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The secret to a successful relationship: lasting chemistry between ascidians and their symbiotic bacteria

Eric W. Schmidt[a]

Department of Medicinal Chemistry, L.S. Skaggs Pharmacy Institute, and Department of Biology, University of Utah, Salt Lake City, Utah 84112, USA

Abstract

Bioactive secondary metabolites are common components of marine animals. In many cases, symbiotic bacteria, and not the animals themselves, synthesize the compounds. Among marine animals, ascidians are good models for understanding these symbioses. Ascidians often contain potently bioactive secondary metabolites as their major extractable components. Strong evidence shows that ~8% of the known secondary metabolites from ascidians are made by symbiotic bacteria, and indirect evidence implicates bacteria in the synthesis of many more. Far from being "secondary" to the animals, secondary metabolites are essential components of the interaction between host animals and their symbiotic bacteria. These interactions have complex underlying biology, but the chemistry is clearly ascidian-species specific. The chemical interactions are ancient in at least some cases, and they are widespread among ascidians. Ascidians maintain secondary metabolic symbioses with bacteria that are phylogenetically diverse, indicating a convergent solution to obtaining secondary metabolites and reinforcing the importance of secondary metabolism in animal survival.

Keywords

natural product; symbiosis; tunicate

Secondary metabolites are common, abundant components of soft-bodied benthic invertebrates, such as ascidians, sponges, and others (Blunt et al. 2014). More than 10,000 distinct compounds have been isolated from marine invertebrates and characterized using chemical methods. Secondary metabolites are used to defend the animals from predation, but other roles exist, such as protection from UV irradiation (Pawlik 1993, 2011; Hay 1996, 2009; Maruyama et al. 2003). Most ecological roles for these compounds have yet to be experimentally determined. Secondary metabolites are often the most abundant soluble components of benthic marine animals, where they commonly comprise >0.1% of the dry weights of the organisms. The compounds are often highly bioactive in assays. These factors argue for the importance of secondary metabolites to animals, even in the vast majority of cases in which the biological roles have not been rigorously defined.

[a]ews1@utah.edu.

Molecular evidence exists showing that some of the abundant animal secondary metabolites from ascidians (Schmidt et al. 2005), sponges (Wilson et al. 2014), bryozoans (Sudek et al. 2007), and some mollusks (Lin et al. 2013) originate in symbiotic bacteria, and not in the animals themselves. This is not to suggest that all secondary metabolites originate in symbiotic bacteria (for other origins, see: Cimino et al. 1999; Schmidt 2008; Schmidt & Donia 2010). The observation that symbiotic bacteria seem to make the potently active and defensive compounds found in marine animals is an interesting one, but it leaves open some very important questions. For example, how did animals acquire specific compounds and specific symbiotic bacteria? Are these specific compounds or symbionts even required, or do animals exploit any appropriate chemical at hand? Are there certain privileged groups of bacteria that participate in these interactions, or are there many different types of bacteria that produce compounds in animals? How does symbiosis govern the distribution of secondary metabolites in nature? What selection process(es) lead to the observed distributions? Answering such questions will lead to better understanding of the basic biology of secondary metabolite-containing animals and to biotechnological applications in areas such as drug discovery.

This review focuses on symbiotic interactions involved in secondary metabolism in ascidians, in which compelling answers to many of these questions are beginning to emerge. The reader should keep in mind that there are many other reasons for which bacteria form symbiotic interactions with animals, and that secondary metabolism may be a relatively rare role. In addition, the review cannot do justice to the outstanding studies of bacterial symbiosis and transmission in ascidians (Hirose 2014; Erwin et al. 2014), except as they apply directly to secondary metabolism. Within ascidians, the focus will be on the family Didemnidae, which is the most prolific ascidian family in terms of secondary metabolism, and specifically on the illustrative example of *Lissoclinum patella* Gottschaldt 1898.

Ascidians contain abundant and bioactive secondary metabolites

Ascidians are excellent model systems for understanding secondary metabolism in animals. Many of the basic principles elucidated in ascidians appear to be applicable to other taxa, such as sponges, as more data is acquired about secondary metabolism in those groups. Although ascidians are a relatively small group in comparison to sponges, more than 1000 secondary metabolites have been isolated from the group (Blunt et al. 2014). Ascidian metabolites are often potently toxic, and they are often the most abundant soluble, discrete compounds of ascidians. The toxicity can be remarkable: in some cases secondary metabolites are present at a concentration that is more than 10^7-fold greater than the lethal concentration to human cell lines (Gouiffes et al. 1988; Kwan et al. 2012). This property has been exploited in the development of two clinically used anticancer drugs (Rinehart et al. 1990; Rinehart 2000). It has also been directly tied to chemical defense and other crucial biological roles in the animals (Jouillé et al. 2003).

Ascidian compounds are highly structurally variable. Classically, nitrogenous metabolites have been associated with ascidian metabolism (Davidson 1993). More recently, however, other types of compounds, such as lipids, isoprenoids, polyketides, and others, are more widely appreciated ascidian products (Schmidt & Donia 2010). Even within the nitrogenous

compounds, there is a large diversity of structural groups. The anticancer didemnins and ecteinascidins are made by the nonribosomal peptide synthetase (NRPS) mechanism (Xu et al. 2012; Rath et al. 2011), while cyclic peptides such as cyanobactins are made by the "RiPP" mechanism (Schmidt et al. 2005; Arnison et al. 2013). Many other types of nitrogenous metabolites, such as pyridoacridines and tyrosine derivatives, are of unknown and difficult-to-anticipate biochemical origins (Marshall & Barrows 2004; Skyler & Heathcock 2002). The many, diverse ascidian compounds have been extensively reviewed elsewhere (Davidson 1993; Blunt et al. 2014).

Didemnidae is the largest ascidian family, comprising 578 out of 2815 described species of ascidians (Shenkar & Swalla 2011), and it is the most chemically rich: ~450 out of >1000 known ascidian metabolites were isolated from didemnids (AntiMarin database search; Blunt et al. 2014). Didemnid metabolites are potently bioactive and biomedically important, as exemplified by the orphan anticancer agent aplidine (Rinehart 2000). Animals containing aplidine relatives are highly deterrent to fish because of the presence of these abundant toxins (Jouillé et al. 2003; Lee et al. 2012). Among the didemnids, *Lissoclinum patella* is the current chemical champion, with this single species containing more than 5% of all known ascidian secondary metabolites (Schmidt et al. 2012). Individual animals do not contain these ~70 or so compounds; rather, an individual colony might contain between ~2-10 compounds. The compounds seem to be randomly distributed, with literature reports of sporadic occurrence of compounds over the range of *L. patella*. However, as described below, compound distribution actually reflects the phylogeny of potentially cryptic species or groups described as *L. patella*.

