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Convergent evolution of signal-structure interfaces for maintaining symbioses

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Symbiotic microbes are essential to the ecological success and evolutionary diversification of multicellular organisms. The establishment and stability of bipartite symbioses are shaped by mechanisms ensuring partner fidelity between host and symbiont. In this minireview, we demonstrate how the interface of chemical signals and host structures influences fidelity between legume root nodules and rhizobia, Hawaiian bobtail squid light organs and *Allivibrio fischeri*, and fungus-growing ant crypts and *Pseudonocardia*. Subsequently, we illustrate the morphological diversity and widespread phylogenetic distribution of specialized structures used by hosts to house microbial symbionts, indicating the importance of signal-structure interfaces across the history of multicellular life. These observations, and the insights garnered from well-studied bipartite associations, demonstrate the need to concentrate on the signal-structure interface in complex and multipartite systems, including the human microbiome.

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Introduction

The diversity and complexity of plants and animals have been shaped, at least in part, through the formation of beneficial associations with symbiotic microbes [1]. The importance of beneficial symbioses in the evolution of plants and animals is illustrated by the virtual ubiquity of symbiotic microbes in aiding digestion across metazoans and by many plants engaging in mutualisms with mycorrhizal fungi. Despite the crucial role symbiotic microbes play in shaping the biology of their hosts, our understanding of the formation, maintenance, and codiversification of symbiotic associations is limited, and largely informed by our understanding of a relatively small number of symbioses.

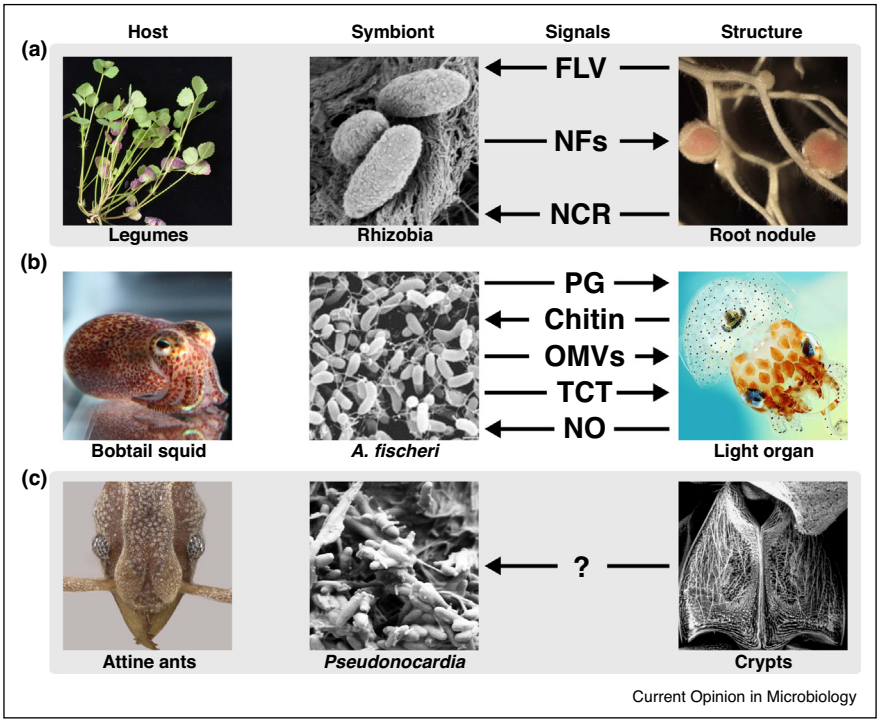
Symbiotic associations, by definition, are interactions that involve two or more species living together in an intimate association. Thus, the host-microbe interface — where the host and symbiont interact directly and exchange complex chemical signals — is fundamental for the establishment and maintenance of the symbiosis. The importance of this interface has been demonstrated through extensive studies of several model bipartite symbiosis, where high host specificity of single symbiont strains greatly facilitates studying how partner fidelity is shaped by chemical signaling and host physiology, behavior, and morphology. Herein, we illustrate the importance of the host-microbe interface in shaping the establishment and maintenance of symbiosis by reviewing three well-studied symbiotic systems involving elaborate structures: legume root nodules and rhizobia, Hawaiian bobtail squid light organs and *Allivibrio fischeri*, and fungus-farming ant crypts and *Pseudonocardia*. Finally, we document the widespread distribution of convergently evolved specialized structures for maintaining microbial symbionts across animals and plants and argue that deeper understanding of the general principles of symbiosis are possible through determining the chemical and physical underpinnings that shape the interface between hosts and symbiotic microbes.

