Global phylogeography of *Oithona similis* s.l. (Crustacea, Copepoda, Oithonidae) - a cosmopolitan plankton species or a complex of cryptic lineages?

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Abstract

Traditionally, many small-sized copepod species are considered to be widespread, bipolar or cosmopolitan. However, these large-scale distribution patterns need to be re-examined in view of increasing evidence of cryptic and pseudo-cryptic speciation in pelagic copepods. Here, we present a phylogeographic study of *Oithona similis* s.l. populations from the Arctic Ocean, the Southern Ocean and its northern boundaries, the North Atlantic and the Mediterrranean Sea. *O. similis* s.l. is considered as one of the most abundant species in temperate to polar oceans and acts as an important link in the trophic network between the microbial loop and higher trophic levels such as fish larvae. Two gene fragments were analysed: the mitochondrial cytochrome oxidase c subunit I (COI), and the nuclear ribosomal 28S genetic marker. Seven distinct, geographically delimitated, mitochondrial lineages could be identified, with divergences among the lineages ranging from 8 to 24 %, thus representing most likely cryptic or pseudocryptic species within *O. similis* s.l. Four lineages were identified within or close to the borders of the Southern Ocean, one lineage in the Arctic Ocean and two lineages in the temperate Northern hemisphere. Surprisingly the Arctic lineage was more closely related to lineages from the Southern hemisphere than to the other lineages from the Northern hemisphere, suggesting that geographic proximity is a rather poor predictor of how closely related the clades are on a genetic level.

Keywords: *Oithona*, Copepoda, species delimitation, biogeography, evolutionary history

Introduction

The existence of widespread or cosmopolitan species is widely accepted in marine plankton due to the lack of apparent geographic barriers and high dispersal potentials. Thus, it has been assumed that allopatric speciation may be less important in pelagic species than sympatric or parapatric speciation (Norris, 2000). During the past years molecular studies have revealed that many supposedly widespread species are mosaics of several cryptic or pseudocryptic species (Andrews et al., 2014; Burridge et al., 2015; Cornils and Held, 2014; Darling et al., 2007; Halbert et al., 2013; Hunt et al., 2010; Miyamoto et al., 2012). The mode of speciation in the pelagic is largely unknown but several features have been suggested as possible barriers to gene flow in the oceans: landmasses or continents (Blanco-Bercial et al., 2011a), oceanic gyres (Andrews et al., 2014), frontal systems (Stupnikova et al., 2013) or temperature and salinity ranges (Chen and Hare, 2011). Also vertical partitioning of cryptic species has been observed (Miyamoto et al., 2010; Weiner et al., 2012). The presence of cryptic or pseudocryptic species within nominal pelagic species provide evidence that traditional species concepts based on morphological identification may have greatly underestimated species richness in the oceans as suggested previously (Norris, 2000).

The cyclopoid copepod *Oithona similis* s.l. Claus, 1866 is accepted as a cosmopolitan species, presumably occurring in all oceanic zones (Razouls et al., 2016) and presumably the most abundant copepod species worldwide, although its importance has long been overlooked due to the use of too coarse mesh sizes for plankton hauls (Gallienne and Robins, 2001; Turner, 2004). Previous investigations have stressed that *O. similis* s.l. is a prominent link between the microbial loop and higher trophic levels (Atkinson, 1995; Castellani et al., 2005; Zamora-Terol et al., 2013). Its preferred habitats are polar and temperate epipelagic marine waters where it is often more abundant than calanoid copepods (Atkinson, 1998; Hopcroft et al., 2010; Pinkerton et al., 2010; Sabatini and Kiørboe, 1994; Zamora-Terol et al., 2014). Furthermore, it has also been found in subtropical and tropical waters (Cepeda et al., 2012; Nishida, 1985), although some of the reported findings seem questionable (Nishida, 1985). Morphological differences have not been detected between *O. similis* s.l. populations across latitudes, except for morphometric differences in the Northern hemisphere,where prosome length and cephalon shape differed significantly among populations from North Atlantic and Arctic water masses (Shuvalov, 1972). He suspected that *O. similis* s.l. is “a polytypic species which comprises various subspecies or intraspecific forms and groups with distinct ecological requirements regarding temperature throughout the life cycle.” A first attempt to investigate the population structure of *O. similis* s.l., using the nuclear molecular marker 28S, revealed low levels of intraspecific variety between North and South Atlantic populations (Cepeda et al., 2012). However, the polar habitats of *O. similis* s.l. were not included in that study.

In polar epipelagic zooplankton communities *O. similis* s.l. is the only dominant copepod species with a presumed cosmopolitan distribution. All other dominant species are restricted to polar and subpolar waters of the respective hemisphere. In the Arctic Ocean, predominant epipelagic species are *Calanus glacialis* Jaschnov, 1955, *Calanus hyperboreus* Kröyer, 1838, *Pseudocalanus* spp. or *Triconia borealis* Sars, 1918 (Ashjian et al., 2003; Hunt et al., 2014; Kosobokova and Hirche, 2000; Madsen et al., 2008), while in the Southern Ocean and adjacent waters *Calanoides acutus* Giesbrecht, 1902, *Calanus propinquus* Brady, 1883, *Calanus simillimus* Giesbrecht, 1892, *Rhincalanus gigas* Brady, 1883 and *Ctenocalanus citer* Heron and Bowman, 1971 account for the majority of abundance and biomass (Atkinson, 1998; Hunt and Hosie, 2006a; 2006b; Park and Ferrari, 2009).

The present study aims at a global assessment of evolutionary lineages within *Oithona similis* s.l. using sequences of two gene fragments: the mitochondrial cytochrome oxidase *c* subunit I (COI), and the nuclear large subunit ribosomal gene (28S). We analysed specimens from both the Southern Ocean and the Arctic Ocean, from different localities in the Atlantic Ocean, and from the Bay of Villefranche in the northwestern Mediterranean Sea near the type locality of *O. similis* s.l. (Claus 1866). The data set was complemented with previously published sequences from the Atlantic and Pacific Ocean. Using several independent species delimitation methods we estimated the existence and number of cryptic or pseudocryptic species within *O. similis* s.l. Additionally, specimens from co-occurring *Oithona* species in the polar systems (*Oithona frigida* Giesbrecht, 1902, *Oithona atlantica* Farran, 1908) and the temperate to subtropical regions (*Oithona nana* Giesbrecht, 1893, *Oithona plumifera* Baird, 1843, *Oithona davisae* Ferrari and Orsi, 1984) were obtained to examine the interspecific variability in the COI and 28S gene.

