Journal of
Plankton Research

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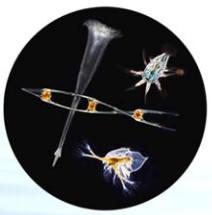

J. Plankton Res. (2017) 39(4): 618–630. First published online July 6, 2017 doi:10.1093/plankt/fbx035

Population genetic structure of *Calanoides natalis* (Copepoda, Calanoida) in the eastern Atlantic Ocean and Benguela upwelling system

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Received March 19, 2017; editorial decision June 1, 2017; accepted June 10, 2010

Corresponding editor: Marja Koski

The population genetic structure of *Calanoides natalis* (ex *Calanoides carinatus*; Copepoda, Calanoida), an ecologically important component of African upwelling systems, was studied in order to (i) search for potential cryptic species, (ii) describe spatial patterns in the distribution of genetic variance and (iii) identify potential barriers to gene flow. Samples were obtained in the eastern Atlantic Ocean from the Iberian Peninsula to Namibia. Analysis of mitochondrial (cytochrome c oxidase subunit I; COI) and nuclear (citrate synthase; CS) marker genes revealed a genetically cohesive population of *C. natalis* with a prevalent shift in allele frequencies. The discovery of a deep split solely present in the mitochondrial dataset does not point to cryptic speciation, but rather suggests the occurrence of nuclear mitochondrial pseudogenes or incomplete reproductive isolation upon secondary contact. Genetic differentiation between the northern and southern hemisphere was significant, which may point to a potential, but permeable barrier close to the equator. No vertical genetic structuring was detected in the northern Benguela implying that horizontal differentiation was more pronounced than vertical structuring. Retention mechanisms and the oxygen minimum zone did not have a strong impact on genetic differentiation of *C. natalis* in the Benguela region.

KEYWORDS: population genetics; zooplankton; ontogenetic vertical migration; cytochrome c oxidase subunit I; citrate synthase

INTRODUCTION

Most of our knowledge on species diversity originates from traditional identification techniques based on species-specific differentiation of morphology. However, recent discoveries of cryptic and pseudo-cryptic species complexes in copepods (Goetze, 2003; Goetze and Ohman, 2010; Laakmann *et al.*, 2012; Andrews *et al.*, 2014; Cornils and Held, 2014) clearly demonstrate that genetic diversity does not necessarily lead to distinguishable morphological features. Additionally, DNA barcoding solely relying on the mitochondrial marker genes such as cytochrome c oxidase subunit I (COI) may lead to the misinterpretation of species diversity (Song *et al.*, 2008).

This phylogeographic study investigated the calanoid copepod *Calanoides natalis* Brady, 1914, an ecologically important species of the herbivorous zooplankton in coastal upwelling areas. Until recently, it was considered to be part of *Calanoides carinatus* (Krøyer, 1849) with a geographical distribution originally described for tropical and temperate upwelling systems ranging from the northeast coast off Somalia, around South Africa to the West African coast, as well as the Brazilian and Argentinian coasts (Peterson, 1998). Based on the analysis of the mitochondrial marker COI, Viñas *et al.* (Viñas *et al.*, 2015) found that the distribution of *C. carinatus* sensu stricto is restricted to the Southwest Atlantic Ocean. Bradford-Grieve *et al.* (Bradford-Grieve *et al.*, 2017) re-described the species in the eastern Atlantic and Indian Ocean as *C. natalis* based on an integrated taxonomic approach combining morphological and molecular analysis.

Calanoides natalis is widely distributed in the eastern Atlantic with highest abundances of >3500 ind. m^{-3} during active upwelling on the Namibian shelf, when reproduction takes place and all ontogenetic stages are present (Verheye *et al.*, 2005). In tropical waters of the Angola Current, only sub-adult copepodite stages V (CV) occur at depths below 400 m with 60 to 400 ind. m^{-3}, whereas 200 nm off Northwest Africa (23.7°N 20.2°W to 15.3°N 20.5°W) CV stages are present below 400 m in low abundances from <1 to 3.7 ind. m^{-3}. Peterson (Peterson, 1998) first suggested that different subpopulations of *C. natalis* might exist along the West African coast arising from its ability to maintain itself within upwelling systems. *Calanoides natalis* undergoes ontogenetic vertical migration, which allows survival during non-upwelling periods and thus low food availability (Verheye *et al.*, 1991). In the Benguela system, the northwestward drift of the active surface population in the Ekman layer may be compensated by the southward flow of diapause stages within the poleward undercurrent (Timonin, 1997; Peterson, 1998; Verheye and Ekau, 2005; Auel and Verheye, 2007). Diel or ontogenetic vertical migrations and their linkage to coastal currents are believed to support the retention of planktonic organisms within upwelling systems (Verheye and Field, 1992; Johnson, 2007; Parada *et al.*, 2008; Harkins *et al.*, 2013; Morgan, 2014; Moyano *et al.*, 2014). Phylogeographic studies in other zooplankton species suggest that retention strategies also promote genetic differentiation (Papetti *et al.*, 2005; Nuwer *et al.*, 2008).

It is not clear, if different life-cycle strategies of *C. natalis* along the African coast represent genetic differentiation or phenotypic plasticity. In equatorial West Africa, a strongly seasonal pattern in the occurrence of actively feeding individuals at the surface and deep-living CV diapause stages has been observed (Binet and de Sainte Claire, 1975; Binet, 1977; Houghton and Mensah, 1978; Dessier, 1979). However, in the Benguela upwelling region, CV diapause stages are permanently present between 200 and 1000 m depth (Kosobokova *et al.*, 1988; Timonin *et al.*, 1992; Arashkevich *et al.*, 1996; Verheye *et al.*, 2005).

