POP2018-06: Protected coral connectivity in New Zealand

Final Report

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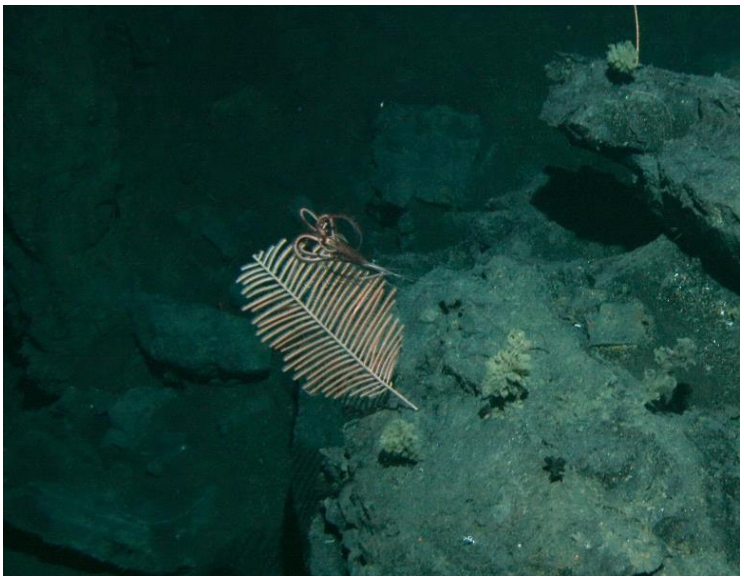

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Cover photo: *Bathypathes patula in situ*. [NIWA, TAN1206]

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Contents

Tables

Figures

Executive summary

The management and conservation of deep-sea coral communities requires an understanding of the extent to which separate coral populations can exchange motile larvae or gametes. This connectivity underpins a population's genetic diversity, which in turn influences adaptive resilience to natural and anthropogenic stresses, including recovery from benthic disturbances such as deep-sea mining and fishing. Knowledge of the dispersal potential of black coral populations is limited however, as is our understanding of the delimitation of what constitutes a genetically and geographically distinct population. This project sought to examine population delimitation and connectivity of a single black coral species – *Bathypathes patula* – by building upon the preliminary results of a previous study, including an increased sample size and testing novel genetic markers for resolution of genetic variation.

We used DNA barcoding to successfully determine the relationships of 77 specimens of *B. patula* housed within the NIWA Invertebrate Collection, using a combination of five genetic markers. Four markers were adapted from previous studies, but one was newly developed for this project and shows promise for distinguishing black coral populations and species. However, our results indicated that, in reality, the tested specimens belonged to a cryptic complex of at least five different genera- not a single species - and no obvious subdivision of these genera into species or populations was discernible from over 2000 base pairs of DNA sequence data. We suggest that this complex warrants a reconsideration of past estimates of anthropogenic effects on *B. patula*, and allowances for hidden diversity should be made during management considerations for black coral species.

Although a population genetic analysis could not be achieved due to multiple species being present in our sample, the hidden taxa uncovered in this study increases our knowledge of black coral diversity in New Zealand and greatly expands the known distribution of one of the cryptic taxa – *Telopathes tasmaniensis* - to include locations across the Exclusive Economic Zone.

We also examined the co-occurrence of *B. patula* and *T. tasmaniensis* and found their geographic and bathymetric distributions to largely coincide. This presents additional difficulty in distinguishing these and other cryptic species, since it appears that gross similarities in their morphology make genetic barcoding the most reliable tool for telling them apart. However, given that we were not able to reliably discriminate multiple species within any of the cryptic genera we sampled, the use of higher-resolution genetic techniques is advisable for future efforts to document species diversity and population connectivity among black corals. While it remains prohibitively expensive for routine identification, genomic approaches comprise the most effective methods for resolving population-level differences for black corals, including connectivity analysis. Given recent reductions in per-sample costs, the ability to resolve relationships at a wide range of taxonomic levels, and amenability to the use of older collections material, we recommend that future attempts to measure the connectivity of black coral populations should employ Ultra-Conserved Elements or RADseq – both are contemporary methods that have shown much promise among related groups of deep-sea corals.

1 Background

The deep seas of New Zealand support many diverse and widespread coral communities (Tracey & Hjorvarsdottir 2019). Under Schedule 7A of the Wildlife Management Act 1953, protection is afforded to groups of corals within these communities, including all hard corals (Order Scleractinia), hydrocorals (Family Stylasteridae), gorgonian octocorals (select groups within the Order Alcyonacea) and black corals (Order Antipatharia), which encompasses groups that display structure-forming or upright tree- and fan-like morphologies. Their fragile growth forms make them susceptible to damage from human activities that disturb the benthos (Probert et al. 1997; Blom et al. 2009), including bottom trawling and mineral exploration and exploitation (Clark & Rowden 2009; Leduc et al. 2015).

The management and conservation of deep-sea corals requires an understanding of their potential for recovery following disturbance. Since corals are sessile, recovery can only occur in the form of growth or reproduction in surviving colonies; survivors may grow to fill-in denuded substrate in their immediate locality, or they may reproduce and release larvae capable of colonizing adjacent areas. New coral growth may also occur from larvae dispersed from more distant populations, provided they can travel sufficient distances to colonize substrate made available by disturbance-related mortality (Palumbi 2003). Such 'connectivity' of populations also serves to minimise localised inbreeding and increases coral genetic diversity, which in turn influence population adaptive resilience and ability to adapt to natural and anthropogenic stresses (Hughes et al. 2008; Sgro et al. 2011).

The re-colonisation potential of protected deep-sea corals in impacted areas is unknown for several key groups in the New Zealand region and highlights an information shortfall when carrying out ecological risk assessments (ERA) (e.g., Clark et al. 2014). Black corals (Hexacorallia: Antipatharia) are one such group of protected corals that are frequently encountered as bycatch in deep-water fisheries (Figure 1; see also Probert et al. 1997). Members of this group can attain large sizes through slow growth rates. Studies using radio-isotopic dating have found colonies regularly exceed a century in age (*Bathypathes*) and colonies in some genera (*Antipatharia* and *Leiopathes*) approach 3000 years old (Hitt et al. 2020; Marriott et al. 2020). Their slow growth, fragility and distribution attributes that make them likely to be encountered (and damaged) by bottom trawling all imply that black corals are at high risk from trawling activity (Clark et al. 2014) and as such they have been designated as indicator species of Vulnerable Marine Ecosystems (FAO 2009). However, assessments of the productivity and recoverability (through growth rates, disturbance resilience, reproduction rates and recolonization potential) of black coral species in New Zealand are hampered by a lack of information on population connectivity. A pilot coral ERA, for example, had to rely on overseas studies for estimates of population connectivity for black corals since no relevant data were available locally for deep-sea black corals at the time (Clark et al. 2014).

Figure 1: Deep-sea black corals observed *in situ* (on left) and as fishery bycatch (at centre & right). Photos: right = NIWA, TAN1503; centre & left = MPI Fisheries Observer Programme.

There have been three genetic studies of black coral connectivity that included populations within the New Zealand Exclusive Economic Zone (EEZ), and two of these are relevant to deep-sea coral ecosystems. Miller (1998) used variations in enzyme expression patterns (allozymes) to examine connectivity between isolated populations of *Antipathes fiordensis* and found restricted gene flow and high levels of genetic structuring within- and between three shallow-water fjord populations. For deep-sea corals, Miller et al. (2010) discovered unique genetic variants in populations of both *Stichopathes variabilis* and *Antipathes robillardi* on Lord Howe Rise, the Norfolk Ridge and the Kermadec Ridge, indicating that tens or hundreds of kilometres of separation is sufficient to limit the dispersal of gametes and larvae between seamount populations of corals. Their study was limited by small sample sizes, however. Most recently, Holland et al. (2020) examined genetic connectivity in *Bathypathes* and *Leiopathes* black corals, using multi-locus DNA sequencing and microsatellite fingerprinting, respectively. No structuring was seen between *Leiopathes secunda* populations, indicating a high level of connectivity across the New Zealand EEZ but the study was also limited by low sample numbers and a lack of detailed identification for many *Leiopathes* specimens. Conversely, northerly, southerly and Antarctic populations of *Bathypathes patula* in the New Zealand region showed limited connectivity and genetic structuring, suggesting a limited exchange of larvae and/or gametes among these populations. This lack of connectivity was ascribed to a barrier effect of the Antarctic Circumpolar Current, which has also been observed for octocorals (Dueñas et al. 2016). However, sample numbers for each of these populations were again low and Holland et al. (2020) advised suggested caution when interpreting the results, until a more in-depth analysis could be conducted.

Given the promise shown in the *Bathypathes patula* dataset of Holland et al. (2020) and the number of additional specimens available in the NIWA Invertebrate Collection, this project attempted to increase the sample size of *B. patula* specimens and add genetic markers with increased resolving power. We sought to spatially define black coral genetic units ('populations') across New Zealand and present initial estimates of connectivity, to improve our understanding of coral community resilience and recovery. However, cryptic diversity hidden within the pool of available *B. patula* samples forced a reconsideration of our primary objectives and indicated that we may currently be underestimating black coral diversity at taxonomic levels above that targeted here.

