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New Gammaproteobacteria Associated with Blood-Feeding Leeches and a Broad Phylogenetic Analysis of Leech Endosymbionts

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Many monophagous animals have coevolutionary relationships with bacteria that provide unavailable nutrients to the host. Frequently, these microbial partners are vertically inherited and reside in specialized structures or tissues. Here we report three new lineages of bacterial symbionts of blood-feeding leeches, one from the giant Amazonian leech, *Haementeria ghilianii*, and two others from *Placobdelloides* species. These hosts each possess a different mycetome or esophageal organ morphology where the bacterial cells are located. DNA sequencing of the bacterial 16S rRNA genes and fluorescent in situ hybridization placed these symbionts in two separate clades in the class *Gammaproteobacteria*. We also conducted a broad phylogenetic analysis of the herein-reported DNA sequences as well as others from bacterial symbionts reported elsewhere in the literature, including alphaproteobacterial symbionts from the leech genus *Placobdella* as well as *Aeromonas veronii* from the medicinal leech, *Hirudo medicinalis*, and a *Rickettsia* sp. detected in *Hemiclepsis marginata*. Combined, these results indicate that blood-feeding leeches have forged bacterial partnerships at least five times during their evolutionary history.

A wide variety of intimate bacterial partnerships with animals, particularly insects, has been described (7, 12). The symbionts often allow their hosts to exploit an otherwise unavailable niche by supplying them with limiting or missing nutrients. Examples of these associations include *Buchnera* spp. in aphids and *Wigglesworthia* spp. in tsetse flies, in which the bacteria supplement monophagous diets of plant sap and vertebrate blood, respectively. Evidence of the significance of the symbionts to their hosts is that the mutualist bacteria are frequently directly transmitted from parent to offspring via vertical transmission and that attempts to "cure" the host of the symbiont typically result in death, sterility, or infertility (15, 23). Many of these obligate symbionts are found in specialized structures or organs, variously termed bacteriocytes or mycetomes (7). Several of these bacterial-eukaryotic partnerships have been intensively studied across coevolutionary (10, 34), developmental (6) and, more recently, genomic (e.g., references 1 and 28) fronts.

Since the 1920s, it has been acknowledged that blood-feeding leeches in the family *Glossiphoniidae* also possess bacterial symbionts that are housed in specialized organs (mycetomes) associated with the esophagus (26). The morphology of these mycetomes is highly variable, however. Species of *Placobdella* that feed on aquatic reptiles and amphibians, for example, have mycetomes consisting of a pair of blind-end sacs that extend laterally from the esophageal lumen (Fig. 1A), the endothelial cells of which are packed with gram-negative rods (31). Bacterial small ribosomal subunit (16S) rRNA and large ribosomal subunit (23S) rRNA genes amplified from DNA extracted from these sacs yielded single genotypes that grouped phylogenetically in the class *Alphaproteobacteria* (31). Fluorescent in situ hybridization (FISH) of bacterial rRNA showed strong signal exclusively within the mycetome epithelial cells. DNA isolates from the mycetomes of three species of *Placobdella* collected in the same lake showed distinct 16S sequences, but symbionts from within a given species of *Placobdella* showed remarkable genetic homogeneity across continental geographic distances (31). This distinct monophyletic clade of bacteria, species of *Reichenowia* (31), comprise the only known *Alphaproteobacteria* that are mutualistic in animals.

Other glossiphoniid leeches have different mycetome morphologies. Leeches of the genus *Placobdelloides* exhibit an "esophageal organ" consisting of a cluster of symbiont-bearing cells encircling the esophagus, just anterior to the gastric tissues (Fig. 1B). Kikuchi and Fukatsu (18) determined that the bacteria isolated from this organ in *Placobdella siamensis* and a *Parabdella* sp. were *Gammaproteobacteria*, closely related to several of the well-described insect symbionts, including *Buchnera* and *Wigglesworthia*. A very different morphology for mycetomal organs, consisting of two pairs of globular sacs connected to the esophagus via thin tubules, is found in *Haementeria ghilianii*, the giant Amazonian leech (Fig. 1C). Although it had been presumed that these sacs contain bacteria (27), this had never been confirmed.

