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Affiliations between bacteria and marine fish leeches (Piscicolidae), with emphasis on a deep-sea species from Monterey Canyon, CA

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Summary

Leeches within the Piscicolidae are of great numerical and taxonomic importance, yet little is known about bacteria that associate with this diverse group of blood-feeding marine parasites of fish and elasmobranchs. We focused primarily on the bacteria from a deep-sea leech species of unknown identity, collected at ~600 m depth in Monterey Canyon, CA, along with two shallow-living leech genera, *Austrobdella* and *Branchellion*, from Los Angeles Harbor, CA. Molecular analysis of all five leech species revealed a dominance of gammaproteobacteria, which were distinct from each other and from previously reported freshwater leech symbionts. Bacteria related to members of the genus *Psychromonas* (99% similarity in 16S rRNA) were dominant in the deep-sea leech species (80–94% of recovered ribotypes) collected over 19 months from two different locations. *Psychromonas* was not detected in cocoons or 2–16 week-old juveniles, suggesting that acquisition is via the environment at a later stage. Transmission electron microscopy did, however, reveal abundant bacteria-like cells near areas of thinning of the juvenile epithelial surface, as well as *Psychromonas* sparsely distributed internally. Electron and fluorescence *in situ* microscopy of adults also showed *Psychromonas*-like bacteria concentrated within the crop. Despite the apparent non-transient nature of the association between *Psychromonas* and the deep-sea leech, their functional role, if any, is not known. The prevalence, however, of an abundant bacterial genus in one piscicolid leech species, as well as the presence of a dominant bacterial species in singular observations of four additional marine species, suggests that members of the Piscicolidae, possibly basal within the class Hirudinea, form specific alliances with microbes.

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Introduction

Leeches (class Hirudinea) are highly unusual annelids that survive mostly on the blood of vertebrates and as a consequence conjure, for most people, unsettling thoughts of bloodletting in the 1700s and scenes from the Amazon. As a group, however, leeches display a diversity of behaviours and lifestyles, ranging from parasitic (sanguivorous) to predaceous (macrophagous, non-sanguivorous). Those that are parasitic have numerous unique adaptations to accommodate blood feeding, including modifications of morphology, biochemistry and microbiology. Morphologically, they possess a highly extensible, diverticulated crop, as well as a strong body wall to withstand stretching during inflation (Sawyer, 1986). Both are significant in that leeches can ingest up to eight times their body weight in blood and feed once every 6–12 months (Khan, 1982; Zebe *et al*., 1986; Graf, 2002). Biochemically, they are well known for their production of vasodilators, anaesthetics and anticoagulants to relieve venous constriction and ensure blood flow, many of which have been used for medical purposes, both as isolated chemical compounds (e.g. hirudin) and as whole leeches applied after microvascular surgery (Fields, 1991). Finally, internal bacterial residents are believed to play an important role in the evolution and nutritional success of leeches, as well as other animals with strategies that involve consumption of unbalanced diets that are difficult to digest, such as blood, but also plant sap and wood.

Hirudo verbana, also sometimes identified as *Hirudo medicinalis*, belongs to the order Arhynchobdellida within the family Hirudinidae, and is the best studied of all leeches (Trontelj *et al*., 2004; Graf *et al*., 2006). Büsing (1951) was the first to identify a non-transient bacterium in the *Hirudo* crop, originally named *Pseudomonas hirudinis*, and later renamed *Aeromonas liquefaciens* (Bullock, 1961). In addition to *Aeromonas*, molecular approaches now suggest the presence, and in some cases dominance, of other additional microflora in *Hirudo*, including *Rikenella* (Bacteriodetes) and members of at least three additional proteobacterial subdivisions, most often found

in the excretory bladders or the intestine (Graf, 2002; Worthen *et al*., 2006; Kikuchi and Graf, 2007; Laufer *et al*., 2008; Kikuchi *et al*., 2009). This relatively simple microbial community is in stark contrast to digestive tract communities in most other animals, and is suggested to result from antimicrobial properties of the ingested vertebrate blood itself (Graf, 1999; 2000; Indergand and Graf, 2000; Rio *et al*., 2009; Silver *et al*., 2011). *Aeromonas*, in particular, has also been observed in other species of *Hirudo*, the North American medicinal leech *Macrobdella*, and the southern African leech *Asiaticobdella* (Wilken and Appleton, 1993; Siddall *et al*., 2007; Laufer *et al*., 2008), as well as mosquitoes (*Culex quinquefasciatus*; Pidiyar *et al*., 2002) and the vampire bat *Desmodus rotundus* (Pinus and Müller, 1980). The pervasive presence of specific bacteria within blood feeders is thought to reflect an important role in the nutritional strategy of these animals.

In the mid 1900s, German researchers were the first to note a lack of both secretory gland cells and endogenous digestive enzymes in the leech gastrodermis and suggested that bacteria, instead, might be involved in the digestive processes in leeches (see Jennings and Van Der Lande, 1967 and references therein). The possibility that leech microflora could compensate for the lack of host digestive capability was supported by evidence that microorganisms isolated from the crop possess, among other enzymes, haemolysins and lecithinases, which are thought to be important in the degradation of haemoglobin and erythrocyte membranes respectively (Jennings and Van Der Lande, 1967; Sawyer, 1986). Additionally, Zebe and colleagues (1986) noted a marked decrease in protein breakdown and subsequent ammonia excretion upon exposure to antibiotics, similar to levels observed in starving leeches, supporting a cessation of digestion in the inferred absence of microflora. It is therefore possible that the hydrolytic activities of internal microbes play an important, albeit variable, role in digestion by leeches. Graf provided evidence, much like the earlier studies of Zebe and colleagues (1986), that *Aeromonas* specifically may play a role in digestion. He noted a decrease in both ammonia excretion and oxygen consumption upon exposure to the antibiotic kanamycin, at a dose that the author claims had no negative effect on the leech host itself (Graf, 2002).

In addition to digestion, leech-associated microflora may also supplement nutrients, such as vitamins within the B complex, which are likely to be deficient in the diet of sanguivorous leeches (Nogge, 1981). Although experimental evidence is still limited, provisioning of B vitamins, including pantothenic acid, pryoxidine, thiamine, biotin and folic acid, by bacterial symbionts has been demonstrated for blood-feeding arthropods, including tsetse flies and ticks (Sang, 1956; Mews *et al*., 1977; Nogge, 1981; Pais *et al*., 2008). This has been investigated much more recently using genomic techniques, which provided evidence that the two vertically transmitted symbionts found in the midgut of tsetse flies (*Wigglesworthia* and *Sodalis*) not only complement the nutritional requirements of the host, but also possibly each other (Akman *et al*., 2002; Toh *et al*., 2006). In particular, *Wigglesworthia* specifically retains, in its reduced genome, pathways for both thiamine and cobalamine biosynthesis (Akman and Aksoy, 2001).

Members of another prominent leech family, the Glossiphoniidae, within the order Rhynchobdellida, have also been shown to commonly associate with bacteria. Bacterial symbionts of these freshwater leeches were found free in the gut lumen and within the excretory system, as described above for *Hirudo*, but also intracellularly in specialized organs called mycetomes. Several mycetome morphotypes have been described, all of which generally attach to the esophagus and contain high densities of bacteria (Kikuchi and Fukatsu, 2002; Siddall *et al*., 2004; Perkins *et al*., 2005; Graf *et al*., 2006). These bacteria have proven to be somewhat diverse among leech species, including gammaproteobacteria closely related to *Wigglesworthia* isolated from the mycetome of *Placobdella* and *Placobdelloides* species (Kikuchi and Fukatsu, 2002; Perkins *et al*., 2005), a distantly related gammaproteobacteria associated with the mycetome of the giant Amazonian leech *Haementeria* (Perkins *et al*., 2005), an alphaproteobacteria related to *Reichenowia* in the mycetomes of other *Placobdella* species (Siddall *et al*., 2004) and *Rickettsia*-like bacteria, not associated with a special organ, in both *Torix* and *Hemiclepsis* genera (Kikuchi *et al*., 2002). A recent genomic investigation showed that *Reichenowia parasitica* possesses genes that code for proteins involved in nitrogen fixation and vitamin B translocation, the former likely as a holdover for their membership within the Rhizobiaceae, but the latter perhaps revealing their involvement in nutrient provisioning to the leech host (Kvist *et al*., 2011).

