GENETIC DIVERGENCE AMONG SYMPATRIC POPULATIONS OF THREE SPECIES OF OYSTER DRILLS (*UROSALPINX*) IN CEDAR KEY, FLORIDA

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ABSTRACT

Three gastropod species of the genus *Urosalpinx* inhabit intertidal and subtidal regions of the southeastern United States. *Urosalpinx cinerea* (Say, 1822) is native to the Atlantic coast from Newfoundland to northeast Florida, while *Urosalpinx perrugata* (Conrad, 1846) and *Urosalpinx tampaensis* (Conrad, 1846) are largely restricted to the Gulf of Mexico. The morphological similarity of *U. cinerea* and *U. perrugata* is so striking that they have been suggested to be subspecies. We used a combination of molecular and morphological methods to examine divergence among populations of these three nominal species in a newly discovered area of sympatry: Cedar Key, Florida. Significant shell morphological differences were detected among the three species, but overlapping ranges prevented unambiguous discrimination of *U. cinerea* and *U. perrugata*. A survey of gene frequencies at nine allozyme-encoding loci in the three Cedar Key populations (together with two allopatric controls) revealed multiple fixed differences between the species, confirming their reproductive isolation. Interspecific mtDNA sequence divergence was extensive (10.3%–17.3%), but well within the range of previously reported congeneric divergences. A neighbor-joining phylogeny grouped *U. cinerea* with *U. perrugata*, while *U. tampaensis* was found to be slightly more divergent. We suggest that the Florida peninsula may have served as a barrier to gene flow, promoting the allopatric speciation of *U. cinerea* and *U. perrugata* sometime after the divergence of *U. tampaensis*.

Oyster drills of the genus *Urosalpinx* have been the focus of substantial research interest for many years, due primarily to their significant impacts on commercial bivalve fisheries. Caging experiments have demonstrated that predation by *Urosalpinx cinerea* (Say, 1822) can push prey populations to local extinction (Katz, 1985). Estimates of realized predation effects on oyster populations depend largely on the density of the drills, but in one Virginia location *U. cinerea* was found to destroy up to 90% of the yearly spatfall (Carriker, 1955). Despite the rich history of biological investigation, however, no previous studies have used modern genetic tools to examine species or population-level divergence within the genus *Urosalpinx*.

Like all muricid gastropods, *Urosalpinx* deposits its eggs in leathery capsules cemented onto solid substrata, developing directly into benthic juveniles. The absence of a planktonic dispersal stage from its life cycle may have limited the natural range of *U. cinerea* in the western North Atlantic (Scheltema, 1989). Human-mediated transport has, however, introduced populations of *U. cinerea* to both the Pacific coast of the United States (Stearns, 1894) and to the eastern North Atlantic (Orton and Winckworth, 1928). It seems likely that populations within the native range of the species may have been homogenized by similar human influences (Carriker, 1955; Elton, 1958).

Three nominal species of *Urosalpinx* are generally recognized in the fauna of the nearshore North Atlantic, distinguished by differing counts of spiral cords and axial ribs on their shells. Abbott (1974) gave the natural range of *U. cinerea* as "Newfound-

land to northeast Florida," the range of *U. perrugata* (Conrad, 1846) as "both sides of Florida," and the range of *U. tampaensis* (Conrad, 1846) as the "west central coast of Florida." The distinction among the species is not clean, however. Abbott (1974) counted 9–11 axial ribs with "numerous" spiral cords for *U. cinerea*, 6–9 axial ribs with "fewer" spiral cords for *U. perrugata*, and 9–11 axial ribs with 9–10 spiral cords for *U. tampaensis*, suggesting as he did that *U. perrugata* might prove to be a subspecies of *U. cinerea*.

In the summer of 2004, we discovered a large population of *U. cinerea* on the northwest coast of Florida at Cedar Key, inhabiting oyster reefs in close sympatry with both *U. perrugata* and *U. tampaensis*. Although there have been some prior reports among hobbyists, ours is the first formal report of a population of *U. cinerea* in the Gulf of Mexico. We speculate that the introduction may have been mediated by oyster or hard clam aquaculture. Commercial-scale aquaculture of Atlantic hard clams [*Mercenaria mercenaria* (Linnaeus, 1758)] in Cedar Key has been ongoing since 1993 (Arnold et al., 2004). Oysters have also almost certainly been transferred from the Atlantic to the Cedar Key area in the past, although records are scarce.

