POLISH POLAR RESEARCH
vol. 35, no. 2, pp. 243–260, 2014 doi: 10.2478/popore-2014-0011

Molecular species delimitation of Icelandic brittle stars (Ophiuroidea)

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Abstract: Brittle stars (Echinodermata: Ophiuroidea) comprise over 2,000 species, all of which inhabit marine environments and can be abundant in the deep sea. Morphological plasticity in number and shape of skeletal parts, as well as variable colors, can complicate correct species identification. Consequently, DNA sequence analysis can play an important role in species identification. In this study we compared the genetic variability of the mitochondrial cytochrome c subunit I gene (COI) and the nuclear small subunit ribosomal DNA (SSU, 18S rDNA) to morphological identification of 66 specimens of 11 species collected from the North Atlantic in Icelandic waters. Also two species delimitation tools, Automatic Barcode Gap Discovery (ABGD) and General Mixed Yule Coalescence Method (GMYC) were performed to test species hypotheses. The analysis of both gene fragments was successful to discriminate between species and provided new insights into some morphological species hypothesis. Although less divergent than COI, it is helpful to use the SSU region as a complementary fragment to the barcoding gene.

Key words: Icelandic waters, ophiuroids, echinoderms, ABGD, COI, GMYC, SSU.

Introduction

Brittle stars are abundant megabenthic taxa in several habitats such as continental slopes and the deep sea floor (Blaber *et al.* 1987; Smith and Hamilton 1988; Piepenburg 1989; Piepenburg and Juterzenka 1994) and they constitute a significant proportion of the biomass on coral reefs (Wray 1999). A total of 241 ophiuroid species has been recorded from the North Atlantic, 180 of which have been reported from bathyal depths (Stöhr *et al.* 2012). Although the fauna of the North Atlantic Ocean is quite well known due to centuries of ocean exploration

from Europe and North America and commercial fisheries, new species of ophiuroids have been described continuously (Paterson 1985; Bartsch 1987; Smith *et al.* 1995; Stöhr 2003; Stöhr and Segonzac 2005; Martynov and Litvinova 2008; Stöhr and Muths 2010; Rodrigues *et al.* 2011), and difficult to identify juvenile stages have been matched to their adults (Sumida *et al.* 1998; Stöhr 2005). The limits of several species are still unclear though, *e.g. Ophiactis abyssicola* (M. Sars, 1861) has been suggested to be a cryptic complex of two species on molecular grounds (Ward *et al.* 2008). *Ophiacantha fraterna* Verrill, 1885 has been separated from *Ophiacantha bidentata* (Bruzelius, 1805) by Martynov and Litvinova (2008) on the basis of morphological differences, but both species may represent the extremes of a morphologically variable species as intermediate forms are common (Stöhr, unpublished results) but these two species have not been analyzed genetically yet. Morphological species identification is mainly based on the number and shape of skeletal parts which change during ontogeny (Sumida *et al.* 1998; Stöhr 2005). Molecular species identification can be highly effective with all life stages of Ophiuroidea, but its overall efficiency needs to be tested further.

The most recommended and widely used gene for metazoan species delimitations is the mitochondrial cytochrome c oxidase subunit I (COI) gene (Hebert *et al.* 2003). The analysed COI barcode region is ~658 base pairs which is located near the 5' end of this gene. This barcode region has shown low divergence (genetic distance) within metazoan species (typically <3%) versus that between species (typically 10–25%; Hebert *et al.* 2003). Also the effectiveness of COI DNA barcoding has been demonstrated for recognition, discrimination, and discovery of ophiuroid species (Ward *et al.* 2008; Corstorphine, unpublished results). In spite of the general preference for the COI gene as the barcoding marker, next to other mitochondrial gene fragments, also nuclear markers have been suggested, for example the 18S or LSU nuclear ribosomal RNA gene (Blaxter 2004; Markmann and Tautz 2005; Monaghan *et al.* 2005; Sonnenberg *et al.* 2007). It is expected that mitochondrial markers provide a better resolution because of the fast fixation of their neutral mutations; however they are not always able to reflect the full correct history of the species (Ballard and Whitlock 2004) while nuclear markers have been shown to be more problematic to trace phylogenetic lineages within species (Sonnenberg *et al.* 2007). Apart from the question of selecting relevant markers, the unreliable use of a single marker (Sonnenberg *et al.* 2007) and challenges in working with COI in echinoderms such as low success in amplification and presence of pseudogenes (Jeffery *et al.* 2003; Clouse *et al.* 2005; Hart and Podolsky 2005; Foltz *et al.* 2007; Hoareau and Boissin 2010) should not be ignored.

Consequently, comparing the sequence variability of the mitochondrial COI gene and a fragment of the nuclear small subunit ribosomal RNA (SSU) in addition to known morphological characterization of established species of ophiuroids results in a better detection of unknown species. This study presents COI and SSU

(18S rDNA) sequences from 66 specimens belonging to 11 morphologically identified species of Ophiuroidea collected from the North Atlantic in Icelandic waters (Table 1). Among them are the above mentioned doubtful species: *Ophiactis abyssicola, Ophiacantha fraterna* and *Ophiacantha bidentata*, the latter two for the first time included in a comparative genetic study. Two recently described bioinformatics approaches, the General Mixed Yule Coalescent (GMYC, Pons *et al.* 2006; Monaghan *et al.* 2009) and the Automatic Barcode Gap Discovery (ABGD, Puillandre *et al.* 2011), are used here to detect conspecific clusters. We present preliminary results of an ongoing molecular genetic species identification approach within the IceAGE project (Icelandic marine Animals: Genetic and Ecology, http://www.iceage-project.org/home.html).

