

# Phylogeographical Patterns among Mediterranean Sepiolid Squids and Their *Vibrio* Symbionts: Environment Drives Specificity among Sympatric Species<sup>∇†</sup>

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**Bobtail squid from the genera *Sepiola* and *Rondeletiola* (Cephalopoda: Sepiolidae) form mutualistic associations with luminous Gram-negative bacteria (*Gammaproteobacteria*: *Vibrionaceae*) from the genera *Vibrio* and *Photobacterium*. Symbiotic bacteria proliferate inside a bilobed light organ until they are actively expelled by the host into the surrounding environment on a diel basis. This event results in a dynamic symbiont population with the potential to establish the symbiosis with newly hatched sterile (axenic) juvenile sepiolids. In this study, we examined the genetic diversity found in populations of sympatric sepiolid squid species and their symbionts by the use of nested clade analysis with multiple gene analyses. Variation found in the distribution of different species of symbiotic bacteria suggests a strong influence of abiotic factors in the local environment, affecting bacterial distribution among sympatric populations of hosts. These abiotic factors include temperature differences incurred by a shallow thermocline, as well as a lack of strong coastal water movement accompanied by seasonal temperature changes in overlapping niches. Host populations are stable and do not appear to have a significant role in the formation of symbiont populations relative to their distribution across the Mediterranean Sea. Additionally, all squid species examined (*Sepiola affinis*, *S. robusta*, *S. ligulata*, *S. intermedia*, and *Rondeletiola minor*) are genetically distinct from one another regardless of location and demonstrate very little intraspecific variation within species. These findings suggest that physical boundaries and distance in relation to population size, and not host specificity, are important factors in limiting or defining gene flow within sympatric marine squids and their associated bacterial symbionts in the Mediterranean Sea.**

Population structure can be determined in nature by a number of intrinsic elements in the environment, including selective pressures resulting from abiotic variables. Many of these factors are the result of physical change in the environment over long periods of time (change in landscape, habitat fragmentation), while some, such as temperature, currents, and salinity, are constantly in flux (3, 7, 21). Small-scale changes in salinity and temperature can have significant effects and are exacerbated in marine environments, where osmotic balance and physical gradients are naturally established. Specifically, temperature may affect the distribution, viability, and fitness of organisms that have not readily adapted to such changing conditions (38, 57). This is pertinent to organisms living in symbiosis with each other, where one partner is dependent upon the other for a number of capabilities, such as physiological functions, nutritional requirements, and predator/prey avoidance (16, 20, 35). Interestingly, very few marine symbioses span across broad geographic ranges, since an enormous spectrum of abiotic and biotic conditions may prevent the ability of the symbiont to move between host populations. Studies of population structure within marine symbioses have increasingly pointed toward the underlying importance of understanding the nonliving elements of the environment as they apply to the

distribution of symbiotic partners (either host or symbiont) across broadly distributed landscapes (42).

Population structure between host and symbionts in many symbioses, particularly mutualistic associations, often produce evidence of cospeciation between established partner lineages in both terrestrial and marine environments (1, 34, 37). The evidence for cospeciation or parallel cladogenesis is often attained through comparative phylogenetic analysis, which may neglect to unveil or account for various environmental factors. Abiotic characteristics of individual habitats may drive ecological or physiological processes, which in turn influence population distribution and interspecies interactions for both host and symbiont, particularly those that are environmentally transmitted. This may also influence either benefits or costs that arise due to the adaptability of each partner to accommodate not only environmental changes but also changes occurring with respect to each other. Considering the potential influencing power of the environment, it is prudent to explore the relative impact of nonliving habitat characteristics on symbiont-host distributions as it applies to the phylogeography and population history of both host and symbiont. This is particularly true for the mutualistic symbiosis between sepiolid squids (Mollusca: Cephalopoda) and bioluminescent bacteria in the genus *Vibrio* (*Gammaproteobacteria*: *Vibrionaceae*).

Symbiosis between sepiolid squids (Cephalopoda: Sepiolidae) and *Vibrio* bacteria has become an ideal model to study environmentally transmitted symbioses between cooperative microbes and their eukaryotic hosts (19, 44, 67). The best-known association within this family of squids is between members of the genus *Euprymna* and its symbiont *Vibrio fischeri* (21,

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TABLE 1. Collection sites of sepiolid squid species and their bacterial symbionts

