Molecular Ecology

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MOLECULAR ECOLOGY

ORIGINAL ARTICLE OPEN ACCESS

Genetic Variation in the Atlantic Bobtail Squid-*Vibrio* Symbiosis From the Galician Rías

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Received: 1 July 2024 | **Revised:** 25 September 2024 | **Accepted:** 10 October 2024

Handling Editor: Kayla King

Funding: This work was funded by NSF-DBI 2214038, NASA EXO-80NSSC18K1053, College of Arts and Sciences at New Mexico State University, and the Quantitative Systems Biology program and School of Natural Sciences at UC Merced.

ABSTRACT

Symbiotic marine bacteria that are transmitted through the environment are susceptible to abiotic factors (salinity, temperature, physical barriers) that can influence their ability to colonize their specific hosts. Given that many symbioses are driven by host specificity, environmentally transmitted symbionts are more susceptible to extrinsic factors depending on conditions over space and time. In order to determine whether the population structure of environmentally transmitted symbionts reflects host specificity or biogeography, we analysed the genetic structure of *Sepiola atlantica* (Cephalopoda: Sepiolidae) and their *Vibrio* symbionts (*V. fischeri* and *V. logei*) in four Galician Rías (Spain). This geographical location is characterized by a jagged coastline with a deep-sea entrance into the land, ideal for testing whether such population barriers exist due to genetic isolation. We used haplotype estimates combined with nested clade analysis to determine the genetic relatedness for both *S. atlantica* and *Vibrio* bacteria. Analyses of molecular variance (AMOVA) were used to estimate variation within and between populations for both host and symbiont genetic data. Our analyses reveal a low percentage of variation among and between host populations, suggesting that these populations are panmictic. In contrast, *Vibrio* symbiont populations show certain degree of genetic structure, demonstrating that the hydrology of the rías is driving bacterial distribution (and not host specificity). Thus, for environmentally transmitted symbioses such as the sepiolid squid-*Vibrio* association, abiotic factors can be a major selective force for determining population structure for one of the partners.

1 | Introduction

In marine ecosystems, species distribution can be affected by biotic factors such as mating systems, life history strategy and dispersal methods (Almeida et al. 2022; Araújo and Louto 2007; Riascos et al. 2020). Abiotic factors, such as marine currents, coastline topography, salinity and temperature can also have dramatic effects on where species are distributed (Kool et al. 2011). Subsequently, the presence of geographic barriers or marine currents in the ocean constitutes an opportunity to examine dispersal and consequently species distribution in a particular location that oftentimes gives rise to new taxa (Strugnell, Allcock, and Watts 2017). Earlier studies have revealed how closely related populations undergo speciation events due to the existence of geographic barriers (Monahan, Pereira, and Wake 2012; Young et al. 2015). However, less is known about how allopatric speciation events affect species that coexist together, as is the case of symbiotic associations (Hellberg et al. 2002; Palumbi 1994).

One approach that is commonly used to discern how various barriers shape the distribution of a species is by analysing the genetic structure of the population (Jahnke et al. 2019; Heras

et al. 2019; Yan et al. 2020). The historic Northwestern coast of the Iberian Peninsula in Galicia has a uniquely jagged coastline, with many inlets or 'rías' that provide an appropriate locality for testing hypothesis on whether such topologies are barriers to gene flow between populations. Examples such as the Mediterranean mussel *Mytilus galloprovincialis* (Diz and Presa 2009), which have planktonic larvae that are transported through currents (Pickett and David 2018), the macro alga *Fucus serratus*, with part of its life cycle including mobile antherozoid gametes (Duarte and Viejo 2018) or the marine seagrass *Zostera noltii* (Coyer et al. 2004), which has a high dispersal potential via vegetative fragmentation (Berković et al. 2014) have shown how gene flow can be influenced by both abiotic and biotic factors. Yet none of these studies have examined whether the microbiota associated with these populations mirror similar genetic fractionation. Therefore, in order to test the hypothesis that symbiotic partners share similar patterns of genetic structuring leading to speciation, we focused on the symbiotic relationship established between *Vibrio* sp. and the Atlantic bobtail squid, *Sepiola atlantica* from the Galician coast.

The Atlantic bobtail squid (*Sepiola atlantica*, d'Orbigny 1839–1842), is a nektobenthic species that is widely distributed in the northeastern Atlantic from latitudes 65° N to 35° N, from Iceland to Morocco (Guerra 1992; Reid and Jereb 2005). This species has been caught unintentionally by fishing nets, preferring sandy bottoms covered by algae ranging from 2 to 40 m in depth. *S. atlantica* inhabits the most central and outer portions of the Rías of Galicia (Figure 1), which are proximal to the open Atlantic Ocean. *S. atlantica* has been found to be more abundant in the northern part of the Rías and are absent from the internal portions furthest away from the coastline (Cacabelos, Gestoso, and Troncoso 2008; Guerra 1986; Rodrigues et al. 2011; Yau and Boyle 1996). Mature males and females can be found throughout the year (Guerra et al. 1986) and their life history strategy has been well documented (Russell 1922; Stephen and Scottish 1944; Rodrigues et al. 2010, 2011; Rodrigues, Guerra, and Troncoso 2012).

Bobtail or bottletail squids (Cephalopoda: Sepiolidae) are cosmopolitan, and form symbiotic associations with bioluminescent bacteria from the genera *Vibrio* and *Photobacterium* (γ-Proteobacteria: Vibrionaceae [Herring 1977]). Juvenile squids are colonized environmentally within hours by *Vibrio* species present in seawater and are housed in a specialised internal organ called the light organ (LO) (McFall-Ngai and Ruby 1991; Montgomery and McFall-Ngai 1994; Nishiguchi 2000). Here, the host provides an appropriate nutrient-rich habitat for the symbiont, while in return, the bacteria provide luminescence to the squid utilised in a behaviour known as counterillumination (Jones and Nishiguchi 2004). Light produced by *Vibrio* bacteria helps camouflaging the squid host silhouette during the evening, increasing the fitness in terms of survivability and predation success during the nocturnal hours (Young and Roper 1976; Pipes and Nishiguchi 2022). At dawn, approximately 95% of the bacteria are vented out into the surrounding seawater, releasing viable Vibrios into the local habitat, while the remaining population (~5%) of bacterial cells inside the light organ multiply during the day, ready for a new cycle the following night (Boettcher, Ruby, and Mcfall-Ngai 1996). Once released, the free-living Vibrios are exposed to a wide range of biotic and abiotic factors that can

