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Genetic divergence across an oxygen minimum zone

A. E. Glazier[1,2,*], R. J. Etter[1]

[1]Biology Department, University of Massachusetts, 100 Morrissey Blvd, Boston, MA 02125, USA

[2]*Present address:* Department of Biology, Temple University, 1900 N. 12th Street, Philadelphia, PA 19122-6078, USA

ABSTRACT: Oxygen minimum zones (OMZs) are large, persistent regions of the world's oceans with oxygen concentrations of ≤0.5 ml l^{-1}. The distribution, abundance, composition, and diversity of benthic fauna shift dramatically where OMZs intersect continental margins, and some species with distributions that extend into the OMZ diverge genetically. These abrupt shifts at the genetic, population, and community levels suggest OMZs might impose a strong selective force on many deep-sea organisms. If larvae are sensitive to hypoxic conditions, the OMZ might preclude dispersal among populations separated by regions of low oxygen and operate essentially as a geographic barrier to gene flow. We investigated this hypothesis by quantifying genetic variation (mitochondrial 16S rRNA) of a wood-boring bivalve, *Xylophaga washingtona*, along a depth gradient (100 to 1829 m) spanning the OMZ in the Northeast Pacific. Two distinct clades were apparent, one spanning the OMZ and the other restricted to within and below it. These clades likely represent independently evolving lineages, suggestive of cryptic species. The bathymetric divergence is consistent with the OMZ impeding gene flow, although we cannot rule out many other environmental, ecological and evolutionary forces that might have led to the observed divergence. Given the predicted expansion of OMZs as well as the deoxygenation of contemporary oceans, it is critical to develop a better understanding of how hypoxic and suboxic regions might influence the evolution of marine organisms.

KEY WORDS: OMZ · Deep sea · *Xylophaga* · Genetic divergence

INTRODUCTION

Oxygen minimum zones (OMZs) are large, persistent midwater features with oxygen concentrations ≤0.5 ml l^{-1} (Levin 2003) that profoundly influence the abundance, distribution, and diversity of benthic organisms (Levin 2003, Gooday et al. 2010). They typically intersect the seafloor at upper bathyal depths (Wyrtki 1966, 1973), leading to significant shifts in community structure, dominance, and composition (Mullins et al. 1985, Levin & Gage 1998, Levin 2002). The ecological and economic effects of OMZs have been intensively investigated (Wishner et al. 1995, Levin 2003, Levin et al. 2013), yet little is known about their evolutionary consequences. OMZs affect the distribution and movement of adults of various species (Vinogradov & Voronina 1962, Saltzman & Wishner 1997), reduce colonization (Levin et al. 2013), and are likely to also impact larval dispersal because hypoxia affects larval behavior, development, and mortality (Widdows et al. 1989, Wang & Widdows 1991). If larvae and adults cannot disperse through the OMZ, gene flow might be inhibited, possibly leading to population differentiation and speciation (White 1987, Wilson & Hessler 1987, Rogers 2000).

Present day OMZs are associated with upwelling systems in the eastern Pacific, Bay of Bengal, southeast Atlantic, and Arabian Sea (Helly & Levin 2004). Upwelled waters carry nutrient-rich bottom water to the euphotic zone, fostering a highly productive ecosystem. The excess organic matter decomposes as it sinks, depleting oxygen and creating an OMZ below. Slow currents at these depths limit horizontal transport and mixing with more oxygen-rich waters. This usually occurs at bathyal depths, from 200 to 1000 m (Wyrtki 1966, 1973), impacting a currently estimated

*Corresponding author: amanda.glazier@temple.edu

1.148×10^6 km^2 of sea floor (Helly & Levin 2004) and 102×10^6 km^3 ocean volume (Paulmier & Ruiz-Pino 2009). These regions differ biogeochemically from more oxygen-rich waters, and are characterized by greater organic carbon preservation (Paropkari et al. 1993, Burdige 2007), metal cycling (Aller 1994, Law et al. 2009), and denitrification (Gruber & Galloway 2008, Gruber 2011). OMZs persist over geologic time scales but fluctuate in strength and extent in response to climatic warming and cooling (von Rad et al. 1995, Reichart et al. 1998, Cannariato & Kennett 1999).

Recent increases in the size, duration, and frequency of regions with low oxygen have been associated with climate change (Stramma et al. 2008, Rabalais et al. 2010, Deutsch et al. 2011). OMZs are shoaling, affecting shallower bathyal and shelf communities (Stramma et al. 2008, 2010), and expanding horizontally (reviewed in Gilly et al. 2013). This expansion has been attributed to changes in circulation (Deutsch et al. 2005), increased stratification due to an increased salinity gradient (Sarmiento et al. 1998, Bopp et al. 2002), alterations in wind systems increasing upwelling (Lovenduski et al. 2008), and decreased oxygen solubility. While global oceanic oxygen as a whole is decreasing (Whitney et al. 2007, Helm et al. 2011), current hypoxic and suboxic regions are more susceptible to further oxygen depletion, are more highly affected by climate change, and are more prone to expansion and contraction than oxygen-rich waters (Deutsch et al. 2011).