The biological species concept emphasizes an inability to interbreed as a criterion for the distinction of species (Mayr, 1942). More recently, Mallet (1995) has suggested that species should be viewed as clusters of genotypes that do not fuse when they occur together. Regardless of the particular definition applied, the co-occurrence of *U. cinerea*, *U. perrugata*, and *U. tampaensis* at Cedar Key constitutes an ongoing natural experiment to assess the species status of these three ecologically and genetically similar marine gastropods. The purpose of the present study is to characterize the levels of genetic divergence among the three species using morphological characters, mtDNA sequences, and allozyme markers, and to look for hybridization between these gastropods in an area of sympatry.

Methods

Sample Collection.—Collections of all three *Urosalpinx* species (CINc, PERc, TAMc) were made on the tidal flats in front of the Beachfront Motel in Cedar Key, Florida (29.135°N, 83.037°W) during field trips in the summers of 2004 and 2005. Sample sizes were N = 37 for CINc, N = 39 for PERc, and N = 52 for TAMc. Collections from Cedar Key were initially grouped by species based on gross morphology. Identifications were later verified on the basis of fixed differences between species for alternate allozyme alleles. Our reference population of N = 30 *U. tampaensis* (TAMp) was supplied by the Gulf Specimen Marine Lab from Fiddler's Point, in Appalachee Bay, Panacea, Florida (30.036°N, 84.385°W). Our reference population of N = 30 *U. cinerea* (CINs) was collected from intertidal oysters and debris in Charleston Harbor at the Fort Johnson Marine Science Center, Charleston, South Carolina (32.753°N, 79.898°W). No reference population of *U. perrugata* was available.

Morphology.—Three shell morphological variables were measured for all 128 individuals sampled from Cedar Key. Shell length was measured with Vernier calipers to the nearest 0.1 mm, and counts of spiral cords and axial ribs were made on the body whorl. A figure diagramming these counts and measurements is available in Robinson (2006). Differences among species in these three variables were assessed statistically using one-way Kruskal-Wallis Rank Sum Tests, a non-parametric analogue to ANOVA. This analysis was chosen because of the discrete nature of our count variables, as well as the low range of values recorded. Statistical analyses were conducted using R statistical computing software (R Development Core Team, 2005).

Allozyme Analysis.—At least 30 individuals per population were removed from the shell and soft tissue was stored at −80 °C in a 0.05 M Tris Phosphate tissue buffer (pH 7.4, 7% sucrose, with xylene cyanole). For analysis of allozyme polymorphism, whole-animal homogenates were centrifuged and the supernatant was resolved using horizontal starch gel electrophoresis (Dillon, 1992). Gels were made with hydrolyzed potato starch (14% weight/volume; 1:1, Electrostarch: Sigma Starch or 12% weight/volume Sigma Starch) and run at approximately 50 mA (voltage varied by buffer system), at 4 °C, for a period of 4 hrs. From an initial screening of 79 stain and buffer system combinations, nine well-resolved enzyme-encoding loci were selected and scored for the sampled populations. The AP6 buffer system of Clayton and Tretiak (1972) was used to resolve leucine aminopeptidase (LAP), aspartate aminotransferase (AAT), and phosphoglucomutase (PGM). A reverse AP6 gel was also used to resolve 6-phosphogluconate dehydrogenase (6PGD), which carried a positive charge at pH 6. The buffer system of Ward and Warwick (1980; WWI) was used to resolve octanol dehydrogenase (OCTDH), mannose-6-phosphate isomerase (MPI), and isocitrate dehydrogenase (ISDH). The TEB9.1 buffer system of Dillon and Davis (1980) was used to resolve xanthine dehydrogenase (XDH) and sorbitol dehydrogenase (SDH). Details of our initial screening of buffers and enzyme systems, together with recipes for all reagents and stains employed in our allozyme analysis, are available in Robinson (2006).

