Molecular Insights into the Calanus species (Crustacea; Copepoda; Calanoida) in the Southern Ocean

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Abstract

The differentiation of marine species continues to pose a challenge despite the growing number of diagnostic metrics and approaches. This study examined the genetic relationship between two sibling species of the genus Calanus (Crustacea; Copepoda; Calanoida), C. simillimus and C. propinquus. In course of the study small and large nuclear ribosomal subunits (18S rDNA and D1 region of 28S rDNA, respectively), nuclear protein-coding gene (histone H3), internal transcribed spacer 1 (ITS1) region and the mitochondrial ribosomal gene (16S), mitochondrial cytochrome c oxidase subunit I (COI), cytochrome B (cytB) for the samples from the Southern Ocean have been defined by means of DNA sequences. Our data offers an interesting insight into mitonuclear discordance in marine copepods, and the failure of traditional mitochondrial markers in separating two similar, albeit morphologically distinct, species. It can be explained by mitochondrial introgression and the presence of pseudogenes (numts).

1. Introduction

The taxonomic relationships of closely related species provide vital information for accurate assessment and preservation of marine biodiversity. However, identifying diagnostic characteristics for species identification and agreeing on exact delimitation of species boundaries is remaining challenging. Molecular phylogenetic analysis provides a reliable and independent mean to evaluate evolutionary and taxonomic relationships and examine species boundaries among closely related and cryptic species (Knowlton, 1993, 2000, Bickford et al., 2007). Molecular systematic and phylogenetic studies of marine zooplankton have resulted in the revision of many pelagic marine taxa (De Vargas et al., 1999, Suatoni et al., 2006), including copepods (Goetze 2003, Adamowicz et al., 2007, Kozol et al., 2012).

Antarctic zooplankton is represented by various groups, but Copepoda is dominant (Atkinson & Peck, 1988, Voronina et al., 1994, Atkinson & Sinclair, 2000). *Calanus propinquus* Brady, 1883 and *Calanus simillimus* Giesbrecht, 1902 are important species in copepod communities of the Southern Ocean (Atkinson, 1998, Atkinson et al., 2012). Although the species are morphologically similar and have comparable life histories, they differ greatly in their abundances depending on the environment. Although their distribution ranges overlap, *C. propinquus* mainly lives in polar seas of higher latitudes (Voronina & Maslennikov, 1993), while *C. simillimus* mainly lives in sub-Antarctic and Polar Frontal Zone (Atkinson & Sinclair, 2000). *Calanus simillimus* is a subantarctic Copepod of Atlantic origin and can be occasionally found in bodies of Atlantic water carried South of the Antarctic convergence. *Calanus propinquus* is a very typical copepod of the Southern epiplankton, but its occurrence is not entirely restricted to the zone enclosed by the Antarctic convergence.

Until 1902, they were considered to be the same species. Now according to morphology, different authors have no doubts that these are valid species. The main differences are conspicuous: *C. propinquus* is bigger than *C. simillimus*, and the structure of the inside of their fifth leg differs (Vervoort, 1951, Bradford-Grieve, 1994, Bradford-Grieve et al., 1999):

Calanus simillimus females vary in length from 2.5 - 3.97 mm (avg 3.235 mm), the males measure 2.62 - 3.42 mm (avg 3.020 mm) (Brun et al., 2017). The adult female has an internal margin of the 1st basal joint of 5th feet with 14-16 distinct, triangular teeth of nearly equal size, the basal 3 or 4 more or less separate from the rest.

Calanus propinquus females vary between 4.75 - 6 mm (avg 5.375 mm), the males between 4.75 - 5.3 mm (avg 5.025 mm) (Brun et al., 2017). The 5th feet, compared with *C. simillimus*, have differently arranged teeth along the inner edge of the 1st basal joint. Each joint has a curved row of 15 small teeth and a basal group of 3 much bigger teeth. *Calanus propinquus* is once recognized by the curious setae on the 1st antennae on all stages.

Apart from these differences, they are very similar, and their larvae are indistinguishable (Hunt & Hosie, 2006), and species identifications are frequently based on individual size and the geographical location of collection.

Active filter-feeding *C. simillimus* and *C. propinquus* have oil sacs that run the length of their body and extended antennae that ‘hang’ them head up in the water column. Both *Calanus* species contained triacylglycerols TAGs as the primary lipid store (Hagen et al., 1993, Pond, 2012). Both *C. simillimus* and *C. propinquus* may show pronounced seasonal vertical migrations (Atkinson & Sinclair, 2000) depending on the region or remain comparatively active throughout the winter (Schnack-Schiel et al., 1991, Atkinson, 1998). It is also possible that they can maintain some of their population in the surface waters in winter, while the remainder descend and possibly enter diapause (Atkinson, 1998).

