Population structure of *Bathymodiolus manusensis*, a deep-sea hydrothermal vent-dependent mussel from Manus Basin

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Deep-sea hydrothermal vents in the western Pacific are increasingly being assessed for their potential mineral wealth. In order to anticipate the potential impacts on biodiversity and connectivity among populations at these vents, environmental baselines need to be established. *Bathymodiolus manusensis* is a deep-sea mussel found in close association with hydrothermal vents in Manus Basin, Papua New Guinea. Using multiple genetic markers (*cytochrome C-oxidase subunit-1* and 8 microsatellites), we examined population structure at two sites in Manus Basin separated by 40 km and near a potential mining prospect, where the species has not been observed. No population structure was detected in mussels sampled from these two sites. The genetic diversity observed can be used as a baseline against which changes in genetic diversity within the population may be assessed following the proposed mining event.

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Abstract

Deep-sea hydrothermal vents in the western Pacific are increasingly being assessed for their potential mineral wealth. In order to anticipate the potential impacts on biodiversity and connectivity among populations at these vents, environmental baselines need to be established. *Bathymodiolus manusensis* is a deep-sea mussel found in close association with hydrothermal vents in Manus Basin, Papua New Guinea. Using multiple genetic markers (cytochrome C-oxidase subunit-1 and 8 microsatellites), we examined population structure at two sites in Manus Basin separated by 40 km and near a potential mining prospect, where the species has not been observed. No population structure was detected in mussels sampled from these two sites. The genetic diversity observed can be used as a baseline against which changes in genetic diversity within the population may be assessed following the proposed mining event.

Introduction

Hydrothermal vents support large, endemic communities fueled by chemoautotrophic primary production (Gage & Tyler 1991; Van Dover 2000), in contrast to the relatively low-biomass deep seafloor. In southwest Pacific back-arc basins, active vents are patchily distributed and subject to local disturbances, including the waxing and waning of hydrothermal flow on short time scales and cessation of flow on millennial timescales (Van Dover 2000; Vrijenhoek 2010). Species demographics may be driven as much by stochastic processes related to disturbance as by response to changing environmental conditions or other ecological phenomena (Vrijenhoek 2010; Thaler et al. 2014). In general, hydrothermal vent communities are thought to be more resilient to disturbance compared to other deep-sea ecosystems (Van Dover 2014).

Deep-sea hydrothermal vents are increasingly being explored for potential mineral extraction (Van Dover 2010). Almost 20% of all known global vent fields currently fall within mining exploration leases (Beaulieu et al. 2013). Establishing baselines for the diversity and connectivity of vent systems is a necessary first step in effective environmental management regimes (Collins et al. 2013). As vents become targets for mineral extraction, managers will need to assess regional biodiversity and connectivity and potential cumulative impacts of multiple mining events in a region (Boschen *et al.* 2013, Van Dover 2014) and design refugia to mitigate the impacts of mining on the vent ecosystem (Collins et al. 2012)**.**

Bathymodiolus manusensis is a deep-sea mussel found at hydrothermal vents in the Manus Basin, Papua New Guinea. It commonly occurs around low-temperature diffuse-flow vent sites on the periphery of active hydrothermal chimneys (Hashimoto & Furuta 2007). Though *B. manusensis* shares close affinity with other bathymodiolin mussels in Lau and North Fiji Basins (*B. brevior*), *B. manusensis* is known only from a few sites within Manus Basin

(Hashimoto & Furuta 2007; Kyuno & Shintaku 2009). *B. manusensis* is one of several habitat-forming mollusks that host chemoautotrophic endosymbionts and derive chemical energy from hydrothermal vent effluent in Manus Basin (Galkin 1997). While other endosymbiont-hosting species at Manus Basin vents, such as *Ifremeria nautilei* and *Alviniconcha* spp. (Urakawa et al. 2005; Bouchet & Waren 1991; Kojima et al. 2001), tend to cluster around orifices where vent effluent is most concentrated, *B. manusensis* occupies the periphery of hydrothermal ecosystems, taking advantage of the less space-restrictive regions around diffuse flow sites (Kyuno & Shintaku 2009).