For all allozyme loci, the most common allele resolved in the South Carolina (CINs) population was given the numerical designation 100 (e.g., MPI 100), with other alleles named by their mobility in reference to this allele. Then the allele frequencies, mean (direct count) heterozygosities, and goodness-of-fit to Hardy-Weinberg expectations (χ^2, with pooling) were calculated using BIOSYS v. 1.7 (Swofford and Selander, 1981). Nei's (1972, 1978) genetic distances and associated standard errors and expected heterozygosities and their standard errors were calculated using DISPAN (Ota, 1993).

mtDNA Analysis.—Ten snails from each population were preserved for mtDNA sequence analysis. An anterior portion of the foot muscle was frozen in > 95% EtOH and stored at −80 °C. DNA isolations were performed using DNeasy Tissue Kits (Qiagen) for all populations, except for the Panacea population (TAMp), from which DNA was extracted using Chelex beads (10% solution). The universal primers of Folmer et al. (1994) were used to amplify a fragment of cytochrome c oxidase subunit I (COI) of the mitochondrial genome, initially with a 40 bp GC-clamp added to the 5′ end of the forward primer (Sheffield et al., 1989). For all PCR amplifications, 25 µL reactions were performed in 200 µL PCR tubes. The reaction solution contained 200 µM dNTPs, 1.5 mM $MgCl_2$, 0.6 µM forward and reverse primers, and 2.5 active units of Taq polymerase (Qiagen HotstarTaq or Promega GoTaq). To this solution, 1 µL of template was added and amplification was carried out using the following cycling protocol: denaturing and activation of HotStarTAQ (Qiagen) at 95 °C (for 15 min), 34 cycles of denaturing at 94 °C (for 30 s), annealing at 46 °C (for 30 s), and elongation at 72 °C (for 1 min), followed by a final elongation cycle at 72 °C (for 7 min) and a 4 °C hold. All amplifications were electrophoresed on a 1% agarose gel, stained with ethidium bromide, and visualized on a BIORAD VersaDoc™ model 1000 imaging system to ensure amplification of the fragment.

Denaturing gradient gel electrophoresis (DGGE) was used as a tool for the initial screening of individuals for differences in COI sequence (Myers et al., 1987; Lessa and Applebaum, 1993). We first ran an optimization gel using samples from all three nominal species to determine the appropriate gradient concentration, as well as the amount of time required for the fragments to reach their denaturing point. Ultimately, our gradients were created down gels of 6.5% polyacrylamide by mixing a polyacrylamide solution containing no urea with a polyacrylamide solution containing 80% urea in a gradient maker (C.B.S. Scientific). Gels were run at 150 V parallel to the denaturing gradient for a period of 6 hrs using a C.B.S. Scientific Co. model number DGGE-4000 denaturing gradient gel electrophoresis system at a constant 60 °C. After electrophoresis, gels were impregnated with silver nitrate and stained to reveal banding patterns, following Bassam et al. (1991).

Ten snails from each of the five sampled populations were initially subjected to analysis on denaturing gradient gels. All samples with the same mobility on DGGE gels were placed into letter-coded haplotype bins, and when possible three individuals bearing haplotypes in each bin were sequenced from each population. Samples for sequencing were reamplified without the GC-clamp, the annealing temperature increased to 48 °C. The amplified DNA fragments were sequenced at either the Molecular Core Facility of Grice Marine Lab (College of Charleston) or the Nevada Genomics Center (University of Nevada, Reno). Sequences were obtained in the forward direction in all cases. This practice provided between 500 and 600 bp of high quality sequence data for each individual. Fragment alignments were performed using Sequencher (Genecodes, Ann Arbor, MI). Kimura 2-parameter nucleotide distances between the unique haplotypes obtained in this study were calculated, and a bootstrapped neighbor-joining phylogeny was constructed using MEGA v. 3.1 (Kumar et al., 2004). *Nucella lamellosa* (Gmelin, 1791), another dispersal-limited muricoid gastropod of the West Atlantic, was chosen as the outgroup because of the close match to *U. cinerea* sequences returned by the nucleotide-nucleotide BLAST utility on the NCBI website (E-score = $1e^{-161}$). The *N. lamellosa* sequence used was submitted by Marko (2004; GenBank Accession #: AY445470).

