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Species delimitation analyses of NE Atlantic *Chaetozone* (Annelida, Cirratulidae) reveals hidden diversity among a common and abundant marine annelid

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ABSTRACT

The polychaetes of the family Cirratulidae (Annelida) are common inhabitants in continental shelf benthic environments and considered an important group of organisms in environmental monitoring surveys. The family represents a taxonomic and systematic challenge, as monophyly of genera and evolutionary relationships within the family remain to be explored in a proper phylogenetic framework. Bitentaculate cirratulids, especially the genus *Chaetozone,* form one of the most species-diverse group of polychaetes worldwide. In this study, we aimed at evaluating the species diversity of the genus *Chaetozone*in benthic environments in the North East Atlantic by molecular means. We tested whether traditional morphological diagnostic characters are able to discriminate between the species hypothesis after species delimitation analyses, and assessed monophyly of the genera involved. Two DNA markers were sequenced from about 200 specimens belonging to *Chaetozone, Aphelochaeta, Dodecaceria, Cirriformia* and *Cirratulus* – the universal mitochondrial barcoding region COI, and the D1-D2 regions of the nuclear 28S rRNA – and analyzed with Bayesian inference, Maximum Likelihood and the species delimitation methods mPTP and GMYC. The first phylogeny of the family Cirratulidae is inferred and the genera *Chaetozone*, *Dodecaceria* and *Cirratulus* are recovered monophyletic. A total of 14 clusters of sequences – corresponding to species of *Chaetozone* – were found in the study area, and only one of them is here referred to a nominal species, *Chaetozone setosa*. Our results reveal several species complexes in the genus *Chaetozone*, that some of these independent lineages are unnamed and undescribed, and that morphological diagnostic features are in most cases unable to discriminate between the most similar species.

1. Introduction

Although estimates of the biodiversity on Earth vary, most studies agree that a majority of it remains to be discovered and described (Mora et al., 2011). This is in particular true for marine life, and our current knowledge represents just a fraction of the actual marine biodiversity (Appeltans et al., 2012). The increasing availability of molecular data, such as the universal DNA barcode cytochrome *c* oxydase 1 (Hebert et al., 2003a, 2003b) combined with novel bioinformatic methods have opened new perspectives to explore biodiversity and biogeographical patterns (Bickford et al., 2007; Nygren, 2014; Cerca et al., 2020). Delimitation of species as "separately evolving metapopulation lineages" (De Quieroz, 2007) through analyses of DNA sequences is now a standard procedure. These molecular based methods allow us to investigate the biodiversity and explore phylogenetic relationships between different genetic lineages and thus lead to a classification that represents the evolutionary history of the group (Schander and Willassen, 2005). By including and analyzing molecular data, "cryptic" or "pseudo-cryptic" diversity is often revealed, in particular within polychaetes, where common and widely distributed morphospecies often turn out to comprise several morphologicaly similar species with more restricted geographical and/or bathymetric distribution (e.g. Nygren and Pleijel, 2011; Nygren, 2014; Brasier et al., 2016; Capa et al., 2013; Aguado et al., 2019; Cerca et al., 2020).

Members of the family Cirratulidae Ryckholt, 1851 are amongst the most common and abundant polychaetes, occurring from the shallow intertidal to the abyss (Blake et al., 2009; Hilbig and Blake, 2000). They constitute a morphologically rather simple and homogenous group of

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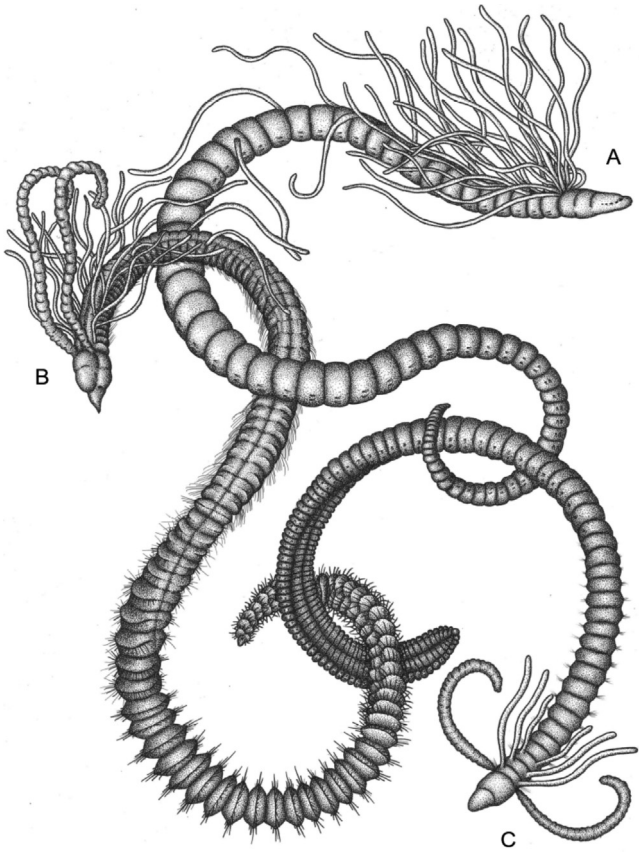

Fig. 1. Family Cirratulidae. (A) Multitentaculate Cirratulidae (*Cirratulus* sp.); (B) soft-bottom bitentaculate Cirratulidae (*Chaetozone* sp.); C: hard-bottom bitentaculate Cirratulidae (*Dodecaceria* sp.).

small annelids (generally less than 2.5 cm long, though sometimes up to 20 cm), characterized by their grooved dorsal tentacles and the long filament-like branchiae that most species bear on many segments (Fig. 1) (Rouse and Pleijel, 2001; Blake and Magalhães, 2017). Cirratulids are typical deposit feeders, being able to collect sediment particles by use of their tentacles (Jumars et al., 2015). Most species live in sediments (Chambers and Woodham, 2003), but members of *Dodecaceria* Ørsted, 1843 bore in calcareous structures (Rouse and Pleijel, 2001; Blake and Magalhães, 2017). Some species are present in great numbers in organically enriched sediments and may act as indicators of organic pollution (Pearson, 1976; Pearson and Rosenberg, 1978; Rygg, 1985; Elías et al., 2003, 2006). However, Cirratulidae are notoriously difficult to identify to species level in biodiversity assessments and monitoring surveys (Elías et al., 2017) as because diagnostic features often are a unique combination of qualitative or quantitative morphological features, that in many cases may vary with growth (e.g. number, segmental origin and even chaetal morphology), preservation procedures (e.g. body shape or prostomial morphology), be subject to interpretation (e.g. type of chaetae or presence of a first achaetous segment) and be difficult to observe (e.g. position of the first pair of branchiae). These difficulties have further hindered a stable and robust classification and resulted in a long and complicated history for the systematics of Cirratulidae (Blake, 1996; Rouse and Pleijel, 2001).