The Solwara 1 vent site in Manus Basin is licensed for extraction of metals associated with seafloor massive sulfides (Coffey Natural Systems 2008). While *B. manusensis* does not occur at Solwara 1, it is abundant at the neighboring Solwara 8 site (40 km distant) and at the proposed set-aside, South Su (2.5 km distant; (Coffey Natural Systems 2008)). Previous studies of connectivity in invertebrate taxa at these sites reveal species-specific patterns of connectivity among sites. *Ifremeria nautilei* and *Chorocaris* sp. 2, two endosymbiont-hosting vent species show no signs of genetic differentiation among Solwara1, Solwara 8, and South Su (Thaler et al. 2011; Thaler et al. 2014) while significant local differentiation was detected in the vent-associated *Munidopsis lauensis* (Thaler et al. 2014). *B. manusensis* has a limited geographic range and is not ubiquitous at active vents in Manus Basin, leading us to anticipate that it might exhibit local-scale genetic differentiation. Because *B. manusensis* is absent from Solwara 1 and because Solwara 1 is situated between Solwara 8 and South Su, we tested the hypothesis that populations from South Su and Solwara 8 are isolated from each other, forming two genetically distinct populations.

Materials and Methods

Sample collection and DNA extraction

Bathymodiolus manusensis were collected from two hydrothermal vent sites in Manus Basin (Solwara 8 and South Su; Figure 1) during the *M/V Nor Sky* research campaign (June-July 2008; Chief Scientist: S. Smith) using an ST200 ROV modified for biological sampling. For *COI* analyses, *B. manusensis* were analyzed from two discrete sulfide mounds at each site (Table 1), with 10 to 43 individuals per mound (Table 2). For microsatellite analyses, up to 142 individuals per locus were analyzed. All sampling was undertaken with the permission of the government of Papua New Guinea and did not involve endangered or protected species.

Mantle tissue was dissected from each individual and preserved in 95% ethanol prior to DNA extraction. Genomic DNA was isolated using a standard Chelex-Proteinase-K extraction (10–30 mg digested with 120 µg Proteinase K (Bioline: Taunton, MA) in 600 µl 10% Chelex-100 resin (Bio-Rad: Hercules, CA) overnight at 60°C, heated to 100°C for 15 min, and centrifuged at 10,000 rpm for 5 minutes; (Walsh et al. 1991). Extracted DNA was stored at 4°C until amplification and archived at -20°C.

COI sequencing and analysis

Bathymodiolus manusensis mitochondrial *COI* fragments were amplified using the following reaction conditions: 10 to 100 ng of DNA template was combined with 2 µL 10x PCR buffer (200 mM Tris, pH 8.8; 500 mM KCl; 0.1% Triton X-100; 0.2 mg/ml BSA), 2 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 µM L*COI*1490 and 0.5 µM H*COI*2198 primers (Folmer et al. 1994), and 1 unit of Taq polymerase in a 20 µL reaction with the following PCR protocol: initial melting temperature of 94°C for 240 seconds; 35 cycles of 94°C for 15 seconds, 48°C for 15 seconds,

72°C for 30 seconds; and a final extension of 72°C for 300 seconds. Reactions were stored at 4°C until purification.

Fourteen µl of PCR product was incubated with 0.2µl 10X ExoAP buffer (500 mM Bis-Tris, 10 mM MgCl2, 1 mM ZnSO4), 0.05µl Antarctic Phosphatase (New England Biolabs: Ipswich, MA), 0.05µl Exonuclease I (New England Biolabs: Ipswich, MA) at 37°C for 60 min followed by 85°C for 15 min to remove unincorporated nucleotides. Sequencing reactions were executed with Big Dye Terminator v3 reactions (Applied Biosystems: Foster City, CA). AMPure magnetic beads (Agencourt: Morrisville, NC) were used to remove excess dye, products were analyzed on an ABI 3730xl DNA Analyzer (Applied Biosystems International), and chromatograms were edited using CodonCode Aligner (version 3.7.1; CodonCode Corporation: Dedham, MA). Consensus sequences were compared against the NCBI GenBank database to confirm identity when available (Benson 1997) and sequence alignments were constructed using the MUSCLE alignment algorithm (Edgar 2004) implemented in CodonCode Aligner. Representative sequences of dominant haplotypes were deposited in GenBank (Accession # KF498731 - KF498847). Full *COI* sequences for each individual are provided as FASTA files (Supplemental File 1).