In the Benguela upwelling system, pronounced differences in CV body mass exist between the active surface population and deeper layers (Auel *et al.*, 2005; Verheye *et al.*, 2005) and vertical migrations may be restricted by the intermediate oxygen minimum layer (Auel and Verheye, 2007). However, it is unknown if these differences are related to different physiological states (Arashkevich *et al.*, 1996) or genetic differentiation.

Besides behavioural aspects, the large-scale oceanography along the West African coast may influence *C. natalis*' population genetic structure. Ocean gyres and the strong Lüderitz upwelling cell have been linked to genetic breaks in the equatorial Atlantic (Norton and Goetze, 2013) and in the Benguela current region (Henriques *et al.*, 2014), respectively. On the other hand, genetic connectivity may be enhanced by poleward flowing undercurrents, which have been associated with the transport of *C. natalis* diapause stages and fish larvae along the West African coast (John *et al.*, 1998, 2000). The advection of diapause stages from other regions may explain the presence of two distinct populations off the shelf of Ghana, differing in their allozyme composition (Sywula *et al.*, 2002).

Although extensive phylogeographic studies have been conducted at the coast of southern Africa (Teske *et al.*, 2011), genetic data of planktonic species with a similar life-cycle strategy as *C. natalis* are essentially lacking. The two phylogenetic studies on *C. natalis* are solely based on the mitochondrial marker gene COI (Viñas *et al.*, 2015; Bradford-Grieve *et al.*, 2017). Primers for the analysis of nuclear marker genes in *C. natalis* can be designed based on known sequences of nuclear-coded genes in closely related species (Bucklin *et al.*, 2003; Goetze, 2003; Unal and Bucklin, 2010; Kozol *et al.*, 2012). The population genetic structure and phylogeography of *C. natalis* in the eastern

Table I: Station data including cruise, region, sampling date, coordinates, sampling depth, number and stage of the analysed individuals of C. natalis

Cruise	Region	Station	Sampling date	Latitude	Longitude	Sampling depth [m]	Individuals analysed	Stage
ANTXXIX/1	Spain	1	01-Nov-2012	37.82°N	12.08°W	500–800	5	CV
ANTXXIX/1	Mauritania	7	07-Nov-2012	18.76°N	20.71°W	600–700	29	CV
ANTXXIX/1	Senegal	8	09-Nov-2012	15.25°N	20.52°W	400–700	28	CV
M103/2	Northern Benguela	80	25-Jan-2014	20.03°S	12.89°E	20–60	32	Female
M103/2	Northern Benguela	88	27-Jan-2014	20.52°S	11.38°E	400–800	29	CV
M103/2	Northern Benguela	121	02-Feb-2014	19.86°S	11.05°E	400–800	29	CV
M103/2	Northern Benguela	135	05-Feb-2014	19.17°S	12.38°E	20–60	33	Female, male
M102	Southern Benguela	2259	19-Dec-2013	32.01°S	16.25°E	0–50	32	CIV, CV, female
M102	Southern Benguela	2265	20-Dec-2013	30.00°S	14.65°E	0–200	10	CIV, CV

Atlantic Ocean is unknown. It remains to be clarified if its life-cycle strategy or environmental barriers have the potential to lead to horizontal or vertical differentiation of populations in this region.

This study aims to identify (i) potential cryptic species within the nominal *C. natalis*, (ii) spatial patterns in the distribution of genetic variance and (iii) potential barriers that may have shaped the genetic structure of the species in the eastern Atlantic Ocean. We analyse a comprehensive dataset of mitochondrial COI and the nuclear marker gene citrate synthase (CS). For horizontal investigation, we compare three regions along the West coast of Africa: Northeast Atlantic (NEA), northern Benguela (NB) and southern Benguela (SB), whereas vertical population structure is analysed in NB.

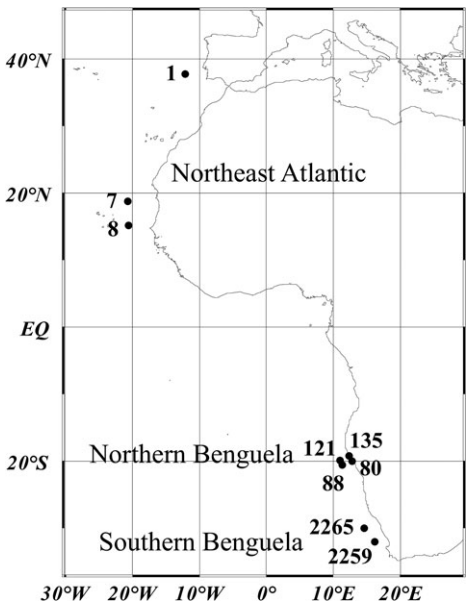

Fig. 1. Station map.

METHOD

Study area and sampling

Specimens of *C. natalis* were collected from depth-stratified net samples from three cruises in the eastern Atlantic between 40°N and 30°S (Table I, Fig. 1). During Polarstern cruise ANTXXIX/1 (October/November 2012), sampling was carried out with a HydroBios Multinet Maxi (0.5 m^2 mouth opening, nine separate nets, 150 µm size). A HydroBios MultiNet Midi (0.25 m^2 mouth opening, five separate nets, 200 µm mesh size) was deployed during RV Meteor cruises M102 (December 2013) and M103/2 (January/February 2014). Immediately after sampling, individuals of *C. natalis* were transferred to Eppendorf tubes and frozen at −80°C. The remaining net sample was preserved in absolute un-denatured ethanol (except for M102, preservation in absolute ethanol only).

Calanoides natalis were found in different depth strata (Table I). Individuals from the 0–200 m layer were sampled at stations 80, 135, 2259 and 2265, mostly adult females and a few developmental stages from stations 2259 and 2265 (Fig. 1). Four males from the surface at station (St.) 135 in the northern Benguela were analysed. Diapausing CV were sampled in deeper layers at stations 1, 7, 8, 88 and 121 (400–800 m depth).