Location and date specimen collected	Host species	Symbiont species	Depth/temp (m/°C)
Banyuls-sur-Mer, France (42°35'29''N, 3°2'22''E), 2003–2008	<i>Sepiola affinis</i>	<i>Vibrio fischeri</i> , <i>V. logei</i>	3–10/20–22
	<i>S. robusta</i>	<i>V. fischeri</i> , <i>V. logei</i>	20/13
	<i>S. intermedia</i>	<i>V. fischeri</i> , <i>V. logei</i>	20–30/13
	<i>Rondeletiola minor</i>	<i>Photobacterium leiognathi</i>	70/13
Bari, Italy (42°35'29''N, 3°2'22''E), 2005	<i>S. affinis</i>	<i>V. fischeri</i> , <i>V. logei</i>	3–10/21–24
	<i>S. robusta</i>	<i>V. fischeri</i> , <i>V. logei</i>	10–20/14–18
	<i>S. intermedia</i>	<i>V. fischeri</i> , <i>V. logei</i>	10–20/14–18
Bay of Biscay, France (43°35'43''N, 01°48'08''E), 2007	<i>R. minor</i>	<i>P. leiognathi</i>	80–120/12–16

29). The symbiosis is established when environmentally transmitted, symbiosis-competent vibrios infect the light organs of newly hatched juvenile squid (30, 53). Upon entrance into the light organ, the bacteria flourish in a relatively nutrient-rich, protected environment and in turn achieve high densities within the organ (59). When a sufficiently high number of symbiont cells is achieved in the light organ, the bacteria become luminescent based on a communication mechanism known as quorum sensing (9, 12, 28, 31). *Vibrio* bacteria then induce a program of drastic morphological changes in the squid host, which helps to establish the symbiont population and completes development of the naïve light organ (33). Development and timing of this *Vibrio* population coincides with the nocturnal hunting behavior of the squid, in which the bacteriogenic luminescence is used in a predator/prey avoidance behavior known as counterillumination (20).

At dawn, the squid bury themselves in the sand and expel 90 to 95% of the symbiotic bacteria from the light organ (25, 54). This expulsion results in a “seeded” environment in which newly hatched, axenic juvenile squids can acquire symbiotically competent *Vibrio* bacteria. The result of this local acquisition of symbionts previously inhabiting hosts from the same population may lead to high degrees of specificity between symbiotic partners (37, 39). In the association between bobtail squids of the genus *Euprymna* (*E. scolopes* and *E. tasmanica* in the Indo-West Pacific Ocean) and *Vibrio fischeri*, this high degree of mutual exclusivity has been observed with *V. fischeri* (43, 67). Although *V. fischeri* is the only symbiont present in most *Euprymna* species, strain variation has been observed within and between populations, indicating that vibrios can migrate among host species complexes, changing the geographic mosaic of these symbionts within their host squids (21, 68). This distribution is affected mainly by other factors, such as temperature, which has been observed to influence the location of other marine populations in similar habitats (21, 22).

In comparison, mutualistic symbioses between Mediterranean squid from the genera *Sepiola* and *Rondeletiola* and Gram-negative bacteria from the genera *Vibrio* and *Photobacterium* (11, 18) demonstrate that species specificity is not always common among all sepiolid genera (17, 38). Furthermore, temperature has been observed to be a major factor determining symbiont distribution both within hosts as well as in their free-living state (22, 38, 57). Therefore, abiotic factors have a much larger influence in the establishment of environmentally transmitted symbiosis than just recognition and spec-

ificity to a particular squid host. The Mediterranean Sea harbors 10 species of squid from the genus *Sepiola* and one species from the genus *Rondeletiola* (*R. minor*), all of which possess a bacteriogenic light organ (6, 38, 41, 55). Sepiolid squid species in the Mediterranean exist in sympatry, with overlapping population boundaries along the northern coast of the Mediterranean (55). Also of interest pertaining to the Mediterranean sepiolid fauna is the fact that each squid can harbor two species of symbionts in their light organs (17, 38). Therefore, the aim of this study was to understand whether the population structure of different sympatric host species had a direct effect on symbiont distribution or if the combined influence of abiotic factors (temperature, water movement, and salinity) and multiple symbiont species influences the association among sympatric populations. This is in contrast to previous studies of allopatric populations, in which *Vibrio* bacteria were specialists with their specific host species (21). We hypothesized that a lack of specificity occurs between host squid and the *Vibrio* species found in Mediterranean sepiolids (*V. fischeri*, *V. logei*, and *Photobacterium leiognathi*) and that temperature was a major force in determining these host-symbiont assemblages. Using a phylogeographical approach, we tested whether any type of genetic structure existed among species of sepiolids in the Mediterranean Sea and if their *Vibrio* symbionts' distribution was influenced by host specificity or environment.

## MATERIALS AND METHODS

**Collection of specimens.** Specimens used in this study were collected from sites in southern France (Banyuls-sur-Mer), eastern Italy (Bari, Italy), and the North Atlantic (Bay of Biscay) (Table 1). Banyuls-sur-Mer is located in the south of France near the Spanish border at the foot of the Pyrenees Mountains. Specimens were collected by trawling (*R. minor*, *S. robusta*, and *S. intermedia*) or by hand during SCUBA dives at night (*S. affinis*). Italian sepiolid squid were collected by trawl net off the coast of Bari, Italy, while samples of *R. minor* were collected via mid-water trawl on several cruises in the Bay of Biscay. Other sequence data were incorporated for individual squid (*S. robusta* and *S. affinis*) from previously published sources as well (27, 40, 41). To increase the sample population size, other hosts and associated symbiotic strains from previous work were incorporated into the overall analysis (27, 40, 41). These samples were included in order to equalize the number of strains and host species for each collection site.