2 Methods

2.1 Pre-existing data and sample selection

At the outset of this project, a preliminary genetic dataset was made available from Holland et al. (2020). The dataset consisted of DNA sequences from three mitochondrial markers (mtDNA) for 59 specimens tentatively identified as *Bathypathes patula* (Family Schizopathidae), including 459 base-pairs (bp) from *16S* rDNA, 374bp from *ND5-igr-ND1* and 558bp from *TrnW-igr-ND2* (see Miller et al. 2010 & Brugler et al. 2013 for discussion of these markers). Initial analysis of this dataset is given in Holland et al. (2020), which informed development criteria and decisions on how best to expand their study to increase the sample size and quantity of informative DNA sequence data from *B. patula*.

Additional samples of *B. patula* were selected from the NIWA Invertebrate Collections for processing and data acquisition. Including the material previously examined, 148 specimens were selected that had been identified as either *Bathypathes patula* or *Bathypathes* 'sp.' by expert international taxonomists or NIWA researchers. Representatives of other genera and species in the Schizopathidae family were also included, for comparative use and as a confirmation of species identity.

2.2 Genetic marker choice and development

The pre-existing dataset of DNA sequences was examined for information content, to determine which (if any) of the three genetic markers presented enough variation to distinguish intra-specific differences between coral populations from inter-specific differences due to specimen misidentification and cryptic diversity ('hidden' species that are otherwise indistinguishable). The *ND5-igr-ND1* mtDNA region was found to have the highest level of variation among included specimens, displaying a maximum pairwise sequence difference of 2.00%, followed by the *TrnW-igr-ND2* mtDNA region (1.97%) and then the *16S* mtDNA region (1.31%). Although combinations of these markers increased the ability to distinguish genetic variants, we decided that *ND5-igr-ND1* and *TrnW-igr-ND2* were sufficient for initial confirmation of species identity, thus we did not expand on the pre-existing *16S* DNA sequence dataset further, although it was included in data analyses (see section 2.4, below).

Alternative, non-mitochondrial loci presented more potential for informative variation between populations and species. We thus explored and developed two additional markers from the nuclear genome: ITS rDNA and the Signal Recognition Particle subunit 54 (SRP54) intron region, which were identified as potentially useful markers of genetic variants by Bo et al. (2018) and Concepcion et al. (2008), respectively.

The ITS rDNA locus (Internal Transcribed Spacers of the ribosomal RNA cistron), which includes approximately 850bp of a ribosomal array consisting of the 3'-end of 18S, the ITS1, 5.8S and ITS2 genes and the 5'-end of 28S, was initially targeted using the primers and methods of Lapian et al. (2007). However, preliminary results indicated that the primers were not specific to black corals and fungal contamination was frequently observed in the resulting DNA sequences. As such, the primers were re-designed to target a smaller proportion (700bp) of the locus, but with higher specificity for *Bathypathes* and other closely related members of the Schizopathidae family (Appendix A1) based on available sequence data in GenBank (www.ncbi.nlm.nih.gov/genbank/) and BLASTn (blast.ncbi.nlm.nih.gov). The resulting primer pair of BTP_ITS_F (GGA AGG ATC ATT ACC GAT CAT CCA

AG) and BTP_ITS_R (GGC CGA GAA TAG AAT GTG TCG CC) was subsequently used in all ITS amplification and sequencing.

Initial attempts to obtain SRP54 sequence data from *Bathypathes* were made by Holland et al. (2020) but were not progressed due to inconsistent amplification success and varied informative content. We used their trial dataset to develop and test a novel pair of PCR primers for a region with reliable sequence data and were able to consistently amplify a locus using the primers BTP_Novo_F (TAA ACT CAA ACA TGG TAA GGA TTA AG) and BTP_Novo_R (TGA GAG TTC AAG TAA AAC TTT TTG TG), which consisted of approximately 350bp (Appendix A2).

2.3 DNA extraction, amplification and sequencing

DNA extracts from samples analysed in Holland et al. (2020) were provided by the authors for this study. These samples used a Geneaid genomic DNA mini kit (Geneaid Biotech Ltd.) to extract genomic DNA from ethanol-preserved tissue samples. Additional ethanol-preserved specimens of *Bathypathes* corals were sampled from the NIWA Invertebrate Collection and were extracted using a modified salting-out procedure (TL Jenkins, Univ. of Exeter, pers. comm.), combined with a DNeasy Blood & Tissue kit (Qiagen Inc.). Samples were digested in a cell lysis buffer (100mM Tris-HCl, 100mM EDTA, 1%SDS, 55mM DTT) with 200-500ug of proteinase K overnight at 56°C, protein was precipitated using 140uL of 7.5M Ammonium Acetate, followed by the manufacturer's recommendations for addition of DNeasy AL buffer, AW1 and AW2 rinses and final elution.

PCR amplifications of each locus were conducted in a 25ul total volume containing 1X MyTaq RedMix (Bioline Inc.), 0.5uM of each primer and 1-4ul of DNA extract. PCR thermocycling conditions for each locus are given in Table 1. Amplification products were visualised on 1% agarose gels via electrophoresis and successful reactions were purified using 0.5 units of ExoSAP-IT (ThermoFisher Sci. Inc.) following the manufacturer's recommendations and were submitted to a commercial facility for DNA sequencing (Macrogen Inc.).

Table 1: Genetic loci targeted for PCR amplification and DNA sequencing. 'Size' = estimated size of the resulting amplicon; 'PCR profile' = optimised PCR thermocycling conditions. '*' = see Results concerning the actual identity of the *SRP54* locus.

Locus Name	Size (bp)	PCR profile
ND5-igr-ND1 mtDNA	500	95°C/3min, (95°C/15s, 55°C/15s, 72°C/20s)^{35}, 72°C/2min
TrnW-igr-ND2 mtDNA	600	95°C/3min, (95°C/15s, 51°C/20s, 72°C/25s)^{35}, 72°C/2min
ITS1-5.8S-ITS2 rDNA	700	95°C/3min, (95°C/15s, 55°C/15s, 72°C/20s)^{35}, 72°C/2min
*SRP54**	350	95°C/3min, (95°C/15s, 57°C/15s, 72°C/15s)^{35}, 72°C/1min

2.4 Data analysis

Results of DNA sequencing were visually inspected for quality and errors and were trimmed and assembled using Geneious Prime software v2019.0.3 (Biomatters Ltd.). Sequences were referenced against sequences in BLASTn, to ensure they did not result from contaminating organisms. For each locus, sequences were aligned using MAFFT v7.388 (Katoh & Standley 2013) and were manually adjusted wherever necessary. Where available, additional sequences of black corals were obtained from GenBank and included in alignments for reference purposes and for outgroup analysis.

Bayesian phylogenetic analysis of aligned DNA sequences was conducted using MrBayes v3.2.6 (Huelsenbeck & Ronquist 2001). Each locus was analysed separately and as a combined (concatenated) dataset using a GTR+G distance correction model, with four chains of 10^6 MCMC steps sampled at 10^3 intervals and 10^5 steps discarded as burn-in. Analysis of the combined multi-locus dataset used partitioned model parameters according to locus, as independent estimates. The posterior output was examined for evidence of convergence in all model parameters. Resulting phylogenetic trees were outgroup-rooted using representatives of other black coral families or, when outgroup family data was absent, other genera of the Schizopathidae.

Genetic variation within *Bathypathes* – particularly *B. patula* – was examined for evidence of contemporary intraspecific genetic variation that could inform a population-level analysis, as compared to interspecific genetic variation attributed to past evolutionary changes in non-interbreeding species and genera. The geographic and bathymetric distribution of genotypes was also examined, to document regional biodiversity patterns and test for differences according to depth, as seen in other deep-sea coral fauna (Miller et al. 2011; Quattrini et al. 2015).

3 Results

3.1 Sequencing success and per-locus rates of variation

A total of 97 specimens (59 from Holland et al. (2020) plus 38 in the current study) identified as *Bathypathes* sp. or *Bathypathes patula* were sampled for genetic analysis. Of these, 20 did not yield data of sufficient quality to warrant inclusion in any analysis. For the remaining specimens, 66 produced data for *TrnW-igr-ND2*, 77 for *ND5-igr-ND1*, 64 for *ITS1-5.8S-ITS2* and 53 for *SRP54* (Table 2). Thirty-eight samples had data for all four loci, 28 contained data for three loci, nine had data for two loci and two had data for a single locus. We also obtained sequence data for all four loci for the holotype specimen of *Telopathes tasmaniensis* (NIWA15339) and *ND5-igr-ND1* data for a specimen of *Lillipathes* sp. (NIWA39093). An additional *Bathypathes* sp. specimen – NIWA38138 – was found to be a likely case of misidentification and was treated as an additional sample of *Lillipathes* sp. or a closely related genus (see phylogenetic results, below).

