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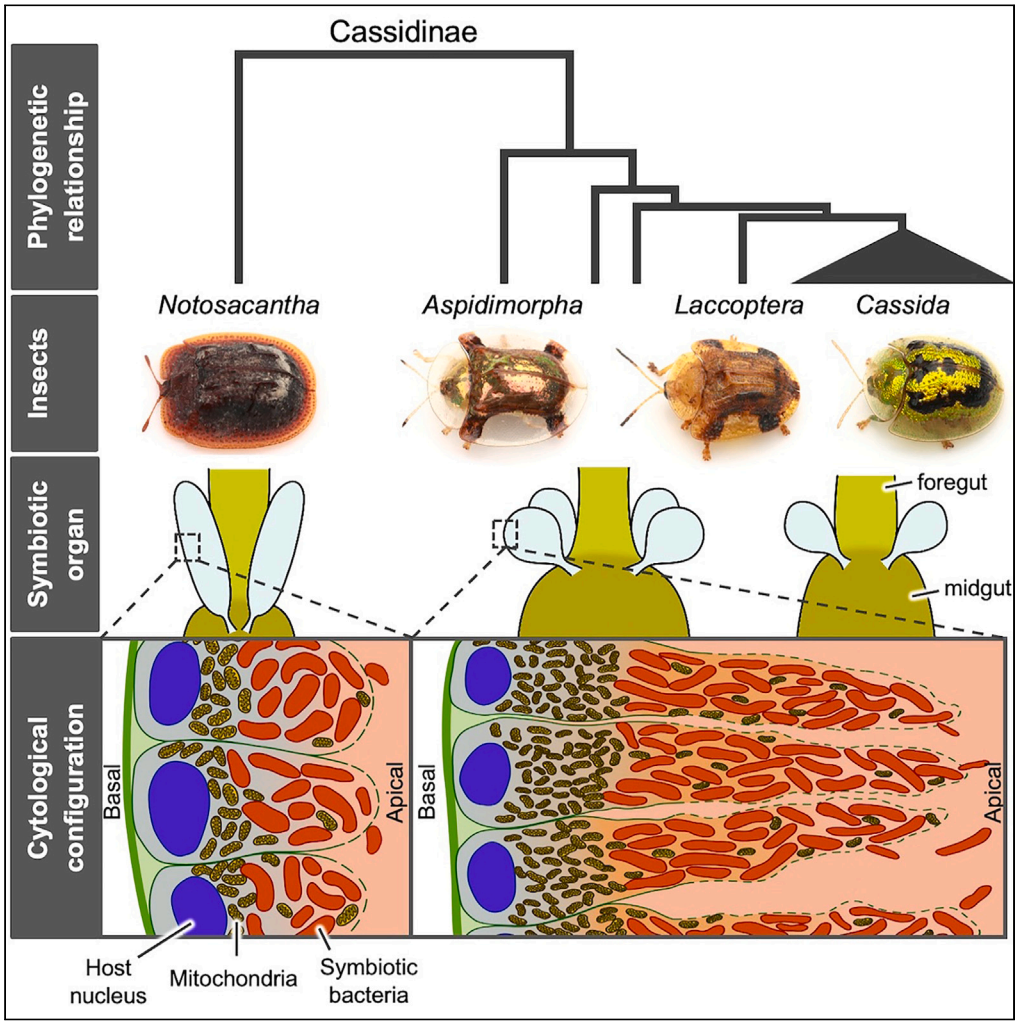


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## Highlights

Pectin-degrading leaf beetle symbionts show both intra and extracellular locations

In the symbiotic organ, a dense mitochondrial zone is adjacent to symbiotic region

Contractile symbiotic organ may drive pectinase/symbiont excretion into gut cavity

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# Intracellularity, extracellularity, and squeezing in the symbiotic organ underpin nurturing and functioning of bacterial symbiont in leaf beetles

Kohei Oguchi,[1,2,10,*] Toshiyuki Harumoto,[3,4] Tatsuya Katsuno,[5,6] Yu Matsuura,[7] Soma Chiyoda,[2] and Takema Fukatsu[1,8,9,*]

## SUMMARY

**Cassidine leaf beetles are associated with genome-reduced symbiotic bacteria *Stammera* involved in pectin digestion. *Stammera* cells appear to be harbored in paired symbiotic organs located at the foregut-midgut junction either intracellularly or extracellularly, whereas the symbiont is extracellular in the ovary-accessory glands of adult females and during caplet transmission in eggs. However, using fluorescence and electron microscopy, an intracellular symbiotic configuration of *Stammera* was observed in *Notosacantha* species. Detailed inspection of other cassidine species revealed fragmented cell membrane and cytoplasm of the symbiotic organs, wherein *Stammera* cells are in an intermediate status between intracellularity and extracellularity. We also identified a mitochondria-rich region adjacent to the symbiont-filled region and well-developed muscle fibers surrounding the whole symbiotic organ. Based on these observations, we discuss why the *Stammera* genome has been reduced so drastically and how symbiont-derived pectinases are produced and supplied to the host's alimentary tract for plant cell wall digestion.**

## INTRODUCTION

Microbial symbioses are ubiquitous phenomena that contribute to the evolution of diverse organisms as a source of novel adaptive traits.[1,2] Insects represent one of the most diverse groups in the terrestrial ecosystem, for which microbial symbionts play important biological roles.[3,4] Some symbionts are essential for growth, survival, and reproduction of their hosts by supplying essential nutrients,[5,6] assisting food digestion,[7,8] and defense against natural enemies.[9,10] In such intimate symbiotic associations, the host insects often develop specialized cells and organs to accommodate the microbial symbionts.[11–13] Some insects retain their symbiotic bacteria extracellularly within the inner cavity of specialized structures associated with their alimentary tract, called crypts or gastric caeca, as in stinkbugs, fruit flies, leaf beetles, etc.[3,14–16] Other insects harbor their symbiotic bacteria intracellularly within the cytoplasm of specialized cells and organs, called bacteriocytes and bacteriomes, as in aphids, weevils, tsetse flies, ants, etc.[3,17–20] The emergence of such sophisticated forms of symbiosis is considered to represent some of the major events in the organismal evolution.[21–24] In this context, evolutionary and developmental connections between extracellular symbiosis and intracellular symbiosis are of interest.

