Cryptic diversity in the leptothecate genera *Laodicea* and *Tiaropsis* (Cnidaria: Hydrozoa)

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Master of Science in Biology - Marine Biology

Autumn 2021

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Lastly, I am forever grateful to my parents and family.

Maryam Rezapoor,

2021 september 1

List of abbreviations

ABGD Automatic Barcode Gap Discovery

BLAST Basic Linear Alignment Search Tool

BIC Bayesian information criterion

BPTP Bayesian Poisson Tree Processes

BOLD The Barcode of Life Data System

http://boldsystems.org

CCDB Canadian Centre for DNA Barcoding

COI Mitochondrial cytochrome c oxidase subunit I gene

ESS Effective sample size

GenBank Genetic sequence database of the National Institute of Health, USA,

http://www.ncbi.nlm.nih.gov/genbank

(HYPNO) Hydrozoan pelagic diversity in Norway

ITS Nuclear ribosomal internal transcribed spacer

16S Ribosomal RNA gene

NCBI National Center for Biotechnology Information

(NorHydro) Norwegian marine benthic Hydrozoa

PCR Polymerase chain reaction

Abstract

Although they are an ecologically and economically important group of animals, hydrozoans are understudied because of their difficult identification due to their small size and fragility. Hydrozoans as a group show a great diversity in life strategies, and often include both a polyp and a medusa stage in their life cycle, which complicates their taxonomy because both stages may be needed for a correct identification. For the two leptothecate hydrozoan genera *Laodicea* (Family Laodiceidae) and *Tiaropsis* (Family Tiaropsidae), the polyp stage is very small and easy to overlook, while the medusa stage is conspicuous and relatively straightforward to identify, at least to genus level. Only one species of each of these genera is believed to occur in Norwegian waters, *Laodicea undulata* (Forbes &Goodsir, 1853) and *Tiaropsis multicirrata* (M. Sars, 1835), but preliminary data suggest that the diversity of these taxa in the region is higher than previously thought. In this study, I used DNA barcoding and different molecular species delimitation methods (based on mitochondrial markers 16S and COI and the nuclear marker ITS) in combination with a detailed morphological analysis of both the hydroid and medusa stages to assess the species diversity of the genera *Laodicea* and *Tiaropsis* in Norway. Based on molecular evidence, *Laodicea undulata*is shown to comprise two molecularly distinct Norwegian clades, which appear not to be sister species. Specimens morphologically identified as *T. multicirrata* split up into three distinct clades in Norway according to the evaluated molecular markers, indicating cryptic diversity in *Tiaropsis* for the studied region. For both *Tiaropsis* and *Laodicea*, the results suggest that the observed clades correspond to undescribed species, but further work is necessary to place them in a broader phylogenetic perspective and to identify any potential morphological characters that define them.

1 Introduction

1.1 Introduction to Phylum Cnidaria, Class Hydrozoa, and Order Leptothecata

Phylum Cnidaria is a broad and heterogeneous group of animals that includes familiar marine organisms such as corals, jellyfish, and sea anemones. Traditionally, this phylum is divided into four classes: Anthozoa, Scyphozoa, Cubozoa and Hydrozoa (Daly *et al.*, 2007), although recent studies suggest that the protozoan-like members of Class Myxozoa are highly modified cnidarians and therefore also belong in the group (Foox and Siddall, 2015). There are more than 11900 extant species of cnidarians (WORMS, 2021), all of them united by the presence of cnidocytes, the 'stinging cells' that constitute the diagnostic feature of the phylum. Other characteristics such as radial symmetry and a life cycle including polyp and jellyfish stages, are usually considered to be typical of Cnidaria, but since they are absent in many species, they should not be considered diagnostic characters (Daly *et al.*, 2007). Most cnidarians are marine, but the group is also present in brackish and freshwater and as endoparasites of other organisms. Because of their high number of species and their widespread distribution in different environments, Cnidaria is often considered one of the major animal phyla.

Class Hydrozoa, with almost 3800 valid species (Schuchert, 2021) and at least 279 species recorded in Norway (Artsdatabanken, 2021), is one of the largest and most diverse groups within Cnidaria. Hydrozoa is regarded as a monophyletic taxon, and this view is supported by both molecular and morphological analysis (Marques and Collins, 2004), but there are no unambiguous diagnostic morphological characters that define the group. Usually, hydrozoans are defined as jellyfish-producing cnidarians in which the medusa stage possess a velum and is generated by budding instead of strobilation (as in Scyphozoa) or metamorphosis (as in Cubozoa), although there are numerous exceptions to these patterns within the class (Bouillon et al., 2006). Two subclasses are commonly recognized within Hydrozoa: Trachylina (approx. 162 nominal species), and Hydroidolina (approx. 3602 nominal species) (Schuchert, 2021).

The life cycle of a hydrozoan typically includes two morphologically different stages (Fig. 1): the polyp (benthic and often colonial, also called **hydroid**) and the medusa (pelagic and solitary, also called **hydromedusa**), but there are many exceptions to this pattern, and it is almost impossible to generalize the life strategies of the class (Boero, Bouillon and Piraino, 1992).When present, the hydromedusa will produce the gametes and is therefore the sexual stage of the life cycle. Fertilization occurs in the water column, resulting (for most hydrozoan species) in a planktonic larval stage known as planula, which is often covered with flagellate cells for swimming. Planulae typically settle and attach to a benthic substrate and start to develop onto the next stage of life as a polyp. Polyps can be solitary or, more often, colonial and characteristically will generate the sexual medusa by asexual budding (Bouillon et al., 2006; Kloc et al., 2018). The medusa stage is free-swimming, soft and transparent, constituted of ca. 95% water, and is colloquially known as “jellyfish” (Schiariti et al., 2018; Cornelius, 1995). Both hydroids and hydromedusae are equipped with nematocysts in different locations, and these are the organelles that inject toxins into their prey (Bouillon et al., 2006; Cornelius_1995a.;Vervoort and Watson, 2003).

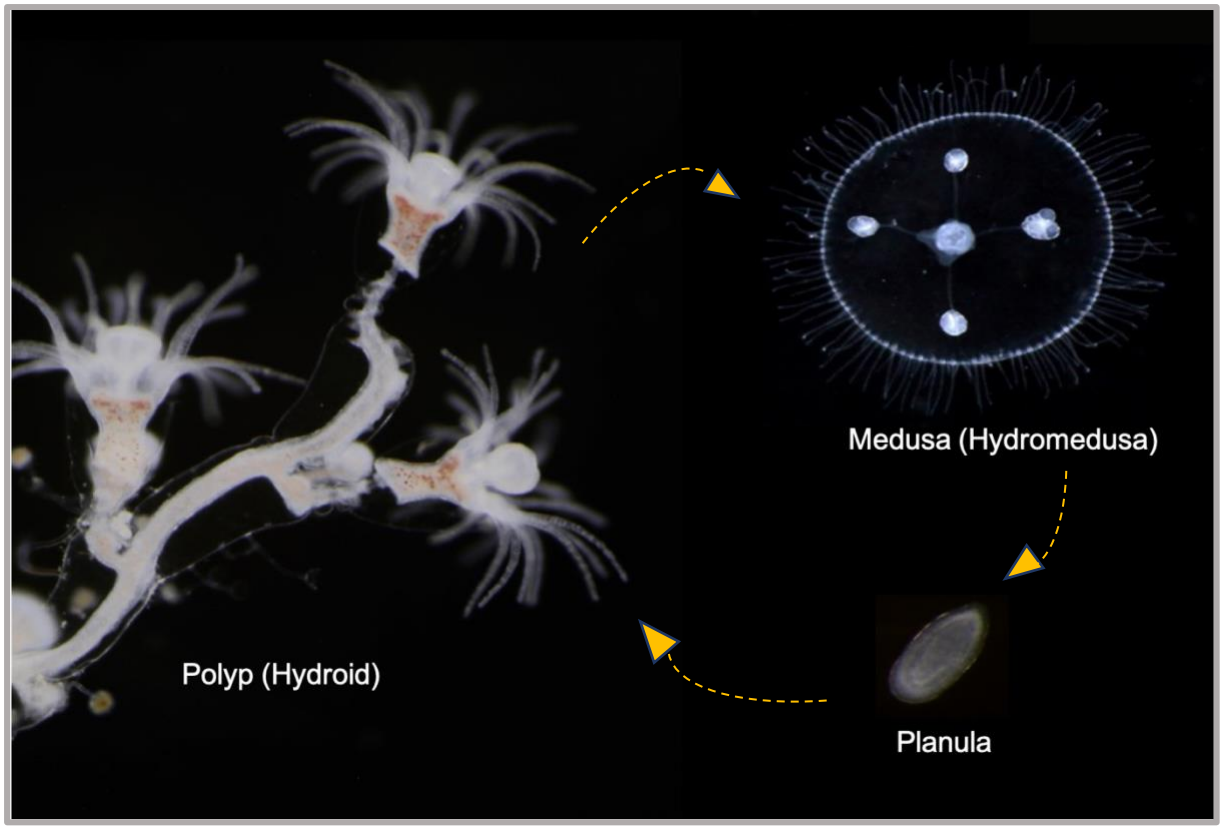


Figure 1. Typical hydrozoan life cycle with alternation of generations/metagenesis between a polyp stage and a medusa stage. The species depicted is the leptothecate *Obelia geniculate* (Linnaeus, 1758).

Hydrozoans are an ecologically and economically important group of animals; however, they are understudied because of their complicated identification and taxonomy (Bouillon et al., 2006). They are present in all oceans and at all latitudes, with some species also inhabiting rivers and lakes, but they are often small and can be inconspicuous and easy to overlook; however, they can considerably affect marine food webs as a result of their roles as predators in the benthos and the plankton. Hydrozoans are usually carnivores and prey on zooplankton, fish larvae, small crustaceans like copepods and other soft-bodied invertebrates, but some species may additionally ingest bacteria, protozoans, and phytoplankton. In turn, several species of snails, sea spiders, and worms graze on hydrozoan polyps, and zooplanktivorous fishes ingest hydromedusae (Bouillon et al., 2006; Bouillon and Boero, 2000). In recent years, researchers have paid additional attention to the impact of jellyfish (including hydromedusae) on human life. The increasing populations of certain jellyfish species in some parts of the world have affected positively and negatively many human life activities such as tourism and fisheries (Brotz *et al.*, 2012).In some cases, human-induced phenomena such as overfishing or global warming have been shown to affect the abundance and distribution of jellyfish and to cause jellyfish blooms (Brotz et al., 2012).

The most speciose order of hydrozoans is Order Leptothecata (Schuchert, 2021). Leptothecata, commonly known as thecate hydroids in the benthic stage and leptomedusae in the planktonic stage, is characterized by 1) the presence of a rigid structure protecting the polyps (the theca) and 2) the position of the gonads in the radial canals of the leptomedusa (Fig 2). All thecate hydroids are colonial, but each colony has at least two different kinds of polyps: those specialized in feeding are called hydranths (and their protective theca is called hydrotheca), while those specialized in producing the leptomedusae are called gonozooids (and are in turn, protected by a gonotheca). In some species, the hydrotheca is large and covers the whole hydranth, but in others the polyps are not completely covered. In some species the hydrotheca has a “lid” (=operculum) that offers further protection for the hydranth. The shape and size of the hydrotheca and gonotheca are important characters for the identification of species in Leptothecata (Cornelius, 1995).

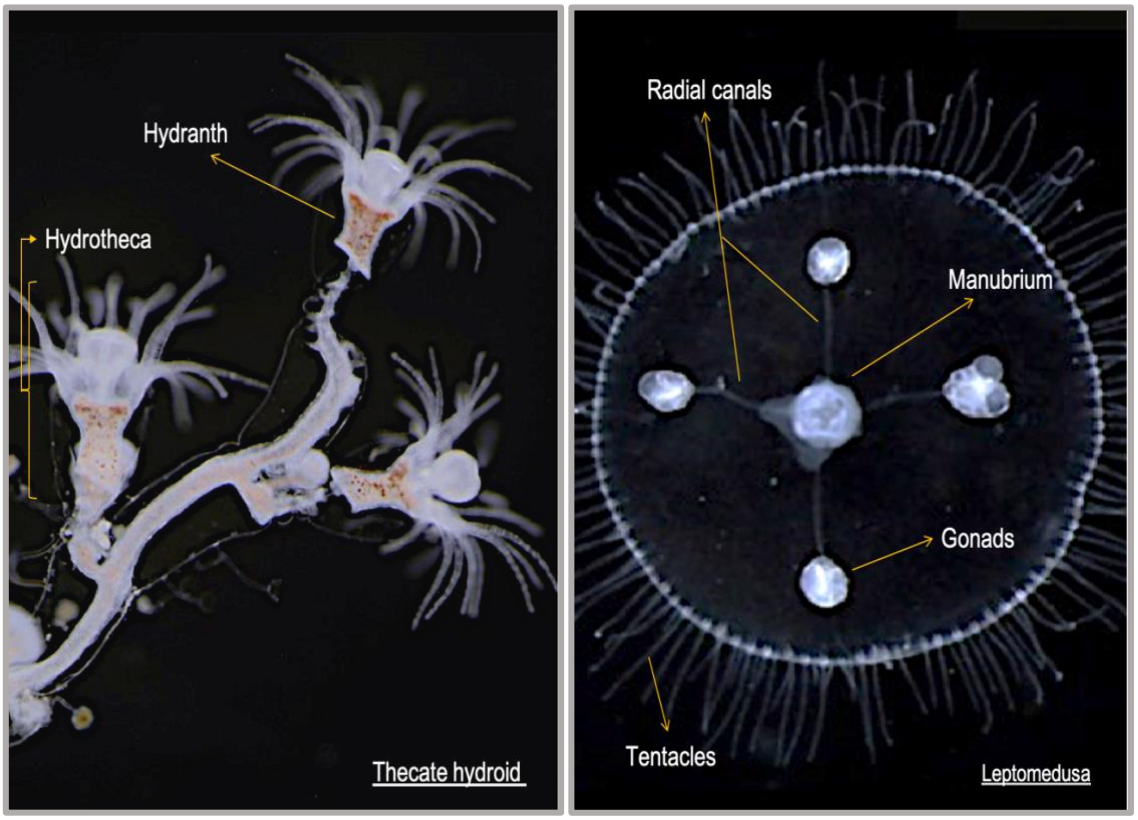


Figure 2. General morphology of thecate hydroids and leptomedusae (Leptothecata).

Leptomedusae have a typical saucer shape, with a body (= umbrella) that is often wider than higher. The bell diameter is generally between 1 mm and 50 mm, but it can be up to 100-200mm in some species. The different body parts of a leptomedusa are connected through a system of canals, one of which runs along the margin of the medusa (circular canal) and 4 or more (the radial canals) that link the circular canal with the mouth and its mounting structure (the manubrium). The umbrella margin generally includes marginal tentacles and may also include cirri and other sense organs. The most common-sense organs for leptomedusae are the statocysts (they can be open or closed) located on the umbrella margin between marginal tentacles, but some groups may have other structures such as cordyli and ocelli. For leptomedusae, the form and position of the gonads, the number and shape of the radial canals, and the number and type of structures along the body margin play an essential role in classification and identification (Cornelius, 1995).

