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The distribution and diversity of species in the genus Owenia (Polychaeta) in Norwegian waters

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Sammendrag

I denne studien ble diversiteten og utbredelsen til *Owenia*-artenesom finnes i norske farvann analysert. Arten *Owenia fusiformis* sammen med slekta *Owenia* har gejjongått en turbulent historie, hvor forfattere beskriver nye arter, hvorpå andre forfattere er uenige, og resultatet er at mange beskrevne arter blir synonymisert med *O. fusiformis*. Norske farvann er intet unntak, og størstedelen av *Owenia*-materialet i norske samlinger idag er fremdeles identifisert til *Owenia fusiformis*. I denne studien vil både morfologiske og molekylære metoder brukes for å løse gåten om den sanne identifikasjonen av *Owenia* i norske farvann. To gensekvenser ble sekvensert, COI of ITS1. En gruppe viste seg å være svært vanskelig å få vellykkede COI-resultater fra, og ingen suksessfulle sekvenser ble oppnådd fra denne gruppen, mens de resterende prøvene var relativt uproblematiske å få akseptable resultater fra. Utifra denne informasjonen reises spørsmål om hvorvidt de benyttede primerene er gode nok. ITS1 var uproblematisk å kopiere opp og sekvensere, men grunnet svært myevariasjon i sekvensene, en stor mengde innsats krevdes for å analysere dataene tilfredsstillende. De morfologiske karrakterene viste seg å inneholde en stor grad av variasjon, så mye at de fleste ikke kunne brukes til å skille mellom artene i studien. Likevel ble det identifisert nok karrakterer, hvor den beste var farging med metylblått, til at det var mulig å skille mellom de forskjellige artene som finnes i norske farvann.Når dette også stemmer overens med de molekylære analysene, ble det konkludert med at minimum to arter, *Owenia borealis* Koh, Bhaud & Jirkov, 2003 og *Owenia polaris* Koh,Bhaud & Jirkov, finnes i norske farvann, og mulighetene er også tilstede for at det finnes ytterligere arter. Typemateriale til *Owenia assimilis* (Sars, 1851) ble også undersøkt, og det ble konkludert med at *O. borealis* er identisk med typematerialet til *O. assimilis*, til tross for at det er muligheter for at typematerialet består av mer enn en art. Dette er dermed et åpen spørsmål, og ytterligere undersøkelser er nødvendig for å oppnå et endelig svar. Materiale fra typelokaliteten til *O. fusiformis* ble også undersøkt, og det ble konkudert med at tilstedeværelsen av den opprinnelige *O. fusiformis* i norske farvann er høyst usannsynlig.

Abstract

In this study the diversity and distribution of *Owenia* species in Norwegian waters are discussed. *Owenia fusiformis* and the *Owenia* genus has gone through a turbulent history, with authors describing several new species, while other researchers have disagreed, resulting in many described species being synonymized with *O. fusiformis*. Norwegian waters are no exception, and the majority of samples in natural history collections are still referred to as *Owenia fusiformis*. In this study both morphological and molecular methods will be used to solve the issue regarding the true identity of *Owenia* in Norwegian waters. Along with morphological character two genes, COI and ITS1, was sequenced and analyzed. COI proved to be very difficult to extract and amplify successfully for one group of specimens, from which no successful sequences were obtained, while being relatively unproblematic in other groups, leading the question if the COI primers are accurate enough. ITS1 was unproblematic to amplify and sequence, but due to highly variable sequences, a lot of effort was needed to analyze the dataset. Morphological characters proved to exhibit high degrees of the variation, resulting in most of the defined characters being useless in differentiating between the species. Still, enough useful morphologic characters, the best one being the methyl blue staining pattern, were defined, making it possible to distinguish between different species occurring in Norwegian waters. This being congruent with the molecular results, led to the conclusion that at least two different species of *Owenia* is present in Norwegian waters, *Owenia borealis* Koh, Bhaud & Jirkov, 2003 and *Owenia polaris* Koh, Bhaud & Jirkov, with results also suggesting there might be more. Type specimens of *Owenia assimilis* (Sars, 1851) were also examined, concluding with this probably being identical to *O. borealis*, although the possibility of the type material being more than one species, leads to this being an open question, in need of more research before an answer can be given. Specimens from the *O. fusiformis* type locality were also examined, concluding with the presence of the true *O. fusiformis* in Norwegian waters being highly unlikely.

Contents

1 Introduction

1.1 Why this study?

In 1985 Nilsen & Holthe (1985) reported *Owenia fusiformis* delle Chiaje, 1841 (Annelida), a species originally described from Naples, Italy, occurring along most of the Norwegian coast (Fig. 1.1). In 2003 Koh et al. (2003) described two new *Owenia* species based on material from the waters around Iceland, the Faroes islands, the North Sea, the Barents Sea and the coasts of Norway and Svalbard. They also resurrected *Owenia assimilis* (Sars, 1851), a species originally described from Norwegian waters, in the areas around Manger and Tromsø, which earlier was synonymized *O. fusiformis*. The did, however, not find *Owenia* fusiformis, which brings us to the interesting questions of what species of *Owenia* do exists in Norwegian waters, and what are their distribution limits? And, do *Owenia* fusiformis occur in Norwegian waters at all?

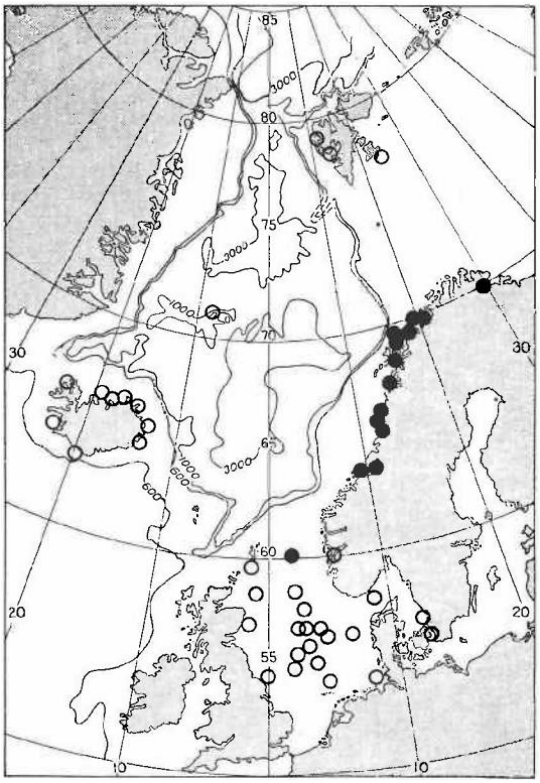

Figure 1.1. Shows the distribution of *Owenia fusiformis* as reported by Nilsen & Holthe 1985. From Nilsen & Holthe 1985 (they did not state the difference between filled and unfilled circles).

1.2 Annelida

Annelida is segmented worms and Polychaeta makes up a large part of the diversity within Annelida (Rouse et al. 2001). Polychaetes are found in almost every marine habitat, from intertidal algal mats to the deepest ocean trenches, including in relation to deep hydrothermal vent systems. Most polychaetes are benthic organisms living in or directly on the bottom, but there are examples of pelagic polychaetes as well. Benthic polychaetes live inside complex

structures like holdfast on kelps, coral reefs or in cracks or calcareous tubes on hard bottoms, while others dig burrows or make tubes in softer sediments. They vary a lot in size from less than a millimeter to more than six meters, and depending on habitat and feeding strategy they can also vary a lot regarding number and type of appendages, like having developed eyes or not, various sensory organs, type of jaws etc. Some actively hunt other organisms, some are scavengers while others are filter feeders.

Linnaeus was the first to formerly describe the first Annelids in 1758, and today more than 21 000 species has been described (Weigert et al. 2014). But even though the Annelid fauna has been studied for more than 250 years, the basal part of the Annelid tree is poorly understood and even if several recent studies (McHugh 2000, Bleidorn et al. 2003, Rousset et al. 2007, Struck et al. 2008, Zrzavy et al. 2009, Struck et al. 2011, Kvist et al. 2013, Weigert et al. 2014) have attempted to sort this out, there are still not consensus regarding the most basal taxa. One of the problems seems to be to acquire enough data, for instance Struck et al. (2011) and Kvist et al. (2013) did not include Oweniidae and Magelonidae in their studies, taxa in which Weigert et al. (2014) found to be the most basal Annelid taxa.

1.3 Family Oweniidae

According to a revision by Capa et al. (2012) Oweniidae Rioja, 1917 is a family consisting of four genera: *Galathowenia* Kirkegaard, 1959; *Myriochele* Malmgren, 1867; *Myriowenia* Hartman, 1960; *Owenia* delle Chiaje, 1841. Other previously described genera which now has been synonymized with one of the four includes *Myrioglobula* Hartman, 1967 (synonymized with *Myriochele* by Capa et al. (2012)), *Ammochares*, *Mitraria* and *Psammocollus*.

Oweniidae is characterized by clearly segmented body, usually more the 15 segments (Rouse et al. 2001). The prostomium and peristomium makes up the head, and are quite variable within the group (Fig. 1.2 illustrates the diversity within Oweniidae). The prostomium is usually a simple rounded lobed-like structure with different additional structures in the different taxa. These structures include ventral grooves, a pair of ventrally grooved palps or a multilobed "crown". In most taxa the peristomium seems to form a complete ring, but in *Owenia* there can look like there has been some fusion with the prostomial crown. A distinct thoracic region is present, consisting of a single segment or up to three segments. All segments are chaetigers, with the thoracic segments being uniramous (u1-u2) with notochaeta only, which are simple capillary chaeta. The rest of the segments are

biramous (b1-bn) with both notopodia and neuropodia. The notopodia are the same as for the thoracic part of the body, the neuropodia are large tori consisting of fields of densely packed hooks, uncini. These hooks are long shafted with two teeth of usually similar size. The pygidium may be a simple lobe, or it can also have a pair of cirri or multiple digitate lobes. Most taxa build distinctive tubes were the sediment particles partly overlap, similar to roofing tiles. The particles can be sand particles, shell fragments, spicules or Foraminifera tests.

Recently, several authors (Cantone et al. 1998, Koh et al. 2001, Parapar 2001, Koh &

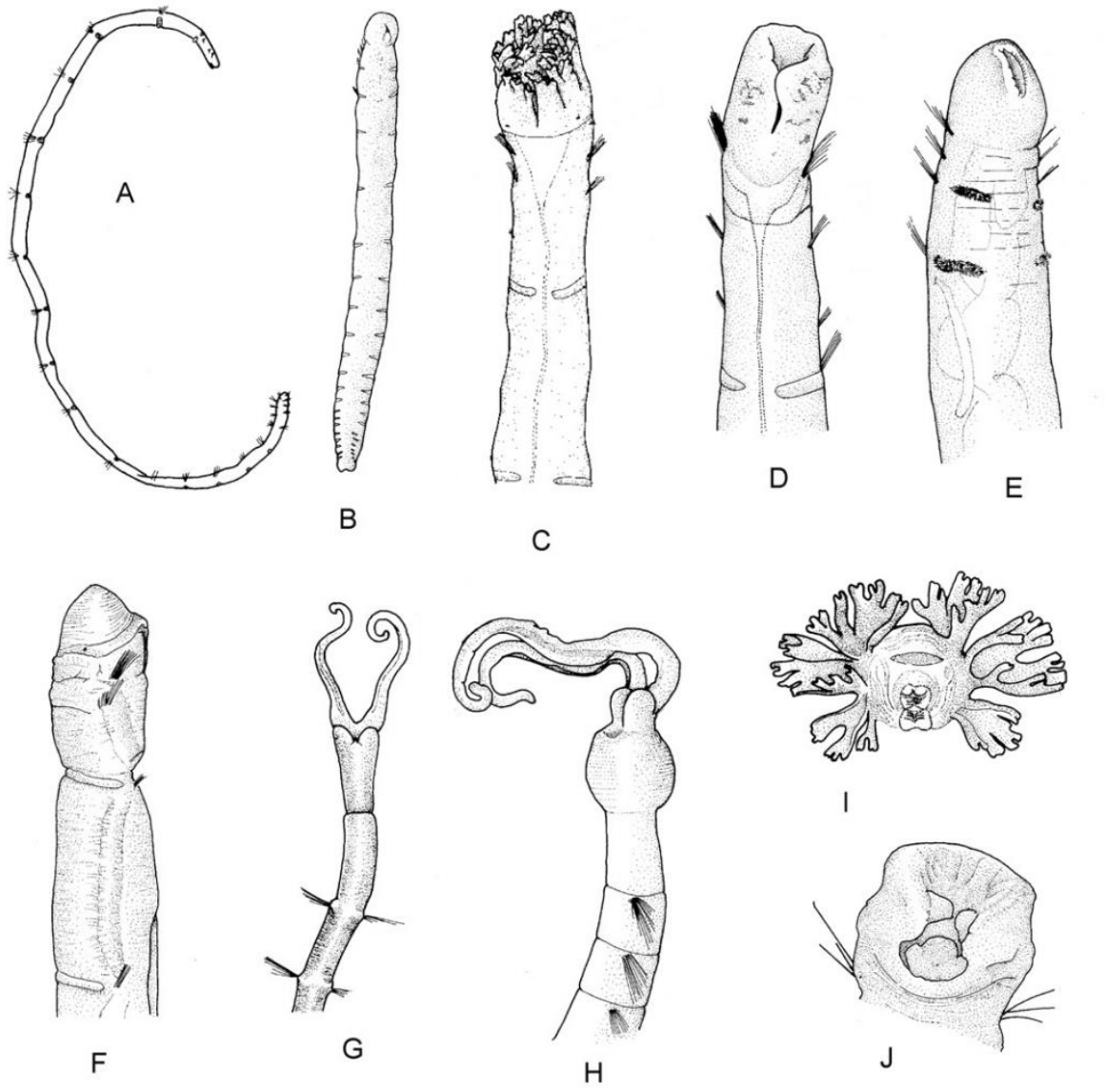

Figure 1.2. Illustrations of the front end of different species in the Oweniidae family. A, *Galathowenia oculata*, entire specimen; B, *Myriochele heeri*, entire specimen; C, *Owenia fusiformis*; D, *Galathowenia oculata*; E, *Myriochele olgae*; F, *Myriochele danielsseni*; G, *Myriowenia gosnoldi*; H, *Myriowenia californiensis*; I, *Owenia fusiformis*, frontal view; J, *Galathowenia oculata*, front view. Modified from Capa et.al 2012.

Bhaud 2003, Koh et al. 2003, Parapar 2003a, b, Ford & Hutchings 2005, Martin et al. 2006, Parapar 2006, Capa et al. 2012, De Leon-Gonzalez et al. 2012) have revised and described new species within the family. Thereby documenting evidence of the challenges with the taxonomy of the group, regarding species complexes and cryptic species, a topic covered further down.

1.4 History of *Owenia*

In the genus *Owenia*, the type species, *O. fusiformis* delle Chiaje, 1841, was described from the Mediterranean. It was later reported from all over the world as a cosmopolitan species, even though several new *Owenia* species was described, for instance *O. brachycera* Marion, 1876, *O. filiformis* Claparede, 1868, *O. collaris* Hartman, 1969 and *O. lobopygidiata* Uschakov, 1950. In her catalogue Hartman (1959) listed the following as valid *Owenia* species:

- *Owenia artifex* (Verrill, 1885); described as *Ammochares artifex* Verrill, 1885
- *Owenia fusiformis* delle Chiaje, 1841; *O. assimilis* Levinsen, 1883, *O. brachycera* and *O. filiformis* was synonomyzed with *O. fusiformis*, together with *Ammochares aedificator* Andrews, 1891, *A. assilmilis* Sars, 1851, *A. ottonis* Grube, 1846, *Ops digitata* Carrington, 1865, *Ammochares brasiliensis* Hansen, 1882, *A. occidentalis* Johnson 1901, *A. sundevalli* Kinberg, 1867 and *A. tegula* Kinberg, 1867.
- *Owenia fusiformis collaris* Hartman, 1955; raised to *O. collaris* Hartman, 1955 by Hartman (1969)
- *Owenia lobopygidiata* Uschakov, 1950
- *Owenia orientalis* (Grube, 1878); described as *Ammochares orientalis* Grube, 1878
- *Owenia tenuis* (Haswell, 1883); described as *Ammochares tenuis* Haswell, 1883

In 1959 *Owenia caudisetosa* Hartmann-Schröder, 1959 was described. *Ammochares orientalis* was synonymized with *O. fusiformis* by Fauvel (1953) and *O. tenuis* was also considered as *O. fusiformis* by several authors (Ehlers 1901, Rullier 1965, Day et al. 1979). Dauvin et al. (1994) did a revision of the *Owenia* genus where they stated that *Owenia artifex* has not been cited after the description and that the species is doubtful thereby probably is a synonym to *O. fusiformis*. They did a morphological study on specimens collected all over the world, concluding that there are two different species of *Owenia*, *Owenia fusiformis* delle Chiaje, 1841 and *Owenia lobopygidiata* Uschakov, 1950.

In the last two decades, several authors (Koh et al. 2001, 2003a, Koh et al. 2003,Ford & Hutchings 2005, Martin et al. 2006) have resurrected previously described species as well as described new *Owenia* species, and thereby strengthening the conclusion that *O. fusiformis* is not a cosmopolitan species. At present, a total of 14 different *Owenia* species is described and considered valid (*O. assimilis* (Sars, 1851), *O. australis* Ford and Hutchings, 2005, *O. bassensis* Ford and Hutchings, 2005, *O. borealis* Koh, Bhaud and Jirkov, 2003, *O. brasiliensis* (Hansen, 1882), *O. collaris* Hartman, 1955, *O. fusiformis* delle Chiaje, 1841, *O. gomsoni* Koh and Bhaud, 2001, *O. johnsoni* Blake, 2000, *O. lobopygidiata* Uschakov, 1950, *O. mirrawa* Ford and Hutchings, 2005, *O. persica* Martin, Koh, Bhaud, Dutrieux and Gil, 2006, *O. petersenae* Koh and Bhaud, 2003 and *O. polaris* Koh, Bhaud and Jirkov, 2003), with the possibility of at least one more valid species (*O. caudisetosa* Hartmann-Schröder, 1959).

1.5 *Owenia* in Norwegian waters

Owenia assimilis was described by Sars (1851) from Norwegian waters (originally described as *Ammochares assimilis*). As stated above this was synonymized with *O. fusiformis* by Hartman (1955). Until recently all *Owenia* in Norwegian waters has been regarded as *O. fusiformis*. Although, during their investigation of oweniids in the North Atlantic, Nilsen & Holthe (1985) suggested that it indeed is more than one species. This was confirmed by Koh & Bhaud (2003) and Koh et al. (2003) with the description of *O. borealis* and *O. polaris* and the resurrection of *O. assimilis*. There has been no study after this to clarify which *Owenia* species is present in Norwegian waters and their distribution.

1.6 Morhological studies

Since Thommasin et al. (1972) first used Scanning Electron Microscopy (SEM) to study oweniids, it has become a valuable tool in taxonomy work on this group, used by many authors (Koh et al. 2001, 2003a, Koh et al. 2003,Ford & Hutchings 2005, Capa et al. 2012). High resolution images make it possible to identify additional microstructures, morphological features witch may be useful in descriptions of species, but are too small to be able to recognize with traditional light microscopy, making it possible to distinguish these highly similar sepecies.

1.7 Characters

In their study Koh & Bhaud (2003) did an extensive examination of both soft and hard structures on specimens identified as *Owenia fusiformis* from 15 different geographical areas. A list of more than 40 different characters was established, and the variation on individual specimens, as well as variation between specimens from the same population was investigated.

In their descriptions of *O. borealis* and *O. polaris* Koh et al. (2003) used these characters: length and diameter of body and tube; number of chaetigerous segments in adult worms; the relative length of the tentacle crown compared to the thorax length; the relative diameter of the tentacle crown compared the thoracic part; tentacle dichotomy; collar edge smoothness; the notch at the collar and the angle it makes relative to the collar direction. The pygidium is described with number of lobes and their placement and whether or not the medioventral slit is clearly visible or not.