Material and Methods

Sampling, and preparation for molecular analysis

For the present study *Oithona* specimens were collected on various research cruises from 2006 to 2013 (Fig. 1, Appendix 1). In the polar regions stratified mesozooplankton samples were collected with a multinet (HydroBios MultiNet Type Midi, 0.25 m^2 aperture), equipped with five nets of 55 to 200 μm mesh-size. During the Polarstern cruises ANT XXIV/2, ARK XXIII/ and ARK XXV/1, specimens were sorted alive under a stereomicroscope immediately after the haul and were transferred to pure ethanol (undenatured 96% EtOH) for genetic analysis. The remaining sample was preserved in 4% formaldehyde seawater solution. All other Antarctic and Arctic specimens used in this study were sorted from mesozooplankton samples preserved in pure ethanol. The ethanol in all samples was changed after 24 hours to ensure optimal preservation for genetic analyses. For comparison, specimens from other

regions were included in the study, e.g. specimens from Villefranche sur Mer (Ligurian Sea) close to the type locality of *O. similis* (Claus, 1866), and from Helgoland Roads (North Sea), the type locality for *Oithona helgolandica* (Claus, 1863). The latter has often been classified as synonymous with *O. similis* (for review see Razouls et al. 2016), but the original description of a male specimen of *O. helgolandica* (Claus 1863) probably refers to *Oithona nana* (Sars, 1918). In this study, we focused on female *O. similis* s.l. in order to eliminate the influence of sexual dimorphisms of body length and morphological characters.

Morphological characterization

Prior to DNA extraction the specimens were placed in distilled water for up to five minutes at room temperature and were identified morphologically under a stereomicroscope under low light conditions. In addition, total length and Prosome:Urosome (P:U) ratios of 50 specimens, representing each of the molecular defined groups, were measured (Appendix 1). To evaluate whether cryptic or pseudocryptic species are found within *O. similis* s.l., three to ten female specimens were set aside for further morphological analysis. These females were dissected and the structure and ornamentation of the swimming legs was studied.

DNA extraction, amplification and sequencing

DNA was extracted from single individuals following the protocol for DNA purification from tissues of the QIAamp DNA Mini Kit (Qiagen) with the following modifications: (1) The individuals were placed in a tube containing ATL buffer and glass beads (diameter: 425 – 600 μm; Sigma G8772), vortexed at maximum speed for five minutes and centrifuged for 10 min at 13,200 rpm (revolutions per minute), before the proteinase K was added. (2) Then the tubes were incubated in a thermo-shaker at 56°C and 550 rpm overnight. (3) 1 μl carrier RNA was applied with the AL buffer. (4) The DNA was eluted in 50 μl AE buffer. DNA samples were stored at −20°C until further analysis.

The mitochondrial protein coding gene COI was amplified using the primer sets LCO1490 and HCO2198 (Folmer et al., 1994). Due to difficulties with the amplification in some specimens a versatile primer set based on a highly conservative mitochondrial DNA region was used (L1384-COI and H2612-COI; (Machida et al., 2004). Polymerase Chain Reaction (PCR) amplifications were performed in 25 µl reaction volumes. For COI the reaction volume included 5 µl of 5x KAPA2G Buffer B (KAPABiosystems), 5 µl of 5x Enhancer 1 (KAPABiosystems), 0.125 µl of 100 µM of each primer, 0.5 µl of 10 mM KAPA dNTP Mix, 0.15 μl of 5 U/μl KAPA2G Robust DNA Polymerase (KAPABiosystems) and 3 µl of DNA template solution. The PCR for COI consisted of 35 cycles of denaturation at 95°C for 20 s, annealing at 49°C for 20 s and extension at 72°C for 20 s. An 800 bp fragment of the large subunit (28S) ribosomal DNA was amplified using the primer set 28SF1 and 28SR1 (Ortman,

2008). PCR amplifications were carried out according to the protocols of (Cepeda et al., 2012).

PCR products were run on a 2% agarose/TBE gel and afterwards stained with ethidium bromide for band characterization. For COI positive results were either purified with the QIAquick PCR purification kit (Qiagen) or with ExoSap-IT (0.25 µl exo, 1 µl SAP). Subsequently they were used for cycle sequencing with Big Dye Terminator Ver. 3.1 (Applied Biosystems Inc., ABI) and the same primers as for the PCR amplifications. The sequences were run on an ABI 3130XL DNA sequencer (Applied Biosystems). In case of 28S positive PCR results were sent for purification and sequencing to Eurofins Genomics (http://www.eurofins.de) for purification with the Agencourt AMPure XP systems (Beckman Coulter) and sequencing on ABI 3730XL DNA sequencer (Applied Biosystems).

Sequence editing and additional sequences

In total, 223 COI and 70 28S sequences were obtained. In CodonCode Aligner Vers. 3.7.1.1 (CodonCode Corporation) both strands were assembled into consensus sequences, aligned and manually checked for sequencing errors. Twenty-one COI sequences labelled *Oithona similis* were added from GenBank and twelve additional 28S sequences under the name of *Oithona helgolandica* (Table 1), resulting in 244 COI and 82 28S sequences for further analyses. Additional COI GenBank sequences labelled "*Oithona similis* " (KC136272 – KC136284) clustered in a preliminary neighbor-joining tree with sequences of *Oithona brevicornis* (GenBank accession numbers AB604190, AB604189) instead of the *O. similis* s.l. and were consequently removed from the dataset.

The aligned sequences were cut to a length of 579 bp (28S) and 438 bp (COI). All 28S sequences had the same length, but 27 sequences of the 244 COI sequences were shorter than 400bp. Additionally, the 28S sequences were aligned with MAFFT v7.157b using the L-INS-I option (Katoh and Standley, 2013). For the phylogenetic analysis and the haplotype networks, the sequences were collapsed to haplotypes with jMOTU (Jones et al., 2011) with zero base cut-off resulting in 41 (COI) and 15 (28S) unique haplotypes.