In recent taxonomical literature (e.g. Blake, 2018), the genera of Cirratulidae have been divided in distinct, though informal, groups depending on the number and morphology of the anterior tentacles. The genera *Cirratulus* Lamarck, 1818, *Cirriformia* Hartman, 1936, *Timarete* Kinberg, 1866, *Fauvelicirratulus* Çinar and Petersen, 2011 and *Protocirrineris* Czerniavsky, 1881, bear numerous grooved tentacular filaments arising from one or several anterior segments, somehow similar in size to the branchiae (not grooved). These genera are referred to as "multitentaculate" Cirratulidae (Fig. 1, A). They typically have a broad and wedge-shaped prostomium, and include the largest specimens of the family, up to 20 cm long. The grooved tentacles of the genera *Aphelochaeta* Blake, 1991, *Caulleriella* Chamberlain, 1919, *Chaetocirratulus* Blake, 2018, *Chaetozone* Malmgren, 1867, *Kirkegaardia* Blake, 2016 and *Tharyx* Webster and Benedict, 1887, arise as two long and thick processes (compared to the branchiae), usually from the back or the posterior margin of the peristomium, owning them the name of "bitentaculate" Cirratulidae (Fig. 1, B). They usually have a narrow and conical prostomium, and are smaller species that rarely size above a couple of centimeters. Members of *Dodecaceria*, constitute their own group. Their paired tentacles, arising laterally, are similar to that of other bitentaculate Cirratulidae. Cirratulidae belonging to this genus are thus referred to as the "hard-bottom bitentaculate" Cirratulidae (Fig. 1, C) as they are exclusively found boring into calcareous constructions. Additionally, recent molecular phylogenies have recovered the family Ctenodrilidae Kennel, 1882 as nested within Cirratulidae (e.g. Weidhase et al., 2016; Magalhães et al., 2017). Ctenodrilidae are small annelids and do not possess the characteristic tentacles of other Cirratulidae While the genera *Raphidrilus* Monticelli, 1910 and *Raricirrus* Hartman, 1961 have similar branchiae to that of other Cirratulidae, the genera *Ctenodrilus* Claparède, 1863, and *Aphropharynx* Wilfert, 1974 do not.

Amongst the 357 extant nominal species of Cirratulidae (based in all/most cases on distinct combinations of morphological features), 227 are bitentaculate, with no less than 91 species and one genus described as new to science in the last 10 years (Read and Fauchald, 2019). It is, however, expected that many species are left to be discovered and described (e.g. Elías et al., 2017; Munari et al., 2017; Chambers, 2000; Chambers and Woodham, 2003). Of the six genera of bitentaculate Cirratulidae, *Chaetozone* is the most diverse with 65 species worldwide. The genus *Chaetozone* is distinguished from the other bitentaculate Cirratulidae by having posterior segments modified into cinctures created by elevated parapodia bearing fascicles of numerous unidentate spines, sometimes with a few bidentate hooks (Blake, 2018). The nature and arrangement of chaetae are the main characters differentiating the bitentaculate genera, as *Aphelochaeta* bears only smooth capillary chaetae, *Kirkegaardia* bears both smooth and serrated capillary chaetae, *Caulleriella* bears capillary chaetae and bidentate hooks, *Tharyx* bears capillary chaetae and knobby tipped spines and *Chaetocirratulus* bears capillary chaetae and a few unidentate spines not arranged in cinctures (Blake, 2018; Blake and Magalhães, 2017). However, the validity of these genera in terms of monophyly has yet to be tested.

Cirratulids are difficult to identify to species because diagnostic features are often a unique combination of qualitative or quantitative morphological features, that in many cases may vary with growth (e.g. number, segmental origin and even chaetal morphology), preservation procedures (e.g. body shape or prostomial morphology), be subject to interpretation (e.g. type of chaetae or presence of a first achaetous segment) and be difficult to observe (e.g. position of the first pair of branchiae). These difficulties have hindered a stable and robust classification and resulted in a long and complicated history for the systematics of Cirratulidae (Blake, 1996; Rouse and Pleijel, 2001).

Many sequenceable specimens (=recently collected and fixed in ethanol) of *Chaetozone* from the North-East Atlantic (NEA) and the Arctic were available from natural history museums in Bergen and Trondheim, Norway. We studied the diversity of the common and broadly distributed genus *Chaetozone* using two molecular markers, the universal barcoding mitochondrial cytochrome *C* oxidase I (COI) and the nuclear 28S ribosomal RNA region D1-D2 (28S). We aimed at uncovering the species diversity in the area and compare molecular results with previously identified morphogroups, in order to evaluate traditional diagnostic features. In order to assess the monophyly of *Chaetozone*, we inferred a phylogeny of the family, based on these two nuclear and mitochondrial markers.

2. Material and methods

2.1. Study area and sampling

The area of study was the North-East Atlantic and Arctic, including the Norwegian Sea, Barents Sea, Greenland Sea and North Sea. The specimens studied were collected mainly from UM/BIO surveys (University Museum and Department of Biology, Bergen), MAREANO project, POLYSKAG (Marine bristle worms (Polychaetes) in coastal waters of Skagerrak), BIOSKAG 2 (Deep Skagerrak), UNIS 2007 and 2015 (University Center in Svalbard) cruises, housed at University Museum of Bergen (ZMBN) and NTNU University Museum (NTNU-VM) (Bakken et al., 2020). Some additional specimens were collected from the Mediterranean Sea (Mallorca, Spain), fixed and preserved in 96% ethanol. Type specimens were made available by The Swedish Natural History Museum (SMNH), the National Museums of Scotland (NMSZ) and the British Museum (Natural History, BMNHN). In total, 306 ethanol fixed specimens were selected for molecular work across the geographic area and across the range of depth covered by the different materials (from 6 to 1256 m).

The study area was divided in six biogeographic regions according to their different oceanographic and topographic characteristics (Blindheim and Rey, 2004; OSPAR, 2010; Yashayaev et al., 2015; Nygren et al., 2018). The Greenland Sea (Fig. 2, cyan stars) is a deep cold-water area and the Barents Sea (Fig. 2, purple dots) is a shallow cold-water area. The Norwegian coast and shelf (Fig. 2, red pentagons), the Skagerrak (Fig. 2, orange triangles) and the North Sea (Fig. 2, blue squares) are shallow areas (less than600 m) with warmer water, while the Norwegian Sea (Fig. 2, green diamonds) is a deep-water area.

A total of 1500 specimens, either fixed and preserved in 96% ethanol or fixed in 10% formalin and preserved in 75% ethanol (mainly from Jan Mayen and the Barents Sea), were examined.

In total, 306 ethanol fixed specimens were selected for molecular work across the geographic area and across the range of depth covered by the different materials (from 6 to 1256 m).

