

## Ecological partitioning among parapatric cryptic species

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### Abstract

Geographic range differences among species may result from differences in their physiological tolerances. In the intertidal zone, marine and terrestrial environments intersect to create a unique habitat, across which physiological tolerance strongly influences range. Traits to cope with environmental extremes are particularly important here because many species live near their physiological limits and environmental gradients can be steep. The snail *Melampus bidentatus* occurs in coastal salt marshes in the western Atlantic and the Gulf of Mexico. We used sequence data from one mitochondrial (COI) and two nuclear markers (histone H3 and a mitochondrial carrier protein, MCP) to identify three cryptic species within this broad-ranging nominal species, two of which have partially overlapping geographic ranges. High genetic diversity, low population structure, and high levels of migration within these two overlapping species suggest that historical range limitations do not entirely explain their different ranges. To identify microhabitat differences between these two species, we modelled their distributions using data from both marine and terrestrial environments. Although temperature was the largest factor setting range limits, other environmental components explained features of the ranges that temperature alone could not. In particular, the interaction of precipitation and salinity likely sets physiological limits that lead to range differences between these two cryptic species. This suggests that the response to climatic change in these snails will be mediated by changes to multiple environmental factors, and not just to temperature alone.

*Keywords:* cryptic species, intertidal gastropod, *Melampus bidentatus*, niche modeling, range limits, temperature

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### Introduction

Interspecific differences in geographical range are driven by a variety of factors, including differences in environmental tolerance, dispersal, biotic interactions and history (Engle & Summers 1999; Gaston 2003; Sexton *et al.* 2009). The ability to tolerate a location's abiotic environment is a minimal requirement for residing there, and the influence of climate on range limits has been shown many times, both through the coincidence of species' ranges with geographic shifts in climate (Root 1988; Rogers & Randolph 1991; Gaston 2003) and with range shifts associated with climate changes over time (Murawski 1993; Whittaker & Tribe 1996; Mehlman

1997; Chen *et al.* 2009). When accounting for physiological causes of range differences, many studies invoke a single niche component (e.g. temperature, Gaston & Spicer 1998; Stillman 2002; salinity, Bertness *et al.* 1992; Chen & Hare 2008; pollutants, Cohen 2002), but ecological differences among species commonly depend on the combined interaction of multiple environmental axes (Hutchinson 1957; Hylleberg & Siegmund 1987; Ackerly 2003; Harmon *et al.* 2005; Alvarez *et al.* 2006; Colwell & Rangel 2009). Among highly similar species, including cryptic species that can only be distinguished genetically, small differences in environmental requirements may only be identified through multidimensional comparisons (Hirzel *et al.* 2002; McCairns & Bernatchez 2008; Nosil 2008; Depraz *et al.* 2009). The identification of cryptic species and their corresponding ecological niches is important to understand the creation and

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maintenance of biodiversity (Bickford *et al.* 2007; Hellberg 2009; Oliver *et al.* 2009); and correctly interpret species relationships and history (Marko & Moran 2009).

In the marine environment, genetic analyses have revealed that many nominal species are actually complexes of cryptic species (Knowlton 1993; Chen & Hare 2008; Hellberg 2009; Marko & Moran 2009). This suggests that some marine species may not be ecological generalists as previously thought, but are instead collections of more ecologically specialized taxa (Grosberg & Cunningham 2001). The large number of cryptic species in the ocean is particularly interesting given that many marine species produce planktonic larvae, the dispersal of which can counteract local adaptation and genetically homogenize populations over large areas (Slatkin 1987; Brown *et al.* 2001; Broquet & Petit 2009; Hellberg 2009). Despite this apparent dispersal ability, larvae are sometimes retained in their natal habitat (Swearer *et al.* 2002), leading to reduced population connectivity and increased local adaptation. Close examination has revealed instances where cryptic marine species partition their habitat by environmental variables, including depth (Prada *et al.* 2008; Vonlanthen *et al.* 2009), temperature (Miller *et al.* 2009b), and salinity (Bertness *et al.* 1992; Chen & Hare 2008; McCairns & Bernatchez 2008).

The role of the environment in setting range limits in the intertidal has been long studied (Connell 1961; Tomanek & Helmuth 2002). This is in part because geographic range changes can often be simplified to one-dimensional shifts in this near-linear habitat (Barry *et al.* 1995; Sagarin *et al.* 1999). Many intertidal species live near their limits of thermal tolerance (Connell 1961, 1972; Helmuth *et al.* 2006b; Somero 2002; Stillman 2002; but see: Gilman 2006; Helmuth *et al.* 2006a), suggesting that small changes in temperature may be lethal and could lead to such range shifts (Barry *et al.* 1995; Dong & Somero 2009; Miller *et al.* 2009a). Over the vertical space between the high and low intertidal, temperature is a major stressor and conditions shift rapidly and unevenly between locales, depending on air and water temperature, submersion period, and the time of day when the tide is lowest (Helmuth *et al.* 2006a). Low tidal elevations require species to be adapted to a largely subtidal lifestyle. At the highest portion of the intertidal, species must endure long dry periods and a wide range of salinities (Bertness 1991; Bertness *et al.* 1992). Outside of the intertidal, the critical temperature (maximum or minimum) that a species can tolerate is often tied to range limits (Gaston 2003), and has been proposed as a factor limiting geographical range in groups including insects (Strathdee & Bale 1998), fish (Cussac *et al.* 2009; Figueira & Booth 2010; Fodrie *et al.* 2010), plants (Lau *et al.* 2008), and amphibians (Wiens *et al.* 2006).

*Melampus bidentatus* (Ellobiidae, Pulmonata, Say 1822) is a pulmonate (air breathing) snail reported from coastal salt marshes and mangrove swamps from southern Canada to the northern Caribbean, a range spanning over 25° of latitude (Martins 1996). Throughout its range, *M. bidentatus* relies on decaying marsh grass (*Spartina alterniflora*) both as its primary food source and as habitat (Rietsma *et al.* 1988; Graca *et al.* 2000; Galvan 2008). Its distribution has been considered to be controlled by tolerance to physiological stressors (Daiber 1982; Bishop & Hackney 1987), although snails can be preyed upon by the killifish *Fundulus heteroclitus* (Vince *et al.* 1976) and by small crabs (Lee & Silliman 2006), and its occurrence at lower tidal elevations may be restricted by competition with the periwinkle *Littoraria irrorata* (Lee & Silliman 2006).

Here, we investigate the environmental factors underlying different geographic distributions for a pair of cryptic species within the nominal species *M. bidentatus*. Physiological limitations are potentially important for these two species because they do not appear to differ in their habitat or diet preferences and appear capable of broad dispersal. Food (*Spartina*) is not a limiting resource in this system (Thompson 1984), thus competition for food is unlikely to have driven cryptic species within *M. bidentatus* to partition the habitat according to diet. The planktonic larval stage of *M. bidentatus* should enable it to disperse widely, and we use genetic estimates of connectivity to test that assumption here. *M. bidentatus* has a high reproductive output (~33 150 eggs/female/year, Apley 1970) and its larvae spend 7–14 days in the plankton (Russell-Hunter *et al.* 1972; Schaeffer *et al.* 1985), suggesting that some larvae likely disperse over large distances every generation.

To identify differences in physiological tolerance that might underlie differences in geographic ranges among cryptic species, we have predicted species distributions using ecological niche models, and defined the differences among the environments these species occupy using a discriminant analysis. Estimates of connectivity and measures of genetic diversity among populations within each species were used to test for evidence of ongoing dispersal and recent population changes. Our results suggest that while temperature plays a major role in shaping range, other components of the environment (precipitation and salinity) explain features of geographic range that temperature alone cannot.

