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Pallenopsis patagonica (Hoek, 1881) – a species complex revealed by morphology and DNA barcoding, with description of a new species of *Pallenopsis* Wilson, 1881

ANDREA WEIS[1]*, ROLAND MEYER[1], LARS DIETZ[2], JANA S. DÖMEL[2], FLORIAN LEESE[2] and ROLAND R. MELZER[1]

[1]*Zoologische Staatssammlung München, Münchhausenstraße 21, 81247 München, Germany*
[2]*Ruhr-Universität Bochum, Evolutionsökologie und Biodiversität der Tiere, Universitätsstraße 150, D-44801 Bochum, Germany*

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Pallenopsis patagonica (Hoek, 1881) is one of the most taxonomically problematic and variable pycnogonid species, and is distributed around the southern South American coast, and the Subantarctic and Antarctic areas. We conducted a phylogenetic analysis of mitochondrial cytochrome *c* oxidase subunit I (*COI*) sequences of 47 *Pallenopsis* specimens, including 39 morphologically identified as *P. patagonica*, five *Pallenopsis pilosa* (Hoek, 1881), one *Pallenopsis macneilli* Clark, 1963, one *Pallenopsis buphtalmus* Pushkin, 1993, and one *Pallenopsis latefrontalis* Pushkin, 1993. Furthermore, we studied morphological differences between the different *COI* lineages using light and scanning electron microscopy, including also material from Loman's and Hedgpeth's classical collections, as well as Hoek's type material of *P. patagonica* from 1881. The molecular results unambiguously reveal that *P. patagonica* is a complex of several divergent clades, which also includes *P. macneilli*, *P. buphtalmus*, and *P. latefrontalis*. Based on the material available, two major clades could be identified, namely a 'Falkland' clade, to which we assign the nominal *P. patagonica*, and a 'Chilean' clade, which is distinct from the 'Falkland' clade. We describe the 'Chilean' clade as new species, ***Pallenopsis yepayekae* sp. nov.** Weis, 2013. All molecular results are confirmed by specific morphological characteristics that are discussed in detail and compared with *Pallenopsis* species closely related to the *P. patagonica* complex. Our results reveal that *P. patagonica* is a species-rich complex that is in need for a thorough taxonomic revision, using both morphological and genetic approaches.

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ADDITIONAL KEYWORDS: biogeography – Chile – *COI* – cryptic species – Pallenopsidae – Pantopoda – Subantarctic.

INTRODUCTION

Pallenopsis patagonica (Hoek, 1881), from the material of the HMS *Challenger* expedition, was, as the name implies, first sampled off southern South American coasts. It represents one of the most taxonomically problematic and variable pycnogonid species known to date. The complexity can already be recognized by the various synonyms that exist for this species, viz. *Pallenopsis glabra* Möbius, 1902, *Pallenopsis hiemalis* Hodgson, 1907, *Pallenopsis meridionalis* Hodgson, 1915, *Pallenopsis moebiusi* Pushkin, 1975, and *Bathypallenopsis meridionalis* (Hodgson, 1927) (Bamber & El Nagar, 2011). In addition, some valid species exist that are morphologically very similar to *P. patagonica*, e.g. *Pallenopsis buphtalmus* Pushkin, 1993. *Pallenopsis patagonica* is known from Antarctic and Subantarctic regions, mainly the Scotia Sea, Ross Sea, Antarctic Peninsula, and South America, including the Magellan Strait, but is also known from the Falkland Islands, South

*Corresponding author. E-mail: andreaweis@gmx.net

Georgia, and shelf regions from the east Antarctic sector (Hoek, 1881; Möbius, 1902; Hodgson, 1907; Loman, 1923a, b; Gordon, 1932; Marcus, 1940; Hedgpeth, 1961; Pushkin, 1975, 1993; Stock, 1975; Müller, 1993; Child, 1995; Munilla & Soler Membrives, 2009; Weis & Melzer, 2012b). Specimens can be found in depths ranging from 3 down to 4540 m (Munilla & Soler Membrives, 2009).

To unscramble the complex taxonomy of *P. patagonica*, and to test whether all morphologically variable specimens available for our analysis represent a single species, we sequenced a fragment of the mitochondrial cytochrome *c* oxidase subunit I (*COI*) gene. This gene is variable, and has been applied successfully for species-level taxonomy in pycnogonids (Mahon, Arango & Halanych, 2008; Krabbe *et al*., 2010; Weis & Melzer, 2012a). Altogether, 39 *P. patagonica* specimens were sampled from 33–72°S and 11–170°W (in a depth range of 3–466 m), with a focus on the area around the southern tip of South America. Furthermore, the morphology of all available specimens was studied in detail with light and scanning electron microscopy (SEM), demonstrating differences among samples from different localities. Morphological analyses include specimens from the type material of *Pallenopsis tumidula* Loman, 1923 (SMNH Type 1293 and syntypes), one specimen of *P. patagonica* (SMNH-125527) from Hedgpeth's collections from the Swedish Museum of Natural History (Loman, 1923b; Hedgpeth, 1961), eight other specimens of *P. patagonica* (SMNH-125445, SMNH-125507, SMNH-125508, SMNH-125509, SMNH-125510), and one unidentified *Pallenopsis* sp. (SMNH-125514). In addition, we also studied/consulted Hoek's type material of *P. patagonica* (BMNH 1881.38, three specimens) and *P. patagonica* var. *elegans* (BMNH 188.38, one specimen), which are kept in the Natural History Museum in London. Furthermore, we analysed three *Pallenopsis notiosa* Child, 1992 specimens, which are housed at the Zoologische Staatssammlung München (ZSM) (Weis & Melzer, 2012b). Our morphological data set includes a total of 61 specimens.

As mentioned in our previous study (Weis & Melzer, 2012a), the southern Chilean coastline provides an interesting opportunity for studying speciation processes. Given that the last glaciation ended only 15 000 years ago, and the low dispersal ability of pycnogonids, haplotypes of cryptic species have only a rather limited geographical distribution, as was the case for *Achelia assimilis* (Haswell, 1885) (Weis & Melzer, 2012a). Whether similar effects can be found concerning the species *P. patagonica* is one aim of the present study.

As yet, further molecular studies focusing on particular groups of pycnogonids have only explicitly been performed for the genera *Colossendeis* (Krabbe *et al*., 2010; Dietz *et al*., 2011; Dietz *et al*., 2013), *Nymphon* (Mahon *et al*., 2008; Arango, Soler-Membrives & Miller, 2011) and *Pseudopallene* (Arango & Brenneis, 2013). With *Pallenopsis* we want to open the field for a further, very complex, variously shaped group, with a focus on southern South American coasts and surrounding areas.

MATERIAL AND METHODS

Specimens and vouchers

Specimens from the Chilean coast were collected by SCUBA diving during expeditions organized by the Huinay Scientific Field Station between 2006 and 2011 (Försterra, 2009). Additionally, we received material from the region of Valparaiso, a more northern area in Chile, from the Falkland Islands, South Georgia, and the Weddell Sea (see Acknowledgements). A detailed overview of the different sample locations of the studied individuals is given in Figure 1. Material was fixed in 96% ethanol to ensure high-quality DNA for genetic analysis. Pycnogonids were identified based on morphology using a variety of literature (Hoek, 1881; Möbius, 1902; Hodgson, 1907; Gordon, 1932, 1944; Stock, 1957, 1975; Pushkin, 1975, 1993; Child, 1995; Weis & Melzer, 2012b). Furthermore, synonyms, depth ranges, and distribution patterns were taken from Müller's (1993) *World Catalogue and Bibliography of the recent Pycnogonida*, Munilla & Soler Membrives (2009), and Pycnobase (Bamber & El Nagar, 2011). All barcoded voucher specimens are kept at ZSM under specific voucher IDs (see Table 1), including PpaE_001–008, PpaE_010, PpaA_001, and PxxE001-002. The respective DNA extract aliquots are stored partially at the Canadian Center for DNA Barcoding (CCDB), the ZSM DNA bank facility, and Ruhr University Bochum. Collection data, BOLD or GenBank accession numbers of all 39 pycnogonid sequences examined in this study, as well as chosen out-group taxa are summarized and listed on Table 1. Some of the specimen details can further be accessed in the Barcode of Life Data Systems (Ratnasingham & Hebert, 2007), under the project Chilean Fjord Pycnogonids (CFAP), as part of the Marine Life (MarBOL) campaign. The sequences FJ969367–69 of *P. patagonica* from the Ross Sea were accessed from GenBank (Nielsen, Lavery & Lörz, 2009). Furthermore, we used five GenBank sequences of *Pallenopsis pilosa* (Hoek, 1881) (PxxE001, PxxE002, KC848052, KC848053, KC848054), one sequence of *P. buphtalmus* Pushkin, 1993 (HM426171), one *Pallenopsis latefrontalis* Pushkin, 1993 (HM426218), and *Pallenopsis macneilli* Clark, 1963 (DQ390086) as outgroups. Although specimens

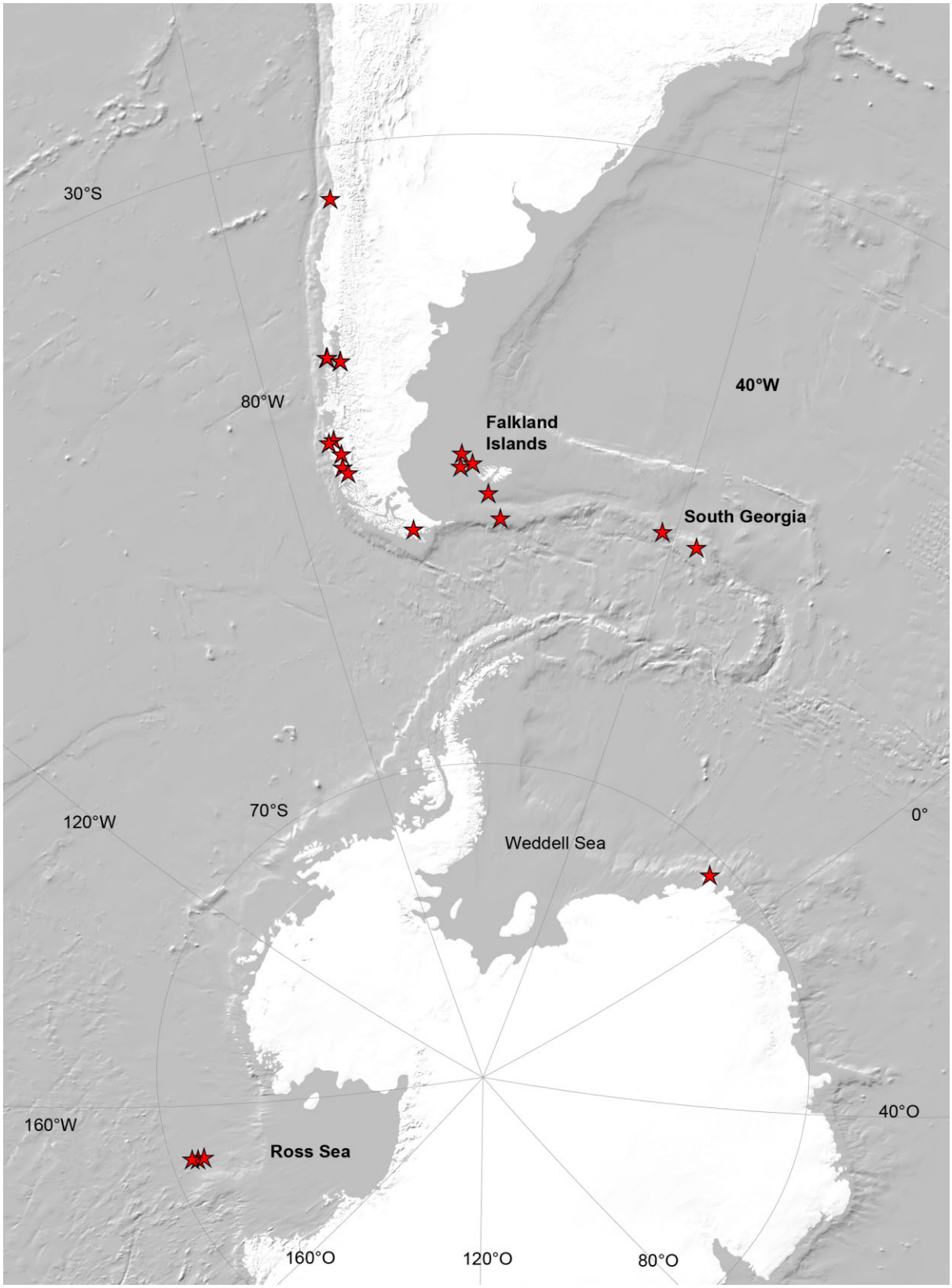

Figure 1. Map of sampling sites of Chilean, Antarctic, and Subantarctic *Pallenopsis* specimens deposited at the Bavarian State Collection of Zoology. Sequences of specimens from the Ross Sea were downloaded from GenBank.