**DNA isolation and sequencing.** All specimens of squid were anesthetized by placing the animal on ice and subsequently removing the light organ through ventral dissection. The light organ from each individual was then homogenized in a 2-ml tube to isolate the symbiotic bacteria inside. Each homogenate was placed through a series of dilutions in sterile filtered seawater before plating the bacteria. These homogenates were spread onto seawater tryptone agar plates (SWT; 0.5% tryptone, 0.3% yeast extract, 70% seawater, 0.3% glycerol, and 15%

agar). After growth on SWT (12 to 24 h, 20 to 28°C), 10 to 15 individual colonies from each plate (representing one squid light organ) were selected, and each colony was then stab inoculated into an SWT agar vial for transport back to New Mexico State University. This sampling regime is applicable to any strain variation that may be observed in an individual squid light organ (68).

DNA was extracted from each individual isolate using the Qiagen DNeasy blood and tissue kit (Qiagen, Valencia, CA). Approximately 5 to 10 ng of the extracted genomic DNA was subsequently used in 4 separate PCRs to amplify the 16S rRNA locus of the symbiotic bacteria using *Vibrio*-specific primer sets (see Table S1 in the supplemental material). A total of 5 to 10 ng of the template DNA was also used to amplify a 650-base-pair region of the glyceraldehyde phosphate dehydrogenase locus (*gapA*) using both previously published *Vibrio*-specific primers (40) and primers newly designed specifically for *V. logei* (see Table S1). The *gapA* locus was used to analyze the bacterial symbiont populations in this study, as it has been shown to be amenable to phylogeographic and phylogenetic analyses for *Vibrio* populations (21, 40). This is due to the fact that the *gapA* locus is less conserved than other metabolic, constitutively expressed genes in the *Vibrio* genus and is capable of differentiating between strains of the same species of bacteria. The *V. logei* primers were designed and utilized due to broad problems associated with amplifying the *gapA* locus in a number of light organ isolates determined to be vibrios. DNA from host squid tissue was extracted by homogenizing a 20- to 25-mg piece of tissue dissected from the anterior mantle. The homogenate was processed using the same DNeasy blood and tissue kit (Qiagen, Valencia, CA). After DNA extraction, approximately 5 ng of host DNA was used in a PCR to amplify the cytochrome *c* oxidase subunit I (COI) and 12S and 16S rRNA loci (21, 27, 41) (see Table S1 in the supplemental material).

PCRs to amplify the above-specified loci from both host squid and *Vibrio* bacteria were performed as 25- $\mu$ l reaction mixtures containing 5 to 10 ng of extract DNA, 0.2  $\mu$ M both forward and reverse primers, 0.2 U of *Taq* polymerase (GoTaq Flexi DNA polymerase; Promega, San Luis Obispo, CA), 2.5 mM magnesium chloride (MgCl<sub>2</sub>), a final concentration of 200  $\mu$ M dGTP, dATP, dCTP, and dTTP (50  $\mu$ M each), and 1 $\times$  buffer (Tris-HCl, KCl, and 0.1% Triton X-100). PCRs were completed in an MJ Research Dyad Disciple thermal cycler (Waltham, MA). Following PCRs, amplified fragments were purified using either a QIAquick PCR purification kit (Qiagen, Valencia, CA) or the QIAEX II gel extraction kit (Qiagen, Valencia, CA). The purified PCR products were used for presequencing reactions in an MJ Dyad Disciple thermal cycler (Waltham, MA) using BigDye Terminator 3.1 (Applied Biosystems, Foster City, CA). Reactions were cleaned of excess dideoxynucleotides and fluorescent dyes by centrifuging the samples through 400  $\mu$ l of dehydrated Sephadex (Sigma, St. Louis, MO) and subsequently collected in a 96-well sequencing plate. All samples were sequenced using the Applied Biosystems 3100 automated capillary sequencer (Applied Biosystems, Foster City, CA) at New Mexico State University. Sequences were aligned and edited using Sequencher, version 4.6 (Gene Codes Corporation, Ann Arbor, MI).

**Analysis of variance.** The baseline variance of initial sample populations of both host squid and symbiotic bacteria was measured using an analysis of molecular variance (AMOVA) using the program ARLEQUIN, version 3.0 (15). Variance calculated in this program was indicative of variation within populations at the different collection sites (North Atlantic, France, and Italy) and between each of the populations sampled. Variation observed within a population at each site was calculated with a base-pair-by-base-pair polymorphic measure, theta.

