Interpreting the polychromatic variation of the European nudibranch *Polycera quadrilineata* (O. F. Müller, 1776), with the description of a new species

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Front cover photo: *Polycera quadrilineata sensu lato* mating. Photo taken in Kristiansund, Norway by Nils Aukan (2011).

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Abstract

The polychromatic variability exhibited by the dorid nudibranch species *Polycera quadrilineata* (O. F. Müller, 1776) (Family Polyceridae) has long fascinated marine scientists. The species was originally described from the Oslofjord (Drøbak) in Norway and is distributed between Lofoten (Northern Norway) throughout the Atlantic to the Iberian Peninsula, the Mediterranean Sea, and the archipelagos of the Azores, Madeira, and Canary Islands, where it often occurs in kelp forests in the vicinity of its bryozoan-prey. The increasing detection of cryptic species in nudibranch gastropods, and other marine invertebrates, has raised the question whether the chromatic variability within *P. quadrilineata* could hide cryptic lineages or still be consistent with the hypothesis of a single entity. In order to test this hypothesis, samples from across the geographical range of *P. quadrilineata* together with representatives from worldwide species, with a focus on the Atlantic diversity, were gathered and studied using an integrative taxonomic approach. Morpho-anatomical characters were investigated by light -and scanning electron microscopy, and novel sequences (66) of the universal barcoding mitochondrial gene cytochrome *c* oxidase subunit I (COI) were generated and gathered from DNA databases (47). Bayesian molecular phylogenetic analysis using MrBayes, the Automatic Barcode Gap Discovery species delimitation method, and a haplotype network analysis using the PopArt software were used to aid delimit species and infer relationships.

The results revealed the existence of a second polychromatic cryptic species within *P. quadrilineata*, here named *Polycera* n. sp., so far only known from Norway where it is sympatric with *P. quadrilineata*. The genetic distance between the two species was estimated to be 9.6–12.4% (COI uncorrected *p*-distance). Chromatically *Polycera* n. sp. differs by exhibiting a black dotted or patchy dotted pattern occasionally with orange/brown blotches, but never black stripes like *P. quadrilineata*. However, the two species share a common colour pattern defined by a whitish base and yellow/orange pigmentation. Anatomically, *Polycera* n. sp. differs by having a weaker labial cuticle, a smaller radula with fewer rows, and only four marginal teeth (instead of the five present in *P. quadrilineata*), and a reproductive system with a shorter penis armed with needle-like and hook-shaped penile spines (whereas *P. quadrilineata* only has needle-like spines).

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1. INTRODUCTION

1.1 What are nudibranch gastropods?

Nudibranchs are marine molluscs belonging to the Class Gastropoda and the Subclass Heterobranchia in the clade Nudipleura and Order Nudibranchia (Thompson, 1976; Wägele & Willan, 2000; Penney et al., 2018; MolluscaBase, 2019b). Despite nudibranchs being primarily macro-faunal and epibenthic (Todd, 1983; Megina et al., 2007), there are groups that have managed to successfully invade the interstitial benthic meiofauna (Todd, 1983; Megina et al., 2007) (*e.g.* Pseudovermidae Thiele, 1931; Swedmark, 1964; Flammensbeck et al., 2019) as well as the pelagic oceanic habitats (Todd, 1983) (*e.g.* Glaucidae Gray, 1827*;* Churchill et al., 2014). Compared to all other gastropods they have perhaps the greatest morphological and ecological disparity with over 4700 species known globally from all the world's oceans and major sea areas (Thompson, 1976; Dean & Prinsep, 2017). In order to move and interact with their environment, nudibranchs use their rhinophores (head tentacles) as chemosensory organs to compensate for their lack of sight (Dean & Prinsep, 2017). Being both specialists or generalists they constitute important predatory consumers within the benthic communities (Carbone et al., 2019) in which the majority are carnivores whose diet mostly consists of sessile invertebrates (Miller, 1961; Todd, 1981; 1983; Megina & Cervera, 2003; Megina et al., 2007; Dean & Prinsep, 2017). In order to feed they use their radula, an internal rasping tongue bearing a ribbon-like membrane covered with robust and tiny, backward-pointing teeth, whose arrangement may additionally be used as a genetic -and species diagnostic trait (Hickman et al., 2014).

1.2 Trophic ecology

Based on prey preferences, nudibranchs can be separated into different ecological categories; porifera-grazers (= sponge feeders), hydroid-grazers (= cnidarian feeders), polyzoan-grazers (= bryozoan feeders), and the miscellaneous feeders (= diverse feeders/generalists) (Miller, 1961; Todd, 1981; 1983; Dean & Prinsep, 2017; Carbone et al., 2019). Additionally, diets may also consist of fish eggs, echinoderms, mollusc eggs, small crustaceans, and other nudibranchs (= cannibalistic hunters) (Todd, 1981; Megina & Cervera, 2003; Carbone et al., 2019).

1.3 Ontogeny

Most sea slugs start their life by hatching as shell-bearing planktonic veliger larvae that undergo metamorphosis, settle in average two to three weeks, before becoming hermaphroditic oviparous (laying eggs produced after internal fertilization) adults (Todd, 1981; 1983; Hayward & Ryland, 1995; Wägele & Willan, 2000). While the majority copulate reciprocally by direct sperm transfer, some species (*e.g. Tenellia fuscata* or *Polycera quadrilineata*; Jörger et al., 2009) transfer sperm through a spermatophore (Pola & González Duarte, 2008; Jörger et al., 2009). As adults, the slugs undergo different types of life cycles. Where the annual life cycle (one-year generation time) is the most common to occur, some undergo biennial (live two years, but only spawn once), or sub-annual (= ephemeral) life cycles (short lived, lasting for about a week or a month) (Todd, 1983).

1.4 Evolving alternative defence mechanisms

The evolutionary reduction and loss of physical protection provided by the external shell, which is only present during the larval stage, is regarded as one of the main reasons behind the diversity and ecological success of these organisms (Thompson, 1976; Todd, 1983; Wägele & Willan, 2000). To compensate for their loss, nudibranchs have evolved alternative defensive mechanisms (Todd, 1983; Carbone et al., 2019) that can be behavioural, morphological and/or chemical, which in turn can be divided into primary –and secondary lines of defence (Todd, 1981).

Primary defence often uses toxins and/or visual communication by colour signalling to deter both competitors and/or predators. As visual communication many species utilize different camouflage strategies such as homochromy, (2) countershading and (3) cryptic or disruptive colouration (Todd, 1981), whereas others utilize aposematic colouration, *i.e.* warning colouration (Todd, 1981; Tullrot & Sundberg, 1991; Tullrot, 1994; Layton et al., 2018). Chemical defence is often attained by incorporating toxins from their diet. However, some also produce their own molecular defences by *de novo* biosynthesis (Carbone et al., 2019). Chemical studies (Dean & Prinsep, 2017; Carbone et al., 2019) conducted on the latter mechanism are still rather scarce but has been shown in a limited number of Polyceridae species that all members of the Polycerinae and Triophinae Subfamilies exclusively have the ability to produce their own bioactive metabolites (= diacylguanidin), namely ‘triophamine’ or ‘limacianine’ (Dean & Prinsep, 2017; Carbone et al., 2019).

In regard to secondary defence, some species (*e.g*. in the *Onchidoris* genus; Penney et al., 2018) possess endoskeletal spicules or other skeletal structures which they use in order to protect their soft, vulnerable bodies against physical damage and predatory attacks. The size, structure, shape, and overall existence of these internal spicules can differ both within and between species (Thompson, 1976; Todd, 1981; Alba-Tercedor & Sánchez-Tocino, 2011; Penney et al., 2018).

1.5 The major clades of the Nudibranchia

The Order Nudibranchia are based on morphological, anatomical, and molecular studies divided into the monophyletic groups Cladobranchia and Doridina (= Anthobranchia) (Wagele & Willan, 2000; Pola & Gosliner, 2010; Dean & Prinsep, 2017; Goodheart, 2017; Carbone et al., 2019). While Cladobranchia comprises the aeolids (*e.g. Flabellina*), arminids (*e.g. Armina*), and dendronatids (*e.g. Doto*) as main taxa (Wagele & Willan, 2000; Pola & Gosliner, 2010; Goodheart, 2017), Doridina (dorid nudibranchs) embraces the majority of all other nudibranchs (Wagele & Willan, 2000; Carbone et al., 2019) such as the current study Family Polyceridae Alder & Hancock, 1845.

Cladobranchia are characterized by having a branched, or sub-divided, digestive gland where most have lost their gills (one exception are the arminids) (Dean & Prinsep, 2017; Goodheart, 2017; Carbone et al., 2019), instead possessing other gas exchanging features like cerata (*e.g.* the aolids; Dean & Prinsep, 2017). Additional characteristics are the possession of cnidocysts (nematocyst sequestration, only found in the aeolids) (Goodheart, 2017). Doridina, on the other hand, are characterized by having a compact digestive gland where most groups are distinguished by a feather-like plume of gills located dorsally (one exception are the Family Phyllidiidae Rafinesque, 1814) like a crown surrounding the anus (Wagele & Willan, 2000; Dean & Prinsep, 2017; Carbone et al., 2019).

1.6 Polymorphic colouration and the cryptic species problem

Chromatic variability, or polymorphic colouration, is a common feature found in many marine invertebrate species and groups, including nudibranchs. Colouration is a trait that occasionally may be used as a good indicator for separating species (Harley et al., 2006; Layton et al., 2018).

Nevertheless, there are situations when congeneric species (species belonging to the same genus) exhibit nearly identical colouration patterns or other morphological traits (Layton et al., 2018) making morphological appearances difficult to use in species recognition. Traditionally, this was attributed to intra-specific variability, restricting the recognition of cryptic and/or pseudo-cryptic species. Alone, morphology can therefore in some cases be insufficient and can lead to other important traits being overlooked (Layton et al., 2018). During recent years, the concept of cryptic species has become a popular and widely used term in modern biodiversity studies, implying that morphologically similar species may only be recognised through molecular analysis (Korshunova et al., 2019). In fact, during recent years, thanks to the advances in molecular phylogenetics, there have been numerous findings of cryptic and pseudo-cryptic species within nudibranchs – *e.g. Aeolidia* Cuvier, 1798 (Carmona et al., 2013), *Glaucus* Forster, 1777 (Churchill et al., 2014), *Anteaeolidiella* M. C. Miller, 2001 (Carmona et al., 2014a), *Spurilla* Bergh, 1864 (Carmona et al., 2014b), *Cratena* Bergh, 1864 (Padula et al., 2014), *Pteraeolidia* Bergh, 1875 (Wilson & Burghardt, 2015), *Felimida* Ev. Marcus, 1971 (Padula et al., 2016), *Chromodoris* Alder & Hancock, 1855 (Layton et al., 2018), *Hypselodoris* Stimpson, 1855 (Epstein et al., 2018), and *Trinchesia* Ihering, 1879 (Korshunova et al., 2019).

The term 'cryptic species' was previously used to refer to situations in which two or more genetically distinct species have been erroneously classified under a single species name due to their morphological resemblance (Bickford et al., 2007; Herron & Freeman, 2014; Layton et al., 2018). However, the term 'cryptic species' have become somewhat controversial (Bickford et al., 2007) and its definition a topic of discussion (Korshunova et al., 2019). It has been asked whether or not cryptic species truly exist as a natural phenomenon, or if they are just temporary taxonomical problems (Heethoff, 2018; Horsáková et al., 2019; Korshunova et al., 2019). According to Bickford et al. (2007), cryptic species may result from erroneous taxonomical conclusions or overlooked morphological characters (Horsáková et al., 2019). The concept of cryptic species becomes even further complicated by the fact that the term is given multiple usages (Korshunova et al., 2019). For example, in behavioural ecological the term 'cryptic species' refers to species being camouflaged and secretive (Todd, 1981; 1983; Claridge, et al., 2005; Bickford et al., 2007). Korshunova et al. (2019), claimed that the term 'cryptic' has been greatly overused, and it does not really help that multiple authors give their own definition to the term in addition to creating other sub-terms like

'pseudo-cryptic' (or falsely cryptic; when species are no longer cryptic due to some obviously morphological differences; Horsáková et al., 2019; Korshunova et al., 2019), 'semi-cryptic' (species that cannot be morphologically distinguished but have distinct geographical distributions or ecology; Vondrák, et al., 2009; Korshunova et al., 2019), 'true cryptic' (or fully cryptic; when no morphological differences have yet been found regardless the distribution and ecology of species; Horsáková et al., 2019; Korshunova et al., 2019), 'quasi-cryptic' (when morphological differences can be recognized), etc. (Korshunova et al., 2019). As a consequence of this confusing situation around the term, it seems that a full consensus on how to actually define 'cryptic' has yet to be reached (Struck et al., 2018; Heethoff, 2018; Horsáková et al., 2019; Korshunova et al., 2019).

The highly polychromatic appearance of the European nudibranch species *Polycera quadrilineata* makes it a potencially interesting case study to address cryptic speciation. It was never thoroughly investigated whether the various colour morphs occuring within this species constitute intra-specific variation or could represent putative cryptic lineages. Moreover, whether or not its polychromatic variation is geographically related or just caused independently and randomly remains unknown. Tullrot & Sundberg (1991) believed the species chromatic variation to be aposematic, functioning as an anti-predator strategy rather than being used for intra-specific communication. Interestingly, preliminary molecular results obtained recently during the course of a research project on sea slugs of Southern Norway conducted at the Department of Natural History, University Museum of Bergen (Malaquias, personal communication) have pointed to the possible occurrence of cryptic species under the name *P. quadrilineata.*

1.7 Study group: the nudibranch species *Polycera quadrilineata* (O. F. Müller, 1776)

1.7.1 Geography

The genus *Polycera* Cuvier, 1816, is distributed globally across the Indo-Pacific and on both sides of the Atlantic with species occurring in shallow waters from boreal seas to the tropics, often associated with encrusting bryozoans (Thompson & Brown, 1984; Thompson, 1988; Martynov et al., 2006; Pola et al., 2014). The target species of the current study – *Polycera quadrilineata* – is widely distributed around Western Europe (Hunnam & Brown, 1975; Hayward & Ryland, 1995;

Rudman, 1999; Martynov et al., 2006; Furfaro & Mariottini, 2016; Telnes, 2018), from Norway, Greenland, Sweden, Denmark, Iceland, Faeroes, all around the British Isles (Thompson & Brown, 1984; Thompson, 1988; Rudman, 1999; Palomar et al., 2014; Moen & Svensen, 2014; Hayward & Ryland, 2017; Artsdatabanken, 2019), the Atlantic coast of France, Spain and Portugal, the Mediterranean Sea (as far as Naples), and the archipelagos of the Canary Islands, Madeira, and the Azores (Bergan & Anthon, 1977; Thompson & Brown, 1984; Thompson, 1988; Cervera et al., 2004; Trainito, 2005; Martynov et al., 2006; Martínez-Pita et al., 2006; Micaroni et al., 2018).
In Norway, the species occur between Lofoten in the North, all the way southwards until reaching the Swedish border (Evertsen & Bakken, 2005; Palomar et al., 2014; Artsdatabanken, 2019).

1.7.2 Taxonomy

The species *P. quadrilineata* belongs to the Gastropoda Subclass Heterobranchia within the Order Nudibranchia, Suborder Doridina and Family Polyceridae. The genus *Polycera*, includes 32 valid species worldwide (Pola et al., 2014; MolluscaBase, 2019c) of which six occur in European waters, namely *P. aurantiomarginata* Garcia-Gómez & Bobo, 1984, *P. elegans* Bergh, 1894, *P. faeroensis* Lemche, 1929, *P. quadrilineata* O. F. Müller, 1776, *P. maculata* Pruvot-Fol, 1951, and *P. hedgpethi* Er. Marcus, 1964, in which the latter is an invasive species native from California and present around the Iberian Peninsula and Mediterranean Sea (Caballer & Ortea, 2002; Cervera et al., 2004; Keppel et al., 2012; Giacobbe & De Mattreo, 2013). *P. quadrilineata* was first described by O. F. Müller (1776) under the species name *Doris quadrilineata* as being "*oblonga, alba, lineic quatuor nigris, auriculis sulphureis*" meaning "*elongated, white, four black lines, yellowish auricles (= probably referring to the yellow patches scattered along the body)*". However, in his 1776'work O. F. Müller did not provide any information about the geography or habitat of this species. It was three years later in an upgraded version of his "Zoologiae Danicae" that he first included the comprehensive description of the species with details about its type locality (Drøbak, Oslofjord, Norway) and habitat (fucoid algae) (O. F. Müller, 1779). Later in 1788 he published an illustration of the species (O. F. Müller, 1788, pl. 17, figs 4–6; see Fig. 1).

Figure 1 – Original illustration of *Polycera* (*Doris*) *quadrilineata* from O. F. Müller (1788).

1.7.3 Morphological features

P. quadrilineata is a highly polychromatic species (Fig. 2) with a translucent creamy-whitish base colour that can be partly or almost entirely covered with continuous or dashed black stripes, or occasionally black or greyish blotches (Thompson & Brown, 1984; Thompson, 1988; Hayward & Ryland, 1995; Moen & Svensen, 2014). Its whitish body surface is covered with dorsal, wart-like tubercles that are either yellow or orange pigmented, often oriented into five or more continuous and longitudinal lines (Eales, 1967; Thompson & Brown, 1984; Thompson, 1988; Moen & Svensen, 2014). Like other *Polycera* species the body is elongated and limaciform (*i.e.* slug-like) with a smooth surface. It can reach up to 30–45 mm in length (Thompson & Brown, 1984; Thompson, 1988; Rudman, 1999; Edwards, 2008; Moen & Svensen, 2014; Telnes, 2018).

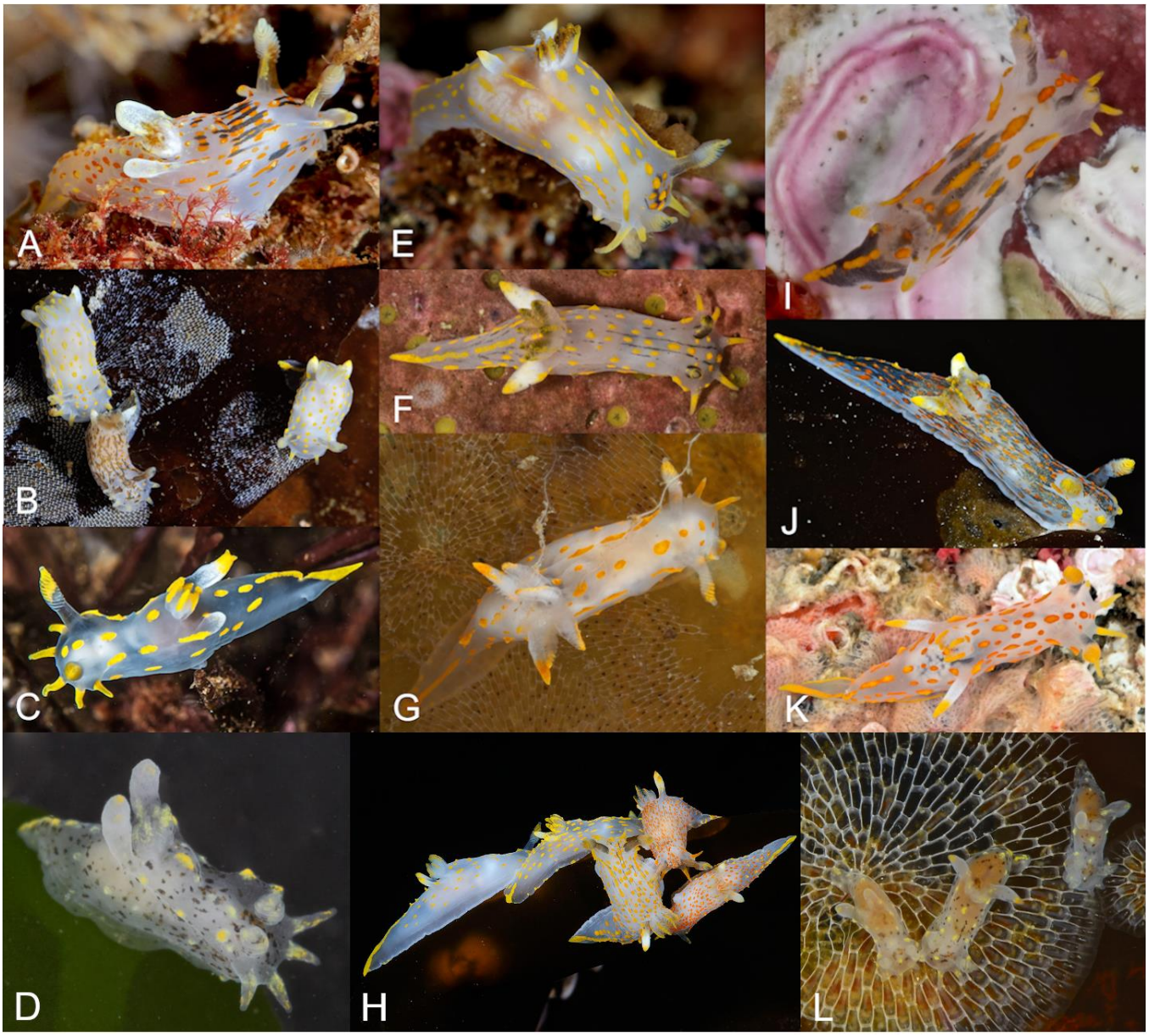


Figure 2 – Polychromatic variation in *P. quadrilineata sensu lato*. **A.** Kristiansund, Møre and Romsdal, Norway, ZMBN 125636, photo by N. Aukan, 2018. **B.** Kristiansund, Møre and Romsdal, Norway, ZMBN 125613, photo by N. Aukan, 2018. **C.** Stavanger, Rogaland, Norway, ZMBN 125688, photo by E. Svensen, 2018. **D.** Hordaland, Norway, ZMBN 106113, photo by K. Kongshavn and M. A. E. Malaquias, 2015. **E.** Flatøy, Hordaland, Norway, photo by N. Aukan, 2018. **F.** Bergen, Hordaland, ZMBN 94139, photo by M. A. E. Malaquias, 2013**. G**. Espegrend, Hordaland, Norway, photo by M. S. Berggren, 2018. **H.** Krifast, Møre and Romsdal, Norway, photo by N. Aukan, 2014. **I.** North side of Baia da Poca, Gracioisa I. Azores, ZMBN 97198, photo by M. A. E. Malaquias, 2014. **J.** Kristiansund, Møre and Romsdal, Norway, ZMBN 125658, photo by N. Aukan, 2018. **K.** Aquàrio dos Mosteiros, Azores, ZMBN 87942, photo by M. A. E. Malaquias, 2011. **L.** Haugesund, Rogaland, Norway, ZMBN 125881, photo by C. Rauch and A. Schouw, 2018.

Among the *Polycera* species present in Norway, *P. quadrilineata* only somewhat resembles *P. faeroensis* (Fig. 3; Moen & Svensen, 2014). Both species are however easily distinguished since the white base colour of *P. faeroensis* completely lacks pigmentation along the dorsum and mid-dorsal row, in addition to having a larger number of veil processes, usually up to 12, as opposed to *P. quadrilineata* who only has four to six (Lemche & Thompson, 1974; Thompson & Brown, 1984; Moen & Svensen, 2014). The veil processes are smooth, lobed and tapering, and can be either yellow, orange or black pigmented, projecting anteriorly out from the frontal veil (Thompson, 1988; Hayward & Ryland, 1995; Moen & Svensen, 2014). The head is equipped with two rhinophores that have a thick stem and a slightly backward leaning cylindrical knob of lamellae on top (Schmekel et al., 1982; Hayward & Ryland, 1995). Posteriorly, near the mid-dorsal section of the body are seven to nine, in a few cases 11, feather-like pinnate gills (Eales, 1967; Schmekel et al., 1982; Todd, 1983; Hayward & Ryland, 1995) surrounding the anal papilla, or anus, in a crown-like fashion (Schmekel et al., 1982; Moen & Svensen, 2014). Like most other dorids *P. quadrilineata* possesses a skeletal network of numerous big, sharpened and calcareous spicules with several axes embedded within their body wall (Thompson & Brown, 1984; Alba-Tercedor & Sánchez-Tocino, 2011; Penney et al., 2018). Description of body features illustrated in Figure 4.

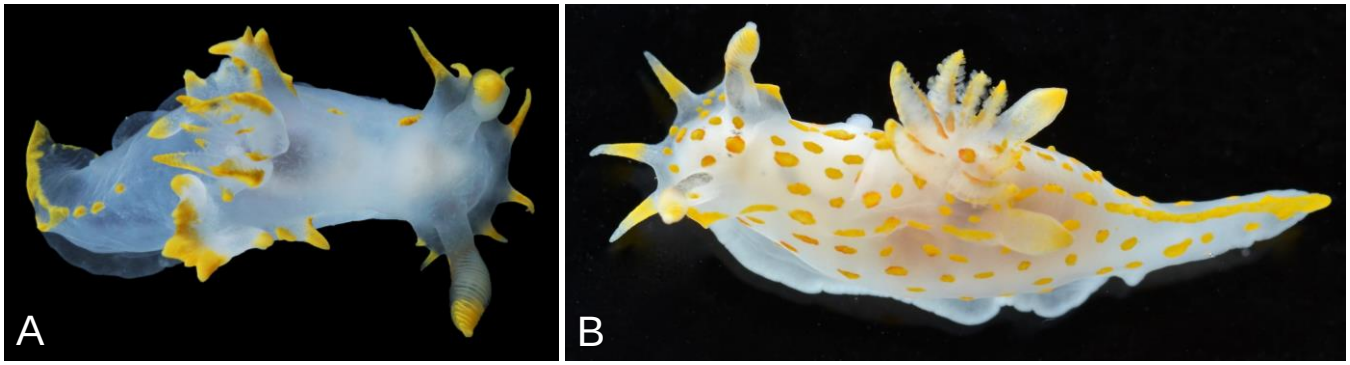


Figure 3 – Morphological comparison between the two Norwegian *Polycera* species (**A**) *P. faeroensis* and (**B**) *P. quadrilineata*. **A.** Trøndelag, Norway, ZMBN 126014**,** photo by V. V. Grøtan, 2018. **B.** Bergen, Hordaland, Norway, ZMBN 127683, photo by C. Rauch, 2019.

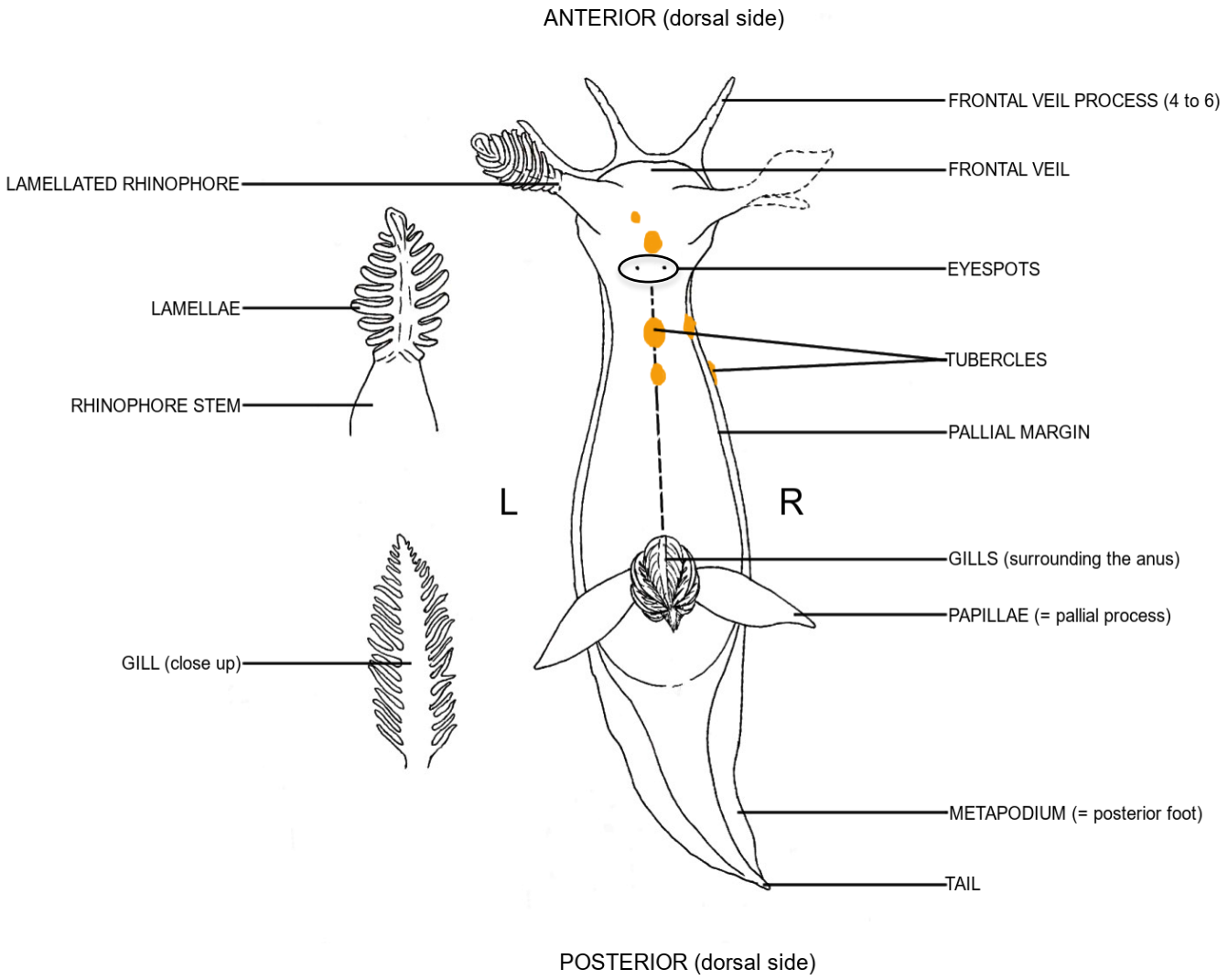


Figure 4 – External morphological illustration of body features found in *P. quadrilineata sensu lato*. L = left. R = right.