Table 1. Summary of collection data and registration of specimens used in this study

Voucher ID	Haplotype	Species	Country/Region	Latitude	Longitude	Depth	BOLD ID/ GenBank ID
ZSMA20111000	HT 7	***Pallenopsis yepayekae* sp. nov.**	Chile; Region de Magallanes y de la Antarctica Chilena	48°44′11.4″S	75°24′53.1″W	15 m	CFAP013-11
ZSMA20111002	HT 6	***Pallenopsis yepayekae* sp. nov.**	Chile; Region de Magallanes y de la Antarctica Chilena	50°50′07.1″S	74°08′20.9″W	25 m	CFAP017-11
ZSMA20111003	HT 3	***Pallenopsis yepayekae* sp. nov.**	Chile; Region de los Lagos	43°25′03.0″S	74°04′51.2″W	25 m	CFAP006-11
ZSMA20111004	HT 1	***Pallenopsis yepayekae* sp. nov.**	Chile; Region de los Lagos	43°24′34.5″S	74°05′00.7″W	9 m	CFAP005-11
ZSMA20111005	HT 4	***Pallenopsis yepayekae* sp. nov.**	Chile; Region de Magallanes y de la Antarctica Chilena	48°44′11.4″S	75°24′53.1″W	23 m	CFAP014-11
ZSMA20111006	HT 1	***Pallenopsis yepayekae* sp. nov.**	Chile; Region de los Lagos	43°25′03.0″S	74°04′51.2″W	20 m	CFAP007-11
ZSMA20111008	HT 28	*Pallenopsis patagonica*	Chile; Region de Magallanes y de la Antarctica Chilena	50°24′52″S	74°33′33″W	15–25 m	CFAP026-11
ZSMA20111009	HT 2	***Pallenopsis yepayekae* sp. nov.**	Chile; Region de los Lagos	43°23′33.4″S	74°07′56.5″W	26 m	CFAP004-11
ZSMA20111012	HT 8	***Pallenopsis yepayekae* sp. nov.**	Chile; Region de los Lagos	43°46′28.5″S	073°02′63.2″W	22 m	CFAP008-11
ZSMA20111016	HT 9	***Pallenopsis yepayekae* sp. nov.**	Chile; Region de Magallanes y de la Antarctica Chilena	48°36′28.7″S	74°53′55.7″W	32 m	CFAP012-11
ZSMA20111017	HT 11	*Pallenopsis patagonica*	Chile; Region de Magallanes y de la Antarctica Chilena	48°36′28.7″S	74°53′55.7″W	32 m	CFAP025-11
ZSMA20111024	HT 10	***Pallenopsis yepayekae* sp. nov.**	Chile; Region de Magallanes y de la Antarctica Chilena	49°34′38.7″S	74°26′49.3″W	28 m	CFAP016-11
ZSMA20111072	HT 29	*Pallenopsis patagonica*	Chile; Region de Valparaiso	33°23′55″S	71°52′78.2″W	339 m	CFAP023-11
ZSMA20111339	HT 5	***Pallenopsis yepayekae* sp. nov.**	Chile; Anihue Raul Marin Balmaceda	43°46′31.35″S	73°01′44.14″W	19 m	CFAP019-11
ZSMA20111340	HT 12	*Pallenopsis patagonica*	Chile; Region de Magallanes y de la Antarctica Chilena	55°00′00.6″S	68°18′88.1″W	24 m	CFAP018-11
ZSMA20111348	HT 14	*Pallenopsis patagonica*	Falkland Islands	50°26′4.00″S	62°46′5.00″W	146–148 m	CFAP027-11
ZSMA20111349	HT 13	*Pallenopsis patagonica*	Falkland Islands	51°16′8.00″S	62°57′8.00″W	171–174 m	CFAP034-11
ZSMA20111350	HT 15	*Pallenopsis patagonica*	Falkland Islands	51°16′8.00″S	62°57′8.00″W	171–174 m	CFAP035-11
ZSMA20111351	HT 20	*Pallenopsis patagonica*	Falkland Islands	51°16′8.00″S	62°57′8.00″W	171–174 m	CFAP036-11
ZSMA20111352	HT 27	*Pallenopsis patagonica*	Falkland Islands	51°16′8.00″S	62°57′8.00″W	171–174 m	CFAP037-11
ZSMA20111354	HT 17	*Pallenopsis patagonica*	Falkland Islands	51°05′8.00″S	61°44′0.00″ W	174–176 m	CFAP028-11
ZSMA20111355	HT 18	*Pallenopsis patagonica*	Falkland Islands	51°05′8.00″S	61°44′0.00″W	174–176 m	CFAP029-11
ZSMA20111357	HT 16	*Pallenopsis patagonica*	Falkland Islands	51°05′8.00″S	61°44′0.00″W	174–176 m	CFAP030-11
ZSMA20111359	HT 18	*Pallenopsis patagonica*	Falkland Islands	51°05′8.00″S	61°44′0.00″W	174–176 m	CFAP031-11
ZSMA20111360	HT 15	*Pallenopsis patagonica*	Falkland Islands	51°05′8.00″S	61°44′0.00″W	174–176 m	CFAP032-11
ZSMA20111361	HT 19	*Pallenopsis patagonica*	Falkland Islands	51°05′8.00″S	61°44′0.00″W	174–176 m	CFAP033-11
PpaE_004	HT 18	*Pallenopsis patagonica*	Falkland Islands	52°57′42″S	60°08′36″W	378 m	KC794961
PpaE_005	HT 15	*Pallenopsis patagonica*	Falkland Islands	52°57′42″S	60°08′36″W	378 m	KC794962
PpaE_006	HT 17	*Pallenopsis patagonica*	Falkland Islands	52°57′42″S	60°08′36″W	378 m	KC794963
PpaE_007	HT 15	*Pallenopsis patagonica*	Falkland Islands	52°57′42″S	60°08′36″W	378 m	KC794964
PpaE_008	HT 15	*Pallenopsis patagonica*	Falkland Islands	52°57′42″S	60°08′36″W	378 m	KC794965
PpaE_010	HT 15	*Pallenopsis patagonica*	Falkland Islands	52°57′42″S	60°08′36″W	378 m	KC794966
PpaE_001	HT 24	*Pallenopsis patagonica*	Subantarctic; West of South Georgia; Shag Rocks	53°46′12″S	41°26′6″W	193 m	KC794959
PpaE_002	HT 25	*Pallenopsis patagonica*	Subantarctic; South Georgia	54°00′59″S	37°26′14″W	78 m	KC794960
PpaE_003	HT 26	*Pallenopsis patagonica*	Subantarctic; Burdwood Bank	54°33′00″S	58°49′20″W	158 m	KC794969
PpaA_001	HT 23	*Pallenopsis patagonica*	Antarctic; Eastern Weddell Sea	71°08′09″S	11°31′37″W	123 m	KC794958
NIWA46256	HT 21	*Pallenopsis patagonica*	Antarctic; Ross Sea	71°15′45″S	170°38′08″W	466 m	FJ969367
NIWA46257	HT 21	*Pallenopsis patagonica*	Antarctic; Ross Sea	72°00′81″S	170°46′47″W	235.5 m	FJ969368
NIWA46258	HT 22	*Pallenopsis patagonica*	Antarctic; Ross Sea	71°37′24″S	170°51′99″W	204.5 m	FJ969369
HM426218		*Pallenopsis latefrontalis*	Antarctic; Eastern Weddell Sea	71°5′31.23″S	11°30′28.8″W	302 m	HM426218
HM426171		*Pallenopsis buphtalmus*	Antarctic; Eastern Weddell Sea	71°19′1.2″S	13°56′31.2″W	848 m	HM426171
DQ390086		*Pallenopsis macneilli*	Australia; Rocky Point, Torquay	38°20′38.07″S	144°19′12.77″E	0.5 m	DQ390086
PxxE001		*Pallenopsis pilosa*	Subantarctic; Bouvet Island	54°21′00″S	3°11′36″E	465 m	KC794967
PxxE002		*Pallenopsis pilosa*	Subantarctic; Bouvet Island	54°21′30″S	3°26′6″E	200 m	KC794968
KC848053		*Pallenopsis pilosa*	Antarctica	66°23′S	140°25′43.87″E	743 m	AAC7281
KC848054		*Pallenopsis pilosa*	Antarctica	65°52′11.81″S	143°0′5.57″E	428 m	AAC7183
KC848052		*Pallenopsis pilosa*	Antarctica	65°51′9.32″S	144°2′23.15″E	1104 m	AAC7182

PxxE001 and PxxE002 were checked for correct determination, we could not access the outgroup specimens KC848052, KC848053, and KC848054 (deposited at the British Antarctic Survey in Cambridge), and HM426171, HM426218, and DQ390086.

For comparative morphological analyses, in addition to our specimens used for DNA sequencing, we investigated 18 specimens from historical collections housed at the Swedish Museum of Natural History and the British Museum of Natural History, i.e. *P. tumidula* (SMNH Type 1293 and seven syntypes), *P. patagonica* (SMNH-125445, SMNH-125507, SMNH-125508, SMNH-125509, SMNH-125510), and one unidentified *Pallenopsis* sp. (SMNH-125514) from the Loman collection, as well as one *P. patagonica* (SMNH-125527) sampled by the Lund University Chile expedition, determined by Hedgpeth (label: det. Hedgpeth 1949). Beyond that we examined Hoek's type material from the HMS *Challenger* expedition, which include three specimens of *P. patagonica* and one specimen designated as *P. patagonica* var. *elegans* (BMNH 1881.38). Furthermore, we studied a related species, *P. notiosa* (ZSMA20111077–79), which is kept at the ZSM, and has been discussed in a previous paper (Weis & Melzer, 2012b).

For morphological documentation we used the following specimens: ZSMA20111000, ZSMA20111002, ZSMA20111004, ZSMA20111006, ZSMA20111009, ZSMA20111016, ZSMA20111348, ZSMA20111350, ZSMA20111357, PpaE007, and PpaE010 for light microscopy; ZSMA20111006, ZSMA20111009, ZSMA20111024, ZSMA20111349, ZSMA20111359, and ZSMA20111360 for SEM studies.

DNA extraction and sequencing

As all the individuals studied were of a suitable size, it was sufficient to take only a piece of leg for DNA extraction. Here, muscle tissue from the tibia was extracted using the DNeasy Mini Kit following the manufacturer's tissue protocol. As a modification from the original protocol, we used only 100 μL of EB buffer for elution. Amplification of a 657-bp fragment of *COI* was performed using standard Folmer primers (Folmer *et al*., 1994) in 25-μL reactions. Individual reactions consisted of 1× polymerase chain reaction (PCR) buffer (5Prime HotMaster), 0.2 mM deoxyribonucleotides (dNTPs), 0.5 μM of each primer, 0.025 U μL^{-1} Taq (5Prime Hotmaster), 1–3 μL extracted DNA (depending on yield), and was filled up to 25 μL with molecular biology-grade H_2O. Cycle conditions were: initial denaturation at 94 °C for 2 min, followed by 36 cycles of 94 °C for 20 s, 48 °C for 30 s, and 65 °C for 80 s. After a final extension at 65 °C for 5 min the reactions were stored at 4 °C. Both DNA extraction and PCR success were checked on a 1% Tris-Borate-EDTA (TBE) agarose gel. 10 μL of the PCR product were purified enzymatically with 0.5 μL Exonuclease I (20 U μL^{-1}) and 1 μL FastAP (1 U μL^{-1}; Thermofisher), by incubating in a thermocycler at 37 °C for 15 min, followed by 96 °C for 15 min prior to sequencing. Sequencing was conducted at GATC (Konstanz, Germany) or performed partially at the CCDB using the standard protocols of IBOL.

