Nemertean Systematics

Usefulness of DNA Barcoding and DNA Taxonomy for Species Identification, Delimitation, and Population Analyses

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Summary

The assessment of phylogenetic relationships and systematics of all metazoan taxa heavily relies on the accurate identification and delimitation of species. For the past two centuries, both species identification and delimitation have mainly been based on morphological data, in many cases accessed through histological sectioning. Although this approach allows comparing various internal characteristics, the definition of these distinctive characteristics can be challenging depending on the investigated taxa and in many cases remains subjective. Moreover, intraspecific variation can complicate the interpretation of morphological data. Therefore, taxonomy has shifted towards the application of molecular data in order to unravel species identities and the relationships within a taxon. In contrast to morphological data, molecular data can rapidly be extracted and often analyzed without expert knowledge. Moreover, molecular sequence data can be used to address numerous different problems with regard to systematics and taxonomy.

Molecular-based taxonomy has been suggested at the beginning of this millennium to facilitate both species identification and delimitation. Based on DNA taxonomy incorporating single gene sequences such as the mitochondrial cytochrome oxidase *c* subunit I (COI), systematics of problematic taxa can be unraveled. One of these problematic and understudied groups is the taxon Nemertea. Due to the lack of a widely applied standard in morphological species descriptions and the presence of only few diagnostic characters, nemertean systematic is still not fully resolved. The application of single gene sequences has helped to answer several taxonomic questions in the past 20 years, but because of the deficiency of taxonomic coverage in online repositories numerous questions remain to be answered. In several taxa, taxonomy has shifted towards the application of next-generation sequencing approaches as an increasing number of genes allow for more accurate analyses. Nevertheless, most recent investigations on nemertean taxonomy still focus on single gene sequence approaches.

This thesis presents three studies that employ single gene sequences to help resolve the muddled taxonomy of Nemertea. All examples aim at highlighting the adequacy and prevailing relevance of single gene data to answer various questions in an understudied taxon such as nemerteans. The data presented in Chapter 1 aimed at delimiting species of a problematic genus with the help of COI data, whereas in Chapter 3 single gene data is used to describe several new species based on a turbotaxonomic or an integrative approach. In contrast to this, haplotype

distribution and population genetics in a cosmopolitan nemertean species is investigated in Chapter 2. Included unpublished results further underline the usefulness of COI sequence data with regard to identification of unknown specimens, detection of cryptic species, delimitation of externally similar specimens, and haplotype distribution along geographically distant populations.

All cases demonstrate the lasting timeliness and usefulness of single sequence gene data. When it comes to the identification and delimitation of nemertean species, molecular single gene approaches are crucial for easily and fast acquired results as both can be achieved by comparing the sequence data. Nevertheless, a lasting problem in this regard is the lacking coverage in online repositories as nemerteans are in many cases understudied. Therefore, it is of utmost importance to increase representation of nemerteans in databases to further add knowledge and facilitate future species identification. When it comes to species descriptions, molecular sequence data alone is in many cases still not sufficient. Therefore, a turbotaxonomic approach as has already been suggested for nemerteans appears to be the best choice. In general, all future species descriptions should at least incorporate a genetic barcode, but an integrative approach combining all available data is favorable if circumstances allow it.

Table of Contents

Die aufgelisteten Manuskripte/ Publikationen sind im Zuge der Dissertation entstanden.. Alle Manuskripte finden sich im Anhang der vorgelegten Arbeit.

Manuskript 1 Sagorny C, Wesseler C, Krämer D, & Döhren J von (2019). Assessing the diversity and distribution of *Cephalothrix* species (Nemertea: Palaeonemertea) in European waters by comparing different species delimitation methods. *Journal of Zoological Systematics and Evolutionary Research*, 57, 497-519. https://doi.org/10.1111/jzs.12266

Manuskript 2 Sagorny C & Döhren J von (in revision). Occasional reproduction significantly affects the population structure of the widespread, predominantly asexually reproducing marine worm *Lineus sanguineus* (Nemertea: Pilidiophora). *Marine Biology*.

Manuskript 3 Sagorny C, Döhren J von, Rouse GW, & Tilic E (in revision). Cutting the ribbon: Bathyal nemerteans from seeps along the Costa Rica margin with descriptions of 3 new genera and 10 new species. *European Journal of Taxonomy*.

1 Introduction

Traditionally, studies on systematics in most metazoan taxa have mainly been based on morphological data (Padial et al. 2010). In some cases, additional information on ecology or life history provided further insight into between-taxa relationships (Boury-Esnault et al. 2013; Chapple & Ritchie 2013). Yet, since the beginning of this millennium, molecular data gained increasing importance in understanding metazoan systematics (Padial et al. 2010). Molecular data developed from the use of single gene sequences to the analysis of several thousand genes per included species (e.g. Weigert & Bleidorn 2016). In contrast to century old morphological methods, molecular datasets can mostly be analysed fast and without expert knowledge (Sundberg et al. 2016a). In general, molecular data allow to analyse sequence datasets to address very different problems. The most commonly applied approach when it comes to single gene sequences is DNA barcoding (Hebert et al. 2003a). This method based on the mitochondrial cytochrome c oxidase subunit I (COI) has initially been suggested as effective tool for the identification of unknown specimens (Hebert et al. 2003b; Hebert et al. 2003a). This approach is nowadays widely applied not only to identify specimens, but also to answer a wide range of ecological, taxonomic, or conservational questions (Kvist 2013). With the advent of DNA barcoding, DNA taxonomy has become possible (Vogler & Monaghan 2007). This approach aims at detecting species boundaries, based on various molecular delimitation methods (Vogler & Monaghan 2007; Fontaneto et al. 2015).

Molecular data can thus be applied to easily identify specimens as representatives of a certain species (Hebert et al. 2003b; Hebert & Gregory 2005). Moreover, it is possible to link males to females in species with strong morphological dimorphisms or to link different life stages of one species to one another (Fišer Pečnikar & Buzan 2014). On the other hand, delimitation between closely related and morphologically often similar individuals can be achieved (Hebert et al. 2004). In addition, population analyses can be performed that might help to detect ongoing speciation or link isolated populations to geographical barriers (e.g. Duran et al. 2004d). A turbotaxonomic approach even allows to describe species based on external morphology and a DNA barcode alone (Butcher et al. 2012).

Thus, molecular approaches nowadays provide powerful tools to identify, describe, and delimit species. Molecular data proved to be especially useful in groups that lack distinct morphological characters (Meyer & Paulay 2005). An example for this is the taxon Nemertea.

This taxon remains severely understudied and many questions remain to be answered. For this reason, nemerteans are a promising study group to verify the usefulness of DNA barcoding and DNA taxonomy with regard to identification, delimitation, and description of species. Moreover, the effectiveness and lasting timeliness of single sequence data can be underlined.

1.1 Introduction Nemertea

Nemertea or ribbon worms are a small group of soft-bodied vermiform animals. To date, around 1,300 species are named but a higher diversity is expected (Gibson 1995; Kajihara et al. 2008; Appeltans et al. 2012). Most nemerteans are marine and can be found in all oceans around the world (Gibson 1972, 1982, 1995). Only few species conquered terrestrial and freshwater habitats (Gibson 1972, 1982; Moore & Gibson 1985; Moore et al. 2001; Sundberg & Gibson 2008). In marine environments, nemertean species can be found from the intertidal zone to hadal depths of ca. 9,500 meters, with *Nemertovema hadalis* CHERNYSHEV & POLYAKOVA 2018 being the deepest sampled named species (Gibson 1972, 1982; Chernyshev & Polyakova 2018b, 2019). Nemerteans succeeded to colonize a great variety of habitats. Interstitial species like *Ototyphlonemertes* DIESING, 1863 are known just as well as fully pelagic species like *Nectonemertes* VERRILL, 1892 (Gibson 1972; Envall 1996; Maslakova & Norenburg 2001), although most species are benthic (Gibson 1972). The most important apomorphy of nemerteans is the eversible proboscis situated in a fluid-filled cavity (rhynchocoel) (Gibson 1972, 1982). This proboscis facilitates a free-living, nocturnal, predatory lifestyle that is exercised by many marine nemertean species (McIntosh 1873-1874; Gibson 1972). By rapidly everting the proboscis, nemerteans can prey on for example small crustaceans and annelids (McDermott & Roe 1985; Thiel & Reise 1993; Thiel 1997).

Nemerteans exhibit a vast size range as interstitial species only measure a few millimetres, whereas *Lineus longissimus* (GUNNERUS, 1770) is regarded as the largest known invertebrate with a length of up to 30 metres (Gibson 1972; Cantell 1976). However, the better part of nemertean species varies between only a few centimetres and 30 cm (Gibson 1972, 1982). Externally, nemerteans possess only little distinct characteristics, a fact that often hampers species identification in this taxon (Gibson 1985; Strand et al. 2014; Sundberg et al. 2016a). Well-visible from the outside is the huge variety of coloration and colour patterns (Gibson 1972).

The taxon Nemertea comprises three major sub-groups: Palaeonemertea, Heteronemertea, and Hoplonemertea (see Fig 1.2). Most species are dioecious and develop via different types of larvae (Gibson 1972). The best-known type of larvae is the conspicuous pilidium larva (e.g. Maslakova 2010). Only few species, such as *Cephalothrix hermaphroditica* GIBSON, SANCHEZ & MENDEZ, 1990 have been found to be hermaphroditic (Gibson et al. 1990). One species, *Lineus pseudolacteus* (GONTCHAROFF, 1951) is even known to reproduce solely asexually (Ament-Velásquez et al. 2016), whereas two species reproduce at least partly asexually: *Lineus sanguines* (RATHKE, 1799) and *Baseodiscus delineatus* (DELLE CHIAJE, 1825) (Gontcharoff 1951; Bierne 1970; Gibson 1972; Ament-Velásquez et al. 2016; Ikenaga et al. 2019). Asexual reproduction results from fissiparity, based on outstanding regenerative capacities (Gontcharoff 1951; Reutter 1967; Gibson 1972). In general, regeneration of posterior body parts is fairly common in nemerteans, whereas only few species, such as *Cerebratulus lineolatus* COE, 1905, *Micrura fasciolata* EHRENBERG, 1828, *Prostoma graecense* (BÖHMIG, 1892), *Tubulanus sexlineatus* (GRIFFIN, 1898), and *Tubulanus ruber* (GRIFFIN, 1898) are capable of regenerating a head (Dalyell 1853; Kipke 1932; Zattara et al. 2018).

As in many other taxa, studying nemerteans is traditionally based on internal organization accessed through histological sectioning (e.g. Gibson 1974; Gibson et al. 1982; Moore & Gibson 1993; Kajihara 2006). In recent years, studying nemertean diversity and systematics has shifted towards the application of molecular methods (Sundberg et al. 2010; Sundberg et al. 2016a). In the following, a brief introduction to nemertean anatomy will be provided in order to assess problems that occur in relation to internal organization as basis for species identification and nemertean systematics.

1.2 Nemertean morphology

In general, nemerteans exhibit a relatively simple internal organization that is amply described in Friedrich (1979) and Gibson (1972, 1982). If not stated otherwise, the description provided in the following is mainly based on these three publications. The anterior region constitutes the head or cephalic lobe that can be demarcated from the rest of the body, whereas the posterior region is utterly unsegmented (Sundberg et al. 2009a; Sundberg et al. 2016a). The cephalic lobe bears horizontal cephalic slits (Heteronemertea) or transversal furrows (Palaeonemertea & Hoplonemertea) that are usually visible from the outside.

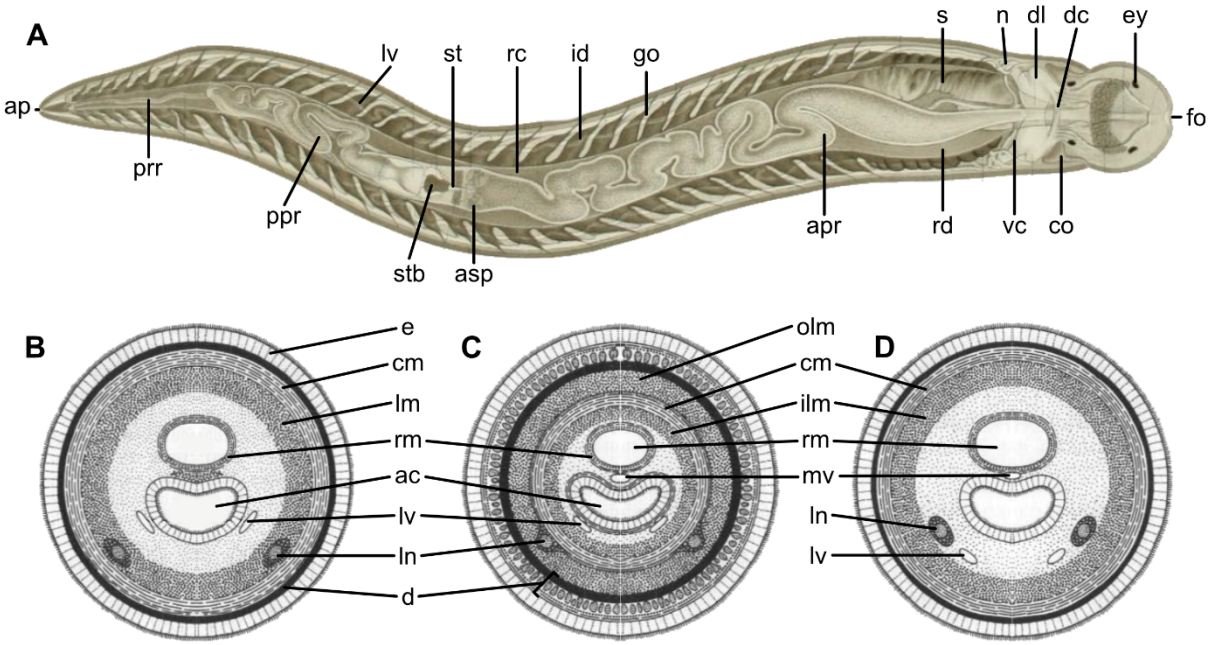


Figure 1.1 Internal anatomy of nemerteans. **A** Schematic drawing of a eumonostiliferous hoplonemerteans showing the general internal organization within Nemertea (modified after Bürger 1895). **B-D** Schematic transverse sections illustrating the organization of the body wall and the position of principal organ systems highlighting differences between Palaeonemertea (**B**), Heteronemertea (**C**), and Hoplonemertea (**D**) (modified after Gibson 1982). Abbreviations: ac, alimentary canal; ap, anal pore; apr, anterior proboscis; asp, accessory stylet pouch; cm, circular musculature; co, cerebral organ; dc, dorsal brain commissure; dl, dorsal brain lobe; e, epidermis; ey, eye; d, dermis; fo, frontal organ; go, gonad; id, intestinal diverticula; ilm, inner longitudinal musculature; lm, longitudinal musculature; ln, lateral nerve cord; lv, lateral vessel; mv, mid-dorsal vessel; n, nephridium; olm, outer longitudinal musculature; ppr, posterior proboscis; prr, proboscis retractor musculature; rc, rhynchocoel; rd, rhynchodaeum; rm, rhynchocoel musculature; s, stomach; st, stylet; stb, stylet bulb; vc, ventral brain commissure.

As mentioned above, the most striking feature is the proboscis apparatus that is only found in nemerteans. It is located dorsal of the alimentary canal and consists of three major components: the eversible proboscis itself, the rhynchocoel (fluid-filled cavity), and the anterior opening, called rhynchodaeum (Fig 1.1A). The extent of the proboscis in relation to the alimentary canal is often regarded as an important diagnostic trait (Sundberg et al. 2009a; Strand et al. 2014). In most hoplonemerteans, the proboscis apparatus is fused with the alimentary canal with one shared opening at the anterior tip of the cephalic lobe. In palaeonemerteans and heteronemerteans, the rhynchodaeum and the alimentary canal open separately, with the proboscis pore located at the anterior tip of the head and the mouth opening on the ventral side in front of the cerebral ganglia. The nemertean proboscis is formed as

invagination of the anterior body wall. Therefore, layering of the proboscis musculature is comparable to body wall musculature. The proboscis can either bear a stylet (Hoplonemertea) or be of a simpler organization (Heteronemertea & Palaeonemertea). In hoplonemerteans, the proboscis can be subdivided into three regions: the anterior muscular tube, the stylet bulb and the blind ending tube (Fig 1.1A). Monostiliferans have one central stylet, whereas Polystiliferans possess several small stylets. In both cases accessory pouches bearing reserve stylets can be present.

Besides the proboscis apparatus, several other characters proved to be important diagnostic traits (Sundberg et al. 2009a; Strand et al. 2014). These include the body wall musculature, the position of the nervous system in relation to the body wall, the composition and position of sensory organs, and the specifications of the blood vascular system. Generally, the body wall musculature reflects the bilaterian Grundmuster with an outer circular and an inner longitudinal layer, but within Nemertea different additional layers can be found. Therefore, the amount and orientation of this additional musculature can be taxon-specific. The nemertean nervous system usually occurs in close contact to the body wall. In palaeonemerteans, it is located either in the epidermis, the dermis, or within the body wall musculature (Fig 1.1B). In heteronemerteans, the nervous system is situated in the circular muscle layer (Fig 1.1C), whereas it is situated internal to the body wall in hoplonemerteans (Fig 1.1D). The central nervous system of nemerteans consists of bilobed, paired cerebral-ganglia and the lateral nerve cords, has a medullary organization, and is surrounded by an outer neurilemma (Bürger 1895; Beckers et al. 2011; Beckers et al. 2013; Beckers 2015; Beckers & Döhren 2016; Beckers et al. 2018). Additionally, a peripheral nervous system is present that comprises various nerves (e.g. cephalic or buccal nerves) and nerve plexus (Beckers et al. 2013). Several different sensory organs can be developed (Beckers et al. 2013). Among these are eyes, as well as cerebral, frontal, lateral, or epidermal sensory organs. The blood vascular system generally consists of a pair of longitudinal vessels that are connected by an anterior cephalic and a posterior anal lacuna. Additionally, a mid-dorsal blood vessel might be present that sometimes penetrates the rhynchocoel wall.

The excretory system is composed of protonephridia in the form of branched tubes that are in close contact with the blood vascular system (Bartolomaeus & Döhren 2010). The reproductive system comprises serially arranged, spherical gonads that are usually alternating with intestinal lateral pouches and open to the exterior via simple gonoducts (Döhren et al. 2010; Döhren 2015).

Due to the relatively simple organization of nemerteans, finding distinctive diagnostic characters and properly describing these is often challenging (Strand et al. 2014). Moreover, a clear definition of important characteristics is often subjective (Sundberg 2015).

1.3 Nemertean phylogeny

The monophyly of Nemertea is well-supported both by molecular and morphological data. But although nemerteans can be easily identified as members of the Spiralia as they undergo spiral cleavage, the exact phylogenetic position is not yet fully resolved (reviewed in Jenner 2004 and Bleidorn 2019). Different hypotheses exist as to the most probable sister group. Early on, nemerteans have been regarded as sister to Platyhelminthes based on morphological similarities (Nielsen et al. 1996; Sørensen et al. 2000). Based on more recent investigations, this hypothesis has been rejected, though (Struck & Fisse 2008). More recent molecular analyses place nemerteans either as sister to Annelida (Kocot et al. 2017), as sister to Mollusca (Podsiadlowski et al. 2009), as sister to Phoronida and/or Brachiopoda (Bleidorn et al. 2009; Nesnidal et al. 2013; Laumer et al. 2015; Luo et al. 2018), or even as sister to all other Trochozoa (Kocot et al. 2017). The most recent analysis suggests a sister group relationship to Platyhelminthes and Annelida (Marlétaz et al. 2019). Based on these conflicting results, the exact relations within Lophotrochozoa remain unclear until further studies support one of the named hypotheses.

1.4 Nemertean systematics

Nemertean systematics have traditionally been based on internal morphology and the different specifications of the characters mentioned above (Bürger 1895, 1904; Coe 1904, 1905; Gibson 1972; Sundberg & Strand 2010). Based on histology, they found three major groupings: Palaeonemertea, Heteronemertea, and Hoplonemertea (Gibson 1972 and references therein). Traditionally, these were classified as Anopla and Enopla (Stiasny-Wijnhoff 1923; Gibson 1972, 1982). The former comprised Heteronemertea and Palaeonemertea and was characterized by an unarmed proboscis and separate proboscis and mouth openings (Stiasny-Wijnhoff 1923; Coe 1943; Gibson 1972, 1982). The latter comprised Hoplonemertea and Bdellonemertea that possess a proboscis equipped with a stylet and a joint mouth and proboscis opening (Stiasny-Wijnhoff 1923; Coe 1943; Gibson 1972, 1982). As the importance of molecular approaches for systematics and phylogenetic estimations has increased in the past decades, nemertean systematics have shifted towards utilizing molecular approaches (Sundberg 2015). Most studies

employing molecular methids aim at creating a stable backbone for nemertean classification (Sundberg 2015). While in the beginning studies on nemertean systematics were based on single genes only, multi-gene approaches or even reconstructions based on whole mitochondrial genomes or transcriptomes have become far more common (Sundberg & Saur 1998; Sundberg et al. 2001; Thollesson & Norenburg 2003; Andrade et al. 2012; Andrade et al. 2014; Kvist et al. 2014; Gonzalez-Cueto et al. 2015; Kvist et al. 2015; Jiang & Deng 2018; Nam & Rhee 2020).

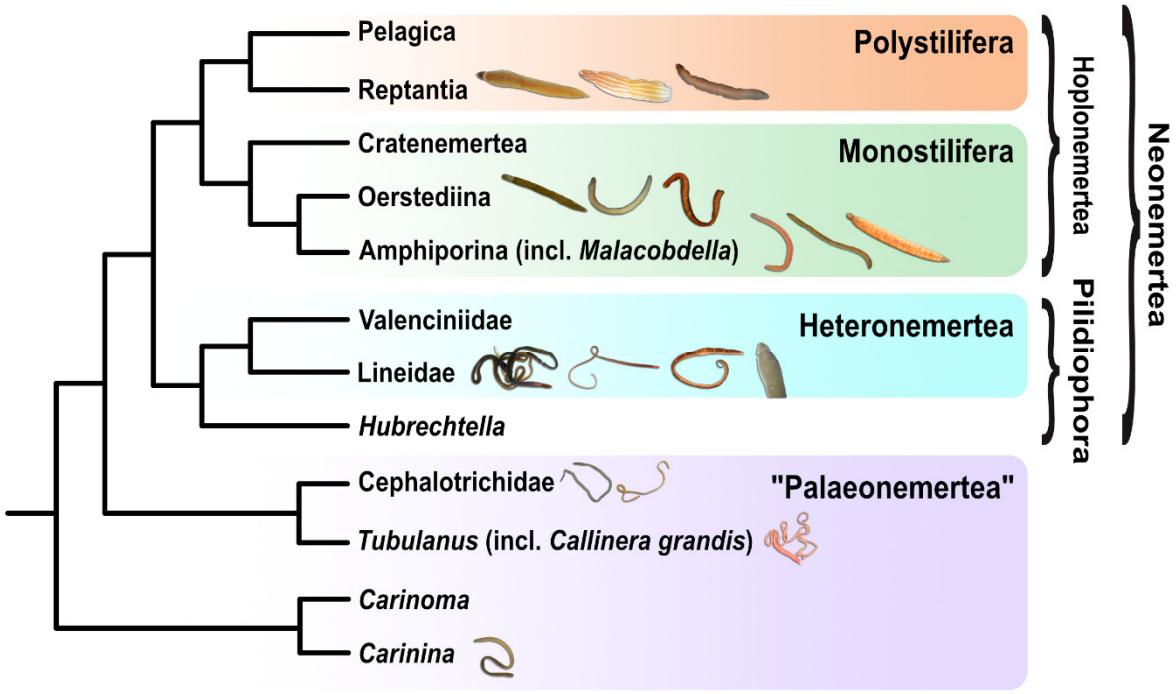


Figure 1.2 Current nemertean phylogeny summarized and redrawn after Andrade et al. (2012; 2014) and Kvist et al. (2014; 2015). Exemplary habitus photographs for nemertean groups investigated in this PhD project are provided **(Reptantia:** *Paradrepanophorus crassus*, *Drepanophorus spectabilis*, deep-sea Reptantia N263; **Oerstediina:** *Oerstedia dorsalis*, *Chernyshevia escarpiaphila*, *Alvinonemertes coralliophila*; **Amphiporina:** *Tetrastemma melanocephalum*, *Amphiporus* cf. *reticulatus*, *Puravida sundbergi*; **Lineidae:** *Riseriellus occultus*, *Lineus sanguineus*, *Micrura fasciolata*, *Cerebratulus marginatus*; **Cephalotrichidae:** *Cephalothrix rufifrons*, *Cephalothrix hermaphroditica*; ***Tubulanus*:** *Tubulanus dariae*; ***Carinina*:** *Carinina ochracea*).

All of these phylogenetic reconstructions aimed at unravelling internal relationships within Nemertea recovered Anopla and Enopla to be unnatural groups: Anopla is paraphyletic, whereas Enopla is synonymous to Hoplonemertea as the only representative of Bdellonemertea is firmly nested within Hoplonemertea (Thollesson & Norenburg 2003; Andrade et al. 2012;

Andrade et al. 2014; Kvist et al. 2014; Kvist et al. 2015). Thus, the names Anopla and Enopla have recently been dismissed (Strand et al. 2019).

In contrast to Anopla and Enopla, the two major nemertean groups Heteronemertea and Hoplonemertea are monophyletic and thus natural groups (Thollesson & Norenburg 2003; Andrade et al. 2012; Andrade et al. 2014; Kvist et al. 2014; Kvist et al. 2015). Based on both molecular data and the similar type of larvae, the family Hubrechtidae is most likely sister to Heteronemertea, thus forming the clade Pilidiophora (Thollesson & Norenburg 2003; Schwartz 2009; Andrade et al. 2012; Döhren 2015; Beckers & Döhren 2016). The clade comprising both Pilidiophora and Hoplonemertea is referred to as Neonemertea (Thollesson & Norenburg 2003) (Fig 1.2).

In contrast to Heteronemertea and Hoplonemertea, the monophyly of Palaeonemertea and the relationship between palaeonemertean genera could not yet be fully resolved (Thollesson & Norenburg 2003; Andrade et al. 2012; Kvist et al. 2014; Kvist et al. 2015). Both transcriptomic and mitogenomic approaches did only include few palaeonemertean species (Andrade et al. 2014; Gonzalez-Cueto et al. 2015; Jiang & Deng 2018; Nam & Rhee 2020). Nevertheless, the transcriptomic approach by Andrade et al. (2014) recovered a monophyletic Palaeonemertea clade based on one species of each of the genera *Tubulanus*, *Cephalothrix*, and *Carinoma*.

Besides this, several uncertainties remain on genus-level. As various genera that were erected more than one century ago often lack distinct diagnoses, numerous species that vaguely resemble their description have been assigned to one of these genera (Strand et al. 2014; Sundberg 2015). In most cases, molecular analyses have shown that these “hold-all” genera are paraphyletic and thus in urgent need of revision (Strand & Sundberg 2005b; Summers et al. 2014; Sundberg 2015). These ambiguous cases often complicate species identification and delimitation in nemerteans.

1.5 Species identification, delimitation and descriptions in nemerteans

Species are the most fundamental units in biology and are the only taxonomic category that can be observed in nature (Mayr 1943; Queiroz 2005, 2007). Among taxonomists, assigning a scientific name is crucial as framework for communication between disciplines (Wheeler 2004; Dayrat 2005; Padial et al. 2010). Nevertheless, definition of a species is far more complex than it seems. To date, several different species concepts exist based on very different assumptions (Mayden 1997; Queiroz 2005). The most commonly accepted concept is the biological species

concept that defines a species as a group of interbreeding individuals that are separated from other species by reproductive isolation (Mayr 1943). This concept is hard to verify in many cases as long ecological studies would be necessary.

Therefore, morphological data is widely accepted as basis of species identification and description (Padial et al. 2010). In many cases, an integrative approach is chosen that includes ecological, ultrastructural, or life-history data (Dayrat 2005; Will et al. 2005; Padial et al. 2010). Unfortunately, morphological methods are often time consuming and only accessible by experts (Sundberg 2015; Sundberg et al. 2016a).

As a result of these deficiencies in identifying species, systematics in many taxa have shifted towards molecular approaches. The advent of DNA based approaches allows to rapidly identify species thus helping to assess species diversity (Hebert et al. 2003a; Hebert et al. 2003b). Therefore, several recent species descriptions that include short diagnoses and descriptions linked with a DNA barcode are regarded as valid (Butcher et al. 2012). This turbotaxonomic approach has been applied in many cases since to describe numerous new species in one publication (e.g. Riedel et al. 2013; Summers et al. 2014; Rouse et al. 2018; Sharkey et al. 2021).

Due to the above-mentioned challenges in nemertean systematics, identifying specimens in this taxon can be tedious. As a result of the simple morphology only few diagnostic characters are accessible (Knowlton 2000; Strand & Sundberg 2005b, 2005a; Chen et al. 2010; Sundberg et al. 2010; Sundberg & Strand 2010; Strand & Sundberg 2011; Fernández-Álvarez & Machordom 2013; Sundberg 2015; Krämer et al. 2017). Traditionally, identification of sampled specimens in nemerteans was often based on internal characteristics assessed via time-consuming histological sectioning (Roe et al. 2007; Sundberg et al. 2010; Sundberg & Strand 2010). This proved to be problematic as Strand et al. (2014) showed that only one third of the commonly investigated characteristics is actually informative for species identification. As a result of this, numerous cryptic species are expected within Nemertea (Appeltans et al. 2012; Sundberg et al. 2016b). Furthermore, many species were described during the 18th and 19th century – a time when species descriptions were sufficient if they included brief descriptions of external morphology (e.g. McIntosh 1873-1874; Verrill 1892). This hampers identification of newly collected specimens of these species as neither diagnosis nor proper character definition is provided (Gibson et al. 1990; Gibson 1995; Sundberg et al. 2009a; Sundberg 2015). In addition, information on type locality and holotype is often lacking (Sundberg 2015). For these reasons, identification based on morphology proved to be problematic. DNA-based analyses

thus have an increasing importance in identifying and also describing species (Sundberg et al. 2010; Strand & Sundberg 2011; Strand et al. 2014). Especially DNA barcoding based on the COI gene fragment proved to be a valuable tool to identify specimens (Hebert et al. 2003a; Hebert et al. 2004; Sundberg et al. 2016b). This approach can for example further enhance the accuracy of species identification in general marine inventories. So far, 95% of all specimens in these inventories are only identified as "Nemertea sp." (e.g. León-Morales & Vargas 1998; Schander & Willassen 2005; Levin et al. 2017).

