SCAR FELLOWSHIP SCHEME 2005-6

Final Report

Fellowship holder: Simone Nunes Brandão, University of Hamburg

Host: Isa Schön, Royal Belgian Institute of Natural Sciences

Eurybathy and circumantarctic distribution in Antarctic benthos? The case of the ostracod family Macrocyprididae (Crustacea)

ABSTRACT

The family Macrocyprididae is used as a model to test the theories predicting circumantartic and eurybathic distribution of benthic species from the Southern Ocean. This family was reported to include five circumantarctic species and one species found in many localities in the Subantarctic and Antarctic regions of the Atlantic sector of the Southern Ocean. Furthermore, eurybathic distributions (depth range from ~1,900 to 3,400m) had been reported for four of these species. Otherwise, a recent taxonomic revision used a narrow morphological species definition and recorded restricted depth and geographic distribution of single species. Herein genetic markers are used to investigate the geographic distribution of species and consequently test the prevailing theories. The genetic results (COI and ITS markers) support the most restricted depth and geographical ranges and confirm the usefulness of the narrow morphological species definition. Therefore, a reevaluation of the prevailing theories on the circumantarctic and eurybathic distribution of Southern Ocean benthic (especially ostracod) species, should be considered.

KEYWORDS: Ostracoda, Southern Ocean, COI, ITS, biogeography, eurybathy, circumantarctic distribution.

INTRODUCTION

Circumantarctic and eurybathic distribution together with high levels of endemism are considered to be important characteristics of the benthos in the Southern Ocean (SO) (e.g. Arntz *et al.*, 1997; Brey *et al.*, 1996). The eastward Antarctic Circumpolar Current as well as the westward Antarctic Coastal Current should facilitate genetic exchange between marine populations living around the Antarctic continent (e.g. Arntz, *et al.*, 1994; Clarke & Johnston, 2003). While explanations for eurybathy include the lack of a thermocline, the sinking of water masses close to the Antarctic continent in the process of deep water formation, as well as the adaptative pressures on organisms related to the glacial-interglacial regime (Brey *et al.*, 1996). Circumantarctic and eurybathic distribution have been consistently and widely

reported in the Southern Ocean (e.g. Clarke & Johnston, 2003; De Broyer *et al.*, 2007; Griffiths *et al.*, 2009; Linse *et al.*, 2006; Peña Cantero & Gili, 2006). However, the conclusions on which such studies are based are extremely and inevitably dependent on the morphological species definition adopted in the many Antarctic faunistic investigations, because genetic analyses on the intraspecific level are still very scarce in the SO (Linse *et al.*, 2007). Species borders applied in taxonomy are flexible and each researcher takes her/his own decisions whether the variability observed between individuals is intra- or interspecific. Furthermore, in the last decades, the general acceptance that SO benthos displays eurybathic and circumantarctic distribution probably influenced taxonomic decisions. In some cases, such as the ostracod *Bairdoppilata simplex*, the isopod *Ceratoserolis trilobitoides*, the bivalve *Lissarca notorcadensis* and the crinoid *Promachocrinus kerguelensis*, the so-called "circumantarctic species" were allowed to present an extremely high level of morphological variation (Brandão, 2008a; Cope & Linse, 2006; Dingle, 2003; Clark & Clark 1967; Wägele, 1986). It is worth noting that previous morphological studies showed that some of these widely distributed SO species are actually groups of distinct species (Brandão, 2008a, 2008b, submitted; Conlan, 1990, Dahl 1990). More importantly, genetic evidence on fishes, isopods, molluscs, and octopuses also demonstrated that some of the so-called circumantarctic or eurybathic species were actually composed of geographically or bathymetrically isolated genetic unities (Allcock *et al.*, 1997; Bernardi & Goswami 1997; Held & Wägele 2005; Linse *et al.*, 2007; Raupach & Wägele, 2006; Raupach *et al.*, 2007). Otherwise, no research on the genetics of Antarctic or deep-sea ostracods (the model organism of the present study) has been published so far. Concerning the three families investigated herein, only four 18S sequences were available in the Genbank (of the National Centre for Biotechnology Information) prior to the present study.

Notably, all these generalizations on the geographic and bathymetric distribution of the SO fauna were based on observations in shallow waters, because the SO deep-sea was, before the ANDEEP project, almost completely unknown (Brandt *et al.*, 2004). The ANDEEP project aimed to fill into the immense gap of knowledge on the deep-sea biology of the Southern Ocean, in order to test existing theories on Antarctic and global patterns of biodiversity and biogeography. Between 2002 and 2005, three cruises on board of the *R.V. Polarstern* provided biological samples from 40 stations (748 to 6,348m water depth) on the Atlantic sector of the SO. Several gears were deployed in order to gather data from a large range of body-sized organisms (bacteria to megafauna) (Brandt *et al.*, 2007). In the current

study, ostracods from a wide depth range collected mostly during the ANDEEP project are analysed.

