Testing the depth-differentiation hypothesis in a deepwater octocoral

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Abstract

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 geographic distances, *Callogorgia delta* was targeted. This species commonly occurs throughout the northern Gulf of Mexico at depths ranging from 400-900 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 geographic 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, *C. americana,* demonstrating the utility of microsatellites in species delimitation of octocorals. Results provided support for the depth-differentiation hypothesis, strengthening the notion that factors co-varying with depth serve as isolation mechanisms in deep-sea populations.

Key Words: deep sea, population genetics, connectivity, adaptive divergence, octocoral, Gulf of Mexico

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, 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 population differentiation with depth, numerous examples have demonstrated

high connectivity across expansive geographic distances (100s to 1000s km) 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 geographic boundaries.

Previous studies have examined population differentiation in mesophotic corals (50-200 m) on the continental shelf [20] and in deep-sea (> 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, southeastern US, 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 Gulf of Mexico (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. [*C. delta* Cairns & Bayer 2002, *C. americana* Cairns & Bayer 2002, *C. gracilis* (Milne Edwards & Haime 1857)] occupying distinct depth zones. Thus depth (and the co-factors 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 *Callogorgia delta* exists on the upper continental slope and is greater over vertical rather than geographic distances. This study also provided the opportunity to examine the level of genetic differentiation between *Callogorgia 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 *Callogorgia 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 *Callogorgia 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* (JSL) during five cruises from 2008-2010 (Fig. 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, and 885, Green Canyon (GC) 235, 246, 249, 338, and Garden Banks (GB) 299 (Fig. 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 -80ºC, preserved in 95% ETOH (stored at -20 ºC) and placed in a high-salt EDTA preservative (stored at -80 ºC). Voucher specimens of each individual colony were preserved in 95% ETOH or dried. Morphological characters and DNA barcodes were used to identify species [see 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) following [26]. Roche 454 reads (Table S1) were input into the software program QDD v2 [27], enabling microsatellite selection and subsequent primer design (see supplemental material). Thirty-nine primer pairs predicted to produce products >120 bp were then tested for amplification across *Callogorgia 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 (Table S2). PCR products were analyzed 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 v6 (created by Biomatters, http://www.geneious.com/) with Gene Scan 500LIZ size standards (Applied Biosystems, Inc.). MICROCHECKER v2.2 [28] was used to check for genotyping errors across all individuals. INEst v2 [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=5,000). 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 if the loci were non-randomly associated with one another. Departures from Hardy-Weinberg Equilibrium and observed and

169 expected heterozygosity at each locus and among populations were tested (GenALEx v
170 6.5) [31]. The statistical power of the microsatellite data was assessed for each species
171 using PowSim v 4.1 [32]. Because of the low statistical power for *C. americana* (see
172 supplemental material), this species was not used to test the depth-differentiation
173 hypothesis.

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

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

186 STRUCTURE v 2.3 [37], a Bayesian model-based clustering approach, was used
187 to determine the number of populations (designated by K) both within *C. delta* and
188 between *C. delta* and *C. americana* by assigning the probability of membership of
189 individuals iteratively to each K. For *C. delta*, model priors included location information
190 [38], an admixture model (i.e., individuals having mixed ancestry), and correlated allele
191 frequencies [39]. The location prior does not bias detecting structure when no actual