DNA barcoding led the way to delimitation of species and the detection of species boundaries, a field of study that is commonly referred to as DNA taxonomy (Monaghan et al. 2006; Pons et al. 2006; Vogler & Monaghan 2007). Delimiting species is an important topic in systematics as it allows to discover monophyletic groups and understand evolutionary processes (Sites & Marshall 2003; Wiens 2007). Species delimitation used to be based on morphology, but due to high levels of intraspecific variations or polymorphisms this sort of delimitation can prove problematic (Envall & Sundberg 1993; Strand & Sundberg 2005b, 2005a; Sundberg et al. 2009b). As a consequence, the importance of molecular approaches is increasing (Pons et al. 2006; Knowles & Carstens 2007). Therefore, several different species delimitation methods have been applied in many taxa, such as spiders (Ortiz & Francke 2016), insects (Pons et al. 2006; Chroni et al. 2017), annelids (Aguado et al. 2019), or lizards (Wiens & Penkrot 2002; Marshall et al. 2006).

Also in nemertean systematics, a set of different molecular species delimitation has been successfully applied (e.g. Strand & Sundberg 2005a; Sundberg et al. 2009b; Chen et al. 2010; Leasi & Norenburg 2014; Sundberg et al. 2016b; Krämer et al. 2017; Chernyshev et al. 2018; Mendes et al. 2018). These methods include non-tree-based delimitation methods such as Automatic Barcode Gap Discovery (ABGD), Nucleotide Divergence Threshold (NDT), and statistical parsimony analyses, as well as tree-based methods like generalized mixed Yule coalescent (GMYC) or multirate Poisson tree processes (mPTP) (Sundberg et al. 2016b). Molecular species delimitation methods can be for example employed to identify meiofaunal specimens (Leasi & Norenburg 2014) or to differentiate between old species names (Krämer et al. 2017). Especially the identification of cryptic species is facilitated by approaches based on DNA taxonomy, as has been shown for different taxa (Leasi & Norenburg 2014; Fontaneto et al. 2015; Leasi et al. 2016; Scarpa et al. 2016; Verdes et al. 2021). Moreover, molecular species delimitation methods can help to reject hypothetical cryptic speciation and provide proof for rather unusual cosmopolitan distributions of species (Sundberg & Strand 2007; Runnels 2013;

Kang et al. 2015). Furthermore, recognizing introduced or even invasive species is made possible by these methods as has been shown for the palaeonemertean species *Cephalothrix simula* Iwata, 1952 (Fernández-Álvarez & Machordom 2013; Kajihara et al. 2013; Faasse & Turbeville 2015). Molecular sequence data furthermore allows to address population structuring and analyse dispersal and gene flow between populations, although only few studies investigating population genetics have been performed for nemerteans (Duran et al. 2004c; Hart & Marko 2010; Alfaya et al. 2013; Runnels 2013).

Although molecular data gained increasing importance in identification and delimitation of nemertean species, descriptions of newly discovered species still heavily rely on morphology. As mentioned above, species descriptions in nemerteans are traditionally strongly based on a combination of external and, more importantly, internal morphology, an approach that came into practice in the late 19th century (compare descriptions in Bürger 1890, 1895; Coe 1904, 1905). These old species descriptions are often poor in details, so that it is often exceedingly difficult to assess whether a specimen should be described under a new species name or whether it merely represents a specimen of a poorly defined species (Gibson 1995). Moreover, the traditional choice of morphological characters employed to describe a species might not be sufficient to differentiate between species (Sundberg et al. 2009a; Strand et al. 2014). An approach based on morphology often heavily relies on the investigated taxa, but also on the taxonomist as definition of "important diagnostic characters" is highly subjective (Sundberg 2015; Sundberg et al. 2016a). Therefore, most researchers choose an integrative approach including morphological, ecological, behavioural or molecular data to describe a new species (Krämer & Döhren 2015; Chernyshev & Polyakova 2021; Mendes et al. 2021).

During the past years, it has been found that external morphology linked with a genetic barcode is in many cases sufficient for species descriptions in nemerteans (Sundberg & Strand 2010; Strand & Sundberg 2011; Strand et al. 2014; Sundberg et al. 2016a). As a consequence, Sundberg et al. (2016a) proposed that species descriptions and re-descriptions can be regarded as valid if they include accurate information on type locality and deposition of type material, a description of external morphology, and a DNA barcode in the form of a COI sequence at least. This approach is in general accordance with the turbo taxonomic approach and allows to rapidly assess species diversity (Butcher et al. 2012).

1.6 Aims and contents of the study

As indicated above, several questions regarding systematics and taxonomy of the taxon Nemertea remain to be answered. In order to answer some of these questions, molecular data are of utmost importance. Despite the presence of next-generation sequencing approaches, most recent investigations concentrating on nemertean taxonomy are still mainly based on DNA barcoding or approaches including up to six different genetic markers (e.g. Chernyshev et al. 2018; Chernyshev et al. 2021c). Therefore, the following series of chapters concentrated on highlighting the usefulness and timeliness of these single gene datasets. Different sets of molecular markers, as well as varying species delimitation methods and phylogenetic reconstructions are applied to unravel ambiguous nemertean relationships. Moreover, different application areas of single gene approaches are highlighted.

The first chapter aimed at testing the usefulness of COI data when it comes to species delimitation in groups that lack distinct morphological characters. As has been shown before, species boundaries in the palaeonemertean genus *Cephalothrix* are not easy to identify based on morphology (Chen et al. 2010; Kajihara et al. 2013). Therefore, cryptic speciation is assumed in this genus. To assess the diversity and distribution of European *Cephalothrix* species, different non-tree-based and tree-based methods were compared.

To provide answers to long lasting questions in nemertean systematics, the fissiparous, partly asexually reproducing heteronemertean *Lineus sanguineus* was investigated for the second chapter. Besides providing evidence for an unusual cosmopolitan distribution of this species, a statistical parsimony analysis of three gene fragments gave insight into haplotype distribution in different populations. Moreover, COI data was used to analyse population structuring in this species based on an AMOVA analysis.

In contrast to this, the third chapter concentrated on species descriptions in nemerteans. Four different gene fragments of more than 80 specimens collected in Costa Rican deep waters were sequenced to identify and describe several new species. Because of the uniform external morphology, additional short descriptions of internal morphology were provided to obtain as much information on the new species as possible.

Moreover, some unpublished results are provided that further underline the usefulness of single gene datasets and provide an outlook for potential future research questions.

2 Material and Methods

2.1 Material examined

Within the framework of this dissertation project, several different, mostly European nemertean species have been examined. In the course of three published/submitted studies, specimens of all three large nemertean groups – Palaeonemertea, Heteronemertea, and Hoplonemertea – have been investigated. The focus of the first study was on the palaeonemertean genus *Cephalothrix* ÖRSTED, 1843. For this, 78 specimens representing seven *Cephalothrix* species have been extracted from coarse sands in the upper littoral from six different localities in Europe between 2011 and 2017 (for detailed specimen information see Sagorny et al. 2019). *Cephalothrix rufifrons* (JOHNSTON, 1837) and *Cephalothrix simula* IWATA, 1952 have been collected from four of the six localities (*C. rufifrons*: Concarneau, Roscoff, Kristineberg, Bergen; *C. simula*: Concarneau, Roscoff, Giglio, Blanes), whereas the remaining five species were sampled from only one locality: *Cephalothrix* cf. *rufifrons* and *Cephalothrix oestrymnica* JUNOY & GIBSON, 1991 were found in Concarneau, *Cephalothrix filiformis* (Johnston, 1828) in Bergen, *Cephalothrix hermaphroditica* GIBSON, SANCHEZ & MENDEZ, 1990 in Roscoff, whereas *Cephalothrix* sp. is only known from Giglio.

For the second study, 108 individuals of the heteronemertean species *Lineus sanguineus* were collected from the coasts of three European countries (Germany, Norway, France). Sampling sites included the eulittoral zone of Helgoland, Sylt, Bergen, Banyuls, Concarneau, Ile de Groix, Roscoff, and Wimereux (for detailed specimen information see Sagorny & Döhren (in revision)). Between 2010 and 2014, specimens were collected from rock fissures, *Mytilus edulis* LINNAEUS, 1758 conglomerations, and under rocks.

Specimens described in the third study represent all three major nemertean groups and were collected at depths between 950 and 2,200 m, mainly along the Costa Rica margin. Sampling of the 84 benthic deep-sea nemerteans occurred between 2009 and 2019 during seven deep-sea expeditions (for detailed specimen information see Sagorny et al. (in revision)). Nemerteans were found at seven localities at the Costa Rica margin (Coco Canyon, Jaco Scar, Mound 11, Mound 12, Mound Jaguar, Parrita Seep, Quepos Plateau) and two localities off the coast of Oregon (Juan de Fuca Ridge, Hydrate Ridge).

Apart from the three published studies, specimens of the heteronemertean genera *Lineus, Micrura*, and *Riseriellus*, as well as of the palaeonemertean genera *Carinoma*, *Carinina*, and

Callinera, were collected in Europe. Numerous small, white palaeonemerteans were extracted from coarse sands in the eulittoral zone of Concarneau (France). These palaeonemerteans can be attributed to one of the following genera: *Carinoma*, *Carinina*, or *Callinera*. In total, eight specimens of the genus *Carinoma*, three specimens of the genus *Carinina*, and ten specimens of the genus *Callinera* were collected. Of the 31 individuals of *Lineus longissimus*, four specimens were collected in Bergen (Norway), whereas 12 and 15 specimens were sampled in Concarneau and Roscoff (France), respectively. Specimens were collected in the eulittoral zone under rocks. Ten specimens of *Riseriellus occultus* ROGERS, JUNOY, GIBSON & THORPE, 1993 were also sampled from the upper intertidal in Concarneau; these were found under stones and in rock crevices. Additionally, two specimens of *Lineus ruber* (MÜLLER, 1774), three specimens of *Lineus clandestinus* KRÄMER ET AL., 2017, and seven specimens of *Lineus viridis* (MÜLLER, 1774) were all collected in Concarneau, except for three *L. viridis* specimens that were sampled in Sylt (Germany). Moreover, 39 specimens of *Lineus* cf. *acutifrons* SOUTHERN, 1913 were collected in coarse sands in the intertidal zone of Concarneau. Both *Micura purpurea* (DALYELL, 1853) and *Micrura fasciolata* EHRENBERG, 1828 were mainly collected in Kristineberg (Sweden; *M. purpurea*: 25 specimens, *M. fasciolata*: four specimens). Additional specimens of *M. pupurea* were collected in France (Wimereux: one specimen, Concarneau: one specimen) and in Norway (Gullesfjorden: one specimen).

For molecular analyses, a tissue sample of each collected specimen was preserved in absolute ethanol (99%). For additional histological investigations, specimens were first relaxed in an equal mix of 7% $MgCl_2$ and seawater. Subsequently, the samples were fixed in 10% formaldehyde in seawater.

2.2 Molecular methods

2.2.1 DNA extraction and amplification

DNA of all the investigated specimens was extracted using two different DNA extraction kits: the DNeasy Blood and Tissue Kit (Qiagen) was used for most specimens exceeding a length of 5 mm, whereas DNA of small specimens (under 5 mm in length) was extracted using the Quick-DNA™ Microprep Plus Kit (Zymo Research). In both cases, extraction followed the manufacturers' protocols. In the course of this dissertation project, various gene fragments were amplified to answer different research questions. Amplified gene regions include both mitochondrial and nuclear gene fragments. Mitochondrial gene regions comprise partial

cytochrome *c* oxidase subunit I (COI) and 16S rRNA. Nuclear genes encompass 18S rRNA and ITS gene fragment (ITS1, 5.8S rRNA, and ITS2). Additionally, histone 3 (H3) was amplified. Primers used for amplification are given in Table 2.2.1. To assess which gene fragments were amplified for the independent studies, see Sagorny et al. (in revision; 2019) and Sagorny & Döhren (in revision). For all additional results, only the mitochondrial COI gene fragment was amplified except for *Lineus* cf. *acutifrons.* For *L.* cf. *acutifrons* the mitochondrial 16S rRNA and nuclear 18S rRNA were amplified in addition.

Table 2.2.1 List of primers used in the course of this dissertation project. Name of the gene fragment, primer name, primer sequence, and reference are provided.

Gene fragment	Primer	Sequence 5'-3'	Reference
16S	arL	CGCCTGTTTATCAAAAACAT	Palumbi et al. 1991
	brH	CCGGTCTGACTCAGATCACGT	Palumbi et al. 1991
COI	LCO1490	GGTCAACAAAATCATAAAGATATTGG	Folmer et al. 1994
	HCO2198	TAAACTTCAGGGTGACCAAAAAATCA	Folmer et al. 1994
H3	aF	ATGGCTCGTACCAAGCAGACVGC	Colgan et al. 1999
	aR	CKYTTIAGIGCRTAIACCACRTCCAT	Colgan et al. 1999
18S	1F	TACCTGGTTGATCCTGCCAGTAG	Giribet et al. 1996
	5R	CTTGGCAAATGCTTTCGC	Giribet et al. 1996
	3F	GTTCGATTCCGGAGAGGGA	Giribet et al. 1996
	bi	GAGTCTCGTTCGTTATCGGA	Whiting et al. 1997
	a2.0	ATGGTTGCAAAGCTGAAAC	Giribet et al. 1999
	9R	GATCCTTCCGCAGGTTCACCTAC	Giribet et al. 1996
ITS	ITS-28S	TTTTCAACTTTCCCTCACGG	Krämer et al. 2017
	ITS-18S	CATTTGAGGAAGTAAAAGTCGTAAC	Krämer et al. 2017

In the course of the dissertation project, different Taq polymerases were applied to perform polymerase chain reactions (PCR). These include Hot-Master Taq polymerase (Invitrogen™), Dream Taq™ PCR Master Mix (Thermo Fisher), and FastGene® Taq ReadyMix (Nippon Genetics). Information on which polymerase was used are given in the respective publications (Sagorny et al. in revision; Sagorny & Döhren in revision; Sagorny et al. 2019). PCR for all additional results was performed using the FastGene Taq polymerase. Thermal PCR cycling mainly followed the programs provided in Table 2.2.2. Possible

deviations from these standard programs are given in the respective publications (Sagorny et al. in revision; Sagorny & Döhren in revision; Sagorny et al. 2019).

Table 2.2.2 Programs of thermal PCR cycling for the five mainly used gene fragments. Temperatures and duration for each step are given.

Step	COI		16S		18S		ITS		H3	
Initiation	94 °C	2 min	94 °C	2 min	94 °C	2 min	94 °C	2 min	94 °C	2 min
# of cycle	40		40		35		40		35	
Denaturation	94 °C	30 s	94 °C	30 s	94 °C	30 s	94 °C	30 s	94 °C	30 s
Annealing	48 °C	60 s	51 °C	60 s	52 °C	30 s	48 °C	60 s	54 °C	60 s
Elongation	72 °C	60 s	72 °C	60s	72 °C	60 s	72 °C	120 s	72 °C	60 s
Final elongation	72 °C	2 min	72 °C	2 min	72 °C	10 min	72 °C	1 min	72 °C	10 min

Amplified products were purified using either the NucleoSpin® Extract II-Kit (Macherey-Nagel GmbH & Co. KG) or the illustra ExoProStar 1-Step (GE Healthcare), in both cases following the manufacturer's instructions. All purified products were Sanger sequenced by LGC Genomics (Berlin, Germany) using either only forward primers (COI, 16S, 18S, H3) or forward and reverse primers (COI, ITS) for sequencing (Sanger et al. 1977).

2.2.2 Sequence analysis

Sequences were edited either with BioEdit version 7.2.5 (Hall 1999) or with Geneious Prime® 2020.0.5 (Biomatters). To verify sequence identity, BLAST searches as implemented in NCBI were conducted for all sequences (Altschul et al. 1990). All sequences obtained during the three studies were deposited in the GenBank database. For accession numbers see the respective publications (Sagorny et al. in revision; Sagorny & Döhren in revision; Sagorny et al. 2019). Sequences were aligned using MAFFT version 7 (Katoh et al. 2002; Katoh & Standley 2013; Kuraku et al. 2013; Katoh et al. 2019), either using the webserver (https://mafft.cbrc.jp/alignment/server/) or the plugin as implemented in Geneious. In both cases, G-INS-I strategy with default parameters (scoring matrix for nucleotide sequences: 200PAM/K=2, gap opening penalty: 1.53, offset value: 0.0) were selected. In order to exclude ambiguous positions, Gblocks version 0.91b (Castresana 2000) was applied to all datasets. If not stated otherwise in the

respective publications, default parameters were chosen. For all analyses, obtained sequences were combined with available sequence data of the investigated taxa taken from GenBank. Furthermore, datasets were completed with additional GenBank sequence data relevant for the respective research question. Information on sequence data taken from GenBank is provided in Appendix I.

2.2.3 Phylogenetic analysis

For all analyses, phylogenetic trees were reconstructed for every single investigated gene fragment. If more than one gene fragment was included, an additional concatenated analysis was performed. For study 2 on *Lineus sanguineus* sequences were concatenated using FASconCAT (Kück & Meusemann 2010), whereas SequenceMatrix (Vaidya et al. 2011) was employed for study 3 on Costa Rican deep-sea nemerteans as well as the study on *Lineus acutifrons*. In studies 1 and 2 and all other additional studies, MrModeltest2 version 2.3 was applied to infer the best-fitting substitution model for phylogenetic reconstruction based on the Akaike information criterion (Nylander 2004). In study 3 and for the study on *L. acutifrons*, PatirionFinder2 was used to select both the partition schemes and the optimal nucleotide substitution models under the "greedy" search scheme (Lanfear et al. 2017). Best-fitting nucleotide substitution models for each additional dataset are given in Appendix 2.

Phylogenetic trees were reconstructed using both Maximum Likelihood (ML) and Bayesian Inference (BI) as optimality criterion. Programs used to calculate phylogenetic ML trees were MEGA version 6.06 (Tamura et al. 2013), RaxML version 8.2.11 as implemented in Geneious (Stamatakis 2014), and iqtree version 1.6.12 (Nguyen et al. 2015). Branch support in the first two programs was estimated using 500 and 1000 bootstrap replicates, respectively (Felsenstein 1985), whereas ultrafast bootstrapping was employed by the latter (Hoang et al. 2018). For all additional studies, iqtree was the program of choice. MrBayes 3.2.7a on the CIPRES Science Gateway was used to reconstruct BI trees (Ronquist et al. 2012). Branch support was estimated using posterior probabilities. For information on the applied programs in studies 1-3, see Sagorny et al. (in revision; 2019) and Sagorny & Döhren (in revision), respectively.

Resulting phylogenetic trees were visualized in SeaView (Gouy et al. 2010) and FigTree version 1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/).

2.2.4 Species delimitation methods

In the course of the project, several different species delimitation methods were employed to infer information on species composition and interspecific diversity in a dataset, as well as on intraspecific diversity and haplotype composition. Pairwise distances within the datasets were calculated using uncorrected p-distances in MEGA. Haplotype networks based on statistical parsimony were calculated either in TCS version 1.21 (Clement et al. 2000) or in PopArt (Leigh & Bryant 2015). Typically, the connection limit was set to 95% (Templeton et al. 1992). Further applied species delimitation methods include Automatic Barcode Gap Discovery (ABGD; Puillandre et al. 2012), nucleotide divergence threshold (NDT; Tang et al. 2012), multi-rate Poisson tree processes (mPTP; Kapli et al. 2017), and Generalized Mixed Yule Coalescent (GMYP). For information on the applied delimitation methods in studies 1-3, see Sagorny et al. (in revision; 2019) and Sagorny & Döhren (in revision), respectively. In all additional studies focussing on European heteronemerteans, only pairwise distances and TCS networks were calculated.

2.2.5 Population analyses

In order to infer information on population structuring in *Lineus sanguineus* and the three species of the *Lineus ruber/viridis* species complex, haplotype and nucleotide diversity were calculated in DnaSP version 6.12.03 (Rozas et al. 2017). An Analysis of Molecular Variance (AMOVA) was performed in GenAlEx version 6.503 (Peakall & Smouse 2006, 2012). Detailed procedures are given in Sagorny & Döhren (in revision).

2.3 Morphological methods

2.3.1 Photography

Prior to photography, all specimens were relaxed in an isotonic mix of 7% $MgCl_2$ with 1:1 seawater. Photographs of the specimens collected in the European intertidal zone were taken with a digital camera (Canon EOS 600D) mounted on a dissection microscope (Zeiss Stemi 2000). Specimens sampled along the Costa Rica margin were photographed alive under Leica MZ8 or MZ9.5 stereomicroscopes with a Canon EOS Rebel T6i camera attachment.

2.3.2 Histology

In order to describe several new nemertean species from Costa Rican deep waters, histological sections were prepared (see Sagorny et al. (in revision)). For this, the relaxed specimens were first fixed in 10% formaldehyde in seawater, before being transferred to 70% EtOH. In the following steps, specimens were dehydrated in an ascending ethanol series, incubated in methybenzoate and butanol, and preincubated in Histoplast (Thermo Scientific) at 60°C. After several days and multiple medium changes, they were embedded in Paraplast (McCormick Scientific). Serial sections of 5 µm thickness were prepared on an Autocut 2050 microtome (Reichert-Jung, Leica, Wetzlar). The resulting sections were transferred to glass slides coated with albumen-glycerin and afterwards stained following the AZAN trichrome staining protocol before being mounted in Malinol (Waldeck).

2.3.3 Light microscopy

Azan-stained histological sections were investigated with an Olympus BX-51 microscope equipped with an Olympus cc12 camera and a dotSlide 2.2 system (Olympus). Digitalized thin sections were aligned with Imod (Kremer et al. 1996) and Imodalign (http://www.q-terra.de/biowelt/3drekon/guides/imod_first_aid.pdf). All image series of the Costa Rican specimens are deposited in https://zenodo.org.

Larvae of *Lineus sanguineus* were mounted in seawater on a glass slide and also examined with an Olympus BX-51 microscope equipped with a ColorView Illu CCD camera (Soft Imaging System).

2.4 Experimental procedures

2.4.1 Hälterung

Specimens of *Lineus sanguineus* were used for experiments on reproductive and fissiparous behaviour and were thus kept at the Institute of Evolutionary Biology and Ecology in Bonn after collection. They were kept at 18°C with 16 hours of light per day. *Tubifex tubifex* (Müller, 1774) was fed once a week and water was exchanged once a month.

2.4.2 Experimental setups

The experimental procedures employed in study 2 to gain information on the influence of temperature and light on reproduction and fragmentation are described in Sagorny & Döhren (in revision). These included different temperatures and light regimes reflecting different seasonal conditions. Each setup included 20 specimens of one of the two investigated populations (Bergen and Concarneau) derived from the same clone and was observed over a period of 6 months. Once a month, regenerated worms and fragments were counted and measured.

Analyses of the obtained results performed in R version 3.6.1 (R Core Team 2019) are given in Sagorny & Döhren (in revision).

2.5 Image processing

Obtained photographs, histological images, and light microscopical images were processed with either Adobe Photoshop CS5 (Adobe) or Affinity Photo version 1.7.1.404 (Affinity). Results of phylogenetic analyses and species delimitation methods were edited in Adobe Illustrator CS5 (Adobe) and Affinity Designer version 1.7.1.404 (Affinity), respectively. All images and data were assembled into plates using the two formerly mentioned programs.

3 Results

3.1 Assessing the diversity and distribution of *Cephalothrix* species (Nemertea: Palaeonemertea) in European waters by comparing different species delimitation methods

Sagorny C, Wesseler C, Krämer D, & Döhren J von (2019). Assessing the diversity and distribution of *Cephalothrix* species (Nemertea: Palaeonemertea) in European waters by comparing different species delimitation methods. *Journal of Zoological Systematics and Evolutionary Research*, 57, 497-519. https://doi.org/10.1111/jzs.12266

The palaeonemertean genus *Cephalothrix* comprises more than 30 described species, with more than half described from European intertidal areas (Gibson 1995; Kajihara et al. 2008). Since all members of the genus are characterized by a pale and translucent body coloration, species delimitation based on external morphology is difficult (Chen et al. 2010; Kajihara et al. 2013). Molecular approaches have already shown that *Cephalothrix* comprises several cryptic species (Chen et al. 2010; Kajihara et al. 2013; Leasi & Norenburg 2014). Moreover, these approaches revealed some problematic species within this genus. These include *Cephalothrix simula* and *Cephalothrix hongkongiensis* (GIBSON, 1990) from both Europe and the NW Pacific, *Cephalothrix filiformis* from Europe and Japanese waters, and *Cephalothrix hermaphroditica* from Chile. Thus, the genus is predestined for application and testing of molecular species delimitation methods.

A combination of multiple non-tree-based and tree-based species delimitation methods, as well as phylogenetic analyses was tested for a dataset comprising only specimens sampled in Europe and for a dataset comprising additional identified or interesting specimens collected from different global localities downloaded from GenBank. The European dataset comprised 215 sequences, whereas the global dataset included 289 sequences.

If the most commonly applied threshold of 5% dissimilarity (Sundberg et al. 2009a; Chen et al. 2010; Kang et al. 2015; Krämer et al. 2017) is assumed sufficient for species separation, all delimitation methods and the phylogenetic analysis identify 12 or 13 distinct groups in the European dataset. The statistical parsimony analysis of the COI dataset yielded 13 unconnected haplotype networks (Fig 3.1.1, Tab 3.1.1). Several networks correspond to those found by Chen

et al. (2010). Only two networks (11: *C. simula* and 25: *C. hermaphroditica*) contain non-European sequences as inferred from the global dataset. Network 1 comprises 41% of all included specimens, represented by one large haplotype and 19 low-frequency haplotypes. All specimens in this network are identified as *Cephalothrix rufifrons* and were sampled in northern Europe. The five unidentified French specimens of Network 10 that represent one haplotype each are separated from *C. rufifrons* by only 32 mutational steps. As these specimens also morphologically resemble *C. rufifrons* this group is referred to as *Cephalothrix* cf. *rufifrons*. The second largest network, Network 11, comprises 31% of all included specimens, represented by one highly-frequent haplotype and five low-frequency haplotypes. Specimens in this network were mostly collected in the southern parts of the North Sea and the Mediterranean and correspond to *C. simula*, a species that was first described from Japanese waters. In addition to the European sampling site, 8 sequences originate from the North West Pacific. This species is assumed to be introduced to Europe from Asian coastal waters (Fernández-Álvarez & Machordom 2013; Kajihara et al. 2013; Faasse & Turbeville 2015). The second network comprising non-European sequence data is Network 25. Besides three specimens collected in France, this network comprises 5 specimens originating from Chile. All Chilean specimens share one haplotype, whereas the French specimens exhibit two haplotypes that are separated from each other by more substitutions than from the Chilean haplotype. The Chilean specimens were described as C. *hermaphroditica*, (Gibson et al. 1990; Kang et al. 2015). The statistical parsimony analysis yielded four further networks: Network 4 comprises the northern European species *C. filiformis*, Network 23 comprises the species *Cephalothrix oestrymnica* that has so far only been collected in France, Network 24 presumably comprises *Cephalothrix linearis* (RATHKE, 1799) that was collected in Norway, and Network 13 comprises three unidentified specimens sampled in Roscoff. Additionally, 5 networks are represented by a single individual each. Two of these were collected in Giglio. 18 substitutional steps are needed to connect these specimens.

