

Cryptic mitochondrial lineages in the widespread pycnogonid *Colossendeis megalonyx* Hoek, 1881 from Antarctic and Subantarctic waters

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Abstract *Colossendeis megalonyx* Hoek, 1881 is a widespread and abundant pycnogonid in the Southern Ocean which has also been reported from the South Atlantic and South Pacific Oceans. Its strictly benthic lifestyle is expected to promote genetic differentiation among populations and ultimately facilitate speciation. On the other hand, the reported eurybathy and unknown larval stages of this species may allow *Colossendeis megalonyx* to maintain genetic continuity between isolated shallow-water habitats by active dispersal through the deep sea or by passive rafting on floating substrates. Thus, it remains unknown whether and to which extent geographically separated populations of *Colossendeis megalonyx* maintain gene flow in the Southern Ocean. We sampled 96 specimens of *Colossendeis megalonyx* from three stations in the Atlantic Sector of the Southern Ocean and one station from the South American continental shelf (Burdwood Bank). The genetic structure of nominal *Colossendeis megalonyx* as well as its phylogenetic position within the genus *Colossendeis* were assessed using a fragment of the cytochrome c oxidase subunit 1 gene. Our data strongly support that nominal *Colossendeis megalonyx* consists of at least five cryptic and one pseudocryptic mitochondrial lineages, four of which appear to be geographically restricted. Two lineages occurred at locations separated by more than

1,000 km in the Antarctic, thus indicating high levels of gene flow or recent colonization. No haplotype sharing across the Polar Frontal Zone was observed. Our results strongly suggest that cryptic speciation occurred within the genus *Colossendeis*. The wide biogeographic distribution range of *Colossendeis megalonyx* and perhaps that of other Antarctic pycnogonids should therefore be regarded with caution.

Keywords Antarctic benthos · Pycnogonida · Circumpolar distribution · Cryptic species · Phylogeography · Deep sea · Colossendeidae

Introduction

The Southern Ocean reveals a species-rich benthic fauna with high degrees of local endemism (Arntz et al. 1994; Brandt et al. 2007; Clarke and Johnston 2003; Gutt et al. 2004; Knox 1994). The high biodiversity is the result of the unique evolutionary history of this ecosystem, being isolated for a period of over 20 million years (Clarke and Crame 1992; Clarke et al. 2004; Crame 1997; Poulin et al. 2002). Several taxa show a particularly high diversity in terms of species number. One of the most prominent examples among the higher taxa is the Pycnogonida. Recent surveys reported 264 species in the Southern Ocean, accounting for 19.6% of the 1,344 species recorded worldwide (Munilla and Soler Membrives 2008). Of these 264 species, 233 (88.3%) are endemic to Antarctic and/or Subantarctic waters (Munilla and Soler Membrives 2008). In the last decade, several molecular genetic studies in the marine Antarctic benthos provided strong evidence that Antarctic biodiversity is severely underestimated due to the presence of multiple morphologically highly similar lineages that most likely represent cryptic species (Allcock

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et al. 1997; Held 2003; Held and Wägele 2005; Hunter and Halanych 2008; Leese and Held 2008; Linse et al. 2007; Raupach and Wägele 2006; Raupach et al. 2007; Thornhill et al. 2008; Wilson et al. 2007, Wilson et al. 2009). A first study on the population structure of *Nymphon australe*, an Antarctic pycnogonid, for which a circumpolar distribution is assumed, revealed that several cryptic lineages co-occur in this taxon, which are likely to represent cryptic species (Mahon et al. 2008). Hence, the underestimation of species diversity appears to be present for Antarctic pycnogonids, too. More importantly, however, the discovery of cryptic species complexes may alter the current view of species' realized distribution ranges. For instance, species with a reported circumpolar distribution may in fact consist of a complex of cryptic species with smaller para- or even allopatric distribution ranges. Yet, no further genetic studies have tested whether cryptic species also occur in other pycnogonids, outlining the importance to investigate the genetic structure of other such taxa. In the Southern Ocean, the particularly species-rich pycnogonid family Colossendeidae Jarzynski, 1870 needs to be evaluated for the presence of cryptic diversity. As very little information on the mode of reproduction and larval development exist for members of the Colossendeidae (Arango pers. comm; Krapp pers. comm.; see Bain 2003) it is difficult to predict a trend for the genetic structure of colossendeids in this region. For several Antarctic pycnogonids, a characteristic egg-carrying behaviour of the males has been reported, which is sometimes referred to as "brooding" (see e.g. Mahon et al. 2008). For *Colossendeis* species this egg-carrying behaviour is also common (e.g. Lehmann et al. 2007), however, the post-egg development of these specimens remains unknown. If no free-swimming larval stages exist, it should generally be expected that gene flow is reduced geographically and strong genetic differences according to an isolation-by-distance model are expected (but see Hunter and Halanych 2008 for a counter example). However, several pycnogonids are reported to have an eurybathic distribution (see e.g. Bamber and El Nagar 2009; Munilla and Soler Membrives 2008) so that stretches of deep sea may represent less of a barrier to gene flow than they are to species that occur in shallow water only. Other pycnogonids, e.g. members of the genera *Anoplodactylus* Wilson, 1878 and *Bathypallenopsis* Stock, 1975, can drift passively with bathypelagic scyphomedusae and thus achieve an extremely broad distribution (see Child and Harbinson 1986; Hedgpeth 1962; Pages et al. 2007). In this respect, dispersal by means of the strong Antarctic Circumpolar Current (ACC) that transports water from the west to the east with high velocities ($20\text{--}40\text{ cm s}^{-1}$, Hofmann 1985; Whitworth et al. 1982) cannot be neglected and might be found to have a strong influence on population structure in the pycnogonids (see Helmuth et al. 1994;

Hunter and Halanych 2008; Leese et al. 2009 for examples outside the Pycnogonida). In summary, due to the lack of knowledge regarding the reproductive mode prevailing in the family Colossendeidae, it is difficult to make predictions about the population genetic structuring of species contained therein.

The aim of this study is to analyse the geographic partitioning of genetic polymorphisms within the pycnogonid *Colossendeis megalonyx* Hoek, 1881 using a mitochondrial DNA marker. According to the current literature, *Colossendeis megalonyx* is regarded as an abundant, eurybathic and circumpolarly distributed pycnogonid species with records inside and outside the Antarctic (Fry and Hedgpeth 1969; Munilla and Soler Membrives 2008). In the context of recent reports on cryptic species within several benthic taxa from the Southern Ocean, we test whether there is evidence for the occurrence of cryptic species in *Colossendeis megalonyx*.

Materials and methods

Sampling

Specimens of *Colossendeis megalonyx* ($n = 96$) as well as other pantopod species ($n = 19$) were collected during two cruises to the Antarctic and Subantarctic. For *C. megalonyx*, collection sites were Burdwood Bank ($n = 15$), the South Sandwich Islands ($n = 10$) and Bouvet Island ($n = 53$) during the ICEFISH 2004 Cruise on the *R/V Nathaniel B. Palmer* and from the tip of the Antarctic Peninsula (Elephant Island; $n = 18$) during the expedition ANT XIV/2 of *R/V Polarstern* (Kattner 1998) (Fig. 1). Species were sampled using Agassiz, Blake or Otter trawls. The depth of sampled sites ranged from 75 to 648 m (Table 1). All samples were immediately fixed in ice-cold ethanol (96%). Information on the other pantopod specimens analysed are listed in Table 1.

