

The Mitochondrial Genome of *Allonautilus* (Mollusca: Cephalopoda): Base Composition, Noncoding-Region Variation, and Phylogenetic Divergence

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ABSTRACT

We used next-generation methods to sequence the mitochondrial genome of *Allonautilus scrobiculatus* from two large, overlapping amplicons generated by PCR. The genome was circular, 16,132 base pairs in length, and possessed the same sequence and orientation of genes as the previously sequenced mitogenome of *Nautilus macromphalus*. These two mitogenomes were approximately 8% divergent overall, but differentiation varied greatly among genes: some tRNA sequences were identical between the two taxa, whereas ATP8 differed by over 15%. The largest of the noncoding regions of the genome included a 62 base pair repeat that was essentially identical between the two genera; however, this repeat was present as six copies in *N. macromphalus*, but varied between four and five among individuals of *Allonautilus*. A 146 base pair deletion (in *Allonautilus* compared to *Nautilus*) included one copy of the repeat plus an adjacent 84 bp; because of this indel, the “CA” microsatellite in *N. macromphalus* was missing from *Allonautilus*. Base composition varied along the *Allonautilus* sequence, and was correlated with the strand on which genes coded. Base composition also varied within the largest noncoding region. A phylogeny of 24 extant cephalopods indicates that there is less molecular divergence between *Allonautilus* and *Nautilus* than there is among congeneric species of *Octopus* and *Sepia*.

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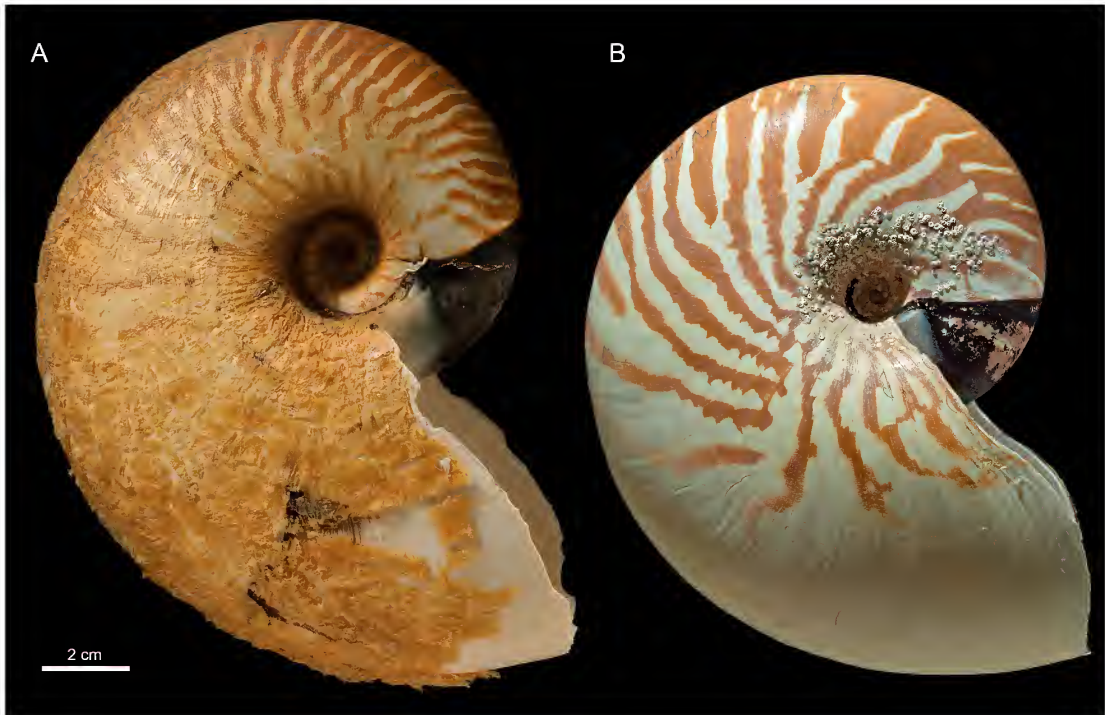


FIGURE 1. *Allonautilus* differs from *Nautilus* in the size and shape of the umbilicus, type of periostracum, and texture of the hood (e.g., Saunders et al., 1987). A. *Allonautilus scrobiculatus*, Little Ndrova Island, Papua New Guinea, AMNH 101045. B. *Nautilus macromphalus*, New Caledonia, AMNH 94104.

