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Phylogenetic Diversity and Cosymbiosis in the Bioluminescent Symbioses of "*Photobacterium mandapamensis*"▽†

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"*Photobacterium mandapamensis*" (proposed name) and *Photobacterium leiognathi* are closely related, phenotypically similar marine bacteria that form bioluminescent symbioses with marine animals. Despite their similarity, however, these bacteria can be distinguished phylogenetically by sequence divergence of their luminescence genes, *luxCDAB*(*F*)*E*, by the presence (*P. mandapamensis*) or the absence (*P. leiognathi*) of *luxF* and, as shown here, by the sequence divergence of genes involved in the synthesis of riboflavin, *ribBHA*. To gain insight into the possibility that *P. mandapamensis* and *P. leiognathi* are ecologically distinct, we used these phylogenetic criteria to determine the incidence of *P. mandapamensis* as a bioluminescent symbiont of marine animals. Five fish species, *Acropoma japonicum* (Perciformes, Acropomatidae), *Photopectoralis panayensis* and *Photopectoralis bindus* (Perciformes, Leiognathidae), *Siphamia versicolor* (Perciformes, Apogonidae), and *Gadella jordani* (Gadiformes, Moridae), were found to harbor *P. mandapamensis* in their light organs. Specimens of *A. japonicus*, *P. panayensis*, and *P. bindus* harbored *P. mandapamensis* and *P. leiognathi* together as cosymbionts of the same light organ. Regardless of cosymbiosis, *P. mandapamensis* was the predominant symbiont of *A. japonicum*, and it was the apparently exclusive symbiont of *S. versicolor* and *G. jordani*. In contrast, *P. leiognathi* was found to be the predominant symbiont of *P. panayensis* and *P. bindus*, and it appears to be the exclusive symbiont of other leiognathid fishes and a loliginid squid. A phylogenetic test for cospeciation revealed no evidence of codivergence between *P. mandapamensis* and its host fishes, indicating that coevolution apparently is not the basis for this bacterium's host preferences. These results, which are the first report of bacterial cosymbiosis in fish light organs and the first demonstration that *P. leiognathi* is not the exclusive light organ symbiont of leiognathid fishes, demonstrate that the host species ranges of *P. mandapamensis* and *P. leiognathi* are substantially distinct. The host range difference underscores possible differences in the environmental distributions and physiologies of these two bacterial species.

"*Photobacterium mandapamensis*" (proposed name) is a widely distributed marine luminous bacterium found in coastal seawater (1, 18). Originally described as a separate species, *P. mandapamensis* overlaps in various taxonomic traits with *Photobacterium leiognathi*, a luminous bacterial symbiont of leiognathid fishes. This similarity led to the synonymization of these two species as *P. leiognathi* (3, 18, 34). Phylogenetic criteria, however, based on the luminescence genes *luxAB*(*F*)*E* separates these bacteria into two evolutionarily distinct lineages that match up with the original species descriptions (1). These contrasting views suggest that *P. mandapamensis* may be either a subspecies of *P. leiognathi* (1) or a separate species. Ecological and further phylogenetic evidence distinguishing these bacteria is presented here, and we therefore refer to them using the original species names, *P. mandapamensis* (18) and *P. leiognathi* (3).

The ability to distinguish *P. mandapamensis* from *P. leiognathi* on the basis of molecular phylogenetic criteria provides the means to determine whether these closely related, phenotypically similar bacteria are ecologically distinct. With respect to bioluminescent symbioses, we hypothesized that *P. mandapamensis* might associate with species of host animals different from those colonized by *P. leiognathi* (1). A difference in host species ranges might reflect other ecological or physiological differences between these bacteria. *Photobacterium leiognathi* has repeatedly been identified as the specific, exclusive light organ symbiont of leiognathid fishes (Perciformes, Leiognathidae), a well-studied group of marine bioluminescent fishes (e.g., see references 1, 3, 6, 9, 36, and 38), and *P. mandapamensis* has not previously been reported to be isolated from leiognathid light organs. Consistent with our hypothesis, a recent report indicates that bacteria provisionally identified as *P. mandapamensis* are light organ symbionts of two other fishes, *Acropoma japonicum* (Perciformes, Acropomatidae) and *Siphamia versicolor* (Perciformes, Apogonidae) (41).

The criteria used for previous identifications of these bacteria, however, i.e., comparisons of phenotypic traits and limited sequence analyses (e.g., see references 6, 9, 36, and 41), lack phylogenetic resolution and therefore leave the actual identities of these bacteria substantially in doubt. Specifically, the light organ bacteria from *A. japonicum*, *S. versicolor*, and cer-

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TABLE 1. Bacterially bioluminescent fishes harboring *P. mandapamensis* as a light organ symbiont

Host family and species	Depth; habitat[a]	Collection location; source	Specimen	Cosymbiont
Moridae				
Gadella jordani	400–760 m; bathydemersal	Tungkang, Taiwan; Philippine Sea	*Gjord*.1	
Acropomatidae				
Acropoma japonicum	100–500 m; bathydemersal	Dahsi, Taiwan; Philippine Sea	*Ajapo*.2	*P. leiognathi*
		Saga, Shikoku, Japan; Tosa Bay	*Ajapo*.3	
			Ajapo.4	*P. leiognathi*
			Ajapo.5	*P. leiognathi*
		Yui, Honshu, Japan; Suruga Bay	*Ajapo*.6	
			Ajapo.7	
			Ajapo.8	
Apogonidae				
Siphamia versicolor	0–68 m; tropical reef	Sesoko Island, Okinawa, Japan	*Svers*.1	
			Svers.3	
			Svers.4	
			Svers.9	
Leiognathidae				
Photopectoralis bindus	10–110 m; coastal demersal	Hama, Okinawa, Japan; Nakagusuku Bay	*Pbind*.5	*P. leiognathi*
Photopectoralis panayensis	10–110 m; coastal demersal	Tigbauan, Panay, The Philippines; Visayan Sea	*Ppana*.1	
			Ppana.2	*P. leiognathi*
			Ppana.3	*P. leiognathi*

[a] Reported depth and habitat data are from R. Froese and D. Pauly (FishBase [www.fishbase.org, version 03/2006]), interspersed with data for *P. panayensis* and *P. bindus* for Leiognathidae in general. Various leiognathid species, e.g., *Leiognathus fasciatus*, *Leiognathus nuchalis*, and *Leiognathus stercorarius*, however, can be caught as adult specimens at 0- to 1-m depths in coastal areas (P. V. Dunlap, personal observation), whereas *P. panayensis* is thought to be an atypically deep-dwelling species (J. Ledesma, personal communication).

tain other *Siphamia* species have been identified variously in the past either as *P. leiognathi* or as *P. mandapamensis* (12, 14, 16, 19, 22, 33, 41, 44). Furthermore, except for leiognathid fishes, relatively few species of host animals have been sampled for luminous bacteria. As a consequence, the host range of *P. mandapamensis* and the extent to which its host preferences and other aspects of its ecology may differ from those of *P. leiognathi* are indefinitely and incompletely known.