Species determination

Pycnogonid specimens were inspected by light microscopy and species were identified using the keys of Fry and Hedgpeth (1969), Pushkin (1993) and Child (1995). Species determination was kindly verified by Franz Krapp (ZFMK Bonn, Germany). Six specimens were deposited in the Zoological Museum Hamburg, Germany.

DNA extraction, PCR and sequencing

Muscle tissue was extracted from the tibia using sterile scalpels and forceps. DNA was isolated from the tissue using the Qiagen QIAamp[®] DNA mini Kit according to the

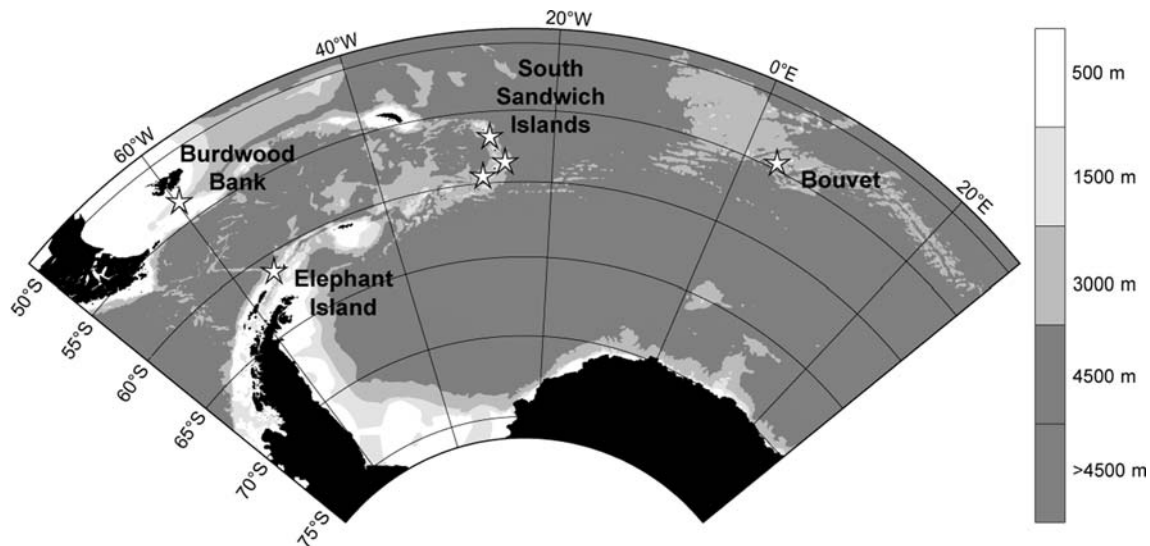


Fig. 1 Sampling sites of *Colossendeis megalonyx*

manufacturer's instructions. Amplification of an 658-bp fragment of the cytochrom c oxidase I (COI) gene was carried out in 25 μ l reactions containing 1 \times Eurotaq PCR buffer, 0.2 mM dNTPs, 0.5 μ M of each primer: LCO1490 5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3' and HCO2198 5'-TAA ACT TCA GGG TGA CCA AAA AAT CA-3' (Folmer et al. 1994), 1 μ l of DNA template, 2.5 mM MgCl₂, 0.5 U Eurotaq (Biotac), filled up to 25 μ l with sterile H₂O. The PCR temperature profile for the COI amplification was: initial denaturation at 94°C, 2 min; 36 cycles of denaturation at 94°C, 20 s, annealing at 46–50°C, 30 s, extension at 72°C, 80 s; final extension at 72°C, 7 min. PCR products were purified using the ExoSAP procedure (Hanke and Wink 1994), using 20 U *ExoI* and 4 U *SAP* (both Fermentas) and an incubation of 30 min at 37°C followed by inactivation at 80°C for 15 min for 22 μ l PCR product. Purified PCR products were bidirectionally sequenced by Macrogen (Seoul, South Korea) and GATC-Biotech (Konstanz, Germany). A subset of samples was sequenced at the University of Guelph, Canada (Barcode of Life) as part of the Census of Antarctic Marine Life (CAML) project.

Analyses

Four pycnogonid species outside the family Colossendeidae (*Nymphon australe*, *Pallenopsis* sp., *Austropallene cornigera*, *Ammothea* sp.), collected at various sampling sites during the ICEFISH 2004 Cruise, were used as outgroup species (Table 1). For the ingroup, sequences of 111 colossendeid specimens were analysed. Sequences were aligned with MUSCLE version 3.6 (Edgar 2004) using the user interface of GENEIOUS 4.6.2 (Biomatters 2005–2008). Alignment of in- and outgroup sequences was trivial

because there were no indels. For the phylogenetic analysis, the alignment of sequences for the 115 specimens was collapsed to a set of distinct haplotype sequences using the software fabox (Villesen 2007).

MEGA version 4.0 (Tamura et al. 2007) was used to calculate descriptive statistics. MRBAYES version 3.1.2 (Huelsenbeck and Ronquist 2001) was employed to estimate a phylogenetic tree. This analysis was conducted by computing 5×10^6 Markov Chain Monte Carlo generations in two parallel runs. Trees were sampled every 100 generations. Convergence was determined for both runs and the first 100 trees were discarded as burnin. A suitable substitution models for the Bayesian analysis was chosen with MRMODELTEST version 2.3 (Nylander 2004). Following the advise in the manual of MrBayes, only the model, but not the parameter values were fixed. Furthermore, Paup 4.b10 (Swofford 2002) was used to calculate a Maximum Parsimony bootstrap tree (1,000 replicates). Alignments and the phylogenetic tree were deposited in TreeBase (www.treebase.org).

TCS version 1.21 (Clement et al. 2000) was used to create a statistical parsimony network for the *Colossendeis megalonyx* specimens with a parsimony connection limit of 95 and 90%. To obtain a single parsimony network the maximum number of connection steps was raised to 49.