The amino acid sequence of the final COI alignment did not reveal any stop codons indicating that pseudogenes probably were not present. Following the advice from (Calvignac et al., 2011) the genetic diversity of each codon position was noted separately revealing that the diversity was highest at the third codon position (1st codon position: 0.0528 ± 0.0109 standard error (S.E.), 2nd codon position: 0.0001 ± 0.0001 S.E. and 3rd codon position: 0.3434 ± 0.0183 S.E.). The new COI and 28S sequences were deposited in GenBank under the accession numbers KU982646 - KU982960. Alignments, models and tree data for Figs. 2 - 4 are available at: https://doi.org/10.1594/PANGAEA.862554.

182
183 **Phylogenetic analysis**
184 To study the interspecific diversity and assess the monophyly of *O. similis* s.l. sequences of
185 other *Oithona* species were added (Table 1, 2; Fig. 2, 3). For the interspecific diversity
186 uncorrected *p*-distances were calculated with MEGA 6.06 (Tamura et al., 2013). For the
187 phylogenetic analysis sequences were analysed both concatenated and separated by gene
188 fragment. The best-fitting partitioning scheme and nucleotide substitution model were
189 determined with PartitionFinder v1.1.0 (Lanfear et al., 2012). For COI each codon position
190 was analysed separately. The selection criterion for the nucleotide substitution model was
191 the corrected Akaike information criterion AICc for small sample sizes. Only the models
192 available in the programs RaxML v 8.0 (Stamatakis, 2014), MrBayes 3.2 (Ronquist et al.,
193 2012) and BEAST 1.8.1 (Drummond et al., 2012) were allowed, resulting in the same
194 partitioning scheme: 28S, COI 1st codon position, COI 2nd codon position, COI 3rd codon
195 position. The best-fitting nucleotide substitution models for RAxML were GTR+I+G for 28S,
196 COI 1st and 2nd codon position, and GTR+G for COI 3rd codon position. For MrBayes the
197 following substitution models were chosen: 28S: GTR+I+G, COI 1st codon position:
198 GTR+I+G, COI 2nd codon position: GTR+I, and COI 3rd codon position: HKY+G. The
199 resulting partitioning schemes and substitution models were then implemented in RAxML
200 and MrBayes to carry out Maximum Likelihood (ML) and Bayesian Inference (BI) analyses,
201 respectively. RAxML was run under the option GTRGAMMAI and a complete random starting
202 tree of 10,000 bootstrap replicates. Best-known likelihood tree search was performed under
203 GTRGAMMAI and a completely random starting tree. The final tree topology was evaluated
204 under GTRGAMMAI to yield stable likelihood values. In MrBayes MCMC analysis was
205 conducted on two independent runs of 10,000,000 generations, each with four chains (1
206 cold, 3 hot), with a sample frequency of 1000 generations. To check for stationary distribution
207 the average standard deviation of the split frequencies were examined for convergence, i.e.
208 the two independent runs converge when the standard deviation of the split frequencies is
209 approaching zero (< 0.01, Ronquist et al., 2011), and the first 25 % of the sampled trees
210 were discarded as “burn-in”. Only Bayesian posterior probability (BPP) values > 0.90 were
211 displayed to show only strongly supported clades. As outgroup sequences of *Mesocyclops*
212 *leuckartii* Claus, 1857 from the most closely related family with published sequences for both
213 genes were chosen (GenBank Accession numbers: KF153692, KF357729).
214
215 **Species delimitation**
216 To test the hypothesis that *O. similis* s.l. is a complex of reproductively isolated species,
217 three independent species delimitation methods were applied to the sequences of both
218 genes: the General Mixed Yule Coalescent Model (GMYC; Pons et al., 2006), Automated

Barcoding Gap Discovery (ABGD; Puillandre et al., 2012) and Rosenberg's probability of reciprocal monophyly (Rosenberg, 2007). GMYC is a maximum-likelihood approach, which measures the transition from intra- to inter-species branching patterns to identify species (Pons et al., 2006). The analysis was conducted with a Bayesian implementation of the GMYC model (bGMYC) (Reid and Carstens, 2012) in the statistical computing language R (R Development Core Team, 2015). This model differs from the original GMYC model as it allows uncertainties in phylogenies and implements flexible prior distributions. Prior to the analysis, ultrametric consensus trees were generated with BEAST 1.8.1. The program was run using the nucleotide substitution model TrN+I+G and the trees were analysed without partitioning the data as suggested by PartitionFinder and a strict molecular clock with a fixed mean substitution rate (1.0). All other tree priors were left at their default values. Two independent analyses of 10,000,000 generations were run in BEAST and sampled every 1,000 generations. The first 1,000 generations were discarded as burn-in. Tracer 1.6 was used to check for a sufficient effective sampling size (ESS) and good mixing. The two runs were then combined with Logcombiner from the BEAST package with a lower sampling frequency to 98 trees for the multi-threshold bGMYC analysis (Monaghan et al., 2009). The analysis was run with multiple thresholds under default settings. bGMYC returns a graph with an ultrametric tree and a heatmap-like figure showing the posterior probabilities of conspecificity (Reid and Carstens, 2012).

ABGD sorts the sequences into groups based on pairwise distances (Puillandre et al., 2012). It is an iterative process and detects significant differences between intra- and interspecific variations (i.e. barcoding gap). The COI alignment was uploaded to the website http://wwabi.snv.jussieu.fr/public/abgd/abgdweb.html and run with the default settings (P (prior limit to intraspecific diversity) ranged between 0.001 and 0.1; X (gap widths) = 1 using the available models JC86 (Jukes-Cantor) and K80 (Kimura).

Rosenberg's probability of reciprocal monophyly was used to determine if the data set is suitable to detect robust monophyletic lineages (Rosenberg, 2007). This method is implemented as "rosenberg" in the R package SPIDER (Brown et al., 2012) and marks dichotomous nodes where the probability of monophyly is significant.