**Nested clade analysis.** Analysis of the sequence data for building haplotype networks was completed in TCS version 1.12 (10). TCS v. 1.12 utilizes a statistical parsimony-based procedure based on Templeton's rules (61). The resulting symbiont and host networks were nested, and potential ambiguities and complementary problems were addressed and resolved using the nesting rules according to Templeton et al. (60, 63). The final nested clade information was then analyzed by the program GEODIS (50). GEODIS analyzes the haplotype network and interprets the nesting to describe intrinsic variation between clades, assuming the specific levels of nesting according to the associated geographical locations.

**Phylogenetic analysis.** Corroboration of the hierarchical nesting scheme that was revealed through the formation of the haplotype network and the nesting of unique clades was generated using the parsimony-based phylogenetics program POY, version 4.1.2 (66). Equal weights for transitions-transversions were assigned for all trees, for both host squid populations and bacterial symbionts, by the use of the respective loci (combined analyses using all molecular loci).

TABLE 2. Symbiont species present at each collection site<sup>a</sup>

Location	Symbiont species (no. of isolates) <sup>b</sup>	% of total symbiont population
Banyuls-sur-Mer, France	<i>V. fischeri</i> (74)	78
	<i>V. logei</i> (16)	16
	<i>P. leiognathi</i> (12)	6
Bari, Italy	<i>V. fischeri</i> (58)	85
	<i>V. logei</i> (10)	15
Bay of Biscay, Atlantic	<i>P. leiognathi</i> (8)	100*

<sup>a</sup> Species identification is based on the 16S rRNA sequence for all symbiotic bacteria isolated from squid light organs. Percentages are based on the total population within one particular site. \*, all symbionts isolated from the *R. minor* host were *Photobacterium leiognathi*. No *Vibrio* species were isolated from these hosts.

<sup>b</sup> *n* = 178.

## RESULTS

**Genetic variation and haplotype prevalence.** Variation in the sequence loci from both host squid and *Vibrio/Photobacterium* symbionts was measured from a subsampling of 178 symbiont strains. *V. fischeri* and *V. logei* strains were isolated from all *Sepiolo* species, and *P. leiognathi* only was isolated from *R. minor* host populations (for a total of 56 individual squids sampled). *Vibrio/Photobacterium* identification was determined using the 1,600-base-pair 16S rRNA locus. After each symbiont strain was identified as either *V. fischeri*, *V. logei*, or *P. leiognathi*, the 650-base-pair *gapA* locus was used in the nested clade analysis for the bacterial symbionts. Numerical distribution of species for each geographical location reflects the relative prevalence of each symbiont species in the populations sampled in this study (Table 2). A high occurrence of *V. fischeri* symbionts was found in both French and Italian populations (Fig. 1).

Both *Vibrio* and *Photobacterium* populations used in this study represented a total of 31 haplotypes. There were, respectively, 23, 6, and 2 species-specific haplotypes found in the analysis belonging to *V. fischeri*, *V. logei*, and *P. leiognathi*. One haplotype (haplotype 24) was shared among *V. logei* symbionts from both French and Italian localities (Fig. 1). In total, 24 strains identified in the initial 16S rRNA species identification were not used in the formation of haplotype networks due to incomplete *gapA* sequence information.

The distribution of haplotypes of symbiotic *V. fischeri* demonstrates grouping in geographical "clusters," which was also reflected in the final nesting. In the Mediterranean Sea, French and Italian clusters were clearly recognizable in the nesting scheme (Fig. 1). The genetic distance between populations of *V. fischeri* from the two Mediterranean sites was comparatively small; the closest association between a French *V. fischeri* haplotype and an Italian *V. fischeri* haplotype was a 3-base-pair difference or 3 unsampled haplotypes (Fig. 1). This sampling discrepancy occurs between the second most numerous French haplotype and the largest Italian haplotype (haplotype 15) (Fig. 1). Final nesting was the most parsimonious arrangement, which resulted in no confirmable false-positive inferences regarding colonization events or other population-level indices (64). Only one closed-loop structure arose in the construction

