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Genomic polymorphism in symbiotic populations of *Photobacterium leiognathi*

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Summary

Photobacterium leiognathi **forms a bioluminescent symbiosis with leiognathid fishes, colonizing the internal light organ of the fish and providing its host with light used in bioluminescence displays. Strains symbiotic with different species of the fish exhibit substantial phenotypic differences in symbiosis and in culture, including differences in 2-D PAGE protein patterns and profiles of indigenous plasmids. To determine if such differences might reflect a genetically based symbiont-strain/host-species specificity, we profiled the genomes of** ***P. leiognathi*** **strains from leiognathid fishes using PFGE. Individual strains from 10 species of leiognathid fishes exhibited substantial genomic polymorphism, with no obvious similarity among strains; these strains were nonetheless identified as** ***P. leiognathi*** **by 16S rDNA sequence analysis. Profiling of multiple strains from individual host specimens revealed an oligoclonal structure to the symbiont populations; typically one or two genomotypes dominated each population. However, analysis of multiple strains from multiple specimens of the same host species, to determine if the same strain types consistently colonize a host species, demonstrated substantial heterogeneity, with the same genomotype only rarely observed among the symbiont populations of different specimens of the same host species. Colonization of the leiognathid light organ to initiate the symbiosis therefore is likely to be oligoclonal, and specificity of the** ***P. leiognathi*****/leiognathid fish symbiosis apparently is maintained at the bacterial species level rather than at the level of individual, genomotypically defined strain types.**

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Introduction

Photobacterium leiognathi forms a species-specific bioluminescent symbiosis with fishes of the family Leiognathidae (Teleostei: Perciformes). The bacterium, which is present in coastal waters and the open ocean (Reichelt and Baumann, 1973; Ruby and Nealson, 1978; Yetinson and Shilo, 1979; Ruby *et al*., 1980), colonizes the nascent circumesophageal light organ of the newly hatched juvenile leiognathid to initiate the symbiosis with each new host generation (Wada *et al*., 1999). The fish develops a dense culture of *P. leiognathi* within the light organ, supplying the bacteria with oxygen and nutrients for growth and luminescence (Harms, 1928; Haneda, 1940; 1950; Boisvert *et al*., 1967; Hastings and Mitchell, 1971; Reichelt *et al*., 1977; McFall-Ngai, 1983a; Dunlap, 1985). Growth of the bacterial population leads to the release of excess cells from the light organ into the gut tract of the fish, from which they are released into the seawater (Haneda, 1950; Ahrens, 1965; Bassot, 1975; Dunlap, 1985). The fish uses a system composed of reflectors, chromatophore-embedded light-organ shutters, transparent and reflective tissues of the gasbladder, and transparent bone, musculature and skin to control, direct and diffuse the intense blue-green bacterial light over its ventral surface (Harms, 1928; Arhens, 1965; Bassot, 1975; McFall-Ngai, 1983b; McFall-Ngai and Dunlap, 1983; Dunlap and McFall-Ngai, 1987). Functions of the bacterial light include camouflage illumination against bottom-dwelling piscivorous fishes and other forms of predator avoidance, attracting prey, schooling and sex-specific signalling (Hastings, 1971; Herring and Morin, 1978; McFall-Ngai, 1983b; McFall-Ngai and Dunlap, 1983; 1984; Dunlap and McFall-Ngai, 1987; McFall-Ngai and Morin, 1991; Woodland *et al*., 2002). Common in shallow tropical and subtropical waters from the east coast of Africa to islands of the mid-Pacific and from Japan to Australia, the Leiognathidae currently is represented by approximately 40 species in three genera, *Gazza*, *Leiognathus* and *Secutor* (e.g. Woodland *et al*., 2001; Froese and Pauly, 200; Kimura *et al*., 2003; J. S. Sparks and P. V. Dunlap, unpubl. data).

Across and within these genera, the light-organ systems of the fish differ anatomically. For example, species-specific differences in the size and shape of light organs between and within the genera have been described (Haneda and Tsuji, 1976; Dunlap and McFall-Ngai, 1984; McFall-Ngai and Dunlap, 1984; Kimura *et al*., 2003; J. S. Sparks and P. V. Dunlap, unpubl. data). Possibly reflecting these differences, *P. leiognathi* strains from different leiognathid fishes exhibit various phenotypic differences. Strains isolated from different host species produce different extracellular enzymes, utilize different growth substrates, or produce different levels of CuZn, Fe and Mn superoxide dismutases (Reichelt *et al*., 1977; Dunlap and Steinman, 1986). Differences have also been noted in the culturability, cell morphology, light production and oxygen uptake rates for *P. leiognathi* cells examined within or directly from the light organs of different leiognathid fishes (Haneda and Tsuji, 1976; Dunlap, 1985; P. V. Dunlap, pers. obs.). Although strain-specific variation in bacteriocuprein has been postulated to relate to the species, geographic source or sex of the host fish (Dunlap and Steinman, 1986), it presently is not known if the observed phenotypic differences are consistent traits of strains from a given species of host fish or whether they relate to symbiosis with individual host species. Nonetheless, these differences presumably reflect genetic differences between strains, and a consistent host species-specific pattern of genetic differences between strains would be strong evidence that the bacterium and fish have undergone a linked evolutionary divergence at the symbiont-strain/host-species level.

To gain insight into this possibility and to characterize the genetic diversity within *P. leiognathi*, we used genomic profiling with pulsed-field gel electrophoresis, to define the genomotypic structure of symbiotic populations from several species of leiognathid fishes from coastal waters of Japan and the Philippines. We report here an extensive genomic polymorphism in *P. leiognathi*, and we present evidence that, despite this polymorphism, specificity in the *P. leiognathi*–leiognathid fish association apparently is maintained at the bacterial species level rather than at the level of individual, genomotypically defined strain types.

Results

Proteomic and genomic polymorphism in P. leiognathi

Photobacterium leiognathi strains from different species of leiognathid fishes have been reported to exhibit various phenotypic differences in laboratory culture and in symbiosis (Haneda and Tsuji, 1976; Reichelt *et al*., 1977; Dunlap, 1985; Dunlap and Steinman, 1986). To gain an overview of the extent of phenotypic differences among strains, we used 2D polyacrylamide gel electrophoresis (2D PAGE, *Experimental procedures*) to analyse the proteomes of *lnuch*.1.1, *lrivu*.1.1 and *lelon*.1.1, isolated from light organs of *L. nuchalis*, *L. rivulatus*, and *L. elongatus*, respectively, from Japan. Visual comparison of the resulting gels revealed that *lnuch*.1.1 and *lrivu*.1.1 (Fig. 1), and *lelon*.1.1 (not shown), whereas producing many apparently identical proteins, each produced a large number of proteins not produced by the other strains, even though the strains were grown under identical conditions. These results, demonstrating that each strain produces several strain-specific proteins, affirm and extend earlier reports of phenotypic variation in *P. leiognathi*.

The proteomic and other, previously described, phenotypic differences are extensive, suggesting that genomic differences are likely to exist among these strains. Detection of such genomic differences would provide a means of rapidly distinguishing and tracking *P. leiognathi* strains independently from phenotypic traits, expression of which in laboratory culture may be variable and for which no link to symbiosis has been established. A genomic approach, by allowing the presence of individual strain types to be identified and monitored in symbiotic populations within and across host specimens and species, would allow the question of specificity between different strains and individual species of the host fish to be addressed in a rigorous manner.

Therefore, to test for genomic differences, we first examined *lnuch*.1.1, *lrivu*.1.1 and *lelon*.1.1 for the presence of indigenous, low molecular weight plasmids, using standard plasmid extraction and separation procedures. Plasmid profiling has been used in various studies (e.g. Liebana *et al*., 2002) to identify and monitor bacterial strains. DNA bands of a size consistent with plasmids were detected for *lnuch*.1.1 and *lrivu*.1.1, ranging from approximately 1.5 kb to more than 23 kb (Fig. 2). Like the proteomic profiles, the plasmid profiles of these strains were distinctly different. Routine subculture of *lnuch*.1.1 did not lead to changes in its plasmid profile, indicating that the plasmids are stable under normal laboratory growth conditions, whereas subculture in the presence of acridine orange led to the loss of certain bands (not shown). In contrast to *lnuch*.1.1 and *lrivu*.1.1, however, *lelon*.1.1 apparently carried no detectable plasmids, giving a plasmid-null profile (Fig. 2). A more extensive analysis revealed that many strains of *P. leiognathi*, even when examined shortly after isolation from light organs of leiognathid fishes, to minimize subculturing, lack detectable plasmids (P. V. Dunlap, unpubl. data). The lack of plasmids in various strains of *P. leiognathi* precludes the use of plasmid profiling as a means of rapidly distinguishing and identifying strains in this species.