Tortoise leaf beetles (Coleoptera: Chrysomelidae) comprise one of the largest leaf beetle groups, the subfamily Cassidinae.[25] Recent studies have shown that cassidine leaf beetles are generally associated with specific symbiotic bacteria, "*Candidatus* Stammera capleta" (hereafter abbreviated as *Stammera*), whose genomes are extremely reduced to around 0.2–0.3 Mb and streamlined for producing pectinases to help host's digestion of plant cell wall.[26–29] Drastic genome reduction down to 0.2–0.3 Mb has been observed among some ancient intracellular insects symbionts, but this level of extreme genome reduction is exceptional among extracellular insect symbionts.[30–32] Previous anatomical and histological studies have identified the following symbiont localizations in cassidine leaf beetles: one or two pairs of symbiotic organs at the foregut-midgut junction of the digestive tract; paired female-specific tubular organs connecting to the reproductive organ for symbiont transmission; and caplets attached on the anterior pole of eggs that newborn larvae consume

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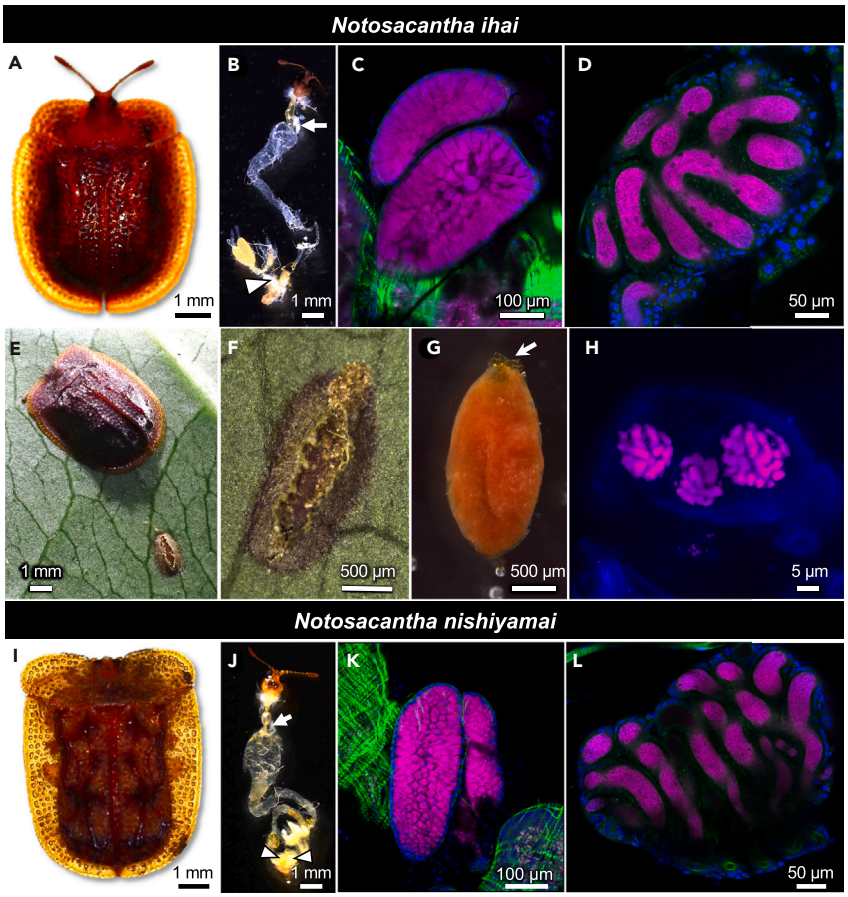


**Figure 1. Morphology, ecology, and symbiotic system of *Notosacantha* leaf beetles**

(A–H) *Notosacantha ihai*. (I–L) *Notosacantha nishiyamai*. (A, I) Adult insects. (B, J) Alimentary tracts dissected from adult females. Arrows show the gut-associated symbiotic organs located at the foregut-midgut junction, whereas arrowheads show the female-specific genital accessory organs for vertical symbiont transmission. (C, K) FISH detection of the symbiotic bacteria in the gut-associated symbiotic organ. (D, L) FISH detection of the symbiotic bacteria in the female-specific transmission organ. (E) An adult female and an egg on the host plant leaf. (F) Enlarged image of the egg inside the leaf crevice. (G) An isolated egg. Arrow indicates symbiont caplets on the anterior pole of the egg. (H) FISH detection of the symbiotic bacteria in the caplets. In (C), (D), (H), (K), and (L), magenta, blue, and green signals show FISH signals targeting symbiont 16S rRNA, DAPI signals targeting host nuclear DNA, and fluorescent phalloidin signals targeting muscular actin filaments, respectively.

to acquire the symbiont.[26,28,33] In the female-specific transmission organs and the egg-associated caplets, the symbiotic bacteria are extracellular for vertical transmission.[26,27,33–35] On the other hand, it has been somewhat unclear whether the symbiotic bacteria in the gut-associated symbiotic organs are either extracellular or intracellular. Early histological observations reported that the symbiont cells are localized to the inner cavity of the symbiotic organs, where the symbiont cells seem associated with the host's degenerate cytoplasm protruding to the inner cavity of the symbiotic organ.[33] Meanwhile, considering that the *Stammera* genomes lack most genes encoding protein secretion machineries except for SecA and CorA,[26,27] the symbiont-produced pectinases may be excreted to host's gut cavity via not only the molecular machineries but also some cellular processes. In recent years, *Stammera* has been mainly regarded as an extracellular symbiont.[16,26–29,33–35]

Leaf beetles of the genus *Notosacantha* constitute an early diverging lineage of the Cassidinae[29] with peculiar biological and ecological properties such as feeding on evergreen tree leaves and larval leaf-mining lifestyle (Figures 1 and S1).[36] In this study, by employing fine cytological and microscopic techniques, we found that in *Notosacantha* species, the *Stammera* cells are intracellular in the apical cytoplasmic region of the epithelial cells of the symbiotic organ. By applying the same cytological and microscopic techniques to the common cassidine leaf beetles representing the genera *Laccoptera*, *Aspidimorpha*, and *Cassida*, we found that the *Stammera* cells appear to exhibit intracellular and extracellular patterns. We also identified remarkable subcellular differentiation of the epithelial cells of the symbiotic organ: the nucleated and symbiont-free basal region; the mitochondria-rich subapical region; and the symbiont-filled apical region whose cytoplasm protrudes to the inner cavity of the symbiotic organ. Based on these findings, we discuss functional, developmental, and evolutionary relevance of the cassidine-*Stammera* symbiosis entailing both intracellular and extracellular associations.

