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PHENOTYPIC AND GENETIC IDENTIFICATION OF *MERCENARIA MERCENARIA*, *MERCENARIA CAMPECHIENSIS*, AND THEIR HYBRIDS

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ABSTRACT The goal of this study was to evaluate the efficacy of phenotypic traits and two genetic assays to distinguish between *Mercenaria mercenaria*, an important aquaculture species, and *Mercenaria campechiensis*, a conspecific species that is partially sympatric, but that has not been successfully farmed. The objectives were to evaluate (1) four established diagnostic phenotypic traits (lunule shape, thickness of concentric shell ridges, prominence of anterior side of the concentric ridges, and presence of purple nacre inside of the shells) to distinguish between species, (2) size polymorphism of PCR fragments from four genes [16S rRNA, 18S rRNA, Internal Transcribed Spacer genes (ITS1 and ITS2)], and (3) PCR-restriction fragment length polymorphism (RFLP) on three of these genes (16S, ITS1, and ITS2). Phenotypic traits correctly distinguished two geographically distant conspecific populations (two locations in Maine for *M. mercenaria* and in Bradenton, FL, for *M. campechiensis*); however, among all other locations, both species had overlapping phenotypic trait values. Although PCR fragments were obtained for all genes tested, size polymorphism was not identified between the two species. Restriction enzyme digestion of PCR fragments resulted in diagnostic fragments distinguishing the two species for 16S, ITS1, and ITS2. Although phenotypic scores failed to delineate between the two species in most locations, PCR-RFLP results failed to identify hybrids in wild or hatchery samples. Further investigation is needed to apply the PCR-RFLP assays on known hybrid offspring from crosses of *M. mercenaria* and *M. campechiensis*. Results have elucidated the difficulties in confidently detecting hybridization in quahogs; however, determining effective methods for species identification and hybrid detection will be important to address issues such as the prevalence and distribution of hybridization in aquaculture and in wild populations, and to determin the presence of *M. campechiensis* which are currently difficult to find.

KEY WORDS: phenotypic score, species identification, PCR-RFLP, northern quahog, *Mercenaria mercenaria,* southern quahog, *Mercenaria campechiensis*

INTRODUCTION

The northern quahog *Mercenaria mercenaria* (family Veneridae) is an important aquaculture and fishery species that is naturally distributed along the Atlantic west coast from the Gulf of St. Lawrence, Canada, south to the Florida Keys, USA (Harte 2001). Since its development as an aquaculture species in the 1970s, *M. mercenaria* has been introduced throughout North America, including California (Murphy 1985), Washington state (Hanna 1966), Puerto Rico (Juste & Cortes 1990), the West Coast of Florida (Arnold et al. 2009), and overseas, including the United Kingdom and France (Chew 2001) and China (Zhang et al. 2003). In the United States, culture of *M. mercenaria* has developed into a $56 million (sales value) industry (USDA 2019), with most production occurring in Virginia and Florida (Yang et al. 2016). Harvest of wild *M. mercenaria* has been an important economic and cultural activity from Maine to Florida (MacKenzie et al. 2001) and is still an important fishery activity with a 7.2-million pounds of meats valued at $52.8 million (National Marine Fisheries Service 2020).

A conspecific, the southern quahog *Mercenaria campechiensis* is distributed along the mid-Atlantic West Coast from the Chesapeake Bay to Florida, and in the Gulf of Mexico from Florida through Texas, and the Yucatán Peninsula to Cuba (Abbott 1954). Notably, there is a considerable broad range overlap with *Mercenaria mercenaria*. On the Gulf Coast of Florida, *M. campechiensis* has been a popular fishery species since 1880, and landings reached 1 million pounds in 1932, but by 1950, the quahog populations had declined dramatically (MacKenzie et al. 1997). Unlike *M. mercenaria*, attempts to culture *M. campechiensis* has not been successful because of its relatively short refrigerated shelf life (Arnold et al. 2009).

These two species are most often peripatrically distributed in the northern-most sympatric areas, with *Mercenaria mercenaria* occurring inshore and *Mercenaria campechiensis* occurring further offshore because of their relative tolerances to salinity and temperature (Dillon & Manzi 1989, Menzel 1989, Arnold et al. 1996). Where they do co-occur, *M. mercenaria* and *M. campechiensis* produce viable hybrids, however, at low frequencies (Dillon & Manzi 1989). In the Indian River Lagoon, FL, these two species were found to occur syntopically in estuarine habitats (Menzel 1989), and hybrids were found at high frequencies (up to 37%) through allozyme analyses (Bert et al. 1993, Bert & Arnold 1995, Arnold et al. 1996).

Although superficially similar in appearance, these two closely related species are distinguished by four morphological characteristics (Dall 1902, Palmer 1927, Abbott 1974, Menzel 1989): (1) lunule shape (Fig. 1A); the lunules for *Mercenaria mercenaria* are typically narrower (width is three-fourth of height) than those for *Mercenaria campechiensis* (width is usually same as height); (2) color of the nacre on the inside of the shells (Fig. 1B); that is, the purple nacre of *M. mercenaria* is distinct from the pure white nacre of *M. campechiensis*; (3) presence/absence of pronounced anterior concentric ridges (Fig. 1C); that is, the anterior concentric ridges of *M. mercenaria* are less prominent than those of *M. campechiensis*; and (4)