Results

Morphology.—Although our three samples of *Urosalpinx* from Cedar Key overlapped substantially in shell length (Fig. 1A), a rank sum test returned significant shell length differences (Kruskal-Wallis $\chi^2 = 12.4608$, df = 2, P = 0.002). Some overlap in the counts of axial ribs and spiral cords was also apparent, especially between *U. cinerea* and *U. perrugata*, although the differences in central tendency were again quite significant. *Urosalpinx perrugata* had significantly fewer axial ribs per body whorl than either of the other two species (Kruskal-Wallis $\chi^2 = 54.4867$, df = 2, P < 0.001, Fig. 1B), and *U. tampaensis* was distinguished by a significantly lower count of spiral cords (Kruskal-Wallis $\chi^2 = 97.1821$, df = 2, P < 0.001, Fig. 1C). Our complete data set for all morphological characters measured on all individuals is available in Robinson (2006).

Allozyme Analysis.—Allele frequencies for the five populations sampled at the nine allozyme-encoding loci are reported in Table 1. No aspartate aminotransferase activity was resolved for populations of *U. tampaensis* with any buffer system employed. Because it seems more likely to us that the AAT gene product was not absent in *U. tampaensis*, but rather so biochemically different that substantially different electrophoretic conditions would have been required to visualize it, this result was scored as the product of a unique allele, "other."

Across all nine loci × five populations, or 45 observations, Table 1 shows 12 polymorphisms by the 95% criterion. No significant deviation from Hardy-Weinberg expectation was apparent at these 12 loci. Genetic distances calculated on the basis of allozyme allele frequencies were small between conspecific populations, the newly discovered population of *U. cinerea* from Cedar Key appearing nearly identical to the reference population collected in South Carolina (Table 2). Genetic distances were quite large between nominal species, however, reflecting the large number of fixed differences observed between species pairs. *Urosalpinx cinerea* and *U. perrugata* shared no alleles at six loci, *U. perrugata* and *U. tampaensis* shared none at seven, and *U. cinerea* and *U. tampaensis* shared no alleles at eight loci.

No putative hybrids were observed. Hybrids should have been readily identifiable by their heterozygosity at any or all of the loci fixed between their pair of parent

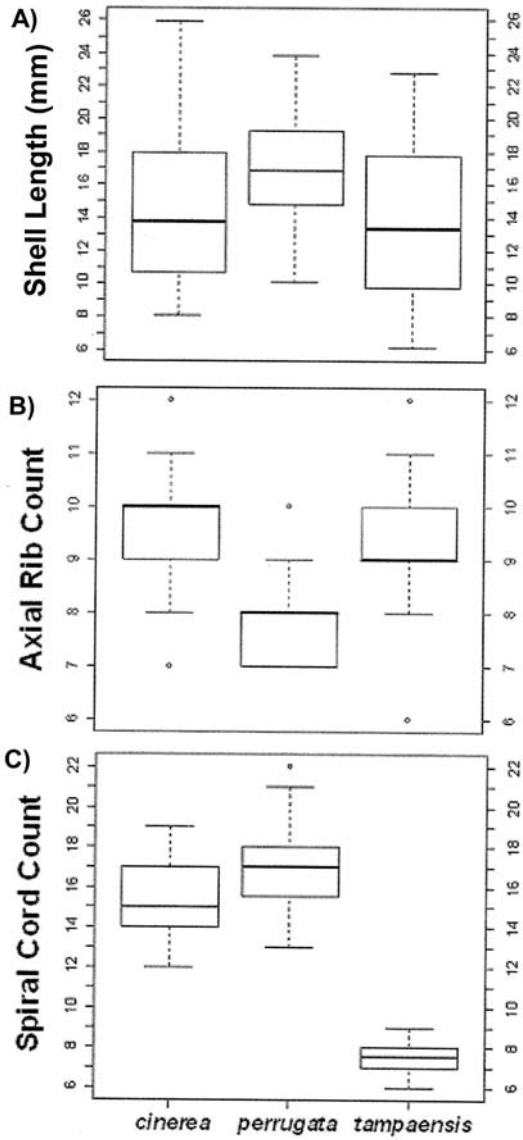

Figure 1. Boxplots of morphological characteristics measured for Cedar Key, Florida, populations of *Urosalpinx*: (A) shell length, (B) count of axial ribs on the body whorl, and (C) count of spiral cords on the body whorl. Median values are indicated by the line in the center of the box, interquartile ranges are the upper and lower faces of the box, ranges are the ends of the extended arms, and outliers are indicated by open circles.

