Natural Merodiploidy of the lux-rib Operon of Photobacterium leiognathi

from Coastal Waters of Honshu, Japan

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Running title: Multiple lux-rib operons in P. leiognathi

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Sequence analysis of the bacterial luminescence (lux) genes has proven effective in helping resolve evolutionary relationships among luminous bacteria. Phylogenetic analysis using lux genes, however, is based on the assumptions that the lux genes are present as single copies on the bacterial chromosome and are vertically inherited. We report here that certain strains of Photobacterium leiognathi carry multiple phylogenetically distinct copies of the entire operon that codes for luminescence and riboflavin synthesis genes, luxCDABEG-ribEBHA. Merodiploid lux-rib strains of P. leiognathi were detected during sequence analysis of luxA. To define the gene content, organization, and sequence of each lux-rib operon, we constructed a fosmid library of genomic DNA from a representative merodiploid strain, lnuch.13.1. Sequence analysis of fosmid clones and genomic analysis of lnuch.13.1 defined two complete, physically separate, and apparently functional operons, designated lux-rib$^1$ and lux-rib$^2$. P. leiognathi strains lelon.2.1 and lnuch.21.1 also were found to carry lux-rib$^1$ and lux-rib$^2$, whereas the type strain ATCC 25521$^T$ apparently carries only lux-rib$^1$. In lnuch.13.1, lelon.2.1, lnuch.21.1, and ATCC 25521$^T$, lux-rib$^1$ is flanked upstream by lumQ and putA and downstream by a gene for a hypothetical multidrug efflux pump. In contrast, transposase genes flank lux-rib$^2$ of lnuch.13.1, and the chromosomal location of lux-rib$^2$ apparently differs in lnuch.13.1, lelon.2.1, and lnuch.21.1. Phylogenetic analysis demonstrated that lux-rib$^1$ and lux-rib$^2$ are more closely related to each other than either is to the lux and rib genes of other bacterial species, which rules out inter-species lateral gene transfer as the origin of lux-rib$^2$ in P. leiognathi; lux-rib$^2$ apparently arose within a previously unsampled or extinct P. leiognathi lineage. Analysis of 170 additional strains of P. leiognathi, for a total of 174 strains examined from coastal waters of Japan, Taiwan, the Philippine Islands, and Thailand, identified 106 that carry only a single lux-rib operon and 68 that carry multiple lux-rib operons. Strains bearing a single lux-rib operon were obtained throughout the geographic sampling range, whereas lux-rib merodiploid strains were found only in coastal waters of central Honshu. This is the first report of merodiploidy of the lux or rib genes in a luminous bacterium and the first indication that a natural merodiploid state in bacteria can correlate with geography.
INTRODUCTION

Luminescence in *Photobacterium leiognathi* and other luminous bacteria is the product of bacterial luciferase, a mixed-function oxidase that uses oxygen, reduced flavin mononucleotide, and a long-chain fatty aldehyde as substrates to produce blue-green luminescence. The genes for bacterial light production are present as an operon, *luxCDABEG*; *luxA* and *luxB* encode the α and β subunits of luciferase, *luxC*, *luxD* and *luxE* specify the enzymatic components of a fatty acid reductase complex necessary for synthesis and recycling of the aldehyde substrate, and *luxG* encodes a flavin reductase (14). Most luminous *Photobacterium* species, i.e., *Photobacterium phosphoreum*, *Photobacterium kishitanii*, and *Photobacterium mandapamensis*, also carry *luxF*, which encodes a non-fluorescent flavoprotein, with a *lux* operon gene order of *luxCDABFEG* (2, 3, 4, 23, 28, 37). Linked to the luminescence genes in some *Photobacterium* species, and apparently co-transcribed with them, are genes involved in synthesis of riboflavin, forming an operon of 10 or eleven genes, *luxCDAB(F)EG-ribEBHA*, which we refer to here as the *lux-rib* operon (23, 26, 27, 34, 37, this study). Upstream of the *lux-rib* operon in *P. mandapamensis*, a species closely related to *P. leiognathi*, are *lumQ* and *lumP* (encoding proteins of the lumazine operon), and these genes are located adjacent to the *putA* gene (encoding proline dehydrogenase) (29, 30, 31).

Phylogenetic analysis of *lux* and *rib* genes, together with housekeeping genes such as the 16S rRNA gene, *gyrB*, *pyrH*, *recA*, *rpoA*, and *rpoD*, has proven helpful in defining evolutionary relationships among luminous bacteria and in the identification of new species (2, 3, 4, 12, 23). Phylogenetic analysis based on *lux* and other genes also has proven effective in providing the bacterial species- and clade-level resolution necessary for testing hypotheses of symbiont-host specificity and evolutionary codivergence in bioluminescent symbioses (16, 23). These studies reveal that the evolutionary divergence of symbiotic luminous bacteria has not followed the evolutionary divergence of their host animals.

Little is known, however, about the evolution of the bacterial luminescence system itself. Based on amino acid sequence identities, a tandem duplication of the ancestral *luxA* gene, followed by sequence divergence in the duplicated gene, is thought to have given rise to *luxB*, leading to formation of the heterodimeric luciferase present in modern-day luminous bacteria. Similarly, a tandem duplication of
luxB is thought to have given rise to the luxF gene (6, 14, 37). The subsequent loss of luxF from the lineage that gave rise to P. leiognathi might reflect the evolutionary divergence of this species from other Photobacterium species (2). Recently, strains of P. mandapamensis bearing non-sense mutations in luxF have been isolated from nature, the first report of naturally occurring mutations in lux structural genes (23). The presence in nature of strains bearing luxF mutations suggests that luxF is less functionally constrained than other lux genes and indicates that this gene does not play an essential role in the free-living ecology and symbiosis of this species.