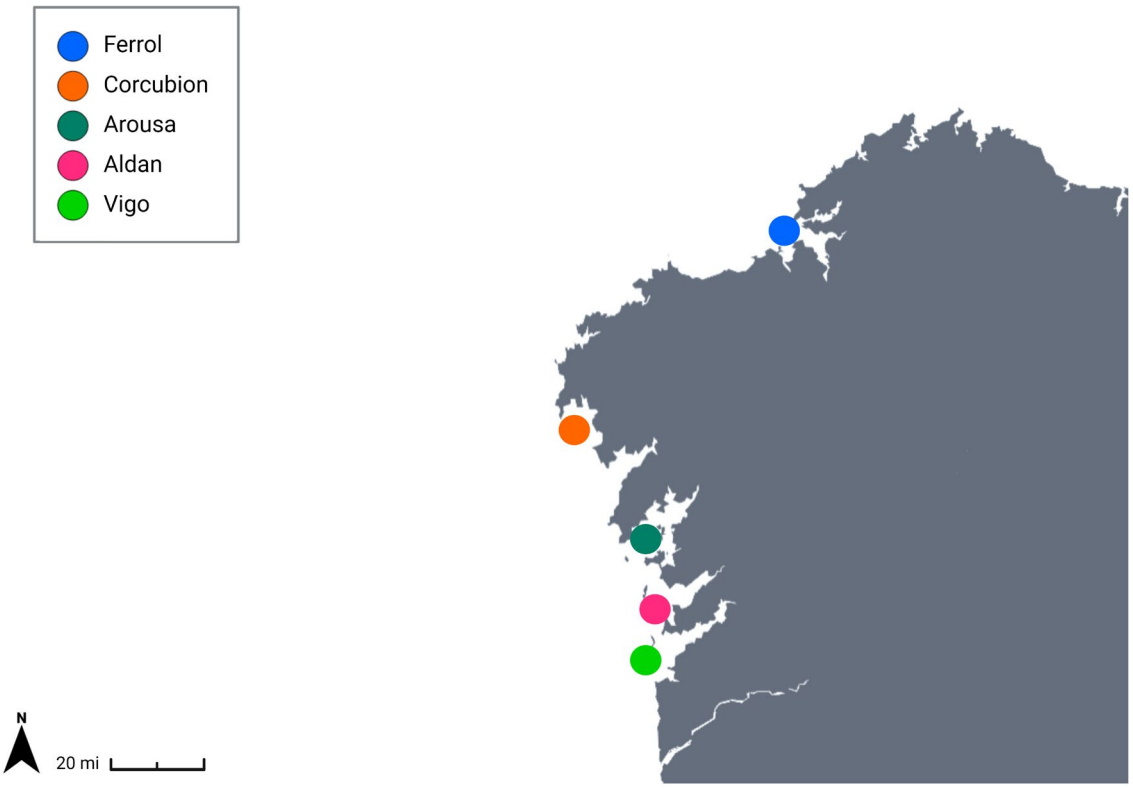

FIGURE 1 | Sampling location in each of the Galician Rías (NW coast of the Iberian Peninsula). Each coloured dot represents the geographical location where samples were taken at each of the five Rías during the years 2007, 2008 and 2009.

affect their predisposition to infect new hosts, and can also migrate to other areas where they colonie different populations of sepiolids (Nyholm et al. 2000; Nyholm and McFall-Ngai 2004; Nyholm and Nishiguchi 2008). Although many studies have focused on the reproduction, behaviour and ecology of *S. atlantica*, very few have focused on the actual symbioses and how both partners shape the association. Therefore, the main objective of this study is to examine the genetic variation in the Atlantic bobtail squid-*Vibrio* symbiosis from the Galician Rías to determine how abiotic factors can affect the genetic structure of both host and symbiont populations using a combined phylogeographic approach. The dynamic geomorphology and subsequent currents present in the area as well as the unique characteristics of each Ría allow for the investigation of what roles abiotic factors or host specificity have in the distribution of these mutualistic associations.

2 | Material and Methods

2.1 | Specimen Collection and Bacterial Isolation

Table 1 lists all sites sampled and analysed in this study. A total of five different populations were collected from waters along the NW Iberian Peninsula in Galicia, Spain (Figure 1). These populations correspond to five different sampling locations (north to south): Ría de Ferrol, Ría de Corcubión, Ría de Arousa, Ría de Aldán and Ría de Vigo. The Southmost Rías, Vigo, Aldán and Arousa are within an area of 30 km, and approximately 100 and 150 km away from Corcubión and Ferrol. Samples were obtained via SCUBA during the daytime at approximately 6–10 m deep at each site. This limited the number of specimens that could be caught since the species is nocturnal and difficult to find due to their burying behaviour during the day (Guerra 1986; Rodrigues et al. 2010). Animals were transported and maintained at the Centro de Investigación Mariña-Estación de Ciencias Mariñas de Toralla (CIM-ECIMAT) facilities and subsequently euthanised 1 day later following protocols previously approved by the Animal Experimentation Ethics Committee from the University of Vigo (Spain) and the Institutional Animal Care and Use Committee (IACUC) at New Mexico State University (NMSU; Andrews et al. 2013; Moltschaniwskyj et al. 2007). Single colonies of *Vibrio* bacteria were isolated from host light organs that were removed via ventral dissection and homogenized and diluted onto seawater tryptone agar (SWT; 0.5% tryptone, 0.3% yeast extract, 0.3% glycerol, 1.5% agar and 70% seawater at 32 ppt). SWT used for light organ isolation at CIA-ECIMAT was made with local seawater, whereas isolations at NMSU were made with artificial seawater containing a mixture of Instant Ocean (21 g/L of seawater; Spectrum Brands, VA) and Marine Mix (7 g/L of seawater; Wiegandt GmbH, Germany). Squid tissues were preserved in 95% ethanol for fixation and subsequent DNA extraction for Sanger sequence analysis at NMSU.

Light organ homogenates isolated at CIA-ECIMAT were grown for 24–48 h. at 15°C, after which ~15 individual colonies from each plate were stab-inoculated into vials containing SWT agar and sealed for transport back to NMSU. After transport, each sample was re-grown on SWT agar plates at 15°C for 24–48 h. Single colonies were isolated and cultured in liquid SWT at 15°C and shaken at 225 rpm for 24–48 h in an Innova 43 shaking incubator (New Brunswick Scientific, NJ). Each overnight culture was sub-cultured and allowed to reach log phase (2–3 h. at 15°C and 225 rpm), and the log-phase cultures were used for DNA extraction and frozen in 40% glycerol for long-term storage at −80°C.

2.2 | DNA Extraction and Amplification

S. atlantica DNA was extracted using approximately 25 mg of ethanol-preserved tissue that was dissected from the gill or mantle of each squid. Dissected tissues were washed with 100 μL of nuclease-free water to remove any residual ethanol. *S. atlantica* DNA was extracted using the DNeasy blood and tissue protocol for animal tissues (Qiagen, Valencia, CA). All genomic DNA extractions were visualized on a 1% agarose gel and quantified using a Nanodrop 9600 (ThermoFisher Scientific, Waltham, MA). Total DNA extracted from each individual squid sample was used to amplify a 658-bp fragment of the cytochrome *c* oxidase subunit I (*COI*, Table S1) (Coryell et al. 2018; Folmer et al. 1994; Jones et al. 2006; Zamborsky and Nishiguchi 2011). The cytochrome *c* oxidase subunit I gene has been shown to be highly variable across invertebrate taxa (Folmer et al. 1994; Jacobs and Grimes 1986) and has been used in previous studies to determine population structure (Calderón, Garrabou, and Aurelle 2006; Fogliata et al. 2022; Wang et al. 2023; Kise et al. 2023). Isolation of DNA from *Vibrio* light organ isolates was completed using the Qiagen DNeasy blood and tissue kit (Valencia, CA) gram-negative bacterial protocol. Approximately 2×10^9 cells were transferred from each log- phase culture for DNA extraction. Purified *Vibrio* DNA was visualized on a 1% agarose gel and quantified using a Nanodrop 9600 (ThermoFisher Scientific). Isolated DNA extracted from

TABLE 1 | Geographic location where *S. atlantica* specimens and their symbionts were collected. Theta, (Θ) a base-pair-by-base-pair measure of polymorphism from the host and the *Vibrio* sp. populations from each location is calculated for each collection site.