1.2 **Problems with hydrozoan classification and taxonomy**

Hydrozoan taxonomy is complex because of the few morphological characters of these animals and the fact that two relatively long-lived life stages are usually involved in hydrozoan life cycles (Bouillon et al., 2006;Calder, 2019; Segura-Puertas et al., 2009). Research on polyps and investigations on medusae have been traditionally independent, and as a result two parallel taxonomies have developed historically. This means that in several cases the same animal has been called with two different names, one for the polyp stage and another for the medusa stage (Schuchert, 1998; Gravili et al., 2018). This is not correct because if polyp and medusa are two stages of the same species, they should have only one name. Therefore, as a community we need to fix all these cases of incongruence one by one in order to achieve a natural classification of hydrozoans. In addition, hydrozoan taxonomy has traditionally been based only on morphological characters, but many characters are difficult to evaluate and not enough is known about morphological variation in most of the species, so there is still much confusion on the identity and boundaries of species in the class (Stream, 1993; Moura *et al.*, 2008; Miglietta *et al.*, 2018; Cunha et al., 2020'1; Mendoza-Becerril *et al.*, 2020).

One of such cases of confusion is the presence of cryptic species and species complexes. **Cryptic species** are distinct species (two or more) that are incorrectly classified due to the fact that they are morphologically indistinguishable. In many cases, the correct placement of a species is impossible to determine only through morphology, and only recently –with the aid of molecular tools– have cryptic species been identified in hydrozoans (Moura *et al.*, 2011; Laakmann and Holst, 2014; Schuchert, 2014). The problem of cryptic species is by no means restricted to hydroids and hydromedusae, and in fact, for many taxa (including most of marine invertebrates), hidden or cryptic diversity has been observed in recent years (Titus et al., 2018; Cerca et al., 2020; Grosse et al., 2020). All around the world, new perspectives and methods for species discovery and identification based on molecular information are allowing scientists to better understand the biological diversity and are transforming the way taxonomy and systematics are conducted (Bickford *et al.*, 2007; Padial *et al.*, 2010; Schlick-Steiner *et al.*, 2010).

1.3 **DNA barcoding and integrative taxonomy**

DNA barcoding is an approach that uses one or more DNA sequences ("molecular markers", usually short fragments of mitochondrial or nuclear genes) which act as a unique barcode to identify an organism to species level(Hebert and Gregory 2005). The most widely used sequence in the majority of animal groups –the so-called universal animal barcode– is a ~550 bp fragment of the mitochondrial cytochrome c oxidase subunit I gene (COI) (Hebert et al. 2003). DNA barcodes are designed to function as internal species tags, allowing a sequence to identify or "barcode" an organism in order to assign it to a certain species(Hebert *et al.*, 2003; Hebert and Gregory, 2005; Hajibabaei *et al.*, 2007). Like any other approach, DNA barcoding has both limitations and advantages that are inseparable elements of the technique. In DNA barcoding, we use barcode reference libraries for comparison, allowing for an efficient taxonomic identification as long as the boundaries between and within species are clear and the reference barcodes are available. Less time is used in DNA barcoding than in traditional morphology methods through the taxonomic assignment and processing of more samples, but on the other hand, access to PCR facilities and reliable markers, additional costs, and the quality of the reference library may limit the usefulness of this approach (Hajibabaei *et al.*, 2006; Ortman *et al.*, 2010).

Integrative taxonomy refers to the use of a combination of molecular, morphological and ecological data in order to classify and identify biodiversity (Wright, 1968; Moura *et al.*, 2008; Padial *et al.*, 2010; Schuchert, Hosia and Leclère, 2017a; Miglietta *et al.*, 2018; Wang *et al.*, 2018).It is a global framework that integrates information from different disciplines in order to provide sufficient rigor for delimiting and defining taxa (Dayrat, 2005; Will, Mishler and Wheeler, 2005). The molecular information used in integrative taxonomy is most often derived from DNA barcoding, which makes it possible to discover and name diverse species even in morphologically indistinguishable groups, thus resolving cases of cryptic lineages (Hebert et al., 2004; Dayrat2005; Padial et al., 2010; Pauls et al., 2010; Brasier et al., 2016; Nygren et al., 2018).

In Hydrozoa, integrative taxonomy and DNA barcoding have traditionally relied on markers different that COI; in particular the mitochondrial16S. Different studies have recommended this marker due to its good resolution for both species delimitation and phylogenetic analyses in the group(Schuchert, 2005; Miglietta *et al.*, 2007; Zheng *et al.*, 2014; Schuchert, Hosia and Leclère, 2017).Despite this, COI sequences have also been shown as phylogenetically informative in the class (Govindarajan, Halanych and Cunningham, 2005; Ortman *et al.*, 2010), in particular when combined with 16S (Govindarajan, Boero and Halanych, 2006; Hemmrich *et al.*, 2007; Martínez *et al.*, 2010). Other markers, such as the internal transcribed spacer (nuclear ITS) are only starting to be used for species delimitation within Hydrozoa and their usefulness seems to be higher when combined with the traditional mitochondrial barcodes(Mendoza-Becerril *et al.*, 2018; Schuchert, 2018).

1.4 **Study cases: the leptothecate hydrozoan genera *Laodicea* and *Tiaropsis***

The two genera that are the object of study of this thesis belong to some of the least studied families within Order Leptothecata (Maronna *et al.*, 2016). Although they are morphologically different and are not closely related, both *Laodicea* and *Tiaropsis* are clear examples of widely distributed genera defined by the characters of their medusae, and in both cases the polyp stage is inconspicuous and has not been used for taxonomic delimitation at any level of classification. In both genera, the bulk of records belong to the type species of the genus, which is defined rather loosely and has a confusing list of synonyms. In addition, the type localities for both taxa are located in the northeastern Atlantic Ocean. Because of these similarities, both genera can be treated in parallel when looking for regional patterns of cryptic diversity and taxonomic incongruences.

1.4.1 **Genus Laodicea Lesson, 1843**

Laodicea Lesson, 1843 is the type taxon of family Laodiceidae Agassiz, 1862, and in turn it is based on the type species *Laodicea undulata* (Forbes &Goodsir, 1853) (Fig. 3). The number of currently recognized valid species in *Laodicea* is 8, including *Laodicea brevigona* (Allwein,1967), *Laodicea fertilis* (Lendenfeld. 1885), *Laodicea fijiana* (Agassiz & Mayer,1899), *Laodicea indica* (Browne,1905), *Laodicea marama* (Agassiz & Mayer, 1899), *Laodicea minuscula* (Vannucci, 1957), *Laodicea pulchra* (Browne, 1902), and *Laodicea undulata* (Forbes & Goodsir, 1853). The diagnostic characters of family Laodiceidae are: medusae with 4 or 8 simple radial canals, mouth 4- sided and attached to subumbrella, manubrium with lobes or manubrial pouches; umbrella with marginal cordyli, with or without cnidocysts; marginal tentacles hollow; gonads on radial canals; with or without marginal cirri; with or without adaxial ocelli; without statocysts; hydrothecae (when known) of *Cuspidella*-type, tubular, lacking a stalk but with operculum with several triangular plates that meet centrally and basally visible crease line(Cornelius, 1995). Within Laodiceidae, genus *Laodicea* is characterized by having medusae with small manubrium and 4 simple radial canals; the gonads are wavy, and the umbrella margin can be with or without cirri, but it always has adaxial ocelli and cordyli between the tentacles. The hydroids (when known) have the characteristics of the family (Cornelius, 1995; Bouillon et al., 2006).

Figure 3. General morphology of (A) a leptomedusa identified as *Laodicea undulata*, and (B) a *Cuspidella*-type hydroid (likely to belong to *Laodicea*). It was not possible to sequence these two specimens, and therefore the identification is uncertain. As a result, they were not included in further analysis, but they are presented here to show the habitus of the two life stages of the genus. Picture credits: (A) Joan J. Soto Ángel. (B) Lara Beckmann.

The type species of the genus, *Laodicea undulata*, is defined by the following characters:

- Medusa with umbrella up to 37 mm wide, usually flatter than a hemisphere, manubrium quadratic, short and attached to subumbrella. The manubrium is small, and it has small perradial lobes. The mouth has 4 folded lips. Four radial canals with simple wavy gonads adjacent to the manubrium and extending nearly to the umbrella margin. The marginal tentacles are hollow, either 200-300 (Russell, 1953a) or up to 400-600 (Kramp,1961), with very little basal swellings on the lateral side of the exumbrella. There are 1-2 spiral cirri between marginal tentacles, and ocelli located on adaxial sides of some marginal tentacle bases usually on each third to fifth tentacle. The ocelli appear as brown or black spots. There is usually one club-shaped cordylus (without nematocyst) between each pair of marginal tentacles. The colour of the manubrium, gonads and tentacle bases is usually light, varying between yellow, brown and pink (Kramp, 1959, 1961; Cornelius, 1995).

- Polyps with hydrotheca growing upward individually from a creeping stolon at varying angles and of different length. Hydroids of "*Cuspidella*" type and impossible to distinct from other *Cuspidella* polyps. The hydranth has 6-8 tentacles where half are short and half long(Russell, 1936; Cornelius, 1995). Hydrotheca length (diaphragm to operculum base) is 280-950 µm, width 90-110 µm, and the length of the opercular cusps is approximately the same as the width of the hydrotheca (Cornelius, 1995a).

This species has been observed in a wide depth range on both coastal and offshore waters, but all the published records are for the medusa stage and the real distribution of the hydroid is not documented. It has been recorded from most of the Atlantic Ocean between ca. 70°N and 55-30°S, including the Adriatic and Black Seas, and also from the China Sea. Its distribution is such that it is thought to occur nearly worldwide in coastal to shelf waters (Zheng *et al.*, 2014; Schuchert, Hosia and Leclère, 2017; Calder, 2019), as evidenced by the 208 records for this species in GBIF (GBIF database, consulted on 27 August 2021). Previous studies (Bouillon 1984a, 1985a; Cornelius_1995a, b) found taxonomic issues associated with the diversity in *Cuspidella*-type hydroids. All these hydroids are known to be difficult to distinguish and identification based on morphology alone is impossible.

1.4.2 **Genus Tiaropsis Agassiz, 1849**

The family Tiaropsidae Boero, Bouillon &Danovaro, 1987 is composed of three genera, including *Tiaropsis* Agassiz, 1849 with the type species *Tiaropsis multicirrata* (M. Sars, 1835) (Fig. 4). The number of currently recognized valid species in *Tiaropsis* is two, namely *Tiaropsis gordoni* (Bouillon & Barnett, 1999) and *Tiaropsis multicirrata* (M. Sars, 1835). The diagnostic characters of family Tiaropsidae are 4-8 radial canals on the medusa and 8 compound sense organs each containing an ecto-endodermal ocellus and an open statocyst. Like *Laodicea*, the hydroids of the Tiaropsidae are tiny, similar to those of "*Cuspidella*" type, with an operculum limited by several flaps that are not clearly divided at the base. The hydranths can retract completely into the hydrotheca (Bouillon and Danovaro, 1987; Bouillon et al., 2006). Within Tiaropsidae, genus *Tiaropsis* is characterized by 4 unbranched radial canals on the medusa stage, and by presenting only one kind of marginal tentacles besides lacking marginal cirri. The hydroids present the diagnostic characters of the family (Cornelius, 1995; Bouillon et al., 2006).

The type species of the genus, *Tiaropsis multicirrata*, is defined by the following characters:

- Medusa strongly flattened at sexual maturity, but even at a young stage the umbrella is flatter than a hemisphere, with thick jelly. Manubrium with small base, attached to subumbrella along the arms of a perradial cross. The mouth has four lips that may be exceedingly folded and frilly. The four radial canals contain the gonads, which are linear and covering ca. along middle of the radial canal's length. The medusa has 200-300 marginal tentacles. The eight compound sense organs each include an open statocyst with about 12-13 concretions and one black ocellus at its base. The medusa is able to swim briskly in any direction especially in young stages of life due to a high umbrella pulsation rate (Zelickman, Gelfand and Shifrin, 1969). The ocelli are present on the subumbrella in an adradial position(Kramp, 1959, 1961; Cornelius, 1995).The diameter recorded for the medusa is about 15-20 mm, sometimes up to 30 mm(Russell, 1953a) and the colour of manubrium, gonads and tentacle bases has been reported as dull yellow, with the tentacle bases with black pigment granules which may also be present in the dorsal walls of the ovaries (Russell, 1953a; Cornelius, 1995a). Otherwise, the manubrium and tentacles have also been described as light brown, with black-tinted gonads (Naumov, 1969; Cornelius, 1995a).

- The polyps are covered by the hydrothecae and grow up to 1 mm in height, arising from a creeping stolon. Hydrothecae tubular, with 1-3 twists on the stalk and conical operculum with 7-11 triangular cusps meeting centrally. Hydranth with16-20 tentacles, gonotheca arising singly from the stolon (Cornelius, 1995a; Rees, W. J. (1941). Height of operculum 80-250 µm; width 100-130 µm; hydrothecal pedicel 60-100 µm long; stolon diameter 50 µm (Cornelius, 1995a; Bouillon, et al 2006).

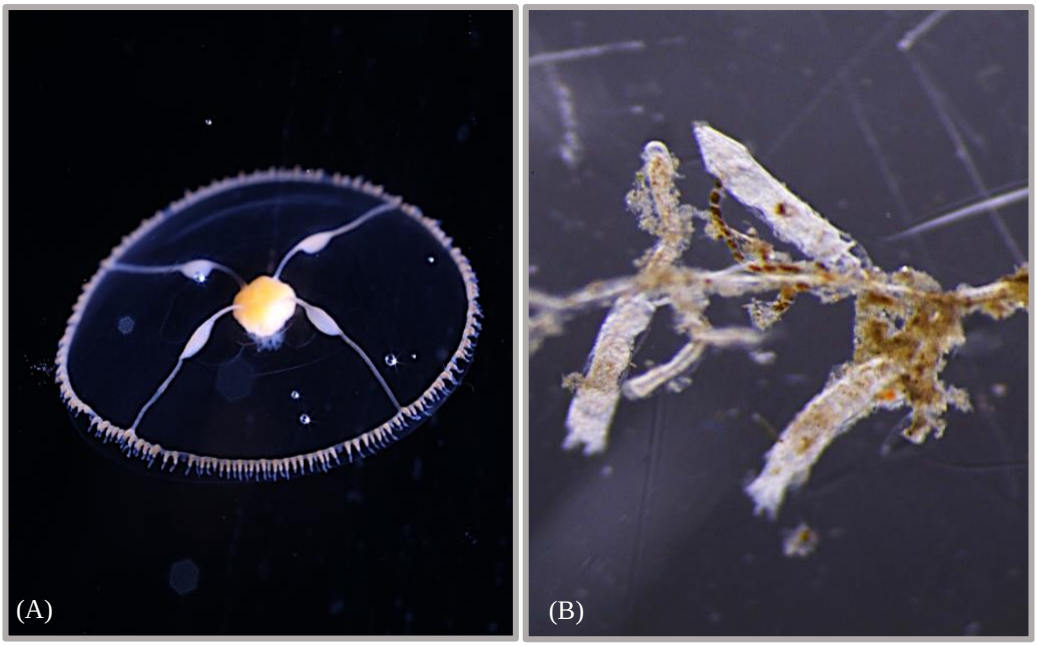


Figure 4. General morphology of (A) a leptomedusa identified as *Tiaropsis multicirrata*, and (B) a *Tiaropsis*-like hydroid. It was not possible to sequence these two specimens, and therefore the identification is uncertain. As a result, they were not included in further analysis, but they are presented here to show the habitus of the two life stages of the genus. Picture credits: (A) Joan J. Soto Ángel. (B) Luis Martell.

