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ORIGINAL PAPER

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Phylogenetic analysis of the *lux* operon distinguishes two evolutionarily distinct clades of *Photobacterium leiognathi*

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Abstract The luminous marine bacterium *Photobacterium mandapamensis* was synonymized several years ago with *Photobacterium leiognathi* based on a high degree of phenotypic and genetic similarity. To test the possibility that *P. leiognathi* as now formulated, however, actually contains two distinct bacterial groups reflecting the earlier identification of *P. mandapamensis* and *P. leiognathi* as separate species, we compared *P. leiognathi* strains isolated from light-organ symbiosis with leiognathid fishes (i.e., ATCC 25521^T, ATCC 25587, *lequu*.1.1 and *lleuc*.1.1) with strains from seawater originally described as *P. mandapamensis* and later synonymized as *P. leiognathi* (i.e., ATCC 27561^T and ATCC 33981) and certain strains initially identified as *P. leiognathi* (i.e., PL-721, PL-741, 554). Analysis of the 16S rRNA and *gyrB* genes did not resolve distinct clades, affirming a close relationship among these strains. However, strains ATCC 27561^T, ATCC 33981, PL-721, PL-741 and 554 were found to bear a *luxF* gene in the *lux* operon (*luxABFE*), whereas ATCC 25521^T, ATCC 25587, *lequu*.1.1 and *lleuc*.1.1 lack this gene (*luxABE*). Phylogenetic analysis of the *luxAB(F)E* region confirmed this distinction. Furthermore, ATCC 27561^T, ATCC 33981, PL-721, PL-741 and 554 all produced a higher level of luminescence on high-salt medium, as previously described for PL-721, whereas ATCC 25521^T, ATCC 25587, *lequu*.1.1 and *lleuc*.1.1 all produced a higher level of luminescence on low-salt medium, a characteristic of *P. leiognathi* from leiognathid fish light organs. These results demonstrate that *P. leiognathi* contains two evolutionarily and phenotypically distinct clades, *P. leiognathi* subsp. *leiognathi* (strains ATCC 25521^T, ATCC 25587, *lequu*.1.1 and *lleuc*.1.1), and *P. leiognathi* subsp. *mandapamensis* (strains ATCC 27561^T, ATCC 33981, PL-721, PL-741 and 554).

Keywords *Photobacterium leiognathi* · *Photobacterium mandapamensis* · 16S rRNA gene · *gyrB* · *lux* operon · *luxF* · Vibrionaceae

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Introduction

Photobacterium mandapamensis, a luminous marine bacterium, was originally described by Hendrie et al. (1970) from strains isolated from coastal seawater in the vicinity of Mandapam, southern India. In a comprehensive analysis of the taxonomy of luminous marine bacteria, Reichelt and Baumann (1973) grouped the Hendrie et al. (1970) strains together with several other phenotypically similar strains under *P. mandapamensis*, choosing strain 480 (ATCC 27561^T) as the type strain. However, recognition that Boisvert et al. (1967) previously had described as *Photobacterium leiognathi* strains from the light organs of leiognathid fish that were phenotypically similar to *P. mandapamensis* led to reassessment of the validity of *P. mandapamensis*. Based on similarities between ATCC 27561^T and *P. leiognathi* strains in mol% G+C content, flagellation, and chemotaxonomic characters, and in accord with nomenclatural priority, *P. mandapamensis* was synonymized with *P. leiognathi*, and one of the light-organ isolates of Boisvert et al. (1967), ATCC 25521^T, was designated as the type (Reichelt and Baumann 1975). This synonymy was supported by DNA reassociation values of 84% for ATCC 25521^T and ATCC 27561^T and 78% for ATCC 25521^T and ATCC 33981 (*P. mandapamensis* strain 391 of Hendrie et al. 1970) (Reichelt and Baumann 1975; Reichelt et al. 1976). Currently, strains originally described as *P. mandapamensis* (e.g., ATCC 27561^T, ATCC 33981), phenotypically similar strains from the surfaces of fish and seawater (e.g., PL-721, PL-741, and 554), and strains from leiognathid light-organ symbiosis (e.g., ATCC 25521^T

and ATCC 25587) are placed in a single species, *P. leiognathi*.

Bacterial luminescence is encoded by genes of the *lux* operon. The *lux* operon gene order most common in luminous bacteria is *luxCDABEG*, with *luxA* and *luxB* encoding the α and β subunits of bacterial luciferase, *luxC*, *luxD* and *luxE* specifying the fatty acid reductase, acyl-transferase, and acyl-protein synthetase components, respectively, of the fatty acid reductase complex of the luminescence system, and *luxG* encoding a flavin reductase (Dunlap and Kita-Tsukamoto 2001). Certain strains of *Photobacterium*, however, bear an additional gene, *luxF*, which encodes a non-fluorescent flavoprotein, with a gene order of *luxCDABFEG* (Meighen and Dunlap 1993). Specifically, *Photobacterium phosphoreum* NCIMB 844 (Soly et al. 1988) and *P. leiognathi* strains PL-741 (Baldwin et al. 1989) and 554 (Illarionov et al. 1990) bear *luxF* (previously referred to as *luxN* and *luxG*, respectively, in the latter two studies). In contrast, *P. leiognathi* ATCC 25521^T lacks *luxF* (Lee et al. 1991), as do *Vibrio fischeri* and all other luminous *Vibrio* species examined to date (Meighen and Dunlap 1993; Dunlap and Kita-Tsukamoto 2001). Sequence variation in *luxA* has been useful for the phylogenetic characterization and identification of luminous bacteria (Haygood 1990; Wimpee et al. 1991; Nealson et al. 1993; Makemson et al. 1997).