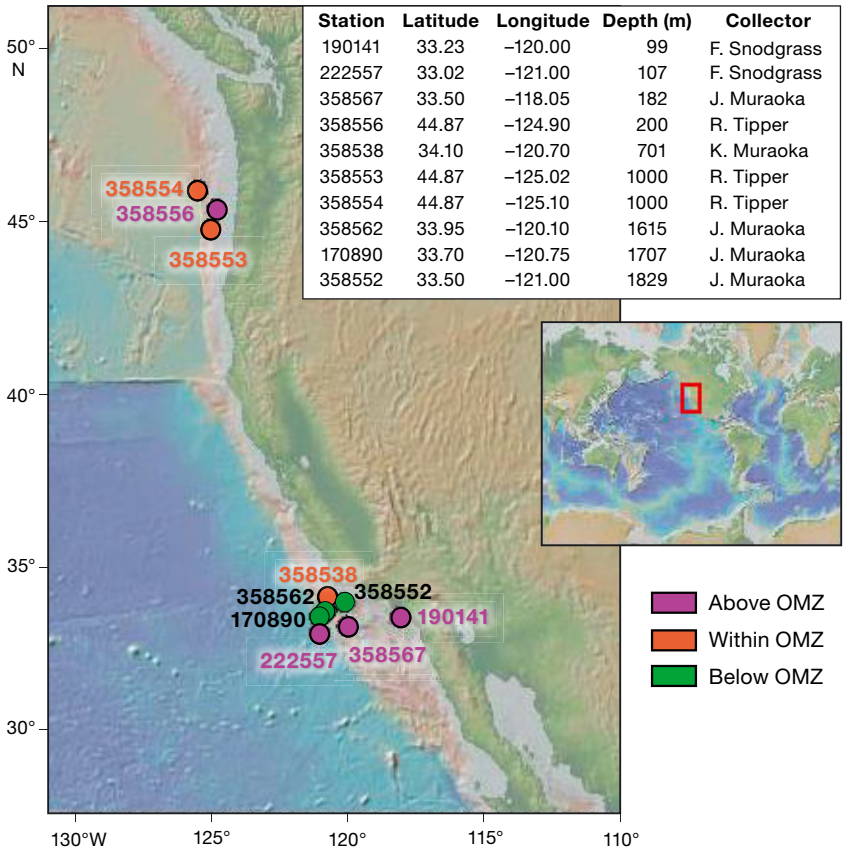
Where OMZs intersect continental margins, the distribution, abundance, composition, and diversity of the benthic fauna change substantially (Levin & Gage 1998, Levin 2003, Gooday et al. 2010) and some evidence suggests that species with distributions that extend into the OMZ diverge genetically (Creasey et al. 1997, 2000). The genetic, population, and community level changes associated with OMZs suggest hypoxic waters might represent an important selective force for many deep-sea organisms. If larvae are sensitive to hypoxic conditions, OMZs might operate as geographic barriers to gene flow, precluding dispersal among populations separated by regions of low oxygen. As OMZs develop and intersect continental margins they would fragment once continuous populations. Because they can persist for thousands of years (Reichart et al. 1998), they could lead to population differentiation and ultimately, speciation (White 1987, Wilson & Hessler 1987, Rogers 2000) from either selective or nonselective processes. On longer time scales, the geographic and bathymetric extent of OMZs change, expanding during periods of global warming and retracting as global temperatures cool. The expansion and contraction of these hypoxic regions might provide shifting barriers to gene flow that lead to repetitive cycles of population differentiation and speciation (White 1987, Jacobs & Lindberg 1998, Rogers 2000). From an evolutionary perspective, OMZs might play a key role in explaining why continental margins support high levels of diversity (Levin 2003, Gooday et al. 2010, Levin & Sibuet 2012) and appear to be important regions for the origin of the deep-water fauna (Etter et al. 2005, 2011).

Here, we explored the possible evolutionary impact of an OMZ by quantifying patterns of genetic variation in the wood-boring bivalve *Xylophaga washingtona* Bartsch (1921) along a depth gradient in the eastern North Pacific. *X. washingtona* has a putative depth range from 20 to 2000 m (Turner 2002), spanning the OMZ of the eastern North Pacific. The genus *Xylophaga* is part of the Family Pholadidae, which utilizes intracellular bacterial endosymbionts within bacteriocytes on the gills to digest wood (Distel & Roberts 1997). Little research has been carried out on the reproductive mechanisms of the group, but it is thought that *X. washingtona* has planktonic larvae (Voight 2009) that do not rise more than 8 m from the seafloor (Tipper 1968). In order to assess gene flow across an OMZ, mitochondrial 16S rRNA was sequenced from individuals sampled above, within, and below the OMZ. Two distinct clades were resolved, one consisting of individuals spanning the OMZ and the other with individuals from within and below the OMZ.

MATERIALS AND METHODS

Samples

A total of 65 formalin-fixed *Xylophaga washingtona* specimens were obtained from the Museum of Comparative Zoology at Harvard University to quantify geographic and bathymetric patterns of genetic variation. All samples were collected from the eastern Pacific by F. Snodgrass (Turner 2002), J. Muraoka (1965), or R. Tipper (1968) (Fig. 1). Samples were assigned to above, within, or below the OMZ based on depths of the 0.5 ml l^{-1} oxygen threshold from Helly & Levin (2004). Specimens from regions shallower than 700 m were considered above the OMZ, those between 700 and 1000 m were considered within the OMZ, and those deeper than 1000 m were considered below the OMZ. Two frozen and 5 ethanol-preserved specimens of *X. washingtona* were obtained from Lisa Levin's collection at the Scripps Institution of Oceano-



Station	Latitude	Longitude	Depth (m)	Collector
190141	33.23	–120.00	99	F. Snodgrass
222557	33.02	–121.00	107	F. Snodgrass
358567	33.50	–118.05	182	J. Muraoka
358556	44.87	–124.90	200	R. Tipper
358538	34.10	–120.70	701	K. Muraoka
358553	44.87	–125.02	1000	R. Tipper
358554	44.87	–125.10	1000	R. Tipper
358562	33.95	–120.10	1615	J. Muraoka
170890	33.70	–120.75	1707	J. Muraoka
358552	33.50	–121.00	1829	J. Muraoka

Fig. 1. Sampled stations off the coast of Oregon and California, USA; colors are based on depth in relation to the oxygen minimum zone. Coordinates are approximate based on available collection data. Dot locations are not to scale

graphy to develop species-specific primers that could then be used to amplify mitochondrial 16S rRNA from formalin-fixed samples.

