Museum genomics illuminate the high specificity of a bioluminescent symbiosis across a genus of reef fish

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Abstract

Symbiotic relationships between bioluminescent bacteria and fishes have evolved multiple times across hundreds of fish taxa, but relatively little is known about the specificity of these associations and how conserved they have been through time. This study describes the degree of specificity of a bioluminescent symbiosis between cardinalfishes in the genus *Siphamia* and luminous bacteria in the Vibrio family. Primarily using museum specimens, we investigate the co-divergence of host and symbiont and test for patterns of divergence that correlate with both biogeography and time. Contrary to expectations, we determined that the light organ symbionts of all 14 *Siphamia* species examined belong to one genetic clade of *Photobacterium mandapamensis* (Clade II), indicating that the association is highly specific and conserved across the host genus. Thus, we did not find evidence of codivergence among hosts and symbionts. We did observe that symbionts hosted by individuals sampled from colder water regions were more divergent, containing more than three times as many single nucleotide polymorphisms than the rest of the symbionts. Overall our findings indicate that the symbiosis between *Siphamia* fishes and *P. mandapamensis* Clade II has been highly conserved across a broad geographic range and through time, despite the facultative nature of the bacterial symbiont. These results suggest that this bioluminescent symbiosis could have played a key role in the evolution of the host genus and that there are conserved mechanisms regulating its specificity that have yet to be defined.

Introduction

Environmentally transmitted microbial symbionts are acquired by a host from a genetically diverse, free-living population of bacteria. These facultative symbionts must retain the genetic machinery necessary to associate with their hosts, while also being able to compete with the rest of the microbial community in the surrounding environment (Bright and Bulgheresi 2010). In the marine environment abiotic factors such as water flow and temperature play critically important roles in structuring the microbial community (Galand et al., 2010, Brown et al., 2012), and accordingly, the available free-living symbiont pool. Even though a colossal diversity of bacteria remains available to marine hosts, the associations between hosts and their microbial symbionts are highly specific; much more so than what would be expected based solely on diversity in the surrounding seawater (Trousselier et al., 2017). Thus, the combined influence of host attributes and abiotic factors contributes to the complexity of the specificity of

environmentally transmitted host-symbiont associations, providing the opportunity to study the evolution of specificity and co-diversification of these critical associations.

Bioluminescent symbioses have evolved multiple times across diverse squid and fish taxa, including at least 17 times in the ray-finned fishes (Davis et al., 2016; Dunlap and Urbanczyk, 2013). Approximately 500 species of fish are known to be symbiotically bioluminescent, but our understanding of specificity between fish hosts and their bacterial symbionts is just emerging. Existing evidence suggests that some level of specificity between host and bioluminescent symbiont is maintained, at least at the host family level. For example, leiognathid fishes exclusively host *Photobacterium leiognathi* and *P. mandapamensis* (Kaeding et al. 2007), and ceratioid anglerfishes, representing four different host families sampled over a broad geographic range, only host two bacterial species, *Enterovibrio escacola* and *E. luxaltus* (Baker et al. 2019). Specificity has been described in 35 additional fish hosts, comprising 7 families (Dunlap et al. 2007). This host family level of bacterial specificity is believed to result from the host fish selecting for its particular symbiont while also preventing other bacteria from colonizing its light organ (Reichelt et al., 1977). Although fish hosts only associate with a narrow range of luminous bacteria, the symbionts are generally not obligately dependent on their host (but see Hendry et al., 2014) and can survive in a variety of other habitats including seawater, sediment, and the surfaces and digestive tracts of various marine organisms. Thus, the specificity of bioluminescent symbioses depends largely on host selectivity and the genetics of the association.

Within the cardinalfish family (Perciformes: Apogonidae), bioluminescence has evolved multiple times, however only species in the genus *Siphamia* rely on a symbiotic relationship with luminous bacteria to produce light; all other bioluminescent cardinalfishes produce their own light, presumably via the acquisition of luciferin from their diet (Thacker and Roje 2009). All 25 species of *Siphamia* are symbiotically bioluminescent (Thacker and Roje 2009; Gon and Allen 2012). The fish possess a ventral light organ connected to the intestine, which functions to host a dense population of luminous bacteria (~10^8 cells) (Fig.1) (Dunlap and Nakamura 2011). The symbionts are ingested by the host during larval development and subsequently colonize the host's light organ (Dunlap *et al.,* 2012).

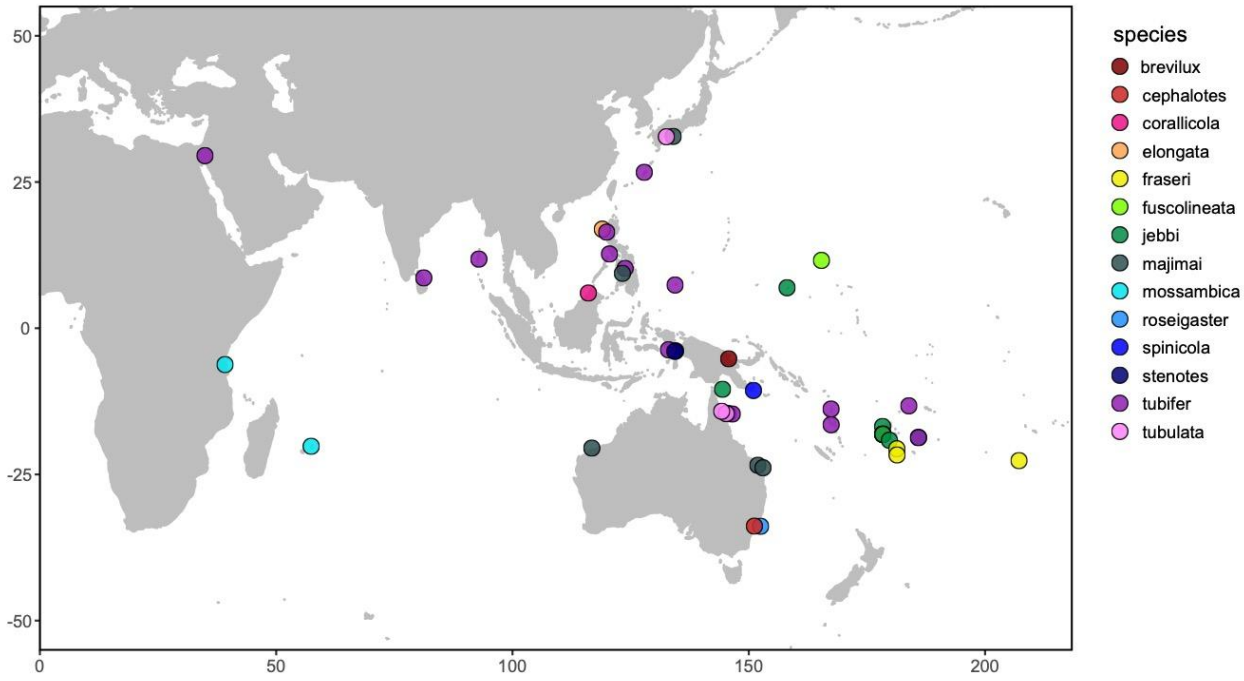

Figure 1. Map depicting the sampling locations of the Siphamia specimens examined in this study. Colors represent different *Siphamia* species as indicated in the figure legend.

The *Siphamia-Photobacterium* symbiosis readily lends itself to study both in the field and in the laboratory because, unlike most bioluminescent fish which occur in deep or open water environments, *Siphamia* reside in shallow waters with high habitat fidelity (Gould *et al* 2014). Furthermore, both host and symbiont can be readily cultured in captivity, making them ideal study organisms for both field and laboratory investigations (Dunlap *et al.,* 2012). However, the luminous symbionts of only one *Siphamia* species, *S. tubifer*, originating from a small geographic region in the Okinawa Islands, Japan, have been characterized to date; the specimens examined were found to host only members of Clade II of *Photobacterium mandapamensis* in their light organs, suggesting a high degree of specificity for this association (Kaeding *et al.,* 2007, Gould and Dunlap 2019). *Siphamia tubifer* is broadly distributed throughout the Indo-Pacific, spanning from eastern Africa to the French Polynesian Islands (Gon and Allen 2012), thus the true degree of specificity across the geographic range of this association remains unknown. Furthermore, the luminous symbionts of the other 24 species in the host genus have yet to be identified.

The primary goals of this study were to characterize the degree of specificity of the bioluminescent symbiosis throughout the *Siphamia* genus and across the broad geographic range of *S. tubifer*. Taking advantage of previous collection efforts, we sampled geographically

and temporally diverse *Siphamia* specimens (Fig. 2) from several natural history museums. Recovering genetic information from wet specimens, particularly those initially fixed in formalin, is a new frontier in museum genomics. Here we present methods for extracting and sequencing the DNA of both a bacterial symbiont and its vertebrate host. Thus, we were able to test for evidence of co-diversification of host and its symbiont, and for patterns of symbiont diversity at the clade-level that correlate with biogeography, temperature, and time.

Figure 2. Photographs of select *Siphamia* specimens from lots used in this study. Specimens a-c represent the *tubifer* subgroup (Gon and Allen 2012), identified by the striated light organ (a) and specimens d-f represent the *tubulata* subgroup, identified by the spotted light organ (d). (a) *S. tubifer* (USNM341595) with insert of light organ detail showing striated morphology. (b) *S. stenotes* (USNM396981, paratype). (c) *S. brevilux* (CAS65338, paratype). (d) *S. tubulata* (CAS28515) with insert of light organ detail showing spotted morphology. (e) *S. corallicola* (USNM203781). (f) *S. jebbi* (CAS223855). Scale bars indicate 1 cm in length.

Methods

Taxon sampling and DNA extraction.