Phylogenetic analysis

A total of 47 pycnogonid sequences were used for the phylogenetic analyses of the 657-bp fragment of *COI*. All 47 DNA sequences were aligned with MUSCLE using GENEIOUS PRO 5.5.4 (Drummond *et al*., 2011). To check for frameshift mutations or stop codons, the *COI* sequences were translated into amino acids using the invertebrate mitochondrial genetic code (translation table 5, available from http://www.ncbi.nlm.nih.gov/Taxonomy/taxonomyhome.html/index.cgi?chapter=cgencodes). After the calculation of 'base pair' frequencies and uncorrected pairwise distances with MEGA 5.05 (Tamura *et al*., 2011), we tested the alignment statistically for substitution saturation in DAMBE 5.2.69 (Xia *et al*., 2003; Xia & Lemey, 2009).

Using MEGA 5.05 software we calculated nucleotide composition, maximum parsimony (MP), and, as we were interested in shallow species-level differences, neighbour-joining (NJ) trees based on the Kimura two-parameter (K2P) model (Kimura, 1980; Saitou & Nei, 1987), with bootstrap values. For maximum likelihood (ML) and Bayesian inference (BI) we first identified the most appropriate substitution model using jMODELTEST 2 and the Akaike/Bayesian information criteria (AIC/BIC; Darriba *et al*., 2012). For ML we used the full set of 88 models, for MrBayes we used the reduced model search scheme (nst = 1, 2, and 6; +I, +G, and +IG). Just as for MP and NJ, we used 1000 bootstrap replicates for the ML analysis under RAxML 7.0.4. The 1000 rapid bootstraps were conducted by using the *–x* option (random seed number). Based on jMODELTEST 2 the best model, according to both AIC and BIC, was GTR+I + G, and this was used in RAxML and the Bayesian analyses with MrBayes 3.2 (Ronquist *et al*., 2012). Bayesian analysis was performed using four independent runs with four independent chains and 5 million Metropolis-coupled Markov chain Monte Carlo (MCMC) generations each. Every 500th tree was saved (giving 10 000 in total). The four independent runs reached stationarity after 0.7–0.9 million generations (average standard deviation of split frequencies below 0.01), and thus the consensus tree was calculated after discarding the first 25% of the trees as burn-in (1.25 million generations). The figure of the recovered phylogenetic tree was made using FigTree 1.4.0.

Search for species boundaries using DNA sequences

To be independent from morphology, we decided to perform molecular analyses on the whole data set, including *P. macneilli*, *P. buphtalmus*, and *P. latefrontalis.* To check for species boundaries in our *P. patagonica* complex, we conducted a general mixed Yule-coalescent (GMYC) analysis (Pons *et al*., 2006; Monaghan *et al*., 2009). As identical sequences cannot be considered by GMYC we removed identical sequences, resulting in a data set of 32 sequences. An ultrametric starting tree was obtained using BEAUTi and BEAST (both versions 1.6.1; Drummond & Rambaut, 2007). The chain length for the MCMC algorithm was set to 10 million generations, sampling trees every 1000 generations. Effective sampling sites and convergence of the parameter estimates was inspected using TRACER 1.5. A consensus tree was obtained using TreeAnnotator 1.6.1. The burn-in was set to 2500, rejecting the first 25% of the trees, and the posterior probability threshold was set to 0.5. The resulting ultrametric tree was subsequently imported into the statistics software R 2.15.2 (http://www.R-project.org/). GMYC analysis was conducted with the R package 'SPLITS' (Species Limits by Threshold Statistics; http://r-forge.r-project.org/projects/splits). We used the single and multiple threshold model for the inference of the number of entities with standard parameters [interval = $c(0, 10)$] and used a likelihood ratio test to select the appropriate model.

Furthermore, we used the freely available software ABGD (Automatic Barcode Gap Discovery; Puillandre *et al*., 2012) for searching barcoding gaps between all 42 sequences (sequences of *P. pilosa* were excluded), and for calculating their intraspecific distance/variance.

COI network

As networks are better suited to visualize the often reticulate relationships within, as well as among, closely related species, we constructed a NeighborNet of all individual *COI* sequences, using SPLITSTREE 4.12 (Huson & Bryant, 2006) and K2P-corrected distances.

Morphological analysis

Specimens were photographed using a Wild M400 photomacroscope, equipped with a digital camera (Nikon D700), by taking several shots focused at different levels along the *z*-axis. To constitute a greater depth of field this series of pictures was then edited and combined to form a single respective image using the computer software Helicon Focus (http://www.heliconsoft.com/). Specimens were prepared for SEM as described by Weis & Melzer (2012a). The editing and composition of both light microscopic and SEM pictures was performed with Adobe PHOTOSHOP CS.

Nomenclatural acts

The electronic edition of this article conforms to the requirements of the amended International Code of Zoological Nomenclature (ICZN), and hence the new name contained herein is available under that code from the electronic edition of this article. This published work and the nomenclatural acts it contains have been registered in ZooBank, the online registration system for the ICZN. The ZooBank life-science identifiers (LSIDs) can be resolved and the associated information viewed through any standard web browser by appending the LSID to the prefix 'http://zoobank.org/'. The LSID for this publication is: urn:lsid:zoobank.org:act:0E39E226-30C7-4853-A6A1-7DD2336F33FE. The electronic edition of this work was published in a journal with an international standard serial number (ISSN), and has been archived and is available from the following digital repositories: PubMed Central and LOCKSS.

RESULTS

Molecular and phylogenetic analysis

The 657-bp *COI* alignment of 47 pycnogonid specimens showed no gaps. Base-pair frequencies indicated an arthropod-typical bias towards adenosine and thymine: A, 31.31%; C, 19.80%; G, 13.95%; and T, 34.68%. The value of substitution saturation (I_{ss}), which was calculated for the whole alignment as well as for the third codon position, was always significantly lower than the critical value ($I_{ss.c}$). An I_{ss} value lower than $I_{ss.c}$ implies only a low level of substitution saturation for the sequences analysed. The 657 base pairs consisted of 410 conserved sites and 247 variable sites, of which 202 were parsimony-informative. Translating the *COI* sequences into amino acid sequences showed neither frame-shift mutations nor stop codons.

Phylogenetic trees constructed using different approaches (BI, MP, NJ, ML) showed no major differences, and therefore we present the Bayesian tree (Fig. 2). Support values for the other methods are also shown on the branches. Minor differences are found with respect to the position of ZSMA20111008 and ZSMA20111072. Both slightly change in position within the tree, but never affect any of the other well-supported clades.

Specimens of *P. patagonica* from Chile (ZSMA20111000, ZSMA20111002–06, ZSMA20111009, ZSMA20111012, ZSMA20111016, ZSMA20111024,

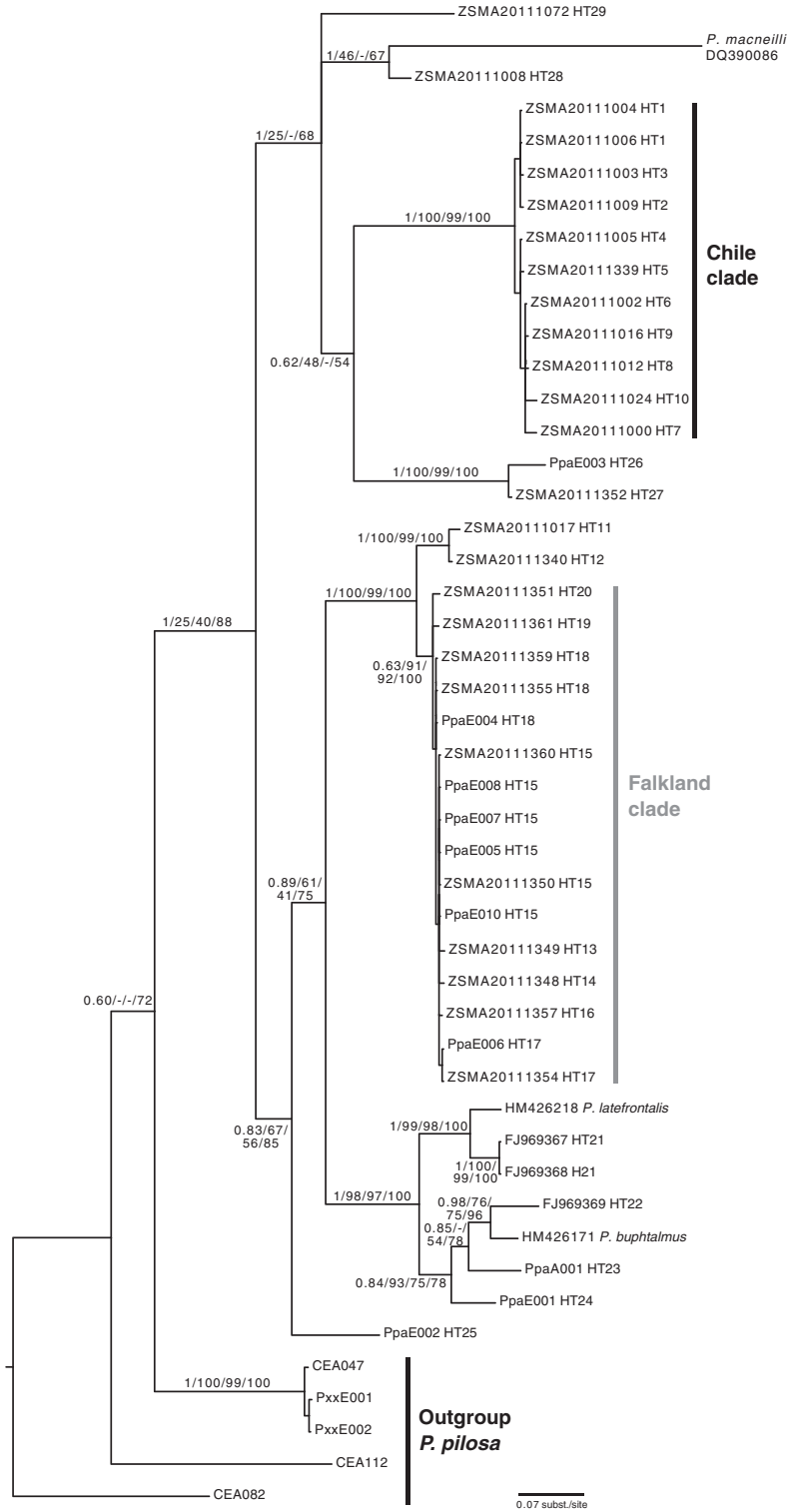

Figure 2. Bayesian phylogenetic tree of cytochrome *c* oxidase subunit I (*COI*) sequences of 28 *Pallenopsis patagonica* (Falkland clade and others), 11 ***Pallenopsis yepayekae* sp. nov.** (Chile clade), one *Pallenopsis macneilli*, one *Pallenopsis buphtalmus*, one *Pallenopsis latefrontalis*, and five *Pallenopsis pilosa*, which serve as the outgroup. Posterior probabilities of the Bayesian inference and bootstrap values of neighbor-joining (NJ), maximum-parsimony (MP), and maximum-likelihood (ML) analyses are displayed above or below branches; branch lengths indicate substitutions per site. Different haplotypes of the studied specimens are defined as HT1–HT29.