192 structure is present, but values of r ≤1 (Fig. S1) signify that the locations are informative
193 to population structure [38]. 1,000,000 MCMC generations were run following a burnin
194 of 250,000 generations. Five independent chains were run to test each value of K (K=1-8
195 for *C. delta*). The level of genetic admixture between *C. delta* and *C. americana* was
196 examined using STRUCTURE with parameters as above without a location prior and
197 K=1-3. STRUCTURE Harvester v0.6 [40] was used to choose K with the delta K
198 criterion [41]. For the chosen K, results from each of the five iterations were aligned
199 using CLUMPP v1.1 [42] and plotted in DISTRUCT v 1.1 [43].
200 To examine the amount of genetic differentiation among populations, F_{ST} [44]
201 was calculated between sites and/or species (GENALEx). Based on the STRUCTURE
202 results for *C. delta*, two sites (GC338 and GC249) located only ~ 5km apart in similar
203 depths, were pooled for these analyses. An Analysis of Molecular Variance (AMOVA)
204 [45] was conducted to test for significance among pairwise F_{ST} values across all sites
205 (GenALEx). Additionally, AMOVA was re-calculated after removing two sites (GC235,
206 GC246) that had small sample sizes. If AMOVA results were significant for *C. delta*,
207 partial mantel tests were used to test for significant correlations between F_{ST} [linearized
208 to $F_{ST}/(1- F_{ST})$] values with vertical distance given geographic distance and with
209 geographic distance given vertical distance (IBD on the Web v3.5, 1,000 randomizations)
210 [46]. Vertical and geographic distances were log transformed. Partial mantel tests were
211 also performed without GC235 and GC246. All analyses were repeated for both
212 candidate neutral loci only and candidate selective loci.
213 BOTTLENECK v 1.2 [47] was used to determine if any *C. delta* populations
214 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 stepwise mutation model (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) (Tables S2-S3). However, CA3 did not amplify well in *C. americana* and CA1 did not amplify well in *C. delta* (Tables S2-S3). No loci were in linkage disequilibrium (all loci, $p>0.05$, Fisher's Exact Test). Three of the same multi-locus genotypes (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 0-5% across all loci (INEST, 'nb' model best for each species, 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, 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 (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 (3 to 7 alleles per

239 locus). Including all loci, the mean number of alleles per locus ranged from 5.2 ± 0.84 SE
240 in *C. americana* to 7.50 ± 1.85 SE in *C. delta*. Without the three loci with the higher
241 number of alleles, the mean number of alleles per locus ranged from 3.86 ± 0.55 SE in *C.*
242 *americana* to 4.14 ± 0.40 SE in *C. delta*.
243 Several loci showed negative fixation (*F)* indices, suggesting heterozygote excess;
244 however, departures from HWE were not significant in most cases (Table S3). Three loci
245 showed significant departures from HWE within a particular site for *C. delta*: CA3 at
246 MC751 and MC885 (Chi Square, $p<0.005$), CA5 at GC235, MC751 and MC885 (Chi
247 Square, $p<0.005$), and CA9 at MC751 for *C. delta* (Chi Square, $p<0.005$). CA5 and CA9
248 were in heterozygote excess at the specific sites. Two loci showed significant departures
249 from HWE for *C. americana*: CA2 and CA7 at GB299 (Chi Square, $p<0.005$); however,
250 CA3 only amplified in five individuals collected from GB299 and was thus not used in
251 analyses.
252 Power analysis indicated that the probability of detecting genetic differentiation in
253 *C. delta* when $F_{ST} \geq 0.0250$ was 100%. The probability of identifying significant genetic
254 structure when the true $F_{ST} = 0$ was <6% in all simulations (Table S5).
255
256 (b) Genetic differentiation between *Callogorgia delta* and *C. americana*
257 STRUCTURE indicated little to no genetic admixture between *C. delta* and *C.*
258 *americana* at eight loci (CA2, CA4-10). Two populations (K=2) corresponded to the two
259 species (Fig. 2). In addition, there was high genetic differentiation between the two
260 species (F_{ST}=0.434, p=0.001; AMOVA, Table S6) and no evidence of hybridization. The
261 proportion of membership to one of the species clusters for each individual was >99%.
262

263 (c) Genetic differentiation within *Callogorgia delta*

264 Genetic differentiation was apparent within *C. delta* sampled from seven sites
265 across the northern GoM using nine loci (CA2-10) (Fig. 2). The F_{ST} value among
266 populations was significant (F_{ST}=0.042, p=0.001) (AMOVA, Table S6). Removing the
267 two sites that had few individuals (GC235 and GC246) from AMOVA analyses still
268 yielded significant overall F_{ST} (F_{ST}=0.042, p=0.001). The overall inbreeding coefficient
269 was low (Avg*Fi*= 0.0065*)* and not a significant component of the model (DIC=3493 for
270 the 'nfb' model, DIC=3491 for the 'nb' model). Inbreeding coefficients were also low for
271 each population (Avg*Fi*=0.011-0.041) and not a significant component of the model for
272 any population based on DIC (INEst, Table S4).