Formalin-fixed samples

As with most deep-sea samples, these were fixed in formalin, which degrades the DNA, making it difficult to quantify genetic variation. We used protocols to extract and amplify (with PCR) mtDNA from small macrofaunal metazoans that were fixed in formalin (described in Chase et al. 1998) to sequence a 350 bp fragment of the variable region of the 16S rRNA mitochondrial gene. Formalin fixation precludes amplification of nuclear loci in a consistent manner for population-level studies, probably because the low copy number of nuclear loci and degradation by the formalin leave few amplifiable fragments. Consequently, our analyses were restricted to a single locus. While we recognize the limitations of evolutionary inferences based on a single locus (e.g. Edwards & Beerli 2000, Avise 2004, Felsenstein 2006), the results provide a novel window into evolution in this vast and remote ecosystem where fresh material is prohibitively expensive to collect, and also provide an informed framework to collect new samples that are more conducive to testing hypotheses with more sophisticated multilocus and genomic analyses.

DNA extraction, PCR amplification and sequence processing

To develop species-specific primers, genomic DNA was extracted from the 2 frozen and 5 ethanol-preserved samples. DNA was extracted from whole individuals using a QiaAMP mini tissue kit (Qiagen) with the default protocol. Partial 16S ribosomal subunit sequences were amplified from the frozen and ethanol-preserved individuals using universal primers (16SarL: 5′-CGC CTG TTT ATC AAA AAC AT-3′ and 16SbrH: 5′-CCG GTC TGA ACT CAG CTC ATG T-3′) (Palumbi et al. 1991). PCR amplification reactions consisted of 1× GoTaq flexi buffer (Promega), 1.2 pmol of each primer, 2 pmol of dNTP, 1.25 µl BSA (10 mg ml^{-1}), 2.5 µl of 25 mM $MgCl_2$, 1 U of GoTaq flexi polymerase (Promega), 2 µl genomic DNA template, and molecular grade water to a final volume of 50 µl. The PCR profile consisted of an initial denaturation cycle of 94°C for 3 min, followed by 35 cycles of 94°C for 30 s, 50°C for 45 s, and 72°C for 1 min, with a final hold at 4°C.

PCR products were checked for the presence of single bands through gel electrophoresis and outsourced to Agencourt (a Beckman-Coulter company) for bi-directional sequencing. The forward and reverse sequences were edited and aligned using Sequencher 5.0.1 (Gene Corp.) and checked by eye. Alignments of multiple individuals were created using ClustalX (Larkin et al. 2007) in BioEdit and MacClade (Maddison & Maddison 2005). The Genbank search tool BLAST was used to ensure these sequences were molluscan and not from the bacterial symbiont. These sequences were aligned and used to develop *Xylophaga*-specific primers to use on the formalin-fixed museum samples since formalin fixation degrades the DNA, which hampers successful

amplification with universal primers (Boyle et al. 2004).

We extracted DNA from 65 formalin-fixed whole individuals (23 above, 21 within, and 21 below the OMZ) using a QiaAMP micro tissue kit with the default protocol preceded by a 96 h incubation in 180 µl ATL buffer and 20 µl proteinase K at 56°C and eluted into 100 µl in buffer AE. The alignment of 16S rRNA sequences from the frozen and ethanol-preserved specimens was used to create species-specific primers (Xyloph16sF: 5′-GTC KGR CCT GCC CGG TG-3′ and Xyloph16sR: 5′-AAT TCA ACA TCG AGG TCG C-3′), that we used to amplify a 350 bp fragment in the formalin-fixed individuals. PCR amplification reactions consisted of 2 U GoTaq flexi polymerase, 2 pmol of each primer and of dNTP, 1.25 µl BSA (10 mg ml^{-1}), 2.5 µl of 25 mM $MgCl_2$, 1× GoTaq flexi buffer, 5 µl genomic DNA template, and molecular grade water to a final volume of 50 µl. The PCR profile consisted of 1 cycle of 94°C for 3 min followed by 45 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min, and a final cycle of 72°C for 3 min. The PCR products were checked for presence of single bands with gel electrophoresis, outsourced to Agencourt for sequencing with no further in lab cleanup or processing, edited and aligned using Sequencher 5.0.1, and alignments of multiple individuals created using ClustalX (Larkin et al. 2007) in BioEdit and MacClade (Maddison & Maddison 2005).

Phylogenetic analyses

Haplotype networks were inferred using statistical parsimony in TCS v.1.21 (Clement et al. 2000). The connection limit was set at 95% and decreased incrementally until all haplotypes were connected.

Phylogenetic relationships were inferred using BEAST v.1.8 (Drummond et al. 2012). Tracer was used to ensure sufficient burn-in and run time based on ESS (estimated sample size) estimations of at least 100. All trees were inferred with a HKY+G+I substitution model based on Akaike's information criterion (AIC) and Bayesian's information criterion (BIC) tests in jModelTest (Guindon & Gascuel 2003, Darriba et al. 2012), uncorrelated lognormal clocks and a Markov chain Monte Carlo (MCMC) chain of 3×10^7 steps, logging every 1000 trees. Starting trees were estimated with UPGMA, and a Yule speciation prior was enforced. Congeneric *Xylophaga* 16S rRNA sequences from *X.* sp1, *X.* sp6, and *X.* cf. *altenai* provided by Takoma Haga (Japan Agency for Marine-Earth Science and Technology) were used for outgroups.

Species delimitation

The phylogenetic analyses indicated *X. washingtona* was comprised of 2 distinct clades, so population genetic analyses were conducted on each of the 2 clades (see 'Results'). Two species delimitation methods were used to test the species status of the 2 clades distinguished in the TCS and BEAST analyses. The generalized mixed yule coalescent (GMYC) method (Pons et al. 2006, Fujisawa & Barraclough 2013) utilizes an ultrametric tree to find the threshold of branching rates between inter- and intraspecific branching events. BEAST v.1.8 was used to create a tree with a single individual per haplotype and 3 congeneric outgroups. Conditions were as described for the phylogenetic analyses but with a strict clock due to ambiguities in the tree with all individuals when a relaxed lognormal clock was used. Both the single threshold and multiple threshold methods were implemented (Fujisawa & Barraclough 2013).