Table 2: Black coral specimens obtained from the NIWA Invertebrate Collections that were genotyped for this study. 'Cat. #' = NIWA Invertebrate Collection catalogue number; 'ID' = original specimen identification; 'Date' = date the specimen was collected; 'Lat.' & 'Long.' = coordinates for the specimen locality; 'Depth' = approximate depth from which the specimen was collected; '16S', 'TrnW', 'ND5', 'ITS' & 'SRP' = indicates for which loci sequence data was obtained.

Cat. #	ID	Date	Lat.	Long.	Depth	16S	TrnW	ND5	ITS	SRP
4294	*Bathypathes patula*	30/01/1977	-28.7	167.9	475		X	X		
4295	*Bathypathes patula*	03/10/1968	-37.3	178.2	1357		X	X	X	X
4296	*Bathypathes patula*	05/02/1988	-31.9	172.4	790		X	X	X	X
4297	*Bathypathes patula*	09/12/1998	-42.9	173.9	1010		X	X	X	
4298	*Bathypathes patula*	03/09/1998	-47.4	147.7	1085		X	X	X	X
14769	*Bathypathes patula*	06/09/1998	-47.5	148.8	890		X	X	X	X
14770	*Bathypathes patula*	09/09/1998	-47.5	148.6	998		X	X	X	X
14771	*Bathypathes patula*	02/12/1998	-50.2	163.6	1006		X	X	X	X
14773	*Bathypathes patula*	10/09/1998	-47.7	147.4	1104		X	X	X	X
14781	*Bathypathes patula*	04/07/2001	-39.7	178.2	957		X	X	X	X
15044	*Bathypathes patula*	15/06/2000	-37.2	167.3	1061		X	X	X	X
15057	*Bathypathes patula*	25/01/2002	-33.8	167.2	313		X	X	X	
15339	*Telopathes tasmaniensis*	27/01/1997	-44.3	147.2	1083		X	X	X	X
24197	*Bathypathes patula*	03/09/1998	-47.4	147.7	1085			X	X	X
24198	*Bathypathes patula*	01/05/2000	-34.8	169.8	790			X	X	X
24221	*Bathypathes patula*	-	-	-	-		X	X	X	
24222	*Bathypathes patula*	-	-	-	-		X	X		
38107	*Bathypathes patula*	03/03/2008	-68.1	-179.3	780		X	X	X	X
38108	*Bathypathes patula*	03/03/2008	-68.1	-179.3	780		X	X	X	X
38138	*Bathypathes*	03/03/2008	-68.1	-179.3	780		X	X	X	X
38161	*Bathypathes patula*	03/03/2008	-68.1	-179.2	879		X	X	X	
39093	*Lillipathes*	13/03/2008	-66.9	170.8	547			X		
39163	*Bathypathes patula*	13/03/2008	-67.1	171.1	566		X	X	X	X
39209	*Bathypathes patula*	14/03/2008	-67.2	171.2	648			X	X	X
39236	*Bathypathes patula*	14/03/2008	-67.2	171.2	648		X	X	X	
40415	*Bathypathes patula*	11/04/2008	-53.7	159.1	1014		X	X	X	X
40488	*Bathypathes*	12/04/2008	-53.7	159.1	770			X	X	
41695	*Bathypathes patula*	08/03/2008	-67.3	-179.9	1130		X	X	X	X
42812	*Bathypathes patula*	19/12/2007	-43.8	-174.3	806		X	X	X	X
42813	*Bathypathes patula*	07/05/2008	-43.9	-174.7	668		X	X	X	
42814	*Bathypathes patula*	20/03/2008	-50.0	163.8	849		X	X	X	X
42815	*Bathypathes patula*	17/04/2008	-43.9	-174.7	628		X	X	X	X
42816	*Bathypathes patula*	20/03/2008	-50.2	163.7	1092		X	X		X
42820	*Bathypathes patula*	19/03/2008	-50.3	163.5	1125		X	X		X
42824	*Bathypathes patula*	19/03/2008	-50.3	163.5	1043		X	X	X	X
42834	*Bathypathes patula*	29/03/2008	-49.8	175.9	849		X	X		
42835	*Bathypathes patula*	17/12/2007	-44.2	-174.4	1202		X	X	X	
42836	*Bathypathes patula*	20/03/2008	-50.0	163.8	849		X	X	X	X
42839	*Bathypathes patula*	14/12/2007	-44.5	-174.8	1288			X		X
43035	*Bathypathes patula*	26/06/1905	-36.9	-169.7	1200		X	X	X	X
43037	*Bathypathes*	25/06/1905	-50.3	163.6	975			X		
45887	*Bathypathes patula*	31/10/2006	-48.7	164.9	911		X	X		

Cat. #	ID	Date	Lat.	Long.	Depth	16S	TrnW	ND5	ITS	SRP
45888	*Bathypathes patula*	22/07/2007	-47.4	178.1	929		X	X	X	X
47191	*Bathypathes patula*	13/03/2008	-67.1	171.1	566		X	X	X	X
47193	*Bathypathes patula*	03/03/2008	-68.1	-179.3	780		X	X	X	X
47195	*Bathypathes patula*	14/03/2008	-67.2	171.2	648		X	X	X	
47196	*Bathypathes patula*	03/03/2008	-68.1	-179.2	879		X	X	X	
47416	*Bathypathes patula*	19/01/2003	-44.2	-174.5	850			X	X	X
47879	*Bathypathes patula*	16/06/2005	-42.8	-177.2	999		X	X	X	X
47911	*Bathypathes patula*	08/03/2008	-44.6	-175.7	758			X	X	X
49168	*Bathypathes patula*	14/03/2008	-67.2	171.2	648		X	X	X	
49169	*Bathypathes patula*	13/03/2008	-67.1	171.1	566	X	X	X	X	
49468	*Bathypathes patula*	26/02/2009	-44.7	-175.4	810	X	X	X		X
53352	*Bathypathes patula*	20/06/2009	-41.8	-179.5	1255	X	X	X	X	X
53374	*Bathypathes patula*	21/06/2009	-41.8	-179.5	1248	X	X	X		X
60355	*Bathypathes patula*	19/03/2008	-50.3	163.5	1043	X	X	X	X	
64561	*Bathypathes*	03/06/2010	-35.4	178.6	1282	X	X	X	X	X
66331	*Bathypathes patula*	14/07/2009	-35.7	165.9	844	X	X	X	X	X
66335	*Bathypathes patula*	30/07/2009	-42.8	-177.2	909		X	X	X	X
66336	*Bathypathes patula*	22/07/2009	-35.9	166.1			X	X		
66337	*Bathypathes patula*	25/10/2009	-44.7	-175.4	1120	X	X	X	X	X
66342	*Bathypathes*	10/10/2009	-44.0	-174.6	700		X	X	X	X
66344	*Bathypathes patula*	29/09/2009	-49.8	175.9	900	X	X	X	X	X
66348	*Bathypathes patula*	15/01/2010	-44.0	-174.6	835	X	X	X	X	X
66349	*Bathypathes patula*	14/01/2010	-43.9	-174.6	650	X	X	X	X	
66354	*Bathypathes patula*	06/03/2009	-43.8	-174.5	810	X	X	X	X	X
69577	*Bathypathes patula*	30/12/2010	-33.6	167.8	841	X	X	X	X	
69648	*Bathypathes patula*	23/01/2009	-44.0	-174.6	659	X	X	X		X
69649	*Bathypathes patula*	06/01/2009	-44.4	-174.8	1070	X	X	X		X
83099	*Bathypathes patula*	27/04/2012	-37.2	176.9	1370	X	X	X	X	
85928	*Bathypathes patula*	25/05/2001	-35.7	178.5	1200			X	X	X
85940	*Bathypathes patula*	27/03/2000	-37.0	176.5	949	X	X	X	X	X
88759	*Bathypathes patula*	17/12/2013	-64.5	177.5	1142		X	X		X
88760	*Bathypathes patula*	11/12/2013	-63.6	175.7	1607		X	X	X	X
106519	*Bathypathes*	07/10/2016	-37.6	169.6	959		X	X	X	X
106520	*Bathypathes*	19/10/2016	-34.0	162.6	503		X	X	X	X
106522	*Bathypathes*	19/10/2016	-34.0	162.6	499		X	X		
118656	*Bathypathes*	29/10/2016	-30.3	-178.2	1431			X		
123390	*Bathypathes*	24/05/2001	-36.1	178.2	750				X	X

Both nuclear markers were found to have rates of variation that exceeded those of the two mitochondrial loci. Among the sequenced specimens of *Bathypathes*, *SRP54* was found to have the highest maximum level of sequence variation (16.9% difference over 245bp), followed by *ITS* (6.9% over 531bp), *ND5-igr-ND1* (2.2% over 354bp) and then *TrnW-igr-ND2* (1.8% over 561bp). However, both nuclear loci displayed several heterozygous positions (conflicting DNA nucleotides = alternative alleles), which created ambiguous base calls at some variable positions and caused a high rate of sequencing failure (ITS=20%, SRP54=38%; likely due to insertion/deletion variants in differing alleles). The *18S-ITS1-5.8S-ITS2-28S* rDNA locus is known to exist in multiple, repeated copies as a 'tandem array'- each copy may potentially be variant (e.g., Forsman et al. 2006), making it less valid for

genetic assessments where homology is assumed for all individuals' DNA sequences (Lam et al. 2006) – this factor was not investigated here as it was outside the scope of the current study.