Several characters on the uncini was also used: the direction of the teeth on the uncini on the first biramous segment relative to the direction of the body; the protrusion of the teeth; squashing of the teeth on the head of the shaft; the distance between the teeth and the stem of the uncini; the curve of the head; angle between the stem and the teeth; whether or not the hook have a shoulder; the shape of the forward facing upper part of the stem (see Fig. 2A in Koh et al. (2003) for more details and illustration of the different characters); in apical view, shape of the slit between the teeth.

Two characters on the chaeta were also used: it they are symmetrical or not and the ratio of the free part to its total length (see Fig. 2B in Koh et al. (2003)).

Characters on the tube were also used: what kind of material the tube particles was made of; how the particles was attached to the tube wall (this comprise several characters like particle size distribution, the direction of the particles, whether the particles smaller or larger surface is attached to the tube wall); the relative diameter of the central lumen compared with total tube diameter.

1.8 Methyl green staining

Koh & Bhaud (2003) did also stain the specimens from 8 different locations with methyl green and described differences in the staining pattern, but Koh et al. (2003) did not use this feature in their description of *O. borealis* and *O. polaris*. However, Blake (2000) did include methyl green staining in both his redescription of *O. collaris* Hartman, 1955 and the decription of *O. johnsoni* Blake, 2000.

1.9 Unknown diversity

Two studies (Holte et al. 1996, Pearson et al. 1996) stated that polychaetes may account for as much as 41-80 % of specimens with up to 8000 ind. m^{-2} in benthic samples, and they also reported *O. fusiformis* as the most abundant polychaete in the North Sea, both in terms and biomass and number of specimens. Estimates from the Norwegian MAREANO (Marine AREAl database for NOrwegian waters) project indicate that as much as 15-25 % of polychaeta in the investigated area have an uncertain taxonomic status (Børge Holthe and Eivind Oug, unpublished data), many as cryptic species complexes.

A cryptic species is "two or more distinct species that are erroneously classified (and hidden) under one species name" (Bickford et al. 2007). This is a relative expression, because when two or more cryptic species are unveiled and described they are, by definition, no longer cryptic species. Once such knowledge is obtained, for instance from molecular data, it is not unusual that distinct morphological differences are found (Saez et al. 2005). The species are now considered to be pseudocryptic (Nygren 2014). Cryptic species may differ on many different biological characters, like reproduction, life history, depth and habitat preferences, feeding strategy, temperature and salinity preferences and tolerance to different water conditions (Nygren 2014, and references within). The reason why they are so important is because some of the complexes are used as bioindicators in environmental monitoring or in bioaccumulation and ecotoxicological studies (Nygren 2014, and references within), and if different species is being used in those kind of studies, the results may not be comparable (Åkesson 1983). This clarifies how important it is to solve these species complexes to be able to get accurate data from environmental surveys, as well as conduct environmental monitoring with as high accuracy as possible.

Several polychaete species complexes have been discovered during the recent years (Mackie et al. 1995, Koh et al. 2003,Ford & Hutchings 2005, Nygren et al. 2009) due to better equipment and techniques, which makes it possible to describe species which one previously was unable to distinguish from one another.

1.10 Molecular studies

Molecular techniques have also been used to reveal and describe cryptic species, where the authors have not been able to distinguish the species based on morphological characters (Westheide et al. 2001, Nygren et al. 2009, 2010, Nygren et al. 2011). Jolly et al. (2006) investigated *Owenia fusiformis* in the area around the English Channel, the Irish Sea, one location in Sweden and one in the Mediterranean sea and found three distinct genetic lineages of *O. fusiformis*.

When phylogenetic analysis from both morphologic and molecular characters supports the same conclusions, the scientific support of the conclusions is solid. This makes combining morphologic and molecular characters a very valuable tool for resolving taxonomic challenges.

1.11 Aim

The aim of the study is to

- investigate the true identity of the species referred to as *Owenia fusiformis*, the most abundant species in Norwegian waters,
- are there more than one *Owenia* species in Norwegian waters, and if so, what are their distribution limits and
- do the true *Owenia fusiformis* occur in Norwegian waters at all?

2 Material and methods

2.1 Material and sampling methods

The material used in this project includes material mostly fixated in formaldehyde and transferred to ethanol for preservation, or some of the recently collected material are both fixated and preserved on ethanol. The material is from the Museum of Natural History and Archaeology, Norwegian University of Science and Technology (NTNU-VM), the University Museum of Bergen, University of Bergen (ZMBN), AKVAPLAN-NIVA in Tromsø and Germany. Specimens have also been collected during the project in the Trondheimsfjord using triangular dredge, the Barents Sea with the MAREANO program using 0.25m^2 van Veen Grab and Beam Trawl and in Kongsfjord and Hinlopenstredet at the west and east coast of Svalbard, respectively, as part of the course AB321 – Marine Benthic Fauna of Svalbard at the University Centre in Svalbard using 0.25m^2 using van Veen Grab.

2.2 Study area

Main study area is the Norwegian waters, including Svalbard (Fig. 2.1A). Some samples from the German (GER) part of the North Sea, Atlantic coast of Spain (ESP) and Naples, Italy (ITA) are also included (Fig. 2.1B, red points). Additional COI sequences downloaded from GenBank are from samples collected mainly in and around the English Channel and the Mediterranean Sea (Fig. 2.1B, yellow points). COI and ITS1 sequences provided by Maria Capa are from six different locations in Australia (Fig. 2.1C).

2.3 Molecular methods

2.3.1 DNA extraction

Extractions were performed using DNeasy Blood and Tissue Kit using the Purification of Total DNA from Animal Tissues (Spin-Column Protocol) protocol. Small tissue samples were placed in 1.5 ml microcentrifuge tubes, and 180 µl ATL buffer and 20 µl proteinase K was

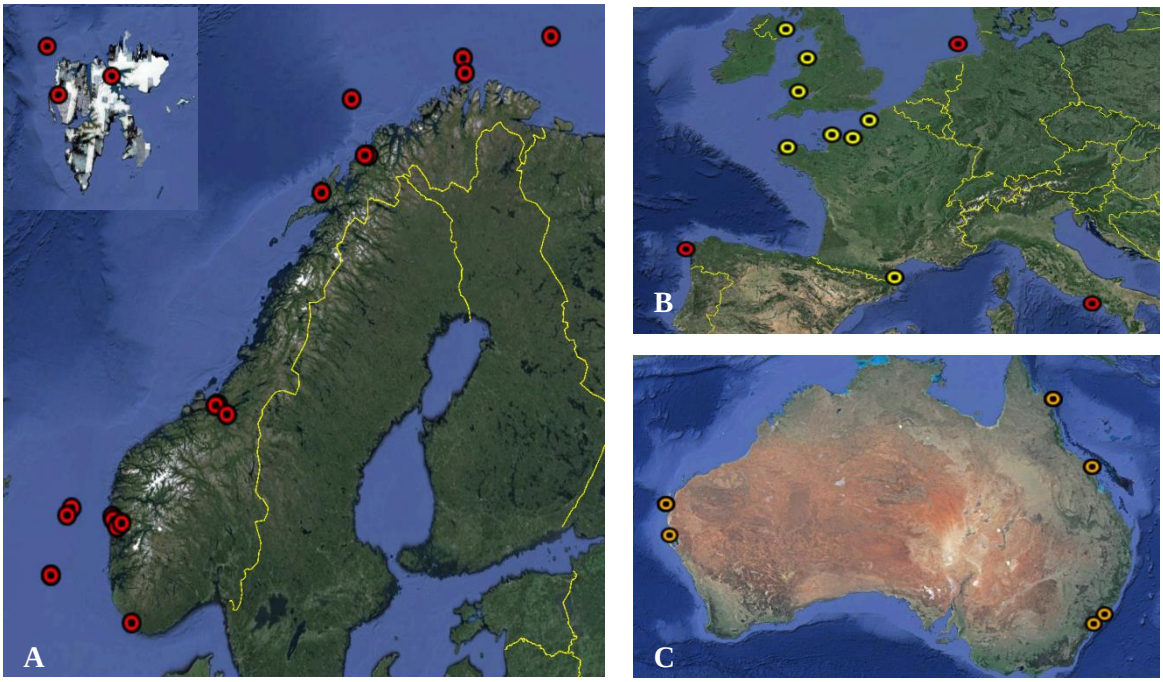

Figure 2.1. The figure marks all locations from which results are presented. A: Norway with Svalbard in the upper left corner; B: Europe; C: Australia. Red markings represent specimens investigated, yellow marking represent sequences downloaded from GenBank and orange marking represent sequences provided by Maria Capa.

added. The samples were mixed thoroughly by vortexing before left for incubating at 56 °C overnight. The samples were thoroughly vortexed again before adding 200 µl AL buffer. Then vortexed again before adding 200 µl ethanol (100%), and vortexed. The mixture was pipetted into DNeasy Mini spin column placed in a 2 ml collection tube and centrifuged at 6000g for 1 minute. The DNeasy Mini spin columns was moved to new collection tubes and 500 µl AW1 buffer was added before centrifuging at 6000g for 1 minute. The DNeasy Mini spin columns was then moved to a new collection tube before adding 500 µl AW2 buffer followed by centrifuging at 20000g for 3 minutes. To elute the DNA the DNeasy Mini spin columns was transferred to 1.5 ml microcentrifuge tubes before 100 µl AE buffer was added, then samples was incubated at room temperature for 1 minute and centrifuged at 6000g for 1 minute.

2.3.2 Amplification of DNA

A fragment of the mitochondrial cytochrome c oxidase subunit I (mtCOI) was amplified using the following PCR kits: TaKaRa Ex Taq™ Hot Start Version; QIAGEN HotStarTaq® *Plus* DNA Polymerase; GE Healthcare illustra™ puReTaq Ready-To-Go PCR Beads. Protocols used were T1, T2, Q1, Q2, Q3 and BEADS (ingredients specified in table 2.1). Primers used was HCO2198 (5'-TAA ACT TCA GGG TGA CCA AAA AAT CA-3') and LCO1490 (5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3') (Folmer et al. 1994). All ingredients except the DNA sample were mixed and 20-24 µl (the volumes varied, used volume was 25µl minus DNA sample volume) was placed in 0.2 ml tube. The DNA samples were added before centrifuging and the tubes were placed in a BIO-RAD C1000™ Thermal Cycler. The PCR programs were CO1, CO1BEAD, CO1TAGR1, CO1TAGR2, CO1TAGR3, CO1TD, TAGRTD and BEADGRTD (conditions specified in table 2) with lid temperature = 105°C.

The internal transcribed spacer 1 (ITS1) between 18S and 5.8S (including parts of both 18S and 5.8S) was amplified using the same technique as with mtCOI. PCR protocols T1 and

Table 2.1. Shows all PCR protocols used and lists the content of each protocol.

PCR protocol name / PCR ingredients	T1	T2	Q1	Q2	Q3	BEADS
DNA grade H2O	var	var	var	var	var	21.0
TaKaRa 10x Taq Buffer	2.5	2.5				
QIAGEN 10x PCR Buffer			2.5	2.5	2.5	
F-primer (10 pmol/µL)	1.0	1.0	1.0	1.0	1.0	1.0
R-primer (10 pmol/µL)	1.0	1.0	1.0	1.0	1.0	1.0
TaKaRa dNTP mix	2.0	2.0	2.0	2.0	2.0	
QIAGEN MgCl2		0.5	1.5	2.0	2.5	
TaKaRa Ex Taq HS	0.2	0.2				
QIAGEN HotStarTaq DNA Polymerase			0.2	0.2	0.2	
DNA sample	var (1-5)	var (1-5)	var (2-4)	var (2-4)	var (2-4)	2.0
Sum	25.0	25.0	25.0	25.0	25.0	25.0

BEADS (ingredients specified in Table 2.1) with primers ITS18SFPOLY (5'-GAG GAA GTA AAA GTC GTA ACA-3') and ITS5.8SRPOLY (5'-GTT CAA TGT GTC CTG CAA TTC-3') (Nygren et al. 2009) was used. PCR programs were ITSBEAD and TAGRTD (conditions specified in Table 2.2). All products were analyzed with gelelectroforesis. The gel was made of 80 ml 1X TAE buffer with 1% agarose and 2.5 µl life technologies invitrogen™ SYBR® Safe DNA gel stain. 4 µl PCR products were mixed with 2 µl Thermo Scientific 6X Orance DNA Loading Dye and 5 µl was pipetted onto the gel. 5 µl Thermo Scientific O'GeneRuler 100 bp DNA Ladder ready-to-use were added to an empty well. The gel was run for 75 minutes on 60 V before the resulting bands were compared to the ladder to estimate the concentration of DNA in the PCR product, if it had the desired length and if there were several bands, indicating bad conditions or contaminations.

Due to several bands in the gel, some samples was extracted from the gel with QIAGEN

Table 2.2. The table shows all different PCR programs used, and the temperature and time for all steps in each program.

PCR program name	PCR program outline
CO1	95°C/240s - (94°C/30s - 48°C/30s - 72°C/60s)*40cycles - 72°C/480s - 12°C/∞
CO1BEAD	95°C/300s - (95°C/30s - 45°C/30s - 72°C/60s)*40cycles - 72°C/480s - 7°C/∞
CO1TAGR1	95°C/240s - (94°C/30s - (gradient45-58°C)/30s - 72°C/60s)*40cycles - 72°C/480s - 12°C/∞
CO1TAGR2	95°C/240s - (94°C/30s - (gradient50.2-54.7°C)/30s - 72°C/60s)*40cycles - 72°C/480s - 12°C/∞
CO1TAGR3	95°C/240s - (94°C/30s - (gradient48.5-53.5°C)/30s - 72°C/60s)*40cycles - 72°C/480s - 12°C/∞
CO1TD	95°C/240s - (94°C/30s - 54°C(-0.5°C pr. cycle)/30s - 72°C/60s)*10cycles - (94°C/30s - 48°C/30s - 72°C/60s)*30cycles - 72°C/480s - 12°C/∞
ITSBEAD	96°C/240s - (94°C/30s - 48°C/30s - 72°C/60s)*40cycles - 72°C/480s - 7°C/∞
TAGRTD	95°C/120s - (94°C/30s - (gradien54-59°C)(-1°C pr. cycle)/30s - 72°C/60s)*5cycles - (94°C/30s - (gradient49-54°C)/30s - 72°C/60s)*34cycles - 72°C/480s - 12°C/∞
BEADGRTD	98°C/10s - 94°C/120s - (94°C/30s - (gradien55-60°C)(-1°C pr. cycle)/30s - 72°C/60s)*5cycles - (94°C/30s - (gradient50-55°C)/30s - 72°C/60s)*34cycles - 72°C/480s - 12°C/∞

MinElute® Gel Extraction Kit in the following manner 1: isolated from the gel using a clean scalpel; 2: placed in a 2.0 ml microcentrifugation tube; 3: 3 volumes of Buffer QG to 1 volume of gel was added; 4: the tubes was incubated at 50°C until all the gel had completely dissolved; 5: 1 gel volume of isopropanol was added before mixing by inverting; 6: the mixtures was transferred to MinElute columns and centrifuged at 10000g for 1 minute; 7: 500 µl Buffer QG was added and the columns was centrifuged again at 10000g for 1 minute; 8: 750 µl Buffer PE was added before centrifuging at 10000g for 1 min; 9: the columns was centrifuged once more for 1 min at 10000g without adding any buffer to remove all buffer residues before: 10: the columns were transferred to a 1.5 ml microcentrifuge tube and 10 µl Buffer EB was placed in the center of the column membrane and; 11: and the column was incubated for 1 min at room temperature before; 12: elution was done by centrifuging at 10000g for 1 min.

In the end the samples were purified using GE Healthcare illustra ExoProStar 1-Step by transferring 15 µl product (diluted were necessary) to a 0.2 µl tube before adding 1 µl of ExoProStar. The samples was then incubated in the BIO-RAD C1000™ Thermal Cycler at 37°C for 15 minutes to purify the PCR product before another step at 80°C for 15 minutes to inactivate the enzymes.

The samples were sent to Eurofins for sequencing.

2.3.3 Sequence quality assessment

DNA Baser Sequence Assembler v4.10.1.13 (Heracle BioSoft 2012) was used to assemble contigs from both directions when possible. The software automatically combines the forward and reverse sequence into a contig, and the resulting contig was inspected and edited manually, poor quality ends were trimmed. In cases were the chromatogram had only one readable direction, this direction was used as the contig. Sequences were both chromatograms were unreadable was not used for further analysis.

All sequences was compared with existing closely related sequences, if found, from GenBank using BLAST (Altschul et al. 1990).

2.3.4 Additional sequences

21 *Owenia fusiformis* COI sequences with representatives from all clades found by (Jolly et al. 2006) was downloaded from GenBank. 1 *Galathowenia oculata* (Zachs 1923) sequence from (Hardy et al. 2011) was a downloaded from GenBank to use as outgroup. An additional 18 unpublished sequences from a total of 13 specimens (6 COI and 10 ITS1 from 11 Australian specimens, 1 COI sequence from a Belgian specimen and 1 COI sequence from a Canadian specimen) were provided by Maria Capa.

2.3.5 Sequence alignments

COI sequences were aligned using MAFFT online (Katoh et al. 2002, Katoh et al. 2008, Kuraku et al. 2013) with default settings except for "UPPERCASE/lowercase" where the "Same as input" option were used, the "Direction of nucleotide sequences" where the "Adjust direction according to the first sequence (accurate enough for most cases)" option was used, the "Strategy" chosen was "Q-INS-i" and the "Scoring matrix for nucleotide sequences" parameter were set to "1PAM / k=2".

Due to highly variable sequences, ITS1 sequences were aligned using MAFFT online with three different settings. Two options varied in the three settings; the "Scoring matrix for nucleotide sequences" parameter which were set to "1PAM / k=2", "1PAM / k=2" and "20PAM / k=2" and the "Gap opening penalty" which were set to 1.53, 1.0 and 1.53, respectively. The remaining options was set to default except for "UPPERCASE/lowercase" where the "Same as input" option were used, the "Direction of nucleotide sequences" where the "Adjust direction according to the first sequence (accurate enough for most cases)" option was used. In addition to MAFFT, both ClustalW and Muscle in MEGA v6.06 was used to align the sequences. In ClustalW a gap opening penalty of 10 and a gap extension penalty of 1 was used, in Muscle the gap opening penalty was set to -200 and the gap extension penalty to 0, the rest of the options was default in both alignments. The online version of Gblocks (Castresana 2000, Talavera et al. 2007) with the "Allow smaller final blocks", the "Allow gap positions within the final blocks" and the "Allow less strict flanking positions" options ticked, to reduce the amount of ambiguous sites in the dataset. *A manual alignment was also made to see if and how it inflicted on the result.*

Three different alignments were made which include both the COI sequences and ITS1 sequences. The ITS1 alignment used was one of the MAFFT alignments described above, where the "Scoring matrix for nucleotide sequences" parameter were set to "1PAM / k=2" and the "Gap opening penalty" were set to 1.53. In one COI+ITS1 alignment all previously used COI and ITS1 sequences were included, another was identical to the one above except some of the sequence downloaded from GenBank was removed so that there was only one downloaded sequence in each clade (the clades was based on previous analysis of the COI dataset) and the last combined alignment only included samples in which both COI and ITS1 sequencing had been successful (alignments referred to as COI+ITS1_MAFFT, COI+ITS1_MAFFT_reduced and COI+ITS1_MAFFT_allCOI+ITS1 respectively in Table 3.1).