Results

Sequences from a fragment of the COI gene were obtained for 115 specimens of Antarctic pycnogonids, resulting in a 561 bp alignment with 41 distinct haplotypes (GenBank Accession numbers GQ386988–GQ387028, Table 1). The fragment was AT-rich with average base pair frequencies

Table 1 Specimens, sampling sites, geographical position, depth information and sampling gear utilised for the colossendeid species analysed in this study

Specimen	Location	Latitude	Longitude	Depth [m]	Gear	Haplotype	Clade	GenBank
<i>Colossendeis megalonyx</i> SSI_A5	South Sandwich	57°03'S	26°44'W	130	Blake Trawl	<i>C. meg.</i> HT03	A	GQ387009
<i>Colossendeis megalonyx</i> EI_N7	Elephant Island	61°46'S	57°32'W	343	Agassiz Trawl	<i>C. meg.</i> HT01	A	GQ387007
<i>Colossendeis megalonyx</i> SSI_L4	South Sandwich Islands	58°57'S	26°27'W	120	Otter Trawl	<i>C. meg.</i> HT01	A	GQ387007
<i>Colossendeis megalonyx</i> EI_N2	Elephant Island	61°47'S	57°30'W	322	Agassiz Trawl	<i>C. meg.</i> HT02	A	GQ387008
<i>Colossendeis megalonyx</i> SSI_S3	South Sandwich Islands	58°57'S	26°27'W	75	Blake Trawl	<i>C. meg.</i> HT01	A	GQ387007
<i>Colossendeis megalonyx</i> SSI_S4	South Sandwich Islands	58°57'S	26°27'W	75	Blake Trawl	<i>C. meg.</i> HT01	A	GQ387007
<i>Colossendeis megalonyx</i> SSI_S5	South Sandwich Islands	58°57'S	26°27'W	75	Blake Trawl	<i>C. meg.</i> HT01	A	GQ387007
<i>Colossendeis megalonyx</i> SSI_S6	South Sandwich Islands	58°57'S	26°27'W	75	Blake Trawl	<i>C. meg.</i> HT01	A	GQ387007
<i>Colossendeis megalonyx</i> BB_J6	Burdwood Bank	54°47'S	59°15'W	300	Blake Trawl	<i>C. meg.</i> HT07	B	GQ387013
<i>Colossendeis megalonyx</i> BB_J7	Burdwood Bank	54°47'S	59°15'W	300	Blake Trawl	<i>C. meg.</i> HT07	B	GQ387013
<i>Colossendeis megalonyx</i> BB_J9	Burdwood Bank	54°47'S	59°15'W	300	Blake Trawl	<i>C. meg.</i> HT07	B	GQ387013
<i>Colossendeis megalonyx</i> BB_O3	Burdwood Bank	52°40'S	60°16'W	202	Otter Trawl	<i>C. meg.</i> HT07	B	GQ387013
<i>Colossendeis megalonyx</i> BB_O4	Burdwood Bank	52°40'S	60°16'W	202	Otter Trawl	<i>C. meg.</i> HT09	B	GQ387015
<i>Colossendeis megalonyx</i> BB_O5	Burdwood Bank	52°40'S	60°16'W	202	Otter Trawl	<i>C. meg.</i> HT05	B	GQ387011
<i>Colossendeis megalonyx</i> BB_O7	Burdwood Bank	52°40'S	60°16'W	202	Otter Trawl	<i>C. meg.</i> HT04	B	GQ387010
<i>Colossendeis megalonyx</i> BB_O8	Burdwood Bank	52°40'S	60°16'W	202	Otter Trawl	<i>C. meg.</i> HT08	B	GQ387014
<i>Colossendeis megalonyx</i> BB_O9	Burdwood Bank	52°40'S	60°16'W	202	Otter Trawl	<i>C. meg.</i> HT08	B	GQ387014
<i>Colossendeis megalonyx</i> BB_O10	Burdwood Bank	52°40'S	60°16'W	202	Otter Trawl	<i>C. meg.</i> HT07	B	GQ387013
<i>Colossendeis megalonyx</i> BB_O11	Burdwood Bank	52°40'S	60°16'W	202	Otter Trawl	<i>C. meg.</i> HT04	B	GQ387010
<i>Colossendeis megalonyx</i> BB_O12	Burdwood Bank	52°40'S	60°16'W	202	Otter Trawl	<i>C. meg.</i> HT06	B	GQ387012
<i>Colossendeis megalonyx</i> BB_P1	Burdwood Bank	52°40'S	60°16'W	202	Otter Trawl	<i>C. meg.</i> HT07	B	GQ387013
<i>Colossendeis megalonyx</i> BB_S1	Burdwood Bank	52°39'S	59°13'W	129	Otter Trawl	<i>C. meg.</i> HT07	B	GQ387013
<i>Colossendeis megalonyx</i> BB_S2	Burdwood Bank	52°39'S	59°13'W	129	Otter Trawl	<i>C. meg.</i> HT08	B	GQ387014
<i>Colossendeis megalonyx</i> BI_F2	Bouvet	54°34'S	3°22'E	648	Otter Trawl	<i>C. meg.</i> HT11	C	GQ387017
<i>Colossendeis megalonyx</i> BI_F6	Bouvet	54°34'S	3°22'E	648	Otter Trawl	<i>C. meg.</i> HT10	C	GQ387016
<i>Colossendeis megalonyx</i> BI_G9	Bouvet	54°34'S	3°22'E	648	Otter Trawl	<i>C. meg.</i> HT10	C	GQ387016
<i>Colossendeis megalonyx</i> BI_H7	Bouvet	54°34'S	3°22'E	648	Otter Trawl	<i>C. meg.</i> HT11	C	GQ387017
<i>Colossendeis megalonyx</i> BI_I2	Bouvet	54°34'S	3°22'E	648	Otter Trawl	<i>C. meg.</i> HT10	C	GQ387016
<i>Colossendeis megalonyx</i> BI_P5	Bouvet	54°34'S	3°22'E	648	Otter Trawl	<i>C. meg.</i> HT10	C	GQ387016
<i>Colossendeis megalonyx</i> BI_Q1	Bouvet	54°34'S	3°22'E	648	Otter Trawl	<i>C. meg.</i> HT11	C	GQ387017
<i>Colossendeis megalonyx</i> BI_Q2	Bouvet	54°34'S	3°22'E	648	Otter Trawl	<i>C. meg.</i> HT10	C	GQ387016
<i>Colossendeis megalonyx</i> BI_Q3	Bouvet	54°34'S	3°22'E	648	Otter Trawl	<i>C. meg.</i> HT10	C	GQ387016
<i>Colossendeis megalonyx</i> BI_Q4	Bouvet	54°34'S	3°22'E	648	Otter Trawl	<i>C. meg.</i> HT11	C	GQ387017
<i>Colossendeis megalonyx</i> EI_J1	Elephant Island	61°12'S	56°01'W	148	Agassiz Trawl	<i>C. meg.</i> HT19	D	GQ387025
<i>Colossendeis megalonyx</i> EI_J2	Elephant Island	61°12'S	56°01'W	148	Agassiz Trawl	<i>C. meg.</i> HT18	D	GQ387024
<i>Colossendeis megalonyx</i> EI_J3	Elephant Island	61°12'S	56°01'W	148	Agassiz Trawl	<i>C. meg.</i> HT19	D	GQ387025
<i>Colossendeis megalonyx</i> EI_J4	Elephant Island	61°12'S	56°01'W	148	Agassiz Trawl	<i>C. meg.</i> HT19	D	GQ387025
<i>Colossendeis megalonyx</i> EI_J5	Elephant Island	61°12'S	56°01'W	148	Agassiz Trawl	<i>C. meg.</i> HT19	D	GQ387025
<i>Colossendeis megalonyx</i> EI_M10	Elephant Island	62°19'S	58°42'W	496	Agassiz Trawl	<i>C. meg.</i> HT13	D	GQ387019
<i>Colossendeis megalonyx</i> EI_M11	Elephant Island	62°19'S	58°42'W	496	Agassiz Trawl	<i>C. meg.</i> HT17	D	GQ387023
<i>Colossendeis megalonyx</i> EI_N9	Elephant Island	62°19'S	58°42'W	496	Agassiz Trawl	<i>C. meg.</i> HT16	D	GQ387022
<i>Colossendeis megalonyx</i> EI_N8	Elephant Island	62°19'S	58°42'W	491	Agassiz Trawl	<i>C. meg.</i> HT13	D	GQ387019
<i>Colossendeis megalonyx</i> EI_N10	Elephant Island	62°19'S	58°42'W	491	Agassiz Trawl	<i>C. meg.</i> HT14	D	GQ387020
<i>Colossendeis megalonyx</i> EI_N11	Elephant Island	62°19'S	58°42'W	491	Agassiz Trawl	<i>C. meg.</i> HT13	D	GQ387019
<i>Colossendeis megalonyx</i> EI_S7	Elephant Island	62°19'S	58°42'W	491	Agassiz Trawl	<i>C. meg.</i> HT12	D	GQ387018
<i>Colossendeis megalonyx</i> EI_S8	Elephant Island	62°19'S	58°42'W	491	Agassiz Trawl	<i>C. meg.</i> HT15	D	GQ387021
<i>Colossendeis megalonyx</i> EI_S9	Elephant Island	62°19'S	58°42'W	491	Agassiz Trawl	<i>C. meg.</i> HT13	D	GQ387019