In general, DNA barcoding is used to identify species within a standard gene region, in this case COI (Hebert et al., 2003). New sequences are compared to a database of known sequences. To test if the lineages identified with GMYC and ABGD can be correctly identified via DNA barcoding the functions "nearNeighbour", "bestCloseMatch" and "threshID" of the R package SPIDER were used. For this, the optimal threshold for species identification was determined with the function "localMinima" from the R package SPIDER, and all sequences were labeled according to the affiliation in the results of GMYC and ABGD. The function "nearNeighbour" identifies the closest specimen and returns the score "true" if the species

label is the same, or “false” if the labels differ. The “bestCloseMatch” (best close match analysis; (Meier et al., 2006) searches for the closest specimens and determines if it is within a given threshold. If it is, then the outcome is scored as “correct”, if it is outside the threshold the outcome is “no ID” (this is also true for singletons). If the closest match includes more than one species the identification is “ambiguous”. When all the matches within the threshold have different species labels the identification is “incorrect”. The third analysis (“threshID”) is threshold-based with a given threshold genetic distance of 1%. This method resembles the BOLD species identification systems (http://www.boldsystems.org/index.php/IDS_OpenIdEngine). The results of this analysis are the same as in the best close match analysis.

Haplotype networks are a common method to visualize genealogical relationships among *O. similis* s.l. TCS haplotype networks (Clement et al., 2000) were created for both genes using POPART (Leigh and Bryant, 2015). For this the 27 sequences shorter than 400bp were removed to gain a better resolution of the relationships between the lineages.

Divergence time estimation

The program DAMBE5 (Xia, 2013) was used to check whether a molecular clock could be used to date some of the nodes in our COI sequences. Using the test of Xia et al. (2003) the program can measure the degree of substitution saturation. High levels of saturation are not suitable for molecular clock estimation (Xia and Lemey, 2009). The program BEAST (settings as above) was used to estimate the time of the most recent common ancestors (tmrca). Fossils of copepods are rare (Harvey and Pedder, 2013; Selden et al., 2010) and therefore to our knowledge no copepod-specific estimates of mutation rates exist (Blanco-Bercial et al., 2011a; Marrone et al., 2013; Thum and Harrison, 2009). Often, tmrcas in copepods were estimated from known sequences divergence of the decapod *Alpheus* spp. with 1.4% per million years (Knowlton and Weigt, 1998). Although this rate has been widely used to estimate molecular evolution in marine invertebrates, there are newer estimates in marine invertebrates showing that substitution rates could also be considerably higher (e.g. Calvo et al., 2015). At present however, we have refrained from using external information to calibrate the tree and only provide estimates of relative divergence times with the 95% highest posterior intervals (HPD) and not absolute node ages.

Results

Both the COI and the 28S phylogenetic trees yielded the specimens morphologically identified as *O. similis* s.l. as a clearly monophyletic group (Fig. 2, 3). While the genetic variation in the nuclear 28S gene fragment was consistently lower within *O. similis* s.l. (0 – 0.010) compared to the overlap with the distances to other *Oithona* species (0.023 – 0.210). In COI genetic distances between *O. similis* s.l. and other *Oithona* species (0.192 – 0.331)

293 overlapped with the genetic variation within *O. similis* s.l. (0 - 0.235), thus suggesting that *O.*
294 *similis* s.l. is more likely a species complex than a panmictic species.
295
296 **Species delimitation**
297 A total of 244 COI sequences worldwide of *O. similis* s.l. were used in the present study.
298 Seven cryptic mitochondrial lineages and two singletons were discovered with the species
299 delimitation methods ABDG and bGMYC (Appendix 4). They were well-supported (posterior
300 probability (PP) > 0.95. For all nodes Rosenberg's probability of reciprocal monophyly was
301 significant (Appendix 4). The three species identification methods (nearest neighbor, best
302 close match and threshold ID) assigned 242 of the 244 COI sequences correctly, using a
303 genetic distance threshold of 0.053. The two singleton sequences from the Chukchi Sea
304 (ARK2 and ARK3) were scored as "no ID" thus indicating that they could not be assigned to
305 any of the discovered species. The maximum genetic distance (uncorrected *p*-distance)
306 within each lineage was always lower than 1%. Genetic divergence among the seven
307 mitochondrial lineages within the *O. similis* species complex ranged between 8.3 and 23.7%
308 (Table 3) and thus they were all more divergent than 8%, the proposed threshold in COI for
309 distinct species in copepods (Bucklin et al., 1999, Hill et al., 2001). Furthermore, the genetic
310 distances among lineages satisfied the 4x rule (Birky et al., 2005), as they were at least 4x
311 higher than genetic distances within lineages. Thus, most probably the seven independent
312 genetic lineages represent cryptic or pseudocryptic species within *O. similis* s.l.
313 The two species delimitation methods bGMYC and ABGD were also applied to the 82
314 sequences of the nuclear 28S gene fragment. For bGMYC no significant lineages were
315 found, while with ABGD three lineages were recovered. The lineages ARK1 is identical with
316 the mitochondrial lineage ARK1. Clade 2 includes all other 28S sequences, except for the
317 two sequences grouped in Clade 3. The latter belong to two specimens from the Polar Front.
318
319 **Phylogeography**
320 Although the sampling in this study may not be high enough to conclusively rule out the
321 presence of lineages or species in an area in which we did not sample it, the pattern
322 emerging from analysing our presence/pseudo-absence data shows seven mitochondrial
323 lineages with regional distributions rather than a single cosmopolitan species (Fig. 4,
324 Appendix 4). Three lineages and the two singletons are localized in the Northern
325 hemisphere. The lineage ARK1 was only found in the Arctic Ocean and its adjacent seas
326 (Chukchi Sea, Beaufort Sea, Canadian Basin, Central Arctic Ocean, Greenland Sea,
327 Norwegian Sea). Two mitochondrial lineages were identified from the North Atlantic (NA) and
328 the North Pacific (NP) respectively, the latter is based only on published sequences from
329 GenBank. The two singletons ARK2 and ARK3 were collected from the Chukchi Sea in the

Pacific part of the Arctic. In the Southern hemisphere four mitochondrial lineages could be recovered with the species delimitation methods. The lineage ANT was found in Antarctic waters (Weddell Gyre, the Coastal Current, the Amundsen Sea and the Polar Front). PF was identified only from samples at the Polar Front of Atlantic and Indian Antarctic sectors. AS was only found in the Amundsen Sea, and BC occurred in the Beagle Channel in Tierra del Fuego (Fig. 1, 4).

