



Biodiversity among luminescent symbionts from squid of the genera *Uroteuthis*, *Loliolus* and *Euprymna* (Mollusca: Cephalopoda)

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Abstract

Luminescent bacteria in the family Vibrionaceae (Bacteria: γ -Proteobacteria) are commonly found in complex, bilobed light organs of sepiolid and loliginid squids. Although morphology of these organs in both families of squid is similar, the species of bacteria that inhabit each host has yet to be verified. We utilized sequences of 16S ribosomal RNA, luciferase α -subunit (*luxA*) and the glyceraldehyde-3-phosphate dehydrogenase (*gapA*) genes to determine phylogenetic relationships between 63 strains of *Vibrio* bacteria, which included representatives from different environments as well as unidentified luminescent isolates from loliginid and sepiolid squid from Thailand. A combined phylogenetic analysis was used including biochemical data such as carbon use, growth and luminescence. Results demonstrated that certain symbiotic Thai isolates found in the same geographic area were included in a clade containing bacterial species phenotypically suitable to colonize light organs. Moreover, multiple strains isolated from a single squid host were identified as more than one bacterial species in our phylogeny. This research presents evidence of species of luminescent bacteria that have not been previously described as symbiotic strains colonizing light organs of Indo-West Pacific loliginid and sepiolid squids, and supports the hypothesis of a non-species-specific association between certain sepiolid and loliginid squids and marine luminescent bacteria.

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Members of the families Loliginidae and Sepiolidae (Mollusca: Cephalopoda) have been previously shown to contain luminous bacteria that reside in specialized light organ complexes. These light organs vary in structure and complexity, from simple, spherically shaped structures [*Rondeletiola* and *Sepiolina*; (Naef, 1912a; Nesis, 1982)], to complex, bilobed organs (Naef, 1912b; McFall-Ngai and Montgomery, 1990; Foster et al., 2002; Nishiguchi et al., 2004).

Squid use the light produced from the bacteria for a behavior known as counterillumination (Young and Roper, 1977; Young et al., 1980; Jones and Nishiguchi, 2004). Luminescence emitted from the light organ reduces the squid's silhouette to match the intensity and wavelength of down-welling light (Young and Roper, 1977). This provides squid with a mechanism that allows them to evade predators by camouflage. All

bacteria housed in the light organs are able to produce light via the *lux* operon both inside the light organs and in their free-living state, although intensity of light and differences between strains of bacteria have never been thoroughly investigated. Likewise, regulation of the *lux* operon inside the light organ of squid has only been extensively studied in the *Euprymna scolopes*–*Vibrio fischeri* symbiosis, where reduction in the amount of light produced affects symbiotic competence (Visick et al., 2000).

Previous investigations have demonstrated that two genera, *Vibrio* and *Photobacterium*, are prevalent in both sepiolid and loliginid squid (Fidopiastis et al., 1998; Nishiguchi et al., 2004). Sepiolid squid primarily contain two species of *Vibrio*, *V. fischeri* and *V. logei* (Fidopiastis et al., 1998; Nishiguchi, 2002), although the genera *Rondeletiola* and *Sepiolina* have been shown to contain *Photobacterium leiognathi* in their light organs (Nishiguchi and Nair, 2003; Nishiguchi et al., 2004). Photobacteria are more commonly found in the genera

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Uroteuthis and *Loliolus* (Nishiguchi, 2002), with no cases of *Vibrio* species detected in bacteriogenic light organ of loliginids. Because there is geographic overlap between several species of *Uroteuthis*, *Loliolus* and *Euprymna* in the Indo-West Pacific, we were interested in isolating and identifying bacteria from these squid, determining whether these strains were phylogenetically related to one another, and whether there was any biogeographical or host-specific patterns evident between the symbionts. We used the complete 16S rRNA sequence from each isolate in a combined analysis with the luciferase α -subunit gene (*luxA*) and the glyceraldehyde phosphate dehydrogenase gene (*gapA*) sequences to determine phylogenetic relationships among closely related species. In addition, we measured both growth and luminescence to determine whether substantial differences occurred between these isolates. Previously isolated squid symbionts from host species collected in close or distant geographic locations were also compared in order to evaluate whether luminous bacteria that have been environmentally transmitted are promiscuous across hosts, or alternatively, whether symbionts exhibit squid family specificity. These comparisons would then allow us to determine if geographic location of the species investigated was a determining factor in predicting the associations between light organ symbionts and loliginid squid.

Materials and methods

Collection of squid and isolation of bacteria from light organs

Squid were collected from either fishermen in the local Thai markets, or by trawl net and brought into the lab for identification. Squid were collected from two geographically separated locations: Rayong, Thailand, which is located on the Gulf of Thailand, and the island of Phuket, in southern Thailand, which is located in the Andaman Sea (Table 1, in bold). Animals collected in Rayong were brought back to the Rayong Coastal Aquaculture and Fisheries Research Center for confirmation of identity. Specimens collected in Phuket were identified at the Phuket Marine Biological Station. Host specimens were used for isolation of symbiotic bacteria according to previous protocols (Nishiguchi, 2002; Nishiguchi and Nair, 2003). Mantle cavities were opened and the entire light organ complex was removed and placed in sterile seawater (700 μ L). Adult light organs were homogenized, serially diluted to 10^{-5} and plated on seawater tryptone (SWT) agar media (Ruby and Asato, 1993). Cultures were incubated overnight at room temperature, and kept at 5 °C upon return to New Mexico State University. Approximately 20–30 light-producing colonies from each plate were subsequently

grown overnight in SWT medium at 20–28 °C, and cultures were either frozen in a glycerol stock for further biological assays or bacterial DNA was extracted for isolate identification and phylogenetic analyses.

