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ORIGINAL ARTICLE

Species delimitation and phylogenetic relationships of the *Prionospio* complex (Annelida, Spionidae) in the Northeast Atlantic

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Abstract

The *Prionospio* complex comprises the most diverse and complex group within the polychaete family Spionidae. The phylogenetic relationships within the group are still poorly understood, and the generic breakdown is unstable. In this study, we assessed the diversity, relationships, and distribution of species of the *Prionospio* complex occurring in Norwegian waters. We analysed mitochondrial genomes and nuclear ribosomal DNA assembled via whole-genome shotgun sequencing, and Sanger sequenced fragments of COI and 16S rDNA. Sanger sequencing proved challenging in the group, where COI was only amplified successfully in 14% of specimens. By molecular species delimitation algorithms, our study revealed the presence of four well-supported but currently undescribed species of *Prionospio* in Norwegian waters. We observed a novel distribution pattern of polychaetes in coastal waters, where certain species demonstrated distribution ranges spanning over 7000 km. Such wide distribution parallels patterns of deep-sea *Prionospio* species, suggesting that factors beyond recent anthropogenic translocations are involved. Our analysis of 38 mitochondrial genomes and ribosomal nuclear DNA enabled us to hypothesise on the phylogenetic relationships of 14 species of the *Prionospio* complex. The analysis suggested that two characters previously used to designate genera: the beginning of the branchiae from chaetiger 3 and the presence of pinnules on the branchiae, might have evolved more than one time within the complex. We return *Aurospio banyulensis* to the genus *Prionospio* according to the diagnosis of *Aurospio* resulting tree where this species was nested among *Prionospio* species. Our findings provide new insights into the diversity and distribution patterns of *Prionospio* species and contribute to a better understanding of marine benthic biodiversity and the importance of taxonomic accuracy in conservation and management practices.

KEYWORDS

biodiversity, cosmopolitan species, phylogeny, *Prionospio*, species delimitation

1 | INTRODUCTION

Marine sediments represent the largest ecosystem on Earth (Snelgrove, 1997). Among the macrofaunal groups inhabiting these sediments, polychaetous annelids are the most abundant (Hutchings, 1998). Despite their significant abundance, our current understanding of polychaete diversity remains incomplete. Only a fraction of the species has been discovered and described, leaving a large portion of the diversity unexplored (Appeltans et al., 2012; Pamungkas et al., 2019). This knowledge gap presents a significant challenge for accurately managing marine ecosystems. Identification of marine macroinvertebrates is one of the most robust and widely used ways to monitor the health of marine benthic communities (Pearson, 1978; Pocklington & Wells, 1992). However, accurate identification relies on a solid foundation of basic knowledge of species. Morphologically similar species might occupy vastly different ecological niches, emphasizing the importance of taxonomic accuracy in conservation and management practices.

In the Northeast Atlantic, polychaetes have been studied by many zoologists, including Linnaeus (1767), Malmgren (1867) and McIntosh (1915, 1922, 1923) among many others. These studies discovered and described a rich diversity of polychaetes in coastal waters, including numerous representatives of one of the largest polychaete families, Spionidae Grube, 1850. However, despite the long history of taxonomic morphological investigations in Europe, modern molecular approaches have shown that our knowledge of diversity is still incomplete. This incompleteness is the result not only of the natural change of marine communities but also of numerous introductions of alien species, as well as a cryptic diversity that cannot alone be revealed by analysis of only morphological features.

Prionospio Malmgren, 1867 and closely related taxa constitute the most diverse and complex group within the polychaete family Spionidae, the so-called *Prionospio* generic complex (Radashevsky, 2015). Systematic treatment of these spionids was reviewed and developed by Foster (1971), Blake and Kudenov (1978), Maciolek (1985), Wilson (1990), Blake (1996), Sigvaldadóttir (1998), and Yokoyama (2007). Various groupings (genera, subgenera, *Prionospio* sensu *lato*, *Prionospio* sensu *stricto*) were proposed by these authors based on different sets of external morphological features of adults and different ideas about their importance for taxonomy. Most relevant for the present study are two taxa first established by Foster (1971) at the generic rank: *Minuspio* Foster, 1971 (later designated as a subgenus of *Prionospio* sensu *lato* and subsequently synonymised with *Prionospio*) characterised by having only apinnate branchiae from chaetiger 2, and *Prionospio* sensu *stricto* with a combination of apinnate and pinnate branchiae from chaetiger 2. Blake et al. (2020) briefly reviewed previous studies on the *Prionospio* complex and provided six morphological features common to the members. However, each of the noted features is homoplastic, also shared by other spionids. Blake et al. (2020) listed 126 species of the complex and grouped them into seven genera, *Apoprionospio* Foster, 1969, *Aurospio* Maciolek, 1981a, *Laubieriellus* Maciolek, 1981b, *Orthoprionospio* Blake & Kudenov, 1978, *Paraprionospio* Caullery, 1914, *Prionospio*, and *Streblospio* Webster, 1879a. *Prionospio* was the largest, comprising 100 species. Considering previous morphological arguments for grouping these species and the results of the first analyses using molecular data by Guggolz et al. (2020) and Abe and Sato-Okoshi (2021), it is likely that the classification adopted by Blake et al. (2020) is convenient for identification purposes but does not reflect the phylogenetic relationships of the species and requires further attention.

Species of the *Prionospio* complex are common inhabitants of soft sediments throughout the world. They often form dense settlements from the intertidal to the abyss both in environments with high oceanic salinity and in estuaries and lakes with brackish or near-fresh water. The complex also includes species classified as opportunistic, as well as species sensitive towards anthropogenic impacts (Borja et al., 2000). Some species have repeatedly been reported as widespread or even cosmopolitan. These "cosmopolitans" are usually species that were briefly described in the 19th century and then identified globally based on a simple set of characters that are common to many other species. Molecular analysis of the members of the complex is still in its initial stage, with only a few species studied.

In Norway, seven species of the *Prionospio* complex have been recorded, including *Aurospio banyulensis* (Laubier, 1966) and six species of *Prionospio* (Mackie, 1984; Pleijel, 1985; Sigvaldadóttir, 1992; Sigvaldadóttir & Mackie, 1993). Of these, *Prionospio fallax* Söderström, 1920 was originally described from Sweden; *Prionospio plumosa* M. Sars in G. O. Sars, 1872 from Norway, and *Prionospio steenstrupi* Malmgren, 1867 from Iceland. Four species were described outside the Northeast Atlantic: *A. banyulensis* from the Western Mediterranean Sea, *Prionospio cirrifera* Wirén, 1883 from the Kara Sea, *Prionospio dubia* Day, 1961 from South Africa, and *Prionospio multibranchiata* Berkeley, 1927 from British Columbia, Canada. Remarkably widespread or cosmopolitan natural distribution of some deep-sea spionids (Guggolz et al., 2020), and secondary, human-mediated distribution of some spionids associated with shells (Radashevsky et al., 2019, 2021,

2022, 2023; Radashevsky, Malyar, et al., 2020) have recently been confirmed by molecular data. However, many other records of species far from their type localities still require verification for their correct identification (Capa et al., 2013; Leaché et al., 2009; Satler et al., 2013). Species of the *Prionospio* complex from the Northeast Atlantic have not been investigated in this way.