Materials and methods

Sample selection and morphological identification. — Specimens were obtained from R/V *Meteor* cruise M85/3 (IceAGE1) in Icelandic waters between August 27th and September 28th, 2011. Stations were sampled around Iceland in the Irminger, Iceland, and Norwegian Basins, the Norwegian Sea, Denmark Strait and Reykjanes Ridge at a depth range of 120 to 2900 meters (Fig. 1). Depending on the substrate conditions (sediment type, rocks and depth, *etc.*), three trawled gears, Epibenthic Sledge (EBS), Agassiz Trawl (AGT) and Triangle dredge (TAD) were deployed to collect samples that contained brittle stars.

Specimens were preserved in 96% ethanol, which was changed after 24 h on board. Ophiuroids were sorted out using a dissecting microscope at the German Center for Marine Biodiversity Research (DZMB) in Hamburg, Germany. Seventy selected specimens were documented by photography for morphological identification. Individuals were isolated in 96% ethanol and stored at -20°C at the DZMB in Wilhelmshaven, Germany, as morphological vouchers for future reference. Specimens were identified to species level using diagnostic morphological characters (Paterson 1985). A list of addressed specimens and station coordinates is given in Supplementary Online Material (SOM) available at http://www.degruyter.com/view/j/popore.2014.35.issue-2/popore-2014-0011/suppl/popore-2014-0011_suppl.pdf and are shown in Fig. 1. For DNA sequence analysis, tissue (arm) was stored as tissue vouchers.

Molecular genetic analysis. — Up to 35 mm of tissue from a single arm of each specimen was dissected using a sterile scalpel. DNA extractions from 70 selected morphologically identified specimens were carried out using 30–40 µl Chelex (InstaGene Matrix, Bio-Rad) according to the protocol and directly used as DNA template for PCR. All DNA samples were stored at -20°C.

Six PCR primers were used, in order to amplify a 658 base-pair region of COI and a 350 base-pair region of 18S rDNA (SSU) in an Eppendorf AG Mastercycler

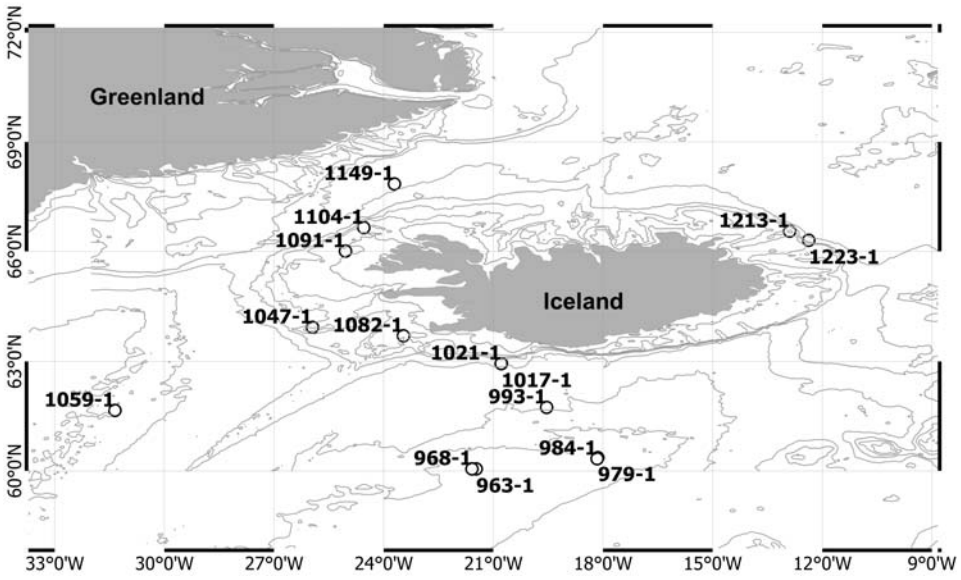

Fig. 1. Sampling sites in the North Atlantic around Iceland. Depth contours are shown at the following water depths: 4000, 3000, 2000, 1000, 500, 300 and 100 meters.

Gradient. For COI, using the primers LCO-1490 and HCO-2198 (Folmer *et al.* 1994), the PCR protocol was 95°C for 5 min, 95°C for 30 s, 45°C for 1 min, and 72°C for 1 min, for 60 cycles (fewer number of cycles can be implemented if the concentration of PCR product reached optimum) and as final elongation 72°C for 7 min. For ophiuroid species that did not work with LCO-1490 and HCO-2198, two different alternative primers were used to obtain COI sequences: HCO-2198 was used together with LCOech1aF1 (Corstorphine, unpublished results) and LCO-1490 together with Nancy (Simon *et al.* 1994). For these reactions the PCR protocol was 95°C for 5 min, then 95°C for 30 s, 42°C for 1 min, 72°C for 1 min for 40 cycles and a final elongation at 72°C for 7 min. A 350 bp of the 18 S was amplified using the universal primers SSU_F04 and SSU_R22 (Blaxter *et al.* 1998) according to the protocol 95°C for 2 min, 95°C for 1 min, 57°C for 45 s, 72°C for 3 min, for 35 cycles, and final elongation time of 72°C for 10 min. The PCR was performed using Illustra PuReTaq Ready-To-Go PCR Beads (GE Healthcare) in 25 µL volume containing 22 µL H_2O, 0.5 µL of each primer (10 pmol/µL) and 2 µL DNA templates. All PCR products were checked by electrophoresis on a 1% agarose/TBE gel containing 1% GelRed.

