts and encode for less recombination machinery (Andersson et al. 1998). In addition, a large proportion of their genomes are made up of pseudogenes and non-coding DNA, which is indicative of genome degradation caused by relaxed selection on genes whose products are substituted by the host, and a deletion bias indirectly caused by smaller effective population sizes (Andersson and Andersson 2001). Generally, *Rickettsia* genomes are more syntenic than closely related symbiont genomes such as *Wolbachia* (K. Fenn personal communication). However, the recently published genomes of *Rickettsia felis* and *Rickettsia bellii* are uncharacteristically larger, less syntenic and have many transposases, proteins with ankyrin repeat domains and tetratricopeptide repeat motifs (both of the latter are involved in protein-protein interactions) (Ogata et al. 2005; Ogata et al. 2006; Darby et al. 2007). These genomes are also associated with genes that encode conjugative machinery.

Conjugation is an ancient mechanism of horizontal gene transfer that occurs between bacteria through cell contact. This transfer is mediated by a plasmid, and regulation, the synthesis of a mating pilus, stabilisation contact and DNA metabolism are encoded by a set of conjugation genes (*Tra* genes) (Clewell 1993). These conjugation genes in *Rickettsia* are encoded either in the chromosome or on a plasmid. *R. belli*, and *Rickettsia massiliae* contain a full complement of chromosomally-encoded conjugation genes, and *Rickettsia canadensis* approximately half this many (Eremeeva et al. 2005; Blanc et al. 2007). *Rickettsia felis* and *Rickettsia monacensis* have plasmid-encoded conjugative genes, although they appear to only have a partial *tra* gene cluster, which, in addition, is non-functional in *R. monacensis* (Ogata et al. 2005; Baldridge et al. 2007). The wealth of

chromosomal-encoded conjugation genes in strains that lack plasmids, has been a matter of speculation, but it appears this may be due to the propensity to lose a plasmid through passage in cell culture before genome sequencing (Baldridge et al. 2008). Indeed, plasmids have now been found in seven strains (although not yet sequenced) (Baldridge et al. 2008).

Many conjugation systems have given rise to Type IV secretion systems (Frank et al. 2005), which are found to function in pathogenesis by delivering effector substances to eukaryotic cells in numerous intracellular pathogenic bacteria (Sexton and Vogel 2002; Segal et al. 2005; Pan et al. 2008). Some of these have only recently evolved from conjugation genes, and still retain the ability to export DNA, as well as proteins (de Felipe et al. 2005; Segal et al. 2005). However, conjugative genes and plasmids have only been found in one other obligate intracellular pathogen (Stephens et al. 1998; Ogata et al. 2005).

The role of conjugative genes in *Rickettsia* biology still needs to be established. The presence of virulence genes on the *R. felis* plasmid suggests that they may play a role in acquiring pathogenicity functions. There are three potential genes on the *Rickettsia felis* plasmid that may be involved in invading host cells: a surface protein and two genes that increase host tissue permeability (Ogata et al. 2005). In addition, there are two genes that encode proteins with ankyrin repeat domains and seven genes with tetratricopeptide repeat motifs (Gillespie et al. 2007). Proteins with ankyrin repeat domains are common in eukaryotic chromosomes but are rare in bacteria. However, they have recently been found in a suite of intracellular bacteria, and are known to be exported protein, which suggests a role in pathogenicity (Iturbe-Ormaetxe et al. 2005; Cho et al. 2007; Pan et al. 2008). They have also attracted attention in *Wolbachia* as variation in a protein with an ankyrin repeat domain associated with a phage has been correlated with different reproductive manipulation phenotypes (Sinkins et al. 2005).

An important step in uncovering conjugation gene function is to link their presence to phenotypic characteristics of the bacterium, and so the main purpose of

this study, is to determine the host range of these conjugation elements. Phylogenetic analyses of the *R. felis* plasmid genes with chromosomal genes yielded a topology that indicates that many of these genes may have come from 'ancestral' *Rickettsia* (Gillespie et al. 2007). I experimentally test this hypothesis by using PCR to detect the presence of conjugation genes in more basal strains of *Rickettsia*. In addition, I show that both plasmid and genome-encoded conjugation gene phylogenies are extensively decoupled from their bacterial host phylogeny, which strongly suggests they are being transferred horizontally between bacterial strains.

8.2 *Methods*

8.2.1 Identification of conjugation genes

Conjugation genes are remarkably conserved for horizontally transferred elements, and so I aligned protein sequences and designed primers in conserved regions to have the best hope of detecting them by PCR. I tested 13 samples given in Chapter 6 (some samples were not included because of a lack of extract) and are given in Figure 8.1. All PCR primers are given in Chapter 2. In addition, as conjugation genes are usually found in close proximity to each other, primers were designed to amplify sequence between the genes detected (as gene orientations from un-sequenced *Rickettsia* are unknown, primers were designed in all orientations). In order to investigate the presence of conjugation genes in *Rickettsia* genomes, I used all currently identified conjugation genes from *Rickettsia* strains (found on strains *R. felis* and *R. bellii*) to perform BLAST searches. The tblastx algorithm, which translates the query sequence and matches it against a translated database, was used to search for possible homologues conserved at the protein level within all known *Rickettsia* genomes (all genomes and search tool at http://patric.vbi.vt.edu/), and in the related genus *Orientia* (http://sourceforge.net/projects/genome-tools/). In addition, tblastx was used for shorter sequences (<300bp) against all nucleotide sequences in Genbank and discontiguous megablast, which allows mismatches in the initial seed (and therefore designed to pick up more dissimilar sequences), was used

for larger sequences to confirm that homologues occur only in *Rickettsia* and *Orientia*.

8.2.2 Phylogeny

The bacterial phylogeny was created in the same way as the MLST analysis in Chapter 6 using the four MLST genes, and all sequenced *Rickettsia* genomes are included (14 in total). The endosymbiont of *Coccidula rufa* was excluded as it is a recombinant strain (Chapter 6). Phylogenies of conjugation genes were constructed for genes that were detected in seven or more strains of bacteria. This included the $TraD_{TI}$, $TraA_{TI}$, and $TraD_{F}$ genes first identified from *Rickettsia felis*, and $TraD_{TI}$, $TraD_{F}$ and $TraB_{F}$ first identified from *Rickettsia bellii*. Although there is overlap in the same class of conjugation genes found on the plasmid of *Rickettsia felis* and the chromosome of *Rickettsia bellii*, the gene sequences are too divergent to be able to align confidently. The exception was $TraD_{F}$ from both types, which could easily be aligned at the protein level and was therefore incorporated in to a single gene tree (shown in Figure 8.4). Model selection was made using the program Modeltest v.3.7 (Posada and Crandall 1998) and models chosen are given in the Figure legends of Figure 8.3 and 8.4. Bayesian phylogenies were created using MrBayes using the MC^3 algorithm for 500,000 generations.

8.3 *Results*

8.3.1 Presence of conjugation genes

Figure 8.1 shows that conjugation genes were detected in 11 of the 13 basal strains of *Rickettsia* tested (not detected in one strain of *Adalia bipunctata* from Moscow or *Halyzia 16guttata*). However, a lack of PCR product is not confirmation that the conjugation genes are not present as they might be too divergent or truncated (as many conjugation genes seem to be in *Rickettsia*).

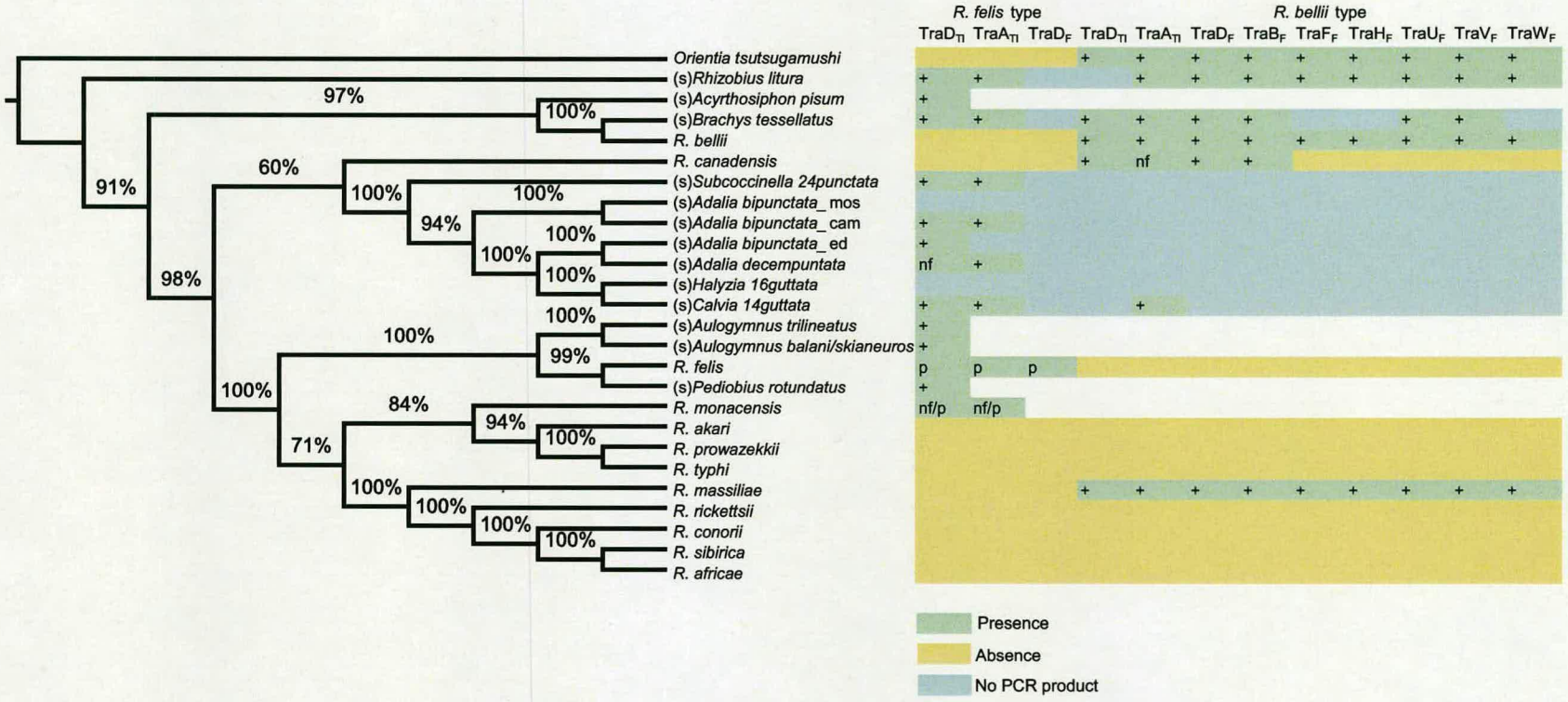


Figure 8.1. The phylogeny of *Rickettsia* indicating the presence or absence of conjugation genes. Subscripts of the different conjugation genes indicate either a similarity to the *Agrobacterium tumificens* plasmid (TI type) or the *Escherichia coli* F plasmid (F type). p donates that the conjugation gene is encoded on a plasmid; nf indicates that the gene does not encode a functional protein. Posterior support is given along the branch length.

