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Testing the depth-differentiation hypothesis in a deepwater octocoral

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The depth-differentiation hypothesis proposes that the bathyal region is a source of genetic diversity and an area where there is a high rate of species formation. Genetic differentiation should thus occur over relatively small vertical distances, particularly along the upper continental slope (200–1000 m) where oceanography varies greatly over small differences in depth. To test whether genetic differentiation within deepwater octocorals is greater over vertical rather than geographical distances, *Callogorgia delta* was targeted. This species commonly occurs throughout the northern Gulf of Mexico at depths ranging from 400 to 900 m. We found significant genetic differentiation ($F_{ST} = 0.042$) across seven sites spanning 400 km of distance and 400 m of depth. A pattern of isolation by depth emerged, but geographical distance between sites may further limit gene flow. Water mass boundaries may serve to isolate populations across depth; however, adaptive divergence with depth is also a possible scenario. Microsatellite markers also revealed significant genetic differentiation ($F_{ST} = 0.434$) between *C. delta* and a closely related species, *Callogorgia americana*, demonstrating the utility of microsatellites in species delimitation of octocorals. Results provided support for the depth-differentiation hypothesis, strengthening the notion that factors covarying with depth serve as isolation mechanisms in deep-sea populations.

1. Introduction

The depth-differentiation hypothesis proposes that the majority of genetic differentiation and biodiversity in the deep sea is generated across the relatively narrow continental slope [1]. Not only do water mass characteristics, pressure and food supply change rapidly with increasing depth across the continental slope, but this region can also exhibit topographic complexity (e.g. submarine canyons, bioherm formation, authigenic carbonates), experience rapid fluctuations in current patterns, be subjected to low levels of dissolved oxygen, and contain high levels of hydrocarbon seepage [2]. Strong environmental gradients with depth coupled with high habitat heterogeneity [3,4] in the bathyal region can create different selective regimes, thus potentially promoting adaptive divergence in deep-sea species.

Several studies have demonstrated high levels of genetic differentiation across relatively small depth ranges in the deep sea, particularly across depth horizons of 1000 and 3000 m [5–9]. Population divergence over relatively narrow bathymetric gradients may be due to either historical patterns in colonization or the environmental changes (e.g. pressure, temperature and dissolved oxygen) that occur with changes in depth [6,7,9]. Few studies, however, have addressed the depth-differentiation hypothesis on species inhabiting the upper continental slope (200–1000 m), yet this region is where environmental changes occur most rapidly. Furthermore, this bathymetric zone may be a source of genetic diversity to down slope areas [1,10] leading to the formation of deeper-occurring populations and species. Thus, it could be expected that isolation by depth and increased genetic diversity would occur over relatively small vertical distances. In contrast to
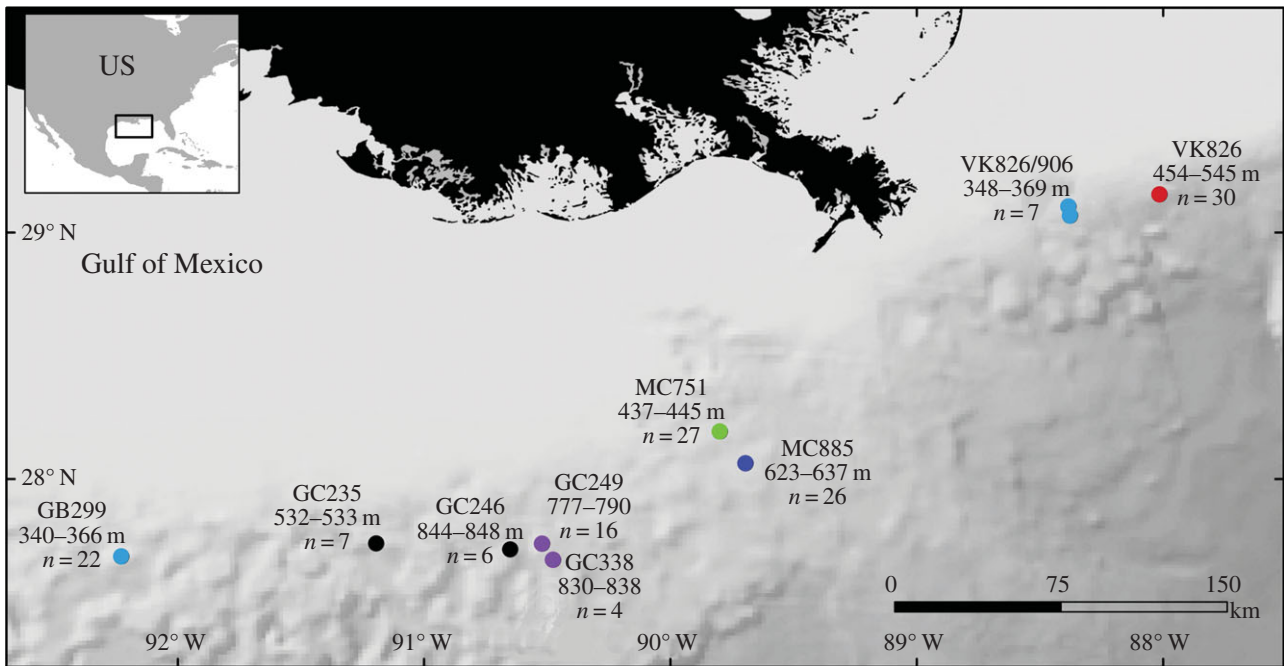
Figure 1. Map of sampling locations of *C. americana* (GB299, VK862/906) and *C. delta* (all other sites). Colours denote clusters identified by STRUCTURE (figure 2). Number of samples (*n*) and depth range of collections are noted. (Online version in colour.)

population differentiation with depth, numerous examples have demonstrated high connectivity across expansive geographical distances (hundreds to thousands of kilometres) in the deep sea [11,12].

Deep-sea corals (including scleractinians, antipatharians and octocorals) occur along the continental slope in a diversity of marine habitats worldwide. These foundation species create habitat for numerous other species and are long-lived and slow-growing [13,14]. Most species reproduce sexually either by broadcast spawning or brooding [15]. Brooding of larvae can occur internally within the polyps or externally on the surface of the colonies [16,17]. Deep-sea corals are susceptible to anthropogenic disturbances, including fishing, hydrocarbon extraction and mining [18,19]. Their prevalence and importance in creating habitat on the continental slope necessitate a better understanding of the level of connectivity among populations occurring across depth and geographical boundaries.

Previous studies have examined population differentiation in mesophotic corals (50–200 m) on the continental shelf [20] and in deep-sea (more than 200 m) corals on the continental slope [21] and seamounts [22]. These studies have indicated different degrees of population connectivity, which can be related to differences in both the reproductive biology and the specific region and depth of sampling of the coral species. Populations of the scleractinian coral *Lophelia pertusa*, a broadcast spawner, were differentiated across regions (e.g. Gulf of Mexico (GoM), southeastern US and northeastern Atlantic), while populations were panmictic in similar depth ranges within each region [21]. In comparison, *Oculina varicosa*, although also a broadcast spawner, exhibited pronounced population divergence across the continental shelf off Florida at a depth of 50 m [20].