Two stations per region were chosen with 30 individuals each, if available (Table I). Only 5 individuals were available from the northernmost region off Spain. In total 227 individuals were analysed taken at overall 9 stations. For the analysis of regional differences, three regions were investigated independently of depth: NEA (St. 1,7,8), NB (St. 80, 88, 121, 135) and SB (St. 2259, 2265). Vertical population structure was examined in NB comparing two shelf stations (St. 80, 135) and two deep stations (St. 88, 121).

DNA extraction, primer design, amplification and sequencing

Genomic DNA from the deep frozen individuals was extracted from whole specimens, except for the individuals

from ANTXXIX/1. To avoid amplification of potential misidentifications, those copepods were cut in halves with a scalpel (cleaned with 70% ethanol and flamed). The forepart was incubated in buffer ATL (Tissue lysis buffer) and proteinase K (Qiagen, Hilden, Germany) until correct identification, and then used for DNA extraction. The posterior part containing the last swimming leg was transferred to absolute ethanol and identified under a stereomicroscope. Individuals preserved in ethanol were rinsed in distilled water to remove ethanol from the specimens before extraction. DNA extraction was carried out with QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) following standard protocols with minor modifications: the individuals were incubated in Buffer ATL and proteinase K for at least 2 h at 56°C and 500 rpm in a Thermomixer comfort (Eppendorf, Wesseling-Berzdorf, Germany). The incubation step after addition of Buffer AL (Lysis buffer) was omitted. After addition of 100 µL elution buffer, the QIAamp Mini spin column was incubated for 20 min at room temperature.

In an attempt to find potential nuclear marker genes for *C. natalis*, we designed primers for the following protein-coding genes: CS, sodium-potassium ATPase α-subunit (NaK), AMP-activated protein kinase (AMPK) and phosphoenolpyruvate carboxykinase (PEPCK). However, we were only able to obtain qualitatively good sequences from CS. Therefore, we focussed on the nuclear marker gene CS for this study.

The CS primers for *C. natalis* were modified from Kozol *et al.* (Kozol *et al.*, 2012) (Table II) and are based on an alignment of available CS sequences in GenBank of the closely related copepods *Calanus finmarchicus* (Crustacea, EL585846), *Calanus sinicus* (Crustacea, JF430060.1) and *Calanus agulhensis* (Crustacea, JF430038.1), and further arthropods: *Anopheles gambiae* (Insecta, XM320478.4), *Mayetiola destructor* (Insecta, KF647629.1), *Ditylenchus destructor* (Nematoda, JN216829.1), *Artemia franciscana* (Crustacea, BQ563145.1), *Lepeophtheirus salmonis* (Crustacea, BT121320.1) and *Bombyx mori* (Insecta, AK385859.1). Alignment was carried out with the program Mesquite Version 2.75 (Maddison and Maddison, 2011, http://mesquiteproject.org) according to the translated protein sequences. Primers were designed with Geneious® version 6.1.4. (Biomatters Ltd., Auckland, New Zealand, www.geneious.com). For the amplification of the COI fragment, the primers LCO1490 (Folmer *et al.*, 1994) and C1-N-2191 (alias Nancy) (Simon *et al.*, 1994) were used (Table II).

PCR amplifications of the marker genes COI and CS were performed in a total reaction volume of 25 µL, with PCR mastermix including 5 µL 5× Colorless GoTaq® Flexi Buffer (Promega, Mannheim, Germany), 2.5 mM Magnesium Chloride Solution (Promega), 0.4 µM primer each, 0.2 mM dNTPs (Carl Roth, Karlsruhe, Germany), 0.65 U GoTaq® G2 Flexi DNA polymerase (Promega) and 2 µL of DNA sample. PCR was carried out in an Eppendorf Masterthermocycler gradient with heated lid (Eppendorf, Hamburg, Germany) with the following temperature profile: initial denaturation (95°C, 5 min), 40 cycles of denaturation (95°C, 30 s), annealing (42°C for COI and 55°C for CS, 1 min), elongation (69°C, 1 min) and final elongation (69°C, 15 min).

The quality of the PCR products was approved via electrophoresis (110–120 V, 30–60 min) on a 2% agarose gel (Agarose MP, AppliChem, Darmstadt, Germany) in TRIS-Borat-EDTA buffer (10× TBE buffer, AppliChem, Darmstadt, Germany) and visualized with the Biovision camera system including darkroom-CN-3000 and the software Vision Capt Version 15.08 (PeqLab, Erlangen, Germany). After quality control, PCR products were sent to Eurofins Genomics (Ebersberg, Germany) for sequencing. Eurofins Genomics purified the PCR products with the Agencourt AMPure XP system (Beckman Coulter, Krefeld, Germany) and carried out a modified version of traditional Sanger sequencing (Sanger *et al.*, 1977) with an ABI 3730XL sequencing machine (Applied Biosystems, Life Technologies GmbH, Darmstadt, Germany).

Editing, alignment and quality control of the sequences

Sequences were aligned and edited with CodonCode Aligner 4.2.7 (CodonCode Corporation, Dedham, Massachusetts, USA, www.codoncode.com). ClustalW (Thompson *et al.*, 1994) was used as alignment algorithm. Sequence control was conducted with the Basic Local Alignment Search Tool (BLAST) provided by the National Center for Biotechnology Information website (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The number of polymorphic sites in the alignment was determined with DnaSP version 5.10.01 (Librado and Rozas, 2009).