In addition to these mycetome-associated symbionts, other bacterial lineages have been characterized in association with leeches. Kikuchi et al. (17) described a *Rickettsia* sp. found in various tissues of two species of Japanese glossiphoniid leeches. These bacteria were located intracellularly in epidermal, esophageal, and intestinal tissues but were not present in all individuals sampled in one population. The medicinal leech, *Hirudo medicinalis*, which belongs to an entirely different suborder, the *Rhyncobdellida*, also maintains a specific bacterium, *Aeromonas veronii* biovar sobria, in its gastric lumen (14). Unlike many of the obligate bacterial symbionts, *A. veronii* can be cultured outside of its host; however, its mode of transmission has yet to be determined (16).

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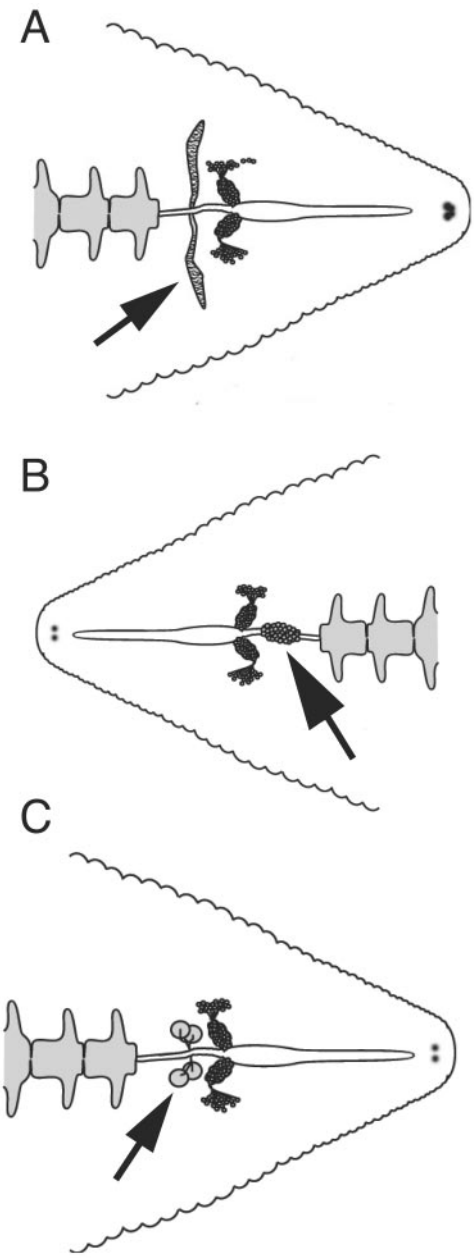

FIG. 1. Schematic illustration of basic morphology of bacterial organs from each of three groups of blood-feeding leeches in the family *Glossophoniidae.* (A) *Placobdella* sp. with blind-end sacs; (B) *Placobdelloides* sp. with small bacteriocytes encircling the leech esophagus; (C) *Haementeria* sp. with large, globular sacs joined to the esophagus via thin ducts.

Here we report three new isolates of mycetome-associated bacterial symbionts of leeches. One lineage comprises the bacterial symbiont found in the globular mycetomes of the giant Amazonian leech, *Haementeria ghilianii*. We also report new results obtained from two additional species of *Placobdelloides*, *Placobdelloides jaegerskioeldi*, the type species of the genus, and *Placobdelloides multistriata*. Classification of these bacteria to class was performed with phylogenetic analyses of the 16S rRNA gene and additionally confirmed with FISH. In an attempt to examine the overall evolutionary history of bacterial symbionts and leeches, we also conducted a broad phylogenetic analysis by combining our new DNA sequence data along with our previously published data from *Reichenowia* (31) and 16S rRNA sequences reported from the other leech bacteria (14, 17, 18).

MATERIALS AND METHODS

Specimens of the giant Amazon leech were collected in the wild in French Guyana in January 2002 and also were obtained from a colony that had been laboratory reared for over a decade (W. Wuttke, personal communication). The two *Placobdelloides* species were collected in South Africa in June 2003. *Placobdelloides jaegerskioeldii* individuals were removed from the rectum of a hippopotamus, and *P. multilineata* was collected under rocks in a pond.