An overlap between the lineages PF and ANT near the Polar Front (Atlantic sector; Appendix 2) revealed evidence for vertical partitioning of the two lineages (Fig. 5). While PF was mainly found in the upper 100 m, ANT occurred mainly deeper than 100 m. Further south, in the Weddell Gyre and the Coastal Current, PF was absent and ANT was distributed from the surface to 250 m depth.

The haplotype network also showed that only the cryptic mitochondrial lineage from the Arctic Ocean (ARK1) is recognized in the nuclear gene fragment 28S. All Arctic 28S shared the same allele. However, most 28S sequences from the Southern Ocean and its boundaries form another 28S allele (ANT, PF, AS, BC, Fig. 4) and sequences from the Northern Atlantic (NA) and from the Southwest Atlantic (published in GenBank) form a third group with several haplotypes.

Divergence Time estimation

The test of Xia et al. (2003) was applied to the COI data set with the proportion of invariable sites as suggested by DAMBE5 (COI: 0.55864). Only fully resolved sites were considered. The index of substitution saturation (ISS) was significantly lower than the critical ISS and the degree of substitution saturation can be considered to be low in the data set. Thus, the data set is suitable for further molecular clock analysis. The effective sampling size (ESS) was higher than 1000 for most parameters, indicating convergence and good mixing of the MCMC chain. The tmrcas were recovered with the 95% HPD. The phylogenetic analysis revealed a strong separation between clades of the Northern hemisphere (NA, NP, ARK2, ARK3) from the polar (ANT, ARK1) and subpolar clades (BC, PF, AS; Fig. 6). Conservative estimates based on mitochondrial substitution rates from other crustaceans (Knowlton and Weigt, 1998) suggest that this separation may have occurred prior to the middle Miocene climate transition, about 21.5 - 13.4 million years ago (mya). Separation of the subpolar clades and the polar groups occurred later in time. The separation of the two polar clades ARK1 and ANT occurred last. Divergence time estimate from other crustaceans (see above) date this event between 5.9 – 11.2 mya. This estimate for the last common ancestor of the two polar lineages is a conservative estimate, if higher substitution rates are assumed (e.g. 5.16%, Calvo et al., 2015) the timing of this split shifts to even more recent estimates (around

2 mya (95% HPD: 1.6 - 3.0 mya)). The lineages in the Northern hemisphere (NP, ARK2, ARK3) and (NA) lineages separated prior to those of the southern hemisphere.

Morphology

During the detailed morphological analysis it could be excluded that the genetic groups found within *O. similis* s.l. were misidentified representatives of the closely related species *Oithona fallax* or *Oithona decipiens*. All analysed individuals only had one outer spine at the outer margin of the third exopod segment (Exp3) of swimming leg two (P2) and no inner marginal spine of the first exopod segment (Exp1) of all four swimming legs (P1 – P4). In *O. fallax* two spines on P2 Exp3 and one spine on P1-P4 Exp1 would be expected. All analysed individuals had one outer marginal spine on the second exopod segment (Exp2) of the first swimming leg (P1), which is missing in *O. decipiens*. Between the genetic groups no differences in swimming leg ornamentation of *O. similis* s.l. could be detected. However, total length measurements prior to DNA extraction of 51 specimens revealed that individuals from lineage ANT were considerably larger than all others groups while the individuals from the northeastern Atlantic were smallest (Appendix 3).

Discussion

The present study provides molecular evidence that *O. similis* s.l. is not a single species with a cosmopolitan distribution, but instead a complex of cryptic or pseudocryptic genetic lineages with much smaller, regional distributions. These lineages are confined to different climate zones (Antarctic, Arctic, Polar Front, temperate Atlantic or Pacific). Genetic divergence has also been reported from other widespread planktonic organisms (Burridge et al., 2015; Casteleyn et al., 2010; Cornils and Held, 2014; Darling et al., 2007; Halbert et al., 2013; Hunt et al., 2010; Kulagin et al., 2013). A recent global plankton metabarcoding survey in the photic zone of the oceans even revealed that only 0.35% of OTUs (operational taxonomic units) are cosmopolitan (de Vargas et al., 2015). These recent findings oppose the assumption that global panmixia is common in planktonic species (e.g. Palumbi, 1992, Norris, 2000).

Taxonomic and phylogenetic implications

It is noteworthy that the all mitochondrial as well as nuclear lineages of *Oithona similis* s.l. form a well-supported monophylum and do not mix with other congeners. This means that according to our present knowledge the species outside *O. similis* s.l. group can be considered as outgroup, but do not have to be included in a reconstruction of evolutionary history inside the group. Our results confirm the close phylogenetic relationships between *O. similis* s.l. and the *O. atlantica* group (Nishida, 1985). Due to the lack of samples the close

relationship between *O. similis* s.l. and *O. decipiens* or *O. fallax* could not be investigated. However, their presence within the *O. similis* s.l. group could be excluded morphologically. The species delimitation methods GMYC and ABGD detected seven cryptic mitochondrial lineages of *O. similis* s.l., but only three clearly distinct nuclear lineages could be recognized using the 28S ribosomal gene. The two genes agreed in the discovery of a cryptic Arctic lineage (ARK1), specimens representing the other six cryptic *O. similis* lineages were poorly resolved and shared seven 28S alleles, allowing no further differentiation. These results indicate that the speciation process in *O. similis* s.l. is probably too recent for the mutation rate within the nuclear 28S gene fragment to be detectable.

