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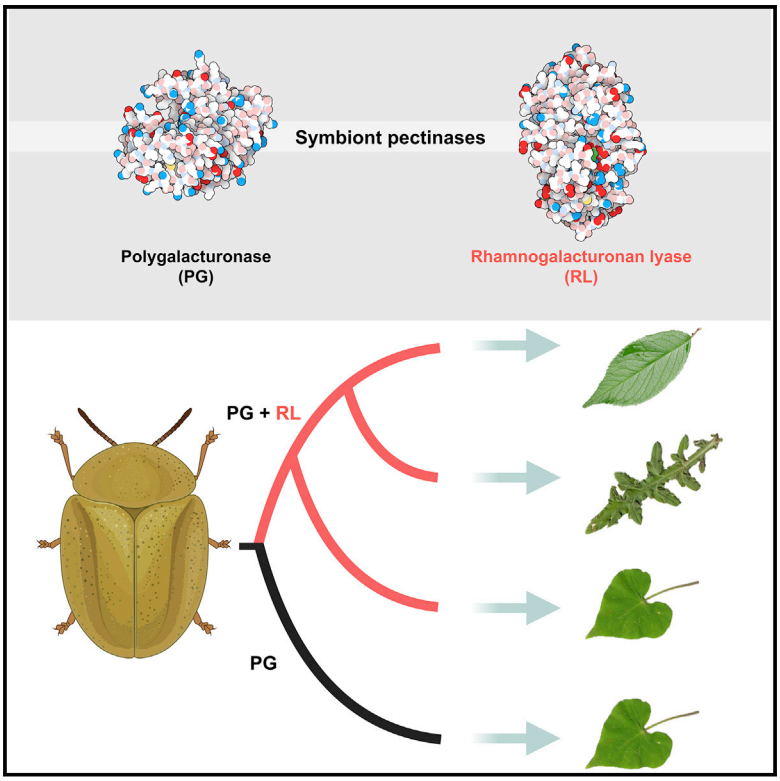
Symbiont Digestive Range Reflects Host Plant Breadth in Herbivorous Beetles

Graphical Abstract



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In Brief

Tortoise leaf beetles rely on the symbiotic bacterium, *Stammera*, to digest foliage rich in pectin. Salem et al. reveal that *Stammera* varies in the pectinases it encodes and supplements. *Stammera* encoding a more dynamic digestive range allows its host to overcome a greater diversity of plant polysaccharides, corresponding to a wider ecological distribution.

Highlights

- *Stammera* genomes are structurally conserved across Cassidinae species
- Symbiont pectinases complement the host's endogenous cellulases and xylanases
- *Stammera* differentially encode pectinases in their reduced genomes
- Symbiont pectinolytic range reflects host plant breadth

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Symbiont Digestive Range Reflects Host Plant Breadth in Herbivorous Beetles

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SUMMARY

Numerous adaptations are gained in light of a symbiotic lifestyle. Here, we investigated the obligate partnership between tortoise leaf beetles (Chrysomelidae: Cassidinae) and their pectinolytic *Stammera* symbionts to detail how changes to the bacterium's streamlined metabolic range can shape the digestive physiology and ecological opportunity of its herbivorous host. Comparative genomics of 13 *Stammera* strains revealed high functional conservation, highlighted by the universal presence of polygalacturonase, a primary pectinase targeting nature's most abundant pectic class, homogalacturonan (HG). Despite this conservation, we unexpectedly discovered a disparate distribution for rhamnogalacturonan lyase, a secondary pectinase hydrolyzing the pectic heteropolymer, rhamnogalacturonan I (RG-I). Consistent with the annotation of rhamnogalacturonan lyase in *Stammera*, cassidines are able to depolymerize RG-I relative to beetles whose symbionts lack the gene. Given the omnipresence of HG and RG-I in foliage, *Stammera* that encode pectinases targeting both substrates allow their hosts to overcome a greater diversity of plant cell wall polysaccharides and maximize access to the nutritionally rich cytosol. Possibly facilitated by their symbionts' expanded digestive range, cassidines additionally endowed with rhamnogalacturonan lyase appear to utilize a broader diversity of angiosperms than those beetles whose symbionts solely supplement polygalacturonase. Our findings highlight how symbiont metabolic diversity, in concert with host adaptations, may serve as a potential source of evolutionary innovations for herbivorous lineages.

INTRODUCTION

Beetles display a remarkable degree of adaptability to exploit a correspondingly diverse flora [1, 2]. As half of all beetles are herbivorous, traits mediating phytophagy are central to the evolutionary success of Earth's most speciose animal group [3]. Recent genomic insights into beetle diversification revealed that the two independent origins of specialized herbivory within the order coincided with the co-option of microbial enzymes to more efficiently digest complex plant polymers, such as cellulose, hemicellulose, and pectin [4–7]. By endowing phytophagous beetles with the catalytic tools to breach the plant cell wall, these enzymes are implicated in the evolution of correspondingly varied and specialized plant-feeding habits [8, 9], fueling the diversification of major herbivorous lineages [4].

Although horizontal gene transfer from bacteria and fungi allowed phytophagous beetles to endogenously maintain a battery of key plant-cell-wall-degrading enzymes [4, 7, 8], novel metabolic features can also be integrated through symbiosis. The pectinolytic phenotype of the tortoise leaf beetle *Cassida rubiginosa* (Coleoptera: Chrysomelidae: Cassidinae) is entirely outsourced to its obligate γ-proteobacterial symbiont, *Candidatus* Stammera capleta (henceforth *Stammera*) [10]. Possessing a highly reduced genome (271 Kb), the symbiont's limited metabolism is streamlined to produce and export pectinolytic enzymes that enable *C. rubiginosa* to process foliage rich in recalcitrant pectins [10, 11]. To ensure that future host generations are endowed with *Stammera*, each egg is maternally provisioned at the anterior pole with a symbiont-harboring caplet as a vehicle for vertical transmission [10]. Infection by *Stammera* is initiated when an emerging larva consumes the caplet. As the sole source of pectinases for its host, the experimental removal of *Stammera* diminishes the digestive capacity of the host beetle, resulting in stunted growth and low survivorship [10].

Our initial characterization of the symbiosis revealed that the thistle-feeding tortoise leaf beetle *C. rubiginosa* relies on two classes of pectinases in its partnership with *Stammera* [10]: a

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Table 1. List of Cassidine Leaf Beetles Included in This Study, Their Host Plants, and the Digestive Enzymes Encoded by Their *Stammera* Symbionts

			Host Plant	*Stammera* Digestive Enzymes		
Beetle Species	Tribe	Location	Clade, Order	Polygalacturonase (GH 28)	Rhamnogalacturonan Lyase (PL 4)	α-Glucuronidase (GH 67)
Cassida versicolor	Cassidini	Japan	Fabids, Rosales [21]	+	+	
Cassida viridis	Cassidini	Germany	Lamiid, Lamiales [22]	+	+	
Cassida vibex	Cassidini	Germany	Campanulids, Asterales [23]	+	+	
Cassida rubiginosa	Cassidini	New Zealand	Campanulids, Asterales [24, 25]	+	+	
Parachirida semiannulata	Cassidini	Panama	Lamiid, Solanales [18]	+	+	
Ischnocodia annulus	Cassidini	Panama	Lamiid, Boraginales [18]	+		+
Stolas discoides	Mesomphaliini	Panama	Lamiid, Solanales [18]	+		+
Chelymorpha alternans	Mesomphaliini	Panama	Lamiid, Solanales [18]	+		+
Acromis sparsa	Mesomphaliini	Panama	Lamiid, Solanales [20]	+		
Cistudinella foveolata	Ischyrosonychini	Panama	Lamiid, Boraginales [18, 26]	+		+
Discomorpha panamensis	Omocerini	Panama	Lamiid, Boraginales [18, 26]	+		+
Agroiconota porpinqua	Cassidini	Panama	Lamiid, Solanales [18]	+		+
Charidotella sexpunctata	Cassidini	USA	Lamiid, Solanales [27]	+		+

Symbol (+) indicates the presence of an encoding gene within the symbiont's genome. GH, glycoside hydrolase; PL, polysaccharide lyase.

polygalacturonase that cleaves homogalacturonan (HG), the homopolymeric backbone of pectin, and a rhamnogalacturonan lyase that targets the heteropolymeric pectic region, rhamnogalacturonan I (RG-I). As the most plentiful polysaccharide of the primary plant cell wall [12], pectin is instrumental for embedding cellulose and xylan into a carbohydrate matrix that ensures the physical integrity of the cell and contributes to adhesion and signal transduction [13]. Although HG is typically more abundant than RG-I, both polysaccharidic sequences are universally present in the primary cell wall and comprise up to 95% of all pectic content in plant tissues [13, 14]. Because pectin degradation facilitates, and typically precedes, the downstream breakdown of other primary cell wall components like cellulose and hemicellulose [15, 16], symbiont-encoded pectinases are critical for *C. rubiginosa* to maximize the dietary value of ingested leaves.

The Cassidinae subfamily includes more than 6,000 species that are all herbivorous [17]. Despite a cosmopolitan distribution and their specializations to exploit a diverse range of angiosperms [17–20], our understanding of the metabolic features behind the origin and radiation of this highly speciose insect lineage is limited, especially in the context of its symbiosis with *Stammera*. Reconciling an extensive record of life history traits for tortoise leaf beetles with comparative genomics, transcriptomics, and biochemical assays, we explore the metabolic features of both host and symbiont to (1) characterize the range of digestive enzymes defining the partnership, (2) demonstrate how variation in symbiotic factors can shape the digestive physiology of the insect host, and (3) highlight how this variation might facilitate niche expansion and adaptation across a diverse group of herbivorous beetles.