Standard summary statistics, including number of haplotypes (*H*), haplotype diversity (*Hd*), nucleotide diversity (π), and Fu's F_S were calculated using DnaSP version 5.10.01 (Librado & Rozas 2009). To detect potential cryptic species, maximum-parsimony phylograms of aligned mitochondrial sequences were assembled in MEGA version 5 (10,000 replicates; Tamura 3-parameter substitution model determined by Mega 5: Find Best-Fit Substitution Model; Tamura et al. 2011). To visualize potential population structure, statistical-parsimony networks were assembled in TCS version 1.21 (default settings; Clement et al. 2000). To detect

120 population structure, Arlequin version 3.5.1.2 (Excoffier et al. 2005) was used to estimate
121 pairwise $\varphi_{ST.}$ Sequential Bonferroni was used in all appropriate comparisons to correct for
122 multiple tests (Rice 1989).
123
124 **Microsatellite genotyping and statistical analyses**
125 Eight microsatellite markers (*Bm17, Bm22, Bm23, Bm53, Bm63, Bm76, Bm81, Bm83*)
126 were amplified from *Bathymodiolus manusensis* in Manus Basin following methods reported in
127 (Schultz et al. 2010). To test whether these markers provided sufficient power to evaluate the
128 null hypothesis of genetic homogeneity, models of the dataset were implemented in POWSIM
129 (Settings based on observed allele distributions, Supplement 1; Ryman and Palm, 2006). Full
130 microsatellite genotypes for each individual are provided as GENPOP files (Supplemental File
131 2).
132 Standard summary statistics, including divergence from expected Hardy-Weinberg
133 Equilibrium (HWE) and allelic richness were assessed using GENEPOP (default settings;
134 version 4.0; Rousset 2008) and Microsatellite Analyzer (version 4.05; Dieringer & Schlötterer
135 2003), respectively. Permutation tests were used to determine significant variation in allelic
136 richness (F-stat; default settings; version 2.9.3.2; Goudet 1995). MicroChecker (version 2.2.3;
137 1000 randomizations; van Oosterhout et al. 2004) was used to detect the potential presence of
138 null alleles, stutter, and large allele dropout. To test for the potential influence of selection, loci
139 were screened using LOSITAN (25,000 simulations; IA and SMM; Antao et al. 2008; Beaumont
140 & Nichols 1996).
141 Pairwise genetic differentiation (F_{ST}) between aggregations, sites, and basins was
142 analyzed using Microsatellite Analyzer. Alpha levels were adjusted via Sequential Bonferroni to

correct for multiple tests (Rice 1989). Structure version 2.3.3 (admixture model, sampling locations as prior distributions; Pritchard et al. 2000) was used to visualize potential population structure. Analyses were conducted with a 1,000,000 step burn-in, 10,000,000 repetitions, and 3 replicates per level from K = 1 to 7. Effective population size was estimated based on microsatellite linkage-disequilibrium using LDNe (default parameters; Waples & Do 2008).

150 **Results**

151 Of 21 *COI* haplotypes (409 bp) identified, 5 were shared at both sites, 3 were only found
152 in Solwara 8 samples, and 13 were only found at South Su. *Bathymodiolus manusensis* from
153 Solwara 8 (47 individuals) and South Su (53 individuals) in Manus Basin (Table 2) comprised a
154 single haplogroup, based on *COI* analysis. A maximum of 5 base-pair mutations separated the
155 most divergent haplotypes (Table 2, Figure 2). The statistical parsimony network for
156 *Bathymodiolus manusensis* has a wheel-and-spoke topology, with a single central dominant
157 haplotype and numerous low-abundance secondary haplotypes (Figure 2). The dominant
158 haplotype is roughly evenly distributed among both sites and all relatively abundant haplotypes
159 ($n \geq 3$) occur at both Solwara 8 and South Su (Figure 2). Fu's F_S values for CO1 sequence data
160 were significantly negative for samples pooled from both sites, as well as within sites and at
161 Mound 1 (Solwara 8) and Mound 4 (South Su; Table 2).

