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Research Article

Molecular Phylogeny and Morphological Distinctions of Two Popular Bivalves, *Ctenoides scaber* and *Ctenoides mitis*

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One of the most well-known species in the bivalve family Limidae (d'Orbigny, 1846) is the brightly colored *Ctenoides scaber* (Born, 1778), commonly known as the rough file clam or flame scallop. Distinguishing this bivalve from its close relative, *C. mitis* (Lamarck, 1807), can be difficult using only morphological features and has led to much taxonomic confusion throughout the literature. In this study, morphological characters were compared to a molecular phylogeny constructed using three genes (COI, 28S, and H3) in order to differentiate *C. scaber* and *C. mitis*. The phylogeny recovered two well-supported clades that differ significantly in shell rib numbers, but not tentacle colors. The two species were then placed in a larger phylogenetic context of the Limidae family, which revealed the need for further systematic revision across genera. As these bivalves are popular in aquaria, cannot be tank-raised, and have been overcollected in the past, proper species identification is important for assessing sustainable collection practices.

1. Introduction

The rapid increase of home aquaria owners (~1.5–2 billion people) and the lucrative global trade, valued at up to $330 million annually [1], have led to the overcollecting of many marine organisms [2, 3]. More than 500 species of marine invertebrates at numbers between 9 and 10 million are traded as ornamental species annually [1]. Although there have been some advances in breeding ornamental marine species [4], the majority are still being collected from the wild. The impacts of ornamental fisheries on invertebrate species with regard to their reproductive age, growth rate, population density/distribution, and population connectivity are largely unknown [5].

One of the obvious challenges for evaluating the impact of ornamental fisheries is to correctly document the numbers of species collected from various localities [6], especially given that cryptic species in marine ecosystems are common [7], and taxonomy in many marine invertebrate groups awaits systematic revision [8]. Currently, a large number of marine invertebrates have not been evaluated for IUCN (International Union for Conservation of Nature) status, despite their popularity in the aquarium trade.

One popular invertebrate group in the ornamental fisheries trade is the bivalve family Limidae, colloquially known as file shells or file clams. They are well-known to SCUBA divers [9] and shell collectors, and several charismatic species are routinely collected for the aquarium trade [10]. Numerous studies have been conducted on their behavior [11–16], morphology [17–19], habitat [20–22], reproduction [23, 24], physiology [25], and vision [26–31]. However, systematics studies have only been done on a small number of species in this family [10, 19, 32–35]. It is also noted that conchological traits alone are not always sufficient to establish clear boundaries between taxa [36].

One of the most well-known species in Limidae is *Ctenoides scaber* (Born, 1778), which in the past has been protected with imposed daily bag limits in the state of Florida due to overcollection [10]. It is known as the "flame scallop" due to its bright red coloration. According to a single retailer (Blue Zoo Aquatics, Hawthorne, CA, USA), around 10 *C. scaber* are sold each month *(pers. comm.)*. Multiplying this

average by the numerous retailers and wholesalers worldwide may warrant unsustainable collection levels. Furthermore, collection of these bivalves is difficult, as they inhabit rocky areas or crevices in corals [24] and substantial damage can be done to the local habitat when they are harvested. Aquaculture of the species is not possible because spawning cannot be induced in the laboratory, and larvae from wild-collected specimens did not survive the past 36 hours [24].

Documentation and identification of *Ctenoides scaber* in aquarium trade appear to be highly inconsistent and inaccurate. For example, the Aquariumtradedata.org database [6] uses shipment declarations and commercial invoices from the United States Fish and Wildlife Service to track aquarium trade data. The database listed 4,820 and 9,670 *C. scaber* being imported to the US in 2008 and 2011, respectively. But in 2009, only 22 individuals were documented. In addition, the documented countries of origin are the Philippines and Indonesia, even though the true *C. scaber* is a Caribbean species.

Compounding the unknown collection rates of *Ctenoides scaber* is the difficulty in differentiating it from the presumably sister species *C. mitis*. The classical distinction between the two species was that *C. scaber* had red pallial tentacles and *C. mitis* had white pallial tentacles [10]. However, this was based only on populations from the Florida Keys, and variation in tentacle color outside the Florida Keys required further scrutiny [10]. Mikkelsen and Bieler [10] found that the number of radial ribs (*C. scaber* 28–78, mean 55, and *C. mitis* 59–149, mean 89) differed between the two species, but they were not able to confirm any of the other previously cited differences, including shell size, shape, opacity, or distribution. In addition, several questions remain unanswered, including how much of tentacle color variations are due to phenotypic plasticity, and whether the two species are actually reproductively isolated.