Host	Population location	Host *N*	Latitude	Longitude	Host theta	Vibrio theta
Sepiola atlantica	Ría de Vigo	2	42.249	8.795	0.00185	0.22434
	Ría de Aldán	3	42.288	8.822	0.00123	0.18521
	Ría de Arousa	2	42.542	8.909	0.0000	0.03494
	Ría de Corcubión	10	42.910	9.148	0.00065	0.06559
	Ría de Ferrol	10	43.447	8.305	0.00065	0.004936

each symbiotic bacterial isolate was used to PCR amplify the 16S rRNA locus for identification and a portion of the glyceraldehyde phosphate dehydrogenase (*gapA*) locus (~600 bp) for the population genetics analysis using previously described *Vibrio*-specific primers (Table S1; Ast, Urbanczyk, and Dunlap 2009; Nishiguchi and Nair 2003). The *gapA* locus has been used reliably to estimate deep phylogenetic connections between bacterial families (Nelson, Whittam, and Selander 1991) within the Vibrionaceae (Nishiguchi and Nair 2003), as well local population structure of symbiotic *V. fischeri* (Coryell et al. 2018; Jones et al. 2006).

Each PCR amplification reaction (25 μL) contained 2–20 ng of template DNA (0.08–0.8 ng/μL), GoTaq DNA polymerase (0.05 U/μL) (Promega, Fitchburg, WI), 5× GoTaq buffer (1×) (Promega, Fitchburg, WI), a 10 mM deoxynucleoside triphosphate mix of each nucleotide (Promega, Fitchburg, WI), and both forward and reverse primers (0.5 μM each) (Table S1). All amplification reactions were run using anobtraine MJ Research Dyad Disciple thermocycler (Waltham, MA) and BIO-RAD C1000 Touch Thermal Cycler (Hercules, CA). PCR conditions for *gapA* locus amplification had an initial denaturation of 2 min at 95°C followed by 30 s at 95°C, annealing temperature 45°C for 1 min, extension temperature for 1 min at 72°C, and a final extension temperature 72°C for 5 min. The PCR conditions for each *COI* locus amplification had an initial denaturation of 2 min at 95°C, followed by 30 s at 95°C, annealing temperature 50°C for 1 min, extension temperature for 1 min at 72°C, and a final extension temperature 72°C for 5 min. Amplicons were purified using QIAquick PCR purification kit (Qiagen) and quantified using a Thermo Scientific NanoDrop One Microvolume UV–Vis Spectrophotometer (ThermoFisher Scientific). Purified amplicons were pre-sequenced using BigDye Terminator v3.1 (Applied Biosystems, Foster City, CA) and amplified on an MJ Research Dyad Disciple thermocycler (Waltham, MA). Pre-sequencing samples were cleaned using 96-well Sephadex plates (Edge Biosystems, St. Louis, MO). Samples were sequenced at the NMSU Molecular Biology Sequencing facility using the Applied Biosystems 3130XL sequencer (Applied Biosystems) and at the UC Berkeley DNA Sequencing Facility (Berkeley, CA). Sequences were assembled and aligned using Molecular Evolutionary Genetics Analysis version 11, MEGA 11 (Tamura, Stecher, and Kumar 2021).

2.3 | Haplotype Networks, Nested Clade and Molecular Variance Analysis

Haplotype networks for both squid and symbiont were generated using TCS v1.12 and ANeCA (Clement, Posada, and Crandall 2000; Panchal and Beaumont 2007) using statistical parsimony methods outlined by Templeton (Templeton and Sing 1993). These haplotype networks were visualised using the software PopArt (Leigh and Bryant 2015). Nested clade analyses were performed using Templeton's nesting algorithm as implemented in GEODIS (Posada, Crandall, and Templeton 2000). Analysis of molecular variance (AMOVA) was executed using the population genetics software platform ARLEQUIN (Excoffier and Lischer 2010) and DNAsp v5 (Librado and Rozas 2009). All analyses were run for measures of within- and among-population variation along with a separate analysis assessing variation by each Ría for both host and symbiont data. Concurrently, theta (Θ), a base- pair- by- base- pair measure of polymorphism was calculated for each mutualist population at each sample site (Table 1).

3 | Results

3.1 | Nested Clade and Molecular Variance Analysis of *S. atlantica*

A total of 27 host cytochrome *c* oxidase subunit I (*COI*) sequences were used to delineate the haplotype network and test the nested clade analysis, yielding a unique network with four different squid haplotypes (Figure 2). Haplotype 1 is the predominant one and includes sequences from all the rías (Vigo, Aldán, Arousa, Corcubión and Ferrol). Sequences from Ferrol and Corcubión located farther north have the greatest number in haplotype 1, along with Arousa and Aldán rías. Haplotype 2 contains a sequence from Ferrol. It is connected to haplotype 1 by one mutational step (Figure 2). Haplotype 3 contains one sequence from Vigo, which is the southernmost ría, and it is also connected to haplotype 1 by one mutational step.

Haplotype 4 contains sequences from Corcubión and Aldán, with both being equally represented in this haplotype. Haplotype 4 is connected to haplotype 1 by one mutational step. Each of the four haplotypes are contained in their own 0-level ranked nest and a higher rank clade, which combines all haplotypes sampled in this study.

The final nested clade analysis of host genetic data was performed with no detectable loops according to the rules established by Templeton and Sing (1993). The nested clade phylogenetic analysis of host genetic data demonstrates that the null hypothesis of panmixia cannot be rejected (Table 2).

Analysis of molecular variance of host genetic data revealed a level of genetic fixation of the population, with an $F_{ST} = 0.0000^*$ (p value < 0.01; Table 3). The within-population variation, reported as Θ, was the same in two locations, Corcubión and Ferrol (0.00065; Table 1). The lowest Θ value ($\Theta = 0$) was reported from Arousa. Interestingly, the highest value of genetic diversity was from Vigo (0.00185).

3.2 | Nested Clade and Molecular Variance Analysis of *Vibrio*

Conversely, the genetic population structure of the *Vibrio* symbionts exhibited a different configuration compared to their host squids. A total of 70 glyceraldehyde dehydrogenase (*gapA*) sequences were analyzed, yielding three non-continuous networks of 39 haplotypes (Figure 3, Table S2). Identical sequences isolated from the same light organ were removed from our analysis. The number of mutational steps and unsampled haplotypes observed are shown in Figure 3. This haplotype network is more diverse and differentiated compared to the one observed from the host populations. Haplotypes 1, 2 and 3 only contain sequences from Arousa.