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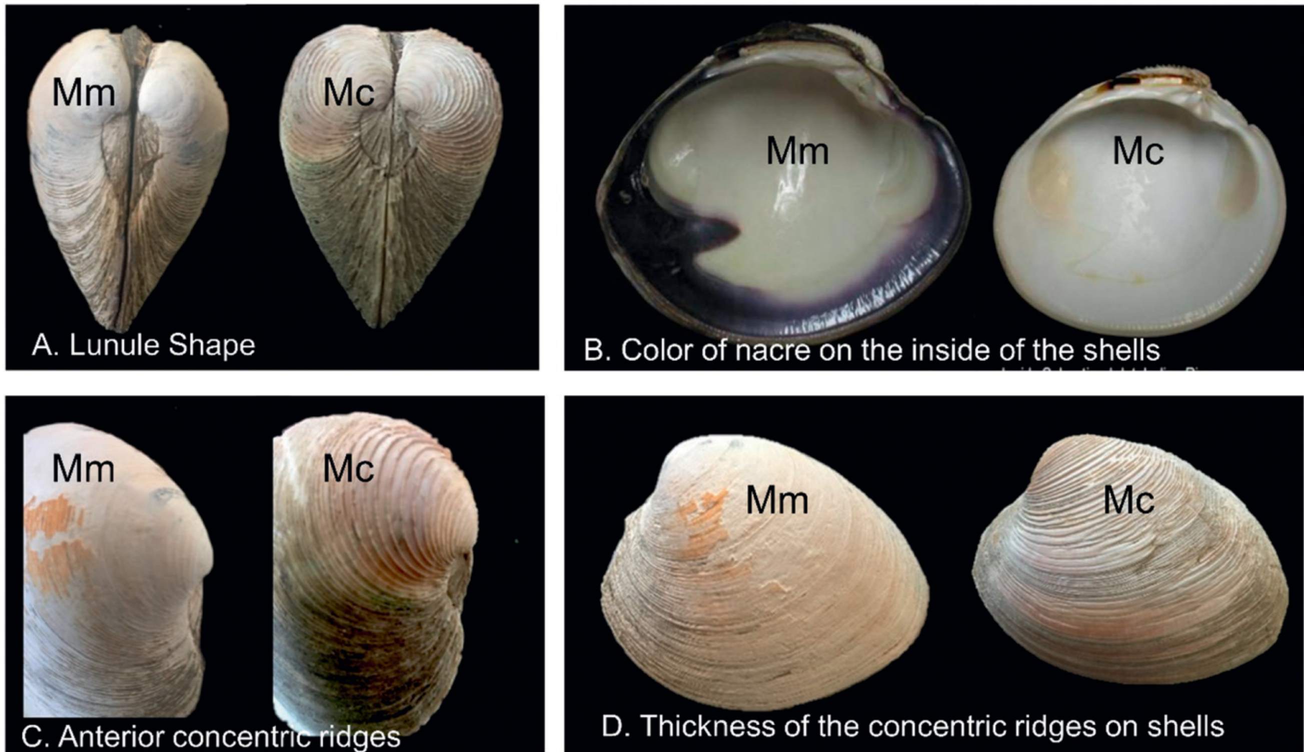

Figure 1. Phenotypic traits of the northern quahog Mm, *Mercenaria mercenaria* (on the left of photos A, B, C, and D) and the southern quahog Mc, *Mercenaria campechiensis* (on the right of photos A, B, C, and D). (A) Lunule shape: narrow lunule in Mm and wide lunules in Mc. (B) Color of the nacre on the inside of the shells: presence of purple color in Mm and absence of purple color in Mc. (C) Anterior concentric ridges: absence of anterior concentric ridges in Mm and presence in Mc. (D) Thickness of the concentric ridges on shells: smooth shell surface in Mm and thick concentric ridges in Mc.

concentric ridges (Fig. 1D); that is, thickness of the concentric ridges on the shell surface of *M. mercenaria* tends to be thin or absent, but pronounced on *M. campechiensis*.

Despite these diagnostic characteristics, significant variation among these traits often makes species identification difficult, especially on the adult stage when their phenotypic characteristics become worn because of their burrow-in-mud lifestyle (Andrews 1971, Dillon & Manzi 1989, Abbott & Morris 1995). Both species are benthic infauna and can be buried in mud and sand flats as deep as 20 cm (Eversole 1987, Roberts et al. 1989). As a result, adult shells typically turn dirt gray in color from siltation (Rosemarie 1984). Where present, hybrids show intermediate characteristics, adding to the difficulty of species identification based on the phenotypic criteria alone.

Attempts to improve stock of *Mercenaria campechiensis* for aquaculture have been explored using enforced hybridization, as hybrids generally show faster growth than pure *Mercenaria mercenaria* (Loosanoff 1954, Chesnut et al. 1956, Haven & Andrews 1956, Menzel 1962, Menzel & Menzel 1965, Menzel 1977). The culture of *M. campechiensis* has been attempted in North Carolina and Virginia but was unsuccessful because of low winter temperatures (Chesnut et al. 1956, Haven & Andrews 1956). Hybridization has also been explored to improve the heat tolerance and shelf-life of aquaculture products with limited documented success (Menzel 1977, Scarpa et al. 2008, Sturmer et al. 2012). Overall, the extent of hybridization and introgression in the aquaculture industry, particularly in Florida, is not well known.

On the Gulf Coast of Florida, *Mercenaria mercenaria* was introduced for aquaculture in the 1990s (Arnold et al. 2009), and the industry has thrived, especially in the vicinity of Cedar Key, FL, where *Mercenaria campechiensis* was once naturally abundant (Dillon & Manzi 1989). As a result, there is the potential for introgression of *M. campechiensis* into broodstock or in the growing areas where occasional damage to grow bags could lead to mixing with the locally distributed *M. campechiensis*. Because it has become difficult to find *M. campechiensis* on the Gulf Coast of Florida, the ability to reliably identify these two quahog species and their hybrids would be a worthwhile diagnostic tool for breeding projects and for the conservation of *M. campechiensis*. Ideally, diagnostic phenotypic characteristics to detect hybrids would be confirmed by genetic assays. To date, limited molecular diagnostic markers have been developed, except several allozymes (Dillon & Manzi 1989) which have been used for the identification of these two species and their hybrids (Dillon & Manzi 1989, Menzel 1989, Dillon 1992, Bert et al. 1993, Arnold et al. 1996).

Direct sequencing of mitochondrial DNA could distinguish between species (e.g., DNA barcoding, Hebert et al. 2003), but would be expensive to do on a large scale and would be ineffective at identifying hybrids. Ideally, a simple PCR-diagnostic tool could be demonstrated to distinguish between *Mercenaria mercenaria* and *Mercenaria campechiensis* based on PCR size polymorphism, for example, where species-specific indels (insertions or deletions of nucleotide bases within homologous regions of DNA) would differentiate among oyster species

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(Wang & Guo 2008). Alternatively, where size polymorphism is absent, restriction digests of PCR products could be an alternative approach to differentiate closely related species (e.g., Aranishi 2005).

The goal of this study was to evaluate the efficacy of phenotypic traits and two genetic assays to distinguish between *Mercenaria mercenaria* and *Mercenaria campechiensis*. The objectives were to evaluate (1) four established diagnostic phenotypic traits (lunule shape, thickness of concentric shell ridges, prominence of anterior side of the concentric ridges, and the presence of purple nacre inside of the shells) to distinguish between species, (2) size polymorphism of PCR fragments from four genes (16S rRNA, 18S rRNA, ITS1, and ITS2), and (3) PCR-restriction fragment length polymorphism (RFLP) on three of these genes (16S, ITS1, and ITS2).