Lissoclinum patella secondary metabolites fall into two biosynthetic categories: 1) cyclic peptides, and 2) carbon-rich compounds known as polyketides (Fig. 1a). Genetic and biochemical studies reveal that *L. patella* cyclic peptides arise from peptides that are synthesized via the normal ribosomal mechanism and then are enzymatically modified, the so-called "RiPP" biosynthetic type (Schmidt et al. 2005). The carbon-rich molecules arise from polyketide synthases (PKSs) (Kwan et al. 2012). Additionally, several other small molecules have been found in *L. patella* (Schmidt et al. 2012).

Ascidian cyanobactins are small, N-C circular peptides such as ulicyclamide, patellamides, trunkamide, and others (Donia et al. 2008; Ireland & Scheuer 1980). Only a subset of these compounds is found in an individual ascidian, so that there is observed chemical variation between individual samples collected in the Great Barrier Reef, Papua New Guinea, Micronesia, Indonesia, and elsewhere. Cyanobactins are most noted for their toxicity to human cell lines, which may have utility in treating some cancers (Carroll et al. 1996; Ireland & Scheuer 1980; Donia & Schmidt 2010, 2011; Sivonen et al. 2010). In addition, some of the compounds are noteworthy for their metal binding properties (Bertram & Pattenden 2007). Their ecological roles in ascidians have not yet been reported.

Only two classes of polyketides are known in *L. patella*: patellazoles and tetrenolin (Corley et al. 1998; Zabriskie et al. 1998; Davidson & Ireland 1990; Gallo et al. 1969). Both compound groups have only rarely been reported, in comparison to the cyclic peptides, which are ubiquitous. Patellazoles are particularly noteworthy for their exceptional potency

(and toxicity) against human cell lines; as such, they may likely serve as defensive metabolites and are potential anticancer agents (Richardson et al. 2005). Patellazoles are found in *L. patella* at concentrations ranging up to ~10^7 times their lethal concentration in human cell-based assays.

Among other compounds are mycosporine-like amino acids (MAAs), which are essential sunscreen components of *L. patella* and other ascidians (Hirose et al. 2004; Maruyama et al. 2003; Hirose & Maruyama 2004). In *L. patella*, MAAs are specifically localized in ascidian cells within the tunic. As will be noted in the section on symbiosis, there are other secondary metabolites now known to occur in *L. patella* and other ascidians that were discovered by metagenome mining, a process that involves sequencing a metagenome, using the information to predict the structures of compounds, and then finding those compounds in the extracts of whole animals.

Symbiotic bacteria make ascidian secondary metabolites

In all cases so far examined, spanning about 80 of the ascidian-derived metabolites, strong evidence shows that defensive compounds and other secondary metabolites are produced by symbiotic bacteria, and not by the animals themselves (Schmidt & Donia 2010; Rath et al. 2011; Schmidt et al. 2012). Indirect but compelling evidence implicates symbiosis in the synthesis of metabolites beyond these 80 (Riesenfeld et al. 2008; Schmidt & Donia 2010; Xu et al. 2012; Schmidt et al. 2012). The metabolites so far studied are synthesized by three widely different species of symbiotic bacteria, which belong to the groups cyanobacteria, alphaproteobacteria, and gammaproteobacteria.

The indirect evidence can be summarized as follows. Many compounds isolated from ascidians are very similar or identical to those isolated from cultivated bacteria. Examples of those compounds include enterocins and didemnins (Piel et al. 2001; Xu et al. 2012). A weakness with those data in terms of assigning source is that the bacteria have not been shown to exist in the host animal, and no biological or other data ties those compounds to bacterial production *in hospite*. There are actually a large number of similar cases of cultivated bacteria that produce identical or similar compounds to those from ascidians (Schmidt & Donia 2010). Therefore, these data are correlative, but compelling. In another compelling case, the palmerolide-containing Antarctic ascidian, *Synoicum adareanum* Herdman 1902 contained certain types of PKS gene fragments that might be related to making the compound, as well as abundant proteobacterial 16S rRNA gene sequences (Riesenfeld et al. 2008).

For other types of ascidian compounds, direct genetic and biological evidence ties the production of specific metabolites to symbiotic bacteria. The non-didemnid ascidian *Ecteinascidia turbinata* Herdman 1880 has been studied in detail because it contains the clinically used anticancer agent, ecteinascidin-743 (also known as ET-743, trabectedin, or Yondelis) (Rinehart et al. 1990). A series of microbiological studies identified a gammaproteobacterium that lives specifically with *E. turbinata*: *Ca*. Endoecteinascidia frumentensis (Moss et al. 2003). A meta-omics project later identified this strain as the producer of ET-743 in a landmark study (Fig. 1) (Rath et al. 2011). The nonribosomal

peptide synthetase (NRPS) genes responsible for ET-743 production were identified by metagenome sequencing. Although the sequences were relatively fragmented, the adjacency of primary metabolic genes to the NRPS sequence allowed it to be tied to *Ca*. E. frumentensis with high confidence. The resulting NRPS proteins were found in the proteome. To demonstrate that the correct biosynthetic genes were likely responsible for ET-743 biosynthesis, a protein was heterologously expressed and assayed with substrate analogs. Taken together, these data support the hypothesis that the gammaproteobacterium produces ET-743.

All of the ~70 compounds from *L. patella* are now known to be made by symbiotic bacteria, and not by the animal itself (Donia et al. 2011a; 2011b; 2011c Kwan et al. 2012), with the exception of tetrenolin, which is rarely found and has not yet been studied. By far, most of the compounds are made by the symbiotic cyanobacteria, *Prochloron didemni* Lewin 1977, except the polyketide patellazoles, which are made by the alphaproteobacteria, *Ca*. Endolissoclinum faulkneri. *Prochloron didemni* is a well known ascidian symbiont that was first studied because of its primary metabolic interchange with the host (Lewin & Cheng 1989; Hirose et al. 2009). Indeed, *P. didemni* is required by many different species of didemnid ascidians (Hirose & Maruyama 2004). *Prochloron didemni* is a sporadic symbiont in Didemnidae and appears to have been convergently acquired by different ascidian species within the group (Münchhoff et al. 2007). It is also casually acquired by other types of organisms.

To determine the origin of secondary metabolites in *L. patella*, four different *L. patella* metagenomes were sequenced (Donia et al. 2011a). In one of these, *L. patella* from Palau, a biosynthetic gene cluster for the cyanobactins patellamides A and C was localized (Schmidt et al. 2005). Genomic analysis showed that the genes were found only in *P. didemni*, and not in other bacteria or the hosts. *Prochloron* has not been cultured. Therefore, to provide functional evidence that the correct gene cluster had been identified, the genes were transferred to *Escherichia coli* (Migula 1895) Castellani and Chalmers 1919, where they were functionally expressed, leading to synthesis of the coral reef metabolites patellamides A and C in laboratory culture.

