ELUCIDATING POPULATION GENETICS OF SHRIMP SPECIES ON UNDERWATER VOLCANOES

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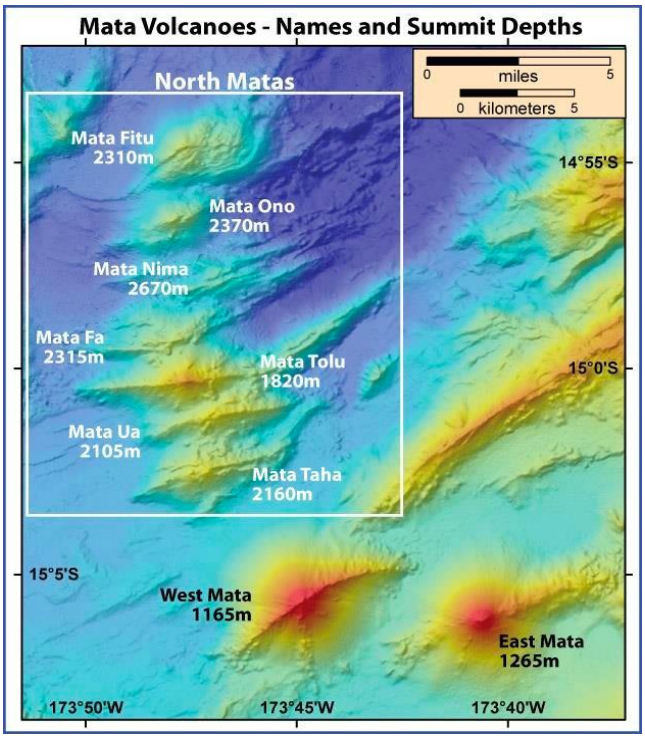
Abstract

To elucidate gene flow between hydrothermal vent communities, shrimp species *Opaepele loihi* and *Rimicaris variabilis* were sampled at the underwater Mata volcanoes near Tonga. A fragment of the mitochondrial cytochrome oxidase I (COI) gene was amplified using HCO/LCO primers from 105 samples. Phylogenetic analysis confirmed the species identity of previously sequenced *O. loihi* and *R. variabilis* samples. Results from AMOVA and nested clade analyses revealed no significant genetic structure across depth or geography supporting high gene flow between vent communities. Future studies with microsatellites and an increased sample size are suggested for further validation, with the goal of offering valuable insights into the management of these distinctive ecosystems, amidst the rise of human interference through activities such as deep-sea mining operations.

Introduction and Background

Hydrothermal vents, underwater geysers, are commonly found thousands of meters below the ocean's surface in areas with little to no light. These extreme environments support diverse marine life through chemosynthetically driven ecosystems, characterized by methane-rich fluid emissions and distinctive sulfur oxidation-reduction reactions (Cong et al., 2022). Located in the Northeast region of the Lau Basin in the South Pacific Ocean, Tonga houses a series of nine underwater volcanoes known as the Mata Volcanoes (**Figure 1**), with depths ranging from 1800 to 2700 meters (Clague et al., 2011) and are spaced 1.5 to 7.5 kilometers apart.

Figure 1. Bathymetric map of the northern range of the Mata Volcanoes.



The dynamic nature of hydrothermal vent ecosystems, characterized by the continual emergence of new vents and the dormancy or destruction of older ones due to volcanic or tectonic activity, profoundly influences the ecological landscape of these environments (Tunnicliffe et al., 1997). The ephemeral nature of these hydrothermal vent ecosystems has directly impacted the existence of

hydrothermal vent species and their genetic population structure as these species come into and out of existence quickly. Particularly, these vent communities face extrinsic pressure affecting the availability and stability of their habitat, which ultimately shapes the frequency and intensity of their demographic patterns (Thaler et al., 2011).

While many species of chemosynthetic bacteria and invertebrates reside in this region, particular species of gastropods and shrimp are notably abundant within these underwater communities. Two species of shrimp, the *Opaepele loihi* and *Rimicaris variabilis*, are native to these hydrothermal vent communities. Like many other shrimp species in these vent communities, they exhibit high connectivity between back-arc basins, which suggests the capacity to disperse over great distances, contributing to their population resilience (Methou et al., 2023). Previous studies have shown that the *O. loihi* and *R. variabilis* species utilize the chemosynthetic bacteria as their main source of energy, due to the rich fatty acid composition of the bacteria (Stevens et al., 2008). As the shrimp matures, a dietary switch from pelagic feeding on photosynthetic material to ingestion of bacteria at vent sites after settlement is observed (Stevens et al., 2008).