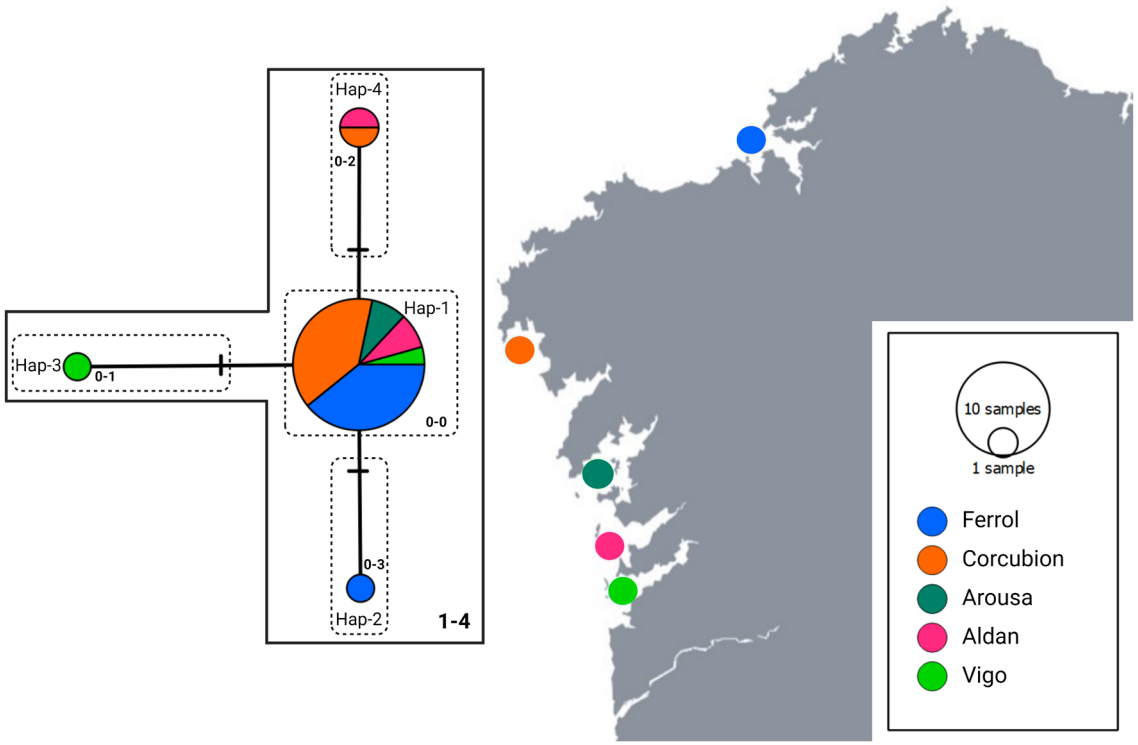

FIGURE 2 | TCS nested haplotype network generated from *S. atlantica* molecular data obtained from the animals captured in the five sampling locations during the years 2007, 2008 and 2009. Each line in the diagram represents one-base-pair mutational step between haplotypes. Black solid circles represent unsampled mutational steps connecting haplotypes. The size of the circles conveys information about the number of sequences that comprise a particular haplotype, while the colour of each circle signifies the geographic source of the sequence data and its relative contribution to the overall haplotype. Each haplotype is denoted by a two-digit code enclosed within dotted lines, which illustrates their hierarchical relationships. Each level of this hierarchy is marked with a two-digit label in a dashed format.

TABLE 2 | *S. atlantica* clades that demonstrated significance during either a permutation contingency analysis or geographic distance analysis. Location of significance is indicated by (D_n), nested clade distance and/or (D_c), the within-clade distance. I–T indicates the average distance between a tip clade and an interior clade. S or L indicates that the distance measure is significantly smaller or larger at the 5% inference level. Inference steps were performed using the automated inference key in GEODIS, part of the AneCA v1.2 population genetics analysis software platform (Posada, Crandall, and Templeton 2000; Panchal and Beaumont 2007).

Clade	Nested clade	Distance	Value (S or L)	Chi square statistics	Inference key steps	Inference
Total cladogram	0–0 T	D_c	38.8831	2.4791	—	Null hypothesis cannot be rejected. Moving on to next clade
		D_c	39.1988			
	0–1 T	D_c	10.1639			
		D_c	0.0			
	0–2 T	D_c	37.4794			
		D_c	28.7409			
	0–3 T	D_c	46.5731			
		D_c	0.0			
	I–T					

Note: "*" means p value < 0.001.

Interestingly, haplotype 1 is nested with haplotype 28 and haplotype 39 that only contain sequences from Ferrol and Corcubión respectively. These are nested in two correlatively ranked clades, 2–14 and 3–9, forming their own separated network. The next largest network (number of haplotypes) contains haplotypes 10 and 11, separated by three mutational steps: haplotype 15, nested with haplotypes 10 and 11 in clade 3–8 and representing sequences only from Aldán,

TABLE 3 | Percentage of variation among and within *S. atlantica* populations.

Source of *Sepiola* sp. variation	df	Sum of squares	Variance components	Percentage of variation
Among population	4	101.367	1.32652*	0.000
Within population	49	247.092	2.12002*	100
Total	51	348.459	3.44654	
Overall (F_{ST})	0.000*			

Note: "*" means p value < 0.001.