There are very few molecular data for these Antarctic copepods. Totally there have been published 13 sequences for *C. propinquus*: COI = 9, 18S = 1, 28S = 3, and 28 sequences for C. *simillimus*: COI = 27, 28S = 1 (Bucklin et al., 2021). The morphological and genetic similarities between *C. propinquus* and *C. simillimus* raise new questions about the taxonomic status of *C. simillimus*. The studies of *Calanus* species based on previous DNA barcoding involving COI raised doubts about the status of *C. simillimus* (Fangping et al., 2014).

Analysis of multiple gene regions is critical for accurate resolution of species relationships. In this study, we analyzed the taxonomic relationship between two sibling species from the Southern Ocean, *C. propinquus* and *C. simillimus*, based on DNA sequences for portions of seven genes: mitochondrial cytochrome c oxidase subunit I (COI), cytochrome B (cytB), 16S ribosomal RNA (16S) and nuclear large (28S) and small (18S) subunits rRNA, nuclear ITS1-5.8S rDNA region (ITS1), histone H3 (H3).

2. Materials And Methods

Sample collection

The samples were collected along the Eastern (SR2) and Western (The Drake Passage) sections of the Atlantic sector of the Southern Ocean (Fig. 1, Table 1).

Each sample was collected with a Juday net from the water layer 0-300 m. Immediately after collection, specimens of *Calanus* spp. were isolated from the samples. The selected animals were fixed in their entirety in 96%

ethanol; a day later, the alcohol in the test tubes with the animals was completely renewed in accordance with the accepted method (Bucklin, 2000). Alcohol samples were stored at -20°C before DNA extraction.

The specimens were identified by the morphological traits (Bradford-Grieve, 1994, Bradford-Grieve et al., 1999). Ensuring the accuracy of identification, sexually mature female individuals were chosen for the experiment, and examined for differences in the fifth legs. In total, 31 individuals of *C. propinquus* and 34 individuals of *C. simillimus* were analyzed (Table 1).

DNA Amplification and Sequencing

Promega Wizard SV Genomic DNA Purification Kit (Promega Corporation, Madison, (USA) was used for lysis and DNA purification following the manufacturer's protocol. DNA sequences for portions of seven fragments (Table 2): other than 16S rDNA and cytochrome oxidase I (COI), cytochrome B, internal transcribed spacer 1 (ITS1), and nuclear 28S rRNA gene, the nuclear 18S rDNA, histone H3 have also been analyzed. The 18S rDNA gene was PCR amplified in three overlapping fragments of about 950, 900 and 850 bp each, using primer pairs 1F-5R, 3F-18Sbi and 18Sa2.0-9R, respectively (see Table 2). Amplifications of the D1 region of 28S, 16S and Histone H3 yielded fragments of approximately 385, 371 and 315 bp, respectively. Loci were amplified using Ready-To-Go PCR Beads (Amersham Pharmacia Biotech, Piscataway, New Jersey). Each 25 µL reaction contained 1 µL of 10 µm of primer pair mix, 1 µL of template and 23 µL of water. Reactions mixtures were heated to 94 °C for 90 s, followed by 35 cycles of 40 s at 94 °C, 40 s at a specific temperature and 45 s at 72 °C, and then a final extension of 7 min at 72 °C on Eppendorf Mastercyclers. Annealing temperature was set to 49 °C for the 18S primer pairs 1F-5R and 18Sa2.0-9R, 52 °C for the 18S primer pair 3F-18Sbi and for the 28S primer pair C1-C2, 45 °C for the 16S primer pair CB and CA and 53 °C for Histone H3 primer pair H3af and H3ar. Amplification products were sequenced in both directions.

Sequences have been processed and aligned in Codone Code Aligner package (Codon Code Corporation, Dedham, Massachusetts). BLAST (Highly similar sequences) match values of CO1 and 16S markers of *Calanus simillimus* from this study were more than 99% with *C. simillimus* and 97% with *C. propinquus* with query Cover 97-100%. There were the same BLAST results for *Calanus propinnquus* mitochondrion markers.

Multiple alignment of the obtained sequences was performed using the ClustalW program (Wang & Jiang, 1994). The trees were constructed using MrBayes 3.2.6 (posterior probability, chain length 2 100 000, G=4, 4 heated chains, hcTemp 0.2, subsampling freq. 200, burn-in length 100 000) and RaxML (with rapid bootstrapping and search for best scouring ML tree, 1000 bootstrap replicates). The topologies of the trees obtained by both methods were identical. Distances of evolutionary divergence K2P were calculated using the Kimura 2 parameters model (Kimura 1980). Evolutionary analyses were conducted in MEGA11 (Tamura et al., 2021).

