Population structure and connectivity in Indo-Pacific deep-sea mussels of the *Bathymodiolus brevior* complex

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ABSTRACT

The discovery of chemosynthetic communities at hydrothermal vents in the late 1970s revolutionized biological concepts on how life can exist and evolve on Earth. Since then more than 550 vent-adapted species have been identified, while the number of novel descriptions continues to increase. Despite recent advances to include molecular approaches in taxonomic investigations, however, many species identifications are still based on morphological characters alone or rely on only one gene. These shortcomings can lead to significant issues in biodiversity and connectivity estimation, if taxa are phenotypically plastic or cryptic or when genes differ in their evolutionary histories. In the present study we analyze species status in the Indo-Pacific *Bathymodiolus brevior* complex (*B. brevior, B. septemdierum, B. elongatus, B. marisindicus*) by screening genetic variation at four nuclear and two mitochondrial loci. Our preliminary results based on three genes show that genetic differentiation is generally low, implying that the investigated mussel populations should be considered as a single species, *B.*

brevior. We suggest that multilocus genotypic features should become the main criteria for defining new species and should be commonly incorporated in taxonomic studies to avoid misclassification due to phenotypic or unigenic characteristics.

INTRODUCTION

Hydrothermal vents were first detected at the Galapagos Rift in 1977 and have ever since changed scientific thinking about the origin and evolution of life (Van Dover 2000). Unlike most other ecosystems, hot vents are based on chemosynthetic primary production, where specialized bacteria oxidize hydrogen sulphide or other reduced chemicals from the hydrothermal fluids to produce energy for carbon fixation. Animals make use of this by harboring the bacteria as endo- or ectosymbionts (Dubilier et al. 2008), which leads to biomass productivities that are among the highest on the planet (Ramirez-Llodra et al. 2010). Due to the dependence on chemosynthesis and the restriction to tectonically active seafloor regions, however, the distribution of hydrothermal habitats is patchy, making migration between vent fields a nontrivial challenge and increasing the vulnerability to environmental disturbances. Furthermore, financial and technical limitations often impede thorough investigations of these remote systems, so that details about connectivity and biodiversity remain poorly understood. While more than 550 novel species have been discovered (Desbruyères et al. 2006a), these assessments are particularly impaired by the fact that taxonomic descriptions are frequently made using morphological parameters, although recent molecular approaches have revealed the occurrence of both cryptic and phenotypically plastic species at vents (Vrijenhoek 2009). Depending on the taxon in question species richness can thus be seriously under- or overestimated. While genetic tools seem to alleviate this problem, they are of little advantage when inappropriately applied. For example, to determine the accurate level of genetic divergence, various molecular markers are necessary, because genes differ in their evolutionary rates and histories and are subject to varying

degrees of sampling bias owing to allelic drop-outs during PCR. Even so, the usage of a single locus (often COI) is still common practice.

Deep-sea mussels of the genus *Bathymodiolus* are among the dominant taxa in hydrothermal ecosystems and were recently found to occur also at vent fields of the Central Indian Ridge and the western Pacific back-arc basins. While researchers were fast in describing four new species based on morphological characteristics (von Cosel et al. 1994; Hashimoto & Okutani 1994: *B. brevior, B. septemdierum, B. elongatus*; Hashimoto et al. 2001: *B. marisindicus*), subsequent work on mitochondrial haplotypes by Kyuno et al. (2009) indicated a rather poor genetic differentiation, suggesting that the morphospecies might be conspecific. In spite of this the species status was not formally questioned or further investigated.

As a case study for the application of molecular taxonomy in the deep-sea, we here use multilocus genotyping to assess phylogenetic relationships and population structure in the Indo-Pacific *Bathymodiolus brevior* complex. Our results imply that the morphological species description is insufficient and that a re-classification of the four morphotypes as *B. brevior* is necessary.