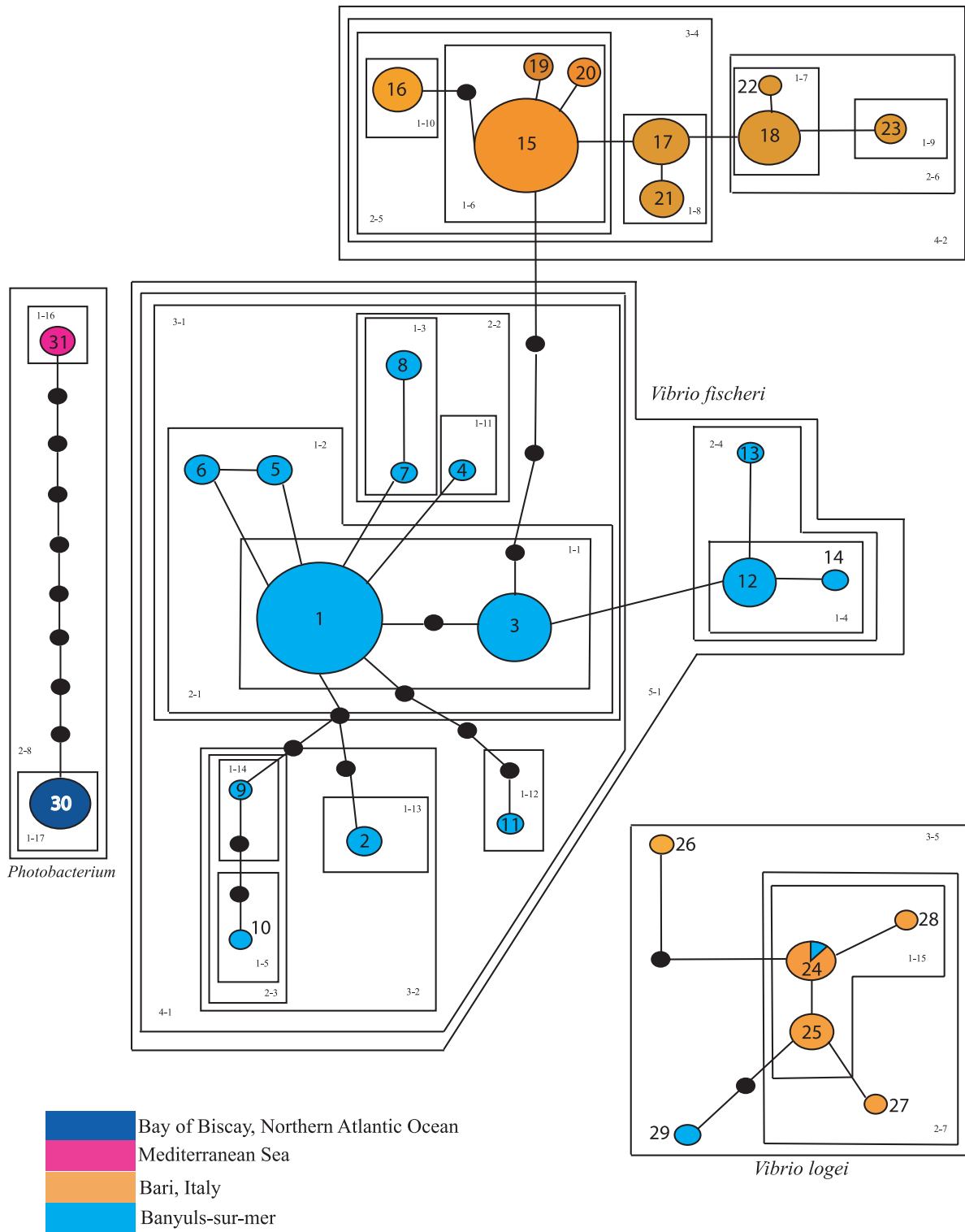


FIG. 1. TCS-generated haplotype network and nested clades for Mediterranean vibrios and *Photobacterium leiognathi* symbionts. Each line represents one base pair difference, with black circles representing an unsampled intermediate haplotype. Each numbered circle represents a distinct haplotype, with color denoting collection site. The size of each circle is proportional to the number of individuals representing each given haplotype. Nested clades are marked by two-number identifiers within each box, signifying the representative nested clade.

of the symbiont haplotype network, which was resolved according to Templeton's rules (62) (Fig. 1).

The *V. logei* network was comparably small, reflecting the total proportion of *V. logei* bacteria within the total symbiont

sample population. This network consists of 6 distinct haplotypes (24–29) (Fig. 1), with one haplotype containing both French and Italian strains (haplotype 24). The nesting of these haplotypes followed an interior-to-exterior scheme, with relat-