RESULTS AND DISCUSSION

Stammera Genomes Are Structurally and Metabolically Conserved

DNA sequencing of the symbiotic organs associated with the foregut of 13 cassidine species, representing four taxonomic tribes and collected from four continents, revealed that all investigated tortoise leaf beetle species maintain a symbiosis with *Stammera* (Table 1). In light of the phylogenetic congruence between Cassidinae beetles and *Stammera* (Figure 1), we applied the reconciliation tool Jane 4 [28] to test for co-speciation. Our analysis outlined nine co-speciation events between host and symbiont (Table S1). Three events were attributed to duplication and host switching, and two were labeled as loss events. No other evolutionary events, such as duplication or failure to diverge, were revealed. Although the general pattern of co-cladogenesis (Figure 1) is in line with cassidines relying on egg caplets to vertically transmit *Stammera* [10], limited horizontal exchange of the symbiont cannot be ruled out. This is consistent with the coevolutionary dynamics reported for many insect groups that extracellularly transfer their symbionts [29], including the hemipteran families Acanthosomatidae [30], Plataspidae [31], and Urostylididae [32].

The assembled genomes of all 13 *Stammera* strains are drastically reduced in size (215–310 Kb) and highly AT-rich (83%–89%; Figure 1; Table S2). In addition to a circular chromosome, each *Stammera* possesses 1 to 2 plasmids that range in size from 3 to 5 Kb (Table S2). Encoding 232–296 putative protein-coding open reading frames (ORFs) with an average size of 912 bp, the resulting coding percentage is between 89% and 92%. All strains possess operons for the three structural

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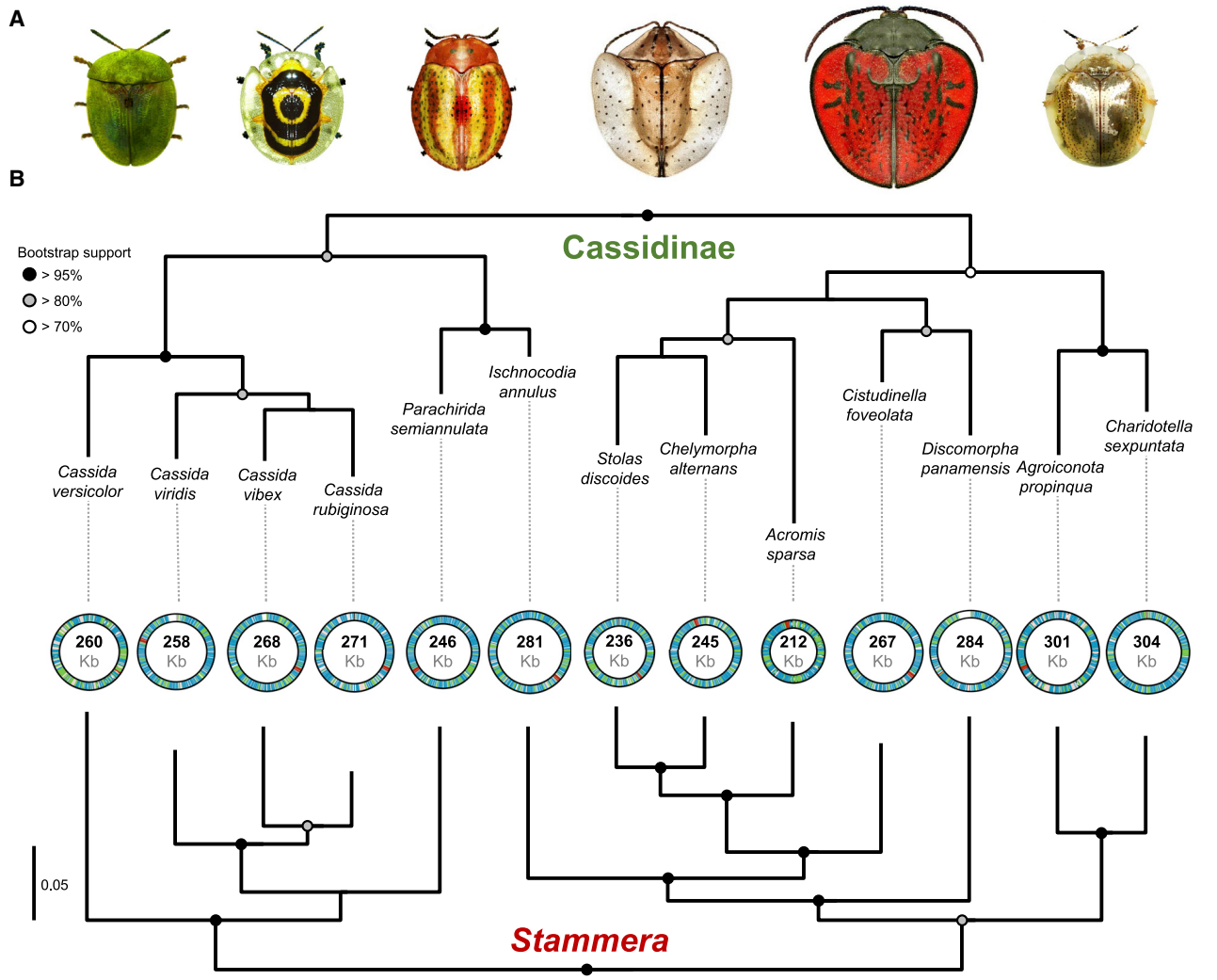

Figure 1. Tortoise Leaf Beetles (Chrysomelidae: Cassidinae) in Co-cladogenesis with Their *Stammera* Symbionts

(A) Cassidinae beetles.

(B) Maximum-likelihood (ML) phylogeny of the host is based on concatenated sequences of 18S and 28S ribosomal RNA and mitochondrial 16S rRNA genes. Symbiont phylogeny is based on ML analysis of a concatenated nucleotide alignment for 30 genes shared by 13 *Stammera* genomes. Diagrams of *Stammera*'s genomes are represented by circles with diameters corresponding to size. Genes coding for rRNA (red), metabolism (blue), and cellular processes (green) are indicated on each circle. Node coloration reflects bootstrap support. Photographs of Cassidinae species from left to right are as follows: *Cassida rubiginosa*; *Ischnocodia annulus*; *Chelymorpha alternans*; *Acromis sparsa*; *Discomorpha panamensis*; and *Charidotella sexpunctata*.

See also Table S1.

ribosomal RNA genes, as well as tRNA genes for all 20 amino acids (Table S2).

Similar to other bacterial symbionts possessing tiny genomes [33], *Stammera* retained gene families involved in the core processes of replication, transcription, and translation (Figures 2 and S1A). Our annotation also revealed that genes related to protein folding and stability are shared throughout, including the *GroES-GroEL* chaperonin complex and the bacterial heat shock protein 70 (Hsp70) (Figure S1A). Suggestive of an important role in the biology of bacterial symbionts [33], such chaperones are among the most highly expressed enzymes within the bacteriomes of aphids [34], cicadas [35], tsetse flies [36], sharpshooters [37], and leaf beetles [11]. In contrast, pathways for the synthesis of essential amino acids, cofactors, and lipids are missing, as are genes encoding signal transduction, cell surface structures, and motility (Figure 2; Data S1).

Energy production and the recovery of reducing equivalents in *Stammera* are achieved through the oxidation of sugars to pyruvate via glycolysis, followed by the fermentation of pyruvate to lactate through the activity of lactate dehydrogenase [11]. The combination of glycolysis and obligate fermentation appears to be a common feature across this γ-proteobacterial lineage (Figure S1B), presumably mediated by the microbe's extracellular localization and relaxed selection to maintain the citric acid cycle. This represents a notable departure from the respiratory metabolism fueling many intracellular symbionts [11], where the citric acid cycle serves as a precursor to produce key micronutrients (e.g., essential amino acids) for their insect hosts