## Methods

### Collections

Snails were morphologically identified as *M. bidentatus* (Say 1822) based on observations of museum collections

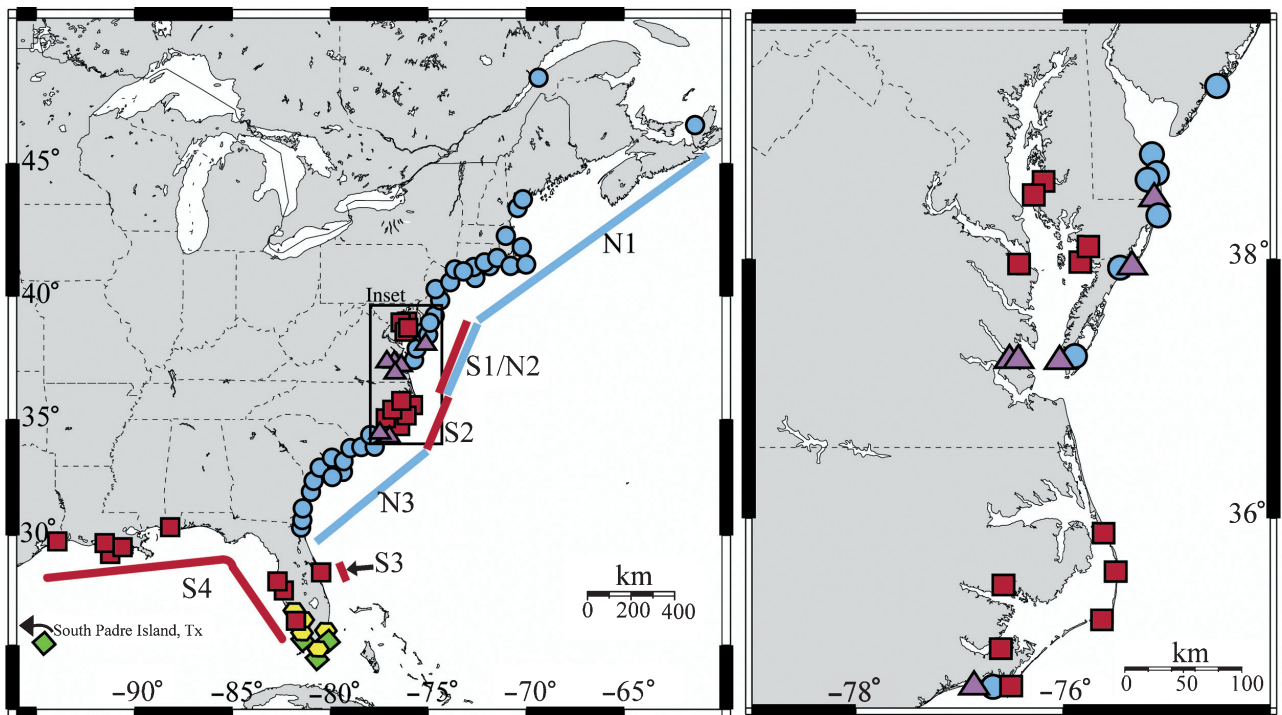


Fig. 1 Collections of cryptic species within *M. bidentatus* as identified by CO1 sequence. Red squares and blue circles represent cryptic species North and South, respectively, and sites where the two occurred in sympatric are indicated by purple triangles. Cryptic species Gulf was collected in sites marked by green diamonds, and the sister species, *M. coffeus*, was found at the sites marked by yellow hexagons. Groupings of collection locales for Migrate-n and AMOVA analyses in North and South are indicated with red (South) and blue (North) lines.

and the descriptions of Martins (1996). Despite previous mention of geographic variation in *Melampus* (Morrison 1950; Martins 1996), no cryptic species could be identified using shell colouring or shape. Collections were made at 74 coastal locales between Cape Breton, Nova Scotia and South Padre Island, TX between February 2005 and August 2008 (Fig. 1, Appendix S1, Supporting Information) and were concentrated where the two Atlantic cryptic species overlap (between Jacksonville, FL and Fenwick Island, DE, see 'Results'). Sequence data from Sapelo Island, GA were kindly provided by John Wares, and are a portion of the *M. bidentatus* sequences used in Robinson *et al.* (2009). The reported (Martins 1996) sister species of *M. bidentatus*, *M. coffeus*, was found at nine locales within the range of *M. bidentatus* and was collected at three additional locales in the southern Caribbean (Appendix S1, Supporting Information). *M. bidentatus* and *M. coffeus* are the only Atlantic representatives of the subgenus *Melampus Melampus* (Beck 1837; Martins 1996). Sequence data from other *Melampus* in the northern Atlantic (*M. Detracia bullaoides*, *M. D. floridanus* and *M. D. monile*) confirm that *Melampus Melampus* is monophyletic and phylogenetically distant from *M. Detracia* sp., and that our collections are not synonymous with any other species

(A. Dennis, unpublished). Whole snails were preserved in 95% ethanol in the field.

#### DNA sequencing, identification of cryptic species and tests for connectivity within species

DNA was extracted using either the QIAamp DNA mini kit, with tissue digested overnight and all other steps according to the manufacturer's instructions (Qiagen #51304), or using Chelex beads (Sigma #C7901), in which  $\sim 1 \text{ mm}^3$  of foot of tissue was incubated in a 20% Chelex solution at room temperature overnight, after which it was heated to 95 °C for 5 min and centrifuged for 5 min at 1500 g prior to PCR.

We sequenced portions of one mitochondrial and two nuclear encoded genes within *M. bidentatus* and *M. coffeus*. A portion of the mitochondrial cytochrome oxidase subunit I (CO1) gene was amplified using primers from Folmer *et al.* (1994): we used their primer HCO1 with a modification of their LCO1 (MCO1: GGTCACAAAT-CATAAGGATATTGG). The length of useable CO1 sequence ranged from 440 to 650 bp, depending on sequence quality and whether an individual was sequenced in both primer directions. To maximize the number of individuals included in the analysis, all

sequences were trimmed to 448 bp. A 316-bp portion of the histone H3 gene was amplified using the primer pair H3F/H3R (Colgan *et al.* 2000). Both CO1 and H3 were amplified using the PCR with an annealing temperature of 50 °C. A third marker was amplified using primers that we designed based on cDNA sequence from *M. bidentatus*. We have tentatively identified this (using blastx) as a portion of the nuclear encoded mitochondrial carrier protein/ADP-ATP transporter and called it MCP. Internal primers designed from this marker (MCPL: GTCTGTTGGCCCACTTGACT and MCPR: TCCTTGCATGGAATAACCAA) were used under standard PCR conditions with an annealing temperature of 52 °C to amplify 128 bp. All sequence data was edited in Geneious (Drummond *et al.* 2009) and aligned by eye; there were no indels. MCP sequences containing more than one ambiguous site were resolved using PHASE v2.1.1 (Stephens *et al.* 2001; Stephens & Donnelly 2003), for which infiles were prepared using SeqPhase (Flot 2010). To resolve sequences with multiple heterozygous sites, PCR products from six individuals (each containing a different combination of two or three heterozygous sites) were cloned using a TOPO TA cloning (Invitrogen Cat# K4575), and sequenced using the M13 forward and reverse primers provided by Invitrogen. There were no ambiguous sites suggesting heterozygosity in either H3 or CO1.

The aim of this sequence analysis was to identify genetically isolated groups rather than to infer relationships among them. To achieve this, 476 CO1 sequences from snails morphologically identified as *M. bidentatus* and *M. coffeus* (Tables 2 and 3) were used to generate an unrooted neighbour joining tree in PAUP (Swofford 2003), and the percent divergence within and between the groups that this generated was calculated in DNAsp using a Jukes and Cantor model of nucleotide substitutions (Rozas *et al.* 1995-2009). The relatively smaller number of differences at the two nuclear markers (H3 and MCP) was summarized based on both the number of fixed differences and the average percent divergence among groups, also calculated in DNAsp. The relationship among alleles for these two markers was visualized using a haplotype network for each gene using TCS (Clement *et al.* 2000), constructed with a limit of five connections. Based on the unambiguous assignment of all *M. bidentatus* individuals to three clades with large (>20%) COI sequence divergence and divergence among the same groups at the two nuclear loci, we designated three groups within *M. bidentatus* as cryptic species and called them North, South and Gulf (see 'Results'). Remaining analyses of population structure were done within or between the cryptic species and *M. coffeus*.

Only two of the cryptic species (North and South; Fig. 1, 'Results') were found at most US locales. To test

whether dispersal limitation can explain the different range limits of these two species, we used 448 bp of CO1 sequence from 407 individuals to estimate connectivity among populations within cryptic species. Estimates of migration among regions were made using the Bayesian inference method in the program Migrate-n version 3.0 (Beerli & Felsenstein 1999, 2001; Beerli 2006). Migrate-n estimates pairwise migration rates scaled by mutation ( $M = m/\mu$ , where  $m$  = migration and  $\mu$  = mutation) among groups and simultaneously calculates  $\theta$  ( $\theta = N_e\mu$  for this haploid mitochondrial marker). Collection locales were grouped for this analysis to determine dispersal patterns among regions, rather than among neighbouring marshes, and to reduce the number of simultaneously estimated parameters. Within North, three groups were designated: N1, Locales above latitude 39°, where North is allopatric to South; N2, between latitudes 34.5° and 39°, where North is sympatric with South; and N3, below latitude 34.5°, where North allopatric to South. Four groups were designated in South: S1, between latitudes 34.5° and 37°, where South is in sympatry with North; S2, below 35° latitude, where South is in sympatry with North in North Carolina; S3, the single collection of South at Cape Canaveral, FL; and S4, South in allopatry in the Gulf of Mexico (Fig. 1, Tables 3 and 4). Each species' transition: transversion ratio was set based on its calculation in the program MEGA 4.1 (Tamura *et al.* 2007). Three preliminary runs, each recording one of every 10 genealogies out of 10 000 000 visited, with a burnin of 50 000 and an adaptive heating scheme using four temperatures (1, 1.5, 3 and 1000) were used to set starting values of  $M$  (South: 174, 58, 78, 171, 574, 302, 339, 333 and 117; North: 195, 864, 110, 280, 239 and 82) and  $\theta$  (South: 0.01963, 1.94, 0.0327 and 2.574; North: 2.35, 2.61 and 0.0256). The final Migrate-n runs recorded 1 000 000 trees from 10 000 000 visited, with a burnin of 10 000 trees per chain and a static heating scheme (temperatures 1, 1.5, 3 and 6), and upper bounds for  $\theta$  and  $M$  of 10 and 1500, respectively. To verify that these sequences are not undergoing recombination (an assumption of Migrate-n) the entire alignment was tested using a maximum chi square test ( $maxchi^2$ , Smith 1992; Piganeau *et al.* 2004).