8.3.2 Synteny of conjugation genes is broken

All attempts but one to amplify sequence between the different genes failed. This implies that the conjugation genes may not be in the same orientation as they are found on the *R. felis* plasmid or the *R. bellii* chromosome. A PCR that targeted the region between $TraA_{TI}$ and $TraD_{TI}$ (felis-type) from the endosymbiont of *Adalia decempunctata* gave a product that was larger than expected. Sequencing the product showed that two additional genes were present between these two genes, which were not present on the *Rickettsia felis* plasmid (Figure 8.2). Therefore the synteny of the *R. felis* plasmid conjugation genes is different in this strain.

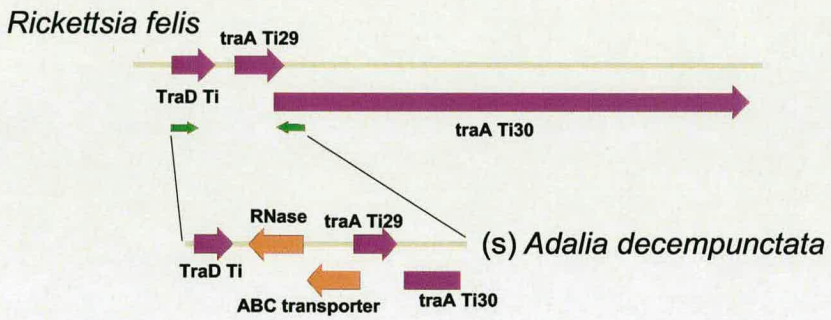


Figure 8.2. The different synteny of conjugation genes between *R. felis* and the symbiont of *A. decempunctata*. Green arrows represent the position of the primer sequences.

In addition, since the $TraA_{TI}$ gene on the *R. felis* plasmid is considerably larger than other conjugation genes (Figure 8.2), primers were designed that targeted both ends. Successful PCR products were obtained for both ends (i.e. 5' and 3') for the symbionts of *Adalia decempunctata*, *Calvia 14guttata* and *Subcoccinella 24punctata*, but all PCR assays which targeted the middle of the gene conducted with specific primers designed within the sequenced ends failed. Although this might be due to experimental error, phylogenetic analysis gave strong support for the different sides of the gene having different evolutionary histories (Figure 8.2b and 8.2c). This

indicates that the two ends are decoupled from one another in some way or that the PCR products come from two different paralogous genes.

8.3.3 Phylogeny of *R. felis* type conjugation genes

To investigate the evolutionary history of the conjugation genes, phylogenetic analysis was conducted on conjugation genes that were detected in seven or more strains of *Rickettsia*. The phylogenies of $TraA_{TI}$ and $TraD_{TI}$, which were first detected on the *R. felis* plasmid, are shown in Figure 8.3 (note that the $TraA_{TI}$ gene is depicted from two different trees – Figure8.3b and 8.3c). There are two cases in Figure 8.3a, where the phylogeny of $TraD_{TI}$ is in agreement with the bacterial phylogeny. First, symbionts of the parasitoid wasp genus *Aulogymnus* group together with strong posterior support (93%), which is also the case on the bacterial phylogeny (Figure 8.1). Second, *R felis* and the symbiont of *Pediobius rotundatus* are positioned in a similar place. However, all other well supported groups on the $TraD_{TI}$ gene tree are not in agreement with the bacterial phylogeny. In many cases, these clusters are made up of bacterial strains that come from the different bacterial groups named in Chapter 6 (Figure 6.1). The only exception is the cluster of conjugation genes that come from the symbionts of the genus *Adalia* (Figure 8.3a). However, the bacterial phylogeny show that the *A. decempunctata* strain is closely related to the *A. bipunctata* strain from Edinburgh (Figure 8.1), whereas the conjugation gene from *A. bipunctata* strain from Edinburgh is closely related to the *A. ipunctata* strain from Cambridge (Figure 8.3a).

A similar pattern is observed in $TraA_{TI}$ where there is a small degree of agreement between conjugation gene and bacteria phylogeny, but is mainly decoupled. There is only one case of agreement where the *Adalia* and *Calvia* $TraA_{TI}$ gene-cluster in Figure 8.3c mimics the bacterial phylogeny (Figure 8.1). Conversely, the $TraA_{TI}$ gene from the symbiont of *S. 24punctata* does not cluster within these strains but appears more related to the symbiont of *Coccidula rufa* and *Rickettsia monacensis*. However, this conjugation gene sequence does cluster with other

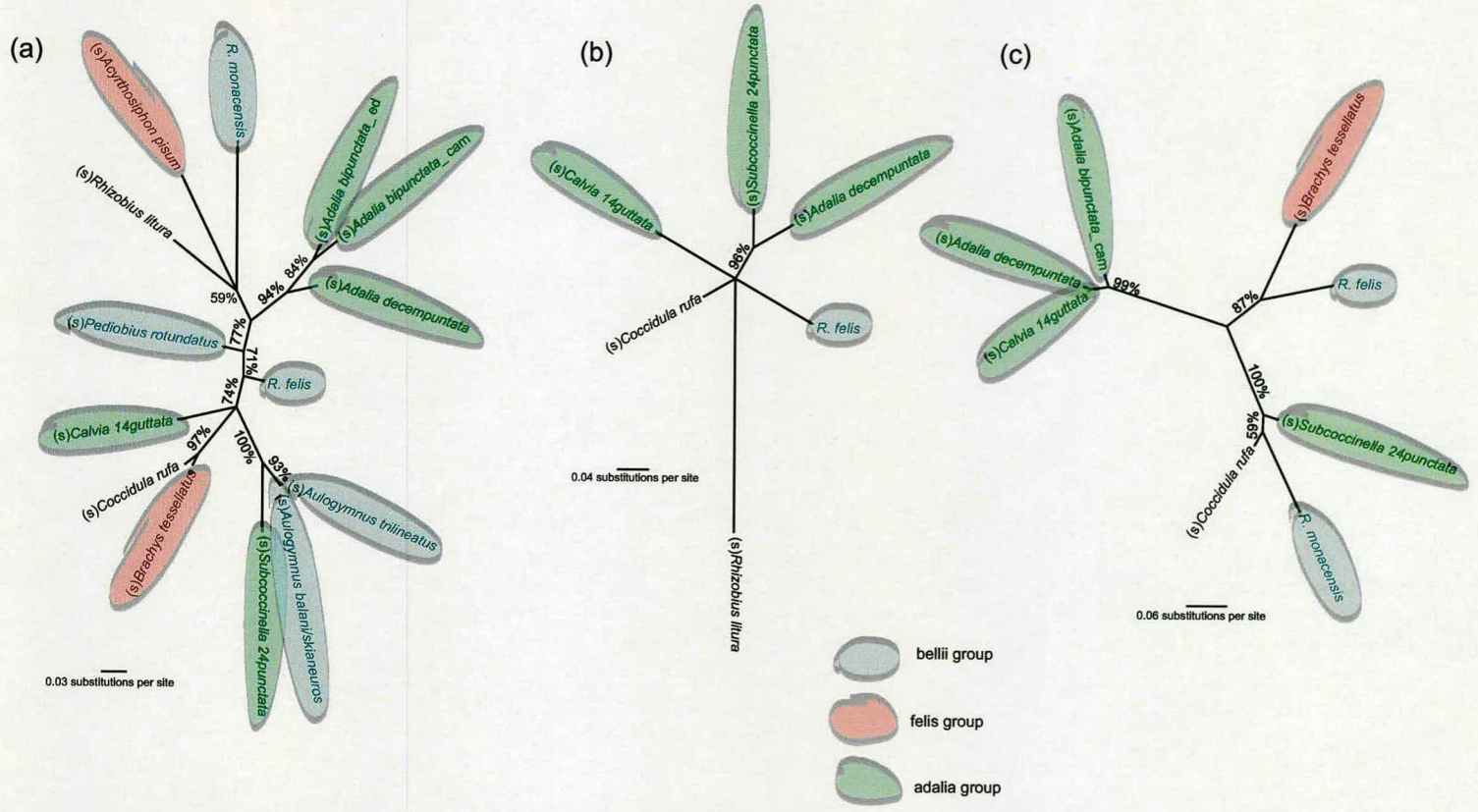


Figure 8.3. Phylogeny of (a) *TraD*$_{TI}$ (b) *TraA*$_{TI}$3'and (c) *TraA*$_{TI}$5'*R. felis*-type conjugation genes. The key represents the bacterial group from Chapter 6 that the conjugation genes were isolated from. Scale bars represent the length of branches. Posterior support is given along the branches. Models of sequence evolution were (a) GTR + Γ (b) F81 (c) HKY + Γ

'adalia' group conjugative elements in Figure 8.3b, but is still not consistent with the rest of the bacterial genome (Figure 8.1).

Finally, the three different gene trees in Figure 8.3 also differ from each other. As mentioned before, the phylogenies of Figure 8.3b and 8.3c are different. The $TraD_{TI}$ gene tree in Figure 8.3a is also different from these two phylogenies. For example, the $TraD_{TI}$ gene from the symbiont of *S. 24punctata* groups with the $TraD_{TI}$ genes from the symbionts of *Aulogymnus* (Figure 8.3a), the $TraA_{TI}$ gene with the symbiont of *A. bipunctata* in Figure 8.3b and the $TraA_{TI}$ gene with *R. monacensis* and the symbiont of *C. rufa* in Figure 8.3c.

8.3.4 Phylogeny of *R. belii* type conjugation genes

Although the $TraB_F$ gene is absent from *R. felis*, the $TraA_{TI}$ and $TraD_F$ genes are present on both the *R. felis* plasmid and in the *R. bellii* chromosome. However, although the genes are orthologus, they are divergent from one another, and in the case of $TraA_{TI}$, too difficult to align. A tblastx search of the $TraA_{TI}$ gene from *R. bellii* indicated a higher similarity to $TraA_{TI}$ genes from numerous other strains of *Legionella*, *Agrobacterium*, *Rhizobium* (to mention just a few) than the $TraA_{TI}$ gene from the *R. felis* plasmid, suggesting independent origins of conjugation genes in to *Rickettsia*. However, a tblastx search of the $TraD_F$ gene from *R. bellii* showed that it is more similar to the $TraD_F$ gene on the *R. felis* plasmid.