For transmission electron microscopy (TEM) of *Haementeria ghilianii* mycetomes, the structures were removed by dissection, fixed in 2.5% glutaraldehyde in 0.2 M phosphate buffer, washed in the same buffer, postfixed in 1% osmium tetroxide in the same buffer, dehydrated through a graded ethanol series, and embedded in Spurr's (32) resin. Sections were cut on a Reichert ultramicrotome, collected on copper grids, stained in uranyl acetate and lead citrate, and examined on a Zeiss LEO 902A transmission electron microscope. In light of having found only two adult specimens of *P. jaegerskioeldi* and one of *P. multistriata*, these specimens were devoted to molecular characterization via DNA sequencing and FISH as described below.

To perform symbiont DNA isolation, bacterial organs of leeches were dissected aseptically and DNA was extracted with the DNeasy extraction kit (QIAGEN, Valencia, Calif.), following the protocol for animal tissues, except resolubilizing in only 50 to 100 μl of buffer. Bacterial rRNA sequences were amplified using bacterial universal primers BSF8 with BSR1541 (http://www.psb.ugent.be/rRNA/primers/BS_lst.html) in 25-μl volumes either with PureTaq Ready-to-Go PCR beads (Amersham Pharmacia, Piscataway, N.J.) with 1 μl template DNA and 1 μl of each of the 10 μM primers, or in 25-μl reaction mixtures with 1 μl of template DNA, 0.13 μl AmpliTaq polymerase (Applied Biosystems, Foster City, Calif.), 0.5 μl of each of the 10 μM primers, 2.5 μl PCR buffer II (Perkin-Elmer), 2.5 μl $MgCl_2$ solution (Perkin-Elmer), and 2 μl of 100 μM deoxynucleoside triphosphate mix. The cycling program consisted of an initial denaturation at 94°for 4 min, 35 cycles of 94° for 15 s, 55° for 15 s, and 72° for 60 s, and then a hold at 72° for 7 min. Amplification products were purified either with the QIAquick PCR purification kit (QIAGEN, Valencia, Calif.) or with the ArrayIt PCR purification kit (TeleChem International, Sunnydale, Calif.) and sequenced using the amplification primers as well as primers BSF517, BSR 534, and BSF1099 (http://www.psb.ugent.be/rRNA/primers/BS_lst.html), BigDye terminator sequencing premix (Applied Biosystems, Foster City, Calif.), and an ABI 3700 automated capillary sequencer. Sequences from opposite strands were reconciled with Sequence Navigator (Applied Biosystems, Foster City, Calif.) or Sequencher (Gene Codes, Ann Arbor Mich.).

The new leech symbiont sequences and a selection of previously published 16S sequences of both other leech bacterial symbionts, other animal symbionts, a general representation of proteobacteria, and two gram-positive taxa that served as the outgroup to root subsequent trees (GenBank accession numbers: *Acyrthosiphon pisum* P symbiont, M27039; *Aeromonas hydrophila*, X74677; *Aeromonas veronii*, AF079299; *Agrobacterium tumifaciens*, ATU389908; *Bacillus anthracis*, AB116124; *Bartonella henselae*, BX897699; *Blochmannia floridanus*, NC_005061; *Bordetella pertussis*, BX640420; *Brucella melitensis*, NC_003318; *Buchnera aphidicola*, NC_004545; *Campylobacter jejuni*, NC_002163; *Caulobacter* sp., AB025196; *Citrobacter freundii*, M59291; *Clostridium tetani*, X74770; endosymbiont of *Parabdella* sp., AB083059; endosymbiont of *Placobdelloides siamensis*, AB083058, *Escherichia coli*, U00096; *Geobacter sulfurreducens*, NC_002939; *Proteus vulgaris*, AJ233425; *Providencia stuartii*, AF008581; *Reichenowia ornatae*, AY316684; *Reichenowia parasiticae*, AY316683; *Reichenowia pictae*, Ay316685; *Rhizobium gallicum*, U86343; *Rhizobium mongolense*, U89820; *Rickettsia* sp. endosymbiont of *Hemiclepsis marginata*, AB113215; *Rickettsia prowazekii*, NC_000963; *Rickettsia* sp. in *Ixodes scapularis*, D84558; *Rickettsia typhi*, U12463; *Shigella dysenteriae*, X96966; *Sinorhizobium meliloti*, NC_003047; *Vibrio cholerae*, AY292952; *Wigglesworthia glossinidia*, AF022879; *Wolbachia* endosymbiont of *Drosophila melanogaster*, NC_002978;) were aligned with ARB (20). All phylogenetic analyses were performed using PAUP*4.0b4 (33) with both parsimony and maximum likelihood (ML) heuristic searches. Unweighted parsimony using tree-bisection-reconnection was employed with 30 replicates of random addition sequences of taxa. ModelTest (25) was run on the data matrix to obtain the most appropriate model for ML parameters. The chosen model was GTR + I + Γ, with a rate