2.3.6 Sequence analysis

COI sequences were analyzed using MEGA v6.06 (Tamura et al. 2013) and MrBayes v3.2.2 (Huelsenbeck et al. 2001, Ronquist et al. 2003). The "Find Best DNA/Protein Models (ML)" function in MEGA was used to find the substitution models which best suited the alignment. MEGA was then used to perform a Maximum Parsimony (MP) (Subtree-Pruning-Regraftig algorithm (Nei et al. 2000) with search level 1 without a starting tree, sites with more than 10% gaps were deleted, bootstrapping with 1000 replicates) and Maximum Likelihood (ML) analysis (HKY substitution model with gamma distribution and invariable sites, sites with more than 5% gaps were deleted, bootstrapping with 1000 replicates). The dataset was also analyzed using Bayesian Inference (BI) in MrBayes v3.2.2 (10 000 000 generations, first 25% discarded as burnin, 2 parallel runs).

ITS1 sequences were analyzed using MEGA v6.06 (Tamura et al. 2013) and MrBayes v3.2.2 (Huelsenbeck et al. 2001, Ronquist et al. 2003). The "Find Best DNA/Protein Models (ML)" function in MEGA was used to find the substitution models which best suited each alignment. The ITS1_MAFFT the ITS1_MAFFT_gblocks alignments was analyzed two times with both Maximum Likelihood (ML) using MEGA (T92 substitution model with gamma distribution and invariable sites, sites with more than 25% and 50% gaps were deleted (one in each run), bootstrapping with 1000 replicates) and Maximum Parsimony (MP) (Subtree-Pruning-Regrafting algorithm (Nei et al. 2000) with search level 1 without a starting tree, sites with more than 25% and 50% gaps (one in each run) were deleted, bootstrapping with 500

replicates), and using Bayesian Inference (BI) in MrBayes v3.2.2 (10 000 000 generations, first 25% discarded as burnin, 2 parallel runs).

The remaining ITS1 alignments was analyzed in MEGA with two ML analyzes each (substitution models and gamma distribution and invariable sites or not according to Table 3.2, sites with more than 25% and 50% gaps were deleted (one in each run), bootstrapping with 500 replicates).

All three COI+ITS1 alignments was tested with the "Find Best DNA/Protein Models (ML)" function in MEGA v6.06 (Tamura et al. 2013) to find the substitution models which best suited each alignment. The COI+ITS1_MAFFT_allCOI+ITS1 alignment were analyzed with Maximum Likelihood (ML) (TN93 substitution model with invariable sites, sites with more than 50% gaps were deleted, bootstrapping with 1000 replicates) and Maximum Parsimony (MP) (Subtree-Pruning-Regraftig algorithm (Nei et al. 2000) with search level 1 without a starting tree, sites with more than 75% were deleted, bootstrapping with 500 replicates) using MEGA v6.06, and Bayesian Inference (BI) using MrBayes v3.2.2 (Huelsenbeck et al. 2001, Ronquist et al. 2003) (10 000 000 generations, first 25% discarded as burnin, 2 parallel runs).

2.4 Morphological methods

Material was observed under a Leica M165C light microscope for macroscopic characters and methyl blue staining. Samples selected for Scanning Electron Microscope (SEM) was washed quickly in Zalo water, then rinsed in water before put in 80 % ethanol. They were dried using either hexamethyldisilazane (HMDS) (after the following protocol: 1:4 HMDS:ethanol for 30 min; 1:2 HMDS:ethanol for 30 min; 3:4 HMDS:ethanol for 30 min; 100% HMDS for 30 min 3 times and then left for evaporation under a lid (not sealed)) or a Polaron Critical Point Dryer E3000, and then coated with Pt/Pd using a Polaron SEM Coating Unit E5100 or a JEOL Fine Coat Ion Sputter JFC-1100. Images were made using a JEOL JSM-6480LV SEM at the Cellular & Molecular Imaging Core Facility (CMIC) at the Norwegian University of Science and Technology (NTNU) and a ZEISS Supra 55VP SEM at the Laboratory for Electron Microscopy, University of Bergen (UiB).

2.4.1 Characters

Images was analyzed and measurements done with ImageJ 1.48v (Rasband, W.S, 1997-2014).

Characters used are according to (Koh et al. 2003) in their description of *O. borealis* and *O. polaris*:

Thoracic characters:

1. Length of branchiae on tentacular crown relative to length of thorax (branchia length/thorax length, measured from base of branchia to the tip, and from the collar notch to first neuropodia on b1)
2. Diameter of tentacular crown relative to the diameter of the thorax
3. Dichotomy on the branchia, placement of the dichotomies (basal, mid or tip) and
4. number of dicothomies
5. The collar angle at the notch
6. The edge of the collar thickness and appearance

Chaeta:

7. Length of the free part of the scale relative to the total length of the scale (fig. 2B in (Koh et al. 2003))

Uncini:

8. Angle of the teeth on the uncini relative to the direction of the body on the first biramous chaetiger, teeth facing directly anteriorly have 0°, then increasing as the teeth rotate ventrally. Measured in the middle between both front and back end of the neuropodia, as well as in the middle between the dorsal and ventral end.
9. Teeth length relative to each other (identical or not)
10. Shape of the slit between the teeths from apical view
11. Teeth position relative to each other (whether the line made between the two tips are vertical, oblique or horizontal when the hook are viewed from the front and standing vertically)
12. Angle between the stem of the hook and the underside of the teeth (distance A in fig. 2A in (Koh et al. 2003))
13. Teeth protrusion from the stem
14. Teeth protrusion relative to the back side of the hook
15. Size of the shoulder (fig. 2A in (Koh et al. 2003))
16. Shape of the top front edge of the shaft

17. Distance between the front edge of the shoulder and the teeth (distance B in fig. 2A in (Koh et al. 2003))

Tube

18. Particle material, the shape and type (what it is made of) of particles
19. Tube lumen diameter, relative to the tube diameter. This is estimated by assuming the lumen diameter is equal to the diameter of the specimen.

2.4.2 Methyl blue staining

Specimens were stained for 3 minutes in methyl blue before they were removed and rinsed two times in 80% ethanol. Samples were analyzed and photographed instantly after rinsing.

2.5 Figures

All figures, unless stated otherwise, was edited in Adobe Photoshop CS4 v11.0 or Adobe Photoshop Ligthroom v3.6.

3 Results

3.1 Molecular results

Out of 64 samples from 40 different specimens 21 successful COI contigs from 20 different specimens was obtained (32.8 % successful sequences) (Table 3.1), 19 from both directions, in 1 sequence only one direction is used. Of 24 different samples from 24 specimens 24 ITS1 contigs were obtained (100 % successful sequences) (Table 3.1**)**, 22 from both directions, and 2 sequences were made from only one direction, showing a high difference in success rate between the two genes.

Table 3.1. The table shows all samples which were attempted to sequence. The number corresponds to how many samples was sent for sequencing for each specimen, red background indicates no successful sequences and green background indicates at least one successful sequence.

Sample / Gene	TH004	TH005	TH007	TH008	TH009	TH013	TH018	TH022	TH026	TH030	TH034	TH035	TH038	TH039	TH044	TH053	TH056	TH059	TH062	TH063	→	
COI	2		3	4	1	3	1	1	2	2	4	5	4	1	1	1	1	1	2	1		
ITS1		1	1	1		1		1	1		1	1	1	1		1	1	1	1			
COI+ITS1								1								1	1	1	1			
→	TH073	TH074	TH075	TH076	TH077	TH078	TH079	TH086	TH087	TH088	TH089	TH090	TH092	TH093	TH094	TH095	TH096	TH097	TH099	TH100	Samples	Sequences
	1	1*	1	1	1	1	1	1	1	3	1	1	1	1	1	1	1	1	2	2	**64**	**20**
	1		1	1					1	1*		1			1	1		1*	1		**24**	**24**
	1		1	1					1			1			1	1		1			**13**	**13**

3.1.1 Alignments and best fit models

The alignment of the COI dataset was unproblematic with no gaps within any sequences, and had 41.5% variable sites and 30.4% parsimony informative sites. The Hasegawa Kishino Yano (HKY) (Hasegawa et al. 1985) model with both gamma distribution and invariant sites came out as best fit model. In ITS1, the 18S section in the front and the 5.8S section in the end are conserved enough for proper alignment (Fig. 3.1A and D), while only small sections within ITS1 align in a credible fashion (Fig. 3.1B and C), indicating a very high mutation rate in ITS1. This is illustrated with the GCGATGGTTTAAA group highlighted in Fig. 3.1C, where the group are shifted right in two sequences even though it clearly align better if aligned together with the group in the rest of the sequences. 11 different ITS1 alignments was made, in which the length of the alignment varied from 970 pb to 373 bp with an average of 674 (Tab. 3.2). The average number of conserved sites are 373 (54% of sites) with 604 (62%) being the highest value and 201 (45%) the lowest. It is important to point out that the highest/lowest value and percentage not necessarily is in the same alignment. There are in average 290 (45%) variable sites with 401 (55%) as high value and 154 (36%) as low. An average of 33 (5%) singleton sites ranging from 96 (10%) to 14 (3%). On average there are 256 (40%) parsimony informative sites in each alignment, with 363 (50%) as high value and 140 (32%) as low. The find best model test resultet in Tamura 3-parameter (T92) (Tamura 1992) with gamma distribution for all ITS1 alignments, plus six of the alignmets should also have invariant sites.

The best model for the COI and ITS1 combined alignments was Generalised time-reversible (GTR) (Tavare 1986) with both gamma distribution and invariant sites for COI+ITS1_MAFFT and COI+ITS1_MAFFT_reduced, and Tamura Nei-model (TN93) (Tamura et al. 1993) with invariant sites for COI+ITS1_MAFFT_allCOI+ITS1 (see table 3.2).

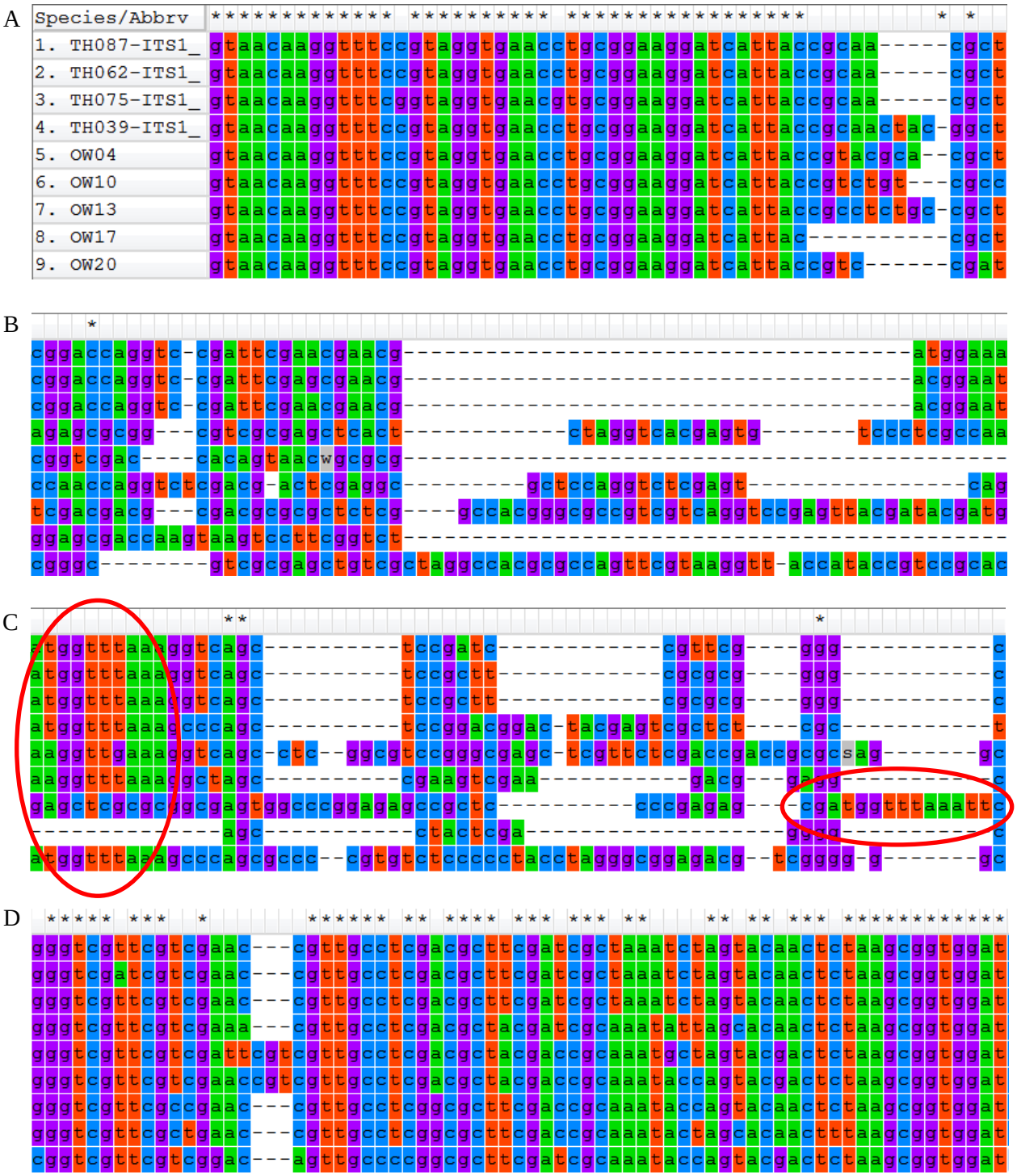

Figure 3.1. Sections of ITS1 alignment ITS1_MAFFT. The alignment is reduced so all clades are represented with only one sequence, conserved sites are marked with a * above the site. A, sequence names and part of 18S in front of ITS1 (note that most sites are conserved); B, typical section of ITS1 (only 1 out of 70 bp are conserved; C, another section of ITS1, highlighted sections illustrates some of the problems with highly variable sequences; D, part of 5.8S after ITS1

3.1.2 Phylogenetic analysis

A total of 12 genetically distinct groups were found in the different phylogenetic analysis excluding the outgroup, 8 in the COI analysis and 9 in the ITS1 analysis, 5 of the groups are found in both genes. 3 groups are represented by singletons. Only two groups are represented in Norwegian waters, one group mainly from Svalbard and the Barents Sea plus one sequence from *Bergen* and one from Germany (group 1), the other group are found from the Trondheimsfjorden area, the area around Bergen and further down to the English Channel and the northern part of Biscaya bay plus one sequence from Portugal (group 2). An additional 2 groups are found in the Atlantic, one group from the English Channel, the Biscaya bay and Portugal (group 3), the other from the English Channel and the Bristol channel (group4). Two of the groups, group 2 and group 3, are pressent in both COI and ITS1 analysis (Fig. 3.2 and 3.3 (bootstrap values from both ML and MP and posterior probability from BI are shown in the figures when a node is identical in the two other trees, if not it is indicated by a "-")), while group1 is only present in the ITS1 dataset (Fig. 3.3), and group4 is only present in the COI dataset (Fig. 3.2). This is due to the COI sequencing was completely unsuccsessful for samples belonging to group 1, and only COI sequences and no ITS1 sequences was available for download from GenBank. There are two groups found in the Mediterranian Sea, one represented in COI sequences from Banyuls Bay (ESP) and in both COI and ITS1 sequences from Naples (ITA) (group 5) (Fig. 3.2 and 3.3) and one group represented with a COI singleton from Banyuls Bay (group 6) (Fig. 3.2). Additional 5 groups are present in specimens from 6 different locations in Australia (Fig. 2.1), 2 groups present in both COI and ITS1 datasets (group 7 and 8) (Fig. 3.2 and 3.3) while 3 groups are only present in the ITS1 dataset (group 9, 10 and 11) (Fig. 3.3). The last group is a singleton from Canada, present in the COI dataset (group 12) (Fig. 3.2).

Alignment		model	Gamma distribution	Invariant sites	Length of alignment	Conserved sites		Variable sites		Singleton sites		Parsimony informative sites		Fractions of bases T	C	A	G	Min. sequence	Max. per sequence
COI_MAFFT		HKY	x	x	506	296 (337)		210 (196)		56 (20)		154 (149)		41	15	20	24	503	3*
ITS1_ClustalW		T92	x	x	839	442		390		27		363		20	30	19	32	551	288
ITS1_ClustalW_ gblocks		T92	x	x	479	216		263		22		241		20	30	18	33	435	44
ITS1_MAFFT		T92	x	x	904	517		374		31		342		20	30	19	32	551	353
ITS1_MAFFT_gblocks		T92	x	x	419	201		218		18		200		21	30	18	32	392	27
ITS1_MAFFT_1.0		T92	x	x	970	604		347		35		311		20	30	19	32	551	419
ITS1_MAFFT_1.0_ gblocks		T92	x		373	219		154		14		140		21	30	19	30	345	28
ITS1_MAFFT_ 20pam/k=2		T92	x	x	949	580		354		31		322		20	30	19	32	551	398
ITS1_MAFFT_ 20pam/k=2_gblocks		T92	x		422	226		196		14		182		21	30	19	31	382	40
ITS1_MUSCLE		T92	x		947	505		401		96		304		20	30	19	32	557	390
ITS1_MUSCLE_ gblocks		T92	x		419	218		201		43		158		20	29	19	32	381	38
ITS1_manual		T92	x		688	498		134		25		109		21	29	19	32	388	300
COI+ITS1_MAFFT		GTR	x	x	1587	911 (694)		645 (592)		104 (58)		540 (533)		33	20	20	27	720	867
COI+ITS1_MAFFT_ reduced		GTR	x	x	1587	913 (967)		643 (589)		109 (62)		533 (526)		31	22	20	28	787	800
COI+ITS1_MAFFT_ allCOI+ITS1		TN93		x	1433	1031		401		17		383		32	21	19	28	1147	286
Alignment sets							%		%		%		%						
All ITS1 align w/o manual	Average				672	373	54	290	45	33	5	256	40	20	29	19	32	462	212
	High				970	604	62	401	55	96	10	363	50					557	419
	Low				373	201	45	154	36	14	3	140	32					345	27
ITS1 align. w/o gblocks and manual	Average				922	530	57	373	41	44	5	328	36	20	30	19	32	552	370
	High				970	604	62	401	46	96	10	363	43					557	419
	Low				839	442	53	347	36	27	3	304	32					551	288
ITS1 align. with gblocks, w/o manual	Average				422	216	51	206	49	22	5	184	43	20	30	18	32	387	36
	High				479	226	59	263	55	43	10	241	50					435	44
	Low				373	201	45	154	41	14	3	140	38					345	27

Table 3.2 (p23). The table shows the best substitution model (HKY: Hasegawa Kishino Yano; T92: Tamura 3-parameter; GTR: Generalized time-reversible; TN93: Tamura Nei-model) for all the different alignments and whether gamma distribution and invariant sites should be included or not. The table also shows number of total sites, conserved sites, variable sites, singleton sites, parsimony informative sites (all numbers in parentheses are without the outgroup, in alignments were parentheses are not present, there are no outgroup), fractions of the different bases, average length of sequences and average number of gaps pr. sequence in all the alignments, as well as all values ITS1 alignments all together and ITS1 alignments with and without gblocks the two latter also without the manual alignment. In the alignment sets the table also show percentage of conserved sites, variable sites, singletons and parsimony informative sites (the max/min value and percent in the same column do not necessarily come from the same alignment) *: gaps are in the beginning of two sequences, no gaps are found within any sequence.

3.1.3 COI analysis

A total of 8 groups were identified in the the COI analysis. Maximum Likelihood (lnL = -2786.86), two out of two Maximum Parsimony (tree length = 503) and Bayesian Inference (mean lnL = -2858.24) all resulted in congruent trees, specially on lower (species) level taxa with good support for the highligthed groups. The ML tree (Fig. 3.2) clearly shows group 2, 3, 5 and 6 as separate groups with good support in all three analyses. They also have a more recent ancestor than the rest of the groups, even though there are less support for this statement. Group 4, 7, 8 and 12 have a common recent ancestor although this are weakly supported, and exactly this grouping do only occur in the ML-tree.