PCR product purifications and sequencing were carried out by Macrogen (Amsterdam, Netherlands). Species which repeatedly failed to amplify or produced multiple bands were not sequenced. Forward and reverse sequences for each individual were assembled, edited and checked for correct amino acid translation frames, using Geneious (version 5.4.5 created by Biomatters; available from

http://www.geneious.com). All sequences were searched against the GenBank nucleotide database using BLASTN (Altschul *et al.* 1990). Edited DNA sequences were aligned using default settings of MUSCLE 3.8.31 (Edgar 2004) and alignments were further edited manually. Maximum and minimum length of COI sequences within the resulting alignment was 680 and 582 bp, respectively and 374 and 371 bp for 18S rDNA (SSU) sequences. In order to compare genetic variation of the two gene fragments, COI and SSU sequences were separately analyzed and compared using the neighbor-joining (NJ) algorithm of the Molecular Evolutionary Genetics Analysis (MEGA 5.2.1) software package (Tamura *et al.* 2011). Uncorrected p-distances were used to investigate and compare the genetic variability on the basis of each gene fragment, within and between species using R 3.0.2 (R core team 2013, www.R-project.org). As phylogenetic inference Bayesian analyses were performed with BEAST v1.7 (Drummond *et al.* 2012) using the best-fitting model for COI sequences GTR+G+I (number of Gamma categories 4, Gamma shape = 0.513, I = 0.671) and K80+G (number of Gamma categories = 4, Gamma shape = 0.1510) for 18S rDNA fragment under Bayesian information criterion (BIC) identified with jModeltest v0.1.1 (Posada 2008). As the best-fitting parameters, a relaxed lognormal clock with a coalescent prior model was used to generate the COI and 18S rDNA (SSU) Bayesian gene trees that were used in conjunction with the GMYC model (Monaghan *et al.* 2009) to delimit species. MCMC analyses were run for 10 million generations whereas all Effective Sample Size (ESS) values calculated with MCMC Trace Analysis Tool (Tracer v1.5.0) software (Rambaut and Drummond 2007) were <200 according to default burn-in. Tree annotator v1.7.5 (Rambaut and Drummond 2007) was used to analyze the MCMC outputs using the default parameters.

Automatic Barcode Gap Discovery and General Mixed Yule Coalescent methods. — DNA barcoding has been proposed as a mean of identifying species based on similarity criterion, when a significant gap is supposed to be between genetic intraspecific and interspecific distances (Mallet 1995). The range of this so called "barcoding gap" can be considered as a threshold for preliminary species delimitation under the assumption that genetic distances between species should be larger than distances within species (Hebert *et al.* 2003). However, in some cases, this barcode gap does not exist or the intra- and interspecific distances overlap. This can happen whenever the sequence of a specimen is more similar to the sequence of another species than to the sequence of other individuals of the same species (Rosenberg and Tao 2008).

The Automatic Barcode Gap Discovery method (ABGD) is an automatic procedure that considers the sequences as hypothetical species based on the barcoding gap. The model first calculates a range of prior intraspecific divergence to obtain the maximum limit of the intraspecific diversity and then partitions the data on the basis of the first significant gap (barcode gap) beyond this limit, which is a potential limit between intra- and interspecific diversity. Inference of the limit and gap

detection are then recursively applied to previously obtained groups to get finer partitions, until there is no further partitioning (Puillandre *et al.* 2011). In conclusion, the ABGD method results in different partitions of species hypotheses which need to be complemented by other taxonomic approaches (Puillandre *et al.* 2011). In this study a genetic distance analysis (neighbor-joining) and a species delimitation tool (GMYC) have been used to complement ABGD results and select the optimal species partition among different prior threshold for two gene fragment analysis. We used the on-line version with ABGD default parameters (http://wwwabi.snv.jussieu.fr/public/abgd/abgdweb.html) to analyze and compare the species partitions that defined by the COI and 18S rDNA (SSU) sequences.

The General Mixed Yule Coalescent model (GMYC) described by Pons *et al.* (2006) and Monaghan *et al.* (2009) can be run on an ultrametric tree by maximizing the sum of the log-likelihoods of waiting times across the entire tree. A key step is fitting the location of the switches from speciation branching event (interspecific relationship) to coalescent branching event (intraspecific relationship). The simple-threshold approach (Pons *et al.* 2006) assumes that there is a threshold time, before which all nodes reflect diversification events and after which all nodes reflect coalescent events. Species in this model are thus delimited by the descendent nodes of branches crossing the threshold. This threshold time can be optimized to find the maximum likelihood solution and hence to estimate the number of species. This approach defines each species on the basis of the most recent common ancestor on the phylogenetic tree and assumes that the most recent diversification event occurred before the oldest within-species coalescent event (Pons *et al.* 2006; Managhan *et al.* 2009). This method uses a pre-existing phylogenetic tree to determine the transition signal from speciation to coalescent branching patterns (Puillandre *et al.* 2012). The GMYC method implemented in the SPLITS package for R was applied to the COI and 18S rDNA (SSU) trees obtained with BEAST.