In the northern GoM, at least 162 species of octocorals contribute to habitat heterogeneity across the continental slope to depths of approximately 3000 m. In particular, species within the genus *Callogorgia* (Family Primnoidae) are the most abundant octocorals inhabiting the upper continental slope in this

region [23]. Strong niche segregation with depth occurs within the genus, with three *Callogorgia* spp. [*Callogorgia delta* Cairns & Bayer 2002, *Callogorgia americana* Cairns & Bayer 2002, *Callogorgia gracilis* (Milne Edwards & Haime 1857)] occupying distinct depth zones. Thus depth (and the cofactors that vary with depth) significantly influences the evolution and ecology of these deep-sea octocorals [23].

Callogorgia delta commonly occurs in the northern GoM at depths of 400–900 m, providing an ideal system in which to examine the depth-differentiation hypothesis at a finer scale within a species inhabiting the bathyal region. If the bathyal region is a source of increased genetic differentiation in the deep sea, then it could be expected that genetic differentiation within *C. delta* exists on the upper continental slope and is greater over vertical rather than geographical distances. This study also provided the opportunity to examine the level of genetic differentiation between *C. delta* and *C. americana*; two species only recently elevated to species status [24]. Closely related octocorals can be difficult to identify visually, and inclusion of samples from several species in a population genetic dataset can lead to erroneous inferences of population connectivity [25]. We thus tested whether the here developed microsatellite markers could distinguish between *C. delta* and *C. americana*, yielding a molecular method for delimiting *Callogorgia* species.

2. Material and methods

(a) Field methods

In the GoM, 116 specimens of *C. delta* and 29 specimens of *C. americana* were collected across nine sites at depths between 340 and 848 m using remotely operated vehicles (i.e. ROVs *Jason II*, *SeaEye Falcon*) and the human-occupied vehicle (HOV) *Johnson-Sea-Link* during five cruises from 2008 to 2010 (figure 1). Surveyed sites were named in accordance with the GoM planning areas and lease blocks (managed by the Bureau of Ocean Energy Management) in which they occur: e.g. Viosca Knoll (VK) 826 and 862/906, Mississippi Canyon (MC) 751,
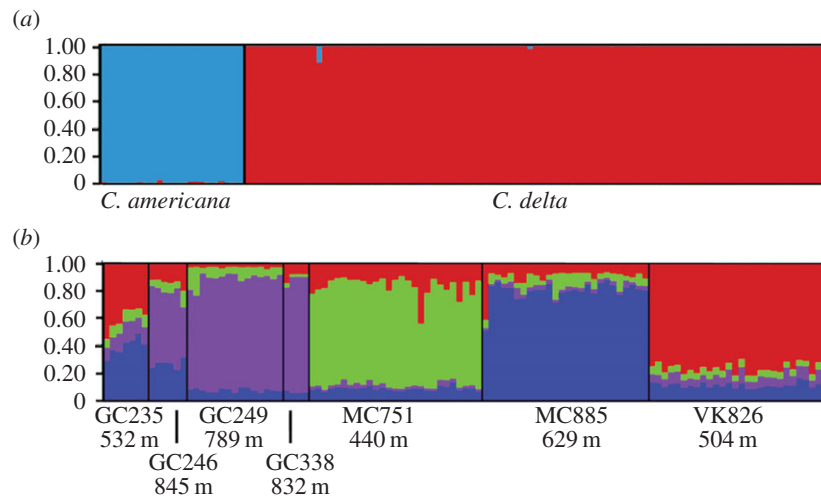
Figure 2. Average probability of membership graphs (STRUCTURE) for (*a*) *Callogorgia* spp. (*n* = 141, *K* = 2) and (*b*) *C. delta* (*n* = 113, *K* = 4). Mean depth of collections for each site is included. (Online version in colour.)

and 885, Green Canyon (GC) 235, 246, 249, 338 and Garden Banks (GB) 299 (figure 1).

Photographs were taken of each coral colony prior to sampling, and branches were subsampled from each colony using the ROV manipulator arm. On the research vessel, branches were divided into 2–3 cm sections and tissue subsamples were subsequently frozen at −80°C, preserved in 95% ETOH (stored at −20°C) and placed in a high-salt EDTA preservative (stored at −80°C). Voucher specimens of each individual colony were preserved in 95% ETOH or dried. Morphological characters and DNA barcodes were used to identify species [23]. A subset of representative voucher specimens were accessioned at the National Museum of Natural History of the Smithsonian Institution, Washington DC, USA (USNM1202708–1202713).

(b) Molecular methods

DNA was extracted using a Qiagen DNeasy kit. Genomic DNA from two individuals of *C. delta* was sequenced on a half plate on Roche-454 (Engencore, Colombia, SC, USA) following [26]. Roche 454 reads (electronic supplementary material, table S1) were input into the software program QDD v. 2 [27], enabling microsatellite selection and subsequent primer design (see the electronic supplementary material). Thirty-nine primer pairs predicted to produce products more than 120 bp were then tested for amplification across *C. delta* and *C. americana* collected from different sites. Of the 39 primer pairs tested, 10 amplified in both species across all sites in the GoM (the electronic supplementary material, table S2). PCR products were analysed on an ABI 3130XL Genetic Analyzer (University of Pennsylvania) with a Gene Scan 500LIZ size standard.

(c) Microsatellite analyses

The quality and applicability of microsatellite markers were assessed using a variety of analyses. First, fragments were sized using the microsatellite plug-in for GENEIOUS v. 6 (created by Biomatters, http://www.geneious.com/) with Gene Scan 500LIZ size standards (Applied Biosystems, Inc.). MICROCHECKER v. 2.2 [28] was used to check for genotyping errors across all individuals. INEST v. 2 [29] was used to check for null alleles while taking into account the possibility of inbreeding within a population. INEST was run using both 'nfb' (accounting for null alleles, inbreeding and genotyping errors) and 'nb '(null alleles and genotyping errors) models on the entire dataset and for each population (ngen = 500 000, burnin = 5000). The deviance information criterion (DIC) was used to determine which model performed better, and thus whether inbreeding was significant in the

populations. Linkage disequilibrium was tested using Fisher's exact test (GENEPOP on the Web) [30] followed by a Bonferroni adjustment among all pairs of loci across all populations to determine whether the loci were non-randomly associated with one another. Departures from Hardy–Weinberg equilibrium and observed and expected heterozygosity at each locus and among populations were tested (GENALEX v. 6.5) [31]. The statistical power of the microsatellite data was assessed for each species using POWSIM v. 4.1 [32]. Because of the low statistical power for *C. americana* (see the electronic supplementary material), this species was not used to test the depth-differentiation hypothesis.

We searched for duplicate genotypes among the samples (GENALEX). Duplicate multi-locus genotypes (MLGs) were subsequently removed from the dataset for further analyses (five total matching genotypes). Probability of identity (*PI* and *PI*sibs) was calculated to determine the probability of multi-locus genotypes matching at random with increasing numbers of loci (GENALEX).

We also searched for outlier loci potentially under selection in *C. delta* using two programs (LOSITAN [33,34] and BAYESCAN [35]), following [36]. LOSITAN implements an F_{ST} outlier test. Simulations were run under infinite allele and stepwise mutation models (SMMs) for 10 000 replications. The Bayesian program BAYESCAN, based on the multinomial Dirichlet model, calculates differences in allele frequencies in each subpopulation from a common migrant gene pool. Default parameters were used and acceptance rates were between 0.25 and 0.45.

