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AWI

Master Thesis

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

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Abstract

Molecular markers have a high potential to resolve genetic differentiation within species and populations in the pelagic realm where barriers to gene flow cannot be easily identified. In the present study *Calanoides carinatus* (Copepoda, Calanoida), an ecologically important component of African upwelling systems, was investigated aiming to (1) describe spatial patterns in the distribution of genetic variance, (2) identify potential barriers that may have shaped the genetic structure of the species and (3) look for cryptic species. Samples were obtained in the eastern Atlantic Ocean from the Iberian Peninsula to Namibia. Analysis of mitochondrial (cytochrome oxidase c subunit I; COI) and nuclear (citrate synthase; CS) marker genes revealed a genetically-cohesive population of *C. carinatus* along the west coast of Africa. Haplotype sharing of *C. carinatus* populations, relatively few private alleles and a prevalent shift in allele frequencies indicated ongoing gene flow within the study area. Significant genetic differentiation of *C. carinatus* populations was discovered between the northern and southern hemisphere which may point to a potential, but permeable barrier close to the equator, possibly triggered by discontinuity of ocean currents in the equatorial region. No vertical genetic structuring in *C. carinatus* was observed among the active surface population and deep-living resting stages in the northern Benguela indicating that horizontal differentiation was more pronounced than vertical structuring of populations. The occurrence of three diverse sequences of mitochondrial COI and their absence within nuclear CS, rather suggests marker evolution of COI than a cryptic species.

Zusammenfassung

Molekulare Marker haben ein hohes Potenzial genetische Differenzierung innerhalb von Arten und Populationen im pelagischen Lebensraum zu erkennen, wo auf den Genfluss wirkende Barrieren nicht leicht bestimmbar sind. Diese Forschungsarbeit beschäftigt sich mit *Calanoides carinatus* (Copepoda, Calanoida), einer ökologisch wichtigen Art afrikanischer Auftriebssysteme, und hat das Ziel (1) räumliche Muster in der Verteilung der genetischen Varianz zu beschreiben, (2) potenzielle Barrieren zu identifizieren, die möglicherweise die genetische Struktur der Art geformt haben und (3) nach kryptischen Arten Ausschau zu halten. Proben wurden im östlichen Atlantik genommen, von der Iberischen Halbinsel bis nach Namibia. Die Analyse des mitochondrialen (Cytochrom-Oxidase c Untereinheit I; COI) und des nuklearen (Cytratsynthase; CS) Markergens zeigte eine genetisch zusammenhängende Population von *C. carinatus* entlang der gesamten Westküste von Afrika. Gemeinsame Haplotypen der *C. carinatus* Populationen, relativ wenige private Allele und eine vorwiegende Verschiebung von Allelfrequenzen weisen auf anhaltenden Genfluss innerhalb des Untersuchungsgebiets hin. Signifikante genetische Unterschiede wurden zwischen der nördlichen und der südlichen Hemisphäre entdeckt, was auf eine potenzielle, jedoch durchlässige, Barriere in der Nähe des Äquators hindeutet, möglicherweise ausgelöst durch die Diskontinuität von Meeresströmungen in diesem Bereich. Vertikale genetische Unterschiede zwischen der aktiven Oberflächenpopulation und den tiefen Ruhestadien im nördlichen Benguela wurden nicht nachgewiesen. Dies lässt vermuten, dass die horizontale genetische Differenzierung generell ausgeprägter ist als die vertikale. Das Auftreten von drei verschiedenartigen COI Sequenzen/Individuen und deren fehlende Variabilität im nuklearen CS-Gen, weist eher auf die Evolution des COI-Markers hin als auf eine kryptische Art.

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List of Abbreviations

COI cytochrome oxidase c subunit I

CS citrate synthase

EUC Equatorial Undercurrent

InDel Insertion/Deletion

MOTU molecular operational taxonomic unit

mtDNA mitochondrial DNA

NB Northern Benguela

NW northwest

NWA Northwest Africa

PCR polymerase chain reaction

SACW South Atlantic central water

SB Southern Benguela

SEC South Equatorial Current

SNPs single nucleotide polymorphisms

1 Introduction

1.1 Processes controlling genetic structuring in the pelagic

Genetic diversity of holoplanktonic organisms such as copepods has been largely underestimated in the past owing to their high dispersal capacities, large population sizes, wide geographic ranges and high tolerance to variable environmental factors (Goetze, 2003). Pelagic species may become geographically segregated (allopatric speciation) by oceanographic barriers, isolation by distance, selection due to environmental adaptations or recent historical events (Palumbi, 1994). Historical events include for instance glaciation events or bottleneck effects followed by a sudden population expansion and establishment of unique haplotypes (Nei et al., 1975; Nuwer et al., 2008; Paiz-Medina and Kochzius, 2013). The bottleneck effect describes the sudden reduction of population size followed by a decrease in heterozygosity and number of alleles which can lead to the formation of a new population out of a few individuals (Nei et al., 1975). Morever, ecology and habitat affinity of populations are probably the main drivers for the speciation of the diverse marine calanoid copepod fauna besides environmental barriers such as continental landmasses and the large-scale ocean circulation (Goetze, 2005). Assortative mating can lead to sympatric and microallopatric speciation (Palumbi, 1992; Dieckmann and Doebeli, 1999; Doebeli et al., 2000) and genetic variability might occur, even though morphological differences are absent leading to the formation of cryptic and pseudo-cryptic species complexes (e.g. Goetze, 2003; Goetze and Ohman, 2010; Laakmann et al., 2012). Furthermore, evolutionary processes are not only driven by selective pressures but also influenced by genetic drift, random changes of gene frequencies in populations (Lande, 1976). However, selection may also play an important role for evolution in marine zooplankton, since their large population size may facilitate the appearance of beneficial mutations and lead to fast evolutionary responses to changes in their environment (Peijnenburg and Goetze, 2013).

1.2 Molecular markers in population genetics and their constraints

Phylogenetic studies in calanoid copepods are mainly based on cytochrome oxidase c subunit I (COI) and 16s ribosomal RNA sequences (e.g. Bucklin et al., 2003; Goetze, 2005; Nuwer et al., 2008; Goetze and Ohman, 2010; Yebra et al., 2011; Blanco-Bercial et al., 2011).

In animals, mitochondria are generally considered to be maternally inherited with scarce recombination events, each organism possessing many identical copies of haploid mitochondrial DNA (mtDNA) (known as homoplasmy) (Birky, 2001). Advantages of mitochondrial markers include sufficient mutation rates for phylogeographic studies and easy amplification with universal primers in many species. However, solely mitochondrial datasets could mask phenomena such as hybridization, asymmetrical mating and migration preferences (Sunnucks, 2000; Teske et al., 2011). Moreover, different evolutionary processes could alter mtDNA sequences and lead to misinterpretation of species diversity (Song et al., 2008). These processes include migration of mitochondrial genes to the nucleus (Song et al., 2008; Buhay, 2009; Calvignac et al., 2011) and the occurrence of multiple differing copies of mtDNA within the mitochondria (so called heteroplasmy) (Barr et al., 2005; Rokas et al., 2003). Moreover, direct and indirect selection acting on mitochondrial marker genes could alter haplotype frequencies within the population and therefore not display the true demographic history of the species (Ballard and Whitlock, 2004). Therefore, additional nuclear markers should be chosen as independent data and compared to the mitochondrial dataset. In copepod studies, the nuclear marker internal transcribed spacer 2 (ITS2) and nuclear coded ribosomal genes such as 18S ribosomal RNA were often investigated in combination with mtDNA (e.g. Bucklin et al., 2003; Goetze, 2003). Nuclear protein-coding genes were also used in crustaceans including phosphoenopyruvate carboxykinase (PEPCK), sodium potassium ATPase α-subunit (NaK) (Tsang et al., 2008; Santamaria et al., 2013) and citrate synthase (CS) in copepods (Unal and Bucklin, 2010; Kozol et al., 2012). Furthermore, Exon Primed Intron Crossing loci (EPIC) may serve as suitable markers (Chenuil et al., 2010). Recent next-generation sequencing approaches also facilitate the discovery of Insertion/Deletion (InDel) markers (Smolina et al., 2014), microsatellites (Leese and Held, 2011) and single nucleotide polymorphisms (SNPs)(Yu et al., 2014).

1.3 Oceanography along the west coast of Africa

The study area, the west coast of Africa, features two of the major eastern boundary upwelling systems, which belong to the most productive marine ecosystems worldwide yielding 20% of the global fish catch over an area less than 1% of the world ocean (Chavez and Messié, 2009). Upwelling systems along the west African coast, driven by equatorward trade winds, are associated to the Atlantic subtropical gyres, the southward flowing Iberia/Canary current in the northern hemisphere and the northward flowing Benguela current in the southern hemisphere (Chavez and Messié, 2009) (Fig. 1).

During periods of strong winds, an offshore Ekman flow develops in the shallow surface layer further inshore deflected by the Coriolis force, and cold, nutrient-rich water is upwelled from the 100-300 m depth layer (Currie, 1953; Mittelstaedt, 1983; Timonin et al., 1992) leading to the enhanced biological productivity of coastal upwelling systems (Pennington et al., 2006). Poleward flowing undercurrents over the shelf slope and bottom are found in both hemispheres in

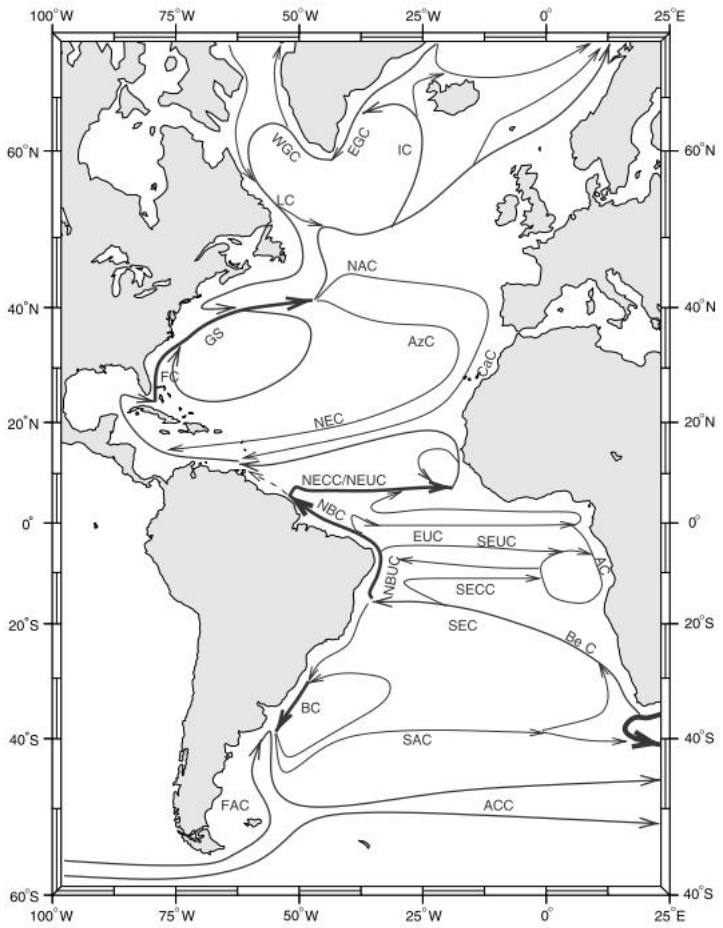

Figure 1: Upper-surface currents in the Atlantic Ocean in northern fall (from Stramma 2001)

the eastern Atlantic Ocean and may reach down to Antarctic Intermediate Water layers around 1000 m depth (Mittelstaedt, 1983; Barton, 1989; Lass et al., 2000; Hagen, 2001; Knoll et al., 2002; Shillington et al., 2006; Mohrholz et al., 2008; Chavez and Messié, 2009; Peña-Izquierdo et al., 2012). During upwelling events, coastal jets develop in the surface layer on the shelf flowing opposite the deep counter-currents (Hagen, 2001). The intensity and frequency of upwelling events depends on the Coriolis parameter and season, the strength of the trades, water column stratification and coastal topography (Chavez and Messié, 2009). Upwelling zones in the northern hemisphere include the northwest (NW) coast of Spain (Ceballos 2004), the northwest African coast from about 15°to 33°N (Hernández-León et al., 2007) and the Gulf of Guinea (Wiafe et al., 2008). Upwelling seasons off Northwest-Africa vary with latitude: south of 20°N during winter and spring, between 20°and 25°N throughout the year with maximum intensity in summer and autumn, north of 25°N predominantly during summer and autumn (maximum around 30°N) (Speth et al., 1978). The major upwelling region of the Benguela upwelling system extends from 19-34°S. Spring and autumn maxima of upwelling occur thoughout the Benguela system, while there is a summer maximum in the extreme south and persistent strong upwelling in Lüderitz and off the Cunene River (17°S) (Hutchings et al., 2006). The occurrence of low oxygen water is a typical feature of the Benguela system (Monteiro and van der Plas, 2006; Hutchings et al., 2009) and especially pronounced in the northern Benguela and

Angola-Benguela front (intermediate oxygen minimum layer in 60-500 m depth) (Ekau and Verheye, 2005). The equatorial Eastern Atlantic features a complex circulation system including amongst others two bands of the westward flowing South Equatorial Current (SEC) and the eastward flowing Equatorial Undercurrent (EUC) at the equator and an opposing current system within Antarctic Intermediate Water layers about 700 m depth (Stramma, 2001). The equatorial region close to the African coast is characterized by the termination and bifurcation of the EUC, the southern branch feeding the Gabon-Congo undercurrent (Wacongne and Piton, 1992) and no cross-equatorial flows have been reported in this region. Still, thermocline convergence, equatorial upwelling and Ekman divergence may lead to cross-equatorial water exchange (Stramma et al., 2003).

