Molecular Species Delimitation and Morphometry in the *Melampus bidentatus* (Panpulmonata, Ellobiidae) Cryptic Species Complex

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Abstract

The coffee bean snail *Melampus bidentatus* occurs in coastal salt marshes along the North American Atlantic and Gulf coasts and in the Caribbean. It was recently found that this large geographical span is actually occupied by a complex of three apparently cryptic species (preliminary called "North", "South", and "Gulf") with partially overlapping distributions. Until now, it was not clear whether there are any morphological differences between the three species or which of the available names can be applied to each of the cryptic species. We used the already known distribution patterns of the cryptic species as well as new barcode sequences to assign available names to the three cryptic species. We then compared morphological characters from 264 specimens using two approaches: an analysis based on 11 landmark points on the shell and another based on the entire shell outline. We were able to assign a nominal name to each of the three cryptic species: *Melampus bidentatus* for "North", *Melampus jauemi* for "South", and *Melampus gundlachi* for "Gulf". The morphometric analyses did not yield any diagnostic differentiating features; these cryptic species are hence diagnosable solely by genetic analysis but may phenotypically differ in some unseen internal features or in their physiology.

Introduction

Cryptic species—defined here as species that have been classified within the same nominal species because they are or seem to be morphologically indistinguishable (Bickford et al., 2007)—have been uncovered at an increasing rate since the advent of genetic sequencing (Bickford et al., 2007; Fišer et al., 2018). Reproductive isolation among and species status of such cryptic lineages can be confirmed through cross-validation of several genetic markers and molecular species delimitation methods (Jörger et al., 2012). However, those species are often not formally described, creating a parallel system of candidate species without proper scientific names (Jörger & Schrödl, 2015). It has thus been proposed that cryptic lineages should be formally described if they can be identified and cross-validated as an independent species with molecular methods alone (Jörger & Schrödl, 2013). Furthermore, it has been shown that following the delimitation of apparently cryptic species, using integrative taxonomic methods (Dayrat, 2005; Schlick-Steiner et al., 2010), previously overlooked morphological characters were discovered to discern these lineages (Horsáková et al., 2020, 2022; Schlick-Steiner et al., 2007; Tan et al., 2010). Such species-diagnostic phenotypic traits could further provide important insights into ecological drivers of speciation, as well as into the conservation status of the respective cryptic species.

The coffee-bean snail, *Melampus bidentatus* Say, 1822, is an air-breathing snail distributed along the North American Atlantic and Gulf coasts and in the Greater Antilles (Martins, 1996). Throughout their range, *Melampus* occur abundantly (reaching densities of hundreds and, occasionally, more than 1000 individuals per m² (Peck et al., 1994; Price, 1980) around the high tide mark in salt marshes and mangroves (Martins, 1996). It lives on decaying marsh grass (*Spartina alternifolia* & *Spartina patens*), which is also its main food source (Galvan, 2008; Graça et al., 2000; Rietzma et al., 1988), though it also forages on animal detritus, filamentous algae, and diatoms (Hausman, 1952; Thompson, 1984). *Melampus bidentatus* is a hermaphrodite (Martins, 1996) that lays its eggs on the ground during spring high tides (during late May to early July in Massachusetts), with the eggs hatching approximately two weeks later following the spring high tides (Russell-Hunter et al., 1972). Individual *M. bidentatus* have a high dispersal potential, as they spend the first few weeks after hatching as veliger larvae in the open ocean before returning to the shore and developing into a largely terrestrial, air breathing snail with a life expectancy of 3-4 years (Russell-Hunter et al., 1972).
Dennis and Hellberg (2010) used three independent genetic markers (one mitochondrial, two nuclear) to identify three genetically well differentiated species, with occasionally overlapping distributional ranges, within the nominal species *M. bidentatus*. Those cryptic species were preliminarily referred to as "North" (distributed from New Brunswick to northern Florida), "South" (distributed in the US part of the Gulf of Mexico, around Cape Canaveral, and from North Carolina to Delaware), and "Gulf" (distributed in southern Florida and southern Texas). For the first two species, high genetic variability, low population structure, and high levels of dispersal were noted. While Dennis and Hellberg (2010) called the three species cryptic, they did not explicitly study their morphology, nor did they make any attempt to assign the three species to the 11 existing synonyms of *M. bidentatus* (Martins, 1996; *Melampus morrisoni* Martins, 1996 was included in *M. bidentatus* here, following Dennis et al., 2014). Because of the known parapatric distribution of the three cryptic species (Dennis & Hellberg, 2010), the type localities of most of the 11 available names lie in regions where only one of the three species occurs. However, in several cases there were no specimens sequenced from type localities, so it was not known which species occupied those regions.