Results

Morphological identification resulted in 70 identified specimens of eleven species (see SOM) of which for 66 individuals we received DNA sequences. DNA sequences for the COI barcode region were obtained for 38 specimens of nine species: *Ophiactis abyssicola, Ophiopholis aculeata* (Linnaeus, 1767), *Ophioscolex glacialis* Müller *et* Troschel, 1842, *Ophiocten hastatum* Lyman, 1878, *Ophiura ljungmani* (Lyman, 1878), *Ophiura sarsii* Lütken, 1855, *Ophiothrix fragilis* (Abildgaard, in O.F. Müller, 1789), *Ophiacantha* cf. *bidentata* and *Ophiacantha* cf. *fraterna* (see SOM). In spite of using four additional COI primers to increase the matching possibility to template DNA and different PCR protocols (see Sec.2.2. Molecular genetic analysis) for 32 specimens we had no amplification success or sequences were concluded to be too short or of low quality (*i.e.* double peaks, background noise), possi-

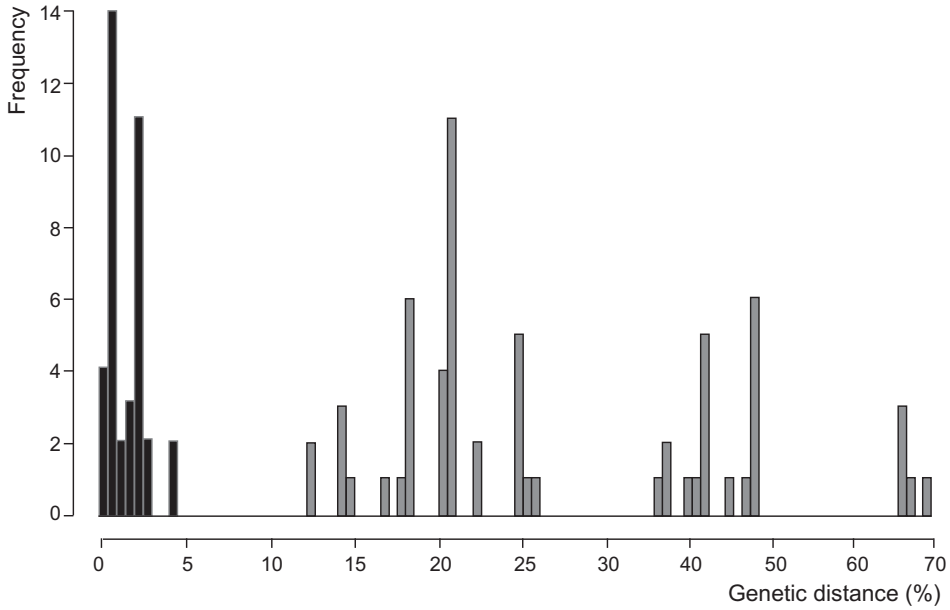

Fig. 2. Histogram shows the percentage of genetic p-distances within and between Ophiuroidea species for the COI gene. The barcoding gap formed by the distributions of intraspecific variation (shown in black) and interspecific variation (shown in grey).

bly representing pseudogenes. Genus *Ophiacantha* has shown to be the most problematic taxon in order to amplify COI. *Ophiacantha abyssicola* G.O. Sars, 1871 failed in COI amplification and among eight specimens of *Ophiacantha* cf. *bidentata* and *Ophiacantha* cf. *fraterna* for two specimens amplification of COI fragment was successful (see SOM). Also *Ophiopleura borealis* Danielssen *et* Koren, 1877 and some specimens of *Ophiura ljungmani* have failed to amplify COI (see SOM).

18S rDNA (SSU) regions was sequenced successfully for 56 specimens in which all 11 selected morphologically identified species represented SSU fragments in the dataset (the COI and SSU amplification results of all specimens are shown in SOM).

Genetic p-distances for pairwise comparisons across the entire COI data revealed distributions in sequence similarity and ranging from 0 to 0.6996 (0–70%) with genetic distance within and between species ranging from 0 to 4.12% (K2P distance: 0–4.23%) and 12.8 to 70%, respectively (Fig. 2). There was no intraspecific variation in 18S level, while the different species differed by 0.002 to 0.0196 (0.3–2%; data not shown).

The optimal gene trees produced by neighbor-joining (NJ) and Bayesian analysis showed nearly identical species delimitation in which the branch tips within species were short, and species were separated by longer branches (Figs 3 and 4 represent COI and SSU trees respectively). Sequences clustered by morphologically identified species in most cases. Ambiguity appeared in *Ophiacantha* species where

