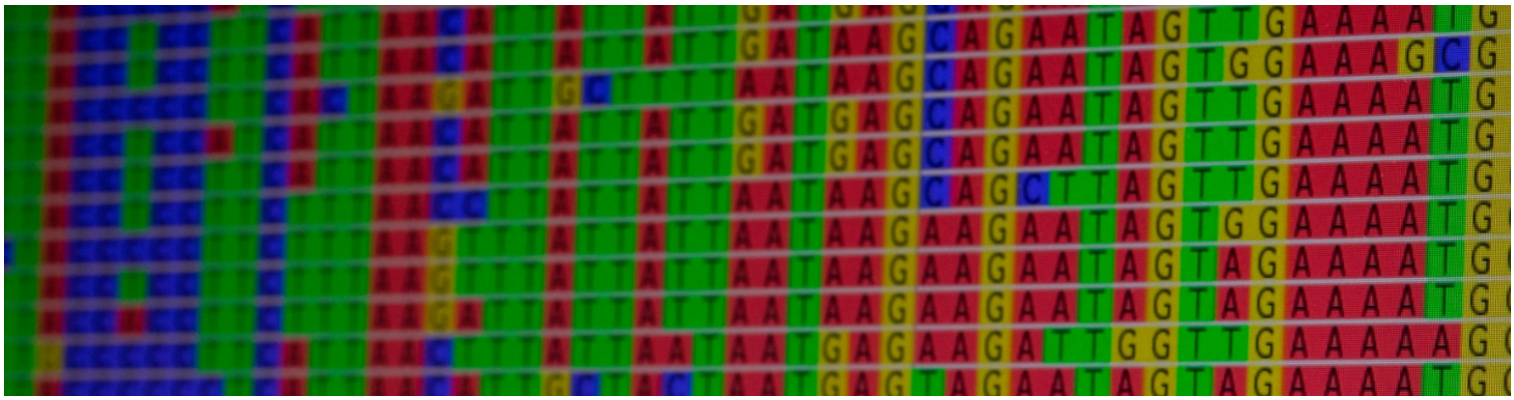

# Speciation and Adaptation in Southern Ocean Sea Spiders

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**Jana Sophie Dömel**
aus Mülheim an der Ruhr

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Title picture was taken by Till-Hendrik Macher.

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1. Gutachter: Prof. Dr. Florian Leese

2. Gutachter: Prof. Dr. Roland R. Melzer

Vorsitzender des Prüfungsausschusses: Prof. Dr. Bernd Sures

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# *Für unsere Zukunft*

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# Table of content

# Abstract

Marine shelf habitats in the Southern Hemisphere have been drastically impacted by glacial periods especially during the Plio- and Pleistocene. Large parts of the sea floor were at least temporarily covered by grounded ice, thereby reducing habitat availability for benthic organisms. Nowadays, an astonishingly high number especially of endemic species has been reported for this shelf fauna. Many of the species have radiated during the Plio- and Pleistocene. Allopatric speciation in independent and isolated refugia has often been postulated as the driving speciation mechanism, especially in the Antarctic, but also at higher latitudes. However alternative driver for speciation have rarely been considered, and especially ecological speciation in sympatry (e.g. shared refugia) due to adaptive divergence seems to be a promising mechanism to address with morphological and genetic data. Hence, one aim of this thesis is to explore evidence for both speciation scenarios focussing on two sea spider species complexes, namely *Pallenopsis patagonica* (Hoek, 1881) and *Colossendeis megalonyx* Hoek, 1881.

The first study addresses genetic diversity within the *P. patagonica* species complex. Previously analysed mitochondrial cytochrome c oxidase subunit I (COI) data of Patagonian and Antarctic specimens was expanded by adding further samples from Patagonia, sub-Antarctica and the Eastern Weddell Sea. Furthermore, sequence data for the nuclear internal transcribed spacer (ITS) were added to obtain more information about the species complex. In fact, a higher number of distinct lineages were detected. Some lineages detected by mitochondrial data were not supported by nuclear data probably due to a lack of resolution (too few substitutions) in the ITS region rather than hybridization and speciation reversal events as reported in an earlier study about the sea spider species complex *C. megalonyx*.

While the first study was based on two single-markers only, in the second study of this thesis an unprecedentedly large genomic data set for specimens of the *P. patagonica* species complex was generated based on a new high-throughput sequencing method, called target hybrid enrichment. Out of 1607 targeted EOGs (Eukaryotic Ortholog Groups, i.e. imputative single-copy genes), 821 EOGs were successfully captured for the *P. patagonica* species complex. Results of phylogenomic analyses supported previously reported lineages but further resolved the species phylogeny and added much higher statistical support to branches. In combination with extensive morphological and morphometric analyses, the large data set enabled to delineate at least eight species. On the basis of this, two new species, namely *P. aulaeosmanorum* sp. nov. Dömel & Melzer, 2019 and *P. obstaculumsuperavit* sp. nov. Dömel, 2019 were formally described. Neither genomic nor morphological data revealed consistent evidence for positive selection, rendering speciation in allopatric glacial refugia, due to genetic drift, as the most likely scenario.

In the third study the target hybrid enrichment approach was applied to the second sea spider species complex *C. megalonyx*. All 1607 targeted EOGs were recovered and a phylogenetic tree with high branch support values was generated based on the genomic data. Phylogenomic analyses supported already reported recent divergences as well as mito-nuclear discordances within this taxon, but resolution and statistical support were improved. Using SNP data inferred from EOGs and their more variable flanking regions, also intraspecific divergence patterns could be resolved. The results indicate restricted gene flow between geographically distinct populations. Morphometric analyses revealed multiple significant differences between lineages, but a clear separation was difficult. Evidence for positive selection was found for four genes associated with structural and neuronal functions. Hence, there is indication for positive selection on genes of the *C. megalonyx* species complex, but its specific contribution to the speciation process remains to be explored further.

In the last study the phylogeny of the group of the longitarsal Colossendeidae which is especially abundant in the Southern Ocean and includes the species complex *C. megalonyx,* was investigated using target hybrid enrichment. Here, also all 1607 targeted EOGs were recovered. A well-resolved phylogeny of the group, which is mostly consistent with morphological data, was obtained. The data supported an Antarctic origin of the longitarsal Colossendeidae and multiple dispersal events to other regions, which occurred at different timescales. This scenario highlights the role of the Southern Ocean as a centre of origin also for non-Antarctic species.

The comparison of phylogenetic trees based on single and target hybrid enrichment marker approaches underlines the benefit of the latter when resolving both ancient and recent speciation events. Analyses of morphological characters performed in this thesis revealed high variation even within lineages of both species complexes.

Although distinct morphological characters suitable for species description and characters with significant differences between lineages were found, none of those seem to have distinct differences of lineages. Hence, no evidence for ecological character displacement and adaptive divergence of lineages that occur in sympatry as found. However, for some genes studied for lineages of the *C. megalonyx* species complex, dN/dS tests revealed codons and branches under selection. Differences in the detection of genes under selection between the two sea spider species complexes are likely based on methodological limitations as the design for target hybrid enrichment approach was based on a transcriptome of *C. megalonyx*. The reduced enrichment of only about half the number of EOGs for the *P. patagonica* species complex, limited the analyses. However, also biological differences, e.g. in reproduction strategies, can be considered as a factor for differences in speciation scenarios. The reproductive mode remains unclear for the *C. megalonyx* species complex but it most likely differs from the brooding strategy of the *P. patagonica* species complex. This can lead to differences in dispersal

capability and reproduction possibility of distinct populations, that are potentially higher in the *C. megalonyx* species complex.

Based on the results of this thesis it is likely that different mechanisms drove speciation events (and speciation reversal) in Southern Ocean sea spiders and future studies should furthermore test different options for drivers of speciation rather than considering that only the impact of glacial cycles formed the species-rich benthic communities in the Southern Hemisphere. While analyses can be improved by adding more genomic regions and improved gene annotations in order to more holistically analyse targeted genes of selection, using target hybrid enrichment enabled to generate the largest genomic data sets available for sea spiders, so far.

# Zusammenfassung

Die marinen Schelflebensräume der Antarktis und Subantarktis wurden vor allem während des Pliozäns und Pleistozäns drastisch von den Eiszeiten beeinflusst. Große Teile des Schelfs waren zumindest vorübergehend mit Eis bedeckt, was die Verfügbarkeit von Lebensräumen für benthische Organismen einschränkte. Heutzutage ist die Schelffauna erstaunlich artenreich und zahlreiche endemischen Arten leben dort. Viele der Arten haben sich während des Pliozäns und Pleistozäns gebildet. Meistens wird eine allopatrische Artbildung in unabhängigen und isolierten Refugien als treibende Kraft für Artbildung angenommen. Eine ökologische Artbildung durch Anpassung von Arten an unterschiedliche Habitate und Ressourcen in Sympatrie stellt einen weiteren, plausiblen Artbildungsmechanismus dar. Dieser wurde bisher aber nur selten adressiert. Daher ist ein Ziel dieser Arbeit, Artbildungsprozesse innerhalb der zwei Asselspinnen Arten *Pallenopsis patagonica* (Hoek, 1881) und *Colossendeis megalonyx* Hoek, 1881 auf Hinweise für beide Artbildungsprozesse zu erforschen. Beide Arten bestehen aus mehrerer genetisch distinkter Linien, d.h. kryptischen Arten und weisen eine weite Verbreitung in der Antarktis und Patagonien auf.

Die erste Studie befasst sich mit der genetischen Vielfalt innerhalb des *P. patagonica*-Artkomplexes. Zuvor analysierte mitochondriale Daten des Gens der Cytochrom-C-Oxidase-Untereinheit I (COI) von patagonischen und antarktischen Proben wurden um weitere Proben aus Patagonien, der Subantarktis und dem östlichen Weddellmeer erweitert. Des Weiteren wurden Sequenzdaten für den Nuclear Internal Transcribed Spacer (ITS) generiert, um mehr und unabhängige Informationen über den Artkomplex zu erhalten. Tatsächlich wurde eine erhöhte Anzahl unterschiedlicher Abstammungslinien festgestellt. Einige Abstammungslinien, die durch mitochondriale Daten entdeckt wurden, wurden durch nukleare Daten nicht unterstützt. Dies kann durch mangelnde Auflösung aufgrund der geringen Anzahl an Substitutionen in der ITS-Region erklärt werden. Hybridisierungsereignisse, die für Arten innerhalb des Artkomplex *C. megalonyx* nachgewiesen sind, wurden hier nicht gefunden.

Während die erste Studie nur auf zwei einzelnen genetischen Markern basierte, wurde in der zweiten Studie dieser Arbeit ein genomischer Datensatz mit tausenden von Gensequenzen für Proben des *P.-patagonica*-Artkomplexes generiert. Dafür wurde eine neue Hochdurchsatz-Sequenzierungsmethode angewandt, die durch Hybridisierung gegen bekannte Genabschnitte zielgerichtet kodierende Genombereiche anreichert. Von den 1607 potentiell anreicherbaren EOGs (Eukaryotische Orthologiegruppen, basierend auf wahrscheinlich nur einfach im Genom vorliegenden Genen mit bekannter Homologie durch Vergleich mit anderen Genomen) wurden für den Artkomplex *P. patagonica* 821 EOGs erfolgreich sequenziert. Die Ergebnisse der phylogenomischen Analysen lösten die Verwandtschaftsverhältnisse der bereits dokumentierten Abstammungslinien weiter auf und bekräftigen diese durch höhere

Unterstützungswerte. In Kombination mit umfangreichen morphologischen und morphometrischen Analysen war die Abgrenzung von mindestens acht Arten möglich. Dazu gehören die zwei neuen Arten, *P. aulaeosmanorum* sp. nov. Dömel & Melzer, 2019 und *P. obstaculumsuperavit* sp. nov. Dömel, 2019 die durch den integrativen Datensatz beschrieben werden konnten. Die genomischen und morphologischen Daten zeigten keine Hinweise für positive Selektion auf, sodass eine Artbildung in allopatrischen Glazialrefugien aufgrund genetischer Drift das wahrscheinlichste Szenario darstellt.

In der dritten Studie wurde der Hybridisierungsansatz auf den zweiten Asselspinnen-Artkomplex *C. megalonyx* angewendet. Alle 1607 EOGs konnten angereichert werden und ein phylogenetischer Baum mit hohen Unterstützungswerten wurde basierend auf den genomischen Daten generiert. Die Analysen unterstützten die bereits bekannten Abstammungslinien, sowie das Auftreten mito-nukleären Diskordanzen. Auch hier wurden eine bessere Auflösung und höhere Unterstützungswerte erhalten. Mit Hilfe der Analyse von Einzelsubstitutionen (SNPs), die aus den EOGs und deren variableren Nachbarregionen abgeleitet wurden, wurde auch die genetische Diversität und Struktur innerhalb einzelner Arten untersucht. Die Ergebnisse belegen einen eingeschränkten Genfluss zwischen geographisch getrennter Populationen. Morphometrische Analysen konnten signifikante Unterschiede zwischen mehreren der genetisch distinkten Abstammungslinien belegen, allerdings waren die Merkmale selten diagnostisch, d.h. eine klare Auftrennung war schwierig. Es wurden Hinweise auf positive Selektion, vor allem in Gene die mit strukturellen und neuronalen Funktionen zusammenhängen, gefunden. Somit gibt es Hinweise auf das Wirken positive Selektion bei dem Artkomplex *C. megalonyx*, aber ihr spezifischer Beitrag zum Artbildungsprozess muss noch weiter untersucht werden.

In der letzten Studie wurde die Phylogenie der Gruppe der im Südpolarmeer besonders häufig vorkommenden longitarsalen Colossendeidae, zu der auch der Artkomplex *C. megalonyx* gehört, mit den 1607 EOGs untersucht. Eine hoch-aufgelöste und statistisch gut unterstützte Phylogenie der Gruppe, die weitgehend mit morphologischen Daten übereinstimmt, wurde erhalten. Die Daten weisen auf einen antarktischen Ursprung der longitarsalen Colossendeidae und zahlreiche Ausbreitungsereignisse in andere Regionen in unterschiedlichen Zeitabständen hin. Dieses Szenario verdeutlicht die Rolle des Südpolarmeeres als Ursprungszentrum auch für nicht-antarktische Arten.

Der Vergleich der Analysen basierend auf Einzelgenen (große Unterschiede zwischen Markern, geringe Auflösung und Unterstützung) im Vergleich zu bis zu tausenden EOG-Sequenzen unterstreicht den Nutzen des Hybridisierungsansatzes, um alte und rezente Artaufspaltungsereignisse zu identifizieren. Die Analysen morphologischer Merkmale belegten große inter- und intraspezifische Variationen für beide Artkomplexe, auch innerhalb distinkter Abstammungslinien.

# General introduction

Speciation is a fundamental source of biodiversity. Increasing biodiversity stabilizes ecosystems, while the loss of biodiversity weakens the adaptability of ecosystems to environmental changes (Chapin III et al. 2000, Novacek and Cleland 2001). Biodiversity in marine ecosystems is threatened by a range of anthropogenic factors, e.g. climate change, pollution, habitat destruction and overfishing (reviewed in Costello et al. 2010). To monitor and predict ongoing and future changes, and to ultimately preserve and sustainably manage these highly diverse ecosystems, a better understanding and documentation of marine biodiversity is necessary. Exploring genetic variation, especially due to the establishment of DNA-barcoding (Hebert et al. 2003), has improved the ability to assess biodiversity. However, the mechanisms responsible for generating biodiversity often remain poorly understood, although it is certain that abiotic as well as biotic factors are relevant (Willig et al. 2003, Allen and Gillooly 2006, Schluter and Pennell 2017). Difficulties in detecting speciation mechanisms arise, in particular, for speciation in natural marine environments in contrast to speciation experiments in defined ambiences that allow controlling at least some impact factors, e.g. abiotic factors such as nutrients and temperature or biotic factors such as food and predation. A unique 'natural laboratory' to assess processes that led and lead to high species diversity is the Southern Ocean (Brandt 1991, Clarke and Crame 1989, Savoye et al. 2008), because it is the most isolated, most difficult to access, and hence least affected marine ecosystem on earth.

## Speciation in marine benthos of the Southern Hemisphere

The marine benthos of the Southern Hemisphere is highly rich in terms of species number and unique with respect to species composition. Especially, the Southern Ocean, i.e. the water south of 60°S, hosts diverse and highly endemic benthos communities that have evolved over tens of millions of years (Knox and Lowry 1977, Clarke and Johnston 2003). This biodiversity consists partly of species that persisted in the Antarctic after the opening of the Drake Passage during the Miocene (about 30 mya) and subsequent establishment of the Polar Front inducing a decrease in temperature, but in larger parts, it arose from the outcome of *in situ* radiations in this cold environment (see Briggs 2003). After major taxonomic groups that could not survive the cooling went extinct, species that had successfully adapted to the changing and extreme conditions could inhabit available habitats (see Knox and Lowry 1977, Clarke et al. 2004 for an overview). Prominent examples include Antarctic icefishes (Notothenioidei, Eastman and McCune 2000), octopuses (Strugnell et al. 2012), and serolid isopods (Brandt 1991, Held 2000; see Convey et al. 2009 and Figure 1 for an overview).

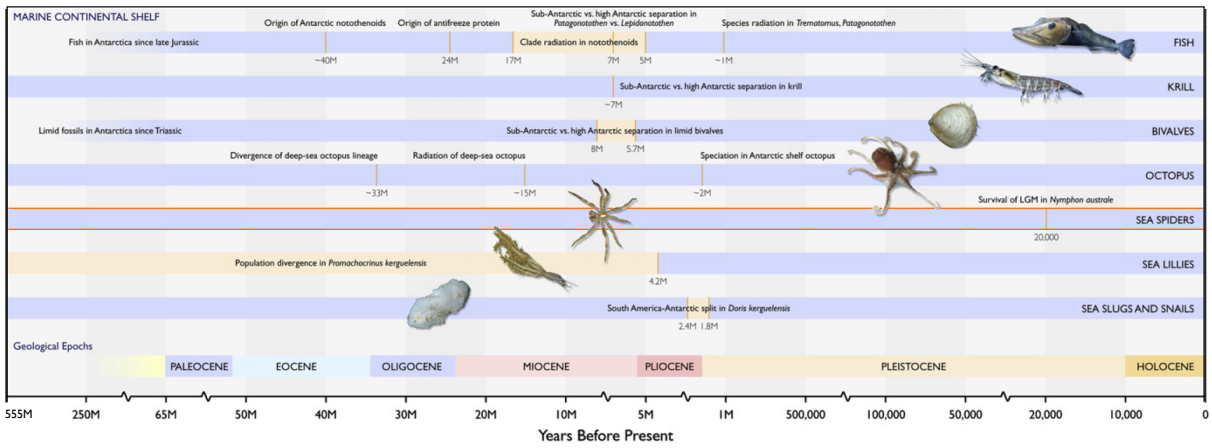


**Figure 1**: Schematic illustration of the supposed evidence for continuous presence of major biological taxa (including sea spiders) of the Southern Ocean since the break-up of Gondwana. Modified after Figure 8 from Convey et al. (2009) in that only marine organisms are shown.

In addition to those radiations during the Miocene, molecular studies have revealed a high number of previously overlooked and more recent divergences during the Pleistocene and late Pliocene (especially 1 to 5 mya) in the Southern Ocean (Held and Wägele, 2005, Raupach and Wägele 2006, Leese and Held 2008, Krabbe et al. 2010, Schüller 2011, Baird et al. 2012, Havermans et al. 2013, Dietz et al. 2015, Galaska et al. 2016; for further discussion, see Janosik and Halanych 2010 as well as Convey et al. 2012), and also at lower latitudes (Leese et al. 2008, Weis and Melzer 2012). As many of these recently diverged species seem to show little or no morphological differentiation and were first detected with the use of genetic markers such as the mitochondrial cytochrome c oxidase subunit 1 gene (CO1), they are often referred to as "cryptic species" (see Held and Leese 2007, Janosik and Halanych 2010, Allcock and Strugnell 2012). The identification of cryptic species has increased the perception of the benthic biodiversity of the Southern Hemisphere. Furthermore, phylogeographic studies have shown that the distribution of many cryptic species is patchy and narrow (Held 2003, Held and Wägele 2005, Wilson et al. 2007, Krabbe et al. 2010). In combination with signatures of population expansion (Leese et al. 2008, Raupach et al. 2010, Strugnell et al. 2012, González-Wevar et al. 2013) and consistent with recent geological data (Anderson et al. 2002, Thatje et al. 2005, 2008, Försterra 2009), these distribution patterns have been interpreted as results of speciation in independent glacial refugia. However, there are different hypotheses that need to be considered especially with respect to speciation of the benthic Southern Ocean fauna, with the most contrary ones being the survival of glacial periods *ex situ* or *in situ*.

The survival of marine life outside of Antarctica (*ex situ*) on the shelves of neighbouring islands or continents seems plausible because grounded shelf ice covered major areas of the Antarctic continental shelf making it uninhabitable for benthic communities (Convey et al. 2009). Support from genetic data for such a scenario was found, e.g. for the shallow water shrimp

*Chorismus antarcticus* (Pfeffer, 1887) which displays very low diversity without differentiation among locations in the Southern Ocean (Raupach et al. 2010). This homogeneous pattern was explained by a limited number of colonists during the recolonization of Antarctica from sub-Antarctic islands (i.e., founder effect). Furthermore, a couple of studies proposed that the sub-Antarctic island South Georgia served as refugia. In the sea spider *Colossendeis megalonyx* Hoek, 1881 that shows high genetic divergence, one lineage (clade A) shared the same mitochondrial haplotype between samples from South Georgia and the Antarctic continental shelf. More importantly, though, is that haplotype diversity was greater in sub-Antarctic compared to Antarctic samples indicating a survival *ex-situ* prior to recolonization of the Antarctic continental shelf (Dietz et al. 2015). A similar pattern was found for the limpet *Nacella concinna* (Strebel, 1908) (González-Wevar et al. 2013). However, especially for species that show small dispersal capabilities, such as benthic brooders, and are restricted and endemic to the Antarctic continental shelf nowadays, a long-term persistence within the Antarctic (*in situ*) is suggested (e.g. Thatje et al. 2005, Fraser et al. 2012). Three hypotheses about how the species could have survived *in situ* are discussed in the literature:

i) *Survival in the Southern Ocean deep-sea:* The deep shelf of the Antarctic continent and periodic destruction of benthic shelf habitats during glacial cycles could have promoted eurybathy, i.e. life in a broad range of depth from shallow waters to the deep-sea. Many species of the Southern Ocean benthos are reported to be eurybathic, e.g. the sea spider *Nymphon australe* Hodgson, 1902 with a reported depth range extending to below 4000 m (Arango et al. 2011) and the deep-sea shrimp *Nematocarcinus lanceopes* Bate, 1888 (Raupach et al. 2010). However, especially for species that are currently not found in greater depths, an escape to the deep-sea followed by a complete re-emergence onto the shelf is unlikely.

ii) *Survival on the Antarctic continental slope:* The slope has been poorly explored but accumulations of detritus that has been moved off the shelf due to glacial motion and expansion of grounded ice sheets, have been suggested to make this habitat unattractive for many benthic invertebrates (Thatje et al. 2005).

iii) *Survival on the Antarctic continental shelf*: During glacial maxima, large parts of the Antarctic shelf were covered by grounded ice. Accessible parts would have experienced strong iceberg scouring, destroying most available habitats for benthic biota. In addition, thick multi-annual pack ice would have limited the primary production and food availability. Hence, survival on the shelf has long been considered unlikely. However, several studies suggest that small ice-free refugia due to diachronous ice-sheet advance and contraction existed and provided opportunities for relict populations to survive on the Antarctic continental shelf (Thatje et al. 2005, 2008, Convey et al. 2009, Fraser et al. 2012). In addition, geothermally

active regions have been postulated for Antarctica, providing even more support for available habitats on the Antarctic continental shelf during glacial maxima (Fraser et al. 2014).

In fact, there is strong evidence for both, the survival of populations on the continental shelf and in deep-sea refugia (Allcock and Strugnell 2012). Reduced population size (especially for populations that survived on the continental shelf) in combination with the assumed limited dispersal potential of many of the holobenthic species (see Thatje 2012 for review), may have promoted the assemblage of genetic changes in isolated populations due to the enhanced probability of bottleneck effects (see Allcock and Strugnell 2012 for discussion). Hence, in the Southern Hemisphere, recent divergences are regarded to be the result of allopatric speciation in multiple glacial refugia (Allcock and Strugnell 2012, Strugnell et al. 2012) with random genetic drift being the main driver of the "diversity pump" (Clarke and Crame 1989, 1992). However, this assumption has never been tested systematically or at least compared against an alternative model in which speciation, particularly in sympatry, has occurred due to ecological diversification ("ecological speciation", Dieckmann et al. 2004, Rundell and Price 2009) (Figure 2).

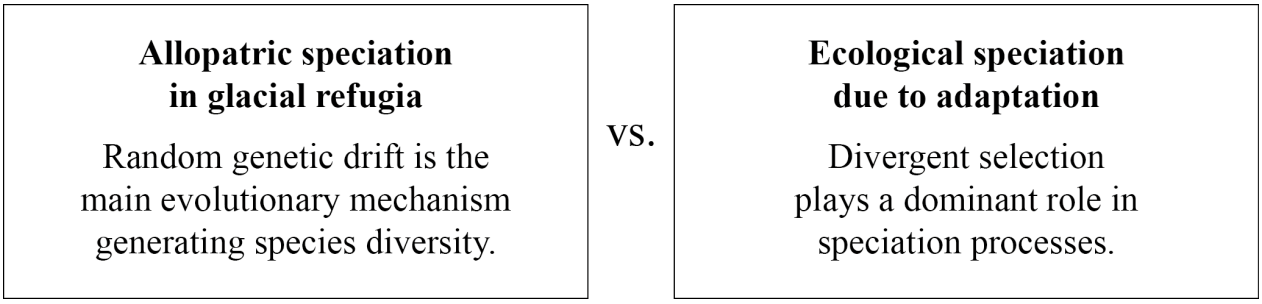


**Figure 2:** Main speciation hypotheses of this thesis.

So far, the role of ecological selection in species divergence processes in the marine environment of the Southern Hemisphere has been poorly studied. One previous study on the Southern Ocean sea slug *Doris kerguelenensis* (Bergh, 1884) considered that interspecific competition (chemical defence against predators) could be involved in speciation (Wilson et al. 2009). Rutschmann et al. (2011) tested whether ecological specialisations into different feeding niches promoted adaptive radiation in notothenioid fishes, and found lineage-independent, ecological differentiation, i.e. food specialisation.

The lack of systematic comparative tests of different speciation models in the Southern Ocean reflects the need for appropriate analyses. With the emergence of high-throughput sequencing technologies (e.g. Illumina; see Metzker 2010), it is now feasible to address ecological and evolutionary questions even in non-model species from a genomic perspective. Ideal candidate species for such an approach should be abundant and typical inhabitants of the Antarctic benthic community. Such taxa often show a holobenthic, brooding lifestyle (Poulin et al. 2002) and

thus have low dispersal capabilities. In this respect, species of sea spiders, or pycnogonids, represent good research candidates.

## Speciation in Southern Ocean sea spiders

Sea spiders (Arthropoda; Chelicerata; Pycnogonida) are extremely diverse in the Southern Hemisphere with high rates of endemism in the Southern Ocean (108 of the 264 reported Antarctic species, Munilla and Soler-Membrives 2009). Sea spiders have comparatively low dispersal rates due to their generally holobenthic lifecycle, during which males carry the eggs and early protonymphon larvae with their ovigers (Arnaud and Bamber 1987). The classical morphology-based taxonomy of Southern Ocean pycnogonids has been well addressed (e.g., Hoek 1881, Calman 1915, Gordon 1932, Fry and Hedgpeth 1969, Pushkin 1993, Child 1995). Nonetheless, many sea spider species from the Southern Hemisphere show high intraspecific genetic distances, e.g. *Achelia assimilis* (Haswell, 1885), *Austropallene cornigera* (Möbius, 1902) and *Nymphon australe,* and several are even proposed to represent complexes of multiple species, e.g. *Colossendeis megalonyx* and *Pallenopsis patagonica* (Hoek, 1881) (Mahon et al. 2008, Krabbe et al. 2010, Weis and Melzer 2012, Weis et al. 2014, Dietz et al. 2015, Dömel et al. 2015). The last two species are widely distributed in Antarctic and sub-Antarctic waters and have members on both sides of the Polar Front, with the northernmost records for *C. megalonyx* at 36°S, and 33°S for *P. patagonica*. In fact, *C. megalonyx* even has the broadest distribution range reported for a pycnogonid in the Southern Ocean (Griffiths et al. 2011). Thus, both species complexes are found along a wide environmental gradient, with genetic data suggesting that *C. megalonyx* has an Antarctic and *P. patagonica* a sub-Antarctic origin (Dietz et al. 2015, Weis et al. 2014). Hence, they represent ideal candidates to compare genomic and morphological signatures of selection promoted by differing impacts of glacial and interglacial cycles.

## Assessing drivers of speciation

Morphological changes, especially ecological character displacement, can occur rapidly in ecological speciation processes (Figure 3; Tautz 2004). Many examples of speciation that include rapid phenotypic changes have been described, e.g. differentiation of the feeding apparatus and colour variation in sympatric cichlid species from lakes in Africa and Central America (e.g. Kocher 2004), and diversification of several morphological traits in three-spined sticklebacks (Hohenlohe et al. 2010, Jones et al. 2012, and references within). Also for the sea spider species complexes *P. patagonica* and *C. megalonyx* potentially adaptive morphological traits have been reported, e.g. difference in shape of proboscis for a species within the

*P. patagonica* species complex, and unique ocular tubercles with rudimentary eyes that lack pigments for a lineage (clade C) within the *C. megalonyx* species complex (Spaak 2010, Weis et al. 2014). However, phenotypic traits under selection are not always easy to identify and the correlation between characters can make the detection even more difficult. Hence, common analyses benefit from genomic data which can be used to test for selection, e.g. by either comparing intraspecific and interspecific variability or the number of non-synonymous against synonymous mutations (dN/dS) in protein-coding genes either per site or set into context with phylogenetic trees (see Nielsen et al. 2005 for review). Comparison of nucleotide diversity and the investigation of differentiation patterns can yield a profound understanding of adaptation processes (e.g. Hohenlohe et al. 2011, Krehenwinkel and Tautz 2013). Detailed insights into adaptation and speciation processes have been achieved in laboratory studies of genomic model organisms such as *Drosophila* or mice (Ihle et al. 2006, Staubach et al. 2012). Further findings, include positive selection, i.e. more non-synonymous mutations than expected under neutral evolution (dN/dS > 1), on traits that are, for example, related to mating such as odorant receptors in orchid bees (Brand et al. 2015) or thermal adaptation and acclimation to heat in rainbow trout (Narum et al. 2013). In sea urchins, positive selection promoted great divergences in habitats for adults (shallow waters vs. deep-sea) but not for larval stages (Oliver 2010). Altogether, several recent studies have documented a large contribution of selection in speciation processes (Schluter 2000, Dieckmann et al. 2004, Coyne and Orr 2004), which makes it even more essential to start investigating adaptive divergence as a potential driver for speciation in benthic organisms of the Southern Hemisphere.

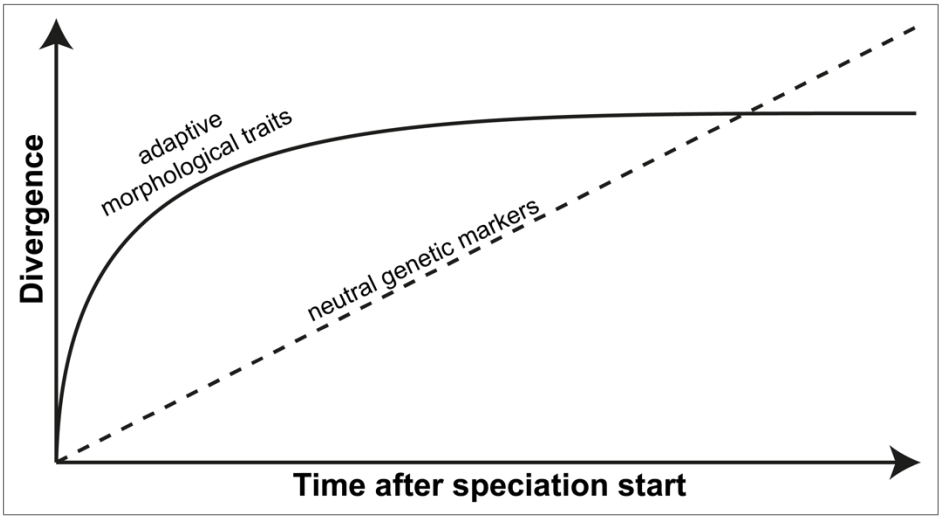


**Figure 3:** Morphological divergence due to ecological selection can develop rapidly during ecological speciation processes ("ecological character displacement"), whereas neutral genetic markers accumulate mutations steady over time. Also, adaptive genetic variation such as non-synonymous mutations accumulates much faster than excepted according to neutral evolution. Modified after Tautz (2004).

## A new generation of genomic analyses

When dealing with difficult biological phenomena such as cryptic species, recent radiation events especially in cases of incomplete lineage sorting (i.e. ongoing speciation) and hybridization, analyses based on commonly used single markers (such as COI) encounter the problem of correctly distinguishing and defining species (e.g. Meyer and Paulay 2005, Lemmon et al. 2012, Dietz et al. 2015). The large and often unknown genetic diversity within species complexes makes it difficult to design primers (e.g. for microsatellite markers) that i) amplify all lineages, and ii) are variable and informative enough to distinguish species. For the Southern Ocean sea spider *Austropallene cornigera*, a species showing high regional differentiation due to very limited gene flow between populations (Dömel et al. 2015), specifically designed primers for a microsatellite approach were tested, but amplified sequences were either too conserved and did not show any variation or genetic regions were too variable and primers did not bind reliably (unpublished data).

With the establishment of "high-throughput sequencing" (HTS) (previously referred to as "next-generation sequencing"; Shendure and Ji 2008), sequence data can be generated at comparably low costs. Consequently, the number of methods to obtain genomic data and bioinformatic pipelines for the analyse of those data has drastically increased over the last years. Full genome sequencing is possible even for non-model organisms and may be the ideal method to apply any genomic analyses (e.g. Ellegren 2014, Nater et al. 2015), but the sequencing costs for multiple samples and especially the challenges to assemble sequences, limit its applications. Transcriptomic analyses are less expensive (e.g. Morin et al. 2008, Jiménez-Guri et al. 2013, Lemer et al. 2015), but the requirement, i.e. high-quality RNA, cannot always be met (e.g. by Antarctic samples with an interrupted cooling chain or old samples from museum collections). Suitable alternatives that require fewer sequences aim to cover a high abundance of genome-wide data. In recent years, restriction site associated DNA sequencing (RAD-seq) was the most commonly used method to analyse single-nucleotide polymorphisms (SNPs) (e.g. Boehm et al. 2015, Suchan et al. 2016, Weigand et al. 2017, Xu et al. 2017). RAD-seq belongs to a range of methods that uses restriction enzymes to digest genomic DNA and analyses specific DNA fragments by subsequent HTS (Baird et al. 2008). As no prior information about the genomic position of loci is needed, RAD-seq can provide a higher and more informative number of neutral markers than traditional mitochondrial gene sequencing or microsatellite markers. Hence, it represents a convenient approach that is widely used to generate genomic datasets for non-model organisms (reviewed by Narum et al. 2013). Several studies have demonstrated the successful application of RAD-seq for species delimitation and studying speciation within species (e.g. Alterman et al. 2014, Weiss et al. 2018, but see detailed list in Lowry et al. 2016

and a summary in McKinney et al. 2016). In contrast, limitations of this technique are that due to fixed mutations loci are not always recovered across distinct species which reduces the number of loci drastically

when analysing different species (e.g. Macher et al. 2015). DdRAD (double-digest RAD, a specific RAD-seq application, Peterson et al. 2012) has been tested for the Antarctic sea spider *Nymphon australe*. In the beginning, the choice of restriction enzymes was difficult due to the lack of genomic background information and also markers obtained were inconsistent and hence uninformative (Dömel 2013). However, another population genomic approach using 2b-RAD successfully recovered more than 3000 single nucleotide polymorphisms (SNPs) for the same species afterwards (Collins et al. 2018).

Another family of HTS methods that allows to rapidly capture hundreds of loci and is useful for both shallow and deep phylogenetic analyses is hybrid enrichment. This method is based on the hybridization of DNA fragments with synthetic oligonucleotides, so-called baits, with subsequent HTS (Gnirke et al. 2009; Figure 4). Harvey et al. (2016) compared RAD-seq and hybrid enrichment, in terms of their applicability in non-model organisms and described a broad concordance. They concluded that hybrid enrichment should be preferred over RAD-seq for intraspecific approaches that use low quality (i.e. highly fragmented) DNA samples (e.g. Guschanski et al. 2013, Blaimer et al. 2016), because of its efficiency to capture also small fragments of targeted regions. Furthermore, the co-enrichment of genes but also the likely enrichment of flanking, i.e. non-coding, regions, offer immense potential to generate data sets for shallow and deep phylogenetics as well as selection analyses. Especially, when analysing ancient radiation RAD-seq typically fails (e.g. Macher et al. 2015). First studies have shown that hybrid enrichment can serve as a robust background for genomic studies (e.g. Abdelkrim 2018) and offers new information about the orthology of genes (Teasdale et al. 2016). The development of the software BaitFisher (Mayer et al. 2016) that infers baits from multiple nucleotide sequence alignments, makes it possible to apply hybrid enrichment to a wide range of species and even to non-model species. Thus, this method which generates thousands of genome-wide markers for multiple specimens in a single run is highly promising to cover multiple aspects of our study.

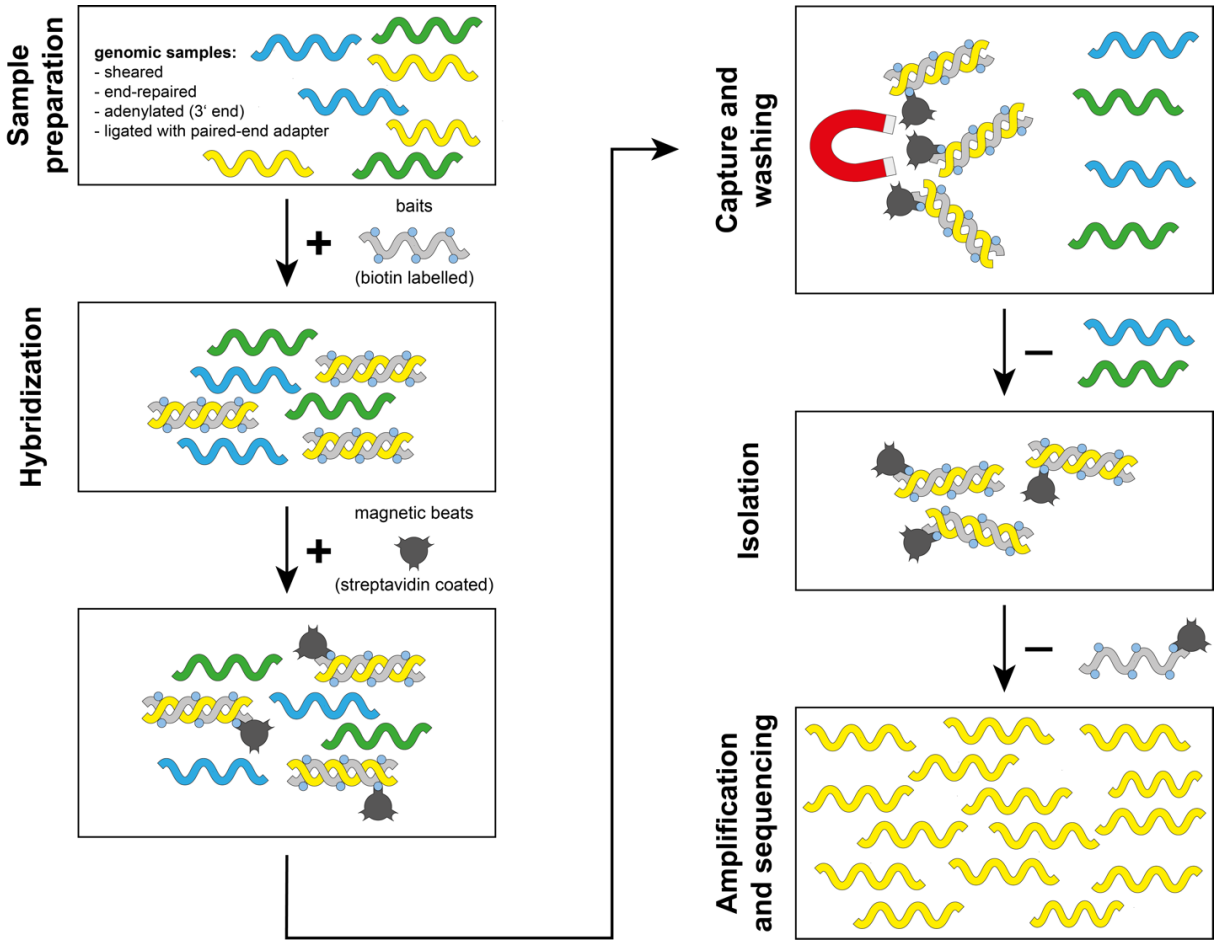


**Figure 4:** Schematic illustration of the laboratory workflow for target hybrid enrichment using biotin labelled baits. Baits (grey) hybridise with targeted fragments (yellow) and are subsequently isolated from non-target fragments (green and blue). Target fragments are amplified before sequencing.

# Aims and outline of this thesis

Despite a growing understanding of the diversity of benthic communities in the Southern Hemisphere, little effort has been made to systematically analyse speciation factors that led to this species-rich environment. Hence, the aim of this thesis was to evaluate diversity within the two sea spider species complexes *Pallenopsis patagonica* and *Colossendeis megalonyx* from the Southern Hemisphere and assess processes potentially leading to recurrent speciation events. For the latter, lineage sorting in independent refugia predominantly induced by genetic drift and adaptive evolution also in sympatry were considered as the two main mechanism. In this context, morphological and genetic patterns between the distinct lineages were assessed and compared. In case of ecological character displacement promoted by positive selection, morphological differences relevant for adaptation to feeding strategies or habitats, e.g. in shape and structure of proboscidea, cheliphores or walking legs, were expected. Also, differences in genes under positive selection were assessed. First, genes were investigated for signatures of positive selection among closely related lineages that also occurred in sympatry. Afterwards genes under selection were analysed and assign to particular functions. Finally, signatures of both sea spider species complex were compared to each other.

As sea spiders are non-model organisms, morphometric analyses, genetic methods and bioinformatics tools to analyse thousands of sequences had to be established first. Afterwards, patterns of genetic divergences and morphological variation within species were studied.

## Aims of this thesis

1. Development of laboratory workflow and bioinformatic analyses to study speciation processes and drivers based on a target hybrid enrichment approach for sea spiders focussing on following taxa:
    a. *Pallenopsis patagonica*
    b. *Colossendeis megalonyx*
    c. longitarsal Colossendeidae

2. Analyses and discussions of genetic and morphological variation in the context of speciation processes addressing the following questions:
    a. *Pallenopsis patagonica*
        i. Can a higher species diversity within the *P. patagonica* species complex be detected when extending the sample set and adding genome-wide data?

ii. Can independently evolving lineages in the *P. patagonica* species complex be distinguished and new species formally be described based on morphometric measurements and morphological characters?
iii. Is there evidence for adaptive divergence especially for lineages that occur in sympatry or do neutral evolutionary processes suffice to explain the observed species diversity within the *P. patagonica* species complex?

b. *Colossendeis megalonyx*
   i. Can a higher species diversity within the *C. megalonyx* species complex be detected when adding genome-wide data?
   ii. Can genome-wide data resolve the level of intraspecific connectivity between population of phylogenetic lineages within the *C. megalonyx* species complex are connected?
   iii. Do lineages within the *C. megalonyx* species complex show differences between morphological characters which enable to distinguish them?
   iv. Are there distinct signatures of positive selection indicating adaptive divergence within the *C. megalonyx* species complex?

c. longitarsal Colossendeidae
   i. Do genome-wide data of the group of longitarsal Colossendeidae resolve the groups phylogeny and serve as backbone for biogeographic analyses?
   ii. Do Antarctic species within the group constitute a monophyletic radiation and thus support an out-of-Antarctica scenario for at least some non-Antarctic species?

## Outline of this thesis

This thesis consists of four chapters corresponding to scientific publications and manuscripts on the evolutionary history of sea spiders from the Southern Hemisphere.

Study design and method development are based on solid background information about species diversity within the species complexes *Pallenopsis patagonica* (chapter I) and *Colossendeis megalonyx* (Dietz et al. 2015). Establishment of laboratory workflow and bioinformatic tools to analyse huge sequencing data sets was performed using both species complexes (chapter II and III). Results provided insights into recent evolutionary processes of the species complexes *P. patagonica* (chapters I and II) and *C. megalonyx* (chapter III). Furthermore, morphometric analyses were conducted for both species complexes highlighting the enormous morphological variation within the species complexes (chapter II and III). Still, two new Southern Ocean sea spider species were described for *Pallenopsis* (chapter II). Finally, genetic methods were applied to a broader evolutionary scale to resolve the taxonomy of species within the longitarsal Colossendeidae (chapter IV).

# CHAPTER I

# Nuclear and mitochondrial gene data support recent radiation within the sea spider species complex *Pallenopsis patagonica*

Jana S. Dömel, Roland R. Melzer, Avril M. Harder, Andrew R. Mahon and Florian Leese

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**Contributions to this manuscript:**

Experimental design and planning: 50%

Laboratory work: 90%

- DNA extraction for 90% of added specimens
- COI sequencing for 100% of added specimens
- ITS sequencing for 90% of specimens

Data analysis: 95%

- Sequence analysis: 95%
- Phylogenetic reconstruction of COI tree: 100%
- Phylogenetic reconstruction of ITS tree: 80%
- ABGD, GMYC and bPTP analyses: 100%
- Barcode gap analyses: 100%

Figures: 70%

- Figure 1: 90%
- Figure 2: 100%
- Figure 3: 50%
- Figure 4: 100%
- Supplementary Material Figure S1: 0%

Tables: 100%

Manuscript writing: 60%

- First draft: 70%
- Editing of first draft and revising after comments in the review process together with the other authors.

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# Nuclear and Mitochondrial Gene Data Support Recent Radiation within the Sea Spider Species Complex *Pallenopsis patagonica*

***Jana S. Dömel*[1]*, *Roland R. Melzer*[2,3,4], *Avril M. Harder*[5], *Andrew R. Mahon*[5] and *Florian Leese*[1,6]**

[1] *Faculty of Biology, Aquatic Ecosystem Research, University of Duisburg-Essen, Essen, Germany,* [2] *Zoologische Staatssammlung München, Munich, Germany,* [3] *Faculty of Biology, Biocenter, Ludwig-Maximilians-University Munich, Munich, Germany,* [4] *GeoBioCenter, Ludwig-Maximilians-University Munich, Munich, Germany,* [5] *Department of Biology, Institute for Great Lakes Research, Central Michigan University, Mount Pleasant, MI, USA,* [6] *Center for Water and Environmental Research, University of Duisburg-Essen, Essen, Germany*

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***Correspondence:**
*Jana S. Dömel*
*jana.doemel@uni-due.de*

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The climate history of the Antarctic continental shelf has formed a diverse benthic ecosystem over evolutionary time scales. The extent of faunal diversity has only recently been unveiled especially by using genetic data. In addition to newly reported species, known species of benthic invertebrates in the Southern Ocean turned out to be in fact species complexes representing genetically very distinct clades. Previous studies have shown that the sea spider *Pallenopsis patagonica* is such a species complex consisting of several divergent mitochondrial clades. However, genetic analyses of another sea spider complex, *Colossendeis megalonyx,* showed that looking at one mitochondrial gene only can lead to overestimation of species number within a species complex and revealed mito-nuclear discordances. In this study we expand the current data set of *P. patagonica* by adding not only samples from Patagonia, the Subantarctic and the Eastern Weddell Sea, but also sequence data for the nuclear internal transcribed spacer (ITS) region to obtain more information about the species complex. In fact, the number of distinct clades is reduced when looking at nuclear data, but there are no cases of mito-nuclear discordance and hence no evidence for hybridization and speciation reversal events between divergent mitochondrial clades as in *C. megalonyx*. As patterns of mitochondrial COI diversity and divergence within *P. patagonica* and *C. megalonyx* are very similar and molecular dating analyses of both species complexes suggest a recent separation of clades during the Pleistocene, different biological processes seem to have led to fast and stable species boundaries in *P. patagonica* as opposed to *C. megalonyx* where hybridization even across major mitochondrial lineages occured.

**Keywords: Southern Ocean, Chelicerata, Pycnogonida, DNA-barcoding, ITS, cryptic species**

## INTRODUCTION

Increased sampling of Southern Ocean habitats and the application of molecular taxonomy uncovered that Antarctic biodiversity has been drastically underestimated (Gutt et al., 2004; De Broyer and Danis, 2011; De Broyer et al., 2014). The main reason for the significant boost in number of newly found species is the detection of morphologically cryptic species in basically all studied taxonomic groups (see Janosik and Halanych, 2010; Kaiser et al., 2013 for reviews). Therefore, paradoxically the Southern Ocean has emerged from being regarded as a biodiversity sink to a center of marine biodiversity in the past two decades. This phenomenon of high *in situ* species diversity has been termed the Antarctic diversity pump (sensu Clarke and Crame, 1989).

Several processes have been discussed as drivers fueling this diversity pump (Clarke and Crame, 1989). In this context, molecular data have led to a paradigm shift: Most of the cryptic species have rendered the distribution ranges of formerly described species from broad (i.e., circum-Antarctic) to small and allopatric (Lörz et al., 2009; Held, 2014). As the timing of many of these divergence events was rather recent in the Plio- or Pleistocene (see Convey et al., 2009 for a review), an influence of the recurrent large-scale glaciations in these periods has been suggested as a main driver fueling the diversity pump, mainly through random genetic drift and lineage sorting in independent glacial refugia (Thatje et al., 2005, 2008; Allcock and Strugnell, 2012). Prominent signatures of population bottlenecks, in particular for shallow-water organisms, have supported that view (e.g., Janko et al., 2007; Raupach et al., 2010).

In this context, Pycnogonida or sea spiders have attracted particular attention, since they show an exceptionally high species diversity in the Southern Ocean (Clarke and Johnston, 2003; Munilla and Soler Membrives, 2009; Griffiths et al., 2011). Moreover, many pycnogonids are benthic brooders with probably limited dispersal capacity. Therefore, lineage sorting events in glacial refugia driving speciation should have been stronger than in other, free-spawning taxa (Allcock and Strugnell, 2012).

In agreement with these predictions, several molecular studies have reported evidence for overlooked diversity in various sea spider species (Mahon et al., 2008; Krabbe et al., 2010; Dietz et al., 2015a,b). Furthermore, intraspecific diversity was found to be significantly partitioned regionally, indicating limited gene flow (Arango et al., 2011; Dietz et al., 2015b; Dömel et al., 2015). Two species complexes stand out by far in terms of their identified numbers of mitochondrial clades revealed by classical DNA barcoding (amplification of cytochrome c oxidase subunit I gene, COI): *Colossendeis megalonyx* (Hoek, 1881) (Krabbe et al., 2010; Dietz et al., 2015b) and *Pallenopsis patagonica* (Hoek, 1881) (Weis et al., 2014; Harder et al., 2016). For *C. megalonyx*, Krabbe et al. (2010) reported the presence of six distinct mitochondrial lineages that likely represent cryptic species with mostly small and allopatric distribution ranges. However, extending the sampling range substantially Dietz et al. (2015b) revealed a much greater number of mitochondrial clades. These also showed mostly circum-Antarctic distribution instead of regional partitioning. Similar findings were made for the crinoid *Promachocrinus kerguelensis* [Wilson et al. (2007): restricted distribution range; Hemery et al. (2012): circumpolar distribution range with extended data set]. Interestingly, analyses of a nuclear gene of *C. megalonyx* indicated that several of the mitochondrial clades do not represent distinct species as they had identical sequences for the otherwise highly variable nuclear internal transcribed spacer region (ITS; Dietz et al., 2015b). This suggests hybridization events between several mitochondrial clades and subsequently speciation reversal after the completion of mitochondrial lineage sorting within *C. megalonyx*.

For *P. patagonica*, Weis et al. (2014) showed that it is also a species complex as has been anticipated by Gordon (1944) and Pushkin (1975, 1993), and described a new species (*P. yepayekae* Weis, 2014 in Weis et al., 2014) using integrative taxonomy combining mitochondrial sequences with morphological characters. Harder et al. (2016) found evidence for even more diversity within the complex by adding further mitochondrial data specifically for Antarctic specimens. As Dietz et al. (2015b) have shown, only looking at mitochondrial data can lead to an overestimation of species number. Therefore nuclear data are needed for *P. patagonica* to explicitly test whether the identified mitochondrial clades reported by Weis et al. (2014) and Harder et al. (2016) are supported by such independent markers. Thus, in this study we analyzed both mitochondrial and nuclear data of the *P. patagonica* complex for a substantially extended data set as compared to Weis et al. (2014) and Harder et al. (2016). We hypothesized that (i) extending the data set for *P. patagonica* by new samples, specifically from previously unsampled locations, reveals further distinct mitochondrial clades, (ii) the number of distinct species is substantially smaller than the number of mitochondrial lineages when analyzing an independent nuclear gene marker, and (iii) the extended data set reveals broader distribution ranges for previously reported clades. Moreover, we addressed the significance of our results in the context of currently discussed evolutionary mechanisms generating Southern Ocean benthic diversity.

## MATERIALS AND METHODS

### Specimens and Sampling Sites

For the remainder of this study, we use the term *P. patagonica* sensu lato (s.l.) when referring to the whole species complex including *P. yepayekae*, because it groups within clades morphologically originally identified as *P. patagonica*. Individuals of *P. patagonica* s.l. from the shelf of South America, Subantarctic islands as well as around the Antarctic continent were analyzed (**Table 1**, **Figure 1**). Chilean specimens were collected by divers during Huinay Fjordos expeditions (HF16, HF21, HF24, and HF26). Falkland samples (ZDLT1) were provided by Vladimir Laptikhovsky (Falkland Islands Fisheries Department, Stanley, Falkland Islands). Samples from the Southern Ocean were collected using different bottom trawls during several cruises on board the RRS *James Clark Ross* (British Antarctic Survey, Cambridge, UK) and the RV *Polarstern* (Alfred Wegener Institute Helmholtz

**TABLE 1 | Specimens list for *P. patagonica* s.l. and outgroups used during study. Species names are only given for outgroups and *P. yepayekae* as other morphological determinations are as yet not possible. Sampling details (location, latitude, longitude, depth) and haplotype information for the specimens analyzed (molecular clade, sequence availability).**

| Species | Clade | Name | Lat | Lon | Depth | ZSM-Voucher Number | COI-GenBank/ BOLD Number | ITS-GenBank/ BOLD Number |
|---|---|---|---|---|---|---|---|---|
| | ANT_A | KT982317 | −68.020 | −67.671 | 208 | | KT982317 | KY272398 |
| | ANT_A | KT982356 | −76.479 | −165.738 | 457 | | KT982356 | KY272399 |
| | ANT_B | FJ969369 | −71.621 | −170.867 | 205 | | FJ969369 | |
| | ANT_C | KT982322 | −64.035 | −56.728 | 220 | | KT982322 | KY272414 |
| | ANT_C | KT982333 | −63.686 | −56.859 | 400 | | KT982333 | KY272415 |
| | ANT_C | KT982334 | −63.686 | −56.859 | 400 | | KT982334 | KY272416 |
| | ANT_C | KT982341 | −63.754 | −55.684 | 334 | | KT982341 | KY272417 |
| | ANT_C | KT982343 | −63.754 | −55.684 | 334 | | KT982343 | KY272418 |
| | ANT_C | PpaA_001 | −71.136 | −11.527 | 123 | | KC794958 | |
| | ANT_C | PS82_121_1 | −76.966 | −32.945 | | ZSM-A20160626 | KY272315 | KY272412 |
| | ANT_C | PS82_143_2_1 | −76.967 | −32.866 | | ZSM-A20160623 | KY272311 | KY272419 |
| | ANT_C | PS82_143_2_3 | −76.967 | −32.866 | | ZSM-A20160625 | KY272319 | KY272405 |
| | ANT_C | PS82_156_2_1 | −75.507 | −27.486 | | ZSM-A20160629 | KY272313 | KY272407 |
| | ANT_C | PS82_156_2_2 | −75.507 | −27.486 | | ZSM-A20160630 | KY272309 | KY272413 |
| | ANT_C | PS82_156_2_3 | −75.507 | −27.486 | | ZSM-A20160631 | KY272310 | KY272410 |
| | ANT_C | PS82_170_1 | −74.906 | −26.685 | | ZSM-A20160632 | KY272318 | KY272411 |
| | ANT_C | PS82_170_2 | −74.906 | −26.685 | | ZSM-A20160633 | KY272317 | KY272409 |
| | ANT_C | PS82_174_3 | −74.491 | −30.977 | | ZSM-A20160637 | KY272312 | KY272403 |
| | ANT_C | PS82_223_1 | −75.522 | −28.973 | | ZSM-A20160730 | KY272308 | KY272408 |
| | ANT_C | PS82_25_2_1 | −74.705 | −29.900 | | ZSM-A20160635 | KY272314 | KY272404 |
| | ANT_C | PS82_25_2_2 | −74.705 | −29.900 | | ZSM-A20160636 | KY272316 | KY272406 |
| | ANT_D.1 | KT982325 | −63.576 | −54.629 | 227 | | KT982325 | KY272396 |
| | ANT_D.1 | KT982326 | −62.442 | −55.459 | 245 | | KT982326 | |
| | ANT_D.1 | KT982330 | −63.389 | −60.120 | 310 | | KT982330 | |
| | ANT_D.1 | KT982331 | −63.389 | −60.120 | 310 | | KT982331 | |
| | ANT_D.1 | KT982346 | −63.834 | −62.664 | 256 | | KT982346 | KY272397 |
| | ANT_D.2 | JR262_1058 | −55.144 | −36.245 | 195.21 | ZSM-A20160708 | KY272301 | |
| | ANT_D.2 | JR262_1319 | −55.002 | −37.272 | 148.81 | ZSM-A20160709 | KY272302 | |
| | ANT_D.2 | JR262_1597_2 | −54.396 | −37.384 | 174.98 | ZSM-A20160710 | KY272305 | |
| | ANT_D.2 | JR262_1903_1 | −53.597 | −41.214 | 132.83 | ZSM-A20160711 | KY272303 | |
| | ANT_D.2 | JR262_48_5_1 | −54.284 | −36.083 | 124.08 | ZSM-A20160712 | KY272297 | |
| | ANT_D.2 | JR262_48_5_2 | −54.284 | −36.083 | 124.08 | ZSM-A20160713 | KY272298 | |
| | ANT_D.2 | JR262_702_1 | −55.166 | −35.485 | 126.99 | ZSM-A20160714 | KY272299 | |
| | ANT_D.2 | JR262_744 | −55.167 | −35.485 | 126.84 | ZSM-A20160715 | KY272304 | |
| | ANT_D.2 | JR262_806_4 | −54.984 | −35.762 | 139.38 | ZSM-A20160716 | KY272300 | |
| | ANT_D.2 | JR287_124_1 | −53.764 | −36.681 | 151 | ZSM-A20160691 | KY272295 | KY272393 |
| | ANT_D.2 | JR287_124_2 | −53.764 | −36.681 | 151 | ZSM-A20160692 | KY272294 | KY272391 |
| | ANT_D.2 | JR287_124_3 | −53.764 | −36.681 | 151 | ZSM-A20160693 | KY272296 | KY272394 |
| | ANT_D.2 | JR287_152 | −53.758 | −36.690 | 145 | ZSM-A20160694 | KY272292 | |
| | ANT_D.2 | JR287_191 | −53.751 | −36.699 | 145 | ZSM-A20160695 | KY272307 | |
| | ANT_D.2 | JR287_59_2 | −54.944 | −35.979 | 246 | ZSM-A20160687 | KY272293 | KY272392 |
| | ANT_D.2 | PpaE_001_HT26 | −53.461 | −41.261 | 193 | ZSM-A20160717 | KC794959 | |
| | ANT_D.2 | PS77_211_6_1_3 | −53.402 | −42.668 | 290.2 | ZSM-A20160696 | KY272306 | KY272395 |
| | ANT_E | KT982297 | −72.177 | −103.514 | 341 | | KT982297 | KY272442 |
| | ANT_E | KT982318 | −68.020 | −67.671 | 208 | | KT982318 | |
| | ANT_F | HM426218 | −71.092 | −11.508 | | | HM426218 | |
| | ANT_F | KT982324 | −63.686 | −56.859 | 400 | | KT982324 | KY272429 |
| | ANT_F | KT982332 | −64.134 | −56.860 | 310 | | KT982332 | |
| | ANT_F | KT982342 | −63.754 | −55.684 | 334 | | KT982342 | |

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**TABLE 1 | Continued**

| Species | Clade | Name | Lat | Lon | Depth | ZSM-Voucher Number | COI-GenBank/ BOLD Number | ITS-GenBank/ BOLD Number |
|---|---|---|---|---|---|---|---|---|
| | ANT_F | PS77_226_7_1_1 | −64.915 | −60.621 | 226.2 | ZSM-A20160648 | KY272331 | KY272434 |
| | ANT_F | PS77_226_7_1_2 | −64.915 | −60.621 | 226.2 | ZSM-A20160649 | KY272334 | KY272430 |
| | ANT_F | PS77_248_2_2 | −65.955 | −60.466 | 212 | ZSM-A20160643 | KY272335 | KY272433 |
| | ANT_F | PS77_248_3_2_1 | −65.924 | −60.332 | 433 | ZSM-A20160644 | KY272337 | KY272436 |
| | ANT_F | PS77_248_3_2_2 | −65.924 | −60.332 | 433 | ZSM-A20160645 | KY272336 | KY272431 |
| | ANT_F | PS77_248_3_2_3 | −65.924 | −60.332 | 433 | ZSM-A20160646 | KY272338 | KY272435 |
| | ANT_F | PS77_248_3_2_4 | −65.924 | −60.332 | 433 | ZSM-A20160647 | KY272332 | |
| | ANT_F | PS77_257_2_2_3 | −64.913 | −60.648 | 152.5 | ZSM-A20160650 | KY272330 | KY272440 |
| | ANT_F | PS77_257_2_2_5 | −64.913 | −60.648 | 152.5 | ZSM-A20160651 | KY272329 | KY272432 |
| | ANT_F | PS77_275 | −70.940 | −10.489 | | ZSM-A20160728 | KY272326 | KY272439 |
| | ANT_F | PS77_291_1_2 | −70.842 | −10.587 | 267.5 | ZSM-A20160642 | KY272333 | KY272437 |
| | ANT_F | PS77_292_2_5 | −70.846 | −10.593 | 243.5 | ZSM-A20160729 | KY272327 | KY272441 |
| | ANT_F | PS82_58_1 | −76.322 | −28.992 | | ZSM-A20160627 | KY272328 | KY272438 |
| | ANT_G | FJ969367 | −71.258 | −170.635 | 466 | | FJ969367 | |
| | ANT_G | FJ969368 | −72.014 | −170.775 | 236 | | FJ969368 | |
| | ANT_H | KT982338 | −63.754 | −55.684 | 334 | | KT982338 | KY272422 |
| | ANT_H | KT982352 | −64.411 | −61.963 | 664 | | KT982352 | |
| | ANT_H | KT982354 | −64.411 | −61.963 | 664 | | KT982354 | |
| | ANT_I | KT982316 | −62.933 | −61.479 | 188 | | KT982316 | KY272423 |
| | ANT_J | KT982293 | −76.998 | −175.093 | 541 | | KT982293 | |
| | ANT_J | KT982294 | −76.904 | 169.965 | 764 | | KT982294 | KY272427 |
| | ANT_J | KT982306 | −76.998 | −175.093 | 541 | | KT982306 | KY272426 |
| | ANT_J | KT982313 | −76.998 | −175.093 | 541 | | KT982313 | KY272428 |
| | ANT_K | PS82_143_2_2 | −76.967 | −32.866 | | ZSM-A20160624 | KY272325 | KY272425 |
| | ANT_K | PS82_244_4 | −72.799 | −19.495 | | ZSM-A20160640 | KY272323 | |
| | ANT_K | PS82_246_2 | −70.928 | −10.475 | | ZSM-A20160641 | KY272324 | KY272424 |
| | ANT_L | PS82_109_2_2 | −77.016 | −33.695 | | ZSM-A20160622 | KY272339 | KY272420 |
| | ANT_L | PS82_34_2 | −76.069 | −30.160 | | ZSM-A20160628 | KY272340 | KY272421 |
| | ANT_M | HM426171 | −71.317 | −13.942 | | | HM426171 | |
| | ANT_M | PS82_183_1_1 | −74.250 | −37.749 | | ZSM-A20160638 | KY272321 | KY272400 |
| | ANT_M | PS82_183_1_2 | −74.250 | −37.749 | | ZSM-A20160639 | KY272320 | KY272401 |
| | ANT_M | PS82_240_2 | −74.660 | −28.763 | | ZSM-A20160731 | KY272322 | KY272402 |
| | ANT_N | PpaE_002_HT25 | −54.016 | −37.437 | 78 | ZSM-A20160718 | KC794960 | |
| | ANT_N | PS77_211_6_1_4 | −53.402 | −42.668 | 290.2 | ZSM-A20160697 | KY272360 | KY272458 |
| | SUB_1 | PS77_208_5_1_1 | −56.168 | −54.548 | 292 | ZSM-A20160726 | KY272289 | KY272367 |
| | SUB_1 | PS77_208_5_1_4 | −56.168 | −54.548 | 292 | ZSM-A20160689 | KY272288 | |
| | SUB_2 | ZSMA20111352_HT27 | −51.269 | −62.952 | 171–174 | ZSM-A20111352 | KF603937/CFAP037-11 | |
| | SUB_2.1 | HF26_254 | −53.007 | −73.923 | 31 | ZSM-A20160456 | KY272290 | KY272368 |
| | SUB_2.2 | PS77_208_3 | −56.152 | −54.530 | 285.5 | ZSM-A20160725 | KY272291 | KY272366 |
| | SUB_3 | ZSMA20111008_HT28 | −50.414 | −74.559 | 15–20 | ZSM-A20111008 | KF603952/CFAP026-11 | KY272390 |
| | SUB_4 | PpaE_004_HT18 | −52.574 | −60.084 | 378 | ZSM-A20160719 | KC794961 | KY272443 |
| | SUB_4 | PpaE_005_HT15 | −52.574 | −60.084 | 378 | ZSM-A20160720 | KC794962 | |
| | SUB_4 | PpaE_006_HT17 | −52.574 | −60.084 | 378 | ZSM-A20160721 | KC794963 | KY272457 |
| | SUB_4 | PpaE_007_HT15 | −52.574 | −60.084 | 378 | ZSM-A20160722 | KC794964 | |
| | SUB_4 | PpaE_008_HT15 | −52.574 | −60.084 | 378 | ZSM-A20160723 | KC794965 | |
| | SUB_4 | PpaE_010_HT15 | −52.962 | −60.143 | 378 | ZSM-A20160724 | KC794966 | |
| | SUB_4 | PS77_208_5_1_2 | −56.168 | −54.548 | 292 | ZSM-A20160727 | KY272356 | |
| | SUB_4 | ZDLT1_889_1 | −50.252 | −61.567 | 159 | ZSM-A20160698 | KY272357 | KY272445 |
| | SUB_4 | ZDLT1_889_2 | −50.252 | −61.567 | 159 | ZSM-A20160699 | KY272358 | KY272446 |

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**TABLE 1 | Continued**

| Species | Clade | Name | Lat | Lon | Depth | ZSM-Voucher Number | COI-GenBank/ BOLD Number | ITS-GenBank/ BOLD Number |
|---|---|---|---|---|---|---|---|---|
| | SUB_4 | ZDLT1_889_3 | −50.252 | −61.567 | 159 | ZSM-A20160700 | KY272359 | KY272444 |
| | SUB_4 | ZSMA20111348_HT14 | −50.434 | −62.768 | 146–148 | ZSM-A20111348 | KF603953/CFAP027-11 | |
| | SUB_4 | ZSMA20111349_HT13 | −51.269 | −62.952 | 171–174 | ZSM-A20111349 | KF603960/CFAP034-11 | |
| | SUB_4 | ZSMA20111350_HT15 | −51.269 | −62.952 | 171–174 | ZSM-A20111350 | KF603961/CFAP035-11 | |
| | SUB_4 | ZSMA20111351_HT20 | −51.269 | −62.952 | 171–174 | ZSM-A20111351 | KF603962/CFAP036-11 | |
| | SUB_4 | ZSMA20111354_HT17 | −51.086 | −61.733 | 174–176 | ZSM-A20111354 | KF603954/CFAP028-11 | |
| | SUB_4 | ZSMA20111355_HT18 | −51.086 | −61.733 | 174–176 | ZSM-A20111355 | KF603955/CFAP029-11 | |
| | SUB_4 | ZSMA20111357_HT16 | −51.086 | −61.733 | 174–176 | ZSM-A20111357 | KF603956/CFAP030-11 | |
| | SUB_4 | ZSMA20111359_HT18 | −51.086 | −61.733 | 174–176 | ZSM-A20111359 | KF603957/CFAP031-11 | |
| | SUB_4 | ZSMA20111360_HT15 | −51.086 | −61.733 | 174–176 | ZSM-A20111360 | KF603958/CFAP032-11 | |
| | SUB_4 | ZSMA20111361_HT19 | −51.086 | −61.733 | 174–176 | ZSM-A20111361 | KF603959/CFAP033-11 | |
| | SUB_5 | HF26_027 | −52.600 | −73.640 | 19 | ZSM-A20160452 | KY272344 | |
| | SUB_5 | HF26_030 | −52.600 | −73.640 | 15–20 | ZSM-A20160448 | KY272343 | |
| | SUB_5 | HF26_059 | −53.007 | −73.923 | 31 | ZSM-A20160457 | KY272349 | |
| | SUB_5 | HF26_086 | −53.357 | −73.087 | 9 | ZSM-A20160465 | KY272341 | KY272448 |
| | SUB_5 | HF26_120 | −53.702 | −72.041 | 22 | ZSM-A20160472 | KY272355 | |
| | SUB_5 | HF26_367 | −53.357 | −73.087 | 20 | ZSM-A20160468 | KY272351 | KY272447 |
| | SUB_5 | HF26_368 | −53.357 | −73.087 | 18 | ZSM-A20160467 | KY272346 | KY272449 |
| | SUB_5 | HF26_369 | −53.357 | −73.087 | 14 | ZSM-A20160466 | KY272353 | |
| | SUB_5 | HF26_373 | −53.379 | −73.159 | 14 | ZSM-A20160488 | KY272347 | KY272453 |
| | SUB_5 | HF26_392 | −53.379 | −73.159 | 17 | ZSM-A20160493 | KY272345 | KY272450 |
| | SUB_5 | HF26_439 | −53.379 | −73.159 | 17 | ZSM-A20160483 | KY272354 | KY272452 |
| | SUB_5 | HF26_451 | −53.379 | −173.159 | 21 | ZSM-A20160490 | KY272352 | KY272451 |
| | SUB_5 | HF26_458 | −53.379 | −73.159 | 14 | ZSM-A20160494 | KY272350 | KY272455 |
| | SUB_5 | HF26_647 | −53.896 | −71.311 | 23 | ZSM-A20160476 | KY272342 | |
| | SUB_5 | HF26_648 | −53.896 | −71.311 | 23 | ZSM-A20160477 | KY272348 | |
| | SUB_5 | KT982315 | −53.270 | −66.386 | 96 | | KT982315 | KY272456 |
| | SUB_5 | ZSMA20111340_HT12 | −55.000 | −68.315 | 24 | ZSM-A20111340 | KF603948/CFAP018-11 | |
| *Pallenopsis yepayekae* | Pye.1 | HF16_171 | −50.338 | −75.381 | 20 | ZSM-A20119982 | KY272271 | |
| *P. yepayekae* | Pye.1 | HF16_187 | −50.359 | −75.339 | 15 | ZSM-A20119968 | KY272266 | KY272369 |
| *P. yepayekae* | Pye.1 | HF16_309 | −50.359 | −75.339 | 20 | ZSM-A20119978 | KY272252 | |
| *P. yepayekae* | Pye.1 | HF16_476_1 | −50.353 | −75.283 | 20 | ZSM-A20119979 | KY272287 | |
| *P. yepayekae* | Pye.1 | HF16_476_2 | −50.353 | −75.283 | 20 | ZSM-A20160580 | KY272283 | |
| *P. yepayekae* | Pye.1 | HF16_476_3 | −50.353 | −75.283 | 20 | ZSM-A20160701 | KY272256 | KY272385 |
| *P. yepayekae* | Pye.1 | HF16_519_1 | −50.412 | −75.345 | 20 | ZSM-A20160702 | KY272262 | KY272370 |
| *P. yepayekae* | Pye.1 | HF16_519_2 | −50.412 | −75.345 | 31 | ZSM-A20160703 | KY272265 | KY272371 |
| *P. yepayekae* | Pye.1 | HF16_520 | −50.412 | −75.345 | 20 | ZSM-A20119986 | KY272260 | |
| *P. yepayekae* | Pye.1 | HF16_521_1 | −50.412 | −75.345 | 19 | ZSM-A20119985 | KY272261 | |
| *P. yepayekae* | Pye.1 | HF16_563 | −50.359 | −75.339 | 20 | ZSM-A20160704 | KY272258 | |
| *P. yepayekae* | Pye.1 | HF21_212 | −45.661 | −73.218 | | ZSM-A20160705 | KY272286 | KY272374 |
| *P. yepayekae* | Pye.1 | HF21_225 | −45.921 | −73.964 | | ZSM-A20160579 | KY272270 | |
| *P. yepayekae* | Pye.1 | HF21_387 | −45.763 | −73.492 | 15 | ZSM-A20160581 | KY272251 | |
| *P. yepayekae* | Pye.1 | HF21_387_2 | −45.763 | −73.492 | 15 | ZSM-A20160583 | KY272285 | KY272375 |
| *P. yepayekae* | Pye.1 | HF21_426_1 | −45.521 | −73.554 | 19 | ZSM-A20160584 | KY272255 | |
| *P. yepayekae* | Pye.1 | HF21_426_3 | −45.521 | −73.554 | 19 | ZSM-A20160706 | KY272253 | KY272388 |
| *P. yepayekae* | Pye.1 | HF21_79 | −45.662 | −73.849 | 20 | ZSM-A20160707 | KY272257 | KY272373 |
| *P. yepayekae* | Pye.1 | HF24_213 | −53.007 | −73.923 | | ZSM-A20160529 | KY272268 | KY272372 |
| *P. yepayekae* | Pye.1 | HF26_029 | −52.600 | −73.640 | 17 | ZSM-A20160450 | KY272281 | |
| *P. yepayekae* | Pye.1 | HF26_031 | −52.600 | −73.640 | 15–20 | ZSM-A20160454 | KY272274 | |

*(Continued)*

**TABLE 1 | Continued**

| Species | Clade | Name | Lat | Lon | Depth | ZSM-Voucher Number | COI-GenBank/ BOLD Number | ITS-GenBank/ BOLD Number |
|---|---|---|---|---|---|---|---|---|
| *P. yepayekae* | Pye.1 | HF26_032 | −52.600 | −73.640 | 15–20 | ZSM-A20160447 | KY272279 | KY272376 |
| *P. yepayekae* | Pye.1 | HF26_090 | −53.379 | −73.159 | 14 | ZSM-A20160499 | KY272254 | KY272384 |
| *P. yepayekae* | Pye.1 | HF26_264 | −52.879 | −74.350 | 20 | ZSM-A20160460 | KY272275 | KY272386 |
| *P. yepayekae* | Pye.1 | HF26_265 | −52.879 | −74.350 | 20 | ZSM-A20160459 | KY272278 | KY272377 |
| *P. yepayekae* | Pye.1 | HF26_306 | −52.879 | −74.350 | 25 | ZSM-A20160458 | KY272282 | |
| *P. yepayekae* | Pye.1 | HF26_363 | −53.007 | −73.923 | 20 | ZSM-A20160462 | KY272284 | |
| *P. yepayekae* | Pye.1 | HF26_376 | −53.379 | −73.159 | 16 | ZSM-A20160527 | KY272276 | |
| *P. yepayekae* | Pye.1 | HF26_378 | −53.379 | −73.159 | 29 | ZSM-A20160498 | KY272277 | KY272387 |
| *P. yepayekae* | Pye.1 | HF26_394 | −53.379 | −73.159 | 17 | ZSM-A20160484 | KY272264 | KY272389 |
| *P. yepayekae* | Pye.1 | HF26_396 | −53.379 | −73.159 | 17 | ZSM-A20160497 | KY272259 | KY272381 |
| *P. yepayekae* | Pye.1 | HF26_397 | −53.379 | −73.159 | 17 | ZSM-A20160496 | KY272272 | KY272382 |
| *P. yepayekae* | Pye.1 | HF26_555 | −53.702 | −72.041 | 21 | ZSM-A20160473 | KY272263 | KY272380 |
| *P. yepayekae* | Pye.1 | HF26_562 | −53.896 | −71.311 | 23 | ZSM-A20160478 | KY272267 | KY272378 |
| *P. yepayekae* | Pye.1 | HF26_563 | −53.818 | −71.056 | 23 | ZSM-A20160479 | KY272280 | KY272379 |
| *P. yepayekae* | Pye.1 | HF26_566 | −53.818 | −71.056 | 7 | ZSM-A20160481 | KY272269 | KY272383 |
| *P. yepayekae* | Pye.1 | HF26_601 | −53.587 | −72.338 | 16 | ZSM-A20160471 | KY272273 | |
| *P. yepayekae* | Pye.1 | ZSMA20111000_HT07 | −48.737 | −75.415 | 15 | ZSM-A20111000 | KF603944/CFAP013-11 | |
| *P. yepayekae* | Pye.1 | ZSMA20111002_HT06 | −50.835 | −74.139 | 25 | ZSM-A20111002 | KF603947/CFAP017-11 | |
| *P. yepayekae* | Pye.1 | ZSMA20111005_HT04 | −48.737 | −75.415 | 23 | ZSM-A20111005 | KF603945/CFAP014-11 | |
| *P. yepayekae* | Pye.1 | ZSMA20111006_HT01 | −43.418 | −74.081 | 20 | ZSM-A20111006 | KF603941/CFAP007-11 | |
| *P. yepayekae* | Pye.1 | ZSMA20111016_HT09 | −48.608 | −74.899 | 32 | ZSM-A20111016 | KF603943/CFAP012-11 | |
| *P. yepayekae* | Pye.1 | ZSMA20111339_HT05 | −43.775 | −73.029 | 19 | ZSM-A20111339 | KF603949/CFAP019-11 | |
| *P. yepayekae* | Pye.2 | ZSMA20111003_HT03 | −43.418 | −74.081 | 25 | ZSM-A20111003 | KF603940/CFAP006-11 | |
| *P. yepayekae* | Pye.2 | ZSMA20111004_HT01 | −43.410 | −74.084 | 9 | ZSM-A20111004 | KF603939/CFAP005-11 | |
| *P. yepayekae* | Pye.2 | ZSMA20111009_HT02 | −43.393 | −74.132 | 26 | ZSM-A20111009 | KF603938/CFAP004-11 | |
| *P. yepayekae* | Pye.2 | ZSMA20111012_HT08 | −43.771 | −73.044 | 22 | ZSM-A20111012 | KF603942/CFAP008-11 | |
| *P. pilosa* | OG | PxxE_001 | −54.350 | 3.193 | | ZSM-A20160732 | KC794967 | KY272459 |
| *P. pilosa* | OG | PxxE_002 | −54.213 | −32.606 | 200 | ZSM-A20160733 | KC794968 | KY272460 |
| *P. pilosa* | OG | PxxE_003 | −54.397 | 3.521 | | ZSM-A20160734 | KY272362 | |
| *P. pilosa* | OG | PxxE_005 | −54.397 | 3.521 | | ZSM-A20160735 | KY272361 | |
| *P. macronyx* | OG | PS42_164_2 | −62.133 | −57.667 | | ZSM-A20160619 | KY272364 | |
| *P. macronyx* | OG | PS42_164_3 | −62.133 | −57.667 | | ZSM-A20160620 | KY272363 | |
| *P. macronyx* | OG | PS42_164_4 | −62.133 | −57.667 | | ZSM-A20160621 | KY272365 | |

Center for Polar and Marine Research, Bremerhaven, Germany). After collection, specimens were stored in ethanol (96%). Specimens were morphologically inspected and assigned to *P. patagonica* s.l. before being molecularly studied.