In this study, we sequenced specimens from the type localities and used this information to assign the synonyms of *M. bidentatus* to the cryptic species detected by molecular analysis. We provide a panel of diagnostic SNPs to differentiate the three species and construct an updated phylogeny to show their relationships to other species of *Melampus*. Finally, we use two morphometrical analyses to compare shell shape among the three cryptic species and their closest relative, *Melampus coffeus*, and correlated those results with relevant environmental variables.

**Materials & Methods**

**Collections**

Twelve newly collected specimens were sequenced for this study. Four of them were analyzed together with sequences from Dennis et al. (2014) and Dennis and Hellberg (2010). New material for this study was collected from Buckroe Beach, Hampton, Virginia on 30 November 2020 by David Johnson (Fig. 11; N; nine of which would be sequenced). In addition, five specimens were collected at Spittal Pond, Bermuda on the 27th of October 2020 by Leocadio Blanco-Bercial (Table 1). Of these five specimens, two were from the *M. bidentatus* complex (Fig. 1; H&I), and the other three were *Melampus bullaoidei*. We also included sequences from two specimens (The Australian Museum, Sydney: C.417369 & C.417370), both from Thirsty Sound, Queensland, Australia, identified as *Melampus ovaloides* Baird 1873 (described in Brenchley, 1873) and *Melampus variabilis* Gassies 1863, respectively. In all cases, fresh samples were preserved in >95% ethanol until DNA extraction.

**Figure 1. Specimens used in the molecular tree.**


**DNA Extraction**

**PCR and Sequencing**

A CO1 (cytochrome c oxidase 1) fragment was amplified using the HCO1 (Folmer et al., 1994) and MCO1 (Dennis & Hellberg, 2010) primers. The PCR was performed with the following admixture: 1μL DNA extract, 17.88μL HPLC-H2O, 5μL 5× MyTaq Buffer, 0.12μL MyTaq polymerase (SU/μL), 0.5μL HCO1 primer (10 μM), 0.5μL MCO1 primer (10 μM). The following PCR protocol was used: first 2:30 min at 94℃, 2 min at 50℃, and 2 min at 72℃; then 35 cycles of 45 s at 94℃, 1 min at 50℃, and 1:15 min at 72℃; and finally 40 s at 94℃, 1 min at 50℃, and 10 min at 72℃. The ramp speed was set to 2℃ per second.

Following PCR, 8 μL of PCR product was mixed with 0.5 μL of Exonuclease I, 0.5 μL of Antarctic Phosphatase, and 1 μL of Antarctic Phosphatase Buffer. The mixture was incubated at 37℃ for 20 minutes and then put at 80℃ for 10 minutes. For the DNA sequencing reaction, 2 μL of the
Table 1. Specimens used in the phylogeny (see also Fig. 1).

<table>
<thead>
<tr>
<th>Species</th>
<th>Site</th>
<th>Coordinates</th>
<th>CO1 accession No</th>
<th>H3 accession No</th>
<th>MCP accession No</th>
<th>First published in; Voucher collection numbers</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Tralia panamensis</em> (C. B. Adams, 1852)</td>
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<td>ON936828</td>
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<td>Dennis et al., 2014; FLMNH 580966</td>
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<td><em>Melampus fasciatus</em> (Deshayes, 1830)</td>
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<td>KM281104</td>
<td>KM281131</td>
<td>–</td>
<td>Romero et al., 2016</td>
<td></td>
</tr>
<tr>
<td><em>Melampus monile</em> (Brugiére, 1789)</td>
<td>Harry Harris Park, Florida</td>
<td>25.024°N, 80.4952°W</td>
<td>KJ609108</td>
<td>KJ609129</td>
<td>ON936832</td>
<td>Dennis et al., 2014; FLMNH 580968</td>
</tr>
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<td>KM281130</td>
<td>–</td>
<td>Romero et al., 2016</td>
<td></td>
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<td><em>Melampus cf. variabilis</em> Gassies, 1863</td>
<td>Thirsty sound, Queensland</td>
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<td>ON936829</td>
<td>ON936834</td>
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<td>ON936830</td>
<td>ON936835</td>
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<td>KJ609127</td>
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<td>ON936831</td>
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<td>Dennis et al., 2014; FLMNH 580969</td>
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<td><em>Melampus olivaceus</em> Carpenter, 1857</td>
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<td>Dennis et al., 2014; FLMNH 580970</td>
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<td>HM153901</td>
<td>Dennis et al., 2014; FLMNH 580973</td>
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<td>Felgates Creek, Virginia</td>
<td>37.2723°N, 76.6026°W</td>
<td>HM154149</td>
<td>HM153891</td>
<td>HM153996</td>
<td>Dennis &amp; Hellberg, 2010; FLMNH 580980</td>
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</table>