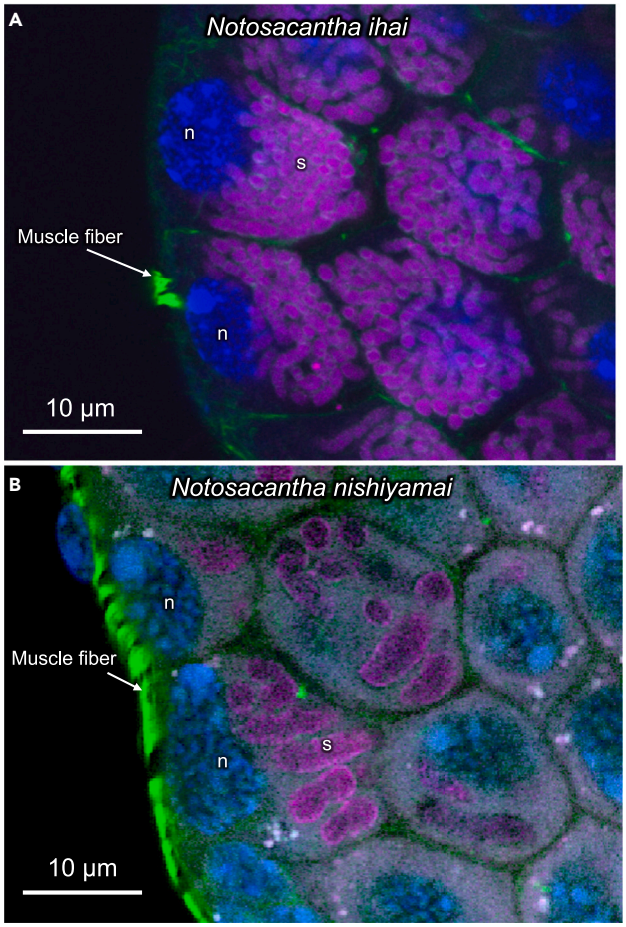


**Figure 2. Intracellular symbiont localization in the symbiotic organ of *Notosacantha* leaf beetles**

FISH visualization of the nucleated and symbiont-free basal region and the symbiont-harboring apical region of the epithelial cells of the gut-associated symbiotic organ of *Notosacantha ihai* (A) and *Notosacantha nishiyamai* (B). Magenta, blue, and green signals show FISH signals targeting symbiont 16S rRNA, DAPI signals targeting host nuclear DNA, and fluorescent phalloidin signals targeting muscular actin filaments, respectively. n, nucleus; s, symbiont.

## RESULTS

### *Stammera* localization in *Notosacantha* species

The localization of *Stammera* was investigated in *Notosacantha ihai* (Figures 1A–1H) and *Notosacantha nishiyamai* (Figure 1I–1L). As shown in our previous work,[28] FISH signals of the symbiont cells were detected in gut-associated paired symbiotic organs at the foregut-midgut junction (Figures 1C and 1K). Specifically in adult females, FISH signals of *Stammera* were also detected in transmission organs associated with the base of the ovary (Figures 1D and 1L). Although larvae of most cassidine species settle on and graze the leaf surface (Figures S1A–S1F), larvae of *Notosacantha* species are, exceptionally, leaf miners that live inside the leaf and consume the internal leaf tissues (Figures S1G–S1I). Accordingly, although adult females of most cassidine species attach their eggs encased in chitinous secretions on the leaf surface (Figures S1C and S1F), adult females of *Notosacantha* species lay their eggs into grazed crevices bored on the leaves (Figures 1E, 1F, and S1I). When the eggs were carefully taken out of the leaf crevices and observed, translucent structures called "caplets" were identified on the anterior pole of the eggs (Figure 1G), in which FISH signals of the symbiotic bacteria were detected (Figure 1H).

### Endosymbiotic features in the symbiotic organ of *Notosacantha* species

In the FISH images, we recognized compartment-like structures within the gut-associated symbiotic organ of the *Notosacantha* species (Figure 2), which prompted us to investigate the internal structure of the symbiotic organ in detail using fine histological and optical sectioning techniques (Figure 3). In adults of both *N. ihai* and *N. nishiyamai*, we observed that (1) muscular cells constituted the outermost layer of the symbiotic organ (Figures 3B and 3H), (2) the basal region of the epithelial cells contained a nucleus, attached to neighboring cells, and thereby constituted the wall of the symbiotic organ together with the muscular layer (Figures 3C–3F, 3I, and 3L), and (3) the apical region of the epithelial cells protruded to and filled up the inner cavity of the symbiotic organ, which were densely populated by *Stammera* cells (Figures 3D, 3E, 3J, and 3K). These observations suggested that the epithelial cell of the symbiotic organ of *Notosacantha* species may be differentiated into

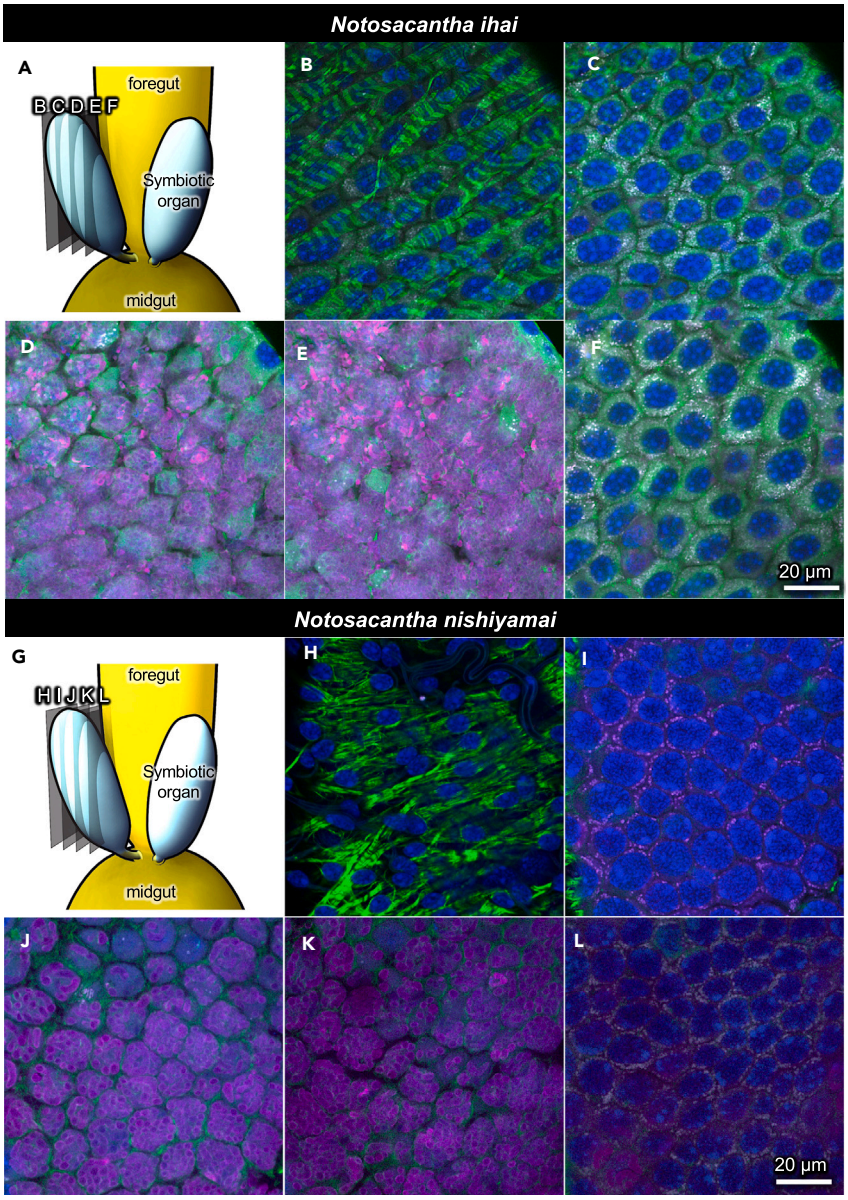


**Figure 3. Confocal optical sections of the gut-associated symbiotic organs of *Notosacantha* leaf beetles**

(A–F) *Notosacantha ihai*. (G–L) *Notosacantha nishiyamai*. (A, G) Schematic diagrams showing the observation image planes in the symbiotic organ. The symbiotic organ was gently compressed between a glass slide and a coverslip, and optical horizontal section images were retrieved continuously.

