

Review Article

Host-Symbiont Interactions for Potentially Managing Heteropteran Pests

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Insects in the suborder Heteroptera, the so-called true bugs, include over 40,000 species worldwide. This insect group includes many important agricultural pests and disease vectors, which often have bacterial symbionts associated with them. Some symbionts have coevolved with their hosts to the extent that host fitness is compromised with the removal or alteration of their symbiont. The first bug/microbial interactions were discovered over 50 years ago. Only recently, mainly due to advances in molecular techniques, has the nature of these associations become clearer. Some researchers have pursued the genetic modification (paratransgenesis) of symbionts for disease control or pest management. With the increasing interest and understanding of the bug/symbiont associations and their ecological and physiological features, it will only be a matter of time before pest/vector control programs utilize this information and technique. This paper will focus on recent discoveries of the major symbiotic systems in Heteroptera, highlighting how the understanding of the evolutionary and biological aspects of these relationships may lead to the development of alternative techniques for efficient heteropteran pest control and suppression of diseases vectored by Heteroptera.

1. Introduction

Insects are the most cosmopolitan, polyphagous, and varied living organisms on Earth, and many species are involved in some kind of symbiotic association with microorganisms, mainly bacteria [1, 2]. Some insects associated with bacteria are also vectors of disease or are important crop pests which increase the relevance of these symbiotic interactions. The widespread distribution of insects may, in fact, be related to bacterial associations that allow host insects to exploit different nutritional sources, such as lignocellulose by termites, or to obtain essential nutrients from their symbionts, as in *Buchnera aphidicola*-aphid symbiosis [3, 4].

According to Moran [2], symbiosis is a “close relationship between two or more individuals.” In insects, there are two major categories of symbiotic associations: obligatory and facultative [5]. Obligatory symbionts (also called primary symbionts) are nutritionally required for the survival of their insect hosts and usually inhabit specialized host cells.

On the other hand, secondary (also known as facultative) symbionts can be beneficial or cause incidental or deleterious infections [1]. The obligatory symbiont, *B. aphidicola*, for example, has never been cultured outside its host and is present intracellularly within specialized cells termed bacteriocytes [1, 5, 6]. Transovarial transmission is often the mode of symbiont transfer from one generation to another, which is a bottleneck that shapes the genome characteristics of the symbiont [7]. Phylogenetic analyses for certain insect families have shown that insects and primary endosymbionts have coevolved for millions of years after a single initial infection [5]. In contrast, secondary symbionts are nonessential to their hosts, may be free living, may not have specialized tissue localization, and occur extracellularly [8]. These secondary symbionts may provide benefits to their hosts such as tolerance to heat stress, compensation for loss of primary symbionts, and resistance to parasites and pathogens [9–13]. Conversely, facultative symbionts can negatively impact the growth, reproduction, and longevity

of their hosts [14]. The evolutionary history of secondary endosymbionts often shows no coevolution with their hosts, suggesting multiple infections and/or horizontal transmission [5, 15]. For example, the secondary endosymbiont of tsetse flies, *Sodalis glossinidius*, can be cultured *in vitro* and apparently has not coevolved with the insect hosts [16]. *Sodalis glossinidius* is closely related to bacterial pathogens of insects suggesting, in this case, that the symbiont evolved from an insect pathogen [16].

In Heteroptera, many species adversely affect humans and their environment by causing direct damage to the crops, or acting as vectors of disease to crops, domestic animals, and humans [17] (however, there are also many agriculturally beneficial predatory heteropterans). For over 50 years, it has been known that insects of the suborder Heteroptera (order Hemiptera) harbor symbiotic microorganisms; however, the significance of the relationship and their role in the host's ecology and evolution are only now being unraveled [1, 9]. Symbiotic bacterial associations occur in all three hemipteran suborders: Sternorrhyncha (e.g., aphids, mealybugs, whiteflies, psyllids, etc.), Auchenorrhyncha (e.g., spittlebugs, planthoppers, leafhoppers, treehoppers, etc.), and Heteroptera (true bugs; Figure 1) [5, 18–21]. Experimental procedures for the phylogenetic tree analysis of Figure 1, the almost complete 16S rRNA gene sequences (1,300 nucleotides) were obtained from GenBank database and were aligned manually using MEGA version 5 software [22]. Phylogenetic trees were inferred by using the maximum-likelihood [23], maximum-parsimony [24], and neighbor-joining [25] tree-making algorithms drawn from the MEGA 5 and PHYML packages [22, 26]; an evolutionary distance matrix for the neighbor-joining algorithm was generated using the Jukes and Cantor [27] model. The topologies of the evolutionary trees were evaluated by a bootstrap analysis [28] of the neighbor-joining method based upon a 1,000 replicates using the MEGA 5 software.