3.2 Identity of the novel nuclear locus

Newly designed primers expected to target the *SRP54* intron produced a PCR-amplicon of the predicted length. However, the amplified region is likely to belong to another nuclear locus, as GenBank-BLASTn queries did not return any previously sequenced *SRP54* data as a significant match. Nearest matches for the sequenced region included short (<90bp) regions of a DNA repair protein (GenBank: XM_015898087) and noncoding RNA (XR_001567584) in *Acropora digitifera*. It is noteworthy that no SRP54 sequence data are available for any member of the Antipatharia and the highly variable nature of the locus may be responsible for a lack of similarity to available DNA sequences in coral orders (Concepcion et al. 2008). However, analysis of the genetic code of our newly obtained black coral DNA sequences indicated the locus is unlikely to be a protein-coding gene (such as *SRP54*). A lack of sequence similarity with common contaminant taxa, plus a closer similarity to hard coral genome data both suggest this locus is derived from the target black coral genome, rather than fungal or microbial associates. It also displays evidence of allele heterozygosity suggestive of a diploid genome. Since further investigation is required, we temporarily refer to this new region as the '*Novel SRP54-like Locus*' (*NSL*) (instead of *SRP54*) until such time as its identity may be more adequately determined.

3.3 Discrimination of taxonomic units

All phylogenetic trees resulting from individual and combined analyses of each genetic locus indicated that the 77 included specimens of '*Bathypathes*' represent a previously unrecognised complex of at least five distinct genera (Figure 2; Appendix B). Comparisons to identified reference sequences deposited in GenBank from previous taxonomic studies indicated most specimens were genetically indistinguishable from either *Bathypathes patula* or *Telopathes tasmaniensis*. In addition, one specimen (NIWA 38138) displayed a close genetic relationship to *Lillipathes* and *Dendrobathypathes*, three specimens (NIWA 4294, 4297, 15057) were related to *Stauropathes*, one specimen (NIWA 83099) represented an unknown lineage (possibly a new, undescribed genus), and one specimen (NIWA 64561) was related to *Bathypathes alternata*. This indicates that there is a surprising level of unresolved higher-level (i.e., at the genus-level) diversity present within what we previously considered to be a single species, which warrants further investigation and consideration in terms of anthropogenic effects on benthic biodiversity.

Within the *B. patula* and *Telopathes* lineages, there was no genetic substructure seen for either mitochondrial marker. Both *ITS* and *NSL* showed some genetic variation within the *Telopathes* and *B. patula* lineages, but there were no well-supported lineages suggestive of partitioning into multiple species or populations (Appendix B). The observed lack of genetic structuring within each genus could indicate either a lack of species- (and population-) level variation in the markers chosen, or it could indicate that only a single species (or meta-population) of each genus was sampled. However, a lack of fine-scale genetic variation makes it impossible to conduct an analysis of connectivity patterns within these newly uncovered taxa. However, we can see from the presence of several cryptic taxa that assessments of the benthic impacts of fishing practises must make allowances for higher than expected diversity among encountered black corals.


JX456459_Myriopathes_japonica
GU296487_Antipathes_grandis
GU296488_Antipathes_grandis
KF054536_Antipathes_grandis
GU296492_Antipathes_caribbeana
GU296491_Antipathes_curvata
KF054546_Cirrhipathes_sp
KF054547_Antipathes_curvata
HM060611_Cirrhipathes_anguina
KF054548_Cirrhipathes_anguina
KF054549_Cirrhipathes_anguina
38138
Lillipathes/Dendropathes
JX560737_Dendrobathypathes_boutillieri
KF054487_Dendrobathypathes_boutillieri
KF054488_Dendrobathypathes_boutillieri
KF054490_Parantipathes_sp
KF054493_Lillipathes_sp
KF054495_Lillipathes_sp
KF054511_Lillipathes_sp_T426-A6
GQ200712_Parantipathes_sp
GQ200713_Parantipathes_sp
GQ200714_Parantipathes_sp
GQ200716_Parantipathes_sp
GQ200717_Parantipathes_sp
GQ200719_Parantipathes_sp
GQ200722_Parantipathes_sp
GQ200723_Parantipathes_sp
GQ200725_Parantipathes_sp
GQ200729_Parantipathes_sp
KF054498_Parantipathes_sp
KF054500_Parantipathes_sp
KF054501_Parantipathes_sp
KF054504_Parantipathes_sp
KF054508_Parantipathes_sp
SCHIZOPATHIDAE
KF054480_Stauropathes_cf_punctata
KF054481_Stauropathes_cf_punctata
KF054482_Stauropathes_cf_punctata
KF054483_Stauropathes_cf_punctata
KF054484_Stauropathes_cf_punctata
64561
Bathypathes alternata
GQ200704_Bathypathes_sp
GQ200705_Bathypathes_sp
GQ200706_Bathypathes_sp
GQ200707_Bathypathes_sp
GQ200710_Bathypathes_sp
4297
4294
15057
(unknown genus)
4295
38107
38108
38161
39163
40415
41695
42815
42835
42836
47191
47195
49168
49169
53352
66331
66349
85940
88759
88760
Bathypathes patula
GQ200697_Bathypathes_sp
GQ200699_Bathypathes_sp
GQ200703_Bathypathes_sp
GQ911222_Bathypathes_sp
KF054475_Bathypathes_patula
KF054476_Bathypathes_patula
KF054477_Bathypathes_patula
KF054479_Bathypathes_sp
83099
(unknown genus)
4296
4298
14769
14770
14771
14773
14781
15044
15339
HOLOTYPE
42812
42813
42814
42816
42824
42834
Telopathes tasmaniensis
45887
45888
47879
49468
53374
60355
66335
66336
66337
66344
66348
66354
69577
69648
69649
GQ200696_Telopathes_magna
GQ200698_Telopathes_magna
GQ200700_Telopathes_magna
GQ200701_Telopathes_magna
GQ200702_Telopathes_magna
KC960443_Telopathes_magna_HOLOTYPE
0.5

A.