To test for differences in genetic diversity among regions that could indicate recent range expansions or genetic bottlenecks in North or in South, haplotype diversity ( $H_d$ ) and Tajima's  $D$  were calculated in DNAsp using the same seven groups of locales as in Migrate-n (Fig. 1, Table 3). We also calculated the effective number of alleles ( $[1/(1-H_d)]$ ; Kimura & Crow 1964; Jost 2007, 2008) to compare the diversity among regions on a linear scale.

High genetic diversity at CO1 meant that few haplotypes were shared within or among collection locales,



and because of this,  $F_{ST}$  was an inappropriate measure of population structure (Hedrick 1999): comparisons within sites were similar to estimates among sites, suggesting complete panmixia ( $F_{ST} \approx 0$ ). Therefore, calculations of genetic structure that incorporated the distance among haplotypes ( $\Phi_{ST}$ ) were used to measure population structure among collection locales and among regions using Arlequin (Excoffier *et al.* 2005). Collection locales were grouped into the same regions used for the Migrate-n analysis (Fig. 1 and Table 3), and pairwise  $\Phi_{ST}$  was also calculated among collection locale within species. Mutational models selected for CO1 by dtmodel (Trn + I + G; Minin *et al.* 2003) were more complicated than what is available in Arlequin, so calculations of  $\Phi_{ST}$  were conducted using the most complex model available (Tamura & Nei 1993), and were calculated using 10 000 replicates.

#### Ecological niche modelling

To compare the environments occupied by cryptic species North and South, we constructed the ecological niche of these two species. Cryptic species composition of 15 locales, in addition to the 65 above, was identified based on sequencing in only one primer direction. These sequences were often relatively short (250–440 bp) and thus were not included in the above analysis of population connectivity. To predict where each species occurs over geographic space, we used a maximum entropy model implemented in the program Maxent (Phillips *et al.* 2006; Phillips & Dudik 2008), in which the probability of a species' occurrence is estimated based on a uniform probability distribution (maximum entropy) and on presence data provided by the user. We chose Maxent because of its performance using presence-only data (Elith *et al.* 2006; Graham & Hijmans 2006; Bigg

*et al.* 2008). Our two northernmost collections (Appendix S1, Supporting Information) were excluded from niche modelling because they were not covered by some environmental layers. Because the cryptic species require identification from sequence data, we did not include collections other than our own (Lozier *et al.* 2009).

We used both marine and terrestrial data to model the intertidal environment. A total of 77 geo-referenced environmental layers were obtained (Table 1). The Worldclim website (Hijmans *et al.* 2005) provided all terrestrial data: monthly maximum and minimum temperatures, monthly precipitation and their 19 bioclimatic variables were downloaded at a 2.5 arc-map unit resolution. Monthly, seasonal and annual sea surface salinity for 2005 were downloaded from the National Oceanographic Data Center's World Ocean Atlas (Antonov *et al.* 2005) and interpolated in Arcmap using the IDW method with a fixed radius of 0.5 (ESRI 1999–2006; Childs 2004). Sea surface temperature (SST) was downloaded as a geoTIFF from the Global Ocean Surface Temperature Atlas Plus (Rayner *et al.* 1997). Mean tidal height was downloaded from the USGS database of Coastal Vulnerability to Sea-Level Rise (Thieler & Hammar-Klose 1999, 2000). All layers were trimmed to an extent that broadly encompassed the reported range of *M. bidentatus* (–113, 12, –52, 56) and converted to a cell size of 0.4167 to match the resolution of the Worldclim layers. Maxent produces ecological niche models only for areas where all environmental data are present, so all predictions resulted in a thin strip of coast (Fig. 3). When the environmental layers covered more land than the intertidal (e.g. the simulations that were run using only terrestrial data covered all of North America), we restricted the simulation to the coast by including an additional, uninformative environmental layer that contained a single datum along the coast.

**Table 1** Summary of environmental layers used in ecological niche modelling

Data	Source	Resolution	Number of layers
Terrestrial temperature: monthly maximum and minimum	Worldclim (Hijmans <i>et al.</i> 2005)	2.5 arc-min	24
Terrestrial precipitation: monthly average	Worldclim (Hijmans <i>et al.</i> 2005)	2.5 arc-min	12
19 bioclimatic variables, terrestrial (Bio1–Bio19)	Worldclim (Hijmans <i>et al.</i> 2005)	2.5 arc-min	19
Ocean salinity: monthly, seasonally and annual averages	National Oceanographic Data Center's World Ocean Atlas (Antonov <i>et al.</i> 2005)	¼ degree	17
Sea surface temperature: seasonal averages.	Global Ocean Surface Temperature Atlas Plus (Rayner <i>et al.</i> 1997)	4 km	4
Mean tidal height	USGS database of Coastal Vulnerability to Sea-Level Rise (Thieler & Hammer-Klose 1999, 2000)	3 arc-min	1

Shaded rows indicate data from the marine environment.

Maxent was run with six configurations of environmental layers to compare the predicted niche resulting from different components of the environment (Table 5). It was first run (1) with all 77 layers. To determine the independent effects of the land and sea, it was then run using (2) only terrestrial layers and (3) only marine layers (Table 1). These first three configurations suggested that temperature played a major role in predicting range. To test for the independent contribution of variables related and unrelated to temperature, variables were separated for additional runs using (4) only temperature layers (maximum and minimum terrestrial temperature, SST and bioclim variables 1–7 and 10–11) and (5) all non-temperature layers (precipitation, mean tidal height, ocean salinity and bioclim 12–17).

To reduce the use of highly correlated environmental layers, a reduced model (6) was built using 19 environmental layers according to the following criteria. Pairwise regression in DIVA (Hijmans & Spooner 2001) showed all maximum and minimum temperature layers to be highly correlated ( $r^2 > 0.9$ ).  $T_{\max}^{10}$  (maximum temperature in October) and  $T_{\min}^2$  (minimum temperature in February) were chosen from these because they repeatedly had the highest contribution when Maxent was run using all variables (based on jackknifing of model gain in the test and training data). SST from April to June (SST2) was chosen because it was the most highly correlated with the other three seasonal averages (respective  $r^2$  values: 0.72, 0.75 and 0.92). All ocean salinity files were also highly correlated with each other ( $r^2 > 0.9$ ), so winter salinity was chosen because it contributed the most to Maxent runs with all variables. Precipitation files were not as highly correlated among months, but in runs with the full model, the highest contribution was made by precipitation in February, March and June and August. Bioclim layers 1–3, 7–9 and 15–19 were used because they are not significantly correlated in North America (Rissler & Apodaca 2007).

All six configurations of the environmental layers were used to construct niche models in Maxent version 3.3.0 (Phillips *et al.* 2006) using the species locations of North and South as shown in Fig. 1 and Appendix S1 (Supporting Information). Maxent was run for 10 replicates with the default settings, except that we randomly set half of the points to build the model and the other half to test it. Models were evaluated based on receiver operating characteristic (ROC) analysis. This generates the AUC (receiver operating statistic) score, a measure of how well the data fits the model; values closer to 1 indicate better fit between the model and the data and 0.5 indicates that the fit is no better than random. For all models, the AUC score was generated for both the random half of the data used to build the model (training) and for the remaining data (test).

The ranges of both North and South contained several features that marked their distributions as being more complex than the simple latitudinal replacements of one another. To examine the presence of these features in the model output, we made a visual comparison between the predicted range generated by Maxent and the known species extent (Fig. 1). In particular, we looked for the following features: (i) correct northern and southern range limits, (ii) the absence of South in the SC-FL region and the (iii) absence of North from locales inside the Chesapeake Bay.

For each model, we compared the outputs using ENMtools (August 2009 version), following the methods of Warren *et al.* (2008). Briefly, we measured how different North and South are from one another (i.e. how different from identical, the 'niche overlap') using Schoener's D and a standardized version of Hellinger distance (calculated as I, see Warren *et al.* 2008). This measure was compared to the test of 'niche identity', which builds niche models based on a set of pseudoreplicates generated from a random sampling from all of our data points, pooled for both species. We generated 100 of these pseudoreplicates and compared the distribution of these simulations to the actual niche overlap Maxent runs that were created using all points to build the model. If the niche identity generated from the combined pool is greater than the actual niche overlap between species, the two ecological niches are less similar than random. We have not included range predictions for the last glacial maximum (Richards *et al.* 2007; Cordellier & Pfenninger 2009) because preliminary runs did not generate distributions that differed between the two species, likely because of the absence of marine data in the available climate models.