The Bayesian Poisson tree process (bPTP) (Zhang et al. 2013) delimits species based on a shift between the number of inter- and intraspecific nucleotide substitutions in a Bayesian framework with a MCMC and a maximum likelihood (ML) framework. The tree with a single individual per haplotype and 3 congeneric outgroups was used. Both models were implemented using the webserver maintained by the Exelixis lab (http://species.h-its.org/). bPTP was carried out with 100 000 MCMC generations and a 10% burn-in.

Genetic diversity and tests of neutrality

Arlequin v.3.5 (Excoffier & Lischer 2010) was used to calculate basic diversity indices and test for neutrality within clades. The numbers of haplotypes, haplotypic diversity, and nucleotide diversity were calculated under a Kimura 2 parameter evolutionary model (K2P). Neutrality was tested using Tajima's D ($p < 0.05$) and Fu's Fs ($p < 0.02$).

For comparison with previous studies, uncorrected *p*-distances were calculated among individuals. Minimum, maximum, and average distances between clades are reported.

We used an analysis of molecular variance (AMOVA) (Arlequin v.3.5) to test for genetic divergence among samples above, within, and below the OMZ within each clade and for all individuals together, and between each clade. AMOVAs were carried out with stations grouped based on OMZ (above, within, and below), geographical location, and pairwise among stations within each clade under

a K2P model of evolution. Significance was determined after a Bonferroni correction for multiple comparisons was enforced.

RESULTS

A total of 64 formalin-fixed individuals were successfully sequenced for a 350 bp fragment of the 16S rRNA gene (Table 1). In all, 11 haplotypes were distinguished; the most prevalent was shared by 27 individuals spanning the OMZ and both geographic locations of our samples (Oregon and California). The second most prevalent haplotype was shared by 23 individuals that were only found within and below the OMZ. The remaining 9 haplotypes were much less common, with 6 as singletons (Fig. 2). Due to the opportunistic nature of the sampling, not all depth–location combinations are represented (Table 1). Sequences were deposited in GenBank under accession numbers KY228416–KY228480.

Phylogenetic analysis

The haplotype network revealed 2 divergent clades separated by 12 substitutions (Fig. 2). Both clades contained individuals from Oregon and California. Clade 1 spanned the OMZ bathymetrically, with 23 individuals above, 6 individuals within, and 7 below the OMZ. Clade 2 was restricted to within and below the OMZ, with 15 within the OMZ and 14 below (Table 1). The average genetic distance (uncorrected *p*-distance) between clades was 0.045. The minimum distance between the 2 clades was 0.034 and the maximum was 0.051.

The BEAST analysis also identified 2 clear clades, with posterior support of 0.94 and 0.85. One clade contained only individuals that occur within and below the OMZ, while the other was a mix of all depths, completely consistent with the 2 clades defined in the haplotype network (Fig. 3).

Species delimitation

The likelihood ratio tests for both the single and multiple thresholds methods from the GMYC species delimitation test were significant ($p < 0.001$), rejecting the null hypothesis that all individuals were from a single species. The single threshold method delimited 2 (CI ± 2) clusters, while the multiple threshold method delimited 2 (CI ± 1) clusters. Both methods support the 2 major clades as independently evolving

Table 1. Number of individuals sequenced for each station in each clade. OMZ: Oxygen minimum zone

Station	Depth (m)	Location	No. in clade 1	No. in clade 2	Total
Above OMZ			23	0	23
190141	99	California	8	0	8
222557	107	California	10	0	10
358567	182	California	2	0	2
358556	200	Oregon	3	0	3
Within OMZ			6	15	21
358538	701	California	6	1	7
358553	1000	Oregon	0	12	12
358554	1000	Oregon	0	2	2
Below OMZ			6	14	20
358562	1615	California	0	4	4
170890	1707	California	6	0	6
358552	1829	California	0	10	10
		Total	35	29	64

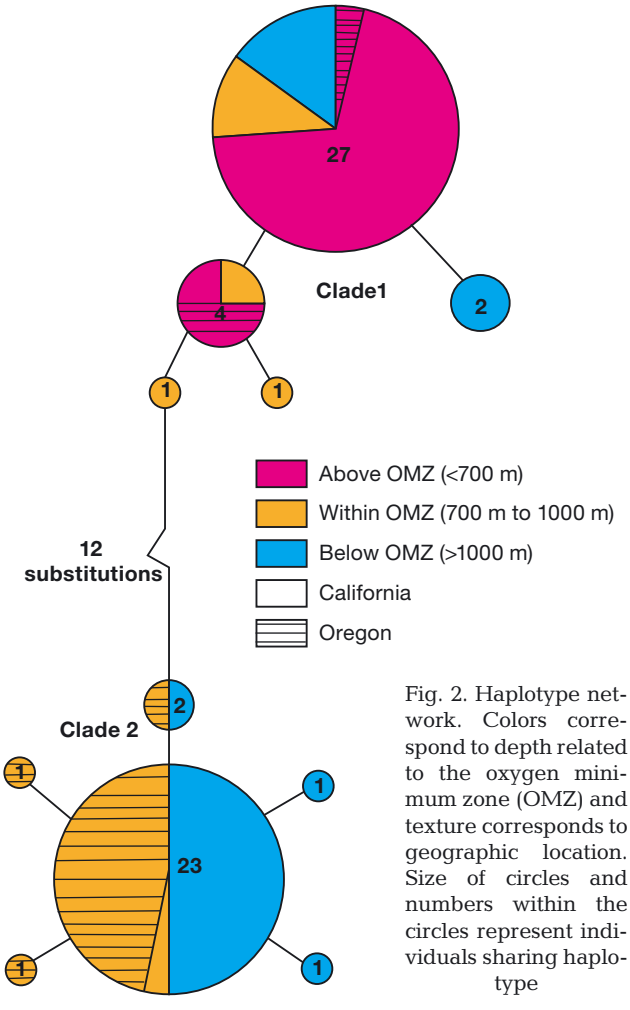


Fig. 2. Haplotype network. Colors correspond to depth related to the oxygen minimum zone (OMZ) and texture corresponds to geographic location. Size of circles and numbers within the circles represent individuals sharing haplotype