Despite a century of research on leech–bacteria relationships, nothing is currently known for the Piscicolidae, a marine family of leeches within the order Rhynchobdellida that includes ~ 200 species (comprising ~ 60 genera), most of which are parasitic on elasmobranchs and teleosts. The Piscicolidae is a candidate for the most ancestral clade within the Hirudinea (Trontelj *et al*., 1999; Utevsky *et al*., 2007), thus their investigation is generally important for determining whether bacterial-mediated digestion and provisioning of complementary nutrients might be a key innovation in all blood-feeding leeches. In this study we provide evidence for an association between a novel deep-sea marine leech from Monterey Canyon and bacteria within the gammaproteobacterial genus *Psychromonas*, using molecular and microscopic techniques. Additionally, we also examined three common shallow-

living piscicolid species within the genera *Austrobdella* and *Branchellion*, along with an additional deep-sea specimen, and suggest that they, too, possess a primary bacterial resident. It was our aim that this study would reveal new leech–microbe alliances and expand our knowledge on the occurrence of bacteria within the Piscicolidae, and leeches in general.

Results

Deep-sea leech distribution, identity and development

Leeches were observed, via submersible, to be strikingly abundant at two sites at ~ 600 m depth in Monterey Canyon (named 'Pebbles' – dives DR093, DR205 and DR235, and 'Grady' – dive DR094, for the artificially implanted whalefalls also present at these sites). These sites are 10 km apart and in different forks of the canyon itself. In both cases, leeches were seen in high abundance on octopus and flat fish (Fig. 1), as well as inanimate objects (i.e. plastic deployments) and organisms unlikely to be prey items (i.e. urchins). Leeches on inanimate objects exhibited 'questing' behaviour in which they waved their anterior ends in the overlaying water column, with the posterior ends securely attached. Presumably, they are able to respond, in this way, to the mechanical stimuli of a passing prey animal. Similar leeches have been documented in Monterey Canyon from sites at different depths (270 m depth on an octopus and 1800 m depth on a flat fish), although at much lower abundance (1–2 per animal, as opposed to ~ 50–100 per animal, Fig. 1). Given this distribution, out of over 18 000 h of video and nearly 4 000 000 video annotations made by the Monterey Bay Aquarium Research Institute (L. Lundsten, pers. comm.), the deep-sea species investigated in this paper appears to be highly concentrated at ~ 600 m, a depth which may be influenced by the abundance of vertebrate prey species. Rockfish (*Sebastes* spp.), Dover sole (*Microstomus pacificus*) and deep-sea sole (*Embassichthys bathybius*), for example, are all most frequently found ~ 600 m depth (L. Lundsten, Monterey Bay Aquarium Research Institute, pers. comm.). *Enteroctopus dofleini*, the giant pacific octopus, was observed on several occasions to be covered in leeches. Although this invertebrate is not likely to be a primary prey species, it too is commonly observed between 300 and 700 m depth. It should be noted that the presence of a whalefall did not directly influence the presence of leeches, since they were not observed at other nearby whalefall sites at depths of 382 m and 1018 m (S. Goffredi, pers. obs.).

Adult leeches were of uniform sizes and measured ~ 1.5 × 18 mm (w × l, including suckers), and were filled, to varying degrees, with blood meal. Like other leeches, the crop appears to be used for storage of blood, and was observed to fill most of the body cavity when engorged (Fig. 1, inset). In one case, the blood meal was identified, via cytochrome c oxidase sequence, as belonging to a *Sebastes* species (data not shown; Hyde and Vetter, 2007). Like other piscicolids, this leech species has a cylindrical, narrowly fusiform body, tapering at both ends, with well-delineated anterior and posterior suckers of different shapes and sizes. Internal reproductive organs could be clearly visualized when blood meal was absent (Fig. 2E) and many specimens were collected with everted male bursa. Tubercles were absent and pigmentation consisted of transverse bands of orange coloration, distinct over the entire length, including suckers, fading ventrally slightly. There appeared to be a crescent-shaped pigmented (orange) area at the dorsal base of the anterior sucker.

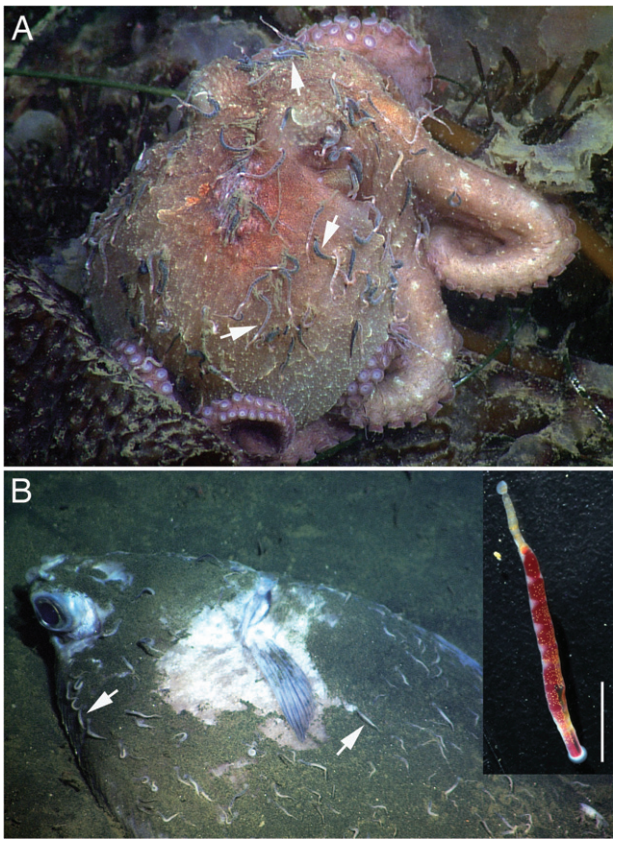

Fig. 1. Deep-sea leech from Monterey Canyon.
A. *Enteroctopus dofleini* covered by leeches (arrows, ~ 85 in this image). Note that some leeches do not appear to be filled with blood.
B. *Microstomus pacificus* with leeches (arrows, ~ 70 in this image). Inset, close-up of a single specimen showing blood-filled crop. Bar, 5 mm.
Photo credits: A and B, Monterey Bay Aquarium Research Institute; C, Dr Greg Rouse (Scripps Institution of Oceanography).

Following the collection in June 2011, adult leeches were maintained in the laboratory, at 4°C, for ~ 4 weeks, at which time it was noticed that cocoons had been

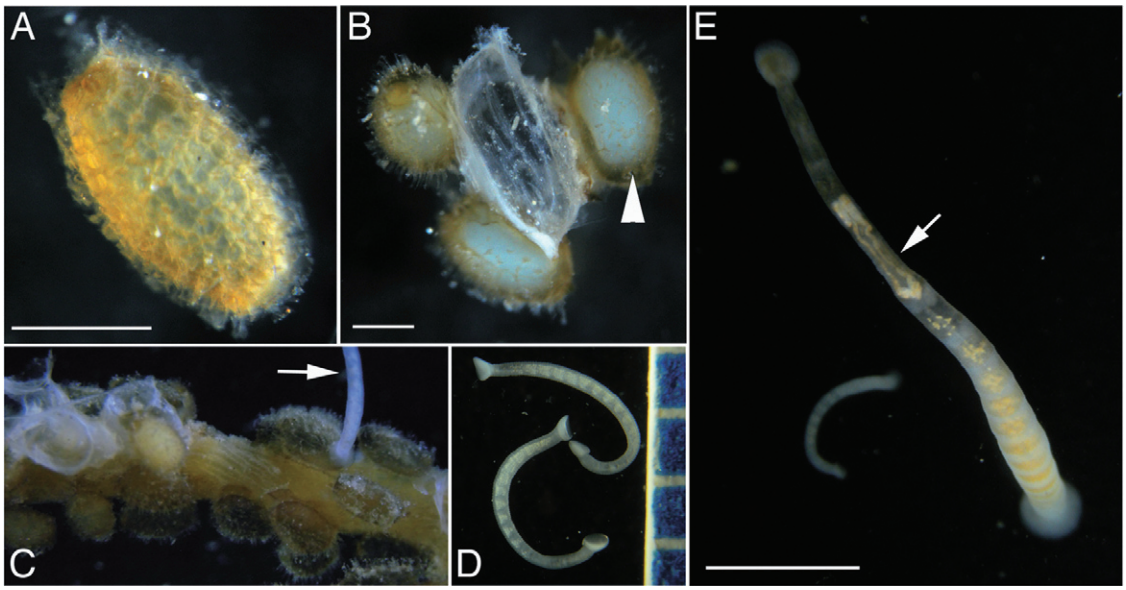

Fig. 2. Developmental series.
A and B. Cocoons deposited by deep-sea leeches on the glass of the aquarium. Notice that the cocoon in A appears empty, while three in B appear to contain a single egg (opaque material, arrowhead). Bars, 500 μm.
C. Cocoons deposited on a biological substrate of unknown nature. The arrow indicates a nearby hatchling.
D. Two hatchling leeches (ruler shows millimetre grade).
E. Hatchling is similar to the nearby adult, with the exception of lack of obvious reproductive organs (arrow in the adult) and lack of pigmentation. Note that the adult is devoid of blood meal after ~ 6 weeks in captivity (compare with blood-filled leech in Fig. 1 at time of collection). Bar, 5 mm.