Determination of bacterial growth and light production

Samples of symbiotic strains were recovered from each stock by streaking onto SWT agar plates and growing for 16 h at 28 °C. One CFU from this culture was used to inoculate 5 mL of seawater tryptone (SWT) broth and grown for 16 h on a shaking incubator (250 r.p.m.) at the same temperature. Ten microliters of this culture was then re-inoculated in 5 mL of SWT broth and incubated for 3 h on a shaking incubator (250 r.p.m.) at 28 °C. The starting aliquot for inoculation of the growth flask containing 50 mL of SWT was taken from this culture. Optical density was measured at 600 nm to guarantee that all strains tested would have the same density at time T_0 by adjusting the volume of each culture. Flasks were maintained in a shaking incubator (250 r.p.m.) at 28 °C for the entire 8- or 16-h period.

Measurements for growth and light production were obtained simultaneously every 30 min for both 8- and 16-h incubation assays. For growth curves, OD_{600} was determined using 1 mL of the initial culture transferred to a 1000 μ L cuvette. Optical density was measured using an Uvikon XL spectrophotometer (Bio-tek Instruments, San Diego, CA, USA) and transferred to a PC using the Laboratory Power Junior 2.06 software. Sterile SWT broth was used as the blank.

For measurements of light emitted by cell suspensions (bioluminescence), 100 μ L of the liquid culture was transferred to a 20 mL disposable scintillation vial and placed in a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA, USA). Light production was measured in light units per milliliter and also indicated as luminescence per cell (luminescence per OD_{600}). The volume of culture was reduced whenever light production exceeded the maximum reading allowed by the luminometer (9999 light units). Light production values were adjusted using dilution factors corresponding to each volume reduction.

Carbon source utilization for phylogenetic analysis

Strains from frozen stock were plated on SWT agar plates and grown overnight at 28 °C. Single colony forming units (CFU) of each strain were recovered after incubation and grown in 10 mL of SWT medium to an OD_{600} of 0.5 (Nishiguchi and Nair, 2003). Bacterial cells were harvested by centrifugation at 5000 r.p.m. (2655 *g*) for 10 min after being washed three times using 5 mL of PBS buffer (pH 7.3). The pellet was finally resuspended in 10 mL of PBS. A 100 μ L aliquot of the cell

Table 1

Bacterial strain, location, host, 16S ribosomal DNA, gapA and luxA accession number. Strains in bold were isolated for this study