#### *Point based analysis of environmental variables*

Despite the use of our 'reduced' model, pairwise comparisons showed some layers were still correlated. To reduce these correlations and to avoid spatial autocorrelation (Legendre 1993), we conducted a principal components analysis (PCA) based on the environmental data extracted for each of our collection points. At each collection point, environmental data from all layers was extracted to a spreadsheet using ArcMap. A detrended correspondence analysis (DCA) conducted in Canoco for Windows 4.5 (ter Braak 1995) produced an axis length less than 2, suggesting that the data is linear and a PCA is appropriate for this data (Lepš & Šmilauer 2003). The PCA was performed in SPSS ((2007) release 16.0.1) with the principal components rotated by the varimax procedure prior to interpretation and extraction to maximize the separation among components. We chose to use the first six components as variables because these were the only

components whose eigenvalues exceeded one, and this value declined quickly in subsequent components (Kaiser 1960). Although there may be problems with using this criterion for variable cutoff with a large number of variables (Netemeyer *et al.* 2007), the scree plot levelled off after the first three components and the first six components explained 96% of the variance, suggesting that these six components largely encompass the of variance of the system (Stevens 2002). These components were extracted as variables in SPSS, thus reducing the 77 environmental variables to a set of six uncorrelated variables.

To quantify the differences between North and South, these six variables were used as independent variables (a requirement of discriminant analysis) in a standardized discriminant function in SPSS, conducted to differentiate the two species. The discriminant analysis uses the environmental variables (now arranged as linear principal components) to build a linear function that defines the difference between two groups. The sign and magnitude of each variable in the discriminant function can be used to interpret their role in differentiating the two cryptic species. Larger magnitude variables are interpreted as having the greatest role in differentiating North and South. Results of the discriminant analysis were evaluated based on the number of collection locales for which the cryptic species they contained were correctly identified.

## Results

### *Cryptic species within M. bidentatus*

A total of 476 individuals morphologically identified as *M. bidentatus* and *M. coffeus* were sequenced for 448 bp

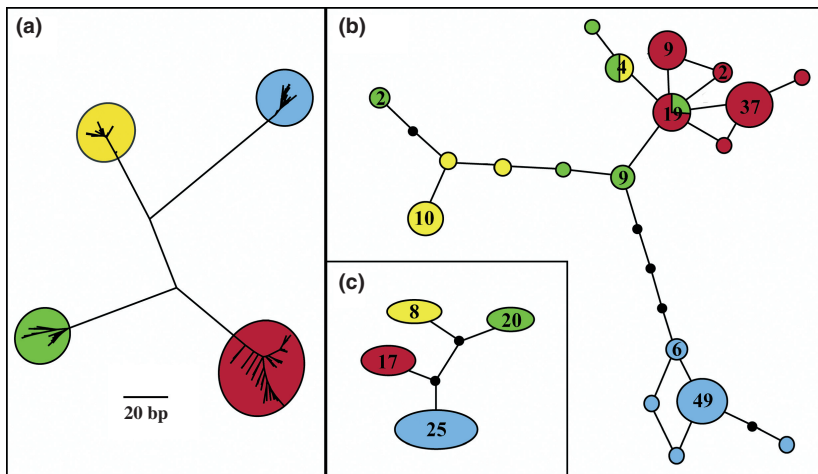
of CO1, 68 individuals were sequenced for 316 bp of H3 and 78 for 128 bp of MCP (GenBank accession numbers HM153824–HM154497). These three genes had 166, 5 and 16 variable sites, respectively, resulting in 269, 4 and 19 haplotypes across all individuals. CO1 sequence from individuals identified as *M. bidentatus* grouped into three monophyletic groups separated by 22–24% sequence divergence (Table 2 and Fig. 2), and these groups have been designated North, South, and Gulf. These same groups were also genetically distinct at the two nuclear loci, H3 and MCP, differing by 0.62–0.98% and 1–5%, respectively (Table 2). From MCP, one haplotype was shared between Gulf and South, and one between Gulf and *M. coffeus* (Fig. 2). There were no shared alleles among species for H3, for which only one haplotype was found in each species. North and South did not share haplotypes at any marker.

Most importantly, the mitochondrially defined North and South that we are comparing using ecological niche modelling differed at both nuclear loci (H3: 0.62% and MCP: 5.45%), although we included individuals from sites where North and South were found in complete sympatry (H3: 6 North and 10 South, MCP: 15 North and 18 South). The levels of mitochondrial divergence among the three major mitochondrial lineages in *M. bidentatus* were equal to (or greater than) those seen in other cryptic species complexes (Barroso *et al.* 2010), including other pulmonate snails, where substitution rates can be high (Chiba 1999; Van Riel *et al.* 2005; Pfenninger *et al.* 2006). Genetic divergence between species was higher than within species (Table 2) and genetic groups were strictly concordant across all three loci, suggesting that there is no gene flow among mitochondrial lineages, as is sometimes seen in terrestrial

**Table 2** Mitochondrial (above) and nuclear (below) sequence divergence among and within (shaded diagonal) *M. melampus* sp.

CO1 H3/MCP	<i>M. bidentatus</i> North (278)	<i>M. bidentatus</i> South (127)	<i>M. bidentatus</i> Gulf (21)	<i>M. coffeus</i> (50)
<i>M. bidentatus</i> North (24/58)	CO1: 1.32% ± 0.04 H3: 0% MCP: 0.28% ± 0.08	CO1: 23.93% ± 0.67 (19)	CO1: 25.07% ± 1.56 (30)	CO1: 23.09% ± 0.96 (27)
<i>M. bidentatus</i> South (17/62)	H3: 0.62% ± 0.19% (1) MCP: 5.46% ± 0.75 (5)	CO1: 2.217% ± 0.09 H3: 0.04% ± 0.03 MCP: 0.64% ± 0.07	CO1: 22.03% ± 1.14 (35)	CO1: 20.91% ± 0.78 (17)
<i>M. bidentatus</i> Gulf (8/20)	H3: 0.96% ± 0.29 (3) MCP: 4.75% ± 0.01 (4)	H3: 0.97% ± 0.31(3) MCP: 1.38 ± 0.27 (0)	CO1: 3.00 % ± 0.25 H3: 0% MCP: 1.04% ± 0.18	CO1: 26.28 % ± 1.54 (39)
<i>M. coffeus</i> (8/16)	H3: 0.96% ± 0.38% (3) MCP: 6.75% ± 1.57 (4)	H3: 0.98% ± 0.39 (3) MCP: 2.50% ± 0.54 (0)	H3: 0.64% ± 0.26 (2) MCP: 2.38% ± 0.55 (0)	CO1: 4.42% ± 0.81 H3: 0% MCP: 1.52% ± 0.37

Percent divergence and standard deviation from one mitochondrial (CO1) and two nuclear (H3 and MCP) markers. Number of alleles sequenced is indicated in parentheses below the species names, and the number of fixed differences between species at the respective marker is indicated in parentheses below their percent divergence.



**Fig. 2** Molecular data used to designate cryptic species. (a) Unrooted neighbour joining tree based on 448 bp of CO1 data, representing 476 individuals identified as *M. bidentatus* and its sister species, *M. coffeus*. (b) and (c) are haplotype networks based on (b) 128 bp of the marker MCP and (c) 316 bp of histone H3. Lines indicate one mutational step and black circles represent inferred haplotypes. Numbers within each circle represent the number of times that allele was found; small circles were represented by one copy. Circle colours are based on cryptic species designations according to neighbour joining analysis using CO1. North in blue, South in red, Gulf in green and *M. coffeus* in yellow.

pulmonates (Thomaz *et al.* 1996; Davison 2000). *Melampus coffeus* was found at several collection locales in south Florida and shared locales with both South and Gulf, but Gulf and South were never found together.

We assigned names to the three groups within the nominal *M. bidentatus* based on their different geographic ranges (Fig. 1). North ranged farthest north (>47°N) and was the only species found above 39°N latitude. Its range extended south to northern Florida, but it did not occur in the Gulf of Mexico. South occurred on the Atlantic coast from Delaware to approximately Cape Canaveral, FL and was also found in the northern Gulf of Mexico. North and South were the only species that occurred regularly in temperate areas. North and South spanned 18° and 12° of latitude, respectively and shared a large portion of their ranges (approximately latitudes 34.5°N to 39°N, Fig. 1). South was not found at any Atlantic localities between 30°N and 34.5°N

despite sampling at 11 locales in this area, including both coastal and low-salinity estuarine sites. The third cryptic species, Gulf, was found only in southernmost Texas and Florida.