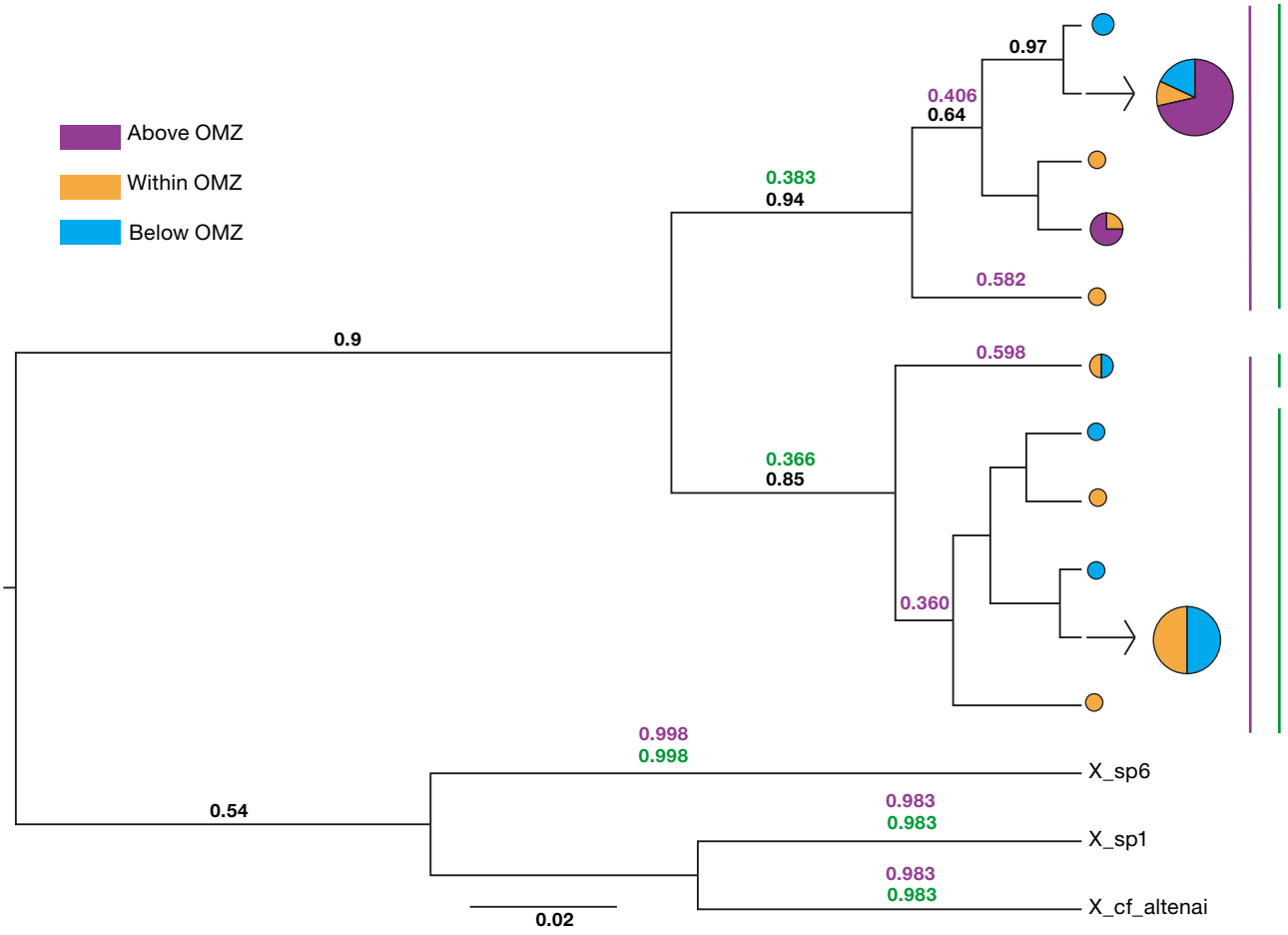


Fig. 3. Phylogeny estimated in BEAST v.1.8 from 16S rRNA sequences. Two well-supported clades were resolved. Pie charts represent number of individuals sharing the haplotype, the smallest corresponding to a single individual; colors correspond to depth related to the oxygen minimum zone (OMZ). Posterior probabilities >0.5 are reported in black on branches. Vertical colored lines represent generalized mixed yule coalescent (GMYC) results; purple: single threshold method; green: multiple thresholds. Maximum likelihood supports from Bayesian Poisson tree process (bPTP) species delimitation are reported in green and Bayesian supports in purple. Branch lengths are proportional to substitutions per site

entities but the multiple thresholds method further separates clade 2 into 2 lineages (Fig. 3).

The bPTP also delimited the 2 clades as independently evolving entities. The ML method identified 2 groups of *Xylophaga washingtona* corresponding to the 2 clades, with support values of 0.383 and 0.366. The Bayesian method identified 4 *X. washingtona* groups; 2 from clade 1 and 2 from clade 2, with support values of 0.406, 0.582, and 0.598, and 0.360 respectively. Support values represent the number of occurrences of the grouping throughout the model sampling.