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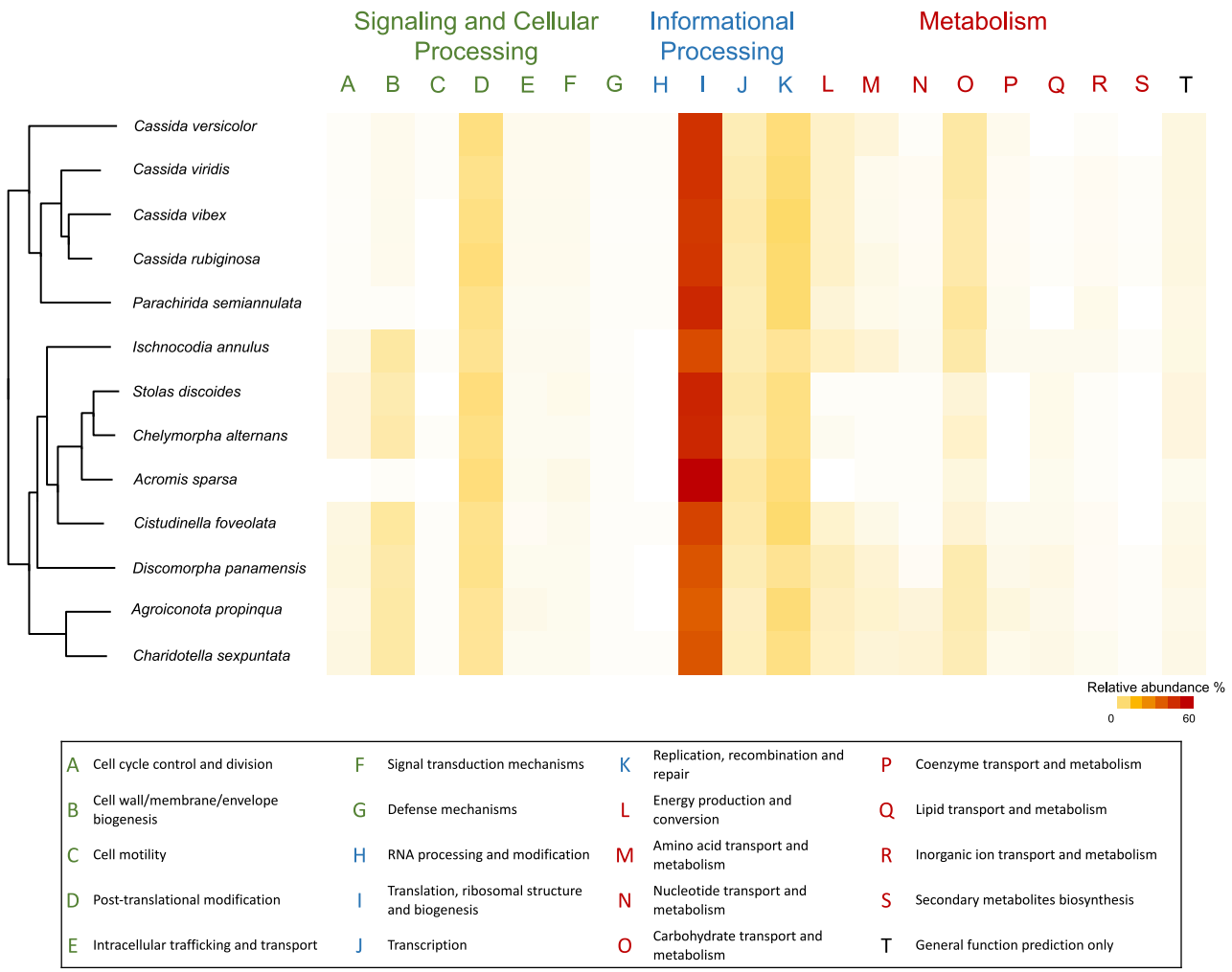

Figure 2. High Metabolic Conservation Identified in the Genomes of *Stammera* from 13 Cassidinae Species

Relative abundance of Clusters of Orthologous Groups (COG)-annotated functional groups is represented as a heatmap. Symbiont phylogeny, as in Figure 1, is based on a ML analysis of a concatenated nucleotide alignment for 30 genes shared by 13 *Stammera* genomes. See also Figure S1, Table S2, and Data S1.

[11, 38, 39]. In the four instances we could not annotate lactate dehydrogenase in *Stammera* (hosts: *Acromis sparsa*; *Chelymorpha alternans*; *Stolas discoides*; and *Cistudinella foveolata*), we also observed an incomplete glycolysis pathway, as well as a missing ATP synthase complex (Figure S1B). Given the monophyly of these four strains within the symbiont's phylogeny (Figures 1 and S1B), it appears that the glycolytic and fermentative pathways were lost once, pointing toward the host presumably supplementing *Stammera* with the necessary energy equivalents as described for other microbes with highly reduced genomes, such as *Carsonella ruddii* in psyllids [39].

Other notable variation across *Stammera* genomes involves genes coding for peptidoglycan biosynthesis (Figure S1B). Although host-restricted symbionts can experience gene losses in all functional categories, pathways underlying production of cell envelope components are commonly depleted [40], as shown in endosymbionts like *Buchnera* in aphids [41], *Hodgkinia* in cicadas [42], and *Carsonella* in psyllids [39]. The differential distribution of these pathways in *Stammera* may indicate some variation in the degree of metabolic dependence and the level of host control over microbial growth and proliferation within the symbiotic organs of cassidine beetles.

Variation in Symbiont-Derived Digestive Enzymes

Our first sequencing and annotation of a *Stammera* genome associated with *C. rubiginosa* revealed the presence of polygalacturonase and rhamnogalacturonan lyase as two pectinases mediating digestion of HG and RG-I, respectively [10]. Polygalacturonase appears foundational to the Cassidinae-*Stammera* symbiosis, evidenced by its distribution across all 13 symbiont genomes (Table 1). As an endo-active enzyme belonging to the glycoside hydrolase family 28 (GH 28), polygalacturonase hydrolyzes the backbone of HG at random intervals to generate galacturonic acid products of various sizes [7, 10].

In contrast to the conservation of polygalacturonase across all 13 genomes, rhamnogalacturonan lyase is encoded by a subset of *Stammera* strains (Table 1). The symbionts supplementing rhamnogalacturonan lyase partner with beetles distributed

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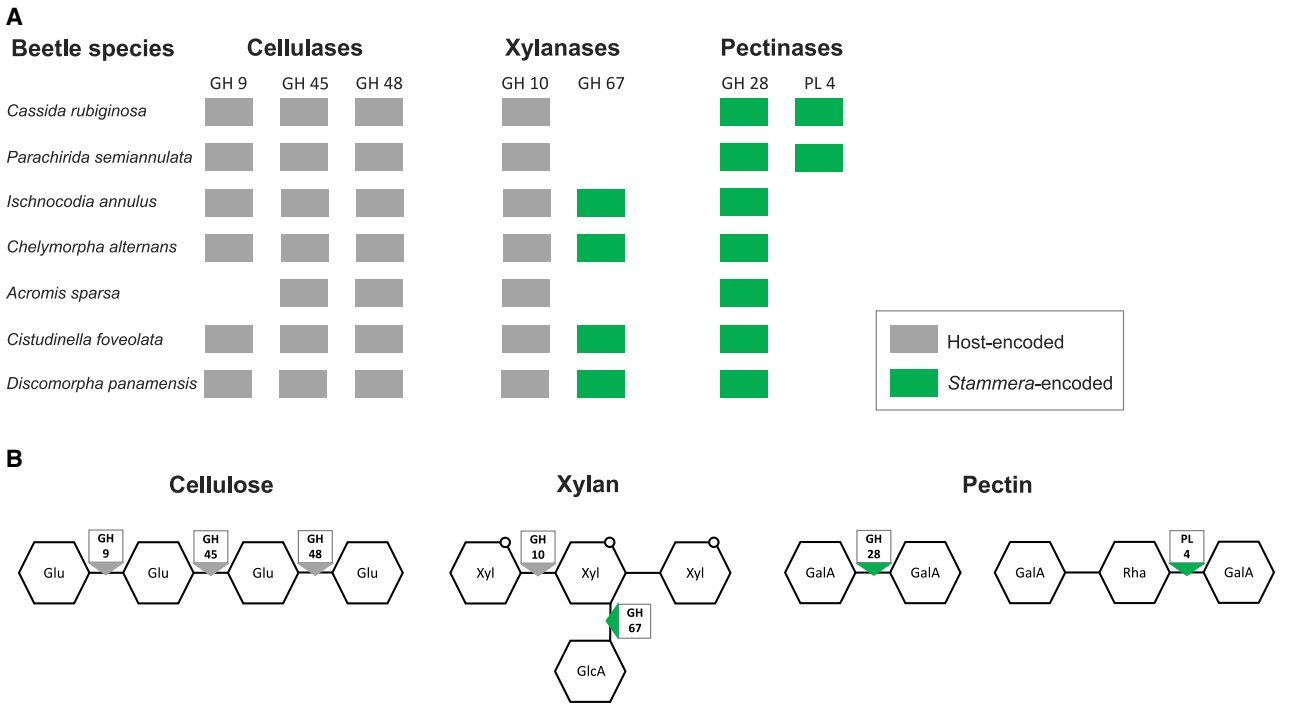

Figure 3. Enzymatic Synergy between Host and Symbiont to Deconstruct the Primary Cell Wall

(A) Host- and *Stammera*-encoded plant-cell-wall-degrading enzymes (PCWDEs) as inferred from transcriptome profiling of seven representative cassidine species. Source of PCWDEs is designated by color, where gray denotes an endogenous beetle gene and green denotes a *Stammera*-encoded enzyme.

(B) Predicted mode of action of the annotated PCWDEs across cellulose, xylan, and pectin.

GalA, galacturonic acid; GH, glycoside hydrolase; GlcA, glucuronic acid; Glu, glucose; PL, polysaccharide lyase; Rha, rhamnose; Xyl, xylose. See also Tables S3 and S4.

across Panama, Germany, Japan, and New Zealand (Table 1). These beetles feed on phylogenetically diverse angiosperms, including plants within the lamiid, campanulid, and fabid eudicot clades (Table 1). Belonging to the polysaccharide lyase family 4 (PL 4), rhamnogalacturonan lyase cleaves the α-(1,4) glycosidic bonds between rhamnose and galacturonic acid of the RG-I backbone through a β-elimination reaction [43].

Furthermore, genome annotation revealed a third class of plant-cell-wall-degrading enzymes in *Stammera*: an α-glucuronidase (GH 67; Table 1). In contrast to the aforementioned pectinases, α-glucuronidase is predicted to cleave the glycosidic bonds linking the xylose backbone to glucuronic acid through a single displacement mechanism [44]. As noted for rhamnogalacturonan lyase, α-glucuronidase is not universally present in all *Stammera* strains (Table 1). But unlike rhamnogalacturonan lyase, the distribution of α-glucuronidase in our survey is restricted to symbionts associated with New World tropical beetle species specializing on host plants within the lamiid eudicot clade (Table 1). The binary distribution of rhamnogalacturonan lyase and α-glucuronidase in this symbiosis is striking (each occurs in the absence of the other), highlighting how *Stammera* can supplement different sets of digestive enzymes to its leaf beetle hosts.