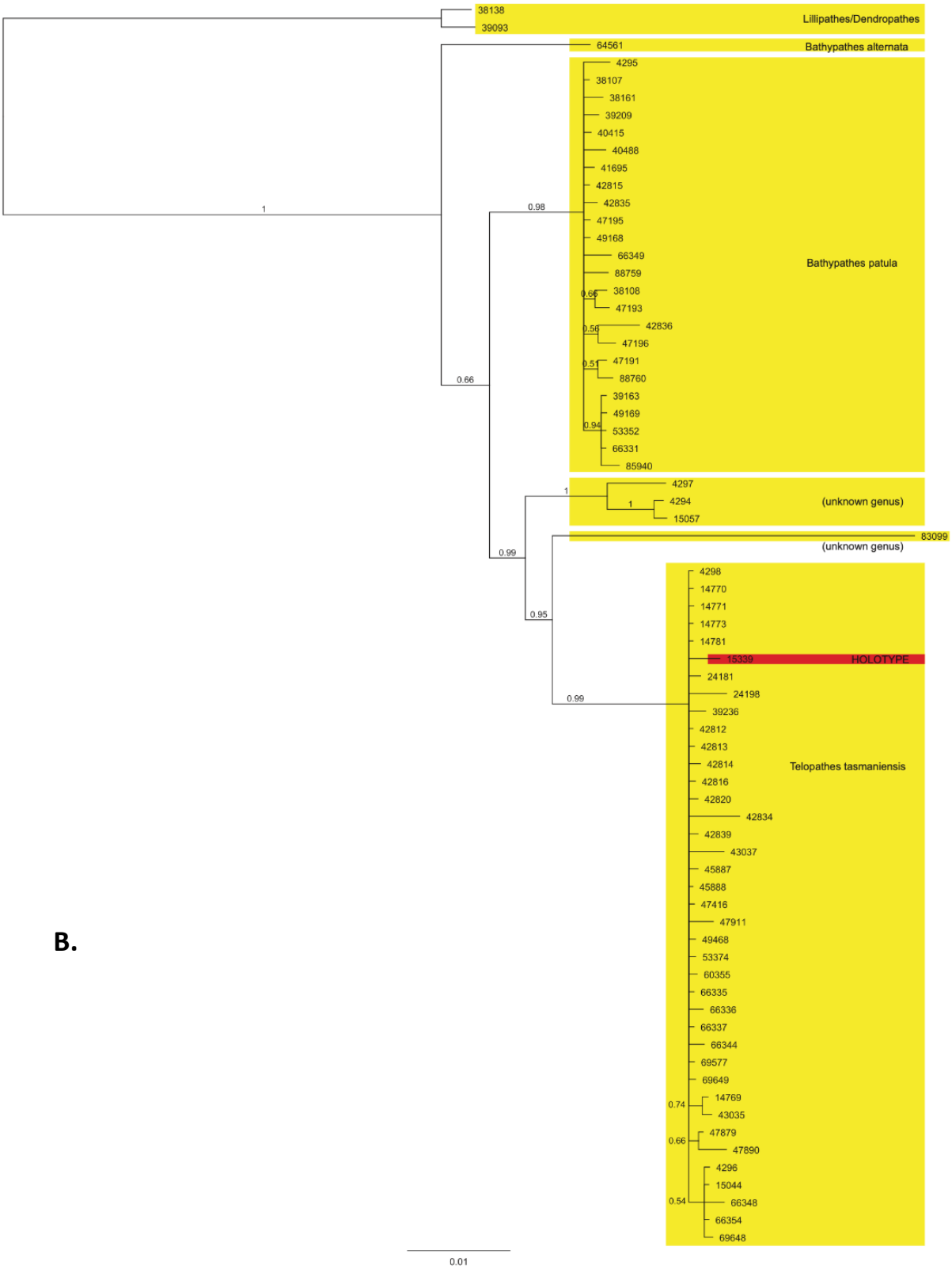

Figure 2: Bayesian phylogenetic reconstructions of black coral relationships based on a) *trnW-igr-ND2* and b) a concatenated analysis of five loci. Trees of the remaining individual loci are shown in Appendix B.

3.4 Geographical and bathymetric patterning

A population genetic analysis was precluded by a lack of population-level sampling for *B. patula* and a lack of species- or population-level genetic structuring among the pre-existing and newly developed markers. We thus focused on comparing distributional patterns in the two most densely sampled taxa, to provide information on regional biodiversity and habitat preferences.

Comparisons of geographic distributions were made using coordinate data associated with each specimen to map its location, as provided by MPI-FNZ Observers. *T. tasmaniensis* and *B. patula* show a similar distribution across the New Zealand EEZ: both are found along Macquarie Ridge to the south, on the Chatham Rise to the east, the outer Bay of Plenty to the North, and along the Lord Howe Rise and West Norfolk Ridge to the northwest (Figure 3). Outside of the EEZ, both extend into the Southern Ocean but only *T. tasmaniensis* is found westward in the Tasman Basin and eastward on the Louisville Seamount Chain. Whether these observations represent actual differences in distributional limits or are an artefact of limited sampling remains to be seen. No other records of *T. tasmaniensis* are available for the New Zealand EEZ and other records of *B. patula* must be treated with caution since they may contain the cryptic taxa uncovered herein. However, it is noteworthy that our new records of *T. tasmaniensis* expand the known geographic distribution of the species significantly and into the New Zealand EEZ, since previously it was only documented from the holotype locality in the Tasman Basin.

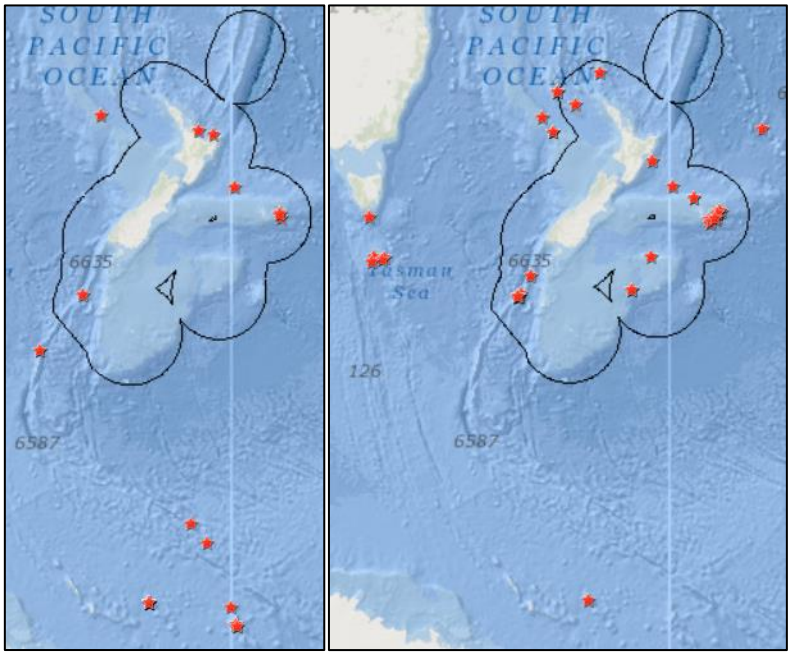

Figure 3: Location of DNA-sequenced specimens of *B. patula* (left) and *T. tasmaniensis* (right).

A comparison of sampling depths for *B. patula* and *T. tasmaniensis* is given in Figure 4. Both taxa displayed a similar bathymetric distribution, with an average of 881m for *B. patula* and 946m for *Telopathes*. Both taxa shared the same minimum depth of 620m, but *B. patula* extended deeper, to a maximum depth of 1700m (*vs*. 1400m for *Telopathes*). *Bathypathes* is known to reach the deepest depths of any black coral genus worldwide (D. Opresko, pers. comm.), but previously the only depth record for *T. tasmaniensis* was for the holotype at 1083m (Opresko 2020). Thus, the new records

presented here significantly expand upon the known bathymetric distribution of this recently described species.

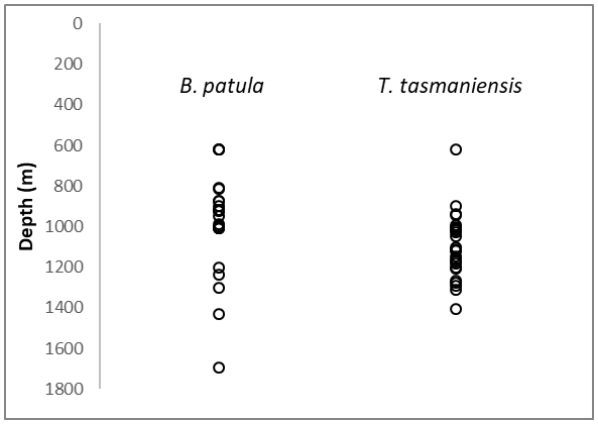

Figure 4: Observed habitat depths for the two cryptic species recovered in phylogenetic analyses. Each point represents the estimated depth of a single sequenced specimen.

4 Summary and Discussion

4.1 Genetic delineation of black corals

Of the 77 specimens originally identified as *B. patula* (or *Bathypathes* sp.) that were genotyped for this study, only 24 were found to genetically match that species. Conversely, more specimens (37) were likely to belong to *T. tasmaniensis*, even though nearly all of these (33) were identified by taxonomic experts for the Antipatharia. This suggests that the morphological characters used as diagnoses of these species may be insufficient to accurately distinguish them, possibly as a result of the re-use of certain growth forms in separate evolutionary lineages (convergent evolution) or excessive variability in a coral colony's growth response to environmental variations (phenotypic plasticity).

Although DNA sequences can also suffer from convergent evolution and plasticity, it is unlikely to be a factor in our analysis since we used over 2000bp of DNA sequence data at four independent loci from two genomic partitions (mtDNA and nDNA) – all of which agreed on the genetic relationships of our study specimens. Many of the sampled specimens were also fragments, with limited morphological features necessary for identification. The integration of genetic barcoding would thus seem prudent when conducting bulk identifications of new collections of black corals. Based on observed patterns of genetic variation plus the discriminatory abilities of the various loci used here, a simple combined barcode of *ND5-igr-ND1* plus *NSL* should be sufficient to delineate all major taxonomic groups uncovered by the larger analysis we have presented, and would represent an affordable option for routine identification.

Previous studies have also indicated a lack of species-level information content in available genetic markers, even when multiple loci were used in combination (MacIsaac et al. 2013; Brugler et al. 2013), but these studies relied primarily on the mitochondrial genome. The only reported uses of nuclear genome-derived markers rely on the *ITS1-5.8S-ITS2* ribosomal cistron, which recovers similar phylogenetic patterns for the Schizopathidae as seen here (MacIsaac et al. 2013; Bo et al. 2018). However, this region is known to exhibit intragenomic variation (Vollmer & Palumbi 2004), which can confound assumptions of homology in phylogenetic analysis when it is treated as a single copy gene in reconstructions of species ancestry (Lam et al. 2006). It is thus preferable to use a nuclear genetic marker that exists as a single copy in the genome, where any observed intragenomic variation would be due to simple diploid heterozygosity. Although the functional identity and single- or multi-copy status of the novel *NSL* marker we have presented here are still unknown, the locus shows promise as a potential marker for population genetic studies as it exhibits numerous SNPs and short (1-3bp) indels along its 250bp length.

