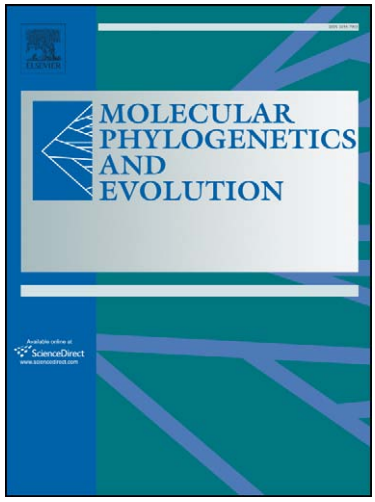

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Hidden diversity in a hyperdiverse gastropod genus: discovery of previously unidentified members of a *Conus* species complex

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Abstract

Molecular sequence data are a powerful tool for delimiting species, particularly in cases where morphological differences are obscure. Distinguishing species in the *Conus sponsalis* complex of tropical marine gastropods has long been difficult, because descriptions and identification has relied exclusively on shell characters, primarily color patterns, and these often appear to intergrade among putative species. Here we use molecular sequence data from two mitochondrial gene regions (16S rRNA and cytochrome oxidase subunit I) and one nuclear locus (a four-loop conotoxin gene) to characterize the genetic discontinuity of the nominal species of this group currently accepted as valid: the Indo-West Pacific *C. sponsalis*, *C. nanus*, *C. ceylanensis*, *C. musicus* and *C. parvatus*, and the eastern Pacific *C. nux*. In these analyses *C. nanus* and *C. sponsalis* resolve quite well and appear to represent distinct evolutionary units that are mostly congruent with morphology-based distinctions. We also identified several cryptic entities whose genetic uniqueness suggests species-level distinctions. Two of these fit the original description of *C. sponsalis*; three forms appear to represent *C. nanus* but differ in adult shell size or possess a unique shell color pattern.

Introduction

Because of the combination of a general dearth of physical barriers within the world's oceans and high dispersal abilities of many marine species (Palumbi, 1992), high rates of gene flow among populations likely buffers rates of speciation in the marine environment. However, molecular-based investigations of several presumably cosmopolitan marine species and species complexes have recently revealed sets of cryptic species that show little or no obvious morphological differentiation (Knowlton 1993; Graves 1998; Simison & Lindberg 1999; Collin 2000; Ridgway *et al.* 2000; Bernardi *et al*. 2003; Hart *et al*. 2003; Meyer *et al.* 2005; Matthews 2006). These studies illustrate that our current understanding of species richness in the world's oceans and the taxonomy of marine organisms remains deficient, and they demonstrate that molecular analyses are important in elucidating the extent as well as the origins and history of marine biodiversity.

In *Conus*, an unusually diverse genus of venomous marine snails, difficult taxonomic problems involve multiple examples of species complexes (Duda & Kohn 2005; Duda & Rolán 2005). With an obligate feeding planktonic larval phase that enhances dispersal in most species (Kohn & Perron 1994) and morphological characteristics ranging from uniform to highly disparate, the problem of species complexes within *Conus* is dramatic. In many cases, morphological characters have not yet permitted satisfactory testing of whether species complexes are single variable species or superspecies (*sensu* Mayr, 1942). The complex of species including *Conus sponsalis* Hwass in Bruguiére, 1792 is

one such troublesome group. Table 1 lists the nominal species described in this complex, and Fig. 1 illustrates their shells. In recent years taxonomists have considered up to six of these species valid (Walls [1979]; Kohn 1992; Röckel *et al*. 1995) (Table 1), but their status has long remained confused. They are alternately recognized as individual species or grouped together as subspecies or forms of *C. sponsalis*. For example, Röckel *et al*. (1995) synonymize *C. nanus* with *C. sponsalis* while Walls ([1979]) considers the former a subspecies of the latter. Kohn (1992) considers *C. nux* distinct, while noting that several other taxonomists regard *C. nux* a subspecies of *C. sponsalis*. Furthermore, *C. musicus* and *C. ceylanensis* are often synonymized (Röckel et al. 1995) and their relation to *C. parvatus* is unresolved.

Much of the difficulty in delimiting members of the *C. sponsalis* complex is due to the lack of clear morphological differences between species. Members of the complex exhibit a continuum in terms of the patterns of variously shaped and colored markings on their shells. Some have reduced or no markings (*C. nanus*), while others show larger, more prominent and somewhat diagnostic markings (*C. ceylanensis*, *C. musicus*, *C. nux*, C. *parvatus*, and *C. sponsalis*) (Fig. 1). Adult shell lengths vary from 15 to 32 mm (Walls [1979]), and although each member of the complex varies less, overlap prevents differentiation on the basis of shell size alone. Geographic distributions of members of the *C. sponsalis* complex often overlap as well (Fig. 2). *C. nanus*, *C. sponsalis*, and *C. musicus* occur widely in the tropical Indo-West Pacific region. *C. parvatus* occurs in the Indian Ocean where it overlaps the ranges of *C. nanus* and *C. sponsalis*. *C. musicus* is more narrowly distributed, entirely within the two prior species. *C. nanus* and *C.*

sponsalis also appear to intergrade with respect to shell color patterns in the Marshall Islands (Röckel *et al.* 1995). *C. nux* is the only member of the complex occurring outside the Indo-West Pacific; it is restricted to the eastern Pacific.

To evaluate the taxonomic status of members of the *C. sponsalis* species complex, we sequenced regions of two mitochondrial genes and a nuclear gene. We constructed gene trees from these data to determine whether the forms represent distinct species that are recognizable as distinct units in these trees. We also compared minimum divergence at cytochrome *c* oxidase I (COI) among members of different clades to the conservative minimum threshold of 4% recommended by Meyer and Paulay (2005) for recognizing evolutionarily significant units.

Methods

We obtained specimens preserved in 70-95% ethanol representing members of the *C. sponsalis* species complex from the locations listed in the Appendix.