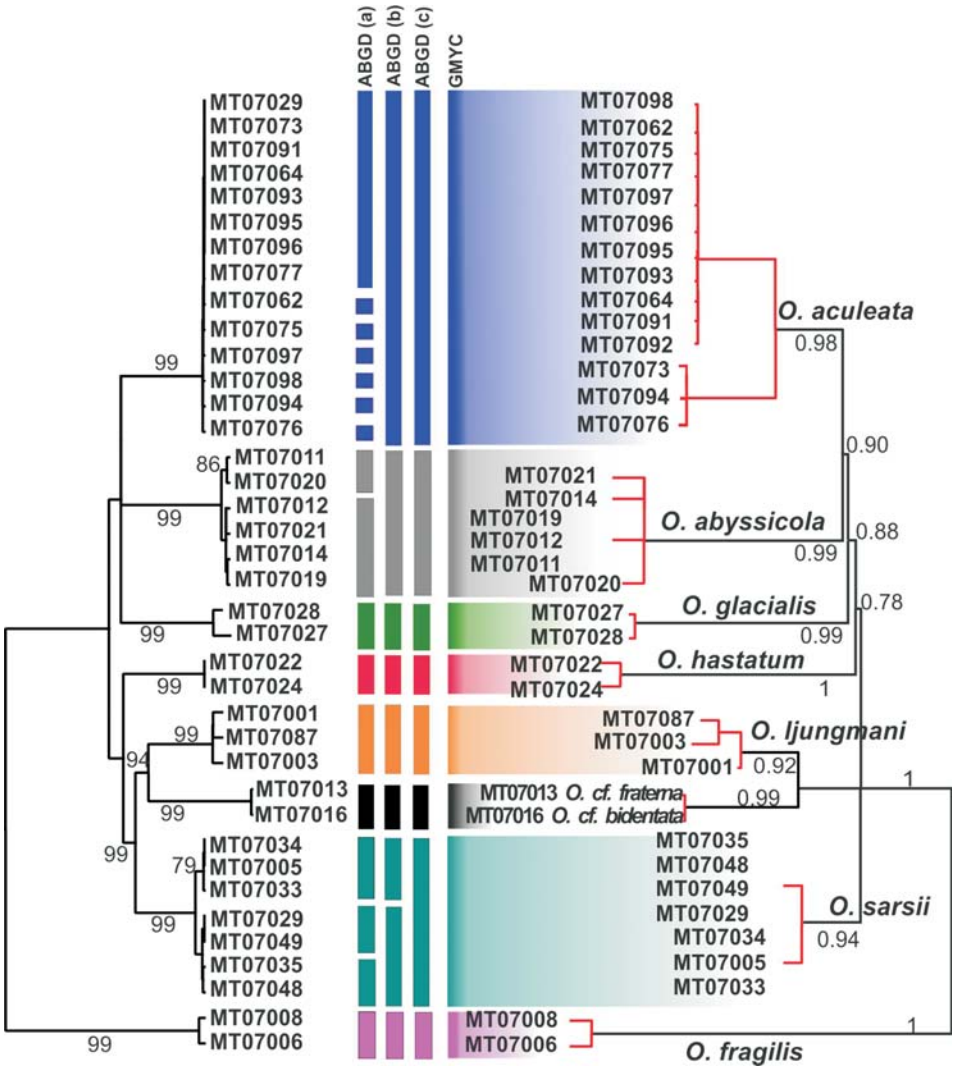

Fig. 3. Gene trees of the neighbor-joining (left) and Bayesian (right) analysis of COI (numbers on branches represent bootstrap values >80% and posterior probabilities >0.70) and species delimitation tools for COI fragment. The four multicolor vertical bars represent alternative taxonomies, respectively supported by Automatic Barcoding Gap Discovery (ABGD) in three different thresholds of prior intraspecific divergence: (a) 0.0001–0.0010, (b) 0.0022–0.0046 and (c) 0.0100–0.100 as well as General Mixed Yule Coalescent (GMYC) (note: the tree does not represent a phylogeny, instead its purpose is to separate species as taxonomic entities).

morphological identification considers them as two separate species (*O.* cf. *fraterna* and *O.* cf. *bidentata*) and both 18S rDNA (SSU) analyses (NJ and Bayesian) recovered two low-supported clades (Fig. 4) with little genetic distance (0.0015), while COI analyses recovered a single highly supported (99% bootstrap value and 0.99 posterior probabilities) cluster, respectively (Fig. 3). The nodes separating species