3. Results

A total of 65 individuals of *Calanus* spp. were analyzed (Table 2): 16S rDNA and cytochrome oxidase I (COI), cytochrome B (cytB), internal transcribed spacer (ITS1), nuclear 28S rRNA, the nuclear 18S rDNA, histone H3.

Mitochondrial DNA

A total of 34 COI sequences for *C. simillimus* and 31 sequences for *C. propinquus* were analyzed, the specimens were collected in different hydrological zones of the South Atlantic (Table 1). In the subsequent analysis, the sequences of the same region of mitochondrial DNA of *C. simillimus* individuals containing in the GenBank NCBI database were additionally used, including individuals from the southwestern Pacific Ocean (GenBank: AF332771) (Hill et al., 2001), from the polar frontal zone of the Drake Passage (KC754439) and from the Indian Ocean: south of The Kerguelen Islands (OK500294) as well as *C. propinquus* individuals from the polar frontal zone of the Drake Passage (KC754440, KC754438), and from the Antarctic zone of the Drake Passage (KC754431 - KC754437). Two clusters are clearly distinguished on the cladogram, the homology coefficient between them is 98%. One of the clades consisted of *C. propinquus* entirely, while the second clade included both species. The homology coefficient between the two species in the second clade reaches 100%. As a result of the joint analysis of the obtained sequences for copepods *C. simillimus* and *C. propinquus*, the presence of a large number of identical haplotypes was established, these haplotypes are carried by individuals collected in the western and eastern parts of the Atlantic sector of the Southern Ocean. During the analysis of the genetic variability of the entire population of the studied individuals of *C. simillimus* and *C. propinquus*, the predominant haplotype conventionally called “haplotype A” was identified, it is possessed by 50 out of 76 individuals. Part of the individuals from the GenBank database also belong to this haplotype. On all cladograms, these individuals are marked in bold.

The cladogram constructed by deleting the individuals with “haplotype A” from the original cladogram (Fig. 2 A) is shown in Fig. 2 B. This cladogram clearly shows the presence of two closely related clades with an intraspecific polymorphism of 0-0.8%. The genetic distance between species was 1.9-3.3%.

All substitutions relative to “haplotype A” in *C. simillimus* are synonymous, except for C53, the substitution of valine (V) for glycine (G).

A similar situation is revealed with the CytB fragment (fig. 3), namely, a clade is formed that combines both species, and a clade with *C. propinquus* is singled out separately, while between clades there are 11 substitutions for 310 bp.

Noteworthy is a separate clade for both COI and CytB, which combines two individuals of *C. propinquus* A75 (Antarctic zone of the Drake Passage) and A96 (Weddell Sea). These two copies are identical to "haplotype A" except for a deletion of 120 nucleotides. COI sequencing revealed that two samples of *C. propinquus* (A75, A96) are lacking a DNA fragment encoding a 44 amino acid protein sequence (fig. 5). Moreover calculation of conservation score for *C. simillimus* CO1 using ConSurf server (Ashkenazy et al., 2016) showed that many of these 44 amino acids are among the most conserved in cytochrome oxidase (fig. 4). The missing fragment corresponds to a loop and two alpha-helices buried in the hydrophobic core of cytochrome oxidase. High conservation of the deleted sequence suggests that the protein without it is either non-functional or has a drastically compromised function, e.g. lacking proton pumping activity.

A completely different result was obtained when analyzing the 16S rDNA fragment (fig. 5). If the data on COI and cytB confirm the identity of mitochondrial DNA of some individuals of *C. simillimus* and *C. propinquus*, then the 16S data do not confirm this – the sample with “haplotype A” and the rest fell into one clade. At the same time, our samples did not unite with a sample from the GenBank database, namely, with *C. simillimus*, for which the entire mitochondrial DNA was sequenced, although COI and cytB completely coincided.

Nuclear DNA

At nuclear DNA (18S, 28S, ITS1, H3) there is very little genetic variation within each species, observed only in the histone-3 (fig. 6). Between the species in the studied fragment, 18S 2 substitutions for 1344 bp, 2 substitutions for 385 bp for 28S, and 2 substitutions for 673 bp for ITS1. Thus, for each of the loci, there are significant differences between the species, confirming that *C. simillimus* and *C. propinquus* are, indeed, distinct, good species. There is not a single case of different species falling into the same clade. And individuals with “haplotype A” and with substitutions are reliably combined.

4. Discussion

As an order, calanoid copepods include numerous groups of sibling species; they are among the most species-rich holoplanktonic invertebrates in the oceans (McGowan, 1971). The genus *Calanus* has been intensely studied because of its ecological importance. The genus *Calanus* comprises 14 species, including 11 that have been assorted into two species groups: the finmarchicus group (*C. finmarchicus*, *C. glacialis*, *C. marshallae*) and the helgolandicus group (*C. helgolandicus*, *C. agulhensis*, *C. australis*, *C. chilensis*, *C. euxinus*, *C. jashnovi*, *C. pacificus*, *C. sinicus*), as well as three ungrouped species (*C. hyperboreus*, *C. simillimus* and *C. propinquus* (Frost, 1974, Bradford, 1988, Hill et al., 2001).