Molecular tools have gained widespread usage for distinguishing species. PCR amplification of a standard gene region ("DNA barcode") or a combination of a few mitochondrial and nuclear markers is the most prevalent approach in molecular analyses. Algorithm-based species delimitation methods are often used to analyse molecular data (e.g., Aguado et al., 2019; Grosse et al., 2020; Hektoen et al., 2022). While such approaches have proven invaluable, they are sensitive to biases and may not always accurately delimit species boundaries (Doorenweerd et al., 2023; Dufresnes & Jablonski, 2022; Funk & Omland, 2003). Different species delimitation algorithms may provide disparate results (Camargo et al., 2012) and it is recommended to employ multiple methods and trust congruent delineations (Carstens et al., 2013). Furthermore, incongruences between mitochondrial and nuclear DNA gene trees can occur, often due to processes where different genes do not share the same evolutionary history (Ballard & Whitlock, 2003). Such complexities underscore the necessity of integrating multiple lines of genetic evidence, alongside morphological data.

To address the limitations of PCR-based methods, such as "universal" primers failing to amplify DNA in many animal groups (e.g., Che et al., 2012; Li et al., 2014), several alternative approaches have been proposed. Shallow shotgun-based whole genome sequencing, colloquially known as "genome skimming", is one of these proposed methods (Coissac et al., 2016; Trevisan et al., 2019). This method involves sequencing bulk DNA at low coverage, enabling retrieval of elements that are abundant in genomic DNA extracts, such as organelle genomes and nuclear ribosomal genes. Genome skimming significantly increases the available genetic data compared to Sanger-based methods and has been successfully employed to delimit species in challenging groups (e.g., Duan et al., 2023; Johri et al., 2020; Maddison & Sproul, 2020). However, in studies of polychaetous annelids, the standard for molecular species delimitation and phylogenetic analyses are still based on Sanger methods (e.g., Aguado et al., 2019; Kupriyanova et al., 2023), and most existing mitogenomes are derived from single-specimen studies (e.g., Li et al., 2016).

The aim of this study was to (1) reveal the species diversity of the *Prionospio* complex occurring in Norwegian waters, (2) assess the extent of the geographical distribution of these species, and (3) provide a hypothesis of their phylogenetic relationships.

2 | MATERIALS AND METHODS

2.1 | Study area and material collection

The study focused on species of the *Prionospio* complex in Norwegian waters. Norwegian samples were collected through projects organised by the NTNU University Museum, Norwegian University of Science and Technology, Trondheim, Norway, and the University Museum in Bergen, University of Bergen, Bergen, Norway, supported by the Norwegian Taxonomy Initiative. Additional fresh samples were collected by Åkerblå AS during routine biomonitoring of finfish aquaculture sites between 2019 and 2021. To assess distribution patterns and identity of the species it was also vital to study specimens from type localities and other geographical areas where they are reported. Thus, African samples were collected during the Guinea Current Large Marine Ecosystem (GCLME) and Canary Current Large Marine Ecosystem (CCLME) Projects between 2005 and 2012. Samples from the Russian Arctic were collected during cruise 72 of the R/V *Akademik Mstyslav Keldysh*. Specimens from British Columbia, the Sea of Japan, and South Korea were acquired on collection trips by one of the authors (VIR). *Aurospio banyulensis* from the type locality was provided by Arne Nygren, Gothenburg University. Specimens were initially examined morphologically, by stereomicroscopy (general morphology), compound microscopy (chaetae), and scanning electron microscopy for fine details. Morphological identifications were done using up-to-date taxonomic literature for the region (Mackie, 1984; Sigvaldadóttir, 1992; Sigvaldadóttir & Mackie, 1993). After examination, all specimens were deposited in the collections at the NTNU University Museum (NTNU-VM) (Bakken et al., 2023), the University Museum of Bergen, University of Bergen (ZMBN), and the Museum of the National Scientific Center of Marine Biology (MIMB), Vladivostok, Russia.

2.2 | DNA extraction, amplification, and sequencing

DNA was extracted from 136 specimens preserved in 96% ethanol (Table S1). Specimens were selected for molecular analyses based on two criteria. First, selection based on covering the greatest geographical region of occurrence of each morphospecies to assess the distribution patterns of each species. Secondly, based on capturing the greatest morphological variation in each morphospecies to uncover potential cryptic species. Total genomic DNA was extracted using the DNeasy Blood & Tissue (Qiagen) or QuickExtract™ (Lucigen) kits, following the manufacturer's protocols. Approximately 1–2 mm^3

tissue (5–15 mg) was used for the DNA extractions, usually a few parapodia to entire lateral sections depending on the size of the specimen. COI and 16S rDNA fragments were initially selected for amplification as previous studies on spionids have shown some success with these markers (Radashevsky et al., 2016; Radashevsky, Pankova, et al., 2020). For 16S rDNA, two different primer sets were used, one amplifying a ~ 550 base pair (bp) region. The second primer pair amplified a shorter ~400 bp region and was used if the first primer pair failed to amplify DNA. For COI, five different primer pairs amplifying the Folmer region were tested (Table S2). All PCR reactions consisted of the following reagents: 15.35–17.35 μL ddH_2O, 2 μL 10× buffer, 2 μL 10 μM dNTP, 2 μL 10 μM forward and reverse primer, 0.15 μL TaKaRa™ taq and 1–3 μL template DNA. PCR products were purified and sequenced by Eurofins Genomics by bi-directional BigDye (v3.1) termination sequencing.

To increase data yield and bypass issues accompanying locus-specific primers, 40 specimens covering all species and regions were selected for shallow whole-genome shotgun sequencing (genome skimming). Genomic DNA was sheared to approximately 350 bp using Covaris Focused-ultrasonicator ME220. Illumina sequencing libraries were prepared using the Blunt-End Single-Tube (BEST) library protocol (Carøe et al., 2018; Mak et al., 2017). We included two negative controls of molecular-grade water to monitor for cross-contamination during the library build process. Sequencing adaptors were ligated to samples and purified using SPRI beads, then indexed P5/P7 adaptors were incorporated by PCR using a custom number of cycles determined with a real-time PCR. Following P5/P7 adaptor incorporation, individual libraries were SPRI bead purified and pooled equimolarly. The pool was sequenced on a Novaseq 6000 PE150 by Novogene Europe with a total data yield of 400GB for the 40 specimens and two negative controls.

2.3 | Bioinformatic treatment

Demultiplexed FASTQ sequence files were delivered by Novogene Europe with sequence error rates between 0.03% and 0.04% for all samples. Adapters were trimmed using cutadapt v.1.8 (Martin, 2011), allowing for 10% sequencing errors in adapters. Two different approaches were employed to retrieve mitochondrial genomes and nuclear ribosomal DNA. Firstly, mitochondrial genomes were extracted using NOVOPlasty v.4.3.1 (Dierckxsens et al., 2017) with standard settings. The mitochondrial genomes of *Boccardiella hamata* (Webster, 1879b) and *Marenzelleria neglecta* Sikorski & Bick, 2004, were used as seeds (GenBank accession MW528029.1 and MK120303.1). Secondly, de novo assembly of each sample was performed using SPAdes v.3.13.0 (Prjibelski et al., 2020) with default settings.