MATERIALS AND METHODS

Samples

Mussel specimens investigated in this study were obtained from twelve vent and seep habitats of the West Pacific and Indian oceans that were visited during multiple expeditions between 1992 and 2007 (Table 1; Figure 1). Upon collection with a ROV or epibenthic sled, samples were preserved in 70% ethanol, frozen whole at -20°C, or dissected and stored at -80°C.

Table 1. *Bathymodiolus brevior* complex sampling localities.

Locality	Abbr.	Latitude	Longitude	Depth (m)	Dive No.*	Date
Central Indian Ridge						
Edmond	ED	23°52.7'S	69°35.8'E	3290-3320	J1:296	04/06/01
Kairei	KA	25°19.2'S	70°02.4'E	2415-2460	J1:301	04/24/01

North Fiji Basin						
White Lady	WL	16°59.5'S	173°54.9'E	1989-1992	J2:149-150	05/28-29/05
Lau Basin						
Kilo Moana	KM	20°3.2'S	176°8.0'W	2612-2622	J2:140-141	05/17-18/05
Tow Cam	TC	20°19.1'S	176°8.3'W	2714	J2:142	05/19/05
Tui Malila	TM	21°59.4'S	176°34.1'W	1845-1900	J2:143-144	05/20-21/05
Hine Hina	HH	22°32.3'S	176°43.0'W	1807-1819	J2:145-146	05/22-23/05
Izu Bonin Arc						
Mariana Trough	MT	18°12.8'N	144°42.4'E	3589	S:140-188	11/14/92
Kermadec Tonga Arc						
Unnamed seep	US	39°58.6'S	178°14.1'E	907	NZ:32050 NZ:32250	04-05/07
Volcano 1	VL	21°09.3'S	175°44.7'W	197-200	PIV:139-142 NZ:32094	06/05 05/07
Kermadec Ridge	KR	25°48.2'S	177°10.2'W	1143	KOK0505:153	04/05
Manus Basin						
South Su	SS	03°48.6'S	152°06.1'E	1300	ST212:36	06-07/08

* Submersibles: J1 = *Jason I*, J2 = *Jason II*, S = *Shinkai 6500*, PIV = *Pisces*, ST = *ST212 trenching ROV*; Epibenthic sleds: NZ, KOK