MATERIALS AND METHODS

Sampling Sites

Quahog samples were collected from eight locations (nine groups) spanning from Maine to Florida, and samples were listed as ''wild'' (wild catch in local areas without aquaculture leases) or ''farmed'' (sampled from aquaculture farms) (Table 1). Wild *Mercenaria mercenaria* were obtained from two sites in Maine, north of any known *M. mercenaria* aquaculture areas. In addition, wild samples and farmed samples were obtained from Martha's Vineyard, MA; wild samples from Savannah, GA; and farmed samples from Cedar Key, FL. Wild *Mercenaria campechiensis* were collected from a site in Bradenton, FL, where no previous or current aquaculture leases are known to have existed. Finally, farmed *M. campechiensis* were obtained from two independent farms near Saint James City, FL (Table 1). These were the third generation of a wild *M. campechiensis* brood (Hurt & Heeb, personal communication).

Clams were transported live to the laboratory at the University of Florida (Gainesville, FL) and maintained in a recirculating system with a bead filter and a UV light until processing for phenotypic scoring and tissue sampling for DNA analysis.

Sample Processing

Whole, live quahogs were weighed to the nearest 0.01 g (Precision Balance ME4002E, Mettler Toledo, Columbus, OH). Shell measurements (length, height, and width) were performed using a Vernier caliper to the nearest 0.01 mm (CEN-TECH digital caliper, Camarillo, CA). Digital photographs of the outside and inside of sample shells were taken to score phenotypic traits (Fig. 1; described in the following text). For DNA extraction, a 1-2 mm^3 piece of adductor muscle was dissected and preserved in 96% ethanol and stored at –20°C in a freezer, after ethanol was replaced twice at 24 and 48 h. All shells were archived for further association analysis.

Phenotypic Identification

Quahogs were scored with the four phenotypic characteristics described earlier and previously used to characterize *Mercenaria mercenaria* and *Mercenaria campechiensis* (Abbott 1954, Abbott 1974, Dillon & Manzi 1989) (Fig. 1):

(1) Lunule shape. The width and height of lunule were first measured on *Mercenaria mercenaria* from Maine and *Mercenaria campechiensis* from Bradenton, FL, to determine whether the trait adequately differentiated these two species, treating these samples as genetically unambiguous representatives for each species. Based on the nonoverlap of the 95% confidence intervals for the mean ratio of lunule width to lunule height that differentiated *M. mercenaria* (Maine) from *M. campechiensis* (Bradenton, FL) (see Results), three categorical phenotypic scores were developed

TABLE 1.

The number and geographic locations of quahogs (family Veneridae) collected for species identification by phenotypic scoring and genotypic analysis.

Species considered	Number	Body sizes (mm)			Population/origin	Wild/farmed	GPS location
		Length	Height	Width			
Mercenaria mercenaria	28	89.65 ± 7.23	75.76 ± 6.07	47.40 ± 3.86	Harpswell, ME	Wild	43° 47′ 26.1″N 69° 57′ 33.7″W
	41	89.53 ± 5.82	76.18 ± 4.77	46.28 ± 4.01	West Bath, ME (low salinity area)	Wild	43° 52′ 32.7″N 69° 51′ 20.2″W
	10	49.47 ± 7.12	42.06 ± 6.39	26.84 ± 3.57	Martha's Vineyard, MA	Wild	41° 22′ 03.1″N 70° 39′ 11.8″W
	26	30.76 ± 5.73	26.48 ± 4.91	16.62 ± 3.32		Farmed	
	50	48.59 ± 10.34	42.57 ± 8.85	27.18 ± 5.86	Savannah, GA	Wild	31° 59′ 25.5″N 81° 01′ 23.3″W
	39	49.92 ± 5.76	43.04 ± 5.37	25.54 ± 3.14	Cedar Key, FL	Farmed	29° 08′ 59.3″N 83° 01′ 50.4″W
Mercenaria campechiensis	26	52.55 ± 7.03	44.57 ± 6.49	29.05 ± 4.56	Saint James City, FL	Farmed*	26° 29′ 55.8″N 82° 03′ 52.2″W
	30	58.05 ± 10.77	50.90 ± 8.92	33.60 ± 6.54	Saint James City, FL	Farmed*	26° 29′ 55.4″N 82° 03′ 52.4″W
	41	70.52 ± 12.36	66.02 ± 11.58	43.86 ± 7.36	Bradenton, FL	Wild	27° 27′ 55.9″N 82° 41′ 46.1″W

* These ''farmed'' samples were from two farms in St. James City, FL, and reported to be the third generation of offspring from several wild quahogs, which were believed to be *Mercenaria campechiensis*, collected locally in St. James City, FL (Hurt & Heeb. personal communications).

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to categorize the remaining samples: "wide"—ratio of lunule width: height ≥ 0.78, "narrow"—ratio of lunule width: height ≤ 0.67, and "intermediate"—ratio of lunule width: height between 0.67 and 0.78.

(2) Color of the nacre on the inside of the shells. A three-category system was used: white, purple, and intermediate (with a small amount of purple around the rim).
(3) Anterior side of the shell concentric ridges. A three-category score was used as thick, smooth, or intermediate.
(4) Thickness of concentric ridges on shell exterior. The concentric ridges were recorded as prominent, smooth, or intermediate.

Size Polymorphism Analysis of PCR (Polymerase Chain Reaction)-Amplified Genes

The 16S ribosomal mitochondrial RNA gene, 18S ribosomal RNA gene, and two internal transcribed spacer genes (ITS1 and ITS2), that have been used to distinguish among oyster species based on size polymorphism (Wang & Guo 2008), were evaluated for gene size polymorphism using universal primers (Table 2).

DNA extraction was performed using the glass fiber plate DNA extraction protocol (Ivanova et al. 2006). PCR was conducted in a 25-µL volume reaction composed of 1.5 µL genomic DNA (20 ng/µL), 5.0 µL of 5 × buffer, 2.5 µL $MgCl_2$ (25 mmol/L), 0.5 µL dNTP (10 mmol/L each), 2.5 µL each primer (10 µmol/L), 0.25 µL Promega GoTaq Flexi DNA Polymerase (5 U/µL), and ddH_2O to 25 µL. Amplifications were conducted in a Mastercycler Pro S thermal cycler (Eppendorf, Hamburg, Germany). The reaction cycle was set as follows: initial denaturation at 95°C for 2 min, followed by 35 cycles of denaturing for 30 s at 95°C, annealing for 30 s at the specific temperature to each primer (Table 2), an extension for 20 s or 30 s at 72°C, and a final extension for 5 min at 72°C. After evaluating varying agarose concentrations (1%, 1.5%, and 2.5%), buffer types [sodium boric acid (Brody & Kern 2004), Tris acetic acid disodium EDTA, and Tris boric acid disodium EDTA], and agarose types (low EEO agarose and the regular), PCR products were run in 1% agarose gels at 110 V for 1 h with Tris boric acid disodium EDTA buffer. A 100-bp size standard was run with each gel, and size polymorphisms were examined visually by staining with ethidium bromide and visualized with a UV light transilluminator (ENDURO GDS Gel Documentation System, Thomas Scientific, Swedesboro, NJ) for photography.