Genetic diversity within species was high for CO1, and most regions contained nearly as many haplotypes as individuals sampled (Table 3). At the same time, shared haplotypes were found across the entire range in both species (e.g. from Virginia and Louisiana in South and from New Brunswick Canada and Florida in North). Estimates of  $\theta$  were highest for the two northern groups of North (N1 and N3), in Gulf of Mexico populations of South (S4), and the North Carolina populations of South (Table S3 and Table 3). Haplotype diversity was greater than 0.9 for most regions, with the exception with the exception of Cape Canaveral collections of South (S3) and the southernmost region of North (N3, Table 3). Among the regions with relatively

**Table 3** Genetic diversity within cryptic species North and South, divided by region

Cryptic species	Group	Region	<i>n</i>	<i>H<sub>d</sub></i>	Effective #alleles	Tajima's <i>D</i>	$\theta$ (95% CI)
<i>M. bidentatus</i> North	N1	Allopatric above 39° latitude	116 (73)	0.968 (0.009)	71.4	-1.97*	2.4 (0, 7.7)
	N2	In sympatry w/South (34.5°-39°)	65 (49)	0.969 (0.013)	32.3	-2.28**	6.1 (1.9, 10)
	N3	Allopatric below 34.5° latitude	97 (42)	0.771 (0.046)	4.4	-2.21**	0.024 (0, 0.18)
	North overall	278 (148)	0.924 (0.013)	13.2	-2.30**		
<i>M. bidentatus</i> South	S1	In sympatry w/North (34.5°-39°)	60 (25)	0.902 (0.024)	10.2	-1.69 ns	0.019 (0, 0.16)
	S2	North Carolina (below 35°)	19 (14)	0.953 (0.036)	21.3	-1.21 ns	4.7 (0.06, 7.5)
	S3	Allopatric at Cape Canaveral, FL	16 (7)	0.625 (0.139)	2.7	-1.76 ns	0.0058 (0, 0.16)
	S4	Allopatric Gulf of Mexico: FL, AL and LA	33 (32)	0.998 (0.008)	500	-1.69 ns	5.9 (1.62, 10)
	South overall	127 (72)	0.961 (0.009)	25.6	-1.89*		

Allopatry and sympatry of North and South are in reference to one another, and groupings are as marked on Fig. 1. All estimates are based on 448 bp of CO1, using the individuals summarized in Appendix A1. Number of individuals, *n*, includes the respective number of haplotypes for that group in parentheses. Mean estimate of  $\theta$  include the 95% confidence intervals and haplotype diversity (*H<sub>d</sub>*) is given with one standard deviation. Significance levels of Tajima's *D*: \*\**P* < 0.01, \**P* < 0.05.



high haplotype diversity, the effective number of alleles was highest in the northernmost group of North (N1, 71.4), and in the Gulf of Mexico populations of South (S4, 500). Among Atlantic populations of South, the North Carolina region contained the highest number of effective alleles (21.3, Table 3).

The AMOVA analysis (Tables S2 and S3) detected significant population structure in both North ( $\Phi_{ST} = 0.23$ ) and South ( $\Phi_{ST} = 0.27$ ), and the largest proportion of the genetic variation came from within populations in both species (76.7% in North, 73.1% in South), followed by variation among groups (i.e. the seven groups used in the Migrate-n analysis: 20.19% in North, 18.92% in South). In North, the average pairwise  $\Phi_{ST}$  among collection locales was 0.17 ( $\pm 0.34$ ), and 172 of 630 of the comparisons were significant; more of the significant comparisons coming from the southernmost locales (N3, Table S3). Within each region, there were locales whose pairwise  $\Phi_{ST}$  with a geographically distant locale was non-significant and nearly zero (e.g. 0.00923 between Sapelo Island, GA and Bath, ME and  $-0.0086$  between Oyster, VA and Hammonasset, CT, Table S2). In South, the average pairwise  $\Phi_{ST}$  was 0.13 ( $\pm 0.25$ ), and 55 of the 210 comparisons were significant (Table S3). More significant values were found in comparisons that included locales from the Gulf of Mexico (S4), and there were again cases where the average pairwise  $\Phi_{ST}$  was small and non-significant across a wide geographic area (e.g. 0.00979 between Dauphin Island, AL and N. of Crisfield, MD).

Migrate-n results suggested that geographically disconnected regions exchange many migrants (Table 4). Although several of the migration estimates had wide confidence intervals that included zero, many estimates were large and did not include zero. In particular, these results suggested ongoing migration in South between the Gulf of Mexico and North Carolina (S4 to S2: 553.5 (534–1000)), a migration event that requires movement around the southern tip of Florida. In addition, estimates between the two allopatric portions of North's range (N3 to N1: 758.4 (549–978)) suggested that migrants are exchanged by geographically separate populations each generation. Pairwise estimates of the effective number of migrants per generation were high for most comparisons (52–932). In both species, northward exchange of migrants was generally lower (North: 52–758; South: 57–291) than southward migration estimates (North: 162–932; South 350–816), but this difference was not significant and confidence intervals were widely overlapping. Migration estimates between the Gulf of Mexico and Atlantic groups of South were also high, particularly the exchange between the Gulf and North Carolina (553 and 346). Values of Tajima's D were significantly negative in North, but not South

**Table 4** Pairwise migration rates within cryptic species

		<i>M. bidentatus</i> NORTH			<i>M. bidentatus</i> SOUTH
N2 → N1	52.3 (0,147)		S2 → S1	165.7 (0, 430)	
N3 → N1	758.4 (549, 978)		S3 → S1	57.4 (0, 164)	
N1 → N2	932.1 (528, 1410)		S4 → S1	72.8 (0, 212)	
N3 → N2	279.4 (39, 534)		S1 → S2	816.9 (566, 1000)	
N1 → N3	162.1 (0, 471)		S3 → S2	291.4 (0, 744)	
N2 → N3	175.4 (36, 336)		S4 → S2	553.5 (534, 1000)	
			S1 → S3	350.3 (0, 844)	
			S2 → S3	405.1 (0, 894)	
			S4 → S3	588.7 (218, 1000)	
			S1 → S4	75.5 (0, 192)	
			S2 → S4	346.1 (72, 630)	
			S3 → S4	88 (0, 184)	

Based on 448 bp of CO1, using the groupings in Fig. 1 and Table 3. Mean estimates of M include the 95% confidence intervals.

(Table 3), consistent with North being in the process of range expansion. Given the inferred southward direction of migration in North and the low variation in the southernmost population, such an expansion would appear to be to the south. The maximum chi-squared test found no evidence of recombination in the CO1 sequence data.

#### Ecological niche modelling

A total of 497 individuals were genetically identified as cryptic species North or South and used to construct ecological niche models. According to the AUC criteria, the best performing models in Maxent (Table 5) were those constructed using only temperature data (Fig. 2, test AUC 0.934 and 0.905) and only terrestrial data (test AUC 0.981 and 0.940). These two models overlapped in 33 variables and were similar, except that the land-only data set predicted North to occur at low levels in the northeastern Gulf of Mexico (not shown). Among other data sets, AUC scores for the test points were around 0.7 (Table 5), suggesting that the predictions of any variable combination we used were better than random (for which AUC = 0.5).

Some major features of the two species' ranges (range limits, the absence of South between SC and FL, and the absence of North in the inner Chesapeake Bay) were not predicted by the models that had the highest AUC scores (Fig. 3). The value of the AUC score as a primary judge of model accuracy has been questioned previously (Lobo *et al.* 2008). Neither the terrestrial-only nor the temperature-only models (AUC scores 0.905–0.994) predicted the absence of South from the southern Atlantic coast between SC and FL (Fig. 3). The predicted northern range limits of North and South were

**Table 5** Summary of ecological niche models generated for cryptic species North and South

Variables used	Species	AUC test (train)	Niche similarity <i>I</i> ( <i>D</i> )
1. Full model	North	0.755 (0.919)	0.656 (0.498)
	South	0.721 (0.864)	
2. Only terrestrial variables	North	0.981 (0.993)	0.703 (0.612)
	South	0.940 (0.994)	
3. Only marine variables	North	0.745 (0.870)	0.699 (0.516)
	South	0.570 (0.704)	
4. Only temperature variables	North	0.934 (0.965)	0.775 (0.642)
	South	0.905 (0.919)	
5. All non-temperature variables	North	0.766 (0.899)	0.7033 (0.550)
	South	0.705 (0.827)	
6. Reduced model	North	0.776 (0.883)	0.691 (0.517)
	South	0.667 (0.813)	

Niche similarity between North and South, measured by *I* (standardized Hellinger distance) and *D* (Schoener's *D*). The shaded rows are pictured in Fig. 3.

the same in the temperature-only model, despite the ~1200 km difference in where these endpoints actually occur. The absence of South in the SC-FL region was best predicted by the model built using all non-temperature variables and the reduced model, whose outputs were similar (the non-temperature output is shown in Fig. 3). The output from the remaining models looked similar to the non-temperature variable model; the full model predicted a lower probability of the occurrence of South throughout the Gulf of Mexico, and the water-only model overpredicted North at southern sites. Interpretation of variable contribution is difficult when there is high correlation among layers, but all simulations that included tidal height (represented by a single layer) ranked it near the top of the variable contribution to the model, according to the gain and jackknife values associated with that variable. Models run without the tidal height layer (not shown) did not predict the absence of South in the SC-FL region.