STRUCTURE v. 2.3 [37], a Bayesian model-based clustering approach, was used to determine the number of populations (designated by *K*) both within *C. delta* and between *C. delta* and *C. americana* by assigning the probability of membership of individuals iteratively to each *K*. For *C. delta*, model priors included location information [38], an admixture model (i.e. individuals having mixed ancestry) and correlated allele frequencies [39]. The location prior does not bias detecting structure when no actual structure is present, but values of $r \leq 1$ (electronic supplementary material, figure S1) signify that the locations are informative to population structure [38]. A total of 1 000 000 MCMC generations were run following a burn-in of 250 000 generations. Five independent chains were run to test each value of *K* (*K* = 1–8 for *C. delta*). The level of genetic admixture between *C. delta* and *C. americana* was examined using STRUCTURE with parameters as above without a location prior and *K* = 1–3. STRUCTURE HARVESTER v. 0.6 [40] was used to choose *K* with the delta *K* criterion [41]. For the chosen *K*, results from each of the five iterations were aligned using CLUMPP v. 1.1 [42] and plotted in DISTRUCT v. 1.1 [43].

To examine the amount of genetic differentiation among populations, F_{ST} [44] was calculated between sites and/or species (GENALEX). Based on the STRUCTURE results for *C. delta*, two sites (GC338 and GC249) located only approximately 5 km apart in similar depths were pooled for these analyses. An analysis of molecular variance (AMOVA) [45] was conducted to test for significance among pairwise F_{ST} values across all sites (GENALEX). Additionally, AMOVA was re-calculated after removing two sites (GC235 and GC246) that had small sample sizes. If AMOVA results were significant for *C. delta*, partial Mantel tests were used to test for significant correlations between F_{ST} [linearized to $F_{ST}/(1 - F_{ST})$] values with vertical distance given geographical distance and with geographical distance given vertical distance (IBD on the Web v. 3.5, 1000 randomizations) [46]. Vertical and geographical distances were log transformed. Partial Mantel tests were also performed without GC235 and GC246. All analyses were repeated for both candidate neutral loci only and candidate selective loci.

BOTTLENECK v. 1.2 [47] was used to determine whether any *C. delta* populations experienced a recent reduction in population size at putative neutral loci. Bottleneck was calculated under three mutation models, including the infinite alleles model (IAM), two-phase model (TPM) and the SMM. A Wilcoxon sign-rank test and the relative distribution of allele frequencies (mode-shift indicator) were used to assess whether any of the populations experienced a recent bottleneck.

3. Results

(a) Microsatellite marker data

Of 39 primer pairs that were tested for amplification across individuals, 10 loci were polymorphic and consistently amplified in either *C. delta* ($n = 116$) or *C. americana* ($n = 29$) (electronic supplementary material, tables S2 and S3). However, CA3 did not amplify well in *C. americana* and CA1 did not amplify well in *C. delta* (electronic supplementary material, tables S2 and S3). No loci were in linkage disequilibrium (all loci, $p > 0.05$, Fisher's exact test). Three of the same MLGs of *C. delta* were found at VK826 and two were found at GC249. Two of the same *C. americana* MLGs were found at GB299. Removing these individuals resulted in 113 *C. delta* and 28 *C. americana* for further analyses. With at least eight loci, the probability of identifying identical multi-locus genotypes at random decreased to 0–0.05% for both species at both probability calculations. Estimates of null allele frequencies ranged from 0 to 5% across all loci (INEST, 'nb' model best for each species; electronic supplementary material, table S4). Except, the null allele estimate was 25% for CA3; however, this locus did not amplify in all individuals. Both BAYESCAN and LOSITAN indicated that two loci (CA3 and CA7) were outliers and thus potentially under selection.

The number of alleles per locus ranged from 3 to 22, and the majority were private alleles within a species (electronic supplementary material, table S2). With the exception of CA3, CA7 and CA10 (14 to 22 alleles per locus), there were few alleles at each of seven loci (three to seven alleles per locus). Including all loci, the mean number of alleles per locus ranged from 5.2 ± 0.84 s.e. in *C. americana* to 7.50 ± 1.85 s.e. in *C. delta*. Without the three loci with the higher number of alleles, the mean number of alleles per locus ranged from 3.86 ± 0.55 s.e. in *C. americana* to 4.14 ± 0.40 s.e. in *C. delta*.

Several loci showed negative fixation (F) indices, suggesting heterozygote excess; however, departures from HWE were not significant in most cases (electronic supplementary material, table S3). Three loci showed significant departures from HWE

within a particular site for *C. delta*: CA3 at MC751 and MC885 (χ^2, $p < 0.005$), CA5 at GC235, MC751 and MC885 (χ^2, $p < 0.005$), and CA9 at MC751 for *C. delta* (χ^2, $p < 0.005$). CA5 and CA9 were in heterozygote excess at the specific sites. Two loci showed significant departures from HWE for *C. americana*: CA2 and CA7 at GB299 (χ^2, $p < 0.005$); however, CA3 only amplified in five individuals collected from GB299 and was thus not used in the analyses.

Power analysis indicated that the probability of detecting genetic differentiation in *C. delta* when $F_{ST} \geq 0.0250$ was 100%. The probability of identifying significant genetic structure when the true $F_{ST} = 0$ was less than 6% in all simulations (electronic supplementary material, table S5).

(b) Genetic differentiation between *Callogorgia delta* and *Callogorgia americana*

STRUCTURE indicated little to no genetic admixture between *C. delta* and *C. americana* at eight loci (CA2, CA4–10). Two populations ($K = 2$) corresponded to the two species (figure 2). In addition, there was high genetic differentiation between the two species ($F_{ST} = 0.434$, $p = 0.001$; AMOVA; electronic supplementary material, table S6) and no evidence of hybridization. The proportion of membership to one of the species clusters for each individual was more than 99%.

(c) Genetic differentiation within *Callogorgia delta*

Genetic differentiation was apparent within *C. delta* sampled from seven sites across the northern GoM using nine loci (CA2–10) (figure 2). The F_{ST} value among populations was significant ($F_{ST} = 0.042$, $p = 0.001$) (AMOVA; electronic supplementary material, table S6). Removing the two sites that had few individuals (GC235 and GC246) from AMOVA analyses still yielded significant overall F_{ST} ($F_{ST} = 0.042$, $p = 0.001$). The overall inbreeding coefficient was low (Avg$Fi = 0.0065$) and not a significant component of the model (DIC = 3493 for the 'nfb' model, DIC = 3491 for the 'nb' model). Inbreeding coefficients were also low for each population (Avg$Fi = 0.011–0.041$) and not a significant component of the model for any population based on DIC (INEST; electronic supplementary material, table S4).

Bayesian clustering analysis (STRUCTURE) with a location prior converged well. Delta K (STRUCTURE Harvester) indicated that the most likely number of population clusters (K) present in the dataset was four (electronic supplementary material, figure S2). Membership in each of these four clusters corresponded well to most of the sites from which individuals were collected: GC249 and GC338, MC751, MC885 and VK826 (electronic supplementary material, table S7). However, at some sites, multiple lineages were represented. A high proportion of individuals from GC235 were assigned to both the VK826 cluster and the MC885 cluster and a high proportion of individuals from GC246 were assigned to both the GC249/338 cluster as well as the MC885 cluster.