INTRODUCTION

The close morphological resemblance of the shells of modern nautilids to those of extinct, shelled cephalopods has given them the cachet of a “living fossil” (Woodruff et al., 1987). *Nautilus* and *Allonautilus* are the only two extant genera of externally shelled cephalopods and comprise the sister taxon to all other living cephalopods, including Sepiida, Octopoda, and Teuthida (Nishiguchi and Mapes, 2008). *Nautilus* is widely distributed at archipelagos throughout the Indo-Pacific. *Allonautilus*, on the other hand, is more restricted in its distribution, and live specimens of this genus have only been collected from Papua New Guinea and the Solomon Islands (Saunders et al., 1987; Ward and Saunders, 1997). The natural history of these animals is poorly known as a result of their relatively deepwater habitat (Saunders and Ward, 1987), but thanks to recent studies, our understanding of their ecology and distribution is vastly improving (Dunstan et al., 2011; Sinclair et al., 2011; Barord et al., 2014).

Fewer than a dozen species of extant nautilids remain of a once large and widespread radiation (Teichert and Matsumoto, 1987), although the exact number of present-day species is still a subject of debate (Bonacum et al., 2011). Many of these species are known only from drift shells (House, 1987), but at least five of them, including *Nautilus macromphalus* Sowerby, 1849, have been caught as live animals (Saunders, 1987). Ward and Saunders (1997) established the

that of the other cephalopods, it is important to determine whether the pattern observed in *N. macromphalus* characterized the rest of the order. Consequently, we sequenced the mitogenome of *Allonautilus scrobiculatus*, which appears to represent the basalmost split among extant members of the order (Bonacum et al., 2011; Bonnaud et al., 2004). Prior to this study, little molecular data were available for *Allonautilus*. Wray et al. (1995) reported some mitochondrial (16S) and nuclear (28S) sequences and Bonnaud et al. (2004) analyzed the 18S sequence, but all these ribosomal genes are conserved across a wide array of taxa. Subsequently, Bonacum et al. (2011) reported on the more rapidly evolving mitochondrial CO1 locus, but the sequences, from three individuals, represented only a fragment of one gene. Thus, a complete mitochondrial sequence can provide useful information for future molecular research on living cephalopods in general and nautilids in particular.

MATERIAL AND METHODS

We obtained tissue samples from three individuals of *Allonautilus scrobiculatus* captured near Little Ndrova Island, Papua New Guinea, in 1984 and 1985. The specimens, collected alive, were preserved in ethanol and maintained in the frozen-tissue collections of the American Museum of Natural History; their accession numbers are: AMCC224107, AMCC224111, and AMCC224119. DNA was extracted from the samples using standard QIAGEN preparations; the extracts were subjected to agarose gel electrophoresis to determine DNA quality.

The published mitogenome of *Nautilus macromphalus* (NCBI Reference Sequence: NC_007980.1) contains six copies of a 62 bp repeat in its largest noncoding region (Boore, 2006). We designed PCR primers (repNmac1: 5'-CAAATCTTCTCGTGCCTCCACA and repNmac2: 5'-GGTTTACAAGGCCGCTACTT) to amplify and sequence this region for three individuals of *A. scrobiculatus* using standard Sanger-sequencing methods on an ABI3730xl DNA Analyzer; the resulting sequences were assembled and aligned by eye using Sequencher, version 4.7 (Gene Codes Corp.).