Morphological Taxonomic Characters

All members of the *C. sponsalis* group share the following shell characters and states, as defined in Röckel *et al*. (1995): The shells are small to moderate-sized, with maximum length 34 mm, and moderately light to moderately solid, with relative weights (shell weight (g)/shell length (mm) of 0.08-0.3. The last whorl is conical to ventricosely conical

with position of maximum diameter (= shell height at maximum diameter/aperture height) of 0.8-0.9, and relative diameter (= maximum diameter/aperture height) of 0.6-0.8. The outline of the last whorl is convex near the shoulder and usually straight anteriorally. The shoulder is rounded to angulate and bears a row of tubercles or nodules. The spire height is low to moderate relative to shell length (0.06-0.18). The teleoconch sutural ramps are flat to slightly concave in outline, with 1-4 spiral grooves, often obsolete on later sutural ramps.

We first attempted to identify specimens based on shell color patterns using the descriptions of members of the *C. sponsalis* complex given in Röckel *et al*. (1995) and Walls ([1979]). We term the following specific names, applied according to these characters, 'pre-identifications.' In the Results section we then test these with the resultant gene trees.

<u>*C. sponsalis*</u>. Ground color of last whorl is white with two spiral rows of reddish brown axial flames of varying size, sometimes fused into continuous bands (Figs. 1B & 1C). Base is purplish blue. Teleoconch has sutural ramps with reddish or dark brown blotches between the tubercles. Aperture is dark bluish violet deep within.

<u>*C. nux*</u>. Shells of are very similar to those of *C. sponsalis* except for the larger size of the flamules on the body whorl (cf. Fig. 1A & 1C). They are allopatric: *C. nux* occurs in the eastern Pacific and *C. sponsalis* occurs in the Indo-West Pacific. Although Walls ([1979])

considers *C. nux* a subspecies of *C. sponsalis*, others regard *C. nux* a valid species (Keen, 1971; Filmer, 2001).

C. nanus. Shells share characteristics of those of *C. sponsalis* except for the reduction in or absence of dark flammules of the body whorl in *C. nanus* (Röckel *et al*. 1995) (Fig. 1). Because *C. nanus* and *C. sponalis* cannot be separated based on shell morphometrics and shells that appear to represent intermediates of these occur in regions where the two forms overlap, Röckel *et al*. (1995) considered *C. nanus* a form of *C. sponsalis*. Others however regard *C. nanus* a valid species (Rehder 1980; Richard 1990) or a subspecies (Walls [1979]). Ground color of last whorl is slightly bluish white with color pattern reduced to a few flecks, a few dotted or dashed spiral lines, or completely absent (Figs. 1D & 1E). Spire color pattern is reduced to dots between tubercles or absent. Aperture is light violet, brown or blue. The form shown in Fig. 1F is small (shell length 9.5 mm), with a more extensive and darker purplish-black area at the base. It seems not to ever have been described as a distinct species. Because its shell form and color pattern are closest to *C. nanus* we refer to it as *C.* sp. A cf. *C. nanus*.

C. musicus. Ground color of shells is white to pale gray. Last whorl has a gray, orange or reddish brown spiral band on each side of the center, with the bands sometimes fusing into one. Shell also has spiral rows of varying numbers of dots and dashes extending from shoulder to base. The dark dots sometimes alternate with white dashes or dots (Fig. 1G). The late teleoconch sutural ramps have dark brown markings between the tubercles. Base

and dorsal part of columella is dark bluish violet. Aperture pale violet to dark bluish violet, usually with lighter bands below shoulder and centrally.

C. ceylanensis. While some taxonomists consider *C. ceylanensis* to be a valid species (Kohn 1960, 1978, 1992; Röckel *et al.* 1995), others have synonymized it with *C. musicus* (Walls [1979]), and some have suggested that it represents a geographic race of *C. sponsalis* (see Kohn 1960, 1978). *C. ceylanensis* is similar in appearance to *C. musicius* but the shell exhibits variably sized reddish-brown to brown blotches throughout center of whorl that are occasionally broken up by a ground color band in the middle (Fig. 1H).

C. parvatus. Tubercles of shoulder are sometimes obsolete. Ground color of shell is white to bluish white or bluish gray, usually lighter toward shoulder. Last whorl has spiral rows of varying numbers of reddish or brown dots and dashes from shoulder or near shoulder to base (Fig. 1I). Outer edges of late teleoconch sutural ramps have a spiral row of reddish brown dots or lines. Base is dark bluish violet. Aperture is pale violet to dark bluish violet, sometimes with lighter bands below shoulder and centrally.

Shell lengths and widths of most specimens were measured to the nearest 0.5 mm with vernier calipers. Most specimens are permanently deposited at the University of Michigan Museum of Zoology (UMMZ), the Florida Museum of Natural History and the Santa Barbara Museum of Natural History.

Sequence data

We extracted DNA from approximately 25 mg of foot tissue with the E.Z.N.A Mollusc DNA Kit (Omega Bio-Tek, Inc.) or with DNAzol (Invitrogen) according to manufacturers' recommendations (we slightly modified Omega Bio-Tek's protocol by extending centrifugation of precipitated DNA to ten minutes and reducing the volume of elution buffer to 40 µl to increase DNA concentration). We prepared cDNA from venom duct mRNA as described previously (Duda & Palumbi 1999).

We amplified regions of the mitochondrial 16S and COI genes with universal 16S (Palumbi *et al*. 1991) and COI (Folmer *et al.* 1994) primers as described previously (Duda *et al.* 2001, Duda & Rolán 2005). These primers amplify approximately 510 base pairs (bp) of the 16S gene and approximately 650 bp of the COI gene (excluding primers). Because sequences of some of the amplification products obtained with the COI primers appeared to be COI sequences that were translocated to the nuclear genome (*i.e.*, numts) based on the presence of frameshift mutations, we designed an internal primer (CTAATCAATTYCCAAATCCYCCAATC) and used this with the LCO1490 primer to preferentially amplify sequences of COI from the mitochondrial genome. These primers amplify 174 bp of the COI gene (exluding primers). We also designed two other primers that sit just inside of the Folmer COI primers and match regions of the COI from various members of the *C. sponsalis* complex (GGTCAACAAACCATAAAGATATTGGGACATT and TATACTTCTGGATGCCCAAAAAATCARAAC) to amplify COI fragments from specimens in which other primers failed to work. While some amplification products of

mitochondrial genes were cleaned using QIAquick (Qiagen) or Wizard columns (Promega), others were prepared for sequencing by diluting 1:5 in water. Sequencing was performed in both directions.