1.7.4 Ecology and Biology

P. quadrilineata is an intertidal and sublittoral species (Bergan & Anthon, 1977; Hayward & Ryland, 1995; 2017) commonly found between the low shore and 30 m depth. However, specimens have been detected down to 60–300 m (Bergan & Anthon, 1977; Thompson & Brown, 1984; Thompson, 1988; Hayward & Ryland, 1995; Evertsen & Bakken, 2005; Edwards, 2008; OBIS, 2014; Hayward & Ryland, 2017). It lives in cold to temperate waters (Betti et al., 2017) ranging from 5–25°C (OBIS, 2014) where it can tolerate salinities between 15–35 PSU (Practical Salinity Unit), although it prefers salinities at 30–35 PSU (Mortensen & Svensen, 2010; OBIS, 2014). It is a specialized

carnivore grazing on encrusted bryozoans (Todd, 1983; Megina et al., 2007; Carbone et al., 2019), particularly on the species *Electra pilosa* and *Membranipora membranacea,* both commonly found along the entire Norwegian coast (Todd, 1981; Thompson, 1988; Mortensen & Svensen, 2010). The species is frequently found in large aggregations of dozens, sometimes hundreds, of specimens whose quantity and general distribution often depends on the food abundance and water temperature (Miller, 1961; Todd, 1983; Evertsen & Bakken, 2005; Edwards, 2008). Although it can be found on both soft and hard bottom substrates, it is most commonly found epiphytically on brown algae such as *Laminaria* kelp or *Fucus*, in addition to some red algae like *Chondrus crispus* (Miller, 1961; Bruce et al., 1963; Todd, 1981; Evertsen & Bakken, 2005; Martynov et al., 2006; Mortensen & Svensen, 2010), in which their bryozoan-prey inhabits (Todd, 1981).

Like most nudibranch species (Todd, 1981) *P. quadrilineata* is believed to undergo an annual life cycle, living about one year. In Norway the species appear to be present along the entire year (Evertsen & Bakken, 2005; Fig. 2 in Appendix 5), with highest abundance during late winter (January and February) and spring (March to May) (Evertsen & Bakken, 2005; Telnes, 2018), while in warmer areas, for example around the Mediterranean Sea, from South-Western Spain (Martínez-Pita et al., 2006) to the North Aegean Sea in the East (Antoniadou et al., 2005), the species is commonly found during spring and summer (April to August). Nevertheless, due to colder water temperatures the species may in these latter areas also be highly abundant during the winter (Betti et al., 2017; Martínez-Pita & García, 2017). Miller (1961) showed that in the British Isles and South-Western Irish Sea, *P. quadrilineata* starts spawning and multiply during late spring and early summer (Miller, 1961; Bruce et al., 1963; Thompson & Brown, 1984) when their prey is most abundant, and starts diminishing around autumn and winter when its food gets scarce due to algae depletion. This is because kelp first starts producing new lamina where the bryozoans grow upon during late winter and early spring (same happens in Norway), creating food for the slugs. It is also common for many species with planktonic larvae to synchronize their spawning period with the algal blooms to secure their offspring's survival since their larvae feeds on phytoplankton (Miller, 1961; Mortensen & Svensen, 2010).

P. quadrilineata is a hermaphroditic species practicing reciprocal copulation with cross-fertilization (Todd, 1981; 1983; Wägele & Willan, 2000; Pola & González Duarte, 2008; Jörger et al., 2009; Moen & Svensen, 2014). Copulation happens through the gonopore opening located on the anterior half

right dorsal side of the slugs (Fig. 5) where each copulating partner exchange spermatophores filled with sperm (Pola & González Duarte, 2008; Jörger et al., 2009).

Figure 5 – Reproductive behaviour of *P. quadrilineata sensu lato*. Photos illustrating the reciprocal copulation by cross-fertilization between individuals, with each gonopore connection being circled in red. Photos taken in Kristiansund, Norway by N. Aukan, 2011.

The species lays white crescent-shaped, gelatinous egg-masses (Fig. 6) which they attach to the algal substratum (Todd, 1981; Martínez-Pita et al., 2006; Moen & Svensen, 2014). The eggs are spherical (Martínez-Pita & García, 2017), small and have been documented to range between 0.06–0.08 mm in diameter (Schmekel et al., 1982; Martínez-Pita et al., 2006; Martínez-Pita & García, 2017), which according to Martínez-Pita et al. (2006) is an egg size typically found in species with planktotrophic larval development.

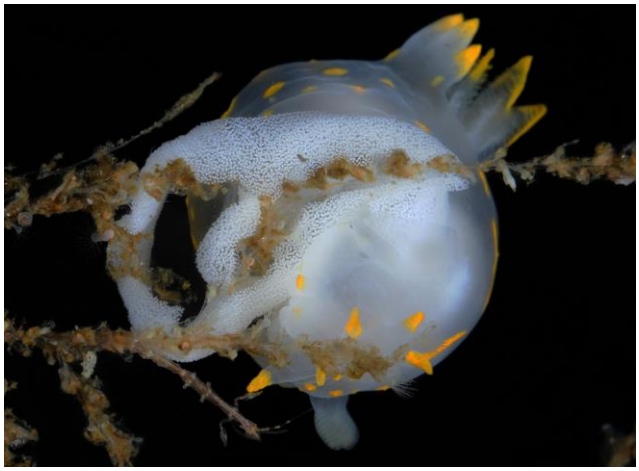


Figure 6 – An individual of *P. quadrilineata sensu lato* laying eggs (Kristiansund, Norway; photo by N. Aukan, 2011).

2. OBJECTIVES

Preliminary results conducted by researchers at the University Museum of Bergen have suggested the possible occurrence of cryptic lineages in the polychromatic nudibranch species *P. quadrilineata*. In this study a combination of morpho-anatomical and DNA characters, using anatomical dissections, scanning electron microscopy, molecular phylogenetics, population genetic analysis, and molecular species delimitation methods are employed to investigate:

(1) The taxonomic status of the species *P. quadrilineata*, *i.e.* whether this species that up to now has been hypothesized to be one single biological lineage with extensive chromatic variability is in fact a single taxon or alternatively comprises a complex of multiple species.
(2) Establish a relation between colour morphs and putative cryptic lineages, and attempt to describe their morpho-anatomical differences.
(3) Define the habitat and geographical distribution of the putative cryptic lineages.

3. MATERIAL AND METHODS

3.1 Taxon sampling

Specimens were gathered from different areas around the Norwegian coast – Haugesund, Stavanger, Trondheim, Egersund, Bergen, Drøbak and Kristiansund, in addition to other European locations in order to cover the geographical distribution of the species in the best possible way (Table 1). Most of the specimens were obtained from the scientific collections of the Department of Natural History, University Museum of Bergen (ZMBN), which were originally collected through SCUBA diving by collaborators of the museum. Additional specimens were collected during fieldwork conducted along the Western fjords outside and around the Espegrend Marine Biological Station (University of Bergen) on board the research vessel 'Hans Brattström' owned by the University of Bergen, using triangular, epibenthic and kelp dredges.

In the latter case, when back at the marine station, living specimens were separated, photographed alive with a digital SLR camera equipped with macro-lens, measured with a ruler (mm), and frozen inside plastic jars overnight in seawater to ensure that the body was kept fully extended for later

possible anatomical studies. Afterwards, the jars were defrosted and the animals fixed and preserved in absolute ethanol (> 96 %). Information regarding the sampling location with geographical coordinates, depth, habitat, and name of collector were databased, and each lot was attributed its own ZMBN voucher number.

3.2 Tissue sampling for DNA extraction

Tissue samples for DNA extraction were gathered from 69 specimens of *P. quadrilineata sensu lato* by cutting a small part of their foot or mantle using forceps or a scalpel inside a Petri dish. Each tissue sample was preserved in a 1.5 ml Eppendorf tube filled with absolute ethanol and given its own sampling number (P1, P2, P3, etc.). In rare cases, when specimens were too small to cut off enough tissue the whole specimen was used. To prevent contamination between the tissue sampling of each specimen, all pieces of equipment were "cleansed" with absolute ethanol.

Table 1 – Material examined and used for molecular analysis, including their sampling locality, habitat, voucher number and GenBank or BOLD accession numbers. The *Polycera* species list summarise which of the individuals turned out to be a new species after DNA sequencing. Specimens (S) from the same lot were coded sequentially with the acronym S1, S2, S3, etc., in the column "Sample no".

Species	Sample no.	Locality	Voucher no.	GenBank/BOLD Ac. No. (COI)
Polycera n. sp.	P2	Norway: Herdla, Askøy, Bergen, Hordaland	ZMBN 125917	*
Polycera n. sp.	P17	Norway: Uthaug, Ørland, Trøndelag	ZMBN 126023	*
Polycera n. sp.	P26	Norway: Steingardsvika Espegrend, Bergen, Hordaland	ZMBN 106115	NBMM034-15
Polycera n. sp.	P34	Norway: Tingelsædet, Egersund, Rogaland	ZMBN 127486	*
Polycera n. sp.	P35	Norway: Uthaug, Ørland, Trøndelag	ZMBN 126025	*
Polycera n. sp.	P37 (S1)	Norway: Tingelsædet, Egersund, Rogaland	ZMBN 127492	*
Polycera n. sp.	P40	Norway: Uthaug, Ørland, Trøndelag	ZMBN 126024	*
Polycera n. sp.	P45 (S1)	Norway: Legern, Haugesund, Rogaland	ZMBN 125855	*
Polycera n. sp.	P46 (S2)	Norway: Legern, Haugesund, Rogaland	ZMBN 125855	*
Polycera n. sp.	P47 (S1)	Norway: Sandhl, Haugesund, Rogaland	ZMBN 125881	*
Polycera n. sp.	P48 (S2)	Norway: Sandhl, Haugesund, Rogaland	ZMBN 125881	*
Polycera n. sp.	P49 (S3)	Norway: Sandhl, Haugesund, Rogaland	ZMBN 125881	*
Polycera n. sp.	P50 (S4)	Norway: Sandhl, Haugesund, Rogaland	ZMBN 125881	*
Polycera n. sp.	P51 (S5)	Norway: Sandhl, Haugesund, Rogaland	ZMBN 125881	*
Polycera n. sp.	P70	Norway: Steingardsvika, Espegrend, Bergen, Hordaland	ZMBN 106113	NBMM032-15
Polycera n. sp.	P54	Norway: Skeisvika, Hundvåg, Stavanger, Rogaland	ZMBN 127607	*
Polycera n. sp.	P55	Norway: Skeisvika, Hundvåg, Stavanger, Rogaland	ZMBN 127608	*
Polycera n. sp.	P68	Norway: Seløysundet, Espegrend, Bergen, Hordaland	ZMBN 127664	*
Polycera quadrilineata	P1	Norway: Flatholmen, Haugesund, Rogaland	ZMBN 125859	*
Polycera quadrilineata	P3	Norway: Brattøya, Kristiansund, Møre and Romsdal	ZMBN 125613	*
Polycera quadrilineata	P4 (S1)	Norway: Hafrsfjord, Sola, Stavanger, Rogaland	ZMBN 125688	*
Polycera quadrilineata	P5	Norway: Breidvika, Drotningsvik, Bergen, Hordaland	ZMBN 125971	*
Polycera quadrilineata	P6	Norway: Seløysundet, Espegrend, Bergen, Hordaland	ZMBN 125032	*
Polycera quadrilineata	P7	Norway: Brattøya, Kristiansund, Møre and Romsdal	ZMBN 125603	*
Polycera quadrilineata	P8	Norway: Sletta, Haugesund, Rogaland	ZMBN 125906	*

Polycera quadrilineata	P9	Norway: Tingelsædet, Egersund, Rogaland	ZMBN 127491	*
Polycera quadrilineata	P10	Norway: Tingelsædet, Egersund, Rogaland	ZMBN 127476	*
Polycera quadrilineata	P11	Norway: Litle Svetlingen, Egersund, Rogaland	ZMBN 127512	*
Polycera quadrilineata	P12	Norway: Litle Svetlingen, Egersund, Rogaland	ZMBN 127511	*
Polycera quadrilineata	P13	Norway: Litle Svetlingen, Egersund, Rogaland	ZMBN 127510	*
Polycera quadrilineata	P14	Norway: Tingelsædet, Egersund Rogaland	ZMBN 127487	*
Polycera quadrilineata	P15	Norway: Drågsvågen, Førde, Sveio, Hordaland	ZMBN 125988	*
Polycera quadrilineata	P16	Norway: Tingelsædet, Egersund, Rogaland	ZMBN 127488	*
Polycera quadrilineata	P18	Norway: Litle Svetlingen, Egersund, Rogaland	ZMBN 127513	*
Polycera quadrilineata	P19 (S1)	Norway: Brattøya, Kristiansund, Møre and Romsdal	ZMBN 125635	*
Polycera quadrilineata	P20 (S1)	Norway: Brattøya, Kristiansund, Møre and Romsdal	ZMBN 125658	*
Polycera quadrilineata	P21 (S2)	Norway: Brattøya, Kristiansund, Møre and Romsdal	ZMBN 125658	*
Polycera quadrilineata	P23 (S2)	Norway: Hafrsfjord, Sola, Stavanger, Rogaland	ZMBN 125688	*
Polycera quadrilineata	P24	Norway: Seløysundet, Espegrend, Bergen, Hordaland	ZMBN 125033	*
Polycera quadrilineata	P22 (S3)	Norway: Brattøya, Kristiansund, Møre and Romsdal	ZMBN 125658	*
Polycera quadrilineata	P27 (S2)	Norway: Brattøya, Kristiansund, Møre and Romsdal	ZMBN 125635	*
Polycera quadrilineata	P28	Azores: Mosteiros, Banco Sabrina, São Miguel Island	ZMBN 87937	*
Polycera quadrilineata	P29	Azores: Baja da Fajã Moinhos, Aquàrio dos Mosteiros	ZMBN 87942	*
Polycera quadrilineata	P30	Azores: Ilhèu dos Mosterios, São Miguel Island	ZMBN 87925	*
Polycera quadrilineata	P32	Azores: North of Baia da Poca, Graciosa I.	ZMBN 97198	*
Polycera quadrilineata	P33	Norway: Tingelsædet, Egersund, Rogaland	ZMBN 127481	*
Polycera quadrilineata	P36	Norway: Uthaug, Ørland, Trøndelag	ZMBN 126017	*
Polycera quadrilineata	P38 (S2)	Norway: Tingelsædet, Egersund, Rogaland	ZMBN 127492	*
Polycera quadrilineata	P39	Norway: Litle Svetlingen, Egersund, Rogaland	ZMBN 127509	*
Polycera quadrilineata	P42	Norway: Drøbak, Akershus, Oslo	ZMBN 125578	*
Polycera quadrilineata	P43	Norway: Egersund havn, Rogaland	ZMBN 125689	*
Polycera quadrilineata	P44	Norway: Nordsundet, Kristiansund, Møre and Romsdal	ZMBN 125636	*
Polycera quadrilineata	P71	Norway: Steingardsvika, Espegrend, Bergen, Hordaland	ZMBN 106114	NBMM033-15
Polycera quadrilineata	P53	Norway: Drøbak, Akershus, Oslo	ZMBN 127587	*
Polycera quadrilineata	P52	Norway: Drøbak, Akershus, Oslo	ZMBN 127600	*
Polycera quadrilineata	P56	Norway: Engøy, Stavanger, Rogaland	ZMBN 127626	*
Polycera quadrilineata	P57	Norway: Engøy, Stavanger, Rogaland	ZMBN 127631	*

Polycera quadrilineata	P58	Norway: Engøy, Stavanger, Rogaland	ZMBN 127633	*
Polycera quadrilineata	P60	Norway: Turøy, Skitholmen, Bergen, Hordaland	ZMBN 127685	*
Polycera quadrilineata	P61	Norway: Turøy, Myrbærholmen, Bergen, Hordaland	ZMBN 127689	*
Polycera quadrilineata	P62	Norway: Turøy, Myrbærholmen, Bergen, Hordaland	ZMBN 127690	*
Polycera quadrilineata	P63	Norway: Turøy, Skitholmen, Bergen, Hordaland	ZMBN 127682	*
Polycera quadrilineata	P64	Norway: Turøy, Skitholmen, Bergen, Hordaland	ZMBN 127678	*
Polycera quadrilineata	P65	Norway: Turøy, Skitholmen, Bergen, Hordaland	ZMBN 127683	*
Polycera quadrilineata	P66	Norway: Turøy, Skitholmen, Bergen, Hordaland	ZMBN 127681	*
Polycera quadrilineata	P67	Norway: Turøy, Skitholmen, Bergen, Hordaland	ZMBN 127676	*
Polycera quadrilineata	P72	Norway: Espegrend, Bergen, Hordaland	ZMBN 94139	NBMM062-15
Polycera quadrilineata	P73	Mediterranean Spain: Mataró, Catalonia	*	*
Polycera quadrilineata	P74	Mediterranean Spain: Roses, Catalonia	*	*
Polycera quadrilineata	P75	United Kingdom: Oban, Scotland	*	EF142907
Polycera quadrilineata	P76	Sweden: Tjärnö	MNCN:15.05/55455	JX274079
Polycera quadrilineata	P77	Sweden: Tjärnö	MNCN:15.05/55460	JX274078
Polycera quadrilineata	P78	Sweden: Tjärnö	MNCN:15.05/55459	JX274077
Polycera quadrilineata	P79	Sweden: Tjärnö	MNCN:15.05/55457	JX274076
Polycera quadrilineata	P80	Sweden: Tjärnö	MNCN:15.05/55464	JX274075
Polycera quadrilineata	P81	Sweden: Tjärnö	MNCN:15.05/55463	JX274074
Polycera quadrilineata	P82	Sweden: Tjärnö	MNCN:15.05/55466	JX274073
Polycera quadrilineata	P83	Sweden: Tjärnö	MNCN:15.05/55456	JX274072
Polycera quadrilineata	P84	Sweden: Tjärnö	MNCN:15.05/55465	JX274071
Polycera quadrilineata	P85	Sweden: Tjärnö	MNCN:15.05/55462	JX274070
Polycera quadrilineata	P86	Sweden: Kristineberg, Bohuslän	*	AJ223275
Polycera capensis	HM162687	South Africa: Hout Bay, Western Cape Province	CASIZ176907	HM162687
Polycera capensis	JX274092	South Africa: False Bay, Western Cape Province	CAS:IZ:176375	JX274092
Polycera capensis	JX274091	South Africa: Oudekraal, Cape Province	CAS:IZ:176280	JX274091
Polycera capensis	JX274083	Australia: Nelson Bay, New South Wales	MNCN:15.05/55470	JX274083
Polycera sp.1	JX274093	USA: Maui, Maalaea Bay, Hawaii,	CAS:IZ:176795	JX274093
Polycera sp.2	JX274090	Pacific Ocean: Kwajalein, Atoll, Marshall Islands	CAS:IZ:120773	JX274090
Polycera faeroensis	JX274089	Portugal: Estacada, Aveiro	MNCN:15.05/55503.2	JX274089
Polycera faeroensis	JX274088	Portugal: Estacada, Aveiro	MNCN:15.05/55503.1	JX274088

Polycera tricolor	JX274087	USA: San Francisco Bay, Marina, California	CAS:IZ:176438a	JX274087
Polycera hedgpethi	JX274086	Morocco: Aghroud	MNCN:15.05/55493	JX274086
Polycera atra	JX274085	USA: San Francisco Bay, Marina, California,	CAS:IZ:170506b	JX274085
Polycera atra	JX274084	USA: San Francisco Bay, Marina, California	CAS:IZ:170506a	JX274084
Polycera sp.A	JX274082	South Africa: Tsitsikamma, Eastern Cape Province,	CAS:IZ:176387	JX274082
Polycera sp.A	JX274081	South Africa: Gordon's Bay, Western Cape Province,	CAS:IZ:176169	JX274081
Polycera aurantiomarginata	JX274069	Morocco: Aghroud	MNCN:15.05/55490	JX274069
Polycera aurantiomarginata	JX274068	Morocco: Aghroud	MNCN:15.05/55492	JX274068
Polycera aurantiomarginata	AJ223274	Spain: Cadiz, Andalusia	*	AJ223274
Palio dubia	KF644300	Canada: Quebec, Baie Ste-Marguerite	CCDB-15498-E04	KF644300
Palio dubia	KF643719	Canada: Quebec, Baie Ste-Marguerite	CCDB-15498-E07	KF643719
Palio dubia	KF643686	Canada: Quebec, Baie Ste-Marguerite	CCDB-15498-E06	KF643686
Palio dubia	AJ223272	Sweden: Kristineberg, Bohuslän	*	AJ223272
Palio dubia	JX274100	Sweden: Gullmaren, Bohuslän	MNCN:15.05/55467	JX274100
Thecacera pennigera	JX274094	South Africa: Oudekraal, Cape Province, Atlantic Coast	CAS:IZ:176285	JX274094
Thecacera pennigera	AJ223277	Spain: Cadiz, Andalusia	*	AJ223277
Thecacera picta	KP871652	USA: California	CAS:IZ:182281	KP871652
Polycerella emertoni	JX274099	Spain: Cadiz, Santi Petri, Pantalan	MNCN:15.05/55482	JX274099
Polycerella emertoni	JX274098	Spain: Cadiz, Santi Petri, Pantalan	MNCN:15.05/55482	JX274098
Polycerella emertoni	JX274097	Spain: Cadiz, Santi Petri, Pantalan	MNCN:15.05/55479.2	JX274097
Polycerella emertoni	JX274096	Spain: Cadiz, Santi Petri, Pantalan	MNCN:15.05/55479.1	JX274096
Polycerella emertoni	AJ223273	Spain: Cadiz, Andalusia	*	AJ223273
Polycerella emertoni	JX274095	Spain: Cadiz, Santi Petri	MNCN:15.05/55480	JX274095
Species outgroup				
Jorunna tomentosa	MG935216	Sweden: Kattegatt	Gastr 8965V	MG935216

3.3 DNA extraction, amplification and purification

DNA was extracted from tissue samples using the 'Qiagen DNeasy Blood and Tissue Kit' (QIAGEN, catalogue no. 69506), following the protocol for 'Purification of Total DNA from Animal Tissues (Spin-Column)' (see Appendix 1.0). Amplification of the barcoding mitochondrial gene cytochrome *c* oxidase subunit I (COI) was performed through Polymerase Chain Reaction (PCR) using the universal primers by Folmer et al. (1994; Table 2), following the standard protocol (Eilertsen & Malaquias, 2013; Austin et al., 2018).

Table 2 – Folmer et al. (1994) universal primers sequences for the COI mitochondrial gene.

Name	Sequence 5'– 3'	Source
COI		
LCO1490 (F)	GGTCAACAAATCATAAAGATATTGG	Folmer et al.,1994
HCO2198 (R)	TAAACTTCAGGGTGACCAAAAATCA	Folmer et al.,1994

Amplifications used a 50 µl volume with 17.5 µl Sigma water (ddH_2O), 5 µl buffer, 5 µl dNTP, 10 µl Q-solution, 7 µl MgCl, 2 µl of each primer (10 µM), 0.5 µl TAQ, and 1 µl DNA. Some amplifications were carried out with only 25 µl volume using the same cocktail mix, but replacing the standard buffer with CoraLLoad (CL) buffer from Qiagen, using only half of each quantity (see Appendix 1.1 and 1.2). PCR reactions were conducted in a BIO-RAD C1000 thermal cycler with an initial denaturation at 95°C for 3 min., followed by 40 cycles of 45 sec. at 94°C (denaturation), 45 sec. at 45°C (annealing), 2 min. at 72°C (extension), and a final extension step at 72°C for 10 min. before cooling down. In order to rule out contamination, a negative and positive control were added to each PCR run. The negative control consisted of distilled water (ddH_2O), whereas the positive control used DNA extract from a previously successfully tested sea slug species, namely *Aplysia punctata.*

Following the amplification, quantity and quality of the PCR products were assessed by gel-electrophoresis (see Appendix 1.3) by adding 4 µl PCR product with 1 µl Ficoll x5 loading buffer, run through a 1.0 % agarose gel based on half-strength TAE buffer solution, together with the staining agent GelRed. For the PCR products already containing a loading buffer (*i.e.* the CL buffer), 5 µl PCR product was added directly into the gel. In both cases, 5 µl FastRuller ladder was added to the agarose gel before running the electrophoresis at 80 V for 30 min.. When finished, the gel was visualized using

the software GeneSnap (v.7.01) with the UV-radiation machine Syngene (Cambridge, UK), and the amount of PCR product (= DNA) that were to be used in the upcoming PCR sequencing reactions were calculated using the software GeneTools (v.4.00; also from Syngene: Cambridge, UK). For the few samples that did not work in the first round, trouble-shooting was carried out by generating new PCR master cocktails with a larger amount of DNA (*i.e.* 4 µl rather than the standard 1 µl). Hence, proportionally less quantity of ddH_2O was added in order to adjust the total volume of the final PCR product to 25 µl.

Successful PCR products were purified using EXOSAP, a combination of the enzymes Exonuclease I (EXO I) and Shrimp Alkaline Phosphatase (SAP), by following the standard purification protocol (see Appendix 1.4). Each EXOSAP master cocktail was prepared in a 1.5 ml Eppendorf tube kept on ice, with each purification samples containing 8 µl PCR product and 2 µl EXOSAP (0.1 µl EXO, 1.0 µl SAP, and 0.9 µl ddH_2O). Final products were incubated for 30 min. at 37°C, followed 15 min. at 85°C (inactivation step), and 4°C for cooling/HOLD in the thermal cycler.

3.4 Preparation of the sequencing reactions

For the sequencing reactions, 1 µl of each purified PCR product was mixed with 6 µl of ddH_2O, 1 µl primer, 1 µl BigDye (BD), and 1 µl sequence buffer. This process was repeated independently for each of the two primers (forward and reverse), and the micro-tubes were labelled accordingly for traceability (see Appendix 1.5). The reactions were conducted in the thermal cycler for 5 min. at 96°C (initial denaturation), followed by 25 cycles of 10 sec. at 96°C (denaturation), 5 sec. at 50°C (annealing), and 4 min. at 60°C, before cooling down at 6°C. Following the thermal cycling, 10 µl ddH_2O were added to each sequencing reaction before being delivered to the sequencing laboratory facility at the Department of Biological Sciences, University of Bergen. Here, Automatic Sanger DNA-sequencing was performed using the capillary-based Applied Biosystem 3730XL Analyzer (University of Bergen).

3.5 Phylogenetic and species delimitation analysis

The software Geneious (v. 11.0.3) was used to inspect, assemble, edit, and cut the chromatograms of the forward and reverse DNA strands. Sequences of each sample were quality checked by careful examination of the chromatograms and trimmed at both ends to remove parts of low quality. To check for contamination the sequences were blasted through the BLAST toll included in the GenBank database, followed by a translation check in Geneious using the invertebrate mitochondrial genetic code to make sure no stop-codons occurred in the sequences. Novel COI sequences (66 seq.) and additional GenBank and BOLD sequences (47 seq.) (Table 1) of *P. quadrilineata sensu lato* and other Polyceridae taxa, representing a total of 17 species, together with the outgroup species *Jorunna tomentosa,* were aligned using the MUSCLE software (Edgar, 2004) implemented in Geneious to check for nucleotide homology. Following the alignment, sequences were trimmed at both ends to a position where at least 50 % of all sequences had nucleotide data. A total of 642 base pairs (bp) of COI remained for use in the phylogenetic analysis.

The MEGA-X software (Kumar et al., 2018) was used to estimate uncorrected pairwise (*p*) distances. From this data, the intra-specific and inter-specific minimum and maximum *p*-genetic distances of all species belonging to the *Polycera* genus were calculated (Table 3) by exporting the matrix into Microsoft Excel. The jModelTest2 software (v. 2.1.10; Darriba et al., 2012) was used to find the best-fit evolutionary model under the Akaike information criterion (AIC; Sakamoto et al., 1986), where the selected model was GTR + I + G. The Bayesian analysis was performed using MrBayes (Huelsenbeck & Ronquist, 2001; Fig. 1 in Appendix 2), with three parallel runs of five million generations each, sampling every 1000 generations, with a burn-in set to 25 %. MrBayes was run through the portal CIPRES (Miller et al., 2010) at https://www.phylo.org/portal2/login!input.action, and the consensus phylogram was converted into a graphical tree in FigTree (v.1.4.3; Rambaut & Drummond, 2016; Fig. 1 in Appendix 3). Species delimitation analysis was conducted through the Automatic Barcode Gap Discovery (ABGD; Puillandre et al., 2012) program performed via the ABGD interphase website at http://wwwabi.snv.jussieu.fr/public/abgd/abgdweb.html. Here, the complete and final COI alignment in fasta format was run through the three evolutionary models available; Simple distance, Kimura (K80) TS/TV [2.0], and Jukes-Cantor (JC69). Each analysis was run separately using standard settings.

3.6 Examination of morpho-anatomical characters

Morphological and anatomical work was done in collaboration with Professor Marta Pola from the University Autonoma of Madrid in Spain, where dissections and scanning electron microscopy (SEM) were carried out according to the standard protocol described below in theme 3.6.1.

3.6.1 Dissection and scanning electron microscopy (SEM)

Anatomical studies were conducted on four *Polycera* specimens, two representatives of each of the two recognized lineages, by the molecular phylogenetic and ABGD analyses. Dissections were done under a stereo microscope Nikon SMZ-1500 equipped with a *camera lucida*. The animals were opened by dorsal incision, and the reproductive system and buccal mass, with the radula and labial cuticle, were removed. The buccal mass was dissolved in a 10 % sodium hydroxide solution until the labial cuticle and radula had been cleansed from their surrounding tissue. These structures were then cleansed with water, and examined and photographed under a light microscope using the Life Science Imaging software *cellSense* (v.1.18). The reproductive systems were drawn using the *camera lucida,* and each penis were isolated and opened so that they could be examined and photographed first using light microscopy followed by scanning electron microscopy (SEM). The labial cuticles and penises were critical point dried using hexamethyldisilazane. All parts (radula, penises and labial cuticles) were mounted on metallic stubs for SEM, and sputter coated with gold-palladium. Observations were done with a Hitachi S-3000N SEM-machine.

3.7 Haplotype network analysis

Haplotype network analysis based on 80 COI sequences were conducted separately for the two lineages recognized within the *P. quadrilineata* complex, here named *Polycera* n. sp. (17 seq.) and *P. quadrilineata* (63 seq.), using the software PopArt (Population Analysis with Reticulate Trees; v. 1.7; Leigh & Bryant, 2015). Prior to PopArt, the COI sequence alignment files had to be renamed and trimmed using the software Notepad++ (v. npp.7.6.6) to remove all unknown nucleotides (N) from both ends, generating a final alignment with 594 bp in length. Sequence P48 was excluded due to its large amount of N and therefore reduced size (541 bp). Notepad++ was additionally used to create separate species alignment files (Fig. 1 in Appendix 7.1) and corresponding trait files (txt.) (Fig. 2 in

Appendix 7.1) containing geographical area codes. As a final step prior to PopArt, each alignment file had to be converted into phylip format (phy) using the software Mesquite (v.3.51; Maddison & Maddison, 2018). After importing the alignment -and trait files into PopArt each file was run through a standard TCS Network analysis (Clement et al., 2002) in order to visualize the genetic relationships and distances between the individual genotypes (see Appendix 7.2). The single specimen obtained from Sveio (Norway) was for the sake of geographical proximity merged with the Haugesund (Norway) specimens represented in the haplotype network of *P. quadrilineata* (Fig. 18). The TCS haplotype networks were later edited for more satisfying visualization using both PopArt (v. 1.7), Adobe Illustrator, CS6 (v.16.0.4) and Gravit Designer (v.2019-2.1) at https://gravit.io/.