Restriction Site Analysis of PCR Products (PCR-RFLP Analysis)

To assess the utility of RFLP to produce diagnostic fragment sizes, the PCR products of three genes (ITS1, ITS2, and 16S rRNA) were Sanger sequenced and aligned, using a minimum of nine consensus sequences per species per gene. PCR amplicons were bidirectionally sequenced using BigDye Terminator v. 3.1 Cycle Sequencing Kit (Applied Bio System, Foster City, CA) in 10 µL volumes: 1.5 µL BigDye Terminator v. 3.1 Ready Reaction Mix, 1.00 µL sequencing buffer 5×, primer 0.16 µL, ddH_2O 6.34 µL, and 1 µL of the template. Cycling conditions were 96°C for 1 min, followed by 25 cycles at 96°C for 30 s, 52°C for 15 s, and 60°C for 4 min. Sequences were cleaned with Sephadex (Sigma-Aldrich) and electrophoresed on an Applied Biosystems 3100 Genetic Analyzer. DNA strands were verified, aligned, and joined into contiguous sequences using SEQUENCHER (ver. 4.2, Gene Codes Corp., Ann Arbor, MI).

The selection of restriction cut sites was determined using the software (visual tool for identifying restriction sites in multiple DNA sequences), which shows a comprehensive restriction map with all the restriction sites of the given DNA sequence (Chen et al. 2009). All enzymes were purchased from NEB (New England Biolabs, Ipswich, ME). Digestions followed manufacturer's temperature recommendations and were conducted in 50 µL reactions containing 3 µL of diluted (1:1 PCR: water) PCR product, 2 µL of the enzyme, and 5 µL enzyme buffer, and 40 µL of ddH_2O. Digestions were run with the same electrophoresis conditions stated earlier for gene polymorphism and photographed for analysis. To verify the DNA sequences of PCR-RFLP products, the target band on the electrophoresis gel was excised and cleaned using QIAquick Gel Extraction Kit (Qiagen) for DNA sequencing.

Genotypic Analysis and Association with Phenotypic Traits

Phenotypic traits were evaluated alongside quahog samples collected from each site and analyzed using the restricted enzymes deemed suitable during the previous step: RsaI for the

TABLE 2.

Candidate genes, related primers, and the annealing temperatures used for PCR amplification for the northern quahog *Mercenaria mercenaria* and the southern quahog *Mercenaria campechiensis*.

Gene	Primer name	Sequences (5′ to 3′) (forward/reverse)	Anneal temperature (°C)	Source
16S rRNA	16Sar	CGCCTGTTTATCAAAAACAT	48	Kappner & Bieler (2006)
	16Sbr	CCGGTCTGAACTCAGATCACGT	–	–
	16SL3-Ven	GCAAYGAGAGTTGTRCTAAGGTAGC	53	–
	16SH1-Ven	ATAATCCAACATCGAGGTCGCAAA	–	–
18S rRNA	18S-F	CTGGTTGATYCTGCCAGT	52	Palumbi (1996)
	18S-R	CYGCAGGTTCACCTACRG	–	–
ITS	ITS1-F	GGTGAACCTGCGGATGGA	55	Cheng et al. (2006)
	ITS1-R	GCTGGCTGCGCTCTTCAT	–	–
	ITS2-F	ATGAAGAGCGCAGCCAGC	58	Winnepenninckx et al. (1998)
	ITS2-R	GGCTCTTCCCGCTTCACTC	–	–

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16S rRNA gene and StyI for the ITS2 gene (see Results). Results from PCR-RFLP analysis were compared with the phenotypic scores of each clam to evaluate as a criterion for species identification. In addition, a total of 50 clams were sampled from one aquaculture farm in Cedar Key, FL, and analyzed genotypically and phenotypically for species identification.

Data Analysis

Data analysis was performed by JMP pro 14 (SAS; Cary, NC). ANOVA analysis was performed to compare the ratios of the lunule width with the length of quahog samples from different locations. A significance level was set as $\alpha = 0.050$.

RESULTS

Phenotypic Identification

Comparison of the lunule shapes (lunule width to height ratios) indicated that lunule shape from wild *Mercenaria mercenaria* collected from West Bath, ME (0.61 ± 0.06, $n = 41$), and Harpswell, ME (0.70 ± 0.11, mean ± SD, $n = 28$), were significantly smaller than that in the wild samples of *Mercenaria campechiensis* from Bradenton, FL (0.82 ± 0.12, $n = 39$) ($P <$ 0.001) (Fig. 2), indicating the lunules in Maine *M. mercenaria* were significantly narrower than those in Florida *M. campechiensis*. Interestingly, the lunule shape in samples from West Bath, ME, was significantly narrower than that in the samples from Harpswell, ME ($P = 0.002$), and the lunule shape in samples from Georgia was similar to that in samples from Bradenton, FL ($P = 0.146$) (Fig. 2). Based on the 95% confidence interval of the lunule shape (mean of lunule width:lunule height ratio) between all wild *M. mercenaria* from Maine (0.62–0.67, 0.64 ± 0.09, mean ± SD, $n = 69$ combining the samples from West Bath and Harpswell, ME) and wild *M. campechiensis* from Bradenton Beach, FL (0.78–0.86, 0.82 ± 0.12, $n = 39$), the working phenotypic scores distinguishing species were defined as lunule width to height ratio ≤ 0.67 for *M. mercenaria*, ≥ 0.78 for *M. campechiensis*, and between 0.67 and 0.78 for unknown species.

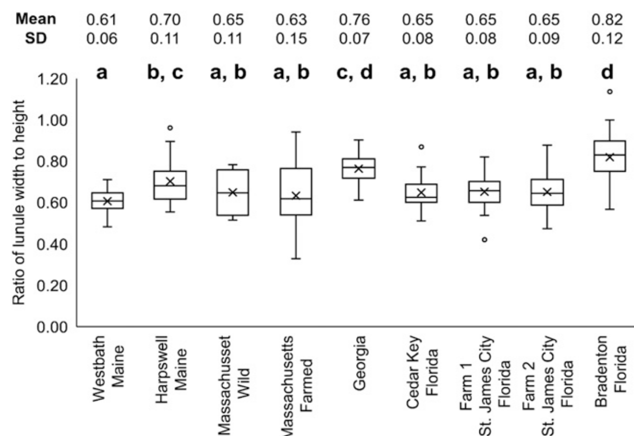

Figure 2. Ratios of lunule width to height in the quahogs collected from different locations. Samples collected from West Bath, ME, and Harpswell, ME, were wild population and identified as *Mercenaria mercenaria* based on other phenotypic scores, and samples collected from Bradenton, FL, were wild and identified as *Mercenaria campechiensis*. The box and whisker plots showed the minimum value, first quartile, median, third quartile, and maximum value. The symbol x in each dataset showed the mean value. The dots indicated the outliers from that dataset. The different letters a, b, c, and d indicated significant differences ($P <$ 0.050) among the lunule ratios in samples from different collection sites.