Tiaropsis multicirrata is distributed in the North Sea, with records both from Norway and around the British Isles (Leclère *et al.*, 2009; Kayal *et al.*, 2015; Schuchert, Hosia and Leclère, 2017). Other records from elsewhere in the Atlantic and Arctic Oceans are also available, including 71 occurrences reported in the GBIF database (consulted on 27 August 2021). The medusa is recorded from shallow coastal waters. Like with *Laodicea*, the hydroid has been found so rarely that its habitat is unknown, and no reliable records exist for the presence of this life stage in the wild (Cornelius, 1995a).

1.5 **AIM**

The main objective of this thesis is to assess the diversity of the genera *Laodicea* and *Tiaropsis* in Norwegian waters by using an integrative approach including molecular and morphological data and looking for potential taxonomic issues associated with cryptic and undescribed diversity. The specific aims of this work are:

1. To test the monophyly of *Laodicea undulata*, *Tiaropsis multicirrata*, Family Laodiceidae, and Family Tiaropsidae through phylogenetic analyses with Bayesian Inference and Maximum Likelihood.
2. To define the observed taxa with the aid of molecular species delimitation tools (ABGD, bPTP) and morphological data, and to test them for cryptic diversity.

3. To determine the distribution of *Laodicea* and *Tiaropsis*in Norwegian waters.

4. To produce complete, high quality DNA barcodes for 3 different markers (mitochondrial 16S and COI, nuclear ITS) for all target taxa.

2 Materials and methods

2.1 Sample collection

The specimens of *Laodicea* spp and *Tiaropsis* spp examined in this project were either borrowed from the Natural History Collections of the University Museum of Bergen or freshly collected from several marine localities along the Norwegian coast (Fig. 5). They were collected as part of the projects "Hydrozoan pelagic diversity in Norway (HYPNO)" and "Norwegian marine benthic Hydrozoa (NorHydro)", funded by the Norwegian Biodiversity Information Centre. The examined material (borrowed + fresh) includes both medusa and polyp stages, collected during the period 2006 – 2020.The sampling localities were determined by opportunity and accessibility but were intended to represent different environments along the coasts of Norway and different geographical regions. For the polyps, different hard substrates likely to harbour hydrozoan colonies were collected with a triangular dredge and examined under the stereomicroscope looking for hydroid colonies. The medusae were collected in vertical tows with either modified WP3 (750 µm mesh) or Nansen (500 µm mesh) plankton nets, or exceptionally through scuba diving by citizen scientists donating samples.

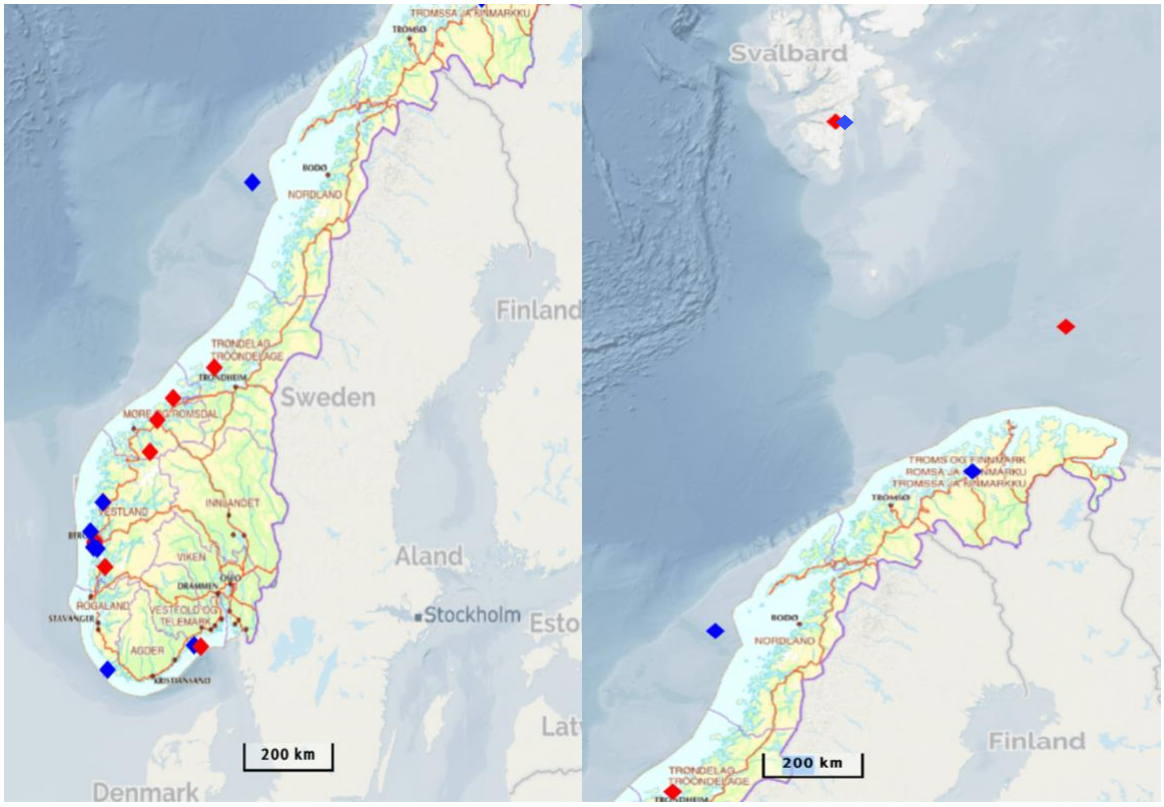


Figure5. Sampling sites for specimens used for the molecular analysis along the Norwegian coast. Red dots represent specimens belonging to families Laodiceidae and Tiarannidae (outgroup). Blue dots represent specimens belonging to families Tiaropsidae and Mitrocomidae (outgroup).

While sampling of hydrozoan polyps is rather straightforward and typical of benthic surveys, the collection of hydromedusae is less intuitive and deserves a more comprehensive explanation. To collect the fragile and delicate medusa stage a modified WP3 net with a mesh size of 750 µm was used, with slow towing speed of ca. 0.3 m/s and a non-filtering cod-end to avoid damage to the animals. The entire sample was gently transferred from the cod-end into a transparent sorting tray and placed on top of a light table (Fig. 6), and the jellyfish specimens were hand-picked with the aid of a broad-ended plastic pipette. The hydromedusae were then brought to the laboratory where they were documented alive for morphology.

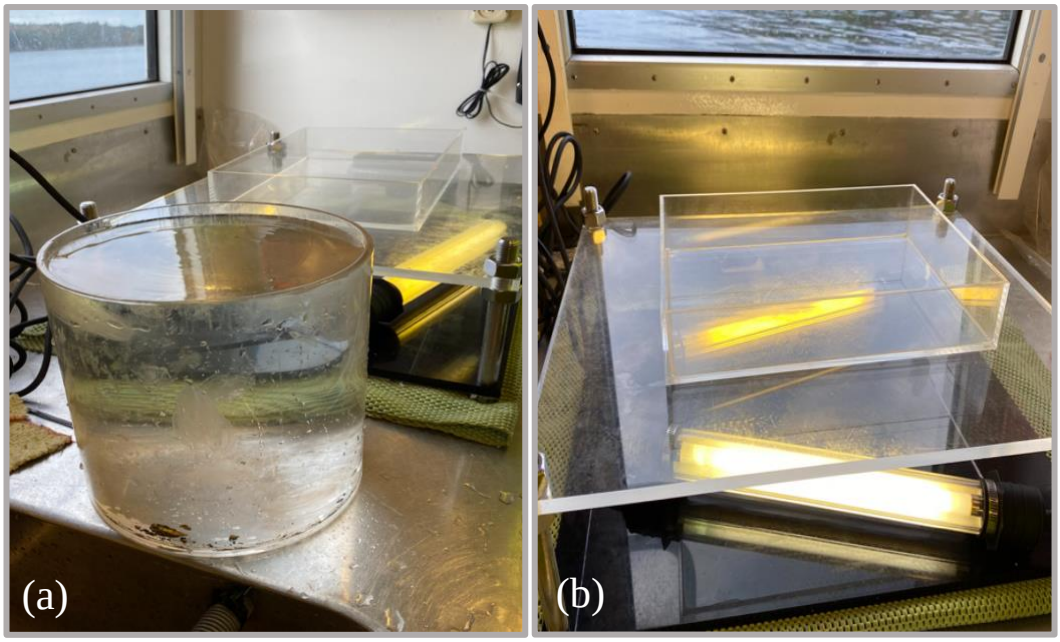


Figure 6. Example of a plankton sample as retrieved from the cod-end of the plankton net (a) and the set-up of the light table(b).

2.2 **Processing & Fixation**

All specimens collected were transported alive to the UMB Invertebrate laboratory and were examined with the aid of a stereomicroscope (OLYMPUS SZX16) and an optical microscope (LEICA DM6000 B). Their general morphology and diagnostic characters were documented through photographs, using a microscope-coupled camera (Canon EOS 6D) and associated software (EOS Utility 3.10.30). Photographic and video vouchers (e-vouchers) were generated for each specimen. A unique ID number was given to each specimen, and this number together with all the information associated with the sampling event (locality, depth, gear, date, etc.) were compiled in an electronic database. After documentation, the specimens intended for molecular analysis were fixated in 96% ethanol, while those to be used for morphological analysis were fixated in ~4 % formaldehyde in sea water buffered with sodium borate.

2.3 **Molecular Analyses**

2.3.1 **Tissue sampling**

Each ethanol-fixed specimen was transferred to a clean Petri dish and clean forceps were employed to take a small amount of tissue for molecular analysis. For polyps, several gonotheca or hydrothecae containing hydranths from the same colony were sampled to obtain approximately 2 mm^3 of tissue. For hydromedusae, 1-3 mm^3 of tissue were obtained from the marginal bulbs and umbrella of each individual for molecular analysis. Disinfection of the tools and working space was conducted between the tissue sampling of each specimen in order to avoid cross-contamination.

2.3.2 **DNA extraction**

Extraction, amplification and sequencing of DNA was carried out either at the DNA laboratory at the University of Bergen or at the Canadian Centre for DNA Barcoding (CCDB - Centre for Biodiversity Genomics, University of Guelph) by the staff at their sequencing facility. The samples sent to CCDB were processed according to the original protocols described in Ratnasingham and Hebert (2007).

In the DNA lab of the UiB, extraction of total DNA from the specimens was performed either following the protocol included by the manufacturer in the Qiagen DNeasy Blood & Tissue Kit (Hilden, Germany) or with the Quick Extract method (Quick Extract TM DNA Extraction Solution) modified after Nygren et al. 2018.

2.3.3 **Amplification of DNA**

Amplification of DNA was done by Polymerase Chain Reaction (PCR) using the enzyme Takara Taq. Three molecular markers (mitochondrial 16S and COI, nuclear ITS) were targeted during amplification (Table .1). Their respective primers and conditions for PCR are explained below:

Table 1. Target markers, primers, and PCR conditions.

Marker	Primer (5'-3')	PCR conditions	Reference
COI	LCO-1490 GGTCAACAAATCATAAAGATATTGG HCO-2198 TAAACTTCAGGGTGACCAAAAAATCA	a)5 min 94°C b)45 sec 94°C c)30 sec 45°C d) 1 min 72°C e) Go to b. and repeat 4x f) 45 sec 94°C g) 30 sec 50°C h)1 min 72°C i) Go to f. and repeat 30x j) 10 min 72°C	(Folmer *et al.*, 1994)
16S	SHA ACGGAATGAACTCAAATCATGT SHB TCGACTGTTTACCAAAAACAT	a)5 min 94°C b)30 sec 94°C c)30 sec 50°C d) 1 min 72°C e) Go to b. and repeat 39 x f)7 min 72°C	(Cunningham and Buss, 1993)
ITS	HITSF GCCGAAAAGTTGACCAAACTTGATC HITSR AGCGGGTAGTCTTGTCTGATCT	a) 5 min 94 °C b) 45 sec 94°C c) 55 sec 55°C d)1.5 min72°C e) Go to b. and repeat 34 x f) 10 min 72°C	(Fontana *et al.*, 2012)

2.3.4 **PCR, Gel electrophoresis and purification**

PCR products were run on agarose(1%inTAEbuffer) gels spiked with Gel red dye to evaluate the successful amplification. The amount of product was then roughly estimated for each PCR product from the UV-visualized gels with the aid of the software packages Gene Snap and Gene Tools (Syngene).

PCR products from successful amplifications (i.e., those that produced clear, correctly sized bands) were subsequently purified with a mixture of the cleaning enzymes EXOI and SAPunder the following conditions:

a) 37 °C for 30 min
b) 85 °C for 15 min

2.3.5 **Sequencing and assembling of contigs / editing of sequences**

The sequencing reactions were performed on ice following the Big Dye v.3.1 protocol of UiB sequencing facility (UIB, 2015). The software Geneious v.11.1.4was used for editing, assembling and pre-analyzing the DNA sequences. The final sequences were run through BLAST (Basic Linear Alignment Search Tool) as implemented in the NCBI website in order to confirm that they belonged to the target taxon (Hydrozoa).

2.4 **Phylogenetic Analysis**

2.4.1 **Alignment**

A FASTA file was created for each combination of marker (COI, 16S, and ITS) and taxon (Laodiceidae, Tiaropsidae) including all our consensus sequences and all additional sequences available in GenBank (http://www.ncbi.nlm.nih.gov) and BOLD (Barcode of Life Data System) (Ratnasingham, S. & Hebert, P. D. N. 2007). Based on the work of Maronna et al. (2016), available sequences belonging to families Tiarannidae and Mitrocomidae were included as outgroups in the FASTA files of Laodiceidae and Tiaropsidae, respectively. In total, this resulted in 6 datasets that were processed following the same pipeline. For each dataset, all sequences were quality checked by eye and contamination was ruled out using the Basic Local Alignment Search Tool (BLAST) as implemented in the NCBI website. The ITS datasets did not pass the quality-check at this stage due to highly ambiguous results and a general lack of sequences for this marker in the GenBank and BOLD databases, so ITS was not further included in the analysis and the rest of the methods correspond only to the COI and 16S datasets. The sequences were subsequently aligned with MUSCLE (version 3.8.31) as implemented in MEGA (Kumar et al., 2018; Stecher et al., 2020). The resulting six alignments were checked by eye and in the case of the two COI datasets they were translated to amino acids to control for stop codons. The complete data matrix with all the specimens included in the analysis and their sequences is shown in Tables2 and 3.