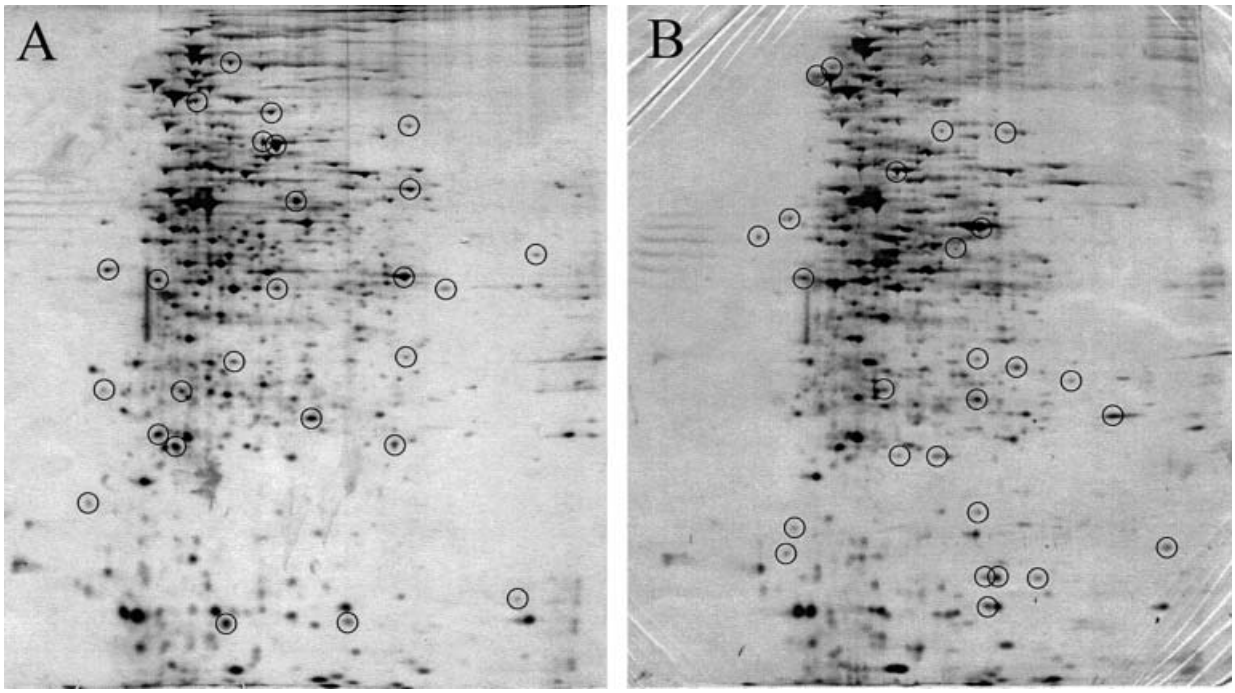

Fig. 1. 2D PAGE of total cellular proteins of *P. leiognathi*. A, *lnuch*.1.1 from *L. nuchalis*; B, *lrivu*.1.1 from *L. rivulatus*. Cells were grown under identical conditions to the same density and their total cellular proteins were then extracted, separated on two dimensions and stained as described in Callahan and Dunlap (2000). Circles indicate visually identified proteins produced by one strain that were not seen for the other strain.

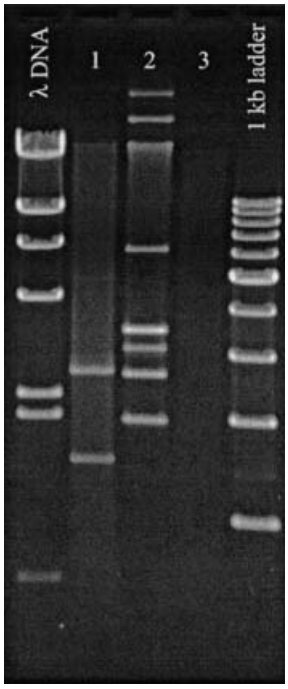

Fig. 2. Plasmid profiles of *P. leiognathi*. Indigenous plasmid DNA of *lnuch*.1.1 from *L. nuchalis*, lane 1; *lrivu*.1.1 from *L. rivulatus*, lane 2; and *lelon*.1.1, from *L. elongatus*, lane 3, was separated and visualized by standard methods (*Experimental procedures*). DNA size standards: λ DNA cut with *Hin*dIII (left); and DNA ladder in increments of 1 kb (right).

To circumvent this problem, we used genomic profiling with pulsed-field gel electrophoresis (PFGE). Pulsed-field gel electrophoresis is a robust method of strain typing that avoids many of the problems associated with other methods of strain typing (e.g. Olive and Bean, 1999; Gürtler and Mayall, 2001). As an initial assessment of genomic diversity in *P. leiognathi* and the applicability of PFGE for typing strains of this species, the genomes of 10 strains, the three reported on above from Japan and seven additional strains, isolated from *G. minuta*, *L. bindus*, *L. equulus*, *L. fasciatus*, *L. jonesi*, *L. leuciscus* and *L. stercorarius* from the Philippines (Table 1), were profiled. Genomic DNA of each strain was digested with restriction endonuclease *Sfi*I, and the resulting DNA fragments were separated by PFGE (*Experimental procedures*). A well-defined fingerprint, or genomic profile, composed of a few to several well-resolved bands, was obtained from the DNA of each strain (Fig. 3). Repeated analysis of each strain consistently gave the same number, sizes and intensities of DNA bands. The genomic profile, i.e. genomotype, of each strain was entirely distinct, with no or very few bands of identical size in common to any two strains. These results demonstrate the presence of substantial genomic polymorphism among *P. leiognathi* strains. Different species of leiognathid fishes harbour genomotypically distinct strains.

Table 1. Leiognathid host species, geographic origin and genomotypes of *P. leiognathi* strains.