Biosynthetic genes for many analogs of patellamides were identified in *P. didemni* genomes from *L. patella*, *Didemnum molle* (Herdmann 1886), and several other *Prochloron*-bearing ascidian species (Donia et al. 2006, 2011a). A second group of related cyanobactins, patellins and trunkamide, is also made by *P. didemni* (Donia et al. 2008). The trunkamide biosynthetic pathway has been reliably produced with increasing yields in *E. coli* culture for the past six years, and at least 12 natural “ascidian” cyanobactins have been successfully produced in *E. coli* culture (Tianero et al. 2012). No cyanobactin genes have been identified outside of *P. didemni* in any of the ascidians so far examined, and the finding of specific sequences within *P. didemni* genomes always reflects the chemistry of the whole animal. Taken together, the evidence clearly demonstrates that *Prochloron* is the sole source of cyanobactins in *L. patella*.

The *P. didemni* genomes have also revealed that *Prochloron* produces a wealth of other ascidian metabolites (Donia et al. 2011a,b). For example, one of the major routes to

cyanobacterial hydrocarbons, the terminal olefins (Mendez-Perez et al. 2011), was identified in the *gaz* cluster from *P. didemni* (Donia et al. 2011a). MAA biosynthetic genes were identified in *Prochloron* genomes, but not in the genome of the host (Donia et al. 2011a). This is intriguing, since the MAAs are localized in specific ascidian cells, as well as being found in *Prochloron* (Maruyama et al. 2003; Hirose et al. 2004). Beyond these, there are many other gene clusters that are putatively involved in secondary metabolism in *Prochloron*, and both new gene clusters and novel *Prochloron*-derived ascidian products continue to be discovered.

Prochloron has been investigated in a series of related didemnid ascidians beyond *L. patella*, where it also produces natural products (Donia et al 2011b,c). For example, *D. molle* contains many novel and potent cyanobactins that are produced by *Prochloron* (Donia et al. 2011b,c). Other compounds classes are produced by *P. didemni* in these other ascidians as well.

The polyketide patellazoles are very potent potential anticancer agents, yet they are rarely found, and the world's supply is insufficient for further development (Richardson et al. 2005). A search for the polyketide patellazoles biosynthetic genes in *Prochloron* genomes revealed that *Prochloron* does not have any genes that could reasonably explain the synthesis of these complex polyketides (Donia et al. 2011a). Animals containing patellazoles were dissected, and different regions of the animal were analyzed by metagenome sequencing (Kwan et al. 2012). Possible patellazoles biosynthetic genes were solely found within the individual zooids within the tunic. Through metagenome sequencing and assembly, the complete genome of *Ca*. E. faulkneri was assembled. This novel alphaproteobacterium contained all of the biosynthetic genes that would be required to synthesize the complex patellazoles. The genome is in a relatively advanced state of degradation, such that the ~65 kbp patellazoles genes occupy about 10% of the coding sequence of the organism. Strikingly, and in contrast to what is found in cultivated bacteria, the patellazoles genes were not found in a contiguous cluster, but instead were distributed in seven different regions around the chromosome, reflecting their ancient origin and likely essential role in the symbiosis.

To verify that the correct genes were identified, neighboring ascidian colonies were analyzed (Kwan et al. 2012). Two colonies were found, one of which contained *Ca*. E. faulkneri, the patellazoles genes, and patellazoles. The second colony lacked all three of these elements, despite being otherwise virtually identical in terms of microbiome with the first sample. This constituted a "virtual knockout" of the patellazoles pathway from an ascidian sample. Additionally, *Ca*. E. faulkneri and the *ptz* genes have been found only in patellazoles-containing ascidians; they are present in all three such ascidians, and they are absent in the many other samples that lack patellazoles.

The bacterial species mentioned above, *P. didemni*, *Ca*. E. faulkneri, and *Ca*. E. frumentensis, produce diverse and important ascidian compounds. However, these specific organisms are not responsible for synthesizing all ascidian metabolites; indeed, metagenomic surveys and metabolomics analyses show that secondary metabolite-containing ascidians have many different types of core microbiomes, and that these three

known secondary metabolite-producing bacterial species have narrow distributions in ascidians (Tianero et al. 2014). The data reveals that ascidians have achieved independent (possibly convergent) solutions to obtaining chemical defenses and other needed secondary metabolites. Such independent solutions may also, in some cases, include synthesis of important metabolites by the animals themselves; there are also many other possible sources of secondary metabolites. A speculative partial list of such sources might include eukaryotic or archaeal symbionts, "collaborative" biosynthesis by multiple organisms, dietary source, non-enzymatic transformation, gene transfer, organelle transfer, mRNA transfer, and so on. The only clear story so far is that there is no single, clear story, and that multiple solutions have evolved to supply secondary metabolites.

The symbiotic bacteria described above have yet to be cultivated and have very defined, specific interactions with their host animals. (This is not to say that they are "uncultivable".) However, there are many reports of cultivated bacteria, isolated from ascidians, sponges, and other organisms, that also produce bioactive secondary metabolites. Identical or closely related bacterial genera are consistently isolated from sponges and ascidians; the isolated genera differ between these groups (Donia et al. 2011a; Abdelmohsen et al. 2014). Are these true symbionts?

From didemnid ascidians, actinomycetes from the renowned secondary metabolite-producing genera *Salinispora* Maldonado 2005 and *Verrucosispora* Rheims 1998 (Udwary et al. 2007; Bister et al. 2004) are consistently isolated. In one case, the same species of *Salinospora* was isolated from the same ascidian species in Japan and in Fiji (Woo et al. 2012; He et al. 2001). The isolates produced the same compounds, lomaiviticins. Thus, this may be more than a chance encounter. Similar *Salinispora* bacteria were also consistently isolated from metagenome-sequenced *L. patella* (Donia et al. 2011a). However, neither the 16S rRNA gene sequences from these genera, nor the potential compounds produced by the bacteria, were identified in extensive analyses of the host animals. This result suggests that these cultivated isolates might result from relatively rare bacterial spores that are associated with the ascidians. Alternatively, there is a known bias against certain sequences in metagenomes that may be interfering with the analysis (Morgan et al. 2010). None of the compounds encoded in these bacteria can be identified in the ascidian extracts, arguing against their importance to the secondary metabolism symbiosis story.

Secondary metabolic integration between symbiont and host

In contrast to cultivated strains that produce compounds largely not found in hosts, secondary metabolites from the uncultivated symbionts are abundant and integrated into host metabolism. The best example of this is the MAAs, which are localized to specialized cells in didemnid ascidians (of diverse species), where they are essential to shielding the animals from UV light (Maruyama et al. 2003). Absent MAAs, the shallow habitat occupied by *L. patella* would expose the animals to excessive irradiation. As mentioned, *Prochloron* makes the MAAs, whereas there is no evidence of MAA biosynthetic genes in the host or in other organisms (Donia et al. 2011a). Therefore, through an unknown mechanism, MAAs must be translocated from producing cyanobacteria to a specific cell type within the animal tunic (Hirose et al. 2004).