ZSMA20111339) and the Falkland Islands (ZSMA 20111348–51, ZSMA20111354–55, ZSMA20111357, ZSMA20111359–61, PpaE004–008, PpaE010) cluster within two well-supported, geographically distinct clades (Figs 2, 3). Several specimens cluster outside these two distinct groups, highlighting the complex nature of *P. patagonica*: ZSMA20111008, ZSMA 20111072 (both from 33°S), [ZSMA20111017 (48°S) and ZSMA20111340 (Region de Magallanes)], and [PpaE003 and ZSMA20111352 (Falklands)]. Specimens from the Ross Sea (FJ969367–69) cluster together with one individual from the eastern Weddell Sea (PpaA001), one from the Shag Rocks (PpaE001), and two from the Southern Ocean assigned to different species (*P. buphtalmus* and *P. latefrontalis*), forming an 'almost Antarctic' clade. The only specimen from South Georgia (PpaE002) clusters basally with the Falkland and 'Antarctic' clades (although with low support). *Pallenopsis macneilli* clusters with ZSMA20111008. The results reveal that specimens initially identified as *P. patagonica* are genetically very heterogeneous, and some show close affinities with specimens identified as different species. Figure 3 shows the NeighborNet of all *Pallenopsis* specimens.

The five specimens of *P. pilosa* selected as the outgroup cluster apart from all other 42 pycnogonids. In the phylogenetic trees, the statistical support for the in-group is good for the model-based inferences (BI, 1; ML, 88), but is poor for the NJ and MP inferences (25 and 40, respectively). Interestingly, the five *P. pilosa* specimens are genetically highly heterogeneous, hinting at further problems with the taxonomy of other *Pallenopsis* specimens. In general, the

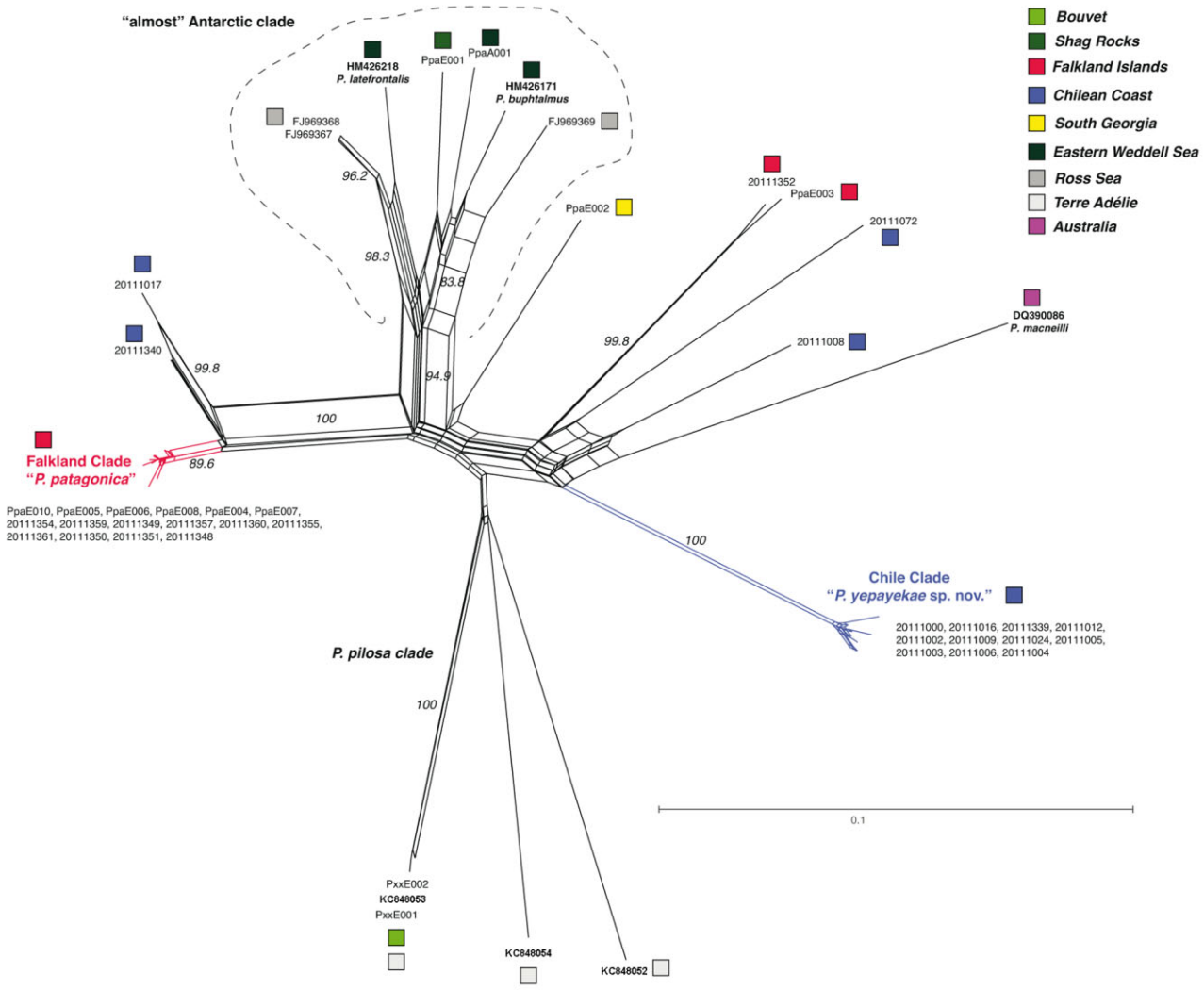

Figure 3. NeighborNet of all individual cytochrome *c* oxidase subunit I (*COI*) sequences, using SPLITSTREE and Kimura two-parameter (K2P) corrected distances.

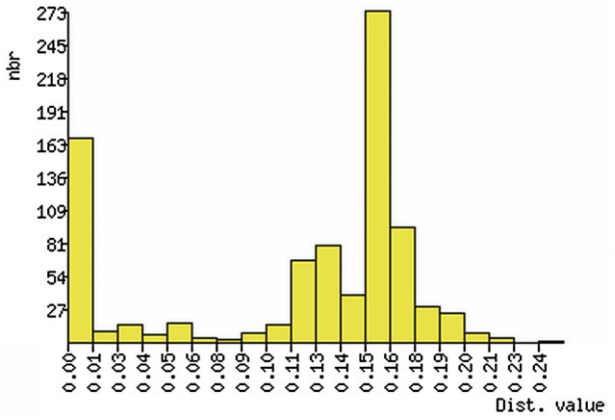

Figure 4. Pairwise genetic distances (Kimura's two-parameter, K2P) for cytochrome *c* oxidase subunit I (*COI*) sequences of *Pallenopsis* specimens used in the present study (with *Pallenopsis pilosa* excluded).

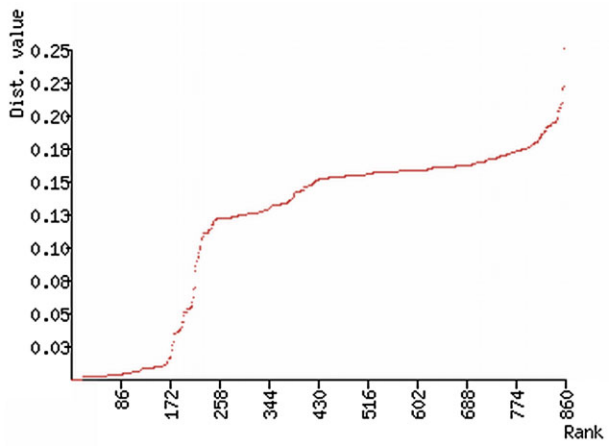

Figure 5. Automatic Barcode Gap Discovery (ABGD) analysis for 42 *Pallenopsis* specimens used in the present study (with *Pallenopsis pilosa* excluded).

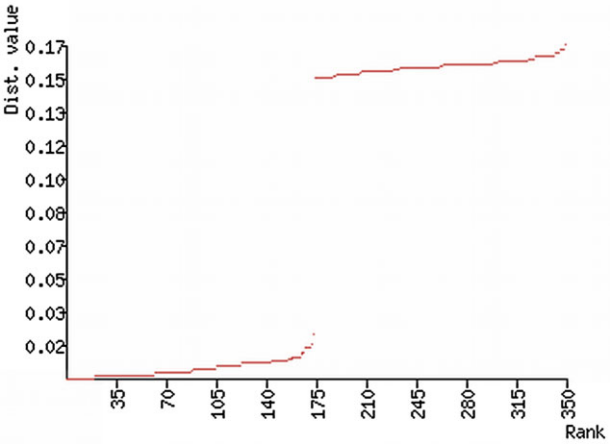

Figure 6. Automatic Barcode Gap Discovery (ABGD) analysis for 27 *Pallenopsis* specimens: 16 specimens from the Falkland clade versus 11 specimens from the Chilean clade.

mitochondrial *COI* fragment is a suitable marker for uncovering lineages previously undetected by morphological analyses (see also Weis & Melzer, 2012a).

To test whether these clusters comprise cryptic or overlooked species we calculated and compared uncorrected pairwise distances between the different specimens/clades (see Table S1). Variation between clades or specimens are high, with a maximum of 23.6% uncorrected pairwise genetic distance. Genetic distances between *P. patagonica sensu stricto* (Falkland clade) and *Pallenopsis yepayekae* sp. nov. (Chilean clade) were high (14.9–19.1%), whereas the variation within these clades was low (0–1.1% and 0–3.5%, respectively).

In addition, we analysed the distance data for distinct barcode gaps using ABGD. Including all 42 pycnogonids studied (five specimens of *P. pilosa* were excluded), no barcode gap is visible (Fig. 5). When ranking the pairwise genetic distances and plotting them there is a large increase at the beginning of the slope, the two horizontal lines are connected by several dots or small clusters of dots; however, when repeating the analyses only including the 11 specimens from the Chilean clade (*Pallenopsis yepayekae* sp. nov.) together with the 16 specimens from the Falkland clade (*Pallenopsis patagonica sensu stricto*) a barcoding gap becomes more obvious (Fig. 6). The two horizontal lines in the distance plot are now clearly separated vertically, without any dots in between them.

For the tree-based assessment of unrecognized species, using the GMYC model with multiple branching events (indicating the presence of several species) was preferred over the null model (single coalescent branching model): likelihood ratio test, $P < 0.001$. This indicates the presence of several species. We also compared the single-threshold model versus the multiple-threshold model, and found support for the single-threshold model $P = 0.861$ ($\chi^2 = 0.751$ and three degrees of freedom). According to the single-threshold GMYC model, the tree consists of 32 haplotypes split into three clusters (confidence interval, 3–5) and 15 distinct GMYC species (ML entities; confidence interval, 11–16). The threshold between Yule speciation and coalescence within populations is indicated by a vertical line in the lineage-through-time plot (LTT) of the Bayesian tree in Figure 7. According to this, the specimens in the Falkland clade and the Chilean clade represent two distinct GMYC species. Furthermore, [FJ969367 and FJ96968 (HT21)] and [ZSMA20111017 (HT11) and ZSMA20111340 (HT12)], and all other 11 specimens represent distinct GMYC species.

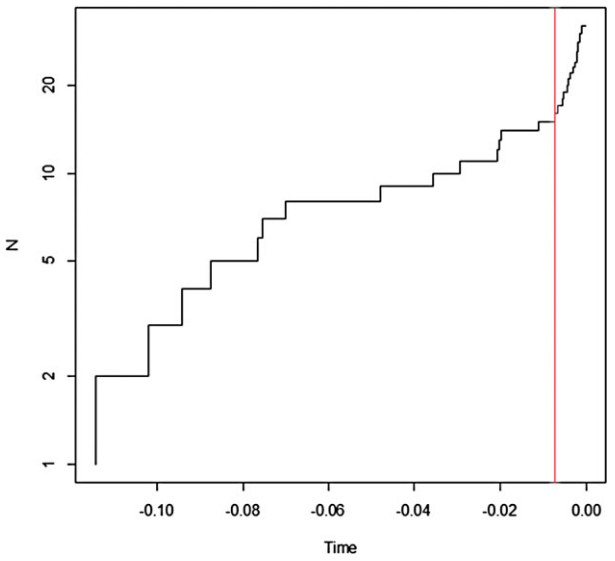

Figure 7. Lineage-through-time plot of the number of lineages (*N*) in the linearized Bayesian haplotype tree (32 unique cytochrome *c* oxidase subunit I, *COI*, barcode sequences). Vertical line represents the single threshold identified by the GMYC model between Yule speciation and coalescence within populations. The number of GMYC species suggested by this analysis was 15.