cemented to the glass of the aquarium, as well as on a biological substrate of unknown nature (Fig. 2A–C). Approximately 200 cocoons were deposited and measured ~ 0.7 × 1.0 mm (w × l). Cocoons were golden brown and covered in a clear, sticky sheath (Fig. 2A). The substrate side was flat with slight concavity, while the rest was covered with projections. Most cocoons appeared empty (Fig. 2A), while a few appeared to still contain contents that were opaque in nature (Fig. 2B). The opaque mass inside of cocoons was assumed to be a single developing egg. In support of this, the number of hatched juveniles was roughly equal to the number of cocoons. Opaque cocoons were monitored over an additional 2 weeks, and although the contents changed in shape, hatching was never observed. Fecundity could not be calculated, given that 10 adults were present and cocoons were deposited on two separate occasions. Hatchling leeches were first measured to be ~ 0.2 × 4.0 mm (w × l; Fig. 2D), at which time they were assumed to be 1–3 weeks old, and were similar in morphology to the adults, with the exception of lack of pigmentation or obvious reproductive organs (Fig. 2E). Over the course of another ~ 5 months, juveniles grew in length to ~ 7 mm, and could stretch to 9 mm. Juveniles not harvested for analysis did not survive beyond ~ 6 months. This was assumed to be due to a lack of blood meal, although we attempted to feed them a live longspine combfish (*Zaniolepis latipinnis*), to which they attached but were never observed to consume blood.

Specimens from all three dives to 'Pebbles' (631 m depth; DR093, DR205 and DR235) as well as those from 'Grady' (587 m depth; DR094) were classified based on mitochondrial cytochrome c oxidase subunit I (COI) and NADH dehydrogenase subunit I (NDI) gene sequences (Apakupakul *et al*., 1999; Williams and Burreson, 2006). These specimens were most closely related to *Johanssonia arctica* (Piscicolinae), previously known as *Oxytonostoma arctica* (Fig. 3). The Monterey Canyon specimens were 90% similar by COI sequence and 92% by NDI sequence to *J. arctica*. Likewise, mitochondrial (12S) and nuclear (18S) rRNA genes were also amplified and found to be closest to *J. arctica* (96% and 99% similar respectively; data not shown; Utevsky and Trontelj, 2004; Williams and Burreson, 2006). It should be noted that very few deep-sea leeches have gene sequences available in public databases; thus, it is not possible to know definitively, even upon morphological examination, whether the deep-sea leech in this investigation is new to science. Interspecific divergence within the deep-sea species was 0.6% for COI and 1.1% for NDI (Fig. 3). Cytochrome c oxidase subunit I sequences acquired for laboratory hatchlings confirmed them to be 99.8% similar to the adults (Fig. 3). The deep-sea leech209 was very different in morphology (no pigmentation, with obvious tubercles;

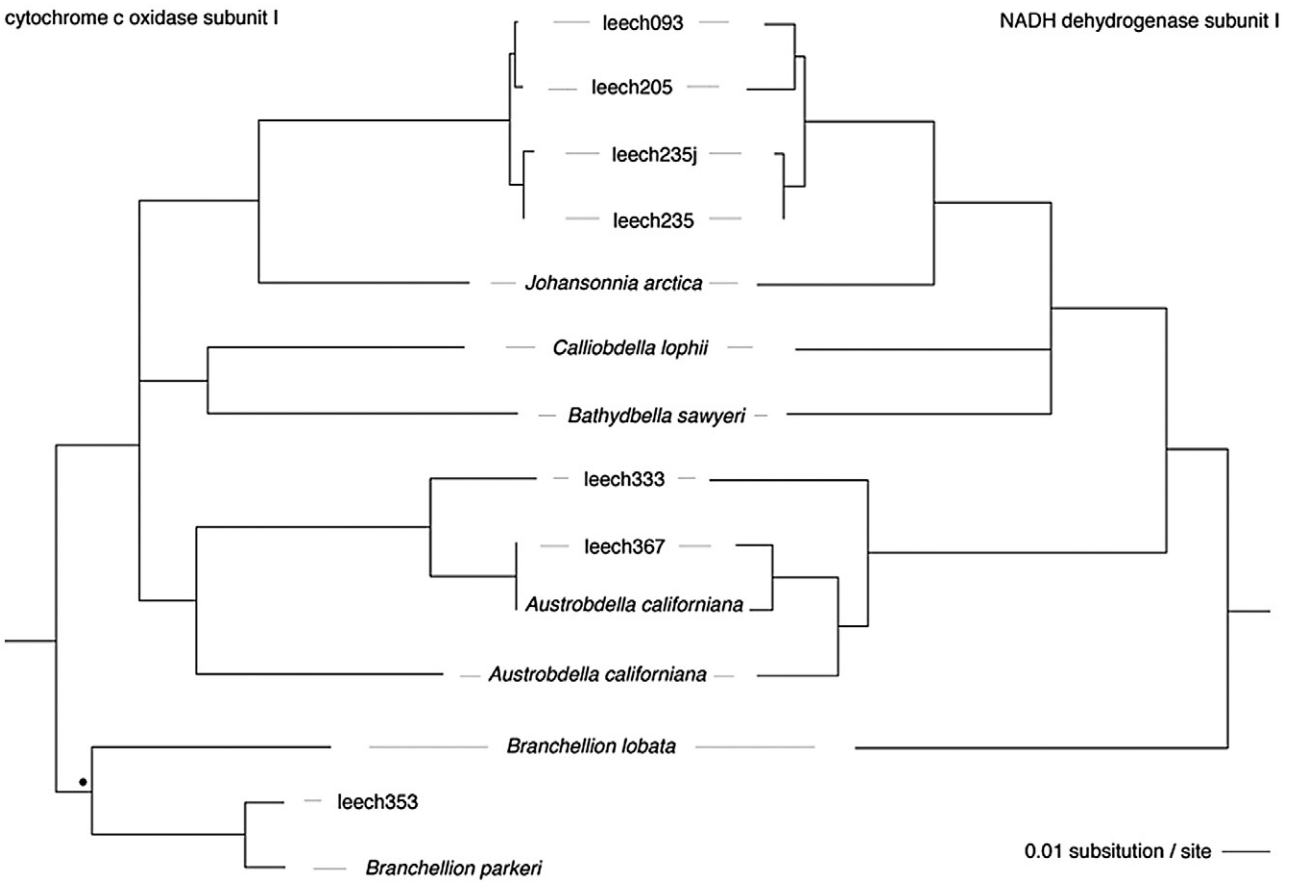

Fig. 3. Leech phylogeny based on mitochondrial cytochrome c oxidase (COI, left) and NADH dehydrogenase (NDI, right) genes. All nodes shown, as neighbour-joining, are supported by bootstrap values > 70% for both parsimony and neighbour-joining distance methods, except the node at the dot (•), which had a parsimony bootstrap of 58. Leech093, 205 and 235 were collected from 631 m depth in Monterey Canyon (leech235j denotes a juvenile hatchling), while the other three leeches examined in this study were collected by trawl from Los Angeles Harbor. The outgroup was an undescribed species of *Branchellion* sp. from Heron Island (not shown).

image not shown) and was < 90% similar to any leech in the public database, including *J. arctica* (only 83–87% similarity by COI and NDI).