A similar translocation is found for toxic secondary metabolites. Prior to molecular studies of *L. patella* secondary metabolism, the origin of metabolites was addressed using cell separation studies. These studies produced conflicting results, in which one study showed cyanobactins were found in *P. didemni*, while another showed that they were distributed through the animals (Degnan et al. 1989a; Salomon & Faulkner 2002). In our hands, it does not matter what portion of the animal is extracted, since similar amounts of compounds are isolated from different regions. This makes it likely that cyanobactins are widely distributed across multiple cell types in the animal. Another study showed that the bistramide polyketides in *Lissoclinum bistratum* Sluiter 1905 were localized to *P. didemni* (Degnan et al. 1989b). We have sequenced the *Prochloron* genome in a bistramides-containing animal, and as in the case of patellazoles, *Prochloron* does not make the bistramides (unpubl. data). Of note, the animals must also be defended from these toxins. Together, these data suggest that, for secondary metabolites beyond MAAs, there is integration between host and symbiont metabolism. The long-term and species-specific associations between host and secondary metabolites, described below, further argues in favor of this integration and a critical role for secondary metabolism.

Chemistry is ancient, ascidian-species specific, and essential to symbiosis

The question of how symbionts and ascidians collaborate to control secondary metabolism has been investigated only in *Ca*. E. faulkneri and *P. didemni*. The most clear cut case involves *Ca*. E. faulkneri, which lives intracellularly in blood cells in the hemocoel of *L. patella* (Kwan & Schmidt 2013; Kwan et al. 2012). Data so far suggest that this strain has been intracellular and strictly vertically transmitted within *L. patella* for >6 million years. In addition, the patellazoles are essential to the survival of the symbiosis.

The lifestyle experienced by *Ca*. E. faulkneri is similar to that found in long-term symbionts of insects (Fig. 2). In the latter cases, extensive experiments by Moran and others show that bacteria experience a population bottleneck every time the host reproduces (McCutcheon & Moran 2011). Because mutations in the bacterial genomes cannot be repaired by recombination with outside populations, the genomes rapidly degrade. Only genes that are essential to survival are maintained between generations, while all others become first pseudogenes and are then eliminated. The initially sequenced *Ca*. E. faulkneri (Fiji L2) had exactly the expected genomic characteristics that indicated long-term maintenance in symbiosis, genome degradation, and strict vertical transmission. While nonessential genes were lacking, the large patellazoles gene cluster occupied 10% of the coding genome. This indicated that the presence of these compounds is somehow essential to the survival of the symbiosis. According to the well-supported model proposed by Moran, nonessential genes would accumulate mutations and disappear. Recently, a related case of genome degradation and maintained secondary metabolism was reported in an insect symbiont (Nakabachi et al. 2013), reinforcing this model.

Three patellazole-containing *L. patella* samples were obtained, from Fiji, the Solomon Islands, and the Eastern Fields of Papua New Guinea (Kwan et al. 2012). *Ca*. E. faulkneri sequences were amplified from all three samples, and in all cases those sequences were ~85% DNA sequence identical in pairwise comparisons. From the Eastern Fields sample, a

second complete *Ca*. E. faulkneri genome ("L5") was assembled and compared to that from the initial Fiji ("L2") genome sample (Kwan & Schmidt 2013). The *Ca*. E. faulkneri genomes were extremely similar and entirely syntenic, but they were only about 85% DNA sequence identical in coding regions (identity was not identifiable except by tBLASTX in intergenic regions). This result confirmed the long-term vertical maintenance of the symbiosis, without horizontal transfer (or even absent sexual reproduction), and further verified the genome degradation model proposed. The patellazoles biosynthetic gene cluster was completely maintained in both samples, despite extensive genome degradation, reinforcing the essential importance of the pathway to the symbiotic relationship. The patellazoles cluster may have originated in horizontal gene transfer prior to the formation of the obligate symbiosis, as it is not found in the closest free-living, genome-sequenced relatives. However, once the symbiosis was established, horizontal transfer apparently no longer occurred.

Ascidians have essentially no fossil record, so speciation is difficult to precisely date. Therefore, molecular methods were employed to provide an estimate of the age of association (Kwan & Schmidt 2013). The complete mitochondrial genomes of the L2, L5, and L6 samples were assembled and compared with each other, reflecting a long evolutionary separation (for example, only ~37% DNA sequence identity in the ribosomal RNA genes). Additionally, the cytochrome C oxidase I (COXI) gene was amplified from other *L. patella* samples and other didemnid ascidians to afford a reasonable estimate of the length of genetic isolation between samples. As an example of one analytical endpoint, the L2 and L5 COXI genes were only about 85% identical, which is a similar distance as found between the symbiotic *Ca*. E. faulkneri genomes in these samples. Based upon various models of COXI gene evolution, the obligate relationship between *L. patella* and *Ca*. E. faulkneri can be estimated to have existed for between 6-31 million years, with the low estimate of 6 million years being based upon the fastest available estimate of possible COXI gene evolution. This age was also reinforced by comparing genome drift rates in known obligate intracellular symbiotic bacteria, which reflect an age of low millions of years. During that time, the patellazoles cluster has been maintained in its modern form, and patellazoles were synthesized and secreted into the host.

The essentiality of the patellazoles to the symbiosis is reflected in the lack of primary metabolic genes that might be important to the ascidian. In many genome-reduced symbioses, bacteria make essential amino acids and vitamins for the host animal (McCutcheon & Moran 2011). In *Ca*. E. faulkneri, many of these pathways have been specifically degraded. (Kwan et al. 2012) The absence of some of the genes that are important to primary metabolism, while 10% of the coding genome directly concerns patellazoles, is telling.

By contrast to the case with *Ca*. E. faulkneri, *P. didemni* is much more complicated; its biology defies expectation and simplistic explanation. In *Ca*. E. faulkneri, genomics indicates that secondary metabolism is the likely primary driving force for the symbiosis. However, the probable major role of *P. didemni* is nutritional (Hirose et al. 2009). For example, *P. didemni* provides the majority of fixed carbon to some ascidians, and it recycles nitrogen in the low-nitrogen tropical environments (Koike & Suzuki 1996; Koike et al.

1993; Pardy & Lewin 1981; Kremer et al. 1982). Many species of ascidians are never found without *P. didemni*, suggesting that absence of *P. didemni* may be lethal. Nonetheless, the secondary metabolism of *P. didemni* is clearly important, and in some cases essential, to the symbiosis. As a good example of essentiality, MAAs are required for survival in the high-UV environment, and they are made by *P. didemni*.