The phylogenies of $TraB_F$, $TraA_{TI}$ and $TraD_F$ genes first detected on the *R. bellii* chromosome are shown in Figure 8.4. Although the $TraA_{TI}$ gene is also present in *Orientia tsutsugamushi*, the portion of the gene sequenced in the strains shown in Figure 8.1 was not present, and therefore, unalignable. The striking characteristic of these gene trees is that the most basal strains are *O. tsutsugamushi*, which is completely consistent with the MLST phylogeny. *Rickettsia canadensis* also seems to be basal to all the other conjugative genes in other strains. This is in disagreement with the MLST phylogeny (Figure 8.1). The posterior support for the placement of

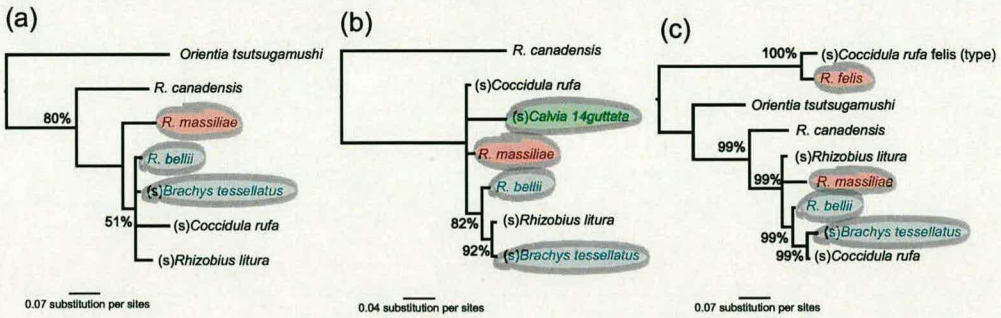


Figure 8.4. Phylogeny of (a) TraB (b) TraA and (c) TraD *R. bellii*-type conjugation genes. Colour represent the groups picture in Figure 8.3. Scale bars represent the length of branches. Posterior support is given along the branches. Trees are rooted according to the roots in mas ref (c) is rooted according to plasmid or chromosome. The models of sequence evolution are (a) F81 Γ (b) F81 Γ (c) HKY

$TraB_F$ gene strains is weak (Figure 8.4a), making it difficult to draw conclusions about the relationship between the strains. However, it does seem to be incongruent with the MLST phylogenies.The phylogenies of the $TraA_{TI}$ and $TraD_F$ genes are also decoupled from the MLST genes shown in Figure 8.1, The $TraA_{TI}$ gene of the symbiont of *Brachys tessellatus* is more closely related to the $TraA_{TI}$ gene from the symbiont of *Rhizobius litura*, whereas the MLST genes are more related to *R. bellii*. Similarly, the $TraD_F$ gene of the symbiont of *Rhizobius litura* is more closely related to the $TraA_{TI}$ gene from *R. massilliae*, than either are to the $TraD_F$ gene from *R. bellii* even though the MLST genes place the symbiont of *Rhizobius litura* basal to *R. bellii,* and *R. bellii* basal to *R. massilliae*. As was shown for the *R. felis* type genes, these gene trees also differ from each other. For example, the conjugation genes from the symbiont of *Brachys tessellatus,* appear in different places in the three trees (Figure 8.4).

8.4 *Discussion*

This study demonstrates that conjugation genes are common among *Rickettsia* and *Orientia*. In addition, phylogeny indicates that they have been horizontally transmitted between strains. This is in stark contrast to the rest of genome, as MLST

analysis suggests that horizontal gene transfer of housekeeping genes is rare (Chapter 6). However, it is not possible to draw extensive conclusions about which strains may have interacted in the past, because many of the genes may be paralogous. In *O. tsutsugamushi* there has been a radiation of these conjugation genes and they are repeated many times throughout the bacterial chromosome (Cho et al. 2007).

The presence of a system that can horizontally transfer DNA between *Rickettsia* species is likely to have an important effect on the evolution of these strains. The intracellular lifestyle and maternal transmission of these bacteria reduces their effective population size and increases their mutation rate. Consequently, endosymbionts suffer the accumulation of deleterious mutations due to Muller's ratchet and interference selection (Moran 1996). Therefore, their small genomes of largely essential genes limit the evolutionary potential to evolve novel traits, which is likely to be a problem for pathogenic bacteria if the selection pressure for host resistance is strong. However, horizontal transfer can counteract these problems by providing novel gene combinations and can purge deleterious mutations.

Figure 8.1 show that most of these conjugation genes still encode functional proteins in many divergent strains. In addition, blast searches indicate that these conjugation genes constitute a *Rickettsia*-specific clade, and have been evolving in *Rickettsia* for some time. Both these factors suggest that these conjugative elements may be adaptive for *Rickettsia.* In addition, it is currently debatable whether plasmids can exist as entirely selfish elements, but if they could, they would require a high degree of infectious transmission (Paulsson 2002), which is impossible given the lifestyle of *Rickettsia.* There seems to be substantial polymorphism in the number of genes between different *Rickettsia* strains, which indicates that there is dynamic loss or gain of these elements, although the absence of a PCR product does not necessarily mean an absence of the gene.

I have experimentally shown that the majority of conjugation genes occur in basal strains. This is consistent with the observation that many of the genes on the *R. felis* plasmid are closely related to the chromosomal genes of *R. bellii* (Gillespie et al.

2007). With the exception of *O. tsutsugamushi*, these basal strains are purely arthropod associated *Rickettsia* (Chapter 6). Horizontally transferred elements in *Wolbachia* have been shown to correlate with the reproductive phenotype of their host (Sinkins et al. 2005), and so it is tempting to speculate that the presence of these conjugative elements may facilitate in the reproductive manipulation of *Rickettsia* hosts. Although this is purely speculative, the lack of these genes in strains that infect vertebrates argues against the role of these genes in vertebrate pathogenicity. Indeed, contrary to other types of bacteria, horizontally transferred elements do not correlate with vertebrate pathogenicity (Darby et al. 2007). This could be because an inactivation in *Rickettsia*-regulatory genes in the strains that infect vertebrates allowed these bacteria to proliferate, increasing the potential to be transmitted infectiously (Darby et al. 2007).

There is evidence for two separate evolutionary origins of conjugative genes in to *Rickettsia*. The genes that are encoded of a plasmid of *R. felis* are more divergent than the *R. bellii* chromosomal genes. The majority of the strains tested here are positive for the *R. felis* plasmid type genes (Figure 8.1). Since these type of conjugation genes have so far only been discovered on a plasmid, this suggests that plasmids may be common. However, further experiments will be needed to establish the physical position of these elements. In conclusion, the presence of plasmids in *Rickettsia* is an exciting discovery that is likely to reveal insights to the biology of the different strains, and will be a useful tool in the genetic manipulation of intracellular bacteria.

Chapter 9. Concluding remarks

The preceding chapters have investigated the incidence and diversity of endosymbiotic bacteria in arthropods. Empirical data from ladybird beetles suggests that many different endosymbionts are common in hosts that have an ecological predisposition to male-killers. In addition, *Wolbachia, Rickettsia* and *Cardinium*, are all known to infect their host species at a low prevalence, which suggests that previous estimates that assess their abundance by examining low numbers of individuals from many species will have almost certainly been an underestimate. However, despite these biases that are likely to be more pronounced for bacteria such as *Rickettsia,* which are not found at high prevalence in host populations, *Wolbachia* still infects more species than other investigated bacteria. What makes *Wolbachia* so successful compared to other symbionts? The answer to this question almost certainly lies in the variety of the phenotypic manipulations that *Wolbachia* has evolved. However, this study predicts that *Cardinium* may have a similar diverse array of phenotypes, which implies that we still have much to learn about the biology and population dynamics of these bacteria.

Although the ecology and genetics of the hosts they infect are major determinants of their incidence, the ability to switch between species will also have an affect on incidence dynamics. The evolutionary origins of *Wolbachia* and *Rickettsia* suggest that frequent switching between related hosts has been an important part of their evolution. This switching is evident on all taxonomic levels, suggesting that host physiology may be an overriding factor in the establishment of a new infection. Although endosymbionts frequently switch between species with a related ecology, it may be genetic relatedness and not ecology that explains this pattern. These studies indicate that the evolution of male-killing has multiple evolutionary origins, (even within a single supergroup of *Wolbachia*), which suggest that the trait may be easy to evolve.

Mobile elements are known to be responsible for the spread of virulence traits. This modular form of evolution means that strains do not have to evolve traits such as male-killing, and could instead acquire them from other strains in the population. This study has found that although most *Rickettsia* genomes seemed to have suffered from the accumulation of deleterious mutations, and are relatively non-recombining, horizontal gene transfer of conjugation genes is widespread. This may allow pathogenic *Rickettsia* a greater capacity to evolve novel traits, thereby increasing its proliferation in host populations.

In conclusion, many endosymbionts are commonly found at very low prevalence within species and this, coupled with the bias of sampling efforts toward arthropod symbionts, suggests there is still a wealth of diversity yet to be uncovered. In addition, an investigation in to the evolutionary potential of *Rickettsia* indicates that arthropod manipulators may be more innovative than previous studies have realised.