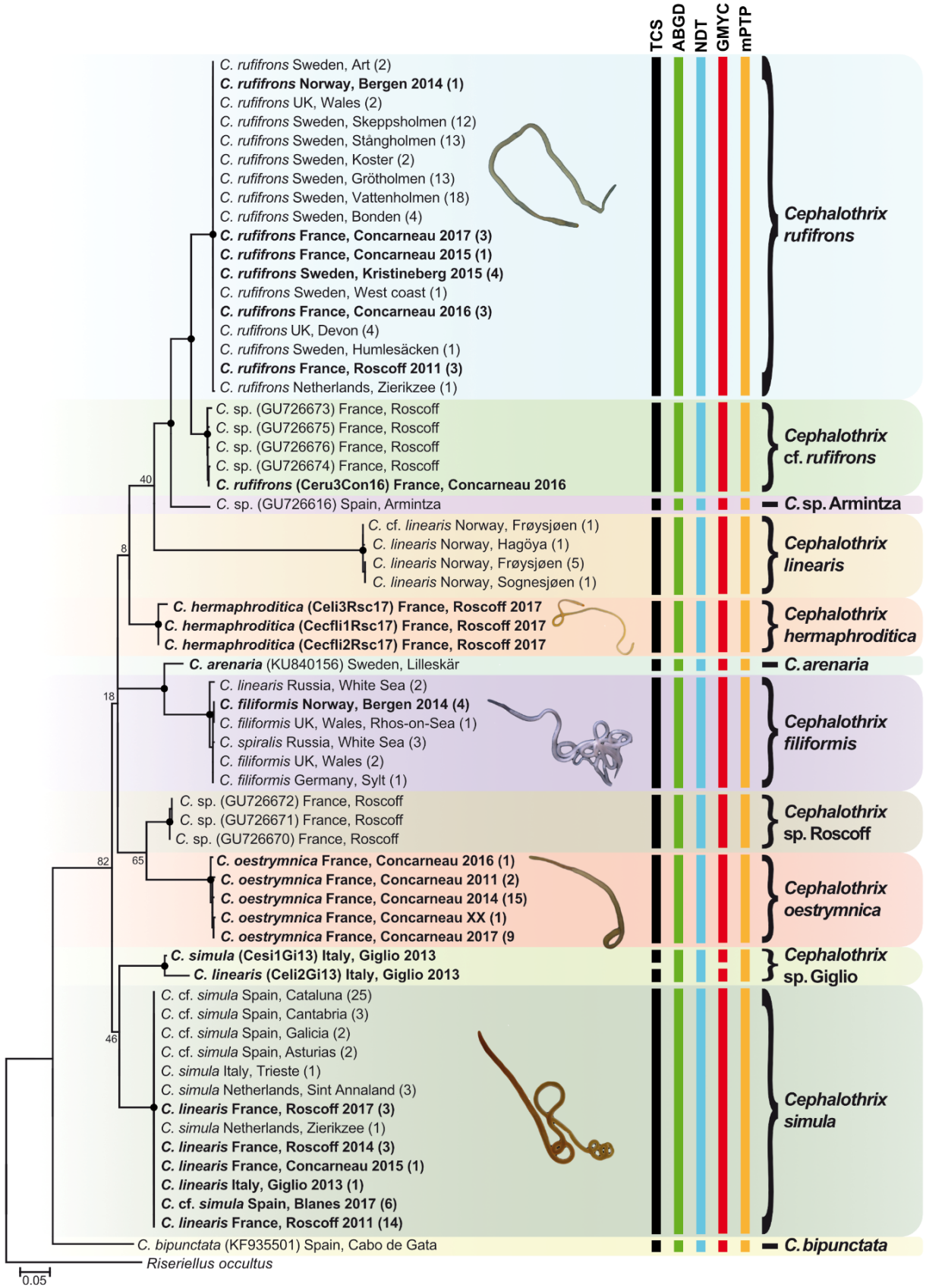
In contrast to the TCS analysis, both Automatic Barcode Gap Discovery (ABGD) and Nucleotide Divergence Threshold (NDT) detected only 12 distinct groups when a threshold of 5% was applied (Fig 3.1.1, Tab 3.1.1). In both analyses, the two specimens from Giglio are part of the same group. This result is supported by uncorrected intraspecific p-distances that are highest between the two specimens from Giglio (3.52%). The ABGD identified a distinct barcoding gap between 2 and 10%, although low interspecific distances between *C. rufifrons* and *C.* cf. *rufifrons* account for a disruption at 6-7%. When the threshold of dissimilarity is

decreased to 1%, all non-tree-based methods yielded more distinct entities than at 5%. An increased threshold (10%) either identified the same amount of entities as a 5% threshold (ABGD, TCS) or a slightly decreased number of entities (NDT).

The tree-based method based on multirate Poisson tree processes (mPTP) also identified 12 groups in the European dataset (Fig 3.1.1, Tab 3.1.1). In contrast to this, the generalized mixed Yule coalescent (GMYC) method identified 13 distinct entities, thus again separating the two specimens from Giglio (Fig 3.1.1, Tab 3.1.1). The maximum-likelihood (ML) analysis resulted in nine distinct lineages with high nodal support and three isolated specimens (Fig 3.1.1, Tab 3.1.1). A clade comprising the closely related *C. rufifrons* and *C.* cf. *rufifrons* specimens and an unidentified individual from Spain is supported by robust nodal support. This is also true for the sister group relation between *C. filiformis* from northern Europe and *Cephalothrix arenaria* HYLBOM, 1957 from Sweden. All other clades lack robust nodal support, so that the exact phylogenetic position of several clades cannot be fully resolved.

Table 3.1.1 Number of entities in the European and the global *Cephalothrix* datasets obtained by non-tree-based delimitation (TCS, ABGD, NDT), phylogenetic analysis and tree-based (mPTP, GMYC) delimitation methods. Entity numbers at three different thresholds are provided for the non-tree-based methods. #E at 5% threshold is given in bold. #E for three ambiguous cases is provided additionally.

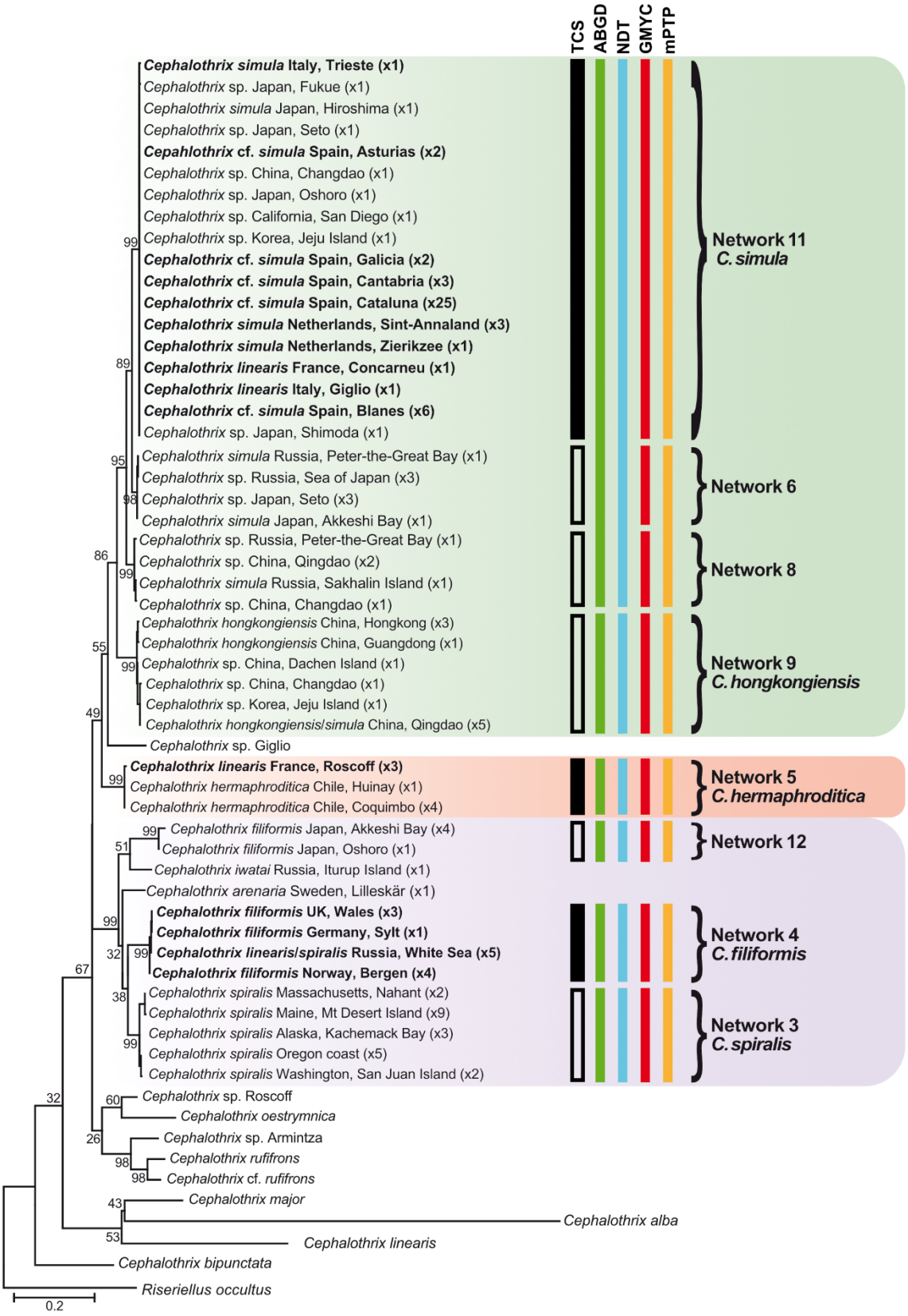
	TCS			ABGD			NDT					
	10%	5%	1%	10%	5%	1%	10%	5%	1%	ML	mPTP	GMYC
#E Europe	13	**13**	17	12	**12**	13	8	**12**	15	**12**	**12**	**13**
#E Global	21	**21**	28	-	**18**	21	10	**18**	28	**20**	**19**	**21**
#E *Cephalothrix* sp. Giglio	2	**2**	2	1	**1**	2	1	**1**	2	**1**	**1**	**2**
#E *C. simula* & *C. hongkongiensis*	4	**4**	4	-	**2**	4	4	**2**	4	**4**	**3**	**4**
#E *C. filiformis* (Europe), *C. filiformis* (Japan) & *C. spiralis*	3	**3**	5	-	**3**	5	1	**3**	7	**3**	**3**	**3**


TCS
ABGD
NDT
GMYC
mPTP
C. rufifrons Sweden, Art (2)
C. rufifrons Norway, Bergen 2014 (1)
C. rufifrons UK, Wales (2)
C. rufifrons Sweden, Skeppsholmen (12)
C. rufifrons Sweden, Stångholmen (13)
C. rufifrons Sweden, Koster (2)
C. rufifrons Sweden, Grötholmen (13)
C. rufifrons Sweden, Vattenholmen (18)
C. rufifrons Sweden, Bonden (4)
C. rufifrons France, Concarneau 2017 (3)
C. rufifrons France, Concarneau 2015 (1)
C. rufifrons Sweden, Kristineberg 2015 (4)
C. rufifrons Sweden, West coast (1)
C. rufifrons France, Concarneau 2016 (3)
C. rufifrons UK, Devon (4)
C. rufifrons Sweden, Humlesäcken (1)
C. rufifrons France, Roscoff 2011 (3)
C. rufifrons Netherlands, Zierikzee (1)
Cephalothrix rufifrons
C. sp. (GU726673) France, Roscoff
C. sp. (GU726675) France, Roscoff
C. sp. (GU726676) France, Roscoff
C. sp. (GU726674) France, Roscoff
C. rufifrons (Ceru3Con16) France, Concarneau 2016
Cephalothrix cf. rufifrons
C. sp. (GU726616) Spain, Armintza
C. sp. Armintza
C. cf. linearis Norway, Frøysjøen (1)
C. linearis Norway, Hagöya (1)
C. linearis Norway, Frøysjøen (5)
C. linearis Norway, Sognesjøen (1)
Cephalothrix linearis
C. hermaphroditica (Celi3Rsc17) France, Roscoff 2017
C. hermaphroditica (Cecfli1Rsc17) France, Roscoff 2017
C. hermaphroditica (Cecfli2Rsc17) France, Roscoff 2017
Cephalothrix hermaphroditica
C. arenaria (KU840156) Sweden, Lilleskär
C. arenaria
C. linearis Russia, White Sea (2)
C. filiformis Norway, Bergen 2014 (4)
C. filiformis UK, Wales, Rhos-on-Sea (1)
C. spiralis Russia, White Sea (3)
C. filiformis UK, Wales (2)
C. filiformis Germany, Sylt (1)
Cephalothrix filiformis
C. sp. (GU726672) France, Roscoff
C. sp. (GU726671) France, Roscoff
C. sp. (GU726670) France, Roscoff
Cephalothrix sp. Roscoff
C. oestrymnica France, Concarneau 2016 (1)
C. oestrymnica France, Concarneau 2011 (2)
C. oestrymnica France, Concarneau 2014 (15)
C. oestrymnica France, Concarneau XX (1)
C. oestrymnica France, Concarneau 2017 (9
Cephalothrix oestrymnica
C. simula (Cesi1Gi13) Italy, Giglio 2013
C. linearis (Celi2Gi13) Italy, Giglio 2013
Cephalothrix sp. Giglio
C. cf. simula Spain, Cataluna (25)
C. cf. simula Spain, Cantabria (3)
C. cf. simula Spain, Galicia (2)
C. cf. simula Spain, Asturias (2)
C. simula Italy, Trieste (1)
C. simula Netherlands, Sint Annaland (3)
C. linearis France, Roscoff 2017 (3)
C. simula Netherlands, Zierikzee (1)
C. linearis France, Roscoff 2014 (3)
C. linearis France, Concarneau 2015 (1)
C. linearis Italy, Giglio 2013 (1)
C. cf. simula Spain, Blanes 2017 (6)
C. linearis France, Roscoff 2011 (14)
Cephalothrix simula
C. bipunctata (KF935501) Spain, Cabo de Gata
C. bipunctata
Riseriellus occultus
40
8
18
82
65
46
0.05

◄ **Figure 3.1.1** Maximum-likelihood tree (GTR+G+I) of the complete European *Cephalothrix* dataset based on COI mtDNA. Numbers above nodes indicate bootstrap support from 500 replicates for each clade; black circles indicate nodal support of 100. *Riseriellus occultus* was used for outgroup rooting. Specimens sequenced for this study are given in bold. Entities detected by non-tree-based (TCS, ABGD, NDT) and tree-based (GMYC, mPTP) delimitation methods are indicated by coloured bars. Names of entities are the results of the detected species in the ML analysis. Exemplary photographs are provided for the most common species collected for this study. Figure modified after Sagorny et al. (2019).

In the global dataset, the statistical parsimony analysis yielded 21 unconnected haplotype networks under a 5% dissimilarity threshold (Fig 3.1.2, Tab 3.1.1). Thirteen networks correspond to the ones already detected in the European dataset. In addition to the European *C. simula* network (Network 11), two further networks (Network 6 and 8) comprise specimens that were previously identified as *C. simula*. Sampling sites for both networks are restricted to the North West Pacific. In some previous studies *C. hongkongiensis* (Network 9) was synonymized with *C. simula*. Based on the TCS analysis *C. hongkongiensis* is a distinct entity. Besides *C. simula*, only *C. hermaphroditica* combines European with non-European (Chile) specimens. Another problematic species is *C. filiformis* as specimens have been sampled under this name both from European as well as eastern Asian waters. Network 12 comprises the *C. filiformis* specimens from Japan, whereas the European network (Network 4) only includes European specimens. Since this species was first described from Europe, the name *C. filiformis* is assigned to Network 4. Fifty additional substitutions are needed to connected both networks. The remaining networks include one group from the north eastern Pacific identified as *Cephalothrix major* COE, 1930, one group from the Caribbean Sea identified as *Cephalothrix alba* GIBSON & SUNDBERG, 1992, and one from both the Atlantic and Pacific coasts of North America identified as *Cephalothrix spiralis* COE, 1930. Additionally, one single haplotype is present that was recently collected from the depths of the Sea of Japan (1494-3334 m) and described as *Cephalothrix iwatai* CHERNYSHEV, 2013.

In contrast to this, both ABGD and NDT combine Networks 6 and 8 with Network 4 (*C. simula*), thus resulting in only 18 distinct clades under a threshold of 5% (Fig 3.1.2, Tab 3.1.1). This again is supported by intraspecific p-distances as *C. simula* and Network 8 differ by only 3.12% to 4.68%, and *C. simula* and Network 6 are separated by a divergence of 4.87% and 5.26%.


TCS
ABGD
NDT
GMYC
mPTP
Cephalothrix simula Italy, Trieste (x1)
Cephalothrix sp. Japan, Fukue (x1)
Cephalothrix simula Japan, Hiroshima (x1)
Cephalothrix sp. Japan, Seto (x1)
Cepahlothrix cf. simula Spain, Asturias (x2)
Cephalothrix sp. China, Changdao (x1)
Cephalothrix sp. Japan, Oshoro (x1)
Cephalothrix sp. California, San Diego (x1)
Cephalothrix sp. Korea, Jeju Island (x1)
Cephalothrix cf. simula Spain, Galicia (x2)
Cephalothrix cf. simula Spain, Cantabria (x3)
Cephalothrix cf. simula Spain, Cataluna (x25)
Cephalothrix simula Netherlands, Sint-Annaland (x3)
Cephalothrix simula Netherlands, Zierikzee (x1)
Cephalothrix linearis France, Concarneu (x1)
Cephalothrix linearis Italy, Giglio (x1)
Cephalothrix cf. simula Spain, Blanes (x6)
Cephalothrix sp. Japan, Shimoda (x1)
Network 11
C. simula
Cephalothrix simula Russia, Peter-the-Great Bay (x1)
Cephalothrix sp. Russia, Sea of Japan (x3)
Cephalothrix sp. Japan, Seto (x3)
Cephalothrix simula Japan, Akkeshi Bay (x1)
Network 6
Cephalothrix sp. Russia, Peter-the-Great Bay (x1)
Cephalothrix sp. China, Qingdao (x2)
Cephalothrix simula Russia, Sakhalin Island (x1)
Cephalothrix sp. China, Changdao (x1)
Network 8
Cephalothrix hongkongiensis China, Hongkong (x3)
Cephalothrix hongkongiensis China, Guangdong (x1)
Cephalothrix sp. China, Dachen Island (x1)
Cephalothrix sp. China, Changdao (x1)
Cephalothrix sp. Korea, Jeju Island (x1)
Cephalothrix hongkongiensis/simula China, Qingdao (x5)
Network 9
C. hongkongiensis
Cephalothrix sp. Giglio
Cephalothrix linearis France, Roscoff (x3)
Cephalothrix hermaphroditica Chile, Huinay (x1)
Cephalothrix hermaphroditica Chile, Coquimbo (x4)
Network 5
C. hermaphroditica
Cephalothrix filiformis Japan, Akkeshi Bay (x4)
Cephalothrix filiformis Japan, Oshoro (x1)
Cephalothrix iwatai Russia, Iturup Island (x1)
Network 12
Cephalothrix arenaria Sweden, Lilleskär (x1)
Cephalothrix filiformis UK, Wales (x3)
Cephalothrix filiformis Germany, Sylt (x1)
Cephalothrix linearis/spiralis Russia, White Sea (x5)
Cephalothrix filiformis Norway, Bergen (x4)
Network 4
C. filiformis
Cephalothrix spiralis Massachusetts, Nahant (x2)
Cephalothrix spiralis Maine, Mt Desert Island (x9)
Cephalothrix spiralis Alaska, Kachemack Bay (x3)
Cephalothrix spiralis Oregon coast (x5)
Cephalothrix spiralis Washington, San Juan Island (x2)
Network 3
C. spiralis
Cephalothrix sp. Roscoff
Cephalothrix oestrymnica
Cephalothrix sp. Armintza
Cephalothrix rufifrons
Cephalothrix cf. rufifrons
Cephalothrix major
Cephalothrix alba
Cephalothrix linearis
Cephalothrix bipunctata
Riseriellus occultus
0.2

◄ **Figure 3.1.1** Maximum-likelihood tree (GTR+G+I) of selected global *Cephalothrix* species based on COI mtDNA. Ambiguous cases including the *Cephalothrix simula/ hongkongiensis* species complex (green), *Cephalothrix hermaphroditica* (orange), and the *Cephalothrix filiformis/spiralis* species complex (purple) are highlighted. Numbers above nodes indicate bootstrap support from 500 replicates for each clade; black circles indicate nodal support of 100. *Riseriellus occultus* was used for outgroup rooting. Specimens sequenced for this study are given in bold. Entities detected by non-tree-based (TCS, ABGD, NDT) and tree-based (GMYC, mPTP) delimitation methods are indicated by coloured bars. Names of entities are based on network numbers as described in Chen et al. (2010). Solid boxes represent TCS networks including European sequence data (see Figure 3.1.1); empty boxes represent networks without European sequence data. Figure modified after Sagorny et al. (2019).

The tree-based delimitation methods yet again suggest the presence of 19 (mPTP) or 21 (GMYC) distinct entities (Fig 3.1.2, Tab 3.1.1). The results of the GMYC analysis are in accordance with the statistical parsimony networks, whereas mPTP combines Network 6 and Network 8, thus resulting in a lower number of detected entities. The ML analysis yielded 20 distinct lineages, thus separating the three "*Cephalothrix simula*" clades. Nevertheless, this grouping is supported by high nodal support, whereas the sister group relationship between this group and *C. hongkongiensis* shows only moderate support. Furthermore, *Cephalothrix filiformis* from Europe is closer related to *C. spiralis* than to the Japanese specimens identified as *C. filiformis*. Nonetheless, a clade combing the former three species as well as *C. iwatai* and *C. arenaria* has robust nodal support. As in the solely European dataset, all other groupings lack robust nodal support.

Overall, at a 5% threshold, all delimitation methods suggested the presence of 12 to 13 European clades, depending on the grouping of the two specimens collected in Giglio. In Europe, the presence of six already described species could be proven. These include *C. rufifrons*, *C. arenaria*, *C. filiformis*, *C. oestrymnica*, *C. bipunctata*, and *C. simula*. Additionally, first evidence of *C. hermaphroditica* – a species so far only known from Chile – in Europe has been provided. Moreover, cryptic speciation was detected as in the case of *C.* cf. *rufifrons*. With regard to several specimens that could not be assigned to a known species, it can be assumed that European species diversity of the genus *Cephalothrix* is indeed higher than previously expected. Including specimen data obtained from non-European localities showed that only two species that can be found in Europe also occur in other coastal regions. Moreover, the limits and problems of the species delimitation methods became visible, especially with regard to the applied threshold in the non-tree-based species delimitation methods (see the case of *C. simula*).

3.2 Occasional reproduction significantly affects the population structure of the widespread, predominantly asexually reproducing marine worm *Lineus sanguineus* (Nemertea: Pilidiophora)

Sagorny C & Döhren J von (in revision). Occasional reproduction significantly affects the population structure of the widespread, predominantly asexually reproducing marine worm *Lineus sanguineus* (Nemertea: Pilidiophora). *Marine Biology*.

The presumably cosmopolitan heteronemertean *Lineus sanguineus* has long been known for its spontaneous fragmentation and regeneration capacities, including regeneration of a new head (e.g. McIntosh 1873-1874; Coe 1929, 1930; Sivaradjam & Bierne 1981; Bierne 1990). These regenerative capacities are thought to provide the basis for asexual reproduction by fissiparity, which appears to be the dominant mode of reproduction (Gontcharoff 1951; Bierne 1970; Gibson 1972). Sexual reproduction or larvae have never been observed before (Coe 1943; Gontcharoff 1951; Riser 1994). Therefore, *L. sanguineus* is a good candidate to infer information on the effects of presumed asexual reproduction on population structuring by AMOVA.

In order to test for a cosmopolitan distribution, a separate maximum-likelihood analysis of the mitochondrial COI and 16S gene fragments and the nuclear ITS gene fragment were performed. Furthermore, a concatenated analysis of all three datasets was conducted. Besides specimens sampled in Europe, the COI and 16S datasets were further combined with specimen data deposited on GenBank, originating from the SW Atlantic, and from the NE, NW and SE Pacific. In all four analyses, all specimens of *L. sanguineus* form a single well supported clade independent of specimen origin. Thus, despite the many existing species names linked to locality, *L. sanguineus* has a cosmopolitan distribution.

The COI dataset comprised 298 sequences in total, sampled in Europe, the Pacific coast of Canada and the US, China, Argentina, and Chile. 15 polymorphic sites were present in the dataset. Haplotype diversity was moderately high (Hd=0.732; SD=0.011), but nucleotide diversity was very low (π=0.00315; SD= 0.00016). A statistical parsimony network yielded one network consisting of 15 haplotypes (Fig 3.2.1 A). 90% of all specimens exhibit one of the three high-frequency haplotypes. Haplotypes are separated by one to three nucleotide substitutions.

Separation of haplotypes by locality could not be detected, only the Mediterranean specimens share the same haplotype (H4).

Table 3.2.1 COI AMOVA values based on regions. Pairwise Φ_{PT} values are given above diagonal; p-values are given below diagonal. Pairwise Φ_{PT} values between Mediterranean and all other populations are given in bold. N Atlantic (=22) contains Norway; NE Atlantic (=106) contains France (=56), Spain (=13), and Wales (=37); North Sea (=30) contains France (=6) and Germany (=24); Mediterranean (=8) contains France (=3) and Spain (=5); SW Atlantic (=8) contains Argentina; NE Pacific (=45) contains Canada (=42) and US west coast (=3); NW Pacific (=69) contains China; SE Pacific (=5) contains Chile.

	N Atlantic	NE Atlantic	North Sea	Mediter-ranean	SW Atlantic	NE Pacific	NW Pacific	SE Pacific
N Atlantic	-	0.030	0.033	0.001	0.023	0.001	0.005	0.001
NE Atlantic	0.048	-	0.016	0.001	0.397	0.001	0.013	0.018
North Sea	0.085	0.016	-	0.001	0.029	0.001	0.003	0.003
Mediterranean	**0.785**	**0.618**	**0.728**	-	0.001	0.001	0.001	0.001
SW Atlantic	0.201	0.000	0.165	**0.692**	-	0.231	0.372	0.225
NE Pacific	0.422	0.230	0.391	**0.730**	0.034	-	0.005	0.330
NW Pacific	0.153	0.037	0.111	**0.634**	0.000	0.099	-	0.262
SE Pacific	0.498	0.188	0.415	**0.725**	0.000	0.000	0.034	-

For a population analysis, close geographical populations were combined into eight regions (Tab 3.2.1). The AMOVA analysis based on the COI dataset detected higher levels of differentiation between populations (Φ_{PT}=0.327; $p<0.05$) than between regions (Φ_{RT}=0.109; $p<0.05$). As 60% of all variation is found within populations and only 29% among populations, gene flow between populations appears to be likely. Variability among regions accounts for only 11% of all variation. Based on pairwise Φ_{PT} values, a high amount of gene flow occurs between the three northern Atlantic regions (Φ_{PT}=0.016-0.085, $p<0.05$) as only low levels of differentiation occur. In contrast to this, high levels of differentiation occur between Norwegian (N Atlantic) and NW Pacific populations, as well as between SE Pacific and northern Atlantic populations (Φ_{PT}=0.415-0.498; $p<0.05$). Only one region, the Mediterranean, exhibits high levels of differentiation from all other regions (Φ_{PT}=0.618-0.785, $p<0.05$). Thus, reduced gene flow is assumed between the Mediterranean populations and populations originating from the remaining seven regions. This result is also reflected by the TCS analysis. All remaining pairwise comparisons resulted in low to moderate levels of differentiation, although not all results are significant due to a small sequence pool for some regions (SE Pacific, SW Atlantic). The only region that shows high levels of differentiation from all other regions is the

Mediterranean, (Φ_{PT}=0.617-0.785, p<0.05). Isolation by distance could be rejected as no significant relationship between geographic and genetic distance could be inferred by the Mantel test (R_{xy}=0.047, p>0.05).

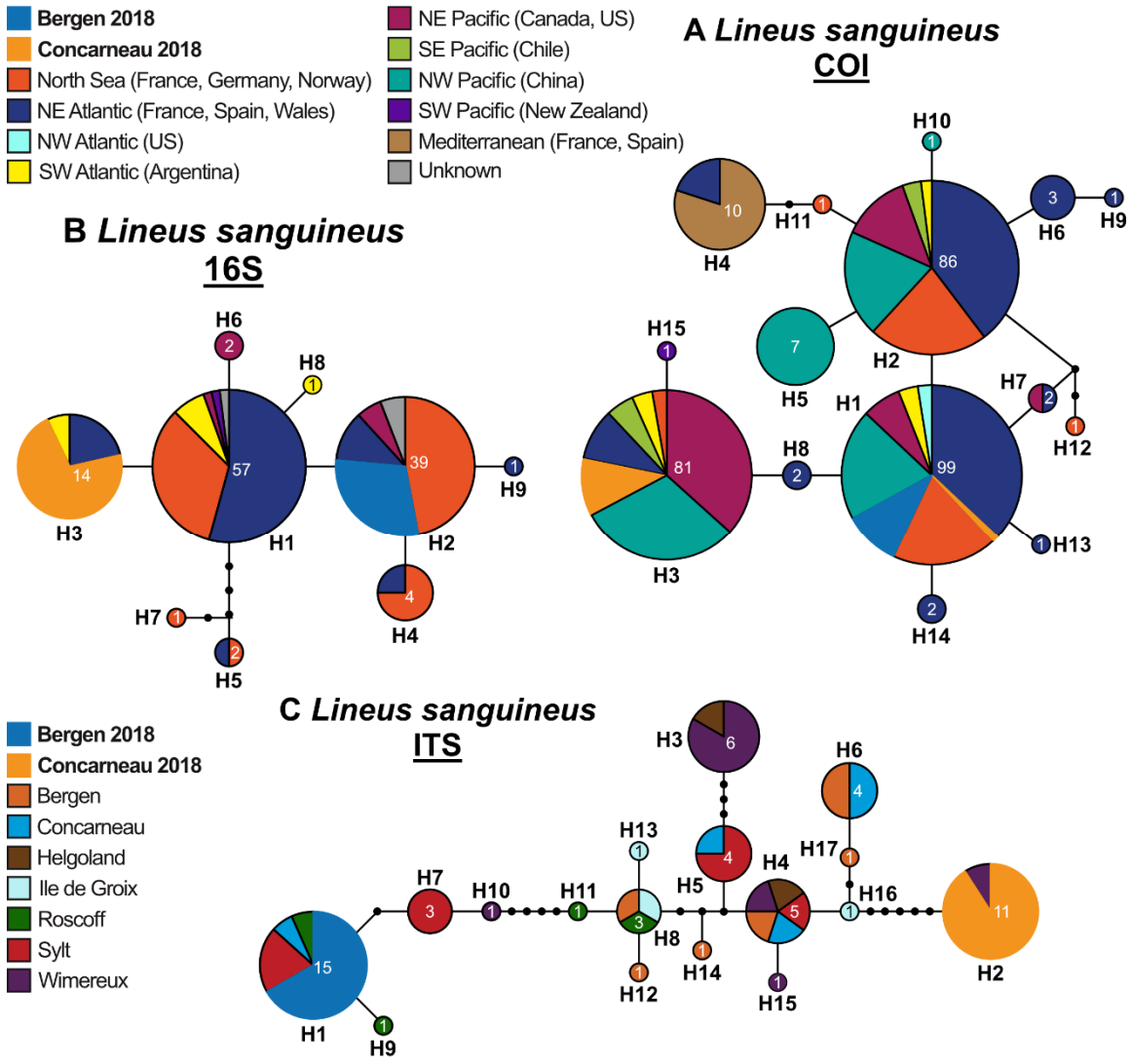
The 16S dataset included 121 specimens from Europe, Argentina, and the Pacific coast of the US and 13 polymorphic sites. Haplotype diversity was moderate (Hd=0.535; SD=0.037), but nucleotide diversity was very low (π=0.00204; SD=0.00037). The TCS analysis yielded one network represented by nine haplotypes (Fig 3.2.1 B). One of two highly frequent haplotypes is present in 80% of all specimens. One to five mutational steps are needed to connect the haplotypes. Populations are not ordered by separated haplotypes.