To begin investigating these issues, we used a multigene phylogenetic approach and examined a wide diversity of deep-water- and shallow-water-dwelling bioluminescent animals for the presence of *P. mandapamensis* as a light organ symbiont. Multiple bacterial strains were isolated from the light organs of each host animal to obtain a sampling of the bacterial diversity present. The strains were then screened using repetitive extragenic palindromic PCR (rep-PCR) genome profiling and sequence-based methods to identify genetically distinct strain types. Representatives of the different types from each light organ were identified phylogenetically based on sequences of the *gyrB*, *luxAB*(*F*)*E*, and *ribBHA* genes, the analysis of which provides unambiguous resolution between *P. mandapamensis* and *P. leiognathi*. We also reconstructed a phylogeny of the host animals found to harbor *P. mandapamensis* to test for cospeciation between the fish and bacterial strains, a possible indication of host-symbiont coevolution. The results reveal a host range for *P. mandapamensis* that is both unexpectedly wide and substantially distinct from that of *P. leiognathi*. They also demonstrate the effectiveness of molecular phylogenetic criteria for examining the ecological specificity of closely related, phenotypically similar bacteria.

MATERIALS AND METHODS

Collection of host specimens. Fishes and squids bearing bacterial light organs were obtained from catches from various shallow coastal and deeper continental shelf locations in the Pacific, Atlantic, and Indian oceans. Specimens were collected at regional wholesale markets on the morning of capture, directly from trawls or trap nets, with handheld lines, and by hand using scuba. When possible, multiple specimens of each species were collected and examined. Results for host species not reported here are described by Dunlap et al. (10). In the present study, specimens of *Acropoma japonicum* and *Gadella jordani* were collected at Dahsi Fish Market on the east coast of Taiwan and at Tungkang Fish Market on the southern coast of Taiwan, respectively, from commercial catches of deepwater benthic trawls at an ~200- to 600-m depth in the northwest portion of the Philippine Sea, east of Taiwan (Table 1). Additional specimens of *A. japonicum* were collected at Saga Market, Shikoku, Japan, from a commercial benthic trawl at a 100- to 300-m depth in Tosa Bay and from a commercial surface set net in Suruga Bay, Honshu, Japan, approximately 1 km from Yumachi. Specimens of *Photopectoralis panayensis* were taken by surface drop net 1 to 2 km from shore in the Panay Gulf (Visayan Sea), the Philippines, in the vicinity of Tigbauan, Panay (Table 1), and specimens of *Photopectoralis bindus* were taken by trap net from Nakagusuku Bay, Okinawa, Japan, landed at Hama Fish Market. Specimens of *Siphamia versicolor* were collected from their protective association with the longspine urchin *Diadema setosum* at a 3- to 5-m depth along coral reefs at Sesoko Island, Motobu, Okinawa, Japan (Table 1). Fishes were identified to the species level by reference to Nakabo (24), Kimura et al. (21), and Sparks et al. (38). Ichthyological nomenclature follows the work of Nelson (27) and Sparks et al. (38). Fish specimen designations follow the work of Dunlap et al. (9). Samples for fish mitochondrial DNA extraction were flank muscle tissue, which was excised free of skin and stored in 90% ethanol at −20°C. DNA was extracted from small chunks of the tissue using the QIAGEN QIAquick tissue extraction kit, according to the manufacturer's protocol. After fish specimens were sampled for muscle tissue and their light organs dissected (see below), they were tagged individually, preserved, and later deposited into the laboratory's permanent specimen collection.

Isolation of bacterial strains. The bacterial strains used in this study are listed in Table 2. Bacteria newly reported here were isolated from the light organs of fishes essentially as previously described (2, 5, 7, 10). Specifically, fish were kept chilled on ice until dissection of the light organ, usually within an hour or two of

TABLE 2. Bacterial species and strains used in this study

Species	Strain	Ecological source[a]	Reference(s)
Photobacterium angustum	ATCC 25915^T	SW	35
Photobacterium damselae subsp. *damselae*	ATCC 33539^T	Skin ulcer, *Chromis punctipinnus*	15
Photobacterium iliopiscarium	ATCC 51760^T	Pyloric cecum, *Clupea harengus*	2, 31
Photobacterium kishitanii	*ahane*.1.1	LO, *Acropoma hanedai*, *Ahane*.1	10
	pjapo.1.1^T	LO, *Physiculus japonicus*, *Pjapo*.1	2, 7
Photobacterium leiognathi	ATCC 25521^T	LO, *Leiognathus splendens*	3
	ATCC 25587	LO, *L. splendens*	3
	lequu.1.1	LO, *Leiognathus equulus*, *Lequu*.1	9
	lleuc.1.1	LO, *Leiognathus leuciscus*, *Lleuc*.1	9
	ajapo.2.16	LO, *Acropoma japonicum*, *Ajapo*.2	This study
	ajapo.4.22	LO, *A. japonicum, Ajapo*.4	This study
	ajapo.5.37	LO, *A. japonicum*, *Ajapo*.5	This study
	lbind.5.1, *lbind*.5.3	LO, *Photopectoralis bindus*, *Pbind*.5	This study
	ppana.1.1–*ppana*.1.20	LO, *Photopectoralis panayensis*, *Ppana*.1	This study
	ppana.2.2, *ppana*.2.3, *ppana*.2.5–*ppana*.2.12	LO, *P. panayensis*, *Ppana*.2	This study
	ppana.3.2, *ppana*.3.6, *ppana*.3.9	LO, *P. panayensis*, *Ppana*.3	This study
Photobacterium mandapamensis	ATCC 27561^T	SW	1, 18
	ATCC 33981	SW	1, 18
	PL-721	Skin, *Coccorella* sp.	1, 26
	AJ-1a	LO, *A. japonicum*	14
	ajapo.2.1–*ajapo*.2.15, *ajapo*.2.17–*ajapo*.2.20	LO, *A. japonicum*, *Ajapo*.2	This study
	ajapo.3.1, *ajapo*.3.7	LO, *A. japonicum*, *Ajapo*.3	This study
	ajapo.4.1, *ajapo*.4.5, *ajapo*.4.10, *ajapo*.4.11, *ajapo*.4.31, *ajapo*.4.40	LO, *A. japonicum*, *Ajapo*.4	This study
	ajapo.5.1, *ajapo*.5.7, *ajapo*.5.21	LO, *A. japonicum*, *Ajapo*.5	This study
	ajapo.6.1–*ajapo*.6.20	LO, *A. japonicum*, *Ajapo*.6	This study
	ajapo.7.1–*ajapo*.7.23	LO, *A. japonicum*, *Ajapo*.7	This study
	ajapo.8.1–*ajapo*.8.24	LO, *A. japonicum*, *Ajapo*.8	This study
	gjord.1.1, *gjord*.1.3, *gjord*.1.5	LO, *Gadella jordani*, *Gjord*.1	This study
	lbind.5.10	LO, *P. bindus*, *Pbind*.5	This study
	ppana.2.1	LO, *P. panayensis*, *Ppana*.2	This study
	ppana.3.1, *ppana*.3.3–*ppana*.3.5, *ppana*.3.7, *ppana*.3.10, *ppana*.3.14	LO, *P. panayensis*, *Ppana*.3	This study
	seafl.1.1, *seafl*.1.3, *seafl*.1.4	SW	1
	svers.1.1, *svers*.1.2, *svers*.1.11	LO, *Siphamia versicolor*, *Svers*.1	This study
	svers.3.2, *svers*.3.7	LO, *S. versicolor*, *Svers*.3	This study
	svers.9.9	LO, *S. versicolor*, *Svers*.9	This study
Photobacterium phosphoreum	ATCC 11040^T	Skin, marine fish	2, 18
Photobacterium profundum	JCM 10084^T	SW	29
Vibrio fischeri	ATCC 7744^T	SW	18