Both MP trees had the same topology except minor differences within the main group. For this reason only one MP with calculated branchlengths are included (Fig. A1). The differences between the ML tree in Fig. 3.2 and the MP tree (Fig. A1) is that in the MP-tree group 1-11 has a more recent common ancestor than if including group 12 (CAN), rather than group 12 having a more recent common ancestor with group 7 (AUS) relative to the the rest of the groups as in the ML tree. The BI tree (Fig. A2) is exactly identical to the ML-tree except node A in Fig. 3.2 is collapsed.

3.1.4 ITS1 analysis

A total of 9 groups were identified in the ITS1 analysis. Analyses resulted in 26 pylogenetic trees, 20 ML-trees, 4 MP-trees and 2 BI-trees. Where possible, the trees are rooted on the Australian groups based on the results in the COI analysis.

3.1.4.1 ML vs. MP vs. BI

The 10 analyses on ITS1_MAFFT and ITS1_MAFFT_gblocks resulted in 4 Maximum Likelihood trees (ITS1_MAFFT, 25% gap-deletion: lnL = -3073.77, 50% gap-deletion: lnL = -2806.90; ITS1_MAFFT_gblocks, 25% gap-deletion: lnL = -2482.75, 50% gap-deletion: lnL = -2431.51), 4 Maximum Parsimony trees (ITS1_MAFFT, 25% gap-deletion: 7 trees, tree length = 548, 50% gap-deletion: 6 trees, tree length = 600; ITS1_MAFFT_gblocks, 25% gap-deletion: 10 trees, tree length = 463, 50% gap-deletion: 10 trees, tree length = 464) and 2 Bayesian Inference trees (ITS1_MAFFT: mean lnL = -4104.28 (Fig. AX); ITS1_MAFFT_gblocks: mean lnL = -2537.95 All 10 (39 if the MP trees are treated as separate) gave identical topolgies except minor differences within the main groups. The ML tree (Fig. 3.3) clearly shows the different groups well supported, although the support weakens further down the tree.

3.1.4.2 Different alignments

In 15 out of 20 (75%) different Maximum Likelihood trees all European and Australian groups separate in two different main groups, if the the Australian sequences (group 7 – 11) are regarded as one group. In 7 of the 15 groups (47%) the topology is identical to Fig. 3.3 (all ITS1_MAFFT alignments; ITS1_MAFFT_20pam/k=2, 25% gap-deletion: lnL = -2428.62, 50% gap-deletion: lnL = -2873.15; ITS1_MAFFT_20pam/k=2_gblocks, 50% gap-deletion: lnL = -2242.69) although the bootstrap support varies from 37 to 90. In 6 of the remaining trees (ITS1_MAFFT_1.0_gblocks, 25% gap-deletion: lnL = -1736.68, 50% gap-deletion: lnL = -1843.41; ITS1_MUSCLE, 25% gap-deletion: lnL = -2581.37; ITS1_MUSCLE_gblocks, 25% gap-deletion: lnL = -2124.59; ITS1_ClustalW, 25% gap-deletion: lnL = -2683.98, 50% gap-deletion: lnL = -3137.95) 4 addional topologies within the

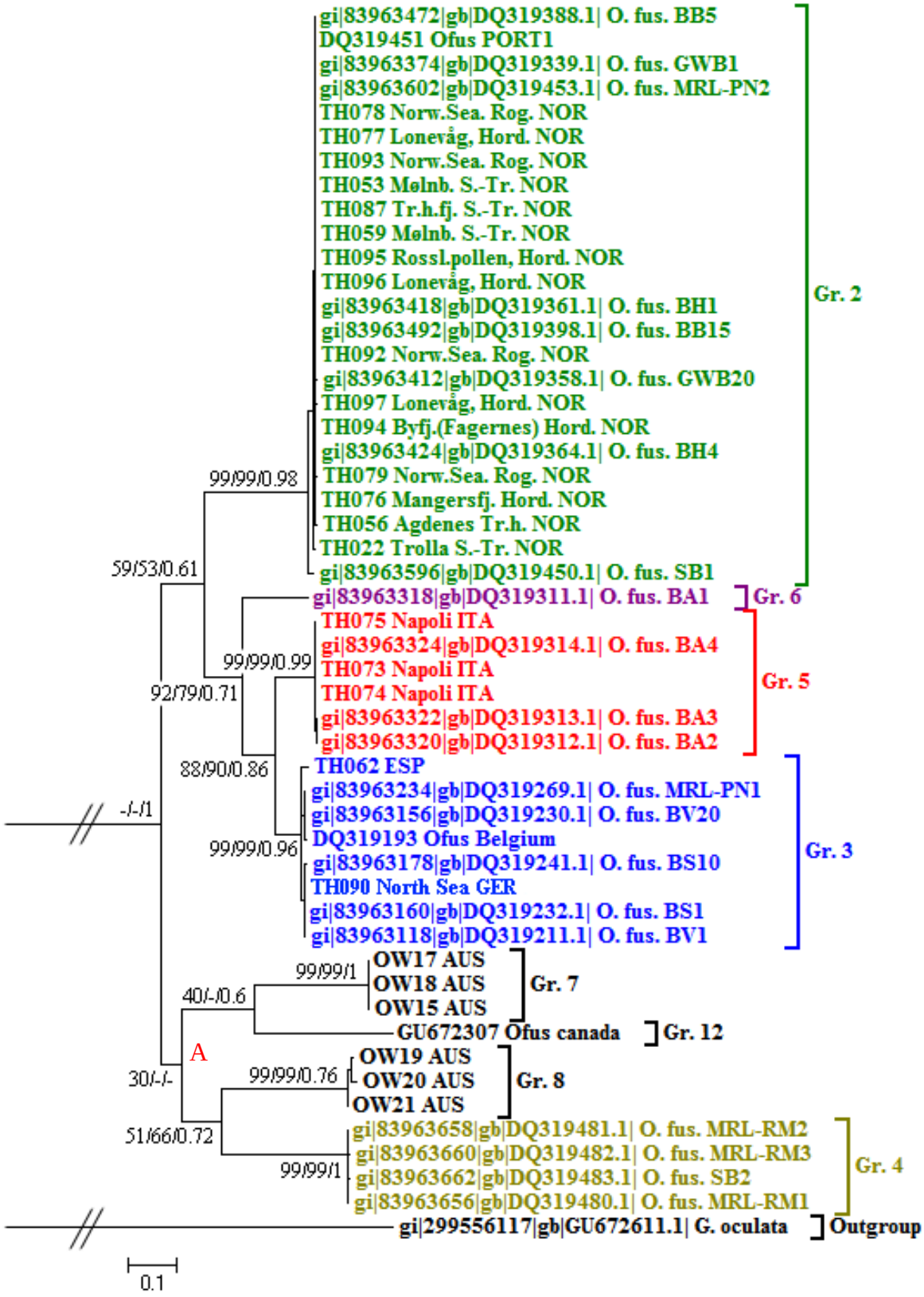

Figure 3.2. Phylogenetic tree from MEGA made with Maximum Likelihood from the COI dataset, lnL = -2786.86. The node values are bootstrap value from ML and the corresponding MP tree and posterior likelihood from the corresponding BI, respectively. If a node doesn't exist or has a bootstrap value in the MP or BI tree it's representet by a "-".

Australian main group are observed, with bootstrap support from 28 to 88. In 2 of the remaining trees (ITS1_ClustalW_gblocks, 25% gap-deletion: lnL = -2401.83, 50% gap-deletion: lnL = -3137.95) group 3 and 5 are clearly separate groups, but they are within group 2, e.g group 2 does not come out as a group different from group 3 and 5 (Fig. A3).

In the last 5 trees the European groups are not monophyletic. 1 or more of the Australian groups fall in together with the European groups, but in four of the trees (ITS1_MAFFT_1.0, 25% gap-deletion: lnL = -2352.16, 50% gap-deletion: lnL = -2788.70; ITS1_MUSCLE, 50% gap-deletion: lnL = -3125.62; ITS1_MUSCLE_gblocks, 50% gap-deletion: lnL = -2360.56) the bootstrap support are very low, ranging from 3 to 35, while in the last tree (ITS1_MAFFT_20pam/k=2, 25% gap-deletion: lnL = -2428.62) it is 71 (Fig. A4).

3.1.4.3 Gblocks

As can be seen in Table 3.2, Gblocks reduce the length of the alignments with more than 50% percent on average, but while the average number of base pairs per sequence is reduced by 30%, as much as 90% of gaps are removed. The number of conserved sites, variable site, singletons and parsimony informative sites are also reduced, but the percentage of parsimony informative sites increase on average with 7 %.

In 5 out of 10 alignments (ITS1_MAFFT 50% and 25% gap-deletion, ITS1_MAFFT_20pam/k=2 50% gap-deletion and ITS1_MUSCLE 50% and 25% gap-deletion) there was no difference in topology between the analyses of alignment without gblocks compared to the analyses of the respective alignments with gblocks.

In 2 alignments (ITS1_ClustalW, 25% gap-deletion and 50% gap-deletion) the European and Australian groups result in two separate main groups without gblocks, while group 3 and 5 are clearly separate groups, but they are within group 2 (Fig. A3) in the trees from both alignments where gblocks is applied.

In 2 of the remaining 3 alignments (ITS1_MAFFT_1.0, 25% gap-deletion and 50% gap-deletion) the alignments where gblocks is applied result in the European and the Australian samples dividing in two separate main groups, while in the trees without gblocks two Australian groups (9 and 10) share a more recent common ancestor with group 2, 3 and 5 than the rest of the groups, although bootstrap support are very weak (3 and 4).

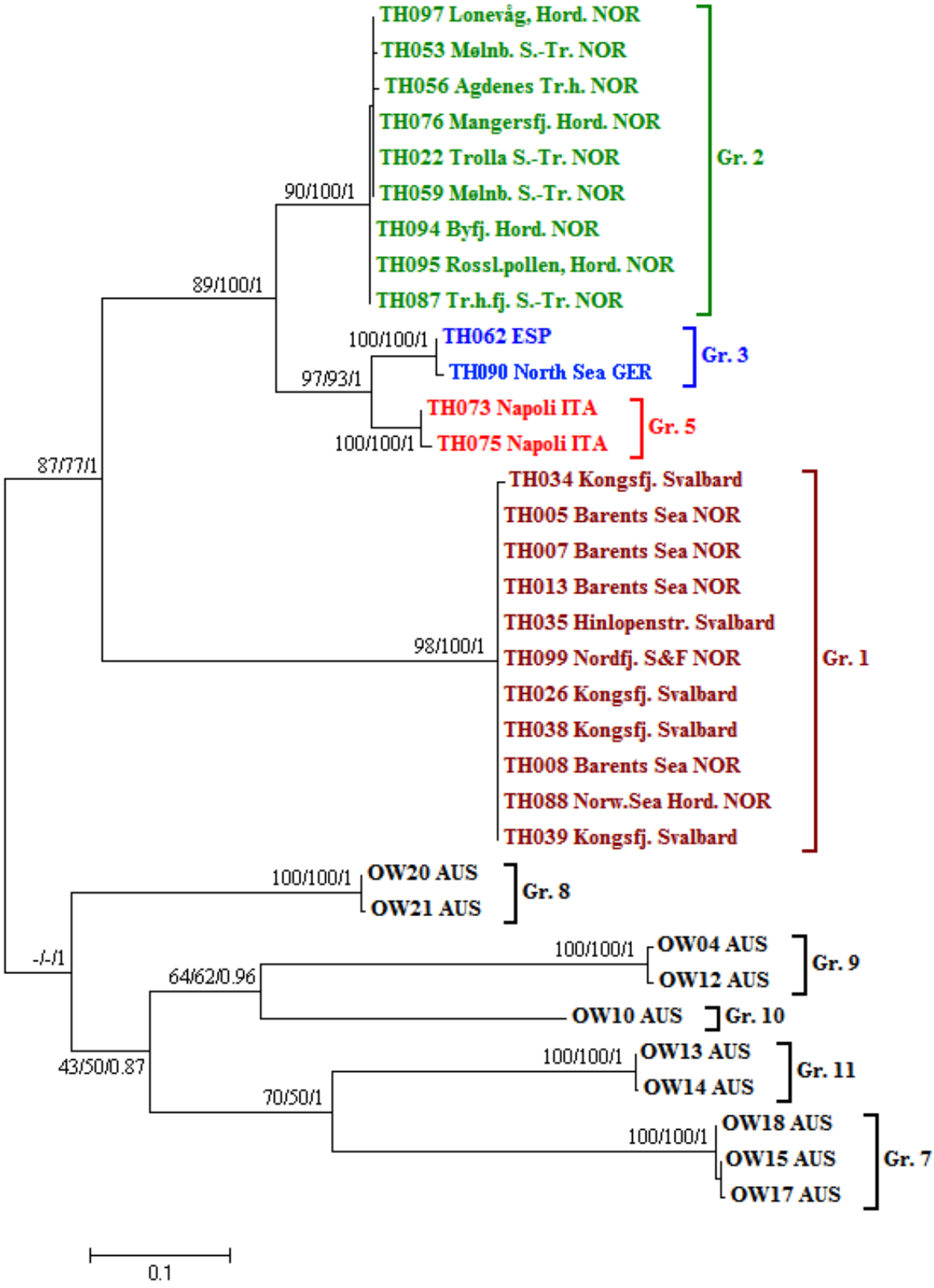

Figure 3.3. Phylogenetic tree from MEGA made with Maximum Likelihood from the ITS1_MAFFT (50% gap-deletion) alignment, lnL = -2806.90. Node values are average bootstrap value from the 4 ML and 4 MP trees and average posterior likelihood from the 2 BI, respectively. If a node doesn't exist or has a bootstrap value in the MP or BI tree it's representet by a "-".

In the last alignment (ITS1_MAFFT_20pam/k=2, 25% gap-deletion) the alignment where gblocks is applied result in group 8 being the closest group to group 1 with a bootstrap support of 32, a boostrap support of 71 support group 8 within the European main group. Without gblocks the European and the Australian samples dividing in two separate main groups.

3.1.4.4 25% vs. 50% gap-deletion

In 7 out of 10 different alignments (ITS1_MAFFT w/o and w/ gblocks, ITS1_MAFFT_1.0 w/o and w/ gblocks, ITS1_MAFFT_20pam/k=2 w/o gblocks and ITS1_ClustalW w/o and w/ gblocks) topologies were identical whether gblocks was used or not.

In 2 alignments (ITS1_MUSCLE w/o and w/ gblocks) group 9 and 10 as one group was a sistergroup to group 1 in both analyses with 50% gap-deletion, while there was two distinct European and Australian groups in both analyses with 25% gap-deletion.

In 1 alignment (ITS1_MAFFT_20pam/k=2 w/ gblocks) the distinct European and Australian groups were present in the the 50% gap-deletion analysis, while group 8 was a sistergroup to group 1 in the 25% gap-deletion analysis.

3.1.4.5 Manual alignment

The manual alignment results in 2 identical trees with both 25% (lnL = -952.90) and 50% (lnL = -1088.09) gap-deletion. The topology in the trees are identical to Fig. 3.3, the only difference being shorter branchlengths (Fig. A5).

3.1.5 COI+ITS1

The COI+ITS1_MAFFT and COI+ITS1_MAFFT_reduced alignments were not analyzed due do to very high portions of gaps in the two alignments (both >50% gaps).

Analyses of the COI+ITS1_MAFFT_allCOI+ITS1 resulted in 5 identical trees (ML: 25% gap-deletion: lnL = -3937.18, 50% gap-deletion: lnL = -4246.02; MP: 25% gap-deletion: 9 trees, tree length = 572, 50% gap-deletion: 3 trees, tree length = 545; BI: mean lnL = -

4641.73), with strong bootstrap support and posterior probability (Fig. 3.4), supporting rooting the ITS1 trees on the Australian group.

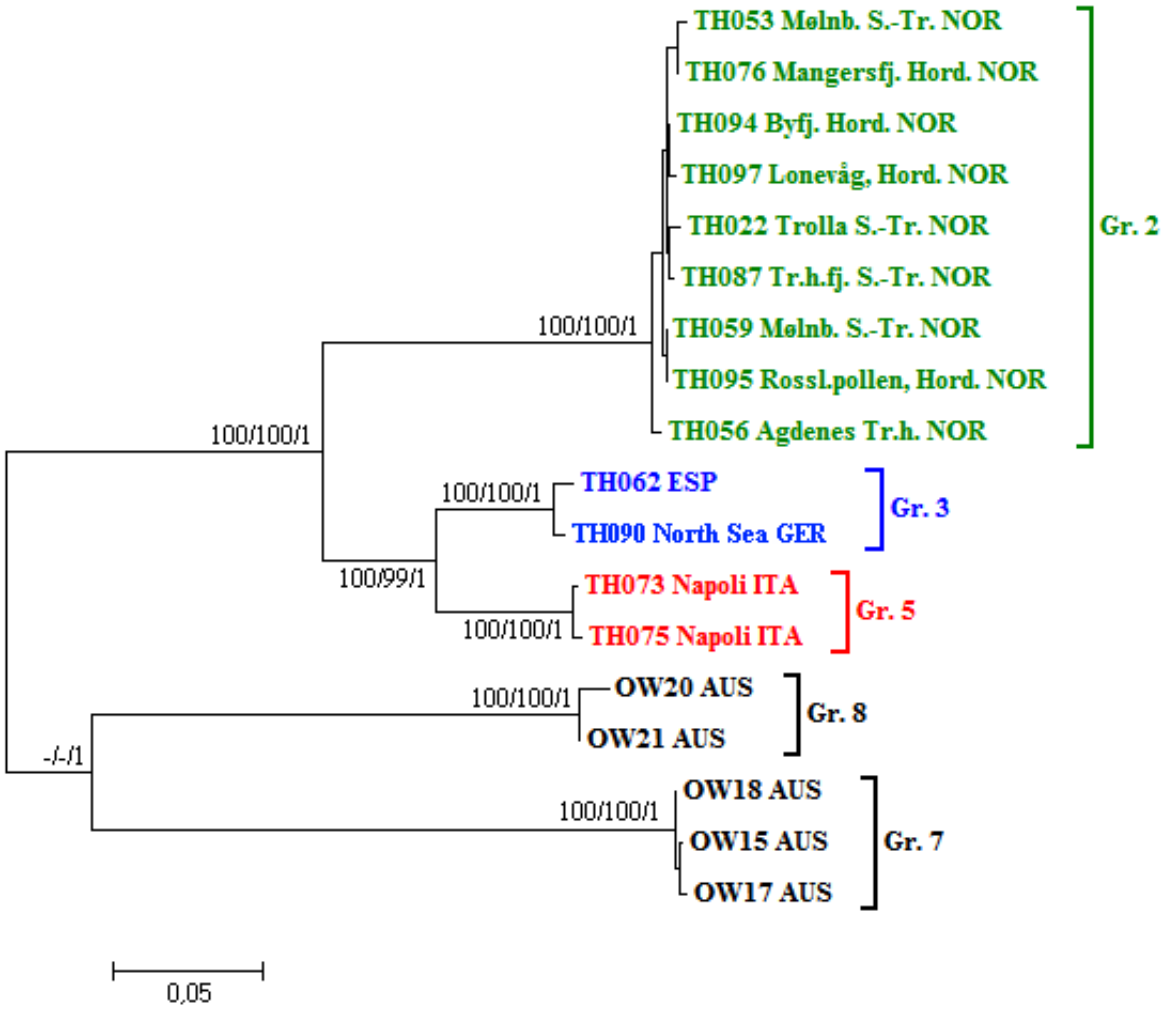

Figure 3.4. Phylogenetic tree from MEGA made with Maximum Likelihood from the COI+ITS1_MAFFT_allCOI+ITS1 (75% gap-deletion) alignment, lnL = -3937.18. Node values are average bootstrap values from the 2 ML and 2 MP trees and average posterior likelihood from the BI tree, respectively.