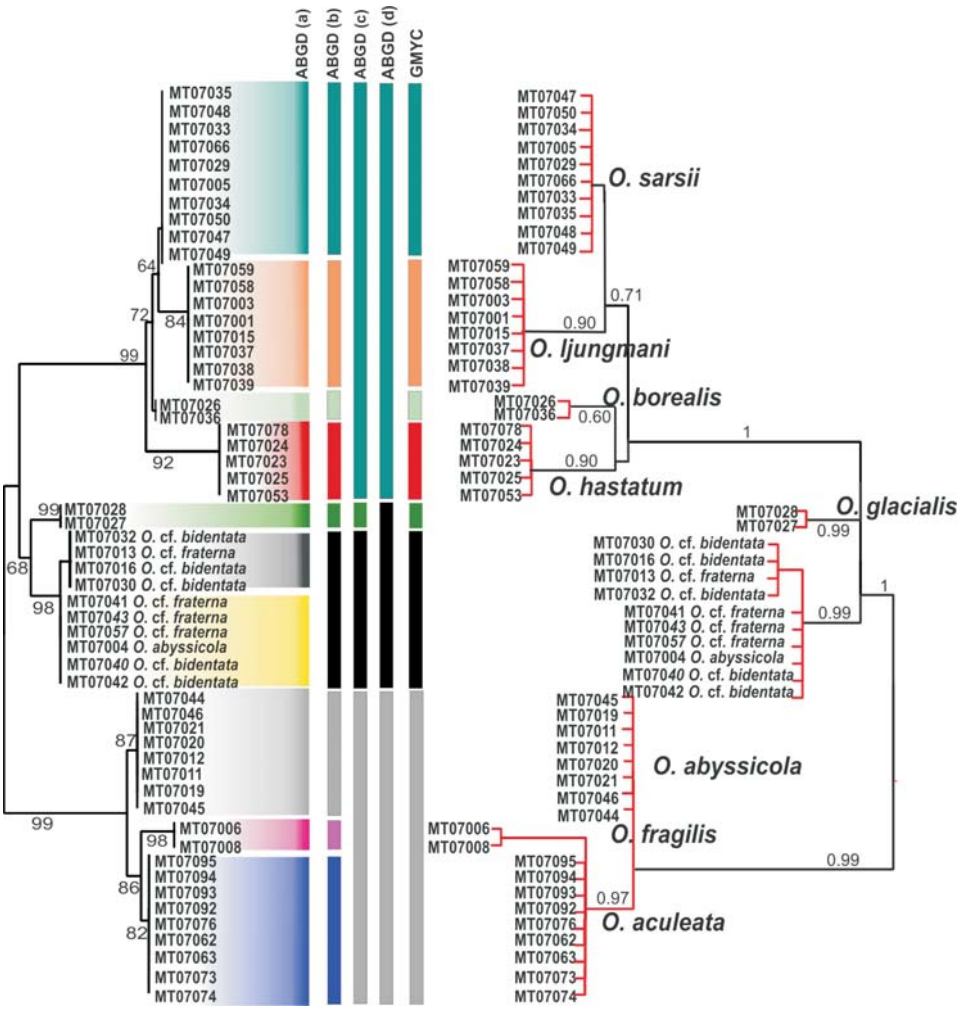

Fig. 4. Gene trees of the Neighbor Joining (left) and Bayesian (right) analysis of 18S rDNA (SSU) fragment (numbers on branches are bootstrap values >60% and posterior probabilities >0.60) and species delimitation tools for the SSU fragment. The five vertical bars represent alternative taxonomies respectively supported by Automatic Barcoding Gap Discovery (ABGD) in four different thresholds of prior intraspecific divergence: (a) 0.0001–0.0022, (b) 0.0046, (c) 0.0100 and (d) 0.0215–0.0464 as well as General Mixed Yule Coalescent (GMYC).

were well supported in the Neighbor Joining and Bayesian analysis of the COI data, while analysis of 18S rDNA (SSU) for most of the species clusters the bootstrap supports and posterior probabilities was a bit lower (Figs 3 and 4).

Species delimitation tools (ABGD and GMYC) were used to cluster sequences of Ophiuroidea specimens for both gene fragments COI and SSU. The likelihood of the GMYC model in the analysis of the mitochondrial gene (COI) was 215.5975, and it was significantly superior to the likelihood of the null model (L0 =208.5693,

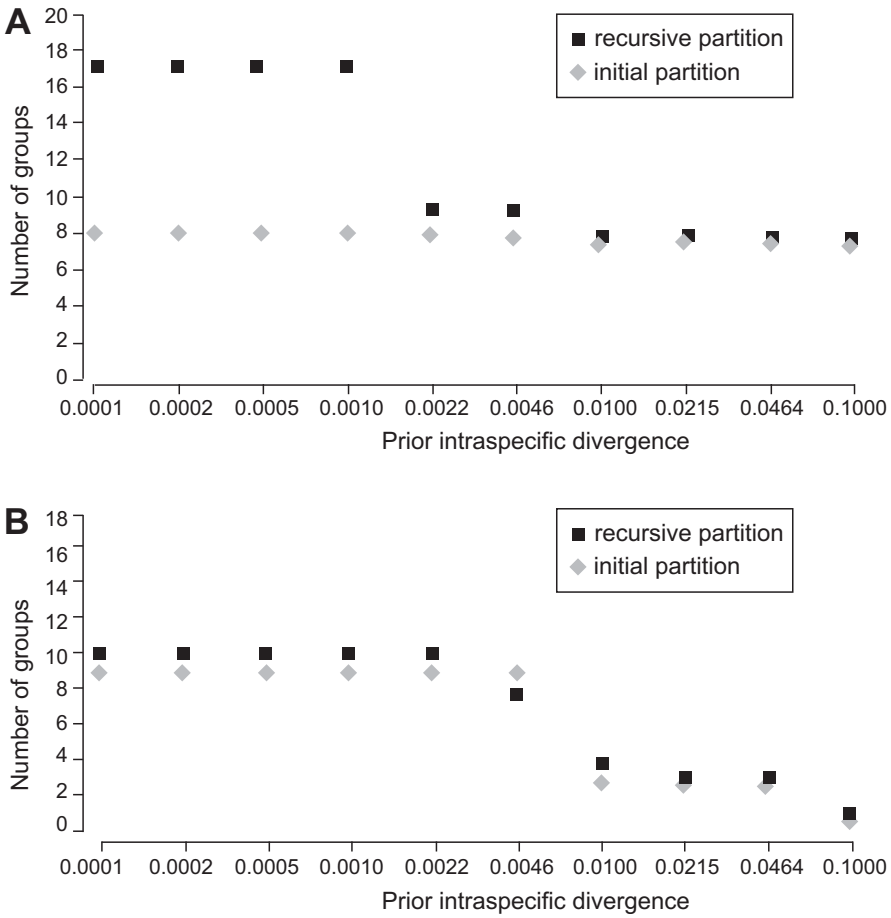

Fig. 5. ABGD results, with the number of partitions obtained in each prior threshold for COI (A) and SSU (B). The model partitions the data on the basis of the first significant barcoding gap according to the maximum limit of the intraspecific diversity (Initial Partition). Inference of the limit and gap detection are then recursively applied to initial partitions to get finer clusters until there is no further partitioning (recursive partitions).