[a] Abbreviations: SW, seawater; LO, light organ.

collection. The ventral light organ (*A. japonicum*, *S. versicolor*, *G. jordani*) or circumesophageal light organ (*P. panayensis*, *P. bindus*) was aseptically dissected from the fish and homogenized in 0.5 ml or 1.0 ml of sterile artificial 70% seawater containing 25 mM HEPES buffer (pH 7.25) (BSW-70) in a sterile handheld Tenbroeck glass tissue grinder. The light organ homogenates were then serially diluted in BSW-70, and portions of one or more of the end dilutions were spread on plates of LSW-70 agar, which contained, per liter, 10 g tryptone, 5 g yeast extract, 350 ml double-strength artificial seawater (25), 650 ml deionized water, and 15 g agar. Dilutions of light organ homogenates, generally to 10^{-5}, and plating volumes, generally 25 to 100 μl, were based on a typical light organ population size of approximately 10^8 cells (5). Plates were incubated for 18 to 24 h at room temperature (typically 18 to 27°C, depending on the season, location, and availability of air conditioning). This plating procedure typically gave rise to approximately 50 to 500 well-isolated luminous colonies per plate. Nonluminous colonies were not observed. Previous comparisons of counts of viable cells and direct counts of bacteria from light organs handled in this way indicated a 100% plating efficiency (5). Ten to 40 colonies (i.e., individual strains) were then picked at random from the plates for each fish specimen, purified on LSW-70 agar plates, and stored as viable cultures at −75°C in cryoprotective medium (8) in the laboratory's permanent strain collection. Bacterial strain designations report the host fish species and specimen number of this laboratory (9); e.g., *ajapo*.2.16 indicates strain number 16 from specimen number 2 of *A. japonicum*. Strain AJ-1a, isolated from the light organ of a specimen of *A. japonicum* collected at Kochi, Shikoku, Japan (14), was a gift of S. Fukasawa. Genomic DNA was purified from 1-ml cultures of strains grown overnight in LSW-70 broth by using the QIAGEN DNeasy tissue extraction kit and by following the manufacturer's protocol for gram-negative bacteria.

Amplification of the *gyrB*, *luxAB*(*F*)*E*, and *ribBHA* genes. The *gyrB*, *luxAB*(*F*)*E*, and *ribBHA* genes code for DNA gyrase subunit B, the α and β subunits of luciferase, a nonfluorescent flavoprotein, a protein involved in the synthesis of aldehyde (a luciferase substrate), and three proteins involved in riboflavin synthesis, respectively. These genes were amplified by PCR, using *Taq* polymerase

along with reagents from either the Eppendorf (Hamburg, Germany) MasterTaq kit or the Promega (Madison, WI) *Taq* DNA polymerase kit, essentially as described previously (1). For details of the amplification protocols and specific primers, see the supplemental material.

Strain typing and phylogenetic screening. DNA genomic profile analysis was carried out with rep-PCR on purified genomic DNA of individual strains using primers REP1R-I (5′-IIIICGICGICATCIGGC-3′) and REP2-I (5′-ICGICTTA TCIGGCCTAC-3′) (40) according to the methods of Di Meo et al. (4) and essentially as described previously (7). For sequence-based screening, PCR products generated with the *luxA* primers CWLAf and CWLAr (43) or CWLAforPl and CWLArevPl (see the supplemental material) or the *luxA-luxB* primers luxAforPlPm (5′-TACAATGARRTTGCRGCWGARCATGG-3′) and luxBrevPhoto (5′-TCRTARCANGCTTCRAATTGYSGYTGYTG-3′) were sequenced. The template for the reactions was prepared directly from cell pellets resuspended in 50 μl of sterile deionized water and repelleted to remove the supernatant. The amplification protocol was as described for the amplification of *gyrB*, with primer annealing at 50°C and a 1-min extension time. Provisional species identifications were based on 90% or greater identity to sequences of the *luxA* amplicon (approximately 650 nucleotides) or the *luxA-luxB* amplicon (approximately 800 nucleotides) of *P. mandapamensis* or *P. leiognathi* from GenBank.

Sequencing and phylogenetic analyses. PCR products were sequenced by staff of the University of Michigan Sequencing Core using the respective amplification primers and dye terminator cycle sequencing on a Perkin-Elmer (Wellesley, MA) ABI 3730 DNA analyzer. The *gyrB*, *luxAB*(*F*)*E*, and *ribBHA* sequences were aligned manually by inferred amino acid sequence. Bacterial phylogeny was reconstructed by parsimony analysis performed with PAUP* (39), using 1,000 heuristic search replicates with tree bisection reconnection branch swapping. Jackknife support was calculated with PAUP* using 1,000 replicates (with 10 heuristic searches per replicate) and 34% deletion, emulating Jac resampling. For strains bearing an insertion in *luxF* (*ajapo*.3.1, *ajapo*.3.7, *ppana*.3.1, and *ppana*.3.14), the portion of *luxF* downstream of the insertion was excluded from the analysis. For nonluminous taxa, *luxABFE* sequences were treated as missing data.

To construct the fish phylogeny, the mitochondrial genes encoding 16S rRNA and cytochrome oxidase subunit I (COI) were amplified and sequenced, using the primer sequences and PCR protocols described by Sparks et al. (38). Sequence data were analyzed by direct optimization as implemented in OY (POY without parallel options) (42). The 100 OY replicates included randomizing outgroup and taxon order input (-replicates 100 -randomizeoutgroup -noonea-sis), with retention of a maximum of two trees per initial build (-buildmaxtrees 2), all changes set to a cost of 1 (-change 1 -gap 1 -extensiongap 1), two iterations of ratcheting with 30% data perturbation saving one tree per iteration (-ratchettbr 2 -rachettrees 1 -ratchetpercent 30), and precise calculations on the optimization down-pass (-exact). Suboptimal trees were evaluated during each iteration using slop commands (-slop 5 -checkslop 10). Jackknife percentages were calculated in PAUP* using 1,000 branch and bound replicates.

Nucleotide sequence accession numbers. Accession numbers for the *gyrB* sequences of newly sequenced *P. leiognathi* and *P. mandapamensis* strains are DQ371341 through DQ371367 and DQ790866 through DQ790884, respectively, and that for *pbind*.5.1 is EF372600. The *luxAB*(*F*)*E* sequences of newly sequenced *P. leiognathi* and *P. mandapamensis* strains have the numbers DQ371368 through DQ371394 and DQ790849 through DQ790865, respectively; EF372601 is the number for the *luxABE* sequence of *pbind*.5.1, and EF372602 is the number for *luxAB* of *pbind*.5.10. The *gyrB* and *luxABFE* sequences of "*Photobacterium kishitanii*" (proposed name) strain *ahane*.1.1 are DQ648287 and DQ648331. Sequences for the *rib* genes (*ribB*, *ribH*, and *ribA*) of *Photobacterium* strains are DQ988873 through DQ988881. For fishes, the accession numbers for the mitochondrial 16S gene and COI, respectively, are as follows: for *Acropoma hanedai*, DQ648414 and DQ648436; for *Acropoma japonicum*, DQ790843 and DQ790845; for *Gadella jordani*, DQ648427 and DQ648449; for *Physiculus japonicus*, DQ648431 and DQ648453, and for *Siphamia versicolor*, DQ790844 and DQ790846. Sequences for *Photopectoralis panayensis* (38) were downloaded from GenBank.