Relevant to both phylogenetic analysis based on lux genes and evolution of the luminescence system is the question of lux gene copy number. The lux and rib genes are tacitly assumed to be present as single copies in P. leiognathi and other luminous bacteria. In contradiction of that assumption, we report here that certain strains of P. leiognathi carry two complete, physically separate, and apparently functional lux-rib operons, one closely associated with putA and the other located elsewhere on the chromosome. The presence of multiple copies of each of the lux and rib genes presumably would provide opportunities for the accumulation of mutations leading to sequence divergence in one or the other copy of each lux and rib gene and opportunities for recombination between the two operons. Instead, we find that both operons are stably inherited and show little or no evidence of mutation or recombination in different merodiploid strains.
MATERIALS AND METHODS

**Bacterial strains and culture conditions.** Bacterial strains used in this study are listed in Table 1 and in the supplemental material. Strains of *P. leiognathi* were isolated from light organs of bacterially luminous marine animals and from seawater collected in various locations in: Honshu, Shikoku, and Okinawa, Japan; Taiwan; the Philippine Islands; and Thailand (2, 8, 15, 16, 18, this study) (see supplemental material for collection details). Bacteria were grown in LSW-70 (15), which contained per liter 10 g tryptone, 5 g yeast extract, 350 ml double-strength artificial seawater (38), 650 ml de-ionized water, and for solid medium, 15 g agar. Genomic DNA was purified from cultures of strains grown overnight in LSW-70 broth using the DNeasy tissue extraction kit (Qiagen). Strains were identified to species by phylogenetic analysis of *lux* and other genes (2, 15, 16, 23, this study).

**DNA amplification and sequencing.** For DNA amplification by PCR, MasterTaq polymerase (Eppendorf) and the following protocol were used: 95°C initial denaturing step for 2 min; 35 cycles with a 94°C denaturing step for 20 sec, a variable temperature annealing step for 15 sec, and an extension step at 68°C for 1 min; a 7 min final extension step at 68°C; snap cooling to 4°C. PCR primers, annealing temperatures and exceptions to this protocol are described in the supplemental material. PCR products were visualized using electrophoresis on 1% agarose gels stained with ethidium bromide and were purified using QIAquick PCR Purification kit (Qiagen) or Montage PCR Filter kit (Millipore). PCR products were sequenced using the respective PCR primers. Sequencing was carried out by the staff of the University of Michigan Sequencing Core using dye terminator cycle sequencing on a Perkin-Elmer ABI 3730 or 3700 DNA Analyzer. Specific primers for species of luminous bacteria other than *P. leiognathi* were designed based on existing publicly available sequences (see supplemental material). Sequences not obtained in this laboratory were downloaded from public databases.

**Cloning luxA and lux-rib operons.** The *luxA* genes of *P. leiognathi Inuch*.13.1, amplified using primers CWLAP1for and CWLAP1rev, were cloned using the TOPO 4.0 cloning kit (Invitrogen). A fosmid library of genomic DNA from strain *Inuch*.13.1 was constructed using a CopyControl Fosmid Library Production kit (Epicentre). The library consists of approximately 3500 clones with inserts of 35–
The library was screened for fosmids containing \textit{lux-rib} sequences by pooling transformant colonies into groups that were subsequently analyzed by PCR using \textit{lux-rib} primers. From these groups, two colonies were identified that each contained a fosmid with a complete \textit{lux-rib} operon, clones B7-25 and C30-24. Fosmids were recovered from colonies using a WizardPlus Miniprep kit (Promega) and sequenced directly using vector-specific and \textit{lux-rib}-specific primers. See the supplemental material for the sequencing strategy. Recovered fosmids were also used as templates for long range PCR using the TripleMaster PCR System (Eppendorf), to determine the orientation of the inserted DNA and approximate position of \textit{lux-rib} in the inserted DNA relative to the vector ends.

\textbf{Plasmid profiling and pulsed-field gel electrophoresis.} Small plasmids in \textit{P. leiognathi} (15) were purified from strains \textit{lelon}.2.1, \textit{lnuch}.13.1, \textit{lnuch}.21.1, and ATCC 25521\textsuperscript{T}, grown overnight in LSW-70 broth, using a WizardPlus Miniprep kit (Promega). The resulting DNA was electrophoresed through a 0.7\% agarose gel (Bio-Rad) and stained with ethidium bromide. Plasmids smaller than 25 kb were present in \textit{lnuch}.13.1, \textit{lelon}.2.1, and \textit{lnuch}.21.1 (data not shown); these plasmids were found to be smaller than the \textit{lux-rib} containing fosmid clones B7-25 and C30-24.

To visualize plasmids larger than 25 kb, we used pulsed-field gel electrophoresis (PFGE) and a BioRad CHEF mapper-chiller system. Genomic DNA in agarose plugs was prepared according to Lucangeli et al. (35) with modifications. Before lysis of the cells embedded in agarose, the plugs were first washed for 1 hour at 37°C in buffer [6 mM TrisHCl (pH7.5), 100 mM EDTA (pH 8.0), 1 M NaCl, 0.5\% Brij58, 0.2 M sodium deoxycholate, 0.5\% N-lauroylsarcosine NL-97] with 0.5 mg.ml\textsuperscript{-1} of lysozme. Genomic DNA was run in 1\% SeaKem agarose gels in 1X TAE buffer. Electrophoresis was for 15 h, at 14°C, gradient 6 V cm\textsuperscript{-1}, angle 120°, initial pulse switch time was 5 s, final pulse switch time was 15 s, with linear ramping. Two plasmids of approximately 180-200 kb, sufficiently large to account for a cosmid clone bearing a \textit{lux-rib} operon, were present in strain \textit{lelon}.2.1, whereas plasmids of 35-40 kb or greater were not detected in \textit{lnuch}.13.1 or \textit{lnuch}.21.1.
Similar PFGE procedures were used to identify the chromosomal locations of lux-rib<sup>1</sup> and lux-rib<sup>2</sup>. Specifically, genomic DNA in agarose plugs was digested for 16 to 18 hours with restriction endonuclease NotI (New England Biolabs) at 37°C in the buffer recommended by the manufacturer.

PFGE gels were 0.8% SeaKem agarose (Bio Whittaker Molecular Applications) or low melting point agarose (Bio-Rad) in 1x TAE buffer. Electrophoresis conditions were: 27 h, at 14°C, gradient 6 V cm<sup>-1</sup>, angle 120°, initial pulse switch time 2.16 s, final pulse switch time 2 m 26.90 s, with linear ramping. Mid Range PFG Marker (New England Biolabs) and Yeast Chromosome Marker (New England Biolabs) were used as size markers. Gels were stained with ethidium bromide to visualize DNA.