P-value = 0.01). There were eight clusters with confidence interval of 3–9. The numbers of species defined using the ABGD method varied with the different prior thresholds ranging from 0.0001 to 0.1000 prior intraspecific divergences (P) for COI gene fragment (Fig. 5A). On the basis of COI, low threshold values ranged from 0.0001 to 0.0010 P and clustered almost each haplotype as a different species (17 species). Moderate threshold 0.0022 to 0.0046 P partitioned nine groups and more conserved partitions with eight species have been clustered through 0.0100 to 0.1000 prior intraspecific divergences (Fig. 5A). High priori thresholds (0.0100–0.1000 P) lead to the same clustering as NJ analysis and GMYC model with eight species (species partitions for ABGD and GMYC delimitation methods on the analysis of COI fragment are detailed in Fig. 3). In conclusion, the eight species clusters

which are defined by ABGD method through high prior threshold for COI analysis are congruent with results of NJ analysis and GMYC model of this gene fragment was selected as the optimum species partition.

The number of species defined with ABGD using 18S rDNA (SSU) sequences varied from ten species to a single species by different prior thresholds (0.0001 to 0.1000) based on the distribution of pairwise genetic distances (Fig. 5B). Low threshold from 0.0001 to 0.0022 p resulted in ten groups while 0.0046 prior threshold partitioned nine species. Prior intraspecific divergence of 0.0100, 0.0215, 0.0464 and 0.1000 clustered four, three, three and one species, respectively (Fig. 5B). The likelihood proportion of 18S rDNA (SSU) sequence analysis using GMYC was 446.643, which was significantly different from the likelihood of the null model (L0 =442.8487, P-value = 0.1). However, the partitions obtained were seven species (confidence interval: 1–15), which are quite different form NJ distance analysis (species partitions for delimitation methods on the analysis of SSU fragment are detailed in Fig. 4). In total, the results of the ABGD delimitation method for SSU sequences using medium prior threshold of prior intraspecific divergence (nine species) can be confirmed by neighbor joining analysis with the same number of clusters. The clustering result of GMYC analysis for SSU fragment is not congruent with other analysis.

In this context, the ability to discriminate between closely related species is the most important criterion for the usefulness of a marker. Although it is clear that the level of divergence in the 18S rDNA (SSU) fragment is much lower than in the COI marker, the same species clusters were identified with both markers in all cases. Also genetic species delimitation agrees with the morphological determination of species, with only exception of *Ophiacantha* species. The conflict with the species of *Ophiacantha* cf. *bidentata* and *O.* cf. *fraterna* is also congruent in both marker analyses. *Ophiacantha abyssicola* is another problematic species which is grouped with *O.* cf. *bidentata* and *O.* cf. *fraterna* by the SSU analysis (unfortunately, the COI amplification process was unsuccessful in *Ophiacantha abyssicola*).

Discussion

This study further supports the validity of using DNA sequence data for species discrimination and identification in marine metazoans. The life-cycle of most echinoderms includes at least two stages respectively: an (often planktonic) larval stage, and a usually benthic adult stage living in different habitats, which is problematic to match juvenile and mature stages (Stöhr 2005). Considering the large potential ambiguity in identifying ophiuroid species caused by phenotypic plasticity (Crawford and Crawford 2007; Owen *et al.* 2009) and sexual dimorphism (Stöhr 2001; Tominaga *et al.* 2004), the high accuracy of the molecular approach is

of great value for discriminating known species. Although low success rate of COI amplification from the ophiuroid specimens of this study further indicates the COI amplification challenges in echinoderms (Jeffery *et al.* 2003; Clouse *et al.* 2005; Foltz *et al.* 2007; Hoareau and Boissin 2010), the analysis of both gene fragments was successful in distinguishing established species.

The range of intraspecific K2P distance of ophiuroids in the COI analysis of our dataset (0–4.23%), was higher than the same values reported by Ward *et al.* (2008) for Echinodermata (0–3.04%) and specifically Ophiuroidea (0–2.23%) and further supports the high genetic diversity within Ophiuroidea species among Echinodermata. Furthermore, 63% of the species displayed higher interspecific divergence than the mean including *Ophiactis abyssicola, Ophiopholis aculeata, Ophiothrix fragilis* and *Ophiocten hastatum*.

In addition, our data confirmed that the 18S rDNA (SSU) fragment can be easily amplified from ophiuroid species with universal primers. However, interspecific distances calculated for the SSU fragment indicate the more conservative status of this fragment, but it seems to show efficient variability to discriminate species, which is comparable to the clustering offered by COI results. It is generally evident that nuclear gene fragments have some highly divergent regions that can discriminate even very closely related species (Sonnenberg *et al.* 2007), hence amplifying and sequencing the complete 18S rDNA fragment during the future continuation of this study can be expected to show higher divergences between and within species. It is indeed clear that the COI fragment is suitable across a broad taxonomic range to delimit species, but also the complementation by a nuclear marker system proved to be of advantage (Weisrock *et al.* 2006; Knowles and Carstens 2007; Boissin *et al.* 2008; O'Meara 2010; Ross *et al.* 2010). Sonnenberg *et al.* (2007) showed that a mitochondrial transfer can be detected using nuclear fragments (D1 and D2 regions) since the nuclear sequence confirmed the taxonomic assignment, whereas the mitochondrial sequence led to the wrong identification.

Thirty eight of ophiuroid specimens sampled in this study could be clustered by COI fragment and 56 specimens by 18S rDNA (SSU). The inability to separate *Ophiacantha* cf. *fraterna* and *Ophiacantha* cf. *bidentata* was congruent in the sequence analysis clustering of both fragments. It appears therefore that SSU represents a complementing marker for DNA barcoding studies based on mitochondrial COI sequences and can prevent problems related to a single-gene approach, such as the presence of pseudogenes (Lorenz *et al.* 2005), incomplete lineage sorting (Funk and Omland 2003) or introgression (Chase *et al.* 2005).