Leiognathid species	Specimen	Geographic origin	Date of collection	Strains examined	Genomotypes and number		Genomotype index strain[a]
Gazza achlamys	*Gachl*.1	Iloilo[b]	07/2000	30	Ga-Ilo-01	23	*gachl*.1.1
					Ga-Ilo-02	6	*gachl*.1.3
					Ga-Ilo-03	1	*gachl*.1.23
	Gachl.2	Iloilo	07/2000	20	Ga-Ilo-04	15	*gachl*.2.1
					Ga-Ilo-05	2	*gachl*.2.2
					Ga-Ilo-06	1	*gachl*.2.7
					Ga-Ilo-07	1	*gachl*.2.16
					Ga-Ilo-08	1	*gachl*.2.19
minuta	*Gminu*.1	Palawan[b]	11/1999	1	Gm-Pal-01	1	*gminu*.1.4
	Gminu.6	Palawan	11/1999	12	Gm-Pal-02	5	*gminu*.6.1
					Gm-Pal-03	6	*gminu*.6.2
					Gm-Pal-04	1	*gminu*.6.3
	Gminu.7	Palawan	11/1999	20	Gm-Pal-05	20	*gminu*.7.1
	Gminu.8	Iloilo	07/2000	28	Gm-Ilo-01	10	*gminu*.8.1
					Gm-Ilo-02	15	*gminu*.8.3
					Gm-Ilo-03	3	*gminu*.8.4
Leiognathus bindus	*Lbind*.1	Palawan	11/1999	1	Lb-Pal-01	1	*lbind*.1.1
elongatus	*Lelon*.1	Shizuoka[b]	09/1983	1	Lel-Shi-01	1	*lelon*.1.1
equulus	*Lequu*.1	Manila[b]	04/1982	1	Le-Man-01	1	*lequu*.1.1
fasciatus	*Lfasc*.1	Manila	04/1982	1	Lf-Man-01	1	*lfasc*.1.1
jonesi	*Ljone*.1	Iloilo	11/1999	30	Lj-Ilo-01	24	*ljone*.1.1
					Lj-Ilo-02	2	*ljone*.1.10
					Lj-Ilo-03	4	*ljone*.1.12
	Ljone.2	Iloilo	11/1999	28	Lj-Ilo-04	4	*ljone*.2.1
					Lj-Ilo-05	20	*ljone*.2.3
					Lj-Ilo-06	4	*ljone*.2.10
	Ljone.4	Iloilo	11/1999	10	Lj-Ilo-07	8	*ljone*.4.1
					Lj-Ilo-08	2	*ljone*.4.4
leuciscus	*Lleuc*.1	Palawan	11/1999	40	Ll-Pal-01	14	*lleuc*.1.1
					Ll-Pal-02	1	*lleuc*.1.31
					Ll-Pal-03	22	*lleuc*.1.3
					Ll-Pal-04	2	*lleuc*.1.5
					Ll-Pal-05	1	*lleuc*.1.33
	Lleuc.2	Palawan	11/1999	42	Ll-Pal-06	4	*lleuc*.2.1
					Ll-Pal-07	34	*lleuc*.2.2
					Ll-Pal-08	1	*lleuc*.2.5
					Ll-Pal-09	1	*lleuc*.2.25
					Ll-Pal-10	2	*lleuc*.2.42
nuchalis	*Lnuch*.1	Kanagawa[b]	06/1980	1	Ln-Kan-01	1	*lnuch*.1.1
	Lnuch.3	Kanagawa	09/1989	5	Ln-Kan-02	2	*lnuch*.3.1
					Ln-Kan-03	3	*lnuch*.3.2
philippinus	*Lphil.1*	Iloilo	07/2000	19	Lp-Ilo-01	19	*lphil*.1.1 [c]
	Lphil.2	Iloilo	07/2000	18	Lp-Ilo-02	18	*lphil*.2.1
					Lp-Ilo-03	2	*lphil*.2.7
	Lphil.3	Iloilo	07/2000	14	Lp-Ilo-04	14	*lphil*.3.1
	Lphil.4	Iloilo	07/2000	14	Lp-Ilo-05	6	*lphil*.4.1
					Lp-Ilo-01	3	*lphil*.4.3 [c]
					Lp-Ilo-06	4	*lphil*.4.4
					Lp-Ilo-07	1	*lphil*.4.7
rivulatus	*Lrivu*.1	Kanagawa	09/1983	1	Lr-Kan-01	1	*lrivu*.1.1
	Lrivu.3	Kanagawa	09/1989	10	Lr-Kan-02	9	*lrivu*.3.1 [d]
					Lr-Kan-03	1	*lrivu*.3.9
	Lrivu.5	Kanagawa	09/1989	20	Lr-Kan-02	20	*lrivu*.5.1[d]
	Lrivu.7	Shizuoka	09/1989	12	Lr-Shi-01	12	*lrivu*.7.1
	Lrivu.8	Shizuoka	09/1989	12	Lr-Shi-02	12	*lrivu*.8.1
	Lrivu.9	Shizuoka	09/1989	20	Lr-Shi-03	20	*lrivu*.9.1
	Lrivu.10	Shizuoka	09/1989	20	Lr-Shi-04	10	*lrivu*.10.1
					Lr-Shi-05	2	*lrivu*.10.5
					Lr-Shi-06	6	*lrivu*.10.6
					Lr-Shi-07	2	*lrivu*.10.14
	Lrivu.11	Shizuoka	09/1989	20	Lr-Shi-08	13	*lrivu*.11.2
					Lr-Shi-09	3	*lrivu*.11.3
					Lr-Shi-10	2	*lrivu*.11.4
					Lr-Shi-11	2	*lrivu*.11.9

Table 1. *cont.*

Leiognathid species	Specimen	Geographic origin	Date of collection	Strains examined	Genomotypes and number		Genomotype index strain[a]
splendens	*Lsple*.1	Gulf of Siam[b]	1967	1	Ls-Tha-01	1	ATCC 25521^T
	Lsple.2	Gulf of Siam	1967	1	Ls-Tha-02	1	ATCC 25587
stercorarius	*Lster*.1	Palawan	12/1999	19	Lst-Pal-01	16	*lster*.1.1
					Lst-Pal-02	3	*lster*.1.13
Secutor insidiator	*Sinsi*.1	Palawan	12/1999	28	Si-Pal-01	15	*sinsi*.1.1
					Si-Pal-02	13	*sinsi*.1.8
megalolepis	*Smega*.1	Iloilo	07/2000	9	Sm-Ilo-01	9	*smega*.1.1
	Smega.2	Iloilo	07/2000	9	Sm-Ilo-02	6	*smega*.2.1
					Sm-Ilo-03	1	*smega*.2.2
					Sm-Ilo-04	2	*smega*.2.5
	Smega.3	Iloilo	07/2000	9	Sm-Ilo-05	6	*smega*.3.1
					Sm-Ilo-06	2	*smega*.3.3
					Sm-Ilo-07	1	*smega*.3.5

a. All strains are from this study except *lfasc*.1.1 (LF-1a) and *lnuch*.1.1 (LN-1a) (Dunlap, 1985) and ATCC 25521^T and ATCC 25587 (Boisvert *et al*., 1967).
b. Iloilo, Manila and Palawan – Philippines; Kanagawa and Shizuoka – Japan; Gulf of Siam – Thailand.
c. Strains *lphil*.1.1 and *lphil*.4.3 exhibited identical genomic profiles.
d. Strains *lrivu*.3.1 and *lrivu*.5.1 exhibited identical genomic profiles.

Specificity of the P. leiognathi*–leiognathid fish association*

The extensive genomic differences among these strains led us to question whether all the strains actually are members of *P. leiognathi*. Previous taxonomic identification of bacteria isolated from the light organs of leiognathids revealed that these fishes harbour *P. leiognathi* as their exclusive bioluminescent symbiotic bacterium (Boisvert *et al*., 1967; Reichelt *et al*., 1977; Dunlap, 1985), but the light organs of relatively few species of leiognathids have been sampled microbiologically. The Leiognathidae is a species-rich and geographically widespread group of fishes, and most of the leiognathid fishes sampled previously were from one location, Ambon, Indonesia (Reichelt *et al*., 1977), with a small number of strains from species captured in other locations (Boisvert *et al*., 1967; Dunlap, 1985). Furthermore, only one symbiotic strain of this species, ATCC 25521^T, previously had been analysed phylogenetically. We therefore sought to test the apparent specificity of *P. leiognathi* for leiognathid fishes by phylogenetically analysing bacterial strains isolated from the light organs of various different leiognathid fishes. Individual strains of the symbiotic bacteria from 14 leiognathid species, the 10 strains above and four additional strains, isolated from *G. achlamys*, *L. philippinus*, *S. insidiator* and *S. megalolepis* from the Philippines (Table 1), were examined. To identify the strains, we used primers 27f and 1492r (Lane, 1991) and internal primers to PCR-amplify and sequence a region of the 16S rDNA (*Experimental procedures*) that distinguishes the known species of luminous bacteria (e.g. Dunlap and Kita-Tsukamoto, 2001). The sequences were then compared to the 16S rDNA sequence of the type strain, *P. leiognathi* ATCC 25521^T, and other luminous bacteria.

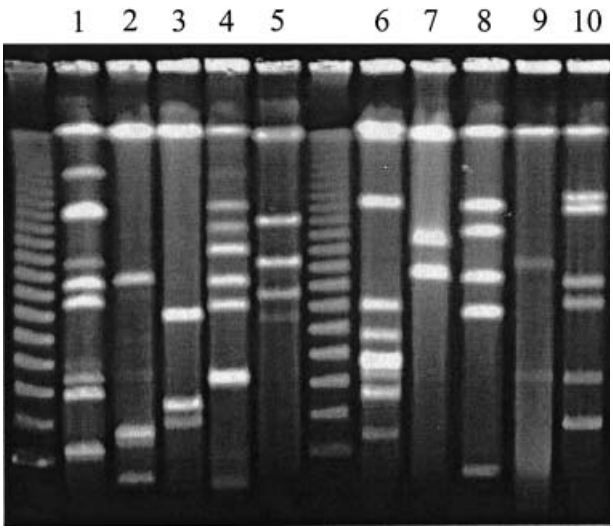

Fig. 3. Genomotypic diversity in *P. leiognathi*. Genomic DNA of individual *P. leiognathi* strains isolated from symbiosis with different species of leiognathid fish was digested with *Sfi*I and the fragments were separated by PFGE (*Experimental procedures*). Lanes: 1, *lnuch*.1.1 from *L. nuchalis*; 2, *lrivu*.1.1 from *L. rivulatus*; 3, *lelon*.1.1, from *L. elongatus*; 4, *lequu*.1.1, from *L. equulus*; 5, *lfasc*.1.1, from *L. fasciatus*; 6, *lbind*.1.1, from *L. bindus*; 7, *lleuc*.1.1, from *L. leuciscus*; 8, *ljone*.1.1, from *L. jonesi*; 9, *lster*.1.1, from *L. stercorarius*; and, 10, *gminu*.1.4, from *G. minuta*. Unmarked lanes are DNA size standards in increments of 48.5 kb.