For the previously reported *lux* sequences in the following strains used in this study, the GenBank accession numbers are as indicated in parentheses: *Vibrio fischeri* ATCC 7744^T (AY341062); *Photobacterium phosphoreum* ATCC 11040^T (AY341063); *P. kishitanii pjapo*.1.1^T (AY341065); *P. leiognathi* ATCC 25521^T (M63594); *P. leiognathi* ATCC 25587 (AY456750); *P. mandapamensis* ATCC 27561^T (AY341067); *P. mandapamensis* ATCC 33981 (AY341068), *lequu*.1.1 (AY341069), and *lleuc*.1.1 (AY341070); and *P. mandapamensis* PL-721 (AY341066), *seafl*.1.3 (AY456752), *seafl*.1.1 (AY456751), and *seafl*.1.4 (AY456753). For the previously reported *gyrB* sequences in the following strains used in this study, the GenBank accession numbers are as indicated in parentheses: *V. fischeri* ATCC 7744^T (AY455874); *Photobacterium damselae* subsp. *damselae* ATCC 33539^T (AY455889); *Photobacterium angustum* ATCC 25915^T (AY455890); *Photobacterium profundum* JCM 10084^T (AY455892); *Photobacterium iliopiscarium* ATCC 51760^T (AY455878); *P. phosphoreum* ATCC 11040^T (AY455875); *P. kishitanii pjapo*.1.1^T (AY455877); *P. leiognathi* ATCC 25521^T (AY455879); *P. leiognathi* ATCC 25587 (AY455880); *P. mandapamensis* ATCC 27561^T (AY455883); *P. mandapamensis* ATCC 33981 (AY455884), *lequu*.1.1 (AY455881), *lleuc*.1.1 (AY455882), *seafl*.1.1 (AY455886), *seafl*.1.3 (AY455887), and *seafl*.1.4 (AY455888); and *P. mandapamensis* PL-721 (AY455885).

RESULTS

Symbiotic host range of *P. mandapamensis*. Light organs of a diversity of deep-water- and shallow-water-dwelling bioluminescent animals were examined for the presence of *P. mandapamensis* and other luminous bacteria. Sequence-based screening and preliminary phylogenetic analyses (see Materials and Methods) presumptively identified *P. mandapamensis* as a light organ symbiont of five species of marine fishes, *Acropoma japonicum*, *Photopectoralis panayensis*, *Photopectoralis bindus*, *Siphamia versicolor*, and *Gadella jordani* (Tables 1 and 2). These fishes, which represent four families in two teleost orders, occur at various depths in a variety of different marine habitats (Table 1). Other animals examined were found to harbor *P. kishitanii*, *P. leiognathi*, or *V. fischeri* (10).

Diversity and cosymbiosis of the light organ symbionts of *Acropoma japonicum*. To conclusively identify the bacterial symbionts of *A. japonicum*, we first examined a representative strain, AJ-1a, from an earlier study in which the bacteria were identified phenotypically as *P. leiognathi* (14). Analysis of the *gyrB* and *luxAB*(*F*)*E* genes identified AJ-1a as a strain of *P. mandapamensis* (Fig. 1). The presence of *luxF*, which discriminates *P. mandapamensis* from *P. leiognathi* (1), in the *lux* operon of AJ-1a confirmed this finding. These results correct an earlier misidentification of the bacteria that are symbiotic with *A. japonicum* (14).

To determine whether other specimens of this fish also harbor *P. mandapamensis* as a symbiont, we isolated bacteria from the light organs of several recently collected specimens of *A. japonicum* (Table 1). The bacteria were screened by rep-PCR genomic profiling or by analysis of *luxA* or *luxA-luxB* sequences to identify genetically distinct types from each light organ, representatives of which were then examined phylogenetically (see Materials and Methods). Substantial bacterial diversity was present in light organs of specimens of *A. japonicum*. For example, at least three genetically distinct strain types, distinguished by rep-PCR profiling, were present among the light organ bacteria of specimen *Ajapo*.2, collected in Taiwan (Fig. 2). The *gyrB* and *luxAB*(*F*)*E* genes of strains representative of each strain type (*ajapo*.2.1, *ajapo*.2.6, *ajapo*.2.16, and *ajapo*.2.19) were sequenced. Strains *ajapo*.2.1, *ajapo*.2.6, and *ajapo*.2.19 were identified as *P. mandapamensis*. These strains formed a clade within *P. mandapamensis*, designated clade II, that is phylogenetically distinct from clade I, formed by AJ-1a and previously identified strains of this species, e.g., ATCC 27561^T, ATCC 33981, and PL-721 (Fig. 1). In contrast, *ajapo*.2.16 was identified as *P. leiognathi* (Fig. 1). Analysis of the *luxF* region confirmed these results; the *lux* operons of *ajapo*.2.1, *ajapo*.2.6, and *ajapo*.2.19 contained *luxF*, whereas *luxF* was absent from the *lux* operon of *ajapo*.2.16. Sequence analysis of the *ribBHA* genes of *ajapo*.2.16 further confirmed