The AMOVA analysis based on the 16S dataset detected moderate levels of differentiation between both populations (Φ_{PT}=0.245; p<0.05) and regions (Φ_{RT}=0.178; p<0.05). As in the COI dataset, variation is highest between individuals (75%), whereas only 7% of all variation is detected among populations. This again points at gene flow between populations. Among region variability accounts for 18% of the detected variation. Close geographical populations were combined into five regions (Tab 3.2.2). The lowest significant level of differentiation occurs between populations from Norway and the North Sea (Φ_{PT}=0.092; p<0.05). Low to moderate levels of differentiation are also present between all other regions, but not all results are significant due to the small sample size. The highest level of differentiation exists between the two northern Atlantic populations, and between N Atlantic and SW Atlantic populations (Φ_{PT}=0.346-0.349; p<0.05).

Table 3.2.2 16S AMOVA values based on regions. Pairwise Φ_{PT} values are given above diagonal; p-values are given below diagonal. N Atlantic (=21) contains Norway; NE Atlantic (=53) contains France (=46), Spain (=5), and Wales (=2); North Sea (=29) contains France (=6) and Germany (=23); SW Atlantic (=5) contains Argentina; NE Pacific (=7) contains US west coast.

	N Atlantic	NE Atlantic	North Sea	SW Atlantic	NE Pacific
N Atlantic	-	0.001	0.023	0.002	0.296
NE Atlantic	0.349	-	0.001	0.289	0.003
North Sea	0.092	0.166	-	0.037	0.225
SW Atlantic	0.346	0.025	0.197	-	0.043
NE Pacific	0.039	0.257	0.032	0.256	-

The ITS dataset included 60 specimens collected in Europe. 26 polymorphic sites were present. Haplotype diversity was high (Hd=0.885; SD=0.024), whereas nucleotide diversity was low (π=0.00527; SD=0.00032). The TCS network comprised 17 distinct haplotypes (Fig 3.2.1 C). In contrast to COI and 16S, there are no highly frequent haplotypes in the ITS dataset, only several medium-frequency haplotypes. Except for H7, all haplotypes are present in specimens from different localities. One to six nucleotide substitutions are needed to connect the haplotypes. Haplotype diversity and structuring is most pronounced in the ITS dataset.



◄ **Figure 3.2.1** Statistical parsimony haplotype networks of all sequences *Lineus sanguineus* specimens with a connection limit of 95% baes on mitochondrial cytochrome *c* oxidase subunit I gene (**A**), mitochondrial 16S rRNA (**B**), and nuclear internal transcribed spacer rRNA (**C**). **A** and **B** include additional sequence data taken from GenBank. Geographic distribution by maritime zones is represented by colour. Specimens from both further examined populations are highlighted (Concarneau, France = orange; Bergen, Norway = blue). Geographic distribution in **C** is based on the sampling sites. Numbers within pie charts represent number of specimens with respective haplotype. Empty lines indicate a single substitution; each black dot indicates one additional nucleotide substitution. Figure modified after Sagorny & Döhren (in revision).

In order to gain information on asexual and sexual reproduction in *L. sanguineus*, additional experiments with varying light and temperature regimes were conducted. Two European populations were chosen for these experiments, one from Concarneau (France) and one from Bergen (Norway). All specimens of one population shared one haplotype in the statistical parsimony analysis based on the COI gene fragment. Both populations were tested for their fragmentary and reproductive behavior under different climatic set-ups. The Bergen population was subjected to two different set-ups ("winter" and "summer"), whereas specimens of the France population were additionally kept at "spring" and "fall" conditions. In the Bergen population, winter conditions (9°C, 8h light) promoted fragmentation, although the number of specimens possessing a head showed no increase over six months (Fig 3.2.2 C). After six months, specimens kept under winter conditions were significantly longer than those kept under summer conditions (18°C, 16h light) (Mann-Whitney U test, W=182, p=0.007) (Fig 3.2.2 D). Summer conditions delayed fragmentation, yielded less fragments, but led to an increase in specimens possessing a head (Figs 3.2.2 C, D).

In the French population, fall conditions (18°C, 8h light) yielded most fragments until month 3 (Fig 3.2.2 A). Afterwards this setup had to be replaced because all specimens of the original setup had deceased. Until month 3, spring (9°C, 16h light) and winter conditions yielded less fragments than both setups with higher temperatures (Fig 3.2.2 A). Under summer conditions, specimens gained length faster than under winter conditions but afterwards these specimens decreased in length (Fig 3.2.2 B). Spring conditions supported the strongest increase in length.

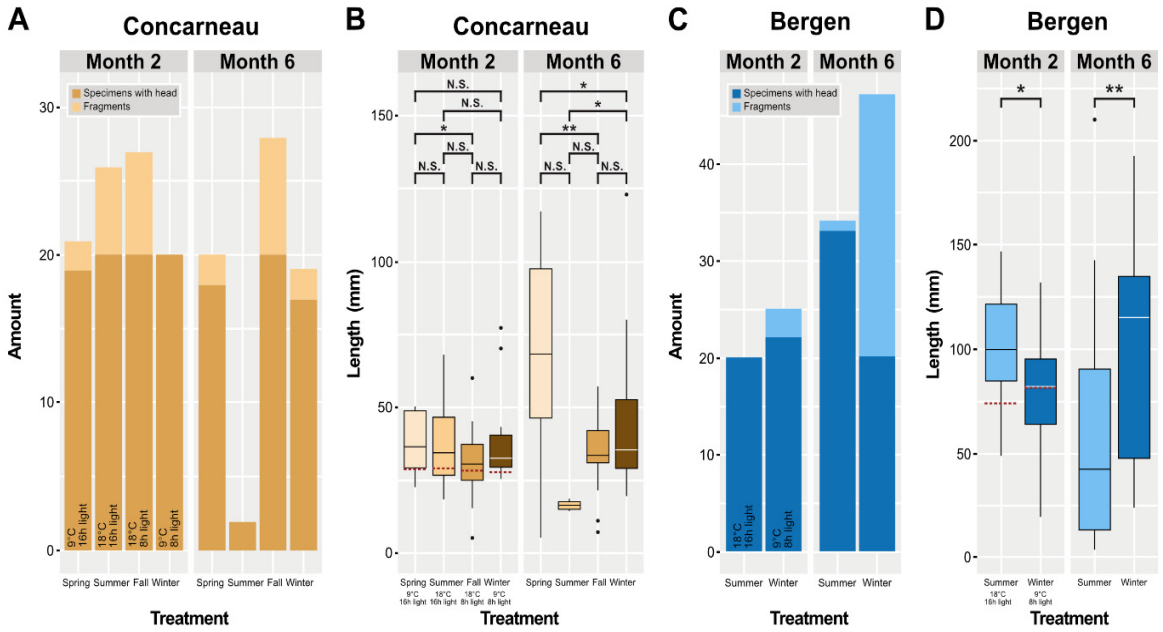


Figure 3.2.2 Fragmentation and growth of *Lineus sanguineus* specimens from the Concarneau population (orange) and the Bergen population (blue). Specimens of the French population (**A** and **B**) were kept at spring (9°C, 16h light), summer (18°C, 16h light), fall (18°C, 8h light), and winter (9°C, 8h light) conditions, whereas specimens of the Bergen population (**C** and **D**) were kept at summer (18°C, 16h light) and winter (9°C, 8h light) conditions. Experimental procedure commenced with 20 individuals for each setup. Complete animals and fragments were counted and measured over a period of six months. Results after one month ("Month 2") and at the end of the experiments ("Month 6") are presented. **A** and **C** Number of fragments and complete animals of *L. sanguineus*. **B** and **D** Length variation of complete specimens. Red dashed lines represent mean value at the beginning of the experiments. Significance was tested based on the Wilcoxon rank sum test (*p < 0.05, **p < 0.01). Figure modified after Sagorny & Döhren (in revision).

At the beginning of the experiments, 2/3 of all specimens of the Bergen population already had gonads (Fig 3.2.3 A). At summer conditions, the number of specimens bearing gonads drastically declined over the course of the experiment (Fig 3.2.3 A). Mature specimens were significantly longer that immature ones (Mann-Whitney U test, W=563.5, p<0.05) (Fig 3.2.3 D). Over the course of the experiments, specimens retained gonads under winter conditions and there was no significant difference in length between sexually mature (bearing gonads) and immature specimens (Mann-Whitney U test, W=1451, p=0.05) (Fig 3.2.3 E). At the end of the experiments, several clutches of eggs could be detected (Fig 3.2.3 A). In contrast to the Bergen population, none of the French specimens showed any signs of gonad maturation during the whole course of the experiment. Nevertheless, few larvae occurred in this population prior to the start of the experiments (Figs 3.2.3 B, C). This is the first evidence of a pilidium

larva for *L. sanguineus*. Exteriorly, these larvae are roughly similar to three to four-day old larvae of *Riseriellus occultus* (Beckers et al. 2015).

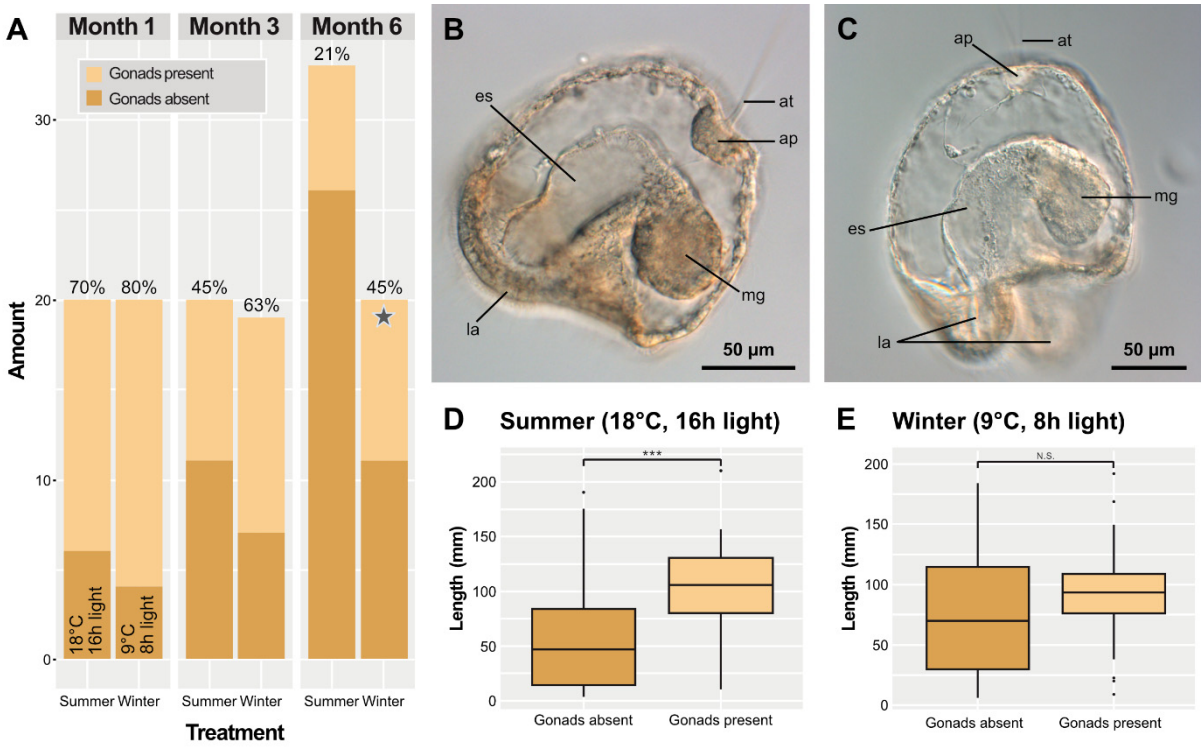


Figure 3.2.3 Sexual maturation and first evidence of pilidium larvae of *Lineus sanguineus*. **A** Number of specimens bearing gonads in the Bergen population at summer (18°C, 16h light) and winter (9°C, 8h light) conditions, examined at the beginning, two months after the start ("Month 3"), and at the end of the experiments. Number of specimens bearing gonads given in percent. Asterisk denotes presence of egg clutches. **B** and **C** Two young pilidium larvae found in the Concarneau population (three to four days post fertilization) of *Lineus sanguineus*. **D** and **E** Length difference of specimens bearing gonads and specimens lacking gonads in the Bergen population. Significance was tested based on the Wilcoxon rank sum test (***$p < 0.001$). At summer conditions (**D**), there is a significant length difference between sexually mature and immature specimens. At winter conditions (**E**), no significant length difference was detected. Abbreviations: *ap* apical plate, *at* apical tuft, *es* oesophagus, *la* lateral lappets, *mg* midgut. Figure modified after Sagorny & Döhren (in revision).

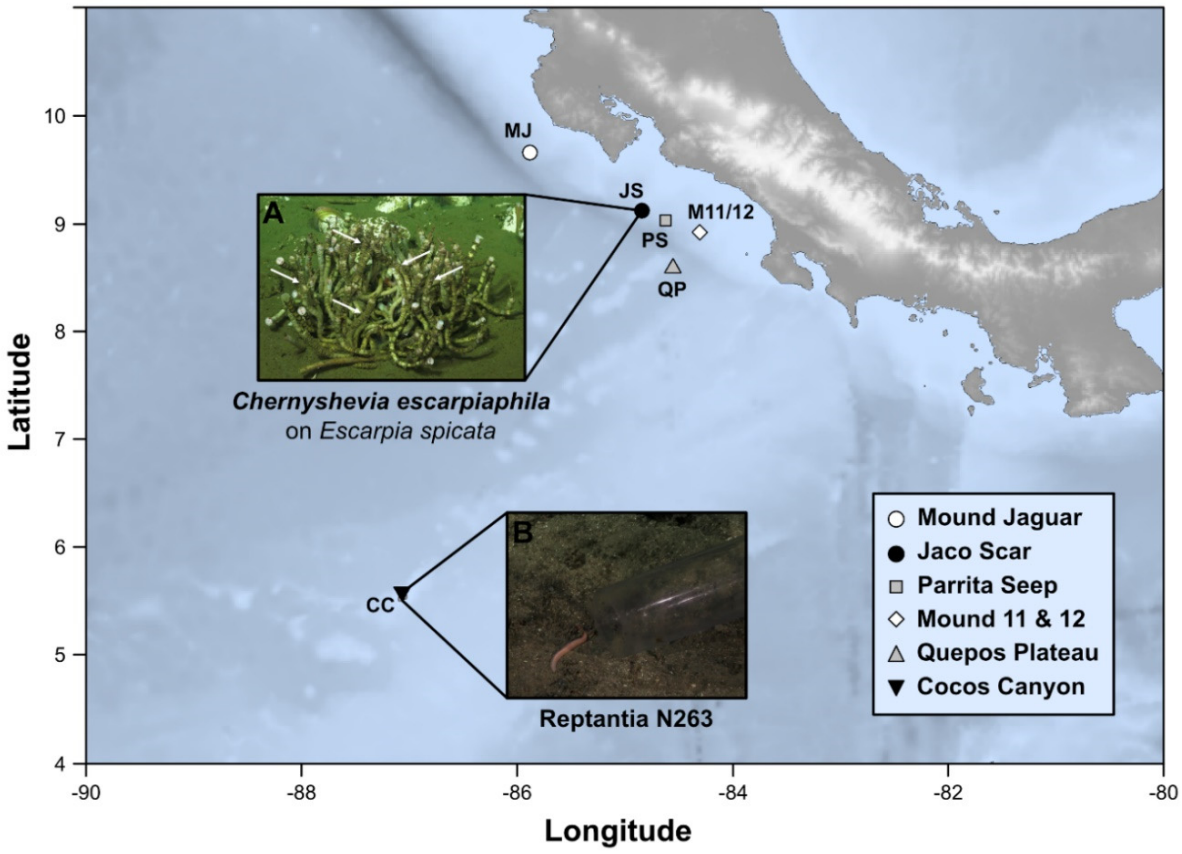
Overall, the results show that *L. sanguineus* is a single cosmopolitan species that mainly reproduces asexually by fissiparity. Both the statistical parsimony analysis and an AMOVA analysis could not support population structuring based on geographic location, thus hinting at gene flow. This can be explained by both dispersal of larvae and dispersal of adult specimens. In general, genetic exchange between global populations is most likely maintained by at least sporadic sexual reproduction via a pilidium larva. Nevertheless, the importance and extent of

adult dispersal by ship could not be finally determined. Sexual maturation appears to be promoted by low temperatures, but other factors apparently also have an influence on sexual reproduction in *L. sanguineus*. This study supports the importance of molecular data, when it comes to determine a species' range of distribution. Moreover, the effectiveness of population analyses in nemertean systematics could be highlighted.

3.3 Cutting the ribbon: Bathyal nemerteans from seeps along the Costa Rica margin, with descriptions of 3 new genera and 10 new species

Sagorny C, Döhren J von, Rouse GW, & Tilic E (in revision). Cutting the ribbon: Bathyal nemerteans from seeps along the Costa Rica margin with descriptions of 3 new genera and 10 new species. *European Journal of Taxonomy*.

Nemertean species often lack distinctive external morphological characters, so that species descriptions are usually still based on internal morphological characters (Gibson 1985; Strand et al. 2014; Sundberg et al. 2016a). This is especially true for specimens collected in the deep sea (Chernyshev 2013; Chernyshev & Polyakova 2018a, 2018b). Lately, several benthic deep-sea nemerteans have been collected and described in the NW Pacific (reviewed in Chernyshev 2020). For the present study, nemertean specimens that were collected in the bathyal zone of the Costa Rica margin are described mainly based on the turbotaxonomic approach, but brief descriptions of internal morphology are included whenever possible.



◄ **Figure 3.3.1** Sampling sites along the Costa Rica margin and exemplary habitat pictures. From North to South, the seven sampling sites include Mound Jaguar (MJ), Jaco Scar (JS), Parrita Seep (PS), Mound 11 (M11), Mound 12 (M12), Quepos Plateau (QP), and Cocos Canyon (CC). **A** The newly described species *Chernyshevia escarpiaphila* was collected on aggregations of the vestimentiferan *Escarpia spicata* at Jaco Scar. Arrows indicate nemertean specimens. **B** The single reptant specimen (SIO-BIC N263) was collected on sandy sediments at Cocos Canyon. Figure modified after Sagorny et al. (in revision).

Five deep-sea expeditions along the Costa Rica margin in the eastern Pacific between 2009 and 2019 yielded a total of 84 benthic nemertean specimens. These were collected at seven different localities in depths between 950 m and 2,200 m (Fig 3.3.1). The specimens are mostly small in size (5-15 mm) and usually have a uniform pale body coloration and a translucent body wall. All three major nemertean groups are represented in the samples. Based on a concatenated analysis (COI, 16S, H3, 18S), 12 distinct species could be identified. Palaeonemerteans are represented by one tubulanid, heteronemerteans by one lineid, and polystiliferous hoplonemerteans by one reptant species. In contrast to this, nine species of monostiliferous hoplonemerteans were recovered in the analyses. In total, eight of the twelve species are described, together with two further benthic species that were collected in the bathyal zone off the coast of Oregon.

The palaeonemertean species is represented by six specimens that were collected at depths around 1,000 m from Mound 11 and Mound 12. Based on a phylogenetic analysis and internal morphology, this species can be clearly identified as a member of the genus *Tubulanus*. The species is named *Tubulanus dariae* sp. nov. and exhibits a bright red body coloration, lacking a conspicuous colour pattern (Fig 3.3.2 A). As typical for deep-sea nemerteans, this species lacks eyes. In the concatenated analysis, *T. dariae* sp. nov. belongs to a well-supported clade containing *Tubulanus ezoensis* YAMAOKA, 1940, *Tubulanus polymorphus* RENIER, 1804, and an undescribed tubulanid from the Sea of Okhotsk (Fig 3.3.3). Except for the latter, these species occur in shallow waters. A clade comprising solely undescribed abyssal and hadal tubulanid specimens is sister to the *T. dariae* clade. If only concentrating on the COI gene, the Swedish shallow water species *Tubulanus lutescens* CANTELL, 2001 is closely related to *T. dariae* with pairwise distances varying between 1-1.3%. Besides dissimilar habitats, both species markedly differ in morphological characters, such as body coloration (bright red in *T. dariae* vs. yellow in *T. lutescens*) and the presence of a lower dorsal nerve (absent in *T. dariae* vs. present in *T. lutescens*). Thus, both species vary sufficiently to justify naming a new species.

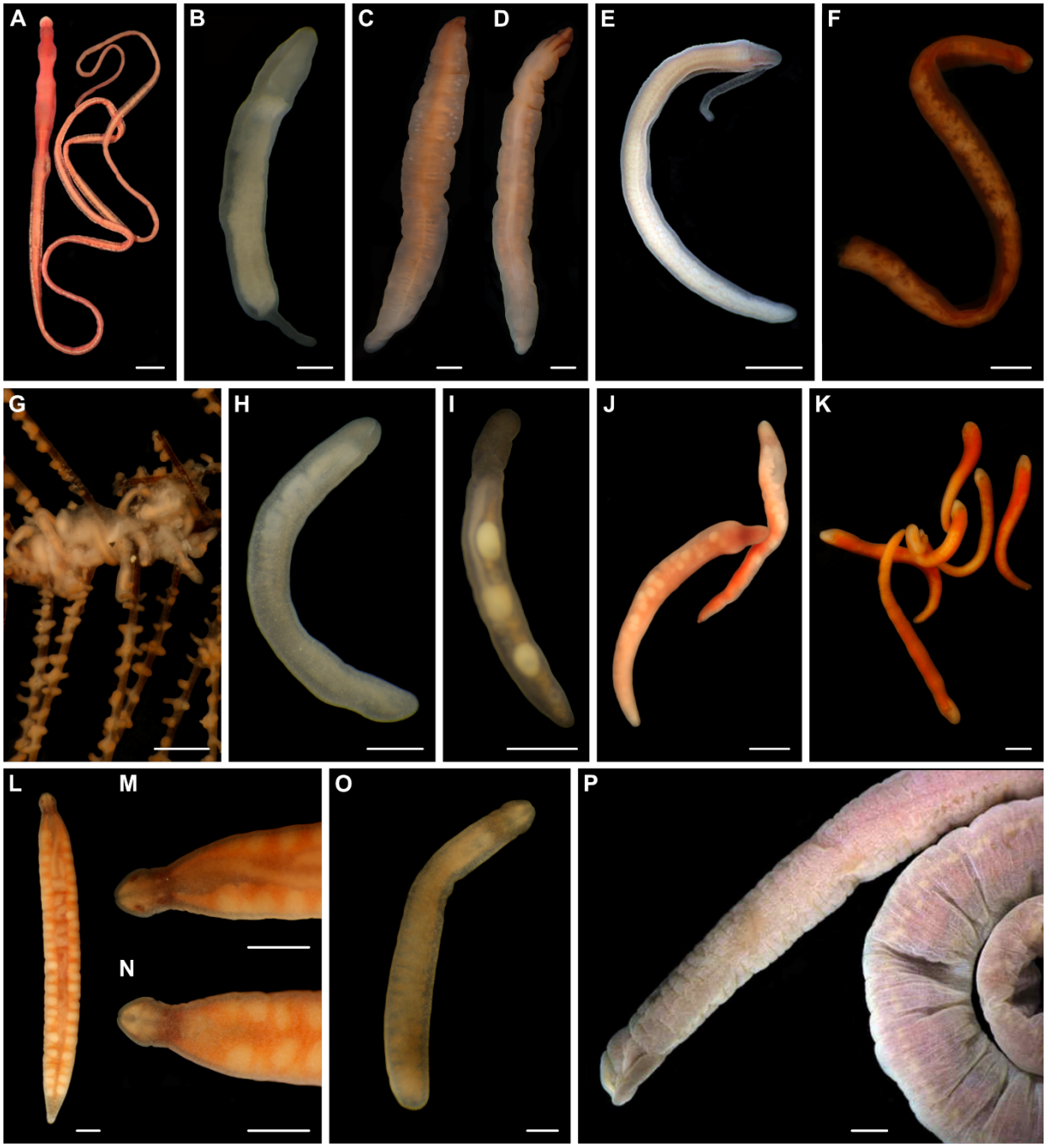
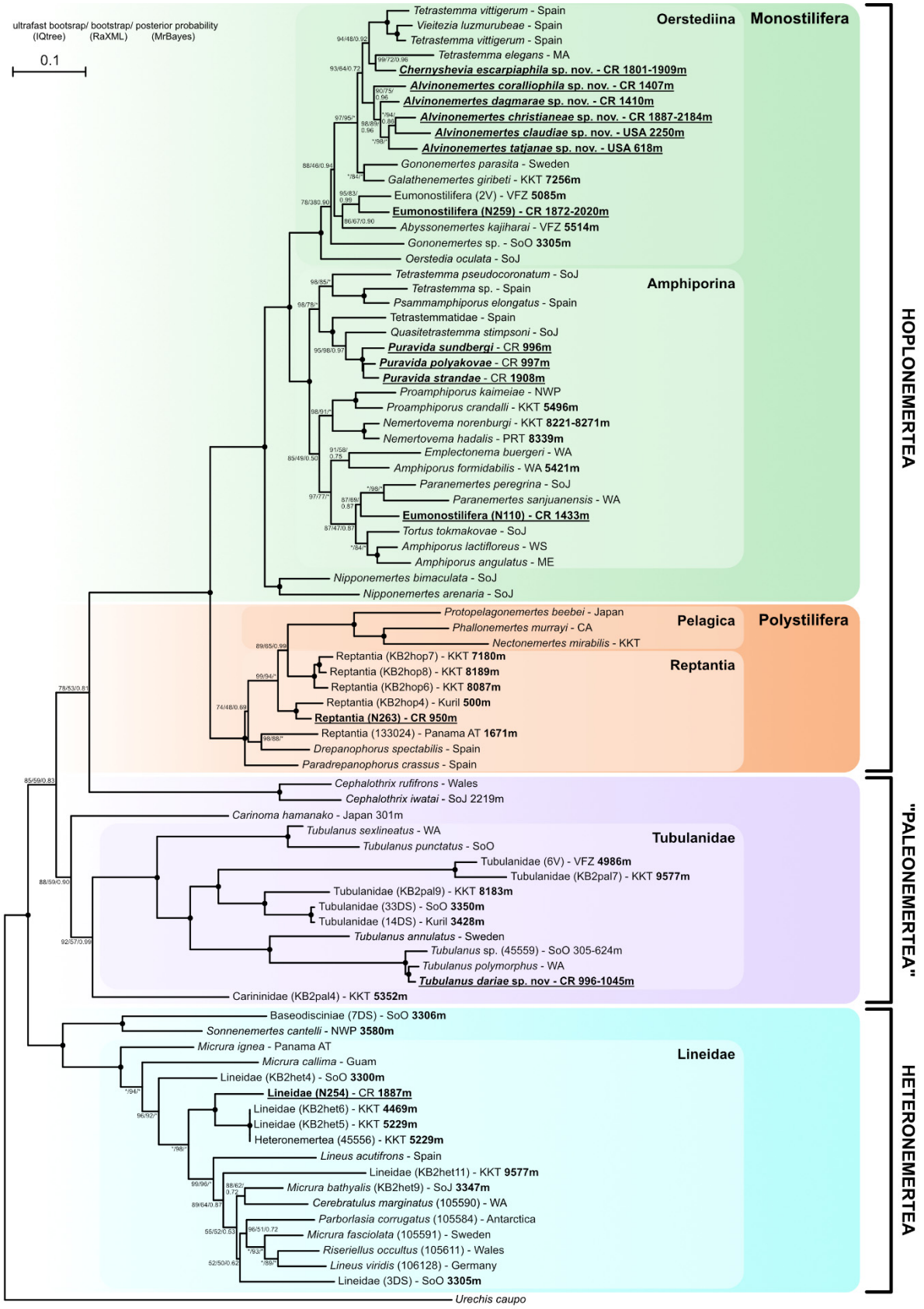


Figure 3.3.2 Habitus photographs of the nemertean specimens collected along the Costa Rica margin. A Palaeonemertea, B Heteronemertea, C-D Polystilifera (Hoplonemertea), E-P Monostilifera (Hoplonemertea). **A** *Tubulanus dariae* sp. nov.; **B** Lineidae SIO-BIC N254; **C, D** Reptantia N263 dorsal (C) and ventral view (D); **E** *Chernyshevia escarpiaphila* sp. nov.; **F** *Alvinonemertes coralliophila* sp. nov.; **G** black coral with specimens of *Alvinonemertes dagmarae* sp. nov.; **H** *Alvinonemertes christianeae* sp. nov.; **I** *Alvinonemertes claudiae* sp. nov.; **J** *Alvinonemertes tatjanae* sp. nov.; **K** Eumonostilifera SIO-BIC N259; **L-M** *Puravida sundbergi* sp. nov. whole animal (L), dorsal view of head (M), and ventral view of head (N); **O** *Puravida polyakovae* sp. nov.; **P** Eumonostilifera SIO-BIC N109. Scale: A-D 5 mm; E, F, I-P 1 mm; H 500 µm; G 2 cm. Figure modified after Sagorny et al. (in revision).