Partner fidelity is maintained through cognate signal exchange in the legume root nodule-rhizobia symbiosis

Many legumes acquire nitrogen through symbiosis with nitrogen-fixing rhizobia, which are housed in specialized structures called root nodules (Figure 1a). These structures accommodate the rhizobial symbionts in an anoxic environment suitable for nitrogenase activity [2]. During the establishment of this symbiosis, the rhizobia gain access into inner cortical root cells through infection threads and are able to directly exchange molecules with the legume host [3,4]. Consequently, partner fidelity is established through legumes screening rhizobial symbionts from the milieu of incompatible microbes and potential pathogens occupying the rhizosphere [5].

Nodulation begins when legume roots release flavonoids, which function as chemoattractants for rhizobia. Notably, different legume species produce distinct suites of flavonoids that attract coadapted rhizobial symbionts (recently reviewed in Ref. [6]). In turn, the coevolved rhizobia

Figure 1



Signal-structure interfaces in bipartite associations between **(a)** legumes and rhizobia, **(b)** Hawaiian bobtail squid and *A. fischeri*, and **(c)** attine ants and *Pseudonocardia*. The arrows indicate the direction of signals from producer to respondent and are arranged from top to bottom in each panel to indicate the order of signaling. FLV, flavonoids; NF, nodulation factors; NCR, nodule-specific cysteine-rich peptides; PG, peptidoglycan; OMVs; outer membrane vesicles; TCT, tracheal cytotoxin; NO, nitric oxide. Image credits: legume, rhizobia, and root nodule, Jean Michel Ané; bobtail squid, Mark Mandell; *A. fischeri* from [28]; light organ from Spencer Nyholm, under the terms of the Creative Commons Attribution License; attine ant, Ted Schultz; *Pseudonocardia,* Cameron Currie; ant crypt, Cameron Currie.

respond to the flavonoid signal by activating expression of *nod* genes to produce lipochitooligosaccharides called Nodulation Factors (NFs). NFs stimulate root hairs to curl and enclose the rhizobia, leading to infection thread formation, differentiation of the rhizobia into bacteroids, and ultimately to root nodulation (reviewed in [7,8]). Rhizobia produce specific NFs consisting of variable numbers of *N*-acetylglucosamine units of variable numbers of N- that may be further modified by the addition of acetyl, carbamoyl, methyl, or variable acyl groups to the terminal non-reducing sugar and addition of acetate, D-arabinose, fucose, glycerol, or sulfate groups to the terminal reducing sugar [9]. Because NFs directly bind to legume membrane receptors called NF Receptors 1 and 5 [10,11], incompatible NF-receptor pairs will fail to initiate signal transduction in the host legume and prevents incompatible symbionts from stimulating nodule formation.