Sequences for four PCR primers for amplifying the mitochondrial genome in two large fragments (Boore et al., 2005) were based on the published (Boore, 2006) *Nautilus* sequence. One primer pair (Nmac32: 5'-GCAGCTAACATGGGAGCCGGACACAT and Nmac61: 5'-CGTGGAGTCCGTGAAAGCCTGT) spanned the region between the ATP6 and CO3 genes, with a predicted size of approximately 7 kb. The other pair (Nmac5: 5'-GAGTT-TAGTCCCTGGCCCTCACAGG and Nmac4: 5'-GGGTGGGATGGGCTGGGGTTG-GTTTC) spanned the region between the CO3 and ND5 genes, with a predicted size of approximately 11 kb. Expected overlaps between the fragments were 1537 and 541 bases. Long-distance PCR amplifications were performed using Promega GoTaq™ Long PCR Master Mix in 50 µl volumes. The two PCR products were cleaned by centrifugation dialysis and combined in approximately equimolar amounts; a total of 500 ng of this mixture was nebulized and prepared as a single DNA library according to protocols for the Roche 454 GS Junior. The sequences resulting from the library were processed using the GS *De Novo* Assembler analysis software using default parameters. The assembled contigs were then imported into Geneious, version 6.1.7 (Biomatters Ltd.), for further analysis and visualization.

The assembled *Allonautilus* genome was aligned with that of *N. macromphalus* and adjusted by eye. Gene delimitation and annotation were based on an analysis using MITOS version 630 (Bernt et al., 2013) and comparison to the published *Nautilus* sequence. The protein-coding loci were translated into amino acids using the standard invertebrate mitochondrial genetic code. For all comparisons, percent sequence divergence was computed as 100 times the number of base pair substitutions divided by the relevant sequence length. For the purposes of assessing genetic divergence between *Allonautilus* and *Nautilus*, we treated the coding loci as comprised of three separate classes: protein-coding genes, ribosomal RNAs, and transfer RNAs. Our assessment of the structural positions of t-RNA substitutions was based on comparison with the secondary structures proposed by Boore (2006).

We assessed base composition heterogeneity around the *Allonautilus* genome by plotting the frequencies of T and G nucleotides in all of the protein and ribosomal genes, as well as in the largest noncoding region, by coding strand. Base composition heterogeneity within the largest noncoding region of the mitogenome was assessed by plotting the frequencies of G and T nucleotides, in a 100 bp sliding window, along the length of that region.

We estimated mitochondrial genetic variation among the three specimens of *Allonautilus* by comparing the Sanger-sequenced fragments of the largest noncoding region for percentage of bases that were variable among individuals and for Nei's (1987) estimator of nucleotide diversity (π); for these estimates, deletions were treated as missing.

In order to place the divergence between *A. scrobiculatus* and *N. macromphalus* within a larger context, we searched GenBank for deposits of complete mitogenomes of cephalopods. For such species, the slower-evolving COX and ATPase genes were aligned with the *N. macromphalus* and newly sequenced *Allonautilus* genes. Those two gene families code on the same DNA strand in mollusks and thus have similar base composition; they also are subject to many fewer alignment ambiguities caused by indels than is the NAD family of genes, particularly with respect to an outgroup. We computed the percentage of sequence divergence among all these taxa, and used PAUP* version 4.0b10 (Swofford, 2001) to infer maximum parsimony trees, using a chiton, *Katharina tunicata*, as an outgroup. In this analysis, we used random addition of taxa, with 25 replicates, and a heuristic search with TBR branch swapping. We also performed a bootstrap analysis, with 1000 replicates, using the same parsimony procedure described above within each replicate.

RESULTS

THE *ALLONAUTILUS* MITOGENOME

The primer pair repNmac1/repNmac2, which was designed to amplify a large portion of the noncoding region, produced fragments of 608 bp and 670 bp, depending on the individual. The PCR products sequenced cleanly only in the forward direction; the presence of a T homopolymer, 11 bases long, near the repNmac2 primer prevented clean reverse reads using Sanger methods. This T polymer was also reported for the *N. macromphalus* mitogenome. These partial sequences of the largest noncoding region for the three specimens have been deposited in GenBank (KP862897–KP862899).