Alternatively, the different patterns of divergence in the mitochondrial and nuclear gene fragments, for example in the Southern hemisphere (ANT, PF, AS, BC) might also be explained by a temporary separation and diversification of *O. similis* populations followed by a period of hybridization. While hybridization has led to the homogenization of ephemeral genetic differences in the nuclear genome, the lack of recombination has led to a persistent record of this period of separation in the mitochondrial genes. According to this interpretation, the coexisting mitochondrial lineages, although not indicating the existence of a second species, would nevertheless count as a record of previous genetic isolation (see Dietz et al., 2015).

Although ribosomal nuclear genes are known to have a low resolution within copepod species (Machida and Tsuda, 2010; Marrone et al., 2013) and are mainly applied to investigate monophyly of higher ranks (e.g. Blanco-Bercial et al., 2011b; Cornils and Blanco-Bercial, 2013), they may include highly variable segments that differentiate between species (e.g. Held, 2000) and the 28S gene fragment has been successfully applied to distinguish between morphotypes of *Oithona dissimilis* Lindberg, 1940 (Ueda et al., 2011) and to identify *Oithona* species, including *O. similis* s.l., in the Atlantic Ocean (Cepeda et al., 2012). In our case however, the genetic differences of 28S were lower than in *O. dissimilis* (*O. dissimilis*: 1.84 – 2.21%; *O. similis* s.l.: 0 – 1.0%). Thus, to investigate the possibility of hybridization nuclear genes with faster mutation rates, such as ITS (internal transcribed spacers) or microsatellites, are needed (e.g. Makino and Tanabe, 2009, Parent et al., 2012).

Identifying taxonomically valid species in *Oithona similis* s.l.

Matching clades in our molecular phylogeny with available taxonomic species is a task in itself and logically separate from demonstrating that the clades are genetically isolated from one another. In this paper, *O. similis* s.l. specimens were taken near the type locality off the coast of Nice (Claus, 1866, Fig. 1), the type locality of *O. similis* s.str. There has however, been a long debate whether an earlier description by the same author of *O. helgolandica* from Helgoland in the North Sea (Claus, 1863) is synonymous with the description from the

Mediterranean (for review see Cepeda et al., 2016). However, both descriptions are very incomplete and the description from the North Sea could also match with *O. nana* (Sars, 1918). Our data show that the specimens from the Mediterranean (Villefranche), Helgoland and other regions in the NE and NW Atlantic form a distinct mitochondrial lineage and may thus all belong to *O. similis* s.str. This separation is also visible in the haplotype network of the ribosomal gene fragment 28S and includes also sequences from the SW Atlantic, taken from Cepeda et al. (2012). The placement of the specimens from the SW Atlantic within *O. similis* s.str. remains unclear until a comparison with COI sequences is possible. Supporting evidence that the Mediterranean and the North Atlantic population may be the same species also comes from ecological research (Castellani et al., 2016) as they respond similarly to temperature. Although a full taxonomic revision of the *Oithona similis* species complex is beyond the scope of this paper but in view of the mounting evidence that it comprises many species we believe that the best way to handle the situation currently is to use *O. similis* s.l. together with a reference to the geographic origin as an interim identification. In order to reconcile these records with a future taxonomic revision of *O. similis* it is advisable to keep voucher samples suitable for molecular analysis.

Biogeography and evolutionary history

Although it is unclear if the clades characterized by diverged COI but uniform 28S sequences have to be interpreted as fully diverged species or not, the differentiation of the mitochondrial haplotypes is nevertheless indicative of historical isolation (Dietz et al., 2015). The distribution of seven mitochondrial lineages in *O. similis* s.l. allows inferences about the processes that have shaped the evolutionary history of this planktonic group that have been obscured previously by treating it as a single evolutionary unit.

The distribution area of each of the newly delineated species or clades in *O. similis* s.l. is considerably smaller but surprisingly geographic proximity is a rather poor predictor of how closely related the clades are on a genetic level. The deepest split in our samples includes a well-supported group of lineages exclusively from the Northern hemisphere. Within it, the Northern Atlantic (NA) lineage is sister to a group of *O. similis* s.l. from the North Pacific (NP) and the Pacific Arctic (ARK2 and ARK3), indicating that the speciation is taking place on an ocean basin scale. The node leading to ARK2 and NP is poorly supported and thus, it is difficult to decide if (1) the Arctic was colonized from the Atlantic and subsequently the North Pacific or (2) the Atlantic and Pacific lineage existed in parallel, in which case the NP lineage would be expected to have given rise to the two Arctic *O. similis* s.l. lineages. There is, however, evidence that planktonic organisms in the North Pacific enter the Pacific Arctic (e.g. Nelson et al., 2009). The two Arctic lineages ARK2 and ARK3 are however, singletons, and it is not known if these lineages do not appear in the subarctic North Pacific as well.

One of the best-supported results is that clearly the Arctic was colonized twice by two very distantly related lineages of *Oithona*. In addition to the two Arctic lineages in the northern hemisphere group (ARK2, ARK3), a third Arctic *Oithona* species candidate (ARK1) is nested deeply within a well-supported group of southern hemisphere group of *O. similis* species candidates. In the nuclear 28S the two singletons share the same allele as the specimens from the Southern hemisphere and not with the other Arctic (ARK1) and the NA assemblage. A close relationship between subpolar North Pacific populations with populations from the Southern hemisphere has also been observed in foraminiferans (Darling et al., 2007).

The lack of lower latitude *O. similis* s.l. in this analysis precludes a definitive statement at this time, but several lines of evidence suggest that the group ARK1 indeed evolved from a cold-adapted ancestor close to the Antarctic and separated from it once it managed the transit to the Arctic Ocean. The relative divergence estimates (Fig. 6) also suggest that the Antarctic and Arctic lineages are the youngest in the tree. This has also been observed in other polar organisms (Held, 2000). If the sequence divergence estimate of 1.4% per mya (*Alpheus* sp., Knowlton and Weigt, 1988) is roughly correct, the split between ARK1 and ANT occurred after the worldwide latitudinal climate gradient had steepened in the upper Miocene with the Arctic and the Southern Ocean being already polar (Zachos et al., 2001). Although this steep climate gradient must have made the dispersal to the other polar ocean difficult, it likely made the chances of survival and successful colonization higher once it arrived there because ARK1 likely inherited adaptions to polar conditions from the ancestral species it shares with ANT.