## Molecular Analyses

Muscle tissue was extracted from the tibia using sterile scalpel and forceps. DNA was isolated from the tissue using a modified salt precipitation protocol after Sunnucks and Hales (1996; see Weiss and Leese, 2016). Extracted DNA was eluted in 100 µl TE minimum buffer (1 mM Tris BASE, 0.1 mM EDTA, pH 8.0). The amplification of the mitochondrial cytochrome c oxidase subunit I gene (COI) and a ribosomal gene region covering the 18S–ITS1–5.8S–ITS2–28S stretch (ITS) was carried out in 25 µl reactions containing 1x (2.5 µl) PCR buffer (5Prime), 0.2 mM dNTPs, 0.5 µM of each primer, 0.025 U/µl (0.125 µl) Hotmaster Taq (5Prime) and 1 µl template DNA, topped up to 25 µl with sterile water. A 658 bp long fragment of the COI was amplified using the common barcoding primer pair HCO2198 and LCO1490 (Folmer et al., 1994). The optimal temperature profile for the PCRs with these primers was an initial denaturation at 94°C for 2 min, followed by 36 cycles of denaturation at 94°C for 20 s, annealing at 46°C for 30 s, extension at 65°C for 60 s, and a final extension at 65°C for 7 min.

For ITS, an approximately 1000 bp long fragment was amplified using primers ITSRA2 and ITS2.2 (Arango and Brenneis, 2013). PCR cycling program was initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 75 s, extension at 65°C for 1 min, with a final extension at 65°C for 5 min.

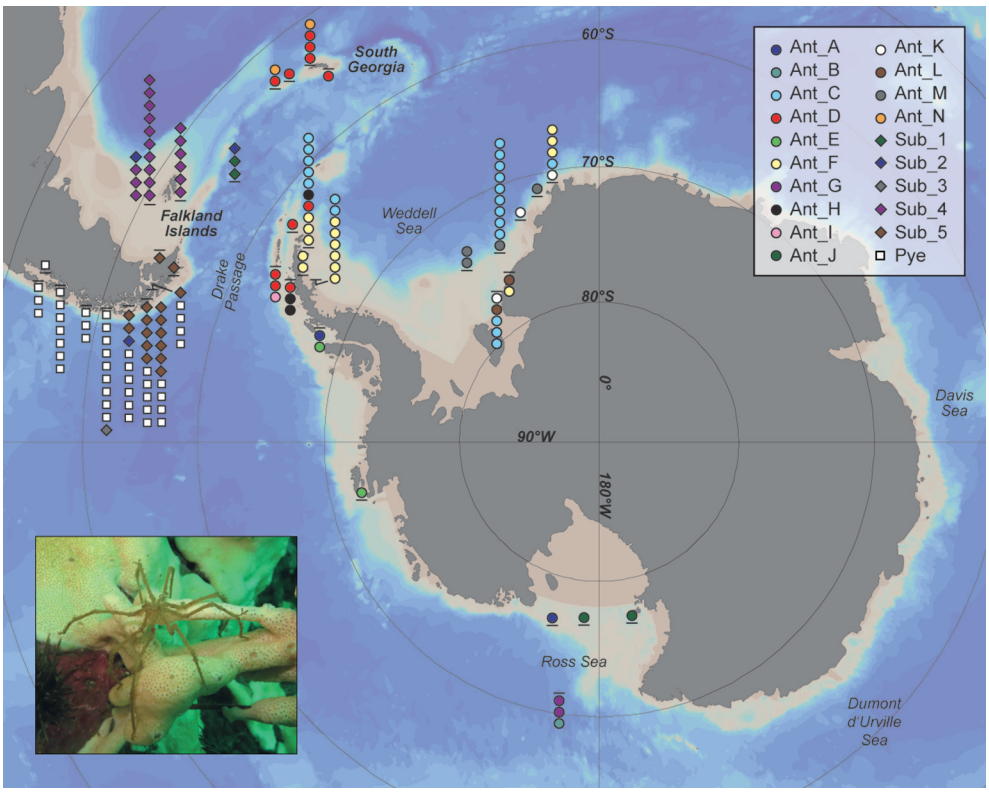


**FIGURE 1 | Sampling sites (dashes) and distribution of Antarctic, Subantarctic, and Patagonian *Pallenopsis patagonica* s.l. specimens and their assignment to molecular clades.** Different clades are represented by different symbols/colors. Each symbol below or above the dash represents one specimen. Picture in lower left corner shows *P. patagonica* in its natural habitat (photo taken by Roland R. Melzer).

For sequencing, 10 U (0.5 µl) Exonuclease I (Thermo Scientific), 1.5 U (1 µl) FastAP Thermosensitive Alkaline Phosphatase (Thermo Scientific) and 9 µl PCR product per reaction were used. The purification mix was incubated for 25 min at 37°C, followed by a denaturation step at 85°C for 15 min. For sequencing at GATC Biotech AG (Cologne, Germany) 5 µl of purified PCR product was mixed with 5 µl of 5 pmol/µl primer. Forward and reverse primers were used to sequence both directions of the DNA strands.

For ITS sequences of samples reported by Harder et al. (2016; herein labeled with GenBank numbers starting with KT98) DNA extraction was performed as stated in Harder et al. (2016). For ITS amplification the same primer pair as mentioned above was used. PCR mixture consisted of 1x PCR buffer, 0.75 U Taq DNA polymerase (5Prime, Hotmaster Taq), 2.5 mM $Mg^{2+}$, 10 nmol of each dNTP, 1 µl of template DNA, 0.5 µM of each primer, and water to 25 µl. PCR cycling program was run, with an initial denaturation at 94°C for 2 min, followed by 37 cycles of denaturation at 94°C for 20 s, annealing at 55°C for 30 s, extension at 65°C for 80 s, with a final extension at 65°C for 10 min. Successful amplification was confirmed by visualizing PCR products on a 1% agarose gel stained with ethidium bromide. Target PCR product was gel extracted and purified using a Qiagen QIAquick® Gel Extraction Kit according to the manufacturer's recommendations. Bidirectional Sanger sequencing of amplicons was performed at High Throughput Genomics Center (Seattle, WA, USA).

## Phylogenetic Analyses

For COI, *P. patagonica* s.l. sequences from Weis et al. (2014; $n = 34$ including five downloaded from NCBI) and Harder et al. (2016; $n = 26$) were added to the final data set. ITS sequences of specimens of both previous studies were generated and also included in the ITS alignment, too.

For both gene regions, sequences were edited with Geneious v. 8.1.3 (Kearse et al., 2012) and aligned in Geneious using MAFFT v. 7.017 Multiple Sequence Alignment (Katoh and Standley, 2013) with default parameters as implemented in Geneious, with a gap opening penalty of 1.53 and offset value of 0.123. For COI, sequences were translated into amino acids using the invertebrate mitochondrial genetic code (transl_table=5) to verify that all codons could be translated without stop codons. For ITS, a version of the alignment where ambiguously aligned regions were removed was produced with Gblocks v. 0.91b (Castresana, 2000) using less stringent parameters (smaller blocks, gaps in final alignment allowed, less strict flanking positions) as has been done in Dietz et al. (2015b). For analyses when only unique copies were needed, sequences were collapsed into unique sequences ("haplotypes" for COI data) with the online tool FaBox v. 1.41 (Villesen, 2007).

For both data sets a maximum-likelihood (ML) analysis was performed with RAxML v. 8.2.4 (Stamatakis, 2014) using the GTRCAT model of sequence evolution and branch support was assessed with 10,000 rapid bootstrap replicates. In addition, for the mitochondrial data set a resolved ultrametric gene tree was calculated using BEAST v. 1.8.3 (Drummond et al., 2012) with the model specified by jModelTest v. 2.1.10 (Guindon and Gascuel, 2003; Darriba et al., 2012). An XML file was created with BEAUti v. 1.8.3 (Drummond et al., 2012) with the following settings: HKY+G+I as substitution models and $80 \times 10^6$ as length of MCMC chain sampling every 1000th tree. Convergence of the likelihood and appropriate effective sampling size (ESS > 200) of parameter estimates were checked using TRACER v. 1.6 (Rambaut et al., 2014), and a consensus tree was calculated using TREEANNOTATOR v. 1.8.3 of the BEAST package. Furthermore, uncorrected pairwise distance matrices were created using MEGA v. 7 (Tamura et al., 2011).

## Species Delimitation Methods

For species delimitation analysis of the COI data set we used ABGD (Automatic Barcode Gap Discovery; Puillandre et al., 2012). As no clear barcode gap was found in the pairwise distance data, ABGD results varied strongly depending on single sequences and run parameters tested. Results presented here are mainly from the default settings but using Kimura-2-parameter (K2P) distance correction. The same settings were applied to the ITS alignment (including and excluding ambiguously aligned regions). Due to the smaller data set for the ITS alignment and the fact that informative alignment gaps cannot easily be interpreted as additional character in tree-based delimitation methods, further species delimitation methods were only applied to the COI data set. The final mitochondrial COI ML tree was used to perform a Bayesian Poisson Tree Processes (bPTP) analysis using the web server (http://species.h-its.org/ptp; Zhang et al., 2013). Furthermore, a Generalized Mixed Yule Coalescent (GMYC) analysis based on the resolved ultrametric gene tree was conducted at the web server (http://species.h-its.org/gmyc; Fujisawa and Barraclough, 2013) using the single-threshold method only (see Fujisawa and Barraclough, 2013).

## Molecular Clock Analysis

A calibrated molecular clock rate for sea spiders has not been reported in previous studies. However, in order to infer possible divergence date ranges for the different clades we applied a widely adopted COI molecular clock rate reported for insects: 1.15% per myr and lineage (Brower, 1994). BEAST v. 1.8.2 was used to estimate divergence times using an HKY+I+G evolution model as well as an uncorrelated local clock model. Analyses were run for $10 \times 10^6$ generations sampling every $1000^{th}$ tree. Convergence of parameter estimates and ESS control and subsequent steps were done as described above.

# RESULTS

## Number of COI Clades

The data set of *P. patagonica* s.l. was extended to a total of 173 specimens including 47 sequences from *P. yepayekae.* For the first time, we studied individuals from the Strait of Magellan and the Eastern Weddell Sea. Further sequences from *P. pilosa* (Hoek, 1881) ($n = 3$) and *P. macronyx* (Bouvier, 1911) ($n = 3$) were added to the data set as outgroups.

The final COI alignment consisted of 426 bp (GC content 32.9%) with 278 identical and 128 parsimony informative sites. Neither stop codons nor frame shift mutations were observed after translation. Both the ML and Bayesian phylogenetic tree (**Figure 2**) resolved *P. patagonica* s.l. as monophyletic and well-separated from the outgroup. In addition, *P. yepayekae* represented a monophyletic group within *P. patagonica* s.l. Moreover, all individuals from the Antarctic shelf formed an "Antarctic super-clade" that also contained one clade with specimens from South Georgia, i.e., one of the Subantarctic islands. The two other specimens collected around South Georgia that did not cluster inside this group represented the basal-most group (Clade N) in the whole *P. patagonica* s.l. group (**Figure 2**). Analysis of the final COI alignment with ABGD using K2P substitution model revealed a steady decrease from 21 to 13 in number of recovered groups between $P = 0.002$ and $P = 0.05$ in the recursive partition. No clear barcode gap was visible when plotting pairwise uncorrected distances between *P. patagonica* s.l. specimens (Supporting information Table S1; see upper diagram in **Figure 3** showing distances between members of the Antarctic clade). When choosing a threshold value of $P = 0.05$ ABGD suggested 13 clades, with several formerly reported clades merged (clade E, F, and G from Harder et al., 2016). At $P = 0.06$ ABGD merged all sequences into one group. Analysis of the Bayesian tree with bPTP, suggested the presence of 20 distinct groups, hence, subdivided five ABGD groupings further resulting in seven additional clades. With 22 groups, GMYC reported the highest number of clades for the ML tree. In contrast to bPTP, GMYC furthermore subdivided *P. yepayekae* and clade ANT_D into two subclades each. Here, we named the clades according to the bPTP results (see Section Discussion for further information). Several "clades" (two for ABGD, four for bPTP and GMYC) consisted of single specimens only (**Figure 2**).

Upon reviewing the data set, the following points are of particular interest to address our hypotheses. Newly collected specimens from the Strait of Magellan that were morphologically determined as *P. yepayekae* clustered with available sequences of *P. yepayekae* (Weis et al., 2014; **Figure 2**). Using the GMYC delimitation method, this species was split into two subclades. One subclade (Pye.2, **Table 1**) included all three haplotypes reported for four specimens sampled in the Chilean region Los Lagos, i.e., at the northernmost occurrence of *P. yepayekae*. Both other delimitation methods resolved *P. yepayekae* as a single clade.

All *P. patagonica* samples from the Strait of Magellan clustered together with sequences that in Weis et al. (2014) formed a sister clade to the Falkland clade (specimens ZSMA20111017 and ZSMA20111340, see Figure 2 in Weis et al., 2014). Average p-distance between this clade and the Falkland clade is 2.7%. The 15 new samples collected around South Georgia clustered together with a specimen from Shag Rocks that was reported as member of the Antarctic clade of *P. patagonica* (PpaE_001, see Weis et al., 2014). All these specimens formed a subclade (ANT_D.2,


**FIGURE 2 | Maximum likelihood tree of COI sequences of *Pallenopsis patagonica* s.l. combining new (black) and previously (gray) reported samples.** *P. pilosa* and *P. macronyx* serve as outgroups. Asterisks (*) indicate availability of ITS sequence data for the respective specimens. Bootstrap/posterior probabilities values above 50/0.5 are provided next to each node. Letters and numbers stand for mitochondrial clades from Antarctica (ANT) and the Subantarctic (SUB), respectively. Columns show results of COI-based species delimitation methods (bPTP, GMYC, and ABGD), number in parentheses denote the total number of predicted species by each method. In addition, results of ABGD when analyzing ITS (see **Figure 4**) are shown in the rightmost column.

**Table 1**) of clade ANT_D that so far consisted of specimens sampled around the tip of the Antarctic Peninsula (ANT_D.2; Harder et al., 2016). One sample from Shag Rocks grouped together with one individual from South Georgia (PpaE_002), which form the most basal clade (ANT_M) within *P. patagonica* s.l. For *P. patagonica* s.l. from the Antarctic shelf, our data set, analyzed with bPTP and GMYC, revealed the presence of three further clades (ANT_K, ANT_L, and ANT_M) in addition to the 10 clades reported from the Antarctic by Harder et al. (2016). These three new clades are exclusively found in the Eastern Weddell Sea (**Figure 1**). The newly found clade ANT_K is sister clade to ANT_J, clade ANT_L to ANT_H, and clade ANT_M to ANT_B (**Figure 2**). The two sequences HM426171 and HM426218 reported in Weis et al. (2014) as *P. buphtalmus* (Pushkin, 1993) and *P. latefrontalis* (Pushkin, 1993) based on provisional ID in BOLD by that time, clustered together with clade ANT_M and clade ANT_F, respectively. Sampling sites of these are also in the same region.

## Nuclear Support for COI Based Species Delimitation

We tested for congruence between the COI and the nuclear ITS clades by analyzing 96 sequences from the majority (bPTP: 18 of 20; GMYC 19 of 22) of reported COI clades (**Figure 2**). Very few ambiguities were included in sequences due to sequence quality issues for some sequences. The ITS sequence for JR287_124_2 was composed only of two shorter single read sequences (forward and reverse) but had no overlap (108 missing data symbols, "?"). The initial alignment was 1071 bp long, but was shortened to 583 bp after filtering for noisy positions with GBlocks. The final alignment consisted of sequences of 344–582 bp in length with 126 sites being identical and 159 parsimony informative. The base composition was very homogenous with A: 23.2%, C: 27.2%, G: 25.8%, and T: 23.8%. The number of ITS haplotypes was 23 representing 18 of the mitochondrial bPTP clades, i.e., four mitochondrial clades (ANT_C, ANT_F, SUB_2, and SUB_5) had two corresponding ITS sequences and *P. yepayekae* had three. ANT_H and ANT_L shared the same sequence. No heterozygous individuals were observed.

The phylogenetic ITS tree is much less resolved than the COI tree (**Figure 4**). However, most samples grouped similar to the COI tree. Separated, albeit poorly supported in the ITS tree, ANT_E and ANT_F clustered together when analyzed with ABGD. Pairwise identity between sequences of the clades was very high (98.9%). Together with ANT_G, for which no ITS data could be obtained, these clades represented a monophyletic group in the mitochondrial tree. In few cases there were minor disagreements in terms of the resolved clades. For examples ANT_L and ANT_H have identical sequences for ITS and hence grouped together. In the COI tree, these two clades represented slightly divergent sister clades. The other dissimilarity between the two phylogenetic trees was a well-supported nuclear clade (bootstrap support of 99%) that included ANT_A and ANT_D as sister groups (no shared haplotypes, however). ABGD distinguished the groups but in the mitochondrial tree ANT_A and ANT_D were not sister groups, yet closely related. In general, ITS showed substantially less variation than COI, and most mitochondrial clades were supported by ITS with minor exceptions mentioned above. However, within the clade SUB_2 we obtained two different ITS sequences from two individuals (PS77_208_3 and HF26_254) that showed much greater nucleotide variation within the ITS than the COI data. Interestingly, in the ITS tree they formed a paraphylum rather than a monophylum as the sequence of another clade (SUB_1) was also included. ABGD split these sequences with low pairwise identity (90.5%) into two separate groups.

## Biogeographic Patterns

The new samples included here prove that *P. yepayekae* also occurs in the Strait of Magellan and here even in sympatry with an other clade of *P. patagonica* s.l. (SUB_5; see **Figure 1**). SUB_5 was represented by only two specimens in Weis et al. (2014) and in contrast to Weis et al. (2014) merged into the "Falkland clade" in Harder et al. (2016). Here, with the additional data, both bPTP and GMYC supported that SUB_5 represents a distinct clade. One formerly reported *P. patagonica* s.l. clade (termed HT25 in Weis et al., 2014), represented by a single specimen found around South Georgia (Ppa_E002), now included one further specimen from the Shag Rocks (PS77_211_6_1_4). This clade is herein referred to as ANT_N. Vice versa, another clade of *P. patagonica* s.l., formerly represented by a single specimen from Shag Rocks (Ppa_E001), now clustered together with newly collected specimens from South Georgia (representing one subclade of ANT_D). ANT_N formed a cluster basal to the split between the Antarctic super-clade (i.e., specimens sampled South of the Polar Front) and the Falkland/Strait of Magellan clade (**Figure 2**). South Georgia individuals belonging to ANT_D thus grouped within the Antarctic clade reported by Weis et al. (2014) and clade D reported by Harder et al. (2016). Hence, these two clades (ANT_N and ANT_D) that occur in the same area, are not sister clades but only distantly related.

The new specimens sampled from the Eastern Weddell Sea grouped into five clades. Two of these clades, ANT_C and ANT_F, were already reported from the Antarctic Peninsula by Harder et al. (2016). Thus, our new data extended the reported distribution range for these two clades to the Eastern Weddell Sea. Specimens of the other three clades have not been reported earlier and were only found in the Eastern Weddell Sea. Specimens of clade ANT_D, found at the northernmost tip of the Antarctic Peninsula, grouped together with the individuals mentioned above from around South Georgia and Shag Rocks (**Figure 2**). No clades with individuals from either side of the Antarctic Polar Front were found in our data set.

## Divergence Dating

The divergence from the most recent common ancestor of *P. patagonica* s.l. occurred 13.6 myr before present [HPD 95% interval: 9.8–17.7 myr before present (BP)]. Also, the divergence of the Antarctic vs. the Falkland/Magellan clade took place in the mid Miocene (9.5 myr BP), 7.2–12.9 myr BP). Divergence of the distinct mitochondrial clades occurred (independently

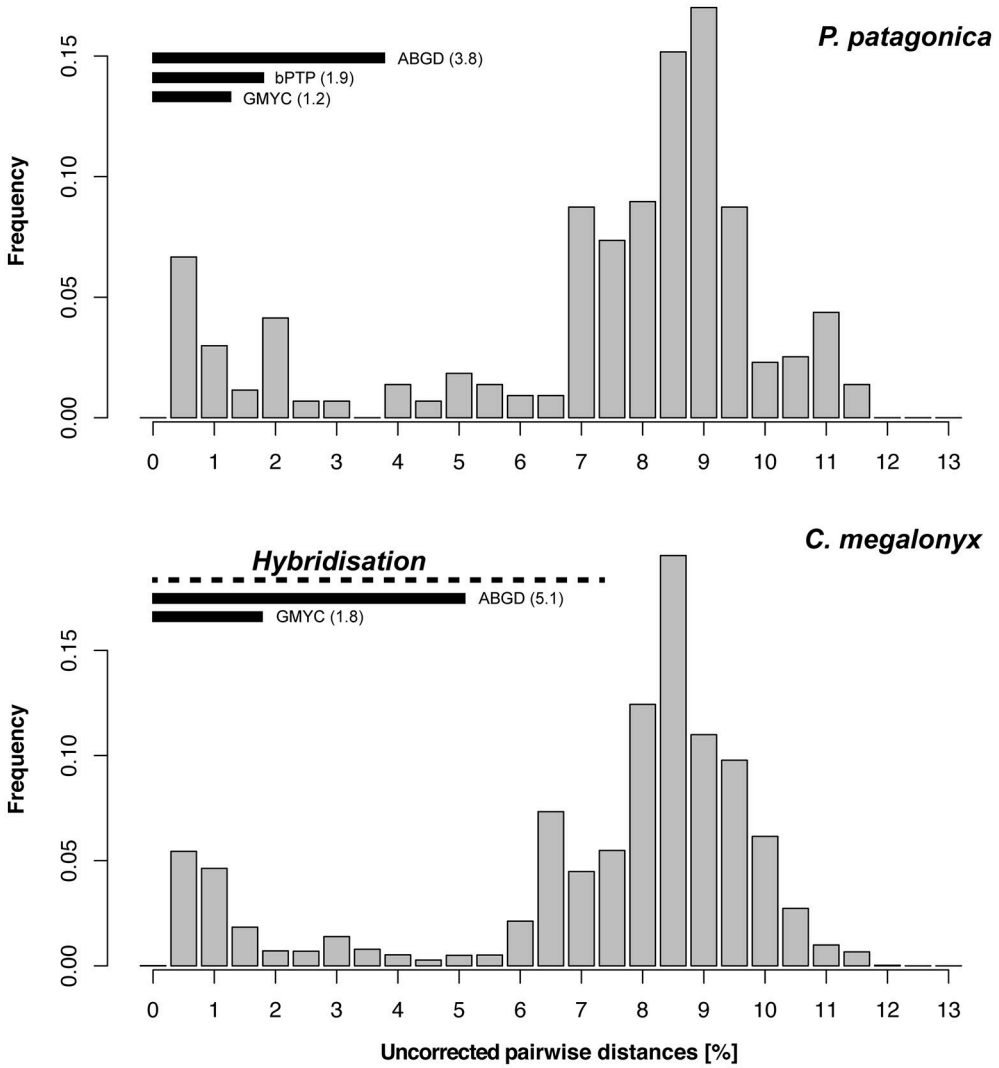


**FIGURE 3 | Barcode gap analysis for Antarctic super-clade of *Pallenopsis patagonica* s.l. (upper) and *Colossendeis megalonyx* (lower) in comparison.** For both data sets, the uncorrected pairwise COI distances between the haplotypes were used. Horizontal bars indicate pairwise distance limits used by delimitation methods to distinguish clades. Dashed line indicates the range of pairwise COI distances across which hybridization was revealed by ITS analyses in *C. megalonyx* (Dietz et al., 2015b).

on whether choosing ABGD, bPTP or GMYC as a delimitating criterion) in the Plio- and Pleistocene, mostly before the last 2 myr BP (Supplementary Figure S1).

# DISCUSSION

## Number of Mitochondrial Clades

As predicted by the first hypothesis, we found additional mitochondrial clades within *P. patagonica* s.l. when analyzing the extended COI data set with bPTP and GMYC. The groupings of bPTP and GMYC were congruent for most clades with the exception that GMYC further subdivided *P. yepayekae* and ANT_D into two geographically separated subclades each. However, when using ABGD with default settings, the number of mitochondrial clades inferred was actually smaller than the number reported by Harder et al. (2016) (**Figure 2**, Supplementary Table S2).

## Which Mitochondrial Clades Can Be Considered As Species?

When trying to find an objective value that best describes the number of species (defined as independently evolving units) with the classical COI barcoding alone, the original approach was to quantify intra- vs. inter-specific genetic distances through a barcoding gap analysis that defines the maximum threshold distance found within a species. For animal taxa, this value has often been found at 2% pairwise distances (e.g., Hebert et al., 2003). Other approaches expect distinct species

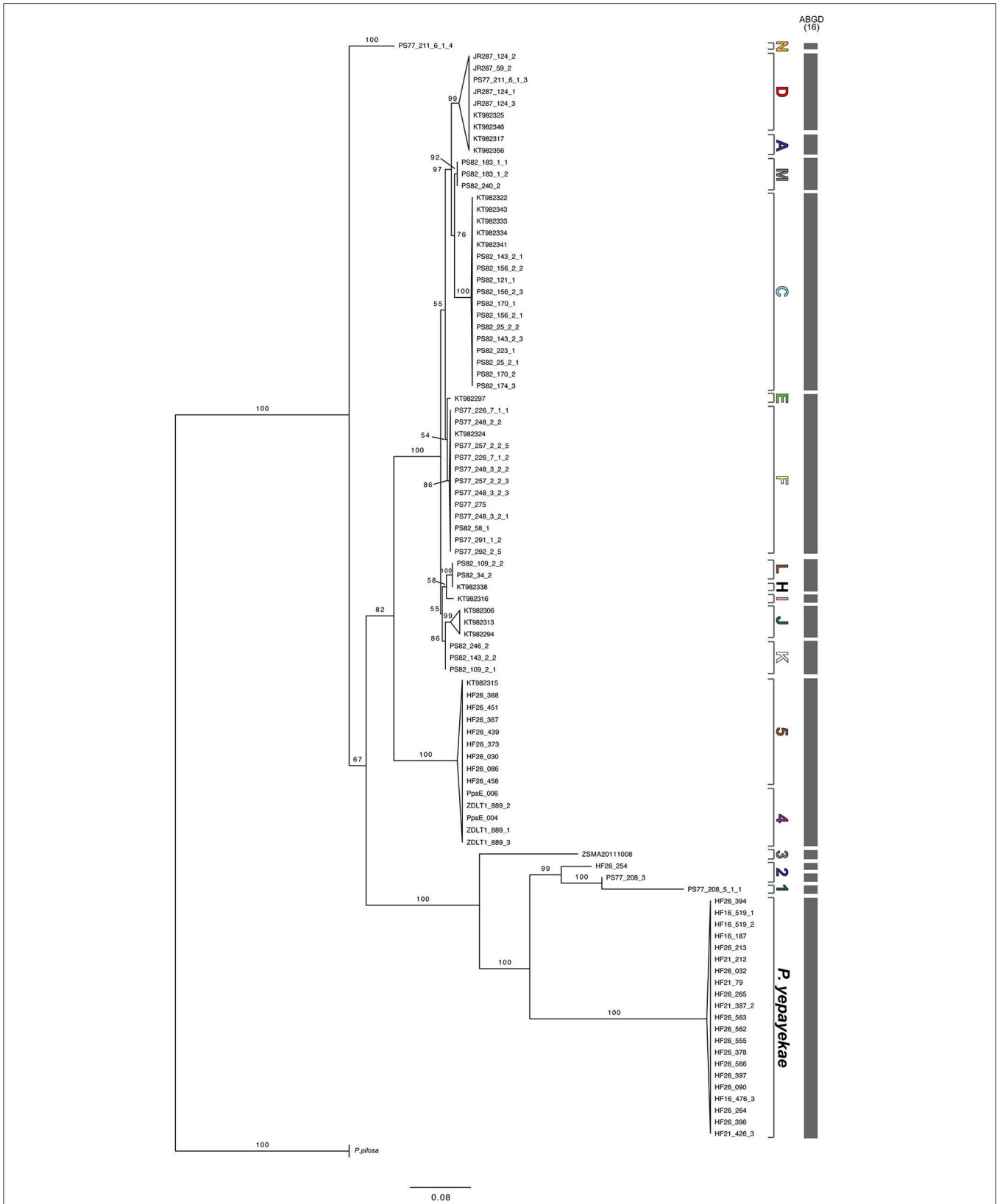


**FIGURE 4 | Maximum likelihood tree of ITS sequences of *Pallenopsis patagonica* s.l.** Bootstrap values above 50 are provided next to each node. Letters on the right correspond to the labels used in the mitochondrial tree (see **Figure 2**). Letters and numbers stand for mitochondrial clades from Antarctica (ANT) and the Subantarctic (SUB), respectively. Bars represent results of the ABGD analysis that was based on the full alignment.

to show a 10x greater divergence than found within species (Hebert et al., 2004). Barcode gap analyses within sea spiders have reported intraspecific threshold distances of up to 5% (Mahon et al., 2008). Here, we could not detect a distinct barcoding gap for the *P. patagonica* species complex but rather a gradient of pairwise distances, though many at low frequency (Supporting Information Table S1). This is similar to results observed within the sea spider complex *C. megalonyx* (Dietz et al., 2015b). Consequently, species delimitation based on COI is not straightforward and we followed several lines of argumentation summarized in Kekkonen and Hebert (2014) in order to discuss whether mitochondrial clades resemble species or not. According to the ideas presented in Kekkonen and Hebert (2014), species status can be assigned to clades with a full match of all different species delimitation methods. This is the case for the following six clades: SUB_3, ANT_C, ANT_H, ANT_I, ANT_L, and ANT_N. For all other clades, only a partial match between the different methods was observed. In such cases, Kekkonen and Hebert (2014) suggest to test whether (a) the resolved clades are monophyletic, (b) individuals are supported by diagnostic characters (nucleotide substitutions, insertions or deletions, see also Jörger and Schrödl, 2013), or (c) specimens of different clades occur in sympatry. All three criteria are based on species concepts. Both, monophyletic entities and diagnostic characters missing in sister taxa matter for the phylogenetic species concept. The biological species concept requires groups that are reproductively isolated, which in nature can only be detected when groups occur in sympatry. Due to a limited sample size and geographical range and hence the potential for unsampled haplotypes leading to ascertainment biases, the criterion of diagnostic characters is not considered here and only the two remaining criteria, monophyly and occurrence in sympatry, are applied to the evaluation of the mitochondrial data set.

The GMYC-subclades for *P. yepayekae* are the only example of the data set where the monophylum criterium cannot be applied, because although the individuals from Los Lagos themselves form a monophylum, the remaining specimens, which represent the majority, would be rendered as paraphyletic. Furthermore, specimens of both subclades do not occur in sympatry. As the Los Lagos specimens represent the northernmost occurrence of *P. yepayekae* known to date, a straightforward explanation of this pattern is isolation-by-distance. In particular because genetic differences between specimens from these two clades (max. uncorrected p-distances observed 0.9%) lie well within the range typically reported as intraspecific for other sea spider species (Mahon et al., 2008; Krabbe et al., 2010; Arango and Brenneis, 2013; Dietz et al., 2015a,b) as well as other arthropods (see Supporting Information Table S1 in Smith et al., 2005) we refrain from assigning species-level status to these two subclades and rather accept the grouping based on bPTP and ABGD.

For clade ANT_D, the found divergence of 1.6% between the two GMYC-subclades (ANT_D.1 vs. ANT_D.2, Antarctic Peninsula vs. South Georgia, respectively) is larger than between the GMYC-subclades of *P. yepayekae*. But because this value is still within the range reported as intraspecific and the subclades occur in different regions it cannot be ruled out that they represent two geographically separated populations. Hence, we refer to these subclades as one clade. ANT_D represents the only reported *P. patagonica* s.l. clade that crossed the deep sea between the continental shelf and the Subantarctic islands, but stayed within the Polar Front. Gene flow between the Antarctic continental shelf and South Georgia has already been reported in a few studies on other benthic invertebrates (Thornhill et al., 2008; Wilson et al., 2009; Dietz et al., 2015a,b).

ABGD merged five of the bPTP/GMYC-clades. All single bPTP/GMYC-clades are reciprocally monophyletic, but only SUB_1 and SUB_2 occur in sympatry. Following the protocol of Kekkonen and Hebert (2014), SUB_1 and SUB_2 would represent two distinct species as revealed by bPTP and GMYC. All other clades merged by ABGD do not occur in sympatry and therefore species assignments are not possible based on the limited data set. ANT_A, ANT_B, and ANT_M were sampled from Ross Sea and Eastern Weddell Sea with not exceedingly high uncorrected pairwise distances ranging from 2.1 to 3.5%. Thus, the grouping suggested by ABGD seems adequate. ANT_E, ANT_F, and ANT_G were sampled from the Western side of the Antarctic Peninsula, on both sides of the Weddell Sea and the Ross Sea. This pattern could be the result of isolation by distance, too. Harder et al. (2016) also found clade G as a distinct clade using bPTP, GMYC, and ABGD as delimitation methods. Clades E and F were separated by bPTP and GMYC, but merged with ABGD. Although Harder et al. (2016) decided to keep all three distinct, we suggest to be more careful here in particular in view of the few specimens available (only two for each of clade E and G) and the shallow divergences. Similar as above, ANT_J and ANT_K from the Ross Sea and Weddell Sea, respectively, have a moderate uncorrected pairwise distance of 2.1%. Hence, they are also not treated as different units here. This is also true for groupings of SUB_4 and SUB_5. Specimens of both again do not occur in sympatry (Strait of Magellan vs. Falkland Plateau), and divergence falls well in the range of values reported as intraspecific (average uncorrected pairwise distance 1.93%). In the reported cases of (partial) mismatch between the three delimitation methods it is difficult to apply a general rule, because no clear barcoding gap is known that allows for a clear cut between intra- and inter-specific genetic distances. The fact that we find clades, e.g., ANT_A and ANT_C, with very low intra-clade divergences (<1%) despite a broad distribution range would suppose that intraspecific genetic distances are small also for species with a broad distribution range (i.e., argument against isolation-by-distance). However, limited sampling size does not allow for further conclusions.

We suggest that for partial matches between delimitation methods every case should be evaluated on its own. Combining all these arguments in a conservative way, we suggest 14 distinct evolutionary units in *P. patagonica* s.l. based on the COI data (**Table 2**, Supporting information Table S2).

## Nuclear Support for COI Based Species Delimitation

As shown in Dietz et al. (2015b) for pycnogonids only looking at mitochondrial data can lead to overestimation and

**TABLE 2 | Comparison of results from species delimitation analyses using mitochondrial (COI) and nuclear (ITS) data of *Pallenopsis patagonica* and a final recommendation for groupings.**

| Clade | COI (14/22) | ITS (16/19) | Final grouping (15) |
|---|---|---|---|
| ANT_A | | | ANT_ABM |
| ANT_B | | NA | |
| ANT_M | | | |
| ANT_C | | | ANT_C |
| ANT_D1 | | | ANT D |
| ANT_D2 | | | |
| ANT_E | | | ANT_EFG |
| ANT_F | | | |
| ANT_G | | NA | |
| ANT_H | | | ANT HL |
| ANT_L | | | |
| ANT_I | | | ANT_I |
| ANT_J | | | ANT_J |
| ANT_K | | | ANT_K |
| ANT_N | | | ANT_N |
| SUB_1 | | | SUB_1 |
| SUB_2 | | | SUB_2.1 |
| | | | SUB_2.2 |
| SUB_3 | | | SUB_3 |
| SUB_4 | | | SUB 4 + 5 (Falkland) |
| SUB_5 | | | |
| Pye.1 | | | *P. vepavekae* |
| Pye.2 | | NA | |

*Supporting Information Table S2 for a detailed list.*

misinterpretation of the actual species number (see Toews and Brelsford, 2012 for a review). Dietz et al. (2015b) also showed that ITS is a suitable marker for sea spiders as unlike the situation reported for other organisms (e.g., Weitemier et al., 2015) no multiple intragenomic variants for this gene could be detected when using high-throughput sequencing data (Leese et al., 2012). Comparing ITS and COI data we first see that contrary to *C. megalonyx* (Dietz et al., 2015b) no mito-nuclear discordances are observed in *P. patagonica* s.l. (**Figures 2**, **4**) This indicates that different processes acted after initial mitochondrial lineage sorting on both sea spider species complexes and will be discussed below. Most importantly, ITS sequences are not shared between different COI clades in *P. patagonica*, with the exception of the mitochondrial sister clades ANT_H and ANT_L (both with a full match when comparing delimitation methods) that show the same ITS sequence. Even though ANT_E and ANT_F do not share one haplotype, ITS sequences are very similar and species delimitation analysis clusters them together. However, the groupings ANT_H and ANT_L as well as ANT_E and ANT_F (including ANT_G) represent a monophylum within the mitochondrial tree each. Whereas, ANT_H and ANT_L both had a full match when comparing mitochondrial data across delimitation methods, ANT_E and ANT_F were grouped together with ANT_G by ABGD. Unfortunately, we were not able to obtain ITS sequences of an individual from ANT_G to analyse whether ANT_G also groups with ANT_E and ANT_F when analyzing ITS. However, ANT_E and ANT_F clustered into one ABGD group in the mitochondrial tree similar to the ITS tree. Given the lack of resolution, we here suggest not proposing species status for clades ANT_H, ANT_L, ANT_E, ANT_F and ANT_G based on mitochondrial results but suggest to refer to the two groups containing ANT_H and ANT_L as well as ANT_E, ANT_F and ANT_G as one clade each. We are aware of the fact that speciation could be recent and thus has not been picked up with ITS (**Table 2**).

Mitochondrial clades considered as one evolutionary unit sometimes comprise up to three different but genetically very similar ITS sequences. These sequences, however, cluster together when using ABGD on the ITS data set. For example, based on COI data clades ANT_J and ANT_K that were both distinguished as separate clades by bPTP and GMYC were treated as one hyper-clade as there was a lack of characters distinguishing them and ABGD clustered them together when analyzing mitochondrial data. But ITS data within this hyper-clade can be assigned to the two different bPTP/GMYC clades. The same holds true for further combinations that are also congruent between ITS and the bPTP/GMYC delimitation of the COI gene (SUB_1 and SUB_2, SUB_4, and SUB_5 as well as ANT_A and ANT_M). In the case of ANT_D, ITS sequences of all available representatives are identical. Thus, the assignment of all individuals to one clade by ABGD and bPTP is congruent with the ITS result. The more resolved delimitation into subclades suggested by the GMYC analysis of the COI data is not supported by the ITS data (Supporting Information Table S2). As above, we here also suggest a conservative approach to not treat these clades as distinct species prior to further evidence.

In view of the evidence from the COI data set and the protocol by Kekkonen and Hebert (2014) as well as the nuclear gene marker results we propose 15 putative evolutionary units for the current data set of *P. patagonica* s.l. (**Table 2**). The number is likely to change should further data become available as major regions of the Southern Ocean, especially East Antarctica, still remain unexplored. More important though, is to add further evidence that helps defining a clear boundary between intra- and interspecific characters of the species complex. COI combined for the first time with ITS data of *P. patagonica* s.l. is a major step forward. However, data are not sufficient for a final delimitation across all clades and additional independent characters (morphology, further genes) are needed to make clear statements. Still, the finding of mito-nuclear agreement supports that in contrast to *C. megalonyx* we can describe the distinct groups contained within *P. patagonica* s.l. reasonably well with the current data available.

## Distribution Ranges

Our data supports a strong barrier effect of the Antarctic Polar Front as we did neither observe sister clades nor clades containing individuals from either side of the Polar Front of *P. patagonica* s.l. Such a pattern has also been observed for many

other benthic invertebrates (e.g., Page and Linse, 2002; Thornhill et al., 2008; Krabbe et al., 2010). The fact that individuals from South Georgia, i.e., a Subantarctic island south of the Polar Front, form one clade with individuals reported from the northernmost tip of the Antarctic Peninsula (ANT_D) and cluster within the Antarctic super-clade of *P. patagonica* s.l. hints at a colonization event of South Georgia from the Antarctic. This direction of gene flow makes sense as it is consistent with a pattern of colonization with the Antarctic Circumpolar Current from West to East (Leese et al., 2010). However, for *C. megalonyx* also shared haplotypes between South Georgia and the Antarctic Peninsula were found for one clade (Clade A; Dietz et al., 2015b). Interestingly, here genetic diversity patterns clearly indicated the opposite pattern, i.e., gene flow from South Georgia to the tip of the Antarctic Peninsula. Dietz et al. (2015b) considered South Georgia as the likely refugium for members of this clade given the exceptionally greater diversity. *Colossendeis* has been reported from bathypelagic samples (Staples, 2007 and references therein) and a distribution with deep-sea currents and not the Antarctic Circumpolar Current are conceivable. It should be noted that whereas haplotypes were shared at least partly for *C. megalonyx* clade A between the two distant regions, no haplotype sharing was observed for *P. patagonica* clade ANT_D here. This indicates that, if at all, gene flow is extremely limited or represented a singular colonization event in *P. patagonica*.

Our enlarged sampling has extended the previously reported distribution ranges of some clades. *P. yepayekae* was found in the Strait of Magellan, which extends the occurrence of this species southwards (Weis et al., 2014). With the first records of specimens of clades ANT_F and ANT_C in the Eastern Weddell Sea, we could extend the distribution range of clades previously only reported from the Antarctic Peninsula (Harder et al., 2016). This is also the case for clade ANT_D where individuals from South Georgia were added to a clade previously only reported from the Antarctic Peninsula. It has been stated that narrow rather than broad distribution ranges might be the rule rather than the exception for sea spiders. Krabbe et al. (2010) postulated that most *C. megalonyx* clades have a narrow and allopatric distribution. However, analyzing more samples lead to the result of clades with a circumpolar distribution with isolation by distance (Dietz et al., 2015b). Thus, for *P. patagonica* s.l. we may also expect much broader distribution ranges when further material especially from unsampled locations is going to be included.

Furthermore, geographic separation between populations within clades (regional pattern) seems likely as we find subclades in several clades that are geographically separated. GMYC analyses revealed a subclade within *P. yepayekae* consisting of four individuals from the same area. A stronger effect of geographic separation can be seen for the second reported subdivision of the bPTP/ABGD clade by GMYC. For ANT_D there is a separation between an island population (South Georgia) and one from the Antarctic shelf (i.e., Antarctic Peninsula). Although supported by nuclear data, SUB_4 and SUB_5 clustered together in the ABGD analysis. Here, SUB_4 is represented by individuals from the Falkland Islands only. Isolation of Falkland Island populations from those found on the rest of the South American continental shelf has also been reported for the isopod *Serolis paradoxa* (Leese et al., 2008).

We also found three new clades in the Eastern Weddell Sea only. It thus might be that different clades have different dispersal capabilities, however, as many habitats around the Antarctic shelf (e.g., Davis Sea and Dumont d'Urville Sea) and Subantarctic islands (e.g., Kerguelen Plateau) have been scarcely sampled, we cannot exclude that distribution ranges are generally broader than currently reported.

## Which Clade Represents *P. patagonica* Sensu Stricto

The type specimen of *P. patagonica* (Hoek, 1881) has been collected from the Atlantic opening of the Strait of Magellan. In the absence of material from the Strait of Magellan, Weis et al. (2014) already proposed the Falkland clade as *P. patagonica* sensu stricto. Adding new samples, the Falkland clade also included samples from the Strait of Magellan, however it should be mentioned that this clade can geographically and genetically be subdivided into two sub clades. Both, GMYC and bPTP divided the Falkland clade into SUB_4 and SUB_5 that respectively included either samples from the Falkland Islands or the Strait of Magellan. Likely, specimens here assigned to clade SUB_5 represent the closest relatives of *P. patagonica* sensu stricto. However, ABGD results for the mitochondrial data combined SUB_4 and SUB_5 into a single clade. This was also supported by ITS data. Further information about the sub clades and the assignment of the type specimen might be obtained by a morphological reinvestigation of the type material in comparison with the new material from the Strait of Magellan.

## Divergence Dates

In the absence of calibrated rates, molecular clock estimates using rates from other taxa can only be regarded as a rough proxy. For cold environments it might be assumed that mutation rate is lower as compared to temperate and tropic regions ("slow-rate hypothesis"; Bargelloni et al., 1994). However, evidence for this is still ambivalent (Held, 2001). Specifically, as we are addressing very recent divergence times it can be assumed that divergence times may be systematically higher than the rates inferred from rather deep calibration points (see Ho et al., 2005). The rate used and the error bars should thus be regarded as a rough orientation helping to interpret the radiation of *P. patagonica*. Even when considering the huge error bars, it is obvious that the divergence of the Subantarctic and the Antarctic super-clades took likely place in the Miocene after the opening of the Drake Passage. This indicates a single colonization event after the onset of the Polar Front and the Antarctic Circumpolar Current. The direction of the colonization (out of or into the Antarctic) remains unsolved in view of the limited number of outgroups. Also, the radiation of the many Antarctic *P. patagonica* species very likely started in the late Pliocene and increased during the Pleistocene (last 2.5 myr). Such patterns have been reported before (e.g., Held, 2000; Page and Linse, 2002; Thornhill et al., 2008; Krabbe et al., 2010; Leese et al., 2010; Hemery et al., 2012; Dietz et al., 2015a,b) suggesting that over evolutionary time scales the Polar Front has not been

an impermeable barrier to gene flow in general, though we did not observe across Drake Passage exchange in more recent clades of *P. patagonica* s.l. However, in view of the above-mentioned limitations of molecular clock calculations, we advise to use the divergence estimates made here with caution.

## Comparing Species Complexes of *P. patagonica* and *C. megalonyx*

It becomes obvious that total divergence contained in *P. patagonica* s.l. exceeds the genetic divergence found within *C. megalonyx* (17 vs. 11% maximum pairwise distances, respectively). This suggests that species delimitation using morphological characters is more advanced in *Colossendeis* as compared to *Pallenopsis*. The description of *P. yepayekae* within *P. patagonica* s.l. shows that morphologically clearly distinguishable species exist, but no similarly detailed morphological inspections as within *Colossendeis* have been performed yet (Hodgson, 1907, 1908; Fry and Hedgpeth, 1969; Pushkin, 1993; Child, 1995; Dietz et al., 2013, 2015a). Thus, when comparing the complexes of *C. megalonyx* and *P. patagonica* side by side a similar proportion of the tree should be taken into consideration. When comparing pairwise COI sequence divergence between *C. megalonyx* (Krabbe et al., 2010; Dietz et al., 2015b) with all representatives of the Antarctic clade within *P. patagonica* s.l. a striking result is that barcode gap patterns look almost identical (**Figure 3**). Also, neither of the two species complexes shows a distinct barcode gap, pairwise sequence distances in the range of 2–5% are found at low frequencies. This is the reason for the more ambiguous ABGD results. However, the majority of inter-clade comparisons for both complexes are in the range of 6.5–9.5% (see **Figure 3**). These are values typically reported as interspecific.

Still, there is a substantial difference between this study and the study on *C. megalonyx* by Dietz et al. (2015b) in that all mitochondrial clades in *P. patagonica* s.l. (with the exception of ANT_L and ANT_H that are identical for ITS) are also supported by diagnostic ITS substitutions (Supporting Information Table S2), whereas in *C. megalonyx* there is strong evidence for hybridization across several clades that have even more than 7% COI divergence. The results for *P. patagonica* thus are similar to most other studies on Southern Ocean biota finding mito-nuclear agreement (e.g., Leese and Held, 2008; Dietz et al., 2015a) and thus support of distinct species. It thus remains subject to discussion whether hybridization among *C. megalonyx* clade members as opposed to *P. patagonica* is possible due to the slower build-up of pre- or post-zygotic reproductive barriers.

One result of the direct comparison of both species complexes with the same molecular markers made here is that similar processes may have led to the divergence of distinct mitochondrial lineages. Assuming similar molecular clock rates, both have likely taken place in the same period (Plio-/Pleistocene) characterized by drastic environmental changes between glacial and interglacial periods (see Thatje et al., 2005; Allcock and Strugnell, 2012). Yet, whereas for *C. megalonyx* hybridization of many of the species has been detected, this is not the case for *P. patagonica* s.l. Given the limited information on the biology of the species reasons for this difference are difficult to estimate. One reason might be differences in the reproductive mode, another one differences in dispersal capabilities. Due to a lack of knowledge about reproduction within different sea spider species a direct comparison is not possible. However, larval stages and egg carrying males have never been reported for the genus *Colossendeis* possibly indicating low reproduction rate, whereas for *Pallenopsis*, males carry the eggs until hatching (benthic brooding) indicating a higher reproduction rate than in *Colossendeis*. Therefore, a low dispersal capability is assumed for *Pallenopsis* (except for occasional dispersal of adults, see below) while the situation in *Colossendeis* is unclear. The data for *C. megalonyx* Clade A (shared haplotypes between South Georgia and the Antarctic Peninsula) as well as most of the circumpolar clades in comparison to the many clades in *P. patagonica* that show rather narrow distribution ranges add further support for this difference in mobility. A lack of dispersal between isolated habitats can in principle promote the rise of reproductive barriers. Other possibilities include e.g., much stronger patterns of sexual selection and thus pre-zygotic mechanisms leading to faster complete lineage sorting. Also, *C. megalonyx* might show generally greater effective population sizes that counteract speciation. In view of similar divergence and diversity patterns for the COI data this seems, however, implausible. Perhaps the Antarctic Peninsula was colonized by active (i.e., walking) migration of *C. megalonyx* Clade A individuals from South Georgia through the deep sea after the end of the last glacial period. *Pallenopsis* has only been reported for the meso-pelagial but also in upper water plankton samples and drifting on jellyfish (Pages et al., 2007 and references therein). However, most likely the Antarctic Circumpolar Current prevents a drift across of the Antarctic Polar Front.

While for *C. megalonyx* we see strong evidence for an *in situ* evolution in Antarctica and migration to the Subantarctic (Clade B, M), this can neither be proved nor rejected for *P. patagonica* yet.

# CONCLUSIONS

The results of our study on the sea spider *P. patagonica* support some, but not all of our initial hypotheses: (1) We find an increase of mitochondrial clades and an extension of distribution ranges with additional sampling. (2) Adding for the first time nuclear ITS data to verify the detected mitochondrial lineages in general found good agreement between both marker systems, i.e., no mito-nuclear discordances. This is in disagreement to a recent report in *C. megalonyx* where strong evidence for hybridization and introgression was reported. Therefore, we suggest that the number of mitochondrial clades likely resembles the number of distinct species. However, application of state of the art species delimitation methods and analysis of both mitochondrial and nuclear genes does not lead to an unequivocal species delineation. Hence, future work needs to include more sets of characters for integrative taxonomy. The application of a molecular clock approach suggests that drivers of the biodiversity pump (speciation drivers) have acted at the same time scales

producing mainly young divergences in both *P. patagonica* s.l. and *C. megalonyx* but led to the formation of new species more efficiently in *P. patagonica* s.l.

## AUTHOR CONTRIBUTIONS

Conceived the study: JSD, FL, and RRM. Laboratory analyses: JSD, AMH, and ARM. Bioinformatic analyses: JSD, FL. Wrote the paper: JSD, FL, and RRM.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fevo.2016.00139/full#supplementary-material

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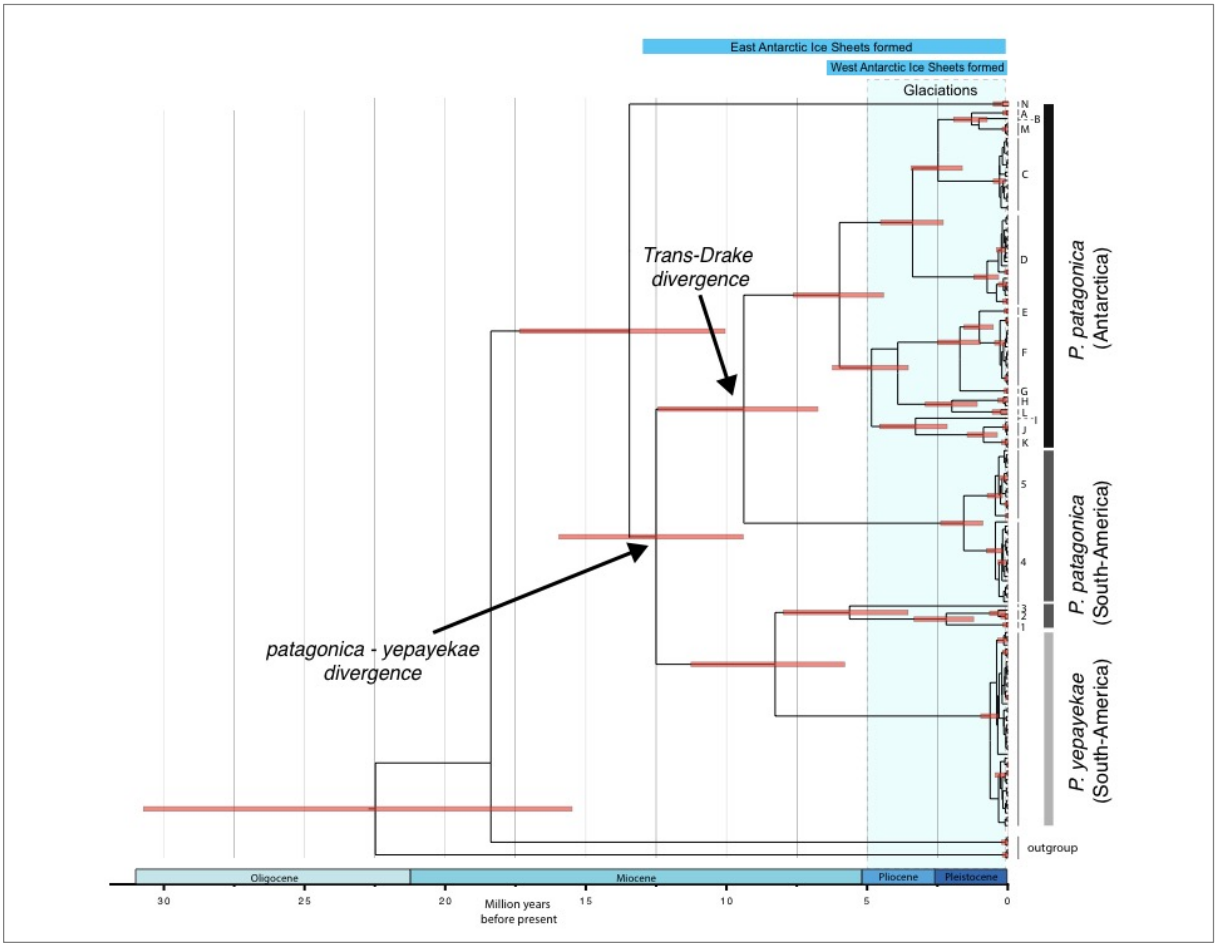
**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## *Supplementary material*

# Nuclear and mitochondrial gene data support recent radiation within the sea spider species complex *Pallenopsis patagonica*




**Supplementary Figure S1:** Time-calibrated COI tree calculated with BEAST using a standard arthropod clock rate of 1.15% per lineage and million years (Brower et al. 1994). Red bars indicate 95% High Posterior Density intervals of divergence dates for nodes with posterior probabilities of >0.7.

**Supporting Information Table S2:** List of diagnostic characters for the final mitochondiral (COI) and nuclear (ITS) data set found for *Pallenopsis patagonica*. Private alleles for individual clades and diagnostic characters for groupings are listed (alignment position: substitution). Asterisk (*) indicates amino acid substitution.

| Clade | Number of individuals | Private alleles | Diagnostic characters for groupings | | ITS | Private alleles | Diagnostic characters for groupings |
|---|---|---|---|---|---|---|---|
| ANT_A | 2 | 97: C | | | 10 | 498: C | |
| ANT_B | 1 | 124: C | | | | | |
| ANT_C | 18 | 228: G; 235: A | | | 13, 14 | 40+41: TG; 46+47: -- (gaps); 465: A | |
| ANT_D.1 | 5 | 136: G | 132: T*; 169: G | | 9 | n.a. | 498: A |
| ANT_D.2 | 17 | n.a. | | | 9 | 499: A | |
| ANT_E | 2 | n.a. | 106: T | | 20 | 62: A | |
| ANT_F | 17 | n.a. | | 334:A | 19 | n.a. | 521: T; 1018: T |
| ANT_G | 2 | 253: G | | | | | |
| ANT_H | 3 | 169: C | | | 15 | 230: T; 526-535: - (gaps) | |
| ANT_I | 1 | n.a. | | | 16 | 841: G | |
| ANT_J | 4 | 289: C | | | 18 | 413: A; 415: C | |
| ANT_K | 3 | 129: C | | | 17 | n.a. | |
| ANT_L | 2 | n.a. | | | 15 | 230: T; 526-535: - (gaps) | |
| ANT_M | 4 | 199: A; 220: -G | | | 12 | 70: C | |
| ANT_N | 2 | 26: T*; 181: T; 244: G; 350: G; 373: A | | | 23 | n.a. | |
| SUB_1 | 2 | 286: G; 397: C | | | 2 | 48:G; 143, 147, 149, 152: C; 162: G; 177: A; and more | |
| SUB_2 | 3 | n.a. | | | 1, 3 | n.a. | |
| SUB_3 | 1 | 18: G; 181: C; 250: G; 252: G; 328: A | | | 5 | n.a. | |
| SUB_4 | 20 | 70: G | 34: G; 199: G; 301: A; 349: T | | 21 | n.a. | 40: C; 87+88: A; 101: G; 135: G; 400+401: GA; 405-412: TTC(T)TTTC; 414: A; 416: C; 420: T; 422: G; 490: -(gap) |
| SUB_5 | 17 | n.a. | | | 22, 26 | 74: G | |
| *P. yepayekae* (Pye.1) | 43 | 426: T* | 41: C; 64: G; 149: A; 154: A; 232: A; 241: T; 277: A; 280: A; 340: G | | 6, 7, 8 | n.a. | |
| *P. yepayekae* (Pye.2) | 4 | n.a. | | | | | |

# CHAPTER II

# Combining morphological and genomic data to resolve species diversity and study speciation processes of the *Pallenopsis patagonica* (Pycnogonida) species complex

Jana S. Dömel, Till-Hendrik Macher, Lars Dietz, Sabrina Duncan, Christoph Mayer, Andrey Rozenberg, Katherine Wolcott, Florian Leese and Roland R. Melzer

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**Contributions to this manuscript:**

Experimental design and planning: 65%

Laboratory work: 100%

Morphological work: 20%

- Morphometric measurements: 0%
- Picture taking: 10%
- Morphological analyses: 50%

Data analysis: 60%

- SNP analyses: 0%
- Orthology: 0%
- Selection tests: 95%
- Morphometric analyses: 80%
- Combining Morphometric and Genetic data: 100%

Figures: 95%

- Figures 1-5, 7, 8, and 10: 100%
- Figure 6: 80%
- Figure 9: 50%
- Figure 11: 80%
- Supplementary Material 1-3, 5, 6: 100% (software output edited)

Tables: 80%

- Table 1: 50%
- Table 2: 20%
- Tables 3 and 4: 100%%
- Supplementary Material 4 and 8: 100%

Manuscript writing: 70%

- First draft: 75%
- Editing of first draft together with the other authors.

# Combining morphological and genomic evidence to resolve species diversity and study speciation processes of the *Pallenopsis patagonica* (Pycnogonida) species complex

Jana S. Dömel*[1], Till-Hendrik Macher[1], Lars Dietz[2], Sabrina Duncan[3], Christoph Mayer[2], Andrey Rozenberg[4], Katherine Wolcott[3], Florian Leese[1,5], Roland R. Melzer[3,6,7]

[1]Aquatic Ecosystem Research, Faculty of Biology, University of Duisburg-Essen, Universitaetsstr. 5, D-45141 Essen, Germany,
[2]Zoological Research Museum Alexander Koenig, Statistical Phylogenetics and Phylogenomics, Adenauerallee 160, D-53113 Bonn, Germany,
[3]Bavarian State Collection of Zoology - SNSB, Muenchhausenstr. 21, D-81247 Munich, Germany,
[4]Technion – Israel Institute of Technology, Faculty of Biology, Haifa 3200003, Israel
[5]Centre for Water and Environmental Research (ZWU), University of Duisburg-Essen, Universitaetsstr. 2, 45141 Essen, Germany
[6]Department Biologie II, LMU Munich, Großhaderner Str. 2, D-82152 Planegg-Martinsried, Germany
[7]GeoBioCenter, LMU Munich, Richard-Wagner-Str. 10 D-80333 Munich, Germany

*Corresponding Author: Jana S. Dömel (jana.doemel@uni-due.de)

## Abstract

***Background*:** *Pallenopsis patagonica* (Hoek, 1881) is a morphologically and genetically variable sea spider species whose taxonomic classification is challenging. Currently, it is considered as a species complex including several genetic lineages, many of which have not been formally described as species. Members of this species complex occur on the Patagonian and Antarctic continental shelves as well as around sub-Antarctic islands. These habitats have been strongly influenced by historical large-scale glaciations and previous studies suggested that communities were limited to very few refugia during glacial maxima. Therefore, allopatric speciation in these independent refugia is regarded as a common mechanism leading to high biodiversity of marine benthic taxa in the high-latitude Southern Hemisphere. However, other mechanisms such as ecological speciation have rarely been considered or tested. Therefore, we conducted an integrative morphological and genetic study on the *P. patagonica* species complex to i) resolve species diversity using a target hybrid enrichment approach to obtain multiple genomic markers, ii) find morphological characters and analyze morphometric measurements to distinguish species, and iii) investigate the speciation processes that led to multiple lineages within the species complex.

***Results*:** Phylogenomic results support most of the previously reported lineages within the *P. patagonica* species complex and morphological data show that several lineages are distinct species with diagnostic characters. Two lineages are proposed as new species, *P. aulaeturcarum* sp. nov. Dömel & Melzer, 2019 and *P. obstaculumsuperavit* sp. nov. Dömel, 2019, respectively. However, not all lineages could be distinguished morphologically and thus likely represent cryptic species that can only be identified with genetic tools. Further, morphometric data of 135 measurements showed a high amount of variability within and between species without clear support of adaptive divergence in sympatry.

***Conclusions*:** We generated an unprecedented molecular data set for members of the *P. patagonica* sea spider species complex with a target hybrid enrichment approach, which we combined with extensive morphological and morphometric analyses to investigate the taxonomy, phylogeny and biogeography of this group. The extensive data set enabled us to delineate species boundaries, on the basis of which we formally described two new species. No consistent evidence for positive selection was found, rendering speciation in allopatric glacial refugia as the most likely model of speciation.

**Key words:** sea spider, marine benthos, Antarctica, Patagonia, integrative taxonomy, target hybrid enrichment, cryptic species, selection.

## 1. Background

The diversity of the marine benthos of the Southern Hemisphere has been influenced by large scale extension of grounded glaciers on the Patagonian and Antarctic continental shelves during repeated glacial cycles in the Plio- and Pleistocene (Thatje et al. 2005, Convey et al. 2009, Försterra 2009). Several studies suggested that benthic life was limited to few isolated refugia in which independent divergence and lineage sorting processes promoted today's high species diversity in Southern Ocean and Patagonian shelf habitats (Clarke and Crame 1989, Leese et al. 2008, Allcock and Strugnell 2012, Fraser et al. 2012, Halanych and Mahon 2018). Molecular taxonomic studies added evidence on the role of glacial impacts on species divergence by reporting many previously unrecognized species (often referred to as "cryptic species") over the last few decades that often show non-overlapping, allopatric distribution ranges (Held and Wägele 2005, Leese et al. 2008, Wilson et al. 2009, Allcock and Strugnell 2012).

One animal group with remarkable (cryptic) species diversity are sea spiders (Mahon et al. 2008, Krabbe et al. 2010, Weis et al. 2014, Dietz et al. 2015a, Dömel et al. 2015, 2017). Sea spiders, or pycnogonids, are a group of exclusively marine arthropods that are especially diverse in the Southern Ocean (Aronson et al. 2007).

One prominent example for high species diversity is the *Pallenopsis patagonica* (Hoek, 1881) sea spider species complex. *Pallenopsis patagonica* has a holobenthic life cycle and is reported to occur with a circumpolar distribution around sub-Antarctic islands and on the continental shelf of Antarctica as well as southern South America (Munilla and Soler-Membrives 2009), i.e. in regions that were strongly impacted by glaciations during the last ice ages (Marden and Clapperton 1995). Since its first description by Hoek (1881), several authors have commented on the high morphological variability of *P. patagonica* and suggested that it represents a species complex (Hodgson 1907, Loman 1923, Gordon 1944, Weis et al. 2014). However, species delineation within this complex is difficult and there is a long history of attempts to resolve this question by either splitting the species when describing new species often based on a small number of specimens (e.g. Möbius 1902, Hodgson 1907, 1915, Pushkin 1975), or by lumping several species together declaring them synonymous (e.g. Child 1995). This culminated in two drastically different surveys by Pushkin (1993) and Child (1995). While Pushkin (1993) described more new species for the species complex, Child (1995) refuted this and instead recognized only one, *P. patagonica*, to which he attributed a high variability to *P. patagonica*. At the moment, four formerly described species are considered synonyms of *P. patagonica*: *P. glabra* Möbius, 1902, *P. hiemalis* Hodgson, 1907, *P. meridionalis* Hodgson, 1915 and *P. moebiusi* Pushkin, 1975 (Child 1995, Bamber et al. 2019). Furthermore, there are more closely related species from the Southern Hemisphere whose relationship to or position within

the species complex is unclear, e.g. *P. buphtalmus* Pushkin, 1993, *P. latefrontalis* Pushkin, 1993, *P. macneilli* Clark, 1963 and *P. notiosa* Child, 1992. Hence, several studies have addressed this issue in recent years by adding genetic data. First, Weis et al. (2014) reported that mostly sub-Antarctic specimens previously assigned to *P. patagonica* can be genetically divided into several groups based on mitochondrial cytochrome *c* oxidase subunit I (COI) data. Weis at al. (2014) also reported high morphological variability within the species complex. Based on the genetic and morphological differences, a new species was described, named *P. yepayekae* Weis, 2014. Further groups within the species complex were suggested based on molecular data reported by Harder et al. (2016) for Antarctic *P. patagonica* specimens. The authors defined ten distinct clades (labelled A-J) using the mitochondrial COI marker (Harder et al. 2016). To validate the proposed number of clades and to exclude mito-nuclear discordances, which can be found in other pycnogonids, e.g. *Colossendeis megalonyx* Hoek, 1881 (Dietz et al. 2015a), Dömel et al. (2017) investigated the highly variable nuclear internal transcribed spacer (ITS) marker for previously studied clades. In contrast to *C. megalonyx*, most lineages of the *P. patagonica* species complex were supported by both markers (only a few recently diverged ones were not). Thus, no evidence for mito-nuclear discordance was found. This suggested that the distinct lineages represented species defined based on the biological species concept. With additional specimens studied by Dömel et al. (2017), additional clades were identified. Altogether, 19 clades with mostly regional distribution patterns were proposed as independently evolving lineages under the specific name *patagonica* (labelled ANT A-N and SUB 1-5 in Dömel et al. 2017, according to their geographic occurrence).

So far, no diagnostic morphological characters are known to delineate clades and characterise new species within the *P. patagonica* species complex, which, however, would be critically important in order to assess the benthic diversity of the Southern Hemisphere and test hypotheses regarding the underlying evolutionary processes.

Many studies on benthic invertebrates, especially on benthic brooders that lack pelagic larval stages like sea spiders, have interpreted the fact that species typically showed allopatric distribution patterns as evidence for lineage sorting in independent ice-free refugia (Held and Wägele 2005, Held and Leese 2007, Leese et al. 2008, Allcock and Strugnell 2012, Soler-Membrives et al. 2017).

However, one study on the sea slug *Doris kerguelenensis* (Bergh, 1884) that occurs in the Southern Ocean, as well as sub-Antarctic waters, suggested that interspecific competition for prey was involved in speciation (Wilson et al. 2009). Similarly, Rutschmann et al. (2011) tested for adaptive speciation and radiation in notothenioid fish and found lineage-independent ecological differentiation into different niches probably as a result of positive selection. This

provides evidence that consideration of genetic drift and independent lineage development in isolated refugia may not suffice to explain the enormous diversity in southern marine benthic habitats (Clarke and Johnston 2003, Griffiths 2010). In fact, Antarctic and sub-Antarctic waters bear such a diverse range of extreme and different habitats and display diverse biotic interactions that speciation due to ecological divergence should more explicitly be explored as a potential process for speciation. In order to test for evidence of selection, quantitative evidence for functionally relevant changes in the genome has to be provided. With the availability of new analytical techniques for morphology (e.g. micro-computed tomography; µCT) and genetics (Next Generation Sequencing (NGS), e.g. target hybrid enrichment; Faircloth et al. 2012, Mayer et al. 2016), it becomes possible to generate large integrative data sets. Target hybrid enrichment, i.e. a technique that captures specific genes with known homology across a taxonomic group using synthetic probes, offers an immense potential to test for genes under selection, especially in poorly studied organisms such as all Southern Hemisphere marine benthic invertebrate species. Hence, this method can also be used to further investigate the species diversity and to test competing hypotheses and compare neutral vs. non-neutral speciation hypotheses, i.e. lineage sorting in bottlenecked refugia vs. adaptive divergence. By combining genomic and morphometric data sets, greater morphological differences are expected especially for taxa living in sympatry in contrast to those living in allopatry due to potential niche specialisation in form of ecological character displacement (Schluter 2000, Coyne and Orr 2004, Dieckmann et al. 2004).

Therefore, in this study we integrate all previous data on the *P. patagonica* species complex, combine them with genomic data obtained via target hybrid enrichment, analyses of morphological features using conventional observation methods and meristic data to study patterns of diversity and underlying evolutionary processes within the *P. patagonica* species complex. Specifically, we address the following questions:

- Do genome-wide data add further information about previously unrecognized species diversity within the *P. patagonica* species complex?
- Do we find morphological characters to distinguish the independently evolving lineages of the *P. patagonica* species complex and formally describe new species?
- Do we find evidence for adaptive divergence at morphological or genetic level or do neutral evolutionary processes suffice to explain the observed species diversity?

## 2. Results

The sample set included specimens of *Pallenopsis buphtalmus* (corresponding to mitochondrial clade ANT_M in Dömel et al. 2017), *P. latefrontalis* (ANT_F), *P. notiosa* (SUB_3) and *P. yepayekae* (Pye.1) as well as of further potential species within *P. patagonica*, i.e. ANT_C, ANT_D, ANT_K, ANT_L, SUB_1, SUB_2, SUB_4 and SUB_5. We refer to this set of putative species as the *P. patagonica* species complex (also *P. patagonica* sensu lato in Dömel et al. 2017), since using the key in Child (1992) would (erroneously) assign all those species to the morphospecies *P. patagonica*.

### 2.1. Genomic Analyses

The obtained dataset consisted of 61 individuals of the *Pallenopsis patagonica* species complex. One individual of *P. pilosa* (Hoek, 1881) genotyped by us and a previously published transcriptome assembly of *Anoplodactylus insignis* (Hoek, 1881) (Fernández et al, 2016) were added as outgroups in genetic analyses. When analyzing all *Pallenopsis* specimens on the nucleotide level, 821 out of 1607 targeted EOGs (Eukaryotic Orthologous Groups), which in our case are putative single-copy groups of orthologous genes, were recovered with a total alignment length of 474,954 bp. The data set used to infer a reliable root by including *A. insignis* was analysed on the amino acid level to reduce the branch length to the outgroup. This alignment included only EOGs for which a sequence of *A. insignis* was present and sites with a sequence coverage of at least 50%, which reduced the data set to 208 EOGs and 22,018 aa (corresponding to 66,054 bp). Furthermore, sequences that were outliers on the amino acid level were excluded. The models of evolution chosen by ModelFinder for the nucleotide data set were GTR+R2 for the first, TIM+R2 for the second and GTR+R4 for the third codon position. For the amino acid alignment including *A. insignis*, JTT+F+R3 was chosen as the best fitting model.

Single-nucleotide polymorphism (SNP) calling for all *Pallenopsis* samples (i.e. including *P. pilosa*) resulted in 2527 SNPs from 168 EOGs. This data set was used for construction of a phylogenetic tree only.

Phylogenetic analyses of the amino acid data set revealed that the *P. patagonica* species complex represents a monophyletic group with *P. pilosa* and *A. insignis* representing a joint outgroup (Supplementary Material 1). In particular *P. pilosa* was shown to be a sister group to the *P. patagonica* species complex, as assumed in previous studies (Weis et al. 2014, Dömel et al. 2017). Further analyses were conducted with the nucleotide data set not including *A. insignis*. Separate phylogenetic analyses based on the EOG alignment (in the following referred to as the

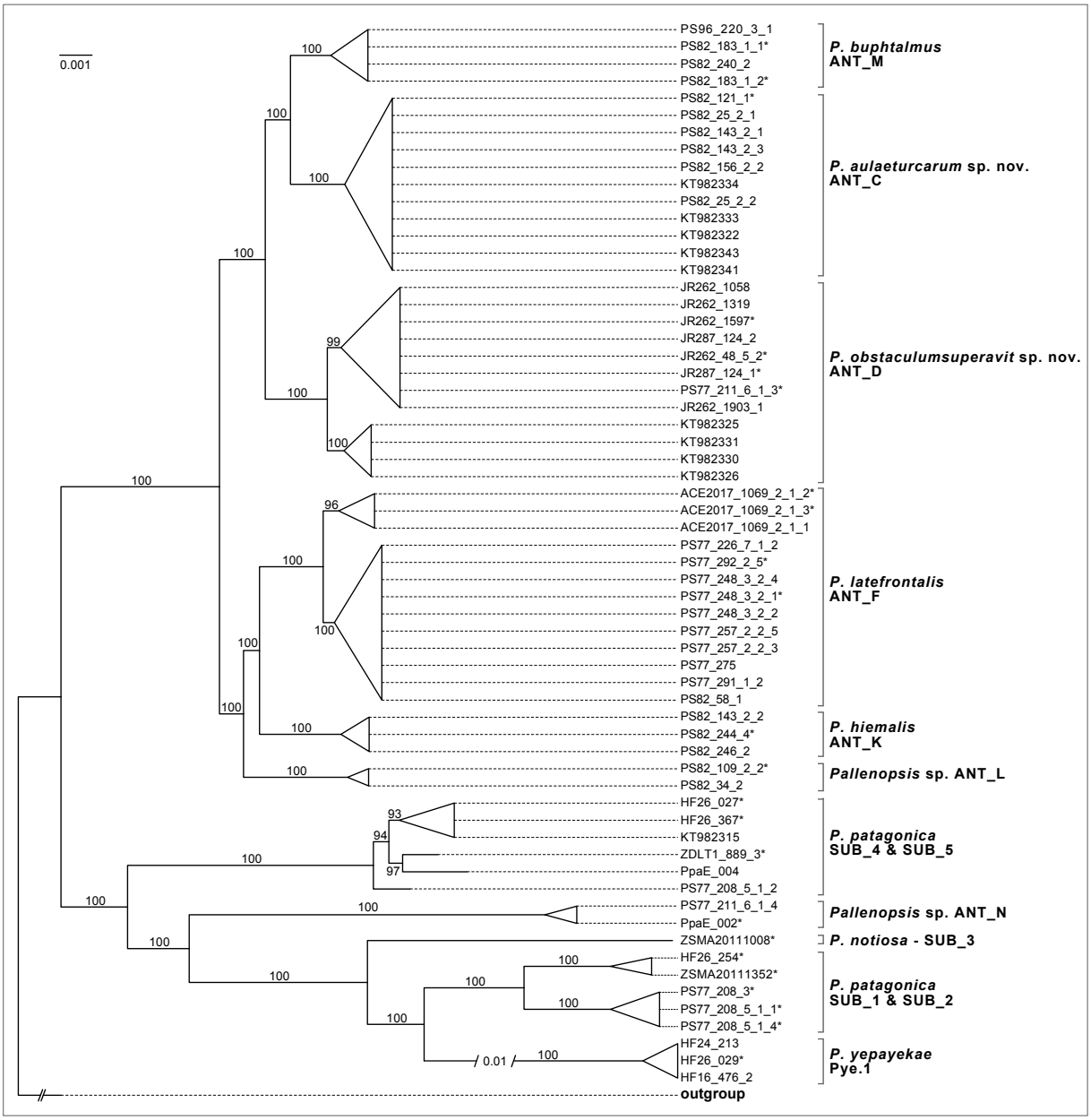


**Figure 1:** Phylogenetic tree of the *Pallenopsis patagonica* species complex. Maximum-Likelihood tree based on concatenated EOG sequences of all *Pallenopsis* samples. Asterisks (*) indicate samples that were also used in morphometric analyses. Bootstrap values are given next to the respective branches.