Deconstructing the Plant Cell Wall Together: A Complementary Role for Host Enzymes

As with many phytophagous beetles [4, 5, 45–47], the tortoise leaf beetle *C. rubiginosa* maintains a range of cellulases and xylanases endogenously [10]. This is attributed to a string of horizontal gene transfer events that coincided with an ancestral transition from fungivory to herbivory in the Coleoptera, currently dated at 250 mya [5, 8]. Acquired from a range of bacterial and fungal donors, many of these enzymes underwent a number duplication events followed by functional diversification [4]. By evolving complementary catalytic activities, they allowed beetles to process cellulose and hemicellulose more efficiently [45–47]. Expanding on our previous transcriptional profiling, we sequenced six additional gut transcriptomes of cassidine beetles to characterize the range of endogenous plant-cell-wall-degrading enzymes in the subfamily and detail the degree of predicted synergy between host and microbe toward deconstructing the complex polysaccharidic networks dominating plant leaves. Conserved across the seven species of cassidines transcriptionally profiled here are four endogenous carbohydrate-active enzymatic families, including cellulases belonging to the GH families 9, 45, and 48, as well as a single xylanase belonging to the family GH 10 (Figure 3; Table S5). Genes encoding these enzymatic families are enriched in most chrysomelid genomes [4, 48].

Heterologous expression of endogenous GHs in phytophagous beetles revealed that GH 45 is functionally active and confers a cellulolytic phenotype, which is in line with descriptions from wider herbivorous taxa, such as nematodes [49], mollusks [50], and rotifers [51]. In contrast, functional characterization of GH 9 failed to confirm the enzyme's predicted cellulolytic activity

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in the Chrysomelidae [45, 46], suggesting that it may have lost its enzymatic potential altogether. The low expression levels of GH 9 in leaf beetle guts relative to other cellulases support the latter hypothesis.

For the endogenous xylanase (Figure 3), GH 10 was previously annotated in the genome of the coffee berry borer, and heterologous expression demonstrated the enzyme's ability to effectively depolymerize the xylan backbone [47]. The optimal activity dynamics of the xylanase was shown to resemble conditions similar to the physiological medium within the beetle's digestive tract [47], allowing the insect to subsist solely on the coffee seed's endosperm, a diet composed primarily of hemicellulose polysaccharides.

Displaying a division of labor that appears to reflect no catalytic overlap (Figure 3), host- and *Stammera*-encoded enzymes contribute to the processing of cellulose, xyloglucan, and pectin: the three most abundant classes of polysaccharides within leaves. However, relative to the conserved battery of digestive enzymes encoded in the genomes of most phytophagous beetles [4], cassidines notably do not encode their own pectinases (Figure 3). Beyond the endogenous cellulases and xylanases, as well as the symbiotic polygalacturonase, rhamnogalacturonan lyase, and α-glucuronidase detected in the reduced genomes of *Stammera*, no additional enzymes involved in the processing of the plant cell wall could be annotated in our transcriptomic survey of the beetles. As our annotations are informed by characterized digestive enzymes spanning public databases, we cannot exclude the possibility that novel catalytic features are additionally encoded.

Two Pectinases Are Better Than One

Given the conservation of host-encoded cellulases and xylanases and variation of plant-cell-wall-degrading enzymes encoded by *Stammera* (Figure 3), we set out to compare the digestive physiology of two leaf beetle species possessing metabolically representative symbionts: *Cassida rubiginosa* and *Chelymorpha alternans*. *Stammera* in *C. rubiginosa* contributes polygalacturonase and rhamnogalacturonan lyase, although *Ch. alternans* harbors a symbiont encoding for polygalacturonase and α-glucuronidase (Table 1; Figure 3).

To confirm *in silico* predictions that *C. rubiginosa* should depolymerize a greater diversity of pectic substrates than *Ch. alternans*, we qualitatively analyzed the breakdown products of HG and RG-I by thin-layer chromatography (TLC). Because both leaf beetle species possess symbionts that encode for polygalacturonase, gut extracts tested against HG revealed that the insects can monomerize the homopolymer into galacturonic acid (Figure 4A). Although the monosaccharide is the sole product accumulating in *C. rubiginosa*, di-galacturonic acid, tri-galacturonic acid, and larger oligosaccharides also appear in assays featuring *Ch. alternans* (Figure 4A). Similarly, and consistent with earlier observations [10], *C. rubiginosa* is also able to depolymerize RG-I, evidenced by the accumulation of monogalacturonic acid as a breakdown product (Figure 4B). This is also the case for other cassidines whose symbionts encode rhamnogalacturonan lyase, including *Cassida vibex* and *Cassida viridis* (Figure S2). In contrast, monogalacturonic acid is not a breakdown product in *Ch. alternans* (Figure 4B). Instead, we observe the accumulation of a larger, uncharacterized molecule, which also appears in *C. rubiginosa* (Figure 4), *C. vibex*, and *C. viridis* (Figure S2). Overall, relative to beetles only supplemented with *Stammera*'s polygalacturonase, the combination of symbiont-encoded primary and secondary pectinases endows a subset of cassidines with a more proficient pectinolytic phenotype across the two polysaccharidic sequences that form the pectic backbone (Figure 4).

The exact mode of action of rhamnogalacturonan lyases in *Stammera* is of particular interest. Its activity in phytopathogens and plants involves cleaving the α-(1,4) glycosidic bond between rhamnose and galacturonic acid at random intervals, producing oligomers of varying sizes at the non-reducing end [14]. Although we do not observe the additional buildup of larger oligosaccharides in our assays, the catalytic phenotype against RG-I is lost following aposymbiosis in *C. rubiginosa* [10], implicating rhamnogalacturonan lyase in this process, either through direct activity or in synergy with other digestive enzymes.

Despite possessing a more limited pectinolytic metabolism relative to *C. rubiginosa*, we hypothesized *Ch. alternans* to display a greater xylanolytic range, owing to its symbiont encoding α-glucuronidase instead of rhamnogalacturonan lyase (Table 1; Figure 3). Testing for activity against xylotriose linked to glucuronic acid, both *Ch. alternans* and *C. rubiginosa* display an ability to depolymerize the hemicellulose's backbone into monomers and oligomers of xylose (Figure 5). This is consistent with the annotation of the endogenous xylanase GH 10 in both species (Figure 3) and the aforementioned catalytic capacity of the enzyme in other phytophagous beetles [47]. Surprisingly, we could not characterize any activity in either species against the glycoside linkage between xylose and glucuronic acid (Figure 5), given the absence of the latter as a breakdown product in the TLC. This is especially notable for *Ch. alternans* in light of the annotation of an α-glucuronidase in its symbiont's genome (Table 1; Figure 3). This indicates that the enzyme does not upgrade *Ch. alternans*' digestive physiology against the hemicellulose. The loss of α-glucuronidase from the symbiont of *Acromis sparsa* suggests that, in at least one lamiid-feeding Cassidinae species, the enzyme may no longer be adaptive.

Reflecting the dominant abundance of HG in angiosperm leaves [12, 13] and the pivotal role polygalacturonases played in the evolution of obligate herbivory in beetles [7], we highlight the conserved distribution of the primary pectinase in *Stammera* (Table 1) and emphasize its importance as a foundational enzyme for the stability of this partnership. Predicted to upgrade the nutritional ecology of herbivorous beetles by facilitating the exploitation of young plant tissues rich in HG (e.g., leaves, stems, seeds, and fruits), the acquisition of polygalacturonases following horizontal gene transfer [4] or symbiosis (Table 1; Figure 3) underscores their adaptive significance for phytophagous taxa [7].

Symbiont Pectinolytic Range Reflects Host Ecological Breadth

As the second most abundant polysaccharide in the plant cell wall, RG-I constitutes 20%–35% of the overall pectic content in plant tissues [13, 14]. Omnipresent in eudicots, an exceedingly diverse and abundant group of angiosperm plants, RG-I contributes to the functionality of the primary cell wall by enhancing its rigidity and firmness [52]. Because RG-I is covalently linked to