Several disputes about species division of the genus have arisen. *Calanus helgolandicus* and *C. euxinus* are probably the same species, based on studies of the COI gene (Unal et al., 2006) and 16S rRNA (Yebra et a., 2011). The status of *C. agulhensis* has also been questioned because of its genetic similarity to *C. sinicus*. Kozol et al. (2012) surveyed genetic differentiation among individuals of two species from different populations using COI, 18S rRNA, and 28S rRNA, and found that genetic differentiation in these gene sequences was small. The results indicated that *C. agulhensis* and *C. sinicus* might be the same species. Therefore, low interspecific genetic differentiation between species of *Calanus* might be common. Based on previous DNA barcoding studies involving COI of *Calanus* species showed doubts about the status of *C. simillimus* (Cheng et al., 2013).

DNA barcoding uses mitochondrial cytochrome c oxidase subunit I (COI) gene sequences to identify species (Hebert et al., 2003). For marine copepods, nucleotide sequences of the mitochondrial 16S rRNA gene have been used most extensively to reveal intraspecific or interspecific variations (Bucklin et al., 1995, 1998, Braga et al., 1999). But in many cases, for example, for mollucks, many crustaceans, etc., there is a problem of mitochondrial pseudogenes (Richly & Leister, 2004). Indeed, early papers by Bucklin et al. have reported variation in both the mitochondrial COI gene (Bucklin et al., 1999) and, subsequently, the nuclear pseudogene copy (Bucklin et al., 2000) using the same PCR primers. As other authors have suggested that they have studied a bona fide mitochondrial COI gene in other copepods within both the genera *Calanus* and *Neocalanus* (Papadopoulos et al., 2005, Kirby et al., 2007) and thus the situation still lacks clarity, we decided instead to use the CYTB region, which, while exhibiting less variation than COI, has still been shown to provide sufficient resolution for intraspecific and intrapopulation studies (Papadopoulos et al., 2005).

In our work, we encountered two phenomena at once. But first, it should be noted that, based on four nuclear fragments and morphology, we obtained evidence of the isolation of two closely related species, *C. simillimus* and *C. propinquus*. Why are these species not confirmed when using mitochondrial fragments, all three in our case, namely COI, cyt B and 16S? We propose the following explanation: here two phenomena intervened at once, disrupting the phylogeny according to the usual mitochondrial markers.

1. Mitochondrial introgression is the presence of the mitochondrial genome of one species (donor) in a population of another species (recipient). A similar phenomenon occurs as a result of interspecific hybridization and subsequent backcrosses. Introgression of foreign mtDNA as a result of hybridization is one of the main causes of paraphyly or polyphyly, found in 23% of animals in the analysis of 2319 phylogenetic trees of individual species (Funk & Omland, 2003). Mitochondrial introgression explains abnormalities in COI cases and cyt B. Mitochondrial genomes of individuals with «haplotype A» from both species are identical to each other, indicating a recent introgression. The absence of a frameshift and stop codons indirectly indicates that in the case of COI and cytB these are no numts.
2. Pseudogenes are non-functional analogues of structural genes that have lost the ability to encode protein and are not expressed in the cell (Vanin, 1985). Pseudogenes of mitochondrial origin (NUMTs - nuclear DNA sequences of mitochondrial origin) are found almost in all mitochondrial genomes from yeast to humans (Bensasson et al., 2001). Their occurrence is explained by the capture of mtDNA fragments and their integration into nuclear chromosomes in the event of double-strand breaks in the latter (Ricchetti et al., 2004, Leister, 2005) numts seem to be especially common in marine crustaceans (Williams & Knowlton, 2001, Buhay, 2009, Schubart, 2009). Sequencing of the pseudogene instead of the 16S mitochondrial region explains the location of both species in the same clade. Also, the large difference from the entire mitochondrial sequence published in Genbank confirms our pseudogene hypothesis. But detection of numts derived from mitochondrial ribosomal RNA (rRNA) or transfer RNA (tRNA) genes is more challenging, since numts may be identified only by changes in the inferred secondary structure of the transcribed RNA (Zhang & Hewitt, 1996).

Mitochondrial DNA is useful for evolutionary studies and for reconstructing the phylogenetic relationships of copepods, which is challenging due to the morphological complexity of their taxonomic features (Blanco-Bercial et al., 2011). Based on studied mitochondrial genome *Calanus* spp. (Kim et al., 2013, Minxiao et al., 2011, Weydmann et al., 2017) genomes exhibit some unusual features, including the longest control region reported for a crustacean, a large tRNA gene cluster, and reversed GC (guanine-cytosine content) skews in most of the protein-coding genes, and the concurrence of multiple non-coding regions and a reshuffled gene

arrangement. Due to these unusual characters, the conservation of mitochondrial gene order in copepods has been questioned or adapted to harsh environments by mitogenome rearrangements.