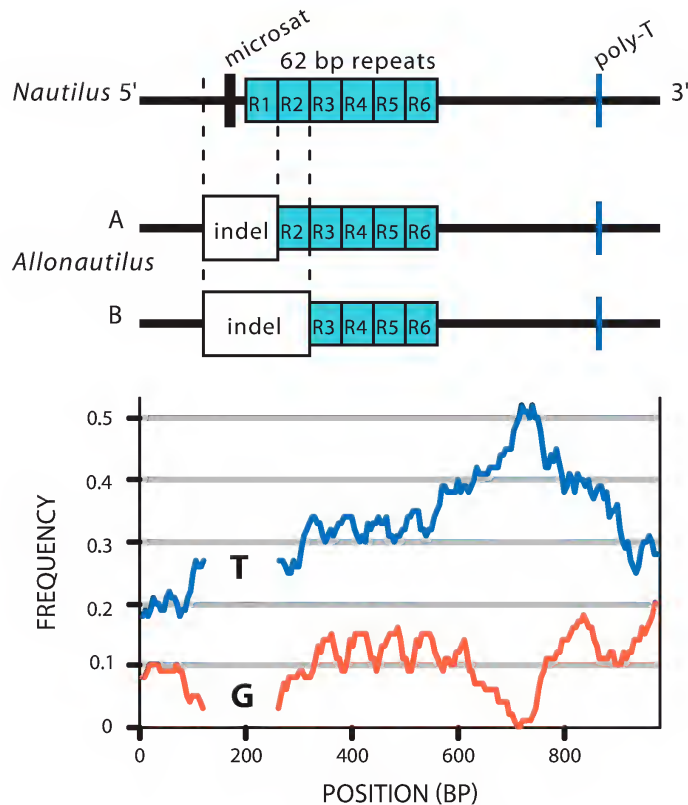


FIGURE 3. Architecture of the large noncoding region of extant nautilid mitogenomes. Features are shown for the strand on which CO1 is coded. *Nautilus macromphalus* (Boore, 2006) was characterized by a microsatellite, six copies of a 62 bp repeat (R1–R6), and a poly-T monomer; in *Allonautilus*, the microsatellite and first copy of the repeat were missing in one individual (indel pattern A), and an additional repeat was missing in two other individuals (indel pattern B); all individuals possessed the poly-T monomer (blue bar). The frequency of T and G nucleotides varied considerably through the noncoding region: their frequencies, in a 100 bp sliding window, are shown in the lower panel.

The specimen of *A. scrobiculatus* with the largest noncoding region (AMCC224119) was used for further sequencing using next-generation techniques. We obtained a single largest contig nearly 17 kb in length containing 10,690 sequences ranging in size from 40 bp to 657 bp, with an average length of approximately 350 bp; overall depth of coverage was approximately 230 \times . The contig was aligned to the published *N. macromphalus* mitogenome and rearranged so that the starting point was the CO1 gene. The assembled genome was circular, 16,132 base pairs in length, and was composed of the same sequence and orientation of 37 genes (fig. 2) previously found in *N. macromphalus* (Boore, 2006). The complete sequence has been annotated and deposited in GenBank (KP892752).

DIVERGENCE BETWEEN *ALLONAUTILUS* AND *NAUTILUS*

We observed a large (146 bp) deletion, compared to *N. macromphalus*, in the largest (826 bp) noncoding region of *Allonautilus* (fig. 3). This deletion, approximately 125 bp from the 5'

TABLE 1. Divergence between *Allonautilus scrobiculatus* and *Nautilus macromphalus* at protein-coding loci.

| Gene | Length (bp) | Number of Substitutions | Number of Transitions | Number of Transversions | Number of Amino Acid Substitutions |
|-------|-------------|-------------------------|-----------------------|-------------------------|------------------------------------|
| ATP6 | 696 | 88 (12.6%) | 68 | 20 | 16 |
| ATP8 | 159 | 24 (15.1%) | 20 | 4 | 7 |
| CO1 | 1533 | 145 (9.5%) | 128 | 17 | 1 |
| CO2 | 682 | 77 (11.3%) | 65 | 12 | 7 |
| CO3 | 780 | 74 (9.5%) | 60 | 14 | 7 |
| Cyt-b | 1133 | 70 (6.2%) | 51 | 19 | 15 |
| ND1 | 933 | 70 (7.5%) | 58 | 12 | 8 |
| ND2 | 1029 | 94 (9.1%) | 83 | 11 | 18 |
| ND3 | 353 | 49 (13.9%) | 43 | 6 | 7 |
| ND4 | 1349 | 125 (9.3%) | 102 | 23 | 21 |
| ND4L | 294 | 21 (7.1%) | 19 | 2 | 4 |
| ND5 | 1719 | 136 (7.9%) | 112 | 24 | 20 |
| ND6 | 505 | 38 (7.5%) | 31 | 7 | 11 |