1.4 Ecology and life strategy of *Calanoides carinatus*

This phylogeographic study is investigating the calanoid copepod *Calanoides carinatus*, an upwelling specialist inhabiting tropical and temperate upwelling systems around Africa, ranging from the northeast coast off Somali, around South Africa to the west coast, as well as the Brazilian and Argentinian coasts (Peterson, 1998). *C. carinatus* is characterized by a crested head and adult females can reach a length up to 2.85 mm (Boltovskoy, 1999)(Fig. 2). Recently, the south west Atlantic population was discovered to be a cryptic sibling species compared to the east Atlantic populations (Vinas et al., unpublished). Since this data has not been published, yet, the species name will still be referred to *C. carinatus* in this study.

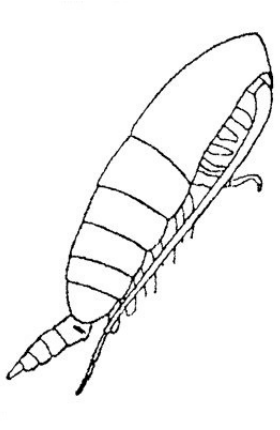

Figure 2: Adult female of *Calanoides carinatus*. Drawing from Giesbrecht (1892). Accessed on Marine Species Identification Portal (http://species-identification.org)

Linking primary production to secondary consumers (e.g. fish larvae), *C. carinatus* is an ecologically important component of the herbivorous zooplankton, e.g. in the Benguela upwelling system (Verheye et al., 2005). However, partly omnivorous feeding and grazing on cilliates by *C. carinatus* has been suggested (Ceballos and Álvarez Marqués, 2006). *C. carinatus* adapted to the highly diffusive and variable upwelling ecosystem performing ontogenetic vertical migration, which allows survival during non-upwelling seasons (Peterson, 1998). *C. carinatus* pursues different life-cycle strategies along the African coast (Fig. 3). In equatorial West Africa, a strongly seasonal pattern has been observed (e.g. Binet and de Sainte Claire, 1975; Binet, 1977; Houghton and Mensah, 1978; Dessier, 1979): With the beginning of the upwelling season and drop in water temperature, CV copepodids from deeper layers start migrating to the shelf and surface waters, moulting into adults and actively feeding

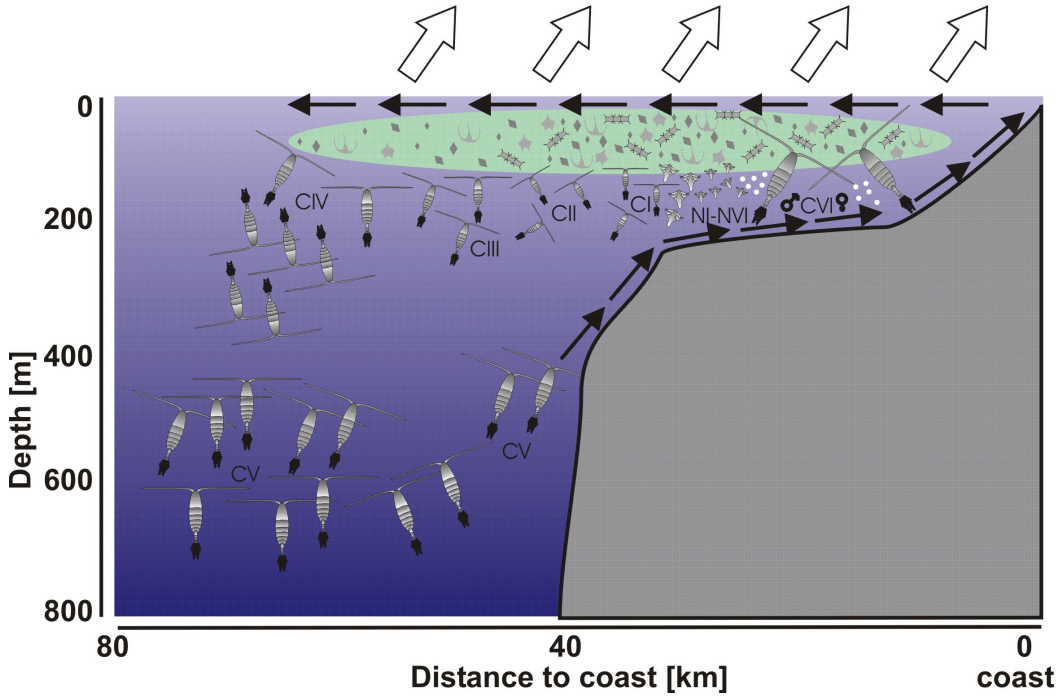

Figure 3: Life cycle of *Calanoides carinatus* associated to the upwelling system (Fig. by Holger Auel)

on the upwelling-associated phytoplankton bloom. They undergo 3-6 generations during the 2.5 - 4 months upwelling period. When temperature rises at the end of the upwelling season, the last generation forms copepodite CV diapause stages, which descend to deeper layers (below 500 m) close to the continental slope and are able to reseed the surface waters during the next upwelling-induced phytoplankton bloom. Interestingly, life cycle strategies of *C. carinatus* seem to be more complex in the Benguela upwelling system where in some regions (Nambian shelf, off southern Angola, southern Benguela) the population of *C. carinatus* consists of two parts throughout the year: the actively reproducing surface population containing all developmental stages with certain seasonal variability in abundance and a presumably permanent stock of CV diapause stages residing in deeper layers between 200 – 1000 m (Kosobokova et al., 1988; Timonin et al., 1992; Arashkevich et al., 1996; Arashkevich and Drits, 1997; Timonin, 1997; Loick et al., 2005; Verheye et al., 2005). In the northern Benguela, upwelling activity, phytoplankton concentration and zooplankton abundance seem to be uncoupled, whereas a clear correlation of these parameters can be observed in the southern Benguela (Hutchings et al., 2006). Diapause stages of *C. carinatus* feature an extremely low metabolism with reduced respiration up to 96 % (Auel et al., 2005), a high body mass and lipid content (Kosobokova et al., 1988; Arashkevich et al., 1996; Arashkevich and Drits, 1997; Verheye et al., 2005). Hence, they are able to outlast extended starvation periods from 149 up to 192 days, in contrast to females and CVs of the active surface population which can survive 10 days at most without feeding (Verheye et al., 2005). Possible external factors for diapause regulation include photoperiod, temperature and population density and internal regulators such as the biological clock, neuro-secretions, lipids and metabolites (Dahms, 1995). However, the triggers for initiation and termination of diapause in *C. carinatus* are still not well understood in upwelling

systems, which are highly variable ecosystems independent of the photoperiod, an important trigger at higher latitudes. Observed moulting of *C. carinatus* in layers below 300 m suggests endogenous triggers for moulting of diapause stages and their active ascent to shoreward-moving layers (Timonin et al., 1992). A combination of internal (e.g. depletion in lipid reserves) and external factors (e.g. physical advection) may lead to successful resettlement during the upwelling season (Idrisi et al., 2004). Recent molecular approaches in *Calanus finmarchicus* uncovered several genes involved in diapause regulation and gave rise to an improved understanding of internal regulation in copepods (Tarrant et al., 2008; Christie et al., 2013a,b). In Antarctic copepods, ammonium accumulation and low pH in the hemolymph were found to play a central role for neutral buoyancy and metabolic depression during diapause (Sartoris et al., 2010; Schründer et al., 2013).

Despite inhabiting a highly dispersive environment, *C. carinatus* is believed to be able to maintain itself in upwelling systems taking advantage of associated currents. In the Benguela system, the northwestward drift of the active surface population in the Ekman layer is assumed to 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). In the Canary current system off NW Africa, a combination of upwelling flows and a cyclonic-anticyclonic eddy dipole may act as a possible retention mechanism (Moyano et al., 2014). Behavioural aspects such as diel or ontogenetic vertical migration have often been described to enhance the maintenance of planktonic organisms in favourable regions taking advantage of associated coastal flows (Verheye and Field, 1992; Johnson, 2007; Parada et al., 2008; Harkins et al., 2013; Morgan, 2014; Moyano et al., 2014). This includes the observation of adults residing in greater daytime depths than early copepodite stages of *C. carinatus* and possible avoidance of the Ekman layer (Verheye and Field, 1992).

However, the rather local maintenance strategy in *C. carinatus* has been repeatedly questioned. Dispersal by undercurrents may play a central role for the life cycle of *C. carinatus* as poleward flowing undercurrents have often been associated with the transport of planktonic organisms and larvae (Auel and Verheye, 2007; Harkins et al., 2013; John et al., 1998, 2000; Peterson, 1998; Timonin, 1997; Verheye and Ekau, 2005). Rather random sources of diapausing stages during the onset of the upwelling season were reported in the diapausing copepod *Calanus pacificus* inhabiting the California Current system (Johnson, 2007). Moreover, the occurrence of two stocks of *C. carinatus* was suggested off the shelf of Ghana during the upwelling season (Sywula et al., 2002). Possible indications for vertical structuring of *C. carinatus* populations include the observation of differences in copepodite CV body mass with lighter individuals at the surface compared to deep-living diapause stages (Auel et al., 2005; Verheye et al., 2005) and the fact that *C. carinatus* might not be able to cross the core of the intermediate oxygen minimum layer in the Angola-Benguela frontal zone and northern Benguela, which could act as a potential barrier for vertical migrations (Auel and Verheye, 2007).

1.5 Aim of the study

This study aims to clarify the population genetic structure of *C. carinatus* in the eastern Atlantic Ocean identifying (1) spatial patterns in the distribution of genetic variance, (2) potential barriers that may have shaped the genetic structure of the species and (3) possible cryptic species within the nominal *C. carinatus*. As marker genes, we chose the mitochondrial COI and the nuclear CS. For horizontal analysis, three regions along the west coast of Africa were compared: Northwest Africa, northern Benguela and southern Benguela, whereas vertical population structure was analysed in the northern Benguela. The three following hypotheses were tested:

I. Populations of *C. carinatus* in the northern hemisphere are different from populations in the southern hemisphere.
Gene flow within hemispheres is enhanced by transport within the surface and undercurrent systems along the west coast of Africa. Opposing current systems at the equator may act as a barrier to gene flow between northern and southern populations.

II. Populations of *C. carinatus* of all three regions differ from each other.
C. carinatus is able to maintain itself in local upwelling systems throughout its life cycle. Gene flow among *C. carinatus* populations is rather restricted owing to either behavioural aspects or limits to transport in large-scale ocean circulation.

III. Active surface individuals and deep living resting stages in the northern Benguela do not belong to the same population.
Upwelling systems are highly diffusive regions and theories of life cycle closure in *C. carinatus* have not been proven, yet. There are several indications that shallow and deep water individuals belong to different populations.