Table II: Primer for amplification, sequence in 5′→ 3′ order and references

Primer	Sequence 5′ → 3′	Source
LCO1490	GGTCAACAAATCATAAAGATATTGG	Folmer *et al.* (1994)
C1-N-2191 (Nancy)	CCCGGTAAAATTAAAATATAAACTTC	Simon *et al.* (1994)
CS_ for	GAAGGTATCAGATTCCGTGG	Modified after Kozol *et al.* (2012)
CS-514 R_ mod	CAAGTACAGTCTCATCAGCTC	Modified after Kozol *et al.* (2012)

In CodonCode Aligner, double peaks in the chromatograms of the diploid CS sequences were converted to ambiguity codes in the consensus sequence. Since genotypes with multiple ambiguous bases occurred, the sequences of the alleles were inferred with the PHASE algorithm (Stephens *et al.*, 2001; Stephens and Donnelly, 2003) as implemented in DnaSP. Although the implementation of the algorithm does not allow the exact identification of allele sequences, it enabled us to capture the variation within CS facilitating the comparison to the mitochondrial COI data. Preliminary analysis of pairwise F_{ST} in the program Arlequin version 3.5.1.2 (Excoffier *et al.*, 2007) revealed that genetic differentiation among stations was more significant in the phased CS dataset (compared to unphased data, see Supplementary Fig. S1). Further phylogeographic analysis was therefore carried out with the phased CS sequences.

Phylogeographic analysis

Haplotypes and haplotype frequencies at the different stations were identified with the online service DNA collapser (Villesen, 2007, http://users-birc.au.dk/biopv/php/fabox/dnacollapser.php). The genetic diversity of COI was determined calculating haplotype and nucleotide diversity (Nei, 1987) of the different regions in the program Arlequin. The COI haplotype network was constructed with the program Haplotype Viewer (available from http://www.cibiv.at/~%20greg/haploviewer) based on the Maximum Parsimony Tree analysis in the program MEGA 6 (Tamura *et al.*, 2013). *Calanoides natalis* COI sequences available on GenBank (accession no. KC287437 – KC287448, KC287453 – KC287461, KC287468 – KC287474, KC287477, KC287476, KC287480, KC287482 – KC287484) were compared to the COI data from this study. For this purpose, sequences were aligned with CodonCode Aligner and cut to equal length of 484 bp. A haplotype network of the combined data was constructed.

MEGA 6 was applied to determine the p-distances among sequences giving percentile genetic differences. Further analysis of neutrality and population genetic structure was carried out with the program Arlequin. The neutrality tests Tajima's D (Tajima, 1989) and Fu's FS (Fu, 1997) were used to test the hypothesis of neutral evolution and look for possible demographic effects within the species. Pairwise F_{ST} were calculated to get a first estimate of genetic differentiation among stations. Genetic differences among regions were tested with the analysis of molecular variance (AMOVA, Excoffier *et al.*, 1992). The statistical significance of the AMOVA results was based on 100,172 permutations.

Isolation by distance was analysed with the Isolation By Distance Web Service, version 3.23 (Jensen *et al.*, 2005, http://ibdws.sdsu.edu/ibdws/) using ϕ_{st} for genetic distance between the sequences and 30 000 randomizations. Geographic distances were obtained with the Geographic Distance Matrix Generator version 1.2.3 (Ersts, 2012).

For determination of the optimal nucleotide substitution model for our sequences, the sequence alignment was tested with the program jModelTest version 2.1.6 (Darriba *et al.*, 2012). Based on the Bayesian information criterion (BIC), the closest nucleotide substitution model that was implemented in the program MrBayes version 3.2.2 (Ronquist *et al.*, 2012) was chosen to build the phylogenetic tree. While the nucleotide substitution model Hasegawa-Kishino-Yano (Hasegawa *et al.*, 1985) with proportion of invariable sites (HKY+I) was used for the COI sequences, the general time reversible model (Tavaré, 1986) with proportion of invariable sites and gamma distributed rate variation among sites (GTR+I+G) was chosen for the CS sequences. Bayesian inference (Markov Chain Monte Carlo method) was performed on two independent runs of 10,000,000 generations, each with four chains (1 cold, 3 hot) and a sample frequency of 1000 generations. The analysis was run until the two runs converged and the standard deviation of the split frequencies approached zero (<0.01) reaching a stationary distribution. The first 25% of the trees were discarded as burn-in fraction. The output quality of the results was checked with the software Tracer v1.6 (Rambaut *et al.*, 2014).

RESULTS

Mitochondrial COI

In total 227 mitochondrial COI sequences were obtained with a length of 676 bp. The alignment contained 573 invariable positions and 103 polymorphic sites. Most of the mutations were silent mutations and therefore, 220 sequences coded for an identical amino acid composition. Compared to the consensus sequence, 7 sequences differed in their translated protein sequence, with partly different chemical properties of the variant amino acids. Four of these sequences had only one different amino acid. Interestingly, the other three sequences were more divergent and revealed a consistent protein sequence with three different amino acids.

Haplotype analysis revealed 40 haplotypes of which two were found over the whole study area and six were shared between NB and SB (Fig. 2). The other haplotypes were restricted to one of the three regions. The haplotype network revealed a deep split within *C. natalis* with three highly divergent individuals (corresponding to divergent translated protein sequence, see above),

66–69 mutational steps away from the prevalent population. Both molecular operational taxonomic units (MOTUs 1 and 2, as defined in Fig. 2) differed between 9.5 and 10.4% in their COI sequences. Variation within the prevalent MOTU 2 ranged from 0.1 to 1.3%, whereas it was higher in MOTU 1 with 0.7 to 5.6% difference. Interestingly, both MOTUs occurred in the whole study area and did not show spatial separation.

For further analysis of regional differences, the three highly divergent sequences were removed from the dataset focussing on the prevalent MOTU. Haplotype diversity was higher in the southern hemisphere (H_d = 0.82–0.83) than in the northern hemisphere (H_d = 0.69). Similar nucleotide diversities (π = 0.0047 – 0.0054) were found over the whole study area. Haplotype and nucleotide diversity of the surface and deep population in NB were similar to each other (both H_d = 0.82, π = 0.0053).