TABLE 2. Divergence between *Allonautilus scrobiculatus* and *Nautilus macromphalus* at ribosomal RNA loci.

| rRNA | Length (bp; in <i>Allonautilus</i>) | Number of Substitutions | Number of Transitions | Number of Transversions | Number of Indels |
|------|--------------------------------------|-------------------------|-----------------------|-------------------------|----------------------|
| 12S | 884 | 55 (6.5%) | 49 | 6 | 3 (1 bp, 1 bp, 7 bp) |
| 16S | 1347 | 120 (9.0%) | 96 | 24 | 1 (1 bp) |

end of the noncoding region, included the “CA” microsatellite reported for *Nautilus*, as well as one copy of a 62 bp repeat. The latter was present as six copies in *N. macromphalus*, but, as a consequence of the deletion, was present as five copies in the completely sequenced *Allonautilus*; in two other specimens there were only four copies. This repeated element was essentially identical to one reported for *N. macromphalus* (Boore, 2006). Aside from the deleted repeats, this large noncoding region of the two genera of nautilus was about 20% divergent for its first 120 bases, and nearly identical thereafter.

As in *N. macromphalus*, four additional, relatively large (> 50 bp), noncoding regions exist in the *Allonautilus* mitochondrial genome (fig. 2). One of these, the nearly 100 bp spacer between the glycine tRNA and ATP6, was 37% divergent from that of *Nautilus*; two others, between the threonine and glycine tRNAs and between the glutamate tRNA and CO3, were 3.3% and 10.7% divergent, respectively. The last large spacer, between the arginine and asparagine tRNAs, was 22 bp longer in *Allonautilus* than *N. macromphalus* due to a large insertion.

The overall sequence divergence (excluding indels) between the *Allonautilus* and *N. macromphalus* mitogenomes was 8.3% for coding genes, but this varied greatly within and among classes of genes. For the 13 protein-coding loci, divergence varied between about 6% and 15%, with a mean of 9.1% (table 1). The two genera were 6.5% and 9.0% divergent for 12S and 16S

TABLE 3. Divergence between *Allonautilus scrobiculatus* and *Nautilus macromphalus* at transfer RNA loci.

| tRNA | Code | Length (bp) | Number of Substitutions | Number of Transitions | Number of Transversions |
|---------------|------|-------------|-------------------------|-----------------------|-------------------------|
| Alanine | A | 66 | 4 (6.1%) | 4 | 0 |
| Arginine | R | 64 | 3 (4.7%) | 2 | 1 |
| Asparagine | N | 65 | 7 (10.8%) | 5 | 2 |
| Aspartic acid | D | 66 | 1 (1.5%) | 0 | 1 |
| Cysteine | C | 62 | 1 (1.6%) | 1 | 0 |
| Glutamic Acid | E | 69 | 4 (5.8%) | 4 | 0 |
| Glutamine | Q | 68 | 6 (8.8%) | 5 | 1 |
| Glycine | G | 66 | 2 (3.0%) | 2 | 0 |
| Histidine | H | 63 | 1 (1.6%) | 1 | 0 |
| Isoleucine | I | 68 | 4 (5.9%) | 4 | 0 |
| Leucine | L1 | 67 | 1 (1.5%) | 1 | 0 |
| Leucine | L2 | 64 | 1 (1.6%) | 1 | 0 |
| Lysine | K | 65 | 0 (0.0%) | 0 | 0 |
| Methionine | M | 67 | 3 (4.5%) | 3 | 0 |
| Phenylalanine | F | 65 | 0 (0.0%) | 0 | 0 |
| Proline | P | 66 | 3 (4.5%) | 3 | 0 |
| Serine | S1 | 67 | 1 (1.5%) | 0 | 1 |
| Serine | S2 | 66 | 2 (3.0%) | 1 | 1 |
| Threonine | T | 68 | 1 (1.5%) | 1 | 0 |
| Tryptophan | W | 66 | 1 (1.5%) | 1 | 0 |
| Tyrosine | Y | 68 | 2 (2.9%) | 2 | 0 |
| Valine | V | 66 | 5 (7.6%) | 5 | 0 |