Both delimitation methods estimate species based on differences between intra- and interspecific variation, and are more powerful with multiple loci and more extensive estimates of interspecific variation of congeners. Given the limitations of working with a single locus and the lack of more extensive genetic data from closely related species, the results from these delimitation methods must be interpreted cautiously and are presented as hypotheses that require further testing with more rigorous multilocus analytical tools.

Genetic diversity and tests of neutrality

Haplotype diversity from stations with 3 or more individuals varied from 0.25 to 0.80 and nucleotide diversity ranged from 0.001 to 0.004. The station with the lowest diversity in clade 1 was above the OMZ, and the highest was from within (Table 2). The stations with the lowest and highest diversity in clade 2 were below the OMZ (Table 2).

Tajima's *D* was nonsignificant for all stations and groupings except clade 2 below the OMZ (Table 2).

Table 2. Diversity indices and tests of neutrality calculated by clade, depth related to the oxygen minimum zone (OMZ) within each haplogroup, and by station within haplogroup with16S rRNA in Arlequin v.3.5. Significant neutrality indices are in **bold**

Station	No. sequenced	No. haplotypes	Haplotype diversity	Nucleotide diversity	Tajima's *D*	Fu's *Fs*
Clade 1	35	5	0.39	0.003	−0.12	−0.67
Above OMZ	23	2	0.24	0.001	−0.21	0.27
190141	8	2	0.25	0.001	−1.06	**−0.18**
222557	10	1	NA	NA	NA	NA
358567	2	1	NA	NA	NA	NA
358556	3	2	0.67	0.002	0.00	0.20
Within OMZ	6	4	0.80	0.004	−0.19	**−1.35**
358538	6	4	0.80	0.004	−0.19	**−1.35**
Below OMZ	6	2	0.53	0.002	15.12	3.15
170890	6	2	0.53	0.002	15.12	3.15
Clade 2	29	6	0.37	0.002	−1.04	**−2.92**
Within OMZ	15	4	0.37	0.001	−0.40	**−0.82**
358538	1	1	NA	NA	NA	NA
358553	12	4	0.45	0.002	0.02	−0.62
358554	2	1	NA	NA	NA	NA
Below OMZ	14	4	0.40	0.001	**−1.48**	**−2.29**
358562	4	2	0.50	0.002	−0.61	0.17
358552	10	3	0.38	0.001	−1.11	**−1.16**
Total	65	11	0.69	0.047	7.25	12.46

Table 3. Analysis of molecular variance (AMOVAs) estimated in Arlequin v.3.5 with 16S rRNA of clade 1. (a) Individuals grouped by station and by depth in relation to the oxygen minimum zone (OMZ); (b) individuals pooled by location in relation to the OMZ. Significant Φ values are in **bold**

Source of variation		df	Sum of squares	Variance component	Percentage of variation
(a)					
Among groups		2	1.70	0.05	17.79
Among populations within groups		3	1.07	0.03	9.99
Within populations		29	6.06	0.21	72.22
Total		34	8.83	0.29	
Φ_{SC}	**0.12**				
Φ_{ST}	**0.28**				
Φ_{CT}	0.18				
(b)					
Among populations within groups		2	1.70	0.07	23.99
Within populations		32	7.13	0.22	76.01
Total		34	8.83	0.29	
Φ_{ST}	**0.24**				

Fu's *Fs* was significant for 2 stations in clade 1 and 1 station in clade 2. When pooled by clade based on the haplotype network and phylogeny, Fu's *Fs* was significant for clade 2 and for samples pooled within and below the OMZ for clade 2 (Table 2).

Population divergence was nonsignificant among depths for clade 1 when stations were grouped by station within each depth (Φ_{CT} = 0.18, p = 0.12) and was significant when individuals were pooled by depth related to the OMZ (Φ_{ST} = 0.24, p = 0.01) (Table 3). It was not significant for any groupings in clade 2 (Table 4). Population divergence among stations with all individuals was nonsignificant (Φ_{CT} = 0.32, p = 0.19) when samples were grouped above, within, and below the OMZ or when grouped by geography (Φ_{CT} = 0.13, p = 0.33) (Table 5). It was significant between clades when individuals were grouped within stations of the respective clade (Φ_{CT} = 0.97, p = 0.003) (Table 5). All AMOVAs estimated within clade should be interpreted with caution due to the small sample sizes and the lack of power (Fitzpatrick 2009).

DISCUSSION

The deep-sea bivalve *Xylophaga washingtona* in the eastern North Pacific is comprised of 2 clades separated by 12 substitutions in the 16S rRNA sequence analyzed here, which, based on our analyses, might represent 2 partially sympatric cryptic species. The genetic distance between the 2 clades is comparable to congeneric pairwise distances in the 16S rRNA of other deep-sea bivalves (Etter et al. 1999, Sharma et al. 2013), and both species delimitation analyses suggest at least 2 lineages. The clades are not segregated geographically; both include individuals from Southern California and Oregon and co-occur at depths within and below the OMZ. One of these clades spans the OMZ while the other is confined to within and below it. Although the observed bathymetric divergence is consistent with what we might expect if the OMZ at some point in the past hampered gene flow, it remains difficult to attribute the divergence directly to the OMZ. Many environmental variables covary with depth, and singly or in combination could provide equally plausible, nonmutually exclusive mechanisms for divergence within the eastern North Pacific.