MORPHOLOGY

To check whether the results of our sequence analyses, i.e. that *P. patagonica* might be a complex of several species, are paralleled by previously undetected morphological differences between these clades we made a detailed analysis of all available specimens. Table S2 displays the enormous morphological variance of the different clades/specimens with respect to their general body size, length of the cement gland tube, leg setation, and auxiliary claw length, but they all fit in the traditional definition of *P. patagonica*. As for most cases only one specimen is available, and because these lack morphological differences that allow us to decide whether they represent variations or putative species-specific features, their analysis will be continued when more specimens are available. Thus we focused our analyses on the two biggest clades, initially referred to as the Chilean clade and the Falkland clade (including 11 and 16 specimens, respectively). Within each of the two clades we observed constant morphological features, which is in accordance with the molecular results. Light-microscopy pictures of individuals from the Falkland and the Chilean clades are shown in Figures 8 and 9. Furthermore, Figure 10 displays detailed SEM studies of the cement gland ducts, female ovigers, and hairs of the second and third coxae from specimens of both clades.

Specimens from the Chilean coast seem to be smaller in their body size compared with the specimens captured from the Falkland Islands and South Georgia (except ZSMA20111352 and PpaE003, from Burdwood Bank; Figs 8A, 9A). Whereas the shape of the proboscis is cylindrical along its length for most specimens (Fig. 8B), individuals from the Chilean clade show a distinct swelling at the middle of the proboscis (Fig. 9C). Also, specimens ZSMA20111008 (Region de Magallanes), ZSMA20111072 (Region de Valparaiso), ZSMA20111352 (Falkland Islands), and PpaE003 (Burdwood Bank) show a light swelling at about half the length of the proboscis. Almost all specimens studied bear an upward-erected slender abdomen (except PpaE002, horizontal), with some short setae. The abdomen from specimens from the Chilean clade is dorsodistally sloped. At the beginning of the slope a rounded edge is found bearing two very prominent spines (Fig. 9D). In contrast, specimens from the Falkland Islands and the Antarctic lack those spines, but show several randomly distributed short setae on the abdomen (Fig. 8C). All individuals examined show a pointed or slightly pointed ocular tubercle. Specimen ZSMA20111008 is the only one with a rounded ocular tubercle.

Furthermore, whereas the length of the cement gland tubes in the Chilean pycnogonids is about three times their diameter (Fig. 10B), specimens from the Falklands and Antarctic area show a very short cement gland tube (Fig. 10A), which is sometimes only hardly visible. Additionally, females of the Chilean clade show a swollen fourth oviger segment, which is not noticeable in the females from the Falkland clade (Figs 8D, 9F, 10C, D). Furthermore, female ovigers from the Chilean clade are eight- to nine-segmented (distal segments often fused), compared with females of the Falkland clade that exhibit a 'ten-segmented' oviger (Fig. 10C, D).

The proportion of the length of the different leg segments is similar throughout all specimens studied, with tibia 2 being the longest. The number of heel spines on the propodus varies between three and four (Fig. 8F). Concerning the leg setae, all individuals have setae not longer than the diameter of the segment upon which they are situated (except ZSMA20111017). The 11 specimens from the Chilean clade show numerous distinct small and stout hairs on the distal ventral side of the second and third coxae (Fig. 9E). Although this characteristic is weakly developed in juveniles, it is already discernable at that stage. This characteristic is not visible or very prominent in any of the other specimens studied (Fig. 8E). Furthermore, the setae themselves show remarkable differences. The setae on the second and third coxae of the specimens from the Chilean clade bear several tiny hairs on their surface (Fig. 10F),

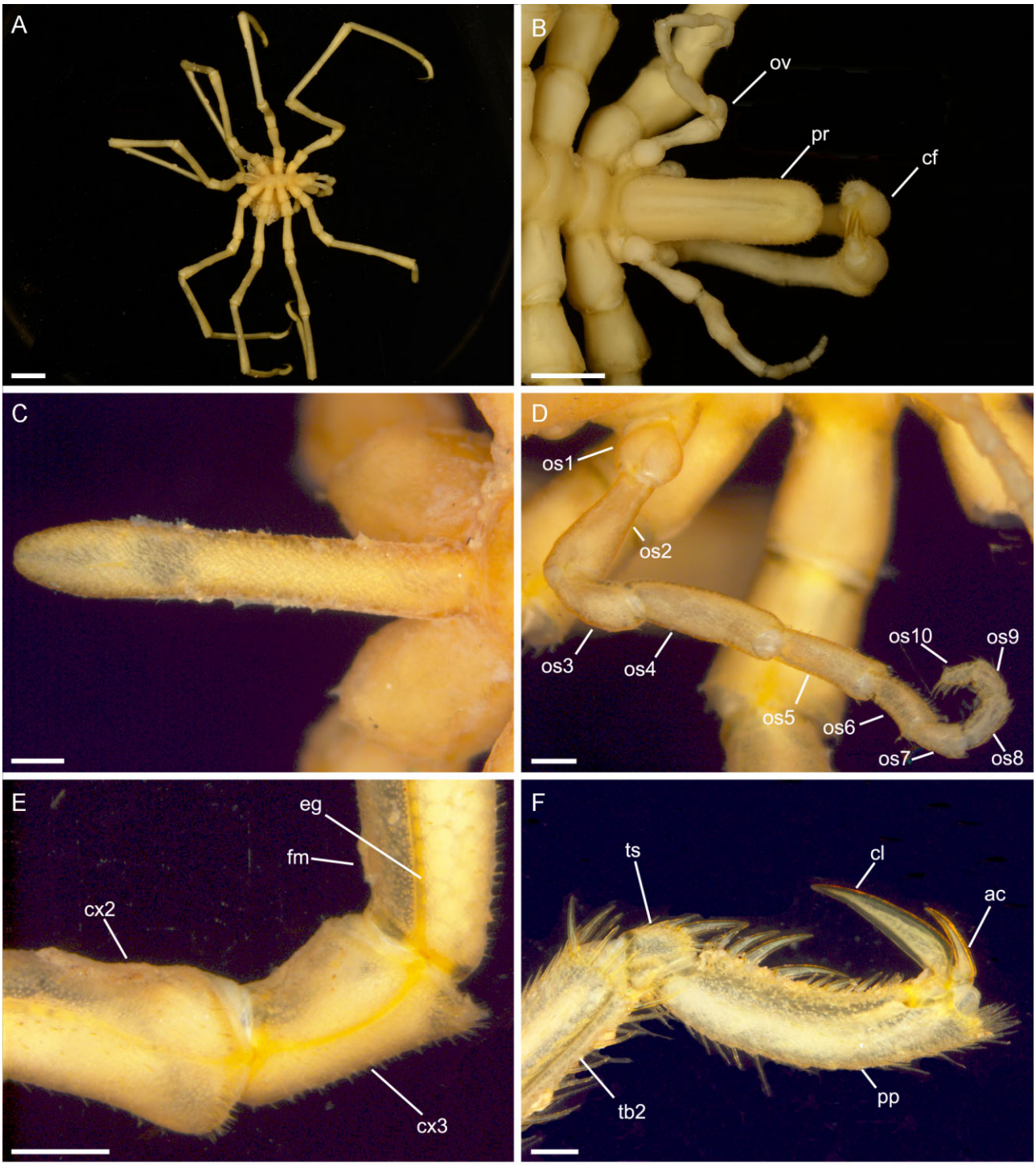

Figure 8. Light microscopy of *Pallenopsis patagonica sensu stricto*. (Falkland clade). A, dorsal view; scale bar = 4 mm. B, ventral view of proboscis; scale bar = 2 mm. C, dorsal view of abdomen; scale bar = 500 µm. D, right oviger (female); scale bar = 500 µm. E, detail view of second and third coxae of left fourth walking leg; scale bar = 1 mm. F, tarsus and propodus with claw and auxiliary claws of right third walking leg; scale bar = 500 µm. A, PpaE007; B, PpaE010; C, ZSMA20111357; D, E, ZSMA20111350; F, ZSMA20111348. Abbreviations: ac, auxiliary claws; cf, chelifore; cl, claw; cx, coxa; eg, eggs; fm, femur; os, oviger segment; ov, oviger; pp, propodus; pr, proboscis; ts, tarsus; tb, tibia.

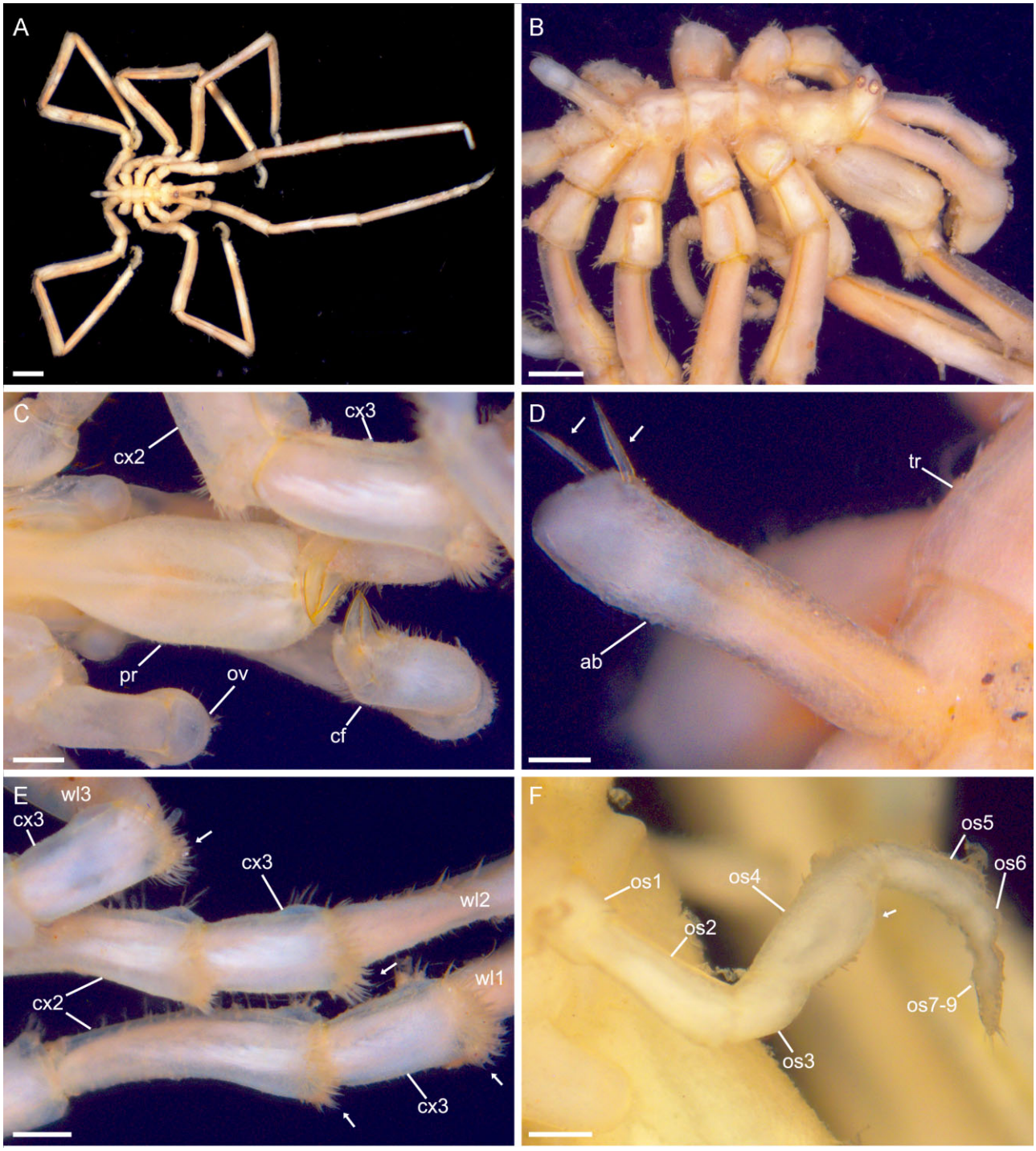

Figure 9. Light microscopy of ***Pallenopsis yepayekae*** **sp. nov.** (Chilean clade). A, dorsal view; scale bar = 2 mm. B, lateral view of trunk; scale bar = 1 mm. C, ventral view of proboscis; scale bar = 500 µm. D, detail view of abdomen, note two prominent spines (arrows); scale bar = 250 µm. E, detail view of second and third coxae of right walking legs, note several short and prominent hairs (arrows); scale bar = 500 µm. F, left oviger (female); scale bar = 250 µm. A, ZSMA20111009; B, ZSMA20111006; C, ZSMA20111000; D, ZSMA20111004; E, ZSMA20111002; F, ZSMA20111016. Abbreviations: ab, abdomen; cf, chelifore; cx, coxa; os, oviger segment; ov, oviger; pr, proboscis; tr, trunk; wl, walking leg.

