

## Cryptic Species Differentiated in *Conus ebraeus*, a Widespread Tropical Marine Gastropod

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**Abstract.** Anomalous mitochondrial and nuclear gene sequences in individuals of the widely distributed tropical marine gastropod *Conus ebraeus* that were not distinguishable by shell shape and color pattern characters suggested the presence of a second, cryptic species. We tested this hypothesis by genetic, morphological, and ecological comparisons of additional individuals from the site in Okinawa where the two forms co-occurred. Radular tooth size and shape, prey type in nature, and microhabitats utilized differed markedly between the two forms. Adults with typical *C. ebraeus* DNA and radular teeth preyed primarily on errant polychaetes (Eunicidae); those with anomalous DNA and teeth ate mainly sedentary capitellids. Juveniles (shell length <13 mm) had more similar teeth and ate primarily syllids. Radular teeth of the anomalous form agreed with those of *Conus judaeus*, distinguished from *C. ebraeus* by Rudolph Bergh in 1895 solely on tooth characters of one specimen from the Philippines. Samples from other widely scattered Pacific localities revealed only typical *C. ebraeus* gene sequences. Both forms occurred in Seychelles (western Indian Ocean), where their radular teeth and diets were consistent with the data from Okinawa, but DNA of available material was degraded. Although *C. judaeus* was long dismissed as an aberrant specimen and junior synonym of *C. ebraeus*, our results support its validity as a distinct species. These results highlight the importance of molecular and

radular tooth characters relative to those of the shell. Moreover, cryptic species could well be important components of species richness in *Conus* specifically and marine molluscan biodiversity more generally.

### Introduction

With some 550 recognized valid extant species, *Conus* is likely the largest genus of animals in the sea. In addition to contributing substantially to marine biodiversity, this circumtropical gastropod genus is important ecologically—up to 36 species co-occur on a single coral reef platform (Kohn, 2001); evolutionarily—its net diversification rate is the highest among gastropods (Stanley 2007); and neurobiologically and medically—because of the many highly specific and potent neuropeptides in its venom (Olivera, 2006).

*Conus ebraeus* Linnaeus, the most widely distributed species of this hyperdiverse genus, occurs in varied shallow-water habitats throughout the tropical Indian and Pacific Oceans, its range spanning more than one-fourth of the world's entire ocean area (Röckel *et al.*, 1995). It is one of only three of the more than 300 Indo-West Pacific *Conus* species that extend to the eastern Pacific region. This unusually broad distribution makes *C. ebraeus* an excellent subject for examining patterns of genetic connectivity of populations and patterns of diversification in the broad Indo-West Pacific marine realm.

In a study to interpret patterns of gene flow, Duda and Lessios (2009) demonstrated a general absence of genetic structure in western and central Pacific populations of *C. ebraeus* by using mitochondrial gene sequences at eight widespread locations, from Hawaii west to the Philippines and from Okinawa south to American Samoa. Also, in a study of the evolution of conotoxins, the small peptides

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*Abbreviations:* DFA, discriminant function analysis; PCA, principal components analysis; SL, shell length; TL, tooth length.

expressed in *Conus* venoms and utilized in prey capture, Duda and Remigio (2008) obtained mRNA sequences from *C. ebraeus* to determine conotoxin expression patterns among a set of closely related *Conus*. These two studies led to the serendipitous discovery that some individuals from Okinawa, Japan, that were identified as *C. ebraeus* from shell characters, possessed very different mitochondrial and conotoxin gene sequences. While the sequences from one group agreed with those obtained from *C. ebraeus* populations at Okinawa and elsewhere, sequences of the other were unique, differing strikingly from other *C. ebraeus* and from all of the more than 200 other *Conus* species whose genes have been sequenced.

We determined morphological and ecological correlates of the genetic differences between the two forms at Okinawa, and we show that these support the hypothesis that a co-occurring cryptic species had been misidentified as *C. ebraeus*. Moreover, analysis of radular tooth morphology supports the hypothesis that the cryptic species masquerading as *C. ebraeus* was described more than a century ago, solely on the basis of its radular tooth characteristics. Thus an available name exists for it in the literature, in contrast to cryptic species identified in recent years from DNA sequence differences in other taxa. We also examined individuals from additional Indo-West Pacific locations in an effort to better understand the distributions of the two species.

## Materials and Methods

### Definitions

Cryptic species are “reproductively isolated species that resemble one another closely and, thus, sometimes remain unrecognized” (Avisé, 2006), “two or more distinct but morphologically similar species that were classified as a single species” (Pfenninger and Schwenk, 2007), or “two or more distinct species that are erroneously classified (and hidden) under one species name (Bickford *et al.*, 2007). We use the term in the sense of these essentially similar recent definitions.

### Specimens

Most specimens were collected from intertidal habitats in Okinawa, Japan. AJK collected 21 specimens from various sites in 1981 in a study of habitats and diets in nature. The initial DNA analyses were carried out on specimens collected at Sesoko Island, Okinawa, near the Sesoko Station of the Tropical Biosphere Research Center, University of the Ryukyus (26°38.0'N, 127°52.4'E) in 2001. Following the discovery of disparate 16S sequences in that material, AJK revisited Sesoko in July 2004 to obtain a larger sample for ecological, morphological, and molecular genetic anal-

yses. The specimens from Okinawa are preserved in the University of Michigan Museum of Zoology.

The strikingly distinct mitochondrial and venom genotypes occurred at Okinawa and the Philippines of the eight Pacific *C. ebraeus* populations examined by Duda and Lessios (2009). Because of the lack of Indian Ocean representatives in these studies, we also examined samples of putative *C. ebraeus* from Republic of Seychelles and its dependencies, collected by AJK during the Yale Seychelles Expedition in 1957–1958 for a comparative ecological study of *Conus* (Kohn, unpubl).

### DNA sequences

We extracted DNA from about 25 mg of foot tissue with the E.Z.N.A Mollusc DNA Kit (Omega Bio-Tek, Inc.) according to manufacturers' recommendations, except that we slightly modified the protocol by extending centrifugation of precipitated DNA to 10 min and reducing the volume of elution buffer to 40  $\mu$ l to increase DNA concentration. We amplified a region of the mitochondrial 16S rRNA gene with universal 16S primers (Palumbi *et al.*, 1991) as described previously (Duda and Kohn, 2005). These primers amplify about 500 basepairs (bp) of the 16S gene. PCR products were cleaned with a QIAquick Multiwell PCR Purification kit (Qiagen), and sequencing was performed in both directions. We examined the chromatograms with Sequencher 4.8 (Gene Codes Corporation), aligned sequences by eye, and constructed a haplotype network using TCS 1.21 (Clement *et al.*, 2000). The sequences recovered comprised two distinct sets of haplotypes that exhibited a fixed difference within a BamHI restriction site. Thus we performed restriction digests on amplification products of additional specimens to infer haplotype identity.

For the large sample collected at Okinawa in 2004, we followed a double-blind protocol in analyzing DNA sequences and radular tooth morphometry. AJK numbered each specimen and sent numbered foot tissue slices to TFD, who extracted and sequenced the DNA at the University of Michigan. AJK prepared and analyzed the similarly numbered radular teeth at the University of Washington. After completing the analyses, we exchanged the resulting data.

### Radular tooth morphometry

Morphometric methods generally followed Nishi and Kohn (1999), except that data on continuously varying radular tooth characters were initially subjected to principal components analysis (PCA), because individual specimens were not preclassified to species prior to discriminant function analysis (DFA). See Table 2 for a list of the characters used. All ratios were arcsin transformed prior to PCA and DFA.