2 Materials and Methods

2.1 Study area and sampling methods

Regions of interest comprised coastal upwelling systems close to Spain and off Senegal/Mauritania in the northern hemisphere and the northern and southern Benguela upwelling systems close to the southwest African coast (Fig. 4).

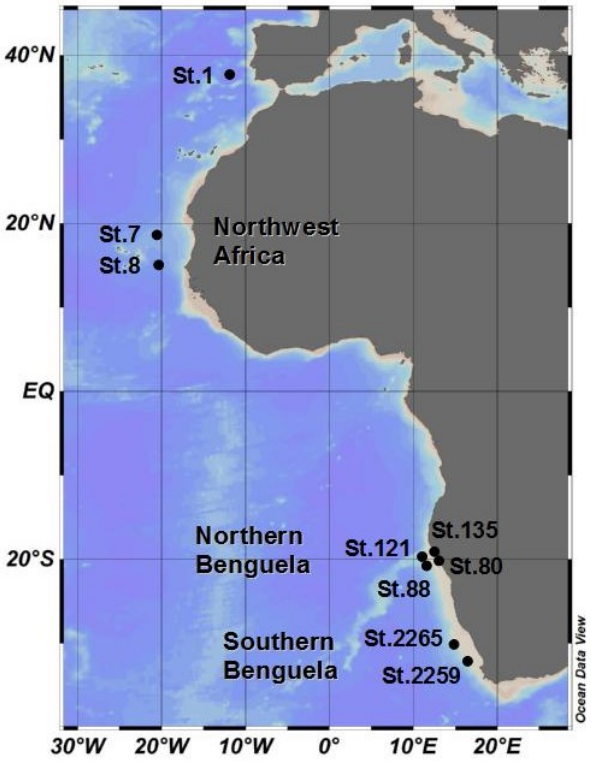

Figure 4: Station map.

Sampling was partially carried out during the RV Meteor cruise M103/2 in January/February 2014 within the GENUS programme (Geochemistry and Ecology of the Namibian Upwelling System). The stations were sampled with a HydroBios MultiNet Midi (0.25 m^2 mouth opening, five separate nets, 200 µm mesh size). Individuals of *C. carinatus* were sorted out immediately after sampling. Circa 5 individuals were transferred to each Eppendorf tube and frozen at -80°C. The rest of the sample was preserved in absolute ethanol. Copepods were identified according to Boltovskoy (1999) to species level with the stereo dissecting microscope Leica MS5 (maximal 40x magnification) (Leica Microsystems, Wetzlar, Germany). Additional samples were provided by the Marine Zoology group, University of Bremen, from the RV Polarstern cruise ANT29/1 in October/November 2012 (Frozen samples obtained with HydroBios Mulitnet Maxi 0.5 m^2 mouth opening, nine separate nets, 150 µm size) and the RV Meteor cruise M102 in December 2013 (Samples obtained with HydroBios Multinet Midi, preserved in absolute ethanol).

Samples were taken from different sampling depths. Individuals from the 0-200 m layer were found at stations 80, 135, 2259 and 2265, mostly adult females and a few developmental stages from St. 2259 and 2265. Four males from the surface station 135 in the northern Benguela

were analysed. Diapausing copepodids CV were sampled in deeper layers at stations 1, 7, 8, 88 and 121 (400 – 800 m depth) (Table 1).

Two stations per region were chosen with ca. 30 individuals each, if available. Only 5 individuals were available from the northernmost region off Spain. In total 227 individuals of *C. carinatus* were analysed taken at overall 9 stations (Fig. 4). For horizontal analysis, three regions were analysed independent of depth: Northwest Africa (NWA), Northern Benguela (NB) and Southern Benguela (SB). Vertical population structure was examined in the northern Benguela comparing two shelf stations and two deep stations (Fig. 5).

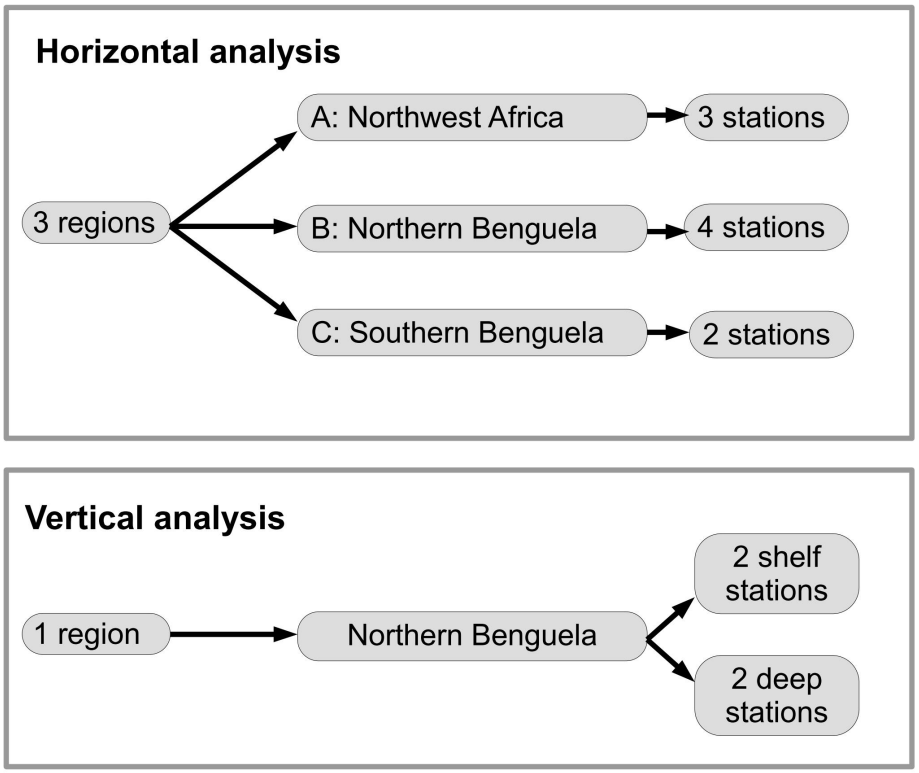

Figure 5: Regions and corresponding number of stations for horizontal and vertical analysis.

Table 1: Station data including cruise, region, coordinates, sampling depth, number and stage of the analysed individuals of *C. carinatus*.

Cruise	Region	Station	Latitude	Longitude	Sampling depth [m]	Individuals analysed	Stage
ANT29/1	Spain	1	37.82°N	12.08°W	500 - 800	5	CV
ANT29/1	Mauritania	7	18.76°N	20.71°W	600 - 700	29	CV
ANT29/1	Senegal	8	15.25°N	20.52°W	200 - 700	5	CV
M103/2	Northern Benguela	80	20.03°S	12.89°E	20 - 60	32	female
M103/2	Northern Benguela	88	20.52°S	11.38°E	400 - 800	29	CV
M103/2	Northern Benguela	121	19.86°S	11.05°E	400 - 800	29	CV
M103/2	Northern Benguela	135	19.17°S	12.38°E	20 - 60	33	female, male
M102	Southern Benguela	2259	32.01°S	16.25°E	0 - 50	32	CIV, CV, female
M102	Southern Benguela	2265	30.00°S	14.65°E	0 - 200	10	CIV, CV

2.2 Molecular methods

2.2.1 DNA extraction

DNA was extracted from whole or half specimens. To avoid potential misidentification in individuals from the training cruise ANT29/1, those copepods were cut in halves with a scalpel (cleaned with 70% ethanol and flamed). The forepart was used for DNA extraction and incubated in buffer ATL (Tissue lysis buffer) and proteinase K (Qiagen, Hilden, Germany) until correct identification. The posterior part containing the last swimming leg was transferred to absolute ethanol and identified with the stereomicroscope Leica MZ16 (maximal 230x magnification) (Leica Microsystems, Wetzlar, Germany). Individuals preserved in ethanol were rinsed in distilled water before extraction. The forceps was cleaned with 70% ethanol and flamed between transfers of the individuals. 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 at least 2 hours at 56°C and 500 rpm. 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 20 min at room temperature.

2.2.2 Primer design

Since this is the first study of the nuclear gene CS in *C. carinatus*, no sequences or primers were available for this species. The primer design was based on the CS GeneBank sequence No. EL585846.1 (GenBank, http://www.ncbi.nlm.nih.gov/genbank/) of the closely related species *Calanus finmarchicus* used for primer design in the study by Kozol et al. (2012). The *C. finmarchicus* CS sequence was aligned to CS sequences available from Genbank comprising *Calanus sinicus* (Crustacea, No. JF430060.1), *Calanus agulhensis* (Crustacea, No. JF430038.1), *Anopheles gambiae* (Insecta, No. XM_ 320478.4), *Mayetiola destructor* (Insecta, No. KF647629.1), *Ditylenchus destructor* (Nematoda, No. JN216829.1), *Artemia franciscana* (Crustacea, No. BQ563145.1), *Lepeophtheirus salmonis* (Crustacea, No. BT121320.1) and *Bombyx mori* (Insecta, No. AK385859.1). Alignment was carried out with the programme Mesquite Version 2.75 (Maddison and Maddison, 2011, http://mesquiteproject.org) according to the translated protein sequences. Primer were designed with Geneious® version 6.1.4. (Biomatters Ltd., Auckland, New Zealand, www.geneious.com). The CS primers designed by (Kozol et al., 2012) were modified for optimal position and length within the *C. finmarchicus* GenBank sequence (No. EL585846.1). The alignment was used to identify regions of the sequence that were mostly conserved over all species to reduce the risk for possible mismatches of the primers in *C. carinatus*. Mutations often occur at the third codon position, so called silent mutations that do not change the amino acid composition. Therefore, the 3' end of the forward primer was adjusted to the second codon position and the 3' end of the reverse primer to the

first codon positon. Optimal primer length and position of both primers conformed to similar melting temperature and the avoidance of self dimers and heterodimers of the primers.

For the amplification of the COI fragment, the universal primers LCO1490 (Folmer et al., 1994) and C1-N-2191 (alias Nancy) (Simon et al., 1994) were used (Table 2).

Table 2: 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-514R_ mod	CAAGTACAGTCTCATCAGCTC	Modified after Kozol et al. (2012)

2.2.3 Amplification and sequencing

The polymerase chain reaction (PCR) was carried out in an Eppendorf Masterthermocycler gradient with heated lid (Eppendorf, Hamburg, Germany) with the GoTaq® G2 Flexi DNA polymerase product (Promega, Mannheim, Germany) containing 5x Colorless GoTaq® Flexi Buffer, 25 mM $MgCl_2$ and 5 U/µL DNA polymerase. A PCR Mastermix was prepared for all samples with final concentrations of 1x PCR buffer, 0.4 µM of each primer, 0.2 mM dNTPs (Carl Roth, Karlsruhe, Germany), 0.65 U DNA polymerase and 2 µL DNA. 2 µL DNA were added to 23 µL Mastermix per sample resulting in a final reaction volume of 25 µL. For the negative control, 2 µL water molecular biology grade (AppliChem, Darmstadt, Germany) were used instead of DNA. For amplification the following temperature profile was applied: initial denaturation (95°C, 5 min), 40 cycles of denaturation (95°C, 30 sec), annealing (42°C, 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 (10x TBE buffer, AppliChem, Darmstadt, Germany). Per sample 4 µL PCR product and 1 µl 6x Orange DNA loading dye (Thermo Scientific, Bremen, Germany) were applied to the gel. The length of the DNA fragments was assessed with the marker Fastruler™ DNA Ruler Low Range (Fermentas, St. Leon-Rot, Germany). The DNA fragments were stained 3-5 min in a 1% ethidium bromide solution (Carl Roth, Karlsruhe, Germany) and de-stained for 30 min. PCR products were 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 a ABI 3730XL sequencing machine (Applied Biosystems, Life Technologies GmbH, Darmstadt, Germany).

2.3 Data analysis

2.3.1 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. The correctness and functionality of protein-coding sequences was tested by translating nucleotide into amino acid sequences and checking for stop codons. 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). For reading frame verification of the translated alignment, the translated nucleotide sequence was compared with the protein database (blastx option). Conspicuous sequences with remarkable changes in nucleotide and amino acid composition were checked with the nucleotide blast option using a nucleotide query against the nucleotide database for correct species identification. The number of polymorphic sites in the alignment was determined with DnaSP version 5.10.01 (Librado and Rozas, 2009).