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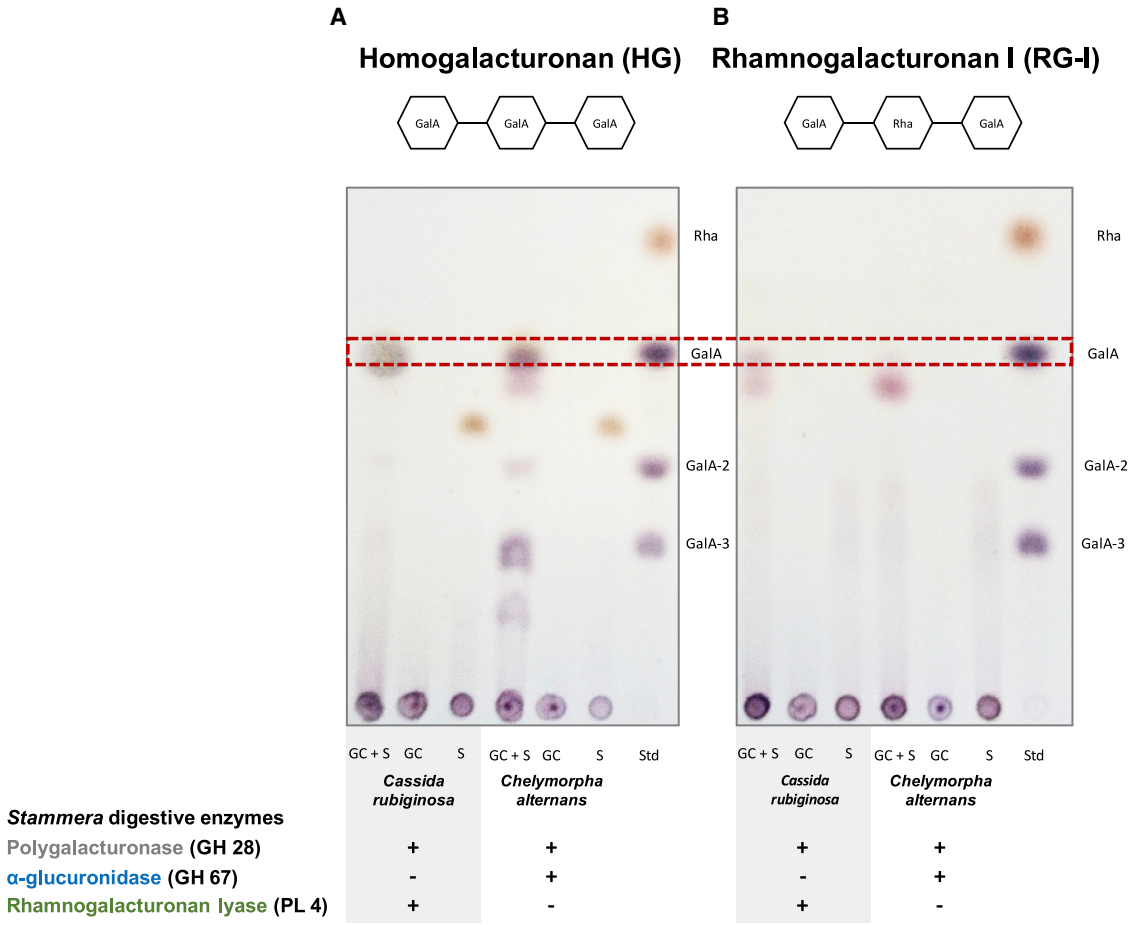

Figure 4. Polygalacturonase and Rhamnogalacturonan Lyase Endow a More Dynamic Pectinolytic Phenotype to Cassidine Leaf Beetles

(A and B) Thin-layer chromatogram (TLC) illustrating the breakdown products of pectinase activity against (A) HG and (B) RG-I using gut contents from *Cassida rubiginosa* and *Chelymorpha alternans*. GC, gut content; S, substrate; Std, standard; GalA, mono-galacturonic acid; GalA-2, di-galacturonic acid; GalA-3, tri-galacturonic acid; Rha, rhamnose. Dashed box highlights GalA differences across treatments. See also Figure S2.

HG, cleavage of the heteropolymeric backbone compromises the stability and mechanical properties of the pectin network [52, 53]. RG-I-degrading enzymes, like rhamnogalacturonan lyase, are typically found in the genomes of plants and their pathogens [54]. Activity of the secondary pectinase in plant roots, leaves, and fruits is tied to developmental modifications requiring plasticity during growth, ripening, and senescing [54]. In phytopathogens like *Dickeya* and *Bacillus*, rhamnogalacturonan lyase activity constitutes a pathogenicity factor by allowing the microbes to break through the primary plant cell wall to initiate intracellular infection [55, 56].

Given that nitrogen is the major nutrient limitation influencing insects exploiting living plant material [57], a more efficient plant-cell-wall-degrading phenotype is predicted to mediate specialization on host plants increasingly divergent in tissue form, composition, and complexity by facilitating access to cytosolic content rich in these compounds [4, 7, 9]. This is evident in the convergent evolution of phytophagy across the Coleoptera, where the independent integration of gene sets encoding for plant-cell-wall-degrading enzymes coincided with the rapid radiation of beetles to exploit angiosperms during the Jurassic [4]. An analogous example for how the integration of novel digestive enzymes can influence ecological range, the evolution of entomopathogenicity in the fungus *Metarhizium* is tied to the horizontal acquisition of a core set of enzymes that facilitate the breakdown of insect cuticular components [58, 59]. Through the complementary activities of horizontally acquired lipid carriers and proteases, *Metarhizium* is able to penetrate the insect exoskeleton by efficiently digesting epicuticular lipids and procuticular proteins [58, 59]. In characterizing the impact of horizontal gene transfer on the pathogenic breadth of *Metarhizium*, generalist species were found to share several genes, including an endoprotease, which lacked homologs in specialist endophytes. Strikingly, experimental transfer of these genes through genome transformation resulted in *Metarhizium* specialists expanding their host range [59].

Coinciding with an extended range of universal plant cell wall polysaccharides that a folivore can metabolize, cassidines whose symbionts additionally provision rhamnogalacturonan lyase appear to have radiated onto host plants spanning a wider phylogenetic breadth (Table 1; Figure 6). Although *Stammera*

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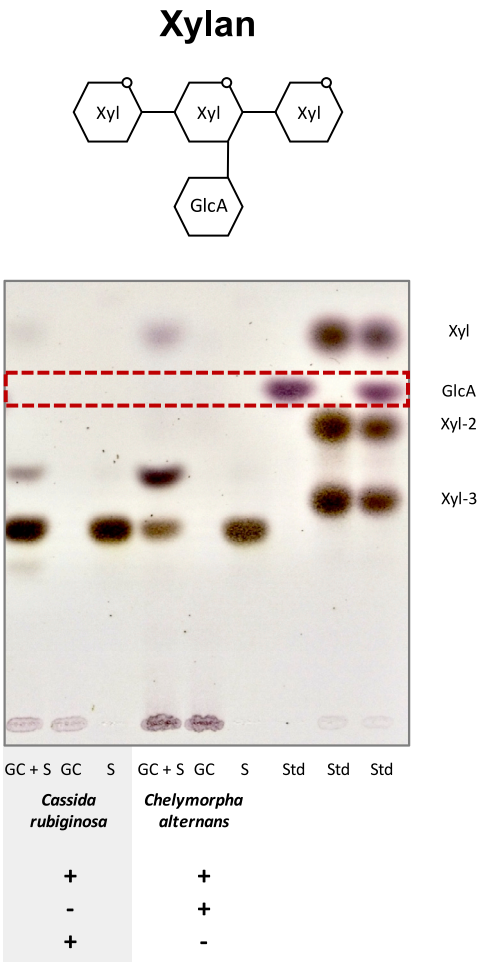

Figure 5. α-Glucuronidase Does Not Upgrade the Xylanolytic Capacity of Cassidine Leaf Beetles

TLC illustrating the breakdown products of xylanase activity assays against xylotriose linked to glucuronic acid using gut contents from *Cassida rubiginosa* and *Chelymorpha alternans*. Xyl, mono-xylose; Xyl-2, di-xylose; Xyl-3, tri-xylose. Dashed box highlights the absence of GlcA buildup across both treatments.

annotated to only express polygalacturonase are restricted to associating with cassidines feeding on plants within the lamiid eudicot clade, beetles symbiotically supplemented with both polygalacturonase and rhamnogalacturonan lyase evolved to specialize on angiosperms across three distinct eudicot clades (lamiids, campanulids, and fabids; Figure 6), which are separated by ~125 Ma of evolution [60].

Radiations to exploit novel ecological niches are rarely a consequence of just a single adaptation but rather an amalgam of several complementary traits, where the evolution of one may facilitate the rise of others that, collectively, drive the origin of novel adaptive forms [61]. Among beetles, the ability to exploit and coevolve with an increasing diversity of angiosperms is attributed to the development of grinding mandibular structures to fragment plant tissues [62], coupled with the acquisition and expansion of enzymatic classes to process toxic secondary compounds and recalcitrant polysaccharides [4]. Consistent with the latter, an upgraded digestive capacity by *Stammera* appears to not only reflect a broader phylogenetic host plant coverage but also an expanded geographical distribution that extends beyond the tropical origins of the Cassidinae [17]. Beetles symbiotically endowed with rhamnogalacturonan lyase and polygalacturonase exploit host plants adapted for temperate climates, such as thistles [23–25], mints [22], and Maleae trees [21], in contrast to the tropical or subtropical distributions of lamiid host plants for cassidines relying solely on polygalacturonase [18–20, 27]. This biogeographical pattern is notable, given that land plants enrich for RG-I in response to drier growth conditions [63, 64]. Because it serves as an important anti-desiccant underlying plant cells' response to turgor changes, plants upregulate the synthesis of RG-I and its arabinan side chains during episodes of osmotic stress [65–69]. Where rainfall and humidity are key determinants of plant adaptation and distribution [70], rhamnogalacturonan lyase may allow beetles to more efficiently exploit temperate plants responding to sharper gradients of osmotic stress than those encountered in the tropics. In outlining an ecological context for the possible adaptive role of rhamnogalacturonan lyase in cassidines, we do not exclude the likelihood that complementary preadaptations may have also contributed to their expanded host plant range.

Following at least two independent horizontal gene transfer events [4], rhamnogalacturonan lyase is endogenously maintained by a subset of beetles belonging to the Buprestidae and Curculionidae families [4, 71–73]. Acquired horizontally from Actinobacteria (Buprestidae) and Proteobacteria (Curculionidae), the secondary pectinase is encoded by beetles specializing on host plants adapted for temperate or arid biomes [4], including pine [74], ash [75], date [76, 77], and citrus trees [78]. In parallel, a symbiosis with *Stammera* may have allowed certain cassidines to do the same. A similar interaction was recently described between cochineal scale insects and their β-proteobacterial symbiont, *Dactylopiibacterium* [79]. By encoding and expressing rhamnogalacturonan lyase and other pectinases, *Dactylopiibacterium* appears to be involved in the digestive capacity of cochineals subsisting exclusively on desert cactuses [79]. Thus, whether endogenously maintained or symbiotically supplemented, the secondary pectinase may be critical for insect herbivores overcoming host plants adapted for drier climates.