Table 1

Microhabitats of *Conus ebraeus* and *C. judaeus* in Okinawa and Seychelles

Microhabitat Type	Okinawa		Seychelles	
	<i>C. ebraeus</i>	<i>C. judaeus</i>	<i>C. ebraeus</i>	<i>C. judaeus</i>
Large sand patch*			2	
Algal-bound sand (1–2 cm deep)	5	59	9	1
Sand in small depression*	2	12	3	
Sand under coral rock*			5	
Thin sand ( $\approx 5$ mm) on limestone bench	6	5	26	10
Coral rubble, rubble + sand*	1	7	3	1
Bare reef limestone, dead coral rock	2	3	13	7
Algae on bare reef limestone	1		1	
Totals	17	86	62	19

\* Indicates predominantly sand substrates that were combined for statistical analysis. (*G* tests for independence): Okinawa:  $G = 12.7$ ;  $df = 3$ ;  $0.005 < P < 0.01$ ; Seychelles:  $G = 5.4$ ,  $df = 3$ ,  $P > 0.1$ .

### Shell morphology and morphometry

Samples of the shells of animals subjected to DNA sequencing were photographed prior to breaking them to extract the bodies for radular tooth analysis. Four shell shape variables were determined from the photographs by using ImageJ: Relative Diameter (=maximum diameter/aperture height), Position of Maximum Diameter (=height of maximum diameter/aperture height), Relative Spire Height (=shell length–aperture height/shell length), and Aperture Shape (aperture width/aperture length). Röckel *et al.* (1995) discuss these in more detail. As for radular tooth morphometry, these ratios were analyzed with PCA followed by DFA after arcsin transformations.

### Ecological analyses

The microhabitat occupied by each specimen, except those collected at Okinawa in 2001, was noted in the field and analyzed according to the microhabitat types categorized by Kohn (1983: Table 1). At Okinawa, population density and distribution of *Conus* across the intertidal zone at the main study site was determined by censusing individuals in six transects, each of 12 or 17 1-m<sup>2</sup> quadrats. No sites in Seychelles were found where abundance was high enough to census quantitatively.

Diets in nature were determined by dissection of alimentary tracts of preserved specimens or from collection of feces from living individuals isolated in small jars of seawater for 24–48 h. Indigestible remains, primarily setae and jaws of polychaetes, were permanently mounted on micro-scope slides and identified to the lowest taxon possible.

## Results

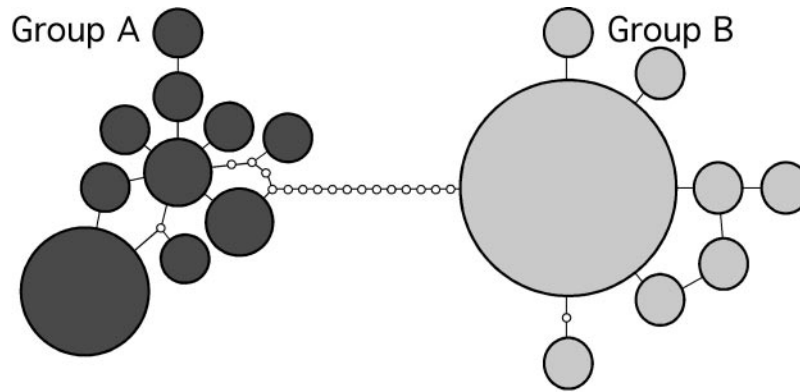
### DNA sequences

We obtained sequences of a 500-bp region of the mitochondrial 16S gene from 45 individuals from Okinawa that

appeared to be specimens of *Conus ebraeus* (Genbank accession numbers AY382011, EU492415–EU492441, EU492443–EU492451, FJ409900–FJ409908). In total, 18 distinct haplotypes were recovered. We obtained two common haplotypes from 7 and 20 individuals respectively. They differed at 17 sites. Only two other haplotypes occurred in more than one individual, and the remaining 14 haplotypes were obtained from single individuals (Fig. 1). These 16 differed from one of the two common haplotypes by 1–5 substitutions. The haplotype network clearly shows two distinct sets of sequences. Sequences from 18 individuals that represent 10 distinct haplotypes occur in one group (Group A), and sequences from the remaining 27 individuals that represent 8 distinct haplotypes occur in the second group (Group B) (Fig. 1). The average pairwise HKY distance (Hasegawa *et al.*, 1985), the most appropriate model of nucleotide substitution determined by Modeltest (Posada and Crandall, 1998), among sequences in these two sets is 0.054 (range: 0.044–0.073); the maximum distance within these groups is 0.011. For comparison, of the 135 *Conus* species with 16S sequences that were examined by Duda and Kohn (2005), 27 pairs exhibited HKY distances less than 0.044, and 42 pairwise distances were less than 0.054. Restriction digests of 16S amplifications of an additional 35 individuals implied that the haplotypes of these individuals are similar to haplotypes that occur in Group B. We hypothesize that *C. ebraeus* has a sibling species.

### Taxonomy of *Conus ebraeus*

Linnaeus (1758) described *C. ebraeus* on the basis of shells in his possession, the collection of the then Queen of Sweden, Louisa Ulrica (Kohn, 1991), and descriptions and figures from prior literature. Kohn (1963) designated as lectotype a shell from Linnaeus's collection now in the Linnean Society of London. Röckel *et al.* (1995) described and illustrated the shell and body color pattern of *C.*



**Figure 1.** Parsimony network of 16S haplotypes of 45 individuals of *Conus ebraeus* from Okinawa. Areas of circles are proportional to the number of individuals with that haplotype (five different sizes of circle are illustrated: the smallest circles each represent one hypothetical haplotype, while the progressively larger circles represent 1, 2, 7, and 20 individuals respectively).

*ebraeus*. Although Linnaeus lacked information about characters other than those of the shell, Troschel (1866), Bergh (1895), Peile (1939), James (1980), and Nybakken (1990) described and illustrated the radular teeth of *C. ebraeus* from diverse geographic areas. These authors' accounts are all consistent, and as radular tooth morphology and morphometry are often species-specific in *Conus* (Kohn *et al.*, 1999; Nishi and Kohn, 1999), we accept them as correctly characterizing the radular teeth of *C. ebraeus*.

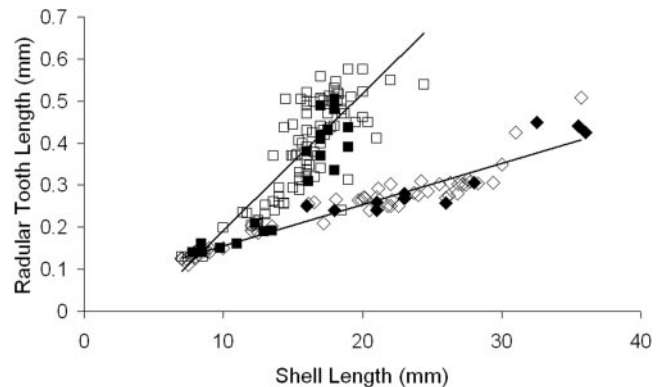
#### Radular tooth morphometry: size, complexity, and shape

Comparison of the radular teeth of specimens characterized by different 16S haplotypes revealed strong correlations between tooth form and haplotype; the teeth of individuals with haplotypes in Group A ( $n = 11$  specimens with both 16S sequences and radular tooth morphometry) differed strikingly from those in Group B ( $n = 23$ ). For specimens of similar body size, the teeth of members of Group B were about twice as large relative to shell length as those in Group A (Figs. 2–4); median tooth lengths were 1.2% and 2.1% of shell lengths, respectively. The larger teeth of Group B members were also more complex, having a well-formed barb and a row of small denticles or serration, characters absent in the teeth of Group A (Fig. 3). Other than tooth size:shell size, none of the shared quantitative characters differed significantly between groups in the small samples with 16S sequences (Table 2).