162 Eight microsatellite loci were amplified from *Bathymodiolus manusensis* (35 to 142
163 individuals per site; Table 3). Alleles per locus ranged from 3 to 20 (mean = 10). Allelic richness
164 (*Rs*) did not vary significantly among mounds or sites (10,000 permutations, $P > 0.05$; Table 3)
165 and neither balancing nor directional selection was detected at any spatial scale (LOSITAN, $P >$
166 0.05). Only one marker deviated from Hardy-Weinberg expectations and showed evidence for
167 heterozygote deficiency at Solwara (*Bm53*; Table 3). POWSIM indicated that the sample set has
168 sufficient statistical power to accept or reject the null hypothesis of genetic homogeneity.
169 MicroChecker indicated that null alleles were present at that loci and were responsible for
170 heterozygote deficiencies. As the presence of null alleles has been shown not to severely bias
171 assignment tests (Carlsson 2008), this marker was included in subsequent analyses.

172 Analysis of Molecular Variance (AMOVA) and pairwise tests for population
173 differentiation (F_{ST} and φ_{ST}) based on *COI* sequences and microsatellite markers indicated no

174 significant genetic differentiation among *Bathymodiolus manusensis* from Solwara 8 and South
175 Su (Table 4). Assignment tests for combined *COI* and microsatellite data placed all *B.*
176 *manusensis* into a single population (Structure, K = 1, data not shown). Effective population size
177 estimated from microsatellite linkage disequilibrium was functionally infinite.
178
179

Discussion

Population structure of *Bathymodiolus manusensis* in Manus Basin

Bathymodiolus manusensis forms a single, coherent population between Solwara 8 and South Su in Manus Basin, Papua New Guinea. No genetic differentiation was detected at any spatial scale using either mitochondrial *COI* or nuclear microsatellite markers. Despite this apparent lack of population structure, *B. manusensis* is absent from Solwara 1, a site that occurs between Solwara 8 and South Su and that is within 2.5 kilometers of South Su. Further, Solwara 1 shares many vent-dependent and vent-associated species with Solwara 8 and South Su (Coffey Natural Systems 2008; Erickson et al. 2009; Thaler et al. 2011; Thaler et al. 2014; Plouviez et al. 2013).

The relatively homogeneous distribution of both *COI* haplotype and microsatellite markers for *Bathymodiolus manusensis* within Manus Basin is consistent with high gene flow between Solwara 8 and South Su. Similar levels of gene flow were observed in *Ifremeria nautilei* (Thaler *et al.* 2011) and *Chorocaris* sp. 2 (Thaler *et al.* 2014), although in both cases, the species were also found at Solwara 1. A significant, negative Fu's F_S indicates that the population may have undergone a recent, rapid expansion, an observation consistent with other species examined from these sites (Thaler *et al.* 2011, Plouviez *et al.* 2013, Thaler *et al.* 2014). *COI* haplotype diversity is higher at South Su (0.59 compared to Solwara 8's 0.45; Table 2), however, there is no consistent pattern of microsatellite richness between the two sites, nor are unique alleles consistently identified at one site over the other.

In more than ten years of exploration and environmental observations, consisting of at least 4 research campaigns, neither *Bathymodiolus manusensis* nor any other mussel in the genus *Bathymodiolus* has been observed at Solwara 1 (Saleu, personal observation). Visual surveys of

the seafloor suggest that adequate habitat exists within the Solwara 1 site for *B. manusensis* to settle (Thaler, personal observation), although the fluid chemistry that might influence mussel recruitment has not been characterized for Manus vents. In a previous study, we identified a similar pattern of presence/absence among populations of *Munidopsis lauensis* at Solwara 8 and South Su (Thaler et al. 2014). One population of *M. lauensis* was found at Solwara 8 and South Su, but absent at Solwara 1, while a second population was restricted to samples from Solwara 1 (Thaler et al. 2014). We hypothesized that sweepstakes effects related to the survival and settlement of recruits at vent sites in Manus Basin was responsible for the observed population structure of *M. lauensis* and that time series sampling would reveal a stochastic, dynamic distribution of these populations throughout the basin (Thaler et al. 2014).