*Note:* The table includes information about species, sites, coordinates, accession numbers for CO1 and H3, and accession numbers for MCP. The table also provides references for the first published date and voucher collection numbers.
cleaned PCR product was mixed with 0.5 μL BigDye v5.1, 2 μL 5x sequencing buffer, 0.5 μL MCO1 Primer (10 μM), and 5 μL HPLC-H2O. The sequencing protocol began with 2 mins at 96° C and then consisted of 30 cycles of 96° C for 15 seconds, 50° C for 10s, and 60° C for 4 mins. The sequenced fragments were purified by passing them through a gel made with Sephadex G-50 Superfine powder and subsequently run on a Genetic Analyser 3500 (Applied Bioscience/Hitachi).

**Nucleotide alignments**

In addition to specimens sequenced in this study, sequences from previously sequenced species were downloaded from GenBank (Table 1). CO1 (448 bp), H3 (Histone 3; 516 bp), and MCP (a nuclear-encoded mitochondrial phosphate carrier protein; 159 bp) sequences were aligned using MAFFT v7.450 (Katoh et al., 2002; Katoh & Standley, 2013) for Windows with the FFT-NS-2 algorithm. *Tralia panamensis* was included in the alignments as an outgroup.

**Phylogenetic relationships among species**

Bayesian and maximum likelihood trees were calculated for each of the three alignments (CO1, H3, and MCP) separately and together (with the markers used as partitions in both the Bayesian and the maximum likelihood trees). MrBayes v3.2.7 (Ronquist et al., 2012) was run using the mixed substitution model (which incorporates model testing into the MCMC), a Markov Chain Monte Carlo (MCMC) with four heated chains with a temperature parameter of 0.2, a length of 10000000 generations, a subsampling frequency of every 1000, and the first 25% of the samples from the cold chain discarded as burn-in. The maximum likelihood tree was calculated using IQTree v1.6.12 (Chernomor et al., 2016; Nguyen et al., 2015) for Windows, including the ModelFinder function (Kalyaanamoorthy et al., 2017) and an ultrafast bootstrap with 1000 replicates (Hoang et al., 2018).

**Genetic Species Description using CO1**

In order to find diagnostic SNPs that could be used for differentiating species, we created an alignment of all CO1 sequences available for each of the three *M. bidentatus* species and *M. coffeus* using ClustalW as implemented in Mega X (Kumar et al., 2018). These alignments were then trimmed to a length of 446 bp, the length of the shortest bulk of CO1 sequences within this species group (published by Dennis & Hellberg, 2010). Based on these alignments, we used pairwise comparisons between any pair of species to identify fixed differences among the two respective species. Position of diagnostic SNPs were noted in reference to Genbank sequences HM154489 ("North"; below assigned to *M. bidentatus*), HM154167 ("South"; *M. jaumei*), and HM154117 ("Gulf"; *M. gundlachi").

**Morphometry**

From the three *M. bidentatus* species and *M. coffeus*, we compared morphological features among 264 specimens from 60 localities. Those specimens were collected and photographed with the aperture facing the camera during the field work of Dennis and Hellberg (2010) and are thus from localities with genetic data. In most cases (at sites with more than one species in all cases), the photographed specimens themselves were genotyped. From these photos, Tps-files were created in tpsUtil version 1.79 (Rohlf, 2019).
We analyzed them using two approaches: based on chosen landmarks or on the shape of the entire shell outline.

**Shell Landmarks**

Eleven landmarks (Fig. 2a) were set in positions related to shape such that they were easily replicated over a large number of samples using tpsDig2 version 2.31 (Rohlf, 2018). The resulting tps-file was imported into MorphoJ (Klingenberg, 2011) and standardized using the Procrustes Superimposition. A Principal Component Analysis (PCA) and Canonical Variance Analysis (CVA, with 10000 permutations) were calculated with the programs default settings to compare the relative distance between landmarks. The CVA was run with the species identities of the specimens as a prior to maximize between-group differences and to minimize within-group differences along CVA axes.

**Shell outline**

In order to ensure that the outlines of the shells can be traced automatically on the pictures, the shells were turned into white shape, while the background was colored black (Fig. 2b). The outlines of the shells were traced with the Outline-tool in tpsDig2, using the apex of the shell as starting point. The number of coordinates (semilandmarks) in the outlines was standardized to 500, which were later converted to landmarks using tpsUtil. The resulting file was imported into MorphoJ, where a PCA and a CVA was conducted in the same manner as with the eleven landmarks.