4. RESULTS

4.1 Molecular phylogenetic analysis

From the 69 samples that were COI sequenced, 64 samples were successfully used in the present study. The remaining five sequences were excluded either due to contamination or poor-quality chromatograms, and two sequences from Catalonia, Spain were added. The molecular phylogenetic analysis run on the total 113 sequences, containing the novel sequences (66 seq.) and GenBank/BOLD sequences (47 seq.), was consistent with a total of 18 species, including *Jorunna tomentosa* (outgroup), supporting the hypothesis of two valid species within *P. quadrilineata*, namely the "true" *P. quadrilineata* and a new undescribed lineage, here referred to as *Polycera* n. sp. (Table 1; Fig. 7; Appendix 3). The *Polycera* genus was not rendered monophyletic due to lack of support (PP = 0.55) and the inclusion of the genera *Polycerella* and *Thecacera*. Nevertheless, a clade with maximum support (PP = 1) with six *Polycera* species was retrieved (Fig. 7; Fig. 1 in Appendix 3). This included the species *P. aurantiomarginata* from Spain and Morocco, which was sister to *P. capensis* from South Africa and Australia (PP = 1). A possible sister relationship between the European *P. faeroensis* and the undetermined *s*pecies *Polycera* sp. A from South Africa (PP = 0.86) were together rendered sister species to the new species *Polycera* n. sp. from Norway (PP = 0.95). The former three species were rendered sister (PP = 0.98) to the common NE-Atlantic and Mediterranean species *P. quadrilineata.*

Additionally, specimens of *Palio dubia* from Canada and Sweden branched off in different parts of the phylogenetic tree, possibly suggesting two separate species (Fig. 7; Fig. 1 in Appendix 3).

Uncorrected pairwise (*p*) genetic distances (Table 3) showed a 9.6–12.4 % difference between *P. quadrilineata* and the new species, and ranged intra-specifically between 0–2.4 % within *P. quadrilineata,* and 0.2–2.3 % within *Polycera* n. sp.. Regarding the inter-specific genetic distance between all included *Polycera* species the estimated maximum uncorrected *p*-distance was between *P. faeroensis* from Portugal, and *P. atra* from California, USA (18.6–19.7 %), whereas the minimum uncorrected *p*-distance was between *P. capensis* from South Africa and Australia, and *P. aurantiomarginata* from Spain and Morocco (4.3–5.8 %). Inter-specific uncorrected *p*-distance between all studied *Polycera* species ranged between 4.3–19.7 %, whereas the intra-specific ranged between 0–2.6 %

4.2 Species delimitation analysis

By using the standard default settings (Pmin = 0.001; Pmax = 0.1) the ABGD analysis retrieved one 'recursive partition' and eight 'initial partitions' with all three models of evolution rendering the same 18 lineages (Figs 1–6 in Appendix 6). The ABGD analysis was fully compatible with the COI Bayesian phylogenetic analysis (Fig. 7) supporting the existence of the same number of species. Only when the prior intra-specific divergence value (P) was above 0.012915 were a lower number of lineages retrieved by the analysis which grouped several of the recognized species together; 17 groups by Kimura, and four groups by Simple distance and Juke-Cantor (Figs 1, 3, 5 in Appendix 6).

Table 3 – Intra –and inter-specific uncorrected pairwise (*p*) distances estimated using MEGA-X (Kumar et al. 2018) between the *Polycera* species. Abbreviation n/a = not applicable.

	Between groups (Inter-specific)											**Within groups (Intra-specific)**	
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**		
1 *P. atra*												*P. atra*	0.8
2 *Polycera* n. sp.	16.7–18.4											*Polycera* n. sp.	0.2–2.3
3 *Polycera* sp. A	17.4–17.8	9.8–11.1										*Polycera* sp. A	0.9
4 *P. faeroensis*	18.6–19.7	9.4–11.8	8.8–9.8									*P. faeroensis*	1.9
5 *P. quadrilineata*	16.5–17.4	9.6–12.4	9.9–11.4	11.8–13.5								*P. quadrilineata*	0.0–2.4
6 *P. aurantiomarginata*	17.4–17.6	10.7–12.6	11.3–12.2	10.9–11.3	9.8–11.8							*P. aurantiomarginata*	0.0–0.8
7 *P. capensis*	16.1–16.5	9.6–12.4	9.8–10.1	9.9–10.5	9.6–11.4	4.3–5.8						*P. capensis*	0.0–2.6
8 *Polycera* sp. 1	18.9–19.3	14.1–15.4	16.3–16.7	15.6–15.8	14.4–15.6	14.4–14.8	13.3–14.3					*Polycera* sp. 1	n/a
9 *Polycera* sp. 2	16.3–16.5	16.5–17.8	17.4–17.6	18.8–18.9	14.6–15.9	17.3–17.8	15.6–15.8	16.1				*Polycera* sp. 2	n/a
10 *P. tricolor*	16.5	17.3–18.8	16.5–16.9	18.6–18.9	15.4–16.9	16.3–16.9	14.4–15.2	16.5	15.9			*P. tricolor*	n/a
11 *P. hedgpenthi*	16.1	13.7–15.0	15.9–16.5	16.5–16.7	16.7–17.8	15.4–15.8	13.7–14.4	16.3	14.3	12.9		*P. hedgpenthi*	n/a

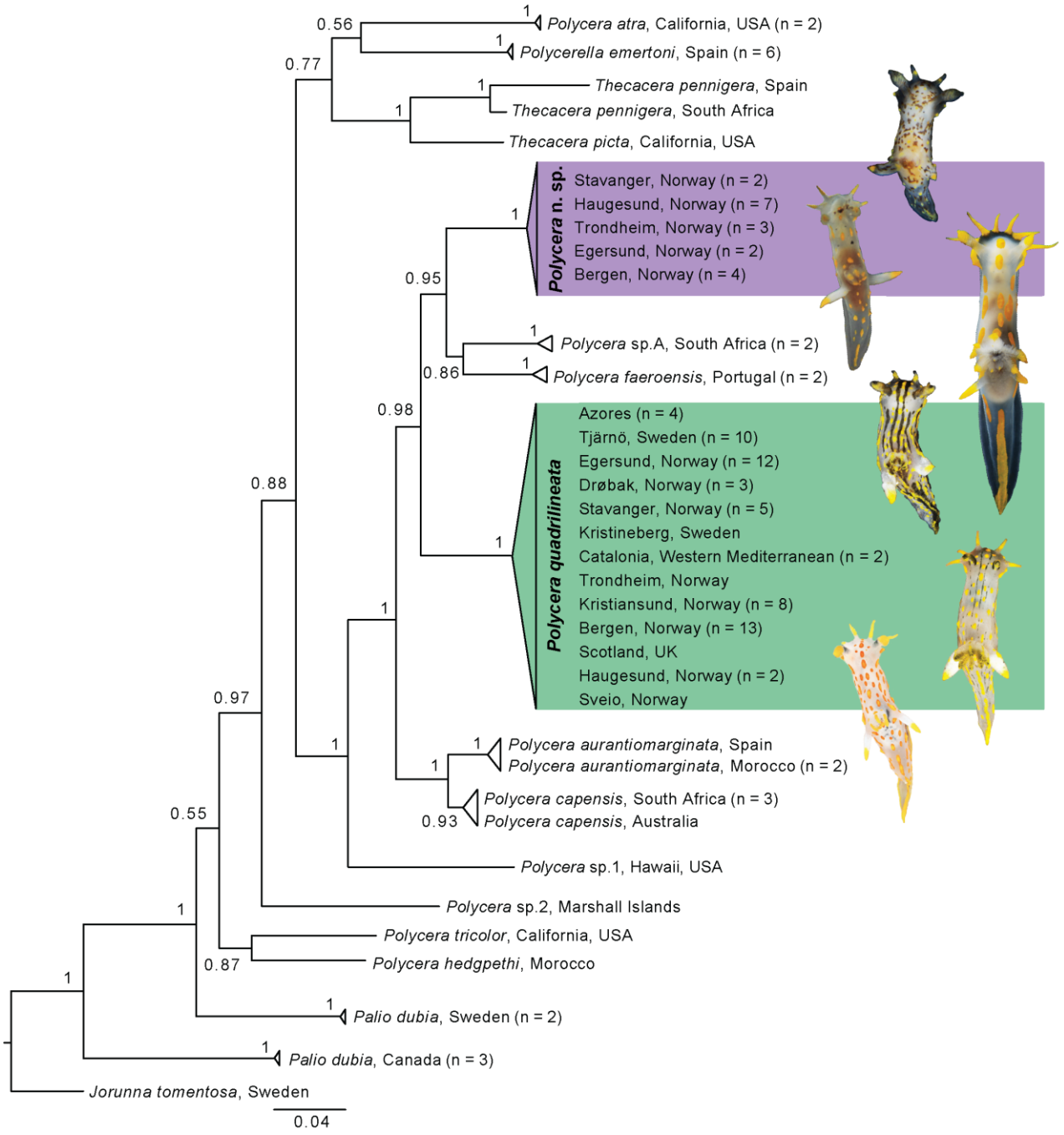


Figure 7 – Bayesian molecular phylogenetic analysis tree based on the COI gene. Numbers on branches represent posterior probabilities (PPs). Tree rooted with *J. tomentosa* (outgroup). Images representing the different morphotypes found within each study species. Green box containing *P. quadrilineata* with its four main morphotypes. Purple box containing *Polycera* n. sp. with its three main morphotypes. Yellow/orange specimen overlapping each species colour-box representing the shared morphotype.

4.3 Systematic descriptions

Abbreviations: UM = University Museum of Bergen, Department of Natural History; UA = University Autonoma of Madrid in Spain; B = BOLD; H = height/length of specimen; spc. = specimen; S.no. = sample code used for each specimen (S) taken from the same lot (*e.g.* S1 = specimen 1).

4.3.1 Family Polyceridea Alder & Hancock, 1845.

Genus *Polycera* Cuvier, 1817.

Diagnosis

Body narrow, elongated, limaciform, highest at middle length; slightly constricted laterally between head and mid-region; notum smooth, partially or entirely papillate or tuberculate. When present, papillae and tubercles show colourful pigmentation. Anterior margin of head expanded with frontal veil bearing numerous, long or short digitate or tuberculate velar processes. Rhinophores perfoliate, non-retractile, with up to 26 lamellae, lacking sheaths. Gills simple, non-retractile into pocket, with up to 11 pinnate to tripinnate gills surrounding the anus in a semi-circle. Oral tentacles short and lobate. Small or large extra-branchial processes absent or present on either side of gill plume. Strong papillae arising from the mantle rim, projecting out from either side of gill plume. Paired jaws conspicuous, sometimes with large wing-like process. Radula up to 20 rows; formula n.2.0.2.n; rachidian tooth (= central tooth) vestigial when present; lateral teeth hamate; second (= outer) laterals larger than first (= inner) laterals; marginal teeth (n) small, simple plates that may vary in number. Prostate gland large; penis acrembolic, armed with spines; spermatheca and spermatocyst semi-serial (Thompson, 1988; Miller, 1996; Hermosillo & Valdés, 2007; Pola et al., 2014).

4.3.2 *Polycera quadrilieata* (O. F. Müller, 1776).

(Fig. 1; Figs 8–10, 14, 15A1; Table 4).

Synonyms

Accessible at WoRMS – 'World Register of Marine Species' (MolluscaBase, 2019a).

Type locality

Drøbak, Akershus, Oslofjord, Norway.

Diagnosis

Body surface smooth, partially tuberculate. Tubercles rounded or pointed, often strongly coloured orange or yellow; background pigmentation translucent white. Some individuals partly or entirely covered with longitudinal black or dark grey continuous or dashed stripes. Frontal veil with four to six long or short veil processes, smooth, tapering distally; somewhat retractile. Rhinophores lamellated with thick stem, leaning slightly forward; number of lamellae varying from six to fifteen. Seven to 11, simple, pinnate, feather-like gills; both small and large gills. Skin embedded with numerous calcareous spines. Oral tube thin, short; buccal bulb muscular, two times longer than the oral tube. Pair of elongated, thin salivary glands attached on either side of buccal bulb at point where oesophagus enters buccal mass. Radular formula 5.2.0.2.5; rachidian tooth absent; laterals elongated, hamate with strong prominent distal cusp and wing-like expansion; outer laterals larger, thicker than inner laterals, over double the size; outer laterals with more pointed and triangular distal cusp; marginal teeth, five small, pseudo-rectangular plates. Labial cuticle large, robust with wing-like processes; well-developed brownish centre with jaw elements. Reproductive system triaulic with long, slender hermaphroditic duct; ampulla large; prostate gland massive; vaginal duct long, folded; penis armed with elongated, chitinous spines; some spines with bifid ends.

Material examined

Norway: Uthaug, Ørland, Trøndelag, 63.726566°N–9.576219°E, 1 spc. sequenced, H = 30 mm, UM (ZMBN 126017). Nordsundet havna, Kristiansund, Møre and Romsdal, 63.12495°N–7.778937°E, 1 spc. sequenced, UM (ZMBN 125636). Brattøy, Kristiansund, Møre and Romsdal, 63.06144°N–7.692341°E, 1 spc. sequenced, UM (ZMBN 125613); 63.06144°N–7.692341°E, 1 spc. sequenced, UM (ZMBN 125603); 63.06144°N–7.692341°E, 1 spc. (S1) sequenced, UM (ZMBN 125635); 63.06144°N–7.692341°E, 1 spc. (S1) sequenced, UM (ZMBN 125658); 63.06144°N–7.692341°E, 1 spc. (S2) sequenced, UM (ZMBN 125658); 63.06144°N–7.692341°E, 1 spc. (S3), sequenced, UM (ZMBN 125658); 63.06144°N–7.692341°E, 1 spc. (S2) sequenced, UM (ZMBN 125635). Breidvika, Drotningsvik, Bergen, Hordaland,

60.368682°N–5.174535°E, 1 spc. sequenced, UM (ZMBN 125971). Seløysundet, Espegrend area, Bergen, Hordaland, 60.24527°N–5.237572°E, 1 spc. sequenced, H = 11 mm, UM (ZMBN 125032); 60.24527°N–5.237572°E, 1 spc. sequenced, H = 8 mm, UM (ZMBN 125033). Steingardsvika, Espegrend area, Bergen, Hordaland, 60.29638°N–5.22144°E, 1 spc. sequenced, B (ZMBN 106114). Espegrend, Bergen, Hordaland, 60.2763°N–5.234900000000039°E, 1 spc. sequenced, B (ZMBN 94139). Skitholmen, Turøy, Bergen, Hordaland, 60.45253333°N–4.9271°E, 1 spc. sequenced, UM (ZMBN 127685); 60.45253333°N–4.9271°E, 1 spc. sequenced, UM (ZMBN 127682); 60.45255°N–4.927333333°E, 1 spc. sequenced, UM (ZMBN 127678); 60.45255°N–4.927333333°E, 1 spc. sequenced, UM (ZMBN 127683); 60.45255°N–4.927333333°E, 1 spc. sequenced, UM (ZMBN 127681); 60.45255°N–4.927333333°E, 1 spc. sequenced, UM (ZMBN 127676). Myrbærholmen, Turøy, Bergen, Hordaland, 60.45396667°N–4.936883333°E, 1 spc. sequenced, UM (ZMBN 127689); 60.45396667°N–4.936883333°E, 1 spc. sequenced, UM (ZMBN 127690). Drågsvågen, Førde, Sveio, Hordaland, 59.606198°N–5.4505°E, 1 spc. sequenced UM (ZMBN 125988). Drøbak, Frogn, Akershus, Oslo, 59.68243°N–10.62352°E, 1 spc. sequenced, H = 14 mm, UM (ZMBN 125578); 59.6822222°N–10.6238056°E, 1 spc. sequenced, UM (ZMBN 127587); 59.6822222°N–10.6238056°E, 1 spc. sequenced UM (ZMBN 127600). Flatholmen, Haugesund, Rogaland**,** 59.64416667°N–5.40333333°E, 1 spc. sequenced, H = 25 mm, UM (ZMBN 125859). Sletta, Skiftesvik, Haugesund, Rogaland, 59.68722222°N–5.35805556°E, 1 spc. sequenced, H = 20 mm, UM (ZMBN 125906). Engøy, Stavanger, Rogaland, 58.981088°N–5.741493°E, 1 spc. sequenced, UM (ZMBN 127626); 58.981088°N–5.741493°E, 1 spc. sequenced, UM (ZMBN 127631); 58.981088°N–5.741493°E, 1 spc. sequenced, UM (ZMBN 127633). Hafrsfjord, Sola, Stavanger, Rogaland, 58.93062°N–5.660183°E, 1 spc. (S1) sequenced and dissected (yellow/orange morphotype), UM (ZMBN 125688); 58.93062°N–5.660183°E, 1 spc. (S2) sequenced, UM (ZMBN 125688). Egersund havn, Rogaland, 58.44928°N–5.990812°E, 1 spc. sequenced, UM (ZMBN 125689). Tingelsædet, Egersund, Rogaland, 5.999357°N–58.415031°E, 1 spc. sequenced and dissected (striped morphotype), H = 20 mm, UM (ZMBN 127491); 1 spc. sequenced, H = 18 mm, UM (ZMBN 127476); 1 spc. sequenced, H = 25 mm, UM (ZMBN 127487); 1 spc. sequenced, H = 23 mm, UM (ZMBN 127488); 1 spc. sequenced, H = 12 mm, UM (ZMBN 127481); 1 spc. (S2) sequenced, H = 17 mm, UM (ZMBN 127492). Litle Svetlingen, Egersund, Rogaland,

5.967477°N–58.396251°E, 1 spc. sequenced, H = 22 mm, sequenced UM (ZMBN 127512); 1 spc. sequenced, H = 15 mm, UM (ZMBN 127511); 1 spc. sequenced, H = 18 mm, UM (ZMBN 127510); 1 spc. sequenced, H = 25 mm, UM (ZMBN 127513); 1 spc. sequenced, H = 20 mm, UM (ZMBN 127509). **Spain:** Roses, Catalonia, 42.1344833°N–3.2661389°E, 1 spc. sequenced, H = 13 mm, UA. Mataró, Catalonia, 41.5241556°N–2.4497500°E, 1 spc. sequenced, H = 6 mm, UA. **Azores (Portugal):** North of Baia da Poca, Gracioisa I., 39.0157000°N–27.9488000°W, 1 spc. sequenced, H = 5 mm, UM (ZMBN 97198). Mosteiros, Banco Sabrina, São Miguel Island, 37.8933778°N–25.8247750°W, 1 spc. sequenced, H = 10 mm, UM (ZMBN 87937). Baja da Fajã Moinhos, Aquàrio dos Mosteiros, 37.8933778°N–25.8247750°W, 1 spc. sequenced, H = 15 mm, UM (ZMBN 87942). Ilhéu dos Mosterios (East side), São Miguel Island, 37.8933778°N–25.8247750°W, 1 spc. sequenced, H = 8 mm, UM (ZMBN 87925).

External morphology (Fig. 8; Table 4)

Length of studied specimens between 5–30 mm (Table 1 in Appendix 4). Specimens with 45 mm have been reported (Edwards, 2008; Moen & Svensen, 2014; Telnes, 2018). Body elongated, limaciform, with distinct marginal ridge; slightly higher than broad; highest and widest at the posterior, mid-dorsal section closer to anus, gills and papillae; ending in an elongated and pointy tail. Foot long and narrow. Body surface smooth, covered with scattered tuberculate blotches; number and size of tubercles varying from few to multiple, small to large; tubercles either smoothly or sharply edged; tubercles sometimes flattened almost merging with the body surface, while others are more protruding and wart-like. Individuals possessing smaller sized tubercles often tend to have larger quantity. Head equipped with four to five, rarely six, smooth, lobed, and digitiform veil processes projecting anteriorly from frontal veil. Frontal veil processes somewhat retractile, with yellow or orange pigmentation; some nearly fully coloured, others only pigmented in the middle; apical tip often whitish. Non-retractile, lamellated rhinophores; stem slightly leaning forward, while the lamellated section slightly leans backwards; stems ticker than lamellated section which ends in a cylindrical knob; number of lamellae on studied specimens in average ten to twelve, but can range between six to fifteen. Eyespots small, dark, circular dots present dorsally, located behind rhinophores; clearly visible on some individuals, nearly invisible on others. Seven to nine, sometimes 11 pinnate, feather-like gills circulating the anus in a crown-like fashion;

individuals may possess both smaller and larger gills at the same time; gills partially retract into pocket. Two elongated, strong, papillae present on each side of the gill circlet, projecting backwards; shape and length of papillae may vary between individuals; some shorter and stubby with rounded apical tip, others slender with sharper apical tip.

Colouration (Figs 8, 14, 15A1)
Species polychromatic with four main morphotypes:

Yellow/orange morphotype (Fig. 8A–C; Fig. 15A1): White, translucent base colour with either yellow or orange, circular to oval tubercles scattered over the body surface. Tubercles strongly pigmented in yellow or orange. Rhinophore stems white with partly yellow or orange lamellae. Frontal veil processes yellow or orange pigmented; sometimes whitish or lacking colouration in apical tips. Lateral papillae yellow or orange in distal half part; proximal half whitish. Often, a mid-dorsal yellow or orange line extends from behind the gills to the tip of the tail. Gills whitish with yellow or orange apical edges.

Black rhinophore morphotype (Fig. 8D–F; Fig. 14C): Like the yellow/orange morphotype but with black, dark brownish or grey, sometimes very light grey (Fig. 8D), rhinophore stems (instead of white). Dark pigmentation most commonly restricted to the frontal side of the rhinophore stems; others being nearly fully pigmented.

Striped morphotypes (Fig. 8G–M; Fig. 14A–B): Like the yellow/orange morphotype, but with additional black or dark grey continuous (Fig. 8J–M) or dashed (Fig. 8G–I) stripes covering the body surface. Thickness and number of dark stripes varies between individuals; some almost fully covered, while others have fewer stripes; stripes either thick or narrow. Dashed stripes often more randomly distributed; some only present in anterior region, while others are more evenly spread. Rhinophore stems often black, dark or light grey, or brownish; lamellae often yellow or orange pigmented, sometimes with additional black pigmentation. Lateral papillae yellow or orange in distal half part; proximal half whitish, sometimes with additional black spots scattered around. Gills whitish with yellow or orange pigmentation; sometimes with few black spots around the edges and apical tip.

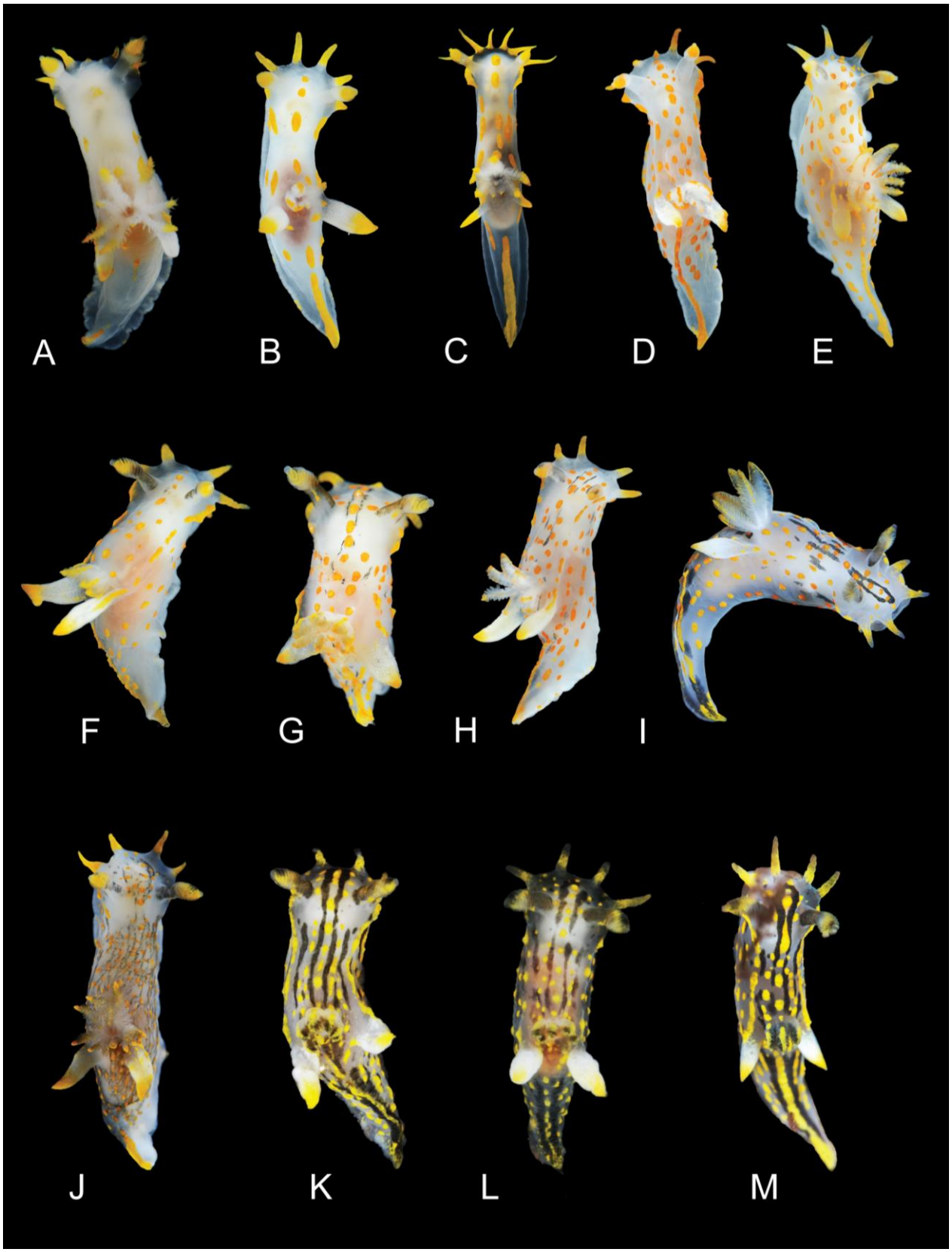


Figure 8 – *P. quadrilineata.* (**A–C**) yellow/orange morphotype, (**D–F**) black rhinophore morphotype, (**G–I**) dashed striped morphotype, (**J–M**) continuous striped morphotype. **A.** Rogaland, Norway, ZMBN 127509, photo by C. Rauch and M. A. E.

Malaquias, 2019. **B.** Hordaland, Norway, ZMBN 127690, photo by C. Rauch, 2019. **C.** Rogaland, Norway, ZMBN 125859, photo by C. Rauch and A. Schouw, 2018. **D.** Hordaland, Norway, ZMBN 127676, photo by C. Rauch, 2019. **E.** Hordaland, Norway, ZMBN 127683, photo by C. Rauch, 2019. **F.** Hordaland, Norway, ZMBN 127677, photo by C. Rauch, 2019. **G.** Hordaland, Norway ZMBN 127678, photo by C. Rauch, 2019. **H.** Hordaland, Norway, ZMBN 127685, photo by C. Rauch, 2019. **I**. Møre and Romsdal, Norway, ZMBN 125635, photo by N. Aukan, 2018. **J.** Rogaland, Norway, ZMBN 127476, photo by C. Rauch and M. A. E. Malaquias, 2019. **K.** Hordaland, Norway, ZMBN 125032, photo by C. G. Sørensen and M. A. E. Malaquias, 2018. **L.** Hordaland, Norway, ZMBN 125033, photo by C. G. Sørensen and M. A. E. Malaquias, 2018. **M.** Rogaland, Norway, ZMBN 125689, photo by E. Svensen, 2017.

Radula (Fig. 9A, B; Table 4)

Radular formula 5.2.0.2.5. Radula elongate, slender. Rachidian tooth absent; laterals elongated, hamate with strong prominent distal cusp; inner laterals small, narrow with distal cusp with somewhat rounded edges; outer laterals larger, thicker than inner, over double the size, distal cusp more pointed and triangular; both laterals with wing-like expansion, somewhat resembling claws, or hooks; wing-like expansion more inconspicuous on inner laterals. Marginal teeth smaller, pseudo-rectangular plates, decreasing in size towards margin; inner marginal with prominent curved spur at anterior end.

Labial cuticle (Fig. 9C–F; Table 4)

Large, robust with two large and elongated lateral wings. Well-developed brownish centre (Fig. 9E) with jaw elements.

Reproductive system (Fig. 10A–D; Table 4)

Triaulic; hermaphroditic duct long, slender. Ampulla large, robust, kidney-shaped; post-ampullary duct bifurcating into short oviduct leading into a large female gland mass and vas deferens through prostrate portion. Prostate gland massive, narrowing towards distal vas deferens, closely attached to bursa copulatrix. Inside the vas deferens a cup-shaped structure indicates the end of the prostatic section. Vas deferens long, narrow, folded before reaching large penile bulb (Fig. 10A). Penis armed with numerous elongated, pointed, chitinous spines, very similar in size along its entire length; some spines bifid in apical tip (Fig. 10B–D). Vaginal duct long, folded, similar in width to vas deferens, connected to bursa copulatrix. Bursa copulatrix very large with two different portions; large elongate proximal portion ending in an oval distal part. Base of bursa copulatrix connected to pyriform, small receptaculum

seminis by long, thin duct. Short uterine duct emerging close to receptaculum seminis and entering female gland, behind elongated portion of bursa copulatrix (Fig. 10A).