Our model organism, the Macrocyprididae, was one of the most abundant ostracod family in the ANDEEP samples (~15% of all ostracod specimens). Previous to the ANDEEP project, six macrocypridid species in two genera had been recorded from the SO: 4 circumantarctic (*Macroscapha inaequalis* (Müller, 1908) (311 to 426m); *Macroscapha inaequata* Maddocks, 1990 (311 to 3694m); *Macroscapha tensa* (Müller, 1908) (68 to 2013m); *Macroscapha turbida* (Müller, 1908) (16 to 494m)) and 2 reported from many localities in the Subantarctic and Antarctic regions of the Atlantic sector of the SO (*Macromckenziea glacierae* Maddocks, 1990 (97 to 3111m); *Macroscapha opaca* Maddocks, 1990 (95 to 2738m) (Hartmann, 1997). The material collected during the ANDEEP project plus the re-study of previously sampled specimens provided several new taxa, increasing the number of Antarctic macrocypridid species to 31 in 4 genera (Brandão, submitted). This augment in the diversity was partly due to the narrower morphospecies definition adopted by myself than the one adopted by previous authors. The 'new' knowledge on Antarctic Macrocyprididae can be summarized as follows. The genus *Macroscapha* (*Mh.*) is the most diverse with 19 species, seven of these studied herein (i.e. *Mh. falcis*, *Mh. solecavai*, *Mh. walterae*, *Mh. inaequata*, *Mh. turbida*, '*Mh. opaca*'). Furthermore, the supposedly circumantarctic *Mh. turbida* was shown to be a complex of at least four morphospecies. *Mh. tensa* was considered a *nomen dubium* and the specimens identified by previous authors as *Mh. tensa* and *Mh. opaca* were re-studied and included in the species complex *Mh. tensa-opaca*, which is composed of at least eight morphospecies (three of these studied herein: *Mh. falcis*, *Mh. solecavai* and '*Mh. opaca*'). One of us (SNB) concluded that the copulatory process of the hemipenis is the best character for the differentiation between seven of the eight morphospecies of the complex *Mh. tensa-opaca*. Otherwise, one of these morphospecies (i.e. '*Mh. opaca*') remains too variable in the size and shape of the valves, but it was not possible to further divide it in discrete morphological groups (especially because the copulatory processes of the hemipenises are similar in all males and because the variation in valve is continuous). The genus *Macromckenziea* presents now two described SO species (i.e. *Mk. glacierae*, *Mk. giambonini*) both investigated herein. The genus *Macrosarisa* was reported for the first time from the SO and is represented by two new species (*Ms. andeep* and *Ms. fahrbachi*), also included in the present study. Finally, the genus *Macropyxis* was represented by seven species, four analysed herein (*Mx. cronini*, *Mx. hornei*, *Mx. jeans*, *Mx. parajeans*).

In the present study, we investigate whether these narrowly defined morphospecies are compatible to genetic species and thus mirror the biological reality, or whether this "new" high biodiversity is an artefact of an over splitting taxonomy, based on, for example, convergent, plesiomorphic or plastic morphological traits. We examine the genetic patterns within and between different taxonomic levels of the ostracod family Macrocyprididae. Moreover, because species delimitation based on DNA datasets is not straightforward, different approaches (i.e. fixed threshold in genetic distances, haplotype networks, phylogenetic reconstructions and a quantitative approach on branching rates) are attempted in the search for the species boundaries. By focusing this study on a circumantarctic and eurybathic species (i.e. *Macroscapha opaca*, 84 to 2921m) the theories which predict circumantarctic and eurybathic distribution for the SO benthos are tested.

MATERIAL AND METHODS

The northern limit of the Southern Ocean is herein defined as the Polar Front. Details of morphological identifications are given in Brandão (submitted).

Sampling

Samples were collected from 19 stations on the Atlantic Sector of the SO during three cruises of the ANDEEP project on board of the *R.V. Polarstern* (Brandt *et al.*, 2007). Additional specimens were collected from the Ross Sea on board of the R. V. *Italica* during the ROSSMIZE project (Rehm *et al*., 2006). The gears used were an epibenthic sledge, a Rauschert dredge, and an Agassiz trawl. When samples arrived on board, they were immediately fixed in pre-cooled (0°C) 96% ethanol and kept at -20°C for at least 48 hours.

DNA extraction, amplification and sequencing

Because other researchers had difficulties in obtaining good quality DNA-extractions from ostracods and other small, deep-sea invertebrates, the developed DNA extraction is explained in more detail. DNA was extracted individually for a total of 293 ostracods specimens (212 with the QIAamp® DNA Mini Kit; 81 kindly provided by Dr Amy Driskell, 'Barcod of Life Initiative') comprising 19 morphospecies, 8 genera, and 3 families. Because an accurate identification of macrocypridid specimens based solely on valves is impossible, a more elaborate DNA-extraction procedure was developed. Previous to the extraction, the valves of most specimens (212) were dissected and transferred to micropaleontological slides;

bi-distilled water was added to the dishes containing each ostracod and left there for at least one hour (up to eight hours) at room temperature (~15-20°C). The animal was dissected and taxonomically important 'soft parts', i.e. hemipenis, furca, male and female appendages V (rarely also the antenna I and II, mandible, maxilla I, and appendages VI and VII) were left in the dish with water. The remaining soft parts were transferred to the lyses buffer, and at least one hour (up to 5 hours) later, protein K was added. The digestion step lasted overnight (at least 12 hours). The taxonomically important soft parts were meanwhile mounted on a glass slide with Hydromatrix permanent medium. The DNA extraction was subsequently performed according to the manufacture's 'Tissue Protocol'. The DNA was eluted twice with 100µl of elution buffer.

For PCR amplification, 2 kits were used: (1) the HotMasterMix (2.5x) (Eppendorf) (HotMaster Taq DNA Polymerase (50 U/ml), 2.5x HotMaster Taq Buffer pH 8.5 with 6.25 mM $Mg(OAc)_2$, 500 µM of each dNTP and stabilizers); and (2) the HotStarTaq Master Mix Kit (Qiagen) (HotStarTaq DNA Polymerase, PCR Buffer with 3 mM $MgCl_2$, and 400 mM each dNTP). Because DNA concentration of extracts was variable (0.51 to 9.21ng/µl), PCR conditions needed to be reoptimized several times. PCR products were checked and photographed on agarose gels stained with SybrSafe. Each PCR product was purified with the 'GFX PCR DNA and Gel Band Purification Kit' (GE Healthcare). 1 to 3µl of purified PCR product plus 0.01µM primer was used with the Big Dye Terminator v. 3.1 cycle sequencing kit (Applied Biosystems) following the manufacture's protocol and analysed on an ABI3130XL sequencer. Identical primers were used for both PCR and sequencing. Sequencing reactions were carried out in both directions.