Following SPAdes assembly, blast databases of scaffold files in each assembled file were generated using Blast v.2.5.0 (Altschul et al., 1990). Blast results were generated for 18S rDNA, 28S rDNA, and the mitochondrial genome using query sequences of *Aurospio foodbancsia* Mincks et al., 2009, for 18S (GenBank accession EU340097.1), *P. dubia* for 28S (GenBank accession EU418867.1), and *B. hamata* and *M. neglecta* for mitochondrial genome. Seqtk v.1.3 (github.com/lh3/seqtk accessed 15.04.2022) was used to extract single fasta consensus sequences from each of the top hit scaffolds of each BLAST search. The top hit sequences for each locus were viewed in Geneious Prime build 2022-11-28 along with the BLAST query sequence. When the BLAST search yielded multiple top hits consisting of multiple shorter sequences for each marker, consensus sequences were made by aligning the shorter sequences with the locus-specific query sequences and complete consensus sequences extracted from other samples, using the MAFFT v7.490 (Katoh & Standley, 2013) implementation in Geneious Prime with default settings. bwa v.0.7.17 (Li & Durbin, 2009) was used to align raw reads to the recovered locus sequences, and SAMtools v.1.7 (Danecek et al., 2021) was used to remove PCR duplicates and report depth at each nucleotide position.

Mitochondrial genomes were annotated through the MITOS2 web server (Donath et al., 2019), and the 13 protein-coding genes and large and small subunit ribosomal genes were extracted for phylogenetic analyses. The annotated gene regions were further edited by aligning them with other published annotated mitochondrial genomes from spionids, and making sure the regions were homologous. Poorly aligned indel regions of the large and small ribosomal RNA subunits for mitochondria (12S, 16S) and nuclear DNA (28S, 18S) were masked in trimAI v1.2 (Capella-Gutierrez et al., 2009) with the "automated1" option prior to downstream analyses. Sanger sequence chromatograms were assembled and quality-controlled in DNA Dragon v.1.5.1 (SequentiX). All Sanger sequences were aligned and concatenated in Geneious Prime.

2.4 | Species delimitation

The species delimitation analyses included both short-read Sanger sequenced data and mitochondrial genomes to assess the maximum number of specimens with the greatest geographical coverage. We also obtained relevant sequences from NCBI GenBank and Barcode of Life Data Systems (BOLD) (Table S1). In the markers where both genome skimmed and Sanger sequenced data was present

(16S rDNA and COI), the sequences were cut to be of the same length. Preliminary analyses were run, and sample collection information was examined to ensure the reliability of the identifications from the public repositories. In total, we included sequences from 155 specimens in the species delimitation analyses.

Different species delimitation methods have been reported to support different delimitations on the same dataset (Camargo et al., 2012; Carstens et al., 2013). Two species delimitation methods were applied to our dataset: The multilocus species delimitation algorithm "for Bayesian Phylogenetics and Phylogeography" (BPP) v.3.4 (Yang, 2015) and the Bayesian implementation of the single locus algorithm Poisson tree processes (bPTP) (Zhang et al., 2013). bPTP was run through the web server (http://species.h-its.org/; accessed 12.12.2022). Analyses were run for 500,000 generations on the mitochondrial genome, COI and 16S rDNA separate, COI and 16S rDNA combined, 18S and 28S combined, and 18S and 28S separate. Duplicate sequences were removed prior to analyses. Thinning was set to 100 and burn-in to 10%. The single-locus input trees were created through MrBayes v.3.2.7 (Ronquist et al., 2012) as described in the next section.

The Joint Bayesian species delimitation and species tree estimation algorithm (A11 analysis) (Rannala & Yang, 2017; Yang & Rannala, 2014) was conducted through BPP rather than just the strict species analysis (A10) to accommodate for uncertainty in the guide tree. BPP required estimations of the population size (θs) and divergence time (τs) parameters. Minimalist BPP was run to estimate these parameters (https://brannala.github.io/bpps/; accessed 22.12.2022) and was set to thetaprior 3 0.029 and tauprior 3 0.96. The analyses were run for 500,000 MCMC iterations with a burn-in of 125,000 and replicated once to check that the results did not diverge significantly between runs. Alignment gaps and ambiguous bases were removed by the program. Delimitation results with a posterior probability (PP) of 0.95 or higher were accepted, while lower PP delimitations were considered unsupported. All species delimitation results were mapped on a phylogram to improve visibility. The occurrences of each delimited species and type localities was plotted on maps using QGIS 3.20.0.

2.5 | Phylogenetic analysis

Three phylogenetic analyses were conducted. First on the concatenated mitochondrial genome and full nuclear ribosomal genes, secondly on the mitochondrial genome only, and third on the nuclear ribosomal genes only. All analyses only used data retrieved from the genome skimming approach. Trees were constructed through RAxML-NG v.1.1.0 (Kozlov et al., 2019) and MrBayes v.3.2.7 (Ronquist et al., 2012) parallel version. Species of *Marenzelleria* Mesnil, 1896, *Lindaspio* Blake & Maciolek, 1992, and *Rhynchospio* Hartman, 1936, were selected as outgroup taxa. Partitions were initially set up for each marker and each codon position for the protein-coding genes. Next, ModelFinder implemented in IQ-TREE 2 multicore version 2.2.2.3 (Chernomor et al., 2016; Kalyaanamoorthy et al., 2017; Minh et al., 2020) was used to infer best-fitting evolutionary models and partitions. ModelFinder utilises a greedy strategy where partitions are merged until the model fit does not increase any further. This resulted in 8 partitions: (1) the first codon position of *cox1*, *cob*, *cox2*, *cox3*; (2) the first codon position of *atp6*, *atp8*, *nad1, nad2*, *nad3*, *nad4*, *nad4l*, *nad5*, *nad6*, and 12S rDNA; (3) the second codon position of *atp6*, *cox1*, *cob*, *cox2*, *cox3*, *nad1*, *nad3*; (4) the second codon position of *atp8*, *nad2*, *nad4*, *nad4l*, *nad5*, *nad6*; (5) the third codon position of all protein-coding mitochondrial genes; (6) 16S rDNA; (7) 18S rDNA; (8) 28S rDNA. A third partition scheme following functional clustering was set up with five partitions: (1) the first codon position of all genes; (2) the second codon position of all genes; (3) the third codon position of all genes; (4) 12S and 16S rDNA; (5) 18S and 28S rDNA. Aikake's information criterion with correction for small sample size (AICc) and the Bayesian information criterion (BIC) was compared between the three partition schemes, where the setup with 8 partitions scored the best and was chosen for use in phylogenetic analyses. In RAxML-NG, evolutionary models for each partition were calculated with ModelFinder in IQ-TREE 2, while MrBayes was set to calculate the substitution model during the run for each partition using the "lset nst=mixed rates=gamma" and "unlink" commands. RAxML-NG was run with 50 parsimony-based and 50 random starting trees. The robustness of the consensus tree was tested by resampling 5000 bootstrap replicates, and subsequently Bootstrap support was mapped onto the best-scoring maximum likelihood tree. MrBayes was run for 85,000,000 generations in two independent runs of one cold chain and three heated chains each. Trees were sampled every 1000th generation, and the first 25% were excluded. The remaining trees were summarised into a majority rule consensus tree with posterior probabilities (PP) indicating the support for each clade. Tracer v. 1.7.1 (Rambaut et al., 2018) ensured the analyses were run long enough by examining the MCMC sampling statistics, where an effective sample size higher than 2000 was considered good. Figtree v. 1.4.4 (Rambaut, 2014) was used to visualise all trees. Bootstrap support higher than 70% and posterior probabilities higher than 0.95 was considered high support.