Therefore, the goal of this study was to assess the phylogenetic status of *Ctenoides scaber* and *C. mitis* and to investigate their diagnostic morphological characteristics. Multigene molecular phylogenies were constructed for the two species as well as the Limidae family. Museum and aquaria specimens were examined to determine tentacle coloration and shell rib counts. Our results will aid proper identification of the two species for future collection management and provide a better understanding of the overall phylogeny of Limidae bivalves.

2. Methods

2.1. Sampling. Museum specimens were loaned from the University of Florida Museum of Natural History (see Supplementary Table 1 for catalogue numbers). Species identifications were given based on museum labels (*Ctenoides scaber*: $n = 3$; *C. mitis*: $n = 8$). Additional "flame scallops" (presumably *C. scaber*, $n = 18$) were purchased through Aqua Imports (Boulder, CO, USA), which were collected in Haiti ($n = 10$) and the Florida Keys ($n = 8$). The outgroups *Ctenoides ales* ($n = 1$) and *Limaria* sp. ($n = 2$) were also purchased through Aqua Imports, which were collected in the Philippines. All store-bought specimens were deposited in the University of Colorado Museum of Natural History (Supplementary Table 1).

2.2. Molecular Data. Genomic DNA was extracted from the mantle edge (*c.* 25 mg) of each specimen using the E.N.Z.A. Mollusc DNA Kit (Qiagen) according to the manufacturer's protocol. The mitochondrial gene COI, the nuclear ribosomal gene 28S, and the nuclear histone protein-encoding gene H3 were amplified for each specimen. Primers used to amplify the genes are listed in Table 1. The GoTaq® Hot Start Green Master Mix was used at concentrations following the manufacturers' instructions. The Polymerase Chain Reaction (PCR) cycling parameters for COI were as follows: initial denaturation of 94°C for 1 min and then 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s, with a final extension of 72°C for 6 min. For 28S, an initial denaturation of 94°C for 4 min was used, followed by 36 cycles of 94°C for 40 s, 53°C for 40 s, and 72°C for 1 min and a final extension of 72°C for 10 min. For H3, an initial denaturation of 94°C for 1 min was used, followed by 35 cycles of 94°C for 30 s, 49°C for 30 s, and 72°C for 30 s with a final extension of 72°C for 6 min. PCR products were visualized using agarose gel electrophoresis (1% agarose). PCR purification and sequencing were conducted by Quintara Biosciences (Berkeley, CA, USA). All sequences were submitted to Genbank and their accession numbers are listed in Supplementary Table 1.

2.3. Phylogenetic Analysis. Sequences of *Ctenoides scaber*, *C. mitis, C. ales,* and *Limaria* sp. were assembled using CodonCode Aligner© software 7.0.1 (Centerville, MA, USA) and aligned using MUSCLE [38]. Gene substitution models were selected using Partitionfinder 2 [39] based on Akaike Information Criterion. The GTR+G model was selected for COI, the GTR+G model was selected for 28S, and the GTR model was selected for H3. Maximum-likelihood (ML) and Bayesian phylogenetic reconstructions were conducted for the three genes individually as well as the concatenated dataset. The ML analyses were conducted using the RAxML BlackBox online server with 100 bootstraps [40, 41]. Bayesian analyses were conducted using MrBayes [42]. For each dataset, two runs were conducted simultaneously and trees were sampled every 10 iterations. Bayesian searches were run for 500,000 iterations for the COI, 28S, and H3 datasets. For the concatenated dataset, the analysis was run for 1,000,000 iterations. Convergence was ensured for each Bayesian run. For all datasets, a 50% majority rule consensus tree was generated after a 10% burnin and visualized in FigTree v1. 3.1 [43] (Figure 1).

To assess the phylogenetic status of *Ctenoides scaber* and *C. mitis* in a larger comparative phylogenetic framework, Genbank sequences of the same three genes from all other available Limidae bivalves were downloaded (Supplementary Table 1). Sequences from two representative *C. scaber* and *C. mitis* individuals (based on the *Ctenoides* phylogeny result), as well as *C. ales* and two *Limaria* sp., were aligned with the Genbank sequences to create a concatenated dataset. ML and Bayesian phylogenies were constructed using this dataset based on methods described above (Figure 2). *Mytilus edulis* and *Pecten maximus* were used as outgroups.

2.4. Morphological Analysis. Specimens were photographed using a Canon EOS Rebel® T5 Digital SLR camera with a

Table 1: PCR primers used for gene amplification on *Ctenoides scaber* and *C. mitis*.