In addition to providing the rhizobia with an environment for nitrogen fixation, the root nodule facilitates the transfer of nodule-specific cysteine-rich (NCR) peptides from the host to the symbiont. These peptides consist of 30–50 variable amino acids with 4 or 6 conserved cysteine residues [12]. As many NCR peptides exhibit antimicrobial activity against rhizobia *in vitro*, it was initially thought NCR peptides function solely in rhizobia population control [13]. However, mutants of the model legume *Medicago truncatula* that are unable to produce specific NCR peptides form defective root nodules and are unable to fix nitrogen [14], suggesting that NCR peptides have additional functions in the symbiosis. Indeed, the antimicrobial activity of NCR peptides occurs at high concentrations [15••], but at sublethal concentrations these peptides alter the bacterial transcriptional program. Specifically, NCR peptides activate regulons that are crucial for symbiosis [15••,16], essential for rhizobia to adapt to the intracellular environment of their legume hosts, and necessary for their transition into differentiated bacteroids [17]. Furthermore, variation in NCR peptide sequence coupled with observations that differences in peptide regioisomeric and reduction state modulate the biological activity of these peptides [15••,18•]. These various modifications bestow NCR peptides with strain-specific activity toward different rhizobial species, which is exemplified by the large number of predicted NCR peptides encoded per genome (often >100) that control the resulting bacteroid's morphology and size [19]. In summary, root nodule symbioses are

formed, and partner fidelity is maintained through production of highly variable and specific chemical signals by both legume hosts and their coevolved rhizobial symbionts.

Non-specific signals and structural adaptations maintain partner fidelity in Hawaiian bobtail squid light organ symbiosis

The nocturnal Hawaiian bobtail squid (*Euprymna scolopes*) masks its shadow from predators using counter-illumination produced by the bioluminescent Gram-negative bacterium *Aliivibrio fischeri* (formerly *Vibrio fischeri*), which colonize a specialized structure called the light organ [20] (Figure 1b). Similar to the legume-rhizobia symbiosis, acquisition of *A. fischeri* by squid is horizontal and dependent upon screening to select symbionts from the greater ocean bacterial community [21]. As the bioluminescence produced by *A. fischeri* is essential for camouflage and bacterial colonization of the light organ is concomitant with irreversible morphological changes [22], there is strong selective pressure for squid to maintain partner fidelity and occlude non-bioluminescent bacteria from persistently colonizing the light organ.

Colonization of the light organ by *A. fischeri* occurs in three phases [23]. In the first phase, newly hatched squid pass microbe-laden seawater through their mantle cavity and over a pair of mucus-coated organs called the ciliated epithelial fields. In response to peptidoglycan derivatives, this appendage secretes mucus stores that facilitate bacterial aggregation on the ciliated epithelial fields [24]. During the second phase of colonization, the squid uses ciliary motions to draw bacterial aggregates toward pores at the base of the ciliated epithelial field and into portal ducts [24]. The final colonization phase occurs when *A. fischeri* migrate in response to a gradient of chitin oligosaccharides (chitobiose) produced in the duct and into the deep crypts of the light organ [23]. Analogous to bacteroid differentiation in the root nodule, when *A. fischeri* cells colonize the light organ, they proliferate and undergo transcriptional reprogramming [21], which results in the loss of flagella [25•] and the release a diaminopimelic acid-type peptidoglycan fragment called tracheal cytotoxin and lipid A. Together these molecules act as synergistic signals to trigger widespread morphological changes that include apoptosis and regression of the ciliated fields [22]. It was recently discovered that during proliferation in the light organ *A. fischeri* shed outer membrane vesicles (OMVs) that contain lipid A, but are devoid of tracheal cytotoxin. These OMVs are sufficient to trigger equivalent morphological differentiation in the squid [26,27]. Moreover, exposure to OMVs shed by non-symbiotic Gram-negative bacteria induces hemocyte migration in squid, which is a hallmark for light organ morphogenesis [26]. Together, the non-specific signal exchange between *A. fischeri* and squid suggest that mechanisms to maintain partner fidelity must occur upstream of light organ colonization.