3 | RESULTS

3.1 | Sequence assembly

DNA was successfully amplified from Sanger sequencing for at least one marker in 112 out of 136 specimens (82%). 16S rDNA amplification was successful in all 112 specimens. Out of these, 67 amplifications were done using the primer pair amplifying a 550 bp region, while the remaining 45 amplifications were done with the primer pair amplifying a 400 bp region. COI was successfully amplified in 19 specimens only (14%) across all five primer pairs.

The genome skimming approach generated between 40 and 110 million raw reads per sample (mean: 68 million, standard deviation: 20 million) for all specimens, while negative controls produced 60 to 80 thousand raw reads. Samples yielded between 0.62 and 1.7 million scaffolds (mean: 1.3 million, standard deviation: 256 thousand). 4355 and 5180 scaffolds were obtained from the negative controls. No *Prionospio* sequences were extracted from the negative controls, and they were not considered further. NOVOPlasty circularised mitochondrial genomes from 15 samples, while SPAdes de novo assembly resolved mitochondrial genomes from 38 samples (including all 15 from the NOVOPlasty assembly). Neither assembly method generated mitochondrial genomes from two samples. In total, 38 out of 40 samples yielded mitochondrial genomes. For downstream analyses, 36 mitogenomes were from the SPAdes de novo assembly and two from the NOVOPlasty assembly. Average read coverage varied between 30 and 1384 among successful samples (mean: 326 mean, 320 standard deviation). Mitochondrial genomes varied in length from 15,007 to 19,439 base pairs. The order of protein-coding and ribosomal genes was the same in all sequenced specimens: *cox1*, *cox2*, *atp8*, *cox3*, *nad6*, *cob*, *atp6*, *nad5*, *nad4l*, *nad4*, *rrnS*, *rrnL*, *nad1*, *nad3*, *nad2*. Differences in mitogenome length between samples were primarily attributable to large un-annotated regions between *atp6* and *nad5*, likely corresponding to the control region. ATG was the initiation codon for all specimens and genes, except one species (specimens MH26, MH28, MH29, MH31), which had the altered start codon GTG in cytochrome b. TAA was the most common stop codon, present in about 60% of all protein-coding genes between all samples, while TAG and incomplete stop codons (TA- and T--) comprised the remaining 40%.

All 22 transfer RNAs (tRNAs) common in invertebrates were annotated in all mitogenomes, including two each for serine (S1 and S2) and leucine (L1 and L2). The tRNAs were ordered in five different arrangements between the samples but were always congruent within species. The two most common RNA orders were shared between 14 samples each. The most frequent differences in gene order were due to trnC(gca) and trnR(tcg) shifting positions (Table S3). All genes and tRNAs were found to be organised on the plus strand of the DNA.

3.2 | Species delimitation

Species delimitation was performed using a combination of genome skimming and Sanger sequencing data. 16S rDNA was cut to 354 bp, and COI to the barcoding region of 658 bp, so all sequences were of the same length. The single locus bPTP and multilocus BPP methods were employed to delimit species. The BPP analysis delimited 14 species with high support (PP > 0.99), while the bPTP analyses delimited between 12 (18S) and 15 (mitochondrial genome, and 16S and COI) species (Figure 1). The analyses based on mitochondrial markers split *Prionospio* sp. 7 in two compared to the BPP analysis. The single-locus 18S analysis was the most restrictive, delimiting 12 species, where *P. cirrifera*, *Prionospio* sp. 1, and *Prionospio* sp. 2 were considered the same species. The bPTP analyses based on 28S only, and 18S and 28S treated as a single locus, both mirrored the multilocus BPP delimitation with 14 supported species.

bPTP analyses of 16S rDNA and COI individually was also conducted, delimiting 14 and 15 species, respectively. In the 16S analysis, *Prionospio* sp. 7 was delimited as a single species, while it was split in two in the COI analysis. The full bPTP results from these two analyses are available in Figures S1 and S2.

From the combined output of all analyses, we assume 14 species, excluding the outgroup in our dataset. This number is consistent with the results of the BPP analysis, and bPTP delimitations of 28S, and combined nuclear ribosomal genes. 10 species were from Norwegian waters (Figure 1). Six of them were identified as previously described species: *Aurospio banyulensis*, *P. cirrifera*, *P.* cf. *dubia*, *P. fallax*, *P. plumosa*, and *Prionospio* cf. *sanmartini* Delgado-Blas et al., 2019. *Prionospio sanmartini* has not previously been recorded from Scandinavian waters. We were unable to identify seven of the delimited species either by morphology or genetic data (*Prionospio* sp. 1–7). Five of them (*Prionospio* sp. 1–3, sp. 6 and sp. 7) occur in East Atlantic or Arctic waters. Morphological characters of these five species are shown in Table S4. Descriptions of these species as well as revision of named species will be provided elsewhere (M. M. Hektoen, V. I. Radashevsky and T. Bakken, in preparation). Two species from our analysis were genetically identical to specimens from Japan sequenced by Abe

