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Molecular and morphometric data suggest the presence of a neglected species in the marine gastropod family Conidae

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ABSTRACT

Knowledge concerning the taxonomic diversity of marine organisms is crucial for understanding processes associated with species diversification in geographic areas that are devoid of obvious barriers to dispersal. The marine gastropod family Conidae contains many species complexes due to lack of clear morphological distinctiveness and existence of morphological intergradations among described species. *Conus flavidus* Lamarck, 1810 and *Conus frigidus* Reeve, 1848 are currently recognized as distinct taxa, but are often difficult to distinguish by morphological characters and include several synonyms, including *Conus peasei* Brazier, 1877. *C. peasei* was originally described by Pease in 1861 (as *Conus neglectus*) based on slight morphological differences of a population of *C. flavidus* from Hawaii that distinguished it from *C. flavidus* from elsewhere. To evaluate the systematics of this group and specifically test the hypothesis of synonymy of *C. peasei* with *C. flavidus*, we examined molecular and morphometric data from specimens of *C. flavidus*, *C. frigidus* and *C. peasei* (i.e., *C. flavidus* from Hawaii). Multiple clades that contain individuals from particular geographic regions are apparent in gene trees constructed from sequences of a mitochondrial gene region. In particular, sequences of *C. peasei* cluster together separately from sequences of *C. flavidus* and *C. frigidus*. Although individuals of *C. peasei*, *C. flavidus* and *C. frigidus* each contain a unique set of alleles for a nuclear locus, a conotoxin gene, alleles of *C. peasei* are more similar to those of *C. flavidus*. In addition, sequences of a region of a second nuclear gene are identical among *C. peasei* and *C. flavidus* though they are distinct from sequences of *C. frigidus*. Morphometric data revealed that shells of *C. peasei* are distinct in some aspects, but are more similar to those of *C. flavidus* than to those of *C. frigidus*. Taken together, these results suggest that *C. peasei* represents a distinct species. Moreover, based on the contradictory relationships inferred from the mitochondrial and nuclear sequences (as well as morphometric data), *C. peasei* may have originated through past hybridization among the ancestral lineages that gave rise to *C. flavidus* and *C. frigidus*.

1. Introduction

Despite the seeming connectivity of marine habitats with few obvious barriers to dispersal, many marine taxa have undergone extraordinary diversification (Sala and Knowlton, 2006; Bowen et al., 2013). Efforts to understand the origins and evolutionary processes that are responsible for this diversification depend on detailed knowledge of the relationships and evolutionary histories of these species. As results from molecular investigations continue to challenge the robustness of taxonomic interpretations (Knowlton, 1986; Bastrop et al., 1998; Lee and Ó Foighil, 2005; Bickford et al., 2006; Mathews, 2006; Payo et al., 2013; Puillandre et al., 2014b; Meyer-Wachsmuth et al., 2014), it has become abundantly clear that our understanding of marine biodiversity remains inadequate.

The predatory marine gastropod family Conidae includes approximately 800 living species (Bouchet and Gofas, 2015) and exhibits a tremendous rate of speciation since its appearance in the Eocene (Stanley, 1979; Kohn, 1990). Members of this family occur throughout tropical to temperate regions of the world's oceans, though the majority of species occur within the Indo-West Pacific (Walls, 1979; Röckel et al., 1995; Duda and Kohn, 2005). Conidae contains several species complexes that show slight morphological differentiation and in some cases intergradation of morphological character states that make species delimitation difficult (Walls, 1979; Röckel et al., 1995). Molecular-based approaches have illuminated multiple instances of previously

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unrecognized cryptic species within several of these complexes (Duda et al., 2008, 2009b; Puillandre et al., 2014a,b), suggesting that the current taxonomy of the group is unreliable and underestimates the actual species richness of Conidae.

One such group for which taxonomy remains unclear is the complex that includes *Conus flavidus* Lamarck, 1810 and *Conus frigidus* Reeve, 1848. The ranges of these species extend throughout most of the Indo-West Pacific and they occur sympatrically throughout much of their ranges in the Pacific (Röckel et al., 1995) (Fig. 1). These species possess rather nondescript yellowish[1] shells and are often very difficult to distinguish (Fig. 2). Nonetheless, certain differences in morphology are generally diagnostic: *C. flavidus* can grow to be larger than *C. frigidus* (maximum shell length 70 mm vs. 54 mm); *C. frigidus* tends to have a slightly higher spire; and the last whorls of *C. flavidus* shells are generally smooth, while those of *C. frigidus* are more granulose (Röckel et al., 1995).

In 1861, Pease recognized slight morphological differences in a population of *C. flavidus* from Hawaii compared to populations of this species elsewhere. Based on the distinctiveness of these features, he described the population from Hawaii as *C. neglectus* to distinguish it from *C. flavidus*. Recognizing that *C. neglectus* was a junior homonym of an Australian cone (*C. neglectus* Adams, 1854), Brazier (1877) renamed the species as *C. peasei*. Nonetheless, Pease's 'neglected cone' has generally been synonymized with the common, broadly distributed *C. flavidus* (Röckel et al., 1995). Intrigued by this history and the challenge of deciphering the species complex, we conducted molecular and morphometric analyses to evaluate the distinctiveness of *C. peasei*. The inclusion of specimens of *C. flavidus* and *C. frigidus* from other regions of the Indo-West Pacific also permitted an assessment of cryptic diversity elsewhere.