We sampled 59 specimens representing 14 *Siphamia* species obtained from the combined wet collections of the California Academy of Sciences, the Australian Museum, and the Smithsonian National Museum of Natural History (Figs. 1-2, Table 1). To extract DNA from these specimens, we adapted the following protocol from two previous methods designed for use with formalin-fixed tissues (Ruane and Austin 2017, Hykin *et al.,* 2015). Light organs were aseptically dissected and individually placed into 1 ml of GTE buffer and allowed to soak for three hours at room temperature. This step was repeated two times after which each light organ was transferred into a final 1 ml aliquot of fresh GTE buffer and left to soak overnight at room temperature. The following morning, each sample was transferred into 1 ml of 100% ethanol for one minute, followed by 1 ml of 70% ethanol for 5 minutes, and 1 ml of nuclease-free water for 10 minutes at room temperature. Light organs were then transferred into 180 ul of pre-heated (98°C) ATL buffer (QIAGEN) and incubated at 98°C for 15 minutes, after which samples were immediately placed on ice for at least 2 minutes. Once cooled, 40 ul of proteinase K was added to each sample and the samples were incubated at 60°C for 48 hours on a shaking heat block. Samples were vortexed periodically and additional 20 ul aliquots of proteinase K were added as needed (up to 100 ul total). Following this incubation period, DNA was extracted using the QIAGEN DNEasy Blood and Tissue Kit as described by the manufacturer. Purified DNA products were eluted into 50 ul of nuclease-free water after a 3-minute incubation at 55°C.

Library preparation and sequencing.

Samples were quantified using the Qubit dsDNA HS Assay Kit on the Qubit 2.0 Fluorometer (Invitrogen) and profiled with an Agilent 2100 Bioanalyzer. Samples with a peak in size distribution greater than 300 bp were sonicated with a Qsonica (Q800R3) for one or two minutes (if peak was greater than 1,500 bp) with a pulse rate of 10-10 seconds and an amplitude of 25%. Samples were then treated with the NEBNext© FFPE DNA Repair Mix following the manufacturer's instructions and DNA libraries were immediately prepped using the NEBNext© Ultra II DNA Library Prep Kit. Samples with low or undetectable quantities of dsDNA were re-quantified using the Qubit ssDNA HS Assay Kit and prepared using the Accel-NGS 1S Plus

DNA Library Kit (Swift Biosciences), which uses both single- and double-stranded DNA as templates. Each sample was uniquely indexed with the NEBNext© Multiplex Oligos for Illumina. Final libraries were cleaned with AMPure XP magnetic beads, pooled, and sequenced as single-end 150 bp (UC Berkeley, QB3) or paired-end 150 bp (NovoGene) reads on the Illumina HiSeq 4000 platform, or as paired-end 150 bp reads on the Illumina NovaSeq S4 platform (Genewiz). Table S1 contains details for each sample and library preparation.

Sequence analysis.

Sequences were demultiplexed, trimmed and quality filtered for a Phred score of 20 or above using Trimmomatic (Bolger *et al.,* 2014). The remaining reads were aligned to the reference genome of *Photobacterium mandapamensis*, isolated from the light organ of *Siphamia tubifer* (Urbanczyk *et al.,* 2011) with BWA-MEM (Li 2013). Unaligned sequences were then processed with MitoFinder (Allio *et al.,* 2020) using the reference mitochondrial genome of the Banggai cardinalfish *Pterapogon kauderni* (Matias and Hereward 2018)). All cardinalfish cytochrome oxidase subunit 1 (*COI*) gene sequences that were recovered aligned using MUSCLE (Edgar 2004) and a maximum likelihood analysis was carried out with raxml-ng (Kozlov*et al.,* 2019) using the evolutionary model TIM2+F+I+G4 which had the lowest BIC score as predicted by IQtree (Nguyen *et al.,* 2015) and 1,000 bootstraps to infer the phylogenetic relationships between host species. *COI* sequences of *Siphamia* spp. from previous studies were also included in the analysis (Table S2). An additional phylogeny was inferred from a supermatrix of 15 mitochondrial genes (*ATP6, ATP8, COXI, COX2, COX3, CYTB, ND1, ND2, ND3, ND4, ND4L, ND5, ND6, 16S, 18S*) identified by MitoFInder that were present in at least 70% of the individuals included in the analysis using the SuperCRUNCH python toolkit (Portik and Wiens 2020). The concatenated supermatrix alignment was used in a maximum likelihood analysis by raxml-ng with 500 bootstrap replicates and the evolutionary model TIM2+F+R4 as predicted by IQtree to infer the phylogenetic relationships between species.

Two approaches were used to determine the identity of the light organ symbionts. First, 16S rRNA gene sequences were extracted from each data set by aligning all light organ sequences to the complete 16S sequence of a free-living strain of *Photobacterium leiognathi* (AY292917)(Nishiguchi and Nair 2003) with BWA-MEM (Li 2013). A sequence similarity search was then performed with the basic local alignment search tool (BLAST) (Altschul *et al.,* 1990) against NCBI's microbial database to identify the known sequence with the lowest E-value and highest

percent identity. Second, the average nucleotide identity (ANI) of each sample was calculated relative to several *Photobacterium* species for which entire genome sequences are available from the NCBI genome database (*P. kishintanii* pjapo1.1 - NZ_PYNK00000000; *P. leiognathi* lrivu4.1 - NZ_BANQ00000000; *P. mandapamensis* ajapo4.1 - NZ_PYNQ01000000; *P. mandapamensis* gjord1.1 - NZ_PYNP00000000; *P. mandapamensis* svers1.1 - NZ_PYNT00000000) with the program fastANI (Jain *et al.,* 2018).

To infer the phylogenetic relationships between symbionts from different hosts, all sequences that aligned to the reference genome of *P. mandapamensis* (Urbanczyk *et al.,* 2011) were also analyzed for sequence variation with the program snippy (Seemann 2015), requiring a minimum depth of 10x and a minimum percent of reads to be 90% to call a variant. A sequence alignment based on a core set of single nucleotide polymorphisms (SNPs) was then created across symbionts with enough genome coverage to produce a core set of at least 1,000 SNPs and including two additional reference genomes of *P. mandapamensis* representing both Clade I (ajapo4.1) and Clade II (Res4.1). The phylogenetic relationships of these bacteria were then inferred with raxml-ng (Kozlov *et al.,* 2019) using the evolutionary model model TVM+F+R3, which had the lowest *BIC* score as predicted by IQtree (Nguyen *et al.,* 2015) and 1,500 bootstrap replicates.

Samples included in both the host and symbiont phylogenies were then compared and tested for co-divergence using the cospeciation function in the R phytools package (Revell 2012). SNPs were annotated with the program SNPeff (Cingolani *et al.,* 2012). Pairwise phylogenetic (patristic) distances between symbionts were calculated with the adephylo package (Jombart and Dray 2008) in R, and pairwise geographic distances were calculated based on each specimen's latitude and longitude using the R package geodist (Padgham and Summer 2020). Tests for correlations between the phylogenetic distances for each pair of symbionts and their geographic distance or difference between sampling years were carried out, and P values were adjusted for multiple comparisons with the Holm method in R.

Results

DNA recovery

Variable amounts of total DNA were recovered from the light organs of preserved *Siphamia* specimens, ranging from undetectable levels (<2 ng) to more than 1,500 ng, and there was no correlation between DNA yield and specimen size (Spearman's rank correlation: rho=0.52, P=0.09). Despite this variability in yield, quality DNA sequences were recovered from several specimens with undetectable levels of starting DNA. In fact, some samples with undetectable levels of input DNA resulted in >90% coverage of the symbiont genome at 10x depth. Of note, many of those sequence libraries were prepared using the Swift Bioscience Accel-NGS 1S Plus DNA Library Kit which uses both double and single stranded DNA as starting template (Table S1).

Host phylogeny

Host *COI* sequences were recovered from 32 samples and analyzed with an additional 12 *Siphamia COI* sequences from previous studies (Table S2) to generate a maximum likelihood phylogeny of 17 *Siphamia* species (Fig 3). The supermatrix of 15 mitochondrial genes from 27 *Siphamia* specimens representing 11 species resulted in a phylogenetic tree with similar, but not identical, topology and stronger bootstrap support at the nodes (Figure S1).

Our phylogenetic hypothesis for *Siphamia* is very similar to that proposed by Gon and Allen (2012) using morphological characters, with slight variations in the placement of specific taxa. Our tree contains a clade that corresponds to Gon and Allen's *S. tubifer* species group, characterized by a striated pattern on the light organ (Fig 2), although one individual *S. majimai* and two *S. jebbi* specimens fell out of this group (Fig 3). Within this group, our trees support the relationships of *S. jebbi* and *S. stenotes* as sister species, as well as *S. tubifer* and *S. fraseri*. The relative placement of *S. mossambica*, *S. majimai,* and *S. goreni* varies among the trees, but there is support for *S. mossambica* and *S. goreni* as sister species in the *COI* tree, *S. mossambica* and *S. majimai* as sisters in the supermatrix tree, and *S. majimai* and *S. goreni* as sisters in the morphological tree (Gon and Allen 2012). As such, it is likely that all three of these species belong to one clade. The relationships among the species outside of the *S. tubifer* group are less certain, with several species clustering into species complexes. However, *S. roseigaster*, *S. cuneiceps*, and *S. cephalotes* consistently fall out as sister taxa to the rest of *Siphamia*, indicating that their lineages diverged earlier.