EOG data set) and the variant calling (in the following referred to as the SNP data set) including all *Pallenopsis* specimens resulted in phylograms with identical topologies (Figure 1 and Supplementary Material 2) but the EOG-based analysis had higher bootstrap support (bs) values and is discussed herein. Two major groups are discernible within the *P. patagonica* species complex, one including specimens assigned to all of the Antarctic clades (ANT) except ANT_N (from now on referred to as the "Antarctic supergroup") and one including specimens from all Patagonian clades (SUB) plus ANT_N (from now on referred to as "Patagonian supergroup"). The "Antarctic supergroup" is comprised of two major lineages, ANT_C/D/M and ANT_F/K/L. More detailed divisions of those groups are in agreement with the clades

delineated in Dömel et al. (2017). There is also a strong support for the geographical divide in ANT_D and *P. latefrontalis* (ANT_F) into specimens from the Antarctic shelf (both 100% bs) and sub-Antarctic islands (South Georgia with 99% bs, and Bouvet Island with 96% bs, respectively). Within the "Patagonian supergroup", SUB_4 and SUB_5 together represent the basalmost group of the "Patagonian supergroup" with SUB_4 being paraphyletic with respect to SUB_5. Analogously, SUB_1 and SUB_2 appear not strictly monophyletic with respect to each other, since specimens from Burdwood Bank belonging to both clades group together. ANT_N is nested within the "Patagonian supergroup", as are *P. notiosa* (SUB_3) and *P. yepayekae*.

For the principal component analyses (PCA) three SNP data sets were analysed. The first data set contained all specimens of the *P. patagonica* species complex and included 2543 SNPs from 175 EOGs. Furthermore, separate data sets for the "Patagonian supergroup" and the "Antarctic supergroup" yielded 2047 SNPs from 183 EOGs and 2487 SNPs from 216 EOGs, respectively. For the first SNP data set (*P. patagonica* species complex), 16 significant axes were found. There is a clear differentiation between five groups (Figure 2A). All Antarctic clades cluster together, with the exception of ANT_N. The Patagonian clades are divided into four groups, SUB_1/2, *P. notiosa* (SUB_3), SUB_4/5 and *P. yepayekae* (Pye.1). Analyses of the data set divided into the two supergroups obtained no significant axes for the "Patagonian supergroup". For the "Antarctic supergroup", the first seven axes were significant and showed a differentiation into the clades previously proposed by Dömel et al. (2017) (Figure 2B).

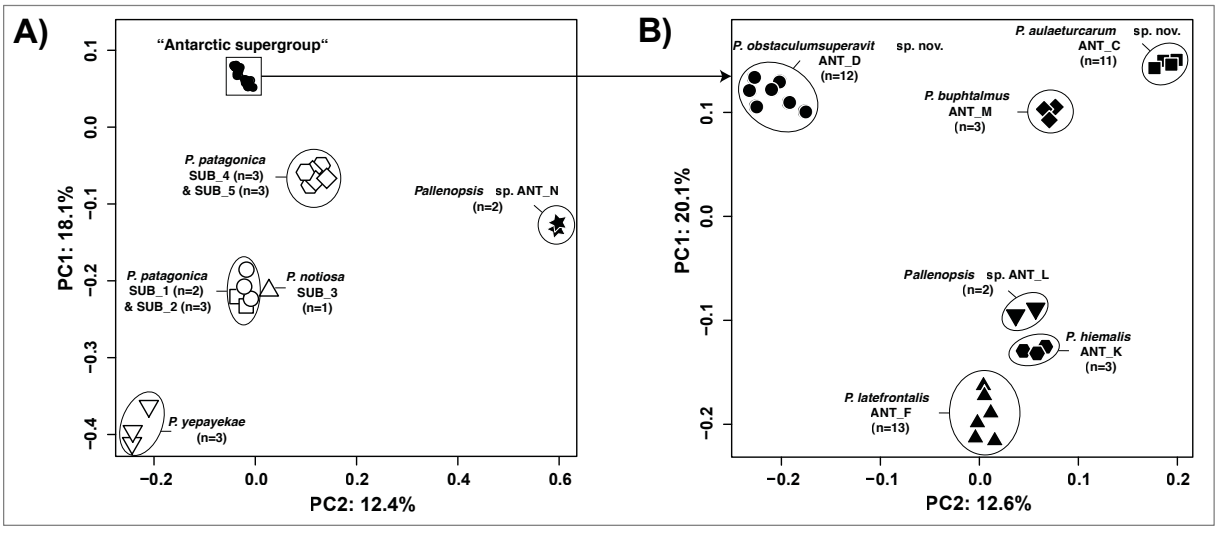


**Figure 2:** PCA from genomic data of the *Pallenopsis patagonica* species complex. PCA plots based on genomic data of **A**) all samples of the *Pallenopsis patagonica* species complex and **B**) samples of the "Antarctic supergroup".

For the clustering analyses, the cross entropy with the lowest median was chosen (Supplementary Material 3). By this criterion, the best number of ancestral populations was seven (K=7). The plot of the sparse nonnegative matrix factorization (sNMF) mostly supported the groupings obtained with the PCA. The differences were that ANT_K and ANT_L as well as SUB_1 and SUB_2 grouped together and showed similar proportions of the same ancestral populations (Figure 3).

For selection tests, a sequence alignment including only positions that were present in at least 50% of the samples was used. This resulted in an alignment of 82,782 bp recovering 293 EOGs. Seventeen codons within 17 EOGs and 49 codons within 38 EOGs under selection using the Fast Unconstrained Bayesian AppRoximation (FUBAR) and the Mixed Effects Model of Evolution (MEME), respectively, were detected. Sixteen codons within 16 EOGs were shared between both methods. Furthermore, no branches under selection were detected, irrespective of the applied test (aBSREL or BUSTED; see Methods).

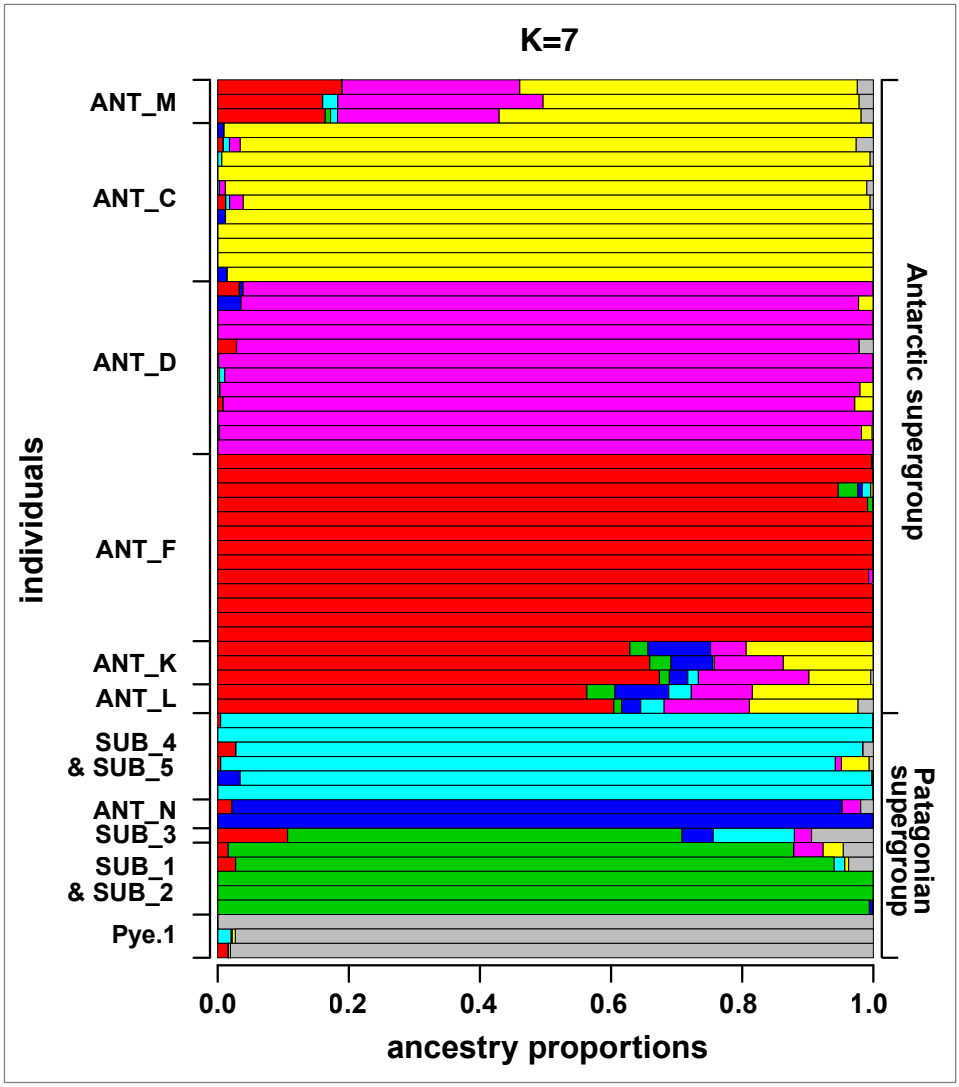


**Figure 3:** sNMF analyses of the *Pallenopsis patagonica* species complex. Graphical illustration of ancestry proportion estimates for all samples with K = 7. Estimated proportions of ancestry populations are illustrated by different colors. Each horizontal bar represents one specimen.

## 2.2. Morphology

### 2.2.1. Morphometrics

Morphometric measurements were taken for 37 individuals (a table including all measurements is provided in Supplementary Material 4) but due to damage during trawling, transport, storage or preceding genetic analysis, distal articles of appendices and hence data for those were often missing. After averaging measurements for bilateral characters, the amount of missing data was reduced by about three quarters. For further analyses, filtered data sets including 38 and 39 characters for the absolute and relative values, respectively, were used. PCA plots using all specimens did not show separation into clades but a trend for a division of sub-Antarctic and Antarctic samples (Supplementary Material 5).

To avoid the problem of overfitting, character sets optimal for species separation in discriminant analysis (LDA) were searched for using a heuristic approach. Therefore, only clades with a minimum of three individuals were included resulting in a data set of seven clades and 29 specimens. Absolute as well as relative values expressed as proportion of the trunk length were used.

For both data sets (absolute and relative values) multiple iterations of character selection were performed and it was recorded how often a character was added to an LDA model in individual optimizations and what its contribution was (see Table 1). The LDA plots of both data sets based on the character combinations with best performance clearly separated all clades from each other, except for clade ANT_D and ANT_F when looking at the absolute values (Figure 4). Furthermore, analysis of cross-validation confusion matrices confirmed that these results were not dominated by overfitting artefacts, with the correctness rate being higher for the relative values (0.83) than for the absolute values (0.76) (Table 2). Here, ANT_F and SUB_5 had many misassignments (absolute data set). Analogously, PCAs for both data sets showed that the clades ANT_D and ANT_F could not be separated from each other for the data set including absolute values (see matrices of all PCs in the Supplementary Material 6).

Significant differences of characters between clades were found for neither of the two data sets after Bonferroni correction. However, 33 and 14 significant differences between specimens from the different geographic regions (SUB and ANT) for absolute and relative value, respectively, were found (Table 1). In all cases, the characters of the Antarctic samples were larger than of the Patagonian ones. As for analyzed specimens, males were more frequent in sub-Antarctic (75%) and females preponderated in Antarctic clades (65%), characters were also tested for significant differences between sexes. There were five and eight significant differences for absolute and relative values, respectively, of which five characters for each data set also showed significant differences between geographic regions (see Table 1).

**Table 1: Results of morphometric analyses of the *Pallenopsis patagonica* species complex for both data sets (absolute and relative values).** Contributions to correctness rate (CR), characters combination (CC) for the best LDA performance and p-values for significant differences between geographic regions and sexes are listed. * p-values are only listed for analyses that showed significant differences. [1]Number of times the character was added and its addition led to a positive increase in cross-validation correctness rate of individual LDA models during repeated character selections (% of total in parentheses). See how the repetitions were organized in Materials and Methods. [2]Average increase in cross-validation correctness rate after addition of the character to an LDA model had a positive effect in the character selections.

| | Absolute | | | | | Relative | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Character | Times the character contributed to an LDA model during character selection[1] | Increase in correctness rate (mean±SD)[2] | CC for best LDA performance | Differences between geographic regions * | Differences between sexes * | Times the character contributed to an LDA model during character selection[1] | Increase in correctness rate (mean±SD)[2] | CC for best LDA performance | Differences between geographic regions * | Differences between sexes * |
| abdomen W | 34 (2.4%) | 0.05 ± 0.03 | | < 0.001 | - | 19 (1.23%) | 0.09 ± 0.04 | | - | - |
| abdomen L | 27 (1.9%) | 0.21 ± 0.15 | | < 0.001 | - | 2 (0.13%) | 0.09 ± 0.01 | | - | - |
| eye H | 115 (8.11%) | 0.22 ± 0.11 | x | < 0.001 | - | 15 (0.97%) | 0.1 ± 0.06 | | - | - |
| eyes distance | 6 (0.42%) | 0.06 ± 0.03 | | - | - | 22 (1.43%) | 0.08 ± 0.03 | | - | - |
| ocular tubercle W | 15 (1.06%) | 0.05 ± 0.03 | x | 0.004 | - | 24 (1.56%) | 0.08 ± 0.05 | | - | - |
| ocular tubercle H | 147 (10.37%) | 0.1 ± 0.07 | x | - | - | 102 (6.63%) | 0.12 ± 0.05 | x | 0.005 | 0.012 |
| ceph. segment | 36 (2.54%) | 0.09 ± 0.08 | | < 0.001 | - | 24 (1.56%) | 0.11 ± 0.06 | | 0.012 | - |
| cheliphore 1 | 136 (9.59%) | 0.2 ± 0.13 | x | < 0.001 | - | 2 (0.13%) | 0.11 ± 0.01 | | 0.048 | - |
| cheliphore 2 | NA | NA | NA | NA | NA | 5 (0.32%) | 0.09 ± 0.03 | x | - | - |
| cheliphore 3 | 5 (0.35%) | 0.05 ± 0.02 | | < 0.001 | - | 1 (0.06%) | 0.1 | | 0.027 | - |
| cheliphore 4 | 8 (0.56%) | 0.06 ± 0.03 | | 0.001 | - | 23 (1.49%) | 0.09 ± 0.04 | | - | - |
| palp | 41 (2.89%) | 0.11 ± 0.1 | | 0.001 | - | 198 (12.87%) | 0.16 ± 0.08 | x | - | - |
| proboscis thick2tip | 136 (9.59%) | 0.11 ± 0.06 | | - | - | 83 (5.39%) | 0.15 ± 0.08 | | - | - |
| proboscis basis | 23 (1.62%) | 0.11 ± 0.07 | | < 0.001 | 0.027 | 6 (0.39%) | 0.06 ± 0.05 | | - | - |
| proboscis thickest | 29 (2.05%) | 0.17 ± 0.14 | | < 0.001 | - | 7 (0.45%) | 0.07 ± 0.05 | | 0.001 | - |
| proboscis L | 2 (0.14%) | 0.02 ± 0.01 | | < 0.001 | - | 13 (0.84%) | 0.06 ± 0.03 | | < 0.001 | - |
| trunk W1 | 90 (6.35%) | 0.11 ± 0.07 | | < 0.001 | - | 41 (2.66%) | 0.11 ± 0.05 | | 0.004 | 0.022 |
| trunk W12 | 30 (2.12%) | 0.09 ± 0.06 | | 0.048 | - | 168 (10.92%) | 0.17 ± 0.09 | x | < 0.001 | - |
| trunk W2 | 8 (0.56%) | 0.07 ± 0.05 | | 0.001 | - | 18 (1.17%) | 0.07 ± 0.05 | | - | - |

**Table 1:** Continued.

| Character | Absolute | | | | | Relative | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Times the character contributed to an LDA model during character selection[1] | Increase in correctness rate (mean±SD)[2] | CC for best LDA performance | Differences between geographic regions * | Differences between sexes * | Times the character contributed to an LDA model during character selection[1] | Increase in correctness rate (mean±SD)[2] | CC for best LDA performance | Differences between geographic regions * | Differences between sexes * |
| trunk W34 | 15 (1.06%) | 0.06 ± 0.03 | | - | - | 73 (4.74%) | 0.11 ± 0.05 | | < 0.001 | 0.042 |
| trunk W4 | 5 (0.35%) | 0.03 ± 0 | | < 0.001 | - | 23 (1.49%) | 0.15 ± 0.09 | | - | 0.037 |
| trunk H | 1 (0.07%) | 0.03 | | - | - | 20 (1.3%) | 0.1 ± 0.04 | | - | - |
| trunk L | 48 (3.39%) | 0.11 ± 0.11 | | < 0.001 | - | NA | NA | | NA | NA |
| forehead H | 69 (4.87%) | 0.07 ± 0.04 | x | 0.029 | - | 45 (2.92%) | 0.09 ± 0.04 | | - | - |
| WL1 coxa1 | 17 (1.2%) | 0.16 ± 0.08 | | < 0.001 | - | 45 (2.92%) | 0.1 ± 0.05 | | - | - |
| WL1 coxa2 | 27 (1.9%) | 0.1 ± 0.09 | | 0.001 | - | 60 (3.9%) | 0.12 ± 0.06 | | 0.012 | 0.034 |
| WL1 coxa3 | 4 (0.28%) | 0.07 ± 0.03 | | 0.002 | - | 3 (0.19%) | 0.09 ± 0.05 | | - | - |
| WL1 femur | 7 (0.49%) | 0.04 ± 0.02 | | < 0.001 | 0.009 | NA | NA | | NA | NA |
| WL2 coxa1 | NA | NA | | NA | NA | 15 (0.97%) | 0.06 ± 0.03 | | - | - |
| WL2 coxa2 | 20 (1.41%) | 0.17 ± 0.12 | | 0.001 | - | 66 (4.29%) | 0.17 ± 0.11 | | 0.041 | 0.016 |
| WL2 coxa3 | NA | NA | | NA | NA | 4 (0.26%) | 0.08 ± 0.04 | | - | - |
| WL3 coxa1 | 7 (0.49%) | 0.11 ± 0.05 | | 0.001 | - | 21 (1.36%) | 0.09 ± 0.04 | | - | 0.023 |
| WL3 coxa3 | 12 (0.85%) | 0.2 ± 0.1 | | < 0.001 | 0.008 | 19 (1.23%) | 0.08 ± 0.05 | | < 0.001 | - |
| WL4 coxa1 | 5 (0.35%) | 0.18 ± 0.13 | | < 0.001 | - | 1 (0.06%) | 0.07 | | - | - |
| WL4 coxa2 | 59 (4.16%) | 0.19 ± 0.14 | | < 0.001 | - | 160 (10.4%) | 0.16 ± 0.1 | x | - | 0.042 |
| WL4 coxa3 | 2 (0.14%) | 0.07 ± 0 | | < 0.001 | 0.038 | 3 (0.19%) | 0.08 ± 0.04 | | - | - |
| WL4 propodus | 8 (0.56%) | 0.14 ± 0.16 | | < 0.001 | - | 22 (1.43%) | 0.09 ± 0.04 | | - | - |
| WL4 tarsus | 11 (0.78%) | 0.1 ± 0.06 | | < 0.001 | 0.025 | 2 (0.13%) | 0.08 ± 0.06 | | - | - |
| WL4 tibia2 | 60 (4.23%) | 0.2 ± 0.13 | | < 0.001 | - | 42 (2.73%) | 0.11 ± 0.06 | | - | - |
| trunk W23 | 55 (3.88%) | 0.08 ± 0.05 | | - | - | 81 (5.26%) | 0.12 ± 0.06 | | < 0.001 | - |
| trunk W3 | 98 (6.91%) | 0.1 ± 0.07 | | < 0.001 | - | 61 (3.96%) | 0.1 ± 0.06 | | 0.034 | - |

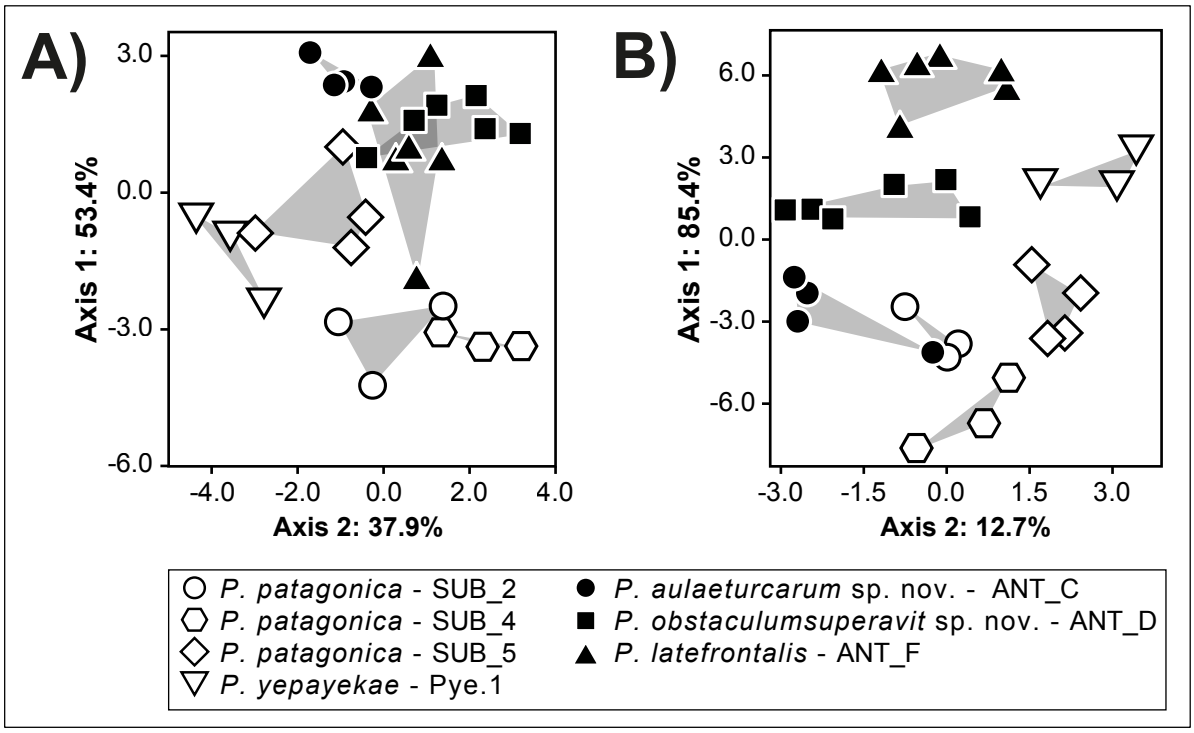


**Figure 4:** LDA of the *Pallenopsis patagonica* species complex. Ordination of the filtered morphometric data set using different combination of characters for a) absolute values (ocular tubercle H, ocular tubercle W, eye H, forehead H, cheliphore 1), and b) relative values (trunk W12, ocular tubercle H, palp, cheliphore 2, WL4 coxa2).

**Table 2:** Cross-validation confusion matrices for morphometric data set of the *Pallenopsis patagonica* species complex using absolute and relative values.

| | **absolute** (correctness rate: 0.76) | | | | | | | **relative** (correctness rate: 0.83) | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **ANT_C** | **ANT_D** | **ANT_F** | **Pye.1** | **SUB_2** | **SUB_4** | **SUB_5** | **ANT_C** | **ANT_D** | **ANT_F** | **Pye.1** | **SUB_2** | **SUB_4** | **SUB_5** |
| **ANT_C** | **4** | 0 | 0 | 0 | 0 | 0 | 0 | **3** | 0 | 0 | 0 | 1 | 0 | 0 |
| **ANT_D** | 1 | **5** | 0 | 0 | 0 | 0 | 0 | 1 | **5** | 0 | 0 | 0 | 0 | 0 |
| **ANT_F** | 1 | 0 | **3** | 0 | 1 | 0 | 1 | 0 | 0 | **6** | 0 | 0 | 0 | 0 |
| **Pye.1** | 0 | 0 | 0 | **3** | 0 | 0 | 0 | 0 | 0 | 0 | **3** | 0 | 0 | 0 |
| **SUB_2** | 0 | 0 | 0 | 0 | **3** | 0 | 0 | 1 | 0 | 0 | 0 | **2** | 0 | 0 |
| **SUB_4** | 0 | 0 | 0 | 0 | 0 | **3** | 0 | 0 | 0 | 0 | 0 | 0 | **2** | 1 |
| **SUB_5** | 1 | 0 | 1 | 1 | 0 | 0 | **1** | 0 | 0 | 0 | 1 | 0 | 0 | **3** |

### 2.2.2. Morphological characters

Using the morphological key for *Pallenopsis* Wilson, 1881 from Child 1995, all specimens analysed were assigned to *P. patagonica*. However, we observed consistent morphological features for several groups. Specimens that occur south of the Antarctic Polar Front are larger in body size and have longer legs in comparison to those from the Patagonian clades. Also, the distance between the lateral processes is longer for the Antarctic specimens. Furthermore, the rudimentary palp is larger for Antarctic individuals (Figure 5).

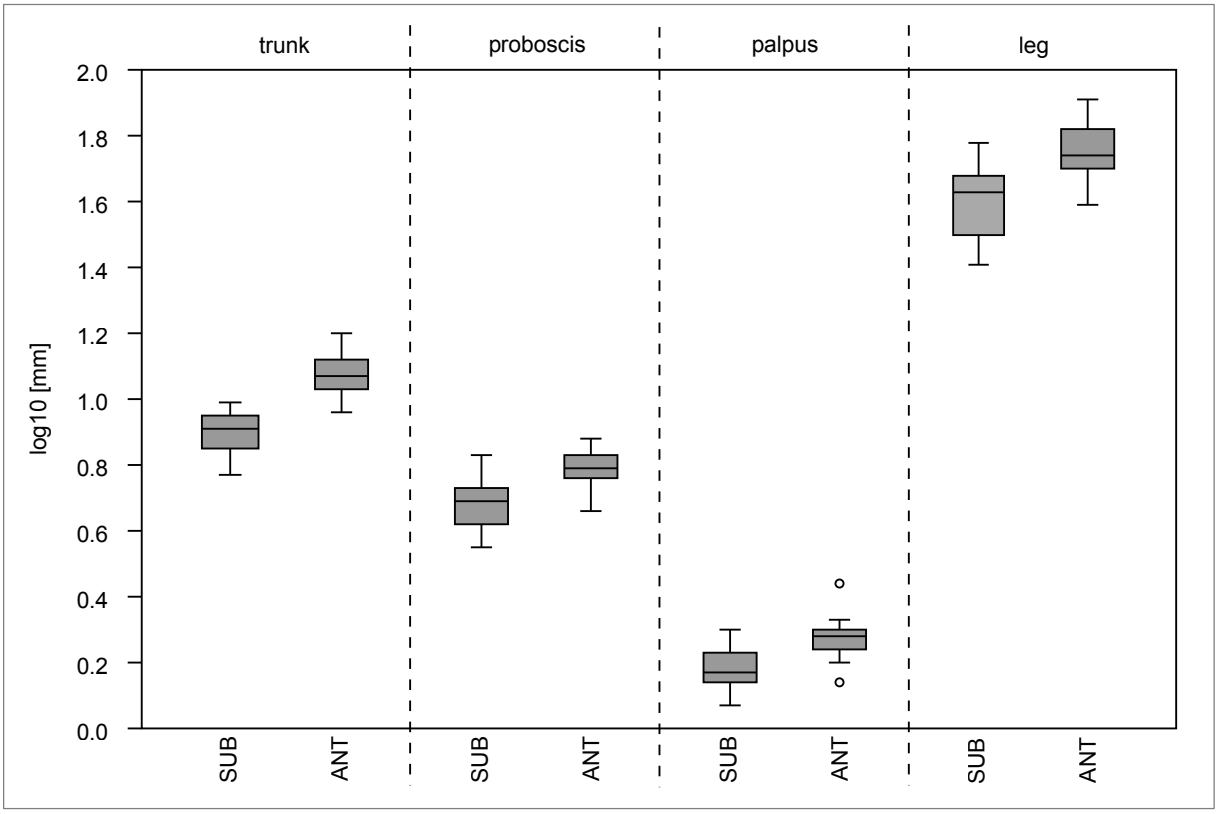


**Figure 5:** Boxplot showing size differences in morphological structures of the *Pallenopsis patagonica* species complex. All comparisons show that characters of samples from ANT (Antarctica) are significantly larger than from SUB (Patagonian) (log10 of absolute values used; $p = 0.0000005$, $p = 0.00008$, $p = 0.00042$ and $p = 0.00012$, respectively).

Specimens from Patagonian clades showed great variation and almost no suitable morphological characters for clade assignments. Only *P. notiosa* (SUB_3) can be distinguished from the others due to its rounded (rather than a pointed or slightly pointed) ocular tubercle and a very long second coxa, which exceeds the combined lengths of the first and third coxae (Figure 6c,e).

Specimens from Antarctica can morphologically be divided into two groups which can be distinguished by the setae patches on the dorso-posterior margin of the trunk segments (Figure 6d), that vary in size for specimens of ANT_C/D/M but are absent in those of ANT_F/K/L and ANT_N. Two Antarctic clades were identified as already described species, namely

*P. buphtalmus* (ANT_M) and *P. latefrontalis* (ANT_L). *Pallenopsis buphtalmus* (ANT_M) can be distinguished from the other Antarctic species due to relatively short accessory claws. For *P. latefrontalis* (ANT_L) the second coxa is characteristically shorter than the combined lengths of the first and third coxae. A straight rather than a curved propodus is distinctive of ANT_K (Figure 6b). Also, the lateral processes in this clade display a dorso-distally located crowning that differs from the frequently occurring but much smaller thickenings (Figure 6a). Those characters were also described for *P. hiemalis* by Hodgson (1907) and Pushkin (1975, 1993), which is generally considered a synonym of *P. patagonica*.

The remaining four Antarctic clades cannot be assigned to any described species and hence are proposed as new species. However, we only have two specimens for each of the clades ANT_L and ANT_N and distinct morphological characters that allow us to claim species-specific features rather than intraspecific variation are lacking. Therefore, we currently refrain from formally describing these two species until more specimens are available to determine the consistency of characters. Instead, they will be referred to as *Pallenopsis* sp. ANT_L and *Pallenopsis* sp. ANT_N, respectively. Worth mentioning is that specimens of *Pallenopsis* sp. ANT_N display bifurcated setae on the second and third coxa (Figure 6f), which are similar to those of the smaller sub-Antarctic species *P. yepayekae*. In addition to the different size of both species, *Pallenopsis* sp. ANT_N also lacks the two setae on the abdomen that are diagnostic for *P. yepayekae*.

Hence, herein we describe two new species of *Pallenopsis*. Specimens of ANT_D stand out because they have a horizontally positioned abdomen in comparison to the common upward orientation. ANT_C exhibits no unique characters which would suffice for its straightforward identification. Instead, the combination of several characters makes it possible to distinguish it from the other closely related congeners. Both newly described species can clearly be attributed to the genus *Pallenopsis* Wilson, 1881 by their slim segmented body, cylindrical proboscis, rudimentary palps, ten-articled ovigera in males, and slender legs with one main and two auxiliary claws (Wilson, 1881).

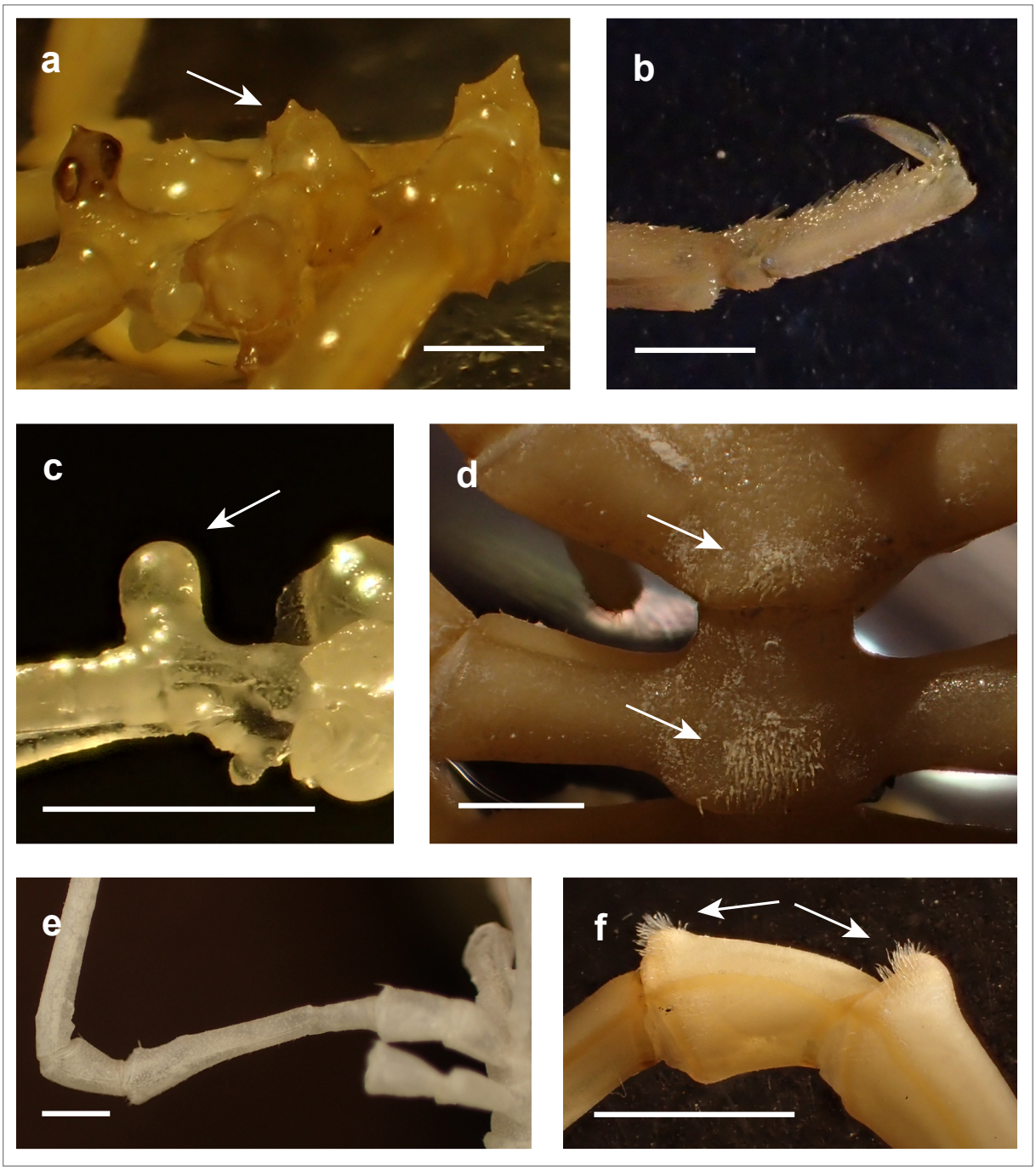


**Figure 6:** Prominent morphological characters of various lineages of the *Pallenopsis patagonica* complex. a, dorso-distally located crowning (see arrow) of lateral processes in PS82_143_2_2 (*P. hiemalis*; ANT_K). b, straight propodus of PS82_143_2_2 (*P. hiemalis*; ANT_K). c, rounded ocular tubercle of ZSM-A20111008 (*P. notiosa*; SUB_3). d, setae patches (see arrows) on dorsal-posterior margin of three trunk segments of JR262_1058 (*P. aulaeturcarum*; ANT_D). e, coxae of ZSM-A20111008 (*P. notiosa*; SUB_3). f, detailed view of second and third coxa with bifurcated setae on distal margins (see arrows) of PS77_211_6_1_4 (*Pallenopsis* sp. ANT_N). Scale bars = 1.5 mm.

## *PALLENOPSIS AULAETURCARUM* SP. NOV. DÖMEL & MELZER

**URN:LSID:ZOOBANK.ORG:ACT:72E41F8B-0A6F-4A5B-815A-1C2CAB65AFA5**

**FIGURES 7 a-g, 9 a-e**

### *Type material*

Holotype: PS82_156_2_1 (ZSM-A20160629), female, Weddell Sea, -75.507 (S), -27.486 (W), January 2014, depth: 281.5 m.

Paratypes: PS82_121_1 (ZSM-A20160626), female, Weddell Sea, -76.966 (S), -32.945 (W), January 2014, depth: 265.2 m. First leg pair and ovigera loose in the jar, proboscis of this individual was used for further analyses with the scanning electron microscope (SEM); PS82_156_2_2 (ZSM-A20160630), female, Weddell Sea, -75.507 (S), -27.486 (W), January 2014, depth: 281.5 m; PS82_223_1 (ZSM-A20160730), male, Weddell Sea, -75.522 (S), -28.973 (W), February 2014, depth: 462 m, both ovigera damaged, cement gland tube used for sex determination; PS82_174_3 (ZSM-A20160637), male, Weddell Sea, -74.491 (S), -30.977 (W), February 2014, depth: 529.7 m, left oviger detached, no morphometric measurements available for this individual.

The type series is deposited in the Bavarian State Collection of Zoology, in the department Arthropoda varia.

### *Distribution*

Weddell Sea, from eastern tip of the Antarctic Peninsula (-63.686, -56.859) to eastern Weddell Sea (-70.940, -10.489), and Bouvet Island (-54.425, 3.524).

### *Diagnosis*

Setae on posterior margin of trunk segments. More rows on ventral side (about three) than on dorsal side (one row). Abdomen oriented upwards.

### *Description (female)*

Size moderate, leg span less than 65 mm. Trunk with distinct segment borders, ridges strongly expressed (Figure 7a,b). Ridges on dorsal side smooth with few setae. Ventral surface covered with 2-3 rows of small clearly apparent spinules. Lateral processes separated by about the size of their diameter, U-shaped (Figure 7a, 9a). Distal margins of all processes display fringe of small spinules. On dorsal side, these spinules are located on slight thickenings (Figure 7b). Ocular tubercle situated on anterior end of cephalic segment. Top of ocular tubercle slightly bend backwards and pointed. Eyes prominent and pigmented, anterior eyes larger than posterior eyes. Proboscis sub-cylindrical, equally thick throughout and slightly directed downwards (Figure 9a,b). It is about half the length of the trunk. Abdomen long, extending from the trunk oriented upwards and covered with few spinules (Figure 7b, 9a). Cheliphores with two-articled

scape, first article longer than second article (Figure 7b). Ultimate cheliphore article (movable finger) equipped with setose pad. Moveable digit slightly longer than fixed digit, its tip curved. Inner margins straight and joined when closed. Setae pad has a triangular shape of which the whole length is attached to chela. Single-articled, laterally placed palp represents the rudimentary state typical for the genus (Figure 7b). It takes the form of an elongated bulb that is twice as long as wide. Female oviger composed of ten articles (Figure 7e). Proximal articles broaden slightly towards the distal part of each article. Second article equal in length to the third article. Fourth oviger article more swollen and the longest of all. From fourth article onwards, article length decreases. Oviger articles are setose, with all setae pointing distally. Legs with several short setae (Figure 7f,g). First and third coxa sub-equal. Second coxa about twice the length of third coxa (Figure 7a,f). Assemblage of short setae on ventral side of second and third coxa (Figure 7h, 9d). Setae without bifurcation. Femur and first tibia about equal in size. Second tibia slightly longer than other leg articles. Tarsus is short and armed with one big spine on the ventral side near its distal part and a couple of smaller lateral spines. Propodus slightly curved, with three to four heel spines that differ insignificantly in length, but the distal spine is the largest (Figure 7i, 9e). The remaining sole is covered with many shorter spines. Claw dorsally curved, its inner margin straight, its tip curved. Two auxiliary claws about half the length of main claw. Sexual pores on all second coxae on ventrodistal surface. In contrast to the male, the female lacks cement gland tubes (see below).

***Measurements*** *(holotype in mm)*

Length of trunk (anterior margin of first trunk segment to distal margin of fourth lateral processes), 9.80; trunk width (across first lateral processes), 3.98; proboscis length, 4.27; abdomen length, 3.91; third right leg, coxa 1, 1.45; coxa 2, 4.68; coxa 3, 2.78; femur, 13.74; tibia 1, 11.66; tibia 2, 15.08; tarsus, 0.32; propodus, 2.46; claw, 1.72; auxiliary claws, 0.63. Different segments were measured in natural posture.

***Male***

The general habitus and size of the male are similar to those of the female. Differences are in the sexual characters: oviger ten-articled (as is typical for the genus), but longer than in the female (Figure 7d). Second articles, nearly twice the length of third article. Fourth and fifth articles the longest and equal in size. Distal articles more setose than proximal articles, with setae pointing in various directions. Long cylindrical cement gland tube is located in the center of the ventral side of the femur in a small recess on top of a little swelling (Figure 7g, 9c). It is about a third of the diameter of the femur and points away from the podomere's surface in a nearly right angle. Sexual pores on ventral side of second coxae of third and fourth pair of legs.

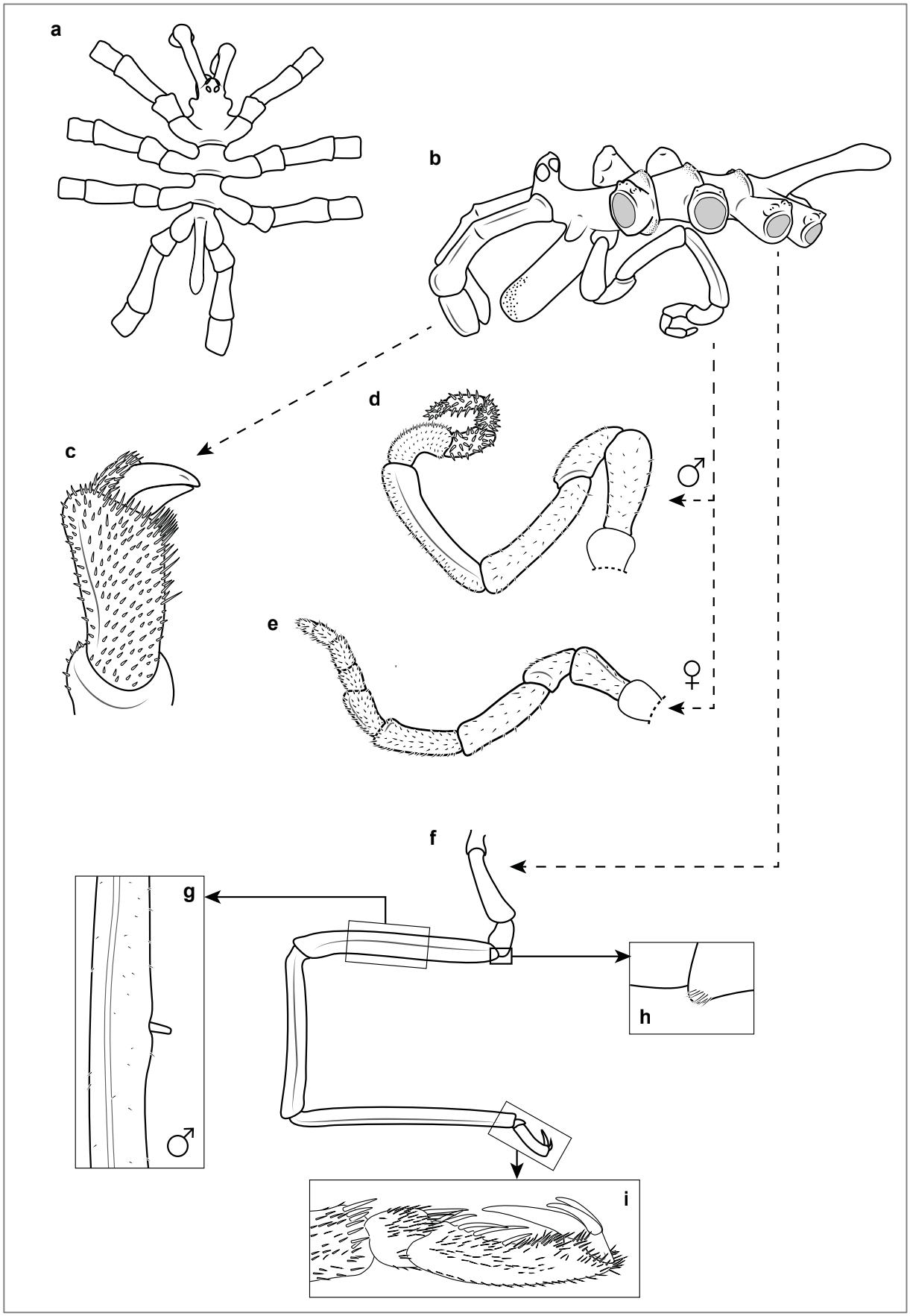


**Figure 7:** *Pallenopsis aulaeturcarum* sp. nov. Dömel & Melzer (clade ANT_C). a, dorsal view. b, lateral view of male .c, cheliphore. d, male oviger. e, female oviger. f, walking leg with enlargement of cement gland tube (g), setae on third coxa (h), and propodus with claw and auxiliary claws (i).

***Etymology***

The specific name *aulaeturcarum* stands for "the yard (aula) of the Turks (turcae)" and is dedicated to the eponymous pub in Munich called "Türkenhof" that was frequently visited to discuss the complex and very variable morphology of *Pallenopsis*. The good atmosphere and drinks definitely improved the spirit and inspired the authors.

***Remarks***

This species belongs to the *Pallenopsis patagonica* s.l. species complex as defined in Dömel et al. 2017 and also analyzed in Harder et al. 2016. In the previous studies, this species was defined as clade ANT_C or C, respectively.

There are no unique characters present for this new species which can be used to separate it from most other species of the genus, but the combination of its several diagnostic characters (shape of cheliphore pad, distances of lateral processes, presence of setae on ventral and dorsal side of trunk, as well as absence of long setae on legs and thickenings on lateral processes) makes it possible to distinguish it from the others.

## *PALLENOPSIS OBSTACULUMSUPERAVIT* SP. NOV. DÖMEL

**URN:LSID:ZOOBANK.ORG:ACT:69F7ADB8-26BB-4183-A178-67EEBABAE8BE**

**FIGURES 8 a-g, 9 f-j**

***Type material***

Holotype: JR262_1058 (ZSM-A20160708), female, South Georgia, -55.144, -36.245, 195.21 m, November/December 2011, missing legs: 3$^{rd}$ and 4$^{th}$ right side, 4$^{th}$ left side; one loose leg in the jar.

Paratypes: JR262_48_5_2 (ZSM-A20160713), female, South Georgia, -54.284, -36.083, 124.08 m, November/December 2011; JR287_124_1 (ZSM-A20160691), male; South Georgia, -53.764, -36.681, 151 m, May 2013; JR287_152 (ZSM-A20160694), female, South Georgia, -53.758, -36.690, 145 m, May 2013, Proboscis of this individual was used for further analyses with the SEM; JR262_1597_2 (ZSM-A20160710), male, South Georgia, -54.396, -37.384, 174.98 m, November/December 2011; PS77_211_6_1_3 (ZSM-A20160696), female, Shag Rocks, -53.402, -42.668, 290.2 m, February 2011.

The type series is deposited in the Bavarian State Collection of Zoology, in the department Arthropoda varia.

***Distribution***

Southern Ocean, from sub-Antarctic islands (South Georgia and Shag Rocks; -53.597, -41.214) as well as the Antarctic continental shelf (west and east of the tip of the Antarctic Peninsula; -63.389, -60.120).

***Diagnosis***

Setae patches of half the width of lateral processes on first trunk segment and with size of width of whole lateral process for second and third trunk segment. Abdomen pointing downwards.

***Description*** *(female)*

Size moderate, leg span less than 85 mm. Trunk with distinct segment borders, ridges strongly expressed (Figure 8a,b). Ridges on dorsal side setae-rich with a setae patch of half the width of lateral processes on first segment and with size of width of whole lateral process for second and third trunk segment. Ventral surface covered with few setae. Lateral processes separated by about the size of their diameter, U-shaped (Figure 8a, 9f). Distal margins of all processes display fringe of small spinules. On dorsal side, these spinules are located on slight thickenings (Figure 8b). Ocular tubercle situated on anterior end of cephalic segment. Top of ocular tubercle slightly bent backwards and pointed. Eyes prominent and pigmented, anterior eyes larger than posterior eyes. Proboscis sub-cylindrical, equally thick throughout and slightly directed downwards (Figure 9f,g). It is about half the size of the trunk. Abdomen long, extending ventrally from the thorax and covered with few spinules (Figure 8a, 9f). Cheliphores with two-articled scape, first article longer than second article (Figure 8c). Ultimate cheliphore article (movable finger) equipped with setose pad. Moveable digit slightly longer than fixed digit, its tip curved. Inner margins straight and joined when closed. Setae pad has a triangular shape of which half the length is attached to chela whereas other half protrudes. Single-articled, laterally placed palp represents the rudimentary state typical for the genus (Figure 8b). It takes the form of an elongated bulb that is twice as long as wide. Female oviger composed of ten articles (Figure 8e). Proximal articles broaden slightly towards the distal part of each article. Second article longer, nearly twice the size of third article. Fourth oviger article more swollen and the longest of all. From fourth article onwards, article length decreases. Oviger articles are setose, with all setae pointing distally. Legs with several short setae (Figure 8f,g). First and third coxa sub-equal. Second coxa about twice the length of third coxa. Assemblage of conspicuous setae on ventral side of second and third coxa, brush-like (Figure 8h, 9i). Setae without bifurcation. Femur and first tibia about equal in size. Second tibia longest leg article. Tarsus is short and armed with one big spine on the ventral side nearer its distal part and a couple of smaller lateral spines. Propodus slightly curved, with three to four heel spines that differ insignificantly in

length, but the distal spine is the largest (Figure 8i, 9j). The remaining sole is covered with many shorter spines. Claw dorsally curved, its inner margin straight, its tip curved. Two auxiliary claws about one-half the length of main claw. Sexual pores on all second coxae on ventrodistal surface. In contrast to the male, the female lacks cement gland tubes (see below).

***Measurements*** *(holotype in mm)*

Length of trunk (anterior margin of first trunk segment to distal margin of fourth lateral processes), 14.33; trunk width (across first lateral processes), 7.40; proboscis length, 6.35; abdomen length, 6.36; third right leg, coxa 1, 2.53; coxa 2, 7.59; coxa 3, 3.2; femur, 19.76; tibia 1, 15.91; tibia 2, 24.50; tarsus, 0.81; propodus, 4.43; claw, 2.46; auxiliary claws, 1.49.
Different segments were measured in natural posture.

***Male***

The general habitus and size of the male is similar to the female. Differences are in the sexual characters: oviger also ten-articled, typical for genus, but longer than female (Figure 8d). Second articles longer, nearly twice the length of third article. Fourth and fifth articles the longest and equal in size. Distal articles more setose than proximal articles, with setae pointing in various directions. Small cylindrical cement gland tube is located in the center of the ventral side of the femur on top of a little swelling (Figure 8g, 9h). It is as high as its diameter and points away from the podomere's surface in a nearly right angle. Sexual pores on ventral side of second coxae of third and fourth pair of legs.

***Etymology***

The specific name *obstaculumsuperavit* stands for "the one that overcame (superare) the obstacle (obstaculum)". *Pallenopsis obstaculumsuperavit* has been reported from the Antarctic continental shelf and South Georgia, which are separated by deep sea representing a barrier for the dispersal of many brooding invertebrates.

***Remarks***

This species belongs to the complex *Pallenopsis patagonica* s.l. defined in Dömel et al. 2017 and also analyzed in Harder et al. 2016. In the previous studies, this species was defined as clade ANT_D or D, respectively.

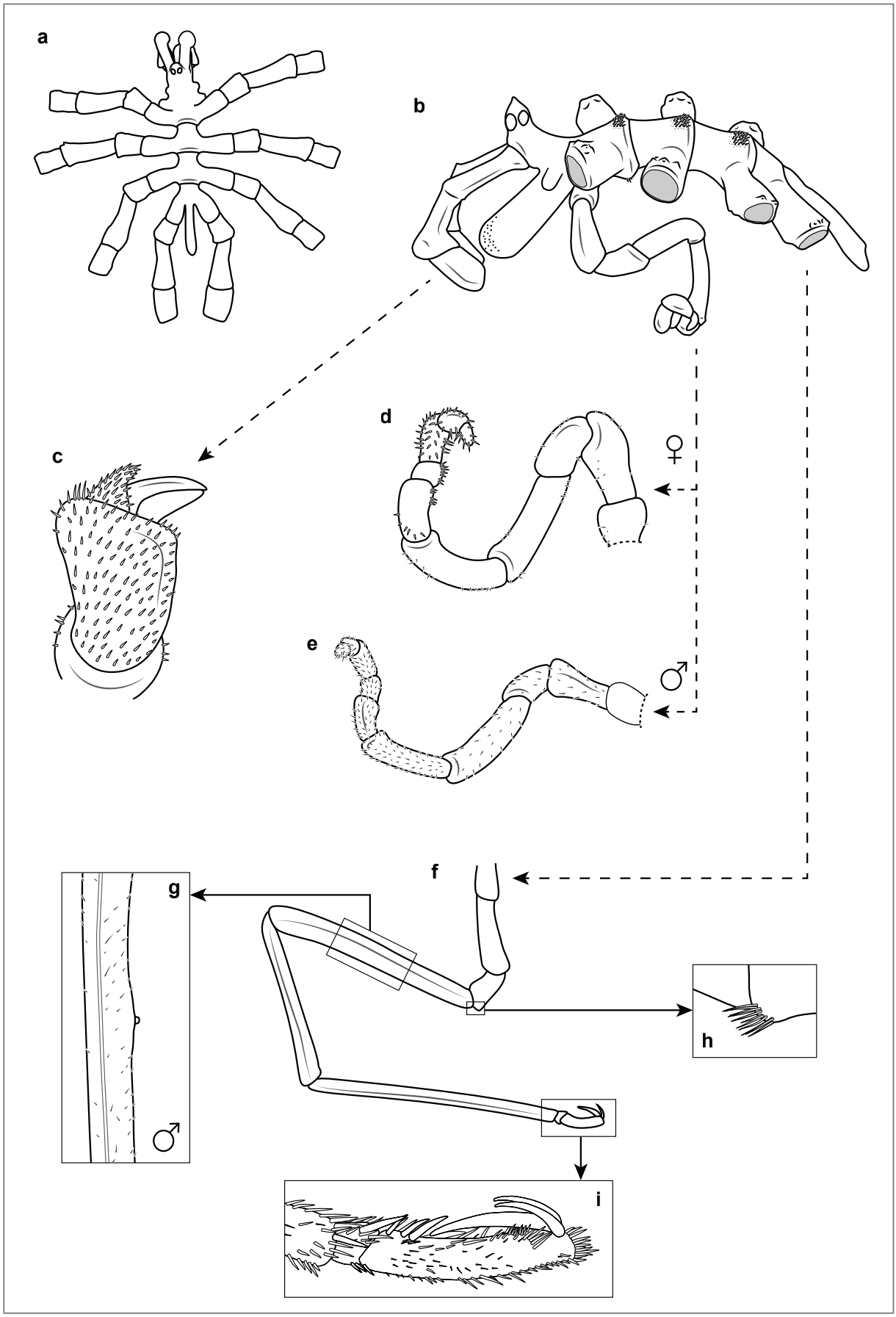


**Figure 8:** *Pallenopsis obstaculumsuperavit* sp. nov. Dömel (clade ANT_D). a, dorsal view. b, lateral view of male. c, cheliphore. d, male oviger. e, female oviger. f, walking leg with enlargement of cement gland tube (g), setae on third coxa (h), and propodus with claw and auxiliary claws (i).

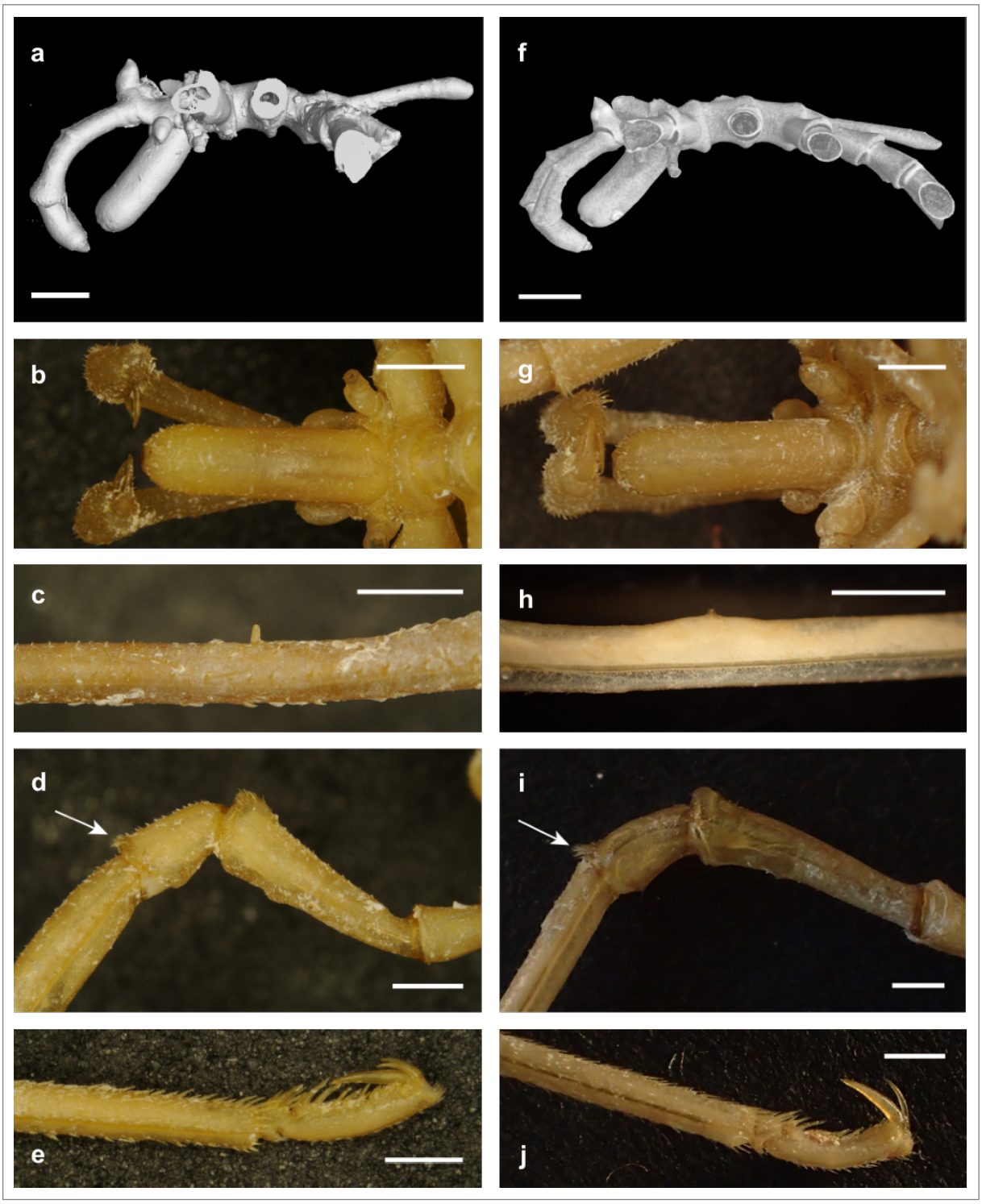


**Figure 9:** *Pallenopsis aulaeturcarum* sp. nov. and *Pallenopsis obstaculumsuperavit* sp. nov. Images of *Pallenopsis aulaeturcaru*m sp. nov. Dömel & Melzer (clade ANT_C) (a-e) and *Pallenopsis obstaculumsuperavit* sp. nov. Dömel (clade ANT_D) (f-j). a, f, micro-computed tomography (µCT) of specimens in lateral view; scale bar = 2.5 mm. b, g, ventral view of proboscis; scale bar = 1.5 mm. c, h, detail view of cement gland tube on femur (male); scale bar = 1.5 mm. d, i, detailed view of coxae with setae on posterior margin of the third coxa (see arrow); scale bar = 1.5 mm. e, j, propodus with claw and accessory claws; scale bar = 1.5 mm. a, PS82_121_1; b, d, e, PS82_156_2_1; c, PS82_185_1; f, JR287_152; g-j, JR287_124_3.

### 2.3. Combining morphological and genetic data

There is a significant positive correlation of greater morphological distances with larger genetic distances for both genetic distances calculated based on COI ($r=0.36$, $p<0.0001$; Figure 10A) and EOG sequences ($r=0.51$, $p<0.0001$; see figure provided in the Supplementary Material 7). There is only a small difference between both correlations and SUB_2 has high intraspecific genetic distances between specimens from Burdwood Bank and the Falkland Islands or the Patagonian shelf (Supplementary Material 7). When dividing the genetic COI distances, which are available for all morphologically analyzed individuals, into ranges (< 2.5% = intraspecific; > 2.5% = interspecific), the morphological distances are always higher for specimens that occur in allopatry than for those in sympatry. However, there is no significant difference between the genetic COI distance ranges, except for genetic distances above 10% (Figure 10B).

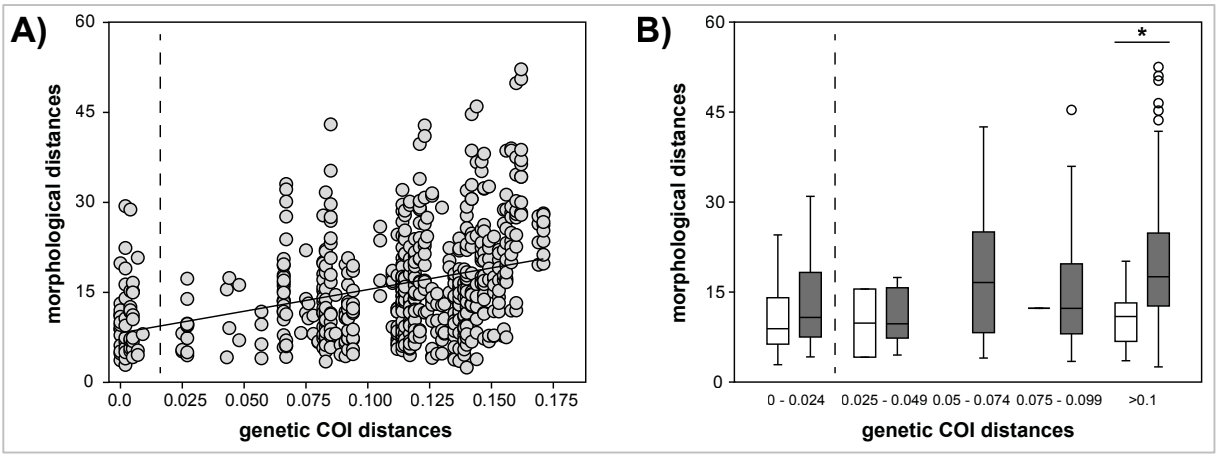


**Figure 10:** Morphological against genetic distances. Morphological distances plotted against uncorrected genetic COI distances a) for each individual with regression line ($r = 0.36$, $p < 0.0001$) and b) for genetic ranges differentiated into sympatric (white) and allopatric (grey) samples of the *Pallenopsis patagonica* species complex. Dashed line separates intraspecific (left) and interspecific (right) genetic distances.

## 3. Discussion

### 3.1. Do genome-wide data add further information about previously unrecognised species diversity within the *P. patagonica* species complex?

We successfully used the target hybrid enrichment method, with baits designed for a different genus (Dietz et al. 2019), to obtain an unprecedented data set to resolve the taxonomy and phylogeny of the *Pallenopsis patagonica* species complex. The genomic data enabled us to obtain better resolved and stronger supported branches in the phylogenetic tree in comparison to the mitochondrial tree published in Dömel et al. 2017. In general, the topologies of the trees were similar except for the placement of the root, which was placed on the branch leading to clade *Pallenopsis* sp. ANT_N in the mitochondrial tree. Genomic data revealed that all clades

from the Patagonian shelf, including SUB_4 and SUB_5 that were found to be paraphyletic with respect to the Antarctic clades in the mitochondrial tree, grouped together in the "Patagonian supergroup". In addition, *Pallenopsis* sp. ANT_N had a well-defined position within the "Patagonian supergroup" instead of being a sister taxon to all other species and clades of the *P. patagonica* species complex as in the previous analysis.

Most of the previously defined mitochondrial clades were well-separated in the multi-gene analyses with the exception of the two pairs of sister clades SUB_1/2 and SUB_4/5 (see table provided in Supplementary Material 8 for an overview). It should be mentioned that the separation of these two pairs of clades was already put into question by the analysis of the ITS sequences in Dömel et al. (2017). There it was shown that specimens from Burdwood Bank (including one single specimen each from SUB_1 and SUB_2) grouped together, but had relatively large genetic distances. This disagreement with the mitochondrial clade assignment might be due to a mito-nuclear discordance, which has also been reported for the sea spider species complex *Colossendeis megalonyx* (Dietz et al. 2015a). Although they were well-defined lineages in the phylogenetic tree, PCA and sNMF plots grouped both clades together. As morphological analyses also showed that there were no recognisable characters to distinguish SUB_1 and SUB_2, they should best be treated as one species. The differentiation between clades SUB_4 and SUB_5 was not supported by ITS data (Dömel et al. 2017). However, as no mito-nuclear discordance was found this could have been due to different mutation rates of the markers. The phylogenetic tree based on target hybrid enrichment revealed that SUB_4 is paraphyletic with respect to SUB_5, which may lead to the conclusion that this group originated on the Falkland Islands and subsequently migrated to the Patagonian shelf. Morphological data did not uncover characters to distinguish the two clades from each other and therefore support the hypothesis that they should still be considered as one species with geographical separation, as proposed by Dömel et al. (2017). Further intraspecific geographic separations were found for *P. obstaculumsuperavit* sp. nov. (ANT_D) and *P. latefrontalis* (ANT_F). For *P. obstaculumsuperavit* sp. nov. (ANT_D) a geographical differentiation has already been assumed between specimens from South Georgia and the Antarctic shelf based on the mitochondrial data set, but samples of *P. latefrontalis* (ANT_F) from Bouvet had not been analysed before. Geographic differentiation between populations from the Antarctic continental shelf and sub-Antarctic islands is known for other sea spiders (Arango et al. 2011, Dömel et al. 2015) as well as further benthic invertebrates (Linse et al. 2007, Thornhill et al. 2008).

Principal component and phylogenetic tree analyses agreed with each other for all other predefined clades. But the cluster analysis showed similar proportions of ancestral populations for the closely related mitochondrial clades ANT_K (*P. hiemalis*, see below) and *Pallenopsis*

sp. ANT_L together. However, the two clades were well separated in the phylogenetic tree and morphological analyses revealed several distinct characters between ANT_K and *Pallenopsis* sp. ANT_L. Therefore, we suggest that these clades represent two distinct species. Most likely, the relatively recent divergence of those two species in combination with a small sample size each (n=3) represented an issue for the cluster analysis. Also, *P. notiosa* (SUB_3) clustered together with SUB_1/2 and again, this might be due to the small sample size especially of *P. notiosa* (SUB_3; n=1).

The results of our analysis also allow to discuss questions on the biogeographic history of the *P. patagonica* species complex. Unlike previous studies based on few genes (Weis et al. 2014, Harder et al. 2016, Dömel et al. 2017), our data clearly show a basal split between a Patagonian and an Antarctic group, whose distributions overlap only in South Georgia. As only little is known about the phylogeny of *Pallenopsis* as a whole and as we do not know exactly which species are the closest relatives of the *P. patagonica* species complex, we cannot assess whether the complex originated within Antarctica or not. However, the Antarctic supergroup shows a pattern of a relatively rapid radiation as opposed to the Patagonian supergroup which demonstrates earlier divergences. This pattern might be due to a rapid radiation after colonisation of the Antarctic, therefore supporting a non-Antarctic origin of the complex.

### 3.2. Do we find morphological characters to distinguish the independently evolving lineages of the *P. patagonica* species complex and formally describe new species?

Using the key for *Pallenopsis* Wilson, 1881 from Child 1995, we would characterize all specimens analyzed as *P. patagonica*. This key, however, only includes nine out of 18 Antarctic and sub-Antarctic species (Munilla and Soler-Membrives 2009). The key given by Pushkin (1993) for ten *Pallenopsis* of the Southern Ocean is misleading and would assign none of the analysed specimens to *P. patagonica*. A recent attempt to update the identification key for Antarctic and sub-Antarctic *Pallenopsis* including all species was performed by Cano-Sánchez and López-González (2019). Still, not all specimens can be assigned correctly to species level. An example is *P. patagonica* (SUB_1/2/4/5), for which the lateral processes do not touch each other (but see Weis et al. 2014).

Morphometric analyses aiming at separating clades were challenging because of limited sample size. In addition, little is known regarding allometric growth in *Pallenopsis* and regression analysis was not possible for the same reason of not having sufficient numbers of individuals of both sexes for each clade (Lovett and Felder 1989). Nevertheless, the simpler approach of taking relative lengths of morphological structures coupled with character selection for discriminant analysis showed that the species can be satisfactorily separated using a small

number of characters with the relative values having better performance in species discrimination. The leg span best represents the actual body size of a sea spider and would have been the preferred reference length but analyses revealed cases of re-grown legs in the data set. Hence, relative values were expressed as proportions relative to the trunk lengths.

Diagnostic characters for at least nine species within the *P. patagonica* species complex, of which five have already been described, were found (see table provided in Supplementary Material 8 for an overview). Additionally, Weis et al. (2014) stated that *P. macneilli* a species found in Australian waters and hence was not included in this study, was also part of the *P patagonica* species complex based on COI data. In general, the morphological distinction between genetic clades is clearer for the Antarctic ones. Weis et al. (2014) already found out that the "Antarctic supergroup" consist of two described species, *P. buphtalmus* and *P. latefrontalis*. Furthermore, Weis et al. (2014) mentioned that one specimen (PpaE002) stood out due to its horizontally positioned abdomen, in comparison to the common upwards oriented abdomen seen in most specimens. The above-mentioned individual has been genetically identified as *P. obstaculumsuperavit* sp. nov. (ANT_D). In fact, the position of the abdomen is a diagnostic character for this newly described species. The individual mentioned in Weis et al. (2014) was reinvestigated and it can be confirmed that the horizontal position of the abdomen described before is actually downwards oriented, too.

*P. aulaeturcarum* sp. nov. (ANT_C) shares many morphological characters with other clades of the "Antarctic supergroup", e.g. spinules on dorsal and ventral surface of the trunk, ratio of claw to accessory claw and propodus, and length of second coxa in relation to the sum of the first and third coxa. It should be stressed that the morphological differentiation would not have been recognised without the knowledge of the genetic background information thus highlighting once again the benefits of an integrative approach.

Cano-Sánchez and López-González (2019) recently described two new species from Victoria Land (Ross Sea), *P. gracilis* Cano-Sanchez & Lopez-Gonzalez, 2019 and *P. rotunda* Cano-Sanchez & Lopez-Gonzalez, 2019. Both can be distinguished from *P. obstaculumsuperavit* sp. nov. (ANT_D) by their upwards oriented abomina. Characters disagreeing with *P. aulaeturcarum* sp. nov. (ANT_C) are the lateral processes that are closer together, even touching, in *P. rotunda* and the forward pointing ocular tubercle of *P. gracialis*.

Specimens from the "Patagonian superclade" were morphologically very similar. In fact, SUB_1/2 and SUB_4/5 look alike and cannot be distinguished morphologically. If we were to consider the morphological result only, we would probably assign those clades to a single species. Strangely enough, within the phylogenetic tree *P. notiosa* (SUB_3), a well-defined species and *Pallenopsis* sp. ANT_N are placed between SUB_1/2 and SUB_4/5. Hence,

SUB_1/2 and SUB_4/5 can be considered as cryptic but not sister species, a phenomenon that has also been observed, e.g. in nematodes (Sudhaus and Kiontke 2007).

Only two individuals from Shag Rocks (south of the Antarctic Polar Front) were available for *Pallenopsis* sp. ANT_N and therefore it was designated as an Antarctic clade by Dömel et al. (2017). Also morphologically, the two individuals were very similar to the Antarctic species (i.e. the distance between the lateral processes is about as long as their diameter and the palps are longer than their diameter). However, phylogenetically, these specimens fell outside the Antarctic radiation and belonged to the "Patagonian supergroup". Furthermore, during more detailed examination of these specimens, bifurcated setae, which are supposed to represent complex structures (Lehmann et al. 2017) with potential for species-specific features and (even if not as prominent) correspond to the character of the Patagonian species *P. yepayekae*, were detected on the second and third coxa. In fact, Weis et al. (2014) described these setae as a unique character of specimens from the Chilean clade (i.e. *P. yepayekae*), and hence a character that can be used to distinguish it from specimens from the Antarctic region or the Falkland Islands. There were two species that were of particular interest, because they partly matched the characteristics of *Pallenopsis* sp. ANT_N: *P. tumidula* Loman, 1923 and *P. candidoi* Mello-Leitao, 1949. Both seemed to exhibit the short setae on the ventral side of the second and third coxa. The latter occurs from South Georgia to South Brazil and hence has a geographical overlap with *Pallenopsis* sp. ANT_N. *Pallenopsis candidoi* can be distinguished from *P. patagonica* s.s. and *P. yepayekae* by the eight-articled oviger in females, and by the auxiliary claws being clearly longer than half the length of the main claw (Weis et al. 2014). The two individuals included in this study were males and no prediction can be made regarding the female ovigera, but the auxiliary claw is approximately half the length of the main claw, rather than longer. *Pallenopsis tumidula* was characterised and drawn by Stock (1957) with so-called 'Fiederdornen' (German for pinnated spine) on the ventral-distal side of the second and third coxa. He mentioned that this feature made *P. tumidula* clearly distinguishable from *P. patagonica*. Confusingly, in the original description of 1923, Loman neither mentioned short setae on the coxa nor depicted them in his drawings. Also, the original description states that the lateral processes are separated by about half their diameter, which is smaller than those displayed by the studied specimens. However, due to the small sample size, we refrain from designating this clade as a new species.

### 3.2.1. Reinstallment of *P. hiemalis*

Specimens assigned to clade ANT_K differed from the others in having a straight rather than curved propodus. Also, the lateral processes had a dorso-distally located crowning of up to three

pointy tubercles that differs from the frequently occurring but much smaller thickenings. Those characters have also been described for *P. hiemalis* by Hodgson (1907) and Pushkin (1975, 1993). However, this species has been synonymised with *P. patagonica* by Child (1995). Cano-Sánchez and López-González (2019) already suggested that *P. hiemalis* is a valid species, however this statement was made without any morphological reinvestigation. There are indeed characters in the original description of *P. hiemalis* that do not fit *P. patagonica* s.s. but are characteristic for Antarctic specimens of the species complex (e.g. "[…] lateral processes rather widely separated" and "Palps, a rather long stump"). Parts of the description that militate against ANT_K specimens being *P. hiemalis* concern the size of the second coxa, i.e. "[…] second [coxa] is fully twice as long as the other two together" (Hodgson 1907). This, however, is an uncommon ratio for *Pallenopsis* and also does not match the description of *P. patagonica* s.s. Hence, this might be a mistake due to a combination of the following phrasings: i) "[…] second [coxa] is twice as long as first or third coxa" and ii) "[...] second [coxa] is fully as long as the other two together". The descriptions of *P. hiemalis* by Hodgson (1907) and Pushkin (1975) differ in their described characters, too. An example of this discordance is the description of a very prominent character of specimens from ANT_K which display three distinct tubercles on the dorsal-distal side of the lateral processes. Those were described as "tricipital tubercles" in Pushkin (1975) but a single "stout tubercle of no great elevation" was described by Hodgson (1907). As Hodgson's description is based on a single specimen, the missing character might be explained by a variation of attributes due to developmental stages. However, the few measurements given in Hodgson (1907) indicate that the individual was full-grown. We herein propose to reinstall *P. hiemalis* Hodgson, 1907 as autonomous species and refer to the species description in Pushkin (1975). *Pallenopsis hiemalis* belongs to *Pallenopsis patagonica* s.l. defined in Dömel et al. 2017 and also analyzed in Harder et al. 2016. In the previous studies, this species was referred to as clade ANT_K or K.

### 3.2.2. Which species is *P. patagonica* s. s.?

The original description of *P. patagonica* (Hoek, 1881) agrees with the morphology of the specimens from clades SUB_4 (Falklands) and SUB_5 (Patagonia). Previous analyses revealed that those two clades can be distinguished with the mitochondrial COI but not with the nuclear ITS marker (Dömel et al. 2017). Further morphometric and morphological analyses detected no distinguishable characters and also the multi-marker analyses revealed that SUB_4 and SUB_5 can be considered as one species. Specimens of SUB_1/2 are very similar to those of SUB_4/5 and as the location of the type material of *P. patagonica* s.s. cannot be defined because the original description records specimens from three different locations in Patagonia (46°53′S

75°11′W, 50°10′S 74°42′W, and 52°20′S 68°0′W) where both species (SUB_1/2 and SUB_4/5) occur, it is difficult to decide which one represents *P. patagonica* s.s. A correct assignment of the species name *P. patagonica* to a genetic clade would therefore necessitate a genetic re-examination of the type series, which may not be obtainable from such old material.