Bacterial strains	Source	Location	Accession numbers		
			16S rRNA	gapA	luxA
<i>Vibrio fischeri</i> CG101	<i>Cleidopus gloriamaris</i>	Australia	AY292939	AY292958	DQ026810
<i>Vibrio fischeri</i> ES 114	<i>Euprymna scolopes</i>	Hawaii	AY292919	AF034845	DQ026811
<i>Vibrio fischeri</i> ES915	<i>Euprymna scolopes</i>	Hawaii	AY292920	AY292953	DQ026812
<i>Vibrio fischeri</i> ET101	<i>Euprymna tasmanica</i>	Australia	AY292923	AF034847	DQ026813
<i>Vibrio fischeri</i> MJ101	<i>Monocentrus japonicus</i>	Japan	AY292946		DQ026814
<i>Vibrio fischeri</i> SL518	<i>Sepiolo ligulata</i>	France	AY292950	AY292962	DQ026815
<i>Vibrio fischeri</i> SR5	<i>Sepiolo robusta</i>	France	AY292926	AF034851	DQ026816
<i>Vibrio fischeri</i> WH1	Free-living	USA	AY292930	AY292955	DQ026817
<i>Photobacterium leiognathi</i> LN101	<i>Uroteuthis noctiluca</i>	Australia	AY292944	AF034849	DQ026808
<i>Photobacterium leiognathi</i> RM1	<i>Rondeletiola minor</i>	France	AY292947		DQ026809
EHR1	<i>Euprymna hyllebergi</i>	Thailand	AY332401		
LAR1	<i>Loliolus affinis</i>	Thailand	AY332398		
EHP8	<i>Euprymna hyllebergi</i>	Thailand	AY332400		
UCP6	<i>Uroteuthis chinensis</i>	Thailand	AY332404		
UDP1	<i>Uroteuthis duvauceli</i>	Thailand	AY332407		
<i>Photobacterium phosphoreum</i> ATCC 11040	Chlorophthalmid fishes		AY341437		AY341063
<i>Photobacterium leiognathi</i> ATCC 25521	<i>Leiognathus splendens</i>	Thailand	D25309		M63594
<i>Vibrio fischeri</i> ET401	<i>Euprymna tasmanica</i>	Australia	AY292943	AY292960	
<i>Vibrio fischeri</i> SA1G	<i>Sepiolo affinis</i>	France	AY292924	AF034848	
<i>Vibrio fischeri</i> SI1D	<i>Sepiolo intermedia</i>	France	AY292948	AY292961	
<i>Vibrio logei</i> ATCC15382	<i>Gadus macrocephalus</i>	USA	AY292932	AY292956	
<i>Vibrio logei</i> SR181	<i>Sepiolo robusta</i>	France	AY292934	AY292957	
<i>Vibrio anguillarum</i> ATCC 19264	<i>Gadus morhua</i>	Denmark	X16895		
<i>Vibrio alginolyticus</i> ATCC 17749	<i>Trachurus trachurus</i>	Japan	X56576		
<i>Vibrio campbellii</i> ATCC 25920	Seawater		X56575		
<i>Vibrio harveyi</i> EH701	<i>Euprymna hyllebergi</i>	Thailand	AY292941		
<i>Vibrio cholerae</i> N16961	Cholera patient	Bangladesh	AE004096	AE004274	
<i>Vibrio fischeri</i> EB12	<i>Euprymna berryi</i>	Japan	AY292921	AY292954	
<i>Vibrio fischeri</i> EM17	<i>Euprymna morsei</i>	Japan	AY292922	AF034846	
<i>Vibrio fischeri</i> ET301	<i>Euprymna tasmanica</i>	Australia	AY292942	AY292959	
<i>Vibrio vulnificus</i> YJ016	Clinical isolate	Taiwan	BA000037	BA000037	
<i>Vibrio parahaemolyticus</i> RIMD 2210633			BA000031	BA000031	
<i>Vibrio salmonicida</i> NCMB 2262	Fanned <i>Salmo salar</i>	Norway	X70643		AF452135
<i>Vibrio pelagius</i> ATCC 25916T	Succinate enriched seawater		X74722		
<i>Salinivibrio costicola</i> ATCC 33508T	Bacon curing brine		X74699		
<i>Photobacterium damsela</i> ATCC 33539T	<i>Chromis punctipinnis</i>	USA	X74700		
<i>Escherichia coli</i> W3110		USA	AC_000091	AP009048	
<i>Photobacterium damsela</i> ATCC 33539	<i>Chromis punctipinnis</i>	USA	AB032015		
<i>Vibrio splendidus</i> ATCC 33125	Marine fish		X74724		
<i>Vibrio lentus</i> 40M4T CECT 5110	Oysters	Spain	AJ278881		
<i>Vibrio orientalis</i> ATCC 33934	Seawater	China	X74719		
<i>Vibrio tasmaniensis</i> LMG 20012	<i>Salmo salar</i>	Australia	AJ316192		
<i>Vibrio rumoiensis</i> S 1FERM P-14531	Fish product processing plant	Japan			AB013297
<i>Vibrio fischeri</i> ES 191	<i>Euprymna scolopes</i>	Hawaii	DQ026825		DQ026819
<i>Vibrio fischeri</i> PP3	Free-living	Hawaii	DQ026824		DQ026818
<i>Vibrio fischeri</i> PP42	Free-living	Hawaii	DQ026826		DQ026820
<i>Vibrio fischeri</i> VLS2	Free-living	Hawaii	DQ026827		DQ026821
EHP1	<i>Euprymna hyllebergi</i>	Thailand	DQ530284	DQ530298	
EHP2	<i>Euprymna hyllebergi</i>	Thailand	DQ530285	DQ520299	
EHP3	<i>Euprymna hyllebergi</i>	Thailand	DQ530286	DQ520300	
EHP4	<i>Euprymna hyllebergi</i>	Thailand	DQ530287	DQ520301	
EHP5	<i>Euprymna hyllebergi</i>	Thailand	DQ530288	DQ520302	
EHR2	<i>Euprymna hyllebergi</i>	Thailand	DQ530289	DQ520303	
EHR3	<i>Euprymna hyllebergi</i>	Thailand	DQ530290	DQ520304	
EHR4	<i>Euprymna hyllebergi</i>	Thailand	AY332402	DQ520305	
LAR2	<i>Loliolus affinis</i>	Thailand	DQ530291	DQ520306	
LAR3	<i>Loliolus affinis</i>	Thailand	DQ530292	DQ520307	
LARS	<i>Loliolus affinis</i>	Thailand	DQ530293	DQ520308	
UCP4	<i>Uroteuthis chinensis</i>	Thailand	DQ530294	DQ520309	

Table 1
Continued

Bacterial strains	Source	Location	Accession numbers		
			16S rRNA	<i>gapA</i>	<i>luxA</i>
UCR4	<i>Uroteuthis chinensis</i>	Thailand	DQ530295	DQ520310	
UCR6	<i>Uroteuthis chinensis</i>	Thailand	DQ530296	DQ520311	
UCR7	<i>Uroteuthis chinensis</i>	Thailand	DQ530297	DQ520312	
<i>Vibrio harveyi</i> ATCC 14126	Amphipod <i>Talorchestia</i> sp.	USA	X74706		AF147084

ATCC: American type culture collection (USA)

LMG: Belgian Coordinated Collections of Microorganisms (BCCM). Division of Bacteria: BCCM/LMG (Belgium) CECT: Coleccion espanola de cultivos tipo (Spanish type culture collection) (Spain)

FERM: Patent and Bio-Resource Center (Japan)

NCMB: National Collections of Industrial and Marine Bacteria Ltd (United Kingdom)

RIMD: Research Institute for Microbial Diseases (Japan)

suspension was added to each of the 96 wells of a GN2 Micro Plate™ (Biolog, Hayward, CA, USA) plate and incubated overnight at 28 °C. Carbon use was determined using an ELx800 absorbance microplate reader (Bio-Tek instruments, Winooski, VT, USA). Each strain was analyzed in duplicate plates. Biolog data were converted into binary information giving a value of 1 for positive results (i.e., use of carbon source) and 0 to the negative ones (i.e., carbon source not utilized). These phenotypic values were then converted into Hennig 86/Nona input data to be used in a combined analysis with the nucleotide data.