ribosomal RNAs, respectively, and 7.8% divergent overall (table 2). For the 22 tRNAs, differentiation varied between zero and 10.8%, averaging 3.7% (table 3). For all 37 coding genes, transitions exceeded transversions by a ratio of five to one.

Fourteen percent of the substitutions between *Allonautilus* and *N. macromphalus* in the protein-coding loci resulted in amino acid changes. Ten of the proteins used ATG start codons and three used GTG codons. These three GTG initiation codons were for the same three genes (ND3, ND4, and ND5) observed to have GTG starts in *Nautilus* (Boore, 2006).

There were 53 changes in tRNA sequences between the two nautilids. Approximately two-thirds of these were in loops or extra arms, and therefore had no effect on the secondary structure of the molecules. Two-thirds of the substitutions occurring within stem regions involved changes between A-U and G-U pairing in the RNA product; two transitions in an arginine tRNA stem compensated for each other.

BASE COMPOSITION

The *Allonautilus* genome was composed of 60.1% A+T bases and 39.9% G+C bases, but this varied considerably along the sequence, as was previously observed in *N. macromphalus*

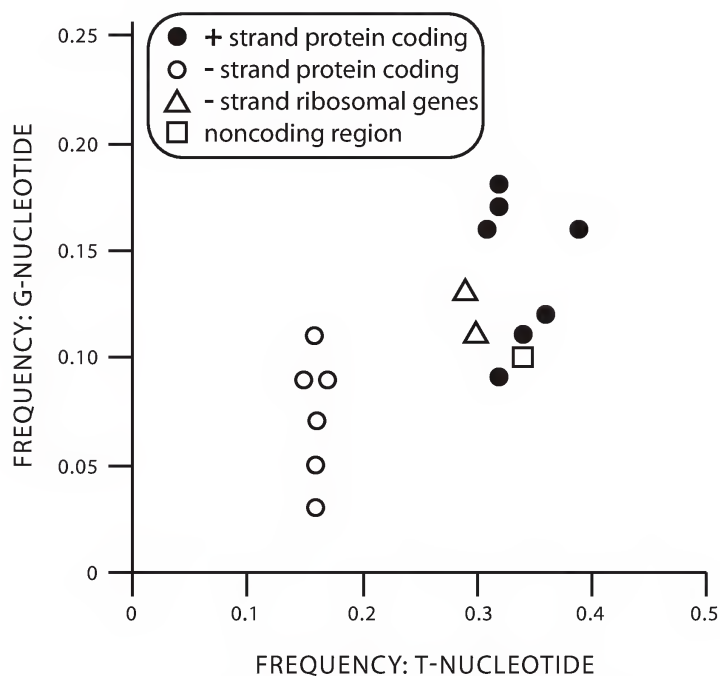


FIGURE 4. Base composition of the major genes (excluding tRNAs) in the mitogenome of *Allonautilus*. Plus-strand (+) defined as the coding strand for CO1.

(Boore, 2006). Protein-coding genes transcribed on the same strand as CO1 were substantially richer in T and G nucleotides than those on the other DNA strand (fig. 4). The two ribosomal genes had approximately the same T and G composition as the CO1-strand proteins, even though they coded on the other strand. The large noncoding region had base composition equivalent to the CO1 family of genes.

The percentage of T and G nucleotides also varied within the large noncoding region. They were approximately 20% and 10%, respectively, at the 5' end of the region, oscillated through the repeat section, and then diverged near the 3' end of the region (fig. 3). At one point there was a section of sequence, more than 100 bp in extent, with no G nucleotides and T content of 50%.