2.2. Morphological studies

Specimens of *Chaetozone* were first sorted into preliminary morphogroups to select a comprehensive number of specimens from different morphologies and biogeographical areas for DNA sequencing. After the molecular analyses, the DNA vouchers were examined more carefully in order to look for morphological patterns representative of each species. Important characters for species identification include the

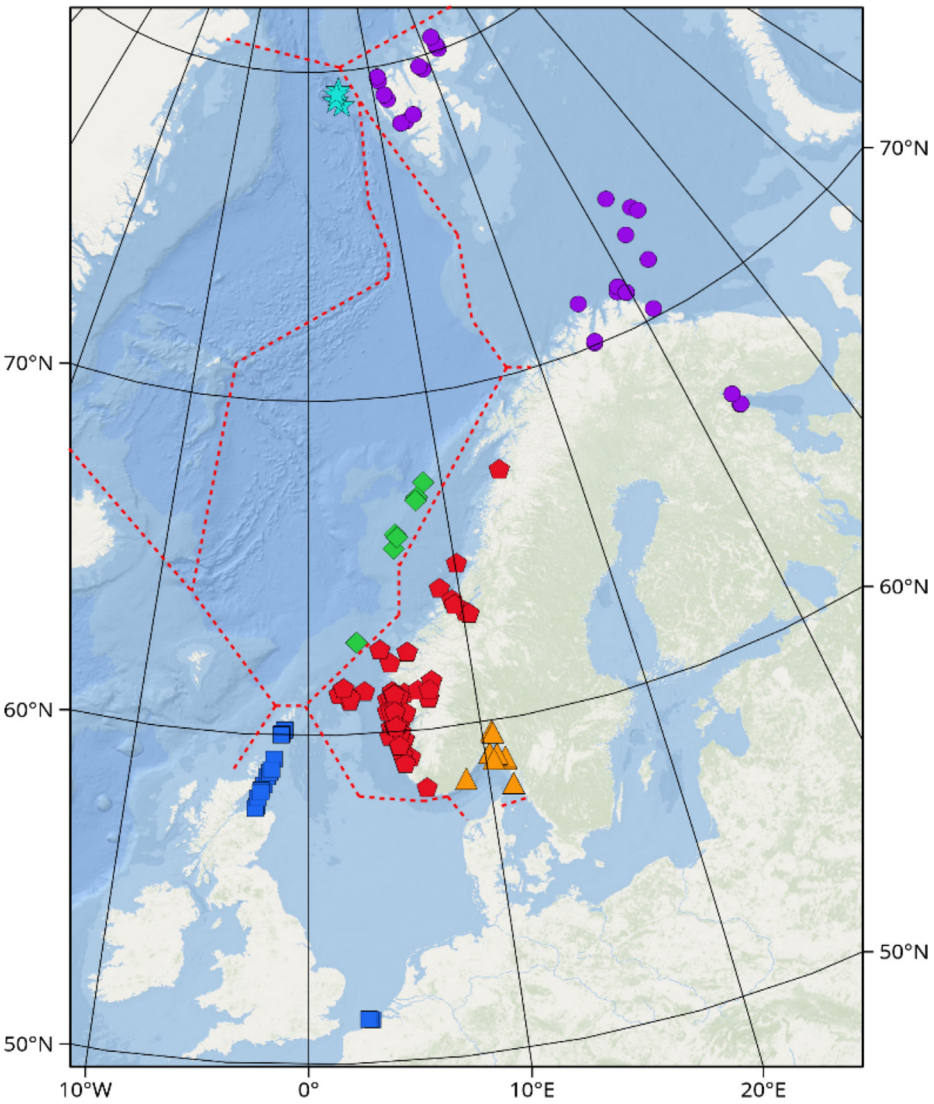

Fig. 2. Sampling sites for molecular studies and biogeographic regions. Delimited by red lines; Greenland Sea (cyan stars), Barents Sea (purple dots), Norwegian Sea (green diamonds), Norwegian coast and shelf, (red pentagons), Skagerrak (orange triangles), North Sea (blue squares). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

general shape of the body, the presence or absence of a dorsal or a ventral groove or ridge along all or part of the body, the shape of prostomium and peristomium (length, presence or absence of peristomial rings or dorsal bump), the nature of the first segment (chaetous or achaetous), the position of the paired tentacles (on a peristomial ring, at the posterior margin of the peristomium or on an anterior segment), the position of the first branchiae (at the posterior margin of the peristomium or on an anterior segment), the nature and arrangement of the chaetae (e.g. length of capillaries, shape of acicular spines, distribution along the body), the shape of the posterior modified segments (high or low, complete or incomplete cinctures) or the shape of the pygidium (Blake, 2015). However, morphology and distribution of chaetae can vary with ontogeny (Elias and Rivero, 2009). All these characters were evaluated for each DNA voucher, and the DNA-based species delimitation served as a fixed reference to discriminate between intra- and inter-specific variation. The goal of these morphological studies was a preliminary comparison of material into morphogroups following traditional diagnostic features. A more detailed revision of morphological attributes will be made when the species are formally described (Grosse et al., in prep).

All specimens were examined using a Leica M165C stereomicroscope and a Leica DM250 compound microscope. Detailed pictures of specimens were taken with a Leica MC170HD camera mounted on a Leica M165C stereomicroscope or Leica DFC420 camera mounted on a Leica MZ16A stereomicroscope. All specimens were stained in a solution of Shirlastain A (SDL International LTD), to enhance contrast at the surface of the specimens and allow an easier and more precise observation of the external morphology. Additionally, some specimens were stained in a methyl blue solution that stains glandular tissues and reveal a distinct pattern in some species (e.g. Doner and Blake, 2006; Dean and Blake, 2009). Parapodia of some specimens, usually an anterior and a posterior one, or complete segments, were mounted on slides in Euparal and photographed with a Leica DFC420 camera mounted on a Leica DM60000 B compound microscope. Some specimens were examined with a Zeiss SUPRA 55VP scanning electron microscope (SEM) at the electron microscopy lab of the University of Bergen. To be certain to link the right morphological characters to the right species previously determined through molecular analyses, some of the DNA vouchers had to be used. The specimens were critically point dried with a Polaron critical point dryer (Watford, England) and coated with gold (40%) and palladium (60%) with a Polaron SC502 coater.

Detailed results of morphological analyses will be part of a subsequent paper describing some of the undescribed species presented here (Grosse et al., in prep).

2.3. DNA sequencing

A few parapodia or a few of the posterior-most segments were taken from the specimens for DNA extraction. Tissue samples from 95 specimens were sent to the Canadian Center for DNA Barcoding, Biodiversity Institute of Ontario, University of Guelph, Guelph, Ontario, that performed sequencing on both strands using the primer pairs polyLCO/polyHCO or ZplankF1_t1/ZplankR1_t1 (Table 1). The rest of the samples (211 specimens) were processed in the facilities of the NTNU University Museum as follows. Tissue samples were placed into 50 µL of QuickExtract (Epicentre) and heated at 65 °C for 60 min followed by 3 min at 95 °C in a thermos-shaker at 300 rpm. These extractions were diluted in 200 µL of EB buffer.

Amplification of the target DNA fragments was done by Polymerase Chain Reaction (PCR). PCR mixtures contained 0.30 µL of each primer, 1.4 µL of DNA template and 10 µL of RedTaq 1.1x MasterMix 2.0 mM MgCl2 (VWR) for a final reaction volume of 12 µL. The different pairs of primers used (jgLCO1490/jgHCO2198, CirrCOIF/CirrCOIR, or polyLCO/polyHCO for COI; and 28SC1′/28SD2 for 28S) and the PCR thermal cycling profiles are shown in Table 1. 1.5 µL of each PCR product was run for 45 min on a 1% agarose gel electrophoresis containing SYBR safe (Invitrogen) for DNA detection and visualized using GeneSnap from SynGene software (Version 6.08, Cambridge, UK). All the successful PCR products were purified with illustra ExoProStar 1-Step (GE Healthcare, Litlle Chalfont, UK). Cycle sequencing was performed on both strands by Eurofins Genomics DNA Sequencing Department (Ebersberg, Germany). Forward and reverse reads were merged into consensus sequences using Geneious 11.0.5 (https://www.geneious.com).