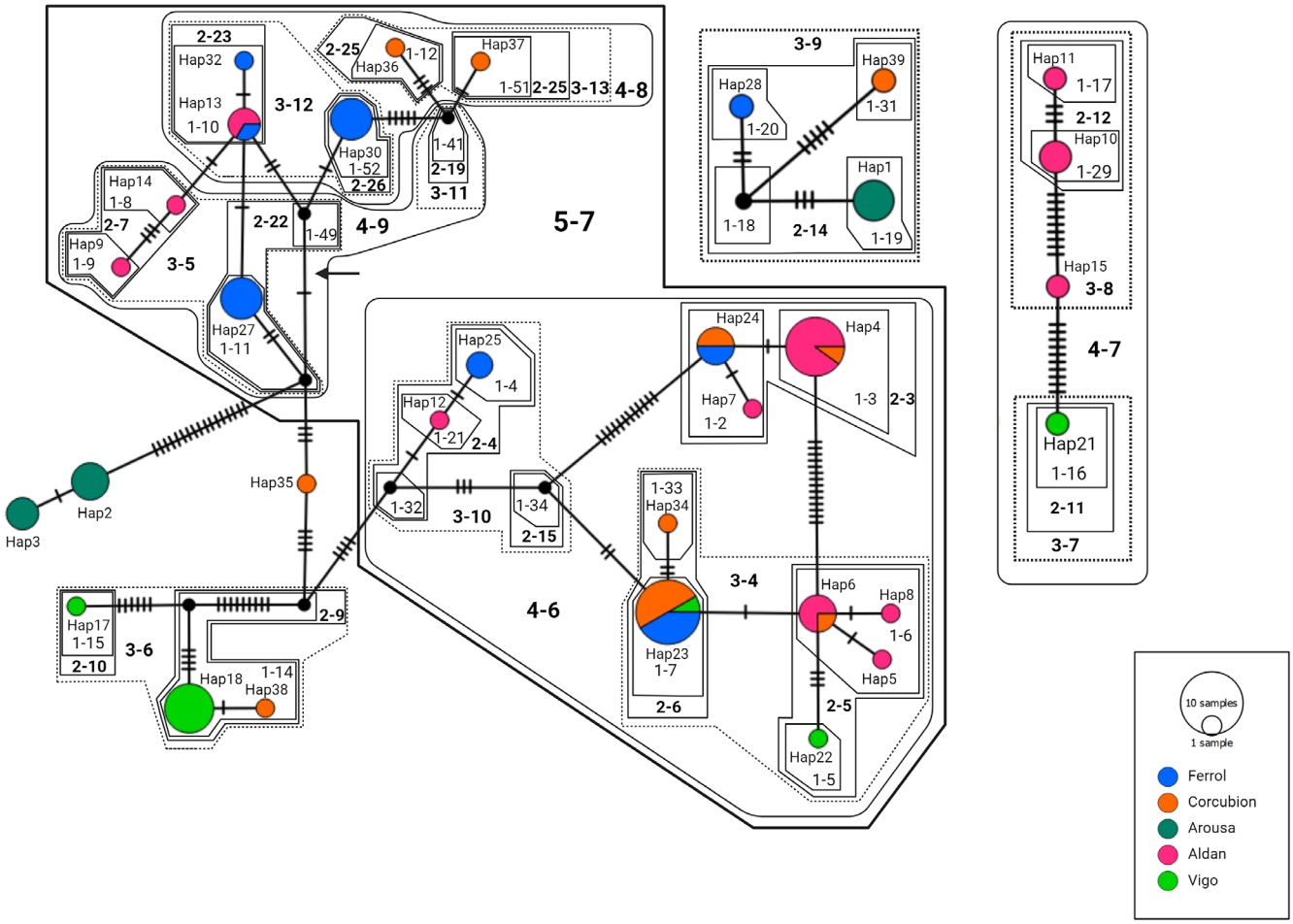

FIGURE 3 | TCS haplotype network generated from *Vibrio* sp. symbionts molecular data acquired from isolates harvested from squid light organs in the Galician Rías during the years 2007, 2008 and 2009. Each line in the diagram represents one-base-pair mutational step between haplotypes. Black solid circles represent unsampled mutational steps connecting haplotypes. The size of the circles conveys information about the number of sequences that compose a particular haplotype, while the colour of each circle signifies the geographic source of the sequence data and its relative contribution to the overall haplotype. Each haplotype is denoted by a two-digit code enclosed within dotted lines, which illustrate their hierarchical relationships. Each level of this hierarchy is marked with a two-digit label in a dashed format. The resolution of the loop structure is resolved at the point marked with an arrow.

and haplotype 21 which possesses sequences only from Vigo nested in its own clade, 3–7. Haplotype 21 is separated by 10 mutational steps from clade 3–8. These four haplotypes form the higher ranked clade 4–7, which constitutes its own network unit.

The biggest network contains the majority of haplotypes and shows the highest rank of nesting in clade 5–7, which groups three separated clades into two large clusters of haplotypes, clade 4–6, 4–8, and 4–9. In clade 4–6 there are two larger haplotypes, haplotype 4 with most sequences isolated from Aldán and a small contribution of sequences from Corcubión. Haplotype 6 shows a similar distribution in terms of abundance of sequences from these two locations, being separated from haplotype 4 by 14 mutational steps. Haplotypes 5 and 8 have sequences only from Aldán and are both connected to haplotype 6 by one mutational

step. Additionally, haplotype 22 which only contains sequences from Vigo is connected to haplotype 6 by three mutational steps. This cluster of haplotypes is nested within clade 2–5, which in turn is nested with clade 2–6 containing haplotypes 23 and 34 in the higher ranked clade 3–4. Haplotype 34 contains only sequences from Corcubión. Haplotype 24 contains the same proportion of sequences from the northernmost rías, Corcubión and Ferrol, and is connected to haplotype 4 and haplotype 7, unique from Aldán, by one mutational step. These three haplotypes form the clade 2–3. Haplotype 12, exclusive from Aldán, and haplotype 25, which contains sequences only from Ferrol, are separated by one mutational step. They are nested within nest 3–10, along with two unsampled haplotypes (Figure 3).

In clade 4–8, no haplotypes containing sequences from Arousa or Vigo are found. Alternatively, one major unique haplotype, 30, with sequences from Ferrol is represented and nested within its own clade, 2–26. Haplotypes 36 and 37, which form their own clade, 3–13, are exclusive from Corcubión and are connected to an unsampled haplotype by three and one mutational steps respectively. This unsampled haplotype is separated by five mutational steps from haplotype 30. Haplotype 13 has sequences from Aldán and Ferrol, with a majority from the first location. Haplotype 13 is connected to haplotype 32 by a single mutational step. Along with haplotype 30, they form clade 3–12.

Within clade 4–9, clade 3–5 nests clades 2–7 and 2–22. Clade 2–7 groups haplotypes 9 and 14, which are unique from Aldán and are separated from each other by three mutational steps. Clade 2–22 contains haplotype 27, with only sequences from Ferrol. It is connected to an unsampled haplotype by two mutational steps, with a second unsampled haplotype by a total of three mutational steps. The loop formed by haplotypes 13 and 27 and the two unsampled haplotypes is resolved at the point the arrow indicates (Figure 3) according to the rules established by Templeton and Sing (1993).

Clade 3–6, which contains haplotypes 17, 18, and 38 and two unsampled haplotypes is not included in the highest rank clade 5–7. Haplotypes 17 and 18 contain sequences uniquely from Vigo, with more sequences from haplotype 18. They are both separated by six and four mutational steps, respectively, from an unsampled haplotype. Haplotype 38 is separated by one mutational step from haplotype 18. Finally, haplotype 35, which is unique from Corcubión is not included in any nest, and is separated by three and four mutational steps from two distinct unsampled haplotypes.

Contingency analyses of symbiotic *Vibrio* nesting exhibited significant evidence of restricted gene flow with isolation by distance in clade 2–5, which includes haplotypes 5, 6, 8, and 22, and comprises a geographical isolated unit encompassing the southern rías Vigo and Aldán, with a small contribution from the centrally located Corcubión ría (Table 4). Clade 3–5 exhibits contiguous range expansion, and comprises populations from Aldán and Ferrol, which are distant rías. Inference from the total cladogram, was inconclusive for past genetic history.

An identical AMOVA performed on symbiont genetic data revealed a higher value ($F_{ST}=0.21314$*, p value <0.001; Table 5) when compared with host genetic data. Base-pair-by-base-pair nucleotide diversity of symbiont populations was highest in samples from Aldán and Vigo (0.18521, 0.22434; Table 1), which are both from the southernmost rías. The lowest amount of genetic diversity was observed in the population from Ferrol (0.004936; Table 1).

4 | Discussion

4.1 | Host Genetic Architecture

Sepiold squids and their *Vibrio* symbionts form highly specific associations, yet both allopatric and sympatric populations are influenced by abiotic factors such as salinity, temperature, marine currents, and changes in the pH due to the transmission (environmental) strategy that they employ (Bongrand et al. 2022; Koch et al. 2014; Nourabadi and Nishiguchi 2021; Soto and Nishiguchi 2021). For example, *Euprymna-Vibrio* populations in the Philippine archipelago demonstrated how marine currents have a central role in the establishment and configuration of these associations (Coryell et al. 2018). These results demonstrate how geographic location impacts the genetic structure of the host, while having less significance for symbiont populations. This is due to differences in the method of dispersal between the two partners of the association, with host squids having limited dispersal range as adults and rarely travel far from where they hatch (Jones et al. 2006; Kimbell, Mcfall-Ngai, and Roderick 2002; Villanueva et al. 2016; Zamborsky and Nishiguchi 2011). In contrast, viable symbiotic *Vibrio* cells are vented every day, and once in the water column they are subject to environmental factors such as dispersal by currents to different locations where they can infect new hosts (Soto, Punke, and Nishiguchi 2012). Similar to the Philippines, the NW coast of Spain is also influenced by oceanographic currents. Particularly, the Eastern North Atlantic Central Water (ENACW) combines two currents, the ENACWp (polar) from the north and ENACWt (tropical) from the south. These two currents converge on the Corcubión ría, creating a vortex which pulls water out to sea and away from the mainland (Figure 4).

Analysis of Molecular Variance (AMOVA) yields interesting results for the genetic structure of *S. atlantica* populations in this area. The F_{ST} value ($F_{ST}=0.0000$, $p<0.0001$, Table 3) observed for host populations indicate that there is gene flow between the populations from the different rías, and it can be considered that the *S. atlantica* population in this area of the Iberian Peninsula is in panmixia. This result reinforces previously reported studies (Villanueva et al. 2016), where they demonstrate that species with planktonic hatchlings reach broader distributional ranges in comparison with species with benthic hatchlings. Species with smaller hatchlings have broader latitudinal distribution ranges, and notably *S. atlantica* has the smallest hatchling size (1.7 mm) among all *Sepiola* members measured (Jones and Richardson 2010; Rodrigues et al. 2011).