Sequence curation, alignment and analyses

Sequences of each specimen were assembled and manually checked in ChromasPro® v 1.41 (Technelysium Pty Ltda). Sequence identity was confirmed with BLASTN (Zhang et al, 2000) and BLASTX searches (Altschul *et al.*, 1997) and sequence alignments were performed with CLUSTALX (Thompson *et al.,* 1994) for all COI sequences and some of the ITS sequences (i.e. alignments involving close species). ITS alignment containing sequences from different genera were performed with Muscle (Edgar, 2004), checked manually in the program MEGA (Tamura *et al.*, 2007), and subsequently cured (deletion of gaps and ambiguous regions, default settings) with the program Gblocks (Castresana, 2000). Modeltest (Posada & Crandall, 1998) was used to identify the model which best fit out dataset. The

programs Muscle and Gblocks were used in the online platform www.phylogeny.fr (Deeper *et al.*, 2008).

Because of the numerous insertions and/or deletions in the macrocypridid, pontocypridid and cypridid ITS sequences, it was not possible to use a single alignment for calculating p-distances between families, genera and species. When the families Pontocyprididae and Cyprididae were included and all gaps and ambiguous sites were removed, only 230 sites were left, which did not provide sufficient differences at the genera and species level. Therefore, only the 164 ITS macrocypridid sequences were aligned and gaps and ambiguous sites were excluded, resulting in a final alignment of 432bp, from which the p- and corrected distances were calculated.

Subsequently, the following analyses were performed:

(I) haplotype networks were generated by TCS (Clement *et al*., 2000) according to the 95% parsimony method of Templeton and colleagues (1992). Because of the large number of deletions/insertions on ITS sequences, here one independent alignment and curation was conducted for each group of similar species. Sites with gaps were excluded from the ITS sequences prior to network calculations. No gaps were present in the COI alignments. For COI, TCS analyses the sequences were conducted according to the genus (*Macroscapha* and *Macromckenziea*).

(II) Uncorrected p-distances and the corrected distances were calculated with Mega and Paup (Swofford, 1998), respectively. Additional to the histograms, TaxonDNA (Meier *et al.*, 2006) was used to identify the threshold for species delimitation in the genera *Macroscapha* and *Macromckenziea*.

In the present study, the term “intraspecific” is used for relationships within morphologically well defined species, i.e. excluding relationships within cryptic species (=species complexes). These last relationships are herein named “intra- or inter-cryptic”. Similarly the term interspecific is used for relationships between morphologically well defined species (i.e. excluding relationships within cryptic species).

(III) Phylogenetic Reconstructions

Parsimony trees with 1,000 bootstraps were calculated with the program TNT (Goloboff *et al.*, 2000) with default settings in the online platform www.phylogeny.fr. Maximum-likelihood (ML) trees with 1,000 bootstrap replicates and also with LR-ELW test (Jobb, 2008) were calculated with Treefinder (Jobb, 2008). For comparisons, additional ML trees were

calculated in the program PhyML (Guindon & Gascuel, 2003) in the online platform www.phylogeny.fr and the reliability of internal branches was assessed using the aLRT test (SH-Like) (Anisimova & Gascuel, 2006). The ultrametric trees were constructed using the Global Rate Minimum Deformation method (GRMD) as implemented in the program Treefinder (Jobb, 2008). This method is trying to keep the real rates as similar as possible to a global rate. The global rate is optimized with the other rates as a free parameter over all edges in the tree. It reflects the assumption that rate is nearly constant over time.

(IV) Pons and colleagues' (2006) algorithm

We used the maximum likelihood approach proposed by Pons and colleagues (2006) to quantitatively search for the species limits. They supposed that there is a change in branching rates at the species boundary. To test for the predicted change in branching rates and to optimize the distance in an ultrametric tree where this change occurred, we used a code (T. Barraclough, pers. comm.) implementing their approach in the statistical software R (R Development Core Team, 2007) using functions from the APE library (Paradis *et al.*, 2004) to test for the predicted change in branching rates and to determine the resulting putative species. Interestingly, this last method does not require prior assumption of population boundaries.

The following abbreviations are used for the macrocypridid genera (Maddocks, 1990): *Mh.*, *Macroscapha*; *Mk.*, *Macromckenziea*; *Ms.*, *Macrosarisa*; *Mx.*, *Macropyxis*.

RESULTS

A total of 173 COI sequences (all 660bp long, no gaps, and no deletions) were obtained from 13 morphospecies in 5 genera and 3 families. Concerning ITS, DNA from165 specimens in 17 morphospecies species from 5 genera and 2 families was successfully amplified and sequenced. The ITS sequences vary from 636 to 733bp in length.

Because no COI and ITS sequences from Macrocyprididae, Pontocyprididae and Bythocytheridae available in Genbank, the BLASTN search of COI sequences resulted in homologies of ~75-90% with hexapods (Lepidoptera, Diptera, Coleoptera) (query coverage 80-95%), and the BLASTX search resulted in ~80% identity (query coverage 80-95%) with an unidentified myodocopid. The BLASTN searches for ITS sequences resulted in very low query coverage 6-10% and 88-100% identity with nematode "partial 18S rRNA gene, ITS1,

5.8S rRNA gene, ITS2 and partial 28S rRNA gene. Otherwise, for the reasons explained in the 'DISCUSSION' section, the possibility of contamination was discarded.

From here onwards, the results are presented for each marker separately.

COI

(I) Haplotype networks

A total of 12 haplotype networks (9 for *Macroscapha*, and 3 from *Macromckenziea*) were obtained from the COI sequences.