Parsimony analysis of the 15 sequences resulted in a single hypothesis of relationships (Fig. 4). This hypothesis was broadly congruent with previous hypotheses for the phylogeny of luminous bacteria based on ribosomal RNA gene sequences (e.g. MacDonnell and Colwell, 1984; Kita-Tsukamoto *et al*., 1993; Ruimy *et al*., 1994). *Photobacterium* was monophyletic, and the 14 newly examined isolates grouped closely with the *P. leiognathi* type strain,

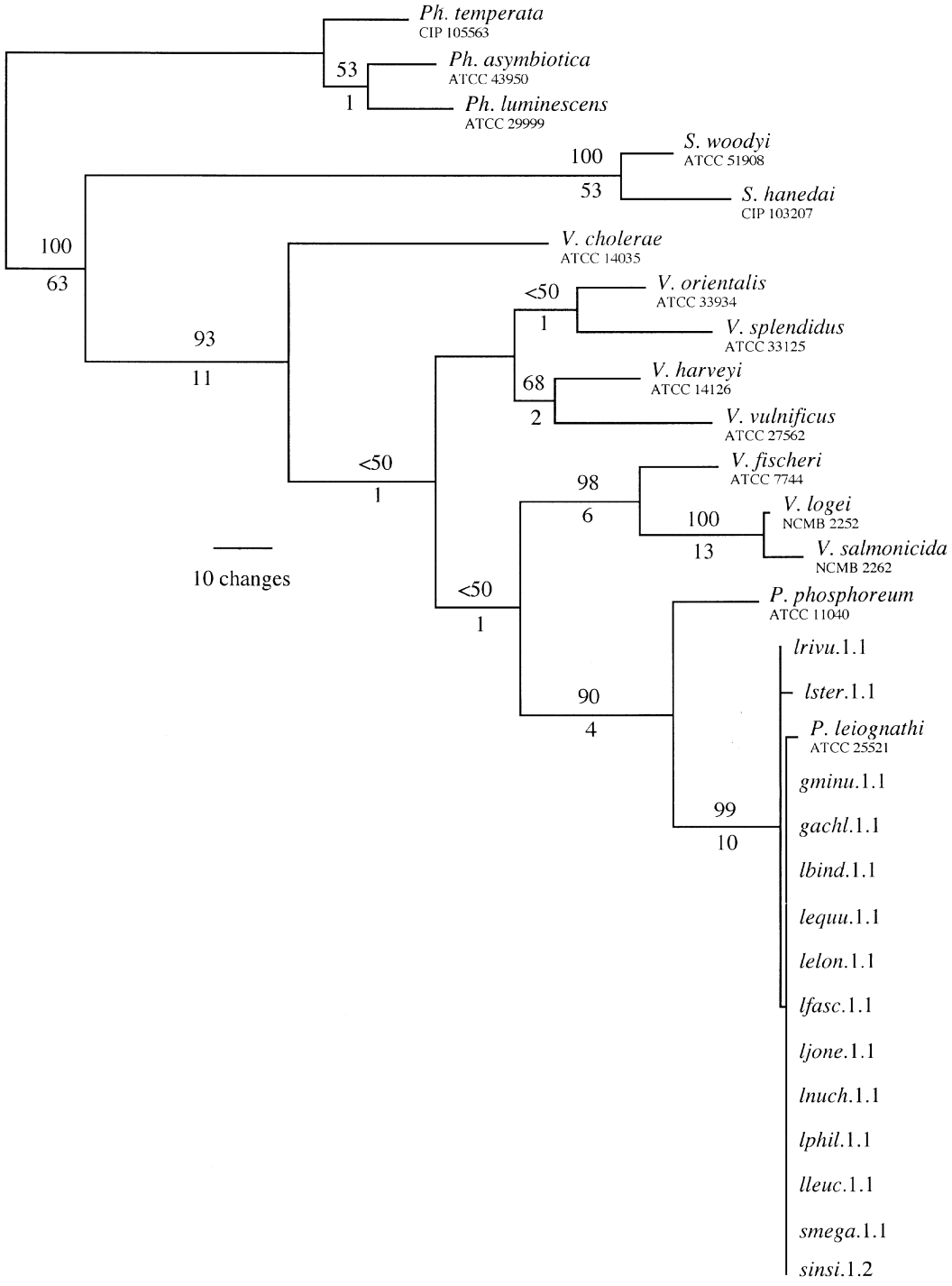

Fig. 4. Phylogram of one of three most parsimonious trees of species of luminous bacteria based on DNA sequences of the 16S rRNA gene (260 informative characters, length = 728, CI = 0.70, RI = 0.79; the two hypotheses not shown differ only in relationships of strains within *Photobacterium leiognathi*). Numbers above the branches indicate jackknife resampling values, and numbers below are Bremer support. See *Experimental procedures* for 16S rDNA sequences of *P. leiognathi* strains and *P. phosphoreum* ATCC 11040^T. Sequence accession numbers for other strains: *Photorhabdus temperata* CIP 105563^T (XINach), AJ007405 (Fischer-Le Saux *et al.*, 1999); *Ph. asymbiotica* ATCC 43950^T, Z76755 (Fischer-Le Saux *et al.*, 1999); *Ph. luminescens* ATCC 29999^T, D78005 (Suzuki *et al.*, 1996); *Shewanella woodyi* ATCC 51908^T, AF003549 (Makemson *et al.*, 1997); *S. hanedai* CIP 103207^T, X82132 (Gauthier *et al.*, 1995); *Vibrio cholerae* ATCC 14035^T, X74695 (Ruimy *et al.*, 1994); *V. orientalis* ATCC 33934^T, X74719 (Ruimy *et al.*, 1994); *V. splendidus* ATCC 33125^T, X74724 (Ruimy *et al.*, 1994); *V. harveyi* ATCC 14126^T, X74706 (Ruimy *et al.*, 1994); *V. vulnificus* ATCC 27562^T, X56582 (Dorsch *et al.*, 1992); *V. fischeri* ATCC 7744^T, X74702 (Ruimy *et al.*, 1994); *V. logei* NCMB 2252^T, AJ437616 (Colquhoun and Sorum, 2002); *V. salmonicida* NCMB 2262^T, X70643 (Wiik *et al.*, 1995); *Photobacterium leiognathi* ATCC 25521^T, X74686 (Ruimy *et al.*, 1994). For other strains of *P. leiognathi* see Table 1.

ATCC 25521^T. Furthermore, the sequences of these strains were nearly identical to each other, whereas they differed by 3% from the 16S rDNA sequence of the most closely related species, *Photobacterium phosphoreum*. These results, which identify the 14 strains as *P. leiognathi* based on their 16S rDNA sequences, confirm and extend to several additional leiognathid species the bacterial specificity of this association first described by Reichelt *et al*. (1977). The association appears to be specific for *P. leiognathi* regardless of the genus, species or geographic source of the leiognathid fish. Therefore, the genomotypic diversity described above characterizes *P. leiognathi*.