Bacteria associated with the deep-sea leech from Monterey Canyon

Transmission electron microscopy (TEM) revealed the presence of bacteria-like cells of a single morphotype, some dividing, within what is assumed to be the crop given the highly microvillus nature and generally convoluted surface of the epithelium (Fig. 4A–D). Bacteria were suspended in the lumen itself and appeared to form a 'bridge' from one side of the membrane to the other, at an apparent constriction of the organ (Fig. 4A). Many were also observed in close association with the microvilli on the inner surfaces (Fig. 4B and D). Transmission electron microscopy of a juvenile hatchling also revealed the presence of bacteria-like cells both externally and internally (Fig. 4E–G). External bacteria, of two morphotypes, appeared to be in a mucous layer, near to areas of dramatic thinning of the leech outer body wall (Fig. 4E and F). Bacteria, ~ 0.5–2 μm in size, were observed within the juvenile leeches, albeit sparsely distributed and extracellular (Figs 4G and 7). It is not clear whether the appearance of the secondary vacuole surrounding each bacterium is real or an artefact of preparation (Fig. 4G).

To determine the identity of microbes associated with the deep-sea leech specimens, we examined 16S ribosomal RNA sequences from total host DNA extracts, some of which were taken from sections primarily through the crop. Bacteria most closely related to the genus *Psychromonas* (subdivision *Gammaproteobacteria*) were directly amplified and sequenced, using general bacterial 16S rRNA primers, from 12 individual adult leeches (Fig. 5). *Psychromonas hadalis* and *Psychromonas profunda* were the closest cultured relatives of the dominant bacterial ribotype found in association with the leeches (97–99% similarity in 16S rRNA; Xu *et al*., 2003; Nogi *et al*., 2007). *Psychromonas*-related bacterial 16S rRNA sequences

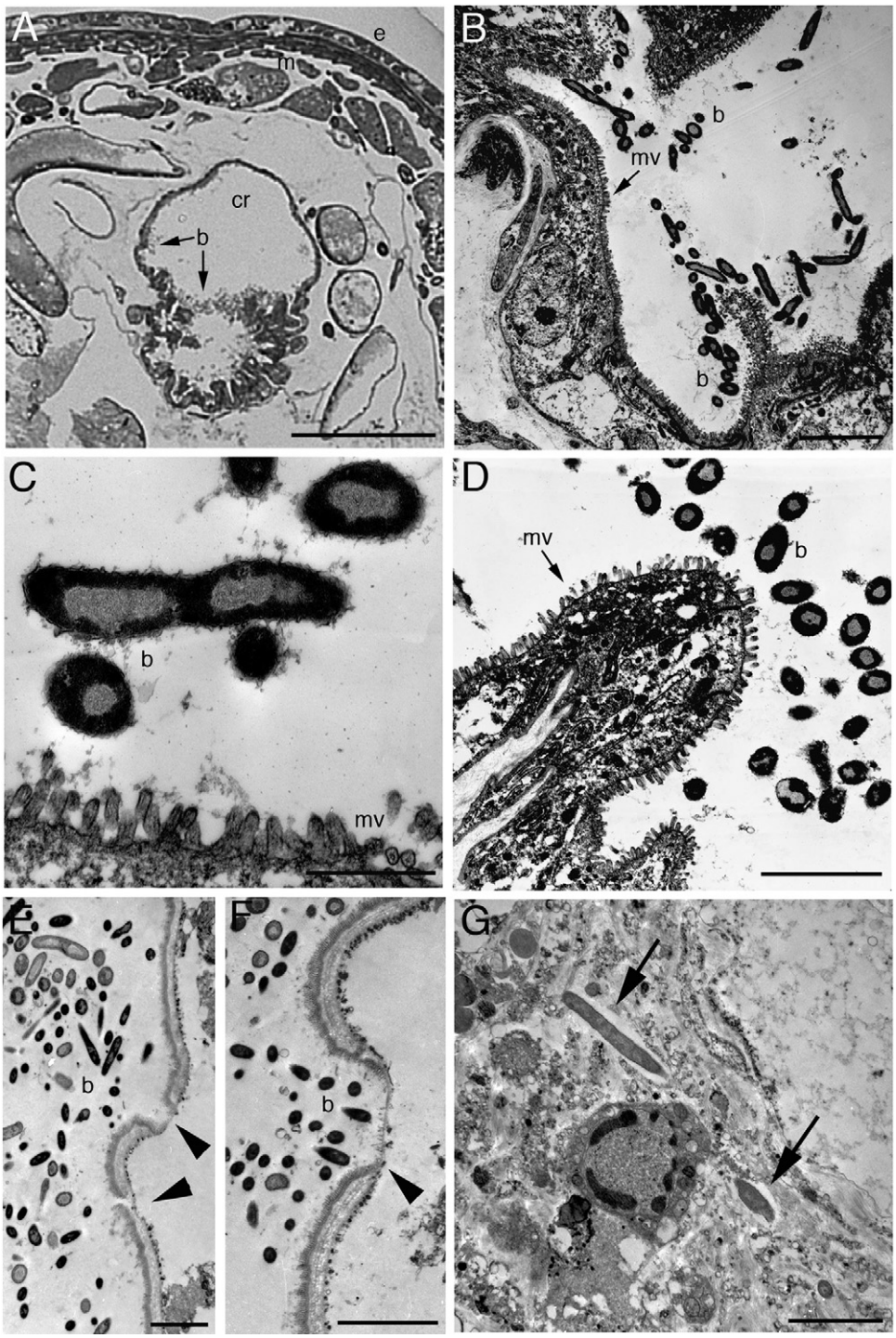

Fig. 4. Leech-associated bacteria, as shown by transmission electron microscopy.
A. Adult leech, in distal cross section, showing central organ, assumed to be the crop (cr) based on the highly microvillus nature and generally convoluted surface of the epithelium. Many bacteria (b) were present within the lumen. e, external surface; m, muscle.
B–D. Close-up images of the same central organ, note microvilli (mv) and numerous bacteria (b), some of which appear to be dividing (C).
E and F. Juvenile leech, showing aggregations of bacteria present in mucous on the outside of the animal (at left in both images). The outer epithelia appear to be thinner near areas of bacterial aggregation (arrowheads).
G. Juvenile leech, showing internal bacteria. It is not clear whether the appearance of the secondary vacuole (arrow) surrounding each bacterium is real or an artefact of preparation. Bars, 5 μm, except for A (100 μm) and C (1 μm).