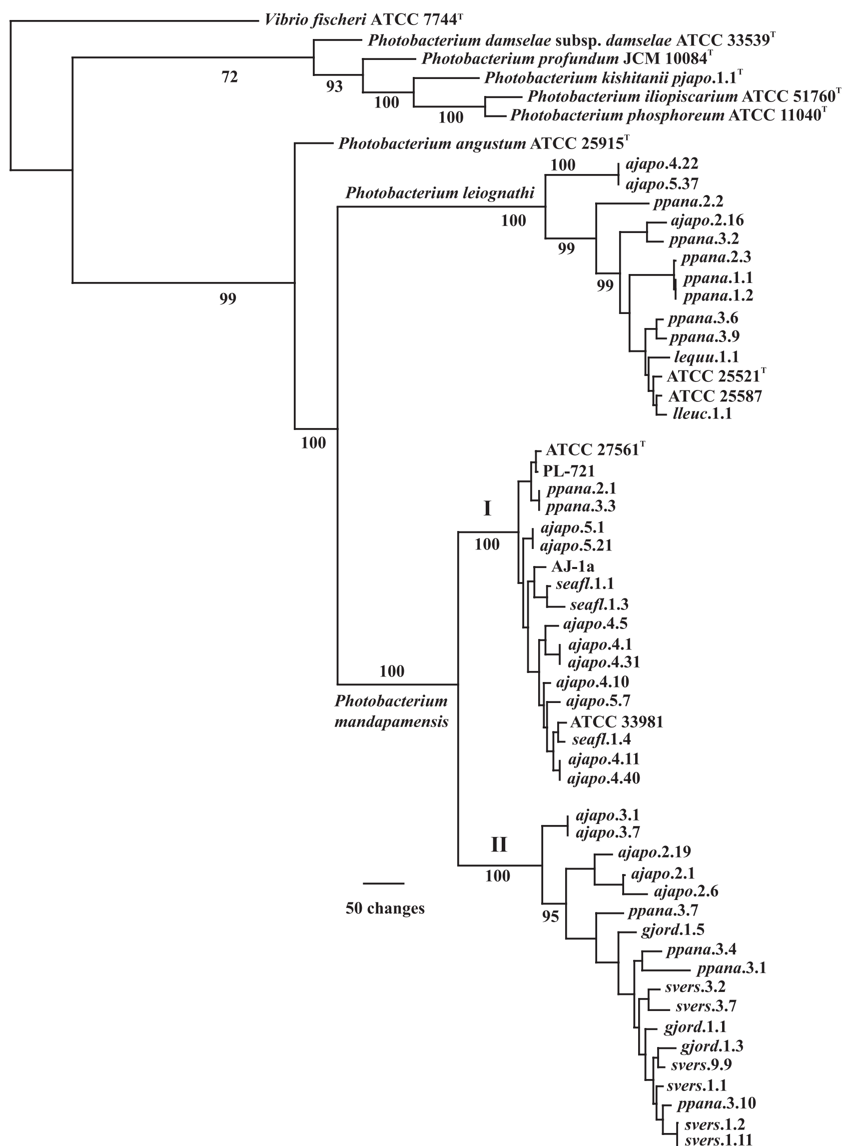

FIG. 1. Phylogram representing 1 of 56 equally most parsimonious hypotheses resulting from combined multilocus analysis of *gyrB* and *luxAB*(*F*)*E* gene sequences of *Photobacterium* species (1,393 informative characters; length, 3,491; consistency index [CI], 0.578; retention index [RI], 0.879). A strict consensus of the 56 trees reduces resolution only among smaller clades near the tips of the tree. The separation of *P. leiognathi* and *P. mandapamensis* results primarily from differences in *lux* operon sequences, as described previously (1), and was confirmed here through analysis of *ribBHA* sequences (Fig. 3). Numbers at the nodes indicate Jackknife resampling values; some values were omitted for clarity. Representative strains from different specimens of *S. versicolor* were used here. Strains whose *gyrB* and *luxAB*(*F*)*E* sequences were identical and that were therefore excluded from this analysis are *gjord*.1.4, *gjord*.1.7, and *gjord*.1.9 (identical to *gjord*.1.1); *gjord*.1.8 (identical to *gjord*.1.3); *gjord*.1.10 (identical to *gjord*.1.5); and *ppana*.3.14 (identical to *ppana*.3.1 [see the text]). Strains with identical rep-PCR profiles and therefore not sequenced or, if sequenced, not used in this analysis due to sequence identity are *gjord*.1.2 (identical to *gjord*.1.1), *gjord*.1.6 (identical to *gjord*.1.4), *ppana*.1.3 through *ppana*.1.20 (identical to *ppana*.1.1 and *ppana*.1.2), *ppana*.2.5 through *ppana*.2.12 (identical to *ppana*.2.3), and *ppana*.3.5 (identical to *ppana*.3.3). For strains from specimen *Ajapo*.2, representatives of each strain type, *ajapo*.2.1, *ajapo*.2.6, *ajapo*.2.16, and *ajapo*.2.19, were sequenced and used in this analysis. Roman numerals I and II refer to *P. mandapamensis* clade I and clade II, respectively (see the text).

this identification (Fig. 3). These results suggest that *P. mandapamensis* is a common light organ symbiont of *A. japonicum*. They also demonstrate that *P. leiognathi* can cooccur with *P. mandapamensis* in light organs of *A. japonicum*. This is the first example of two bacterial species cooccurring in the light organ of a fish, a situation we term cosymbiosis.

To investigate how commonly *P. mandapamensis* and *P. leiognathi* occur as cosymbionts of *A. japonicum* and to further assess the extent of phylogenetic diversity within *P. mandapamensis*, we examined the light organ bacteria of several additional specimens of this fish species (Table 1). The sequence of *luxA* or *luxA-luxB* was used to provisionally identify strains from each fish (see Materials and Methods). Of the 120 strains examined from three specimens of *A. japonicum* from Tosa Bay, Shikoku, Japan, *Ajapo*.3, *Ajapo*.4, and *Ajapo*.5, two strains, *ajapo*.4.22 and *ajapo*.5.37, were provisionally identified as *P. leiognathi*. The remaining 118 strains were identified as *P. mandapamensis*. All 40 strains from specimen *Ajapo*.3 were

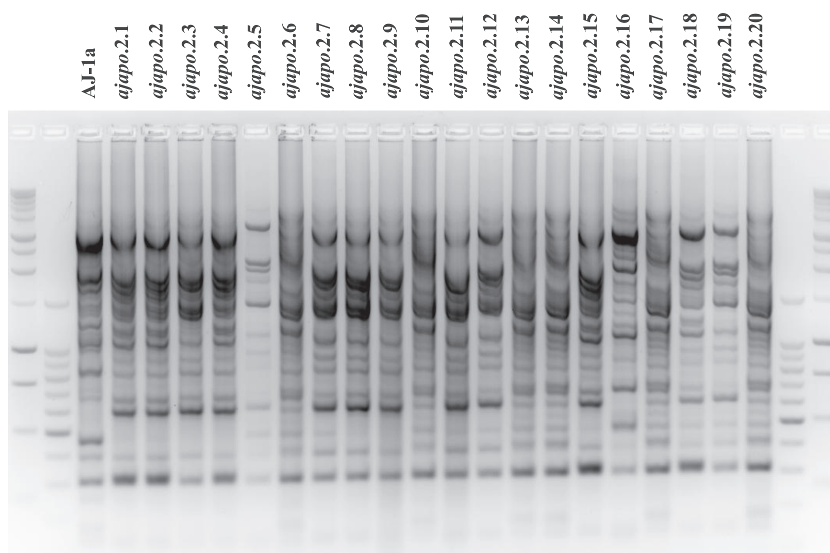

FIG. 2. rep-PCR genomic profiling of strains from *A. japonicum* specimen *Ajapo*.2. Three distinct strain types were identified: (i) strains *ajapo*.2.1 to *ajapo*.2.5, *ajapo*.2.7 to *ajapo*.2.9, *ajapo*.2.11, *ajapo*.2.12, *ajapo*.2.15, *ajapo*.2.18, and *ajapo*.2.19 (strains *ajapo*.2.1 and *ajapo*.2.19 [identified as *P. mandapamensis*] represent the minor genetic variation among these 13 strains); (ii) *ajapo*.2.6, *ajapo*.2.10, *ajapo*.2.13, *ajapo*.2.14, *ajapo*.2.17, *ajapo*.2.20 (with identical rep-PCR profiles, represented by *ajapo*.2.6 [*P. mandapamensis*]); and (iii) *ajapo*.2.16 (*P. leiognathi*). Included for comparison is strain AJ-1a from the study of Fukasawa et al. (14), identified here as *P. mandapamensis*. Flanking unlabeled lanes are 1-kb and 100-bp DNA size standard ladders.