3.2 Morphological analysis

To distinguish between *Owenia* species based on morphological features proves to be very difficult. Only soft features are big enough to be vissible without SEM and only a few characters are possible to use for identification, and these are mostly continous charaters leaving it up to the researcher to decide the borders between the different states. With the use of staining pattern and the macrocharacters at least two different groups were possible to distinguish. One corresponding to group 1 in the molecular analyses, and the other corresponding to group 2, and possibly also group 3 and 5. The characters observed with SEM proves to be highly variable, not showing separate character states in the different groups. Due to these difficulties, descriptions such as each species are not given, but rather an evaluation of the characters used by Koh et al. (2003) when discribing the species.

3.2.1 Character sets

The characters used are divided in four sets. Macrocharaters are characters big enough to be observed in a normal light microscope. Microcharacters are smaller characters, in which a high magnification microscope like SEM are required for observation. The metyl blue staining pattern is regarded as a character set and the last set of characters are features on the tube.

3.2.1.1 Macrocharacters

Only six different macrocharacters are investigated in this study, character 1-6 as defined in 2.4.1. They are all features on soft bodyparts, meaning they may be susceptible for fixation method, and may be affected by the stress level in the specimens prior to fixation. Except for the number of dichotomies, all macrocharacters are continous characters, meaning no natural limits between states are present, leaving it to the researcher to deside the different states. Nevertheless, two main groups were identified based on macrocharacters in combination with methyl blue staining pattern.

3.2.1.2 Microcharacters

A total of 18 specimens were investigated with the use of Scanning Electron Microscopy (Table A3), and an additional 15 speciemens were dried and coated, but due to a power blackout resulting in the SEM breaking down, no imges were obtained from those samples.

In total 17 different characters were analyzed and measured on the SEM samples; the six characters examined as macrocharacters plus an additional 11 characters: characters 7-17 as defined in 2.4.1. A great amount of variation were registered, both within samples (Fig. 3.5A), locations and groups.

Obtaining good SEM results require very clean specimens. Specimens used in this study was rinsed in Zalo water and brushed with a tiny brush before drying, but there was still large amount of mucus in several of the samples (Fig. 3.5E). It is also important to use clean water and ethanol to prevent small dust particles from sticking to the specimen, especially the chaeta and uncini. Another observation done in this study is that samples should be dried prior to drying. This will reveal eventual remains of the tube on the animal, which can be virtually impossible to see through a light microscope, but will cover the surface of the specimen where present (Fig. 3.5F).

3.2.1.3 Methyl blue staining

Two distinctly different patterns are described. The staining pattern vary, especially the intensity of the staining, resulting in the groups not necessarily being simple to distinguish from one another even though the patterns are clearly different. Variations in the patterns may also be an issue. A possibly distinct pattern for *O. fusiformis* and *Owenia* sp. A (group 5 and 3, respectively, in section 4.1) are are also described, but the differences described are somewhat uncertain due to very similar patterns and the descriptions being based on very few specimens.

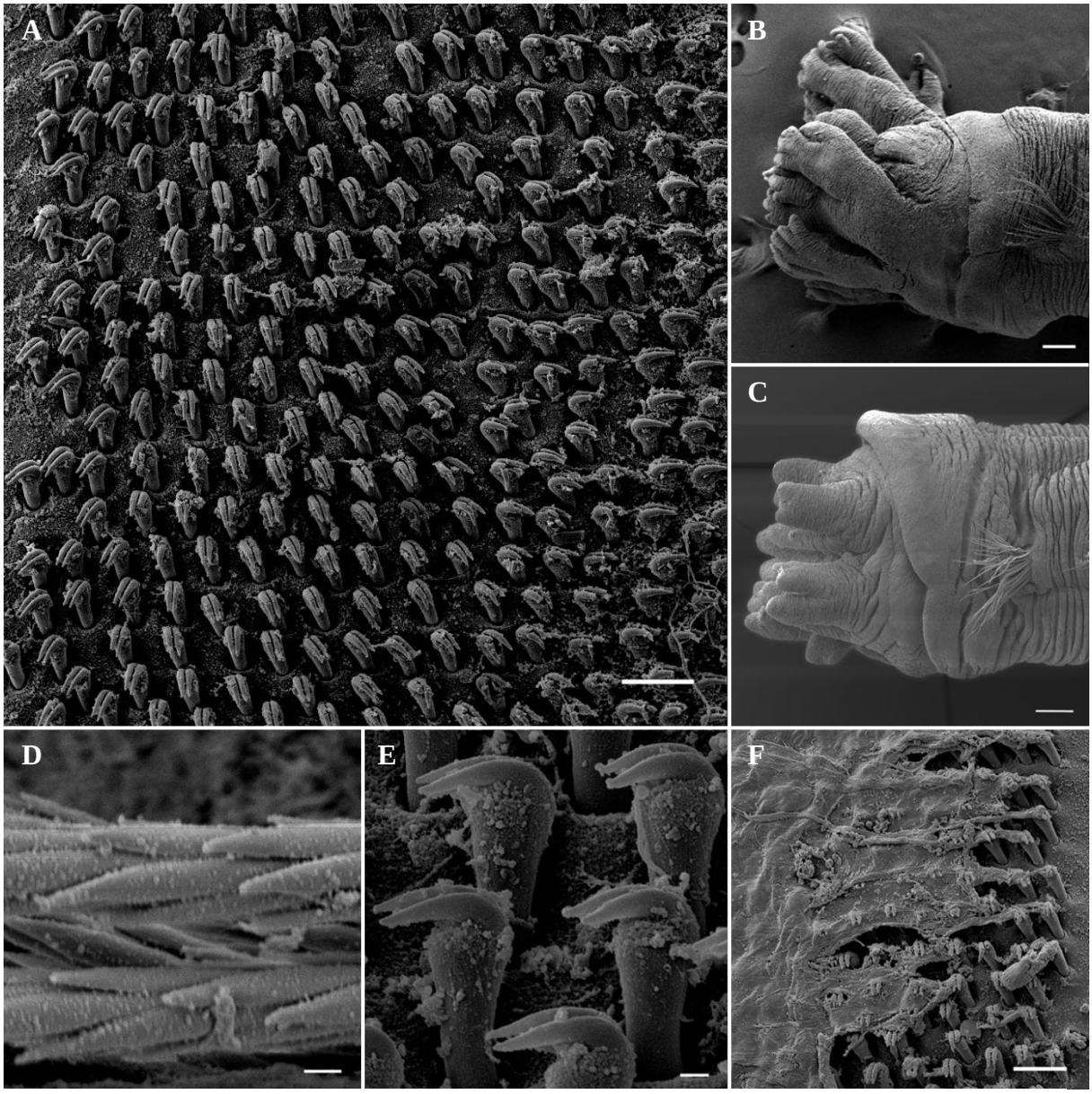

Figure 3.5. The figure illustrates different aspects of the SEM imaging. A: large variation in character state; B: thin, sharp collar; C: thick, rounded collar; D: varying characters; E: mucus due to improper cleaning; F: residual part of the tube covering the hooks on the neuropodia. Scale bars: A, F: 100 μm; B, C: 10 μm; D, E: 1 μm.

3.2.1.4 Tube

The particles which are embedded in the tubes can be categorized in four different groups with two different variables: the material of which the particles is made of and the shape of the particles (Fig. 3.6). The results indicate that the different groups have preferences in selecting which particles to use during tube construction, but in some cases specimens are forced to use what is in the sediment even though it is not the prefered particles.

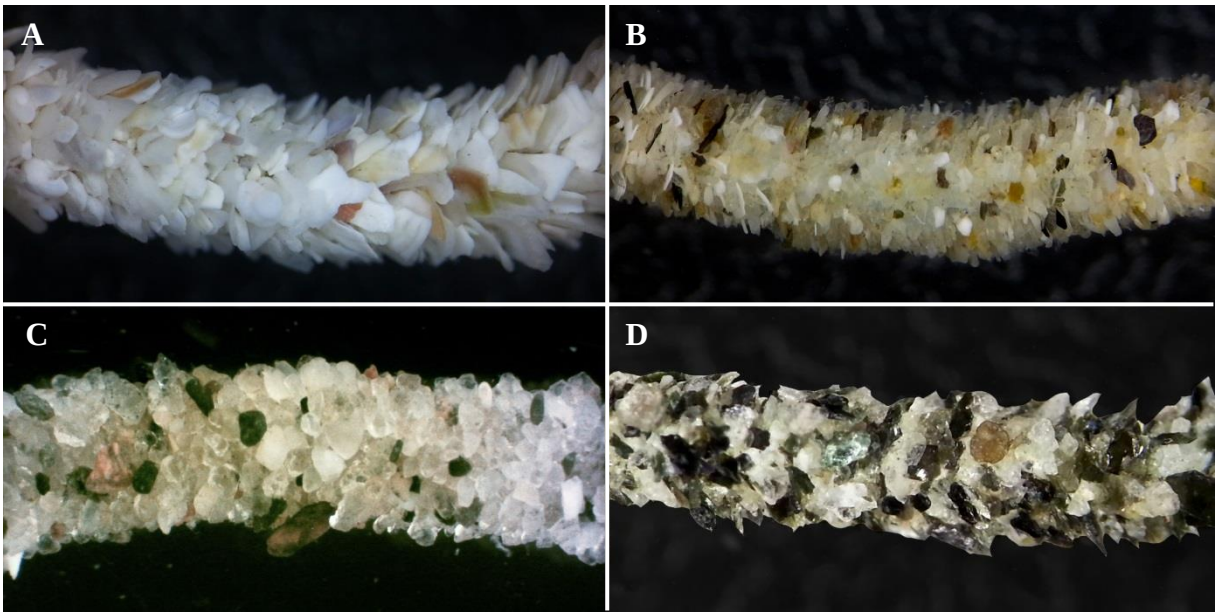

Figure 3.6. The figure illustrates different sediment particle use in the tubes. A: Only flat calcareous partiles; B: a mix between round mineral and flat calcareous particles; C: only round mineral particles; D: flat mineral particles particles

3.2.2 *Owenia assimilis* (Sars, 1851)

Material examined

From a total of 40 type specimens 6 speciemens were selected for staining and measurement of macrocharacters. Specimens are collected at three different locations (Table A1 and A2).

Macrocharacters

Specimens are characterized by tentacle crown diameter which are slightly smaller than the diameter of the thorax, the branchiae length relative to the length of the thorax ranges from 0.22 to 0.38 and the branchiae have from 2 to at least 4 dicotomies from the base to the tip. The collar are thin, and appear to be slightly rounded. Collar angle are measured on only two specimens, being 150° and oblique.

Methyl blue staining pattern

The methyl blue staining pattern are highly similar to the staining pattern of *O. borealis* (described in detail below), with one specimen showing some differences on the ventral "V" shaped pattern.

Tube

The tubes from 4 of the 6 specimens were examined. None of the samples had the whole tube, resulting in no maximum length measurement being possible. Diameter of the tubes vary from 2.1 mm to 3.4 mm, with the lumen diameter relative to the total diameter of the tube being from 0.27 to 0.43. All tubes had calcareous flat particles in them making a overlapping, roof tile-like pattern, with no observable mineral particles (see Fig. 3.6A).

Remarks

This is type specimens described in Sars (1851). Samples are collected at three different localities, one widely separated from the other two. Due to the condition of the specimens the six specimens which were most suitable for measurements were selected.

There are evidence suggesting that the type material consists of more than one species, but further analyses are required to solve this problem.

3.2.3 *Owenia borealis* Koh, Jirkov & Bhaud, 2003

Material examined

Material examined in detail according to table A1, A2 and A3.

Macrocharacters

The relative diameter of the tentacle crown are close to, or equal to, the diameter of the thorax, and the collar appears to be very thin with a sharp edge lying close to the base of the tentacle crown (Fig. 3.5B). The specimens have predominantly dichotomies from approximately the middle of the branchiae and towards the tips, although several specimens have dichotomies at the base as well. The number of dichotomies varies from 1 to at least 4. The colar angle ranges from 140° to 170°, with one specimen having a perpendicular collar with no angle between the collar above and below the notch.

Microcharacters

Due to the SEM breakdown, microcharacters from only 6 specimens from this group were examined. In 5 specimens the ratio of the free part of the scales on the chaetae to the total scale length varies from 0.40 to 0.50, although one specimen shows variation from 0.37 to 0.70 (Fig. 3.5D). The angle of teeth on the uncini compared to the length axis of the specimens shows a high degree of variability, ranging from 10° to 120° (Fig. 3.5A), with one specimen having a difference of 120° from hooks on the anterior end to hooks on the posterior end of the neuropodia. In all specimens the length of the teeth on the hooks are the same, relative to each other, and in 5 specimens the slit between them widens, starting at the outer 1/3 to ½ of the teeth, in one specimen, the slit has parallel edges, being considerable wider than the inner part of the rest of the specimens. From frontal view the tips of the teeth are oblique relative to each other in all specimens. The angle between the frontal edge of the hook stem and the underside of the teeth varies from 75° to 85°. The protrusion of the teeth varies from 2.00 μm to 2.85 μm, and the protrusion relative to the back side of the hook, varies from 0.38 to 0.46. The shoulder on the hooks ranges from barely visible to covering 2/3 of the stem, and the front of the stem are straight in all but one specimen, were a slight curve is noticeable. The height between the shoulder and the underside of the teeth varies between 0.73 μm and 1.15 μm.

Methyl blue staining pattern

The pattern (Fig. 3.7) is characterized by continous blue on the collar from notch to notch, via the dorsal side. At the notch, a blue line along both edges of a slit going from the notch in the posterior direction of the specimen. The lower line gradually fades away as it goes ventral (Fig. 3.7A), and it also has a larger blue area on the lower side with no clear edge, fading away in all directions. The upper line goes all the way back to the notopodia in u1, via the chaeta on b1, b2 and b3. On the ventral side of the specimen V/U-combined pattern is clearly vissible with a lighter area below the tips of the arms (Fig. 3.7C), giving the appearance of to dots, although how clear this feature is varies between specimens. Four weak blue lines are also vissible going down the length of the animal, two dorsal and two ventral, the dorsal lines separated more than the ventral lines.

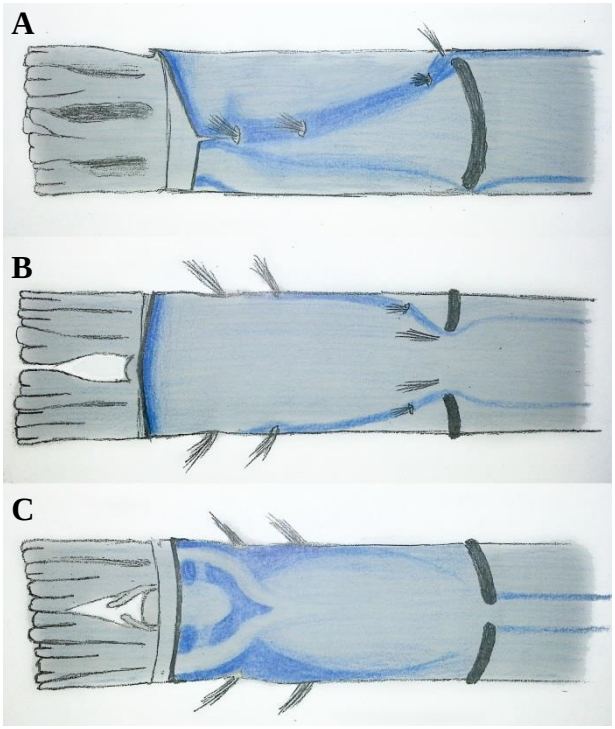

Figure 3.7. Schematic drawing of an *Owenia* colored blue according to observed patterns in *Owenia borealis* after methyl blue staining. A, lateral view; B, dorsal view; C, ventral view.

Tube

The tubes are up to 90.5 mm long, and the particles are mostly flat calcareous particles, but specimens with both flat and round mineral particles, as well as a mixture of the two, are also observed (Fig. 3.6A-D) The flat particles are arranged in the typical roof tile-like pattern. The diameter of the tube varies from 1.3 mm to 3.4 mm, with the lumen being from 37% to 75% of the total diameter.

Remarks

The identification to *O. borealis* is based on the macrocharaters, specifically relative diameter og the tentacle crown and thickness and shape of the collar. The tube characters are also taken into account, although not given much weight in the identifiacation. The rest of the characters used displayes too much variation to be informative when differentiating between the groups and comparing with the descriptions in Koh et al. (2003). This group linked to the methyl blue staining pattern allows for connecting *O. borealis* to group 2 in section 3.1.

Distribution

Accoring to this study and Koh et al. (2003), and when connecting this species to clade 2 in Jolly et al. (2006), the southern distribution limit of *O. borealis* is Portugal, going all the way north to the western part of the Barents Sea, specifically the area around Bjørnøya, Norway. The eastern reporting of the species is Kosterfjorden in Sweden, and the westernmost report of the species is the west coast of Iceland. The depth distribution ranges from 5 meters down to 1350m.

3.2.4 *Owenia fusiformis* delle Chialje, 1841

Material examined

Material from *O. fusiformis* type locality, Naples, Italy, have been examined. The description is based on only four specimens, due to the condition of the samples (Table A1 and A2).

Macrocharacters

The relative diameter of the tentacle crown is equal to the diameter of the thorax, the collar edge is thin (Fig. 3.5B), and the collar is oblique (only measured on one specimen). The relative length of the branchiae varies from 0.27 to 0.54, with 2 or more dichotomies from the brachiae base to the tip. The only feature differing this group from the one above is that the branchaie protrudes further out from thorax dorsally than ventrally, but the branchiae still have about the same length.

Methyl blue staining pattern

The staining pattern is very similar to the *O. borealis* pattern and could easily be mistaken, but some differences do exist. Dorsally, directly behind the collar, there is an area (Fig. 3.8B) with stronger staining, althoug this is is not very clear on all specimens. Two of the specimens also has a dark triangle shaped pattern dorsally between u2 and b1. Between u2 and u3 there might be a double line, compared to the *O. borealis* pattern, where there is a single, thicker line. There may also be line going ventrally from u2 towards the ventral end of the neuropodia on b1. The ventral "V" shaped pattern differs from the one in Fig. 3.7 by having straight lines, rather than curved. The pattern has a darker/more blue apperance than the *O. borealis* pattern.

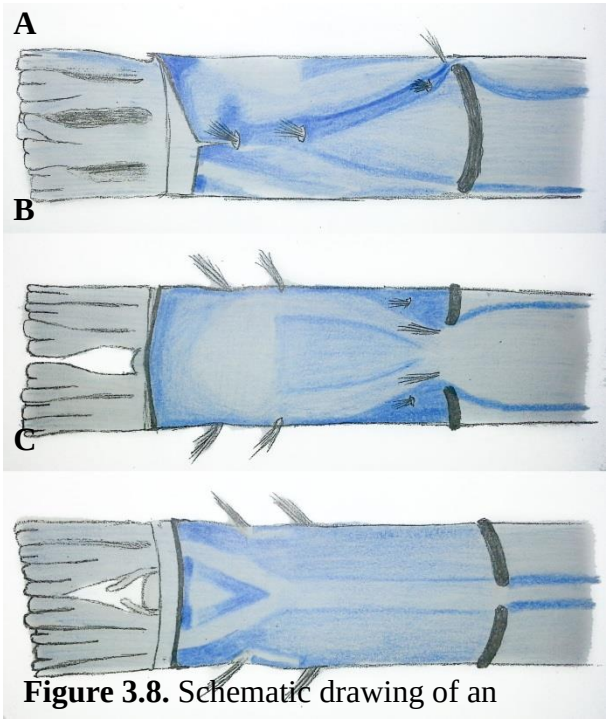

Figure 3.8. Schematic drawing of an *Owenia* with the blue staining pattern illustrated according to observed patterns in *Owenia fusiformis*. A, lateral view; B, dorsal view; C, ventral view.

Tubes

The tubes are made from a mixture of mineral and calcareous particles (Fig. 3.6B) which are both flat and round in shape. No specimens had the tube intact, so no maximum length of the tube was measured. The flat particles are arranged in the roof tile-like pattern, in the same way as the *O. borealis* tubes. The tube diameter varies from 1.9 mm to 3.0, with the lumen making up 37% to 58% of this diameter.