2. Methods

2.1. Specimens

We obtained specimens from collections of the Mollusk Division of the University of Michigan Museum of Zoology, Ann Arbor, MI (UMMZ) and the Museum of Comparative Zoology, Cambridge, MA, USA (MCZ). The present study included specimens of *C. peasei* from Hawaii (n = 39), *C. flavidus* from American Samoa (n = 9) and Guam (n = 4), and *C. frigidus* from American Samoa (n = 17) and Okinawa, Japan (n = 2) (Appendix A). We incorporated GenBank sequences of *C. flavidus* from Hawaii (=*C. peasei*) (n = 1; GenBank accession number AY588180); *C. flavidus* from French Polynesia (n = 1; KJ549909); *C. frigidus* from American Samoa (n = 1; KJ549912), Vanuatu (n = 3; KJ550263-KJ550266), Guam (n = 2; KJ549913, KJ551262), Madagascar (n = 1; KJ550267), and Reunion (n = 1; KJ549914). We also included sequences of *Conus* species that showed close phylogenetic affinity to focal taxa [*C. emaciatus* (KJ49903, KJ550231–KJ550233), *C. moreleti* (KJ549960), *C. kintoki* (KJ549934), *C. coelinae* (KJ550176–KJ550177)] as well as a selection of additional species that are more distantly related [*C. gladiator* (AY588185), *C. mus* (AY588206), *C. regius* (AY588216), *C. brunneus* (AY588161), *and C. bartschi* (AY588159)] (see Puillandre et al., 2014a) for rooting purposes. Although the identity of *C. flavidus* and *C. frigidus* specimens from which GenBank sequences were derived cannot be confirmed with certainty in all cases (e.g., the occurrence of *C. frigidus* in the Indian Ocean is questionable (Röckel et al., 1995)), their inclusion provides a broader geographic framework for evaluation of the species complex.

[1] For interpretation of color in Fig. 2, the reader is referred to the web version of this article.

2.2. Molecular data

We extracted genomic DNA from 15 to 25 mg of foot tissue for all specimens except for two in which only fecal material was available using the E.Z.N.A. Mollusk DNA kit (Omega Bio-Tek Inc.) following the manufacturer-recommended protocol. We amplified approximately 650 base pair (bp) of a region of the mitochondrial gene cytochrome oxidase c subunit I (COI) using general primers LCO1490 and HCO2198 (Folmer et al., 1994). Additionally, we amplified regions of two nuclear loci: 'FTX' and internal transcribed spacer region 2 (ITS2). Locus FTX is a member of the A-Superfamily of conotoxins, a suite of loci that encode neurotoxins and are characterized by their unique cysteine framework pattern. Amplification of a 118 bp region of conotoxin locus FTX was achieved using A-superfamily conotoxin primers 'Apref3' and 'CTXAR1' (Chang and Duda, 2012). Although these primers tend to amplify multiple loci from other species at low annealing temperatures (i.e., 45 °C) (see Chang and Duda, 2012), at a relatively high annealing temperature (i.e., 55 °C) they appear to amplify a single orthologous locus from *C. flavidus*, *C. frigidus* and *C. peasei* given the similarity in sequences obtained from these species (see Results). For ITS2 amplification, we utilized primers from Xu et al. (2001). We used the following amplification cycles: 94 °C for 30 s; 45 °C (COI), 55 °C (FTX) and 60 °C (ITS2) for 30 s; and 72 °C for 30 s for a total of 40 cycles.

We sequenced amplified fragments in both directions using amplification primers as sequencing primers at the University of Michigan DNA Sequencing Core. We visualized chromatograms and edited them in Sequencher (Gene Codes Corporation, Ann Arbor, Michigan, USA). To determine nuclear alleles from resultant sequences, we first identified alleles from homozygous individuals (i.e., chromatograms of these individual showed no overlapping peaks) and heterozygous individuals with a single polymorphic site (as determined from single overlapping peaks in chromatograms). We then examined chromatograms with more than one set of overlapping peaks to determine if the overlapping peaks could be fully explained by a combination of two of the previously recognized alleles. If ambiguity remained after following the steps above, we did not include these sequences in subsequent analyses.

We assembled phylogenetic trees of COI sequences using Bayesian approaches in MrBayes (Ronquist et al., 2011). We utilized the GTR substitution model based on available models in MrBayes and results from analyses of substitution models in MEGA v.5.2.1 (Tamura et al., 2011) and performed two parallel runs for 3,000,000 generations with a 25% burnin; otherwise, we used default values. We evaluated convergence based on measurements of the average standard deviation of split frequencies among runs.

To further evaluate species delimitation in this group, we analyzed COI sequences from *C. flavidus*, *C. frigidus*, and *C. peasei*, excluding outgroups with the Automatic Barcode Gap Discovery (ABGD) approach (Puillandre et al., 2011a). The program was run on the ABGD graphic web interface with the default Kimura 2-parameter model.

Because sequence diversity was low for FTX and ITS2, we constructed statistical parsimony networks (Templeton et al., 1992) for these genes with TCS 1.21 (Clement et al., 2000) to visualize the relationships of alleles. For the FTX locus, which showed distinct alleles for the populations/species examined, we also calculated Φ_{ST} values to determine the extent and pattern of partitioning of genetic variance with Arlequin 3.5.2.2 (Excoffier and Lischer, 2010).