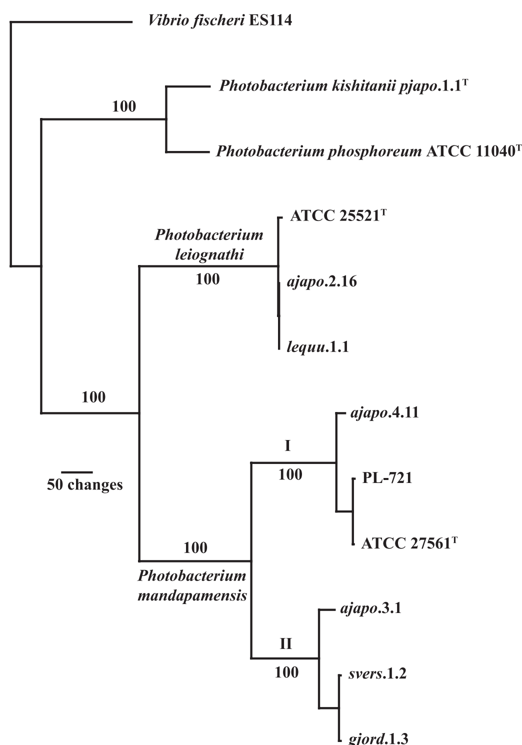

FIG. 3. Phylogram of the single most parsimonious tree resulting from analysis of the genes *ribB*, *ribH*, and *ribA* (868 informative characters; length, 1,603; CI, 0.812; RI, 0.861). Sequences were aligned by inferred amino acid sequences and analyzed using the branch-and-bound algorithm as implemented in PAUP* (39). Jackknife resampling values appear at the nodes and were calculated using 1,000 branch-and-bound replicates with 34% deletion, emulating Jac resampling. Roman numerals I and II refer to *P. mandapamensis* clade I and clade II, respectively. Compare the figure with Fig. 1.

members of *P. mandapamensis* clade II, whereas all 78 *P. mandapamensis* strains from specimens *Ajapo*.4 and *Ajapo*.5 were members of clade I. Of the 67 strains examined from three specimens of *A. japonicum* from Suruga Bay, Honshu, Japan, *Ajapo*.6, *Ajapo*.7, and *Ajapo*.8, all were identified as *P. mandapamensis*. All 20 strains from *Ajapo*.6 were members of clade II, and all 23 strains from *Ajapo*.7 were members of clade I. Of the 24 strains from specimen *Ajapo*.8, 5 were members of clade I and 19 were members of clade II. The provisional identifications were confirmed by sequence analysis of the *gyrB* and *luxAB*(*F*)*E* genes for 13 representative strains from specimens *Ajapo*.3, *Ajapo*.4, and *Ajapo*.5 (Fig. 1). Furthermore, analysis of the *ribBHA* genes of *ajapo*.3.1 and *ajapo*.4.11 confirmed the placement of these strains in clade II and clade I, respectively, of *P. mandapamensis* (Fig. 3). We note parenthetically here that the *luxF* genes of *ajapo*.3.1 and *ajapo*.3.7 contained insertions that apparently inactivate this gene (see the supplemental material), the first demonstration of nonsense mutations in the *lux* genes of bacteria from nature.

These results reveal the presence of substantial phylogenetic diversity among the symbionts of *A. japonicum*, with strains representing three phylogenetically distinct clades, *P. mandapamensis* clade I and clade II and *P. leiognathi*. Furthermore, they demonstrate that cosymbiosis of *P. mandapamensis* and *P. leiognathi*, while not frequently observed, might not be rare.

Diversity and cosymbiosis of the light organ symbionts of *Photopectoralis panayensis* and *Photopectoralis bindus*. Cosymbiosis of *P. mandapamensis* and *P. leiognathi* was also found in the light organs of two leiognathid fishes, *P. panayensis* and *P. bindus*. rep-PCR analysis identified the presence of a single strain type among the 20 strains of specimen *Ppana*.1, three types among the 11 strains of *Ppana*.2, eight among the 10 strains of *Ppana*.3, and three among the 10 strains of *Pbind*.5

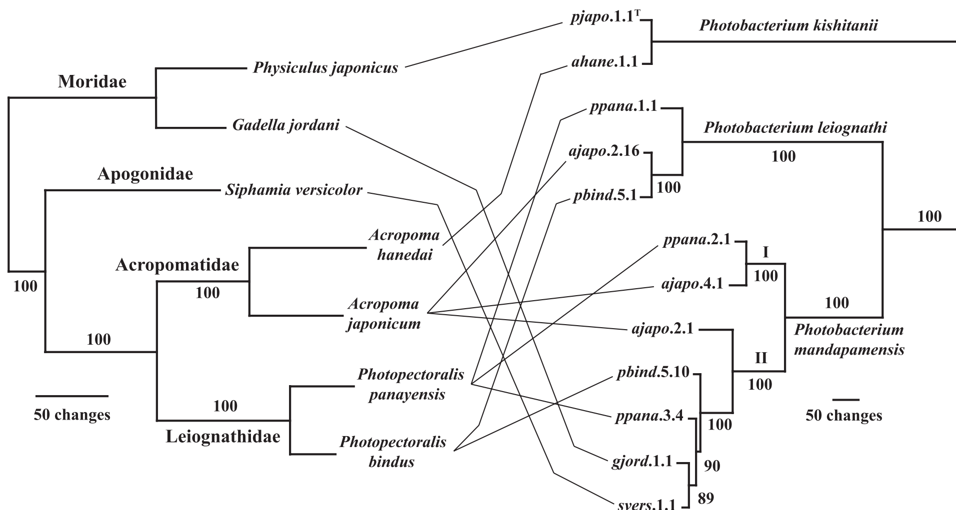

FIG. 4. Test of cospeciation between symbiotic luminous bacteria and their fish hosts. Fish specimens from seven species in four families from two teleost orders and bacterial strains representative of the symbionts of these fishes (Fig. 1) were tested for phylogenetic congruence. Genes analyzed were mitochondrial 16S rRNA and COI genes for the fish and *gyrB* and *luxAB*(*F*)*E* for the bacteria. The fish data were analyzed with OY (see Materials and Methods for settings), which resulted in a single most parsimonious hypothesis (330 informative characters; length, 829; CI, 0.813; RI, 0.678). The data for bacteria (1,407 informative characters) were analyzed by an exhaustive search in PAUP in which all possible tree topologies were evaluated; the single most parsimonious hypothesis (length, 2,001; CI, 0.870; RI, 0.911) is shown. Strains of *P. kishitanii* were isolated from the morid fish *Physiculus japonicus* (7) and the acropomatid fish *Acropoma hanedai* (10). Numbers at major nodes are jackknife support values (see Materials and Methods), and Roman numerals I and II refer to *P. mandapamensis* clades I and II, respectively.

(data not shown). The *gyrB* and *luxAB*(*F*)*E* genes of representatives of these strain types were sequenced for identification. Strains identified as *P. leiognathi* were *ppana*.1.1 and *ppana*.1.2; *ppana*.2.2 and *ppana*.2.3; *ppana*.3.2, *ppana*.3.6, and *ppana*.3.9; and, *pbind*.5.1 and *pbind*.5.3. Strains identified as *P. mandapamensis* were *ppana*.2.1 (clade I); *ppana*.3.1 (clade II), *ppana*.3.3 (clade I), *ppana*.3.4 (clade II), *ppana*.3.5 (clade I), *ppana*.3.7 (clade II), *ppana*.3.10 (clade II), and *ppana*.3.14 (clade II); and *pbind*.5.10 (clade II) (Fig. 1). We note parenthetically here a rare instance of different fish specimens harboring the same strain type (7, 9); strain *ppana*.2.3 was identical in rep-PCR profile and in *gyrB* and *luxABE* sequence to *ppana*.1.1 and other strains from specimen *Ppana*.1 (Fig. 1). Furthermore, strains *ppana*.3.1 and *ppana*.3.14 were found to bear a transposase-containing insertion in *luxF* that inactivates the gene (see the supplemental material).