(B and H) Outermost sections of the symbiotic organ, in which actin fibers of the muscular cells (green) and nuclei of the basal region of the epithelial cells (blue) are seen.

(C and I) Slightly inner sections of the symbiotic organ, in which host cell nuclei (blue) are arranged in the basal region of the epithelial cells constituting the wall of the symbiotic organ, whereas no symbiotic bacteria are detected.

(D and J) Deeper sections of the symbiotic organ, in which the apical cytoplasmic regions of the epithelial cells are full of symbiotic bacteria (magenta) and devoid of host cell nuclei. (E, K) Sections of the opposite side of the symbiotic organ corresponding to (D, J). (F, L) Sections of the opposite side of the symbiotic organ corresponding to (C, I). In (B–F) and (H–L), magenta, blue, and green signals show FISH signals targeting symbiont 16S rRNA, DAPI signals targeting host nuclear DNA, and fluorescent phalloidin signals targeting muscular actin filaments, respectively.

two distinct regions, namely the nucleated and symbiont-free basal region and the symbiont-harboring apical region protruding toward the inner cavity. Similar cytological configuration was also observed in the gut-associated organ of larvae of *N. ihai* (Figure S2A). TEM observations of the epithelial cells of the symbiotic organ of *N. ihai* uncovered that (1) pleomorphic *Stammera* cells were intracellularly packed in the apical region (Figures 4A and S3A), (2) the basal nucleated region was symbiont-free and entailed a zone with numerous mitochondria adjacent to the *Stammera*-filled apical region (Figures 4B and S3A), (3) *Stammera* cells were often closely associated with mitochondria in the host

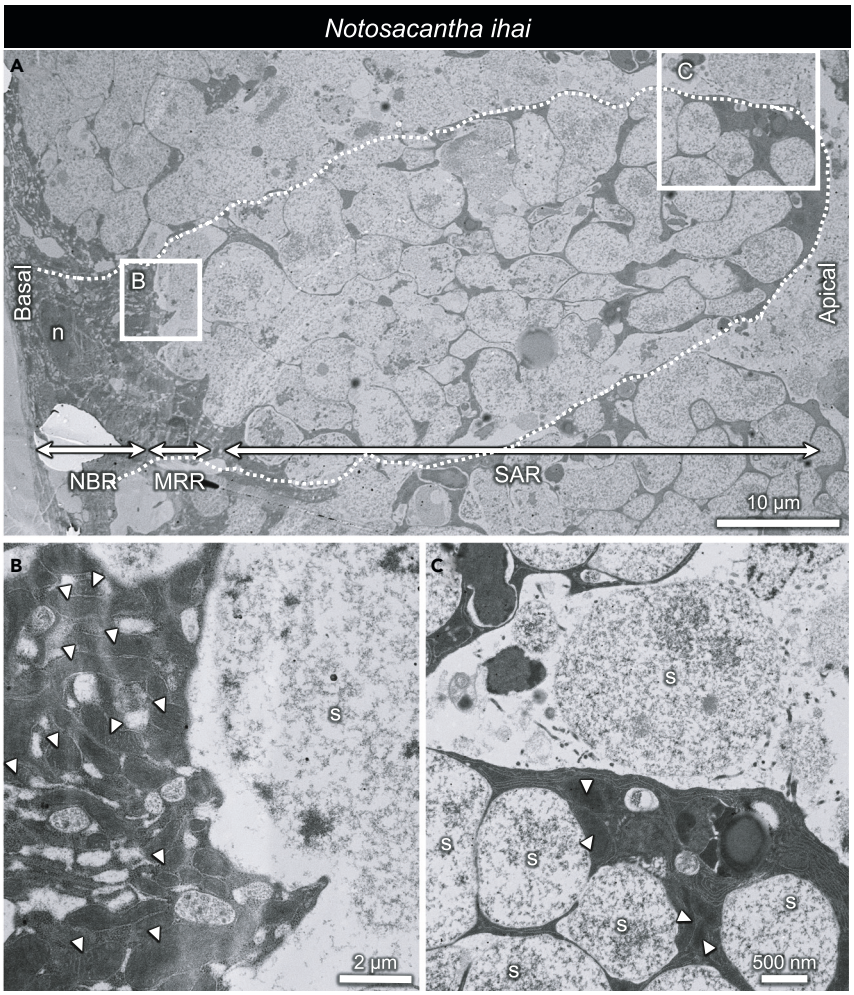


**Figure 4. TEM images of ultrathin tissue sections of the gut-associated symbiotic organ of *Notosacantha ihai***

(A) The whole image of the epithelial cell, in which the nucleated and symbiont-free basal region (NBR), the mitochondria-rich and symbiont-free region (MRR), and the symbiont-filled voluminous apical region (SAR) are seen. A dotted circle highlights an epithelial cell of the symbiotic organ, whereas squares indicate the areas enlarged in (B) and (C).

(B) An enlarged image of the interface between the symbiont-free basal region and the symbiont-harboring apical region, in which a large number of mitochondria (arrowheads) concentrate, thereby constituting the MRR.

(C) An enlarged image of the tip of the SAR, in which the symbiont cells are evidently within the host cytoplasm in association with mitochondria (arrowheads). Note that there are also extracellular symbiont cells. n, nucleus; s, symbiont.

cytoplasm (Figures 4C and S3A), and (4) *Stammera* cells were also detected in the cavity of the symbiotic organ extracellularly (Figure 4C). These observations indicated that, in the gut-associated symbiotic organ of *Notoscantha* species, the epithelial cells are differentiated into the symbiont-free basal region, the mitochondria-rich region, and the symbiont-filled apical region, in the last of which *Stammera* cells are mainly localized within the cytoplasm intracellularly.

## Endosymbiotic features in the symbiotic organ of other cassidine species

Next, we conducted detailed FISH observations of the gut-associated symbiotic organ of adult insects of *Laccoptera nepalensis* (Figure 5A), *Aspidimorpha difformis* (Figure 5B), *Cassida circumdata* (Figure 5C), and *Cassida versicolor* (Figure 5D), which phylogenetically form the most species-rich clade in the Cassidinae.[28] In all the species, the epithelial cells of the symbiotic organ were differentiated into two distinct regions, namely the nucleated and symbiont-free basal region and the symbiont-harboring apical region protruding toward the inner cavity, but the cellular boundaries of the apical region looked somewhat obscure (Figure 5). In larvae of *L. nepalensis*, similar cytological configuration was observed (Figure S2B). TEM observations of the epithelial cells of the symbiotic organ of adult *L. nepalensis* uncovered that the cell membrane, and the cytoplasm of the apical region was highly degenerate and, consequently, the *Stammera* cells looked almost extracellular, although remnant cell membranes and mitochondria were associated with them by which the integrity of the apical region seemed to be