2.3.2 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 haplotype network of the COI data was constructed with the programme Haplotype Viewer (available from http://www.cibiv.at/~%20greg/haploviewer) based on the Maximum Parsimony Tree analysis in the programme MEGA 6 (Tamura et al., 2013). MEGA 6 was also applied to determine the p-distances among sequences giving percentile genetic differences.

The programme Arlequin version 3.5.1.2 (Excoffier et al., 2007) was used for further analysis of neutrality and population genetic structure. The neutrality tests Tajima's D (Tajima, 1989) and Fu's FS (Fu, 1997) were carried out to test hypothesis of neutral evolution and to 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 results was based on 100172 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 30000 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 programme jModelTest version 2.1.6 (Darriba et al., 2012). Based on the Bayesian information criterion (BIC), the closest nucleotide substitution model,

which was implemented in the programme MrBayes version 3.2.2 (Ronquist et al., 2012), was chosen to build the phylogenetic tree.

Gene specific methods

The genetic diversity of COI was determined calculating haplotype and nucleotide diversity (Nei, 1987) of the different regions in the programme Arlequin. *C. carinatus* 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.

In CodonCode Aligner, double peaks in the chromatograms of the CS sequences were converted to ambiguity codes in the consensus sequence. Haplotypes of the diploid CS sequences were inferred with the PHASE algorithm (Stephens et al., 2001; Stephens and Donnelly, 2003) as implemented in DnaSP version 5.10.01 Further phylogeographic analysis was carried out with the phased sequences.

3 Results

3.1 Mitochondrial COI

3.1.1 Genetic diversity

In total 227 mitochondrial COI sequences were obtained with a length of 676 bp. The alignment contained 573 invariable positions and 103 polymorphic sites. Since most of the mutations were silent mutations, the translated amino acid composition was largely identical. Four individuals differed in one amino acid. Three individuals were more divergent in their nucleotide composition and had three different amino acids in comparison to the consensus sequence.

Haplotype analysis revealed 40 haplotypes whereof two were found over the whole study area and six were shared between the northern and the southern Benguela (Fig. 6). The other haplotypes were restricted to one of the three regions. The haplotype network revealed a deep split within *Calanoides carinatus* with three highly divergent individuals, 66 – 69 mutational steps away from the prevalent population. Both molecular operational taxonomic units (MOTUs) differed between 9.5 to 10.4% in their COI sequences. Variation within the prevalent MOTU ranged from 0.1 to 1.3%. The three divergent individuals had 0.7 to 5.6% sequence differences. Interestingly, both MOTUs occurred in the whole study area and did not show spatial separation.

Further analysis of regional differences was focussed on the prevalent MOTU and the three highly divergent sequences were removed. 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 (Table 3). Haplotype and nucleotide diversity of the surface and deep population in the northern Benguela did not differ from each other (H_d = 0.82, π=0.0053).

Table 3: Molecular diversity indices of mtCOI (Haplotype diversity H_d, nucleotide diversity π) calculated for the three regions.

	NWA	NB (total)	SB	NB (surface)	NB (deep)
Sequences analysed	61	121	42	65	56
H_d	0.69	0.83	0.82	0.82	0.82
π	0.0049	0.0054	0.0047	0.0053	0.0053

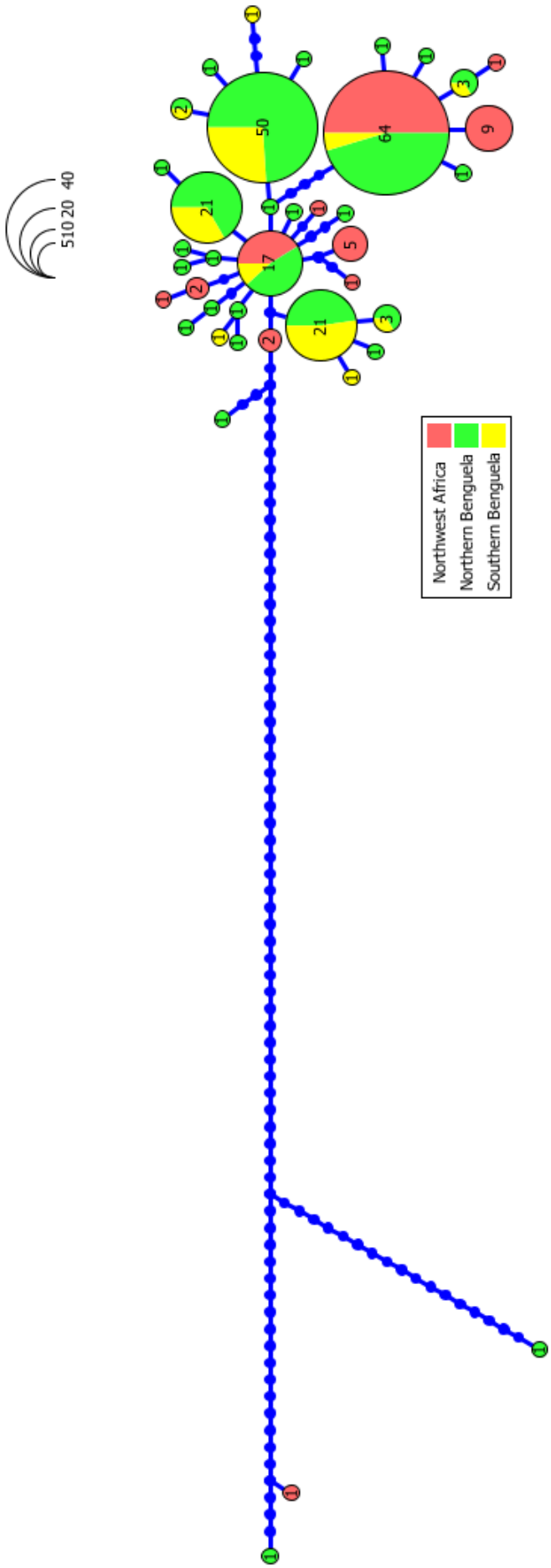

Figure 6: Haplotype network of COI data. Circles present the haplotypes which are connected by mutational steps (blue points). The size of the circle corresponds to the number of individuals that share the same haplotype. The pie charts represent the proportion of orgin of the individuals.

3.1.2 Neutrality tests

Tajima's D and Fu's FS test did not reveal any significant departure from neutrality (Table 4). It confirmed the theory of neutral evolution of our sequences, which is a necessary assumption for the following population genetic tests.

Table 4: Results from the neutrality tests Tajima's D and Fu's FS of the COI sequence data. Non-significant values for all stations (p>0.16).

	St.1	St.7	St.8	St.80	St.88	St.121	St.135	St.2259	St.2265
Sample size	5	28	28	32	27	29	33	32	10
Tajima's D	0.91	0.27	-0.28	-0.13	0.54	0.24	-0.97	0.03	0.44
Fu's FS	1.77	1.09	0.79	0.32	1.00	-2.20	-2.23	-0.53	0.94

3.1.3 Population structure

Analysis of pairwise F_{ST} gave first insight to genetic differentiation between stations (Fig. 7). 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). Station 1 (Spain) did not show such a strong differentiation to the southern hemisphere as St.7 and 8. Interestingly, the surface stations in the Northern Benguela (St.80 and 135) seemed to be less differentiated to the northern hemisphere than the deep NB stations (St.88 and 121). F_{ST} between station 2259 (southern Benguela) and station 88 (deep NB) was moderate and significant. Geographically close stations had low to non-significant F_{ST} values and therefore less genetic differences.

Hierarchical AMOVA confirmed genetic differentiation within the whole study area with a significant global F_{ST} value (Table 5). Genetic variance was apparent within populations (stations) indicated by the significant F_{ST} values in all tests. Genetic differentiation among populations within groups was observed, when testing the northern against the southern hemisphere ((NWA) $\leftrightarrow$ (NB +SB)), NWA against NB, and combined NWA and NB against SB (see significant F_{SC} values table 5). For horizontal analysis, highly significant differences between the northern and southern hemisphere (F_{CT} = 0.21*) as well as among the three regions (F_{CT} = 0.17**) were found. Northwest Africa significantly differed from Northern Benguela (F_{CT} = 0.18*). Testing the combined regions NWA and NB against SB did not result in a significant difference. No significant differences were found testing the other regions separately (NWA $\leftrightarrow$ SB, NB $\leftrightarrow$ SB). Moreover, the surface population and deep-living diapause stages in the northern Benguela did not significantly differ from each other.

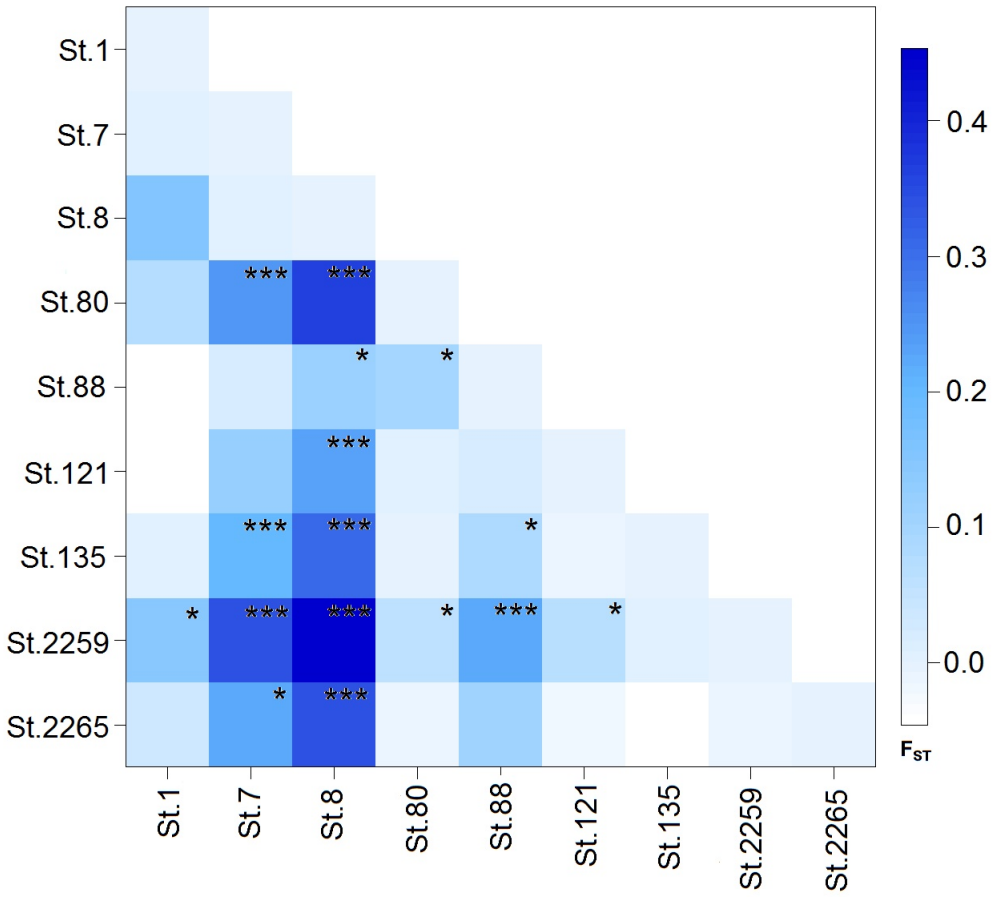

Figure 7: Matrix of population pairwise F_{ST} between the stations based on mtCOI sequences. Significant results are indicated with *(p<0.05), ** (p<0.01) and ***(p<0.001).

Table 5: Hierarchical AMOVA results for COI with fixation indices indicating genetic differentiation within the population (F_{ST}), among populations within groups (F_{SC}) and among groups (F_{CT}). Regions are abbreviated as follows: Northwest Africa (NWA), Northern Benguela (NB), Southern Benguela (SB). Significant results in bold, significance level *(p< 0.05), **(p<0.01) and ***(p<0.001).