### 3.2.3. Polar gigantism

The phenomenon that Antarctic specimens are unusually large is commonly known as polar gigantism (Chapelle and Peck 1999). The morphometric analyses revealed that all specimens within the Antarctic Polar Front are significantly larger than the Patagonian ones. This could have been biased by the fact that males dominated in sub-Antarctic and females dominated in Antarctic specimens. Size differences between male and female with the latter being the larger ones have been reported for many species (Arnaud and Bamber 1988), however dimorphism did not seem to influence our results.

Morphometric analyses alone would probably have led to incorrect conclusions regarding the phylogenetic position as one would have probably assumed that *Pallenopsis* sp. ANT_N is more closely related to the "Antarctic superclade". However, looking at *Pallenopsis* sp. ANT_N in more detail, we detected bifurcated setae on the second and third coxa, which (even if not as prominent) is very similar to the character of the Patagonian species *P. yepayekae*. This can be seen as evidence for at least two independent events of polar gigantism within the genus *Pallenopsis*.

## 3.3. Do we find evidence for adaptive divergence at morphological or genetic levels or do neutral evolutionary processes suffice to explain the observed species diversity?

Target hybrid enrichment can be used to specifically target coding regions and hence is a useful technique to test a large number of genes for selection (Irisarri et al. 2018). For the *P. patagonica* species complex, only a few genes were found to be under selection. In addition, no branch under selection was detected and delineation due to selection pressure on any of the detected genes can be excluded. However, the bait set used here was not tailored to *P. patagonica* but was based on a transcriptome of the Southern Ocean sea spider *Colossendeis megalonyx*. Whereas for *C. megalonyx* all bait regions were recovered (Dietz et al. 2019), for the *P. patagonica* species complex on average only 30% (max. of 35%) of all bait regions were successfully enriched. Most likely those loci represent well-conserved genes that show relatively little variation across families or genera of sea spiders and recently evolved genes that could have been of further relevance were not analyzed within this study.

Furthermore, there is no clear evidence for selection when analyzing morphological and genetic data together. In the case of sympatric speciation and adaptation to different ecological niches, one would expect high morphological differences also for recently diverged species, i.e. genetic distances just above 2.5%, and especially when they occur in sympatry (ecological character displacement). This does not appear to be the case in the *P. patagonica* species complex, because regardless of whether the species occur in sympatry or not, morphological distances were similarly high throughout the range of genetic distances. Only at very high genetic distances, for specimens living in allopatry morphological distances were significantly higher than for specimens living in sympatry. At the same time, specimens from the same area tended to be more similar to each other among species, which may be explained by their similar adaptations to the same environment.

Among the characters which were found to contribute to species separation we find some with potential ecological significance (like the absolute and relative length of the proboscis and first cheliphore article), which might indicate the existence of differences in food preferences between the species. The proboscis is known to have a diverse range of shapes and sizes among sea spiders indicating differences in feeding strategies (Wagner et a. 2017, Dietz et al. 2018), but also the cheliphores can be relevant features as they are used to capture or cut the prey (Arnaud and Bamber 1988). Yet, these characters are accompanied by other ones with supposedly little or no role in ecological differentiation. We might thus tentatively hypothesise that minor ecological differences between the species do exist, but they reflect local adaptation or even non-selective variation, since no character displacement in sympatry is observed. Especially species that occur in sympatry were expected to form different ecological niches. As the Antarctic continental shelf is relatively uniform in terms of geological structures and large regions that have been influenced by grounded ice shelfs or even iceberg ploughing are plain and dominated by gravel, food sources seem to be a major cause for specialisation. As this does not seem to be the case in the *P. patagonica* species complex, this might indicate that there is no competition for food. Jones (1972) found a similar case were four species of the *Jaera albifrons* group (Crustacea; Isopoda) displayed identical mouthparts although they occurred in sympatry and concluded that food was not an isolating factor. It does not appear to be the case that the scarce morphological characters that differentiate the species of the complex, like position of the abdomen, distances between the lateral processes or shape of the setae patch on the cheliphores are of significant biological relevance and hence could be subject to selection.

## 4. Conclusion

Combining genome-wide molecular sequence data with extensive morphological and morphometric analyses, we generated an unprecedented data set for members of the *P. patagonica* sea spider species complex. We established a well-resolved phylogeny based on target hybrid enrichment data and delineated species boundaries within the taxonomically difficult group which led to the reinstallment of *P. hiemalis* as well as the description of two new species, namely *P. aulaeturcarum* and *P. obstaculumsuperavit*. Contrary to previous studies, our results supported the division of the species complex into an Antarctic and a Patagonian group. Concerning speciation processes, our data supports the hypothesis of speciation in independent glacial refugia, as we found no consistent evidence for adaptive divergence. The latter aspect, however, can only be answered conclusively when more specimens from the different lineages and areas as well as more genomic loci become available.

## 5. Methods

### 5.1. Material

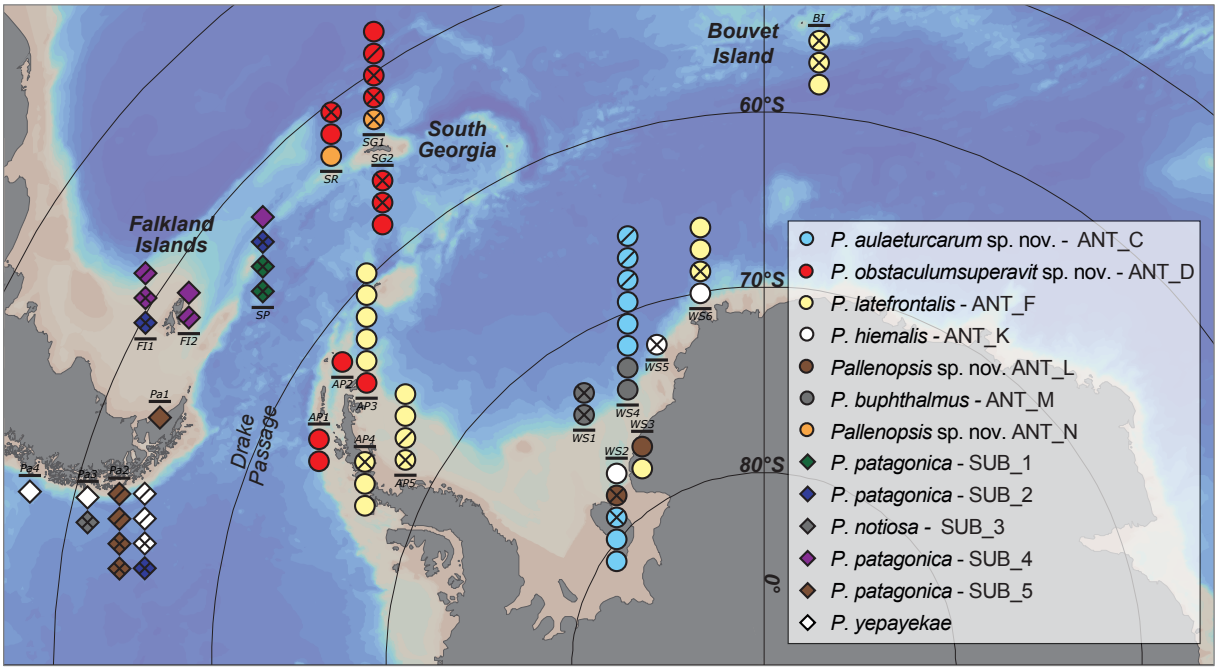


**Figure 11:** Specimen map. Sampling sites of Antarctic, sub-Antarctic and Patagonian specimens of the *Pallenopsis patagonica* species complex and their assignment to species or mitochondrial clades. Each symbol below or above the line and locality ID represents one specimen. Different clades are represented by different symbols/colors. Analysis methods are indicated for each individual within a symbol (slash: morphological analyses only; no indication: genetic analyses only; cross: genetic and morphological analyses).

A subset of specimens already included in Dömel et al. (2017) was studied including individuals from the Antarctic continental shelf and the shelf of sub-Antarctic islands, the Falkland Islands and Patagonia (Figure 11) (for further details of sampling and storage see Dömel et al. 2017). Up to three individuals per species or clade were analyzed. For morphological measurements, 37 specimens were used. For genetic analyses, more samples of three lineages (ANT_C, ANT_D, *P. latefrontalis* (ANT_F)) with Antarctic distribution ranges were included and additional samples of *P. latefrontalis* (ANT_F) from Bouvet Island were added to improve the geographical coverage. Hence, the final genetic dataset consisted of 62 individuals of the *P. patagonica* species complex and a single individual of *P. pilosa* (Hoek, 1881) as an outgroup (Table 3).

### 5.2. Bait Enrichment

For genetic analyses, a target hybrid enrichment approach was chosen. For the present analyses we used the bait set designed in Dietz et al. (2019) using the workflow described by Mayer et al. (2016). Briefly, the baits were constructed based on an assembly of transcriptomic data of the sea spider *Colossendeis megalonyx* and included a total number of 12,014 baits covering 3682 bait regions from 1607 single-copy EOGs present in all spider genomes. See Dietz et al. (2019) for details and bait sequences. Baits were manufactured by Agilent Technologies (Waldbronn, Germany).

Sample preparation was conducted following a slightly modified version of Agilent's protocol "200 ng DNA sample" for "Agilent's SureSelect Target Enrichment System". A detailed written protocol is provided in Supplementary Material 9, Protocol 1. After the enrichment steps, samples were pooled in equimolar ratios for sequencing. Two pools were prepared, containing 32 samples each. Libraries were sent to GATC Biotech GmbH (Konstanz, Germany) for sequencing on an Illumina MiSeq platform using the V2 2x250 bp paired-end sequencing kit. 5% PhiX spike-in was added to each run to increase sequencing diversity and hence improve the signal of sequences. Upon delivery, the NGS reads were adapter- and quality-trimmed with fastq-mcf r. 488 (Aronesty 2011). The raw data are available from NCBI Sequence Read Archive (BioProject ID PRJNA544606). We used two complementary approaches to construct data sets, SNP and EOG, from the reads for different purposes. The SNP approach was used to call variants of different sample sets and also include flanking regions. The EOG approach is solely based on orthologous regions and hence is supposed to cover genes only.

**Table 3:** List of specimens for the *Pallenopsis patagonica* species complex and outgroup used for target hybrid enrichment and morphometric measurements in this study. Species names are given if possible. Sampling details (location, latitude, longitude, depth in m), specimen information (ID, voucher number, molecular clade and sequence availability) and analyses applied to each individual (G: genomic target hybrid enrichment; M: morphometric analyses) are listed.

| Species | Clade | ID | Location | Lat | Lon | Depth [m] | ZSM-Voucher Number | GenBank number | | G | M |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | COI | ITS | | |
| *Pallenopsis aulaeturcarum* sp. nov. | ANT_C | KT982322 | AP3 | -64.035 | -56.728 | 220 | | KT982322 | KY272414 | x | |
| *P. aulaeturcarum* sp. nov. | ANT_C | KT982333 | AP3 | -63.686 | -56.859 | 400 | | KT982333 | KY272415 | x | |
| *P. aulaeturcarum* sp. nov. | ANT_C | KT982334 | AP3 | -63.686 | -56.859 | 400 | | KT982334 | KY272416 | x | |
| *P. aulaeturcarum* sp. nov. | ANT_C | KT982341 | AP3 | -63.754 | -55.684 | 334 | | KT982341 | KY272417 | x | |
| *P. aulaeturcarum* sp. nov. | ANT_C | KT982343 | AP3 | -63.754 | -55.684 | 334 | | KT982343 | KY272418 | x | |
| *P. aulaeturcarum* sp. nov. | ANT_C | PS82_25_2_1 | WS4 | -74.705 | -29.900 | 406.2 | ZSM-A20160635 | KY272314 | KY272404 | x | |
| *P. aulaeturcarum* sp. nov. | ANT_C | PS82_25_2_2 | WS4 | -74.705 | -29.900 | 406.2 | ZSM-A20160636 | KY272316 | KY272406 | x | |
| *P. aulaeturcarum* sp. nov. | ANT_C | PS82_121_1 | WS2 | -76.966 | -32.945 | 265.2 | ZSM-A20160626 | KY272315 | KY272412 | x | x |
| *P. aulaeturcarum* sp. nov. | ANT_C | PS82_143_2_1 | WS2 | -76.967 | -32.866 | 293.7 | ZSM-A20160623 | KY272311 | KY272419 | x | |
| *P. aulaeturcarum* sp. nov. | ANT_C | PS82_143_2_3 | WS2 | -76.967 | -32.866 | 293.7 | ZSM-A20160625 | KY272319 | KY272405 | x | |
| *P. aulaeturcarum* sp. nov. | ANT_C | PS82_156_2_1 | WS4 | -75.507 | -27.486 | 281.5 | ZSM-A20160629 | KY272313 | KY272407 | | x |
| *P. aulaeturcarum* sp. nov. | ANT_C | PS82_156_2_2 | WS4 | -75.507 | -27.486 | 281.5 | ZSM-A20160630 | KY272309 | KY272413 | x | |
| *P. aulaeturcarum* sp. nov. | ANT_C | PS82_156_2_3 | WS4 | -75.507 | -27.486 | 281.5 | ZSM-A20160631 | KY272310 | KY272410 | | x |
| *P. aulaeturcarum* sp. nov. | ANT_C | PS82_223_1 | WS4 | -75.522 | -28.973 | 462 | ZSM-A20160730 | KY272308 | KY272408 | | x |
| *P. obstaculumsuperavit* sp. nov. | ANT_D.1 | KT982325 | AP3 | -63.576 | -54.629 | 227 | | KT982325 | KY272396 | x | |
| *P. obstaculumsuperavit* sp. nov. | ANT_D.1 | KT982326 | AP2 | -62.442 | -55.459 | 245 | | KT982326 | | x | |
| *P. obstaculumsuperavit* sp. nov. | ANT_D.1 | KT982330 | AP1 | -63.389 | -60.120 | 310 | | KT982330 | | x | |
| *P. obstaculumsuperavit* sp. nov. | ANT_D.1 | KT982331 | AP1 | -63.389 | -60.120 | 310 | | KT982331 | | x | |
| *P. obstaculumsuperavit* sp. nov. | ANT_D.2 | JR262_1058 | SG2 | -55.144 | -36.245 | 195.2 | ZSM-A20160708 | KY272301 | | x | x |
| *P. obstaculumsuperavit* sp. nov. | ANT_D.2 | JR262_1319 | SG2 | -55.002 | -37.272 | 148.8 | ZSM-A20160709 | KY272302 | | x | |
| *P. obstaculumsuperavit* sp. nov. | ANT_D.2 | JR262_1597_2 | SG2 | -54.396 | -37.384 | 174.9 | ZSM-A20160710 | KY272305 | | x | x |
| *P. obstaculumsuperavit* sp. nov. | ANT_D.2 | JR262_1903_1 | SR | -53.597 | -41.214 | 132.8 | ZSM-A20160711 | KY272303 | | x | |
| *P. obstaculumsuperavit* sp. nov. | ANT_D.2 | JR262_48_5_2 | SG1 | -54.284 | -36.083 | 124.1 | ZSM-A20160713 | KY272298 | | x | x |
| *P. obstaculumsuperavit* sp. nov. | ANT_D.2 | JR287_124_1 | SG1 | -53.764 | -36.681 | 151 | ZSM-A20160691 | KY272295 | KY272393 | x | x |
| *P. obstaculumsuperavit* sp. nov. | ANT_D.2 | JR287_124_2 | SG1 | -53.764 | -36.681 | 151 | ZSM-A20160692 | KY272294 | KY272391 | x | |
| *P. obstaculumsuperavit* sp. nov. | ANT_D.2 | JR287_152 | SG1 | -53.758 | -36.690 | 145 | ZSM-A20160694 | KY272292 | | | x |

**Table 3:** Continued.

| Species | Clade | ID | Location | Lat | Lon | Depth [m] | ZSM-Voucher Number | GenBank number | | G | M |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | COI | ITS | | |
| *P. obstaculumsuperavit* sp. nov. | ANT_D.2 | PS77_211_6_1_3 | SR | -53.402 | -42.668 | 290.2 | ZSM-A20160696 | KY272306 | KY272395 | x | x |
| *P. latefrontalis* | ANT_F | PS77_226_7_1_2 | AP4 | -64.915 | -60.621 | 226.2 | ZSM-A20160649 | KY272334 | KY272430 | x | x |
| *P. latefrontalis* | ANT_F | PS77_248_3_2_1 | AP5 | -65.924 | -60.332 | 433 | ZSM-A20160644 | KY272337 | KY272436 | x | x |
| *P. latefrontalis* | ANT_F | PS77_248_3_2_2 | AP5 | -65.924 | -60.332 | 433 | ZSM-A20160645 | KY272336 | KY272431 | x | |
| *P. latefrontalis* | ANT_F | PS77_248_3_2_3 | AP5 | -65.924 | -60.332 | 433 | ZSM-A20160646 | KY272338 | KY272435 | | x |
| *P. latefrontalis* | ANT_F | PS77_248_3_2_4 | AP5 | -65.924 | -60.332 | 433 | ZSM-A20160647 | KY272332 | | x | |
| *P. latefrontalis* | ANT_F | PS77_257_2_2_3 | AP4 | -64.913 | -60.648 | 152.5 | ZSM-A20160650 | KY272330 | KY272440 | x | |
| *P. latefrontalis* | ANT_F | PS77_257_2_2_5 | AP4 | -64.913 | -60.648 | 152.5 | ZSM-A20160651 | KY272329 | KY272432 | x | |
| *P. latefrontalis* | ANT_F | PS77_275 | WS6 | -70.940 | -10.489 | 225.5 | ZSM-A20160728 | KY272326 | KY272439 | x | |
| *P. latefrontalis* | ANT_F | PS77_291_1_2 | WS6 | -70.842 | -10.587 | 267.5 | ZSM-A20160642 | KY272333 | KY272437 | x | |
| *P. latefrontalis* | ANT_F | PS77_292_2_5 | WS6 | -70.846 | -10.593 | 243.5 | ZSM-A20160729 | KY272327 | KY272441 | x | x |
| *P. latefrontalis* | ANT_F | PS82_58_1 | WS3 | -76.322 | -29.002 | 228.5 | ZSM-A20160627 | KY272328 | KY272438 | x | |
| *P. latefrontalis* | ANT_F | ACE2017 1069_2_1_1 | BI | -54.425 | 35.241 | 327 | ZSM-A20190284 | | | x | |
| *P. latefrontalis* | ANT_F | ACE2017 1069_2_1_2 | BI | -54.425 | 35.241 | 327 | ZSM-A20190285 | | | x | x |
| *P. latefrontalis* | ANT_F | ACE2017 1069_2_1_3 | BI | -54.425 | 35.241 | 327 | ZSM-A20190286 | | | x | x |
| *P. hiemalis* | ANT_K | PS82_143_2_2 | WS2 | -76.967 | -32.866 | 293.7 | ZSM-A20160624 | KY272325 | KY272425 | x | |
| *P. hiemalis* | ANT_K | PS82_244_4 | WS5 | -72.799 | -19.495 | 739.7 | ZSM-A20160640 | KY272323 | | x | x |
| *P. hiemalis* | ANT_K | PS82_246_2 | WS6 | -70.928 | -10.475 | 213.5 | ZSM-A20160641 | KY272324 | KY272424 | x | |
| *Pallenopsis* sp. ANT_L | ANT_L | PS82_34_2 | WS3 | -76.069 | -30.160 | 473 | ZSM-A20160628 | KY272340 | KY272421 | x | |
| *Pallenopsis* sp. ANT_L | ANT_L | PS82_109_2_2 | WS2 | -77.016 | -33.695 | 435.2 | ZSM-A20160622 | KY272339 | KY272420 | x | x |
| *P. buphtalmus* | ANT_M | PS82_183_1_1 | WS1 | -74.250 | -37.749 | 833.5 | ZSM-A20160638 | KY272321 | KY272400 | x | x |
| *P. buphtalmus* | ANT_M | PS82_183_1_2 | WS1 | -74.250 | -37.749 | 833.5 | ZSM-A20160639 | KY272320 | KY272401 | x | x |
| *P. buphtalmus* | ANT_M | PS82_240_2 | WS4 | -74.660 | -28.763 | 769 | ZSM-A20160731 | KY272322 | KY272402 | x | |
| *P. buphtalmus* | ANT_M | PS96_220_3_1 | WS4 | -74.657 | -26.896 | -421.8 | ZSM-A20190287 | | | x | |
| *Pallenopsis* sp. ANT_N | ANT_N | PpaE_002_HT25 | SG1 | -54.016 | -37.437 | 78 | ZSM-A20160718 | KC794960 | | x | x |
| *Pallenopsis* sp. ANT_N | ANT_N | PS77_211_6_1_4 | SR | -53.402 | -42.668 | 290.2 | ZSM-A20160697 | KY272360 | KY272458 | x | |
| *P. patagonica* | SUB_1 | PS77_208_5_1_1 | SP | -56.168 | -54.548 | 292 | ZSM-A20160726 | KY272289 | KY272367 | x | x |
| *P. patagonica* | SUB_1 | PS77_208_5_1_4 | SP | -56.168 | -54.548 | 292 | ZSM-A20160689 | KY272288 | | x | x |

**Table 3:** Continued.

| Species | Clade | ID | Location | Lat | Lon | Depth [m] | ZSM-Voucher Number | GenBank number | | G | M |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | COI | ITS | | |
| *P. patagonica* | SUB_2 | ZSMA20111352_HT27 | FI1 | -51.269 | -62.952 | 174 | ZSM-A20111352 | KF603937 | | x | x |
| *P. patagonica* | SUB_2 | HF26_254 | Pa2 | -53.007 | -73.923 | 31 | ZSM-A20160456 | KY272290 | KY272368 | x | x |
| *P. patagonica* | SUB_2 | PS77_208_3 | SP | -56.152 | -54.530 | 285.5 | ZSM-A20160725 | KY272291 | KY272366 | x | x |
| *P. notiosa* | SUB_3 | ZSMA20111008_HT28 | Pa3 | -50.414 | -74.559 | 20 | ZSM-A20111008 | KF603952 | KY272390 | x | x |
| *P. patagonica* | SUB_4 | PpaE_004_HT18 | FI2 | -52.574 | -60.084 | 378 | ZSM-A20160719 | KC794961 | KY272443 | x | |
| *P. patagonica* | SUB_4 | PpaE_007_HT15 | FI2 | -52.574 | -60.084 | 378 | ZSM-A20160722 | KC794964 | | | x |
| *P. patagonica* | SUB_4 | PS77_208_5_1_2 | SP | -56.168 | -54.548 | 292 | ZSM-A20160727 | KY272356 | | x | |
| *P. patagonica* | SUB_4 | ZDLT1_889_2 | FI1 | -50.252 | -61.567 | 159 | ZSM-A20160699 | KY272358 | KY272446 | | x |
| *P. patagonica* | SUB_4 | ZDLT1_889_3 | FI1 | -50.252 | -61.567 | 159 | ZSM-A20160700 | KY272359 | KY272444 | x | x |
| *P. patagonica* | SUB_5 | HF26_027 | Pa2 | -52.600 | -73.640 | 19 | ZSM-A20160452 | KY272344 | | x | x |
| *P. patagonica* | SUB_5 | HF26_367 | Pa2 | -53.357 | -73.087 | 20 | ZSM-A20160468 | KY272351 | KY272447 | x | x |
| *P. patagonica* | SUB_5 | HF26_373 | Pa2 | -53.379 | -73.159 | 14 | ZSM-A20160488 | KY272347 | KY272453 | | x |
| *P. patagonica* | SUB_5 | HF26_392 | Pa2 | -53.379 | -73.159 | 17 | ZSM-A20160493 | KY272345 | KY272450 | | x |
| *P. patagonica* | SUB_5 | KT982315 | Pa1 | -53.270 | -66.386 | 96 | | KT982315 | KY272456 | x | |
| *P. yepayekae* | Pye.1 | HF16_476_2 | Pa3 | -50.353 | -75.283 | 20 | ZSM-A20160580 | KY272283 | | x | |
| *P. yepayekae* | Pye.1 | HF24_213 | Pa4 | -46.723 | -75.255 | 31.4 | ZSM-A20160529 | KY272268 | KY272372 | x | |
| *P. yepayekae* | Pye.1 | HF26_029 | Pa2 | -52.600 | -73.640 | 15-20 | ZSM-A20160450 | KY272281 | | x | x |
| *P. yepayekae* | Pye.1 | HF26_363 | Pa2 | -53.007 | -73.923 | 20 | ZSM-A20160462 | KY272284 | | | x |
| *P. yepayekae* | Pye.1 | HF26_378 | Pa2 | -53.379 | -73.159 | 29 | ZSM-A20160498 | KY272277 | KY272387 | | x |
| *P. pilosa* | outgroup | PS96_004_3 | | | | | ZSM-A20190288 | | | x | |

### 5.3. SNP analyses

As there is no reference genome for sea spiders available, a *de novo* reference based on all raw reads from the samples of the *P. patagonica* species complex was generated with a pipeline of custom Bash shell scripts including quality filtering, sequence editing and assembly. Further information is provided in Supplementary Material 9, Protocol 2. SNPs were called separately for three different data sets: i) all samples belonging to the *P. patagonica* species complex, ii) *P. patagonica* samples belonging to the "Patagonian supergroup", and iii) *P. patagonica* samples belonging to the "Antarctic supergroup"; see result section for group assignment) to maximize the number of group-specific SNPs (see Supplementary Material 9, Protocol 3 for more information). To analyze the genetic structure, PCAs were conducted using the R-package SNPRelate v. 1.12.2 (Zheng et al. 2012) with default parameters. sNMF-plots were calculated to investigate the number of genetic clusters within the dataset, using the LEA package v. 2.0.0 (Frichot and Francois 2015). A range of K values (number of ancestral populations) in the interval of 1-20 were tested. The number of repetitions was set to 40 with 40,000 iterations and the lowest cross-entropy per K value was determined and plotted to choose the most likely K value. To also analyse the relationships between clusters, a maximum likelihood tree based on the SNP data was obtained with SNPhylo v. 20140701 (Lee et al. 2014).

### 5.4. Orthology assignment and phylogenetic analyses

For the bait construction, Dietz et al. (2019) had searched OrthoDB 9.1 (Zdobnov et al. 2017) for orthologous single-copy genes present in all four spider (Araneae) genomes. Using Orthograph v. 0.5.14 (Petersen et al. 2017) these genes were aligned on the amino acid level and hidden Markov models (HMMs) were created. With the aid of Orthograph, these HMMs were then reused to mine the transcriptome of *P. patagonica* for the EOGs of interest, as was previously done for *C. megalonyx* (Dietz et al. 2019). As the baits were originally designed for *Colossendeis*, the *Pallenopsis* genes were aligned with their *Colossendeis* homologs using MAFFT v. 7.305b (Katoh and Standley 2013). Regions that were aligned to the *Colossendeis* bait sequences and which were at least 30 bp in length were extracted. The trimmed Illumina reads were mapped against these regions with the BWA-MEM algorithm in bwa v. 0.7.17 (available from: https://sourceforge.net/projects/bio-bwa/files). Default parameters were used, except that the minimum match length was set to 30 bp. Successfully mapped reads were mapped again against the full coding sequences from the corresponding contigs with bwa as described above. Diploid consensus sequences of the regions matching the reference were generated for each specimen with samtools v. 1.6 (Li et al. 2009) and bcftools v. 1.6 (available from: https://github.com/samtools/bcftools). As the consensus sequences were already aligned

to the reference sequence, no further alignment was necessary and all sequences were already in the correct reading frame. All gene alignments were then concatenated to one supermatrix of nucleotide sequences, which was used in a maximum likelihood phylogenetic analysis with IQ-TREE v. 1.5.4 (Nguyen et al. 2015). The alignment was partitioned by codon positions and the optimal partitioning scheme was selected with an algorithm implemented in ModelFinder (Chernomor et al. 2016, Kalyaanamoorthy et al. 2017) using the Bayesian Information Criterion. A phylogenetic tree search was conducted with IQ-TREE using the selected models, and branch support values were determined from 1000 ultrafast bootstrap replicates.

For rooting the tree, we mined the published transcriptome of *Anoplodactylus insignis* (NCBI accession number SRX2544807) for the genes of interest using Orthograph with the same procedure as described above. *Anoplodactylus insignis* belongs to the Phoxichilidiidae, a family thought to be related to the Pallenopsidae (Arango and Wheeler 2007, Sabroux et al. 2017). Amino acid sequences of *A. insignis* were added to the translated genes alignments with MAFFT using the –add option. EOGs for which no *A. insignis* sequence was found and positions present in less than 50% of the taxa were removed. Outlier sequences were excluded with the OLIinSeq program by CM (available upon request) as described in Dietz et al. (2019). After the root of the tree was determined, further analyses were carried out with the nucleotide data sets excluding *A. insignis*.

### 5.5. Selection tests

Comparative sequence analyses based on stochastic evolutionary models within HyPhy v. 2.3.13 (Kosakovsky Pond et al. 2005) were used to test for selection. The alignment described in the previous section excluding *A. insignis* was used, additionally filtering out all positions present in less than 50% of the samples. All analyses were based on the phylogenetic tree obtained with IQ-TREE (see above), as we expect all genes to have evolved according to the same phylogeny. Furthermore, either the default or settings recommended by the authors of the programs were used. FUBAR (Murrell et al. 2013) and MEME (Murrell et al. 2012) were used to test for selection across sites. Genes with codons under selection (FUBAR: pp $\geq$ 0.99; MEME: p $\leq$ 0.01) that were recognised with both methods were used for further branch-site tests, namely, aBSREL (Smith et al. 2015) and BUSTED (Murrell et al. 2015). Here, both terminal and internal branches were tested.

### 5.6. Morphology

Specimens from the different mitochondrial clades of the *P. patagonica* species complex were studied using light microscopy and µCT. For identification, preparation and analyses of

individuals, Leica DMRD and Leica DM5000B microscopes were used. Accurate pictures were taken using the Olympus Stylus TG-4 camera (Microscope mode for automatic generation of extended depth of field images). To obtain a 3D reconstruction of one individual per clade without damaging the specimen, a Phoenix Nanotom (GE Sensing & Inspection Technologies, Wunstorf, Germany) cone beam CT scanner was used at voltages of 80 kV to 120 kV and currents of 90 to 140 µA for 53 min. 1440 radiographs were saved and analysed with the integrated software and VGStudio Max v. 2.2.2 (64 bit; Isosurface and Volume Rendering).

Morphometric body measurements were carried out using the digital caliper from MarCal IP67 (Mahr Metrology, Germany). Measurements follow those applied by Dietz et al. (2013, 2015b), with a focus on characters evaluated as useful for species delimitation, and characters that are potentially linked to fitness differences. The latter include i) the proboscis with terminal mouth, which takes up and processes food; ii) the cheliphores, which function as devices to hold the prey/food and moving it to the mouth opening; and iii) the walking legs. When all limbs were present, up to 135 measurements per specimen were taken (Table 4). However, due to damage during trawling, transport, storage or preceding genetic analysis, distal leg articles were often missing and as a result, not all limbs could be measured. Due to the bilateral symmetry of the body, the averaged measurements of the left and right appendages (legs, palps and cheliphores) were used to reduce the amount of missing values. Ovigeral articles, which are appendices specific to sea spiders and used by males to carry fertilized eggs, were excluded from further analyses, to avoid a bias caused by sexual dimorphism.

For analyses of morphometric measurements Past v. 3.18 (Hammer et al. 2001) was used. First, measurements were tested for normality distribution using the Anderson-Darling, Shapiro-Wil, and Jarque-Bera tests. PCAs were performed to visualise the clustering of specimens regardless of predefined clades and missing values were handled as “iterative imputation” as recommended in Past Manual (Hammer and Harper 2006). In addition, row-wise bootstrapping was carried out using N=1000. Also, LDAs was performed and confusion matrices calculated. To cope with the missing data points and to limit the analysis to clades with a minimal representation, we pre-filtered the data set to leave only clades with a minimum of three individuals and characters with not more than 10% missing values. Remaining missing values were imputed using Predictive Mean Matching. Analyses were performed using both absolute values of measurements, and relative lengths of measurements expressed as proportion of the trunk to reduce biases caused by different absolute sizes.

Since the number of the characters was large with respect to the number of the individuals, a selection of characters for LDA was performed to avoid model overfitting. The heuristic search

for the optimal sets of characters was carried out by iteratively using the stepclass function from the R package klaR v. 0.6-14 with forward-backward selection direction, cross-validation correctness rate as the optimality criterion (taking ten folds) and 5 as the maximum number of characters in a set. The search was organized by picking each one of the characters as starting variable and repeating the procedure ten times. The performance of the character sets was recorded and the best set was used for a final LDA.

**Table 4: List of characters measured for morphometric analyses**. Description of how characters were measured and abbreviations for all as used in Table 3 and Supplementary Material 4.

| Abbreviation | Description |
|---|---|
| trunk L | total length of trunk |
| ceph. segment | length of cephalic segment |
| trunk W1 | diameter of lateral process of 1$^{st}$ trunk segment |
| trunk W12 | width of trunk between 1$^{st}$ and 2$^{nd}$ lateral processes |
| trunk W2 | diameter of lateral process of 2$^{nd}$ trunk segment |
| trunk W23 | width of trunk between 2$^{nd}$ and 3$^{rd}$ lateral processes |
| trunk W3 | diameter of lateral process of 3$^{rd}$ trunk segment |
| trunk W34 | width of trunk between 3$^{rd}$ and 4$^{th}$ lateral processes |
| trunk W4 | diameter of lateral process of 4$^{th}$ trunk segment |
| trunk H | height of trunk |
| abdomen L | length of abdomen |
| abdomen W | width of abdomen |
| ocular tubercle H | height of ocular tubercle |
| ocular tubercle W | width of ocular tubercle |
| eye H | height of anterior eye |
| forehead H | distance between eyes and apex of ocular tubercle |
| eyes distance | distance between eyes |
| proboscis L | proboscis length |
| proboscis basis | diameter of proboscis at proximal basis |
| proboscis thickest | diameter of proboscis at thickest part of proboscis |
| proboscis thick2tip | distance between tip of proboscis and thickest part |
| proboscis thinnest | diameter of proboscis at thinnest part of proboscis |
| proboscis thin2tip | distance between tip of proboscis and thinnest part |
| l/r palp | length of palp bulb |
| l/r cheliphore 1-3 | length of first 3 cheliphore articles; left and right |
| l/r cheliphore 4 | ultimate cheliphore article (moveable finger) |
| l/r oviger 1-10 | length of all 10 ovigeral articles; left and right |
| l/r WL1-4 coxa1 | length of 1$^{st}$ coxa for all 4 pairs of walking leg; left and right |
| l/r WL1-4 coxa2 | length of 2$^{nd}$ coxa for all 4 pairs of walking leg; left and right |
| l/r WL1-4 coxa3 | length of 3$^{rd}$ coxa for all 4 pairs of walking leg; left and right |
| l/r WL1-4 femur | length of femur for all 4 pairs of walking leg; left and right |
| l/r WL1-4 tibia1 | length of 1$^{st}$ tibia for all 4 pairs of walking leg; left and right |
| l/r WL1-4 tibia2 | length of 2$^{nd}$ tibia for all 4 pairs of walking leg; left and right |
| l/r WL1-4 tarsus | length of the tarsus for all 4 pairs of walking leg; left and right |
| l/r WL1-4 propodus | length of the propodus for all 4 pairs of walking leg; left and right |
| l/r WL1-4 claw | length of the claw for all 4 pairs of walking leg; left and right |
| l/r WL1-4 aux. claw | length of auxiliary claw for all 4 pairs of walking leg; left and right |

Finally, nonparametric unifactorial Kruskal-Wallis H in combination with Dunn's post hoc test (Bonferroni-corrected) were used to test for significant differences between geographic (sub-Antarctic vs Antarctic) and genetic groupings as well as sexes.

### 5.7. Combining morphological and genetic data

To test whether there are greater morphological differences for taxa living in sympatry in contrast to those living in allopatry, which can be expected in case of adaptive divergence, pairwise morphological distances were calculated in Past. Subsequently, those were compared with uncorrected pairwise genetic distances calculated in MEGA7 (Kumar et al. 2016). To be able to calculate genetic distances between all morphologically analysed specimens, COI sequences were used as enrichment data were not available for all specimens. In addition, genetic distances between specimens that were also used for target hybrid enrichment were calculated using the EOG sequence alignment. Linear regression between values and significant differences between ranges of genetic distances were again evaluated in Past.

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## 6. Declarations

### Ethics approval and consent to participate

Research followed all applicable laws and ethical guidelines per individual countries' requirements. Permits for sampling during Antarctic and Patagonian expeditions were obtained. As no vertebrate animals were impacted by the research no special ethical approvals were needed for this work.

### Consent for publication

Not applicable.

### Availability of data and material

The datasets generated and analyzed during the current study are included in this published article or available from the corresponding author on reasonable request.

### Competing interests

The authors declare that they have no competing interests.

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### Authors' contributions

JD, FL and RM designed and directed the project. JD performed lab work, analyzed major parts of the data and led the manuscript writing together with FL and RM. LD and TM analyzed sequence data with support from CM and AR. SD and KW assisted with morphological measurements and took morphological images. JD and AR performed the PCA and LDA analyses on morphometric data. JD and RM prepared picture plates. All authors contributed to the final discussion of the data and commented on the manuscript

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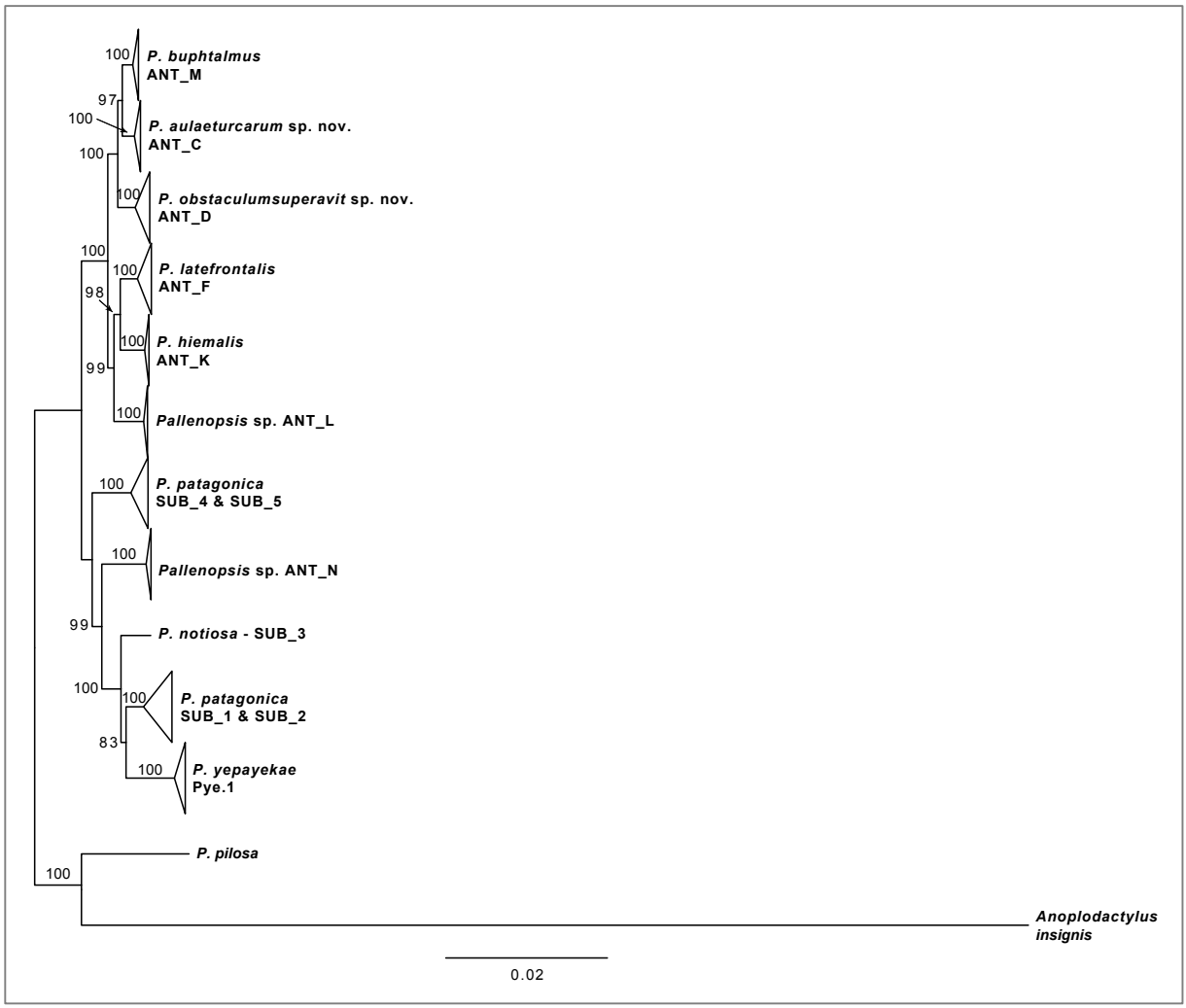
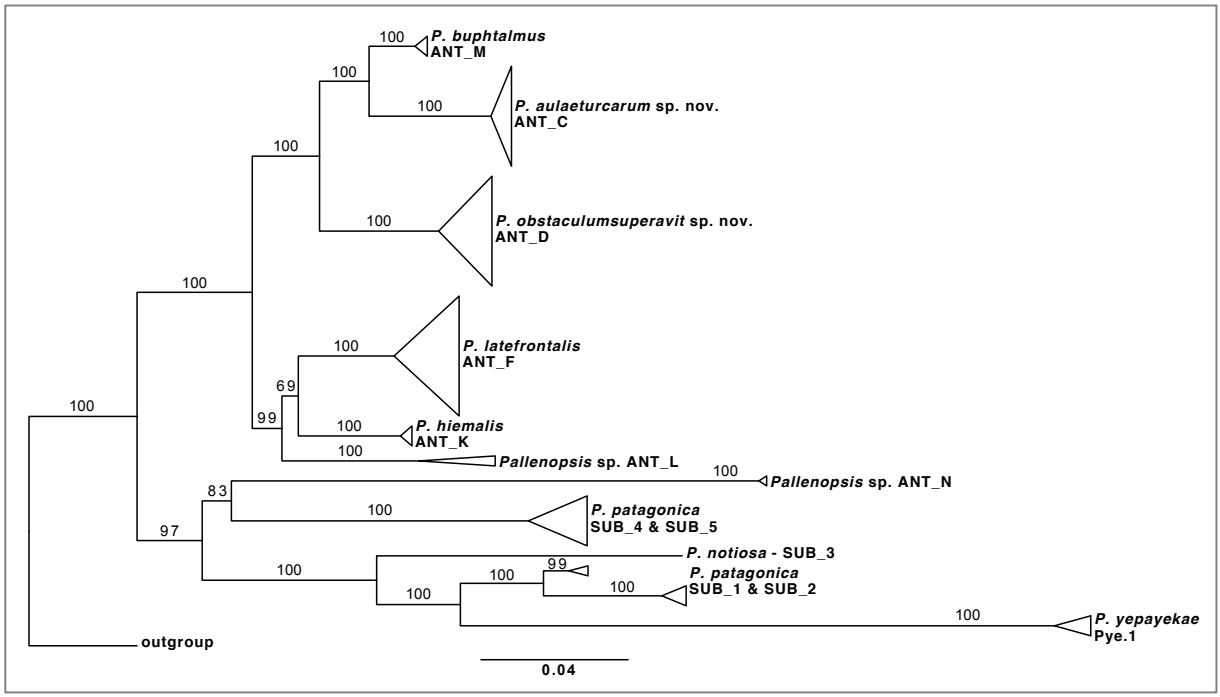
## *Supplementary material*

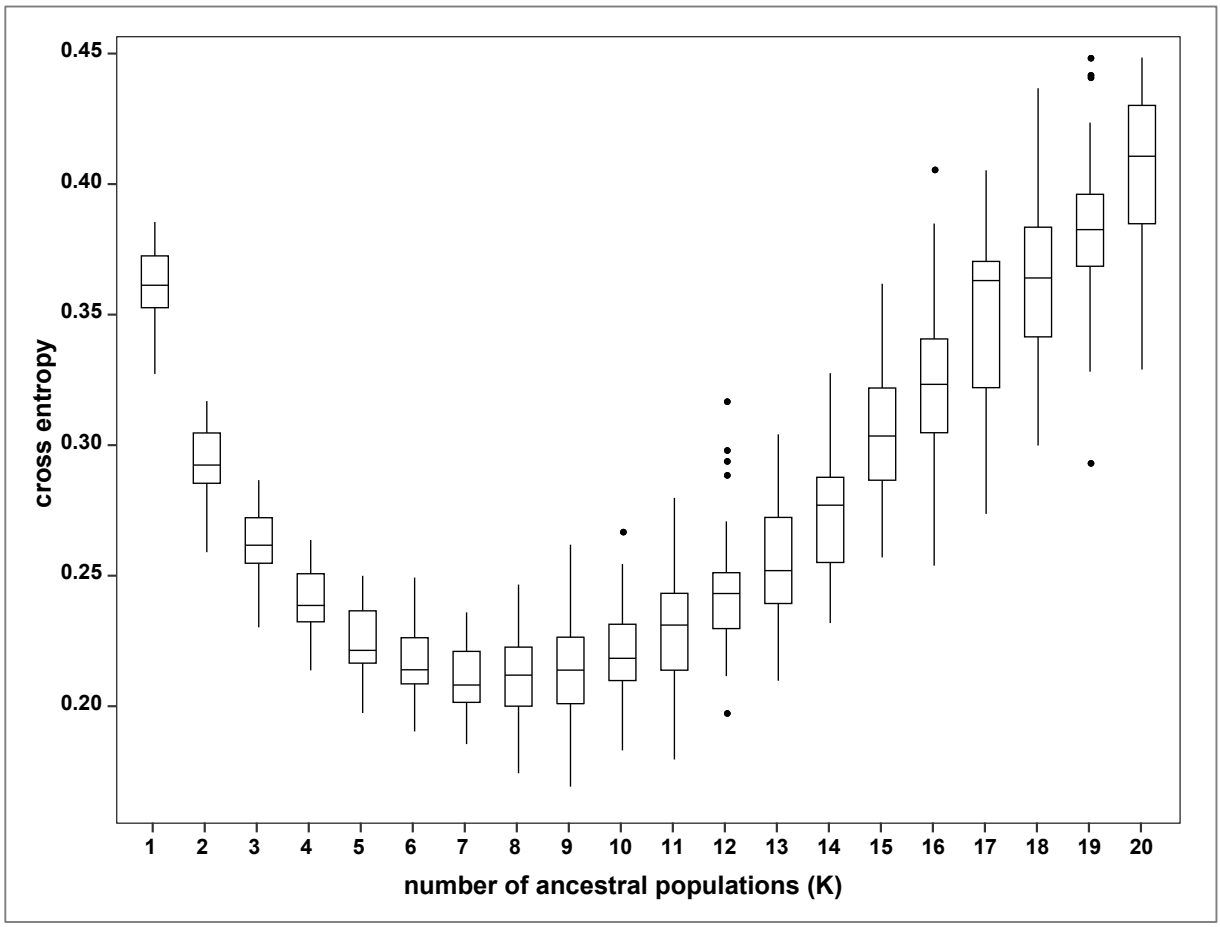
# Combining morphological and genomic evidence to resolve species diversity and study speciation processes of the *Pallenopsis patagonica* (Pycnogonida) species complex




**Supplementary Material 1:** Phylogenetic EOG tree of the *Pallenopsis patagonica* species complex. Maximum-Likelihood tree based on concatenated EOG sequences of all samples using *P. pilosa* and transcriptomic data of *Anoplodactylus* insignis as outgroup. Bootstrap values are given next to the respective branches.

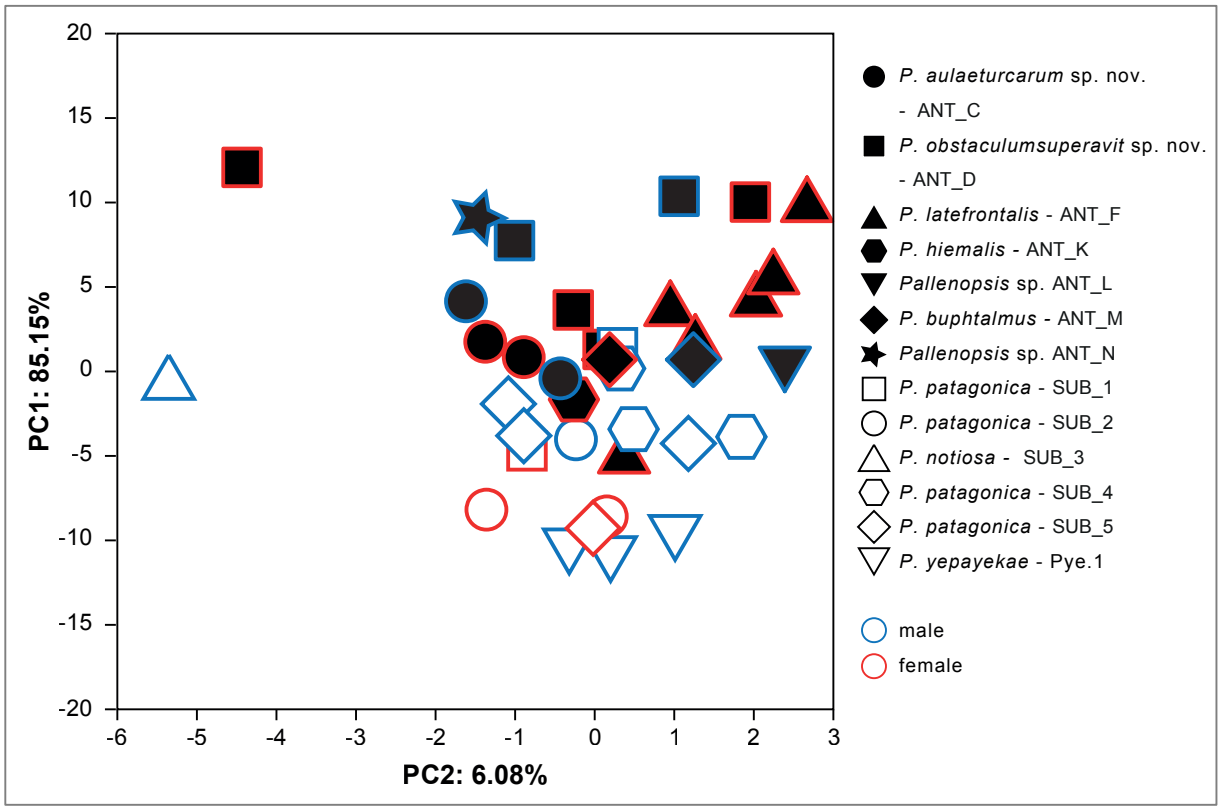



**Supplementary Material 2:** Phylogenetic SNP tree of the *Pallenopsis patagonica* species complex. Maximum-Likelihood tree based on aligned SNP data of all Pallenopsis samples. Bootstrap values are given next to the respective branches.

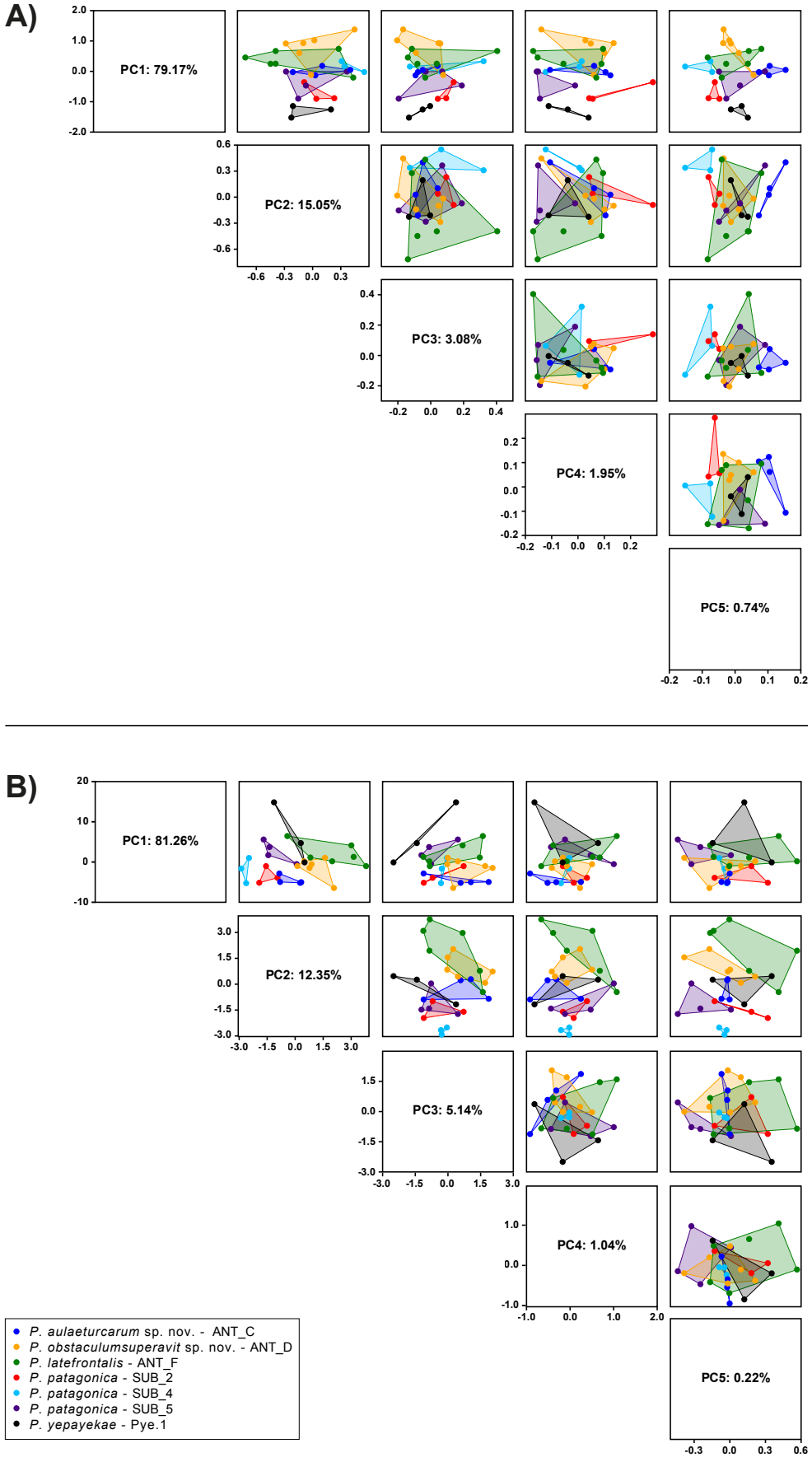



**Supplementary Material 3:** Cross-entropy estimates of genomic sNMF analysis of the *Pallenopsis patagonica* species complex for 1 to 20 ancestral populations (K value).

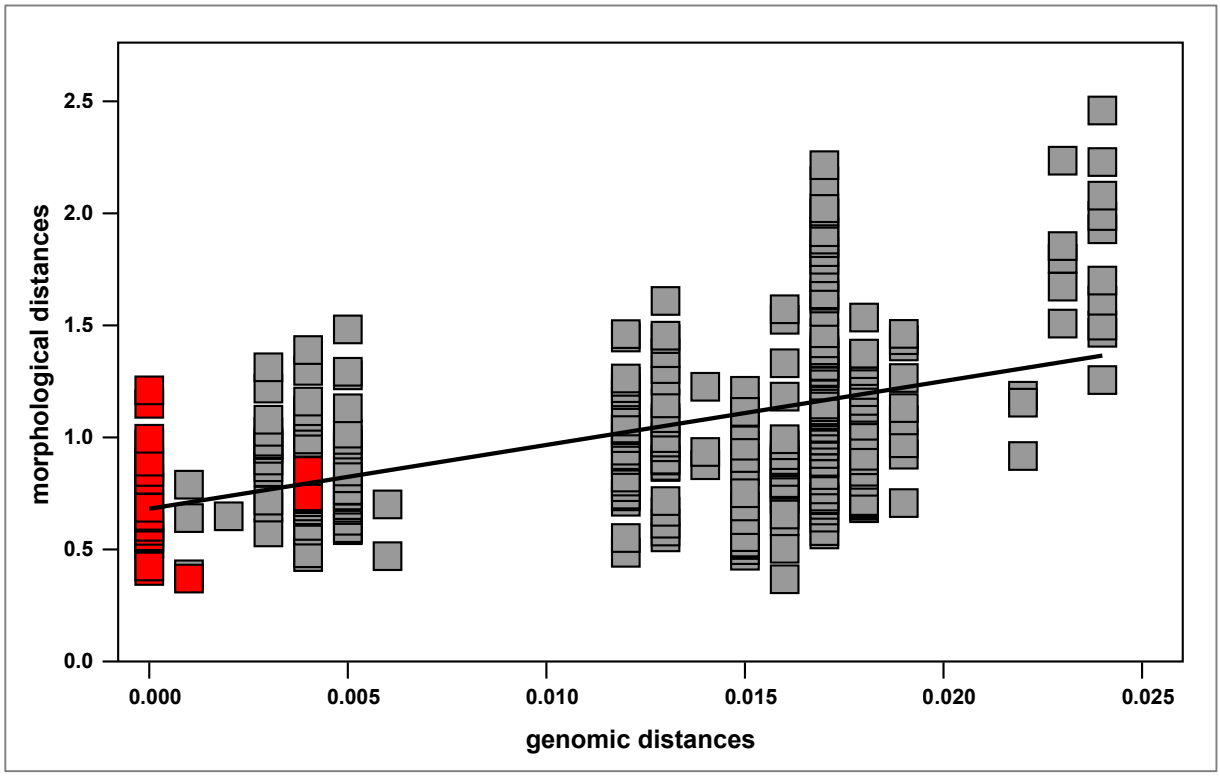
**Supplementary Material 4:** Morphological measurements of the *Pallenopsis patagonica* species complex. *Provided as electronic supplement of this thesis.*




**Supplementary Material 5:** PCA from morphological data of the Pallenopsis patagonica species complex. PCA plots based on morphological measurements. All mitochondrial clades are indicated by different symbols. Symbols of samples from Patagonian (SUB) have no filling, in contrast to the filled symbols of Antarctica (ANT). Male specimens have a blue, female a red outline.




**Supplementary Material 6: Matrices of PCA plots based on reduced morphometric data sets of the *Pallenopsis patagonica* species complex.** All combinations of all five axes (PCs) are represented for data sets including A) absolute and B) relative values. Each colour represents a different clade (see legend).




**Supplementary Material 7:** Morphological distances against genomic distances. Morphological distances plotted against genomic distances (based on target hybrid enrichment data) between individuals of the *Pallenopsis patagonica* species complex. Red: intraspecific distances (the rightmost red squares represent intraclade distances of SUB_2); grey: interspecific distances. Linear regression line is given ($r=0.51$, $p<0.0001$).

**Supplementary Material 8:** Summary of information used for species delimitation of the *Pallenopsis patagonica* species complex. For species delimitation, morphological and genetic analyses were considered. Previously published results are also included. Black filling indicates missing data. *Provided as electronic supplement of this thesis.*

**Supplementary Material 9**

**Protocol 1: Bait enrichment**

For genetic analyses, a target hybrid enrichment approach was chosen and a bait set especially designed for sea spiders was used, but see Dietz et al. (2019) for further details about bait design. The total number of baits was 12,014 in 3,682 bait regions. Baits were produced by Agilent Technologies (Waldbronn, Germany).

Sample preparation was conducted following a slightly modified version of Agilent's protocol "200 ng DNA sample" for "Agilent's SureSelect Target Enrichment System". 100 ng per 10 µl were used for DNA fragmentation in the Bioruptor Pico Sonicaton System (Diagenode SA). One cycle consisted of 30 seconds of ultrasound shearing followed by a 30 second pause (cooling). The optimal number of shearing cycles was determined by the proportion of fragments smaller than 500 bp as determined with Fragment Analyzer using the High Sensitivity NGS Kit (DNF-474-33, Advanced Analytical Technologies). A percentage of short fragments greater than 95% was generally regarded as optimal, but a few difficult samples were accepted with 85% for further preparation. Afterwards, samples were topped off with HPLC water to a total of 10 µl, then stored at 4°C until further preparation. Unless otherwise indicated, the following steps only used half the volumes mentioned in the original protocol. Reaction products for all steps involved in the hybrid enrichment were purified using magnetic beads (Agentcourt AMPure XP Kit, Beckman Coulter Genomics). To enrich for fragments smaller than 500 bp a volume of beads equal to the reaction volume was added to the reaction itself. Steps prior to hybridization included end repairing, adenylation of the 3' end, and adaptor ligation. DNA concentration was measured using the High Sensitivity Kit for Qubit (Thermo Fisher Scientific). Depending on the concentration, amplification of adaptor-ligated samples was performed with 10 to 20 cycles of PCR. A minimum of 375 ng per sample was required for the hybridization step. Samples were dried overnight in a thermal block at 40°C and eluted with HPLC water to a concentration of 220 ng/µl. 1.7 µl was then used for hybridization, performed at 60°C for 24 hours. In contrast to the original protocol, targeted sequences were caught using 15 µl streptavidin-coated magnetic beads (Dynabeads MyObe Streptavidin C1, Thermo Fisher Scientific). Post-capture PCR with 18 cycles was performed to amplify captured and indexed fragments of each sample individually. Concentrations were measured with Qubit's dsDNA BR Array Kit (Thermo Fisher Scientific). Afterwards, samples were pooled in equal quantities for sequencing. Each pool contained 32 samples (1st library 12.6 ng/µl and 2nd library 21.8 ng/µl). Libraries were sent to GATC Biotech GmbH (Konstanz, Germany) for

sequencing on an Illumina MiSeq platform using the V2 2x250 bp paired-end sequencing kit. 5% PhiX spike-in was added in each run.

**Protocol 2: *De novo* reference**

Since no sea spider genome is currently available, a *de novo* reference had to be produced to be able to map the enrichment data and align sequences of different individuals. The final *de novo* reference was based on the assembly of 63 complex paired-end read samples of the *P. patagonica* species. All pairs of NGS raw read files were initially quality checked using FastQC v. 0.11.5 (Andrews et al. 2010) and then filtered and trimmed with cutadapt v. 1.14 (Martin et al. 2011) (minimum length = 35; Error-rate = 0.1; quality cutoff = 15; overlap minimum length = 3). The trimmed paired reads were assembled with Trinity v. 2.5.1 (Grabherr et al. 2011), using a minimum coverage of 10 and the standard kmer length of 25. As Trinity is an RNA assembler, the output contains isoforms of the same contig. A maximum of two isoforms per contig were allowed to account for potentially heterozygous loci, while contigs with three or more isoforms were removed from the data set to avoid chimeras and paralogous genes. Two isoforms in a pair were aligned using the water program from EMBOSS v. 6.5.7.0 (Li et al. 2015). Consensus sequences were called with consambig from EMBOSS and added to the final *de novo* reference data set alongside single-isoform contigs. The final assembly for the *de novo* reference.

**Protocol 3: SNP calling**

Trimmed raw reads of all samples were mapped to the reference assembly using BWA mem (Li 2013). Resulting alignment files were subsequently processed with samtools v. 1.6 (Li et al. 2009, Li 2011), by adding read groups, to uniquely identify each read, and marking duplicates to reduce redundancy on the level of individual libraries. Variant calling was conducted with HaplotypeCaller from the GATK v. 4.0.3.0 package (McKenna et al. 2010). Variant calling was performed for three different sample sets. For further analysis, SNPs were extracted using GATK SelectVariants and filtered with VariantFiltration according to the GATK best practices workflow (DePristo et al. 2011) by applying the following thresholds: QualByDepth < 2.0, FisherStrand > 60.0, RMSMappingQuality < 40.0, StrandOddsRatio > 3.0, MappingQualityRankSum < -12.5 and ReadPosRankSumTest < -8.0. In addition to the 0/0 homozygous and 0/1 heterozygous genotypes filtered by VariantFiltration, the resulting variant files were filtered to include 0/0 homozygous genotypes with VCFFilterJS r. f4c7a81 (Lindenbaum et al. 2018). VCFtools v. 0.1.13 (Danecek et al. 2011) was used to retain only bi-allelic sites with a maximum missing rate of 20% across individuals within a data set.

Furthermore, additional vcf files with thinned sites, i.e. one SNP per contig only, were produced. Invariant sites with missing samples, which were neither considered by VCFFilterJS nor by GATK, were filtered with custom script. The vcf files were used as a basis for all subsequent analyses.

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# CHAPTER III

# Analyzing drivers of speciation in the Southern Ocean using the sea spider species complex *Colossendeis megalonyx* as a test case

Jana S. Dömel, Lars Dietz, Till-Hendrik Macher, Andrey Rozenberg, Christoph Mayer, Johanna M. Spaak, Roland R. Melzer and Florian Leese

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Experimental design and planning: 65%
Laboratory work: 100%
Morphometric measurements: 0%
Data analysis: 65%

- Tree reconstruction: 0%
- Selection test: 85%
- Gene Ontology: 90%
- SNP analyses: 40%
- Selection tests: 95%
- Morphometric analyses: 70%

Figures: 80%

- Figure 1: 100%
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- Supplementary Material Table S3: 5%

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- First draft: 95%
- Editing of first draft together with the other authors.

# Analyzing drivers of speciation in the Southern Ocean using the sea spider species complex *Colossendeis megalonyx* as a test case

Jana S. Dömel*[1], Lars Dietz[2,3], Till-Hendrik Macher[1], Andrey Rozenberg[4], Christoph Mayer[2,3], Johanna M. Spaak[5], Roland R. Melzer[6,7,8], Florian Leese[1,9]

[1]Aquatic Ecosystem Research, Faculty of Biology, University of Duisburg-Essen, Universitaetsstr. 5, D-45141 Essen, Germany,
[2]Zoological Research Museum Alexander Koenig, Statistical Phylogenetics and Phylogenomics, Adenauerallee 160, D-53113 Bonn, Germany
[3]Faculty of Mathematics and Natural Sciences, University of Bonn, D-53012 Bonn, Germany
[4]Technion – Israel Institute of Technology, Faculty of Biology, Haifa 3200003, Israel
[5]Biological Sciences, School of Science, University of Waikato, Hamilton 3216, New Zealand
[6]Bavarian State Collection of Zoology - SNSB, Muenchhausenstr. 21, D-81247 Munich, Germany
[7]Department Biologie II, Ludwig Maximilians University of Munich, Großhaderner Str. 2, D-82152 Planegg-Martinsried, Germany
[8]GeoBioCenter, LMU Munich, Richard-Wagner-Str. 10 D-80333 Munich, Germany
[9]Centre for Water and Environmental Research (ZWU), University of Duisburg-Essen, Universitaetsstr. 2, 45141 Essen, Germany

*Corresponding Author: Jana S. Dömel (jana.doemel@uni-due.de)

## Abstract

*Colossendeis megalonyx* Hoek, 1881 has the broadest distribution of all sea spiders in the Southern Ocean. Previous studies have detected several evolutionarily young lineages within this taxon and interpreted them as a result of allopatric speciation in a few shelf refuges during glacial maxima. However, alternative scenarios such as ecological speciation in sympatry have rarely been considered or tested. Here, we generated the most extensive genomic and morphometric data set on the *C. megalonyx* species complex to i) comprehensively resolve species diversity, ii) explore intraspecific connectivity between populations located around Antarctica, and iii) systematically test for positive selection indicative of adaptive speciation. We successfully applied a target hybrid enrichment approach and recovered all 1607 genes targeted. Phylogenomic analysis was consistent with previous findings and, moreover, increased the resolution of branching within lineages. We found phylogenetically well-separated lineages occurring in sympatry to be genetically distinct from each other and the gene flow between geographically distinct populations to be restricted. Evidence for positive selection was found for four genes associated with structural and neuronal functions. Hence, there is an indication for positive selection in the *C. megalonyx* species complex, yet its specific contribution to the speciation process remains to be explored further. Finally, morphometric

analyses revealed multiple significant differences between lineages, but a clear separation proved difficult. Our study highlights that positive selection is a relevant factor as a driver for speciation in the Southern Ocean.

**Keywords:** Antarctic benthos, target hybrid enrichment, positive selection, integrative taxonomy, cryptic species.

## 1. Introduction

The Southern Ocean accommodates a unique, speciose and highly endemic benthic community (Knox and Lowry 1977; Clarke and Johnston 2003; Aronson et al. 2007; Clarke 2008) that has evolved through *in situ* radiations after the opening of the Drake Passage (about 30 mya) and the subsequent establishment of the Polar Front (see Poulin et al. 2002; Briggs 2003; Convey et al. 2009). Prominent examples of early *in situ* radiations during the Mio- and Pliocene include the Antarctic icefishes (Notothenioidei, Eastman and McCune 2000), octopuses (Strugnell et al. 2012), and serolid isopods (Brandt 1991; Held 2000). In addition to these ancient radiations, molecular studies revealed a high number of recent divergences that probably occurred in the Plio- and Pleistocene (e.g. Convey et al. 2009; Fraser et al. 2012; Halanych and Mahon 2018 for overviews). For these, recurrent glaciations are seen as important drivers of speciation (Clarke and Crame 1989; Thatje et al. 2005). Grounded shelf ice covered major areas of the continental shelf during glacial maxima, thereby making it uninhabitable for benthic communities (Convey et al. 2009). At the same time, recent evidence suggests that small ice-free refugia existed and that relict populations could survive on the continental shelf (see Thatje et al. 2005, 2008 and Fraser et al. 2012, 2014 for overviews). The assumed limited dispersal potential of many Southern Ocean benthic species in combination with the higher rates of random genetic drift in small, isolated populations, likely triggered rapid lineage sorting in independently evolving populations and ultimately allopatric speciation. The often allopatric occurrence of cryptic species adds strong support for this hypothesis (e.g. Held 2003; Held and Wägele 2005; Wilson et al. 2007).

However, several studies have documented that divergent selection also has a large contribution to speciation processes (Schluter 2000; Dieckmann et al. 2004; Coyne and Orr 2004), for example in cichlid fishes in African and central American lakes (e.g. Kocher 2004), or the marine snail *Littorina saxatilis* Olivi, 1792 (Johannesson et al. 2017). Interestingly, the role of selection in Southern Ocean speciation processes has rarely been addressed. Rutschmann et al. (2011) tested for evidence of ecological specialisation underlying the adaptive radiation of notothenioid fishes and found a lineage-independent, ecological differentiation into different niches for more ancient speciation. A study on the Southern Ocean sea slug *Doris kerguelenensis* (Bergh, 1884) reported evidence that interspecific competition (predation) was involved in more recent speciation during the Plio- and Pleistocene (Wilson et al. 2009). More intense analyses including more markers found distinct anti-predatory secondary metabolites in distinct cryptic lineages, thereby supporting that defence against predation was a (differential) mechanism leading to adaptive speciation (Wilson et al. 2013). Those results already show that recently diverged inhabitants of the Southern Ocean benthos represent

suiteable evolutionary test cases to address the possible impacts of selection in lineage diversification.

One taxon consisting of a remarkable amount of recently diverged lineages are pycnogonids (Mahon et al. 2008; Krabbe et al. 2010; Weis and Melzer 2012; Weis et al. 2014; Dietz et al. 2015; Dömel et al. 2015, 2017). Pycnogonids, also called sea spiders, are a group of exclusively marine arthropods that are especially diverse in the Southern Ocean (Aronson et al. 2007), showing high endemism to this region (Munilla and Soler-Membrives 2009). Thus, sea spiders are characteristic representatives of the Southern Ocean benthos suitable to study drivers of recent speciation processes under the extreme conditions in Antarctica. A prominent example of a sea spider species complex is *Colossendeis megalonyx* Hoek, 1881. *C. megalonyx* is one of the most broadly distributed sea spiders in the Southern Ocean (Griffith et al. 2011; Dietz et al. 2015). It occurs in shallow and deep Antarctic and sub-Antarctic benthic habitats as well as around South America, South Africa and Madagascar (Munilla and Soler-Membrives 2009). The feeding spectrum seems to be broad and even pelagic invertebrates are frequently consumed (Moran et al. 2018). However, due to the fact that many cryptic species are known investigations of food preference have to be specified for each lineage before the above mentioned observations can be generalized (Dietz et al. 2018). Furthermore, especially the reproduction mode remains a mystery, as no larval stages for *Colossendeis* have ever been reported (Arnaud and Bamber 1988). Previous studies found several cryptic lineages within *C. megalonyx* (Krabbe et al. 2010; Dietz et al. 2015). Depending on the marker those studies looked at, the proposed number of delineated lineages ranged from 15 to 20 (clade A to O) for the mitochondrial cytochrome c oxidase subunit I (COI) and six (I to VI) for the highly variable nuclear internal transcribed spacer region (ITS) for over 300 analysed specimens (Dietz et al. 2015). Also, the nuclear gene for histone H3 was tested for a subsample of specimens and revealed similar groupings as proposed for ITS (Krabbe 2010). At the same time, comparisons of trees reconstructed from nuclear versus mitochondrial markers revealed mito-nuclear discordance in *C. megalonyx* (Dietz et al. 2015). This was explained by speciation reversals induced by hybridisation between members of formerly distinct mitochondrial clades. While recombination leads to gradual homogenization of nuclear genomes in hybridizing lineages, the deep mitochondrial divergences remain in the absence of recombination in mitochondrial DNA. Distribution ranges differed between clades with some being local but many others being circum-polarly distributed and members of several lineages occur in sympatry (Dietz et al. 2015). Here the question arises, how coexistence is maintained, and ecological character displacement to minimize interspecific competition and allow for stable co-existence is expected. Previous work already reported morphological differences for some of the

mitochondrial clades, e.g. specimens of clade C lack pigmented eyes, and specimens of clade F are larger than others (Krabbe et al. 2010; Dietz et al. 2015). Hence, *C. megalonyx* offers an ideal test case to investigate the impact of ecological divergence and the action of positive, divergent selection for recently diverged species that occur in sympatry.

New genomic tools allow testing for neutral against adaptive divergence. With the establishment of high-throughput sequencing technologies and bioinformatic programs, these tools become accessible even for non-model species (e.g. Faircloth et al. 2012; Lemmon et al. 2012; Hugall et al. 2015; Mayer et al. 2016; Weiss et al. 2018; Breinholt et al. 2018). Although full genome sequencing followed by annotation and detailed analysis is the ideal method to perform genomic analyses and test for selection (e.g. Ellegren 2014; Nater et al. 2015), the high costs for high-quality genomes of multiple samples limit its application. Other, less expensive approaches are transcriptomic analyses (e.g. Morin et al. 2008; Lemer et al. 2015), yet the downside for studies on organisms from remote marine habitats is that often no material with well-preserved RNA is available. A suitable alternative is target hybrid enrichment (Faircloth et al. 2012), which allows capturing hundreds of target genes by hybridizing genomic DNA against specifically designed probes, so-called baits. Another advantage of target hybrid enrichment is that low quality and quantity of DNA are sufficient and therefore the method can also be applied to degenerated samples (Mayer et al. 2016; Wood et al. 2018). This makes this technique a good candidate to be applied for Southern Ocean benthos studies, where the limitation of well-preserved material is obvious. First studies have shown that the technique can offer new insights into the phylogeny and diversification of non-target species (e.g. Abdelkrim et al. 2018; O'Hara et al. 2019). Also population genetic patterns within lineages can be assessed especially when including co-enriched flanking regions of targeted genes (Dömel et al. in press). Furthermore, the method explicitly targets coding regions, and sequences of these can be analyzed for signatures of selection, e.g. through dN/dS tests (Teasdale et al. 2016).

In this study, we examined the power of target hybrid enrichment to explore drivers of speciation in the recently diverged *C. megalonyx* species complex. Specifically, we aimed to i) comprehensively resolve species diversity within the complex, which is still under debate, ii) explore intraspecific connectivity for a subset of lineages, and iii) test for positive selection in the different lineages. Data were complemented by new morphological data to search for significant differences between molecularly separated lineages and to explore whether morphological characters also hint at selection favouring divergent phenotypes of genetically distinct species in sympatry. Finally, results were compared to a study of another sea spider

species complex *Pallenopsis patagonica* (Hoek, 1881) that addressed similar questions (Dömel et al. in press).

## 2. Material and methods

### 2.1. Material

For genetic analyses of *Colossendeis megalonyx,* a sub-set of specimens analyzed in Dietz et al. (2015) was selected including individuals from the Antarctic continental shelf and waters around sub-Antarctic and Patagonian islands (Table 1). Furthermore, new samples from the South Sandwich Islands were included. Specimens belonged to seven different mitochondrial clades (A-F and I) and five nuclear groups (I-IV and VI) after Dietz et al. (2015). To test if sympatrically occurring lineages show marked differences in genes due to positive selection, we focused especially on specimens from the two mitochondrial clades D1 and E1, which are reported to be sympatric in East Antarctica (Dietz et al. 2015). Hence, 50 individuals of those two clades from three different locations were included in this study. In total, 64 samples were analyzed using target hybrid enrichment (Figure 1).

We also included morphological measurements of 103 specimens from ten mitochondrial clades. Here, in addition to the genetic dataset, clades G, N and O were analyzed.

### 2.2. Methods

#### 2.2.1. Target hybrid enrichment

For target hybrid enrichment, a specific bait set designed for *C. megalonyx* following the workflow described by Mayer et al. (2016) based on an assembly of transcriptomic data of *C. megalonyx* was used. It included a total number of 12,014 baits covering 3,682 bait regions from 1,607 eukaryotic ortholog genes (EOGs), i.e. putative single-copy genes (see Dietz et al. 2019 for details and bait sequences). Baits and target enrichment kit were ordered from Agilent Technologies (Waldbronn, Germany).