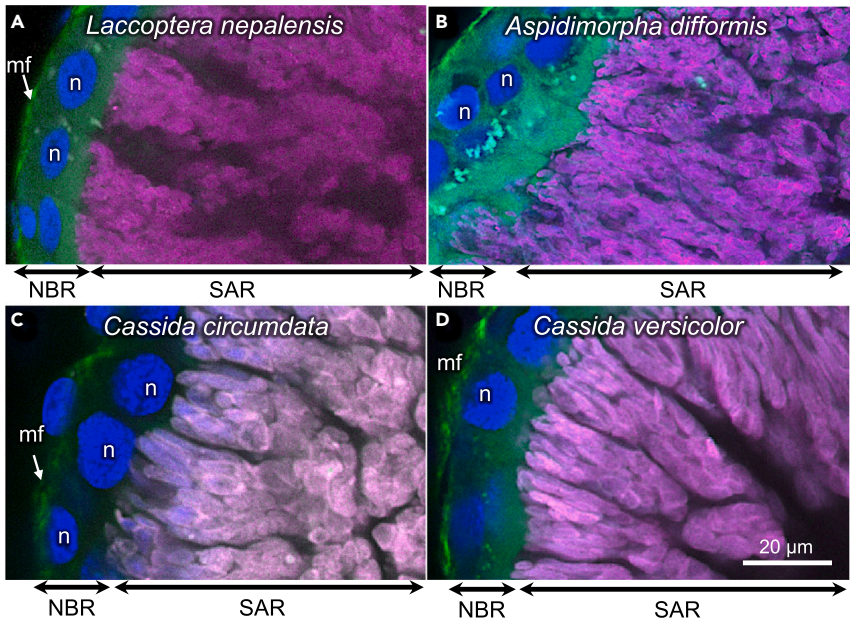


**Figure 5. FISH visualization of the nucleated and symbiont-free basal region and the symbiont-harboring apical region of the epithelial cells of the gut-associated symbiotic organ of common cassidine leaf beetles**

(A) *Laccoptera nepalensis*. (B) *Aspidimorpha difformis*. (C) *Cassida circumdata*.

(D) *Cassida versicolor*. Magenta, blue, and green signals show FISH signals targeting symbiont 16S rRNA, DAPI signals targeting host nuclear DNA, and fluorescent phalloidin signals targeting muscular actin filaments, respectively. n, nucleus; mf, muscle fiber; NBR, nucleated and symbiont-free basal region; SAR, symbiont-filled voluminous apical region.

maintained (Figures 6A–6D, 6F, and S3B; Video S1). The basal region contained a nucleus and rough ER, and strikingly, a very dense population of mitochondria occupied the region between the nucleated basal region and the symbiont-harboring apical region, thereby constituting a mitochondria-rich subapical region (Figures 6A, 6B, 6E, and S3B; Video S1). Here, it should be noted that the degenerative features of the cell membrane and the cytoplasm of the apical region may vary among individuals (Figure S4). Similar ultrastructural traits were also observed in the gut-associated symbiotic organ of *A. difformis* (Figure S5). On the basis of these observations, we suggest that, in the gut-associated symbiotic organ of typical cassidines of the genera *Laccoptera*, *Aspidimorpha*, and *Cassida*, the epithelial cells are also differentiated into the symbiont-free basal region, the mitochondria-rich subapical region, and the symbiotic apical region, in the last of which *Stammera* cells appear to be localized both extracellularly and intracellularly. Here, it is also notable that the intracellular-extracellular *Stammera* cells often exhibit pleomorphic and degenerative cytology.

### Musculature surrounding the symbiotic organ of cassidine species

In observing the phalloidin-stained symbiotic organs of *Notosacantha* species, we discovered a unique arrangement of muscle fibers wrapping around the whole organ on the outermost layer (Figures 7A and 7B). Similar muscular arrangement was also observed on the symbiotic organs of *L. nepalensis* and *A. difformis* (Figures 7C and 7D). This muscular arrangement strongly suggested that muscular contraction of the whole gut-associated symbiotic organ expels the content of the inner cavity, namely symbiont cells and symbiont-produced pectinases, into the main tract of the gut lumen that contains leaf fragments to be digested.

## DISCUSSION

Previous histological studies reported that, in the life cycle of cassidine leaf beetles, although being unequivocally extracellular in the female-specific transmission organs and the egg-associated caplets, it has been obscure whether *Stammera* is either extracellular or intracellular in the gut-associated symbiotic organs.[26,28,33] In this study, we conducted detailed histological and cytological observations of the symbiotic system of diverse cassidine species, which enabled us to gain some insight into functional, physiological, and evolutionary aspects of the cassidine-*Stammera* association.

Why are the symbiotic organs located at the foregut-midgut junction? Considering that the primary biological function of *Stammera* is the pectinase production to assist host's digestion of plant cell wall in the digestive tract,[26,27] it seems quite likely, though speculative, that *Stammera* is evolutionarily derived from a gut bacterial associate and thus was originally extracellular. In this context, the location of the symbiotic organs as symbiont reservoirs at the foregut-midgut junction makes sense in that the symbiotic bacteria are stably maintained in the upstream