Heteropteran symbionts are found in the gut lumen, as in Reduviidae, or in gastric caecae as in Acanthosomatidae, Alydidae, Coreidae, Parastrachiidae, Pentatomidae, Pyrrhocoridae, Plataspidae, and Scutelleridae. As opposed to symbionts of Sternorrhyncha and Auchenorrhyncha, the symbionts of Heteroptera are not passed to the next generation from mother to offspring in a transovarial manner. The posthatch transmission mechanisms may involve egg surface contamination (= smearing on egg surface), coprophagy (= proctophagy: feeding on excrement), capsule transmission, or acquisition from the environment “*de novo*” every generation after the nymphs hatch [1, 18, 19, 21, 29–39]. This curious mode of transmission is challenging to both the host and symbiont. In particular for the symbiont, external transmission may require high genome stability to prevent the loss of genes required for living outside the host in a variable environment, and the challenge for the host bugs is to reinoculate themselves each generation. These challenges may be responsible for the multiple acquisition of the symbiont or low cospeciation observed in Heteroptera [21, 29, 40].

Not all heteropteran microbial associations are beneficial to the host; at times the bacteria may be pathogenic and

reduce fitness (i.e., reproduction, mortality, and longevity) [41]. The beneficial and pathogenic aspects of symbiosis have been studied in the past, but now novel molecular approaches are being applied to these systems. With the advent of molecular approaches, it is increasingly clear that manipulation of symbiotic interactions can contribute to the development of new strategies for pest control, including the use of modified symbionts to control insects (paratrangensis) [30, 42], replacement of native symbionts with genetically modified symbionts via genetic drive [43, 44], and a technique called “incompatible insect” [45]. Additionally, Broderick et al. [46] showed that *Bacillus thuringiensis*, widely applied in biocontrol projects, only kills the lepidopteran larvae if the insects harbor a gut-associated microbial community, highlighting the importance of gut-associated bacteria to pest control.

2. Reduviidae

The vectors of the Chagas disease pathogen are bloodsucking Reduviidae in the subfamily Triatominae. For example, *Rhodnius prolixus* (Stal) is a blood-sucking triatomine that is a common vector of Chagas disease, the incurable illness damaging the heart and nervous system that afflicts millions of people in Central and South America [42]. This insect acquires its bacterial symbiont, *Rhodococcus rhodnii*, soon after the first instar bug hatches. In triatomines, generally, the aposymbiotic (without symbiont) first instar nymphs hatch and probe for their bacterial symbionts that are acquired orally through “contamination” by feces on or nearby egg masses [1, 30, 47]. This insect-symbiont association has been exploited by paratrangensis, in which the symbiont has been genetically transformed to negatively interfere with the survivorship of the Chagas disease agent, *Trypanosoma cruzi* [48]. Genetically modified *R. rhodnii* symbionts expressing a selectable gene product were stably maintained in *R. prolixus* without deleterious effects on host survival and fitness, thereby substantiating the paratrangenic approach.

According to Hurwitz et al. [49] the paratrangenic strategy has been used with other vector-borne disease systems such as sandfly-mediated leishmaniasis and sharpshooter-mediated Pierce's disease [50–52]. Additionally, the authors highlight the main strategies for the success of the paratrangensis strategy.