For sample preparation, the DNA extracts from Dietz et al. (2015) were used when possible (see methods in Dietz et al. 2015 for DNA extraction details). For DNA samples with low quantity (for enrichment 100 ng per 10 µl sample were needed) and newly included individuals, DNA was extracted following the salt precipitation protocol after Sunnucks and Hales (1996; see Weiss and Leese 2016). For all DNA samples, RNase digestion was conducted using 1 µl RNase A per 50 µl DNA sample. Purification was performed using the NucleoSpin Gel and PCR Clean-up kit (Macherey and Nagel, Düren, Germany) and DNA were subsequently eluted in 10 µl HPLC. 1 µl DNA was used to measure concentration with the Qubit Fluorometer using

**Table 1: Specimens list for the *Colossendeis megalonyx* species complex used during study.** Sampling details (location, latitude, longitude, depth, cruise), mitochondrial clade assignment, voucher number for Bavarian State Collection (ZSM) and GenBank as well as use for genomic and/or morphometric analyses.

| ID | Location | Lat | Lon | Depth [m] | Cruise | COI Clade | ZSM-Voucher Number | COI GenBank Number | Genomic analyses | Morphometric analyses |
|---|---|---|---|---|---|---|---|---|---|---|
| #87-21 | | | | | | A | ZSM-A20171215 | KT201823 | | x |
| 41BT22-1 | South Georgia | -54.22 | -36.53 | 230 | ICEFISH | A | ZSM-A20171302 | KT201815 | x | x |
| 76OT50-1 | Bouvet Island | -54.64 | 3.30 | 648 | ICEFISH | E1 | ZSM-A20171231 | KT202070 | | x |
| 76OT50-2 | Bouvet Island | -54.64 | 3.30 | 648 | ICEFISH | E1 | ZSM-A20171230 | KT202068 | | x |
| ACE2017_830_1_3 | South Sandwich Islands | -57.16 | -26.79 | 326 | ACE 2017 | A | | | x | |
| ACE2017_962_1_1_2 | South Sandwich Islands | -59.47 | -27.33 | 130 | ACE 2017 | E1 | | | x | x |
| AGT42/164-1 | South Shetland Islands | -62.13 | -57.67 | 555 | PS42 | D1 | ZSM-A20171329 | KT202010 | | x |
| AGT42/164-4 | South Shetland Islands | -62.13 | -57.67 | 555 | PS42 | N3 | ZSM-A20171352 | KT202131 | | x |
| AGT42/175-1 | South Shetland Islands | -62.32 | -58.70 | 496 | PS42 | F | ZSM-A20171298 | | x | x |
| AGT42/175-2 | South Shetland Islands | -62.32 | -58.70 | 496 | PS42 | O | ZSM-A20171260 | KT202139 | | x |
| AGT42/175-5 | South Shetland Islands | -62.32 | -58.70 | 496 | PS42 | F | ZSM-A20171258 | KT201964 | | x |
| AGT42/175-8 | South Shetland Islands | -62.32 | -58.70 | 496 | PS42 | O | ZSM-A20171259 | KT202140 | | x |
| AGT42/175-9 | South Shetland Islands | -62.32 | -58.70 | 496 | PS42 | D1 | ZSM-A20171309 | KT202033 | | x |
| IU-2007-130 | Terre Adelie | -67.00 | 145.30 | 1352 | CEAMARC | E1 | | | | x |
| IU-2007-248 | Terre Adelie | -65.70 | 140.57 | 545 | CEAMARC | E1 | | KT202089 | x | |
| IU-2007-249b | Terre Adelie | -65.70 | 140.57 | 545 | CEAMARC | D1 | | KT202060 | x | |
| IU-2007-249g | Terre Adelie | -65.70 | 140.57 | 545 | CEAMARC | E1 | | KT202090 | x | |
| IU-2007-255 | Terre Adelie | -66.34 | 139.99 | 568 | CEAMARC | D1 | | KT202046 | x | |
| IU-2007-287 | Terre Adelie | -66.75 | 145.33 | 604 | CEAMARC | E1 | | KT202100 | | x |
| IU-2007-31b(1) | Terre Adelie | -65.87 | 144.11 | 1096 | CEAMARC | D1 | | KT202047 | x | |
| IU-2007-31b(2) | Terre Adelie | -65.87 | 144.11 | 1096 | CEAMARC | E1 | | KT202096 | x | |
| IU-2007-31b(3) | Terre Adelie | -65.87 | 144.11 | 1096 | CEAMARC | E1 | | KT202097 | x | |
| IU-2007-31b(4) | Terre Adelie | -65.87 | 144.11 | 1096 | CEAMARC | E1 | | KT202094 | x | |
| IU-2007-31b(6) | Terre Adelie | -65.87 | 144.11 | 1096 | CEAMARC | D1 | | KT202048 | x | |
| IU-2007-31b(8) | Terre Adelie | -65.87 | 144.11 | 1096 | CEAMARC | E1 | | KT202092 | x | |
| IU-2007-31b(9) | Terre Adelie | -65.87 | 144.11 | 1096 | CEAMARC | E1 | | KT202104 | x | |
| IU-2007-37d | Terre Adelie | -65.47 | 139.36 | 799 | CEAMARC | E1 | | KT202095 | x | |
| IU-2007-39 | Terre Adelie | -66.00 | 143.00 | 470 | CEAMARC | E1 | | KT202098 | x | |
| IU-2007-41a | Terre Adelie | -66.45 | 140.53 | 1204 | CEAMARC | F | | KT201965 | | x |

**Table 1: Specimens list for the *Colossendeis megalonyx*.** Continued.

| ID | Location | Lat | Lon | Depth [m] | Cruise | COI Clade | ZSM-Voucher Number | COI GenBank Number | Genomic analyses | Morphometric analyses |
|---|---|---|---|---|---|---|---|---|---|---|
| IU-2007-50 | Terre Adelie | -66.00 | 142.66 | 447 | CEAMARC | D1 | | KT202049 | x | |
| IU-2007-51b | Terre Adelie | -66.00 | 142.66 | 447 | CEAMARC | E1 | | KT202105 | x | |
| IU-2007-62(1) | Terre Adelie | -66.33 | 143.27 | 698 | CEAMARC | E1 | | KT202108 | x | |
| IU-2007-62(3) | Terre Adelie | -66.33 | 143.27 | 698 | CEAMARC | E1 | | KT202091 | x | |
| IU-2007-62(4) | Terre Adelie | -66.33 | 143.27 | 698 | CEAMARC | E1 | | KT202101 | x | |
| IU-2007-73 | Terre Adelie | -66.75 | 145.00 | 648 | CEAMARC | E1 | | KT202093 | x | |
| PA_E005 | South Sandwich Islands | -57.06 | -26.75 | 130 | ICEFISH | A | | GQ387009 | | x |
| PA_E010 | South Sandwich Islands | -58.20 | -26.10 | 400 | ICEFISH | E1 | ZSM-A20171305 | GQ387026 | | x |
| PB_E002 | South Sandwich Islands | -58.20 | -26.10 | 400 | ICEFISH | E1 | ZSM-A20171266 | GQ387026 | x | x |
| PF_E001 | Bouvet Island | -54.64 | 3.30 | 78 | ICEFISH | E1 | ZSM-A20171267 | GQ387026 | | x |
| PF_E002 | Bouvet Island | -54.64 | 3.30 | 648 | ICEFISH | C | ZSM-A20171286 | GQ387017 | x | x |
| PF_E003 | Bouvet Island | -54.64 | 3.30 | 648 | ICEFISH | E1 | ZSM-A20171288 | GQ387026 | x | x |
| PF_E004 | Bouvet Island | -54.64 | 3.30 | 648 | ICEFISH | E1 | | GQ387026 | | x |
| PF_E005 | Bouvet Island | -54.64 | 3.30 | 648 | ICEFISH | E1 | ZSM-A20171287 | GQ387026 | x | |
| PF_E006 | Bouvet Island | -54.64 | 3.30 | 648 | ICEFISH | C | ZSM-A20171210 | GQ387016 | | x |
| PF_E007 | Bouvet Island | -54.64 | 3.30 | 648 | ICEFISH | E1 | ZSM-A20171335 | GQ387026 | x | |
| PF_E009 | Bouvet Island | -54.64 | 3.30 | 648 | ICEFISH | E1 | ZSM-A20171334 | GQ387026 | x | |
| PF_E010 | Bouvet Island | -54.64 | 3.30 | 648 | ICEFISH | E1 | ZSM-A20171333 | GQ387026 | x | |
| PF_E011 | Bouvet Island | -54.64 | 3.30 | 648 | ICEFISH | E1 | ZSM-A20171350 | GQ387026 | | x |
| PG_E003 | Bouvet Island | -54.64 | 3.30 | 648 | ICEFISH | E1 | ZSM-A20171269 | GQ387026 | x | x |
| PG_E005 | Bouvet Island | -54.64 | 3.30 | 648 | ICEFISH | E1 | ZSM-A20171270 | GQ387026 | x | |
| PG_E006 | Bouvet Island | -54.64 | 3.30 | 648 | ICEFISH | E1 | ZSM-A20171271 | GQ387026 | x | |
| PG_E009 | Bouvet Island | -54.64 | 3.30 | 648 | ICEFISH | C | ZSM-A20171211 | GQ387016 | | x |
| PG_E010 | Bouvet Island | -54.64 | 3.30 | 648 | ICEFISH | E1 | ZSM-A20171285 | GQ387026 | x | x |
| PG_E011 | Bouvet Island | -54.64 | 3.30 | 648 | ICEFISH | E1 | ZSM-A20171336 | GQ387026 | x | |
| PH_E004 | Bouvet Island | -54.64 | 3.30 | 648 | ICEFISH | E1 | ZSM-A20171289 | GQ387026 | x | |
| PH_E005 | Bouvet Island | -54.64 | 3.30 | 648 | ICEFISH | E1 | ZSM-A20171342 | GQ387026 | | x |
| PH_E007 | Bouvet Island | -54.64 | 3.30 | 648 | ICEFISH | C | ZSM-A20171277 | GQ387017 | x | x |
| PH_E011 | Bouvet Island | -54.64 | 3.30 | 648 | ICEFISH | E1 | ZSM-A20171339 | GQ387026 | | x |
| PH_E012 | Bouvet Island | -54.64 | 3.30 | 648 | ICEFISH | E1 | ZSM-A20171290 | GQ387026 | | x |
| PI_E002 | Bouvet Island | -54.64 | 3.30 | 648 | ICEFISH | C | | GQ387016 | | x |

**Table 1: Specimens list for the *Colossendeis megalonyx*.** Continued.

| ID | Location | Lat | Lon | Depth [m] | Cruise | COI Clade | ZSM-Voucher Number | COI GenBank Number | Genomic analyses | Morphometric analyses |
|---|---|---|---|---|---|---|---|---|---|---|
| PI_E003 | Bouvet Island | -54.64 | 3.30 | 648 | ICEFISH | E1 | ZSM-A20171351 | GQ387026 | | x |
| PI_E005 | Bouvet Island | -54.64 | 3.30 | 648 | ICEFISH | E1 | ZSM-A20171301 | GQ387026 | x | x |
| PJ_E001 | South Shetland Islands | -61.20 | -56.02 | 148 | ICEFISH | D1 | | GQ387025 | | x |
| PJ_E002 | South Shetland Islands | -61.20 | -56.02 | 148 | ICEFISH | D1 | ZSM-A20171321 | GQ387024 | | x |
| PJ_E003 | South Shetland Islands | -61.20 | -56.02 | 148 | ICEFISH | D1 | ZSM-A20171313 | GQ387025 | | x |
| PJ_E004 | South Shetland Islands | -61.20 | -56.02 | 148 | ICEFISH | D1 | ZSM-A20171322 | GQ387025 | | x |
| PJ_E005 | South Shetland Islands | -61.20 | -56.02 | 148 | ICEFISH | D1 | ZSM-A20171323 | GQ387025 | | x |
| PJ_E006 | Burdwood Bank | -54.78 | -59.25 | 300 | ICEFISH | B | ZSM-A20171311 | GQ387013 | | x |
| PJ_E007 | Burdwood Bank | -54.78 | -59.25 | 300 | ICEFISH | B | ZSM-A20171310 | GQ387013 | | x |
| PJ_E009 | Burdwood Bank | -54.78 | -59.25 | 300 | ICEFISH | B | ZSM-A20171312 | GQ387013 | | x |
| PL_E004 | South Sandwich Islands | -58.95 | -26.45 | 120 | ICEFISH | A | ZSM-A20171220 | GQ387007 | | x |
| PM_E008 | South Shetland Islands | -62.32 | -58.70 | 491 | ICEFISH | F | ZSM-A20171256 | GQ387027 | | x |
| PM_E010 | South Shetland Islands | -62.32 | -58.70 | 496 | ICEFISH | D1 | ZSM-A20171326 | GQ387019 | | x |
| PM_E011 | South Shetland Islands | -62.32 | -58.70 | 496 | ICEFISH | D1 | ZSM-A20171274 | GQ387023 | | x |
| PN_E002 | South Shetland Islands | -61.78 | -57.50 | 322 | ICEFISH | A | ZSM-A20171216 | GQ387008 | | x |
| PN_E007 | South Shetland Islands | -61.77 | -57.53 | 343 | ICEFISH | A | ZSM-A20171300 | GQ387007 | x | x |
| PN_E008 | South Shetland Islands | -62.32 | -58.70 | 491 | ICEFISH | D1 | ZSM-A20171324 | GQ387019 | | x |
| PN_E009 | South Shetland Islands | -62.32 | -58.70 | 496 | ICEFISH | D1 | ZSM-A20171275 | GQ387022 | | x |
| PN_E010 | South Shetland Islands | -62.32 | -58.70 | 491 | ICEFISH | D1 | ZSM-A20171282 | GQ387020 | x | x |
| PN_E011 | South Shetland Islands | -62.32 | -58.70 | 491 | ICEFISH | D1 | ZSM-A20171325 | GQ387019 | | x |
| PN_E012 | South Shetland Islands | -62.32 | -58.70 | 491 | ICEFISH | F | ZSM-A20171222 | | | x |
| PO_E003 | Burdwood Bank | -52.67 | -60.27 | 202 | ICEFISH | B | ZSM-A20171319 | GQ387013 | | x |
| PO_E004 | Burdwood Bank | -52.67 | -60.27 | 202 | ICEFISH | B | | GQ387015 | | x |
| PO_E005 | Burdwood Bank | -52.67 | -60.27 | 202 | ICEFISH | B | ZSM-A20171296 | GQ387011 | x | x |
| PO_E006 | Burdwood Bank | -52.66 | -60.29 | 202 | ICEFISH | B | ZSM-A20171320 | | | x |
| PO_E007 | Burdwood Bank | -52.67 | -60.27 | 202 | ICEFISH | B | ZSM-A20171317 | GQ387010 | | x |
| PO_E008 | Burdwood Bank | -52.67 | -60.27 | 202 | ICEFISH | B | ZSM-A20171318 | GQ387014 | | x |
| PO_E009 | Burdwood Bank | -52.67 | -60.27 | 202 | ICEFISH | B | ZSM-A20171315 | GQ387014 | | x |
| PO_E010 | Burdwood Bank | -52.67 | -60.27 | 202 | ICEFISH | B | ZSM-A20171253 | GQ387013 | | x |
| PO_E011 | Burdwood Bank | -52.67 | -60.27 | 202 | ICEFISH | B | ZSM-A20171316 | GQ387010 | | x |
| PO_E012 | Burdwood Bank | -52.67 | -60.27 | 202 | ICEFISH | B | ZSM-A20171297 | GQ387012 | | x |

**Table 1: Specimens list for the *Colossendeis megalonyx*.** Continued.

| ID | Location | Lat | Lon | Depth [m] | Cruise | COI Clade | ZSM-Voucher Number | COI GenBank Number | Genomic analyses | Morphometric analyses |
|---|---|---|---|---|---|---|---|---|---|---|
| PP_E001 | Burdwood Bank | -52.67 | -60.27 | 202 | ICEFISH | B | ZSM-A20171314 | GQ387013 | | x |
| PP_E002 | Bouvet Island | -54.64 | 3.30 | 648 | ICEFISH | E1 | ZSM-A20171280 | GQ387026 | x | |
| PP_E003 | Bouvet Island | -54.64 | 3.30 | 648 | ICEFISH | E1 | ZSM-A20171338 | GQ387026 | x | x |
| PP_E004 | Bouvet Island | -54.64 | 3.30 | 648 | ICEFISH | E1 | ZSM-A20171284 | GQ387026 | | x |
| PP_E005 | Bouvet Island | -54.64 | 3.30 | 648 | ICEFISH | C | ZSM-A20171207 | GQ387016 | | x |
| PP_E006 | Bouvet Island | -54.64 | 3.30 | 648 | ICEFISH | E1 | ZSM-A20171283 | GQ387026 | x | x |
| PP_E009 | Bouvet Island | -54.64 | 3.30 | 648 | ICEFISH | E1 | ZSM-A20171281 | GQ387026 | | x |
| PQ_E001 | Bouvet Island | -54.64 | 3.30 | 648 | ICEFISH | C | ZSM-A20171276 | GQ387017 | | x |
| PQ_E002 | Bouvet Island | -54.64 | 3.30 | 648 | ICEFISH | C | ZSM-A20171208 | GQ387016 | | x |
| PQ_E003 | Bouvet Island | -54.64 | 3.30 | 648 | ICEFISH | C | ZSM-A20171209 | GQ387016 | | x |
| PQ_E004 | Bouvet Island | -54.64 | 3.30 | 648 | ICEFISH | C | ZSM-A20171206 | GQ387017 | | x |
| PQ_E005 | Bouvet Island | -54.64 | 3.30 | 648 | ICEFISH | E1 | ZSM-A20171225 | GQ387026 | | x |
| PQ_E006 | Bouvet Island | -54.35 | 3.17 | 458 | ICEFISH | E1 | ZSM-A20171226 | GQ387026 | | x |
| PQ_E009 | Bouvet Island | -54.35 | 3.17 | 458 | ICEFISH | E1 | ZSM-A20171227 | GQ387026 | | x |
| PR_E001 | Bouvet Island | -54.35 | 3.17 | 458 | ICEFISH | E1 | ZSM-A20171229 | GQ387026 | | x |
| PR_E002 | Bouvet Island | -54.35 | 3.17 | 458 | ICEFISH | E1 | ZSM-A20171228 | GQ387026 | | x |
| PS_E001 | Burdwood Bank | -52.65 | -59.22 | 129 | ICEFISH | B | ZSM-A20171272 | GQ387013 | x | x |
| PS_E002 | Burdwood Bank | -52.65 | -59.22 | 129 | ICEFISH | B | ZSM-A20171273 | GQ387014 | | x |
| PS_E003 | South Sandwich Islands | -58.95 | -26.45 | 75 | ICEFISH | A | ZSM-A20171268 | GQ387007 | x | x |
| PS_E004 | South Sandwich Islands | -58.95 | -26.45 | 75 | ICEFISH | A | ZSM-A20171217 | GQ387007 | | x |
| PS_E005 | South Sandwich Islands | -58.95 | -26.45 | 75 | ICEFISH | A | ZSM-A20171306 | GQ387007 | | x |
| PS_E006 | South Sandwich Islands | -58.95 | -26.45 | 75 | ICEFISH | A | | GQ387007 | | x |
| PS_E007 | South Shetland Islands | -62.32 | -58.70 | 491 | ICEFISH | D1 | ZSM-A20171278 | GQ387018 | | x |
| PS_E008 | South Shetland Islands | -62.32 | -58.70 | 491 | ICEFISH | D1 | ZSM-A20171279 | GQ387021 | | x |
| PS_E009 | South Shetland Islands | -62.32 | -58.70 | 491 | ICEFISH | D1 | ZSM-A20171332 | GQ387019 | | x |
| PS61_45_3 | South Shetland Islands | -60.99 | -55.19 | 196 | PS 61 | A | ZSM-A20171213 | KT201911 | | x |
| PS61_45_4 | South Shetland Islands | -60.99 | -55.19 | 196 | PS 61 | A | ZSM-A20171214 | KT201912 | | x |
| PS77_208_5_2 | Burdwood Bank | -54.55 | -56.17 | 294 | PS77 | B | ZSM-A20171293 | KT202115 | x | x |
| PS77_257_2_5_1 | Eastern Antarctic Peninsula | -64.91 | -60.65 | 158 | PS77 | N3 | ZSM-A20171308 | KT202129 | | x |
| PS77_257_2_5_2 | Eastern Antarctic Peninsula | -64.91 | -60.65 | 158 | PS77 | E1 | ZSM-A20171292 | KT202102 | x | x |
| PS77_260_6_14 | Eastern Weddell Sea | -70.84 | -10.60 | 252 | PS77 | I | ZSM-A20171294 | KT201735 | x | x |

**Table 1: Specimens list for the *Colossendeis megalonyx*.** Continued.

| ID | Location | Lat | Lon | Depth [m] | Cruise | COI Clade | ZSM-Voucher Number | COI GenBank Number | Genomic analyses | Morphometric analyses |
|---|---|---|---|---|---|---|---|---|---|---|
| PS77_260_6_15 | Eastern Weddell Sea | -70.84 | -10.60 | 252 | PS77 | I | ZSM-A20171307 | KT201749 | | x |
| PS77_260_6_17 | Eastern Weddell Sea | -70.84 | -10.60 | 252 | PS77 | I | ZSM-A20171295 | KT201736 | | x |
| PS77_260_6_22 | Eastern Weddell Sea | -70.84 | -10.60 | 252 | PS77 | I | ZSM-A20171247 | KT201740 | | x |
| PS77_260_6_3 | Eastern Weddell Sea | -70.84 | -10.60 | 252 | PS77 | G | ZSM-A20171252 | KT201805 | | x |
| PS77_260_6_7 | Eastern Weddell Sea | -70.84 | -10.60 | 252 | PS77 | G | ZSM-A20171223 | KT201807 | | x |
| PS77_260_6_8 | Eastern Weddell Sea | -70.84 | -10.60 | 252 | PS77 | I | ZSM-A20171240 | KT201746 | | x |
| PS77_260_6_9 | Eastern Weddell Sea | -70.84 | -10.60 | 252 | PS77 | I | ZSM-A20171248 | KT201784 | | x |
| PS77_286_1_3_2 | Eastern Weddell Sea | -70.84 | -10.60 | 248 | PS77 | N2 | ZSM-A20171255 | KT202124 | | x |
| PS77_286_1_5 | Eastern Weddell Sea | -70.84 | -10.60 | 248 | PS77 | F | ZSM-A20171254 | KT201957 | | x |
| YPM48435-1 | South Orkney Islands | -60.40 | -46.76 | 280 | Yale | I | | KT201775 | x | |
| YPM48435-2 | South Orkney Islands | -60.40 | -46.76 | 280 | Yale | D1 | | KT201973 | x | |
| YPM48435-3 | South Orkney Islands | -60.40 | -46.76 | 280 | Yale | D1 | | KT202024 | x | |
| YPM48435-4 | South Orkney Islands | -60.40 | -46.76 | 280 | Yale | D1 | | KT201977 | x | |
| YPM48435-5 | South Orkney Islands | -60.40 | -46.76 | 280 | Yale | D1 | | KT202012 | x | |
| YPM48435-7 | South Orkney Islands | -60.40 | -46.76 | 280 | Yale | D1 | | KT201979 | x | |
| YPM48435-8 | South Orkney Islands | -60.40 | -46.76 | 280 | Yale | D1 | | KT201983 | x | |
| YPM48435-9 | South Orkney Islands | -60.40 | -46.76 | 280 | Yale | D1 | | KT202050 | x | |
| YPM48435-15 | South Orkney Islands | -60.40 | -46.76 | 280 | Yale | D1 | | KT202011 | x | |
| YPM48435-17 | South Orkney Islands | -60.40 | -46.76 | 280 | Yale | D1 | | KT201980 | x | |
| YPM48435-23 | South Orkney Islands | -60.40 | -46.76 | 280 | Yale | D1 | | KT201975 | x | |
| YPM48435-27 | South Orkney Islands | -60.40 | -46.76 | 280 | Yale | D1 | | KT201984 | x | |
| YPM48435-38 | South Orkney Islands | -60.40 | -46.76 | 280 | Yale | D1 | | KT201967 | x | |
| YPM48435-39 | South Orkney Islands | -60.40 | -46.76 | 280 | Yale | D1 | | KT201991 | x | |
| YPM48435-40 | South Orkney Islands | -60.40 | -46.76 | 280 | Yale | D1 | | KT201992 | x | |

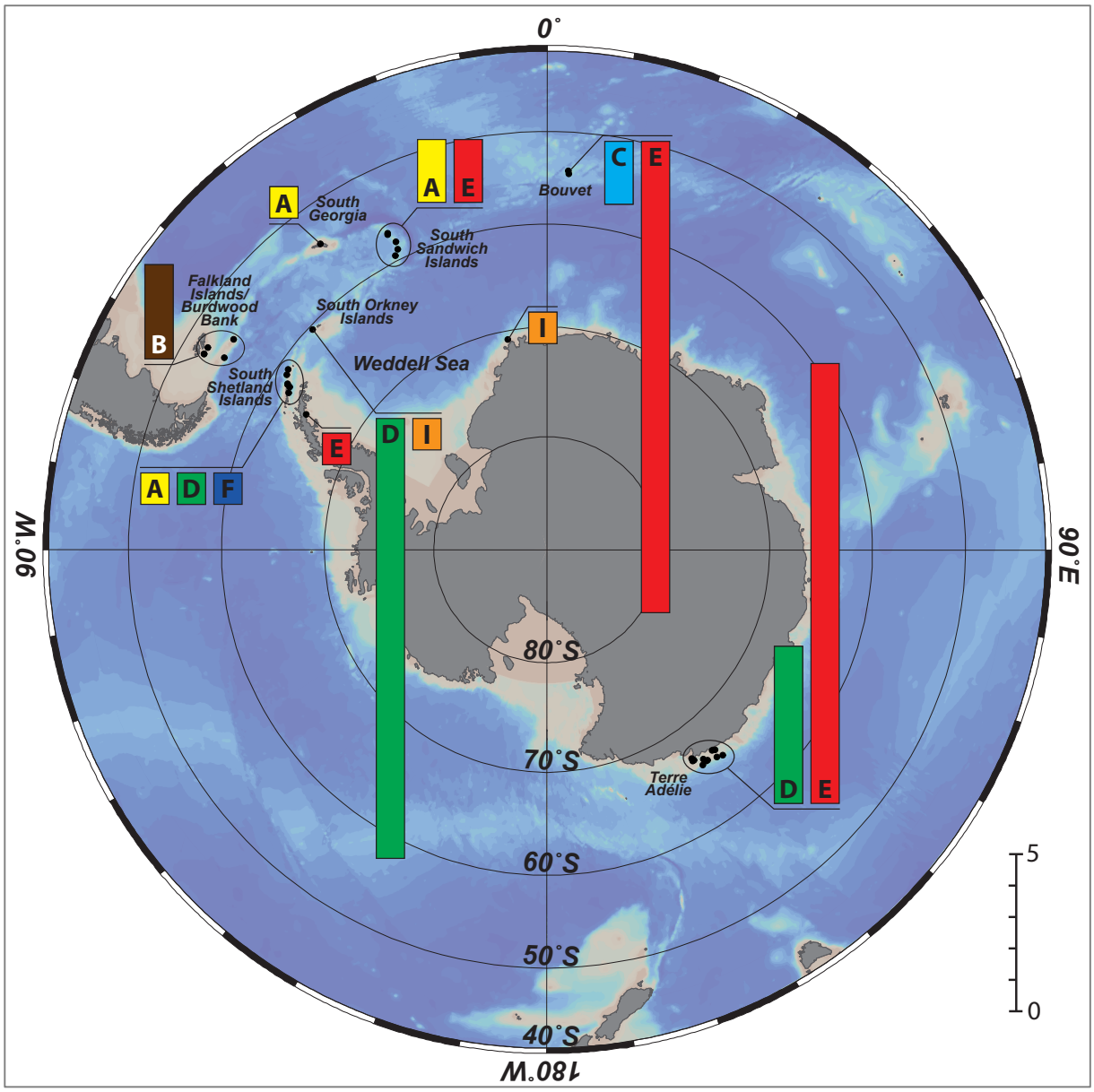


**Figure 1: Sample localities and specimens.** Black dots represent sampling sites of Antarctic and sub-Antarctic specimens of the *Colossendeis megalonyx* species complex used for genetic analyses. For each region, each COI clade (identity indicated by the letter) is represented by a single colored bar. Height of the bar represents the sample size (see the scale at lower right part).

the dsDNA BR Array Kit (Thermo Fisher Scientific, Waltham, USA). For qualitative analyzes, 2 µl DNA was used on a Fragment Analyzer (Agilent Technologies, Santa Clara, USA) using the Standard or High Sensitivity Genomic DNA kits (DNF-487-33/ DNF-487-33). Sample enrichment and sequencing details were performed described in Dömel et al. (in press). Two libraries containing 32 samples each were sent to GATC Biotech GmbH (Konstanz, Germany) for sequencing on an Illumina MiSeq platform using the V2 2x250 bp paired-end sequencing kit. 5% PhiX spike-in was added to each run to increase sequencing diversity and hence improve the signal of sequences. Upon delivery, the NGS reads were adapter- and quality-trimmed with fastq-mcf r. 488 (Aronesty, 2011). The raw data are available from the NCBI

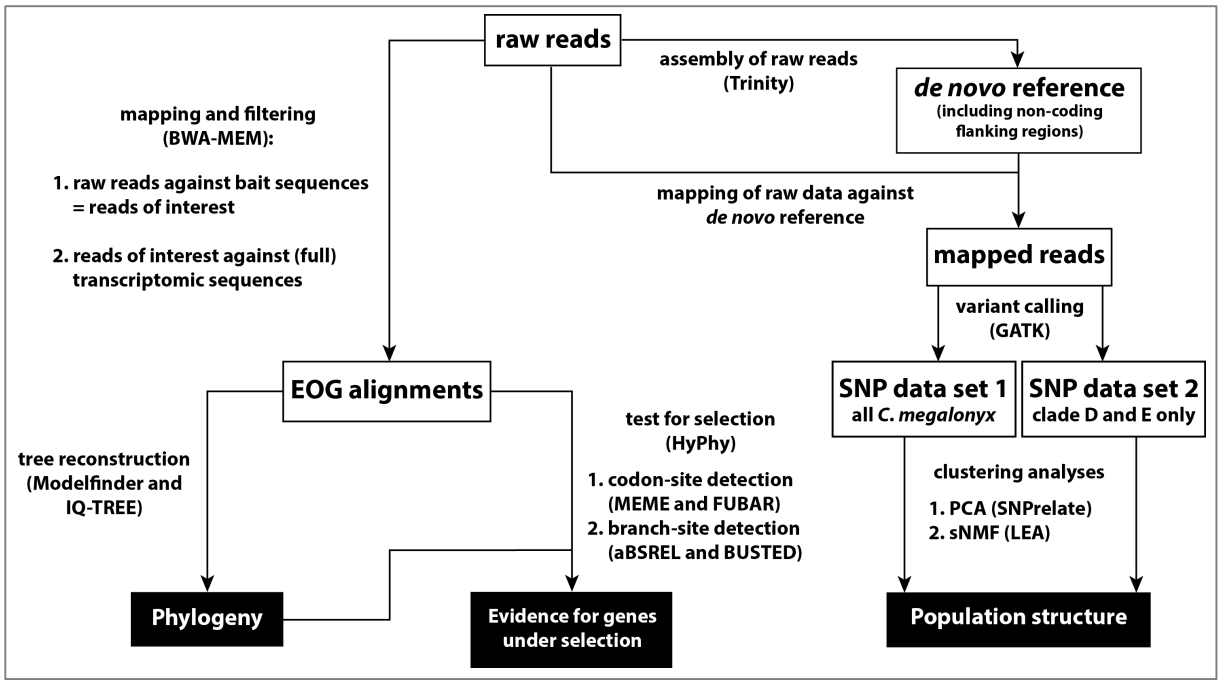


**Figure 2: Flow chart of bioinformatics analyses starting from target enrichment raw reads.** Overview of analyses conducted using target hybrid enrichment data generated during this study.

Sequence Read Archive (SRA: BioProject ID PRJNA545212). In the following, we used two complementary approaches to construct data sets for different purposes (see Figure 2 for an overview). First, EOGs only were used to infer a robust species phylogeny based on orthologous regions as well as to search for signatures of selection (dN/dS tests). Secondly, two data sets were designed using a single nucleotide polymorphisms (SNPs) approach.

### 2.2.2. Tree reconstruction and test for selection

For details about phylogenetic tree reconstruction and subsequent tests for selection, see Dömel et al. (in press). The raw reads were mapped against the bait sequences using BWA-MEM algorithm with bwa v. 0.7.17 (available from http://bio-bwa.sourceforge.net) and then reads for which mapping was successful were mapped against the full coding region obtained from the *C. megalonyx* transcriptome.

A maximum likelihood phylogenetic analysis was performed on the concatenated alignment with IQ-TREE v. 1.5.4 (Nguyen et al. 2015) using ultrafast bootstrapping with 1000 replicates for estimating nodal support. The most likely model of evolution was selected with ModelFinder (Kalyaanamoorthy et al. 2017), a tool integrated into IQ-TREE. The alignment was partitioned by codon positions, and the optimal partitioning scheme was selected with the algorithm implemented in ModelFinder (Chernomor et al. 2016). For rooting the phylogenetic tree, enrichment data of one individual each of *C. angusta* Sars, 1877 and *C. scotti* Calman, 1915 already used in Dietz et al. (2019) were included as outgroup as that study already showed

that those two species were suitably outgroups to root the phylogeny within *C. megalonyx*. Additionally, a filtered alignment, excluding positions present in less than half of the samples, was generated and another phylogenetic tree was calculated as described above to serve as background information to test for selection.

Further, selection analyses were conducted with HyPhy v. 2.3.13 (Kosakovsky Pond et al. 2005) using default settings recommended by the authors. Evidence for selection for each codon site was tested with a 'Fast, Unconstrained Bayesian AppRoximation' (FUBAR; Murrell et al. 2013). FUBAR assumes that the selection pressure is constant along the entire phylogeny. In addition to FUBAR, we also used the 'mixed effects model of evolution' (MEME; Murrell et al. 2012) approach. MEME can detect episodic selection, i.e. sites evolving under positive selection for a subset of the data set. Genes with codons reported under selection by both methods (FUBAR: $pp \geq 0.99$; MEME: $p \leq 0.01$) were further used to identify branches of the phylogenetic tree (rather than sites within the alignment) under positive selection. Therefore, the 'Branch-Site Unrestricted Statistical Test for Episodic Diversification' (BUSTED; Murrell et al. 2015) at a minimum of one site or branch of a gene was applied using these genes. Finally, the 'Adaptive Branch-Site Random Effects Likelihood' (aBSREL; Smith et al. 2015) model was used to test for each branch whether a subset of sites has evolved under positive selection. Here, both terminal and internal branches were tested.

Genetic GTR distance was calculated in PAUP* v. 4.0a165 (Swofford 2003).

### 2.2.3. Gene Ontology

Genes potentially under selection reported by FUBAR and MEME were functionally characterised using Gene Ontology (GO) categories. Therefore, the sequence of each gene from the *C. megalonyx* transcriptome was analysed using Blast2GO (Götz et al. 2008). First, a blastx-fast search using the NCBI metazoan database for genes was conducted for gene identification, allowing an E-Value of $10^{-5}$. Subsequently, hits were used for annotation and assignment of GO terms for each gene. GO-terms were further analysed with REVIGO (Supek et al. 2011), to reduce and combine closely related gene terms and visualise results. Fisher`s Exact test was conducted using Blast2GO with a false discovery rate of $\leq 0.05$ to test whether GO-terms of genes potentially under selection were significantly enriched compared to all genes analysed.

### 2.2.4. SNP analyses

Variant calling was conducted as described in Dömel et al. (in press). In brief, a reference assembly based on all raw reads from the samples of *C. megalonyx* was generated with Trinity v. 2.5.1 (Grabherr et al. 2011). Non-coding and more variable flanking regions were included

in the data set. Trimmed raw reads of all samples were mapped to the reference assembly using BWA mem (Li 2013) and processed with samtools v. 1.6 (Li et al. 2009; Li 2011). Variant calling was conducted with HaplotypeCaller from the GATK v. 4.0.3.0 package (McKenna et al. 2010). Variant calling was performed for two separate data sets: i) all *C. megalonyx* samples (SNP data set 1, Figure 2), and ii) sample from clade D1 and clade E1 (excluding PS77_257_2_5_2) only (SNP data set 2). The latter was done specifically to obtain SNPs for population genetic analyses (aim ii in the introduction).

To analyze the genetic structure, principal component analyses (PCA) were conducted using the R-package SNPRelate v. 1.12.2 (Zheng et al. 2012) with default parameters. For SNP data set 2, plots of the sparse nonnegative matrix factorization (sNMF) were calculated to get proportions of the degree of admixture between clades, using the LEA package v. 2.0.0 (Frichot and Francois 2015). A range of K values (number of ancestral populations) in the interval of 1-20 was tested. The number of repetitions per K was set to 40 with 40,000 iterations. The lowest cross-entropy per K value was determined and plotted to choose the most likely K value.

### 2.2.5. Morphology

Morphometric measurements of *C. megalonyx* specimens were carried out using a digital calliper (MarCal IP67, Mahr Metrology, Germany). In total, 133 characters were measured (see Supplementary material 1 for a list of characters).

To test for significant differences of morphological characters between mitochondrial clades, nonparametric unifactorial Kruskal-Wallis H test in combination with Dunn's post hoc test (Bonferroni-corrected) were used in Past v. 3.18 (Hammer et al. 2001).

As the morphometric data set included many missing values, due to articles that broke off during storage, further analyses were based on characters and individuals that had a maximum of 10% missing values. Remaining missing values were imputed using Predictive Mean Matching. Furthermore, only clades with a minimum of three individuals were kept, and clades G, N and O were excluded from analyses. In addition, relative lengths of all measurements were expressed as a proportion of the trunk length of each specimen. Consequently, two reduced data sets consisting of 92 individuals each were analysed. Number of characters included was 44 for actual values of measurements (left and right averaged) and 43 for relative lengths. A selection of characters for Linear Discriminant Analysis (LDA)was performed to avoid model overfitting. The heuristic search for the optimal sets of characters was carried out by iteratively using the stepclass function from the R package klaR v. 0.6-14 with forward-backwards selection, cross-validation correctness rate as the optimality criterion. The search was organised by picking each of the characters as the starting variable and repeating the procedure ten times.

The performance of the character sets was recorded, and the best set was used for a final LDA which was performed using Past.

Morphological distances between mitochondrial clades were calculated as Δp (Safran et al. 2012) using MATLAB R2018b (The MathWorks, Natick, 2014).

## 3. Results

### 3.1. Enrichment and genomic analyses

Sequencing of target hybrid enrichment libraries of specimens of the *Colossendeis megalonyx* species complex resulted in an average number of 426,047 (standard deviation (SD) 67,634) reads per individual. All 1607 EOGs were recovered. The average number of recovered genes per individual was 1603.13 genes (99.7%, SD 18%). All genes were found in at least 49 individuals (51 when the outgroups were included). On average, each gene was recovered in 63.75 individuals (99.6%). The shortest gene alignment was 60 bp and the longest 8103 bp.

The length of the concatenated alignment was 1,078,695 bp and it contained 15.9% missing data (ranging from 11.24% to 47.8%). In the optimal partitioning scheme selected by ModelFinder, all three codon positions were treated as separate partitions. The best model of evolution according to BIC was GTR+R2 for first and second codon positions and GTR+R3 for third codon positions. The phylogenetic tree (Figure 3) supported the mitochondrial COI clades identified by Dietz et al. (2015) except for clades D1 and E1. Clade D1 was recovered as paraphyletic concerning most specimens of clade E1. Clade E1 was polyphyletic, as it was divided into two major branches that grouped in different parts of the tree. One branch was represented by one individual from the Eastern Antarctic Peninsula (PS77_257_2_5_2) only. This individual belongs to the nuclear ITS group III, in contrast to the other samples from clade E1 analysed which were assigned to ITS group II. However, also clade C was assigned to ITS group II, but is clearly separated from the other specimens of this group (clades D1 and E1).

The tree presented herein recovered the larger groupings A+F+I, B+C and D+E, which were consistent with the COI tree (Dietz et al. 2015). Within the specimens belonging to the mitochondrial clades D1 and E1, three geographically restricted clusters per clade were identified. All geographic clusters are maximally supported with bootstrap values of 100%. Nearly all Scotia Arc individuals formed the most basal group of clade D1. Within that group, the single individual from Elephant Island (PN_E010) grouped outside those from the South Orkney Islands (n=13). A single individual from the South Orkneys (YPM48435-9), however, clustered with individuals from Terre Adélie (n=5). Within clade E1, individuals from Terre

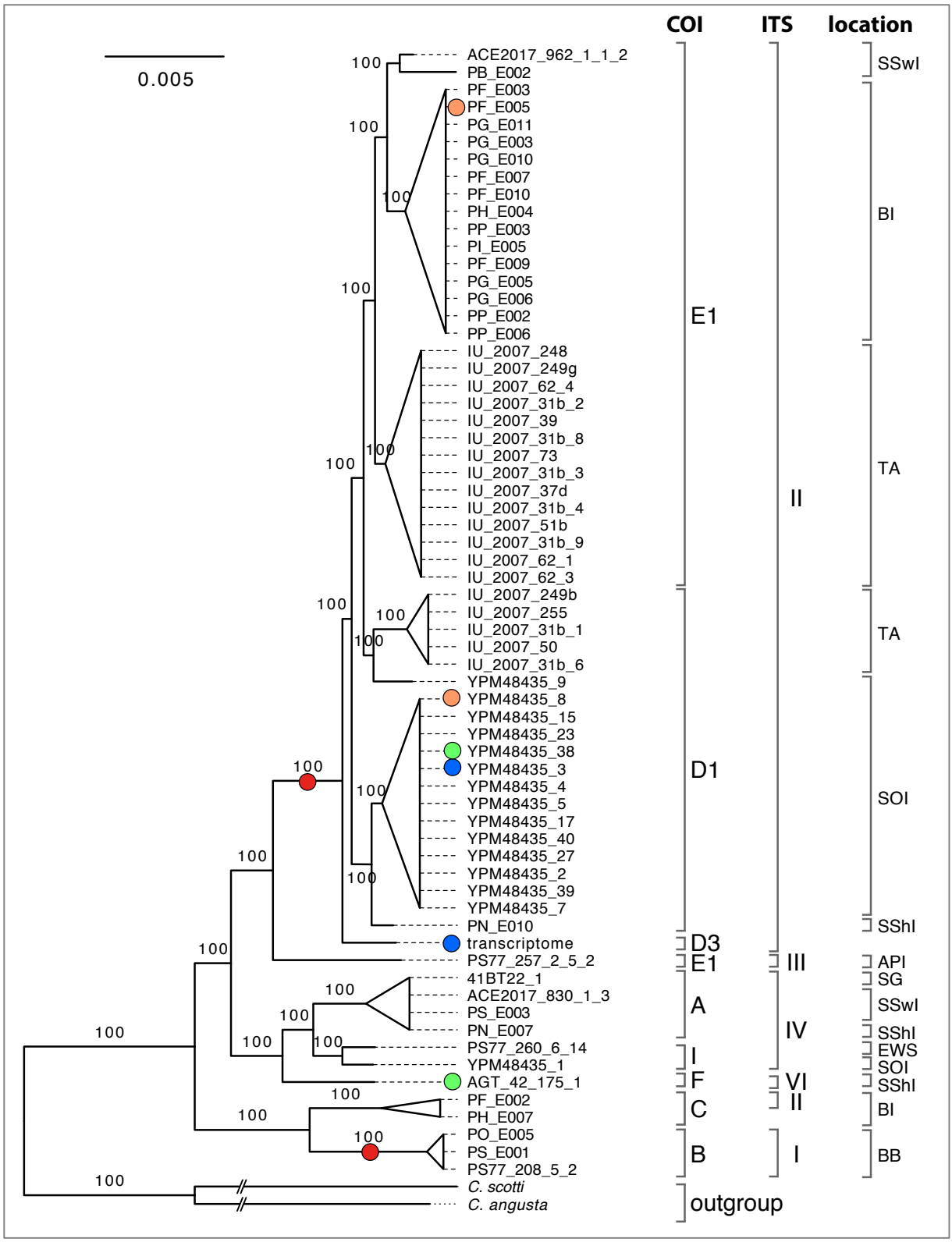


**Figure 3: Maximum-likelihood tree based on concatenated EOG sequences of all genetic samples of the *Colossendeis megalonyx* species complex.** Affiliation to COI and ITS groups (Dietz et al. 2015) as well as geographical sampling location are shown on the right side (API: Antarctic Peninsula; BI: Bouvet; BB: Burdwood Bank; EWS: Eastern Weddell Sea; SG: South Georgia; SOI: South Orkney Islands; SShI: South Shetland Islands; SSwI: South Sandwich Islands; TA: Terre Adélie). Coloured dots highlight branches for which genes under selection were found with aBSREL and BUSTED. Each colour represents one gene found to be under selection (green: EOG091000CK; blue: EOG0910000D; orange: EOG0910003N; red: EOG0910019Q).

**Table 2:** Morphometric (upper right) and genomic (lower left) distances between lineages of the *Colossendeis megalonyx* species complex represented as heat maps (red: high distances; blue/green: small distances). n: number of individuals analyzed for each clade and data set.

| | E (n=28) | D (n=15) | A (n=13) | I (n=6) | F (n=8) | C (n=10) | B (n=17) |
|---|---|---|---|---|---|---|---|
| E1 (n=31) | | 95.57 | 107.12 | 369.95 | 151.79 | 72.69 | 72.21 |
| D (n=17) | 0.0027 | | 149.87 | 219.12 | 134.60 | 72.86 | 131.62 |
| A (n=4) | 0.0091 | 0.0091 | | 109.61 | 213.49 | 114.63 | 72.01 |
| I (n=2) | 0.0085 | 0.0085 | 0.0040 | | 276.43 | 179.92 | 118.31 |
| F (n=1) | 0.0092 | 0.0091 | 0.0060 | 0.0055 | | 124.19 | 186.58 |
| C (n=2) | 0.0116 | 0.0115 | 0.0119 | 0.0113 | 0.0118 | | 94.07 |
| B (n=3) | 0.0126 | 0.0126 | 0.0126 | 0.0121 | 0.0125 | 0.0076 | |

Adélie (n=14) were the sister group to those from the South Sandwich Islands (n=2) and Bouvet Island (n=15). Although individuals of both mitochondrial clades D1 and E1 occur in Terre Adélie, they represented two separated groups in the phylogenetic tree. Genetic distances between clades ranged from 0.0027 between clade D and E to 0.0126 between B and E/D/F (Table 2).

Variant calling for SNP data set 1 (all *C. megalonyx*) obtained 13,611 SNPs. PCA of those data had 14 significant axes and showed differentiation into five groups, i.e. clades A, B, F, I, and a group consisting of clades D1 and E1 (excluding PS77_257_2_5_2) (Figure 4A). The remaining individuals, two of clade C and PS77_257_2_5_2 (clade E1), did not cluster with the other groups or each other. For SNP data set 2 (clade D1 and E1 only) 14,904 SNPs were found. PCA of those data revealed six significant axes and showed differentiation into eight groups that mostly show a geographic separation (Figure 4B). The sNMF analysis supported the results of the SNP-PCA and reported K=4 as the most likely number of clusters (Figure 5A).

Both mitochondrial clades D1 and E1 were further subdivided into two geographical groups (Figure 5B). For clade E1, individuals from Terre Adélie and Bouvet Island showed a high proportion of one ancestral population each. Individuals from the South Sandwich Islands showed a high admixture of both of those ancestral populations. For clade D1, individuals from the South Orkney Islands and Terre Adélie showed a high proportion of one ancestral population each. One specimen from the South Shetland Islands was mostly assigned to the same ancestral population as those from the South Orkney Islands but also showed a small proportion of ancestry shared with individuals from Terre Adélie of clade E1. One individual from the South Orkney Islands (YPM48435-9) was assigned in similar proportions to all four ancestral populations. This pattern (a similar proportion to all ancestral populations) remained constant with increasing K. Only at a value of K=11 the individual was assigned a separate population.

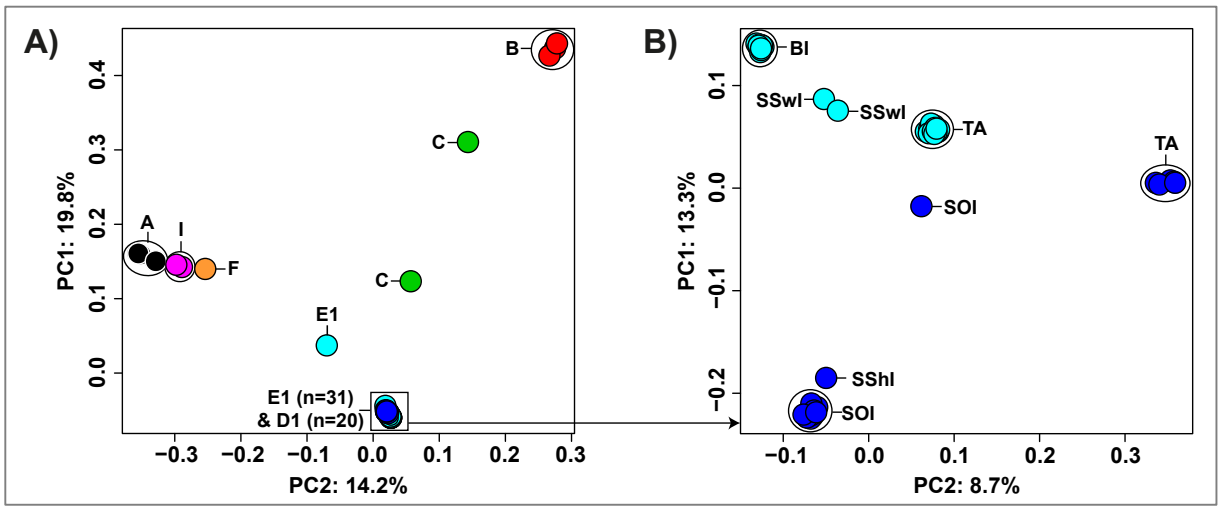


**Figure 4: PCA from genomic data of the *Colossendeis megalonyx* species complex.** PCA plots based on genomic data of A) all samples and B) samples of the clades D1 (blue) and E1 (aqua) with the latter excluding specimen PS77_257_2_5_2 that was assigned to another ITS group in Dietz et al. (2015). API: Antarctic Peninsula; BI: Bouvet; BB: Burdwood Bank; EWS: Eastern Weddell Sea; SG: South Georgia; SOI: South Orkney Islands; SShI: South Shetland Islands; SSwI: South Sandwich Islands; TA: Terre Adélie.

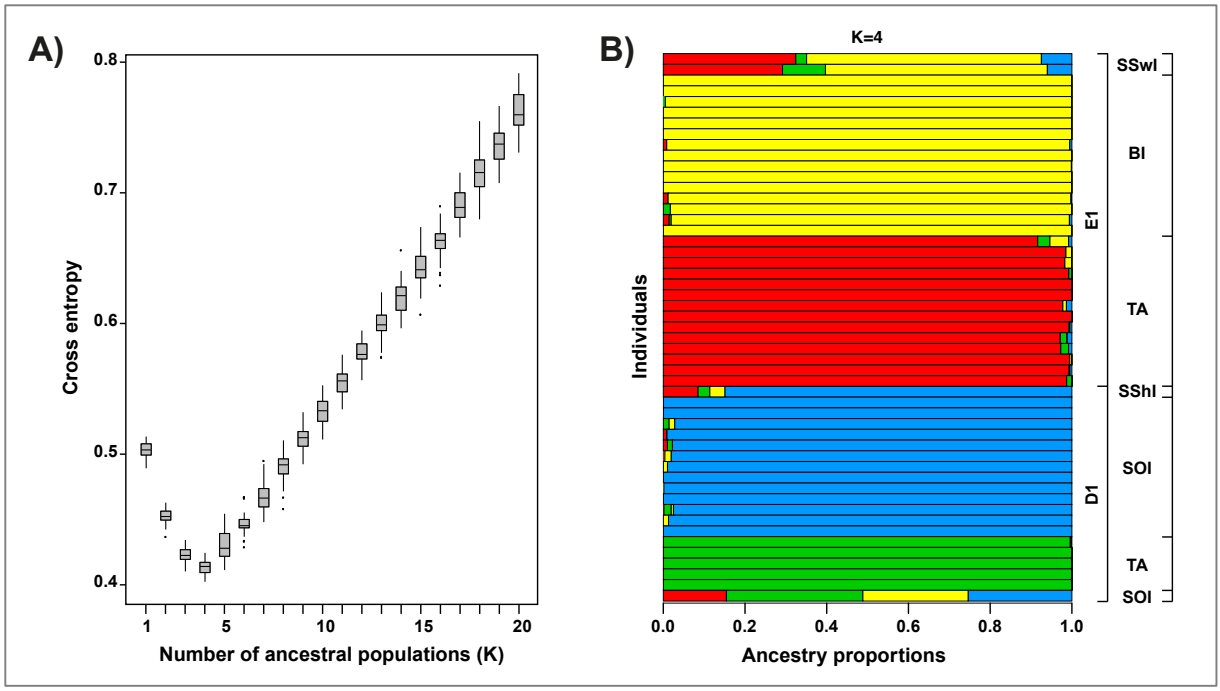


**Figure 5**: **sNMF analyses of individuals from clades D1 and E1.** A) Cross-entropy estimates of 1 to 20 ancestral populations (K value). B) Graphical illustration of ancestry proportion estimates with K = 4. Estimated ancestry proportions for each specimen are represented by horizontal bars. Clade assignment and sampling location are shown on the right side (BI: Bouvet; SOI: South Orkney Islands; SShI: South Shetland Islands; SSwI: South Sandwich Islands; TA: Terre Adélie).

### 3.2. Genes under selection

FUBAR and MEME identified 473 codons in 342 genes and 199 codons in 126 genes, respectively, to be under selection. Of these, 139 codons and 124 genes were shared between the two methods. GO terms were assigned to 93 of the 124 genes under selection. Blast2GO found 265 hits, of which 84 were relevant for biological processes (P), 79 for cellular components (C), and 102 for molecular functions (F). Hits resulted in 119 different GO-terms that were further combined to 107 GO-terms (P: 39; C: 21; F: 47) by REVIGO (see Supplementary material 2 and 3 for figures and tables, respectively).

When testing the 124 shared genes identified with MEME and FUBAR using BUSTED, evidence for selection in 20 genes was supported. Six genes were found to be under selection on 11 branches with the aBSREL model. Of those, six branches (3 genes) corresponded to terminal branches, and five branches were internal ones. Of the latter, only three branches (2 genes) were well supported in the phylogenetic tree (pp 100%). Four genes were found with both branch-site tests, including those where terminal branches within different clades were supposed to be under selection and one where two internal nodes were supposed to be under selection (Figure 3). The four genes found to be under selection in all selection tests were further investigated (Table 3). For three of these genes, GO-terms could be determined.

**Table 3: List of genes potentially under selection.** Details (extracted from Blast2GO) about the four genes detected to be under selection with all methods used.

| Gene ID | Amino acid position | Description (Blast x-fast) | InterPro GO IDs and names |
|---|---|---|---|
| EOG091000CK | 208, 226 | Hexosaminidase D-like | • 0004553 (F: hydrolase activity, hydrolyzing O-glycosyl compounds)<br>• 0005975 (P: carbohydrate metabolic process)<br>• 0015929 (F: hexosaminidase activity) |
| EOG0910000D | 3145, 6556 | Nesprin-1 isoform X7 | • 0005515 (F:protein binding)<br>• 0005635 (C:nuclear envelope)<br>• 0034993 (C:meiotic nuclear membrane microtubule tethering complex)<br>• 0051015 (F:actin filament binding)<br>• 0090286 (P:cytoskeletal anchoring at nuclear membrane) |
| EOG0910003N | 166 | SLIT-ROBO Rho GTPase-activating protein 1-like isoform X5 | • 0005515 (F:protein binding)<br>• 0007165 (P:signal transduction) |
| EOG0910019Q | 532 | neuroglian-like isoform X2 | No GO term |

### 3.3. Morphometric analyses

Morphometric measurements were taken for 103 individuals (a table including all measurements is provided in Supplementary material 4). After the averaging of bilateral characters, the final data set consisted of 76 characters per specimen. Of those, 68 characters had significant differences between mitochondrial clades (see Supplementary material 5 for list of characters and significant p-values). The 8$^{th}$ and the 10$^{th}$ (most distal) article of the palpus and the 5$^{th}$ article of the oviger had the most differences between mitochondrial clades (n=8). The clades between which most differences were found were clades A and B (n=25) and clades C and I (n=24). Morphometric distances between clades ranged from 72.02 between A and B to 369.95 between clade E and I (Table 2).

The best character combination for the absolute lengths consisted of the trunk length, 'WL2 femur' (length of femur of second walking leg) and four palp articles (palp 3, 6, 8 and 10). For the relative lengths the best character combination consisted of 'proboscis thickest' (diameter of proboscis at thickest part) and six palp articles (palp 1, 3, 4, 6, 8, and 10) (see Supplementary material 6 for detailed result of selection analyses based on morphometric data). Cross-validation confusion rate was higher for relative lengths (0.90) in comparison to the one of the absolute lengths (0.81) (Table 4). Miss-assignment in the cross-validated confusion matrices did not exceed two, except for clade E with nine based on the absolute lengths and seven based on the relative lengths (Table 4). In LDA plots, clades were difficult to separate, except for clade I which was separated from the other clades in all plot for the absolute length that did not include the forth axis and in the plot for the relative length of the second and third axes (Figure 6).

**Table 4: Cross-validation confusion matrices for morphometric data set of the *Colossendeis megalonyx* species complex using absolute and relative lengths.**

| | **absolute** (correctness rate: 0.82) | | | | | | | **relative** (correctness rate: 0.90) | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **clade A** | **clade B** | **clade C** | **clade D** | **clade E** | **clade F** | **clade I** | **clade A** | **clade B** | **clade C** | **clade D** | **clade E** | **clade F** | **clade I** |
| **clade A** | **11** | 0 | 0 | 1 | 1 | 0 | 0 | **10** | 0 | 1 | 0 | 1 | 0 | 1 |
| **clade B** | 1 | **14** | 0 | 1 | 0 | 0 | 0 | 1 | **14** | 0 | 1 | 0 | 0 | 0 |
| **clade C** | 0 | 0 | **10** | 0 | 0 | 0 | 0 | 0 | 0 | **10** | 0 | 0 | 0 | 0 |
| **clade D** | 1 | 0 | 2 | **11** | 0 | 0 | 0 | 1 | 0 | 1 | **11** | 1 | 0 | 0 |
| **clade E** | 3 | 0 | 2 | 1 | **19** | 0 | 1 | 2 | 0 | 0 | 4 | **19** | 0 | 1 |
| **clade F** | 0 | 0 | 0 | 1 | 1 | **5** | 0 | 0 | 0 | 0 | 1 | 1 | **5** | 0 |
| **clade I** | 0 | 0 | 0 | 0 | 0 | 0 | **6** | 0 | 0 | 0 | 0 | 0 | 0 | **6** |

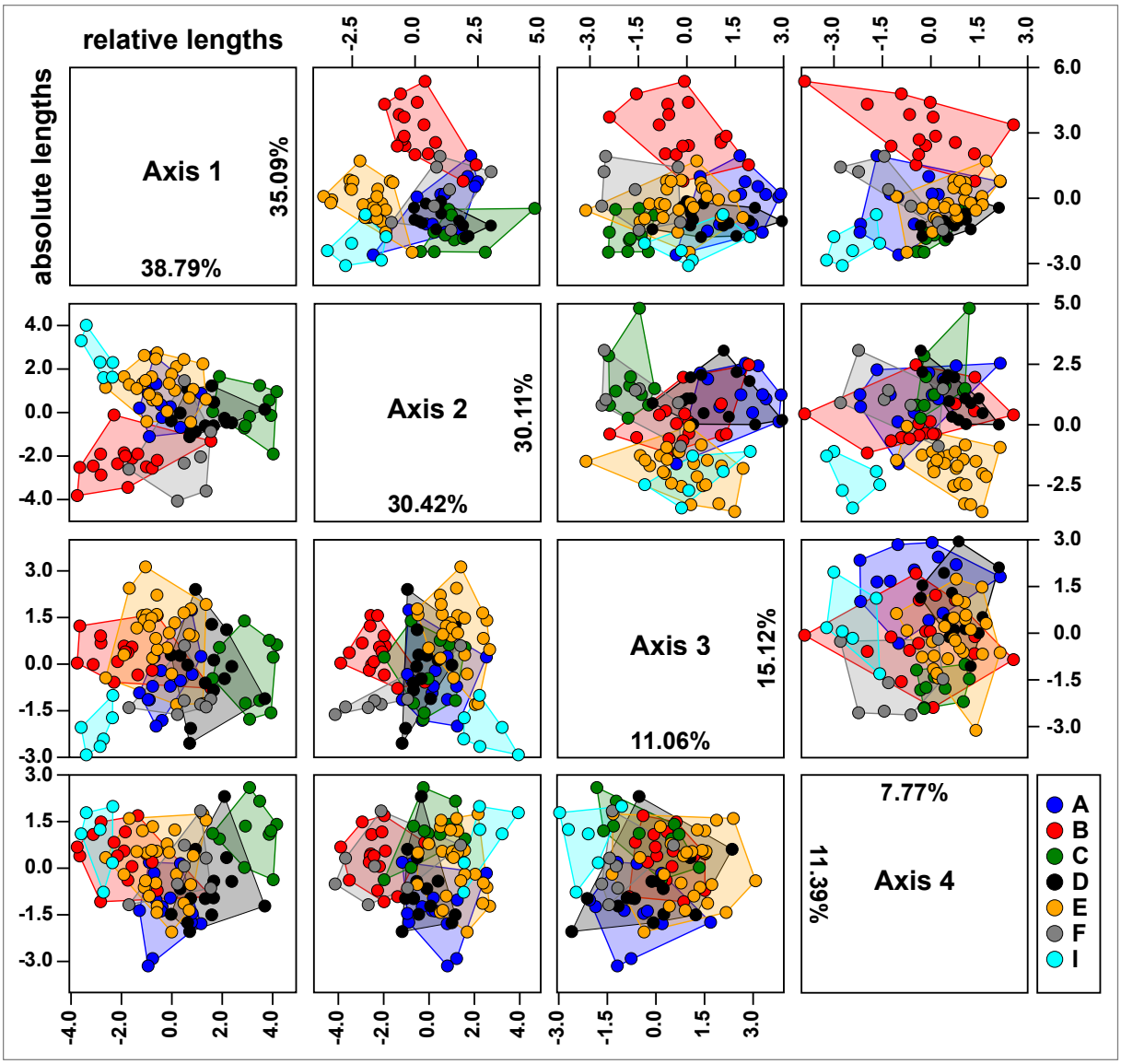


**Figure 6: LDA from morphometric data of the *Colossendeis megalonyx* species complex using the best combination for absolute and relative lengths.** LDA plots show all four axes of the same analysis conducted with the reduced morphometric data set for absolute (lower left) and relative (upper right) length each. Color code for clade assignment on the right.

## 4. Discussion

### Species diversity within the species complex *Colossendeis megalonyx*

We substantially advance the knowledge of the phylogeny of the *Colossendeis megalonyx* species complex with the genomic target hybrid enrichment data in terms of resolution and support. The topology of our tree resembled the phylogeny inferred by Dietz et al. (2015) to a large degree. For mitochondrial clade E1, there was one individual (PS77_257_2_5_2) that did not group with the other individuals assigned to clade E1. However, it was the only individual of this clade belonging to ITS group III. This mito-nuclear discordance has been reported before by Dietz et al. (2015). The ITS sequence of this individual was very similar to those of others found in the same location (Eastern Antarctic Peninsula), which belonged to the mitochondrial

clade N3. The position of clade N3 in the Bayesian mitochondrial tree by Dietz et al. (2015) was similar to that of the discussed individual in the phylogenomic tree (sister to clades D and E). This confirms that the misleading placement of PS77_257_2_5_2 in the mitochondrial tree is a result of introgression of mitochondrial DNA. Another clade with mito-nuclear discordance according to Dietz et al. (2015) was clade C, for which the ITS sequence of only one (PF_E002) of the two analysed individuals is known. In contrast to other members of clade C, PF_E002 belongs to ITS group II, that also includes clade E1 individuals from the same location. Here our results agree with the mitochondrial data, as clade C is resolved as sister to clade B, suggesting that the ITS sequence in this individual is a result of hybridization.

An additional benefit of the genomic data is the increased resolution of specimens assigned to mitochondrial clades D1 and E1. These were both assigned to ITS group II in Dietz et al. (2015). However, target hybrid enrichment data clearly show that D1 is paraphyletic with respect to E1. One possible explanation for this is that the group ancestrally had the mitochondrial DNA of clade D, and a subgroup of it acquired the mitochondrial DNA of clade E by introgression from an unknown source. In contrast to ITS data that suggest one species for D1 and E1, target hybrid enrichment data detect clear signatures of divergence even in sympatry (Terre Adélie). Trawls are often dragged for a few kilometers and potentially cover multiple habitats. However, the multiple occurrences in one trawl are most likely due to habitat sharing. As the clades D and E were distinguished by both mitochondrial and nuclear data, they are genetically clearly separated and reproductively isolated from each other. Kekkonen and Hebert (2014) stated that reproductively isolated groups that occur in sympatry represent different species (Biological Species Concept). By this criterion, the two groups should be treated as distinct species. Hence, we can assume that the benthos of Terre Adélie represents a diverse habitat offering potential for multiple niches and also served as a secondary contact zone where differentiated clades met again after allopatric divergence. Similarly, in the South Orkney Islands, two clearly differentiated lineages belonging to the mitochondrial haplogroup D1 are present according to our data. If these are seen as distinct species, our phylogeny suggests that the other geographically restricted lineages between this group are also separate species, suggesting that the number of species within the *C. megalonyx* complex may have been strongly underestimated with mitochondrial data. As not all clades included in the species complex *C. megalonyx* were analyzed within the present study, no conclusion about the actual number of species within the complex can be made. However, within the subsample used, there are already more lineages than previously detected due to further differentiation within mitochondrial clades, e.g. clade E1.

Differentiation of lineages from the species complex *C. megalonyx* using morphometric data was possible but difficult. This might be explained by high morphological variation within clades. High intraspecific morphological variation has also been reported for Antarctic nematodes (Hauquier et al. 2017). A previous study revealed that there is a high degree of sexual dimorphism within members of the different mitochondrial clades (Spaak 2010). For clades C and E, 30% of all analyzed characters differed significantly between sexes. For clades A, B and D, this value was a lot lower (4%) (Spaak 2010). We did not reveal obvious separation of male and female measurements within the present study, but the increased variability due to sexual dimorphisms might represent an issue for PCA based on morphometric analyses. However, Dietz et al. (2013) used similar characters and analyses and were able to distinguish *C. tenera* Hilton, 1943 from the species complex *C. megalonyx*. Hence, the shortage of morphological differences is most likely due to the more recent divergence of lineages within this species complex.

Due to the sampling, our results cannot clearly resolve whether *C. megalonyx* originated inside or outside the Antarctic. However, Dietz et al. (2019) recently found that the taxon is nested within a large Antarctic radiation which diversified mostly within the Antarctic, dispersing to other regions multiple times. This supports the scenario that *C. megalonyx* is originally an Antarctic taxon, containing only one clade which dispersed to South America, as indicated by the mitochondrial tree with a larger sampling within the complex (Dietz et al. 2015a).

**Intraspecific connectivity within the species complex *Colossendeis megalonyx***

Population genetic analyses using SNP data revealed that intraspecific connectivity among geographically distinct populations is restricted. But even more, our data hints at further divergence within locations. Within clade D1, one individual sampled from the South Orkney Islands, YPM48435_9 is clearly separated from the other 14 specimens of the same mitochondrial clade and location, being closely related to the Terre Adélie specimens of clade D1. In the sNMF analysis, the specimen was assigned to all four ancestral clusters with similar probability each. This can have three different reasons: i) sample contamination, ii) hybridization or iii) specimen belongs to a separate population despite the COI sequence being identical to other specimens from the South Orkney Islands. Contamination is ruled out by the heterozygosity of this individual that was not higher than in the other samples. If a DNA mixture of two (or more) specimens had occurred, fixed differences between the two specimens would have artificially increased heterozygosity. This was not the case. The individual could also be a hybrid of D1 and E1 individuals. However, hybridization between four distinct clusters, as suggested by the sNMF results, is unlikely. Furthermore, we would expect higher

heterozygosity in a hybrid specimen. Therefore, we suggest that this specimen may represent an independently evolving lineage, with the identical COI haplotype being the result of mitochondrial introgression. In Dietz et al. (2015), the samples from the South Orkney Islands already showed a high species diversity, and nine different mitochondrial clades occur in sympatry. This makes the presence of several intraspecific populations and introgression more likely. In general, the Scotia Sea has been proposed as a hotspot for speciation especially for brooding species (Allcock and Strugnell 2012 and demonstrated for the bivalve *Lissarca notorcadensis* Melvill and Standen, 1907 (Linse et al. 2007). Nothing is known about the reproduction mode of *Colossendeis*, but the limited connectivity between several populations also within proposed lineages leads to the assumption that more species than previously expected, exist within the species complex *C. megalonyx*. Only genomic data as used in this study can unveil these very shallow divergences and possible young speciation events, yet more material is needed (see below).

**Evidence of positive selection in the different lineages of *Colossendeis megalonyx***

For the first time, we explicitly searched for signatures of positive selection in the *C. megalonyx* species complex and reported evidence for this in about 8% of all genes analyzed. Genes identified covered a broad range of biological processes, cellular components and molecular functions. However, many of these genes had only rather higher-level GO-categories, i.e. assignments to specific gene functions were not possible. GO-term enrichment did not support the dominance of any particular group of functional pathways in the data set. However, it should be noted that for the design of the baits, the availability of genes was already limited to 1607 EOGs only. The latter was owed by the facts that bait design was based on a single (incomplete) transcriptome and that GO terms were based on distantly related spider genomes.

Nevertheless, while it might be difficult to assign specific functions to the genes analyzed to be under selection, the fact that the same genes were reported to be under positive selection on different branches suggests that they are candidate genes that were potentially involved in the adaptive radiation into independently evolving lineages. We focused our analyses on branches leading to distinct lineages to test for the role of positive selection rather than interpreting evidence in terminal branches. With this approach still about 0.4% of all analyzed genes were found to be under selection. Those genes included one gene (EOG0910000D) associated with nesprin that is part of the LINC (Linker of Nucleoskeleton and Cytoskeleton) complex and one gene (EOG091000CK) relevant for fundamental processes associated with hexosaminidase which plays a role in the carbohydrate metabolic process. No direct biological relevance of these two genes for speciation processes can be deduced at this stage. Another gene of interest

was EOG0910019Q as it was found to be under selection in two branches at deeper level within the phylogeny (one branch that grouped all individuals from clade D and E1 as well as another branch that grouped all individuals from clade B). BLAST search assigned this gene isoform to neuroglian, associated with neuroglia formation and thus probably relevant for the neural system. The importance of adaptation to the cold in neural systems has been shown for fish. Antarctic fishes adapted to their constantly cold environment and showed a relatively high neuronal conduction velocity also at temperatures below zero degrees Celsius (Macdonald 1981; Montgomery and Macdonald 1990). Of course, neural systems in invertebrates differ from those in vertebrates, but similar challenges to adapt to the cold can be assumed. In fact, positive selection in this gene was detected for the branch of clade B, the only clade analyzed that occurs outside the Antarctic convergence (Burdwood Bank and the Falkland Islands) where the water temperature is several degrees higher than within. However, an assumption of adaptation due to temperature cannot be made as to the clades, and E1 occur only south of the Antarctic convergence, like other analyzed clades in which no evidence for selection could be found. Hence, we refrain from more detailed discussions about functional aspects at this point because without a larger data set (sample size and several genes analyzed) and functional validation of genes, these observations are first evidence, and it is highly recommended not to start story-telling about underlying principles (Pavlidis et al. 2012). Hence, although the importance of adaptation for speciation remains unclear, it has to be considered as a relevant factor and there is urgent need to improve the data basis for such studies (see below).

Assuming that adaptive processes took place, differences in morphological traits are expected, too. Indeed, morphological differences between individuals of distinct mitochondrial clades can be detected using morphometric measurements. Characters supposed to be influenced by selection (e.g., proboscis, eyes or claws) did not stand out in terms of numbers of significant differences between clades, but if significant differences were found, they always distinguish only in one or two specific clades from the others. For example, significant differences in morphometric measurements of proboscis characters were only found between clade B and other clades. Also, significant differences in the relative claw length were found only between clade B or I and other clades. Finally, significant differences in eye structures were predominantly found between clade A and other clades. However, the clades with which significant differences were found varied between characters and did not show a clear pattern.

Other characters potentially relevant for selection that should be considered for future studies include the inner structures of the proboscis and smaller structures also of other body parts. Wagner et al. (2017) analyzed proboscides of various genera and found remarkable differences between taxa, most likely dependent on prey preferences. Also, setae and pores scattered across

the body could be of interest. Setae are found to be expressed in various forms and have sensory functions (Lehmann et al. 2017). The pores represent gland openings (Heß et al ca 1996; Lehmann et al 2017), however they also act as part of the respiratory systems and are responsible for cutaneous gas exchange. Lane et al. (2017) described limitations of body size in pycnogonids due to the need to take up sufficient amounts of oxygen. This limit, however, probably varies between species that occur inside and outside the Antarctic convergence as well as for eurybathic and stenobathic species due to different amounts of available oxygen.

**Comparing the sea spider species complexes of *Colossendeis megalonyx* and *Pallenopsis patagonica***

The *C megalonyx* species complex was compared with the sea spider species complex *Pallenopsis patagonica* based on results of single-marker analyses by Dömel et al. (2017). That study revealed that both species complexes occur in the Southern Ocean and show comparable genetic distances for the distinct lineages based on COI. A recent study on the *P. patagonica* species complex with a similar focus as this study assessed genetic divergence by conducting a target hybrid enrichment approach using the same bait set as used herein for the *C. megalonyx* complex. In combination with morphological variation, species diversity within the species complex was analyzed and factors leading to recurrent speciation events were assessed. The comparison of the phylogenomic trees of both species complexes underlines the benefit of analyzing thousands of genome-wide markers obtained from target hybrid enrichment in comparison to single marker (e.g. COI and ITS) approaches with respect to the resolution and support of both shallow and deep nodes. When comparing previous studies there was a notable difference between the species complexes because mito-nuclear discordance was only found for the *C. megalonyx* species complex. Target hybrid enrichment data revealed one case of mito-nuclear discordance in lineages that, however, are closely related to each other for the *P. patagonica* species complex, hence this pattern was explained due to a lack of resolution rather than hybridization as proposed for the *C. megalonyx* species complex. A further difference between the *C. megalonyx* and *P. patagonica* species complexes is the evidence for positive selection, which is so far lacking for the latter. This might be based on the fact that only half the genes targeted could be recovered for the *P. patagonica* species complex probably due to the fact that baits were originally designed for the *C. megalonyx* species complex. But also biological differences, e.g. in reproduction strategies, should be considered as a factor for differences in speciation scenarios. The reproductive mode remains unclear for *Colossendeis* but it most likely differs from the brooding strategy of *Pallenopsis* for which eggs-carrying males were caught frequently (Hübner et al. 2017). Especially if *Colossendeis* larvae (which

have never been reported) were detach and free-floating, geographic separation would represent smaller barriers for this taxa than for the brooder, *Pallenopsis*. This would explain the more frequent detection of speciation reversal within the *C. megalonyx* species complexes in comparison to the *P. patagonica* species complex, but not the strong geographic divergence within lineages. Alternatively, pre-zygotic barriers might be less stable among lineages within the *C. megalonyx* species complexes which hybridise with each other at times. In the case that there is no fitness reduction for the hybrids, speciation reversal occurs.

**Limitations and recommendations for future research**

*Limitations of samples from remote habitats*

One limitation when performing evolutionary studies in remote areas and inaccessible habitats such as the Antarctic benthos is the availability of specimens (Kaiser et al. 2013). Furthermore, the quick processing and correct preservation of samples are substantial, especially for genomic or transcriptomic studies. This holds for sea spiders because often single specimens do not contain DNA of suitable quality and quantity for genomic analyses (especially long read sequencing or de novo genome sequencing). Three attempts for de novo sequencing were unsuccessful as part of this project and thus gene annotation information and bait design options were limited.

*Recommendations for analyses of non-model organisms*

The findings of our study can advise future research on non-model organisms. Depending on the question, target hybrid enrichment as conducted here has obvious strengths but also limitations. If the aim is to screen for candidate genes under selection as broadly as possible, a better gene coverage than available for *C. megalonyx* should be aimed for. Therefore, different techniques not relying on sparse reference data are advised, if sufficient funding and adequate material for analysis is available (transcriptome sequencing, shallow genome resequencing). However, target hybrid enrichment represents a good alternative if that is not the case. A strength of the method is the power to resolve phylogenies. Thus, for studies that aim to resolve species diversity, our results demonstrate the exceptional benefit of target hybrid enrichment data, as even with few specimens, the resolution is much better and more robust than earlier results based on a few genes (Krabbe et al. 2010, Dietz et al. 2015). For the inference of population genetic processes, this study provides a glimpse into the yet largely unrecognized power of SNPs obtained from flanking regions co-enriched with targeted gene sequences. Even with few specimens, as were available here, clear patterns of population structure could be derived. Furthermore, individual outliers could be confidentially identified as such, because the

large number of markers provided a great credibility. This strength has also been reported by a simulation study indicating that the number of markers partly compensates for the number of available specimens (Willing et al. 2012). Still, while we find distinct population patterns, the lack of potential source or stepping stone populations limits the interpretation of the patterns. Thus, while this technique allows for robust and highly resolved data even for n=1 population data, we recommend to invest on maximizing sample size per population to derive conclusions about demographic processes.

## 5. Conclusion

We successfully applied target hybrid enrichment analyses on the Southern Ocean sea spider species complex *C. megalony*x. With the data, we reconstructed the best resolved and supported species tree available until now, showing additional evidence of species divergence inside the *C. megalonyx* species complex. Furthermore, we derived population structure within species and could show limited gene flow among geographically isolated populations, for which previous data lacked resolution. Thereby, our study supports the assumption that populations of lineages of the *C. megalonyx* species complex are not very well connected but rather strongly isolated. Finally, we identified candidate genes under positive selection.

