Chapter 2
Genetics and Evolution of Deep-Sea Chemosynthetic Bacteria and Their Invertebrate Hosts

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Contents

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2.1 Introduction

The earth scientists who discovered deep-sea hydrothermal vent communities along the Galápagos Rift in 1977 set the stage for revealing chemosynthetic symbioses in animals. They found high concentrations of hydrogen sulfide in the vent effluents and hypothesized that "a significant proportion of the organic carbon utilized within these hot spring regions could be produced by chemolithotrophic sulfur-oxidizing bacteria" (Corliss et al. 1979, p. 1079). Chemolithotrophic microbes are faced with the problem of extracting energy from narrow redox zones in marine environments. The free-living species typically occur in biofilms on sulfidic rocks or in filamentous mats like *Beggiatoa* spp. (Fig. 2.1a, Plate 3a), absorbing reduced gases from the substrate below and oxygen from the ambient water above. Symbiotic microbes, however, can span broader oxic-anoxic boundaries by exploiting the behavior, physiology and morphology of their animal hosts (Stewart et al. 2005). For example, the small mobile shrimp *Rimicaris exoculata* (Fig. 2.1b, Plate 3b), which lives at the interface between hot sulfidic vent water and ambient seawater, circulates vent fluids through a gill chamber that is packed with sulfur-oxidizing (thiotrophic) episymbionts. In contrast, the sedentary vesicomyid clam, *Calyptogena magnifica* (Fig. 2.1c, Plate 3c), spans a broad redox zone by growing up to 30 cm in length. The clam protrudes its highly vascularized and extensible foot deeply into small rocky fissures to absorb dissolved sulfides, and it uses its siphon to circulate ambient seawater to gills housing intracellular thiotrophic endosymbionts. The sessile vestimentiferan tubeworm, *Riftia pachyptila* (Fig. 2.1d, Plate 3d), grows up to 1.5 m in length. It absorbs dissolved sulfide and oxygen from the ambient bottom water with a feathery plume (the obturaculum) and delivers the gases through its circulatory system to the trophosome, a specialized organ housing thiotrophic endosymbionts. *Riftia* has a leathery tube that allows it to flex and relocate its plume among water masses that are variably sulfidic or oxygenated. Other species (Fig. 2.1e, Plate 3e) have rigid tubes that penetrate deeply into anoxic sediments, allowing absorption of sulfides through the worm's posterior end (Freytag et al. 2001). The vent mussel, *Bathymodiolus azoricus* (Fig. 2.1f, Plate 3f), is more versatile, because it absorbs sulfide and methane from venting waters and hosts thiotrophic and methanotrophic endosymbionts in its gills. Soon after the discovery of hydrothermal vents, related taxa were found at cold-water hydrocarbon seeps, in anoxic basins, on whale- and wood-falls, and in coastal reducing sediments (reviews in Sibuet and Olu 1998; Tunnicliffe et al. 1998; Smith and Baco 2003). I subsequently use the term "chemosynthetic" (as defined in Dubilier et al. 2008) to describe these ecosystems and the animals and microbes that are supported mainly by thiotrophic (i.e. chemolithoautotrophic) or methanotrophic (i.e. chemoorganoautotrophic) primary production.

Marine chemosynthetic symbioses have been characterized now from seven invertebrate phyla (Dubilier et al. 2008). Though many invertebrate taxa have independently evolved chemosynthetic symbioses, six families dominate the biomass at deep-sea vents, seeps, wood- and whale-falls: polychaete annelids in the families Siboglinidae and Alvinellidae; bivalve molluscs in the families Vesicomyidae and Mytilidae; gastropod molluscs in the family Provannidae; and decapod crustaceans in

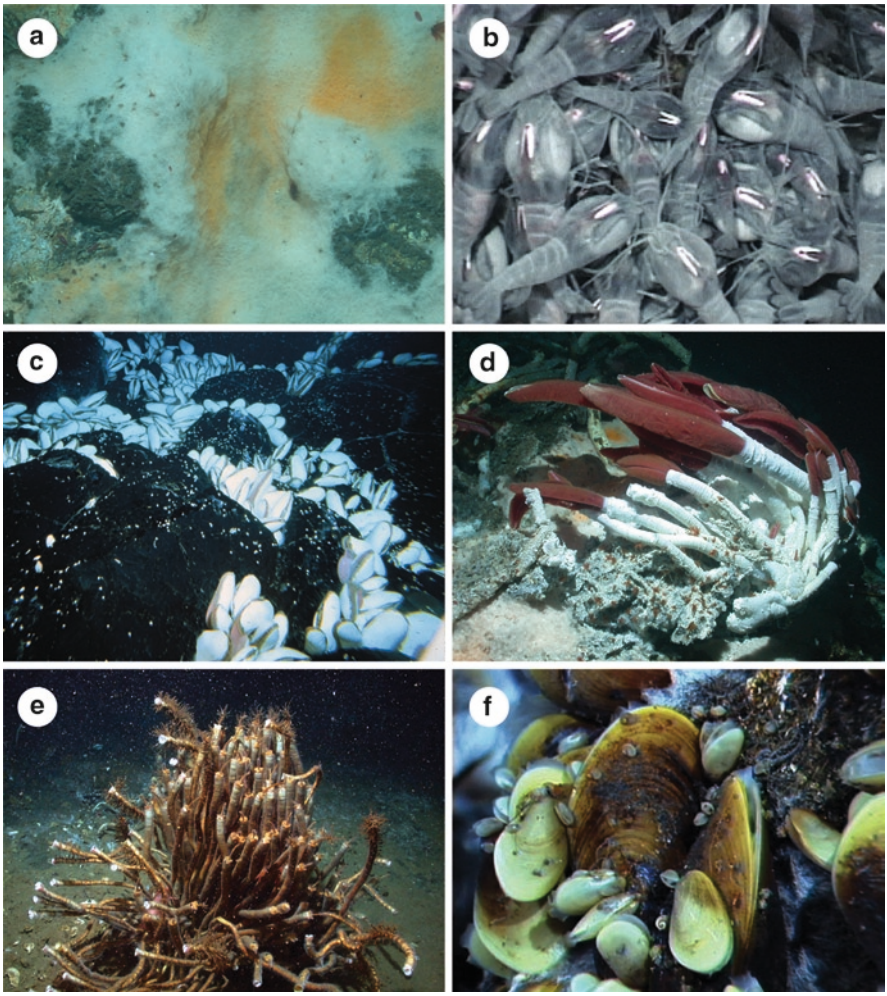

Fig. 2.1 Chemosynthetic taxa found at hydrothermal vents and seeps. (**a**) *Beggiatoa* mat growing on sulfides in the Gulf of California. (**b**) *Rimicaris* shrimp swarming at a hydrothermal vent on Central Indian Ridge. (**c**) *Calyptogena magnifica* clams clustering in basaltic cracks at 21°N latitude on the East Pacific Rise. (**d**) *Riftia pachyptila* cluster growing at the base of a hydrothermal vent chimney in the Gulf of California. (**e**) A tubeworm cluster composed of *Escarpia spicata* and *Lamellibrachia barhami* growing in soft sediments at cold seeps in the Gulf of California. (**f**) A cluster of *Bathymodiolus azoricus* mussels (courtesy of C. Van Dover) from the Snake Pit vent locality on the Mid-Atlantic Ridge. A color plate of this figure can be found in Appendix (Plate 3)

the shrimp family Alvinocarididae. A variety of ε-Proteobacteria dominate the episymbiont populations associated with decapod crustaceans, alvinellid worms and provannid snails. *Alvinella pompejana* worms host a complex array of ε-Proteobacteria on their bodies (Cary et al. 1997). These and other bacteria pack the gill chamber of *Rimicaris* shrimp (Zbinden et al. 2008). The "hairy" snail, *Alvinoconcha hessleri*, is also associated with external ε-Proteobacteria (Urakawa et al. 2005). Filamentous ε-Proteobacteria form dense tufts that attach to long hair-like structures on the legs of

the Yeti crab, *Kiwa hirsuta* (Goffredi et al. 2008). The roles that these episymbionts play in providing nutritional support to their hosts remains unclear in most cases, and too little is known about their biogeographical diversity to consider these bacteria further in the present context. Current information about the phylogenetic diversity of bacterial epi- and endosymbionts associated with decapod crustaceans, the bivalve families Solemyidae, Lucinidae and Thyasiridae, the gastropods families Provannidae and Lepetodrilidae, and a variety of annelids, sponges and protists can be found elsewhere (Bright and Giere 2005; DeChaine and Cavanaugh 2005; Cavanaugh et al. 2006; Dubilier et al. 2008). Because more is known about their transmission modes, genetics and evolution, I focus instead on the chemosynthetic γ-Proteobacteria associated with three taxa, the vesicomyid clams, vestimentiferan tubeworms and bathymodiolin mussels. A comparison of genetic, demographic and evolutionary processes affecting these endosymbionts and their hosts should provide a foundation for assessing lesser-known symbioses in the other taxa.

The consequences of various symbiont transmission modes must be considered with respect to one of greatest challenges facing deep-sea chemosynthetic organisms – finding suitable island-like habitats in vast ocean basins. Hydrothermal vents are distributed intermittently along global mid-ocean ridge system, in back arc basins and on volcanically active seamounts (Van Dover et al. 2002). Distances between active vent fields are typically on the order of tens to hundreds of km, and isolation-by-distance affects some vent taxa with relatively limited dispersal distances (Vrijenhoek 1997). Topographical structures such as large transform faults displace adjacent spreading centers and limit opportunities for along-axis dispersal (Young et al. 2008). Hydrocarbon seeps are distributed as discrete localized patches, mostly along continental margins (Sibuet and Olu 1998). Whale carcasses occur at breeding and feeding grounds and along migratory pathways, and wood-falls that exist mostly along terrestrial margins (Smith and Baco 2003; Pailleret et al. 2007).