Table 2. Specimens of Laodiceidae and Tiarannidae used for the molecular and morphological analysis.

Specimen ID	Bold ID	Scientific name	Life stage	Locality / Area of collection	Latitude	Longitude	GenBank ID COI	GenBank ID 16S	ITS	Collection date	Refrence
HYPNO_0295	_	M_octocostatum	medusa	Korsfjord	60.184633	5.19595	_	_	_	14-Jun-16	This study
HYPNO_0363	_	L_undulata	medusa	Korsfjordouter	60.15195	5.099117	_	_	*	03-Aug-16	This study
HYPNO_0687	_	L_undulata	medusa	Korsfjordmid	60.184633	5.19595	_	_	*	10-Aug-17	This study
HYPNO_0949	_	Cuspidellasp	polyp	Vatlestraumen	60.33865	5.18607	_	_	*	26-Apr-17	This study
NH0519	_	L_undulata	medusa	Hardangerfjord	_	_	_	_	*	_	This study
NH0520	_	L_undulata	medusa	Hardangerfjord	60.25725	5.139333	_	_	*	06-Apr-17	This study
_	_	L_undulata	_	Mediterranean	_	_	MG811653	_	_	_	Schuchertunpub
_	GBCI9694-19	L_undulata	_	Sweden	58.2438	11.4323	MF000514	_	_	_	Schuchertunpub
_	GBCI2369-13	L_undulata	_	China	_	_	JQ716121	_	_	_	Zhengetal2014
_	GBCI2370-13	L_undulata	_	China	_	_	JQ716120	_	_	_	Zhengetal2014
HYPNO_0258	_	Laodiceasp	medusa	Korsfjordmid	60.184633	5.19595	_	_	*	28-Apr-16	This study
HYPNO_0637	_	Laodiceasp	medusa	Fanafjord	60.247283	5.286917	_	_	*	18-May-17	This study
NH0399	_	Laodiceasp	medusa	Kristiansund	63.0621807	7.6947671	_	_	*	06-Jun-19	This study
NH0400	_	Laodiceasp	medusa	Kristiansund	63.1152025	7.6649692	_	_	*	09-Jun-19	This study
NH0401	_	Laodiceasp	medusa	Kristiansund	63.1152025	7.6649692	_	_	*	09-Jun-19	This study
_	_	Laodiceasp	_	Hawaii	_	_	MW278608	_	_	_	ND
_	_	Laodiceasp	_	Hawaii	_	_	MW278609	_	_	_	ND
HYPNO_0948	_	Cuspidellasp	polyp	Korsfjord	60.14	5.000	_	_	_	26-Apr-17	This study
HYPNO_0189	_	P_crocea	medusa	Raunefjord	60.2573	5.13933	KY570314	KY570305	_	_	This study
HYPNO_0252	_	P_crocea	medusa	Korsfjord	60.184633	5.19595	_	_	_	28-Apr-16	This study
HYPNO_0286	_	P_crocea	medusa	Raunefjord	60.2573	13.7358	KY570318	KY570309	_	04-May-16	This study
HYPNO_0314	_	P_crocea	medusa	Raunefjord	60.2573	5.13933	KY570313	KY570304	_	14-Jun-16	This study
HYPNO_0355	_	P_crocea	medusa	Raunefjord	60.25725	5.139333	_	_	_	01-Jul-16	This study
HYPNO_0052	_	P_lactea	medusa	Svalbard	79.77866	18.115667	_	_	_	20-Aug-15	This study
HYPNO_0053	_	P_lactea	medusa	Svalbard	79.77866	18.115667	_	_	_	20-Aug-15	This study
HYPNO_0415	_	P_lactea	medusa	Svalbard	80.041	17.3315	_	_	_	09-Sep-16	This study
HYPNO_0416	_	P_lactea	medusa	Svalbard	80.041	17.3315	_	_	_	09-Sep-16	This study
HYPNO_0730	_	P_lactea	medusa	Svalbard	76.925	15.238333	_	_	_	13-Jul-16	This study
_	_	P_lactea	_	unknloc	_	_	_	KT809322	_	_	Kayaletal2015
HYPNO_0791	_	S_mertensii	medusa	Sotra	60.276125	5.116674	_	_	_	03-May-18	This study
HYPNO_0793	_	S_mertensii	medusa	Sotra	60.276125	5.116674	_	_	_	03-May-18	This study
_	_	S_mertensii	_	unknloc	_	_	_	KT809332	_	_	Kayaletal2015
_	_	S_mertensii	_	Fanafjord	_	_	MF000507	_	_	_	Schuchertunpub
_	HA703_14	Staurostomasp	_	AtlanticCanada	_	_	MF000508	_	_	_	ND
_	KHA704_14	Staurostomasp	_	AtlanticCanada	_	_	MF000509	_	_	_	ND
_	KHA705_14	Staurostomasp	_	AtlanticCanada	_	_	MF000510	_	_	_	ND
_	KHA706_14	Staurostomasp	_	AtlanticCanada	_	_	MF000511	_	_	_	Ortmanetal2010
_	_	C_rubrum	_	unknloc	_	_	MF000512	_	_	_	ND

HYPNO_0190	_	M_rotunda	medusa	Raunefjord	60.2573	5.13933	_	_	_	04-Apr-16	This study
HYPNO_0331	HYPNO184-16	M_rotunda	medusa	Sognefjord	61.0434	5.43153	_	_	_	21-Jun-16	This study
HYPNO_0350	HYPNO265-17	M_rotunda	medusa	Korsfjordout	60.152	5.09912	_	_	_	01-Jul-16	This study
HYPNO_0367	HYPNO236-17	M_rotunda	medusa	Korsfjordmid	60.1846	5.19595	_	_	_	03-Aug-16	This study
HYPNO_0845	_	M_rotunda	polyp	AgdenesTrondelag	63.59465	9.50252	_	_	_	26-Oct-16	This study
HYPNO_0853	_	S_plicatile	polyp	AgdenesTrondelag	63.65323	9.76393	_	_	_	24-Oct-16	This study
HYPNO_0871	_	P_crocea	polyp	Kristiansund	63.574	7.42167	_	_	_	04-Oct-13	This study
HYPNO_0875	_	S_plicatile	polyp	Skagerrak	58.79	9.6767	_	_	_	27-Jun-06	This study
HYPNO_0882	_	S_plicatile	polyp	Skagerrak	10.2639	50.000	_	_	*	16-May-09	This study
_	SWEMA1056_15	S_plicatile	_	Sweden	_	_	MG935063	_	_	_	unpub
_	SWEMA1055_15	S_plicatile	_	Sweden	_	_	MG935003	_	_	_	unpub
_	_	S_plicatile	_	Flesland	_	_	JN109191	_	_	_	Schuchertunpub
HYPNO_0872	_	S_plicatile	polyp	BarentsSea	72.10667	34.30033	_	_	*	05-Aug-13	This study
HYPNO_0873	_	S_plicatile	polyp	BarentsSea	71.77683	33.54033	_	_	_	07-Aug-13	This study
HYPNO_0874	_	S_plicatile	polyp	BarentsSea	72.5805	32.38367	_	_	_	03-Aug-13	This study
_	_	L_undulata	_	Mediterranean	_	_	_	FJ550471	_	_	Leclereetal2009
_	_	L_undulata	medusa	Mediterranean	43.6963	7.3075	_	KY363967	_	25-Apr-16	Schuchertetal2017
_	_	L_undulata	medusa	Sweden	58.2438	11.4323	_	KY363963	_	03-Oct-14	Schuchertetal2017
_	_	L_undulata	_	China	_	_	_	JQ715947	_	_	Zhengetal2014
_	_	L_undulata	_	China	_	_	_	JQ715946	_	_	Zhengetal2014
_	_	Melicertissasp	_	unknloc	_	_	_	AY512515	_	_	Collinsetal2005
_	_	P_crocea	medusa	Korsfjord	60.1846	5.196	_	KY363959	_	14-Jun-16	Schuchertetal2017
_	_	P_crocea	medusa	Korsfjord	60.1846	5.196	_	KY363953	_	14-Jun-16	Schuchertetal2017
_	_	P_crocea	medusa	Korsfjord	60.1846	5.196	_	KY363958	_	14-Jun-16	Schuchertetal2017
HYPNO_0287	_	P_crocea	medusa	Raunefjord	60.2573	5.13933	_	KY570310	_	04-May-16	This study
_	_	P_crocea	_	unknNAtlantic	_	_	_	KJ866187	_	_	Licandroetalunpub
HYPNO_0801	_	S_mertensii	medusa	BarentsSea	ND	ND	_		_	ND	This study
HYPNO_0802	_	S_mertensii	medusa	BarentsSea	ND	ND	_		_	ND	This study
_	_	S_mertensii	_	Fanafjord	_	_	_	KY363948	_	_	Schuchertunpub
_	_	Staurostomasp	_	unknNorway	_	_	_	MF662613	_		Halsbandetal2017
HYPNO_0914	_	M_rotunda	polyp	MoreogRomsdal	62.75167	6.94083	_		_	12-Oct-05	This study
_	_	M_rotunda	_	Mediterranean	_	_	_	FJ550476	_	_	Leclereetal2009
_	_	M_rotunda	_	GulfCadiz	_	_	_	JN714674	_	_	Mouraetal2012
_	_	S_bathyale	_	Madeira	_	_	_	JN714679	_	_	Mouraetal2012
_	_	S_bathyale	_	Azores	_	_	_	JN714678	_	_	Mouraetal2012
_	_	S_bathyale	_	Lisbon	_	_	_	JN714680	_	_	Mouraetal2012
HYPNO_0884	_	S_plicatile	polyp	Vatlestraumen	60.33788	5.18403	_	_	_	06-Feb-06	This study
_	_	S_plicatile	_	Raunefjord	_	_	_	FJ550513	_	_	Leclereetal2009
_	_	S_plicatile	polyp	Korsfjord	60.1673	5.2541	_	KY363944	_	04-May-10	Schuchertetal2017
_	_	S_plicatile	_	Antarctica	_	_	_	FN424152	_	_	PenaCanteroetal2010

Table 3. Specimens of Tiaropsidae and Mitrocomidae used for the molecular and morphological analysis.

Specimen ID	Bold ID	Scientific name	Life stage	Locality/Area of collection	Latitude	Longitude	GenBank ID COI	GenBank ID 16S	ITS	Collection date	Refrence
HYPNO_0361	_	L_undulata	medusa	Raunefjord	60.25725	5.139333		_	_	03-Aug-16	This study
HYPNO_0189	_	P_crocea	medusa	Raunefjord	60.2573	5.13933	KY570314	KY570305	_	_	This study
_	GBCI9667-19	Tmulticirrata	_	Iceland	64.0401	_	MF000487	_	_	18-May-2000	Schuchertunpub
_	GBCI9668-19	Tmulticirrata	_	Iceland	64.0401	-22.7152	MF000488	_	_	18-May-2000	Schuchertunpub
_	GBCI9683-19	Tmulticirrata	_	Raunefjord	60.274	5.2035	MF000503	_	_	15-Jun-2006	Schuchertunpub
_	_	Tmulticirrata	_	unkloc	_	_	KT809326	_	_		Kayaletal2015
HYPNO_0020	_	Tmulticirrata	medusa	Skagerrak	57.6997	9.78533	_	_	_	26-Apr-15	This study
HYPNO_0025	_	Tmulticirrata	medusa	Skagerrak	57.8337	6.94883	_	_	_	27-Apr-15	This study
HYPNO_0513	_	Tmulticirrata	medusa	Fanafjord	60.247283	5.286917	_	_	_	06-Apr-17	This study
HYPNO_0521	_	Tmulticirrata	medusa	Raunefjord	60.25725	5.139333	_	_	_	06-Apr-17	This study
HYPNO_0569	_	Tmulticirrata	medusa	NorthSea	59.9446	5.077767	_	_	_	30-Apr-17	This study
HYPNO_0735	_	Tmulticirrata	medusa	Svalbard	76.828425	15.488108	_	_	_	14-Jul-16	This study
HYPNO_0814	_	Tmulticirrata	medusa	Hjeltefjord	60.39605	5.148117	_	_	*	23-May-18	This study
HYPNO_1149	_	Tmulticirrata	medusa	Raunefjord	60.274	5.203485	_	_	_	15-Jun-06	This study
NH0042	_	Tmulticirrata	polyp	NorthSea	60.238033	5.2054	_	_	*	29-Mar-19	This study
_	KHBC218-13	Tmulticirrata	_	VancouverAquarium	_	_	_	_	_	_	ND
_	GBCI8838-19	Mpolydiademata	_	unknloc	_	_	KU710349	_	_	_	Kayaletal2015
_	_	Mpolydiademata	_	HudsonBayAtlanticCanada	_	_	MG423333	_	_	_	ND
_	GBCI9688-19	Mpolydiademata	_	Fanafjord	60.2408	5.2294	MF000508	_	_	23-Apr-15	Schuchertunpub
_	GBCI9681-19	Mpolydiademata	_	Scotland	56.455	-5.434	MF000501	_	_	11-May-2004	Schuchertunpub
HYPNO_0001	HYPNO001-15	Mpolydiademata	_	Skagerrak	58.882	9.686	_	_	_	25-Apr-15	This study
HYPNO_0229	HYPNO256-17	Mpolydiademata	_	NorthSea	57.000	3.65	_	_	_	15-Apr-16	This study
HYPNO_0951		Mpolydiademata	_	Skagerrak	58.6342	10.2639	_	_	_	16-May-09	This study
_	GBCI9692-19	Mniwai	_	NewZealand	-36.8123	174.803	MF000512	_	_	31-Jul-2002	Schuchertunpub
_	GBCI9686-19	Hocellata	_	Raunefjord	60.2748	5.2027	MF000506	_	_	22-Apr-15	Schuchertunpub
HYPNO_0262	HYPNO144-16	Hocellata	_	midKorsfjord	60.1846	5.19595	_	_	_	28-Apr-16	This study
HYPNO_0470	HYPNO241-17	Hocellata	_	Svalbard	78.92	12.1866	_	_	_	25-Aug-16	This study
_	_	Mbrownei	_	Roscoff	_	_	MF000485	_	_	_	Schuchertunpub
HYPNO_1444	_	Epanicula	medusa	NorwegianSea	ND	ND	_	_	_	ND	This study
HYPNO_1448	_	Epanicula	polyp	Valencia	39.492557	0.158656	_	_	_	07-Aug-17	This study
HYPNO_1449	_	Epanicula	polyp	Valencia	39.504465	0.02172	_	_	_	10-Aug-17	This study
HYPNO_0247	_	panicula	medusa	midKorsfjord	60.184633	5.19595	_	_	_	28-Apr-16	This study
HYPNO_0282	_	Epanicula	medusa	Raunefjord	60.25725	5.139333	_	_	_	04-May-16	This study
HYPNO_0594	_	Epanicula	medusa	NorthSea	60.738633	4.744667	_	_	_	01-May-17	This study
HYPNO_0618	_	Epanicula	medusa	Sognefjorden	61.37275	7.38715	_	_	_	05-May-17	This study
HYPNO_0324	_	Cproducta	medusa	midKorsfjord	60.184633	5.19595	_	_	_	14-Jun-16	This study
HYPNO_0325	_	Cpilosella	medusa	midKorsfjord	60.184633	5.19595	_	_	_	14-Jun-16	This study
HYPNO_0570	_	Cproducta	medusa	NorthSea	59.9446	5.077767	_	_	_	30-Apr-17	This study