Remarks

No true identification of the specimens have been performed. Koh et al. (2003) do not give a thorough description, and their specimens are collected in Banyuls Bay, a locality in which results in Jolly et al. (2006) indicates more than one species may be present. What is presented in this study merely describes the existence of an *Owenia* group from the type localiy at Naples, which is different from the other *Owenia* groups described in this study. This goup is presented as group 5 in section 3.1.

Distribution

According to this study, and when compared with the sequences from Jolly et al. (2006), *O. fusiformis* seems to be endemic to the Mediterranean Sea. Depth distribution is unknown.

3.2.5 *Owenia polaris* Koh, Jirkov & Bhaud, 2003

Material examined

Material examined in detail according to table A1, A2 and A3.

Macrocharacters

The relative diameter of the thorax are smaller than the diameter of the thorax, as well as the collar being thick and rounded **(**Fig. 3.5C). The diameter of the tentacle crown is considerably less than the diameter of thorax. The relative length of the branchiae of the tentacle crown varies between 0.17 and 0.69. The specimens have predominantly basal dichotomies on the brachiae, but severeal specimens have dichotomies from the base to the tip, the number of dichotomies vary from 1 to 3. The angle the collar forms in the notch varies from 140° to 160°.

Microcharacters

A total of 12 specimens were examined using SEM. The ratio of the free part to total length of the chaeta scales varies from 0.34 to 0.62, with the majority of samples having a ratio around 0.5. In one specimen the ratio ranges from 0.36 to 0.62 (Fig. 3.5D). The angle of teeth on the uncini compared to the length axis of the specimen shows a high degree of variability, ranging from -5° to 170° (Fig. 3.5A), the biggest variation found within one specimen is a difference of 160° from hooks on the front end to hooks on the back end of the neuropodia. In half of the specimens the length of the teeth relative to each other are identical, in the other half the teeth are not the same length. The slit between the teeth widens gradually in all specimens, continually widening in one of the specimens, the rest widens in the outer part of the teeth. From frontal view the tips of the teeth are oblique relative to each other in all specimens. The angle between the frontal edge of the hook stem and the underside of the teeth varies from 60° to 90°. The protrusion of the teeth ranges from 1.60 μm to 2.25 μm, and the protrusion relative to the back side of the hook, varies from 0.33 to 0.42. The shoulder on the hooks ranges from barely visible to covering 3/4 of the stem, and the front of the stem are

straight in all but two specimens, were a slight curve is noticeable. The height between the shoulder and the underside of the teeth varies between 0.42 µm and 1.10 µm.

Methyl blue staining pattern

The pattern observed (Fig. 3.9) is characterized by continous blue along the edge of the collar about ¾ around, missing only on the ventral side. From the notch a thick blue line goes back to the first row of chaetae where a thinner blue line stretches dorsally about halfway up to the middle of the specimen, a smaller weaker line also streches ventrally, but noticable shorter. A thinner weaker line also stretches from the chaetae on u1 to u2 and further to u3. A thin line might be vissible from u3 to the notopodia on b1 as well. On the ventral side there is a characteristic V-shaped pattern with the ends of the "V" widening out almost closing the lighter area inside the "V". Four weak blue lines are also vissible going down the length of the animal, two dorsal and two ventral, the dorsal lines separated more than the ventral lines.

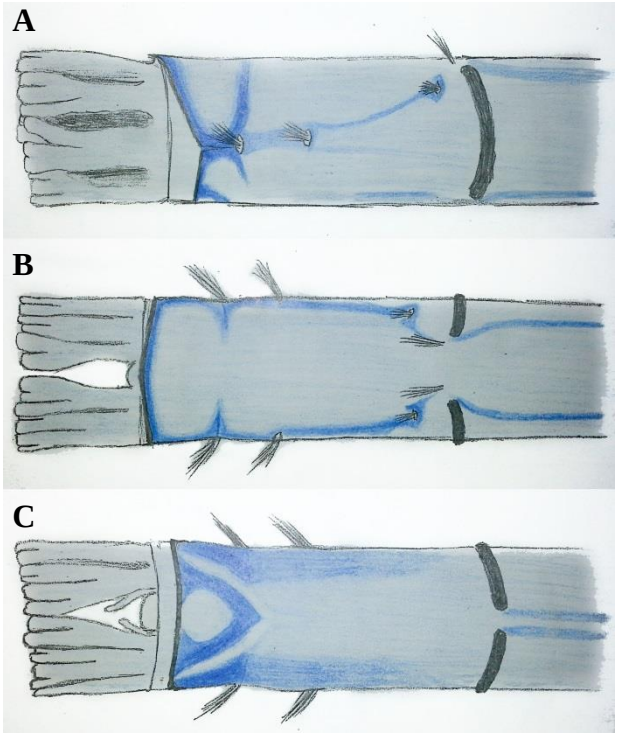

Figure 3.9. Schematic drawing of an *Owenia* colored blue according to observed patterns in *Owenia polaris* after methyl blue staining. A, lateral view; B, dorsal view; C, ventral view.

Tube

The tubes are up to 49.4 mm long, consisting of round mineral particles (Fig. 3.6C). The diameter varies from 0.7 mm to 3.0 mm, with the fraction being the lumen is 0.40 to 0.69.

Remarks

The identification to *O. polaris* is based on the macrocharacters, specifically relative diameter og the tentacle crown and thickness and shape of the collar. The tube characters are also taken into account, although not given much weight in the identifiacation. The rest of the characters used displayes too much variation to compare with the descriptions in Koh et al. (2003) or is identical to *O. borealis*, not being informative when differentiating between the two species. This group linked to the methyl blue staining pattern allows for connecting *O. polaris* to group 1 in section 3.1.

Distribution

According to Koh et al. (2003) and this study *O. polaris* has a southern distribution limit to the Norwegian Sea outside the west coast of Norway, and are rather common in Troms and Finnmark Counties in Norway. The reported easterly distribution limit is according to Koh et al. (2003) Pechora Sea, southeast of the southern tip of Novaja Semlja in Russia. The northernmost reported locality is northwest of the northwestern tip of Spitzbergen, Svalbard. The depth distribution is 12 to 930 meters (Koh et al. 2003).

3.2.6 *Owenia* sp. A

Material examined

Material examined in detail according to table A1 and A2.

Macrocharacters

The diameter of the tentacle crown are identical to the diameter of the thorax, with a thin, sharp collar (Fig. 3.5B). The angle at the notch was not possible to measure, due the condition of the samples. The branchiae length relative to the thorax length is 0.35 and 0.50, and branchiae has 3 or more dichotomies from the base to the tips.

Methyl blue staining pattern

The staining pattern for this group is very smiilar to both *O. borealis* and *O. fusiformis*. They main difference being a darker area dorsally behind the collar (Fig. 3.10A and B), the line between u2 and u3 are thicker, and the ventral "V" shape has more curved edges,

especially compared to the *O. fusiformis* pattern, and the "V" shape is also longer, streching almost down to an imaginary line between the two chaeta on u2.

Tube

Both tubes contains flat, calcareous particles arranged in the roof tile-like pattern (Fig. 3.6A). No samples had the entire tube, so maximum length was not measured. The diameter of the tubes were 3.0 mm and 4.7 mm, with the lumen being 50% and 57% of the total diameter, respectively.

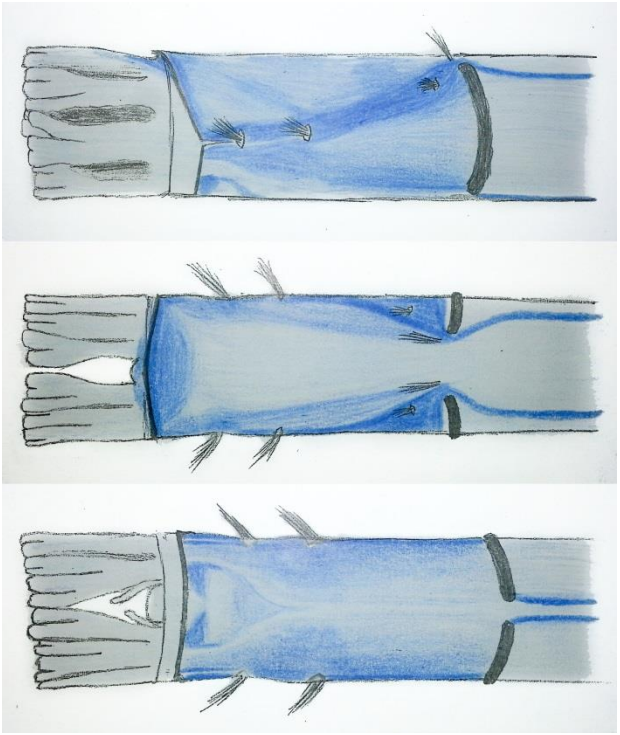

Figure 3.10. Schematic drawing of an *Owenia* with the pattern formed by staining specimens from group 3 identified. A, lateral view; B, dorsal view; C, ventral view.

Remarks

The description is based on two specimens, one of with is broken. This group is separated from the other species by a sligthly different staining pattern, as well as the molecular results indicate that it is a separate group. This group is presented as group 3 in section 3.1.

Distribution

According to this study and Jolly et al. (2006), the distribution of this species is limited to Portugal in the south to the German part of the North Sea. Only one depth record is available, 39 meters depth.

4 Discussion

4.1 Molecular

4.1.1 DNA amplification and sequencing

The fact that no successful sequences were obtained from the Svalbard and Barents Sea areas might be an indication that the primers are not optimal for this group. As much as six different PCR protocols made from three different PCR kits, as well as eight different PCR programs, including several gradients in an attempt to optimize the annealing temperature and several touch-down programs to make the primers more specific in the first cycles, did not result in any successful sequences. There was PCR products after the PCR reactions, but gelelectroforesis revealed that these normally consisted of DNA segments with two or more lengths. The desired band was also extracted from the gel to remove unwanted DNA fragments, but these were not successful either. In total 32 samples from the Svalbard and Barents Sea area was sequenced, without positive result, while all ITS1 PCR products from the same specimens were successfully sequenced. The COI other than those from Svalbard and the Barents Sea was successful in 21 out of 32 samples (66%) leading to the conclusion that the primers might not bind to the target template, or are not specific enough, either way resulting in non-successful sequences.

4.1.2 DNA alignments and analyses

The COI sequences were unproblematic to align, and all three analyses performed resulted in the same main groups. All Atlantic groups have high bootstrap support and posterior probabilities in lower taxonomic level, with the support decreasing on higher level taxa. When ML, MP and BI were compared they gave mostly, congruent results, except the placement of the Canadian sequence (group 12), supporting the Atlantic groups being closer related to each other, rather than with any of the Australian or the Canadian group. The five European groups all include sequences downloaded from GenBank, while three of the groups include sequences from samples used in this project.

ITS1 sequences had a large amount of variation, as illustrated in Fig 3.1. This resulted in alignments with large amounts of gaps. Because of this, several different methods and settings were applied wen aligning the sequences. Gblocks were applied in an attempt to remove as much as possible of the non-homologous sites, and because of the huge number of gaps in each sequence different deletion limits were used to see how this affected the results. As with COI results there are high support values in the lower taxa part of all ITS1 trees. There are, however, differences among the higher taxa. All European groups in the ITS1 analyses consist only of sequences from samples used during this project, and no outgroup were available.

When ML, MP and BI are compared, it results in identical trees, again supporting the Atlantic groups being closer related to each other, rather than with any of the Australian or the Canadian group, with higher support than in the COI tree. The Svalbard and Barents Sea group (group 1) is also present, coming out as a sister group to the rest of the European groups.

When the different alignments are tested, the factor which affects the results the most is the gap opening penalty. A low gap opening penalty, results in one or more Australian groups mixing with the European groups. In only one alignment without relatively low gap opening penalty one Australian group mixed in the European group.

In analyses were Gblocks were applied, Gblocks have no effect on main topology in 7 out 10 (70%) of the analyses. However, in 3 out of 10 analyses information seems to have been lost when Gblocks are applied, resulting in loosing resolution in the trees, and in 2 out of 10 analyses it is the other way around, Gblocks seems necessary to structure the data.

When 25% gap deletion was compared to 50% gap deletion, there was no difference in 7 out of 10 analyses, indicating the gap deletion limit has little effect on this dataset.

The manual alignment was made to how big difference it would make if everything which did not seem correctly aligned was either moved or deleted. As seen in Table 3.2 the manual alignment are 688 bp long, containing only 109 parsimony informative sites, which are the lowest value of all ITS1 alignments. The only difference registered from this alignment was shorter branch lengths in the tree.

All in all, the decisive factor for this dataset is the alignment method, although no well supported changes occur with any of the different alignments, resulting in the trees presented are considered as correct regarding lower level taxa and the separation of the European group from the Australian in ITS1 results as well as in COI results.

The COI+ITS1 analysis shows group 2, 3 and 5 as one European group and group 7 and 8 as the Australian group with very high support, both from bootstrap values and posterior

probability, supporting the decision to use the Australian group as an outgroup in the ITS1 analyses.

4.2 Morphological

As stated in the results, differentiating between the different *Owenia* species based solely on morphologic characters is very hard. There are few characters which are clearly visible through normal stereo and light microscope, and those are variable, and no clear differences between species have been defined in this study. This, though, is not surprisingly when looking at the history of the *Owenia* genus. There have been several descriptions of new species, and there have been revisions in which the species has been synonymized with *O. fusiformis*. There are only in the last few decades there has been agreement among researchers that *Oweina fusiformis* is a species complex, rather than a cosmopolitan species. A part of the reason for this is due to better equipment and methods, especially the scanning electron microscope, which open up for being able to use microstructures which previously was too small.

But even though SEM makes it possible to study small features, it does not mean it is easy to delimit different species. As shown in this study, as well as discussed by Ford & Hutchings (2005), characters can show a high degree of variation, making it them very difficult or impossible to use in delimiting species. In this study none of the microcharacters used was useful in delimiting the different species; they were either consistent over all species or was too variable with much overlapping between species.

4.2.1 Usefulness of different characters

The most informative characters in this study were the macrocharacters in combination with the methyl blue staining pattern, although some of the macrocharacters, especially the relative length of the branchiae compared to the length of the thorax and placement of the dichotomies did not match or had too much variation compared with the descriptions given in Koh et al. (2003). A personal observation done during field work for this thesis, illustrates how the soft bodied characters might be susceptible to stress level: some samples were stored

alive in the fridge overnight after the tube was removed, resulting in the tentacle crown had been retracted considerably, making in much shorter the morning after.

The microcharacters studied were in general too conserved or too variable to be useful in describing the species in this study. Koh et al. (2003) describes the chaeta scales as an useful character to differentiate between *O. borealis* and *O. polaris*, with the free part making out either 1/3 or 1/2 of the total length of the scale, respectively, while in this study the rate in *O. borealis* is 0.40 to 0.50, being less than 1/2 of the scale, with one specimen covering more than this interval (0.37 to 0.50). *O. polaris* shows greater variability in this study, with rats ranging from 0.34 to 0.62, covering al rates between almost 1/3 to almost 2/3. Another example of great variation is the angle between the direction of the teeth on the hooks and the axis following the length of the body. Koh et al. (2003) describes *O. borealis* as having a 45° angle, and *O. polaris* as having angles between 0° and 20°. Measurements done in this study concludes with *O. borealis* ranging from 10° to 120°, with as much as 120° variation across the band of hooks, and *O. polaris* having variation between -5° to 170°, with 160° variation across the band in one specimens. This clearly shows that this is not a useful character on the specimens in this study, both covering a very large interval, as well as both species covering the same interval.

Some characters do not have much variation, examples being the slit between the teeth, the position of the tips of the teeth relative to each other and the angle between the front part of the stem of the hook and the underside of the teeth. All these characters either show no variation, or they show some variation, but they overlap perfectly, meaning there is no way of using them to distinguish the species. But this does not necessarily mean that the characters are useless, they might be useful to distinguish the specimens from other locations or other species.

The characters identified on the tubes gave clear differences between the species, but as Ford & Hutchings (2005) also discuss, it is hard to say for certain that this is species specific, rather than an artifact reflecting available particles in the sediment. In this study *O. polaris* consistently had tubes were round, mineral particles were used, but in *O. borealis* some tubes were made of flat calcareous particles, while other tubes were made of mineral particles, although these seem to be predominantly flat.

4.2.2 Species present in Norwegian waters

As the results show, two, maybe three, *Owenia* species are present in Norwegian waters, these being *Owenia borealis* Koh, Bhaud & Jirkov, 2003, *Owenia polaris* Koh, Bhaud & Jirkov, 2003, and possibly *Owenia assimilis* (as described in Koh et al. (2003)). The presence of both *O. borealis* and *O. polaris* are supported in both morphological and molecular results, with group 1 from the molecular results corresponding with *O. polaris*, and group 2 corresponding with *O. borealis*.

Investigation of *O. assimilis* (Sars, 1851) type material, however, suggests that *O. borealis* Koh, Bhaud & Jirkov is incorrectly described as a new species. Even though there is a possibility the type material is more than one species, the majority of the specimens are identical to *O. borealis* as described by Koh et al. (2003). One specimen in the type material might be identical to *O. assimilis* as described by Koh et al. (2003), clearly illustrating the need for further and more thorough work on both the *O. assimilis* type material and the species described by Koh et al. (2003).

On the question of whether or not the true *Owenia fusiformis* is present in Norwegian waters, the answer is almost without any doubt "no". Both molecular and morphological evidence presented in this study suggests that *O. fusiformis* is endemic to the Mediterranean Sea, which supports earlier studies by Koh & Bhaud (2003) and Jolly et al. (2005). Although, it is important to notice that this is provided the specimens examined in this study is the true *O. fusiformis*.

4.2.3 Molecular results combined with morphological results

In this study, both molecular and morphological data has been used to answer the same questions. When congruent, this gives the conclusions good support, making the findings solid. Molecular data has the potential to uncover complexities which otherwise would have been overlooked and probably regarded as variation, but in no way should it replace morphological studies. Both are valuable tools, and should be used together, making the findings as well supported as possible.

5 Conclusions

Based on evidence presented in this study and from other literature, it is safe to say that there are more than one *Owenia* species present in Norwegian waters. Three different *Owenia* species is reported from Norwegian waters by Koh et al. (2003): *Owenia assimilis* (Sars, 1851), *Owenia borealis* Koh, Bhaud & Jirkov, 2003 and *Owenia polaris* Koh, Bhaud & Jirkov, 2003. There is no doubt that the species referred to as *O. borealis* and *O. polaris* by Koh et al. (2003) are present in Norwegian waters, the question is whether *O. borealis* is the correct name. Investigation of *Owenia assimilis* (Sars, 1851) type material reveals uncertainties whether the type material consists of one or more species. The species referred to as *O. assimilis* by Koh et al. (2003) was not found during this study, except for one possible identification in the *O. assimilis* type material. Further and more thorough studies are needed to resolve this matter.

Regarding the distribution limits of the two certain species in Norwegian waters, *O. borealis* (name as used by Koh et al. (2003)) and *O. polaris*, *O. borealis* is recorded from Rogaland county in the south to Bjørnøya in the north, with Troms county being the eastern distribution limit, while *O. polaris* mainly occur in the area along Troms and Finnmark counties, the eastern parts of the Barents Sea and around the Svalbard archipelago, although recorded as far south as the Norwegian Sea of the coast of Hordaland county.

As presented in this study, no record of the true *Owenia fusiformis* delle Chiaje, 1841 has been recorded outside of the Mediterranean Sea, making it highly unlikely that the true *O. fusiformis* is present in Norwegian waters.

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Appendix

Table A1. The table shows all samples which have been investigated and from which samples have been picked for further analyses. The species, locality, if it is a type locality, or type material, the position, sampling date and depth are shown if the information excist.