species. For example, F1 hybrid progeny from a cross of *U. cinerea* and *U. perrugata* would be expected to express both allozymes at six loci, 100/125 at MPI, 100/95 at AAT, and so forth. But none of the more than 90 drills analyzed from Cedar Key displayed such heterozygosity at any locus.

Individuals collected at Cedar Key and previously classified by us (on the basis of shell morphology) to *U. cinerea*, *U. perrugata*, and *U. tampaensis* were found to carry only those allozyme alleles expected for their species, with two exceptions. In

Table 1. Allele frequencies at nine enzyme loci and overall heterozygosities for five populations of *Urosalpinx*. Population abbreviations consist of three upper-case letters for each species (CIN–*U. cinerea*, PER—*U. perrugata*, TAM—*U. tampaensis*) followed by a single letter identifying the population (s—South Carolina, c—Cedar Key, p—Panacea, FL). Mean heterozygosities were calculated using the direct count (D.C.) method.

	Population				
Allele (sample size)	CINs	CINc	PERc	TAMc	TAMp
ISDH					
(N)	30	30	30	32	26
100	0.867	0.933	0.000	1.000	0.962
95	0.133	0.067	0.000	0.000	0.038
92	0.000	0.000	0.133	0.000	0.000
90	0.000	0.000	0.833	0.000	0.000
85	0.000	0.000	0.033	0.000	0.000
PGM					
(N)	29	30	31	33	28
100	1.000	0.983	0.000	0.000	0.000
98	0.000	0.017	0.000	0.000	0.000
95	0.000	0.000	0.903	0.000	0.000
93	0.000	0.000	0.097	0.000	0.000
90	0.000	0.000	0.000	0.909	0.732
87	0.000	0.000	0.000	0.091	0.268
6PGD					
(N)	30	30	31	32	28
105	0.000	0.250	0.000	0.000	0.000
100	1.000	0.750	1.000	0.000	0.000
95	0.000	0.000	0.000	0.625	0.446
90	0.000	0.000	0.000	0.375	0.554
MPI					
(N)	30	29	31	33	28
125	0.000	0.000	1.000	1.000	1.000
100	1.000	1.000	0.000	0.000	0.000
AAT					
(N)	30	30	31	30	30
100	1.000	1.000	0.000	0.000	0.000
95	0.000	0.000	1.000	0.000	0.000
Other	0.000	0.000	0.000	1.000	1.000
LAP					
(N)	30	30	31	33	28
110	0.000	0.000	1.000	1.000	1.000
100	1.000	1.000	0.000	0.000	0.000
SDH					
(N)	30	30	27	32	28
103	0.000	0.000	0.352	0.000	0.000
100	1.000	1.000	0.648	0.000	0.000
97	0.000	0.000	0.000	1.000	1.000
XDH					
(N)	30	30	31	33	24
103	0.000	0.000	0.000	1.000	1.000
100	1.000	1.000	1.000	0.000	0.000
OCTDH					
(N)	30	29	31	33	28
115	0.000	0.000	1.000	0.000	0.000
110	0.000	0.000	0.000	1.000	1.000
105	0.317	0.483	0.000	0.000	0.000
100	0.683	0.517	0.000	0.000	0.000
Exp. H ± SE	0.075 ± 0.052	0.117 ± 0.069	0.104 ± 0.057	0.072 ± 0.054	0.109 ± 0.066
Mean H (D.C.)	0.064 ± 0.043	0.104 ± 0.056	0.088 ± 0.046	0.069 ± 0.050	0.104 ± 0.062

Table 2. Nei's (1972) genetic distances (± SE) (Nei, 1978) calculated from allele frequencies at nine allozyme-encoding loci for five *Urosalpinx* populations.