Based on the thickness of concentric ridges (anterior side and on shell exterior) and nacre color diagnostics, wild clam samples from Harpswell, ME, and West Bath, ME, were phenotypically scored as 100% *Mercenaria mercenaria*, whereas wild samples from Bradenton, FL, were phenotypically scored as 100% *Mercenaria campechiensis* (Table 3).

Amplicon Size Polymorphism

PCR amplification of 16S rRNA, 18S rRNA, and ITS1 and ITS2 genes from genome DNA was successful in both species (Fig. 3), but no length polymorphism was observed between the two species for these tested genes, regardless of electrophoresis conditions (not shown). The approximate lengths of these amplicons were 16S rRNA: 500 bp, 18S rRNA: 1,500 bp, ITS1: 800 bp, and ITS2: 550 bp.

Restriction Site Analysis of PCR Products (PCR-RFLP Analysis)

The PCR products of three genes were sequenced: (1) ITS1 for *Mercenaria mercenaria* (746 bp, $n = 11$) and *Mercenaria campechiensis* (746 bp, $n = 10$), (2) 16S rRNA for *M. mercenaria* (508 bp, $n = 10$) and *M. campechiensis* (522 bp, $n = 9$), and (3) ITS2 for *M. mercenaria* (576 bp, $n = 12$) and *M. campechiensis* (576 bp, $n = 10$).

Digestion profiles of the candidate restriction enzymes for each gene could yield different DNA fragments (Table 4). For the ITS1, BpmI generated two restriction fragments for *Mercenaria mercenaria* and three restriction fragments of *Mercenaria campechiensis*, allowing the delineation of the two species (Fig. 4A). Restriction profiles generated by BstAPI (Fig. 4B), AccI (Fig. 4C), and BsoBI (Fig. 4D) did not generate restriction fragments that can be used to identify the two clam species. For 16S, restriction enzyme RsaI generated two DNA fragments in *M. mercenaria* and *M. campechiensis* with different sizes (Fig. 4E), allowing the identification of the two species. For ITS2, restriction enzyme StyI did not cut *M. mercenaria* but produced three bands for *M. campechiensis* (Fig. 4F). One of these three bands was the same size as the uncut PCR product, whereas the other two bands were approximately 200 and 400 bp, suggesting that there was a single cut site recognized by StyI. The largest fragment from the StyI digestion of *M. campechiensis* ITS2 was sequenced, and a transversion on the StyI digestion site (A → C) was found, indicating a heterozygous allele in the ITS2 gene in *M. campechiensis*.

Genotypic Analysis and Association with Phenotypic Traits

Genetic analyses of quahog samples indicated that all of the wild samples from Harpswell, ME, and West Bath, ME, were identified as *Mercenaria mercenaria*, and samples from Bradenton Beach, FL, were identified as *Mercenaria campechiensis*. Similar to the results by phenotypic scoring, samples from other locations identified by PCR-RFLP analysis showed as either *M. mercenaria* or *M. campechiensis*, but no hybrids were found even for samples with intermediate phenotypic characters. The detailed results were

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TABLE 3.

Phenotypic scoring of quahogs (hard clams) collected from different geographical locations based on the morphological criteria shown in Figure 1.

			Phenotypic character and species recognition							
Location	Number	Scores	Lunule (width/ length)		Purple color inside		Concentric ridges on shell		Anterior concentric ridges	
Harpswell, ME	28 Wild	Yes	6	Mc	28	Mm	0	Mc	0	Mc
		No	11	Mm	0	Mc	28	Mm	28	Mm
		INTMD	11	Un	0	Un	0	Un	0	Un
West Bath, ME	41 Wild	Yes	0	Mc	41	Mm	0	Mc	0	Mc
		No	36	Mm	0	Mc	41	Mm	41	Mm
		INTMD	5	Un	0	Un	0	Un	0	Un
Martha's Vineyard, MA	10 Wild	Yes	0	Mc	5	Mm	0	Mc	0	Mc
		No	6	Mm	2	Mc	10	Mm	10	Mm
		INTMD	4	Un	3	Un	0	Un	0	Un
	26 Farmed	Yes	5	Mc	1	Mm	0	Mc	2	Mc
		No	17	Mm	8	Mc	23	Mm	24	Mm
		INTMD	3	Un	17	Un	3	Un	0	Un
Savannah, GA	50 Wild	Yes	21	Mc	10	Mm	0	Mc	4	Mc
		No	8	Mm	22	Mc	39	Mm	46	Mm
		INTMD	21	Un	18	Un	11	Un	0	Un
Cedar Key, FL	39 Farmed	Yes	2	Mc	0	Mm	0	Mc	0	Mc
		No	25	Mm	39	Mc	39	Mm	39	Mm
		INTMD	8 + 4*	Un	0	Un	0	Un	0	Un
Bradenton, FL	41 Wild	Yes	25	Mc	0	Mm	41	Mc	41	Mc
		No	4	Mm	41	Mc	0	Mm	0	Mm
		INTMD	10	Un	0	Un	0	Un	0	Un
St. James City, FL	30 Farmed	Yes	1	Mc	2	Mm	30	Mc	30	Mc
		No	18	Mm	15	Mc	0	Mm	0	Mm
		INTMD	7	Un	13	Un	0	Un	0	Un
	26 Farmed	Yes	1	Mc	1	Mm	26	Mc	26	Mc
		No	20	Mm	14	Mc	0	Mm	0	Mm
		INTMD	9	Un	11	Un	0	Un	0	Un

The definitions of the phenotypic scores were as follows: (1) lunule shape: yes, wide lunule with ratio of lunule width to lunle height ≥ 0.78; no, narrow lunule with ratio of lunule width: lunule height ≤ 0.67; Intermediate (INTMD) with ratio of lunule width: lunule height fell between 0.67 and 0.78. (2) Color of the nacre: yes, purple; no, white; INTMD, a small amount of purple around the rim. (3) Anterior side of the shell concentric ridges: yes, thick; no, smooth; INTMD, fall between thick and smooth. (4) Thickness of shell concentric ridges: yes, prominent (or say thick); no, smooth; INTMD, fall between thick and smooth. Species identification based on phenotypic scores were annotated as Mm, *Mercenaria mercenaria*; Mc, *Mercenaria campechiensis*; and Un, unknown.
* Four samples had no lunule measurements in this group.

shown in Table 5. Association of phenotypic traits and genetic analyses demonstrated that phenotypic traits of "presence or absence of anterior concentric ridges" was a more reliable score for species identification than other phenotypic traits.