**Correlations between environment and morphology**

To determine if the axes from the PCA and CVA correlate with known environmental variables, two datasets each were created from the landmarks and the outline analyses: One that included all specimens from all sites (henceforth called “All”) and one that only included sites for which salinity data was available. Included were axes from the PCA and the CVA as well as species identity, centroid size, latitude, and longitude (and salinity in the “Salinity” datasets). These datasets were imported into R 3.6.3 (R Core Team, 2020) for further analysis and plotting.

For each dataset, linear models were created for each CV-axes and the first five PC-axes, as well as centroid size, which were used as response variables, using the lm() function. Species identity, latitude, longitude, and centroid size (the latter not when it was used as a response variable) were used as explanatory variables. The best model was selected from the initial model using stepwise regression as implemented in R with the step() function. The P-values of that model were then read from the table created with the anova() function, the adjusted R² from the output of the summary() command.

**Results**

Genetic analysisThe topologies of both the maximum likelihood tree (Fig. 3) and the Bayesian tree are largely congruent to each other and to the one published in Dennis et al. (2014), including the topology of the *M. bidentatus* species complex and the position of *M. coffeus* within that complex. The only difference between the trees is that, in the maximum likelihood tree, the position of *Melampus floridanus* was as a sister species to a group containing the three cryptic species currently lumped together under *M. bidentatus* (“North,” “South,” “Gulf”; referred to as “M. bidentatus,” “M. jaumei,” and “M. gundlachi,” respectively in Fig. 3). *M. coffeus*, *M. carolinus*, and *M. olivaceus*, while in the other trees this species was recovered as a sister group of the species pair *M. carolinus* and *M. olivaceus*. All species were recovered as monophyletic with significant node support (Bayesian posterior probabilities (BPP) > 0.95; maximum likelihood bootstrap support (MLBS) > 85%). Many of the relationships between species were recovered with moderate node support values (BPP > 0.8 (usually more); MLBS considerably above 50%) in at least one tree. There were two groups where some of the node support values were weak (BPP < 0.80 or absent, MLBS around 50% or lower). The first one is the group around *M. bidentatus* in the wider sense, including *M. coffeus*, where the relationships between species were not resolved with significant node support, though the species themselves were recovered as monophyletic. The second one is the group around *M. fasciatus*, *M. monile*, *M. bullalooides*, *M. cf. variabilis*, and *M. cf. ovoides*, where the two most basal nodes—only recovered in the maximum likelihood tree—delineating the relationships between *M. fasciatus*, *M. monile*, and the clade includ-
Figure 3. Maximum likelihood tree of the genus *Melampus*.

Node support values depicted are maximum likelihood bootstrap support and Bayesian posterior probabilities. Colored branches: Species group around *M. bidentatus* (Blue: *M. bidentatus*; Red: *M. jaumei*; green: *M. gundlachi*; yellow: *M. coffeus*). Bold type: Specimens sequenced for this study.
ing *M. bullaoides*, *M. cf. variabilis*, and *M. cf. ovoides* only had a bootstrap support of slightly over 50% each.

All of the nine specimens sequenced in this study from Hampton, Virginia (only one of which is shown in the tree, Fig. 3; for the rest, see Supplementary alignment 1), were identified as belonging to the cryptic species "South." We cannot say with full certainty that the species composition in Hampton today mirrors that of the site at the time of the species description (with "North" living at sites nearby) as the whereabouts of the collection of Hippolite Mitré (1811-1851) are unknown (Breure, 2017). Nonetheless, repeated sampling suggests that species composition in parapatric regions does not rapidly change across years. Taken together with the moderate salinity at this site (approximately 20 ppt; CBNERR-VA VIMS 2020), there is reasonable support that it consistently contains "South." Therefore, based on these sequences we propose that the name *M. jaumei* (Mitré, 1841) is used for "South." Of the two "*M. bidentatus*" specimens from Bermuda, we were able to generate a CO1 sequence from one (the shell coloration of this specimen is largely consistent with the descriptions of *redfieldi* Pfeiffer, 1854 and *alternatus* Davis, 1904). This specimen was identified as "Gulf." The names described from the Bermuda Islands should therefore be associated with this species as junior synonyms (the oldest name for this species is *M. gundlachi* Pfeiffer, 1853; see below). Also assigned to "Gulf" in our tree is a specimen from Jamaica sequenced in Dayrat et al. (2011; GenBank: HQ660006), which was hitherto identified as "*M. bidentatus.*"

The two specimens from Thrusty Sound, Australia were recovered as conspecific and as the sister taxon of *Melampus bullaoides*. The identity of these specimens needs to be further investigated and compared to specimens from the type localities of the both species (*ovoides* Baird 1873: Tuvalu, American Samoa (Syntypes: Natural History Museum, London (NHM) 1968873); *variabilis* Gassies 1865: New Caledonia (Syntypes: NHM 1883.11.10.76–78 & Muséum National d'Histoire Naturelle, Paris MNHN-IM-2000-5129)). This was beyond the scope of this paper but would need to be mainly based on molecular methods as specimens identified as *M. ovoides* and *M. variabilis* from this site did have some apparent morphological differences (2 visible parietal teeth and a lower spire in *M. cf. ovooides*; one visible parietal tooth and a higher spire in *M. cf. variabilis*), which might indicate that the characters used thus far to differentiate the two species might be subject to intraspecific variation.