The *O. loihi* and *R. variabilis* species fall in the Arthropoda phylum, Alvinocarididae family, and the Bresilioidea superfamily. *Opaepele loihi* are characterized by a compressed carapace, evenly rounded abdomen, smooth base of obsolescent spine, and gills like the *Rimicaris* genus (Williams & Dobbs, 1995). *Rimicaris* genus have been characterized by reduced and dorsoventrally flattened rostrum, an absent post-rostral carina on the carapace (Komai & Giguère, 2019), and enlarged visual organs, which able to detect light in very dim environments (Chamberlain, 2000; Nuckley et al., 1996). The *Rimicaris* genus includes several major species, *R. exoculata, R. chacei, R. hybisae,* and *R. kairei,* along with *R. variabilis,* while the *Opaepele* genus has only recorded one known species: *O. loihi.* Both genera are endemic to their specific vent communities worldwide and only reside at these hydrothermal vent sites.

Recent studies have challenged the preceding belief that these shrimp species exhibit seasonal spawning behavior. While the reproductive strategies of bresiliid shrimp have been underexplored, recent findings indicate that their reproductive patterns vary depending on the shrimp family. Emerging evidence suggests that some species exhibit seasonal reproduction, while others demonstrate aperiodic reproductive cycles, displaying varied reproductive patterns, which can be possibly influenced by thermal preferences (Methou et al., 2023). Further studies have revealed that the females within the *Rimicaris* genus exhibit a unique brooding behavior by carrying their eggs beneath the abdomen (Methou et al., 2023), subsequently releasing hatched larvae into the ocean. These findings challenge previous assumptions about the reproductive process of these species, which were believed to release gametes instead. The demographic pattern of distribution of this shrimp varies according to their life stage, as juveniles tend to occupy cooler regions, while brooding females tend to stay closer to the hot vent fluid emissions (Methou et al., 2023). It is important to note that our understanding of the behavioral patterns of the Alvinocarididae genus primarily stems from observing Atlantic hydrothermal vent shrimp, leaving considerable gaps in knowledge regarding shrimp from other regions.

The mitochondrial genome is commonly employed in studying population genetics due to its versatility when studying replication and identification of mutations in mitochondrial genes. This can be attributed to the fact that they are maternally inherited, are highly mutable, and are relatively smaller in size, when compared to nuclear DNA. A molecular marker frequently used in evolutionary research is the cytochrome oxidase I (COI) gene, which is derived from mitochondrial DNA. This gene is relatively short (700 bp) and lacks introns, allowing for easy collection and analysis of data. Additionally, the COI houses many copies in each cell, evolves quickly, and undergoes haploid inheritance without recombination (Raupach et al., 2015), which is why the COI gene was utilized in this study to assess relatedness between different shrimp populations at hydrothermal vents, as they come in and out of existence relatively quickly.

Methods

Sample Collection and Storage

The 105 total shrimp samples were collected in 2017, on the R/V Falcor to Samoa expedition to the Mata Volcanoes, through the Schmidt Ocean Institute. The main tool used for sample collection was the remotely operated vehicle (ROV) SuBastian. The latitude, longitude, depth, and number of samples from each site can be found in *Table 1*. Upon collection, these samples were preserved with 95% EtOH, RNALater, then frozen at sea. In the lab, the samples were saved in a -70 °C freezer for long-term storage, and a -20 °C freezer for day-to-day storage.

DNA extractions were completed using the *DNEasy* extraction kit manufactured by Qiagen according to manufacturer protocols. To prepare a usable DNA sample, samples were taken out of the -70 °C freezer.

Their initial concentrations and DNA quality after extraction were measured with a NanoDrop 2000c spectrophotometer (ThermoFisher Scientific, Waltham, MA).

Table 1. Metadata for all shrimp collection sites.