2.3. Morphological data

We obtained simple linear shell measurements using calipers with "rhinoplasty" (Kohn and Riggs, 1975). In particular, we

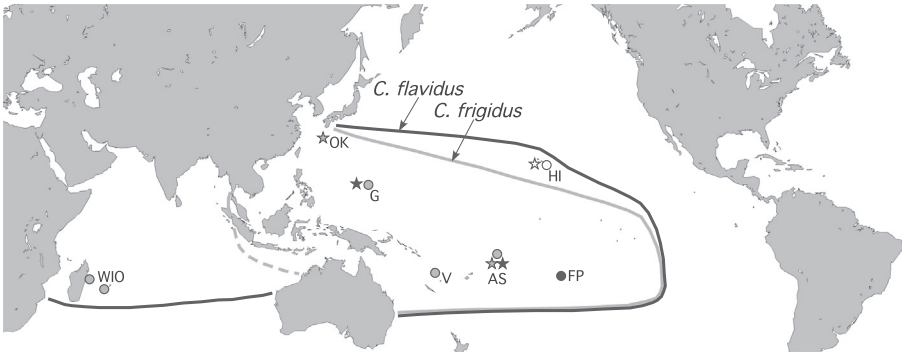

Fig. 1. Ranges of *C. flavidus* and *C. frigidus* in the Indo-West Pacific (Röckel et al., 1995); dashed line indicates uncertainty about the range boundaries of *C. frigidus* in the Indian Ocean. Stars indicate sampling locations of specimens we analyzed; circles denote sources of specimens of which sequences from GenBank were obtained; *C. frigidus* (gray fill), *C. flavidus* (black fill) and *C. peasei* (no fill). WIO = Western Indian Ocean (samples from Madagascar and Reunion), OK = Okinawa, G = Guam, V = Vanuatu, AS = American Samoa, HI = Hawaii, and FP = French Polynesia.

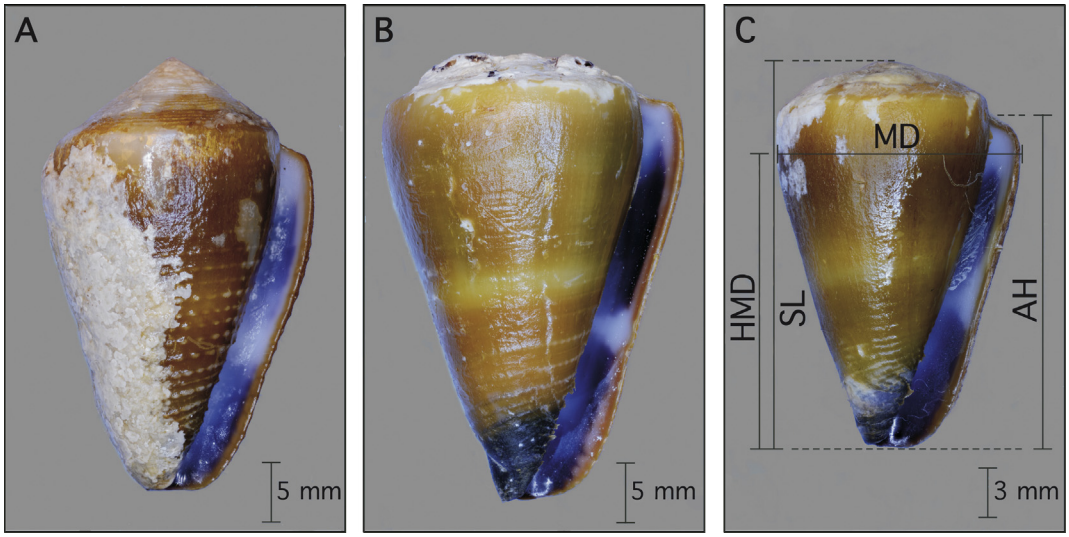

Fig. 2. Representative specimens of (A) *C. frigidus* (American Samoa), (B) *C. flavidus* (American Samoa) and (C) *C. peasei* (Hawaii) and morphometric measurements utilized; SL = shell length, MD = maximum diameter, AH = aperture height, and HMD = height of maximum diameter.

recorded shell length (SL), aperture height (AH), maximum diameter (MD), and height of maximum diameter (HMD) (see Fig. 2) as described by Röckel et al. (1995). With these data we calculated relative diameter (RD = MD/AH), position of maximum diameter (PMD = HMD/AH), length-width ratio (LW = SL/MD), and relative spire height (RSH = (SL − AH)/SL). We log transformed these proportions and compared them among populations of *C. frigidus* and *C. flavidus* using independent sample *t*-tests in SPSS Statistics (IBM Analytics). Because we were limited to specimens with available and intact shells, the morphometric study included three groups that were comprised of nine *C. flavidus* specimens from American Samoa, 38 specimens of *C. peasei* from Hawaii, and ten specimens of *C. frigidus* from American Samoa, respectively. Sample sizes for PMD analysis were smaller due to incomplete shell fragments without recorded HMD data (*C. flavidus*: n = 5, *C. peasei*: n = 16, *C. frigidus*: n = 2).