Previously, we reported that strain PL-721, isolated from a deep-sea evermannelid fish and identified as *P. leiognathi* (Nealson and Hastings 1977), differed in its growth and luminescence physiology in response to salt content of the medium compared to strains of *P. leiognathi* isolated from the light organs of leiognathid fishes (Dunlap 1985). These physiological differences and the different ecological origins of strains currently identified as *P. leiognathi* led us to test the possibility that this species contains two phylogenetically distinct groups of strains. Based on sequence analysis of regions encoding 16S rRNA, DNA gyrase subunit B (*gyrB*) and Lux proteins (*luxAB(F)E*), together with distinguishing phenotypic traits, we report here that strains originally described as *P. mandapamensis* and later synonymized with *P. leiognathi* (i.e., ATCC 27561 and ATCC 33981) and certain strains originally identified as *P. leiognathi* (i.e., PL-721, PL-741, 554) form a phylogenetically coherent clade that is distinct from other *P. leiognathi* strains and from other *Photobacterium* species. These distinctions demonstrate the presence of two subspecies within *P. leiognathi*, specifically *P. leiognathi* subsp. *leiognathi* and *P. leiognathi* subsp. *mandapamensis*.

Materials and methods

Cultivation and isolation of bacteria. Bacterial strains used in this study and their sources are listed in Table 1. For routine culture, cells were grown at 22–24°C in a seawater-based medium (Dunlap et al. 2004) containing 10 g tryptone, 5 g yeast extract, and 700 ml artificial seawater per liter (LSW-70), with 15 g agar per liter for solid medium. To test for maximum temperature of growth, strains were grown for 48 h in 3 ml of LSW-70 broth in a temperature-controlled water bath shaker with moderate aeration. To test for minimal growth temperature, strains were plated on LSW-70 agar and incubated at 6–8°C for one week; growth data for other temperatures (4 and 11°C) were taken from the literature (Table 2). Minimal medium, for testing response to salt, contained 15 mM NH_4Cl, 0.2 mM α-glycerophosphate, 20 mM glycerol, 10 μg ferric ammonium citrate per milliliter, 20 mM HEPES (pH 7.25), 15 g agar per liter, and either 400 ml/l (BM-40) or 925 ml/l (BM-100) of artificial seawater. The minimal medium was supplemented with 0.03% yeast extract, inclusion of which allowed all strains to grow well and at similar rates on the low-salt and high-salt media.

To isolate luminous bacteria, seawater samples were aseptically collected from coastal seawater at Ft. Lauderdale, FL, and plated on LSW-70 or *Photobacterium* broth (Difco) agar. Luminous colonies arising on these plates were then picked and purified on LSW-70 agar. Luminous strains exhibiting colony characteristics on LSW-70 of the *Vibrio harveyi* group (large colonies, forming brownish pigmentation in older cultures, producing strong odor) or of *V. fischeri* (colonies forming yellow cell-associated pigmentation) (Reichelt and Baumann 1973; P.V. Dunlap, pers. obs.) were excluded from further screening.

Sequencing and phylogenetic analysis. Phylogenetic analysis involved comparisons of 16S rRNA gene sequences, *gyrB* sequences, organization of the *luxAB(F)E* genes (i.e., presence or absence of *luxF*), and sequences of the *luxAB(F)E* region. Nucleotide sequences were obtained by PCR-amplification of bacterial genomic DNA using standard methods (see Supplementary Table 1 for primer sequences). Previously reported sequences were downloaded from GenBank or were taken from the published literature, as indicated in Table 1. Sequencing of PCR products was carried out by staff of the University of Michigan Sequencing Core using dye terminator cycle sequencing. 16S rRNA gene sequences were aligned by eye, and *gyrB* and *luxAB(F)E* sequences were aligned by inferred amino acid sequence. Parsimony analyses were performed with PAUP* (Swofford 2003) using 1,000 heuristic search replicates with TBR branch swapping. Jackknife support was calculated with PAUP* using 1,000 replicates emulating Jac resampling. Bremer support was calculated with the aid of TreeRot (Sorenson 1999). Sequences of the 16S rRNA gene, *gyrB* and *luxABE* region of *V. fischeri* ATCC 7744^T were used to root the phylogenetic hypotheses. GenBank accession numbers for sequences obtained in this study for the 16S rRNA gene, *gyrB* and *luxAB(F)E* (see Table 1) are as follows. ATCC 25915^T (AY455890), ATCC 33539^T (AY455889), ATCC 51760^T (AY455878), ATCC 25521^T (AY455879), ATCC 25587 (AY455870, AY455880, AY456750), *lequu*.1.1 (AY455881, AY341069), *lleuc*.1.1 (AY455882, AY341070), ATCC 27561^T (AY341441, AY455883, AY341067), ATCC 33981 (AY341442, AY455884, AY341068), PL-721 (AY341440, AY455885, AY341066), *seafl*.1.1 (AY455871, AY455886, AY456751), *seafl*.1.3 (AY455872, AY455887, AY456752), *seafl*.1.4 (AY455873,

Table 1 Bacterial species and strains used in this study[a]

Species	Strain	16S rRNA gene	*gyrB*	*luxAB(F)E*	Habitat and geographic origin
Photobacterium angustum	ATCC 25915^T	D25307	*	–	SW, Hawaii
Photobacterium damselae subsp. *damselae*	ATCC 33539^T	AB032015	*	–	Skin ulcer, *Chromis punctipinnis*, California
Photobacterium damselae subsp. *piscida*	NCIMB 2058	X78105	AJ249849	–	Spleen, *Seriola queradiata*, Kochi Prefecture, Japan
Photobacterium iliopiscarium	ATCC 51760^T	AB000278	*	–	Pyloric cecum, *Clupea harengus*, Norway
Photobacterium leiognathi subsp. *leiognathi*	ATCC 25521^T	X74686	*	M63594	LOS, *Leiognathus splendens*, Gulf of Thailand
	ATCC 25587	*	*	*	LOS, *Leiognathus splendens*, Gulf of Thailand
	lequu.1.1	AY204492	*	*	LOS, *Leiognathus equulus*, Manila, Philippines
	lleuc.1.1	AY204495	*	*	LOS, *Leiognathus leuciscus*, Iloilo, Philippines
Photobacterium leiognathi subsp. *mandapamensis*	ATCC 27561^T [b]	*	*	*	SW, Banda Island, Indonesia
	ATCC 33981[c]	*	*	*	SW, Mandapam, southern India
	554	–	–	X08036	SW, Indian Ocean
	PL-721	*	*	*	SAP?, *Coccorella* sp., Sulu Sea
	PL-741	–	–	[d]	UNK (SAP? *Coccorella* sp.?, Sulu Sea?)[e]
	seafl.1.1	*	*	*	SW, Ft. Lauderdale, FL
	seafl.1.3	*	*	*	SW, Ft. Lauderdale, FL
	seafl.1.4	*	*	*	SW, Ft. Lauderdale, FL
Photobacterium phosphoreum	ATCC 11040^T	*	*	*	UNK
	NCIMB 844	*	*	*	UNK, *Merluccius capensis*, Cape Town, South Africa
	pjapo.1.1	*	*	*	LOS, *Physiculus japonicus*, Odawara, Japan
Photobacterium profundum	JCM 10084^T [f]	D21266	*	–	SW, Ryukyu Trench
	SS9	AB003191	*	–	SW, Sulu Trough
Vibrio fischeri	ATCC 7744^T	*	*	*	SW, UNK