To identify putatively orthologous conotoxin loci, we amplified O-superfamily conotoxins from cDNA with general four-loop conotoxin primers (Tox1 and Tox2; Duda & Palumbi 1999) from several members of the *C. sponsalis* complex, including *C. nanus*, *C. nux* and *C. sponsalis*. These primers amplify approximately 250 bp of O-superfamily transcripts. Amplifcation products were cloned and inserts of expected size were sequenced. We identified a putative orthologous toxin gene that was apparently expressed by *C. nanus* and *C. sponsalis* but not *C. nux* and designed a primer (GAAACTCCATGTTAACCAAGAAGCG) just upstream of the coding region of the mature conotoxin peptide to be used in conjunction with the general four-loop conotoxin primers that matches a region of the 3' untranslated region of these genes (*i.e.*, Tox2). These primers amplify approximately 125 bp of the conotoxin locus that was identified from analyses of cDNA. Amplifications of the conotoxin locus were cloned using a TA cloning kit (Invitrogen) and colonies were screened using M13 and M13R primers. Amplification products of up to eight positive screens were cleaned using QIAquick columns (Qiagen) and sequenced to identify both alleles from heterozygous individuals.

Gene trees

We inspected chromatograms of new sequences and aligned these sequences with published sequences of 16S, COI and four-loop conotoxin genes from other *Conus*

species, including published sequences of members of the complex, with Sequencher 4.6 (Gene Codes Corporation). Ultimately we excluded sequences from all species except those from members of the *C. sponsalis* complex and sequences from *C. abbreviatus*, a fairly close relative of members of this complex (Duda & Kohn 2005). We used MODELTEST 3.7 (Posada & Crandall 1998) to choose the best model of nucleotide substitution for each dataset. These models were used with PAUP* 4b10 (Swofford 2002) to reconstruct gene trees using neighbor-joining of maximum-likelihood distances. Support for nodes was estimated with 1000 bootstrap replicates. We also examined posterior probabilities of support for nodes in gene trees using Bayesian analysis with MrBayes v.3.1.2 (Huelsenbeck & Ronquist 2001; Ronquist & Huelsenbeck 2003). We examined sequences from each gene region separately, but also examined combined sequence data of the mitochondrial genes. For combined Bayesian analyses, sequences were partitioned and separate models of nucleotide substitution were used for each data partition.

Results

Sequence data

We obtained sequences of a region of the COI gene from 61 individuals, including 27 partial sequences that were amplified with an internal primer, and included these with six sequences from GenBank (GenBank accession numbers are given in the Appendix). We also obtained sequences of a region of the 16S gene from 63 individuals, and included

these with six sequences from GenBank (see Appendix). Sequences of the four-loop conotoxin locus were obtained from 30 individuals; sequences from seven individuals were determined from cDNA and sequences from the other 23 individuals were obtained from amplifications of genomic DNA (see Appendix for GenBank accession numbers). 17 individuals were heterozygous and 13 were homozygous at the conotoxin locus. Although we attempted to obtain sequences of each of the three gene regions from every individual, we did not recover completely overlapping datasets for all 76 individuals examined.

Gene trees

We obtained conotoxin sequences of 16 individuals pre-identified as *C. nanus*, two as *C. nux* and 10 as *C. sponsalis*. The K81uf model was chosen for sequences of the conotoxin locus (base frequencies: A = 0.1783, C = 0.2756, G = 0.2011, T = 0.3450; rate matrix: [A-C] = 1.0000, [A-G] = 9.4460, [A-T] = 6.7369, [C-G] = 6.7369, [C-T] = 9.4460, [G-T] = 1.0000). The tree constructed using this model exhibits three main groups (Fig. 3). The first group (I, Fig. 3) contains only individuals from Hawaii, Guam, and Samoa that were initially recognized as *C. nanus*. The second group (II, Fig. 3) contains two individuals of *C. nanus*, both *C. nux,* and all but one of the individuals that were pre-classified as *C. sponsalis*. The only *C. sponsalis* individual that did not join group II possessed a conotoxin sequence quite divergent from all other sequences (III, Fig. 3).

We obtained 16S and at least partial COI sequences from one individual pre-identified as *C. ceylanensis*, two pre-identified as *C. parvatus*, and multiple individuals pre-identified

as *C. musicus* (*n* = 6 and 4 for 16S and COI sequences respectively), *C. nanus* (*n* = 29 and 30), *C. nux* (*n* = 10), and *C. sponsalis* (*n* = 21 and 20). The HKY+G model was determined to be most appropriate for the 16S data (base frequencies: A = 0.3294, C = 0.1679, G = 0.2018, T = 0.3009; transition to transversion ratio = 8.3458; gamma distribution shape parameter = 0.0000). The HKY+G model was also determined to be most appropriate for the COI data (base frequencies: A = 0.2506, C = 0.1646, G = 0.2115, T = 0.3733; transition to transversion ratio = 6.8599; gamma distribution shape parameter = 0.2052). Trees reconstructed from analysis of individual data sets (not shown) exhibited similar topologies as those obtained from combined data. Bayesian and distance methods also produced similar trees.