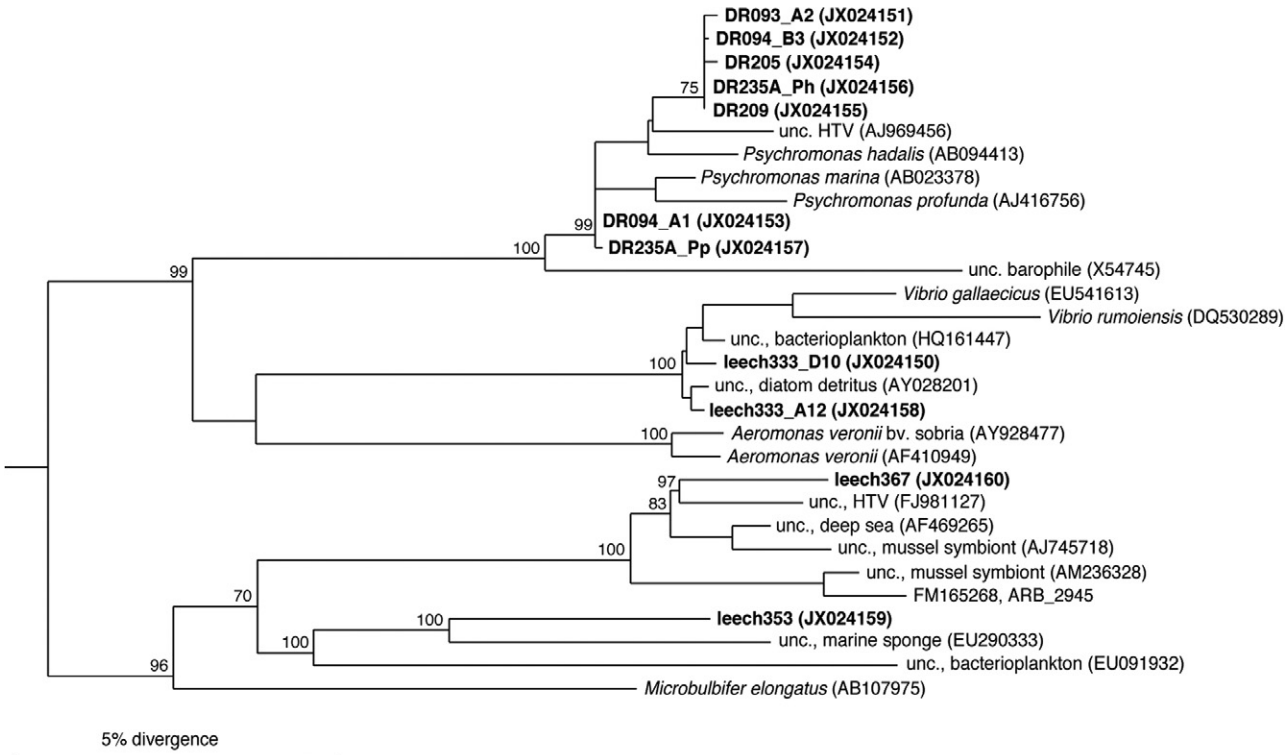

Fig. 5. Phylogenetic relationships of gammaproteobacteria associated with leeches, based on sequence divergence within the 16S rRNA gene. Additional sequences were obtained from GenBank and compiled and aligned with our 16S rRNA sequences (in bold) using the ARB automated alignment tool with subsequent manual refinements. For near full-length representatives and closest relatives, neighbour-joining analysis was conducted with Olsen distance correction. *Desulfocapsa sulfexigens* (Y13672) was used as an outgroup. Numbers next to nodes correspond to bootstrap values > 70, based on and 5000 neighbour-joining replicates. unc., uncultured.

recovered from many leeches collected at the same locality over a time span of 19 months (six individuals from DR093, three from DR205 and two from DR235), as well as from a site ~ 10 km away (three individuals from DR094), revealed nearly identical ribotypes (99.9% similarity; Fig. 5), suggesting both the non-transient and specific nature of this leech–microbe association. Even leech209, a presumably different species collected from a site at 1018 m depth, possessed a dominant *Psychromonas*-like symbiont that was 99.8% similar to the 600 m species (Fig. 5). *Psychromonas* was not detected, using general bacterial and *Psychromonas*-specific primers, in either the cocoons, which appeared to contain eggs, or 2–16 week-old juveniles; thus, it remains unknown when the bacteria become associated with the leeches.

Comparison of cloned sequences ($n = 36$–87 per individual) from four additional leeches, plus the one specimen from a deeper depth (leech209), revealed that *Psychromonas*-related bacteria comprised the vast majority (~ 80–94%) of associated bacteria (Table 1). Two

Table 1. Summary of bacterial ribosomal 16S rRNA clone library results for deep-sea leeches (shown as % of each library).

	Leech ID[a]				
Closest bacterial group	DR093	DR094	DR205	DR235	DR209
Gammaproteobacteria					
Psychromonas-like	94	80	85	91	92
Alteromonadaceae[b]	–	13	–	–	–
Other[c]	6	7	15	9	8
Total # of clones	36	87	45	82	48

a. Leeches from dives DR093, 094, 205 and 235 are all the same species collected from ~ 600 m depth in Monterey Canyon, CA. Leech209 (DR209) is a different species collected from 1018 m depth.
b. Alteromonadaceae included both *Marinobacterium* and *Microbulbifer*-related ribotypes.
c. Other recovered ribotypes were related to *Flavobacterium* and *Lentisphaera* genera.

Psychromonas ribotypes were commonly present and for two leeches it was determined, via restriction fragment length polymorphism (RFLP) and sequencing, that bacteria most closely related to *P. hadalis* were dominant (62–70%), as compared with *P. profunda* (16–20%), with a consistent ratio of ~ 3.5:1. Additional recovered ribotypes (6–20% by abundance, 'other' in Table 1) were related to other gammaproteobacteria (e.g. *Marinobacterium*), as well as *Flavobacterium* and *Lentisphaera* genera.

Fluorescence *in situ* hybridization (FISH) results complemented the TEM results by revealing bacteria both within a crop-like organ, and sparsely distributed in other areas of adult leeches. Via FISH, bacteria were visualized within cells near the crop, which could be seen clearly due to autofluorescence of the blood meal (Fig. 6A–C). Bacteria, via FISH, also measured ~ 0.5–1.0 μm in length. In general, FISH imagery of bacteria seemed best when leeches were devoid of blood meal. The presence of the predominant *Psychromonas*-like bacteria in a highly convoluted and microvillus structure, possibly the very distal end of the crop, was also observed (Fig. 6D–F). Additionally, aggregations of *Psychromonas*-like bacteria were observed in a posterior sucker of a juvenile hatchling (Fig. 7).

Bacteria associated with shallow-living marine leeches

Three shallow-living marine leeches, collected in Los Angeles Harbor by trawl, were also investigated for bacterial presence (Fig. 8). Two specimens, leech367 and leech333, were most closely related to *Austrobdella californiana* (Platybdellinae; 99–100% and 95–96%, by COI and NDI gene sequences respectively), yet ~ 6% divergent from each other (Fig. 3). In a previous study, three different *A. californiana* were found to be up to 13% divergent based on COI and NDI; thus, a detailed investigation of this particular species complex is still necessary (Williams and Burreson, 2006). Leech353 was most closely related to *Branchellion parkeri* (Piscicolinae; 98% similar in COI sequence; Fig. 3). There is no NDI sequence for *B. parkeri* in GenBank and we, too, were also unable to amplify NDI from leech353, using the previously published primer set (Light and Siddall, 1999).

With regard to bacterial presence, a single gammaproteobacterial ribotype was abundant (83–94% of recovered ribotypes) in all shallow-water leeches (Table 2). For the two cryptic species of *A. californiana*, the dominant bacterial ribotypes were different from each other. The main bacterial ribotype associated with leech333 was most closely related (97% similarity in 16S rRNA) to *Vibrio* isolated from other invertebrates, including cultured clams and a spider crab (Fig. 5; Beaz-Hidalgo *et al*., 2009; Gomez-Gil *et al*., 2010). The dominant bacterial ribotype associated with leech367 was most closely related (96% similarity in 16S rRNA) to the thiotrophic symbiont isolated from hydrothermal vent bivalves, designated gamma type A (Table 2; Duperron *et al*., 2005). The species of *Branchellion*, leech353, was also found to associate with a dominant gammaproteobacteria, only distantly related to any bacteria in the database (< 89% similarity in 16S rRNA), including *Microbulbifer elongatus*, and those isolated from marine sediment and a sponge (designated gamma type B; Ohta *et al*., 2004; Sipkema and Blanch, 2010; Table 2; Fig. 5). Additional recovered ribotypes (6–17% by abundance, 'other' in Table 2) were primarily related to uncultured epsilonproteobacteria and Bacteroidetes.

Discussion

The prevalence of one bacterial genus in a deep-sea piscicolid leech species, as well as the presence of a dominant bacterial species in singular observations of four additional marine species, supports the widespread occurrence of bacteria in the Hirudinea. Association of each leech species with an abundant, albeit variable, gammaproteobacteria is in agreement with previous observations for freshwater leeches, in that most species observed to date, including two other main leech families (Hirudinidae and Glossiphoniidae), have all revealed a common bacterial type, which differs depending on leech species (Jennings and Van Der Lande, 1967; Kikuchi *et al*., 2002; 2009; Siddall *et al*., 2004; Graf *et al*., 2006). The variable nature of the primary bacterial species in leeches is similar to that observed for the nutritional symbionts of weevils, which have undergone substitution many times during their evolution (Lefèvre *et al*., 2004; Conord *et al*., 2008). Although Toju and Fukatsu (2011) provided evidence that infection frequencies of the various symbionts in weevils were significantly correlated to climatic and ecological influences, factors contributing to symbiont replacement, whether internal (host physiology) or external (the differential occurrence of locally adapted bacterial species), largely remain unknown.