[a]All strains on which sequencing was conducted in this study were obtained from the ATCC except: *lequu*.1.1 and *lleuc*.1.1 (Dunlap et al. 2004); PL-721 (provided by K. Nealson); *seafl*.1.1, *seafl*.1.3, *seafl*.1.4 and *pjapo*.1.1 (this study); NCIMB 844 (provided by C. Miyamoto and E. Meighen); and JCM 10084^T and SS9 (provided by D. Bartlett). GenBank accession numbers for previously obtained 16S rRNA gene, *gyrB* and *luxAB(F)E* sequences are listed in the table.
[b]Strain 480 of Reichelt and Baumann (1973).
[c]Strain NCIMB 391 of Hendrie et al. (1970) and 477 of Reichelt and Baumann (1973).
[d]Sequence from Baldwin et al. (1989).
[e]The origin of PL-741 is reported in Ruby and Morin (1978) to be Reichelt et al. (1977), but strain PL-741 is not cited therein.
[f]JCM 10084^T=DSJ4. Symbols: ^{T}, type strain; *, sequence obtained in this study (see "Materials and methods" for GenBank accession numbers); –, sequence not available (i.e., either non-luminous or strain not available); SW, seawater isolate; UNK, habitat or geographic origin unknown; LOS, light-organ symbiont; SAP, saprophyte.

AY455888, AY456753), ATCC 11040^T (AY341437, AY455875, AY341063), NCIMB 844 (AY341438, AY455876, AY341064), *pjapo*.1.1 (AY341439, AY455877, AY341065), JCM 10084^T (AY455892), SS9 (AY455891), ATCC 7744^T (AY341436, AY455874, AY341062).

Results

To test the possibility that *P. leiognathi* as now formulated contains two distinct bacterial clades, we examined several representatives of *P. leiognathi* for differences in phylogenetically informative loci. Included in the analysis were the type strains for *P. leiognathi*, ATCC 25521^T, and *P. mandapamensis*, ATCC 27561^T, together with several additional strains.

16S rRNA gene and gyrB analysis. Comparison of the aligned 16S rRNA gene from species of *Photobacterium* resulted in a single phylogenetic hypothesis, with no clear distinctions among the strains identified as *P. leiognathi* from light organs of leiognathid fishes (ATCC 25521^T,

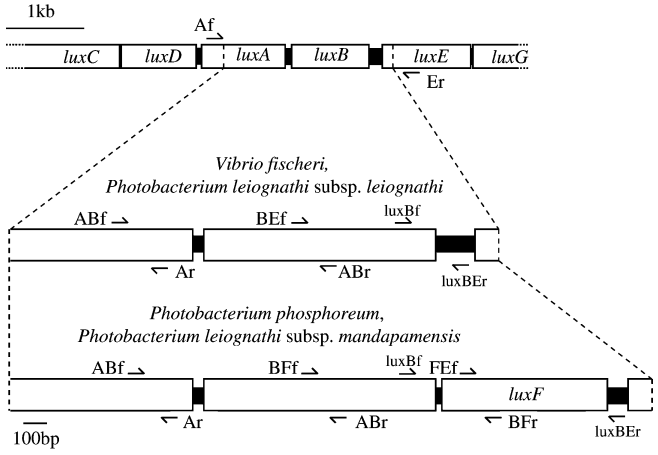

Fig. 1 Organization of the *luxCDAB(F)E* genes in luminous bacteria. Designated are the oligonucleotide primers used for PCR-amplification of specific fragments of the *luxAB(F)E* region of *V. fischeri*, *P. leiognathi* subsp. *leiognathi*, *P. leiognathi* subsp. *mandapamensis* and *P. phosphoreum*. Each primer designation represents at least two species-specific sequences. See Supplementary Table 1 for the sequences of primers used for each species. Presence of the *luxF* gene in *P. phosphoreum* and *P. leiognathi* subsp. *mandapamensis* and its absence in *V. fischeri* and *P. leiognathi* subsp. *leiognathi*, based on sequence analysis (see Fig. 2 and Supplementary Fig. 3), is indicated

ATCC 25587, *lequu*.1.1, *lleuc*.1.1) and from other habitats (ATCC 27561^T, ATCC 33981, PL-721) (Supplementary Fig. 1). Although clear distinctions were apparent for most *Photobacterium* species, the 16S rRNA gene sequences of the *P. leiognathi* strains were nearly identical, differing by 0.6% or less in pair wise comparisons. Like the 16S rRNA gene, *gyrB* also provided little insight into possible distinctions among *P. leiognathi* strains. We found that sequence differences in *gyrB*, while more numerous than in the 16S rRNA gene and supporting species-level distinctions among other members of *Photobacterium*, did not provide sufficient information for phylogenetic resolution of strains currently grouped within *P. leiognathi* (Supplementary Fig. 2).

Analysis of the luxAB(F)E region. To assess the possible value of the *lux* region for distinguishing closely related strains of *P. leiognathi*, we examined the gene organization and sequence of *luxAB(F)E*. First, to determine whether the presence of a *luxF* gene is characteristic of *P. phosphoreum* and as a consequence could provide a comparison with *P. leiognathi* strains lacking or bearing this gene, we examined strains ATCC 11040^T and *pjapo*.1.1. Sequence analysis of the *lux* operons of both strains revealed a *luxF* gene between *luxB* and *luxE* (Fig. 1).