DNA extraction, amplification and sequencing of bacterial genes

To extract DNA from isolates, bacteria were grown overnight at 28 °C at 250 r.p.m. in 5 mL of SWT. 2 mL of each culture was centrifuged for one minute, and media removed. Bacterial DNA was isolated using the DNAeasy kit (Qiagen Inc., Valencia, CA, USA) according to manufacturer's instructions. One to 10 ng of template DNA was used for polymerase chain reaction (PCR) amplifications. For the 16S rRNA locus, four sets of primers (each amplifying approximately 420 bp) were used to determine the entire 16S rRNA sequence (~1600 bp). Primer sequences were obtained from Nishiguchi and Nair (2003) and are shown in Table 2. A single PCR reaction (50 µL) contained 2.5 mM of MgCl₂, 0.5 mM dNTPs (25 µM each; Promega, Madison, WI, USA), 0.2 µM of forward and reverse primer, 10 × reaction buffer (10 mM Tris-HCl, pH 9.0, 50 mM KCl, and 0.1% Triton X-100) and 0.05 U/µL of Taq DNA Polymerase (Promega; Madison, WI), AmpliTaq Gold® or AmpliTaq® DNA polymerase (Applied Biosystems, Foster City, CA, USA).

The *luxA* gene was used as a second molecular marker for additional phylogenetic analyses. A group of primers was used for the amplification of this locus that codes for the α-subunit of luciferase. PCR protocol and

conditions for each of these sets of primers are shown in Table 2. PCR products were cleaned with Gene Clean II DNA purification kit (Q-biogene, Irvine, CA, USA).

gapA is a gene that codes for the glyceraldehyde phosphate dehydrogenase enzyme, and was used as the third phylogenetic marker in the analysis. For its amplification, primers *gapA* F and *gapA* R were used. Primer sequences and conditions for amplification are shown in Table 2.

PCR products were sequenced using Applied Biosystems Big Dye® (v.3.1). Excess fluorescently labeled dNTPs were removed by spin column or plate (Edge Biosystems, Gaithersburg, MD, USA) according to manufacturer instructions. Samples were sequenced in an Applied Biosystems 3100 automated capillary sequencer. Independent forward and reverse sequences obtained were combined and edited using Sequencher v. 4.6. (Gene Codes™, Ann Arbor, MI, USA). Edited sequences were imported to BBEdit v.7.0.3 and converted into UNIX format to be revised, prealigned, and compared through the Genetic Data Environment v.2.2. (Smith et al., 1994).

Phylogenetic analysis

Sequences were analyzed using the direct optimization method described by Wheeler (Wheeler, 1996, 1998) and implemented in the computer program POY (Wheeler, 1995; Wheeler et al., 2002). The method assesses the number of transformations in DNA sequences required by a phylogenetic topology without using pair-wise optimization through multiple sequence alignment. POY treats insertions and deletions as processes, which place it apart from multiple sequence alignment (Wheeler, 1998).

Several analyses were implemented with character transformations weighted differently to determine how different phylogenetic hypotheses were affected (sensitivity analysis *sensu* Wheeler, 1995). Final analysis was performed with characters equally weighted, using a