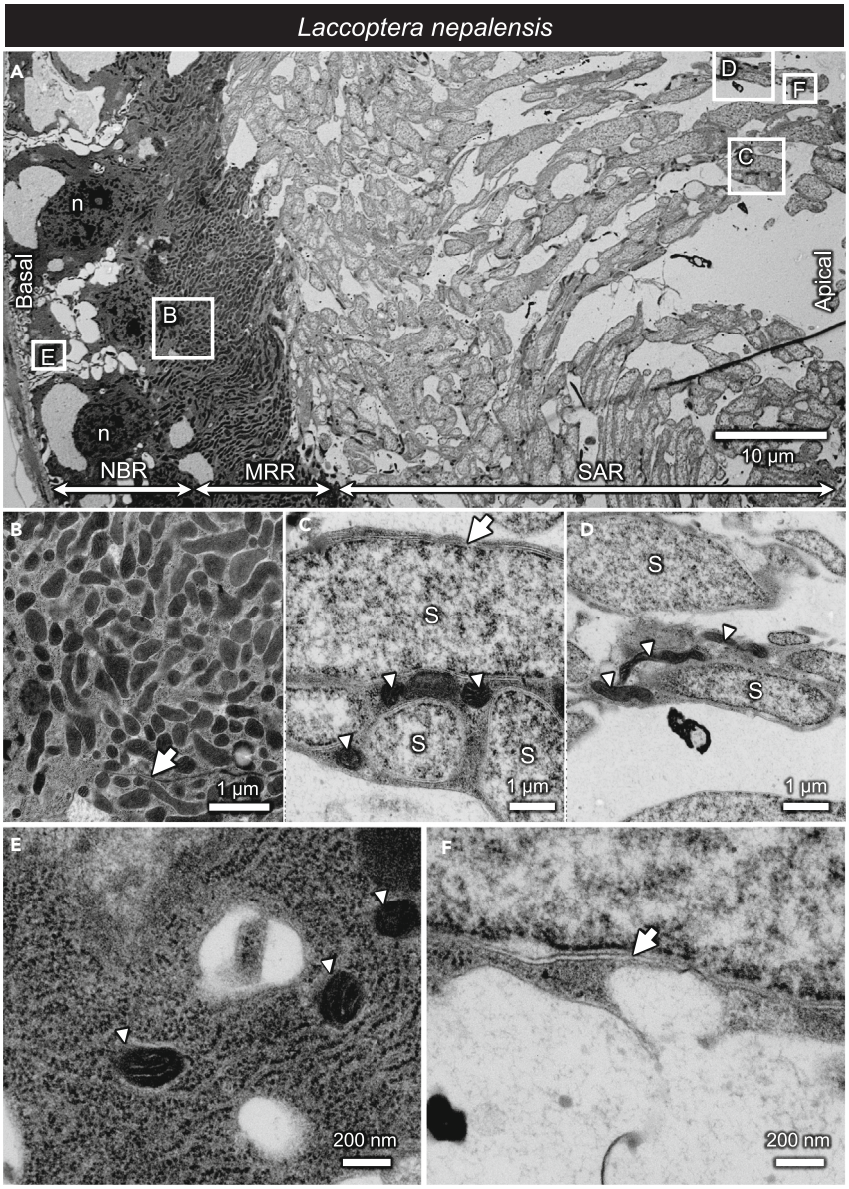


**Figure 6. TEM images of ultrathin tissue sections of the gut-associated symbiotic organ of *Laccoptera nepalensis***

(A) The whole image of the epithelial cells, in which the three distinct subcellular regions are visualized: the nucleated and symbiont-free basal region (NBR), the mitochondria-rich subapical region (MRR), and the symbiont-filled apical region (SAR) in which numerous symbiont cells are surrounded by and associated with degenerate cytoplasm with cell membrane and mitochondria.

(B) An enlarged image of the MRR. (C) An enlarged image of symbiotic bacteria in the middle of the SAR.

(D) An enlarged image of symbiotic bacteria in the tip of the SAR. Note that, in (C) and (D), the symbiont cells are associated with fragmented host cytoplasm, cell membrane, and mitochondria, being in an intermediated status between endocellularity and extracellularity.

(E) Enlarged image of MRR. In the basal region, there are many dot-like structures (rough endoplasmic reticula) in and around the mitochondria.

(F) Magnified image of SAR. n, nucleus; s, symbiont. Arrows and arrowheads indicate cell membranes and mitochondria, respectively.

reservoirs and, whenever necessary, the symbiont-produced pectinases are supplied to the midgut main tract by squeezing the sac-like symbiotic organs.

Why, then, is *Stammera* intracellular specifically in the symbiotic organ? A conventional idea is that, because *Stammera* shows extreme genome reduction down to 0.2–0.3 Mb,[26,27,29] the fragile symbiont cells are retained within the host cytoplasm where environmental fluctuation and stressors are less severe in comparison with extracellular localities. This hypothesis is, however, countered by the fact that *Stammera* extracellularly persists in the female-specific transmission organs and the egg-associated caplets, although extracellularity during vertical transmission is widely observed, at least transiently, among diverse intracellular symbiotic bacteria of insects.[3,12,18] Of course, it seems

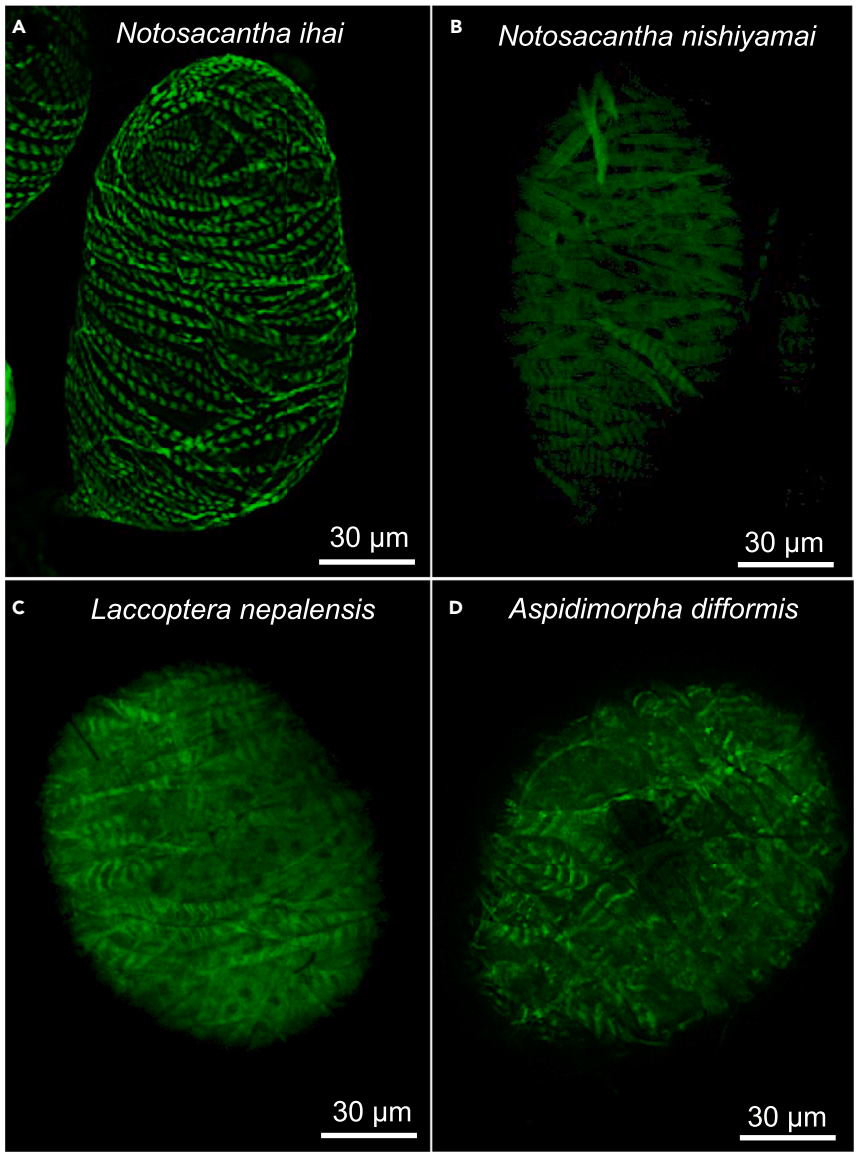


**Figure 7. Muscle fibers covering the gut-associated symbiotic organ of cassidine leaf beetles**

(A) *Notosacantha ihai*. (B) *Notosacantha nishiyamai*. (C) *Laccoptera nepalensis*.
(D) *Aspidimorpha difformis*. Actin fibers are visualized by fluorescent phalloidin in green.