#### Point-based analysis

The PCA produced six components with eigenvalues greater than 1, which together explain >96% of the variance (Table 6). Eigenvalues of additional components were small (each contributed <1% to the remaining variance) and were therefore excluded from subsequent analyses. The six principal components can be lumped into categories according to the environmental variables with large loading values (>0.6) in the component matrix (Table S1, Supporting Information). We summarized the components to represent: (i) land and SST (as well as a few precipitation layers, mostly from warm months), (ii) ocean salinity, (iii) precipitation, (iv) mean

diurnal temperature (bioclim2), (v) temperature of the driest quarter (bio 9), and (vi) contained some precipitation, but had no variables strongly correlated with it. PCA using only the 'reduced' model (of 19 variables, not shown) yielded similar results, except salinity and temperature were grouped into the same component, likely because the reduced model included only one data point representing salinity.

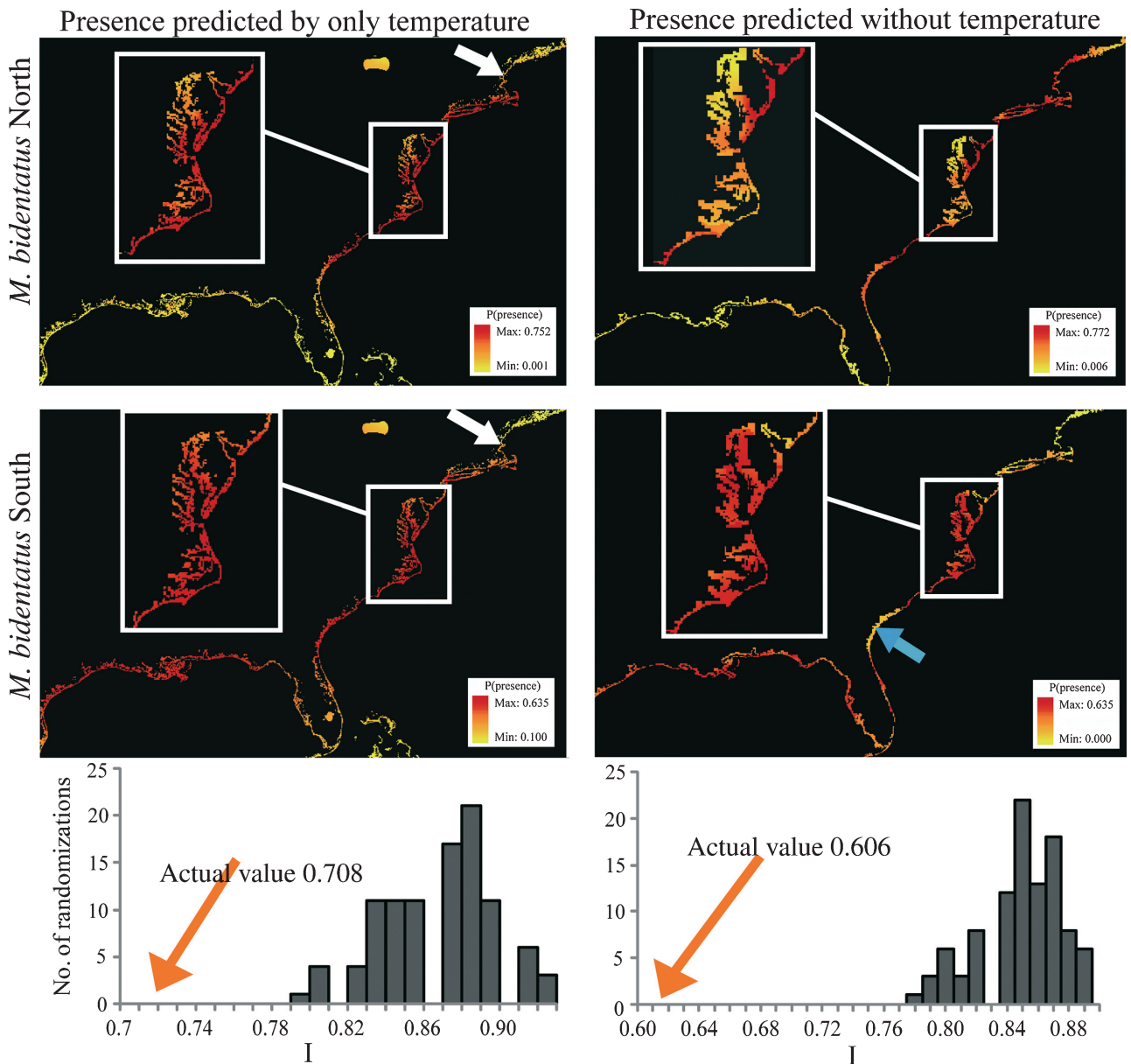
The two species were separated in one dimension by a discriminant analysis (Wilks'  $\lambda = 0.548$ ,  $\chi^2 P < 0.0001$ ). The first five principal components appeared to be important in separating the two species (absolute value of standardized canonical discriminant function coefficients between 0.257 and 0.846, Table 6). The positive values associated with temperature (component 1), precipitation (component 3) and diurnal temperature range (component 4) suggest that increases in their values are associated with South, whereas increases in ocean salinity (component 2) and temperature of the driest quarter (component 5) are associated with North (Table 6). Components 1 (temperature) and 2 (salinity) had the strongest influence on the discriminant equation (0.846 and -0.684, respectively). The contribution of each variable in the structure matrix paralleled that of the discriminant coefficients (not shown). The contribution of the tidal height layer was almost evenly distributed among all principal components (Component 1: -0.304, Component 2: 0.444, Component 3: -0.194, Component 4: 0.308, Component 5: -0.151, Component 6: 0.126).

Although the discriminant function was able to differentiate North from South, it placed 6 of 26 South locales and seven out of 46 North locale into the wrong group. Of these 13 misdiagnoses, six were from collection locations that contained both species in sympatry. Three of these sympatric locales were scored as North, three were scored as South. The remaining miscalled locales were in the region of range overlap (four in North Carolina) or at the southern range limit of North (two Florida locales).

#### Discussion

##### *Geographic ranges of the cryptic species within M. bidentatus*

Sequence data from samples collected throughout the geographical range of the nominal species *M. bidentatus* distinguished three cryptic species. We focused on the relative ranges of two of these species, North and South, because they were abundant in the temperate zone and the forces underlying their partial overlap were not immediately apparent (Fig. 1). The third cryptic species, Gulf, was only sampled from two disjunct sites (South Padre Island, TX and Southern Florida). Its



**Fig. 3** Predicted distributions of cryptic species North and South generated in Maxent. Highest probability of occurrence is shown in red, fading to areas with no predicted occurrence, shown in yellow. Distributions on the left were generated using only temperature data (test AUC 0.934 North, 0.905 South). Distributions on the right were generated using all variables besides temperature: precipitation, ocean salinity and tidal height (test AUC 0.766 North, 0.705 South). The white arrows indicate incorrectly predicted northern range limits from the temperature-only model. The blue arrow indicates the correct prediction of the absence of South's in the Georgia/South Carolina area, a feature predicted only by models that included tidal height. Inset maps show the Chesapeake Bay and Pamlico Sound area. Below the images are null distributions showing the overlap statistic ( $I$ ) for North and South generated using 100 randomizations. The orange arrow indicates the value of  $I$  in actual Maxent runs using all locality points, showing that the predictions by Maxent are less similar than expected by chance.

replacement of the temperate species (North and South) along the Florida Coast is consistent with the faunal transition from temperate to tropical biogeographical provinces in this region and coincides with many marine and coastal taxa that do the same (Engle & Summers 1999; Wares 2002). Based on these shared

distributional patterns, we predict that the nominal *M. bidentatus* found in the southern Gulf of Mexico and the northern Caribbean (primarily Cuba and Mexico) will turn out to be this Gulf form.