Within the 446 bp long CO1 fragment, 267 positions (~60%) were conserved across all sequences and species. 43 of the remaining positions (or SNPs) were found to be useful in separating one or more species pair from one another. Only two of these 43 positions (96 & 415) lacked any intraspecific variation in any of the four species. We identified between 5 and 18 diagnostic SNPs for each species pair (see taxonomic consequences & supplementary alignment 2). There were nine positions where one species had a unique SNP ("North:" 2 positions; "South:" 0; "Gulf:" 6; *M. coffeus*: 1).

**Application of names**

Dennis and Hellberg (2010) sampled the type locality of *Melampus bidentatus* Say, 1822 (Jacksonville, Florida, "North"), which is the most senior name assigned to the cryptic species "North." They also sampled the very vaguely defined type regions of two available names (*Melampus obliquus* Say, 1822, South Carolina, "North;" *Auricula cornea* Deshayes, 1830, New York, "North"). The specific identity of three available names can be inferred using specimens sampled from sites reasonably close to their type locality (*Melampus bidentatus* var. *lineatus* Say, 1822, Bliveree, New Jersey, "Gulf;" *Melampus gundlachi* Pfeiffer, 1853, Cayo Blanco, Cuba, "Gulf;" *Melampus morrisoni* Martins, 1996, Key West, Florida, "Gulf"). While there is no genetic record from Cayo Blanco, Cuba, the type locality of the latter name, there are genetic records from directly across the Florida straits (on Sugarloaf Key and on Key Biscayne) that were identified as "Gulf." With none of the other species being reported that far south and the high rate of dispersal that has been shown for species within this species complex (Dennis & Hellberg, 2010), it can be reasonably assumed that the species occurring at the type locality would be the same as the one occurring in the nearby Florida Keys.

**Morphometry**

**Plots**

We will only discuss the results for the first two axes and the plot that combines the two for each analysis as the other axes, with the exception of the CV-axis 3 of the outline-based dataset, did not show any further potential for separating the cryptic species.

In the PCA based on the 11 landmarks (Supplementary Fig. 1a–c), all cryptic species and *M. coffeus* overlapped with each other to a degree that a separation was not possible, though axis 1 showed a trend of grouping the species by their latitudinal distribution: *M. coffeus* specimens were distributed at the low values, while *M. bidentatus* specimens tended to be located at higher values, with *M. jaumei* and *M. gundlachi* being located roughly in between. The shape changes along axis 1 went from broad and low spired individuals at low values to slender and high spired at high values; the shape changes along axis 2 went towards a more shouldered body whorl and a high spire at high values.

In the CVA based on the 11 landmarks (Supplementary Fig. 1d–f), there was a very large overlap of *M. bidentatus* and *M. jaumei* on both axes, with *M. coffeus* being better separated on axis 1. *Melampus gundlachi* overlapped completely with the other three species. The shape changes on axis 1 went towards a low spire and a shouldered body whorl at high values, and the shape changes on axis 2 towards a less shouldered body whorl, broader aperture, and the columellar tooth being located closer to the lower end of the aperture at high values.

The PCA based on the outline (Fig. 4a–c) was functionally identical to the one based on the 11 landmarks. The shape changes along axis 1 went from broad and low spired...
individuals at low values to slender and high spired at high values; the shape changes along axis 2 go from a more or less slender body whorl towards a more shouldered one.

In the CVA based on the outline (Fig. 4d–f), the first two axes separated the four species into four clusters with only very little overlap. Axis 1 separated M. gundlachi and—to a lesser extent—M. coffeus from M. bidentatus and M. jaumei. The shape changes along axis 1 went from broad and low spired to slender and high spired. Axis 2 separated M. gundlachi and M. bidentatus from M. jaumei and M. coffeus, though there was still a large overlap between M. bidentatus and M. coffeus. The shape changes along it went from a less shouldered body whorl and a higher spire at low values to a more shouldered body whorl and a lower spire at high values. Axis 3 (Supplementary Fig. 2) separated M. gundlachi from M. bidentatus and M. coffeus, but some overlap with M. jaumei remained. The shape changes along it went towards a more ovoid shape at high values. Generally (and especially on axis 3), the shape changes along the axes in this analysis were rather minuscule and unlikely to rise above the intraspecific variation that is intentionally minimized in the CVA.