Site #	Site Name	Dive	Station	Latitude South	Longitude West	Depth (m)	*O. loihi*	*R. variabilis*
1	Mata Tolu	S94	North, Tall, Handsome	-15.00449	-173.79359	1823	1	34
2	West Mata	S103	40 m SE of WP18	-15.0932943	-173.7457246	1289	1	0
3	West Mata	S103	29m NW of WP20	-15.093796	-173.7462064	1267	3	0
4	West Mata	S85	10m NE of Marker 13	-15.0943195	-173.748284	1185	10	0
5	West Mata	S85	East edge of summit ridge	-15.0939	-173.7476	1189.9	2	0
6	West Mata	S85	Near Summit close to WP17	-15.0941	-173.746	1283	2	0
7	West Mata	S87	Midway between WP16 and WP16	-15.0942	-173.74805	1185	10	0
8	West Mata	S87	20m NW of WP19	-15.0946175	-173.7463428	1276	2	0
9	West Mata	S87	10m south of WP13	-15.094047	-173.748133	1196	10	0
10	West Mata	S103	Spatter Mound	-15.089261	-173.738921	1520	1	1
11	West Mata	S103	20m E of WP20	-15.088127	-173.737943	1589	0	1
12	Mata Fitu	S97	-	-14.915482	-173.773712	2758	0	11
13	Mata Ua	S100	-	-15.016681	-173.78693	2334	0	9
14	Mata Ono	S102	-	-14.9405805	-173.799559	2360	0	4

Amplification of COI Gene

Polymerase Chain Reaction, or PCR, was used to amplify the gene of interest in the shrimp samples. The primers used for our experiment were HCO/LCO, which targeted a portion of the COI gene, were used for the *Opaepele loihi* and *Rimicaris variabilis* species (Folmer, 1994). Two Thermocycler programs were used, depending on the PCR kit. When using the Promega Core System I kit, the following PCR parameters were used:

PCR parameters for HCO/LCO (Folmer, 1994) are:

1. 95°C for 2:00 minutes
2. 95°C for 1:00 minute
3. 52°C for 1:00 minute
4. 72°C for 1:30 minute
5. 72°C for 7:00 minutes
6. 4°C for indefinite minutes

Steps 2-5 were repeated for 34 more cycles

When using the BioRad Reliance One-Step Multiplex Supermix PCR kit, the following PCR parameters were utilized:

1. 95°C for 10:00 minutes
2. 95°C for 1:00 minute
3. 48°C for 1:00 minute
4. 60°C for 1:30 minute
5. 4°C for indefinite minutes

Steps 2-4 were repeated for 30 more cycles

Confirmation of Successful Amplification

To confirm that the PCR reaction properly amplified the targeted gene, a gel electrophoresis run was performed. The gel itself was made of 1.5% agarose gel. A band at ~ 700 bp confirmed that the correct portion of the COI gene was present.

Purification of PCR Product

Following a successful amplification of the COI gene, indicated by a bright band on the gel, the PCR products underwent a purification process. The PCR product for each sample was purified using Qiagen's QIAquick PCR purification kit according to manufacturer protocols. Nuclease free water was used to elute the DNA off the membrane into a fresh microcentrifuge tube. From there, the concentration of DNA in the purified product was determined using the ThermoFisher Scientific Nanodrop 2000 spectrophotometer and then the purification products were stored in the -20 °C freezer, to await sequencing.

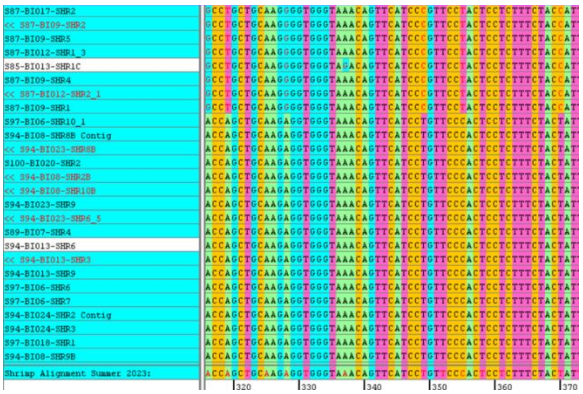


Figure 2. Subsection of the final alignment of all successfully sequenced shrimp samples. Each color is assotiated with a different nucleotide allowing for discrepancies to be easily identified and assessed. Red letters indicated intersequence differences.

Sanger Sequencing

A 96 well plate was used to prepare and transport the purified PCR products to Eurofins Genomics for Sanger sequencing. The purified PCR product for each sample was deposited into a pair of wells, each well receiving 8 µL of the product and 4 µL of primer. Each well only contained one primer, either LCO or HCO, but never both. This allowed for a separate sequence file to be generated from each well, one with the forward primer sequence and one with the reverse primer sequence, which would be used to achieve long and clean contiguous sequences.