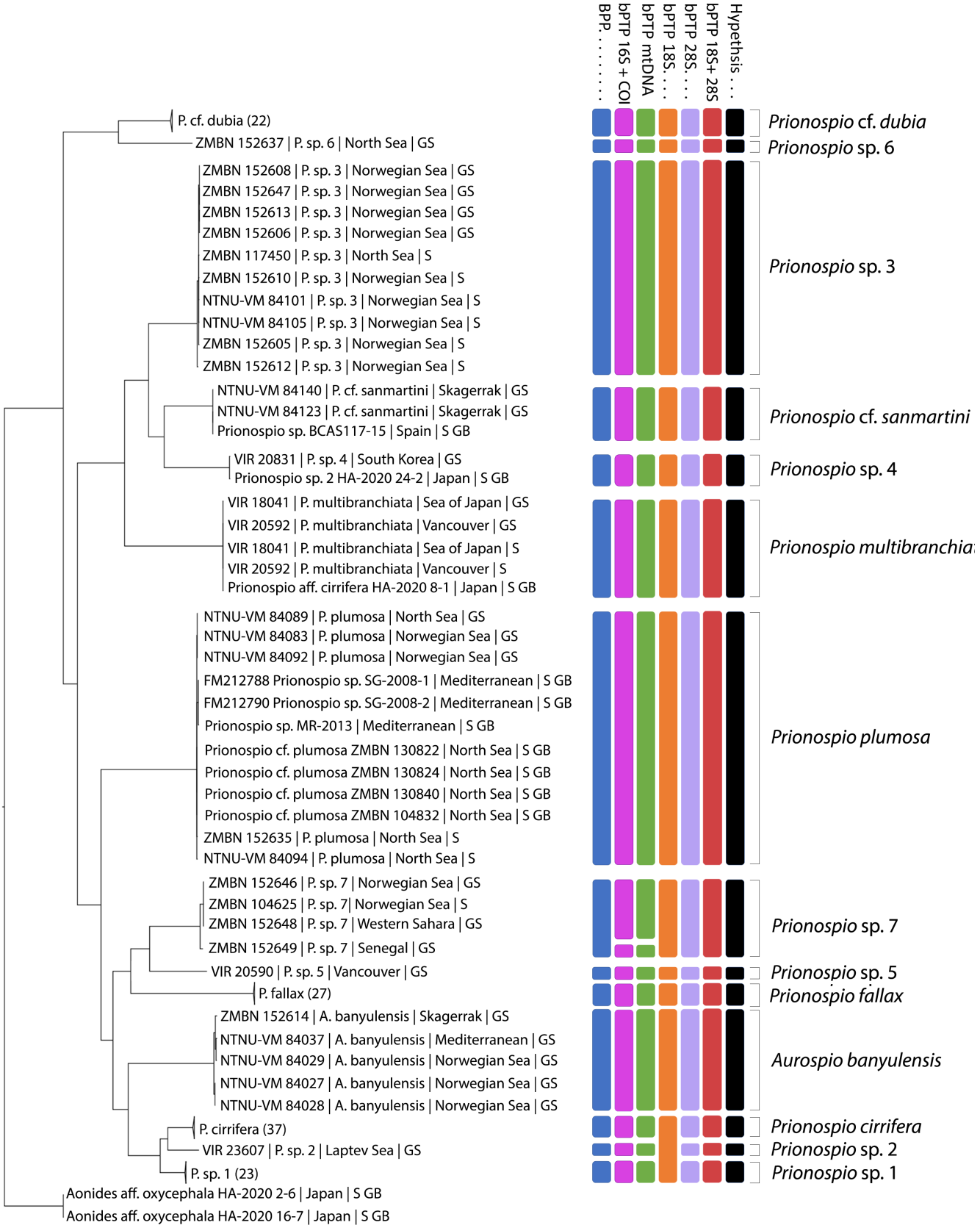

FIGURE 1 Molecular species delimitation results mapped on a phylogram. Clades containing more than 20 terminal nodes have been collapsed for readability, and number of sequences in collapsed clades are given in parenthesis. Terminal names are given as museum codes or personal database numbers of Vasily Radashevsky (VIR) where applicable, followed by species name, and geographic location. Letter codes at the end of terminals indicate data source. GB, GenBank; GS, Genome Skimming; S, Sanger sequencing. Putative species inferred by species delimitation algorithms are indicated by coloured bars on the right. The rightmost set of bars suggest the final hypothesis of species borders.

and Sato-Okoshi (2021): their *Prionospio* aff. *cirrifera* was identical to *P. multibranchiata*, and their *Prionospio* sp. 2 was identical to *Prionospio* sp. 4 from our analysis. Sequences from unidentified polychaete larvae from the eastern Mediterranean (Gaudron et al., 2010) clustered with *P. plumosa* in our analysis.

3.3 | Geographical distribution of *Prionospio* and *Aurospio*

Genetic data in this study provide new knowledge about the geographical distribution of all examined species and confirm wide distribution for some of them. *Prionospio* cf. *dubia* exhibited the widest distribution from Northern Norway to the Democratic Republic of Congo in the Gulf of Guinea (Figure 2). *Prionospio* sp. 7 was found in both West African and Norwegian offshore waters (Figure 2). *Prionospio cirrifera* was in samples from the Laptev Sea to Skagerrak (Figure 3), and *P. plumosa* was found along the Norwegian coast and in deep waters in the eastern Mediterranean (Figure 2). Specimens of *A. banyulensis* were found in the Western Mediterranean (type locality) as well as in Skagerrak, and the northern Norwegian Sea (Figure 2). *Prionospio multibranchiata* was not confirmed in Atlantic waters but was found on both sides of the North Pacific Ocean. *Prionospio fallax* (Figure 3), *Prionospio* sp. 3 (Figure 3),

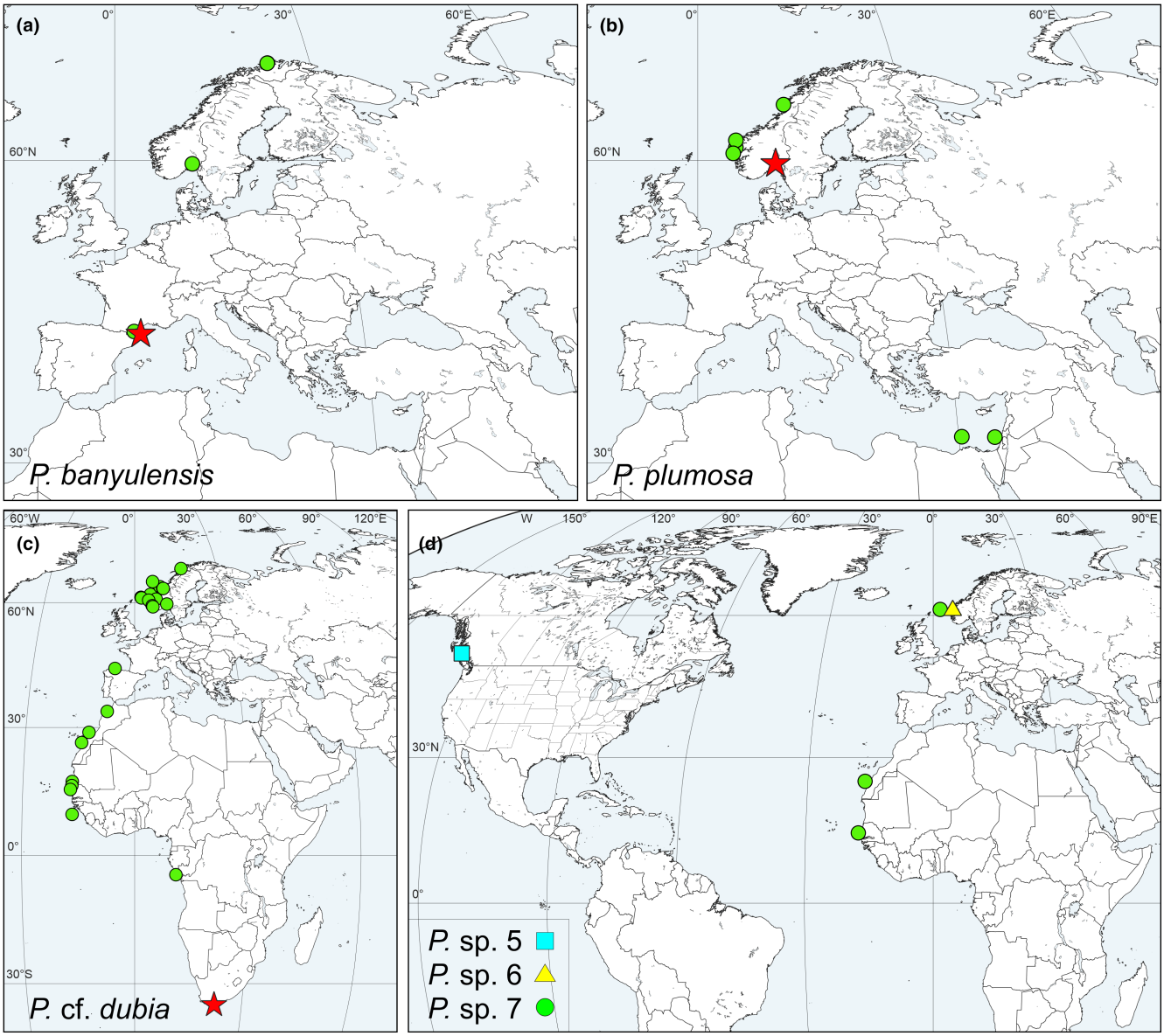

FIGURE 2 Map showing distribution of delimited species. (a) *Prionospio banyulensis* (green circles). (b) *Prionospio plumosa* (green circles). (c) *Prionospio* cf. *dubia* (green circles). (d) *Prionospio* sp. 5 (blue square), *Prionospio* sp. 6 (yellow triangle), *Prionospio* sp. 7 (green circles). Type localities are marked with red stars.