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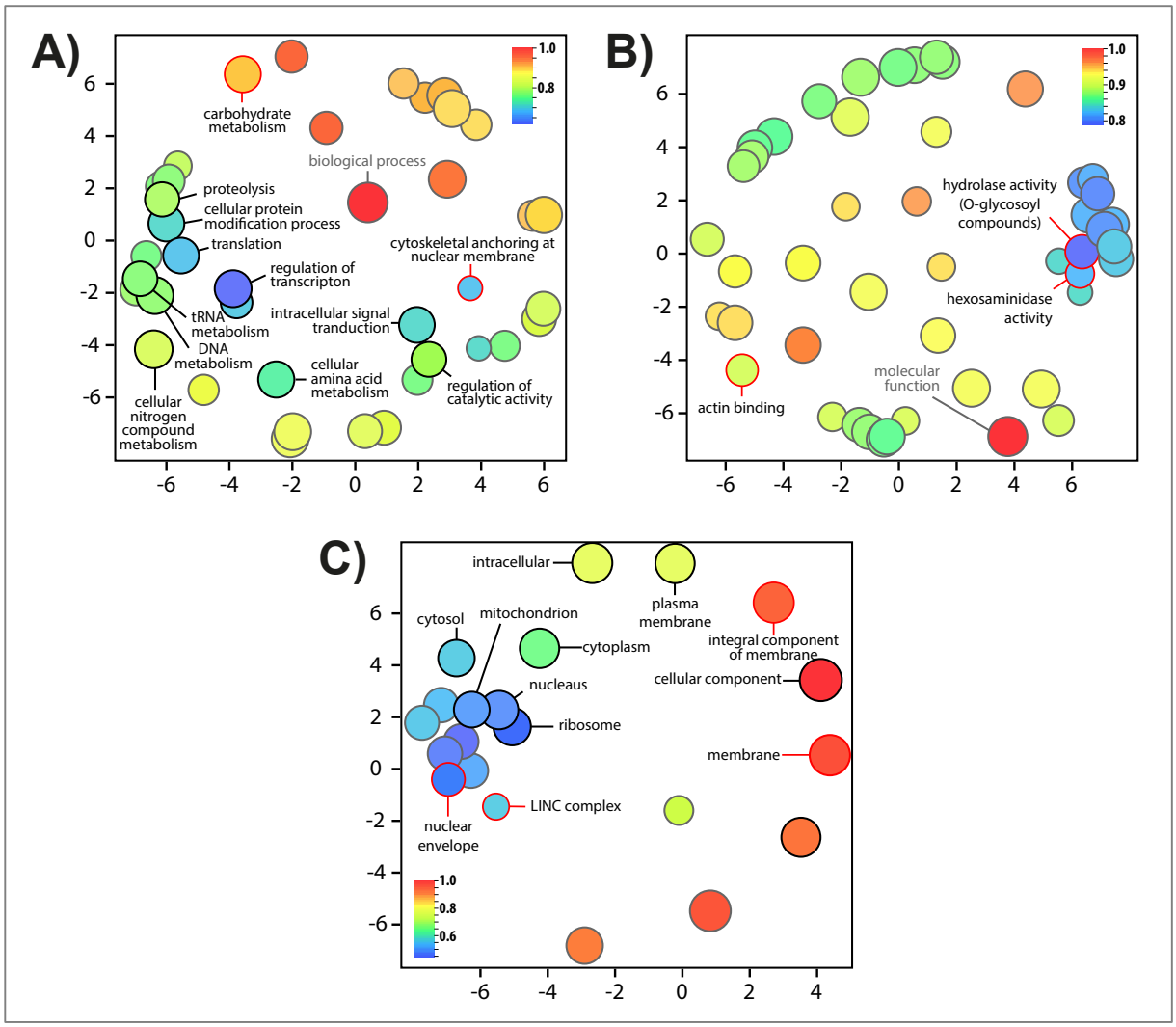
## *Supplementary material*

# Nuclear and mitochondrial gene data support recent radiation within the sea spider species complex *Pallenopsis patagonica*

**Supplementary material 1: List of characters measured for morphometric analyses of *Colossendeis megalonyx*.**

| Abbreviation | Description |
|---|---|
| trunk L | total length of trunk |
| ceph. segment | length of cephalic segment |
| trunk W1 | diameter of lateral process of 1st trunk segment |
| trunk W2 | diameter of lateral process of 2nd trunk segment |
| trunk W23 | width of trunk between 2nd and 3rd lateral processes |
| trunk H | height of trunk |
| abdomen L | length of abdomen |
| abdomen W | width of abdomen |
| ocular tubercle H | height of ocular tubercle |
| ocular tubercle W | width of ocular tubercle |
| eye H | height of eye |
| forehead H | distance between eyes and apex |
| eyes distance | distance between eyes |
| proboscis L | proboscis length |
| proboscis basis | diameter of proboscis at proximal basis |
| proboscis thickest | diameter of proboscis at thickest part of proboscis |
| proboscis thinnest | diameter of proboscis at thinnest part of proboscis |
| proboscis distal | diameter of proboscis at distal end |
| proboscis thick2tip | distance between tip of proboscis and thickest part of proboscis |
| l/r palp 1-10 | length of all palp articles; left and right |
| l/r basal article | diameter of basal article (joint article of palps and ovigera); left and right |
| l/r oviger 1-10 | length of all 10 ovigeral articles; left and right |
| l/r WL1-4 coxa1 | length of 1st coxa for all 4 pairs of walking leg; left and right |
| l/r WL1-4 coxa2 | length of 2nd coxa for all 4 pairs of walking leg; left and right |
| l/r WL1-4 coxa3 | length of 3rd coxa for all 4 pairs of walking leg; left and right |
| l/r WL1-4 femur | length of femur for all 4 pairs of walking leg; left and right |
| l/r WL1-4 tibia1 | length of 1st tibia for all 4 pairs of walking leg; left and right |
| l/r WL1-4 tibia2 | length of 2nd tibia for all 4 pairs of walking leg; left and right |
| l/r WL1-4 tarsus | length of the tarsus for all 4 pairs of walking leg; left and right |
| l/r WL1-4 propodus | length of the propodus for all 4 pairs of walking leg; left and right |
| l/r WL1-4 claw | length of the claw for all 4 pairs of walking leg; left and right |




**Supplementary material 2:** Scatterplots to visualize and summarize gene ontology of genes form the *Colossendeis megalonyx* species complex under positive selection divided into A) biological processes, B) cellular components, and C) molecular functions. Position of circles to each other represent the relation to each other. Size of circles represent frequency (logarithmic). Color indicate uniqueness of GO-term (the lower the more specific the term). Red lines indicate GO terms of genes found using branch-site selection models.

**Supplementary material 3a: List of genes under positive selection associated with biological processes.** Description, frequency, uniqueness and dispensability of each GO-term are listed.

| term ID | dispensable term ID | description | frequency | uniqueness | dispensability |
|---|---|---|---|---|---|
| GO:0002098 | | tRNA wobble uridine modification | 0.09% | 0.76 | 0.61 |
| GO:0002376 | | immune system process | 0.60% | 0.96 | 0 |
| GO:0005975 | | carbohydrate metabolic process | 5.26% | 0.9 | 0.04 |
| GO:0006030 | | chitin metabolic process | 0.08% | 0.85 | 0 |
| GO:0006259 | | DNA metabolic process | 5.61% | 0.78 | 0.37 |
| GO:0006355 | | regulation of transcription, DNA-templated | 9.92% | 0.61 | 0.51 |
| GO:0006355 | GO:0010468 | regulation of gene expression | 10.82% | 0.68 | 0.83 |
| GO:0006397 | | mRNA processing | 0.56% | 0.78 | 0.11 |
| GO:0006399 | | tRNA metabolic process | 2.50% | 0.77 | 0.35 |
| GO:0006412 | | translation | 5.69% | 0.69 | 0.61 |
| GO:0006464 | | cellular protein modification process | 7.73% | 0.71 | 0.57 |
| GO:0006486 | | protein glycosylation | 0.32% | 0.69 | 0.4 |
| GO:0006508 | | proteolysis | 5.22% | 0.79 | 0.34 |
| GO:0006511 | | ubiquitin-dependent protein catabolic process | 0.58% | 0.77 | 0.25 |
| GO:0006520 | | cellular amino acid metabolic process | 5.59% | 0.73 | 0.33 |
| GO:0006810 | | transport | 17.62% | 0.89 | 0.45 |
| GO:0006825 | | copper ion transport | 0.08% | 0.91 | 0 |
| GO:0006879 | | cellular iron ion homeostasis | 0.11% | 0.76 | 0.23 |
| GO:0006879 | GO:0045454 | cell redox homeostasis | 0.86% | 0.72 | 0.75 |
| GO:0006898 | | receptor-mediated endocytosis | 0.10% | 0.91 | 0.25 |
| GO:0006913 | | nucleocytoplasmic transport | 0.24% | 0.89 | 0.27 |
| GO:0006950 | | response to stress | 4.58% | 0.89 | 0 |
| GO:0007049 | | cell cycle | 1.89% | 0.84 | 0.22 |
| GO:0007051 | | spindle organization | 0.08% | 0.76 | 0.4 |
| GO:0007051 | GO:0007015 | actin filament organization | 0.24% | 0.75 | 0.73 |
| GO:0007155 | | cell adhesion | 0.54% | 0.96 | 0 |
| GO:0008150 | | biological process | 100.00% | 1 | 0 |
| GO:0009058 | | biosynthetic process | 31.61% | 0.94 | 0.02 |
| GO:0016192 | | vesicle-mediated transport | 1.09% | 0.9 | 0.25 |
| GO:0016579 | | protein deubiquitination | 0.20% | 0.77 | 0.49 |
| GO:0018342 | | protein prenylation | 0.03% | 0.8 | 0.07 |
| GO:0034641 | | cellular nitrogen compound metabolic process | 34.14% | 0.83 | 0.19 |

**Supplementary material 3a: Biological processes.** Continued.

| term ID | dispensable term ID | description | frequency | uniqueness | dispensability |
|---|---|---|---|---|---|
| GO:0035556 | | intracellular signal transduction | 4.00% | 0.71 | 0.59 |
| GO:0035556 | GO:0007165 | signal transduction | 6.62% | 0.69 | 0.85 |
| GO:0042254 | GO:0022618 | ribonucleoprotein complex assembly | 0.36% | 0.81 | 0.82 |
| GO:0042254 | | ribosome biogenesis | 1.42% | 0.84 | 0 |
| GO:0044281 | | small molecule metabolic process | 15.14% | 0.85 | 0.42 |
| GO:0045087 | | innate immune response | 0.15% | 0.91 | 0.38 |
| GO:0050790 | GO:0010951 | negative regulation of endopeptidase activity | 0.16% | 0.67 | 0.75 |
| GO:0050790 | GO:0043547 | positive regulation of GTPase activity | 0.47% | 0.8 | 0.84 |
| GO:0050790 | | regulation of catalytic activity | 1.58% | 0.79 | 0 |
| GO:0051301 | | cell division | 1.23% | 0.84 | 0 |
| GO:0055114 | | oxidation-reduction process | 15.06% | 0.85 | 0.13 |
| GO:0061024 | | membrane organization | 0.76% | 0.82 | 0.49 |
| GO:0090286 | | cytoskeletal anchoring at nuclear membrane | 0.00% | 0.69 | 0.56 |
| GO:2000601 | | positive regulation of Arp2/3 complex-mediated actin nucleation | 0.00% | 0.71 | 0.56 |

**Supplementary material 3b: List of genes under positive selection associated with cellular components.** Description, frequency, uniqueness and dispensability of each GO-term are listed.

| term ID | dispensable term ID | description | frequency | uniqueness | dispensability |
|---|---|---|---|---|---|
| GO:0000139 | | Golgi membrane | 0.40% | 0.46 | 0.68 |
| GO:0005575 | | cellular component | 100.00% | 1 | 0 |
| GO:0005576 | | extracellular region | 2.38% | 0.91 | 0 |
| GO:0005622 | | intracellular | 41.18% | 0.77 | 0.14 |
| GO:0005623 | | cell | 53.55% | 0.96 | 0 |
| GO:0005634 | | nucleus | 8.97% | 0.48 | 0.55 |
| GO:0005635 | | nuclear envelope | 0.28% | 0.49 | 0.62 |
| GO:0005730 | | nucleolus | 0.66% | 0.51 | 0.46 |
| GO:0005737 | | cytoplasm | 26.02% | 0.65 | 0.28 |
| GO:0005739 | | mitochondrion | 2.16% | 0.5 | 0.48 |
| GO:0005773 | | vacuole | 0.46% | 0.55 | 0.4 |
| GO:0005794 | | Golgi apparatus | 0.97% | 0.44 | 0.66 |
| GO:0005794 | GO:0005783 | endoplasmic reticulum | 1.33% | 0.43 | 0.78 |
| GO:0005829 | | cytosol | 2.55% | 0.56 | 0.11 |
| GO:0005840 | | ribosome | 4.20% | 0.47 | 0.57 |
| GO:0005886 | | plasma membrane | 10.51% | 0.77 | 0.2 |

**Supplementary material 3b: Cellular components.** Continued.

| term ID | dispensable term ID | description | frequency | uniqueness | dispensability |
|---|---|---|---|---|---|
| GO:0016020 | | membrane | 61.59% | 0.96 | 0 |
| GO:0016021 | | integral component of membrane | 55.87% | 0.94 | 0 |
| GO:0031410 | | cytoplasmic vesicle | 0.73% | 0.54 | 0.42 |
| GO:0032991 | | macromolecular complex | 14.01% | 0.92 | 0 |
| GO:0033588 | | Elongator holoenzyme complex | 0.03% | 0.74 | 0 |
| GO:0034993 | | LINC complex | 0.01% | 0.56 | 0.29 |

**Supplementary material 3c: List of genes under positive selection associated with molecular functions.** Description, frequency, uniqueness and dispensability of each GO-term are listed.

| term ID | dispensable term ID | description | frequency | uniqueness | dispensability |
|---|---|---|---|---|---|
| GO:0000166 | | nucleotide binding | 20.19% | 0.88 | 0.45 |
| GO:0003674 | | molecular function | 100.00% | 1 | 0 |
| GO:0003677 | | DNA binding | 12.55% | 0.87 | 0.49 |
| GO:0003682 | | chromatin binding | 0.22% | 0.9 | 0.05 |
| GO:0003700 | | transcription factor activity, sequence-specific DNA binding | 4.22% | 0.96 | 0 |
| GO:0003723 | | RNA binding | 5.28% | 0.88 | 0.32 |
| GO:0003735 | | structural constituent of ribosome | 2.68% | 0.94 | 0 |
| GO:0003756 | | protein disulfide isomerase activity | 0.03% | 0.94 | 0.02 |
| GO:0003779 | GO:0051015 | actin filament binding | 0.07% | 0.88 | 0.76 |
| GO:0003779 | | actin binding | 0.33% | 0.9 | 0.05 |
| GO:0004180 | GO:0004181 | metallocarboxypeptidase activity | 0.08% | 0.82 | 0.73 |
| GO:0004180 | | carboxypeptidase activity | 0.29% | 0.8 | 0.53 |
| GO:0004252 | | serine-type endopeptidase activity | 0.81% | 0.8 | 0.6 |
| GO:0004322 | | ferroxidase activity | 0.02% | 0.94 | 0 |
| GO:0004386 | | helicase activity | 1.22% | 0.82 | 0.28 |
| GO:0004553 | | hydrolase activity, hydrolyzing O-glycosyl compounds | 1.49% | 0.79 | 0.28 |
| GO:0004649 | | poly(ADP-ribose) glycohydrolase activity | 0.01% | 0.84 | 0.56 |
| GO:0005044 | | scavenger receptor activity | 0.04% | 0.96 | 0 |
| GO:0005507 | | copper ion binding | 0.32% | 0.88 | 0.05 |
| GO:0005509 | | calcium ion binding | 0.97% | 0.88 | 0.3 |
| GO:0005525 | | GTP binding | 1.78% | 0.87 | 0.27 |
| GO:0008061 | | chitin binding | 0.11% | 0.92 | 0.04 |

**Supplementary material 3c: Molecular functions** Continued.

| term ID | dispensable term ID | description | frequency | uniqueness | dispensability |
|---|---|---|---|---|---|
| GO:0008135 | | translation factor activity, RNA binding | 0.91% | 0.88 | 0.05 |
| GO:0008168 | | methyltransferase activity | 2.78% | 0.88 | 0.33 |
| GO:0008233 | | peptidase activity | 4.05% | 0.82 | 0.32 |
| GO:0008270 | | zinc ion binding | 4.51% | 0.86 | 0.56 |
| GO:0008289 | | lipid binding | 0.52% | 0.93 | 0 |
| GO:0008318 | | protein prenyltransferase | 0.02% | 0.91 | 0.02 |
| GO:0008378 | | galactosyltransferase activity | 0.05% | 0.9 | 0.21 |
| GO:0015929 | | hexosaminidase activity | 0.05% | 0.82 | 0.67 |
| GO:0016301 | | kinase activity | 6.06% | 0.86 | 0.67 |
| GO:0016491 | | oxidoreductase activity | 12.78% | 0.92 | 0.05 |
| GO:0016740 | | transferase activity | 21.04% | 0.92 | 0.09 |
| GO:0016757 | | transferase activity, transferring glycosyl groups | 1.99% | 0.88 | 0.2 |
| GO:0016779 | | nucleotidyltransferase activity | 1.95% | 0.87 | 0.31 |
| GO:0016787 | | hydrolase activity | 22.29% | 0.92 | 0.08 |
| GO:0016798 | | hydrolase activity, acting on glycosyl bonds | 1.82% | 0.83 | 0.29 |
| GO:0016810 | | hydrolase activity, acting on carbon-nitrogen (but not peptide) bonds | 1.79% | 0.83 | 0.04 |
| GO:0016853 | | isomerase activity | 2.69% | 0.93 | 0.02 |
| GO:0016874 | | ligase activity | 3.54% | 0.93 | 0.04 |
| GO:0016887 | | ATPase activity | 4.56% | 0.81 | 0.67 |
| GO:0017056 | | structural constituent of nuclear pore | 0.03% | 0.94 | 0.56 |
| GO:0019843 | | rRNA binding | 1.41% | 0.87 | 0.62 |
| GO:0022857 | | transmembrane transporter activity | 5.87% | 0.96 | 0 |
| GO:0030234 | GO:0004867 | serine-type endopepti-dase inhibitor activity | 0.07% | 0.91 | 0.74 |
| GO:0030234 | GO:0005096 | GTPase activator activity | 0.18% | 0.9 | 0.8 |
| GO:0030234 | | enzyme regulator activity | 0.86% | 0.9 | 0 |
| GO:0033925 | | mannosyl-glycoprotein endo-beta-N-acetyl-glucosaminidase activity | 0.01% | 0.84 | 0.57 |
| GO:0036459 | | thiol-dependent ubiquitinyl hydrolase activity | 0.18% | 0.82 | 0.23 |
| GO:0043167 | | ion binding | 33.49% | 0.91 | 0.09 |
| GO:0046872 | | metal ion binding | 15.43% | 0.86 | 0.63 |

**Supplementary material 4: Morphological measurements of the *Colossendeis megalonyx* species complex.** Measured characters of all individuals used for morphometric analyses. Detailed information about ID, voucher number, sex as well as species and mitochondrial clade assignment is given. Missing values are substituted with a question mark (?). *Provided as electronic supplement of this thesis.*

**Supplementary material 5: Significant differences of morphometric measurements of the *Colossendeis megalonyx* species complex.** Overview of results for significant tests between all possible clades' combinations. Only significant p-values are given. *Provided as electronic supplement of this thesis.*

**Supplementary material 6: Results of morphometric analyses of the *Colossendeis megaolnyx* species complex for both data sets (absolute and relative lengths).** Contributions to correctness rate and standard deviation (SD) are listed. [1]Number of times the character was added and its addition led to a positive increase in cross-validation correctness rate of individual LDA models during repeated character selections (% of total in parentheses). See how the repetitions were organized in Materials and Methods. [2]Average increase in cross-validation correctness rate after addition of the character to an LDA model had a positive effect in the character selections

| | **Absolute** | | **Relative** | |
|---|---|---|---|---|
| **Character** | Times the character contributed to an LDA model during character selection[1] | Increase in correctness rate (mean±SD)[2] | Times the character contributed to an LDA model during character selection[1] | Increase in correctness rate (mean±SD)[2] |
| **trunk L** | 136 (12.9%) | 0.10 ± 0.04 | - | - |
| **trunk W23** | 5 (0.47%) | 0.10 ± 0.04 | 4 (0.43%) | 0.06 ± 0.01 |
| **proboscis L** | 56 (5.3%) | 0.15 ± 0.04 | 7 (0.76%) | 0.11 ± 0.02 |
| **proboscis thickest** | 100 (9.5%) | 0.09 ± 0.03 | 91 (9.9%) | 0.09 ± 0.03 |
| **basal article** | 3 (0.28%) | 0.07 ± 0.02 | - | - |
| **palp 1** | - | - | 1 (0.11%) | 0.07 |
| **palp 10** | 70 (6.6%) | 0.16 ± 0.06 | 66 (7.2%) | 0.20 ± 0.07 |
| **palp 3** | 85 (8.1%) | 0.09 ± 0.04 | 104 (11.3%) | 0.15 ± 0.07 |
| **palp 4** | 25 (2.4%) | 0.07 ± 0.02 | 23 (2.5%) | 0.07 ± 0.01 |
| **palp 5** | - | - | 3 (0.33%) | 0.08 ± 0.02 |
| **palp 6** | 24 (2.3%) | 0.07 ± 0.02 | 47 (5.1%) | 0.09 ± 0.03 |
| **palp 7** | 3 (0.28%) | 0.07 ± 0.02 | 24 (2.6%) | 0.12 ± 0.08 |
| **palp 8** | 52 (4.9%) | 0.13 ± 0.06 | 10 (1.1%) | 0.06 ± 0.01 |
| **palp 9** | 220 (20.9%) | 0.21 ± 0.04 | 214 (23.2%) | 0.23 ± 0.06 |
| **WL1 coxa1** | 21 (2.0%) | 0.08 ± 0.02 | - | - |
| **WL1 coxa3** | 6 (0.57%) | 0.06 ± 0.01 | 8 (0.87%) | 0.07 ± 0.01 |
| **WL1 femur** | 31 (2.9%) | 0.11 ± 0.04 | 54 (5.9%) | 0.08 ± 0.03 |
| **WL1 tibia1** | 31 (2.9%) | 0.13 ± 0.04 | 16 (1.7%) | 0.18 ± 0.05 |
| **WL2 coxa1** | 7 (0.66%) | 0.08 ± 0.02 | 1 (0.11%) | 0.05 |
| **WL2 coxa2** | 3 (0.28%) | 0.06 ± 0.01 | 3 (0.33%) | 0.07 ± 0.02 |
| **WL2 coxa3** | - | - | 20 (2.2%) | 0.06 ± 0.01 |
| **WL2 femur** | 51 (4.8%) | 0.10 ± 0.04 | 42 (4.6%) | 0.10 ± 0.04 |
| **WL2 tibia1** | 30 (2.8%) | 0.11 ± 0.04 | 45 (4.9%) | 0.12 ± 0.05 |
| **WL3 coxa1** | 9 (0.85%) | 0.06 ± 0.01 | 1 (0.11%) | 0.06 |
| **WL3 coxa2** | - | - | 1 (0.11%) | 0.07 |
| **WL3 coxa3** | 3 (0.28%) | 0.06 ± 0.01 | 1 (0.11%) | 0.06 |
| **WL3 femur** | 14 (1.3%) | 0.12 ± 0.03 | 11 (1.2%) | 0.10 ± 0.03 |
| **WL3 tibia1** | 55 (5.2%) | 0.12 ± 0.04 | 119 (12.9%) | 0.13 ± 0.04 |
| **WL4 coxa1** | 9 (0.85%) | 0.06 ± 0.01 | 1 (0.11%) | 0.06 |
| **WL4 coxa2** | 1 (0.09%) | 0.09 | 3 (0.33%) | 0.07 ± 0.00 |
| **WL4 coxa3** | 3 (0.28%) | 0.07 ± 0.02 | 1 (0.11%) | 0.08 |

# CHAPTER IV

# Phylogenomics of the longitarsal Colossendeidae: the evolutionary history of an Antarctic sea spider radiation

Lars Dietz, Jana S. Dömel, Florian Leese, Andrew R. Mahon and Christoph Mayer

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# Phylogenomics of the longitarsal Colossendeidae: The evolutionary history of an Antarctic sea spider radiation

Lars Dietz[a,b,*], Jana S. Dömel[c], Florian Leese[c,d], Andrew R. Mahon[e], Christoph Mayer[a,b]

[a] Zoological Research Museum Alexander Koenig, Statistical Phylogenetics and Phylogenomics, Adenauerallee 160, D-53113 Bonn, Germany
[b] Faculty of Mathematics and Natural Sciences, University of Bonn, D-53012 Bonn, Germany
[c] Aquatic Ecosystem Research, Faculty of Biology, University of Duisburg-Essen, Universitätsstr. 5, D-45141 Essen, Germany
[d] Centre for Water and Environmental Research (ZWU), University of Duisburg-Essen, Universitätsstr. 2, D-45141 Essen, Germany
[e] Department of Biology, College of Science and Technology, Central Michigan University, 200 Library Drive, Mount Pleasant, MI 48859, USA

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ABSTRACT

Sea spiders (Pycnogonida) constitute a group of marine benthic arthropods that has a particularly high species diversity in the Southern Ocean. The "longitarsal" group of the sea spider family Colossendeidae is especially abundant in this region. However, this group also includes some representatives from other oceans, which raises the question where the group originates from. Therefore, we here investigated the phylogeny of the group with a hybrid enrichment approach that yielded a dataset of 1607 genes and over one million base pairs. We obtained a well-resolved phylogeny of the group, which is mostly consistent with morphological data. The data support an Antarctic origin of the longitarsal Colossendeidae and multiple dispersal events to other regions, which occurred at different timescales. This scenario is consistent with evidence found in other groups of marine invertebrates and highlights the role of the Southern Ocean as a source for non-Antarctic biota, especially of the deep sea. Our results suggest an initially slow rate of diversification followed by a more rapid radiation possibly correlated with the mid-Miocene cooling of Antarctica, similar to what is found in other taxa.

## 1. Introduction

Sea spiders (Pycnogonida or Pantopoda) are a group of marine benthic arthropods which occur in all oceans, but are particularly species-rich in the Southern Ocean. Of the 1350 species occurring worldwide, about 18% are present in the Antarctic (Munilla and Soler-Membrives, 2009), which is the highest percentage among all higher taxa of benthic animals (Aronson et al., 2007). Pycnogonids appear to be the sister group of the other chelicerate arthropods (Meusemann et al., 2010; Borner et al., 2014; Sharma et al., 2014), but the internal phylogeny of the group is currently not resolved (Arango and Wheeler, 2007; Nakamura et al., 2007; Arabi et al., 2010; Sabroux et al., 2017). One group of particular phylogenetic interest is the family Colossendeidae Hoek, 1881, which appears to have diverged early within the phylogeny of Pycnogonida (Arango and Wheeler, 2007; Dietz et al., 2011). The Colossendeidae occur mostly in the deep sea, but are also found in shallower waters especially in the Arctic and Antarctic. The family is subdivided into the mostly Indo-Pacific Hedgpethiinae Pushkin, 1990 and the cosmopolitan Colossendeinae Hoek, 1881 (Bamber et al., 2018). Within the Colossendeinae, it is possible to distinguish a "longitarsal" group, in which the most distal three leg articles (tarsus, propodus, claw) taken together are at least 75% as long as the second tibia, and a "brevitarsal" group, in which they are proportionally much shorter (Calman, 1915). The monophyly of the longitarsal group has been confirmed by molecular data (Dietz et al., 2015a). While the brevitarsal group is found worldwide, especially in the deep sea, the longitarsal group occurs mostly in the Antarctic. It includes widespread taxa such as *Colossendeis megalonyx* Hoek, 1881 and *C. robusta* Hoek, 1881, which are both complexes that include several morphologically similar species (Krabbe et al., 2010; Dietz et al., 2013, 2015a,b). The family also includes *Decolopoda* Eights, 1835 and *Dodecolopoda* Calman & Gordon, 1933, currently classified as separate genera, but phylogenetically nested within *Colossendeis* Jarzynsky, 1870 (Arango and Wheeler, 2007; Dietz et al., 2015b), which are anomalous in having five or six pairs of walking legs instead of four, and in retaining chelifores as adults. While most of the longitarsal species are restricted to the Antarctic, some also occur in other regions such as South America (e.g. *C. megalonyx*) or Kerguelen (e.g. *C. robusta*). Furthermore, the group also includes species that do not occur in the Antarctic, such as *C. scoresbii* Gordon, 1932 from South America, *C.*

* Corresponding author.
*E-mail address:* ldietz@uni-bonn.de (L. Dietz).

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*tenera* Hilton, 1943 from the South Pacific, and *C. angusta* Sars, 1877 with a worldwide distribution. The placement of these species within the longitarsal group is supported by molecular data (Dietz et al., 2013, 2015a). The type species of the genus *Colossendeis*, the Arctic *C. proboscidea* (Sabine, 1824), also has longitarsal walking legs (Sars, 1891) but has not yet been investigated with molecular methods.

As the longitarsal group includes a number of morphologically diverse species within the Southern Ocean, it is possible that it constitutes an in situ radiation. Such radiations are particularly numerous within the Southern Ocean (see Chenuil et al., 2018 for a review) due to its geological and climatic history. Most importantly, the continent and its surface waters are strongly isolated. This isolation is caused by the Antarctic Circumpolar Current, the strongest ocean current in the world, which has existed for about 25 million years (Pfuhl and McCave, 2005), and by the sharp drop in temperature at the Polar Front (Belkin, 2007). Besides this, the Antarctic shelf is surrounded by the deep sea on all sides, which prevents the dispersal of stenobathic benthic organisms between it and other shelf regions. The onset of evolutionary radiations in Antarctic taxa can often be dated to the middle Miocene (about 14 Ma), a time of rapid cooling (Crame, 2018). Examples of such radiations include notothenioid fishes (Matschiner et al., 2015), epimeriid amphipods (Verheye et al., 2017) or serolid isopods (Held, 2000). In pycnogonids, a probable Antarctic radiation was already identified by Stock (1957) within the genus *Austrodecus* based on morphological evidence. It has also been shown that some non-Antarctic taxa are phylogenetically nested inside Antarctic radiations, i.e. they colonized other regions from the Southern Ocean. Such "out-of-Antarctica" scenarios have been demonstrated e.g. for some octopuses (Strugnell et al., 2008) and gastropods (Göbbeler and Klussmann-Kolb, 2010).

So far, the phylogeny of the longitarsal group has only been investigated with a single locus, the mitochondrial cytochrome c oxidase subunit 1 (CO1) gene (Krabbe et al., 2010; Dietz et al., 2015a,b). While the resulting phylogeny clearly supports the monophyly of the group itself, it offers little information about the relationships of species within the group. With CO1, *C. glacialis* Hodgson, is recovered as sister to all other longitarsal species, followed by *C. australis* Hodgson, 1907 (both Antarctic species), but otherwise the resolution is poor. Therefore, the data published so far are not suitable to reliably test whether the Antarctic species within the group indeed constitute a monophyletic radiation, and whether an out-of-Antarctica scenario is supported for at least some non-Antarctic species.

Since it is unlikely that single genes resolve these questions, we here investigated the phylogeny of the longitarsal Colossendeidae with a multi-locus approach. Specifically, we used Target Hybrid Enrichment, a technique in which a large number of loci is captured by hybridizing genomic DNA with specially designed probes ("baits") based on reference sequences from known genomes or transcriptomes (Mayer et al., 2016). We used the resulting phylogenetic tree to test the Antarctic vs. non-Antarctic origin of the longitarsal group and various sea spider taxa within it by reconstructing the ancestral distribution areas of those clades and by assessing and comparing the likelihood of different models of dispersal and vicariance.

## 2. Materials and methods

### 2.1. Transcriptome sequencing

As reference for bait design, we used transcriptomes of two *Colossendeis megalonyx* (clade D3 of Dietz et al., 2015b) individuals from the Amundsen Sea. As an outgroup, a transcriptome of a *Pallenopsis patagonica* s.l. (clade ANT_M of Dömel et al., 2017) individual from the Eastern Weddell Sea was used. Transcriptomes were generated following Havird et al. (2014) by placing a single individual in ice-cold Trizol and homogenizing it via bead-beating. Total RNA was extracted and purified using an RNeasy kit (Qiagen) and DNAse I treatment according to the manufacturer's instructions, with RNA quality assessed on a Bioanalyzer 2100 (Agilent).

Libraries were constructed using the SMART cDNA construction kit (Clontech) with the provided 3′ oligo replaced by the Cap-Trsa-CV oligo. cDNA was purified with a QIAquick PCR purification kit (Qiagen) and adapters trimmed via digestion with SfiI (Clontech). The library was then sequenced at the Genomic Services Lab at the HudsonAlpha Institute for Biotechnology in Huntsville, Alabama, USA using Illumina Tru-Seq technology. Paired-end (PE) reads of 100 bp were received in FASTQ format and assembled.

The PE reads were normalized using the normalize-by-median.py script (https://github.com/dib-lab/khmer) prior to assembly with Bridger (Chang et al., 2015) and Trinity version r20131110 (Grabherr et al., 2011) to improve computational efficiency (Haas et al., 2013) under default parameters and 6 CPU cores.

### 2.2. Hybrid enrichment

For bait design, sets of orthologous genes suitable as targets for sequencing were identified with OrthoDB 9.1 (Zdobnov et al., 2017). As no pycnogonid genome is currently available, genes were used that are single copy and present in all four spider (Araneae) genomes present in OrthoDB, resulting in a total number of 2198 genes, in the following referred to as EOGs (Eukaryotic Ortholog Groups). Orthograph 0.5.14 (Petersen et al., 2017) was used to search the *C. megalonyx* transcriptome for orthologs of these genes and to predict the sequences of the coding regions. As the two individuals whose transcriptomes were sequenced had identical CO1 sequences, and were from the same sampling station, the two transcriptomes were pooled. For 1700 of the spider EOGs (77.3%), homologs were found in the *C. megalonyx* transcriptome. A BLASTx search as implemented in Geneious 9.1.6 (Kearse et al., 2012) was performed against the NCBI protein database to exclude contaminations. Similarly, 454 sequencing datasets of *Austropallene cornigera* (Möbius, 1802) and *Pallenopsis patagonica* (Hoek, 1881) originally published by Leese et al. (2012) and an EST dataset of *Endeis spinosa* (Montagu, 1808) available on GenBank (LIBEST_025662) were searched for sequences orthologous to the spider EOGs with Orthograph. The resulting sequences of each identified EOG were aligned with MAFFT 7.305b (Katoh and Standley, 2013) using the L-INS-I algorithm. Based on these alignments, bait sequences were designed with a modified version of BaitFisher 1.2.7 (Mayer et al., 2016) that allowed to include multiple bait regions per gene alignment. Search for bait regions was restricted to parts of the alignment in which the transcriptomic sequence of *C. megalonyx* was present using the required taxa option in the BaitFisher configuration file. The bait length was set to 120 and the clustering threshold to 0.15. Bait regions consisted of 3 baits per region with an offset of 40 bp and the minimum distance between bait regions on the same gene was set to 200. Baits were quality filtered using BaitFilter 1.0.5 (Mayer et al., 2016). For 1607 genes (94.5%), it was possible to successfully design baits. The total number of baits was 12,014 in 3682 bait regions. Bait sequences are provided in Supplementary File S1. Baits were produced by Agilent Germany. They were hybridized with DNA from 32 individuals belonging to 14 currently recognized species of longitarsal colossendeids and two individuals of the brevitarsal species *Colossendeis macerrima*, Wilson, 1881 (see Table 1). The resulting enriched DNA was sequenced at GATC Biotech (Konstanz, Germany) with an Illumina MiSeq Personal Sequencer, using paired-end technology (v2, read length: 2 * 250 bp) with 5% PhiX control DNA. Adapter sequences and low-quality regions were removed with fastq-mcf (Aronesty, 2011). Gene regions that were used for bait design were then extracted from the coding sequences predicted by Orthograph with a custom Perl script. The trimmed reads were mapped against these regions with the BWA-MEM algorithm in bwa 0.7.17 (available from: http://bio-bwa.sourceforge.net). Default parameters were used, except that the minimum match length was set to 30. Reads for which the mapping was successful were then extracted from the resulting SAM file using a custom Perl script and mapped

**Table 1**
Specimens sampled for this study. "Accession" refers to GenBank accession number of the specimen's CO1 sequence.

| Specimen number | Species | Accession | Region | Cruise | Collection |
|---|---|---|---|---|---|
| 19080-2 | *C. angusta* | KC462560 | North Atlantic | IceAGE | UDE |
| 19305-1 | *C. angusta* | KC462561 | North Atlantic | IceAGE | UDE |
| 19305-3 | *C. angusta* | KC462565 | North Atlantic | IceAGE | UDE |
| 59BT40 | *C. bouvetensis* | KT201727 | Bouvet Island | ICE FISH | UDE |
| AGT42/164 | *C. glacialis* | KT202214 | S. Shetlands | PS42 | UDE |
| AGT42/175-1 | *C. megalonyx* | KT201963 | S. Shetlands | PS42 | ZSM |
| IU-2007-4795 | *C. robusta* | KT201701 | Kerguelen | POKERII | MNHN |
| IU-2007-5035 | *Decolopoda* | KT202182 | Kerguelen | POKERII | MNHN |
| IU-2013-15806 | *C. australis* | KT202208 | Terre Adélie | REVOLTA | MNHN |
| IU-2013-15808 | *C. glacialis* | KT202209 | Terre Adélie | REVOLTA | MNHN |
| IU-2013-15812 | *C. glacialis* | KT202211 | Terre Adélie | REVOLTA | MNHN |
| PA_E003 | *C. bouvetensis* | GQ386999 | S. Sandwich | ICE FISH | UDE |
| PB_E004 | *C. scotti* | KT202167 | S. Sandwich | ICE FISH | UDE |
| PJ_E008 | *C. scoresbii* | GQ387001 | Burdwood Bank | ICE FISH | UDE |
| PJ_E010 | *C. scotti* | KT202164 | S. Georgia | ICE FISH | UDE |
| PL_E003 | *C. wilsoni* | | S. Sandwich | ICE FISH | UDE |
| PM_E001 | *Decolopoda* | GQ386995 | Elephant Island | ICE FISH | UDE |
| PM_E002 | *C. australis* | KT202202 | Elephant Island | ICE FISH | UDE |
| PM_E003 | *Dodecolopoda* | GQ386992 | Elephant Island | ICE FISH | UDE |
| PN_E003 | *C. tortipalpis* | KT202142 | S. Shetlands | ICE FISH | UDE |
| PS_E001 | *C. megalonyx* | GQ387013 | Burdwood Bank | ICE FISH | ZSM |
| PS77-226.7.2 | *C. bouvetensis* | KT201724 | E. Antarctic Peninsula | PS77 | UDE |
| PS77-226.7.3 | *C. robusta* | KT201710 | E. Antarctic Peninsula | PS77 | UDE |
| PS77-233.3.1.1 | *C. drakei* | KT201715 | E. Antarctic Peninsula | PS77 | UDE |
| PS77-233.3.1.2 | *C. drakei* | KT201714 | E. Antarctic Peninsula | PS77 | UDE |
| PS77-260.6.1 | *C. cf. scotti* | KT202178 | E. Weddell Sea | PS77 | UDE |
| PS77-260.6.19 | *C. wilsoni* | | E. Weddell Sea | PS77 | UDE |
| PS77-265.2.1 | *C. tortipalpis* | KT202153 | E. Weddell Sea | PS77 | UDE |
| PS77-292.2.3 | *Decolopoda* | KT202186 | E. Weddell Sea | PS77 | UDE |
| PS77-300.1.4 | *C. glacialis* | KT202215 | E. Weddell Sea | PS77 | UDE |
| ZSMA20111336 | *C. macerrima* | KF603928 | South Chile | INSPIRE | ZSM |
| ZSMA20111353 | *C. scoresbii* | KF603934 | Falklands | ZDLT1 | ZSM |
| ZSMA20160003 | *C. macerrima* | | North Chile | | ZSM |
| ZSMA20161881 | *C. proboscidea* | | Beaufort Sea | | ZSM |

against the full coding sequence with bwa as described above. Diploid consensus sequences of the regions matching the reference were generated with samtools 1.6 (Li et al., 2009) and bcftools 1.6 (available from: https://github.com/samtools/bcftools). As the consensus sequences were already aligned to the reference sequence by the mapping process, no further alignment was necessary.

All sequence data are accessible from the NCBI Sequence Read Archive (SRA: BioProject ID PRJNA533448). A sequence alignment partitioned by genes is available as Supplementary Information S2.

### 2.3. Phylogenetic and biogeographic analyses

The gene alignments were concatenated and the resulting alignment was partitioned by codon positions. A translated version of the concatenated alignment was also constructed using AliView 1.18.1 (Larsson, 2014). In addition to the hybrid enrichment data, sequences from the reference transcriptome itself were included in the analyses. For both nucleotide and aminoacid alignments, maximum-likelihood phylogenetic analyses were carried out with IQ-TREE 1.6.3 (Nguyen et al., 2015). The model of evolution was selected with ModelFinder (Kalyaanamoorthy et al., 2017) using the Bayesian Information Criterion (BIC) and the optimal partitioning scheme was chosen by the partition finding algorithm implemented in ModelFinder (Chernomor et al., 2016). Branch support was assessed by computing ultrafast bootstrap trees (Hoang et al., 2018) within IQ-TREE.

For rooting the tree, EOGs homologous to those used in *Colossendeis* were extracted from the *Pallenopsis* transcriptome with Orthograph and added to the translated alignment with MAFFT using the –-add option. A visual inspection of the alignment showed that, for some genes, clearly nonhomologous regions had been aligned. Therefore, for each gene, outliers were excluded using the OLIinSeq program by CM (available upon request). This program uses a sliding window approach to detect outlier sequences. Briefly, in each window, for each sequence the mean BLOSUM62 score to other sequences is calculated. For the set of mean scores, the interquartile distance (IQD) is calculated and sequences for which the mean score is lower than the lower quartile by an amount of at least IQD * f, where f is a specified factor, are considered as outliers. Here, we used the default parameters, with a sliding window of length 30 and f = 1.5. Taxa which were classified as outliers in more than 50% of the windows were removed from the alignment. The resulting alignments were then concatenated and analyzed with IQ-TREE as described above. Due to the comparatively long branch leading to the outgroup, only sequences of colossendeids were included in further analyses, with the rooting specified by the analysis including *Pallenopsis*.

A multi-species coalescent tree was constructed with ASTRAL 5.6.1 (Zhang et al., 2018). First, for each gene alignment, individual taxa were excluded if more than half of the sequence was missing. Furthermore, we excluded genes for which less than half of the included 35 taxa were present. For the remaining 1487 genes, maximum-likelihood trees were computed with IQTREE, using the model GTR + I + G. Branches with bootstrap-support values below 10% were collapsed with TreeCollapserCL 4 (available from: http://emmahodcroft.com/TreeCollapseCL.html). The resulting gene trees were then input into ASTRAL and a species tree was inferred from them using default parameters.

For biogeographic testing, a Bayesian ultrametric tree was computed with BEAST 2.5.0 (Bouckaert et al., 2014) using the model GTR + I + G, a log-normal relaxed clock with an arbitrarily chosen clock rate of 1.0, and a Yule tree prior with uniform birth rate. The analysis was conducted with an MCMC chain length of 10,000,000 and a sampling frequency of 1000. The ingroup was constrained as monophyletic, but apart from this, the topology was not constrained. As no *Colossendeis* fossils are known, no calibration was done. After

convergence was checked using Tracer 1.6 (Rambaut et al., 2018), the first 40% of the chain was discarded as burn-in, and a maximum clade credibility tree was calculated with TreeAnnotator 2.5.0 (Bouckaert et al., 2014). Biogeographic analyses were done with BioGeoBEARS (Matzke, 2013) based on the BEAST tree. As the dispersal and extinction parameters in BioGeoBEARS are rates of events per time and have to be within a specified range, it is necessary to assume realistic time scales. Therefore, the branch lengths of the tree were multiplied by 1000 to give more realistic estimates of divergence time, as the root can be assumed to have an age of several millions of years. Species-level clades in this tree were collapsed manually. A species-area matrix was constructed in which the presence or absence of each species was denoted for the following areas: (1) the Antarctic, including the Scotia Arc, (2) the South American shelf, (3) the Kerguelen plateau, (4) the non-Antarctic deep sea, and (5) the Arctic. Species distributions were taken from Munilla and Soler Membrives (2009), taking into account the taxonomic revisions by Dietz et al. (2015a). Ancestral area reconstruction was done using the DEC, DIVALIKE and BAYAREALIKE models implemented in BioGeoBEARS, each with and without a "jump" parameter J which allows speciation by the founder effect after colonization of a new area, as well as with and without a "range switching" parameter A that allows a lineage to completely switch its range from one area to another. In the DEC model, after speciation one daughter lineage always occupies only a single region, and sympatric speciation is possible. The DIVALIKE model allows speciation by vicariance in which both daughter lineages may occupy more than one region, but does not allow sympatric speciation. In BAYAREALIKE, the range does not change during speciation. Default ranges were assumed for the parameters. Distribution ranges consisting of non-adjacent areas (i.e. combining the Arctic with any other area except for the deep sea) were excluded. The most appropriate model was then selected with the corrected Akaike Information Criterion (AICc). In addition, likelihood ratio tests were also carried out to test whether the addition of the parameters J and A lead to a significantly better fit.

## 3. Results

### 3.1. Enrichment results

The sequencing resulted in an average number of 477,365 reads per individual. In total 1607 genes were recovered in at least 5 of the examined 34 individuals, leading to a total sequence length of 1,025,670 bp. The average number of individuals for which each gene was recovered was 31.79 (93.49%). For each individual, on average 1502.38 genes were recovered. The total amount of missing data in the concatenated alignment varied between taxa from 9.45% to 66.7% (average 24.5%).

We found *Pallenopsis* homologs for 1262 genes (78.53%), of which 553 (43.82%) were excluded as outliers.

### 3.2. Phylogenetic analysis

For the nucleotide dataset, the optimal partitioning scheme chosen by ModelFinder treated all three codon positions as separate partitions. The models chosen by ModelFinder were GTR + R3 for the first position, GTR + R2 for the second position, and GTR + R4 for the third position. For the translated alignment, HIVb + F + R4 was chosen as the best model.

In our phylogenetic analyses we obtained well-resolved trees in which almost all groupings have 100% bootstrap support (Fig. 1). In the analysis including the outgroup, *C. macerrima* was resolved as sister to all other colossendeid species with full support, i.e. the longitarsal taxa included in our dataset form a monophyletic group. Therefore, in further analyses, trees were rooted with *C. macerrima*. Topologies of the nucleotide and amino acid trees are identical. Except for *C. wilsoni* Calman, 1915, all traditionally recognized species are recovered as monophyletic. Within the longitarsal clade, *C. glacialis* is the first species to branch off, followed by *C. australis* and *C. proboscidea*. A group consisting of *C. drakei* Calman, 1915, *C. robusta*, *C. wilsoni*, and *C. bouvetensis* Dietz, Pieper, Seefeldt & Leese, 2015, here called the "*robusta* group", is sister to a group consisting of the remaining species, here called the "*megalonyx* group". Within the latter group, *C. megalonyx* is sister to the non-Antarctic species *C. angusta* and *C. scoresbii*, while *C. scotti* Calman, 1915 and *C. tortipalpis* Gordon, 1932 are sister to the multi-legged taxa *Decolopoda* and *Dodecolopoda*.

The results agree well with the CO1 tree (Dietz et al., 2015a,b), in which the monophyly of the longitarsal clade, the early diverging position of *C. glacialis* and *C. australis* within it, and the monophyly of the "*robusta* group" and the "*megalonyx* group" were also recovered. However, these relationships have lower support in the CO1 tree, which furthermore did not recover other groupings found in the multi-gene tree. Within the "*megalonyx* group", the CO1 and multi-gene phylogenies only agree in recovering the groups *C. scotti* + *C. tortipalpis* and *Decolopoda* + *Dodecolopoda*. The present phylogeny also agrees with the well-supported nodes of the tree based on nuclear ITS sequences (Dietz et al., 2015b).

The recovered results within *C. glacialis* are inconsistent with the CO1 data, where the specimens AGT 42/164 and PS77-300.1.4 both belong to Clade 1 (Dietz et al., 2015a), while in the multi-gene tree PS77-300.1.4 is closer to specimens belonging to the mitochondrial clade 6. *C. wilsoni*, for which we lack CO1 data, is not recovered as monophyletic, as the specimen PS77-260.6.19 is closer to *C. bouvetensis* than to PL_E003. Other intraspecific relationships agree with the CO1 tree in cases where this can be assessed.

The species tree recovered by the ASTRAL analysis (Supplementary File S3) is mostly identical to the ML trees based on the concatenated dataset. The only exception is the position of the *C. bouvetensis* specimen 59BT40, which is present in only 446 of the included 1487 gene trees. The specimen groups as sister to all other included representatives of *C. wilsoni* and *C. bouvetensis*, and not within *C. bouvetensis* as in the ML trees. All branches have a support value of 1, except for the one including the *C. wilsoni/bouvetensis* specimens other than 59BT40.

### 3.3. Biogeography

The topology of the ultrametric tree calculated by BEAST is identical to that of the ML tree, and all nodes have a posterior probability of 1. Of the models tested with BioGeoBEARS, DEC + A had the highest AICc score, but DEC, DEC + J, DIVALIKE + A and DIVALIKE + J also had AICc weights greater than 0.05 (Table 2). With a likelihood ratio test we found that for the base models DEC ($p = 0.015$) and DIVALIKE ($p = 0.0096$), the +A model variant is significantly better. Similarly, the +J model variant was found to be significantly better only for DEC ($p = 0.035$) and DIVALIKE ($p = 0.022$). For all +J+A models, except for BAYAREALIKE + J, the value of the J parameter was optimized as zero, making their results identical to those of the equivalent +A models without the J. As the BAYAREALIKE models can be rejected based on the AICc comparison, they will not be discussed further.

The DEC model infers that the common ancestor of the longitarsal clade most likely had a wide distribution (Antarctic, deep sea and Arctic) and most of the internal nodes within that clade had a range including all these areas as well as South America. Several other ancestral distribution ranges could not be excluded, all of them including multiple areas. In contrast, DIVALIKE infers an exclusively Antarctic range for most internal nodes, although the distribution of the common ancestor of *C. proboscidea* and its sister group includes the Arctic, the deep sea and the Antarctic. In models including the parameters J and/or A, an exclusively Antarctic distribution is inferred for all internal nodes that include Antarctic taxa (see Fig. 2 for the reconstruction using DEC + A). The range of the ancestor of *C. scoresbii* and *C. angusta* is inferred to include the Antarctic and South America (DEC models) or

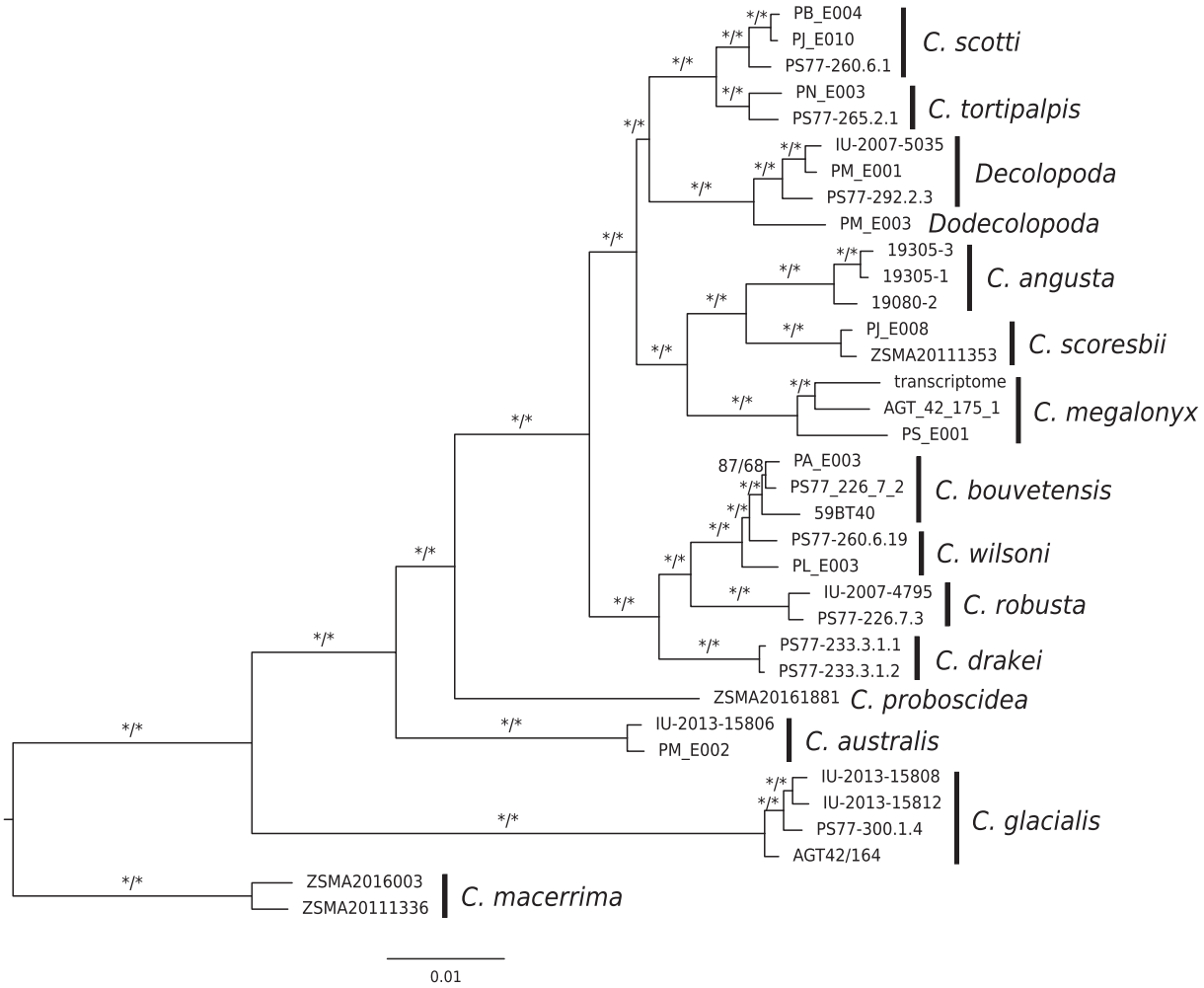


**Fig. 1.** Maximum-likelihood phylogenetic tree of the Colossendeidae based on the concatenated 1607-gene dataset. Numbers above branches are bootstrap support percentages based on nucleotide/aminoacid analyses. Asterisks indicate a bootstrap support of 100.

only South America (DIVALIKE models).

## 4. Discussion

### 4.1. Phylogeny and taxonomy

All applied methods, including nucleotide- and protein-based ML analyses using concatenated alignments filtered by different criteria, as well as coalescent-based species trees and the Bayesian ultrametric tree, recovered the same phylogeny (except for the position of one taxon for which a large proportion of the data is missing). Furthermore, most of the nodes are maximally supported. The phylogeny is also consistent with the well-supported nodes in the CO1 tree (Dietz et al., 2015b). This provides a consistent phylogenetic picture of the group, not biased by factors affecting only specific types of analyses such as incomplete lineage sorting which is expected to effect concatenated analyses more than multi-species coalescent analyses (Kubatko and Degnan, 2007).

The phylogeny is broadly consistent with morphological data.

**Table 2**
Models tested in BioGeoBEARS, with parameter estimates and AICc test results. Models with +J allow founder effect speciation, those with +A allow range switching of a lineage. −lnL: negative logarithm of likelihood, params: number of free parameters, d: dispersal parameter, e: extinction parameter, j: jump parameter, a: range switching parameter, AICc: corrected Akaike Information Criterion score, weight: model weight calculated form AICc score.

| Model | −lnL | Params | d | e | j | a | AICc | Weight |
|---|---|---|---|---|---|---|---|---|
| DEC | 38.14 | 2 | 0.013 | 0 | 0 | 0 | 81.38 | 0.099 |
| DEC + A | 35.16 | 3 | 0.012 | 0 | 0 | 0.0026 | 78.72 | 0.38 |
| DEC + J | 35.92 | 3 | 0.012 | 0 | 0.024 | 0 | 80.24 | 0.18 |
| DEC + J + A | 35.16 | 4 | 0.012 | 0 | 0 | 0.0026 | 82.76 | 0.05 |
| DIVALIKE | 39.39 | 2 | 0.018 | 0.0087 | 0 | 0 | 83.88 | 0.029 |
| DIVALIKE + A | 36.04 | 3 | 0.013 | 0 | 0 | 0.0027 | 80.47 | 0.16 |
| DIVALIKE + J | 36.79 | 3 | 0.013 | 0 | 0.024 | 0 | 81.97 | 0.074 |
| DIVALIKE + J + A | 36.04 | 4 | 0.013 | 0 | 0 | 0.0027 | 84.52 | 0.021 |
| BAYAREALIKE | 40.78 | 2 | 0.017 | 0.036 | 0 | 0 | 86.65 | 0.0071 |
| BAYAREALIKE + A | 39.24 | 3 | 0.014 | 0.01 | 0 | 0.003 | 86.89 | 0.0063 |
| BAYAREALIKE + J | 39.41 | 3 | 0.013 | 0.0033 | 0.04 | 0 | 87.22 | 0.0054 |
| BAYAREALIKE + J + A | 39.25 | 4 | 0.014 | 0.0083 | 0.0001 | 0.0031 | 90.94 | 0.0008 |

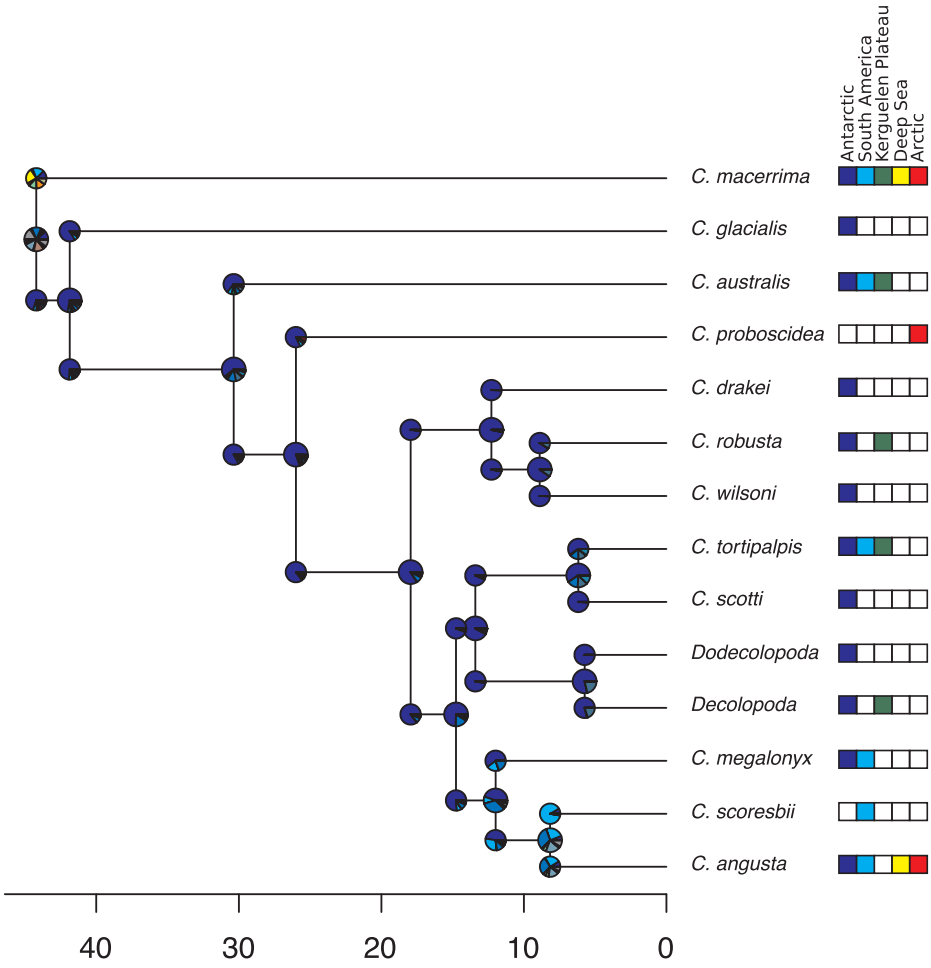


**Fig. 2.** Bayesian clock-like tree of the Colossendeidae generated by BEAST. Pie charts show ancestral range estimates inferred in BioGeoBEARS under the DEC + A model. Filled squares show the distribution of species, and colors correspond to those in the pie charts. Time units are not calibrated due to a lack of fossils. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

*Decolopoda* and *Dodecolopoda*, which are characterized by one and two additional pairs of walking legs, respectively, and the retention of chelifores in adults, form a well-supported clade that is deeply nested within the longitarsal Colossendeidae. Species of the "*robusta* group" have several morphological similarities, such as a relatively short proboscis (about as long as the trunk), also found in *C. glacialis*, and the more or less equal length of the palp articles 5 to 9 (counted according to Cano-Sánchez and López-González, 2016), which is also found in *C. glacialis* and in *Decolopoda* + *Dodecolopoda* (Fry and Hedgpeth, 1969; Child, 1995). In the plesiomorphic state, as found in the brevitarsal group as well as in *C. australis* and *C. proboscidea*, article 6 is much longer than articles 5 and 7–9. While *C. glacialis* is morphologically similar to the *robusta* group, and has even been synonymized with *C. robusta* (Fry and Hedgpeth, 1969), our results strongly support its placement outside that group, as was already found by Dietz et al. (2015a). The "*megalonyx* group" is more difficult to characterize morphologically, but in all species except *Decolopoda* + *Dodecolopoda*, palp article 6 is also the longest, and article 7 is shorter than 8 and 9.

The paraphyly of *C. wilsoni* with respect to *C. bouvetensis* raises questions concerning the species-level taxonomy of these taxa. As we lack CO1 data for the *C. wilsoni* specimens, we cannot assess whether they belong to any of the mitochondrial clades identified by Dietz et al. (2015a) on the basis of publicly available CO1 sequences. *C. wilsoni* is unique within the genus *Colossendeis* in having only eight palp articles, which makes its paraphyly unlikely. However, in all other characters, *C. wilsoni* appears very similar to *C. lilliei* Calman, 1915, which seems to be closely related to *C. bouvetensis* based on morphological characters (Dietz et al., 2015a) and in which the terminal palp article is often reduced (Calman, 1915). This suggests that the taxonomy of the *C. wilsoni/lilliei/bouvetensis* clade is in need of revision, and a greater taxonomic coverage of samples is needed to clarify the situation.

The phylogenetic position of *Decolopoda* and *Dodecolopoda* shows that the genus *Colossendeis* is paraphyletic, as already found by Krabbe et al. (2010). This suggests that the retention of chelifores by adults is a derived character. The paraphyly of *Colossendeis* in relation to *Decolopoda* and *Dodecolopoda* also raises nomenclatural issues. To keep *Colossendeis* monophyletic, the other two genera should ordinarily be merged with it. However, since the name *Decolopoda* Eights 1835 is older than *Colossendeis* Jarzynsky 1870, keeping all genera monophyletic either requires all *Colossendeis* species to be renamed as *Decolopoda*, or the genus *Colossendeis* to be broken up into a large number of genera that are morphologically difficult to distinguish. As this would cause a large amount of nomenclatural instability, at present we prefer not to modify the current classification, and to keep a paraphyletic genus *Colossendeis*.

The disagreements of the interspecific phylogeny recovered here with that based on the CO1 gene (Dietz et al., 2015a,b) can probably be explained by the lower resolution offered by the CO1 gene tree due to the much shorter alignment (545 bp). All well-supported interspecific groupings within the CO1 tree agree with the multi-gene tree, and for other groupings the resolution is too low to assess whether the disagreements are caused by genuine discrepancies between the mitochondrial phylogeny and the species tree. However, the disagreement within *C. glacialis*, where specimens belonging to a well-supported

mitochondrial clade do not group together in the multi-gene analysis, is probably best attributed to introgression of mitochondrial DNA between different lineages, which has also been shown to occur in *C. megalonyx* (Dietz et al., 2015b).

The divergence between the three *C. megalonyx* specimens included in our dataset is about as high as that between species universally regarded as distinct, such as *C. scotti* and *C. tortipalpis*, or *Decolopoda* and *Dodecolopoda*. Our results therefore support the hypothesis that there are several currently unrecognized species within *C. megalonyx* (Krabbe et al., 2010; Dietz et al., 2015b). As some of the divergent mitochondrial clades within this species complex apparently hybridize (Dietz et al., 2015b), a larger sampling will be necessary to clarify the taxonomy of the group. However, our data confirm that *C. scoresbii*, which was synonymized with *C. megalonyx* by Fry and Hedgpeth (1969), is not part of that species complex.

### 4.2. Biogeographic history

The results of the biogeographic models rated as most likely by AICc and likelihood ratio tests suggest that the longitarsal Colossendeidae originated in the Southern Ocean, and that the non-Antarctic taxa within that group are examples of an out-of-Antarctica dispersal. This scenario is supported by all models with AICc weight > 0.05, except for the DEC model (AICc weight = 0.099), which infers a global distribution for most internal nodes. We regard a global distribution as very unlikely, not only since models supporting this scenario are rejected by likelihood ratio tests and together have a much smaller AICc weight than those favoring an out-of-Antarctica dispersal, but also since this scenario requires that despite the global distribution, species divergence events occur almost exclusively within the Antarctic. Therefore, we regard the results of the more complex models DEC + A and DEC + J as more likely. Based on these results, we conclude that the origin and most of the evolution of the longitarsal group occurred in the Antarctic, from where other regions of the world's oceans were repeatedly colonized. This scenario resembles the evolutionary history of other marine organisms where non-Antarctic lineages originated in the Antarctic. In many cases, these are deep-sea organisms such as octopuses (Strugnell et al., 2008) or pleurobranch gastropods (Göbbeler and Klussmann-Kolb, 2010), which probably colonized the deep sea via the "thermohaline expressway" (Strugnell et al., 2008), i.e. the flow of deep water from the Antarctic northwards. However, there are also cases where shelf areas of the Subantarctic were colonized by Antarctic species, e.g. notothenioid fish (Dornburg et al., 2017) or the gastropod *Nacella* Schumacher, 1817 (González-Wevar et al., 2016). Švara and Melzer (2016) also found a probable case of northwards dispersal from the Southern Ocean in the pycnogonid genus *Austrodecus*, which was identified as a Southern Ocean radiation by Stock (1957). These dispersal events occurred on very different evolutionary scales, ranging from the Oligocene (Göbbeler and Klussmann-Kolb, 2010) to the Pleistocene (González-Wevar et al., 2016). This is also the case in *Colossendeis*, where e.g. the entirely non-Antarctic *scoresbii/angusta* clade appears to be several million years old, while the intraspecific out-of-Antarctica dispersals within *C. robusta* and *Decolopoda australis* clearly occurred much more recently due to the close relationship between Antarctic and Kerguelen individuals (Fig. 1).

Our results identify one supraspecific clade consisting of exclusively non-Antarctic taxa, namely *C. angusta* and *C. scoresbii*. The sister group to this clade is *C. megalonyx*, which occurs both in the Antarctic and southern South America. It is likely that the common ancestor of *C. megalonyx* and the *angusta-scoresbii* clade already had adaptations that facilitated the dispersal to non-Antarctic environments. While *C. scoresbii* is limited to southern South America, *C. angusta* has a worldwide distribution in the deep sea. However, the species *Colossendeis tenera* from the Pacific coast of North and Central America as currently recognized is paraphyletic with respect to *C. angusta* according to CO1 data (Dietz et al., 2013), suggesting that *C. angusta* colonized the deep sea from that region. Therefore the common ancestor of the *angusta-scoresbii* clade may have lived on the South American Shelf and dispersed out of Antarctica via the Drake Passage. A deep sea origin is unlikely due to the presence of pigmented eyes in some members of the clade. However, an unidentified colossendeid species from Kerguelen morphologically similar to *C. megalonyx* groups close to *C. scoresbii* in the CO1 tree (Dietz et al., 2015b), suggesting that it also belongs to this clade, and which therefore has a wider distribution than is represented in this study.

Other clades also contain non-Antarctic taxa. Within *C. megalonyx*, the mitochondrial sister clades B and M (Dietz et al., 2015b) occur in South America. While the representative of Clade B (PS_E001) is sister to the other *C. megalonyx* specimens in our limited sampling, other data indicate that Clades B + M are nested within *C. megalonyx* as sister to the Antarctic deep-sea clade C (Krabbe et al., 2010; Dietz et al., 2015b), suggesting an Antarctic origin of *C. megalonyx* as a whole. The *Decolopoda* specimen from Kerguelen is nested within an otherwise Antarctic clade, indicating that its ancestors dispersed from the Antarctic relatively recently. As the species does not occur in South America, dispersal via the Drake Passage is unlikely in this case. The *robusta* group also includes non-Antarctic members from the deep sea of the Southern Hemisphere, such as *C. stramenti* Fry and Hedgpeth, 1969 from South America, which is sister to *C. drakei* according to CO1 data. Probably the Indo-Pacific species *C. bruuni* Fage, 1956 and *C. curtirostris* Stock, 1963 also belong to this group (Dietz et al., 2015a). Since we lack molecular data for these species, we currently cannot assess their relationships with the Antarctic representatives of that clade. *C. australis* and *C. tortipalpis* are also known from the Subantarctic (Munilla and Soler-Membrives, 2009), however, as the taxonomy of these taxa is rather complex and many supposedly widespread pycnogonid species actually contain multiple cryptic taxa (e.g. Dietz et al., 2015a; Weis et al., 2014; Dömel et al., 2017), we cannot be certain whether the Subantarctic specimens have been correctly assigned to these species.

Finally, the Arctic species *C. proboscidea* is also part of the longitarsal clade. Its phylogenetic position also suggests an Antarctic origin, but due to its phylogenetically isolated position it is not possible to draw any detailed conclusions from its biogeographic history. As it lacks pigmented eyes (Sars, 1891), a deep-sea origin is likely.

### 4.3. An Antarctic radiation?

Species-rich clades that diversified within an isolated geographic region are often called evolutionary radiations. However, this term implies an initial fast diversification after the origin of the clade. While the Antarctic origin of the longitarsal colossendeids is clearly supported by our data, it is questionable whether the clade can be considered to have emerged from an evolutionary radiation, as there is no evidence for a rapid diversification soon after its origin. This result is comparable with that reported for epimeriid amphipods by Verheye et al. (2017) and may be interpreted as the result of extinctions of several early diverging lineages caused by later environmental changes such as the Pleistocene glaciations, in which most of the Antarctic shelf was covered by ice (Fraser et al., 2012). However, the clade consisting of all longitarsal species other than *C. glacialis*, *C. australis* and *C. proboscidea* seems to represent a secondary radiation with several branching events in a relatively short time. Pearse et al. (2009) suggest that Antarctic conditions facilitate evolutionary diversification due to colonization of new habitats via the Antarctic Circumpolar Current (ACC) and repeated habitat fragmentation during the ice ages. The latter explanation probably applies mostly to intraspecific divergences, but the former is consistent with our results as it predicts that speciation was ongoing since the onset of the ACC and that the Scotia Arc region should be a hotspot of diversity, which is indeed the case for pycnogonids in general (Griffiths et al., 2010).

If the origin of the longitarsal clade itself is connected to the isolation of the Antarctic and forming of the ACC, this secondary radiation

may have taken place during the Middle Miocene Climate Transition (MMCT), which was characterize by a sharp drop in temperature (Crame, 2018). Such a pattern of high diversification rates during the MMCT and observed low rates during the formation of the ACC is also observed in notothenioid fish (Near et al., 2012). It should however be noted that, in the absence of fossils useful for calibrating the phylogeny, any attempt to correlate the diversification of the group with events in geological history must remain speculative.

## 5. Conclusions

The present study shows that the hybrid enrichment method is suitable for inferring well-resolved phylogenies on the family level within sea spiders. With this data we produced the first completely resolved phylogenetic tree of the longitarsal Colossendeidae, confirming some previous phylogenetic hypotheses but also adding significant new data about taxonomic relationships. Our results support the scenario of an Antarctic origin of the group with multiple dispersals to other regions, contributing to the understanding of the history of the Antarctic benthos as a whole.

## Acknowledgements

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## Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ympev.2019.04.017.

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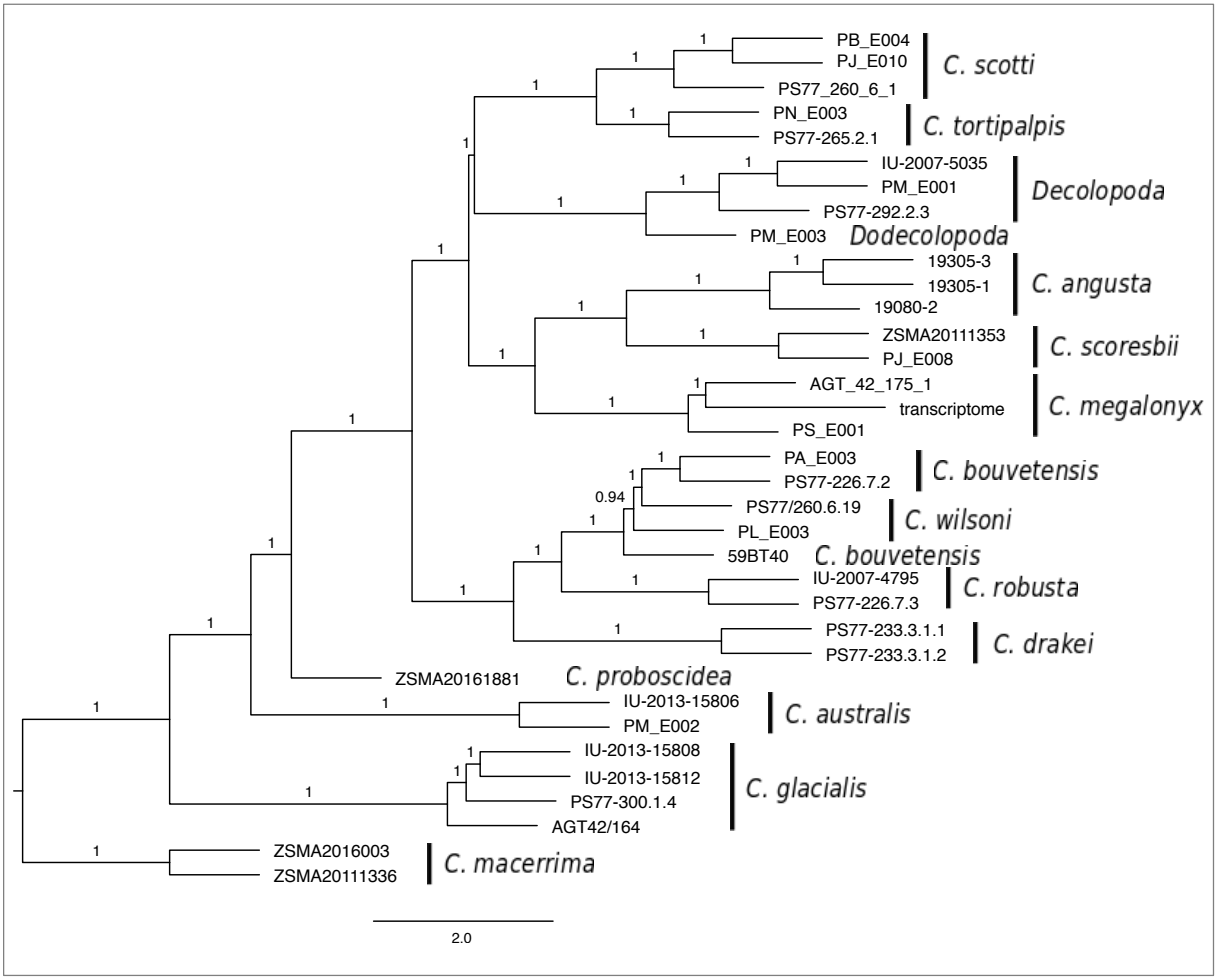
# *Supplementary material*

# Phylogenomics of the longitarsal Colossendeidae: the evolutionary history of an Antarctic sea spider radiation

**Lars Dietz, Jana S. Dömel, Florian Leese, Andrew R. Mahon and Christoph Mayer**

**Supplementary File S1:** Sequences of baits used for capturing DNA. (Provided as electronic supplement of this thesis.)

**Supplementary File S2:** Alignment of gene sequences obtained in this study, in NEXUS format partitioned by genes. (Provided as electronic supplement of this thesis.)




**Supplementary Figure S3:** Multi-species coalescent tree constructed with ASTRAL using a maximum-likelihood phylogenetic tree of the Colossendeidae based on the filtered concatenated dataset including 1487 genes as input. Numbers above branches are bootstrap support percentages based on nucleotide analyses.

# Cumulative discussion

The results of this thesis provide an advanced contribution to the research of the diversity and evolutionary history of benthic communities of the Southern Hemisphere. A laboratory workflow and bioinformatic pipeline for genomic analyses were developed and successfully tested for species with limited genetic background information.