HYPNO_0947	_	Cproducta	polyp	Skagerrak	58.755	9.6567	_	_	_	27-Jun-06	This study
_	_	Tmulticirrata	medusa	Raunefjord	60.274	5.2035	_	KY363943	_	15-Jun-2006	Schuchertetal2017
_	_	Tmulticirrata	_	Iceland	_	_	_	FJ550468	_	_	Leclereetal2009
_	_	Tmulticirrata	_	unkloc	_	_	_	KT809326	_	_	Kayaletal2015
_	GBCI8838-19	Mpolydiademata	_	unkloc	_	_	_	KU710349	_	_	Kayaletal2015
_	_	Mpolydiademata	medusa	Fanafjord	60.2408	5.2294	_	KY363949	_	23-Apr-15	Schuchertetal2017
_	_	Mpolydiademata	medusa	Scotland	56.455	5.434	_	KY363939	_	11-May-2004	Schuchertetal2017
_	_	Hocellata	medusa	Raunefjord	60.2748	5.2027	_	KY363947	_	22-Apr-15	Schuchertetal2017
_	_	Mniwai	_	NewZealand	_	_	_	FJ550473	_	_	Leclereetal2009
_	_	Cpilosella	medusa	Korsfjord	60.1846	5.196	_	KY363955	_	14-Jun-16	Schuchertetal2017
_	_	Mbrownei	_	Roscoff	_	_	_	KX355404	_	_	Schuchertunpub
_	_	Cpanicula	_	GulfCadiz	_	_	_	JN714650	_	_	Mouraetal2012
_	_	Cpanicula	_	AlboranSea	_	_	_	JN714649	_	_	Mouraetal2012
_	_	Cpanicula	_	GulfCadiz	_	_	_	JN714648	_	_	Mouraetal2012

2.4.2 **Selection of the best evolution model and construction of tres**

For each alignment, the best evolution model was identified with the use of Model Finder (Kalyaanamoorthy et al. 2017) as implemented in the IQ-TREE online web server, version 1.6.12 (Nguyen, et al. 2015). In all cases, the best model was selected following the Bayesian Information Criterion (BIC). The selected models for each dataset are shown in Table 4. Phylogenetic trees were constructed following both the Maximum Likelihood (ML) and Bayesian approaches.

Table 4. Selected evolution models:

Best evolution models **Laodiceidae + Tiarannidae**		**Criterion** Bayesian information criterion (BIC)
Gene / partition	**Best model**	**Input data**
16S	K3Pu+F+I+G4	57 sequences with 604 nucleotide sites
COI	TIM2+F+I+G4	54 sequences with 658 nucleotide sites

Best evolution models **Tiaropsis + Mitrocomidae**		**Criterion** Bayesian information criterion (BIC)
Gene / partition	**Best model**	**Input data**
16S	TIM2+F+I+G4	40 sequences with 589 nucleotide sites
COI	GTR+F+I+G4	39 sequences with 657 nucleotide sites

2.4.3 **Maximum Likelihood (ML)**

ML analyses were performed through the IQ-TREE online web server (Nguyen, et al. 2015) for each marker (COI and 16S)/case study. The following settings were used: substitution models were correspondingly set; gamma rate categories were set to 4 and support values were estimated with 1000 ultrafast bootstrap (UFBoot) replicates (Hoang et al. 2017). FigTree 1.4.4 was used for visualizing and editing the phylogenetic trees.

2.4.4 **Bayesian Inference (BI)**

Bayesian inference was performed with MrBayesv.3.2.7 (Ronquist et al. 2012) for each marker/case study. Each run of the Markov chains consisted of 500,000 generations, by which time < 0.01 average standard deviation of split frequencies was achieved. Convergence of each run was checked by Tracer 1. 7. 1 (Rambaut et al. 2018). Effective Sample Size (ESS)was >200 for each parameter. Burn-in was set at 25% in all cases. Posterior probabilities were calculated for all nodes on the resulting trees. FigTree 1.4.4 was used for visualizing and editing the phylogenetic trees.

2.5 **Molecular Species delimitation**

The molecular data were further analyzed through two species delimitation methods: ABGD (Puillandre *et al.*, 2012) and bPTP (Zhang *et al.*, 2013).

ABGD (Automatic Barcode Gap Discovery) is a method used to detect a gap between intra and interspecific data. In this work, each alignment was analyzed with ABGD using the online web service (https://bioinfo.mnhn.fr/abi/ public/abgd/abgdweb.html) with the following parameters: Pmin = 0.001; Pmax = 0.1; Steps = 10; X (relative gap width) = 1, model Kimura 2-parameter.

BPTP (Bayesian Poisson Tree Processes)(Zhang *et al.*, 2013) was performed on the Exelis lab web server (http://species.h-its.org/ptp) using the Bayesian inferred trees as input. The analysis was run for 200000 MCMC generations, with a thinning value = 100 and burn-in of 25%.

2.6 **Morphological Analysis**

The specimens, electronic vouchers and photographs were examined thoroughly in order to identify the set of distinctive taxonomic features that characterized each of the recovered clades. All observations were done with the aid of a stereomicroscope (OLYMPUS SZX16) and/or an optical microscope (LEICA DM6000 B) using a microscope-coupled camera (Canon EOS 6D). For each specimen, the following diagnostic characters were evaluated:

a) For the polyps: shape of the hydrotheca, number of tentacles, shape of opercular plates
b) For the leptomedusae: shape and size of umbrella, number of marginal tentacles, shape of manubrium, position of gonads, number of cordyli, position of ocelli.

A database was constructed with the morphological information per specimen, and it was analyzed in search of patterns that allow the characterization of the identified clades.

3 Results and Discussion

A total of 129 specimens were examined in this work.78 of them belonged to the study case 1 (Laodiceidae + outgroup), while 51 belonged to the study case 2 (Tiaropsidae + outgroups). Although both the medusa and polyp stages were represented in the examined material, the percentage of individuals in each stage was very different relative to the total data set (43.41% and 13.17%, respectively). This predominance of hydromedusae in collections of Laodiceidae and Tiaropsidae is in agreement with previous studies. For example, for *Laodicea*, the only previous records of the polyp stage are those of Hincks (1868), Russell (1936), Ramil and Vervoort (1992), Ramil, Ansín and Fernández (1998), and De Vito *et al.*(2006), and for *Tiaropsis* the polyp stage has only been found before by (Rees and Sc, 1941).

3.1 **DNA barcodes and sequencing success**

In the present work, complete, quality-checked DNA barcodes were produced for all the target taxa for the mitochondrial markers COI and 16S. On the other hand, the use of ITS as a barcode for species in Laodiceidae and Tiaropsidae was unsuccessful and not a single barcode was obtained for this marker. ITS has successfully been used for species delimitation in several hydrozoan taxa such as *Hydra*, *Pandeidae* and *Aglaopheniidae* (Schwentner and Bosch, 2015; Postaire *et al.*, 2016; Schuchert, 2018), but most of these cases are restricted to the Anthothecata. This marker has also been useful for species delimitation in other marine invertebrate taxa such snails and polychetes (Armbruster and Bernhard, 2000; Nygren, 2014). For *Laodicea* and *Tiaropsis*, obtaining informative ITS sequences were not possible due to the presence of different copies of this gene in the same individual. This has also been reported for other marine animal taxa and the conclusion seems to be that ITS produces mixed results and it is useful for species delimitation of some groups but not for others. This is obviously related to the fact that some marker works better than others in some taxa (Zheng *et al.*, 2014).

3.2 **Genus *Laodicea* in Norway**

Based on molecular evidence, *Laodicea undulata*, the only valid species of *Laodicea* previously recorded in the area, is shown to comprise two distinct clades in Norwegian waters, and these clades appear not to be sister species (Figs.7&8). At a worldwide scale, three clades were identified with marker 16S and four clades with marker COI, providing strong evidence for the existence of cryptic diversity in this taxon. The phylogenetic results from both markers in both ML and BI approaches are highly congruent and all the clades are strongly supported in all trees with posterior probabilities/ bootstrap values of ≥0.99 and ≥78, respectively. The two molecular delimitation methods (ABGD and bPTP) were also concordant in their results for COI and 16S, suggesting two different potential species under the name *Laodicea undulata* in Norway and 3-4 worldwide (Figs.7 & 8).

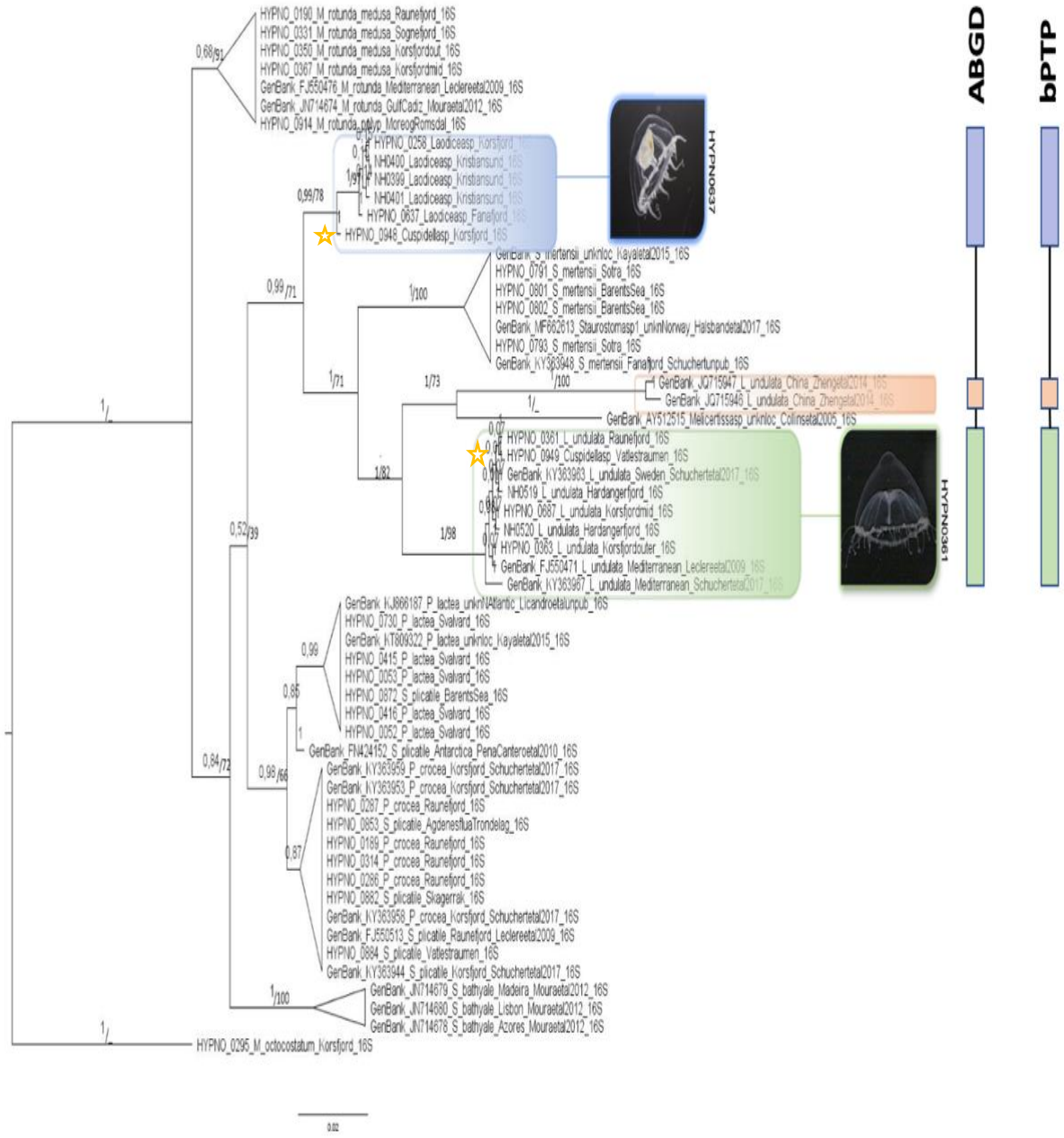


Figure 7. Bayesian Inference gene tree for 16S and species delimitation results of ABGD and bPTP for the Laodiceidae. The Maximum Likelihood tree had the same topology so only the BI results are shown. However, both posterior probabilities and bootstrap values are included for each node. ☆ Represent the polyp stage.