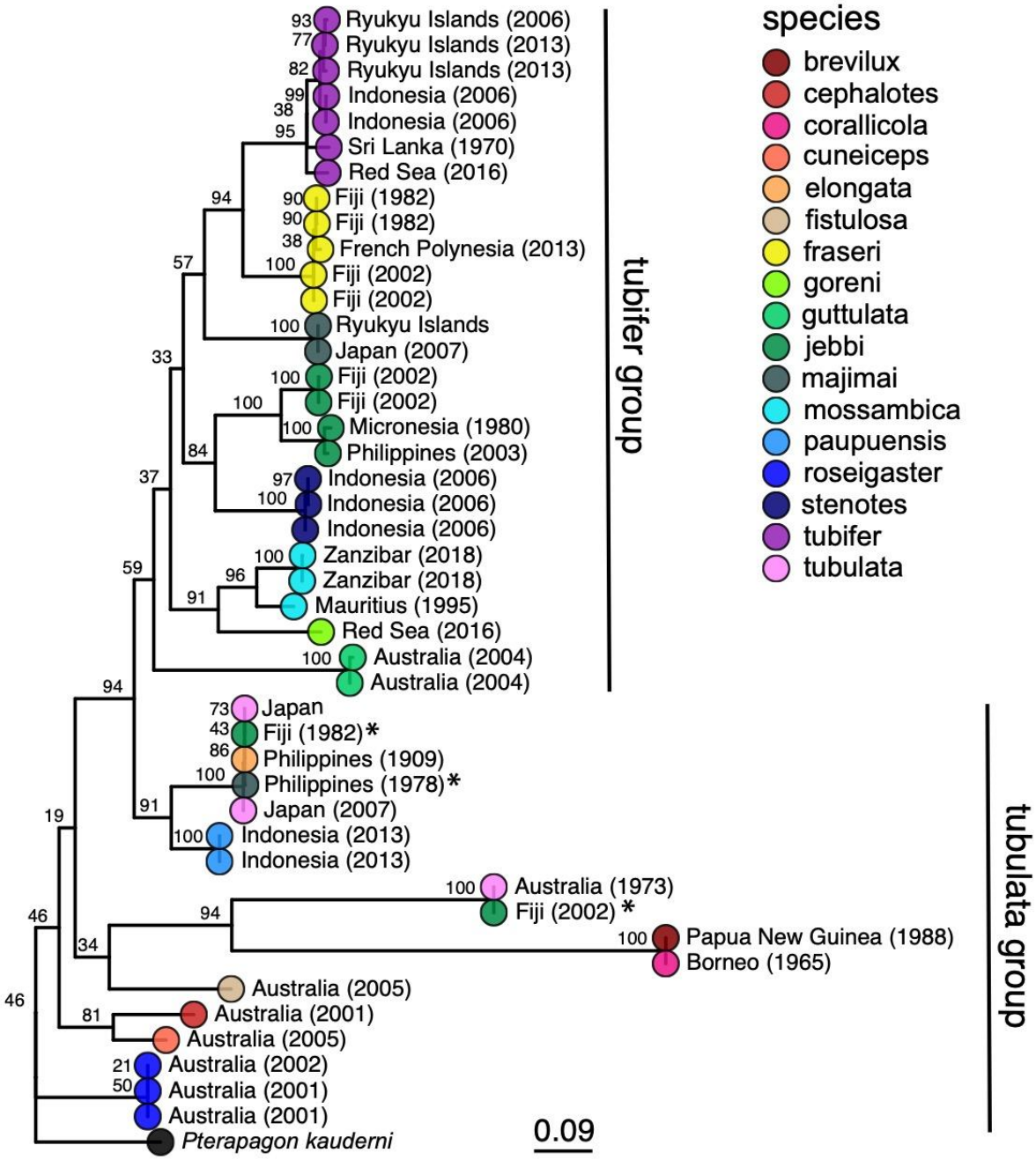

Figure 3. Maximum likelihood phylogeny of *Siphamia* based on *COI* gene sequences. Species identities are indicated by the branch tip colors and the sampling location and year of each specimen is listed in the branch label. Bootstrap support values are indicated at each node. The Banggai cardinalfish, *Pterapogon kauderni,* was used as the outgroup. The *tubifer* and *tubulata* subgroups within *Siphamia* (Gon & Allen 2012) are highlighted with vertical lines to the right of the tree. Specimens that fall outside of their designated subgroup based on species identities are indicated with an *.

Symbiont identification and phylogeny

To identify the light organ symbionts of the *Siphamia* hosts, we recovered 16S rRNA sequences from the shotgun sequence data. 93% of all samples had >95% coverage of the 16S rRNA gene at 10x read depth. Samples were putatively identified by matching sequences against those in the NCBI database and 67% had *P. mandapamensis* as their top hit (Table S3.). All other symbionts were identified as *P. leiognathi.* However, previous analyses of 16S rRNA gene sequences could not resolve *P. leiognathi* from *P. mandapamensis* (Ast and Dunlap 2004, Wada *et al.,* 2006*).* Therefore, to confirm the identities of the light organ symbionts, we also calculated the average nucleotide identity (ANI) of each symbiont relative to several *Photobacterium* strains for which whole genomes are available. 86% of the symbionts examined had ANI values relative to *P. mandapamensis* strains in Clade II (gjord1.1 and svers1.1) of 95% or greater (Table 2), which is the recommended value to delimit bacterial species (Goris *et al.,* 2007). All remaining samples also had the highest ANI values relative to *P. mandapamensis* Clade II, with the exception of one sample (AMI18740-066), which was most similar to *P. mandapamensis* Clade I, however many of these samples had low genome coverage (Table S1). None of the symbionts had higher ANI values relative to *P. leiognathi.*

Single nucleotide polymorphisms (SNPs) were detected for the light organ symbionts from most of the specimens sampled, but this number varied greatly and correlated with the variability in genome coverage (Spearman's rank correlation: rho=0.84, P<0.001, Table S1). Samples with greater than 50% symbiont genome coverage at 10x read depth had an average of 23,221 SNPs relative to the reference genome of *P. mandapamensis* (Urbanczyk *et al.,* 2011). A core set of 1,471 SNPs were identified across 32 specimens that represent 11 *Siphamia* host species and included reference genomes from both Clade I and Clade II of *Photobacterium mandapamensis.* 68% of these SNPs were synonymous, and the remaining non-synonymous SNPs were found in 288 distinct genes. None of the core SNPs were located in the *lux* operon, composed of the genes responsible for light production. However, two non-synonymous SNPs were detected in the *rpoN* gene, which is known to play a role in biofilm formation, bioluminescence, and symbiosis initiation for *Aliivibrio fischeri* (Wolfe *et al.,* 2004)*,* the luminous symbiont of many squid and other fish species. No other SNPs were detected in genes of known function for the bioluminescent symbiosis between *A. fischeri* and the squid host *Euprymna scolopes* (Norsworthy and Visick 2013).

A maximum likelihood phylogeny was inferred for the bacterial symbionts using full sequence alignments that included the core set of SNPs described above. This analysis confirmed that all *Siphamia* light organ symbionts examined belong to Clade II of *P. mandapamensis* and that the reference strain of *P. mandapamensis* representing Clade I (ajapo4.1) was a clear outgroup (Fig 4). The majority of symbionts analyzed were closely related to the reference strain svers1.1 of *P. mandapamensis,* although several symbionts fell out in a group with *P. mandapamensis* strain Res 4.1. However, both reference strains are members of Clade. There were three additional symbionts, all from different host species, that did not belong to either of these subgroups, but are still clearly members of Clade II.

No clear patterns of symbiont divergence that corresponded with host species, geography, or collection year emerged. There was no correlation between phylogenetic distance and geographic distance (Spearman's rank correlation: rho=-0.013, P_{corr}=1) and there was a slightly negative correlation between phylogenetic distance and time in years (Spearman's rank correlation: rho=-0.17, P_{corr}=0.006). In fact, the oldest specimen for which informative sequence data was retained was collected in 1931 and it had luminous bacteria in its light organ that was highly similar to symbionts from specimens collected more than eighty years later. Similarly, *Siphamia* specimens collected from locations in the western Indian Ocean had symbionts that were closely related to those from locations as far east as Fiji and even French Polynesia. With respect to *S. tubifer*, which has the broadest geographic distribution of all *Siphamia* species, the symbionts of all ten specimens included in the phylogeny fell out in Clade II of *P. mandapamensis* and showed no pattern of strain diversity by geography, confirming the high degree of specificity of this association, even across a broad geographic range.

The bacterial symbionts from four distinct host species had notably longer branches than the others, two of which were closely related to reference strain Res 4.1 (NZ_PYNS00000000), an isolate from the light organ of *S. tubifer* collected in Okinawa, Japan in 2014. Corresponding with longer branch lengths, these four symbionts had more than 3 times as many SNPs than any other sample, ranging between 66,583 and 72,219 SNPs (Table S1). Interestingly, these four specimens were collected from two locations, Sydney, Australia and Kochi, Japan, which had the lowest minimum annual temperatures of all locations in this study (Table 1). Furthermore, there were 20,082 SNPs in common among these samples that were not present in the core set of SNPs identified across all samples.