- (1) In order to perform genetic manipulation, it is necessary that the symbiont can be cultured.
- (2) The symbiont must be identified within a certain disease-transmitting vector.
- (3) After the genetic manipulation, insect and symbiont fitness should not be negatively impacted.
- (4) The transgene product expressed must interfere with pathogen development in the vector, but should not affect the fitness of the vector.
- (5) The technique used to spread the genetically modified symbiont/commensal to naturally occurring vector populations should minimize the spread of

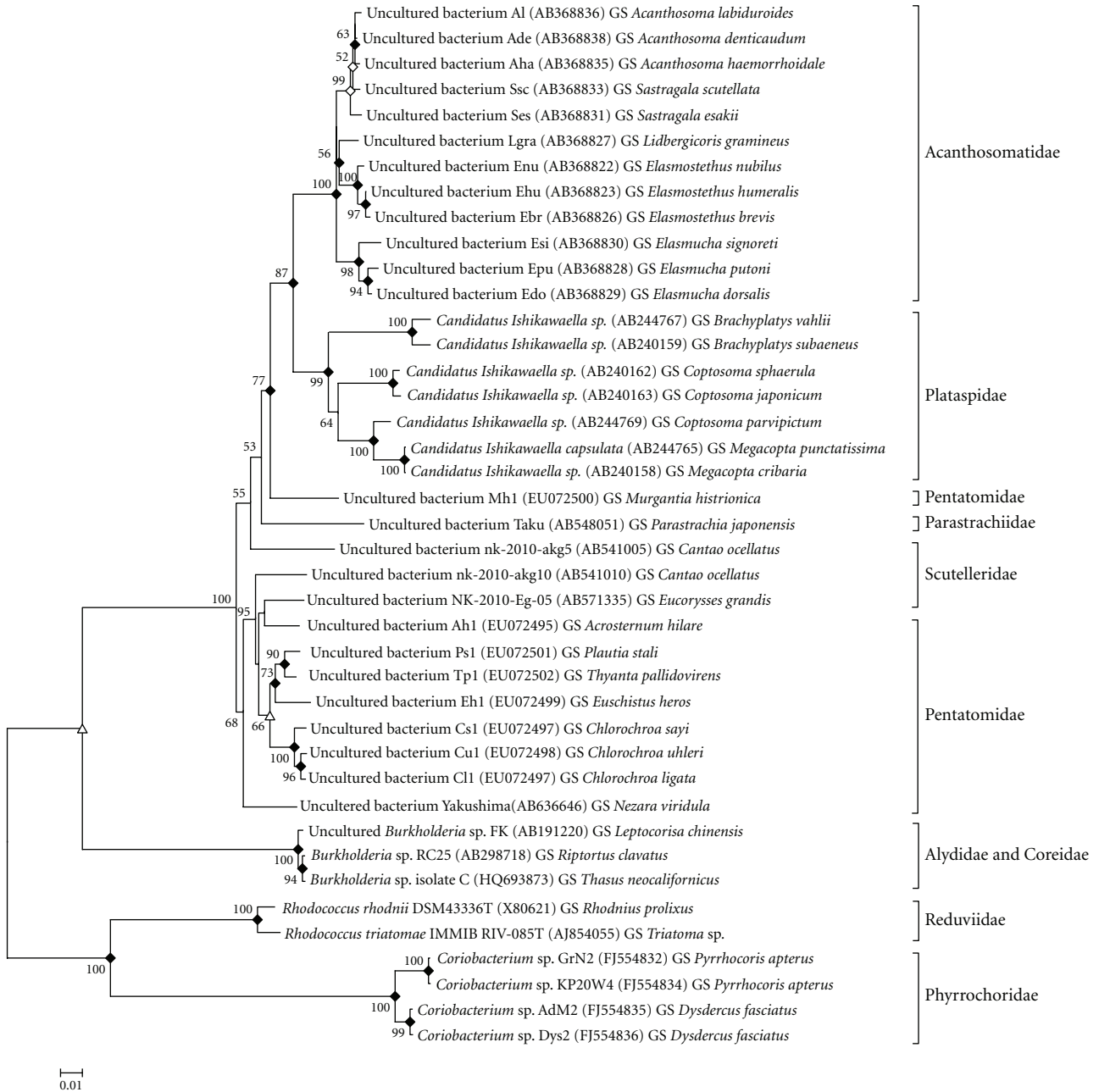


FIGURE 1: Neighbor-joining tree based on nearly complete 16S rRNA gene sequences (1,300 bp) showing phylogenetic relationships between gut symbionts (GS) of heteropteran species. Insect families are indicated after brackets. Black diamonds indicate branches of the tree that were also recovered with the maximum-likelihood and maximum-parsimony tree-making algorithms; white triangle and diamonds stand for branches that were recovered by the maximum-likelihood or by the maximum-parsimony tree-making algorithms, respectively. Numbers at the nodes are percentage bootstrap values based on a neighbor-joining analysis of 1,000 resampled datasets; only values above 50% are given. Bar 0.01 substitutions per nucleotide position.

the transgene to other organisms in the vector's environment, which include both the nontarget microbes inside the host/vector and other organisms that live in the same ecological niche.

3. Acanthosomatidae

This family, a member of the stink bug or shield bug Pentatomoidea superfamily, is characterized by social behavior,

which features the maternal instinct to guard eggs and nymphs against possible predators [53]. The genetic and evolutionary characterization of the caeca-associated symbionts in 14 different species of Acanthosomatidae, representing a total of five genera (*Elasmostethus*, *Lindbergicoris*, *Elasmucha*, *Sastragala*, and *Acanthosoma*), has been elucidated [33]. Acanthosomatid bugs harbor extracellular symbionts of a specific clade of Gammaproteobacteria in midgut crypts.

In Acanthosomatidae, both host and symbiont have co-specified together, and the vertical symbiont transmission is via egg surface contamination [33].