Eight main sets of sequences are present in the tree derived from analysis of combined 16S and COI sequence data (Fig. 4). Group I, containing individuals pre-identified as *C. nanus*, *C. parvatus*, and *C. sponsalis*, corresponds to Group I in the conotoxin phylogram (Fig. 3) based on overlapping memberships of these groups. The second group comprises all six individuals that were pre-identified as *C. musicus*. The individual pre-identified as *C. ceylanensis* is distinct as sister to the remaining species of Groups I and II (Group III, Fig. 4). The fourth group contains two individuals from Cook Islands pre-identified as *C. nanus* and 17 pre-identified as *C. sponsalis*. The fifth group comprises all ten individuals pre-identified as *C. nux*. Groups IV and V occur in a well-supported group that corresponds to Group II in the conotoxin tree (Fig. 3). The sixth group contains four specimens pre-identified as *C. nanus*. Of these, the two with conotoxin sequences were in Group II on that tree. The seventh group contains four individuals referred to as *C.* sp. A

cf. *C. nanus* that exhibit a distinct shell color pattern from *C. nanus* (see Fig. 1F and below). The eighth group contains one individual from Oman pre-identified as *C. nanus* and five as *C. sponsalis*; the only member of this group with conotoxin sequence (from Guam, pre-identified as *C. sponsalis*) comprises Group III in the conotoxin tree (Fig. 3).

Taxonomic considerations

C. nanus. We obtained COI, 16S and conotoxin sequences from multiple specimens pre-identified as *C. nanus*, but these did not form a single clade in any gene tree. They occurred in two of the three groups in the conotoxin tree and five of the eight groups in the 16S+COI tree (Figs. 3, 4). In the conotoxin tree, all but two specimens clustered in one group (I, Fig. 3); the two exceptions, 'nanus CspHx1' and 'nanus CspHx2', occurred in a second group with individuals of *C. nux* and *C. sponsalis* (II, Fig. 3). These *C. nanus* individuals also cluster with other specimens of *C. nanus* in the 16S+COI tree (Group VI, Fig. 4) for which conotoxin sequences were not obtained. Minimum divergence between individuals of Groups I and VI of the 16S+COI tree at COI is 0.0477 (HKY+G distance), a value greater than the conservative minimum threshold value of 0.04 that was recommended for distinguishing evolutionarily significant units by Meyer and Paulay (2005). Two members of Group VI with shell measurements are both 23 mm in length (see Fig. 1E and Appendix); shells of other specimens identified as *C. nanus* that occur in other clades (specifically Groups I of the conotoxin and 16S+COI trees; Figs. 3, 4) are 4.5-18.0 mm long. These large individuals better fit Sowerby's (1833) description of *C. nanus*, and the size (length = 22 mm, width = 13 mm) of the lectotype of this species

(British Museum (Natural History), No. 198170) is more similar to the large specimens (Kohn 1992, see Appendix).

Four individuals that were pre-identified as *C.* sp. A cf. *C. nanus* and exhibit a distinctive shell color pattern (see Fig. 1F) did not cluster with other specimens pre-identified as *C. nanus*. We do not possess conotoxin sequences of these specimens, but these individuals are clearly differentiated from the other members of the *C. sponsalis* complex at 16S and COI (Group VII; Fig. 4) and the lowest HKY+G distance to other specimens is 0.0421. The anterior third to half of their shells has a broad brown and black spiral band, while the posterior portion of the last whorl is white to beige without darker markings (Fig. 1F). Shells of all other members of the *C. sponsalis* complex are dark blue or black only at the anterior tip (Fig. 1). These individuals are also quite small (mean shell length 9 mm; range 7-10 mm) (see Appendix). We are unaware of any other *Conus* species that possess this shell color pattern; further study may reveal this form to be a new species.

Other *C. nanus* individuals occur together in a single clade in the conotoxin tree and their conotoxin sequences diverge considerably from other members of the complex. These individuals cluster with *C. parvatus* and a specimen that was pre-identified as *C. sponsalis* in the 16S+COI tree (Group I; Fig. 4). The small individuals that were pre-identified as *C. nanus* appear to be members of an evolutionarily distinct unit of this complex, but separation of this form from *C. parvatus* is not apparent from our results even though they exhibit relatively distinct shell color patterns (see Figs. 1D and 1I). Small-sized cone snails with whitish or bluish shells that have weak to no markings (Fig.

1D) have almost always been identified as *C. nanus.* Clearly additional work is needed to determine whether these small forms are distinct from *C. parvatus*, but our results explicitly show that they are genetically distinct from individuals that most closely match Sowerby's (1833) original description and lectotype of *C. nanus*.

C. nux. Individuals of *C. nux* do not cluster separately from individuals of *C. sponsalis* in the conotoxin gene tree (Fig. 3). However, *C. nux* specimens exhibit reciprocal monophyly, albeit weakly, in the 16S+COI tree (Fig. 4). Genetic distances (HKY+G) among individuals of *C. nux* and *C. sponsalis* (Groups IV and V of the 16S+COI tree, Fig. 4) range from 0.0173 to 0.0443. Because of their allopatric distributions and similar though distinct shell color patterns, *C. sponsalis* and *C. nux* at least represent allospecies (*sensu* Mayr & Diamond 2001), but their reciprocal monophyly in the 16S+COI suggests that they may be reproductively isolated evolutionarily significant units.

C. sponsalis. Specimens of *C. sponsalis* are distributed throughout the gene trees, but most occur in one of two distinct clades. The minimum genetic divergence among members of these clades at COI (HKY+G distance) is 0.1132. Members of one of these groups (Group II of the conotoxin tree, Fig. 3; Group IV of the 16S+COI tree, Fig. 4) appear to be restricted to the western and central Pacific (Appendix). The members of this clade exhibit a mean shell length of 15.4 mm (range = 9-25 mm) (see Appendix) and match the description of *C. sponsalis* Hwass in Bruguiére, 1792.