That *Bathymodiolus manusensis* shares the same pattern of presence/absence with one population of *Munidopsis lauensis* suggests that the apparent exclusion of certain species or populations from Solwara 1 may be the result of a consistent, species- and population-dependent, dispersal barrier, rather than stochastic recruitment events. Other "leaky" dispersal barriers have been observed for hydrothermal vent populations across the equatorial East Pacific Rise (Plouviez et al. 2010; Plouviez et al. 2009; Vrijenhoek 2010), but those sites were separated by thousands of kilometers, whereas the Manus Basin sites are 2.5 to 40 km apart. In order to determine if there is a barrier restricting some, but not all, species (or populations) from recruiting to Solwara 1, we would need to sample additional species to identify consistent patterns across multiple taxa and sample the same species at additional time points to establish if observed patterns are temporally stable. The alternative hypothesis that species and populations are adapted to particular environmental conditions that are not always present at a site remains plausible (and not mutually exclusive), especially given well-documented evidence for such

circumstances in *Alviniconcha* species in Lau Basin (Beinart et al. 2012) and the lack of fluid chemistry data from evident and putative mussel habitats at Manus Basin vents.

Implications for management strategies

The limited distribution of *Bathymodiolus manusensis* and of a *Munidopsis lauensis* population within Manus Basin underscores the potential complexity of connectivity and habitat availability within vent ecosystems and the value of comprehensive environmental baselines prior to the initiation of an extractive regime (Collins et al. 2012; Collins et al. 2013; Thaler et al. 2014; Boschen et al. 2016). It is possible that multiple mining events in Manus Basin could affect source-sink dynamics of *Bathymodiolus manusensis* and other taxa, resulting in regime shifts in vent communities of Manus Basin as has been noted in other marine ecosystems (Scheffer & Carpenter 2003). A similar phenomenon was observed in Moorea coral reef communities, where persistent disturbance, caused, in this case, by invasive crown-of-thorn starfish resulted in permanent changes in community structure as opportunistic recruits occupied newly exposed ecologic niches (Berumen & Pratchett 2006).

The potential for regime shifts, where species not present at the disturbance site but occurring at neighboring sites establish a foothold following anthropogenic impacts, creates a challenge for environmental management and mitigation programs. At the very least, there is a need to understand if such a regime shift constitutes a significant adverse impact that should trigger a management response. Managers need to understand the extent of local variation in population and community structure to anticipate cumulative impacts and ecological consequences of regime changes following disturbance.

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377

Tables

Table 1. Bathymodiolus manusensis sampling locations in Manus Basin.

Site	Mound	Latitude	Longitude	Depth (m)
Solwara 8	Mound 1	3° 43.740'S	151° 40.404'E	1720
	Mound 2	3° 43.824'S	151° 40.458'E	1710
South Su	Mound 3	3° 48.564'S	152° 6.144'E	1300
	Mound 4	3° 48.492'S	152° 6.186'E	1350

383 **Table 2.** *Bathymodiolus manusensis*. Summary statistics for *COI* sequences (409 bp) from
384 Manus Basin. N: number of individuals, H: number of haplotypes, *Hd*: haplotype diversity, F_S:
385 Fu's F_S. Significant Fu's F_S indicated in bold.

386

Location	N	H	*Hd*	F_S
Manus Basin (total)	100	20	0.52	**-25.60**
Solwara 8	47	9	0.45	**-7.29**
Mound 1	34	9	0.46	**-8.12**
Mound 2	13	4	0.42	-1.66
South Su	53	16	0.59	**-18.32**
Mound 3	10	3	0.38	-1.16
Mound 4	43	14	0.63	**-13.97**

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389 **Table 3.** Summary statistics for eight microsatellite loci amplified from *Bathymodiolus*
390 *manusensis* from Manus Basin. n = number of individuals, a = number of alleles, *Rs* = allelic
391 richness, H_E = expected heterozygosity, H_O = observed heterozygosity (bold = significant
392 heterozygote deficiency).