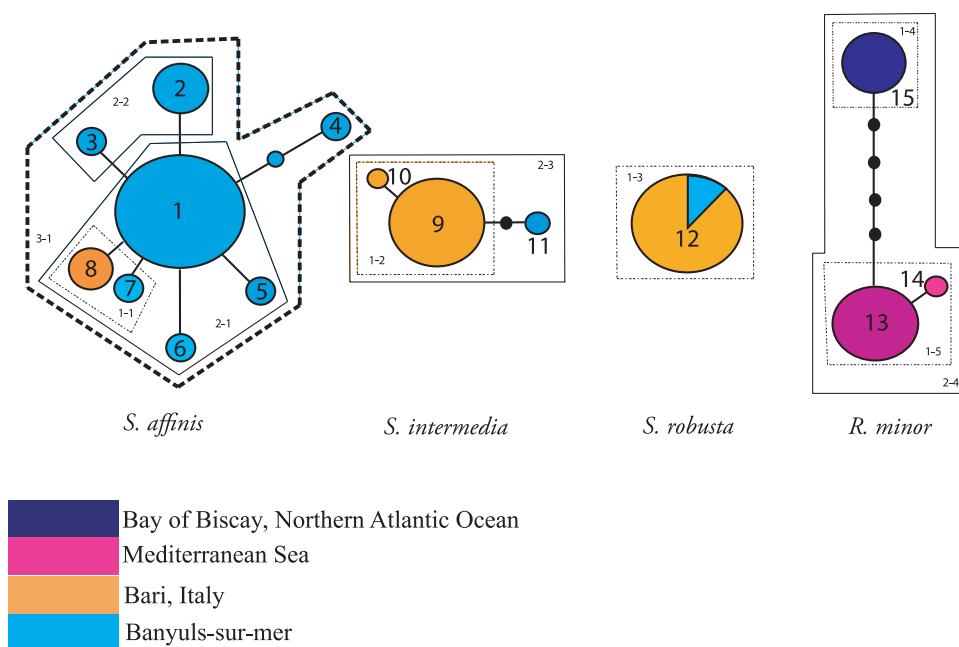


FIG. 2. Haplotype network and corresponding nesting schematic of individual species haplotype networks for both Mediterranean and Northern Atlantic (Bay of Biscay) squids. All species were genetically distinct at the COI, 12S, and 16S loci by a 10 bp-difference, precluding any connection between separate species.

edness, not geography, being the most parsimonious nesting result.

Complete cladograms for symbiotic bacteria from the sampled populations infer a lack of evidence for restriction of gene flow between populations (Fig. 1). Clades 1-3, 1-4, and 2-3 show potential restriction in gene flow and possible habitat fragmentation, which may be a reflection of the terminal, exterior location of these clades within the total cladogram. Overall, the nesting diagrams for the symbiont populations demonstrate geographical structure. Nesting at the fourth level (clades 4-1 and 4-2) (Fig. 1) is indicative of an association between genetic relatedness and local environment.

All *Vibrio* symbionts formed one clade in the phylogenetic analysis based on location (see Fig. S1 in the supplemental material). This confirms the geographical bias of the haplotype network and subsequent nesting diagrams (Fig. 1). Interestingly, *Vibrio* isolates (both *V. fischeri* and *V. logei*) were contained with the *Photobacterium* clade, with Italian *V. logei* sister to Italian *V. fischeri*. All French *V. fischeri* isolates analyzed were sister to the Italian *V. fischeri/V. logei* clade.

For host squids, a total of 15 haplotypes were found in all sample populations (Fig. 2). Initial networks that were calculated using a single-gene model (COI) revealed a relatively restricted network, supporting strong species delineation. To diversify the network, two additional genes (12S and 16S rRNA) were incorporated into the analysis to uncover unique variation inherent in other genes within species and among host populations. *S. affinis* had the largest number of COI haplotypes from all host species sampled, with a total of 9 distinct haplotypes, resulting in the most diverse and possibly least parsimonious set of haplotypes of all genes examined. Both 12S and 16S rRNA data had a more limited set of haplotypes for *S. affinis* (6 and 7 haplotypes, respectively), com-

pared to 8 when using all 3 loci (Fig. 2). *S. intermedia* and *R. minor* each showed a relatively low number of representative haplotypes. These particular species demonstrate a smaller, more inclusive set of haplotypes, with both *S. intermedia* and *R. minor* having only 3 haplotypes. *R. minor* haplotypes are split between two distant geographic locales, Banyuls-sur-Mer and the Bay of Biscay (Fig. 2). Interestingly, all *S. robusta* hosts (8 total) sampled in this study possessed the same haplotype for all three loci investigated (haplotype 12). Haplotypes were all species specific, with no introgression of any individual haplotype between squid species sampled for all loci. Additionally, combined phylogenetic analysis of all loci confirmed the structure and orientation of the nested haplotypes as they occur within the populations sampled, with *S. affinis* and *S. intermedia* sister to *S. robusta* and with all *R. minor* individuals being sister to the *Sepiolo* clade (see Fig. S2 in the supplemental material).

Host squid networks and nested diagrams demonstrated no introgression between sympatric species (Fig. 2). No connectable distances were found between species, regardless of distance or geographical location. The largest number of base pair differences among any one species was two in the population of *S. affinis*, nesting at the highest level of 3 (clade 3-1) (Fig. 2). Evidence for range expansion or fragmentation was unsubstantiated in this analysis due to a lack of direct evidence for the geographical separation of any of the host species (recall that all species are sympatric).

Variation within total populations of both hosts and symbionts was calculated using the measurement theta ( $\theta$ ). Symbiont theta values indicate higher per-base-pair variation, ranging from 0.00 to 0.0331. All symbiont populations, except the relatively small sample size of *P. leiognathi*, demonstrated some degree of genetic variation within each population (Table 3).