Oligoclonal structure of P. leiognathi *populations in leiognathid fishes*

The initial genomic profiling results (Fig. 3), indicating that different species of leiognathid fishes harbour genomotypically distinct strains, are consistent with the possibility that a symbiont-strain/host-species specificity characterizes this symbiosis. Analysis of single strains from different fish species, however, is not sufficient to establish that specificity. Convincing evidence of specificity would be the fulfillment of two criteria: first, that the population of *P. leiognathi* cells within light organs of individual fish is composed wholly or substantially of strains exhibiting a single genomotype, which would be strong evidence of specificity in the association at the strain level; and second, that different specimens of the same host species harbour strains of the same types, which would confirm that specificity. Failure to fulfill either the first or the second criterion would argue against this level of specificity. With respect to these criteria, Reichelt *et al*. (1977) previously showed through phenotypic analysis that one or two strains numerically dominated *P. leiognathi* populations symbiotic with individual leiognathid fishes, but that different fish specimens of the same species often harboured bacteria that differed in phenotypic traits. However, the relationship between phenotypic variation among strains, symbiosis with different fish species, and possible genetic differences among strains is unknown for *P. leiognathi*. No information has been available on the genetic structure of the bacterial populations symbiotic with leiognathid fishes, within individual host specimens or across host species, with which to address these questions. Therefore, to test the first criterion at the genetic level and to begin defining the natural genetic diversity within symbiotic populations of *P. leiognathi*, we profiled multiple strains from individual fish specimens, to determine the extent of genomotypic variation of *P. leiognathi* within the light organs of individual fishes. Included in this analysis, like that of Reichelt *et al*. (1977), were specimens of several different host species, representing each of the three leiognathid genera, *Gazza*, *Leiognathus* and *Secutor*.

We found that one or two strain types numerically dominated the symbiotic populations of individual fishes. For some host specimens, all the examined strains were of the same genomotype. An example of this uniformity is shown in Fig. 5; the 19 strains examined from *L. philippinus* specimen 1 (*Lphil*.1) were all of the same type. Additional examples of genomotypic uniformity are listed in Table 1. More commonly, however, two to five genomotypes were present, with one or two types comprising a high percentage of the strains. Two types, for example, were present in *Gachl*.1, more that 75% of which were represented by strain *gachl*.1.1 (Fig. 6). Similarly, for a specimen of *L. jonesi* (*Ljone*.1), three types were present among the 30 examined strains, with one type, Lj-Ilo-1, accounting for the majority of the strains. In specimens of *L. nuchalis* (*Lnuch*.3) and *Secutor insidiator* (*Sinsi*.1), two types were present, in approximately equal numbers. We also examined additional strains from certain host specimens reported on above. For *Lleuc*.1, four additional genomotypes were identified; two of the five types, Ll-Pal-01 and Ll-Pal-03, represented by *lleuc*.1.1 and *lleuc*.1.5, respectively, accounted for 90% of the 40 examined strains. For *Lster*.1, the genomic profiles of 18 additional strains were examined; the profiles of 15 of the additional strains were identical to that of *lster*.1.1 (Fig. 3), and three were of a second type (Table 1). Additional examples are

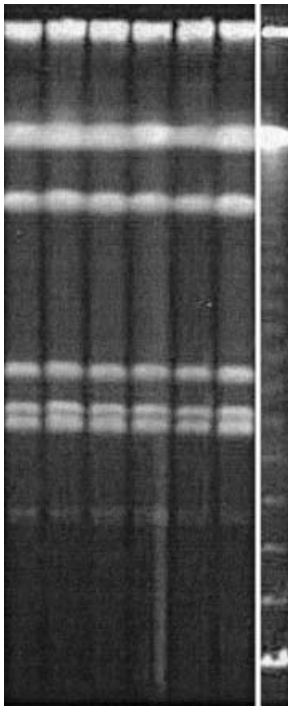

Fig. 5. Genomotypic uniformity of *P. leiognathi*. Genomic profiles were obtained as described in *Experimental procedures*. Shown are genomic profiles of six of 19 strains, *lphil*.1.1 to *lphil*.1.19, from specimen 1 of *L. philippinus*; all 19 profiles were identical. Lane at the right contains DNA size standards in increments of 48.5 kb.

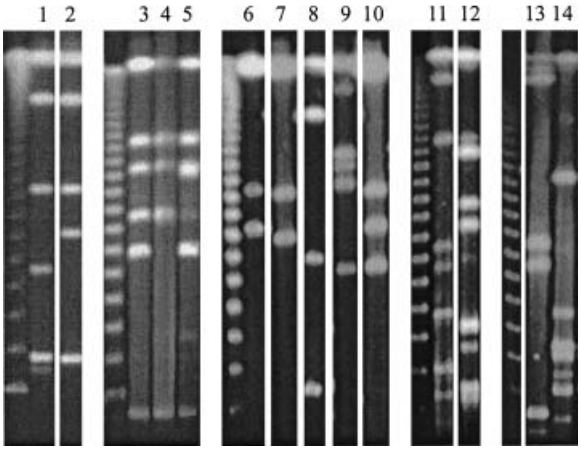

Fig. 6. Oligoclonal structure of symbiotic populations of *P. leiognathi*. For the 30 strains from *Gachl*.1 from Iloilo, Philippines (lanes 1 and 2), three genomotypes were seen: lane 1, Ga-Ilo-01 (represented by *gachl*.1.1, 1.2, 1.4 through 1.10, 1.13 through 1.18, 1.20 through 1.22, 1.24, 1.26, and 1.28 through 1.30); lane 2, Ga-Ilo-02 (represented by *gachl*.1.3, 1.11, 1.12, 1.19, 1.25, and 1.27); and Ga-Ilo-03 (not shown) (represented by *gachl*.1.23). For the 30 strains from *Ljone*.1 from Iloilo, Philippines (lanes 3–5), three genomotypes were seen: lane 3, Lj-Ilo-01 (represented by *ljone*.1.1 through 1.9, 1.11, 1.14 through 1.16, 1.19 through 1.22, 1.24, 1.25, 1.27, 1.29, and 1.30); lane 4, Lj-Ilo-02 (represented by *ljone*.1.10 and 1.18); and lane 5, Lj-Ilo-03 (represented by *ljone*.1.12, 1.13, 1.17, 1.23, and 1.28). For the 40 strains from *Lleuc*.1 from Palawan, Philippines (lanes 6–10), five genomotypes were seen: lane 6, Ll-Pal-01 (represented by *lleuc*.1.1, 1.2, 1.4, 1.6, 1.8 through 1.12, 1.19, 1.23, 1.28, 1.35, and 1.36); lane 7, Ll-Pal-02 (represented by *lleuc*.1.31); lane 8, Ll-Pal-03 (represented by *lleuc*.1.3, 1.7, 1.13 through 1.18, 1.20 through 1.22, 1.24 through 1.27, 1.29, 1.30, 1.32, and 1.37 through 1.40); lane 9, Ll-Pal-04 (represented by *lleuc*.1.5 and 1.34); lane 10, Ll-Pal-05 (represented by *lleuc*.1.33). For the five strains from *Lnuch*.3 from Kanagawa, Japan, two genomotypes were seen: lane 11, Ln-Kan-02 (represented by *lnuch*.3.1 and 1.5); and, lane 12, Ln-Kan-03 (represented by *lnuch*.3.2 through 3.4). For the 28 strains from *Sinsi*.1 from Palawan, Philippines (lanes 13 and 14), two genomotypes were seen: lane 13, Si-Pal-01 (represented by *sinsi*.1.1 through 1.4, 1.7 through 1.11, 1.20 through 1.22, 1.24, 1.25, and 1.27); and, lane 14, Si-Pal-02 (represented by *sinsi*.1.5, 1.6, 1.12 through 1.19, 1.23, 1.26, and 1.28). Unmarked lane to the left of each set of profiles contains DNA size standards in increments of 48.5 kb.

listed in Table 1. These results confirm and extend to the genomic level to the results of Reichelt *et al*. (1977). They demonstrate that the symbiont population of an individual leiognathid fish can be composed of more than one genomotypically distinct strain type and sometimes is composed of several types, but that one or two types typically dominate the population. We propose the term 'oligoclonal' to describe the genomotypic structure of symbiotic populations of *P. leiognathi*. These results also extend to additional host species the finding above (Fig. 3) that different species of leiognathid fishes harbour genomotypically distinct strains.

Within-host-species genomic heterogeneity of P. leiognathi

The oligoclonal structure of symbiont populations within individual fish specimens, together with the genomotypic differences between strains from different host species, appears consistent with a symbiont-strain/host-species specificity in this association. Fulfillment of the second criterion, that different specimens of the same host species harbour strains of the same genomotype, would confirm that specificity. To test this criterion, we profiled and compared the symbiont populations of multiple specimens of various individual fish species.