3. Results

3.1. Mitochondrial DNA

We obtained COI sequences from 49 specimens of *C. flavidus* and *C. frigidus* (GenBank accession numbers KU856983–KU857030, KX811539) and obtained 10 sequences of these species from GenBank; GenBank sequences of 15 *Conus* species were used for rooting purposes. Phylogenetic reconstruction of COI haplotypes of *C. peasei*, *C. flavidus* and *C. frigidus* (Fig. 3) revealed multiple well-supported clades, including those containing *C. peasei* (clade I); *C. frigidus* from Madagascar, Reunion, Vanuatu and American Samoa (clade II); *C. flavidus* from Guam, American Samoa, and French Polynesia (clade III); and *C. frigidus* from the northwestern Pacific (Okinawa and Guam) (clade IV). COI haplotypes of *C. peasei* in clade I are more similar to those of *C. frigidus* in clade II than to those of *C. flavidus* from other locations (clade III) (Fig. 3). Average genetic distances (Tamura and Nei (1993)) among sequences within each clade range from 0.005 (*C. peasei*; clade I) to 0.011 (*C. flavidus*; clade III). The average genetic distance (Tamura-Nei) among sequences of the clades of *C. peasei* (clade I) and *C. frigidus* in clade II is 0.053 (range: 0.043–0.080), while the average among *C. peasei* and *C. flavidus* clades (i.e., clades I and III) is 0.105 (range: 0.098–0.113).

Consistent with these results, ABGD analysis detected four groups with prior intraspecific divergence (P) of 0.0129 or greater. These groups correspond to *C. peasei* (clade I), *C. flavidus* (clade III), *C. frigidus* from clade II, and *C. frigidus* from clade IV. In recursive partitions at P = 0.0077 through P = 0.001, additional groups within the *C. frigidus* clades were detected. Throughout all partitions, *C. peasei* (clade I) constituted a single, unique group.

Fig. 3. Phylogram of COI sequences constructed using Bayesian approaches. Average standard deviation of split frequencies among two runs after three million generations was 0.0053. Posterior probability support values are provided for major clades, but are not included for internal branches within these clades. Sequence names include the identity of the specimen based on shell morphology, a code representing the specimen from which the sequence was derived or the accession number for sequences from GenBank, and an abbreviation for the source location of the specimen (Mad = Madagascar, Reu = Reunion, OK = Okinawa, G = Guam, V = Vanuatu, AS = American Samoa, HI = Hawaii, and FP = French Polynesia) (see Appendix A for additional details).

3.2. Nuclear DNA

For conotoxin locus 'FTX', we recovered 12 unique alleles from 21 individuals that occur in COI clades I, II and III (GenBank accession numbers KU856952–KU856982). These included sequences from seven individuals of *C. peasei* (i.e., members of COI clade I); two specimens of *C. flavidus* (one each from American Samoa and Guam) (i.e., COI clade III), and 12 specimens of *C. frigidus* from American Samoa (i.e., COI clade II). In accordance with the reciprocal monophyly observed in COI trees, we observed no shared alleles at FTX among *C. peasei*, *C. frigidus* (from COI clade II), and *C. flavidus* (Fig. 4A). As evident in the haplotype network, population genetic analyses revealed that there is less differentiation at FTX between *C. peasei* and *C. flavidus* (Φ_{ST} = 0.350) than between *C. peasei* and *C. frigidus* (Φ_{ST} = 0.834).

We sequenced ITS2 from a total of 15 individuals (GenBank accession numbers KU856950–KU856951). These included sequences from seven individuals of *C. peasei* (i.e., members of COI clade I), four of *C. flavidus* from American Samoa (i.e., COI clade III), and four of *C. frigidus* from American Samoa (i.e., COI clade II). Chromatograms of ITS2 sequences contained multiple similarly sized products that differed at insertions/deletions that made interpretation of these sequences difficult. Nonetheless, we were able to interpret 71 bp of these sequences with confidence from the specimens examined. Sequences of *C. peasei* and *C. flavidus* are identical and differed from sequences of *C. frigidus* at two positions, an insertion/deletion and a transversion (Fig. 4B).

3.3. Shell morphometry

The relative diameters of shells of *C. peasei* (i.e., members of COI clade I), *C. flavidus* (i.e., COI clade III), and *C. frigidus* (i.e., COI clade II) all differed significantly at the 5% level, with shells of *C. peasei* being the narrowest, followed by *C. flavidus*, and then *C. frigidus*

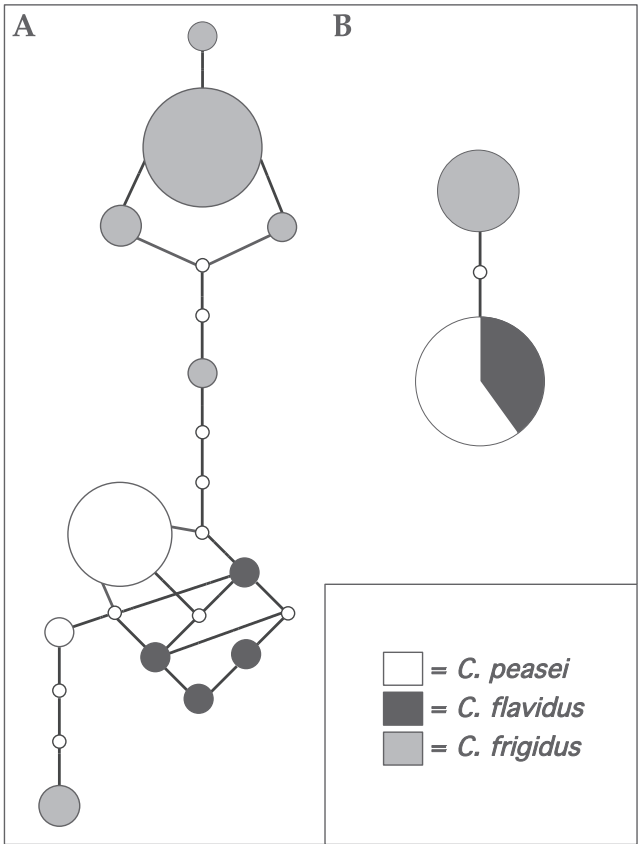

Fig. 4. Haplotype networks showing the relationships of (A) FTX and (B) ITS2 alleles of populations of *C. peasei*, *C. flavidus* and *C. frigidus* (i.e., members of COI clade II, Fig. 3). Colored circles represent observed alleles and are area-proportional to the frequency of the allele. Each step to a circle along the network indicates a change in one nucleotide base; small white circles represent hypothetical intermediate alleles.