2.4. Phylogenetic analyses

In addition to the sequences produced in the present study, 202 published and unpublished sequences of COI (194 sequences) and 28S D1-D2 (8 sequences) were downloaded from the Barcode of Life Data System (BOLD, Ratnasingham and Hebert, 2007) and GenBank (Benson et al., 2008). These sequences were from specimens identified as members of *Aphelochaeta*, *Caulleriella*, *Chaetozone*, *Cirratulus*, *Cirriformia*, *Ctenodrilus*, *Dodecaceria*, *Kirkegaardia*, *Protocirrineris*, *Raricirrus*, and *Timarete*, as well as three outgroups: *Flabelligera affinis*, *Glyphanostomum* sp. and *Polycirrus* sp. (following Rouse and Pleijel, 2001; Weidhase et al., 2016; Magalhães et al., 2017)

COI sequences were aligned with MUSCLE (Edgard, 2004) implemented in Aliview 1.25 (Larsson, 2014). 28S D1-D2 sequences were aligned with MAFFT 7 online version (Katoh et al., 2017) with the algorithm Q-INS-i, that considers the secondary structure of RNA, using the 200PAM/k = 2 scoring matrix and a gap penalty of 1.53.

Best fitting models for each marker were selected using PartionFinder 2.1.1 (Lanfear et al., 2016; Guindon et al., 2010) with the Bayesian Information Criterion. The number of variable and parsimony-informative sites was calculated with MEGA 10.0.5 (Kumar et al., 2018). Nucleotide divergence (p-distance and K2P distances) over sequence pairs within and between the well supported lineages after the phylogenetic analyses and species delimitation analyses was estimated in MEGA X 10.0.5 (Kumar et al., 2018). Paired positions containing gaps and missing data were removed.

To test the monophyly of the different genera, Maximum likelihood (ML) and Bayesian inference (BI) analyses were performed on each marker independently, as well as on a combined dataset including only the specimens for which both markers were available. Two more dataset were created containing respectively all *Chaetozone* COI and 28S sequences. Duplicate sequences were removed from these two datasets for downstream species delimitation analyses and they were analyzed with BI.

Both the COI and the 28S datasets contained missing data and the 28S dataset also contained some poorly aligned regions. Gblocks 0.91b (Castresana, 2000) was used to remove these regions in a repeatable way and test their influence. Gblocks was used with its softest parameters: allowing smaller blocks, gaps within the final blocks, and less strict flanking conditions.

ML analyses were conducted using IQ-TREE 1.6.10 (Nguyen et al., 2015). A GTR + Γ model with four Γ category count was used for the COI datasets and TN93 + Γ model with four Γ category counts was used for the 28S datasets. For the combined analysis, each partition was allowed to have its own set of branch length. Support values were estimated with a 10,000 ultrafast bootstraps (Hoang et al., 2018). BI analyses were conducted using BEAST2 (Bouckaert et al., 2014). A GTR + Γ model with four Γ category count was used for the COI partition and an TN93 + Γ model with four Γ category counts was used for the 28S partition. A strict clock was assumed for both partitions. A Yule model was used as tree prior with a default Γ distribution as birth rate prior for both partitions. A lognormal distribution with M = 1.0 and S = 1.25 for kappa parameters prior of the 28S partition (Drummond and Bouckaert, 2015). For the family-wide COI analysis, a monophyly constraint was put on the ingroup excluding the outgroup. Trees of both partitions were linked in the combined analysis. All analyses were run

Table 1
PCR Primers: The different primer pairs used to amplify both markers used in this study and their respective cycles.

Region	Name	Source	Sequence 5′-3′	Cycle	
COI	jgLCO1490	(Geller et al. 2013)	TITCIACIAAYCAYAARGAYATTGG		3 min 96 °C
	jgHCO2198	(Geller et al. 2013)	TAIACYTCIGGRTGICCRAARAAYCA	34x	60 s 95 °C
					60 s 48 °C
					60 s 72 °C
					6 min 72 °C
	CirrCOIF	(Weidhase et al. 2016)	TTTTTCTACTAACCATAAAGACATTG		60 s 96 °C
	CirrCOIR	(Weidhase et al. 2016)	CCGAGGAAGTGTTGAGGGA	34x	60 s 94 °C
					60 s 53 °C
					60 s 72 °C
					5 min 72 °C
	polyLCO	(Carr et al. 2011)	GAYTATWTTCAACAAATCATAAAG		60 s 96 °C
	polyHCO	(Carr et al. 2011)	TAMACTTCWGGGTGACCAAARAATCA	5x	40 s 95 °C
					40 s 46 °C
					60 s 72 °C
				35x	40 s 94 °C
					40 s 51 °C
					60 s 72 °C
					7 min 72 °C
	ZplankF1_t1	(Prosser et al. 2013)	TCTASWAATCATAARGATATTG		60 s 95 °C
	ZplankR1_t1	(Prosser et al. 2013)	TTCAGGRTGRCCRAARAATCA	29x	40 s 94 °C
					40 s 51 °C
					60 s 72 °C
					5 min 72 °C
28S	28SC1	(Le et al. 1993)	ACCCGCTGAATTTAAGCAT		60 s 96 °C
	28SD2	(Le et al. 1993)	TCCGTGTTTCAAGACGG	29x	30 s 95 °C
					60 s 62 °C
					60 s 72 °C
					7 min 72 °C

with a chain length of 50,000,000. Convergence of each run and parameter was checked using Tracer 1.7.1 (Rambaut et al., 2018). A maximum clade credibility was obtained with Treeannotator (Bouckaert et al., 2014) after discarding 20% of the trees as burnin. All phylogenetic analyzes were performed on Cypres Science Gateway (Miller et al., 2010). Trees were visualized and edited using FigTree 1.4.4 (Rambaut, 2014) and LibreOffice Draw.

2.5. Species delimitation

All trees after BI analyses of the *Chaetozone* dataset were analyzed for species delimitation using two methods: the multi-Rate Poisson Tree Process (mPTP, Kapli et al., 2017) and the General Mixed Yule Coalescent model (GMYC, Pons et al., 2006; Fujisawa and Barraclough, 2013). mPTP 0.2.4 was applied through its webserver (https://mptp.h-its.org) and GMYC was implemented in R (R Core Team, 2015) with the packages ape 5.3 (Paradis and Schliep, 2018), MASS 7.3-45 (Venables and Ripley, 2002), Paran 1.5.2 (Dinno, 2018) and splits 1.0–19 (Ezard et al., 2017).

3. Results

3.1. Phylogenetic results

For the present study, 175 new COI sequences (GenBank accession numbers (AC): MT06591-MT066031, MT361869-MT361871, BOLD AC in Supplementary material 1) and 122 new 28S D1-D2 sequences (GenBank AC: MT365538-MT365659) were obtained. A total of 369 sequences were used to infer the COI gene tree and 127 for the 28S gene trees (Supplementary Material 1). The COI dataset was 658 bp long and contained 438 variable sites, 388 of which were parsimony-informative. The 28S alignment was 770 bp long, contained 342 variable sites, 249 of which were parsimony-informative. Both markers were available for 65 specimens (outgroups excluded).