These chemosynthetic habitats are variably ephemeral. The vent fields found along rapidly spreading ridge axes persist for only a few decades before they are eliminated, but fields found at slow-spreading axes can persist for thousands of years (Lalou and Brichet 1982; Lalou et al. 1993). Seeps probably are not as ephemeral, but they tend to be patchy, even at local scales (Barry et al. 1996). Whale-falls last for varying periods depending apparently on depth, dissolved oxygen and the assemblage of decomposer organisms (Smith and Baco 2003; Braby et al. 2007). Metapopulation processes, governed by the frequency of local habitat extinctions, sources of colonizing species, and rates of dispersal will affect both the richness of species and the diversity of genotypes found in chemosynthetic communities (Vrijenhoek 1997). Perhaps many of the chemosynthetic organisms that live at ephemeral vents and whale-fall habitats should be considered "weedy species" (sensu Baker 1965) that must grow fast, reproduce early and disperse effectively to colonize new habitats before their local habitat is extinguished. On the other hand, some seep habitats appear to support chemosynthetic animals that grow very slowly and live long, raising the possibility that these animals and their associated microbes are subject to very different metapopulation processes (Berquist et al. 2000).

2.2 Some General Consequences of Symbiont Transmission Modes

The modes by which symbionts are transmitted to offspring will profoundly affect the demography and evolution of the microbes and their hosts. Vertical transmission from parent to offspring (Fig. 2.2a) can occur via large gametes such as eggs (transovarial), by subsequent inoculation of retained zygotes or larvae in brooding organisms, or by feeding as in some social insects (Douglas 1989). Horizontal transmission (Fig. 2.2b) can occur directly via host-to-host transfers or indirectly via infections by a free-living stage of the microbe. Among the chemosynthetic invertebrates that dominate vent, seep, and whale-fall communities, transovarial transmission has been convincingly documented only for vesicomyid clams (Endow and Ohta 1990; Cary and Giovannoni 1993), though it is suspected in solemyid bivalves (Krueger et al. 1996). Horizontal transmission via an apparently free-living infectious phase (also known as environmental acquisition) has been convincingly documented in vesti-

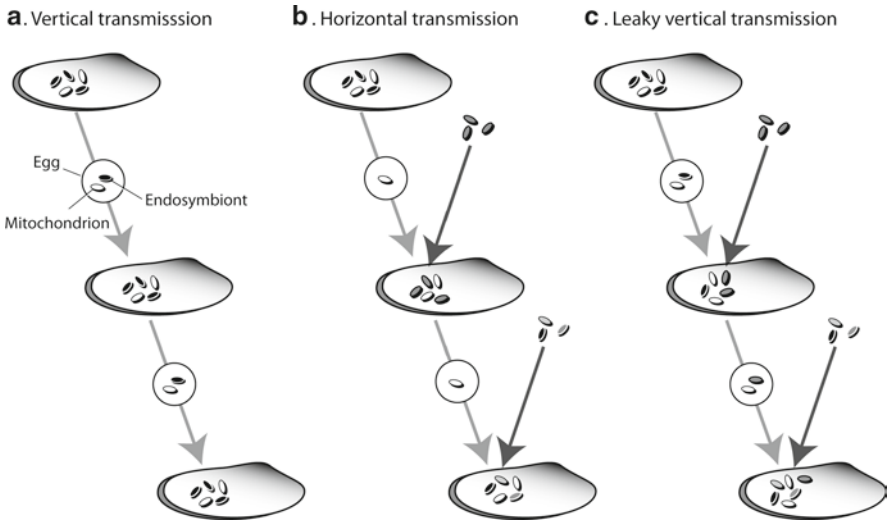

Fig. 2.2 Transmission modes that have been documented or inferred in various chemosynthetic organisms, illustrated for a hypothetical bivalve mollusc. The white ovals represent mitochondria. The black and shaded ovals represent endosymbiotic bacteria. (**a**) Under obligately vertical transmission, the symbiont is inherited maternally along with the mitochondria, leading to genetic coupling of mitochondrial and endosymbiont genotypes. (**b**) Under horizontal transmission, the bacteria are acquired anew in each generation, decoupling the cytoplasmic organelles from bacterial genotypes. Furthermore, infections involving environmental bacteria will likely lead to mixed symbiont genotypes (strains) within a host. (**c**) Leaky vertical transmission is predominantly vertical with occasional environmental acquisition, or vertical with massive environmental swamping. In either case, the vertical transmission component will create a small lag-time in the decoupling of host mitochondrial and symbiont genotypes. The horizontal component, depending how prevalent, can create mixed-strain infections. A color plate of this figure can be found in Appendix (Plate 4)

mentiferan tubeworms. Mixed transmission modes (Fig. 2.2c) also exist, in which vertical transmission is supplemented by host-to-host transfers or de novo environmental infections with free-living bacteria. Before critically evaluating what we know about symbiont transmission in deep-sea chemosynthetic organisms, it will be helpful to consider some general consequences of various transmission modes.

2.2.1 *Symbiont Assurance Versus Locally Optimal Symbionts for the Host*

Vertical transmission provides the host with symbiont assurance. Dispersing propagules arriving at a nascent vent, seep or whale-fall habitat already carry the right kind of chemosynthetic symbiont needed to grow and prosper. Nonetheless, symbiont assurance carries a hidden cost because dispersing propagules might not carry the optimal bacterial strain for the particular habitat in which they settle. In contrast, host species that acquire symbionts after settlement have the potential to adopt bacterial strains that are optimally adapted to the local environment (Won et al. 2003b). Yet, environmental acquisition poses a number of risks. Perhaps most significantly, dispersing host propagules might fail to be infected altogether. Environmental acquisition can also lead to mixed symbiont genotypes within a host individual creating opportunities for the evolution of selfish "cheaters" that exploit the host by providing less nutrition (Frank 1996). Finally, environmental acquisition creates potential pathways for infection that can be exploited by pathogens. Host species employing leaky vertical systems of transmission still face the risks of infection by cheaters and pathogens, but they also enjoy the benefits of symbiont assurance and the capacity to acquire locally optimal strains.

2.2.2 *A Dispersal Benefit for the Symbionts*

If microbial dispersal is limited in deep-ocean basins, vertically transmitted symbionts might benefit by hitchhiking along with host propagules, thereby gaining access to new chemosynthetic habitats. A persistent dictum in microbiology is that "everything is everywhere, and nature selects" (Beijerinck 1913), but molecular studies have made it apparent that microbial populations are often spatially subdivided (Papke et al. 2003; Whitaker et al. 2003). Local-scale differentiation can result from limited dispersal abilities of free-living microbe (Harvey and Garabedian 1991; Schloter et al. 2000). For example, isolation-by-distance occurs on very small scales for *Pseudomonas* (Cho and Tiedje 2000), but local differences might not be adaptively neutral. The fitness of *Bacillus* strains decays exponentially when strains are grown in soil extracts taken only meters away from their home site (Belotte et al. 2003). Living in many places at the same time decreases the risk of extinction due to local catastrophes, so the dispersal benefit provides an advantage to facultatively vertical

symbionts that recycle to the ambient environment. The evolutionary fate of obligately vertical symbionts, however, is linked inexorably to the fate of their host.

2.2.3 Genetic Consequences of Transmission Modes

Obligately vertical transmission (Fig. 2.2a) is expected to result in genetic homogeneity of the symbiont lineages found within host individuals. The theoretical framework developed to explain the clonality of transovarial symbionts was developed for the maternally inherited cytoplasmic genomes of the chloroplasts and mitochondria in eukaryotes (Birky et al. 1983, 1989). Individual animals typically possess only one haploid mitochondrial genotype (haplotype) that was inherited from the mother, a state referred to as homoplasmic. Countless numbers of mitochondria exist among the cells of an individual, but only the germ-line mitochondria are transmitted to eggs. A series of intra-individual bottlenecks occur as germ-line cells divide and sort a subset of the mitochondria into daughter cells. For example, after about 25 cell divisions in the germ-line of a *Drosophila* fly, genetic drift among its mitochondrial genomes will result in eggs that are homoplasmic. The rate at which homoplasmy is reached in a species will be a function of the mean number of mitochondria transmitted during each cell division and the number of germ-line cell divisions. Transovarial endosymbionts will similarly achieve clonal homogeneity within a host lineage at a rate determined primarily by its transmission numbers through each egg generation of the host (Rispe and Moran 2000).

Horizontal transmission, on the other hand, can retain symbiont heterogeneity (mixed infections), depending on several factors. The diversity of mixed strains within host individuals will be a function of the diversity of infectious strains in the ambient environment or in the co-occurring pool of hosts if transmission is contagious. Intra-host symbiont diversity should also be affected by length of time that a window for infection remains open during host development. In the squid-*Vibrio* symbiosis this window for infection is open for a very brief "permissive" phase during early embryogenesis, which limits the number of bacteria establishing a symbiotic association (reviewed by Nyholm and McFall-Ngai 2004). A brief permissive phase also occurs in the larvae of vestimentiferan tubeworms, but the entire lifespan of bathymodiolin mussels might be permissive to infections (discussed below).

