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Extreme mitochondrial DNA divergence within populations of the deep-sea gastropod *Frigidoalvania brychia*

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Abstract The deep sea supports a diverse and highly endemic invertebrate fauna, the origin of which remains obscure. Little is known about geographic variation in deep-sea organisms or the evolutionary processes that promote population-level differentiation and eventual speciation. Sequence variation at the 16 S rDNA locus was examined in formalin-preserved specimens of the common upper bathyal rissoid *Frigidoalvania brychia* (Verrill, 1884) to examine its population genetic structure. The specimens came from trawl samples taken over 30 years ago at depths of 457–1,102 m at stations in the Northwest Atlantic south of Woods Hole, Massachusetts, USA. Near the upper boundary of its bathymetric range (500 m), extremely divergent haplotypes comprising three phylogenetically distinct clades (average uncorrected sequence divergence among clades ~23%, ~3% within clades) were found at stations separated by a maximum distance of ~80 km, suggesting the presence of high levels of intraspecific divergence or the possibility of morphologically cryptic species. Only one of these clades was found at two stations in the mid- to lower part of *F. brychia*'s depth distribution (800–1,100 m), suggesting lower clade diversity with increasing depth, although among-sample divergence, with a single exception, was minimal. One station was genetically divergent from all others sampled, containing a unique suite of haplotypes including two found only at this site. Steep vertical selective gradients, major oceanographic changes during the late Cenozoic, and habitat fragmentation by submarine canyons might have contributed to an upper bathyal region that is highly conducive to evolutionary change.

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Introduction

During the last several decades, much has been learned about the interactions of mutation, selection, migration and random genetic drift, and their impacts on population-level differentiation (e.g. Hartl and Clark 1997; Li 1997). Genetic structure is the most basic information for documenting the degree of population-level divergence and inferring its cause(s). The evolutionary processes of population differentiation, speciation, adaptive radiation, and the geographic spread of taxa can be reconstructed by associating divergence patterns with spatial and/or temporal changes in the environment (Avise 2000).

The deep sea is the largest and most recently explored environment on Earth. Long thought to be unfavorable to life, sampling has instead revealed a rich, endemic invertebrate fauna (Hessler and Sanders 1967; Sanders 1968; Smith et al. 1998). While patterns of biodiversity at the species level have been documented from local to global scales (Rex et al. 1997, 2000), research on the genetic foundation of this biodiversity is just beginning. Genetic population structure is known for only a few invertebrate species from soft-sediment habitats (e.g. France and Kocher 1996a; Chase et al. 1998a; Creasey and Rogers 1999), largely through the development of techniques to extract and sequence DNA from formalin-fixed archived material (France and Kocher 1996b; Chase et al. 1998b). With little comparative genetic data for deep-sea organisms, the mechanisms of population differentiation and the potential nature and role of geographic isolating barriers in the deep-sea ecosystem remain speculative.

In the present study we assessed the genetic population structure of the upper bathyal snail *Frigidoalvania*

brychia, by using sequence variation in mitochondrial 16 S rDNA isolated from formalin-preserved specimens. Mitochondrial 16 S rDNA has been shown to be sufficiently polymorphic for similar studies within deep-sea taxa (e.g. Chase et al. 1998a; Etter et al. 1999). *F. brychia* is one of four rissoid species that dominate the upper-slope snail fauna and is the most abundant gastropod at upper bathyal depths in the western North Atlantic, south of New England (Rex and Warén 1982). The continental margin in the region is topographically complex with the slope face cut deeply by submarine canyons. Canyons are formed by a combination of erosional and depositional processes that were particularly active during Pleistocene and Pliocene glaciations, and continue today. Geographic variation in shell form can be extensive within *F. brychia* (Rex et al. 1988). At the upper reaches of its depth range (about 500 m) little variation in shell morphology is apparent; whereas, in the deepest part of its range (about 1,100 m), shell variation is greater, but still appears to be continuous. *F. brychia* increases in size with depth, which has been interpreted to reflect strong depth-correlated selective gradients in the upper bathyal zone (Rex and Etter 1998). With lecithotrophic larval development in an egg capsule and a very short or nonexistent planktonic phase (Warén 1974), there is seemingly little potential for dispersal and gene flow in *F. brychia*.