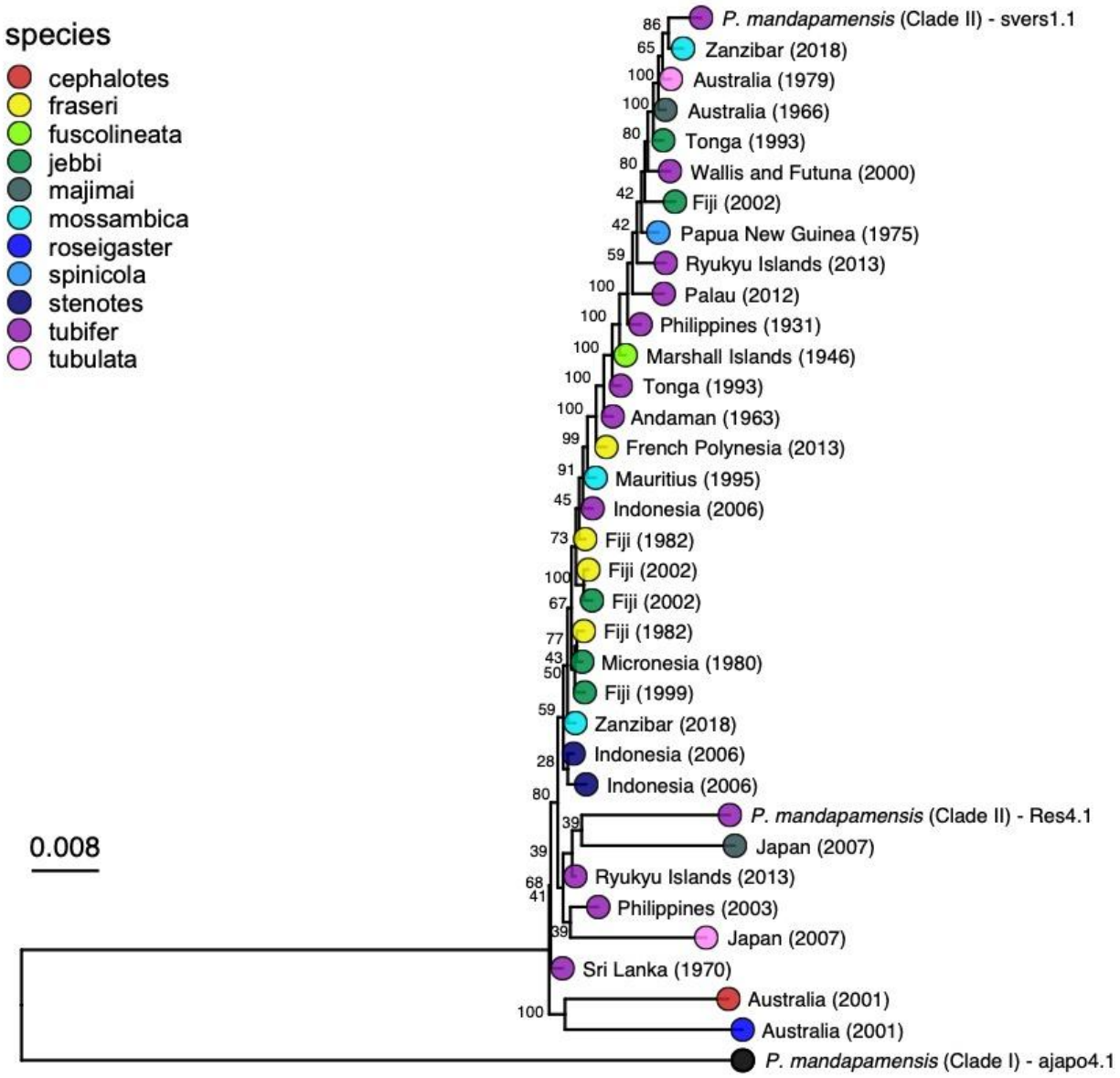

Figure 4. Maximum likelihood phylogeny of the light organ symbionts of various *Siphamia* species constructed from a core set of 1,471 single nucleotide polymorphisms. Corresponding host species are indicated by the branch tip colors and the sampling location and year of each specimen is listed in the branch label. Bootstrap support values are indicated at each node.

Analysis of co-divergence.

Twenty specimens had informative sequence information for both the host and symbiont, and thus, we were able to carry out an analysis of co-divergence based on the host COI phylogeny and corresponding symbiont phylogeny for these individuals. This analysis revealed no evidence of co-divergence of *Siphamia* hosts and their light organ symbionts (P=0.13) as seen in Figure 5. However, *S. roseigaster* and *S. cephalotes* fall out as sister lineages relative to the

rest of *Siphamia*, and their symbionts follow a similar pattern, forming a sister clade to the rest of *P. mandapamensis* Clade II.

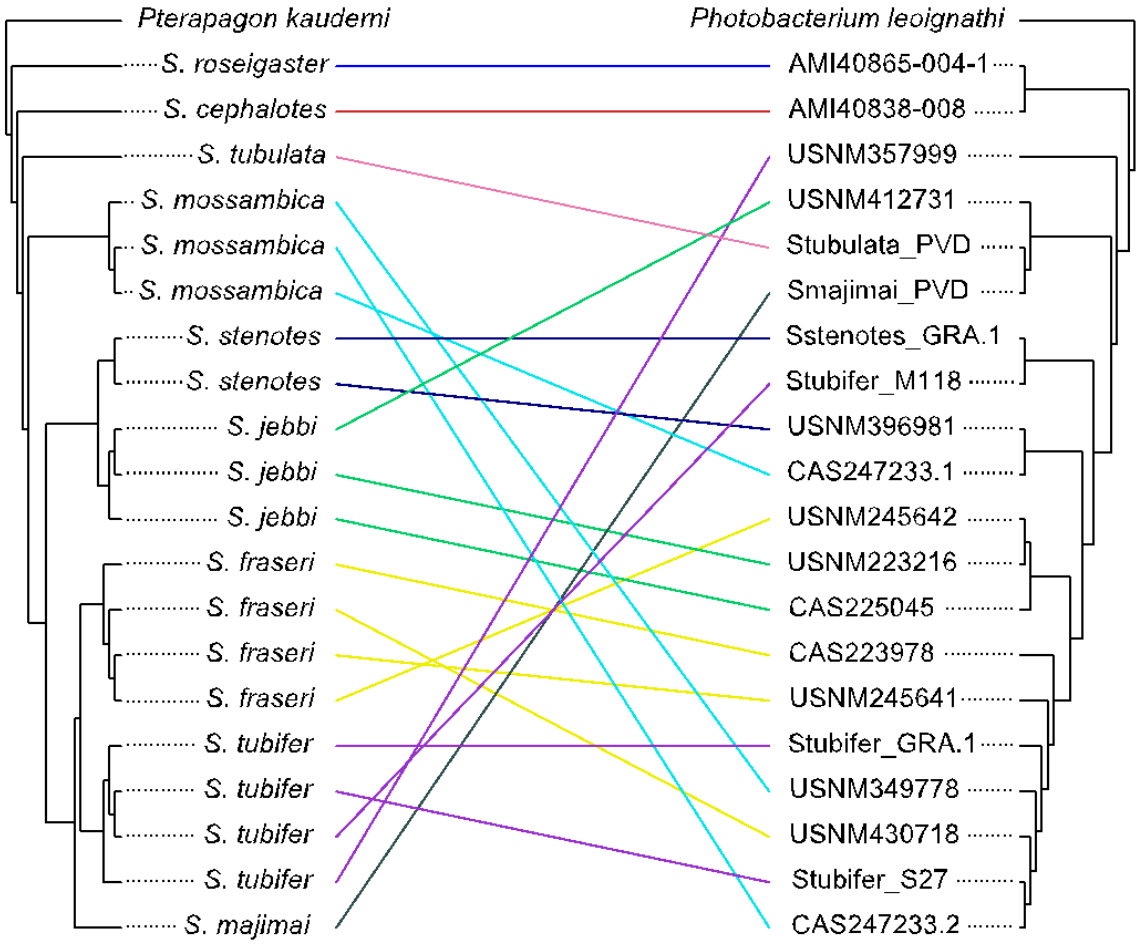

Figure 5. Analysis of the phylogenetic relationships of *Siphamia* hosts (left) and their light organ symbionts (right) revealed no evidence of co-divergence. The host cladogram is based on *COI* gene sequences and the symbiont cladogram is based on a core set of 1,471 single nucleotide polymorphisms. Linkages between individual hosts and their symbionts are shown and colored according to host species.

Discussion

Our results indicate that the symbiosis between cardinalfishes in the genus *Siphamia* and the luminous bacterium *Photobacterium mandapamensis* is highly conserved across host species, over geographic space, and through time. All light organ symbionts examined were identified as strains belonging to Clade II of *P. mandapamensis.* This high degree of specificity is surprising given the facultative symbiotic life history of the bacterium and the broad geographic and

temporal ranges examined. Such a high degree of specificity is expected for vertically transmitted symbioses in which a host directly transfers its symbiotic bacteria to its offspring (Moran 2006). For environmentally transmitted symbioses, where the specific association must be re-established by each new host generation we expected a lower degree of specificity, similar to what has been documented for most other symbiotically luminous fishes such as the leiognathid fishes (Kaeding *et al.,* 2007). Thus, the highly conserved relationship between *Siphamia* hosts and *P. mandapamensis* (Clade II) indicates there may be unique mechanisms in the host and/or symbiont that contribute to maintaining the specificity of the association.

Host larvae only take up symbionts in the pelagic phase, when their light organ becomes receptive to colonization (Dunlap *et al.,* 2012). Yet, *Photobacterium* normally occurs in relatively low concentrations in the pelagic environment, and even more so at the sub-clade level. For the larval host to rely on this improbable encounter in the open water would be considered a very risky strategy. However, it has been shown that established populations of Siphamia hosts regularly excrete/shed their specific symbiont, thereby enriching its population in the immediate environment (Trousselier *et al.,* 2017). Indeed, a previous study of *S. tubifer* symbiont genomics revealed fine-scale population structure among geographic locations, indicating that symbiont populations are heavily influenced by their local hosts. Thereby this local enrichment may be a key mechanism/factor in mitigating the risk of relying on environmental transmission in the Siphamia-symbiont dependency (Gould and Dunlap 2019), and for *Siphamia*, ensures that *P. mandapamensis* (Clade II) will be readily available to new recruits anywhere that *Siphamia* species already occur.

The apparent preference to associate with *P. mandapamensis* Clade II over strains in Clade I also suggests that there are critical strain level differences between members of these clades that could be of consequence to the host. However, most studies of microbial symbioses overlook symbiont strain variation, even though this variation can be of huge consequence for a host, and merits further investigation. For example, in *A. fischeri*, the primary symbiont for most *Euprymna* squid species, patterns of strain variation have been observed within and between host populations (Jones *et al.,* 2006, Wollenberg and Ruby 2009), and can have different colonization efficiencies (Lee and Ruby 1994, Bongrand *et al.,* 2016), mechanisms of biofilm formation during host colonization (Rotman *et al.,* 2019), and could have variable fitness consequences to their host (Koch *et al.,* 2014).

In this study we characterized strain variation in *P. mandapamensis* associated with various *Siphamia* hosts. There was no distinct correlation between symbiont strain and host species with respect to time or geography, although we did observe some strain divergence associated with colder temperatures. Four of the *Siphamia* specimens examined had more than three times as many symbiont SNPs as the others. Interestingly, these four individuals were all collected from more temperate regions in Japan and Australia with the lowest minimum annual temperatures of all locations in this study (Table 1). Temperature is a driving factor of the distribution of bacteria in the marine environment (Sul *et al.,* 2013*)*, and has been shown to affect the distribution of the luminous vibrio symbionts of *Sepiolid* squid (Nishiguchi 2000) and to regulate the symbiotic associations of other marine taxa, such as cnidarians (Herrera *et al.,* 2020). Thus the symbionts associated with these four specimens might have some genetic adaptations to slightly cooler temperatures. Future studies investigating the influence of temperature on strain diversity and host colonization efficiency would help to elucidate the role that temperature might play in the *Siphamia-Photobacterium mandapamensis* symbiosis.

Our primary objective of this study was to sequence the symbionts found in the light organs of various *Siphamia* species, but we were able to recover enough host sequence data to also construct a reasonably well-supported host phylogeny. This allowed us to examine co-diversification of hosts and their microbial symbionts. Although we found no evidence of co-diversification, the high degree of specificity maintained for this symbiosis across host species over space and time suggests that this association is genetically constrained by the host. This host-mediated selection poses the question of whether *P. mandapamensis* (Clade II) provides a fitness advantage to the host compared to other bacteria moving through the gut of *Siphamia*, including other luminous bacteria. It should also be noted that a lack of co-diversification does not preclude a history of co-evolution of host and symbiont in the system (Moran 2006) and members of Clade II of *P. mandapamensis* are likely have specific adaptations that provide them with a fitness advantage inside the light organ environment of *Siphamia* fishes.