Table 1 continued

Specimen	Location	Latitude	Longitude	Depth [m]	Gear	Haplotype	Clade	GenBank
<i>Colossendeis megalonyx</i> SSI_A10	South Sandwich	58°24'S	26°12'W	400	Blake Trawl	<i>C. meg.</i> HT20	E	GQ387026
<i>Colossendeis megalonyx</i> SSI_A11	South Sandwich	58°24'S	26°12'W	400	Blake Trawl	<i>C. meg.</i> HT20	E	GQ387026
<i>Colossendeis megalonyx</i> SSI_A12	South Sandwich	58°24'S	26°12'W	400	Blake Trawl	<i>C. meg.</i> HT20	E	GQ387026
<i>Colossendeis megalonyx</i> SSI_B2	South Sandwich	58°24'S	26°12'W	400	Blake Trawl	<i>C. meg.</i> HT20	E	GQ387026
<i>Colossendeis megalonyx</i> BI_F1	Bouvet	54°34'S	3°22'E	78	Otter Trawl	<i>C. meg.</i> HT20	E	GQ387026
<i>Colossendeis megalonyx</i> BI_F3	Bouvet	54°34'S	3°22'E	648	Otter Trawl	<i>C. meg.</i> HT20	E	GQ387026
<i>Colossendeis megalonyx</i> BI_F4	Bouvet	54°34'S	3°22'E	648	Otter Trawl	<i>C. meg.</i> HT20	E	GQ387026
<i>Colossendeis megalonyx</i> BI_F5	Bouvet	54°34'S	3°22'E	648	Otter Trawl	<i>C. meg.</i> HT20	E	GQ387026
<i>Colossendeis megalonyx</i> BI_F7	Bouvet	54°34'S	3°22'E	648	Otter Trawl	<i>C. meg.</i> HT20	E	GQ387026
<i>Colossendeis megalonyx</i> BI_F9	Bouvet	54°34'S	3°22'E	648	Otter Trawl	<i>C. meg.</i> HT20	E	GQ387026
<i>Colossendeis megalonyx</i> BI_F10	Bouvet	54°34'S	3°22'E	648	Otter Trawl	<i>C. meg.</i> HT20	E	GQ387026
<i>Colossendeis megalonyx</i> BI_F11	Bouvet	54°34'S	3°22'E	648	Otter Trawl	<i>C. meg.</i> HT20	E	GQ387026
<i>Colossendeis megalonyx</i> BI_G1	Bouvet	54°34'S	3°22'E	648	Otter Trawl	<i>C. meg.</i> HT20	E	GQ387026
<i>Colossendeis megalonyx</i> BI_G2	Bouvet	54°34'S	3°22'E	648	Otter Trawl	<i>C. meg.</i> HT20	E	GQ387026
<i>Colossendeis megalonyx</i> BI_G3	Bouvet	54°34'S	3°22'E	648	Otter Trawl	<i>C. meg.</i> HT20	E	GQ387026
<i>Colossendeis megalonyx</i> BI_G4	Bouvet	54°34'S	3°22'E	648	Otter Trawl	<i>C. meg.</i> HT20	E	GQ387026
<i>Colossendeis megalonyx</i> BI_G5	Bouvet	54°34'S	3°22'E	648	Otter Trawl	<i>C. meg.</i> HT20	E	GQ387026
<i>Colossendeis megalonyx</i> BI_G7	Bouvet	54°34'S	3°22'E	648	Otter Trawl	<i>C. meg.</i> HT20	E	GQ387026
<i>Colossendeis megalonyx</i> BI_G8	Bouvet	54°34'S	3°22'E	648	Otter Trawl	<i>C. meg.</i> HT20	E	GQ387026
<i>Colossendeis megalonyx</i> BI_G10	Bouvet	54°34'S	3°22'E	648	Otter Trawl	<i>C. meg.</i> HT20	E	GQ387026
<i>Colossendeis megalonyx</i> BI_G11	Bouvet	54°34'S	3°22'E	648	Otter Trawl	<i>C. meg.</i> HT20	E	GQ387026
<i>Colossendeis megalonyx</i> BI_G12	Bouvet	54°34'S	3°22'E	648	Otter Trawl	<i>C. meg.</i> HT20	E	GQ387026
<i>Colossendeis megalonyx</i> BI_H3	Bouvet	54°34'S	3°22'E	648	Otter Trawl	<i>C. meg.</i> HT20	E	GQ387026
<i>Colossendeis megalonyx</i> BI_H4	Bouvet	54°34'S	3°22'E	648	Otter Trawl	<i>C. meg.</i> HT20	E	GQ387026
<i>Colossendeis megalonyx</i> BI_H5	Bouvet	54°34'S	3°22'E	648	Otter Trawl	<i>C. meg.</i> HT20	E	GQ387026
<i>Colossendeis megalonyx</i> BI_H6	Bouvet	54°34'S	3°22'E	648	Otter Trawl	<i>C. meg.</i> HT20	E	GQ387026
<i>Colossendeis megalonyx</i> BI_H8	Bouvet	54°34'S	3°22'E	648	Otter Trawl	<i>C. meg.</i> HT20	E	GQ387026
<i>Colossendeis megalonyx</i> BI_H9	Bouvet	54°34'S	3°22'E	648	Otter Trawl	<i>C. meg.</i> HT20	E	GQ387026
<i>Colossendeis megalonyx</i> BI_H10	Bouvet	54°34'S	3°22'E	648	Otter Trawl	<i>C. meg.</i> HT20	E	GQ387026
<i>Colossendeis megalonyx</i> BI_H11	Bouvet	54°34'S	3°22'E	648	Otter Trawl	<i>C. meg.</i> HT20	E	GQ387026
<i>Colossendeis megalonyx</i> BI_H12	Bouvet	54°34'S	3°22'E	648	Otter Trawl	<i>C. meg.</i> HT20	E	GQ387026
<i>Colossendeis megalonyx</i> BI_I3	Bouvet	54°34'S	3°22'E	648	Otter Trawl	<i>C. meg.</i> HT20	E	GQ387026
<i>Colossendeis megalonyx</i> BI_G6	Bouvet	54°34'S	3°22'E	648	Otter Trawl	<i>C. meg.</i> HT20	E	GQ387026
<i>Colossendeis megalonyx</i> BI_I5	Bouvet	54°34'S	3°22'E	648	Otter Trawl	<i>C. meg.</i> HT20	E	GQ387026
<i>Colossendeis megalonyx</i> BI_P2	Bouvet	54°34'S	3°22'E	648	Otter Trawl	<i>C. meg.</i> HT20	E	GQ387026
<i>Colossendeis megalonyx</i> BI_P3	Bouvet	54°34'S	3°22'E	648	Otter Trawl	<i>C. meg.</i> HT20	E	GQ387026
<i>Colossendeis megalonyx</i> BI_P4	Bouvet	54°34'S	3°22'E	648	Otter Trawl	<i>C. meg.</i> HT20	E	GQ387026
<i>Colossendeis megalonyx</i> BI_P6	Bouvet	54°34'S	3°22'E	648	Otter Trawl	<i>C. meg.</i> HT20	E	GQ387026
<i>Colossendeis megalonyx</i> BI_P9	Bouvet	54°34'S	3°22'E	648	Otter Trawl	<i>C. meg.</i> HT20	E	GQ387026
<i>Colossendeis megalonyx</i> BI_P10	Bouvet	54°34'S	3°22'E	648	Otter Trawl	<i>C. meg.</i> HT20	E	GQ387026
<i>Colossendeis megalonyx</i> BI_P11	Bouvet	54°34'S	3°22'E	648	Otter Trawl	<i>C. meg.</i> HT20	E	GQ387026
<i>Colossendeis megalonyx</i> BI_P12	Bouvet	54°34'S	3°22'E	648	Otter Trawl	<i>C. meg.</i> HT20	E	GQ387026
<i>Colossendeis megalonyx</i> BI_Q5	Bouvet	54°34'S	3°22'E	648	Otter Trawl	<i>C. meg.</i> HT20	E	GQ387026
<i>Colossendeis megalonyx</i> BI_Q6	Bouvet	54°21'S	3°10'E	458	Otter Trawl	<i>C. meg.</i> HT20	E	GQ387026
<i>Colossendeis megalonyx</i> BI_Q9	Bouvet	54°21'S	3°10'E	458	Otter Trawl	<i>C. meg.</i> HT20	E	GQ387026
<i>Colossendeis megalonyx</i> BI_R1	Bouvet	54°21'S	3°10'E	458	Otter Trawl	<i>C. meg.</i> HT20	E	GQ387026
<i>Colossendeis megalonyx</i> BI_R2	Bouvet	54°21'S	3°10'E	458	Otter Trawl	<i>C. meg.</i> HT20	E	GQ387026
<i>Colossendeis megalonyx</i> EI_M8	Elephant Island	62°19'S	58°42'W	491	Agassiz Trawl	<i>C. meg.</i> HT21	F	GQ387027