Thus, our results illustrate that mitochondrial DNA alone is not sufficient and may lead to wrong results to identify species of marine invertebrates. Our results resolve the issue of the taxonomic status of *C. simillimus*.

Declarations

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Data Availability Statement: Nucleotide sequences are submitted in GenBank https://www.ncbi.nlm.nih.gov/ at numbers ON645225-ON645226, ON641072-ON641085, ON637122-ON637133, ON637066-ON637103, ON631753-ON631764, ON646048-ON646076, ON705020-ON705023.

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Tables

Table 1 is available in the Supplementary Files section.

Table 2. PCR primers used in amplification and sequencing

Loci	Number	Final length	Outgroup	Primer name	Primer sequence	Reference
CO1	76	479 bp	*Calanoides carinatus* MH159133	LCOI 1490	GGTCAACAAATCATAAAGATATTGG	(Folmer et al., 1994)
				HCOI 2198	TAAACTTCAGGGTGACCAAAAAATCA	
ITS1	14	673 bp	*Cosmocalanus darwinii* KT389867	LR1	GGTTGGTTTCTTTTCCT	(Gardes, & Bruns, 1993)
				SR6R	AAGWAAAAGTCGTAACAAGG	
16S	13	371 bp	*Calanoides carinatus* FR849642	CB	ATTCAACATCGAGGTCACAA	(Bucklin et al., 2000)
				CA	TGTTAAGGTAGCATAGTAAT	
Cyt b	19	323 bp	*Acartia tonsa* MH710532	cytb424	GGWTAYGTWYTWCCWTGRGGWCARAT	(Staton et al., 2005)
				cytb876	GCRTAWGCRAAWARRAARTAYCAYTCWG	
H3	11	315 bp	*Calocalanus pavo* JQ911997	H3af	ATGGCTCGTACCAAGCAGACVGC	(Colgan et al., 1998)
				H3ar	ATATCCTTRGGCATRATRGTGAC	
28S	7	385 bp	*Calanoides carinatus* AF385465	C1	ACCCGCTGAATTTAAGCAT	(Vân et al., 1993)
				C2	TGAACTCTCTCTTCAAAGTTCTTTTC	
18S	12	1344 bp	*Calanoides carinatus* GU969155	1F	TGTAAAACGACGGCCAGTTACCTGGTTGATCCTGCCAGTG	(Giribet et al., 1996)
				5R	CAGGAAACAGCTATGACC TTGGCAAATGCTTTCGC	
				2.0	TGTAAAACGACGGCCAGT ATGGTTGCAAAGCTGAAA	
				9R	CAGGAAACAGCTATGACGATCCTTCCGCAGGTTCACCTAC	
				3f	TGTAAAACGACGGCCAGT GTTCGATTCCGGAGAGGGA	
				bi	CAGGAAACAGCTATGACGGT CTCGTTCGTTATCGGA	

Figures

Figure 1

Geographic locations of the samples analyzed for this study.

● – samples from R/V Akademik Ioffe 30 voyage (December 2009 - January 2010)

◊ – samples from R/V Akademik Sergey Vavilov 31 voyage (October-November 2010)