The morphospecies *Mh. inaequata*, *Mh. turbida*, *Mh. walterae* and *Mh. aff. walterae* are isolated from each other in the 4 haplotype. The 108 sequences from the three morphospecies of the '*Macroscapha tensa-opaca*' species complex (i.e. *Mh. falcis*, '*Mh. opaca*' and *Mh. solecavai*) revealed 79 haplotypes in five isolated haplotype networks.

Regarding the genus *Macromckenziea*, the two similar morphological species (*Mk. glacierae* and *Mk. giambonini*) collected from the Eastern and Western Weddell Sea (stations k and r) were studied. Three separated networks were obtained from the 17 COI sequences (14 haplotypes). The first network involves 12 of the 13 haplotypes of the morphospecies *Mk. glacierae*. The Eastern and Western populations do not share haplotypes.

(II) Genetic distances

COI p-distances between genera (*Mh.*, *Mk.*, and *Ms.*) fluctuate between 13.60 and 20.30%, those between the families Macrocyprididae, Pontocyprididae and Bythocytheridae vary from 19.80 to 27.60%. Interspecific distances vary from 8.30% to 18.50% within the genus *Macromckenziea*. Intraspecific p-distances within the genera *Macroscapha* and *Macromckenziea* range from 0.00 to 2.30%, with the exception of the cryptic species. In the species complex *Macroscapha tensa-opaca,* the distances between specimens included in different haplotype networks are between 1.80% to 7.70%. The p-distances between *Mh. walterae* and *Mh.* aff. *walterae* are from 4.70 to 5.00%. In the genus *Macroscapha*, interspecific p-distances range from 7.40 to 13.00%. The results on the HKY distances between the different taxa are similar to the ones from the p-distances.

In order to search for a threshold for intraspecific genetic distances, each of the species complexes (i.e. '*Mh. tensa-opaca*'; and all specimens of *Mk. glacierae*) was considered as a single species. If we chose as threshold (6.21%) the value calculated on the overlap between

intra- and interspecific borders with a 5% error margins of the intrageneric sequences (as implemented by Meier *et al.*, 2006), our identifications were not efficient: 108 (62.42%) sequences were correctly identified; 63 (36.41%) were ambiguous; 2 (1.15%) sequences could not be matched; and 1 (0.57%) sequence was incorrectly identified.

(III) Phylogenetic Reconstructions

Considering a threshold for the bootstrap supports of 70%, the COI ML tree presents three major clades: (1) *Mh. inaequata* plus *Mh. walterae* and *Mh. aff. walterae*; (2) *Macroscapha tensa-opaca* species complex; and (3) *Mh. turbida*.

The 108 sequences from the three morphospecies of the *Macroscapha tensa-opaca* species complex - i.e. *Mh. falcis*, '*Mh. opaca*' and *Mh. solecavai* – form a well supported major clade, which is further subdivided into five clades. These five clades are equivalent to the five haplotype networks.

Each of the morphospecies *Mh. inaequata*, *Mh. walterae* and *Mh. turbida* are monophyletic. As could be anticipated by their morphological similarity, *Mh. walterae* and *Mh. aff. walterae* are sister groups, and these last species are together a sister group to *Mh. inaequata*. In this tree, the position of *Mh. turbida* is uncertain.

The parsimony tree displays similar patterns as the ML tree although some subclades are less well supported.

(IV) Pons and colleagues' (2006) algorithm

When applying the algorithm by Pons and colleagues (2006) to the COI phylogeny, the likelihood of the tree without a transition between coalescent and speciation lineage branching processes is significantly smaller than the one taking these transitions into account. The solution with the highest likelihood provided 10 entities, 9 clusters. Other solutions within 2 log likelihood units of the maximum (as an approximation of a confidence interval of 95% (Edwards, 1972 apud Pons *et al.*, 2006)) show the number of entities fluctuating between 9 and 21.

ITS

(I) haplotype networks

A total of 6 haplotype networks (4 for *Macroscapha*, and 2 from *Macromckenziea*) were obtained from the ITS sequences.

All 102 ITS sequences of the species complex '*Macroscapha tensa-opaca*' group together in one haplotype network. Similar to COI, the ITS sequences of the three morphospecies *Mh. inaequata*, *Mh. turbida* and *Mh. walterae* are isolated from each other, but *Mh. aff. walterae* cluster with *Mh. walterae* in one network.

Concerning the genus *Macromckenziea*, the two morphospecies (*Mk. glacierae* and *Mk. giambonini*) appear in two distinct networks.

(II) Genetic distances

The p-distances between genera range from 8.80 to 20.90%. The intraspecific p-distances vary from 0.00 to 0.95% in *Macroscapha*; from 0.00 to 1.94% in *Macromkenziea*, between 0.47 and 0.95% in *Macrosarisa;* and 0.00% in *Macropyxis.* The p-distances within cryptic species range from 0 to 2.45%. Distances between species from the same genus fluctuate from 2.10 to 8.40% in *Macroscapha*, 5.80 to 6.00 in *Macromkenziea*; 21.60 to 22.00% in *Macrosarisa*; and 0.00 to 7.40% in *Macropyxis*. The HKY distances are similar to the p-distances.

Since the ITS sequences present numerous deletions and insertions, making alignment between species impossible, we do not perform analyses on distance thresholds for species delimitations.

(III) Phylogenetic analyses

In accordance with COI, all morphospecies (except by '*Mh. opaca*') are monophyletic in the ITS tree. Again considering a threshold for the support of 70%, the major clades, the *Macroscapha tensa-opaca* species complex and *Mh. turbida* are highly supported. But contrary to COI, the grouping of *Mh. inaequata*, *Mh. walterae* and *Mh. aff. walterae* remains uncertain. Otherwise, the monophyly of each of these last three morphospecies is highly supported. Regarding the '*Macroscapha tensa-opaca*' species complex, both *Mh. falcis* and *Mh. solecavai* are monophyletic and also form together a highly supported. Further relationships among the subclades of the '*Mh. tensa-opaca*' species complex are puzzling.