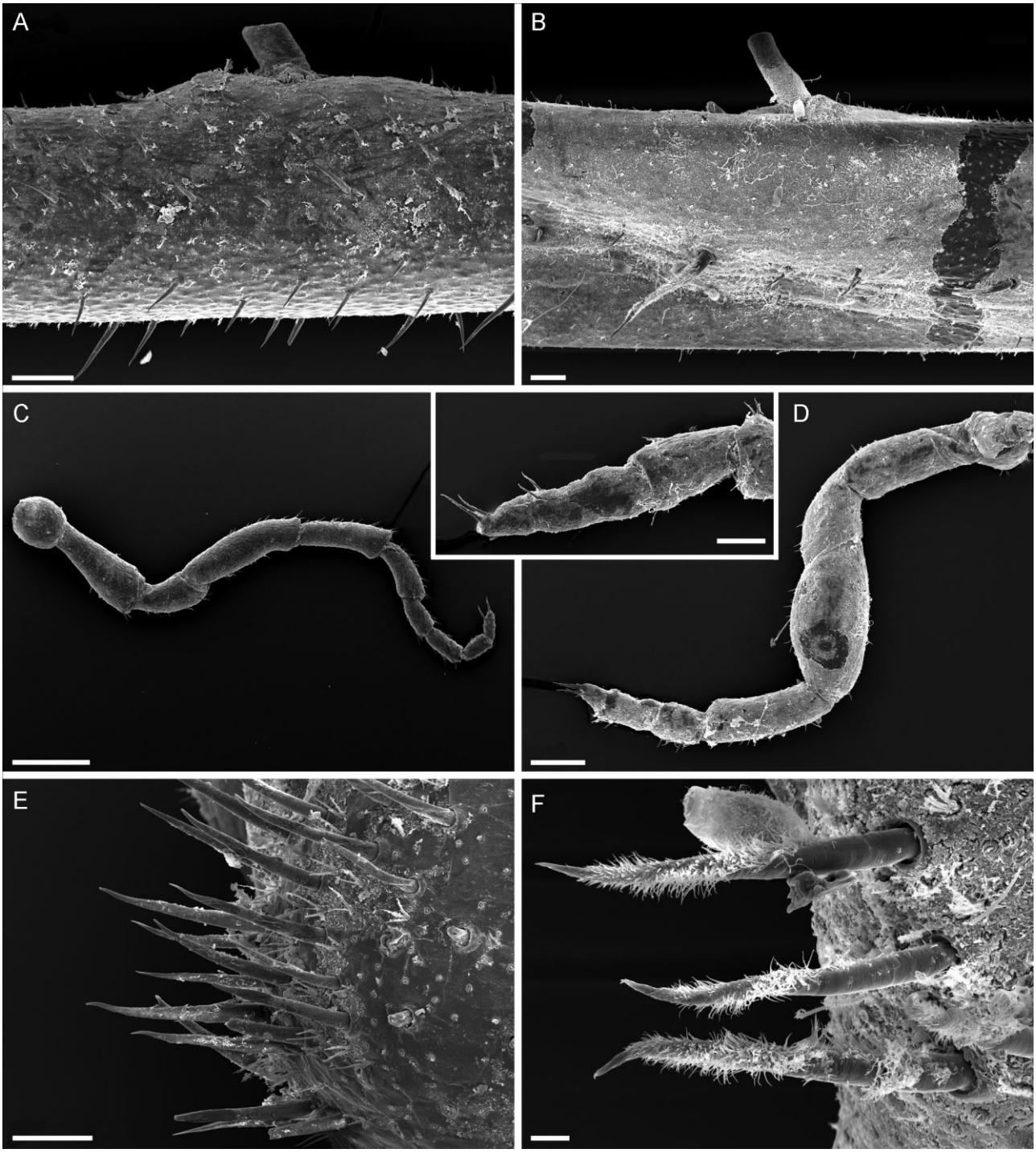

Figure 10. Scanning electron microscopy (SEM) of *Pallenopsis patagonica* (A, C, E) and ***Pallenopsis yepayekae* sp. nov.** (B, D, F). A, detail view of cement gland tube of left first walking leg; scale bar = 200 μm. B, detail view of cement gland tube of left second walking leg; scale bar = 100 μm. C, right oviger (female); scale bar = 1 mm. D, right oviger (female); scale bar = 200 μm [insert: detail view of distal oviger segments (female); scale bar = 100 μm]. E, detail view of hairs on third coxa of left fourth walking leg; scale bar = 100 μm. F, detail view of hairs on second coxa of left second walking leg; scale bar = 20 μm. A, ZSMA20111360; B, F, ZSMA20111006; C, ZSMA20111349; D, ZSMA20111009, insert: ZSMA20111024; E, ZSMA20111359.

whereas the setae from the other specimens are 'smooth' or rather normally developed (Fig. 10E).

The length of the auxiliary claws varies between one-third and one-half the length of the main claw, without distinction between specimens from different areas. Only specimen ZSMA20111008 from the Chilean fjord region at 50°S bears extremely short auxiliary claws, being one-quarter the length of the main claw.

Pallenopsis patagonica specimens from the Swedish Museum of Natural History, determined by Loman, show similar morphological characteristics to those of our specimens from the Falkland clade. Loman's specimens were collected by the Swedish South Polar Expedition (1901–1903) at the Graham Region, South Georgia, and the Falkland Islands. The undetermined *Pallenopsis* sp. (SMNH-125514) was collected at the Patagonia Archipelago (Tierra del Fuego) 55°10′S, 66°15′W, and is in good accordance in morphology with our Chilean clade. This specimen, an ovigerous male, shows the characteristic hairs on the ventral side of the second and third coxae, has a long cement gland tube (more than three times its width), and a proboscis with a light swelling at half of its length.

The specimen of Hedgpeth (SMNH-125527) appears to be a female and was collected by the Lund University Chile Expedition (1948–49) at Canal San Antonio 41°47′S, 73°15′W. This is the exact region where samples from our Chilean clade are from. Also this specimen shows the same morphological characteristics as our specimens from the Chilean fjords: a nine-segmented oviger (with the fourth oviger segment swollen), a proboscis with a slight swelling at the middle, and prominent brush-like setae on the ventral side of the second and third coxae.

Reinvestigation of Hoek's type material

Hoek's type material consists of three female specimens: one bigger specimen, upon which his type determination is based, and two smaller specimens that he designated as juveniles. The three individuals were sampled from three different stations, namely station 304, 308, and 313 (located at 46°53′S, 75°11′W, 50°10′S, 74°42′W, and 52°20′S, 68°0′W, respectively). Unfortunately it is not known which specimen was captured from which sample site, as the specimen labels don't contain this information. Whereas the bigger specimen and one of the smaller ones are morphologically identical with the individuals of our Falkland clade, the other one resembles completely the specimens from our Chilean clade. It shows distinct prominent features: (1) a proboscis slightly swollen at the middle; (2) an eight- to nine-segmented oviger, with the fourth oviger segment thickened; and (3) several short brush-like setae at the ventral side of the second and third coxae. Also, the structure of these hairs accords well with that described for the individuals of our 'Chilean clade'. The abdomen shows the same shape bearing two spines on the rounded edge of the beginning of the dorsodistal slope. One of the spines on the dorsal side is broken and the other is not as prominent as in most of the individuals from our 'Chilean clade', but nevertheless is clearly visible.

Moreover, Hoek's material also contains a specimen called *P. patagonica* var. *elegans* from station 320 near the La Plata estuary in Argentina (37°17′S, 53°52′W). As Hoek already mentioned, this individual resembles a variety of *P. patagonica*, i.e. our Falkland clade, with only a more slender appearance.

The results of our morphological analyses as well as our molecular data strongly indicate that the Chilean clade, i.e. the 11 specimens collected at the southern Chilean coast, represents a new species that is described below.

Pallenopsis yepayekae sp. nov. Weis

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Figures 9A–F, 10B, D, F, 11A–F

The new species can clearly be attributed to the genus *Pallenopsis* Wilson, 1881 by its slender segmented body, cylindrical proboscis, rudimentary palps, ten-segmented ovigers in males, and slender legs with claws and auxiliary claws (Wilson, 1881).

The species description of *P. yepayekae* sp. nov. is based altogether on 14 specimens: 11 specimens collected by the 'Huinay Fjordos' expeditions 2006–2011, one specimen (SMNH-125514) that was only determined to genus level by Loman (1902), and two further specimens that were initially determined as *P. patagonica*, namely SMNH-125527 from Hedgpeth (1949) and BMNH-1881.38 from Hoek (1881).

Types

Holotype: Male (ZSMA20111002), Chile, Hanover area, Canal Pitt Chico, 50°50′07.1″S, 74°08′20.9″W, 25 m, 07.03.2006, leg. R. Melzer, M. Schrödl.

Paratypes

Four males: ZSMA20111000, Chile, Western Katalalixar, Canal Castillo, 48°44′11.4″S, 75°24′ 53.1″W, 15 m, 12.03.2006, leg. R. Melzer, M. Schrödl; ZSMA20111006, Chile, Fjords of region X, Inio 4, 43°25′03.0″S, 74°04′51.2″W, 20 m, 24.02.2008, leg. G. Försterra; ZSMA20111339, Chile Anihue Raul Marin Balmaceda, Islas Tres Hermanas, 43°46′ 31.35″S, 73°01′44.14″ W, 19 m, 17.01.2011, leg. V. Häussermann; SMNH-125514, South Atlantic Ocean, Argentina, Patagonia archipelago (Tierra del Fuego),

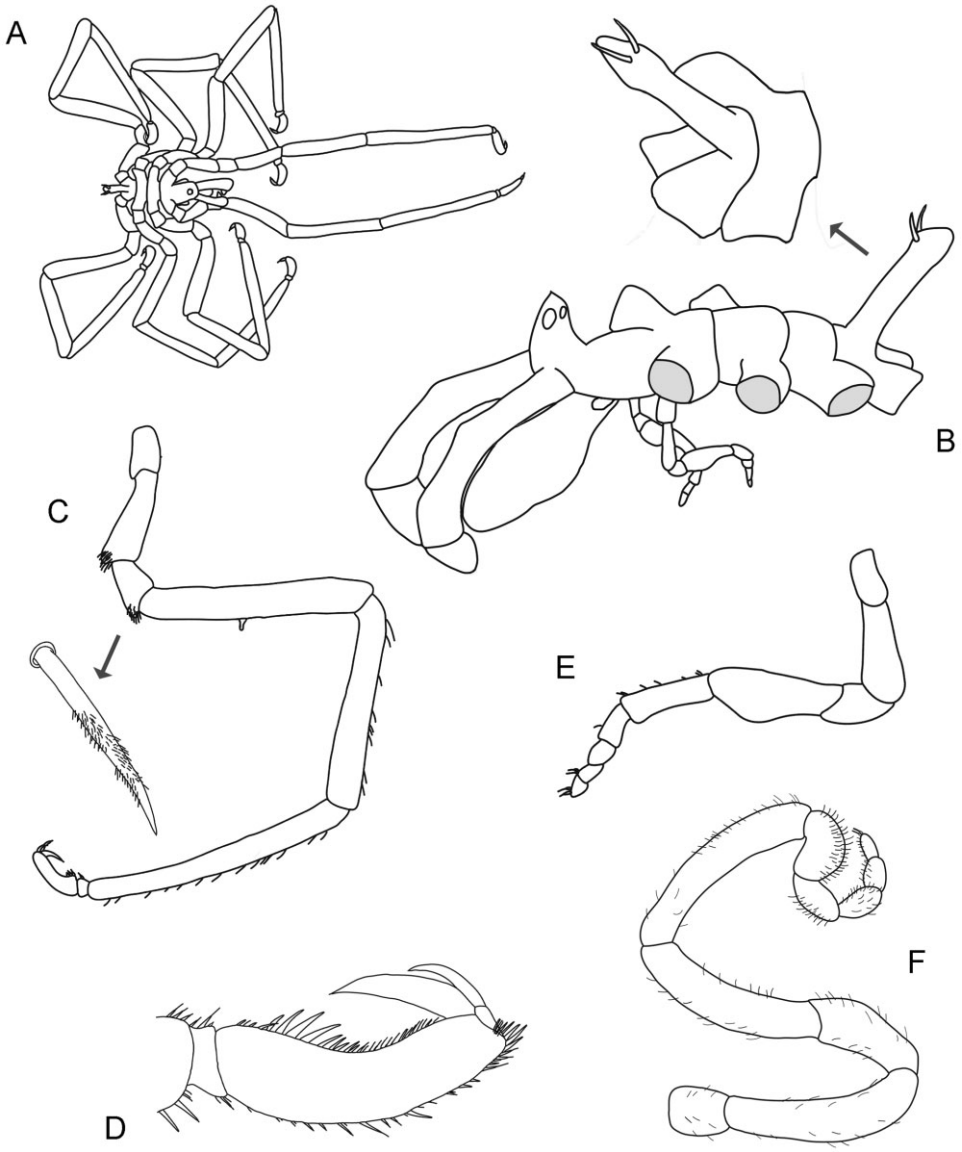

Figure 11. Drawings of ***Pallenopsis yepayekae*** **sp. nov.** A, dorsal view. B, lateral view of female and detailed view of abdomen. C, walking leg, with enlargement of setae of coxae 2 and 3. D, propodus with claw and auxiliary claws. E, female oviger. F, male oviger.