Siphamia, the only symbiotically luminous genus of cardinalfish, is monophyletic and divergent from the rest of the Apogonidae (Thacker and Roje 2009). The absence of this symbiosis in all other cardinalfish genera, including the other bioluminescent genera, brings up intriguing questions regarding the role of the symbiosis in the evolution of the Siphamia genus, specifically whether this association is a form of speciation by symbiosis (Wallin 1927), endowing *Siphamia* species with a key innovation that helped them persist and perhaps even proliferate. Parallel

examples have been documented in damselfishes' (Pomacentridae) mutualism with sea anemones, proposed to be the key innovation leading to the radiation of anemonefishes (Amphiprioninae; Litsios et al., 2012). Similarly, symbiosis with zooxanthellae may be a key attribute in enhancing adaptive radiation for the heterobranch genus *Phyllodesmium* (Wagele 2004). In *Siphamia*, there seems to be a rigorous mechanism of maintaining symbiont specificity across the host genus, presumably driven by the host. Therefore, understanding the genetic architecture of the Siphamia symbiont selection mechanism may be key to deciphering the highly specific nature of the association.

We also highlight the potential for formalin-fixed, fluid-preserved museum specimens to be used to study microbial symbioses. Adapting recently developed molecular techniques to extract and prepare DNA from these specimens for sequencing, including the use of single-stranded DNA as templates to construct sequence libraries, we recovered informative sequence data for both the host and its bacterial symbiont. This process allowed us to identify and compare strain level differences between the bacterial symbionts of many host species collected over nearly a century throughout the Indo-Pacific. We saw no clear correlation between sequence quality or yield and variables such as specimen age, size, or DNA input. It is likely that the observed variability between samples is largely due to the initial preservation method and long-term storage conditions of the specimens. For example, the quality (buffered or unbuffered) and concentration of the formalin solution used to initially fix a specimen can have variable effects on DNA quality (Hykin *et al.,* 2015), as can the length of time a specimen remained in formalin before being transferred to its long-term storage solution. Unfortunately, many specimen records lack such information. Moving forward, it would be beneficial for researchers to have access to such information for specimens archived in natural history museums. Nevertheless, with the advancement of new genomic techniques and sequencing technologies, the ability to retrieve informative genetic information for both a host animal and its symbiotic bacteria from these specimens will continue to advance our understanding of these critical associations.

Tables

Table 1. Information for the *Siphamia* specimens sampled in this study. Listed are each specimen's catalog number or unique identifier, species identification, sampling location and year, the minimum and maximum temperatures at that location, and the standard length of the individual sampled. Specimens with decimals after their catalog number or unique identifier indicate that more than one individual was sampled from the same specimen lot. Sea surface temperatures from the topmost meter of water at the geographical point of specimen collection were calculated as the temporal minimum and maximum from monthly climatologies (2002-2009) extracted from the Aqua-MODIS database available on Bio-ORACLE. (Tyberghein *et al*., 2012)

Specimen ID	Species	Location	Year	Min Temp	Max Temp	Length (cm)
AMI18353-041	jebbi	Fiji	1974	31.06	25.57	1.69
AMI18740-066	jebbi	Australia	1975	29.48	24.69	1.46
AMI19450-018.1	tubifer	Australia	1975	29.94	24.09	2.94
AMI19450-018.2	tubifer	Australia	1975	29.94	24.09	3.62
AMI20353-001	majimai	Australia	1972	31.76	22.61	1.67
AMI20753-031	tubulata	Australia	1979	30.04	23.54	2.56
AMI33715-016	jebbi	Australia	1993	29.70	25.08	1.46
AMI37933-007	tubifer	Vanuatu	1997	30.18	27.30	2.19
AMI40838-008	cephalotes	Australia	2001	23.03	15.49	3.07
AMI40865-004.1	roseigaster	Australia	2001	24.16	18.64	4.56
AMI40865-004.2	roseigaster	Australia	2001	24.16	18.64	4.55
AMIB4208	majimai	Australia	1958	28.11	21.64	2.31
AMIB4247	tubifer	Vanuatu	1959	29.64	26.23	2.01
CAS247233.1	mossambica	Zanzibar	2018	31.06	25.92	2.55
CAS247233.2	mossambica	Zanzibar	2018	31.06	25.92	2.92
CAS222309	jebbi	Fiji	2002	30.28	26.16	-
CAS223855	jebbi	Fiji	2002	29.88	25.89	-
CAS223939.1	jebbi	Fiji	2002	29.88	25.89	2.35
CAS223939.2	jebbi	Fiji	2002	29.88	25.89	1.81
CAS223978.1	unknown	Fiji	2002	29.88	25.89	3.68
CAS223978.2	unknown	Fiji	2002	29.88	25.89	4.05
CAS223979.1	fraseri	Fiji	2002	29.88	25.89	2.8
CAS223979.2	fraseri	Fiji	2002	29.88	25.89	3.04
CAS225045	jebbi	Fiji	1999	29.88	25.89	-
CAS27441	tubifer	Philippines	1931	30.77	27.89	3.26
CAS28515	tubulata	Australia	1973	30.31	23.60	-
CAS84356	tubifer	Palau	2012	30.33	28.57	1.9

Stubifer_M118	tubifer	Ryukyu Islands	2013	29.60	20.83	1.3
Smajimai_PVD	majimai	Japan	2007	28.75	18.68	2.61
Stubulata_PVD	tubulata	Japan	2007	28.28	18.02	2.12
Stubifer_S27	tubifer	Ryukyu Islands	2013	29.60	20.83	2.65
Sstenotes_GRA.1	stenotes	Indonesia	2006	30.80	26.42	1.89
Sstenotes_GRA.2	stenotes	Indonesia	2006	30.80	26.42	1.98
Stubifer_GRA.1	tubifer	Indonesia	2006	30.88	26.60	2.39
Stubifer_GRA.2	tubifer	Indonesia	2006	30.88	26.60	2.85
USNM112099	elongata	Philippines	1909	30.74	27.58	3.46
USNM142281.1	fuscolineata	Marshall Islands	1946	29.70	27.05	2.2
USNM142281.2	fuscolineata	Marshall Islands	1946	29.70	27.05	2.76
USNM203781	corallicola	Borneo	1965	31.33	28.34	2.58
USNM223216	jebbi	Micronesia	1980	30.73	28.31	1.74
USNM245638	jebbi	Fiji	1982	29.10	25.04	2.07
USNM245641	fraseri	Fiji	1982	28.60	24.12	4.13
USNM245642	fraseri	Fiji	1982	28.08	23.32	3.65
USNM298542	brevilux	Papua New Guinea	1988	30.83	28.73	2.24
USNM341594	jebbi	Tonga	1993	29.02	25.28	1.91
USNM341595	tubifer	Tonga	1993	29.59	25.63	3.87
USNM349778	mossambica	Mauritius	1995	28.76	23.68	2.36
USNM357884	tubifer	Philippinnes	1980	30.60	27.53	3.68
USNM357889	spinicola	Papua New Guinea	1975	29.83	25.76	3.11
USNM357892	tubifer	Red Sea	1969	28.10	21.47	3.35
USNM357897	tubifer	Andaman	1963	31.15	27.89	4.09
USNM357999	tubifer	Sri Lanka	1970	30.58	27.33	2.94
USNM358001	majimai	Philippines	1978	30.06	27.19	2.1
USNM374480	majimai	Australia	1966	27.85	21.93	1.97
USNM374837	unknown	Wallis and Futuna	2000	30.27	28.26	1.96
USNM396981	stenotes	Indonesia	2006	31.57	27.68	1.89
USNM412731	jebbi	Philippines	2003	30.79	28.54	1.73
USNM430718	fraseri	French Polynesia	2013	27.80	23.49	3.34

Table 2. Average nucleotide identities (%) of the light organ symbionts of the *Siphamia* specimens sampled in this study relative to several *Photobacterium* species for which entire genomes are available

from NCBI: *P. kishitanii* (pjapo1.1), *P. leiognathi* (lrivu4.1), *P. mandapamensis,* Clade I (ajapo4.1), *P. mandapamensis*, Clade II (gjord1.1, svers1.1). Also listed is each specimen's catalog number or unique identifier and the symbiont's percent genome coverage at 10x sequencing depth relative to *P. mandapamensis* (svers1.1)