Vertical and horizontal symbionts are expected to differ greatly in their capacity to acquire foreign genetic material through lateral gene transfer or homologous recombination. The clonality of obligately vertical symbionts greatly limits these opportunities unless occasional leakage of symbionts occurs between hosts (Stewart et al. 2008). Nonetheless, natural selection can only discriminate among the recombinational variants that are permissible within the obligate host's environment. This limitation on the scope for selection also exists for horizontally infectious symbionts that lack a free-living phase altogether. In contrast, facultatively vertical and horizontal symbionts that retain a free-living phase will be exposed to mixed infections and thereby have greater opportunities for recombination, increasing the scope for selection (Jiggins et al. 2001).

2.2.4 Demographic Consequences of Transmission Modes for the Symbionts

Transovarial transmission dramatically reduces the genetically effective size (N_e) of the symbiont population. The bottlenecking process reduces N_e to N_{fem}, the effective number of breeding females in the host population (Rispe and Moran 2000). This reduction has profound evolutionary consequences. According to the "Nearly Neutral" theory of molecular evolution, genetic drift in small populations leads to the fixation of slightly deleterious mutations and a concomitant acceleration of nucleotide substitution rates (Ohta 1987). Bacterial genomes are predominantly clonal, so they also are subject to Muller's ratchet – i.e. mutational decay due to an accumulation of slightly deleterious mutations in small populations (Muller 1964). Indeed, nucleotide substitution rates are faster in the transovarial *Buchnera* endosymbionts of aphids than in their free-living counterparts (Moran 1996). The observed mutations are considered slightly deleterious because they are expected to destabilize the secondary structure of rRNA molecules (Lambert and Moran 1998) and increase amino acid substitutions in protein-coding loci (Wernegreen and Moran 1999).

Conversely, horizontal symbionts are expected to exhibit lower substitution rates than vertical symbionts because they have potentially much larger population sizes. If the bacteria recycle between host-associated and free-living phases, then N_e will be equal the sum of the effective number of bacteria transmitted to the next generation through each phase ($N_{sym} + N_{free}$). However, if the symbionts are entirely devoured by the host and fail to recycle, then $N_e = N_{free}$. Alternatively, if the bacteria do not reproduce in a free-living phase, but only occur as spores or dormant infectious stages, then $N_e = N_{sym}$. These demographic considerations warrant further investigation because the fitness contributions of these bacterial life phases will likely determine possible paths of coevolution (see for example Genkai-Kato and Yamamura 1999).

2.2.5 Cytoplasmic Cotransmission and Genetic Hitchhiking

Transovarial symbionts of animals are inherited maternally in tandem with other cytoplasmic factors such as mitochondria (Funk et al. 2000). Cytoplasmic cotransmission effectively couples the host and symbiont genomes (Fig. 2.2a), which is manifested as covariance (gametic phase disequilibrium) in the frequencies of host mitochondrial and symbiont genetic polymorphisms (Hurtado et al. 2003). In contrast, meiosis randomly shuffles the nuclear genome in sexually reproducing populations; consequently, nuclear polymorphisms should not exhibit associations with cytoplasmic genes (cytonuclear disequilibrium). Natural selection can tighten associations between cytoplasmically cotransmitted factors. A beneficial new mutation arising in a symbiont genome might lead to a selective sweep that carries along a

cotransmitted mitochondrial haplotype (Hurst and Jiggins 2005), a process known as genetic hitchhiking (Maynard Smith and Haigh 1974). Conversely, a selective sweep involving a beneficial mitochondrial variant will cotransmit a coupled symbiont genome. In either case, one might expect lower genetic diversity among the cytoplasmic factors of species with vertically transmitted obligate symbionts (Hurst and Jiggins 2005).

2.2.6 Host-Symbiont Cospeciation

As a host species spreads geographically and splits during cladogenic events, the obligately vertical (heritable) symbiont lineages will split, as well. Cladogenic events in the host and symbiont lineages will be congruent and their phylogenetic trees should exhibit parallel topologies and congruent evolutionary ages of the internal nodes (Huelsenbeck et al. 1997). With time and increasing divergence, hybridization becomes improbable, and cospeciation is inevitable, unless occasional leakage of symbionts occur via host transfers. Once reproductive isolation completely blocks opportunities for hybridization and recombination among the nuclear genomes of the host species, these genes too will begin to covary with changes in the genes of heritable symbionts.

Though it is more likely, cospeciation does not require vertical transmission. Obligate horizontal symbionts can also speciate in synchrony if splitting events involve geographic isolation. For example, chewing lice are obligate parasites of pocket gophers (Hafner and Nadler 1988). The lice spread by host-to-host transfers, so allopatric speciation events in the host will also lead to corresponding isolation of the parasites. Tight adaptive constraints forced on obligate horizontal symbionts by their specific hosts also contributes to cospeciation (Clayton et al. 2003). Caution is warranted, however, in considering reported examples of cospeciation in horizontal symbionts. For example, Atlantic and Pacific species of sepiolid squid host corresponding Atlantic and Pacific strains of luminescent *Vibrio* endosymbionts that are acquired infectiously (Nishiguchi et al. 1998). Though extraordinary evidence for coevolution has been documented in the squid-*Vibrio* association, parallel phylogenies might result from underlying geographic covariance, which should always be factored-out of studies on host–symbiont cospeciation (Nieberding et al. 2008; Won et al. 2008).

2.2.7 Symbiont Genome Reduction

Genome reduction is expected when endosymbionts become vertical and lose their free-living phase entirely (Andersson 2006; Pál et al. 2006). Vertical symbionts are enslaved in the host environment and face no selective pressures to maintain unnecessary or redundant functions. Mitochondria, for example, are cytoplasmic organelles descended from proteobacterial endosymbionts (Gray et al. 1999). Animal

mitochondria retain a small number of functional genes that encode a minimal set of transfer RNAs, large and small subunit ribosomal RNAs and some essential respiratory chain proteins. The genes required for replication of mitochondrial DNA and metabolic functions exist in the cell's nucleus. When free-living bacteria make the transition to obligate intracellular symbionts, genes that were needed to survive in the external environment may no longer be useful in the intracellular environment and can therefore degenerate or be deleted. Adaptively neutral gene losses will accumulate because of genetic drift, and slightly deleterious deletions can accrue due to the actions of Muller's ratchet in small populations, but deletion mutations might benefit the symbionts if they lead to more efficient replication.

Significant reductions in genome size are well documented in a number of obligate, intracellular pathogens and mutualists (reviewed in Moran 2002). Obligate nutritional endosymbionts of insects provide extreme examples among the γ-Proteobacteria. *Buchnera aphidicola* strain BCc, which infects aphids, has only ~416 Kb of DNA encoding ~362 proteins (Perez-Brocal et al. 2006), and the *Carsonella rudii* strain that infects psyllids is even smaller, having only ~160 Kb of DNA encoding 182 proteins (Nakabachi et al. 2006). For comparison, free-living *Escherichia coli* contain an order of magnitude more DNA, ~4,600 Kb and ~4,300 protein-coding genes (Blattner et al. 1997). The highly reduced insect endosymbionts obtain most of the compounds needed for intermediate metabolism from their host cells, and many of the genes responsible for DNA replication and cell membrane components are missing. Shifts in nucleotide composition to higher AT content and a loss of genes involved in DNA repair are hypothesized to accelerate genomic erosion in these obligate symbionts (Moran et al. 2009). Nonetheless, *Buchnera* and *Carsonella* retain subsets of genes responsible for the biosynthesis of critical amino acids that their hosts are unable to obtain from strict diets of plant saps.

In contrast, horizontally transmitted and facultative endosymbionts that retain a metabolically active free-living stage are expected to retain the genes that control motility, cell division, and cellular metabolism. Indeed, some horizontal symbionts have evolved enlarged genomes to deal with the diverse contingencies of living in the host-associated and ambient environments. For example, a horizontally transmitted nitrogen-fixing rhizobium associated with legumes contains an enlarged chromosome and plasmids that carry accessory genes involved in nitrogen fixation, nodule formation and proteins that facilitate the infection of root tissues (reviewed in Downie and Young 2001).

2.3 Vesicoymid Clams and Vertical Transmission

The vesicomyid clam, *Calyptogena magnifica* Boss and Turner (1980), has a reduced digestive system and obtains its nutrition from the thiotrophic γ-proteobacterial endosymbiont, Candidatus *Ruthia magnifica* Newton et al. (2007), which lives in the clam's gills. Endow and Ohta (1990) were first to suggest that vesicomyid symbionts are vertically transmitted. They identified microscopic, rod-shaped, bacterial

inclusions in the primary oocytes and follicle cells of *C. soyoae*. Earlier oogonial stages did not contain the inclusions; so, the authors hypothesized that a transmission stage of the gill symbiont is delivered to follicle cells and eventually to eggs during a narrow developmental window. Soon after, Cary and Giovannoni (1993) examined bacterial 16S rRNA sequences from vesicomyid symbionts and verified the presence of these sequences in ovarian tissues of *C. magnifica* and several other vesicomyids. Their symbiont-specific 16S probes hybridized with the bacterial inclusions in the follicle cells, but unlike Endow and Ohta, they saw no inclusions in the oocytes. Cary and Giovannoni (ibid. pp. 5698–5699) suggested, "nutritive [follicle] cells would provide the ideal mechanism for inoculation of the developing eggs." To date, the mechanisms that these clams use to inoculate their eggs or adhering cells remain a mystery, as do pathways they might use to translocate a putative transmission stage of the symbiont from bacteriocytes in the gill to follicle cells in the ovary.