	CINs	CINc	PERc	TAMc
CINc	0.010 ± 0.007			
PERc	1.130 ± 0.506	1.206 ± 0.518		
TAMc	2.264 ± 1.007	2.167 ± 0.998	1.412 ± 0.644	
TAMp	2.276 ± 1.009	2.183 ± 1.000	1.392 ± 0.640	0.006 ± 0.004

both of these cases, snails initially classified as *U. perrugata* were identified on the basis of their allozyme phenotype as *U. cinerea*. Both of these individuals had oyster spat cemented to their shells, hindering initial identification based on gross shell morphology. These snails were reclassified in our records as *U. cinerea* before further analyses were conducted.

mtDNA Analysis.—Nine haplotype bins were created on the basis of differing mobility in denaturing gradient gels: three unique to South Carolina *U. cinerea* (A4, B1, and C1; preserving the haplotype nomenclature of Robinson, 2006), one shared between South Carolina *U. cinerea* and *U. tampaensis* (D), one unique to Cedar Key *U. cinerea* (E1), one unique to *U. tampaensis* (F), and three unique to *U. perrugata* (G–I). Direct sequencing of three individuals from each haplotype bin yielded twelve *U. cinerea* sequences (three from Cedar Key, FL and nine from South Carolina), six *U. perrugata* sequences, and eight *U. tampaensis* sequences (five from Cedar Key, FL and three from Panacea, FL). The bin-D haplotypes putatively shared by *U. cinerea* and *U. tampaensis* were revealed to differ by 13.1% (67 of 563 bp mismatch). These haplotypes were relabeled D1 (*U. cinerea*) and D2 (*U. tampaensis*). Two additional haplotypes (D3 and D4) were discovered in the *U. tampaensis* D-bin, one additional haplotype was found in the *U. tampaensis* F-bin (F2), and one additional haplotype was discovered in the G-bin of *U. perrugata* (G2). All 14 unique sequences obtained in this study are available via GenBank (Accession #s: DQ868949–DQ868962).

The neighbor-joining phylogeny constructed on the basis of these COI sequence data separated the three *Urosalpinx* species into three distinct clades (Fig. 2). Interior nodes show high bootstrap support values (> 95%) in all cases. Kimura 2-parameter nucleotide distances between lineages within species ranged from 0.2% to 1.6%, while interspecific divergence ranged from 10.3 to 11.3% for the comparison of Cedar Key *U. cinerea* and *U. perrugata*, 15.8 to 17.3% for the comparison of *U. perrugata* and *U. tampaensis*, and 13.1 to 13.6% for the comparison of *U. cinerea* and *U. tampaensis*.

Discussion

Shell morphological criteria, specifically the counts of spiral cords on the body whorl, were sufficient to distinguish *U. tampaensis* from the two other species of *Urosalpinx* inhabiting Cedar Key with 100% accuracy. *Urosalpinx perrugata* was generally distinguishable by a significantly lower axial rib count, although some overlap was apparent between *U. perrugata* and *U. cinerea* in all three of the shell characters we examined.

Gene frequencies at allozyme-encoding loci revealed striking genetic divergence among all three species. Lewontin (1991) has noted that such data are especially well suited for assessing reproductive isolation among sympatric populations because the codominant nature of allozyme markers allows easy visual identification of hybrid

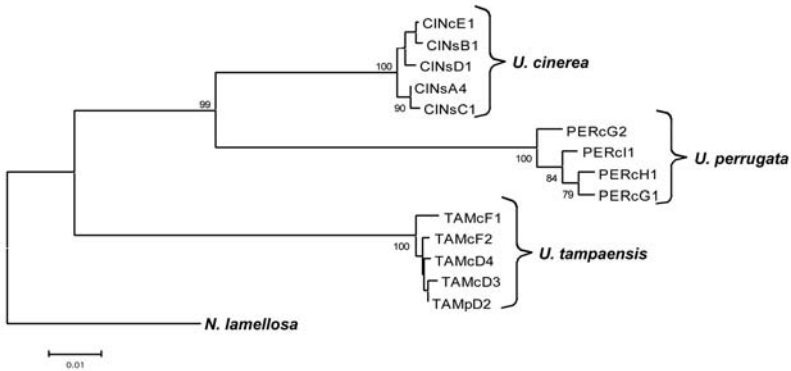

Figure 2. Neighbor-joining phylogeny of the 14 unique sequences obtained in this study, with *Nucella lamellosa* as outgroup. Numbers at each node give percent bootstrap support.