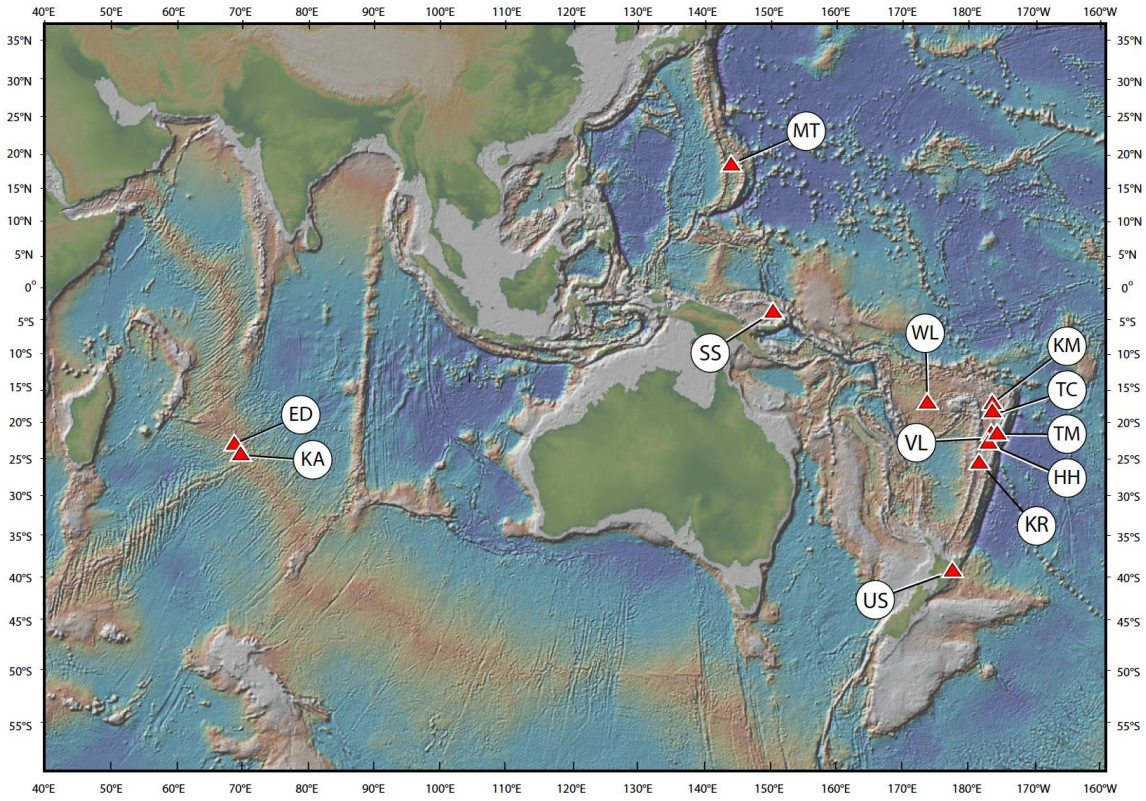

Figure 1. Sampling sites of the *B. brevior* complex in the Indo-Pacific. See Table 1 for abbreviations.

DNA isolation, PCR and sequencing

DNA was extracted with the DNeasy Blood & Tissue Kit (QIAGEN, Valencia, CA) according to manufacturer's instructions except that 2x100 µl were used for elution. Mussels were subsequently genotyped at four nuclear (Cat, Col-1, EF1α, H3) and two mitochondrial (ND4, COI) loci that were found to contain polymorphic sites in *B. brevior* (Table 2). Reaction mixtures for PCR consisted of 12.5 µl Amplitaq Gold Fast PCR Master Mix (Life Technologies Corp., Carlsbad, CA), 1 µl of each primer (10 pmol/µl), 2.5 µl 10X BSA and 1-5 µl (>20 ng/µl) template or sterile water (as negative control) in a volume of 25 µl. In the case of nuclear genes and ND4, templates were initially denatured for 10 min at 96°C, and then subjected to 35 cycles of 96°C for 3s, 50-55°C for 3s, 68°C for 15s on a Veriti thermal cycler (Life Technologies Corp., Carlsbad, CA). The final extension was done at 72°C for 7 min. PCR procedures for amplification of COI are given in Johnson et al. (2013). Prior to sequencing, all PCR products were diluted in 50 µl MilliQ H_2O and purified with a Multiscreen HTS PCR 96 vacuum manifold system (Millipore Corp. Billerica, MA). Sequencing reactions were run in both directions utilizing BigDye Terminator v3.1 chemistry (0.5 µl BigDye v3.1, 1.75 µl 5X sequencing buffer, 0.5 µl primer (3.2 pmol/µl), 2 µl PCR product, 5.25 µl sterile water; Life Technologies Corp., Carlsbad, CA) and the following cycle conditions: initial denaturation of 96°C for 1 min and 28 cycles of 96°C for 10 s, 50°C for 5s, 60°C for 1:15 min. Products were precipitated via a modified ethanol/EDTA/sodium acetate protocol and dissolved in 10 µl HiDi formamide before sequencing on an ABI 3130 Genetic Analyzer (Life Technologies Corp., Carlsbad, CA).

Table 2. Primer pairs for the seven gene loci analyzed in this study. COI = Cytochrome-c-oxidase subunit-I, ND4 = NADH dehydrogenase subunit 4, Cat = Catchin, Col-1 = Collagen type XIV, EF1α = Elongation factor 1α, H3 = Histone 3.