Table 2
Oligonucleotide primers and PCR conditions used in this study

Primer name and amplified region	Primer sequence	Cycle					
		Cycles	Hot start	Denaturation	Annealing	Extension	Final extension
16S rRNA**	IF 5'-AGA GTT TGA TCM TGG CTC AG-3' 4R 5'-AGG CCT TCT TCA TAC ACG CG-3'	25	94 °C 2 min	94 °C 15 s	49 °C 15 s	72 °C 15 s	72 °C 7 min
16S rRNA**	2F 5'-GCA AGC CTG ATG CAG CCA TG-3' 3R 5'-ATC GTT TAC GGC GTG GAC TA-3'	25	94 °C 2 min	94 °C 15 s	49 °C 15 s	72 °C 15 s	72 °C 7 min
16S rRNA**	3F 5'-AAA CAG GAT TAG ATA CCC TG-3' 2R 5'-CTG GTC GTA AGG GCC ATG AT-3'	25	94 °C 2 min	94 °C 15 s	49 °C 15 s	72 °C 15 s	72 °C 7 min
16S rRNA**	4F 5'-AGG TGG GGA TGA CGT CAA GT-3' 1R 5'-AAG GAG GTG WTC CAR CC-3'	25	94 °C 2 min	94 °C 15 s	49 °C 15 s	72 °C 15 s	72 °C 7 min
<i>luxA</i> -specific*	F 5'-GTTCTTAGTTGGATTATTGG-3'	29–33	94 °C 2 min	94 °C 15 s	44 °C 30 s	72 °C 30 s	72 °C 7 min
Positions 568–995	R 5'-TCAGTCCCATTAGCTTCAAATCC-3'	30	94 °C 2 min	94 °C 15 s	55 °C 30 s	72 °C 30 s	72 °C 7 min
1st <i>luxA</i> †	F 5'-CAGAATCACCAAAAAGGAATAGGT-3'	26–30	94 °C 2 min	94 °C 15 s	50 °C 30 s	72 °C 30 s	72 °C 7 min
Positions 1–590	R 5'-CAATTCATTATAGAGTCCATCTGTG-3'	25–30	94 °C 2 min	94 °C 15 s	47 °C 30 s	72 °C 30 s	72 °C 7 min
2nd <i>luxA</i> †	F 5'-GTTCTTAGTTGGATTATTGGTA-3'	25–30	94 °C 2 min	94 °C 15 s	43 °C 30 s	72 °C 30 s	72 °C 7 min
Positions 450–1068	R 5'-AACTTGTCCCTTATTCTCTAGTAT-3'	28	94 °C 2 min	94 °C 15 s	42.5 °C 30 s	72 °C 30 s	72 °C 7 min
<i>luxA</i> 1–569†	F 5'-GCGAAGATTAAAGAAATAAAA-3'	28	94 °C 2 min	94 °C 15 s	48.4 °C 30 s	72 °C 30 s	72 °C 7 min
Positions 1–569	R 5'-TTCTTTAGAGTACTTTTGTGG-3'	25–30	94 °C 2 min	94 °C 15 s	43 °C 30 s	72 °C 30 s	72 °C 7 min
<i>luxA</i> 400–1168†	F 5'-ATTTAATTTGGTGTGTTA-3'	25–30	94 °C 2 min	94 °C 15 s	43 °C 30 s	72 °C 30 s	72 °C 7 min
Positions (400–1168)	R 5'-TTAATTTTGGAGTTCITTTA-3'	25–30	94 °C 2 min	94 °C 15 s	43 °C 30 s	72 °C 30 s	72 °C 7 min
<i>luxA</i> 401–1168 ⁶	F 5'-TTAATTTTGGTGTGTTT-3'	28	94 °C 2 min	94 °C 15 s	42.5 °C 30 s	72 °C 30 s	72 °C 7 min
Positions 401–1168	R 5'-TTAATTTTGGAGTTCITTTA-3'	28	94 °C 2 min	94 °C 15 s	48.4 °C 30 s	72 °C 30 s	72 °C 7 min
<i>luxA</i> ext†	F 5'-AAATTTTGGTGTGTTTAAAG-3'	28	94 °C 2 min	94 °C 15 s	48.4 °C 30 s	72 °C 30 s	72 °C 7 min
<i>luxA</i> int1†	R 5'-AAATTTTGGTGTGTTTAAAG-3'	28	94 °C 2 min	94 °C 15 s	48.4 °C 30 s	72 °C 30 s	72 °C 7 min
<i>luxA</i> int2†	F 5'-CTATAATCAACACGTCGATTS-3'	28	94 °C 2 min	94 °C 15 s	48.5 °C 30 sec	72 °C 30 s	72 °C 7 min
<i>luxA</i> int3†	R 5'-KTTTGATACATATGGACSTT-3'	28	94 °C 2 min	94 °C 15 s	47.5 °C 30 s	72 °C 30 s	72 °C 7 min
Pleioleio γ A†	F 5'-TGATAATCRTAACCCWCGAGT-3'	25–30	94 °C 2 min	94 °C 15 s	56 °C 30 s	72 °C 30 s	72 °C 7 min
Pleioleio γ AB†	R 5'-GTTTAAAGATCAACTGTCTAAAGRCG-3'	25–30	94 °C 2 min	94 °C 15 s	60 °C 30 s	72 °C 30 s	72 °C 7 min
Pphosp A†	F 5'-CGTCGCCGACTTGATTAACGTAAC-3'	25–30	94 °C 2 min	94 °C 15 s	58 °C 30 s	72 °C 30 s	72 °C 7 min
Pphosp AB†	R 5'-ATCCTTTCTGCTGCCATTC AAC-3'	25–30	94 °C 2 min	94 °C 15 s	55.4 °C 30 s	72 °C 30 s	72 °C 7 min
<i>luxA</i> 12§	F 5'-TTTATAGATCCAAATGCAAAAGCCG-3'	30	94 °C 2 min	94 °C 15 s	58 °C 30 s	72 °C 30 s	72 °C 7 min
<i>luxA</i> 12§	R 5'-TCCAAACGATATGTTAGTGAAGC-3'	30	94 °C 2 min	94 °C 15 s	58 °C 30 s	72 °C 30 s	72 °C 7 min
<i>luxA</i> 12§	F 5'-AAACGTCGTTGATATAGCAACG-3'	30	94 °C 2 min	94 °C 15 s	56 °C 30 s	72 °C 30 s	72 °C 7 min
<i>luxA</i> 12§	R 5'-GANCANCANTINACNGAGTTTGG-3'	29	94 °C 2 min	94 °C 15 s	40 °C 15 s	70 °C 1 min	70 °C 7 min
<i>luxA</i> 12§	F 5'-ATTCNTCTTCAGNCCATTNGCTCAAANCC-3'	29	94 °C 2 min	94 °C 15 s	40 °C 15 s	70 °C 1 min	70 °C 7 min
<i>luxA</i> 12§	R 5'-CTACTGATCAAATGTCAAAGGA-3'	29	94 °C 2 min	94 °C 15 s	40 °C 15 s	70 °C 1 min	70 °C 7 min
<i>luxA</i> 12§	F 5'-TCAGAAACCGTTTGTCTCAAACCC-3'	29	94 °C 2 min	94 °C 15 s	40 °C 15 s	70 °C 1 min	70 °C 7 min
<i>luxA</i> 12§	R 5'-GTG TAC TTC GAG CGT TAT AC-3'	29	94 °C 2 min	94 °C 15 s	40 °C 15 s	70 °C 1 min	70 °C 7 min
<i>gapA</i> **	5'-GCC CAT TAC TCA CCC TTG TT-3'	29	94 °C 2 min	94 °C 15 s	40 °C 15 s	70 °C 1 min	70 °C 7 min

* Lee and Ruby, 1995.
 † Oligonucleotide primers designed in this study.
 ‡ Ast and Dunlap, 2004.
 § Modified from Budsberg *et al.*, 2003.
 ¶ Wimpee *et al.*, 1991.
 ** Nishiguchi and Nair, 2003.

molecular matrix in which a value of 1 was given to each transition, transversion, insertion, deletion and gap. Combined phylogenetic analysis produced a total of 54 875 156 trees examined in TBR after the initial construction of a total of 3948 trees. In total, there were 54 879 104 trees examined in all branch swapping strategies. This process took a total of 102.8 h. A total of 241 838 974 alignments were performed by direct optimization during this time.