Pairwise F_{ST} values among sites harbouring *C. delta* ranged between 0.021 and 0.078 with p-values ranging from 0.002 to 0.126 (AMOVA, table 1). After a Bonferroni correction, F_{ST} values between only a few pairs of sites remained significant ($p < 0.003$, AMOVA, table 1). The strongest differences were observed between MC751 with VK826, MC885 and GC249/338 ($F_{ST} = 0.033–0.053$, $p < 0.003$). Relatively high F_{ST} values were also found between VK826 and

Table 1. Pairwise F_{ST} values for *C. delta* among sites at all loci. F_{ST} values in bold are significant (AMOVA, Bonferroni adjustment, $p \leq 0.003$). *P*-values are indicated above diagonal. $n =$ number of unique multi-locus genotypes used in analyses. Depth range of collected specimens is noted.

	GC235 532–533 m n = 7	GC246 844–848 m n = 6	GC249 777–790 m n = 15	GC338 830–838 m n = 4	GC249/338 777–838 m n = 19	MC751 437–445 m n = 27	MC885 623–637 m n = 26	VK826 454–545 m n = 28
GC235	—	0.109	0.058	0.123	0.081	0.044	0.029	0.126
GC246	0.039	—	0.048	0.257	0.071	0.010	0.073	0.029
GC249	0.038	0.048	—	0.239	—	0.004	0.021	0.001
GC338	0.051	0.026	0.020	—	—	0.016	0.043	0.056
GC249/338	0.035	0.038	—	—	—	0.002	0.007	0.007
MC751	0.038	0.078	0.048	0.078	**0.053**	—	0.002	0.002
MC885	0.041	0.032	0.029	0.057	0.034	**0.033**	—	0.006
VK826	0.021	0.054	**0.056**	0.056	0.052	**0.044**	0.040	—

MC885 ($F_{ST} = 0.040$, $p = 0.004$) and VK826 and GC249/338 ($F_{ST} = 0.052$, $p = 0.007$); however, these results did not remain significant after the Bonferroni adjustment. Greater genetic differentiation was evident at increasing differences in depth, given geographical distance (partial Mantel test, $r = 0.61$, $p = 0.002$, figure 3). By contrast, no significant correlation was found with F_{ST} and geographical distance given vertical distance ($r = 0.21$, $p = 0.22$). Partial Mantel tests were also conducted following the removal of the two sites with low sample sizes (GC235 and GC246). In this analysis, a relatively high, but non-significant ($r = 0.68$, $p = 0.08$) correlation was found with F_{ST} and vertical distance given geographical distance. However, a significant correlation in F_{ST} with geographical distance given vertical distance was also evident ($r = 0.78$, $p = 0.03$).

Because both BAYESCAN and LOSITAN indicated that two loci were outliers and thus potentially under selection, we re-analysed the F_{ST} data using either the two candidate loci under selection or the seven putative neutral loci. Pairwise F_{ST} values at the two candidate loci were much higher ($F_{ST} = 0.012$–0.166) compared with the putative neutral loci ($F_{ST} = 0.000$–0.086) (electronic supplementary material, table S8). After a Bonferroni correction, numerous F_{ST} values between pairs of sites at the two candidate loci were significant ($p < 0.003$, AMOVA), whereas none were significant at the neutral loci ($p > 0.003$, AMOVA). Greater genetic differentiation with increasing vertical distance given geographical distance was evident at the two candidate loci (partial Mantel test, $r = 0.56$, $p = 0.008$, figure 3). No genetic differentiation with geographical distance given vertical distance was evident at these loci ($r = 0.25$, $p = 0.24$). Removing the two sites with small sample sizes revealed a significant correlation of F_{ST} with both vertical distance ($r = 0.97$, $p = 0.04$) and geographical distance ($r = 0.93$, $p = 0.04$). In comparison, no significant genetic divergence with vertical distance given geographical distance ($r = 0.33$, $p = 0.08$) or geographical distance given vertical distance ($r = -0.05$, $p = 0.57$) was evident at the seven neutral loci. Removing the two sites with small sample sizes resulted in no significant correlations of F_{ST} with vertical distance ($r = 0.33$, $p = 0.27$) or geographical distance ($r = -0.11$, $p = 0.72$).

BOTTLENECK results indicated that there was a shifted mode distribution of allele frequencies for *C. delta* at GC235 and GC249/338. In addition, there was significant hetero-zygote excess (Wilcoxon sign-rank test, $p < 0.05$) at both sites calculated under the IAM, TPM and SMM models.

4. Discussion

The hypothesis that there is no genetic differentiation within *C. delta* across the northern GoM can be rejected. Rather, results indicated that there is weak, but significant genetic differentiation across the sites surveyed. Different evolutionary processes could lead to the genetic differentiation observed within *C. delta*, including genetic drift due to a past reduction in population size, limited gene flow among sites and adaptive divergence in the presence of gene flow across a gradient of depth. Regardless of the precise mechanism, depth is an important factor influencing the population structure of *C. delta* across the slope in the northern GoM. A higher degree of genetic differentiation over vertical rather than geographical distance within *C. delta* re-enforces the importance of the environmental factors associated with depth as important abiotic gradients influencing the evolution of deep-sea populations and species.