The 16S sequences of all individuals cited as *C. ebraeus* in prior papers (Duda *et al.*, 2001; Duda and Kohn, 2005) belonged to Haplotype Group A. In addition, the prior descriptions of *C. ebraeus* radular teeth by the authors listed in the previous section conformed with descriptions of the teeth of members of Haplotype Group A, and we attribute them to *C. ebraeus*. In contrast, the teeth of Group B

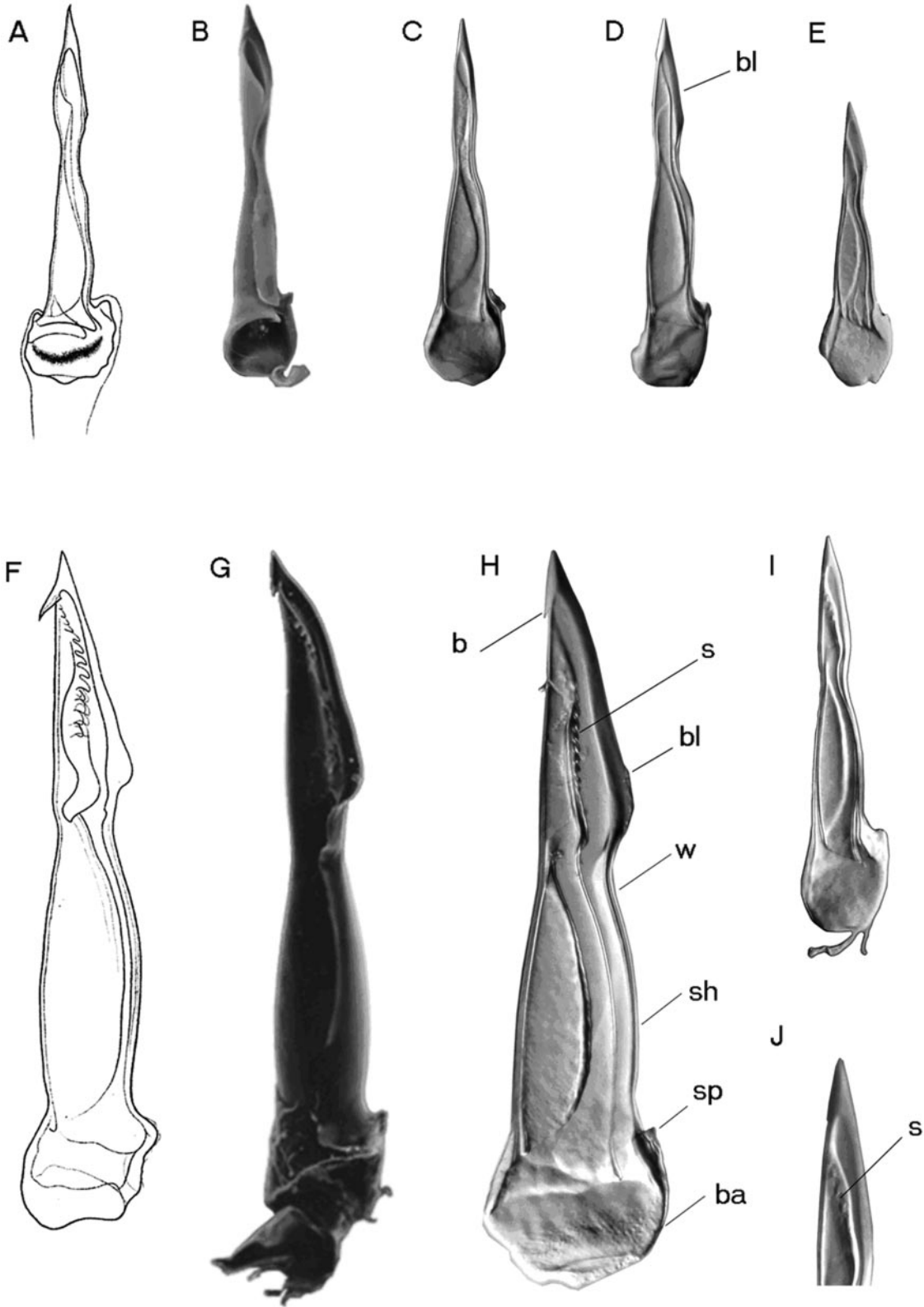
matched those described and illustrated for *C. judaeus* Bergh, 1895.

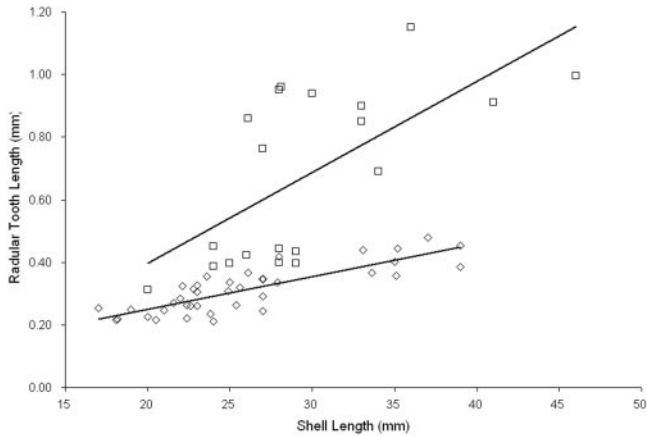
Bergh (1895) described most functional systems of 33 species of *Conus*, including *C. ebraeus*, in a detailed comparative anatomical study that remains the standard work. He described *C. judaeus* solely because the radular teeth of the one specimen he examined differed strikingly from those of *C. ebraeus* (Fig. 3). Bergh undoubtedly had to break the shell to remove the body for anatomical study, and nothing of it has been found to survive (A. Vedelsby, Zoological Museum, University of Copenhagen, pers. comm.).



**Figure 2.** Linear relationships of radular tooth length (TL) to shell length (SL) in specimens from Okinawa. Solid symbols represent specimens with 16S rDNA sequences. Diamonds: Haplotype Group A; Squares: Haplotype Group B. Group A:  $TL = 0.01SL + 0.02$ ;  $n = 11$ ;  $R^2 = 0.84$ . Group B:  $TL = 0.03SL - 0.13$ ;  $n = 23$ ;  $R^2 = 0.85$ . The power function  $TL = 0.006SL^{1.46}$  fits the data more closely ( $R^2 = 0.89$ ) than the linear function. Open symbols represent specimens without 16S rDNA sequences assigned to *Conus ebraeus* (squares) or *C. judaeus* (diamonds) based on radular tooth morphology only. The regression lines shown encompass all data for each species. *C. ebraeus*:  $TL = 0.01SL + 0.06$ ;  $n = 68$ ;  $R^2 = 0.89$ . *C. judaeus*:  $TL = 0.03SL - 0.13$ ;  $n = 128$ ;  $R^2 = 0.70$ . The power function  $TL = 0.006SL^{1.49}$  fits the data more closely ( $R^2 = 0.79$ ) than the linear function.







**Figure 4.** Relationship of radular tooth length (TL) to shell length (SL) in *Conus ebraeus* (diamonds) and *C. judaeus* (squares) from Seychelles and Dependencies. *C. ebraeus*:  $TL = 0.01SL + 0.04$ ;  $n = 39$ ;  $R^2 = 0.69$ . *C. judaeus*:  $TL = 0.03SL - 0.19$ ;  $n = 20$ ;  $R^2 = 0.42$ .

However, Bergh's descriptions are generally thorough and accurate, and his drawings are clear. In this case, his illustrations (Bergh, 1895: pl. 6, figs. 128–131) are the only representations of the holotype of *C. judaeus* known to exist. His detailed two-page comparison (Bergh, 1895: 161–163) noted differences only in radular tooth characters except for what he considered minor variations—for example, in surface texture of the digestive gland.

Because Bergh (1895) described the *C. judaeus* radular tooth in such detail, and because those of Haplotype Group B specimens agree in all particulars and match no other known *Conus* radular tooth, we assign the cryptic species characterized by Haplotype Group B to *C. judaeus* Bergh, 1895.

Table 2 compares the samples generated by assigning specimens whose DNA was not sequenced, based on their radular tooth characters (presence or absence of a barb and serration and tooth size relative to body size). Analysis of the quantitative morphometric characters in these larger

samples indicated highly significant differences in five of the six quantitative characters (Table 2: bottom row); shaft width relative to tooth length was the exception.

We also assigned the 162 specimens from Okinawa whose radular teeth we had examined but whose 16S rDNA was not sequenced, according to the tooth characters summarized above and in Table 2. Of these, 57 had teeth conforming with those of Haplotype Group A and were assigned to *C. ebraeus*, and 105 had teeth conforming with those of Haplotype Group B and were assigned to *C. judaeus*.