VARIATION WITHIN *ALLONAUTILUS*

We sequenced 80% or more of the large noncoding region of the mitogenome for three specimens of *Allonautilus*. Two individuals had four copies of a 62 bp repeat; the third individual had five copies. Additional base pair substitutions at five sites resulted in three haplotypes for the three specimens. The percentage of variable sites was 0.82% and nucleotide diversity was 0.0055.

EVOLUTIONARY RELATIONSHIPS

We inferred the phylogenetic relationships among *Allonautilus* and those cephalopods whose complete mitogenomes had been deposited in GenBank. The GenBank sequences

included representatives of the squids, bobtail squids, cuttlefish, octopi, and vampire squids, as well as of *Nautilus*; the GenBank accession numbers of the sequences used are available from the authors. Because the base composition of loci on opposite coding strands varied greatly (fig. 4) and there were many overlapping indels in the NAD genes, we prepared a Nexus file of the COX and ATPase genes coding on the same strand for our analysis (3843 bp). The PAUP* analysis resulted in a single most-parsimonious tree (fig. 5). The tree had a total length of 11,019 steps and a consistency index (excluding uninformative characters) of 0.3421.

An uncorrected matrix of percent sequence divergences (not shown) indicated that variation (for the five COX and ATPase loci) varied between approximately 6% and 34% among cephalopods, and between 34% and 39% between cephalopods and *Katharina*.

DISCUSSION

THE *ALLONAUTILUS* MITOGENOME

The *A. scrobiculatus* genome was found to have a gene order arrangement (fig. 2) identical to that of the previously sequenced *N. macromphalus*. Consequently, it appears that the novel arrangement reported for *N. macromphalus* may characterize the extant nautilids. There were a few length differences between the two genera in noncoding regions due to insertions or deletions; notably, a microsatellite as well as one of the repeats present in *N. macromphalus* were absent from the *Allonautilus* mitogenome. Overall sequence divergence between the two nautilids was approximately 8.3%.

VARIATION WITHIN *ALLONAUTILUS*

We observed a modest amount of genetic diversity in the large noncoding region among three individuals of *Allonautilus scrobiculatus* that we sampled. Because neutral variation is expected to scale approximately with population size (Kimura, 1983), this result suggests that substantial numbers of individuals of this species must exist, in spite of their limited geographic range. The census data available with regard to population sizes of these organisms is limited and ambiguous (Dunstan et al., 2011; Barord et al., 2014). Woodruff et al. (1987) documented genetic variation in *A. scrobiculatus* using an indirect electrophoretic approach that assays protein, rather than DNA, variation. Our results are consistent with that prior investigation. The presence of variation in the number of long (62 bp) repeats suggests that a simple PCR system could be designed to assay genetic diversity of populations in the species, using electrophoresis of PCR products, without the necessity for sequencing.

PHYLOGENY AND GENETIC DIVERGENCE

Our estimate of the phylogenetic relationships among those cephalopods whose complete mitogenomes are known (fig. 5) is largely concordant with prior results (e.g., Akasaki et al., 2006; Nishiguchi and Mapes, 2008; Allcock et al., 2011; Kawashima et al., 2013). Each of the ordinal-level taxa was monophyletic with moderate to high bootstrap support, and *Allonautilus* and *Nautilus* were united with 100% support. There was also support for some supraordinal relation-