△ – samples from GenBank

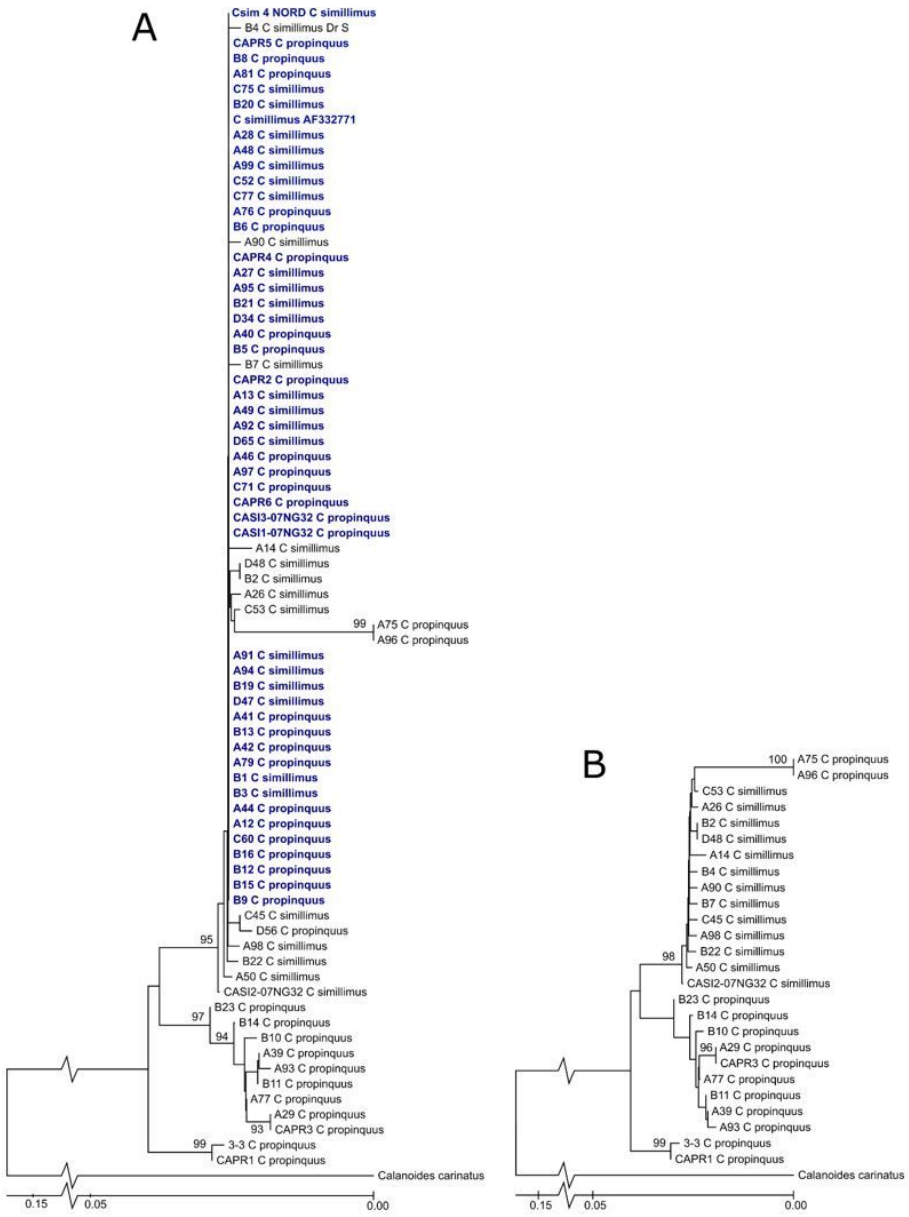

Figure 2

Maximum likelihood tree based on CO1 sequence variation showing relationships among *Calanus*spp., with GenBank sequences AF332771, OK500294, KC754431 - KC75440 included for comparison. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown above the branches, only values greater than 90 are shown. Sequences belonging to the same haplotype (haplotype A) are marked in blue. A - general cladogram, B - except for "haplotype A".