(Table 1 and Fig. 5). We found a significant difference at the 1% level between the relative spire height of *C. peasei* and *C. frigidus*, but no difference between *C. peasei* and *C. flavidus* (Table 1). For the length-width ratio, the only significant difference occurred for the comparison between samples of *C. peasei* and *C. frigidus* (Table 1). We observed no significant differences in the position of the maximum diameter (Table 1).

4. Discussion

We used phylogenetic, population genetic and morphometric approaches to evaluate the presence of cryptic species of *C. flavidus* and *C. frigidus* in the Indo-West Pacific. Indeed, COI sequences of specimens of these species fall out into multiple, well defined clades that largely correspond with geographic regions: *C. peasei* from Hawaii (i.e., COI clade I, Fig. 3); *C. frigidus* from the western Indian Ocean and southwestern Pacific (i.e., COI clade II); *C. flavidus* from Guam, American Samoa and French Polynesia (i.e., COI clade III); and *C. frigidus* from the northwestern Pacific (i.e., COI clade IV) (Fig. 3). The lack of shared alleles of one of the nuclear genes (i.e., FTX) also supports the genetic distinctiveness of *C. peasei*. Interestingly, nuclear and mitochondrial markers reveal conflicting patterns in terms of which species, *C. flavidus* (i.e., COI clade III) or *C. frigidus* (i.e., COI clade II), is more closely related to *C. peasei* (i.e., COI clade I). Moreover, *C. peasei* is more similar in shell morphology to *C. flavidus* than it is to *C. frigidus* (i.e., COI clade I); however, shells of *C. peasei* can be distinguished from those of *C. flavidus* by their smaller relative diameter.

4.1. Removal of C. peasei from synonymy with C. flavidus

We advocate the taxonomic separation of the population of *C. flavidus* at Hawaii from other populations of *C. flavidus*. Following taxonomic precedence, we recognize it as *Conus peasei* Brazier, 1877. Our rationale for this recommendation follows.

First, the presence of reciprocally monophyletic clades in the phylogenetic reconstruction based on COI sequences strongly delineates *C. peasei* from all other specimens examined (Fig. 3). When subjected to ABGD analysis, the divergence between the COI sequences of these monophyletic clades is sufficient to distinguish them as separate groups. The presence of divergent COI haplotypes has revealed the existence of cryptic forms of other Conidae species, including *C. judaeus* (Duda et al., 2009a), members of the *C. sponsalis* complex (Duda et al., 2008) and *C. conco* (Puillandre et al., 2014b). Moreover, many widespread species of Conidae exhibit very low levels of genetic differentiation at COI over vast geographic scales, but no evidence of reciprocally monophyletic clades (except for cryptic species; see below) (Duda and Lee, 2009; Duda and Lessios, 2009; Duda et al., 2012).

Second, FTX alleles of *C. peasei* are distinct from those from *C. flavidus* and *C. frigidus*, although they are more similar to alleles of *C. flavidus* (Fig. 4A). Although our sample size is small, these results corroborate the observed distinctiveness of COI haplotypes from these species and provide support for separation of *C. peasei* from *C. flavidus*. As described below, allelic diversity of FTX of *C. peasei* and *C. flavidus* is not consistent with observed patterns of intraspecific diversity of conotoxin loci of other species. Although populations of widespread *Conus* species often show differentiation at conotoxin loci, this largely from differences in allelic frequencies and few alleles are private among populations (Duda and Lee, 2009; Duda et al., 2009a). For example, a population of *C. miliaris* at Easter Island that was described as a subspecies (*C. miliaris pascuensis*) by Rehder (1980) is genetically differentiated from populations elsewhere in the Indo-West Pacific at COI and two conotoxin loci, but unlike the situation with *C. peasei*, the subspecies of *C. miliairis* at Easter Island shares haplotypes at these loci with other populations of this species (Duda and Lee, 2009). On the contrary, ITS2 sequences of *C. peasei* are identical to those of *C. flavidus* (Fig. 4B). This marker though has low resolution in some Neogastropoda taxa (Oliverio et al., 2002) and so the observed sharing of alleles may simply reflect the lack of divergence at this locus.

Third, linear and simple ratio comparisons of morphometric measures of shells of *C. peasei* (i.e., COI clade I, Fig. 3), *C. flavidus* (i.e., COI clade III) and *C. frigidus* from the western Indian Ocean and southwestern Pacific (i.e., COI clade II) showed that members

Table 1
Shell morphometrics of populations of *C. frigidus* from American Samoa (AS) and *C. flavidus* from AS and Hawaii (HI). Averages are listed with standard errors in parentheses.