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

282 Pairwise F_{ST} values among sites harboring *C. delta* ranged between 0.021 and
283 0.078 with p-values ranging from 0.002 to 0.126 (AMOVA, Table 1). After a Bonferroni
284 correction, F_{ST} values between only a few pairs of sites remained significant (p<0.003,
285 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 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 geographic distance (partial mantel test, r=0.61 , p=0.002, Fig. 3). In contrast, no significant correlation was found with F_{ST} and geographic 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 geographic 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-analyzed 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) (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 geographic distance was evident at the two candidate loci (partial mantel test, r=0.56, p=0.008, Fig. 3). No genetic differentiation with geographic 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 geographic distance (r=0.93, p=0.04). In comparison, no significant genetic divergence with vertical distance given geographic distance (r=0.33, p=0.08) or geographic 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 geographic 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 heterozygote 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 *Callogorgia delta* across the northern Gulf of Mexico (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 geographic 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 MYA [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].

(b) Genetic differentiation across the GoM continental slope

The results from 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 geographic 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 geographic distance were found, suggesting that geographic 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 Viosca Knoll (VK) and Mississippi Canyon (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 as 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 life spans 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 200-400 m; Tropical Atlantic water (TAW) dominates depths ranging from 400-600 m; Antarctic Intermediate water (AAIW) dominates depths ranging from 600-1000 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

401 *Callogorgia* by entraining larvae and inhibiting the relatively simplistic planula larvae
402 from physically settling out of the water column. In addition, dispersing larvae may not
403 be able to physiologically tolerate environmental parameters (pressure, temperature,
404 dissolved oxygen) that change at water mass boundaries. Physiological intolerances of
405 echinoderm larvae to temperature and/or pressure have been shown to limit distribution
406 of bathyal species into either shallower or deeper depths depending upon their adult depth
407 ranges [58-59].

408 Larvae that pass through water mass barriers and successfully form adult colonies
409 may have a different range of physiological tolerances to environmental conditions than
410 their source population. This could lead to selection, and thus adaptive divergence among
411 populations occupying different depths [60]. Pre-reproductive selection could be common
412 in sessile animals, as sessile species are not able to move away from environmental
413 pressures once settled [60]. For *Callogorgia* occupying this area of the continental slope,
414 variability in temperature and dissolved oxygen may be major factors influencing either
415 larval or post-settlement survival. Where *C. delta* occurred, temperatures ranged from
416 5.0-10.0°C and dissolved oxygen from 1.5-3.5 ml/l [see 23]. Changes in only a few
417 degrees of temperature could lead to adaptive protein changes and thus could influence
418 species distributions [61]. The potential presence of microsatellite markers under
419 selection (or linked to loci under selection) supports the idea that these processes may be
420 at work here. Therefore, adaptive divergence in the presence of gene flow could lead to
421 the weak, but significant genetic differentiation with depth observed in *C. delta* across the
422 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 Green Canyon (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 vs. 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

446 examined population differentiation in the deep-sea fish *Coryphaenoides rupestris*, one
447 microsatellite locus (out of 16 total) was found to be under selection with genetic
448 differentiation greater at this locus across a depth boundary of 1200 m [36]. In our study
449 two loci, CA3 and CA7, were designated as outliers and thus potential candidates for
450 selection; however, it is noted that these two loci had much higher allelic diversities and
451 larger motif sizes than the putative neutral loci. Including more loci spread across the
452 genome would help resolve whether CA3 and CA7 are in fact under selection or linked to
453 loci under selection. Increasing the number of loci used would yield a more robust
454 estimate of the average genetic differentiation among loci, and thus allow for more
455 confidence when designating outliers. Nevertheless, our results provide evidence that
456 environmental conditions are shaping the pattern of genetic differentiation in *C. delta*
457 across a gradient of depth.

458 The pattern of genetic differentiation with depth has emerged as an important
459 feature of coral population structure. Population differentiation across depth has been
460 indicated in several species of shallow and mesophotic corals that exhibit different
461 reproductive modes. This includes three species of broadcast spawning corals: *Oculina*
462 *varicosa* across the Florida continental shelf (<2 to 80 m) [20], *Eunicea flexuosa* across
463 the Caribbean basin (<5 to 25 m) [60], and *Montastraea cavernosa* off Florida (<10 to 25
464 m) [26]. Similarly, populations of the octocoral *Paramuricea clavata*, a brooding species,
465 were differentiated over both markedly short vertical (10 to 40 m) and horizontal (<400
466 km) distances in the Mediterranean [65]. In comparing these examples with this study, a
467 stronger pattern of isolation by depth compared with distance further suggests that

adaptive divergence in the presence of gene flow is could lead 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.