4. Alydidae and Coreidae

Alydidae (called broad-headed bugs) and Coreidae (leaf-footed bugs) are relatively small, principally herbivorous families. Alydid and coreid bugs feed mostly on seeds, less often on the phloem, of various plants; alydids also occasionally exhibit coprophagy and carrion feeding [17]. The broad-headed bugs, *Riptortus clavatus* (Thunberg), *Leptocoris chinensis* (Dallas), and the giant mesquite bug *Thasus neocalifornicus* Brailovsky and Barrera, harbor symbionts of the *Betaproteobacteria* type in the genus *Burkholderia*. These insects acquire symbionts from the soil (i.e., horizontally) in each generation and harbor the bacteria in the lumen of crypts situated along the midgut [15, 54, 55]. Inoculation of aposymbiotic nymphs with cultured symbiotic microorganisms and comparison with aposymbiotic adults reared under sterile conditions suggest that the absence of the symbiont decreases host fitness [15, 54]. Moreover, phylogenetic analysis shows that the *Burkholderia*-like sequences from the digestive tract of *T. neocalifornicus* are closely related to those found in *L. chinensis* and *R. clavatus*, data acquired by amplifying the 1.5-kb segment of the eubacterial 16S rRNA gene [15].

5. Parastrachiidae

In Parastrachiidae (another pentatomoid family), *Parastrachia japonensis* Scott is monophagous, feeding of drupes of the deciduous tree *Shoepfia jasminodora* (Santalales: Olacaceae) [56]. The parastrachiid mother provides food for her nymphs, and the ensuing adults enter into diapause for 9 months, surviving only on water. Molecular phylogenetic analyses of *P. japonensis* symbionts revealed that they constitute a distinct phyletic line in the Gammaproteobacteria 16S rRNA gene subclade. This parastrachiid symbiont has no close relatives, but is allied with gut symbionts of acanthosomatid and plataspid bugs, as well as with endocellular symbionts of sharpshooters, tsetse flies, and aphids [36]. According to Kashima et al. [57], this symbiont might be involved in the uric acid recycling system due to the increased mortality of the adults when they were treated with antibiotic during the nonfeeding period.