Specimen ID	pjapo1.1	lrivu4.1	ajapo4.1	gjord1.1	svers1.1	10x coverage
Stubulata_PVD	80.1	92.9	96.7	97.4	97.4	95.1%
Smajimai_PVD	80.1	92.8	96.7	97.4	97.3	94.8%
AMI40838-008	80.2	92.8	96.5	97.2	97.1	92.3%
AMI40865-004.1	80.1	92.9	96.5	97.1	97.2	93.4%
Stubifer_GRA.2	79.8	92.2	96.0	96.8	97.2	52.0%
CAS84356	80.0	92.1	96.0	96.6	97.1	64.8%
CAS247233.1	80.0	92.0	95.8	96.6	97.0	76.9%
Stubifer_S27	80.0	92.1	95.7	96.5	96.9	88.9%
USNM412731	80.2	91.9	95.6	96.3	96.4	95.9%
CAS27441	80.0	91.8	95.5	96.2	96.6	95.3%
AMI40865-004.2	79.7	92.0	95.7	96.0	96.2	5.9%
Sstenotes_GRA.1	80.1	91.8	95.4	96.1	96.2	95.8%
Sstenotes_GRA.2	79.6	91.6	95.2	95.9	96.5	0.5%
USNM245638	79.7	91.4	95.3	96.0	95.9	35.6%
USNM245641	79.9	91.4	95.1	95.7	95.9	97.5%
USNM245642	79.9	91.4	95.1	95.7	95.8	96.2%
CAS222309	79.9	91.3	95.0	95.7	95.8	95.2%
USNM223216	80.0	91.3	94.8	95.5	95.6	96.5%
USNM142281	79.7	91.8	95.7	96.3	94.6	96.1%
USNM374837	79.7	91.2	94.9	95.6	95.7	22.8%
USNM357999	79.9	91.2	94.9	95.5	95.6	95.6%
AMIB4247	79.7	91.2	94.8	95.5	95.7	9.3%
AMI33715-016	79.7	91.2	94.9	95.5	95.6	13.7%
USNM203781	79.6	91.2	95.0	95.6	95.6	2.5%
CAS28515	79.6	91.1	94.9	95.5	95.6	5.6%
Stubifer_GRA.1	79.9	91.1	94.7	95.4	95.5	95.8%
CAS247233.2	79.9	91.1	94.7	95.4	95.5	96.4%
USNM357892	79.6	91.0	94.7	95.4	95.6	3.9%
Stubifer_M118	80.0	91.0	94.6	95.3	95.3	96.2%
CAS225045	80.1	91.0	94.7	95.3	95.3	96.1%
AMI18353-041	79.4	91.1	94.8	95.5	95.5	2.6%
USNM349778	79.9	91.0	94.6	95.2	95.3	95.8%
CAS223855	79.4	91.0	94.7	95.3	95.5	6.8%
USNM396981	79.9	90.9	94.6	95.2	95.3	96.1%
USNM341594	79.8	90.9	94.6	95.2	95.3	61.4%
AMI19450-018.2	79.4	91.1	94.7	95.4	95.4	45.3%
USNM430718	79.8	91.0	94.5	95.2	95.3	95.3%
USNM357884	79.6	90.8	94.5	95.2	95.5	33.3%
CAS223939	79.8	91.3	95.0	93.2	95.8	12.0%
USNM357889	79.5	90.8	94.5	95.1	95.3	42.7%
USNM358001	79.3	90.6	94.5	95.1	95.3	3.2%
AMI20753-031	79.6	90.7	94.3	94.9	95.1	14.9%
USNM341595	79.8	90.5	94.0	94.6	94.7	95.5%
USNM374480	79.6	90.5	94.0	94.6	94.8	40.5%
AMI37933-007	79.5	90.2	94.0	94.6	95.0	1.9%
CAS223978	79.9	91.0	94.6	88.6	95.4	95.4%
CAS223979	78.9	89.5	93.3	93.9	94.2	17.7%
USNM298542	78.6	89.4	93.3	93.7	94.0	0.1%
USNM357897	79.4	89.4	93.0	93.5	93.7	95.0%
USNM112099	78.0	89.3	93.3	93.8	94.1	3.7%
AMI19450-018.1	78.1	89.4	93.1	93.6	94.0	2.6%
AMIB4208	80.7	90.3	94.0	94.6	90.0	6.7%
AMI20353-001	77.5	87.8	91.8	92.1	92.6	2.3%
AMI18740-066	80.4	83.3	86.8	84.8	85.4	2.1%

Data Availability Statement

Sequence data associated with this study will be made publicly available prior to publication.

Author Contributions

ALG conceived of the project and secured funding for the work. AF-P and ALG carried out the genomic methods and analyses. AMG assisted with the identification of specimens and analysis of their associated metadata. All authors contributed to the discussion and interpretation of the results and to writing the manuscript. All authors approve of the submitted version of this manuscript.

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Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary Information

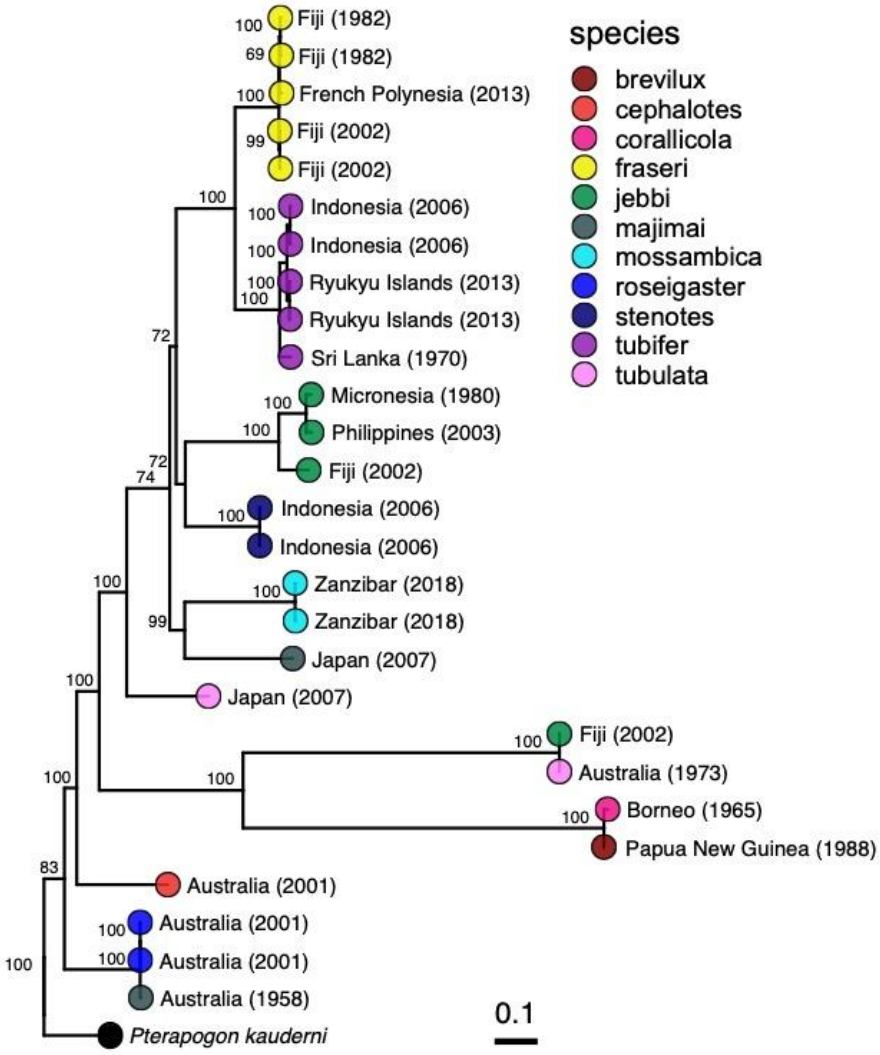

Figure S1. Maximum likelihood phylogeny of *Siphamia* based on a concatenated supermatrix of 15 mtDNA gene sequences (*ATP6, ATP8, COXI, COX2, COX3, CYTB, ND1, ND2, ND3, ND4, ND4L, ND5, ND6, 16S, 18S*). Species identities are indicated by the branch tip colors and the sampling location and year of each specimen is listed in the branch label.

Table S1. Information for the *Siphamia* specimens sampled and their corresponding sequence information. Listed are each specimen's catalog number or unique identifier, *s*pecies identification, sampling location and year, the standard length of the individual sampled, the total amount of double stranded DNA extracted from the light organ, the raw number of sequence reads, the number of reads that passed quality filtering and were trimmed, the number of reads that aligned to the symbiont reference genome (*P. mandapamensis* strain svers1.1), the percent of the symbiont reference genome covered at 10x sequence read depth, the total number of SNPs identified for each symbiont relative to the reference genome, the type of sequencing that was carried out, and the kit used for sequence library preparation. Specimens with decimals after their catalog number or unique identifier indicate that more than one individual was sampled from the same specimen lot.

Specimen ID	Species	Location	Year	Length (cm)	Total dsDNA (ng)	Raw	Trimmed	Aligned	%10x	SNPs	Sequence Run	Library Prep
AMI18353-041	jebbi	Fiji	1974	1.69	<2	28258958	26861780	187425	0.5	0	HiSeq 2x150	Swift
						27257752	26799476	407326	1	166	NovaSeq 2x150	Swift
AMI18740-066	jebbi	Australia	1975	1.46	<2	40906024	39209732	306641	2.1	0	HiSeq 2x150	Swift
AMI19450-018.1	tubifer	Australia	1975	2.94	3	58651761	56851489	442960	2.6	0	HiSeq 2x150	Swift
AMI19450-018.2	tubifer	Australia	1975	3.62	3.5	33895456	32610468	1337245	23.2	10	HiSeq 2x150	NEB Ultra II
						24526375	23858943	1042258	16.4	5,435	NovaSeq 2x150	NEB Ultra II
AMI20353-001	majimai	Australia	1972	1.67	<2	39503975	38494612	344674	2.3	1	HiSeq 2x150	Swift
AMI20753-031	tubulata	Australia	1979	2.56	<2	35965027	35217790	1085963	14.9	6,916	NovaSeq 2x150	Swift
AMI33715-016	jebbi	Australia	1993	1.46	2.2	35229540	33924547	359824	1.9	0	HiSeq 2x150	Swift
						33504802	33190608	655297	5	862	NovaSeq 2x150	Swift
AMI37933-007	tubifer	Vanuatu	1997	2.19	52.1	3604557	2090383	219645	0.1	47	NovaSeq 2x150	NEB Ultra II
						49624926	48314012	498902	1.9	0	HiSeq 2x150	Swift
AMI40838-008	cephalotes	Australia	2001	3.07	52.8	34771013	34092047	5099255	92.3	70,709	NovaSeq 2x150	NEB Ultra II
AMI40865-004.1	roseigaster	Australia	2001	4.56	74.1	91467705	89885499	6944692	93.4	72,219	NovaSeq 2x150	NEB Ultra II
AMI40865-004.2	roseigaster	Australia	2001	4.55	4.6	48426323	46531047	654416	5.9	1,588	HiSeq 2x150	Swift