3 Results


ultrafast bootsrap/ bootstrap/ posterior probability
(IQtree) (RaXML) (MrBayes)
0.1
Monostilifera
Oerstediina
Tetrastemma vittigerum - Spain
Vieitezia luzmurubeae - Spain
Tetrastemma vittigerum - Spain
Tetrastemma elegans - MA
Chernyshevia escarpiaphila sp. nov. - CR 1801-1909m
Alvinonemertes coralliophila sp. nov. - CR 1407m
Alvinonemertes dagmarae sp. nov. - CR 1410m
Alvinonemertes christianeae sp. nov. - CR 1887-2184m
Alvinonemertes claudiae sp. nov. - USA 2250m
Alvinonemertes tatjanae sp. nov. - USA 618m
Gononemertes parasita - Sweden
Galathenemertes giribeti - KKT 7256m
Eumonostilifera (2V) - VFZ 5085m
Eumonostilifera (N259) - CR 1872-2020m
Abyssonemertes kajiharai - VFZ 5514m
Gononemertes sp. - SoO 3305m
Oerstedia oculata - SoJ
Amphiporina
Tetrastemma pseudocoronatum - SoJ
Tetrastemma sp. - Spain
Psammamphiporus elongatus - Spain
Tetrastemmatidae - Spain
Quasitetrastemma stimpsoni - SoJ
Puravida sundbergi - CR 996m
Puravida polyakovae - CR 997m
Puravida strandae - CR 1908m
Proamphiporus kaimeiae - NWP
Proamphiporus crandalli - KKT 5496m
Nemertovema norenburgi - KKT 8221-8271m
Nemertovema hadalis - PRT 8339m
Emplectonema buergeri - WA
Amphiporus formidabilis - WA 5421m
Paranemertes peregrina - SoJ
Paranemertes sanjuanensis - WA
Eumonostilifera (N110) - CR 1433m
Tortus tokmakovae - SoJ
Amphiporus lactifloreus - WS
Amphiporus angulatus - ME
Nipponemertes bimaculata - SoJ
Nipponemertes arenaria - SoJ
Polystilifera
Pelagica
Protopelagonemertes beebei - Japan
Phallonemertes murrayi - CA
Nectonemertes mirabilis - KKT
Reptantia
Reptantia (KB2hop7) - KKT 7180m
Reptantia (KB2hop8) - KKT 8189m
Reptantia (KB2hop6) - KKT 8087m
Reptantia (KB2hop4) - Kuril 500m
Reptantia (N263) - CR 950m
Reptantia (133024) - Panama AT 1671m
Drepanophorus spectabilis - Spain
Paradrepanophorus crassus - Spain
Cephalothrix rufifrons - Wales
Cephalothrix iwatai - SoJ 2219m
Carinoma hamanako - Japan 301m
Tubulanidae
Tubulanus sexlineatus - WA
Tubulanus punctatus - SoO
Tubulanidae (6V) - VFZ 4986m
Tubulanidae (KB2pal7) - KKT 9577m
Tubulanidae (KB2pal9) - KKT 8183m
Tubulanidae (33DS) - SoO 3350m
Tubulanidae (14DS) - Kuril 3428m
Tubulanus annulatus - Sweden
Tubulanus sp. (45559) - SoO 305-624m
Tubulanus polymorphus - WA
Tubulanus dariae sp. nov - CR 996-1045m
Carininidae (KB2pal4) - KKT 5352m
Baseodisciniae (7DS) - SoO 3306m
Sonnenemertes cantelli - NWP 3580m
Lineidae
Micrura ignea - Panama AT
Micrura callima - Guam
Lineidae (KB2het4) - SoO 3300m
Lineidae (N254) - CR 1887m
Lineidae (KB2het6) - KKT 4469m
Lineidae (KB2het5) - KKT 5229m
Heteronemertea (45556) - KKT 5229m
Lineus acutifrons - Spain
Lineidae (KB2het11) - KKT 9577m
Micrura bathyalis (KB2het9) - SoJ 3347m
Cerebratulus marginatus (105590) - WA
Parborlasia corrugatus (105584) - Antarctica
Micrura fasciolata (105591) - Sweden
Riseriellus occultus (105611) - Wales
Lineus viridis (106128) - Germany
Lineidae (3DS) - SoO 3305m
Urechis caupo
HOPLONEMERTEA
"PALEONEMERTEA"
HETERONEMERTEA

◄ **Figure 3.3.3** Maximum likelihood (ML) tree including all deep-sea nemertean specimens collected along the Costa Rica margin and selected, comparable sequences taken from GenBank based on a concatenated 4 gene dataset (16S, COI, 18S, H3). Numbers beside nodes indicate support values (ultrafast ML bootstrap value (IQtree)/ ML bootstrap value (RAxML)/ Bayesian posterior probability (MrBayes)). Black circles indicate nodal support of 100/100/1.0; asterisks indicate nodal support of either 100/ or /1.0. *Urechis caupo* was used for outgroup rooting. Specimens belonging to new species are underlined and given in bold. Sampling localities and depths (for deep-sea species only) are provided for all included specimens. Figure modified after Sagorny et al. (in revision).

Only a single heteronemertean specimen could be collected at 1,887 m depth at Jaco Scar. Based on outer morphology and the phylogenetic analysis, this specimen belongs to the Lineidae (Figs 3.2.2 B; 3.3.3). This 45 mm long, pale whitish specimen is sister to three undescribed abyssal lineids collected at the Kuril-Kamchatka-Trench. A description of this specimen is hindered by the problematic taxonomy of Lineidae with three large, presumably paraphyletic "hold-all" genera and numerous small genera that only comprise one species.

As for heteronemerteans, only a single specimen of polystiliferous hoplonemerteans was sampled. This specimen was collected at a depth of 950 m at Cocos Canyon (Fig 3.3.1 B). Thus, this is the only specimen that was not collected directly along the Costa Rica margin. This 65 mm long, pale pinkish, and dorso-ventrally flattened specimen is sister to an undescribed reptant specimen collected at 500 m depth near the Kuril Islands (Figs 3.3.2 C, D; 3.3.3). Interestingly, this clade is sister to a group containing all Pelagica on the one hand and three further unidentified hadal reptant specimens from the Kuril-Kamchatka-Trench. Therefore, the results suggest that Reptantia is indeed a paraphyletic group. Because of the convoluted taxonomy of Reptantia combined with only few available sequence data, naming of the Costa Rican reptant species is postponed.

With 76 specimens, the better part of the collected nemertean specimens encompasses eumonostiliferous hoplonemerteans. These represent nine distinct species, of which seven are described as new to science (Fig 3.3.3). The species belong to three genera, all of which are newly erected. In addition, two species from the north eastern Pacific are identified as members of Eumonostilifera. These are closely related to specimens collected along the Costa Rica margin. Except for Mound 11 and Cocos Canyon, eumonostiliferans were collected at all remaining five localities. Of the nine Costa Rican species, four can be attributed to the clade Oerstediina. The remaining four species are part of the clade Amphiporina.

Within Oerstediina, two new genera are described: *Chernyshevia* gen. nov. and *Alvinonemertes* gen. nov. *Chernyshevia* is a so far monotypic genus that comprises the type

species *Chernyshevia escarpiaphila* sp. nov. With 48 specimens, this species is the most abundant species along the Costa Rican margin and was collected at Jaco Scar and Mound Jaguar at depths around 1,800 m. Numerous individuals of *C. escarpiaphila* could be observed on the tubes of the annelid *Escarpia spicata* JONES, 1985 (Fig 3.3.1 A). Specimens are up to 10 mm in length and have a pale white to pinkish body coloration (Fig 3.3.2 E). The lateral nerve cords bear a small accessory nerve and the mid-dorsal blood vessel lacks a vascular plug. The rhynchocoel extends to the posterior end of the body. One pair of ventro-lateral cephalic furrows is present that continues as unforked ciliated canal and open into the cerebral organs located close in front of the brain. In a concatenated analysis, this species is sister to *Tetrastemma elegans* (GIRARD, 1852) (Fig 3.3.3). Together with *Tetrastemma vittigerum* (BÜRGER, 1904) and *Vieitezia luzmurubeae* JUNOY, ANDRADE & GIRIBET, 2010, these species belong to a well-supported clade. As the genus *Tetrastemma* is most likely paraphyletic and some morphological differences are visible between *V. luzmurubeae* and *C. escarpiaphila* sp. nov., the new species is assigned to a newly erected genus. Morphological differences include body coloration (pale white in *C. escarpiaphila* vs. pale yellow with four dorsal, brown longitudinal bands in *V. luzmurubeae*) and the presence of an accessory nerve (present in *C. escarpiaphila* vs. absent in *V. luzmurubeae*).

The aforementioned clade is sister to the second newly erected genus, *Alvinonemertes* gen. nov. This genus comprises the five species *Alvinonemertes coralliophila* sp. nov., *Alvinonemertes dagmarae* sp. nov., *Alvinonemertes. christianeae* sp. nov., *Alvinonemertes claudiae* sp. nov., and *Alvinonemertes tatjanae* sp. nov. None of the species exceeds 20 mm length, and all are characterized by a pale, translucent body (Figs 3.3.2 F-J). The proboscis is equipped with a central stylet and two accessory stylet pouches, each bearing 2-5 accessory stylets. Comparable to *Chernyshevia*, an accessory nerve is present. The two species *A. coralliophila* sp. nov. and *A. dagmarae* sp. nov. were both found on corals, maybe indicating a symbiotic relationship. Furthermore, the two north eastern Pacific species, *A. claudiae* and *A. tatjanae*, can be attributed to the genus *Alvinonemertes*. In the concatenated analysis, the genus *Alvinonemertes* is sister to the clade comprising *Chernyshevia*, *Vieitezia*, and the aforementioned "*Tetrastemma*" species (Fig 3.3.3).

Additionally, seven further eumonostiliferan specimens were collected at Quepos Plateau, representing one species (Fig 3.3.2 K). This species is sister to two unidentified nemerteans from the abyssal zone of the Vema Fracture Zone. This group forms a well-supported clade with *Abyssonemertes kajiharai* CHERNYSHEV & POLYAKOVA, 2017 (Fig 3.3.3).

In order to assign the new species to the genus *Abyssonemertes*, additional specimens would be necessary for histological sectioning.

Based on molecular data, three new species are described in the newly erected genus *Puravida* gen. nov. within Amphiporina. All three species are less than 15 mm in size and lack distinct coloration (Figs 3.3.2 L-O). In contrast to *Alvinonemertes*, members of the genus *Puravida* lack an accessory nerve and the proboscis bears only two accessory stylets per pouch. Moreover, the mid-dorsal blood vessel forms a vascular plug. Specimens of the species *Puravida sundbergi* sp. nov. and *Puravida polyakovae* were collected at depths of approximately 1,000 m at Mound 12, whereas specimens of *Puravida strandae* were collected at 1,900 m depths at Jaco Scar. With robust support, the three species are sister to *Quasitetrastemma stimpsoni* CHERNYSHEV, 1992 (Fig 3.3.3). The new genus can be differentiated from *Quasitetrastemma* by the absence of eyes and the absence of an accessory nerve in *Puravida*.

Moreover, one further specimen that has been collected at Parrita Seep is part of Amphiporina (Fig 3.3.2 P). This specimen shares a clade with several *Paranemertes* species, *Tortus tokmakovae* CHERNYSHEV, 1991, and two *Amphiporus* species (Fig 3.3.3). The exact position within this clade cannot fully be resolved.

Overall, our data support the taxonomic diversity of bathyal nemerteans as all major groups were represented. As has been shown previously, palaeonemerteans and especially eumonostiliferous hoplonemerteans are most abundant in depths below 1,000 meters. Interestingly, some taxa are absent from the Costa Rica margin, including the palaeonemertean genera *Cephalothrix*, *Carinina*, and *Carinoma*, as well as heteronemerteans of the family Valenciniidae. Moreover, no Cratenemertea could be collected. This study underlines the effectiveness of a turbo taxonomic approach when it comes to describing several species new to science, but it also highlights the shortcomings of this approach when it comes to species that possess only scarce external characteristics.

4 Unpublished Results

4.1 Species identity of palaeonemerteans of the genera *Carinoma*, *Callinera*, and *Carinina* that share a similar external morphology

Based on external morphology, palaeonemerteans of the genera *Carinoma*, *Callinera*, and *Carinina* are difficult to distinguish from each other (Gibson 1982). All genera are characterized by a pale translucent body coloration, a thin body, and the absence of eyes (Gibson 1982). Thus, identification in the field is problematic. Several specimens of small, white palaeonemerteans that lack eyes have been sampled along the coasts of Concarneau and Roscoff in France. As external morphology is lacking, these have been preliminarily identified as *Carinoma armandi* (McIntosh, 1875).

A maximum-likelihood analysis of COI sequences of 21 specimens sampled in France combined with all sequence data available on GenBank for the genera *Carinoma*, *Callinera*, and *Carinina* and some *Tubulanus* sequences showed that the sampled individuals indeed belong to five distinct species. The ML analysis supports three large monophyletic clades: the first comprises species representing the genus *Carinoma*, the second species representing the genus *Carinina*, and the third clade comprises species of the genus *Callinera*, as well as the genus *Tubulanus* and some unidentified palaeonemerteans. The *Carinina* clade is sister to the *Callinera* clade, whereas the *Carinoma* clade is sister to the former two.

To date, the genus *Carinoma* comprises ten described species (Gibson 1995, Kajiahara et al. 2008). In contrast to this, the ML analysis of our dataset distinguishes 12 species (Fig 4.1.1). All species have robust nodal support, whereas relationships between species lack robust support so that predictions regarding their exact phylogenetic position remain questionable. The better part of the included specimens lacks proper species identification and are thus only referred to as *Carinoma* sp. Identified species include *Carinoma mutabilis* Griffin, 1898, *Carinoma tremaphoros* Thompson, 1900, and *Carinoma hamanako* Kajihara, Yamasaki, & Andrade 2011. The latter species appears to be monophyletic, whereas *C. mutabilis* and *C. tremaphoros* appear to hide cryptic species. The individuals sampled in France constitute two species of *Carinoma*. The four specimens NE91, JK1, JK2, and CA4 presumably represent the species *C. armandi*. This clade is sister to a clade comprising three unidentified specimens from Oregon (bootstrap support: 100). Together, both clades are sister to a lineage comprised by four

additional specimens collected in France (bootstrap support: 83). Thus, the number of *Carinoma* species most likely exceeds the so far accepted number of ten species and cryptic speciation seems to be prevalent in the genus.

In contrast to this, 19 species of the genus *Carinina* are accepted so far. The ML analysis estimated five well-supported species based on the available sequence data. Support between the species is also high. Four species only comprise unidentified specimens, either from Oregon or from Russia. The only identified specimens belong to the species *Carinina ochracea* SUNDBERG ET AL., 2009. Specimens originate from Spain and Sweden. In addition, three specimens collected in France are part of this clade. This is the only case, where specimens sampled in France belong to the same clade as specimen data taken from GenBank. No other specimens sampled in France belong to this genus. The high number of unidentified specimens underlines the difficulty when it comes to identifying species that lack distinct external characteristics.

The genus *Callinera* comprises nine described species and is commonly regarded as paraphyletic (Kvist et al. 2015). In the ML analysis, five species that have been identified as *Callinera* are part of the same clade as the three included *Tubulanus* species, thus supporting the paraphyly of the genera *Callinera* and *Tubulanus*. Additionally, this group includes a clade identified as *Carinina plecta* KAJIHARA, 2006. Either, the genus *Carinina* is paraphyletic, or the specimens have been misidentified. Again, all species have robust nodal support, but relationships between genera remain questionable. Two species of *Callinera* include only unidentified specimens. Identified species include *Callinera kasyanovi* CHERNYSHEV, 2008, *Callinera grandis* BERGENDAL, 1903, and *Callinera emiliae* KAJIHARA, 2007. Two specimens collected in France are sister to *C. grandis*, although this relationship is only weakly supported (bootstrap value: 58).

Eight specimens collected in France form one well-supported group. This group is sister to the remaining *Callinera* and *Tubulanus* species. This grouping has high nodal support. Based on external characteristics and the ML analysis, this clade cannot be unequivocally assigned to any of the three investigated palaeonemertean genera. Further data of related species and further genetic markers would be necessary to resolve this question.

Generally, small white palaeonemerteans should not be assigned to a species based on external morphology alone as this study proved that the collected specimens belong to five different species in three or even four different genera. This emphasises the utility of DNA barcoding for the identification of nemertean species.

Figure 4.1.1 Maximum-likelihood tree (GTR+G+I) of all available COI sequences representing the genera *Carinoma*, *Carinina*, and *Callinera*. Numbers beside nodes indicate support values (ultrafast bootstrap values). Black circles indicate nodal support of 100. *Lineus acutifrons* was used for outgroup rooting. Specimen-ID and sampling locality are provided.

4.2 Haplotype diversity in the longest known invertebrate *Lineus longissimus* in European waters

The heteronemertean *Lineus longissimus* is regarded as the longest known invertebrate with a length of up to 30 metres (Gibson 1972; Gittenberger & Schipper 2008). Moreover, it is the type species of the genus *Lineus* (Ament-Velásquez et al. 2016). The species is commonly found in the North Sea, the Baltic and the northern European Atlantic coasts (Gibson 1982, 1995). It is also frequently encountered along the Breton coast in France.

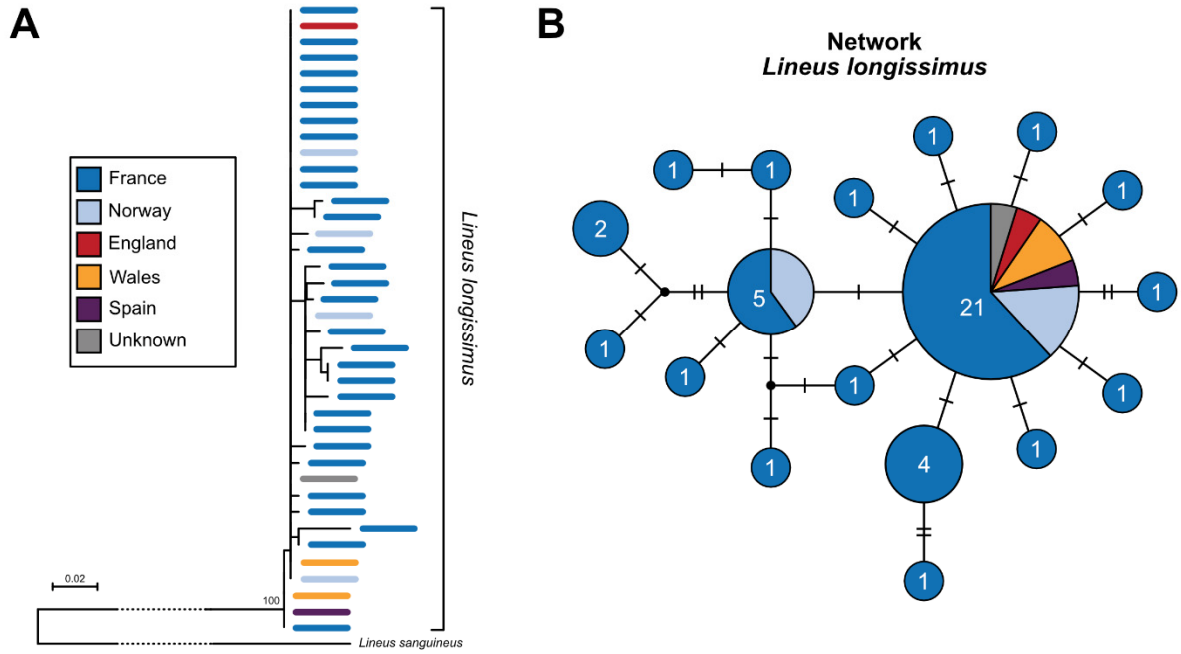


Figure 4.2.1 Specimens of the European *Lineus longissimus* sampled at 5 different localities show no separation by locality. **A** Maximum-likelihood tree (HKY+G+I) of 39 European specimens. Numbers beside nodes indicate support values (ultrafast bootstrap values). All included specimens belong to a single clade. *Lineus sanguineus* was used for outgroup rooting. Specimens are colour-coded based on sampling locality. **B** Statistical parsimony haplotype network of 46 *L. longissimus* specimens based on mitochondrial COI sequences with a connection limit of 95%. Sampling localities are represented by colour. Numbers within pie charts represent number of specimens with respective haplotype. Each bar indicates a single substitution.

Mitochondrial COI sequences of thirty-one specimens sampled in France were combined with 15 sequences taken from GenBank. These originate from Norway, England, Wales, and Spain. An ML analysis of the combined dataset shows that all specimens belong to a single well

supported clade (Fig 4.2.1 A). Accordingly, external morphology appears to be sufficient to identify this species in the field. Cryptic speciation seems to be absent.

A statistical parsimony analysis yielded one network including 19 haplotypes (Fig 4.2.1 B). One highly frequent haplotype is found in 21 out of 46 specimens. Specimens sharing this haplotype originate from all included localities: France, Norway, Wales, and Spain. The second most frequent haplotype is shared by five specimens. Three of these five specimens originate from France, whereas the remaining two specimens were collected in Norway. Furthermore, two haplotypes are found in four or two specimens, respectively, that were all collected in France. In addition, there are 14 low-frequency haplotypes that are represented by one specimen from France each. All haplotypes are connected by one to three mutational steps. There is no separation of haplotypes by locality, thus supporting the results of the ML analysis.

4.3 Differences in sampling locality and habitat choice are not reflected by molecular data in the heteronemertean *Riseriellus occultus*

The heteronemertean species *Riseriellus occultus* has been described from northern Wales and north western Spain (Rogers et al. 1993; Gibson 1995). In Wales, the species can be collected in the intertidal under stones and rocks, whereas in Spain they occur imbedded in sand (Sundberg & Strand 2007). Despite these vast differences in habitat and geography, there is no genetic difference between specimens from the two localities, thus excluding cryptic speciation (Sundberg & Strand 2007). *Riseriellus occultus* can also commonly be found under rocks and stones along the Breton coast (Concarneau and Roscoff).

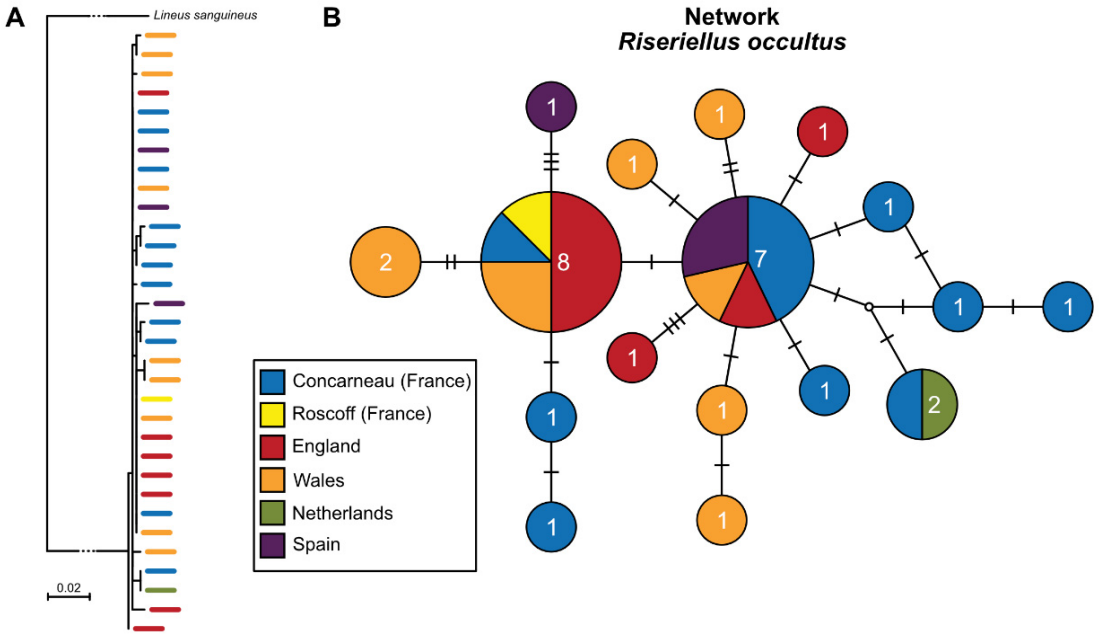


Figure 4.3.1 Specimens of the heteronemertean *Riseriellus occultus* sampled in 5 different European countries show no separation by locality or habitat. **A** Maximum-likelihood tree (HKY+G+I) of 32 European specimens. All included specimens belong to a single clade. *Lineus sanguineus* was used for outgroup rooting. Specimens are colour-coded based on sampling locality. **B** Statistical parsimony haplotype network of all *R. occultus* specimens based on mitochondrial COI sequences with a connection limit of 95%. Sampling localities are represented by colour. Numbers within pie charts represent number of specimens with respective haplotype. Each bar indicates a single substitution.

In total, twelve specimens of *R. occultus* were collected in France. These were combined with sequence data of 20 specimens taken from GenBank. These were collected in Spain, Wales, England and the Netherlands. An ML analysis based on the COI gene yielded one well supported clade including all sampled localities (Fig 4.3.1 A). This finding is also supported by a TCS analysis. One network was recovered that is represented by 17 haplotypes (Fig 4.3.1 B). Two frequent haplotypes are shared by seven and eight specimens, respectively. Two haplotypes are present in two specimens, whereas the remaining 13 haplotypes are only found in single specimens. The haplotypes are interconnected by one to three mutational steps. The most-frequent haplotype is found in specimens from England, both French localities and Wales. The second-most frequent haplotype is found in specimens from Concarneau, Spain, Wales and England. Additionally, the only specimen sampled in the Netherlands shares a haplotype with a French specimen. Thus, there is no separation of haplotypes by habitat or locality. The results suggest that all populations of *R. occultus* are genetically close despite their different habitat preferences.

These results support the findings of Sundberg & Strand (2007) as no separation by sampling locality could be detected. Based on the results, different populations of *R. occultus* share a recent common ancestor, so that ecological differences have not yet led to speciation.

4.4 Population structuring in the *Lineus viridis*/*ruber* species complex based on locality

The *Lineus viridis* and *Lineus ruber* species complex has long been problematic (Gontcharoff 1951; Gibson 1982, 1995). Recent molecular investigations proved that both are indeed separate species (Rogers et al. 1995; Krämer et al. 2017; Cherneva et al. 2019). Moreover, it was shown that another cryptic species is present that was described as *Lineus clandestinus* (Krämer et al. 2017). The investigations by Krämer et al. (2017) and Cherneva et al. (2019) both showed differences in haplotype structuring between the three species: *Lineus clandestinus* exhibits the least structuring, whereas the remaining two species show roughly the same amount of structuring.

These trends were also obvious in a TCS network analysis based on the COI gene (Fig 4.4.1). All available sequence data for the three species was taken from GenBank. Additionally, two specimens of *L. ruber*, three specimens of *L. clandestinus,* and seven specimens of *L. viridis* were added to the dataset. All individuals were collected in Concarneau, except for 3 *L. viridis* specimens that originate from Sylt. The total dataset comprised 138 specimens of *L. viridis,* 52 specimens of *L. clandestinus*, and 130 specimens of *L. ruber*. The statistical parsimony analysis yielded three unconnected networks representing each of the three species.

The *Lineus viridis* network is represented by 31 distinct haplotypes (Fig 4.4.1 A). The three most frequent haplotypes are found in 22%, 20%, and 15% of the specimens included in this network. Three medium-frequency haplotypes are shared by 8% or 6% of the specimens, respectively. The remaining haplotypes are found in single individuals or are shared by no more than three specimens. There is no separation of haplotypes by locality as the most frequent haplotypes are shared by specimens from all sampling localities. The only exception to this are the specimens collected in Wimereux (France). None of these share one of the most frequent haplotypes and one haplotype is only found in 11 specimens from Wimereux. Nevertheless, only two mutational steps separate this haplotype from the most frequent haplotype.

In contrast to this, the *L. clandestinus* network is represented by only eight haplotypes (Fig 4.4.1 B). 81% of all included specimens share one highly-frequent haplotype. The remaining haplotypes are found in two or one specimens, respectively. Haplotypes are separated by one mutational step. There is no separation by locality.

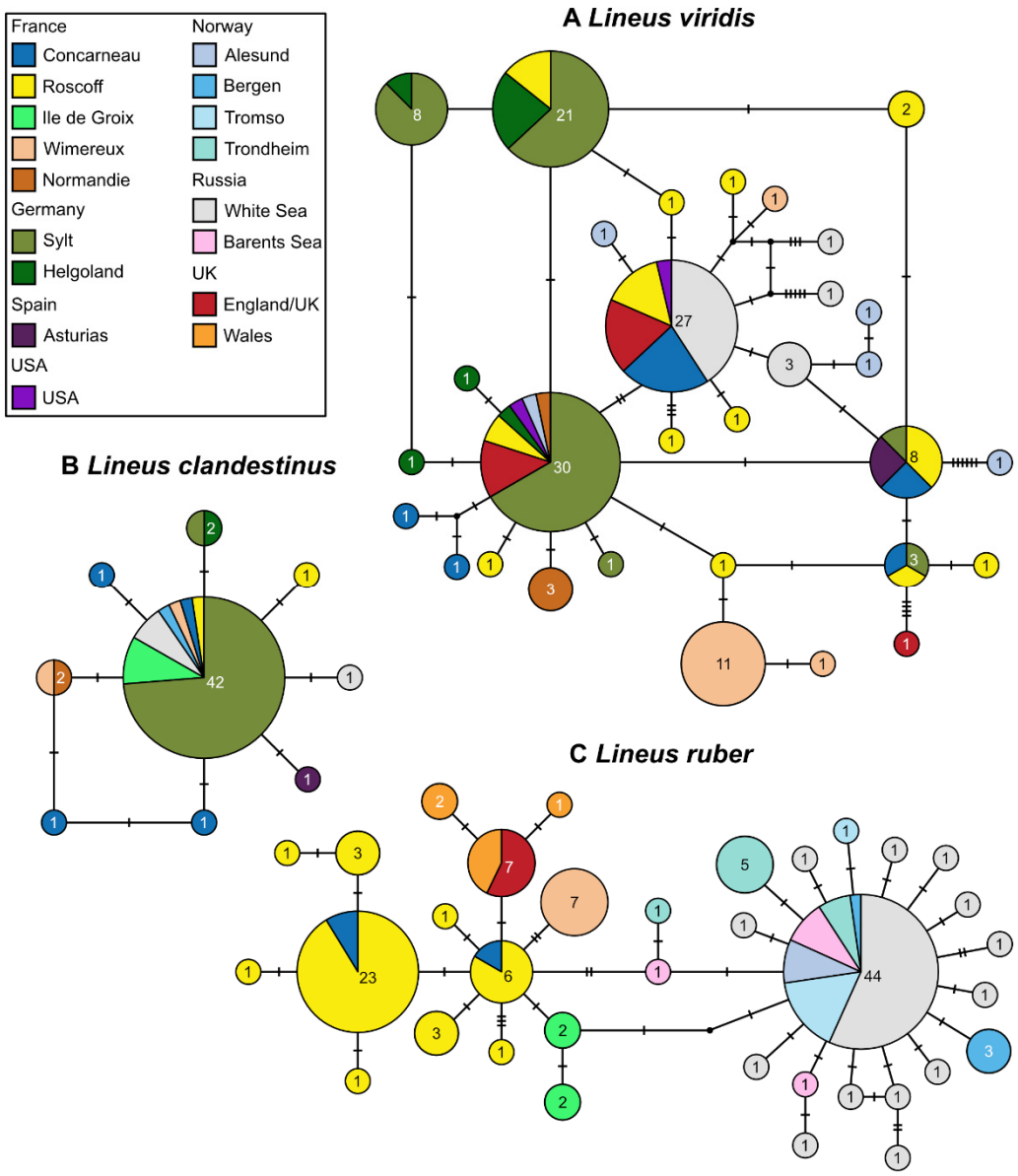


Figure 4.4.1 Statistical parsimony haplotype networks of all *Lineus viridis* (**A**), *Lineus cladestinus* (**B**), and *Lineus ruber* (**C**) specimens based on mitochondrial COI sequences with a connection limit of 95%. Geographic distribution by sampling sites in each country is represented by colour. Numbers within pie charts represent number of specimens with respective haplotype. Each bar indicates a single substitution.