6. Pentatomidae

Within Heteroptera, the Pentatomidae (the true “stink bugs”) is one of the largest families with over 4000 species [17]. Many pentatomid insects are polyphagous, feeding on a diverse range of plants. Stink bugs are economically important pests throughout the world on a multitude of crops, including soybeans, rice, pecan, cocoa, and macadamia nuts to name a few [17, 58]. They can cause direct and indirect damage, as can other heteropteran, by feeding on plant tissue with needle-like stylets, injecting digestive enzymes into plant tissue, or providing free access

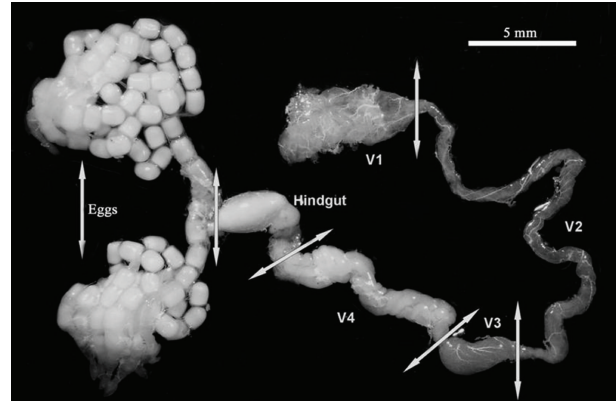


FIGURE 2: Detailed figure of the midgut of *Nezara viridula* divided into four ventricles. V4 is the gastric caeca, where the symbionts are located [21].

to microbial infection [17, 59]. The economic importance of stink bugs is magnified as they are usually difficult to control [60]. Recently, it was shown that the stink bugs, *Acrosternum hilare* (Say), *Chlorochroa ligata* (Say), *Chlorochroa sayi* (Stal), *Chlorochroa uhleri* (Stal), *Dichelops melacanthus* (Dallas), *Edessa meditabunda* (F.), *Euschistus heros* (Fabricius), *Loxa deducta* Walker, *Murgantia histrionica* (Hahn), *Nezara viridula* (L.), *Pellaea stictica* (Dallas, 1851), *Piezodorus guildinii* (Westwood), *Plautia stali* Scott, *Thyanta pallidovirens* (Stal), and, *Thyanta perditor* (F.) are associated with plant pathogens (*Pantoea* spp.) contained in the gastric caecal region (ventricula 4; Figures 2 and 3) of their midguts [21, 34, 35, 40, 61, Prado, S.S. unpublished data]. In general, stink bug symbionts are polyphyletic, although some degree of monophyly has also been observed suggesting that the symbionts were probably acquired and occasionally replaced by other bacteria over evolutionary time [34, 35]. Smearing of symbionts on the egg surface by ovipositing females and subsequent acquisition of the symbiont by aposymbiotic first instar nymphs appears to be the mechanism of vertical transmission.

The cosmopolitan pentatomid, *Nezara viridula*, is both generally and obligatorily associated with a gut symbiont; however, it seems that the type and duration of the association is somewhat different between populations based on the geographical region where the insect is found [20, 21, 39]. At 30°C, *N. viridula*'s symbiont maintenance is affected and insect development is accelerated [40]. Insects free of the symbionts reared at 20°C had longer mean nymphal developmental time, and females never laid eggs [21, 40].

In addition, *P. stali*, when deprived of its gut-associated symbiont, has a slower developmental time than individuals with the symbiont [29]. For *A. hilare*, the elimination of the symbiont by surface sterilization of egg masses negatively impacted development and reproduction [35]. Conversely, the absence of *M. histrionica*'s gut symbiont seems to have no effect on the development of the insect host; however, when both species (*A. hilare* and *M. histrionica*) were reared at 30°C, each lost their respective symbiont [61].