More recently, developments in genomics combined with increased DNA sequencing coverage provided by Next Generation Sequencing (NGS) technologies have provided an alternative to traditional PCR amplification and DNA sequencing approaches to genotyping specimens for biodiversity measurements and population genetics. The use of targeted NGS methods (without resorting to expensive whole genome sequencing) for the identification and sequencing of thousands of SNP loci in corals has been accomplished via RADseq (Herrera et al. 2016) and, most recently, enrichment for informative Ultra-Conserved Elements (UCEs) (Quattrini et al. 2017). Both methods excel at delineating genetic relationships at a wide variety of taxonomic levels – from species (Quattrini et al. 2019) to orders (Quattrini et al. 2017). UCEs are currently being applied to the Antipatharia to evaluate the broad-scale systematic relationships of families, genera and species and per-sample costs have made this an attractive approach for detailed analysis of black coral

biodiversity patterns, including connectivity analysis (J. Horowitz, pers. comm.). At a sequencing cost of NZD$100 per sample, compared to <$5 per locus per sample for traditional DNA sequencing, a UCE (and RADseq) approach still remains prohibitively expensive for routine identification. However, it is fast becoming the method of choice for biodiversity and population genetic analyses of corals and is currently the most effective approach to accomplish our original goals, although it was not feasible within the scope of this project, nor available at its outset.

4.2 Cryptic diversity of the Schizopathidae

Unfortunately, an assessment of the genetic connectivity of *B. patula* populations within New Zealand was not achievable within the limits of this study. Although *B. patula* initially represented the most numerous deep-sea black coral species within the extensive NIWA Invertebrate Collection, the hidden diversity among these specimens resulted in fewer than half the original number, with few instances of multiple samples from a given region that could be considered as a 'population'. However, we were instead able to document a wide range of diversity within the Schizopathidae that will serve as a framework for further molecular systematic and biogeographic research.

The new records of *T. tasmaniensis* indicate its distribution is much wider and deeper than recently documented (Opresko 2020). Within the New Zealand EEZ and adjacent regions, it occurs sympatrically with *B. patula* (Figure 4), which may partially explain why the two have been confused. Both form pinnate ostrich-feather-like colonies, but *Telopathes* is distinguished by branching of the main stem (corallum), whereas *Bathypathes* is normally unbranched (Figure 5). However, for young (or damaged or highly fragmented specimens), this distinguishing character is not apparent, and accurate identification must rely on either close examination of microscopic features, or on the use of molecular systematics.

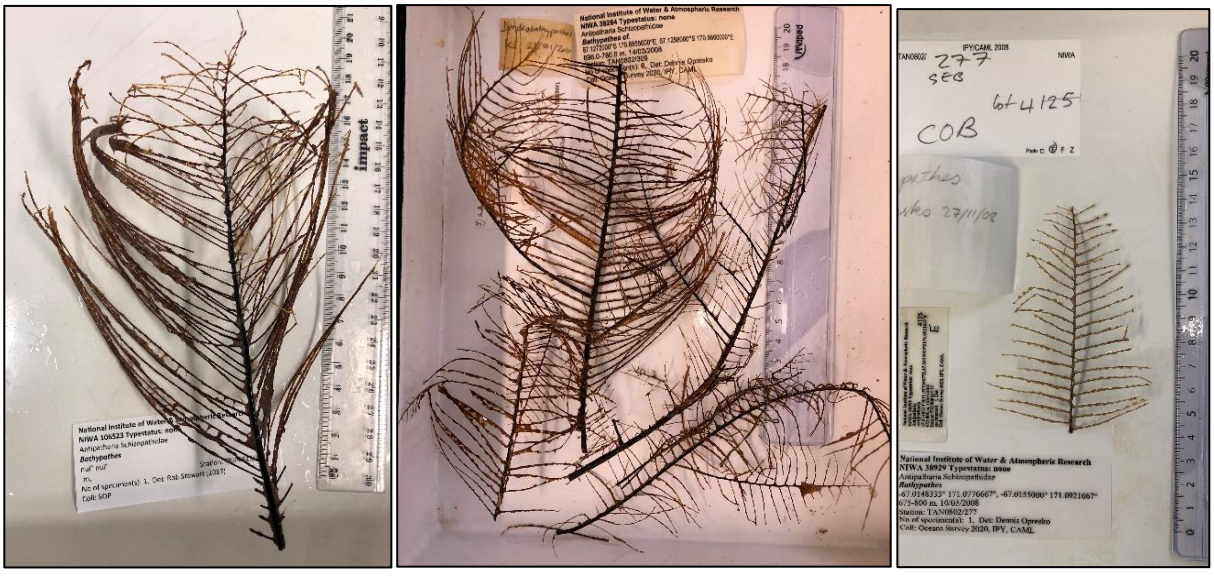

Figure 5: Gross similarities of *Bathypathes* (left) and *Telopathes* (centre) growth forms. Note that branching is often absent in young colonies (right), making the two genera indistinguishable without DNA sequencing or Scanning Electron Microscopy.

In addition to elucidating the relationships of *B. patula, B. alternata* and *T. tasmaniensis*., we have uncovered novel lineages that do not readily adhere to other known taxa. Three specimens belong to a lineage that shows affinity to *Stauropathes* in the *ND5-igr-ND1* analysis, but is resolved as a separate, distinct clade in the *ITS* and *trnW-igr-ND2* trees. None of these specimens produced PCR products for *NSL*, which suggests they may share a novel mutation in a primer binding site that prevents amplification from initiating.

A single specimen was also identified as a candidate for a new genus. NIWA 83099 shared a sister relationship to *T. tasmaniensis* in results for both mitochondrial loci, whereas *ITS* was unable to resolve its position relative to *Bathypathes* and *Telopathes*. This specimen would also not amplify at the *NSL* locus despite repeated attempts, indicating it may also be too divergent for *NSL* primers to anneal. Based on the available results, this single specimen either represents a new genus or is at the least a second species of *Telopathes*. The latter is less likely since genetic variation within *Telopathes* appears to be low, based on our observations that *T. magna* – a North Atlantic species – is nearly genetically identical to *T. tasmaniensis* found in the SW Pacific for both mitochondrial loci. The level of genetic distance between NIWA 83099 and *Telopathes* is consistent with a separate genus. It remains to be determined whether it is a new genus, or an example of a described genus that has not previously been sequenced.

Since the recognition and description of *Telopathes* as a distinct genus in 2013, there have been three molecular systematic studies that have shown the relationships of similar genera within the Schizopathidae (Figure 6). The original description and analysis of *Telopathes* also included *Stauropathes* and two lineages of *Bathypathes*, but no other closely related genera, whereas an examination of Mediterranean black coral relationships by Bo et al. (2018) also presented the same four lineages. However, a recent study by Barrett et al. (2020) based on whole mitochondrial genome data has shown two additional relatives: a third *Bathypathes* lineage plus a second *Stauropathes* lineage. Whether these additional two lineages are related to either of the two novel lineages identified in the current study remains to be determined, but it is further evidence that the Schizopathidae still contains undescribed, potentially cryptic genera.

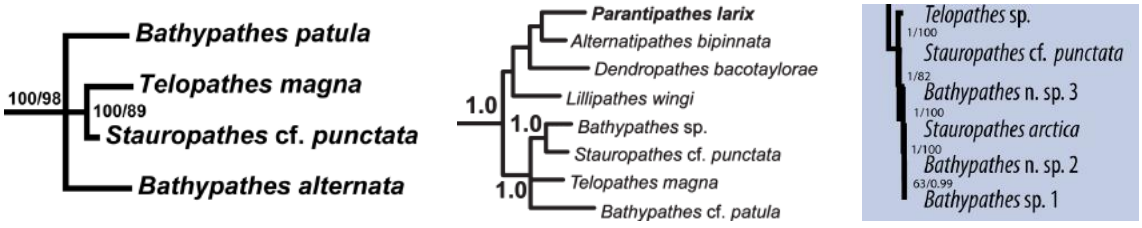

Figure 6: Phylogenetic results of previous studies that include *Telopathes*. Left = MacIsaac et al. 2013; Centre = Bo et al. 2018; Right = Barrett et al. 2020.

Given the degree of misidentification which appears to have occurred within the New Zealand Schizopathidae collections, combined with the number of potentially new taxa that have been uncovered from a relatively small pool of specimens, it appears that we currently do not have an adequate understanding of the diversity of Schizopathidae within New Zealand and nearby regions. Although a population genetic analysis would provide crucial productivity information for an Environmental Risk Assessment of demersal fishing practises, caution must be taken to first confirm that sufficient numbers of conspecifics are available for the task. The preliminary analyses of *B. patula* given by Holland et al. (2020) are an example of how pre-existing misidentification of study specimens can mislead a population genetic analysis. Their finding that 'southern', 'northern' and

'Antarctic' populations are genetically structured and exhibit low levels of connectivity appears to be driven by the cryptic supra-specific diversity uncovered here – the southern 'population' is actually the species *T. tasmaniensis* and the Antarctic and northern populations are contained within *B. patula* plus another species lineage that shares some affinity for *Stauropathes*. Instead of three structured populations of a single species, we have three distinct (and presumably non-interbreeding) genera, separated by evolutionary timescales. Given that this study has effectively halved the highest number of specimens available among any species of deep-sea black coral in New Zealand, it seems that future attempts to delineate populations and intra-specific connectivity patterns will require increased sampling, as well as integrated morphological-genetic identification systems.