The *L. ruber* network is represented by 35 haplotypes and exhibits strong haplotype structuring (Fig 4.4.1 C). The two most frequent haplotypes are found in 34% and 18% of all specimens. Four medium-frequency haplotypes are found in 4% or 5% of the included specimens, respectively. The remain 29 haplotypes are found in single individuals or are shared

by no more than three specimens. Haplotypes are separated by one to three mutational steps. In contrast to the other two networks, the haplotypes in the *L. ruber* network are separated by locality. The most frequent haplotype and the connected low-frequency haplotypes are present in specimens sampled in Norway, the White Sea, and the Barents Sea. The second-most frequent haplotype on the other hand is connected to the medium-frequency haplotypes including specimens sampled in France and the UK. Both haplotype groups are connected via two specimens sampled on the Ile de Groix (France) and one specimen collected in the Barents Sea.

Table 4.4.1 COI AMOVA values for *Lineus viridis* based on sampling sites. Pairwise Φ_{PT} values are given above diagonal; p-values are given below diagonal. High levels of differentiation ($\Phi_{PT} > 0.5$) are given in bold. All other populations show low levels of differentiation. Number of specimens per sampling site are given in parentheses.

	US (3)	UK (10)	Hel (9)	Sylt (43)	Con (12)	Ros (20)	Wim (14)	Nor (4)	Åle (5)	WS (16)	Ast (2)
US	-	0.367	0.044	0.015	0.314	0.414	0.005	0.084	0.284	0.025	0.228
UK	0.000	-	0.001	0.001	0.363	0.253	0.001	0.008	0.060	0.004	0.178
Helgoland	0.309	0.336	-	0.328	0.002	0.014	0.001	0.002	0.003	0.001	0.015
Sylt	0.324	0.373	0.000	-	0.001	0.001	0.001	0.002	0.001	0.001	0.006
Concarneau	0.000	0.000	0.387	0.432	-	0.177	0.001	0.005	0.109	0.017	0.131
Roscoff	0.000	0.019	0.147	0.189	0.034	-	0.001	0.034	0.018	0.001	0.391
Wimereux	**0.582**	**0.536**	**0.660**	**0.656**	**0.561**	0.442	-	0.001	0.001	0.001	0.022
Normandie	0.308	0.300	0.487	0.420	0.354	0.186	**0.672**	-	0.021	0.001	0.059
Ålesund	0.040	0.143	0.416	**0.572**	0.127	0.153	**0.582**	0.308	-	0.002	0.420
White Sea	0.319	0.191	**0.624**	**0.661**	0.138	0.277	**0.709**	**0.619**	0.273	-	0.018
Asturias	0.284	0.261	**0.549**	0.488	0.247	0.011	**0.703**	**0.791**	0.00	**0.590**	-

The same trend is visible in an AMOVA analysis. The AMOVA analysis of the *L. viridis* dataset detected medium levels of differentiation between populations (Φ_{PT}=0.393; $p<0.05$). Variation within populations (61%) is higher than variation among populations (39%), thus indicating a lack of population structuring. Regarding pairwise Φ_{PT} values, there are low to medium levels of differentiation and thus gene flow between most included populations, although not all values are significant due to a small sequence pool for some populations (Tab 4.4.1). The only population that shows high levels of differentiation from all other populations is Wimereux (Φ_{PT}=0.536-0.709, $p<0.05$), a trend that could also be observed in the TCS analysis. Moreover, high levels of differentiation are present between the White Sea population

and populations from Germany and northern France (Φ_{PT}=0.619-0.709, p<0.05). The highest level of differentiation occurs between populations from the Normandie and Asturias (Spain), although this result is not statistically supported (Φ_{PT}=0.791, p=0.08). As expected, isolation by distance could be rejected as no significant relationship between geographic and genetic distance could be inferred by the Mantel test.

As for *L. viridis,* the AMOVA analysis of the *L. clandestinus* dataset yielded medium levels of differentiation between populations (Φ_{PT}=0.440; p<0.05). Again, variation within populations (56%) exceeds variation among populations (44%) accounting for little population structuring. Pairwise Φ_{PT} values reveal low to medium levels of differentiation between populations (Tab 4.4.2). As only the Sylt population is represented by more than four specimens, most results lack statistical support. Only the high levels of differentiation between the population from Sylt and populations from Concarneau and Wimereux, respectively, are significant (Φ_{PT}=0.630-0.812, p<0.05). As for *L. viridis*, the Mantle test revealed no significant relationship between geographic and genetic distance.

Table 4.4.2 COI AMOVA values for *Lineus clandestinus* based on sampling sites. Pairwise Φ_{PT} values are given above diagonal; p-values are given below diagonal. High levels of differentiation (Φ_{PT} > 0.5) are given in bold. All other populations show low levels of differentiation. Number of specimens per sampling site are given in parentheses.

	Sylt (32)	Roscoff (2)	Concarneau (4)	Ile de Groix (4)	Wimereux (3)	White Sea (4)
Sylt	-	0.125	0.004	0.116	0.002	0.220
Roscoff	**0.712**	-	0.526	0.344	0.314	0.594
Concarneau	**0.630**	0.040	-	0.422	0.370	0.454
Ile de Groix	0.000	0.385	0.167	-	0.130	1.000
Wimereux	**0.812**	0.323	0.084	0.579	-	0.140
White Sea	0.328	0.111	0.133	0.000	0.376	-

In contrast to this, differentiation levels are high between populations of *L. ruber* (Φ_{PT}=0.725, p<0.05). Therefore, variation is higher among populations (73%) than within populations (27%), providing evidence for significant population structuring in this species. As already implied by the results of the statistical parsimony analysis, low to medium levels of differentiation are present between populations from Norway, the White Sea and the Barents Sea (Φ_{PT}=0.127-0.449, p<0.05), although not all results are statistically significant (Tab 4.4.3).

As expected, differentiation levels between the former populations and populations from France and the UK are high (Φ_{PT}=0.586-1.00, p<0.05). The highest levels of differentiation occur between the population from Ålesund and populations from Wimereux and England (Φ_{PT}=1.000, p<0.05). Interestingly, differentiation levels are similarly high between the different French and British populations (Φ_{PT}=0.575-1.000, p<0.05). The populations from Wimereux and England show the highest level of differentiation (Φ_{PT}=1.000, p<0.05). A Mantle test revealed a significant medium level relationship between geographic and genetic distance, thus indicating a possible isolation by distance (R_{XY}=0.344, p<0.05)

Table 4.4.3 COI AMOVA values for *Lineus ruber* based on sampling sites. Pairwise Φ_{PT} values are given above diagonal; p-values are given below diagonal. High levels of differentiation (Φ_{PT} > 0.5) are given in bold. All other populations show low levels of differentiation. Number of specimens per sampling site are given in parentheses.

	Trom (8)	Ber (4)	Åle (4)	Tron (10)	WS (38)	BS (6)	Ros (37)	IdG (4)	Con (3)	Wim (7)	Eng (4)	Wal (6)
Tromsø	-	0.014	0.414	0.033	0.098	0.299	0.001	0.001	0.010	0.001	0.002	0.001
Bergen	0.278	-	0.136	0.010	0.002	0.041	0.001	0.018	0.029	0.006	0.023	0.007
Ålesund	0.000	0.667	-	0.329	0.461	0.658	0.001	0.028	0.030	0.001	0.001	0.004
Trondheim	0.127	0.301	0.057	-	0.001	0.085	0.001	0.003	0.006	0.001	0.002	0.001
White Sea	0.044	0.332	0.000	0.188	-	0.465	0.001	0.001	0.001	0.001	0.001	0.001
Barents Sea	0.000	0.449	0.000	0.099	0.000	-	0.001	0.008	0.031	0.001	0.004	0.002
Roscoff	**0.751**	**0.786**	**0.766**	**0.729**	**0.774**	**0.747**	-	0.001	0.379	0.001	0.001	0.001
Ile de Groix	**0.635**	**0.821**	**0.867**	**0.586**	**0.696**	**0.765**	**0.602**	-	0.034	0.002	0.032	0.006
Concarneau	**0.717**	**0.871**	**0.927**	**0.660**	**0.780**	**0.818**	0.000	**0.692**	-	0.008	0.019	0.023
Wimereux	**0.864**	**0.971**	**1.000**	**0.819**	**0.857**	**0.939**	**0.718**	**0.935**	**0.935**	-	0.001	0.001
England	**0.793**	**0.947**	**1.000**	**0.736**	**0.817**	**0.897**	**0.575**	**0.867**	**0.837**	**1.000**	-	0.462
Wales	**0.770**	**0.862**	**0.881**	**0.728**	**0.813**	**0.830**	**0.591**	**0.737**	**0.630**	**0.887**	0.046	-

4.5 Evidence for cryptic speciation in the European heteronemertean *Lineus acutifrons*

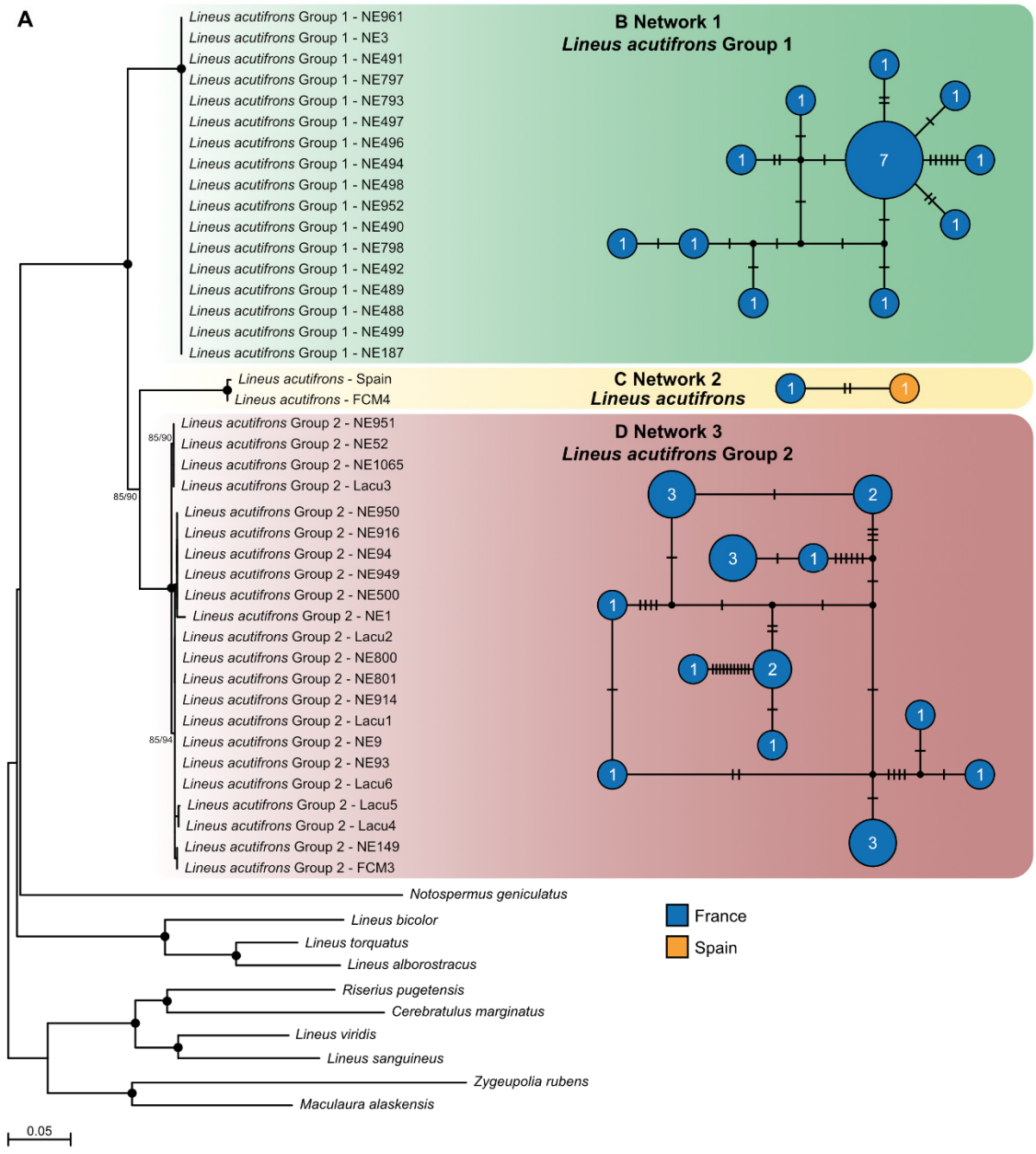
The heteronemertean *Lineus acutifrons* has been first incompletely described by Southern (1913). 96 years later, several new specimens were collected along the coasts of Galicia (Puerta et al. 2010; Puerta & Junoy 2011). The species is characterized by a pink to red body coloration, the lack of eyes, an acutely pointed head that is clearly demarcated from the remaining body and a caudal cirrus (Southern 1913; Puerta et al. 2010; Puerta & Junoy 2011). Nemerteans fitting this description can also commonly be found in France. Over the years, 41 specimens were collected along the Breton coast. Based on external morphology, all specimens were identified as *L. acutifrons*.

Nevertheless, a concatenated maximum-likelihood analysis of all French specimens combined with the only specimen deposited in GenBank (COI, 16S, 18S) resulted in three distinct clades (Fig 4.5.1). The sister group relationship of all three clades is well-supported. The first clade comprises 17 specimens sampled in France. It is sister to the two other "*L. acutifrons*" clades. The second clade comprises only two specimens, one sampled in France and one specimen from Spain. The Spanish sequence data was obtained from the re-description of *L. acutifrons* in 2010. The sister group to this clade comprises 22 specimens sampled in France. The results of the ML analysis hint at cryptic speciation as all specimens exhibit similar external characteristics. Nevertheless, it can be shown that the species *Lineus acutifrons sensu stricto* is not restricted to Galicia, but can also be found in Brittany, although in smaller numbers than first expected.

A statistical parsimony analysis of the whole dataset yielded three distinct networks. Network 1 is consistent with the first clade sustained in the ML analysis and is represented by 11 haplotypes. One haplotype is shared by seven individuals, the remaining 10 haplotypes can only be found in single individuals. The haplotypes are interconnected by one to six mutational steps. Network 2 comprises two specimens and is consistent with the *L. acutifrons sensu stricto* clade Both haplotypes are separated by two mutational steps. Network 3 represents the third clade recovered in the ML analysis and includes 12 haplotypes. This network shows the highest amount of haplotype structuring as three haplotypes are shared by three specimens, two haplotypes are shared by two specimens, and seven haplotypes are only found in individual

specimens. One to eleven mutational steps are needed to connect the haplotypes. In order to connect Networks 1 and 3, 60 mutational steps are necessary, whereas Networks 2 and 3 are separated by 71 mutational steps. This again hints at the presence of three distinct species.

The results underline the usefulness of phylogenetic analyses and molecular species delimitation methods to uncover cryptic species in groups were specific external differences are scarce.



◄ **Figure 4.5.1** Nemerteans showing characteristics of *Lineus acutifrons* hide at least two cryptic species. **A** Maximum-likelihood tree (GTR+G+I) of all specimens identified as *Lineus acutifrons* based on a concatenated 3 gene dataset (16S, COI, 18S). Numbers beside nodes indicate support values (ultrafast bootstrap values/alRT). Black circles indicate nodal support of 100. Various lineid species were used for outgroup rooting. The three clades are coded by colour. **B-D** Statistical parsimony haplotype networks of all specimens of the *L. acutifrons* type based on mitochondrial COI sequences with a connection limit of 95%. Geographic distribution by sampling sites in each country is represented by colour. Numbers within pie charts represent number of specimens with respective haplotype. Each bar indicates a single substitution. Network 1 is represented by 11 haplotypes (**B**), Network 2 (= *L. acutifrons*) is represented by 2 haplotypes (**C**), and Network 3 is represented by 12 haplotypes (**D**).

4.6 Haplotype diversity in the two most prominent European *Micrura* species: *Micrura fasciolata* and *Micrura purpurea*

To date, the genus *Micrura* comprises 55 described species and, along with *Lineus* and *Cerebratulus*, belongs to the largest heteronemertean genera (Schwartz & Norenburg 2005; Hiebert & Maslakova 2015). The latest molecular investigations suggest that the genus is indeed paraphyletic (Thollesson & Norenburg 2003; Schwartz 2009; Andrade et al. 2012; Kvist et al. 2014). Thus, only the species that are closely related to the type species *Micrura fasciolata* should be included in the genus *Micrura*. In northern Europe, the most abundant species of the genus are *Micrura purpurea* and *M. fasciolata* (Gibson 1982, 1995). Both species can be easily identified based on external morphological characters. The conspicuous colour pattern of *M. fasciolata* includes numerous, white transverse bars that are distributed over the whole dorsal surface (Gibson 1982). *Micrua purpurea* on the other hand, exhibits a bright yellow transverse band at the very anterior part of the head (Gibson 1982).

Both species can be commonly found along the southern Swedish coasts, including Kristineberg. In total, 25 specimens of *M. purpurea* and 4 specimens of *M. fasciolata* were collected in Kristineberg. Additionally, two specimens of *M. purpurea* were collected in Wimereux and Concarneau, respectively. Both species were combined with sequence data deposited at GenBank (19 sequences of *M. facsiolata* and 36 sequences of *M. purpurea*). Furthermore, exemplary sequences of all identified *Micrura* species were added to the dataset. An ML analysis based on the COI barcoding gene yielded two large well-supported clades, reflecting *M. fasciolata* and *M. purpurea* (Fig 4.6.1 A). External morphology appears to be indeed sufficient to identify both species, as all specimens belong to the species they were identified as. No cryptic speciation occurs. The *M. fasciolata* clade is sister to *Micrura varicolor* PUNNETT, 1903 with robust nodal support. In contrast to this, the sister group relationship between *M. purpurea* and *Micrura wilsoni* (COE, 1904) is only moderately-well supported. With lacking nodal support, the latter two species are sister to a clade comprising *Micrura dellechiajei* (HUBRECHT, 1879) as sister to the well-supported *Micrura chlorapardalis* SCHWARTZ & NORENBURG, 2005/*Micrura rubramaculosa* SCHWARTZ & NORENBURG, 2005 clade. The low nodal support between this larger clade and the *M. fasciolata* clade might indicate the presence of two different genera, although further species need to be included to

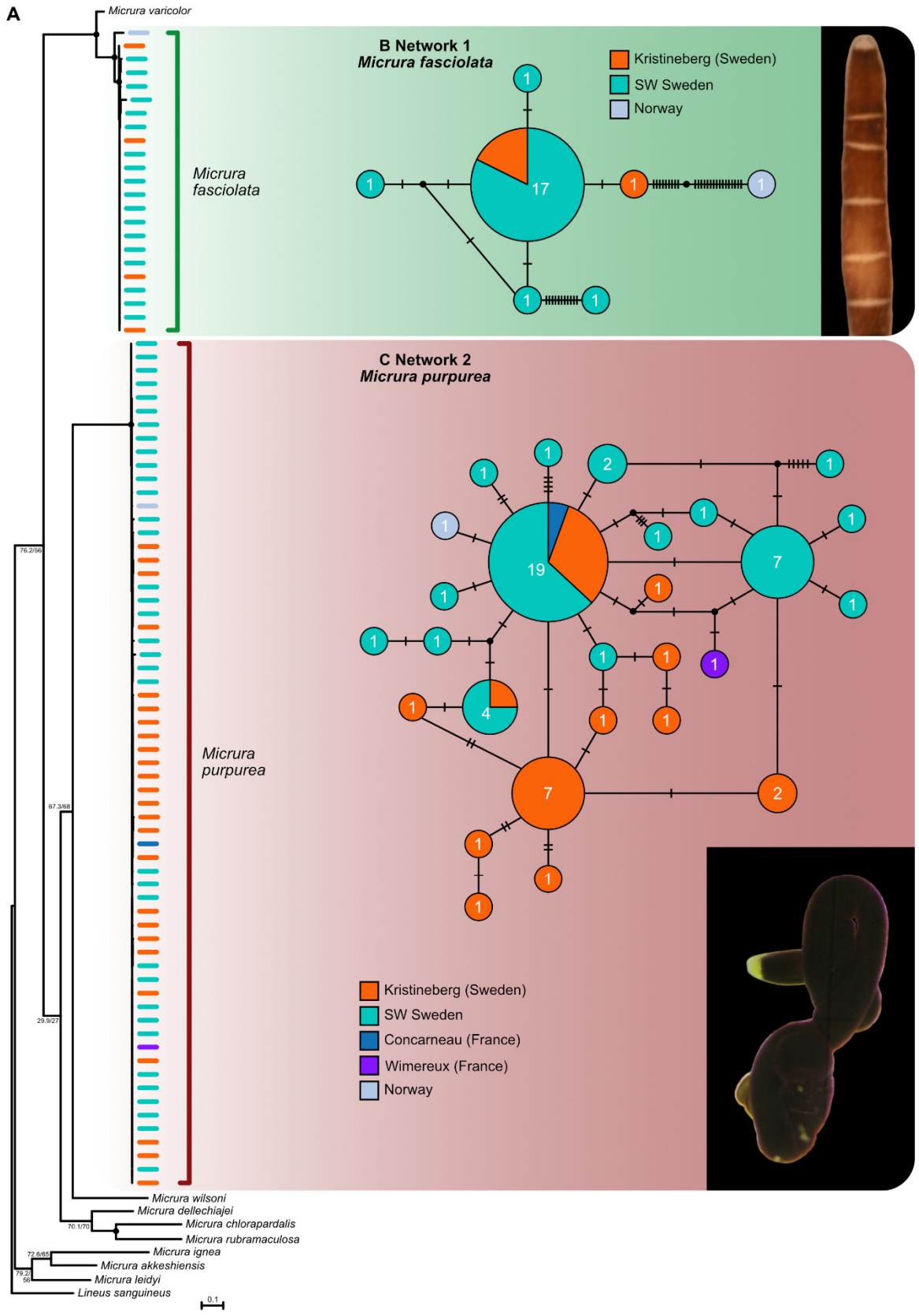
verify this hypothesis. The three species *Micrura ignea* SCHWARTZ & NORENBURG, 2005, *Micrura akkeshiensis* YAMAOKA, 1940, and *Micrura leidyi* (VERRILL, 1892) belong to a third clade that appears to be only loosely related to the other two clades.

A TCS analysis yielded one network representing *M. fasciolata* and one network representing *M. purpurea*. The *M. fasciolata* network is represented by 7 haplotypes (Fig 4.6.1 B). One large haplotype is found in 17 specimens collected in Kristineberg and other Swedish sampling localities. The remaining 6 haplotypes are only present in single individuals. Four specimens from Sweden and Kristineberg are connected to the most frequent haplotype by one to two nucleotide substitutions. One Swedish specimen is separated by 11 nucleotide substitutions, whereas the only specimen originating from Norway is separated by 25 nucleotide substitutions from one haplotype from Kristineberg.

In contrast to this, the *M. purpurea* network has higher degree of haplotype structuring (Fig 4.6.1 C). In total, 27 haplotypes are present. One frequent haplotype (19 specimens) is found in specimens from Sweden (including Kristineberg) and the only specimen from Concarneau. Additionally, three moderately frequent haplotypes are shared by seven and four specimens, respectively. The remaining 23 haplotypes are present in two or only one specimen each. Haplotypes are interconnected by one to six nucleotide substitutions. The only specimen from Norway is separated from the main haplotype by one mutational step, whereas the specimen from Wimereux is separated from the main haplotype by 3 and one of the medium-frequency haplotypes by 2 mutational steps.

The results show that specimens of the most commonly found European *Micrura* species can be identified based on external morphology. Nevertheless, the genus is most likely in need of revision as it appears to be paraphyletic.

► **Figure 4.6.1** Species identification based on external characteristics is a valid tool to discriminate between the two most common European species *Micrura fasciolata* and *Micrura purpurea*. **A** Maximum-likelihood tree (HKY+G+I) of all European *M. fasciolata* and *M. purpurea* specimens based on the COI gene fragment. Selected *Micrura* species were added to infer information on relationshiphs within the genus. *Lineus sanguineus* was used for outgroup rooting. Numbers beside nodes indicate support values (ultrafast bootstrap values). Specimens are colour-coded based on sampling locality. **B, C** Statistical parsimony haplotype networks of *M. fasciolata* (**B**) and *M. purpurea* (**C**) based on mitochondrial COI sequences with a connection limit of 95%. Geographic distribution is represented by colour. Numbers within pie charts represent number of specimens with respective haplotype. Each bar indicates a single substitution. Exemplary habitus photographs for both species are provided.


A
Micrura varicolor
Micrura fasciolata
B Network 1
Micrura fasciolata
Kristineberg (Sweden)
SW Sweden
Norway
17
C Network 2
Micrura purpurea
19
7
4
7
2
Micrura purpurea
Kristineberg (Sweden)
SW Sweden
Concarneau (France)
Wimereux (France)
Norway
76.2/56
67.3/68
29.9/27
70.1/70
72.6/65
79.2/
58
Micrura wilsoni
Micrura dellechiajei
Micrura chlorapardalis
Micrura rubramaculosa
Micrura ignea
Micrura akkeshiensis
Micrura leidyi
Lineus sanguineus
0.1

5 General Discussion

5.1 Usefulness of DNA barcoding in nemertean systematics

To date, the application of molecular single gene sequences is still the approach of choice when it comes to identification of unknown specimens. The main advantage is the easy and fast extraction of these gene fragments. In the animal kingdom, the most commonly used gene fragment for identification and DNA barcoding still is the cytochrome oxidase c subunit I (COI) (Hebert et al. 2003a; Hebert & Gregory 2005). Over the years, numerous further applications of single sequence data besides identification that are of greater implications could be added (Kvist 2013). This kind of data facilitates molecular species delimitation within a genus and might even be employed in population analyses (Vogler & Monaghan 2007; Kvist 2013). Moreover, DNA barcoding can be applied to uncover cryptic species, or enhance the rate of species discovery (Kvist 2013; Sundberg et al. 2016b).

For several centuries, species identification and taxonomy were solely based on morphological data. With the advent of DNA barcoding at the beginning of the last millennium, molecular data gained increasing importance in solving numerous variable taxonomic questions (Hebert et al. 2003b; Hebert et al. 2004; Hebert & Gregory 2005). As a result, the systematics of several taxa has experienced considerable revision (e.g. Weigert & Bleidorn 2016 for Annelida). This is also true for nemertean systematics (Andrade et al. 2012; Sundberg 2015). In this group, the single-locus COI sequence can be successfully employed as species identifier (Sundberg et al. 2016b), especially when it comes to the presence of new or cryptic species (Sundberg et al. 2009b; Chen et al. 2010; Strand & Sundberg 2011; Leasi & Norenburg 2014). Nevertheless, two main prerequisites must be fulfilled for effective species identification based on DNA barcoding: firstly, the presence of labelled target sequences deposited in online repositories, and secondly, the presence of a barcoding gap in the investigated taxon (Kvist 2013; Sundberg et al. 2016b).

The first prerequisite needs to be fulfilled to allow for comparison of a query sequence to several different target sequences of the same taxon (Kvist 2013). Thus, successful species identification is dependent on representation of target sequences in online repositories such as NCBI or BOLD (Kvist et al. 2010). Generally, taxonomic coverage in these databases is still poor as, on average, only 20% of all species per phylum were represented roughly 10 years ago (Kwong et al. 2012; Kvist 2013). Coverage in online databases is highly dependent on the

studied taxon, but also on the investigated geographic location (Ramirez et al. 2020). In general, taxa that are targets of the BOLD campaigns are better represented than non-target taxa (Ratnasingham & Hebert 2007; Kwong et al. 2012). Weigand et al. (2019) showed that in aquatic biota the gaps in barcode references libraries is largest for invertebrates. This is also true for the taxon Nemertea with an assumed representation of only 17% (Kvist 2013; Weigand et al. 2019). In order for DNA barcoding to work properly, a representation of each species, preferably with several specimens spanning the species' distributional range, would be desirable (Weigand et al. 2019).