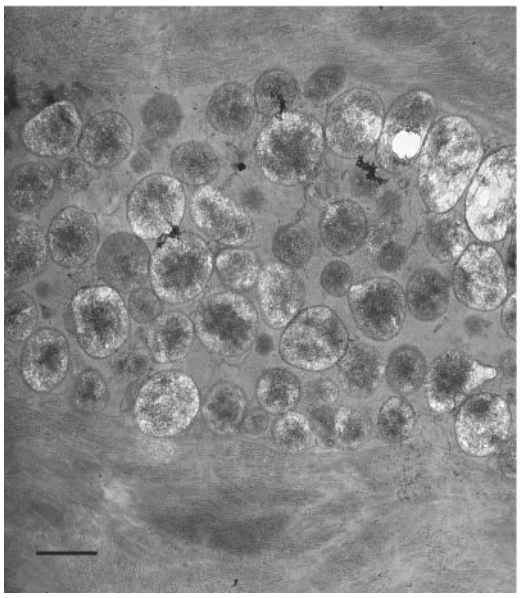

FIG. 2. Transmission electron micrograph showing pleomorphic bacterial cells separated by connective tissue in epidermal layer of *H. ghilianii* mycetome. Bar, 1 μm.

matrix of $a = 1.0197$, $b = 2.8384$, $c = 1.5644$, $d = 0.9358$, $e = 4.3810$, and $f = 1.0000$, proportion of invariable sites (I) of 0.3854, and a gamma distribution shape parameter of 0.7361. Nodal support of the parsimony tree was determined via jackknife with 37% deletion of characters in each round under the full heuristic search with 30 random addition sequences.

Fluorescent in situ hybridizations were performed as previously described (27). Briefly, leeches were fixed in paraformaldehyde, dehydrated in an ethanol series, embedded in Paraplast PLUS (Kendall Healthcare, Mansfield, Mass.), and sectioned. The 5- to 7-μm sections were then hybridized with eubacterial (3), alphaproteobacterial (21), or gammaproteobacterial (21) probes labeled with Cy3.

RESULTS

As previously anticipated, TEM clearly revealed bacterial symbionts in the organs of the giant Amazonian leeches collected from French Guyana. However, unlike the rod-shaped symbionts found inside mycetomal cells in species of *Placobdella*, the bacteria from *H. ghilianii* were pleomorphic and embedded in a collagenous extracellular matrix surrounding the periphery of the mature organ (Fig. 2). Amplification of the DNA extracted from the mycetomes of *H. ghilianii* with bacterium-specific 16S rRNA primers yielded a single sequence (GenBank accession number AY999969) that was 93% identical to the *Providencia stuartii* 16S rRNA sequence, clearly placing the symbiont in the class *Gammaproteobacteria*. The 16S rRNA sequences from separate DNA extractions performed on the anterior and posterior mycetome pairs isolated from the same leech were identical. Furthermore, the sequences from the symbionts of the wild-caught and lab-raised leeches were >99% identical.