(a) Utility of microsatellite markers in species delimitation

Determining species boundaries within octocorals has been problematic due to morphological gradations [48], phenotypic plasticity [48], the slow evolutionary rate of mitochondrial genomes [49] and the lack of phylogenetically informative, single copy nuclear loci that can be amplified across the clade [50]. Our microsatellite analyses indicated the utility of these loci in resolving species boundaries of octocorals that have been separated for millions of years. *Callogorgia delta* and *C. americana* were only recently elevated from sub-species to species status [24], with an estimated time since divergence of approximately 19 Ma [23]. Yet, the majority of microsatellite markers consistently amplified across *C. delta* and *C. americana*, resulting in a high F_{ST} value (0.434, $p = 0.001$) between species. Previous studies have also indicated the utility in using similar numbers of microsatellite loci to delimit species of scleractinian corals [51,52].
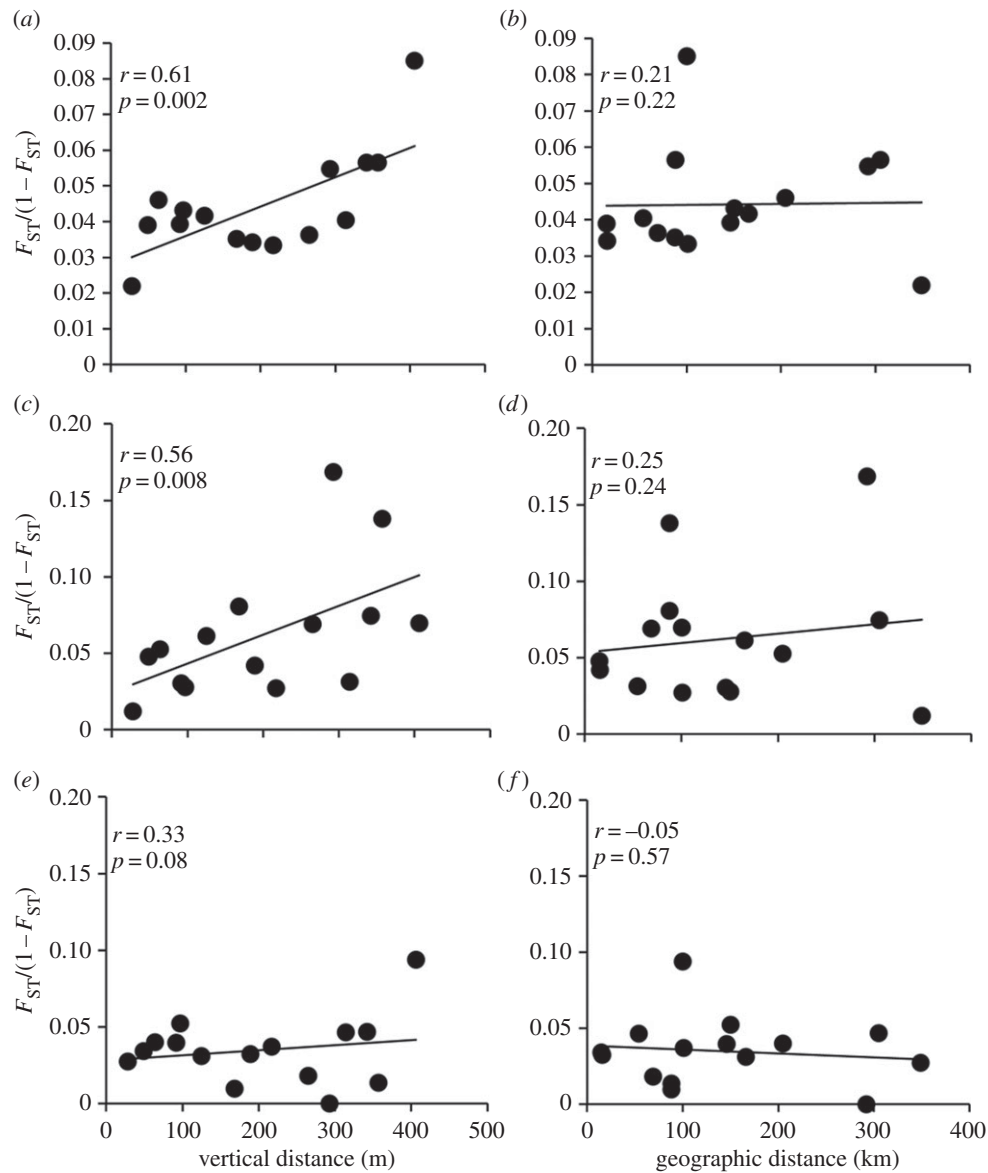
Figure 3. Scatterplots of pairwise F_{ST} for *C. delta* with respect to vertical and geographical distance at (a,b) all nine loci; (c,d) two loci candidates for selection and (e,f) seven putative neutral loci.

(b) Genetic differentiation across the Gulf of Mexico continental slope

The results from the STRUCTURE analyses suggested that genetic differentiation occurs among populations of *C. delta* across 400 m of depth and 400 km of distance in the northern GoM, with population clusters apparent at MC751, VK826, MC885 and GC249/338 (in order from shallowest to deepest). The remaining two sites, GC246 and GC235, contained individuals that were admixed across a few other populations, but this could be indicative of the low sample sizes ($n < 7$) collected from these two sites. This population structure pattern was further supported by F_{ST} results, as numerous pairwise comparisons showed significant differentiation between sites.

Our results also indicated that *C. delta* is more strongly isolated by depth rather than by geographical distance in the GoM. F_{ST} values were significantly correlated with larger differences in vertical rather than horizontal distances. This is further supported by the STRUCTURE analyses and the low and non-significant, F_{ST} value (0.021, $p = 0.126$) between two sites (VK826 and GC235) that were located

400 km apart yet in similar depth ranges. In comparison, MC751 (440 m) and MC885 (629 m) were significantly divergent ($F_{ST} = 0.033$, $p = 0.002$), despite the fact that these two sites were spatially only separated by 15 km. When the sites with small sample sizes were excluded from the analysis, high correlations with both depth and geographical distance were found, suggesting that geographical distance also has a role in isolating populations. These results, however, are confounded by the fact that the deeper site, GC249/338, was also the furthest from VK and MC areas.

We acknowledge that few *C. delta* individuals were sampled in the GC area. Obtaining samples from multiple localities in the deep sea is more difficult than in shallow-water environments [53], particularly as depth of sampling increases. We stress that more samples are needed to corroborate the pattern of isolation by depth in this study. However, if these results hold in the presence of additional samples, then the observed patterns of isolation by depth would strongly indicate that depth is a significant factor shaping populations in the deep sea. Recent studies [8,12] using microsatellite markers to distinguish populations of *Lamellibrachia* tubeworms in the deep (300–2600 m) northern GoM yielded similar results to

those presented in this study. Depth-dependent gene flow was evident in *Lamellibrachia* spp., whereas continuous gene flow was apparent across 650 km in the GoM.

Gene flow in *C. delta* may be limited across the GoM through a number of different mechanisms. If long-distance dispersal via horizontal transport occurs, suitable substrate may not be available within a given depth range. Lack of habitat availability would result in a lack of successful recruitment. Sub-optimal habitat would lead to increased mortality, thus limiting gene flow. Alternatively, shorter larval lifespans along with slow current flow or mesoscale circulations (e.g. eddies) may result in local retention [54], thereby limiting connectivity. Although reproduction has not been studied in the genus *Callogorgia*, species within the family Primnoidae either broadcast spawn (e.g. *Primnoa* spp. [55]) or internally brood their larvae (e.g. *Thouarella, Fannyella*, [17,56]). Most planula larvae of brooding octocorals appear to settle shortly after release [16,56]. Thus, the dispersal distance of brooding species could be reduced if larvae settle close to the colonies, ultimately leading to limited gene flow between sites. If *Callogorgia* also brood larvae, this could contribute to limited larval dispersal among sites. However, if this were the case, we would suspect that either inbreeding would be significant at some sites or that isolation by distance would be evident using the putative neutral loci.

Limited gene flow could also occur across depth in the GoM due to the existence of water mass boundaries. In the GoM, four water masses dominate the continental slope. Sargasso Sea Water is predominant from 200 to 400 m; Tropical Atlantic Water (TAW) dominates depths ranging from 400 to 600 m; Antarctic Intermediate Water (AAIW) dominates depths ranging from 600 to 1000 m; and a mixture of North Atlantic Deep Water and Caribbean Water occurs below 1000 m [57]. These water mass boundaries (particularly across TAW to AAIW) could create a barrier to gene flow within *Callogorgia* by entraining larvae and inhibiting the relatively simplistic planula larvae from physically settling out of the water column. In addition, dispersing larvae may not be able to physiologically tolerate environmental parameters (pressure, temperature and dissolved oxygen) that change at water mass boundaries. Physiological intolerances of echinoderm larvae to temperature and/or pressure have been shown to limit distribution of bathyal species into either shallower or deeper depths depending upon their adult depth ranges [58,59].