Within Nemertea, vast differences occur in the effectiveness of species identification as success is heavily dependent on the investigated subgroups. While heteronemerteans and eumonostiliferous hoplonemerteans are quite well-represented, labelled sequences of polystiliferous hoplonemerteans are for the most part missing (Maslakova & Norenburg 2001; Kajihara & Yamaguchi 2020). According to the World Register of Marine Species (WoRMS), approximately 50 species representing polystiliferous Reptantia are currently recognized in 20 genera. In contrast to this, only 16 COI reptant sequences are deposited in GenBank. Of these, only two sequences are identified to species level, most of the remaining sequences are labelled as "Repantia sp." (Chernyshev & Polyakova 2019; Kajihara 2020). This is also true for the second group of polystiliferous nemerteans: the taxon Pelagica comprises nearly 100 described species in 40 genera, yet only 12 sequences representing three identified species and three taxa identified to genus level were published (Thollesson & Norenburg 2003; Bucklin et al. 2010; Kajihara et al. 2011). To make matters worse, the bulk part of polystiliferous nemerteans were described at the beginning of the last century, often based on only single specimens (Maslakova & Norenburg 2001; Kajihara & Lindsay 2010). Due to these often-incomplete species descriptions and the fact that many species have only been collected once, identification of unknown polystiliferous specimens based on molecular data alone is difficult (Maslakova & Norenburg 2001; Kajihara & Yamaguchi 2020). Therefore, the deep-sea reptant specimen in Chapter 3.3 could not be described. In order to properly identify and describe new species and answer taxonomic questions, a revision of this group might be needed (see Chapter 3.3).

In general, large and wide-spread genera are more likely well-represented in repositories than smaller, less-easily identifiable genera. This is for example true for most palaeonemertean genera, except for *Cephalothrix* and *Tubulanus*. These specimens often closely resemble each other as they are all part of the interstitial fauna (Gibson 1982). Therefore, good representation in databases would be crucial for species identification and detection of cryptic species.

Unfortunately, most sequences deposited in GenBank are only identified to genus level (see Chapter 4.1). These cases highlight the importance of properly curated online repositories as, at the moment, identification based on sequence data alone might be inconclusive (Meyer & Paulay 2005; Kvist 2013).

Successful application of DNA barcoding is further impeded by misidentifications in sequence databases as wrongly identified sequences negatively affect the search for a barcoding gap (Kvist et al. 2010; Kvist 2013; Sundberg et al. 2016b). The barcoding gap is defined as the gap between maximum intraspecific and minimum interspecific divergences (Sundberg et al. 2016b). In nemertean taxonomy, this gap usually lies between 4% and 10% and indicates that DNA barcoding as such is successful in nemerteans (Sundberg et al. 2016b). The presence of such a gap is assumed to be important for accurate and effective barcoding, although the size of this gap cannot be uniformly defined for all taxonomic groups (Čandek & Kuntner 2015; Kvist 2016). Nevertheless, some authors argued that the presence of a barcoding gap is merely the result of insufficient taxon sampling (Meyer & Paulay 2005; Meier et al. 2006; Wiemers & Fiedler 2007; Meier et al. 2008). If a barcoding gap is assumed to be present, misidentified sequences therefore might lead to low interspecific and high intraspecific divergences, thus giving wrong impressions on speciation and molecular evolution in the investigated groups (Collin & Cruickshank 2013; Kvist 2013). Examples of misidentified sequences include sequences tagged with the same name although belonging to different taxa as is prevalent in the large hold-all genera like *Lineus*, *Cerebratulus*, or *Oerstedia* (Sundberg et al. 2016b). This is for example the case for *Lineus ruber* and *Lineus sanguineus* that are commonly confused with each other and thus mislabelled (Kang et al. 2015; Krämer et al. 2017). Moreover, it is possible that sequences are not only assigned to the wrong species but also to the wrong genus, as has been shown for sequences labelled as *Cephalothrix linearis* (see Chapter 3.1, Kajihara 2019).

If misidentified sequences were excluded, it has been shown for several taxa, including nemerteans, that 3% intraspecific variation in the COI gene fragment is sufficient to distinguish between species (Smith et al. 2005; Sundberg et al. 2016b). The case of European *Cephalothrix* species further confirmed this (see Chapter 3.1).

Although some obstacles remain, DNA barcoding can mostly replace identification based on internal morphology and substantiate external morphological characters (Butcher et al. 2012; Sundberg et al. 2016a).

5.2 Shortcomings of molecular data in species descriptions

While identification of unknown specimens can be easily achieved via DNA barcoding, species descriptions still heavily rely on traditional methods, like histological sectioning (Padial et al. 2010). Describing a new species based on histology is a time-consuming process that can only be performed by experts (Roe et al. 2007; Sundberg et al. 2010; Sundberg 2015; Sundberg et al. 2016a). Nevertheless, this approach has been employed for the last centuries in nemertean species descriptions (e.g. Verrill 1892; Bürger 1895; Coe 1904; Kirsteuer 1963; Krämer & Döhren 2015). Problems with this approach arise when it comes to the more or less subjective definition of characters (Sundberg 2015; Sundberg et al. 2016a). Moreover, several morphological characters used for species delimitation exhibit high levels of intraspecific variation (Strand et al. 2014; Sundberg et al. 2016a). In order to solve these problems and gain a standardized approach to nemertean descriptions, a character matrix with clearly defined characters and their states was proposed by Sundberg et al. (2009a). Several species descriptions have been based on this checklist (e.g. Taboada et al. 2013; Strand et al. 2014). Albeit the usefulness of this proposal, its standard use in nemertean descriptions could not be enforced so far. In general, there is no scientific evidence that species identifications are more accurate when based on internal characters as opposed to external (Kvist 2013). Despite the mentioned problems with regard to histological sectioning, nemertean species descriptions are still predominantly based on internal characteristics, although molecular data is usually included (e.g. Chernyshev et al. 2015; Krämer & Döhren 2015; Chernyshev & Polyakova 2019; Hookabe et al. 2020).

Nevertheless, following the proposal by Sundberg et al. (2016a), a short description of external characteristics linked with a DNA barcode is sufficient for nemertean species descriptions. Valid species descriptions excluding histology have since then been provided for several nemertean species (e.g. in Strand & Sundberg 2011; Kajihara 2015; Kajihara et al. 2018; Chernyshev et al. 2020) following a general plea for DNA taxonomy (Tautz et al. 2003). As has been shown, even the identification of cryptic species is possible without histology as external characters, if distinguishable, are of higher importance than detailed anatomical data (Krämer et al. 2017; Chernyshev et al. 2018). Moreover, re-descriptions of species that have been insufficiently described in past centuries and that lack a deposited holotype, can be easily achieved in this way (Sundberg et al. 2016a). In the process, old names can be re-assigned to sampled specimens before new names are assigned for a species (Martinsson 2016).

Unintentional descriptions of a known species as a new one can be prevented in this way and future synonymizations can be avoided (Kajihara et al. 2008). Following the proposal by Sundberg et al. (2016a), the neotype of *Cephalothrix linearis* could be successfully assigned (Kajihara 2019). Despite the general acceptance of the proposal, many species descriptions still include histological data (Strand et al. 2014). This multidisciplinary approach including data from various sources, such as morphology, biogeography, and ecology is known as integrative taxonomy (Padial et al. 2010; Boury-Esnault et al. 2013; Chapple & Ritchie 2013). As argued by Tautz et al. (2003), taxonomy should always include DNA sequence data (Sundberg et al. 2010). In nemertean taxonomy, an integrative approach is still the most commonly applied way of describing a species (e.g. Hiebert & Maslakova 2015; Krämer & Döhren 2015; Chernyshev & Polyakova 2021; Mendes et al. 2021). Although the importance of histological data in nemertean systematics is diminishing, investigating internal morphology might prove useful for descriptions in some cases as it is desirable to include as much information on species as possible. According to Chernyshev & Polyakova (2018b), morphological data is crucial to identify and describe deep-sea nemerteans. For this reason, an integrative approach was chosen in this study in order to describe several deep-sea species collected along the Costa Rica margin (Chapter 3.3). The latest take at an integrative approach not only includes traditional data like morphology and mitochondrial gene fragments, but also genomic data in the form of several thousand single nucleotide polymorphism (SNP) variants (Mendes et al. 2021).

As the majority of taxonomic knowledge is based on morphology, a combination of morphological and molecular approaches might be the most reasonable choice for future nemertean species descriptions, although the advent of histology free descriptions should be further supported wherever no important information is lost (Sundberg et al. 2016a). These descriptions following a turbotaxonomic approach can help gain a better understanding of species diversity, especially in the light of biodiversity loss and habitat degradation (Butcher et al. 2012).

5.3 DNA taxonomy and species delimitation

DNA barcoding can provide the basis for numerous further applications, including DNA taxonomy (Vogler & Monaghan 2007). DNA taxonomy aims at detecting species boundaries, based on various molecular delimitation methods and has been applied in many different taxa (Monaghan et al. 2006; Pons et al. 2006; Vogler & Monaghan 2007; Fontaneto et al. 2015).

Based on non-tree-based and tree-based delimitation methods, molecular sequences are grouped into molecular operational taxonomic units (MOTUs) that are equivalents to traditional 'species' (Godfray et al. 2004; Vogler & Monaghan 2007). Vogler & Monaghan (2007) have shown that sequence variation in mitochondrial genes, like the COI gene region, can serve as basis for species delimitation in understudied groups (Tautz et al. 2003; Pons et al. 2006). This approach differs from DNA barcoding as it does not aim at species identification by sequence similarity but at delineation of species based on species concepts (Hebert et al. 2003a; Hebert & Gregory 2005; Vogler & Monaghan 2007).

DNA taxonomy proved especially valuable in the identification of cryptic species that cannot easily be distinguished based on morphological characters. Numerous studies have shown that the number of predicted species in a dataset is higher than the number of included unique taxon labels (Sundberg et al. 2009b; Leasi & Norenburg 2014; Sundberg et al. 2016b; Krämer et al. 2017; Chernyshev et al. 2018; Mendes et al. 2018). This phenomenon is especially prevalent in nemerteans as species identification and differentiation based on external morphology is in many cases unambiguous (Gibson 2002; Strand & Sundberg 2005a; Sundberg et al. 2009b; Sundberg 2015). In the course of this PhD project, several examples of cryptic speciation could be detected (see Chapter 3.1, Chapter 4.1, and Chapter 4.5). Due to their simple external morphology, cryptic species seem to be prevalent in palaeonemerteans (see Chapter 3.1 for *Cephalothrix*, and Chapter 4.1 for *Carinina*, *Carinoma*, and *Callinera* species). In addition, specimens identified as *Lineus acutifrons* most likely represent at least three different species that are congeneric in respect to habitat (Chapter 4.5).

In order to detect cryptic species and uncover species boundaries, numerous different molecular species delimitation methods can be applied. The effectiveness of these methods has already been tested in numerous different taxa (e.g. Wiens & Penkrot 2002; Ortiz & Francke 2016; Chroni et al. 2017; Aguado et al. 2019). Nevertheless, the number of detected entities can extensively vary between different tree-based and non-tree-based delimitation methods based on the investigated gene fragments and the applied threshold (see Chapter 3.1).

This threshold applied for separating entities in non-tree-based delimitation methods is a major point of concern (Sundberg et al. 2016b; Sagorny et al. 2019). The most commonly applied threshold to separate species is 5% (Sundberg et al. 2009b; Chen et al. 2010; Kang et al. 2015; Krämer et al. 2017). Although results of analyses with a threshold of 5% are in most cases congruent with the results obtained by tree-based methods, it remains an arbitrarily set limit that does not necessarily represent natural groupings, i.e. species (Sagorny et al. 2019).

According to Sundberg et al. (2016b), already 3% dissimilarity are enough to separate two species (see above). Thus, many species delimitation methods remain somewhat subjective.

The most widely used molecular species delimitation method in nemerteans proved to be the statistical parsimony analysis. In this approach, sequences are grouped by haplotypes to form networks representing a species (Templeton et al. 1992). It has been shown that these TCS networks significantly coincide with Linnean names (Hart & Sunday 2007). This method has been successfully applied to delimit species in all three major nemertean groups: Palaeonemertea (e.g. Chen et al. 2010 for *Cephalothrix*), Heteronemertea (e.g. Kang et al. 2015 and Krämer et al. 2017 for different *Lineus* species, Sundberg et al. 2010 and Verdes et al. 2021 for *Cerebratulus*), and Hoplonemertea (e.g. Sundberg et al. 2009b for *Oerstedia*, Andrade et al. 2011 and Leasi et al. 2016 for *Ototyphlonemertes*, Hao et al. 2015 for *Paranemertes*, Strand & Sundberg 2005a 2005a for *Tetrastemma*). The estimation of haplotype networks enables the user to distinguish between individuals belonging to different and distant populations and might hint at gene flow between these populations (Templeton et al. 1992). In the case of *Riseriellus occultus*, for example, this method has helped to uncover that haplotypes are not congruent with geographic localities or different habitat preferences (Sundberg & Strand 2007). In the course of the present PhD project, statistical parsimony networks were calculated in order to delimit species (as in Chapter 3.1 for European *Cephalothrix* species and in Chapter 4.5 for cryptic species of the *Lineus acutifrons* type), as well as to gain information on haplotype distributions along populations (as in Chapter 3.2 for *Lineus sanguineus*, Chapter 4.2 for *Lineus longissimus*, Chapter 4.3 for *Riseriellus occultus*, Chapter 4.4 for species of the *Lineus ruber* complex, and Chapter 4.6 for two species of *Micrura*).

DNA taxonomy based on the single locus COI barcoding gene proved to be a valuable tool to identify species, detect species boundaries, uncover cryptic speciation, and verify distributional ranges in nemerteans (Sundberg et al. 2016b). Nevertheless, incorporating more than one gene region can facilitate the reconstruction of a solid phylogeny and the uncovering of species diversity (Leasi et al. 2016). An additional application of species delimitation methods to more than one gene dataset might further help to verify the results of the COI gene fragment and exclude ambiguous results based on potential wrong marker choice (see Chapter 3.2).

5.4 Reconstructing phylogenetic relationships

Besides the identification and delimitation of species, molecular sequence data nowadays composes the main backbone for the reconstruction of phylogenetic relationships (Vogler & Monaghan 2007; Weigert & Bleidorn 2016). With the advent of molecular methods, phylogenies were first reconstructed based on single gene sequences (e.g. Winnepenninckx et al. 1995; Kim et al. 1996; Winnepenninckx et al. 1998; Eeckhaut et al. 2000). As molecular methods became more accessible, additional gene fragments, including mitochondrial and nuclear genes, as well as histones were incorporated in order to gain a more accurate result and achieve higher resolution (e.g. Brown et al. 1999; Adkins et al. 2001; Whiting 2002; Mattern 2004; Yu & Zhang 2006; Struck et al. 2007). With the introduction of next-generation sequencing techniques, a higher amount of molecular data became accessible by increasing the speed and extent of the analyses (Weigert & Bleidorn 2016). These approaches for example allow for the fast study of whole mitochondrial genomes (e.g. Cameron et al. 2012; Perseke et al. 2013; Aguado et al. 2015; Yang et al. 2015; Lee et al. 2019). In order to recover a robust phylogeny, the application of transcriptomic data became most favourable within the last decade as large amounts of genes and species can be incorporated (e.g. Borner et al. 2014; Peters et al. 2014; Peters et al. 2018; Stiller et al. 2020; Wen et al. 2020). For Annelida, this transition from single gene phylogenies to an transcriptomic approach has helped to resolve the monophyly of two large subgroups and the position of some early branching species (reviewed in Weigert & Bleidorn 2016).

The reconstruction of phylogenetic relationships within nemerteans also started with the application of only single genes like mitochondrial 16S rRNA or nuclear 18S rRNA (Sundberg & Saur 1998; Sundberg et al. 2001). Estimations based on a multi-gene approach have become far more common since then (Thollesson & Norenburg 2003; Andrade et al. 2012; Kvist et al. 2014; Kvist et al. 2015). Therefore, both mitochondrial genes (usually 16S rRNA, COI) and nuclear genes (18S rRNA, 28S rRNA, ITS) as well as histones (H3, H4) are commonly employed (Thollesson & Norenburg 2003; Andrade et al. 2012; Kvist et al. 2014; Kvist et al. 2015). To date, only few studies in nemertean taxonomy have aimed at a next-generation approach in order to reconstruct phylogenetic relationships.

Within the last decade, roughly 20 whole mitochondrial genomes have been sequenced (Chen et al. 2009; Podsiadlowski et al. 2009; Chen et al. 2011; Chen et al. 2012; Xu et al. 2012; Sun et al. 2014; Sun & Sun 2014; Gonzalez-Cueto et al. 2015; Shen et al. 2015; Shen & Shi-

Chun 2016; Sun et al. 2016; Jiang & Deng 2018; Redak & Halanych 2019; Nam & Rhee 2020). These represent the three large nemertean subgroups, although palaeonemerteans and polystiliferous hoplonemerteans are vastly underrepresented. Thus, relationships within the groups could not be fully resolved (see Fig 5.1). Moreover, not enough sequences were included to reconstruct important splits within the three groups (Nam & Rhee 2020). Nevertheless, a comparison of gene arrangement showed striking differences between the three large groups (Podsiadlowski et al. 2009; Chen et al. 2012; Chen et al. 2014; Sun et al. 2014; Gonzalez-Cueto et al. 2015).

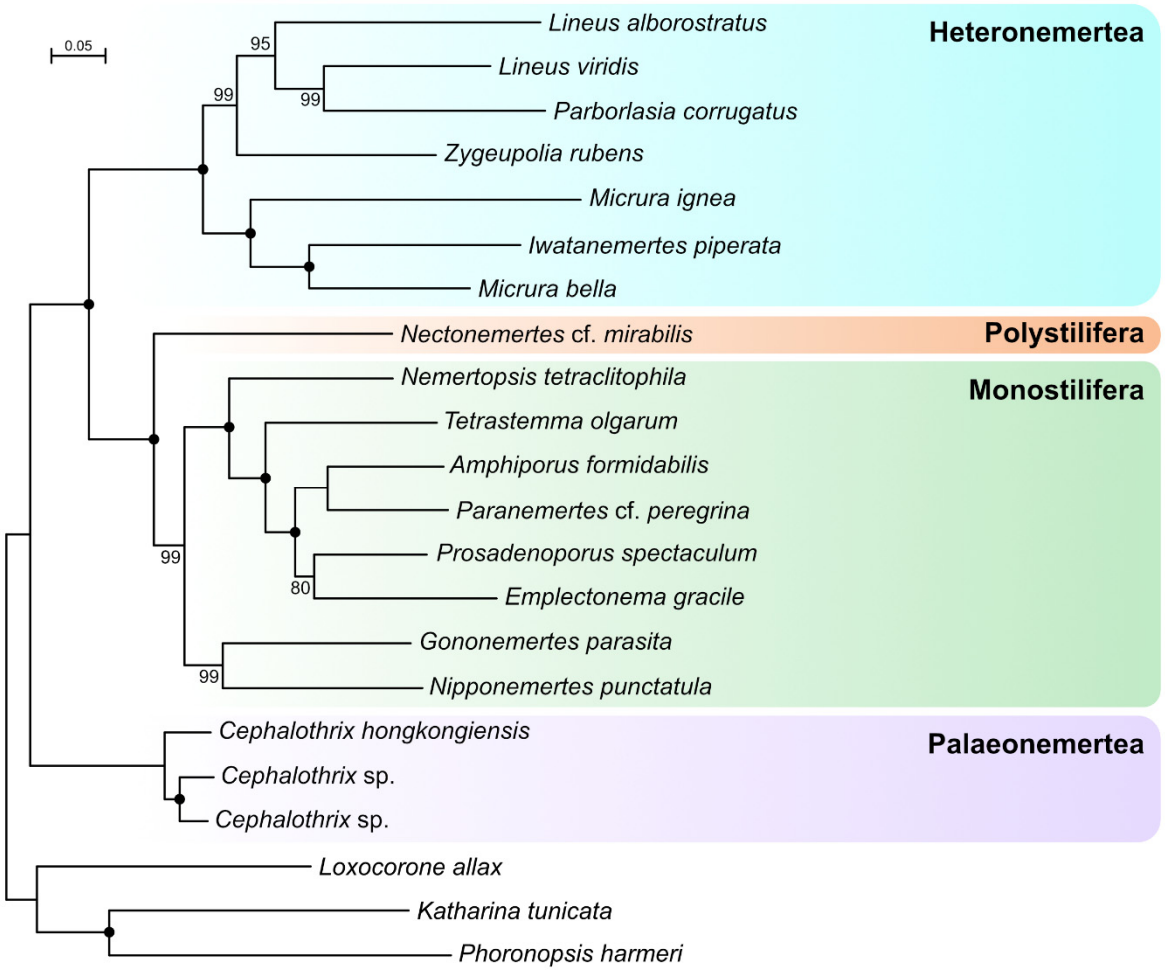


Figure 5.1 Maximum-likelihood (ML) phylogeny of 19 nemertean species. Numbers beside nodes indicate posterior probability. Black circles indicate nodal support of 100 (redrawn after Nam & Rhee 2020).

The only transcriptomic approach was based on 12 specimens again representing the three main groups (Dunn et al. 2008; Riesgo et al. 2012; Andrade et al. 2014). This analysis

confirmed the monophyly of heteronemerteans and hoplonemerteans that was already assumed based on previous multi-gene approaches (Thollesson & Norenburg 2003; Andrade et al. 2012; Kvist et al. 2014; Kvist et al. 2015). Moreover, the monophyly of palaeonemerteans was recovered (Andrade et al. 2014). Based on multi-gene approaches no unambiguous consensus could be reached with regard to the monophyly of this group (Thollesson & Norenburg 2003; Andrade et al. 2012; Kvist et al. 2014; Kvist et al. 2015). Thus, transcriptomic approaches can help to unravel problematic relationships and to recover a robust phylogeny (Andrade et al. 2014). In order to fully resolve the nemertean phylogeny, further transcriptomic data needs to be sequenced and incorporated.

Despite these NGS approaches, most recent phylogenetic reconstructions that aim at resolving relationships within Nemertea are still based on a multi-gene approach (e.g. Chernyshev & Polyakova 2019; Chernyshev et al. 2021b). This might be the case because traditional Sanger sequencing is more cost efficient and less labour intensive if less than five markers and less than 100 specimens are investigated (Sonet et al. 2018). Therefore, depending on the research question, multi-gene approaches are still the most appropriate choice in nemertean systematics. This is also true when it comes to the identification of unknown species and their taxonomic position as has been shown for deep-sea nemerteans (Chapter 3.3). Nevertheless, deep splits can only be resolved with approaches based on larger amounts of genes, especially when it comes to ambiguous taxa such as the genus *Lineus* (Strand et al. 2014).

5.5 Problematic nemertean taxonomy and the example of *Lineus*

As shown above, nemertean taxonomy is rather difficult to resolve (Sundberg et al. 2010). This is partly due to the guidelines that have to be followed to name a species. In order to assign a scientific name to a species, international codes of nomenclature have to be followed (Barnett 2019). To date, there are five international codes such as the International Code of Zoological Nomenclature that provide certain rules. These include naming new taxa as species in genera in a binomial format (ICZN 1999). As genera names often change over time, or species names are synonymized, this system leads to instability of names impeding communication and identification of individuals (Tautz et al. 2003). Following the rigid codes and binomial format puts severe constraints on naming of species as appropriate genus names need to be provided (Tautz et al. 2003). Numerous nemertean species have been described in the past two centuries (e.g. Bürger 1895, 1904; Coe 1904, 1905). Therefore, genus names have often been changed

with increasing knowledge of nemertean systematics (Gibson 1995; Kajihara et al. 2008). Moreover, many species have only been poorly described based on few morphological characteristics (Sundberg et al. 2016a). Thus, it is not uncommon that one species has been described several times under different species names as has for example been shown for *Lineus sanguineus* (see Chapter 3.2, Rathke 1799; Leidy 1855; Bürger 1893; Coe 1931; Gibson 1995; Kang et al. 2015). As a consequence of both, numerous synonyms might be present for some species (Gibson 1995). This name instability further increases the already problematic nemertean taxonomy.

Moreover, characters are often insufficiently defined in these century-old species descriptions (Sundberg 2015; Sundberg et al. 2016a). This lack of a standard approach led to the use of a specific name whenever a specimen vaguely resembled a given description, thus impeding delimitation of similar looking species (Sundberg 2015). While this phenomenon can be observed in many nemertean species (e.g. *Oerstedia dorsalis* (ABILDGAARD, 1806) Sundberg et al. 2009b), it is also true on genus-level.

Some genera that have been erected more than one century ago comprise hundreds of species and act as "hold-all" genera that are in most cases paraphyletic, such as *Lineus*, *Micrura*, *Cerebratulus*, or *Tetrastemma* (Strand & Sundberg 2005b; Sundberg 2015; Chernyshev et al. 2021b). Therefore, a current trend is the erection of monotypic genera for every newly described species (Strand et al. 2014). By now, more than 60% of all nemertean genera are monotypic (Strand et al. 2014).

As a result of only brief and very general genus diagnoses, these hold-all genera comprise large numbers of unrelated species (Strand & Sundberg 2005a; Sundberg 2015). Thus, these genera are in urgent need of revision. First of all, I argue that genera such as *Lineus* need new and clearly defined diagnoses based on the respective type species so that no uncertainties remain when assigning a species to this genus. Moreover, all species so far assigned to a genus should be revised and compared to the genus diagnosis even if this approach might lead to the loss of some monotypic genera. In some cases, re-descriptions of species might become necessary if they lack a clear diagnosis.

The problematic taxonomic relations within nemertean systematics can be emphasized when regarding the oldest known nemertean taxon, *Lineus*, that currently comprises around 80 species accounting to 20% of all described heteronemertean species (Gibson 1995). The genus is only poorly defined in terms of morphology (Schwartz 2009). In recent years, some species

have been reallocated to other genera based on anatomical differences (e.g. *Lineus torquatus* COE, 1901 has been assigned to the new genus *Kulikovia* (Chernyshev et al. 2018)).

Only species closely related to the type species *Lineus longissimus* should be included in the revised genus, whereas the rest should be moved to new or existing genera (Puerta et al. 2010). Molecular approaches, even those based on one single gene region, facilitate the identification of closely related species of one genus and can help to assign species to different genera that then represent natural groups. Accordingly, all three "*Lineus acutifrons*" species need to be assigned to new genera (Chapter 4.5).

Yet still, several uncertainties remain with regard to species that should be included in the genus *Lineus*. Based on different studies, eight species could be assigned to the *L. longissimus* clade. Traditionally, the clade comprises the species *Lineus ruber*, *Lineus viridis*, and *Lineus clandestinus* (Puerta et al. 2010). Furthermore, the three species *Ramphogordius lacteus* RATHKE, 1843, *Ramphogordius pseudolacteus*, and *Ramphogordius sanguineus* are often included in the genus *Lineus* sensu stricto, as well as *Riseriellus occultus* (Ament-Velásquez et al. 2016; Chernyshev et al. 2018) The latter four species have originally been assigned to different genera based on morphological differences (Rathke 1843; Rogers et al. 1993). In spite of this, it is argued that *Ramphogordius* should be synonymized with *Lineus* according to several molecular phylogenies (Thollesson & Norenburg 2003; Ament-Velásquez et al. 2016). This should also be true for *R. occultus* as this species also falls into the *L. longissimus* clade (Sundberg & Saur 1998; Strand et al. 2005). Based on mitochondrial COI alone, *Lineus* should encompass all eight aforementioned species as *L. longissimus* is sister to a clade comprising those species (Fig 5.2). Despite this, the only approach incorporating transcriptomic data resulted in a divergent composition of the genus *Lineus* (Ament-Velásquez et al. 2016). Based on their results, *L. longissimus* is sister to "*Ramphogordius*" (Ament-Velásquez et al. 2016). Thus, *Ramphogordius* should be treated as junior-synonym of *Lineus*. As opposed to this, the taxonomic placement of both *R. occultus* and the *L. ruber*/*viridis* species complex still needs to be resolved (Ament-Velásquez et al. 2016).

In order to revise the genus *Lineus* more sequence data in public repositories is necessary as only 9 out of the ca. 80 recognized species are represented on GenBank (Chernyshev et al. 2018). This again highlights the problems of DNA barcoding and molecular methods when it comes to taxonomic coverage (Kvist 2013). This problem exemplified for the genus *Lineus* can be applied for numerous further nemertean genera and again underlines the need for a thorough revision based on high amounts of genes.

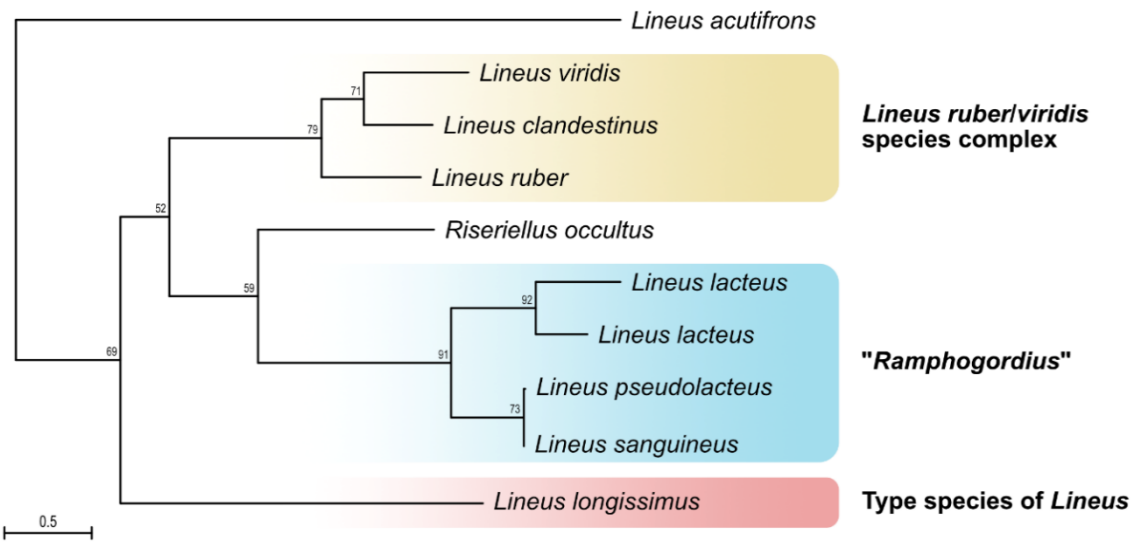


Figure 5.2 Maximum-likelihood (ML) tree of the nine assumed *Lineus* species based on mitochondrial COI sequences. Numbers beside nodes indicate ultrafast bootstrap support values. The type species *Lineus longissimus* is given in red, the *Lineus ruber*/*viridis* species complex in yellow, and the genus "*Ramphogordius*" in blue.