The relative ranges of North and South along the Atlantic coast were more unusual. South was the only

**Table 6** Results of principal components and discriminant analyses

Principal component	Eigenvalue	Percent of variance	Cumulative percent	Variables	Canonical discriminant function coefficient
1	41.6	54.1	54.1	Temperature (water and air), Precipitation: June and July	0.958
2	19.2	24.9	79.0	Ocean salinity	-0.768
3	7.9	10.3	89.3	Precipitation	0.265
4	2.1	2.7	92.0	Diurnal temp. range (Bio2)	-0.358
5	1.6	2.1	94.0	Temperature of driest quarter (Bio 9)	0.088
6	1.5	2.0	96.0	Primarily precipitation variables	0.439

species found in the northern Gulf of Mexico, but it also occurred on the mid-Atlantic coast in Florida and between North Carolina and Delaware. The two species also overlapped in the Chesapeake Bay region (Fig. 1). At the six locales where the two co-occurred they were in complete sympatry (found clumped together under the same plant) and could not be distinguished morphologically. At neighbouring locales, some as little as 15 km apart, only one of the two species was found. This mosaic distribution likely explains allozyme divergence previously noted between populations inside of and outside of the Chesapeake Bay (Schaeffer *et al.* 1985). Although the northern range limit of South (around latitude 39°N) coincides with the range limits of a number of species and with a decrease in water temperature and salinity (Engle & Summers 1999; Wares 2002), South was not found along a large portion of the coast south of this barrier (between South Carolina and northern Florida, Fig. 1). The northern range endpoint for North was farther north (by at least 10 latitudinal degrees) than that for South, suggesting that North can better survive colder temperature, specifically the cooler conditions found north of Cape Cod, a common barrier to a number of species (Engle & Summers 1999; Wares 2002; Wethey 2002; Vermeij 2005; Sanford *et al.* 2006). However, North also dominated the Atlantic Coast between South Carolina and northern Florida, where South was absent, suggesting that there may be some environmental factor in that region, presumably other than temperature, which favours North over South.

#### *High connectivity among populations within cryptic species*

Estimates of migration rates (Table 4) and of populations structure across each species' range (Tables S2 and S3) suggested that North and South are both capable of dispersal across broad swathes of their range, including regions where they were never found. This is consistent with the predicted dispersal capabilities in *M. bidentatus*. Larval hatches timed with spring tides

likely help flush them from their natal shores (a conclusion supported by the presence of swimming veligers in the outgoing tides of a tidal inlet, Apley 1970) and *M. bidentatus* often occurs near the outer coast, where it is less likely to experience the restricted dispersal often seen in strictly estuarine species (Kelly *et al.* 2006). Most notably, levels of long distance dispersal in South between the disjunct North Carolina and the Gulf of Mexico populations (around the southern tip of Florida, Table 4) were high and significantly greater than zero, suggesting that larvae from South are capable of dispersing over large distances (Table 3).

However, South was never found along the swath of coast from South Carolina to the north of Florida. Thus, migrants in South appear to bypass an area where they are absent. In other cases where genetic connectivity has been found across a range discontinuity, areas of species absence have been attributed to ecological processes (Bernardi *et al.* 2003; Keever *et al.* 2009), and this is likely the case here as well. Small genetic distances among samples of *M. coffeus* collected in Florida, Panama, Curaçao and Puerto Rico (Table 2) also suggest that *Melampus* species are capable of long distance dispersal. Thus, dispersal limitation alone does not explain the differences in geographic range between North and South.

#### *Historical contributions to contemporary ranges*

During the last glacial maximum, ice sheets and cold temperatures made most habitats in the Atlantic intertidal unsuitable above 40°N, causing southward range contractions in many marine species (Wares & Cunningham 2001; Stuart *et al.* 2008; Kenchington *et al.* 2009). Other species, however, persisted in northern refugia in the Atlantic intertidal (Bigg *et al.* 2008; Maggs *et al.* 2008), notably *F. heteroclitus* (Haney *et al.* 2009), a fish that co-occurs with *Melampus* (Vince *et al.* 1976; Allen *et al.* 1994) and which likewise shows disjunct differentiated genetic lineages (Duvernell *et al.* 2008). North may have persisted through the last glacial maximum in such northern refugia; haplotype diversity



(0.968), the effective number of alleles (71.4), and  $\theta$  (2.4) were high to the north of 40°N. The lowest diversity in North ( $H_d = 0.766$ , effective number of alleles = 4.4, and  $\theta = 0.024$ ) was found in the southernmost populations, despite high collection effort in this region ( $n = 97$  across 12 locales). If North is expanding in any direction, then it is moving to the south.

Within South, the single collection locale at Cape Canaveral had low diversity ( $H_d = 0.635$ , effective number of alleles = 2.7, and  $\theta = 0.0058$ ), but Tajima's  $D$  was not significantly negative. Because the Cape Canaveral population lies between two regions with which it exchanges migrants, this is more likely a region whose reduced genetic diversity is due to low habitat availability and smaller population sizes than it is an area of expansion. The high diversity ( $H_d = 0.902$  and  $0.953$ , effective number of alleles 10.2 and 21.3) of the other two Atlantic groups of South suggests that populations in these regions have been large for a longer time, although the diversity was greatest in the Gulf of Mexico ( $H_d = 0.998$ , effective number of alleles = 500), suggesting that this region has been occupied by South for the longest period of time. However, based on high estimates of  $\theta$  (4.7) in the North Carolina region, South could have survived the last glacial maximum around this latitude, which was to the south of glacial ice.

#### *Microhabitat differences between North and South*

Given their differing northern range limits, it is not surprising that temperature (both land and sea surface) was the most important variable separating North and South. The role of temperature in setting range limits has been proposed for many taxa, including insects (Addo-Bediako *et al.* 2000), fish (Cussac *et al.* 2009) and frogs (Graham *et al.* 2004). However, predictive models built in Maxent using temperature could not differentiate the northern range limits of these two species, and overpredicted the northern range limit of South by over 500 km (Table 5, Fig. 3). Northern range limits and the absence of South between SC and FL were best predicted by a model built using all non-temperature variables (precipitation, salinity and tidal height, Fig. 3). In particular, increased mean tidal height best explained the occurrence of North in the southern Atlantic area where South was not found. *M. bidentatus* occupies portions of the marsh that are at and above the high tide mark (Apley *et al.* 1970; Russell-Hunter *et al.* 1972). To avoid long periods of submersion, snails may move to higher elevations in the marsh when the tidal amplitude is high. These higher portions of the marsh, however, experience less frequent tidal inundations, and thus increased tidal height may cause greater desiccation stress to *Melampus*.

These results suggest that North may dominate in the geographic span between South Carolina and northern Florida (the mid-Atlantic region with the highest tidal differences) because it is more resistant to desiccation than South. An interaction between desiccation and freeze tolerance has been shown in both marine and terrestrial snails (Roland & Ring 1977; Miller *et al.* 2009a), so a higher desiccation tolerance in North could also explain the additional 10° of northern latitude occupied by North. Tolerance of higher salinity may also play a role. Estuarine invertebrates tend to be less freeze tolerant than those living in full strength sea water (Aarset 1982). Higher salinities may alter cells osmotically in a way that increases freeze tolerance (Ansart & Vernon 2003), and acclimation to higher salinities has been shown to aid freeze tolerance in mussels (Murphy & Pierce 1975). The distribution of South around low salinity sites in the Gulf of Mexico (e.g. around the Mississippi River outflow, Antonov *et al.* 2005) and the inner Chesapeake Bay also suggest that South may better cope with the challenges posed by lower salinities than North. The stresses of temperature likely interact with many aspects of the environment to physiologically limit each species. In molluscs, temperature tolerance is linked to both salinity acclimation (Hylleberg & Siegismund 1987; Rajagopal *et al.* 2005) and desiccation stress (Miller *et al.* 2009a). In the habitat occupied by *Melampus*, moisture may buffer thermal stresses (Ansart & Vernon 2003), in which case the importance of tidal height and precipitation could be a reflection of microhabitat differences in temperature that are not measured in our geographically broad environmental layers (Helmuth *et al.* 2006a).

Other unmeasured differences among regions could reinforce these range differences, and these unknown differences could explain why the ecological niche modelling and the discriminant analysis could not correctly assign the species identity at every locale. The reported biotic pressures on *Melampus* (predation and competition) do not vary geographically in a pattern that correlates with variation in the ranges of North and South, but there may be habitat differences that we have not considered. For example, North and South may partition the environment in relation to the strong population structure and clinal variation in the cordgrass, *Spartina* (Blum *et al.* 2007) that these snails live on. As their primary source of both food and shelter, preferences for different genetic varieties could underlie range differences among cryptic species of *M. bidentatus* (Sotka & Hay 2002). Any such ecological differences between these two species may have evolved in the different historical environments occupied by these two species.

## Conclusion

Our results suggest that cryptic species North and South occupy different environmental niches. This implies that these species may differ in their physiological tolerance of the intertidal environment. Many species are expected to shift their ranges poleward as temperatures rise (Sagarin *et al.* 1999; Herbert *et al.* 2003; Hellmann *et al.* 2008; Chen *et al.* 2009), but our findings suggest that the response of *Melampus* will not be a simple latitudinal range shift. Under a warming climate, change in the intertidal will be influenced by changes in a suite of environmental components in addition to temperature including ocean pH, sea level, precipitation and water currents (Harley *et al.* 2006; Helmuth *et al.* 2006a,b). In *Melampus*, changes in terrestrial precipitation and in ocean salinity might alter the way these two species partition their environment. For example, increases in local rainfall could favour South by reducing desiccation and salinity stress, or less frequent rainfall could extend dry periods, potentially favouring North over South.