The topologies of the most parsimonious tree are congruent with the ML tree, but the bootstraps values tend to be lower in the MP.

(IV) Pons and colleagues' (2006) algorithm

Also with the ITS dataset, the likelihood of the tree without a transition between coalescent and speciation lineage branching processes is significantly smaller than for the one taking these transitions into account. The solution with the highest likelihood provides 15 entities, 12 clusters. Other solutions within 2 log likelihood units of the maximum (i.e. 92.99084 and 94.83776) (as an approximation of a confidence interval of 95% (Edwards, 1972 apud Pons *et al.*, 2006)) show the number of identified entities fluctuating from 7 to 24. The solution with the highest likelihood provides a puzzling picture, which fits those of the haplotype networks and the ML tree.

DISCUSSION

Sequence identity

In the case of the ITS sequences, which all gave the highest BLAST scores with nematodes, we can discard the possibility of contamination because the query coverage was very low (6-10%) and only comprised about 60 nucleotides; and all 165 sequences gave similar results in BLASTN searches. It is rather unlikely that all samples would be contaminated by ITS of nematodes but not COI, for which we also used universal primers

(Folmer *et al.*, 1994). Moreover, the phylogenetic relationships based on these ITS sequences are in accordance with morphological and COI phylogenies (see above).

Morphological and genetic species delimitation

Two genetic methods tested herein proved useful in providing species limits. In general, the results from the haplotype networks and the phylogenetic reconstructions reveal a strong correlation between molecular divergence and the narrow species morphological definition adopted herein. In the present study, both morphology and molecules support the monophyly of the following. *Macroscapha* taxa: *Mh. inaequata*, *Mh. walterae*, *Mh. aff. walterae*, *Mh. turbida*, *Mh. falcis*, *Mh. solecavai* and also the '*Mh. tensa-opaca*' species complex ('*Mh. opaca*' plus *Mh. falcis* plus *Mh. solecavai*).

At a lower taxonomic level (i.e. within the species complex '*Mh. tensa-opaca*'), ITS data support the monophyly of the two morphospecies *Mh. falcis* and *Mh. solecavai* but no phylogeographic pattern among 'populations' of the '*Mh. opaca*' can be verified. A possible explanation is the incomplete lineage sorting (Wahlberg *et al.* 2003) in the most genetically diverse '*Mh. opaca*'.

As expected for mitochondrial markers with their generally higher rates of evolution than nuclear markers in ostracods (Schön et al. 2003), COI sequences provide a finer resolution within the species complex '*Mh. tensa-opaca*'. Previous investigations concluded that the morphological characters used by previous authors (Hartmann, 1997; Maddocks, 1990) are insufficient for the identification of the species within this complex and that the copulatory process of the hemipenis should also be studied (Brandão, submitted). Otherwise, '*Mh. opaca*' as defined by the hemipenis (including the copulatory process) presents an extremely wide variation in size and valve morphology. This large morphological variability indicates (after my (SNB) narrow species definition) that '*Mh. opaca*' is composed by more than one species, and the phylogenetic analyses confirm this supposition.

Additionally, genetic species delimitation based on a fixed threshold (3% or 6.21%) as previously proposed (Hebert, *et al.*, 2003; Meier *et al.*, 2006) does not seem appropriate, mostly because this method necessitates a gap between intraspecific and interspecific genetic distances (Hebert, *et al.*, 2003a, b; Meyer and Paulay, 2005). In the present study, distances between newly diverged species (herein exemplified by the *Mh. tensa-opaca* species complex) tend to lie between the intra- and interspecific values what blurs the methodology based on a fixed threshold. Herein ~35% of the specimens could not be unambiguously

identified. Lefébure and colleagues (2006) analysed thousands of crustacean COI and 16S sequences and also concluded that fixed thresholds are not efficient in delimiting species.

Finally, the analyses of branching rates as proposed by Pons and colleagues (2006) aim "to classify the observed branching time intervals defined by the nodes in a clock-constrained phylogram to either being the result of inter-specific ("diversification") or intraspecific ("coalescent") processes of lineage branching". Concerning the COI sequences of the genus *Macroscapha*, the entities provided in the solution with the highest likelihood exactly matches the morphospecies and the haplotype networks. Otherwise, when this last analysis was repeated with a smaller data set (i.e. excluding the morphospecies *Mh. inaequata*, *Mh. turbida*, *Mh. walterae*, and *Mh. aff. walterae*), the specimens of the species complex '*Mh. tensa-opaca*', which had previously been divided in six entities, were further divided into nine entities (not shown). The sensitivity of the methods to the dataset summed to the extreme variability in the number of entities obtained in a single analysis considering the 95% confidence interval (9 to 21 for COI; 7 to 24 for ITS) shows that the applicability of Pons and colleagues' (2006) algorithm is limited.

Testing the theories on eurybathic and circumantarctic distribution

The genetic data presented herein plus the morphological data presented elsewhere (Brandão, submitted) show that the supposedly widely distributed and eurybathic species '*Mh. opaca*' and '*Mh. tensa*' are actually a complex of at least eight morphospecies. Furthermore, one of these morphospecies (the most morphologically variable and most widely distributed) is composed by at least four isolated genetic entities or cryptic species. These genetic entities are furthermore restricted to different regions of the Southern Ocean. Similar results on genetic isolated unities composing the supposedly 'circumantarctic species' were previously found in different taxonomic groups. The relatively well studied crinoid *Promachocrinus kerguelensis* had previously been reported from several locations around Antarctica and also from a number of Subantarctic Islands. Genetic analyses showed that '*P. kerguelensis*' from the Northern Antarctic Peninsula, Scotia Arc and Bovet Island consist of multiple distinct lineages and that genetic flux between these lineages is restricted (Wilson *et al.*, 2007). Specimens of the bivalve *Lissarca notorcadensis* collected from the Scotia Arc were show to be genetically isolated from the ones living in the Weddell Sea (Linse *et al.*, 2007). Cryptic speciation based on genetics accompanied by morphological differences was also recorded for

the large-sized 'circumantarctic' isopods *Glyptonotus antarcticus* and *Ceratoserolis trilobitoides* (Held, 2003; Held & Wägele, 2005).