Linear modeling to test for the influence of species and environment on shape

To check for any correlation between the PC- and CV-axes, simple linear models were constructed. Among explanatory variables, "Species" was strongly significant in the models of Centroid Size, all three CV-axes most PC-axes in both the landmark (supplementary table 1), and the outline based analyses (supplementary table 2). Other explanatory variables were rarely eliminated from the models during the stepwise regression, though many of them were only occasionally significant. "Latitude" was, for instance, highly significant (and generally with a high correlation, $R^2$) when the response variable was Centroid Size or an axis, where the shape change went from broad and low spired to slender and high spired (such as the PC1- and CV1-axes in both the landmark and the outline dataset). Salinity was a significant explanatory variable, if Centroid Size was the response variable, both in the landmark ($p = 5.052e-07, R^2 = 0.15$) and the outline dataset ($p = 7.515e-07, R^2 = 0.166$).

Taxonomic consequences

Following the results above, we hereby formally re-describe the three cryptic species and their associated names. The morphological descriptions and size ranges are based on the individuals that were also used for the morphometrical analyses above.

Differences to Melampus coffeus: Even though M. coffeus was recovered in the same clade as the M. bidentatus group in our phylogeny and in spite of its great morphological similarities, it has generally been considered as a distinct species (see Martins (1996) for an exhaustive taxonomic history of these groups). Martins (1996) differentiated M. coffeus through its accentuated conical shape, compared to the more slender profile of similarly sized specimens from what he considered M. bidentatus. He also noted that M. bidentatus has 1-3 spiral grooves on the whorls of the spire and the shoulder of the body whorl, while M. coffeus only has them on the first four whorls. We additionally observed some morphological traits that overlap with M. bidentatus but might be useful for a quick preliminary differentiation. Firstly, M. coffeus more frequently has a high number (>10) of palatal lamellae (though M. bidentatus specimens from lower latitudes seem to have more lamellae as well (Martins, 1996)). The coloration tends to be somewhat different as well; specimens without any horizontal bands are much rarer in M. coffeus than in M. bidentatus, and even in uni-colored M. coffeus there are hints toward lighter colored bands. These stripes are usually whitish/beige on a dark background (usually the opposite around in M. bidentatus, which also rarely exists in M. coffeus), and the color of the bands contrast more sharply with the background color than in M. bidentatus as well.

Examined museum specimens for M. coffeus: Florida, Harry Harris Park (FLMNH 580973); Panama: Bocas del Drago (FLMNH 580974).

Melampus bidentatus Say, 1822

1822 Melampus bidentatus Say, An account of some marine shells of the U.S., p. 245. (Type locality: Mouth of St. John's river, near Jacksonville, Florida; Neotype: National Museum of Natural History (USNM), Washington D.C. 859014)

1822 Melampus bidentatus var. lineatus Say, An account of some marine shells of the U.S., p. 246. (Type locality: Bi-valve, New Jersey; Neotype: USNM 859013)

1822 Melampus obliquus Say, An account of some marine shells of the U.S., p. 377. (Type locality: South Carolina; Type material lost.)

1830 Auricula cornea Deshayes, Encyclopédie méthodique. Histoire naturelle des vers oar Brugiére et de Lamarck, continué par Mr. G.P. Deshayes, p. 90. (Type locality: New York; location of type material unknown)

1856 Melampus bidentatus var. borealis "Conrad" Pfeiffer, Monographia Auricularceorum viventium, p. 46. (Type locality: Georgia; type in Cuming collection, vide Pfeiffer, not found at The Natural History Museum, London, U.K. (Martins, 1996)) non Melampus borealis Conrad 1832
Measurements (n= 109): Shell height: 3.7–13.5 mm (mean: 9.05 mm); shell width: 2.3–7.8 mm (mean: 5.16 mm).

Description: Shell shape from slender-conical and high-spired (mostly at the northern edge of the distribution area) to low-spired and ovoid. Aperture slender, size about half to three quarters the shell height, with two parietal teeth (of which one is often visible from the aperture or absent) and one columellar tooth, 0–10 palatal lamellae (usually 5 or less). Shell color beige to dark brown, with most beige specimens having somewhat narrow red-brown horizontal bands or (rarely) dense and frequently wavy vertical bands of the same color.


Unique SNPs: positions 27 (bid: T, rest: A or G), 96 (bid: A, rest: G)

Distribution: The known distribution of this species reaches from New Brunswick and the St. Lawrence Stream in the north to the Atlantic coast of northern Florida (around Jacksonville) in the south (Dennis & Hellberg, 2010; Martins, 1996). Its exact distribution within Florida is unclear due to a dearth of genetic sequences from the Atlantic coast.