Nuclear and mitochondrial sequences of the other clade (Groups III and VIII in Figs. 3, 4 respectively) are quite divergent from sequences of these genes from other members of the *C. sponsalis* complex, including other specimens pre-identified as *C. sponsalis* that exhibit similar shell color patterns and shapes as these individuals. Shell lengths of specimens in this clade range from 9.0 to 22.5 mm (mean = 17.0 mm) (see Appendix) and so considerably overlap with the shell sizes of members of the other main clade of *C. sponsalis* as discussed above. Despite our failure to identify any diagnostic morphological characters to distinguish members of this clade from those of the other, this clade appears to represent an evolutionarily distinct lineage within the *C. sponsalis* species complex based on the mitochondrial and nuclear gene trees (Figs. 3, 4). Because members of this clade also appear to fit the original description of this species, it is unclear which clade requires a new species description. This clade likely occurs throughout the Indo-West Pacific and its distribution overlaps considerably with that of the other clade, although thus far it appears to be absent from Hawaii.

C. ceylanensis and *C. musicus*. Although we did not recover conotoxin gene sequences from specimens pre-identified as *C. ceylanensis* and *C. musicus*, we obtained 16S and COI sequences from one *C. ceylanensis* and several *C. musicus*. All specimens pre-identified as *C. musicus* cluster together uniquely in the tree constructed from combined 16S+COI data (Group II, Fig. 4). The minimum genetic distance at COI (HKY+G distance) between members of Groups I and II of the 16S+COI tree is 0.0201. Support for Group II is weak and the sole individual pre-identified as *C. ceylanensis* falls out basal to this clade. We obtained COI and 16S data from only one individual pre-identified as *C.*

ceylanensis. Minimum genetic divergences at COI (HKY+G distances) among this individual and members of Groups I and II of the 16S+COI tree are 0.0158 and 0.0188 respectively. We were only able to obtain a 174 bp fragment of the COI gene from four individuals pre-identified as *C. musicus* while approximately 650 bp of this gene was obtained from *C. ceylanensis* (see Appendix). Thus, we are not confident that *C. ceylanensis* and *C. musicus* represent evolutionarily distinct units and suggest that additional work on these species be conducted to more appropriately determine the validity of these species.

C. parvatus. We obtained COI and 16S sequences for only two specimens pre-identified as *C. parvatus*. Sequences of these individuals were similar to sequences of some of the specimens pre-identified as *C. nanus* (Group I, Fig. 4). Our results were thus equivocal with regards to the separation of *C. nanus* and *C. parvatus*. Additional specimens and markers should be analyzed to more thoroughly evaluate their evolutionary distinctiveness.

Problems with specimen pre-identifications

Two individuals that were pre-identified as *C. nanus*, 'nanus KR1' and 'nanus KR2' from the Cook Islands, occur with individuals of *C. sponsalis* in the 16S+COI tree (Group IV, Fig. 4). Another specimen that was pre-identified as *C. nanus*, 'nanus 3122', occurs in a separate group with individuals of *C. sponsalis* in the 16S+COI tree (Group VIII, Fig. 4). Conotoxin sequences were not obtained from these individuals. Nonetheless, these specimens exhibit shell color patterns that fit the description of *C. nanus* and their shell

sizes overlap those of both *C. nanus* and *C. sponsalis* (Fig. 5A-C and see Appendix). Another specimen, 'sponsalis CspS1', that was pre-identified as *C. sponsalis* based on the presence of small flammules on its shell (Fig, 5D), occurs in a clade that contains primarily specimens of *C. nanus* (Group I of the 16S+COI tree, Fig. 4).

Discussion

Examination of 166 sequences from 76 individuals of the *Conus sponsalis* species complex revealed that the members of this assemblage comprise several well-supported groups in gene trees constructed from sequence data of one nuclear locus and two mitochondrial gene regions (Figs. 3 & 4). Although the different gene regions differ in their ability to resolve relationships among members of this complex, group membership is entirely consistent among the various trees that were reconstructed (*i.e.,* an individual is not a member of different groups in different gene trees) (Figs. 3, 4). The rapid rate of conotoxin gene evolution in *Conus* (Duda & Palumbi 1999), had led us to predict that these sequences would detect genetic differences among evolutionarily distinct units more clearly than mitochondrial sequences. However, the 16S+COI tree (Fig. 4) as well as trees reconstructed from each mitochondrial gene region exhibited much more structure than the conotoxin tree. The improved resolution of the COI and 16S trees presumably reflects higher rates of evolution and greater variation of the mitochondial genes compared to the conotoxin locus.

Our analyses show that the *C. sponsalis* species complex corresponds to at least five and as many as eight evolutionarily significant units that perhaps represent distinct species, including three species that have not yet been described. Two members of this complex, *C. nanus* and *C. sponsalis*, likely consist of two sets of "pseudo-cryptic" species that are distinguishable at mitochondrial and in some cases nuclear sequences. We have not yet identified any clear differences in shell morphology among the members of these sets aside from differences in adult shell size and shell color patterns of specimens that were pre-identified as *C. nanus*. These results are particularly astonishing because the members of this complex with cryptic forms are common, intertidal species and taxonomic crypsis had not before been suspected. The discovery of these pseudo-cryptic species emphasizes the importance of molecular studies for deciphering the biodiversity of the world's oceans and suggests that many additional cryptic species still lurk in *Conus* as well as in other taxa. We anticipate that *Conus* is much more species-rich and that the diversification of this group was much more rapid than previously reported (see Kohn 1990, Röckel *et al.* 1995).

Our work provides a phylogenetic basis for evaluating morphological criteria to distinguish members of the *C. sponsalis* species complex. Most specimens that we pre-identified based on shell color patterns occurred in groups with similarly named specimens. Members of two of the distinct evolutionary units we identified (Groups VI and VII, Fig. 4) also exhibit unique shell patterns and shell sizes. This implies that aspects of the shell morphology of members of the *C. sponsalis* species complex are reasonably diagnostic and can delimit the members of this complex. However, several

specimens did not group with similarly named specimens (*e.g.*, 'nanus KR1', 'nanus KR2', 'nanus 3122' and 'sponsalis CspS1'; see Figs. 4 & 5) and members of the two main clades that contained specimens pre-identified as *C. sponsalis* (Groups II & III in Fig. 3 and Groups IV and VIII in Fig. 4) do not exhibit any apparent morphological differences. Thus, additional work is clearly needed to delimit several of the members of these clades based on morphological criteria alone.