Tree search was implemented by TBR (tree bisection and reconnection) branch-swapping on the best of 100 random addition replicates, holding 1000 trees per round and performing one round of tree-fusing (Goloboff, 1999). At the same time, the command “exact” was used to guarantee a more accurate calculation of tree costs for direct optimization on the downpass; it also performs a complete Sankoff optimization (Sankoff and Rousseau, 1975). Binary trees were plotted in TreeView 0.4.1 and the consensus tree was calculated using PAUP*4.0.10 (Swofford, 2002). Analysis was executed in the NetBSD biology computer system at New Mexico State University.

All newly sequenced 16S rRNA, *gapA* and *luxA* loci have been deposited in GenBank with the accession numbers shown in Table 1 (strains isolated from this study are in bold type in Table 1). Previously determined gene sequences were obtained from GenBank.

Results and discussion

Quantification of bacterial numbers of loliginid and sepiolid light organ symbionts from Thailand

Colonies isolated from light organ homogenates of either *Uroteuthis*, *Loliolus* or *Euprymna* hosts were luminescent on SWT plates. In average, 10 light organs were extracted from this location in Thailand and used for strain isolation. A total of 40 isolates from each host specimen were extracted, with one to five isolates randomly selected for analyses (Table 1). Colony forming units (CFUs) from each light organ homogenate were enumerated to calculate the total number of CFUs present in each squid light organ. Adult light organs from both *U. chinensis* and *U. duvauceli* ranged from 10^{11} to 10^{12} CFU/light organ, whereas *E. hyllebergi* light organs contained approximately 10^9 – 10^{10} CFU/light organ.

Bacterial growth and light production

Results of the luminescence assays are shown in Table 3. Growth and light production clearly demonstrated that peak luminescence (the point of time in which the amount of light units per milliliter reaches its maximum), coincides in most cases with a period at

Table 3
Light emission of bacterial isolates

Bacterial isolate	Maximum luminescence (light units/mL)	Bacterial isolate luminescence (luminescence/cell)	Time (min) of maximum luminescence
<i>Vibrio fischeri</i> ES114	0.045	0.023	600
<i>Vibrio fischeri</i> ET101	0.59	0.52	420
<i>Vibrio harveyi</i> EH701	3490	7759.6	420
<i>Vibrio fischeri</i> WH1	159.1	158.9	480
<i>Vibrio fischeri</i> CG101	4.25	3.24	540
<i>Vibrio fischeri</i> VLS2	136.9	91.6	450
<i>Photobacterium leiognathi</i> RM1	123.4	227.1	300
<i>Vibrio fischeri</i> PP3	2.05	1.39	330
<i>Vibrio fischeri</i> PP42	0.27	0.17	330
UCP1	119.8	68.25	420
UCR1	134.9	91.74	300
UCR4	195.2	107.4	240
UCR6	3011	1347.6	420
UCR7	9998	4489.3	270
EHR1	44.14	19.48	300
EHR2	16.13	7.61	270
EHR3	29.1	13.06	330

which bacteria are in stationary phase. Strains in Table 3 showed a unique peak of maximum luminescence occurring in culture. From this group of strains, symbionts from *Euprymna* (ES114 and ET101) exhibited the lowest light production, registering between 0.045 and 0.6 light units per milliliter. Strains PP3 and PP42, both free-living *V. fischeri* strains from Hawaii, emitted their peak luminescence simultaneously at 330 min, but their intensities varied slightly, with PP3 higher (~2 light units per milliliter). It is noticeable that strains with the lowest light production grouped together in the combined phylogenetic tree (Fig. 1), in a clade containing other sepiolid squid symbionts, such as *V. fischeri* ES191, ES915 and EM17.

The remaining free-living strains VLS2 and WH1 exhibited similar values of light production (Table 3). Both strains produced peak luminescence after 450 min and production of light was similar: 140 and 160 light units per milliliter for VLS2 and WH1, respectively.

Even though UCP1 and UCR1 displayed relatively similar luminescence, their peak luminescence occurred at different times during growth. UCP1 isolated from *Uroteuthis chinensis* from Phuket, emitted its peak