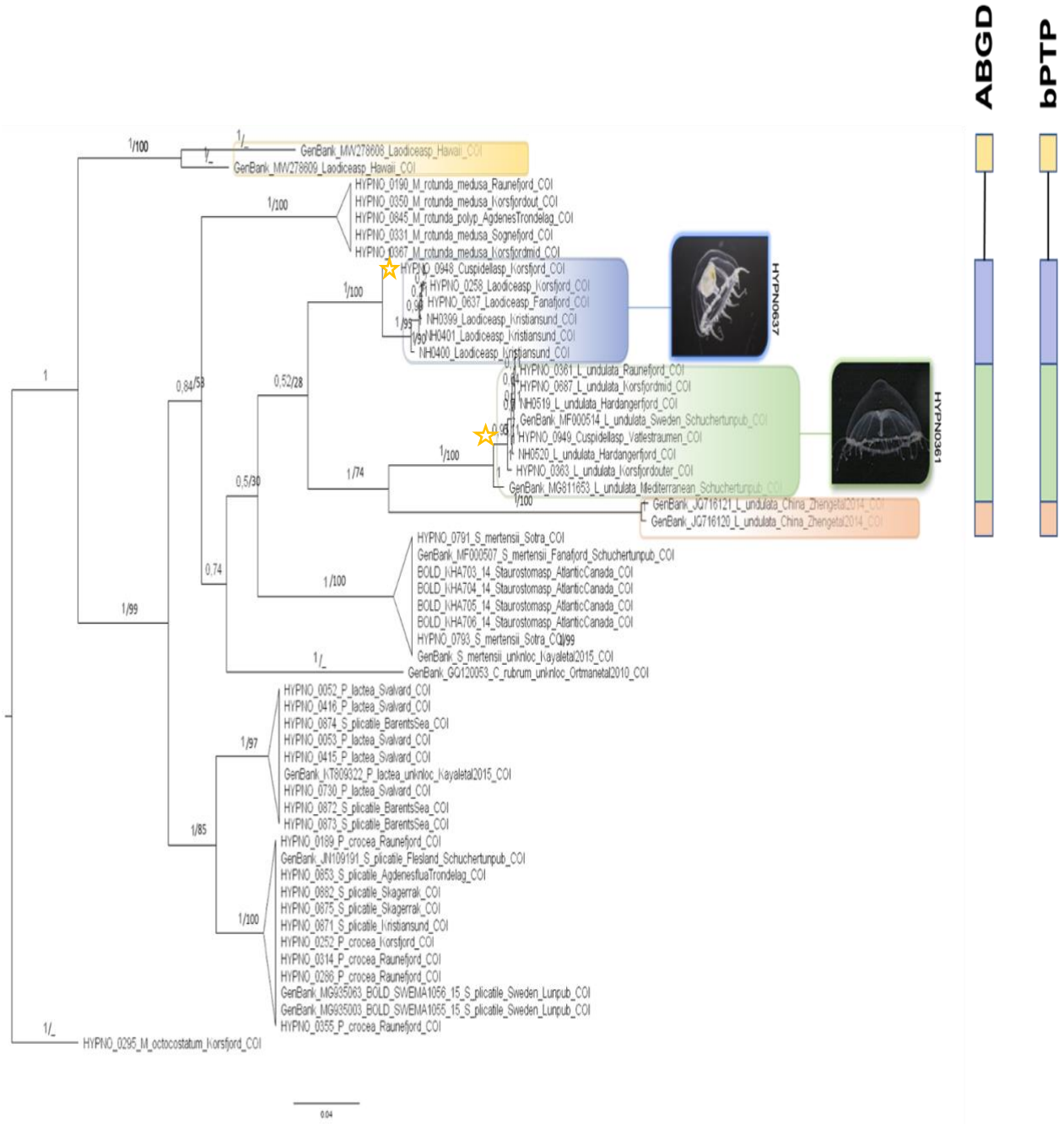


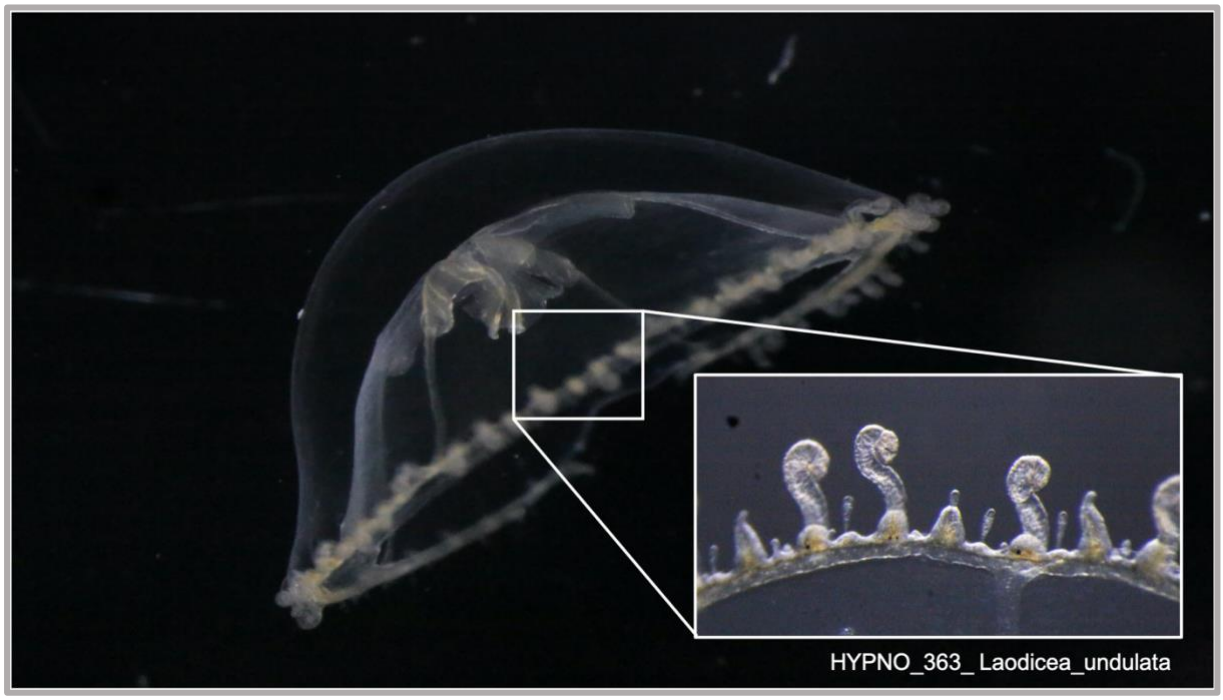
Figure 8. Bayesian Inference gene tree for COI and species delimitation results of ABGD and bPTP for the Laodiceidae. The Maximum Likelihood tree had the same topology so only the BI results are shown. However, both posterior probabilities and bootstrap values are included for each node. ☆ Represent the polyp stage.

The occurrence of more than one species of *Laodicea* in Norway is surprising because all the previous records of the genus in the country correspond to *Laodicea undulata*. Previously, this species was observed for example in Korsfjord (Hosia and Båmstedt, 2007), Tromsø (Kramp&Damas1925), and in Hadselfjord (Kramp&Damas1925). In fact, *L. undulata* is the most commonly reported species of *Laodicea* in the entire north-eastern Atlantic Ocean. In this area the records of the species are numerous and are summarized by Kramp (1961). Other nominal species of *Laodicea* have been reported from the NE Atlantic, but all of them are either synonymised with *L. undulata* or are considered *taxa inquirenda* (WORMS, 2021). A *taxon inquirendum* refers to a taxon that is not correctly defined and its validity in the taxonomic structure is still uncertain so there is no possibility to identify the taxon completely. The records for these species are very few, restricted to their description, and are all older than 1950.These species are *Laodicea ocellata* Babnik, 1948 and *Laodicea bigelowi* Neppi& Stiasny, 1911 (type locality in the Adriatic Sea), *Laodicea ulothrix* Haeckel, 1879 (type locality Canary Islands), *Laodicea cruciata* Forsskål, 1775 (type locality likely southern North Sea, but unrecognizable species), and *Laodicea chapmani* Günther, 1903 (type locality Western Ireland). Based on their original descriptions all these species are unrecognizable from the current concept of *Laodicea undulata* and it cannot be evaluated whether they are valid or not until a worldwide review of the genus is conducted. The specimens examined in this work do not fit any of these species given the few characters included in their descriptions.

Both the family Laodiceidae and the genus *Laodicea* are not recovered as monophyletic in the analysis. Including more markers and more samples from all over the world is necessary to evaluate correctly the monophyly of these taxa, but my results suggest that some members of the family Tiarannidae belong inside the family Laodiceidae. This is not surprising as the jellyfish from both families (for example the jellyfish of the tiarannid *Modeeria* and the jellyfish of the laodiceid *Ptychogena*) are very similar and both families are united by the presence of the cordyli, which are absent from all other families of Leptothecata (Cornelius, 1995).

No morphological diagnostic characters were identified for any of the clades of *Laodicea* present in Norway, and the genus could therefore be considered a species complex. The morphology of the specimens of *Laodicea* is very variable and a larger number of individuals from different developmental stages is perhaps required to correctly define these taxa morphologically.

A selection of the morphological variation in the recovered clades is presented in Figs. 9-11.



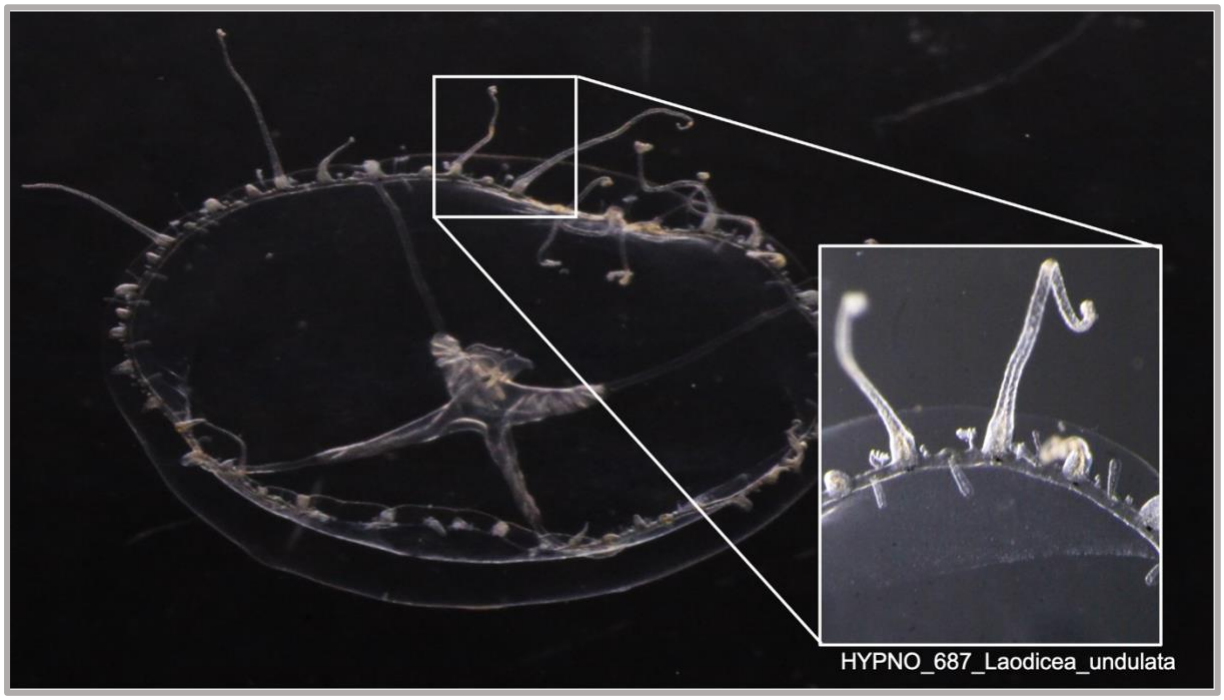


Figure 9. General morphology of two leptomedusae specimens corresponding to clade 1 of *Laodicea undulata* (temporarily named *Laodicea undulata*). Details of the umbrella margin are included.

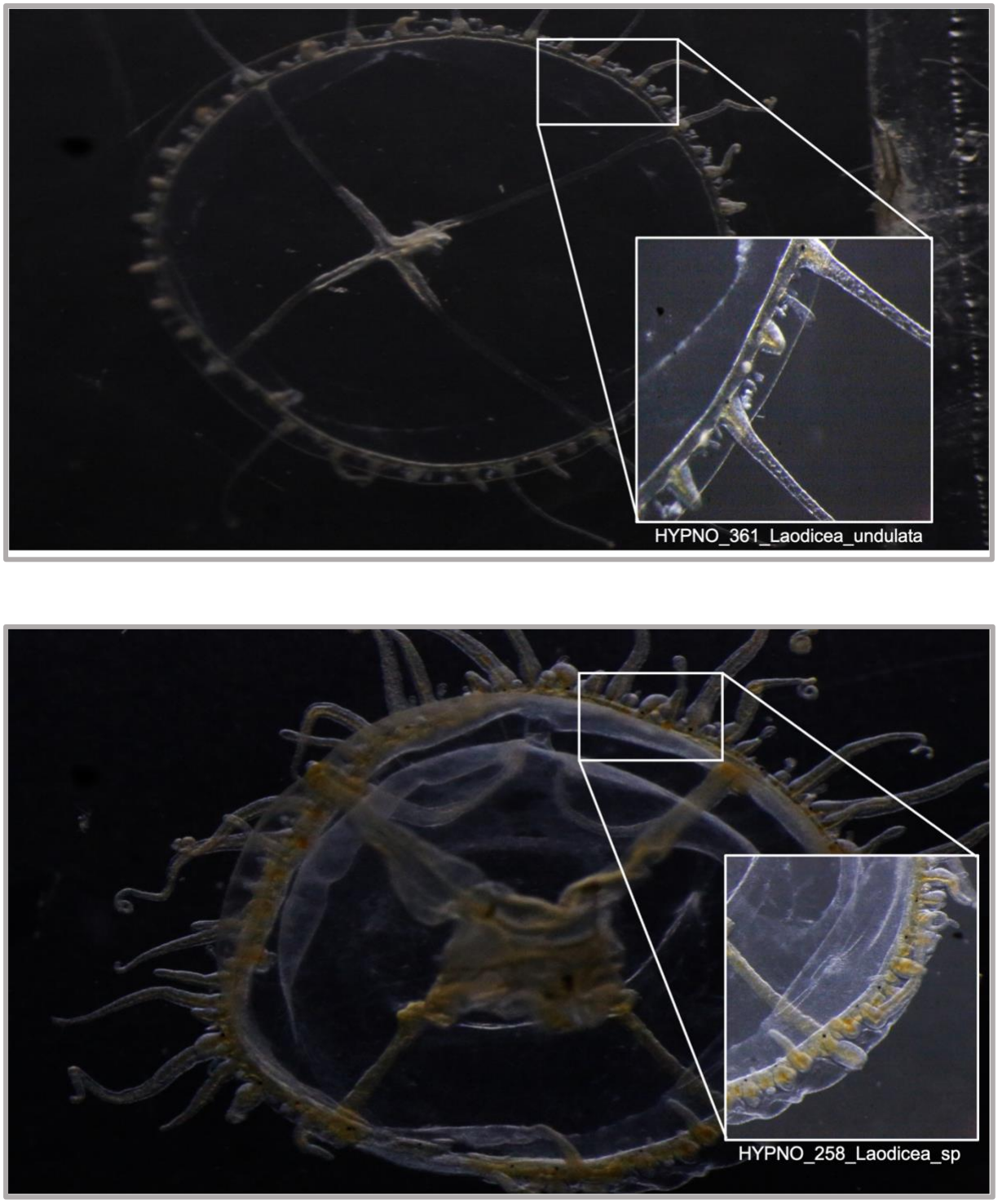


Figure 10. General morphology of two leptomedusae specimens corresponding to clade 1 (top) and clade 2 (bottom) of *Laodicea undulata* (the clades are temporarily named *Laodicea undulata* and *Laodicea* sp., respectively). Details of the umbrella margin are included.