In conclusion, we have uncovered significant genus-level diversity among a large and disparate collection of specimens previously identified as *Bathypathes patula*. This assemblage includes black coral species previously unknown from the New Zealand region and requires a reconsideration of the distributional limits and prevalence of *B. patula* – particularly as it pertains to the frequency and severity of anthropogenic impacts. We need to identify the implications of moving from our previous concept of *B. patula* as a widespread, but often solitary sentinel species to a paradigm in which our current records actually represent multiple cryptic genera and unknown numbers of species – some of which may be locally rare. For the moment, it seems we now know even less about protected black coral populations than we did previously, although we have gained an increased appreciation for the complexities of their apparent diversity.

5 Recommendations

- Investigate the feasibility of employing genetic barcoding during routine identification of new NIC black coral specimens (especially the Schizopathidae). Ideally barcoding should be accomplished using at least two loci (*ND5-igr-ND1* mtDNA and *NSL* nDNA loci). This would avoid underestimates of diversity and assist with assessments of how many conspecifics are available for genetic connectivity analyses.
- A genetic connectivity assessment of a New Zealand black coral species is still needed, which is contingent upon increased sampling plus development of a suitable genetic methodology. The most cost-effective and powerful approach to genetic connectivity analysis would be to employ a UCE genome-scale method, which may partially overcome the limitations of small sample sizes and older archival material.
- A reassessment of the distributional limits and prevalence of *B. patula* and *T. tasmaniensis* are needed, in light of the current study. An in-depth taxonomic assessment is required of the other cryptic taxa uncovered here, to determine their identity and occurrence before a similar distributional study can be undertaken.

6 Acknowledgements

We acknowledge the invaluable assistance and expert opinion of numerous individuals in the formulation and progression of this research. Lyndsey Holland (MPI-FNZ) generously shared datasets and DNA extracts from a previous study and was key in recognising and formulating the original project objectives, as well as improving the report. Jonathan Gardner (VUW) provided input into research planning and provided storage and access for existing DNA extracts. We also thank Rob Stewart (NIWA) for his advice, identification expertise and for providing photographs of black corals *in situ*. Conversations with Jeremy Horowitz (James Cook Univ.) provided valuable advice and insights into UCE technology and applications, and Mercer Brugler (NYC College of Technology) provided crucial encouragement and advice in our sampling and sequencing design. We also thank Sadie Mills and the NIWA Invertebrate Collection staff for their help with Specify database extracts, Amalia Calle (NIWA intern) for her assistance with specimen selection, sampling and DNA extractions, and Megan Wintringham (NIWA) and Shannon Weaver (DOC-CSP) for administrative support. The advice of Judy Sutherland (NIWA) in reviewing and improving this manuscript is also greatly appreciated. Finally, we thank the Conservation Services Program, DOC for providing essential funding for the study of New Zealand protected corals.

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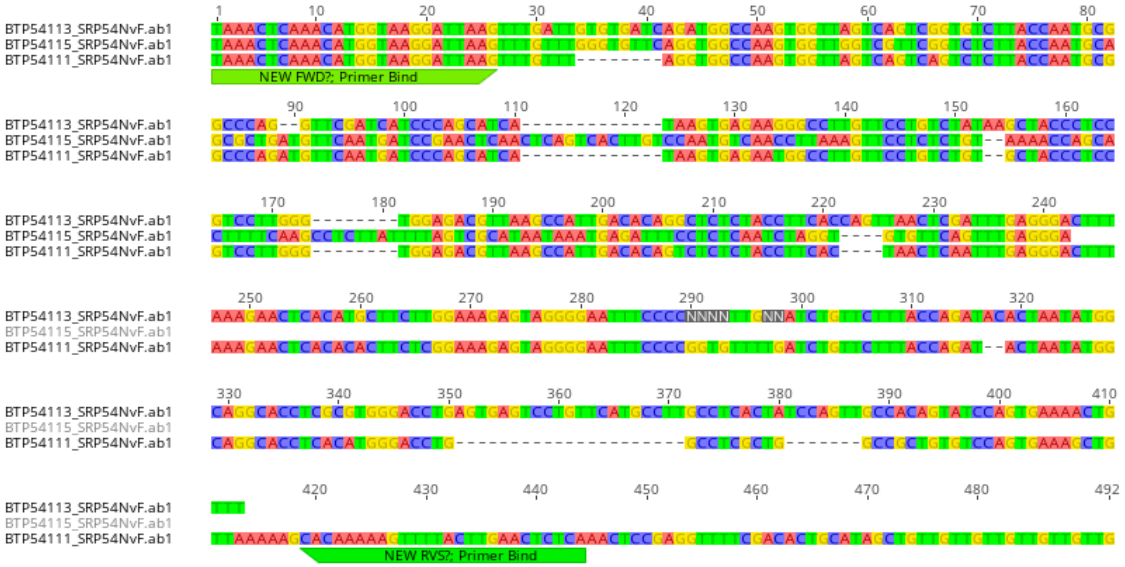
Appendix A Primer development for nuclear markers

A1: New primer design for *ITS1-5.8S-ITS2* rDNA locus, based on GenBank sequences of *Bathypathes* and related schizopathid genera.

Green blocks indicate the location of the newly designed primers used in the current study.

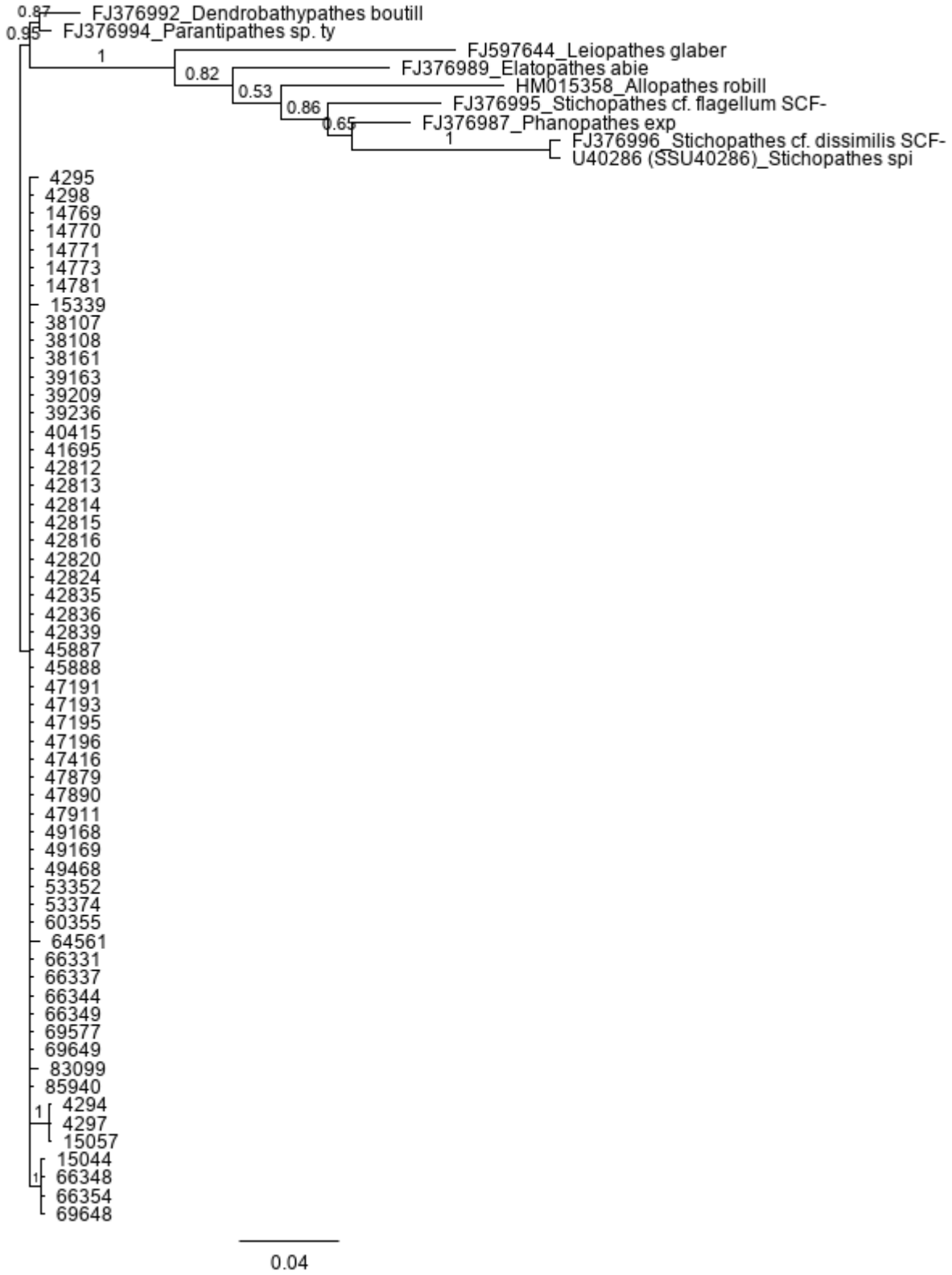
A2: New *B. patula* primers for putative *SRP54* nuclear locus, using three sequences obtained during preliminary analyses by Holland et al. (2020).