Although the radular teeth of adults of *C. ebraeus* and *C. judaeus* are easily distinguishable, those of juvenile specimens of the two species (with shell length [SL] <13 mm) were more similar to each other than the adult teeth. The Okinawa sample contained enough juveniles to show that the teeth of the two species follow distinct growth trajectories. The linear functions of the two (Fig. 2) cross at  $SL \approx 8$  mm, and tooth length as a proportion of shell length was indistinguishable in juveniles. The teeth of the smallest *C. judaeus* specimens sequenced (SL 7–12 mm) also resembled those of *C. ebraeus* because most had not yet formed the barb, or serration, characteristic of adult teeth. However, as *C. judaeus* grows, its teeth grow about three times as rapidly relative to body size as those of *C. ebraeus*. Their growth rate also accelerates relative to shell length, with an allometric coefficient of about 1.5, whereas tooth and shell growth is isometric in *C. ebraeus* (Fig. 2). *C. judaeus* teeth formed when the shell reaches 12–13 mm also add serration and a barb, and their general shape changes (Fig. 3). Simpler in form because they lack a barb and serration, *C. ebraeus* teeth are also more petite, and relative to tooth length, they have narrower waists and broader bases than those of *C. judaeus* (Table 2). The sample from the main granitic islands of the Republic of Seychelles and its atoll dependencies southwest of the Seychelles Bank comprised 62 individuals. They had been collected 50 years earlier and fixed in formalin, and DNA could not be extracted from any.

**Figure 3.** Radular teeth of Haplotype Groups A and B from Okinawa and those of *Conus ebraeus* and *C. judaeus* as characterized by Bergh (1895). (A–E) *C. ebraeus*. (F–J) *C. judaeus*. (A) Diagram of a radular tooth of *C. ebraeus*, reproduced from Bergh (1895), pl. VI, fig. 125). Tooth length (TL) = 0.37 mm; shell length (SL) = 34 mm. (B) Scanning electron microscope (SEM) image of a radular tooth of *C. ebraeus* (Haplotype A) from Okinawa (No. 9945, TL = 0.43 mm; SL = 31 mm). (C) Nomarski differential interference contrast (DIC) image of a radular tooth of *C. ebraeus* (Haplotype Group A) from Okinawa (No. 9990, TL = 0.25 mm; SL = 16 mm). (D) Another tooth from the same individual as in (C), oriented to show the blade (bl). (E) Radular tooth of a smaller *C. ebraeus* specimen (Haplotype Group A) from Okinawa (No. 10004, TL = 0.19 mm, SL = 12 mm). (F) Diagram of radular tooth from the holotype specimen of *C. judaeus*, reproduced from Bergh (1895), pl. VI, fig. 129). TL = 0.68 mm; SL = 32 mm. (G) SEM image of a radular tooth of *C. judaeus* (Haplotype Group B) from Okinawa (No. 10034, TL = 0.45 mm; SL = 20 mm). (H) DIC image of a radular tooth of *C. judaeus* (Haplotype Group B) from Okinawa (No. 10092, TL = 0.37 mm; SL = 15 mm). (I) DIC image of a radular tooth of a smaller *C. judaeus* specimen (Haplotype Group B) from Okinawa (No. 10088, TL = 0.21 mm; SL = 12 mm), with incipient serration. (J) Enlargement of the tip of tooth shown in (I) to indicate serration. This tooth had not yet formed a barb. b, barb; ba, base; bl, blade; s, serration; sp, spur; w, waist.

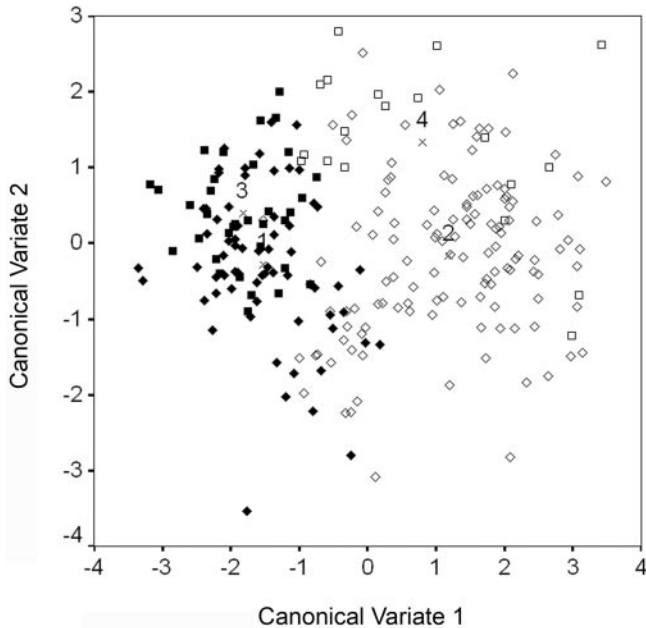
Table 2

		Qualitative characters		Quantitative characters						
		Barb	Serration	Tooth Length/Shell Length	Blade Length	Waist Length	Waist Width/Tooth Length	Shaft Width/Tooth Length	Base Width/Tooth Length	
<b>OKINAWA</b>										
Haplotype Group A ( <i>C. ebraeus</i> )										
	W/16S only ( <i>n</i> =11)	-	-	0.013; 0.012	0.336; 0.341	0.382; 0.381	0.063; 0.064	0.135; 0.123	0.242; 0.233	
	mean; median range			0.010-0.016	0.225-0.400	0.326-0.418	0.047-0.075	0.116-0.170	0.208-0.295	
	All data ( <i>n</i> =68)									
	mean; median range	-	-	0.014; 0.013	0.306; 0.318	0.370; 0.366	0.059; 0.059	0.142; 0.143	0.253; 0.250	
	W/16S only ( <i>n</i> =23)	-	-	0.010-0.018	0.225*-0.400	0.321-0.418	0.040-0.084	0.103-0.181	0.187-0.307	
Haplotype Group B (= <i>C. judaeus</i> )										
	mean; median range	+	+	0.021; 0.021	0.361; 0.354	0.381; 0.395	0.070; 0.071	0.141; 0.138	0.237; 0.238	
	All data ( <i>n</i> =127)	+	+	0.015-0.029	0.286-0.439	0.300-0.459	0.043-0.107	0.113-0.179	0.188-0.276	
	mean; median range	+	+	0.023; 0.024	0.370; 0.365	0.396; 0.403	0.076; 0.077	0.147; 0.143	0.232; 0.229	
	<i>C. ebraeus</i> vs. <i>C. judaeus</i>	+	+	0.013-0.035	0.247-0.473	0.277-0.473	0.043-0.115	0.095-0.220	0.167-0.302	
	<i>U</i> tests (W/16S)			<0.001	0.22	0.71	<0.31	0.21	<0.60	
	<i>U</i> tests (All data)			<0.001	<0.001	<0.001	<0.001	0.18	<0.001	
<b>SEYCHELLES</b>										
<i>C. ebraeus</i>										
	( <i>n</i> =34)	-	-	0.012; 0.012	0.332; 0.331	0.387; 0.381	0.064; 0.064	0.152; 0.156	0.266; 0.262	
	mean; median range			0.009-0.015	0.248-0.383	0.333-0.432	0.047-0.085	0.113-0.200	0.216-0.316	
<i>C. judaeus</i>										
	( <i>n</i> =19)	+	+	0.022; 0.020	0.360; 0.355	0.424; 0.429	0.082; 0.080	0.163; 0.165	0.228; 0.226	
	mean; median range	+	+	0.014-0.033	0.290-0.390	0.334-0.480	0.058-0.105	0.130-0.210	0.175-0.261	
<i>C. ebraeus</i> vs <i>C. judaeus</i> <i>U</i> tests				<0.001	0.001	<0.001	<0.001	0.17	<0.001	

In the upper section of the table, labeled "OKINAWA," the upper rows give the results of the double-blind tests that compared haplotypes and radular tooth morphometry of the same specimens. The consistency of the radular tooth character differences between Haplotype Groups A and B supports the hypothesis that these represent *C. ebraeus* and *C. judaeus*, respectively. The lower rows give all results, including specimens whose DNA was not sequenced but whose radular teeth conformed to those of Haplotypes A and B, respectively. The lower section of the table, labeled "SEYCHELLES," gives the results of the sample from that region, based solely on radular tooth morphometry because DNA could not be extracted from the specimens.