AMIB4208	majimai	Australia	1958	2.31	<2	36877472	34915450	467033	6.4	2	HiSeq 2x150	Swift
						87751	55995	12134	0	0	NovaSeq 2x150	Swift
AMIB4247	tubifer	Vanuatu	1959	2.01	<2	28358555	27422784	269641	2	0	HiSeq 2x150	Swift
						23520680	23130431	584903	2.9	1,633	NovaSeq 2x150	Swift
CAS247233.1	mossambica	Zanzibar	2018	2.55	542	5478617	5384608	454932	76.9	8,112	HiSeq 1x150	SparQ
CAS247233.2	mossambica	Zanzibar	2018	2.92	1530	75836474	73645413	33775734	96.4	15,579	NovaSeq 2x150	NEB Ultra II
CAS222309	jebbi	Fiji	2002	-	10.2	50665038	45699796	28523692	95.2	21,238	NovaSeq 2x150	NEB Ultra II
CAS223855	jebbi	Fiji	2002	-	<2	44113541	41855479	377420	2.4	3	HiSeq 2x150	Swift
						33488272	33006205	576345	2.8	469	NovaSeq 2x150	Swift
CAS223939.1	jebbi	Fiji	2002	2.35	11.8	28012894	21498132	225577	4.3	63	HiSeq 1x150	SparQ
CAS223939.2	jebbi	Fiji	2002	1.81	8.9	11046726	10551137	716650	12	7,698	NovaSeq 2x150	NEB Ultra II
CAS223978.1	unknown	Fiji	2002	3.68	178.5	330421	323250	32364	0.1	10	HiSeq 1x150	SparQ
CAS223978.2	unknown	Fiji	2002	4.05	50.5	23063434	21244678	10747171	95.4	18,773	NovaSeq 2x150	NEB Ultra II
CAS223979.1	fraseri	Fiji	2002	2.8	9.2	24192691	22748353	328578	0.9	0	HiSeq 2x150	NEB Ultra II
						26735318	25635641	457797	1.1	7	NovaSeq 2x150	NEB Ultra II
CAS223979.2	fraseri	Fiji	2002	3.04	15.5	10467851	9981063	381078	17.7	72	HiSeq 1x150	SparQ
CAS225045	jebbi	Fiji	1999		3.4	21590245	20304117	15184111	96.1	18,316	NovaSeq 2x150	NEB Ultra II
CAS27441	tubifer	Philippines	1931	3.26	1.8	18607665	18124484	1483134	95.3	18,841	HiSeq 1x150	SparQ
CAS28515	tubulata	Australia	1973	-	<2	36559960	33708337	213670	1	2	HiSeq 2x150	Swift
						35922973	35323462	589696	3.1	1,301	NovaSeq 2x150	Swift
CAS84356	tubifer	Palau	2012	1.9	38.9	48287910	38767642	6258850	64.8	13,661	HiSeq 1x150	SparQ
Stubifer_M118	tubifer	Ryukyu Islands	2013	1.3	26.5	75237177	72661256	57068084	96.2	16,828	HiSeq 2x150	Swift
Smajimai_PVD	majimai	Japan	2007	2.61	8949	36930530	35957911	26787137	94.8	70,889	NovaSeq 2x150	NEB Ultra II

Stubulata_PVD	tubulata	Japan	2007	2.12	1225.5	34195315	33077310	21855337	95.1	66,583	NovaSeq 2x150	NEB Ultra II
Stubifer_S27	tubifer	Ryukyu Islands	2013	2.65	-	7403111	7277173	517331	88.9	19,790	HiSeq 1x150	SparQ
Sstenotes_GRA.1	stenotes	Indonesia	2006	1.89	115.9	22315393	24640343	21426979	95.8	24,716	NovaSeq 2x150	NEB Ultra II
Sstenotes_GRA.2	stenotes	Indonesia	2006	1.98	308	467708	426870	85208	0.5	23	HiSeq 1x150	SparQ
Stubifer_GRA.1	tubifer	Indonesia	2006	2.39	96	23365446	22298866	6159687	95.8	18,701	NovaSeq 2x150	NEB Ultra II
Stubifer_GRA.2	tubifer	Indonesia	2006	2.85	95.9	9455319	8509771	452475	52	3,041	HiSeq 1x150	SparQ
USNM112099	elongata	Philippines	1909	3.46	<2	49945895	48231023	430697	3.7	8	HiSeq 2x150	Swift
USNM142281.1	fuscolineata	Marshall Islands	1946	2.2	<2	16213148	15893522	6118510	96.1	15,366	NovaSeq 2x150	Swift
USNM142281.2	fuscolineata	Marshall Islands	1946	2.76	10.1	39670892	27192174	695872	62.8	381	HiSeq 1x150	NEB Ultra II
USNM203781	corallicola	Borneo	1965	2.58	<2	33588104	32488841	640895	0.6	0	HiSeq 2x150	Swift
						56446868	51658133	1237586	1.2	178	NovaSeq 2x150	Swift
USNM223216	jebbi	Micronesia	1980	1.74	7	181380283	186040521	176746273	96.5	14,693	NovaSeq 2x150	NEB Ultra II
USNM245638	jebbi	Fiji	1982	2.07	2.2	36426337	34932628	508273	9.2	38	HiSeq 2x150	Swift
						27073152	26801480	647893	13	2,188	NovaSeq 2x150	Swift
USNM245641	fraseri	Fiji	1982	4.13	5.3	864701674	829515210	714629012	97.5	17,070*	NovaSeq 2x150	NEB Ultra II
USNM245642	fraseri	Fiji	1982	3.65	13	35743393	35774905	33716527	96.2	16,396	NovaSeq 2x150	NEB Ultra II
USNM298542	brevilux	Papua New Guinea	1988	2.24	21.1	662337	482006	148333	0.1	23	NovaSeq 2x150	NEB Ultra II
						49597285	47386221	486582	2.4	0	HiSeq 2x150	Swift
USNM341594	jebbi	Tonga	1993	1.91	<2	27318206	27030306	1313295	61.4	7,995	NovaSeq 2x150	Swift
USNM341595	tubifer	Tonga	1993	3.87	7.8	18908174	17504661	4858215	94.2	725	HiSeq 2x150	NEB Ultra II
						11846770	11271717	2974122	88.1	17,900	NovaSeq 2x150	NEB Ultra II
USNM349778	mossambica	Mauritius	1995	2.36	15	33471029	32308812	12943283	95.8	18,219	NovaSeq 2x150	NEB Ultra II
USNM357884	tubifer	Philippinnes	1980	3.68	7.3	18374904	17191172	759889	8.5	1	HiSeq 2x150	NEB Ultra II

						13974418	12217495	879577	11.9	4,148	NovaSeq 2x150	NEB Ultra II
USNM357889	spinicola	Papua New Guinea	1975	3.11	4.1	21853900	21329666	2042137	42.7	12,366	NovaSeq 2x150	NEB Ultra II
						46425	42876	22	0	0	HiSeq 2x150	NEB Ultra II
USNM357892	tubifer	Red Sea	1969	3.35	<2	35509177	33451543	215301	0.7	1	HiSeq 2x150	Swift
						27788827	27434566	542781	1.7	1,107	NovaSeq 2x150	Swift
USNM357897	tubifer	Andaman	1963	4.09	3.9	26178035	25619280	9568200	95	17,295	NovaSeq 2x150	NEB Ultra II
USNM357999	tubifer	Sri Lanka	1970	2.94	<2	54251311	53731832	26374339	95.6	22,417	NovaSeq 2x150	Swift
USNM358001	majimai	Philippines	1978	2.1	<2	38324209	36313447	254445	0.8	2	HiSeq 2x150	Swift
						33631537	33293817	428773	1.1	151	NovaSeq 2x150	Swift
USNM374480	majimai	Australia	1966	1.97	2.1	63381524	62370257	1400945	40.5	8,157	NovaSeq 2x150	Swift
USNM374837	unknown	Wallis and Futuna	2000	1.96	12.1	10549216	7951371	985084	22.8	9,465	NovaSeq 2x150	NEB Ultra II
						41856128	40302628	595029	10.7	11	HiSeq 2x150	Swift
USNM396981	stenotes	Indonesia	2006	1.89	153.9	36161679	35712980	34096922	96.1	16,892	NovaSeq 2x150	NEB Ultra II
USNM412731	jebbi	Philippines	2003	1.73	23.6	27071135	25964385	16604538	95.9	32,687	NovaSeq 2x150	NEB Ultra II
USNM430718	fraseri	French Polynesia	2013	3.34	58.9	18468382	17513981	2806955	95.3	20,592	NovaSeq 2x150	NEB Ultra II

Table S2. Information for the *Siphamia COI* sequences that were used to construct the host phylogeny. Listed are each specimen's catalog number or unique identifier, *s*pecies identification, sampling location, exact latitude and longitude, year, and the source of the sequence.

Specimen ID	Species	Location	Latitude	Longitude	Year	Source
AMI40838-008	cephalotes	Australia	-33.840	151.185	2001	this study
AMI40865-004-1	roseigaster	Australia	-33.865	152.000	2001	this study
AMI40865-004-2	roseigaster	Australia	-33.865	152.000	2001	this study

AMI41858-030	roseigaster	Australia	-29.417	153.356	2002	Mabuchi *et al.* 2014
AWCF412	goreni	Red Sea	25.707	36.622	2016	Atta *et al.* 2019
AWCF713	tubifer	Red Sea	25.707	36.622	2016	Atta *et al*. 2019
BW-A5255	fistulosa	Australia	-16.896	146.447	2005	International Barcode of Life
CAS223855	jebbi	Fiji	-18.151	178.360	2002	this study
CAS223978	fraseri	Fiji	-18.100	178.360	2002	this study
CAS223979	fraseri	Fiji	-18.100	178.360	2002	this study
CAS225045	jebbi	Fiji	-18.145	178.369	1999	this study
CAS247233.1	mossambica	Zanzibar	-6.220	39.171	2018	this study
CAS247233.2	mossambica	Zanzibar	-6.220	39.171	2018	this study
CAS28515	tubulata	Australia	-14.202	144.260	1973	this study
CSIRO-H-6648-02 (BW-A12333)	guttulata	Australia	-17.106	146.005	2004	International Barcode of Life
CSIRO-H-7457-03 (BW-A12338)	guttulata	Australia	-12.579	143.478	2004	International Barcode of Life
CSIRO-H-8482-02 (BW-A5590)	cuneiceps	Australia	-22.123	150.334	2005	International Barcode of Life
FAKU73087	tubulata	Japan	32.743	132.561	-	Mabuchi *et al.* 2014
FAKU78690	majimai	Ryukyu Islands	30.432	130.400	-	Mabuchi *et al.* 2014
KU_Tissue4631 (CAS222309)	jebbi	Fiji	-17.324	178.238	2002	Mabuchi *et al.* 2014
Stubifer_M118	tubifer	Ryukyu Islands	26.656	127.880	2013	this study
Smajimai_PVD	majimai	Japan	32.800	133.500	2007	this study
Stubulata_PVD	tubulata	Japan	32.743	132.561	2007	this study
Stubifer_S27	tubifer	Ryukyu Islands	26.635	127.866	2013	this study
SAIAB194663	paupuensis	Indonesia	-2.220	130.564	2013	Gon *et al*. 2014
SAIAB194704	paupuensis	Indonesia	-2.965	131.334	2013	Gon *et al*. 2014