2.3.1 Symbiont Homogeneity and Genetic Drift

The number of symbionts transmitted through a clam's eggs also is unknown, but the transmitted bacteria are undoubtedly a miniscule subset of the number that lived in the clam's gills. Bottlenecking and genetic drift during translocation to the ova are inevitable; so symbiont clonality is expected. Conversely, an observation of mixed symbiont genotypes within a host individual suggests horizontal transmission or occasional symbiont leakage. As predicted, the vertically transmitted symbionts of vesicomyid clams appear to be clonal within individuals of nearly all the vesicomyid species examined to date. Hurtado et al. (2003) examined *R. magnifica* symbionts from five hydrothermal vent areas along the Galápagos Rift (GAR) and East Pacific Rise (EPR) between 21°N and 17°S latitude. All the clams examined from this 4,800 km range hosted a single 16S rRNA phylotype, but the ITS region of the ribosomal operon exhibited 11 subtypes that varied within and among the sampled geographical localities. Nonetheless, each individual clam hosted only a single subtype. Five ITS subtypes were found in different clams from the 17°S EPR locality, so mixed genotypic infections should have been detectable had they occurred there. Goffredi et al. (2003) similarly examined the symbionts hosted by three morphologically indistinguishable species of the *Vesicomya* (=*Calyptogena*) *pacifica* cryptic-species complex. Though these clams occupy a variety of vent and cold seep habitats along the western American margin, the species segregate mostly according to depth. Each of the clams examined in this study was associated with only one host-specific ITS genotype. A caveat exists, however, regarding the detection of ITS heterogeneity via the polymerase chain reaction (PCR), a run-away process that depends on primer specificity and the starting numbers of various target sequences in a potential mixture of sequences. If a minority genotype occurs in a mixture, it might not amplify in sufficient quantity to be evident in DNA sequence traces or in studies that involve cloning of PCR products (e.g., Stewart et al. 2008).

As previously argued, rates of Nearly Neutral nucleotide substitution should increase in small populations. Consequently, vertical symbionts that have very low N_e are expected to exhibit accelerated evolutionary rates compared to free-living bacteria. Peek et al. (1998a) found that vesicomyid endosymbionts have significantly accelerated substitution rates for 16S rRNA sequences. Nearly Neutral theory also predicts that mutations should be more pronounced in regions of molecules that are subject to lower selective constraints. As expected, substitution rates were consistently greater in the unconstrained loop regions of the rRNA molecule versus the pair-bonded stem regions. Various domains of the rRNA molecule also experience different selective constraints. Domain II for example is highly constrained and no significant difference in substitution rates existed between vertical symbionts and free-living bacteria. Domains I and III, however, are less constrained and there the majority of substitutions fell into the loop regions.

2.3.2 Cytoplasmic Cotransmission and Rare Leakage

Obligately vertical symbionts are inherited maternally along with other cytoplasmic factors such as mitochondria, which should lead to gametic phase disequilibrium between these factors. Nuclear polymorphisms, on the other hand, should not exhibit cytonuclear disequilibrium in a randomly mating population. Hurtado et al. (2003) examined these predictions in a *Calyptogena magnifica* population from 17°S along the EPR (Table 2.1). As predicted, the symbiont ITS polymorphisms were significantly associated with mitochondrial COI haplotypes, whereas the nuclear alleles varied independently of both cytoplasmic factors.

Cytoplasmically cotransmitted genomes can be decoupled, however, if vertical transmission is leaky (Fig. 2.2c). Stewart et al. (2008) reported that an unnamed member of the *V. pacifica* cryptic species complex (sp. mt-II) was infected with a foreign vesicomyid symbiont. They examined 118 clams from the Endeavor segment (47°58′N) of the Juan de Fuca Ridge and all but two of the clams hosted symbiont type A (*symA*), which occurs elsewhere in *V.* sp. mt-II. The two unusual clams hosted a highly divergent strain (*symB*). Recent evidence based on strain-specific

Table 2.1 Likelihood ratio contingency tests of gametic phase disequilibrium between host and symbiont genetic markers. Three tests were conducted: (i) symbiont versus host mitochondria; (ii) host nuclear versus host mitochondria; and (iii) host nuclear versus symbiont. Expected numbers appear in parentheses. Each gametic combination is labeled with *S* representing 17°S-endemic variants and *N* representing alternative variants occurring in northern populations (From Hurtado et al. 2003)

		Frequency of gametic combinations					
Test	Contrast	*N/N*	*N/S*	*S/N*	*S/S*	$G_{(1\ df)}^2$	*P*
(i)	*sym*ITS/*mt*COI	8 (6.1)	0 (1.9)	21 (22.9)	9 (7.1)	4.951	0.026*
(ii)	*Cmg24/mt*COI	5 (5.9)	3 (2.1)	47 (46.1)	15 (16.0)	0.612	0.434
(iii)	*Cmg24/sym*ITS	2 (1.6)	6 (6.4)	12 (12.4)	50 (49.6)	0.134	0.714

PCR primers and pyrosequencing methods indicates that mixed (*symA/symB*) infections are more frequent than reported in the earlier study (Stewart and Cavanaugh 2009). The source of *symB* infections and the mechanism of host transfer remain unknown, but the evidence of symbiont leakage in vesicomyids is intriguing. Rare horizontal transfers create possibilities for genetic recombination among endosymbiont strains. Recombination could obscure patterns of cospeciation and generate variability that might act to retard genome reduction in these symbionts (Stewart et al. 2009). Nonetheless, recent genomic studies reveal significant reductions genome size and strongly support the hypothesis that vertical transmission is the predominant mode of symbiont acquisition in vesicomyids (summarized below).

2.3.3 Cospeciation

Despite the potential for symbiont leakage in some vesicomyids a general pattern of host-symbiont cospeciation is observed. Peek et al. (1998b) found that the 16S rRNA gene tree derived from the bacterial endosymbionts was not significantly different in topology from the mitochondrial COI gene tree derived from nine vesicomyid species (Fig. 2.3). Some minor discrepancies exist

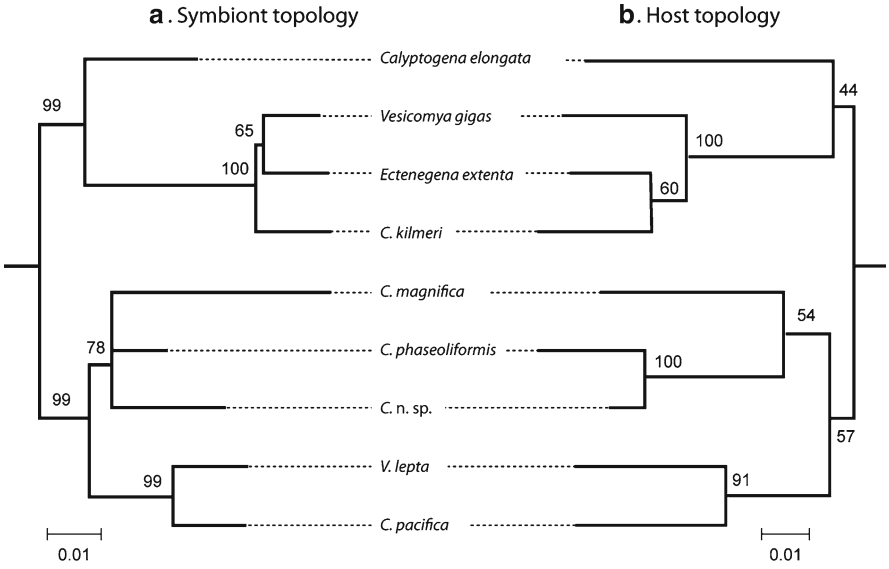

Fig. 2.3 Cospeciation between endosymbionts and nine vesicomyid clams species (Redrawn from Peek et al. 1998b). (**a**) The maximum likelihood (ML) phylogram for the bacteria was based on 16S rRNA sequences. (**b**) The ML phylogram for the vesicomyids was based on combined data from portions of the mitochondrial COI gene and the large subunit 16S rRNA gene. The numbers at nodes represent bootstrap support values. See original publication for statistical tests of cospeciation

between the two trees, but the differences can be more parsimoniously explained by statistical uncertainties in the inferred trees rather than symbiont leakage. Additional gene sequences involving more clam species are currently being examined to produce a more robust host tree (A. Audzijonyte, personal communication, 2010). All vesicomyid symbionts that have been examined to date comprise a discrete monophyletic clade that excludes related γ-Proteobacteria found in other bivalves or local marine environments (Dubilier et al. 2008). So, if occasional leakage occurs it must result from host-to-host transfers within the Vesicomyidae and not from environmental infections by free-living strains or host transfers from other molluscan families.

This vesicomyid-endosymbiont association appears to be young compared to the 200 million year old association between aphids and *Buchnera* endosymbionts (Moran et al. 1993). The oldest confirmed vesicomyid shells (late middle Eocene, 45 million years ago) are attributable to the genus *Archivesica* (summarized in Amano and Kiel 2007). Two other genera also appeared during the late Eocene, so the last common ancestor of all vesicomyids must have lived at least 45 million years ago. These fossil data are consistent with molecular estimates for a relatively young common ancestor for vesicomyids, 22–44 million years ago (Peek et al. 1997; Little and Vrijenhoek 2003). Previously, these estimates of evolutionary age appeared to be at odds with reports of "vesicomyid" shells from Cretaceous cold seep deposits in Japan (Kanie et al. 1993; Kanie and Nishida 2000), but this fossil evidence for ~100 million year old vesicomyids is now disputed (Amano et al. 2008). New fossil specimens from Hokkaido, Japan, revealed that a putative Cretaceous "*Vesicomya*" lacks the characteristic hinge morphology distinguishing vesicomyids from other heterodonts and is instead a member of the family Lucinidae (Amano et al. 2008). Other putative Cretaceous vesicomyids appear to be misidentified solemyid shells (Kiel et al. 2008). For now, the fossil and molecular evidence together suggest that a conservative estimate for the origin of vesicomyid clams is about 45 million years ago. A molecular clock method for estimating the evolutionary age of the vesicomyid symbionts is difficult to develop, but a current estimate broadly encompasses this young age (Peek et al. 1998b).