The data provided by the nested clade analysis reinforces our earlier findings where the major haplotype in this area is haplotype 1 (Figure 2). This haplotype is distributed among individuals mostly belonging to the Ferrol and Corcubión rías, where the majority of sequences were from these two rías (Table 1).

TABLE 4 | *Vibrio* clades that demonstrated significance during either a permutation contingency analysis or geographic distance analysis. Location of significance is indicated by (D_n), nested clade distance and/or (D_c), the within-clade distance. I–T indicates the average distance between a tip clade and an interior clade. S or L indicates that the distance measure is significantly smaller or larger at the 5% inference level. Inference steps were performed using the automated inference key in GEODIS, part of the AneCA v1.2 population genetics analysis software platform (Posada, Crandall, and Templeton 2000; Panchal and Beaumont 2007).

Clade	Nested clade	Distance	Value (S or L)	Chi square statistics	Inference key steps	Inference
2–3	1–2 T	D_n	49.85*l	7.95*	1-2 IO	I–T Status undetermined: Inconclusive outcome
		D_c	38.9164			
	1–3 T	D_n	30.1481*s			
		D_c	15.6138*s			
2–5	1–5 T	D_n	14.5031	16.6667*	1-2-3-4 NO	Restricted gene flow with isolation by distance
		D_c	0.0			
	1–6 I	D_n	36.4419			
		D_c	23.8062*s			
	1–22 I	D_n	50.2167*l			
		D_c	33.1001			
	I–T	D_n	26.5304			
		D_c	26.9042			
3–5	2–7 T	D_n	60.5609*l	8.0*	1-19-20-2-11-12 NO	Contiguous range of expansion
		D_c	0.0			
	2–22	D_n	13.4493*s			
		D_c	0.0*s			
	I–T	D_n	−47.1116*s			
		D_c	0.0		1-2 IO	
Total Cladogram	5–7	D_n	40.5398*l	153.2982*		I–T Status undetermined: Inconclusive outcome
		D_c	36.3931			

Note: "*" means p value < 0.001.

TABLE 5 | Percentage of variation among and within *Vibrio* sp. populations.

Source of *Vibrio* sp. variation	df	Sum of squares	Variance components	Percentage of variation
Among population	4	112.367	2.16499*	21.314
Within population	55	167.750	3.05000*	78.686
Total	59	280.117	5.21499	
Overall (F_{ST})	0.21314*			

Note: "*" means p value < 0.001.

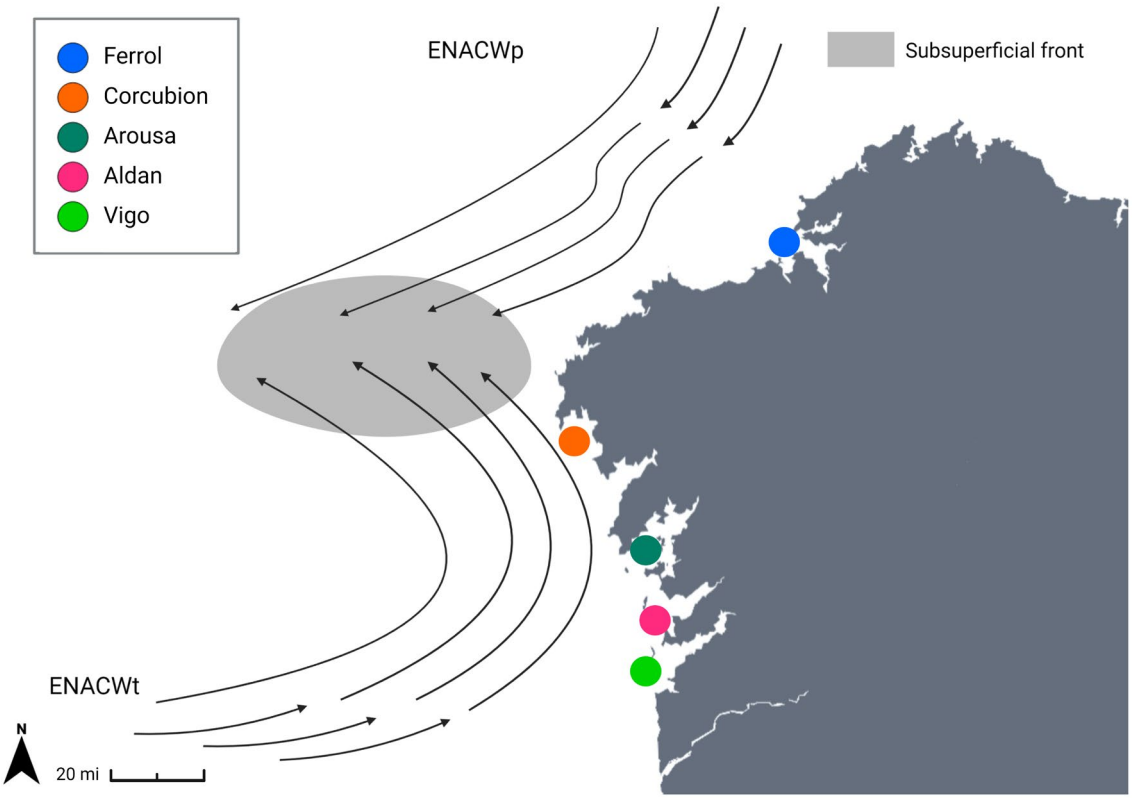

FIGURE 4 | (Modified from Ríos et al. 1992). The Eastern North Atlantic Central Water Current (ENACW) with its two components from polar origin (ENACWp) and tropical origin (ENACWt). The subsuperficial front is shaded in grey, marking the area of confluence of the two components.

Sequences belonging to individuals from Arousa, Aldán and Vigo were also found in haplotype 1.

The influence of the polar component of the ENACW whose north–south direction could be distributing *Sepiola* juveniles in a southerly direction may have an important role in the passive migration of this species. This may facilitate encounters with other juveniles from the southern rías of Vigo, Aldán and Arousa. In contrast, the tropical component of the ENACW, which in turn moves individuals from the southern areas corresponding to Vigo, Aldán, and Arousa rías, may facilitate the mixing of individuals from the south with those from the north in the Corcubión confluence area (Figure 4). The confluence of these two currents suggests a substantial amount of gene flow occurring between both northern and southern populations.

It is interesting to mention the presence of two local haplotypes with unique sequences from the most distant rías, haplotype 2 corresponding to Ferrol, the northernmost ría, and haplotype 3 corresponding to Vigo, the southernmost ría. The fact that these two rías are the farthest from the area of current convergence in Corcubión may have contributed to the emergence of these unique haplotypes. However, the fact that they are separated from the main haplotype (1) by only one mutational step and the nested clade analysis together with the contingency analysis did not yield significant results suggests that the individuals belonging to these unique haplotypes are not isolated. Further studies in the area are needed in the future to determine whether these haplotypes have established themselves significantly in the respective rías to ascertain if the distance from the area of current convergence in Corcubión plays any role in the process of allopatric speciation. This supports the

idea that despite the presence of physical facilitators such as water movement, the distances between populations, and the existence of geographical barriers such as the rías have an important role in shaping the genetic structure of host-symbiont populations.