55°10′S, 66°15′W (st. no. 60 of Swedish South Polar Expedition 1901–03), 100 m, 15.09.1902, leg. J. C. C. Loman.

Seven females: ZSMA20111003, Chile, Fjords of region X, Inio 4, 43°25′03.0″S, 74°04′51.2″W, 25 m, 24.02.2008, leg. RF; ZSMA20111004, Chile, Fjords of region X, Inio 5, 43°24′34.5″S, 74°05′00.7″W, 9 m, 24.02.2008, leg. NR; ZSMA20111009, Chile, Fjords of region X, Inio 3, 43°23′33.4″S, 74°07′56.5″W, 26 m, 24.02.2008, leg. V. Häussermann; ZSMA20111016, Chile, Western Katalalixar, Canal Adalberto, 48°36′28.7′S, 74°53′55.7′W, 32 m, 12.03.2006, leg. R. Melzer, M. Schrödl; ZSMA20111024, Chile, Messier Channel and Fjords, Paso del Abismo, 49°34′38.7′S, 74°26′49.3′W, 28 m, 10.03.2006, leg. R. Melzer, M. Schrödl; SMNH-125527, South Pacific Ocean, Chile, Canal Chacao, Canal San Antonio, 41°47′40″S, 73°15′40″W (st. no. M109 of Lund University Chile Expedition 1948–49), 36 m, 06.05.1949; BMNH-1881.38, either from station 304, 308, or 313 of the HMS *Challenger* expedition 1872–76 between 46°53′S, 75°11′W and 52°20′S, 68°0′W, 82–320 m, 31.12.1875–20.01.1876.

Two juveniles: ZSMA20111005, Chile, Western Katalalixar, Canal Castillo, 48°44′11.4″S, 75°24′53.1″W,

23 m, 12.03.2006, leg. V. Häussermann; ZSMA20111012, Chile, Raul Marin, Las Hermanas, 22 m, 11.03.2007, leg. R. Meyer, K. Jörger.

Beside the specimens there are also DNA aliquots (including ten paratypes plus holotype) stored under specific voucher IDs at ZSM (see also Table 1) and CCDB.

Etymology

In Kawésar language, yepayek is the name of the ciprés de las güaitecas (*Pilgerodendron uviferum*). If one looks at the fine ramification of the branches of a cypress-like tree, the similarity to the structure of the setae of the ventral side of the second and third coxae of the new species described here becomes obvious. The name of the species also refers to the *Yepayek*, a ranger boat of the Corporación Nacional Forestal (CONAF) named after the tree, which carried the scientists to the different places in the Chilean fjords sampled during 'Huinay fjordos' expedition 3. It is the *Yepayek* and its always friendly and cooperative crew to whom we owe the chance to collect this new species; therefore, we decided to name the species *Pallenopsis yepayekae* sp. nov. and also to keep in mind the adventurous trip through the labyrinth of the Chilean fjords.

Diagnosis

Compared with *P. patagonica*, a rather small species of smooth habitus, and in a few individuals the legs show red stripes. Proboscis (Fig. 11B) with distinct swelling at the middle. Abdomen (Fig. 11B) erect (about 45°) and dorsodistally sloped. The beginning of the slope shows a rounded edge on which two very prominent spines are sited (Fig. 11B). Second and third coxae with many conspicuous short brush-like setae on the ventral side (Fig. 11C). Oviger of the females eight- to nine-segmented with the fourth oviger segment being swollen (Fig. 11E). Cement gland duct of males relatively long, measuring about three times the length of its diameter.

Description

Male: Size moderate to small, leg span less than 60 mm. Trunk glabrous with distinct segment borders, lateral processes separated by about one-third their diameter (Fig. 11A, B). Ocular tubercle at anterior portion of cephalic segment, slightly pointed (Fig. 11B). Eyes prominent, pigmented with posterior eyes smaller than anterior eyes. Proboscis slightly directed downwards, swollen at middle (Fig. 11B). Abdomen erect, somewhat extending beyond the distal margins of the lateral processes, dorsodistally sloped, with two very prominent spines on the dorsal side (Fig. 11B).

Chelifores with movable finger equipped with setose pad. Tips overlap when closed, inner edges join when closed. Lateral palp buds have the form of short knobs (Fig. 11B).

Oviger ten-segmented, typical for genus (Fig. 11F). Distal segments more setose than proximal segments, with setae pointing in various directions.

Legs (Fig. 11C) with several setae not longer than the diameter of the segment upon which they are situated. Coxae 1 and 3 subequal. Second coxa about twice the length of third coxa. Second and third coxae with many conspicuous short brush-like setae on the ventral side (Fig. 11C). Femur and tibia 1 of about equal size. Tibia 2 longest leg article. Tarsus short, armed with one bigger spine on the ventral side. Propodus (Fig. 11D) slightly curved, with three or four heel spines. Sole with many shorter spines. Claw robust, slightly curved, auxiliary claws about one-third to one-half of main claw length.

Cement gland tube about three times as long as its diameter, medioventrally on femur on slightly raised surface. Sexual pores on ventral side of second coxae of third and fourth pair of legs.

Measurements (holotype, in mm): Length of trunk (anterior margin of first trunk segment to distal margin of fourth lateral processes), 4.82; trunk width (across first lateral processes), 2.94; proboscis length, 2.29; abdomen length, 1.81; third leg, coxa 1, 0.85; coxa 2, 2.58; coxa 3, 1.23; femur, 5.90; tibia 1, 5.49; tibia 2, 7.06; tarsus, 0.27; propodus, 1.44; claw, 0.76; auxiliary claws, 0.50. Different leg segments were measured in natural posture.

Female: General habitus and size similar to male. Differences are only in the sexual characters: oviger (Fig. 11E) eight- to nine-segmented, with fourth oviger segment swollen; distal oviger segments fused and less setose than in the male; all setae pointing distally. Sexual pores on all second coxae on ventrodistal surface.

Distribution: Chilean fjord region 41°47′40″–55°10′S and 66°15′–75°24′53.1″W; depth range 9–100 m.

As Hoek's syntypes series of *P. patagonica* includes one specimen of *P. yepayekae* sp. nov., a lectotype for *P. patagonica* must be designated. Of the two specimens from the BMNH-1881.38 material of the HMS *Challenger* expedition, the larger specimen, upon which Hoek's description is based, shall be the lectotype, and the smaller specimen the paralectotype. The lectotype of *P. patagonica* can clearly be distinguished from the new species *P. yepayekae* sp. nov. by the following characteristics: abdomen without two prominent spines on the dorsal side, ten-segmented oviger in females, second and

third coxae without conspicuous short brush-like setae on the ventral side, and a cylindrical proboscis without a swelling at the middle.

DISCUSSION

The results of our study indicate great morphological as well as genetic variation in the individuals examined, indicating that *P. patagonica sensu lato* is a good example for studying species complexes.

To avoid circular reasoning by mixing morphology-based considerations and molecular results, all molecular analyses were performed using the whole data set, and checked against the morphological results later. Correspondingly, the morphology of the specimens was analysed without taking sequence-defined groupings into account. After the first morphological determinations all specimens studied could be assigned to *P. patagonica* according to the hitherto existing definitions (Gordon, 1932; Stock, 1957; Pushkin, 1975, 1993; Child, 1995). We also decided to include the available sequences of *P. macneilli*, *P. buphtalmus*, and *P. latefrontalis* in our studies, owing to their close relationship with *P. patagonica.* Furthermore, as we did not have these three specimens at hand to check whether the determinations and the genetic data show their affinities to the *P. patagonica* complex, we treated them as neutrally as possible and considered them also as possible *P. patagonica* specimens.

Molecular analysis

Regarding the molecular results presented in this study, different clades are supported by high bootstrap or posterior probability values. Regarding all *Pallenopsis* specimens studied, two bigger clades can be clearly distinguished: the Chilean clade with 11 specimens and the Falkland clade with 16 specimens. This is not surprising, as our morphological data already grouped the Chilean and Falkland specimens separately (see Table S2).

Combining all evidence of our results, in particular the extremely high intraspecific distances of 23%, and also considering the high 'intraspecific' variation of 10.4% for *P. patagonica* reported in our previous study (Weis & Melzer, 2012a), we conclude that *P. patagonica* might represent a large species complex, potentially hiding several undescribed new species.

In contrast to our previous study of *Achelia assimilis* (Haswell, 1885), where we assumed subspecies because of the geographic pattern (possible allopatric speciation process), in *P. patagonica* we find another case. As seen in the network and the phylogenetic tree, there is geographic overlap between the single clades, i.e. haplotypes of different subnetworks are present at the same location (see Fig. 3). The same pattern has been observed at several locations for the giant sea spider *Colossendeis megalonyx* Hoek, 1881 (Krabbe *et al*., 2010). To confirm this finding, more sequences from specimens from South Georgia, Antarctica, and more northern areas of the Chilean coast are required.

Again, as in other pycnogonids, in *P. patagonica* we observe very high interspecific distances compared with other taxa (Hebert *et al*., 2004; Lefebure *et al*., 2006; Raupach *et al*., 2010). Either the number of undescribed species in Pycnogonida is higher than in other taxa, or there is a peculiar 'pycnogonid' phenomenon not understood at the moment.

Furthermore, the tree-based GMYC modelling analyses, a recently developed species delimitation method (Pons *et al*., 2006; Monaghan *et al*., 2009) that has been used in several groups of organisms (Barraclough *et al*., 2009; Bode *et al*., 2010; Esselstyn *et al*., 2012; Williams *et al*., 2012), reveal the presence of about 15 distinct GMYC species, of which only two are represented by our two bigger clades (Falkland and Chilean clades). This suggests the presence of possibly unrecognized species; however, further sampling is needed to test explicitly for this phenomenon.

Morphological analysis

As for most of the clusters/clades only a few or even only one specimen is available at the moment, more specimens from these scattered clades are needed to unravel this complex phenomenon. However, there are enough specimens in the Falkland and Chilean clades for making conclusions regarding their species status. As the original description of *P. patagonica* (Hoek, 1881) fits perfectly with the morphology of the 16 specimens from the Falkland clade, they must be the *P. patagonica sensu stricto*. In contrast, specimens from the Chilean clade show several morphological and molecular differences, which leads us to the description of a species new to science.