Also in contrast to *Ca*. E. faulkneri, *P. didemni* is found extracellularly in most ascidians (Hirose 2014). In different species, different levels of both vertical and horizontal transmission are found. There is no apparent co-speciation of *P. didemni* with ascidians (Donia et al. 2011; Münchhoff et al. 2007), and there is also no apparent co-adaptation on the part of *P. didemni*. Phylogenetic analysis of *P. didemni* using 16S approaches reveals no correlation with host taxonomy. This is true even within subpopulations of single ascidian species: *P. didemni* 16S rRNA gene sequences are no more identical and contain no more similar SNPs within a subpopulation than they do to *P. didemni* from any other ascidian species. The same holds for other marker gene sequences, including genes encoding primary metabolic proteins. These results indicate that there is likely no co-evolution of symbiont with host.

Alignment of four sequenced *P. didemni* genomes from different *L. patella* hosts showed that the genomes are very highly related (Donia et al. 2011a). All of the central genes responsible for metabolism and for providing nutrients to host ascidians are virtually identical in the four strains, with at least >95% or higher DNA sequence identity. Most of the differences between genomes lie in abundant tracts of repetitive DNA, which is very similar to what is found in many cyanobacterial genomes (Mazel et al. 1990). *Prochloron* also contains abundant repeats. These consist of intergenic (noncoding) sequences, multiple paralogous genes that are largely uncharacterized or cell-surface associated genes, and repetitive units found embedded within genes that vary in copy number and location between genomes. Similar patterns are observed in *P. didemni* genome sequences from other ascidian species (unpubl. data).

Beyond repetitive DNA, the only major differences between *P. didemni* genomes lie in secondary metabolism (Fig. 3). The four *P. didemni* genomes showed differences in the combinations of secondary metabolic genes (Donia et al. 2011a). Different combinations of those genes were present in each of the four samples, leading to differences in observed chemistry in the whole animals. This observation was extended by analyzing *P. didemni* from 20 different ascidian samples from collections across the western tropical Pacific (Donia et al. 2011b). These included ascidians from diverse species, including *L. patella*, *D. molle*, *Diplosoma* sp., and others. A sporadic variation of biosynthetic genes was observed, where different combinations of secondary metabolic pathways were found in *P. didemni* in different ascidians. There was no observable pattern of co-occurrence of pathways when examining variables such as ascidian host species, location in the tropical Pacific, or ascidian habitat. Therefore, pathways sporadically co-occur, leading to different combinations of chemicals in the host animals. When a specific biosynthetic pathway is found, it is 99-100% identical at the DNA sequence level to the same biosynthetic pathway from another *P. didemni* strain, and there is no pattern of SNP occurrence that reflects

phylogeny either of host or symbiont. These data indicate that the constantly shuffling combination of pathways arises by horizontal gene transfer between *P. didemni* cells.

There is also a remarkable variation in the cyanobactin cyclic peptide biosynthetic pathways, which strongly supports this horizontal transfer model (Donia et al. 2008, 2006). One group of cyanobactins is produced by the *pat* pathway, which makes patellamides. When this pathway is found, it is 99-100% identical between strains. The actual natural product is a highly modified, ribosomally encoded peptide of 6-8 amino acids. This peptide is hypervariable. It is undergoing rapid horizontal gene transfer, as reflected by the genetics (Donia et al. 2006). A second group of cyanobactins is produced by the *tru* pathway. There is a crossover between *pat* and *tru*; outside of this cross, these pathways are identical, but they average about ~50% identity within the cross (Donia et al. 2008). There is a constant shuffling between *pat* and *tru*, as evidence by the fact that the boundary between the two clusters differs by up to 200 bp on each side of the insertion in different *P. didemni* strains.

A population model was proposed based upon these data (Donia et al. 2011b). In this model, *P. didemni* in the ocean comprises a panmictic population that exchanges genetic material, which prevents sequence divergence. The major differences between genomes are the precise focus on secondary metabolic exchange, and the role of secondary metabolism in symbiosis.

Given the sporadic variation observed, how can secondary metabolism be central to acquisition and maintenance of the *P. didemni* symbiosis? To investigate this phenomenon in *L. patella*, the biogeography of the species was compared to secondary metabolite profiles (Fig. 4) (Kwan et al. 2014). By examining ascidian phylogenetic markers, such as the COXI and the 18S rRNA genes, it was apparent that *L. patella* may be comprised of several distinct species or populations. Strikingly, the secondary metabolites found in the *L. patella* samples exactly mapped to the animal phylogenetic tree. Despite the fact that *P. didemni* does not co-speciate with host animals, the *P. didemni*-synthesized secondary metabolites are highly host-specific. This is not a geographical effect, since several different host types and secondary metabolite types closely coexist. This led to the proposal that the host somehow controls secondary metabolite content in *L. patella*.

The *L. patella* phylogenetic study also revealed a deep divergence between the patellazole-containing group and the non-patellazole-containing ascidians (Kwan et al. 2014). They are branched deeply enough in the COXI tree that, in most types of animals, they would certainly be considered different species. The two potentially different species co-occur in parts of their range. However, the lack of a molecular clock in ascidians, combined with the lack of appropriate biological studies, makes this conclusion tentative. One clear outcome of this finding is that extinction of cryptic subspecies may likely lead to a permanent loss of chemical diversity.

Convergent solutions to secondary metabolism in ascidians

Ascidians have found many different solutions to obtaining secondary metabolites. Many different didemnid ascidian species harbor *P. didemni* (and sometimes other cyanobacteria), which produce natural products. The distribution of *Ca*. E. frumentensis and *Ca*. E. faulkneri

is much more restricted, being so far limited to *E. turbinata* and the "group B" *L. patella* clade, respectively. This represents just a small subset of ascidian diversity, perhaps ~1% of ascidian species. Do the data obtained so far reflect widespread trends in ascidians?

To investigate this question, 32 different ascidian samples representing 15 different species were examined by comparing microbiomes and metabolomes (Tianero et al. 2014). Ascidians were examined spanning a wide geographical and temperature range, including Florida, California, and the tropical Pacific. It was found that the ascidian microbiomes were similarly diverse across all ranges and habitats. As found in other ascidian microbiome-focused studies (e.g., Erwin et al. 2014), the ascidians contained a large diversity of different types of bacterial strains. The most abundant strains in each ascidian comprised an ascidian species-specific core microbiome. Secondary metabolism was also highly ascidian species-specific. There was little similarity between ascidian species in terms of core bacterial strains, except in the subset of *Prochloron*-containing ascidians. The data included samples of *L. patella* and *E. turbinata*, ensuring their robust relationship to current knowledge about secondary metabolite symbiosis. The results indicated that some of the conclusions from examining *P. didemni*, *Ca*. E. faulkneri, and *Ca*. E. frumentensis and their hosts are likely transferrable to many other ascidian species. For example, secondary metabolism is highly correlated to host phylogeny within "*L. patella*"; in this study, the correlation was extended to 14 further ascidians from different classes. A species-specific distribution of symbiotic bacteria has been found in individual ascidian species; the study showed that this is a general trend among secondary metabolite-rich ascidians. Although not all secondary metabolites are likely to be made by symbiotic bacteria, the results showed that ascidians have found many independent, possibly convergent, solutions to obtaining the needed secondary metabolites. Based upon the small number of symbioses that have been studied in detail, there are likely to be many other, interesting stories found by further examining ascidians.