Larvae that pass through water mass barriers and successfully form adult colonies may have a different range of physiological tolerances to environmental conditions than their source population. This could lead to selection, and thus adaptive divergence among populations occupying different depths [60]. Pre-reproductive selection could be common in sessile animals, as sessile species are not able to move away from environmental pressures once settled [60]. For *Callogorgia* occupying this area of the continental slope, variability in temperature and dissolved oxygen may be major factors influencing either larval or post-settlement survival. Where *C. delta* occurred, temperatures ranged from 5.0 to 10.0°C and dissolved oxygen from 1.5 to 3.5 ml l^{-1} [23]. Changes in only a few degrees of temperature could lead to adaptive protein changes and thus could influence species distributions [61]. The potential presence of microsatellite markers under selection (or linked to loci under selection) supports the idea that these processes may be at work here. Therefore, adaptive divergence in the presence of gene flow

could lead to the weak, but significant genetic differentiation with depth observed in *C. delta* across the continental slope of the northern GoM.

Callogorgia delta may also have undergone a recent population bottleneck, as indicated by the low allelic diversity and heterozygote excess at several loci, specifically at the GC sites. Allelic diversity can often be reduced faster than heterozygosity, particularly if heterozygotes have a selective advantage [47,62]. It is possible that there was a decrease in the effective population size at sites in the GC region in the past. This region experienced fluctuations in the amount of freshwater discharge and water depth during glacial and inter-glacial periods over at least the past 16 000 years, which could have led to habitat fragmentation in the region through anoxic events or temperature and salinity fluctuations [63,64]. Alternative to the population bottleneck scenario, it is possible that the significant results are spurious due to poor sampling of individuals at the GC sites as statistical power increases with more loci and more individuals.

Disentangling the evolutionary mechanisms (e.g. selection versus limited gene flow) causing genetic differentiation across the GoM in *C. delta* will likely require additional data, as any of the scenarios described above could result in the pattern observed. Determining whether species in the genus *Callogorgia* are brooders or broadcast spawners would provide insight into their effective dispersal distances and whether reproductive mode could limit gene flow. However, our results indicated that the most likely scenario for the genetic differentiation in *C. delta* across depth is adaptive divergence in the presence of gene flow. First, there was no evidence for significant inbreeding within any site. Second, F_{ST} values calculated using only the candidate selective loci revealed much higher genetic differentiation between sites, whereas no genetic differentiation was evident at the seven neutral loci. In a similar study that examined population differentiation in the deep-sea fish *Coryphaenoides rupestris*, one microsatellite locus (out of 16 total) was found to be under selection with genetic differentiation greater at this locus across a depth boundary of 1200 m [36]. In our study, two loci, CA3 and CA7, were designated as outliers and thus potential candidates for selection; however, it is noted that these two loci had much higher allelic diversities and larger motif sizes than the putative neutral loci. Including more loci spread across the genome would help resolve whether CA3 and CA7 are in fact under selection or linked to loci under selection. Increasing the number of loci used would yield a more robust estimate of the average genetic differentiation among loci, and thus allow for more confidence when designating outliers. Nevertheless, our results provide evidence that environmental conditions are shaping the pattern of genetic differentiation in *C. delta* across a gradient of depth.

The pattern of genetic differentiation with depth has emerged as an important feature of coral population structure. Population differentiation across depth has been indicated in several species of shallow and mesophotic corals that exhibit different reproductive modes. This includes three species of broadcast spawning corals: *O. varicosa* across the Florida continental shelf (less than 2 to 80 m) [20], *Eunicea flexuosa* across the Caribbean basin (less than 5 to 25 m) [60] and *Montastraea cavernosa* off Florida (less than 10 to 25 m) [26]. Similarly, populations of the octocoral *Paramuricea clavata*, a brooding species, were differentiated over both markedly short vertical (10–40 m) and horizontal (less than 400 km) distances in the Mediterranean [65]. In comparing these examples with this

study, a stronger pattern of isolation by depth compared with distance further suggests that adaptive divergence in the presence of gene flow is likely leading to depth differentiation in *Callogorgia* across the upper continental slope of the GoM.

(c) Further considerations

Adaptive divergence with depth is becoming increasingly recognized as an important process shaping population and species evolution in the deep sea [7–9]. Our data and results from recent studies [7–9] reveal the importance of considering different environmental conditions associated with depth that could lead to population isolation in the deep sea. These depth-related patterns necessitate additional research to disentangle the mechanisms responsible for population divergence in the deep-sea environment. Furthermore, our results have significant implications for conservation efforts. With the increasing potential for anthropogenic impacts to deep-sea communities [18–19], future design of protected areas in the deep sea could beneficially incorporate a variety of depth ranges and habitat types in order to capture the diversity within and among vulnerable marine ecosystems.

References

1. Rex MA, Etter RJ. 2010 *Deep-sea biodiversity: pattern and scale*. Cambridge, MA: Harvard University Press.

2. Levin LA, Etter RJ, Rex MA, Gooday AJ, Smith CR, Pineda J, Stuart CT, Hessler RR, Pawson D. 2001 Environmental influences on regional deep-sea species diversity. *Annu. Rev. Ecol. Syst.* **132**, 51–93. (doi:10.1146/annurev.ecolsys.32.081501.114002)

3. Levin LA, Sibuet M. 2012 Understanding continental margin biodiversity: a new imperative. *Annu. Rev. Mar. Sci.* **4**, 79–112. (doi:10.1146/annurev-marine-120709-142714)

4. Cordes EE, Cunha MM, Galeron J, Mora C, Olu-Le Roy K, Sibuet M, Van Gaever S, Vanreusel A, Levin L. 2010 The influence of geological, geochemical, and biogenic habitat heterogeneity on seep biodiversity. *Mar. Ecol.* **31**, 51–65. (doi:10.1111/j.1439-0485.2009.00334.x)

5. Etter RJ, Rex MA, Chase M, Quattro J. 2005 Population differentiation decreases with depth in deep-sea bivalves. *Evolution* **59**, 1479–1491. (doi:10.1111/j.0014-3820.2005.tb01797.x)

6. Zardus JD, Etter RJ, Chase MR, Rex MA, Boyle EE. 2006 Bathymetric and geographic population structure in the pan-Atlantic deep-sea bivalve *Deminucula atacellana* (SCHENCK 1939). *Mol. Ecol.* **15**, 639–651. (doi:10.1111/j.1365-294X.2005.02832.x)

7. Jennings RM, Etter RJ, Ficarra L. 2013 Population differentiation and species formation in the deep sea: the potential role of environmental gradients and depth. *PLoS ONE* **8**, e77594. (doi:10.1371/journal.pone.0077594)

8. Cowart DA, Halanych KM, Schaeffer SW, Fisher CR. 2014 Depth-dependent gene flow in Gulf of Mexico cold seep *Lamellibrachia* tubeworms (Annelida, Siboglinidae). *Hydrobiologia* **736**, 139–154. (doi:10.1007/s10750-014-1900-y)

9. Glazier AE, Etter RJ. 2014 Cryptic speciation along a bathymetric gradient. *Biol. J. Linn Soc.* **113**, 897–913. (doi:10.1111/bij.12389)

10. Jablonski D, Bottjer DJ. 1990 Onshore–offshore trends in marine invertebrate evolution. In *Causes of evolution: a paleontological perspective* (eds RM Ross, WD Allmon), pp. 21–75. Chicago, IL: University of Chicago Press.