2.3.4 Genome Reduction

An evolutionary history of obligately vertical transmission is expected to result in genomic reduction for the symbionts. Two complete genomes have been sequenced, to date – the endosymbionts *Ruthia magnifica* from *Calyptogena magnifica* (Newton et al. 2007) and *Vesicomyiosocious okutanii* from *Calyptogena okutanii* (Kuwahara et al. 2007). When compared to the free-living thiotroph, *Thiomicrospira crunogena*, the two symbiont genomes are roughly half the size, have less than half of the protein-coding genes, and have reduced GC contents (Newton et al. 2008). Genes for externally expressed structures such as flagella and pili are missing in the symbionts. The absence of *ftsZ* and related genes in *V. okutanii* (Kuwahara et al.

2007) suggests that the bacterium has ceded control of cell division to the host. Nonetheless, the symbionts retain most of the metabolic functions of *T. crunogena* and have added genes that encode various sulfur oxidation pathways. Both the number of protein-coding genes and order of these genes in the genome appear to be highly conserved between *R. magnifica* and *V. okutanii*. Differences exist, however, for genes that encode cell envelope components and proteins involved in nitrate reduction. Comparative genomics involving additional vesicomyid endosymbionts will help to resolve questions regarding the phylogenetic pathways of genome reduction and gene retention in these bacteria. Whole genome studies might also help to resolve whether the apparent "leakage" reported for *Vesicomya* cf. *pacifica* sp II has contributed to recombination among the symbiont lineages in different hosts, thereby providing some resistance to the genomic degradation of these symbionts (Stewart et al. 2009).

2.4 *Riftia* and Environmental Infections

Adults of the vestimentiferan tubeworm *Riftia pachyptila* Jones (1981) lack a digestive system entirely and have instead a specialized organ, the trophosome, that houses the sulfur-oxidizing, intracellular, γ-Proteobacteria endosymbiont, Candidatus *Endoriftia persephone* Robidart et al. (2008). *Riftia* acquires its *E. persephone* symbionts horizontally from the local environment in which the worm larvae settle. The rod-shaped symbionts are not observed in *Riftia*'s eggs, sperm or freshly settled larvae (Cavanaugh et al. 1981; Cary et al. 1989, 1993; Nussbaumer et al. 2006). Molecular probing suggests that bacteria bearing very similar, if not identical, 16S rRNA sequences occur in sea-water and biofilms from the hydrothermal vent environment (Harmer et al. 2008), but direct linkages to a competent infectious phase have not been made. In a landmark field experiment, Nussbaumer and coworkers (2006) found that freshly settled vestimentiferan larvae are free of endosymbionts (aposymbiotic) and have a rudimentary gut. Unlike frenulate siboglinids that appear to acquire endosymbionts via the digestive tract (Southward 1988), *Riftia* larvae are infected by rod-shaped bacteria that penetrate the epidermis. Then the bacteria migrate to the dorsal mesentery where they are enclosed in vacuoles within mesodermal cells, a process that initiates development of the trophosome and metamorphosis to a juvenile stage, when subsequent infections are halted by massive apoptosis of the skin.

2.4.1 Absence of Cospeciation

If the environmental infection model of Nussbaumer et al. (2006) can be generalized to all vestimentiferans, symbiont phylotypes should be associated with local environments where the worm larvae settle rather than a particular host species.

Indeed, no evidence is found for cospeciation between vestimentiferan hosts and their symbionts (Feldman et al. 1997; Nelson and Fisher 2000; McMullin et al. 2003). Host and symbiont phylogenies are broadly incongruent (Fig. 2.4). Instead, the symbionts are associated with types of habitats (basaltic vents versus sedimented seeps) and broad biogeographical regions. The bacterial 16S sequences amplified from the trophosomes of all the vestimentiferan species examined to date reveal two primary phylotypes of the thiotrophic symbiont (Feldman et al. 1997; Di Meo et al. 2000; Nelson and Fisher 2000; McMullin et al. 2003; Vrijenhoek et al. 2007). Sequence divergence between phylotypes I and II is 4.3% on average, so they might be considered legitimate bacterial species according to the criteria of many microbial systematists (e.g., Stackebrandt and Goebel 1994). This degree of sequence divergence suggests that phylotypes I and II diverged more than 200 million years ago (Feldman et al. 1997), long before the radiation of their vestimentiferan hosts, most probably about 60 million years ago (Black et al. 1997; Chevaldonné et al. 2002; Little and Vrijenhoek 2003). Previous evidence for "vestimentiferan" tubes in Jurassic hydrothermal vent deposits (Little et al. 2004) is now disputed (Kiel and Dando 2009), so it seems likely that these worms have radiated during the Cenozoic Era.

Phylotype I is globally widespread, occurring in five vestimentiferan genera (*Lamellibrachia*, *Escarpia*, *Seepiophila*, *Arcovestia* and *Alaysia*) that occupy cold seep environments in the Atlantic, Gulf of Mexico and Pacific, and occupy vents only in the western Pacific (S. Johnson, MBARI, 2010). Minor 16S subtypes of phylotype I (Fig. 2.4a) appear to segregate according to depth of the seep habitats in the Gulf of Mexico (McMullin et al. 2003). Phylotype II, or *E. persephone* Robidart et al. (2008), is less variable and more narrowly distributed, occurring in four vestimentiferan genera: *Riftia*, *Tevnia*, *Oasisia* and *Ridgeia*, found at eastern Pacific hydrothermal vents. Phylotypes I and II occur in close proximity at vents and cold seeps in the Gulf of California, but were never found together in the same habitat (Vrijenhoek et al. 2007). On the other hand, some vestimentiferan species will switch symbiont phylotypes based on the habitats in which their larvae settle. The eastern Pacific seep worm *Escarpia spicata* ordinarily hosts phylotype I, but specimens found adjacent to a hydrothermal vent in the Gulf of California hosted phylotype II, just like its *Riftia* neighbors (Di Meo et al. 2000).

2.4.2 Shared Hosts and Mixed Symbionts

According to the environmental infection model, different vestimentiferan species that settle together in the same habitat should be infected by the same local strains of the symbiont. Furthermore, mixed-strain infections are expected within individual hosts if the window for infection is open for an appreciable period during larval development. Indeed, *Riftia*, *Tevnia*, and *Oasisia* sampled from the same eastern Pacific vent fields appear to share the same symbiont phylotype (Feldman et al.

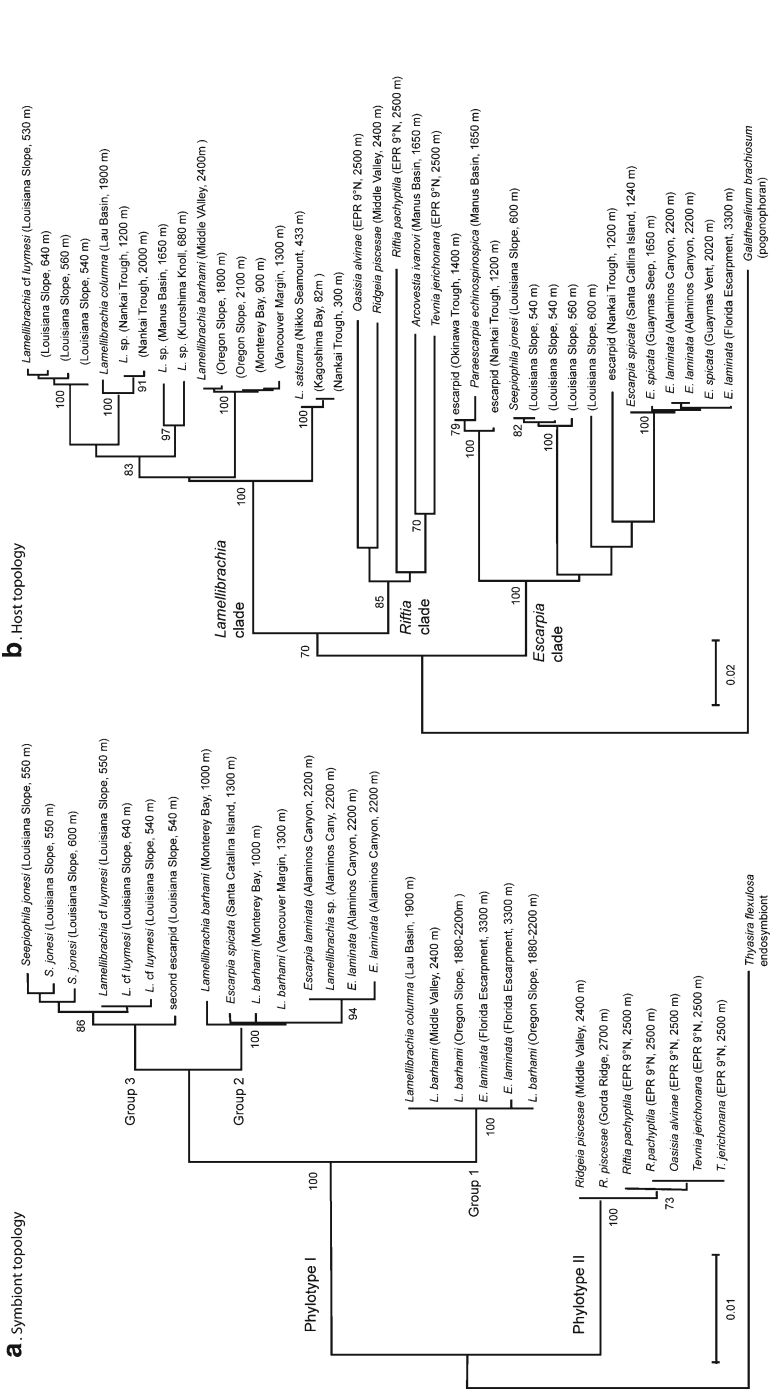

Fig. 2.4 Absence of cospeciation between endosymbionts and vestimentiferan tubeworm species (Redrawn from McMullin et al. 2003). (**a**) Neighbor-joining (NJ) phylogram for the bacteria was based on 16S rRNA sequences. (**b**) NJ phylogram for the worms was based on mitochondrial COI sequences. The numbers at nodes represent bootstrap support values from original publication. Note that the taxa do not correspond between the two trees so no attempt was made to connect hosts and symbionts. Eastern Pacific members of the *Riftia* clade only host symbiont phylotype II. Phylotype I symbionts can be further subdivided into three subgroups