Perspective

Symbiotic bacteria make many of the most abundant compounds found in host ascidians. Although the underlying pattern of symbiosis can be complex, the chemistry found in ascidians is species-specific to the animal. Ascidians have formed long-term relationships with bacteria, enabling them to acquire potently bioactive compounds, many of which are likely protective. There has been convergence in acquiring this trait, since different bacteria are responsible for making different compounds in different hosts. These results reveal essential biological roles for secondary metabolites in ascidians.

Although much is known about secondary metabolite symbiosis in ascidians, most of the interesting biological questions about these interactions have yet to be addressed. For example, what are the ecological, biochemical, and physiological factors that determine which bacteria/metabolites are stabilized in a symbiotic interaction? How do the resulting molecules, which are often very toxic, affect the host community and the surrounding ecology? Even more fundamentally, are these compounds really defensive? Although much is said about the issue, we do not yet really know what possible roles they might play in the environment. Finally, as mentioned above, the origin of only about 8% of ascidian metabolites has been experimentally defined. From the microbiome-metabolome

comparison data, there are certainly many other interesting biological sources and symbiosis stories that are yet to be discovered, and the most interesting questions await answers.

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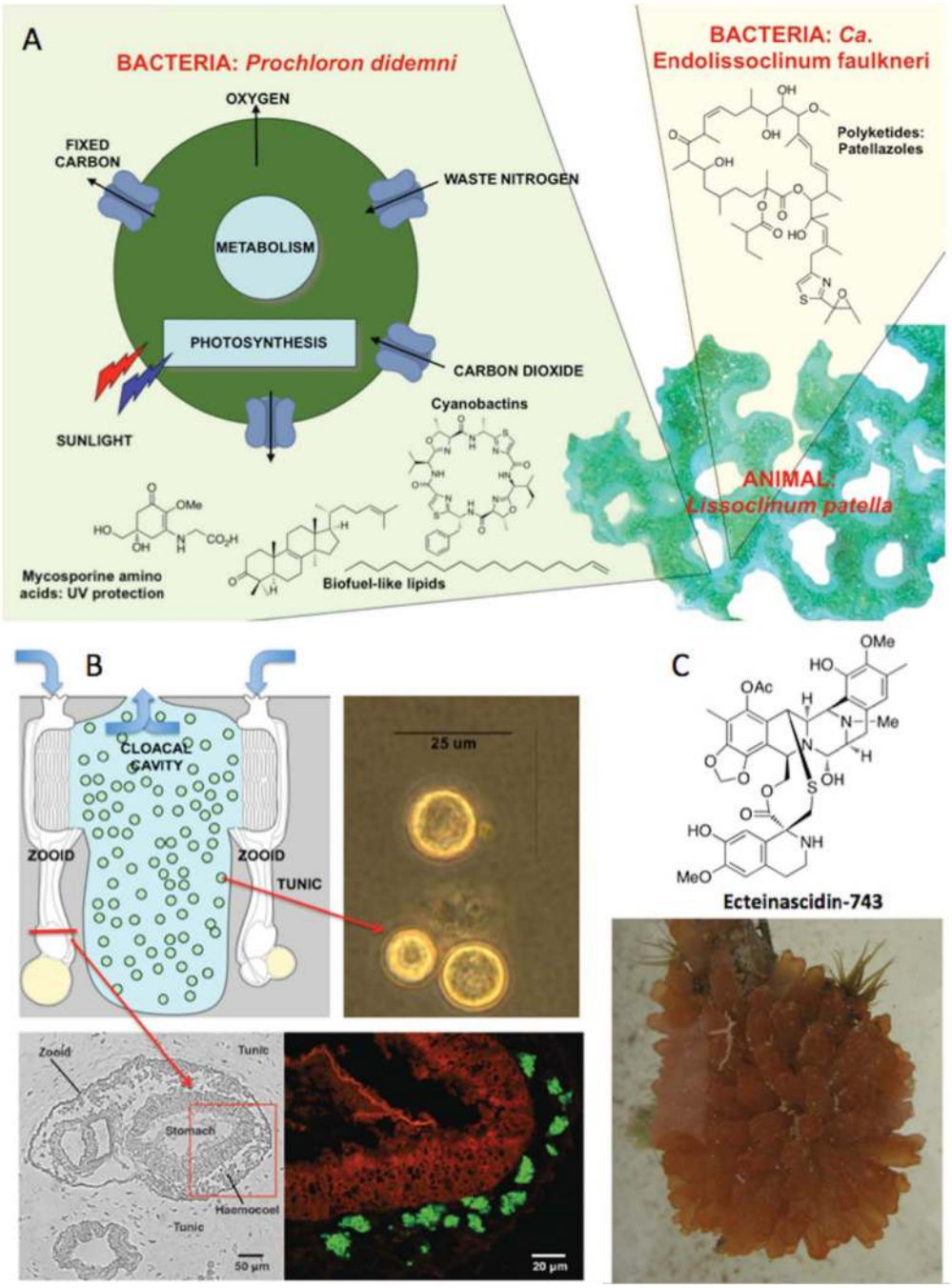

Fig. 1.

Symbiosis and secondary metabolites in ascidians. **A**. *Lissoclinum patella* forms symbioses with two known bacterial strains, which produce different sets of secondary metabolites. **B**. Within *L. patella*, *Prochloron didemni* cyanobacteria (green spots in cartoon and in micrograph to right) are localized to the cloacal cavity. The symbiotic alphaproteobacteria *Ca*. Endolissoclinum faulkneri are found within blood cells, as shown at bottom in the light micrograph of a slice of *L. patella* and in a fluorescent in situ hybridization experiment (green fluorescence) of the same tissue slice focused on the red-boxed region. **C**.

Ecteinascidins are produced by symbiotic *Ca*. Endoecteinascidia frumentensis in the ascidian *Ecteinascidia turbinata*. Portions of this figure are reproduced from Kwan et al. (2012). I thank M. Haygood for the photograph of *E. turbinata*.