Appendices

Appendix 1

Table S1.1 The distribution of *Rickettsia* in arthropods

taxon	location	*R*
Malacostraca		
Isopoda		
unknown sp.	Chile	-
Arachnida		
Araneae		
unknown sp.	Mexico	-
Clubionidae	Mexico	-
Gnaphosidae sp.1	Mexico	-
Gnaphosidae sp. 2	Mexico	-
Salticidae	Mexico	-
Salticidae	Mexico	-
Holothyrida		
unknown sp.	Mexico	-
Entognatha		
Collembola		
unknown sp. 1	Chile	-
unknown sp. 2	Chile	-
unknown sp. 3	Chile	-
unknown sp. 4	Chile	-
unknown sp. 5	South Africa	-
Insecta		
Blattodea		
unknown sp. 1	Ghana	-
unknown sp. 2	Chile	-
unknown sp. 3	Chile	-
Blattidae sp. 1	Mexico	-
Blattidae sp. 2	Mexico	-
Coleoptera		
unknown sp. 1	Mexico	-
unknown sp. 2	Mexico	-
unknown sp. 3	New York, USA	-
unknown sp. 4	Mexico	-
unknown sp. 5	Ghana	-
unknown sp. 6	Ghana	-
unknown sp. 7	Mexico	-
unknown sp. 8	India	-
unknown sp. 9	Ghana	-
unknown sp. 10	Mexico	-
unknown sp. 11	Mexico	-
unknown sp. 12	Mexico	-
unknown sp. 13	Mexico	-
unknown sp. 14	Panama	-
unknown sp. 15	Panama	-
unknown sp. 16	Panama	-
unknown sp. 17	Panama	-
unknown sp. 18	Panama	-
unknown sp. 19	Panama	-
unknown sp. 20	Panama	-
unknown sp. 21	Panama	-
Anthicidae	Mexico	-
Anthribidae sp. 1	Ghana	-
Anthribidae sp. 2	Mexico	-
Anthribidae sp. 3	Mexico	-
Anthribidae sp. 4	Mexico	-
Brentidae sp. 1	Ghana	-
Brentidae sp. 2	Papua New Guinea	-
Buprestidae sp. 1	Mexico	-
Buprestidae sp. 2	Mexico	-
Buprestidae sp. 3	Ghana	-
Carabidae sp. 1	New York, USA	-
Carabidae sp. 2	New York, USA	-
Carabidae sp. 3	Mexico	-
Carabidae sp. 4	Mexico	-
Carabidae sp. 5	Mexico	-
Carabidae sp. 6	Mexico	-
Carabidae sp. 7	Mexico	-
Carabidae sp. 8	Mexico	-
Carabidae sp. 9	Mexico	-
Carabidae sp. 10	Mexico	-
Carabidae sp. 11	Mexico	-
Carabidae sp. 12	Mexico	-
Carabidae sp. 13	Mexico	-
Carabidae sp. 14	Mexico	-
Carabidae sp. 15	Mexico	-
Carabidae sp. 16	Mexico	-
Carabidae sp. 17	Mexico	-
Carabidae sp. 18	Mexico	-
Carabidae sp. 19	Mexico	-
Carabidae sp. 20	Mexico	-
Carabidae sp. 21	Mexico	-
Carabidae sp. 22	Mexico	-
Carabidae sp. 23	Mexico	-
Carabidae sp. 24	Mexico	-
Carabidae sp. 25	Mexico	-
Carabidae sp. 26	Mexico	-
Carabidae sp. 27	Mexico	-
Carabidae sp. 28	Mexico	-
Carabidae sp. 29	Mexico	-
Carabidae sp. 30	Mexico	-
Carabidae sp. 31	Ghana	-
Carabidae sp. 32	Ghana	-
Carabidae sp. 33	Ghana	-
Carabidae sp. 34	Ghana	-
Carabidae sp. 35	Ghana	-
Carabidae sp. 36	Ghana	-
Carabidae sp. 37	Ghana	-
Carabidae sp. 38	Ghana	-
Carabidae sp. 39	Ghana	-
Carabidae sp. 40	India	-
Carabidae sp. 41	India	-
Carabidae sp. 42	India	-
Cerambycidae sp. 1	Mexico	-
Cerambycidae sp. 2	Ghana	-
Cerambycidae sp. 3	India	-
Cerambycidae sp. 4	Panama	-
Cerambycidae sp. 5	Papua New Guinea	-
Cerambycidae sp. 6	Papua New Guinea	-
Chrysomelidae sp. 1	Mexico	-
Chrysomelidae sp. 2	Mexico	-
Chrysomelidae sp. 3	New York, USA	-
Chrysomelidae sp. 4	Ghana	-
Chrysomelidae sp. 5	India	-
Chrysomelidae sp. 6	South Africa	-
Chrysomelidae sp. 7	South Africa	-
Chrysomelidae sp. 8	South Africa	-
Chrysomelidae sp. 9	South Africa	-
Chrysomelidae sp. 10	South Africa	-
Chrysomelidae sp. 11	South Africa	-
Chrysomelidae sp. 12	South Africa	-
Chrysomelidae sp. 13	South Africa	-
Chrysomelidae sp. 14	South Africa	-
Chrysomelidae sp. 15	Papua New Guinea	-
Chrysomelidae sp. 16	Papua New Guinea	-
Cleridae sp. 1	Mexico	-
Cleridae sp. 2	Ghana	-
Cleridae sp. 3	Papua New Guinea	-
Cleridae sp. 4	Papua New Guinea	-
Coccinellidae sp. 1	New York, USA	-
Coccinellidae sp. 2	Mexico	-
Curculionidae sp. 1	Mexico	-
Curculionidae sp. 2	India	-
Curculionidae sp. 3	India	-
Curculionidae sp. 4	South Africa	-
Curculionidae sp. 5	South Africa	-
Curculionidae sp. 6	Chile	+
Curculionidae sp. 7	Papua New Guinea	-
Curculionidae sp. 8	Papua New Guinea	-
Curculionidae sp. 9	Papua New Guinea	-
Dytiscidae sp. 1	India	-
Dytiscidae sp. 2	India	-
Elateridae sp. 1	Mexico	-
Elateridae sp. 2	Mexico	-
Elateridae sp. 3	Ghana	-

taxon	location	*R*	taxon	location	*R*	taxon	location	*R*	taxon	location	*R*
Elateridae sp. 4	Ghana	-	*Scarabaeidae sp. 6*	Ghana	-	*Tenebrionidae sp. 7*	Ghana	-	*unknown sp. 27*	Mexico	-
Elateridae sp. 5	Ghana	-	*Scarabaeidae sp. 7*	Ghana	-	*Tenebrionidae sp. 8*	Ghana	-	*unknown sp. 28*	Mexico	-
Elateridae sp. 6	Papua New Guinea	+	*Scarabaeidae sp. 8*	India	-	*Tenebrionidae sp. 9*	Ghana	-	*unknown sp. 29*	Mexico	-
Endomychidae	Ghana	-	*Scarabaeidae sp. 9*	India	-	*Tenebrionidae sp. 10*	Ghana	-	*unknown sp. 30*	Mexico	-
Erotylidae sp. 1	Mexico	-	*Scarabaeidae sp. 10*	India	-	*Tenebrionidae sp. 11*	India	-	*unknown sp. 31*	Mexico	-
Erotylidae sp. 2	Ghana	-	*Scarabaeidae sp. 11*	India	-	*Trogidae sp. 1*	Mexico	-	*unknown sp. 32*	Mexico	-
Heteroceridae	India	-	*Scarabaeidae sp. 12*	India	-	*Trogidae sp. 2*	Mexico	-	*unknown sp. 33*	New York, USA	-
Hydrophilidae sp. 1	India	-	*Scarabaeidae sp. 13*	Panama	-	*Trogidae sp. 3*	Mexico	-	*unknown sp. 34*	New York, USA	-
Hydrophilidae sp. 2	India	-	*Scarabaeidae sp. 14*	Panama	-	Dermaptera			*unknown sp. 35*	New York, USA	-
Hydrophilidae sp. 3	India	-	*Scarabaeidae sp. 15*	Panama	-	*unknown sp. 1*	Ghana	-	*unknown sp. 36*	New York, USA	-
Hydrophilidae sp. 4	India	-	*Scarabaeidae sp. 16*	Panama	-	*unknown sp. 2*	Chile	-	*unknown sp. 37*	Panama	-
Hydrophilidae sp. 5	India	-	*Staphylinidae sp. 1*	Mexico	-	*unknown sp. 3*	unknown	-	*unknown sp. 38*	Panama	-
Hydrophilidae sp. 6	India	-	*Staphylinidae sp. 2*	Mexico	-	Diptera			*unknown sp. 39*	Papua New Guinea	-
Hydrophilidae sp. 7	India	-	*Staphylinidae sp. 3*	Ghana	-	*unknown sp. 1*	Ghana	-	*unknown sp. 40*	California, USA	-
Hydrophilidae sp. 8	Ghana	-	*Staphylinidae sp. 4*	Ghana	-	*unknown sp. 2*	Mexico	-	*unknown sp. 41*	California, USA	-
Hydrophilidae sp. 9	Ghana	-	*Staphylinidae sp. 5*	Ghana	-	*unknown sp. 3*	Mexico	-	*unknown sp. 42*	California, USA	-
Hydrophilidae sp. 10	Mexico	-	*Staphylinidae sp. 6*	Ghana	-	*unknown sp. 4*	Mexico	-	*unknown sp. 43*	California, USA	-
Lagriidae sp. 1	Papua New Guinea	-	*Staphylinidae sp. 7*	Ghana	-	*unknown sp. 5*	Mexico	-	*unknown sp. 44*	California, USA	-
Lagriidae sp. 2	Papua New Guinea	-	*Staphylinidae sp. 8*	Ghana	-	*unknown sp. 6*	Mexico	-	*Anthomyiidae sp. 1*	New York, USA	-
Lampyridae sp. 1	Chile	-	*Staphylinidae sp. 9*	Ghana	-	*unknown sp. 7*	Mexico	-	*Anthomyiidae sp. 2*	New York, USA	-
Lampyridae sp. 2	New York, USA	-	*Staphylinidae sp. 10*	Ghana	-	*unknown sp. 8*	Mexico	-	*Anthomyiidae sp. 3*	Mexico	-
Lampyridae sp. 3	Panama	-	*Staphylinidae sp. 11*	India	-	*unknown sp. 9*	Mexico	-	*Anthomyiidae sp. 4*	Mexico	-
Lampyridae sp. 4	Panama	-	*Staphylinidae sp. 12*	India	-	*unknown sp. 10*	Mexico	-	*Asilidae sp. 1*	New York, USA	-
Lycidae	Papua New Guinea	-	*Staphylinidae sp. 13*	India	-	*unknown sp. 11*	Mexico	-	*Asilidae sp. 2*	New York, USA	-
Meloidae sp. 1	Mexico	-	*Staphylinidae sp. 14*	India	-	*unknown sp. 12*	Mexico	-	*Asilidae sp. 3*	Mexico	-
Meloidae sp. 2	Mexico	-	*Staphylinidae sp. 15*	India	-	*unknown sp. 13*	Mexico	-	*Asilidae sp. 4*	Mexico	-
Meloidae sp. 3	Mexico	-	*Staphylinidae sp. 16*	South Africa	-	*unknown sp. 14*	Mexico	-	*Bombyliidae sp.1*	Mexico	-
Meloidae sp. 4	Panama	+	*Staphylinidae sp. 17*	South Africa	-	*unknown sp. 15*	Mexico	-	*Bombyliidae sp.2*	Mexico	-
Mordellidae sp. 1	Mexico	-	*Staphylinidae sp. 18*	South Africa	-	*unknown sp. 16*	Mexico	-	*Bombyliidae sp.3*	Mexico	-
Mordellidae sp. 2	Ghana	-	*Staphylinidae sp. 19*	Chile	-	*unknown sp. 17*	Mexico	-	*Bombyliidae sp.4*	Mexico	+
Mordellidae sp. 3	Ghana	-	*Staphylinidae sp. 20*	Chile	-	*unknown sp. 18*	Mexico	-	*Bombyliidae sp.5*	Mexico	-
Nitidulidae	Mexico	-	*Staphylinidae sp. 21*	Chile	-	*unknown sp. 19*	Mexico	-	*Bombyliidae sp.6*	Mexico	-
Ochodaeidae	Mexico	-	*Staphylinidae sp. 22*	Panama	-	*unknown sp. 20*	Mexico	-	*Bombyliidae sp.7*	Mexico	-
Passalidae	Panama	-	*Tenebrionidae sp. 1*	Mexico	-	*unknown sp. 21*	Mexico	-	*Bombyliidae sp.8*	Mexico	+
Scarabaeidae sp. 1	Mexico	-	*Tenebrionidae sp. 2*	Mexico	-	*unknown sp. 22*	Mexico	-	*Bombyliidae sp.9*	Mexico	-
Scarabaeidae sp. 2	Mexico	-	*Tenebrionidae sp. 3*	Ghana	-	*unknown sp. 23*	Mexico	-	*Calliphoridae sp. 1*	Ghana	-
Scarabaeidae sp. 3	Mexico	-	*Tenebrionidae sp. 4*	Ghana	-	*unknown sp. 24*	Mexico	-	*Calliphoridae sp. 2*	Ghana	-
Scarabaeidae sp. 4	Mexico	-	*Tenebrionidae sp. 5*	Ghana	-	*unknown sp. 25*	Mexico	-	*Cecidomyiidae sp. 1*	Ghana	-
Scarabaeidae sp. 5	Ghana	-	*Tenebrionidae sp. 6*	Ghana	-	*unknown sp. 26*	Mexico	-	*Cecidomyiidae sp. 2*	Ghana	-