Analyses of the different datasets before and after Gblocks presented very little differences. The bootstrap values and posterior probabilities did not vary significantly (1–10%). The results presented here are those of the analyses of the whole datasets without removal of data by Gblocks (Fig. 3, Supplementary Material 2).

For the sake of clarity, the results are presented using the final species hypotheses and a numbering system. *Chaetozone* species are numbered from 1 to 25 (Fig. 4). *Chaetozone* is recovered monophyletic in all analyses, except in the COI dataset analyzed with ML. However, two species, 17 and 22 previously identified as *Tharyx* sp. (Carr et al., 2011) and *Aphelochaeta* sp. respectively, are nested within *Chaetozone* and assumed to be misidentifications. *Aphelochaeta* is also recovered monophyletic in all analyses. The genus *Cirratulus* is recovered monophyletic in all analyses, except in the 28S dataset analyzed with ML, and the one sequence labelled as *Protocirrineris* nested within is assumed to be a misidentification. The genus *Raricirrus* Hartmann, 1961, is recovered monophyletic in the COI analyses, and no 28S sequences were available for this genus. *Ctenodrilus* Claparède, 1863 and *Dodecaceria* form together a monophyletic group in the COI analyses, but though *Ctenodrilus* is recovered as monophyletic, it remains unclear whether it is within or sister to *Dodecaceria*. No 28S sequences were available for *Ctenodrilus*. The genera *Timarete* and *Cirriformia* are recovered paraphyletic. At a higher level, the multitentaculate cirratulids are supported as monophyletic, nested within bitentaculates (Fig. 3).

3.2. Species diversity

For *Chaetozone*, GMYC recovers 26 clusters from the COI dataset, and 11 from the 28S dataset. mPTP recovers 24 clusters from the COI dataset, and 9 from the 28S dataset (Fig. 4). There are few differences between the outcomes of these four analyses, and for the sake of simplicity we will present them relatively to the final species hypotheses. The reasoning behind the final hypotheses of species delimitations is presented in the discussion. The results of mPTP and GMYC analyses of COI are identical for 22 of the putative species and differ on the boundaries within species 8 and 23. mPTP groups 18 sequences in species 8, where GMYC makes two clusters: one with 17 sequences and another with just one. A similar pattern emerges for species 23, where mPTP clusters together three sequences and GMYC divides them in two distinct clusters. This species 23 belongs to the Pacific Ocean and is not

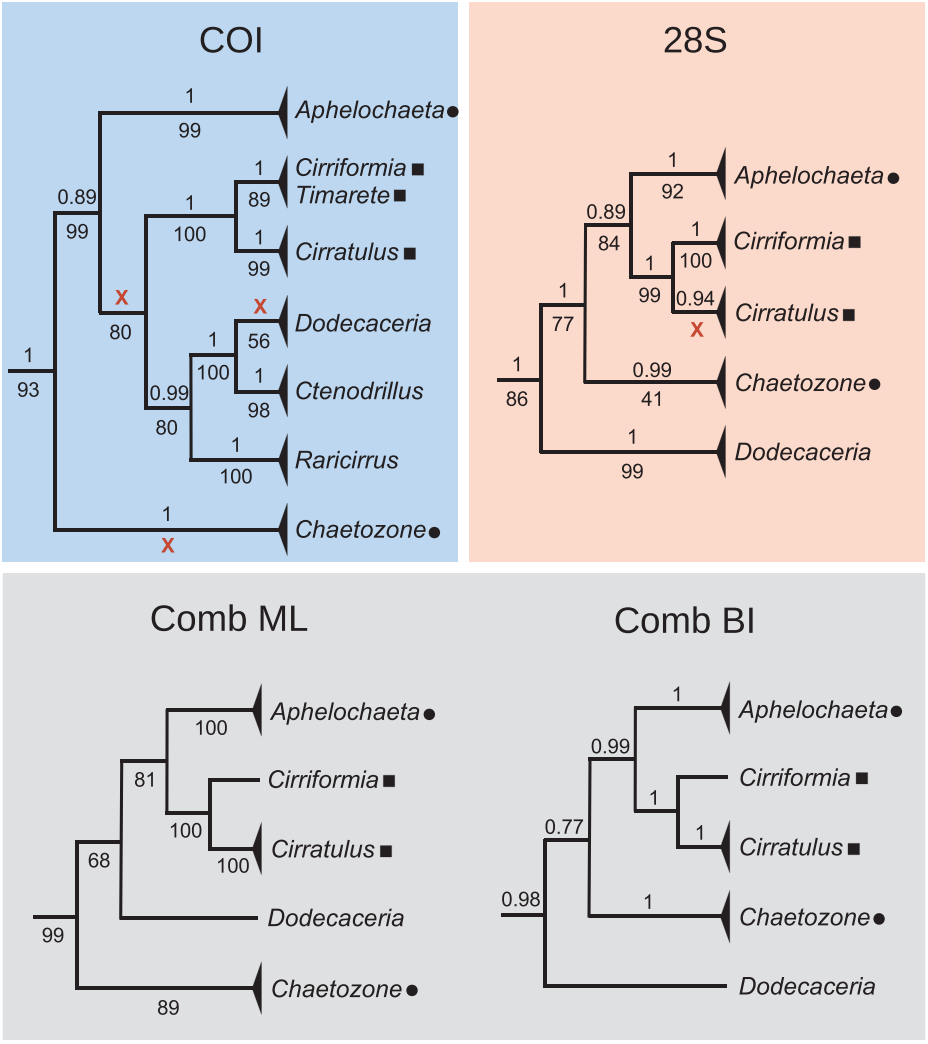

Fig. 3. Phylogeny of Cirratulidae. Upper left: COI gene tree; Upper right: 28S gene tree; Bottom: ML and BI analyses of the combined dataset. Posterior probabilities and bootstrap values are indicated above and below branches respectively. Red crosses above or below branches indicate that the clade was not recovered in BI or ML analyses respectively. Black circles indicate soft-bottom bitentaculate Cirratulidae. Black squares indicate multitentaculate Cirratulidae. Branch lengths are arbitrary. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

part of this study. Since there is no additional data to support one or the other molecular results, therefore we tentatively and conservatively consider it as one species. The results of mPTP and GMYC analyses of the 28S dataset are identical for eight of the resulting putative species and differ on the delineation of species 7 and 8. mPTP lumps species 7 and 8 together, while GMYC separates them and further divides species 7 in two clusters. Only one of these clusters matches one from the COI dataset. Though species 7 and 8 are well supported, further branching within them, as picked up by GMYC, is not. Both mPTP and GMYC analyses of the 28S dataset lump together species 9 and 10 and also species 11 and 12.

In total, we find 25 species of *Chaetozone,* and 14 are from the study area.