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		Bm17	*Bm22*	*Bm23*	*Bm53*	*Bm63*	*Bm76*	*Bm81*	*Bm83*
Solwara 8	n	140	137	126	133	91	136	129	142
	a	3	17	8	5	20	6	16	12
	Rs	3.00	15.74	6.04	4.66	13.85	5.70	14.14	8.75
	as	264-368	236-284	238-270	243-264	198-262	189-206	197-245	206-224
	H_O	0.24	0.91	0.55	**0.44**	0.78	0.64	0.86	0.58
	H_E	0.29	0.89	0.61	**0.57**	0.85	0.65	0.86	0.55
South Su	n	65	63	53	61	35	58	60	61
	a	3	18	5	4	13	7	13	8
	Rs	3.00	18.00	5.00	4.00	13.00	7.00	13.00	8.00
	as	264-268	228-287	238-265	251-264	198-263	189-210	188-258	210-223
	H_O	0.29	0.89	0.62	0.43	0.94	0.71	0.73	0.54
	H_E	0.28	0.86	0.62	0.47	0.88	0.69	0.85	0.56

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397 **Table 4.** Pairwise comparisons of *Bathymodiolus manusensis* from two mound each of two sites
398 in Manus Basin. F_{ST} from microsatellites above the diagonal, φ_{ST} from partial *COI* below the
399 diagonal. No pairwise estimates of population differentiation were significant ($P < 0.05$).

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	Solwara 8 Mound 1	Solwara 8 Mound 2	South Su Mound 3	South Su Mound 4
SW8 Mound 1	-	0.00	0.00	0.00
SW8 Mound 2	0.00	-	0.00	0.00
SSU Mound 3	0.00	0.02	-	0.00
SSU Mound 4	0.00	0.00	0.00	-

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Figure Legends

Figure 1. Sampling locations in Manus Lau Basin. Figure adapted from one originally published in Thaler *et al.* (Thaler et al. 2011).

Figure 2. *Bathymodiolus manusensis*. Statistical parsimony network for *COI* haplotypes from samples collected at Solwara 8 and South Su, Manus Basin. Large circles represent a single individual unless noted on the figure. Small black circles represent inferred haplotypes not observed in this data set. Solwara 8 represented by gray circles. South Su represented by white circles. Each node represents 1 base pair difference.