The bacterial 16S sequences obtained from the esophageal organs of *P. multristriata* (GenBank accession number AY999970) and *Placobdelloides jaegerskioeldi* (GenBank accession number AY999971) were approximately 94% similar to the gammaproteobacterial symbionts previously reported from leeches in the same genus (18). FISH performed with eubacteria- and gammaproteobacteria-specific probes supported these classifications and suggested relatively low concentrations of the symbiotic bacteria in *H. ghilianii* mycetomes, consistent with the TEM images (Fig. 3A) but large numbers of symbionts within each esophageal organ cell in *P. jaegerskioeldi* (Fig. 3B). Neither *H. ghilianii* nor *P. jaegerskioldi* preparations showed any hybridization to the alphaproteobacterial probe; however, it should be noted that these results cannot at present rule out the presence of minor species of bacteria also housed in the mycetome structures.

The phylogenetic analysis of the new leech mycetome-associated symbiont 16S sequences combined with those from the other leech symbionts and a set of broadly representative bacteria yielded two equally parsimonious trees; the strict consensus of these is illustrated in Fig. 4. Leech bacterial symbionts appear as five distinct lineages or clades in this phylogeny of proteobacteria. The *Reichenowia* bacteria here group with the human and animal pathogens *Brucella* and *Bartonella* and the plant symbiont *Sinorhizobium*, with a moderate jackknife value (83%) on this node. The *Placobdelloides* symbionts comprise a monophyletic clade of gammaproteobacteria that, in turn, is part of a larger clade of gammaproteobacterial animal mutualists. Our new *H. ghilianii* symbiont lineage did not cluster with the *Placobdelloides* leech bacteria but, rather, was basal to the clade containing these symbionts and the insect obligate

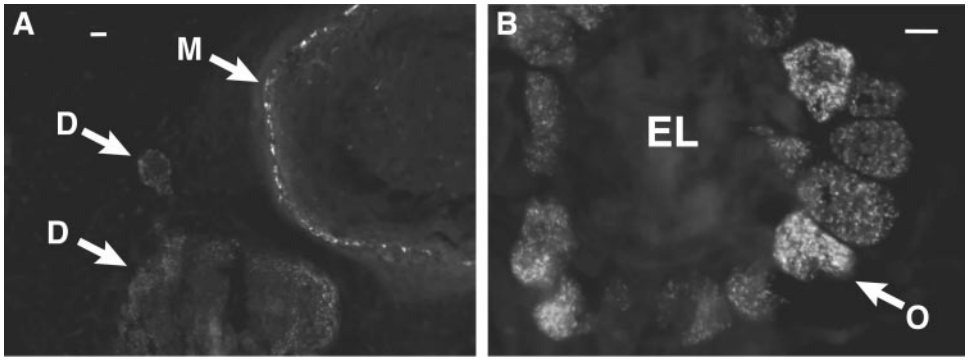

FIG. 3. FISH localizing bacterial cells in leech mycteomes. (A) *H. ghilianii* symbionts hybridized with gammaproteobacterial probe. M, mycetome structure; D, duct. (B) *P. jaegerskeoldi* esophageal organ symbionts hybridized with gammaproteobacterial probe. O, esophageal organ cell; EL, esophageal lumen. No fluorescence was observed with alphaproteobacterial probes on either of these leech species. Bar, 20 μm.