TH-nr.	Species	Locality	Type-loc.	Pos. (lat.)	Pos. (long.)	Date	Depth
1		Barents Sea		71°54.6277'N	033°27.6418'E		
2		Barents Sea		71°54.6277'N	033°27.6418'E		
3		Barents Sea		71°54.6277'N	033°27.6418'E		
4		Barents Sea		71°54.6277'N	033°27.6418'E		
5	*O. polaris*	Barents Sea		71°54.6277'N	033°27.6418'E		
6		Barents Sea		71°54.6277'N	033°27.6418'E		
7	*O. polaris*	Barents Sea		71°54.6277'N	033°27.6418'E		
8	*O. polaris*	Barents Sea		71°54.6277'N	033°27.6418'E		
9		Barents Sea		71°54.6277'N	033°27.6418'E		
10		Barents Sea		71°54.6277'N	033°27.6418'E		
11		Barents Sea		71°54.6277'N	033°27.6418'E		
12		Barents Sea		71°54.6277'N	033°27.6418'E		
13	*O. polaris*	Barents Sea		71°54.6277'N	033°27.6418'E		
14		Barents Sea		71°54.6277'N	033°27.6418'E		
15		Barents Sea		71°54.6277'N	033°27.6418'E		
16		Barents Sea		71°54.6277'N	033°27.6418'E		
17	*O. borealis*	North Sea (Osebergfeltet)		60° 35,80'N	002° 47,36'E	1996-05-17	
18	*O. borealis*	North Sea (Osebergfeltet)		60° 35,80'N	002° 47,36'E	1996-05-17	
19		North Sea (Osebergfeltet)		60° 35,80'N	002° 47,36'E	1996-05-17	
20		North Sea (Osebergfeltet)		60° 35,80'N	002° 47,36'E	1996-05-17	
21		North Sea (Osebergfeltet)		60° 35,80'N	002° 47,36'E	1996-05-17	

22	*O. borealis*	Trolla, Tr.h.fj., Trondheim		63°27.1192'N	010°18.9124'E	2013-08-31	5
24		North Sea (Osebergfeltet)		60° 35,80'N	002° 47,36'E	1996-05-17	
25		Kongsfjord, Svalbard		78°56.8651'N	011°55.7134'E	2013-09-26	294
26	*O. polaris*	Kongsfjord, Svalbard		78°56.8651'N	011°55.7134'E	2013-09-26	294
27		Kongsfjord, Svalbard		78°56.8651'N	011°55.7134'E	2013-09-26	294
28		Kongsfjord, Svalbard		78°56.8651'N	011°55.7134'E	2013-09-26	294
29		Kongsfjord, Svalbard		78°56.8651'N	011°55.7134'E	2013-09-26	294
30		Kongsfjord, Svalbard		78°56.8651'N	011°55.7134'E	2013-09-26	294
31		Kongsfjord, Svalbard		78°56.8651'N	011°55.7134'E	2013-09-26	294
32		Kongsfjord, Svalbard		78°56.8651'N	011°55.7134'E	2013-09-26	294
33		Kongsfjord, Svalbard		78°56.8651'N	011°55.7134'E	2013-09-26	294
34	*O. polaris*	Kongsfjord, Svalbard		78°56.8651'N	011°55.7134'E	2013-09-26	294
35	*O. polaris*	Hinlopen strait, Svalbard		79°37.0486'N	018°57.7345'E	2013-10-01	332
37		Kongsfjord, Svalbard		78°56.8651'N	011°55.7134'E	2013-09-26	294
38	*O. polaris*	Kongsfjord, Svalbard		78°56.8651'N	011°55.7134'E	2013-09-26	294
39	*O. polaris*	Kongsfjord, Svalbard		78°56.8651'N	011°55.7134'E	2013-09-26	294
40		Kongsfjord, Svalbard		78°56.8651'N	011°55.7134'E	2013-09-26	294
41		Kongsfjord, Svalbard		78°56.8651'N	011°55.7134'E	2013-09-26	294
42		Kongsfjord, Svalbard		78°56.8651'N	011°55.7134'E	2013-09-26	294
43		Kongsfjord, Svalbard		78°56.8651'N	011°55.7134'E	2013-09-26	294
44		Kongsfjord, Svalbard		78°56.8651'N	011°55.7134'E	2013-09-26	294
45		Kongsfjord, Svalbard		78°56.8651'N	011°55.7134'E	2013-09-26	294
46		Kongsfjord, Svalbard		78°56.8651'N	011°55.7134'E	2013-09-26	294
47		Kongsfjord, Svalbard		78°56.8651'N	011°55.7134'E	2013-09-26	294
48		Kongsfjord, Svalbard		78°56.8651'N	011°55.7134'E	2013-09-26	294
49		Kongsfjord, Svalbard		78°56.8651'N	011°55.7134'E	2013-09-26	294
50		Kongsfjord, Svalbard		78°56.8651'N	011°55.7134'E	2013-09-26	294
51		Kongsfjord, Svalbard		78°56.8651'N	011°55.7134'E	2013-09-26	294
52		Mølnbukt, S.-Tr. NOR		63°37,625'N	09°37,900'E	2013-06-28	100-50
53	*O. borealis*	Mølnbukt, S.-Tr. NOR		63°37,625'N	09°37,900'E	2013-06-28	100-50

54		Mølnbukt, S.-Tr. NOR		63°37,625'N	09°37,900'E	2013-06-28	100-50
55		Mølnbukt, S.-Tr. NOR		63°37,625'N	09°37,900'E	2013-06-28	100-50
56	*O. borealis*	Agdenes, S.-Tr. NOR		63°39,028'N	09°46,083'E	2013-06-19	
57		Mølnbukt, S.-Tr. NOR		63°37,625'N	09°37,900'E	2013-06-28	100-50
58		Agdenes, S.-Tr. NOR				2013-06-19	
59	*O. borealis*	Mølnbukt, S.-Tr. NOR		63°37,625'N	09°37,900'E	2013-06-28	100-50
60		Mølnbukt, S.-Tr. NOR		63°37,625'N	09°37,900'E	2013-06-28	100-50
61		Mølnbukt, S.-Tr. NOR		63°37,625'N	09°37,900'E	2013-06-28	100-50
62	*Owenia* sp. A	NW Spain		42°55'18"N	009°17'41"W		
63		Jan Mayen					
65	*O.* cf. *borealis*	Tromsøflaket, Troms NOR		71°07,5615'N	16°55,4431'E		560
66	*O. borealis*	Stord, Hord. NOR		59°45,507	05°29,504		
67	*O. borealis*	Stord, Hord. NOR		59°45,507	05°29,504		
68	*O. polaris*	Nordkapp bank, Finnm. NOR		71°46,69'N	25°59,69'E		323
70	*O. polaris*	Nordkapp bank, Finnm. NOR		71°24,5819'N	25°58,0135'E		276
73	*O. fusiformis*	Naples, Italy	x	40°49'55"N	014°15'23"E		
74	*O. fusiformis*	Naples, Italy	x	40°49'55"N	014°15'23"E		
75	*O. fusiformis*	Naples, Italy	x	40°49'55"N	014°15'23"E		
76	*O. borealis*	Mangersfjorden, Hord. NOR		60°38.178'N	004°57.350'E	2004-02-07	
77	*O. borealis*	Lonevåg, Hord. NOR		60°31.859'N	005°29.378'E		
78	*O. borealis*	Norwegian Sea, Rog. NOR		58,88251°N	2,37308°E	2008-02-07	110
79	*O. borealis*	Norwegian Sea, Rog. NOR		58,88324°N	2,37507°E	2008-02-07	112
80		Eidsfjord, Nordl. NOR		68.71999°N	15.0888°E	2008-10-07	107
81		Akvaplan					
82		NW of Spitzbergen, Svalbard		80.11755°N	8.77875°E		512
84		Tr.h.fj. S.-Tr. NOR					
85		Tr.h.fj. S.-Tr. NOR					
86		Tr.h.fj. S.-Tr. NOR					
87	*O. borealis*	Tr.h.fj. S.-Tr. NOR					
88		Norwegian Sea, Hord. NOR		60°23.77'N	002°37.59'E		93

90	*Owenia* sp. A	North Sea, GER		54°19.73'N	006°59.68'E		39
92	*O. borealis*	Norwegian Sea, Rog. NOR		58,88324°N	2,37507°E	2008-02-07	112
93	*O. borealis*	Norwegian Sea, Rog. NOR		58,88324°N	2,37507°E	2008-02-07	112
94	*O. borealis*	Byfjorden, off Fagernes, Hord. NOR		60°24.970'N	05°17.580'E		259
95	*O. borealis*	Rosslandspollen, Hord. NOR		60°33.808'N	05°02.292' E		233
96		Lonevåg, Hord. NOR		60°31.859'N	005°29.378'E		
97	*O. borealis*	Lonevåg, Hord. NOR		60°31.859'N	005°29.378'E		
98		Lonevåg, Hord. NOR		60°31.859'N	005°29.378'E		
99	*O. borealis*	Nordfjord, S&F NOR				2001-08-18	
100	*O. borealis*	Nordfjord, S&F NOR				2001-08-18	
101		Tromsø area, Troms, NOR					
105		Sørfjord, Troms NOR		69.60336°N	18.29335°E		50
106	*O. polaris*	Sørfjord, Troms NOR		69.60336°N	18.29335°E		50
107	*O. polaris*	Eidsfjord, Nordl. NOR		68.71999°N	15.0888°E	2008-10-07	107
108	*O. polaris*	Eidsfjord, Nordl. NOR		68.71999°N	15.0888°E	2008-10-07	107
110	*O. polaris*	Brensholmen, Troms NOR		69.60875°N	18.04148°E		20
111		Brensholmen, Troms NOR		69.60875°N	18.04148°E		20
112	*O. polaris*	Brensholmen, Troms NOR		69.60875°N	18.04148°E		20
113		Nordfjord, S&F NOR				2001-08-18	
114	*O. borealis*	Nordfjord, S&F NOR				2001-08-18	
116		Flekkefjord, V.-Agd. NOR		58°13,72'N	06°40,95'E		78
117	*O. borealis*	Flekkefjord, V.-Agd. NOR		58°13,72'N	06°40,95'E		78
120	*O. polaris*	Tromsøflaket, Troms NOR		70°56,76'N	17°05,53'E		583
121	*O. polaris*	Sørfjord, Troms NOR		69.60336°N	18.29335°E		50
124		North Sea (Osebergfeltet)		60° 35,80'N	002° 47,36'E	1996-05-17	
125		North Sea (Osebergfeltet)		60° 35,80'N	002° 47,36'E	1996-05-17	
126		North Sea (Osebergfeltet)		60° 35,80'N	002° 47,36'E	1996-05-17	
132		Flekkefjord, V.-Agd. NOR		58°13,72'N	06°40,95'E		
134	*O. fusiformis*	Naples, Italy	x				
135	*O. assimilis*	Herlø, Manger , Hord. NOR	x (type material)			1849	

136	*O. assimilis*	Tromsø, Troms, NOR	x (type material)			1849	
137	*O. assimilis*	Tromsø, Troms, NOR	x (type material)			1849	
138	*O. assimilis*	Manger, Hord. NOR	x (type material)			1849	
139	*O. assimilis*	Manger, Hord. NOR	x (type material)			1849	
140	*O. assimilis*	Herlø, Manger , Hord. NOR	x (type material)			1849	

Table A2.Shown the results of the analyses of the macrocharaters in all measured specimens. Characters 1-6 as described in section 2.4.1. When a question marks is used it is uncertainties of the measurement or the evaluation of the character.

Sample	Methyl blue staining	Tube				Specimen		Tentacular crown					
		Length (mm)	Diam. (mm)	Material & shape	Lumen prop. of tot. wall diam.	Length (mm)	Diam. (mm)	Rel. length (mm)	Diam.	Dichotomy	Number of dichotomies	Angle at collar notch (°)	Edge
5	O. pol.	54	2.5	Min, round	0.52	19	1.3	0.47	<	mid/tip	2	160	mid
7	O. pol.			Min, round				0.29	<<	mid/tip	2	165	thick
8	O. pol.	40	1.7	Min, round	0.53	12.9	0.9	0.36	<	tip		170	thick/mid
13	O. pol.							0.19	<<				thick
17								0.69	=	base/mid/tip	4	140?	thin, sharp
22	O. bor. ?	19.2	1.3	Min/calc, flat	0.46	12.9	0.6	0.36	<	base/mid/tip?	2+		thin
26	O. pol.	25.4	2.4	Min, round				0.21	<<	base/mid	2	160	mid
34	O. pol.	32.7	3	Min, round	0.57	29.5	1.7	0.28	<<	base/mid/tip	3	160	thick/mid
35	O. pol.	31.4	3	Min/Foram., round	0.40	15.9	1.2	0.27	<<<	tip	1		mid?
38	O. pol.	46.3	2.6	Min, round	0.46	20.2	1.2	0.13	<<			160	thick/mid
39	O. bor. ?	49.4	2.8	Min, round	0.46	17.7	1.3	0.14	<	tip	1	160	thick, rounded
53			1.1	Min/calc, flat	0.55	13.3	0.6						
59	O. bor.		1.6	Calc, flat	0.75		1.2	0.42	<				thin, (rounded)
62	O. sp. A?		4.7	Calc, flat	0.57		2.7	0.5	=	base/mid/tip	3+		thin
64			1.3		0.69	14.2	0.9	0.31	<<	base/mid/tip	2-3	150	thick, rounded?
65			2.3	Min/calc?, flat	0.39		0.9	0.24	<<	mid/tip	2	150	?
66			1.5	Calc, flat	0.53		0.8	0.34	=	mid/tip	3-4	150	thin, sharp
67						22.8	1.1	0.48	=	mid/tip	3-4	160	thin, sharp
68			1.1	Min, round?	0.55	9.3	0.6	0.29	<?	mid/tip	1-2	160	mid, rounded?
70			0.7	Min, round?	0.57	8.5	0.4	0.25	<<	mid	1	140	thick, rounded?

73	O. fus.							0.44 (uneven)	=	base/mid/tip	3+		thin
74	O. fus.		3	Min/calc, round/flat	0.37		1.1	0.54 (uneven)	=	mase/mid/tip?	3+		thin
75	O. fus.		2.8	Min/calc, round/flat	0.43		1.2	0.41 (uneven)	=	mase/mid/tip?	3+	oblique	thin?
77	O. bor.			Min, round/flat				0.26		mid/tip	2		thin
79	O. bor.		1.7	Min, round	0.53		0.9	0.36	=				thin
82								0.27	<	base/mid/tip	3	140	thick, rounded?
84	O. bor.			Min, flat				0.34	=	mid/tip	2	150	thin
85	O. bor.		2.2	Calc, flat	0.45		1	0.42	=	base/mid/tip	3		thin
87	O. bor.	33.8	1.9	Calc, flat	0.37	18.4	0.7	0.23	=	base/mid/tip	3		thin
88			1.8	Min, round	0.50	34.7	0.9	0.3	=	mid/tip	3		thin
90	O. sp. A		3	Calc, flat	0.50		1.5	0.35	=	base/mid/tip	4		thin
92	O. bor. (?)		1.2	Min, flat	0.50		0.6	0.33	=	mid/tip	2		thin
93	O. bor.		1.1	Min, flat	0.45		0.5	0.26	=				thin
94	O. bor.	90.5	3	Calc, flat	0.43		1.3	0.36	<	mid/tip	3		mid
95	O. bor.	66.6	2.9	Calc, flat	0.00			0.63	=	base/mid/tip	4	170	thin
97	O. bor.		1.6	Min?, flat	0.44		0.7	0.45	=	mid/tip	3	150	thin
99	O. bor.	67.5	3.3	Min, flat	0.42	49.2	1.4	0.17	<	tip	1	160	thin
100	O. bor.	59.3	2.4		0.50	26.1	1.2	0.26	=	tip	1	0 (perp)	thin
102								0.21	<<	base/mid/tip	3	160	thick, rounded?
106								0.31	<<	mid/tip	2	150	thick, rounded?
107								0.27	<	mid/tip	1	150	thick, rounded?
108								0.25	<	mid/tip	1	160	mid, rounded?
110								0.25	<<<	mid/tip	1-2	150	mid, rounded?
112								0.28	<<	base/mid/tip	1	160	mid, rounded?
114								0.25	<	base/mid/tip	3	150	thin, rounded?
117	O. bor.							0.39	<	mid/tip	2	140	thin, sharp/rounded?
120	O. bor. ?							0.4	<	mid/tip	2-3	150?	mid/thin
121	O. bor. ?							0.41	=	mid/tip	2+	150?	mid/thin

129	O. bor.							0.52	=	tip	2+	160	thin
134	O. fus.		1.9	Min/calc, flat	0.58	33.3	1.1	0.27 (uneven)	=	mid/tip	2+	?	thin
135	O. bor.		3.4	Calc, flat	0.35		1.2	0.28	<	base/mid/tip	3?	?	mid
136	O. bor. ?							0.35	<	base/tip	2?	?	mid
137	O. bor. ?		3	Calc, flat	0.27		0.8	0.34	<	base/tip	2?	150	mid
138	O. bor.							0.22	<	tip	2?		thin
139	O. bor.		2.1	Calc, flat	0.43		0.9		<	Mid/tip	4	?	mid
140	O. bor, with different "V"		2.8	Calc, flat	0.29		0.8	0.38	=	Mid/tip	2?	oblique	thin, smooth

Table A3. The table shows the results from the measurements of characters 7-17 as described in section 2.4.1

Sample	Chaeta	Uncini									
	fp:tl	Angle rel. to bode direction (°)	Rel. teeth length	Slit between teeth	Rel. teeth tip orientation	Teeth angle (°)	Protrusion (µm)	Rel. protrusion	Shoulder	Front of stem	Gap shoulder-teeth (µm)
117	0.40	50-70 (50)	=	wide, paralell	oblique	80-85	2.25	0.38	2/3	straight	1.15
17	0.54		=	widens from 1/3	oblique	80	2.85	0.46	v small, 1/3	straight	0.86
65	0.50	15-30 (15)	=	widens from 1/3	oblique	75-85	2.10	0.45	1/2	straight	0.73
66	0.37-0.55	10	=	widens from 1/2	oblique	80	2.40	0.42	v small 1/3	straight	0.8
67	0.45	15	=	widens from 1/3	oblique				2/3	straight	
114	0.42	60-180 (120)	=	widens from 1/2	oblique	80-85	2.00	0.38	?	small curve	0.85
39	0.50	20-170 (60)	=	widens from 1/2	oblique	70-80	2.26	0.40	3/4	straight	0.56
26	0.56	25	not=	widens from 1/3	oblique	80	1.60	0.33	2/3 / absent	small curve	0.81
64	0.45	-5-10 (5)	not=	widens from 1/2	oblique	90				small curve	
68	0.50	5-10	=	widens from 1/2	oblique	70-85	1.68	0.38	2/3	straight	0.61
70	0.40	0	not=	widens gradually	oblique	60-75	1.62	0.42	v small, 1/3	straight	0.63
82	0.34-0.49	0-25	not=	widens from 1/3	oblique	70-90	2.09	0.42	v small 1/3	straight	0.63
102	0.46-0.58	30	=	widens from 1/2	oblique	80-90	1.85	0.37	1/3	straight	0.42
106	0.36	15	n/a	n/a	oblique	n/a	2.15	0.41		straight	
107	0.39-0.52	30	=	widens from 1/3	oblique	75-85	1.90	0.41	2/3	straight	0.67
108	0.47	20-40 (20)	=	widens from 1/2	oblique	85	2.08	0.36	2/3	straight	0.53
110	0.50	90-170	=	widens from 1/3	oblique	75-85	1.92	0.37	v small, 1/3	straight	
112	0.36-0.62	0-15 (15)	not=	widens from 1/2	oblique	85-90	1.90	0.37	1/2	straight	1.10