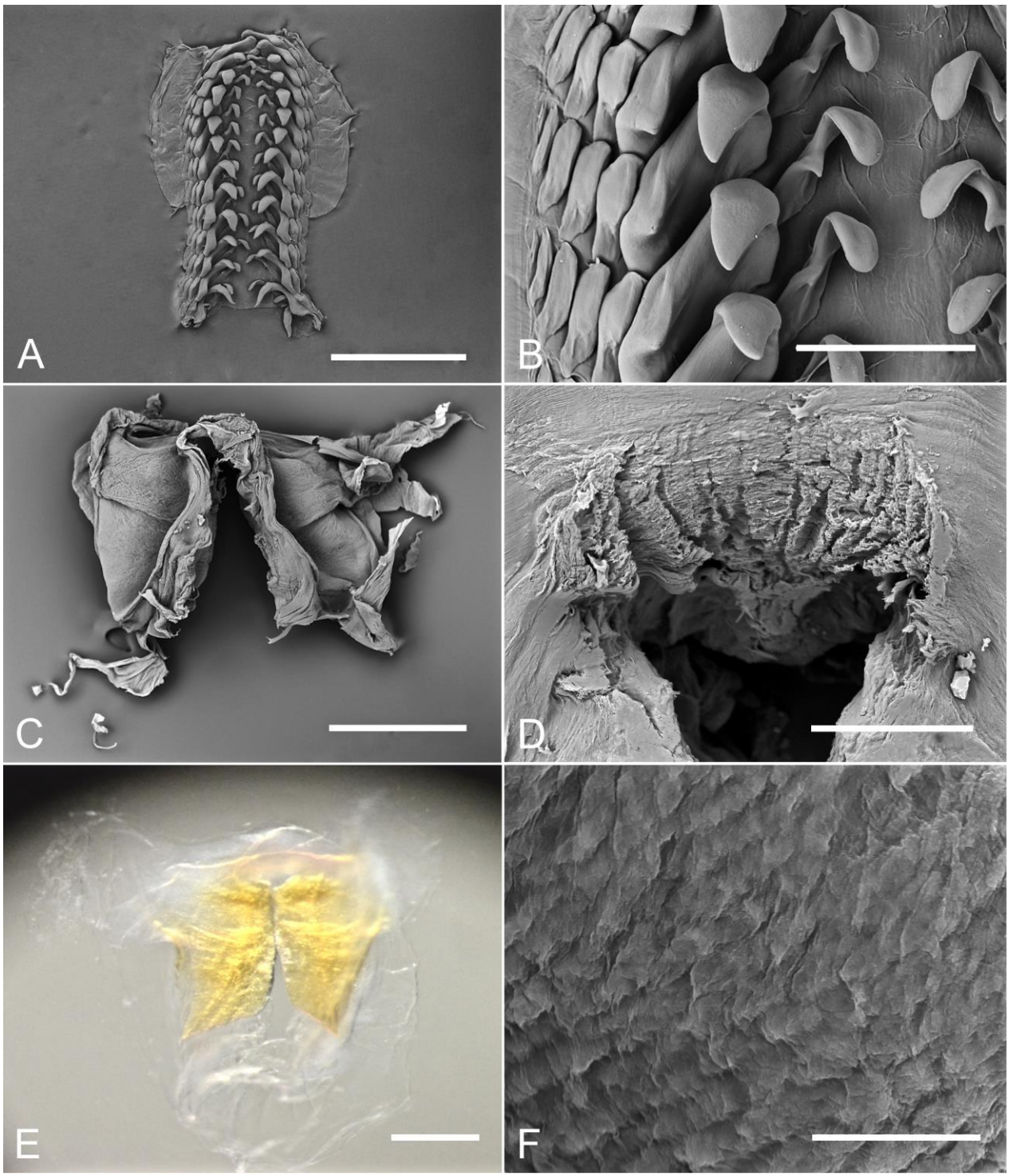


Figure 9 – Scanning electron micrographs of *P. quadrilineata.* **A.** complete radula (ZMBN 127491). **B**. detailed view of the left side of the radula (ZMBN 125688). **C.** labial cuticle (ZMBN 125688)**. D**. detail of central region of labial cuticle (ZMBN

127491). **E.** optical microscopy picture of labial cuticle (ZMBN 127491). **F.** close up of tissue from labial cuticle wall (ZMBN 125688). Scale bars: **A** = 1 mm, **B** = 300 μm, **C** = 500 μm, **D** = 100 μm, **E** = 1 mm, **F** = 30 μm.

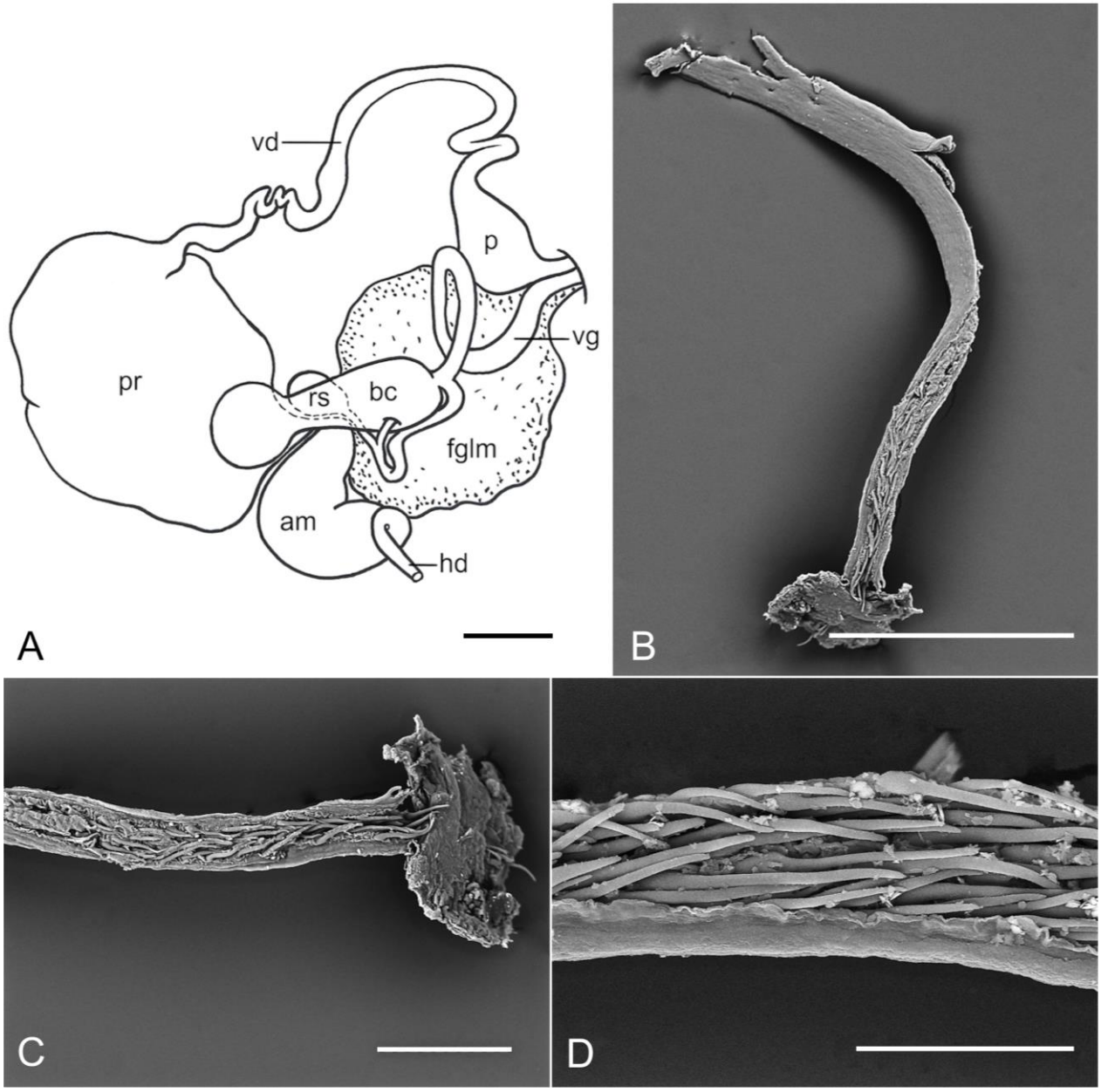


Figure 10 – Reproductive system and scanning electron micrographs of the penis of *P. quadrilineata.* **A.** reproductive system (ZMBN 125688). **B.** whole penis (ZMBN 127491). **C.** detail of penis close to genital aperture (ZMBN 127491). **D.** detail of penile spines (ZMBN 125688). Fglm = female gland mass; hd = hermaphroditic duct; pr = prostrate; vd = vas deferens; p = penis; vg = vagina; bc = bursa copulatrix; rs = receptaculum seminis; am = ampulla. Scale bars: **A** = 1 mm, **B** =300 μm, **C** = 100 μm, **D** = 50 μm.

Ecology

Intertidal and sub-littoral species commonly found in shallow waters associated with algae, frequently hiding among kelp feeding on encrusted bryozoans like *E. pilosa* and *M. membranacea.* It has been reported from depths up 300 m (Bergan & Anthon, 1977). Lives in cold to temperate waters between 5–25°C.

Distribution (Table 1; Fig. 18; Table 1 in Appendix 7.1)

Widely distributed across Western Europe from Norway with Lofoten as its northernmost limit, Greenland, Sweden, Denmark, Iceland, Faeroes, all around the British Isles, southwards to the Mediterranean Sea, Iberian Peninsula, and archipelagos of the Azores, Madeira and the Canary Islands (Thompson & Brown, 1984; Thompson, 1988; Moen & Svensen, 2014).

Remarks

Difficult to give an exact number of gills and lamellae since these features frequently vary in number and were only roughly studied from taxon sampling pictures. Nevertheless, the number of gills varying from 7–11 (Table 4) seems to agree with the literature. According to Schmekel et al., (1982) *P. quadrilineata* has 12 lamellae on the rhinophores which was the case for most individuals currently studied, whose number ranged from 10–12. However, total estimated lamellae number ranged from 6–15 (Table 4). Tubercles possessed by individuals with the striped morphotypes frequently tended to be more orange than yellow. Tubercles frequently seemed more smoothly rounded than those on *Polycera* n. sp.. The degree of translucent base pigmentation of the body varied between individuals, making some more translucent than others. The various morphotypes currently studied (Figs 8, 11, 14–16) partly violates earlier literature (Thompson & Brown, 1984; Thompson, 1988; Hayward & Ryland, 1995; Moen & Svensen, 2014) where *P. quadrilineata* is diagnosed with dark stripes or blotches, in which the latter case only was discovered in the new species (Figs 11, 16). Body size seems to be comparatively larger than *Polycera* n. sp.. Specimens may reaching up to 30 mm with an average length of ~16.6 mm, whereas *Polycera* n. sp. has an average size of 5 mm (Table 4; Table 1 in Appendix 4).

Compared to *Polycera* n. sp., the radula of *P. quadrilineata* is larger, thicker, more elongated, with a greater number of rows, with the inner laterals closer together (Fig. 9A, B vs. Fig. 12A,

B). The lateral teeth are thicker and wider than in the new species, especially the inner laterals which are straighter in *Polycera* n. sp.. Wing-like expansion on the outer laterals more apparent on *P. quadrilineata*, which has an additional marginal tooth on each row side (Fig. 9B vs. Fig. 12B). Labial cuticle thicker, more robust and with a stronger appearing brownish centre than that of *Polycera* n. sp. (Fig. 9C–F vs. Fig. 12C–F). Penile spines more elongated (Fig. 10B–D vs. Fig. 13B–D), disagreeing with Thompson & Brown (1984) who describes the spines as hooked, which is a feature only found in the new species (Table 4). In Norway *P. quadrilineata* is sympatric with *Polycera* n. sp., but the former has a much broader geographic range (Figs 17, 18; Table 1 in Appendix 7.1). For the seasonal abundance in Norway (Figs 1, 2 in Appendix 5) *P. quadrilineata* have a more or less evenly distributed abundance throughout the entire year but with an apparent peak between January and May (Fig. 2 in Appendix 5).

4.3.3 *Polycera* n. sp.

(Figs 11–13, 15A2, 16; Table 4)

Diagnosis

Body surface smooth, partially tuberculate. Tubercles rounded or pointed, some more protruding than others; tubercles with yellow, light-yellow or orange pigmentation; base colour translucent white. Colour pattern with randomly distributed black, dark grey or brown dots and orange/brown patches; patches most common in head and "neck" region. Frontal veil with four to six long or short processes, smooth, tapering distally; somewhat retractile. Rhinophores lamellated with thick stem, leaning slightly forward; around six to ten lamellae. Roughly seven to nine pinnate, feather-like gills. Radular formula 4.2.0.2.4; rachidian tooth absent; laterals elongated, hamate with hook-like shaped structures and strong prominent triangular distal cusp; inner laterals narrow, straight; outer laterals thicker, broader than inner, at least twice the size; four marginal teeth, small, nearly quadrangular plates. Labial cuticle small, weak, with two lateral short wings and a weakly apparent brownish centre with jaw elements. Reproductive system triaulic with long, slender hermaphroditic duct. Ampulla small. Penis armed with two types of chitinous spines; one

type resembling curvy hooks, the other type being more elongated. Vaginal duct elongated, bent, shorter than vas deferens but about same width.

Material examined

Norway: Uthaug, Ørland, Trøndelag, 63.727012°N–9.572252°E, 1 spc. sequenced, H = 3 mm, UM (ZMBN 126023); 63.727012°N–9.572252°E, 1 spc. sequenced, H = 8 mm, UM (ZMBN 126025); 63.727012°N–9.572252°E, 1 spc. sequenced, H = 7 mm, UM (ZMBN 126024). Askøy, Bergen, Hordaland, 60.561459°N–4.961138°E, 1 spc. sequenced, UM (ZMBN 125917). Steingardsvika, Espegrend, Bergen, Hordaland, 60.29638°N–5.22144°E, 1 spc. sequenced, B (ZMBN 106115); 60.29638°N–5.22144°E, 1 spc. sequenced, B (ZMBN 106113). Seløysundet, Espegrend, Bergen, Hordaland, 60.24135°N–5.240833333°E, 1 spc. sequenced, UM (ZMBN 127664). Legern, Haugesund, Rogaland, 59.51139°N–5.242222°E, 1 spc. (S1) sequenced, H = 2 mm, UM (ZMBN 125855); 59.51139°N–5.242222°E, 1 spc. (S2) sequenced, H = 2 mm, UM (ZMBN 125855). Sandhl, Haugesund, Rogaland, 59.4275°N–5.44°E, 1 spc. (S1) sequenced, H = 3 mm, UM (ZMBN 125881); 59.4275°N–5.44°E, 1 spc. (S2) sequenced, H = 3 mm, UM (ZMBN 125881); 59.4275°N–5.44°E, 1 spc. (S3) sequenced, H = 3 mm, UM (ZMBN 125881); 59.4275°N–5.44°E, 1 spc. (S4) sequenced, H = 3 mm, UM (ZMBN 125881); 59.4275°N–5.44°E, 1 spc. (S5) sequenced, H = 3 mm, UM (ZMBN 125881). Skeisvika, Hundvåg, Stavanger, Rogaland, 59.006475°N–5.719213°E, 1 spc. sequenced, UM (ZMBN 127607); 59.006475°N–5.719213°E, 1 spc. sequenced, UM (ZMBN 127608). Tingelsædet, Egersund, Rogaland, 5.999357°N–58.415031°E, 1 spc. sequenced and dissected (yellow/orange morphotype), H = 14 mm, UM (ZMBN 127486); 5.999357°N–58.415031°E, 1 spc. (S1), sequenced and dissected (patchy dotted morphotype), H = 9 mm, UM (ZMBN 127492).

External morphology (Fig. 11; Table 4)

Length of studied specimens between 2–14 mm (Table 1 in Appendix 4). Body structure bearing strong morphological resembles to *P. quadrilineata*; elongated, limaciform, with distinct marginal ridge; slightly higher than broad; highest and widest at the posterior, mid-dorsal section close to anus, gills and papillae; ending in an elongated and pointy tail. Foot long and narrow. Body surface smooth, covered with scattered tubercles; number and size of tubercles varying from few to multiple, small to large; tubercles sometimes flattened, while

others are more protruding and wart-like. Individuals with dotted morphotype tend to have rounded, slightly protruding, pearl-shaped tubercles with light-yellow pigmentation. Head equipped with four to six, sometimes seven, smooth, lobed, and digitiform veil processes projecting anteriorly from frontal veil. Frontal veil processes somewhat retractile, with varying coverage of yellow or orange pigmentation; some nearly fully covered, others only pigmented in the mid-section or with yellow spots randomly scattered around; apical tip lacking other pigmentation than the whitish base. Non-retractile, lamellated rhinophores; stems slightly leaning forward, while the lamellated section slightly leans backwards; stalk stems ticker than the lamellated section which ends in a cylindrical knob; number of lamellae varying approximately between six to ten. Eyespots small, dark circles located behind rhinophores; clearly visible on some individuals, nearly invisible on others. Seven to nine pinnate, simple, feather-like gills circulating the anus in a crown-like fashion near the mid-dorsal section of the body; individuals may possess both smaller and larger gills; gills can partially retract into pocket. Two elongated, narrow, papillae on each side of the gills either projecting backwards or out to the sides; shape and length of papillae may vary between individuals; often shorter and stubby with rounded apical tip on the individuals with dotted morphotype; slender with sharper apical tip often on individuals with yellow/orange or patchy dotted morphotypes.

Colouration (Figs 11, 15A2, 16)
Species polychromatic with three main morphotypes:

Yellow/orange morphotype (Fig. 11A–B; Fig. 15A2): as in the yellow/orange morphotype described for *P. quadrilineata.*

Patchy dotted morphotype (Fig. 11C–D; Fig. 16A): Much like the yellow/orange morphotype only with few patchy black or dark grey dots; most frequently scattered around head and "neck" region. Size and intensity of the dark dots may vary; some being weak with large distal space, while others are intensely pigmented due to denser aggregations. Dark dots occur on gills, lamellae and frontal veil processes. Veil processes often less yellow/orange pigmented.

Dotted morphotype (Fig. 11E–H; Fig. 16B): White, translucent base colour with few light-yellow, small, circular, slightly protruding, rounded tubercles scattered over surface. Body covered with black, dark to light grey or brownish dots randomly scattered over entire surface; orange/brown to brown patches often present dorsally on head region between the rhinophores; some individuals being nearly fully covered with orange/brown to brown patches. Pail white rhinophores and frontal veil processes; lamellae sometimes with a weak hint of light-yellow pigmentation. Frontal veil processes often pigmented with few, small, yellow patches randomly distributed. Lateral papillae yellow or orange pigmented in last distal third; first two thirds whitish, with yellow, orange or black patches. Apical edges of gills weakly yellow or orange, some with few additional black spots. Gills sometimes with the same colour as the base. Tail often with mid-dorsal line coloured in yellow or dotted.

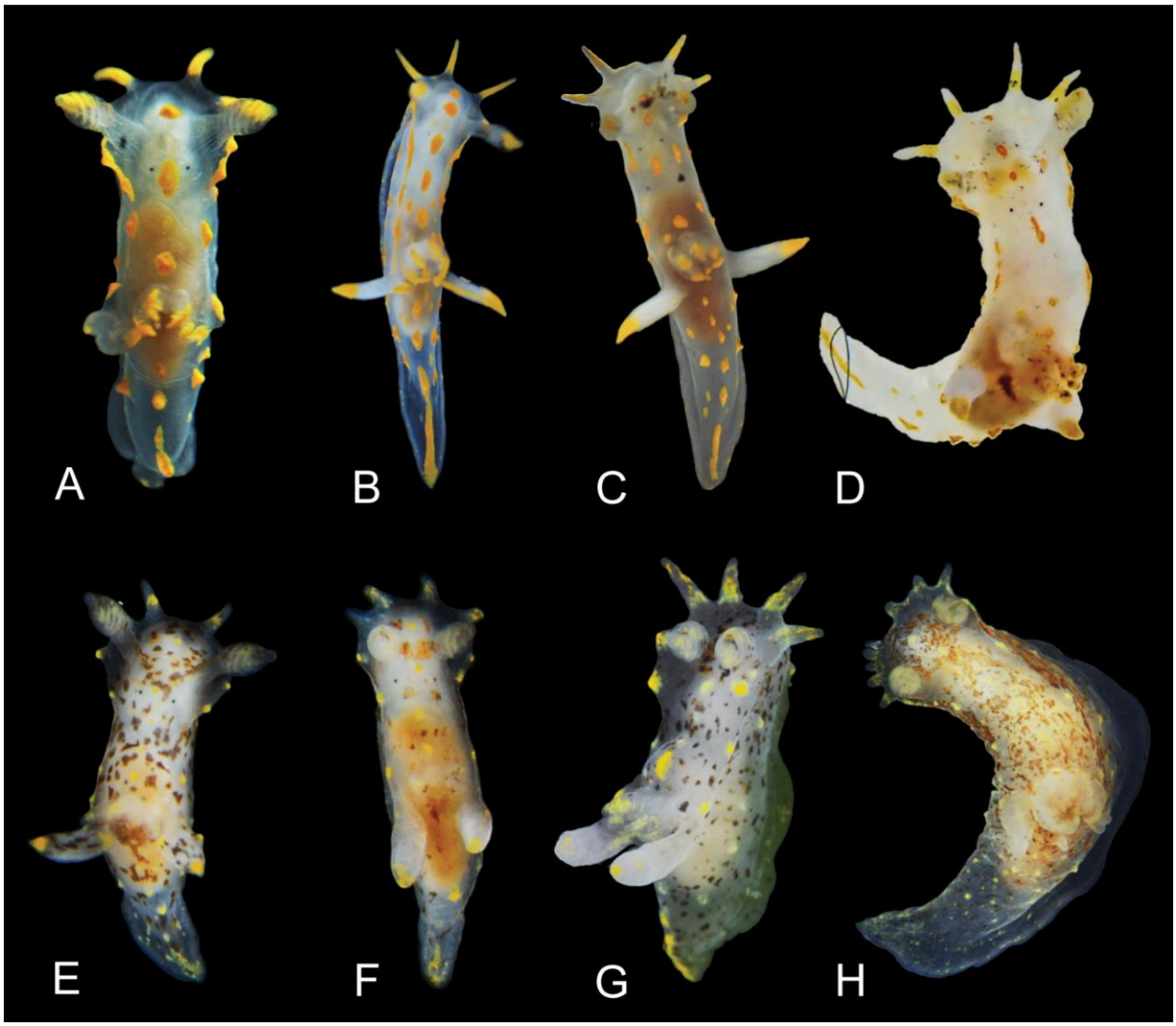


Figure 11 – *Polycera* n. sp.. (**A–B**) yellow/orange morphotype, (**C–D**) patchy dotted morphotype, (**E–H**) dotted morphotype. **A.** Trøndelag, Norway, ZMBN 126023, photo by V. V. Grøtan, 2018. **B.** Trøndelag, Norway, ZMBN 126025**,** photo by V. V. Grøtan, 2018. **C.** Trøndelag, Norway, ZMBN 126024, photo by V. V. Grøtan, 2018. **D.** Rogaland, Norway, ZMBN 127608, photo by O. Meldahl, 2018. **E.** Rogaland, Norway, ZMBN 125881, photo by A. Schouw and C. Rauch, 2018. **F.** Rogaland, Norway, ZMBN 125855, photo by A. Schouw and C. Rauch, 2018. **G.** Hordaland, Norway, ZMBN 106113, photo by K. Kongshavn and M. A. E. Malaquias, 2015. **H.** Hordaland, Norway, ZMBN 125917, photo by A. Schouw and C. Rauch, 2018.

Radula (Fig. 12A, B; Table 4)

Radula short, wide, with radular formula 4.2.0.2.4. Rachidian tooth absent; laterals elongated, hamate with strong prominent distal cusp; inner laterals smaller, narrower with triangular, slightly rounded distal cusp, hook-like shaped structure present along mid-height on outer edge; outer laterals larger, at least twice the size of inner laterals, with triangular pointed

distal cusp, base broad, hook-like shaped structure near base on outer edge. Marginal teeth small, plate-like, elongated, pseudo-quadrangular, decreasing in size towards the margin; inner marginal with prominent, weakly curved spur at anterior end.

Labial cuticle (Fig. 12C–F; Table 4)

Small, weak, with two short lateral wings; weakly apparent brownish centre (Fig. 12F) with jaw elements.

Reproductive system (Fig. 13A–D; Table 4)

Triaulic; hermaphroditic duct elongate, thin. Ampulla small, kidney-shaped; post-ampullary duct bifurcating into short oviduct leading into a large female gland mass and short vas deferens through prostrate portion. Prostate gland massive, narrowing towards distal vas deferens, surrounding bursa copulatrix. Inside vas deferens a cup-shaped structure indicates the end of prostatic section. Vas deferens short, narrow, folded before reaching genital pore. Penile bulb not well developed (Fig. 13A). Penis armed with two types of chitinous spines; spines closest to prostate more elongate; spines closest to genital opening hook-shaped (Fig. 13B–D). Vaginal duct elongate, bent, shorter than vas deferens, but with similar width. Vagina ends in large oval bursa copulatrix. Shape of bursa copulatrix may appear elongate due to pressure exerted by the prostate. Base of bursa copulatrix connected to pyriform, small receptaculum seminis by short, thin duct. Short uterine duct emerging close to receptaculum seminis, entering female gland (Fig. 13A).

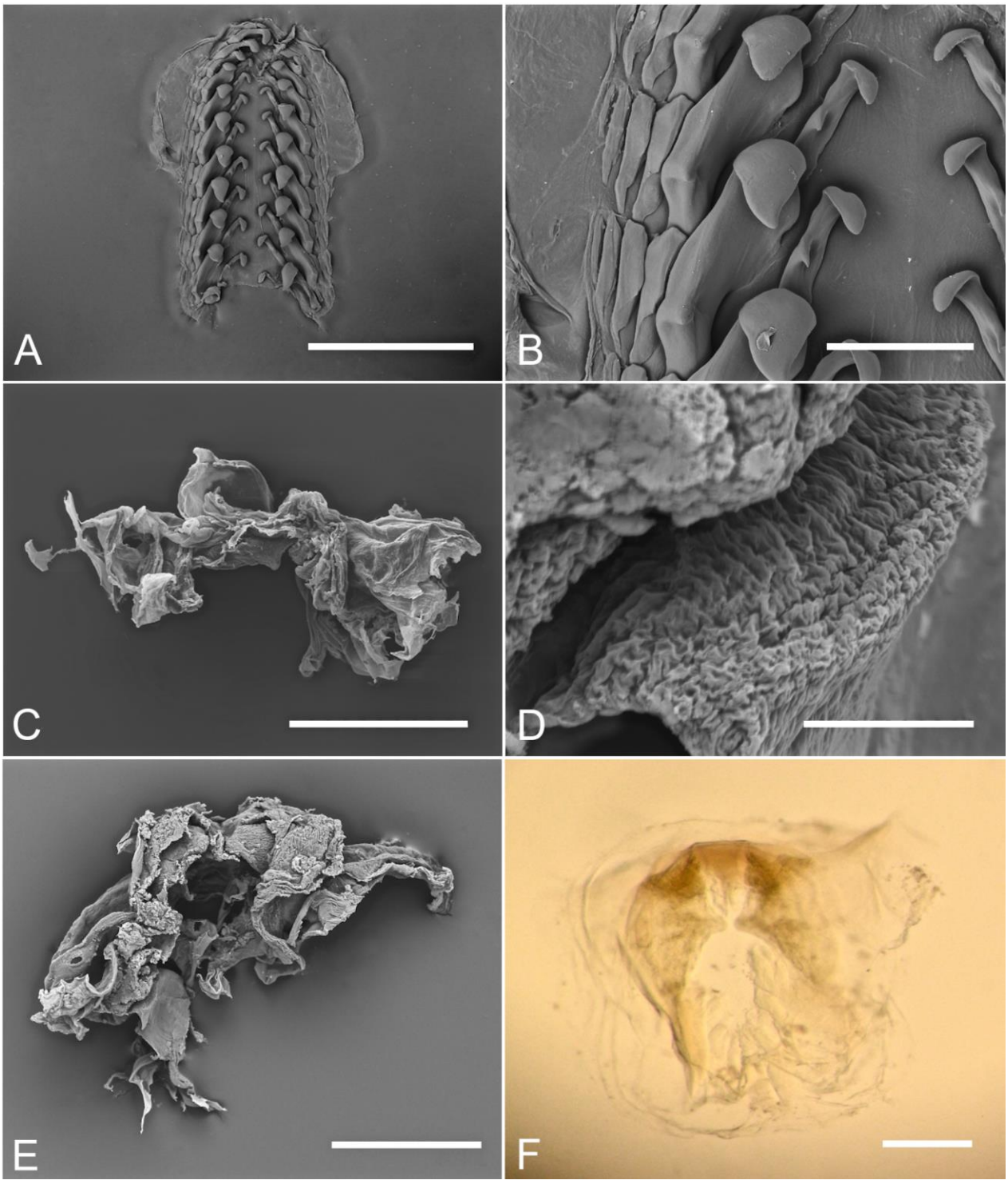


Figure 12 – Scanning electron micrographs of *Polycera* n. sp.. **A**. radula (ZMBN 127486). **B.** detailed view of the left side of the radula (ZMBN 127486). **C.** Labial cuticle (ZMBN 127486). **D**. close up of tissue from the labial cuticle wall (ZMBN 127492). **E.** Labial cuticle (ZMBN 127492). **F.** optical microscopy picture of labial cuticle (ZMBN 127492). Scale bars: **A** = 1 mm, **B** = 200 µm, **C** = 500 µm, **D** = 20 µm, **E** = 300 µm, **F** = 1 mm.