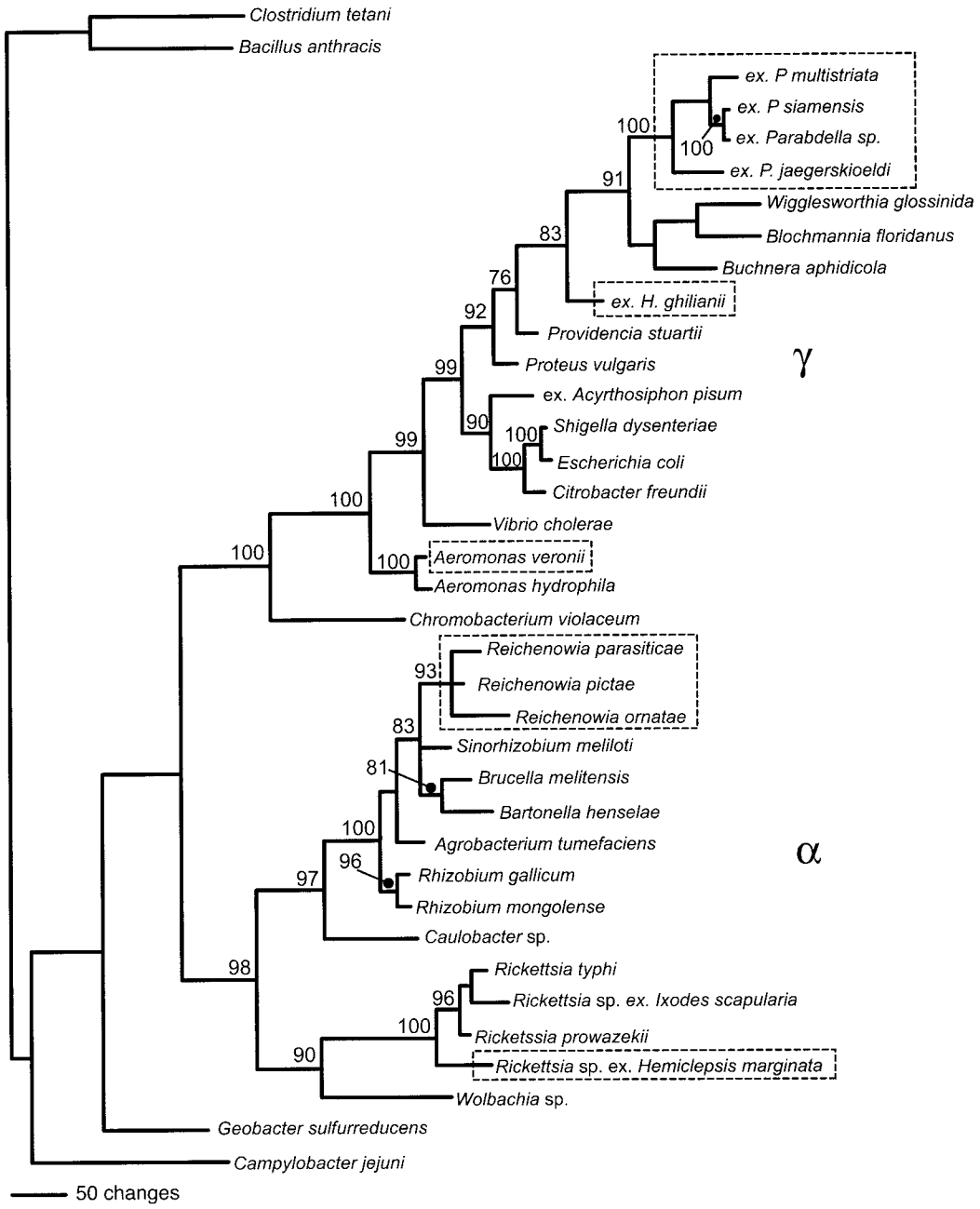

FIG. 4. Phylogeny of 16S rRNA sequences of leech-associated symbionts in the context of other animal symbionts and various proteobacteria, rooted with gram-positive taxa. The tree is a strict consensus of two equally parsimonious trees depicted as a phylogram. Jackknife support values greater than 75% are indicated. The five bacterial clades or lineages associated with blood-feeding leeches are outlined with dashed boxes.

symbionts. The alphaproteobacterial *Rickettsia* sp. from the Japanese glossiphoniid leeches clusters with the other *Rickettsia*, as expected; however, we did not recover a close relationship with the *Ixodes* tick *Rickettsia* sp. as Kikuchi et al. (17) did. Finally, *Aeromonas veronii* from *Hirudo medicinalis* clusters predictably with *A. hydrophila* in a very basal gammaproteobacterial clade. The tree generated with ML was not statistically different from the two parsimony-based topologies (Shimodaira-Hasegawa test; $P = 0.163$ and 0.179) and so is not shown. All of the above statements hold true for the ML except that although the symbiont from *Haementeria ghilianii* is still basal to the other gammaproteobacterial animal symbionts, it clusters with *Proteus* and *Providencia*.