A major determinant of squid colonization specificity is biofilm formation by *A. fischeri* [28,29]. Furthermore, squid also contribute to partner fidelity with *A. fischeri* through specialized mucosal structures that function as intrinsic physical barriers to colonization [30] and by generating environmental conditions to select *A. fischeri* over non-symbiotic bacteria [31]. For instance, though peptidoglycan isolated from distantly related bacteria indiscriminately induces mucus secretion by the squid, these secretions contain galaxin protein EsGal1, which contains an antimicrobial repeat domain that inhibits the growth of Gram-positive bacteria *in vitro* [32]. During colonization, bacteria are exposed to nitric oxide produced by squid [33]. By producing flavohemoglobin [34] and an alternative oxidase [35] *A. fischeri* survive oxygen radical stress, which inhibits competing bacteria. In addition, nitric oxide may disperse *A. fischeri* biofilms and promote entry of *A. fischeri* into the light organ ducts [36]. In specific response to small aggregates of *A. fischeri* ($\leq$5 cells) the squid produce and secrete an enzyme called chitotriosidase into the mucus fields and ducts. This enzyme is an endochitinase that produces chitobiose, which primes *A. fischeri* for chemotaxis toward light organ ducts [37]. In conclusion, by coupling signaling to specialized structures, the squid maintains partner fidelity with *A. fischeri*, despite the reliance on generic microbial signals, such as lipopolysaccharide and peptidoglycan in the microbial world.

Behavioral and structural adaptations maintain partner fidelity in the Attine ant-Pseudonocardia symbiosis

Attine ants engage in an ancient, coevolved, and obligate mutualism with fungi they cultivate for food in specialized gardens. The cultivated fungi are host to coevolved and specialized fungal parasites in the genus *Escovopsis* [38], which function as 'crop diseases', directly consuming the fungal cultivar [39,40]. To help defend their fungus garden from *Escovopsis*, many attine ants engage in a mutualism with bacteria in the genus *Pseudonocardia*, which occur as exosymbionts, growing directly on the cuticle of worker ants and queens [41,42] (Figure 1c). *Pseudonocardia* produce antifungal antibiotics with potent and directed antagonistic properties towards *Escovopsis* [43,44].

New adults emerge from their pupal casing aposymbiotic, immediately after which *Pseudonocardia*-covered workers inoculate the new adult's exoskeleton with the symbiont [45]. If a new adult worker does not acquire *Pseudonocardia* within the first two hours after emerging, it is no longer able to associate with the exosymbiont [45]. Thus, the specialized transmission behavior and narrow temporal window for acquisition helps ensure the specificity and maintenance of ant-*Pseudonocardia* symbiosis, as the chance for introduction of other microbial symbionts or opportunists is greatly reduced. Indeed, there is high

partner fidelity between attine ants and *Pseudonocardia*: a single strain of *Pseudonocardia* colonizes worker ants and queens within an individual colony [46,47]. Across generations, partner fidelity is supported by maternal transmission: *Pseudonocardia* are transferred vertically between generations by colony-founding queens [41]. As predicted by vertical transmission, there is high relatedness of *Pseudonocardia* both between ant colonies within populations as well as across species within the attine ant genera [48,49], indicating that this partner fidelity is maintained over large evolutionary scales.