Next, we examined *P. leiognathi* strains not previously tested for the presence of *luxF*, including ATCC 27561^T, ATCC 33981, PL-721, ATCC 25587, *lequu*.1.1, and *lleuc*.1.1. As found for the *P. phosphoreum* strains, sequence analysis of the *lux* operons of strains ATCC 27561^T, ATCC 33981, and PL-721 revealed the presence of *luxF*, with a gene order of *luxABFE*. In contrast, however, *luxF* was not present in the *lux* operons of strains ATCC 25587, *lequu*.1.1 and *lleuc*.1.1 (Fig. 1), which were isolated from leiognathid fish light organs. Furthermore, we tested several additional strains of *P. leiognathi* isolated from leiognathid fish light organs, including strains from a recent study of genomotypic diversity in this species (Dunlap et al. 2004); all lacked a *luxF* gene (J.C. Ast and P.V. Dunlap, unpublished data).

To test the phylogenetic relationships among these strains, we next analyzed the sequence of *luxAB(F)E*, examining individual protein coding regions (Fig. 1) in a combined analysis. Parsimony analysis resulted in a single hypothesis, which resolved *P. phosphoreum* and two closely related but phylogenetically distinct clades within what is currently referred to as *P. leiognathi* (Supplementary Fig. 3). To examine the relationship of these strains together with other *Photobacterium* species, some of which are non-luminous, we then carried out a combined multilocus analysis, using the 16S rRNA gene and *gyrB*, together with the genes and inter-genic spacers of the *lux* region for luminous species. The multilocus analysis resolved *P. phosphoreum* and two *P. leiognathi* clades (Fig. 2) in a manner very similar to that of *luxAB(F)E* alone. Analysis of 16S and *gyrB*, however, contributed little to the distinction between strains, as indicated in Fig. 2, whereas gene organization and sequence of the *lux* region diagnosed the two *P. leiognathi* clades. One clade contains strains originally described as *P. leiognathi* (ATCC 25521^T and ATCC 25587) and other strains from leiognathid light-organ symbiosis (*lequu*.1.1 and *lleuc*.1.1). The other clade contains strains originally described as *P. mandapamensis* (ATCC 27561^T and ATCC 33981) and certain strains identified after the 1975 synonymy (Reichelt and Baumann 1975) as *P. leiognathi* (PL-721, PL-741, and 554). The Bremer support and jackknife resampling values (Fig. 2) indicate very strong support for the two clades. These results indicate that *P. leiognathi* is composed of two evolutionarily distinct groups, designated here as the *leiognathi* clade (*P. leiognathi* subsp. *leiognathi*; Boisvert et al. 1967; strains ATCC 25521^T, ATCC 25587, *lequu*.1.1 and *lleuc*.1.1) and the *mandapamensis* clade (*P. leiognathi* subsp. *mandapamensis*; Hendrie et al. 1970; strains ATCC 27561^T, ATCC 33981, PL-721, PL-741, and 554).

Distinguishing the leiognathi and mandapamensis clades. With this phylogenetic resolution, we were in a position to reassess the possibility that phenotypic characters might allow members of the two clades to be distinguished, despite their substantial similarity. However, a comprehensive re-examination of the published chemotaxonomic characters reported for strains of *P. leiognathi* and *P. mandapamensis* (Hendrie et al. 1970; Reichelt and Baumann 1973, 1975; Reichelt et al. 1976; Ruby and Morin 1978) failed to identify characters that would consistently distinguish members of the two clades.

We therefore sought to identify new phenotypic characters for this purpose. Initially, we examined minimal and maximal temperatures for growth. Certain strains of each clade, however, failed to grow at the low and the