Data Accessibility

Microsatellite allele calls are deposited in Dryad (doi:10.5061/dryad.fq7d1)

Competing Interests

We have no competing interests.

Author Contributions

AMQ and EEC conceived and designed the study. AMQ performed the research, analyzed the data and wrote the article with contributions from E.E.C. IBB helped with microsatellite marker design. IBB, TMS, and CLM helped with population genetic analyses and edited the manuscript. All authors gave final approval for publication.

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Figure Captions

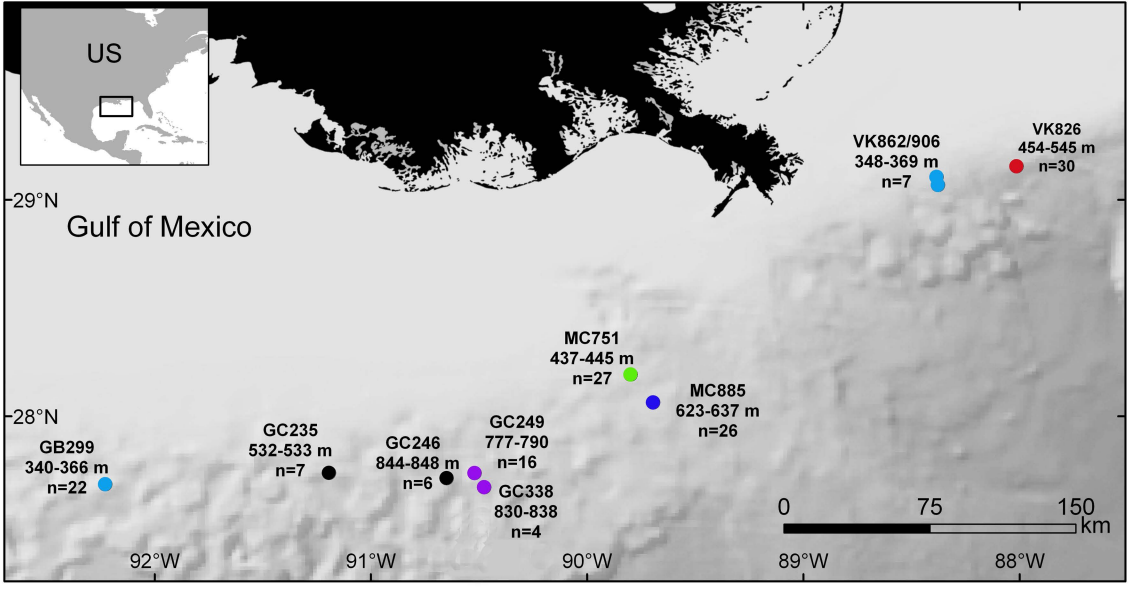
Figure 1. Map of sampling locations of *Callogorgia americana* (blue symbols) and *C. delta* (all other colored symbols). Colors denote clusters identified by STRUCTURE (see Fig. 2). Number of samples (n) and depth range of collections are noted.