Etter and Rex (1990) have proposed the depth-differentiation model, suggesting that the upper bathyal zone is particularly conducive to evolutionary diversification in the deep sea. *F. brychia*, like most upper bathyal snails, occupies a restricted depth range that extends as a narrow ribbon along the North American continental margin (Rex 1977; Rex et al. 1988). During glacial–interglacial cycles, its vertical range might have expanded, shifted deeper with the descent of deep-sea conditions that attended lower sea level, or contracted in response to the combined circumstances of lower sea level and shoaling of deep currents. We examined within- and among-sample genetic variation in *F. brychia* along a depth gradient and evaluated its consistency in light of these potential patterns.

Materials and methods

Molecular methods

Samples of *Frigidoalvania brychia* (Verill, 1889) came from five epibenthic sled (Hessler and Sanders 1967) samples taken from depths of 457–1,102 m in the western North Atlantic, south of Woods Hole, Massachusetts (Table 1; Fig. 1). Total DNA was extracted from individuals as described by Chase et al. (1998b). PCR amplifications were in 50-µl reaction volumes consisting of 10 µl template, 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100, 2.5 mM $MgCl_2$, 0.2 mM each dNTP, and 25 pmol each forward and reverse primer. Reactions were heated to 95°C for 5 min prior to the addition of 2.5 U of *Taq* DNA polymerase and then cycled 40 times (conditions: 95°C for 1 min, 50°C for 1 min, and 72°C for 1 min).

Primer design for the 16 S rDNA fragments generated in this study followed the general methods of Chase et al. (1998a). A forward primer (mollusk 16F; 5′-RRG CTK GWA TGA ATG GTT TG-3′) was designed by aligning 16 S sequence data from one gastropod and two protobranchs: *Nucella lapillus*, *Nucula proxima*, and *Solemya velum*. Template DNA was amplified with mollusk 16F and primer 16R3 (Chase et al. 1998a) and sequenced. Sequence generated was then used to design the primer Fb16F (5′-AAA TAA ATA TTT AGG TGA AGA AGC-3′). Although the size of the amplification product decreased when Fb16F was paired with 16R3, we found that this primer pair generated more consistent PCR amplifications from archival specimens than amplifications with the mollusk 16F/16R3 pair. These conditions were used to assay 110 museum individuals, 63 of which resulted in PCR product suitable for sequencing. PCR products were sequenced using standard solid phase methods (Salminen 1992) or with a *Taq* dye deoxy termination cycle sequencing kit (Applied Biosystems) and run on an Applied Biosystems model 377 automated DNA sequencer. Both strands were sequenced to confirm mutations in every individual deviating from the most common haplotype. Sequences generated in this study have been submitted to GenBank (accession numbers AY037050–AY037061).

We evaluated the possibility of PCR or fixation (i.e. formalin exposure) artifacts contributing to the observed genetic diversity using several methods. For example, PCR amplifications of DNA extracted from the same tissue source on different days and repeated amplifications using template DNA from a single extraction invariably produced identical sequences. These results strongly suggest that artifacts are not a significant source of sequence variation in this study, consistent with previous studies using archival specimens (e.g. France and Kocher 1996b; Chase et al. 1998b).

Data analyses

Sequences were aligned with ClustalW using the default multiple alignment settings (Thompson et al. 1994; alignment available from the senior author). Taxa with identical sequences were filtered and then combined in MacClade (version 3.05; Maddison and Maddison 1992) to determine the number of haplotypes. Relationships among haplotypes were estimated using the maximum-parsimony algorithms implemented in PAUP* (version 4.0b; Swofford 1999). Heuristic searches were used to find the most parsimonious trees, and ten random additions of the input taxa were used to improve the accuracy of each search. Alignment gaps were treated as missing data, and substitutions were weighted equally regardless of position (i.e. in stem and loop regions; Alves-Gomes et al. 1995). Sensitivity analyses of the data were assessed by performing heuristic searches with transversion:transition ratios weighted from 1:1 to 10:1. Weighting schemes had little effect on the resulting topology (data not shown). Therefore, a weighting scheme of 2:1

Table 1 *Frigidoalvania brychia*. Station number, locality data, and sample size for *F. brychia* collected from the upper slope south of New England

Station	Latitude (N)	Longitude (W)	Depth (m)	Sample size
346	39°54.0′	70°10.7′	457	11
96	39°55.2′	70°39.5′	498	15
105	39°56.6′	71°03.6′	530	16
207	39°51.3′	70°54.3′	808	11
87	39°48.7′	70°40.8′	1,102	10
Total				63