individuals. Allozyme electrophoresis has indeed proven useful for studies of hybridization in oysters (Allen and Gaffney, 1991), mussels (Bates and Innes, 1995), and hard clams of the genus *Mercenaria* (Dillon and Manzi, 1989a; Arnold et al., 2004). Given the fixed differences at multiple allozyme-encoding loci shown in Table 1, our failure to identify a single putative hybrid in a combined sample of 90+ drills collected from close sympatry strongly suggests that reproductive isolation among the three species is complete.

The levels of COI sequence divergence among populations of *U. cinerea*, *U. perrugata*, and *U. tampaensis* also confirm the species status of these three taxa. In a combined analysis of over 1100 congeneric species pairs of mollusks, Hebert et al. (2003) found that 67.5% showed sequence divergences between 8 and 16%. Our sequence divergence values range from 10 to 17%, supporting the present taxonomic status (separate species within the same genus) of these members of the genus *Urosalpinx*.

The congruence we have documented among the morphological, allozyme, and mtDNA datasets analyzed in this study is not trivial. Previous investigations have often yielded conflicting results between mitochondrial and allozyme or other nuclear genetic markers. Examples of such studies include oysters (Buroker, 1983; Karl and Avise, 1992; Hare and Avise, 1996), hard clams (Dillon and Manzi, 1989b; Ó Foighil et al., 1996), and whelks of the genus *Busycon* (Wise et al., 2004). In the case of *Urosalpinx*, however, no discrepancy is apparent among the three categories of data, all three species distinguishable using morphological, nuclear, and mitochondrial criteria.

The performance of DGGE in resolving differences between amplified DNA sequences was less than satisfactory in this study. A different approach to optimization might have led to better success, perhaps involving a less concentrated gradient of urea and a standardization on intraspecific, rather than interspecific, variation. But it is difficult to reconcile the equivalent migration down the denaturing gradient we observed in the *U. cinerea* and *U. tampaensis* D-bin amplicons, given their extensive sequence divergence. Although DGGE and related techniques [e.g., single-stranded conformation polymorphism (SSCP)] certainly have a potential to reduce the high cost of DNA sequencing in large-scale studies (see Sunnucks et al., 2000), it must be recognized that there are risks to be weighed against the benefits.

Although collections at the Florida Museum of Natural History report *U. perrugata* from the east coast of Florida as far north as Palm Beach County (http://www.flmnh.ufl.edu), we have been unable to personally confirm *Urosalpinx* populations of any species inhabiting south Florida. *Urosalpinx cinerea* seems locally common along the Atlantic coast as far south as Cape Canaveral. But our field observations suggest that both *U. cinerea* and its congeners are ecologically replaced by other gastropods, including the buccinid drills (*Cantharus*) and the crown conch (*Melongena*), between Cape Canaveral and Tampa Bay. Low salinities in the 10,000 islands region of Florida (M. Finn, pers. comm.) may also limit the distribution of *Urosalpinx* in south Florida, given their inability to tolerate prolonged exposure to salinities lower than about 15 psu (Carriker, 1955). We therefore suggest that the *Urosalpinx* species may have diverged allopatrically, with periods of lower sea level, or other barriers, effectively isolating populations in the Gulf of Mexico over an extended period of time.

The peninsula of Florida presents a well-documented barrier to gene flow for a great variety of organisms inhabiting the Atlantic and Gulf coasts of the United States (Avise, 2000). Genetic divergence across the south Florida ecotone has been demonstrated for the toadfish *Opsanus* (Freshwater et al., 2000), the mole crab *Emerita talpoida* (Say, 1817; Tam et al., 1996), the squid genus *Loligo* (Herke and Foltz, 2002), the eastern oyster *Crassostrea virginica* (Gmelin, 1791; Karl and Avise, 1992; Hare and Avise, 1996), the mussel *Geukensia* (Sarver et al., 1992), and several gastropods, including sinistral *Busycon* spp. (Wise et al., 2004) and members of the *Crepidula plana* Say, 1822 complex (Collin, 2000). Only rarely have genetic studies failed to uncover a barrier effect attributable to the Florida peninsula (Felder and Staton, 1994; Dayan and Dillon, 1995).