Locus	Primer	Sequence	Reference
mtDNA			
COI	COIG	5'-GTATTGAATTAGCACGTCCTGGAA-3'	Genio et al. 2008
	COIH	5'-ATACTATTCCAAACCCGGGTAAAAT-3'	
ND4	ArgBL	5'-CAAGACCCTTGATTTCGGCTCA-3'	Bielawski & Gold 1996
	NAP2H	5'-TGGAGCTTCTACGTGRGCTTT-3'	Arevalo et al. 1994

nDNA			
Cat	CatF Cat2R	5'-GAGYGTCTTTCMAAGATCATCTCCA-3' 5'-CATTTYCTGATGTTWCGCTGGAT-3'	Johnson et al. 2013
Col-1	Col160F Col1R	5'-GGTTCACGAYCGGAWGTTCCC-3' 5'-TCTCCTTCGCTATTTTTGTGG-3'	Johnson et al. 2013 Faure et al. 2009
EF1α	EF1αF EF1αR	5'-ACGCCTGGGTATTGGACAAAC-3' 5'-CCAAGAGGGGTCGTACAAATT-3'	Faure et al. 2009
H3	H3F H3R	5'-ATGGCTCGTACCAAGCAGACV-3' 5'-ATATCCTTRGGCATRATRGTG-3'	Colgan et al. 1998

Sequence analysis

Forward and reverse sequences for each sample and gene were quality trimmed, clipped and paired applying the De Novo Assemble tool with highest sensitivity in Geneious v7.1.5 (http://www.geneious.com/). If required, base calls were corrected manually. Subsequently, consensus sequences were multiple aligned with the integrated MUSCLE program using 20 iterations. In order to check for the accuracy of the PCR amplifications, the resulting nucleotide alignment was compared against the nr database with MEGABLAST choosing an e-value of 1e-20, a low complexity filter and a minimum similarity of 75%. Nuclear alleles in heterozygous individuals were further resolved with PHASE v2.1.1 (Stephens et al. 2001; Stephens & Donnelly 2003). For ensuring the reliability of the results, we used 10000 iterations of the MCMC chain, a burnin of 1000 and five different seeds for the random number generator. Since sequence checks indicated problems with DNA quality and contamination, samples from the Kermadec-Tonga-Arc and the Manus Basin were excluded from all further analyses.

Statistical analyses

We used the MJ method in Network v4.6.1.2 (www.fluxus-engineering.com; Bandelt et al. 1999; Forster et al. 1996; Saillard et al. 2000) to reconstruct phylogenies for mtDNA and nDNA sequence data. Weights of characters were set to 10, while those of mutation types were set to 1:1 (transitions:transversions). All calculations were repeated three times with the epsilon parameter fixed at values of 0, 10 and 20 to validate the consistency of the results. Finally, redundant median vectors were removed from all networks using the MP option (Polzin & Daneschmand 2003).

RESULTS

As the internship duration was not sufficient to process all data in time, we will report some initial results for the mitochondrial genes and the nuclear locus H3. Phylogenetic networks (Figure 2) showed a clear differentiation of Indian and Pacific Ocean based on ND4 and COI haplotypes, although sequence divergence was not strong enough to regard populations as distinct species (<4% difference between most divergent haplotypes). Haplotype diversity was generally high with lots of unique variants for both loci. By contrast, polymorphisms at locus H3 were shared between both ocean basins, while allelic diversity was much smaller than in the case of mtDNA.