Sstenotes_GRA.1	stenotes	Indonesia	-3.870	133.981	2006	this study
Sstenotes_GRA.2	stenotes	Indonesia	-3.870	133.981	2006	this study
Stubif_Kaeding	tubifer	Ryukyu Islands	26.635	127.866	2006	Kaeding *et al.* 2007
Stubifer_GRA.1	tubifer	Indonesia	-3.680	133.728	2006	this study
Stubifer_GRA.2	tubifer	Indonesia	-3.680	133.728	2006	this study
USNM112099-2	elongata	Philippines	16.930	120.233	1909	this study
USNM203781	corallicola	Borneo	6.015	116.059	1965	this study
USNM223216	jebbi	Micronesia	6.930	158.100	1980	this study
USNM245638	jebbi	Fiji	-19.161	179.756	1982	this study
USNM245641	fraseri	Fiji	-20.620	181.330	1982	this study
USNM245642	fraseri	Fiji	-20.620	181.330	1982	this study
USNM298542-2	brevilux	Papua New Guinea	-5.230	145.750	1988	this study
USNM349778	mossambica	Mauritius	-20.188	57.400	1995	this study
USNM357999	tubifer	Sri Lanka	8.602	81.226	1970	this study
USNM358001	majimai	Philippines	9.383	123.258	1978	this study
USNM396981	stenotes	Indonesia	-3.960	134.355	2006	this study
USNM412731	jebbi	Philippines	12.693	120.522	2003	this study
USNM430718	fraseri	French Polynesia	-22.641	207.178	2013	this study

Table S3. Results of the nucleotide BLAST search of symbiont 16S rRNA genes. Listed are each specimen's catalog number or unique identifier, the percent of the reference 16S rRNA gene sequence (*Photobacterium leiognathi*, AY292917) covered at 10x sequence depth, the top matching sequence from the NCBI database including its accession number in parentheses, and the corresponding query coverage, E-value, and percent identity relative to that sequence.

Specimen ID	%10x	Top hit	Query coverage	E-value	% identity

AMI18353-041	88.49	Photobacterium leiognathi subsp. mandapamensis strain MahLm3 (JN380344.1)	91%	0.0	86.08
AMI18740-066	89.2	Photobacterium leiognathi (AY292917.1)	93%	0.0	79.97
AMI19450-018.1	98.25	Photobacterium leiognathi strain LC1-277 (AB243248.1)	90%	0.0	86.48
AMI19450-018.2	99.94	Photobacterium leiognathi strain ljone1.1 (AY204494.1)	94%	0.0	95.96
AMI20353-001	95.02	Photobacterium leiognathi strain W214 (MF554624.1)	89%	0.0	83.82
AMI20753-031	99.94	Photobacterium leiognathi strain ljone1.1 (AY204494.1)	94%	0.0	95.14
AMI33715-016	100	Photobacterium mandapamensis seafl.1.4 (AY455873.1)	95%	0.0	84.58
AMI37933-007	99.81	Photobacterium mandapamensis seafl.1.4 (AY455873.1)	95%	0.0	95.93
AMI40838-008	100	Photobacterium leiognathi subsp. mandapamensis strain ATCC 27561 (NR_115206.1)	95%	0.0	99.93
AMI40865-004.1	99.94	Photobacterium leiognathi subsp. mandapamensis strain ATCC 27561 (NR_115206.1)	95%	0.0	99.86
AMI40865-004.2	99.94	Photobacterium leiognathi strain AK-MIE (MH746214.1)	91%	0.0	99.01
AMIB4208	100	Photobacterium leiognathi strain LC1-283 (AB243249.1)	90%	0.0	90.74
AMIB4247	99.94	Photobacterium leiognathi strain LC1-283 (AB243249.1)	90%	0.0	92.75
CAS222309	100	Photobacterium leiognathi subsp. mandapamensis strain ATCC 27561 (NR_115206.1)	95%	0.0	100.00
CAS223855	100	Photobacterium leiognathi strain LC1-283 (AB243249.1)	88%	0.0	86.35
CAS223939.1	99.94	Photobacterium leiognathi strain ljone1.1 (AY204494.1)	94%	0.0	99.04
CAS223939.2	99.94	Photobacterium mandapamensis seafl.1.1 (AY455871.1)	94%	0.0	97.67
CAS223978.1	29.61	Photobacterium leiognathi subsp. mandapamensis strain ATCC 27561 (NR_115206.1)	95%	0.0	99.73
CAS223978.2	100	Photobacterium mandapamensis seafl.1.1 (AY455871.1)	94%	0.0	100.00
CAS223979.1	99.94	Photobacterium leiognathi strain LC1-277 (AB243248.1)	90%	0.0	94.88
CAS223979.2	99.94	Photobacterium leiognathi subsp. mandapamensis strain ATCC 27561 (NR_115206.1)	95%	0.0	100.00
CAS225045	100	Photobacterium leiognathi subsp. mandapamensis strain ATCC 27561 (NR_115206.1)	95%	0.0	100.00
CAS247233.1	100	Photobacterium mandapamensis seafl.1.1 (AY455871.1)	94%	0.0	100.00

CAS247233.2	99.94	Photobacterium leiognathi subsp. mandapamensis strain ATCC 27561 (NR_115206.1)	95%	0.0	100.00
CAS27441	100	Photobacterium leiognathi subsp. mandapamensis strain ATCC 27561 (NR_115206.1)	95%	0.0	100.00
CAS28515	100	Photobacterium leiognathi subsp. mandapamensis strain MahLm3 (JN380344.1)	92%	0.0	84.13
CAS84356	99.94	Photobacterium leiognathi subsp. mandapamensis strain ATCC 27561 (NR_115206.1)	95%	0.0	99.93
Smajimai_PVD	100	Photobacterium leiognathi subsp. mandapamensis strain ATCC 27561 (NR_115206.1)	95%	0.0	99.93
Sstenotes_GRA.1	100	Photobacterium leiognathi subsp. mandapamensis strain ATCC 27561 (NR_115206.1)	95%	0.0	100.00
Sstenotes_GRA.2	99.94	Photobacterium leiognathi subsp. mandapamensis strain ATCC 27561 (NR_115206.1)	95%	0.0	99.73
Stubifer_GRA.1	100	Photobacterium leiognathi subsp. mandapamensis strain ATCC 27561 (NR_115206.1)	95%	0.0	100.00
Stubifer_GRA.2	99.94	Photobacterium leiognathi subsp. mandapamensis strain ATCC 27561 (NR_115206.1)	95%	0.0	99.93
Stubifer_M118	100	Photobacterium leiognathi subsp. mandapamensis strain ATCC 27561 (NR_115206.1)	95%	0.0	99.93
Stubifer_S27	99.94	Photobacterium leiognathi subsp. mandapamensis strain ATCC 27561 (NR_115206.1)	95%	0.0	100.00
Stubulata_PVD	100	Photobacterium leiognathi subsp. mandapamensis strain ATCC 27561 (NR_115206.1)	95%	0.0	99.93
USNM112099	92.18	Photobacterium leiognathi strain AK5 (AB243232.1)	90%	0.0	85.57
USNM142281.1	99.94	Photobacterium leiognathi subsp. mandapamensis strain ATCC 27561 (NR_115206.1)	95%	0.0	100.00
USNM142281.2	100	Photobacterium leiognathi strain ljone1.1 (AY204494.1)	93%	0.0	94.36
USNM203781	100	Photobacterium leiognathi strain LC1-277 (AB243248.1)	90%	0.0	90.68
USNM223216	100	Photobacterium leiognathi subsp. mandapamensis strain ATCC 27561 (NR_115206.1)	95%	0.0	100.00
USNM245638	100	Photobacterium leiognathi strain AK5 (AB243232.1)	90%	0.0	93.11
USNM245641	100	Photobacterium leiognathi subsp. mandapamensis strain ATCC 27561 (NR_115206.1)	95%	0.0	99.93
USNM245642	100	Photobacterium leiognathi subsp. mandapamensis strain ATCC 27561 (NR_115206.1)	95%	0.0	100.00
USNM298542	99.94	Photobacterium leiognathi subsp. mandapamensis strain MahLm3 (JN380344.1)	91%	0.0	82.60
USNM341594	100	Photobacterium leiognathi subsp. mandapamensis strain MahLm3 (JN380344.1)	92%	0.0	93.40
USNM341595	100	Photobacterium leiognathi subsp. mandapamensis strain ATCC 27561 (NR_115206.1)	95%	0.0	100.00

USNM349778	100	Photobacterium leiognathi subsp. mandapamensis strain ATCC 27561 (NR_115206.1)	95%	0.0	99.93
USNM357884	100	Photobacterium mandapamensis seafl.1.1 (AY455871.1)	94%	0.0	100.00
USNM357889	100	Photobacterium mandapamensis seafl.1.4 (AY455873.1)	95%	0.0	97.90
USNM357892	100	Photobacterium leiognathi strain LC1-277 (AB243248.1)	90%	0.0	91.53
USNM357897	100	Photobacterium leiognathi subsp. mandapamensis strain ATCC 27561 (NR_115206.1)	95%	0.0	99.93
USNM357999	100	Photobacterium leiognathi subsp. mandapamensis strain ATCC 27561 (NR_115206.1)	95%	0.0	99.93
USNM358001	100	Photobacterium leiognathi strain LC1-277 (AB243248.1)	90%	0.0	87.62
USNM374480	100	Photobacterium leiognathi strain LC1-283 (AB243249.1)	90%	0.0	91.82
USNM374837	100	Photobacterium leiognathi subsp. mandapamensis strain MahLm3 (JN380344.1)	91%	0.0	87.07
USNM396981	100	Photobacterium leiognathi strain Ileuc1.1 (AY204495.1)	94%	0.0	100.00
USNM412731	100	Photobacterium leiognathi subsp. mandapamensis strain ATCC 27561 (NR_115206.1)	95%	0.0	100.00
USNM430718	99.94	Photobacterium leiognathi subsp. mandapamensis strain ATCC 27561 (NR_115206.1)	95%	0.0	100.00