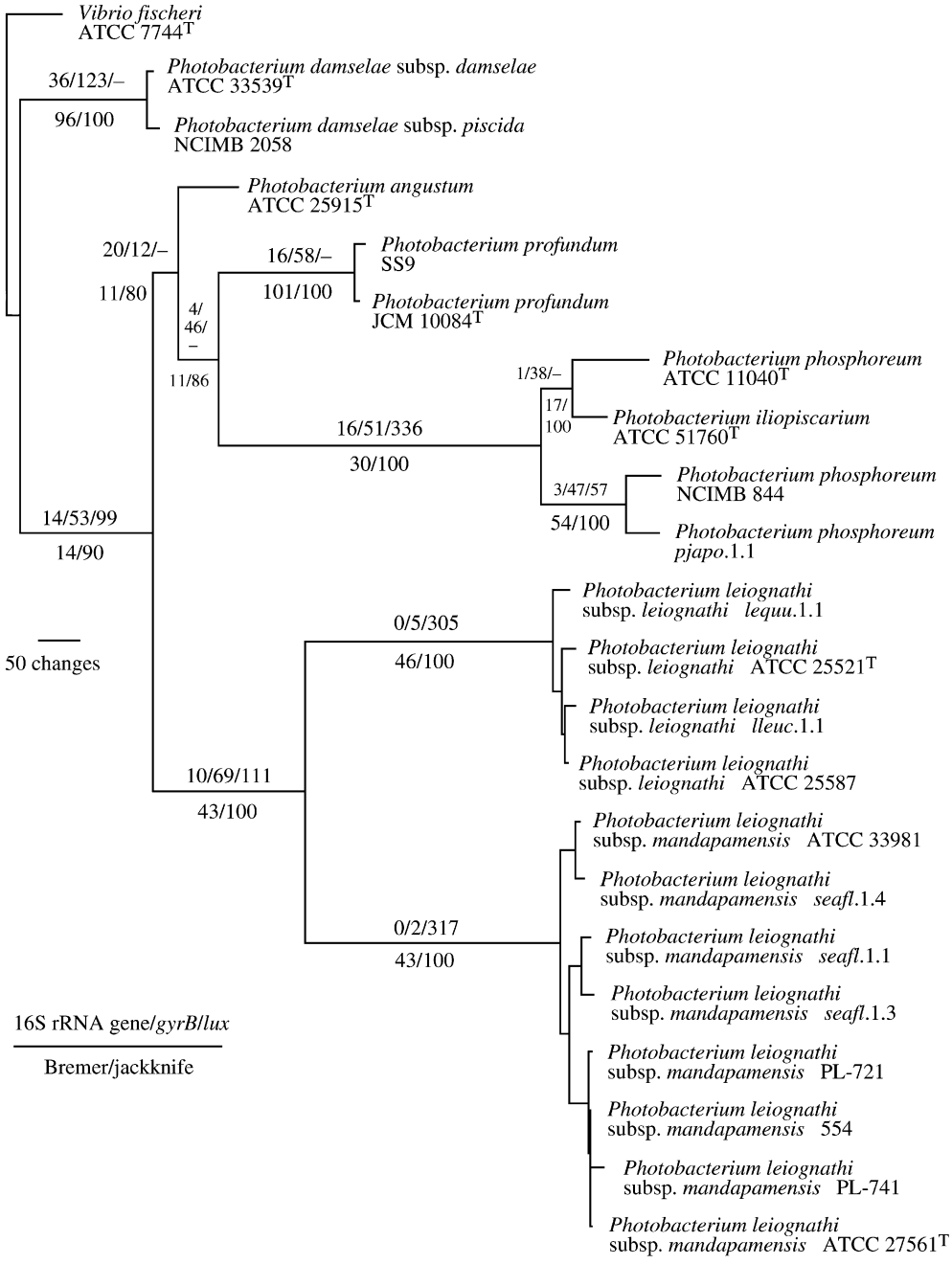

Fig. 2 Phylogram of the most parsimonious hypothesis resulting from combined multilocus analysis of 16S rRNA gene, *gyrB*, and *luxAB(F)E* sequences of *Photobacterium* species (1,429 informative characters, length=2,627, CI=0.76, RI=0.87). Numbers below branches are Bremer support and jackknife resampling values. Numbers above the branches indicate the quantitative contribution of each locus, 16S rRNA gene, *gyrB*, and *luxAB(F)E*, to branch length. For example, for the *P. leiognathi* subsp. *leiognathi* clade, the values of 0/5/305 represent no steps contributed by 16S rRNA gene, five steps contributed by *gyrB*, and 305 steps contributed by *luxAB(F)E*. See Table 1 for strain information

high temperatures examined (Table 2). Thus, minimal and maximal growth temperatures apparently are not effective for distinguishing members of the *leiognathi* and *mandapamensis* clades.

We next compared the luminescence responses of strains of the two clades grown on a minimal medium containing either a low level (BM-40) or a high level (BM-100) of seawater salts. Strains ATCC 27561^T, ATCC 33981 and PL-721 produced a higher level of luminescence on BM-100 compared to BM-40. In contrast, strains ATCC 25521^T, ATCC 25587, *lequu*.1.1 and *lleuc*.1.1 produced a higher level of luminescence on BM-40 compared to BM-100 (Table 2). These results indicate that comparison of relative luminescence intensity on low and high salt-containing minimal medium can distinguish members of the *mandapamensis* clade from those of the *leiognathi* clade. Furthermore, strain PL-721 (*mandapamensis* clade) was noted earlier to produce luminescence that is bluer in color than the blue–green luminescence of *P. leiognathi* from leiognathid fish light organs (Dunlap 1984). We therefore screened strains ATCC 27561^T, ATCC 33981, PL-721, ATCC 25521^T, ATCC 25587, *lequu*.1.1 and *lleuc*.1.1 for luminescence color. Strongly luminous strains of the *mandapamensis* clade were distinctly bluer in color than strongly luminous strains of the *leiognathi* clade (Fig. 3).

Table 2 Phenotypic characters distinguishing the *mandapamensis* and *leiognathi* clades of *P. leiognathi*

	mandapamensis clade			*leiognathi* clade			
	ATCC 27561^T	ATCC 33981	PL-721	ATCC 25521^T	ATCC 25587	*lequu*.1.1	*lleuc*.1.1
Growth at							
4°C	–[a]	–[a]	+[b]	–[a]	ND[c]	ND	ND
6–8°C	–	–	+	–	–	–	–
11°C	+[b]	+[b]	+[b]	ND[c]	ND	ND	ND
35°C	+	+	+	+	+	+	+
37°C	+	+	–	–	+	+	+
39°C	–	–	–	–	–	–	–
Luminescence[d]							
BM-40	√	+	++	+++	++++	+++++	++++
BM-100	++	+++	+++	++	–	+++	–

[a] (–) No growth at the indicated temperature. Data from Reichelt and Baumann (1975).
[b] (+) Growth at the indicated temperature. Data from Ruby and Morin (1978).
[c]ND, no data, but strains likely to be members of the *leiognathi* clade were reported not to grow at 4°C and to grow at 11°C (Ruby and Morin 1978).
[d]Luminescence, relative light production ranging from none observed after 10 min dark adaptation (–), to trace (√), faint (+), and progressively stronger (++,+++, etc.). Boldface indicates the medium, low-salt (BM-40) or high-salt (BM-100), on which a strain produced more light.