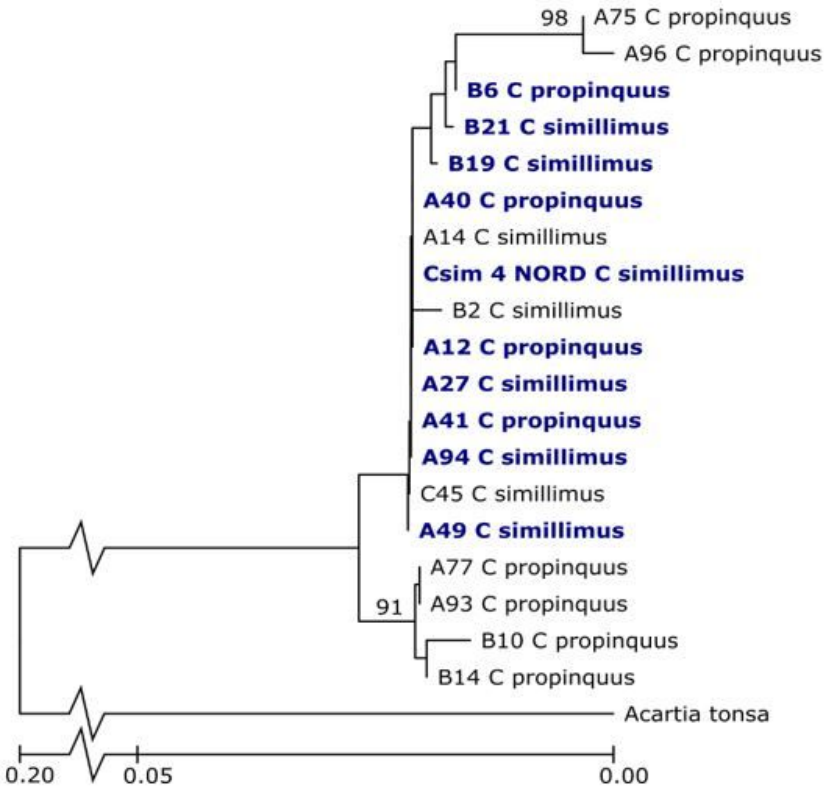

Figure 3

Maximum likelihood tree based on CytB sequence variation showing relationships among Calanus spp., with GenBank sequence OK500294 included for comparison. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown above the branches, only values greater than 90 are shown. Sequences belonging to "haplotype A" for CO1 (fig. 2A) are marked in blue.

```
Haplotype A      VTAHAFIMIFFMVMPILIGGFGNWLVPLMLGAADMAFPRMNNMSFWFLMPALIMLLSSSLVESGAGTGWTVYPPLSSNIAHAGASVDFAIFSLHLAGVSSILGAVNFISTLGNLRVFGMLMDRMPLFAWAVLITAVLLLLSLPVLAGAITMLLTD
A75 C propinquus VTAPSMD----------------------------------------LMPALIMLLSSSLVESGAGTGWTVYPPLSSNIAHAGASVDFAIFSLHLAGVSSILGAVNFISTLGNLRVFGMLMDRMPLFAWAVLITAVLLLLSLPVLAGAITMLLTD
A96 C propinquus VTAPSMD----------------------------------------LMPALIMLLSSSLVESGAGTGWTVYPPLSSNIAHAGASVDFAIFSLHLAGVSSILGAVNFISTLGNLRVFGMLMDRMPLFAWAVLITAVLLLLSLPVLAGAITMLLTD
```

Figure 4

Conservation of amino acids in cytochrome oxidase of *C. simillimus* CO1 sequence (UPP55814). Missing fragment of *C. propinquus* (A75, A96) samples is between position 57-101. ConSurf output: b - A buried residue according to the neural-network algorithm. f - A predicted functional residue (highly conserved and exposed). s - A predicted structural residue (highly conserved and buried).

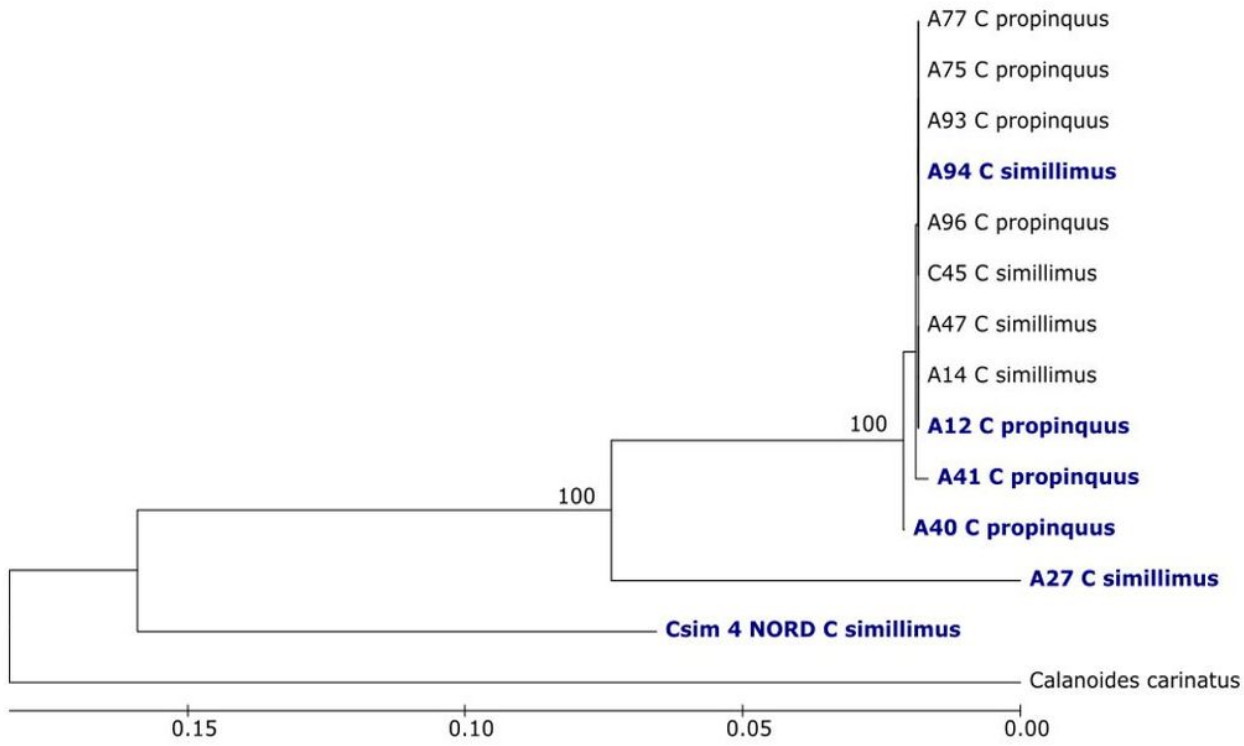

Figure 5

Maximum likelihood tree based on 16S sequence variation showing relationships among *Calanus* spp., with GenBank sequence OK500294 included for comparison. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown above the branches, only values greater than 90 are shown. Sequences belonging to "haplotype A" for CO1 (fig. 2A) are marked in blue.