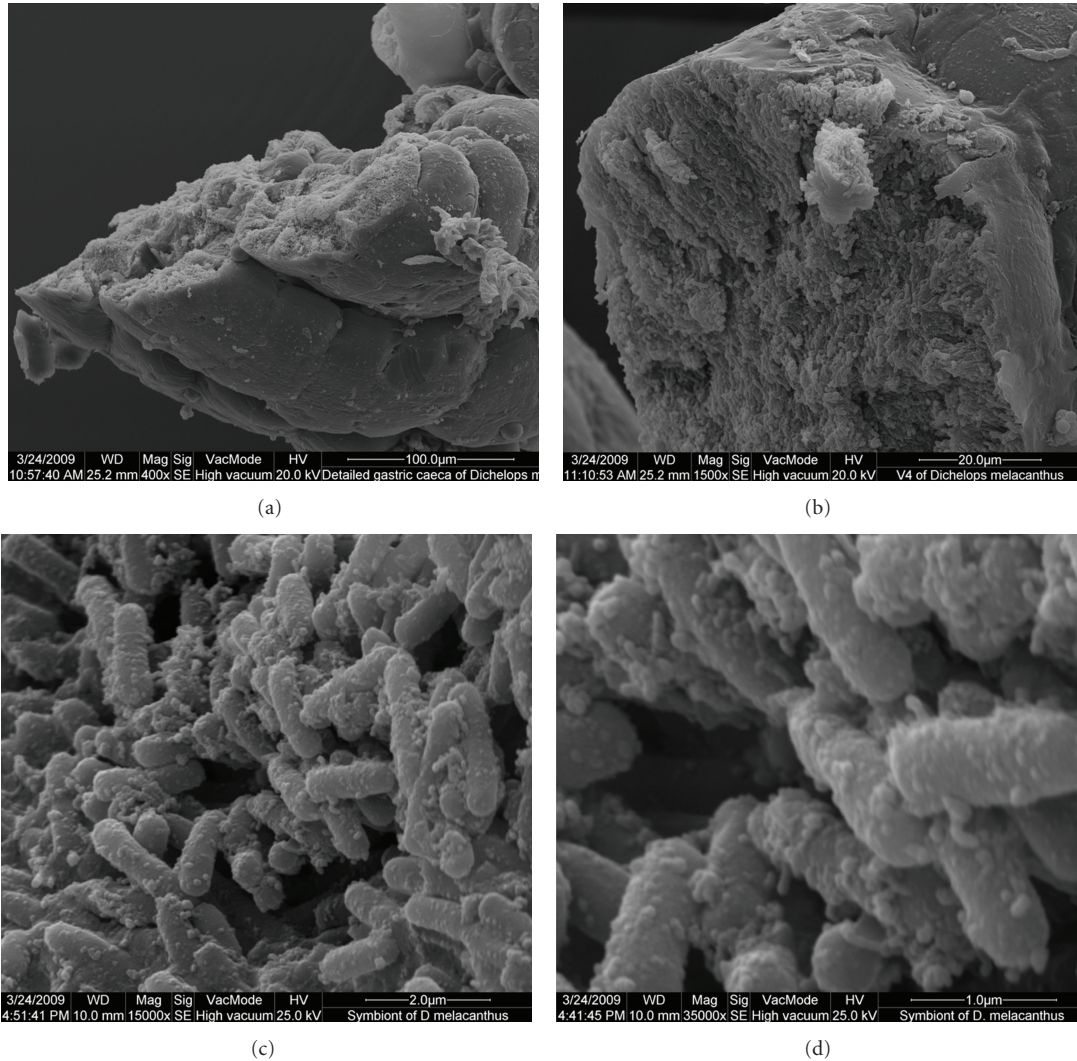


FIGURE 3: Detailed figure of scan electron microscopy (SEM) of the gastric caeca of *Dichelops melacanthus* in (a), detail of the amount of bacteria inside the gastric caeca in (b), and bacteria detail in (c) and (d).

The impact of surface sterilization on the maintenance of the symbionts and on the development of *E. heros*, *D. melacanthus*, and *P. stictica* is being evaluated by Prado et al. (unpublished). Data thus far has shown that a decrease in host fitness was associated with, and probably mediated by, symbiont loss at 30°C. This suggests that, not only egg mass sterilization, but also higher temperature may affect population performance of the insects directly or indirectly through mediated effects on their mutualists [35, 61]. The role of these vertically transmitted pentatomid gut symbionts, therefore, appears to vary for different bug host species. For instance, the cabbage stink bugs, *Eurydema rugosa* Motschulsky and *Eurydema dominulus* (Scopoli), also have symbionts associated with their gastric caecae; absence of the caeca-associated symbiont due to surface sterilization of egg masses caused retarded growth, reduced body weight, and abnormal body color [62].