The deep-sea leech investigated in this study is so far known only from ~ 600 m depths in Monterey Canyon, CA. A formal species description for the deep-sea leech in this study is still necessary, and will include specifics of the male and female anatomy, coelomic and excretory system architecture, and number of eyespots on annuli and suckers, to name a few (Sawyer and Chamberlain, 1972; Borda and Siddall, 2004; Williams *et al*., 2007). For now, we can only comment on the external features of the species, as well as its early life stages, including cocoons. The deep-sea leech in this study reproduces by depositing golden brown oval-shaped sticky, robust cocoons, which appeared to contain only a single egg. Juveniles, which were estimated to have hatched 2–4 weeks post deposition, roughly resembled the adults. The species

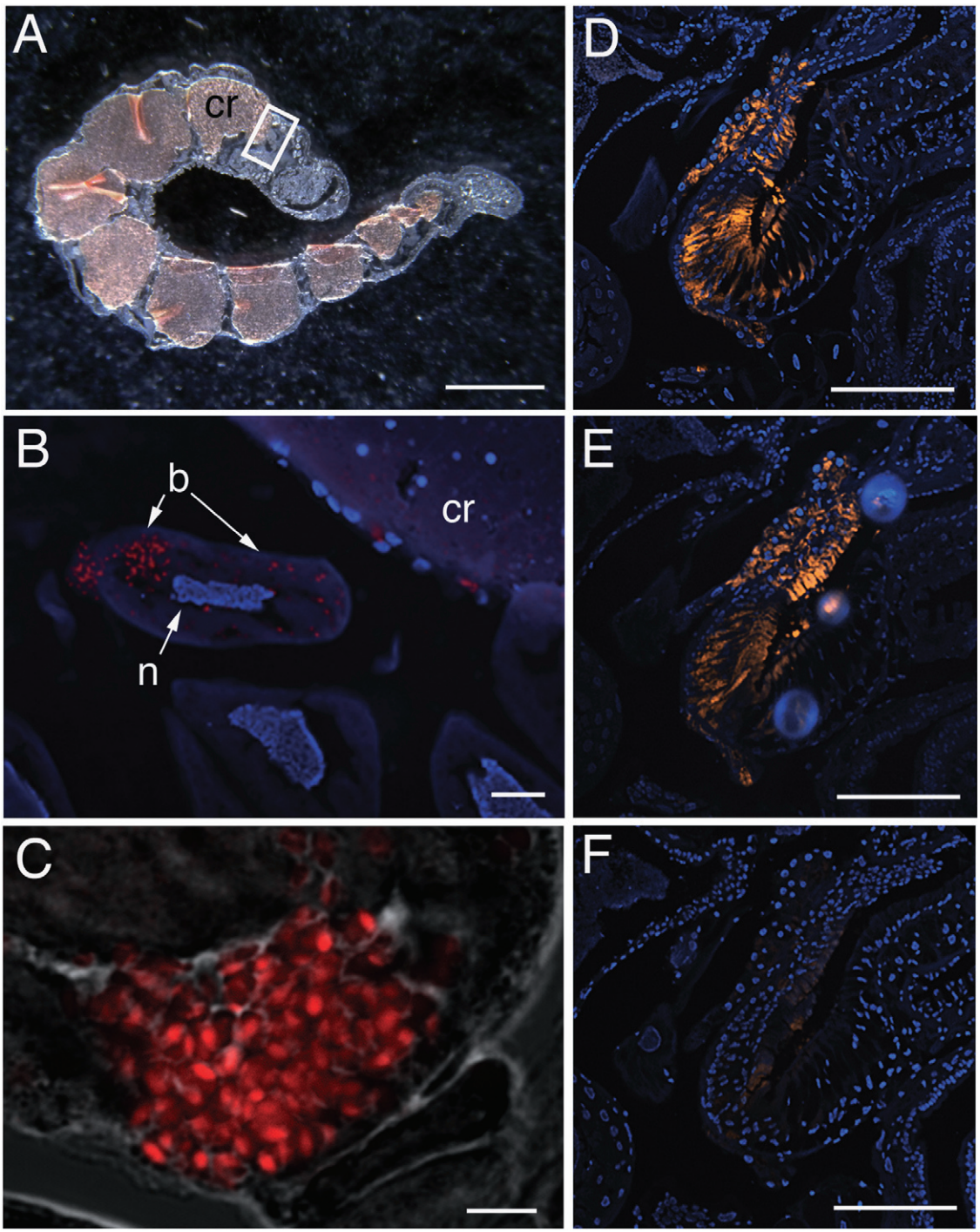

Fig. 6. Bacteria associated with an adult leech, as shown by fluorescence *in situ* hybridization microscopy (FISH).
A. Light image of a leech thick section embedded in, and then removed from, Steedman's wax prior to hybridization. The box denotes the area shown in B. Bar, 1 mm. cr, crop.
B. Close-up of the crop (note the autofluorescent nature) and bacteria-filled cell (n, nucleus), hybridized with the Eub338 probe set labelled with Cy3 (pink) and counter-stained with DAPI. Bar, 10 μm.
C. Close-up of a cell containing bacteria (possibly a haemocyte), hybridized with the Eub338 probe set, overlayed with differential interference contrast imagery, taken with a Leica TCS SP5 II confocal microscope. Bar, 5 μm.
D–F. Leech sections hybridized with the Eub338 probe set, *Psychromonas*-specific probe (NOR2-1453) and a 'nonsense' Arc915 probe respectively, showing bacteria in a highly convoluted, crop-like organ. Bars, 100 μm.

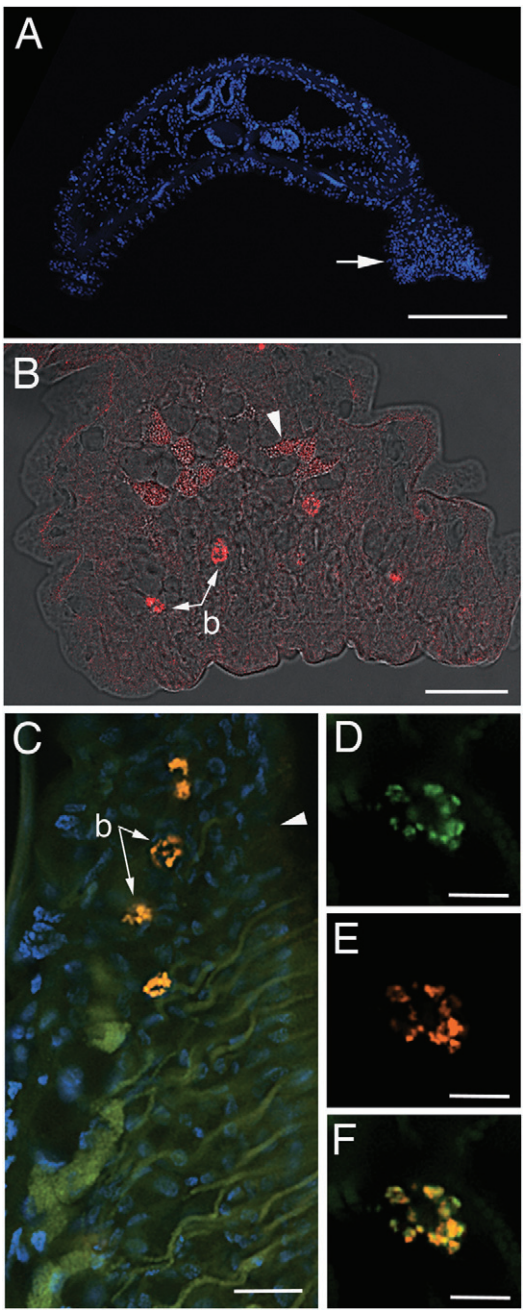

Fig. 7. Bacteria associated with a juvenile leech, as shown by fluorescence *in situ* hybridization microscopy (FISH).
A. Whole juvenile in longitudinal cross section, stained with DAPI. The arrow denotes the posterior sucker. Bar, 100 μm.
B. Close-up of posterior sucker showing bacteria (b), hybridized with the Eub338 probe set, labelled with Cy3, overlayed with differential interference contrast imagery. Taken with a Leica TCS SP5 II confocal microscope. Note the surrounding body wall and autofluorescent nature of very different nearby structures (arrowhead), presumed to be newly formed reproductive tissue. Bar, 25 μm.
C. Close-up of the sucker showing bacteria (b), similarly hybridized with the Eub338 probe set (pink) and counter-stained with DAPI. The arrowhead denotes the body wall. Autofluorescence is shown in green. Bar, 10 μm.
D–F. Close-up of a bacterial aggregation, hybridized with the Eub338 probe set, *Psychromonas*-specific probe (NOR2-1453) and with both channels overlayed respectively, showing complete overlap of signals. Bars, 5 μm.

appears by molecular analysis to cluster within a well-supported group that includes *Johanssonia*, *Calliobdella* and *Bathybdella* (Williams and Burreson, 2006), and to be most closely related to *J. arctica*, from off of Newfoundland (Khan, 1982). The cold-water lifestyle of *J. arctica* is consistent, as is cocoon and adult morphology (Khan, 1982); however, 10% divergence in COI gene sequence suggests that they are not the same species. Other deep-sea leeches have been described; however, none, with the exception of *Bathybdella sawyeri*, have available molecular data with which to infer relatedness (Richardson and Meyer, 1973; Burreson, 1981; Janssen, 1993). *Glyptonotobdella antarctica*, based on behaviour, depth (300–665 m), cold-water lifestyle and coloration, remains a possible close relative; however, cocoon morphology was markedly different (Janssen, 1993). Observations made during three research expeditions to two sites revealed them to be abundant on most surfaces, both biological (ex. octopus and urchins) and inanimate. Both *J. arctica* and *G. antarctica* have been observed to move between invertebrates (octopuses, urchins and pycnogonids) and their actual fish prey (Janssen, 1993) and other well-known species, such as *Notostomum* and *Megaliobdella* spp., have often been collected separately from prey animals (Sloan *et al*., 1984; Utevsky *et al*., 2007), as was the case for the deep-sea leech examined in this study.