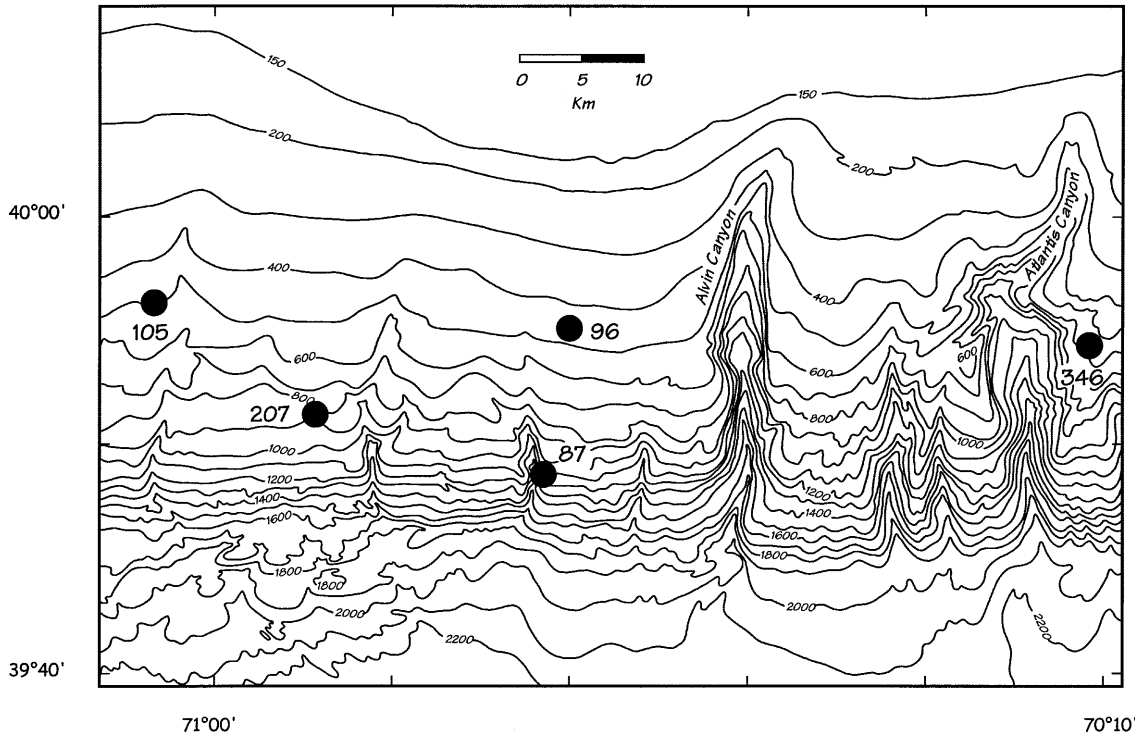

Fig. 1 *Frigidoalvania brychia*. Bathymetry of the continental slope south of New England, showing five sampling stations for *F. brychia* analyzed in this study (after NOAA, National Ocean Survey). Depth contours in meters. See Table 1 for station data

(transversion:transition) was used in all subsequent parsimony analyses.

Neighbor-joining trees were generated with MEGA (Kumar et al. 1993). Jukes–Cantor distances were calculated with the complete-deletion option for gaps and missing data. A partial 16 S sequence from the gastropod *Turritella communis* (Lieberman et al. 1993) was used as an outgroup in all phylogenetic analyses. Bootstrapping (Felsenstein 1985) was used to estimate the degree of support for individual groupings. We used the exact test of Raymond and Rousset (1995) as implemented in Arlequin (version 1.1; Schneider et al. 1997) to test the null hypothesis that observed haplotype distribution is random with respect to sampling location. The significance of individual tests was estimated by comparison to simulated distributions constructed from 1,000 random permutations of the original data matrix.

Results

We sequenced 136 aligned positions (including insertions/deletions) of the mitochondrial 16 S locus from 63 of 110 individual *Frigidoalvania brychia*. However, the rate of successful amplification from formalin-fixed tissues varied among stations sampled: successful amplifications were observed in specimens sampled from stations 346, 96, and 87 approximately 50% of the time, while about 80% of the extractions produced amplification products at the two intermediate depth stations (105 and 207). Of 136 base pairs (bp), 56 nucleotide positions were variable, and 51 base positions were phylogenetically informative. Base substitutions at the 56 variable positions defined 12 unique haplotypes (Table 2). We estimated the relationship among the 12 haplotypes using heuristic searches under the parsimony criterion, and recovered two trees of 151 steps, which differed only in the placement of haplotype E within clade I (Fig. 2). Neighbor-joining analyses produced concordant topologies regardless of the distance measure employed (data not shown). Both phylogenetic methods revealed evidence for three distinct haplotypic clades (clades I, II, and III in Fig. 2). Monophyly of each group was highly supported by bootstrapping, although relationships among clades were not significantly resolved. Average pairwise divergence within clades was minimal (uncorrected divergence ~3%) when compared to average divergence among the three groups (~23%).