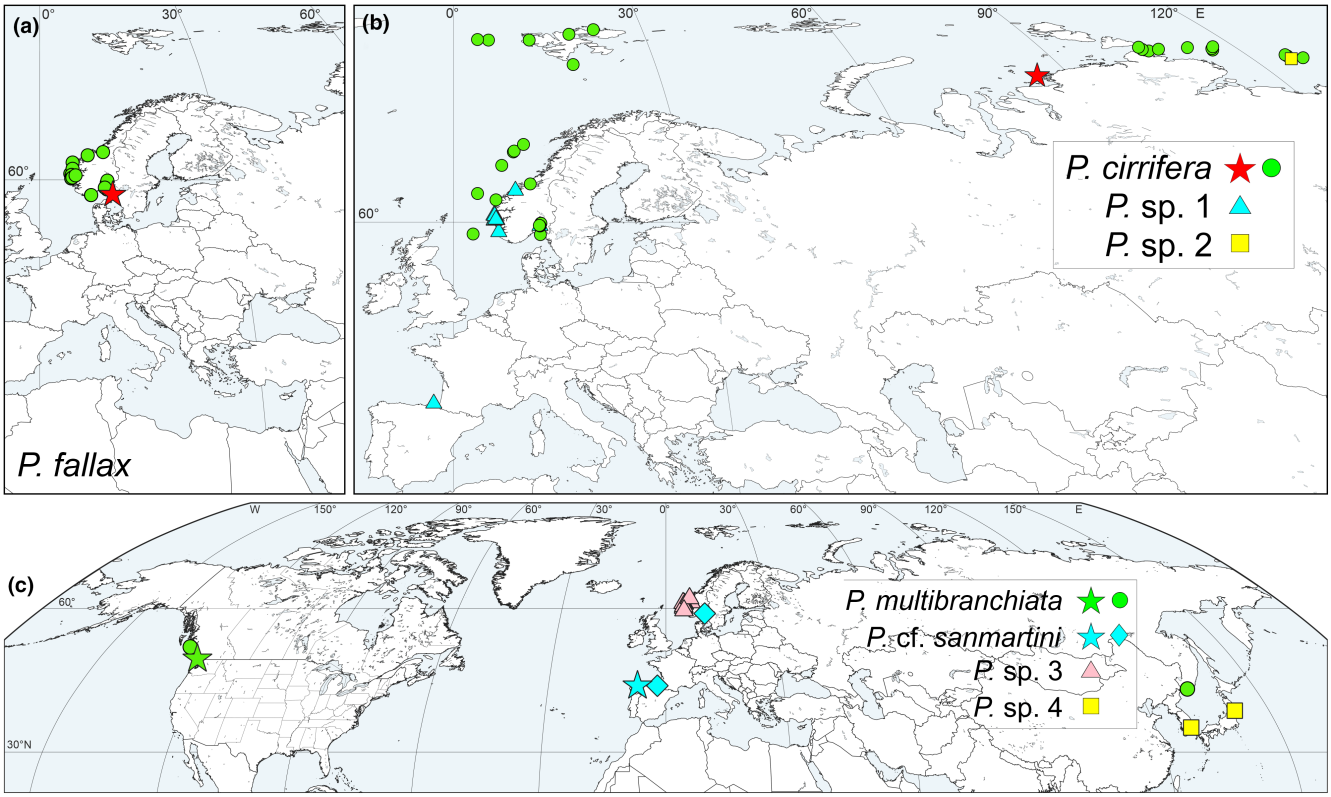

FIGURE 3 Map showing distribution of delimited species. (a) *Prionospio fallax* (green circles). (b) *Prionospio cirrifera* (green circles), *Prionospio* sp. 1 (blue triangles), *Prionospio* sp. 2 (yellow square). (c) *Prionospio* multibranchiata (green circles), *Prionospio* cf. *Sanmartini* (blue rhombi), *Prionospio* sp. 3 (pink triangles), *Prionospio* sp. 4 (yellow squares). Type localities are marked with red stars (a, b) or green and blue stars (c).

and *Prionospio* sp. 6 (Figure 2) were only present in samples from the southwestern coast of Norway.

3.4 | Phylogenetic relationships

Three sets of phylogenetic analyses were run using both Maximum Likelihood (ML) and Bayesian Inference (BI): (1) fully concatenated analysis of both mitochondrial and nuclear markers (18,456 bp); (2) protein-coding and ribosomal mitochondrial genes only (13,413 bp); and (3) the nuclear genes 18S and 28S rDNA (5033 bp). The trees from the fully concatenated analysis and mitochondrial genome analysis had the same topology and were both well-supported (Figure 4a, Figure S3).

The tree derived from nuclear ribosomal genes was poorly supported and exhibited a different topology to the mitochondrial and combined analyses (Figure 4b). Three species were placed differently in the nuclear gene tree compared to the mitochondrial. *Prionospio multibranchiata* was sister to *Prionospio* sp. 3 in the nuclear analysis and sister to a clade containing *Prionospio* sp. 3, *Prionospio* sp. 4, and *P.* cf. *sanmartini* in the mitochondrial and combined analyses. *Prionospio plumosa* was sister to the clade comprising *P. cirrifera* and two other morphologically similar species in the nuclear analysis and placed more basally in the mitochondrial and combined analysis. Finally, *P. cirrifera* was sister to *Prionospio* sp. 2 in the nuclear analysis and sister to *Prionospio* sp. 1 in the mitochondrial and combined analyses.

In all analyses, *A. banyulensis* was nested within *Prionospio*, sister to a clade comprising *P. cirrifera* and related species. Taxa with a combination of pinnate and apinnate branchiae (*Prionospio* sensu stricto after Foster, 1971), with only apinnate branchiae (*Minuspio* sensu Foster, 1971), and with branchiae from chaetiger 3 (*Aurospio* sensu Sigvaldadóttir, 1998) were mixed in both topologies.

4 | DISCUSSION

4.1 | Species delimitation

Using a molecular species delimitation approach, our study revealed five well-supported but currently undescribed species of *Prionospio* in East Atlantic and Arctic