NotI digestion fragments of DNA were recovered using PFGE carried out in gels of low melting point agarose. Individual bands were excised, and the agarose was melted (65°C) and digested using β-agarase (New England Biolabs) as recommended by manufacturer. DNA fragments were purified using phenol/chloroform extraction, recovered by ethanol precipitation, and suspended in distilled water. NotI digestion of <i>P. leiognathi</i> strain Inuch.13.1 genomic DNA produced seven fragments that ranged in size from approximately 1500 kb to 220 kb. PCR was performed using the individual DNA fragments as template and with lux-rib<sup>1</sup>-specific and lux-rib<sup>2</sup>-specific luxD-luxA primers.

**Phylogenetic analysis.** Sequences of the lux-rib operon genes of <i>Photobacterium</i> strains were aligned to the sequences of lux and rib genes from other luminous species (Table 2), with the nucleotide alignment based on inferred amino acid sequences; data were analyzed simultaneously with the parsimony criterion using PAUP* (48). Spacer regions between genes in the lux and rib operons were omitted from the analysis, and inferred deletions were treated as missing data. Jackknife support percentage values (17) were calculated in PAUP* using 1000 replicates with 10 heuristic searches per replicate, a jackknife resampling value of 34%, and emulating JAC resampling, which re-samples characters independently. Housekeeping genes (gapA, gyrB, recA, rpoA, and rpoD) were amplified, sequenced, and analyzed by similar methods, to test the relationships within <i>Photobacterium</i> determined by lux-rib analysis.
Analysis of recombination. To test for recombination between the genes of lux-rib<sup>1</sup> and lux-rib<sup>2</sup> in lnuch.13.1, lelon.2.1 and lnuch.21.1, we developed a partition congruence analysis, as described here (script to produce PAUP* commands available on request). Included in the analysis were lux-rib<sup>1</sup> of <i>P. leiognathi</i> ATCC 25521<sup>T</sup>, the lux-rib operon of <i>P. mandapamensis</i> ATCC 27561<sup>T</sup>, and, as an outgroup, the lux-rib operon of <i>P. kishitanii</i> ATCC BAA-1194<sup>T</sup> (a.k.a. <i>pjapo</i>.1.1<sup>T</sup>; 4). Omitted from the analysis were non-coding intergenic spacer regions, the luxF gene in <i>P. mandapamensis</i> and <i>P. kishitanii</i>, and the first 341-bp of luxC sequence, which is not available for the lux-rib<sup>2</sup> operons of lelon.2.1 and lnuch.21.1 (Fig. 1). The resulting aligned lux-rib<sup>1</sup> and lux-rib<sup>2</sup> coding regions of each strain, 8732 nucleotides, were analyzed in a progressive 200 nucleotide window that shifted 50 nucleotides with each analytical run (171 200-character partitions and one partition of 182 characters). Each partition was subjected to an exhaustive search using PAUP*, with gaps treated as a fifth nucleotide. Each phylogenetic analysis resulted in up to four equally parsimonious trees, yielding a total of 258 trees. The topology of each tree was then compared with that predicted from phylogenetic analysis of entire lux-rib<sup>1</sup> and lux-rib<sup>2</sup> operons.

Six of the 172 partitions yielded an incongruent tree, and the alignments of these partitions were then visually inspected. Five of the incongruent topologies (four overlapping partitions within the luxA-luxB region and one in luxD) were ascribed to a lack of character change rather than recombination, whereas one showed a possible recombination event between the luxB<sup>1</sup> and luxB<sup>2</sup> genes of strain lnuch.21.1 (see Results).

Screening for lux-rib merodiploidy. In addition to lnuch.13.1, lelon.2.1, lnuch.21.1, and ATCC 25521<sup>T</sup>, one hundred seventy strains of <i>P. leiognathi</i>, isolated from various geographic locations (see Fig. 5; supplemental material), were assayed for the presence of single or multiple lux-rib operons using luxA PCR amplification and sequencing. The PCR primers were those that detected multiple luxA sequencing chromatogram peaks in <i>P. leiognathi</i> lnuch.13.1, lelon.2.1, and lnuch.21.1. The resulting amplicons were then sequenced and the resulting chromatograms examined for single or multiple luxA sequence peaks.

GenBank accession numbers and sources of sequence data. GenBank accession numbers for DNA sequence data obtained or used in this study are listed in Table 2. Other sources of DNA sequence
data used in the analysis shown in Fig. 1 are: *P. leiognathi* ATCC 25521<sup>T</sup>, *luxCDABEG* (28), *ribEBHA* (27); *P. mandapamensis* PL-741, *lumP*, *luxD* (9), *luxC* (29), *lumQ* (30), *putA* (31), *luxE* (32), *luxG* (33), *ribEBHA* (34); *P. mandapamensis* 554, *luxABF* (22), *lumP* to *luxC* (unpublished GenBank submission, Illarianov, 1991); *P. angustum* SKA34 (http://www.moore.org/microgenome/detail.aspx?id=36); and, *P. angustum* S14 (http://www.moore.org/microgenome/detail.aspx?id=8). Annotation of open reading frames (ORFs) for *P. angustum* SKA34 and S14 genome sequences in GenBank as of April 2007, including gene identifying number, cluster of orthologous groups (COG) number, and COG inferred protein group are: ORF1, SKA34_07688 and VAS14_15429, COG1566, hypothetical multi-drug resistance efflux pump; ORF2, SKA34_07693 and VAS14_15434, COG3158, hypothetical potassium ion transporter (COG identification applies to SKA34 only); ORF5 (found in SKA34 only), SKA34_07678, hypothetical protein; ORF6, SKA34_07683 and VAS14_15424, COG2207, hypothetical transcriptional regulator, AraC/XylS family protein. ORF3 and ORF4 are hypothetical bacterial transposases and are not homologous to any sequences proximal to the *lux-rib* regions of any other strains examined here.
RESULTS

Multiple luxA genes in P. leiognathi. The sequencing chromatograms of luxA PCR amplicons of some P. leiognathi strains contained multiple discrete peaks. Exhaustive attempts to eliminate these anomalies through attempts to identify possible sources of DNA contamination were unsuccessful, which suggested that some strains carry multiple luxA genes. To test this possibility, we cloned the luxA PCR amplicon from lnuch.13.1, a strain that showed multiple chromatogram peaks. Individual recovered clones were found to carry one or the other of two different luxA sequences, which demonstrated the presence of two luxA genes in lnuch.13.1.