The neutrality tests Tajima's D and Fu's FS did not reveal any significant departure from neutrality (non-significant values for all stations, $P > 0.16$, for detailed results see Supplementary Table SI). This confirmed the theory of neutral evolution of our sequences, which was a necessary assumption for the following population genetic tests.

Higher F_{ST} values and thereby higher genetic differentiation between stations were observed among stations that were more distant (St. 7 and 8, Senegal/Mauritania, compared to stations from the southern hemisphere, St. 80 – 2265; Fig. 3). The lower F_{ST} values of St. 1 (Spain) compared to the southern hemisphere may be explained by the low sampling effort at this station (Table I). Interestingly, the NB surface stations (St. 80 and 135) seemed to be less differentiated to the northern hemisphere than the NB deep stations (St. 88 and 121). F_{ST} between St. 2259 (SB) and St. 88 (deep NB) was moderate and significant. Geographically close stations had low to non-significant F_{ST} values and therefore less genetic differentiation.

Hierarchical AMOVA confirmed genetic differentiation within the whole study area with a significant global F_{ST} value (Table III). Genetic variance was apparent within sampling stations indicated by significant F_{ST} values in all tests. Genetic differentiation among stations within sampling regions was observed, when testing the northern (NEA) against the southern hemisphere (NB + SB), NEA against NB, and combined NEA and NB against SB (see Table III, significant F_{SC} values). For horizontal analysis of genetic differentiation amongst regions (see Table III, F_{CT} values), highly significant differences between the northern and southern hemisphere as well as among the three regions were found. Moreover, NEA significantly differed from NB. No significant differences were found testing the combined regions NEA and NB against SB and the other regions separately (NEA ↔ SB, NB ↔ SB). Analysing vertical population structure in NB, the surface population and deep-living diapause stages did not significantly differ from each other, which did not support our hypothesis of vertical segregation of *C. natalis* populations in the NB.

Linear regression analysis of genetic distance (φ_{st}) and geographic distance among all stations resulted in a relatively low regression (r = 0.45). However, the Mantel test confirmed a significant correlation of genetic and geographic distance among the stations (Z = 20004.58, r = 0.45, P = 0.02). In general, geographically closer populations tended to have smaller genetic distances. More distant populations had a wide range of genetic distances, which consequently influenced the regression analysis. When analysing isolation by distance solely in the southern hemisphere stations, no correlation between genetic and geographic distance was found (Z = 552.90, r = 0.19, P = 0.33).

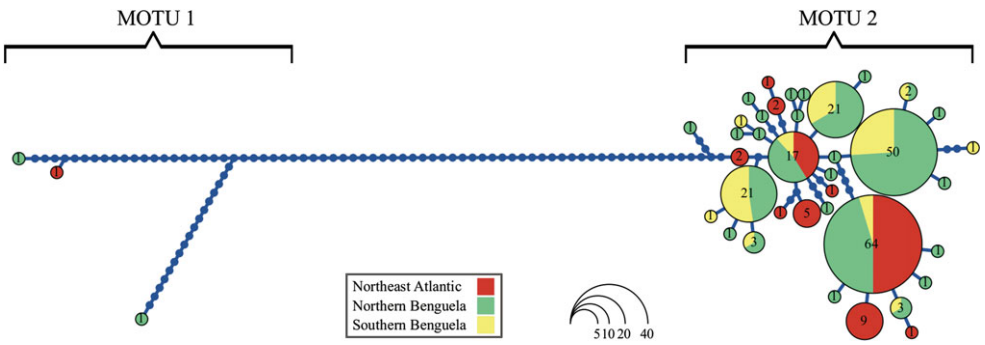

Fig. 2. Haplotype network of COI data. Circles present the haplotypes, which are connected by mutational steps (points). The size of the circle corresponds to the number of individuals that share the same haplotype. The pie charts represent the distribution of a certain haplotype within the different regions. Brackets define MOTU 1 and 2.

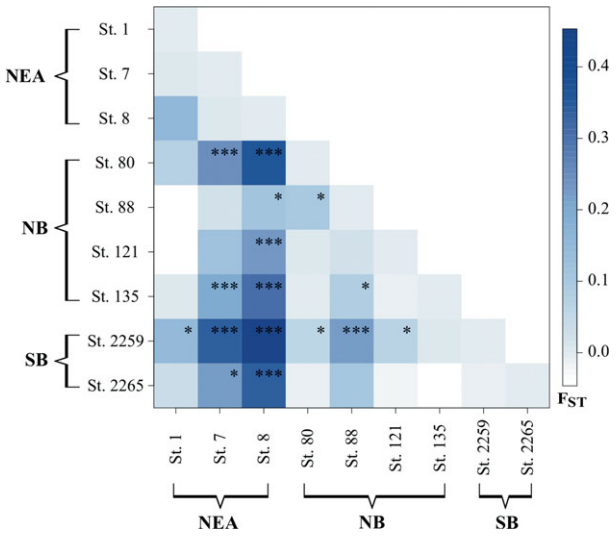

Fig. 3. Matrix of station pairwise F_{ST} based on mtCOI sequences. Significant results are indicated with *($P < 0.05$) and ***($P < 0.001$). Brackets specify the corresponding regions.

Table III: Hierarchical AMOVA results for COI with fixation indices indicating genetic differentiation within stations (F_{ST}), among stations within regions (F_{SC}) and among regions (F_{CT})

Grouping	F_{ST}	F_{SC}	F_{CT}
Global	**0.15*** **		
(NEA) ⇔ (NB + SB)	**0.25*** **	**0.04****	**0.21***
(NEA) ⇔ (NB) ⇔ (SB)	**0.19*** **	0.03	**0.17****
(NEA) ⇔ (NB)	**0.20*** **	**0.03***	**0.18***
(NEA) ⇔ (SB)	**0.36*** **	0.02	0.34
(NB) ⇔ (SB)	**0.07****	0.03	0.04
(NEA + NB) ⇔ (SB)	**0.19*** **	**0.12*** **	0.08
(surface NB) ⇔ (deep NB)	**0.04***	0.01	0.04

Significant results in bold, significance level *($P < 0.05$), **($P < 0.01$) and ***($P < 0.001$).