Bacteria related to members of the genus *Psychromonas* were observed to comprise the vast majority of bacteria associated with 17 deep-sea leech specimens, collected from two different locations (~ 10 km apart) over the course of 19 months. Affiliation to the genus *Psychromonas* is consistent with the temperature of the deep-sea habitat, which was 4–5°C at the collection sites in this study. In fact, this bacterial genus is considered to live primarily in permanently cold environments, such as polar regions and deepwater environments (Lauro and Bartlett, 2008). The closest known cultured relatives to the leech-associated bacteria are *P. profunda* and *P. hadalis*, both psychrophilic heterotrophs isolated from deep-sea sediments (Xu *et al*., 2003; Nogi *et al*., 2007).

Specific *in vivo* localization, transmission mechanism and biological function of the *Psychromonas*-like bacteria have yet to be established. The fact that *Psychromonas*-like ribotypes were not observed in cocoons or juveniles implies environmental acquisition of the locally adapted microbe by the deep-sea leech species. This is different from the *Hirudo*–bacterial symbiosis, in which symbionts

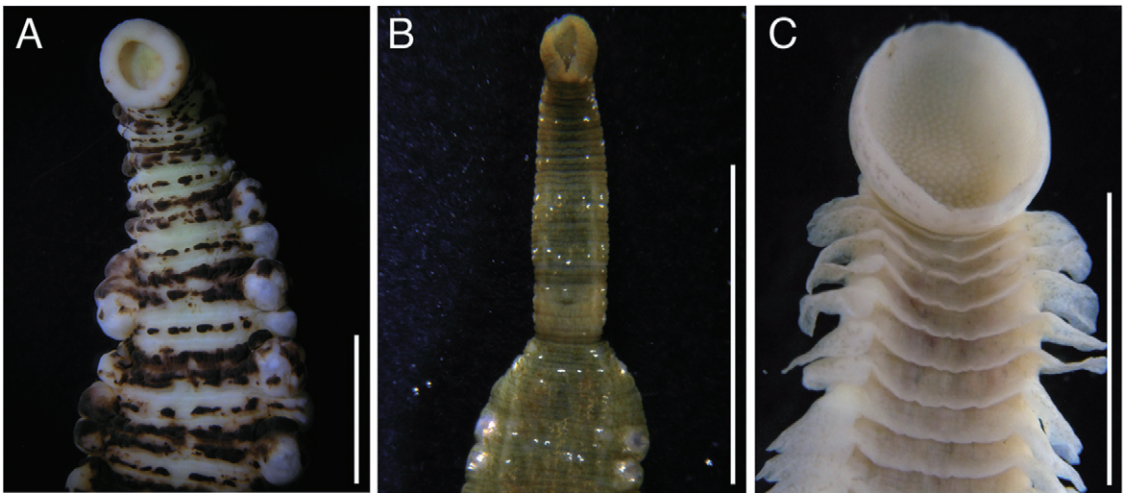

Fig. 8. Shallow-water leeches, collected from Los Angeles Harbor, used in this study.
A and B. (A) Leech367 and (B) Leech333 were most closely related to the *Austrobdella californiana* species complex (note the very different morphology).
C. Leech353 was most closely related to *Branchellion parkeri*. Bars, 10 mm.

were present in the cocoon fluid immediately following deposition (Graf *et al*., 2006; Rio *et al*., 2009). It should also be noted that other bacteria may be present in the deep-sea leech, given the tendency for the polymerase chain reaction to underestimate, or miss, altogether, the less abundant microbial members of a community.

Similar to the *Aeromonas* symbionts of freshwater leeches, *Psychromonas* species are found ubiquitously in the environment but appear to also associate with animals (Graf, 2002; Lauro and Bartlett, 2008; Zbinden *et al*., 2010). As with *Aeromonas*, the pervasive nature of the association between *Psychromonas*-like bacteria and the deep-sea leech suggests biological significance, and we assume the function may be either enhanced digestion or nutritional compensation for an unbalanced diet, similar to that suggested and shown for freshwater leeches. Of course, vitamin requirement may vary considerably depending on feeding habits, specific prey species, and host and symbiont metabolic rates; thus, it may remain difficult to assign a specific improvement on leech fitness by association with bacteria.

If microbes either aid in the digestion of blood meal or possibly provide nutrients to their host, then their presence has great implications for the evolution of blood feeding in general. Others have noted the inherent plasticity in blood-feeding behaviour and that this capability has been lost at least fours times during leech evolution (Apakupakul *et al*., 1999; Borda and Siddall, 2004). Perkins and colleagues (2005) imply further that leeches must have then lost and found bacterial partnerships at least that many times during their evolutionary history. Since blood feeding is a variable character state among leeches (Siddall and Burreson, 1995; Apakupakul *et al*., 1999), then so too must be their association with specific bacterial symbionts. If, indeed, leeches have a common origin in an ancestral sanguivorous leech (Apakupakul *et al*., 1999; Borda and Siddall, 2004), then it becomes important to also investigate associated microbes in

Table 2. Summary of bacterial ribosomal 16S rRNA clone library results for shallow-water leeches (shown as % of each library).

Closest bacterial group	*Austrobdella*[a] 333	*Austrobdella*[a] 367	*Branchellion*[b] 353
Gammaproteobacteria			
Vibrio	94	–	4
Gamma type A	–	83	–
Gamma type B	–	–	88
Other[c]	6	17	8
Total # of clones	46	42	25

a. Leech333 and leech367 were most closely related to the *Austrobdella californiana* species complex (91–98% similar in COI/ND1), but were 5.5% divergent from each other.
b. Leech353 was most closely related to *Branchellion parkeri* (91% similar in COI sequence).
c. Other recovered ribotypes were related to uncultured epsilonproteobacteria and Bacteroidetes.

additional divergent leech species, especially those considered to be basal. On the other hand, the alternative hypothesis of multiple independent origins of blood feeding, with a macrophagous animal as an ancestor (Sawyer, 1986; Siddall and Burreson, 1996), would also benefit from a concerted effort to screen relevant leech lineages for associated bacteria. On a smaller phylogenetic scale, this has been done for non-blood-feeding freshwater glossiphoniids, with neither of the lineages possessing bacterial symbionts (Apakupakul *et al*., 1999; Siddall *et al*., 2004). Conversely, but perhaps just as informative, Jennings and Van Der Lande (1967) noted that the predatory leech *Erpobdella octoculata* harboured a more diverse bacterial community than the sanguivorous leeches examined, all of which yielded only one dominant species. They suggested that engulfment of whole prey allows for increased exposure and consumption of microorganisms, some of which may become at least transiently established (Jennings and Van Der Lande, 1967). Very little is still known about microbial presence in marine leeches, but it follows that certain leech lineages could be examined with the hypothesis that a truly sanguivorous lifestyle results in the presence of a dominant bacterial species, as has been now observed for at least three major families within the Hirudinea, including the Piscicolidae.