Analysis of COI Sanger Sequences

Using the software CodonCode Aligner, each nucleotide was checked by eye. The ends of the chromatographs had a higher frequency of erroneous stutter nucleotide repeats and uncalled bases, which required them to be trimmed. Once the forward and reverse sequences had been cleaned and trimmed for a given sequence, they were formed into a contiguous sequence, called a contig. This was repeated for each shrimp sample that produced a quality nucleotide sequence through Sanger sequencing. Once each sample had been individually examined and successfully formed into a contig, all of the contigs for each sample were compiled into an alignment. This allowed each sample to be organized and compared against one another. The alignment was created using the software CodonCode Aligner with the Muscle alignment parameters. Like the editing of the individual nucleotide sequences, the alignment also required each nucleotide discrepancy to be checked by eye. The alignment's ends were trimmed to be even, resulting in an alignment 699 nucleotides in length and 124 sequences representing 105 shrimp samples. Duplicates of sequences were removed, leaving only the 105 unique shrimp sequences in the alignment. (**Figure 2**).

Making the Phylogenetic Tree

Once the alignment was trimmed and the legitimacy of all discrepancies was evaluated, the alignment was exported into a text file. In addition to the 105 shrimp sequences, known COI gene sequences for *R. variabilis, O. loihi, N. saintlaurentae, S. leurokolos, R. exoculate, A. Formosa,* and *R. chacei* were added to the text file. Including many known species of deep-sea shrimp allowed for the unidentified shrimp sequences to be quickly identified once the file was made into a phylogenetic tree. This method is referred to as binning. The phylogenetic tree was created in the software program MEGA11 using the statistical method of Neighbor-joining (Kumar et al., 1994). It was run with 500 bootstrap replications and utilized the Kimura 2-parameter model.

Creating a Haplotype Network

Haplotype networks are a way to visualize the correlation between genetic variation and geographical distribution. This analysis was performed for both the *R. variabilis* and the *O. loihi* samples to preliminarily demonstrate any potential genetic structure within the populations. TCS-1.21 (Clement et al., 2000) and TCS beautifier (Santos et al., 2016) were both used to create the haplotype networks (**Figure 4** and **Figure 5**).

Performing the Nested Clade Analysis

To test genetic structure within each species, a Nested Clade Phylogenetic Analysis (NCPA) was performed using the Java Script software ANeCA 1.2 (Panchal, 2007).

Performing the AMOVA

The Analysis of Molecular Variance (AMOVA) was used to test for the influence of geography and depth on population structure. The AMOVA was run on the software program Arlequin 3.5 (Excoffier and Lischer, 2010) using the Kimura 2-parameter model which penalizes transversions more than transitions. To do this, the Phylip files of both the *O. loihi* and *R. variabilis* shrimp sequences associated with their collection sites were converted to Arlequin files using the software DnaSP.

Performing the Mantel Test

The Mantel test is used in this study to assess any potential genetic isolation of the populations of shrimp based on their geographical distribution. The Mantel test was run for both *O. loihi* and *R. variabilis* using the Arlequin 3.5 software (Excoffier and Lischer, 2010).

Analysis & Results

Phylogenetic Analysis

This analysis confirmed that three species were present within the sample set collected. Multiple phylogenetic trees were made throughout the analysis process, but the one shown in **Figure 3** only includes the three species that were identified within the shrimp samples (*R. variabilis, O. loihi, N. saintlaurentae*) as well as one outgroup (*M. fortunata voucher*).

While three separate species were identified, *N. saintlaurentae* (n = 4) lacked a robust sample size and therefore was excluded from further data analysis.