Identification of two complete luxCDABEG-ribEBHA operons. PCR and sequence analysis of lnuch.13.1 genomic DNA revealed that other lux genes were associated with each luxA gene, which suggested that some or all of the lux operon, luxCDABEG, might be present in two copies. Despite a concerted effort based on PCR and sequence analysis, however, we were unable to find a close physical linkage between the two putative lux operons, as might have resulted from a tandem duplication of all or part of the lux operon. This observation suggested that if two operons were present, they were not closely associated. To test this possibility and to define the gene content, organization, and sequence of each putative lux operon, we constructed a fosmid library of lnuch.13.1 genomic DNA. Sequence-based screening of the recovered fosmid clones identified two, B7-25 and C30-24, with different lux gene sequences. For both clones, the region containing and immediately flanking the lux operon was sequenced (see supplemental material for PCR primers and sequencing strategy).

Both clones contained a complete luxCDABEG operon (Fig. 1). Furthermore, luxC was preceded in both by the lumQ gene. A short region with homology to part of lumP also was identified in B7-25 but was absent from C30-24. The ribEBHA genes also were present downstream of luxG in both clones. The individual lux and rib genes were of the same lengths in both clones, but the lengths of the individual intergenic spacer regions varied, especially in the AT-rich region between lumQ and luxC (Fig. 1). These results establish the presence of two distinct luxCDABEG-ribEBHA operons in P. leiognathi lnuch.13.1,
designated here as lux-rib\(^1\) (from fosmid B7-25) and lux-rib\(^2\) (from fosmid C30-24). Fosmid maps with additional details are presented in the supplemental material. In addition to having the same gene content and organization, the two operons are of similar length, approximately 11 and 11.5 kb, respectively, their lux and rib gene coding regions are approximately 90% identical in nucleotide sequence overall, and the genes of both operons are complete and translatable. This is the first example of multiple lux-rib operons (or multiple lux genes or multiple rib genes) in a luminous bacterium.

**Chromosomal locations of lux-rib\(^1\) and lux-rib\(^2\).** The absence in \(\text{lnuch.13.1}\) of a plasmid large enough to account for either fosmid clone B7-25 or C30-24 (Materials and Methods) suggested that both lux-rib operons were likely to be chromosomal. To test this possibility, we examined genomic DNA of \(\text{lnuch.13.1}\) using PFGE and PCR amplification. Analysis of undigested genomic DNA demonstrated the presence of two apparently circular chromosomes in \(\text{lnuch.13.1}\), one larger than the other (data not shown), as reported previously for \(P.\) leiognathi and other examined members of \(Vibrionaceae\) (40). Digestion of \(\text{lnuch.13.1}\) genomic DNA with restriction endonuclease NotI yielded seven fragments, which ranged in size from approximately 1500 kb to 220 kb (Fig. 2). The genome size of \(\text{lnuch.13.1}\) was therefore estimated to be approximately 5.3 megabases. The seven NotI digestion fragments were purified and used as templates for PCR amplification of each of the two lux-rib operons, with primers for a luxD-luxA region that is specific in sequence to each operon. The lux-rib\(^1\)-specific primers amplified a product only from fragment NotI-g, whereas the lux-rib\(^2\)-specific primers amplified a product only from fragment NotI-b (Fig. 2). Sequence analysis confirmed that the two amplification products were luxD-luxA of lux-rib\(^1\) and of lux-rib\(^2\), respectively. These results demonstrate that the two lux-rib operons of \(\text{lnuch.13.1}\) are located on different NotI chromosomal digestion fragments. Given the sizes of the fosmid clones B7-25 and C30-24 and the lack of DNA in common to them, the two lux-rib operons are separated at least by 9 kb of DNA.

Consistent with these observations, DNA flanking lux-rib\(^1\) in \(\text{lnuch.13.1}\) was distinct from that flanking lux-rib\(^2\) (Fig. 1). For lux-rib\(^1\), the putA gene was present upstream of lumQ, and a gene for a hypothetical multidrug resistance efflux pump (ORF1) was present downstream of ribA. In
*Photobacterium angustum* strains SKA34 and S14, which lack *lux* genes, *putA* and the gene for ORF1 are present, and they are near each other, separated by less than 2 kb of DNA; this DNA codes for two unidentified ORFs that are unrelated to *lux* or *rib* genes (Fig. 1). As in *P. leiognathi*, the *lux-rib* genes in *P. mandapamensis* are flanked by *lumP*, *lumQ*, and *putA*, and by the gene for ORF1 (Fig. 1).

In contrast, the DNA flanking *lux-rib*² of *lnuch.13.1* contained neither *putA* upstream of *luxC* nor the gene for ORF1 downstream of *ribA*. Instead, homologs of putative bacterial transposase genes flanked *lux-rib*². Specifically, the region upstream of *luxC* in *lux-rib*² contained a sequence homologous to a putative transposase gene identified from the genome of *Photobacterium profundum* SS9, and the region downstream of *ribA* in *lux-rib*² contained a homolog to bacterial transposase genes of the IS66 family (Fig. 1). These results establish that different, non-homologous DNA flanks *lux-rib*¹ and *lux-rib*².

**Presence and chromosomal locations of multiple *lux-rib* operons in other strains of *P. leiognathi***. Using the information from *lnuch.13.1*, we next asked if other strains of *P. leiognathi* carry the two *lux-rib* operons and if the chromosomal locations of the operons were the same as in *lnuch.13.1*. We examined in detail two strains, *lelon.2.1* and *lnuch.21.1*, that like *lnuch.13.1* showed multiple *luxA* chromatogram peaks, and one strain, ATCC 25521T, the sequenced *luxA* amplicon of which consistently exhibited only single peaks. Primers based on *lnuch.13.1* *lux-rib*¹ and *lux-rib*² were used for amplification of *lux-rib* sequences from genomic DNA of these strains. Two different *luxA* sequences were identified in *lelon.2.1* and *lnuch.21.1*, indicating the presence of two *lux-rib* operons, whereas only a single *luxA* sequence was identified in ATCC 25521T. Complete sequencing of the *lux-rib* operons from *lelon.2.1* and *lnuch.21.1* (see supplemental material for PCR primers and sequencing strategy) demonstrated the presence of two distinct *luxCDABEG-ribEBHA* operons in each of these strains (Fig. 1). As in *lnuch.13.1*, the genes of both operons in *lelon.2.1* and *lnuch.21.1* are, with one exception, intact and translatable. The exception is the *luxC* gene in *lux-rib*² of *lelon.2.1*, which has a single nucleotide deletion; the deletion results in a translational stop codon approximately mid-way into the gene (Fig. 1). From DNA of ATCC 25521T, however, no amplicon was generated by any primers based on sequences of *lux-rib*² of *lnuch.13.1*. These PCR and sequencing chromatogram results demonstrate that both *lux-rib*¹
and lux-rib\(^2\) are present in some strains of \(P.\) leiognathi, e.g., \(lnuch.13.1,\) \(lelon.2.1,\) and \(lnuch.21.1,\) whereas other strains, e.g., ATCC 25521\(^\text{T}\), apparently have only \(lux-rib\(^1\).\) These results also establish that the presence of multiple \(lux\) chromatogram peaks, versus single \(lux\) chromatogram peaks, provides a reliable indication of the presence of multiple versus single \(lux-rib\) operons in \(P.\) leiognathi.