Acknowledgments

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Table 1. Nominal species comprising the *Conus sponsalis* complex, listed in chronological order. Taxa in boldface have been considered valid by late 20th-Century workers. For images of type specimens and complete original citations and descriptions, see The *Conus* Biodiversity Website (http://biology.burke.washington.edu/conus/).

***C. sponsalis* Hwass in Bruguière, 1792**

***C. musicus* Hwass in Bruguière, 1792**

C. puncturatus Hwass in Bruguière, 1792

***C. ceylanensis* Hwass in Bruguière, 1792**

C. maculatus Bosc, 1801

***C. nux* Broderip, 1833**

***C. nanus* Sowerby, 1833**

C. pusillus Reeve, 1843

C. mighelsi Kiener, 1845

C. acutus Sowerby, 1857

***C. parvatus* Walls, 1979**

Figure legends

Fig. 1. Photographs of shells of members of the *Conus sponsalis* complex. Scale bars indicated on left hand side of images are in millimeters. A. *C. nux*; B. *C. sponsalis* (M6); *C. C. sponsalis* (Csp6); D. *C. nanus* (M41); E. *C. nanus* (Hx1); F. *C.* sp. A cf. *C. nanus* (89040); G. *C. musicus*; H. *C. ceylanensis* (3142); I. *C. parvatus* (3143) (code names of specimens used in analyses are indicated in parentheses). See appendix for measured sizes and geographic sources of specimens. Photographs of A-G courtesy of Liath Appleton (UMMZ Mollusk Division); photos of H-I from CPM.

Fig. 2. Map of the Indian and Pacific Oceans with distributions of members of the *Conus sponsalis* complex. Distributions inferred from Röckel *et al*. (1995). Dashed lines indicate unverified yet probable ranges. 1: *C. nux*, 2: *C. sponsalis*, 3: *C. nanus*, 4: *C. musicus*, 5: *C. ceylanensis*, 6: *C. parvatus*.

Fig. 3. Conotoxin gene tree of members of the *Conus sponsalis* complex. Individual names include the specimen's pre-identification followed by its collection site and code (see Appendix). Bootstrap values and posterior probabilities from Bayesian analysis are indicated above and below branches, respectively. Names of sets of individuals are given based on morphological pre-identifications of individuals in each set. Sources of sequences (cDNA or gDNA) are given with specimen names.

Fig. 4. Phylogeny of the *Conus sponsalis* complex constructed from analysis of combined 16S and COI data sets for all individuals surveyed. Posterior probabilities from Bayesian analysis are indicated on branches; posterior probabilities in brackets were determined from analysis of data from the subset of individuals in which both 16S and COI were recovered (dashes indicate that the clade was not resolved in this analysis). Specimen names and names of sets of individuals are given as in Fig. 3.

Fig. 5. Photographs of specimens that did not occur with similarly pre-identified specimens in the phylogenetic reconstructions. A. 'nanus KR1' from the Cook Islands that was pre-identified as *C. nanus*, B. 'nanus KR2' from the Cook Islands that was pre-identified as *C. nanus*, C. 'nanus 3122' from Oman that was pre-identified as *C. nanus*, D. 'sponsalis CspS1' from American Samoa that was pre-identified as *C. sponsalis*.

Appendix. Specimen data for members of the *Conus sponsalis* complex examined in this work. Dashes are given when data were not recovered. GenBank accession numbers of COI sequences in which only partial sequences were obtained are denoted with asterisks. GenBank accession numbers of conotoxin sequences from cDNA (c) and gDNA (g) are identified with superscripts; two accession numbers are given for individuals in which we recovered two alleles.

				GenBank Accession Number(s)		
Morphological ID	Specimen code	Collection Site	Shell length x width (mm)	16S	CO1	conotoxin
C. ceylanensis	3142	Thailand	12x7.5	EU423321	EU423417	--
C. musicus	X	--	--	AF144004	--	--
C. musicus	CmusicBA1	Bismarck Archipelago	15x8	EU584419	EU584387*	
C. musicus	CmusicBA2	Bismarck Archipelago	9x5	EU584420	EU584388*	
C. musicus	CmusicBA3	Bismarck Archipelago	13x7.5	EU584421	EU584389*	
C. musicus	Cmusic1	Papua New Guinea	10.5x6	AF174185	--	--
C. musicus	CmusicPI1	Philippines	15x8.5	EU584422	EU423418*	--
C. nanus	CnanS1	American Samoa	12x9	EU423322	EU584390*	--
C. nanus	CnanS2	American Samoa	13x8	EU423323	EU584391	--

C. nanus	CnanS3	American Samoa	12x8	EU423324	EU584392*	EU423370[c], EU423371[c]
C. nanus	CnanS9	American Samoa	11x6.5	EU423325	AY588207	--
C. nanus	KR1	Cook Islands	14.5x8	EU423326	EU423422	--
C. nanus	KR2	Cook Islands	12.5x7.5	EU423327	EU423423	--
C. nanus	CspEI2	Easter Island	10.5x5	EU423328	EU584395*	--
C. nanus	JK4 7	French Polynesia	--	EU423331	--	--
C. nanus	M13	Guam	6.5x3	EU423332	EU589330*	EU423384[g], EU423385[g]
C. nanus	M14	Guam	7x3.5	--	EU584397*	EU423386[g]
C. nanus	M23	Guam	9.5x5	EU423333	EU584398*	EU423387[g], EU423388[g]
C. nanus	Csp1	Hawaii	--	AF174199	EU584388*	--
C. nanus	Csp2	Hawaii	--	EU423334	--	--
C. nanus	CspH1	Hawaii	17.5x11	EU423335	EU584400*	EU423372[c], EU423373[c]
C. nanus	CspH2	Hawaii	16x10	EU423336	EU584401*	--
C. nanus	CspH3	Hawaii	18x12.5	EU423337	EU584402*	EU423374[c], EU423375[c]
C. nanus	CspH5	Hawaii	16x10	EU423338	EU584403	EU423376[c], EU423377[c]
C. nanus	CspHx1	Hawaii	23x14	EU423339	EU423425	EU423389[g]
C. nanus	CspHx2	Hawaii	23x15	EU423340	EU423426	EU423390[g]