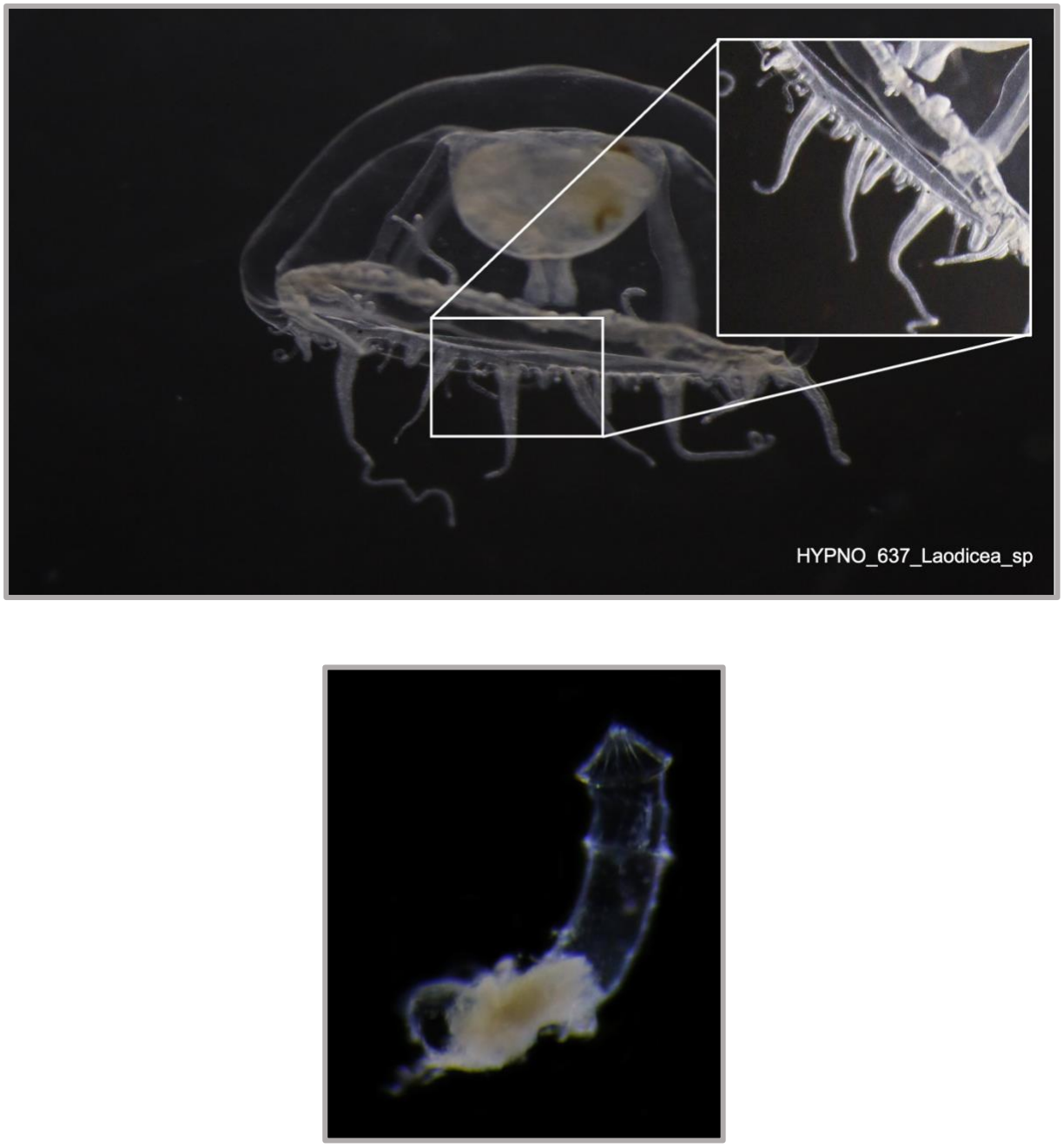


Figure 11. General morphology of one leptomedusa (top) and one polyp(bottom) specimen corresponding to clade 2 of *Laodicea undulata* (this clade is temporarily named *Laodicea* sp.). Details of the umbrella margin are included for the leptomedusa.

This work represents the first unambiguous record of the polyp stage of *Laodicea undulata* in the sea. Previously the observations of the polyps were made based on hydroids obtained from rearing the medusae in the laboratory (Russell, 1936). The polyps of *Laodicea* are very small, easy to overlook, and cannot be identified with certainty based only on morphological characters (Cornelius, 1995). The use of DNA barcodes was necessary for detecting the presence of the polyps in marine benthic habitats, and this highlights how the assessment of biodiversity needs to use integrative approaches to reach a comprehensive list of the taxa present in a given locality.

3.3 **Genus *Tiaropsis* in Norway**

Tiaropsis multicirrata is the only species of genus *Tiaropsis* that has been recorded previously in Norway. The results of the present study show that instead of only one, three distinct clades of this taxon are present in Norwegian waters (Figs. 10 & 11). The splitting of *Tiaropsis* in three clades was recovered in both molecular markers 16S and CO1, indicating the presence of cryptic diversity in this genus in the studied region. Very few sequences are available for *Tiaropsis* in GenBank and BOLD, and all of them come from individuals collected in the northeastern Atlantic Ocean. These sequences fell into the clades present in Norway. All the analyses show a strong support for each of the three *Tiaropsis* clades (posterior probabilities and bootstrap values of 1 and ≥95, respectively), and they were recovered as different potential species in the two molecular delimitation methods (ABGD and bPTP) (Figs.12&13).

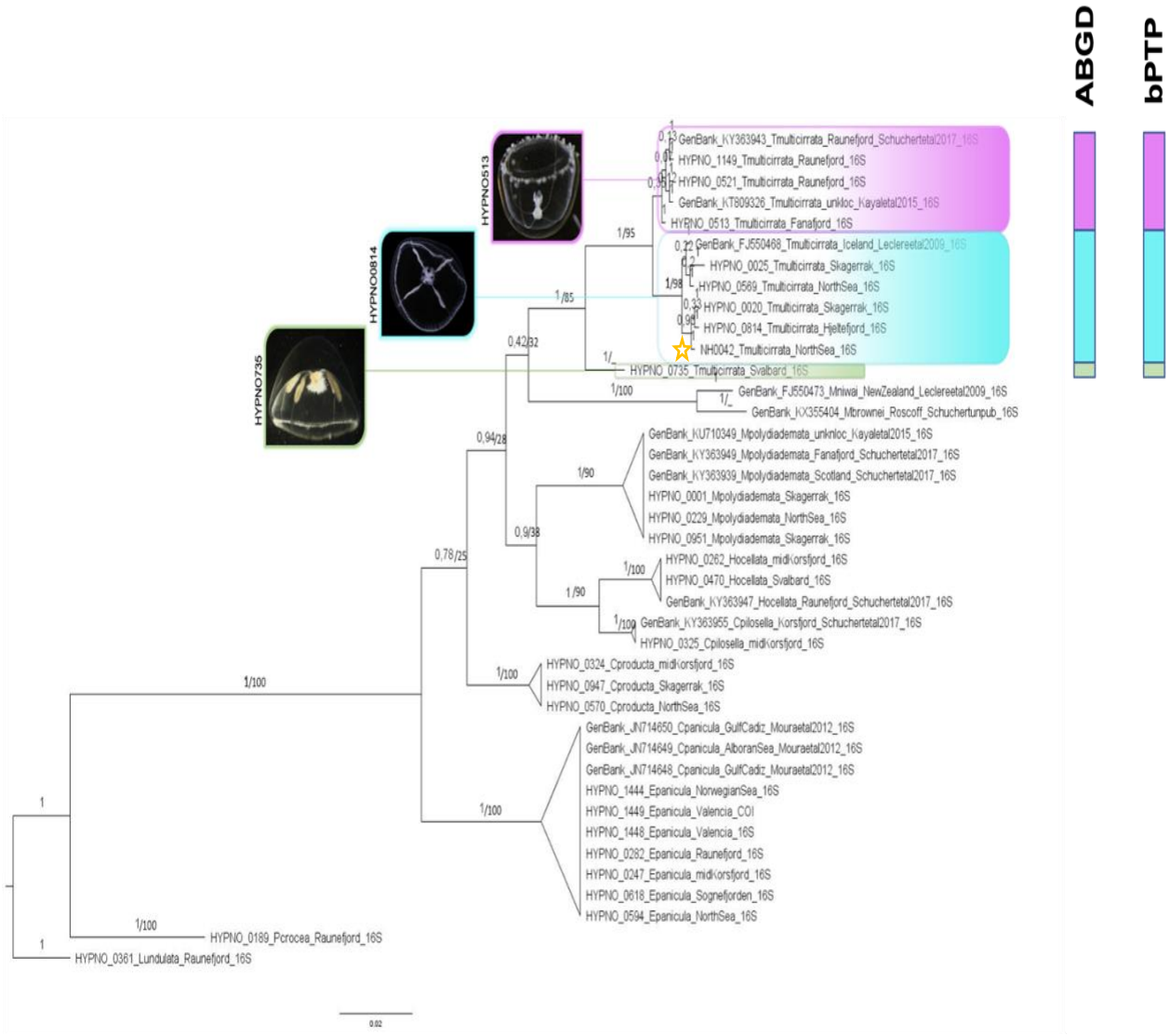


Figure 12. Bayesian Inference gene tree for 16S and species delimitation results of ABGD and bPTP for the Tiaropsidae. The Maximum Likelihood tree had the same topology so only the BI results are shown. However, both posterior probabilities and bootstrap values are included for each node. ☆ Represent the polyp stage.

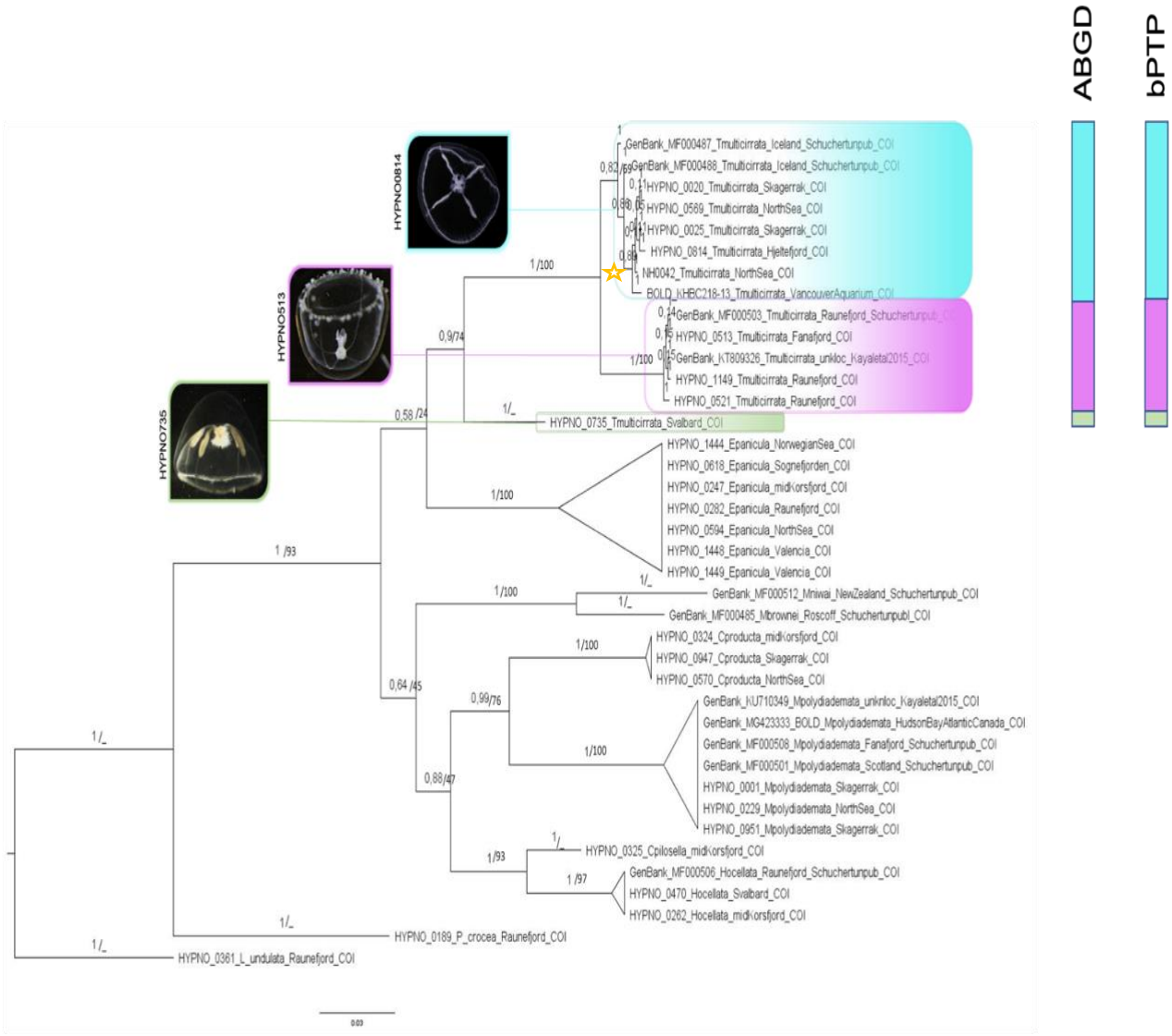


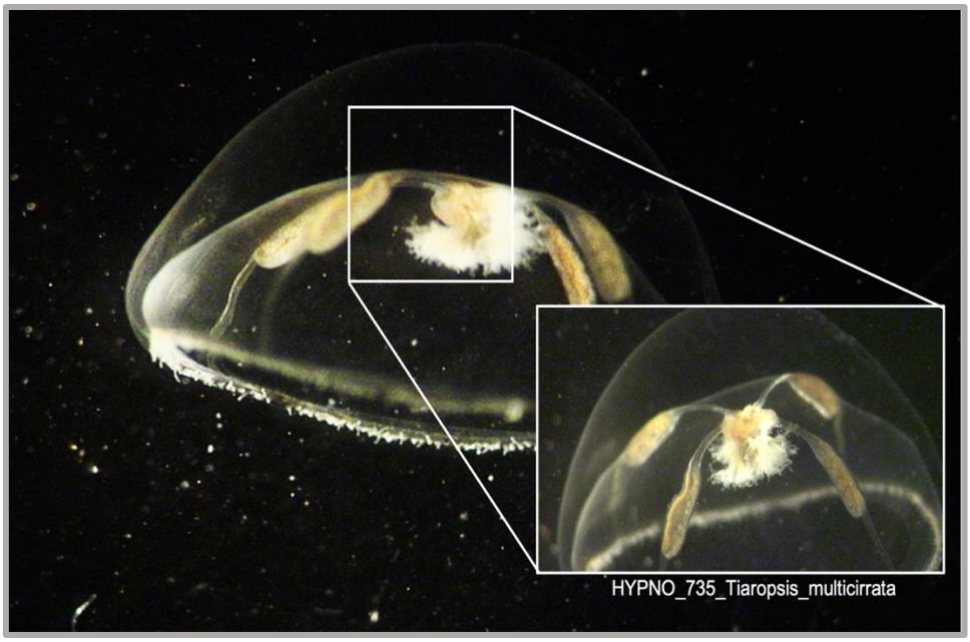
Figure 13. Bayesian Inference gene tree for COI and species delimitation results of ABGD and bPTP for the Tiaropsidae. The Maximum Likelihood tree had the same topology so only the BI results are shown. However, both posterior probabilities and bootstrap values are included for each node. ☆ Represent the polyp stage.