Here, we were able to use publicly available environmental data to tease apart the niches of two very similar species that occur at the intersection of two habitats (terrestrial and marine, in the case of the intertidal).

Our approach could be similarly applied to other species that are restricted to the intersection of habitats (e.g. the edge of a forest) or to a specific range of conditions (e.g. high elevation forest or the deep sea). We have used this to identify a suite of variables from both terrestrial and marine environments (temperature, salinity, precipitation and tidal height) that likely act together to limit the range of *M. bidentatus*. How each of these variables interacts to limit the occurrence of each species will be best understood through direct physiological comparisons.

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## Appendix A1

Collection locales and the species they contained.

Collection locale	Collection date	Latitude	Longitude	<i>n</i> (included in population structure)	Species
New River, New Richmond Quebec*	5/13/1965	48.58	-68.78	4 (4)	North
Cape Breton Island, Nova Scotia*	12/10/2006	46.25	-60.58	3 (3)	North
Bath, ME	6/21/2006	43.87	-69.91	7 (6)	North
Cousins Island, ME	6/21/2006	43.75	-70.14	9 (8)	North
Newbury, MA	6/7/2005	42.74	-70.85	7 (5)	North
Mayo Beach, MA	6/22/2006	41.93	-70.07	9 (8)	North
West Dennis, MA	6/22/2006	41.66	-70.07	8 (8)	North
West Falmouth, MA	6/8/2005	41.60	-70.65	11 (10)	North
Conanicut Island, RI	6/12/2007	41.49	-71.39	8 (5)	North
New London, CT	6/13/2007	41.39	-72.10	7 (5)	North
BeeBe Cove, CT	6/13/2007	41.33	-71.99	5 (4)	North
Hammonasset, CT	6/19/2006	41.27	-72.66	10 (7)	North
Lordship, CT	6/9/2005	41.16	-73.11	8 (8)	North
Orient, NY	6/14/2007	41.14	-72.38	7 (6)	North
Bridgeport, CT	6/19/2006	41.14	-73.29	6 (5)	North
Whitestone, NY	6/10/2005	40.77	-73.75	9 (9)	North
Brunswick, NJ	6/11/2005	40.46	-74.25	9 (9)	North
Pt. Pleasant, NJ	6/17/2007	40.07	-74.04	8 (7)	North
Ocean City, NJ	6/17/2007	39.30	-74.59	10 (0)	North
Plum Island, DE	6/18/2007	38.81	-75.19	5 (0)	North
Indian River, DE	6/11/2005	38.61	-75.07	14 (14)	North
Indian River Bay, DE	6/18/2007	38.59	-75.13	3 (3)	North

## Appendix A1 (Continued)

Cambridge, MD	6/19/2007	38.57	-76.07	1 (1)	South
Ragged Point, MD	6/19/2007	38.55	-76.27	6 (5)	South
Fenwick Island, DE	6/20/2007	38.46	-75.08	7 (7) North 7 (7) South	North & and South
Ocean City, MD	6/20/2007	38.40	-75.06	9 (0)	North
N. of Crisfield, MD	6/20/2007	38.01	-75.85	4 (0)	South
Crisfield, MD	6/12/2005	37.99	-75.86	11 (10)	South
Chincoteague, VA	6/20/2007	37.94	-75.37	9 (2) North 1 (0) South	North & and South
Wallops Island, VA	6/20/2007	37.94	-75.41	5 (5)	North
Oyster, VA	6/12/2005	37.29	-75.92	9 (9)	North
Cape Charles, VA	6/21/2007	37.28	-76.02	2 (2) North 3 (3) South	North & and South
Williamsburg, VA	4/9/2005	37.27	-76.58	13 (11) North 45 (30) South	North & and South
Hayes, VA	6/21/2007	37.27	-76.40	1 (1) North 1 (1) South	North & and South
Smithfield, VA	5/24/2007	37.01	-76.60	1 (0)	South
Roanoke Island, NC	6/22/2007	35.89	-75.64	4 (0)	South
Pea Island, NC	6/22/2007	35.68	-75.48	3 (2)	South
Bellhaven, NC	6/23/2007	35.54	-76.62	2 (2)	South
Near Hatteras, NC	6/22/2007	35.22	-75.66	7 (5)	South
South River, NC	6/15/2005	34.95	-76.59	8 (8)	South
Morehead City, NC	6/24/2007	34.73	-76.73	9 (0) North 1 (0) South	North & and South
Beaufort, NC	6/13/2005	34.72	-76.68	11 (9)	North
Harkers Island, NC	6/24/2007	34.71	-76.58	9 (1)	South
Wilmington, NC	8/4/2008	34.24	-77.95	10 (0)	North
Carolina Beach, NC	8/7/2008	34.00	-77.89	9 (0)	North
Oak Island Bridge, NC	5/21/2007	33.73	-78.07	10 (1)	North
Myrtle Beach, SC	8/4/2008	33.68	-78.89	9 (0)	North
Georgetown, SC	8/4/2008	33.37	-79.26	5 (0)	North
McClellanville, SC	8/4/2008	32.94	-79.66	7 (0)	North
Near Savannah, GA	8/5/2008	32.79	-80.14	8 (8)	North
Downtown Charleston, SC	8/5/2008	32.78	-79.95	6 (0)	North
Grice Marine Lab, SC	6/17/2005	32.75	-79.90	21 (19)	North
N. of Savannah, GA	8/5/2008	32.60	-80.76	10 (9)	North
Savannah, GA	8/5/2008	32.02	-81.00	8 (8)	North
Brunswick, GA	8/5/2008	31.18	-81.39	8 (7)	North
Sapelo Island, GA	See Robinson <i>et al.</i> 2010;	24 (24)	North		
Amelia Island, FL	8/5/2008	30.67	-81.44	10 (10)	North
Jacksonville, FL	8/6/2008	30.41	-81.58	9 (9)	North
Dauphin Island, AL	4/11/2005	30.26	-88.12	9 (9)	South
Sabine, LA	5/2/2005	29.90	-93.37	5 (5)	South
Grand Isle, LA	2/15/2005	29.26	-89.95	5 (4)	South
LUMCON, LA	11/12/2006	29.25	-90.66	12 (6)	South
Port Fourchon, LA	2/18/2005	29.11	-89.95	4 (4)	South
Cape Canaveral, FL	8/6/2008	28.64	-80.78	16 (15)	South
Clearwater, FL	12/14/2005	27.86	-82.85	2 (2) South 1 (0) <i>M. coffeus</i>	South & and <i>M. coffeus</i>
Naples, FL*	8/8/2008	27.49	-82.70	10 (9)	<i>M. coffeus</i>
Sarasota, FL*	8/9/2008	27.33	-82.54	8 (5)	<i>M. coffeus</i>
Sanibel Island, FL*	8/8/2008	26.49	-82.01	8 (3)	<i>M. coffeus</i>
Miami, FL*	8/8/2008	26.33	-81.84	10 (10)	<i>M. coffeus</i>
Bonita Beach, FL	8/8/2008	26.33	-81.84	2 (2) South 7 (4) <i>M. coffeus</i>	South & and <i>M. coffeus</i>
South Padre Island, TX*	5/16/2006	26.14	-97.18	10 (10)	Gulf
Key Biscayne, FL*	7/21/2005	25.68	-80.17	1 (1) Gulf 1 (1) <i>M. coffeus</i>	Gulf & and <i>M. coffeus</i>
Sugarloaf Key, FL*	7/20/2005	24.65	-81.57	3 (3) Gulf	Gulf
Key Largo, FL*	7/19/2005	25.02	-80.50	9 (9) Gulf 1 (1) <i>M. coffeus</i>	Gulf & and <i>M. coffeus</i>
Holmes Beach, FL	8/9/2008	27.49	-82.70	1 (1) South 8 (8) <i>M. coffeus</i>	South & and <i>M. coffeus</i>
La Parguera, Puerto Rico*	7/2008	17.97	-67.05	11 (10)	<i>M. coffeus</i>
St. Joris Bay, Curacao*	7/1/2005	12.12	-68.82	2 (2)	<i>M. coffeus</i>
Bocas del Drago, Panama*	1/2005	9.38	-81.30	3 (1)	<i>M. coffeus</i>

Locales with an "\*" were not included in ecological niche modelling. Individuals that have been identified but were not used to infer population structure were identified based on sequences between 200 and 440 bp.

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### Supporting Information

Additional supporting information may be found in the online version of this article.

**Table S1** Principal component matrix produced using a varimax rotation and Kaiser normalization. This shows the correlation among each value and that principal component

**Table S2** Pairwise calculations of  $\Phi_{ST}$  for *Melampus bidentatus* North

**Table S3** Pairwise calculations of  $\Phi_{ST}$  for *Melampus bidentatus* South

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