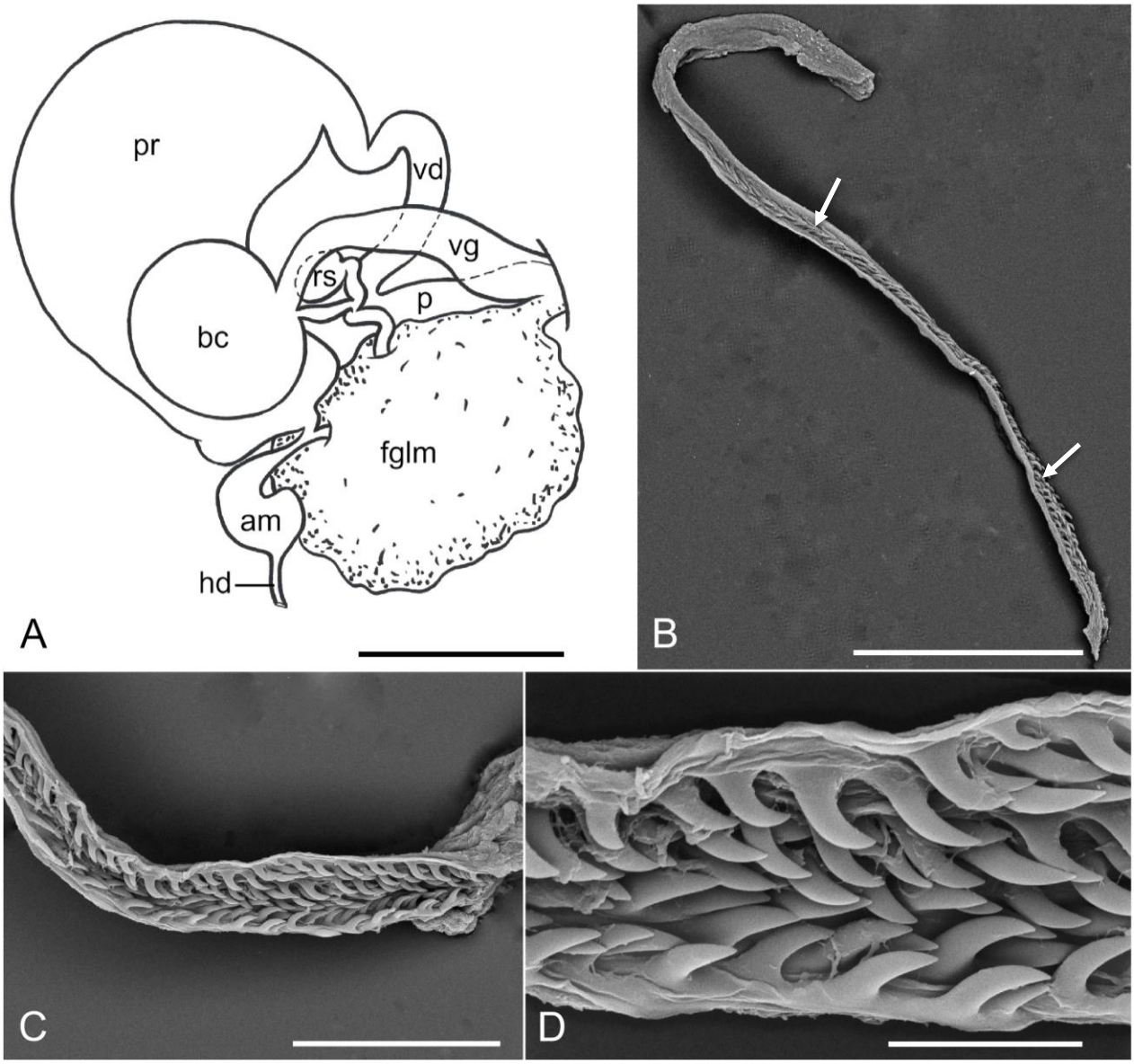


Figure 13 – Reproductive system and scanning electron micrographs of the penis of *Polycera* n. sp.. **A.** reproductive system (ZMBN 127492). **B.** whole penis (ZMBN 127486). **C.** detail of penis close to genital aperture (ZMBN 127486). **D**. close up of penile spines (ZMBN 127486). Upper arrow = elongated penile spines. Lower arrow = hook-shaped penile spines. Fglm = female gland mass; hd = hermaphroditic duct; pr = prostrate; vd = vas deferens; p = penis; vg = vagina; bc = bursa copulatrix; rs = receptaculum seminis; am = ampulla. Scale bars: **A** = 1 mm, **B** = 300 µm, **C** = 100 µm, **D** = 20 µm.

Ecology

Sub-littoral species occurring between 2–15 m, often on kelp (*Laminaria*) feeding on encrusted bryozoans.

Distribution (Table 1; Fig. 17; Table 1 in Appendix 7.1)

Know from Northern Norway to Southern-Western Norway, where it has only been found in Haugesund, Stavanger, Bergen, Egersund and Trondheim.

Remarks

Besides the colour patterns, another external difference between *Polycera* n. sp. and *P. quadrilineata* is the lower average number of lamellae on the rhinophores (6–10 in the former and 10–12 in the latter; Table 4). Gills seemingly the same as *P. quadrilineata* by the quantity (7–9 in *Polycera* n. sp., and 7–11 in *P. quadrilineata*), and by possessing both smaller and larger gills at the same time. Body size generally smaller than *P. quadrilineata* ranging from 2–14 mm in length with 5 mm as the average size documented (Table 1 in Appendix 4). Only two specimens possessed the patchy dotted morphotype, which seems to be a less common morph of the species. Tubercles on the yellow/orange morphotype frequently seemed more pointy-edged than *P. quadrilineata.*

Compared to *P. quadrilineata*, the radula of *Polycera* n. sp. was shorter, wider, with fewer rows, and more spaced out lateral teeth. Inner laterals comparatively narrower and smaller. Only four marginal teeth present rather than five like in *P. quadrilineata* (Fig. 12A, B vs. Fig. 9A, B). Labial cuticle thinner, weaker, with less apparent structures (Fig. 12C–F vs. Fig. 9C–F). Penis shorter, with two types of spines that are both elongated and hooked (Fig. 13B–D vs. Fig. 10B–D; Table 4). *Polycera* n. sp. seems to be less common than *P. quadrilineata* and geographically restricted to Norway (Figs 17, 18; Table 1 in Appendix 7.1) where it is sympatric with *P. quadrilineata. Polycera* n. sp. seems to be more abundant during December, but specimens were also found during January, February, July and October (Fig. 1 in Appendix 5).

Table 4 – Comparative summary of the diagnostic characters of *P. quadrilineata* and *Polycera* n. sp..

Character	***P. quadrilineata***	***Polycera* n. sp.**
External morphology		
No. of main morphotypes	4	3
Distinctive morphotypes	Continuous or dashed stripes	Dotted or patchy dottet
No. lamellae	6–15. Most commonly 10–12	6–10
No. gills	7–11	7–9
Body size	5–30 mm with average size ~ 16.6 mm (45 mm reported)	2–14 mm with average size = 5 mm
Papillae	Short, stubby with rounded apical tip, or slender with sharp apical tip (regardless of morphotype)	Short, stubby with rounded apical tip (dotted morphotype). Slender with sharp apical tip (yellow/orange or patchy dotted morphotype)
Radula		
No. of rows	13–14	8–9
Inner lateral teeth	Broad base with large distal cusp	Narrower base with small distal cusp
Marginal teeth	5	4
Labial cuticle		
Structure	Large, robust with strong brownish centre	Small, fragile with weak brownish centre
Reproductive system		
Ampulla	Large	Small
Bursa copulatrix	Large	Large
Receptaculum seminis	Small with long, thin duct	Small with short, thin duct
Vaginal duct	Elongated	Elongated
Prostate gland	Massive	Massive
Vas deferens	Long	Short
Penile bulb	Large	Little developed
Penile spines	One type; elongated, needle-like; some with bified apical tip	Two types; elongated closest to prostrate; hooked closest to genital opening

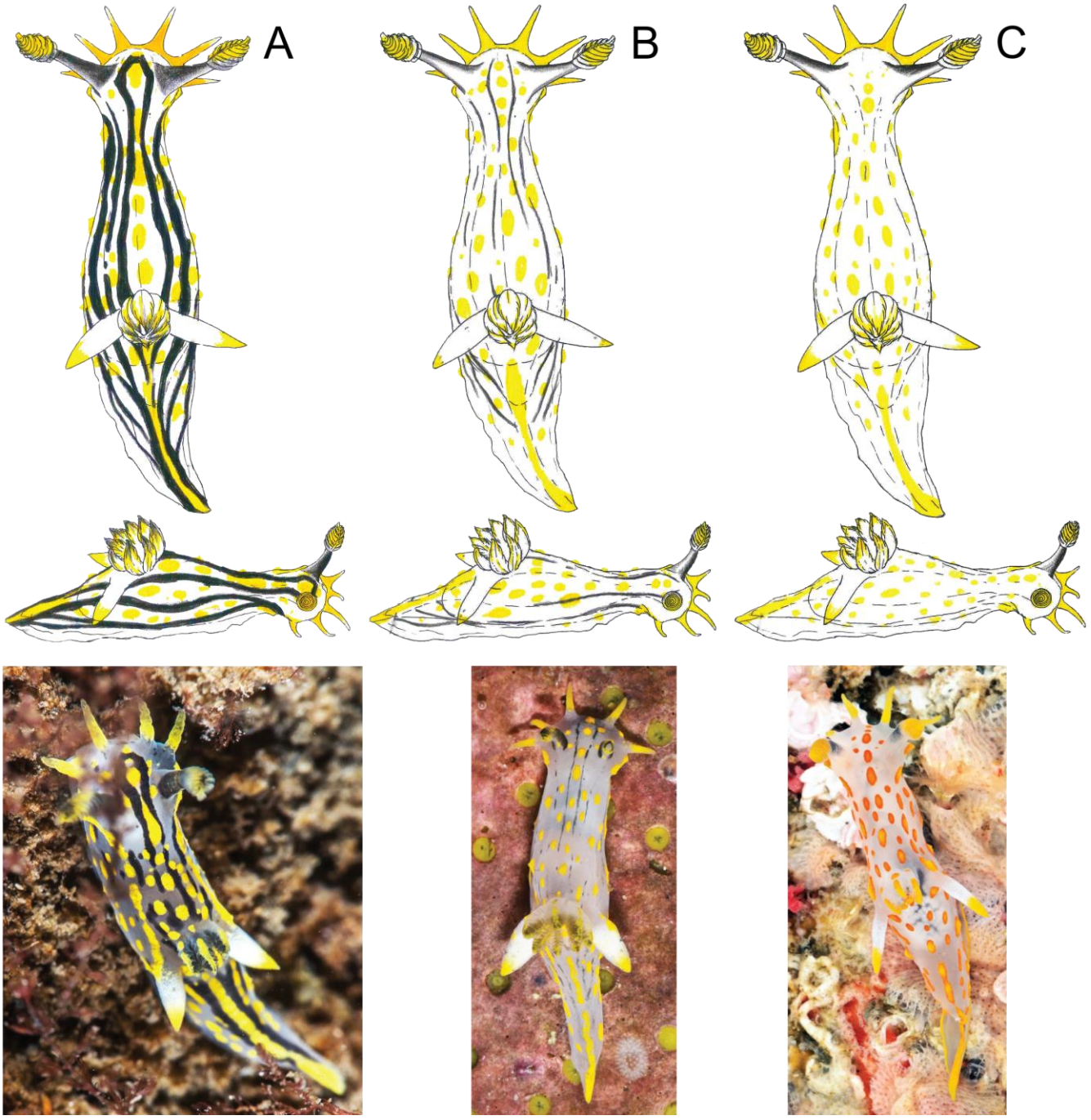


Figure 14 – Main morphotypes found in *P. quadrilineata*. **A.** continuous striped morphotype. **B**. dashed striped morphotype. **C.** black rhinophore morphotype. **A.** Rogaland, Norway, ZMBN 125689, photo by E. Svensen, 2017. **B.** Hordaland, Norway, ZMBN 94139, photo M. A. E. Malaquias, 2013. **C.** Aquàrio dos Mosteiros, Azores, ZMBN 87942, photo by M. A. E. Malaquias, 2011. Drawings by C. G. Sørensen, 2019.

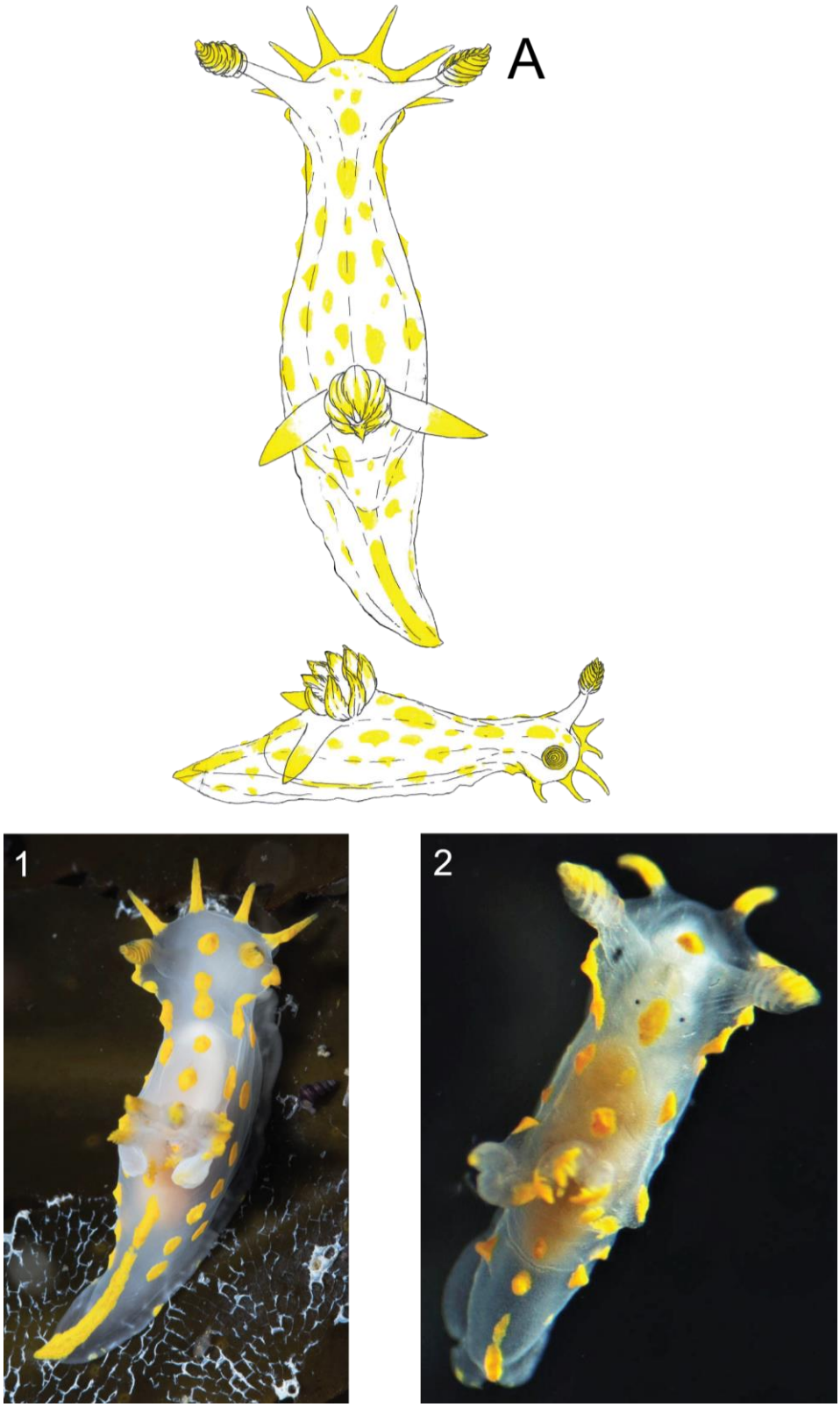


Figure 15 – Main morphotype shared by *P. quadrilineata* (**A1**) and *Polycera* n. sp. (**A2**), namly the yellow/orange morphotype. **A1.** Akershus (Drøbak), Norway, ZMBN 127600 photo by H. Jensen, 2019. **A2.** Trøndelag, Norway, ZMBN 126023, photo by V. V. Grøtan, 2018. Drawing by C. G. Sørensen, 2019.

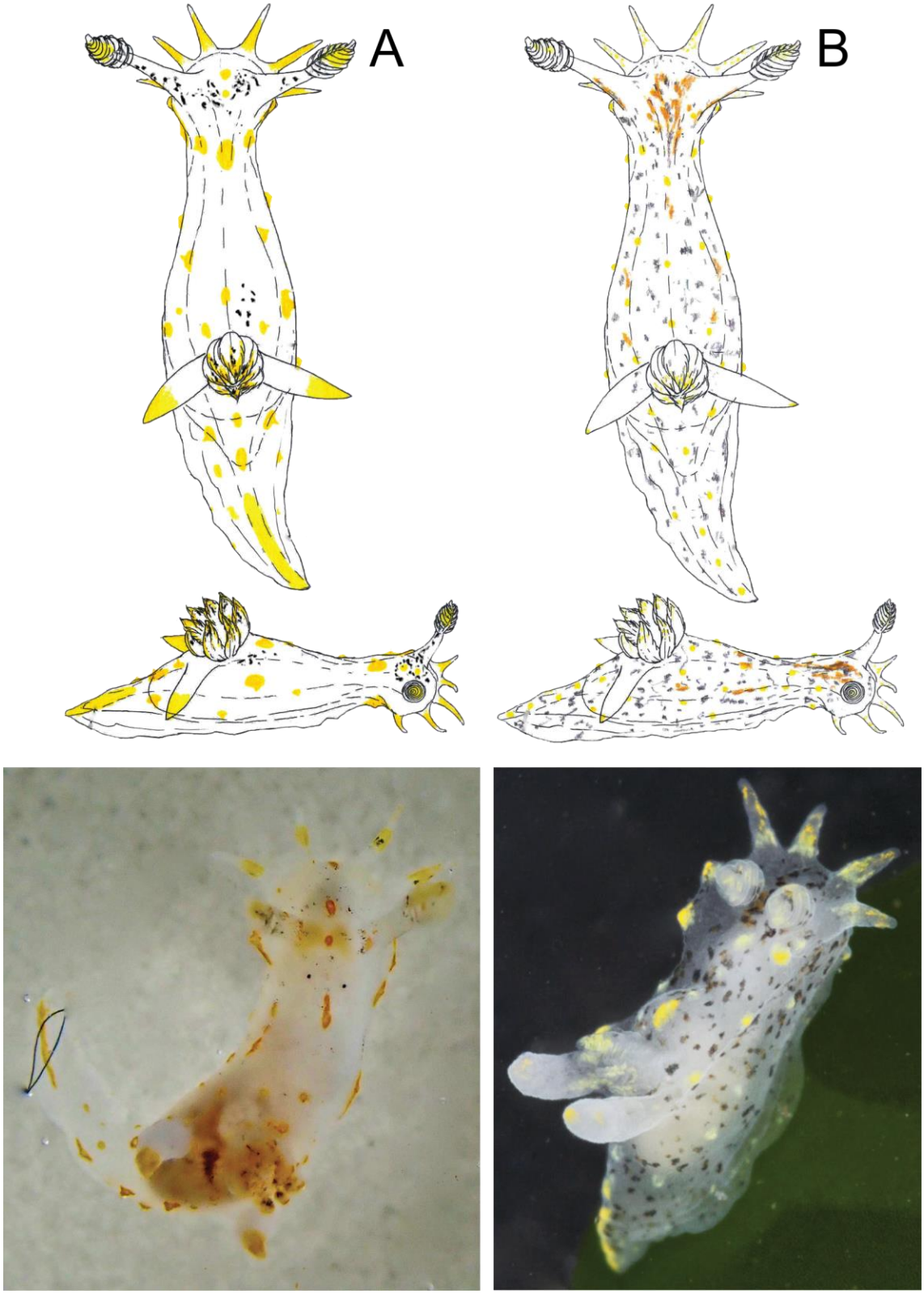


Figure 16 – Main morphotypes found in *Polycera* n. sp.. **A.** patchy dotted morphotype. **B.** dotted morphotype. **A.** Rogaland, Norway, ZMBN 127608, photo by O. Meldahl, 2018. **B.** Hordaland, Norway, ZMBN 106113, photo by K. Kongshavn and M. A. E. Malaquias, 2015. Drawings by C. G. Sørensen, 2019.

4.4 Haplotype network analysis

The TCS haplotype network of *Polycera* n. sp. (Fig. 17), represented by 17 specimens (three from Northern Norway and 14 from Southern-Western Norway; Table 1 in Appendix 7.1), showed high genetic diversity, in fact no haplotypes were shared among individuals. There was no geographical structure in the network.

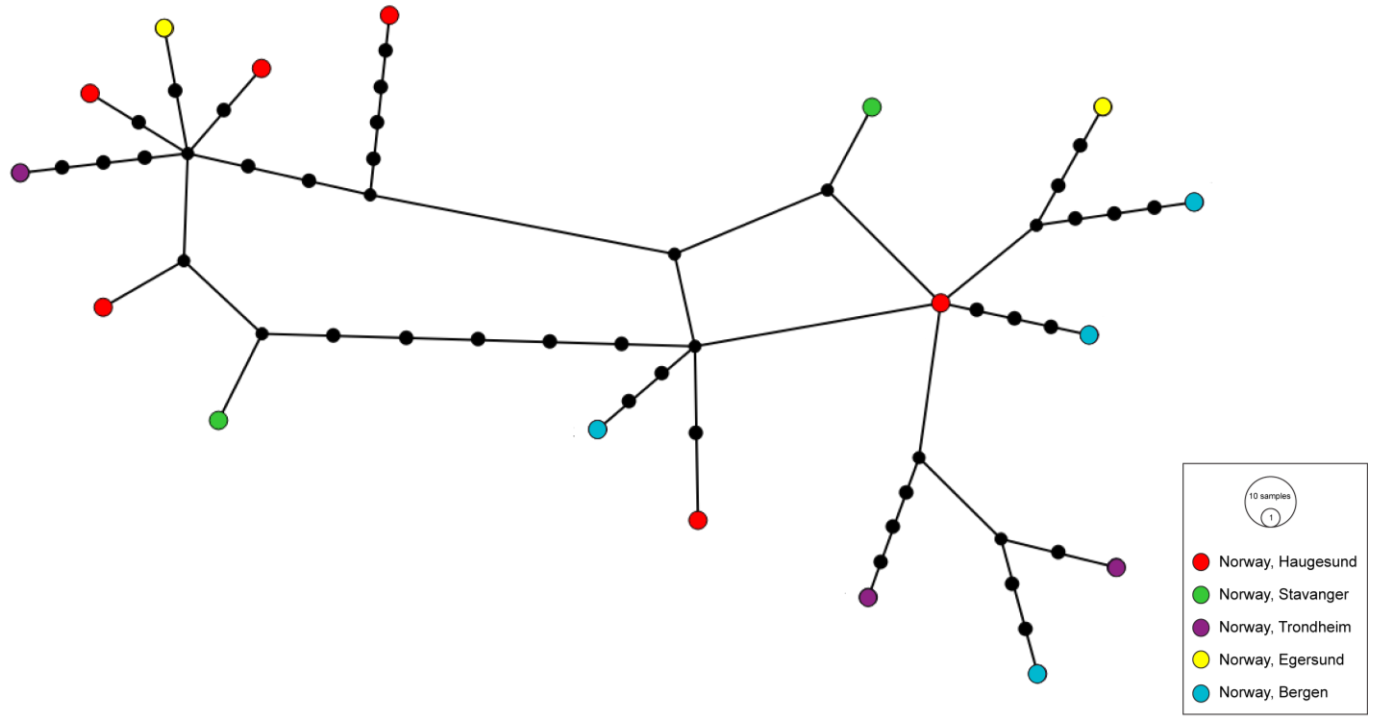


Figure 17 – TCS haplotype network analysis based on the COI gene generated in the programme PopArt, including sequences from 17 specimens of *Polycera* n. sp.. Lines between black dots represent one mutation, while black dots represent hypothetical haplotypes. Each coloured circle represents a unique haplotype, and the size of each circle indicates how many specimens share that haplotype. Different colours represent geographical locations.

For *P. quadrilineata* (Fig. 18), represented by 63 specimens gathered from areas around Scandinavia, the Mediterranean Sea and the Azores, the TCS network also showed high genetic diversity. However, nine haplotypes were shared by multiple individuals between regions (Table 1, 2 in Appendix 7.1). Among these, four haplotypes were shared by two individuals each, two were shared by three individuals, one by four individuals, one by five, and the most common haplotype by 11 individuals (Fig. 18; Table 2 in Appendix 7.1). Several haplotypes were found in multiple localities within Norway, and three haplotypes were also found in Sweden (Fig. 18; Table 2 in Appendix 7.1). However, there

was no clear geographical structure in the network apart from the specimens from the Azores (n = 4), which clustered together (Fig. 18). The haplotypes from the Azores are between 2–3 base pairs different (0.3–0.5%), and the difference to the closest haplotypes outside of the Azores (two haplotypes from Norway) are 4 base pairs (0.7%).

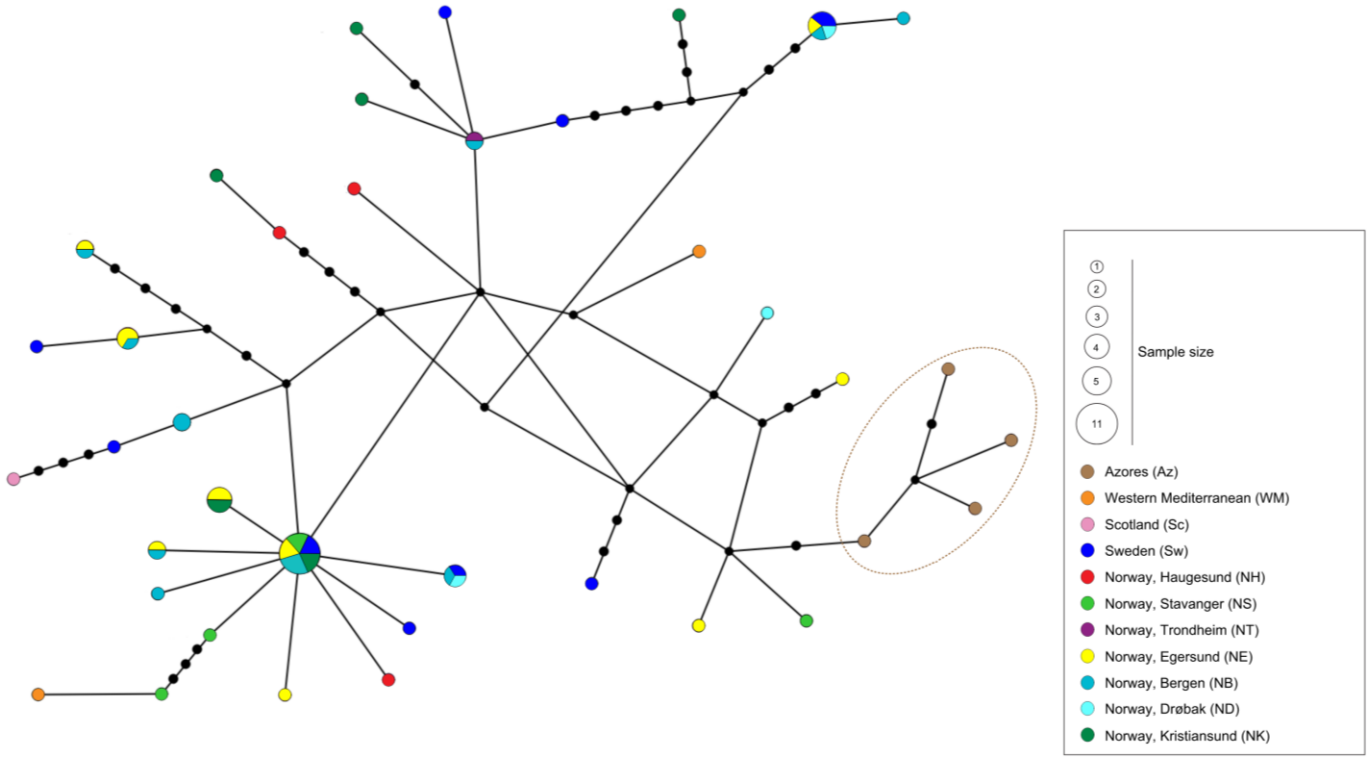


Figure 18 – TCS haplotype network analysis based on the COI gene generated in the programme PopArt, including sequences from 63 specimens of *P. quadrilineata*. Lines between black dots represent one mutation, while black dots represent hypothetichal haplotypes. Each coloured circle represents a unique haplotype, and the size of each circle indicates how many specimens share that haplotype. Different colours represent geographical locations.

5. DISCUSSION

5.1 Species diversity and molecular variation: a new species of *Polycera*

The COI gene is a fast evolving, standard mitochondrial gene used for molecular barcoding of marine invertebrates (Hebert et al., 2003; Dawnay et al., 2007; Geller et al., 2013; Ratnasingham & Hebert, 2013), that allows discrimination between species. It is extensively used for studying systematics, phylogeography and population genetics (Dawnay et al., 2007; Geller et al., 2013), which is why it was

chosen and implemented in this study. The combination of the Bayesian phylogenetic COI gene tree analysis (Fig. 7; Fig. 1 in Appendix 3) together with the genetic distances (Table 3) and species delimitation analysis (Figs 1–6 in Appendix 6), clearly supported the occurrence of a second lineage of *Polycera* species in Norway which up until now was masked under the species name *P. quadrilineata* assumed to be a part of this species natural chromatic variability. The specimens here ascribed to *P. quadrilineata* received maximum support in the Bayesian analysis (Fig. 7; Fig. 1 in Appendix 3), and were attributed to this species since it included representatives from the type locality, namely Drøbak in the Oslofjord, Norway, and matched the original description by O. F. Müller (1776; 1779; 1788). Interestingly, the new species identified in this work, which has been confused historically with *P. quadrilineata*, was rendered sister to a clade containing the European species *P. faeroensis* (also present in Norway) and the undetermined *Polycera* sp. A from South Africa. Despite sharing a more recent common ancestry with this three former species, *P. quadrilineata* seems to be closer related to *P. aurantiomarginata* from Spain and Morocco, and *P. capensis* from South Africa and Australia (Fig. 7; Fig. 1 in Appendix 3; Table 3).

All but one sister pair of *Polycera* species studied in this work have an inter-specific genetic variability ranging between 8.8–19.7 % (Table 3) and intra-specific variability ranging from 0–2.6 %, showing the existence of a clear DNA barcode gap between species. The genetic distance between *P. capensis* and *P. aurantiomarginata* is considerably lower compared to the rest of the species, ranging from 4.3–5.8 %. Potentially this could be due to the fact that the taxonomic status of *P. capensis* is still debatable, and it haven been suggested that this species might not be valid (Palomar et al., 2014). Nevertheless, even if the latter distance is considered, there is still a clear molecular barcode gap between sister species among the *Polycera* species studied in this work (Table 3; Figs 1, 3, 5 in Appendix 6). Furthermore, the genetic variability between *P. quadrilineata* and the new lineage recognized in this work was estimated to 9.6–12.4 % (Table 3), supporting their distinct taxonomic status. Additionally, the molecular species delimitation analysis using the three evolutionary models implemented supported the presence of two separate species within *P. quadrilineata* (*P. quadrilineata* and *Polycera* n. sp.) regardless the *P*-value (ABGD; Figs 1, 3, 5 in Appendix 6).

5.2 Cryptic species or overlooked species?

The term 'cryptic species' is as previously mentioned somewhat confusing by being largely given various sub-designations and multiple usages (Korshunova et al., 2019). Based on the current discovery of morphotypes that easily distinguish *P. quadrilineata* (dark continuous or dashed stripes, and black rhinophores; Figs 8, 14) from the new species (dark dotted or patchy dotted; Figs 11, 16) it seems reasonable to think of these species as being either 'pseudo-cryptic' or 'quasi-cryptic' due to their recognizable morphological differences (Horsáková et al., 2019; Korshunova et al., 2019). Alternatively, they may even be considered not cryptic, but instead holding differences which have been previously overlooked and confused. However, this is not the case when the two species possesses their shared (yellow/orange) morphotype (Fig. 15) which makes them undistinguishable and hence clearly cryptic.