Table 4. Analysis of molecular variance (AMOVAs) estimated in Arlequin v.3.5 with 16S rRNA of clade 2. (a) Individuals grouped by station and by depth in relation to the oxygen minimum zone (OMZ); (b) individuals pooled by location in relation to the OMZ. Significant Φ values are in **bold**

Source of variation		df	Sum of squares	Variance component	Percentage of variation
(a)					
Among groups		1	0.10	0.01	6.80
Among populations within groups		3	0.26	−0.03	−16.21
Within populations		24	4.41	0.18	109.41
Total		28	4.77	0.17	
Φ_{SC}	−0.17				
Φ_{ST}	−0.09				
Φ_{CT}	0.07				
(b)					
Among populations within groups		1	0.10	−0.01	−2.92
Within populations		27	4.67	0.17	102.92
Total		28	4.77	0.17	
Φ_{ST}	−0.03				

Historical allopatry

The 2 partially sympatric clades identified from species delimitation methods are strongly divergent at the 16S rRNA gene, but it remains unclear how divergence originated given their potential dispersal abilities and the lack of obvious contemporary isolating barriers. One possibility is that the clades represent populations isolated in oxygenated refugia during a time of increased OMZ strength, that came into secondary contact as the OMZ weakened. This situation has been found frequently in many shallow water and terrestrial

Table 5. Analysis of molecular variance (AMOVAs) estimated in Arlequin v.3.5 with 16S rRNA for all individuals. Grouped by (a) depth related to the oxygen minimum zone (OMZ); (b) geographic location; (c) clade; and (d) pairwise by station. Grey cells: within depth regime relative to the OMZ. Significant Φ values are in **bold**

Source of variation		df	Sum of squares	Variance component	Percentage of variation
(a)					
Among groups		2	123.27	1.67	31.87
Among populations within groups		7	125.82	3.12	59.63
Within populations		54	24.02	0.44	8.50
Total		63	273.10	5.24	
Φ_{SC}	**0.87**				
Φ_{ST}	**0.92**				
Φ_{CT}	0.32				
(b)					
Among groups		1	53.00	0.66	12.85
Among populations within groups		8	193.93	4.06	78.58
Within populations		54	23.89	0.44	8.57
Total		63	270.82	5.16	
ΦSC	**0.90**				
ΦST	**0.91**				
ΦCT	0.13				
(c)					
Among groups		1	254.74	8.02	97.26
Among populations within groups		9	3.13	0.03	0.34
Within populations		53	10.47	0.20	2.40
Total		63	268.34	8.24	
Φ_{SC}	**0.13**				
Φ_{ST}	0.98				
Φ_{CT}	**0.97**				

(d)

	190141	222557	358567	358556	358538	358553	358554	358552	170890	358562
190141	–									
222557	0.03	–								
358567	−0.32	0.00	–							
358556	0.39	0.78	0.37	–						
358538	0.08	0.17	−0.20	−0.17	–					
358553	**0.98**	**0.99**	**0.98**	0.97	**0.84**	–				
358554	0.99	1.00	1.00	0.98	0.69	−0.33	–			
358552	**0.99**	**1.00**	0.99	**0.99**	**0.83**	−0.01	−0.32	–		
170890	0.17	0.31	−0.09	0.42	0.09	**0.98**	0.98	**0.99**	–	
358562	0.98	**0.99**	0.98	0.97	0.74	−0.09	−0.26	0.08	**0.97**	–

sympatric divergent clades associated with glacial refugia (Hewitt 1999, Maggs et al. 2008). Gene pools might have been isolated in large expanses of oxygenated waters on either side of an OMZ or within oxygenated pockets surrounded by hypoxia (White 1987, Jeppsson 1990, Armstrong 1995).

OMZs, including that of the northeast Pacific (McKay et al. 2005, Cartapanis et al. 2011), have fluctuated in strength and size with periods of climatic warming and cooling (von Rad et al. 1995, Reichart et al. 1998, Cannariato & Kennett 1999). For example, during the past 70 ky the OMZ off of California has increased in strength in association with deglaciation, warm interstadials, and the Bølling-Ållerød event, and decreased during cooler stadials (Gardner & Hemphill-Haley 1986, Cannariato & Kennett 1999, Cartapanis et al. 2011). Interstadials were times of increased global temperatures with associated changes in ocean productivity. The Bølling-Ållerød event was an interstadial during the last glacial period and occurred from 14 700 to 12 700 yr BP. Based on foraminiferan assemblages, the OMZ core shoaled and had a deeper lower limit than present day during interstadials 16/17, 14, 11, and 8, the Bøllinger-Ållerød event, and the early Holocene (Cannariato & Kennett 1999). The increase in strength and range of the OMZ has been attributed to decreased ventilation by North Pacific Intermediate Water (NPIW) (Cartapanis et al. 2011), decreased oxygen content of NPIW (Crusius et al. 2004), and increased productivity due to increased upwelling (McKay et al. 2005, Cartapanis et al. 2011). Evidence from laminated sediments suggest a strengthened OMZ from the last deglaciation to the early Holocene, which slowly weakened beginning about 5000 yr BP to the present state (Gardner & Hemphill-Haley 1986). Increased strength of the OMZ would present harsher conditions for survival within it, precluding taxa able to withstand contemporary conditions.

Because OMZs have fluctuated through geological time in strength and geography, we used a molecular clock to estimate the potential timing of interclade divergence. There are no estimates of a molecular clock for 16S in *X. washingtona* or for pholad bivalves in general, so we used an estimate based on pteriomorph bivalves (Page & Linse 2002), which was similar to estimates from other mollusks (Canapa et al. 1996, Reid et al. 1996). Molecular clocks based on different time frames and from distantly related taxa can be problematic (Ho et al. 2008), so inferences based on these estimates should be interpreted with caution. Based on this clock, the 2 clades diverged between 30 and 42 mya using all substitutions, or between 10 and 14 mya using just transversions. If increased OMZ strength led to the divergence of these clades, we would expect at least one of these time intervals to coincide with a warm period. Both atmospheric and deep ocean temperatures were considerably warmer during the late Oligocene and early Miocene (15 to 30 mya) relative to the present (Zachos et al. 2001, Hansen et al. 2013) suggesting OMZs might have been more intense and more expansive (Norris et al. 2013). Estimates of divergence time based on a molecular clock therefore coincide with a warmer ocean and potentially a more intense OMZ that might have been inhospitable for *X. washingtona* and represented a much more formidable barrier to dispersal than present conditions.