3.3. Morphology of Chaetozone

The first round of morphological examinations revealed that the majority of specimens could be assigned to three species, all previously reported in the study area: *Chaetozone setosa* Malmgren, 1867, *C. jubata* Chambers and Woodham, 2003 and *C. zetlandica* McIntosh, 1911. However, some characters showed great variation between and within each of these preliminary morphogroups, in particular in the general body shape, the presence/absence of a ventral/dorsal groove or ridge, the shape of the head and the length of the chaetae. This variation, often continuous, was difficult to interpret either as inter- or intra-specific variation, or even fixation artefact, as many specimens were partially collapsed.

More detailed morphological analyses were compared with the molecularly delimited species to discriminate between inter- and intra-specific variation. Characters like the position of the paired tentacles and of the first pair of branchiae do not seem to show intra-specific variation, but are often shared between several species. Other characters such as the general body shape (e.g. some swollen anterior segments) or the shape of the peristomium and the prostomium (e.g. degree of fusion or separation of both, length, obvious peristomial rings) can show rather important variability in some species and none in other. Few species are identifiable through a unique morphological feature, but rather a combination of characters is needed to identify a species or species complex. Other specimens could not be attributed to any described species. More detailed morphological analyses were compared with the molecularly delimited species to discriminate between inter- and intra-specific variation. At least seven distinct morphogroups can be distinguished. Species 7 and 8 are morphologically identical, and correspond to the morphological *C. setosa*. Species 8 is the only species sampled in Svalbard, including the type locality: Spitsbergen. It is also found in the Barents Sea and in the White Sea

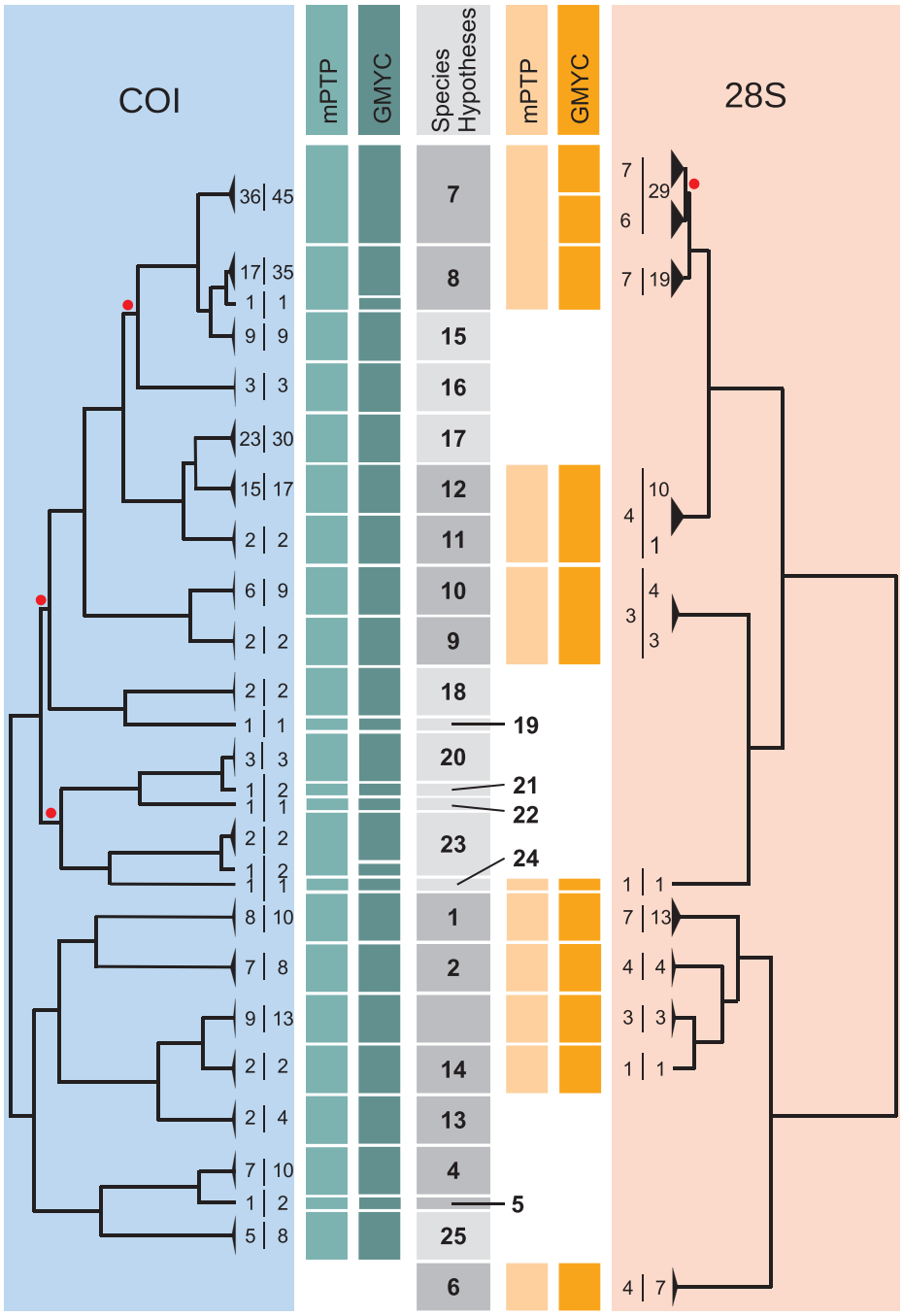

Fig. 4. Species delimitation in *Chaetozone*. From left to the middle: Bayesian COI gene tree, number of unique haplotypes per clade (=sequences in the analyses), total number of sequences (=specimens) per clade, mPTP clusters, GMYC clusters. From Right to the middle Bayesian 28S gene tree, total number of sequences (=specimens) per clade, number of unique haplotypes per clade (=sequences in the analyses), GMYC clusters, mPTP clusters. The final species hypotheses are indicated in the middle. Species present in the study area are highlighted in dark gray. Red dots indicate branches with a support value below 0.8. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(Fig. 5). Species Seven in found on the shelf along the Norwegian coast, in the North Sea and in the Skagerrak (Fig. 5). Therefore, we conclude that species 8 is in fact the true *C. setosa*. Species 9 and 10 are also identical and are similar to *Chaetozone zetlandica*. They are found in the same area (Norwegian coast and shelf) (Fig. 5) and in the same depth range (6–95 m). It not possible at this stage to determine which one of them, if any, is in fact the original *C. zetlandica* as its type locality is Shetland (Scotland). Species 2 and 4 present considerable intra-specific variation but are not morphologically different from one another. Species 1 is nearly identical to species 2 and 4 except for the presence of long segmented capillary chaetae. These three species are similar to *Chaetozone jubata*. They are found in the same area (Norwegian coast and shelf) (Fig. 5) and in the same range of depth (mostly between ~200 and ~600 m). It is not possible at this stage to determine which one of them, if any, is *C. jubata* as its type locality is the Faeroe-Shetland channel. Species 3, 5 and 12 have also been examined in detail and each present a unique morphology and are interpreted to be new to science. These species are currently being described and will be the subject of a following paper (Grosse et al., in prep.). For other species, the specimens are too few or in too poor conditions to draw any significant conclusions.