11. Thaler AD, Zelnio K, Saleu W, Schultz TF, Carlsson J, Cunningham C, Vrijenhoek R, Van Dover CL. 2011 The effects of spatial scale on the population dynamics of *Ifremeria nautilei*, a hydrothermal vent endemic gastropod from the southwest Pacific. *BMC Evol. Biol.* **11**, 372. (doi:10.1186/1471-2148-11-372)

12. Cowart DA, Huang C, Arnaud-Haond S, Carney SL, Fisher CR, Schaeffer SW. 2013 Restriction to largescale gene flow versus regional panmixia among cold seep *Escarpia* spp. (Polychaeta, Siboglinidae). *Mol. Ecol.* **22**, 4147–4162. (doi:10.1111/mec.12379)

13. Andrews AH, Cordes EE, Mahoney MM, Munk K, Coale KH, Cailliet GM, Heifetz J. 2002 Age, growth and radiometric age validation of a deep-sea, habitat-forming gorgonian (*Primnoa resedaeformis*) from the Gulf of Alaska. *Hydrobiologia* **471**, 101–110. (doi:10.1023/A:1016501320206)

14. Roark EB, Guilderson TP, Dunbar RB, Ingram B. 2006 Radiocarbon-based ages and growth rates of Hawaiian deep-sea corals. *Mar. Ecol. Prog. Ser.* **327**, 1–14. (doi:10.3354/meps327001)

15. Kahng SE, Benayahu Y, Lasker HR. 2011 Sexual reproduction in octocorals. *Mar. Ecol. Prog. Ser.* **443**, 265–283. (doi:10.3354/meps09414)

16. Cordes EE, Nybakken JW, VanDykhuizen G. 2001 Reproduction and growth of *Anthomastus ritteri* (Octocoraillia: Alcyonacea) from Monterey Bay, California, USA. *Mar. Biol.* **138**, 491–501. (doi:10.1007/s002270000470)

17. Orejas C, López P, Gili JM, Teixidó N, Gutt J, Arntz W. 2002 Distribution and reproductive ecology of the Antarctic octocoral *Ainigmaptilon antarcticum* in the Weddell Sea. *Mar. Ecol. Prog. Ser.* **231**, 101–114. (doi:10.3354/meps231101)

18. Ramirez-Llodra E et al. 2011 Man and the last great wilderness: human impact on the deep sea. *PLoS ONE* **6**, e22588. (doi:10.1371/journal.pone.0022588)

19. White HK et al. 2012 Impact of the Deepwater Horizon oil spill on a deep-water coral community in the Gulf of Mexico. *Proc. Natl Acad. Sci. USA* **109**, 20 303–20 308. (doi:10.1073/pnas.1118029109)

20. Eytan RI, Hayes M, Arbour-Reily P, Miller M, Hellberg ME. 2009 Nuclear sequences reveal mid-range isolation of an imperilled deep-water coral population. *Mol. Ecol.* **18**, 2375–2389. (doi:10.1111/j.1365-294X.2009.04202.x)

21. Morrison CL, Ross SW, Nizinski MS, Brooke S, Järnegren J, Waller RG, Johnson RL, King TL. 2011 Genetic discontinuity among regional populations of *Lophelia pertusa* in the North Atlantic Ocean. *Conserv. Genet.* **12**, 713–729. (doi:10.1007/s10592-010-0178-5)

22. Baco AR, Shank TM. 2005 Population genetic structure of the Hawaiian precious coral *Corallium lauuense* (Octocorallia: Coralliidae) using microsatellites. In *Cold-water corals and ecosystems* (eds A Freiwald, JM Roberts), pp. 663–678. Berlin, Germany: Springer.

23. Quattrini AM, Georgian SE, Byrnes L, Stevens A, Falco R, Cordes EE. 2013 Niche divergence by deep-sea octocorals in the genus *Callogorgia* across the continental slope of the Gulf of Mexico. *Mol. Ecol.* **22**, 4123–4140. (doi:10.1111/mec.12370)

24. Bayer FM, Cairns SD, Cordeiro RTS, Pérez CD. 2014 New records of the genus *Callogorgia* (Anthozoa: Octocorallia) in the western Atlantic, including the description of a new species. *J. Mar. Biol. Assoc. UK*, 1–7. (doi:10.1017/S0025315414001957)

25. Pante E *et al.* 2015 Species are hypotheses: avoid connectivity assessments based on pillars of sand. *Mol. Ecol.* **24**, 525–544. (doi:10.1111/mec.13048)

26. Serrano X, Baums IB, O'Reilly K, Smith TB, Jones RJ, Shearer TL, Nunes FLD, Baker AC. 2014 Geographic differences in vertical connectivity in the Caribbean coral *Montastraea cavernosa* despite high levels of horizontal connectivity at shallow depths. *Mol. Ecol.* **23**, 4226–4240. (doi:10.1111/mec.12861)

27. Meglécz E, Costedoat C, Dubut V, Gilles A, Malausa T, Pech N, Martin JF. 2010 QDD: a user-friendly program to select microsatellite markers and design primers from large sequencing projects. *Bioinformatics* **26**, 403–404. (doi:10.1093/bioinformatics/btp670)

28. Van Oosterhout C, Hutchinson WF, Wills DP, Shipley P. 2004 MICROCHECKER: software for identifying and correcting genotyping errors in microsatellite data. *Mol. Ecol. Notes* **4**, 535–538. (doi:10.1111/j.1471-8286.2004.00684.x)

29. Chybicki IJ, Burczyk J. 2009 Simultaneous estimation of null alleles and inbreeding coefficients. *J. Heredity* **100**, 106–113. (doi:10.1093/jhered/esn088)

30. Raymond M, Rousset F. 1995 GENEPOP (v. 1.2): population genetics software for exact tests and ecumenicism. *J. Heredity* **86**, 248–249.

31. Peakall ROD, Smouse PE. 2006 GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. *Mol. Ecol. Notes* **6**, 288–295.

32. Ryman N, Palm S. 2006 PowSim: a computer program for assessing statistical power when testing for genetic differentiation. *Mol. Ecol. Notes* **6**, 600–602. (doi:10.1111/j.1471-8286.2006.01378.x)

33. Antao T, Lopes A, Lopes RJ, Beja-Pereira A, Luikart G. 2008 LOSITAN: a workbench to detect molecular adaptation based on a F_{ST}-outlier method. *BMC Bioinformatics* **9**, 323. (doi:10.1186/1471-2105-9-323)

34. Beaumont MA, Nichols RA. 1996 Evaluating loci for use in the genetic analysis of population structure. *Proc. R. Soc. Lond. B* **263**, 1619–1626. (doi:10.1098/rspb.1996.0237)

35. Foll M, Gaggiotti OE. 2008 A genome scan method to identify selected loci appropriate for both dominant and codominant markers: a Bayesian perspective. *Genetics* **180**, 977–993. (doi:10.1534/genetics.108.092221)

36. White TA, Stamford J, Rus Hoelzel A. 2010 Local selection and population structure in a deep-sea fish, the roundnose grenadier (*Coryphaenoides rupestris*). *Mol. Ecol.* **19**, 216–226. (doi:10.1111/j.1365-294X.2009.04446.x)

37. Pritchard JK, Stephens M, Donnelly P. 2000 Inference of population structure using multilocus genotype data. *Genetics* **155**, 945–959.