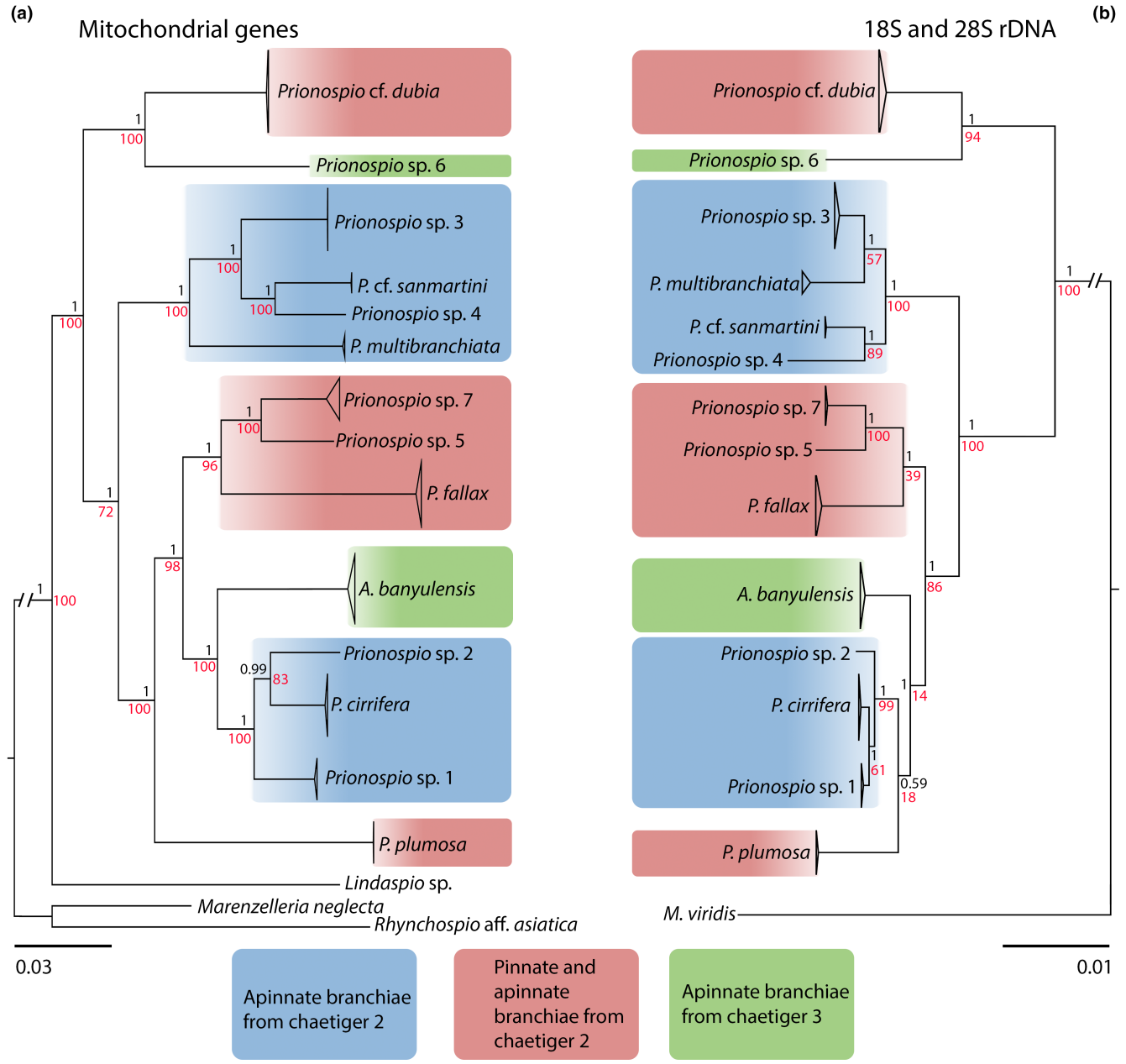

FIGURE 4 Consensus phylogenetic trees from Bayesian and maximum likelihood analyses. (a) concatenated mitochondrial genes. (b) Nuclear ribosomal 18S and 28S rDNA. Black numbers on nodes indicate posterior probabilities from the Bayesian analysis, red numbers indicate bootstrap support from the maximum likelihood analysis. Clades are coloured based on the morphological characters indicated at the bottom.

waters (*Prionospio* sp. 1–3, sp. 6, sp. 7), four of which are present in Norway (sp. 1, sp. 3, sp. 6, sp. 7). We could not definitely assess whether two species from the Pacific Ocean (*Prionospio* sp. 4 and sp. 5) represent undescribed species or not due to limited material for morphological study. *Prionospio multibranchiata* and *P. steenstrupi* have previously been reported from Norwegian waters but were not detected in the present study. Based on available data, we cannot confidently draw a conclusion regarding their presence, but, based on morphological characters, we suspect that they do not occur in the region. Taken together, our study increases the number of species from Norwegian waters from 7 to 10. These findings continue the trend that molecular species delimitation studies are useful in uncovering previously unknown and cryptic diversity, even in well-studied regions such as the Northeast Atlantic (Grosse et al., 2020; Nygren & Pleijel, 2011). Two species in our analysis, *P.* cf. *dubia* and *P.* cf. *sanmartini*, are only tentatively named. *Prionospio* cf. *dubia* due to incomplete species description and lost type material, and *P.* cf. *sanmartini* due to discrepancies between the species description and type material. Molecular data is absent

from the type localities of both species. Further discussion of the morphology and identity of these species will be provided elsewhere (M. M. Hektoen et al. in preparation).

While molecular species delimitation can be subject to bias when interpreting results in an unguided manner (Carstens et al., 2013), our delimitation results were largely convergent between the methods with few exceptions. The single-locus analysis of mitochondrial genes divided one putative species in two, whereas the 18S rDNA analysis lumped three putative species. 18S rDNA is a slowly evolving gene, and due to this may not always distinguish closely related polychaete species (Halanych & Janosik, 2006; Meißner et al., 2017). In contrast, mitochondria evolve at a faster rate and are only inherited maternally. Analyses solely based on mitochondrial markers may artificially separate polychaete species (Dellicour & Flot, 2018). bPTP does not implement a strict cutoff value for support but other lines of evidence should be investigated if delimitations receive low support. The number of species delimited in our separate analyses varied between 12 and 15, and our results reiterate the importance of using both mitochondrial and nuclear markers, and multiple algorithms when utilizing species delimitation methods.

4.2 | Distribution of *Prionospio* and *Aurospio*

We could assess the geographical distribution of the species occurring in Norwegian waters more accurately than in previous studies by including specimens from type localities and regions outside the Northeast Atlantic. Our results present a novel pattern of widely distributed polychaete species in shelf waters, demonstrated by *P.* cf. *dubia* occurring across a latitudinal range of at least 8500 km and *P. multibranchiata* across a longitudinal range of at least 7000 km. We also found that three species occur in both Norwegian and Mediterranean or West African waters. *Prionospio* cf. *sanmartini* was found in Skagerrak, the first time outside of its type locality in Northern Spain. However, the sequence from Northern Spain was a short-read COI metabarcode which is not always sufficient to distinguish between closely related species, and the identity of the Skagerrak species is still in question. Widely distributed species are often found to comprise cryptic species complexes (Barroso et al., 2010; Bleidorn et al., 2006; Hutchings & Kupriyanova, 2018; Nygren et al., 2018; Radashevsky et al., 2014; Seixas et al., 2021; Simon et al., 2019). Our findings are thus in contrast to many contemporary diversity studies.