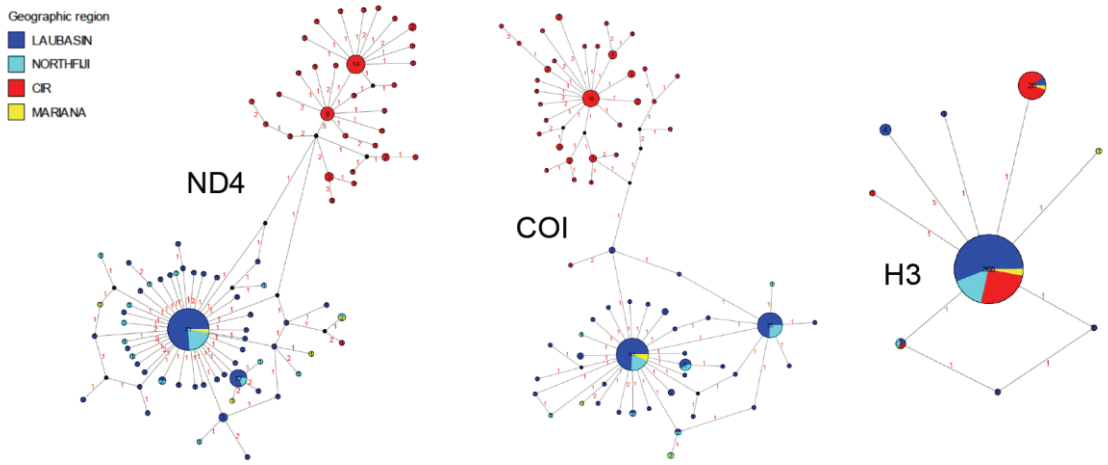

Figure 2. Phylogenetic networks for ND4, COI and H3. Pie sizes are proportional to the frequency of the respective gene variant. Numbers within pies indicate the number of individuals carrying the haplo- or genotype, while numbers on the branches show the number of mutations between alleles.

DISCUSSION

Our preliminary data on the genetic variation in Indo-Pacific *Bathymodiolus* populations suggest that the four morphologically described species *B. brevior*, *B. septemdierum*, *B. elongatus* and *B. marisindicus* are the same taxon and should be merged under the scientific name of *Bathymodiolus brevior*. Although this conclusion is based on results obtained from all applied markers, it is interesting to note that the signal of genetic (non-)divergence differed between nDNA and

mtDNA. Whereas the nuclear gene H3 did not indicate any differentiation at all, the two mitochondrial loci ND4 and COI were weakly divergent between the Indian Ocean mussels and the western Pacific populations. Several scenarios exist that can account for these discrepancies. Given the slower evolutionary rate of (conserved) nuclear genes (lower genetic diversity; Brown et al. 1979) and the essential function of H3 in DNA packaging (evolutionary constraints), the absence of genetic differences might be a matter of incomplete lineage sorting or stabilizing selection. Contrary to this, the mitochondrial divergence could be due to a founder event caused by past immigration of Central Indian Ridge mussels into the West Pacific (or vice versa; Van Dover et al. 2001; Desbruyères et al. 2006b) or differential selection of haplotypes, if habitats vary in their ecological settings. While our data do not allow rejecting any of these hypotheses, the mentioned scenarios are not mutually exclusive. The question remains whether the observed genetic structure is a result of historic or contemporaneous events. As suggested by Hessler & Lonsdale (1991) a direct ridge link north of New Guinea might have enabled communication between the Indian and Pacific Ocean more than 55 million years ago, whereas Desbruyères et al. (2006b) proposed exchanges through a southern route near the Macquarie Ridge Complex. While these connections would generally support the low genetic divergence found in our study, sea level drops during past glacial periods are likely to have temporarily isolated Indian and Pacific populations (Barber et al. 2000), which could explain the slight differences seen in mitochondrial haplotypes. Considering the community similarities between both ocean basins, however, current migration between Indian and Pacific systems is probable, even though immediate geological connections have vanished (Desbruyères et al. 2006b). Present connectivity might be achieved through planktonic larvae that disperse with ocean currents and through so far unknown chemosynthetic sites that serve as stepping stones (Wood et al. 2014).

CONCLUSIONS/RECOMMENDATIONS

Using multilocus molecular biological techniques, we provide evidence that mussels of the *Bathymodiolus brevior* complex are conspecifics in spite of differing significantly in their phenotypes. Moreover, levels of divergence varied between different marker types (nuclear vs mitochondrial). These findings have important ramifications for other taxonomic studies that still use morphological characters or a single gene as species descriptors, since these approaches will most likely give inaccurate results. To prevent misclassifications and creation of confusing synonyms we recommend applying various genetic markers before defining a novel species.

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