Subtle morphological differences between *P. quadrilineata* and *Polycera* n. sp. were detected (summarized in Table 4), but due to some overlapping characters they seem to reflect more a trend rather than discrete characters. For example, the number of gills and lamellae in the rhinophores (7–11 and 6–15, respectively in *P. quadrilineata,* and 7–9 and 6–10, respectively in *Polycera* n. sp.). However, the information about these characters was collected from taxonomical pictures rather than living specimens and could therefore have been a limiting factor, particularly when counting the number of gills. Nevertheless, as illustrated in the results the two species are both genetically and anatomically distinct (Table 3; Figs 9, 10, 12, 13). In this study *P. quadrilineata* has an average body size (16.6 mm) larger than the new species here described (5 mm) (Table 1 in Appendix 4), yet the total number of specimens studied (18 for *Polycera* n. sp., and 63 for *P. quadrilineata*) maybe account for some bias in the measurements since only few were actually measured (12 measured for *Polycera* n. sp., and 24 measured for *P. quadrilineata*; Table 1 in Appendix 4). Also, despite *P. quadrilineata* having a larger average body size, one specimen (P34) showed that the new species may reach up to 14 mm, questioning the conclusion about *P. quadrilineata* attaining a larger body length.

5.3 Mimicry in *Polycera* species?

The aposematic colouration of *P. quadrilineata* (Tullrot & Sundberg, 1991; Tullrot, 1994), and possibly *Polycera* n. sp., questions whether or not their morphological resemblance is due to some

kind of mimicry. In Batesian mimicry, a toxic or distasteful organism gets mimicked by an "imposter", the so called 'mimic species', in order to reduce its own predation (Davies, 2012) (classical example is the king snake mimicking the poisonous coral snake). In Müllerian mimicry on the other hand, unpalatable species that can be either related or unrelated help each other by performing reciprocal mimicry against a common predator (*e.g.* between insects like the cuckoo bee and yellow jacket wasp) (Campbell et al., 2015).

It is still unknown exactly what type of deterrent chemicals *P. quadrilineata* may possess, only that predators find it repulsive (Tullrot & Sundberg, 1991; Tullrot, 1994). It is also hard to say whether or not *Polycera* n. sp. may share this aposematic behaviour with *P. quadrilineata,* or if it just uses it to its own advantage by looking morphologically similar. Hence, so far it is difficult to determine if mimicry does occur between these *Polycera* species or not, and if so what type of mimicry. This line of thought is merely speculative, but the topic could be interesting to pursue in a future study

5.4 Trophic ecology and seasonal occurrence

From the material examined (Table 1) both *P. quadrilineata* and *Polycera* n. sp. proved to be sympatric in Norway, living side by side, sharing the same habitat and apparently diet, namely the encrusted bryozoans *Membranipora membranacea* and *Electra pilosa* that mostly grow on the lamina of *Laminaria* kelp. However, considering their digestive system differences (Figs 9, 12) it is possible that they in fact use different food resources. While *Polycera* n. sp. has a weaker labial cuticle (Fig. 12C–F) and a radula with a smaller number of rows and teeth organized more far apart from each other (Fig. 12A, B), *P. quadrilineata* have a thicker, more robust labial cuticle (Fig. 9C–F) and a radula with more rows and teeth (Fig. 9A, B). However, this was not evaluated in the present study.

Comparing the seasonal abundance of the two species in Norway (Figs 1, 2 in Appendix 5) based on studied material (13 specimens of the new species; 41 specimens of *P. quadrilineata*), and data from Evertsen & Bakken (2015) for *P. quadrilineata* (25), it is possible to see that the new species was only present during a few months of the year during different seasons (Fig. 1 in Appendix 5), whereas *P. quadrilineata* has an overall more evenly distributed abundance across the different seasons, being more abundant along the entire year (Fig. 2 in Appendix 5). The seasonal data showed highest

abundance of *P. quadrilineata* during late winter to the middle of summer, and the highest abundance of *Polycera* n. sp. during fall and early to late winter. However, these results can most likely be an artefact of sampling bias since the collection of sea slugs was not designed to evaluate the annual dynamic of the *Polycera* populations. It is not clear whether the fact that *Polycera* n. sp. had a much higher abundance in November and December than *P. quadrilineata* reflects its natural dynamic or again is an artefact of sampling. It is therefore based on the present data difficult to confidently determine whether or not one species is more commonly found during a specific time of the year than the other. Future studies to specifically address this matter would have to be conducted. Nevertheless, it is clear that both species can be found together at certain months, namely January to February, June to August, the end of September to the end of October, and in December (Figs 1, 2 in Appendix 5).

5.5 Population structure analysis

The population structure analysis showed a high genetic diversity and a lack of geographical structure within both species (Figs 17, 18). However, due to the overall low sampling number in each population of the current study it is difficult to say anything about the mutation rate and gene flow (Table 1; Table 1 in Appendix 7.1). In order to better understand the population structure of each species it is therefore necessary to taken a larger sampling number for each of the sampling sites into account in future studies.

The fact that the Azorean specimens showed a structural grouping in the haplotype network of *P. quadrilineata* could potentially indicate some degree of genetic isolation (Fig. 18). This is not surprising given the geographic placement of the Azores being far away from the other localities, in addition to this area having a more temperate climate than Scandinavian countries. Among the nine haplotypes of *P. quadrilineata* found in multiple localities, specimens from Bergen (Norway) and Egersund (Norway) appeared in nearly all cases, and while all shared haplotypes contained Norwegian specimens, only three included specimens from Sweden as well (Fig. 18; Table 1, 2 in Appendix 7.1). This might indicate some form of gene flow between populations from Norway and Sweden, which is not surprising considering their close geographical boarders. Additionally, the fact that Bergen and Egersund are the geographical areas with most shared haplotypes is probably caused

by a sampling artefact since these areas had an overall larger sampling number compared to the other localities included in this study (Table 2 in Appendix 7.1).

Polycera n. sp. so far appears to be restricted to Norwegian waters between Northern Norway to Southern-Western Norway (Fig. 17). The fact that no haplotypes were shared within the new species in addition to almost all specimens being found in Haugesund could be because of the reduced number of specimens included in the analysis and/or a potential sampling artefact. The same could be said for the fact that no specimens were found outside of Norway (Fig. 17; Table 1 in Appendix 7.1). This remains to be further investigated.

6. CONCLUSION

The possible occurrence of cryptic species within *P. quadrilineata sensu lato* was strongly supported by the COI Bayesian analysis and the ABGD species delimitation analysis which were compatible with the occurrence of a second species, here named *Polycera* n. sp.. These two species were estimated to be 9.6–12.4 % genetically distinct (COI uncorrected *p*-distance). The COI gene tree further suggested a sister relationship between the new species and a clade (PP = 0.95) containing the European *P. faeroensis* and an undetermined *Polycera* species (*Polycera* sp. A) from South Africa.

Both *P. quadrilineata* and *Polycera* n. sp. are polychromatic, in which four colour morphs occur in the former species and three in the latter, with one colour morph being common to both species. Although, these species are anatomically distinct with different radula, labial cuticles and penile ultrastructures.

Both seem to occur in the same ecological niche, but they may feed on distinct food items. This needs to be evaluated with additional research. Geographically, *P. quadrilineata* is a worldwide species occurring from Scandinavia southwards to the Mediterranean Sea, Iberian Peninsula, and the archipelagos of the Azores, Madeira, and Canary Islands, whereas the new *Polycera* species here recognized only is confirmed in the Northern to Southern-Western coast of Norway where it is sympatric with *P. quadrilineata*.

7. REFERENCES

Agusti, A., Morla, M., Sauleda, J., Saus, C., & Busquets, X., (2004), "*NF-κB activation and iNOS upregulation in skeletal muscle of patients with COPD and low body weight*". Thorax, 59(6), p.483–487. Available at: https://thorax.bmj.com/content/thoraxjnl/59/6/483.full.pdf (2019).

Alba-Tercedor, J. & Sánchez-Tocino, L., (2011), "*The use of the SkyScan 1172 high-resolution micro-CT to elucidate if the spicules of the sea slugs (Mollusca: Nudibranchia, Opisthobranchia) have a structural or a defensive function"*. In SkyScan Users Meeting, Vol. 2011, p.113–121.

Antoniadou, C., Koutsoubas, D., & Chintiroglou, C. C., (2005), "*Mollusca fauna from infralittoral hard substrate assemblages in the North Aegean Sea*". Belgian Journal of Zoology, 135(2), p.119.

Artsdatabanken (2019). "*Polycera quadrilineata (O. F. Müller, 1776)*" [Internet]. Available at: https://www.artsdatabanken.no/Taxon/Polycera%20quadrilineata/108635 (2019)

Austin, J., Gosliner, T., & Malaquias, M. A., (2018), "*Systematic revision, diversity patterns and trophic ecology of the tropical Indo-West Pacific sea slug genus Phanerophthalmus A. Adams, 1850 (Cephalaspidea, Haminoeidae)*". Invertebrate Systematics, 32(6), p.1336–1387.

Bergan, K., & Anthon, H., (1977) "*Livet i Fjæra*" (7th ed.). Oslo, J. W. Cappelens Forlag A. S., p.38, 113.

Betti, F., Bava, S., & Cattaneo-Vietti, R., (2017), "*Composition and seasonality of a heterobranch assemblage in a sublittoral, unconsolidated, wave-disturbed community in the Mediterranean Sea*". Journal of Molluscan Studies, 83(3), p.325–332. Available at: https://academic.oup.com/mollus/article/83/3/325/3862017 (2019)

Bickford, D., Lohman, D. J., Sodhi, N. S., Ng, P. K., Meier, R., Winker, K., ... & Das, I., (2007), "*Cryptic species as a window on diversity and conservation*". Trends in Ecology & Evolution, 22(3), p.148–155.

Bruce, J. R., Jones, N. S., & Colman, J. S., (1963), "*Marine fauna of the Isle of Man and its surrounding seas*", (2th ed. revised and reset. ed.). L. M. B. C. memoirs on typical British marine plants and animals. Liverpool. Vol. 36, p.202.

Burn, R. (2015), "*Nudibranchs and Related Molluscs"*. Melbourne: Museum Victoria, [Internet book], p.25. Available at: https://books.google.no/books?hl=no&lr=&id=r0teDQAAQBAJ&oi=fnd&pg=PR8&dq=Nudibranchs+and+Related+Molluscs&ots=dge2QjFzV&sig=BcscAI162mDV2vYpa_w3hTrdIq4&redir_esc=y#v=onepage&q&f=false (2019).

Caballer, M., & Ortea, J. (2002), "*Primera cita de Polycera hedgpethi Marcus, 1964 (Mollusca: Opisthobranchia) para la península Ibérica*". Noticiario de la Sociedad Española de Malacología, 37, p.55–56.

Campbell, N., Reece, J., Urry, L., Cain, M., Wasserman, S., & Minorsky, P., (2015). "*Biology: A global approach"* (10th ed., Always learning: G-22 Glossary). Boston: Pearson, p.1285.

Carbone, M., Herrero-Barrencua, A., Ciavatta, M. L., Castro, J. J., Cervera, J. L., & Gavagnin, M., (2019), "*Occurrence of symmetrical diacylguanidines triophamine and limaciamine in three polyceridae species from Canary Islands: Are they chemical markers of these nudibranchs*?". Biochemical Systematics and Ecology, 83, p.62–65. Available at: https://doi.org/10.1016/j.bse.2019.01.005 (2019)

Carmona, L., Pola, M., Gosliner, T. M., & Cervera, J. L., (2013), "*A tale that morphology fails to tell: a molecular phylogeny of Aeolidiidae (Aeolidida, Nudibranchia, Gastropoda)*". PLoS ONE, 8(5), E63000. Avalable at: https://doi.org/10.1371/journal.pone.0063000 (2019)

Carmona, L., Bhave, V., Salunkhe, R., Pola, M., Gosliner, T. M., & Cervera, J. L., (2014**a**). "*Systematic review of Anteaeolidiella (Mollusca, Nudibranchia, Aeolidiidae) based on morphological and molecular data, with a description of three new species*". Zoological Journal of the Linnean Society, 171(1), p.108–132. Available at: https://doi.org/10.1111/zoj.12129 (2019)

Carmona, L., Lei, B. R., Pola, M., Gosliner, T. M., Valdés, Á., & Cervera, J. L., (2014**b**). "*Untangling the Spurilla neapolitana (Delle Chiaje, 1841) species complex: a review of the genus Spurilla Bergh, 1864 (Mollusca: Nudibranchia: Aeolidiidae): the species compex*". Zoological Journal of the Linnean Society, 170(1), p.132–154. Availabe at: https://doi.org/10.1111/zoj12098 (2019)

Cervera, J. L., Calado, G., Gavaia, C., Malaquias, M. A., Templado, J., Ballesteros, M. B. V., ... & Megina, C., (2004). "*An annotated and updated checklist of the ophisthobranchs (Mollusca: Gastropoda) from Spain and Portugal (including islands and archipelagos)"*. Boletín del Instituto Español de Oceanografía, 2004, vol. 20, num. 1–4, p.1–122.

Churchill, C., Valdés, &., & Ó Foighil, D., (2014). "*Molecular and morphological systematics of neustonic nudibranchs (Mollusca : Gastropoda : Glaucidae:), with descriptions of three new cryptic species".* Invertebrate Systematics, 28(2), p.174–195.

Claridge, A. W., Mifsud G., Dawson, J. & Saxon, M. J., (2005), "*Use of infrared digital cameras to investigate the behaviour of cryptic species*". Wildlife Research, 31(6), p.645–650. Available at https://doi.org/10.1071/WR03072 (2018)

Clement M, Snell Q, Walke P, Posada D, Crandall, K., (2002). "*TCS: estimating gene genealogies*". Proc (16th ed.) Int Parallel Distrib Process Symp Vol.2, p.184.

Darriba, D., Taboada, G. L., Doallo, R., & Posada, D., (2012). "*jModelTest2: more models, new heuristics and parallel computing*". Nature methods, 9(8), p.772.

Davies, N., Krebs, J., & West, S., (2012). "*An introduction to behavioural ecology"* (4th ed.). Hoboken: John Wiley & Sons. Cap 4, p.98–102.

Dawnay N., Ogden R., Mcewing R., Carvalho G., & Thorpe R., (2007). "*Validation of the barcoding gene COI for use in forensic genetic species identification*". Forensic Science International, 173(1), 1–6. Available at: https://doi.org/10.1016/j.forsciint.2006.09.013 (2019).

Dean, L. J., & Prinsep, M. R., (2017), "*The chemistry and chemical ecology of nudibranchs*". Natural Product Reports, 34(12), p.1359–1390. Available at: https://pubs.rsc.org/en/content/articlehtml/2017/np/c7np00041c (2019)

Eales, N. (1967),*"*The littoral fauna of the British Isles: A handbook for collectors" (4th ed.). Cambridge: Cambridge University Press. p.205–206.

Edgar, R. C., (2004). "*MUSCLE: multiple sequence alignment with high accuracy and high throughput*". Nucleic Acids Research, 32(5), p.1792–1797.

Edwards, R. V., (2008) "*Polycera quadrilineata*: *A sea slug*". In Tyler-Walters H., and Hiscock K., (eds). Marine Life Information Network: Biology and Sensitivity Key Information Reviews, [Internet]. Plymouth: Marine Biological Association of the United Kingdom. Available at: https://www.marlin.ac.uk/species/detail/1982 (2018)

Eilertsen M. H., & Malaquias M. A. E., (2013) "*Systematic revision of the genus Scaphander (Gastropoda, Cephalaspidea) in the Atlantic Ocean, with a molecular phylogenetic hypothesis"* (Report). Zoological Journal of the Linnean Society, 167(3), p.389–429.

Epstein, H., Hallas, J., Johnson, R., Lopez, A., & Gosliner, T., (2018). "*Reading between the lines: Revealing cryptic species diversity and colour patterns in Hypselodoris nudibranchs (Mollusca: Heterobranchia: Chromodorididae)*". Zoological Journal of the Linnean Society, 186(1), p.116–189. Available at: https://doi.org/10.1093/zoolinnean/zly048 (2019)

Evertsen, J., & Bakken, T., (2005). "*Nudibranch diversity (Gastropoda, Heterobranchia) along the coast of Norway*". Fauna norvegica, 25, p.1–37. Available at: https://www.researchgate.net/profile/Torkild_Bakken/publication/257442633_Nudibranch_diversity_Hetero

branchia_Gastropoda_along_the_coast_of_Norway/links/0deec525c487f92c61000000/Nudibranch-diversity-Heterobranchia-Gastropoda-along-the-coast-of-Norway.pdf (2019)

Flammensbeck, C. K., Haszprunar, G., Korshunova, T., Martynov, A. V., Neusser, T. P., & Jörger, K. M., (2019). "*Pseudovermis paradoxus 2.0—3D microanatomy and ultrastructure of a vermiform, meiofaunal nudibranch (Gastropoda, Heterobranchia)*". Organisms Diversity & Evolution, 19(1), p.41–62. Available at: https://link.springer.com/content/pdf/10.1007%2Fs13127-018-0386-2.pdf (2019)

Folmer, O., Black, M., Hoeh, W., Lutz, R. & Vrijenhoek, R., (1994). "*DNA primers for amplification of mitochondrial cytochrome c oxydase subunit I from diverse metazoan invertebrates*". Molecular Marine Biology and Biotechnology, 3(5), p.294–299. Available at: https://www.mbari.org/wp-content/uploads/2016/01/Folmer_94MMBB.pdf (2019)

Furfaro, G., & Mariottini, P., (2016). "*Check-list of the Nudibranchs (Mollusca Gastropoda) from the biodiversity hot spot "Scoglio del Corallo" (Argentario promontory, Tuscany)*". Biodiversity Journal, 7(1), p.67–78.

Geller, J., Meyer, C., Parker, M., & Hawk, H., (2013). "*Redesign of PCR primers for mitochondrial cytochrome c oxidase subunit I for marine invertebrates and application in all-taxa biotic surveys"*. Molecular Ecology Resources, 13(5), p.851–861. Available at: https://doi.org/10.1111/1755-0998.12138 (2019)

Giacobbe, S., & De Matteo, S., (2013). "*The potentially invasive opisthobranch Polycera hedgpethi Er. Marcus, 1964 (Gastropoda Nudibranchia), introduced in a Mediterranean coastal lagoon*". Biodiversity Journal, 4(2), p.359–364.

Goodheart, J. A., (2017). "*Insights into the systematics, phylogeny, and evolution of Cladobranchia (Gastropoda: Heterobranchia)*". American Malacological Bulletin, 35(1), p.73–82.

Harley, C., Pankey, M., Wares, J., Grosberg, R., & Wonham, M., (2006). "*Color Polymorphism and Genetic Structure in the Sea Star Pisaster ochraceus*". The Biological Bulletin, 211(3), p.248–262. Available at: https://www.journals.uchicago.edu/doi/abs/10.2307/4134547 (2019)

Hayward, P. J., & Ryland, J. S., (1995). "*Handbook of the Marine Fauna of North-West Europe*". Oxford: Oxford University Press, [Internet book], p.539–543 & p.558–560. Available at: https://books.google.no/books?id=sZ8mdRT4jbIC&pg=PA558&lpg=PA558&dq=phanerobranchiates&source=bl&ots=Xx51_772BU&sig=ACfU3U02kFEZZiWsBNeb6uIFcD9meEnA&hl=no&sa=X&ved=2ahUKEwjd87CmpdrjAhUj4aYKHbJZDvgQ6AEwAHoECAAQAQ#v=onepage&q=phanerobranchiates&f=false (2019)

Hayward, P. J., & Ryland, J. S., (2017). "*Handbook of the Marine Fauna of North-West Europe*", (2th ed.). Oxford: Oxford University Press, [Internet book], p.528–529. Available at: https://books.google.no/books?id=Ltv_DQAAQBAJ&pg=PA528&dq=polycera+quadrilineata&hl=no&sa=X&ved=0ahUKEwjltKLG5ZvlAhWpwsQBHUbAA1cQ6AEIPTAC#v=onepage&q=polycera%20quadrilineata&f=false (2019)

Hebert, P. D., Ratnasingham, S., & De Waard, J. R., (2003). "*Barcoding animal life: cytochrome c oxidase subunit 1 divergences among closely related species*". Proceedings of the Royal Society of London, series B: Biological Sciences, 270(Suppl_1), p.96–99. Available at: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1698023/pdf/12952648.pdf (2019).

Heethoff, M., (2018). "*Cryptic Species – Conceptual or Terminological Chaos? A Response to Struck et al.".* Trends in Ecology & Evolution, 33(5), p.310. Available at: https://www.cell.com/action/showPdf?pii=S0169-5347%2818%2930038-7 (2019)

Hermosillo, A., & Valdés, Á., (2007), "*A new Polycera (Opisthobranchia: Mollusca) from Bahía de Banderas, México"*. Proceedings of the California Academy of Sciences, Vol. 58(23), p.477–484.

Herron, J. & Freeman, S. (2014). "*Evolutionary analysis"*, (5th ed., Glossary). Boston: Pearson, p.830.

Hickman, Jr., C. P., Roberts, L. S., Keen S. L., Eisenhour, D. J., Larson A., & I'Anson, H., (2014), "*Integrated Principles of Zoology"*, (16th ed.). 2 Penn Plaza, New York: McGraw-Hill Education, p.332.

Horsáková, V., Nekola, J. C., & Horsák, M., (2019). "*When is a "cryptic" species not a cryptic species: A consideration from the Holarctic micro-landsnail genus Euconulus (Gastropoda: Stylommatophora)*". Molecular phylogenetics and evolution, 132, p.307–320. Available at: https://doi.org/10.1016/j.ympev.2018.12.004 (2019)

Huelsenbeck, J. P., & Ronquist, F., (2001). "*MRBAYES: Bayesian inference of phylogenetic trees*". Bioinformatics, 17(8), p.754–755. Available at: http://146.6.100.192/users/CH391L/Handouts/MRBAYES.pdf (2019)

Hunnam, P., & Brown, G., (1975). "*SUBLITTORAL NUDIBRANGH MOLLUSCA (SEA SLUGS) IN PEMBROKESHIRE WATERS*". Field Studies, 4, p.131–159. Available at: https://fsj.field-studies-council.org/media/350665/vol4.2_101.pdf (2019)

Jörger, K. M., Heß, M., Neusser, T. P., & Schrödl, M., (2009), "*Sex in the beach: Spermatophores, dermal insemination and 3D sperm ultrastructure of the aphallic mesopsammic Pontohedyle*

milaschewitchii (Acochlidia, Opisthobranchia, Gastropoda)". Marine Biology, 156(6), p.1159–1170. Available at: https://link.springer.com/article/10.1007/s00227-009-1158-5 (2019)

Keppel, E., Sigovini, M., & Tagliapietra, D., (2012). "*A new geographical record of Polycera hedgpethi Er. Marcus, 1964 (Nudibranchia: Polyceridae) and evidence of its established presence in the Mediterranean Sea, with a review of its geographical distribution".* Marine Biology Research, 8(10), p.969–981. Available at: https://doi.org/10.1080/17451000.2012.706306 (2019)

Khan Academy (2019) "*Polymerase Chain Reaction (PCR)*" [Internet]. Available at: https://nb.khanacademy.org/science/biology/biotech-dna-technology/dna-sequencing-pcrelectrophoresis/a/polymerase-chain-reaction-pcr (2019)

Knowlton, N. (1986); "*CRYPTIC AND SIBLING SPECIES AMONG THE DECAPOD CRUSTACEA*". Journal of Crustacean Biology, 6(3), p.356–363. Available at: https://doi.org/10.1163/193724086X00208 (2018)

Korshunova, T., Picton, B., Furfaro, G., Mariottini, P., Pontes, M., Prkić, J., ... & Martynov, A., (2019). "*Multilevel fine-scale diversity challenges the 'cryptic species' concept*". Scientific reports, 9(1): 6732, p.1–23.

Kumar, S., Stecher, G., Li, M., Knyaz, C., & Tamura, K., (2018), "*MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms*". Molecular Biology and Evolution, 35(6) p.1547–1549.

Layton, K. K., Gosliner, T. M., & Wilson, N. G., (2018). "*Flexible colour patterns obscure identification and mimicry in Indo-Pacific Chromodoris nudibranchs (Gastropoda: Chromodorididae)*". Molecular Phylogenetics and Evolution, 124, p.27–36. Available at: https://doi.org/10.1016/j.ympev.2018.02.008 (2019)

Leigh, J. W., & Bryant, D., (2015). "*Popart: Full-feature software for haplotype network construction*". Methods in Ecology and Evolution, [Internet], 6(9), p.1110–1116. Available at: http://popart.otago.ac.nz/index.shtml (2019)

Lemche, H., & Thompson, T. E., (1974). "*Three Opisthobranch gastropods new to the British fauna*". Journal of Molluscan Studies, 41(3), p.185–193. Available at: https://doi.org/10.1093/oxfordjournals.mollus.a065268 (2019).

Maddison, W. P. & D.R. Maddison (2018). "*Mesquite: a modular system for evolutionary analysis*". Version 3.51. [Internet]. Available at: http://www.mesquiteproject.org (2019)

Martínez-Pita, I., Sánchez-España, A. I., & García, F. J., (2006). *"Some aspects of the reproductive biology of two Atlantic species of Polycera (Mollusca: Opisthobranchia)".* Journal of the Marine Biological Association of the United Kingdom, 86(2), p.391–399. Available at: https://doi.org/10.1017/S0025315406013245 (2019).

Martínez-Pita, I., & García, F. J., (2017). "*Embryonic Abnormalities of Two Sea slug Species from South Spain*". Thalassas: An International Journal of Marine Sciences, 33(2), p.109–115.

Martynov, A. V., Korshunova, T. A., & Savinkin, O. V., (2006) "*Shallow-water opisthobranch molluscs of the Murman coast of the Barents Sea, with new distributional data and remarks on biology*". Ruthenica, 16(1–2), p.59–72.

Megina, C & Cervera, J. L., (2003). "*Diet, prey selection and cannibalism in the hunter opisthobranch Roboastra europaea*". Journal of the Marine Biological Association of the United Kingdom, 83(3), p.489–495.

Megina, C., Gosliner, T. M., & Cervera, J. L., (2007). "*The use of trophic resources by a generalist eolid nudibranch: Hermissenda crassicornis (Mollusca: Gastropoda)*". Cahiers de biologie marine, 48(1): p.1–7. Available at: https://www.researchgate.net/publication/262314042 (2018)

Micaroni, V., Strano, F., Di Franco, D., Crocetta, F., Grech, D., Piraino, S., & Boero, F., (2018). "*Project 'Biodiversity MARE Tricase': A biodiversity inventory of the coastal area of Tricase (Ionian Sea, Italy) – Mollusca: Heterobranchia*". The European Zoological Journal, 85(1), p.179–192. Available at: https://www.tandfonline.com/doi/pdf/10.1080/24750263.2018.1462413?needAccess=true (2019)

Miller, M. C., (1961) "*Distribution and Food of the Nudibranchiate Mollusca of the South of the Isle of Man*". Journal of Animal Ecology, 30(1), p.95–116.

Miller, M. C., (1996) "*A new species of the dorid nudibranch genus Polycera Cuvier, 1816 (Gastropoda: Opisthobranchia) from New Zealand*". Journal of Molluscan Studies, 62(4), p.443–450. Available at: https://doi.org/10.1093/mollus/62.4.443 (2019)

Miller, M. A., Pfeiffer, W., & Schwartz, T. (2010) "*Creating the CIPRES Science Gateway for inference of large phylogenetic trees*". In Proceedings of the Gateway Computing Environments Workshop (GCE), New Orleans, LA, [Internet], p.1–8. Available at: https://www.phylo.org/portal2/login!input.action (2019)

Moen, F. E. & Svensen, E., (2014), "*Dyreliv i havet; Nordeuropeisk Marin Fauna*", (6th ed.). Kristiansund: Kom forlag. p.403, 410, 411.

MolluscaBase (2019**a**), "*Polycera quadrilineata (O. F. Müller, 1776)*". [Internet], Accessed through: World Register of Marine Species (WoRMS). Available at: http://www.marinespecies.org/aphia.php?p=taxdetails&id=140838 (2019).

MolluscaBase (2019**b**), "*Heterobranchia*". [Internet], Accessed through: World Register of Marine Species (WoRMS). Available at: http://www.marinespecies.org/aphia.php?p=taxdetails&id=14712 (2019).

MolluscaBase (2019**c**). "*Polycera Cuvier, 1816*". [Internet], Accessed through: World Register of Marine Species (WoRMS). Available at: http://www.marinespecies.org/aphia.php?p=taxdetails&id=138369 (2019).

Mortensen S., & Svensen E., (2010), "*Livet i Fjæra*". Oslo: Kom Forlag, p.31, 32, 42, 51, 80–87, and p.247–248.