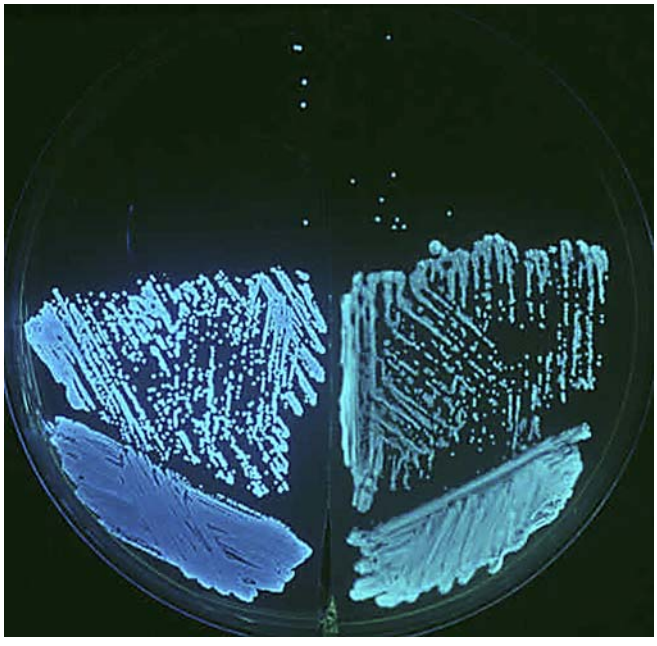

Fig. 3 Luminescence color of members of the *leiognathi* and *mandapamensis* clades of *P. leiognathi*. Strains PL-721 (*left*: *mandapamensis* clade) and *lequu*.1.1 (*right*: *leiognathi* clade) were grown overnight on LSW medium and photographed by the light produced by the cells. The image was captured with Fujichrome Sensia 400 daylight film exposed to the plate in complete darkness at an f11 for ~5 min using a Nikon N70 SLR camera fitted with a Nikon Nikkor AF Micro 60 mm 1:2.8D lens. The bluer color was characteristic of strains ATCC 27561^T, ATCC 33981 and PL-721, whereas the more blue–green color was characteristic of strains ATCC 25521^T, ATCC 25587, *lequu*.1.1 and *lleuc*.1. Distinctions between strains were made most readily on strongly luminous strains

The qualitative nature of these phenotypic differences, however, and the inter-strain variations in levels of light, which can complicate comparisons of luminescence intensity and color, led us to seek a more definite means of distinguishing these strains. To take advantage of the difference in the presence of *luxF*, we designed a primer pair (luxBf/luxBEr; Fig. 1 and Supplementary Table 1) that specifically amplifies the region of the *lux* operon spanning *luxB* and *luxE*. The expectation was that a larger PCR product, approximately 1,140 bp long, would be generated by the luxBf/luxBEr primer pair from strains bearing *luxF*, whereas a smaller PCR product, approximately 500 bp long, would be generated for strains lacking *luxF*. To test the ability of the primer pair to distinguish between strains of the two clades, we used them to direct the amplification of DNA from ATCC 27561^T, ATCC 33981, PL-721, ATCC 25521^T, ATCC 25587, *lequu*.1.1 and *lleuc*.1. A smaller product, approximately 500 bp in length, was recovered from ATCC 25521^T, ATCC 25587, *lequu*.1.1 and *lleuc*.1, whereas a larger product, approximately 1,140 bp in length, was recovered from ATCC 27561^T, ATCC 33981 and PL-721 (Fig. 4). These results establish the ability of the luxBf/luxBEr primer pair to distinguish members of the two clades.

Identification of unknown strains. To test the effectiveness of these phenotypic screening and PCR procedures on unidentified bacteria, we examined previously uncharacterized strains of luminous bacteria recently isolated from coastal seawater in Florida. Three of the strains, *seafl*.1.1, *seafl*.1.3, and *seafl*.1.4 (Table 1), were found through phenotypic screening to exhibit stronger luminescence on BM-100 compared to BM-40 and to produce a bluer color of luminescence. When tested with the luxBf/luxBEr primer pair, each generated a product of approximately 1,140 bp long (data not shown), consistent with presence of a *luxF* gene and indicating membership in the *mandapamensis* clade.

To test clade membership of these strains, we analyzed their 16S rRNA gene, *gyrB* and *luxAB(F)E* sequences. The 16S rRNA gene and *gyrB* sequences of *seafl*.1.1, *seafl*.1.3, and *seafl*.1.4, as found for ATCC 27561^T, ATCC 33981,

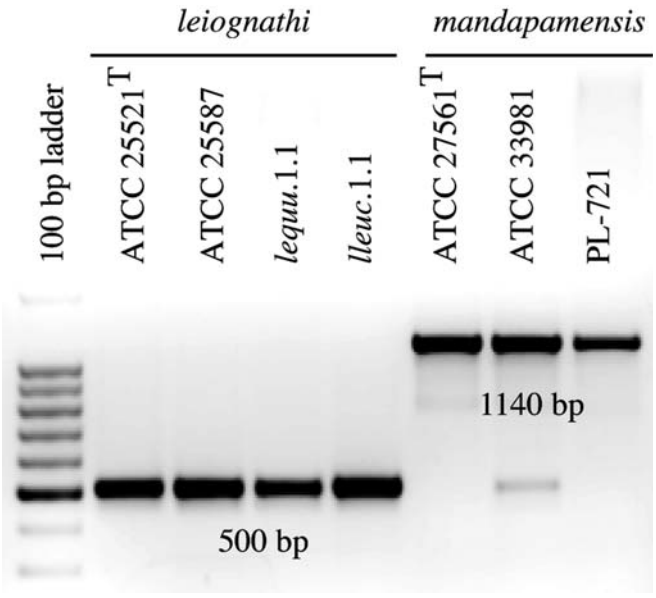

Fig. 4 PCR-based differentiation between members of the *leiognathi* and *mandapamensis* clades of *P. leiognathi*. Amplification of DNA was directed by the luxBf/luxBEr primer pair (Supplementary Table 1). The presence of *luxF* (strains of the *mandapamensis* clade) results in a larger PCR product, approximately 1,140 bp, whereas the absence of *luxF* (strains of the *leiognathi* clade) results in a smaller PCR product, approximately 500 bp. See Fig. 1 for gene organization

PL-721, ATCC 25521^T, ATCC 25587, *lequu*.1.1 and *lleuc*.1, placed them within *P. leiognathi* but did not resolve clade membership (Supplementary Figs. 1 and 2). Analysis of the *lux* region, however, confirmed the presence of *luxF*, and sequence analysis of the *luxABFE* region resolved these three strains to the *mandapamensis* clade (Supplementary Fig. 3), as did a multilocus phylogenetic analysis based on sequences of the 16S rRNA gene, *gyrB* and *luxABFE* (Fig. 2).