Our dataset also indicates that the isolated 'genetic entities' of '*Mh. tensa-opaca*' are not only geographically but also bathymetrically isolated. The only study involving genetics of deep-sea, SO organisms (i.e. the isopod *Betamorpha fusiformis*) reported a clear phylogeographic pattern (Raupach *et al.*, 2007). From the seven monophyletic groups of 16S rDNA haplotypes, six haplotypes were bathymetrically restricted to a depth range smaller than 200m, while only one haplotype occurred in a relatively wide depth range (2,660 to 4,890m) (Raupach *et al.*, 2007).

All these genetic analyses mentioned above indicate that distinct genetic entities comprise several 'circumantarctic or eurybathic species' and that gene flow around the Antarctic continent is probably far from homogenous (i.e. panmixia probably does not occur), even in animals with putative high dispersal (as the crinoid '*P. kerguelensis*'). The complex climatic history of the SO, with its advances and retreats of ice sheets, probably caused geographical isolation of previously linked populations (Anderson *et al.*, 2002). Consequently, as genetic data show, speciation probably took place. We interpret all these results above as strong evidence that prevailing theories which predict circumantarctic and eurybathic distributions of the SO benthic species need reevaluation.

Conclusion

The correlation between the narrow morphological species definitions and genetics (herein and from previous publications) provides evidence that the former reflects the 'biological species'. It also indicates that the biodiversity of Antarctic benthos and also that of deep-sea ostracods, which is based on a considerably wider morphological species definition, is underestimated. Furthermore, a reevaluation on prevailing theories on the circumantarctic and eurybathic species distribution should be considered.

REFERENCES

Allcock, A.L., Breirley, A.S., Thorpe, J.P. & Rodhouse, P.G. (1997) Restricted gene flow and evoltuionary divergence between geographically separated populations of the Antarctic octopus *Pareledone turqueti*. *Marine Biology*, 129, 97–102.

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W. & Lipman, D.J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acid Research*, 25, 3389-3402.

Anderson, R.F., Chase, Z., M.Q., F. & J., S. (2002) The Southern Ocean´s biological pump during the Last Glacial Maximum. *Deep-Sea Research II*, 49, 1909–1938.

Anisimova, M. & Gascuel, O. (2006) Approximate likelihood ratio test for branches: A fast, accurate and powerful alternative. *Systematic Biology*, 55, 539–552.

Arntz, W.E., Brey, T. & Gallardo, V. (1994) Antarctic zoobenthos. *Oceanography and Marine Biology: an Annual Review*, 32, 241-304.

Arntz, W.E., Gutt, J. & Klages, M. (1997) Antarctic marine biodiversity: an overview. *In:* Battaglia, B., Valencia, J. & Walton, D.W.H. (Eds.) *Antarctic communities Species, structure and survival*. Cambridge University Press, Cambridge, pp. 3–14.

Ayress, M., Neil, H., Passlow, V. & Swanson, K.M. (1997) Benthonic ostracods and deep watermasses: a qualitative comparison of southwest Pacific, southern and Atlantic Oceans. *Palaeogeography, Palaeoclimatology, Palaeoecology*, 131, 287-302.

Bernardi, G. & Goswami, U. (1997) Molecular evidence for cryptic species among the Antarctic fish *Trematomus bernachii* and *Trematomus hansoni*. *Antarctic Science*, 9, 381–385.

Brandão, S.N. (2008) New species of Bairdioidea (Crustacea, Ostracoda) from the Southern Ocean and discussions on *Bairdoppilata simplex* (Brady, 1880), *?Bairdoppilata labiata* (Müller, 1908) and *Bythopussella aculeata* (Müller, 1908). *Zootaxa*, 1866, 373–452.

Brandão, S.N. (submitted) Diversity and distribution of Macrocyprididae Müller, 1912 (Crustacea, Ostracoda) from the Southern Ocean. *Zoological Journal of the Linnean Society*.

Brandt, A., De Broyer, C., Gooday, A.J., Hilbig, B. & Thomson, M.R.A. (2004) Introduction to ANDEEP (ANtarctic benthic DEEP-sea biodiversity: colonization history and recent community patterns)—a tribute to Howard L. Sanders. *Deep-Sea Research II*, 51, 1457–1465.

Brandt, A., Gooday, A.J., Brandão, S.N., Brix, S., Brokeland, W., Cedhagen, T., Choudhury, M., Cornelius, N., Danis, B., De Mesel, I., Diaz, R.J., Gillan, D.C., Ebbe, B., Howe, J.A., Janussen, D., Kaiser, S., Linse, K., Malyutina, M., Pawlowski, J., Raupach, M. & Vanreusel, A. (2007)

First insights into the biodiversity and biogeography of the Southern Ocean deep sea. *Nature*, 447, 307-311.

Brey, T., Dahm, C., Gorny, M., Klages, M., Stiller, M. & Arntz, W.E. (1996) Do Antactic benthic invertebrates show an extended level of eurybathy? *Antarctic Science*, 8, 3-6.

Castresana, J. (2000) Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. *Journal Molecular Biology and Evolution*, 17, 540–52.

Clark, A.H. & Clark, A.M. (1967) A monograph of the existing crinoids 1(5). *US National Museum Bulletin*, 82, 1–860.