Gene	Primer name	Sequence (5′-3′)	Source
COI	LCO1490	GGT CAA CAA ATC ATA AAG ATA TTGG	Folmer et al. (1994)
	HCO2198	GTA AAT ATA TGR TGD GCTC	Folmer et al. (1994)
	CTE 54F	AAG GGG GAT TGC CTT TAG CC	This Study
	CTE 601R	GCC GTG TTT ACA TGG CGA TCG GT	This Study
	CTE 32F	TGT TGG GGT TTT GGT CGT CT	This Study
	CTE 664R	GGT ACA AAA CAG GGT CCC CC	This Study
28S	Limoida_121F	TCA GAC GAG ATT ACC CGC TGA ATT TAA GC	This Study
	Sc28S_950R	TCT GGC TTC GTC CTA CTC AAG CAT AG	This Study
H3	H3aF	ATG GCT CGT ACC AAG CAG ACV GC	Colgan et al. (1998)
	H3aR	ATA TCC TTR GGC ATR ATR GTG AC	Colgan et al. (1998)

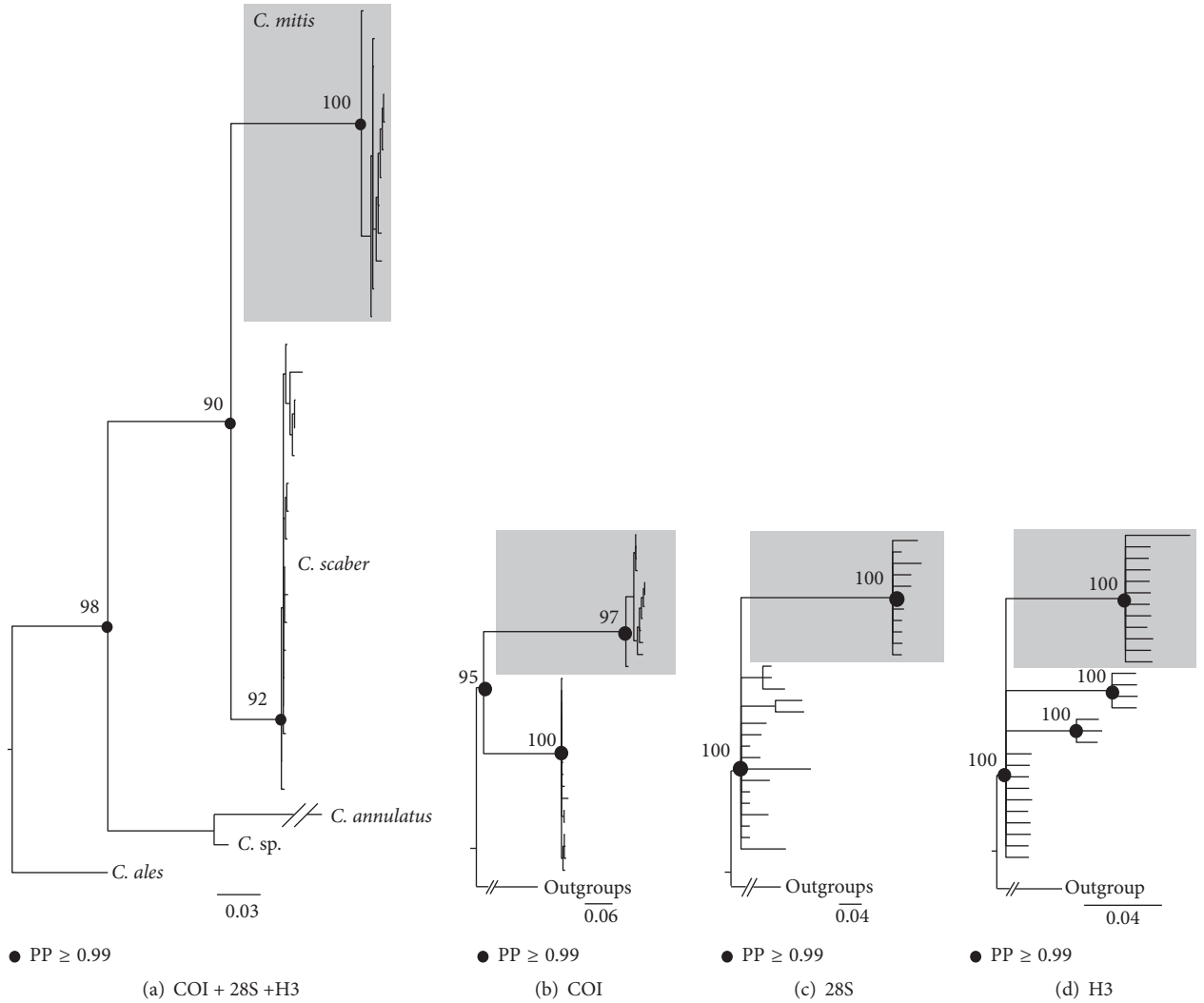


Figure 1: Bayesian topologies of *Ctenoides scaber* and *C. mitis*. (a) Concatenated gene tree. (b)–(d) Individual gene tree for COI, 28S, and H3, respectively. Shaded individuals belong to the *C. mitis* clade and nonshaded ones belong to the *C. scaber* clade. Posterior probabilities (PP) that are greater than 99% were denoted with black dots on the nodes. Bootstrap values that are greater than 90% (based on maximum likelihood analysis) were labeled above the branches.