Grouping	F_{ST}	F_{SC}	F_{CT}
Global	**0.15*** **		
(NWA) ↔ (NB +SB)	**0.25*** **	**0.04****	**0.21***
(NWA) ↔ (NB) ↔ (SB)	**0.19*** **	0.03	**0.17****
(NWA) ↔ (NB)	**0.20*** **	**0.03***	**0.18***
(NWA) ↔ (SB)	**0.36*** **	0.02	0.34
(NB) ↔ (SB)	**0.07****	0.03	0.04
(NWA+NB) ↔ (SB)	**0.19*** **	**0.12*** **	0.08
(surfaceNB) ↔ (deepNB)	**0.04***	0.01	0.04

3.1.4 Isolation by distance

Linear regression analysis of genetic distance (ϕ_{st}) and geographic distance among all stations resulted in a relatively low regression (r=0.45) (Fig. 8). 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. 9).

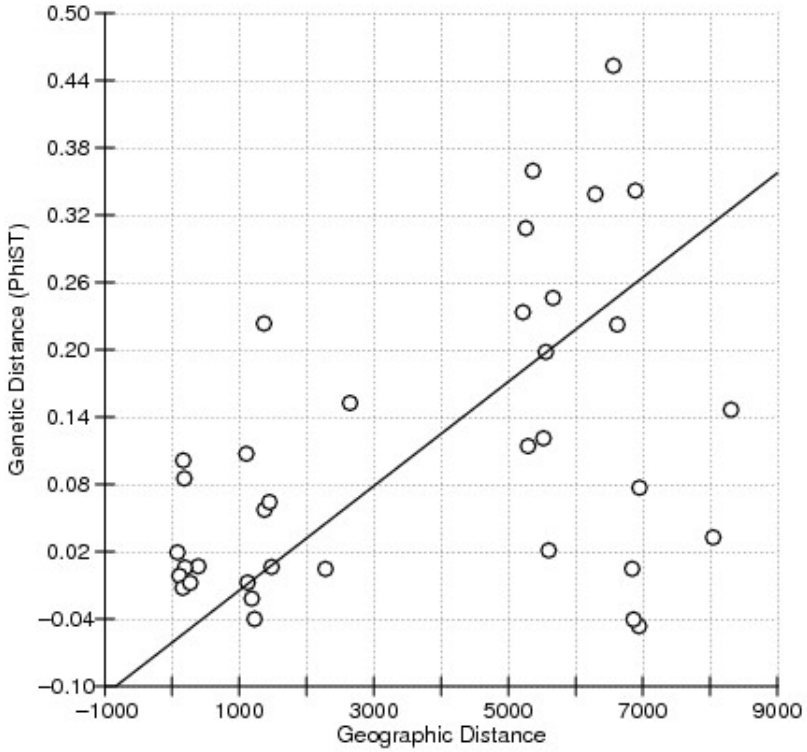

Figure 8: Genetic distance among stations (ϕ_{st}) plotted against geographic distance including regression line (analysis of all sequences).

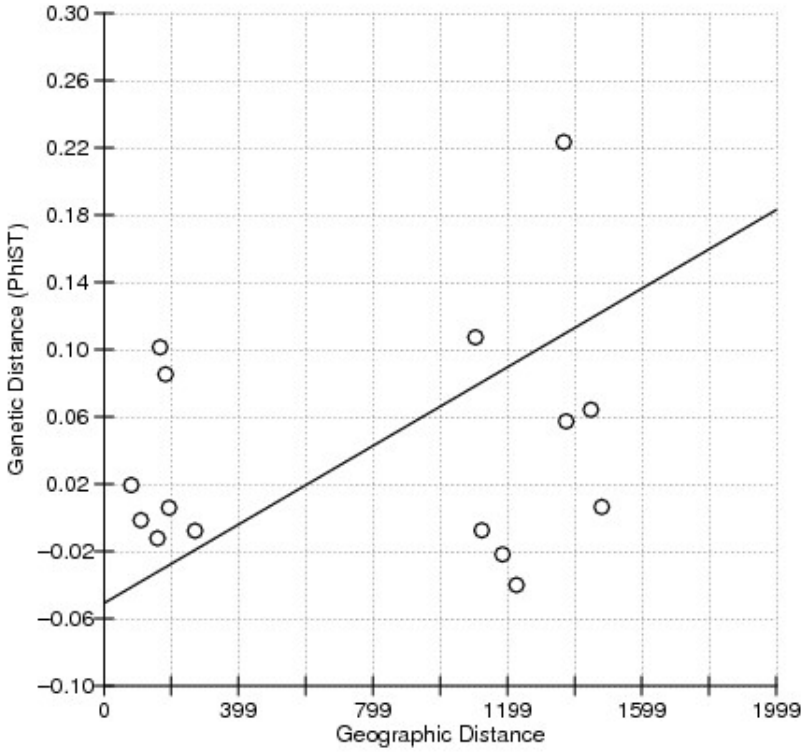

Figure 9: Genetic distance among stations (ϕ_{st}) plotted against geographic distance including regression line (analysis of COI sequences from southern hemisphere).

3.2 Nuclear CS

3.2.1 Genetic diversity

After alignment and quality control of the sequences, 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 alleles was mostly similar except 15 alleles which differed in one amino acid from the consensus sequence.

The 324 alleles were collapsed into 153 haplotypes. Three haplotypes were found in all study regions. Additionally, 5 haplotypes were shared between NWA and NB and 11 shared haplotypes were found between NB and SB. Sequence differences ranged from 0.3 to 6.1%.

3.2.2 Neutrality tests

Tajima's D and Fu's FS test for neutral evolution of the marker gene CS had different results (Table 6). For Tajima's D, no significant results were obtained for all stations. On the other hand, Fu's FS revealed significant departure from neutrality for most of the stations (except St.2259 and 2265). Significant negative results of neutrality tests indicate an excess number of alleles.

Table 6: Results from the neutrality tests of CS sequence data. Tajima's D and Fu's FS. Significant results in bold, significance level *(p< 0.02), **(p<0.01) and ***(p<0.001).

	St.1	St.7	St.8	St.80	St.88	St.121	St.135	St.2259	St.2265
Sample size	10	44	38	46	44	28	52	42	20
Tajima's D	-0.19	-1.04	-0.63	-1.02	-0.55	-0.84	-0.99	-0.25	0.11
Fu's FS	-2.26	**-17.36*** **	**-8.02** **	**-10.95** **	**-18.82*** **	**-9.47** **	**-24.46*** **	-4.18	-0.38

3.2.3 Population structure

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 southern Benguela stations (St.2259 and 2265) (Fig. 10). Moreover, St.7 and 8 were moderately differentiated from the northern Benguela, with more or less genetic differences to stations located in the northern hemisphere. 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 from St.7 and 8.

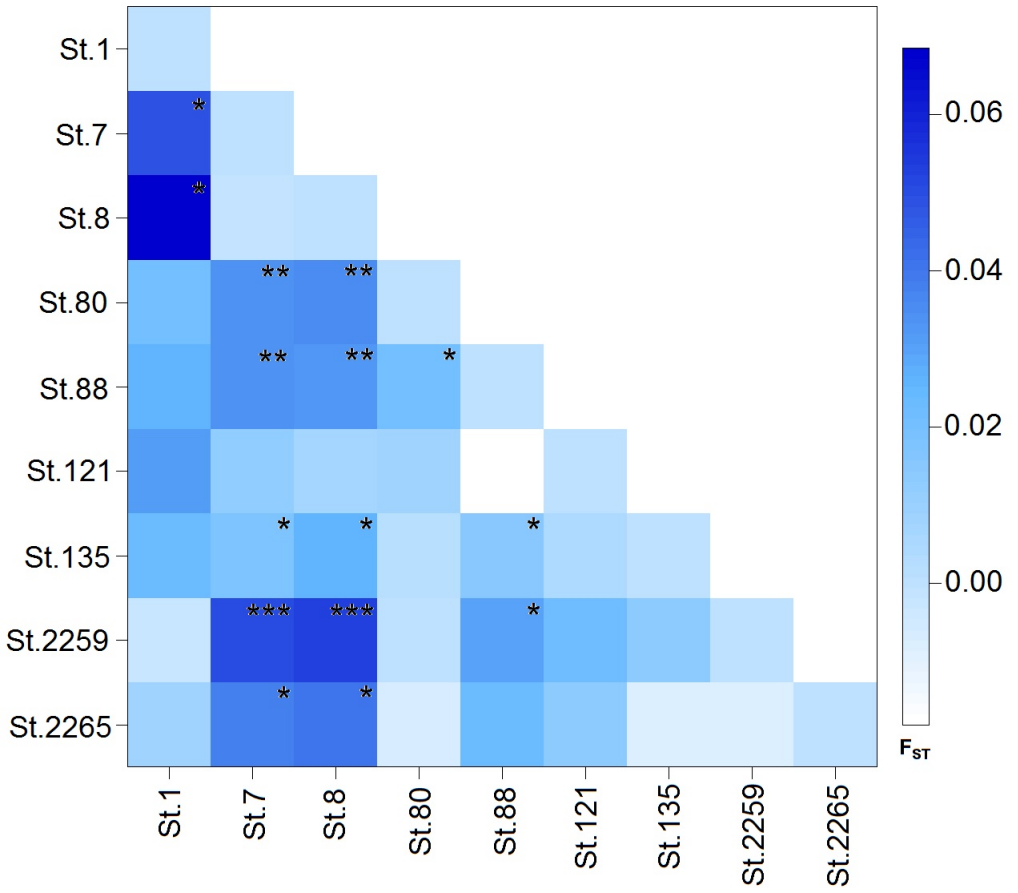

Figure 10: Matrix of population pairwise F_{ST} between the stations based on CS sequences. Significant results are indicated with *(p<0.05), ** (p<.0.01) and ***(p<0.001

AMOVA analysis of the CS marker revealed a signifcant global F_{ST} value of 0.02 (p<0.001) which indicates genetic differentiation within all samples (Table 7). Genetic differences within the populations were found in all AMOVA tests, except the ones only testing in the Benguela region (see F_{ST} values table 7). Genetic differentiation among populations within groups (F_{SC}) was proven for AMOVA tests between northern and southern hemisphere, NWA and NB, and (NWA+NB) against SB. Significant differences between regions (F_{CT}) were found between the northern and the southern hemisphere (F_{CT}=0.02, p<0.05) and among the three regions (F_{CT}=0.02, p<0.05). Separate testing for differences between two regions did not show any significant results. The combined regions NWA and NB tested against SB did not significantly differ from each other. Moreover, no vertical genetic structuring was evident between the surface and deep-living population in the NB.

Table 7: Hierarchical AMOVA results for CS with fixation indices indicating genetic differentiation within the population (F_{ST}), among populations within groups (F_{SC}) and among groups (F_{CT}). Regions are abbreviated as follows: Northwest Africa (NWA), Northern Benguela (NB), Southern Benguela (SB). Significant results in bold, significance level *(p<0.05), **(p<0.01) and ***(p<0.001).

Grouping	F_{ST}	F_{SC}	F_{CT}
Global	**0.02*** **		
(NWA) $\leftrightarrow$ (NB +SB)	**0.03*** **	**0.01***	**0.02***
(NWA) $\leftrightarrow$ (NB) $\leftrightarrow$ (SB)	**0.02*** **	0.01	**0.02***
(NWA) $\leftrightarrow$ (NB)	**0.02*** **	**0.01***	**0.01***
(NWA) $\leftrightarrow$ (SB)	**0.04*** **	0.01	0.04
(NB) $\leftrightarrow$ (SB)	0.01	<0.01	0.01
(NWA+NB) $\leftrightarrow$ (SB)	**0.03*** **	**0.02****	0.01
(surfaceNB) $\leftrightarrow$ (deepNB)	0.01	-0.01	0.02

3.2.4 Isolation by distance

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 (Z=3435.38, r=0.44, p=0.03)(Fig. 11) 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 (Z=120.43, r=0.32, p=0.08)(Fig. 12).