Müller, O. F., (1776). "*Zoologiae Danicae prodromus: seu Animalium Daniae et Norvegiae indigenarum; characteres, nomina, et synonyma imprimis popularium*". Hallageriis; Havniae (Copenhagen), p.229. Available at: https://www.biodiversitylibrary.org/item/47550#page/265/mode/1up (2019)

Müller, O. F., (1779). "*Zoologia Danica, seu, Animalium Daniae et Norvegiae rariorum ac minus notorum descriptiones et historia*". Sumtibus Weygandinis: Hauniae et Lipsiae (Copenhagen) Vol. 1, (C3) p.37. Available at: https://archive.org/details/zoologiadanicase01mlle/page/36 (2019)

Müller, O. F., Abildgaard, P. C., Müller, C. F., Holten, J. S., Vahl, M., & Rathke, J., (1788). "*Zoologia Danica, seu Animalium Daniae et Norvegiae rariorum ac minus notorum descriptiones et historia*". Typis N. Mölleri aulae regiae typographi et filii: Havniae (Copenhagen). Plate 17 (XVII), figs 4–6. Vol. 2, p.38. Available at: https://archive.org/details/zoologiadanicase02ml/page/n37 (2019)

OBIS, (2014). "*Polycera quadrilineata (O. F. Müller, 1776)*". Records obtained from Ocean Biogeographic Information System. Intergovernmental Oceanographic Commission of UNESCO, [Internet]. Available at: https://obis.org/taxon/140838 (2019)

Padula, V., Araujo, A. K., Matthews-Cascon, H., & Schrödl, M., (2014). "*Is the Mediterranean nudibranch Cratena peregrina (Gmelin, 1791) present on the Brazilian coast? Integrative species delimitation and description of Cratena minor n. sp.".* Journal of Molluscan Studies, 80(5), 575-584. Available at: https://doi.org/10.1093/mollus/eyu052 (2019)

Padula, V., Bahia, J., Stöger, I., Camacho-García, Y., Malaquias, M. A. E., Cervera, J. L., & Schrödl, M., (2016). "*A test of color-based taxonomy in nudibranchs: Molecular phylogeny and species delimitation of the Felimida clenchi (Mollusca: Chromodorididae) species complex*". Molecular Phylogenetics and Evolution, 103, p.215–229. Available at: https://doi.org/10.1016/j.ympev.2016.07.019 (2019)

Palomar, G., Pola, M., & Garcia-Vazquez, E., (2014). "*First molecular phylogeny of the subfamily Polycerinae (Mollusca, Nudibranchia, Polyceridae)*". Helgoland Marine Research, 6*8*(1), p.143–153. Available at: https://hmr.biomedcentral.com/articles/10.1007/s10152-013-0374-z (2019)

Penney, B., Ehresmann, K., Jordan, K., & Rufo, G., (2018). "*Micro-computed tomography of spicule networks in three genera of dorid sea-slugs (Gastropoda: Nudipleura: Doridina) shows patterns of phylogenetic significance*". Acta Zoologica, p.1–19. Available at: https://doi.org/10.1111/azo.12266 (2019)

Pola, M., & González Duarte, M. M., (2008), "*Is self-fertilization possible in nudibranchs?*". Journal of Molluscan Studies, 74(3), p.305–308. Available at: https://academic.oup.com/mollus/article/74/3/305/1022306 (2019)

Pola, M., & Gosliner, T. M., (2010). "*The first molecular phylogeny of cladobranchian opisthobranchs (Mollusca, Gastropoda, Nudibranchia)*". Molecular Phylogenetics and Evolution, 56(3), 931–941. Available at: https://doi.org/10.1016/j.ympev.2010.05.003 (2019)

Pola, M., Sánchez-Benítez, M., & Ramiro, B., (2014). "*The genus Polycera Cuvier, 1817 (Nudibranchia: Polyceridae) in the eastern Pacific Ocean, with redescription of Polycera alabe Collier & Farmer, 1964 and description of a new species*". Journal of Molluscan Studies, 80(5), p.551–561. Available at: https://doi.org/10.1093/mollus/eyu049 (2019)

Puillandre, N., Lambert, A., Brouillet, S., & Achaz, G., (2012), "*ABGD, Automatic Barcode Gap Discovery for primary species delimitation"*. Molecular Ecology, 21(8), p.1864–1877.

Rambaut, A., & Drummond, A. J., (2016). "*FigTree vertion 1.4.3*", [Internet]. Available at: http://tree.bio.ed.ac.uk/software/figtree/ (2019)

Ratnasingham, S., & Hebert, P. D., (2013). "*A DNA-based registry for all animal species: the Barcode Index Number (BIN) system*". PLoS ONE, 8(7), E66213. Available at: https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0066213 (2019).

Ronquist, F., Huelsenbeck, J., & Teslenko, M., (2011). "*Draft MrBayes version 3.2 Manual: Tutorials and Model Summaries"*. Distributed with the software from http://brahms. biology. rochester.

edu/software. html. Cap.2, p.9–30. Avalable at: http://bioinformatics.se/tools/mrbayes/mb3.2_manual.pdf (2019)

Rudman, W. B., (1999). "*Polycera quadrilineata (Muller, 1776)*", [Internet], Sea Slug Forum. Australian Museum, Sydney. Available at: http://www.seaslugforum.net/factsheet/polyquad (2019)

Sakamoto, Y., Ishiguro, M., & Kitagawa, G., (1986). "*Akaike information criterion statistics*". Dordrecht, The Netherlands: D. Reidel, 81.

Schmekel, L., Portmann, A., Richter, I., & Bousani-Baur, S., (1982). "*Opisthobranchia des Mittelmeeres; Nudibranchia und Saccoglossa"*. Berlin Heidelberg New York, Springer-Verlag, p.110–111.

Struck, T., Feder, J., Bendiksby, M., Birkeland, S., Cerca, J., Gusarov, V., . . .& Dimitrov, D., (2018), "*Finding Evolutionary Processes Hidden in Cryptic Species*". Trends in Ecology & Evolution, 33(3), p.153–163. Available at: https://www.sciencedirect.com/science/article/pii/S0169534717302902 (2019)

Swedmark, B., (1964). "*The interstitial fauna of marine sand*". Biological Reviews, 39(1), p.1–42.

Tarjuelo, I.; Posada, D.; Crandall, K. A.; Pascual, M. & Turon, X. (2004) "*Phylogeography and speciation of colour morphs in the colonial ascidian Pseudodistoma crucigaster*". Molecular Ecology, 13(10), p.3125–3136.

Telnes, K. (2018), *"Nudibranch – Polycera quadrilineata".* [Internet], SeaWater: The Marine Flora & Fauna of Norway. Available at: http://www.seawater.no/fauna/mollusca/quadrilineata.html (2019)

Thermo Fisher Scientific (2019). "*Loading Dyes and Buffers-Thermo Scientific: Buffer for electrophoresis*". [Internet]. Available at: https://www.thermofisher.com/no/en/home/brands/thermo-scientific/molecular-biology/thermo-scientific-nucleic-acid-electrophoresis-purification/dna-electrophoresis-thermo-scientific/loading-dyes-buffers.html#buffers (2019)

Thompson, T. E., (1976), "*Biology of Opisthobranch Molluscs: 151*". London: Ray Society, Vol. I: p.15, 59, 103.

Thompson, T. E., (1988), "*Molluscs: Benthic Opisthobranchs (Mollusca: Gastropoda): Keys and notes for the identification of the species"*. Synopses of the British Fauna (New Series), (2th ed.). Leiden: Brill, Vol. 8: p.202–204.

Thompson, T. E., & Brown G. H., (1984), "*Biology of Opisthobranch Molluscs: 156*". London: Ray Society, Vol. II: p.67–69.

Todd, C. D. (1981), "*The Ecology of Nudibranch Molluscs*". Oceanography and Marine Biology: An Annual Review. Vol. 19: p.141–233.

Todd, C. D. (1983), "*Reproductive and trophic ecology of nudibranch molluscs*". The Mollusca: Ecology Vol. 6: p.225–259.

Trainito E., (2005), "*Nudibranchi del Mediterraneo. Guida ai molluschi opistobranchi*" Il Castello, p.96.

Tullrot, A. & Sundberg, P. (1991), "*The conspicuous nudibranch Polycera quadrilineata: Aposematic colouration and individual selection*". Animal Behavior, 41(1), p.175–176.

Tullrot, A. (1994), "*THE EVOLUTION OF UNPALATABILITY AND WARNING COLORATION IN SOFT-BODIED MARINE INVERTEBRATES".* Evolution, 48(3), p.925–928. Available at: https://www.jstor.org/stable/2410499?seq=1#metadata_info_tab_contents (2018)

Vondrák, J., Říha, P., Arup, U., & Søchting, U., (2009). "*The taxonomy of the Caloplaca citrina group (Teloschistaceae) in the Black Sea region; with contributions to the cryptic species concept in lichenology*". The Lichenologist, 41(6), p.571–604.

Wägele, H., & Willan, R.C., (2000). "*Phylogeny of the Nudibranchia*". Zoological Journal of the Linnean Society, 130(1), p.83–181. Available at: https://doi.org/10.1111/j.1096-3642.2000.tb02196.x (2019)

Wilson, N. G., & Burghardt, I., (2015). "*Here be dragons-phylogeography of Pteraeolidia ianthina (Angas, 1864) reveals multiple species of photosynthetic nudibranchs (Aeolidina: Nudibranchia)*". Zoological Journal of the Linnean Society, 175(1), p.119–133. Available at: https://doi.org/10.1111/zoj.12266 (2019)

APPENDIX 1: MOLECULAR WORK

1.0 DNA extraction using the Qiagen DNeasy Blood and Tissue Kit (Ref. No. 69506)

Prior to DNA extraction all tissue samples had to be dried to remove access ethanol from earlier conservation in order to prevent the ethanol from inhibiting the DNA extraction process, thereby contaminating the experiments. When dried, 180 µl of lysis ATL buffer and 20 µl proteinase K was added to each 1.5 ml Eppendorf tube containing the tissue. ATL buffer helps open up the cells through lysis in order to extract the DNA, while proteinase K breaks down the proteins. To prevent contamination between samples the pipette tip was changed during each content transfer. Mixing of tissue samples and chemicals were done thoroughly by vortexing (3 sec.) and spinning, before transferring each of the Eppendorf tubes into a 56°C heat block incubator where they were kept overnight for lysis processing.

Following day, each samples were initially given a 15 sec. vortexing and a quick spin before being added 200 µl AL buffer (immediately vortexed for 5 sec. for it to yield a homogenous solution) and 200 µl absolute ethanol (immediately mixed for 5 sec.). After mixing, all Eppendorf content were pipetted over to separate 'DNeasy mini spin column tubes' (= filtering tubes) kept in 2.0 ml 'collecting tubes'. The AL buffer helps attaching DNA to the filter, while ethanol helps coiling the DNA so that it more easily attaches to the filter. From here each filtering tube were centrifuged at 8000 rpm (rotation per minute) for 1 min. Leftover content that had gone through the filters were discarded, while the filters themselves, containing the DNA, were transferred into new 2.0 ml collecting tubes and added 500 µl AW1 washing buffer, followed by 1 min. centrifugation at 8000 rpm, and run-through discarding. Further cleansing were conducted following the same procedure as before, except this time each filters were first added 500 µl AW2 washing buffer and centrifuge for 1 min. at 8000 rpm, followed by discarding the leftovers before centrifuging the filters a second time, only this time at full speed 13 000 rmp for 3 min. to remove all liquid contamination and ethanol leftovers. Fully cleansed filters were transferred into new 1.5 ml Eppendorf tubes. For the first round 200 µl Buffer AE was added directly onto the DNeasy membrane filter, and incubated for about 2–5 min. in room temperature (approximately 25°C) in order for the DNA to be released before centrifugation in 1 min. at 8000 rpm. The AE buffer helps the DNA dis-attached from the filter more easily. For the seconds round the exact same procedure was followed, except this time with only 100 µl Buffer AE.

By adding 100 µl instead of 200 µl it increases the final DNA concentration which would be used for the Polymerase Chain Reaction (PCR), and decreases the overall DNA yield. The DNA extracts was kept cold in the refrigerator while starting the initial PCR preparations.

1.1 Preparing the PCR (Polymerase Chain Reaction) amplification samples

The PCR amplifications was run on the mitochondrial COI gene, who's universal primers were initially diluted from 100 µM to 10 µM (100 µl) concentration solutions created in separated 1.5 ml Eppendorf tubes by adding 10 µl primer and 90 µl ddH_2O, while kept on ice. Each amplification samples were to contain a total volume of 50 µl (49 µl master cocktail and 1 µl DNA extract). Exact amounts of each mix ingredient were estimation by multiplying their standard amount on the according number of samples that were to be amplified, including one positive control and one negative control, and one for pipetting error. While creating the master cocktails each chemical first had to be properly defrosted, and it was here utterly important to add TAQ for last, leaving it in the freezer until the very end to prevent the enzyme from overheating (very heat sensitive), thereby activating too earlier. In order to keep it inactive the whole mixing process was prepared in 1.5 ml Eppendorf tubes kept on ice or in a cooling tube-box. When fully prepared, each tube was mixed by vortexing (3 sec.) followed by a quick spin before pipetting 49 µl of master cocktail into new 0.2 ml microfuge tubes for the according number of samples. Finally, before transferring the PCR products into the thermal cycler, each sample were added 1 µl corresponding DNA extract, respectively.

1.2 Adjustments in the molecular lab

Smaller quantities of PCR product proved to work just as well for the PCR amplification after a few runs. Hence, in order to save chemicals and economical expenses, the initial 50 µl PCR product was halved down to 25 µl (*i.e.* 24 µl master mix and 1µl DNA) during the later runs. Standard quantities reduced to 8.25 µl ddH_2O, 2.5 µl buffer, 2.5 µl dNTP, 5 µl Q-solution, 3.5 µl MgCl, 1 µl per primer, and 0.25 µl TAQ. Further adjustments was done by replacing the standard buffer that were used in the two first PCR amplifications with a CoraLLoad (CL) buffer. Instead of having to add a loading buffer into the PCR samples before adding them to the gel-electrophoresis, the CL buffer already includes a loading buffer thereby saving a lot of lab work, time and money.

1.3 Post-PCR

1.3.1 Preparation of agarose gel

Prior to electrophoresis, a total volume of 200 ml, 1.0% TAE (Tris-acetate-EDTA) gel solution was prepared by adding 2.0 g agarose (in dry weight) and 200 ml 1x TAE buffer into a glass bottle. TAE is a standard buffer mixture consisting of tris base, acetic acid and EDTA, and is a common buffer used when running agarose electrophoresis on bigger DNA fragments (*i.e.* longer nucleic acid fragments) since it's compatible with the enzyme reactions and works by separating the DNA and/or RNA (Thermo Fisher Scientific, 2019). Processing of the agarose gel were initiated using microwave heating (approximately 30 sec. or 1 min.), followed by magnetic mixing using the IKA^{R}RCT basic safety control mixer and a magnet. Processing was repeated until the agarose gel had become properly mixed, getting as clear as possible. Note, that while blending, the lid on the glass bottle was kept slightly upon due to gas exchange and it was necessary to use heat protective gloves.

1.3.2 Electrophorese

DNA electrophorese was conducted using a fitting electrophorese chamber and a UVT gel-tray. The tray was properly placed into the chamber in a casting position where it worked as an agarose gel platform preventing the gel from leaking out into the chamber pools. One to two combs (depending on the chamber size and number of samples), each comb consisting of 10 arms, were placed in the tray and used to create smaller wells, or reservoirs, in the gel for the according number of PCR samples, including a ladder marker. For the first round a mix of agarose gel (warm and fluent) and GelRed (GR) was added to the tray. For the runs including 18 or more samples, 50 ml agarose gel and 3 µl GR was added, whereas the smaller runs including only 10 samples was added 30 ml agarose gel and 1µl GR. Before handling GR it was important to use protective gloves. After adding the mix, the gel rested in about 15 min. (depends on chamber and tray size) in order for the agarose gel to stiffen into a firm gel plate.

When stiffened, the gel tray was carefully placed in a running position and the comb(s) were removed exposing the newly created wells. For the next step it was important to use the exact same buffer as before, namely 1x TAE buffer, which was added to each chamber pools until the whole agarose gel was covered, drowning the wells. Since the PCR products themselves were not heavy enough to sink

into the wells they had to be mixed with a Ficoll 5x (bromophenol blue dye) loading dye. Note, for the later runs containing the CoraLLoad buffer this step was neglected. Mixing was done by placing 1 µl Ficoll 5x droplets onto a piece of tape (notably with good distance to prevent DNA mixing) for the according number of samples, where each droplet was mixed with 4 µl PCR product, including the negative –and positive controls, respectively, leaving 5 µl of final mixed solution. Before transferring the mixed products into separate gel wells, leaving the last well for the negative control, each well row was added 5 µl of FastRuller ladder marker into the first well. The ladder marked was used as a reference to whether or not the DNA fragments would get the right band length, as well as how much PCR product (= DNA) each samples contained. When all wells had been filled the electrophorese chamber was closed and the electrophorese run set to 80 volt for 30 min..

During the run the gel was added electrical currents which started pulling the PCR products, or DNA fragments, that had been added to the wells through the agarose gel (Khan Academy, 2019). After 30 min. the gel plate was transferred and laid out on a UV-radiation Syngene (Cambridge, UK) machine equipped with a chemiluminescence sensitive video camera (Agusti et al., 2004). With the aid of the software programs GeneSnap (v.7.01) and GeneTools (v.4.00), both from Syngene, the gel bands were visualized and estimated for DNA quantity through densitometry (Agusti et al., 2004). GeneSnap is a camera program connected to Syngene used for studying the film containing the PCR results by visualizing the UV-radiated gel bands showing if the samples worked (giving of a light band) or not (shows nothing). Radiation exposure set to 400 ms (milliseconds). GeneTool is a calculation program that was used to estimate the DNA content of each band. In general, too much DNA in the PCR sequencing reactions can result in deficits of the BigDye products, resulting in the excessive DNA quantity using up all the BigDye before all samples have been sequenced. From the working samples, both band length (converted into number of base pairs = bp) and light intensity (converted into nano gram showing the DNA content in each band) was taken into account when estimating the exact amount of PCR product that were to be used in the upcoming PCR sequencing reactions.

1.4 Purification of PCR products protocol

In order to only sequence clean DNA, PCR products were purified using EXOSAP, a combination of Exonuclease I (EXO I) and Shrimp Alkaline Phosphatase (SAP). These enzymes are rather heat

sensitive, especially SAP. The enzymes were taken out of the freezer only right before use and put back immediately after the needed volume had been aliquoted. The EXOSAP master cocktail was prepared in a 1.5 ml Eppendorf tube kept on ice, where standard ingredients was; 0.1 µl EXO, 1.0 µl SAP, and 0.9 µl ddH_2O. To create enough master cocktail, each standard quantity were multiplied by the according number of samples that were to be purified including one, sometimes two, for compensating pipetting error(s). After mixing, the Eppendorf content was blended by flickering and given a quick spin. New 0.2 ml microfuge tubes were added 2 µl EXOSAP master mix and 8 µl PCR product, respectively. To avoid vortexing, mixing was done by either pipetting or flicking, followed by a quick spin before transferring the samples into the thermal cycler for enzyme cleansing using following conditions; 37°C for 30 min., 85°C for 15 min., and 4°C for cooling/HOLD.

1.5 Preparing sequencing reactions

For the final DNA sequencing products each primer were diluted from their original 10 µM concentrations down to 3.2 µM by following the standard lab protocol. Exact volume of diluted primers were estimated by the formula; C1V1 = C2V2. Dilution of each primer was done in separate 1.5 ml Eppendorf tubes (one per primer) by adding 34 µl of ddH_2O and 16 µl of diluted primers (those previously prepared for the PCR amplification).

In order for the universal primers to be sequenced separately so that their sequencing chromatograms (forward 5' → 3' against reverse 3' → 5') later could be compared, each primer were created their own separate master cocktail. Each master cocktails were prepared in 1.5 ml Eppendorf tube kept on ice and added the standard ingredients; 6 µl of ddH_2O, 1 µl diluted primer, 1 µl BigDye (BD), and 1 µl buffer. Like before, these quantities were multiplied by the according number of samples, including two extra in case pipetting error. PCR samples with high light intensity bands were diluted by creating 10 µl solutions consisting of 9 µl ddH_2O and 1 µl of PCR product, whereas PCR samples with natural or weak light bands were used directly. New 0.2 ml microfuge tubes for the according number of each primer were labelled with their own sequence ID (CGS1, CGS2, etc.) and added 9 µl master cocktail and 1 µl PCR product, respectively. Initially preparing all samples for the forward primer LCO1490 (F) before preparing those for the reverse primer HCO2198 (R). Each sample were mixed by flickering, given a quick spin, and transferred into the thermal cycle for about 2 hours. Last step before delivery was to add 10 µl ddH_2O to each sequencing sample.

APPENDIX 2: DATA SCRIPT FOR BAYESIAN ANALYSIS IN MrBAYES

```
BEGIN MrBayes;

set autoclose=yes nowarn=yes;

        lset nst=6 rates=invgamma;

        mcmc ngen=5000000 nruns=3 relburnin=yes burninfrac=0.25 samplefreq=100 printfreq=10000 nchains=4 savebrlens=yes starttree=random;

        sump burnin=12500;

        sumt burnin=12500 contype=halfcompat;

END;
```

Figure 1 – Script code for MrBayes.

'lset nst=6 rates=invgamma' = setting the evolutionary model to the General Time Reversible model accounting for gamma-distribution (G) across sites and a proportion of invariable sites (I).

'mcmc ngen=5000000' = total number of generations run per analysis.

'nruns=3' = number of parallel runs conducted on each analysis.

'relburnin=yes' = yes to using the burning percentage.

'burninfract=0.25' = summarizing the burn in to 25% which implies eliminating the first 25% of tree generated by the analysis.

'samplingfreq=100' = determining how often the chain is sampled (in this case once) for every 100 generation.

'printfreq=10000' = parameter frequency control. Indicates the frequency that information is shown in the screen, in this case every 10000 generations.

'nchains=4' = defines the number of parallel chains in each run of the analysis.

'savebrlens=yes' = Indicates that branch length information should be saved.

'starttree=random' = Indicates that the first tree saved is generated by random clumping/agglutination of samples.

'sump burnin=12500' = Indicates the exact number of trees to each probability values should be excluded.

'sumt burnin=12500' = Indicates the exact number of trees topologies to be excluded.

'contype=halfcompat' = defines the type of consensus tree. 'Halfcompat' results in a 50 majority rule tree. (Ronquist et al., 2011).

APPENDIX 3: THE COMPLETE BAYESIAN TREE

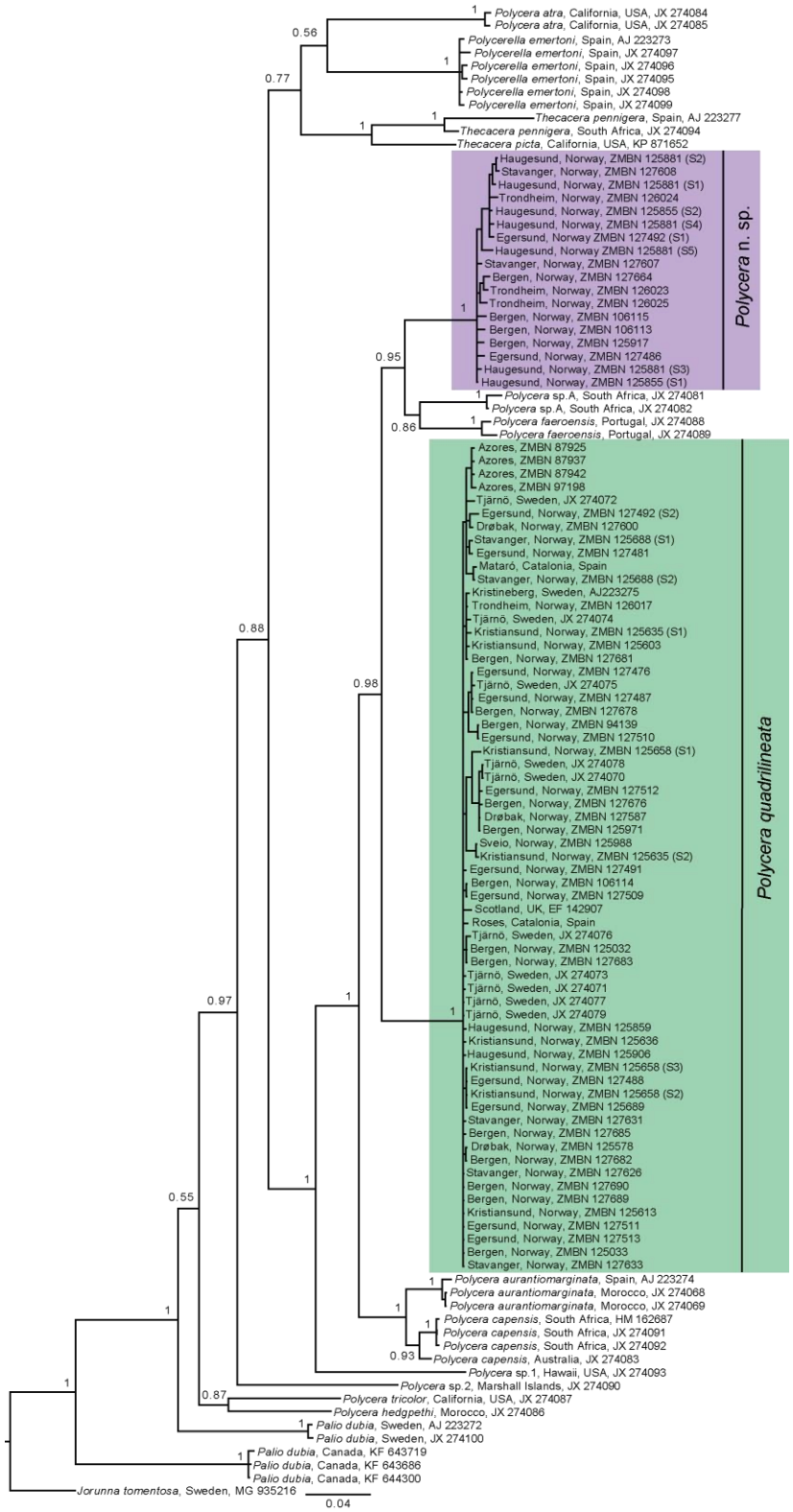


Figure 1 –Bayesian tree based on the COI gene. Numbers above branches are posterior probabilities. Tree rooted with the nudibranch *Jorunna tomentosa*. Green box containing *P. quadrilineata.* Purple box containing *Polycera* n. sp..

APPENDIX 4: TOTAL LENGTH OF SPECIMENS STUDIED

Table 1 – Size measuring data of *Polycera* n. sp. and *P. quadrilineata.* Size measured in millimetre length. Specimens (S) from the same lot were coded sequentially with the acronym S1, S2, S3, etc., in the column "Voucher no & spc. code".

Species	Sample no.	Length (mm)	Voucher no. & spc. code
Polycera n. sp.	P17	3	ZMBN 126023
Polycera n. sp.	P34	14	ZMBN 127486
Polycera n. sp.	P35	8	ZMBN 126025
Polycera n. sp.	P40	7	ZMBN 126024
Polycera n. sp.	P45	2	ZMBN 125855 (S1)
Polycera n. sp.	P46	2	ZMBN 125855 (S2)
Polycera n. sp.	P47	3	ZMBN 125881 (S1)
Polycera n. sp.	P48	3	ZMBN 125881 (S2)
Polycera n. sp.	P49	3	ZMBN 125881 (S3)
Polycera n. sp.	P50	3	ZMBN 125881 (S4)
Polycera n. sp.	P51	3	ZMBN 125881 (S5)
Polycera n. sp.	P37	9	ZMBN 127492 (S1)
Average size =		**5**	
Polycera quadrilineata	P1	25	ZMBN 125859
Polycera quadrilineata	P4	18	ZMBN 125688 (S1)
Polycera quadrilineata	P6	11	ZMBN 125032
Polycera quadrilineata	P8	20	ZMBN 125906
Polycera quadrilineata	P9	20	ZMBN 127491
Polycera quadrilineata	P10	18	ZMBN 127476
Polycera quadrilineata	P11	22	ZMBN 127512
Polycera quadrilineata	P12	15	ZMBN 127511
Polycera quadrilineata	P13	18	ZMBN 127510
Polycera quadrilineata	P14	25	ZMBN 127487
Polycera quadrilineata	P15	23	ZMBN 125988
Polycera quadrilineata	P18	25	ZMBN 127513
Polycera quadrilineata	P24	8	ZMBN 125033

Polycera quadrilineata	P28	10	ZMBN 87937
Polycera quadrilineata	P29	15	ZMBN 87942
Polycera quadrilineata	P30	8	ZMBN 87925
Polycera quadrilineata	P32	5	ZMBN 97198
Polycera quadrilineata	P33	12	ZMBN 127481
Polycera quadrilineata	P36	30	ZMBN 126017
Polycera quadrilineata	P38	20	ZMBN 127509
Polycera quadrilineata	P39	14	ZMBN 125578
Polycera quadrilineata	P42	17	ZMBN 127492 (S2)
Polycera quadrilineata	P73	6	*
Polycera quadrilineata	P74	13	*
Average size =		**16.58**	

APPENDIX 5: SEASONAL RECORDS

Graphics only representing specimens gathered from Norway. Azores specimens (n = 4) found in July. Catalonian, Spain specimens (n = 2) found in early spring (April and May).

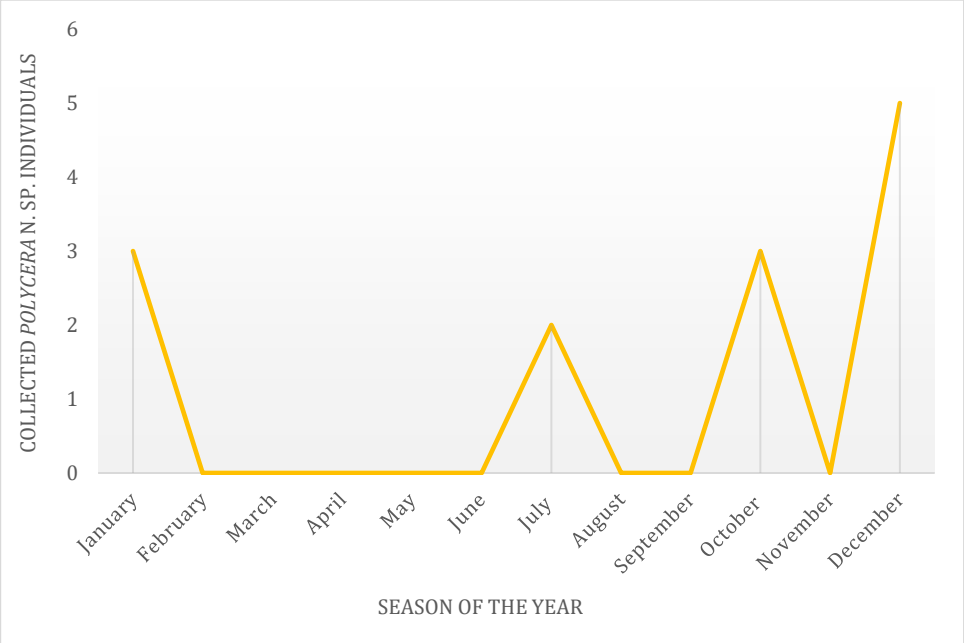


Figure 1 – Seasonal abundance of *Polycera* n. sp. specimens collected in Norway from 2015–2019. Data obtained from own study material (n = 13). Chart showing highest abundance during the winter between December and January. Additional high abundances in the middle of July and October.