+, present; -, absent. Fig. 3 illustrates the characters used.

\*- Two specimens (7.0 and 8.0 mm long) had not yet developed blades.



**Figure 5.** Discriminant function analysis (DFA) of radular teeth of *Conus ebraeus* and *C. judaeus* from Okinawa and Seychelles, based on the six shared quantitative characters shown in Table 2. Solid squares: Okinawa *C. ebraeus* (1); solid diamonds: Seychelles *C. ebraeus* (3); open diamonds: Okinawa *C. judaeus* (2); open squares: Seychelles *C. judaeus* (4). Numbers and X's indicate group centroids. CV1 accounts for 88% of the variance and is most strongly and positively correlated ( $R = 0.9$ ) with tooth length/shell length (TL/SL). CV2 explains 9% of the remaining variance. On CV1, both *C. judaeus* samples differed from both *C. ebraeus* samples at  $P < 0.001$ , and the two geographically separated pairs of conspecific samples were statistically identical. For *C. ebraeus*,  $P = 0.96$  and for *C. judaeus*,  $P = 0.70$  (ANOVA with Bonferroni correction). The DFA correctly identified 98% of *C. ebraeus* and 85% of *C. judaeus* individuals. Most of the ambiguous points in the zones of overlap between species in the figure represent juvenile specimens (SL < 10 mm) whose teeth were not yet fully differentiated.

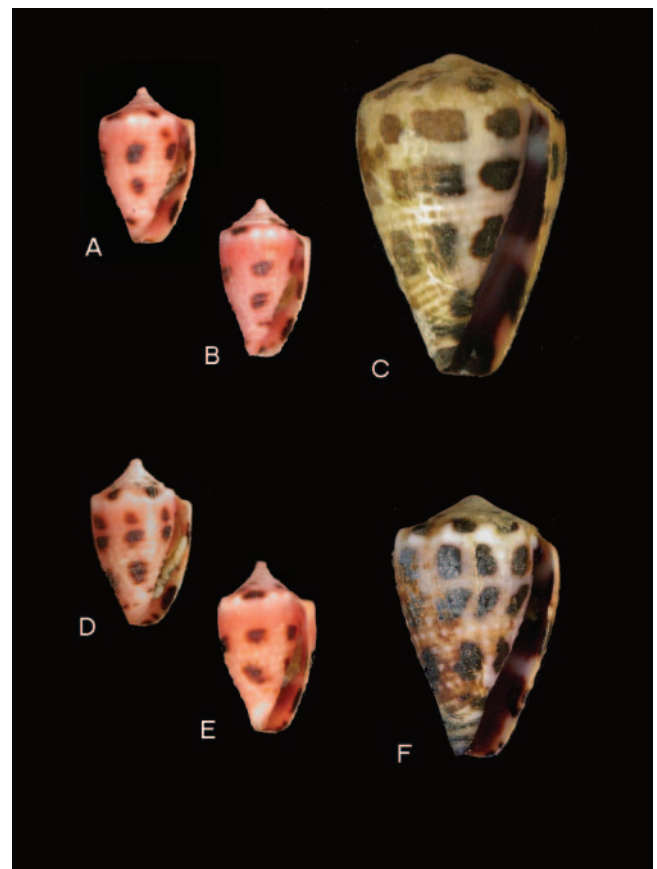
Identifications were thus based solely on analyses of the radular tooth morphometric characters indicated in Table 2. The results were essentially identical to those from Okinawa. The two species differed significantly with respect to the same five univariate radular characters (Table 2), and the rates of change in radular tooth length relative to shell length were identical for the two species at both sites (Figs. 2, 4).

PCA of the six shared quantitative characters (Table 2) clearly indicated separation of *C. ebraeus* from *C. judaeus*, but did not distinguish samples of the same species between Okinawa and Seychelles. Multivariate DFA of these data clearly separated the species, with minimum overlap due mainly to the general similarity of juvenile teeth, and correctly predicted species membership in most cases (Fig. 5).

In the remaining Results sections we thus distinguish *C. ebraeus* and *C. judaeus* and compare and contrast their morphological and ecological characteristics.

### Shell morphometry

We were unable to visually distinguish the shells of either adults or juveniles of the two species identified from disparate DNA sequences by color pattern or shape (Fig. 6). Adult shells of both have black markings on a white ground. The ground color of juveniles (SL  $\leq 10$  mm) is red; it gradually fades to white. We thus subjected shells to multivariate statistical analyses of shape to detect any measurable but subtle interspecific differences. In PCA of four combined shell shape variables, *C. ebraeus* ( $n = 7$ ) and *C. judaeus* ( $n = 38$ ) had statistically identical scores on PC1 ( $t = 0.9$ ;  $P = 0.35$ ). It accounted for 41% of the variance; relative spire height and relative diameter were the individual variables most highly correlated with PC1. On PC2 (30% of the variance), influenced mainly by position of maximum diameter (+) and aperture shape (-), *C. ebraeus* had higher scores than *C. judaeus* ( $t = 1.9$ ;  $P = 0.06$ ). DFA correctly classified 6 of 7 specimens of *C. ebraeus* (85%) and 28 of 38 of *C. judaeus* (74%). Although the *C. ebraeus*



**Figure 6.** Shells of juvenile and adult *Conus* specimens from Okinawa with Haplotypes of Groups A (A–C) and B (D–F). Shell lengths: (A) 7.8 mm; (B) 7.9 mm; (C) 17.2 mm; (D) 8.4 mm; (E) 8.5 mm; (F) 17.5 mm. A–C are identified as *C. ebraeus*; D–F are identified as *C. judaeus*. (See text). Juvenile specimens of both typically have red ground color, while that of adults is white.



sample was quite small, the results suggest subtle interspecific differences in shell shape, but that they are unlikely to be adequate to distinguish shells of the two species.

### Specimens

In the large Okinawa sample from 2004, *C. judaeus* was almost twice as abundant as *C. ebraeus*, but the latter varied more in shell length and reached a larger size (SL 36 mm) than *C. judaeus* (24 mm). Despite the larger proportion of juveniles ( $\leq 12$  mm), overall *C. ebraeus* (19%) shells were significantly larger (Fig. 7A).

The proportions of *C. ebraeus* and *C. judaeus* were reversed in the sample from Seychelles. Shell lengths of the two species differed significantly, but *C. judaeus* was larger, in contrast to Okinawa (Fig. 7A). Moreover, no juveniles  $\leq 15$  mm in shell length were found in Seychelles. *C.*

*judaeus* attained a maximum SL of 46 mm, while the largest *C. ebraeus* specimen was 39 mm long (Fig. 7B).

Both species occurred at all Seychelles islands where at least 10 individuals were collected (the main islands Mahé, Frigate, and Silhouette, and the dependency Cosmoledo). Shell lengths of both species were larger on the fringing reefs of the main granitic islands than the dependencies' atoll reefs (*C. ebraeus*: ( $t = 1.3$ ;  $P = 0.09$ ); *C. judaeus* ( $t = 2.2$ ;  $P = 0.04$ )).