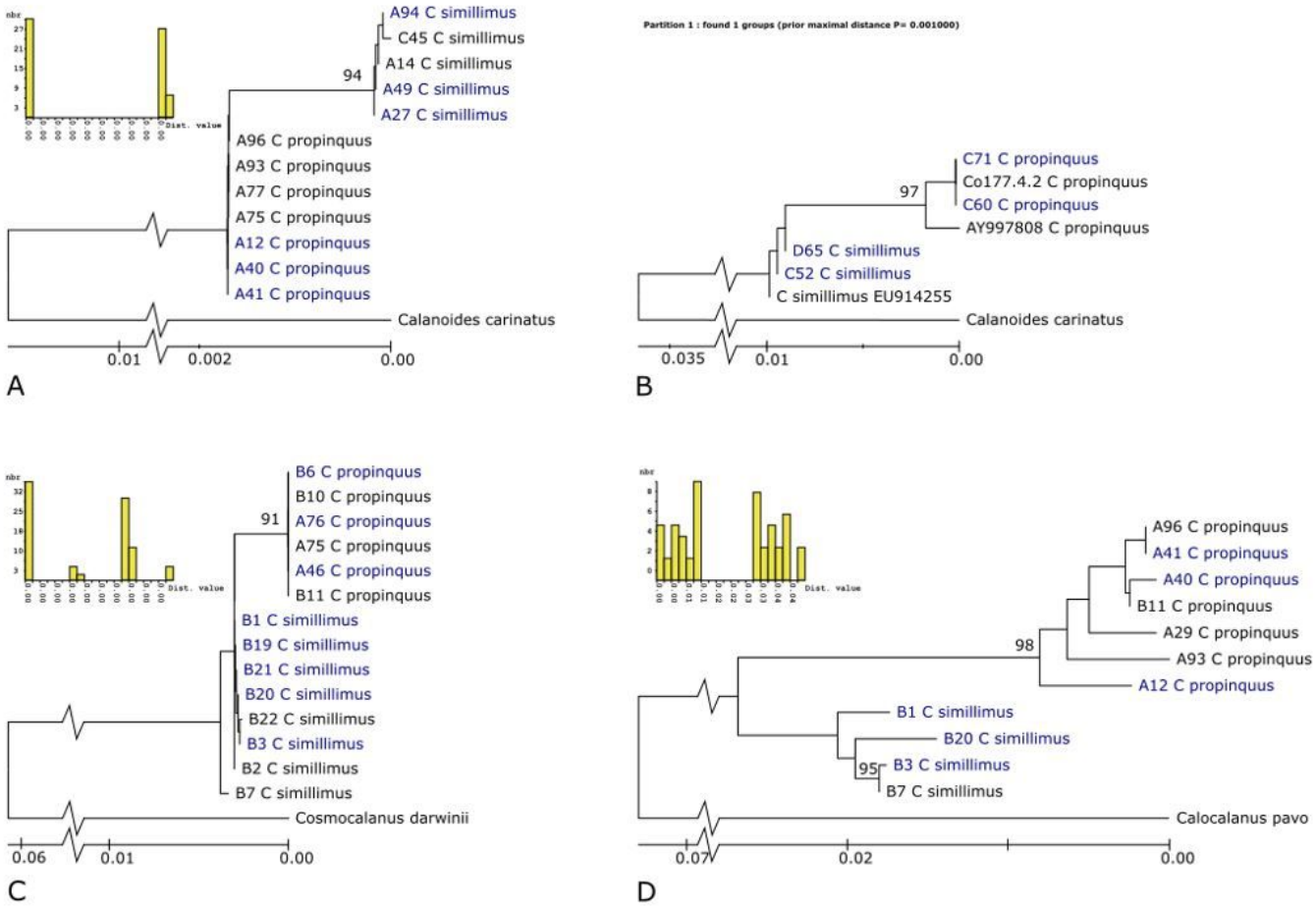

Figure 6

Maximum likelihood tree based on sequence variation showing relationships among *Calanus*spp.: A – nuclear small subunit rRNA (18S), B – nuclear large subunit rRNA (28S), C – internal transcribed spacer 1 rDNA region (ITS1), D – histone H3 (H3). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown above the branches, only values greater than 90 are shown. Sequences belonging to "haplotype A" for CO1 (fig. 2A) are marked in blue. Histograms show barcode gaps (Puillandre et al., 2012).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Table1.docx