In contrast to an expectation of high cross-specimen genomotypic uniformity, we found that strains with the same genomotype were only rarely present in light organs of different specimens of the same host species. An example of the same strain type in different specimens of the same host species was seen with *L. philippinus*; the genomotype of *lphil*.1.1 (Lp-Ilo-01), which represented 19 of 19 strains in *Lphil*.1, was identical to that of *lphil*.4.3, which represented three of 14 strains in *Lphil*.4 (Fig. 7). However, other strains of the four examined *L. philippinus* specimens, which were captured at the same location, were genomotypically distinct. Another example was seen

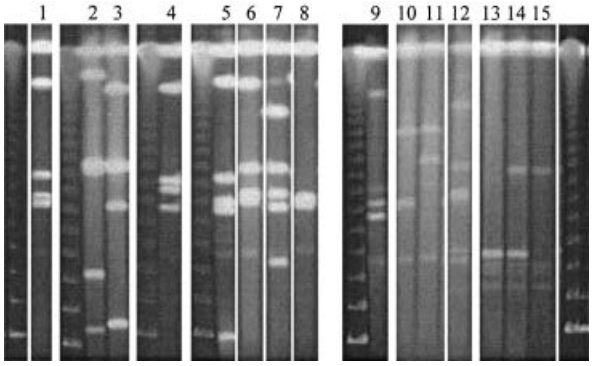

Fig. 7. Genomotypic heterogeneity of symbiotic populations of *P. leiognathi*. Genomic profiles of strains from four specimens of *L. philippinus* and from three specimens of *S. megalolepis*, from Iloilo, Philippines, were compared. For the 19 strains from *Lphil*.1, one genomotype was seen: lane 1, Lp-Ilo-01 (represented by *lphil*.1.1 through 1.19). For the 18 strains from *Lphil*.2, two genomotypes were seen: lane 2, Lp-Ilo-03 (represented by *lphil*.2.7 and 2.12); and, lane 3, Lp-Ilo-02 (represented by *lphil*.2.1 through 2.6, 2.8 through 2.11, and 2.13 through 2.18). For the 14 strains from Lphil.3, one genomotype was seen: lane 4, Lp-Ilo-04 (represented by *lphil*.3.1 through 3.14). For the 14 strains from *Lphil*.4, four genomotypes were seen: lane 5, Lp-Ilo-05 (represented by *lphil*.4.1, 4.2, 4.5, 4.10, 4.11, and 4.14); lane 06, Lp-Ilo-01 (represented by *lphil*.4.3, 4.6, and 4.8) (profile identical to that of *lphil*.1.1); lane 7, Lp-Ilo-06 (represented by *lphil*.4.4, 4.9, 4.12, and 4.13); and, lane 8, Lp-Ilo-07 (represented by *lphil*.4.7). For the nine strains from *Smega*.1, one genomotype was seen: lane 9, Sm-Ilo-01 (represented by *smega*.1.1 through 1.9). For the nine strains from *Smega*.2, three genomotypes were seen: lane 10, Sm-Ilo-02 (represented by *smega*.2.1, 2.3, 2.4, and 2.7 through 2.9); lane 11, Sm-Ilo-03 (represented by *smega*.2.2); and, lane 12, Sm-Ilo-04 (represented by smega.2.5 and 2.6). For the nine strains from *Smega*.3, three genomotypes were seen: lane 13, Sm-Ilo-06, represented by *smega*.3.3 and 3.8); lane 14, Sm-Ilo-05 (represented by *smega*.3.1, 3.2, 3.4, 3.6, 3.7, and 3.9); and, lane 15, Sm-Ilo-07 (represented by *smega*.3.5). Unmarked lanes contain DNA size standards in increments of 48.5 kb.

with *L. rivulatus*; the genomotype of *lrivu*.3.1 (Lr-Kan-02), which represented nine of 10 strains from *Lrivu*.3, was identical to that of *lrivu*.5.1, which represented the 20 strains isolated from *Lrivu*.5; however, strains from other specimens of *L. rivulatus*, taken at the same and different locations, were genomotypically distinct (Table 1). More typical was the situation represented by *S. megalolepis*; three specimens caught at the same location each harboured a distinct symbiont population, with none of the strain types occurring in more than one fish (Fig. 7). Also, of the 42 strains isolated from a second specimen of *L. leuciscus* (*Lleu*.2), 34 were of one type, four of a second, two of a third, and one each of a fourth and fifth types; none of these strains, however, matched the types present in *Lleu*.1. Furthermore, two specimens of *G. minuta* from the same general location harboured genomotypically distinct symbiont population (Table 1), with none of the strains occurring in more than one fish. As an additional test, we profiled the two *P. leiognathi* strains isolated by Boisvert *et al*. (1967), ATCC 25521^T and ATCC 25587, each from the light organ of a different specimen of a leiognathid fish tentatively identified as *L. splendens*. Like the majority of results obtained with our isolates from different specimens of the same host species, these two strains were genomotypically distinct. Additional examples are listed in Table 1. We conclude that the same strain type sometimes is present in different specimens of a host species, but not often. In most cases, host specimens of the same species, even from the same geographic location, harbour symbiont populations that are substantially distinct genetically. These results, in failing to fulfill the second criterion, clearly contradict the hypothesis that specificity between genomotypically defined strain types and individual host species characterizes the *P. leiognathi*/leiognathid fish association.

Discussion

This study reports an extensive genomic polymorphism in *P. leiognathi*, the bioluminescent light-organ symbiont of leiognathid fishes. Phenotypic variation among *P. leiognathi* strains symbiotic with different species of leiognathid fishes (Haneda and Tsuji, 1976; Reichelt *et al*., 1977; Dunlap, 1984; 1985; Dunlap and Steinman, 1986), including substantial differences in 2D PAGE protein patterns and profiles of indigenous plasmids, introduced the possibility of specificity in this association at the symbiont-strain/host-species level. Consistent with this level of specificity, genomic profiling demonstrated that different host species harbour genetically distinct strains of the bacterium, which by 16S rDNA sequence analysis nonetheless were found to be members of *P. leiognathi*, and revealed a genomotypically oligoclonal structure for the populations of *P. leiognathi* of individual fishes. However, analysis of the genomotypic structure of symbiont populations across multiple specimens demonstrated that different specimens of the same species of host fish harbour symbiont populations that are substantially distinct genetically. This finding and the extensive nature of genomotypic polymorphism in *P. leiognathi* indicate that genetic processes leading to specificity between the symbiotic bacterium and host animal in this association, although operating at the bacterial species level, apparently do not operate at the level of genomotypically defined strain types.

The very substantial genomotypic diversity described here and the rareness with which the same strain type of *P. leiognathi* was found within symbiotic populations of different fish specimens, despite the large number of fishes and strains examined, suggests that the extent of natural genomotypic diversity in this bacterium is enormous. An extensive genomic polymorphism has been reported also for *Vibrio harveyi* (Suwanto *et al*., 1998), a luminous bacterium that infects crustaceans but is not known to form a bioluminescent symbiosis (Dunlap and Kita-Tsukamoto, 2001), and for various other species of bacteria that associate with animal hosts (e.g. Buchrieser *et al*., 1995; Morton *et al*., 2001; Singh *et al*., 2001; Wallace *et al*., 2002). Furthermore, we have extended the work reported here to symbiotic associations between *Vibrio fischeri* and its various cephalopod and fish hosts and find a similarly extensive genomic polymorphism (P. V. Dunlap, M. M. Pearce and J. C. Ast, unpubl. data). The patterns described here for *P. leiognathi* symbiotic with leiognathid fishes therefore may be typical of bacteria forming other luminescent mutualisms.

Genomotypic diversity is likely to arise in *P. leiognathi* through the gradual accumulation of changes at the nucleotide level, the majority of which presumably are neutral, leading individual strain types to diverge genomotypically in a progressive manner over time. The length of time necessary for these changes to generate the extensive diversity described here is not yet obvious. We do know from preliminary analysis, however, that genomotypic changes of the kind reported do not occur readily during prolonged continuous-culture of *P. leiognathi* under laboratory conditions (M. M. Pearce and P. V. Dunlap, unpubl. data). Nonetheless, we have noted some examples of a high degree of genomotypic similarity among strains from the same symbiont populations (e.g. Figure 7, lanes 10 and 11 and 13–15), which introduces the possibility that genomotypic divergence might occur during the course of the symbiosis. It is interesting in this regard to speculate on the extent of and possible selective limits to genomotypic divergence.