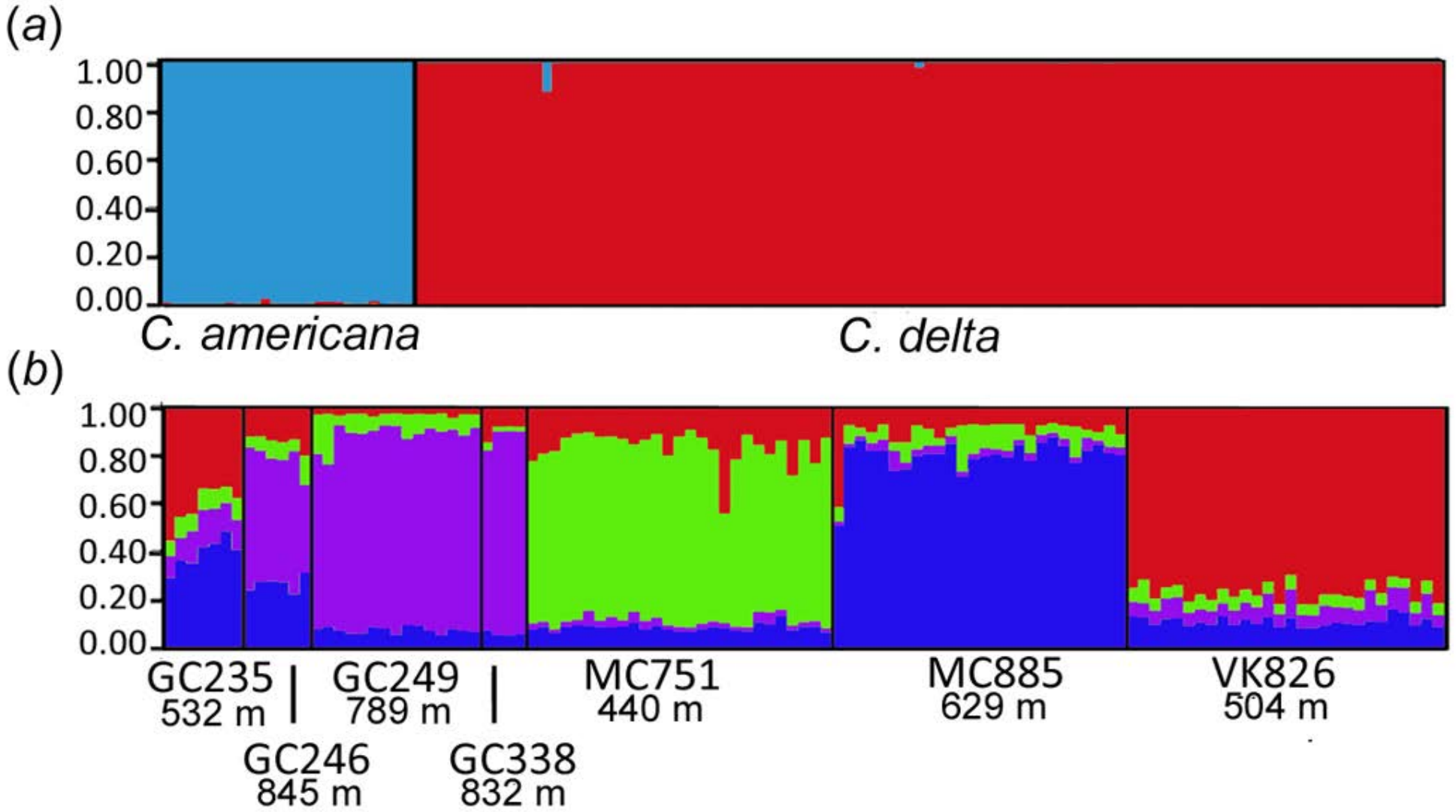
Figure 2. Average probability of membership graphs (STRUCTURE) for (*a*) *Callogorgia* spp. (K=2, n=141), and (*b*) *Callogorgia delta* (n=113, K=4). Mean depth of collections for each site is included.