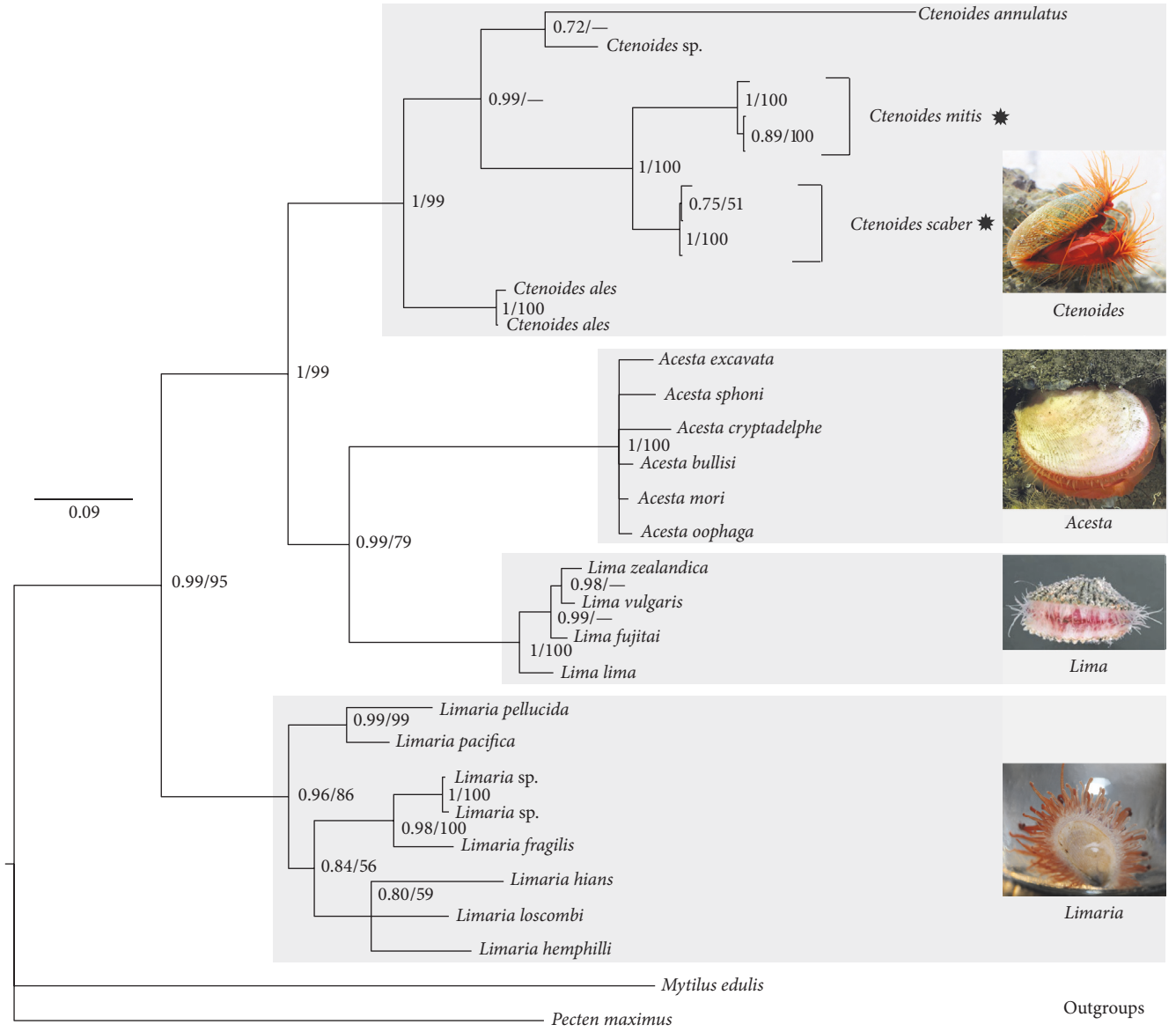


Figure 2: Bayesian phylogeny based on a concatenated genes (COI, 28S, and H3) showing phylogenetic position of *Ctenoides scaber* and *C. mitis* (black stars) within the Limidae family. Node labels indicating posterior probabilities and bootstrap values. The *Ctenoides* photo *(C. scaber)* was taken by J. Li. The *Acesta* photo *(Acesta* sp.*)* is Figure 2C of Gagnon et al. [37] and was reprinted with permission from the authors. The *Lima* photo (*L. caribaea*) was reprinted with permission from the American Museum of Natural History. The *Limaria* photo *(Limaria* sp.*)* was taken by L. Dougherty.