We have not attempted to examine here the phylogenetic relationships among the different strain types based

on genomic profile analysis. Analyses of similarity based on DNA banding patterns are difficult to interpret phylogenetically due to a lack of resolution afforded by the limited number of bands and to the unsubstantiated assumption of homology among DNA bands of the same size. Nucleotide sequence-based analyses, which are necessary for meaningful characterization of the evolutionary relationships among strains, will be presented elsewhere. Here, we focus on what symbiont genomotypic diversity reveals about the initiation and specificity of the association.

Analysis of the genomic structure of symbiont populations within individual fishes demonstrated an apparent numerical dominance of one or two genomotypes in each fish specimen examined. We interpret this pattern as indicating that the symbiont populations of leiognathid fishes typically are oligoclonal, a pattern that is very similar to the phenotypic clustering of *P. leiognathi* strains from leiognathid fishes reported by Reichelt *et al*. (1977). The complementary nature of genomic oligoclonality and phenotypic clustering suggests that the phenotypic variation described for *P. leiognathi* (Reichelt *et al*., 1977; Dunlap and Steinman, 1986) results at least in part from differences between strains at the genome level. However, much additional work, beyond the scope of this report, will be necessary to establish the extent and nature of the link between phenotypic differences and the genomotypes of these strains. We do not know at this time, for example, whether the genomotypic differences reported on here correlate with possible phenotypic differences among these strains or whether such phenotypic differences might be important in the symbiosis. Nonetheless, the presence of apparently similar phenotypic clustering of bacteria symbiotic with families of fishes harbouring other species of luminous bacteria (Ruby and Nealson, 1976; Ruby and Morin, 1978) indicates that genomotypic oligoclonality might be typical of bioluminescent symbioses.

A possible criticism of the interpretation of oligoclonality is that it is based on examination of a small number of strains, in our study and in the study by Reichelt *et al*. (1977), relative to the total symbiont population size. Leiognathids typically harbour in their light organs approximately 10^8 bacteria (Dunlap, 1984). However, the consistency with which oligoclonality was observed, over a large number of fish specimens and regardless of the host taxon or geographic source of specimens, argues against that criticism. Even when the genomic profiling was extended to 40 or more strains per fish, the same pattern of one or two numerically dominant strains was evident. It should be noted that the isolation procedure used in this study, which is similar to that used in the study by Reichelt *et al*. (1977) and which involves a serial dilution of a homogenate of the fish's light-organ, would tend to dilute out less numerous strain types if present, thereby introducing a sampling bias that could limit the genomotypes observed to the more numerous types present. The full extent of symbiont genomic polymorphism within each light organ therefore may be greater than reported here. This bias, however, would tend to emphasize rather than obscure the observed numerical dominance of one or two genomotypes. Substantial naturally occurring genomotypic diversity is captured by this approach, and the pattern of oligoconality appears robust.

Oligoclonality has important implications for understanding the process by which the leiognathid light organ is colonized by *P. leiognathi*. Symbiont transmission in leiognathid fishes is horizontal; eggs within reproductively mature female leiognathids apparently lack *P. leiognathi* cells (P. V. Dunlap, pers. obs.), and the nascent light organ of the aposymbiotic juvenile fish is colonized by *P. leiognathi* from seawater (Wada *et al*., 1999). The presence of one to a few genomotypically distinct strains within individual light organs demonstrates that the symbiont population typically is founded by more than one *P. leiognathi* cell. Furthermore, these cells may be either of the same or different genomotypes. Whether colonization actually is oligoclonal, however, with each different colonizing strain establishing itself and persisting as a founding member of the symbiont population, or possibly is polyclonal, with competition between multiple colonizing strains leading to the oligoclonal state by extinction of certain genomotypes, is not known conclusively at this time. Nonetheless, the data presented here more readily support the simpler, neutral model in which colonization of the light organ is effected by cells of one to a few of the many different genomotypes present in the oceanic population, with the progeny of those founder cells persisting and establishing the symbiont population.

Microarchitecture of the light organ and growth dynamics of the symbiont population support this oligoclonal colonization scenario. The light organ tubules, which form the bulk of the light organ, release supernumerary bacterial cells, generated during growth and division of *P. leiognathi* in the light organ tubules, into a reservoir, which empties via ducts into the esophagus (Ahrens, 1965). The ducts presumably block the entry of secondary colonizers, i.e. immigrants from the oceanic population, once the light organ becomes colonized and develops. Furthermore, secondary colonization may be unlikely given the exceptionally high density of *P. leiognathi* cells within the light organ tubules (Harms, 1928; Dunlap, 1984), the growth of the *P. leiognathi* cells within the tubules (Dunlap, 1984), and the resulting flux of cells out of the tubules (Harms, 1928; Ahrens, 1965; Bassot, 1975; Dunlap, 1984). Alternatively, however, periodic secondary colonization of the light organ by *P. leiognathi* cells from a genomotypically diverse oceanic population cannot be ruled out at this time.

The patterns of genomic polymorphism and oligoclonality described here appear consistent with a symbiont-strain/host-species specificity in the *P. leiognathi*–leiognathid fish association. Specificity of this type would be intriguing because it presumably would indicate that the bacterium and fish have undergone a linked evolutionary divergence, i.e. co-speciation at the symbiont-strain/host-species level, as the Leiognathidae speciated (J. S. Sparks and P. V. Dunlap, unpubl. data). The anatomical differences in the light organs of different leiognathid species (Haneda and Tsuji, 1976; McFall-Ngai and Dunlap, 1984; Kimura *et al*., 2003; J. S. Sparks and P. V. Dunlap, unpubl. data) and the potentially different physiological conditions these anatomical differences present within the light organ provide a possible rationale for genetic adaptation of *P. leiognathi* strains to individual host species. Furthermore, evidence suggestive of co-evolution or parallel evolution has been obtained for the uncultured luminous symbionts of anomalopid fishes (Wolfe and Haygood, 1991) and for *V. fischeri* colonizing light organs of sepiolid squids (Nishiguchi *et al*., 1998).

A reasonable expectation, however, for the co-speciation scenario is that the same genomotypically identified *P. leiognathi* strain types would be found consistently in specimens of the same leiognathid host species. Contradicting that expectation, we found an extensive genomic polymorphism among *P. leiognathi* strains from specimens of the same host species, even when those specimens were from the same geographic location. Nonetheless, it is possible that specificity at the symbiont-strain/host species level exists in this association, but that strain typing by genomic DNA profiling does not characterize differences among strains with a resolution sufficient to identify it. A greater knowledge of genomic and phenotypic differences among strains and the possible relationship between those differences and symbiosis with individual host species (Dunlap and Steinman, 1986) would provide insight on this issue.

Alternatively, specificity in this association might exist only at the bacterial-species/fish-family level, with the genomotypic polymorphism described here reflecting natural fine-scale variation that is not relevant to host species-specificity in the symbiosis. In this regard, the extensive polymorphism in *P. leiognathi* and the remarkable rareness with which the same strain type is found within symbiotic populations of different fishes suggest that the genomotypic diversity of the global oceanic population of *P. leiognathi* is enormous. Colonization therefore might be random, with aposymbiotic hatchling fishes being colonized by whatever strains of *P. leiognathi* are present in their local environment. If so, then ecological factors that determine the local and regional incidence of different strain types in the oceanic population may play a defining role in the genomotypic structure of symbiotic populations of *P. leiognathi*.

Experimental procedures

Collection and identification of leiognathid fish

Leiognathid fishes were collected at local fish markets and from coastal waters in various locations in Japan and the Philippines (Table 1). For market specimens, the geographic source of the fish is given if known. Fish were identified to species according to the descriptions provided in Monkolprasit (1973), Abe (1976), Dunlap and McFall-Ngai (1984), Mochizuki and Hayashi (1989), Yamashita *et al*. (1998), Kimura *et al*. (2000), and Woodland *et al*. (2001). Professor S. Kimura, FRLM, kindly confirmed the identification of *Secutor* specimens used in this study as *S. insidiator* and *S. megalolepis* and identified the specimens of *L. jonesi* and *L. philippinus*. Specimens of fishes used in this study have been retained for taxonomic reference.