Representative haplotypes from each of the three major clades were not randomly distributed with respect to sampling location (depth). Haplotypes in clades II and III only occurred in samples from the three shallower stations (< 530 m) and were relatively rare among the 63 individuals (3% and 12%, respectively, Table 2). In contrast, clade I comprised the greatest haplotype diversity, was found in higher frequencies among the individuals sampled, and occurred in all samples regardless of depth (Table 2).

Because of the distinctive distributions and large number of mutational steps between clades I, II, and III, we did not pool all observed haplotypes from each sampling location for population-level analyses. Instead, only clade I had sufficient individuals ($n = 53$) and a wide enough depth distribution to permit tests of population differentiation across the depth gradient. Estimates of gene diversity (h), a measure of within-population haplotype variation (Nei 1987), were similar across all five stations (Table 2). However, the results of an exact

Table 2 *Frigidoalvania brychia*. Mitochondrial 16 S rDNA diversity in the deep-sea rissoid. See Fig. 1 for sampling stations. Shown are variant bases relative to the most common haplotype (E) observed; *h* (gene diversity) and its standard error shown by station

Haplotype 10 20 30 40 50		Station					Total
		346	96	105	207	87	
E AATTAATTAACTCT-TGCTTCTGAATGCATTTTTC-TAG ACTATCATGCGACTTTA		4	6	10	8	6	34
A .....A-.A.....-.....G.A.....		5	2	0	3	4	14
B .....G...A-.A.....-.....G.A.....		0	1	0	0	0	1
C .....A-.A.....-.....A.....C.		0	1	0	0	0	1
D .....A-.A.....-.....A.....		0	0	2	0	0	2
F .....A-.A.....-.....A.C.....		0	1	0	0	0	1
G .TA.GT..C.T.AAA.C..GGGAGG..TTGGA.AAA..A.T..AGG.T.ATTA..C		1	0	0	0	0	1
H .TA.GT..C.T.AAA.C..GGGAGG..TTGGA.AAA..A.T..AGG.T.ATTA...		0	1	0	0	0	1
I G..AC.CAT..G-CAGTAA....T.CA...A.G.ATAGATTA.AAGAT..C..C..		0	2	0	0	0	2
J G..AC.CAT..G-CAGTAA....T.CA...A.G.ATAGATTA.AAGATT.C..C..		0	1	0	0	0	1
K G..AC.CAT..G-CAGTAA....T..A...A.G.ATAGATTA.AAGAT..C..C..		0	1	0	0	0	1
L G..AC.CAT..AGCCGTTA....G..A...A.A.ACA.ATTA.AA.AT..T.....		1	0	3	0	0	4
	h	0.56	0.64	0.41	0.44	0.53	
	SE	0.09	0.15	0.15	0.13	0.09	

test for population differentiation using only clade I individuals revealed significant heterogeneity in the distribution of haplotypes across samples ($P < 0.001$). Pairwise tests for differentiation among samples revealed that significant values occurred in comparisons involving station 105 and all other stations sampled (all $P < 0.001$); no other pairwise comparison was significant (all $P > 0.32$). Inspection of Table 2 reveals that haplotypes D and F were unique to station 105 and that haplotype A, a commonly observed variant at all other stations, was absent from this sample.

Discussion

We used genetic analyses to address questions of differentiation in *Frigidoalvania brychia* across a depth gradient. Our results revealed a surprising level of intraspecific variation within and among samples taken from around 500 to 1,100 m. At the upper end of its bathymetric range, *F. brychia* comprises several morphologically cryptic but genetically distinct clades. However, the distribution of these clades was decidedly nonrandom with respect to depth. Two clades (clades II and III) were relatively rare and were restricted to depths < 530 m, while one clade (clade I) was relatively common (50% of individuals) and found throughout the depth gradient sampled.