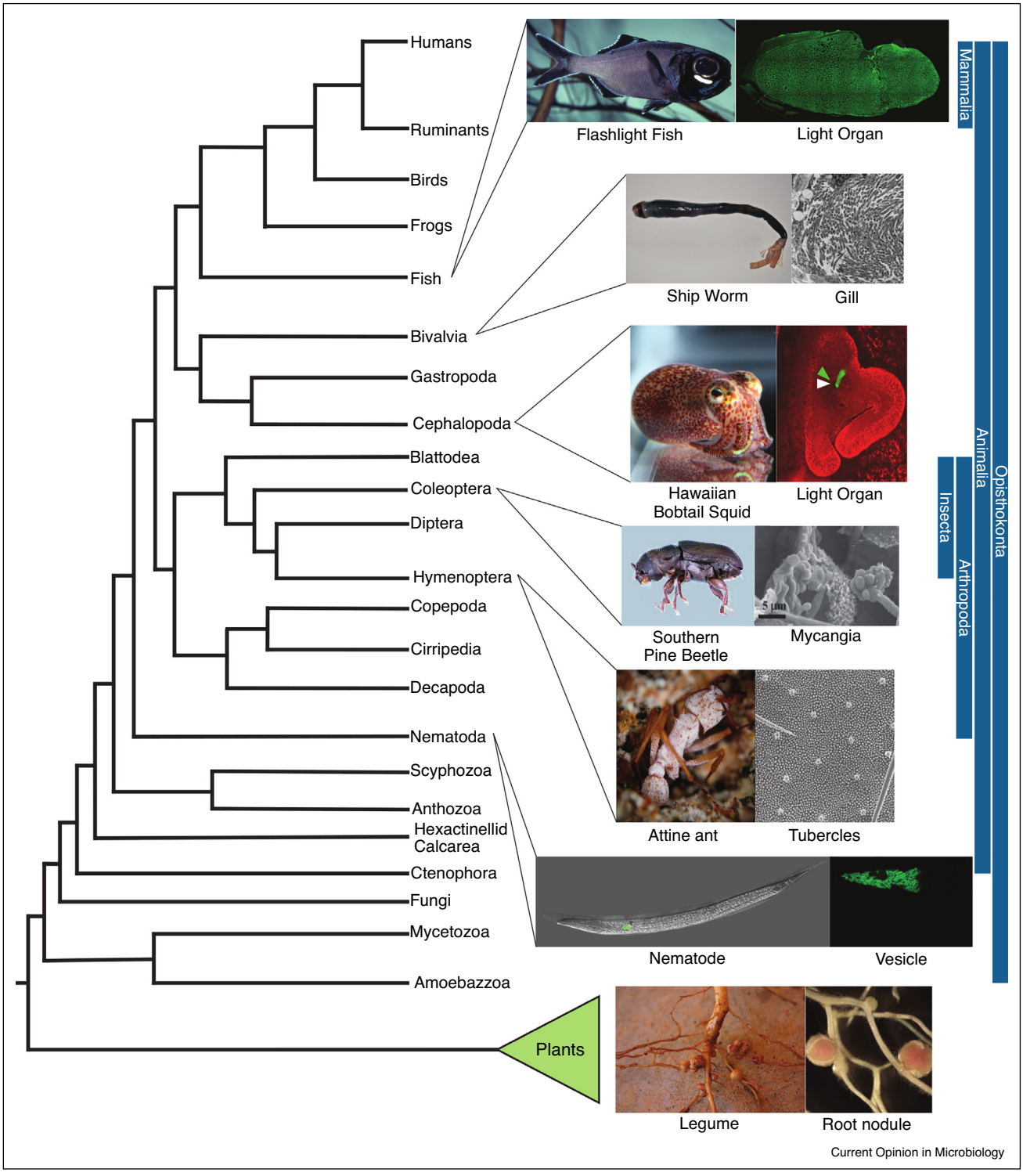
Just as with legumes and squid, attine ants have specialized structures and glands associated with the ant-*Pseudonocardia* interface. The structures can be elaborate, including large crypts or invaginations in the ant exoskeleton [42,50••]. In most genera, the structures include tubercles, on which *Pseudonocardia* directly grow. These tubercles are connected to internal glands that provide nutrients for *Pseudonocardia* growth [51]. Although the chemical signals involved at the ant-*Pseudonocardia* interface remain to be determined, it is clear that this interface is crucial to the stability and fidelity of the association. First, amino acid based stable isotope studies indicate that *Pseudonocardia* obtain all their nutrition directly from ants [51]. Second, by restricting the temporal window for *Pseudonocardia* acquisition before newly emerged adult workers leave the relatively hygienic environment of the fungus garden, it is improbable that these ants will become colonized by non-partner *Pseudonocardia* or other non-symbiotic microbes. Though the precise mechanisms are still under investigation, this narrow acquisition window is likely controlled by a programed disruption of gland cells that feed the *Pseudonocardia*. Third, ant-*Pseudonocardia* switching experiments have demonstrated significant reductions in *Pseudonocardia* acquisition in non-native pairings [52], indicating nutritional and glandular secretion coadaptation at finer phylogenetic levels. Fourth, phylogenomic analyses show that the anatomical modification to house *Pseudonocardia* has arisen independently at least three times throughout evolutionary history of attine ants [50••]. Taken together, the above evidence indicates that the maintenance, stability, and fidelity of the ant-*Pseudonocardia* association is largely mediated by the chemical signaling occurring embedded within the interface of the symbiosis on the ant exoskeleton.