DISCUSSION

The evolution of bacterial symbioses in the context of their leech hosts can be readily understood insofar as the phylogenetic relationships of leeches are well understood (4, 5, 30). Of the various symbiotic lineages identified above, most are con-

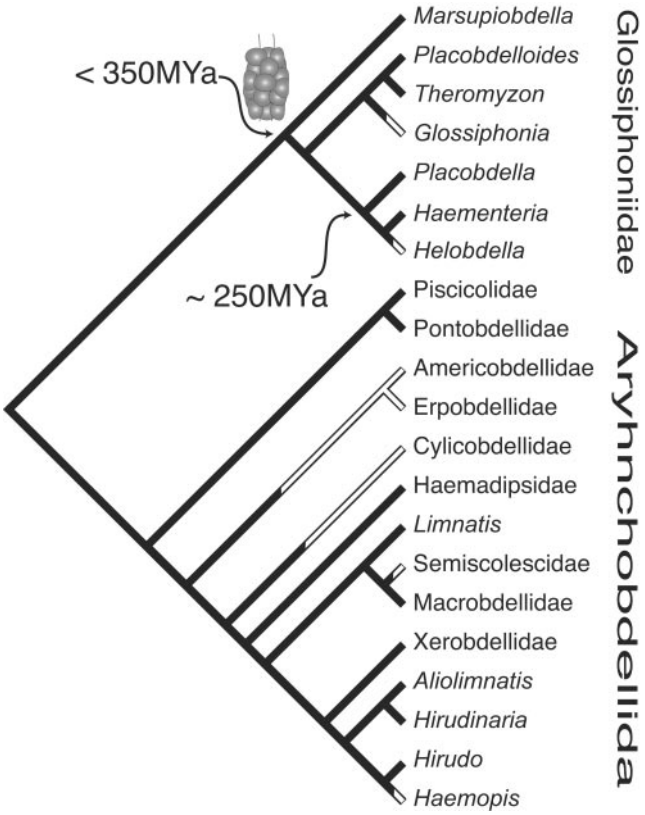

FIG. 5. Generalized phylogeny of the major groups of leeches (after reference 29). Losses of blood feeding are depicted here with white branches leading to taxa. The date of divergence of *Placobdella* spp. is estimated to be 250 Mya, at the point of separation of North and South America. The origin of the glossiphoniids is estimated to be ~350 Mya, and the ancestral morphology of bacteria-associated organs is hypothesized to be a cluster of small cells encircling the esophagus, as is seen in present-day *Marsupiobdella* and the *Placobdelloides* spp.

centrated in the family *Glossiphoniidae* (Fig. 5) (29), with the exception of *Aeromonas veronii* in *Hirudo medicinalis* in the very distantly related family *Hirudinidae*. On the whole, however, leeches in the remaining families have yet to be examined. In terms of associations in the *Glossiphoniidae*, only the non-blood-feeding lineages (*Helobdella* and *Glossiphonia*) lack esophageal-associated symbionts (29). Species of *Marsupiobdella*, the most basal lineage in the group, possess esophageal organ structures identical to those seen in *Placobdelloides*, substantiating the notion that this is the ancestral condition for glossiphoniid leeches. From the ancestral state, there appear to have been two independent symbiont replacements, each correlated with a radically different mycetome organ and occupied by phylogenetically independent bacterial symbionts. The genus *Placobdella* has a North American origin (29) and so must have acquired the alphaproteobacterial symbionts in North America after Laurasia split off from Gondwana during the opening of the Tethys Sea about 250 million years ago (Mya). Meanwhile, in what would become South America, the ancestral *Haementeria* acquired a different gammaproteobacterium than that which was present in its predecessors, whereas the *Helobdella* lineage gave up blood feeding and the bacterial symbionts altogether (Fig. 5). The ultimate origin of the esophageal symbiosis can be inferred from our analyses to have originated in a freshwater context, probably in an amphibian-feeding leech sometime after the origin of freshwater tetrapods about 350 Mya (8).