TABLE 3. Within-population variance (theta,  $\theta$ ) for each host species at each respective collection site<sup>a</sup>

Host species	Collection site	No. of hosts	Host $\theta$	Symbiont $\theta$
<i>S. affinis</i>	Banyuls-sur-Mer	18	0.0292	0.0331
	Bari	9	0.0220	0.0297
<i>S. intermedia</i>	Banyuls-sur-Mer	4	0.0026	0.0055
	Bari	7	0.0000	0.0082
<i>S. robusta</i>	Banyuls-sur-Mer	4	0.0000	0.0071
	Bari	6	0.0000	0.0025
<i>R. minor</i>	Banyuls-sur-Mer	4	0.0184	0.0000*
	Northern Atlantic	4	0.0000	0.0000*

<sup>a</sup> Values were calculated only for hosts from which at least two *Vibrio* or *Photobacterium* strains were isolated. \*, all symbionts are *P. leiognathi*. Variation among this species was observed only between populations in the Mediterranean versus the North Atlantic (Bay of Biscay).

The largest amount of within-population variation ( $\theta = 0.0331$ ) was observed with *V. fischeri* sampled from Banyuls-sur-Mer, France, from *S. affinis* (Table 3). Conversely, no genetic variation was found in symbiotic *P. leiognathi* isolated from the Bay of Biscay (Table 3). Additionally, no significant variation within populations of *P. leiognathi* was observed over the time period sampled, as only 3 derivative novel haplotypes arose chronologically in the course of this study (Fig. 1).

Theta values for host squids ranged from 0.00 to 0.0292 (range indicative of all 3 loci sampled for the entire host population) (Table 3). *S. robusta* hosts from both French and Italian sample sites demonstrated no genetic variation among individuals and therefore have a  $\theta$  value of 0. Relatively low theta values for host variation across all loci indicate little or no divergence within populations of species, regardless of the location of populations.

## DISCUSSION

**Symbiont population structure.** Symbiotic bacteria that were sampled from collected host squids exhibited much more dynamic patterns across geographical locations than their respective host squids. Only one closed-loop structure was observed with the initial haplotype network construction within the *V. fischeri* population from Banyuls-sur-Mer (France). This ambiguity occurred between the most highly represented haplotype (Fig. 1, haplotype 1) and less-represented satellite haplotypes (haplotypes 5 and 6). These structures in future haplotype networks need to be carefully considered due to the potential for false-positive results (23, 48).

The most obvious trend apparent in the nesting of individual haplotypes is a strong correlation between relatedness and geography, with different amounts of variation depending on the species sampled. In *V. fischeri* populations, geographical grouping is evident (Fig. 1; see also Fig. S1 in the supplemental material). Within this species, no haplotypes were shared between Banyuls-sur-Mer and Bari. This suggests that genetic differences are due to geographical boundaries for symbiotic bacteria. In *V. logei* populations, a shared haplotype between sample sites was found, indicating that a small amount of introgression has occurred between geographical locations and

that not all populations are in complete isolation from each other. *V. logei* is a known psychrophile and has been shown to prefer colder habitats (17, 38, 65), which may reduce the amount of movement during warmer seasons. Differences also exist between *V. fischeri* and *V. logei* in their concentration throughout different times of the year (22). Thus, introgression of certain haplotypes may occur when abiotic factors may be conducive for bacteria to have higher residence times in the water column when habitats share similar features. Since seasonal temperature fluctuations seem to be driving population dynamics within other symbiotic *Vibrio* communities (21, 58), it is likely that introgression may readily occur in Mediterranean vibrios when habitats are similar.

Niche-specific differences driven by abiotic factors like temperature may also play a major role in defining specific genetic differences which have ultimately led to a clear distinction between the two *Vibrio* species in the northern Mediterranean (57, 58). This has direct implications for the population structure of the squid-*Vibrio* symbiosis. Deeper-dwelling, colder-water sepiolid species (i.e., *S. robusta* and *S. intermedia*) may be discriminately exposed to a higher proportion of *V. logei*, thereby favoring colonization by one *Vibrio* species over another (38, 42, 58). Although this *in situ* evidence is only suggestive, additional studies of such populations could add evidence as to how symbiont structure is formed in these particular sepiolids over both time and habitat preference.

*P. leiognathi* isolates from Mediterranean *R. minor* hosts all shared the same haplotype (Fig. 1, haplotype 29) compared to those from the North Atlantic (Fig. 1, haplotypes 29 and 30). This genetic distinction may be due to differences in abiotic factors that shape their ecology, as well as a large geographical distance between sample sites (Atlantic versus Mediterranean seas). The locational disparity may also be exacerbated by the very narrow Strait of Gibraltar, which symbiotic bacteria would need to pass through to access either host site while adjusting to differential conditions between the Atlantic Ocean and the Mediterranean Sea (Table 1) (45, 46). Additionally, water movement into the Mediterranean Sea from the Atlantic Ocean would most likely prevent significant physical movement and gene flow of most organisms out of the Mediterranean (2, 5, 26, 32, 57).