Canon EFS F/2.8 60 mm Macro Lens. Variations in tentacle coloration were recorded for live specimens or museum vouchers that contained photographs (Figure 3). Shell height and rib number were analyzed using ImageJ version 1.51n [44]. The shell height of each individual was measured as the average value (mm) of the left and right valves from the umbo to the furthest distal point. The shell rib number of each individual was recorded as the average of the left and right valves, including the auricles, and was counted near the growth edge on the main body of the shell. Shell height versus shell rib number was plotted for all individuals based on phylogroups and tentacle colors. The Mann-Whitney *U* test was used to determine whether the ratios were significantly different between groups.

3. Results

3.1. Phylogenetic Analysis. For the *Ctenoides* only analyses, both the concatenated tree (Figure 1(a)) and the COI gene tree (Figure 1(b)) recovered two strongly supported clades. One clade contained individuals with the classic *Ctenoides mitis* coloration (pure white tentacles) and the other contained specimens with the classic *C. scaber* coloration (pure red tentacles). Therefore, the two groups were tentatively defined

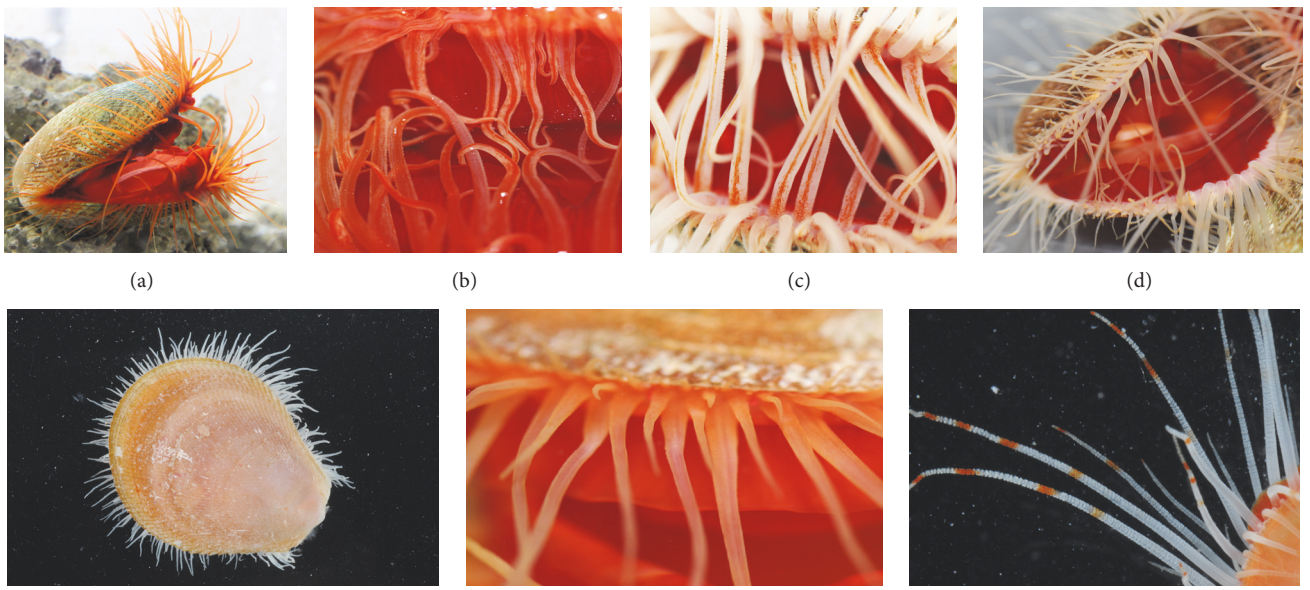


FIGURE 3: Tentacle color variations in *Ctenoides scaber* and *C. mitis*. *C. scaber* colorations included red/orange (a), red/orange with white stripes (b), white with red/orange stripes (c), or white (d). *C. mitis* colorations were white (e), white/orange (f), or white with red bands (g). Photo credit: L. Dougherty and J. Li.

as the *C. mitis* and *C. scaber* clades, respectively (Figure 1). However, it is worth noting that both clades also contained individuals without the typical coloration of their corresponding species (Figure 2, discussed in the next section). For the museum specimens, one preidentified *C. scaber* was placed in the *C. mitis* clade. For the aquarium store "*C. scaber*" specimens, 14 were placed in the *C. scaber* clade while the other 4 were placed in the *C. mitis* clade.