Species	Relative diameter	Relative spire height	Length-width ratio	Position of maximum diameter
C. peasei	0.692 (0.0046)	0.112 (0.0051)	1.632 (0.012)	0.881 (0.0098)
C. flavidus	0.719 (0.0098)	0.135 (0.012)	1.611 (0.0093)	0.854 (0.014)
C. frigidus	0.764 (0.012)	0.169 (0.0098)	1.579 (0.028)	0.906 (0.0094)

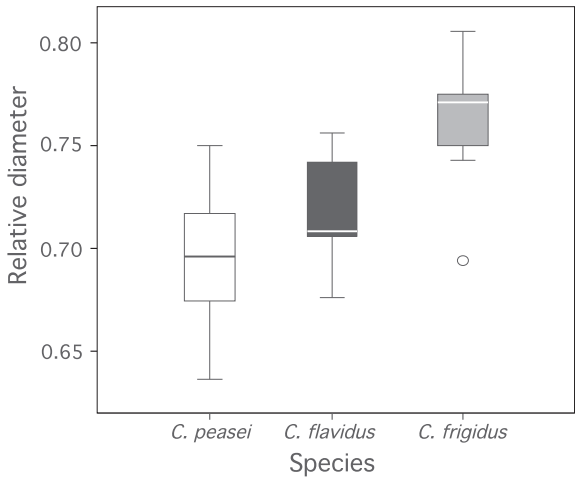

Fig. 5. Box plot representation of the relative diameters of *C. peasei*, *C. flavidus* and *C. frigidus* (members of COI clade II; Fig. 3). All measures are significantly different at the 5% level. The circle represents an outlier individual of *C. frigidus* with a relative diameter of 0.69.

of the three clades can largely be distinguished based on relative diameters of shells (Table 1 and Fig. 5). Additional anecdotal evidence comes from Pease's original interpretation that the distinctiveness of *C. peasei* warranted a species description. Although he did not elaborate on the features that distinguished it from *C. flavidus* in his brief description, Brazier (1877) cites a more narrow aperture and shell width, bright orange edge of lip, interior with large deep purple spots, and thicker and rougher epidermis as differentiating *C. peasei* from *C. flavidus*. With the exception of the orange lip, these qualitative assessments generally hold true among the specimens used in this study, especially the more narrow shell width. While species of Conidae have traditionally been determined on the basis of shell morphology alone (Röckel et al., 1995), the recent availability of genetic data has illuminated the unreliability of this approach within hyperdiverse genera (Cruz et al., 2011; Fedosov et al., 2011; Puillandre et al., 2011b). However, the inclusion of morphological data with molecular data can be useful. Here, the concordance between molecular data and relative diameter of shells strengthens the case for taxonomic separation and provides a means to potentially distinguish these species with morphological characters.

Hawaii's relative isolation in the Indo-West Pacific offers a plausible scenario for the origination of *C. peasei*. While endemism of marine taxa in the Hawaiian Archipelago is lower than that of terrestrial groups (Kay and Palumbi, 1987), there is evidence for barriers to gene flow in some marine taxa due to geographic isolation, current patterns, and dispersal capabilities (Hourigan and Reese, 1987; Santamaria et al., 2013). In particular, some 162 Hawaiian-endemic molluscs, including several species of Conidae, have been proposed (Kosuge, 1969). Moreover, a number of species that are widespread in the Indo-West Pacific, including *C. frigidus* and now also *C. flavidus*, do not occur in the Hawaiian Archipelago and *C. sanguinolentus* shows a phylogeographic break at Hawaii (Duda et al., 2012). We posit that a chance dispersal event of *C. flavidus* to Hawaii and subsequent lack of gene flow between this population and populations elsewhere facilitated the origination of *C. peasei* and its separation from *C. flavidus*.

Sample sizes used in this study are certainly not large for all populations nor for all loci examined, but because all individuals sampled from Hawaii (n = 40) were identified as *C. peasei*, it is unlikely that *C. flavidus* occurs here in great frequency. While we detected no *C. peasei* outside of Hawaii, sample sizes were too small to exclude the possibility that *C. peasei* has a more extensive range than is assumed here. Although Brazier (1877) reports *C. peasei* from Darnley Island in the Torres Straits, it is unclear if this taxon is the same as recognized by Pease or that we recognize here. Thus, without further analyses, we do not know if the ranges of *C. peasei* and *C. flavidus* overlap in the Indo-West Pacific. Molecular genetic and morphological assessments at additional locations are necessary to address this concern.

4.2. Resolving the mitochondrial and nuclear conflict

Inferred relationships from the mitochondrial gene tree and levels of similarity among alleles of the two nuclear markers are contradictory (Figs. 3 and 4). COI haplotypes from *C. peasei* (i.e., COI clade I, Fig. 3) are more similar to those of *C. frigidus* (i.e., COI clade II), while nuclear markers reveal genetically similar (FTX) or identical (ITS2) alleles between *C. peasei* and *C. flavidus* (Fig. 4). Presumably either the mitochondrial or nuclear gene trees reflect the species tree. This complication is not exceptional among taxa, as other organisms show discordance among mitochondrial and nuclear gene trees (Nolte et al., 2005; Adams et al., 2003; Rognon and Guyomard, 2003). Two hypotheses may explain the discordance: 1) incomplete lineage sorting of ancestral polymorphisms and 2) introgression of genes through a past hybridization event (Fig. 6).

One scenario (Fig. 6A) assumes that the common ancestor of *C. flavidus*, *C. frigidus*, and *C. peasei* contained multiple alleles for either COI or ITS2 and FTX. As the common ancestor of these species separated into two and finally three species, the polymorphism was retained and alleles were fixed randomly in daughter species. Fig. 6A depicts this scenario for one of the nuclear loci we examined, but incomplete lineage sorting would have had to occur at the COI locus (i.e., the entire mitochondrial genome) or both ITS2 and FTX loci to explain the discordance.