1997; McMullin et al. 2003), but the 16S sequences examined in these studies are too conservative to reflect differences that might exist among co-infecting symbiont strains (Di Meo et al. 2000). Nucleotide sequences from a portion of the RuBisCO gene (*Rbc*) distinguish subtype strains of phylotype I that occur in *Lamellibrachia barhami* and *Escarpia spicata* from the Gulf of California (Vrijenhoek et al. 2007). These two species are commonly found living together in mixed aggregations. Nearly half of the *L. barhami* and *E. spicata* individuals sampled from one of these aggregations also hosted mixed symbiont strains. When aggregations were sampled from discrete environmental patches only tens of meters apart, the frequencies of *Rbc* subtypes differed significantly, but the co-occurring worms hosted the same local strains in the same frequencies. Worms living 7 km apart hosted a distinct *Rbc* subtype, which again was shared by both host species. *Lamellibrachia barhami* and *E. spicata* belong to two very distinct clades that were separated soon after the origin of vestimentiferan siboglinids, yet no evidence was found for discrimination among these subtypes by these highly divergent worm genera.

Though vestimentiferans do not appear to differentiate among local strains of the symbiont, it would be wrong to infer that they are not selective regarding the kinds of bacteria that they incorporate. Only phylotype I bacteria were detected in the *L. barhami* and *E. spicata* specimens examined in our study, so the worm larvae must be highly effective at sequestering the right mutualistic symbiont and rejecting potential pathogens. It seems improbable that the *Rbc* alleles assessed in our study might affect external phenotypes that could be detected by the host during bacterial infections. Nevertheless, the nucleotide substitutions distinguishing these RuBisCO subtypes differ by as many as four amino acids and might not be adaptively neutral if they affect enzyme activities and bacterial maintenance during the symbiotic phase. Additionally, the *Rbc* subtypes might hitchhike along with variation in linked genes that confer local adaptive benefits to the bacteria. Various geochemical factors might favor distinct strains in different environmental patches or at different times. The frequencies of various symbiont strains might also vary spatially within the trophosome of an individual worm as it spans a geochemical gradient. Perhaps the footprints of natural selection still reside in the *Rbc* sequences or those of linked genes. Tests of this adaptive hypothesis are underway in my laboratory. Nonetheless, it also is possible that the observed subtype variation is adaptively neutral and nothing more than a consequence of genetic drift and small-scale subdivision of microbes with very limited dispersal capacity. These horizontal symbionts would not obtain the dispersal benefits afforded by vertical transmission.

2.4.3 Population Size of Horizontal Symbionts

The genetically effective size of horizontal symbiont populations is composed of two components, $N_e = N_{sym} + N_{free}$. A free-living form of *E. persephone* (phylotype II) settles in biofilms on basaltic surfaces and has been filtered from seawater

sampled up to 100 m from an eastern Pacific vent, which "suggests a potentially large environmental pool of symbionts" (Harmer et al. 2008, p. 3897). Genomic evidence (Robidart et al. 2008) also suggests that *E. persephone* can persist as a heterotroph while living in the ambient environment, but the contribution of heterotrophic nutrition to N_{free} is unknown. In addition, no mechanism is known for vestimentiferan endosymbionts to escape the host-associated environment and invade the ambient environment. If the endosymbionts are completely exploited by the host (incorporated, cultivated, and entirely digested), N_{sym} is zero and the host-associated phase contributes nothing to N_e and bacterial fitness. Bacterial co-evolution should not occur under a scenario of complete exploitation, but mathematical models involving very low fitness payoffs ($N_{sym} > 0$) suggest that coevolution is possible for rapidly evolving facultative symbionts (Frean and Abraham 2004). To avoid complete exploitation, some of the host-associated bacteria must escape and re-inoculate the ambient environment or infect other hosts (see Section 2.4.4, below). Perhaps molecular footprints for the evolution of cooperation versus coercion can be found in the genomic and proteomic sequences of *E. persephone* (Robidart et al. 2008). Excellent models for the evolution of cooperation with very low payoffs exist in the plant-rhizobium and aphid-*Buchnera* systems (reviewed by Simms and Taylor 2002; Moya et al. 2008).

Though we do not know how N_{sym} or N_{free} contribute to the total population size of *E. persephone*, a tangential approach offers some insight. Very slow rates of nucleotide substitution are observed in the horizontal endosymbionts hosted mostly by vestimentiferans (Peek et al. 1998a). This deceleration relative to vertically transmitted symbionts could be interpreted as evidence for a greatly enhanced N_e, according to the Nearly Neutral model of molecular evolution. The distribution of mutations observed in various domains of the 16S rRNA molecule is consistent with expectations of the model. An alternative model, however, suggests that infectious horizontal symbionts should decelerate evolutionary rates because they are subject to severe constraints that limit any form of change. According to the Red King model, the slowest runner wins a coevolutionary race involving mutualists (Bergstrom and Lachmann 2003). Changes in a mutualist are perceived as potential threats (an invasive pathogen for example) by the host, so they will be purged by purifying selection. The Red King model is the converse of the Red Queen model (Van Valen 1973) for antagonistic relationships, wherein evolutionary rates are accelerated due to an arms race between the antagonists. Perhaps a good example of a Red Queen process is hypervariability in the externally expressed, central portion of the flagellin gene (*fliC*) of pathogenic *Escherichia coli* (Reid et al. 1999). The pathogen must change its external appearance frequently to stay ahead of the host's capacity to recognize and develop mechanisms to exclude the pathogen. Mutualists, on the other hand, must avoid any changes that might be recognized as threatening; so decelerated nucleotide substitution rates are expected. Though the 16S sequences examined by Peek et al. (1998a) evolved more slowly in the horizontal endosymbionts, these data alone are not sufficient to discriminate between the Nearly Neutral and Red King models. Future studies should examine the evolutionary rates of genes that are externally expressed and potentially involved in

host–symbiont signaling and recognition. Now that the genomes from several vertical and horizontal symbionts have been sequenced, it may be possible to target a number of genes that might shed light on this matter.

2.4.4 Escape Strategies and the Absence of Genome Reduction

As evidenced by the enlarged genomes of nitrogen fixing rhizobium (Downie and Young 2001), horizontal transmission should not result in genome reduction if the symbionts face conflicting selective pressures imposed by host-associated and free-living environments. Present genomic evidence suggests that both the free-living and host-associated phases contribute to the overall fitness of *E. persephone*. Early research revealed that genome size (~3.3 Mb) and GC (~58%) content of the *Riftia* endosymbiont are about what is expected for free-living bacteria (Nelson et al. 1984). A recent metagenomic analysis reveals that *E. persephone* has retained the functional versatility needed to survive and reproduce in the ambient environment and also adapt to the symbiont lifestyle (Robidart et al. 2008). *E. persephone* has retained a full suite of genes needed for heterotrophic metabolism, including all the genes needed for glycolysis, fructose degradation and the Kreb's cycle. The genome also contains the requisite components for autotrophic carbon fixation via partial Calvin-Benson and reverse tri-carboxylic acid (rTCA) cycles. However, several key enzymes of the Kreb's cycle were not detected in a proteomic analysis of host-associated *E. persephone*, suggesting that the bacterium can adaptively regulate heterotrophic metabolism when it occurs in the symbiont phase (Markert et al. 2007). *E. persephone* retains suites of genes involved in cellular motility, signal transduction and specific genes that control cell division, a critical fitness-related function. In most respects, the symbiont's genome resembles that of the free-living thiotroph *Thiomicrospira crunogena* in the functional categories of genes it contains; however, it appears to have elevated contents of genes involved in combating host defenses against pathogens and in energy production, critical functions needed to invade and prosper in the host environment. These apparently coevolved changes suggest that the host-associated phase contributes significantly to overall fitness in *E. Persephone*, because the evolution of such differences seems improbable if the host-associated phase is just a demographic dead-end.

A search for exit strategies from vestimentiferan hosts is warranted. Perhaps a transmissive phase of *E. persephone* invades the host's circulatory system to exit via respiratory structures, through excretory pores or along with gametes through gonopores. We do not know what happens to *E. persephone* when *Riftia* dies. I have observed that the trophosome, and presumably its content of symbionts, shrinks notably in size when the worms are starved of sulfides at waning vents (unpublished data). Damage to the host due to predation by bythograeid crabs (Micheli et al. 2002) and polynoid annelids (personal observations) might also create opportunities for the bacteria to re-inoculate the environment.