Recently, using genus-specific primers and appropriate PCR conditions, Zucchi et al. [63] characterized an earlier

unnoticed community of actinobacteria inhabiting the gastric caeca of several pentatomid species (Table 1). Although only a few insect species have been reported associated with *Actinobacteria* [64], the best-known case involving nutrient provision (see Section 2), studies on pentatomids have pointed to an alternative beneficial association in which the actinobacteria produce an antibiotic barrier against pathogens [65, 66]. The role of these actinobacteria in the midgut of the stink bug is still unknown, but Zucchi et al. [63] speculate that the actinobacteria byproducts may regulate the gastric caecal bacterial community.

7. Phyrrochoridae

There is at least 262 pyrrochorid species distributed in 29 genera, with *Dysdercus* being the most important and largest genus [17]. These bugs are called cotton stainers because their excreta, plus the disease organisms they admit, stain cotton fiber. An actinobacterium, *Coriobacterium glomerans*, has been described as the extracellular gut-associated

TABLE 1: Actinobacteria diversity inhabiting the midgut of Pentatomidae.

Family	Genus	Host
<i>Actinomycetaceae</i>	<i>Actinomyces</i> spp.	<i>Thyanta perditor</i>
<i>Brevibacteriaceae</i>	<i>Brevibacterium</i> spp.	<i>Dichelops melacanthus</i> <i>Dichelops melacanthus</i>
<i>Corynebacteriaceae</i>	<i>Corynebacterium</i> spp.	<i>Edessa meditabunda</i> <i>Thyanta perditor</i> <i>Dichelops melacanthus</i>
<i>Dietziaceae</i>	<i>Dietzia</i> spp.	<i>Loxa deducta</i> <i>Pellea stictica</i> <i>Loxa deducta</i> <i>Pellea stictica</i>
<i>Intransporangiaceae</i>	<i>Ornithinimicrobium</i> spp.	<i>Dichelops melacanthus</i> <i>Edessa meditabunda</i> <i>Edessa meditabunda</i> <i>Dichelops melacanthus</i> <i>Edessa meditabunda</i> <i>Loxa deducta</i> <i>Pellea stictica</i>
<i>Kineosporiaceae</i>	<i>Kineococcus</i> spp.	<i>Piezodorus guildinii</i> <i>Thyanta perditor</i>
<i>Microbacteriaceae</i>	<i>Microbacterium</i> spp. <i>Arthrobacter</i> spp.	<i>Dichelops melacanthus</i> <i>Edessa meditabunda</i> <i>Edessa meditabunda</i> <i>Dichelops melacanthus</i> <i>Edessa meditabunda</i> <i>Loxa deducta</i> <i>Pellea stictica</i>
<i>Micrococcaceae</i>	<i>Citrococcus</i> spp.	<i>Piezodorus guildinii</i> <i>Thyanta perditor</i>
<i>Mycobacteriaceae</i>	<i>Mycobacterium</i> spp.	<i>Dichelops melacanthus</i> <i>Piezodorus guildinii</i> <i>Thyanta perditor</i>
<i>Propionibacteriaceae</i>	<i>Propionibacterium</i> spp.	<i>Dichelops melacanthus</i> <i>Piezodorus guildinii</i>
<i>Streptomycetaceae</i>	<i>Streptomyces</i> spp.	<i>Nezara viridula</i>

Source: modified from Zucchi et al. [63].

symbiont in *Pyrrhocoris apterus* (L.) and *Dysdercus fasciatus* Sign. [32, 67, 68]. Recently, Kaltenpoth et al. [32] showed that the bacterial symbionts are located mainly in the third part of the midgut (V 3), with cells found connected to the epithelium and swimming freely in the gut. The symbionts are primarily transmitted vertically by egg smearing, but horizontal transmission also occurs [32]. The bacterial cells can form long chains in the gut of the insects, where they are assumed to aid in digestion [67].