Nuclear CS

After alignment and quality control, 162 CS sequences with a length of 328 bp were obtained for further analysis. Double peaks of bases were detected in chromatograms of several sequences indicating heterozygous DNA with two different alleles per individual. In the alignment, 236 invariable sites and 92 polymorphic sites were detected. Phasing the diploid sequences into alleles resulted in 324 sequences. Amino acid composition of the sequences was identical, except for 15 sequences, which differed in one amino acid from the consensus sequence.

The 324 phased sequences were collapsed into 153 unique haplotypes for further analysis. Three haplotypes were found in all study regions. Additionally, five haplotypes were shared between NEA and NB and eleven shared haplotypes were found between NB and SB. Sequence differences ranged from 0.3 to 6.1%.

Tajima's D and Fu's FS test for neutral evolution of the marker gene CS disagreed in their results (for detailed results, see Supplementary Table SII). For Tajima's D, no significant departure from neutrality was detected for all stations. On the other hand, Fu's FS revealed significant results for most of the stations ($P < 0.01$), apart from St. 2259 and 2265 with non-significant results.

Pairwise F_{ST} among stations based on the nuclear CS sequences revealed significant differentiation of St. 7 and 8 (region: Senegal/Mauritania) to the most distant SB stations (St. 2259 and 2265) (Fig. 4). Moreover, St. 7 and 8 were moderately differentiated from NB. In general, geographically close stations seemed to be more similar to each other. Remarkably, St. 1 (Spain) was more similar to the southern hemisphere, but showed high genetic differentiation to St. 7 and 8 from the northern hemisphere.

AMOVA analysis of the CS marker revealed a significant global F_{ST} value (Table IV), which indicates genetic differentiation in the whole dataset. Genetic differences within stations were found in all AMOVA tests, except the ones only testing in the Benguela region (see F_{ST} values Table IV). Genetic differentiation among stations within sampling regions (F_{SC}) was proven for AMOVA tests between northern and southern hemisphere, NEA and NB, and combined NEA and NB against SB. Significant differences between regions (F_{CT}) were found between the northern and the southern hemisphere and among the three regions (for detailed results see Table IV). Separate testing for differences between two regions did not show any significant results. The combined regions NEA and NB tested against SB did not significantly differ from each other. Moreover, no vertical genetic differentiation was evident between the surface and deep-living individuals in the NB.

Linear regression analysis of genetic distance (φ_{st}) based on CS sequences and geographic distance among stations revealed a similar pattern as COI, with relatively low, but significant correlation ($\chi = 3435.38$, $r = 0.44$, $P = 0.03$) confirmed by the Mantel test. Isolation by distance analysis of stations from the southern hemisphere (excluding the northern hemisphere) did not reveal significant correlation of genetic and geographic distance in the Benguela region ($\chi = 120.43$, $r = 0.32$, $P = 0.08$).

Phylogenetic tree analysis of both marker genes

The phylogenetic tree of COI haplotypes showed clear differentiation of the highly diverse sequences from the prevalent MOTU. The branch had 100% support and a remarkably long genetic distance to the other sequences

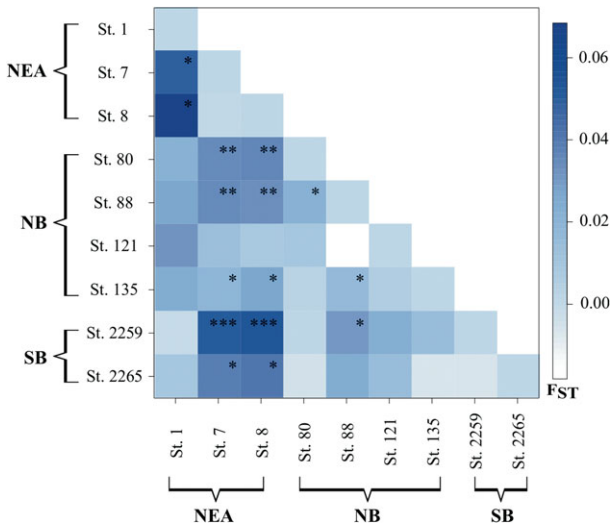

Fig. 4. Matrix of station pairwise F_{ST} based on phased CS sequences. Significant results are indicated with *($P < 0.05$), **($P < 0.01$) and ***($P < 0.001$). Brackets specify the corresponding regions.

Table IV: Hierarchical AMOVA results for phased CS with fixation indices indicating genetic differentiation within stations (F_{ST}), among stations within regions (F_{SC}) and among regions (F_{CT})

Grouping	F_{ST}	F_{SC}	F_{CT}
Global	**0.02*** **		
(NEA) ⇔ (NB + SB)	**0.03*** **	**0.01***	**0.02***
(NEA) ⇔ (NB) ⇔ (SB)	**0.02*** **	0.01	**0.02***
(NEA) ⇔ (NB)	**0.02*** **	**0.01***	**0.01***
(NEA) ⇔ (SB)	**0.04*** **	0.01	0.04
(NB) ⇔ (SB)	0.01	<0.01	0.01
(NEA + NB) ⇔ (SB)	**0.03*** **	**0.02****	0.01
(surface NB) ⇔ (deep NB)	0.01	−0.01	0.02

Significant results in bold, significance level *($P < 0.05$), **($P < 0.01$) and ***($P < 0.001$).

as it has already been shown in the haplotype network (see Supplementary Fig. S2). In contrast, this distinct cluster was not observed, when analysing the phylogeny of the CS haplotypes, which revealed high similarities of CS sequences within the whole dataset. The corresponding CS haplotypes of the three individuals with highly divergent COI sequences were distributed within several branches and clustered together with other sequences with up to 100% support (see Supplementary Fig. S3).