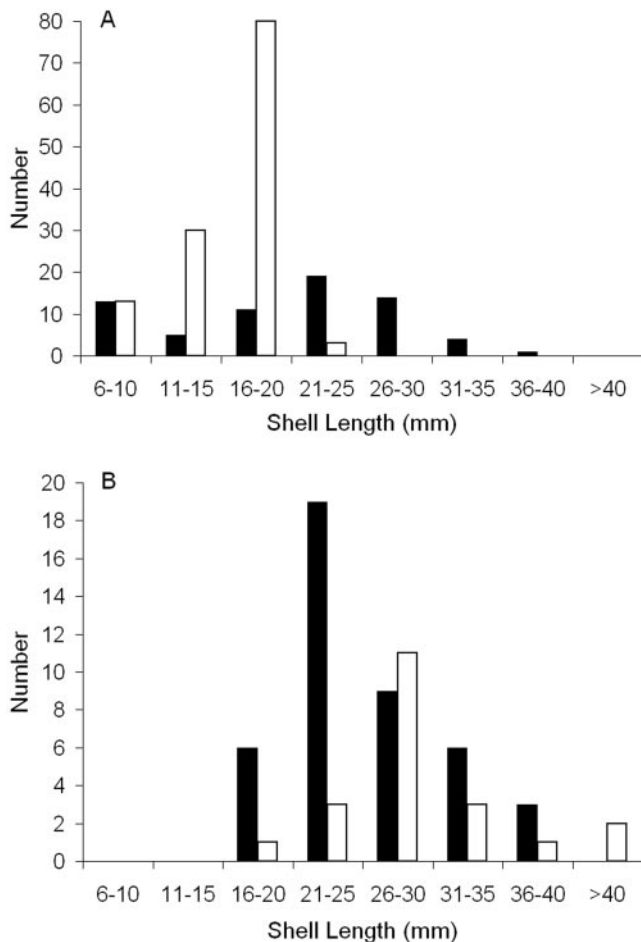
### Ecology: *Conus* diversity, density, and distribution

In Okinawa the main field study in July 2004 was made at an intertidal limestone bench 12–17 m wide between the shore and reef just north of the Sesoko Station, fronting the site of the former laboratory visited in 1981. A bright green algal mat, primarily of *Ulva conglobata* and *Enteromorpha clathrata* affixed to the limestone bench, occupies the shoreward two-thirds of the bench and the holdfasts of the algae bind sand to a depth of 1–2 cm over much of this area. Depressions in the bench contain deeper pockets of sand. The outer third of the bench is lower and topographically more irregular, as it is excavated into burrow pits and channels by the grazing of abundant sea urchins (*Echinometra* spp.). Living corals (*Goniastrea* sp.) first occur at the seaward edge of the bench.

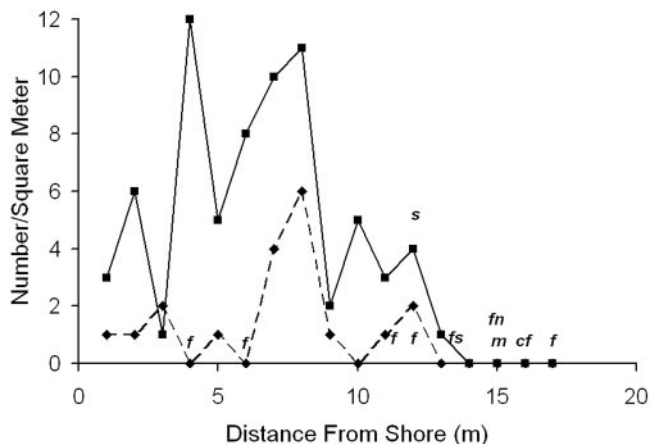
Six transects comprising 1-m<sup>2</sup> quadrats across the bench from shore to the first living corals supported 115 *Conus* individuals of seven species, an overall density of 1.2/m<sup>2</sup> (Fig. 8). During the field observations, 78% (1.0/m<sup>2</sup>) appeared to be *C. ebraeus*. Subsequent examination of gene sequences of a sample and radular teeth of all revealed about three-fourths (71/91) of these to be *C. judaeus*. Nine individuals of the third commonest species (8%), *C. frigidus*, were present, and there were six *C. sponsalis* (5%), two *C. nanus*, and one each of *C. miliaris* and *C. coronatus*. In the more extensive but nonquantitative observations of a somewhat broader area made in 1981, three additional species, *C. chaldaeus*, *C. lividus*, and *C. emaciatus*, were also observed.

Distributions across the bench from shore to seaward edge of *C. judaeus* and *C. ebraeus* coincided with the area where algal-bound sand predominated and were statistically identical (Fig. 8; Mann-Whitney *U* test:  $z = 0.78$ ;  $P = 0.43$ ). The five less common species predominantly (16/19 individuals) occupied the outer 5 m of the platform, where the limestone was bare of macroalgae and sand, and where its surface was topographically more complex due to urchin burrows. Only a few *C. frigidus* occurred on the inner portion (Fig. 8).

Both *C. ebraeus* and *C. judaeus* occurred predominantly on sand substrates (Table 1), but their occupation of different microhabitats differed significantly (*G* test for independence:  $0.005 < P < 0.01$ ). *C. ebraeus* individuals were more



**Figure 7.** Shell length-frequency distributions of *Conus ebraeus* (solid bars) and *C. judaeus* (open bars). (A) Okinawa. *C. ebraeus*: mean = 20.2 mm; median = 21 mm;  $n = 67$ . *C. judaeus*: mean = 15.7 mm; median = 16 mm;  $n = 126$ .  $t = 4.3$ ;  $P < 0.001$ . (B) Seychelles. *C. ebraeus*: mean = 26.0 mm; median = 24 mm;  $n = 41$ . *C. judaeus*: mean = 29.7; median = 28;  $n = 21$ .  $t = 2.3$ ;  $P = 0.024$ .



**Figure 8.** Distribution of *Conus* species across an intertidal limestone bench at Sesoko Island, Okinawa. *C. ebraeus* (diamonds and dashed line;  $n = 19$ ) and *C. judaeus* (squares and solid line;  $n = 71$ ). *C. frigidus* (f); *C. sponsalis* (s); *C. nanus* (n); *C. miliaris* (m); *C. coronatus* (c).

evenly distributed across the different microhabitats occupied, while *C. judaeus* more often occupied deeper sand accumulations bound in place by algae or in small depressions on the bench.

In Seychelles as in Okinawa, *C. ebraeus* occupied a broader array of microhabitats. However, the microhabitats of both species differed, as they occurred less often on deeper sand and more frequently on a thin (<5 mm) layer of sand on limestone substrate, or on bare limestone. The two species' use of microhabitat types did not differ statistically in Seychelles (Table 2).

*Ecology: Food habits in nature in Okinawa*

As expected from prior studies of *C. ebraeus* elsewhere (e.g., Kohn, 1959, 2001), both it and *C. judaeus* preyed exclusively on polychaete annelids. While the prey of juveniles (SL < 13 mm) were essentially identical, at least at the family level, the diets of adults of the two species diverged strikingly (Table 3). Juveniles of both species consumed primarily Syllidae: 91% in *C. ebraeus*, 82% in *C. judaeus* ( $G$  test for independence:  $G = 1.3$ ;  $P > 0.5$ ). In contrast, the eunicid *Palola siciliensis* composed 82% of the prey of adult *C. ebraeus*, while a capitellid, probably *Dasybranchus caducus*, composed 66% of the prey of adult *C. judaeus*.

The differences in taxon composition of the diets between *C. ebraeus* and *C. judaeus* (Table 3) are highly significant both for individuals of all sizes and for adults (SL  $\geq$  13 mm) only, at the family level or when errant families (Syllidae, Nereididae, and Eunicidae) are combined and contrasted with the sedentary family Capitellidae (Table 3, data rows 5 and 6). In all cases,  $G > 16$  and  $P < 0.001$ ). In both species, the shifts in prey taxon composition of the diet with change in body size were highly significant (details shown in Appendix Fig. 1).