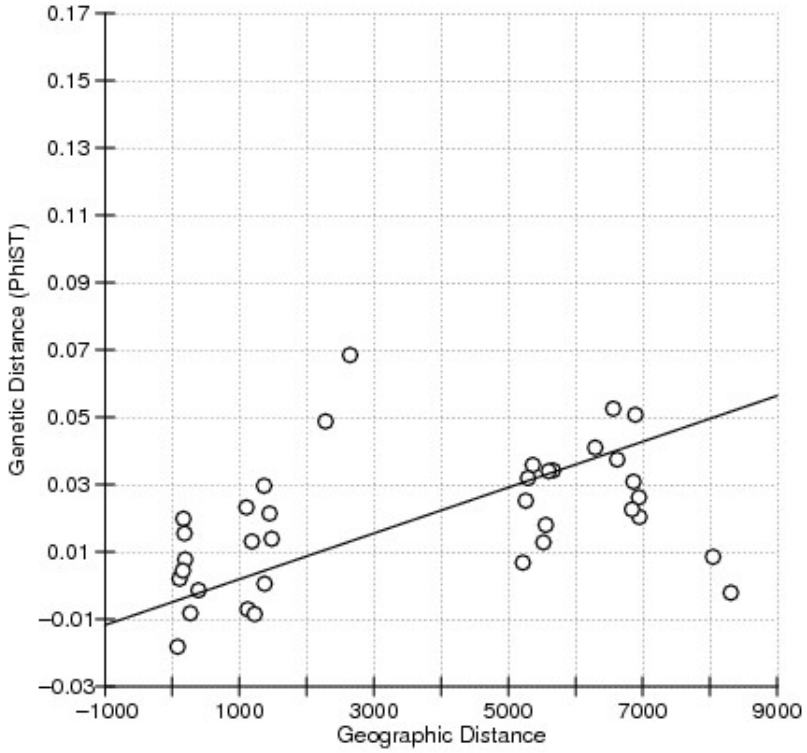

Figure 11: Genetic distance among stations (ϕ_{st}) plotted against geographic distance including regression line (analysis of all sequences).

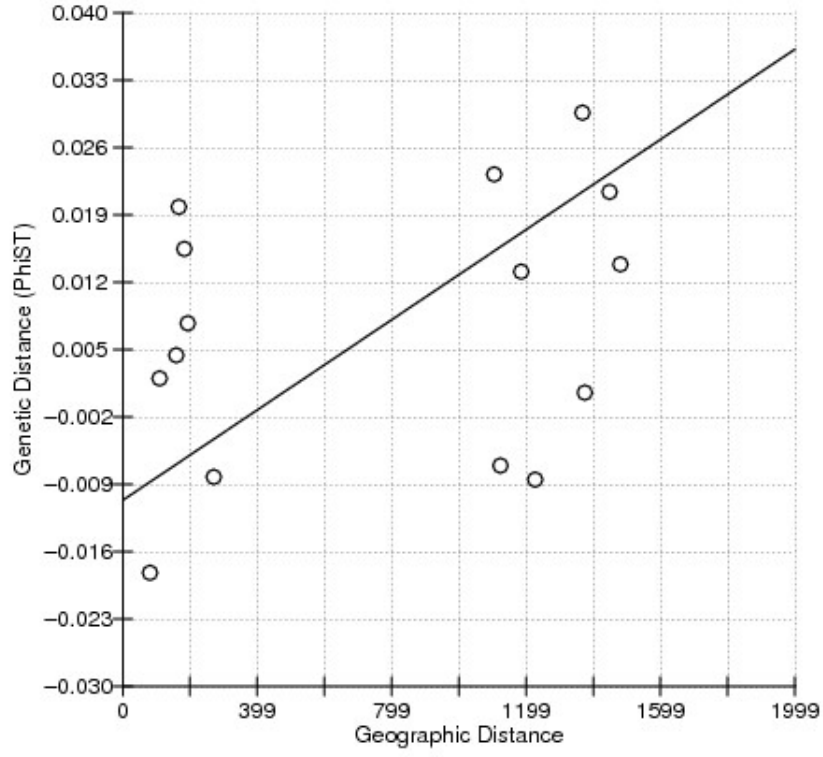

Figure 12: Genetic distance among stations (ϕ_{st}) plotted against geographic distance including regression line (analysis of CS sequences from southern hemisphere).

3.3 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 was supported by 100% probability and had a remarkably long genetic distance to the other sequences (Fig. 13) as has already been observed in the haplotype network. In contrast, this distinct cluster was not observed, when analysing the phylogeny of the CS haplotypes (Fig. 14, 15). The corresponding CS haplotypes are distributed within several branches and cluster together with other sequences with up to 100% probability.

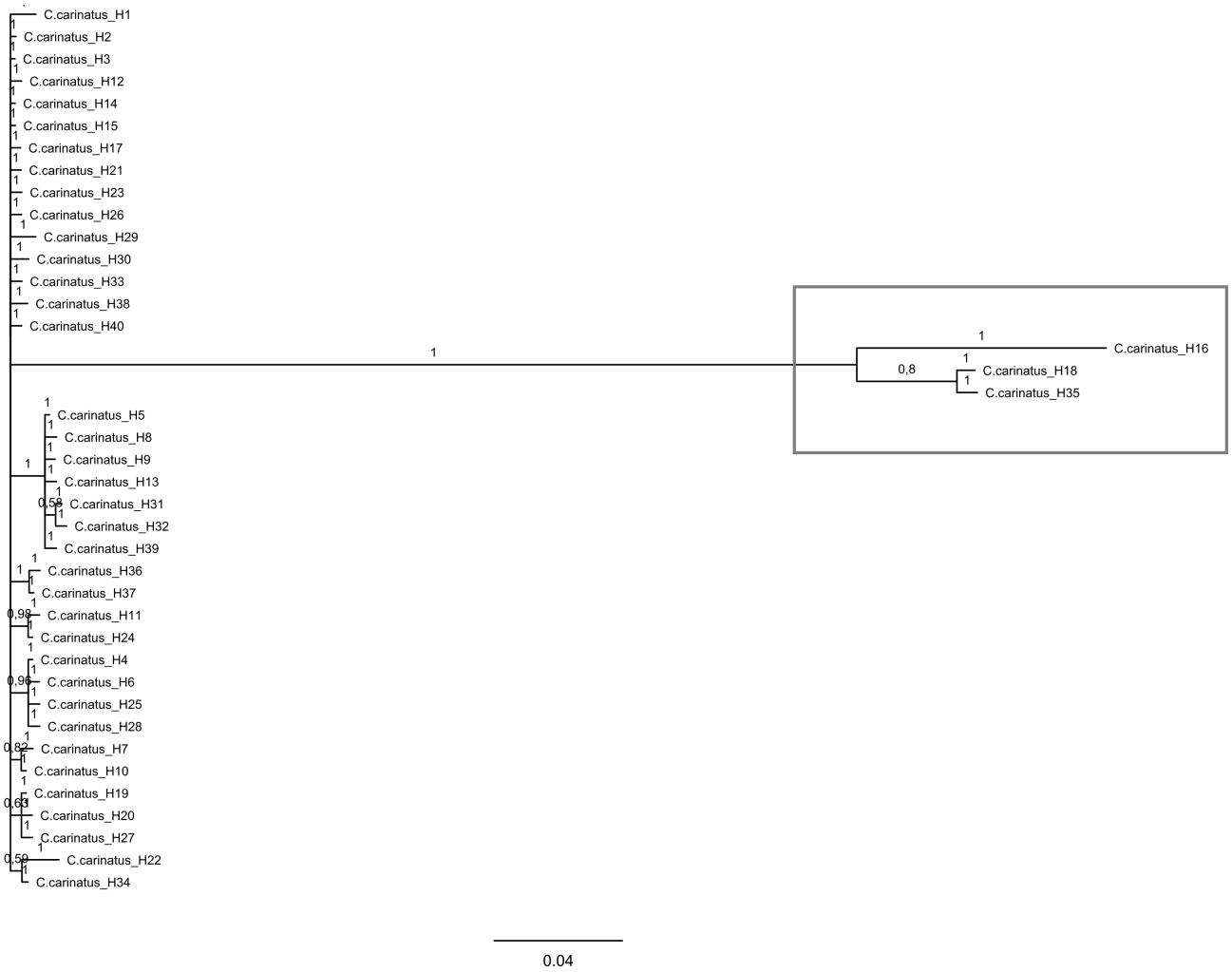

Figure 13: MrBayes tree of COI haplotypes. The highly diverse sequence are labelled with a grey rectangle. Branch probabilities are shown in decimal format.

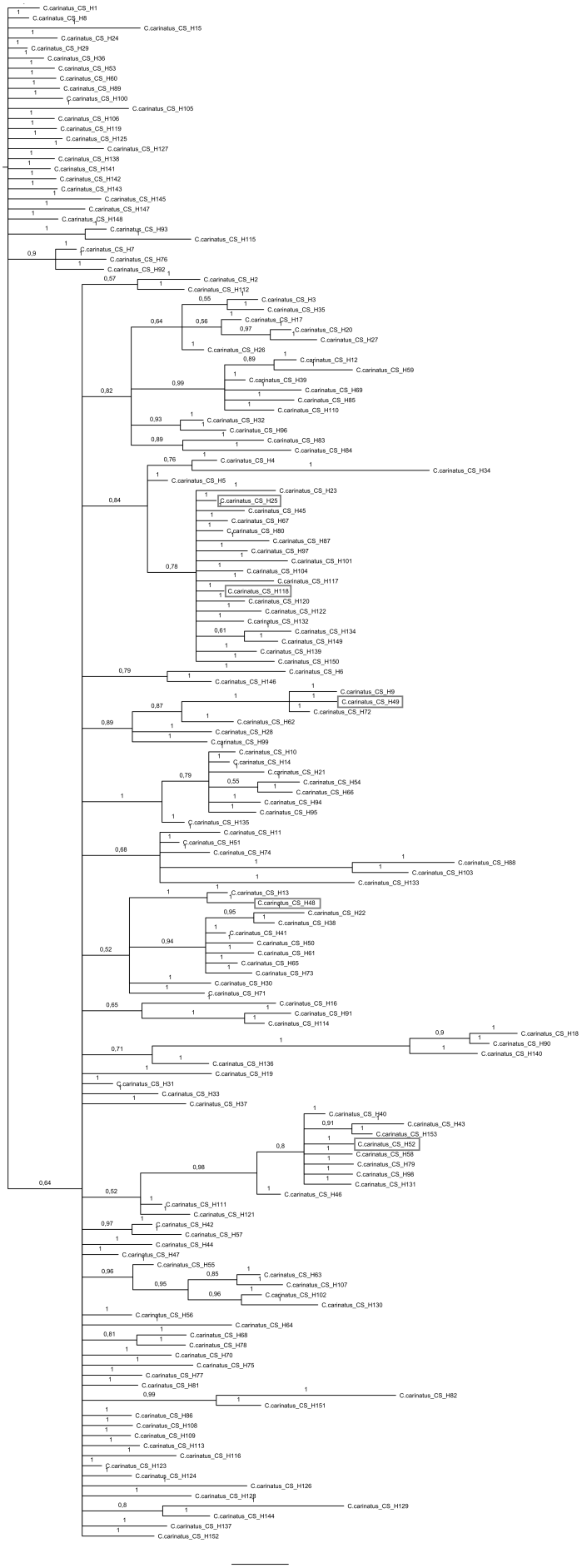

Figure 14: Phylogenetic tree of CS haplotypes. CS sequences corresponding to the aberrant COI sequences are labed with a grey rectancle. Branch probabilities are shown in decimal format.