taxon	location	*R*	taxon	location	*R*	taxon	location	*R*	taxon	location	*R*
Cecidomyiidae sp. 3	Mexico	-	*Drosophilidae sp. 1*	Chile	-	*Phoridae sp. 13*	South Africa	-	*Tabanidae sp. 1*	New York, USA	-
Ceratopogonidae sp. 1	Mexico	-	*Drosophilidae sp. 2*	South Africa	-	*Pipunculidae*	Michigan	-	*Tabanidae sp. 2*	New York, USA	-
Ceratopogonidae sp. 2	Mexico	-	*Drosophilidae sp. 3*	South Africa	-	*Platypezidae*	New York, USA	-	*Tabanidae sp. 3*	New York, USA	-
Chironomidae sp. 1	Mexico	-	*Empididae sp. 1*	Mexico	-	*Platystomatidae*	Mexico	-	*Tabanidae sp. 4*	Mexico	-
Chironomidae sp. 2	Mexico	-	*Empididae sp. 2*	Mexico	-	*Psilidae sp. 1*	Mexico	-	*Tachinidae sp. 1*	Mexico	-
Chironomidae sp. 3	California, USA	-	*Ephydridae sp. 1*	Mexico	-	*Psilidae sp. 2*	Mexico	-	*Tachinidae sp. 2*	Mexico	-
Chloropidae sp. 1	Mexico	-	*Ephydridae sp. 2*	Mexico	-	*Sarcophagidae*	Mexico	-	*Tachinidae sp. 3*	Mexico	-
Chloropidae sp. 2	Mexico	-	*Haliplidae*	Mexico	-	*Scatopsidae*	Mexico	-	*Tachinidae sp. 4*	Mexico	-
Chloropidae sp. 3	Mexico	-	*Lauxaniidae sp. 1*	Mexico	-	*Sciaridae sp. 1*	New York, USA	-	*Tachinidae sp. 5*	Mexico	-
Chloropidae sp. 4	Mexico	-	*Lauxaniidae sp. 2*	Mexico	-	*Sciaridae sp. 2*	Mexico	-	*Tachinidae sp. 6*	Mexico	-
Chloropidae sp. 5	Mexico	-	*Longchopteridae sp. 1*	New York, USA	-	*Sciaridae sp. 3*	Mexico	-	*Tachinidae sp. 7*	Michigan	-
Chloropidae sp. 6	Mexico	-	*Longchopteridae sp. 2*	New York, USA	-	*Sciomyzidae sp. 1*	New York, USA	-	*Tachinidae sp. 8*	Michigan	-
Chloropidae sp. 7	Mexico	-	*Longchopteridae sp. 3*	New York, USA	-	*Sciomyzidae sp. 2*	New York, USA	-	*Tachinidae sp. 9*	Michigan	-
Chloropidae sp. 8	Mexico	-	*Muscidae sp. 1*	Mexico	-	*Sciomyzidae sp. 3*	New York, USA	-	*Tachinidae sp. 10*	Ghana	-
Chloropidae sp. 9	Mexico	-	*Muscidae sp. 2*	Mexico	-	*Sciomyzidae sp. 4*	New York, USA	-	*Tephritidae sp. 1*	Mexico	-
Chloropidae sp. 10	Ghana	-	*Muscidae sp. 3*	Mexico	-	*Sepsidae sp. 1*	New York, USA	-	*Tephritidae sp. 2*	Mexico	-
Chloropidae sp. 11	New York, USA	-	*Muscidae sp. 4*	Mexico	-	*Sepsidae sp. 2*	New York, USA	-	*Tephritidae sp. 3*	Mexico	-
Conopidae	Mexico	-	*Muscidae sp. 5*	Mexico	-	*Simuliidae sp. 1*	Ghana	-	*Tephritidae sp. 4*	Mexico	-
Culicidae sp. 1	New York, USA	-	*Muscidae sp. 6*	Mexico	-	*Simuliidae sp. 2*	Ghana	-	*Tephritidae sp. 5*	Mexico	-
Culicidae sp. 2	New York, USA	-	*Muscidae sp. 7*	Mexico	-	*Simuliidae sp. 3*	Mexico	-	*Tephritidae sp. 6*	Mexico	-
Culicidae sp. 3	Michigan	-	*Muscidae sp. 8*	Mexico	-	*Sphaeroceridae sp. 1*	New York, USA	-	*Tephritidae sp. 7*	Mexico	-
Culicidae sp. 4	Mexico	-	*Muscidae sp. 9*	Mexico	-	*Sphaeroceridae sp. 2*	Mexico	-	*Tephritidae sp. 8*	Mexico	-
Culicidae sp. 5	Mexico	-	*Muscidae sp. 10*	Mexico	-	*Stratiomyidae sp. 1*	Mexico	-	*Tephritidae sp. 9*	Mexico	-
Culicidae sp. 6	Mexico	-	*Muscidae sp. 11*	Chile	-	*Stratiomyidae sp. 2*	Mexico	-	*Tephritidae sp. 10*	Mexico	-
Culicidae sp. 7	Mexico	-	*Muscidae sp. 12*	Chile	-	*Syrphidae sp. 1*	New York, USA	-	*Therevidae sp. 1*	Michigan	-
Culicidae sp. 8	California, USA	-	*Muscidae sp. 13*	Michigan	-	*Syrphidae sp. 2*	New York, USA	-	*Therevidae sp. 2*	Chile	-
Dolichopodidae sp. 1	New York, USA	-	*Muscidae sp. 14*	California, USA	-	*Syrphidae sp. 3*	New York, USA	-	*Tipulidae sp. 1*	New York, USA	-
Dolichopodidae sp. 2	New York, USA	-	*Phoridae sp. 1*	Mexico	-	*Syrphidae sp. 4*	New York, USA	-	*Tipulidae sp. 2*	New York, USA	-
Dolichopodidae sp. 3	New York, USA	-	*Phoridae sp. 2*	Mexico	-	*Syrphidae sp. 5*	New York, USA	-	*Tipulidae sp. 3*	New York, USA	-
Dolichopodidae sp. 4	New York, USA	-	*Phoridae sp. 3*	Mexico	-	*Syrphidae sp. 6*	Mexico	-	*Tipulidae sp. 4*	New York, USA	-
Dolichopodidae sp. 5	New York, USA	-	*Phoridae sp. 4*	Mexico	-	*Syrphidae sp. 7*	Mexico	-	*Tipulidae sp. 5*	Ghana	-
Dolichopodidae sp. 6	Mexico	-	*Phoridae sp. 5*	Mexico	-	*Syrphidae sp. 8*	Mexico	-	*Ulidiidae*	Mexico	-
Dolichopodidae sp. 7	Mexico	-	*Phoridae sp. 6*	Mexico	-	*Syrphidae sp. 9*	Mexico	-	Hemiptera		
Dolichopodidae sp. 8	Mexico	-	*Phoridae sp. 7*	Mexico	-	*Syrphidae sp. 10*	Mexico	-	*unknown sp. 1*	unknown	-
Dolichopodidae sp. 9	Mexico	-	*Phoridae sp. 8*	Ghana	-	*Syrphidae sp. 11*	Mexico	-	*unknown sp. 2*	Mexico	-
Dolichopodidae sp. 10	Mexico	-	*Phoridae sp. 9*	Chile	-	*Syrphidae sp. 12*	Mexico	-	*unknown sp. 3*	New York, USA	-
Dolichopodidae sp. 11	Mexico	-	*Phoridae sp. 10*	Chile	-	*Syrphidae sp. 13*	Mexico	-	*unknown sp. 4*	Mexico	-
Dolichopodidae sp. 12	Mexico	-	*Phoridae sp. 11*	South Africa	-	*Syrphidae sp. 14*	Mexico	-	*unknown sp. 5*	Mexico	-
Dolichopodidae sp. 13	South Africa	-	*Phoridae sp. 12*	South Africa	-	*Syrphidae sp. 15*	Michigan	-	*unknown sp. 6*	Mexico	-