The 10.3%–17.3% mtDNA sequence divergences we here report among *Urosalpinx* species are higher than those recorded from similar studies analyzing organisms with greater dispersal potential. *Mercenaria* spp., which undergo planktonic development, show divergences ranging from 2.08 to 3.56% around Florida (Ó Foighil et al., 1996). Toadfish, which do not disperse in the plankton but which have greater adult movement than *Urosalpinx*, show divergences of 3.9%–5.5% between species (Freshwater et al., 2000). Dispersal characteristics have been shown to impact the levels of population differentiation in many taxa (e.g., plants—Hamrick and Godt, 1996; blennies—Riginos and Victor, 2001; bryozoans—Watts and Thorpe, 2006). It seems reasonable to expect that dispersal ability might also explain a large portion of the genetic differentiation among congeners as well.

Molecular clocks have been widely employed for timing divergence events in marine environments (e.g., Knowlton and Weigt, 1998; Hellberg and Vacquier, 1999; Wares, 2000, 2001; Marko, 2002; Donald et al., 2005). The estimation of mutation rate is usually accomplished by comparing DNA sequences from species pairs that were separated by a major geological event, such as the rise of the Isthmus of Panama. Some variation among taxa is to be expected, but these rates are typically on the order of one or a few percent divergence per million years. Wares (2000) used the divergence between Atlantic and Pacific members of the muricid gastropod genus *Nucella*, combined with the timing of the opening of the Bering Strait (3.5 Mybp) to estimate a mutation rate for this genus of 1.21×10^{-8} per site per generation, or about 2.42% per Myr. Given the confamilial relationship of *Nucella* and *Urosalpinx*, we would suggest that this rate and its corresponding error (2.71×10^{-9} or 0.542%)

would be appropriate for estimating divergence times among members of the genus *Urosalpinx*.

Using all three positions to date interspecific divergence, the mean COI sequence difference of 10.7% we obtained for the split between *U. cinerea* and *U. perrugata* would imply a divergence time between 3.6 and 5.7 Mybp, in the early to mid-Pleiocene. The weighted mean 14.9% sequence difference we observed between *U. tampaensis* and the *cinerea/perrugata* average would suggest that *U. tampaensis* diverged from a hypothetical *cinerea/perrugata* stem in the late Miocene, between 5 and 7.9 Mybp. These estimates agree fairly well with those from the fossil record. Petuch (2004) suggested that *Urosalpinx* probably diversified in the mid-Pliocene about 3.6–2.6 Mybp. Gene trees are expected to diverge before the appearance of the species they represent in the fossil record, which could explain the slightly earlier dates estimated from sequence data.

Our estimates of time since divergence suggest that sea level fluctuation and the presence of the Sewanee Strait might have been involved in speciation in the genus *Urosalpinx*. This prehistoric seaway through the northern part of the Florida peninsula has been implicated as a factor in speciation of stone crabs (*Menippe* spp.) in the western Atlantic (Bert, 1986). Its presence could have allowed populations of *Urosalpinx* to exchange migrants between the Atlantic and Gulf of Mexico during the late Miocene and (after its closure) isolated these populations to speciate allopatrically (Palumbi, 1994).

In the present day, however, our results confirm that a large and viable population of *U. cinerea* has secondarily colonized the coastal waters of the Gulf of Mexico in the vicinity of Cedar Key, and is now reproductively isolated from both *U. perrugata* and *U. tampaensis*. Although allozyme allele frequencies show a high level of similarity between the Cedar Key and South Carolina populations of *U. cinerea*, the former population carries a 6PGD allele in high frequency not detected in the latter. The two sets of CO1 sequences are also similar but distinct, the single haplotype identified from Cedar Key differing by but a single nucleotide from one of the four haplotypes sequenced from South Carolina. Future research on genetic variation among populations of *U. cinerea* sampled throughout its native range on the Atlantic coast may yield additional clues regarding the precise origin of the Cedar Key population.

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