Bacteria that provide scarce or unavailable nutrients to their hosts will evolve to become vertically transmitted as their presence is required for host survival and reproduction. It has been predicted that these symbionts eventually will resemble organelles such as mitochondria and chloroplasts, and so their study can offer glimpses into what might have occurred in the evolution of these more ancient symbionts (13). As of yet, it has not definitively been shown that the bacteria found in leech mycetomes are vertically transmitted from a hermaphroditic parent to its offspring; however, multiple lines of evidence seem to support it. First, these bacteria are found intracellularly and in specialized structures, the presence of which has been termed a "key lifestyle feature" of obligate, vertically transmitted symbionts (35). Second, symbionts of *Placobdella* species from geographically isolated populations (in Ontario, Michigan, and Texas) showed less genetic differentiation than those taken from different *Placobdella* species collected from the same lake (31), and although much more sampling is clearly needed to confirm this result, these results are consistent with vertical transmission. Third, independent results have detected bacteria in leech offspring at early stages. Kikuchi and Fukatsu (18), using PCR, detected the same bacteria as had been observed in the adult in 10 of 10 eggs removed from a *P. siamensis* individual. In our previous studies, we used FISH to detect large populations of *Reichenowia* in very young *P. parasitica* leeches that had never fed on blood and that were removed from the ventral surface of their brooding parent (31). These results suggest that the bacteria are not acquired from blood meals, though it is possible that vertical transmission is effected by the parents by regurgitating symbiotic bacteria when depositing cocoons. Finally, the fact that the symbiont sequences from wild-caught *Haementeria ghilianii* and those from the same species that had come from laboratory colonies kept for over a decade were >99% identical strongly suggests a pattern of vertical transmission. Chen et al. (10) reported a very similar result in an analysis of *Wigglesworthia* from colony-reared and field-collected tsetse flies.

It is not currently known what role any of these leech bacterial symbionts play. Like the *Wigglesworthia* symbionts of tsetse flies, the symbionts, particularly those associated with mycetomes or other specialized cells, may supply their hosts with B vitamins. These nutrients are scarce in vertebrate blood, and so those organisms that are hematophagous throughout their lives (in contrast to blood-feeders, such as mosquitoes and fleas, which as larvae are carnivores or detritovores) must obtain these nutrients from a symbiotic partner that has retained the metabolic capability to synthesize the nutrients (24). Traditionally, this question has been answered with elaborate experiments involving treating the host animal with antibiotics to remove symbionts and then systematically augmenting the hosts' diet with additional nutrients until comparable fitness to that of symbiont-possessing animals is obtained (12). Now, whole genomic sequences from symbionts can be examined to gain insight into the nature of the relationship. Bacterial endosymbionts, over time, experience genome reduction, losing genes that are either redundant or code for products that can

be provided by the host cell (22). Thus, the presence of complete biosynthetic pathways can suggest important roles in supplementing nutrients to the host.

The role for the other bacterial species that have been found in leeches is also uncertain. There have been numerous suggestions as to what the role of the *Aeromonas* bacteria might play in medicinal leeches, including not only synthesizing B vitamins but also aiding in the digestion of blood and preventing the growth of other bacterial species in the digestive tract (16). It is possible that the *Rickettsia* reported from the glossophoniid leeches might be secondary symbionts, which are common in many of the insects with primary (obligate) symbionts, including aphids (9) and tsetse flies (11). These S symbionts are nonessential and may not be present in all host individuals or populations, though they are typically vertically transmitted from parent to offspring (2, 13). Kikuchi et al. (17) provided some evidence that the *Rickettsia* organisms found in *Torix tagoi* were vertically transmitted; however, closely related leeches did not appear to be infected, and thus the pattern is more reminiscent of S symbionts than obligate mutualists. The advantage to the host of possessing these S symbionts is not certain; however, several suggestions have been made. One study found that the pea aphid secondary symbiont was able to "rescue" its host from negative survival and fitness effects if the primary symbiont *Buchnera* was lost (19). These authors proposed that their results suggest a potential for repeated symbiont replacements and that bacterial partnerships could be "open for renewal and improvement over evolutionary time," something that might help to explain the phylogenetic diversity of primary symbionts seen in closely related insects. Perhaps acquisition of transient bacterial species by leeches, originally functioning as secondary symbionts and later establishing more obligate, primary roles in the host, is similar in this respect and can also help explain the rather unexpected high diversity of bacterial partners in these blood-feeding hosts as well.

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