This long-distance dispersal may be less unlikely than it may appear at first sight because at about the same time two more independent splits occurred between a species pair that today comprises a polar specialist and their closest relative that is geographically and climatically distant, namely (AS+(PF+BC)) and (ARK2 and NP). It is reasonable to assume that the *O. similis* s.l. species in the upper Miocene were more widely distributed than today making long distance dispersal between climatic zones as well as across hemispheres rare but not impossible before the climatic gradient steepened to the extent seen today.

Our data clearly show that the Antarctic (ANT) and the Arctic (ARK1) lineages are highly divergent, which has also been shown for other common polar epipelagic species, e.g. the pteropod *Limacina helicina* (Hunt et al., 2010) and foraminifera (Darling et al., 2007), and can also be seen in the morphologically similar polar species of *Calanus* spp. (Kozol et al., 2012). These results emphasize that cosmopolitan or bipolar distribution in epipelagic planktonic species may be much less common than previously thought, possibly due to dietary or reproductive requirements. Within the epi- to mesopelagic genus *Microcalanus* bipolar and even cosmopolitan cryptic mitochondrial lineages have been found (Cornils, unpublished data). In tropical regions these species show a meso- to bathypelagic distribution. Thus,

514 gene flow between the polar ecosystems may only be feasible if species are able to
515 submerge to deeper (colder) water layers in the tropics (Allcock, 2014).
516 Geographic overlaps of the lineages occurred only at the Antarctic Polar Front (ANT – PF,
517 ANT - AS) and in the Chukchi Sea (ARK1 – ARK2, ARK1 – ARK3). Our data indicate that the
518 apparent co-occurrence of the lineages ANT and PF is possibly a product of the encounter of
519 two different water masses, the colder waters of the East-Wind-Drift (Antarctic Intermediate
520 Water) and the warmer surface waters of the Antarctic Circumpolar Current (ACC). The
521 lineage PF may be associated with the warmer water masses as it was mainly found in the
522 surface waters, while ANT occurred below 100 m depth and thus, could be associated with
523 the deeper Antarctic Intermediate Water (Fig. 5). The latter is also known from the other
524 dominant epipelagic Antarctic copepod species: *Calanoides acutus*, *Rhincalanus gigas* or
525 *Calanus propinquus* can also be found as far north as the Polar Front, but they appear to be
526 associated with the deeper Antarctic Intermediate Water (Park and Ferrari, 2009). These
527 results reinforce the notion that the Antarctic Polar Front is an important ecological barrier for
528 zooplankton species (Marin, 1987). The same mechanism of different vertical distributions
529 may also account for the seemingly co-occurring mitochondrial lineages ANT and AS in the
530 Amundsen Sea.
531 In the Northern Atlantic our mitochondrial sequences did not reveal any geographic overlap
532 between the lineages ARK1 and NA. However, early morphological observations on *O.*
533 *similis* s.l. in the Northern hemisphere revealed considerable differences in size and in the
534 curvature of the cephalosome between Arctic specimens and boreal forms from the North
535 Atlantic and North Pacific (Shuvalov, 1972). Shuvalov observed that the Atlantic *O. similis* s.l.
536 form could be found as far north as Svalbard or the Barents Sea. These findings indicate that
537 the transport of the lineage NA via the North Atlantic Current to the Arctic Ocean would be
538 possible. This phenomenon is also known for other boreal species such as *Calanus*
539 *finmarchicus* (Hirche and Kosobokova, 2007). 28S sequences of *O. similis* s.l. from the
540 Irminger Current (Cepeda et al., 2012) and from the Norwegian Sea (present study) were
541 placed in the variable Atlantic group of the haplotype network indicating that the NA lineage
542 may also occur at higher latitudes.
543
544 **Ecological implications and response to environmental change**
545 In polar and temperate habitats *O. similis* s.l. is a prominent link between the microbial loop
546 and higher trophic levels such as fish larvae (Checkley, 1982; Paradis et al., 2012; Turner,
547 2004). Although we assume that the ecological role in the different pelagic communities
548 remains similar among the cryptic lineages there may be behavioral, ecological or
549 physiological differences between the lineages as it has been observed in marine protists
550 (Darling et al., 2007). Shuvalov (1972) found many dead *O. similis* s.l. specimens in abruptly

changing water masses at the interface of the Arctic and the North Atlantic Ocean and assumed that temperature tolerance differs between the polar and the subpolar/temperate specimens, since the seasonal cycle of water temperature varies greatly between high and lower latitudes. Generally it has been observed that temperature and food availability influence the fecundity of *O. similis* s.l. (Castellani et al., 2005; Ward and Hirst, 2007). Unfortunately, large-scale studies comparing temperature tolerance of *O. similis* s.l. from different latitudes are lacking so far. Thus, we will use the intensely studied globally distributed *Calanus* species complex as a model for the *O. similis* s.l. complex. The *Calanus* species are morphological very similar, but they exhibit differences e.g. in their reproductive cycles (Niehoff et al., 2002). In the northern Atlantic latitudinal shifts within the *Calanus* spp. have been observed during the past 40 years, probably associated with ocean warming (Beaugrand et al., 2003, Chust et al., 2014). Temperature has been identified as the critical factor that makes marine systems vulnerable to global warming and the zooplankton composition, abundance and trophic efficiency is highly linked to it (Richardson, 2008). We assume that the lineages associated with temperate water masses have a higher tolerance to changing temperatures due to the pronounced seasonality compared to the stenotherm polar lineages ARK1 and ANT. Future warming of the polar oceans could thus initiate a poleward shift of the subpolar lineages of *O. similis* s.l. to the high Antarctic or an invasion of the temperate Atlantic or Pacific lineages to the Arctic. These invasions could cause severe changes in the seasonal availability of *Oithona* in the polar oceans and thus, alter the food availability for higher trophic levels such as fish larvae. In the North Sea the boreal *Calanus finmarchicus* is gradually replaced by the warm-temperate *Calanus helgolandicus* which has lead to severe consequences for the recruitment of Atlantic cod (Beaugrand et al., 2003). Therefore, understanding of the present environmental factors and historical events that shape the biogeography of widespread zooplankton organisms is critical to analyse the impact of climate change on higher tropic levels.