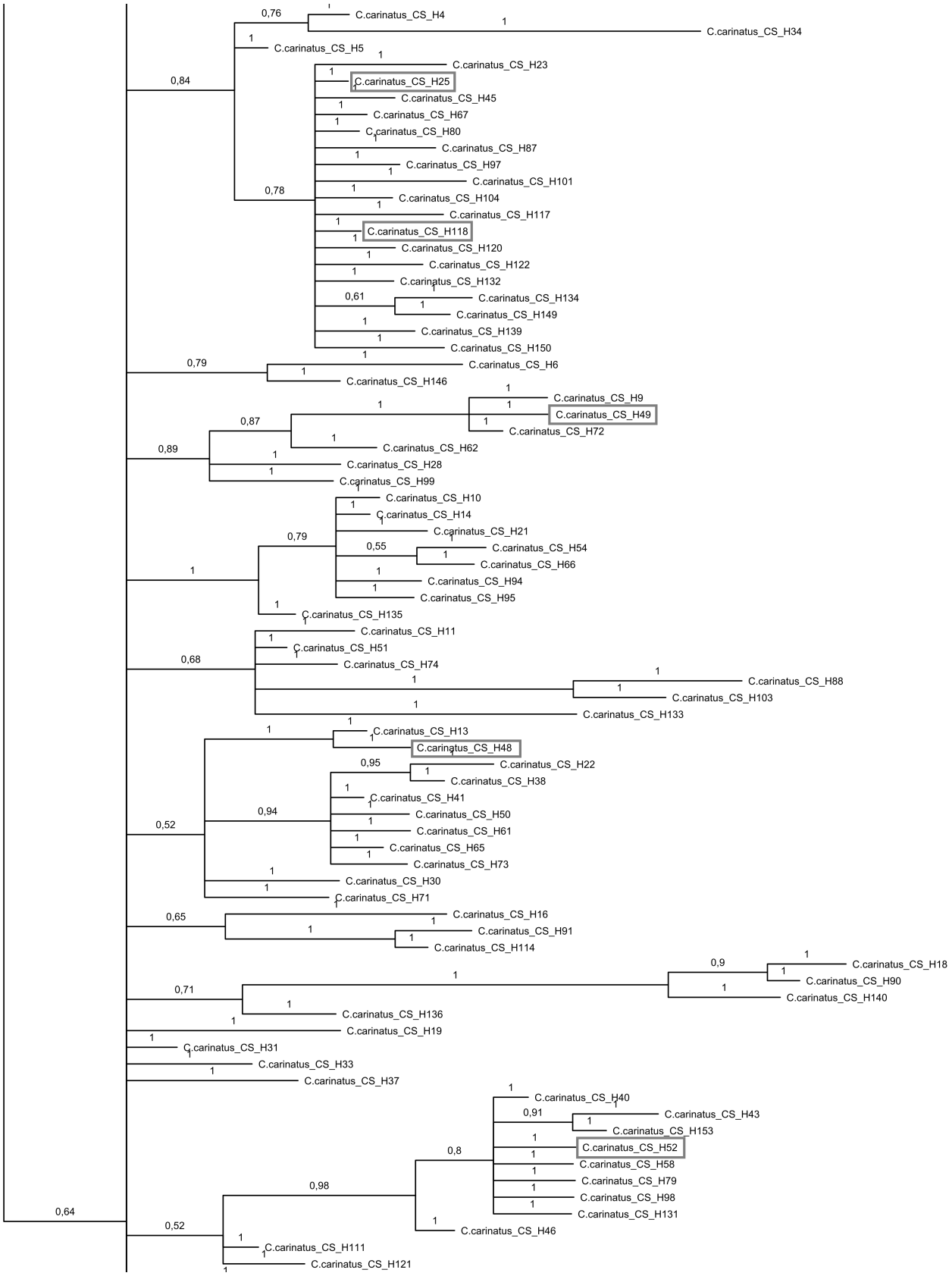

Figure 15: Detailed view of phylogenetic tree of CS haplotypes. CS sequences corresponding to the aberrant COI sequences are labed with a grey rectancle. Branch probabilities are shown in decimal format.

4 Discussion

4.1 Genetic diversity and population structure of *C. carinatus*

Genetic diversity of COI was within the range for planktonic copepods (Holmborn et al., 2011 and references therein). Nucleotide diversity remained constant over the whole study region and within a mid-range compared to literature. Haplotype diversity was high in both regions, but more diverse haplotypes occurred in the southern hemisphere, which may suggest a gene diversity loss in the northern hemisphere. The comparison of haplotype and nucleotide diversity among the surface and deep population in the northern Benguela (NB) did not reveal vertical differences in population structure. Haplotype sharing over the whole study area observed in both markers and only rare occurrence of private alleles with prevailing shifts in haplotype frequencies within COI indicate ongoing gene flow among populations.

Remarkably, phylogeographic analysis of both genetic markers had highly similar results for *C. carinatus*. Significant genetic structuring of *C. carinatus* was found within the study area as well as within populations. In some cases, genetic differentiation was observed between populations within groups. This may either indicate an inappropriate testing scheme or genetic structuring within regions, which could not be further dissolved in this study. Horizontal analysis revealed pronounced interhemispheric differences of *C. carinatus* populations, with less differentiation between NB and southern Benguela (SB). The positive result of the three-regions-test may rather be biased by the strong differences of Northwest Africa (NWA) to the southern hemisphere, as NWA significantly differed from NB but no difference was found when testing NB and SB separately. Even though differentiation of NWA to the SB could not be proven in this study, the pairwise F_{ST} between stations strongly suggest that there is distinct genetic differentiation of SB populations to the northern hemisphere.

The significant correlation of genetic and geographical distance within the whole study area indicates that geographically closer populations tend to be less differentiated than geographically distant populations. Low regression of the isolation by distance analysis may be affected by the broad haplotype sharing observed in *C. carinatus* populations. Independent analysis of the Benguela region did not reveal significant isolation by distance correlation which confirms the assumption of mostly interhemispheric genetic differentiation.

Vertical analysis of surface and deep *C. carinatus* populations did not reveal significant differences in genetic population structure that could be resolved with the molecular markers used

in this study. Therefore, horizontal differentiation of *C. carinatus* populations was more pronounced than vertical structuring in this study. Moreover, the observed differentiation among the hemispheres suggests a potential, but permeable barrier to gene flow close to the equator.

4.2 Possible drivers for the phylogeographic structure of *C. carinatus*

As dispersal capacity and oceanographic barriers may play a central role for genetic differentiation in copepods (Goetze, 2011; Norton and Goetze, 2013), the population genetic structure of *C. carinatus* could be driven by the oceanography along the west coast of Africa. Since *C. carinatus* performs ontogenetic migration, two vertically separated populations exist: the active shelf population and deep living resting stages along the continental slope down to 1000 m depth (Kosobokova et al., 1988; Timonin et al., 1992; Arashkevich et al., 1996; Arashkevich and Drits, 1997; Timonin, 1997; Loick et al., 2005; Verheye et al., 2005). Therefore, dispersal could be governed either by surface currents close to the African coast or by deep countercurrents along the shelf edge. Surface transport in the northern hemisphere may be facilitated by the southward flowing Canary current, the Guinea dome and the eastward flowing Guinea current, whereas the northward flowing Benguela current and the Angola gyre may play role for dispersal in the southern hemisphere (Houghton, 1976; Stramma, 2001). Moreover, upwelling systems are associated with offshore Ekman transport and equatorward flowing coastal jets over the shelf (Hagen, 2001). On the other hand, gene flow among *C. carinatus* populations may be strongly influenced by poleward undercurrents which have often been related to the transport of planktonic organisms and larvae (Timonin, 1997; John et al., 1998; Peterson, 1998; John et al., 2000; Verheye and Ekau, 2005; Auel and Verheye, 2007; Harkins et al., 2013). They are found in both hemispheres in the eastern Atlantic Ocean and may reach down to Antarctic intermediate water layers around 1000 m depth (Mittelstaedt, 1983; Barton, 1989; Wacongne and Piton, 1992; Lass et al., 2000; Hagen, 2001; Knoll et al., 2002; Shillington et al., 2006; Mohrholz et al., 2008; Chavez and Messié, 2009; Peña-Izquierdo et al., 2012). Moreover, *C. carinatus* resting stages may be transported by undercurrents over long distances due to their extremely reduced metabolism, high body mass and lipid content (Kosobokova et al., 1988; Arashkevich et al., 1996; Arashkevich and Drits, 1997; Verheye et al., 2005) which enables them to outlast extended starvation periods from 149 up to 192 days (Verheye et al., 2005). In contrast, active surface individuals can only survive 10 days at most without feeding which restricts their dispersal capacity to short time periods or upwelling blooms.

Seasonal variations of the current system along the west African coast may also influence dispersal and gene flow among *C. carinatus* populations. For instance, seasonal reversal of intermediate water flow has been discussed at latitudes higher than Cap Blanc (21°N) (Elmoussaoui et al., 2005; Peña-Izquierdo et al., 2012). In the northern Benguela, hypoxic South

Atlantic central water (SACW) originating from the Angola gyre intrudes to the south in austral summer, whereas in winter the equatorward flow of oxygen-rich Eastern SACW shifts further to the north (Mohrholz et al., 2008). Overall, gene flow among *C. carinatus* populations seems to be quite possible within the hemispheres considering coastal surface and undercurrent systems. The equatorial region in the eastern Atlantic is characterized by the termination and bifurcation of the eastward flowing EUC (Wacongne and Piton, 1992; Stramma, 2001) and cross-equatorial flow mainly occurs in the western Atlantic as part of meridional overturning circulation (Stramma et al., 2005; Garzoli and Matano, 2011). Still, cross-equatorial water exchange may happen due to thermocline convergence, equatorial upwelling and Ekman divergence (Stramma et al., 2003). This strongly supports the idea of a potential, but permeable barrier close to the equator shaping the genetic population structure of *C. carinatus*. Moreover, genetic breaks across the equator have already been found in other copepod species in the Atlantic Ocean (Norton and Goetze, 2013; Paiz-Medina and Kochzius, 2013). These authors suggest that the differentiation between northern and southern populations is mainly driven by the Atlantic gyres.

However, *C. carinatus* features a completely different life cycle strategy including dormancy and its high dependency on coastal upwelling systems and is therefore more likely influenced by the near-shore currents along the African coast described above. Even though *C. carinatus* has been repeatedly described to be able to maintain itsself in the upwelling system (Timonin, 1997; Peterson, 1998; Verheye et al., 1991; Verheye and Field, 1992; Verheye and Ekau, 2005; Auel and Verheye, 2007) habitat affinity does not seem to have a strong impact on genetic structuring of *C. carinatus* populations. The finding that NB and SB populations were similar in their genetic structure suggests ongoing gene flow between the regions. To date, strong genetic differences of coastal zooplankton species have only been observed across ocean basins (Nuwer et al., 2008; Harkins et al., 2013). Random sources for reseeding of the surface population during upwelling events has also been suggested for *Calanus pacificus* (Johnson, 2007) which follows a similar life strategy as *C. carinatus*. Furthermore, *C. carinatus* did not reveal any vertical structuring in the NB implying that the active surface population and the deep-living resting stages do belong to the same population. Therefore, observed CV body mass differences within *C. carinatus* (Auel et al., 2005; Verheye et al., 2005) may rather display an adaptation to different life cycle stages within the same genetic background. Moreover, the oxygen minimum zone in the northern Benguela does not seem to act as a strong barrier between *C. carinatus* populations as proposed by Auel and Verheye (2007).

Overall, maintenance mechanisms of *C. carinatus* do not seem to have a strong impact on genetic differentiation considering the high connectivity of the Benguela populations. Population genetic structure may be predominantly formed by large-scale ocean circulation. Surface and deep currents along the west African coast are likely conveyors for *C. carinatus* which may enhance gene flow within hemispheres. However, ocean circulation close to the equator may restrict dispersal of *C. carinatus* leading to the remarkable differentiation between both hemispheres. Even though present ocean-circulation serves as a good explanation for genetic

differentiation in *C. carinatus*, it should be kept in mind that genetic differences may also display a historic pattern that has persisted to present time (Palumbi, 1994; Papadopoulos et al., 2005; Blanco-Bercial et al., 2011; Henriques et al., 2014).

4.3 Cryptic species or marker evolution?

The analysis of mitochondrial COI revealed the occurrence of two molecular operational taxonomic units (MOTUs) along the west coast of Africa. Haplotypes within the prevalent MOTU were weakly differentiated (0.1 -1.3%), but a high magnitude of differentiation between both MOTUs occurred (9.5 – 10.4%) lacking intermediate values. Interestingly, both MOTUs occurred over the whole study area and did not have a clear spatial separation. Most of the individuals belonged to the prevalent MOTU, whereas only three highly diverse sequences from the other MOTU were found. These findings are in accordance with the study by Vinas et al. (unpublished) who described a single genetically-cohesive population of *C. carinatus* in the NE/SE Atlantic, but also found a few highly diverse sequences off the Spanish coast. Moreover, the comparison of available GenBank sequences to the COI data of this study confirmed the results. The construction of a haplotype network of the combined COI sequences revealed a perfect match of both datasets (see Fig. 16) which proved that the highly divergent sequences were not just an artefact in the dataset, but a real feature within *C. carinatus*.