taxon	location	R	taxon	location	R	taxon	location	R	taxon	location	R
unknown sp. 7	Mexico	-	*Cicadellidae sp. 19*	Panama	-	*Pyrrhocoridae*	Ghana	-	*Andrenidae sp. 7*	Mexico	-
unknown sp. 8	Mexico	-	*Cicadellidae sp. 20*	Panama	-	*Reduviidae sp. 1*	Mexico	-	*Andrenidae sp. 8*	Mexico	-
unknown sp. 9	Mexico	-	*Cicadellidae sp. 21*	Papua New Guinea	-	*Reduviidae sp. 2*	Ghana	-	*Andrenidae sp. 9*	Mexico	-
unknown sp. 10	Mexico	-	*Cicadidae sp. 1*	Ghana	-	*Reduviidae sp. 3*	Ghana	-	*Andrenidae sp. 10*	Mexico	-
unknown sp. 11	Mexico	-	*Cicadidae sp. 2*	Ghana	-	*Reduviidae sp. 4*	Ghana	-	*Andrenidae sp. 11*	Mexico	-
unknown sp. 12	Mexico	-	*Coreidae*	Papua New Guinea	-	*Reduviidae sp. 5*	Mexico	-	*Andrenidae sp. 12*	Mexico	-
unknown sp. 13	Mexico	-	*Cydnidae sp. 1*	India	-	*Reduviidae sp. 6*	New York, USA	-	*Andrenidae sp. 13*	Mexico	-
unknown sp. 14	Mexico	-	*Cydnidae sp. 2*	India	-	*Reduviidae sp. 7*	Panama	+	*Andrenidae sp. 14*	Mexico	-
unknown sp. 15	Mexico	-	*Cydnidae sp. 3*	India	-	*Ricaniidae*	Papua New Guinea	-	*Apidae sp. 1*	India	-
unknown sp. 16	Mexico	-	*Cydnidae sp. 4*	India	-	*Saldidae*	India	-	*Apidae sp. 2*	Mexico	-
unknown sp. 17	Mexico	-	*Delphacidae*	India	-	Hymenoptera			*Apidae sp. 3*	Mexico	-
unknown sp. 18	Mexico	-	*Derbidae sp. 1*	Papua New Guinea	-	*unknown sp. 1*	Spain	-	*Apidae sp. 4*	Mexico	-
unknown sp. 19	Mexico	-	*Derbidae sp. 2*	Papua New Guinea	-	*unknown sp. 2*	unknown	-	*Apidae sp. 5*	Mexico	-
unknown sp. 20	Mexico	-	*Fugoridae sp. 1*	Panama	-	*unknown sp. 3*	Mexico	-	*Apidae sp. 6*	Mexico	-
unknown sp. 21	Panama	-	*Fugoridae sp. 2*	Panama	-	*unknown sp. 4*	Mexico	-	*Apidae sp. 7*	Mexico	-
unknown sp. 22	Panama	-	*Fugoridae sp. 3*	Panama	-	*unknown sp. 5*	Mexico	-	*Apidae sp. 8*	Mexico	-
unknown sp. 23	Panama	-	*Fugoridae sp. 4*	Papua New Guinea	-	*unknown sp. 6*	Mexico	-	*Apidae sp. 9*	Mexico	-
Aphididae sp. 1	Chile	-	*Gelastocoridae*	Ghana	-	*unknown sp. 7*	Mexico	-	*Apidae sp. 10*	Mexico	-
Aphididae sp. 2	South Africa	-	*Lygaeidae*	Papua New Guinea	-	*unknown sp. 8*	Mexico	-	*Apidae sp. 11*	Mexico	-
Aphididae sp. 3	Chile	-	*Membracidae sp. 1*	Mexico	-	*unknown sp. 9*	Mexico	-	*Apidae sp. 12*	Mexico	-
Cercopidae	Ghana	+	*Membracidae sp. 2*	Mexico	-	*unknown sp. 10*	Mexico	-	*Apidae sp. 13*	Mexico	-
Cicadellidae sp. 1	Mexico	-	*Membracidae sp. 3*	Mexico	-	*unknown sp. 11*	Mexico	-	*Apidae sp. 14*	Mexico	-
Cicadellidae sp. 2	Mexico	-	*Membracidae sp. 4*	Mexico	-	*unknown sp. 12*	Mexico	-	*Apidae sp. 15*	Mexico	-
Cicadellidae sp. 3	Chile	-	*Membracidae sp. 5*	Mexico	-	*unknown sp. 13*	Mexico	-	*Apidae sp. 16*	New York, USA	-
Cicadellidae sp. 4	South Africa	-	*Membracidae sp. 6*	Mexico	-	*unknown sp. 14*	Mexico	-	*Apidae sp. 17*	New York, USA	-
Cicadellidae sp. 5	South Africa	-	*Membracidae sp. 7*	Panama	-	*unknown sp. 15*	Mexico	-	*Apidae sp. 18*	New York, USA	-
Cicadellidae sp. 6	Chile	-	*Miridae sp. 1*	Mexico	-	*unknown sp. 16*	Mexico	-	*Apidae sp. 19*	New York, USA	-
Cicadellidae sp. 7	Chile	-	*Miridae sp. 2*	South Africa	-	*unknown sp. 17*	Mexico	-	*Apidae sp. 20*	California, USA	-
Cicadellidae sp. 8	Chile	-	*Miridae sp. 3*	South Africa	-	*unknown sp. 18*	Mexico	-	*Braconidae sp. 1*	Mexico	-
Cicadellidae sp. 9	Chile	-	*Nepidae*	Ghana	-	*unknown sp. 19*	Mexico	-	*Braconidae sp. 2*	Mexico	-
Cicadellidae sp. 10	Panama	-	*Pentatomidae sp. 1*	Mexico	-	*unknown sp. 20*	Mexico	-	*Chalcidae sp. 1*	Mexico	-
Cicadellidae sp. 11	Panama	-	*Pentatomidae sp. 2*	Mexico	-	*unknown sp. 21*	Mexico	-	*Chalcidae sp. 2*	Mexico	-
Cicadellidae sp. 12	Panama	-	*Pentatomidae sp. 3*	Ghana	-	*unknown sp. 22*	South Africa	-	*Chalcidae sp. 3*	Mexico	-
Cicadellidae sp. 13	Panama	-	*Pentatomidae sp. 4*	Mexico	-	*Andrenidae sp. 1*	Mexico	-	*Chalcidae sp. 4*	Mexico	-
Cicadellidae sp. 14	Panama	-	*Pentatomidae sp. 5*	India	-	*Andrenidae sp. 2*	Mexico	-	*Chalcidae sp. 5*	Mexico	-
Cicadellidae sp. 15	Panama	-	*Pentatomidae sp. 6*	Chile	-	*Andrenidae sp. 3*	Mexico	-	*Chalcidae sp. 6*	Mexico	-
Cicadellidae sp. 16	Panama	-	*Pentatomidae sp. 7*	New York, USA	-	*Andrenidae sp. 4*	Mexico	-	*Chalcidae sp. 7*	Mexico	-
Cicadellidae sp. 17	Panama	-	*Pentatomidae sp. 8*	Panama	-	*Andrenidae sp. 5*	Mexico	-	*Chalcidae sp. 8*	Mexico	-
Cicadellidae sp. 18	Panama	-	*Pentatomidae sp. 9*	Papua New Guinea	-	*Andrenidae sp. 6*	Mexico	-	*Chalcidae sp. 9*	Mexico	-