For \(lelon.2.1,\) \(lnuch.21.1,\) and ATCC 25521\(^\text{T}\), the order and orientation of genes flanking \(lux-rib\(^1\)\) were found to be the same as in \(lnuch.13.1.\) Specifically, the DNA upstream of \(luxC\) contained a short region with some homology to \(lumP,\) followed by \(lumQ\) and \(putA,\) and the region downstream of \(ribA\) contained the gene for ORF1 (Fig. 1). These results demonstrate positional homology of \(lux-rib\(^1\) in the four tested strains of \(P.\) leiognathi. In contrast, positional homology of the \(lux-rib\(^2\) operons in \(lnuch.13.1,\) \(lelon.2.1,\) and \(lnuch.21.1\) could not be demonstrated. Attempts to amplify the regions upstream of \(luxC\) and downstream of \(ribA\) in \(lelon.2.1\) and \(lnuch.21.1,\) using primers based on the sequences from fosmid clone C30-24 (\(lnuch.13.1,\) \(lux-rib\(^2\)), failed to produce the predicted products. Therefore, either the chromosomal locations of \(lux-rib\(^2\) or the sequences flanking \(lux-rib\(^2\) differ in these three strains.

**Evolutionary origin of \(lux-rib\(^2\).** With the complete sequences of \(lux-rib\(^1\) from \(lnuch.13.1,\) \(lelon.2.1,\) \(lnuch.21.1,\) and ATCC 25521\(^\text{T}\), and of \(lux-rib\(^2\) from \(lnuch.13.1,\) \(lelon.2.1,\) and \(lnuch.21.1,\) we were in a position to define the extent of phylogenetic divergence between genes of the two operons. We hypothesized that \(lux-rib\(^2\), which is flanked by putative bacterial transposase genes in \(lnuch.13.1,\) was acquired by lateral gene transfer from another species of luminous bacteria.

To test this hypothesis and gain insight into the evolutionary origin of \(lux-rib\(^2\), we carried out a parsimony analysis of \(lux-rib\(^1\), \(lux-rib\(^2\), and \(lux\) and \(rib\) genes of other species of luminous bacteria in Vibrionaceae, Shewanellaceae, and Enterobacteriaceae. Instead of a close relationship between \(lux-rib\(^2\) and the \(lux\) and \(rib\) genes of one of these other species of bacteria, we found that \(lux-rib\(^2\) was most closely related to \(lux-rib\(^1\) of \(P.\) leiognathi (Fig. 3). These results rule out lateral transfer from another bacterial species as the origin of \(lux-rib\(^2\) in \(P.\) leiognathi. Instead, they indicate that \(lux-rib\(^2\) originated from within \(P.\) leiognathi. Separate phylogenetic analysis of each \(lux\) and \(rib\) gene resolved the same relationships as those in Fig. 1 (see the supplemental material).
This analysis also revealed that the lux-rib\(^2\) operons of \textit{lnuch}.13.1, \textit{lelon}.2.1, and \textit{lnuch}.21.1 are very closely related to each other, as are the lux-rib\(^1\) operons of these strains and of ATCC 25521\(^T\) (Fig. 3). Therefore, although lux-rib\(^1\) and lux-rib\(^2\) are distinct phylogenetically from each other, each has diverged very little among the different strains carrying them. It should be noted in this regard that the merodiploid strains were collected from different geographic locations and as much as twenty years apart (Table 1).

**Recombination between lux-rib\(^1\) and lux-rib\(^2\).** The presence in strains of \textit{P. leiognathi} of two complete lux-rib operons with a high level of sequence identity presumably would provide opportunities for recombination. We therefore tested for recombination between lux-rib\(^1\) and lux-rib\(^2\) by carrying out a partition congruence analysis on the sequences of the aligned lux-rib\(^1\) and lux-rib\(^2\) coding regions of \textit{lnuch}.13.1, \textit{lelon}.2.1 and \textit{lnuch}.21.1. Analysis of the partition trees revealed that nearly all of them, 166 of 172, were topologically congruent, indicating no detectable recombination between the two operons. Six partitions did exhibit incongruence, but for five of these, the incongruence could be ascribed to a lack of character change rather than recombination. One partition, however, for the luxB\(^1\) and luxB\(^2\) genes in strain \textit{lnuch}.21.1, showed evidence consistent with recombination, with a portion of luxB\(^1\) apparently exchanged for luxB\(^2\) sequence (Fig. 4). These results indicate that, despite the extensive homology, recombination between genes of the lux-rib\(^1\) and lux-rib\(^2\) operons of merodiploid strains of \textit{P. leiognathi} apparently occurs only rarely.