Discussion

The results of this study demonstrate the effectiveness of sequence analysis of the *lux* region for distinguishing closely related luminous bacteria. Two evolutionarily distinct clades of bacteria within *P. leiognathi* were resolved based on presence of the *luxF* gene and sequence of the *luxAB(F)E* region. One clade (*mandapamensis*; ATCC 27561^T, ATCC 33981, PL-721, PL-741, and 554) comprises strains bearing *luxF*, whereas strains of the other clade (*leiognathi*; ATCC 25521^T, ATCC 25587, *lequu*.1.1, *lleuc*.1.1) lack *luxF* (Baldwin et al. 1989; Illarionov et al. 1990; Lee et al. 1991; this study). A PCR method based on presence or absence of *luxF* was developed and its efficacy, in combination with phenotypic differences, for provisional clade assignment of strains was demonstrated with previously unstudied luminous bacteria. Clade membership was then confirmed with sequence analysis of 16S rRNA gene, *gyrB* and *lux*. DNA reassociation values of 78 and 84% between members of the *leiognathi* and *mandapamensis* clades previously placed these bacteria within a single species, *P. leiognathi* (Reichelt and Baumann 1975; Reichelt et al. 1976). However, the phylogenetic differences described here demonstrate the presence of evolutionarily distinct lineages consistent with two subspecies, *P. leiognathi* subsp. *leiognathi* and *P. leiognathi* subsp. *mandapamensis*. Further study might reveal these differences to be indicative of a species-level divergence.

Sequence divergence in *luxA* was used previously to assess the phylogenetic relationships between uncultured luminous bacteria symbiotic with anomalopid fish and known luminous bacteria (Haygood 1990) and as the basis for a hybridization method for distinguishing different species of luminous bacteria (Wimpee et al. 1991; Nealson et al. 1993). More recently, the sequence of *luxA* has been analyzed in the context of the description of a new species of luminous bacterium, *Shewanella woodyi* (Makemson et al. 1997), and in that report differences were noted in the *luxA* sequences of strains characterized more fully here at the sequence level, ATCC 25521^T and PL-721. In the present study, we used the sequence of the *luxAB(F)E* region, which in contrast to sequences of 16S rRNA gene and *gyrB* provided substantial phylogenetic insight, to distinguish two evolutionarily distinct clades within the species *P. leiognathi*. The similarity of the combined 16S rRNA gene, *gyrB* and *lux* analysis to that of *lux* alone demonstrates the phylogenetic resolving power of *luxAB(F)E*, a locus that appears to be very effective for examining fine-scale within-species and between-species divergence among luminous bacteria.

Recently, the *gyrB* locus, which specifies the B subunit of gyrase, an enzyme essential for DNA replication, has been found to be more effective for resolving relationships among various bacteria than the 16S rRNA gene (Yamamoto et al. 2000; Dauga 2002; Yáñez et al. 2003). Consistent with these reports, *gyrB* provides good species resolution in *Photobacterium* (Supplementary Fig. 2), diagnosing most of the currently recognized species in this genus. However, we demonstrate here that *gyrB*, like the 16S rRNA gene, does not distinguish members of the *leiognathi* and *mandapamensis* clades. The inability of both 16S rRNA and *gyrB* to distinguish between members of these clades affirms a close evolutionary relationship. Analysis of *gyrB* therefore may prove most effective for resolving more distantly related species.

The results of this study have implications for the phylogeny of other species of *Photobacterium*. Based on analysis of the 16S rRNA gene, Urakawa et al. (1999) moved *Photobacterium iliopiscarium* from *Vibrio* to *Photobacterium*. In the present study, using multiple strains of *P. phosphoreum* and a combined 16S rRNA gene/*gyrB* analysis, we find that *P. iliopiscarium* is nested among, rather than sister to, *P. phosphoreum*. We find also that *Photobacterium damselae* is sister to all other *Photobacterium* species, as previously reported (Dunlap and Kita-Tsukamoto 2001), rather than sister to *P. leiognathi* (e.g., Nogi et al. 1998; Urakawa et al. 1999). It is likely, however, that with additional study, especially of more strains