Clarke, A. & Johnston, N.M. (2003) Antarctic marine benthic diversity. *Oceanography and Marine Biology: an Annual Review*, 41, 47–114.

Clement, M., Posada, D. & Crandall, K. (2000) TCS: a computer program to estimate gene genealogies. *Molecular Ecology*, 9, 1657-1660.

Conlan, K.E. (1990) Revision of the crustacean amphipod genus *Jassa* Leach (Corophioidea: Ischyroceridae). *Canadian Journal of Zoology*, 68, 2031-2075.

Cope, T. & Linse, K. (2006) Morphological differences in *Lissarca notorcadensis* Melvill and Standen, 1907 from the Scotia, Weddell and Ross Seas. *Deep-Sea Research II*, 53, 903–911.

Dahl, E. (1990) Records of Nebalia (Crustacea Leptostraca) from the Southern Hemisphere-a critical review. *Bulletin of the British Museum of Natural History, Zoology*, 56, 73-91.

De Broyer, C., Lowry, J.K., Jazdezewski, K. & Robert, H. (2007) Census of Antarctic Marine Life. Synopsis of the Amphipoda of the Southern Ocean. Volume 1: Part 1. Catalogue of the Gammaridean and Corophiidean Amphipoda (Crustacea) of the Southern Ocean with distribution and ecological data. *Bulletin de l'Institut Royal de Sciences Naturelles de Belgique*, 77 (supplement 1), 1-325.

Dereeper, A., Guignon, V., Blanc, G., Audic, S., Buffet, S., Chevenet, F., Dufayard, J.F., Guindon, S., Lefort, V., Lescot, M., Claverie, J.M. & Gascuel, O. (2008) Phylogeny.fr: robust phylogenetic analysis for the non–specialist. *Nucleic Acids Research*, 36 (Web Server issue), W465–469.

Dingle, R.V. (2003) Recent Subantarctic benthic ostracod faunas from the Marion and Prince Edward Islands Archipelago, Southern Ocean. *Revista Española de Micropaleontologia*, 35, 119–155.

Edgar, R.C. (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research*, 32, 1792–1797.

Edwards, A.W.F. (1972) *Likelihood*. The John Hopkins University Press, London.

Etter, R.J., Rex, M.A., Chase, M.C. & Quattro, J.M. (1999) A genetic dimension to deep-sea biodiversity. *Deep-Sea Research*, 46, 1095–1099.

Etter, R.J., Rex, M.A., Chase, M.R. & Quattro, J.M. (2005) Population differentiation decreases with depth in deep-sea bivalves. *Evolution*, 59, 1479–1491.

Folmer, O., Black, M., Hoeh, W., Lutz, R. & Vrigenhoek, R. (1994) DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology*, 3, 294-299.

France, S.C. & Kocher, T.D. (1996) Geographic and bathymetric patterns of mitochondrial 16S rRNA sequence divergence among deep-sea amphipods, *Eurythenes gryllus*. *Marine Biology*, 126, 633–643.

Goffredi, S.K., Hurtado, L.A., Hallam, S. & Vrijenhoek, R.C. (2003) Evolutionary relationships of deep-sea vent and cold seep clams (Mollusca: Vesicomyidae) of the ‘‘pacifica/lepta’’ species complex. *Marine Biology*, 142, 311-320.

Goloboff P, Farris S, Nixon K (2000) TNT (Tree analysis using New Technology) (BETA) ver. Published by the authors, Tucumán, Argentina.

Griffiths, H.J., Barnes, D.K.A. & Linse, K. (2009) Towards a generalized biogeography of the Southern Ocean. *Journal of Biogeography*, 36, 162-177.

Guindon, S. & Gascuel, O. (2003) A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Systematic Biology*, 52, 696-704.

Hartmann G (1997) Antarktische und Subantarktische Podocopa (Ostracoda). In: Wägele JW, Sieg J, editors. Synopses of the Antarctic benthos. Koenigstein: Koeltz Scientific Books. pp. 240-255.

Hasegawa M, Kishino H, Yano K (1985) Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. *Journal of Molecular Evolution* (22): 160-174.

Hebert, P.D.N., Cywinska, A., Ball, S.L. & deWaard, J.R. (2003) Biological identifications through DNA barcodes. *Proceedings of the Royal Society of London B*, 270, 313-321.

Hebert, P.D.N., Ratnasingham, S. & deWaard, J.R. (2003) Barcoding animal life: cytochrome c oxidase subunit 1 divergences among closely related species. *Proceedings of the Royal Society of London B*, Suppl. 27, S96–S99.

Held, C. (2003) Molecular evidence for cryptic speciation within the widespread Antarctic crustacean *Ceratoserolis trilobitoides* (Crustacea, Isopoda). *In:* Huiskes, A.H.L., Gieskes, W.W.C., Rozema, J., Schorno, R.M.L., van der Vies, S.M. & Wolff, W.J. (Eds.) *Antarctic Biology in a Global Context*. Backhuys Publishers, Leiden, pp. 135-139.

Held, C. & Wägele, J.W. (2005) Cryptic speciation in the giant Antarctic isopod *Glyptonotus antarcticus* (Isopoda: Valvifera: Chaertiliidae). *Scientia Marina*, 69, 175–181.

Horne, D.J., Cohen, A. & Martens, K. (2002) Taxonomy, Morphology and Biology of Quaternary and Living Ostracoda. *In:* Holmes, J. & Chivas, A. (Eds.) *The Ostracoda. Applications in Quaternary Research. Geophysical Monograph*. American Geophysical Union, Washington, DC, pp. 5-36.

Jobb, G. TREEFINDER version of October 2008. 2008. Munich, Germany. Distributed by the author at www.treefinder.de.