**Non-random geographic distribution of merodiploid \textit{P. leiognathi}**. We noted that the collection locations of lux-rib merodiploid strains, \textit{lnuch}.13.1, \textit{lelon}.2.1, and \textit{lnuch}.21.1, all from Honshu, Japan, differed from the collection location, the Gulf of Thailand, of ATCC 25521\(^T\), which carries only lux-rib\(^1\). This difference suggested that strains bearing multiple lux-rib operons might have a non-random geographic distribution. To test this possibility, we examined 170 additional strains of \textit{P. leiognathi} collected from various locations from: Honshu, Shikoku, and Okinawa, Japan; Taiwan the Philippine Islands; and the Gulf of Thailand (Fig. 5; Supplemental Table 2). These strains were tested for single or multiple lux-rib operons using the primers that revealed multiple luxA chromatogram peaks for
*lnuch*.13.1, *lelon*.2.1, and *lnuch*.21.1. One hundred five of the additional strains were found to carry a single *lux-rib* operon, whereas 65 of the additional strains carried multiple *lux-rib* operons. Strains bearing a single *lux-rib* operon were obtained throughout the geographic sampling range, but strains bearing multiple *lux-rib* operons were found only in coastal waters of central Honshu (Fig. 5). Thus, despite an extensive sampling, we were unable to find *P. leiognathi* strains bearing multiple *lux-rib* operons south of Honshu, Japan. Therefore, *lux-rib* merodiploid strains have a geographic distribution that apparently is limited to coastal waters of Honshu. This is the first indication that a natural merodiploid state in a bacterial species can correlate with geography.
DISCUSSION

Certain strains of *P. leiognathi*, a coastal marine luminous bacterium, carry two intact and apparently functional *luxCDABEG-ribEBHA* operons. Merodiploidy extends to an eleventh gene, *lumQ*, which is closely associated with but transcribed divergently from the *lux-rib* operons. The two *lux-rib* operons are distinct in sequence and chromosomal location. One operon, *lux-rib*\(^1\), is flanked by *putA* and the gene for a hypothetical multidrug resistance efflux pump (ORF1). Positional homology of *lux-rib*\(^1\) in strains of *P. leiognathi*, of the *lux-rib* operon of *P. mandapamensis*, and of flanking DNA in *P. angustum* suggest that the site between *putA* and the ORF1 gene is the ancestral chromosomal location of the *lux-rib* operon in *P. leiognathi* and *P. mandapamensis*. The second operon, *lux-rib*\(^2\), is present in many but not all strains of *P. leiognathi*, and it is flanked by genes specifying transposases, not by *putA* and the ORF1 gene.

The *lux-rib* merodiploidy reported here is the first example of multiple copies of *lux* or *rib* genes in a luminous bacterium. Natural merodiploidy is not uncommon in bacteria, but it usually involves individual genes or small genetic regions and is thought to arise typically by tandem duplication (e.g., 1, 19, 45, 54). The actinomycete *Actinomadura* sp., for example, carries on the same 3 kb genomic DNA fragment two *rpoB* genes that are 93% identical; both copies are stably inherited without recombination (50). It is known also that many bacteria carry multiple copies of the *rrn* operon, with the number of operons apparently corresponding to the rate of response to resource availability (24). The situation in *P. leiognathi*, however, differs markedly and apparently is unique; the two *lux-rib* operons are well separated, each contains 11 genes, and merodiploidy apparently did not arise by a tandem duplication of one of the operons. Furthermore, *lux-rib* merodiploidy is common in *P. leiognathi*, having been detected in 68 of 174 tested strains, but the merodiploid state is not found in all strains of this species.

The presence of transposase genes flanking *lux-rib*\(^2\) indicates a potential for this operon to transfer among *P. leiognathi* strains and to other species. Despite the potential for mobility, *lux-rib*\(^2\) apparently was not acquired by *P. leiognathi* by lateral transfer from another species of luminous bacteria (Fig 3). The two operons are phylogenetically distinct but are more closely related to each other than
either is to the *lux* or *rib* genes of any other known species of luminous bacteria. The evolutionary
divergence between *lux-rib*\(^1\) and *lux-rib*\(^2\), which is evident both at the level of the whole operon and at the
level of individual genes (Fig. 3; supplemental material), demonstrates also that *lux-rib*\(^2\) is not likely to
have arisen by a recent duplication of *lux-rib*\(^1\). Furthermore, the merodiploid state appears to be stable;
more than 20 years separate the collection of merodiploid strains of *P. leiognathi* from the environment
(Table 1; supplemental material) with little sequence divergence over that time for either operon. We
found also that an extended period of growth of *lnuch*.13.1 in continuous culture (approximately 400
generations) did not lead to loss or altered chromosomal location of *lux-rib*\(^2\) (data not shown). It is
possible therefore that *lux-rib*\(^2\) arose in the distant past within, and was acquired by transposon-mediated
transfer from, a lineage of *P. leiognathi* that either has not yet been sampled or has gone extinct. If borne
out by future studies, this scenario would represent the first documented instance of intra-specific transfer
of the *lux-rib* genes in luminous bacteria. With respect to phylogenetic analysis based on *lux* and *rib*
genes, the presence of two distinct *lux-rib* operons in certain strains of *P. leiognathi* adds complexity to
analyses based on these genes, but it does not prevent discrimination of this species from other luminous
bacteria.

The putative transposases flanking *lux-rib*\(^2\) show similarity to IS elements characteristic of some
plasmids (10) and pathogenic islands (20) in *Vibrionaceae*. In this regard, Rajanna et al. (42) have
proposed that the *V. cholerae* pathogenicity island (VPI) is excised from the chromosome and transferred
between cells as an extrachromosomal circular molecule, thereby spreading VPI-encoded genes among
strains. One possibility therefore is that *lux-rib*\(^2\) was transferred among some strains of *P. leiognathi*,
possibly during light organ symbiosis with fishes and squids, where bacterial population densities are
high, by a mechanism similar to that involved in the transfer of pathogenic islands. However,
determining the frequency, mechanism, and conditions under which *lux-rib*\(^2\) is transferred will require
much additional work. Given the potential for mobility of *lux-rib*\(^2\), we also cannot exclude the possibility
that some strains of *P. leiognathi* carry more than two *lux-rib* operons.
The presence of multiple copies of the lux-rib operon in a cell presumably provides opportunities for recombination and for the accumulation of non-sense mutations in the duplicate genes. These events presumably could lead to changes in the gene content and gene order of the lux operon of P. leiognathi and possibly to the evolution of novel functions in the duplicate genes. Recombination between the genes of lux-rib\(^1\) and lux-rib\(^2\), however, apparently is rare. Only one apparent instance of possible recombination, between the luxB\(^1\) and luxB\(^2\) genes in strain lnuch.21.1, was identified (Fig. 5). Similarly, mutations leading to loss of function in the genes of either lux-rib operon are remarkably infrequent.