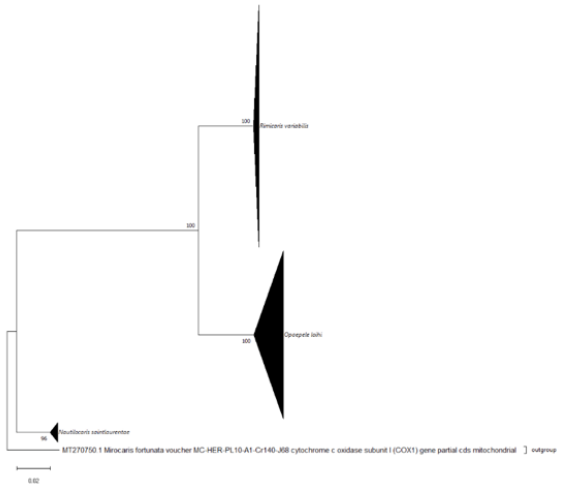


Figure 3. Phylogenetic tree representing 105 shrimp samples and 4 GenBank sequences (*R. variabilis, O. loihi, N. saintlaurentae*). Run with 500 bootstrap replicates with the support for each node indicated by the number at the branch points. Outgroup was M. fortunata voucher.

Haplotype Network Analysis

Within the haplotype networks, each color represents a site in which a sample was collected, and each node represents a haplotype. The size of the nodes indicates the

quantity of samples that were found with a given haplotype. Larger nodes correlate to a higher number of samples. The largest node is the ancestral node which is hypothesized to be the oldest haplotype within each population. The small, colorless nodes represent haplotype variants that were not found within the samples but are represented to accurately depict the genetic distance between some nodes. **Figure 4** and **Figure 5** show the haplotype networks generated for both the *R. variabilis* and *O. loihi* species respectively.

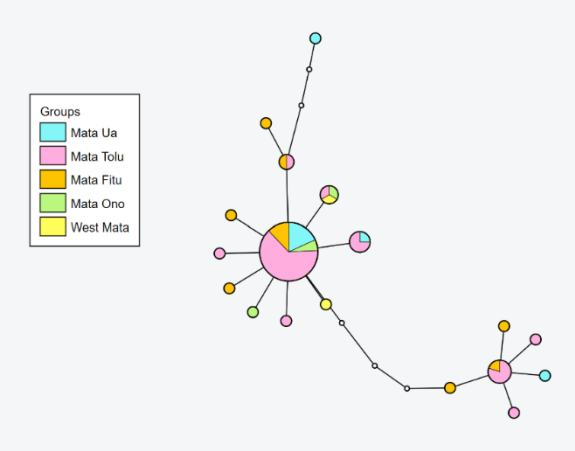


Figure 4. Haplotype network of R. variablilis shrimp samples. Each color represents a different site of collection. No significant geographical groupings of alleles are observed.

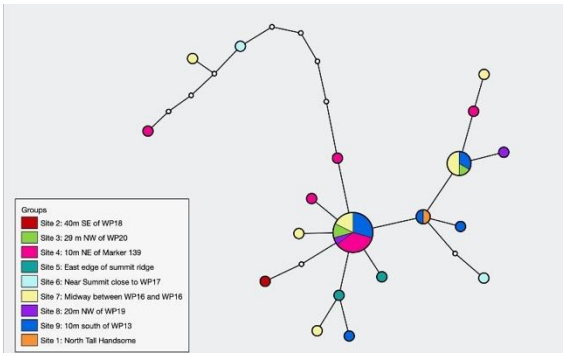


Figure 5.Haplotype network for O. loihi shrimp samples. Each color represents a different site of collection. No significant geographical groupings of alleles are observed.

Nested Clade Analysis

The results of the Nested Clade Phylogenetic analysis (NCPA) are summarized in *Table 2*. The NCPA categorizes the nodes of the haplotype networks into nests and can show differences in phylogeographical associations due to recurrent but restricted gene flow versus the influence of historical events operating at the population level (e.g. past fragmentation, colonization, or range expansion events). The first layer of nests (1-X) groups the nodes that are separated by only one node. The second layer of nests (2-X) groups the first clades together. **Figure 6** shows the nests determined by the NCPA for the *R. variabilis* samples. All nests were insignificant, except 1-2, which had significant support for continuous range expansion. The NCPA for the *O. loihi* showed no significance for any of the nests within the haplotype network.