Hypoxic effects on larvae and rafting

Even if adults can survive within the OMZ, their larvae might be less tolerant of hypoxic waters limiting connectivity among populations separated by an OMZ. Effects of hypoxia on larvae are varied and species-specific (Vaquer-Sunyer & Duarte 2008, Eerkes-Medrano et al. 2013) but evidence suggests larval development, movement (Widdows et al. 1989, Wang & Widdows 1991), settling ability (Baker & Mann 1992), and survival (Eerkes-Medrano et al. 2013) are impacted by hypoxia. Adult populations tolerant of hypoxic conditions can be maintained within the OMZ through rafting on wood, similar to long-distance hitchhiking of shallow-water invertebrates (Helmuth et al. 1994, Kano et al. 2013). In this manner, adults could persist within the OMZ but gene flow would be restricted because any larvae spawned in the hypoxic conditions would not survive and dispersal through the OMZ would be unsuccessful.

Environmental gradients with depth

Many environmental gradients parallel changes in depth and might singly or in combination affect the viability of adults or their larvae. In addition to oxygen, the environmental gradients with the greatest potential to impact species ranges and divergence are temperature and hydrostatic pressure. Differential tolerance to thermal stress can occur across life history stages (Stillman & Somero 2000, Somero 2002) and appears to affect bathymetric zonation (Somero 2002). Populations and species might also adapt to specific hydrostatic pressure regimes due to the similar molecular and cellular effects of de-

creased temperature and increased pressure (reviewed in Brown & Thatje 2014). The combined effects of hydrostatic pressure and temperature appear to play a complex role in setting bathymetric range limits (Sébert 2002, Brown & Thatje 2011, 2014), and might affect population connectivity among depth regimes within *X. washingtona*.

Divergence along ecological and environmental gradients, referred to as ecological speciation (Nosil 2012), plays an important role in population differentiation and speciation in a variety of ecosystems (Nosil 2012, Shafer & Wolf 2013) and is likely to be equally important in the deep sea. Environmental gradients have been suggested to drive depth-related divergence in the deep sea (e.g. France & Kocher 1996, Etter et al. 2005, Jennings et al. 2013, Quattrini et al. 2013, Glazier & Etter 2014), particularly those that attend changes in depth such as temperature, particulate organic carbon (POC) flux, hydrostatic pressure, and oxygen availability. Evidence that genetic divergence is frequently associated with depth is increasing, and suggests depth-related environmental gradients might play a fundamental role in the evolution of the deep-sea fauna.

Cryptic species in the deep sea

The 2 clades of *X. washingtona* reported here represent the first documentation of genetically distinct evolutionary lineages associated with an OMZ. The low support for species delimitations in the bPTP analysis and the large confidence intervals of potential species in the GMYC analysis likely reflects the use of a single locus. Since the 16S locus alone does not contain enough information to confidently delimit cryptic species, more independent loci will be necessary to determine if the 2 clades have diverged sufficiently to warrant species status (Pante et al. 2015a,b).

Visual inspection of individuals from the 2 clades did not reveal any clear morphological divergence. *X. washingtona* was originally described in 1923 based on morphological characters. Turner's (2002) higher grouping system was based on morphological characters, namely the mesoplax, siphon, and cirri morphology, and placed the *Xylophaga* species into 6 groups. Group 5 is the '*X. washingtona*' group, which originally consisted of 9 morphologically similar species. Voight (2007) and Voight (2009) described 1 and 2 new species respectively that share morphological characteristics with this group. The splitting of *X. washingtona* into 2 cryptic species would add a 13th species.

The discovery of morphologically indistinguishable, yet genetically distinct, lineages such as these has become common in the deep sea with the increased use of genetic analyses. Cryptic species and species complexes have been associated with environmental gradients that attend changes in depth (Brandão et al. 2010, O'Loughlin et al. 2011, Jennings et al. 2013, Glazier & Etter 2014), geography (Brandão et al. 2010), differential reproductive timing (Brandão et al. 2010), currents (O'Loughlin et al. 2011), historical refugia (O'Loughlin et al. 2011, Hemery et al. 2012), and environmental heterogeneity (Knox et al. 2012). Sympatric cryptic species have been suggested in amphipods (Baird et al. 2011), assellote isopods (Raupach & Wägele 2006, Raupach et al. 2007), brittle stars (Hunter & Halanych 2008), and a crinoid (Hemery et al. 2012) and have been associated with niche separation (Baird et al. 2011), competitive exclusion (Baird et al. 2011), prey specialization (Baird et al. 2011), and historical refugia followed by secondary contact (Hemery et al. 2012). The strong prevalence of morphologically similar yet genetically distinct lineages suggests deep-sea biodiversity has been underestimated and geographic and bathymetric distributions overestimated.

CONCLUSIONS

We found clear genetic divergence in 16S rRNA between 2 clades of *X. washingtona* arrayed along a depth gradient in the eastern North Pacific. One clade is distributed throughout the OMZ while the other is restricted to depths within and below. Many possible mechanisms might account for the observed divergence, including the contemporary effects of hypoxia on developing larvae or a more intense OMZ in the remote past that created isolated refugia that promoted divergence. Our results suggest the OMZ in the northeast Pacific might limit species' ranges and gene flow in the deep sea and might play an important role in the evolution of the deep-water fauna contributing to the high biodiversity on continental margins. As oceanic oxygen concentrations decrease with global climate change, and hypoxic and anoxic regions increase in number and size (Diaz & Rosenberg 2008, Keeling et al. 2010, Helm et al. 2011), gene flow in other regions and depths might become inhibited in the same way, impacting ecological and evolutionary processes as well as population differentiation and species formation.

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