Both 28S and H3 gene trees supported the monophyly of the *C. mitis* clade but could not resolve the relationships between the *C. mitis* clade and the *C. scaber* individuals (Figures 1(c) and 1(d)), possibly due to the slower rate of evolution of the two genes.

The family level phylogeny for Limidae (Figure 2) showed strong support for the genera *Limaria*, *Lima*, *Acesta*, and *Ctenoides*. However, internal relationships within these genera were occasionally poorly resolved, especially in *Acesta*. The *C. mitis* and *C. scaber* clades were recovered as sister groups to one another.

3.2. Morphological Analysis. In the *Ctenoides scaber* clade, four variations in tentacle coloration were observed: solid red tentacles ($n = 5$, Figure 3(a)), red tentacles with white stripes ($n = 2$, Figure 3(b)), white tentacles with red stripes ($n = 4$, Figure 3(c)), and solid white tentacles ($n = 3$, Figure 3(d)). The red coloration in *C. scaber* varied from dark orange to red but is recorded throughout as "red" for simplicity. In the *Ctenoides mitis* clade, three variations in tentacle coloration were observed: solid white tentacles ($n = 4$, Figure 3(e)), light orange tentacles ($n = 1$, Figure 3(f)), and white tentacles with red bands (banded), which may be a coloration observed only in juveniles ($n = 2$, Figure 3(g)).

Tentacle coloration patterns of museum and aquarium store specimens based on locality and phylogenetic position are summarized in Table 2. Based on the two classical morphologies (red tentacles for *C. scaber* and white tentacles for *C. mitis*), of the 10 Florida Keys specimens, 8 fell into their expected clade. Exceptions were two *C. mitis*, a light orange individual and a banded individual (likely a juvenile phenotype). Of the 12 Caribbean specimens, only two fell into the expected clades (one *C. scaber* and one *C. mitis*). The other specimens had tentacle color variations that were not typical of either group (Table 2). The atypical colorations did not show any obvious phylogenetic signals.

Shell rib analysis revealed that the distribution of shell rib numbers in the *C. scaber* clade ($n = 17$) was significantly lower than that of the *C. mitis* clade ($n = 12$, Figure 4(a), Mann-Whitney U Test, $P < 0.00001$). Rib numbers of individuals from the *C. scaber* clade ranged from 37 to 58 (mean 48.3) and rib numbers in the *C. mitis* clade ranged from 73 to 103 (mean 85.8). Red and striped tentacles corresponded to the low shell rib number group, while light orange and banded tentacles corresponded to the high shell rib group (Figure 4(b)). White tentacles corresponded to both the low and high shell rib groups. Figure 5 shows a representative shell from *C. scaber* (a) and *C. mitis* (b).

In summary, our phylogenetic analysis recovered two well-supported clades corresponding to *Ctenoides scaber* and *C. mitis*. Genetic variations within each clade are relatively low, but that could be the result of limited geographical sampling. Morphological analyses showed that tentacle color alone cannot be used to determine species, as colors vary inter- and intraspecifically (Figure 3). Shell rib counts gave statistically different distributions for the two species (Figure 4), but the two distributions have some degree of overlap.

Table 2: Tentacle color patterns based on locality and phylogenetic position.

	Florida Keys	Caribbean
Ctenoides scaber clade	Solid red ($n = 4$)	Solid red ($n = 1$) Red/white stripes ($n = 2$) White/red stripes ($n = 5$) Solid white ($n = 2$)
Ctenoides mitis clade	Solid white ($n = 4$) Light orange ($n = 1$) White/red bands ($n = 1$)	Solid white ($n = 1$) White/red bands ($n = 1$)