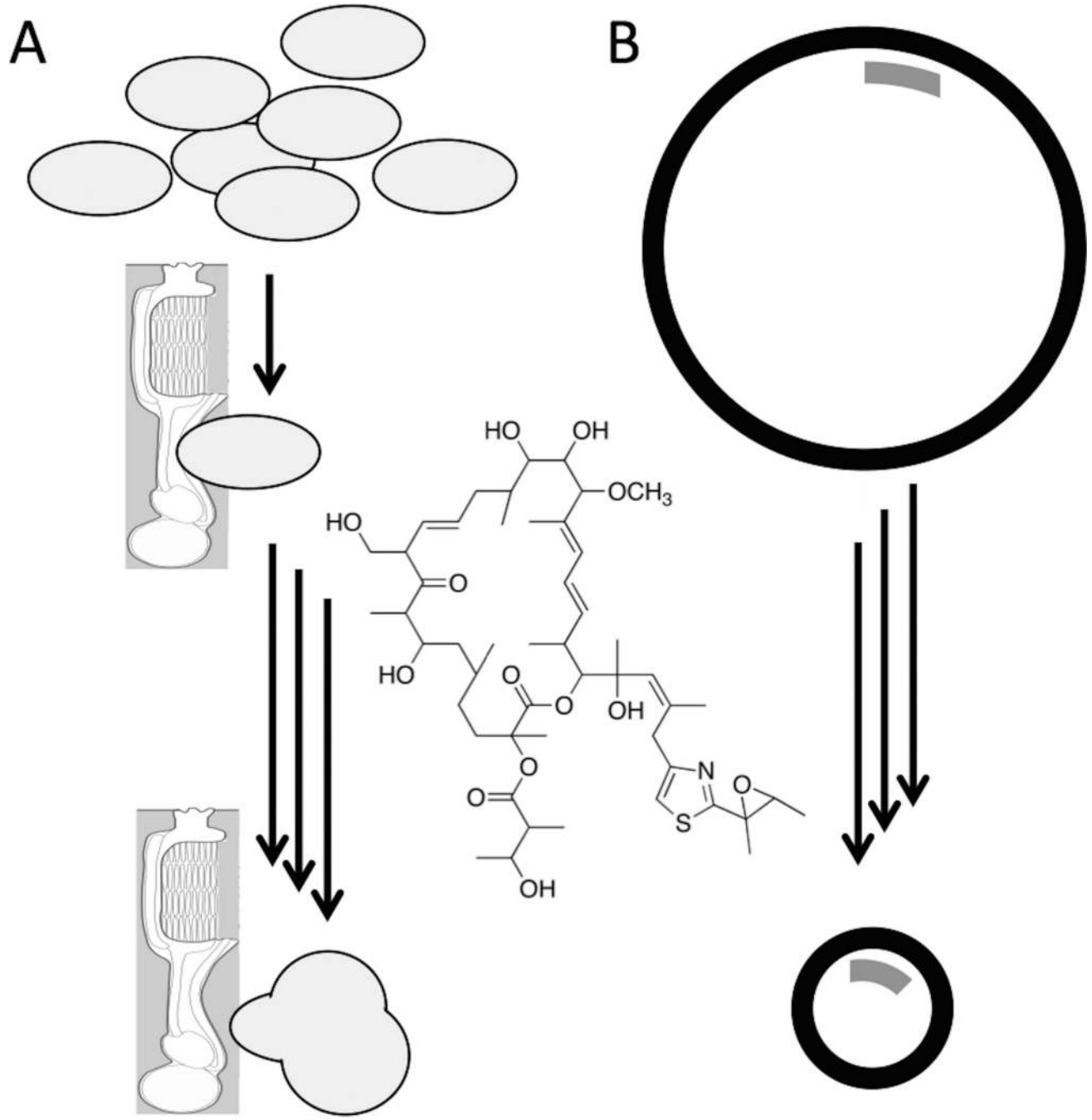

Fig. 2.

Model of ancient symbiosis leading to patellazoles production in *Lissoclinum patella*. **A**. An alphaproteobacterium related to *Ca*. Endolissoclinum faulkneri lived in the ocean and acquired genes for production of the polyketides, patellazoles. The bacteria associated with the zooids of *L. patella*. Over time (estimated 6-31 million years), due to a population bottleneck resulting from strict vertical transmission, the bacteria lost their normal shape and became obligate symbionts of *L. patella*, *Ca*. E. faulkneri. **B**. The ancestor of *Ca*. E. faulkneri, which had a large genome, acquired the patellazoles biosynthetic gene cluster

(light gray). At that time, the patellazoles cluster might have represented just 1% of the genome. Over time, due to the population bottleneck, the genome degraded such that >10% of the coding genome was represented by the patellazoles biosynthetic pathway. Patellazole C, one of the products that has been maintained over the 6-31 million year estimated evolutionary history, is shown at center.