*Ecology: Food habits in nature in Seychelles*

As at Okinawa, the prey taxa utilized by the two *Conus* species differed strikingly in Seychelles, and the pattern was very similar, although *C. ebraeus* was much more abundant than *C. judaeus* and the sample size of the former was thus larger. *C. ebraeus* fed exclusively on errant polychaetes of

**Table 3**

*Diets of Conus ebraeus and C. judaeus in nature at Okinawa and Seychelles*

	Okinawa						Seychelles	
	<i>C. ebraeus</i>			<i>C. judaeus</i>			<i>C. ebraeus</i>	<i>C. judaeus</i>
	SL<13 mm	SL $\geq$ 13 mm	Total	SL<13 mm	SL $\geq$ 13 mm	Total		
No. Examined	19	53	72	22	108	130	99	20
Prey Family								
Syllidae	10	0	10	14	1	15		
Eunicidae	0	14	14		2	2	46	
Nereididae	1	1	2	3	9	12	60	4
Capitellidae	0	2	2		23	23		8
Total Errant	11	15	26	17	12	29	106	4
Total Sedentary		2	2		23	23		8
Total	11	17	28	17	35	52	106	12

The body of the table shows the number of prey organism remains of the polychaete family at left recovered from the specimens of categories listed above. Syllidae were not further identified. At Okinawa, all Eunicidae eaten by *C. ebraeus* were *Palola siciliensis*; *C. judaeus* ate one *Lysidice collaris* and one *L. ninetta*. Most Nereididae were not further identified, but *C. ebraeus* ate 2 *Pseudonereis* sp., and *C. judaeus* ate 1 *Perinereis* sp., and 1 *Pseudonereis* sp. At Seychelles, Eunicidae eaten by *C. ebraeus* comprised 33 *Palola siciliensis* and 9 *Nematonereis unicornis*; Nereididae comprised 51 *Nereis falcaria*, 10 *Perinereis* spp., and 8 others. All Nereididae eaten by *C. judaeus* at Seychelles were *Perinereis* spp. All Capitellidae had hooded hooks that conformed with those of *Dasybranchus caducus*; it is probably that species, but the absence of intact worms precluded confident determination.

the families Nereididae and Eunicidae, while most *C. judaeus* consumed the capitellid *Dasybranchus caducus* (Table 3). No specimens  $\leq 15$  mm SL of either species were observed in Seychelles, likely accounting for the absence of Syllidae in the diets.

Despite the smaller ranges of *Conus* body size than in Okinawa, prey species composition of both species also shifted with increasing shell length. Smaller individuals of *C. ebraeus* (SL 16–30 mm) ate primarily *Nereis falcaria*, other nereids, and the small eunicid *Nematonereis unicornis*, while the proportion of the eunicid *Palola siciliensis* increased with increasing body size. In *C. judaeus*, only individuals of SL 21–35 mm ate nereids (*Perinereis* spp.), while larger individuals (SL 26–45 mm) ate all of the capitellids recorded. The shift in prey species composition with increasing size was highly significant in *C. ebraeus*, but the *C. judaeus* sample size was small and the difference between members of the two prey families consumed by individuals differing in size was not statistically significant (App. Fig. 2).

### Discussion

As in most higher taxa of metazoans, the number of cryptic species reported in the molluscan class Gastropoda as a whole approximates the expected proportion of its number of described species (Pfenninger and Schwenk, 2007: fig. 1). In our previous study (Duda *et al.*, 2008) mtDNA sequence data revealed the presence of several cryptic species in the *Conus sponsalis* species complex. However, correlations with other character sets remain to be detected in that group. The present report is the first to differentiate cryptic species of *Conus* by morphological and ecological characteristics that support the initial distinction by molecular genetic data. To our knowledge, these are also the first reports of cryptic species in any of the hyperdiverse genera of neogastropods that contribute importantly to marine biodiversity, especially in tropical coral reef environments. In addition to *Conus*, these include *Mitra*, *Nassarius*, *Oliva*, *Terebra*, and *Vexillum*, each with 100–400 recognized valid species. Because the genes of few individuals of species in these taxa have been sequenced to date, cryptic species could well be more prevalent within them, resulting in their biodiversity being seriously underestimated.

The cryptic species reported here is also unusual and perhaps unique in that it appears to have been recognized and described more than a century ago. We thus recognize the prescience of Ludvig Sophus Rudolph Bergh (1824–1909), who distinguished *C. judaeus* from *C. ebraeus* on the basis of his study of a single specimen of the former published in 1895. A highly respected Danish physician and zoologist, Bergh was well known in two major fields. He published more than 50 papers on venereal diseases, and he

established a hospital for their treatment in Copenhagen that later bore his name and only recently ceased operation.

Bergh's systematics research on molluscs throughout the latter half of the 19th century was extensive, covering more than 2000 pages, and incisive (Winckworth, 1946). Most focused on nudibranch gastropods that lack shells as adults, and this likely influenced Bergh to attach more value to anatomical than shell characters when he began to study shelled prosobranchs. He published nearly 90 papers on this group, including what remains today the most thorough comparative anatomical study of *Conus*, treating 33 species. In this monograph, Bergh (1895) described only the one new species, based entirely on his anatomical study of a single specimen reportedly from the Philippines. The specific name *C. judaeus* perpetuated a tradition established by Linnaeus (1758: *C. ebraeus*) and continued by Röding (1798: *C. chaldaeus*) of applying names of biblical lands and peoples to *Conus* shells with black markings on a white background that Linnaeus is supposed to have thought resembled Hebrew letters. Bergh's thorough 700-word description of his single specimen of *C. judaeus* occupies two full pages of his monograph.

Nothing of the specimen Bergh dissected and described is known to have survived, but his descriptions are generally thorough and accurate and his drawings clear. Bergh (1895: pp. 161–163) distinguished *C. judaeus* from *C. ebraeus* solely on the basis of differences in radular tooth characters. Except for these (Fig. 3), his anatomical comparisons noted only minor variations between the two species—for example, different surface texture of the digestive gland. Bergh (1895) apologized for describing *C. judaeus* from a single specimen and character set, but he considered the differences so major that they must indicate species-level distinction from *C. ebraeus*. Subsequent to Bergh's description, taxonomists who have specialized in prosobranch taxonomy used shell characters exclusively, and those who considered both nominal species (including AJK) dismissed *C. judaeus* as an aberrant *C. ebraeus* and its name as a junior synonym (Röckel *et al.*, 1995; Filmer, 2001).

We have now demonstrated that these authors, unduly influenced by the absence of differentiating shell characters, appear to have too quickly dismissed Bergh's conclusion, and that he was in fact correct. Molecular and ecological differences as well as the radular morphological characters identified by Bergh support the hypothesis that *C. judaeus* Bergh, 1895, is a valid species distinct from but partially sympatric with and occupying the same habitat as *C. ebraeus* Linnaeus, 1758.

The structural and size differences between the teeth of *C. ebraeus* and *C. judaeus* may provide insights into the evolution of characters of the highly specialized hypodermic radular teeth of *Conus*. While some aspects of *Conus* radular tooth ontogeny are known from a broad survey of species (Nybakken, 1990), piscivorous species have been

studied in most detail (Rolán, 1986; Nybakken and Perron, 1988; Rolán and Boyer, 2000). The results all suggest what may well be a general pattern in the temporal order of appearance of tooth structures.

Nybakken (1990: fig. 8) illustrated juvenile and adult teeth of *C. ebraeus* that conform in all respects with those in our study, and he showed that the tooth of juvenile *C. patricius*, another vermivore, lacks barb, blade, and serration, while adult teeth possess these structures (Nybakken, 1990: fig. 9). In the juvenile piscivore *C. ermineus* of  $SL < 10$  mm, the tooth has a simple shaft of nearly uniform diameter, a sharp apical tip, and a spur, but no barb, blade, or serration. A very small barb and the first serration appear when the shell is about 10 mm, and a blade and well-developed barb and a row of serration appear nearly simultaneously at about 13 mm (Rolán and Boyer, 2000: figs. 2–5). At this stage of its life, *C. ermineus* is vermivorous, but as growth continues the tooth changes form profoundly. Teeth that develop after  $SL \approx 14$  mm retain the first barb, but serration and blade are transient features. Serration no longer forms, and the blade changes to a second barb. A larger third barb characteristic of many piscivorous species is also added proximally (Nybakken and Perron, 1988; Rolán and Boyer, 2000). The tooth thus changes from a typically vermivorous to a typically piscivorous weapon.