Additionally, *S. atlantica* abundance is low year-round, and seasonal behaviour is variable (Rodrigues et al. 2011). As previously reported by Yau and Boyle (1996) and Guerra et al. (1986), the abundance of *S. atlantica* in Firemore Bay (west coast of Scotland) and in the ría of Vigo varied from 0.006 to 0.001 individuals per square meter (individuals/m^2). This is a scarce species whose variability in number of individuals over time can also be influenced by biological processes such as predation, competition, recruitment, or abiotic factors. Although our study involved only a singlea fe year of sampling (2011), cohort effects are unlikely to influence our results. A comparable study in a similar system involving the bobtail squid *Euprymna albatrossae* and their *Vibrio fischeri* symbionts in the Philippine archipelago (Coryell et al. 2018) found that genetic variation in symbiont populations was driven primarily by abiotic factors, such as geographic barriers, water temperature, salinity, and marine currents rather than temporal effects. Despite conducting sampling over multiple years (2010–2015), no significant temporal variation was detected in the genetic structure of the symbionts. This suggests that cohort effects were not a significant factor in the system, as abiotic factors played a more dominant role in determining the genetic structure of the population of both the symbiont and the host.

During the summer months, adults migrate to deeper and colder waters in this area (Guerra 1986) and to the North Sea (Oesterwind et al. 2010). Although no direct observations have been made, it has been hypothesized that these same populations migrate to a geographical location close to their origin, facilitating increased genetic exchange. *Rossia macrossoma* (Delle Chiaje, 1830) and *Rondeletiola minor* (Naef, 1912) two closely related sepiolids that primarily live in deeper waters of the Atlantic Ocean and Mediterranean Sea, have also exhibited the same migratory behaviour (Lefkaditou and Kaspiris 2005). These species concentrate in deeper waters during summer and early autumn, whereas recruitment is mainly observed in November at depths shallower than 200 m. Given the geographic topology of this area, Atlantic bobtail squid species provide a clear example of allopatric speciation, with individual genetic units observed in each of the rías. However, our results from both AMOVA and NCA indicate the genetic variability found within *S. atlantica* is minimum due to prevailing seasonal currents in the area.

4.2 | Symbiont Genetic Architecture

Symbiont genetic data indicates that *Vibrio* bacteria are subject to certain barriers that restrict their genetic exchange. This is contrary to most hypotheses regarding bacterial plankton distribution in the ocean, where most microorganisms are subjected to currents and other abiotic factors (Abreu et al. 2023; Sousa et al. 2021). Open marine environments have few physical barriers for dispersal and microbes are expected to exist as well-mixed populations, based on the classical assumption in microbial biogeography that 'everything is everywhere, but the environment selects' (Baas Becking 1934). However, AMOVA of total symbiont genetic data reveals that a higher percentage of variation relies among populations (df = 4, SS = 112.367, VC = 2.16499, PV = 21.314%; Table 5, Figure S1) when compared to the host (df = 4, SS =101.367, VC = 1.32652, PV = 0%; Table 3). The level of genetic diversity for the total symbiont population in the region value is indicative of more restricted gene flow between populations ($F_{ST} = 0.21314$, $p < 0.0001$, Table 5, Figure S1) than the F_{ST} value reported for host populations ($F_{ST} = 0.000$, $p < 0.0001$, Table 3). One main contiguous haplotype network was detected in the symbiont genetic data revealing connections between *Vibrio* populations from all the rías (Figure 3); however, two more networks were revealed with no connections with the main one or between them. The existence of three haplotype networks in this area for the symbiont suggests that there are at least three different genetic units. The distance between them in terms of mutational steps is > 12, so the ANeCA software categorizes them as separate networks. The network corresponding to clade 4–7 encompasses haplotypes from Aldán and Vigo. These rías are closer to each other than to any other ría, which may facilitate the movement of bacterial cells between these rías. This connection would make it possible for the symbionts present in this area to adapt to local salinity and temperature conditions, facilitating the emergence of unique haplotypes. Additionally, if we observe the nested clades within clade 4–7, clade 3–7 contains a unique haplotype from Vigo, haplotype 21, while clade 3–8 comprises haplotypes present only in Aldán. This suggests that even at the level of individual rías, the unique environmental conditions of each one can lead to the emergence of unique haplotypes corresponding to locally adapted populations.

The smallest network, which corresponds with clade 3–9, contains unique haplotypes from Arousa, Corcubión and Ferrol, with these rías geographically situated consecutively from south to north. The configuration of this network can be explained by the same reasons that shape the network of clade 4–7. The proximity of these rías would have facilitated the movement of symbionts between them, promoting the emergence of genetically similar haplotypes. The presence of the tropical component of the ENACW would transport bacterial cells from Arousa northward, while the polar component of the ENACW would transport bacterial cells from the populations of the Ferrol southward, favouring the mixing of these populations in the convergence area of Corcubión. This is where the tropical and polar components of the Eastern North Atlantic Central Water (ENACW) current meet, which acts as a geographical barrier between the southern and northern rías, preventing, but not entirely precluding, the mixing of *Vibrio* populations from the northern and southern rías. The observed patterns of genetic variation follow the direction of these ocean currents, indicating that passive transport through the water is a key driver of genetic connectivity between populations. For instance, haplotypes found in northern and southern rías align with the direction of current flow, suggesting that the mixing of bacterial populations is facilitated by abiotic factors (i.e., the currents) rather than solely by genetic drift due to geographic isolation. If genetic drift were the primary driver, we would expect more random divergence

patterns that do not align with the directionality of currents. Instead, the structured pattern of haplotype distribution, especially across physically separated but hydrologically connected rías, strongly suggests that abiotic factors, such as currents, play a significant role in shaping the genetic diversity of these *Vibrio* populations.

Results of the permutation contingency analysis revealed that the formation of clade 2–5 is determined by restricted gene flow with isolation by distance (Table 4). This clade contains unique haplotypes from Vigo and Aldan, as well as a dominant haplotype with sequences mostly from Aldán and a small contribution from Corcubión. This result reinforces the hypothesis of geographical isolation of this area from the other rías. Compared to the haplotype network of the host, *S. atlantica*, it is interesting to note that while the *S. atlantica* population is in panmixia, the *Vibrio* symbiont population is affected by abiotic factors such as ocean currents and the geographical configuration of the habitat. Given that the entire Galician coast is an upwelling zone, a phenomenon that affects sea surface temperature, content of organic matter in the water column and sediments, and other important abiotic conditions, these factors can also influence the settlement of cephalopods and consequently their *Vibrio* populations. *S. atlantica* prefers exposed coasts, typically in the outer parts of ría where the sediment is coarser than the inner part, with a preference for grain sizes between 0.25–0.125 mm. (fine sand). Depth varies between 3 and 12 m, and the temperature ranges from 11°C–19°C, with low percentages of organic matter in the sediment. Essentially, all Galician estuaries have the same granulometric distribution, following a gradient from coarse sand at the most exposed areas of the ría mouth to mud in the internal areas. This gradient means that locally, although separated by several kilometres, we find optimal benthic bottoms similar from north to south for the settlement of different populations. The Corcubión Inlet, oriented southwest, is the western-most point of the Iberian Peninsula. It serves as a transition zone separating the Rías Baixas from the Costa da Morte and Rías Altas. Although it is not an estuarine system, some locations, due to currents, granulometric conditions, and other abiotic variables, make this inlet suitable for settlement of *Sepiola*. All locations in the southern rías (Arousa, Aldán and Vigo) have a southwest orientation, except for the northern Ría de Ferrol, which is oriented east to west. Thus, the panmixia observed in *Sepiola* populations may be due to habitat preference, and reinforces the observation that the genetic structure of the symbiont population is directly influenced more by abiotic factors and not to host location in the Iberian Peninsula.