Isolation of P. leiognathi *strains, media and culture conditions*

Photobacterium leiognathi strains were isolated from light organs of leiognathid fish essentially as described in Dunlap (1984). Freshly caught fish were kept chilled until removal of the light organ, generally within 1–2 h from the time of collection. Light organs were dissected aseptically from the fish and homogenized in 1 ml of sterile artificial 40% seawater in a sterile hand-held Ten Broeck tissue homogenizer to release the bacteria. Homogenates were then serially diluted in sterile artificial 40% seawater and spread on plates of 40% seawater agar (LSW-40 agar), which contained 10 g tryptone, 5 g yeast extract, 400 ml artificial seawater, 600 ml deionized water and 15 g agar per litre. Plates were incubated for 18–24 h at room temperature (24–27°C). Dilutions of the light-organ homogenates, generally to 10^{-5}, and plating volumes, generally 25–100 μl, were based on a typical light-organ population size of approximately 10^8 cells (Dunlap, 1984); spread plating typically gave rise to 100–500 well-isolated colonies per plate. Single colonies (i.e. individual strains) were then picked at random from the plates, purified on LSW-40 agar and stored at −75°C, as described in Dunlap and Kita-Tsukamoto (2001).

Fish host specimen, bacterial strain and genomotype nomenclature

Fish specimen designations use the capitalized first letter of the genus name and the first four letters of the species name. Thus, *Leiognathus jonesi* specimen 1 is designated *Ljone*.1. Bacterial strain designations follow the fish specimen designation, except with a lower case letter for the host genus, to indicate a bacterial strain, and with sequential numbering indicating multiple strains from that host specimen. Thus, strains isolated from the light organ of *Gazza minuta* specimen 3 (*Gminu*.3) are designated *gminu*.3.1, *gminu*.3.2, and so on. Previously described strains from this laboratory were *Inuch*.1.1 (a.k.a. LN-1a) and *Ifasc*.1.1 (a.k.a. LF-1a) (Dunlap,

1985). The two ATCC strains of *P. leiognathi* used in this study, ATCC 25521^T and 25587 (Boisvert *et al.*, 1967), retain their original strain numbers. PFGE genomotypes of strains were assigned sequentially based on host species and geographic location (see Table 1).

2D PAGE and plasmid profiling

For 2D PAGE analysis of proteins, strains were grown in LSW-40 broth to an absorbance at 660 nm of 1.0, and total cellular proteins were extracted, separated and visualized essentially according to the procedures of O'Farrell (1975), as described in Callahan and Dunlap (2000). To profile indigenous plasmids, strains were grown in LSW-40 broth overnight, and plasmid DNA was extracted by standard alkaline-lysis methods, separated on 0.7% agarose Tris-acetate-EDTA gels, and stained with eithidum bromide.

Pulsed-field gel electrophoresis (PFGE)

For the preparation of genomic DNA for PFGE, strains were grown with aeration in 3 ml of LSW-40 broth at 27°C to an absorbance at 660 nm of approximately 1.0, which corresponded to approximately 10^8 cells ml^{-1}. Cells from 1 ml volumes were then pelleted at 3500 *g* at 4°C for 5 min, washed twice and resuspended in 500 µl pulsed-field buffer I [PFI; 10 mM Tris HCl (pH 7.5) and 1 M NaCl]. Five hundred microlitres of cell suspension was then mixed with 500 µl of 1% InCert agarose (FMC) at 50–60°C, added to molds and allowed to solidify for 10 min at 4°C. The agarose plugs were then incubated in 1 ml pulsed-field buffer II [PFII; 6 mM Tris HCl (pH 7.5), 100 mM EDTA (pH 8.0), 1 M NaCl, 0.5% Brij-58, 0.2% deoxycholate, 0.5% *N*-lauroylsarcosine NL-97, 1 mg ml^{-1} lysozyme and 20 µg ml^{-1} RNAse A], for 3 h at 37°C with shaking (170 r.p.m.). The plugs were then washed three times with 8 ml of $T_{10}E_1$ [10 mM Tris-HCl (pH 7.5), 1 mM EDTA (pH 8.0)] for 30 min at 37°C with shaking. The plugs were then incubated twice overnight in 1 ml pulsed-field buffer III [PFIII; 100 mM EDTA (pH 8.0), *N*-lauroylsarcosine NL-97, and 1 mg ml^{-1} Proteinase K] at 37°C with shaking, washed three times with $T_{10}E_1$ and three times with $T_{10}E_{50}$ [10 mM Tris-HCl (pH 7.5), 50 mM EDTA (pH 8.0)] for approximately 30 min each at 37°C with shaking, and then stored in $T_{50}E_{10}$ at 4°C.

For the digestion of DNA, portions of individual plugs were washed three times with $T_{10}E_1$, and then incubated with 300 ml of the appropriate restriction enzyme buffer at room temperature for one hour. For digestion with *Sfi*I, 100 µg ml^{-1} BSA was included in the buffer. The buffer was then replaced with 300 ml of fresh buffer containing 20 U of the appropriate restriction enzyme. Incubations were for 16 h at 50°C for *Sfi*I and at 37°C for other enzymes. To stop the digestion, a similar volume of PF III was added. The restriction digestion buffer was then replaced with 500 ml of $T_{10}E_1$ and the plugs were incubated at 50°C for 2 h, after which the buffer was replaced with 500 ml fresh $T_{10}E_1$ and the plugs were held at 4°C before gel electrophoresis.

For electrophoresis, gels [1% SeaKem GTG agarose (FMC)] in 0.5× TBE [100 mM Tris (pH 7.5), 100 mM Boric acid, 0.2 mM EDTA (pH 8.0)] were pre-electrophoresed in 2 L of running buffer (0.5× TBE containing 1 ml l^{-1} of 100 mM thiourea) at 14°C for 1 h. Portions of the plugs were then loaded into the wells and the wells sealed with 1% agarose. Plugs containing 48.5 kb Lamda DNA ladder served as size standards. Electrophoresis was conducted with a CHEF Mapper XA Pulsed field Electrophoresis System (Bio-Rad) at 14°C for 23 h using the auto algorhythm mode and set for separation of DNA fragments ranging from 40 to 800 KB. Following electrophoresis, gels were stained with SYBR green (FMC) and scanned as digital files.

The effectiveness of various restriction enzymes for cutting *P. leiognathi* genomic DNA was assessed. *Sfi*I generally gave the highest number and best resolution of DNA bands and therefore was used in the work reported here. *Not*I, though effective in generating a cleanly resolved genomic fingerprint, generally gave fewer bands. In contrast, *Avr*II gave only a partially resolved fingerprint under the conditions used here.

16S rDNA sequence analysis

For *P. leiognathi* strains reported here, bacterial chromosomal DNA from cells grown overnight in LSW-40 broth was purified using the Wizard genomic DNA purification kit (Promega) and used as the template for standard PCR-amplification of 16S rDNA with universal primers 27f (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492r (5′TACG GYTACCTTGTTACGACTT-3′) (Lane, 1991) and internal primers 669f (5′-TAGCGGTGAAATGCGTAG-3′) and 772r (5′-AATCCTGTTTGCTCCCCACG-3′). Polymerase chain reaction products were purified using the QIAquick gel extraction kit (Qiagen). Sequencing of PCR products was carried out by staff of the University of Michigan Core Facility. Identical procedures were followed for *P. phosphoreum* ATCC 11040^T, except that cells were grown in LSW-70 broth, containing 700 ml artificial seawater per litre (otherwise identical to LSW-40 broth). For *P. leiognathi* ATCC 25521^T and strains of other bacterial species, 16S rDNA sequences were downloaded from GenBank (see legend for Fig. 1). Initial alignment of the 1.3–1.5 kb sequences was performed using CLUSTALX and was refined by eye. Phylogenetic sequence analysis of the resulting 168 informative sites was performed using PAUP* (Swofford, 2002). For parsimony analysis, 1000 heuristic search replicates with TBR branch swapping were used. Jackknife values were calculated using 1000 replicates of 10 heuristic searches each. Bremer support values were calculated using TreeRot (Sorenson, 1999) in conjunction with PAUP*. GenBank accession numbers for the *P. leiognathi* strains reported here are: *gachl*.1.1, AY204488; *gminu*.1.1, AY204489; *lbind*.1.1, AY204490; *lelon*.1.1, AY204491; *lequu*.1.1, AY204492; *lfasc*.1.1, AY204493; *ljone*.1.1, AY204494; *lleuc*.1.1, AY204495; *lnuch*.1.1, AY204496; *lphil*.1.1, AY204497; *lrivu*.1.1, AY204498; *lster*.1.1, AY204499; *sinsi*.1.2, AY204500; and, *smega*.1.1, AY204501.

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