In conclusion, *O. similis* s.l. is not a single species with a circumglobal distribution. Instead, it consists of at least seven distinct mitochondrial lineages that most likely represent cryptic or pseudocryptic species. This study provides further evidence that widespread epipelagic copepod species are often a complex of cryptic species with deep divergences in mitochondrial genes.

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Figure captions

Figure 1: Station map for *Oithona similis* sampling locations. The samples from the type localities of *Oithona similis* (Mediterranean Sea) and *Oithona helgolandica* (North Sea) are marked in bold letters.Yellow dots (North Pacific Ocean) and the purple dot (Polarfront) south of Australia are based on location derived from GenBank COI sequences (Table 1). The geographic information of the 28S sequences from GenBank are not included, but can be viewed in Cepeda et al. (2012).

Figure 2: Bayesian phylogenetic tree of COI haplotypes for the genus *Oithona*. Bayesian posterior probabilities (BPP) and maximum likelihood bootstrap values (BS) are placed at the above and below nodes. Only values above 0.9 (BPP) and 50 (BS) are displayed. Colour bars represent the sampling localities of *Oithona similis* s.l. with the number of sequences (n). *O. similis* s.l. specimens from the type localities are marked in bold letters.

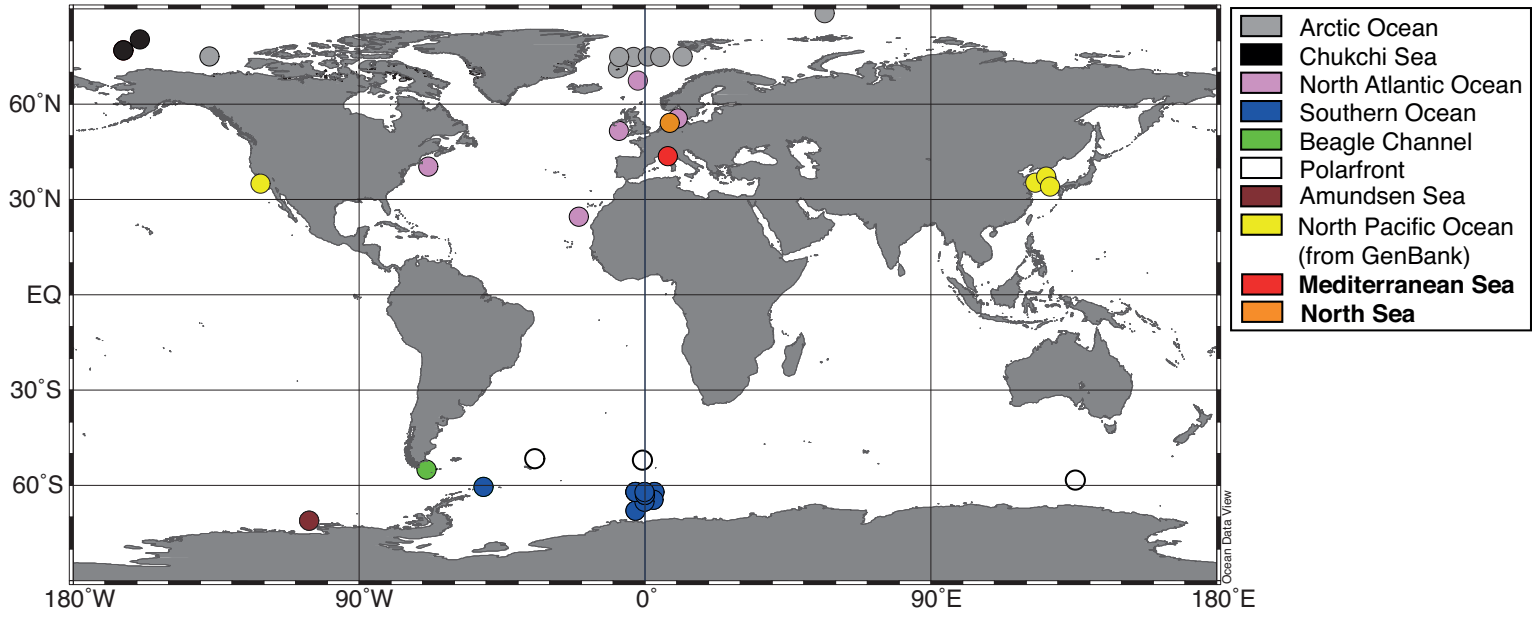
Figure 3: Bayesian phylogenetic tree of 28S alleles for the genus *Oithona*. Bayesian posterior probabilities (BPP) and maximum likelihood bootstrap values (BS) are placed at the above and below nodes. Only values above 0.9 (BPP) and 50 (BS) are displayed. Colour bars represent the sampling localities of *Oithona similis* s.l. with the number of sequences (n) *O. similis* s.l. specimens from the type localities are marked in bold letters.

Figure 4: TCS haplotype networks of COI and 28S for *O. similis* s.l., generated using PopArt. Numbers represent mutational steps that separate haplotypes. Circle size of the haplotypes indicate relative haplotype frequency. Colours represent the sampling locations. The number of total haplotypes differs from Table 3 due to PopArt's exclusion of nucleotide positions with missing and ambiguous data. 27 COI sequences with less than 400 bp and eight 28S sequences with ambuiguities were removed to get a higher resolution of the phylogeography of the discovered lineages.

Figure 5: Vertical distribution of the specimens of the cryptic species PF and ANT at the station of the Polar Front in the Atlantic sector. Red lines show the vertical temperature profiles of the sampled stations (from Strass, 2010, see Supplementary Table for stations).

Figure 6: Relative timing of the diversification of *O. similis* s.l. based on COI with terminal lineage names and 95% HPD node bars. Arctic lineages (ARK) are marked in bold letters.

Figure 1


60˚N
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180˚W
90˚W
0˚
90˚E
180˚E
Ocean Data View
Arctic Ocean
Chukchi Sea
North Atlantic Ocean
Southern Ocean
Beagle Channel
Polarfront
Amundsen Sea
North Pacific Ocean
(from GenBank)
Mediterranean Sea
North Sea