taxon	location	*R*	taxon	location	*R*	taxon	location	*R*	taxon	location	*R*
Chalcidae sp. 10	Mexico	-	*Formicidae sp. 9*	Mexico	-	*Halictidae sp. 22*	Mexico	-	*Pompilidae sp. 6*	Mexico	-
Chalcidae sp. 11	Mexico	-	*Formicidae sp. 10*	Mexico	-	*Halictidae sp. 23*	Mexico	-	*Pompilidae sp. 7*	Mexico	-
Chalcidae sp. 12	Mexico	-	*Formicidae sp. 11*	Mexico	-	*Halictidae sp. 24*	Mexico	-	*Pompilidae sp. 8*	Mexico	-
Chalcidae sp. 13	Mexico	-	*Formicidae sp. 12*	Mexico	-	*Halictidae sp. 25*	New York, USA	-	*Pompilidae sp. 9*	Mexico	-
Chrysididae sp. 1	Mexico	-	*Formicidae sp. 13*	Mexico	-	*Halictidae sp. 26*	New York, USA	-	*Pompilidae sp. 10*	Mexico	-
Chrysididae sp. 2	Mexico	-	*Formicidae sp. 14*	Mexico	-	*Ichneumonidae sp. 1*	Mexico	-	*Pompilidae sp. 11*	Mexico	-
Colletidae sp. 1	Mexico	-	*Formicidae sp. 15*	Mexico	-	*Ichneumonidae sp. 2*	Mexico	-	*Pompilidae sp. 12*	Mexico	-
Colletidae sp. 2	Mexico	-	*Formicidae sp. 16*	Mexico	-	*Ichneumonidae sp. 3*	Mexico	-	*Pompilidae sp. 13*	Mexico	-
Colletidae sp. 3	Mexico	-	*Formicidae sp. 17*	Mexico	-	*Ichneumonidae sp. 4*	Mexico	-	*Pompilidae sp. 14*	Mexico	-
Crabronidae sp. 1	Mexico	-	*Formicidae sp. 18*	Mexico	-	*Ichneumonidae sp. 5*	Mexico	-	*Pompilidae sp. 15*	Mexico	-
Crabronidae sp. 2	Mexico	-	*Formicidae sp. 19*	Mexico	-	*Ichneumonidae sp. 6*	New York, USA	-	*Pompilidae sp. 16*	Mexico	-
Crabronidae sp. 3	Mexico	-	*Formicidae sp. 20*	Mexico	-	*Ichneumonidae sp. 7*	Papua New Guinea	-	*Pompilidae sp. 17*	Mexico	-
Crabronidae sp. 4	Mexico	-	*Formicidae sp. 21*	Mexico	-	*Leucospidae*	Mexico	-	*Pompilidae sp. 18*	Mexico	-
Crabronidae sp. 5	Mexico	-	*Formicidae sp. 22*	Mexico	-	*Megachilidae sp. 1*	Mexico	-	*Pteromalidae sp. 1*	Florida, USA	-
Crabronidae sp. 6	Mexico	-	*Formicidae sp. 23*	Papua New Guinea	-	*Megachilidae sp. 2*	Mexico	-	*Pteromalidae sp. 2*	Florida, USA	-
Crabronidae sp. 7	Mexico	-	*Formicidae sp. 24*	Rochester	-	*Megachilidae sp. 3*	Mexico	-	*Pteromalidae sp. 3*	Florida, USA	-
Crabronidae sp. 8	Mexico	-	*Gasteruptiidae sp. 1*	Mexico	-	*Megachilidae sp. 4*	Mexico	-	*Pteromalidae sp. 4*	France	-
Crabronidae sp. 9	Mexico	-	*Gasteruptiidae sp. 2*	New York, USA	-	*Megachilidae sp. 5*	Mexico	-	*Pteromalidae sp. 5*	France	-
Crabronidae sp. 10	Mexico	-	*Halictidae sp. 1*	Mexico	-	*Megachilidae sp. 6*	California, USA	-	*Pteromalidae sp. 6*	Kazakhastan	-
Crabronidae sp. 11	Mexico	-	*Halictidae sp. 2*	Mexico	-	*Mutillidae sp 1.*	Mexico	-	*Pteromalidae sp. 7*	Mexico	-
Crabronidae sp. 12	Mexico	-	*Halictidae sp. 3*	Mexico	-	*Mutillidae sp 1.*	Mexico	-	*Pteromalidae sp. 8*	Russia	-
Crabronidae sp. 13	Mexico	-	*Halictidae sp. 4*	Mexico	-	*Mutillidae sp 1.*	Mexico	-	*Scoliidae sp. 1*	Ghana	-
Crabronidae sp. 14	Mexico	-	*Halictidae sp. 5*	Mexico	-	*Mutillidae sp 1.*	Mexico	-	*Scoliidae sp. 2*	Ghana	-
Crabronidae sp. 15	Mexico	-	*Halictidae sp. 6*	Mexico	-	*Mutillidae sp 1.*	Mexico	-	*Sphecidae sp. 1*	Mexico	-
Crabronidae sp. 16	Mexico	-	*Halictidae sp. 7*	Mexico	-	*Mutillidae sp 1.*	Mexico	-	*Sphecidae sp. 2*	Mexico	-
Crabronidae sp. 17	Mexico	-	*Halictidae sp. 8*	Mexico	-	*Mutillidae sp 1.*	Mexico	-	*Sphecidae sp. 3*	Mexico	-
Crabronidae sp. 18	Mexico	-	*Halictidae sp. 9*	Mexico	-	*Mutillidae sp 1.*	Mexico	-	*Sphecidae sp. 4*	Mexico	-
Diapriidae sp. 1	India	-	*Halictidae sp. 10*	Mexico	-	*Mutillidae sp 1.*	Mexico	-	*Sphecidae sp. 5*	Mexico	-
Diapriidae sp. 2	India	-	*Halictidae sp. 11*	Mexico	-	*Mutillidae sp 1.*	Mexico	-	*Sphecidae sp. 6*	Mexico	-
Eurytomidae	Mexico	-	*Halictidae sp. 12*	Mexico	-	*Mutillidae sp 1.*	Mexico	-	*Sphecidae sp. 7*	Mexico	-
Figitidae	New York, USA	-	*Halictidae sp. 13*	Mexico	-	*Mutillidae sp 1.*	Mexico	-	*Sphecidae sp. 8*	Mexico	-
Formicidae sp. 1	Ghana	-	*Halictidae sp. 14*	Mexico	-	*Mutillidae sp 1.*	Mexico	-	*Sphecidae sp. 9*	Mexico	-
Formicidae sp. 2	India	-	*Halictidae sp. 15*	Mexico	-	*Mutillidae sp 1.*	Mexico	-	*Sphecidae sp. 10*	Mexico	-
Formicidae sp. 3	India	-	*Halictidae sp. 16*	Mexico	-	*Pemphredonidae*	Mexico	-	*Sphecidae sp. 11*	Mexico	-
Formicidae sp. 4	India	-	*Halictidae sp. 17*	Mexico	-	*Pompilidae sp. 1*	Mexico	-	*Sphecidae sp. 12*	Mexico	-
Formicidae sp. 5	India	-	*Halictidae sp. 18*	Mexico	-	*Pompilidae sp. 2*	Mexico	-	*Sphecidae sp. 13*	Mexico	-
Formicidae sp. 6	Mexico	-	*Halictidae sp. 19*	Mexico	-	*Pompilidae sp. 3*	Mexico	-	*Sphecidae sp. 14*	Mexico	-
Formicidae sp. 7	Mexico	-	*Halictidae sp. 20*	Mexico	-	*Pompilidae sp. 4*	Mexico	-	*Sphecidae sp. 15*	Mexico	-
Formicidae sp. 8	Mexico	-	*Halictidae sp. 21*	Mexico	-	*Pompilidae sp. 5*	Mexico	-	*Sphecidae sp. 16*	Mexico	-

taxon	location	*R*
Sphecidae sp. 17	Mexico	-
Sphecidae sp. 18	Mexico	-
Sphecidae sp. 19	Mexico	-
Sphecidae sp. 20	Mexico	-
Sphecidae sp. 21	Mexico	-
Sphecidae sp. 22	Mexico	-
Sphecidae sp. 23	California, USA	-
Tiphiidae sp. 1	Mexico	-
Tiphiidae sp. 2	Mexico	-
Vespidae sp. 1	Mexico	-
Vespidae sp. 2	Mexico	-
Vespidae sp. 3	Mexico	-
Vespidae sp. 4	Mexico	-
Vespidae sp. 5	unknown	-
Lepidoptera		
unknown sp. 1	Chile	-
unknown sp. 2	Chile	-
unknown sp. 3	Mexico	-
unknown sp. 4	Mexico	-
unknown sp. 5	Mexico	-
unknown sp. 6	Mexico	-
unknown sp. 7	Mexico	-
unknown sp. 8	Mexico	-
unknown sp. 9	New York, USA	-
unknown sp. 10	South Africa	-
Arctiidae sp. 1	Ghana	-
Arctiidae sp. 2	Ghana	-
Arctiidae sp. 3	India	-
Arctiidae sp. 4	Mexico	-
Arctiidae sp. 5	New York, USA	-
Geometridae sp. 1	New York, USA	-
Geometridae sp. 2	New York, USA	-
Lycaenidae sp. 1	Mexico	-
Lycaenidae sp. 2	Mexico	-
Lycaenidae sp. 3	New York, USA	-
Lycaenidae sp. 4	New York, USA	-
Noctuidae	Papua New Guinea	+
Nymphalidae sp. 1	Panama	-
Nymphalidae sp. 2	Panama	-
Pieridae	New York, USA	-
Saturniidae	Ghana	-
Mantodea		
unknown sp.	South Africa	-
Neuroptera		
Chrysopidae sp. 1	Mexico	+
Chrysopidae sp. 2	Mexico	-
Myrmeleontidae sp. 1	Mexico	-
Myrmeleontidae sp. 2	Mexico	-
Odonata		
Coenagrionidae sp.1	Mexico	-
Coenagrionidae sp.2	Mexico	-
Coenagrionidae sp.3	Mexico	-
Coenagrionidae sp.4	Mexico	-
Coenagrionidae sp.5	Mexico	-
Zygopteraidae sp. 1	Spain	-
Zygopteraidae sp. 2	Spain	-
Zygopteraidae sp. 3	Spain	-
Orthoptera		
unknown sp. 1	Spain	-
unknown sp. 2	Spain	-
unknown sp. 3	Mexico	-
Acrididae	Ghana	-
Gryllidae sp. 1	Mexico	-
Gryllidae sp. 2	South Africa	-
Mantidae	Panama	-
Tettigoniidae sp. 1	Panama	-
Tettigoniidae sp. 2	Mexico	-
Psocoptera		
unknown sp.	Chile	-
Siphonaptera		
unknown sp.	Chile	-
Strepsiptera		
unknown sp.	Chile	-
Thysanoptera		
unknown sp. 1	Mexico	-
unknown sp. 2	Chile	-
unknown sp. 3	South Africa	-
unknown sp. 4	South Africa	-
unknown sp. 5	South Africa	-
unknown sp. 6	South Africa	-
unknown sp. 7	South Africa	-
unknown sp. 8	Chile	-
Thripidae	Papua New Guinea	-
Trichoptera		
unknown sp.	South Africa	-

Appendix 2

The assumed distribution of prevalences among species is the Beta distribution:

$$P(q \mid \mu,\rho) = \frac{q^{\mu(\rho^{-1}-1)-1}(1-q)^{(1-\mu)(\rho^{-1}-1)-1}}{B\left(\mu(\rho^{-1}-1),(1-\mu)(\rho^{-1}-1)\right)}$$

where B(.,.) is the Beta function. This distribution has mean μ and variance $\mu(1-\mu)\rho$, but is often written as a function of two shape parameters, $a=\mu(\rho^{-1}-1)$ and $b=(1-\mu)(\rho^{-1}-1)$.

For each species we have random sample of n individuals, of which k were found to be infected. The complete Beta-binomial likelihood surface is then:

$$L(data \mid \mu,\rho) = \int_0^1 P(q \mid \mu,\rho) \prod_i^{species} \binom{n_i}{k_i} q^{k_i}(1-q)^{n_i-k_i}\, dq$$

$$= \prod_i^{species} \binom{n_i}{k_i} \frac{B\left(\mu(\rho^{-1}-1)+k_i,(1-\mu)(\rho^{-1}-1)+n_i-k_i\right)}{B\left(\mu(\rho^{-1}-1),(1-\mu)(\rho^{-1}-1)\right)}$$

Note that for single-individual screens, which have $n_i=1$, and k_i =0 or 1, the parameter ρ cancels from the equation, confirming that between-species variance in prevalence cannot be estimated from single-individual studies.

Given ML estimates of μ and ρ, the ML estimate of the proportion of species with prevalence greater than c is simply $\hat{x}_c = \int_c^1 P(q \mid \hat{\mu},\hat{\rho})dq$.