Examined museum specimens: Québec: New Richmond (Smithsonian National Museum of Natural History 827125); Massachusetts, Newbury (FLMNH 580982); Virginia, Jaxport Creek (FLMNH 580984); Florida, Jaxport Cruise Terminal (FLMNH 580893).

**Melampus gundlachi** Pfeiffer, 1853

1853 *Melampus gundlachi* Pfeiffer, Neue Auricularcen, p. 126. (Type locality: Cayo Blanco, Cuba; location of type unknown)

1854 *Melampus redfieldi* Pfeiffer, Neue Auricularcen, p. 112. (Type locality: Bermuda; location of type unknown)

1904 *Melampus coffeus* var. *bishopi* Davis, Notes on the Mollusca of the Bermuda Islands, p. 127, plate 4 fig. 15. (Type locality: Bermuda; Lectotype: Academy of Natural Sciences of Philadelphia (ANSP) 86925)

1904 *Melampus coffeus* var. *verticalis* Davis, Notes on the Mollusca of the Bermuda Islands, p. 127, plate 4, fig. 12. (Type locality: Bermuda; Lectotype: ANSP 86927)

1904 *Melampus coffeus* var. *alternatus* Davis, Notes on the Mollusca of the Bermuda Islands, p. 127, plate 4, fig. 11. (Type locality: Bermuda; Lectotype: ANSP 86926)

1951 *Detracia clarkii* Morrison, Two new Western Atlantic species of pulmonate mollusks of the genus *Detracia* and two old ones (family Ellibiidae), p. 18, figs. 2, 6. (Type locality: Key West, Florida; USNM 594588, non *Melampus clarkii* White 1895)

1996 *Melampus morrisoni* Martins, Anatomy and Systematics of Western Atlantic Ellibiidae, p. 297. (Type locality: Key West, Florida; USNM 594588)

Measurements (n= 15): shell height: 3.8–14.7 mm (mean: 8.75 mm); shell width: 2.1–9.2 mm (mean: 5.12 mm)

Description: Shell shape conical with a usually low spire, aperture narrow and high (half to three quarters of the shell height), two parietal teeth (of which one is not always visible in the aperture or absent), one columellar tooth, 0–10 palatal lamellae. Shell color beige, often with somewhat narrow red-brown horizontal bands.


Distribution: Are there genetic records of this species from three sites in Florida (Key Largo, Sugarloaf Key, Key Biscayne), from one site in Texas (South Padre Island), from Bermuda, and from Jamaica (Dayar et al., 2011; Dennis & Hellberg, 2010). The records from the Greater Antilles, Bahamas, Mexico, and Belize listed in Martins (1996) are likely to belong to this species as well. A wider distribution in Texas and Florida is likely as well, but the extent of this distribution would require further genetic studies to separate this species from *M. jaumei*.

Remarks: Martins (1996) classified *M. morrisoni* in the subgenus *Detracia* (Gray 1840; which we were not able to recover as a monophyletic group) together with four other species (*M. bulboides* (type species of *Detracia*), *M. floridanus*, *M. paranus*, *M. monile*) based on shell morphological (strong columellar tooth) and anatomical characters (greater length of the pallial gonoducts, pouch-like mantle organ, medial nodes on the central tooth of the radula) but also noted the close similarity of the species to *M. bidentatus*. Dennis et al. (2014) found that specimens identified as *Melampus morrisoni* Martins, 1996 from Sugarloaf Key, Florida, clustered with this species (which they called "*M. bidentatus Gulf*"). They thus concluded that those two forms were conspecific. We follow here their assessment as we lack any further new data on that matter.

Examined museum specimens: Texas, South Padre Island (FLMNH 580978); Florida, Sugarloaf Key (FLMNH 580977, FLMNH 580979); Bermuda, Spittal Pond (FLMNH 580975, FLMNH 580976).

**Discussion**

Herein we were able to apply names that had previously been synonymized with *M. bidentatus* to three cryptic species. All but two of these synonyms could be assigned based on the distribution data collected by Dennis and Hellberg (2010), and the last two were assigned based on new collections and Sanger sequencing of a partial sequence of CO1. We therefore were able to assign names to the cryptic species that were hitherto preliminarily called "North" (now *M. bidentatus*), "South" (now *M. jaumei*), and "Gulf" (now *M. gundlachi*). Assignment of the species based on the newly constructed phylogenies was generally consistent with the phylogeny published by Dennis et al. (2014). We also found 46 SNPs in the 446 bp CO1 fragment used in our analysis which can be used to differentiate between *M. bidentatus*, *M. jaumei*, *M. gundlachi*, and *M. coffeus*.