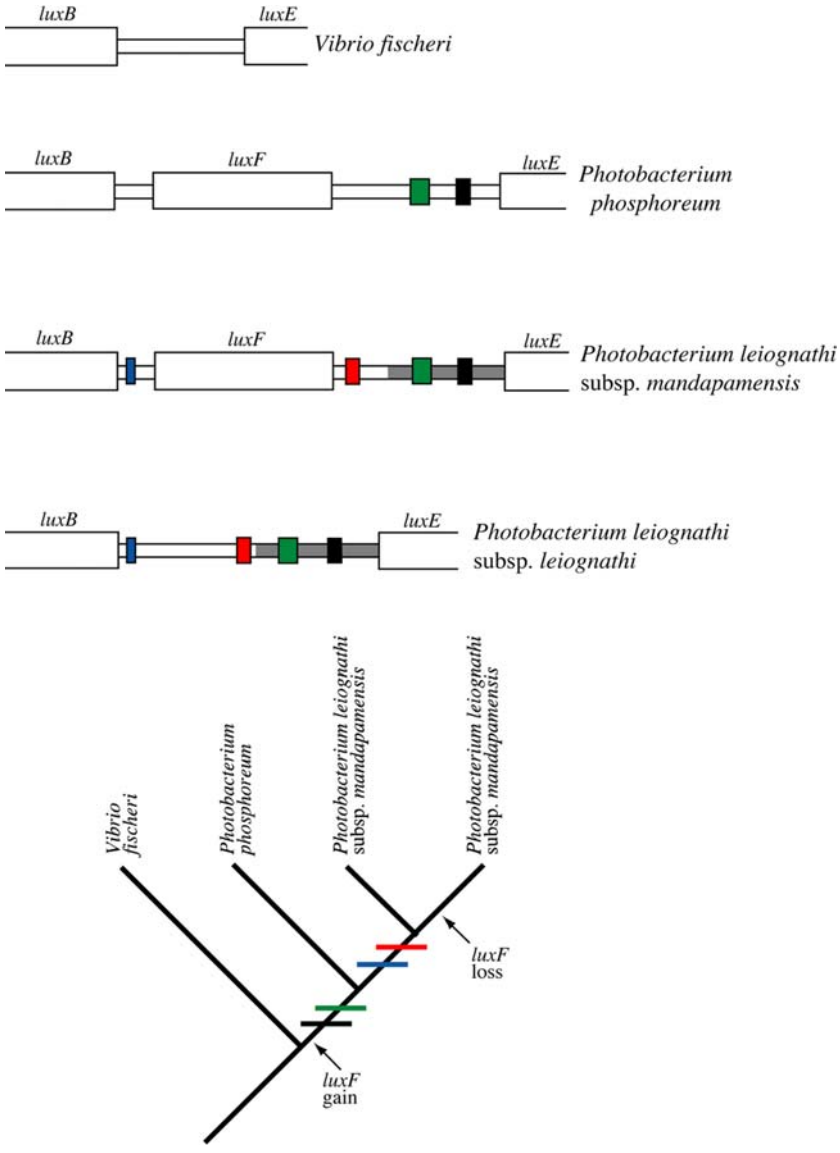

Fig. 5 Organization of the *luxB(F)E* region, including inter-genic spacer regions in *V. fischeri* and *Photobacterium* species. Identical sequences of DNA in the spacer regions (drawn to scale) are indicated by *colored rectangles*; the *gray shaded region* shared by *P. leiognathi* subsp. *mandapamensis* and *P. leiognathi* subsp. *leiognathi* indicates an area that, while not identical in sequence, is readily alignable, whereas the *unshaded white regions* indicate areas that could not be aligned. The cladogram maps characters onto the hypotheses presented in Fig. 2 and shows the hypothesized sequence of events in *Photobacterium* evolution: *luxF* and the *green* and *black regions* are acquired before the divergence of *P. phosphoreum* and the ancestor of *P. leiognathi* subsp. *leiognathi* and *P. leiognathi* subsp. *mandapamensis*; the *red* and *blue regions* are acquired after the divergence of *P. phosphoreum* but before the divergence of *P. leiognathi* subsp. *leiognathi* and *P. leiognathi* subsp. *mandapamensis*; finally, *luxF* is lost in *P. leiognathi* subsp. *leiognathi* after its divergence from *P. leiognathi* subsp. *mandapamensis*

of each species, the view presented here of evolutionary relationships among members of the *Photobacterium* clade (Fig. 2) will require further modification.

Presence of *luxF* in the three examined strains of *P. phosphoreum*, NCIMB 844 (Soly et al. 1988), ATCC 11040^T and *pjapo*.1.1 (this study), suggests that *luxF* is characteristic of *P. phosphoreum*. We were interested therefore in the possibility that the presence of *luxF* provides insight into speciation in luminous bacteria. The *luxF* gene is thought to have arisen in the ancestor of *Photobacterium* through a *luxB* gene duplication event (Meighen and Dunlap 1993). Based on the results presented here, we propose that following this duplication, *luxF* was subsequently lost from strains that became *P. leiognathi* subsp. *leiognathi* while being retained by strains that became *P. phosphoreum* and *P. leiognathi* subsp. *mandapamensis* (Fig. 5). This proposed loss of *luxF* therefore would mark a major evolutionary divergence in the *Photobacterium* lineage. An alternative possibility is that *luxF* arose independently in *P. phosphoreum* and *P. leiognathi* subsp. *mandapamensis*. This alternative scenario, however, requires two separate gene acquisition events and is not well supported at the sequence level. Specifically, the non-coding region 3′ of *luxB* in *P. leiognathi* subsp. *leiognathi* has regions identical to the non-coding regions in *P. leiognathi* subsp. *mandapamensis* between *luxB* and *luxF* and between *luxF* and *luxE* (Fig. 5). These sequence identities indicate a closer relationship between members of the *leiognathi* and *mandapamensis* clades than between *P. leiognathi* subsp. *mandapamensis* and *P. phosphoreum*. It is interesting to speculate that the absence of *luxF* in members of the *leiognathi* clade, and the resulting possible functional differences in its luminescence system, relate in some way to a difference in the native ecology of this bacterium compared to members of the *mandapamensis* clade.

An inference arising from this study is that members of the *leiognathi* and *mandapamensis* clades may be ecologically distinct. While *P. leiognathi* (*leiognathi* and *mandapamensis* clades) can be isolated from seawater (Baumann and Baumann 1981; Hastings and Nealson 1981; Dunlap

and Kita-Tsukamoto 2001), strains examined in this study lacking *luxF* (*leiognathi* clade) all were isolated from the light organs of leiognathid fishes, whereas the strains bearing *luxF* (*mandapamensis* clade) were isolated from seawater and the surface of marine fish, but not from light organs of leiognathid fishes (Table 1). Whether members of the *mandapamensis* clade enter into a bioluminescent symbiosis is not known at this time, but the more blue color of luminescence produced by members of this clade suggests that such an association might be found with animals occurring at greater depths in the ocean than with the shallow-dwelling leiognathid fishes. Assessment of previous reports of the incidence of these two bacteria (e.g., Baumann and Baumann 1981) is complicated, however, by their prior synonymy and by the previous inability to distinguish them phenotypically. Establishment of *P. leiognathi* subsp. *mandapamensis* as a subspecies phylogenetically distinct from *P. leiognathi* subsp. *leiognathi* and application of the methods described here for differentiating between strains of the *mandapamensis* and *leiognathi* clades, using previously isolated strains and new isolates, should allow rapid progress now to be made in understanding the extent to which the ecologies of these two closely related luminous bacteria differ. Specifically, for example, we can state with reasonable confidence based on the results presented here that *P. leiognathi* subsp. *mandapamensis* is geographically widespread, with phylogenetically very similar strains occurring in seawater from locations as distant as southern India (Hendrie et al. 1970) and southern Florida (this study). *P. leiognathi* subsp. *mandapamensis* therefore may be cosmopolitan in its distribution.

The inability of methods considered standard for bacterial species identification (i.e., mol% G+C, DNA reassociation, 16S rRNA gene sequence, chemotaxonomic characters) to distinguish members of the *leiognathi* and *mandapamensis* clades (Reichelt and Baumann 1973, 1975; this study) is intriguing in light of the distinct separation provided by *luxAB(F)E* analysis. The shortcomings of the standard methods have been noted previously, and there is growing recognition of the importance of multilocus sequence analysis, as used here, in bacterial systematics for species identification (e.g., Palys et al. 1997; Stackebrandt et al. 2002). Based on the example presented here, our view is that analysis of inherited characters holds great promise for providing the information, sensitivity and precision necessary to capture an accurate picture of bacterial species diversity. Analysis of the *lux* operon, a locus that has diverged more rapidly than the 16S rRNA gene or *gyrB*, may have special value for resolving questions of species identity, clade membership and geographic distribution in luminous bacteria.

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