DISCUSSION

The coexistence of these two quahog species and their capability of hybridization, as reported in the Indian River Lagoon of Florida (Arnold et al. 1996), have contributed to some confusion about species identification. Therefore, accurate recognition of these two quahog species is essential for breeding, conservation, and understanding of their biological and genetic differences.

Hybrids of closely related species often possess intermediate morphological traits, including body shape and other meristic traits (e.g., Zigler et al. 2012, Konopinski & Amirowicz 2018, Elliott et al. 2019). In many cases, minor deviations away from one or the other parental species can lead to difficulty in correctly diagnosing pure from F1 individuals, particularly when relying on morphology alone (e.g., Lessios 2007). It is not known what the implication of seeding with F1 hybrid quahogs would be, although evidence of reduced fitness of hybrids has been documented (Bert et al. 1993). The potential decrease in the viability of offspring owing to excessive mortality rates can result in low production, and in turn financial losses. In addition, the identification of pure and hybrid lineages is fundamental in developing policies for the conservation and management of native species (Allendorf et al. 2001), particularly in cases where there is widespread introduction of nonnative species into the range of native congeners (Bert et al. 1993). The natural interactions of hybrids with native populations are problematic to forecast (Todesco et al. 2016), and native wild populations face an additional high impact risk due to the genetic contamination with high incidences of hybrids (Melo et al. 2009). Currently, pure *Mercenaria campechiensis* on the Florida West Coast are difficult to find, which directly impacts its conservation efforts, and fertile hybrids (Menzel 1977) of these two closely related species have the possibility of genetic introgression, which may be a reason for the extinction of

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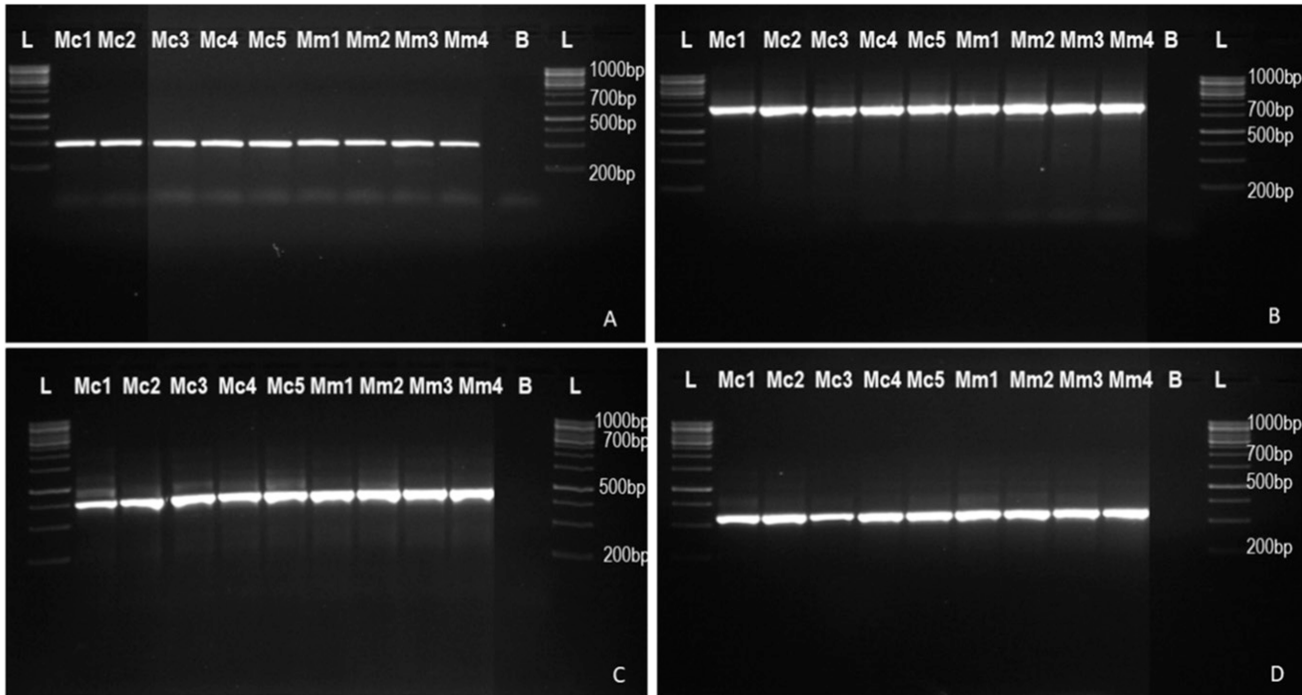

Figure 3. PCR amplification of genes from the southern quahog Mc, *Mercenaria campechiensis* and the northern quahog Mm, *Mercenaria mercenaria*. (A) 100-bp ladder (L) and a blank lane (labeled B) were run as controls. Electrophoresis gel was made of 1% agarose and run at 110 V for 1 hr. No differences in the PCR product sizes in the following four genes between Mm and Mc were identified. (A) 16S rRNA. (B) 18S rRNA. (C) ITS1. (D) ITS2.

pure species from natural populations. Therefore, pure wild populations need to be identified accurately for conservation activities.

Phenotypic Scoring of Mercenaria mercenaria *and* Mercenaria campechiensis *and Hybrids*

Phenotypic features have been traditionally used to differentiate *Mercenaria mercenaria* and *Mercenaria campechiensis*, including the four traits used in this study and the overall shell morphology (*M. mercenaria* tends to be flat and longish, whereas *M. campechiensis* tends to be round) (Abbott 1954, Abbott 1974). Based on shell morphology, plus frequency analyses of seven allozyme loci, *M. mercenaria* and *M. campechiensis* and their hybrids were distinguished, and a high percentage of hybrids (up to 37%) were found in the Indian River Lagoon, FL (Arnold et al. 1996). Three phenotypic traits, thickness of centric ridges, nacre color, and multivariate morphometrics were considered as the effective criteria for species identification, but lunule morphology was not (Dillon & Manzi 1989). Results presented here reflected similar results: lunule morphology was not a reliable phenotype for species identification, although the lunule in *M. mercenaria* was indeed significantly narrower than that in *M. campechiensis*. The thickness of centric ridges and the anterior ridges was also shown to be a reliable criterion. The interior shell nacre color in this study was not 100% reliable, and intermediate scores with only a tiny amount of purple near the shell edge were identified as either *M. mercenaria* or *M. campechiensis*. This was in agreement with one previous publication in which 80% of *M. mercenaria* were strongly purple, whereas 92% of *M. campechiensis* were purely white (Dillon & Manzi 1989).