Specimens described by Hoek have a cylindrical proboscis without swelling at half of its length and a ten-segmented oviger in females. The bigger female Hoek describes has a body length of about 16 mm, which is similar to our specimens from the Falkland Islands, South Georgia and Antarctica. Hoek mentions some small and stout hairs at the swollen extremity of the second, third, and fourth joint of the leg (meaning coxa 2, coxa 3, and femur, respectively). Perhaps this could be the setae that we describe in the specimens from the Falkland clade on the ventral side of coxae two and three. However, these hairs are not visible in his drawings (see Hoek, 1881: plate XII, figs 6–9), implying that they are not as prominent as,

for example, in our individuals studied from the Chilean coast. Hoek's specimens were captured by the HMS *Challenger* at station 304 (46°53′S, 75°11′W), station 308 (50°10′S, 74°42′W), and station 313 (52°20′S, 68°0′W). Fortunately two of our specimens, namely ZSMA20111008 and ZSMA20111002, are from almost exactly the same location as HMS *Challenger* station 308. Regrettably Hoek did not mention which of the three specimens is from which sample location. We assume that the only adult female, on which his description and drawings are also based, has been captured east of Chile in the Atlantic at station 313, as this description matches much better with our specimens from the Falkland Islands and surrounding area (see above).

If one follows the first description given by Hoek (1881) under the synonym *Phoxichilidium patagonicum*, the specimens from the Falkland Islands and Antarctica would match better than those from the Chilean clade. Hoek focused his description on just the bigger individual, and denominated the smaller ones as juveniles, without giving them any more attention. In our opinion these two specimens are adult females as well, as both are already carrying eggs inside the femur. After specific study, one of the smaller females is shown to resemble exactly *P. yepayekae* sp. nov. Furthermore, one of Hoek's sample locations (station 308) falls exactly in the area of the sample sites given for *P. yepayekae* sp. nov. Hence, we assume that this individual of Hoek's material derives from station 308. Unfortunately, we cannot deduce, either from Hoek's descriptions or from his material that we have at hand, which specimen was captured at which station. The bigger specimen and the one that resembles *P. yepayekae* sp. nov. are both kept in the same tube labelled with station 313, which is obviously wrong as according to Hoek's original data these samples come from two different locations. Also, the sample site of the third specimen is not well documented.

Later, Möbius (1902), Hodgson (1907), Hodgson (1915), Bouvier (1913), Calman (1915), Loman (1923a), Gordon (1932), Marcus (1940), Hedgpeth (1961), Pushkin (1975, 1993), Stock (1957), and Child (1994) also described several further specimens and synonyms of *P. patagonica*. The specimens were mainly captured from the Southern Ocean, including Bouvet and South Georgia, or from the Falkland Islands and the Atlantic coast of South America. With every newly added description the species *P. patagonica*, with its various existing synonyms, became more and more diverse and variable. The morphological frame under which one could assign a pycnogonid to this species became broader and more ambiguous. Hence, it is not surprising that in a broader sense all of our studied specimens match with the characterization of *P. patagonica*.

To check that there are no other species hidden behind the 39 specimens studied, we chose *P. pilosa* as an out-group, as well as *P. buphtalmus*, *P. latefrontalis*, and *P. macneilli*, and examined and compared the descriptions of other *Pallenopsis* species found in this area with our individuals. Child (1992) described two new *Pallenopsis* species from Chile, namely *P. notiosa* and *P. truncatula*. The latter one has very short auxiliary claws (about 0.15 the length of the main claw), well-separated lateral processes, a glabrous abdomen, a very short cement gland tube in males, and a ten-segmented oviger in females. None of our individuals show all of these features in combination. For example ZSMA20111008 is the only specimen bearing such short auxiliary claws, but in contrast to *P. truncatula* it has a rounded ocular tubercle, a setose abdomen, and a femur being as long as tibia 1 (femur is shorter than tibia 1 in *P. truncatula*). Also, *P. notiosa* can be excluded concerning our specimens, as it has a rounded ocular tubercle, well-separated lateral processes, and a very long second coxa (about three times coxa 3; see Weis & Melzer, 2012b). Our specimens have a slightly conical or pointed ocular tubercle, only little-separated lateral processes, and a second coxa being about twice the length of the third coxa. ZSMA20111008, for example, has a rounded ocular tubercle, but the other characteristics do not match. Furthermore, in neither of the two species Child mentions are there prominent hairs on the ventral side of the second and third coxae, which occur in our Chilean specimens. *Pallenopsis macneilli*, which is closest to ZSMA20111008 in the tree, does not fit with our material because of its horizontal abdomen, relatively long auxiliary claws, and its distribution area, which is located in Australia.

Two other interesting possible species could be *Pallenopsis tumidula* Loman, 1923 and *Pallenopsis candidoi* Mello-Leitao, 1949, as both seem to exhibit the short hairs on the ventral side of the second and third coxa. However, the latter has an eight-segmented oviger in females, and auxiliary claws clearly longer than half the length of the main claw, which differs from our specimens. Furthermore, *P. candidoi* is only sampled from South Georgia to South Brazil so far. *Pallenopsis tumidula* is characterized and drawn by Stock (1957) with 'Fiederdornen' on the ventral distal side of coxae 2 and 3. He mentions that this feature makes *P. tumidula* clearly distinguishable from *P. patagonica*. Confusingly, if one regards the original description of 1923, Loman neither mentions short hairs on the coxae nor shows them in his drawings. Furthermore, the type material we had at hand from the Swedish Museum of Natural History didn't show any prominent hairs on the coxae. Only our specimens from the

Chilean clade show this kind of 'Fiederdornen', and in contrast to *P. tumidula* they have eight- or nine-segmented ovigers in females, whereas Loman mentions a 'ten-segmented' oviger in his individuals. One drawing by Loman of a young female shows the last oviger segments to be fused, which could be more consistent with our specimens. But this would mean that all our specimens from the Chilean clade would be just juveniles, which can be excluded for example by the visible eggs inside the femur of females, indicating an adult state. Furthermore, Loman does not mention any setae on the abdomen. Besides several short setae, our specimens also show two very prominent larger spines on the distal end of the abdomen. Another fact that should be kept in mind is that *P. tumidula* has only been captured from North Argentina so far. All this leads us to the decision that our specimens can not be *P. tumidula*.

Concerning our specimens from the Falkland clade, on the first view one possible candidate could be *Pallenopsis kupei* Clark, 1971; however, the auxiliary claws, being more than half as long as the main claw (Clark, 1971), and the Macquarie and New Zealand Plateau distribution of this species (Child, 1995), separate it from *P. patagonica*.

Furthermore, analysing Loman's *P. patagonica* collection and one *P. patagonica* specimen of Hedgpeth from the Swedish Museum of Natural History confirms our considerations. Eight specimens (SMNH-125445, SMNH-125507, SMNH-125508, SMNH-125509, SMNH-125510) captured from the Graham region, South Georgia and Falkland Islands, determined as *P. patagonica* by Loman, are perfectly in accordance with the morphology of our specimens from the Falkland clade. In contrast, the specimen SMNH-125527, determined as *P. patagonica* by Hedgpeth, and collected at 41°47′S, 73°15′W, fits better with the description of the specimens of our Chilean clade. This would mean that this specimen is not a *P. patagonica*, but a *P. yepayekae* sp. nov. Furthermore, the only specimen undetermined by Loman (SMNH-125514), which was collected at Tierra del Fuego (55°10′S, 66°15′W), shows the same characteristics as *P. yepayekae* sp. nov., here described as a new species. This also explains why Loman determined this specimen only to genus level. He seemed to see the differences to *P. patagonica*.

For *P. patagonica*, however, a broad variability concerning different characteristics is discussed. Gordon (1932) notices that the gap between the lateral processes ranges from being little separated to separated by about their own diameter. Furthermore, the spination of the propodus varies greatly in numbers and length, bearing two, three, or four spines, for example (Gordon, 1944). Whereas Stock (1975) describes the propodus as more heavy and robust, it is considered long by Child (1995).

The length of the auxiliary claw is given as either one-third the length of the main claw (Stock, 1957), half the length of the main claw (Möbius, 1902; Hodgson, 1907; Calman, 1915; Gordon, 1944), or even longer (Pushkin, 1975; Pushkin, 1993). Except for one specimen (ZSMA20111008), our studied specimens have auxiliary claws reaching one-third to one-half the length of the main claw.

Whereas Stock (1957) remarks that *P. patagonica* lacks 'Fiederdornen' (stellate setae) on the second and third coxae of the legs, some kind of short hairs are mentioned in Pushkin (1975): '. . . The few very small spines are located along the ventral surface of the 2nd and 3rd segments. Similar spines surround the genital pore and form a small cluster on the ventral dilatation of the distal part of the third segment.' Here, specimens from the Chilean clade are distinguishable from specimens from the Antarctic region or Falkland Islands by their 'Fiederdornen'.

Another very variable characteristic affects the cement gland of the males. Whereas the cement gland tube itself, when present, is always very short, the ventral pore can be on a flat surface, on a broad raised surface, or something in between (Child, 1995). Our specimens show a mixture of everything: sometimes the cement gland tube is hardly visible (PpaE_001–002, PpaA_001), is short (specimens from the Falkland Islands), or is three times its own width (which is the case for the Chilean clade). Concerning the orientation of setae of the ovigers, we could detect the same sexual dimorphism as mentioned in Bamber (2002). There are no differences between *P. yepayekae* sp. nov. and *P. patagonica*.

Moreover, the abdomen of *P. patagonica* can be long and erect or be shorter and horizontal (Child, 1995). The only specimen with a straight horizontal abdomen is PpaE_001 from the Shag Rocks, near South Georgia. All other individuals have an upward-erected abdomen. As the morphological differences among the corresponding specimens lie well within the broad variation described in the literature, we assigned all of our studied specimens (except those assigned to *P. yepayekae* sp. nov.) tentatively to *P. patagonica*; however, in parallel with our molecular results this pronounced morphological variability in many features indicates that *P. patagonica* is a species complex.

CONCLUSION

To summarize, we could not assign our specimens (except *P. yepayekae* sp. nov. described in the present paper) to any of the described/known *Pallenopsis* species other than *P. patagonica* occurring near the

studied area with sufficient certainty. It seems necessary to include, beyond the morphological description, another level/source of information, i.e. a data set independent of morphology, as is presented here. With our molecular data, this is the first attempt/step to unravel the species complex of *P. patagonica* with a wider set of techniques. But the molecular data also confirm the variability of the species, resulting in different clades supported by high bootstrap values.

As already discussed in our previous study (Weis & Melzer, 2012a), with a focus on *Achelia assimilis*, the distribution area of *P. yepayekae* sp. nov. corresponds well with the area covered by glaciers during the last ice age. However, the *Pallenopsis* habitat extends to much deeper waters (down to 3500 m) than for *Achelia* (about 900 m) (Child, 1994). Therefore, the present-day distribution was either achieved by recolonization from deeper waters or by leading-edge recolonization from more northern, ice-free habitats. The diversity of different haplotypes does not imply that there was a strong bottleneck; however, further specimens are needed to verify this assumption. The extremely high genetic distances between the Falkland 'patagonica' clade and the Chilean 'yepayekae' clade indicate that these do not resemble populations that are geographically isolated. Over a long geographic gradient, genetic distances within *P. yepayekae* sp. nov. were low. Therefore, an allopatric speciation, possibly influenced by the massive glaciations, may be a likely explanation for the speciation.

The morphological and molecular results strongly support the hypothesis that the specimens from the Chilean clade represent a species new to science, described here as *P. yepayekae* sp. nov. The decision to erect *P. yepayekae* sp. nov. as a new species is also supported by the eleven individuals that do not differ strongly, both genetically and morphologically. It is known from previous works (for example, Hebert *et al*., 2004) that in less extensively studied invertebrate taxa (such as pycnogonids) hidden biological diversity, in the form of cryptic or overlooked species, is often the rule rather than the exception. Investigating how many further species may be hidden behind the *Pallenopsis* complex remains beyond the scope of this paper. This will be an interesting question for further analyses with hopefully more specimens available from the Southern Ocean.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. Range of uncorrected pairwise distances (min-max) between specimens and clades (below diagonal) and the respective standard error (above diagonal).

Table S2. Morphological characteristics of specimens that were available for morphological studies.