Green blocks indicate the location of the newly designed primers used in the current study.



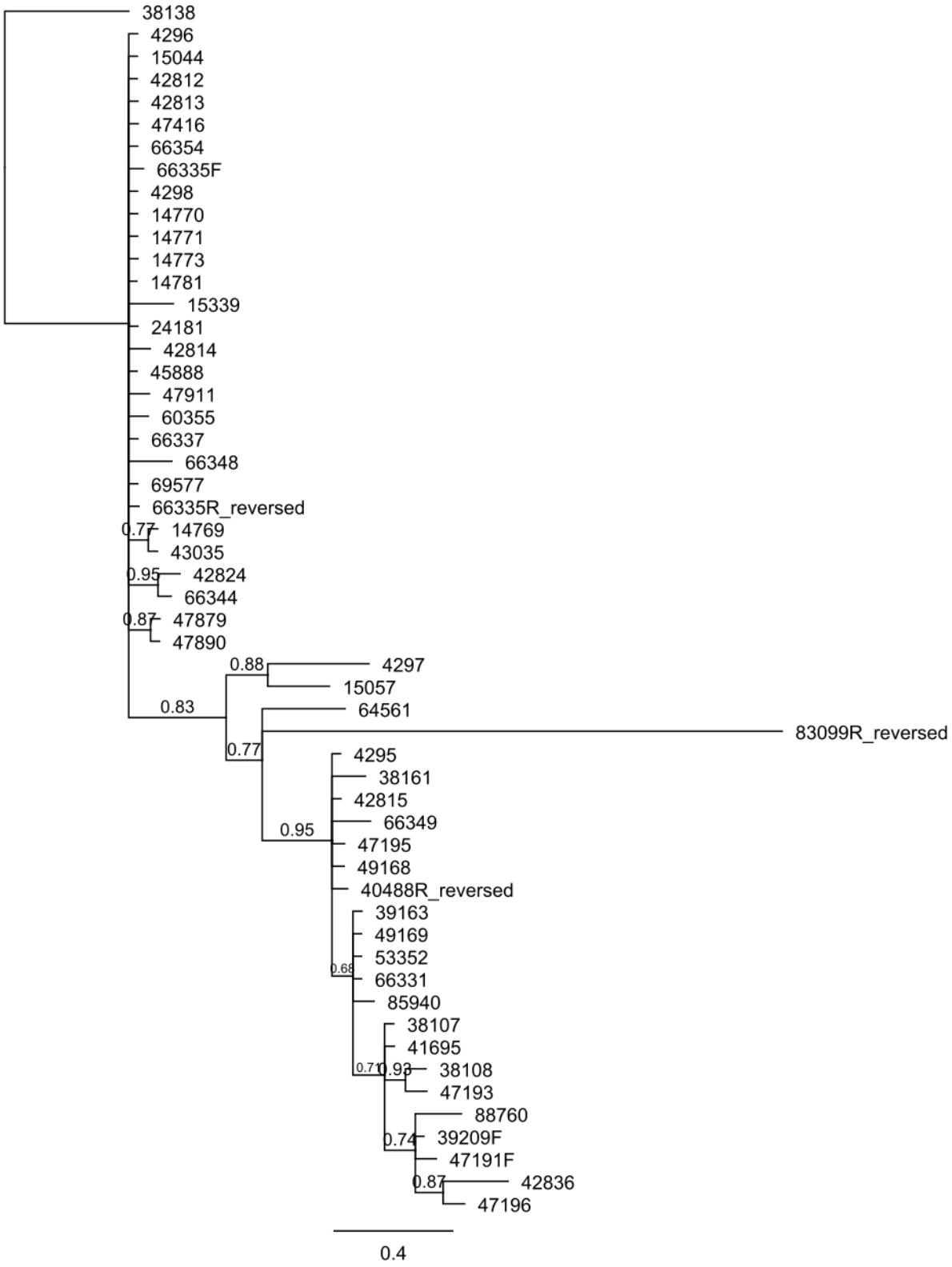
Appendix B Raw phylogenetic results for individual loci

B1: *16S* mtDNA

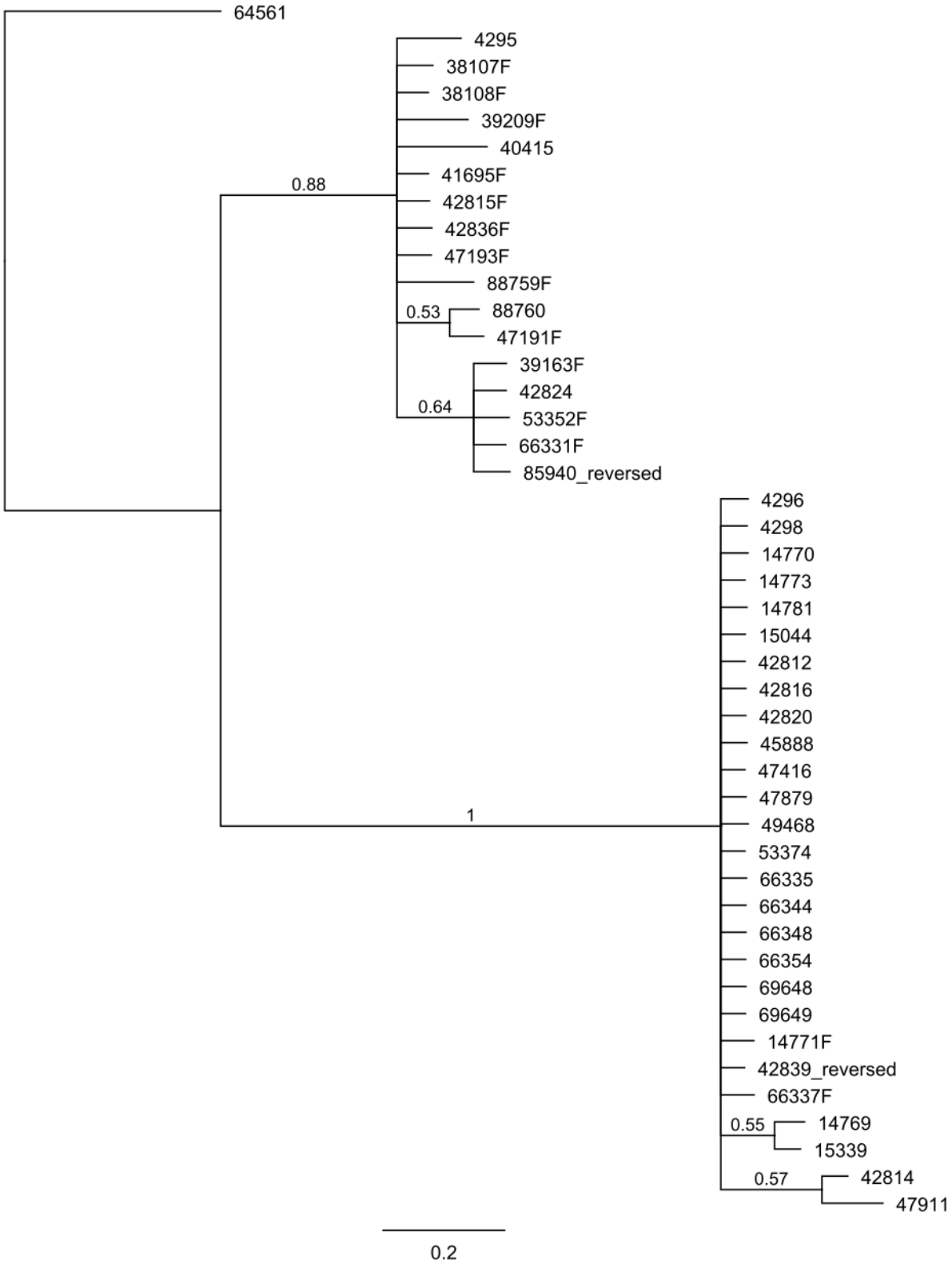


B2: *ND5-igr-ND1* mtDNA

B3: *ITS1-5.8S-ITS2* rDNA



B4: *NSL* nDNA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42824
53352F
66331F
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4296
4298
14770
14773
14781
15044
42812
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42820
45888
47416
47879
49468
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66335
66344
66348
66354
69648
69649
14771F
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66337F
14769
15339
42814
47911
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0.53
0.64
1
0.55
0.57
0.2