TABLE 4.

Restriction enzymes, number of cutting site, and expected number of PCR-RFLP fragments for genes (ITS1, ITS2, and 16S) in Mm and Mc for species identification based on sequencing data.

		Number of cutting site		PCR-RFLP fragments	
Enzyme	Gene	Mm	Mc	Mm	Mc
BpmI	ITS1	1	2	2	3
BsoBI	ITS1	2	0	3	1
BstAPI	ITS1	2	1	3	2
RsaI	16S rRNA	2	1	3	2
AccI	ITS1	0	1	1	2
StyI	ITS2	0	1	1	2

Mm, *Mercenaria mercenaria*; Mc, *Mercenaria campechiensis*.

Polymorphism of Conserved Gene Length by PCR

Species-specific multiplex PCR has been a common approach for species identification because of its high accuracy, sensitivity, and convenience (one-step PCR), and has been applied to many species by use of conserved mitochondrial or ribosomal genes (Glass & Donaldson 1995). For molluscan bivalves, species-specific PCR has been applied for multiple oyster species identification (Klinbunga et al. 2003, Cross et al. 2006, Wang & Guo 2008), razor clam identification (Fernández-Tajes et al. 2010), and cockle species identification (Freire et al. 2011), as well as nonnative oyster contamination (Melo et al. 2013). Species-specific PCR assays for the two quahog species were conducted but failed to identify length polymorphism of the tested genes. Attempts were

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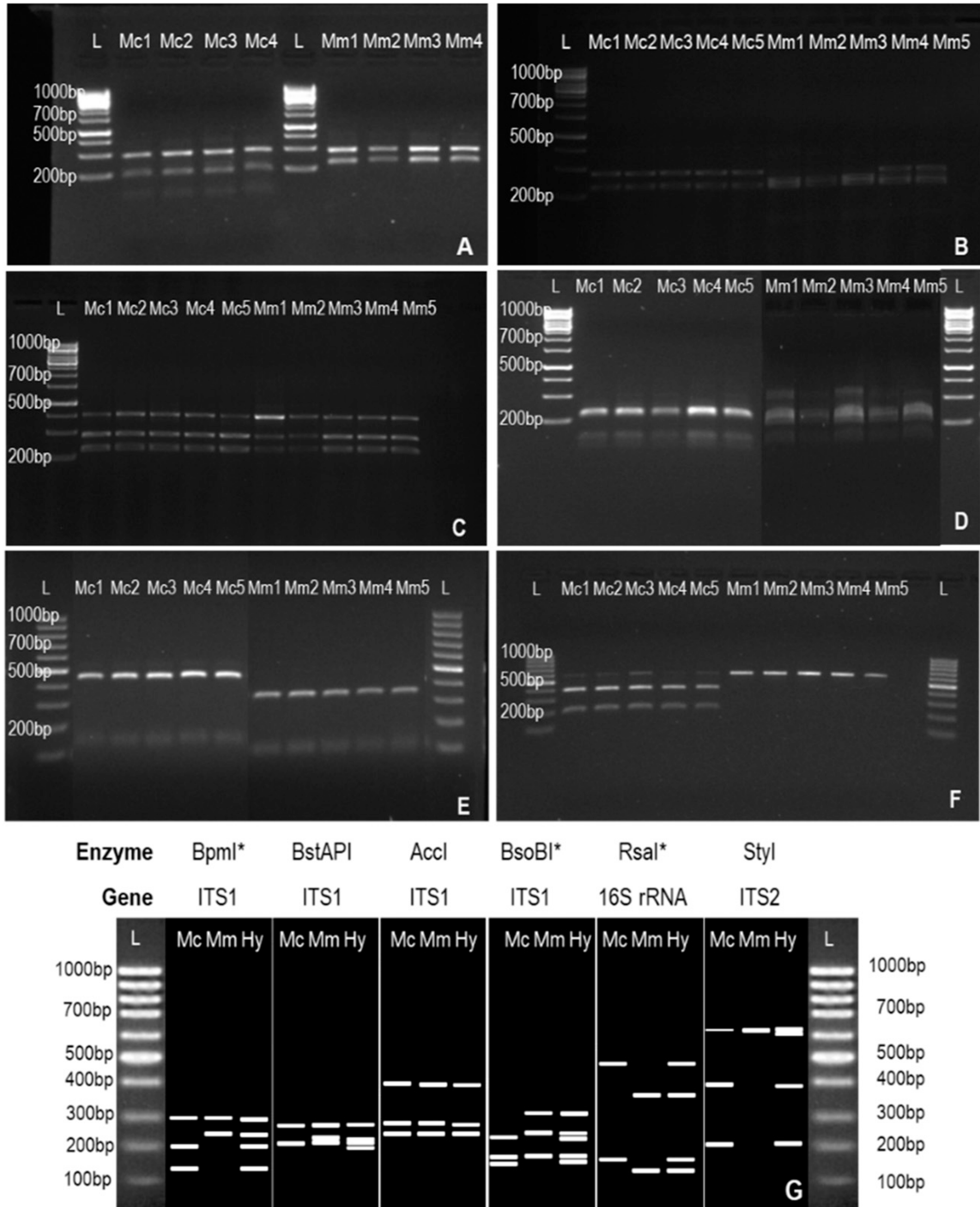

Figure 4. Restriction enzyme digestion profiles for quahog identified by phenotypic scores (Mc, *Mercenaria campechiensis*; Mm, *Mercenaria mercenaria*; U = unknown). (A) BpmI for ITS1 gene produced three bands for Mc and two bands in Mm, (B) BstAPI for ITS1 gene produced two bands for both, (C) AccI for ITS1 gene produced three bands at similar sizes for both species, (D) BsoBI for ITS1 gene produced three bands for both species, (E) RsaI for 16S rRNA gene produced two different sized bands for each species, (F) StyI for ITS2 gene produced three bands in Mc and one band in Mm. The largest size band in Mc was dim and often even invisible in some individuals, and resequencing of this fragment reflected heterozygous alleles that occurred with one base transverse on the StyI cutting site, and (G) schematic PCR-RFLP analysis of expected hybrid band. Considering the PCR product sizes and band number, the combination of enzyme and gene suitable for species identification were marked with *, and two fair ones were marked with **.

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TABLE 5.

Genotypic analysis of the quahog samples for species identification and comparison with phenotypic scores.