*C. judaeus* radular teeth follow the first part of the *C. ermineus* trajectory very closely. Juveniles  $\leq 12$  mm long often lack barb, serration, or both, acquiring them at similar body size to young *C. ermineus*. *C. judaeus* tooth length increases more rapidly than does shell length; the power function fits the data more closely than the linear function (Figs. 2, 4). In contrast, *C. ebraeus* teeth maintain a more constant TL/SL ratio (Fig. 2); the linear function accounts for 89% of the variance. *C. ebraeus* teeth may be paedomorphic, as neither barb nor serration ever form in adults, and the waist remains narrower relative to its length throughout life. Although tooth ontogeny in closely related species has not been examined, studies on the development in tooth characters in piscivorous species show that their structures form in a definite order (Nybakken and Perron, 1988; Rolán and Boyer, 2000).

The similarity in tooth form and size in juveniles and the differences in adults of *C. ebraeus* and *C. judaeus* appear closely correlated with their feeding biology. Juveniles of both species preyed primarily on polychaetes of the family Syllidae (Table 3, App. Fig. 1), many of which attain a maximum length of only a few millimeters. Shifts in prey species composition with increasing size of *Conus* is known in the few species studied over a considerable range of body size, including *C. ebraeus* (Kohn and Nybakken, 1975; Kohn, 1978), and Nybakken (1990) associated dietary shifts generally with ontogenetic change in radular tooth form. Larger individuals of *C. judaeus* prey predominantly on Capitellidae (Table 3, App. Fig. 2), sedentary deposit-feed-

ing polychaetes that typically burrow in sand, and the barbed teeth may aid extraction of the prey from its burrow. In contrast, larger *C. ebraeus* prey primarily on errant, mainly herbivorous Nereididae and Eunicidae (Table 3, App. Figs. 1, 2) that spend some of their time in tubes or burrows, respectively, but may be more vulnerable while foraging among algae.

Geographically the distribution of *C. judaeus* remains poorly known, but it is clearly far narrower than that of *C. ebraeus* as it has not been detected east of the Philippines. Bergh (1895) noted that his original specimen of *C. judaeus* was collected in the Philippines by the English professional collector and naturalist Hugh Cuming (1791–1865). This statement is likely accurate, because Cuming spent the years 1836–1840 in the Philippines, where he collected widely, amassing specimens of more than 2500 species of marine molluscs as well as many thousands of birds, insects, and other natural history specimens that he shipped to England for sale (Dance, 1986). Two of the eight individuals of *C. ebraeus* from the Philippines that were examined by Duda and Lessios (2009) possessed 16S sequences that were identical to the common haplotype of Haplotype Group B, although radular teeth from these specimens have not yet been studied. This supports the inference that Bergh's specimen of *C. judaeus* may indeed have been collected in the Philippines. As yet, the present account provides the only other locality records, from Okinawa in the north Pacific and Seychelles and its dependencies in the south Indian Ocean. However, the latter also remain unverified by molecular genetic data.

Documentation of the existence of a cryptic species pair in *Conus* suggests that this genus may be even more species-rich than its presently recognized 500+ valid species. In a meta-analysis, Bickford *et al.* (2007: fig. 1) documented the rapidly increasing recognition of cryptic species in general, and Pfenninger and Schwenk (2007) demonstrated their widespread occurrence among marine invertebrate taxa. A recent study of the same Okinawa habitats as ours including Sesoko Island presents strong molecular genetic evidence for four co-occurring sibling species in the photosymbiotic tunicate *Didemnum molle* (Hirose *et al.*, 2009).

Our study demonstrates congruent differences between the co-occurring cryptic species *C. judaeus* and *C. ebraeus* in gene sequences, radular tooth morphology, and ecological resource partitioning. It also vindicates Bergh's weighting of radular over shell characters, as well as his reliance on a single but thoroughly studied specimen in this case. Although shell characters have been used almost exclusively to classify *Conus* ever since Linnaeus, the hypothesis of the primacy of radular characters in taxonomy and phylogeny has recently gained support. In a detailed study of the radiation and phylogeography of a group traditionally classified as comprising four closely related *Conus* species in the Cape Verde Islands, Cunha *et al.* (2008) investigated



similar questions to those posed here using different but related morphometric methods. Their results also demonstrated closer agreement of molecular inferences with radular tooth than with shell morphometry. Studies of other sections of *Conus* as well as in the closely related family Turridae are also revealing congruence of molecular with radular but not shell characters (Kohn and Meyer, 2007; Kantor *et al.*, 2008). Our continuing investigations will seek to further test the generality of this hypothesis. In the future, searches for cryptic species elsewhere in *Conus* may also contribute to understanding the evolution of high species diversity, prey specialization associated with diversification of conotoxins, and also the medical applications of the toxins.

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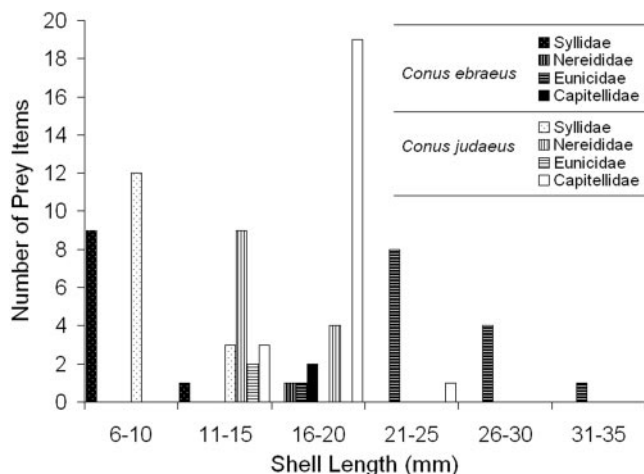
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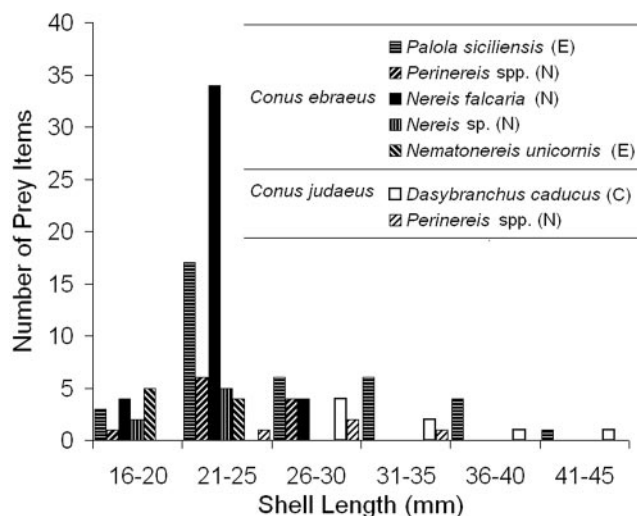
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## Appendix

The figures show the shifts in diet composition in nature with growth of *Conus ebraeus* and *Conus judaeus*. Each bar represents the number of prey organisms of one family of polychaetes recovered from the alimentary tracts of *Conus* in the size-groups on the abscissa.



**Appendix Figure 1.** Shifts in prey composition with body size in *Conus ebraeus* (histograms with black backgrounds;  $n = 27$ ) and *C. judaeus* (histograms with white backgrounds;  $n = 53$ ) at Okinawa. For both, prey taxa are highly significantly associated with predator body size (Kruskal-Wallis ANOVA; *C. ebraeus*:  $\chi^2 = 20.3$ ;  $P < 0.001$ ; *C. judaeus*:  $\chi^2 = 36.1$ ;  $P < 0.001$ ).



**Appendix Figure 2.** Prey species consumed by *Conus ebraeus* (histograms with black backgrounds;  $n = 106$ ) and *C. judaeus* (histograms with white backgrounds;  $n = 12$ ) of different body sizes at Seychelles. E, Eunicidae; N, Nereididae; C, Capitellidae. The shift in prey species with size in *C. ebraeus* is highly significant (Kruskal-Wallis ANOVA:  $\chi^2 = 29.6$ ;  $P < 0.001$ ). The trend of dietary shift with size in *C. judaeus* is not significant (*U* test:  $U = 10$ ;  $P \approx 0.2$ ).