Experimental procedures

Sample collection

Deep-sea leeches were collected, using the ROV 'Doc Ricketts' (owned and operated by the Monterey Bay Aquarium Research Institute) from two whalefalls in Monterey Canyon off the coast of California; dive DR093 (16 November 2009), DR205 (26 October 2010) and DR235 (5 June 2011) to the 'Pebbles' site, 36.802N, 121.994W, 631 m depth; dive DR094 (16 November 2009) to the 'Grady' site, 36.712N, 121.996W, 587 m. The 'Pebbles' site is ~ 10.4 km north of 'Grady', on the other side of the canyon. An additional deep-sea leech was collected at the 'Francisco' site in Monterey Canyon, 36.772N, 122.083W, 1018 m depth (dive DR209, 29 October 2010). Shallow leeches were collected, incidentally, by trawl in Los Angeles Harbor (33.710N, 118.268W, 8–19 m depth) on March 2009 (leech353), October 2009 (leech367) and November 2010 (leech333). Leech353 was collected directly from a host fish, *Rhinobatos productus*. All shallow leeches were preserved in cold 70% ethanol immediately upon collection and stored at 4°C until processing. Deep-sea specimens were surface-treated with 100% ethanol and frozen at –80°C or preserved directly in 4% sucrose-buffered paraformaldehyde for FISH microscopy or 3% sucrose-buffered glutaraldehyde for electron microscopy (see specifics below). Specimens, and cocoons, were initially examined using a Leica S8APO stereomicroscope and photographed with a Nikon Coolpix P6000 digital camera (Figs 1, 2, 6 and 8). Following dive DR235 (June 2011), ~ 20 deep-sea adult leeches were collected and maintained in the laboratory for ~ 25 days, at which time they deposited ~ 200 cocoons. Hatchling leeches were collected, weekly, in the same manner as above until all had either been harvested or died (~ 6 months). In all cases, we digested the entire leech, except where sub-dissected, or for large shallow leaches (in which case the body was dissected in cross section, near the area of the crop).

Molecular analyses

The DNEASY kit (Qiagen, Valencia, CA, USA) was used to extract total DNA from leech specimens. For the deep-sea species, entire leeches were extracted (thus including crop and intestinum), while in the large shallow leaches, except the smaller leech334, the body was dissected in cross section, near the area of the mid-crop. Identities of the leeches were determined by phylogenetic comparisons with previously published mitochondrial COI and NDI sequences. The COI gene was amplified using one of two previously published sets of primers; COIF (5′-TCMACTAATCAYAARGAYA-TTGGNAC-3′) and COIR (5′-CCDCTTAGWCCTARRAARTGTTGNGG-3′; Nelson and Fisher, 2000) or LCO1490 (5′-GGTCAACAAATCATAAAGATATTGG-3′) and HCO2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′; Folmer *et al*., 1994). A partial fragment of the NDI gene was amplified using the primers LND300 (5′-TGGCA-GAGTAGTGCATTAGG-3′) and HND1932 (5′-CCTCA-GCAAAATCAAATGG-3′), published previously (Light and Siddall, 1999). Thermal cycling conditions included 60 s each of denaturation at 94°C, annealing at 50°C and 45°C, for COI and NDI, respectively, elongation at 72°C (25 cycles), and a final extension at 72°C for 6 min. Polymerase chain reaction products were sequenced directly (see details below).

To assess whether leeches hosted bacteria, we examined 16S rRNA sequences amplified either directly from DNA extracts or via clone library analysis of bacterial 16S rRNA genes. A 1465 bp fragment of the bacterial 16S rRNA gene was generated using bacteria-specific primers (27F, 1492R; Lane, 1991). In some cases, a previously published FISH probe (NOR2-1453; 5′-GGTCATCGCCATCCCC-3′; Eilers *et al*., 2000) was used as a reverse PCR primer, paired with 27F, to selectively amplify a 1426 bp region of 16S rRNA specific to the genus *Psychromonas*. Following sequencing, this primer sequence was determined to be an exact match to *Psychromonas*-related bacteria recovered from the deep-sea leeches and allowed for rapid screening of the presence of *Psychromonas*-like bacteria in multiple specimens. Thermal cycling conditions included 60 s each of denaturation at 94°C, annealing at 54°C and 65°C, for general and *Psychromonas*-specific 16S rRNA respectively, elongation at 72°C (25 cycles) and a final extension at 72°C for 6 min. Polymerase chain reaction products were sequenced directly, in 12 specimens, or cloned first (five specimens). PCR products were pooled and cloned using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA, USA). Clone libraries of PCR-amplified bacterial 16S rRNA genes were constructed from each leech, with 36–87 clones analysed for each library (Table 1). Transformants were grown overnight (in LB broth with 50 $\mu g\ ml^{-1}$ kanamycin), and screened directly for the presence of inserts using M13F and M13R

vector primers and the thermal cycling conditions of an 8 min initial denaturation, followed by 30 s each of denaturation at 94°C, annealing at 54°C, elongation at 72°C (30 cycles) and a final 6 min of elongation at 72°C. In all cases, M13 amplicons were digested first with *HaeIII* (according to manufacturer's instructions; New England Biolabs) in order to observe diversity, including ratios of *P. hadalis* versus *P. profunda*, and select unique samples for sequencing. M13 amplicons, or 16S rRNA gene products sequenced directly, were cleaned prior to sequencing with MultiScreen HTS plates (Millipore Corporation, Bedford, MA, USA). Samples were sequenced either via ABI sequencing technology (Laragen, Culver City, CA, USA) or using the Genome Laboratory DTCS Quick Start Kit (Beckman Coulter, Fullerton, CA, USA), precipitated with glycogen and sodium acetate, resuspended in 40 μl of formamide and run on a CEQ 8800 Genetic Analysis System (Beckman Coulter, Fullerton, CA, USA). Sequences were assembled, edited and aligned using Sequencher v4.10.1 (GeneCodes Corp.). Closest relatives were acquired using the GenBank Basic Local Alignment Search Tool, BLASTn (Altschul *et al*., 1997), and identified using phylogenetic analysis. GenBank accession numbers for sequences obtained in this study are JX024151–JX024161 for bacterial 16S rRNA, JX024162–JX024168 for leech COI and JX024169–JX024174 for leech ND1.

Electron microscopy

For examination by TEM, samples (~ 1 mm^3) were fixed in 3% glutaraldehyde buffered with 0.1 M phosphate and 0.3 M sucrose (pH 7.8). Following a wash in 0.1 M sodium cacodylate containing 24% sucrose, samples were post-fixed with 1% OsO_4 in 0.1 M sodium cacodylate for 1 h, stained *en bloc* in 3% uranyl acetate in 0.1 M sodium acetate buffer for 1 h, dehydrated through an ethanol series, then infiltrated and embedded in Spurr's resin (Ted Pella, Redding, CA, USA). Thick (0.4 μm) and thin (70 nm) sections were stained with methylene blue and lead citrate respectively, then examined and photographed using a Zeiss Labrolux 12 light microscope and Zeiss EM109 TEM.

Fluorescence in situ *hybridization microscopy*

Leeches, initially preserved in cold paraformaldehyde for 24 h, were rinsed twice with 1× phosphate-buffered saline, transferred to 70% ethanol and stored at −20°C. Samples were embedded in Steedman's wax (one part cetyl alcohol was added to nine parts polyethylene glycol (400) distearate, mixed at 60°C; Steedman, 1957) and added to the sample in an ethanol : resin gradient of 3:1, 2:1 and 1:1, according to Pernthaler and Pernthaler (2005). Samples eventually embedded in full-strength wax were allowed to solidify and sectioned (5–10 μm-thick) using a Leica RM2125 manual microtome and placed onto Superfrost Plus slides (Fisher Scientific). Samples were de-waxed by three rinses in 100% ethanol (10 min each), followed by rehydration in 70% ethanol (10 min). Hybridization and wash buffers were made as described previously (Pernthaler and Pernthaler, 2005), using 35% formamide in the hybridization buffer and 450 mM NaCl in the wash solution. A universal bacterial probe set (EUB338I-III; Amann *et al*., 1990; Daims *et al*., 1999), along with a genus-specific *Psychromonas* probe (NOR2-1453, 5′-GGTCATCGCCATCCCC-3′; Eilers *et al*., 2000), and an archaea-specific probe (Ar915, 5′-GTGCTCCCCCGCCAATTCCT-3′; Stahl and Amann, 1991), used as a negative control, were labelled with Cy3. Hybridizations were typically 3–8 h, followed by washes of 15 min. Tissues were counter-stained with a dilute 4′6′-diamidino-2-phenylindole (DAPI) solution (5 mg ml^{-1}) for 1 min and examined under epifluorescence microscopy using a Nikon E80i epifluorescence research microscope with a Nikon DS-Qi1Mc high-sensitivity monochrome digital camera.

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