DISCUSSION

Recently, it has been shown that the distribution of *C. carinatus* s.s. is restricted to the Southwest Atlantic Ocean (Viñas *et al.*, 2015) and its sibling species in the eastern Atlantic has been re-described as *C. natalis* (Bradford-Grieve *et al.*, 2017). In the present study, we investigate the population genetic structure and phylogeography of *C. natalis* in the eastern Atlantic Ocean by using enhanced sampling effort and the combined analysis of mitochondrial COI and nuclear CS. Our results show that horizontal genetic differentiation in this species seems to be more pronounced than vertical structuring. Moreover, the observed differentiation among the hemispheres suggests a potential, but permeable barrier to gene flow close to the equator.

Interestingly, a few highly divergent sequences of mitochondrial COI are found in both hemispheres in the eastern Atlantic Ocean, without being spatially separated from the prevalent MOTU. A similar observation is made by Viñas *et al.* (Viñas *et al.*, 2015) who described a single genetically cohesive population of *C. natalis* in the NE/SE Atlantic, but also found a few highly diverse sequences off the Spanish coast. We constructed a combined COI haplotype network, which reveals a perfect match of both datasets (see Supplementary Fig. S4). Even though, the magnitude of genetic distance between the aberrant sequences and predominant MOTU is consistent with the species threshold of 8–24.9% differentiation found in calanoid copepods (Bucklin *et al.*, 1999; Hill *et al.*, 2001; Bucklin and Frost, 2009), it is highly unlikely that we are actually looking at a cryptic species of *C. natalis*. The pattern of two distinct groups disappears when analysing the nuclear marker CS and, instead, reveals high genetic similarity within the whole dataset.

A possible explanation may be the presence of multiple variants of the COI gene within the mitochondrial DNA of *C. natalis*. Typically, identical copies of mtDNA sequences in animals (known as homoplasmy) (Birky, 2001) serve as a crucial assumption in population genetic studies based on mtDNA, even though mitochondrial heteroplasmy and recombination may be more common than previously thought (Rokas *et al.*, 2003; Barr *et al.*, 2005). One mechanism leading to heteroplasmy, doubly uniparental inheritance has been found in many bivalves (Saavedra *et al.*, 1997; Breton *et al.*, 2007; Theologidis *et al.*, 2008; Dégletagne *et al.*, 2016) where genetically distinct male and female mitochondrial lineages exist. However, a strict separation of gender-specific lineages does not seem to be common in *C. natalis*, as males were found to have similar haplotypes as females in this study.

This finding may rather display effects of marker evolution within the mitochondrial genome. COI-like sequences have often been reported as a problematic feature in molecular genetics (Buhay, 2009) as unnoticed

co-amplification of highly divergent paralogous copies can lead to misinterpretation of species diversity (Song *et al.*, 2008). They have been found in a wide range of crustacean taxa including calanoid copepods (Bucklin *et al.*, 1999, 2000; Machida and Lin, 2017) and diaptomid calanoids (Thum and Harrison, 2009; Marrone *et al.*, 2013). The aberrant sequences found in this study do not contain any stop codons or frame shifts, and therefore do not resemble classical nuclear mitochondrial pseudogenes (so called numts) that often contain InDels (Insertion/Deletions) and mutations at any codon position (Song *et al.*, 2008; Buhay, 2009; Calvignac *et al.*, 2011). Still, the pronounced nucleotide diversity of the sequences may indicate a recent translocation of a COI copy to the nucleus. Machida and Lin (Machida and Lin, 2017) made a similar observation of functional variants of COI sequences in single individuals of *Neocalanus plumchrus* indicating the amplification of nuclear mitochondrial pseudogenes. The recombination of pseudogenes and potential hybridization events with other copepod species (Machida and Lin, 2017) may have the potential to further enlarge variability of COI in *C. natalis*.

The highly diverse sequences may also point to a past event of genetic differentiation in *C. natalis*, yet with incomplete reproductive isolation due to secondary contact. The resulting genetic differentiation might have eroded away through hybridization among the three groups in the nuclear genome but might have persisted in the mitochondrial genome due to the absence of recombination. This underlines the necessity to analyse a combination of mitochondrial and nuclear markers in *C. natalis* to be able to interpret its phylogeography.

For further analysis of the population genetic structure of *C. natalis*, we decided to exclude the aberrant COI sequences, to make both the mitochondrial and the nuclear dataset comparable. The analysis of both datasets reveals highly consistent results, which strongly suggests that we are indeed looking at the true evolutionary history of *C. natalis* in the eastern Atlantic Ocean. This is also supported by the neutrality tests on COI and partly on CS, which do not point to selective pressures acting on the marker genes. The excess number of alleles of CS, partly indicated by Fu's FS neutrality test, may rather suggest a recent population expansion in these regions. The haplotype diversity of CS may also be overestimated by the haplotype inference algorithm used in this study, which may lead to uncertainties in haplotype sequences. The sample size per region was more than adequate for phylogeographic analysis, as more than 30 sampled individuals are considered to display natural haplotype frequencies with 95% probability in diploid populations (Fung and Keenan, 2014). Still, greater sampling efforts within some stations may have favoured the sampling of singleton haplotypes, which influences haplotype diversity and further statistical tests. Moreover, different evolutionary rates and possible resolution limits of our marker genes may have influenced the results. As our study did not resolve vertical genetic differentiation in the northern Benguela, we assume temporal stable populations of *C. natalis*, independent of sampling depth and time, for the interpretation of its horizontal population structure in the eastern Atlantic Ocean.