Whether these genetically distinct clades represent morphologically cryptic species of *F. brychia* is difficult to ascertain with the present data (Etter et al. 1999). Although the average genetic divergence among clades was approximately an order of magnitude greater than divergence within any individual clade, biometric variation within *F. brychia* was minimal at < 530 m where all three genetic clades were found. Thus, there is no discernible morphological evidence for species-level divergence at these shallow stations (Rex et al. 1988). Large-scale mtDNA divergences either within and/or among populations of marine and terrestrial taxa are not uncommon (e.g. Burton and Lee 1994; Foltz et al. 1996; Frati et al. 1997; Munstermann and Conn 1997; Schizas et al. 1999), particularly within other deep-sea "species" (Bucklin and Wiebe 1987; Vrijenhoek et al. 1994; Craddock et al. 1995; France and Kocher 1996a; Chase et al. 1998a; Etter et al. 1999). Although many of these extreme divergences have been attributed to the presence of cryptic species, other explanations including rapid mutation rates, selection on certain mtDNA haplotypes,

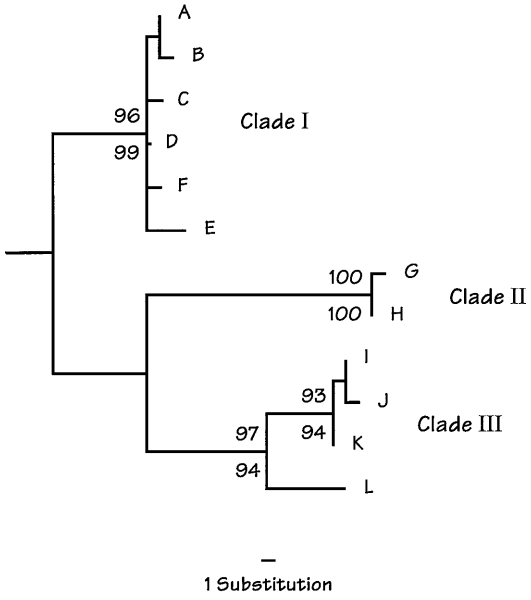

Fig. 2 *Frigidoalvania brychia*. Phylogeny of 12 haplotypes of the mitochondrial 16 S ribosomal gene in five populations collected from the upper continental slope south of New England. *Numbers* show bootstrap support for specific nodes (percentage of 100 replicates): maximum-parsimony above each node, neighbor-joining below. For clarity only nodes supported by > 90% of 100 bootstrap replicates are shown. Phylogeny was rooted with an orthologous sequence from *Turritella communis* (not shown). See Table 2 for haplotype distributions within and among samples

and retention of ancestrally related polymorphisms might also explain the suite of divergent haplotypes detected in *F. brychia*. It is possible that an examination of other independent, unlinked genetic markers, in particular nuclear loci, might help differentiate among these hypotheses (e.g. Grady and Quattro 1999).

Alternatively, the three divergent clades might represent large-scale sequence divergence within populations of a single species. Extreme intraspecific variation like that observed in *F. brychia* has been observed within other species, particularly among populations of the tidepool copepod *Tigriopus californicus* (Burton and Lee 1994). Of particular relevance to our studies, extremely divergent mtDNA haplotypes have been sampled within populations of a pulmonate land snail, *Cepaea nemoralis* (Thomaz et al. 1996), and the deep-sea protobranch *Deminucula atacellana* (Chase et al. 1998a). Thomaz et al. (1996) argue that the population structure of *C. nemoralis* favors the retention of ancestral genotypes, leading to semi-isolated populations that harbor highly divergent, presumably ancient mtDNA haplotypes. Whether similar mechanisms promote the retention of ancestral mtDNA variants within *F. brychia* is at present difficult and perhaps impossible to evaluate without detailed knowledge of the population-level dynamics of this deep-sea taxon.

The question of cryptic species versus the retention of ancestral polymorphisms notwithstanding, we were most interested in exploring genetic divergence within *F. brychia* along a depth gradient. Population-level analyses were restricted to those individuals from clade I, since individuals harboring clade I haplotypes occurred in all samples regardless of depth. Estimates of within-sample haplotype diversity were very similar across all five stations, providing no evidence for an association between depth and genetic diversity like that observed for phenotypic characters. In contrast, we detected significant heterogeneity in the distribution of haplotypes across stations. Pairwise tests for differentiation among samples revealed that this heterogeneity stems from comparisons involving station 105 (530 m). No other pairwise comparison was significant. Station 105 contained a relatively unique suite of haplotypes, including two found only at this site. Further, haplotype A, which was found at all other sample stations, regardless of depth, was not detected at this station.