The identification of *P. mandapamensis* as a light organ symbiont of *P. panayensis* and *P. bindus*, 9 of 43 strains examined at the sequence level, provides the first demonstration that *P. leiognathi* is not the exclusive bioluminescent symbiont of leiognathid fishes. Furthermore, these results confirm and extend to two additional fish species the finding that *P. mandapamensis* and *P. leiognathi* occur as cosymbionts.

Phylogenetic clustering of the light organ symbionts of *Siphamia versicolor*. In contrast to the diversity described above, a high degree of phylogenetic clustering was found for the light organ symbionts of *S. versicolor*. All of the 92 bacterial strains isolated from light organs of the four examined specimens of this fish were presumptively identified by *luxA* or *luxA-luxB* sequence screening as *P. mandapamensis*. Phylogenetic analysis based on sequences of the *gyrB* and *luxAB*(*F*)*E* genes of six representative strains unambiguously identified these strains as *P. mandapamensis*, and all were members of clade II (Fig. 1). The presence of *luxF* in the *lux* operons of these strains confirmed this identification, as did sequence analysis of the *ribBHA* genes of *svers*.1.2 (Fig. 3).

Identification of the light organ bacteria of *Gadella jordani*. Phylogenetic clustering was found also for the light organ bacteria of *G. jordani*. Screening by rep-PCR of 10 representatives of the light organ population of specimen *Gjord*.1, the single specimen obtained, revealed the presence of four different strain types (data not shown). Sequence analysis of *gyrB* and *luxAB*(*F*)*E* and the presence of *luxF* identified the eight examined strains as *P. mandapamensis*, and all were members of clade II (Fig. 1). Sequence analysis of the *ribBHA* genes of *gjord*.1.3 confirmed this identification (Fig. 3).

Test of symbiont-host cospeciation between *P. mandapamensis* and luminous fishes. The identification of five fish species that harbor *P. mandapamensis* as a light organ symbiont and the ability to discriminate phylogenetically between this bacterium and other luminous bacteria allowed us to test the hypothesis that *P. mandapamensis* and its hosts have cospeciated. Cospeciation, visualized as a pattern of matching host and symbiont phylogenies, can reflect host-symbiont coevolution (32). Therefore, to test for cospeciation and ask whether coevolution might explain the host preferences of *P. mandapamensis*, we used mitochondrial 16S rRNA and COI gene sequences to reconstruct a phylogeny of the fishes harboring *P. mandapamensis* and compared it with a phylogeny of representative bacterial strains from these fishes.

The relationships among the fishes were unambiguously resolved, and a robust clade structure was evident both for the fishes and for the bacteria (Fig. 4). However, the clade structure of the bacteria did not topologically match that of the fish. Specifically (i) the distantly related fishes *A. japonicum* (Perciformes: Acropomatidae) and *G. jordani* (Gadiformes: Mori-

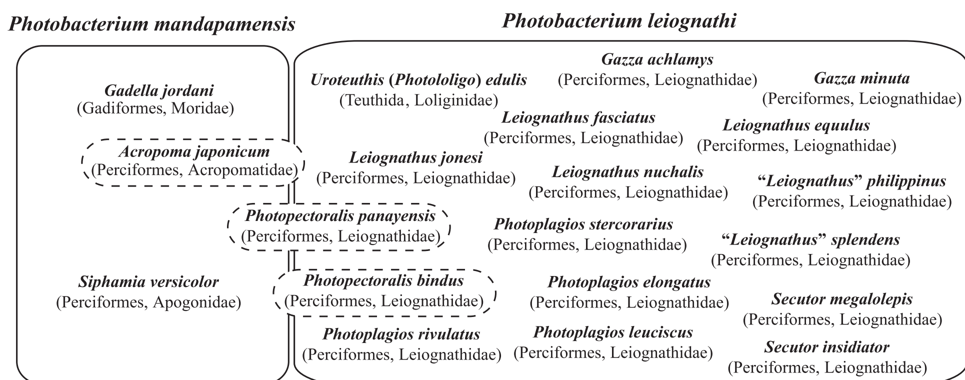

FIG. 5. Host species ranges of the light organ symbionts *P. mandapamensis* and *P. leiognathi*. Species of fish that harbor *P. mandapamensis* and species of fish and squid that harbor *P. leiognathi* are shown with host family and order in parentheses. Hosts reported are those for which detailed analysis of bacteria has been carried out using methods that discriminate between *P. mandapamensis* and *P. leiognathi*. Most leiognathids harbor apparently only *P. leiognathi*, whereas all examined specimens of *S. versicolor* and the one available specimen of *G. jordani* harbored only *P. mandapamensis*. In light organs of some fishes (*A. japonicum*, *P. panayensis*, and *P. bindus*), *P. mandapamensis* and *P. leiognathi* occur as cosymbionts. Nonetheless, the host ranges of these two bacterial species are substantially distinct.

dae) harbor the same species of bacteria as light organ symbionts; (ii) closely related fishes, i.e., in the same genus or family, namely, *A. japonicum* and *A. hanedai* and *G. jordani* and *P. japonicus*, harbor different bacterial species; and (iii) specimens of some fishes, *A. japonicum*, *P. panayensis*, and *P. bindus*, harbor members of two or three different bacterial clades. The absence of topological congruence between host and symbiont phylogenies refutes the hypothesis that *P. mandapamensis* and its host fishes have cospeciated. We conclude that factors other than symbiont-host coevolution account for the host preferences of *P. mandapamensis*.

DISCUSSION

Despite their phenotypic similarity and close evolutionary relationship, *P. mandapamensis* and *P. leiognathi* can be distinguished by molecular phylogenetic criteria. Using these criteria, we demonstrate here that their symbiotic host species ranges, a major aspect of the ecology of these bacteria, are substantially distinct (Fig. 5). To date, five fishes, *Acropoma japonicum* (Perciformes: Acropomatidae), *Photopectoralis panayensis* and *Photopectoralis bindus* (Perciformes: Leiognathidae), *Siphamia versicolor* (Perciformes: Apogonidae), and *Gadella jordani* (Gadiformes: Moridae) harbor *P. mandapamensis* as a light organ symbiont. In specimens of two of these fishes, *S. versicolor* and *G. jordani*, *P. mandapamensis* appears to be the sole bacterial species present, whereas in light organs of the other fishes, *P. mandapamensis* occurs together with *P. leiognathi* as a cosymbiont. Molecular phylogenetic analysis also revealed the presence of two distinct clades of *P. mandapamensis*, the possible significance of which for bioluminescent symbioses or other aspects of the ecology of this bacterium is not yet obvious. In contrast, *P. leiognathi* occurs as the apparently exclusive light organ symbiont of most leiognathid fishes and the loliginid squid *Uroteuthis* (*Photololigo*) *edulis* (Fig. 5) (13).