Species delimitation methods. — In this study, two gene fragment datasets of ophiuroids were analyzed with two species delimitation methods (ABGD and GMYC). GMYC is generally considered an effective method to detect species boundaries (Leliaert *et al.* 2009); although it has been argued that in some cases it

could lead to an overestimation of the number of species (Lohse 2009). ABGD detects the breaks in the distribution of genetic pairwise distances, by means of barcode gap (Hebert *et al.* 2003), relying exclusively on genetic distance between DNA sequences. Overall, both methods resulted in almost the same partitions in COI and 18S rDNA (SSU), as all species were confirmed as valid except in the *Ophiacantha fraterna/bidentata* clade where both results supported the hypothesis of a single species whereas the topology of SSU gene tree resulted in two low supported clades (for details see Sec. 4.2.) Conflicting results were recorded when three morphologically well supported species (*Ophiothrix fragilis*, *Ophiopholis aculeata* and *Ophiactis abyssicola*) defined by ABGD in both COI and SSU fragments, clustered into one species in GMYC in the analysis by SSU fragment (Fig. 4). Compared to ABGD which determines the set of partitions on the basis of pairwise genetic distance, GMYC considers the evolutionary process and needs pre-analysis of an ultrametric tree which may not reflect the correct divergence between species (Puillandre *et al.* 2012), especially in short fragments (350 bp) such as the here analyzed SSU fragment. However, as underlined in the results, several prior thresholds can interpret different patterns of partition in ABGD methods in case of one or two species in both fragments. Hence all these observations further confirmed that ABGD and GMYC are complementary methods which should be performed together to decrease the risk of conflicting hypotheses obtained with a single method and increase the final resolution of species delimitation (Puillandre *et al.* 2012). In conclusion, ABGD and GYMC could be misleading when only a few specimens are used to represent each species (Lohse 2009; Puillandre *et al.* 2011).

Problematic species clusters. – DNA sequence analysis and species delimitation tools on the COI fragment clustered the two species *O.* cf. *fraterna* and *O.* cf. *bidentata* in one clade with considerably high support value (99%); meanwhile Neighbor Joining and Bayesian analysis of the SSU fragment partitioned a complex of three *Ophiacantha* species in two clades in which the inter-clade is not well-supported with a genetic distance of 0.0027 (Fig. 4). These two species *O. fraterna* and *O. bidentata* have been controversial since 1885. Verrill (1885 and 1899) and Koehler (1914) considered these two nominal species as a single morphologically variable species, segregated by depth. Most studies maintained *O. fraterna* just as a variety of *O. bidentata* (Farran 1913; Grieg 1921; Mortensen 1933). Baranova and Kunzevich (1969) and Paterson (1985) did not find any reason to separate a deep-water species and considered *O. fraterna* as a synonym of *O. bidentata*. Finally, Martynov and Litvinova (2008) distinguished these two North Atlantic species on the basis of some morphological characters such as (1) the spicules of the disk have a rosette of denticles at the top in *O. fraterna* whereas in *O. bidentata* they are more club-shaped and along the sides of the spicules, (2) the outer oral papilla and tentacle is widened in *O. bidentata* but narrow in *O. fraterna* and (3) *Ophiacantha fraterna* is smaller than *O. bidentata*.

The species delimitation tools used in this study as first molecular investigation on these two species supported their sequence similarity and may indicate the fact that this is a young species pair. The level of genetic distance on 18S rDNA (SSU) is very low, but indicates different species even if the two different clades do not comprise the nominal species, respectively. Hence, it is possible that we are dealing with regional morphological variants, thus different morphotypes live at different depths of the North Atlantic. The presence of specimens with intermediate characters (Stöhr, unpublished) may suggest either that this is a single species with great morphological ecological plasticity (mainly in response to depth) or that these are indeed two valid species that hybridize at medium depths. More investigations are desired to increase the amplification efficiency of the COI fragment as well as sequencing the complete 18S rDNA in order to decide between these hypotheses.

The third problematic species, *Ophiacantha abyssicola,* was recovered in the same cluster as *O.* cf. *fraterna* and *O.* cf. *bidentata* in the genetic distance analysis as well as with the species delimitation analysis conducted on SSU fragment (COI is not available from this species due to amplification problems). As the sequence of the SSU fragment is available only from a single specimen of this species, confirming the actual status of this species is not yet possible. Although further studies need to focus on more specimens and gene fragments, these considerations show that the sequences of ribosomal genes may provide additional information for species delimitation.

The 11 species studied here are common and widespread in the North Atlantic Ocean and are all known from Iceland (Paterson 1985). They were collected from a relatively limited area, which may explain the lack of differentiation between the problematic species. Additional specimens from a greater geographic and depth range need to be analyzed.

Acknowledgments. — We gratefully acknowledge the captain and crew members of the R/V *Meteor* (cruise No. 85 Leg 3, 2011) and German Center for Marine Biodiversity Research team for help in providing and sorting samples as well as laboratory assistance. Financial support was provided by the German Science Foundation (DFG) under the grant number BR3843/4-1. Also, we would like to express our sincere thanks to the reviewers who identified areas of our manuscript that needed corrections or modification.

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Received 20 February 2014
Accepted 22 April 2014