We suggest, that the population genetic structure of *C. natalis* may be driven by past and present oceanography along the West coast of Africa, as dispersal capacity and oceanographic barriers have been described to play a central role for genetic differentiation in copepods (Goetze, 2011; Norton and Goetze, 2013). Our observation of a genetically cohesive population of *C. natalis* in the eastern Atlantic Ocean confirms the findings by Viñas *et al.* (Viñas *et al.*, 2015). Bradford-Grieve *et al.* (Bradford-Grieve *et al.*, 2017) conclude that the species integrity of *C. natalis* is maintained by the population in the Indian Ocean due to the leakage of water into the Atlantic Ocean in the Agulhas Retroflection.

In this study, we discovered genetic differentiation within *C. natalis* between the northern and southern hemisphere. Genetic breaks across the equatorial Atlantic have already been found in other copepod species and they are mostly explained by the adherence of populations within Atlantic gyres (Norton and Goetze, 2013). However, *C. natalis* features a completely different life-cycle strategy as an upwelling specialist, which is highly dependent on coastal upwelling blooms and performs ontogenetic vertical migration to survive non-upwelling periods. Therefore, its dispersal capacity is likely restricted to near-shore surface and deep counter-currents (see compiled view of ocean currents along the West African coast, by John *et al.* (John *et al.*, 2000)). Surface transport may be facilitated by large-scale oceanographic features e.g. the Canary and Benguela Currents (Houghton, 1976; Stramma, 2001) or upwelling-associated currents such as offshore Ekman transport and equatorward flowing coastal jets over the shelf (Hagen, 2001). On the other hand, gene flow between different regions may be strongly influenced by poleward undercurrents, which have already been related to the transport of planktonic organisms and larvae (Timonin, 1997; John *et al.*, 1998, 2000; Peterson, 1998; Verheye and Ekau, 2005; Auel and Verheye, 2007; Harkins *et al.*, 2013). This is underlined by the fact that *C. natalis* resting stages may be transported by undercurrents over long distances due to their extremely reduced metabolism (Auel *et al.*, 2005), high body mass and lipid content (Kosobokova *et al.*, 1988; Arashkevich *et al.*, 1996; Arashkevich and Drits, 1997; Verheye *et al.*, 2005). However, the current system along

the West African coast has mainly the potential to enhance gene flow within hemispheres. Major cross-equatorial currents are missing in the eastern Atlantic Ocean and water exchange may just be accomplished through thermocline convergence, equatorial upwelling and Ekman divergence (Stramma *et al.*, 2003). This strongly supports our suggestion of a potential, but permeable barrier close to the equator, which leads to some extent to genetic differentiation between hemispheres in *C. natalis*.

Our findings reveal that habitat affinity does not seem to have a strong impact on genetic structuring of *C. natalis*. The observed genetic connectivity rather shows that its retention strategy (Peterson, 1998) does not have the potential to actually restrict gene flow between local upwelling systems. These findings agree with phylogeographic studies of other coastal zooplankton species that have only shown strong genetic differentiation across ocean basins (Nuwer *et al.*, 2008; Harkins *et al.*, 2013). Our analysis of the vertical population structure of *C. natalis* in the northern Benguela suggests that the active surface individuals and deep-living resting stages seem to belong to the same population. The CV body mass differences observed by Auel *et al.* (Auel *et al.*, 2005) and Verheye *et al.* (Verheye *et al.*, 2005) may rather display an adaptation to different life-cycle stages within the same genetic background. The oxygen minimum zone may indeed restrict vertical migrations as proposed by Auel and Verheye (Auel and Verheye, 2007), but does not seem to act as a strong barrier to gene flow. The active surface population may effectively originate from a random reseeding event, as it has already been proposed for the ontogenetically migrating copepod *Calanus pacificus* (Johnson, 2007). This may be especially common in the Benguela region where a certain flexibility of migration behaviour (Timonin, 1997) and permanent stock of diapause stages (Verheye *et al.*, 2005) have been reported.

CONCLUSIONS

This study gives first insight into the population structure and phylogeography of *C. natalis* in the eastern Atlantic Ocean and the potential impact of behavioural aspects on genetic differentiation in an ontogenetically migrating copepod and upwelling specialist. The combined analysis of mitochondrial and nuclear marker genes revealed a genetically cohesive population in the eastern Atlantic Ocean with some differentiation between the northern and southern hemisphere suggesting active gene flow between upwelling systems and a not particularly effective barrier close to the equator. Gene flow may be influenced by the large-scale ocean-circulation along the West African coast, which promotes conveyance by surface and undercurrents within hemispheres, but hinders cross-equatorial transport. The transport of long-living diapause stages in poleward undercurrents may be especially effective in enhancing genetic connectivity within hemispheres. Retention mechanisms of *C. natalis* and the oxygen minimum zone in the northern Benguela do not seem to have a strong impact on genetic differentiation. A highly diverse signal in the mitochondrial marker COI may either point to the presence of nuclear mitochondrial pseudogenes or a past event of genetic differentiation that has not persisted in the nuclear marker gene. This underlines the importance of studying both mitochondrial and nuclear markers to avoid an overestimation of species diversity.

SUPPLEMENTARY DATA

Supplementary data is available at *Journal of Plankton Research* online.

FUNDING

This project was supported by the German Federal Ministry for Education and Research (03F0650E) BMBF as part of the GENUS project (Geochemistry and Ecology of the Namibian Upwelling System).

ACKNOWLEDGEMENTS

We thank the crew of RV Meteor, Dr. Anna Schukat and Dr. Anneke Denda, for their help during the sampling on the cruise M103/2. Sincere thanks goes to Andrea Eschbach for technical support in the laboratory. The authors would like to thank Ryuji Machida and one anonymous reviewer for their valuable comments on the manuscript.

DATA ARCHIVING

The DNA sequences of *C. natalis* found in this study were archived in GenBank under the accession numbers KY700868-KY701094 (COI) and KY701095-KY701256 (CS).

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