2.5 Bathymodiolin Mussels and Dual Symbiosis

Mussels in the genus *Bathymodiolus* Kenk and Wilson (1985) and related genera of the subfamily Bathymodiolinae (Bivalvia: Mytilidae) are also among the dominant constituents of chemosynthetic environments (Jones et al. 2006). Bathymodiolins appear to be more versatile than vesicomyids and vestimentiferans, because the mussels are mixotrophic, retaining a functional digestive tract while hosting nutritional endosymbionts (Page et al. 1990). The majority of bathymodiolin species host only intracellular thiotrophic γ-Proteobacteria in their gill tissues, but several species are known to host only intracellular methanotrophic γ-Proteobacteria, and other species can host both types simultaneously, a phenomenon known as dual symbiosis (Fisher et al. 1993). Some members of the genus *Idas* host a high diversity of extracellular sulfur-oxidizing symbionts on their gill filaments (Duperron et al. 2008a). The distribution of symbiont types among various mussel hosts has been summarized elsewhere (DeChaine and Cavanaugh 2005; Won et al. 2008). Recently *B. heckerae* was reported to host four bacterial phylotypes (Duperron et al. 2007) and a newly discovered *Idas* species from the Mediterranean was reported to host six (Duperron et al. 2008b). Little is known about the genetics and evolution of these bacteria, and no genome sequences are available from any of the mussel endosymbionts.

The preponderance of evidence strongly suggests that bathymodiolins acquire their thiotrophic endosymbionts locally from the environment in which they live; nonetheless, evidence for vertical transmission also exists. Cytological investigations of *Bathymodiolus thermophilus* sperm and eggs revealed no evidence for bacteria (Herry and Le Pennec 1986); consequently Le Pennec et al. (1988) were first to suggest that *B. thermophilus* acquires its endosymbionts horizontally by endocytosis through gill epithelium. Subsequent weak inferences, however, generated momentum for an alternative hypothesis that the symbionts were vertically transmitted. Cary and Giovannoni (1993, p. 5699) concluded their seminal publication on vesicomyid symbiont transmission with the statement, "Recent preliminary studies have revealed a similar transovarial transmission mechanism in the symbiont of the mytilid bivalve *Bathymodiolus thermophilus*." Their molecular probes hybridized with bacteria in the gonad-bearing mantle tissue of *B. thermophilus*, but the background signal was too noisy to localize the bacteria in eggs or ovarian nurse cells (S.C. Cary, personal communication, 2003). Unlike vesicomyids, the bathymodiolin symbionts are not restricted to gills (Salerno et al. 2005). Cary and Giovannoni's concluding statement and a subsequent report of apparent congruence between host and symbiont phylogenies among several bivalve taxa including vesicomyids, lucinids, thyasirids, solemyids and *B. thermophilus* (Distel et al. 1994, p. 540), led other researchers to infer vertical transmission in mussels (Nelson and Fisher 1995; Peek et al. 1998a; Trask and Van Dover 1999). The methanotrophic endosymbionts associated with bathymodiolins have not been studied in similar depth, so even less is known about their potential modes of transmission.

2.5.1 Mixed Transmission and Cytonuclear Decoupling in a Hybrid Zone

A fortuitous discovery allowed us to test some predictions of vertical and horizontal transmission hypotheses for bathymodiolins. Closely related northern (*N*) and southern (*S*) species (*B. azoricus* and *B. puteoserpentis*, respectively) that occupy the Mid-Atlantic Ridge host dual symbionts (Fisher et al. 1993). These mussels hybridize along an intermediate portion of the ridge axis (O'Mullan et al. 2001; Won et al. 2003a). Host mitochondrial and symbiont genetic polymorphisms would be decoupled in the hybrid zone if the symbionts were acquired horizontally from the local environment. Alternatively, cytoplasmic co-transmission would maintain symbiont-mitochondrial coupling in hybrids if symbiont transmission were strictly vertical. *Bathymodiolus azoricus* females would co-transmit *mtN* mitochondria and *symN* bacteria to their offspring and *B. puteoserpentis* females would cotransmit *mtS/symS* to their offspring. Cytoplasmic co-transmission would also occur in the hybrid females as long as it is strictly maternal, so the *mtN/symN* and *mtS/symS* cytotypes should remain coupled in F_1 hybrids and subsequent hybrid generations. Won et al. (2003b) were able to test these predictions, because the thiotrophs segregated into distinct *N* and *S* subtypes based on ITS sequences. A sample of mussels from the hybrid zone included 24 individuals with the southern cytotype *mtS/symS*, 18 with a recombinant cytotype *mtN/symS*, and five mixed infections with the northern mitotype *mtN*/(*symN* + *symS*). The 18 recombinant cytotypes and five mixed infections were not consistent with predictions of strictly vertical transmission. Environmental acquisition is the simplest explanation for these data, but anomalies existed. No individuals had the *mtN/symN* coupling type or the *mtS/symN* recombinant type. If the *symS* and *symN* bacterial strains both occurred locally as free-living infectious agents, a few *mtN/symN* and *mtS/symN* cytotypes would be expected, but their absence might be a consequence of sample size ($n = 47$ mussels from the hybrid zone). We tested for PCR bias against the *symN* type as a likely explanation for the absence of *mtN/symN* and *mtS/symN* by probing the mussels with sensitive molecular hybridization methods, but without success. An alternative hypothesis emerged, however, upon examination of nuclear-encoded allozyme polymorphisms: the latter could recombine with the cytotypes of subsequent hybrids and gradually eliminate cytonuclear coupling in an old demographically stable hybrid population. Apparent F_1 hybrids and beyond were identified in the sample, but the five *mtN*/(*symN* + *symS*) mussels had nuclear genotypes that would be expected for first generation immigrants from the north. Consequently, Won et al. (2003b) hypothesized that these putative immigrants might have transported the *symN* thiotrophs from their natal sites, before emigrating to the south, where they were subsequently infected by local *symS* thiotrophs. Electron microscopy and molecular evidence are consistent with this hypothesis, as thiotrophic bacteria were found in pediveliger larvae and the earliest juveniles of *B. azoricus* settling at Mid-Atlantic vents (Salerno et al. 2005).

Several lines of evidence support the hypothesis that mussel symbionts can be replaced throughout the host's lifespan. Cytological evidence exists for the apparent endocytosis of free-living bacteria through the gill membranes of adult mussels (Le Pennec et al. 1988; Won et al. 2003b; Salerno et al. 2005) and other bivalves (Gros et al. 1996, 1998). A bacterial exit strategy for re-inoculation of the local environment may also exist. Salerno et al. (2005) suggested that the pit-like structures in gill epithelium might involve exocytosis of the bacteria. Exo- and endocytosis are suggested in a recent experiment reported by Kádár et al. (2005). They "cleared" *B. azoricus* mussels of thiotrophic endosymbionts by exposing them to sulfide-free seawater for a period of 30 days in the laboratory. A combination of microscopy and the application of symbiont-specific PCR primers indicated that the mussels appeared to be aposymbiotic, though the authors could not be entirely sure of this matter. Then they exposed the "cleared" mussels to sulfide-enriched seawater and newly acquired symbiont-bearing mussels. Thiotrophic bacteria soon grew again in gills of the "cleared" mussels. Kádár et al. (2005) hypothesized that the bacteria exited the newly acquired mussels and entered the gills of "cleared" mussels through pit-like structures in the epithelium. They interpreted the new infections as evidence that the thiotrophs have the capacity to exit the host environment and revert to an infectious stage. Though research that is more definitive is needed to resolve this matter, present evidence suggests that bathymodiolin symbionts might obtain a significant dispersal benefit if their hosts engage in some degree of vertical transmission. Dispersing along with the host larvae would allow the symbionts to "seed" bacterial populations at nascent chemosynthetic sites and possibly leave a historical imprint that covaries with the history of host dispersal.

2.5.2 *Absence of Cospeciation in Thiotrophs*

Environmental acquisition alone, however, appears to provide the simplest explanation for the phylogeographic distribution of the thiotrophic endosymbionts (Won et al. 2008). The gene tree constructed for the thiotrophs associated with 15 host species was not congruent with the phylogeny of corresponding host species (Fig. 2.5). Pairwise genetic distances among the 15 thiotrophs were not correlated with genetic distances among host species, but they were positively correlated with geographical distances among host localities. This evidence for isolation-by-distance among the thiotrophs was not observed among the mussels – genetic distances did not correspond at all with geographical distances. In some cases closely related hosts like *B. azoricus* and *B. puteoserpentis* live near one another, yet other related species like "*Bathymodiolus*" *tangaroa* and "*B.*" *mauritanicus* occur in different ocean basins on the opposite sides of the globe.[1] Conversely,

[1] The quotes denote the dubious assignment of this genus name to these species (Jones and Vrijenhoek 2006).