With a single exception, all the genes of both operons in lnuch.13.1, lelon.2.1, and lnuch.21.1 are intact and translatable. This retention of functionality suggests the possibility of selection for the physiological function of the genes of both lux-rib operons. In this regard, it is known that selection can protect both copies of duplicated genes under conditions in which expression benefits the cell (25) and that selection for increased levels of gene products can lead to retention of duplicated genes (e.g., 54). The number of genes involved and the substantial energetic costs associated with lux gene expression and the activity of Lux and Rib proteins (e.g., 13) argue for this selection hypothesis.

Increases in gene copy number would be expected to increase the cellular levels of the protein products of those genes (45). The presence of two functional lux-rib operons in merodiploid P. leiognathi therefore presumably would result in higher cellular levels of the Lux and Rib proteins than in strains carrying a single operon. Possibly reflecting lux-rib merodiploidy, two different luciferases, one soluble and the other apparently associated with the cytoplasmic membrane, were reported many years ago for P. leiognathi strain S-1 (5, 7). Whether retention of the lux-rib merodiploid state is subject to selection, however, and what the possible physiological benefits may be for cells producing higher levels of Lux and Rib proteins are not obvious at this time. The limited geographic distribution of lux-rib merodiploid strains (see below) is intriguing in this regard and suggests a possible connection with environmental conditions in coastal waters of Honshu, Japan, possibly the colder winter water temperatures there (49) compared to warmer coastal areas south of Honshu. Molecular genetic and biochemical analyses will be needed to determine whether both operons are actually expressed, whether their expression may be
regulated differently, and what the effects on cellular levels of Lux and Rib proteins may be. At this time, essentially nothing is known about the regulation of luminescence in \textit{P. leiognathi} except that tested strains exhibit a population density responsive induction of luminescence and luciferase synthesis that does not appear to be dependent on acyl-homoserine lactones (11). The demonstration here of multiple \textit{lux-rib} operons in certain strains of \textit{P. leiognathi} provides the genetic foundation for analysis of these issues.

The non-random geographic distribution of merodiploid \textit{P. leiognathi} strains (Fig. 5) is unexpected. Bacterial distributions in nature are widely considered to be cosmopolitan, although non-random geographic patterns, especially in the presence of physical barriers to dispersal, have been identified (e.g., 41, 44, 46, 51, 52). In the case of \textit{P. leiognathi}, which exists in the fluid marine environment, no physical barrier to dispersal is evident, yet our extensive sampling has found merodiploid strains only in coastal waters of central Honshu. One explanation for the non-random distribution of merodiploid strains is simple historical contingency (36); the merodiploid state, assuming it arose relatively recently in cells in coastal waters of central Honshu, may not have had sufficient time to disperse to the south. Northward flow of the Kuroshio Current (49) might delay or prevent southerly dispersal of merodiploid strains. Alternatively, strains carrying multiple \textit{lux-rib} operons might have a physiological advantage in coastal waters of Honshu, as mentioned above, thereby allowing them to persist despite the presence of strains that carry only \textit{lux-rib} \textsuperscript{1}. Much additional work will be needed to test these notions and determine the possible environmental conditions involved in the maintenance and limited geographic distribution of \textit{lux-rib} merodiploid strains of \textit{P. leiognathi}.
ACKNOWLEDGEMENTS

We thank the following individuals for assistance in field collection work: S. Kimura, Y. Tachibana, and the captain and crew of the T/S Seisui-maru, Mie University, Japan; T. Adachi, Fukui Prefectural Fisheries Experimental Station, Japan; A. Fukui, Tokai University, Japan; C. Lavilla-Pitogo, SEAFDEC, Philippine Islands; J. Ledesma, Tigbauan, Philippine Islands; T. Yoshino and H. Ishimori, University of the Ryukyus, Japan; Y. Haneda (deceased), Yokosuka City Museum, Japan; and Y. Machida and H. Endo, Kochi University, Japan. K. Davis, A. Kaeding, and A. Gorog, University of Michigan, provided technical assistance, and A. Kondrashov provided helpful advice. DNA sequencing was carried out by staff of the University of Michigan Sequencing Core.

This work was supported by grant DEB 0413441 from the National Science Foundation.
REFERENCES


Mikrobiol. 94:283-330.


Table 1. Bacterial strains used in this study.

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain</th>
<th>Habitat, geographic source, isolation year</th>
<th>Reference</th>
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<td><em>Photobacterium leiognathi</em></td>
<td>ATCC 25521&lt;sup&gt;T&lt;/sup&gt;</td>
<td>LO&lt;sup&gt;a&lt;/sup&gt;, Gulf of Thailand, ca. 1967</td>
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<td></td>
<td><em>lelon</em>.2.1</td>
<td>LO, Sagami Bay, Honshu, Japan, 1983</td>
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<td><em>Inuch</em>.13.1</td>
<td>LO, Wakasa Bay, Honshu, Japan, 2003</td>
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<td><em>Inuch</em>.21.1</td>
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<td><em>Photobacterium kishitanii</em></td>
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<td>LO, Sagami Bay, Honshu, Japan, 1982</td>
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<td><em>Photobacterium mandapamensis</em></td>
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<td>PL-721</td>
<td>FS&lt;sup&gt;a&lt;/sup&gt;, Sulu Sea, 1975</td>
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<sup>a</sup> LO, light organ of fish; SW, seawater; FS, skin of marine fish.
Table 2. GenBank accession numbers for DNA sequences used in this study.

<table>
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Photorhabdus luminescens

subsp. laumondii   TTO1   luxCDABE and ribE, ribB,

subsp. luminescens ATCC 29999T   luxCDABE   M90093

Shewanella

hanedai   ATCC 33224T   luxCDABEG   AB058949

woodyi   ATCC 51908T   luxA   DQ322063

Vibrio

cholerae   NCIMB 41T   luxCDABE   AB115761

fischeri   ES114   luxCDABE   NC_006841

ribEBH   NC_006840

MJ-1   luxCDABE   AF170104

harveyi   B-392   luxD   J03950

luxAB   M10961

luxE   M28815

luxG–ribB   M27139f

logei   ATCC 29985T   luxAB   EF576941

orientalis   ATCC 33934T   luxDA   AB058948

salmonicida   NCIMB 2262T   luxCDABEG   AF452135

splendidus   ATCC 33125T   luxAB   EF536342
a The designation lux-rib indicates the luxCDABEG-ribEBHA genes, and lux-rib\(^1\) and lux-rib\(^2\)
indicate luxCDABEG-ribEBHA operon-1 and operon-2, respectively, of P. leiognathi (see text).