Alternatively, the ancestor of *C. peasei* may have originated through introgression of the mitochondrial genome of *C. frigidus* onto the nuclear background of *C. flavidus* (Fig. 6B). This may have

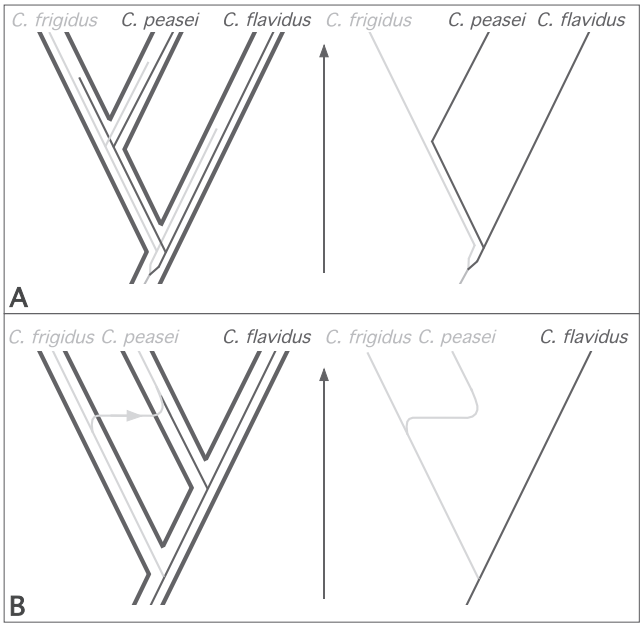

Fig. 6. Illustrations of hypotheses to explain the discordance in relationships inferred from mitochondrial and nuclear data. Left: black outlines represent the species trees and lines denote alleles present within the species at a given time. Right: gene trees. (A) Ancestral polymorphism with incomplete lineage sorting of nuclear alleles. (B) Introgression of mitochondrial genome.

occurred through hybridization between the two parental species and subsequent reproductive isolation of the new daughter species based on the observed patterns of divergence at COI and FTX. Although Fig. 6B illustrates introgression of the mitochondrial genome, introgression of the nuclear loci could also be responsible for the discordance among relationships inferred from nuclear and mitochondrial sequences. While the prevalence of hybrid speciation in metazoans remains controversial, the number of putative examples, including fish (e.g. Rognon and Guyomard, 2003; Nolte et al., 2005), insects (ex: Buckley et al., 2006; Mavárez et al., 2006; Fontaine et al., 2015), and birds (ex: Brelsford et al., 2011) has expanded in recent years. It is becoming increasingly apparent that hybridization, through its addition of genetic variation, is a more important driver of speciation in metazoans than previously assumed (Mallet, 2007; Mavárez and Linares, 2008; Abbott et al., 2013).

4.3. Implications for the taxonomy of Conidae

This study provides an improved understanding of the evolutionary history of the species complex that includes *C. flavidus* and *C. frigidus*. We uncovered novel genetic variation of *C. flavidus* from Hawaii that was previously recognized though disregarded because of the population's morphological similarities with *C. flavidus* elsewhere in the Indo-West Pacific. While the number of specimens examined from northwestern Pacific *C. frigidus* populations is not large enough to warrant the same conclusion, interpretation of the COI tree (Fig. 3) and separation of *C. frigidus* clades in ABGD analysis suggest that an additional cryptic member of this complex may occur there as well (i.e., members of COI clade IV). Genetic exploration of other complexes in Conidae has also revealed cryptic members of species complexes that were not apparent based on examination of shell morphology. For example, Duda et al. (2008) found cryptic diversity in the species complex including *Conus sponsalis* Hwass in Bruguiére, 1792, identifying up to eight species in the complex, three of which had been previously unrecognized. Moreover, Duda et al. (2009b) used molecular, morphological and ecological data to uncover a cryptic species that had long been synonymized with *C. ebraeus*. Together, these cases suggest that species delineations may be less forthright than have been traditionally accepted, especially in diverse genera with various degrees of morphological disparity. They call for the genetic analysis of Conidae species complexes to re-evaluate outdated taxonomy based solely on shell morphology. Improved knowledge of the phylogeny of Conidae is vital for understanding the evolutionary history of this hyperdiverse family. With this as our framework, we will be better equipped to inform conservation strategies and address questions about the origins of marine biodiversity.

5. Conclusion

We demonstrated the presence of hidden diversity within the *C. flavidus*-*C. frigidus* species complex, supporting the resurrection of an endemic Hawaiian *Conus* species, *C. peasei*. The origin of this species remains ambiguous due to discordance between results from analyses of mitochondrial and nuclear sequences and morphometric data. In addition to the presence of a cryptic species at Hawaii, our results suggest *C. frigidus* may include additional cryptic taxa (i.e., COI clade IV, Fig. 3), further illuminating the underestimation of the diversity in Conidae, and in marine organisms in general.