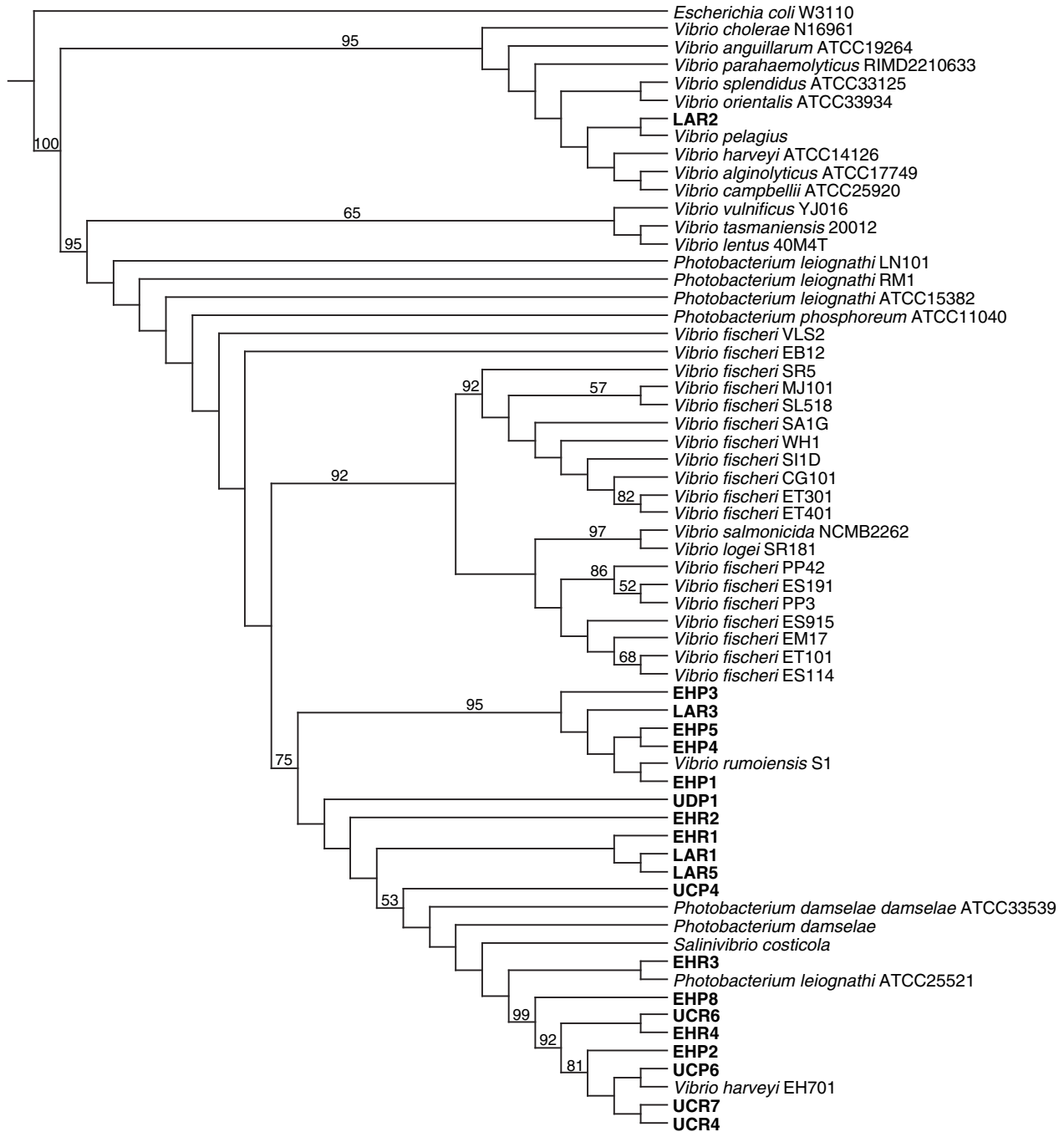


Fig. 1. Phylogenetic tree of *Vibrionaceae* species and isolates, based on DNA sequences of three loci and 95 biochemical characters. The tree was obtained with insertions, deletions, transitions and transversions equally weighed. Jackknife percentages of more than 50% are shown as numbers on nodes. Strains isolated from this study are in bold.

luminescence at 420 min, whereas UCR1, also from the same squid species but collected in a different location (Rayong), emitted higher luminescence at 300 min. UCR4, an isolate from *U. chinensis* (Rayong), displayed 195.2 light units/mL (specific luminescence of 107.4). This value is close in range to ones emitted by other

isolates from the same host, but peaked earlier in the assay. Also, light production values from Thailand strains were comparatively higher than levels exhibited by symbiotic *V. fischeri* strains ES114, ET101, CG101 and free-living *V. fischeri* strains PP3 and PP42. High values of light produced by VLS2 are due to the very

luminous phenotype of this *V. fischeri* strain isolated from Hawaii (Lee and Ruby, 1995).

Thailand strains showing high levels of bioluminescence are found forming a group that also includes *Vibrio harveyi* EH701. Previous research has shown that luminescence in *V. harveyi* strain B392 reaches a higher intensity than *V. fischeri* strain ATCC 7744 (Meighen, 1999). These results indicate that Thailand strains are comparable with *V. harveyi* in that their luminescence is greater than *V. fischeri*. Measurements of light intensity from this study may indicate that Thailand symbiotic strains are more closely related to *V. harveyi* than to *V. fischeri*, which is confirmed by the phylogenetic data (see next section) in Fig. 1.

Combined phylogenetic analysis of 16S rRNA, gapA, luxA sequences and biological data

Using several loci and combining molecular information with biochemical attributes has been described as an appropriate approach to understanding relationships among members of a group of taxa (Giribet and Wheeler, 2002; Nishiguchi and Nair, 2003). By exploring our data set using a sensitivity analysis it was possible to generate hypotheses of relationships and at the same time evaluate their stability.

Direct optimization produces more parsimonious and congruent explanations of sequence variation than multiple sequence alignment (Giribet et al., 2001). Without the use of multiple sequence alignment, the method assesses the number of transformations in DNA sequence required by a phylogenetic topology (Giribet and Wheeler, 2002). Therefore, it is clear that the most parsimonious hypothesis is represented by the phylogeny reported here.

The most parsimonious tree (Fig. 1) produced from the analyses using all parameter sets that minimized overall incongruence among the 16S rRNA, *gapA*, *luxA* genes and phenotypic data, demonstrates that the clade containing symbiotic *Vibrio fischeri* is not monophyletic. A number of internal clades contain symbiotic isolates, irrespective of host or geographic location, although support for some internal nodes is below the 50% threshold. This group also includes free-living bacteria, as well as both fish and squid symbionts, indicating that symbiosis in these *V. fischeri* strains has arisen multiple times. *Vibrio salmonicida* and *V. logei* SR181 group in a single clade for both 16S rRNA analysis (data not shown) and the combined phylogenetic analysis (Fig. 1). Previous molecular and biochemical data (Fidopiastis et al., 1998; Nishiguchi and Nair, 2003) has demonstrated that *V. salmonicida* is a close relative (and possible sibling species) to *V. logei*, which provides additional evidence that these species of bacteria may share a common ancestor that was psychrophilic in nature.