5.6 Population analyses and biogeography

With the advent of molecular techniques, addressing population structuring and dynamics has also become possible (Duran et al. 2004d). Molecular population genetics allows to analyse dispersal, colonization patterns and gene flow between populations (Hart & Marko 2010; Palumbi 2020). In marine environments barriers to gene flow appear to be absent so that marine species should show less genetic structuring between populations separated by large distances (Duran et al. 2004c). Nevertheless, several studies have shown that hydrological and ecological barriers exist that might impede long-distance dispersal (Duran et al. 2004c). Several mechanisms facilitate dispersal between marine populations spanning a wide geographic range (Cowen & Sponaugle 2009). Possible means of dispersal include accidental transport by ship or the development via long lived planktonic larvae (Hedgecock 1986; Cowen & Sponaugle 2009; Bhattachan et al. 2020). In general, it is assumed that marine benthic invertebrate species that develop via long-lived planktonic larvae have higher dispersal capacities than species lacking this type of larvae (Crisp 1978; Hedgecock 1986; Collin 2001; Romiguier et al. 2014). Moreover, the fecundity of a species might influence genetic variability (Romiguier et al. 2014). Besides abiotic characteristics, larval dispersal in marine species is also dependent on biological

species-specific characteristics (Takabayashi et al. 2003). Generally, a correlation between larval longevity and dispersal distance can be observed (Ayre & Hughes 2000). Thus, a higher level of gene flow and a lower level of genetic differentiation is expected in species with planktonic larvae (Crisp 1978; Hedgecock 1986; Palumbi 1992; Avise 2000). Moreover, a larger population size yields a higher genetic diversity (Casillas & Barbadilla 2017). Usually it can be assumed that genetic diversity is dependent on the position within a species' distributional range, with highest diversity in the centre and decreasing diversity in the periphery (Lal et al. 2017).

When it comes to the analysis of population dynamics, also the biogeography of a species has to be considered. This field of study is concerned with the geographic distribution of a species in relation to its ecology and evolution (Huggett 2004). Although there are less biogeographical barriers in marine environments than in terrestrial environments, several open-ocean barriers such as ocean currents or environmental gradients pose restraints on a species' distributional range (Thornhill et al. 2008; Leasi et al. 2016). Therefore, the distribution of a species is not only restricted by ecological reasons (such as dispersal capacity, life history or biotic and abiotic factors), but also by historical reasons (such as centres of origin or climatic and geological changes) (Huggett 2004). Among others, historical biogeography states that species originate in one place and from there spread to other places (Huggett 2004). This approach might also provide hints at the artificial introduction of species as has been shown for *Cephalothrix simula* and *Emplectonem gracile* (JOHNSTON, 1837) (Turbeville 2011; Fernández-Álvarez & Machordom 2013; Faasse & Turbeville 2015) In marine environments, the geographic range of a species is often limited by the presence of favourable habitats (Norris 2000). An often-observed phenomenon when studying biogeography is that molecular data uncover a less wide distribution of a species in comparison to the respective morphotypes, thus hinting at cryptic speciation (Leasi & Norenburg 2014). In nemerteans, biogeographic studies have shown that only few truly cosmopolitan species occur in disjunct oceans (Leasi & Norenburg 2014). Moreover, some biogeographic patterns have been revealed for meiofaunal nemertean species (Leasi & Norenburg 2014; Leasi et al. 2016).

Population analyses are traditionally based on single sequence data such as mitochondrial COI and nuclear ITS regions, or on microsatellites (Estoup & Angers 1998). Examinations of all three approaches have been made in the sponge *Crambe crambe* (SCHMIDT, 1862) (Duran et al. 2004a; Duran et al. 2004d; Duran et al. 2004c). The results of the mitochondrial dataset revealed low variability and low nucleotide diversity (Duran et al. 2004d). Nevertheless, more

detailed observations hinted at low gene flow between populations (Duran et al. 2004d). This result was supported by both nuclear single sequence data and microsatellites that both revealed high population structuring due to isolation by distance (Duran et al. 2004a; Duran et al. 2004c). Several recent studies include both mitochondrial and microsatellite data (Yan et al. 2020; Park et al. 2021). In general, microsatellites are assumed to be most informative as even subtle differences can be assessed (Estoup & Angers 1998; Duran et al. 2004c; Yan et al. 2020). In recent years, restriction site-associated DNA (RAD) sequencing that concentrates on large amounts of single nucleotide polymorphisms (SNPs) has become available – an approach that proved to be an even more powerful approach for studying population structure than microsatellites (Vendrami et al. 2017).

Despite the effectiveness of microsatellites and RAD sequencing, numerous studies addressing population structuring revert to the application of single sequence data. An often-used tool in this respect is the analysis of molecular variance (AMOVA) based on haplotype divergences (Excoffier et al. 1992). This analysis allows to infer information on phylogeography, the assumed link between geography and intraspecific phylogenies (Avise 2000; Uthicke & Benzie 2003). AMOVA based on mitochondrial data has already been successfully applied to analyse genetic differentiation and gene flow in numerous marine benthic taxa, including crustaceans (Samadi et al. 2006; Yan et al. 2020), molluscs (Collin 2001; Samadi et al. 2006; Einfeldt et al. 2017; Blakeslee et al. 2021), echinoderms (Uthicke & Benzie 2003; Duran et al. 2004b), and ascidians (Bhattachan et al. 2020; Haye et al. 2021). This method may also help to infer information on gene flow between native and invasive populations of a species (Bhattachan et al. 2020).

With regard to population genetics in nemerteans, only few studies have been performed and these studies are limited to few species (Alfaya et al. 2013; Runnels 2013). Investigated species include the heteronemertean species *Lineus ruber* and *Lineus viridis* (Rogers et al. 1997), *Parborlasia corrugatus* (McIntosh, 1876) (Rogers et al. 1998; Thornhill et al. 2008), *Lineus sanguineus* (Runnels 2013), and various species of the genus *Lineus* (Ament-Velásquez et al. 2016), as well as the hoplonemertean species *Malacobdella arrokeana* Ivanov et al., 2002 (Alfaya et al. 2013), *Emplectonema gracile* (Delaney 2019) and different species of the genus *Ototyphlonemertes* (Andrade et al. 2011; Tulchinsky et al. 2012). Species with long-lived planktonic larvae, such as *P. corrugatus* and *E. gracile*, exhibit high levels of genetic connectivity over large geographical distances (Rogers et al. 1998; Thornhill et al. 2008; Delaney 2019). Interestingly, also little genetic structuring was observed between the various

Ototyphlonemertes species, although these species are interstitial, and thus lack a larval dispersal stage (Andrade et al. 2011; Tulchinsky et al. 2012).

Despite large geographic distances, Rogers et al. (1997) found little evidence of genetic differentiation between North American and European populations of *L. ruber* and *L. viridis*, respectively. The results for *L. viridis* could be supported by the AMOVA analysis presented in Chapter 4.4 that also indicates a lack of population structuring between wide spread populations. In contrast to the results of Rogers et al. (1997), there is clear evidence for population structuring in *L. ruber* (see Chapter 4.4). The results suggest that isolation by distance occurs, especially between populations from northern and western European localities. This trend could not be observed by Rogers et al. (1997), because only western European populations were investigated. In all three species investigated in Chapter 4.4, the AMOVA results are congruent with the haplotypes recovered by the statistical parsimony analysis. In contrast to this, the results for *L. sanguineus* as presented in Chapter 3.2 differ from the results obtained by Runnels (2013). This could be due to the differing employed genetic markers and the different origin of analysed populations. The results nevertheless show that single gene data can successfully be applied to study population dynamics and structuring in an understudied taxon like nemerteans where information on population dynamics is still vastly lacking.

5.7 Conclusions

I conclude that single gene data or data based on only few gene fragments is still appropriate to answer numerous different questions in an understudied taxon such as Nemertea. First of all, identification of specimens based on DNA barcoding was successful in all investigated taxa, except for the newly described deep-sea species. Nevertheless, some problems occurred in some genera regarding representation in online repositories. Moreover, DNA taxonomy based on the COI barcoding gene can help detect species boundaries and cryptic speciation as has been shown for European *Cephalothrix* species (Chapter 3.1). In this regard, the statistical parsimony analysis has proved to be especially helpful as it not only allows to delimit species but also to analyse haplotype distribution within one species. Based on the COI gene, this TCS analysis has provided meaningful results for the heteronemertean species *Lineus sanguineus* (Chapter 3.2), *Lineus longissimus* (Chapter 4.2), *Riseriellus occultus* (Chapter 4.3), and the two *Micrura* species (Chapter 4.6). In addition, COI data can be used to address population genetics as has been shown for *L. sanguineus* (Chapter 3.2) and the *Lineus ruber*/*viridis* species complex

(Chapter 4.4). Nevertheless, including more than one marker might strengthen the informative value of the obtained results, especially if nuclear genes are included. Generally, the results have shown that the use of the COI gene alone is not sufficient for phylogenetic reconstructions as this gene fragment is relatively unconserved and has many neutral mutations (Strand et al. 2014). In the phylogenetic analyses of European *Cephalothrix* species (Chapter 3.1) and several other European palaeonemertean species (Chapter 4.1), the relationships between species could not be resolved.

Moreover, a combination of more than one genetic marker proved to be helpful in the identification of distributional patterns as has been shown for *L. sanguineus* (Chapter 3.2). Additionally, the identification of cryptic species (see Chapter 4.5 for *Lineus acutifrons*) and the assignment of newly described species to higher taxa (see Chapter 3.3 for Costa Rican deep-sea nemerteans) is facilitated by incorporating more than one genetic marker. Employed markers should always include at least one nuclear marker. Within nemertean systematics, standard markers include mitochondrial COI and 16S rRNA, nuclear 18S rRNA, 28S rRNA and ITS, and histones H3 and H4.

When it comes to species descriptions, morphological data is typically still included. Chapter 3.3 shows that molecular data in combination with descriptions of external morphology is in many cases sufficient to identify and also to describe a new nemertean species. This is especially relevant in small and rare nemerteans (e.g. deep-sea species), as often not enough material is present to allow for both molecular and histological analysis. Nevertheless, I argue that, whenever enough specimens are available, histological data or other relevant biological data (e.g. ecology or life-history) should be included in future species description to allow for a preferably complete species record.

Although the use of single gene data is somewhat outdated, the presented datasets highlight their usefulness in addressing numerous taxonomic questions. Therefore, I conclude that these data can still help uncover species identity, boundaries, distribution, population genetics and to some extent even relationships within Nemertea. Nevertheless, including more than one genetic marker will strengthen the obtained results. Especially when it comes to the reconstruction of a solid phylogeny, more genomic data should be sequenced in the future to obtain a good resolution between genera and the various subgroups of Nemertea.

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Appendix

A

I. Supplementary Material Chapter 4

Supplementary Table 1 List of all sequence data taken from GenBank for the unpublished results. Species name, sampling locality, GenBank accession numbers (COI; 16S and 18S where applicable), chapter in which the sequences were incorporated, and reference are provided.

Species	Locality	COI	16S	18S	Chapter	Reference
PALAEONEMERTEA						
Callinera emiliae	Philippines	KU839771	-	-	4.1	Sundberg et al. 2016
	Philippines	KU839772	-	-	4.1	Sundberg et al. 2016
	Philippines	KU840154	-	-	4.1	Sundberg et al. 2016b
Callinera grandis	Sweden	EU489491	-	-	4.1	Sundberg et al. 2009a
	Sweden	HQ848626	-	-	4.1	Andrade et al. 2012
	Sweden	KU840132	-	-	4.1	Sundberg et al. 2016
	-	KU840141	-	-	4.1	Sundberg et al. 2016
	-	KU840142	-	-	4.1	Sundberg et al. 2016b
	Sweden	KU840288	-	-	4.1	Sundberg et al. 2016b
Callinera kasyanovi	Russia	KP270865	-	-	4.1	Kvist et al. 2015
Callinera sp.	Russia	KP270864	-	-	4.1	Kvist et al. 2015
Carinina ochracea	Sweden	EU489492	-	-	4.1	Sundberg et al. 2009
	Sweden	HQ848627	-	-	4.1	Andrade et al. 2012
	Spain	KM487741	-	-	4.1	Fernández-Álvarez et al. 2015
	Spain	KM487742	-	-	4.1	Fernández-Álvarez et al. 2015
	Sweden	KU840085	-	-	4.1	Sundberg et al. 2016
Carinina plecta	Japan	EU489493	-	-	4.1	Sundberg et al. 2009
	Japan	KU840453	-	-	4.1	Sundberg et al. 2016
Carinina sp.	Russia	KP270863	-	-	4.1	Kvist et al. 2015
Carinina sp.	USA, OR	KU197653	-	-	4.1	Hiebert & Maslakova unpublished
	USA, OR	KU197659	-	-	4.1	Hiebert & Maslakova unpublished
	USA, OR	KU197660	-	-	4.1	Hiebert & Maslakova unpublished
Carinina sp.	USA, OR	KU197654-KU197658	-	-	4.1	Hiebert & Maslakova unpublished
	USA, OR	MT843571	-	-	4.1	Chernyshev et al. 2021a
Carinoma hamanako	Japan	HQ848628	-	-	4.1	Andrade et al. 2012
	Japan	HQ848629	-	-	4.1	Andrade et al. 2012
	Japan	KF935500	-	-	4.1	Kvist et al. 2014
Carinoma mutabilis	USA, OR	KU197666-KU197669	-	-	4.1	Hiebert & Maslakova unpublished

A Appendix

Species	Locality	COI	16S	18S	Chapter	Reference
Carinoma mutabilis	USA, WA	AJ436942	-	-	4.1	Thollesson & Norenburg 2003
	USA, OR	KU197665	-	-	4.1	Hiebert & Maslakova unpublished
Carinoma sp.	USA, OR	KU197670	-	-	4.1	Hiebert & Maslakova unpublished
Carinoma sp.	USA, OR	KU197674-KU197676	-	-	4.1	Hiebert & Maslakova unpublished
Carinoma sp.	USA, OR	KU197661-KU197664	-	-	4.1	Hiebert & Maslakova unpublished
Carinoma sp.	USA, MD	MH235806	-	-	4.1	Aguilar et al. unpublished
	USA, MD	MH235811	-	-	4.1	Aguilar et al. unpublished
	USA, MD	MH235833	-	-	4.1	Aguilar et al. unpublished
	USA, MD	MH235836	-	-	4.1	Aguilar et al. unpublished
	USA, MD	MH235845	-	-	4.1	Aguilar et al. unpublished
	USA, MD	MH235856	-	-	4.1	Aguilar et al. unpublished
	USA, MD	MH235900	-	-	4.1	Aguilar et al. unpublished
	USA, MD	MH235939	-	-	4.1	Aguilar et al. unpublished
	USA, MD	MH235941	-	-	4.1	Aguilar et al. unpublished
Carinoma sp.	USA, OR	KU197671-KU197673	-	-	4.1	Hiebert & Maslakova unpublished
Carinoma tremaphoros	USA, FL	AJ436943	-	-	4.1	Thollesson & Norenburg 2003
Carinoma tremaphoros	USA, FL	HQ848630	-	-	4.1	Andrade et al. 2012
Tubulanus polymorphus		KP697783	-	-	4.1	Strand unpublished
Tubulanus ruber		KX853122	-	-	4.1	Krämer et al. unpublished
Tubulanus sp.		KU197703	-	-	4.1	Hiebert & Maslakova unpublished
HETERONEMERTEA						
Lineus acutifrons	Spain	GU590937	JF277573	JF304778	4.5	Puerta et al. 2010; Andrade et al. 2012
Lineus clandestinus	Sylt	KM878417-KM878431	-	-	4.4	Krämer et al. 2017
	Concarneau	KM878432	-	-	4.4	Krämer et al. 2017
	Sylt	KM878433-KM878437	-	-	4.4	Krämer et al. 2017
	Ile de Groîx	KM878438	-	-	4.4	Krämer et al. 2017
	Sylt	KM878439-KM878441	-	-	4.4	Krämer et al. 2017
	Ile de Groîx	KM878442	-	-	4.4	Krämer et al. 2017
	Sylt	KM878443	-	-	4.4	Krämer et al. 2017
	Sylt	KM878444	-	-	4.4	Krämer et al. 2017
	Ile de Groîx	KM878445	-	-	4.4	Krämer et al. 2017
	Sylt	KM878446-KM878449	-	-	4.4	Krämer et al. 2017
	Ile de Groîx	KM878450	-	-	4.4	Krämer et al. 2017

A Appendix

Species	Locality	COI	16S	18S	Chapter	Reference
Lineus clandestinus (continued)	Sylt	KM878451	-	-	4.4	Krämer et al. 2017
	Helgoland	KM878452	-	-	4.4	Krämer et al. 2017
	Sylt	KM878453	-	-	4.4	Krämer et al. 2017
	Wimereux	KM878454	-	-	4.4	Krämer et al. 2017
	Roscoff	KM878455	-	-	4.4	Krämer et al. 2017
	Sylt	KM878456	-	-	4.4	Krämer et al. 2017
	Roscoff	KM878457	-	-	4.4	Krämer et al. 2017
	Wimereux	MK047694	-	-	4.4	Cherneva et al. 2019
	Asturias	MK047695	-	-	4.4	Cherneva et al. 2019
	White Sea	MK078735-MK078738	-	-	4.4	Cherneva et al. 2019
	Bergen	MK078739	-	-	4.4	Cherneva et al. 2019
Lineus longissimus	Wales	AJ436935	-	-	4.2	Thollesson & Norenburg 2003
	Wales	DQ911372	-	-	4.2	Sundberg & Strand 2007
	Wales	DQ911374	-	-	4.2	Sundberg & Strand 2007
	Spain	DQ911376	-	-	4.2	Sundberg & Strand 2007
	Norway	GU392023	-	-	4.2	Strand & Sundberg 2011
	Norway	KP697738	-	-	4.2	Strand unpublished
	Norway	KP697739	-	-	4.2	Strand unpublished
	Norway	KU839761	-	-	4.2	Sundberg et al. 2016
	France	KX261784	-	-	4.2	Ament-Velásquez et al. 2016
	France	KX261785	-	-	4.2	Ament-Velásquez et al. 2016
	France	KX261786	-	-	4.2	Ament-Velásquez et al. 2016
	France	KX261787	-	-	4.2	Ament-Velásquez et al. 2016
	France	KX261788	-	-	4.2	Ament-Velásquez et al. 2016
	France	KX261789	-	-	4.2	Ament-Velásquez et al. 2016
	-	KY561813	-	-	4.2	Chernyshev et al. 2018
Lineus ruber	Wales	GU733828	-	-	4.4	Chen et al. 2010
	England	KC812595	-	-	4.4	Strand et al. 2014
	England	KC812602	-	-	4.4	Strand et al. 2014
	Ile de Groîx	KM878458-KM878461	-	-	4.4	Krämer et al. 2017
	Roscoff	KM878462	-	-	4.4	Krämer et al. 2017
	Concarneau	KM878463	-	-	4.4	Krämer et al. 2017
	Roscoff	KM878464-KM878486	-	-	4.4	Krämer et al. 2017
	Wimereux	KM878487-KM878492	-	-	4.4	Krämer et al. 2017
	Roscoff	KM878493	-	-	4.4	Krämer et al. 2017
	Roscoff	KM878494	-	-	4.4	Krämer et al. 2017
	Tromsø	KP697740-KP697747	-	-	4.4	Strand unpublished
	England	KR606056	-	-	4.4	Kang et al. 2015

A Appendix

Species	Locality	COI	16S	18S	Chapter	Reference
Lineus ruber (continued)	Wales	KU840090-KU840094	-	-	4.4	Sundberg et al. 2016
	England	KU840260	-	-	4.4	Sundberg et al. 2016
	England	KU840261	-	-	4.4	Sundberg et al. 2016b
	Roscoff	KX261741-KX261747	-	-	4.4	Ament-Velásquez et al. 2016
	Wimereux	KX261748	-	-	4.4	Ament-Velásquez et al. 2016
	Roscoff	KX261749-KX261753	-	-	4.4	Ament-Velásquez et al. 2016
	Trondheim	MK078657-MK078664	-	-	4.4	Cherneva et al. 2019
	Ålesund	MK078665-MK078668	-	-	4.4	Cherneva et al. 2019
	Trondheim	MK078669	-	-	4.4	Cherneva et al. 2019
	Ålesund	MK078670	-	-	4.4	Cherneva et al. 2019
	White Sea	MK078671-MK078675	-	-	4.4	Cherneva et al. 2019
	Barents Sea	MK078676-MK078681	-	-	4.4	Cherneva et al. 2019
	White Sea	MK078682-MK078713	-	-	4.4	Cherneva et al. 2019
Lineus viridis	USA	EF124970	-	-	4.4	Schwartz & Norenburg unpublished
	USA	EF124974	-	-	4.4	Schwartz & Norenburg unpublished
	UK	KC812596	-	-	4.4	Strand et al. 2014
	UK	KC812597	-	-	4.4	Strand et al. 2014
	Helgoland	KM878335	-	-	4.4	Krämer et al. 2017
	Helgoland	KM878336	-	-	4.4	Krämer et al. 2017
	Sylt	KM878337-KM878340	-	-	4.4	Krämer et al. 2017
	Helgoland	KM878341	-	-	4.4	Krämer et al. 2017
	Sylt	KM878342-KM878344	-	-	4.4	Krämer et al. 2017
	Helgoland	KM878345	-	-	4.4	Krämer et al. 2017
	Sylt	KM878346	-	-	4.4	Krämer et al. 2017
	Sylt	KM878347	-	-	4.4	Krämer et al. 2017
	Roscoff	KM878348	-	-	4.4	Krämer et al. 2017
	Sylt	KM878349	-	-	4.4	Krämer et al. 2017
	Roscoff	KM878350	-	-	4.4	Krämer et al. 2017
	Sylt	KM878351-KM878353	-	-	4.4	Krämer et al. 2017
	Roscoff	KM878354	-	-	4.4	Krämer et al. 2017
	Sylt	KM878355	-	-	4.4	Krämer et al. 2017
	Roscoff	KM878356	-	-	4.4	Krämer et al. 2017
	Roscoff	KM878357	-	-	4.4	Krämer et al. 2017
	Sylt	KM878358-KM878367	-	-	4.4	Krämer et al. 2017
	Helgoland	KM878368	-	-	4.4	Krämer et al. 2017

A Appendix

Species	Locality	COI	16S	18S	Chapter	Reference
Lineus viridis (continued)	Sylt	KM878369-KM878376	-	-	4.4	Krämer et al. 2017
	Helgoland	KM878377	-	-	4.4	Krämer et al. 2017
	Sylt	KM878378-KM878383	-	-	4.4	Krämer et al. 2017
	Helgoland	KM878384	-	-	4.4	Krämer et al. 2017
	Sylt	KM878385	-	-	4.4	Krämer et al. 2017
	Helgoland	KM878386	-	-	4.4	Krämer et al. 2017
	Roscoff	KM878387-KM878389	-	-	4.4	Krämer et al. 2017
	Concarneau	KM878390	-	-	4.4	Krämer et al. 2017
	Sylt	KM878391	-	-	4.4	Krämer et al. 2017
	Roscoff	KM878392	-	-	4.4	Krämer et al. 2017
	Concarneau	KM878393	-	-	4.4	Krämer et al. 2017
	Roscoff	KM878394	-	-	4.4	Krämer et al. 2017
	Roscoff	KM878395	-	-	4.4	Krämer et al. 2017
	Wimereux	KM878396-KM878408	-	-	4.4	Krämer et al. 2017
	Roscoff	KM878409	-	-	4.4	Krämer et al. 2017
	Roscoff	KM878410	-	-	4.4	Krämer et al. 2017
	Concarneau	KM878411	-	-	4.4	Krämer et al. 2017
	Roscoff	KM878412	-	-	4.4	Krämer et al. 2017
	Concarneau	KM878413	-	-	4.4	Krämer et al. 2017
	Wimereux	KM878414	-	-	4.4	Krämer et al. 2017
	Roscoff	KM878415	-	-	4.4	Krämer et al. 2017
	Roscoff	KM878416	-	-	4.4	Krämer et al. 2017
	UK	KU840097	-	-	4.4	Sundberg et al. 2016
	UK	KU840098	-	-	4.4	Sundberg et al. 2016
	UK	KU840242-KU840247	-	-	4.4	Sundberg et al. 2016b
	Roscoff	KX261754-KX261758	-	-	4.4	Ament-Velásquez et al. 2016
	USA	MK047696	-	-	4.4	Cherneva et al. 2019
	Ålesund	MK078714-MK078718	-	-	4.4	Cherneva et al. 2019
	White Sea	MK078719-MK078734	-	-	4.4	Cherneva et al. 2019
Micrura fasciolata	Sweden	GU392020-GU392022	-	-	4.6	Strand & Sundberg 2011
	Sweden	HQ848577	-	-	4.6	Andrade et al. 2012
	Sweden	HQ848578	-	-	4.6	Andrade et al. 2012
	Norway	KP697749	-	-	4.6	Strand unpublished
	Sweden	KU839812	-	-	4.6	Sundberg et al. 2016
	Sweden	KU839813	-	-	4.6	Sundberg et al. 2016
	Sweden	KU839816	-	-	4.6	Sundberg et al. 2016
	Sweden	KU839818	-	-	4.6	Sundberg et al. 2016

A Appendix

Species	Locality	COI	16S	18S	Chapter	Reference
Micrura fasciolata (continued)	Sweden	KU839867	-	-	4.6	Sundberg et al. 2016
	Sweden	KU839893	-	-	4.6	Sundberg et al. 2016
	Sweden	KU839899	-	-	4.6	Sundberg et al. 2016
	Sweden	KU839921	-	-	4.6	Sundberg et al. 2016
	Sweden	KU839951	-	-	4.6	Sundberg et al. 2016
	Sweden	KU839960	-	-	4.6	Sundberg et al. 2016
	Sweden	KU839973	-	-	4.6	Sundberg et al. 2016
	Sweden	KU839983	-	-	4.6	Sundberg et al. 2016
	Sweden	KU840150	-	-	4.6	Sundberg et al. 2016
Micrura purpurea	Sweden	GU392018	-	-	4.6	Strand & Sundberg 2011
	Sweden	GU392019	-	-	4.6	Strand & Sundberg 2011
	Sweden	HQ848586	-	-	4.6	Andrade et al. 2012
	Sweden	KU839803	-	-	4.6	Sundberg et al. 2016
	Sweden	KU839815	-	-	4.6	Sundberg et al. 2016
	Sweden	KU839819	-	-	4.6	Sundberg et al. 2016
	Sweden	KU839826	-	-	4.6	Sundberg et al. 2016
	Sweden	KU839868-KU839870	-	-	4.6	Sundberg et al. 2016
	Sweden	KU839890	-	-	4.6	Sundberg et al. 2016
	Sweden	KU839892	-	-	4.6	Sundberg et al. 2016
	Sweden	KU839894	-	-	4.6	Sundberg et al. 2016
	Sweden	KU839898	-	-	4.6	Sundberg et al. 2016
	Sweden	KU839907	-	-	4.6	Sundberg et al. 2016
	Sweden	KU839918-KU839920	-	-	4.6	Sundberg et al. 2016
	Sweden	KU839922	-	-	4.6	Sundberg et al. 2016
	Sweden	KU839935	-	-	4.6	Sundberg et al. 2016
	Sweden	KU839936	-	-	4.6	Sundberg et al. 2016
	Sweden	KU839943	-	-	4.6	Sundberg et al. 2016
	Sweden	KU839946	-	-	4.6	Sundberg et al. 2016
	Sweden	KU839949	-	-	4.6	Sundberg et al. 2016
	Sweden	KU839975	-	-	4.6	Sundberg et al. 2016
	Sweden	KU839980	-	-	4.6	Sundberg et al. 2016
	Sweden	KU839985	-	-	4.6	Sundberg et al. 2016
	Sweden	KU839990	-	-	4.6	Sundberg et al. 2016
	Sweden	KU840011-KU840013	-	-	4.6	Sundberg et al. 2016
	Sweden	KU840015	-	-	4.6	Sundberg et al. 2016
	Sweden	KU840017-KU840019	-	-	4.6	Sundberg et al. 2016
Riseriellus occultus	Wales	DQ911378	-	-	4.3	Sundberg & Strand 2007
	Wales	DQ911380	-	-	4.3	Sundberg & Strand 2007
	Wales	DQ911382	-	-	4.3	Sundberg & Strand 2007

A Appendix

Species	Locality	COI	16S	18S	Chapter	Reference
Riseriellus occultus (continued)	Wales	DQ911384	-	-	4.3	Sundberg & Strand 2007
	Wales	DQ911386	-	-	4.3	Sundberg & Strand 2007
	Wales	DQ911389	-	-	4.3	Sundberg & Strand 2007
	Wales	DQ911391	-	-	4.3	Sundberg & Strand 2007
	Spain	DQ911393	-	-	4.3	Sundberg & Strand 2007
	Spain	DQ911395	-	-	4.3	Sundberg & Strand 2007
	Spain	DQ911397	-	-	4.3	Sundberg & Strand 2007
	Wales	HQ848581	-	-	4.3	Andrade et al. 2012
	Wales	HQ848582	-	-	4.3	Andrade et al. 2012
	France	KM878496	-	-	4.3	Krämer et al. 2017
	England	KU839738	-	-	4.3	Sundberg et al. 2016
	England	KU839739	-	-	4.3	Sundberg et al. 2016
	England	KU839742	-	-	4.3	Sundberg et al. 2016
	England	KU839745	-	-	4.3	Sundberg et al. 2016
	England	KU839747	-	-	4.3	Sundberg et al. 2016
	England	KU839750	-	-	4.3	Sundberg et al. 2016
	England	KU840241	-	-	4.3	Sundberg et al. 2016
	France	KX261790	-	-	4.3	Ament-Velásquez et al. 2016
	Netherlands	MK160498	-	-	4.3	Faasse et al. 2018