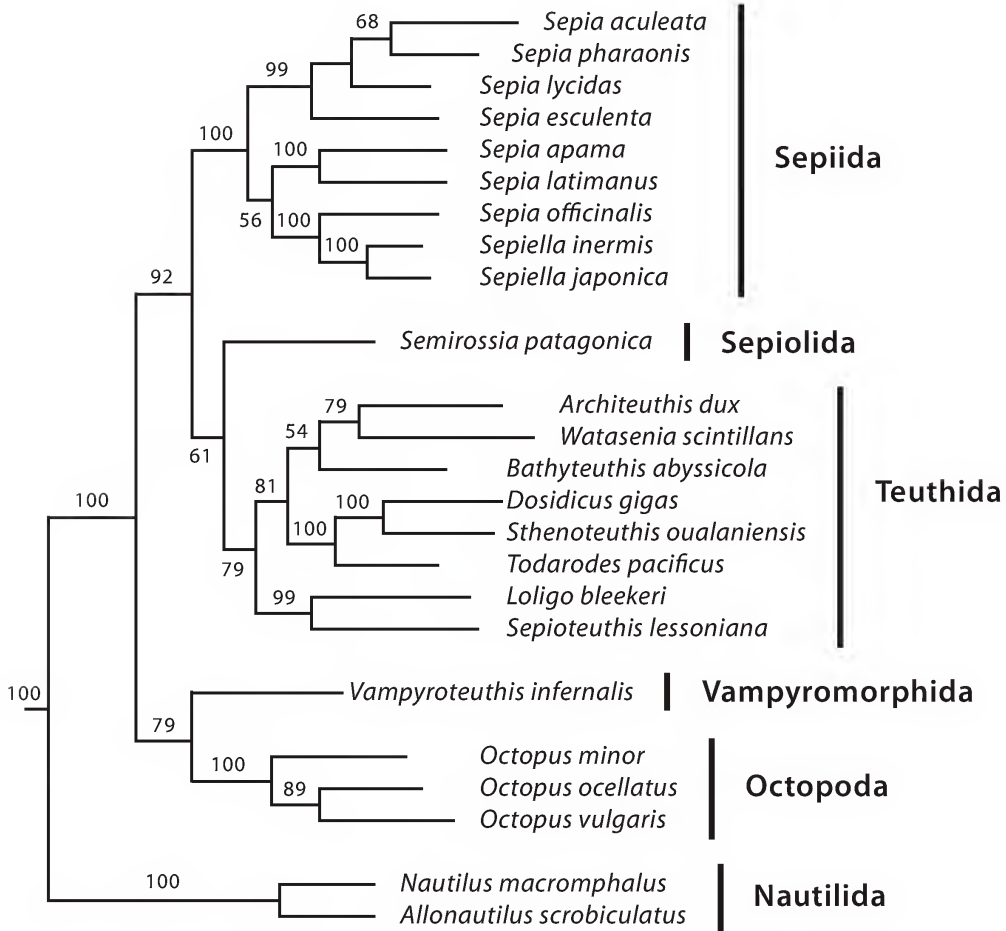


FIGURE 5. Phylogenetic relationships among some cephalopod species (and their orders) based on mitochondrial DNA sequences. Parsimony phylogram is based on COX and ATPase genes for cephalopod species whose mitogenomes have been sequenced; *Katharina tunicata* was used as an outgroup (not shown). Bootstrap values are shown along branches.

ships: Sepiida, Sepiolida, and Teuthida were united with 92% support; these comprise the traditional superorder Decapodiformes. The placement of Vampyromorphida as the sister taxon to the Octopoda was 79%; these two represent the traditional Octopodiformes. This latter relationship has been difficult to resolve in some prior molecular studies (e.g., Stöger and Schrödl, 2013). There was 100% support for the reciprocal monophyly of Coleoidea and Nautiloidea.

As is apparent from the phylogram shown in figure 5, there was less evolutionary divergence between *Allonautilus* and *Nautilus* than there was among species within the genus *Sepia* or species in the genus *Octopus*. In particular, for the relatively easily aligned COX and ATPase loci, the divergence between *Allonautilus* and *Nautilus* was 10.5%. Among three species of *Octopus*, divergences ranged between 14.2% and 16.1%; among 7 species of *Sepia*, divergences ranged between 11.4% and 18.8%. The only comparison in our study showing less divergence was that between the two species of *Sepiella*, 6.1%. Consequently, there was less divergence between the two genera of nautilids than between all but one pair of con-

genera in our survey, and all other intergeneric comparisons exceeded the nautilid divergence. Thus, the morphological divergence between the genera (fig. 1) is not accompanied by substantial mitochondrial differentiation, as previously noted by Woodruff et al. (1987) and Wray et al. (1995). If genetic divergence scales approximately linearly with time, then the divergence between several important coleoid genera predates the divergence between the two nautilid genera. Nuclear DNA sequences may provide an additional perspective on the consequent taxonomic issue.

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