Table 1 continued

Specimen	Location	Latitude	Longitude	Depth [m]	Gear	Haplotype	Clade	GenBank
<i>Colossendeis megalonyx</i> EI_N12	Elephant Island	62°19'S	58°42'W	491	Agassiz Trawl	<i>C. meg.</i> HT22	F	GQ387028
<i>Colossendeis lillei</i> 1	South Georgia	55°04'S	35°12'W	117	Blake Trawl			GQ387004
<i>Colossendeis lillei</i> 2	South Georgia	55°04'S	35°12'W	117	Blake Trawl			GQ387005
<i>Colossendeis lillei</i> 3	South Sandwich	57°03'S	26°44'W	130	Blake Trawl			GQ387006
<i>Colossendeis robusta</i> 1	South Sandwich	56°15'S	27°34'W	336	Blake Trawl			GQ387001
<i>Colossendeis robusta</i> 2	Bouvet	54°34'S	3°22'E	648	Otter Trawl			GQ386999
<i>Colossendeis robusta</i> 3	Burdwood Bank	54°47'S	59°15'W	300	Blake Trawl			GQ387000
<i>Colossendeis scoresbii</i> 1	South Georgia	54°25'S	35°55'W	100	Blake Trawl			GQ386997
<i>Colossendeis scoresbii</i> 2	South Sandwich	56°15'S	27°34'W	336	Blake Trawl			GQ386998
<i>Colossendeis wilsoni</i> 1	South Sandwich	58°24'S	26°12'W	400	Blake Trawl			GQ387002
<i>Colossendeis wilsoni</i> 2	Elephant Island	61°16'S	56°31'W	412	Otter Trawl			GQ387003
<i>Decolopoda australis</i> 1	Shag Rocks	53°47'S	41°22'W	195	Blake Trawl			GQ386993
<i>Decolopoda australis</i> 2	Elephant Island	60°56'S	55°42'W	136	Otter Trawl			GQ386994
<i>Decolopoda australis</i> 3	Elephant Island	60°55'S	55°44'W	196	Otter Trawl			GQ386995
<i>Decolopoda australis</i> 4	South Sandwich	58°24'S	26°12'W	400	Blake Trawl			GQ386996
<i>Dodecolopoda mawsoni</i>	Elephant Island	60°55'S	55°44'W	196	Otter Trawl			GQ386992
<i>Ammothea</i> sp.	South Sandwich	57°03'S	26°44'W	130	Blake Trawl			GQ386988
<i>Pallenopsis</i> sp.	South Sandwich	56°15'S	27°34'W	336	Blake Trawl			GQ386989
<i>Nymphon australe</i>	South Sandwich	58°24'S	26°12'W	400	Blake Trawl			GQ386990
<i>Austropallene cornigera</i>	South Sandwich	57°03'S	26°44'W	130	Blake Trawl			GQ386991

For the *Colossendeis megalonyx* specimens the haplotype number and the respective phylogenetic clade are indicated. GenBank accession numbers for the 561 bp COI fragment are listed in the last column

of A 29.2%, C 17.2%, G 13.4% and T 40.2%. Of the 561 aligned positions 258 were variable, 214 were parsimony informative and 44 were singleton sites (autapomorphies). A total of 63 replacement substitutions were found in the amino acid alignment.

Among the 96 sequences of nominal *Colossendeis megalonyx*, we observed 103 variable positions in the 561 bp COI alignment (96 parsimony informative, 7 singleton sites). Only six amino acid replacement substitutions were found in the *Colossendeis megalonyx* amino acid alignment. Positions with replacement substitutions involved only amino acids with similar chemical characteristics.