Polychaetes with wide distribution ranges are more common in the deep sea (abyssal depths and below) than in coastal waters, possibly due to the relatively homogenous environmental conditions over large regions and low temperatures facilitating longer planktonic larval phases (McClain et al., 2009; O'Connor et al., 2007). Deep sea *Prionospio* and *Aurospio* species with wide distribution ranges have been reported previously (e.g., Maciolek, 1981a; Mincks et al., 2009; Paterson et al., 2016), some species even occur with a pan-oceanic distribution (Guggolz et al., 2020). This study is the first to confirm similar widespread distribution of *Prionospio* in coastal waters. Such distribution patterns in shallow water are often attributed to anthropogenic translocations via bait trade (Bergamo et al., 2019), symbionts of organisms reared for aquaculture (Radashevsky, Malyar, et al., 2020), fouling on ship hulls (Lewis et al., 2006) or transport via ballast water (Abe & Sato-Okoshi, 2021; Carlton, 1996; Carlton & Geller, 1993). Nonetheless, the similar patterns exhibited by *Prionospio* and *Aurospio* in the deep sea most likely indicate that recent anthropogenic translocation is not the single leading cause of the distribution discovered in coastal species of these genera. Like many spionid taxa, *Prionospio* has a planktonic larval stage, facilitating widespread distribution. *Prionospio* larvae have been studied morphologically (e.g., Abe & Sato-Okoshi, 2021; Hannerz, 1956; Radashevsky et al., 2006), however little is known about pelagic propagule duration for different species (Shanks, 2009). Considering the large distances these species are distributed, it is likely that gene flow occurs between distant populations through ocean currents over multiple generations, connecting different regions (McClain & Hardy, 2010; Rex & Etter, 2010). *Prionospio* cf. *dubia* has occasionally been recorded from abyssal depths (Maciolek, 1985), but it is unlikely that any species discussed in this study are common in the deep sea as they have not previously been reported from deep-sea specific studies. Several species discussed in this study have also been reported from the West Atlantic (Maciolek, 1985), East Pacific (Blake, 1996), and Australian waters (Wilson, 1990). However, due to our focus on the East Atlantic, the potential distribution patterns of these species in other global waters could not be thoroughly evaluated.

In our dataset we also had species with perhaps more limited distribution, such as *Prionospio* sp. 6. This species was only represented by a single specimen in the current analysis, and only four specimens in total have been found, all from a small region off the southwestern coast of Norway. Rare species with limited distributions are particularly important to characterise, as they are vulnerable to habitat change from anthropogenic factors (Gaston, 1994). Even though *Prionospio* sp. 6 is partly sympatric with *P.* cf. *dubia* and could have been misidentified as *P. dubia* in the past, they may not necessarily share the same ecological

niche. Further study of such taxa could therefore increase the accuracy of, for example, biomonitoring schemes. This is also the case for *P.* cf. *sanmartini* and *Prionospio* sp. 3, which likely have been confused with *P. multibranchiata* in the past, given their morphological similarity. These were not found to be sympatric and are less likely to share ecological niches.

4.3 | Phylogenetic relationships in *Prionospio* complex

Analysis of mitochondrial genomes and nuclear ribosomal DNA yielded information regarding the phylogenetic relationships of species in the *Prionospio* complex. *Aurospio banyulensis* was found nesting among *Prionospio* species. The monotypic genus *Aurospio* was erected for *Aurospio dibranchiata* Maciolek, 1981a, possessing three morphological characters unique for the *Prionospio* complex: two pairs of branchiae on chaetigers 3 and 4, branchiae being thin and flat, and branchiae basally fused to the notopodial postchaetal lamellae. Subsequent authors simplified the diagnosis of *Aurospio* to include all species with branchiae beginning from chaetiger 3. Blake et al. (2020, 58–59) re-established the original diagnosis of *Aurospio* and noted that "... subsequent researchers (Mincks et al., 2009; Paterson et al., 2016; Sigvaldadóttir, 1998) have misconstrued the differences between *Aurospio* and *Prionospio* and have taken species that clearly belong to *Prionospio* and referred them to *Aurospio*." Nevertheless, Blake et al. (2020, 59) included all of them in the list of *Aurospio* species (six in total) with a comment that the "issue will be addressed more fully in a subsequent study". Guggolz et al. (2020) analysed 16S rDNA from 21 deep-sea species of the *Prionospio* complex, including *A.* cf. *dibranchiata, A. foodbancsia*, and four unidentified species with branchiae from chaetiger 3, which they referred to *Aurospio*. Although the analysis was not well supported, they found *Aurospio* species appearing in four different clades mixed with *Prionospio* species, indicating that branchiae from chaetiger 3 probably do not mark a monophyletic clade. Together with two other species referred to *Aurospio*, *A.* cf. *dibranchiata* formed a well supported clade, which, however, was deep inside among *Prionospio* species (Guggolz et al., 2020, fig. 2). No unique morphological character shared by members of this clade was noted. We studied two species with branchiae from chaetiger 3 (*A. banyulensis* and *P.* sp. 6) and found them nested among different *Prionospio* species, thus showing support to the idea that branchiae from chaetiger 3 evolved more than one time within *Prionospio*. We did not include *A. dibranchiata* in our analysis, and therefore we cannot comment any further on the status of the genus. Nevertheless, at this point, we suggest returning *banyulensis* to *Prionospio* as it was originally assigned by Laubier (1966).

Generic divisions within the *Prionospio* complex have historically been established primarily based on morphological characters of branchiae. Within *Prionospio* sensu *lato*, worms with pinnate and apinnate branchiae from chaetiger 2 were assigned to *Prionospio*, while those with only apinnate branchiae from chaetiger 2 were assigned to *Minuspio*. However, our analysis of molecular data proposed that this crucial character has evolved (or been lost) more than once in the evolution of *Prionospio*. Difficult-to-place species are often referred to *Prionospio* (e.g., Peixoto & Paiva, 2019) which could artificially inflate the number of species in the genus. *Prionospio* sp. 6 in our analyses represents such a species, with characters typical for *Prionospio*, *Aurospio*, and *Laubieriellus*. We tentatively consider this species as a member of *Prionospio* due to its sister relationship with *P.* cf. *dubia*. Still, it illustrates problems with the generic systematisation of the *Prionospio* complex.

Incongruence between mitochondrial and nuclear gene trees has commonly been reported in phylogenetic studies (e.g., Platt et al., 2018), and can usually be attributed to complex evolutionary processes such as incomplete lineage sorting (Pamilo & Nei, 1988), lack of recombination (Ballard & Whitlock, 2003), and introgression (Toews & Brelsford, 2012). These processes can cause the evolutionary history of the mitochondrion to not accurately reflect the group's evolutionary history (Edwards & Bensch, 2009). In the present study, the incongruence was minor, where only three species were placed differently between the mitochondrial and nuclear trees, and both analyses show traditional taxonomic groups to be polyphyletic. The incongruence could also be an artefact of the conserved nature of 18S and 28S rDNA, and indicates the need for expanded nuclear datasets in future research. All issues in molecular (Guggolz et al., 2020; Abe & Sato-Okoshi, 2021; present study) and morphological (Sigvaldadóttir, 1998; Yokoyama, 2007) analyses show that the phylogenetic relationships within the *Prionospio* complex are still poorly understood and the position of members of this complex requires further study.

5 | CONCLUSION

The use of HTS approaches in polychaete studies has been increasing but is still mostly limited to family or higher phylogenies where one specimen per group is included (e.g., Zhang et al., 2018). Here, we show whole-genome sequencing to be a suitable approach to investigate intrageneric relationships. This is especially valuable in groups

like *Prionospio* where standard PCR-based approaches were largely unsuccessful due to primer specificity. Future research should focus on incorporating mitochondrial and nuclear genome data from other members of the complex and conducting more comprehensive taxon sampling from geographic areas not included in this study such as the West Atlantic and Australian waters.

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DATA AVAILABILITY STATEMENT

All sequences have been submitted to public repositories and specimens to museum collections. See Table S1 for detailed sample information. Raw sequencing data from whole genome shotgun sequencing is available in the European Nucleotide Archive (ENA) under project number PRJEB62452. Annotated mitochondrial genomes (accession numbers OR935903-OR935940), 18S rDNA (accession numbers OR243450-OR243488) and 28S rDNA (accession numbers OR243562-OR243599), and sequences acquired through Sanger sequences (accession numbers OR243489-OR243560) are available in NCBI GenBank.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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