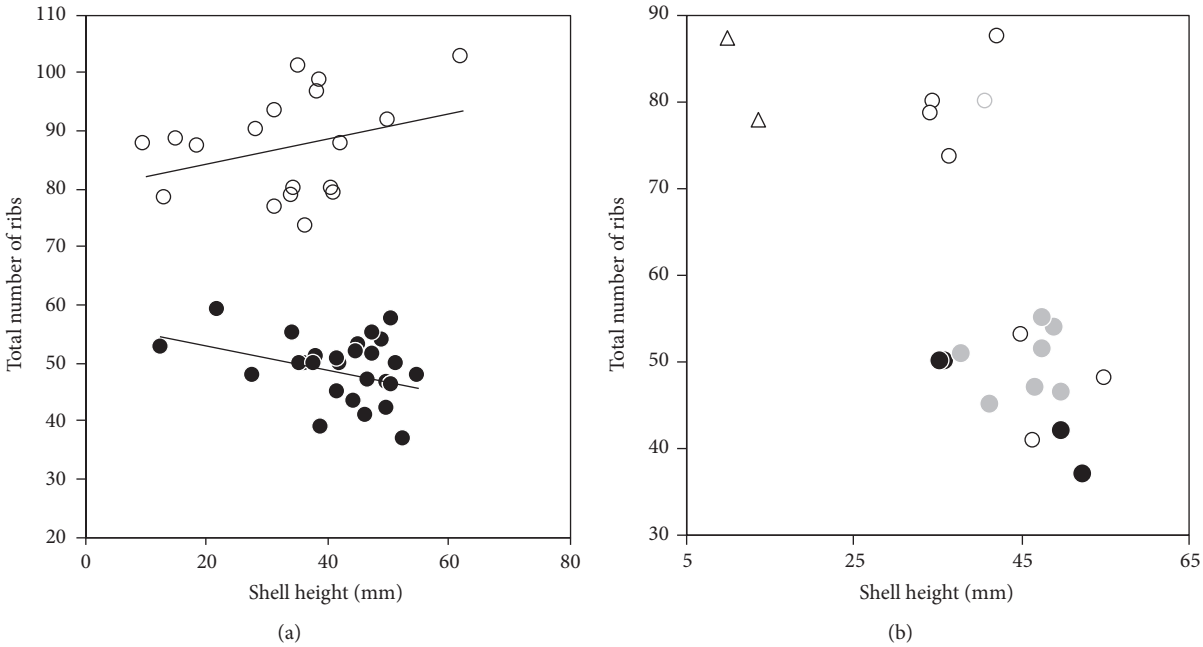


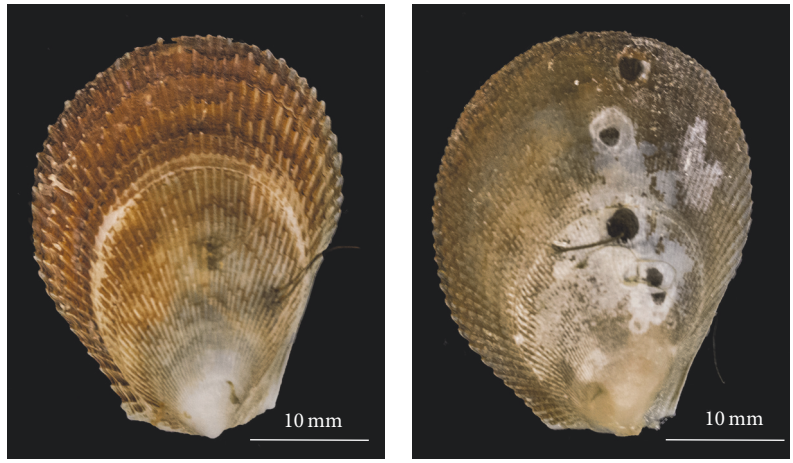
Figure 4: (a) Relationships between shell rib numbers and shell height. Black dots represent individuals belonging to the *Ctenoides scaber* clade and open circles represent those from the *C. mitis* clade. The distributions are significantly different ($P < 0.00001$). (b) Number of shell ribs versus shell height for individuals with tentacle color information available. Red, white, striped, light orange, and banded tentacles are represented by black dots, open circles, grey dots, grey open circles, and triangles, respectively.

4. Discussion

Distinguishing *Ctenoides scaber* from *C. mitis* is challenging, as is evidenced by observed misidentification in museum specimens and by the hobbyist community. The two species have many characteristics in common; they share ecological similarities with similar maximum depths and overlapping geographical distributions, and they are often found in the same location, suggesting overlapping ecological niches [10]. Internal anatomy, including mantle and eye structures, is also similar between the two species [17, 29, 30, 45].

Both *C. scaber* and *C. mitis* exhibit tentacle color variations. Mikkelsen and Bieler [10] placed high confidence in species identification using tentacle coloration in the Florida Keys. Our results confirmed the white and very light orange tentacle colorations in *C. mitis*, and the red coloration in *C. scaber* from the Florida Keys. However, outside of the Florida Keys, *C. scaber* exhibits many tentacle color variations without showing obvious genetic distinction from the red individuals (Figure 3).