The high divergence of the sequences and the absence of intermediate values may suggest the presence of a cryptic species, which is a common phenomenon in copepods (Goetze, 2003; Goetze and Ohman, 2010; Laakmann et al., 2012; Cornils and Held, 2014). The magnitude of genetic distance is well in line 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). However, after comparison to the nuclear data set, this hypothesis had to be rejected. When analysing the phylogenetic tree for CS, the pattern of two distinct groups disappeared. CS sequences from the divergent individuals clearly clustered together with other sequences contradicting the COI data. Therefore, it is highly unlikely that a cryptic species was found. The incident 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 coamplification 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 such as *Calanus finmarchicus* (Bucklin et al., 1999, 2000) or diaptomid calanoids (Thum and Harrison, 2009; Marrone et al., 2013). Since “classical” nuclear mitochondrial pseudogenes, so called numts, often contain InDels and mutations at any codon position which lead to a shift in reading frame and to the presence of stop codons, they can be easily identified by rigorous qualitity control (Song et al., 2008; Buhay, 2009; Calvignac et al., 2011). However, the aberrant sequences found in this study did not contain any stop codons or frame shifts, solely three differing amino acids. Still, the pronounced nucleotide diversity of the

sequences may indicate a recent translocation of a COI copy to the nucleus which most of the time remains undetected (Calvignac et al., 2011).

Heteroplasmy, the co-existence of several variants of plastids in an organelle, cell, tissue or individual, is another possibility to explain the occurrence of highly variant sequences. 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. However, Barr et al. (2005) and Rokas et al. (2003) pointed out that mitochondrial heteroplasmy and recombination might be more common than previously thought. One mechanism leading to heteroplasmy, "doubly uniparental inheritance" (DUI) was found in many bivalves (e.g. Saavedra et al., 1997; Theologidis et al., 2008, review by Breton et al., 2007) where genetically distinct male and female mitochondrial lineages exist. Moreover, considering that genes coding necessary enzymes for recombination were found in animal mitochondria (Thyagarajan et al., 1996) and experimental evidence for recombinant mtDNA is growing (e.g. Ladoukakis and Zouros, 2001; Ladoukakis et al., 2011; Chen, 2013), multiple variants of mitochondrial COI might also appear in *C. carinatus*.

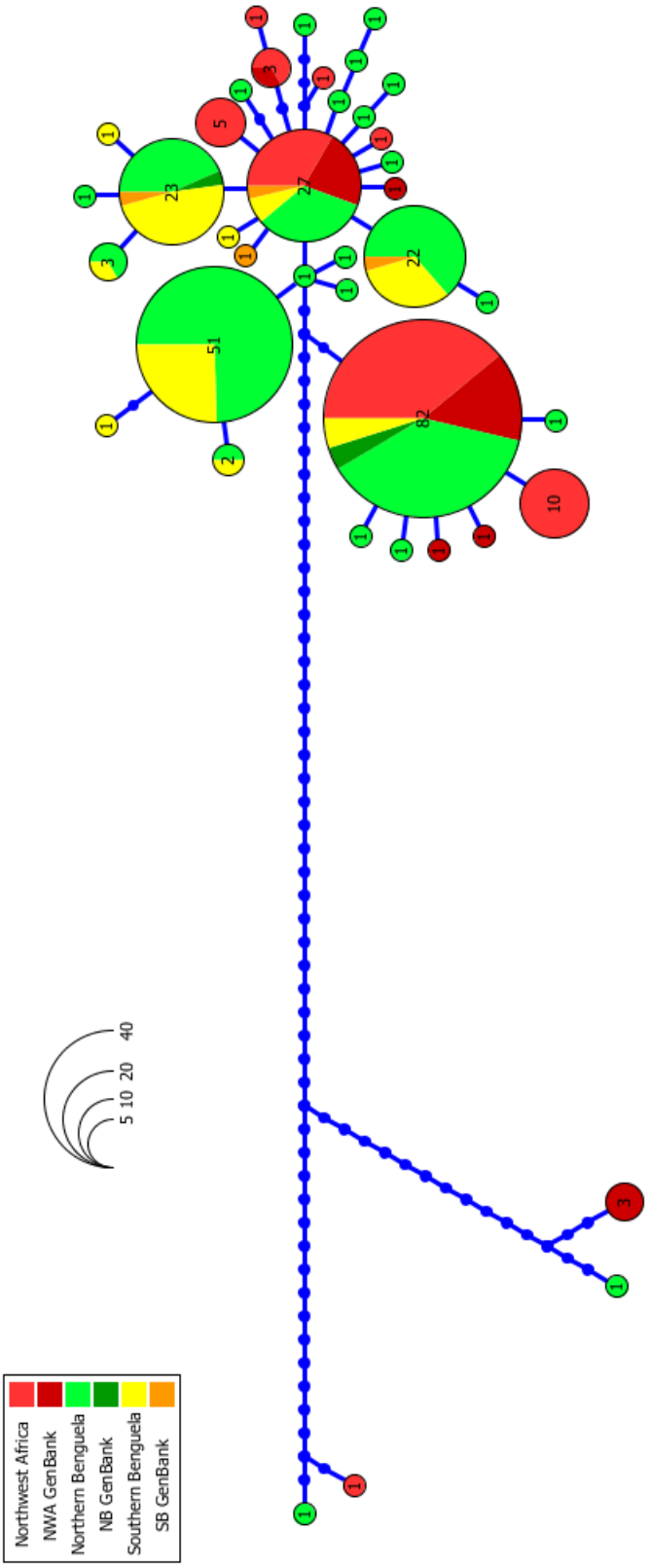

Figure 16: Haplotype network of the 484 bp COI fragment. Combined data set of this study and available sequences on GenBank.

4.4 Quality control of the sequences

Accurate quality control was carried out to ensure correctness and sufficient quality of sequences that were finally used for phylogeographic analysis. This also includes the approval of correct species identification and the control for possibly occurring pseudogenes, non-functional copies in the nuclear genome, which could bias the final analysis. Translated amino acid sequences of both marker genes did not contain any stop codons which implies that functional copies of the genes were found. Bias of misidentification or by non-functional copies of the marker genes can therefore be excluded as source of error. Recurring amplification and sequencing errors, misinterpretation of single bases by the editing programme or human error may still have an effect on sequence quality.

4.5 Possible factors biasing the analysis

Several factors could have influenced the genetic diversity and population structure found for both markers. For mitochondrial COI, these processes include hybridization, assymetrical mating and migration preferences (Sunnucks, 2000; Teske et al., 2011), direct selection of the marker gene, or indirect selection due to strong linkage within mtDNA, inter-nuclear-mitochondrial gene coupling (Ballard and Whitlock, 2004) or maternally inherited symbionts (Hurst and Jiggins, 2005) as for instance observed in isopods (Marcadé et al., 1999; Rigaud et al., 1999). However, a possible sex change mechanism in the familiy Calanidae (Fleminger, 1985) would rule out the possibility of female associated migration preferences. Moreover, the analysis of neutral evolution did not show a significant departure from neutrality for all stations. Therefore, it is highly unlikely that the genetic pattern of mitochondrial COI in *C. carinatus* was shaped by direct selective pressures. The nuclear gene CS was chosen as independent marker to prove the genetic pattern observed within mitochondrial COI. Since recombination occurs more often in the nucleus, nuclear genes do not face the problem of strong linkage to other genes. On the other hand, allele sequences of heterozygous DNA cannot be determined just by sequencing. The numerical approach for haplotype inference used in this study can lead to uncertainties in haplotype sequences and therefore bias the phylogegraphic analysis for the nuclear marker CS. Moreover, the significant negative values of Fu's FS neutrality test (Fu, 1997) indicated an excess number of alleles for most of the stations, which may suggest either population expansion or selection acting on the marker gene. However, the striking consistency of the results observed in both markers strongly suggests that we are indeed looking at the true evolutionary history of *C. carinatus* and not at a genetic pattern shaped by selective pressures.

It should also be noticed that sampling was not optimal to answer the intended research questions. Different sampling efforts were applied in the three regions and led to unequal number of individuals analysed for the different areas. Since stations were pooled to regions, 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 could have favoured the sampling of singleton haplotypes, which might have influenced haplotype diversity. Station-wise analysis for neutrality and pairwise F_{ST} was most probably influenced by sample size, especially considering St.1, which only contained 5 individuals. The contradictory AMOVA results may also be a consequence of differing sample size especially regarding the comparison of NWA and SB, which clearly indicated strong differentiation within the pairwise F_{ST} analysis, but did not reveal significant differences within the AMOVA. Moreover, samples were obtained in different years and seasons considering that temporal changes may influence population genetic structure of *C. carinatus*. Vertical structuring of populations was only tested in one region, the NB where both surface individuals and deep-living resting stages were obtained. Individuals from the northern hemisphere originated from deep water strata and for SB solely surface individuals were obtained. Hence, for horizontal analysis of the data set, we assumed temporal stable populations of *C. carinatus* and the absence of vertical differences over the whole study area.

Most importantly, differing tendencies of the molecular markers for population genetic structure of *C. carinatus* can be explained by different evolutionary rates of the genes. Moreover, non-significant results do not necessarily mean that no differences exist. It may just indicate that the resolution limit of the marker gene is reached and differences are not well displayed within the population genetic context any more.

5 Conclusion

In a nutshell, a genetically-cohesive population of *C. carinatus* ocurred along the west coast of Africa. Distinct genetic and geographical differentiation among the northern and southern hemisphere was discovered with a potential barrier close to the equator, confirming the first hypothesis. Haplotype sharing across the entire study area indicated that this barrier was not particularly effective and suggests active gene flow among populations. Genetic differentiation among both hemispheres may be best explained by large scale ocean-circulation along the west African coast which promotes conveyance by surface and undercurrents within hemispheres, but hinders cross-equatorial transport. The second hypothesis suggesting populations restricted to rather local systems could not be consistently proven. This especially concerns the high similarities among the Benguela regions, which implicates frequent gene flow among these regions. Moreover, a difference between the active surface population and the deep-living resting stages in the northern Benguela was not proven rejecting the third hypothesis. However, considering the high similarity of northern and southern Benguela, maintenance mechanisms of *C. carinatus* seem to play a minor role for genetic structuring and rather support the idea of frequent exchange via currents among populations within the Benguela region. Evidence for a cryptic sibling species was not detected within the study area. Highly diverse sequences within mitochondrial COI might rather display effects of marker evolution like heteroplasmy or a paralogous copy recently translocated to the nuclear genome.

5.1 Future prospects

Future studies should focus on getting a better understanding of the vertical and horizontal genetic population structure of *C. carinatus* as well as connectivity among populations and detailed analysis of life cycle strategies in different regions. This includes an elaborate sampling scheme comprising all major upwelling systems along the coast (including Guinea). If possible, both the surface and deep population should be sampled taking the upwelling seasons of the different regions into account. Moreover, Sabatini et al. (2007) pointed out that the original species description of *C. carinatus* most probably relied on a specimen from the SW Atlantic, which was recently identified as a cryptic sibling species (Vinas et al., unpublished). Therefore, thorough morphological examination of the SE Atlantic population should be considered. Combined with genetic analysis and possibly non-destructive extraction methods (Cornils, pers.

comm.), this may finally prove the origin of the highly diverse sequences found in the mitochondrial COI gene. For better resolution of the phylogeographic structure of *C. carinatus*, a set of multiple markers should be chosen for analysis. This may include a next-generation sequencing approach, which will facilitate the identification of potential marker genes such as InDel markers, microsatellites or SNPs. In addition, the obtained data may be further used to answer other ecological research questions concerning performance or life cycle strategies of *C. carinatus*. Structure analysis (Pritchard et al., 2000) of multiple locus data, without a priori defined groups, may be able to further dissolve population structure of *C. carinatus*. Moreover, demographic analysis and migration tests may clarify migration rates among regions and demographic patterns such as recent population expansion, which was also indicated by some of the neutrality tests. Demographic analysis may include skyline plots (Ho and Shapiro, 2011) that can estimate past changes in population size over time and may give an improved understanding of the demographic history of the species in different regions. Finally, computer modelling may further help to understand which currents play major roles for transport of *C. carinatus*.

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I declare that I have authored this thesis independently, that I have not used other than the declared sources/resources, and that I have explicitly marked all material which has been quoted either literally or by content from the used sources.

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