Strikingly, the permutation contingency analysis demonstrated that the formation of clade 3–5 is due to contiguous range expansion (Table 4). This pattern refers to a population expanding its range continuously and uniformly from an ancestral location. This infers that as the population expands, new individuals colonize adjacent areas, leading to a scenario of continuous and gradual spread rather than isolated jumps or long-distance dispersal events. However, the haplotypes found in this clade are unique and belong to two geographically distant locations, Aldán and Ferrol (Figure 3). Therefore, the presence of the contiguous range expansion pattern could be explained by a possible event of local extinctions in the rías existing between Ferrol and Aldán, after a historical range expansion event. The population might have historically expanded continuously across the entire area, but subsequent local extinctions in the intermediate areas could have eliminated those haplotypes. Due to the dynamic nature of bacterial populations and their ease of transport in marine environments, the ocean currents present in the area could have transported individuals from locally adapted populations between the rías of Ferrol and Aldan and the rías between them, persisting only in the estuaries with suitable conditions for these locally adapted populations, in this case, Ferrol and Aldán.

Another explanation to this gap existing between Ferrol and Aldán in a clade that shows continuous range of expansion can be the presence of the ENACW current in this area, which would prevent migration among the rías. Therefore, any genetic exchange between bacteria of these two geographical units is highly unlikely since the vortex created between the two components of the current (ENACWt and ENACWp) would passively transport *Vibrio* cells out to sea. Additionally, each ría is subject to different hydrological regimes (Álvarez et al. 2003; DeCastro et al. 2000) that feature different depths and bottom configurations, which are each affected by different sources of fresh water (DeCastro et al. 2006). These intrusions of fresh water, such as the mouth of nearby rivers, can alter the pattern of circulation within each estuary (Alvarez et al. 2006). For example, the proximity of the Miño river to the Vigo ría can reverse the pattern of water circulation in the Vigo and Pontevedra rías, making fresh water intrude superficially into them and preventing the movement of microorganisms through this route (Sousa et al. 2013, 2014). The Arousa ría specifically has a series of characteristics that make it unique compared to its neighbouring Aldán and Vigo rías. The southern orientation and shallowness at the mouth of the estuary contribute more fresh water that is cooler in winter, with upwelling events less frequent in summer (Alvarez et al. 2007), potentially preventing the recruitment of new *Vibrio* cells from the open ocean. Characteristics such as salinity and temperature, along with isolation from the ocean, can lead to populations of symbionts locally adapted to those conditions to form a unique genetic unit, like haplotypes 2 and 3 which are unique to Arousa (Soto and Nishiguchi 2021; Soto et al. 2009).

Our study is not the first study demonstrating the genetic structure in a beneficial symbiont population and the low genetic variability observed in their corresponding host populations. Previous studies of zoantharian endosymbionts demonstrated high genetic variation over small geographic scales around the Okinawa-jima Island in Japan (Noda et al. 2017). Gould and Dunlap (2019) used a genomic approach to measure how populations of the facultative light organ symbiont, *Photobacterium mandapamensis* exhibit genetic structure at spatial scales of tens of kilometres despite the absence of physical barriers to dispersal and in contrast to the population structure of its coral reef fish host *Siphamia tubifer*. In the Galician rías region, general physical barriers such as the geomorphology of the region and the specific conditions of each ría such as temperature differences, salinity gradients, and different hydrological regimes change throughout each season. These conditions shape unique environments leading to locally adapted *Vibrio* strains in each ría, creating a different pool of symbionts over time. Studies

in other symbiotic systems demonstrate how locally adapted symbiont strains in deep-sea hydrothermal vents are specifically associated with their hosts with no host differentiated populations across an ~800 km. gradient (Breusing et al. 2022). Additionally, polyclonal symbiont populations in hydrothermal vent tubeworms had less variability than their free-living counterparts (Polzin et al. 2019). For bobtail squids, *Vibrio* bacteria are acquired from the environment within the first few hours post hatching, with 95% of the population subsequently vented each day at dawn. The vented *Vibrio* bacteria can then diversify before infecting a new host, which may explain the higher genetic diversity observed in these beneficial symbionts (Soto and Nishiguchi 2021; Soto, Rivera, and Nishiguchi 2014; Soto et al. 2019).

In conclusion, we observed how the influence of abiotic factors, in particular seasonal marine currents, influence the configuration of the genetic structure of both Atlantic bobtail squid and their beneficial symbiotic *Vibrio* counterparts. For bobtail squids, marine currents seem to be facilitating the encounter of juveniles coming from northern and southern rías increasing their genetic variability. In *Vibrio* bacteria, marine currents, along with the particular isolation and conditions of each ría could be influencing the existence of locally adapted symbionts which remain isolated from one another. These findings are in accordance with previous studies of host-symbiont co-speciation, and increase our understanding of the evolution of beneficial symbioses in the marine environment (Jones et al. 2006; Zamborsky and Nishiguchi 2011; Soto, Punke, and Nishiguchi 2012; Coryell et al. 2018). In particular, our study is a fresh perspective examining both host and environmentally transmitted symbionts and the roles that the environment has on establishing the symbiosis. These results have the potential to establish a fundamental basis for how environmentally transmitted symbioses are affected by changes in seasonal and subsequent dramatic abiotic fluctuations, and eventually global climate change. Understanding the dynamics between beneficial bacteria and their animal hosts has greater consequences on how ecosystems are impacted by climate change and the bacteria that are responsible for the overall health and survival of all living eukaryotes.

Author Contributions

P. A. Pérez-Ferrer performed the research, analysed the data, and wrote the paper. M. Ashraf performed the research, M. Rodrigues performed the research, J. Troncoso contributed reagents and provided research space and M. K. Nishiguchi designed the research, contributed new reagents and analytical tools, and wrote the paper.

Acknowledgements

We thank the staff at the University of Vigo marine station for help in collecting and husbandry of all sepiolids collected in this study. This work was funded by NSF-DBI 2214038, NASA EXO-80NSSC18K1053, College of Arts and Sciences at New Mexico State University, and the Quantitative Systems Biology program and School of Natural Sciences at UC Merced.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Genetic data: Raw sequence reads are deposited in the SRA (BioProject *Sepiola*- PRJNA1081930). Individual genotype data are http://datadryad.org/stash/share/b5YjT7zpdDBnXonlqlj6M0g4CN0ScGB-NEd6oPS9mPY and doi:10.5061/dryad.9cnp5hqsz. Unique haplotype data are deposited to NCBI Nucleotide Database. *Vibrio* haplotype accession numbers are SAMN42497480—SAMN42497518 and SAMN40347782—SAMN40347870/. *Sepiola* haplotype accession numbers are SAMN42469291—SAMN42469294 and SAMN40194858—SAMN40194884.

Benefit sharing statement: Benefits Generated: A research collaboration was developed with scientists from the countries providing genetic samples; all collaborators are included as co-authors; the results of research have been shared with the provider communities and the broader scientific community (see above), and the research addresses a priority concern, in this case the conservation of organisms being studied. More broadly, our group is committed to international scientific partnerships, as well as institutional capacity building. Benefits from this research accrue from the sharing of our data and results on public databases as described above.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.