We note that our current study of this pectinolytic symbiosis does not extend to monocot-feeding, leaf-mining beetles. Previously a separate subfamily under the Hispinae, this group was recently reclassified as part of the Cassidinae [17]. Microbial

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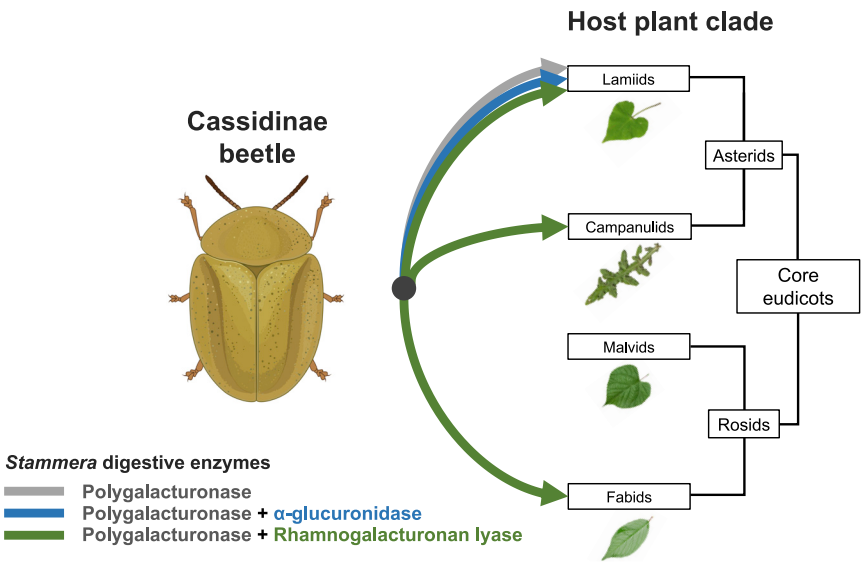

Figure 6. Cassidines Endowed with *Stammera*'s Polygalacturonase and Rhamnogalacturonan Lyase Have Radiated onto Host Plants Spanning a Wide Phylogenetic Range

Representative enzymatic combinations encoded by *Stammera* and the corresponding host plant range of cassidines. Eudicot phylogeny is adapted from Chase et al. [26].

profiling of these monocot-feeding taxa revealed the absence of *Stammera* and host symbiotic organs in *Cephaloleia* species [80], as well as in the palm pest *Octodonta nipae* [81]. This indicates that the symbiosis with *Stammera* is not shared by all members of the expanded subfamily. Toward understanding when *Stammera* was initially acquired, a broader sampling of monocot-feeding taxa is necessary and may offer new insights into the evolutionary transition and adaptation to eudicots within the Cassidinae.

The obligate symbioses insects form with micro-organisms serve as some of the most striking examples of beneficial partnerships in nature [82]. Endowing many key adaptations, these interactions are cited in spurring evolutionary innovation by expanding the availability of resources that may be evolutionarily exploited [83, 84]. Offsetting the loss of endogenous pectinases in cassidines, we demonstrate how polygalacturonase is a defining and consistent feature of the symbiosis with *Stammera*. Contrasting a conserved catalytic capacity against HG, cassidines additionally supplemented with rhamnogalacturonan lyase display a more-proficient digestive phenotype against RG-I. As the pectic heteropolymer is dynamically regulated by eudicots to contend with abiotic stress throughout development, we offer a framework for how a symbiont may modulate plant-herbivore interactions by expanding the range of universal plant cell wall polymers that its host can readily overcome.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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  - Enzymatic assays

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.cub.2020.05.043.

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AUTHOR CONTRIBUTIONS

H.S. and N.M.G. conceived of the study. H.S., N.M.G., D.W., R.K., Y.P., T.F., and A.B. designed the experiments. H.S., A.B., K.F., and M.M. performed genome sequencing, assembly, and analysis. R.K. and Y.P. carried out transcriptome sequencing, assembly, and analysis. R.K. and Y.P. performed the enzymatic assays. M.C. contributed insect specimens for initial assays. H.S. wrote the manuscript. All authors edited and commented on the paper.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and Virus Strains		
Candidatus Stammera capleta	This study and [10]	NCBI Taxonomy ID: 2608262
Biological Samples		
Cassida versicolor collected in Tsukuba, Japan	This paper	N/A
Cassida viridis collected in Jena, Germany	This paper	N/A
Cassida vibex collected in Jena, Germany	This paper	N/A
Cassida rubiginosa collected in Jena, Germany	This paper	N/A
Parachirida semiannulata collected in Gamboa, Panama	This paper	N/A
Ischnocodia annulus collected in Gamboa, Panama	This paper	N/A
Stolas discoides collected in Gamboa, Panama	This paper	N/A
Chelymorpha alternans collected in Gamboa, Panama	This paper	N/A
Acromis sparsa collected in Gamboa, Panama	This paper	N/A
Cistudinella foveolata collected in Gamboa, Panama	This paper	N/A
Discomorpha panamensis collected in Gamboa, Panama	This paper	N/A
Agroiconota porpinqua collected in Gamboa, Panama	This paper	N/A
Charidotella sexpunctata collected in Gamboa, Panama	This paper	N/A
Chemicals, Peptides, and Recombinant Proteins		
RNAlater	Sigma-Aldrich	Cat#R0901
TURBO DNase	Thermo Fisher Scientific	Cat#AM2238
TLC plates Silica gel 60	Merck	Cat#116835
rhamnogalacturonan I	Megazyme	Cat#P-RHAM1
Polygalacturonic acid	Megazyme	Cat#P-PGACT
Galacturonic acid	Santa Cruz	Cat#91510-62-2
di-galacturonic acid	Sigma-Aldrich	Cat#D4288
Tri-galacturonic acid	Sigma-Aldrich	Cat#T7407
rhamnose	Sigma-Aldrich	Cat#83650
Xylose with glucuronic acid decoration	Megazyme	Cat# O-XUX
Xylose	Sigma-Aldrich	Cat#47253
di-xylose	Megazyme	Cat#O-XBI
Tri-xylose	Megazyme	Cat#O-XTR
Glucuronic acid	Sigma-Aldrich	Cat#G5269
Critical Commercial Assays		
innuPrep DNA/RNA Mini kit	Analytik Jena	Cat# 845-KS-2080050
RNeasy MinElute Clean up Kit	Quiagen	Cat# 74204
RNA 6000 Nano LabChip kit	Agilent	Cat#5067-1511
QIAGEN RNA Extraction kit	QIAGEN	Cat# 74104
QIAGEN DNeasy Blood & Tissue Kit	QIAGEN	Cat# 69506
Taq DNA polymerase	VWR	Cat# 89167-762
Deposited Data		
Transcriptome sequencing Bioproject ID	This paper	NCBI:PRJNA561700
Genome sequencing Bioproject ID	This paper	NCBI:PRJNA561424

(*Continued on next page*)

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental Models: Organisms/Strains		
Cassida viridis collected in Jena, Germany	This paper	N/A
Cassida vibex collected in Jena, Germany	This paper	N/A
Cassida rubiginosa collected in Jena, Germany	This paper	N/A
Chelymorpha alternans collected in Gamboa, Panama	This paper	N/A
Software and Algorithms		
CLC Genomics Workbench v11.0	Quiagen	Cat#832001
Trimmomatic v0.36	[85]	http://www.usadellab.org/cms/index.php?page=trimmomatic
Trinity v2.5.1	[86]	https://github.com/trinityrnaseq/trinityrnaseq/releases/tag/Trinity-v2.5.1
Bowtie2	[87]	http://bowtie-bio.sourceforge.net/bowtie2/index.shtml
Spades	[88]	http://bioinf.spbau.ru/spades
GapFiller	[89]	https://www.baseclear.com/genomics/bioinformatics/basetools/gapfiller
RAST	[90]	http://rast.nmpdr.org
MUSCLE	[91]	http://phylogeny.lirmm.fr/phylo_cgi/one_task.cgi?task_type=muscle
PartitionFinder 2	[92]	http://www.robertlanfear.com/partitionfinder/
Jane 4	[28]	https://www.cs.hmc.edu/~hadas/jane/

RESOURCE AVAILABILITY

Lead Contact

Further information and requests for resources (e.g., available reference specimens), pectic substrates and commercial reagents should be directed to and will be fulfilled by the Lead Contact, Hassan Salem (hassan.salem@tuebingen.mpg.de).

Materials Availability

This study did not generate new unique reagents.

Data and Code Availability

Genomic and transcriptomic sequencing data generated in this study are available at the National Center for Biotechnology Information under BioProjects PRJNA561424 and PRJNA561700, respectively.

BioProjects can be accessed at:

PRJNA561424: https://dataview.ncbi.nlm.nih.gov/object/PRJNA561424?reviewer=24lm5sv3qppb252f3uuv0kt50e
PRJNA561700: https://dataview.ncbi.nlm.nih.gov/object/PRJNA561700?reviewer=i7r9617ob3a4a6ap4ot835degl

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Adult Cassidinae specimens were collected in Germany, Panama, the United States of America, Japan and New Zealand between 2016 and 2018. The insects were submerged in 70% ethanol until they were processed ahead of DNA extraction. Ten adults were included per sample. Representative specimens were identified based on morphological characters and their identity was confirmed by partial sequencing of the mitochondrial 16S rRNA gene, as well as the nuclear 18S and 28S rRNA genes. For enzymatic assays, *Cassida rubiginosa, Cassida vibex* and *Cassida viridis* were collected from leaves of *Cirsium arvense, Cirsium oleraceum* and *Mentha arvensis* in Jena, Germany; whereas *Chelymorpha alternans* are continuously reared at the Max Planck Institute for Chemical Ecology on *Ipomoea batatas*.