Phylogenetic and phylogeographic analysis

Appropriate substitution models for our data set were determined using hierarchical likelihood ratio tests and the Akaike information criterion implemented in MrMODELTEST, which proposed, respectively, the GTR + G and the HKY + G-model. Bayesian trees reconstructed using these two models had the same topology, but slightly more conservative posterior probabilities were obtained with the GTR + G-model. Since the philosophy of Markov Chain Monte Carlo methods is to vary all parameters

independently, the posterior probabilities found by the GTR + G-model should be considered more realistic. The maximum parsimony bootstrap tree was less resolved, with lower support values on most clades, as compared to the Bayesian tree. Both tree topologies, however, showed no direct conflicts (Fig. 2).

The family Colossendeidae is resolved as a well-supported monophylum (posterior probability of 1.0, Fig. 2). Within the Colossendeidae all species are supported with high posterior probability values (0.99 or 1.0) with the exception of *Colossendeis robusta* that consists of two specimens with very similar and one with a strongly diverged sequence (Fig. 2). *Colossendeis megalonyx* is subdivided into six distinct and well-supported clades (posterior probabilities of 1.0). Clade A consists of three haplotypes (HT01–HT03) that differ by a maximum of two substitutions (Fig. 3, Table 1). Clade B consists of six different haplotypes (HT04–HT09) and clade C of two haplotypes that differ by one substitution only (HT11/12). Clade D consists of 14 specimens with eight different haplotypes (HT12–19), whereas clade E comprises 47 specimens representing a single haplotype (HT20). Finally, clade F is composed of two specimens with two haplotypes that are distinguished by seven substitutions (HT21/22). Analysis of the separate *Colossendeis megalonyx*

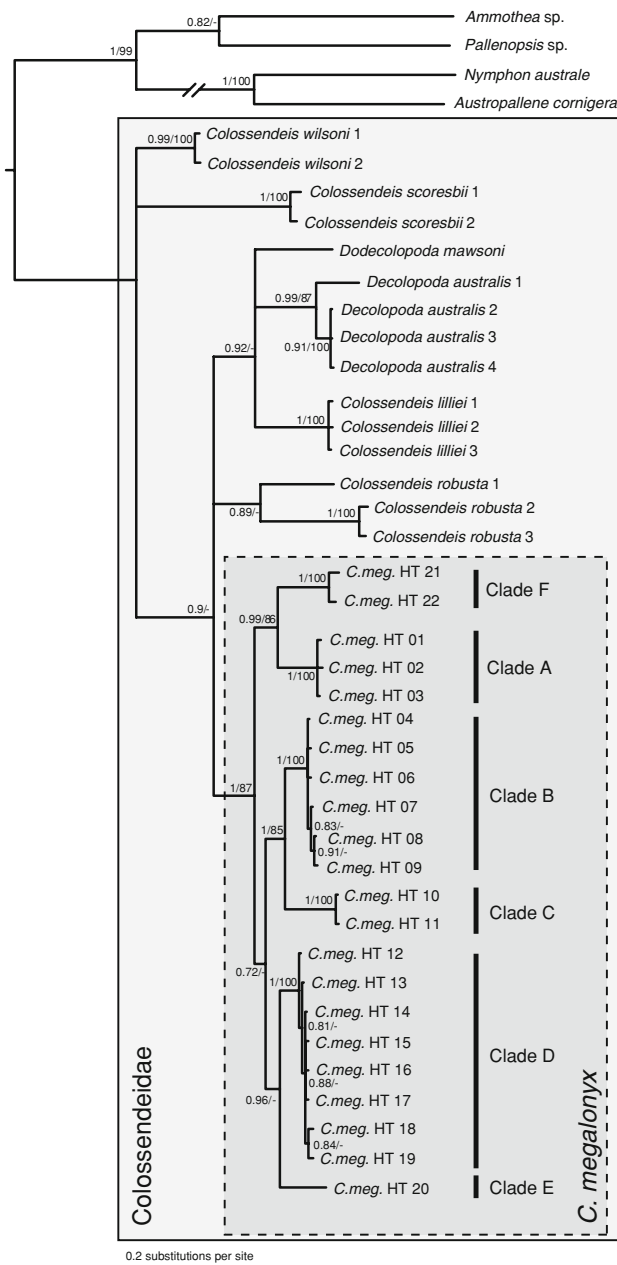


Fig. 2 Bayesian phylogenetic tree of the 41 distinct haplotypes of the pycnogonid species analysed in this study, based on a 561 bp alignment of the cytochrome c oxidase I gene. The monophyletic clades within nominal *Colossendeis megalonyx* are named A–F. Clades with posterior probabilities below 0.7 have been converted to polytomies. Branch labels show posterior probabilities/parsimony bootstrap values if they are above 0.7/70, respectively

alignment (clades A–F) using TCS with a 95% as well as a 90% statistical parsimony connection limit resulted in six separate networks that represent the six clades (A–F) found in the phylogenetic tree (Fig. 2). Setting the number of allowed steps to 49 resulted in one statistical parsimony haplotype network in which the six groups are interconnected by long branches (Fig. 3). Clades B and D show a

greater diversity than the other clades that have a maximum of three distinct haplotypes. Amino acid replacement substitutions are found at the long branches between the different clades and also within clade A, B and D (Fig. 3). The average uncorrected pairwise distances between the six different clades were large, ranging from 5.9 to 10.5% (see Table 2). Variation within clades was small, reaching a maximum of 1.6% uncorrected genetic distance.

Only relatively few specimens belonging to clades A–D and F were sampled and thus genetic diversity estimates may be biased due to undersampling. All 47 specimens of clade E are genetically identical.