To investigate possible morphological variation, we clustered samples using both a PCA and CVA on morphometrical datasets based on 11 landmarks and the shell outline. These results were correlated with several environmental variables. We found that, although correlated, the PC- and the CV-axes were not able to separate the cryptic species. The significant correlation between the "Species" explanatory variable and most of our response variables (Centroid Size, PC-axes 1 & 2, all CV-axes in both the 11 landmark and the outline datasets) as well as the result of the outcome CVA suggest that there are some differences in shell shape between the species. However, as the CV-axes suggest, those differences are rather small. A visualization of this variation (Fig. 4e & f) illustrates how small these variations are, and thus they may not be usable in a practical manner. It should, however, be noted that in the PCA the R²-values are only high for PC-axis 1 in both datasets, and these axes also had a highly significant correlation with Latitude (with a similarly high R²-value as for the "Species" variable; see Supplementary tables 1 & 2). Given that the four species analyzed morphometrically herein have a very different latitudinal distribution (Dennis & Hellberg, 2010), the significant correlations of the "Species" explanatory variable with the PC1 response variables might be best explained with an already described (Martins, 1996) morphological gradient in shell shape (and thickness) from north to south. This increasing thickness could be a response to temperature or to increasing predation by crabs, which has been shown to increase shell thickness in other gastropods (Moody & Aronson, 2012).

Taken together, our morphological analyses suggest that there is currently no reliable way to separate *M. bidentatus*, *M. jaumei*, and *M. gundlachi* from each other based on morphology. Other possible ways to separate the three cryptic species not studied here are shell microstructure, genital anatomy, and karyotyping. Shell microstructure was used by Martins (1996) to separate what he recognized as *M. bidentatus* from *M. coffeus*. He included figures depicting specimens from the distribution areas of the three species delineated here (*M. bidentatus*: Frias Martins, 1996, Fig. 278; *M. jaumei*: Martins, 1996, Fig. 277; *M. gundlachi*: Martins, 1996, Figs. 279, 280, 365–367), which show little variation between the species. This same publication also included drawings of the genital anatomy (*M. bidentatus*: Martins, 1996, Fig. 287a; *M. jaumei*: Martins, 1996, Fig. 287b; *M. gundlachi*: Martins, 1996, Figs. 287d & 374) that
might show small differences (e.g., a strongly enlarged mucous gland in a specimen that might be identified as a putative *M. jaumei* based on its origin), but these appear to also be small differences and would need further research in a dedicated study. Natarajan and Burch (1966) noted a difference in the karyotype of *Melampus* specimens from Tybee Island and Jekyll Island in Georgia (which they identified as *M. coffeus*, but which are likely *M. bidentatus* as there is no reliable record of *M. coffeus* in Georgia; 2n = 38) and from Mollusk, Virginia (identified there as *Melampus bidentatus lineatus*, likely *M. jaumei* based on Dennis and Hellberg (2010); 2n = 56). Further research is necessary to confirm that observation. While karyotype would not be any more easily diagnosed in the field than a CO1 barcode, such karyotype differences could contribute to reproductive isolation and hence be drivers of speciation (see e.g., Ravaoarimanana et al., 2004).

Cryptic species complexes have been repeatedly shown to be less cryptic when their morphological characters were reevaluated in later integrative studies (e.g., Horsáková et al., 2022). Morphology-based identification of species within a newly discovered cryptic species complex would greatly facilitate the review and correct assignment of type material and other older museum material as well as the reevaluation of past studies on life history, ecology, or behavior. However, in this case, we could not identify phenotypic characteristics to support our formal diagnosis based on SNPs. In the case of the *M. bidentatus* species complex, Dennis and Hellberg (2010) found no indication that any of the cryptic species hybridize with each other, even where they live sympatrically. The regionalized distribution patterns of the three distinct lineages point towards a separation on species level as well, given that the veliger larvae of *Melampus* is a good disperser (Dennis & Hellberg, 2010; Russell-Hunter et al., 1972). In fact, the regionalized distribution patterns point towards physiological differences; Dennis and Hellberg (2010) attributed the distribution gap of *M. jaumei* in Georgia and South Carolina to an interplay of temperature, salinity, and desiccation stress based on niche modeling between the three species. We have therefore separated the three lineages into three species based on diagnostic SNPs and reassigned various junior synonyms to the relevant species. We await any possible characters that could reliably separate the three cryptic species with great interest.

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Data Availability

New sequences were deposited in GenBank (see table 1 for accession). The Supplementary alignments, as well as the data used for the phylogeny and the morphometrical analyses were deposited on Dryad (https://doi.org/10.5061/dryad.3j9kd51q9).
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Supplementary Materials

Supplementary Material