We are presently unable to identify the population-level forces responsible for significant genetic divergence involving comparisons between station 105 and all other sampling locations. Altered gene flow across large geographical distances has been implicated in genetic discontinuities in many systems (e.g. Avise 1994). However, in this case the geographic distance between station 105 and other sampling stations is no more extreme than that between pairs of samples from other stations; though station 105 is located at the western extremity of the region surveyed. Similarly, we can detect no obvious association between depth and genetic divergence; station 105 is very similar in depth to stations 96 and 346. However, the power of any measure of association applied to the current data is low given the small number of populations sampled and lack of statistically significant estimates of genetic divergence between populations.

Etter and Rex (1990) suggested that the upper bathyal region of the western North Atlantic is particularly conducive to population differentiation. The present genetic data lend some support to this contention, primarily since upper bathyal samples of *F. brychia* include two genetically distinct clades that were not sampled at deeper stations (and thus, given reservations regarding the number of individuals surveyed per site, do not exist or are more rare in deeper stations). Also, our population-level data indicate some measure of among-population differentiation involving a single sample collected near the upper extreme of *F. brychia*'s depth range. However, lack of any other evidence for significant between-station differentiation precludes any firm conclusions regarding an association of genetic divergence with depth in this species.

It is difficult to explain the genetic heterogeneity in *F. brychia*, but the recent revolution in paleoclimatic and paleoceanographic research indicates that the upper bathyal region imposes the high spatio-temporal environmental variability and habitat fragmentation that generally promote population differentiation. While the current population divergence observed in *F. brychia* does not correspond in any obvious way with known habitat fragmentation, it is possible that genetic variability and divergence patterns might have built up over a long period of environmental change during which populations separated and coalesced in complex and dynamic ways. Glaciation during the Pliocene and particularly the Pleistocene must have strongly impacted the upper bathyal environment. Indeed, data from deep seabed cores show that benthic foraminiferans (Kurihara and Kennett 1988) and ostracods (Cronin and Raymo 1997) experienced bathymetric distributional changes during glacial cycles, but no record is available for macrofaunal elements not preserved in fossil sediments like *F. brychia*. At the five glacial maxima of the last 500,000 years, sea level was 120–140 m lower than today (Rohling et al. 1998) and would have eliminated much of the extensive and productive environment of the adjacent continental shelf and displaced deep-sea conditions downward on the continental slope. The deep thermohaline circulation also weakened and shoaled during glacial maxima (Adkins et al. 1997; Raymo et al. 1998; Weaver et al. 1998).

Recent evidence suggests that within the 100,000-year-long cycles of major glaciation there is extreme global climate instability on millennial time scales, and that the associated climatic transitions are abrupt, lasting only decades (Severinghaus et al. 1998). These short-term oscillations caused reorganization of the deep circulation that is reflected in changes in the species composition of foraminiferal assemblages (Cannariato

et al. 1999), and changes in isotopic ratios of deep-sea coral skeletons (Smith et al. 1997) and foraminiferan tests (Marchitto et al. 1998; Oppo et al. 1998; McManus et al. 1999). Thus, it is likely that upper bathyal communities experienced environmental fluctuations on decadal to glacial (10^4 years) time scales during the last several million years of the Pliocene and Pleistocene. Potentially, this would have meant exposure to very different physical factors and biotic interactions as bathymetric distributional ranges were displaced. Population distribution would have expanded and contracted with the depth of occupancy and variation in surface production associated with the advance and retreat of ice sheets (Slowey and Curry 1992, 1995; Bard et al. 2000). These population and environmental changes, coupled with the isolating effects of canyon formation, may have caused genetic heterogeneity by both selective and non-selective mechanisms. Similar, if less severe, environmental changes affecting deep-sea species may extend back to the mid-Cenozoic (Kaiho 1994; Miller et al. 1996; Zachos et al. 1997; Lear et al. 2000), as a consequence of episodic global cooling.

In conclusion, mitochondrial DNA in the common deep-sea snail *F. brychia* shows high sequence divergence among three distinct genetic clades across small spatial scales near the upper reaches of its bathymetric range. Genetic variation attenuates with depth to a single clade at 1,100 m; clade diversity appears inversely related to phenotypic variation in shell architecture. The coexistence of three distinct genetic clades in morphologically coherent populations at 500 m indicates a high reservoir of genetic "biodiversity", with the possibility of cryptic species. The fragmentation and dynamic history of the upper bathyal zone might make it an important site of genetic differentiation in deep-sea organisms. While evidence from just five populations of a single species should be interpreted with caution, results are consistent with the view that the upper bathyal zone of the western North Atlantic is an active site of population differentiation in deep-sea mollusks.

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