Sample		Genetic/RFLP analysis			Lunule shape			Nacre color			Concentric ridge			Anterior concentric ridge		
Location	N	Mm	Mc	Unknown	Mm	Mc	Unknown	Mm	Mc	Unknown	Mm	Mc	Unknown	Mm	Mc	Unknown
Harpswell, ME	19	19	0	0	8	0	11	19	0	0	19	0	0	19	0	0
West Bath, ME	20	20	0	0	18	0	2	20	0	0	20	0	0	20	0	0
Martha's Vineyard,	7 Wild	7	0	0	4	0	3	6	1	0	7	0	0	7	0	0
MA	6 Farmed	6	0	0	4	2	0	3	3	0	5	0	1	6	0	0
Savannah, GA	24	19	3	2*	2	13	9	15	9	0	21	0	3	21	3	0
Cedar Key, FL	30	27	3	0	23	1	6	30	0	0	30	0	0	30	0	0
Bradenton beach, FL	32	0	32	0	4	20	8*	0	32	0	0	32	0	0	32	0
Saint James City, FL	21	0	21	0	16	1	4	10	11	0	0	21	0	21	21	0
Saint James City, FL	18	0	18	0	12	0	6	10	8	0	0	18	0	0	18	0

Mm, *Mercenaria mercenaria*; Mc, *Mercenaria campechiensis*. Numbers represent the count of specimens examined for each category out of the maximum sampled (*N*).
* No effective data were collected from these samples—either no DNA bands were obtained, or the shells were broken for lunule identification.

made to increase the resolution of electrophoresis by adjusting agarose concentration and type (low EEO agarose), electrophoresis voltage, running time, and buffer system of the submerged gel electrophoresis (Brody & Kern 2004), and the length polymorphism of selected genes were not resolved. Further investigation is needed with different primers or genes.

Use of PCR-RFLP for Detecting Closely Related Species and Their Hybrids

PCR-RFLP analysis is an approach by using restriction enzymes to digest DNA and create DNA fragments with varying sizes among individuals, populations, and species, and is the first generation of a genetic marker (Grodzicker et al. 1974). For hybrid identification, RFLP is a prevalent marker, especially after Southern blotting is replaced by PCR (Liu & Cordes 2004). For molluscan bivalves, PCR-RFLP analysis of the 5S rDNA gene has been used on razor clams for the identification of *Solen marginatus*, *Ensis siliqua*, *Ensis arcuatus*, *Ensis macha*, *Ensis directus*, and their hybrids (Fernández-Tajes & Méndez 2007). PCR-RFLP was also used to differentiate four scallop species through analysis of the ITS region (López-Piñón et al. 2002), and for identifying mussel species (Santaclara et al. 2006). As a co-dominant marker, RFLP can uncover both alleles of an individual and be used for the identification of hybrids. In the current study, restriction enzymes, BpmI for ITS1, RsaI for 16S rRNA, and StyI for ITS2, could identify two species by generating different size or different numbers of fragments for each species.

Besides PCR-RFLP, microsatellite markers and single-nucleotide polymorphism (SNP) have also been applied to species identification. Microsatellite markers are characterized by high abundance, high polymorphism, and easy access; are capable of differentiating homozygous and heterozygous individuals (Miah et al. 2013); and have been developed for *Mercenaria mercenaria* (Wang et al. 2010) for population diversity analysis (Hargrove et al. 2015). Microsatellite markers have been applied to the European oyster *Ostrea edulis* for larval identification (Morgan & Rogers 2001) and Zhikong scallop *Chlamys farreri* for genetic mapping (Zhan et al. 2005). Single-nucleotide polymorphism markers are based on single-nucleotide polymorphism, and thus are sensitive and well suited for high-throughput large-scale genotyping analysis, such as haplotype mapping, linkage disequilibrium studies, and disease diagnosis (Vignal et al. 2002). The SNP markers have also been used for species and hybrid identification in mussels *Mytilus edulis*, *Mytilus coruscus*, and *Perna viridis*, (Chen et al. 2020) and in oysters (Jin et al. 2015) and their hybrids (Xu et al. 2014) using high-resolution melting curve analysis (Wang et al. 2014).

Hybrids play an important role in altering the genetic structure of native habitats of parental species (Bartley et al. 2000), but accurate identification of hybrids is usually difficult, particularly for hybrids beyond the F1 generation (Sanz et al. 2009, Hashimoto et al. 2012), which may be affected by environmental factors such as temperature, salinity, and dissolved oxygen (Lindsey 1988), and more sensitive methods for species identification are needed. In this study, no hybrids were found, although many clams had intermediate or mixed phenotypic characteristics. In addition, the ITS2 locus in *Mercenaria campechiensis* after StyI enzyme digestion showed heterozygous alleles which could be difficult to use to distinguish hybrids. Intensive hybridization has been reported in quahogs in the Indian River Lagoon, FL, based on morphological and allozyme analyses (Dillon & Manzi 1989, Bert et al. 1993, Arnold et al. 1996), but only minimal hybrids (0.1%) were reported in wild samples from North Carolina by the same author (Dillon 1992), and it was proposed that clear reproductive isolation may exist between the wild populations of these two species. Further sensitive markers, such as SNP markers, for hybrids of these two species need to be developed by using the known hybrids from artificial hybridization of confirmed *Mercenaria mercenaria* and *M. campechiensis* with clarified phenotypic and genetics analyses.

CONCLUSION

Four phenotypic traits, which have been traditionally used for the identification of two quahog species, were used in this

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study, to differentiate northern and southern quahogs (*Mercenaria mercenaria* and *Mercenaria campechiensis*) in wild populations from Maine and Florida. Samples from Maine were identified as *M. mercenaria*, and samples from Bradenton, FL were identified as *M. campechiensis*. Samples from other locations showed mixed results with intermediate scores of phenotypic traits. PCR fragment length polymorphism of 16S rRNA, 18S rRNA, ITS1, and ITS2 genes was not sensitive to identify the two quahog species because of the low base pair size differences. PCR-RFLP analysis was an effective method to identify these two species: restriction enzymes, BpmI for ITS1 and RsaI for 16S, could differentiate between the two quahog species and have the potential to identify their hybrids. StyI for ITS2 and BsoBI for ITS1 gene could identify both quahog species, and hybrids may display bands with similar sizes because ITS2 gene in *M. campechiensis* was detected to have heterozygous alleles. Association analyses of phenotypic traits and genetic analyses indicated that phenotypic traits of "presence or absence of anterior concentric ridges" was reliable for the identification of these two species. Lunule morphology is not a reliable phenotypic trait for species identification although the average lunule in *M. mercenaria* was significantly narrower than that in *M. campechiensis*.

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