Knowlton, N. (1993) Sibling species in the sea. *Annual Review of Ecology and Systematics*, 24, 189-216.

Le Goff-Vitry, M.C., Pybus, O.G. & Rogers, A.D. (2004) Genetic structure of the deep-sea coral Lophelia pertusa in the northeast Atlantic revealed by microsatellites and internal transcribed spacer sequences. *Molecular Ecology*, 13, 537–549.

Lefébure T., Douady C.J., Gouy M., Gibert J. (2006) Relationship between morphological taxonomy and molecular divergence within Crustacea: Proposal of a molecular threshold to help species delimitation. *Molecular Phylogenetics and Evolution* 40: 435–447.

Linse, K., Cope, T., Lörz, A.N. & Sands, C. (2007) Is the Scotia Sea a centre of Antarctic marine diversification? Some evidence of cryptic speciation in the circum-Antarctic bivalve *Lissarca notorcadensis* (Arcoidea: Philobryidae). *Polar Biology*, 30, 1059–1068.

Linse, K., Griffiths, H.J., Barnes, D.K.A. & Clarke, A. (2006) Biodiversity and biogeography of Antarctic and sub-Antarctic Mollusca. *Deep-Sea Research II*, 53, 985–1008.

Maddocks, R.F. (1990) Living and fossil Macrocyprididae (Ostracoda). *The University of Kansas Paleontological Contributions, Monograph*, 2, 1-404.

Meier, R., Shiyang, K., Vaidya, G. & Ng, P.K.L. (2006) DNA Barcoding and Taxonomy in Diptera: A Tale of High Intraspecific Variability and Low Identification Success. *Systematic Biology*, 55, 715–728.

Meyer, C.P. & Paulay, G. (2005) DNA Barcoding: Error Rates Based on Comprehensive Sampling. *PLoS Biology*, 3, 2229-2238.

Paradis, E., Claude, J. & Strimmer, K. (2004) APE: Analyses of Phylogenetics and Evolution in R language. *Bioinformatics*, 20, 289-290.

Peña Cantero, A.L. & Gili, J.M. (2006) Benthic hydroids (Cnidaria, Hydrozoa) from off Bouvet Island (Antarctic Ocean). Polar Biology, 29, 764–771.Pons, J., Barraclough, T.G., Gomez-Zurita, J., Cardoso, A., Duran, D.P., Hazell, S., Kamoun, S., Sumlin, W.D. & Vogler, A.P. (2006) Sequence-based species delimitation for the DNA taxonomy of undescribed insects. *Systematic Biology*, 55, 595–609.

Posada, D. & Crandall, K.A. (1998) Modeltest: testing the model of DNA substitution. *Bioinformatics*, 14, 817-818.

Raupach, M.J., Malyutina, M., Brandt, A. & Wägele, J.W. (2007) Molecular data reveal a highly diverse species flock within the munnopsoid deep-sea isopod *Betamorpha fusiformis* (Barnard, 1920) (Crustacea: Isopoda: Asellota) in the Southern Ocean. *Deep-Sea Research II*, 54, 1820–1830.

Raupach, M.J. & Wägele, J. W. (2006) Distinguishing cryptic species in Antarctic Asellota (Crustacea: Isopoda) - a preliminary study of mitochondrial DNA in Acanthaspidia drygalskii. *Antarctic Science*, 18, 191–198.

Rehm, P., Thatje, S., Arntz, W.E., Brandt, A. & Heilmayer, O. (2006) Distribution and composition of macrozoobenthic communities along a Victoria-Land Transect (Ross Sea, Antarctica). *Polar Biology*, 29, 782-790.

Schön, I., Martens, K., Van Doninck, K. & Butlin, R.K. (2003) Evolution in the slow lane: molecular rates of evolution in sexual and asexual ostracods (Crustacea, Ostracoda). *Biological Journal of the Linnean Society of London*, 79, 93-100.

Swofford, D. L., 1998. Paup. Phylogenetic Analysis Using Parsimony (and other methods), version 4.0. Sunderland: Sinauer Assoc.

Tamura, K., Dudley, J., Nei, M. & Kumar, S. (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) Software Version 4.0. *Molecular Biology and Evololution*, 24, 1596–1599.

Templeton, A.R., Crandall, K.A. & Sing, C.F. (1992) A cladistic analysis of phenotypic associations with haplotypes inferred from restriction endonuclease mapping and DNA sequence data. III. Cladogram estimation. *Genetics*, 132, 619–633.

Thompson, J.D., Higgins, D.G. & Gibson, T.J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions–specific gap penalties and weight matrix choice. *Nucleic Acids Research*, 22, 4673–4680.

Tsukagoshi, A. (1988) Reproductive character displacement in the ostracod genus Cythere. *Journal of Crustacean Biology*, 8, 563-575.

Wägele, J.W. (1986) Polymorphism and distribution of *Ceratoserolis trilobitoides* (Eights, 1833) (Crustacea, Isopoda) in the Weddell Sea and synonymy with *C. cornuta* (Studer, 1879). *Polar Biology*, 6, 127-137.

Weinberg, J.R., Dahlgren, T.G., Trowbridge, N. & Halanych, K.M. (2003) Genetic differences within and between species of deep-sea crabs (Chaceon) from the North Atlantic Ocean. *The Biological Bulletin*, 204, 318–326.

Wilson, N.G., Hunter, R.L., Lockhart , S.J. & Halanych, K.M. (2007) Multiple lineages and absence of panmixia in the “circumpolar” crinoid *Promachocrinus kerguelensis* from the Atlantic sector of Antarctica. *Marine Biology*, 152, 895–904.

Zhang, Z., Schwartz, S., Wagner, L. & Miller, W. (2000) A greedy algorithm for aligning DNA sequences. *Journal of Computational Biology*, 7, 203-214.