38. Hubisz M, Falush D, Stephens M, Pritchard J. 2009 Inferring weak population structure with the assistance of sample group information. *Mol. Ecol. Res.* **9**, 1322–1332. (doi:10.1111/j.1755-0998.2009.02591.x)

39. Falush D, Stephens M, Pritchard JK. 2003 Inference of population structure: extensions to linked loci and correlated allele frequencies. *Genetics* **164**, 1567–1587.

40. Earl DA, vonHoldt BM. 2012 STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output and implementing the Evanno method. *Conserv. Genet. Res.* **4**, 359–361. (doi:10.1007/s12686-011-9548-7)

41. Evanno G, Regnaut S, Goudet J. 2005 Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. *Mol. Ecol.* **14**, 2611–2620. (doi:10.1111/j.1365-294X.2005.02553.x)

42. Jakobsson M, Rosenberg NA. 2007 CLUMPP: a cluster matching and permutation program for dealing with label switching and multimodality in analysis of population structure. *Bioinformatics* **23**, 1801–1806. (doi:10.1093/bioinformatics/btm233)

43. Rosenberg NA. 2004 DISTRUCT: a program for the graphical display of population structure. *Mol. Ecol. Notes* **4**, 137–138. (doi:10.1046/j.1471-8286.2003.00566.x)

44. Weir BS, Cockerham CC. 1984 Estimating F-statistics for the analysis of population structure. *Evolution* **38**, 1358–1370. (doi:10.2307/2408641)

45. Excoffier L, Smouse PE, Quattro JM. 1992 Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. *Genetics* **131**, 479–491.

46. Jensen JL, Bohonak AJ, Kelley ST. 2005 Isolation by distance, web service. *BMC Genetics* **6**, 13. (doi:10.1186/1471-2156-6-13)

47. Piry S, Luikart G, Cornuet JM. 1999 *BOTTLENECK: a program for detecting recent effective population size reductions from allele data frequencies*. Montpellier, France: CBGP. See http://www1.montpellier.inra.fr/CBGP/software/Bottleneck/pub.html.

48. Sánchez JA, Aguilar C, Dorado D, Manrique N. 2007 Phenotypic plasticity and morphological integration in a marine modular invertebrate. *BMC Evol. Biol.* **7**, 122. (doi:10.1186/1471-2148-7-122)

49. Shearer TL, Van Oppen MJH, Romano SL, Wörheide G. 2002 Slow mitochondrial DNA sequence evolution in the Anthozoa (Cnidaria). *Mol. Ecol.* **11**, 2475–2487. (doi:10.1046/j.1365-294X.2002.01652.x)

50. McFadden CS, Benayahu Y, Pante E, Thoma JN, Nevarez PA, France SC. 2011 Limitations of mitochondrial gene barcoding in Octocorallia. *Mol. Ecol. Res.* **11**, 19–31. (doi:10.1111/j.1755-0998.2010.02875.x)

51. Baums IB, Johnson ME, Devlin-Durante MK, Miller MW. 2010 Host population genetic structure and zooxanthellae diversity of two reef-building coral species along the Florida Reef Tract and wider Caribbean. *Coral Reefs* **29**, 835–842. (doi:10.1007/s00338-010-0645-y)

52. Boulay JN, Hellberg ME, Cortés J, Baums IB. 2014 Unrecognized coral species diversity masks differences in functional ecology. *Proc. R. Soc. B* **281**, 20131580. (doi:10.1098/rspb.2013.1580)

53. Gage JD, Tyler PA. 1991 *Deep-sea biology: a natural history of organisms at the deep-sea floor*. Cambridge, UK: Cambridge University Press.

54. Cowen RK, Lwiza KM, Sponaugle S, Paris CB, Olson DB. 2000 Connectivity of marine populations: open or closed? *Science* **287**, 857–859. (doi:10.1126/science.287.5454.857)

55. Feehan KA, Waller RG. 2015 Notes on the reproduction of eight species of eastern Pacific cold-water octocorals. *J. Mar. Biol. Assoc. UK*, 1–6. (doi:10.1017/S002315415000053)

56. Brito TAS, Tyler PA, Clarke A. 1997 Reproductive biology of the Antarctic octocoral *Thouarella variabilis* Wright and Studer 1889. In *Proc. 6th Int. Conf. Coelenterate Biology, The Leeuwenhorst, Noordwijkerhout, The Netherlands, 16–21 July 1995*, pp. 63–69. Leiden, The Netherlands: Natural History Museum of Leiden.

57. Rivas D, Badan A, Ochoa J. 2005 The ventilation of the deep Gulf of Mexico. *J. Phys. Oceanogr.* **35**, 1763–1781. (doi:10.1175/JPO2786.1)

58. Young CM, Tyler PA, Gage JD. 1996 Vertical distribution correlates with pressure tolerances of early embryos in the deep-sea asteroid *Plutonaster bifrons*. *J. Mar. Biol. Assoc. UK* **76**, 749–757. (doi:10.1017/S002531540003143X)

59. Tyler PA, Young CM. 1998 Temperature and pressure tolerances in dispersal stages of the genus *Echinus* (Echinodermata: Echinoidea): prerequisites for deep-sea invasion and speciation. *Deep Sea Res. II* **45**, 253–277. (doi:10.1016/S0967-0645(97)00091-X)

60. Prada C, Hellberg ME. 2013 Long prereproductive selection and divergence by depth in a Caribbean candelabrum coral. *Proc. Natl Acad. Sci. USA* **110**, 3961–3966. (doi:10.1073/pnas.1208931110)

61. Somero GN. 1995 Proteins and temperature. *Annu. Rev. Phys.* **57**, 43–68. (doi:10.1146/annurev.ph.57.030195.000355)

62. Cornuet JM, Luikart G. 1996 Description and power analysis of two tests for detecting recent population bottlenecks from allele frequency data. *Genetics* **144**, 2001–2014.

63. Leventer A, Williams DF, Kennett JP. 1983 Relationships between anoxia, glacial meltwater and microfossil preservation in the Orca Basin, Gulf of Mexico. *Mar. Geol.* **53**, 23–40. (doi:10.1016/0025-3227(83)90032-4)

64. Poore RZ, Dowsett HJ, Verardo S, Quinn TM. 2003 Millennial to century-scale variability in Gulf of Mexico Holocene climate records. *Paleoceanography* **18**, 1048. (doi:10.1029/2002PA000868)

65. Mokhtar-Jamaï K, Pascual M, Ledoux JB, Coma R, Féral JP, Garrabou J, Aurelle D. 2011 From global to local genetic structuring in the red gorgonian *Paramuricea clavata*: the interplay between oceanographic conditions and limited larval dispersal. *Mol. Ecol.* **20**, 3291–3305. (doi:10.1111/j.1365-294X.2011.05176.x)

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