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Appendix A. Specimen information

ID code	Museum catalog number[b]	Species ID	Locality	GenBank accession number		
				COI	FTX[c]	ITS2
CflAS011[a,d]	UMMZ 303891	*flavidus*	American Samoa	KU857023		KU856950
CflAS012[a,d]	UMMZ 303883	*flavidus*	American Samoa	KU857024		KU856950
CflAS033[d]	UMMZ 303248	*flavidus*	American Samoa	KU857025	KU856952 KU856953	KU856951
CflAS082[d]	UMMZ 303264	*flavidus*	American Samoa	KU857026		KU856950
CflAS001[d]	UMMZ 301307	*flavidus*	American Samoa			
CflAS004[d]	UMMZ 301309	*flavidus*	American Samoa			
CflAS097[d]	UMMZ 303270	*flavidus*	American Samoa			
CflAS099[d]	UMMZ 303271	*flavidus*	American Samoa			
CflAS102[d]	UMMZ 303272	*flavidus*	American Samoa			
CflG1	MCZ 318156	*flavidus*	Guam	KU857027		
CflG2	MCZ 318156	*flavidus*	Guam	KU857028	KU856973 KU856974	
CflG3	MCZ 318156	*flavidus*	Guam	KU857029		
CflG4	MCZ 318156	*flavidus*	Guam	KU857030		
CflAS013[d]	UMMZ 303038	*frigidus*	American Samoa	KU857006	KU856961	KU856951
CflAS014[d]	UMMZ 303039	*frigidus*	American Samoa	KU857007	KU856962	
CflAS015[d]	UMMZ 303053	*frigidus*	American Samoa	KU857008		

(*continued on next page*)

Appendix A. (*continued*)

ID code	Museum catalog number[b]	Species ID	Locality	GenBank accession number		
				COI	FTX[c]	ITS2
CflAS018[d]	UMMZ 303054	*frigidus*	American Samoa	KU857009	KU856963 KU856978	KU856951
CflAS028[d]	UMMZ 303171	*frigidus*	American Samoa	KU857010		
CflAS034	UMMZ 303249	*frigidus*	American Samoa	KU857011	KU856964	
CflAS036[d]	UMMZ 303250	*frigidus*	American Samoa	KU857012		
CflAS037	UMMZ 303251	*frigidus*	American Samoa	KU857013	KU856971 KU856976	
CflAS045	UMMZ 303252	*frigidus*	American Samoa	KU857014	KU856972 KU856982	
CflAS052	UMMZ 303253	*frigidus*	American Samoa	KU857015	KU856965	
CflAS053	UMMZ 303254	*frigidus*	American Samoa	KU857016	KU856979 KU856966	
CflAS055	UMMZ 303255	*frigidus*	American Samoa	KU857017	KU856969 KU856981	
CflAS058[d]	UMMZ 303256	*frigidus*	American Samoa	KU857018	KU856967 KU856975	KU856951
CflAS062	UMMZ 303258	*frigidus*	American Samoa	KX811539	KU856970 KU856980	
CflAS069[a,d]	UMMZ 305078	*frigidus*	American Samoa	KU857019		
CflAS072[d]	UMMZ 303260	*frigidus*	American Samoa	KU857020	KU856968	KU856951
CflAS003[d]	UMMZ 301310	*frigidus*	American Samoa			
CflJ002	UMMZ 318157	*frigidus*	Okinawa	KU857021		
CflJ005	UMMZ 318157	*frigidus*	Okinawa	KU857022		
CflH008[d]	UMMZ 302340	*peasei*	Hawaii	KU856983		
CflH021[d]	UMMZ 302345	*peasei*	Hawaii	KU856984		
CflH024[d]	UMMZ 302348	*peasei*	Hawaii	KU856985	KU856954	KU856950
CflH026[d]	UMMZ 302350	*peasei*	Hawaii	KU856986	KU856955	KU856950
CflH028[d]	UMMZ 302351	*peasei*	Hawaii	KU856987		
CflH029d	UMMZ 302352	*peasei*	Hawaii	KU856988	KU856956	KU856950
CflH032[d]	UMMZ 302354	*peasei*	Hawaii	KU856989		
CflH037[d]	UMMZ 302356	*peasei*	Hawaii	KU856990		
CflH040[d]	UMMZ 302358	*peasei*	Hawaii	KU856991	KU856957	KU856950
CflH048[d]	UMMZ 302361	*peasei*	Hawaii	KU856992		
CflH051[d]	UMMZ 302364	*peasei*	Hawaii	KU856993		
CflH052[d]	UMMZ 302365	*peasei*	Hawaii	KU856994		
CflH053[d]	UMMZ 302366	*peasei*	Hawaii	KU856995		
CflH057	UMMZ 302368	*peasei*	Hawaii	KU856996		
CflH060[d]	UMMZ 302371	*peasei*	Hawaii	KU856997		
CflH065[d]	UMMZ 302374	*peasei*	Hawaii	KU856998		
CflH069[d]	UMMZ 302375	*peasei*	Hawaii	KU856999	KU856958 KU856977	KU856950
CflH070[d]	UMMZ 302376	*peasei*	Hawaii	KU857000		
CflH099[d]	UMMZ 302382	*peasei*	Hawaii	KU857001		
CflH101[d]	UMMZ 302384	*peasei*	Hawaii	KU857002		
CflH103[d]	UMMZ 302385	*peasei*	Hawaii	KU857003	KU856959	KU856950
CflH107[d]	UMMZ 302389	*peasei*	Hawaii	KU857004		
CflH109[d]	UMMZ 302390	*peasei*	Hawaii	KU857005	KU856960	KU856950
16 specimens[d]	MCZ 318158	*peasei*	Hawaii			

[a] DNA obtained from feces.
[b] MCZ: Museum of Comparative Zoology (Harvard University); UMMZ: University of Michigan Museum of Zoology.
[c] Two accession numbers provided for heterozygous individuals.
[d] Specimen(s) used for morphometric work.

Appendix B. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2017.02.011.

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