## Establishment of target hybrid enrichment for sea spiders

The enrichment efficiency even of small DNA fragments was one reason to apply target hybrid enrichment to sea spider species of the Southern Hemisphere in this thesis, because those samples often show a high degree of degeneration of the DNA. Indeed, target hybrid enrichment was successfully established and genomic data could be enriched and analysed for all studied specimens and species. For specimens of the Colossendeidae (including *Colossendeis megalonyx* Hoek, 1881) all targeted 1607 EOGs (Eukaryotic Orthologous Groups; which in the case of this thesis are putative single-copy groups of orthologous genes) were recovered. Target hybrid enrichment of the species complex of *Pallenopsis patagonica* (Hoek, 1881) yielded a lower recovery rate, but still, 821 EOGs were enriched. This reduced recovery for the *P. patagonica* species complex can be explained by the fact that the design of the sea spider bait set was based on a single transcriptome of *C. megalonyx* in combination with four spider (Arachnida) genomes. So far, genes enriched for both sea spider species complexes have not been compared with each other, but based on enrichment success they must feature at least some more conserved parts that allow baits to be applied among genera and families. Whereas phylogenomic analyses show that variation among genes analysed even within recently diverged species complexes was sufficient enough to resolve intraspecific differentiation. Bait regions of some genes, however, differed too much and could only be recovered for the Colossendeidae, but not for the *P. patagonica* species complex. Overall, target hybrid enrichment added an enormous amount of genome-wide data for all taxa analysed and contributed to good resolutions for shallow and deeper phylogeny.

## Genetic diversity of sea spiders of the Southern Hemisphere

The monophyletic group referred to as the *P. patagonica* species complex, consists of multiple species. Using an extended sample set (in terms of number and geographic coverage) the number of previously reported species by Weis et al. (2014) increased and eleven species were proposed for the species complex within this thesis (chapter I and II; Dömel et al. 2015, in

press). A recent study of Cano-Sánchez and López-González (2019) described two further Antarctic species of *Pallenopsis* from the Ross Sea demonstrating an even higher diversity for Antarctic *Pallenopsis*. Apparently, species descriptions in Cano-Sánchez and López-González (2019) were based on morphological data only and the relation to or within the *P. patagonica* species complex remains unclear. However, it can be assumed that more species will be discovered when analysing further samples, especially from the Antarctic continental shelf.

In comparison to the two single-marker approaches (cytochrome c oxidase subunit I, COI, and internal transcribed spacer, ITS) from the initial study (Dömel et al. 2017), target hybrid enrichment did not detect less of more diversity for the *P. patagonica* species complex, but particularly improved the understanding of phylogenetic relationships between lineages due to a better resolution. Therefore, a clear split between species from the Antarctica continental shelf and Patagonian can be defined with the only distribution overlap of different species found around the sub-Antarctic island South Georgia. Distribution patterns of species of the *P. patagonica* species complex range from narrow to broad, although gene flow for the latter seems to be limited especially between populations from the Antarctic continental shelf and sub-Antarctic islands. The latter was also supported by analyses conducted based on SNP data recovered from the EOGs. Intraspecific geographic separations have also been detected for other sea spiders (Arango et al. 2011, Dömel et al. 2015). One inconsistency between mitochondrial and target hybrid enrichment analyses was found. In the phylogenetic target hybrid enrichment tree, one specimen assigned to the mitochondrial clade SUB_2 formed a monophyletic group with specimens from the mitochondrial clade SUB_1, while the other specimens from SUB_2 formed a sister group to the monophyletic group mentioned first. All specimens of the first group were collected around Burdwood Bank (Patagonian shelf). However, the two lineages are closely related to each other and the pattern can best be explained due to a lack of resolution rather than hybridization as has been reported for the sea spider species complex *C. megalonyx* (Dietz et al. 2015).

Prior to this thesis, Krabbe et al. (2010) and Dietz et al. (2015) detected several clades within *C. megalonyx* using mitochondrial COI and nuclear ITS sequences. Phylogenetic reconstructions based on both markers showed different topologies and also the number of species proposed differed between 20 (mitochondrial) clades to six (nuclear) groups. This difference was explained by speciation reversal, i.e. hybridization and eventually merging of temporary isolated groups, due to a discontinuity of reproduction barriers (Dietz et al. 2015).

Within this thesis, not all lineages reported for the *C. megalonyx* species complex were analysed using target hybrid enrichment and no final conclusion about the actual number of species within this complex can be made (chapter III, Dömel et al. in prep.). However, greater

differentiation in comparison to Dietz et al. (2015) due to limited gene flow among populations from distant localities within previously defined mitochondrial clades, is a key result of this thesis. For several clades, regional differentiation could be detected. Moreover, for one occasion gene flow even between individuals of the same group (based on COI and ITS) from the same location (South Orkney Islands) is restricted. This supports the assumption of this region being a hotspot for speciation especially for brooding species (Allcock and Strugnell 2012). Mito-nuclear discordance within the *C. megalonyx* species complex was also found in the phylogenomic tree based on target hybrid enrichment data. Specimens assigned as mitochondrial clade E1 represented two groups that were paraphyletic and corresponded to the ITS groups II and III. However, due to another focus of this study, only one individual that showed mito-nuclear discordance in previous studies was analysed and further assumptions about events of speciation reversal are omitted.

*C. megalonyx* was also analysed in the context of the longitarsal Colossendeidae. The longitarsal group is named after its character that the length of the most distal three leg articles (tarsus, propodus, and claw) are, taken together, at least 75% as long as the second tibia (Calman 1915). The longitarsal Colossendeidae occur mostly in the Antarctic. Target hybrid enrichment was conducted for 14 species of the longitarsal Colossendeidae and one other (brevitarsal) species as outgroup which resulted in a well-resolved phylogeny (chapter IV, Dietz et al. 2019). The divergence between specimens of the *C. megalonyx* species complex that belonged to three different mitochondrial clades (clades B, D and F) included in the analyses was about as high as that between distinct species, such as *C. scotti* Calman, 1915 and *C. tortipalpis* Gordon, 1932. This further advocates the findings that there are several distinct lineages within the *C. megalonyx* species complex (Krabbe et al. 2010, Dietz et al. 2015, Dömel et al. in prep.). Furthermore, the data support an Antarctic origin of the longitarsal Colossendeidae and multiple dispersal events to other regions, which occurred at different timescales. This migration direction is consistent with evidence found in other groups of marine invertebrates and highlights the role of the Southern Ocean as diversity pump (Clarke and Crame 1989) also for non-Antarctic biota. One example of the *C. megalonyx* species complex for such a migration out of Antarctica is the mitochondrial clade B that occurs on Burdwood Bank only.

In conclusion, the comparison of the phylogenetic trees of all taxa studied underlines the benefit of analysing thousands of genome-wide markers obtained from target hybrid enrichment in comparison to single marker (e.g. COI and ITS) approaches with respect to the resolution of shallow and deep nodes. Thus, target hybrid enrichment proves as a useful method to analyse phylogenetic relationships of ancient (e.g. family level) and recently diverged species (e.g. cryptic species).

## Morphological diversity of sea spiders of the Southern Hemisphere

Analyses of morphological characters performed on specimens of the *P. patagonica* species complex revealed high variation, even within lineages. There were significant differences between morphometric measurements of the Antarctic and the sub-Antarctic but no significant differences between more recently evolved lineages were found. However, diagnostic characters for at least nine species were found which could partly be assigned to already described species. In addition, two new species, namely *P. aulaeosmanorum* sp. nov Dömel & Melzer 2019 and *P. obstaculumsuperavit* sp. nov. Dömel, 2019, could be described.

For the *C. megalonyx* species complex, many significant differences between morphometric characters of lineages were found. Differentiation of lineages of the species complex was difficult though as variation within lineages was high and no clear divergence between lineages could be detected due to multiple overlaps of measurements.

Although there was no obvious bias caused by dimorphism for neither species complex the increased variability when including males and females for analyses might represent an issue for morphometric analyses. In addition, the high number of potential species within the species complexes in combination with often few available specimens per lineage made a statistically robust separation difficult. Finally, species could only be distinguished with the help of prior knowledge about lineages or morphological distinct characters. Lineages analysed, that did not show any morphological differences (e.g. SUB1, SUB2, SUB4 and SUB4 of the *P. patagonica* species complex) represent actual cryptic species. The fact that more actual species can be named for the *P. patagonica* species complex indicates that more extensive work on the genus *Pallenopsis* itself has to be conducted to obtain a taxonomic awareness comparable to the one of the more popular "giant sea spider" *C. megalonyx*.

## Selection within sea spiders of the Southern Hemisphere

Although distinct morphological characters suitable for separation and descriptions of species within the *P. patagonica* species complex were found and significant differences between lineages for the *C. megalonyx* species complex were analysed for many characters, none of those seems to imply important functions for the biology of the (potential) species that could have been subject to selection pressure.

Genetic analyses detected genes under positive selection. However, for the *P. patagonica* species complex, no branch under selection was detected (Dömel et al. 2019). For the *C. megalonyx* species complex, four genes showed both evidence for positive selection and at least one branch that had experienced positive selection. The majority were terminal branches

of single specimens, but for one gene deeper branches were involved, too. Predictions of the genes' function were based on their homology to distantly related spider (Arachnida) genomes that were used for EOG identification for bait design in the first place. Genes were associated with structural and neuronal aspects, however, assignments to gene functions were often vague, i.e. at a rather general metabolic description level. Therefore, linking the observed evidence for positive selection to any specific mechanism involved in evolutionary processes should be treated with caution before functional validation of genes.

In a case of sympatric speciation and adaptation to different ecological niches, high morphological differences also for recently diverged species, especially when they occur in sympatry (ecological character displacement) are expected. This does not appear to be the case for the *P. patagonica* species complex, because regardless of whether the species occur in sympatry or not, morphological distances were similarly high. Such a comparison for lineages within the *C. megalonyx* species complex was not possible due to a limited set of samples that were analysed morphometrically and genomically.

The difference in the detection of evidence for selection between the *P. patagonica* and *C. megalonyx* species complexes might be due to the fact that less genes could be recovered for the *P. patagonica* species complex which can also be based on methodological issues (but see the section about suitability of target hybrid enrichment below). However, also biological differences, e.g. in reproduction strategies, can be considered as a factor for differences in speciation scenarios. The reproductive mode remains unclear for *C. megalonyx* but it most likely differs from the brooding strategy of *Pallenopsis* for which eggs-carrying males were caught frequently. Especially if larvae of *C. megalonyx* (which have never been reported) were detached and free-floating, geographic separation would represent smaller barriers. This would also explain the more frequent detection of speciation reversal within the *C. megalonyx* species complexes in comparison to the *P. patagonica* species complex. Although the drivers of speciation within these two sea spider species complexes remain unclear, they most certainly underwent different evolutionary histories, i.e. for the *P. patagonica* species complex, an origin outside Antarctica is supposed whereas for species of the Colossendeidae an origin inside Antarctica has been analysed (Dömel et al. 2017, Dietz et al. 2015, 2019).

With respect to the two speciation processes addressed in this thesis (allopatric speciation in glacial refugia vs. ecological speciation due to adaptation), the allopatric speciation scenario that is mostly promoted by random genetic drift seems to contribute in major parts to speciation processes in the Southern Ocean. However, especially for the *C. megalonyx* species complex,

the data show evidence for positive selection. Thus, at the moment, there is rather little support for ecological speciation but, given the methodological limitations discussed below, it should be considered further when testing mechanism that led and leads to high benthic biodiversity within the Southern Hemisphere.

## Suitability of target hybrid enrichment for studies of the Southern Ocean benthos

Little is known about the size of sea spider genomes but our studies probably include only a small proportion of the whole genome (see Leese et al. 2012 for assumptions based on 454 data). Hence, the amount of recovered genes only represents a small proportion of the sea spiders genome. A better genomic scaffold, i.e. a more complete transcriptome or genome of preferably several species, will increase the number of genes targeted, the knowledge about genes variability to design suitable baits and finally the likelihood to find the genes that are under selection. However, the sequencing and assemblage of genomes or transcriptomes is often expensive, time-consuming and dependent on good sample quality. Anyway, the assignment of gene functions will remain subject to speculation until the functional validation of genes at least for closer relatives of the studied species.

Minor adjustments in bait design are recommended for similar studies that lack sufficient genomic background information to avoid several difficulties during bioinformatic analyses, especially for population genomic approaches. Herein, baits were designed using a three-tiling approach, i.e. the design of up to three baits (each 120 bp long) with an offset of 40 bp within one bait region (up to 200 bp long), to increase the binding efficiency despite the potential presence of intronic regions. In fact, sequencing data showed that there were intronic regions within bait regions. Processing of those reads was difficult because the bait regions to which the reads were mapped to did not have any information about intronic or flanking regions and correct alignment of reads was not possible. Hence, for studies that aim to use target hybrid enrichment to also enrich flanking regions for population genomic studies and base their bait design on transcriptomic data only, a single tiling approach is recommended to avoid mentioned issues when mapping and aligning reads.

In addition to the methodological issues that can partly be avoided in future studies, it should be mentioned that target hybrid enrichment is an expensive approach. The kits for DNA preparation, hybridization and tagging with sequencing primers cost about 6000 € for 16 samples (375 €/sample). Additional costs arise for DNA extraction, quality and quantity assessment of DNA samples, magnetic beads and high throughput sequencing. Hence, due to budget limitations, only a restricted number of samples per species complex could be analysed and the coverage of lineages within the complexes as well as the geographic coverage samples

from good geographical coverage was not adequate for all planned analyses. Therefore, while the method itself has immense potential to study inter- and intraspecific variation, the cost-benefit ratio has to be taken into account for future projects.

Beyond the fact that expeditions to the Southern Ocean are very costly, the limited number of samples from benthic communities of the Southern Hemisphere also relies on habitat accessibility. Consequently, only limited material is available (Kaiser et al. 2013). Observation and documentation methods of marine environments are constantly improving, but the Antarctic environment itself, such as ice sheet expansion and extreme weather conditions, remains an uninfluenceable factor. Hence, research in this remote and extreme location bears both the continuous challenge of facing unexpected problems but moreover the opportunity to explore an exciting and almost unspoiled 'natural laboratory'.

## Outlook

Apart from improvements suggested for target hybrid enrichment, further observational methods and analyses to test for adaptation in sea spiders, in particular when focusing on adaptations in feeding ecology, can be considered. As especially Southern Ocean sea spiders do not have any predators that they have to defend themselves from, one interesting factor that could cause divergent speciation is competition for food (Dietz et al. 2018 – see Appendix I). In fact, Wagner et al. (2017 – see Appendix II) investigated the inner shape and structure of the proboscis, i.e. the sea spider's organ of food uptake, of different sea spider taxa and sets of characters that vary between taxa were established. One very interesting part of the proboscis that showed intertaxon differences was the so-called "oyster basket" which is located at the proximal base of the proboscis (Arnaud and Bamber 1987). The oyster basket consists of varying numbers of rows of setae that can be furcated and exhibited different lengths in relation to the total proboscis length. Therefore, images of proboscides using microcomputed X-ray tomography (μCT) were taken for both sea spider species complexes (Figure 1). This imaging method has the advantage of causing fewer damages to the specimens than, for example, scanning electron microscopy (SEM) for which proboscides are bisected and coated with a golden layer. However, the resolution was too low to interpreted filigree structures such as single setae of the oyster basket. Hence, a project associated with this thesis, also analysed proboscides of all available mitochondrial clade reported for the *P. patagonica* species complex

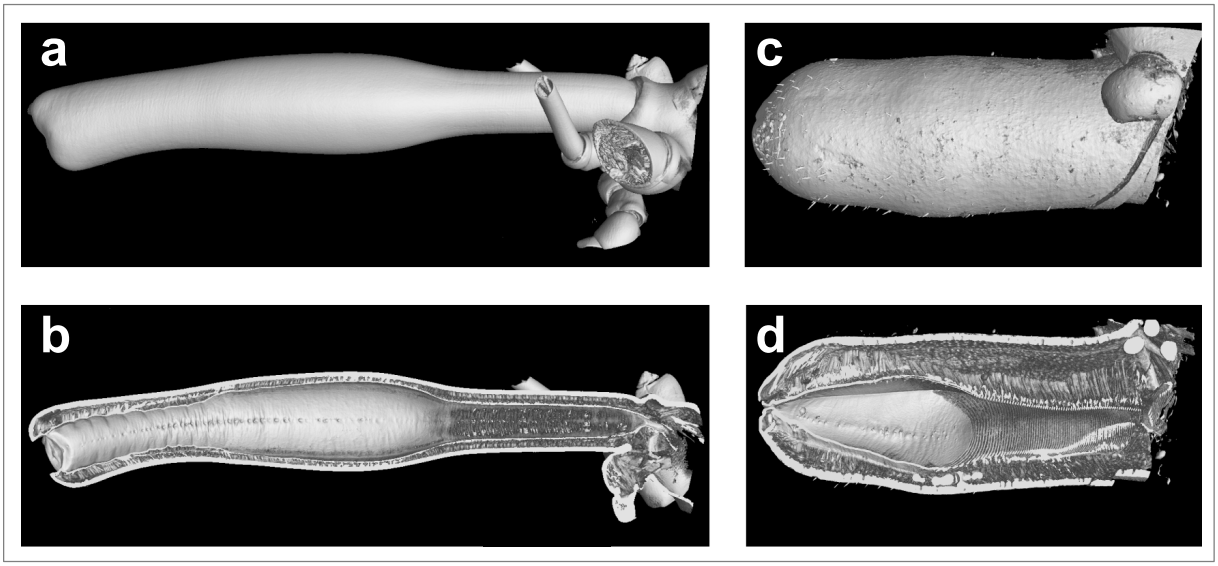



**Figure 1:** Images of µCTs for proboscides of representatives of the species complexes *Colossendeis megalonyx* (a,b) and *Pallenopsis patagonica* (c,d) illustrated from different prospectives (whole, a,c; bisected: b,d).

using SEM. Unfortunately, conclusions about lineage-specific differences in the inner pharynx armatures could not be made so far.

Another completely different approach to analyse food preferences within sea spiders would be to analyse the gut content of species. Especially molecular gut content analyses have improved the study of food webs and feeding interactions and have also been used in arthropods (e.g. Roualdes et al. 2016, Macias-Hernandez et al. 2018). For this method, DNA of the gut content is extracted, amplified and sequenced using a metabarcoding approach.

This was also tested for the sea spider species complex *C. megalonyx* during a project associated with this thesis but did not succeed (Macher 2016). One initial problem was the preparation of the gut. Due to the limited space within the trunk, the digestive system of sea spiders expands in form of digestive diverticula into the legs, often as far as the tarsus (Arnaud and Bamber 1987, Fahrenbach and Arango, 2007) and it is difficult to estimate which part of the digestive tract would be most informative to infer diet composition. The latter has also been investigated by Macias-Hernandez et al. (2018) in spiders which have a similar expansion of the digestive tract. They found out that prey DNA can be detected in all body parts, but is most abundant in the opisthosoma (probably most comparable with the trunk of sea spiders). Hence, for gut content analyses in sea spiders, DNA should best be extracted from the trunk. Still, morphological differentiation of the digestive tract especially of conserved specimens proved difficult and a co-extraction of sea spider DNA had to be assumed. Therefore, similar to an approach in fishes (Su et al. 2018) blocking primers were developed for *C. megalonyx* to reduce the amplification of predator DNA and simultaneously enhance the resolution of prey DNA. However, no reduction of predator DNA amplification using those blocking primers could be retained and further analyses have been procrastinated. Hence, metabarcoding primers

specialized for potential sea spider prey (after Helfer and Schlottke 1935) were designed but have not been tested so far. Further concerns are that Fahrenbach and Arango (2007) hypothesise that the food digestion begins in the proboscis and prey cells are already reduced to subcellular dimensions and it is unclear if cells of prey reach the sea spiders gut at all. In this case, the detectability of prey DNA in the gut content would be dramatically decreased. In addition to that, the duration between ingestion events can be long and survival without food uptake, particularly for Southern Ocean sea spiders, over several months and up to years is possible (personal communication with the aquarium manager Rebecca Smith of the British Antarctic Survey, Cambridge, UK) which would decrease the detectability even more.

However, there is one further promising method that could be applied to investigate interaction in food webs: stable isotope signatures. The two most commonly used stable isotope ratios in food web analyses are those of nitrogen ($^{15}N/^{14}N$) and carbon ($^{13}C/^{12}C$). Predators typically show a higher ratio in nitrogen relative to their prey or food and consequently, nitrogen isotope measurements serve as indicators for trophic positions (McCutchan et al. 2003, Vanderklift and Ponsard 2003). In contrast, values of carbon isotopes vary little along the food chain and are mainly used to determine primary sources in a trophic network (Kelly 2000, McCutchan et al. 2003). In the marine environment, values of carbon isotopes can also indicate pelagic versus benthic contribution to food intake (Hobson et al. 1994). Analyses of stable isotope signatures would be particularly interesting for species that occur in sympatry (e.g. *P. patagonica* s.s. and *P. yepayekae* Weis, 2014 in Patagonian, *P. aulaeturcarum* sp. nov. and *P. buphthalmus* in the Eastern Weddell Sea, clade E and D of the *C. megalonyx* complex from Terre Adélie, and the hot spot being the Scotia Arc where eight mitochondrial clades of the *C. megalonyx* complex have been reported for).

# Conclusion

**Development of laboratory workflow and bioinformatic analyses of a target hybrid enrichment approach for sea spiders based on a single transcriptome to study speciation processes and drivers.**

A laboratory workflow and a pipeline for bioinformatic analyses for the application of target hybrid enrichment was successfully established for all sea spiders analysed.

**Analyses and discussions of genetic and morphological variation in the context of speciation processes.**

a. *Pallenopsis patagonica*

*i.* *Can a higher species diversity within the* P. patagonica *species complex be detected when extending the sample set and adding genome-wide data?*

- Additional samples especially of locations previously not included in analyses uncovered a higher species diversity within the *P. patagonica* species complex. Genome-wide data did not yield a higher species diversity but provided a better resolution to understand the phylogenetic relationship between multiple lineages. A clear split between species from the Antarctica continental shelf and Patagonian was detected.

*ii.* *Can independently evolving lineages in the* P. patagonica *species complex be distinguished and new species formally be described based on morphometric measurements and morphological characters?*

- Diagnostic characters to distinguish species were found for the majority of lineages. Based on those, two new species could be described for the *P. patagonica* species complex. Further lineages could be assigned to already existing species. However, a few lineages could not be distinguished morphologically and hence represent cryptic species.

*iii.* *Is there evidence for adaptive divergence especially for lineages that occur in sympatry or do neutral evolutionary processes suffice to explain the observed species diversity within the* P. patagonica *species complex?*

- No evidence that would explain speciation due to adaptive speciation could be found. Therefore, allopatric speciation is proposed as the main speciation process that led to multiple lineages within the *P. patagonica* species complex.

b. *Colossendeis megalonyx*

*i. Can a higher species diversity within the* C. megalonyx *species complex be detected when adding genome-wide data?*

- Genome-wide data substantially improved the resolution of the phylogenetic relationships within the *C. megalonyx* species complex.

*ii. Can genome-wide data resolve the level of intraspecific connectivity between population of phylogenetic lineages within the C. megalonyx species complex are connected?*

- Low intraspecific connectivity between geographically separated populations could be detected.

*iii. Do lineages within the* C. megalonyx *species complex show differences between morphological characters which enable to distinguish them?*

- Several significant differences of characters between species were detected. However, diagnostic characters or sets of characters that would help to distinguish species morphologically could not be defined.

*iv. Are there distinct signatures of positive selection indicating adaptive divergence within the* C. megalonyx *species complex?*

- Target hybrid enrichment enabled to test for selection in over thousand genes of the *C. megalonyx* species complex. Furthermore, a few genes were found to be under positive selection but interpretations of genes function were vague and conclusions about changes in specimen's biology relevant for speciation processes could not be made. Therefore, there are indications that also ecological speciation, in addition to allopatric speciation, plays a role in species divergences of the *C. megalonyx* species complex.

c. longitarsal Colossendeidae

*i. Do genome-wide data of the group of longitarsal Colossendeidae resolve the groups phylogeny and serve as backbone for biogeographic analyses?*

- Genome-wide data revealed good resolutions for the monophyletic group of longitarsal Colossendeidae. Results of the phylogenomic tree were used to apply biogeographic analyses.

*ii. Do Antarctic species within the group of longitarsal Colossendeidae constitute a monophyletic radiation and thus support an out-of-Antarctica scenario for at least some non-Antarctic species?*

- Analyses of the longitarsal Colossendeidae support an Antarctic origin and multiple dispersal events to other regions.

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# Feeding ecology in sea spiders (Arthropoda: Pycnogonida): what do we know?

Lars Dietz[1,2*], Jana S. Dömel[3], Florian Leese[3], Tobias Lehmann[4,5] and Roland R. Melzer[4,5,6]

**Abstract:** Sea spiders (Pycnogonida) are a widespread and phylogenetically important group of marine arthropods. However, their biology remains understudied, and detailed information about their feeding ecology is difficult to find. Observations on pycnogonid feeding are scattered in the literature, often in older sources written in various languages, and have never been comprehensively summarized. Here we provide an overview of all information on feeding in pycnogonids that we have been able to find and review what is known on feeding specializations and preferences in the various pycnogonid taxa. We deduce general findings where possible and outline future steps necessary to gain a better understanding of the feeding ecology of one of the world's most bizarre animal taxa.

**Keywords:** Pantopoda, Marine arthropods, Food chain, Benthos, Community ecology

## Background

Sea spiders (Pycnogonida) are a phylogenetically distinct group of marine arthropods with about 1500 species. General reviews of their biology were provided by King [1] and Arnaud & Bamber [2]. Almost all species have a holobenthic lifestyle. They are particularly abundant and species-rich in the polar regions, where genetic studies have identified several cases of unrecognized diversity [3, 4].

Although pycnogonids are widespread in all oceans and have been known to science for over 250 years, the feeding habits of most taxa remain poorly studied and a detailed review on the feeding ecology of pycnogonids has, to our knowledge, never been published. Observations on this topic are generally scattered throughout the literature, and especially publications written in languages other than English are often difficult to find. General textbooks usually only state that pycnogonids feed mostly on sessile prey, such as coelenterates, sponges and bryozoans (e.g., [5]).

In the present paper, we review all available observations published in the last two centuries including both detailed studies and preliminary notes, thus providing a state of the art summary of known food preferences for this bizarre and highly understudied group of exclusively marine arthropods. Additionally, we discuss morphological correlates of different feeding preferences and the occurrence of generalism vs. specialization in various pycnogonid taxa.

## Morphological features for food uptake

A pycnogonid that features all appendages used for feeding (*Nymphon gracile*) is pictured in Fig. 1c. As the main organ for food uptake, pycnogonids have a unique triradially symmetric proboscis with a terminal mouth surrounded by three movable lips and gland openings probably secreting saliva [6]. The proboscis musculature allows suction and pumping of food, mostly in liquid form. Moreover, the proximal part of the proboscis contains the pharyngeal filter, also termed "oyster basket" or "Reusenapparat" (in old literature in German, e.g. [7]), which is composed of densely packed bristles that are used to filter out or grind ingested solid particles. Recently Wagner et al. [8] have compared pharynx inner surfaces of various pycnogonids using scanning electron microscopy and showed taxon-specific features of the filter bristles and other pharynx armatures, e.g.

* Correspondence: ldietz@uni-bonn.de
[1]Zoological Research Museum Alexander Koenig, Statistical Phylogenetics and Phylogenomics, Adenauerallee 160, D-53113 Bonn, Germany
[2]Faculty of Mathematics and Natural Sciences, University of Bonn, D-53012 Bonn, Germany
Full list of author information is available at the end of the article

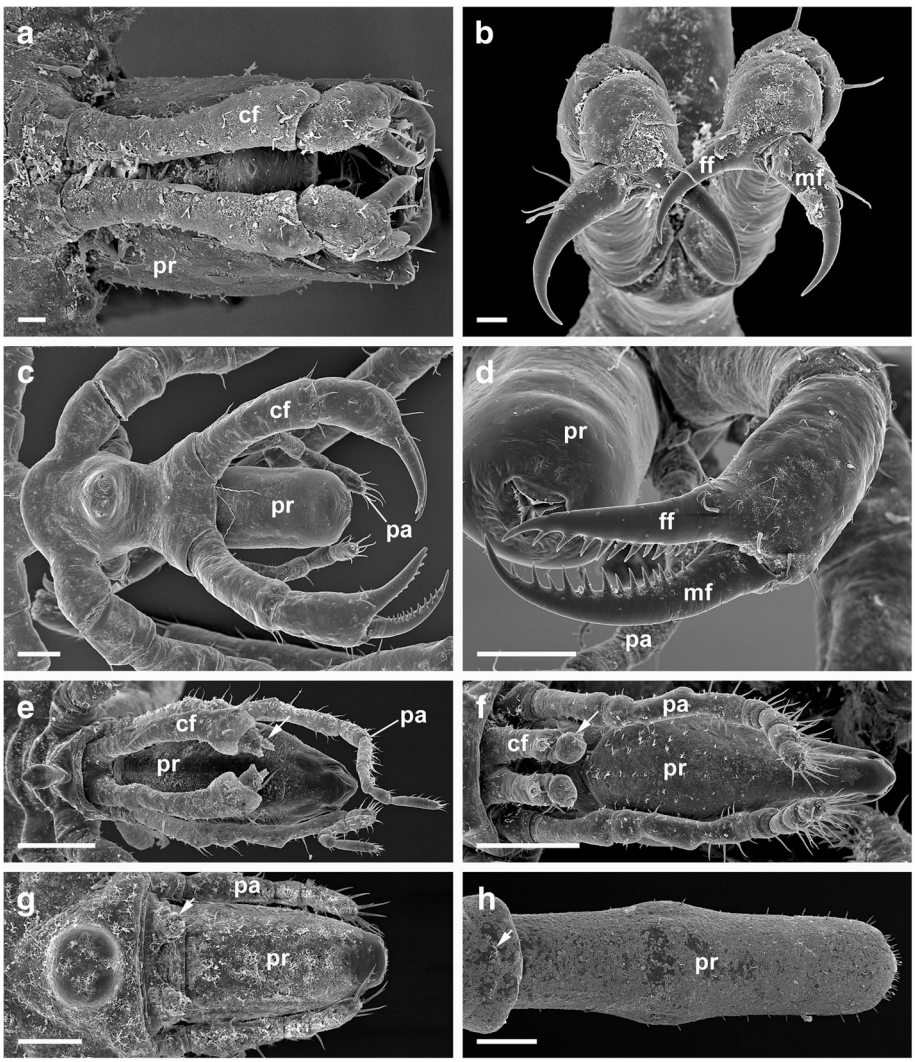


**Fig. 1** Chelifores and palps of different pycnogonid families showing different morphologies. Originals, except B after [115]. **a** *Anoplodactylus angulatus*, with dorsally positioned chelifores, palps absent. Bar 20 μm. **b** *Anoplodactylus petiolatus*, detail of chelifore with unarmed fixed and movable finger. Bar 20 μm. **c** *Nymphon gracile*, with laterally positioned chelifores and dorsally positioned palps. Bar 100 μm. **d** *Nymphon gracile*, detail of chelifore with toothed fixed and movable finger. Bar 100 μm. **e** *Ammothella appendiculata*, with reduced chela. Fixed and movable finger still present (arrow). Palps long, extending beyond proboscis. Bar 200 μm. **f** *Achelia echinata*, with reduced chela. Fixed and movable finger fused to small bud (arrow). Palps with approx. Same length of proboscis. Bar 200 μm. **g** *Tanystylum conirostre*, chelifore reduced to small bud with seta (arrowhead). Palps shorter than proboscis. Bar 100 μm. **h** *Endeis spinosa*, chelifore reduced protuberance with seta (arrowhead). Palps absent. Bar 200 μm. cf., chelifore; ff, fixed finger; mf, movable finger; pa, palpus; pr, proboscis

denticle arrays. However, as differences in feeding ecology between pycnogonid taxa are so far poorly known, no definite conclusions on correlation with feeding modes could be made. The morphology of the mouth opening also differs, as the lips are often fringed with microtrichia of various numbers and lengths (Fig. 2). In some cases, these are reduced or lost, and the lips are either fringed with papillae (some ammotheids) or not armed at all, as in *Anoplodactylus*. Pycnogonid taxa also differ in whether the mouth is surrounded by setae, as in *Endeis* (Fig. 2a), or not, as e.g. in *Ammothella* (Fig. 2f). In *Endeis*, which lacks palps, the setae have a tactile function [1]. This indicates that different pycnogonid taxa have different "toolboxes" for handling food, though in a superficial inspection the general morphology of their feeding apparatus looks quite uniform. Ammotheids and ascorhynchids, most of which lack functional chelifores and feed on hydroids, often have a more mobile proboscis than

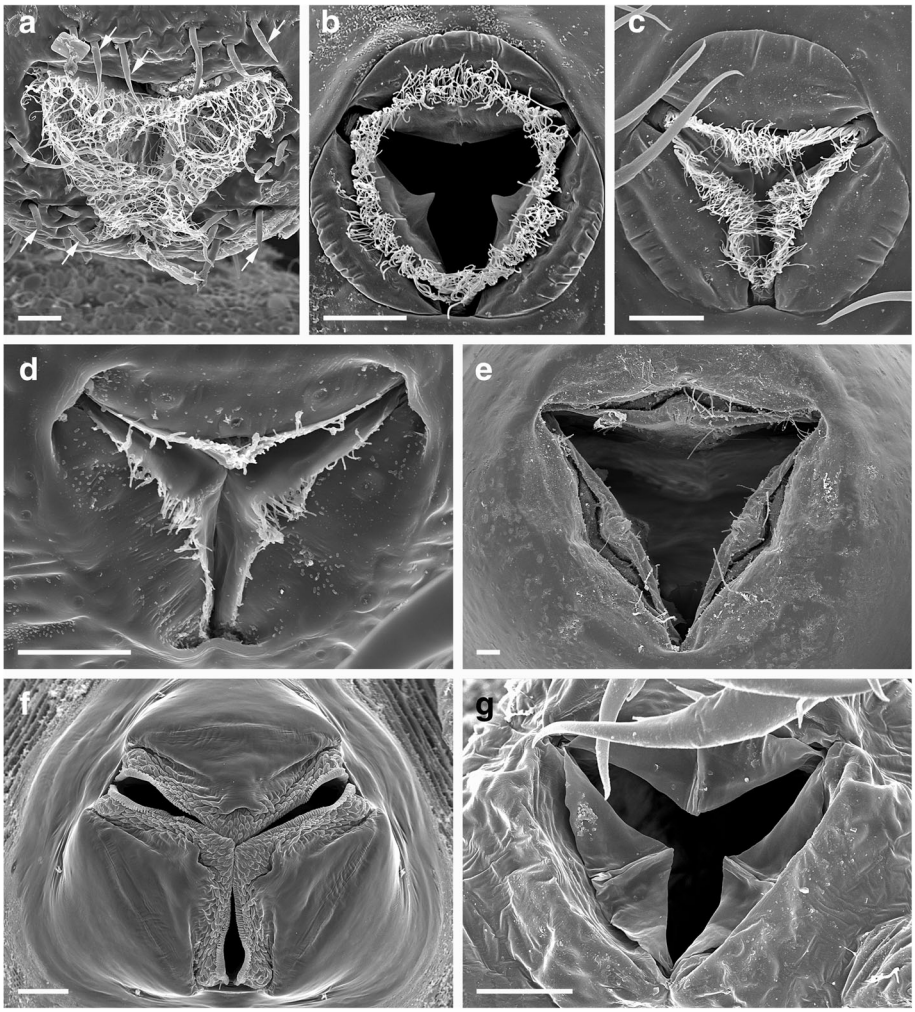


**Fig. 2** Mouth openings of different pycnogonid families showing different morphologies. Dorsal is up. Originals, except A, B, C, F, G after [119]. Bars 20 μm. **a** *Endeis spinosa,* mouth surrounded by setae (arrows) and lips fringed with many microtrichia. **b** *Callipallene tiberi,* mouth closed, lips fringed with microtrichia. **c** *Callipallene phantoma*, mouth open, lips fringed with microtrichia. **d** *Nymphon gracile*, lips fringed with few microtrichia. **e** *Pycnogonum littorale*, lips occasionally fringed with microtrichia. **f** *Ammothella appendiculata*, mouth without seta or microtrichia, but fringed with papillae. **g** *Anoplodactylus angulatus*, mouth equipped with three valves

nymphonids and other taxa with chelifores [1]. However, this does not apply to taxa without chelifores that are parasitic on much larger animals (Pycnogonidae) or detritivorous (*Endeis*).

Other organs important for feeding in pycnogonids are the chelifores and palps, which are homologous to the arachnid chelicerae and pedipalps, respectively [9]. The chelifores consist of a scape and a chela with a movable and an immovable finger and are used for cutting off and macerating pieces of the prey organism and leading them to the proboscis (Fig. 1). The chelifores can be placed dorsally (e.g. in the Phoxichilidiidae, Fig. 1a,b) or laterally (e.g. in *Nymphon*, Fig. 1c,d) of the proboscis. According to Wyer & King [10], only species with laterally positioned chelifores use them to macerate prey, as they are more mobile than dorsally placed ones. For this purpose, when the chelifores are laterally positioned, they often have serrated chelae (Fig. 1d). In the adults of some taxa, the chelifores are highly reduced (many Ammotheidae, Fig. 1e-g) or lost (Austrodecidae, Colossendeidae, Rhynchothoracidae, Pycnogonidae, Endeidae, Fig. 1h). The palps are, besides their tactile function, also used to hold the prey items or guide the proboscis. Palps differ between taxa in the degree of robustness and supination as well as in their length relative to the proboscis and the number and proportion of articles

(Fig. 1e-g). In some taxa they are reduced or lost (Pycnogonidae, Callipallenidae, Pallenopsidae, Phoxichilidiidae, Endeidae, Fig. 1a,h). The walking legs, of which there are four (rarely five or six) pairs, can also be used to hold prey, and the morphology of their distal parts also differs between taxa. The prey is held between the claw and the propodus, which often has spines on its ventral surface. In some pycnogonids, such as *Nymphon brevirostre* and members of the Phoxichilidiidae, the tarsus is extremely short and the propodus is curved, apparently as an adaptation for climbing among hydroids, on which they feed [11].

The digestive system of pycnogonids was described by Fahrenbach & Arango [6]. It is divided into a foregut within the proboscis, where food processing and filtering take place as described above, a midgut where the food is digested and absorbed, and a hindgut covered by cuticle in the reduced abdomen. The midgut is remarkable in that it has diverticula extending into the walking legs and chelifores, which in most, but not all species reach almost to the tips of these appendages. The mechanism of digestion was described by Richards & Fry [12]. Intracellular digestion occurs exclusively by pinocytosis, i.e. only liquid material is taken up.

## What do sea spiders eat?

Pycnogonids are usually described as predatory or parasitic. The difference between these terms is that, while predators kill their prey and often consume all or most of the organism, parasites usually do not directly kill their host [13]. Under this definition, most pycnogonids can be described as parasitic. Parasitism in pycnogonids was reviewed by Staples [14], who also treated feeding on hydroids and other colonial organisms as parasitism, not as predation. While infestations occasionally lead to the death of the host (e.g. [15]), this also occurs in other parasite-host relationships. However, there are some cases of predation by pycnogonids, in which entire animals (e.g. annelids; [16, 17]) were consumed. In almost all cases, parasitism by adult pycnogonids can be categorized as ectoparasitism, although some instances of endoparasitism in the pallial cavity of molluscs and in actinians are known. Other pycnogonids can be described as herbivorous [10] or detritivorous (e.g. [17]). Pycnogonid larvae are either obligate parasites or lecithotrophic and can be either ecto- or endoparasitic (see overview in [18]). Chelifores, palps and ovigera are already present in the earliest larval stages and are used for attachment to the host (Fig. 3d-f).

**Box 1 First reports were often erroneous**

The first records of pycnogonid feeding were erroneous. To our best knowledge, Linnaeus [120] was the first who mentioned a pycnogonid, identified as *Phoxichilidium femoratum* by Calman [121], feeding by drilling holes with its proboscis into the shells of mussels (*Mytilus* spp.). However, this way of feeding appears to be physically impossible, as the tissue of the proboscis lips is certainly not hard enough to drill into a molluscan shell. Similarly erroneous was the claim by Lamarck [122] and others that *Pycnogonum* is parasitic on whales, which was based on confusion with cyamid amphipods [123]. The first reliable observations on pycnogonid feeding were given by Zenker [19], who reported on food being found in the dissected proboscis of *Nymphon gracile*. Parasitism on hydroids by pycnogonid larvae was first documented by Allman [124]. Adult pycnogonids have often been found on hydroids and other sessile organisms (e.g. [7]), but the first documented observation of feeding was published by Cole [36] for *Anoplodactylus lentus*. Further detailed observations on the feeding mode of several pycnogonid species belonging to different families were recorded by Prell [39]. Later, some authors also performed experiments in which the food preference of different pycnogonid species, mostly from the North Sea [16, 25], but also from the Southern Ocean [26] was tested.

A synopsis of pycnogonid-host associations was given by Helfer & Schlottke [116], however, not in all cases the pycnogonid can be assumed to be feeding on the organism on which it was found (Fig. 4 shows some associations of pycnogonids with other organisms, and it is unclear whether any of these are used as a food source). Some entries in their table are also erroneous, with the original sources actually describing epibionts or predation on pycnogonids. King [1] updated this synopsis, distinguishing between associations of larval and adult pycnogonids with their hosts and cases where the pycnogonids were actually observed feeding. A further short review of pycnogonid feeding was provided by Arnaud & Bamber [2] as part of their general review of pycnogonid biology.

## Feeding specializations

In the following section, published records of feeding by pycnogonids on different types of prey are summarized (see also Table 1) and possible specializations of various taxa are discussed.

### Algae

Zenker [19] reported about finding tissue of probably brown algal origin in the proboscis of *Nymphon gracile*. Wyer & King [10] mentioned *Ammothella longipes* feeding on the red alga *Mastocarpus*

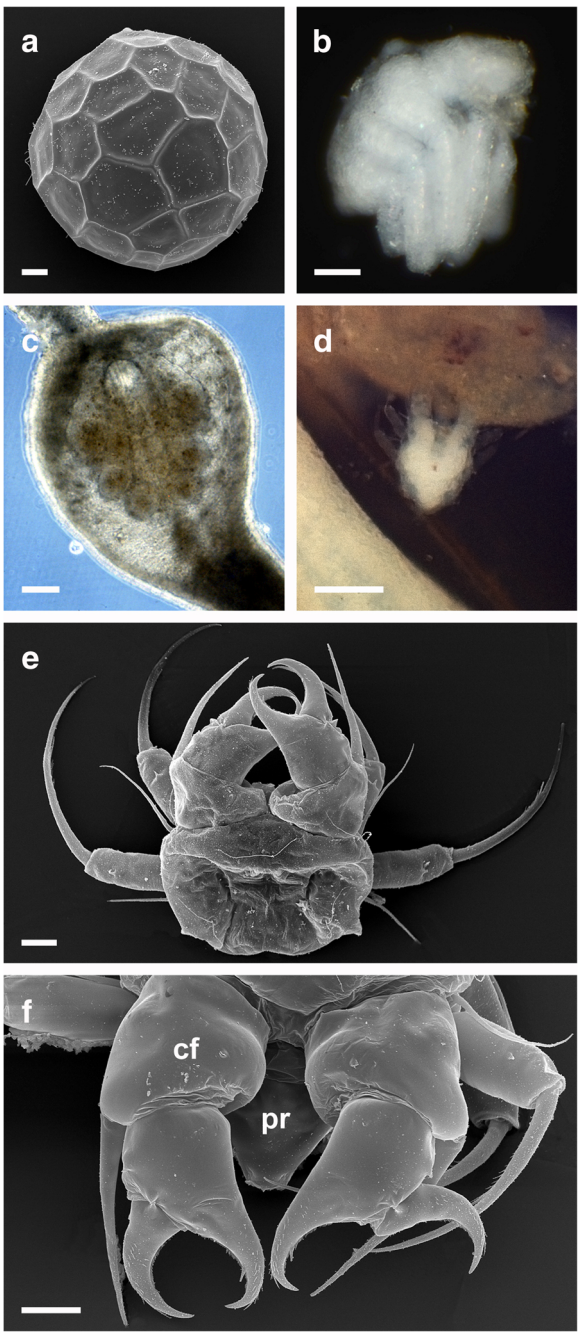


**Fig. 3** Feeding and morphological features of protonymphon larva and subadults. Originals, except C after [42]. **a** *Callipallene spectrum*, SEM micrograph of Egg. Bar 20 μm. **b** *Callipallene producta*, newly hatched postlarva. Bar 40 μm. **c** *Anoplodactylus petiolatus*, larva in a gallzooid of *Hydractinia echinata*. Bar 50 μm. **d** *Achelia spec.*, protonymphon detached on host organism. Bar 100 μm. **e** *Achelia echinata*, SEM micrograph of protonymphon, dorsal view. Bar 20 μm. **f** *Achelia echinata*, SEM micrograph of chelifore and proboscis. Bar 20 μm. cf., chelifore; pr, proboscis

*stellatus*. In the case of *A. longipes* on brown algae (*Halopteris*), Soler-Membrives et al. [17] found this species not actually consuming the algae, but the detritus accumulated on them. Bamber & Davis [20] showed that *Achelia echinata* feeds on the green alga *Ulva* and the red alga *Griffithsia* by labelling the algae radioactively. *Ulva* seems to be preferred. From the paucity of observations, we conclude that algae or detritus from algal structures seem to be a food source of minor importance, although they are consumed by several phylogenetically distantly related sea spiders. It is possible that algal tissue is sometimes ingested when pycnogonids are feeding on organisms living on the algae, or as part of the gut content of their prey.

### Sponges

Marcus [21] observed a specimen of *Ascorhynchus corderoi* feeding on an unidentified sponge. Dayton et al. [22] recorded *Ammothea striata* feeding on a sponge, which also was not identified. *Colossendeis* was observed carrying a piece of possible sponge underneath its body [23]. Cuartas & Excoffon [24] reported that *Tanystylum orbiculare* and *Anoplodactylus petiolatus* fed on the demosponge *Hymeniacidon perlevis* when their preferred hydroid prey was not available. In conclusion, sponges appear to be uncommon as a pycnogonid food source, although they are often mentioned as such in more general reviews. However, it should be noted that pycnogonid feeding on sponges is understudied, as most of the studies investigating food preference in pycnogonids did not include sponges as a possible prey item (e.g. [25]). The results of the only study known to us that does include them [26] were inconclusive as to whether the pycnogonids actually fed on the sponges.

### Hydroids

Associations of pycnogonid larvae with their (mostly hydroid) hosts have been summarized by King [1] and Staples & Watson [27]. The larvae of some phoxichilidiids and ammotheids are endoparasites forming galls in the gastral cavity of hydroid polyps. Hodge [28] first observed this for *Phoxichilidium femoratum* on *Coryne eximia* and Semper [29] documented the development of the same species in more detail on *Hydractinia echinata*. Dogiel [30] also found a similar mode of development in *Endeis spinosa*, whose larva develops attached to the hydranth of *Obelia* sp. Since then, such a relationship has also been found in many other species (see overview in [31]). In most Ammotheidae and Pycnogonidae as well as in *Nymphon gracile* [32], the larvae are ectoparasites of hydroids, although in the Pycnogonidae the adults feed mostly on actinians [30, 33]. Russel & Hedgpeth

**Table 1** Summary of known food sources for pycnogonid family-level taxa

| | Algae | Sponges | Hydroids | Actinians | Corals | Medusae | Bryozoans | Mollusks | Annelids | Crustaceans | Echinoderms | Detritus |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Austrodecidae | | | + | | | | + | | | | | |
| Colossendeidae | | ? | + | + | + | + | | + | + | | | + |
| Rhynchothoracidae | | | + | | | | + | | | | | |
| Pycnogonidae | | | | + | | + | + | | | | + | + |
| Ascorhynchidae | | + | | | ? | | | + | | | | |
| Nymphonidae | ? | | + | + | ? | | + | + | + | + | | + |
| Callipallenidae | | | + | | | | + | | + | | | |
| Pallenopsidae | | | ? | | + | + | | | | | | + |
| Phoxichilidiidae | | + | + | | | + | + | + | + | + | + | |
| Endeidae | | | + | + | + | | | | | | | + |
| Ammotheidae | + | + | + | + | + | + | + | + | + | + | + | + |
| Incertae sedis | | | | + | | | | | + | | | |

A plus sign indicates a definitive feeding association, a question mark indicates an association not confirmed by direct observations of feeding or gut content

[34] reported on the presence of larvae of two ammotheid species on the hydroid *Orthopyxis everta*, the ectoparasitic *Ammothea hilgendorfi* and the endoparasitic, gall-forming *Tanystylum duospinum*. Adults of both species are also found on the hydroid. Often the larvae appear to be host-specific and development can differ even between closely related species, e. g. *Anoplodactylus pygmaeus* larvae form galls in the gastral cavity of *Obelia* polyps, while those of the closely related *A. petiolatus* live attached to the manubrium of medusae from the same genus [35].

Feeding of adult pycnogonids on hydroids also has often been documented. Cole [36] observed adults of *Anoplodactylus lentus* feeding on *Eudendrium ramosum*. The hydranths were cut off with the chelifores and placed in front of the mouth. Loman [37] reported the same for *Phoxichilidium femoratum* feeding on *Tubularia*, with gonophores being preferred as food over other parts of the hydroid. According to Loman [38], *Nymphon brevirostre* feeds on the same species. Prell [39] reported that several *Nymphon* species from the North Sea feed almost exclusively on thecate hydroids (*Lafoea* in the wild, *Campanularia* in an aquarium setting). The hydrothecae are led to the mouth without breaking them off using the chelifores. Athecate hydroids are consumed only in case of extreme starvation. Agreeing with this, according to Schlottke [40], *N. brevirostre* prefers the thecate *Obelia geniculata* to the athecate *Coryne pusilla*. He also observed *Anoplodactylus pygmaeus* and *Phoxichilidium femoratum* feeding on various hydroid species. Wyer & King [10] observed several species of North Atlantic pycnogonids (*Nymphon gracile*, *Phoxichilidium femoratum*, *Anoplodactylus petiolatus* and *Achelia echinata*) feeding on *Dynamena pumila*, while *Nymphon brevirostre* fed on various hydroids epizoic on the bryozoan *Flustra foliacea*. They noted that in *N. gracile* the (laterally positioned) chelae were used to macerate the prey whereas this is not the case in the phoxichilidiids, where they are dorsally positioned and only used for grasping. *A. echinata*, which has reduced chelifores, grasps hydroid tentacles and pulls them off with the proboscis lips. Lotz [16] found that *Achelia echinata*, *Nymphon brevirostre* and *Callipallene brevirostris* do not accept non-hydroid food, and starve if no hydroids are present. However, *Anoplodactylus petiolatus*, which normally also feeds on hydroids, does accept other food. Stock [25] showed *Nymphon gracile*, *N. brevirostre* and *Endeis spinosa* are chemically attracted to various hydroid species. While *N. brevirostre* and *E. spinosa* prefer *Laomedea*, *N. gracile* prefers *Dynamena*. Staples & Watson [27] documented multiple cases of pycnogonid-hydroid association in Australia and New Zealand. Particularly notable is the association of *Austrodecus frigorifugum* with *Dictyocladium monilifer*. The pycnogonid, which lacks chelifores, inserts its very narrow proboscis, guided by its palps, into the hydrothecae and gonothecae of the hydroid. In contrast, the related Antarctic species *A. glaciale* feeds mostly on bryozoans [26]. According to Staples & Watson [27], the pointed proboscis of *Achelia transfugoides* is adapted for feeding on the hydrothecae of *Stereotheca elongata* and *Sertularia marginata*. They also report that *Parapallene australiensis* occurs in such great numbers on *Halopteris glutinosa* that they infer an obligatory association, and the same appears to be the case for *Tanystylum* sp. and *Pennaria wilsoni*. According to Varoli [41], both *Anoplodactylus stictus* and *Tanystylum isabellae* accept *Sertularia* as food, but not *Dynamena*. Both hydroids belong to the family Sertulariidae. Heß & Melzer [42] reported on the feeding of *Anoplodactylus petiolatus* on *Hydractinia echinata*. The pycnogonid feeds mostly at night and avoids touching the hydroid polyps, feeding mostly on

the tips of spines. However, even pycnogonids that are almost completely engorged by the polyps are able to pull themselves out using their legs.

As pycnogonids are particularly common in the Southern Ocean, many observations of their feeding on hydroids are also recorded from there. Hodgson [43] mentioned that the Antarctic pycnogonid *Decolopoda* was observed holding *Tubularia* hydranths in its chelae (note that *Decolopoda* and *Dodecolopoda* are unusual among the Colossendeidae by their presence of chelifores). According to Dayton et al. [22], *Colossendeis robusta* and *C. megalonyx* were also seen feeding exclusively on hydroids, mostly on a small unidentified species growing on sponges. An unidentified species of *Colossendeis* was also photographed feeding on a solitary hydroid in the North Central Pacific [44]. Fry [26] found that, when provided with a diverse selection of food items, *Rhynchothorax australis* preferred hydroids, especially *Eudendrium tottoni*. The preference of *R. australis* for *E. tottoni* was explained by the fact that this was the only athecate among the tested hydroid species, and that its hydranths are therefore less protected. However, this explanation seems to be contradicted by the observation that *Nymphon* follows the opposite pattern [39]. *Austrodecus glaciale* also fed on hydroids, although its preferred food was bryozoans. Stout & Shabica [45] also recorded several other Antarctic species (*Austrodecus* sp., *Pentanymphon antarcticum*, *Nymphon* sp., *Achelia* sp.) associated with or feeding on hydroids. Richards [46] reported that *Nymphon australe* was found with hydroid colonies grasped in its chelifores. *Pallenopsis yepayekae* was photographed on a plumulariid hydrozoan (this paper, Fig. 4c), but it cannot be determined whether feeding actually took place. In conclusion, hydroids seem to be a food item of major importance for most pycnogonid groups. We found more records of pycnogonids feeding on hydroids than on any other type of prey. It is possible that, in some cases, pycnogonids attack hydroids to feed on their gut content, as has been observed for sea anemones (see below). This behaviour would be a type of kleptoparasitism, or if the hydroid is also consumed, kleptopredation, as has been observed in nudibranchs [47].

### Actinians

Pycnogonids in the family Pycnogonidae appear to be specialist feeders on actinians. The wide proboscis and the ability to open the mouth widely can be interpreted as specializations for ingesting large amounts of soft-bodied animal tissue. Although associations between Pycnogonidae and anemones had been observed earlier, the feeding mechanism of *Pycnogonum* was first documented by Prell [39] for *P. litorale* on *Metridium* and *Urticina crassicornis*. According to him, the animal feeds mostly on the pedal disk of the actinians, using its first pair of legs to span the skin before inserting its proboscis (Pycnogonidae lack chelifores and palps). The same was

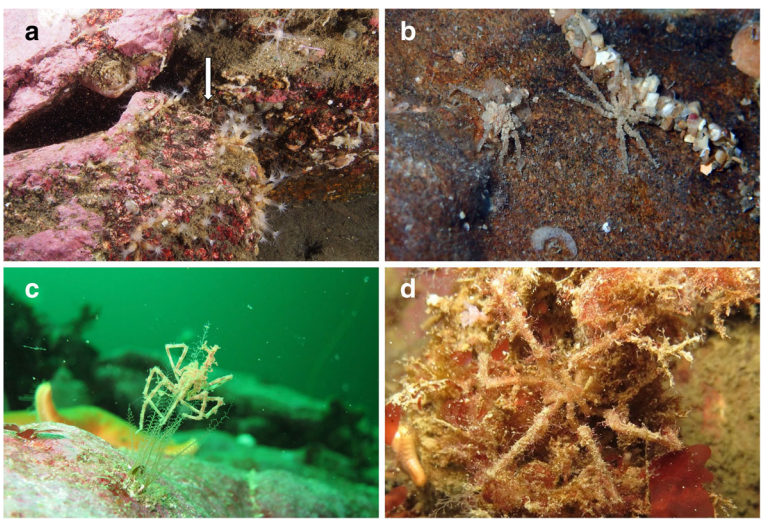


**Fig. 4** Pycnogonids in their natural environment, near possible food sources. **a** *Callipallene margarita* and its surroundings mainly built-up by red algae, *Clavularia* octocorals, and organic debris; Southern Chilean fjords, photo: Kaitlin McConnell. Pycnogonid indicated by arrow. **b** Female (right) and male (left) *Achelia langi* under a stone in wave dominated upper infralitoral near a *Polycirrus* polychaete; note male carrying fertilized eggs; Northern Adriatic, photo: Roland Melzer. **c** and **d** *Pallenopsis yepayekae*; C on a plumulariid hydrozoan. The pycnogonid may be feeding on the polyps, but this cannot be certainly determined. Southern Chilean fjords, photo: Roland Meyer. D On red algae, well camouflaged by a "roof-garden". Southern Chilean fjords, photo: Roland Melzer

observed by Wyer & King [10] for *P. litorale* feeding on various actinian species. Arndt [48] reported an individual of the same species with its proboscis bored into a tentacle of *Edwardsiella loveni*. Wilhelm et al. [33] documented that, after the transition from larval to juvenile stage, *P. litorale* immediately shifts from its original hydroid host to the actinian *Metridium senile*. Bamber [49] showed that *P. litorale* had a preference for some anemones (*Calliactis* and *Adamsia*) over others (*Actinia* and *Tealia*). In the case of *Adamsia,* the entire anemone was consumed. These observations are difficult to explain as the preferred anemones are symbionts of hermit crabs and therefore normally inaccessible to the pycnogonids. Other species of *Pycnogonum*, such as *P. stearnsi* [26] and *P. benokianum* [50] have also been documented as actinian predators.

Other pycnogonids have also been documented feeding on actinians. Stock [25] showed that *Nymphon brevirostre*, and possibly *Endeis spinosa*, can discern the presence of actinians in seawater by chemical cues and are attracted to them, although they are not the preferred food. *Artemidactis victrix* is the preferred food of *Ammothea striata* according to Stock [22]. Wyer & King [10] reported *Nymphon gracile* feeding on *Actinia equina*. In most cases, the feeding mechanism was similar to that of *Pycnogonum*, but occasionally tentacles or other pieces of the actinian were removed with the chelifores. Richards [46] observed *Ammothea carolinensis* feeding exclusively on anemones. *Nymphon orcadense*, *N. hirtipes* and *Decolopoda australis* were also observed feeding on actinians in an aquarium setting. *A. carolinensis* inserted its proboscis into the mouth opening of the anemone, leading to the suggestion that it feeds only on the gut contents (kleptoparasitism). *D. australis* was observed carrying the anemone around in its proboscis after separating it from the rock. This behavior is also visible in a photograph by Wu [51] showing an Antarctic pycnogonid identifiable as belonging to the *Colossendeis megalonyx* complex. Braby et al. [52] observed *Colossendeis minuta* and *C. colossea* feeding on the anemones *Anthosactis pearseae* and *Liponema brevicorne*. While the smaller *A. pearseae* was always consumed in its entirety after separating it from the rock, in *L. brevicollis* sometimes autotomized tentacles were consumed. *Colossendeis* sp. was also observed feeding on actinostolid anemones in the Southern Ocean [53]. Mercier et al. [54] also observed *N. hirtipes* feeding on the actinian *Stephanauge nexilis* in the wild. Mercier & Hamel [15] reported on the small pycnogonid *Pigrogromitus timsanus* parasitizing the actinian *Bartholomea annulata*, leading to the host's death. The pycnogonids were found more frequently on the column than on the tentacles, which would enable them to feed on the gonads. This agrees with other observations (e.g. [37]) that pycnogonids preferentially feed on the gonadal tissues of coelenterates. Endoparasitism of actinians (*Entacmaea quadricolor*) by juvenile pycnogonids (*Ammothella biunguiculata*) has also been documented [55]. Therefore, actinians are an important food source mostly for members of the Pycnogonidae, as well as some pycnogonids belonging to other taxa.

### Other cnidarians

Pycnogonids have also been documented to feed on medusae of various taxa. Prell [39] mentioned *Pycnogonum litorale* feeding on the stauromedusa *Lucernaria*. *Phoxichilidium femoratum* also fed on *Lucernaria*, cutting off branched tentacles with the chelifores. A similar technique is used by other species, although younger larvae appear to use their chelifores only for clinging to the host [10]. Uchida & Hanaoka [56] reported ammotheids feeding on the stalked medusa *Manania distincta*. An unidentified species of *Colossendeis* was photographed feeding on a coronate medusa in the North Atlantic [44]. *Colossendeis* was also observed feeding on medusae entrapped by sea anemones (Moran, pers. comm. cited by [44]). Lebour [57] found larvae of *Anoplodactylus petiolatus* on five different species of medusa, most frequently on *Obelia* sp.. Wyer & King [10] reported larvae of the same species from the medusa *Clytia hemispherica*. Okuda [58] recorded larvae of *Achelia alaskensis* developing on the hydromedusa *Polyorchis karafutoensis*. Mauchline [59] found unidentified juvenile pycnogonids attached to the medusa *Periphylla periphylla*, and Child & Harbison [60] recorded both adults and juveniles of *Bathypallenopsis scoparia* from the same species. Examination of the gut contents suggested that the adult had eaten the tentacles, but the juveniles fed on the gonads or the contents of the gastrovascular sinus. Similarly, Pagès et al. [61] reported *B. tritonis* attached to *Pandea rubra*. *Bathypallenopsis calcanea* was found on the medusa *Aeginura grimaldii*, but no evidence of feeding by the pycnogonid was observed [62]. Other species of pycnogonids found in bathypelagic samples (*Bathypallenopsis* spp. and *Colossendeis gardineri*) are probably also associates of medusae or other pelagic organisms [63]. Unlike some other animals associated with medusae, e.g. some copepods [64], the morphology of these pycnogonids does not appear to be greatly modified.

There have been several reports of pycnogonids associated with corals, e.g. *Boehmia chelata* and alcyonarians [65]. Stephensen [66] noted that *Nymphon hirtipes* is only found where the soft coral

*Eunephthya* occurs, while *Boreonymphon robustum* is probably associated with *Umbellula encrinus*. He noted that the peculiar shape of the *Boreonymphon* chelae may be adapted to grasping *Umbellula* tentacles, and specimens carrying juveniles were often found in places with smaller coral species. The ammotheid *Tanystylum grossifemorum* has been recorded from several octocoral species [67]. Child [68] found several species associated with the scleractinian coral *Oculina varicosa*. In none of these cases, pycnogonids were directly observed feeding on the corals. However, corals are known to be hosts of pycnogonid larvae. Moseley [69] found cysts containing unidentified pycnogonid larvae in the gastric cavity of gastrozooids of the hydrocoral *Pliobothrus symmetricus*. Stock [70] described galls containing larvae probably belonging to *Ascorhynchus* in the soft coral *Chrysogorgia papillosa*. Feeding of adult pycnogonids on corals was to our knowledge first reported by Slattery & McClintock [71], who found *Colossendeis megalonyx* to feed on the soft corals *Alcyonium antarcticum* and *Clavularia frankliniana*. *Colossendeis robusta* was also found feeding on the latter species, while *Ammothea* sp. fed on *Gersemia antarctica*. Arango [72] recorded *Endeis mollis* feeding on the hydrozoan coral *Millepora exaesa* and the zoanthid *Palythoa caesia* and *E. biseriata* feeding on the zoanthid *Protopalythoa* sp. A pycnogonid probably identifiable as *Bathypallenopsis mollissima* has been observed feeding on an unidentified bamboo whip coral (Isididae) according to Watling et al. [73]. Feeding of adult pycnogonids on corals, therefore, appears to be little documented, although it may be especially common in deep-sea forms.

### Bryozoans

Predation of pycnogonids on bryozoans has been reviewed by Ryland [74] and Key et al. [75]. Prell [39] mentioned, without further details, *Phoxichilidium femoratum* feeding on the bryozoan *Crisia*. Fry [26] found that both *Austrodecus glaciale* and *Rhynchothorax australis* fed on all five bryozoan species that were presented to them, but they were not among the preferred foods of *Rhynchothorax*, while *Austrodecus* showed a strong preference for the bryozoan *Cellarinella roydsi*. He pointed out that the extremely thin distal proboscis of austrodecids appears to be an adaptation for feeding on bryozoan zooids through the frontal wall pores. *Cellarinella roydsi* is the only one of the tested bryozoan species that has numerous frontal pores. However, according to Ryland [74], it is also possible that the pycnogonid feeds through the peristome, as the species does not have an operculum. Most of the pores also do not penetrate the entire frontal wall [76]. The spiny palps of *Austrodecus* are probably used to guide and strengthen the proboscis [77]. Wyer & King [10, 78] recorded *Achelia echinata* feeding on *Flustra foliacea*, inserting the proboscis through the operculum. However, *Ammothella longipes* would not feed on the bryozoans even when the zooids were extended, instead preferring the red algae growing on the bryozoan colony. *Pycnogonum litorale* was observed feeding on the rotting edge of a colony of the same species. *Nymphon gracile* was observed feeding on *Amathia imbricata*, using the same method as on hydroids. Varoli [41] reported that both *Anoplodactylus stictus* and *Tanystylum isabellae* would feed on *Amathia distans*. Sherwood et al. [79] showed that *Stylopallene longicauda* sequesters amathamine alkaloids from *Amathia wilsoni*, therefore demonstrating that this bryozoan is a food source of the pycnogonid. The alkaloids are probably used as a chemical defense. According to Staples [80], the digitiform chelae of *Pseudopallene watsonae* larvae are probably used to manipulate the manubrium of bryozoan zooids before inserting the proboscis. In the adult, however, the chelae are robust as in other species of *Pseudopallene* and appear more suited to crushing bryozoan zooids. It, therefore, appears that bryozoans are an important food source for many different pycnogonid taxa, and bryozoan feeders often show clear specializations such as an extraordinarily thin proboscis or chelifores suitable for crushing.

### Mollusks

Parasitism of pycnogonids on mollusks was reviewed by [81]. Merton [82] recorded a nymphonid, which he named *Nymphon parasiticum*, parasitic on the nudibranch *Tethys fimbria*. However, no fully grown specimen was found, and the species was to our knowledge never recorded again. Similarly, Ohshima [83] recorded a juvenile ammotheid parasitic on the nudibranch *Armina variolosa*. Stock [68] recorded a juvenile of an unidentified species of *Ascorhynchus* parasitic on the gills of the nudibranch *Aplysia dactylomela*. Edmunds [84] found unidentified pycnogonids feeding on the nudibranchs *Cuthona perca* and *Spurilla neapolitana*. In one case the proboscis was inserted into the liver duct. Piel [85] reported *Anoplodactylus californicus* preying on the nudibranch *Dondice occidentalis*, grabbing cerata with the chelicerae, causing ceratal autotomy and consuming them. Rogers et al. [86] observed that *Anoplodactylus evansi* consumed 13 different species of opisthobranchs in an aquarium setting. The species would consume almost no other prey that was offered. Whole animals

were consumed after immobilizing them with the claws of the front legs. Arango & Brodie [87] recorded *A. longiceps* preying on the nudibranch *Okenia* sp., and Mercier et al. [51] reported about a specimen of *Nymphon hirtipes* feeding on a nudibranch (*Tritonia* sp.), which was shredded and ingested completely.

Pycnogonids have also been recorded feeding on shelled gastropods. Shabica [88] mentioned *Colossendeis megalonyx*, *C. robusta* and *Pentanymphon* sp. as predators of the Antarctic limpet *Nacella concinna*, and Bain [89] observed *Anoplodactylus californicus* feeding on the prosobranch snail *Pleurobranchus digueti*. The species *Ascorhynchus endoparasiticus* is parasitic in the pallial cavity of the opisthobranch *Scaphander punctostriatus* [90].

Bivalves are also known to be a food source for pycnogonids. The ascorhynchid *Nymphonella tapetis* is an economically important parasite of various bivalve species in the Northwest Pacific [91]. Only juveniles are parasitic. Curiously, in other *Nymphonella* species, which may be synonymous with *N. tapetis*, endoparasitism has never been recorded [92]. *Nymphonella* is phylogenetically nested within *Ascorhynchus*, which includes other mollusk-feeding species [92]. Arnaud & Bamber [2] reported the presence of juveniles of two different unidentified *Ascorhynchus* species as endoparasites in *Tellina perna*. Benson & Chivers [93] recorded an infestation of the mussel *Mytilus californianus* by the normally free-living species *Achelia chelata*. Tharme et al. [94] reported an unidentified pycnogonid, represented by larvae as well as adults, living parasitically on the bivalve *Donax serra*. Lotz [16] mentioned that *Anoplodactylus petiolatus* would consume *Mytilus* tissue when the preferred food was not available. The same was observed by Bain [89] for *A. californicus* and by Varoli [41] for *Tanystylum isabellae*. While mollusks can be consumed by a variety of pycnogonid taxa, only a few species, mostly ascorhynchids, are specialized molluscan parasites.

### Annelids

While there are several records of pycnogonids on tubicolous polychaetes (e.g. [45]), it was not clarified whether they feed on the polychaetes themselves or on their epibionts. However, Wyer & King [10] recorded *Nymphon gracile* feeding on an unidentified sedentary polychaete. Richards [46] recorded that an unknown sedentary polychaete living on red seaweed seemed to be the preferred food of the Southern Ocean species *Nymphon orcadense*, and was also accepted by starved specimens of *N. australe*. *Nymphon molleri* was observed feeding on the spionid polychaete *Polydorella stolonifera*, *Anoplodactylus evansi* on an unidentified small polychaete and *Ammothea australiensis* on the tubicolous polychaete *Galeolaria caespitosa* [95]. The latter species prevented the polychaete from retracting by placing its palps behind the branchial crown and operculum. Shabica [96] recorded *Colossendeis megalonyx* feeding on tubicolous polychaetes in a tank setting. *Achelia simplissima* feeds on the spirorbid *Spirorbis bifurcatus* [97]. Salazar-Vallejo & Stock [98] recorded the larvae and juveniles of a pycnogonid tentatively identified as *Ammothella spinifera* developing on *Sabella melanostigma*. The abdominal segments of the host, which contain the reproductive tissue, were preferred to the thoracic ones.

Pycnogonids have also repeatedly been reported to feed on errant annelids. Hilton [99] recorded a callipallenid identified only as "*Pallene*" "devouring a soft annelid worm". Similarly, Lotz [16] recorded *Anoplodactylus petiolatus* eating errant polychaetes in an aquarium setting, fully ingesting them. Rogers et al. [86] also found *A. evansi* eating an unidentified errant polychaete. Stock [100] recorded a juvenile, tentatively referred to *Hannonia* (a genus of uncertain placement) as parasitic on the polychaete *Cirriformia capensis*. *Ammothella longipes* was recorded feeding on nereid polychaetes [17, 101]. The species appears to be carnivorous during spring and summer and detritivorous in the winter based on fatty acid analyses [102]. It appears that annelids are a food source of medium importance used by many different species but there are few annelid specialists.

### Crustaceans

Richards [46] mentioned that *Nymphon orcadense*, in the absence of its preferred polychaete food, would consume dead amphipods. Lotz [16] reported that, in the absence of its favored food source (hydrozoans), *Anoplodactylus petiolatus* would catch and eat copepods of the species *Tisbe furcata*. When a copepod touches the pycnogonid's body, it is caught with the claw of a walking leg. It is then placed in front of the proboscis opening first using the claws of both legs of a pair and then using the chelifores, before being sucked out. Bain [89] reported *Anoplodactylus californicus* feeding on brine shrimp (Anostraca), which were caught directly from the water column with the chelifores. Varoli [41] reported that dead specimens of the amphipods *Apohyale media* and *Caprella danilevskii* and the anostracan *Artemia salina* were accepted by *Anoplodactylus stictus* and *Tanystylum isabellae*, but living ones were not. Soler-Membrives et al. [17] recorded *Ammothella longipes* holding caprellid amphipods, but it was not observed whether

they were actually feeding on them. Thus, crustaceans seem to be a food source only in few cases, and probably mainly dead amphipods or copepods are important in that respect.

#### Echinoderms

Stock [102] described the species *Pycnosomia asterophila*, which was found only on the oral surface of the asteroid *Calliaster corynetes*. Nakamura & Fujita [103] found juveniles and adults of *Ammothea hilgendorfi* on *Coscinasterias acutispina*, mostly on the aboral and lateral surfaces.

Sloan [104] recorded the species *Anoplodactylus ophiurophilus*, which is exclusively found attached to the oral side of ophiuroids of the genus *Ophiocoma*. The species *O. doederleini* appears to be preferred. The pycnogonid evidently feeds on the oral mucus which the ophiuroids produce to entrap particles.

Losina-Losinsky [105] found specimens of *Pycnosomia strongylocentroti* attached to the spines and pedicellariae of an echinoid (*Strongylocentrotus*) with their legs. He noted that the propodus of this species appears specialized for such an attachment.

Prell [39] reported one case where *Pycnogonum litorale*, which is normally specialized on actinians, fed on the holothurian *Cucumaria frondosa*. Ohshima [106] reported juveniles of *Ammothella biungiuculata* and *Ammothea hilgendorfi* associated with the holothurians *Apostichopus japonicus* and *Holothuria lubrica*, respectively, although actual feeding was not observed. Echinoderms, therefore, seem to be a food source of minor importance, which is used mostly by a few specialized phoxichilidiid species.

#### Sediment and detritus as a food source

Pycnogonids have also been observed as sediment feeders. Stout & Shabica [45] recorded the Antarctic species *Decolopoda australis* and *Pallenopsis* cf. *patagonica* "feeding in the soft sediments". Similarly, photographs of Antarctic *Colossendeis* specimens with their proboscis inserted into sediment led Hedgpeth [107] to conclude that these animals feed on the meiofauna living in the uppermost sediment layers. While this seems likely in this case, pycnogonids were also observed to feed on organic detritus. Wyer & King [10] observed starved specimens of *Nymphon gracile* feeding on the detritus that had accumulated on their bodies, removing it with the ovigera and transferring it to the mouth via the chelifores. *Achelia echinata*, *Endeis laevis* and *Pycnogonum litorale* were found feeding on detritus that had accumulated on various substrates such as bryozoan colonies. In the case of *Endeis*, the detritus was first broken down with the spines surrounding the mouth. Similar observations were reported on *Ammothella longipes* and *Endeis spinosa* by Soler-Membrives et al. [17], who found the latter species to be exclusively detritivorous. This might explain the loss of chelifores in that genus as opposed to the related Phoxichilidiidae, which have well-developed chelifores. Richards [46] reported *Nymphon orcadense* feeding on detritus of unidentified animal origin. Therefore, while specialized detritivory seems to occur only in *Endeis*, many pycnogonids appear to be able to feed on detritus when no other food is available.

#### Other prey

Richards & Fry [12] suggested that pycnogonids might feed by filtering particle-rich water, suggesting that *Nymphon orcadense* uses this behavior when its preferred polychaete prey is not available. They noted that during these times the pycnogonid was observed to feed on other prey, but much less frequently than would be expected. Such a mode of feeding would also explain the observation that *Colossendeis proboscidea* was seen rapidly opening and closing its proboscis lips in "goldfish fashion" [12]. They also suggested that pycnogonids may be able to take up nutrients through the cuticle, which however has, to our knowledge, not yet been demonstrated.

Based on stable isotope analyses, Bergquist et al. [108] inferred that the hydrothermal vent species *Sericosura verenae* is mostly bacterivorous, while other *Sericosura* species may combine bacterivory with detritivory. Based on the same method, Cordes et al. [109] also inferred bacterivory in *Anoplodactylus* sp. from cold seeps.

Animal taxa other than those discussed in the previous section were also found to be pycnogonid prey. Zenker [19] found benthic foraminiferans in the proboscis of *Nymphon gracile*, which were probably ingested by consuming detritus. Shabica [96] recorded *Pentanymphon antarcticum* feeding on a small ctenophore. Richards [46] observed *Nymphon orcadense* feeding on the nemertean *Antarctonemertes valida*. Shabica [96] found *Colossendeis* sp. feeding on the nemertean *Parbolasia corrugatus* in the Antarctic. Soler-Membrives et al. [17] recorded two occurrences of predation by *Ammothella longipes* on unidentified nematodes. King & Crapp [110] found *N. gracile* feeding on eggs of the gastropod *Nucella*. Kott [111] found a specimen of *Ammothea carolinensis* whose proboscis was inserted into the branchial cavity of an ascidian (*Pyura georgiana*), apparently to feed on its genital products after release from the gonads. Lebrato & Jones [112] observed *Colossendeis* sp. feeding on pyrosome carcasses (*Pyrosoma atlanticum*). Leigh-Sharpe [113] recorded a specimen of *Pycnogonum*

*litorale* found on the gills of a fish (*Merlangius merlangus*). Arnaud [114] and Arnaud & Bamber [2] recorded eight Antarctic pycnogonid species (*Nymphon australe*, *Pentanymphon antarcticum*, *Ammothea carolinensis*, *A. clausi*, *A. glacialis*, *Colossendeis megalonyx*, *C. robusta*, *C. scotti*) feeding on seal meat in fish traps. In an aquarium setting, *Nymphon orcadense* fed on a mixture of minced limpet, squid and spratt [46]. Richards [46] also observed that *Colossendeis* and/or *Decolopoda* apparently fed on smaller pycnogonids (*Nymphon orcadense*) in an aquarium setting. These observations demonstrate that many pycnogonids are generalist feeders, which are able to use a wide variety of food sources on which they are not specialized.

## General findings

Our review documented observations of feeding for only approximately 100 of the about 1500 species (Table 1, Additional file 1). Thus, the most important finding is that for most pycnogonid species, the feeding mode and preferred food still remains unknown. This is especially true of deep-sea forms as well as those of the Antarctic, which include about 20% of the known pycnogonid species [115]. Therefore, taxonomic groups which are typical of these regions, such as the Colossendeidae and Pallenopsidae, are also underrepresented here.

However, for those species where details about feeding items are known, the data reviewed here confirm the generally accepted view that pycnogonids feed mostly on sessile organisms such as hydroids, actinians and bryozoans. King [1] stated that littoral pycnogonids feed on hydroids, bryozoans and sponges "in about that order of frequency". The data reviewed here show that hydroids are indeed the most common food source, being eaten by members of almost all pycnogonid families. It is also confirmed that the second most common food source is bryozoans, which are also consumed by a wide variety of pycnogonid species. However, there are only very few records of littoral pycnogonids feeding on sponges (e.g. [21]), which suggests that they are not among the preferred prey. Sponges might be a more common food source for deep-sea forms [1], although, so far, the data are insufficient. Other types of prey are used less commonly, often by specialist feeders (e.g. Pycnogonidae as actinian specialists). Sediment feeding appears to be especially common in deep-sea forms, about whose behavior little is known, and may be an important but underestimated part of pycnogonid feeding ecology, as already suggested by King [1]. Food sources of juvenile and adult pycnogonids should be distinguished, as there are several species (mostly ammotheids and ascorhynchids) which are parasitic even as late-stage juveniles but free-living as adults, such as the bivalve parasite *Nymphonella tapetis*.