Appendix 3

Table S2.1 Accession numbers of strains used in Chapter 6

Strain name	*16S* gene	*gltA* gene	*AtpA* gene	*CoxA* gene
Orientia tsutsugamushi	AM494475	-	AM494477	AM494478
(s)Deep sea octacoral	DQ395479	-	-	-
(s)*Haplosporidium sp.*	AJ319724	-	-	-
(s)Melted red snow	AJ867656	-	-	-
(s)Mountain snow	AJ867656	-	-	-
(s)*Hydra oligactis*	EF667896	-	-	-
(s)Acid impacted lake	EF520410	-	-	-
(s)Pasture water	EF074039	-	-	-
(s)Rice roots	AM159487	-	-	-
(s)Forested wetland	AF523878	-	-	-
(s)Kalahari water	DQ223223	-	-	-
(s)*Diophrys appendiculata*	AJ630204	-	-	-
(s)Artic tundra	AM945518	-	-	-
(s)*Torix tagoi*	AB066351	-	-	-
(s)*Limonia chorea*	AF322443	-	-	-
(s)Indoor dust	AM697554	-	-	-
(s)*Cerobasis guestfalica*	DQ652596	-	-	-
(s)*Lutzomyia apache*	EU223247	-	-	-
(s)*Nuclearia pattersoni*	AY364636	-	-	-
(s)*Kytorhinus sharpianus*	AB021128	-	-	-
(s)Curculionidae	FJ609387	-	-	FJ666773
(s)*Rhizobius chrysomeloides*	FJ609388	FJ666753	FJ666796	FJ666774
(s)Meloidae	FJ609389	FJ666754	FJ666797	FJ666775
(s)*Bemisia tabaci*	DQ077707	DQ077708	-	-
(s)*Empoasca papayae*	U76910	U76908	-	-
(s)*Tetranychus urticae*	AY753175	-	-	-
(s)Bombyliidae	FJ609390	FJ666755	FJ666798	FJ666776
(s)*Acyrthosiphon pisum*	FJ609391	FJ666756	FJ666799	FJ666777
(s)Bombyliidae	FJ609392	FJ666757	FJ666800	FJ666778
(s)*Brachys tessellatus*	FJ609393	FJ666758	FJ666801	-
(s)Reduviidae	FJ609394	-	-	FJ666779
(s)Chrysopidae	FJ609395	FJ666759	-	FJ666780
R. bellii	CP000849	CP000849	CP000849	CP000849
R. bellii	CP000087	CP000087	CP000087	CP000087
(s)Elateridae	FJ609396	FJ666760	FJ666802	FJ666781
(s)Noctuidae	FJ609397	FJ666761	FJ666803	FJ666782
(s)*Onychiurus sinensis*	AY712949	-	-	-
(s)*Subcoccinella vigintiquattuorpunctata*	FJ609398	FJ666762	FJ666804	FJ666783
(s)*Scymnus suturalis*	FJ609399	-	FJ666805	FJ666784
(s)*Adalia bipunctata* (Moscow)	FJ609400	FJ666765	FJ666807	FJ666787
(s)*Adalia bipunctata* (Cambridge)	FJ609401	FJ666764	FJ666808	FJ666786
(s)*Adalia bipunctata* (Ribe)	-	FJ666763	-	-
(s)*Halyzia sedecimguttata*	FJ609402	FJ666766	FJ666809	FJ666788
(s)*Calvia quattuordecimguttata*	FJ609403	FJ666767	FJ666810	FJ666789
(s)*Adalia bipunctata* (Edinburgh)	-	-	FJ666806	FJ666785
(s)*Adalia decempuntata*	FJ609404	FJ666768	FJ666811	FJ666790
(s)*Coccotrypes dactyliperda*	AY961085	-	-	-
R. canadensis	CP000409	CP000409	CP000409	CP000409
R. tarasevichiae	AF503168	AF503167	-	-
R. helvetica	L36212	U59723	DQ821790	-
(s)*Ixodes scapularis*	AB001518	-	-	-
R. montanensis	L36215	U74756	AY124737	-
R. massiliae	CP000683	CP000683	CP000683	CP000683
R. japonica	L36213	U59724	DQ821776	-
R. peacockii	DQ062433	DQ100162	-	-
R. rickettsii	CP000848	CP000848	CP000848	CP000848
R. conorii	AE008647	AE008647	AE008647	AE008647
R. sibirica	AABW00000000	AABW00000000	AABW00000000	AABW00000000
R. typhi	AE017199	AE017199	AE017199	-
R. prowazekii	AJ235272	AJ235272	AJ235272	AJ235272
R. australis	U17644	U59718	DQ821777	-

Strain name	*16S* gene	*gltA* gene	*AtpA* gene	*CoxA* gene
R. akari	CP000847	CP000847	CP000847	CP000847
(s)Cercopidae	-	-	-	FJ666791
(s)*Aulogymnus trilineatus*	FJ609405	FJ666769	FJ666812	FJ666792
(s)*Aulogymnus balani/skianeuros*	FJ609406	FJ666770	FJ666813	FJ666793
R. felis	CP000053	CP000054	CP000055	CP000056
(s)*Liposcelis bostrychophila*	DQ407743	-	-	-
(s)*Liposcelis bostrychophila*	DQ652592	-	-	-
(s)*Pediobius rotundatus*	FJ609407	FJ666771	FJ666814	FJ666794
(s)*Neochrysocharis formosa*	AB231472	-	-	-
(s)*Coccidula rufa*	FJ609408	FJ666772	FJ666815	FJ666795

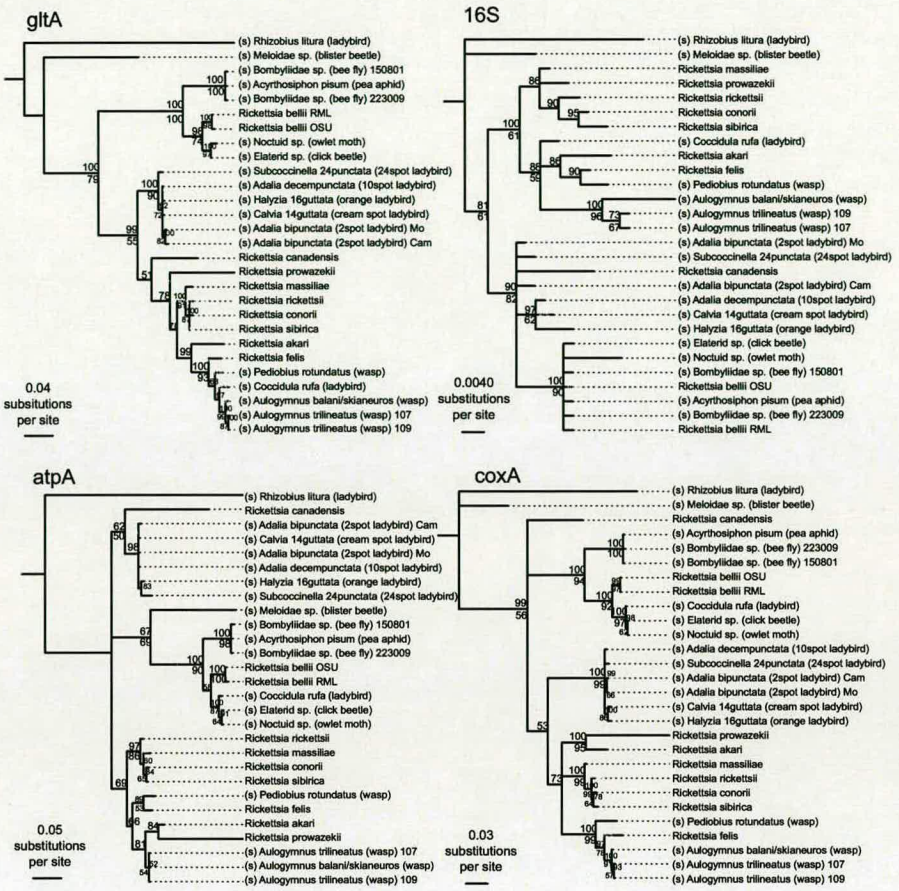


Figure S2.1 Phylogenetic trees of each of the individual genes used in the study. Posterior probabilities are given above the node and maximum likelihood values are given below. Branch lengths are indicated by the scale bar of substitutions per site at the bottom left of each gene tree.

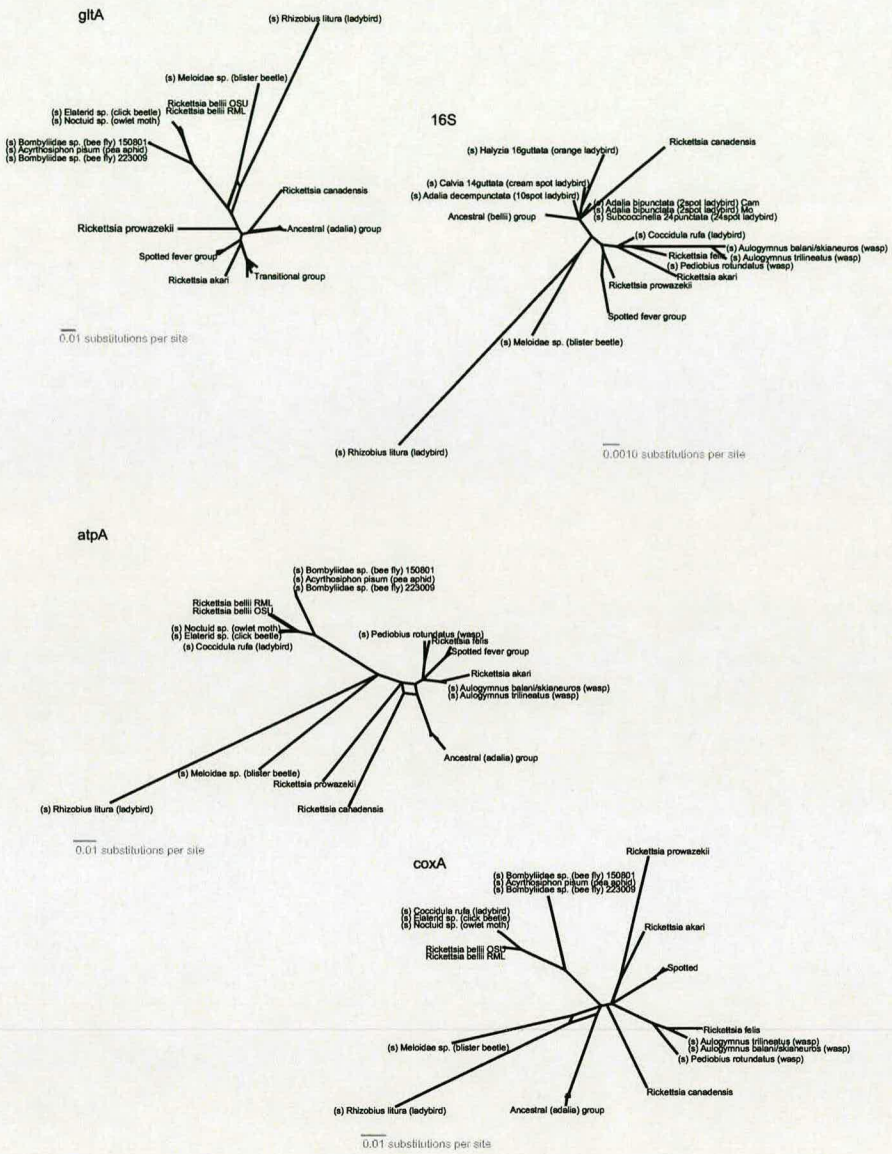


Figure S2. Split networks for each of the individual genes used in the study. A test of tree-likeness was carried out on each of the individual gene and only the 95% confidence network is shown, indicating only the statistically significant splits. Branch lengths are indicated by the scale bar of substitutions per site at the bottom left of each gene tree.

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