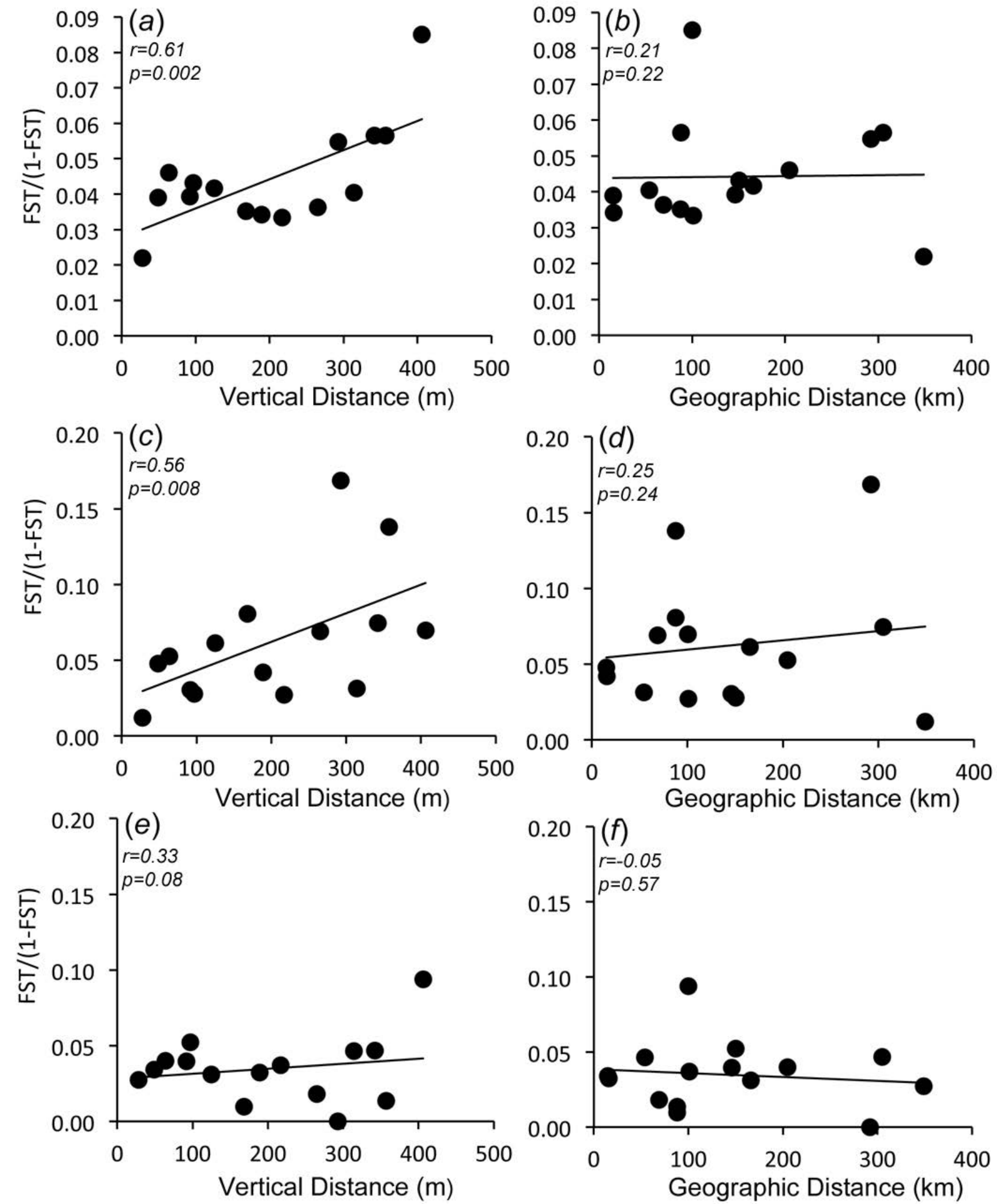
Figure 3. Scatterplots of pairwise F_{ST} for *Callogorgia delta* with respect to vertical and geographic distance at (*a-b)* all nine loci; (*c-d*) two loci candidates for selection; and (*e-f*) seven putative neutral loci.

Table 1. Pairwise F_{ST} values for *Callogorgia delta* among sites at all loci. F_{ST} values **in bold** are significant (AMOVA, Bonferroni adjustment, p≤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	--


US
29°N
Gulf of Mexico
28°N
VK862/906
348-369 m
n=7
VK826
454-545 m
n=30
MC751
437-445 m
n=27
MC885
623-637 m
n=26
GB299
340-366 m
n=22
GC235
532-533 m
n=7
GC246
844-848 m
n=6
GC249
777-790
n=16
GC338
830-838
n=4
0
75
150
km
92°W
91°W
90°W
89°W
88°W


(a)
1.00
0.80
0.60
0.40
0.20
0.00
C. americana
C. delta
(b)
1.00
0.80
0.60
0.40
0.20
0.00
GC235
532 m
GC246
845 m
GC249
789 m
GC338
832 m
MC751
440 m
MC885
629 m
VK826
504 m


(a)
r=0.61
p=0.002
FST/(1-FST)
Vertical Distance (m)
(b)
r=0.21
p=0.22
Geographic Distance (km)
(c)
r=0.56
p=0.008
Vertical Distance (m)
(d)
r=0.25
p=0.24
Geographic Distance (km)
(e)
r=0.33
p=0.08
Vertical Distance (m)
(f)
r=-0.05
p=0.57
Geographic Distance (km)