C. nanus	Hd1	Hawaii	10.5x6.5	EU423341	EU423419*	EU423391[g], EU423392[g]
C. nanus	Hd2	Hawaii	8.5x4.5	--	EU423420*	EU423393[g], EU423394[g]
C. nanus	Hd3	Hawaii	6.5x4	EU584423	EU423421	EU423395[g]
C. nanus	M33	Hawaii	6.5x3	EU423342	EU584404*	EU423396[g]
C. nanus	M34	Hawaii	7x3	EU423343	EU584405	EU423397[g], EU423398[g]
C. nanus	M35	Hawaii	6x3	EU423344	EU584406	EU423399[g], EU423400[g]
C. nanus	M41	Hawaii	4.5x2	EU423345	--	EU423401[g]
C. nanus	3122	Oman	20.5x12.5	EU423346	EU423427	--
C. nanus	Csp3	Papua New Guinea	--	EU423347	--	--
C. nanus	Csp4	Papua New Guinea	14.5x8.5	EU423348	--	--
C. nux	Cnux1	Mexico	21x14	AF174186	EU584407*	--
C. nux	Cnux2	Mexico	22.5x15	EU423350	EU584408*	--
C. nux	3121	Panama	--	EU423351	EU423428	--
C. nux	CnuxP3	Panama	15x10	EU423352	AY588210	EU423402[g]
C. nux	CnuxP11	Panama		EU584424	EU584409*	
C. nux	CnuxP12	Panama		EU584425	EU584410	

C. nux	CnuxP13	Panama		EU584426	EU584411	
C. nux	CnuxP14	Panama		EU584427	EU584412	
C. nux	Pd1	Panama	20.5x13.5	EU423353	EU584413	EU423403[g]
C. nux	Pd2	Panama	21.5x13	EU423354	EU584414	--
C. parvatus	3126	Madagascar	17x11	EU423355	EU423429	--
C. parvatus	3143	Reunion	12.5x8	EU423356	EU423430	--
C. sp. A cf. *C. nanus*	CnanusBA1	Bismarck Archipelago	7x4	--	EU584393*	--
C. sp. A cf. *C. nanus*	CnanusBA2	Bismarck Archipelago	9.5x4.5	--	EU584394*	--
C. sp. A cf. *C. nanus*	3139	French Polynesia	10x6	EU423329	EU423424	--
C. sp. A cf. *C. nanus*	89040	French Polynesia	9.5x5.5	EU423330	EU584396	--
C. sponsalis	X	--	--	AF143993	--	--
C. sponsalis	CspS1	American Samoa	16x9.5	EU423349	--	--
C. sponsalis	CspS3	American Samoa	18x11.5	EU423357	EU584415*	EU423407[g]
C. sponsalis	CspS4	American Samoa	18.5x12.5	EU423358	EU584416*	EU423378[c], EU423379[c]
C. sponsalis	CspS5	American Samoa	22x15	EU423359	EU584417	EU423380[c], EU423381[c]
C. sponsalis	CspS6	American Samoa	22.5x15	EU423360	EU584418	EU423408[g], EU423409[g]

C. sponsalis	CspS7	American Samoa	18x12	EU423361	EU589329	EU423410[g], EU423411[g]
C. sponsalis	M3	American Samoa	9x5	EU423362	EU423431	EU423404[g], EU423405[g]
C. sponsalis	M4	American Samoa	11x6	--	--	EU423406[g]
C. sponsalis	AWS4	Fiji	--	EU423364	EU423437	--
C. sponsalis	AWS5	Fiji	--	EU423365	EU423438	--
C. sponsalis	3104	French Polynesia	--	--	EU423436	--
C. sponsalis	KR3	French Polynesia	--	EU423363	--	--
C. sponsalis	M7	Guam	14.5x9	EU584432	EU423441	--
C. sponsalis	M8	Guam	13.5x9	EU423366	EU423442	EU423414[g], EU423415[g]
C. sponsalis	M9	Guam	14x9	EU423367	EU423443	EU423416[g]
C. sponsalis	M16	Guam	13x9	EU584428	EU423432*	EU423412[g]
C. sponsalis	M17	Guam	11.5x6	EU584429	EU423433*	--
C. sponsalis	M19	Guam	9x5	EU584430	EU423439	EU423413[g]
C. sponsalis	M21	Guam	9.5x6	EU584431	EU423440	--
C. sponsalis	CsponsH7	Hawaii	25x15	AY382026	AY588220	EU423382[c], EU423383[c]
C. sponsalis	Csp5	Palau	22.5x12	EU423368	EU423434*	--
C. sponsalis	Csp6	Papua New Guinea	16x10	EU423369	EU423435	--