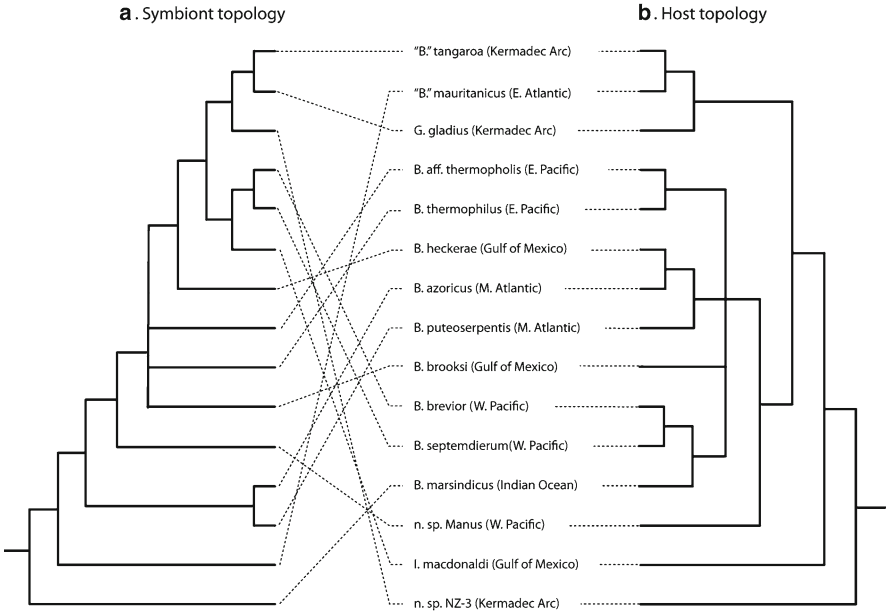

Fig. 2.5 Absence of cospeciation between endosymbionts and 15 species of bathymodiolin mussels (Redrawn from Won et al. 2008). (**a**) Bayesian cladogram of the bacteria was based on 16S rRNA. (**b**) Bayesian cladogram of the host species was based on combined data from three genes: mitochondrial COI, ND4 and nuclear encoded 18S rRNA. See original publication for the bootstrap support values and statistical tests of cospeciation

very distantly related hosts *Idas macdonaldi*, *B. brooksi* and *B. heckerae* all occur in the Gulf of Mexico. Nominal species like *B. brevior*, *B. septemdierum* and *B. marisindicus* appear to be synonymous (Jones et al. 2006). *Bathymodiolus brevior* and *B. septemdierum* occur in the western Pacific and share closely related symbionts, but *B. marisindicus* occurs in the Indian Ocean and hosts the most divergent symbiont seen in this study.

Won et al. (2008) hypothesized that the mussel thiotrophs began to diverge and spread around the globe approximately 112–160 million years ago, long before the continents reached their present locations about 50 million years ago. They would have passed through more continuous ocean basins than exist today. The host phylogeny, on the other hand, suggests that bathymodiolins probably began to radiate about 60 million years ago, so their paths to spread globally were probably very different from the ancient paths used by the symbionts. Regardless, it may be safe to conclude that vertical transmission, if it occurs at all in these mussels, has left no historical imprint on the phylogeographic distribution of the thiotrophic symbionts. Similar phylogeographic studies involving 16S rRNA and other genetic markers remain to be conducted with the methanotrophs, but these studies will be more difficult because the methanotrophs exhibit a greater diversity of genotypes than found in the mixed thiotroph hosted by individual mussels.

2.6 Conclusions

A large number of observed and expected differences exist between the obligately vertical endosymbionts hosted by vesicomyid clams and the horizontal endosymbionts hosted by vestimentiferans tubeworms (summarized in Table 2.2). The situation in bathymodiolin mussels is more complex, however. The mussels acquire thiotrophic symbionts from the local environment, but a component of vertical transmission might also exist. Transmission modes of methanotrophs and other bacterial endosymbionts remain unknown (Duperron et al. 2008b).

Symbiont infections involving multiple strains as seen in vestimentiferans and bathymodiolins appear to be a good indicator of horizontal transmission. Environmental infections are hypothesized to create opportunities for exploiting locally optimal symbiont strains (Won et al. 2003b), despite the associated risks. Pathogenic microorganisms might evolve to exploit the host acquisition pathways, requiring investments by the host in surveillance and active defense mechanisms that do not discriminate against mutualists. *Riftia* opens a time-limited window for environmental infections and then closes it by destroying subsequently infected epidermal tissues (Nussbaumer et al. 2006). *Bathymodiolus*, on the other hand, might be susceptible to environmental infections throughout its adult life (Kádár et al. 2005). To assess whether horizontal transmission carries a significant *pathogen penalty*, it might be valuable to compare the diversities and densities of non-mutualist microbes associated with horizontal and vertical hosts. Evidence exists for a variety of other microbes associated with the tissues of chemosynthetic organisms (e.g., Naganuma et al. 1997; Elsaied et al. 2002; Goffredi et al. 2004), but their roles are unknown. Pathogenic fungi are known to infect *Bathymodiolus* mussels (Van Dover et al. 2007). Horizontal symbionts should bear a significant cost associated with enhanced host surveillance of potential pathogens. The *Riftia* endosymbiont, *E. persephone*, has an elevated content of genes that appear to be involved in combating host defenses, as in many pathogenic bacteria (Robidart et al. 2008). Similar elevations are not evident in the vertical symbionts *Ruthia magnifica* and *Vesicomyiosocious okutanii*. Studies of the trade-offs between acquisition of locally optimal symbionts and the risk of pathogens deserve more attention.

Mixed environmental infections also engender risks of within-host symbiont competition. To provide the host with nutrition, chemosynthetic symbionts must be devoured via intracellular autophagy or they must “leak” nutrients to the host, or both (reviewed in Cavanaugh et al. 2006). Competition among multiple symbiont genotypes within a host should favor cheaters that contribute less to the host while gaining access to a broad redox zone. How can honest symbionts evolve in such mixtures unless their contribution to the host also increases their own fitness? Trying to envision the evolution of cooperative mutualists in mixed genotypic infections is difficult, but a number of theoreticians have attempted to address this problem (e.g., Frank 1996; Doebeli and Knowlton 1998; Frean and Abraham 2004). Cheaters who exploit the host too intensively will decrease their own fitness if the host dies and the bacteria fail to escape to the ambient environment

Table 2.2 Three model systems involving deep-sea chemosynthetic endosymbionts and a summary of the genetic, demographic and evolutionary consequences of their transmission modes. For lines 16–22, designations of "lost", "retained", "reduced" or "enhanced" pertain to comparisons with the free-living thiotroph *Thiomicrospira crunogena*

Host	*Calyptogena magnifica*[a]	*Riftia pachyptila*	*Bathymodiolus azoricus*
1. Endosymbiont	*Ruthia magnifica*	*Endoriftia persephone*	Undescribed
2. Chemosynthetic type	Thiotroph	Thiotroph	Dual symbiosis[b]
3. Symbiont location	Gill bacteriocytes	Trophosome bacteriocytes	Gill bacteriocytes[c]
4. Transmission mode	Transovarial	Environmental	Environmental[d]
5. Symbiont assurance for the host	Yes	No	Potentially[e]
6. Dispersal benefit for the symbiont	Yes	No	Potentially[e]
7. Locally optimal symbionts	No	Yes	Yes
8. Mixed symbionts	No	Yes	Yes
9. Symbiont recombination[f]	Unlikely[g]	Possible	Possible
10. Maternal inheritance	Yes	No	Unclear
11. Expectation of symbiont N_e	Very small	Potentially very large	Large
12. Expectation of mutational decay	Yes	Unlikely	Unlikely
13. Observed nucleotide substitution rates	Very fast	Very slow	Unclear
14. Evidence for cytoplasmic coupling	Yes	No	Unclear
15. Host-symbiont cospeciation in family	Yes	No	No
16. Symbiont genome sequenced	Yes	Yes, but not closed[h]	No
17. Symbiont genome reduction	By about one-half	No	?
18. Symbiont motility genes	Lost	Retained	?
19. Symbiont central metabolism genes	Retained	Retained	?
20. Symbiont energy production genes	Enhanced	Enhanced	?

21. Symbiont DNA replication genes	Reduced	Retained	?
22. Symbiont GC content	Reduced	Enhanced	?
23. Symbiont competition	No	Yes	?
24. Risk of cheaters	Limited	Yes	Yes

[a] *Calyptogena okutanii* and Candidatus *Vesicomyiosocious okutanii* provide an equally valid model.

[b] γ-proteobacterial thiotrophs and methanotrophs in gill.

[c] Bacteriocytes also occur in other tissues including mantle and gonad.

[d] A vertical component of transmission might also exist.

[e] Potentially if a vertical component of transmission does exists.

[f] Recombination might occur through transformation, transduction and conjugation in mixed symbiont infections. It might also occur in vertical symbionts if they occasionally are leaky.

[g] Exceptions appear to exist (Stewart et al. 2009).

[h] A consequence of mixed infections involving multiple symbiont genotypes.

(Genkai-Kato and Yamamura 1999). On the other hand, if reproduction in the host and re-inoculation of the ambient environment enhances bacterial fitness even slightly, cooperation with the host might be expected. Experimental studies with legume-rhizobium mutualists reveal that the plant host exerts sanctions against bacterial cheaters by controlling the distribution of critical resources to less productive modular tissues (root nodules) (Kiers et al. 2003). Similar experiments are impossible with most deep-sea vent and seep mutualists, but they are warranted for chemosynthetic mutualists that could be cultured from shallower environments.

Enslavement of chemosynthetic symbionts through obligately vertical transmission avoids of the problems of mixed symbiont genotypes and intra-host symbiont competition. The relatively recent (~45 million year old) enslavement of intracellular γ-Proteobacteria by vesicomyid clams might be considered analogous to the symbiogenic origin of organelles in eukaryotes (Vetter 1991). The origins of mitochondria and chloroplasts by symbiogenesis are now well established though the order of events leading to eukaryotes is still debated (Cavalier-Smith and Lee 1985; Embley and Martin 2006). The extreme genome reduction reported for the insect endosymbiont *Carsonella rudii* suggest that this bacterium exists in a state "between living cell and organelles" (Tamames et al. 2007). Nonetheless, "full-fledged" organelles like mitochondria and chloroplasts have evolved sophisticated mechanisms to import proteins encoded by the nuclear genome and synthesized in the cellular cytoplasm (Cavalier-Smith and Lee 1985; Theissen and Martin 2006). It will be interesting to see if the vertically transmitted vesicomyid endosymbionts have evolved even rudimentary mechanisms for protein transport. Clearly there is much to be learned about evolution from the genome sequencing and proteomic analyses of additional chemosynthetic endosymbionts.

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