**Host population structure.** The population structure of host squid species used in this study suggests strong species delineations within sympatric populations (Fig. 2; see also Fig. S2). Individual squids within each species shared similar haplotypes regardless of collection site (Fig. 2). There was also no significant temporal or geographical change in host populations based on the genetic information, considering squid were sampled within a period of 6 years (Table 1). Lack of significant change over time (between 3 to 7 years) suggests a slow mutation rate in the COI and rRNA-coding loci or active gene flow between populations such that all species populations are homogeneous (8).

Genetically similar populations occurring in geographically distinct areas suggest a number of possible evolutionary scenarios. One is a recent dispersal or radiation event which separated genetically similar populations into various geographical locations. Since the Mediterranean Sea has been shown to have surface water currents driven by strong seasonal changes, this may provide a mechanism for juvenile squids to

be moved to different locations at specific times of the year (56). Another possible consideration is that certain loci do not provide sufficient phylogenetic resolution to expose fine differences between geographically different populations of host squid (4). This second scenario is less likely, given that previous studies of Indo-West Pacific sepiolids and other Mediterranean fauna using mitochondrial loci provide strong support for determining population structure (13, 14, 21, 47). Additional mitochondrial genes (12S and 16S rRNA) reinforce this species exclusivity through parallel networks resulting in only two additional haplotypes when combined with the COI sequence data (haplotypes 9 and 13) (Fig. 2).

**Influence of abiotic factors.** The Mediterranean Sea is a relatively shallow, contained body of ocean, which is accessed by other bodies of water at only a small number of places: the Strait of Gibraltar, the Dardanelles, and the Bosphorus. This results in the lack of water movement between the Mediterranean and any other significantly large body of water. The northern Mediterranean is also absent of ocean-like currents, which could facilitate movement around the coastal margins of the Mediterranean (49, 51). Previous work evaluating the population genetic structure of sepiolid squid and their symbionts in the Indo-West Pacific demonstrated some specificity between allopatric *Euprymna* species and their *Vibrio* symbionts, with observed movement of *V. fischeri* strains between host populations that had similar environmental regimes (21). This supports the idea that although *Euprymna* hosts can share *V. fischeri* from other *Euprymna* hosts (host specificity) throughout the Indo-West Pacific, *Vibrio* bacteria have the ability to move around and infect new populations if environmental factors facilitate their transport, survival, and fitness (57, 58).

Thus, one major difference between the Indo-West Pacific and the Mediterranean that may lead to a lack of specificity between host squids and their vibrio symbionts is temperature. The Mediterranean Sea is subject to shallow thermoclines, especially during summer months, which can drastically change temperatures within the water column (Table 1) (52). These seasonal temperature differences may create population differences due to temperature adaptation that separates the habitat ranges of prospective symbionts (22, 38, 57). Thus, particular temperature conditions of host habitats may directly influence which species/strain of symbiont predominates in their light organs (38, 39, 57, 58). Newly hatched juveniles of species which are found at relatively deeper depths, such as *S. ligulata*, *S. intermedia*, and *S. robusta*, are more likely to be inoculated by *V. logei*, which has a selective advantage at colder temperatures (38). Since sepiolid squids are found continuously year-round, various generations of *Sepiolo* may have been colonized by different *Vibrio* bacteria, depending on when and where they were infected (6, 36, 38). In our study, the ratio of *V. fischeri* to *V. logei* found in *S. affinis* (found in shallower, warmer waters) (Table 1) was significantly higher, suggesting that the warm-water-preferring *V. fischeri* has an advantage in terms of its potential to form symbioses with sepiolid squids in those areas. This has also been observed in laboratory experiments, in which temperature rather than host specificity is a strong force in forming these associations (38).

The influence of abiotic factors on the establishment and persistence of this symbiosis in sepiolid squid has only recently emerged as a major force in determining the natural structure

of the symbiosis (57, 58). This study has demonstrated a lack of specificity between sympatric species of light organ-bearing sepiolid squids and their *Vibrio* symbionts in the Mediterranean. Adaptation to multiple squid populations in the Mediterranean seems to be driven mainly by environmental determinants, but host specificity is still important in the establishment of the population genetic structure, since *Vibrio* or *Photobacterium* species are the sole symbionts of sepiolid squids in both the Indo-West Pacific and Mediterranean seas (41). The combined influence of host specificity and abiotic factors contribute to the complexity of the population genetic structure of environmentally transmitted host-symbiont associations. Understanding the behavior of symbiotic bacteria among eukaryotic hosts and the phenomena that determines their phylogeographical matrix may help aid in our understanding of the population dynamics of other related *Vibrio* species in nature (including pathogens such as *V. cholerae* and *V. parahaemolyticus*) and what factors are important for their survival in both their free-living and symbiotic states.

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