4. Discussion

4.1. Phylogeny of Cirratulidae

The hypothetical close relationship between Ctenodrillidae and *Dodecaceria* recovered in our study is congruent with previous analyses including these taxa (e.g. Bleidorn et al., 2003; Weidhase et al., 2016) that suggest *Ctenodrilus* as an ingroup or a sister group of *Dodecaceria*.

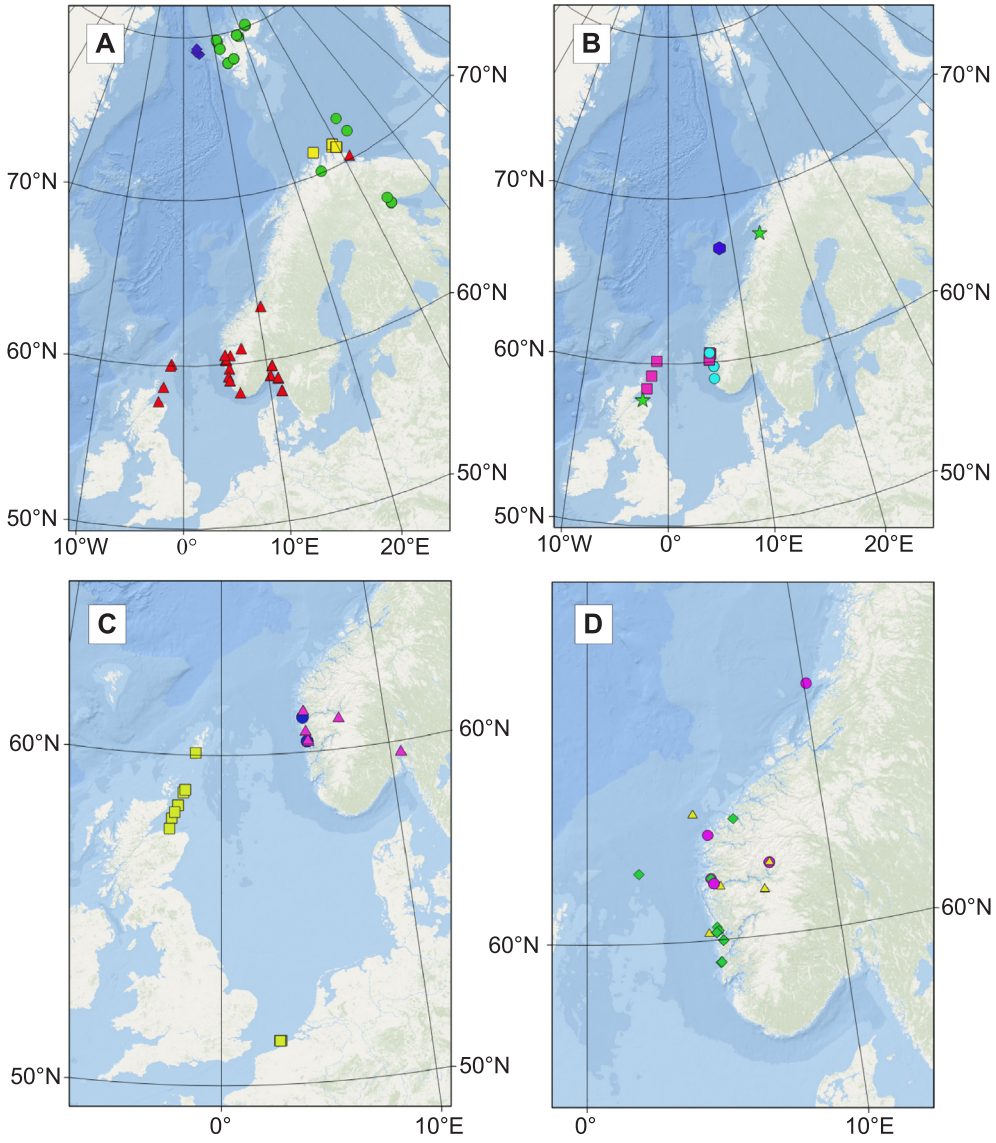

Fig. 5. Distribution of *Chaetozone* species. A: Species 3 (yellow squares), Species 7 (red triangles), Species 8 (green circles), Species 13 (blue squares); B: Species 9 (turquoise circles), Species 10 (pink squares), Species 11 (green stars), Species 14 (blue hexagon); C: Species 5 (blue circles), Species 6 (pink triangles), Species 12 (square triangles); D: Species 1 (green squares), Species 2 (yellow triangles), Species 4 (pink circles). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Our results are also in line with that of Magalhães et al. (2017) and Choi et al. (2018) who suggest that *Timarete* and *Cirriformia* are closely related and probably not reciprocally monophyletic. The three genera of multitentaculate Cirratuliade included in this study - *Cirratulus*, *Cirriformia* and *Timarete* - cluster together, which indicates the multitentaculate Cirratulidae seem to be a natural grouppresence of multiple tentacles is a synapomorphy of member of this clade. Concerning the soft-bottom bitentaculate Cirratulidae, the genera *Aphelochaeta* and *Chaetozone* have not been recovered as sister groups, therefore, bitentaculate Cirratulidae are shown to be paraphyletic. The presence numerous unidented spines, arranged in cinctures and characteristic of the genus *Chaetozone* is indeed an autapomorphy of this group.

A few conflicts appear between the different methods (BI vs ML) and datasets (COI, 28S, combined). In particular, the relation of *Dodecaceria* and *Chaetozone* with the rest of the family is not the same, due to incongruence between the resolution at the base of the topology after analyses of the mitochondrial and nuclear markers. However, when analyzing the combined dataset, none of these topologies are particularly strongly supported (Fig. 3). Moreover, no reliable sequences are currently available for the genera *Kirkegaardia*, *Protocirrineris*, *Chaetocirratulus*, *Caulleriella, Fauvelcirratulus* and *Tharyx*. Therefore it is not possible to fully assess the phylogeny of the diversity of the family at this point. Another drawback is the identification of sequences downloaded from GenBank, that can often not be verified. While most of the *Cirriformia* and *Timarete* sequences came from recently described and redescribed species and judged reliable, this was not the case for all downloaded sequences. We regarded species 17 and 22, previously identified as *Tharyx* sp. (Carr et al., 2011) and *Aphelochaeta* sp., as misidentifications. We were not able to examine the vouchers, but their sequences were recovered nested within clades clearly presenting diagnostic characters of *Chaetozone*. Therefore, an identification error seemed the most likely explanation. The same reasoning is applied to the one sequence labelled as *Protocirrineris* nested within *Cirratulus*. This highlights the importance of depositing vouchers in museums, linked to the sequences deposited in GenBank (Pleijel et al., 2008). The addition of molecular data from more specimens, carefully identified and characterized, representing the full scope of morphological characters found in Cirratulidae is needed to get a clearer idea of the validity of all

Cirratulidae genera, their morphological diagnostic and their relationships.

4.2. Species delimitation

Overall the two species delimitation methods reached similar results when analyzing the *Chaetozone* COI datasets and disagreed only on two species: 8 and 23. Species 7 as inferred by mPTP was deemed the most reasonable after considering the molecular distance between the specimens (Supplementary material 3), their morphology and their geographical origin. In the case of species 23, the sequences were downloaded from BOLD and the vouchers are from the East Pacific. As such, they are out of the scope of this study and we conservatively treat them as one species, keeping in mind that these results may indicate cryptic diversity and warrant further investigation.