Conclusion

Signal-structure interfaces between hosts and their microbial symbionts are crucial for maintaining legume-rhizobia, squid-*A. fischeri*, and ant-*Pseudonocardia* partner fidelity (Figure 1). Integral to each symbiosis is the presence of modified morphological structures that are essential for maintaining the microbial symbionts and mediating host-symbiont signal exchange. Similar to these three well-studied bipartite associations highlighted above, elaborate structural modifications for housing microbial symbionts occur across plants and animals (Figure 2). For example, crypt structures that house fungal symbionts — mycangia — have evolved numerous times within beetles [53] and the crypts that house *Pseudonocardia* in attine ants evolved at least three independent times [50••]. Similar to the light organ of bobtail squid, an analogous structure has convergently evolved in flashlight fish for harboring bioluminescent bacteria, including *A. fischeri* [54]. Notably, biofilm formation by *A. fischeri* is required for successful colonization of both squid and fish light organs. However, production of biofilm exopolysaccharide is regulated by distinct *A. fischeri* lineage-specific pathways, suggesting specific adaptations have arisen to modulate the signal-structure interface across different hosts [55••]. In addition, there are many more examples of structural modifications that house symbiotic microbes across eukaryotes, including the gills of shipworms [56] and the vesicles of nematodes [57]. Taken together, this suggests host structural modification are crucial to the host-microbe interface within bipartite symbioses.

Beyond bipartite host-microbe systems, within the widespread occurrence of microbiomes associating with diverse hosts, the presence of structural modifications at the host-microbe interface is much less recognized. These modifications may not be as well characterized in part because the associations between hosts and their microbiomes appear more plastic than bipartite associations. In animals, the majority of microbe-host interfaces occur in the gastrointestinal tract, which may harbor structural modifications to accommodate microbial symbionts that are analogous to the modifications observed in bipartite systems [58–60,61•,62,63••,64]. For example, both ruminants (Class Mammalia) and the hoatzin (Class Aves) have convergently evolved specialized structures, the rumen and the crop, respectively, in their foreguts that facilitate the growth of communities of fermentative microbes [65]. Furthermore, examples showing concordance between host phylogeny and microbiome composition [66] and co-diversification of microbial symbionts with their hosts [67] suggest that there are suitable frameworks in place for the evolution of signal-structure interfaces in all animals. For instance, it is tempting to hypothesize that the suite of antimicrobial peptides produced by the human immune system may be analogous to NCR peptides in modulating the physiology of microbes and to promote the growth of specific symbionts, as recently uncovered in amphibian systems [68]. Nevertheless, it is clear that signal-structure interfaces influence partner fidelity across the tree of life and these interfaces likely occur not only in bipartite associations, but also within more complex microbiomes. We believe more concerted efforts examining the signal-structure interfaces that occur across phylogenetically diverse host-microbe associations will help identify the general principles that govern the maintenance, stability, and coevolution of symbiotic systems.

Figure 2



Convergent evolution of structures to maintain microbial symbionts across the eukaryotic tree of life. The phylogenetic tree shows the relationships between different eukaryotic lineages. Each inset represents an example of a eukaryotic host (left) from the specified lineage that uses a specialized structure (right) to establish and maintain a bipartite association with a microbial symbiont. Image credits: flashlight fish, Stefan Herlitze; flashlight fish light organ adapted from [69] under the terms of the Creative Commons Attribution License; ship worm and gill, Margo Haygood; bobtail squid and light organ, Mark Mandel; southern pine beetle, Erich Vallery under the terms of the Creative Commons Attribution License; mycangia, Kier Klepzig [70]; attine ant, Don Parsons; ant crypt, Cameron Currie; nematode and vesicle modified from [71] under the terms of the Creative Commons Attribution License; legume and root nodule, Jean Michel Ané.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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