plausible that, in these organs, the fragile symbiont cells are stabilized by some host-derived factors as reported in stinkbugs and other insects.[37,38] Meanwhile, our finding of the "mitochondria-rich zone" at the interface of the symbiont-free basal region and the symbiont-filled apical region of the epithelial cells of the symbiotic organs has led to an alternative hypothesis: intracellularity of *Stammera* in the symbiotic organ is for efficient nutritional and energy provisioning for supporting massive symbiont proliferation and pectinase production. Both larval and adult cassidine beetles consume large amounts of plant leaves, and thus much symbiont-derived pectinases are needed to process the food material. We hypothesize that, although speculative, by incorporating the symbiont cells within the host cytoplasm and placing them in the proximity of energy-producing mitochondria, the leaf beetles enable efficient symbiont proliferation and resultant pectinase production that may be not feasible when the symbiotic bacteria that depend on glycolysis and fermentative metabolism and lack the TCA cycle are harbored extracellularly.[39]

Why is the *Stammera*-harboring apical cytoplasm degenerate and fragmented in most cassidine species? In the extremely reduced *Stammera* genomes, most genes encoding transporter machineries have been lost, whereas secA, which is a general translocation membrane protein reported to involve extracellular export of pectinase,[40] corA, which is a membrane transporter known to regulate pectinase production,[41] and a few other transporter-related genes are retained.[26,27,29] Hence, it is predicted that *Stammera*-produced pectinases are excreted to the

extracellular milieu via the transporters in the symbiotic organ.[26,29] On the other hand, considering the massive plant feeding by larval and adult leaf beetles, we propose that the cassidine beetles have evolved an additional mechanism for massive pectinase export in necessity. For supplying sufficient amounts of symbiont-derived pectinases for plant cell wall digestion, the leaf beetles efficiently nurture the symbiotic bacteria within the apical cytoplasm of the symbiotic organ, liberate the proliferated symbiont cells into the inner cavity by fragmenting the cytoplasm, and excrete them to the midgut main tract by squeezing the whole symbiotic organ.

Why, then, is the symbiont-harboring apical cytoplasm neither degenerate nor fragmented in *Notosacantha* spp.? At present, we do not know why, but point out several potentially relevant factors. The genus *Notosacantha* constitute an early diverging lineage of the Cassidinae,[29] and thus the endosymbiotic configuration may reflect an ancestral character of the symbiotic system. Alternatively, the possibility that the endosymbiotic configuration evolved specifically in the *Notosacantha* lineage cannot be excluded. The *Notosacantha* species are relatively rare in Japan, and their geographic distribution is restricted to southwestern subtropical regions, where they exhibit peculiar biological and ecological properties such as feeding on evergreen tree leaves (see Figures 1 and S1).[36] Note that the majority of the other cassidine species in Japan feed on leaves of herbs or deciduous trees (see Figure S1).[42] It should be also noted that because of their rarity, only a limited number of sampling opportunities were available for the *Notosacantha* species, and specimens collected in different seasons and at different developmental stages might uncover different endosymbiotic configurations.

Finally, on account of the discovery of the intracellular symbiotic configuration of *Stammera* in *Notosacantha* spp., which represent an early diverging lineage of the Cassidinae, we point out the possibility that, although *Stammera* has been conventionally regarded as an extracellular symbiont,[16,26–29,33–35] it might have virtually evolved as an intracellular symbiont within the epithelial cells of the symbiotic organs in the evolutionary course of the Cassidinae. Certainly *Stammera* cells are often observed extracellularly in the ovary-associated transmission organs, the egg-associated caplets, and the inner cavity of the symbiotic organs in the life cycle of cassidines,[26,28,33–35] but we hypothesize that the main location of growth and proliferation of *Stammera* may be the cytoplasm of the epithelial cells of the gut-associated symbiotic organs. Assuming this, the drastic genome reduction in *Stammera* down to 0.2–0.3 Mb may be not regarded as exceptional among extracellular symbionts but aligned to the evolutionary consequence observed among some ancient intracellular symbiotic bacteria of insects and other organisms.[30–32] Even so, it seems remarkable that the tiny-genome *Stammera* cells can withstand a variety of extracellular conditions in the transmission organs, in the egg-associated caplets, during oral ingestion until colonization to the symbiotic organs, etc. for extended periods, whose underlying mechanisms are to be pursued in future studies.

In conclusion, we demonstrated that both intracellular and extracellular associations with the genome-reduced and pectinase-producing bacterial symbiont *Stammera* are found in cassidine leaf beetles and discussed how these endosymbiotic associations have been established and diversified during the host-symbiont coevolution. Figure 8 summarizes the hypothetical model as to how the gut-associated symbiotic organs develop and function in accordance with the digestive physiology of cassidine leaf beetles. More extensive studies on the configuration, development, and functioning of the symbiotic organs in more diverse cassidine lineages will shed further light on evolutionary aspects of the beetle-*Stammera* associations (see Graphical abstract).

### Limitations of the study

This study demonstrates that both intracellular and extracellular symbiont localizations are found in cassidine leaf beetles associated with a genome-reduced pectin-degrading bacterial symbiont. This study provides detailed histological and cytological descriptions of the symbiotic system, whereas underlying mechanisms and biological functions are merely based on speculation. For example, in the epithelial cells of the gut-associated symbiotic organ, we identified a mitochondria-rich subapical region between the symbiont-free basal region and the symbiont-filled apical region and suggested that the mitochondria-rich region may enable efficient energy provisioning for massive symbiont proliferation and pectinase production, but this idea is just a speculation.

## STAR★METHODS

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

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- RESOURCE AVAILABILITY
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  - Materials availability
  - Data and code availability
- EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS
- METHOD DETAILS
  - Fluorescent *in situ* hybridization (FISH)
  - Transmission electron microscopy (TEM)
  - Scanning electron microscopy array tomography (SEM-AT)

## SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2024.109731.

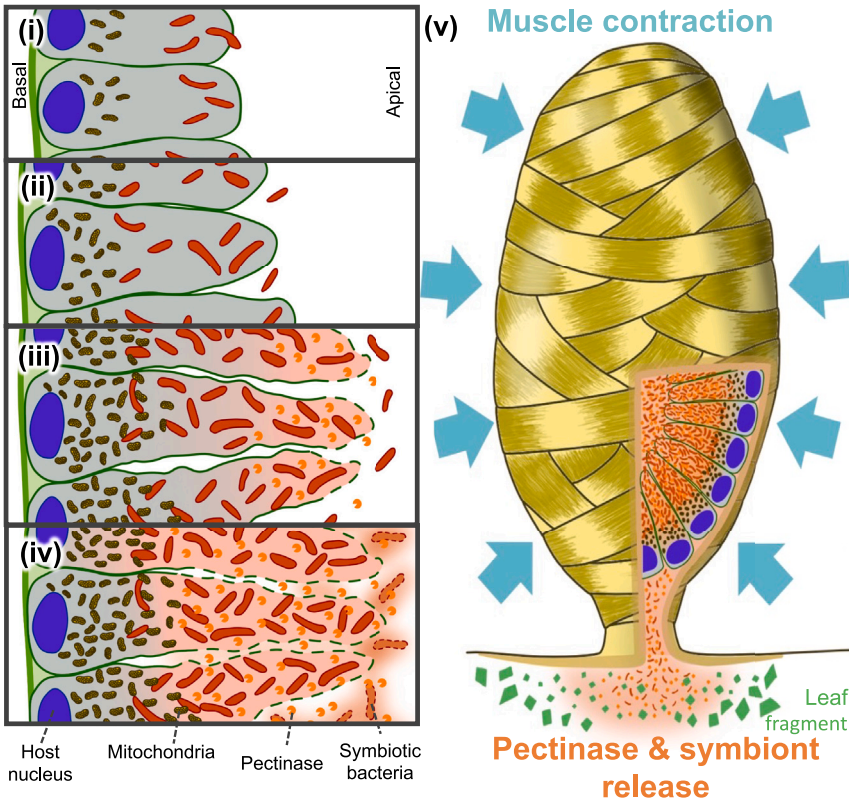


**Figure 8. Symbiotic dynamics of *Stammera* and functional aspects of symbiotic organ in cassidine leaf beetles**

(i) *Stammera* enter the gut-associated symbiotic organs intracellularly; (ii) host mitochondria support *Stammera* proliferation and pectinase production; (iii) *Stammera* proliferate and produce large amounts of pectinase; (iv) the cell membrane of the apical region degenerates and many symbiotic bacteria leak out of the host cell, and the cell membrane of extracellular symbiotic bacteria also degenerates and pectinase enzymes leak out of the bacteria; (v) contraction of the muscle layer pushes out the content of the symbiotic organ, containing *Stammera* cells and pectinase enzymes, into the midgut digestive tract.

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## AUTHOR CONTRIBUTIONS

K.O. conducted histological and cytological analyses. T.H. performed TEM and SEM-AT preparation and analyses with support by T.K. K.O., Y.M., C.S., and T.F. collected samples and took photographs of the insects. K.O. and T.F. wrote the paper. All authors contributed to the article and approved the submitted version.

## DECLARATION OF INTERESTS

The authors declare that they have no competing interests.

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## STAR★METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| Biological samples | | |
| *Notosacantha ihai* | Field collection | N/A |
| *Notosacantha nishiyamai* | Field collection | N/A |
| *Aspidimorpha difformis* | Field collection | N/A |
| *Laccoptera nepalensis* | Field collection | N/A |
| *Cassida versicolor* | Field collection | N/A |
| *Cassida circumdata* | Field collection | N/A |
| Chemicals, peptides, and recombinant proteins | | |
| DAPI | Thermo Fisher Scientific | Cat#62247 |
| Alexa Fluor™ 488 Phalloidin | Thermo Fisher Scientific | Cat#A12379 |
| Software and algorithms | | |
| Array Tomography Supporter software | Japan Electron Optics Laboratory | N/A |
| Stacker *NEO* software | Japan Electron Optics Laboratory | N/A |
| Dragonfly Pro software | Object Research Systems | N/A |
| Measurement Adviser software | System In Frontier | N/A |

### RESOURCE AVAILABILITY

See "key resources table".

#### Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact Kohei Oguchi (k.ohgreen226@gmail.com).

#### Materials availability

This study did not generate new unique insect strain and reagents.

#### Data and code availability

- All figures and supplementary figures are as listed. Raw microscopy data will be shared by the lead contact upon request.
- Code: This study does not report original code.
- All other requests: Any additional information required will be shared by the lead contact upon request.

### EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

The samples of cassidine leaf beetles used in this study are listed in Table S1. The live adult insects were immediately used for experiments. Among the collected insects, *Laccoptera nepalensis* was reared successively by feeding fresh leaves of the sweet potato *Ipomoea batatas*. Larvae of L. *nepalensis* immediately after hatching were also used. The insects were dissected in phosphate-buffered saline (PBS: 0.8% NaCl, 0.02% KCl, 0.115% $Na_2HPO_4$, and 0.02% $KH_2PO_4$) using fine tweezers under a dissection microscope (M165FC, Leica).

### METHOD DETAILS

#### Fluorescent *in situ* hybridization (FISH)

Whole-mount FISH targeting bacterial 16S rRNA was performed essentially as described previously.[28] The dissected insect tissues were fixed in 4% paraformaldehyde solution in PBS for 3 h and then thoroughly washed three times in PBT (0.3% Triton X-100 in PBS). The fixed insect tissues were washed twice in a hybridization buffer (20 mM Tris-HCl, 0.9 M NaCl, 0.01% SDS, 30% formamide). For detection of bacterial 16S rRNA, the general probe EUB338 (5′-GCT GCC TCC CGT AGG AGT-3′) 5′-labeled with Alexa Fluor 555 was used. The samples were incubated overnight at room temperature in the hybridization buffer containing 50 nM of the probe. After washing twice in PBT, nuclear DNA

and filamentous actin were stained with 4.5 μM 4′,6-diamidino-2-phenylindole (DAPI) (Thermo Fisher Scientific) and 1.5 μM AlexaFluor 488-labeled phalloidin (Thermo Fisher Scientific), respectively, for 1 h at room temperature, followed by washing with PBT again.[18] Then, the samples were washed twice in PBT, mounted in 50% glycerol in PBS, and observed under a confocal laser scanning microscope (CLSM 700; Carl Zeiss, Germany and FV3000; Olympus, Japan).

### Transmission electron microscopy (TEM)

The insects were dissected in cold PBS to extract the gut-associated symbiotic organs attached to the foregut-midgut boundary. The samples were fixed overnight at 4°C in the mixture of 4% paraformaldehyde (Electron Microscopy Sciences, 15710) and 2% glutaraldehyde (Sigma-Aldrich, G5882) in PBS, incubated with 2% osmium tetroxide in distilled water at 4°C for 2 h, dehydrated in a graded ethanol series (50%, 60%, 70%, 80%, 90%, 95%, 99%, and 100%), treated with 100% propylene oxide followed by Epon 812, and embedded in Epon 812. Ultrathin sections of 70 nm thickness were cut using a diamond knife on an ultramicrotome (ARTOS 3D, Leica), collected on single-hole grids with a support membrane, stained with uranyl acetate and lead citrate, and observed by a transmission electron microscope (Hitachi, H-7650).

### Scanning electron microscopy array tomography (SEM-AT)

The samples were fixed, treated, embedded and sectioned as described above using a diamond knife (SYM jumbo, 45°, SYNTEK) to obtain 300 nm serial sections. The serial sections were collected on a cleaned silicon wafer strip held by a micromanipulator (MN-153, NARISHIGE). The sections were stained at room temperature using 2% (w/v) aqueous uranyl acetate for 20 min and Reynolds' lead citrate for 2 min. The sections were imaged using a scanning electron microscope with a backscattered electron (BSE) detector (JSM-7900F, JEOL) supported by Array Tomography Supporter software (version 1.3.1.0, System In Frontier Inc.) that enables automated imaging. For 3D reconstruction, the images were stacked in order by Stacker *NEO* software (System In Frontier Inc.) and the resultant image stacks were processed using Dragonfly Pro software (ORS Inc.). For 2D stitching, the images were aligned by Measurement Adviser software (System In Frontier Inc.).