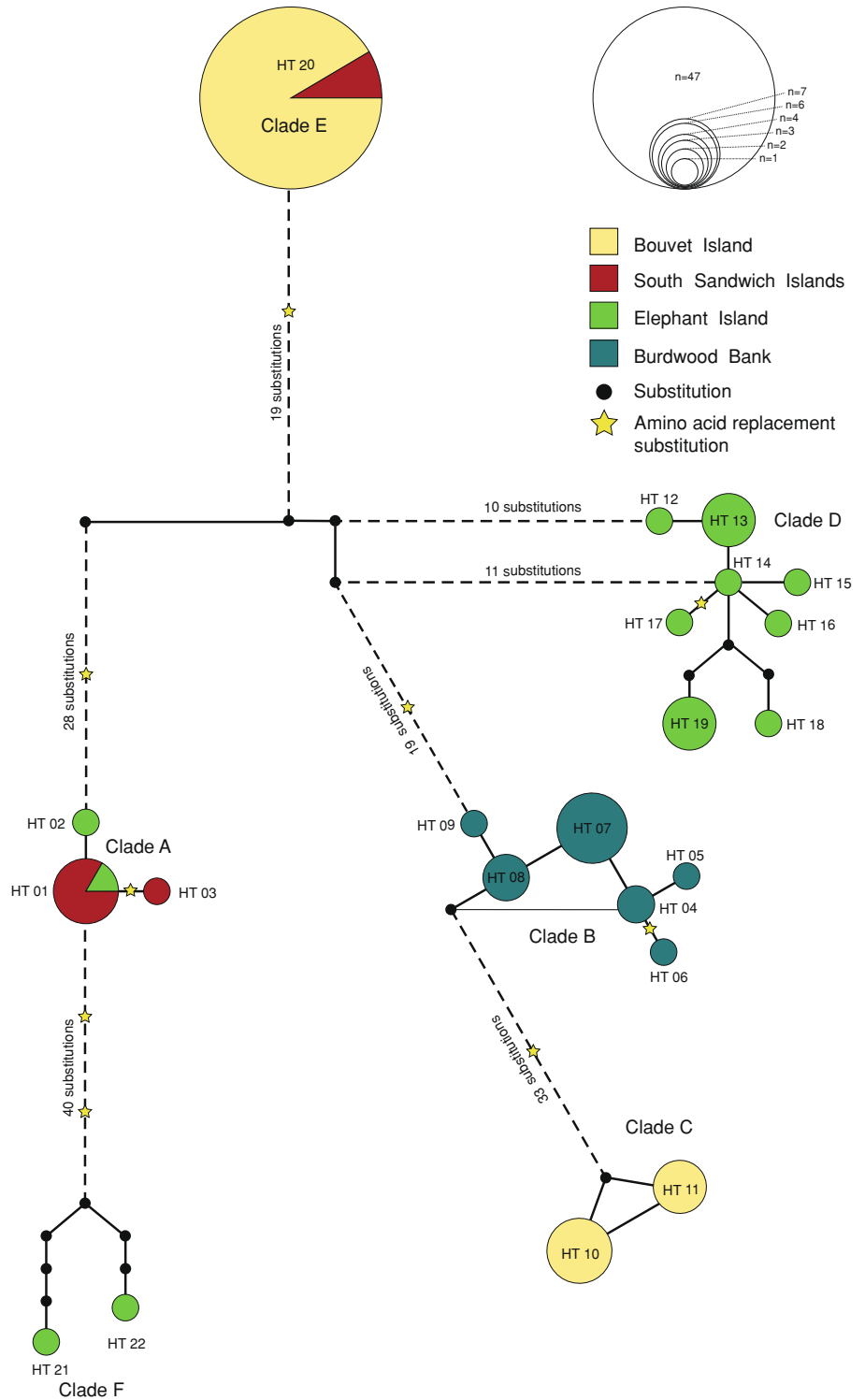
Geographic and depth distribution of *Colossendeis megalonyx* clades

Specimens of clades B, C, D and F occurred in only in one location whereas specimens from clades A and E were sampled from multiple locations (Fig. 3). Conversely, specimens from the South Sandwich Islands group in clade A and E, specimens from Bouvet Island in clades C and E, specimens from the Antarctic Peninsula in clade A, D and F. All 15 specimens sampled outside the Antarctic convergence at Burdwood Bank group into clade B. No consistent depth preference can be determined for the clades based on the data available, with the exception of clade C (all sampled at a depth of 648 m; Table 1). Inspection of specimens by light microscopy revealed that all specimens of clade C lacked eyes, whereas eyes were present in specimens of all other clades (unpigmented for two specimens in clade E though).

Discussion

Our data support the existence of six reciprocally monophyletic *Colossendeis megalonyx* phylogroups. Several characteristics strongly support that these six phylogroups may represent five putative cryptic and one pseudocryptic species within the morphospecies *Colossendeis megalonyx*: First, the genetic distance between the six clades is an order of magnitude higher than the distance found within clades (criterion adopted by Hebert et al. 2004; Held 2003). Second, comparisons of other well distinguished pycnogonid species also showed uncorrected genetic distances above 4% (Mahon et al. 2008, with the exception of *Nymphon australe* and *Nymphon paucituberculatum*). The same magnitude of interspecific dissimilarity was also found for other chelicerates investigated so far (Barrett and Hebert 2005; Wilcox et al. 1997). Thus, with magnitudes in uncorrected pairwise genetic distances ranging from 5.9 to 10.5%, our data strongly hint at the presence of distinct species. Third, a statistical parsimony analysis using TCS

Fig. 3 Statistical parsimony network (allowing a maximum number of 49 steps) of the *Colossendeis megalonyx* (*C. meg.*) haplotypes. The diameters of the circles represent the number of specimens sampled with this haplotype. *Dashed lines* represent branches with a connection limit of <90% (i.e., a probability of homoplasious character states >10%) that therefore result in separated subnetworks using the default settings in TCS



yielded completely separated networks for the six clades at a 95% as well as a 90% connection limit. In previous analyses, this level of separation has frequently been shown to be in accordance with the definition of biological species (Hart and Sunday 2007).

Given the geographically limited sampling scheme we propose that a more comprehensive sampling, encompassing the entire reported distribution range of nominal *Colossendeis megalonyx*, is very likely to uncover even more distinct phylogroups.

Table 2 Mean uncorrected pairwise genetic distances (in percent) among members of the six clades detected (diagonal) and between the different clades (below diagonal)

Clade	A	B	C	D	E	F
A	0.1					
B	8.4	0.2				
C	9.4	6.5	0.1			
D	8.1	6.2	7.5	0.5		
E	8.8	7.5	9.1	5.9	0	
F	8.2	8.7	10.5	8.6	9.6	1.6

From a phylogeographic perspective it is interesting to note that specimens from clade B are found exclusively outside the Antarctic convergence, whereas specimens from all other clades were sampled within the Antarctic (see Fig. 2). A similar observation has been made for benthic Antarctic taxa without and with pelagic distributional stages (Hunter and Halanych 2008; Linse et al. 2007; Thornhill et al. 2008; Wilson et al. 2009), indicating that the Polar Frontal Zone and the Antarctic Circumpolar Current system may constitute a major isolating barrier to gene flow between the regions at least on ecological, but not necessarily on evolutionary time scales (Page and Linse 2002; Wilson et al. 2009).

From a phylogenetic perspective, it is interesting to note that Clade B does not form the outgroup to the Antarctic clades (A, C–F). Hence, our data do not support that *Colossendeis megalonyx* has its historic origin on the South American shelf and colonized the Antarctic subsequently. Our data show that clade B obtains a derived position within the Antarctic clades, suggesting a scenario of Antarctic radiation of nominal *Colossendeis megalonyx* and a subsequent expansion to Subantarctic and temperate regions which has been postulated earlier for several pycnogonids (Arnaud 1987; Hedgpeth 1969; Munilla 2001) and also for other Antarctic taxa (see Wilson et al. 2009). A more complete data set comprising samples from other regions of the Antarctic is needed to clarify the routes of colonization of the Antarctic shelf.

The observation that all species from clade C, sampled at a depth of 648 m, have no eyes is particularly remarkable. Indeed, the absence of eyes is a typical feature also of other deep-sea arthropod species (Raupach et al. 2009). Presumably, eyes have been lost due to the lack of selective advantage in the dark environment.