Table 2. Intraclade Genetic Distances

Species	Genetic Distance (%)
Nautilocaris saintlaurentae	0.0095144
Opaepele loihi	0.0063229
Rimicaris variabilis	0.0036243

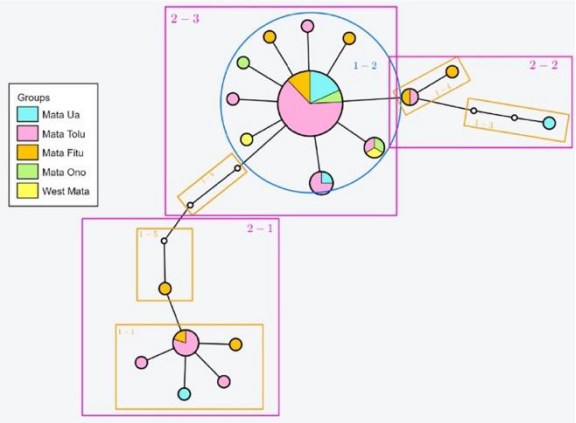


Figure 6. Haplotype network for R. variabilis with nests indicated as determined by Nested Clade Phylogenetic Analysis. All nests found to be insignificant with the exception of 1-2 which has some support for continuous range expansion.

Analysis of Molecular Variance

Since most of the *O. loihi* shrimp samples were collected from West Mata alone (n = 40), they required further geographical breakdown into the sites to run the AMOVA. The latitudes and longitudes for 4 sites on the Eastern front of West Mata were selected to be compared to 4 sites on the Western front of West Mata. A summary of these p-values can be found in *Table 3*.

Table 3. Results of the AMOVA from multiple comparisons of different groupings of sampling populations of *O. loihi* and *R. variabilis* by geography and depth.

Geographic Comparisons	Depth Range Comparisons (meters)	Among Regions	Between Populations Within A Region	Within Populations
Eastern sites vs. Western sites of West Mata (*O. loihi*)	1100 m - 1199 m vs. 1200 m - 1299 m (*O. loihi*)	0.24733±0.00286	0.39721±0.00313	0.64929±0.00293
Northern volcanoes vs. Southern volcanoes vs. West Mata (*R. variabilis*)		0.46599±0.00346	0.43607±0.00346	0.73199±0.00321
	1500 m - 1999 m vs. 2000 m - 2500 m vs. >2500 m (*R. variabilis*)	0.45525±0.00357	0.46878±0.00345	0.26970±0.00307

The *R. variabilis* samples were split up into Northern Mata Volcanoes, Southern Volcanoes, and West Mata to run the AMOVA. The p-value when comparing the regions was calculated to be 0.46599±0.00346. The p-value when comparing the populations within each respective region was calculated to be 0.43607±0.00346. And the p-value when comparing within populations was calculated to be 0.73199±0.00321. Since none of the p-values were less than 0.05, there was no significant difference in molecular variation due to location found between the various regions and populations of *R. variabilis* (*Table 3*).

In addition to the distance-based AMOVA for the R. variabilis, a depth-based analysis was performed. For this analysis, West Mata (1589 m) and Mata Tolu (1813 m) were grouped together and compared against Mata Fitu (2758 m), Mata Ua (2355 m) and Mata Ono (2360 m). The AMOVA p-values comparing regions (deep vs shallow) was calculated to be 0.45525±0.00357. The p-value for the comparison of populations within the regions was 0.46878±0.00345. And the p-value for the comparison within populations was determined to be 0.26970±0.00307. None of these values are less than 0.05, indicating no molecular variance due to the depth of the collection sites (*Table 3*).

Mantel Test

The Mantel test results indicated that the populations of shrimp were not genetically isolated by distance. The p-values were determined to be 0.923000 and 0.551000 for the *O. loihi* and *R. variabilis* samples respectively. Neither p-value was less than 0.05, indicating no significant support for potential genetic isolation by distance between the regions and populations of both species.

Discussion

This study confirmed the identity of the two main species of shrimp found at the hydrothermal vents at the Mata Volcanoes as *O. loihi* and *R. variabilis*, along with one smaller population of shrimp, known as *N. saintlaurentae*. There were 42 *O. loihi* samples, 59 *R. variabilis* samples and 4 *N. saintlaurentae* samples in this study. The majority of the genetic analysis techniques revealed that there was no significant population structure between any of the shrimp populations, including comparisons of populations by geographic location or depth (Tables 1). These findings are expected given the ephemeral nature of these hydrothermal vent communities, which often emerge and vanish unpredictably. It may be concluded that the individuals within these communities heavily rely on larvae with wide dispersal abilities to ensure their survival amidst their harsh and unpredictable environment.