METHOD DETAILS

Symbiont genome sequencing, assembly, and annotation

Symbiotic organs were dissected from ten individuals of each of the 13 cassidine species, and DNA was purified using the QIAGEN DNeasy Blood & Tissue Kit (Hilden, Germany) according to the manufacturer's instructions. Sequencing was performed on a HiSeq 2500 Sequencing System from Illumina (https://www.illumina.com/systems/sequencing-platforms/hiseq-2500.html), utilizing the

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paired-end 150 bp technology and at a depth of ~40 million reads. Adaptor sequences were trimmed with Trimmomatic v0.03 using the default parameters [85]. To filter beetle-, plant-, and *Wolbachia*-associated sequences, the quality-controlled reads were mapped using Bowtie2 [87] to the publicly available sequences of closely related species. Assembly was performed using Spades under default parameters [88]. This resulted in ~40,000 contigs per assembly. After assembly, the contigs were screened for GC content and taxonomic identity to Enterobacteriaceae via BLAST to filter out remaining contaminant sequences. Contigs were then further assembled into scaffolds using GapFiller [89]. Resulting genomic scaffolds were subsequently connected and circularized by PCR and Sanger sequencing where necessary. The final genome sequences were automatically annotated using RAST [90] (as implemented in KBase [93]) and according to the genetic code 4 (TGA encoding tryptophan) to computationally translate the predicted protein-coding genes (Data S1).

Phylogenetic reconstruction

The phylogenetic relationships among the Cassidinae were reconstructed using a mitochondrial (16S RNA) and two nuclear genes (18S and 28S rRNA) for all host species. Toward inferring the relationships among the different *Stammera* strains, a set of reference alignments based on highly conserved Clusters of Orthologous Groups (COG) families was used to identify 30 protein-coding genes shared by all taxa. Host and symbiont genes were aligned using MUSCLE [91] ahead of concatenation. PartitionFinder 2 [92] was used to select the best partitioning scheme. Applying the greedy algorithm resulted in 3 and 28 partitions for host and symbiont alignments, respectively (Table S5). Maximum likelihood (ML) tree inference was performed using GARLI 2.0 [94] as implemented in Cipres Science Gateway [86]. The identified partitioning schemes were used in the ML analyses, and each subset was assigned the model suggested by PartitionFinder 2. For each analysis, branch support was estimated following 1,000 bootstrap replicates.

The phylogenetic reconciliation tool Jane 4 [28] was applied to describe the coevolutionary association between Cassidinae beetles and *Stammera*, using default cost settings. This analysis leverages five evolutionary scenarios to characterize host-symbiont interactions: cospeciation (joint speciation with the host lineage); duplication (both symbionts are kept in the same host); duplication and host switch (symbionts are duplicated and transferred from one host species to another); losses (loss of symbiont); and failure to diverge. Accounting for possible vector combinations, our optimal reconstructions reported a total vector cost of 8. The statistical significance of the total cost was tested using the null distribution of cost values based on 1,000 randomly generated trees. Following these randomizations, 100% had a total vector cost value higher than that of our predicted results, indicating that the obtained reconstructions were not randomly attributed.

Transcriptome sequencing, assembly, and annotation

Dissected beetle midguts from six representative cassidines were preserved in RNAlater. Preserved midguts were extracted, snap-frozen in liquid nitrogen and stored at −80°C. Total RNA was extracted from three beetles using the innuPrep DNA/RNA Mini kit (Analytik Jena, Jena, Germany) following the manufacturer's instructions. Genomic DNA contamination was removed by DNase treatment (TURBO DNase, Invitrogen, Carlsbad, CAL, USA) for 30 min at 37°C. Total RNA samples were further purified by using the RNeasy MinElute Clean up Kit (QIAGEN, Hilden, Germany) following the manufacturer's protocol, except for elution to 20 μL volume. The integrity and quality of the RNA samples were determined using the RNA 6000 Nano LabChip kit on an Agilent 2100 Bioanalyzer (both Agilent Technologies, Santa Clara, CAL, USA) according to the manufacturer's instructions.

RNA-Seq was performed at the Max Planck Genome Center (Cologne, Germany). There, poly(A)$^+$ enriched RNA was fragmented to an average size of 300-350 nucleotides and a TruSeq compatible, directional library was prepared for each sample. Libraries were tagged using dual indexing and were multiplexed on the same sequencing lane. Sequencing was carried out on a HiSeq3000 sequencing platform (Illumina, CA, USA) using paired-end (2 × 150 bp) reads. Quality control measures, including the filtering of high-quality reads based on fastq file scores, the removal of reads containing primer/adaptor sequences and trimming of the read length, were carried out using CLC Genomics Workbench v11.0 (QIAGEN, Hilden, Germany). Multiple assemblies were performed for each sample whereby an increasing number of randomly selected read pairs were included per assembly (Table S4).

The RNA-Seq data for *Chelymorpha alternans* were generated in a similar way, except that the library was tagged using mono indexing and was multiplexed with other beetle-derived libraries on the same sequencing lane. Illumina-specific adapters, tags and low-quality bases were removed using Trimmomatic v0.36. The resulting clean reads were assembled using Trinity v2.5.1 using default parameters. We faced problems of cross-contamination of the sequencing data after de-multiplexing, which made subsequent analyses difficult. We cured these RNA-Seq data from cross-contamination using the protocol described by Peters et al. [95]. Briefly, we compared the *C. alternans* transcriptome assembly with all other assemblies sequenced in the same run using BLASTN. The coverage depth of BLASTN identified transcripts that shared nucleotide sequence identity of at least 98% over a length of at least 180 bp was then compared. If the relative coverage of two transcripts originating from two different assemblies differed >2-fold, the transcript with the lower relative coverage was assumed to be a contaminant and was removed from the corresponding assembly. Plant cell wall degrading enzymes (PCWDEs) were annotated in the assembled transcriptomes by BLAST-searches using representative PCWDE sequences from insects, nematodes, bacteria, and fungi, and as previously described in Kirsch et al. [7] and Busch et al. [46].

Enzymatic assays

Qualitative analysis of breakdown products was performed by thin layer chromatography (TLC) of 20 μL enzyme assays set up as follows: 14 μL of crude gut extract and pooled from *Cassida rubiginosa*, *Cassida vibex, Cassida viridis* or *Chelymorpha alternans*

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(three beetles each) were incubated with either 0.2% polygalacturonic acid, rhamnogalacturonan I or xylotriose linked with glucuronic acid in 20 mM citrate/phosphate buffer pH 5.0 at 40°C for 16 h. The whole assay volumes were used for TLC afterward. Samples were applied to TLC plates (Silica gel 60, 20 cm, Merck) in 4 μL steps, and enzymatic breakdown products were separated using the following mobile phase: ethyl acetate:glacial acetic acid:formic acid:water (9:3:1:4) for approximately 60 min. After drying, carbohydrates were stained by spraying the plates with a solution containing 0.2% (w/v) orcinol in methanol:sulfuric acid (9:1), followed by a short heating until spots appeared. The reference standard either contained 2 μg each of galacturonic, di-galacturonic, tri-galacturonic acid and rhamnose, or 2 μg each of xylose, di-xylose, tri-xylose and glucuronic acid.

Current Biology, Volume *30*

Supplemental Information

Symbiont Digestive Range Reflects Host Plant Breadth in Herbivorous Beetles

Hassan Salem, Roy Kirsch, Yannick Pauchet, Aileen Berasategui, Kayoko Fukumori, Minoru Moriyama, Michael Cripps, Donald Windsor, Takema Fukatsu, and Nicole M. Gerardo