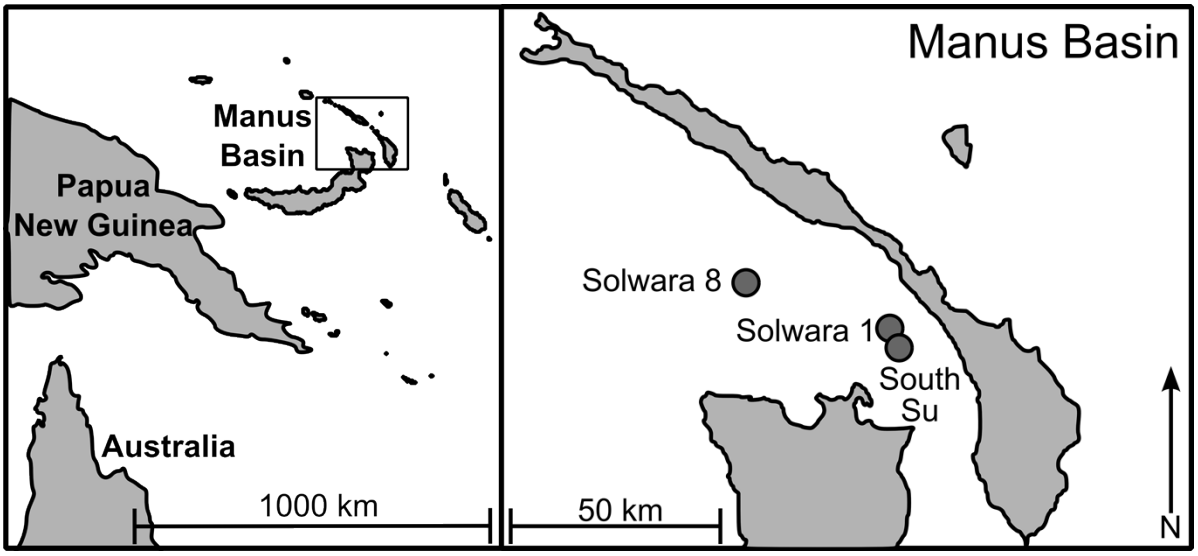
Supplemental Files

Supplemental Data 1. FASTA format file for all *Bathymodiolus manusensis COI* sequences.

Supplemental Data 2. GENPOP format file of all *Bathymodiolus manusensis* microsatellite markers.

426 **Figures**

427



428

429 Figure 1.

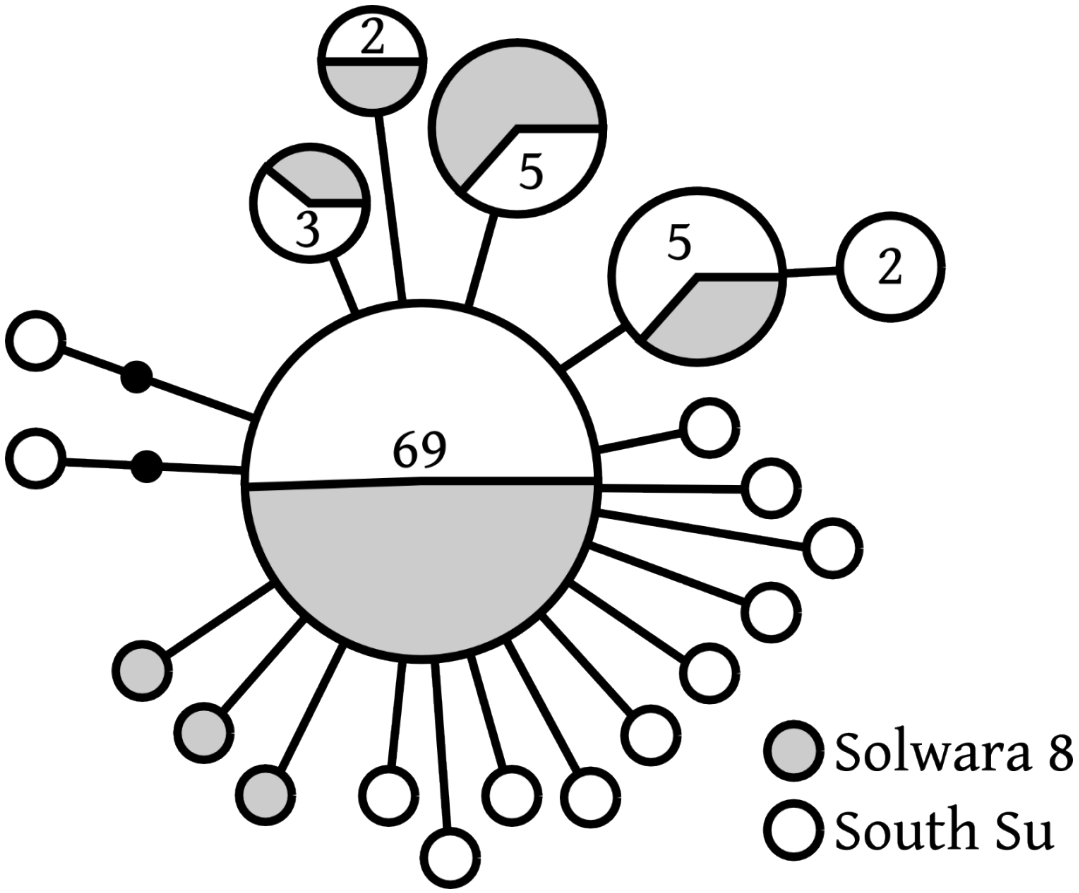


Figure 2.