The phylogenetic analysis confirmed that our sample of shrimp were identified to be the following species: *O. loihi, R. variabilis, N. saintlaurentae.* The hypothesized phylogenetic tree was run against 500 pseudo-replicates resulting in the *O. loihi* and *R. variabilis* clades being grouped together 100% of the time while the *N. saintlaurentae* clade came out 96% of the time when grouped together. The resultant p-values from the Nested Clade Phylogenetic Analysis (NCPA) of the *O. loihi* samples confirmed no significant differences between genetic factors and environmental factors' role in influencing the genetic structure of the *O. loihi* shrimp populations, indicating high gene flow between all populations. The NCPA of the R. variabilis also confirmed that there were no significant differences between genetic factors and environmental factors' role in influencing the genetic structure, except nest 1-2. The analysis showed support for this nest's range expansion, including the ancestral node and multiple single mutation individuals. Range expansion for the ancestral haplotype is an expected pattern for any newly founded population, which aligns with the fact that these shrimp populations are ephemeral.

The colors within the haplotype networks indicate a given location or site that the samples were obtained from, with the nodes indicating various haplotypes of the COI gene. Clustering of the colors among nodes would indicate a level of genetic isolation between populations, which can be explained by geographical conditions and/or mode of reproduction. However, no clustering was observed in either haplotype network (**Figures 4 and 5**), further supporting the hypothesis of high gene flow among both the *R. variabilis* and *O. loihi* populations. These results show that the most frequent haplotypes found within the two species are widespread throughout the different sites and volcanoes. They also indicate that neither genetic mutation. nor spatial arrangements of the shrimp are responsible for their unique haplotype network configurations.

The AMOVA and Mantel tests for *O. loihi* analyses were larger than 0.05, resulting in that there is no statistically significant difference between the populations located at West Mata, as illustrated by Table 2. These results only included *O. loihi* samples that came from West Mata (40 individuals) and excluded samples collected from other sites, as they lacked robust enough sample sizes. These results further support the hypothesis of high gene flow amongst these populations, as there are no significant genetic differences between the regions, the populations, or within the populations. The Mantel test also confirmed that there was no isolation by distance.

For the *R. variabilis,* the geography based AMOVA comparing the Northern Matas, Southern Matas, and West Mata. All the calculated p-values were insignificant, which indicates there is no significant molecular variance due to the distance between the Northern Matas, Southern Matas, or West Mata. This supports the hypothesis of high gene flow within and between the shrimp populations. Additionally, the p-value for the Mantel test was also insignificant, which indicates that there is no isolation by distance for the populations of the *R. variabilis* shrimp.

The results of this study are consistent with the study conducted by Thaler et al. (2012), which performed similar genetic analyses using *Chorocaris* species. The result of Thaler's study indicated no significant differentiation at any spatial scale, both with the COI gene or microsatellite markers. More recently, Methou et al. (2023) conducted a study analyzing the size frequency distributions and reproductive outputs of several Pacific Alvinocaridid sp., including *O. loihi*, reporting that the population demographics differed among each species. Additionally, one study done by Beedessee et al. (2013) in the Central Indian Ridge involving shrimp species *R. kairei,* as well as other endemic organisms such as *Alviniconcha sp.* and *A. rodrigeuzensis* found high connectivity in slowly spreading ridge systems. Although more studies would need to be done to confirm, we can hypothesize that deep-sea vent communities that have little spatial separation are more likely to house species with high gene flow.

Future studies could include using microsatellites, a higher resolution genetic marker, to both confirm the findings in this study and further study the population structure of these communities. Expanding the scope of our investigation by incorporating a larger sample size, which would encompass various sites and volcanoes, could significantly increase the validity of the findings from this study. Additionally, further research regarding the reproductive mechanisms and patterns of *O. loihi* and *R. variabilis* would aid in elucidating the factors that contribute to the observed high gene flow among populations. Increasing the sample size would also capture a more comprehensive representation of the two shrimp populations and might include deeper analyses of the third shrimp species found in this study, *N. saintlaurentae*.

The research conducted in this study holds great importance to better understand the fundamental population dynamics of hydrothermal vent communities. In recent years, there has been debate regarding the actions and implications towards mining activities in the vicinity of these vent communities for valuable metals and minerals, which could potentially impact the biodiversity inhabiting these environments (Thaler et al., 2012). The genetic findings can serve as a reminder to evaluate these species' susceptibility to the disruptive effects of mining operations or future volcanic eruptions. Moreover, studying the ecosystems on and around hydrothermal vents may grant legislators greater insights as to how to protect these communities when considering mining.

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