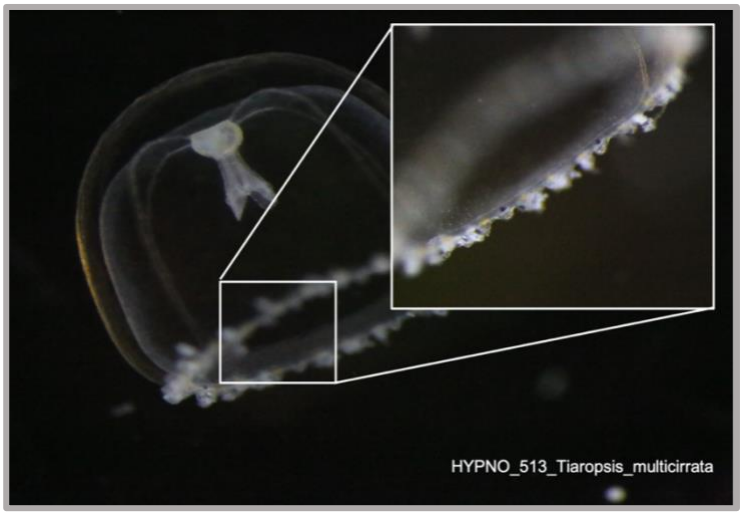
The diversity of *Tiaropsis* is very low, as the genus has only two valid species: *T. multicirrata* (present in the North Atlantic), and *Tiaropsis gordoni* Bouillon and Barnett, 1999 (recorded only in New Zealand). However, my results suggest that this view may be an artefact caused by the similar morphology of all *Tiaropsis* medusae and the existence of only a few morphological characters used to delimit the species (Cornelius, 1995). A couple of other nominal species exist in *Tiaropsis*, but the current consensus is to regard all of them as synonyms of *T. multicirrata* and an assessment of their original descriptions revealed that it is not possible to separate them from each other and from *T. multicirrata* based on the available evidence. These species are *Thaumantias pattersoni* Green, 1857 (type locality Dublin coast), *Tiaropsis diademata* L. Agassiz, 1849 (type locality Massachusetts, USA), and *Thaumantias melanops* Forbes, 1848 (type locality British coasts). The specimens examined in this work did not fit any of these species given the few characters included in their descriptions.

Previously, this species was observed for example in Bergen (Kramp, 1920; Rees, 1941) and Raunefjord (Schuchert, Hosia and Leclère, 2017). The monophyly of genus *Tiaropsis* was recovered in all the trees, and it was strongly supported in the 16S trees, but not in the COI trees (Figs. 10 & 11). Morphologically, *Tiaropsis* is a well-defined taxon that differs from *Octogonade* Zoja, 1896 in the number of radial canals (4 vs 8, respectively), and from *Tiaropsidium* Torrey, 1909 in the number of tentacle types (all tentacles identical in *Tiaropsis*, 2 different types in *Tiaropsidium*). Unfortunately, there are no available sequences for these other two tiaropsid genera, so the monophyly of the genus cannot be correctly evaluated at present. The difference in the support for monophyly of *Tiaropsis* between 16S and COI may be due to the higher degree of variation in the latter marker, which reduces how informative it is for deeper nodes in the phylogeny. In fact, COI is never used alone for systematic analysis in Hydrozoa, and it is preferred for species delimitation and DNA barcoding, while 16S has been used successfully for both kinds of analyses (Zheng et al., 2014).

One interesting result from the phylogenetic analysis was that the family Mitrocomidae appears as non-monophyletic in all the trees. The results suggest that in order for it to be monophyletic it should include also the genus *Tiaropsis*. Including more markers and more samples of all the genera in Mitrocomidae and Tiaropsidae is necessary to evaluate correctly the monophyly of these taxa, but my results contradict the results of Maronna et al. (2016), who found that Mitrocomidae was monophyletic. The latter authors, however, only included representatives of genus *Mitrocomella* in their analysis and their results may be an artefact of this reduced taxonomic sampling. The close relationship between Mitrocomidae and Tiaropsidae is not surprising because both families share the presence of open statocysts in their jellyfish (Cornelius, 1995).

No morphological diagnostic characters were identified for any of the clades of *Tiaropsis* present in Norway, and in the same way as *Laodicea undulata*, *T. multicirrata* can be considered a species complex. The morphology of the specimens of *Tiaropsis* is variable and a larger number of individuals from different developmental stages is necessary to define these clades morphologically. A selection of the morphological variation in the recovered clades of *Tiaropsis* is presented in Fig. 14-16.





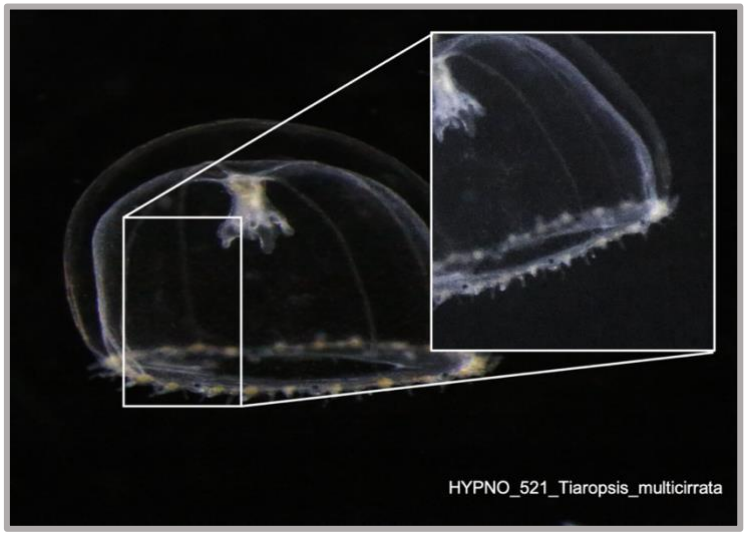


Figure 14. General morphology of three leptomedusae specimens corresponding to clade 1 (top) and clade 2 (middle and bottom) of *Tiaropsis multicirrata* (the clades correspond to clade green and clade pink in Figs. 12 and 13, respectively). Details of the umbrella margin are included.

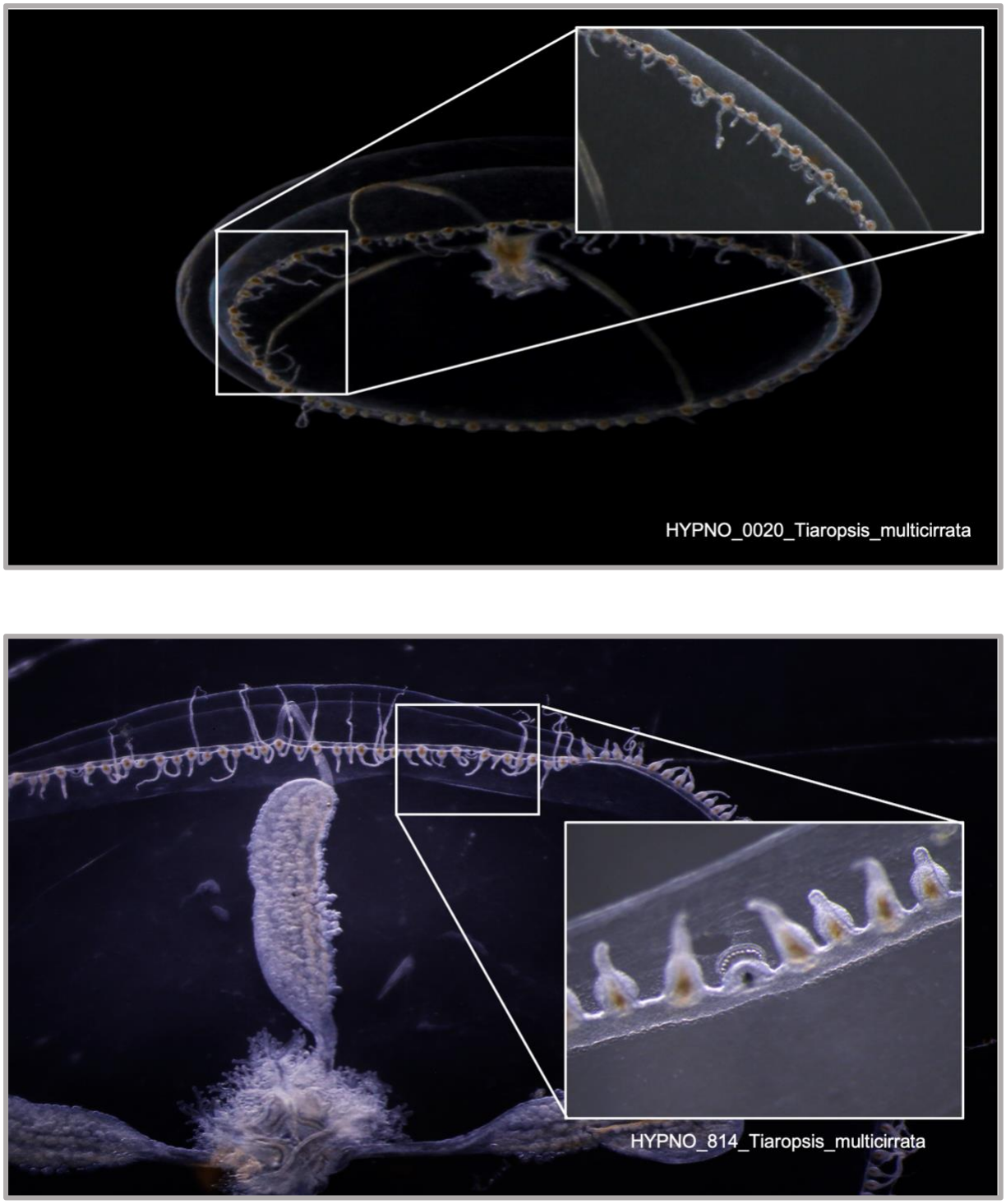


Figure 15. General morphology of two leptomedusae specimens corresponding to clade 3 of *Tiaropsis multicirrata* (this clade corresponds to clade blue in Figs. 12 and 13). Details of the umbrella margin are included.

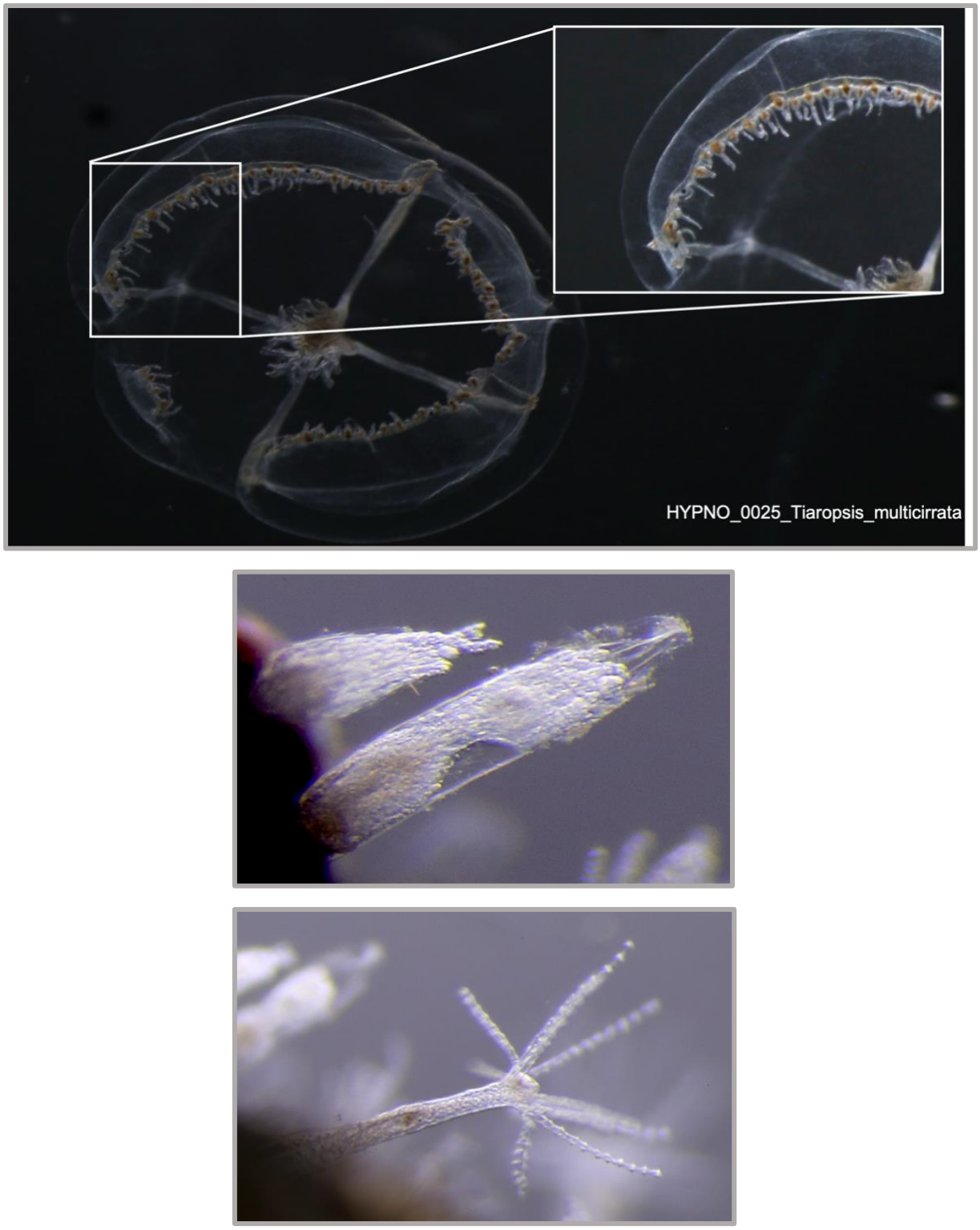


Figure 16. General morphology of one leptomedusa (top) and one polyp (bottom) specimens corresponding to clade 3 of *Tiaropsis multicirrata* (this clade correspond to clade blue in Figs. 12 and 13). Details of the umbrella margin are included for the leptomedusa.

4 Conclusions

In this study, the combination of molecular and morphological analysis (the so-called integrative taxonomy) allowed to detect cryptic speciation in two genera of hydrozoans occurrying in Norway: *Laodicea* and *Tiaropsis*. The mitochondrial markers COI and 16S were both informative of the relationships observed, contrary to the marker ITS that was impossible to obtain for the analyzed specimens.

Based on molecular evidence, *Laodicea undulata* comprises 2 molecularly distinct clades in Norwegian waters, and these clades appear not to be sister species. On the other hand, the specimens morphologically identified as *T. multicirrata* split up into three distinct clades in Norway. For both *Tiaropsis* and *Laodicea*, the results suggest that the observed clades correspond to undescribed species, but further work is necessary to place them in a broader phylogenetic perspective. The morphological comparison was primarily conducted on the medusa stage, as we had few samples from the hydroid stage. Morphological analysis did not identify any potential morphological characters that define the different clades, and Laodicea undulata and Tiaropsis multicirrata are, in consequence, regarded as species complexes. The potential differences between the hydroid stages of the putative species, therefore, remain largely unexplored.

The monophyly of genus *Laodicea* and of families Laodiceidae, Tiarannidae and Mitrocomidae is challenged by the results of this work. Mitrocomidae becomes monophyletic when Tiaropsidae is included, and this is supported by the morphological character of open statocysts. Tiarannidae and Laodiceidae are both united by the presence of cordyli and may form a monophyletic group when put together.

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