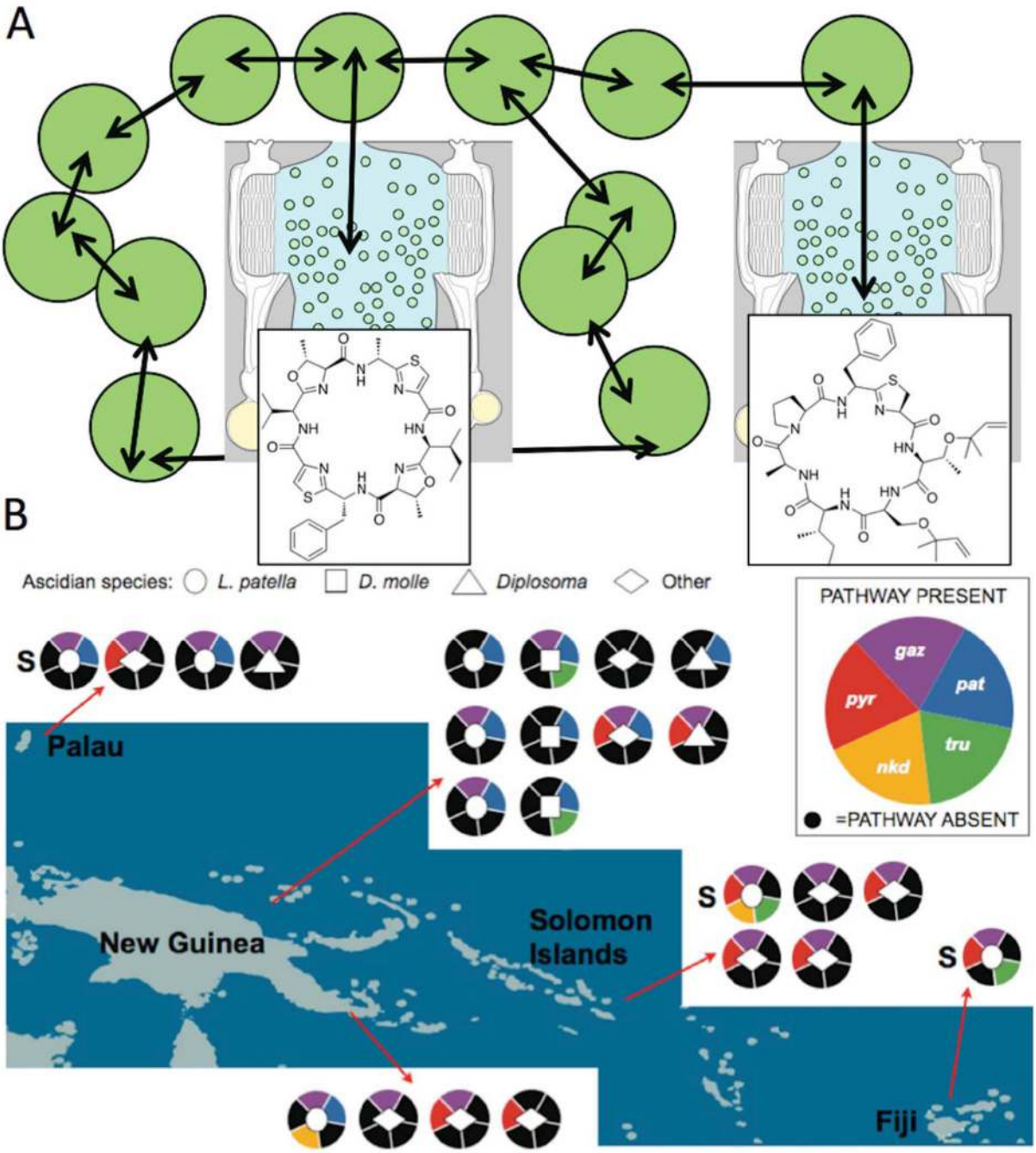

Fig. 3.

Secondary metabolic exchange in *Prochloron didemni*. **A**. *Prochloron didemni* (shown as green circles) forms a population in the ocean that undergoes genetic exchange (indicated by arrows). The variable components are localized to secondary metabolites, such as patellamides (left) or trunkamide (right), which are found in abundance in host ascidians. **B**. A schematic representation of genomes of *P. didemni* from various ascidians in the tropical Pacific. The pie-shaped circles with wedges represent genomes of *P. didemni*; each slice of

the pie represents the discrete presence or absence of a specific biosynthetic pathway to a secondary metabolite. Figure 3B modified from Donia et al (2011b).

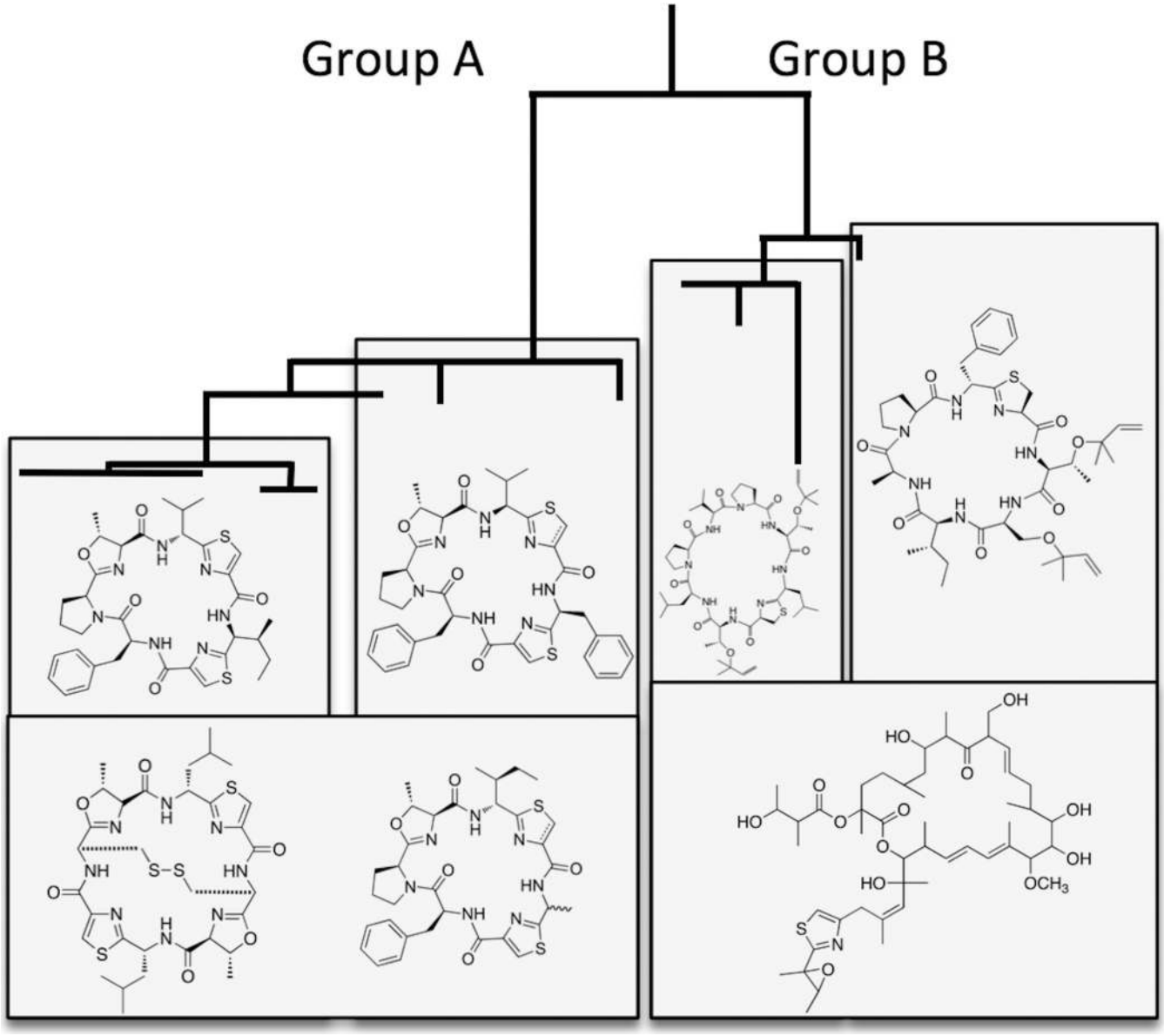

Fig. 4.

Despite sporadic exchange of secondary metabolites in symbiotic *Prochloron didemni*, *Lissoclinum patella* natural products are highly host-specific. This pattern is true for many other ascidian species as well. Shown is a phylogenetic tree of *L. patella*, where Group A and Group B *L. patella* may be different species. Clade-specific compounds are shown in boxes.