The geographic and bathymetric sympatry of the different clades in all locations, with the exception of clade B from Burdwood Bank, raises the question how such strong differences have developed on evolutionary timescales in an eurybathic taxon such as the *Colossendeis megalonyx* complex. While at first sight sympatric differentiation or putative speciation may be regarded as possible

explanations, the retention of ancestral polymorphisms, which have developed in allopatry is another. The presence of shared haplotypes at the South Sandwich Islands and Bouvet for clade E and the presence of closely related haplotypes at the South Sandwich Islands and Elephant Island (clade A) suggest that migration of *Colossendeis megalonyx* over several thousands of kilometres is possible and may occur repeatedly. Alternatively, Bouvet and the South Sandwich Islands might have been colonized only recently in a single event (see clades A and E in Fig. 3). In this case, the insufficient time for subsequent differentiation would account for the limited number (clade A) or even the lack of observable differences (clade E) between specimens sampled from Bouvet/South Sandwich Islands and the possible source populations.

In the absence of samples of *Colossendeis megalonyx* from depths below 648 m it remains an open question if active dispersal across the deep sea or passive dispersal by rafting or transportation on a different host species in surface waters are the most plausible explanations for the occurrence of some clades at geographically distant sampling sites (clades A and E). Both mechanisms have been reported for other Antarctic taxa (Hedgpeth 1964; Helmuth et al. 1994; Lehmann et al. 2007; Sirenko 2000).

While from an evolutionary perspective, an allopatric scenario can well explain the geographic partitioning of genetic polymorphisms, fundamental ecological questions concerning the coexistence of two or more such similar “life forms” remain. Pre- or postzygotic reproduction barriers have presumably evolved (Coyne and Orr 2004) and niches must be partitioned to avoid competition among these benthic carni-/detritivores. According to the competitive exclusion principle (Hardin 1960) it is expected that species with very similar demands will not be able to co-exist over evolutionary timescales in sympatry. Thus, if the genetically strongly differentiated clades in fact represent different *Colossendeis* species it must be assumed that they differ in other, yet undiscovered ecological niche parameters, e.g. in prey specialisation.

To estimate the levels of gene flow between strongly isolated regions, subsequent studies must directly focus on the population structure, e.g. of the large clade E consisting of only one COI haplotype. Applying fast evolving molecular markers such as microsatellites could be able to resolve this structure (Held and Leese 2007).

Taxonomic implications

The taxonomic status of *Colossendeis megalonyx* Hoek, 1881 has been discussed controversially. Whereas Fry and Hedgpeth (1969) subdivided *Colossendeis megalonyx* into four distinct subspecies, Pushkin (1993) and Child (1995) used the original description of *Colossendeis megalonyx*

Hoek, 1881. Cano and Lopez-Gonzalez (2007) and Bamber and El Nagar (2009) are using this species name as well. Currently, *Colossendeis megalonyx* encompasses three synonymised taxa (Bamber and El Nagar 2009): *Colossendeis frigida* Hodgson, 1907, *Colossendeis rugosa* Hodgson, 1907 (which was originally distinguished as a separate species due to its spiny legs) and *Colossendeis orcadensis* Hodgson, 1909 (which is blind and has a prolonged fifth palp segment, see Fry and Hedgpeth 1969).

As *Colossendeis megalonyx* was originally described from the South American shelf between the Falkland Islands and Patagonia (Hoek 1881), which is in close proximity to Burdwood Bank, it is likely that *Colossendeis megalonyx* is represented by specimens from clade B but evaluation of the type material is pending. If this was true, the Antarctic clades might represent formally undescribed species or, as suggested by the lack of eyes in all specimens from clade C, belong to *Colossendeis orcadensis*, which currently is a junior synonym of *Colossendeis megalonyx*. No conspicuous morphological differences could be observed between specimens of the clades (A, B, D–F) by light-microscopical inspection. However, possible future studies using scanning electron microscopy, other morphological imaging methods or morphometric approaches may be capable of tracing reliable morphological differences between the genetically defined clades (A, B, D–F) and thus disclosing the different clades as pseudocryptic rather than cryptic species. Also, the reported variability within nominal *Colossendeis megalonyx* (see e.g. Fry and Hedgpeth 1969) should be reanalysed in the context of our new findings. A taxonomic revision must include more than a single character as well as further morphological characters, but this is beyond the scope of this paper.

Colossendeis scoresbii constituted an isolated and distinct lineage within the Colossendeidae, which supports its status of currently being regarded as a separate species and not as a subspecies of *Colossendeis megalonyx* (see Fry and Hedgpeth 1969). Specimens of *Colossendeis lilliei* Calman, 1915 were identified according to the keys of Fry and Hedgpeth (1969) and Pushkin (1993) and formed a well-supported lineage (Fig. 2). This finding challenges the current treatment of *Colossendeis lilliei* as a junior synonym of *Colossendeis robusta* (Munilla and Soler Membreives 2008; Bamber and El Nagar 2009) and supports recent morphological evidence that suggests that *Colossendeis lilliei* should be regarded as a separate species (Cano and Lopez-Gonzalez 2007). Similarly, *Colossendeis robusta* from outside the Southern Ocean is genetically distinct from *Colossendeis robusta* inside the Southern Ocean. The order of magnitude of these differences is also of the same magnitude as reported for reproductively isolated pycnogonid species (Mahon et al. 2008). Hence, our data provide additional evidence that *Colossendeis robusta*

consists of several distinct phylogroups that are likely to represent different cryptic species according to the criteria defined above. In conclusion, our data suggest that multiple, phylogenetically very distinct lineages are present within the nominal taxon *Colossendeis robusta* (Fig. 2) and we feel that a taxonomic revision of the genus *Colossendeis* may be in order.

Noteworthy, *Decolopoda australis* and *Dodecolopoda mawsoni* cluster within the genus *Colossendeis* (see Fig. 2). If this position should be supported in future studies, a taxon comprising at least the *Colossendeis* species contained in this study and should also include *Decolopoda* and *Dodecolopoda* in order to avoid paraphyly of the genus *Colossendeis* (see Nakamura et al. 2007 for similar results). In its current composition, *Colossendeis* Jarzinsky, 1870 would be a junior synonym of *Decolopoda* Eights, 1835 but a final conclusion will have to wait until other possible members of this taxon have been evaluated in detail.

When comparing the phylogenetic positioning of the outgroups to *Colossendeis megalonyx* in this study it becomes obvious that within the Colossendeidae several phylogenetic and taxonomic questions remain and must be clarified using a combination of both traditional morphological analyses and DNA barcoding techniques as proposed in this study.

Conclusions

Our analyses show that genetic variability is highly structured within nominal *Colossendeis megalonyx* and the magnitude of genetic differences strongly support that the different genetic clades represent a further example of a cryptic/pseudocryptic Antarctic species complex. Some clades show geographically restricted distribution ranges whereas others occur over large distances. As a consequence the reported distribution ranges of *Colossendeis megalonyx* and possibly several other pycnogonids with similar life history traits should be re-evaluated.

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