In situation of conflicts between COI and 28S analyses, the COI based delimitation was preferred, as COI was the chosen marker for species delimitation (e.g. Vogler and Monaghan, 2006; Vitecek et al., 2017; Nygren et al., 2018; Aguado et al., 2019).

4.3. Diversity of Chaetozone

In Europe, a total of eleven species of *Chaetozone* are reported: *C. setosa* Malmgren, 1867; *C. abranchiata* (Hansen, 1879); *C. caputesocis* (Saint-Joseph, 1894); *C. carpenteri* McIntosh, 1911; *C. zetlandica* McIntosh, 1911; *C. corona*, Berkeley and Berkeley, 1941; *C. vivipara* Christie, 1984; *C. gibber*, Woodham and Chambers, 1994; *C. christiei,* Chambers, 2000; *C. jubata*, Chambers and Woodham, 2003; *C. elakata*, Blake and Lavesque, 2017. However, the identity and generic position of some of these species (e.g., *C. abranchiata*, *C. caputesocis*, *C. zetlandica* and *C. vivipara*) need to be assessed, as descriptions often too succint (Petersen, 1999; Blake and Lavesque, 2017; Le Garrec et al., 2017).

Although several of the molecularly delimited species examined fit relatively well published descriptions, only one of the species could, with some degree of confidence, be associated with a valid species name: species 8 (Fig. 4) as *Chaetozone setosa*. This was only possible because recently collected material from the type locality was available. In the case of species 9 and 10, that are similar to *C. zetlandica*, or species 2, 4 and 1, that could be identified as *C. jubata*, these names cannot be attributed unequivocally to one of these species, because they seem to belong to complexes of cryptics pecies, without comparing the molecular data obtained herein to that of type material or at least material from their respective type localities. This highlights the need to obtain molecular data of specimens from type localities, which are capital to be able to resolve species complexes.

In addition to the aforementioned species complexes, at least three other new morphotypes are found in Europe and will be described as new species in an upcoming paper (Grosse et al., in prep). This will hopefully aid in better recognition of at least some *Chaetozonegroups of* species, which will help biodiversity assessment and monitoring programs. All in all, the number of species of *Chaetozone* known from the NEAthis area has increased by at least 11. With the possible resolution of the species complexes of *C. jubata* and *C. zetlandica,* even more species could be added to that number. As the example of *C. setosa* in this study clearly shows, this can be achieved by an extensive sampling, including type localities, and the use of molecular data. However, morphological identification of species stays difficult. While methyl green or methyl blue staining has shown some efficiency in distinguishing between species of *Aphelochaeta* (e.g., Doner and Blake, 2006), no pattern was found for the *Chaetozone* specimens stained in this study. Therefore, it becomes necessary to rely on traditional combination of characters, which do not allow to recognize cryptic species, or molecular data, which require additional efforts and expertise. As more species are discovered through molecular data, they are also described with sequences or molecular characters as only diagnostics (Nygren and Pleijel, 2011). This allows to have a name available for every species, especially in the case of species complexes, but it also means that molecular analyses are necessary in order to identify them and the use of molecular data.

Chaetozone setosa is one of many early described species that have been recorded all over the world (Chambers, 2000; Oug et al., 2014). The species was redescribed by Blake (2015), who concluded that C. setosa is restricted to Arctic and subarctic waters in Northern Europe. Our molecular data support this conclusion, with confirmed barcoded records from Svalbard, the Barents Sea and the White Sea. Records of *C. setosa* in the Norwegian Sea, the North Sea and the Skagerrak belong to a related species (species 7 in this paper) that will be described and delineated in an upcoming paper (Grosse et al., in prep). Records of *C. setosa* from other geographical areas most likely refer to morphological similar species. Specimens from the Canadian Arctic, were identified as *C. setosa* and sequenced as part of a previous study (Carr et al., 2011). Although these specimens are probably morphologically very similar (if not identical) to the two species from the North-East Atlantic, they cluster in a third species (Species 15 in this paper) closely related to them. The revision of putative cosmopolitan or broadly distributed species such as *Chaetozone setosa* is needed, as was recently pointed out by Hutchings and Kupriyanova (2018). Many of the current "cosmopolitan" polychaete species, were described in the early days, had succinct descriptions and were for decades the only available names when identifying species, even in regions distant from their type localities. As for several of these supposedly cosmopolitan species (e.g. Bleidorn et al., 2006; Álvarez-Campos et al., 2017; Simon et al., 2019), it is expected that most widely distributed species of Cirratulidae will be revealed to be complexes of regionally distributed species, except maybe for deep sea species (Elías et al., 2017). In the case of potential bioindicators, identifying the correct species during environmental monitoring is particularly important.

The majority of *Chaetozone* species (e.g. species 1, 2, 4, 5, 6, 7, 9, 10 and 11) occurs on the Norwegian coast and shelf, which present the most important diversity, followed by the North Sea (Fig. 5). Species 3 and 8 (*C.setosa*) are endemic to the Barents Sea, where only three species in total are present. Species 7 is the species with the broadest distribution, from the Barents Sea to the Skagerrak. Species of *Chaetozone* are also distributed differently in the water column. Species 9, 10, 11 and 12 are from relatively shallow waters, from the littoral to around 60 m deep. Species 5 and 7 occupy slightly deeper waters, from around 30 to 160 m deep, while species 1, 2 and 4 occupy deep water from 200 to 1200 m deep inside the fjords. These last species are morphologically similar to *C. jubata*, which has been described as a deep water species (> 500 m, Chambers, 2003; Chambers et al., 2007). A single species has been discovered from the deep waters of the Greenland Sea, and another one from the Norwegian Sea where they are respectively restricted, but considering the extremely low sampling in these areas, it is not possible to draw ant conclusion about the diversity in these regions.

Knowledge of species distribution, but also the discovery of new species are directly related to the sampling effort (e.g. Nygren et al., 2018). Though the material in this study tried to cover a wide geographic area and a large range of depth, most of the data obtained was from the southern and rather shallow parts of the Norwegian coast and shelf, while several regions, in particular the deeper waters of the North East Atlantic were barely sampled. Even though a greater number of species than expected was revealed within *Chaetozone* in this work, it is therefore probable that an even higher diversity might be uncovered when studying further these parts of the ocean.

Even in well studied geographic regions, there is still a great amount of unknown diversity that can only be revealed through integrative taxonomy. Indeed, we are convinced that molecular taxonomy will be the key to solve the systematic puzzle that are European bitentaculate Cirratulidae.

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Maël Grosse: Conceptualization, Software, Formal analysis, Investigation, Data curation, Writing - original draft, Visualization. **Torkild Bakken:** Conceptualization, Investigation, Resources, Data curation, Writing - review & editing, Supervision. **Arne Nygren:** Conceptualization, Investigation, Resources, Writing - review & editing. **Jon A. Kongsrud:** Conceptualization, Investigation, Resources, Data curation, Writing - review & editing, Supervision. **María Capa:** Conceptualization, Investigation, Resources, Writing - review & editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ympev.2020.106852.

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