Our analysis also resolved the placement of *V. fischeri* fish isolates CG101 and MJ101 within a more uniform group with strains from France (SR5 and SL518) and Australia (ET401 and ET301) as well as the free-living strain WH1. Interestingly, symbiotic strain LAR2, isolated from *Loliolus affinis* (Loliginidae), did not group with clades containing the remaining squid strains. This clade contains only *Vibrio* species, including recently described species found either associated with or causing disease to marine organisms (Banatvala et al., 1997; Thompson et al., 2001, 2003). Additionally the node was well supported, with a jackknife value of 95% (Fig. 1). Species *V. vulnificus*, *V. tasmaniensis* and *V. lentus* also grouped in one clade with 65% support. Thompson et al. (2004) reported a close relationship between *V. lentus* and *V. tasmaniensis*, which coincides with the phylogenetic hypotheses presented here that these species are sister taxa.

All Thailand strains isolated from either *Uroteuthis*, *Loliola* or *Euprymna* squid hosts (with the aforementioned exception of isolate LAR2) grouped in one separate clade with *Vibrio harveyi* EH701 (previously described by Nishiguchi and Nair, 2003), *Vibrio rumoiensis*, some *Photobacterium* species and *Salinivibrio costicola* (Fig. 1). This result suggests that Thailand molecular variants, found colonizing light organs in loliginid and sepiolid squid are neither *Vibrio fischeri* nor *Vibrio logei*, which are the bacterial species commonly found as light organ symbionts. This is a relationship not previously reported in squid.

Our combined analysis produced a phylogeny that was able to resolve Thailand isolates with a relatively high bootstrap value (75%). Within this clade, there is one node (95% jackknife support), containing isolates EHP3, LAR3, EHP5, EHP4 and EHP1, which includes the recently described species *Vibrio rumoiensis* (Yumoto et al., 1999). This facultative psychrophilic bacterium is known to exhibit high catalase activity, which may explain why Thailand squid symbionts exhibit a closer relationship to identified strains of *V. rumoiensis*. Previous studies with *E. scolopes* light organs have demonstrated high levels of halide peroxidase present in this organ (Small and McFall-Ngai, 1998). This enzyme produces potent microbicides in response to pathogens by generating hypohalous acids from hydrogen peroxide. In bacteria, catalase is used to detoxify any host generated hydrogen peroxide (Ruby and McFall-Ngai, 1999) therefore giving an enormous ecological advantage to those bacteria exhibiting high catalase activity in the light organ. Production of this enzyme would allow bacteria to combat any immunological response, and successfully colonize light organ tissue. Any host-generated hydrogen peroxide would therefore be neutralized before it has the chance to enter the bacterium. Ecological variants related to *V. rumoiensis* and possibly

sharing this beneficial defensive phenotype will easily colonize squid light organs.

With respect to isolates UDP1, EHR2, EHR1, LAR1, LAR5 and UCP4, there is no clear resolution in the phylogenetic tree. However, they are clearly part of the clade containing the Thailand isolates, which separate them from the *V. fischeri* group.

In addition, *Vibrio harveyi* EH701 is also included in the Thailand clade, indicating a possible relationship between luminescent symbionts and *V. harveyi*. *V. harveyi* has been found to produce much higher luminescence in culture than *V. fischeri* (Meighen, 1999), which may increase its ecological advantage over potential but low light producing symbionts. *V. harveyi* has been recognized as a cause of disease in marine invertebrates, particularly crustaceans used for aquacultural farms. Similarly, a *V. harveyi*-like organism, classified as *Vibrio carchariae* has been isolated from sharks (Austin and Zhang, 2006). Thus, *V. harveyi* is classified as a pathogen of invertebrates and vertebrates. Furthermore, Pizzutto and Hirst (1995) reported the high diversity within the species by genetic fingerprinting and protein profiling in an attempt to differentiate virulent from avirulent strains of *V. harveyi*. The authors concluded that virulence and phylogenetic history are not associated, which led them to consider that virulence was acquired by association with mobile genetic elements, such as plasmids or transposons. It is then possible that the Vibrionaceae strains found colonizing light organs in Thai squid correspond to a non-virulent form of *V. harveyi* with the conserved ecological advantage of being highly luminescent.

Within this clade other species such as *Salinivibrio costicola*, *Photobacterium damsela*, and *Photobacterium leiognathi* are also found, supporting previous tested hypotheses proposed by Kita-Tsukamoto et al. (1993), Alsina and Blanch (1994) and Ruimy et al. (1994).

Of importance to this study is the fact that isolates obtained from a common host found in the same geographic area are embedded within a large clade that contains a variety of bacterial species with characteristics that make them excellent candidates as bacterial symbionts. However, strains isolated from a particular squid host are not found to be phylogenetically related to a unique bacterial species. This result indicates a possible non-specific association between Thai squid with marine luminescent bacteria. The results presented here clearly suggest that symbioses between Vibrionaceae bacteria and their squid hosts are family specific and not strain specific. This is in contrast to earlier studies of sepiolid squid symbionts (Nishiguchi et al., 1998), where parallel cladogenesis occurred between native strains and their host squid. More recently, better taxon sampling among strains within and between populations of host squid has provided a more complex picture of *Vibrio*-sepiolid squid dynamics than previ-

ously thought (Jones et al., 2006). Introgression of shared haplotypes among strains isolated from different host species seems to be more common than previously thought, with genetic breaks observed at locations where abiotic factors (i.e., temperature) seem to be a major influence. As sepiolid and loliginid squid in Thailand are colonized by more than one species from the Vibrionaceae, this suggests that specificity for genera of this family still exists, but is influenced by additional factors besides host species. Future studies to determine whether homologous genes are co-opted for similar functions between these bacteria will help determine the level of specificity that is required for symbiosis within the Vibrionaceae, and whether abiotic or biotic factors have an effect for determining species level colonization in this environmentally transmitted association.

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