### Food specialization as a rule?

Many pycnogonids appear to be specialized for feeding on a single taxonomic group such as thecate or athecate hydroids, actinians, or bryozoans (Additional file 1). Individuals of these species may even be unable to survive the absence of their preferred food [16]. Like other specialized feeders, these pycnogonids may be vulnerable to environmental change if the frequency of their prey item is reduced.

However, the claim [1] that no pycnogonids are dependent on a single host species (rather than a larger taxonomic group) appears to be correct. Hydroid feeders seem to be the most common group in temperate shallow seas, and feeding on hydroids is therefore particularly intensively studied. The feeding mechanisms of actinian specialists (Pycnogonidae) and detritivores (some *Endeis* species) have also been well studied. Other pycnogonids, especially members of the Phoxichilidiidae such as *Phoxichilidium* and *Anoplodactylus*, appear to be generalist feeders able to live on a wide variety of prey. Prell [39] already noted that *Phoxichilidium femoratum* is a voracious predator ("ein arges Raubtier") of many different animals, and the observations of Lotz [16] and others on *Anoplodactylus* agree with this. It is notable that, even within a genus, the feeding preferences may vary widely. Examples are *Anoplodactylus*, which contains generalists as well as obligatory echinoderm commensals, *Endeis*, which includes detritivores and coral feeders, and *Austrodecus*, which includes bryozoan and hydroid feeders. Helfer & Schlottke [116] stated that pycnogonids, due to being incapable of making fast movements, are only able to feed on slow-moving or sessile prey. While this appears to be generally true, there are exceptions. Several pycnogonid species were observed to capture and eat errant polychaetes, and *Anoplodactylus* also consumes free-swimming crustaceans (see above).

Differences in feeding preference often correspond to differences in morphology. There are variations, especially in the morphology of the proboscis and chelifores, which can be assumed to correlate with feeding preferences, such as extremely thin proboscides in bryozoan-feeding austrodecids and *Stylopallene*, or the very robust chelifores of *Pseudopallene* and related genera used to crush bryozoan zooids. In *Anoplodactylus*, the lips appear to be specialized for cutting tissue, which would be useful for its generalist predatory lifestyle. The chelifores are well developed in most hydroid feeders, which use them to grasp

stems or hydrothecae and lead them to the mouth. In animals living parasitically on much larger hosts, such as Pycnogonidae on actinians, and in detritivores such as *Endeis* they are reduced. The proboscis is much more mobile in ammotheids and ascorhynchids than in most other forms, which fits with the fact that these animals seem to be mostly hydroid feeders that cannot hold their prey in their small chelifores. However, these correlations may not be perfect. As an example, a preference for bryozoans has been shown for only one Antarctic austrodecid species [26], while the Australian *Austrodecus frigorifugum* feeds on hydroids [27]. For most taxa, the correlation between morphology and feeding has yet to be investigated. This is especially true of internal anatomy, where Wagner et al. [8] have found significant differences between taxa in the proboscis, and the detailed anatomy of the digestive system has been studied only for very few taxa.

### Cryptic species and food specialization

Morphological correlates of different feeding habits thus exist, but the question arises what those differences actually are. In other words, there is a considerable gap in the current knowledge of pycnogonid feeding ecology that needs to be closed. This is of particular interest since in the past decade molecular and morphological studies, especially in the Southern Ocean, have shown that pycnogonids are a useful model taxon for analyses of speciation and phylogeography of holobenthic marine organisms [3, 4]. However, these studies have focused exclusively on genetic drift as speciation motor, while selection (with food preferences as a major cue) has hardly been considered so far. Besides, differences in food preference between closely related species are little known. To deepen knowledge of pycnogonid feeding ecology would, therefore, be an important contribution to marine evolutionary biology, especially of high-latitude environments.

### New methods provide new insights

While most observations and experiments were conducted using classical setups, mostly by direct observation of feeding, only a few studies have been undertaken using novel techniques such as fatty acid analyses [17, 101] or stable isotopes [108, 109, 117]. Molecular content analyses of pycnogonid gut content have to our knowledge never been published. A metabarcoding approach, in which standard barcoding markers are amplified from bulk samples and sequenced with next-generation methods, has been successfully used for identifying gut contents in several taxa (e.g. [118]), and could also be useful in pycnogonids. However, as the cellular material is already processed and filtered in the pycnogonid proboscis, it does not enter the midgut [6]. Therefore, a metabarcoding approach might be less successful than in animals where cellular prey tissue is found in the gut. When genomic or transcriptomic data of pycnogonids become available (several transcriptomes already exist in unpublished form), they should be checked carefully for the presence of non-pycnogonid DNA, which could be an important source of new data on pycnogonid feeding. Preliminary results by J. Dömel and T. Macher (in prep.) for two Antarctic pycnogonid species have already confirmed the presence of several taxonomic groups known to be pycnogonid prey.

## Outlook

Despite the fact that pycnogonids have been observed for almost two centuries, information about the feeding habits of more than 90% of the species is missing. Hence, one of the tasks for future studies will be to keep going the “naturalist path”, i.e. observation of pycnogonids in their habitats in order to record their actual food preferences. Moreover, previous analyses of morphological adaptations of the organs of food assimilation (chelifores, palps, proboscis lips, proboscis inner structures) to the type of nourishment proved fruitful and therefore should be made for many more species. Apart from analyses of these structure-function relationships, there are three approaches using modern techniques that have been neglected until now, i.e. fatty acid and stable isotope analyses as well as DNA sequencing of gut contents. However, these results are needed to analyse the relative contribution of selection for pycnogonid speciation processes next to the typically discussed allopatric scenarios fuelled by genetic drift and lineage sorting.

## Conclusions

1. Pycnogonids feed on a wide variety of prey, mostly on sessile animals, but also detritus and other food sources.
2. Hydroids appear to be the most common food source of pycnogonids, followed by bryozoans and actinians. Other food sources are less common.
3. Many pycnogonids are generalist feeders, but a number of taxa are specialized in a particular food source, e.g. actinians for members of the Pycnogonidae.
4. Pycnogonid taxa often show clear adaptations to their preferred food, especially in the morphology of the proboscis and chelifores.

5. For most pycnogonids, especially deep-sea forms, the preferred food source is still unknown. More research on pycnogonid feeding ecology could reveal mechanisms of differentiation between closely related species and therefore of evolutionary radiations.

## Additional file

**Additional file 1:** Feeding-table. Summary of known food sources for pycnogonid species. Reference numbers are the same as in the main text. (XLSX 50 kb)

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#### Availability of data and materials
The data presented here are extracted from the published literature.

#### Authors' contributions
LD collected a majority of the data and wrote most of the text. TL and RRM contributed the figs. FL, JSD and RRM discussed the data with LD and contributed to the structuring of the manuscript and presentation of the data. All authors read and approved the final manuscript.

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#### Author details
[1]Zoological Research Museum Alexander Koenig, Statistical Phylogenetics and Phylogenomics, Adenauerallee 160, D-53113 Bonn, Germany. [2]Faculty of Mathematics and Natural Sciences, University of Bonn, D-53012 Bonn, Germany. [3]Aquatic Ecosystem Research, Faculty of Biology, University Duisburg-Essen, Universitätsstr. 5, D-45141 Essen, Germany. [4]Bavarian State Collection of Zoology – SNSB, Münchhausenstraße 21, 81247 Munich, Germany. [5]Department Biologie II, Ludwig-Maximilians-Universität München, Großhaderner Straße 2, 82152 Planegg-Martinsried, Germany. [6]GeoBioCenter LMU, Richard -Wagner-Str. 10, 80333 Munich, Germany.

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ORIGINAL ARTICLE

ORGANISMS DIVERSITY & EVOLUTION

# Comparative study of bisected proboscides of Pycnogonida

**Philipp Wagner**[1,2] · **Jana S. Dömel**[3] · **Michaela Hofmann**[1,2] · **Jeremy Hübner**[1,2] · **Florian Leese**[3] · **Roland R. Melzer**[1,2,4]

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**Abstract** To comparatively describe "inner" pharynx armatures in pycnogonids, we bisected proboscides of *Achelia langi* (Dohrn, 1881), *Anoplodactylus californicus* Hall, 1912, *Ascorhynchus castellioides* Stock, 1957, *Austrodecus glaciale* Hodgson, 1907, *Callipallene margarita* (Gordon, 1932), *Colossendeis macerrima* Wilson, 1881, *Endeis spinosa* (Montagu, 1808), *Nymphon macronyx* Sars, 1877, *Pallenopsis patagonica* (Hoek, 1881), *Pantopipetta* sp., *Pigrogromitus timsanus* Calman, 1927, and *Pycnogonum litorale* (Strøm, 1762) and analyzed them with the scanning electron microscope (SEM). Moreover, proboscides were stained with actin green to visualize the muscle arrangement with fluorescence and confocal microscopes (*A. langi*) and were analyzed with microcomputed X-ray tomography (μCT; *Ascorhynchus japonicus*). As a result of our observations, sets of characters that vary between taxa are established. These traits include length and width of proboscis, shape and structure of the inner mouth opening, borders and armature of the antimeres, shape and position of denticle arrays and rows, relative length of pharyngeal filter apparatus, and arrangement and structure of the filter bristles. Analyses of these characters indicate a substantial variability on the pantopod's proboscis inner surface probably as an adaptation to different food sources. Finally, we suggest that the presence of the oyster basket represents a ground pattern character of Pycnogonida.

**Keywords** Pharynx · Comparative morphology · Scanning electron microscopy · Sea spider

✉ Roland R. Melzer
melzer@zsm.mwn.de

[1] Biocenter, Department Biologie II, Ludwig-Maximilians-Universität München, Großhaderner Straße 2, 82152 Planegg-Martinsried, Germany

[2] Zoologische Staatssammlung, Münchhausenstraße 21, 81247 Munich, Germany

[3] Fakultät für Biologie, Aquatische Ökosystemforschung, Universität Duisburg-Essen, Universitätsstraße 5, 45151 Essen, Germany

[4] Geobio Center LMU, Richard-Wagner-Straße 10, 80333 Munich, Germany

## Introduction

The pycnogonid's primary organ for food uptake, i.e., the proboscis or trunk, has attracted particular interest of every generation of researchers who dedicated their work to this group of marine arthropods. The reason for this is probably the uncommon structure and enigmatic evolutionary origin. Already in his early masterpiece, Dohrn (1881) made detailed analyses of the proboscides of some Mediterranean species including structure of the three antimeres forming the proboscis; three lip lobes equipped in many species with external setae; and "jaws," musculature, and a triradial inner pharynx or foregut surface revealing the presence of a complex "Reusenapparat," nowadays referred to as oyster basket or pharyngeal filter. Dohrn suggested that only liquid food is used for nutrition, while food particles are prevented from entering the esophagus. Among the elements of the oyster basket already described by Dohrn are densely arranged filter bristles ("Stachel") originating from basal annular ribs (also called bands or plates), areas armed with denticles, and rows of platelets or teeth. Dohrn also established the hypothesis that the outer surface of the proboscis is solid and the inner surface or pharynx is flexible with muscle strands inserting at the corners of the triangular pharynx (radial muscles sensu Fry 1965) and those on the bow-shaped sides (interradial muscles sensu Fry 1965) acting as antagonists resulting in the suction power needed for food uptake and alternating

upward and downward movement of the filter bristles in the Reusenapparat. In addition to these muscle bundles, Dohrn identified longitudinal muscles for the lips ("Retraktoren der Lippen," see also Dencker 1974 for an excellent 3D drawing of lip muscles). Some of the proboscis features mentioned by Dohrn have also been observed by Hoek (1881) in his study on the "Challenger" pycnogonids.

Fry (1965), Dencker (1974), and Richards and Fry (1978) made important amendments to these ideas. First, it was suggested that contraction of radial and interradial muscles induces a change from trifoliate to triangular pharynx cross section representing the primary pump mechanism. Second, presence of a fourth type of muscle, i.e., circular muscles in the proximal thinner part of the proboscis, was identified and presumed to be antagonists of the flexible annular ribs of the oyster basket. Contrary to the earlier studies, these studies considered that not only liquid food, but also small food particles might pass the filter apparatus during the bristle's movement, thus suggesting that the oyster basket is not only a filter, but also a grinding mill used for maceration of food particles. The question whether the oyster basket is a filter (Dohrn 1881) or a filter and grinding apparatus (Fry 1965) has not been satisfactorily solved until present. Of relevance in this aspect is maybe a little-noticed study by Lotz (1968) who observed the actual grinding activity and transport of food particles across the oyster basket in *Anoplodactylus petiolatus* in situ.

While functional morphology and feeding ecology are undoubtedly relevant and understudied topics until today, a second big topic for morphologists was the evolutionary origin of the proboscis. While Dohrn (1881) saw the proboscis as an organ *sui generis* not homologous to other mouthparts, various hypotheses were formulated by other authors on putative homologs of the antimeres in other arthropod groups, e.g., the rostrum, labrum, pedipalps, and other appendages (e.g., Wirén 1918; reviews in Helfer and Schlottke 1935; Hedgpeth 1954; Dunlop and Arango 2005; Machner and Scholtz 2010). In support of Dohrn's anticipatory interpretation, recent developmental studies have shown that the proboscis is an outgrowth of the anterior body region not showing any correspondence to these putative homologs (Brenneis et al. 2011; see also Meisenheimer 1902). This is of vast impact on ideas on the phylogenetic position of Pycnogonida, as all major euarthropod lineages possess a labrum, while pycnogonids seem to lack this structure, and this would support molecular phylogenetic studies suggesting that Pycnogonida are the sister group of all other Euarthropoda (Giribet et al. 2001; Dunlop and Arango 2005; Scholtz and Edgecombe 2006), rather than being a basal offspring of Chelicerata (Wheeler and Hayashi 1998; Dunn et al. 2008; Regier et al. 2010). Extending sets of morphological characters as has been done here for the proboscides will help to solve this point in the future.

In addition to previously mentioned studies, recent works using modern techniques have sought to extend knowledge on proboscis structure and functional morphology. Using transmission EM, Fahrenbach and Arango (2007) have revealed numerous salivary glands around the mouth opening in several species of pycnogonids indicating a chelicerate-like extraintestinal digestion. Furthermore, a fascinating ultrastructure and highly regular arrangement of the filter bristles have been documented in *Nymphopsis spinosissima* and *Ammothea hilgendorffi* with branchings of the bristles or barbules having a diameter of only ca. 150 nm (70 nm at the tip), suggesting a filter mesh size/grating of the whole apparatus of only 100 nm. In this study, also two arrays of teeth located anterior to the Reusenapparat on the otherwise smooth pharynx surface were documented. In addition, Soler-Membrives et al. (2013) related feeding habits of *Ammothella longipes* and *Endeis spinosa*, i.e., carnivorous and detritivorous forms, to different structures of the digestive system, showing that the main differences are found in the pharynx, mouth, and adjacent structures. Miyazaki (2002) made histological analyses of proboscis cross sections and the arrangement of muscle strands of representatives of all extant families, thus reviving Dohrn's (1881) and Fry's (1965) approach. Moreover, it is noteworthy that taxonomic drawings sometimes show pharynx inner structures, but not at an adequate resolution.

While these studies have considerably extended our knowledge on functional morphology of the proboscis over the course of the past 130 years, a comparative analysis of the armatures of the inner surface of the proboscis using an imaging technique of high-resolution power is missing. Therefore, the aim of the present study was to fill this knowledge gap by using the scanning EM for analyses of bisected proboscides of various representatives that cover most extant pycnogonid families accepted at present. In addition, we have used microcomputed X-ray tomography (μCT) in *Ascorhynchus japonicus* and fluorescence and confocal microscopy of stains of musculature with actin green in *Achelia langi* to reveal the basic elements of proboscides.

## Material and methods

### Species studied

All studied proboscides originate from the coll. Arthropoda varia at the Zoologische Staatssammlung München (ZSM) and include representatives of nearly all extant pycnogonid families (in brackets, ZSM collection numbers are given):

Ammotheidae: *Achelia langi* (Dohrn, 1881): (ZSMA20051966/ZSMA20051899/ZSMA20051900)

Phoxichilidiidae: *Anoplodactylus californicus* Hall, 1912: (ZSMA20111250/ZSMA20111248)

Ascorhynchidae: *Ascorhynchus castellioides* Stock, 1957: (ZSMA20071706), *A. japonicus* Ives, 1891: (ZSMA19040001)

Austrodecidae: *Austrodecus glaciale* Hodgson, 1907: (ZSMA20100166), *Pantopipetta* sp. (SokhoBio AK Lavrentyev71)

Callipallenidae: *Callipallene margarita* (Gordon, 1932): (ZSMA20111172/ZSMA20111175)

Colossendeidae: *Colossendeis macerrima* Wilson, 1881: (ZSMA20119959).

Endeidae: *Endeis spinosa* (Montagu, 1808): (B15-725515/ZSMA20042371).

Nymphonidae: *Nymphon macronyx* Sars, 1877: (PRÜ1V678/PRÜ2V678/PRÜ3V678/PRÜ4V678)

Pallenopsidae: *Pallenopsis patagonica* (Hoek, 1881): (ZSMA20111359).

Ascorhynchoidea family incertae sedis: *Pigrogromitus timsanus* Calman, 1927: (ZSMA20071660)

Pycnogonidae: *Pycnogonum litorale* (Stroem, 1762): (ZSMA20051979)

### Preparation and SEM

We cut off the proboscides of the specimens and bisected them using scalpels and pieces of razor blades. Afterwards, they were dehydrated in acetone (80% 10 min, 90% 10 min, 3 × 100% 20 min) and critical point dried in a Baltec CPD 030. After mounting and orientating them on self-adhesive carbon stickers on SEM holders, they were coated with gold on a Polaron E 5100 sputter coater. For SEM, we used a Leo 1430 VP at acceleration voltages between 15 and 30 kV and SE detector (300 V). For some pictures, photos at different focus were stacked and processed with CombineZ, because of the limited field depth of single shots.

### Stains with fluorescence markers

Dissected proboscides of *A. langi* were preserved in 2% formalin in phosphate-buffered saline (PBS 0.1 m, pH 7.1) for between 1 and 2 h, rinsed several times in PBS, and stained with actin green 488 Ready Probes for 2 h (one drop actin green in PBS per 1.5-ml Eppendorf vial. After rinsing in PBS, specimens were embedded in fluorescence mount with DAPI as counterstain for nuclei (Roth, Karlsruhe). Specimens were analyzed with Leica SP5 confocal microscope. Scans were done using two different settings: (1) lens: Leica HCX IRAPO L 25 × 0.95 water; excitation I 405 nm, detection wavelength range I 415–485 nm, excitation II 488 nm, and detection wavelength range II 500–590 nm; voxel size: xy orientation 1005 nm; and z-orientation 2010 nm (average 3) and (2) lens: Leica HXL PLAPO CS 63 × 1.20 Water UV; excitation I 405 nm, detection wavelength range I 415–530 nm and excitation II 488 nm, and detection wavelength range II 500–590 nm; voxel size: xy orientation 400 nm; and z-orientation 800 nm (average 8).

### Microcomputed X-ray tomography

One specimen of *A. japonicus* was analyzed non-invasively as a whole mount with a Phoenix Nanotom (GE Sensing & Inspection Technologies, Wunstorf, Germany) cone beam CT scanner at a voltage of 100 kV and a current of 80 μA for 47 min. One thousand four hundred forty radiographs were registered and analyzed with the integrated software and with VGStudio Max 2.2.2 64 bit (volume rendering). To increase contrast, the specimen was stained for 24 h in Lugol's solution prior to scanning. Afterwards, the solution was removed again by rinsing several times in 75% ethanol for several days.

## Results

### General morphology of proboscides

To depict the general morphology of pycnogonid proboscides, we have used μCT and fluorescence stains (Fig. 1, see also Fig. 8) and provided surveys of all SEM preparations of proboscides studied (Fig. 2). In *A. japonicus* (Fig. 1a–f), the general features of the proboscis are shown, with triradiate mouth and lips (Fig. 1a), triradiate pharynx with radial and interradial muscles (Fig. 1b–d, h), and antimere borders running longitudinally along the pharynx' inner surface (Fig. 1b–f). Moreover, the pharynx lumen in this species consists of a distal, broad section and a proximal slim section containing the oyster basket. In Fig. 1f, the main characteristics of the proboscides that show intertaxon differences are indicated with circles. These are specifically the following: the mouth section, anterior and posterior denticle fields, and oyster basket that exhibited very different lengths in relation to the total proboscis length (Fig. 2). In *A. langi*, in addition to the regularly arranged radial and interradial muscles (Fig. 1g–h), fine muscle strands projecting anteroposteriorly were observed, representing most probably the lip musculature (Fig. 1h). In the same area, dense accumulation of nuclei indicates presence of numerous gland organs and sensory cells around the lips and mouth (Fig. 1g). In the following, the specific features that were observed for each representative are dealt with (for survey, see Table 1).

### Proboscis length and width

The length of the examined proboscides varied strongly between the studied species. The shortest proboscis was that of *P. timsanus* and was shorter than 1 mm. The length of the proboscides of *A. langi*, *A. californicus*, *A. castellioides*, *A. glaciale*, and *C. margarita* ranged from around 1 to 1.5 mm. *E. spinosa*, *N. macronyx*, *P. patagonica*, and *P. litorale* showed longer proboscides with a range from around 2.0 to 3.5 mm. All of them were surpassed by *C. macerrima* with a proboscis length of almost 10 mm (Fig. 2).

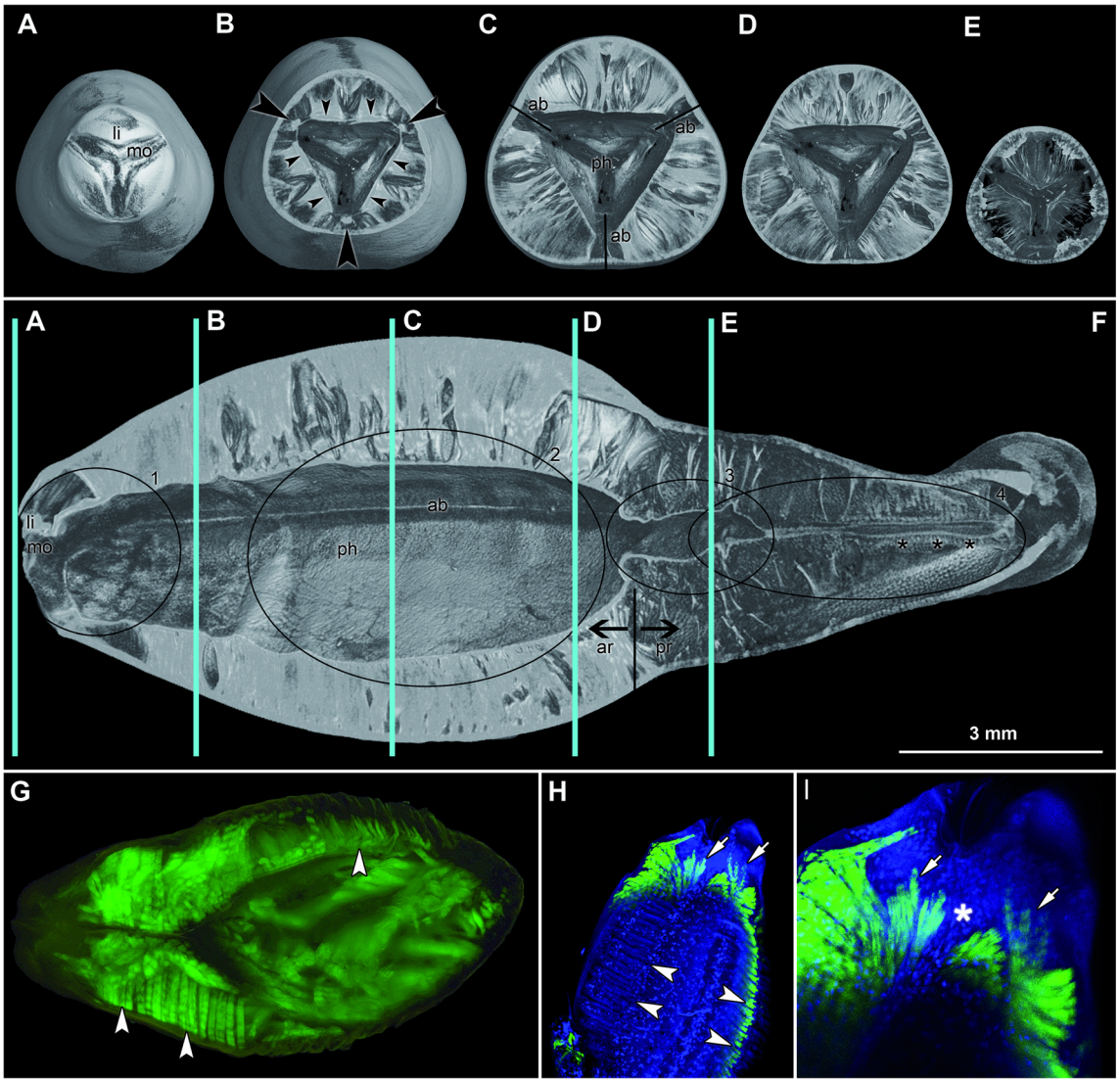


**Fig. 1** General features of proboscides using μCT (**a**–**f**), fluorescence (**g**), and confocal microscopy (**h**, **i**): **a**–**f** *Ascorhynchus japonicus*; **a**–**e** cross section of volume generated from μCT scan in different focus layers; **f** parasagittal section with positions of **a**–**e** marked with *vertical lines*, anterior is left; **g** actin green stain of nuclei in *Achelia langi*; **h** actin green and DAPI stain of musculature in *Achelia langi*, details of anterior region; and **i** actin green and DAPI stain of musculature in *Achelia langi*, details of mouth section; *ab* antimere border; *ar* anterior region; *li* lips; *mo* mouth opening; *ph* pharynx; *pr* posterior region; *small black arrowheads* radial muscles; *big black arrowheads* interradial muscles; *small white arrows* lip musculature; *big white arrowheads* radial musculature; *white asterisk* dens accumulation of nuclei close to the mouth; *black asterisk* region of the filter bristles; *circle 1*, region of lips, mouth opening, and sealing lip; *circle 2* region of anterior field of denticles; *circle 3* region of posterior field of denticles; *circle 4* region of the filter apparatus

The proboscides width/diameter varied from 140 μm to 1.2 mm. *A. glaciale* showed a very elongated proboscis but with the smallest measured width of 140 μm at the thickest and of 50 μm at the slimmest section near the mouth opening. This proboscis showed more special features in having the annulated, "pseudo segmented" look as is typical for Austrodecidae, with staggered indentions on the outer and inner surface (Fig. 2d, e). These indentions and pseudo segmented organization were not found in any other species, which all showed smooth proboscis surfaces. *A. langi*, *A. californicus*, and *C. margarita* had narrow proboscides with width of about 170 to 250 μm (Fig. 2a, b, f). The proboscides of *A. castellioides*, *E. spinosa*, *N. macronyx*, and *P. timsanus* were in an intermediate position with widths of 380 to 550 μm (Fig. 2c, h, i, k). Very broad proboscides were measured in *C. macerrima*, *P. patagonica*, and *P. litorale* with widths of 1 mm and wider (Fig. 2g, j, l).

## Lips, mouth opening, and "bulge"

All examined specimens possessed a mouth opening with three anterior lips except for *A. glaciale* that had a ventral, slit-shaped mouth opening (Fig. 3d). Small bristles were found on the lips or mouth openings of some species, viz.

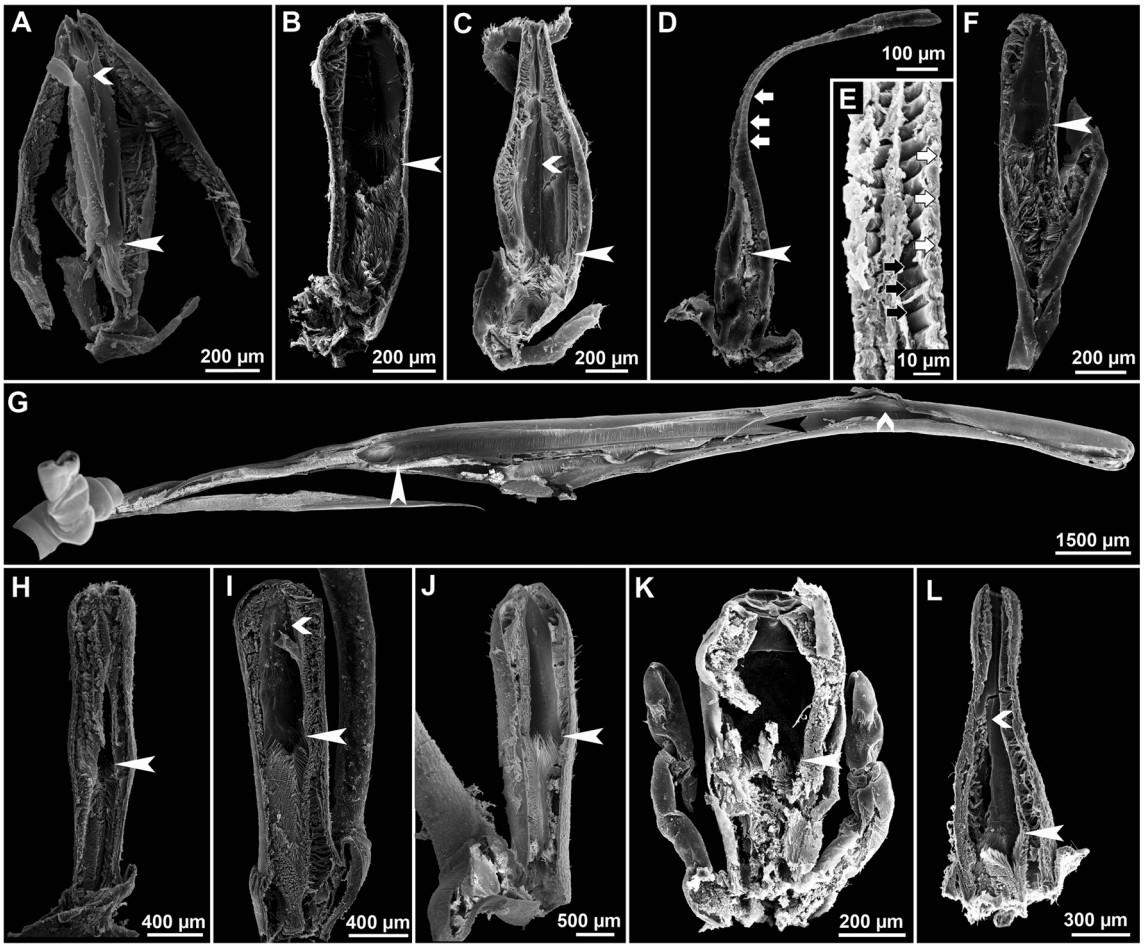


**Fig. 2** SEM images of bisected proboscides, anterior is up except for **g** where anterior is right. **a** *Achelia langi*. **b** *Anoplodactylus californicus*. **c** *Ascorhynchus castellioides*. **d** *Austrodecus glaciale*. **e** Detail of "pseudo segmented" proboscis of *Austrodecus glaciale*. *Small white arrows* indentation on outer surface of the proboscis; *small black arrows* indentation on inner surface of proboscis. **f** *Callipallene margarita*. **g** *Colossendeis macerrima*. **h** *Endeis spinosa*. **i** *Nymphon macronyx*. **j** *Pallenopsis patagonica*. **k** *Pigrogromitus timsanus*. **l** *Pycnogonum litorale*; *large white arrowheads* start of the oyster basket; *large black arrowhead* start of first filter bristle rows in *Colossendeis macerrima*; *small white arrowheads* antimere border; *small white arrows* annulated "pseudo segmented" look as is typical for Austrodecidae

*A. langi*, *A. californicus*, and *N. macronyx* (Fig. 3a, b, h). In *A. castellioides*, the lips were not covered by bristles; instead, small flaps were present (Fig. 3c). Bristles surrounding the mouth opening were found in *A. californicus*, *C. margarita*, *C. macerrima*, *E. spinosa*, *P. patagonica*, and *P. litorale* (Fig. 3b, e–g, i, k). Proximal to the mouth opening on the inner surface, all species contained a ring- or funnel-shaped bulge or swelling extending into the lumen and thus formed an annulus restricting the pharynx diameter. Its surface was smooth and very similar to the surrounding pharynx surface. This bulge was very prominent in *A. langi*, *A. californicus*, *A. castellioides*, *C. margarita*, *E. spinosa*, *P. timsanus*, and *P. litorale* (Fig. 3a–c, e, g, j, k). In *C. macerrima*, *N. macronyx*, and *P. patagonica*, it was less developed (Fig. 3f, h, i). For *A. glaciale*, only a description from the outside was possible, since bisection of the tip was technically impossible due to the very thin proboscis. However, we can state that there are lips without bristles and that also the mouth opening is bristle free (Fig. 3d). The notch was not visible from the outside so we cannot make any descriptions here.

## Antimere borders

Well-visible antimere borders were found in all species, except in *A. glaciale* (Figs. 1a–f, 2a, c, g, i, l, and 7a). They proceeded as a longitudinal invagination or groove from the anterior mouth opening from the corners between the lips to the proximal part of the pharynx. Further proximally, they passed across the filter apparatus. Distal to the oyster basket, the antimere borders were smooth or deprived of denticles in all species examined.

The antimere borders were also found within the oyster basket. In *A. langi*, *A. glaciale*, and *C. macerrima*, they proceeded as longitudinal invaginations, with no denticles

**Table 1** Proboscis and pharynx characteristics for each species studied revealed by SEM analysis

| Proboscis and pharynx characteristics for each species studied | *Achelia langi* (Ammotheidae) | *Anoplodactylus californicus* (Phoxichilidiidae) | *Ascorhynchus castellioides* (Ascorhynchidae) | *Austrodecus glaciale* (Austrodecidae) | *Callipallene margarita* (Callipallenidae) |
|---|---|---|---|---|---|
| Length of proboscis | 1300 μm | 1000 μm | 1500 μm | 1100 μm | 1100 μm |
| Width of proboscis | 170 μm | 250 μm | 450 μm | 140 μm | 250 μm |
| Triradiate lips | x | x | x | ? | x |
| Lips with bristles | x | x | Small flaps | ? | No |
| Triradiate mouth opening | x | x | x | No | x |
| Mouth opening with bristles | No | x | No | ? | x |
| Ring- or funnel-shaped bulge | x | x | x | ? | x |
| Denticle rows on midline between antimere borders | No | Few, small, pointed denticles form row between antimere borders pointing anteriorwards | No | No | No |
| Armature of antimere borders within filter apparatus | No | Ribs with triangular denticles pointing anteriorwards | Not visible | No | Ribs with triangular denticles pointing anteriorwards |
| Denticle array 1 | No | x | No | ? | x |
| Form of denticles | | Triangular pointing posteriorwards | | | Long, pointed pointing posteriorwards |
| Form of denticle array 1 | | Broad, but thin | | | Thin ring around inner surface, interrupted by antimere borders |
| Denticle array 2 | x | No | No | ? | x |
| Form of denticles | Big, round, pointing posteriorwards | | | | Triangular pointing posteriorwards |
| Length of filter apparatus | 1/4 | 1/2 | 1/4 | <1/10 | 1/2 |
| Length of filter bristles | 105 μm | 75 μm | 80 μm | 25 μm | 90 μm |
| Width of filter bristles | 2 μm | 2 μm | 2 μm | 1 μm | 2 μm |
| Side branches | x | x | x | No | x |
| Length of side branches | 4 μm | 5 μm | 6 μm | | 3 μm |
| Shape of filter bristles | Feather-like filter bristles with long side branches | Feather-like filter bristles with long side branches | Feather-like filter bristles with long side branches | Spine-shaped filter bristles consisting only of the central axis without side branches | Feather-like filter bristles with short side branches |
| Space between side braches | medium | Medium | Medium | | Small |
| Basal rib | x | x | x | x | x |
| Space between filter bristles | No space | Big space | Small space | Big space | Big space |

| *Colossendeis macerrima* (Colossendeidae) | *Endeis spinosa* (Endeidae) | *Nymphon macronyx* (Nymphonidae) | *Pallenopsis patagonica* (Pallenopsidae) | *Pigrogromitus timsanus* (Ascorhynchoidea family incertae sedis) | *Pycnogonum litorale* (Pycnogonidae) |
|---|---|---|---|---|---|
| >10,000 μm | 2200 μm | 2400 μm | 3500 μm | 840 μm | 3400 μm |
| 1000 μm | 550 μm | 520 μm | 1200 μm | 380 μm | 1000 μm |
| x | x | x | x | x | x |
| No | No | x | No | No | No |
| x | x | x | x | x | x |
| x | x | No | x | No | x |
| x | x | x | x | x | x |

**Table 1** (continued)

| *Colossendeis macerrima* (Colossendeidae) | *Endeis spinosa* (Endeidae) | *Nymphon macronyx* (Nymphonidae) | *Pallenopsis patagonica* (Pallenopsidae) | *Pigrogromitus timsanus* (Ascorhynchoidea family incertae sedis) | *Pycnogonum litorale* (Pycnogonidae) |
|---|---|---|---|---|---|
| Few, small, pointed denticles form row between antimere borders pointing anterior and continue between the rows of filter bristles pointing posteriorwards | No | No | Few, big, roundish denticles form row between antimere borders pointing anteriorwards | No | No |
| No | Ribs with triangular denticles pointing anteriorwards | Not visible | Not visible | Not visible | Not visible |
| No | No | x<br>Long, triangular pointing posteriorwards<br>Broad, but thin | x<br>Small, triangular pointing posteriorwards<br>Broad and very long field | x<br>Triangular pointing posteriorwards<br>Anterior pointing v-shaped array, tip between antimere borders | No |
| No | x<br>Small, slim pointing posteriorwards | x<br>Slim, pointed pointing posteriorwards | x<br>Few small, pointed pointing posteriorwards | No | No |
| <1/2 | 1/2 | 1/2 | 1/2 | 1/2 | 1/4 |
| 350 μm | 115 μm | 140 μm | 100 μm | 40 μm | 60 μm |
| 2 μm | 3 μm | 2 μm | 2 μm | 4 μm | 1 μm |
| different | x<br>3 μm | x<br>3 μm | x<br>3 μm | x<br>7 μm | No |
| Filter bristles consisting of splitting axis | Feather-like filter bristles with long side branches | Feather-like filter bristles with short side branches | Feather-like filter bristles with short side branches | Filter bristles similar to a pipe cleaner with long side branches | Spine-shaped filter bristles consisting only of the central axis without side branches |
| Small | Small | Small | Small | Small | |
| x | x | x | x | x | x |
| No space | Big space | Small space | Small space | Big space | Big space |

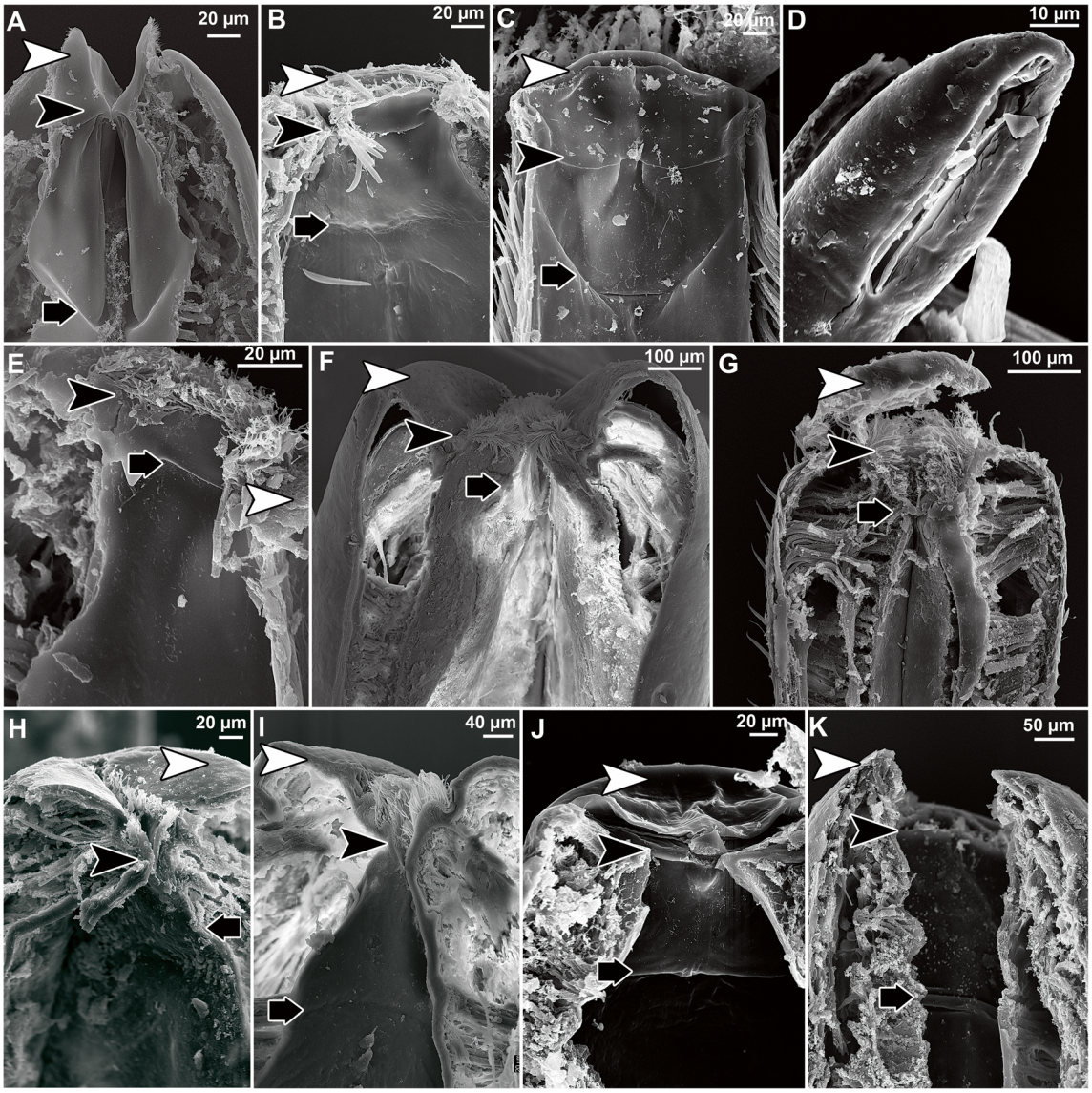


**Fig. 3** SEM images of mouth openings, anterior is up; **a** *Achelia langi*. **b** *Anoplodactylus californicus*. **c** *Ascorhynchus castellioides*. **d** *Austrodecus glaciale*. **e** *Callipallene margarita*. **f** *Colossendeis macerrima*. **g** *Endeis spinosa*. **h** *Nymphon macronyx*. **i** *Pallenopsis patagonica*. **j** *Pigrogromitus timsanus*. **k** *Pycnogonum litorale*; *large white arrowheads* lip, *large black arrowheads* mouth opening, *small black arrows* roundish or funnel-shaped bulge

visible as in the distal sections of these invaginations. In *A. californicus*, *C. margarita*, and *E. spinosa*, however, ribs with triangular denticles on top were found (Fig. 7a). In contrast to the denticle arrays described in the following, with denticles pointing posteriorly, the tip of these denticles pointed anteriorly. In our preparations of *A. castellioides*, *N. macronyx*, *P. patagonica*, *P. timsanus*, and *P. litorale*, the antimere borders were not visible, due to filter bristles covering the respective areas.

### Longitudinal denticle rows

Single longitudinal denticle rows were present on the midline of the three antimeres in some species. In *A. californicus* and *C. macerrima*, this row contained small, pointed denticles (Fig. 7b); in *P. patagonica*, they were much larger and roundish. The denticles in all three species were oriented anteriorwards. All other species lacked denticles parallel to the antimere borders. In *A. glaciale*, no antimere borders were visible due to the small size of the specimen.

### Denticle arrays

Two different denticle arrays were found on the inner surface of the proboscides. A more anterior array, at about half way between mouth and filter apparatus, is referred to as denticle array 1 and a second, more posterior array, located close to the

beginning of the filter apparatus, as denticle array 2 within this study (Fig. 4).

Denticle array 1 was found in five species, with the denticles always pointing posteriorwards. A broad but thin (in sagittal orientation) array was found in *A. californicus* with triangular denticles (Fig. 4a) and also in *N. macronyx* with long and pointed denticles. In *C. margarita*, the denticles were also long and pointed, and the array formed a thin ring around the inner surface, only interrupted by the antimere borders. Small triangular denticles, which formed a broad and very long field, were visible in *P. patagonica*. In *P. timsanus*, the triangular denticles formed an anteriorly pointing V-shaped array, with the tip lying between the antimeric borders (Fig. 4b). In all other species, denticle array 1 was not present.

Denticle array 2, with denticles pointing posteriorwards, was also found in five species. Like in denticle array 1, the denticles were of different shape: In *A. langi*, they were large and roundish (Fig. 4c); in, *C. margarita*, they were triangular; in *E. spinosa*, they were small and slim (Fig. 4d); in *N.* macronyx, they were pointed (Fig. 4e), and in *P. patagonica*, a few small, pointed denticles were present.

Due to the small size, it was not possible to localize a denticle array in *A. glaciale*.

## Oyster basket and filter bristles or barbules

In all examined species, the prominent filter apparatus was found in the proximal pharynx section but extended over different contingents or percentage of pharynx length (Fig. 2). In *A. glaciale*, with its very long and thin proboscis, the filter apparatus was less than one tenth of the proboscis length (Fig. 2d). In *A. langi* (Fig. 2a), *A. castellioides* (Fig. 2c), and *P. litorale* (Fig. 2l), the filter apparatus inhabited around one fourth and in *A. californicus* (Fig. 2b), *C. margarita* (Fig. 2f), *E. spinosa* (Fig. 2h), *N. macronyx* (Fig. 2i), *P. patagonica* (Fig. 2j), and *P. timsanus* (Fig. 2k), around half of the proboscis length. In *C. macerrima*, more than half of the proboscis was occupied by the oyster basket (Fig. 2g).

The size of the filter bristles ranged from 25- to 350-μm length and 1 to 4 μm in width. Five types of filter bristles were

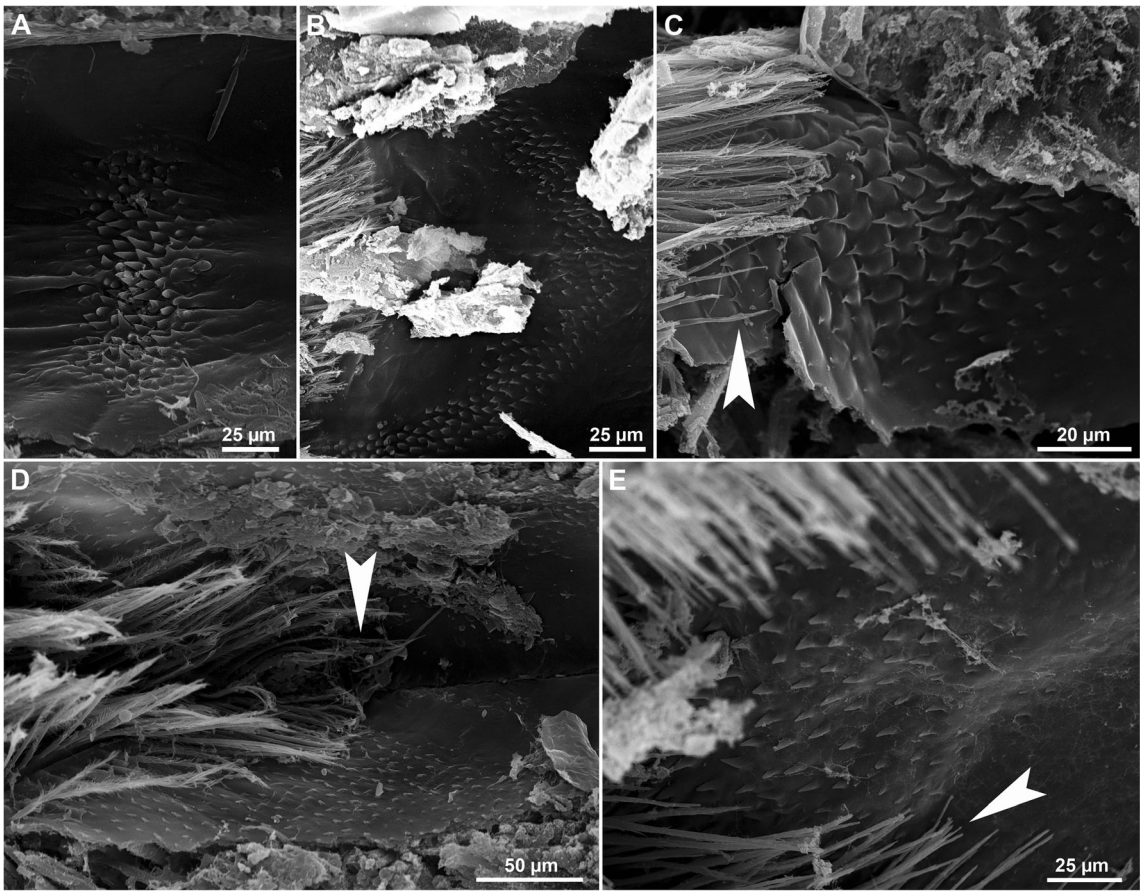


**Fig. 4** SEM images of shape and position of denticle arrays, anterior is right; **a**, **b** anterior field of denticles in distance to filter apparatus; **c**–**e** posterior field of denticles, close to the filter apparatus. **a** *Anoplodactylus californicus*, triangular denticles. **b** *Pigrogromitus timsanus*, triangular denticles. **c** *Achelia langi*, big, round denticles. **d** *Endeis spinosa*, small, slim denticles. **e** *Nymphon macronyx*, slim, pointed denticles; *large white arrowheads* anterior filter bristles showing a "used look"

found: feather-shaped bristles with (i) short and (ii) long side branches inserted at different angles along the main axis, (iii) spine-shaped bristles without side branches, (iv) filter bristles consisting of a main axis splitting distally into longitudinal subbranches, and (v) filter bristles with a pipe cleaner-like shape with long side branches inserted all around the main axis.

*C. margarita* (Figs. 5e and 6e), *N. macronyx* (Figs. 5h and 6h), and *P. patagonica* (Figs. 5i and 6i) possessed feather-like filter bristles of type (i) with short side branches. The lengths of the bristles ranged from around 90 μm in *C. margarita*, 100 μm in *P. patagonica* to 140 μm in *N. macronyx* and were all about 2 μm wide. Many side branches with a length of about 3 μm were found on both sides of the axis with small spaces between them.

*A. langi* (Figs. 5a and 6a), *A. californicus* (Figs. 5b and 6b), *A. castellioides* (Figs. 5c and 6c), and *E. spinosa* (Figs. 5g and 6g) showed more or less similar filter bristles of type (ii). The filter bristle length ranged from 75 μm in *A. californicus*, 80 μm in *A. castellioides*, 105 μm in *A. langi* to 115 μm in *E. spinosa*. The width of the bristles was around 2 μm except for *E. spinosa* with bristles around 3 μm wide. In all four species, the filter bristles were feather-like shaped with several side branches on both sides of the bristle's axis. The side branches were around 4 μm long in *A. langi* and *E. spinosa*, 5 μm long in *A. californicus*, and around 6 μm long in *A. castellioides*. In *E. spinosa*, the space between the bristles was smaller than in the other three species, resulting in more side branches and a fluffier look of the filter bristles.

*A. glaciale* (Figs. 5d and 6d) and *P. litorale* (Figs. 5k and 6k) showed spine-shaped filter bristles consisting only of the central axis without side branches (type (iii)). The filter bristles of *A. glaciale* were very short with around 25 μm; in *P. litorale*, they were around 60 μm long. In both species, the bristles were around 1 μm wide.

*C. macerrima* (Figs. 5f and 6f) showed a different shape of the filter bristles (type (iv)). Side branches were not found, but instead, the axis of the bristles split and formed long strands of a longitudinal orientation. The bristles were around 350 μm long and 2 μm wide. In all species except for *C. macerrima*, the anterior end of the oyster basket formed a ring-shaped distinct border (Fig. 2), while in *C. macerrima*, a few bristle rows including the basal bars bent anteriorwards and extended to the anterior part of the pharynx close to the antimere borders. These structures shaped like longitudinal combs

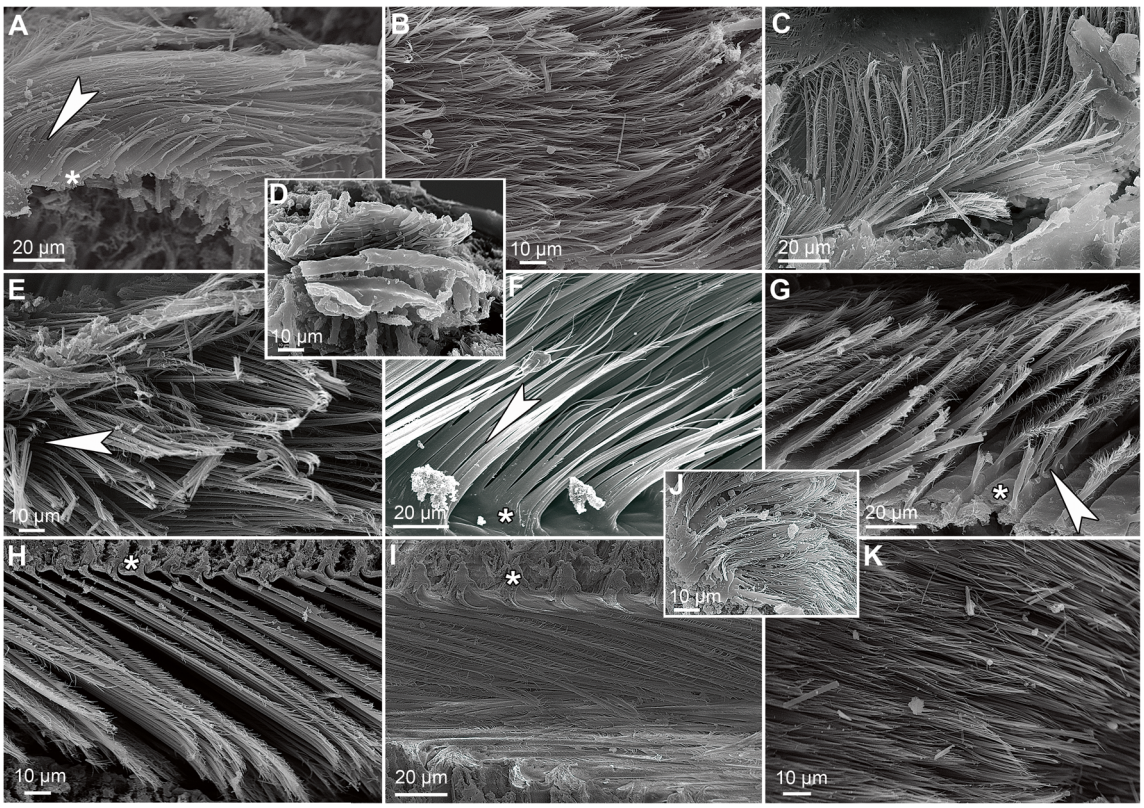


**Fig. 5** SEM images of filter bristles, anterior is right; **a**–**c**, **g** feather-like filter bristles with long side branches; **d**, **k** spine-shaped filter bristles consisting only of the central axis without side branches; **e**, **h**, **i** feather-like filter bristles with short side branches; **f** filter bristles consisting of splitting axis; **j** filter bristles similar to a pipe cleaner with long side branches. **a** *Achelia langi*. **b** *Anoplodactylus californicus*. **c** *Ascorhynchus castellioides*. **d** *Austrodecus glaciale*. **e** *Callipallene margarita*. **f** *Colossendeis macerrima*. **g** *Endeis spinosa*. **h** *Nymphon macronyx*. **i** *Pallenopsis patagonica*. **j** *Pigrogromitus timsanus*. **k** *Pycnogonum litorale*; *white asterisks* basal part of the filter bristles arranged in rips; *large white arrowheads* spaces between the bases of the filter bristles on the bars

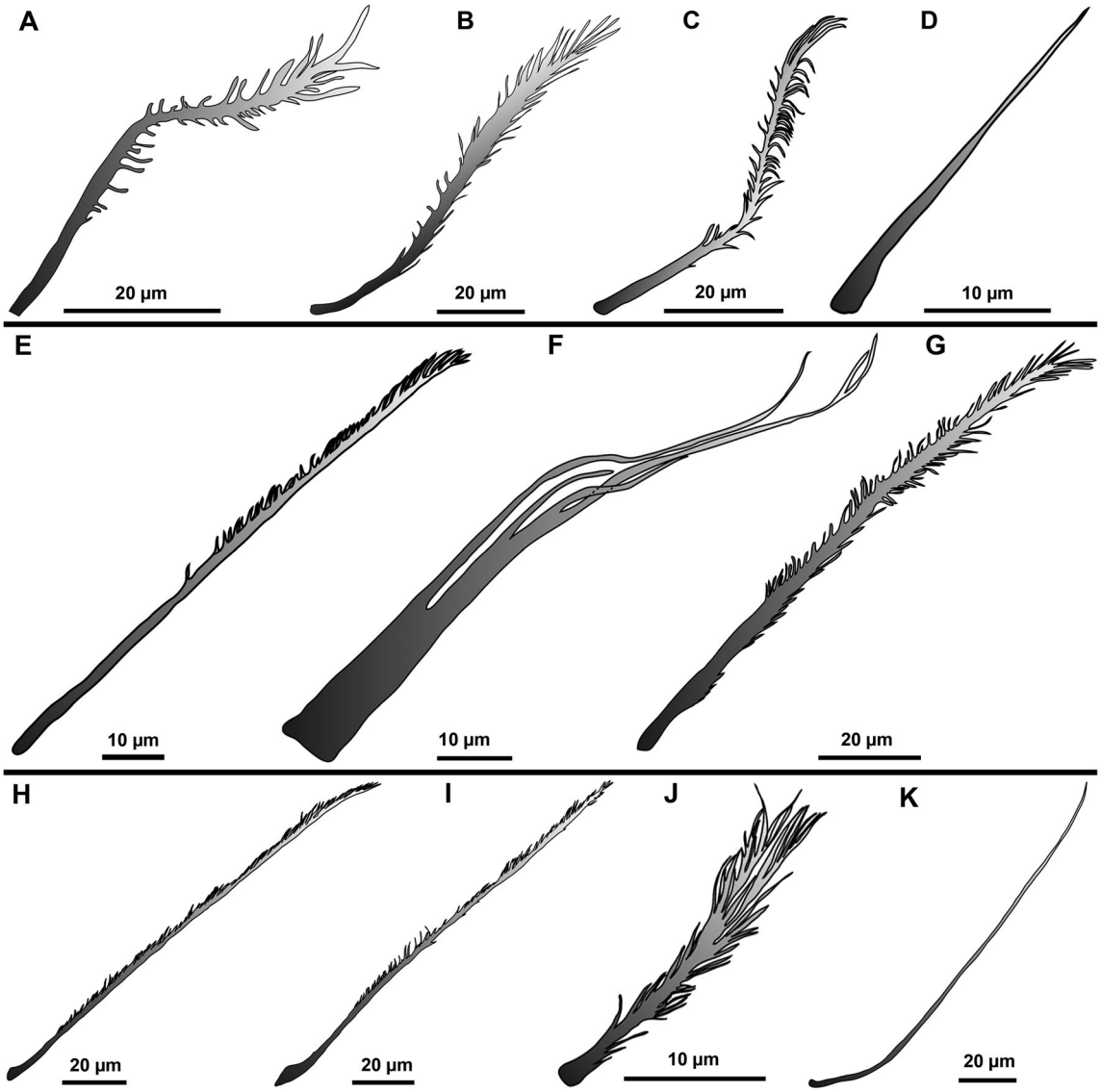


**Fig. 6** Drawings of filter bristles from the middle region of the filter apparatus (to exclude the used ones from the beginning of the filter apparatus); **a**–**c**, **g** feather-like filter bristles with long side branches; **d**, **k** spine-shaped filter bristles consisting only of the central axis without side branches; **e**, **h**, **i** feather-like filter bristles with short side branches; **f** filter bristles consisting of splitting axis; **j** filter bristles similar to a pipe cleaner with long side branches. **a** *Achelia langi*. **b** *Anoplodactylus californicus*. **c** *Ascorhynchus castellioides*. **d** *Austrodecus glaciale*. **e** *Callipallene margarita*. **f** *Colossendeis macerrima*. **g** *Endeis spinosa*. **h** *Nymphon macronyx*. **i** *Pallenopsis patagonica*. **j** *Pigrogromitus timsanus*. **k** *Pycnogonum litorale*

extended to around two thirds of the pharynx length. These filter bristles showed no splitting axis, but a single axis instead, forming one long strand (Fig. 7d).

Filter bristles similar to a pipe cleaner with long side branches all around the axis (type (v)) were found in *P. timsanus* (Figs. 5j and 6j). The bristles were only 40 µm long, but 4 µm wide. The side branches were long with very small spaces between them and were present around the whole axis, giving the filter bristle the pipe cleaner-like look.

Two features were shared by all the examined species. Firstly, at the most anterior part of the filter apparatus, the bristles showed a used look (Fig. 4c, d, e). They were shorter and had less or no side branches, and hence, this section of oyster basket was not used to assign the bristles to one of the five types. Secondly, numerous basal bars orientated one after another in transversal planes all along the oyster basket were found in all studied species; i.e., they formed rings only interrupted by the antimere borders. These represented the structures at which the filter bristles were inserted (Fig. 5g, h, i). In *N. macronyx* (Fig. 7e) and *A. langi* (Fig. 7f), the three-dimensional arrangement of the bars and their articulation could be analyzed. *N. macronyx* showed bars with two hooks, an

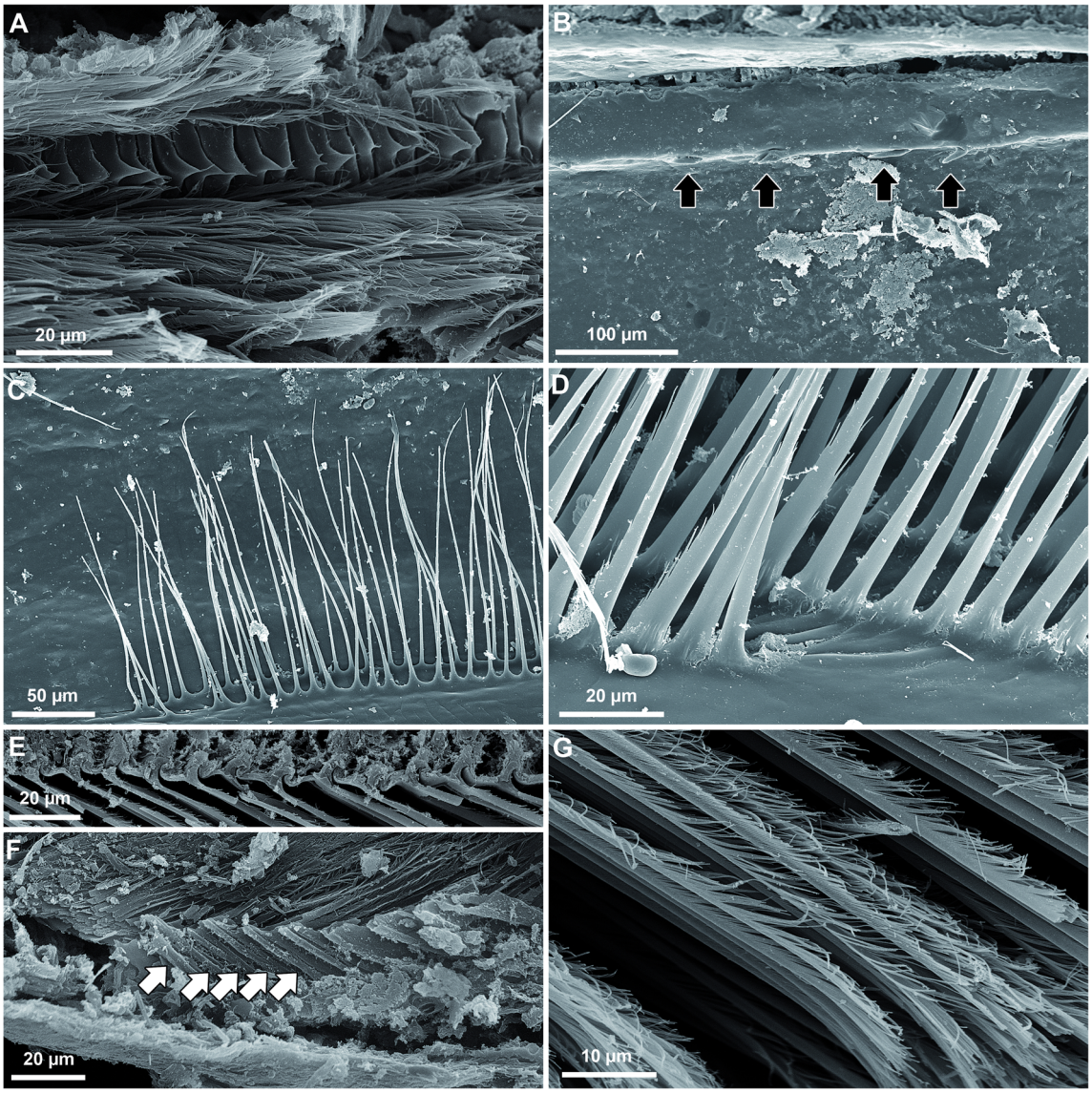


**Fig. 7** SEM images of details; **a** *Endeis spinosa*, border between two antimeres with denticles within the filter apparatus. **b** *Colossendeis macerrima*, row of denticles at antimeres outside the filter apparatus. **c** *Colossendeis macerrima*, start of the filter bristles sitting on bars. **d** *Colossendeis macerrima*, basis of filter bristles arranged on bars. **e** *Nymphon macronyx*, longitudinal section of the basis of filter bristles, section through bars. **f** *Achelia langi*, top view on the bars and filter bristles. **g** *Nymphon macronyx*, feather-like structure of the filter bristles; *black arrows* denticles at antimeres outside the filter apparatus; *white arrows* top view on bars within the filter apparatus

anterior and a posterior one. These hooks interlocked with those of the prior anterior bar and those of the following posterior bar. In *A. langi*, these bars were absent. In all other species, the bars were present but could not be examined to this detail.

Three different types were found when looking on the spaces between the bases of the filter bristles on the bars. In *A. langi* and *C. macerrima*, no spaces were found between the basal parts of filter bristles. Small spaces were found in *A. castellioides*, *N. macronyx*, and *P. patagonica*. Bigger spaces, measuring about the width of a filter bristle, were found in *A. californicus*, *A. glaciale*, *C. margarita*, *E. spinosa*, *P. timsanus*, and *P. litorale* (Fig. 5a, e–g).

## Discussion

With the present study, we made the first attempt to visualize pycnogonid pharynx armatures across taxa using SEM. The pictures indicate that pharynx armatures are highly variable, as has been suggested by Fry (1965), and it seems reasonable to assume that they are related to different feeding behavior and preferred nutrition of the different pycnogonid lineages. Details on feeding habits are known for several pycnogonids (e.g., Fry 1965; Lotz 1968; Richards and Fry 1978; Heß and Melzer 2003; Soler-Membrives et al. 2013; Mercier et al. 2015; see also summary of early observations in Helfer and Schlottke 1935), but for many taxa, knowledge is cursory and

relies on occasional and partly contradictory observations. Thus, it seems too early for detailed analysis of structure-function relationships between pharynx structures and nutrition. However, we think that consideration of aspects of functional morphology of the pharynx structures based on our results seems adequate at the current state of knowledge.

According to our results, it is suggested that all pycnogonids share the same three sections of pharynx, i.e., (i) the lip and mouth part used for food uptake, followed by (ii) a "food handling" section characterized by specific denticle arrangements that extends over large parts of the pharynx, and (iii) the oyster basket section (Fig. 8). Each section bears characteristic structural elements, most of which have already been described for some species in the early works of Dohrn and Hoek (both 1881). This also accounts for the triradiality of the pharynx and its musculature providing suction power, lip, and oyster basket movement, as is now known for various pycnogonids (e.g., Miyazaki 2002). It should also be noted that our results fully support the triradiate, Y-shaped pharynx form and orientation of the antimeres and muscles as reviewed in this recent survey and many older studies. However, confusion has arisen in literature based on an illustration in King (1973) where the pharynx is erroneously shown in upside-down orientation, and regrettably, this went straight into phylogenetic analyses (see Miyazaki 2002 for details).

Among the elements of section 1, most interesting seems the structure described here as bulge that we found in all of the studied pycnogonids except *Austrodecus* but is not mentioned in earlier studies. Its location and shape suggest that together with the inner part of the lips, it might function as a sealing lip needed to produce suction power for food uptake.

Section 2, here referred to as food handling section, seems to form a special functional unit that has not been considered before as a distinct part of the pharynx. According to our results, its maximum equipment includes armatures that have either a posterior or anterior orientation, i.e., denticle row (anterior orientation) and denticle areas 1 and 2 (both with posterior denticle orientation; see also Fahrenbach and Arango 2007). We suggest that this is an indication of the presence of larger food particles in this part of the pharynx, as

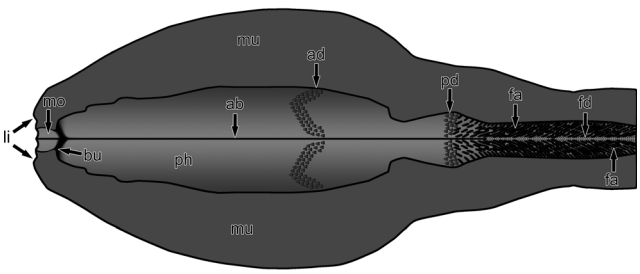


**Fig. 8** Drawing of general features of proboscides: *ab* antimere border, *ad* anterior field of denticles, *bu* bulge, *fa* filter apparatus, *fd* denticles within the filter apparatus, *li* lips, *mo* mouth opening, *mu* musculature, *pd* posterior field of denticles, *ph* pharynx

otherwise, the denticles would not be needed. These denticle areas could contribute to handling and milling of food. On the other hand, the denticle areas with their denticles oriented posteriorwards could prevent food from being squeezed back to the mouth and the denticle rows oriented anteriorwards could prevent particular food from entering the oyster basket untimely. Hence, section 2 might be a region where food is prepared for the oyster basket, e.g., by continuing the predigestion process started by secretion around the lips (Fahrenbach and Arango 2007) or by providing first steps of milling with the help of the denticles. Conversely, in cases where section 2 is completely smooth, we would expect at least a high share in fluid nutrition. In the pantopods studied here, almost all variations in these types of armature occur: All three denticles areas are found in *P. patagonica*, and a completely smooth section 2 is found in *A. castellioides* (and eventually *A. glaciale*). In between these two extreme versions of armature, all possible combinations of elements have been seen: e.g., the two denticle arrays but no denticle row are found in *N. macronyx*; denticle row and denticle array 1 (but not 2) are found in *A. californicus*. *C. macerrima* has only the denticle row but none of the denticle areas, and *A. langi* has only denticle area 2 but neither denticle row nor denticle area 1.

Compared to section 2, section 3, the oyster basket, is the part of pharynx that has been most intensely studied in the past, though only for a limited set of taxa (Dohrn 1881; Hoek 1881; Fry 1965; Dencker 1974; Richards and Fry 1978; Fahrenbach and Arango 2007; Soler-Membrives et al. 2013). In the present study, we give further evidence that the oyster basket can be found in representatives of all extant pycnogonid main lineages and that its position is always in the proximal-most section of pharynx. This includes ubiquity of more features related to oyster basket, i.e., basal ribs, filter bristles, and musculature allowing for bristle movement for the filtering and/or grinding mill function. In addition to these general features, we observed two sets of characters that vary strongly between species: (i) the relative length of oyster basket in relation to pharynx length and (ii) the fine structure and dimensions of filter bristles. As shown in Table 1, relative length of oyster basket varies between 1/10 (*A. glaciale*) and one half of pharynx length (e.g., *A. californicus*). Between the extremes are pycnogonids with filter apparatus exhibiting ca. one-fourth pharynx length, i.e., *A. langi*, *A. castellioides*, and *P. litorale*. Already, these differences should result in different filters and/or milling properties and intensities, respectively. In this context, Soler-Membrives et al. (2013) suggested that carnivorous and detritivorous pycnogonids differ in the relative length of oyster basket.

The second feature, i.e., fine structure of the filter bristles, also showed a high degree of variability between taxa indicating differences in the filtering and milling properties. In earlier studies (Fahrenbach & Arango 2007), only bristles with regular, feather-like lateral branches have been described using

the transmission EM. These correspond to types 1 and 2 in this study that mainly differ in the length of the two rows of side braches on each bristle. Though these two types are found in most of the studied species, there are at least three more conspicuous bristle types that differ from the "feather-shaped" form. While the pipe cleaner type found in *P. timsanus* is also characterized by presence of side branches, but in radial rather than feather-shaped arrangement on the main axis, the unbranched spine-like bristles found in *A. glaciale* and *P. litorale* differ strongly, indicating a change in functional properties. Interestingly, the largest pycnogonids of the family Colossendeidae, here represented by *C. macerrima*, also have bristles without side branches. Instead, they show a longitudinal splitting of the main branch into numerous longitudinal filaments. In addition, *C. macerrima* is the only species studied here with bristle rows twisted into the anterior-posterior axis of pharynx extending into the anterior pharynx portion far distal to the oyster basket. s. str.

For analyses of the oyster basket's function, it seems relevant that in many specimens, we observed abraded filter bristles in its anterior-most part, indicating that the filtering and/or milling activity leads to deterioration. Fahrenbach and Arango (2007) who observed unornamented anterior-most barbs in *A. hilgendorffi* have described a similar effect. For comparison of filter bristles, only "fresh" ones located at the middle or proximal part should therefore be included in analyses.

Apart from the filter bristle's structure, also their different lengths are suggested to indicate food differences. It is maybe not a surprise that *C. macerrima*, the largest species and the one with the biggest proboscis, has also the longest filter bristles and is presumably capable of taking up the largest food particles. Similarly, the shortest, spine-like bristles are found in a very small species, *A. glaciale*, followed by the pipe cleaner-like bristles in *P. timsanus*. However, the relation between body and filter size and presumed food size is not necessarily linear. For example, species like *A. langi*, *E. spinosa*, and *N. macronyx* have longer bristles than *P. patagonica*, the second largest species in our selection, and those of *C. margarita*, a species that is also small compared to *Pallenopsis*, are almost as long as these.

Relevant for the understanding of the oyster basket's function are also the anterior and posterior hooks that connect consecutive ribs, i.e., the sockets for the filter bristles. These are documented here for the first time for *A. langi* and *N. macronyx*. Their way of interlocking indicates that they provide articulation and power transmission. We suggest therefore that together with the contraction of radial and interradial muscles, they allow for a consorted up and down movement of filter bristles "rib by rib." In contrast to other studies (Fry 1965; Dencker 1974), we could not locate circular muscles in our actin stains of *A. langi* proboscides.

The pycnogonid's pharynx features studied in this paper offer great potential for phylogenetic hypotheses, exhibiting the typical pattern of features evolving during phylogeny including presence and absence of structures, changes in form and size, etc. As only one species per main lineage within pycnogonids has been analyzed in this study, it would be premature to try a detailed cladistic analysis of the features. However, we are convinced that several of the herein identified morphological characteristics of the proboscis are worth a closer consideration in a phylogenetic context.

Among these, distribution of bristle types among taxa might be relevant. In this study, we find the feature "absence of lateral bristle branches" in all three representatives of the Austrodecidae + Pycnogonidae + Colossendeidae clade in Arango & Wheeler (2007), i.e., putative sister group to all other Pycnogonida. This clade is well supported by molecular results, but there has been no morphological support until now (Arango and Wheeler 2007). If so, bristles with lateral branches as we found in all other studied taxa would be the alternative character state.

Moreover, as the oyster basket made of filter bristles is found in all taxa examined—including even Austrodecidae—it is suggested that it represents a ground pattern feature of Pycnogonida useful for phylogenetic considerations and thus could complement recent studies (Arango 2002; Dunlop and Arango 2005). However, the species studied here cover only Stiripasterida and Eupantopodida, i.e., the main extant pycngonid lineages, while the various extinct forms are not accessible for such an analysis. Moreover, it should be noted that the presence of an oyster basket, and also triradiality of the whole pumping-filtering-milling mechanism of the pharynx, could be plesiomorphic. As reviewed in Miyazaki (2002), various arthropods possess a triradial pharynx, and even in non-arthropods, this character state is found (see also Nielsen 2013). This also holds true for the presence of pharyngeal filter apparatus. These are common in Arachnida, e.g., solifuges (Klann and Alberti 2010), ricinuleids (Talarico et al. 2011), and tetrapulmonate arachnids (Dunlop 1994; Dunlop et al. 2006), i.e., taxa with extraintestinal predigestion and uptake of liquefied food as in pycnogonids. In Xiphosura and Scorpiones, two early offshoots of the chelicerate stem lineage with different feeding habits, pharyngeal filters are not found.

In conclusion, it seems worthwhile to expand our approach on more representatives of the pycnogonid's main lineages in the future to learn more about the variability of different features described here and to learn more about the evolution of the oyster basket. A second scope for future studies could be the question of homology or non-homology of pycnogonid oyster basket and arachnid filters. Though the "uniqueness" of pycnogonid's proboscis as inferred already by Dohrn (1881) and supported by recent studies (Brenneis et al. 2011) seems now well established, the presence of the filter apparatus could be plesiomorphic and therefore represent a link between the pharynx of pycnogonids located in the proboscis and that of other Chelicerata located also in the pharynx, but deeper in the anterior body section.

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