8. Plataspidae

Insects of this family are almost entirely from the tropical old world most species are Oriental and; this is one of a few groups that feed most of the time on legumes [17]. The Japanese common plataspid, *Megacopta punctatissima* (Montandon), harbors the bacterial symbiont *Candidatus Ishikawaella capsulata* in its gastric caeca [19]. Fukatsu and Hosokawa [18] showed that after hatching, the aposymbiotic first instar nymphs immediately probe small brownish capsules attached to the eggs masses laid by the females in order to acquire their symbiont [18, 19, 41]. In addition, Hosokawa et al. [19] used phylogenetic reconstruction to show that both insect and symbiont have undergone cospeciation and when deprived of its symbiont, *M. punctatissima*'s growth and survival are negatively influenced [18, 19, 41]. The plataspid

bug, *Megacopta cribraria* (Fabricius), also has an obligatory relationship with its primary endosymbiont, which is similar to that first described for *M. punctatissima* [69]. Recently, Hosokawa et al. [41] showed by experimentally exchanging the obligatory gut-associated symbiont between *M. punctatissima* and *M. cribraria*, that the success of the important pest species on legumes (*M. punctatissima*) was negatively impacted due to high nymphal mortality before or upon hatching. Conversely, *M. cribraria*, which is considered a nonpest species of the legume crop, when carrying *M. punctatissima*'s obligatory symbiont, exhibited the attributes of the naturally pestiferous species. These exciting findings raise new hypotheses on the evolutionary origin of an insect pest, which may lead to the development of alternative methods to control and manage species considered pests [41].

9. Scutelleridae

Within the Pentatomoidea, this family (commonly called shield bugs) is most closely related to Pentatomidae. All scutellerids are phytophagous, but only a few have been reported as pests [17]. The giant jewel shield bugs, *Cantao ocellatus* (Thunberg) and *Eucorysses grandis* (Thunberg), possess a gammaproteobacterial primary gut symbiont and a *Sodalis*-allied secondary symbiont [37, 38]. The specific

bacterium from *E. grandis* was consistently identified in insects from five different geographic regions and was detected in 100% of the insects surveyed from three host populations. Molecular phylogenetic analysis clearly showed that the primary gut-associated symbiont of *E. grandis* constitutes a distinct lineage in the *Gammaproteobacteria*, and is closely related neither to the gut symbiont associated with *C. ocellatus*, nor to gut symbionts of other stink bugs, suggesting that scutellerid symbionts have multiple evolutionary origins [38].

10. Concluding Remarks

In this paper, we reviewed major trends in symbiotic association for diverse members of the Heteroptera. These interactions have been primarily studied for their ecological interest regarding insect development. In the past decade, it has become increasingly clear that exploiting these relationships may be a fruitful alternative type of biological control; paratransgenesis [42] and specific elimination of the essential bacteria of the pest or vector insect [70] demonstrated promising results.

Endosymbiotic bacteria of insects have received considerable attention in the past few decades. Many studies focused on the intimate associations of intracellular symbionts, their hosts and the degree of mutual interdependence of these symbioses. Most of these studies focused on obligatory symbionts that are difficult or so far impossible to cultivate. Successful attempts at pest control or disease management, such as those demonstrated by the paratransgenesis of the endosymbiont actinobacteria from triatomine species, should become more common once efforts to identify other bacterial symbionts for other heteropteran hosts are more successful, particularly with secondary symbionts. Furthermore, recent studies have shown that extracellular gut symbionts of insects can engage in symbiotic interactions of similar intimacy and specificity with their hosts, and may exhibit similar evolutionary and genomic consequences of the symbiotic lifestyle [19].

There may also be negative impacts on the symbiont-host relationship caused by global warming changes and, consequently, interference in insect survivorship and ecology from elevated global temperatures, encouraging more research on these associations [9, 12, 14, 15, 71].

At the time of writing, only a few examples of insect-microorganism associations are effectively being explored for control of pests or human diseases. This is still an open area of research with great potential for control of insect pests and vectors of disease, as the cases mentioned earlier using the paratransgenic strategy in the systems of sandfly/leishmaniasis and sharpshooter/Pierce's disease represent [50–52]. In fact, it is only recently that considerable information has been gathered to permit the design of alternative methods of control. Studies on different bacterial groups, such as actinobacteria, reminds us how intricate and complex the associations between stink bugs and microorganisms are. Further comprehension of their biological, physiological, and ecological features is necessary to have a better picture

of the evolution of these interactions and to devise a more effective pest and disease control programs.

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