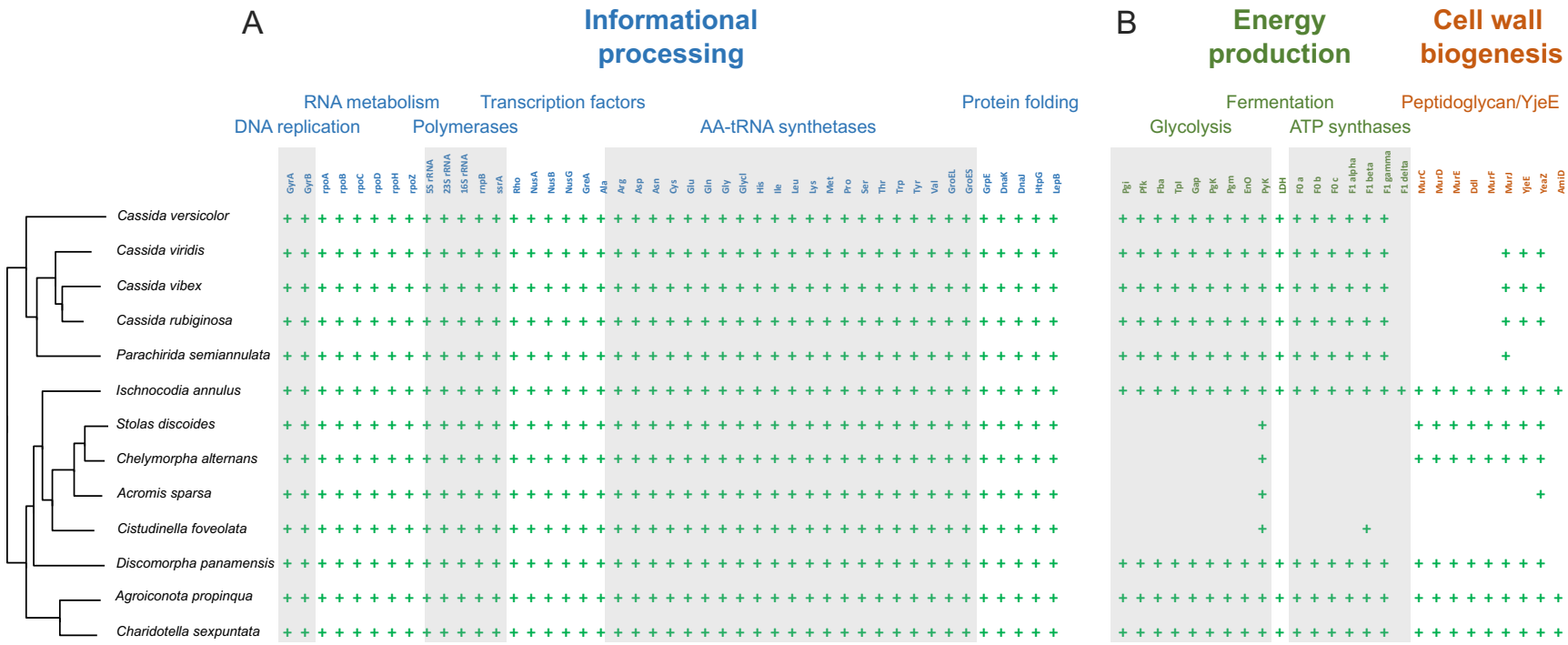

Figure S1. List of protein-coding and RNA genes implicated in (A) informational processing (blue), (B) energy production (green), and cell wall biosynthesis (orange) in the genomes of *Stammera.* Related to Figure 2. Symbol (+) indicates the presence of an encoding gene within the symbiont's genome. Symbiont phylogeny, as in Figure 1, is based on ML analysis of a concatenated nucleotide alignment for 30 genes shared by 13 *Stammera* genomes.

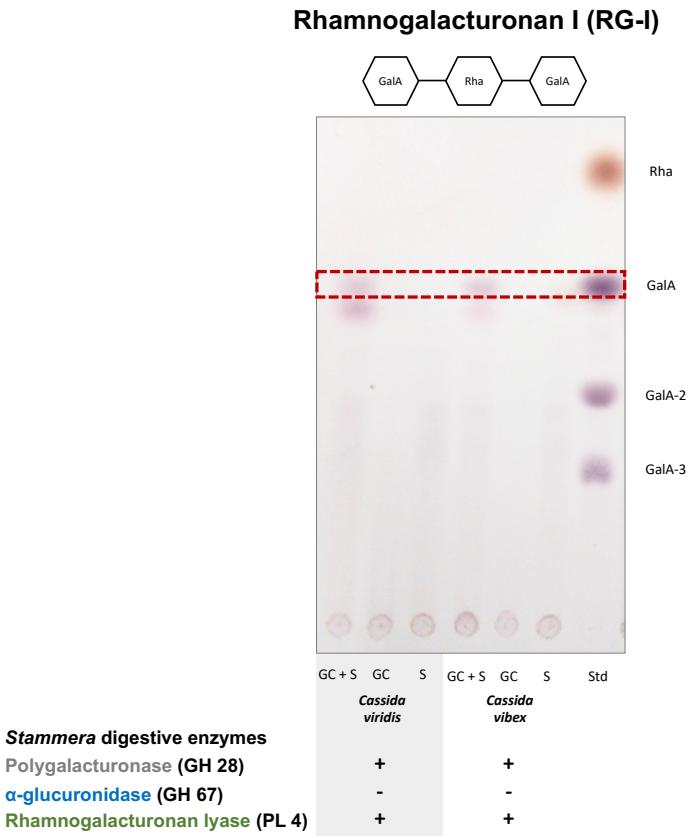

Figure S2. Digestive activity against rhamnogalacturonan I (RGI) is conserved across cassidines endowed with *Stammera*'s rhamnogalacturonan lyase. Related to Figure 4. Thin layer chromatogram (TLC) illustrating the breakdown products of pectinase activity in *Cassida viridis* and *Cassida vibex* assays RG-I. Abbreviations: GC, gut content; S, substrate; Std., standard; Gal, mono-galacturonic acid; Gal-2, di-galacturonic acid; Gal-3, tri-galacturonic acid; Rha, rhamnose; GH, glucoside hydrolase; PL, polysaccharide lyase. Dashed box highlights Gal differences across treatments.

Coevolutionary events					Vector cost
# Cospeciations	# Duplications	# Duplications & host switches	# Losses	# Failure to diverge	
9	0	3	2	0	8

Table S1. **Results of cospeciation analysis performed in Jane 4 indicating the number of inferred events of each type that best explain the relationship between Cassidinae beetles and *Stammera* using the default cost scheme. Related to Figure 1.**

Beetle host	Size (bp)	Plasmids	AT Content (%)	ORFs	Ribosomal RNAs	Transfer RNAs
Cassida versicolor	267,621	2	16.2	279	3	29
Cassida viridis	266,618	1	16.0	280	3	29
Cassida vibex	268,511	1	15.2	281	3	29
Cassida rubiginosa	271,175	0	15.4	271	3	29
Parachirida semiannulata	251,963	2	15.2	273	3	29
Ischnocodia annulus	288,199	1	15.2	303	3	29
Stolas discoides	241,886	1	14.2	254	3	28
Chelymorpha alternans	249,326	1	15.6	262	3	29
Acromis sparsa	215,782	1	13.9	236	3	29
Cistudinella foveolata	273,119	1	15.4	287	3	29
Discomorpha panamensis	291,581	1	16.6	309	3	28
Agroiconota propinqua	290,077	1	17.2	309	3	29
Charidotella sexpuntata	310,648	1	18.0	327	3	29

Table S2. General genomic features of *Stammera* determined in this study. Related to Figure 2.

	Cellulases				Pectinases			Hemicellulases			
Beetle species	**GH 9**	**GH 45**	**GH 48**	**GH 5_2**	**GH 28**	**CE 8**	**PL 4**	**GH 10**	**GH 11**	**GH 5_8**	**GH 5_10**
Cassida rubiginosa	✚	✚	✚					✚			
Parachirida semiannulata	✚	✚	✚					✚			
Ischnocodia annulus	✚	✚	✚					✚			
Chelymorpha alternans	✚	✚	✚					✚			
Acromis sparsa		✚	✚					✚			
Cistudinella foveolata	✚	✚	✚					✚			
Discomorpha panamensis	✚	✚	✚					✚			

Table S3. Distribution of endogenous Plant Cell Wall Degrading Enzymes (PCWDEs) in cassidine leaf beetles following transcriptomic profiling. Symbol indicates the presence (✚) of a gene. Abbreviations: GH, glycoside hydrolase; CE, carbohydrate esterase; PL, polysaccharide lyase. Related to Figure 3.

Beetle species	Number of reads (bp)	Average read length (bp)	Number of contigs	N50
Parachirida semiannulata	56,000,884	149.33	30,460	1,468
Ischnocodia annulus	85,215,626	149.88	46,903	1,491
Chelymorpha alternans	68,384,501	149.50	24,280	2,105
Acromis sparsa	87,678,778	149.79	43,563	1,836
Cistudinella foveolata	84,597,262	150.13	43,664	2,013
Discomorpha panamensis	87,668,350	150.14	33,764	1,648

Table S4. **Assembly statistics of cassidine transcriptomes generated in this study**. **Related to Figure 3.**

Alignment	Subset	Partitions by nucleotide position	Best model
Host	1	1-526, 2-526, 3-526	GTR+I+G
	2	527-2375, 528-2375, 529-2375	GTR+I+G
	3	2376-3032, 2377-3032, 2378-3032	GTR+I+G
Stammera	1	1-2900, 10013-10403	GTR+I+G
	2	2-2900	GTR+I+G
	3	3-2900	GTR+I+G
	4	2901-5313	GTR+G
	5	2902-5313	GTR+I+G
	6	2903-5313	GTR+G
	7	6751-7580, 5314-6749, 11946-13273	GTR+I+G
	8	11423-11943, 11944-13273, 5315-6749, 11945-13273	GTR+I+G
	9	9635-10010, 5316-6749	GTR+G
	10	6750-7580	GTR+I+G
	11	16178-16547, 15773-16175, 16550-16853, 6752-7580	GTR+I+G
	12	7581-8222	GTR+G
	13	15062-15458, 8225-8821, 7582-8222, 9633-10010, 10011-10403	GTR+G
	14	7583-8222, 10012-10403, 9634-10010	GTR+I+G
	15	8223-8821, 8823-9260	GTR+I+G
	16	8224-8821	GTR+I+G
	17	8822-9260	GTR+G
	18	8824-9260	GTR+G
	19	15459-15770, 14577-15059, 9261-9632, 14028-14576	GTR+I+G
	20	16177-16547, 15772-16175, 14029-14576, 15061-15458, 9262-9632, 14578-15059	GTR+I+G
	21	14027-14576, 9263-9632, 13276-14026, 14579-15059	GTR+G
	22	10404-11420, 10406-11420	GTR+G
	23	10405-11420	GTR+G
	24	11421-11943, 15060-15458	GTR+G
	25	11422-11943, 13275-14026,16549-16853, 15460-15770	GTR+I+G
	26	13274-14026, 15771-16175	GTR+G
	27	15461-15770	GTR+G
	28	16548-16853, 16176-16547	GTR+I+G

Table S5. Partitioning schemes and the best-fitting models identified by PartitionFinder 2. Related to STAR Methods.