Bacterial symbionts of *G. jordani* and *P. panayensis* previously had not been examined, and for *P. bindus* only a single bacterial strain from the light organ of a single specimen had been identified (9). Furthermore, for the symbionts of *A. japonicum* and *S. versicolor*, only phenotypic and limited sequence analysis had been carried out (14, 16, 19, 22, 33, 41, 44), leaving the identities of these bacteria in substantial doubt. In contrast, the extensive sampling and multigene phylogenetic analysis described here establishes *P. mandapamensis* as a light organ symbiont of these fishes, either as the apparently exclusive symbiont or as a cosymbiont with *P. leiognathi*. Therefore, five species of luminous bacteria, *P. kishitanii*, *P. leiognathi*, *P. mandapamensis*, *V. fischeri*, and *Vibrio logei*, have been documented to date as bioluminescent symbionts of marine animals (Fig. 1) (8, 10).

The difference in the host ranges of *P. mandapamensis* and *P. leiognathi* indicate that these bacteria might differ in other ways as well, such as in their environmental distributions or physiologies. Such differences presumably would relate in some way to the hosts that they preferentially colonize. Phenotypically, in their growth and luminescence responses to salinity and the color of light produced, these bacteria also differ (1). These ecological and phenotypic differences are underscored at the phylogenetic level by the robust and unambiguous divergence of the sequences of their *lux* and *rib* genes, as well as by the presence of *luxF* in all tested strains of *P. mandapamensis* and its absence in all tested strains of *P. leiognathi*. Despite the large number of strains examined here and previously (1, 9), no phylogenetically intermediate strains have been found. Together, these lines of evidence indicate that *P. mandapamensis* and *P. leiognathi*, despite the many phenotypic and genotypic traits they share (34), are biologically distinct. The ecological and phylogenetic differences described here appear consistent with their original descriptions as separate species (3, 18).

The presence of two bacterial species within an animal's light organ, termed here cosymbiosis, was first reported for sepiolid squids, certain specimens of which harbor both *V. fischeri* and *V. logei* in their light organs (11, 28). Cosymbiosis indicates that symbiotic associations between luminous bacteria and their fish and squid hosts are more accommodating of bacterial diversity than previously thought (8, 17). A strict host

family-bacterial species specificity therefore does not characterize bioluminescent symbioses (10). Nonetheless, these associations do exhibit a high degree of symbiont specificity, with different bacterial species exhibiting substantially different symbiotic host ranges.

The use here and previously of methods to screen for genetic diversity in populations of light organ bacteria, rep-PCR genomic profiling and sequence analysis of the *luxA* gene or *luxA-luxB* genes, has led to the identification of unexpected intraspecies genetic diversity within light organ populations (Fig. 2; 7, 9) and to the finding that multiple bacterial species occur cosymbiotically in fish light organs. This diversity can easily be missed when only single strains are analyzed from a host specimen (e.g., see references 9 and 41). However, it is important to note that no obvious correlation was found between rep-PCR DNA fingerprints and *luxAB*(*F*)*E* sequences. Strains with generally similar DNA fingerprints, such as AJ-1a and *ajapo*.2.16 (Fig. 2), were found by sequence analysis to be different species, and strains with very different fingerprints often were found here to be the same species. The exceptions to this lack of correlation are those strains with the same DNA fingerprint, which we consistently find have identical or very nearly identical sequences for multiple genetic loci. Therefore, DNA fingerprint analysis, while highly effective for typing strains and for grouping them as genetically identical or distinct in ecological surveys of bacterial populations and communities, does not appear to reliably distinguish between or group strains phylogenetically.

The identification of nonsense mutations in *luxF* as small insertions or the presence of a putative transposase gene (see Fig. S1 in the supplemental material) in some strains of *P. mandapamensis* is the first example of nonsense mutations in the *lux* operons of luminous bacteria from nature. The mutations indicate that the LuxF protein is not necessary for the survival or reproduction of *P. mandapamensis* or for its ability to form bioluminescent symbioses. The mutations provide a natural test of the functional role of LuxF in luminescence. LuxF, a nonfluorescent flavoprotein (FP_{390}), exhibits substantial amino acid sequence similarity to the α and β subunits of luciferase, especially to the β subunit, and it is coexpressed with LuxA, LuxB, and other *lux* operon proteins, suggesting that LuxF plays a role in light emission (20, 23, 30, 37). We found that strains carrying nonsense mutations in *luxF* are luminous, but less strongly so than other strains, and that the lower production of light probably is not due to a limitation for aldehyde, a substrate for the luminescence reaction (see the supplemental material). It therefore appears that while *luxF* is not required for luminescence in *P. mandapamensis*, it might contribute in some way to luminescence intensity.

The diversity of bacteria within light organs of *A. japonicum*, *P. panayensis*, and *P. bindus* has implications for the ecological interactions of these host animals with their luminous symbionts. First, it indicates that strains of both clades of *P. mandapamensis* and of *P. leiognathi* were likely to be present together in the environments where light organs of aposymbiotic juveniles of these fishes became colonized. This interpretation is based on the assumption that colonization occurs only during a short period early in the fish's development; however, later secondary colonization, as the fish matures, may be a possibility and could account for the observed diversity. The ability to discriminate between these bacteria using molecular phylogenetic criteria provides a means now to begin defining their specific ecological distributions and relative numbers in other habitats, such as seawater. Second, this diversity demonstrates that these fishes do not strictly discriminate among closely related but phylogenetically distinct bacteria. It is possible, therefore, that genetic and physiological differences between *P. mandapamensis* and *P. leiognathi* are unimportant for symbiosis; alternatively, bacterial attributes important for symbiosis might be shared by these bacteria. The predominance of *P. mandapamensis* in light organs of *A. japonicum* and of *P. leiognathi* in light organs of *P. panayensis* and *P. bindus*, however, introduces the possibility of competitive interactions that, over the life of the host animal, could result in changes in the presence and in numbers of one bacterial species over the other within individual light organs. The apparent lack of bacterial diversity in light organs of *S. versicolor* and *G. jordani* presents a potentially interesting contrast to the situation found for the other fishes, but it may simply be a consequence of limited sampling.

The identification of five fish species that harbor *P. mandapamensis* as a light organ symbiont and the ability to discriminate phylogenetically between this bacterium and other luminous bacteria allowed us test the hypothesis that *P. mandapamensis* and its hosts have cospeciated. The topological congruence expected for fish and bacterial phylogenies of symbiotic partners that have cospeciated, however, was not observed (Fig. 4). The results clearly contradict the suggestion, based on limited data, that the symbionts of certain of these fishes have diverged in a host-dependent manner (41). Instead of identifying a matchup between host and symbiont phylogenies, this analysis indicates that *P. mandapamensis* may be more of a generalist as a light organ symbiont, as reflected by the phylogenetic diversity of its host fishes. In colonizing host species in four families of two teleost orders, *P. mandapamensis* apparently is second only to *P. kishitanii* among light organ symbiotic bacteria in the breadth of its host species range (10). Furthermore, because coevolution, a process of reciprocal genetic adaptation between host and symbiont, is difficult to envision in the absence of cospeciation (32), the results presented here are consistent with the view that coevolution apparently is not the basis for this bacterium's host preferences. The factors that allow *P. mandapamensis* to affiliate with phylogenetically divergent hosts that inhabit widely different marine habitats are not known at this time. An intriguing possibility, however, is that of host selection for luminous bacteria (36) together with an overlap in the ecological distributions of *P. mandapamensis* and the host animals whose aposymbiotic juveniles this bacterium colonizes (2, 10).

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