C. sponsalis	3138	Reunion	--	--	EU423444	--

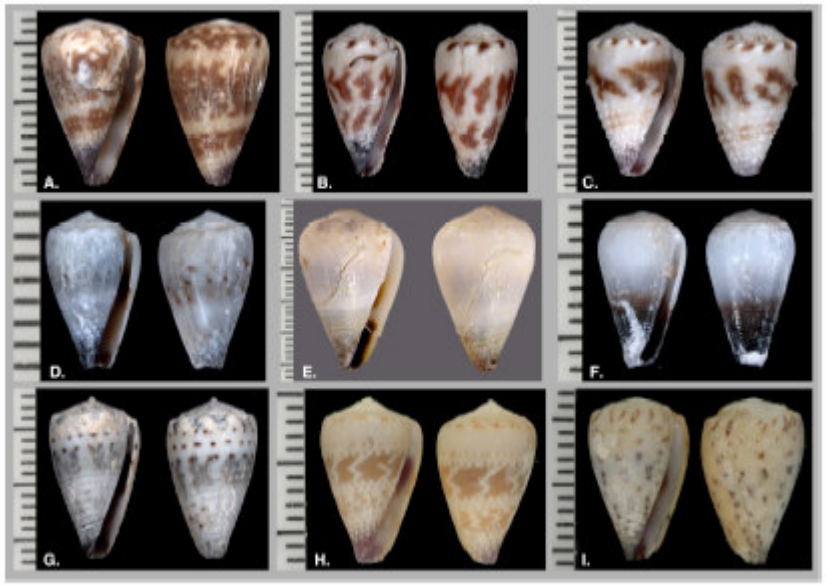

Fig.1

Fig.2

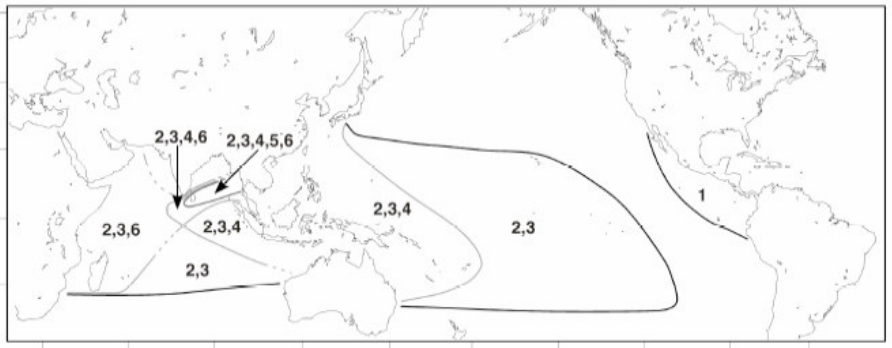

Fig.3

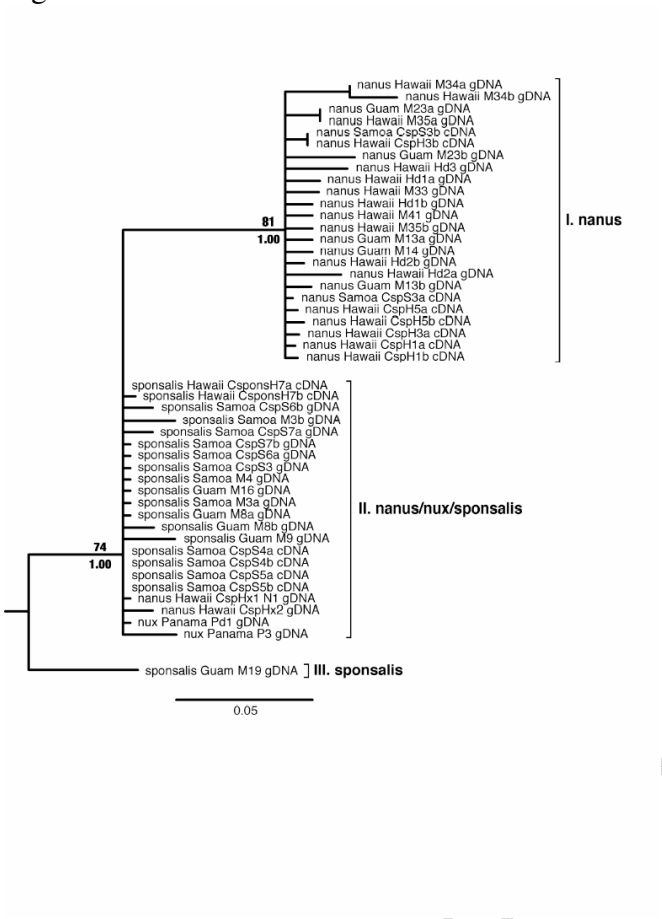

Fig.4

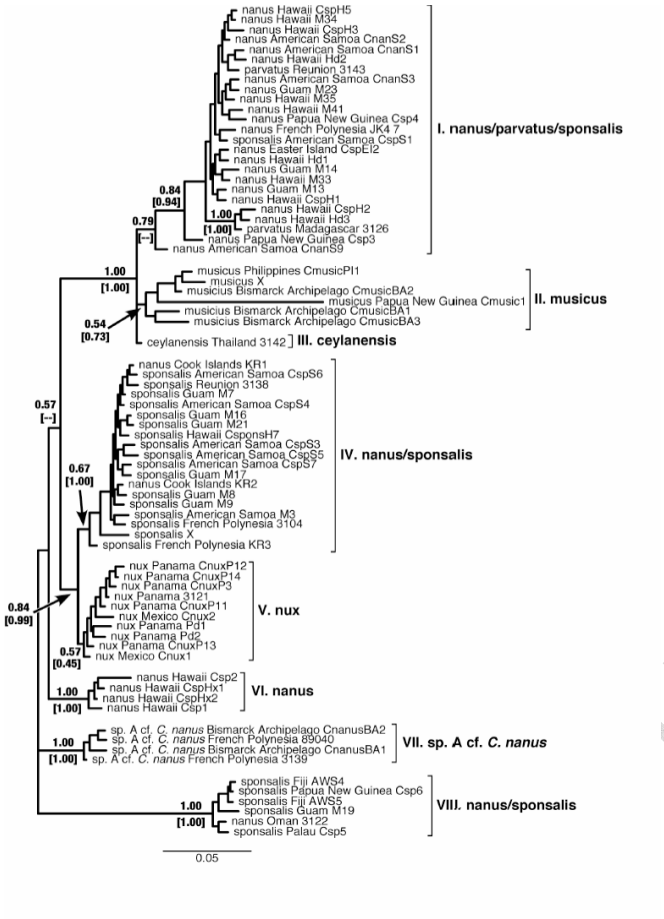

Fig.5