As individuals of both species can possess solid white tentacles, tentacle color alone is not sufficient to determine species. This study confirmed that the *C. scaber* group has significantly fewer shell ribs than the *C. mitis* group. However, the overlap between the two species (between 59 and 78 ribs) means that no universal rule can be established to determine species based on shell ribs alone. Shell roughness is another characteristic which may be used to distinguish the two species, as the radial ribs of *C. mitis* are finer than *C. scaber* (Figure 5). However, shell roughness, size, and shape do vary to some extent across individuals, and, without instrumentation, species differentiation based on roughness could be challenging. Overall, the morphological diversity of shell ribs combined with the variety of tentacle coloration schemes stresses the importance of using molecular phylogenetics to verify species identification in future studies.



(a) (b)

Figure 5: (a) Representative valve of *C. scaber*; individual UCM 48137 (University of Colorado Museum of Natural History). Shell height 35.4 mm; shell ribs $n = 50$. (b) Representative valve of *C. mitis*; individual UCM 48138. Shell height 34.7 mm; shell ribs $n = 80$. Scale bar = 10 mm.

It is unclear whether the differential rib numbers between the two species has ecological significance. One possible hypothesis is that the high and low rib numbers are related to different predator defense mechanisms. The bright coloration (the result of carotenoids) in the tentacles of *C. scaber* [46] is hypothesized to be warning coloration for distasteful tissue. As *C. mitis* generally lacks brightly colored tentacles, they might have developed higher number of shell ribs to increase shell strength [47, 48]. However, this hypothesis does not explain the presence of white-colored *C. scaber,* so the effects of rib number and rib "roughness" on shell strength need to be examined further.

Based on the phylogenetic results, it is likely that *C. scaber* and *C. mitis* are recently diverged sister species. However, the speciation mechanism between the two has not been well studied. Ecological speciation has been demonstrated in numerous shallow-water marine invertebrates, with a divergence in life history being the most conspicuous difference between sibling species [7]. In bivalves, these differences include bathymetry limits, unique dietary adaptations, or host/symbiont associations [49]. *Ctenoides scaber* and *C. mitis* have overlapping geographical ranges and are often collected from the same substrate and at the same depth [10]. This strong similarity in microhabitat makes it difficult to hypothesize what ecological factor(s) could result in divergent selection between the two species. One potential difference is the prevalence of whiter tentacles in *C. mitis*, which may be the result of fewer carotenoids in the diet. Future research is needed to determine if the two species have different dietary adaptations and how they utilize carotenoids.

Alternatively, the speciation could have been allopatric rather than ecological, with geographical isolation occurring following secondary contact. Although absolute geographical barriers are fewer in the sea, abiotic factors such as ocean currents or temperature can result in population isolation [50]. More information regarding each species' historical geographical range would be necessary to examine this hypothesis. It should be noted that the sampling in this study did not cover the entire geographical range of both species, so it is possible that additional genetically distinct clades or morphotypes may exist.

Although our family-level phylogeny based on limited taxon sampling was able to resolve phylogenetic relationships between *Ctenoides scaber* and *C. mitis*, it demonstrates the need for a more comprehensive phylogenetic reconstruction. To our knowledge, phylogenetic relationships among/within the nine extent Limidae genera are not well established. This is partially because taxon coverage in past studies are poor and partially because taxonomical classification of Limidae is largely based on shell morphologies, some of which may not reflect true phylogenetic signals. For example, a recent study shows that the genera *Limatula* and *Antarctolima* are actually nested within *Ctenoides* [35]. We have also found that taxonomical inconsistencies are common in Genbank entries of Limidae species.

Limidae species exhibit many morphological and behavioral traits that are quite unique for bivalves. For example, a diverse spectrum of visual abilities can be found in the family, ranging from eyeless *(Limaria)* to pit eyes *(Lima)* to closed-lens eyes *(Ctenoides)* [29–31]. Members of the family also show distinct defense mechanisms, including nest-building behavior [20], swimming, tentacle autotomy [14, 18, 20, 21], and, potentially, defensive chemicals [30]. The evolution of these traits cannot be addressed without a comprehensive phylogeny.

Finally, conservation concerns must be addressed. *Ctenoides scaber* is highly collected for the aquarium trade, but estimates of their abundance are lacking. How many of the collected file clams are actually *C. scaber* and not *C. mitis* is unknown, as is the abundance of both of the species throughout the western Atlantic. Considering the popularity of these organisms with shell collectors, SCUBA divers, and

within the aquarium trade, a closer look at their conservation status is warranted.

Ethical Approval

The authors complied with all ethical standards in conducting this research.

Conflicts of Interest

The authors have no conflicts of interest.

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Supplementary Materials

Supplemental Table 1. Specimen information and Genbank accession numbers of all individuals used in the phylogenetic analyses. Sequences newly generated in this study are highlighted in grey. Where necessary, species names were updated to the currently accepted taxonomy. UF = University of Florida; UCM = University of Colorado Museum of Natural History. *(Supplementary Materials)*

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