b P. angustum and P. profundum do not have lux genes; these sequences were used to
demonstrate homology to sequences found flanking the lux-rib\(^1\) operon.

c Sequence from fosmid B7-25.

d Sequence from fosmid C30-24.

e luxF is designated as luxG in this record.

f ribB is designated as luxH in this record.
Figure Legends

**Fig. 1.** Gene organization of the *lux-rib*<sup>1</sup> and *lux-rib*<sup>2</sup> operons of *P. leiognathi*. Genes and spacer regions are drawn to scale. Arrows above genes indicate direction of transcription. Dashed rectangles between *lumQ* and *luxC* in *P. leiognathi* *lux-rib*<sup>1</sup> sequences indicate an approximately 200 bp region that is alignable to the *lumP* sequence of *P. mandapamensis*. Genes shaded grey have homologs in *P. leiognathi*, *P. mandapamensis*, and *P. angustum*. Small shaded rectangles outside of genes indicate non-coding intergenic sequences of *P. leiognathi* and/or *P. mandapamensis* that are alignable to sequences in *P. angustum* strains SKA34 and S14, including approximately 80 bp before the *P. leiognathi* *putA* start codon that aligns to sequence within *putA* in *P. angustum*. Genes shaded dark grey in *P. angustum* indicate regions (ORF5, ORF6, and 759 bp from the start codon of *putA*) that are not alignable with a gene or intergenic sequence in *P. leiognathi* or *P. mandapamensis*. Double hash marks in the *P. angustum* sequences indicate contiguous sequences that have been separated to indicate the position of the *lux-rib* operon in *P. leiognathi* and *P. mandapamensis*. Blank regions in *P. mandapamensis* strains indicates that the sequence is not available; dashed lines in *P. mandapamensis* ATCC 27561<sup>T</sup> indicate regions where the DNA was amplified in this study but not sequenced. The hashed rectangles indicate ORFs (ORF3 and ORF4) flanking the *lux-rib*<sup>2</sup> operon of fosmid C30-24 that are apparently homologous to bacterial transposases (see text). In *lux-rib*<sup>2</sup> of *P. leiognathi lelon*.2.1, a dashed vertical line in the region homologous to *luxC* indicates the site of a single nucleotide deletion that causes a frameshift of *luxC* in this strain, resulting in a stop codon 18 codons later. See the supplemental material for complete maps of the fosmids.

**Fig. 2.** PFGE analysis of the chromosomal locations of *lux-rib*<sup>1</sup> and *lux-rib*<sup>2</sup>. A. *NotI* digestion of *lnuch*.13.1 genomic DNA. B. PCR amplification using *lux-rib*<sup>1</sup>–specific primers (luxDfor1 and luxArevsec#3, supplemental material) with genomic fragments *NotI*-a through *NotI*-g as templates. C. PCR amplification using *lux-rib*<sup>2</sup>–specific primers (luxDfor1 and luxArevprim#3, supplemental material)
and genomic fragments NotI-a through NotI-g as templates. Genomic DNA of lnuch.13.1 was used as a positive control (data not shown).

**Fig. 3.** Phylogenetic hypothesis of relationships among luminous bacteria based on lux and rib gene sequences. The tree is unrooted because bacterial lux genes are present only in members of Vibrionaceae, Shewanellaceae, and Enterobacteriaceae; no outgroup bearing the lux genes is known. The total number of aligned nucleotide positions in the dataset is 11021; exclusion of non-coding spacer regions and parsimony-uninformative characters results in 4745 nucleotides for analysis. The single most parsimonious hypothesis is shown (length = 13739, consistency index = 0.615, retention index = 0.712). Numbers at nodes are jackknife-resampling values. The two lux-rib operons from P. leiognathi strains lnuch.13.1, lelon.2.1, and lnuch.21.1 (circled with dashed line) have distinct sequences, but are each other's closest relatives (circled inset below main figure; the asterisk indicates that the jackknife value is for the same branch on each part of the figure). The primary lux-rib operon (lux-rib\(^1\)) is proximal to putA in all strains, whereas the secondary lux-rib operon (lux-rib\(^2\)) is flanked by putative bacterial transposases in strain lnuch.13.1. Phylogenetic analysis based on housekeeping genes (i.e., gapA, gyrB, recA, rpoA, and rpoD) of Photobacterium species yielded trees consistent with the lux-rib hypothesis shown here.

**Fig. 4.** Alignment of a region of luxB from lux-rib\(^1\) and lux-rib\(^2\) of P. leiognathi lnuch.21.1. A. Nucleotide alignment. B. Amino acid alignment. Asterisks highlight differences between the sequences; variable nucleotides are shown in bold.

**Fig. 5.** Map of Japan and Southeast Asia, showing the geographic origins of P. leiognathi strains bearing single or multiple lux-rib operons. Scale bar is approximately 500 km. Insert shows an enlarged map of the main islands of Japan (some landmasses were omitted for clarity). Locations: a) Sagami Bay, Kanagawa Prefecture, Honshu, Japan; b) Suruga Bay, Shizuoka Prefecture, Honshu, Japan; c) Wakasa Bay, Fukui Prefecture, Honshu, Japan; d) Ago Bay, Mie Prefecture, Honshu, Japan; e) Tosa Bay, Kochi
Prefecture, Shikoku, Japan; f) Nakagusuku Bay, Okinawa-honto, Okinawa Prefecture, Japan; g) Funauki Bay, Iriomote Island, Okinawa Prefecture, Japan; h) Taipei, Taiwan; i) Dahsi, Taiwan; j) Manila Bay, Luzon, Philippine Islands; k) Iloilo, Panay, Philippine Islands; l) Palawan, Philippine Islands; m) Gulf of Thailand. Numbers next to each location indicate the number of strains identified as bearing single (white area in circle) or multiple (gray area in circle) lux-rib operons.
**P. leiognathi lux-rib**

- *lnuch.13.1 (B7-25)*
- *lelon.2.1*
- *lnuch.21.1* (ATCC 25521\(^ \text{T}\))

**P. leiognathi lux-rib\(^2\)**

- *lnuch.13.1 (C30-24)*
- *lelon.2.1*
- *lnuch.21.1*

**P. angustum**

- *SKA34*
- *S14*

**P. mandapamensis**

- *ATCC 27561\(^ \text{T}\)*
- *PL-741*
- *554*