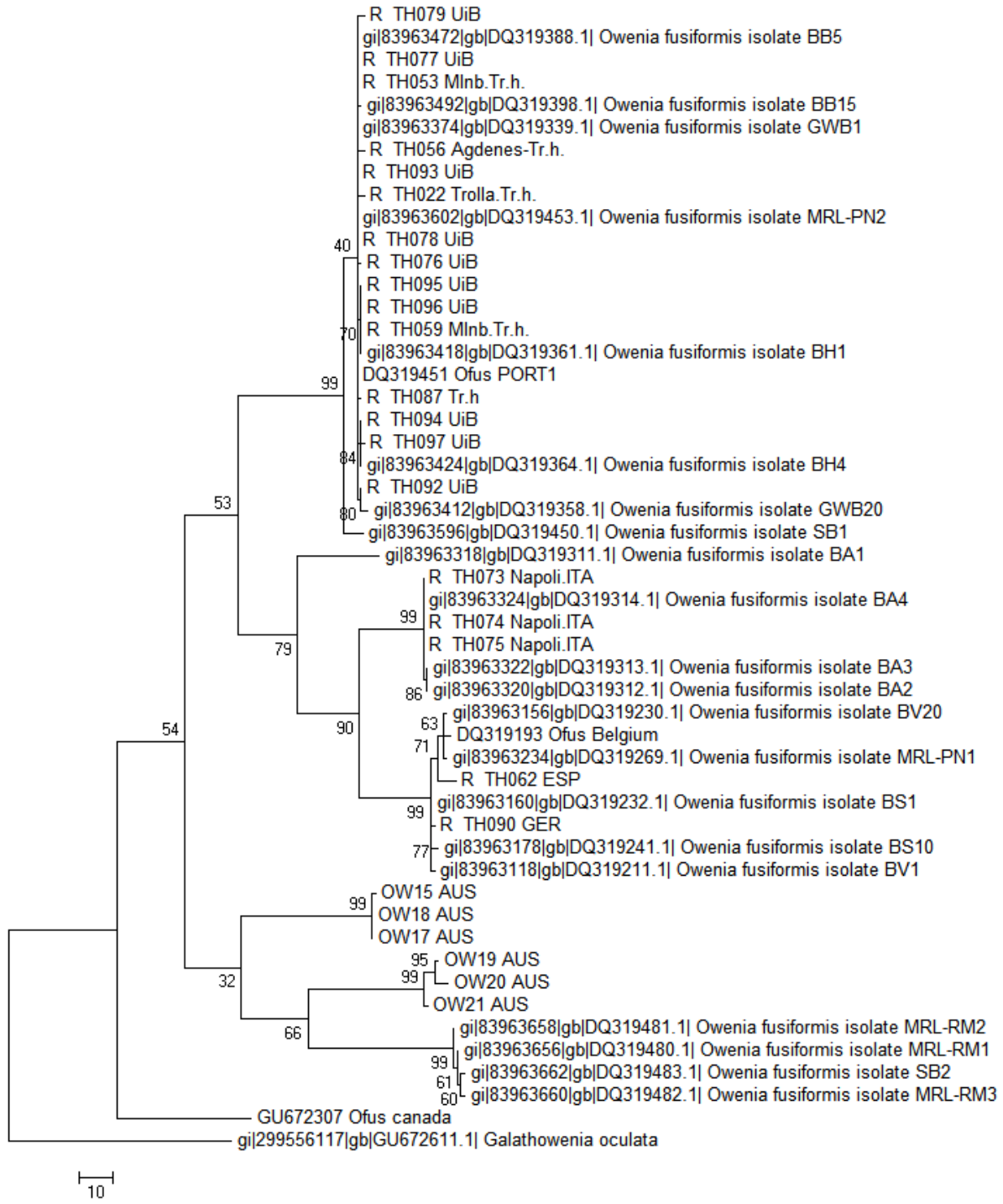

Figure A1. One out of two equally parsimonious trees made with Maximum Parsimony in MEGA6. The tree is obtained using the Subtree-Pruning-Regrafting method with search level 1 without a starting tree, 1000 bootstrap replicates and sites with more than 10 % gaps were deleted. Tree length: 503 bp.

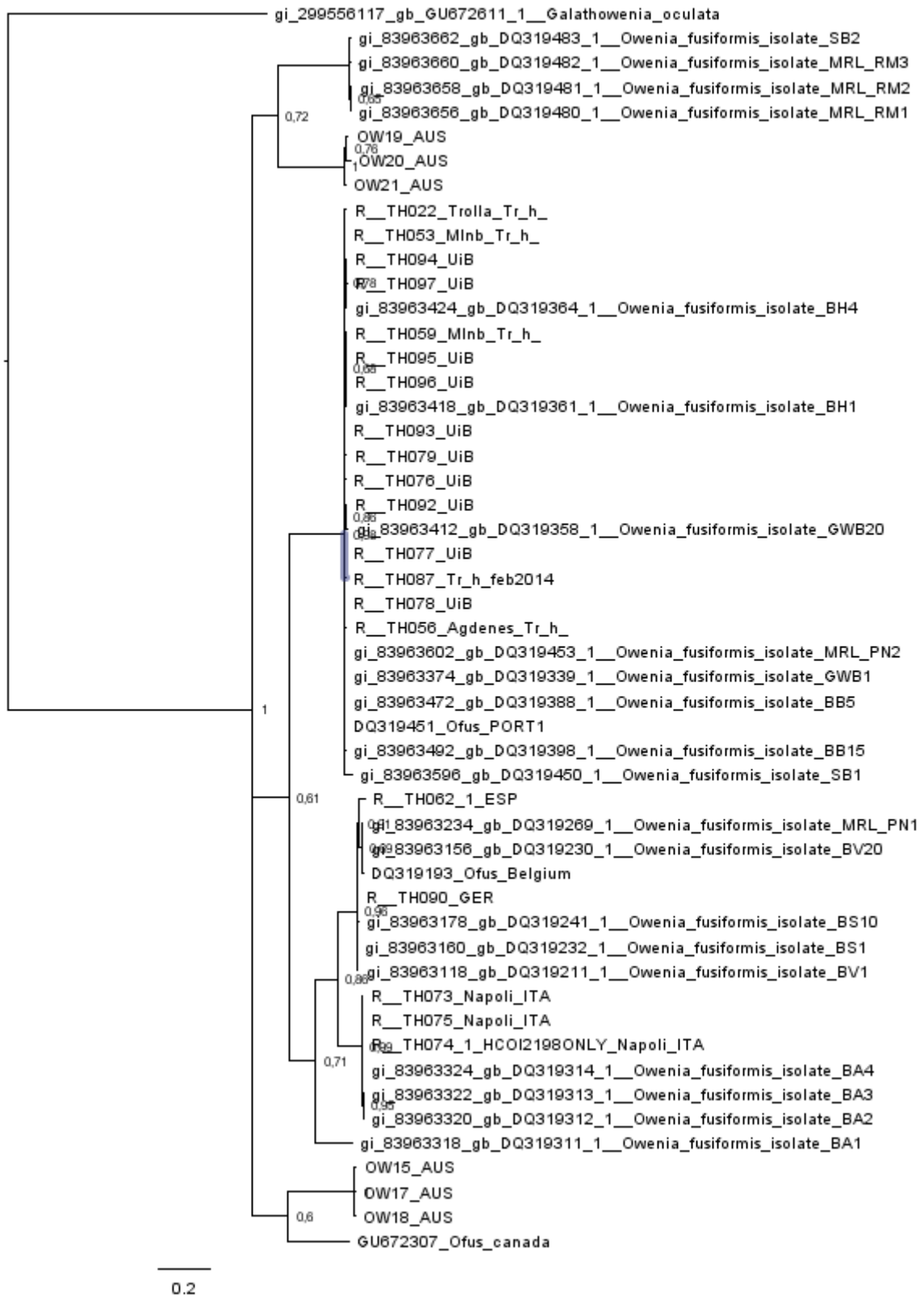

Figure A2. Bayesian Inference tree obtained analyzing the COI dataset using MrBayes (10 000 000 generations, 25% discarded as burnin. Mean lnL from to parallel runs: -2858.24. Node values are posterior likelihood.

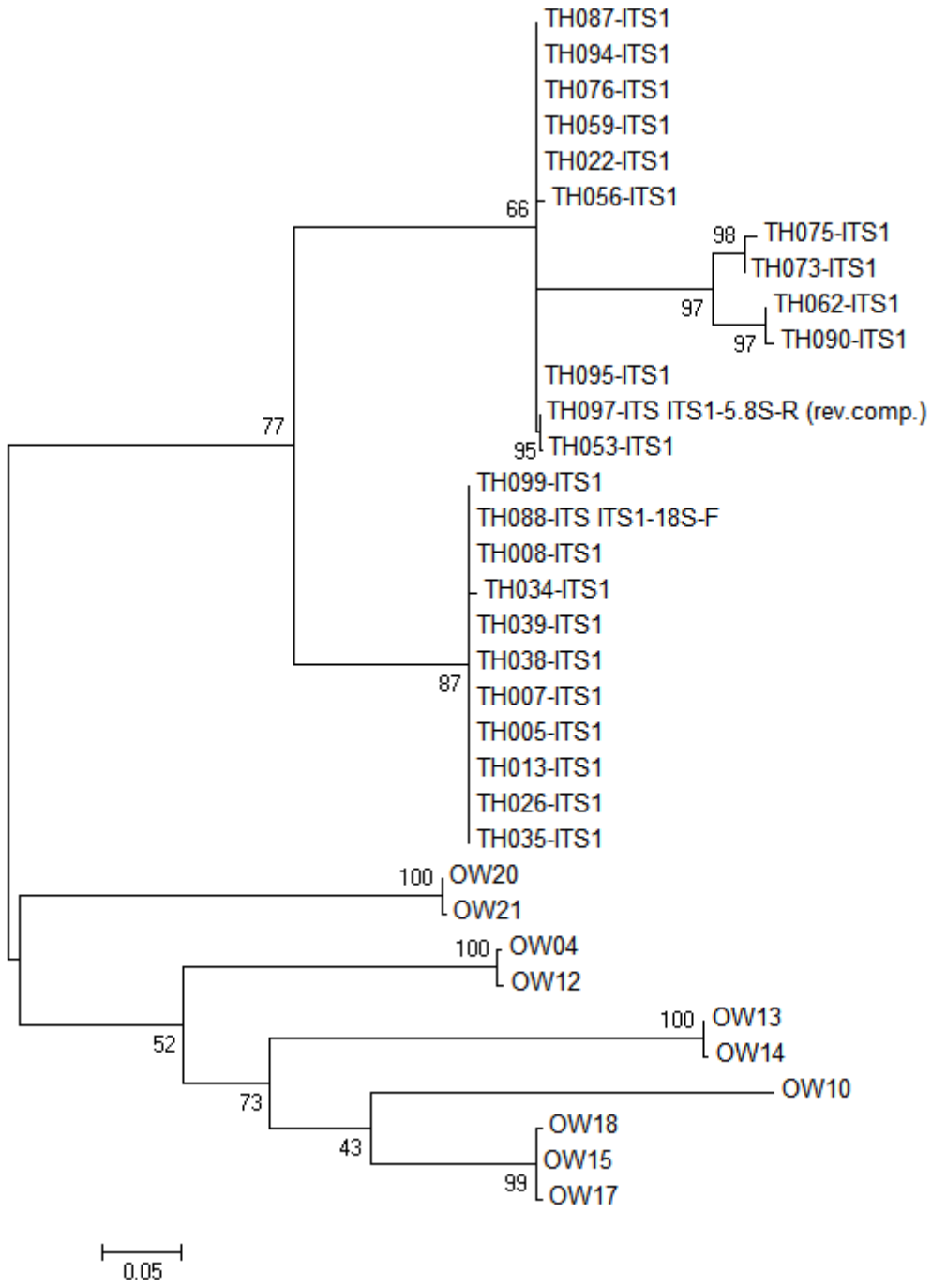

Figure A3. Maximum Likelihood tree from the ITS1_ClustalW_gblocks alignment made in MEGA6. 1000 bootstrap replicates and sites with more than 50 % gaps were deleted. lnL = -3137.95

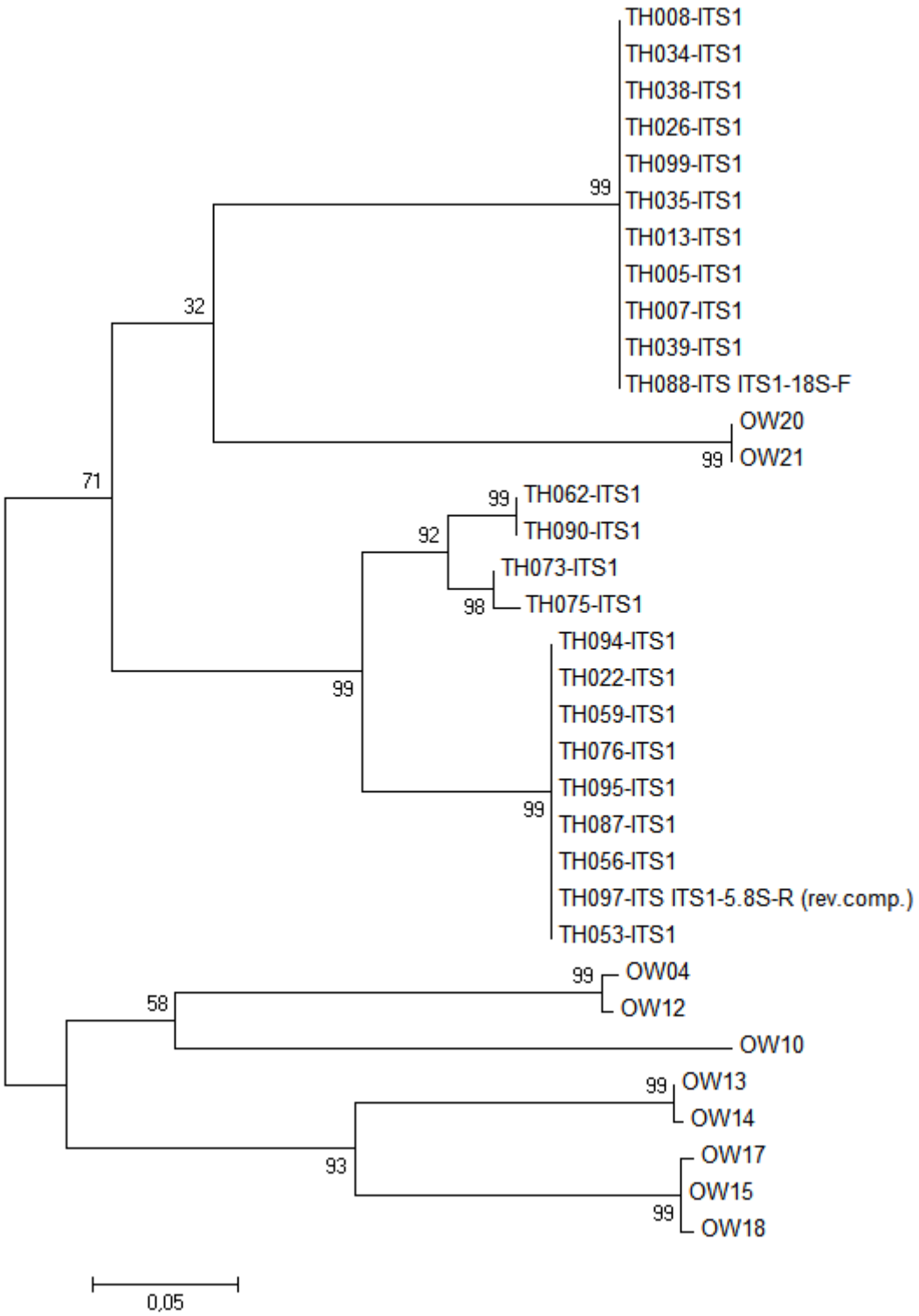

Figure A4. Maximum Likelihood tree from the ITS1_MAFFT_20pam/k=2_gblocks alignment made in MEGA6. 1000 bootstrap replicates and sites with more than 25 % gaps were deleted. lnL = -2428.62

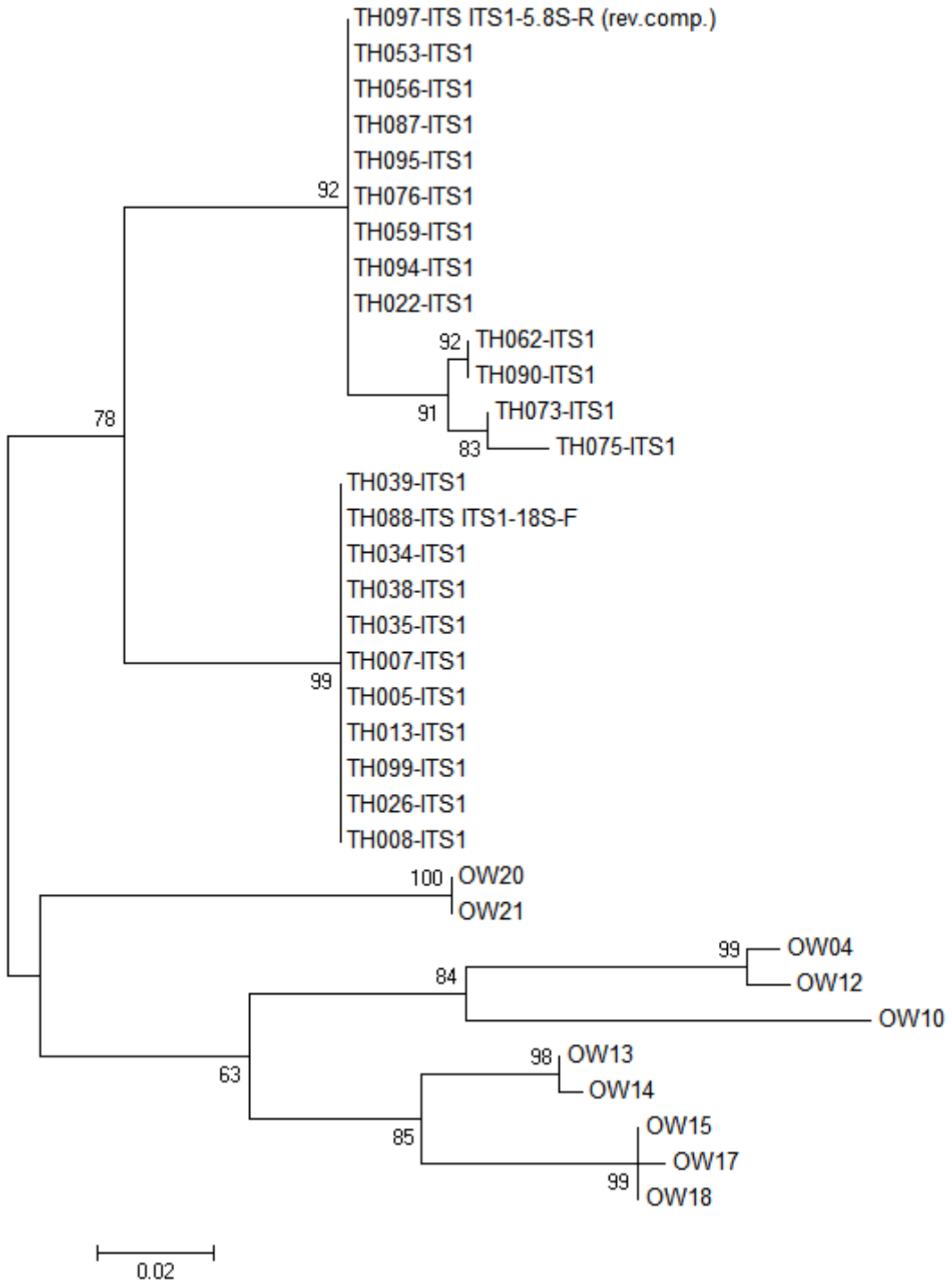

Figure A5. Maximum Likelihood tree from the ITS1_manual alignment made in MEGA6. 1000 bootstrap replicates and sites with more than 50 % gaps were deleted. lnL = -1088.09