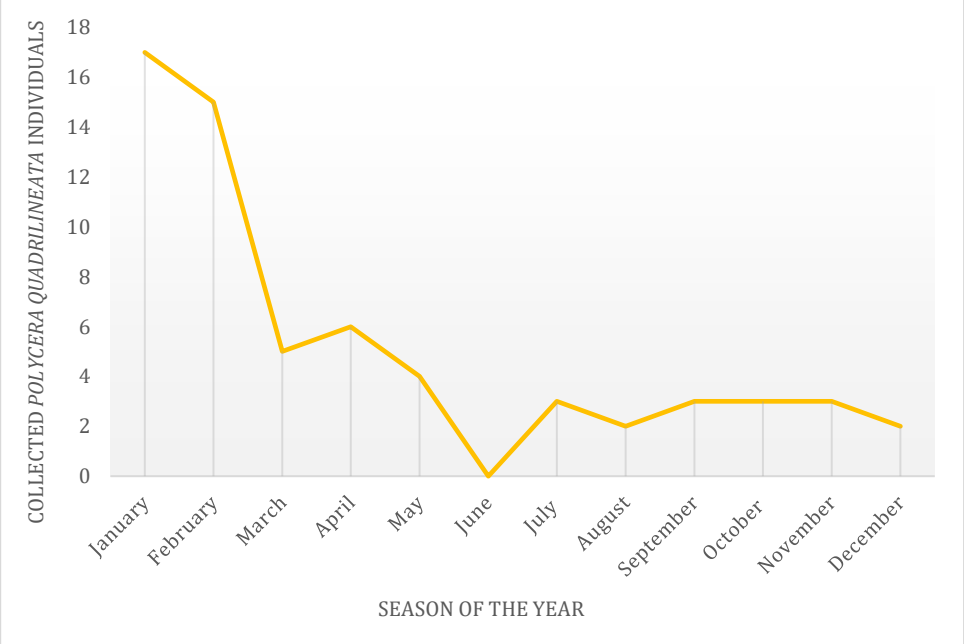


Figure 2 – Seasonal abundance of *P. quadrilineata* specimens collected in Norway from 2013–2019. Data obtained from own study material (n = 41) in addition to Evertsen & Bakken (2005) (n = 25). Chart showing highest abundance during January towards the end of May. Overall good distributed abundance throughout the entire year.

APPENDIX 6: SPECIES DELIMITATION ANALYSIS

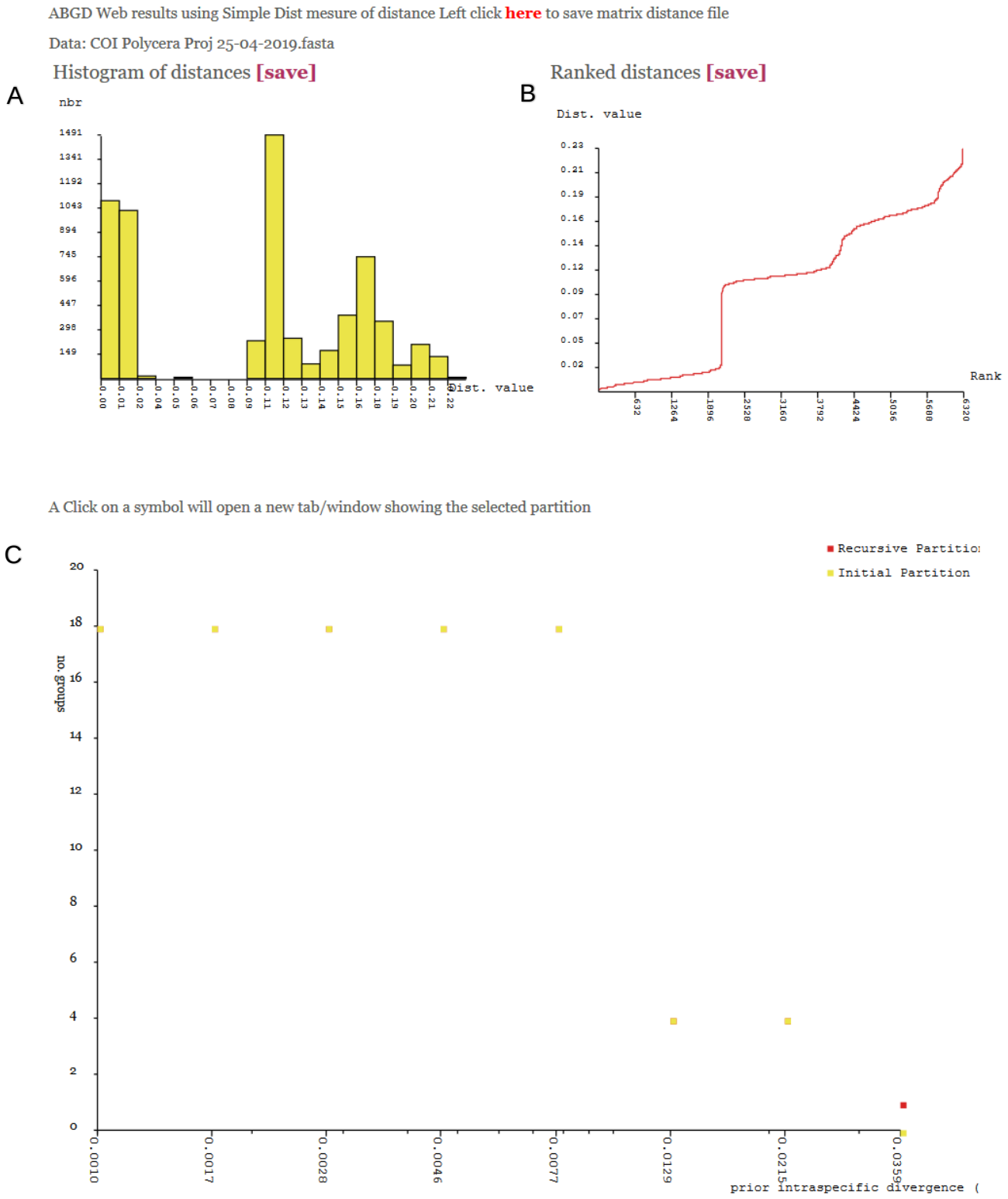
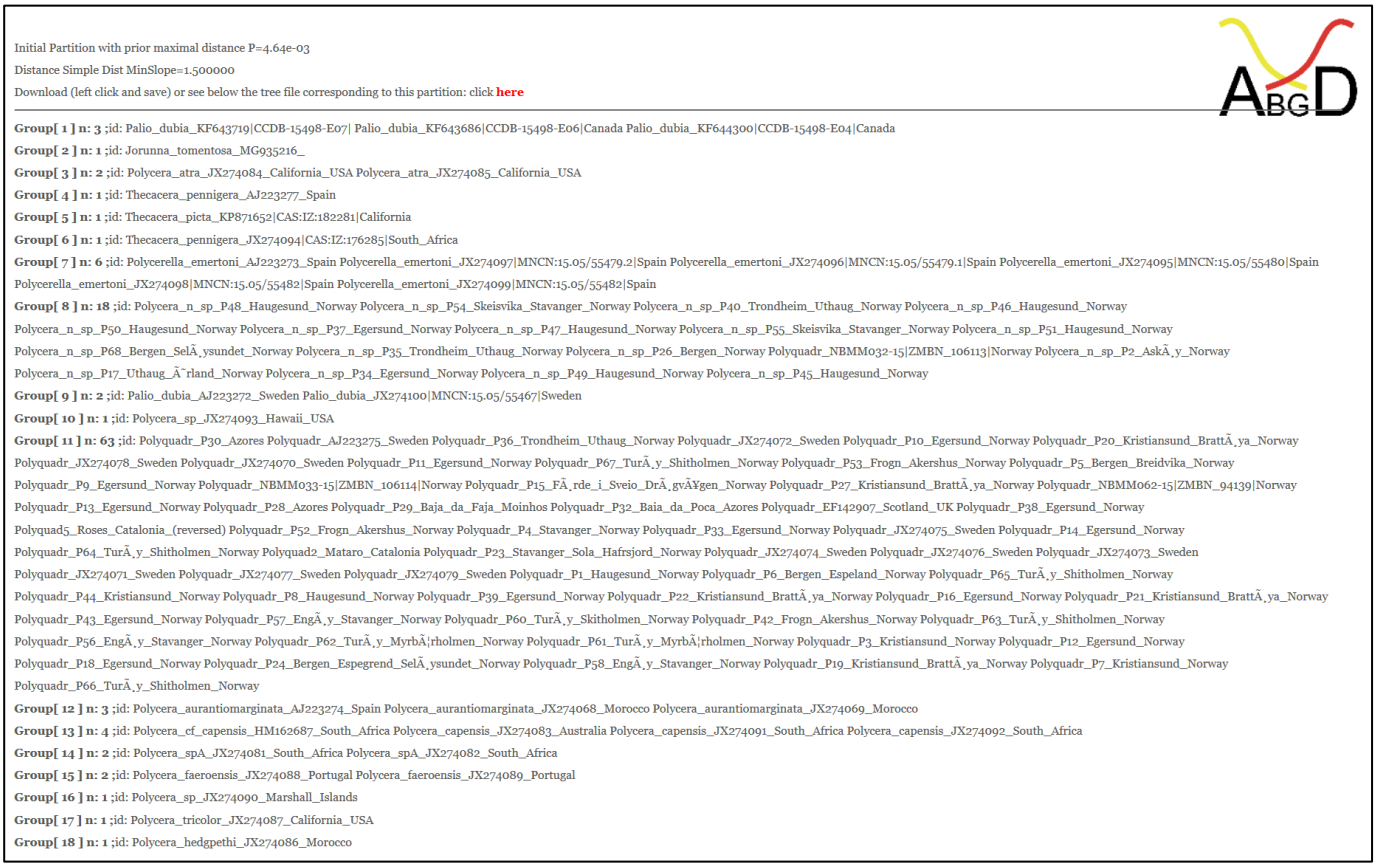


Figure 1 – Schematic illustration of the Automatic Barcoding Gap Discovery (ABGD) using 'Simple distance' analysis. **A.** distribution of pairwise differences with count-values in the vertical direction and distance values in the horizontal direction (Puillandre et al., 2012).

Low divergence presumes intra-specific divergence, whereas higher divergence represents inter-specific divergence. **B.** ranked pairwise differences (Puillandre et al., 2012). Has the same representative principle as the latter one. **C**. slope of ranked pairwise differences. Here the method automatically finds the first statistical significant slope peak. A barcoding gap will appear where there is a sudden increase in slope region. A clear barcoding gap can be seen in **A–C**, illustrated by the dashed line, supporting the hypothesis about separate species. Screen-shot taken from the 'abgd interphase website' at; http://wwwabi.snv.jussieu.fr/public/abgd/abgdweb.html.



Initial Partition with prior maximal distance P=4.64e-03
Distance Simple Dist MinSlope=1.500000
Download (left click and save) or see below the tree file corresponding to this partition: click **here**

Group[1] n: 3 ;id: Palio_dubia_KF643719|CCDB-15498-E07| Palio_dubia_KF643686|CCDB-15498-E06|Canada Palio_dubia_KF644300|CCDB-15498-E04|Canada
Group[2] n: 1 ;id: Jorunna_tomentosa_MG935216_
Group[3] n: 2 ;id: Polycera_atra_JX274084_California_USA Polycera_atra_JX274085_California_USA
Group[4] n: 1 ;id: Thecacera_pennigera_AJ223277_Spain
Group[5] n: 1 ;id: Thecacera_picta_KP871652|CAS:IZ:182281|California
Group[6] n: 1 ;id: Thecacera_pennigera_JX274094|CAS:IZ:176285|South_Africa
Group[7] n: 6 ;id: Polycerella_emertoni_AJ223273_Spain Polycerella_emertoni_JX274097|MNCN:15.05/55479.2|Spain Polycerella_emertoni_JX274096|MNCN:15.05/55479.1|Spain Polycerella_emertoni_JX274095|MNCN:15.05/55480|Spain Polycerella_emertoni_JX274098|MNCN:15.05/55482|Spain Polycerella_emertoni_JX274099|MNCN:15.05/55482|Spain
Group[8] n: 18 ;id: Polycera_n_sp_P48_Haugesund_Norway Polycera_n_sp_P54_Skeisvika_Stavanger_Norway Polycera_n_sp_P40_Trondheim_Uthaug_Norway Polycera_n_sp_P46_Haugesund_Norway Polycera_n_sp_P50_Haugesund_Norway Polycera_n_sp_P37_Egersund_Norway Polycera_n_sp_P47_Haugesund_Norway Polycera_n_sp_P55_Skeisvika_Stavanger_Norway Polycera_n_sp_P51_Haugesund_Norway Polycera_n_sp_P68_Bergen_SelÃ¸ysundet_Norway Polycera_n_sp_P35_Trondheim_Uthaug_Norway Polycera_n_sp_P26_Bergen_Norway Polyquadr_NBMM032-15|ZMBN_106113|Norway Polycera_n_sp_P2_AskÃ¸y_Norway Polycera_n_sp_P17_Uthaug_Ã¯rland_Norway Polycera_n_sp_P34_Egersund_Norway Polycera_n_sp_P49_Haugesund_Norway Polycera_n_sp_P45_Haugesund_Norway
Group[9] n: 2 ;id: Palio_dubia_AJ223272_Sweden Palio_dubia_JX274100|MNCN:15.05/55467|Sweden
Group[10] n: 1 ;id: Polycera_sp_JX274093_Hawaii_USA
Group[11] n: 63 ;id: Polyquadr_P30_Azores Polyquadr_AJ223275_Sweden Polyquadr_P36_Trondheim_Uthaug_Norway Polyquadr_JX274072_Sweden Polyquadr_P10_Egersund_Norway Polyquadr_P20_Kristiansund_BrattÃ¸ya_Norway Polyquadr_JX274078_Sweden Polyquadr_JX274070_Sweden Polyquadr_P11_Egersund_Norway Polyquadr_P67_TurÃ¸y_Shitholmen_Norway Polyquadr_P53_Frogn_Akershus_Norway Polyquadr_P5_Bergen_Breidvika_Norway Polyquadr_P9_Egersund_Norway Polyquadr_NBMM033-15|ZMBN_106114|Norway Polyquadr_P15_FÃ¸rde_i_Sveio_DrÃ¸gvÃ¥gen_Norway Polyquadr_P27_Kristiansund_BrattÃ¸ya_Norway Polyquadr_NBMM062-15|ZMBN_94139|Norway Polyquadr_P13_Egersund_Norway Polyquadr_P28_Azores Polyquadr_P29_Baja_da_Faja_Moinhos Polyquadr_P32_Baia_da_Poca_Azores Polyquadr_EF142907_Scotland_UK Polyquadr_P38_Egersund_Norway Polyquad5_Roses_Catalonia_(reversed) Polyquadr_P52_Frogn_Akershus_Norway Polyquadr_P4_Stavanger_Norway Polyquadr_P33_Egersund_Norway Polyquadr_JX274075_Sweden Polyquadr_P14_Egersund_Norway Polyquadr_P64_TurÃ¸y_Shitholmen_Norway Polyquad2_Mataro_Catalonia Polyquadr_P23_Stavanger_Sola_Hafrsjord_Norway Polyquadr_JX274074_Sweden Polyquadr_JX274076_Sweden Polyquadr_JX274073_Sweden Polyquadr_JX274071_Sweden Polyquadr_JX274077_Sweden Polyquadr_JX274079_Sweden Polyquadr_P1_Haugesund_Norway Polyquadr_P6_Bergen_Espeland_Norway Polyquadr_P65_TurÃ¸y_Shitholmen_Norway Polyquadr_P44_Kristiansund_Norway Polyquadr_P8_Haugesund_Norway Polyquadr_P39_Egersund_Norway Polyquadr_P22_Kristiansund_BrattÃ¸ya_Norway Polyquadr_P16_Egersund_Norway Polyquadr_P21_Kristiansund_BrattÃ¸ya_Norway Polyquadr_P43_Egersund_Norway Polyquadr_P57_EngÃ¸y_Stavanger_Norway Polyquadr_P60_TurÃ¸y_Skitholmen_Norway Polyquadr_P42_Frogn_Akershus_Norway Polyquadr_P63_TurÃ¸y_Shitholmen_Norway Polyquadr_P56_EngÃ¸y_Stavanger_Norway Polyquadr_P62_TurÃ¸y_MyrbÃ¦rholmen_Norway Polyquadr_P61_TurÃ¸y_MyrbÃ¦rholmen_Norway Polyquadr_P3_Kristiansund_Norway Polyquadr_P12_Egersund_Norway Polyquadr_P18_Egersund_Norway Polyquadr_P24_Bergen_Espegrend_SelÃ¸ysundet_Norway Polyquadr_P58_EngÃ¸y_Stavanger_Norway Polyquadr_P19_Kristiansund_BrattÃ¸ya_Norway Polyquadr_P7_Kristiansund_Norway Polyquadr_P66_TurÃ¸y_Shitholmen_Norway
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Group[14] n: 2 ;id: Polycera_spA_JX274081_South_Africa Polycera_spA_JX274082_South_Africa
Group[15] n: 2 ;id: Polycera_faeroensis_JX274088_Portugal Polycera_faeroensis_JX274089_Portugal
Group[16] n: 1 ;id: Polycera_sp_JX274090_Marshall_Islands
Group[17] n: 1 ;id: Polycera_tricolor_JX274087_California_USA
Group[18] n: 1 ;id: Polycera_hedgpethi_JX274086_Morocco

Figure 2 – Output data from the 'Simple Distance' ABGD analysis showing the total number of 18 groups, with n = number of sequences/specimens that exists within each group. An overview of the species included in the phylogenetic analysis. The output supports the hypothesis about having two separate species – *Polycera* n. sp. (n = 18) and *P. quadrilineata* (n = 63).

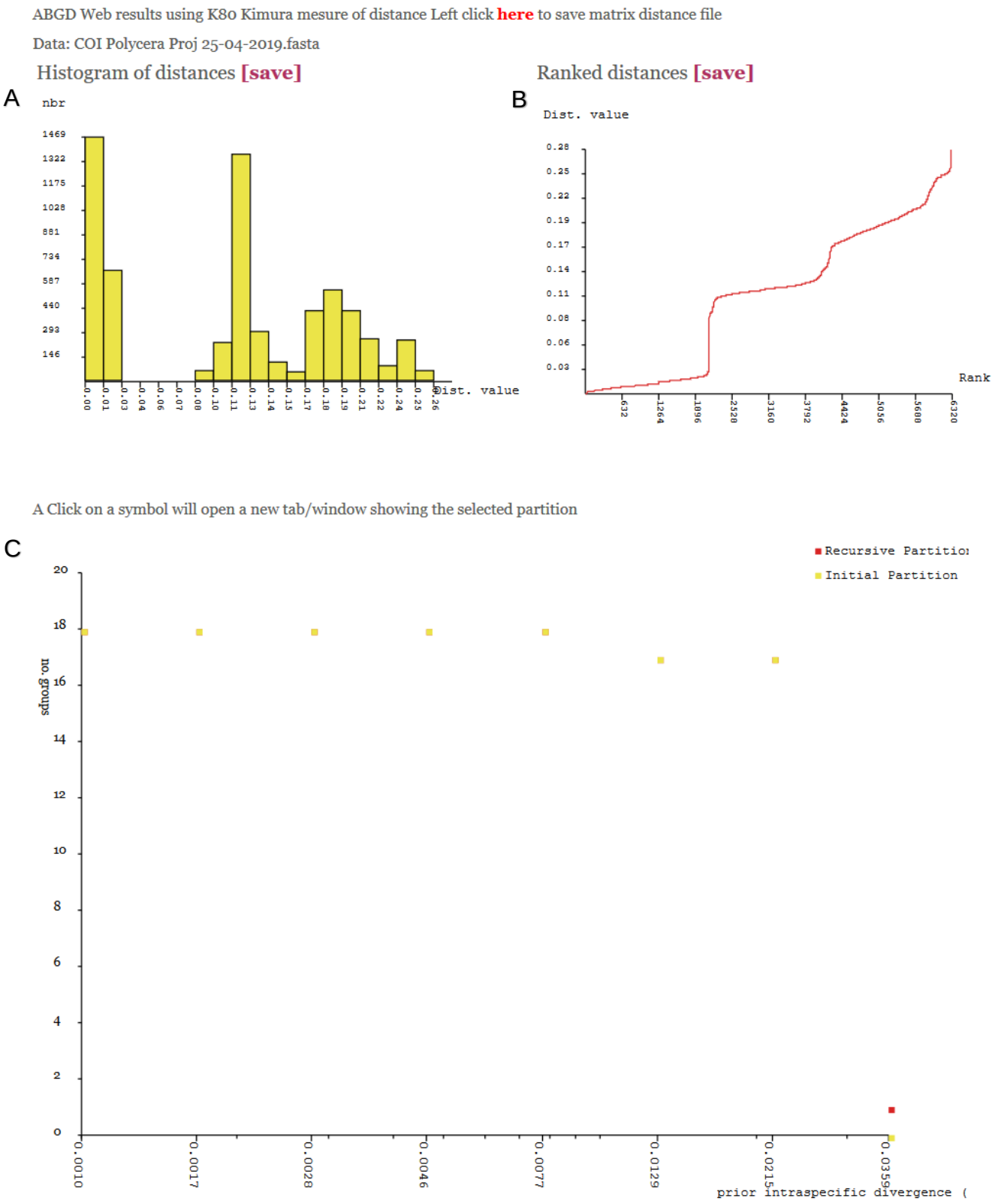
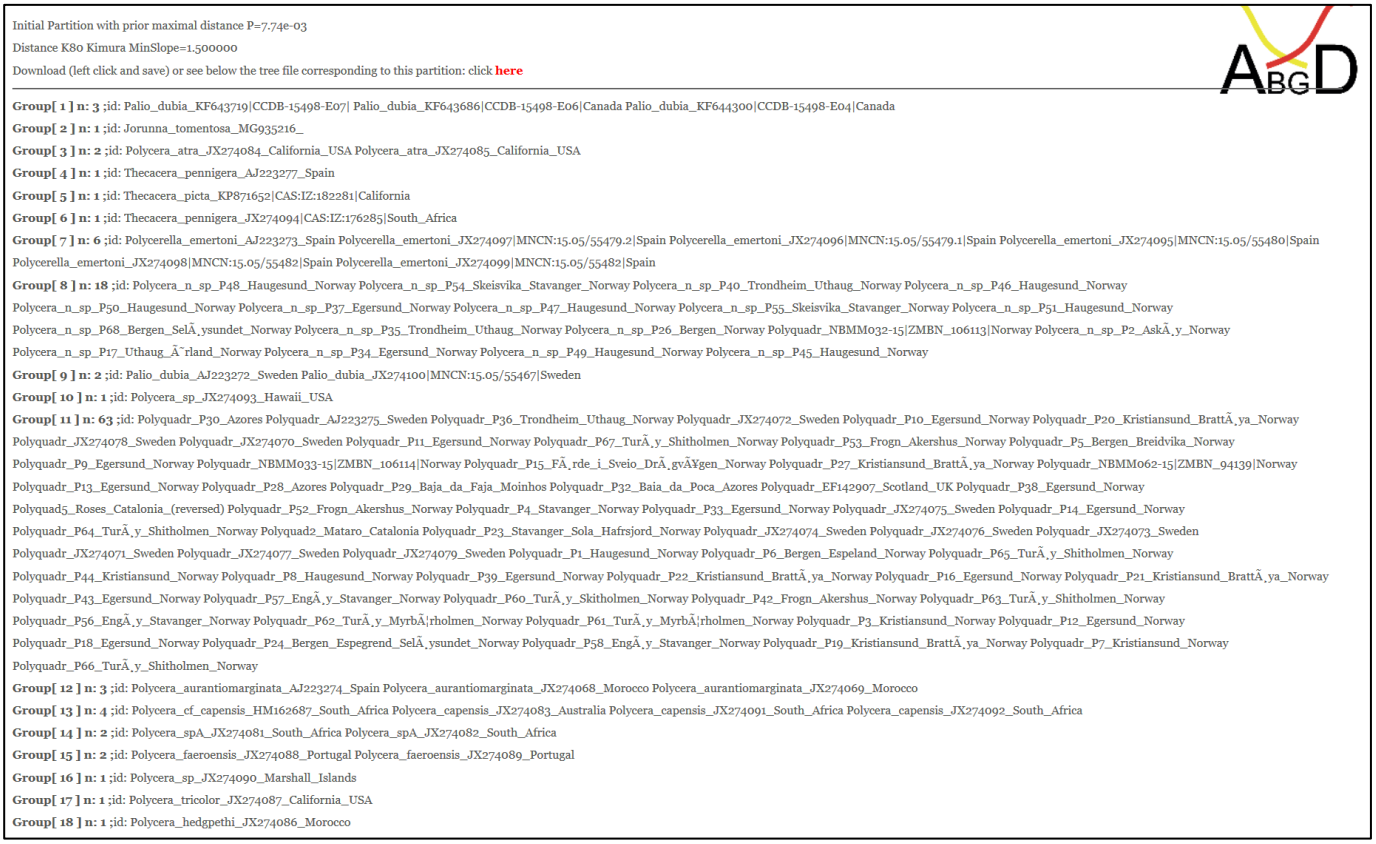


Figure 3 – Schematic illustration of the Automatic Barcoding Gap Discovery (ABGD) using 'Kimura (K80) TS/TV' [2.0]' analysis. **A.** distribution of pairwise differences with count-values in the vertical direction and distance values in the horizontal direction

(Puillandre et al., 2012). Low divergence presumes intra-specific divergence, whereas higher divergence represents inter-specific divergence. **B.** ranked pairwise differences (Puillandre et al., 2012). Has the same representative principle as the latter one. **C**. slope of ranked pairwise differences. Here the method automatically finds the first statistical significant slope peak. A barcoding gap will appear where there is a sudden increase in slope region. A clear barcoding gap can be seen in **A–C**, illustrated by the dashed line, supporting the hypothesis about separate species. Screen-shot taken from the 'abgd interphase website' at; http://wwwabi.snv.jussieu.fr/public/abgd/abgdweb.html.



Initial Partition with prior maximal distance P=7.74e-03

Distance K80 Kimura MinSlope=1.500000

Download (left click and save) or see below the tree file corresponding to this partition: click **here**

Group[1] n: 3 ;id: Palio_dubia_KF643719|CCDB-15498-E07| Palio_dubia_KF643686|CCDB-15498-E06|Canada Palio_dubia_KF644300|CCDB-15498-E04|Canada

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Group[5] n: 1 ;id: Thecacera_picta_KP871652|CAS:IZ:182281|California

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Group[12] n: 3 ;id: Polycera_aurantiomarginata_AJ223274_Spain Polycera_aurantiomarginata_JX274068_Morocco Polycera_aurantiomarginata_JX274069_Morocco

Group[13] n: 4 ;id: Polycera_cf_capensis_HM162687_South_Africa Polycera_capensis_JX274083_Australia Polycera_capensis_JX274091_South_Africa Polycera_capensis_JX274092_South_Africa

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Group[17] n: 1 ;id: Polycera_tricolor_JX274087_California_USA

Group[18] n: 1 ;id: Polycera_hedgpethi_JX274086_Morocco

Figure 4 – Output data from the 'Kimura (K80) TS/TV' [2.0]' ABGD analysis showing the total number of 18 groups, with n = number of sequences/specimens that exists within each group. An overview of the species included in the phylogenetic analysis. The output supports the hypothesis about having two separate species – *Polycera* n. sp. (n = 18) and *P. quadrilineata* (n = 63).

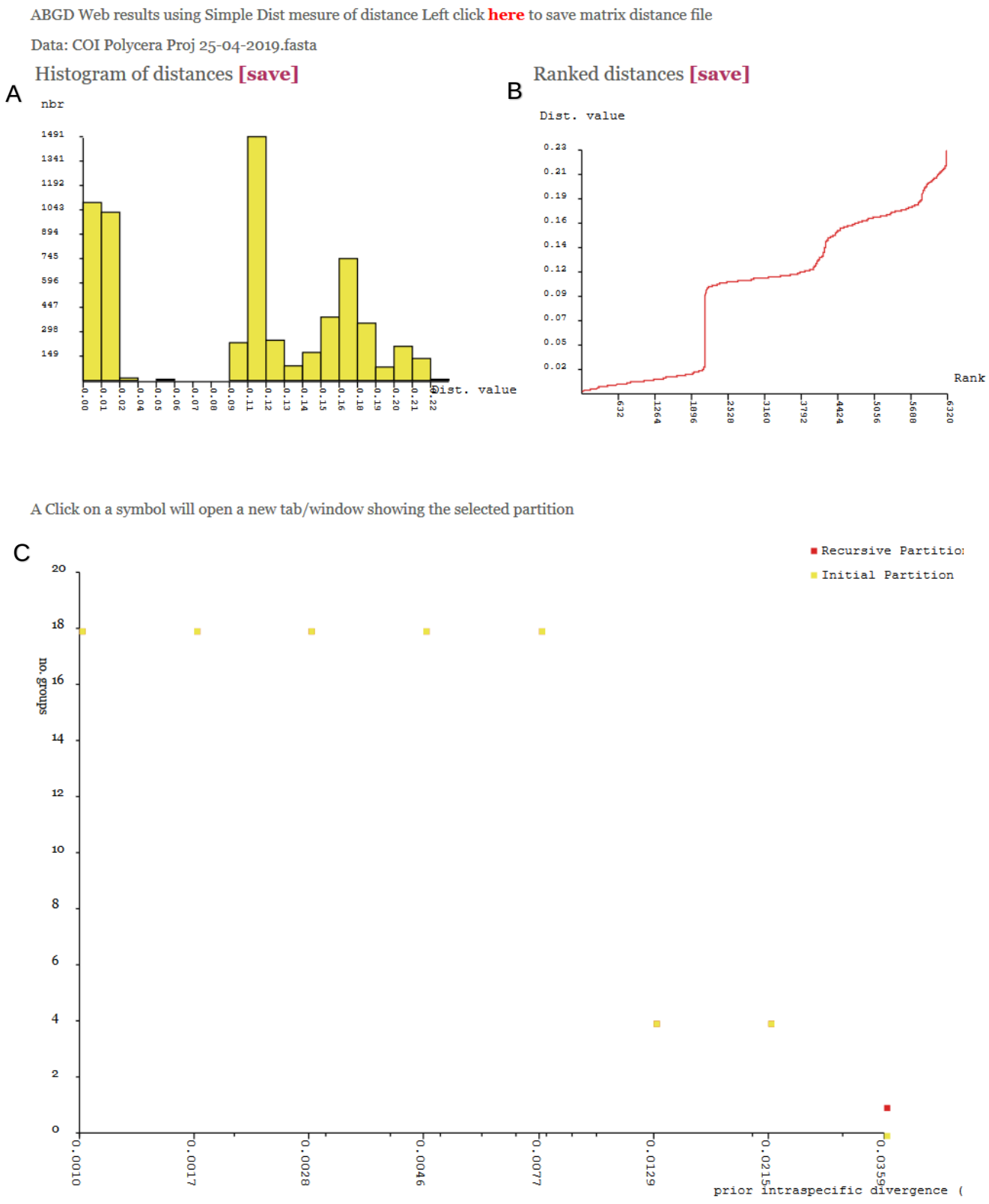
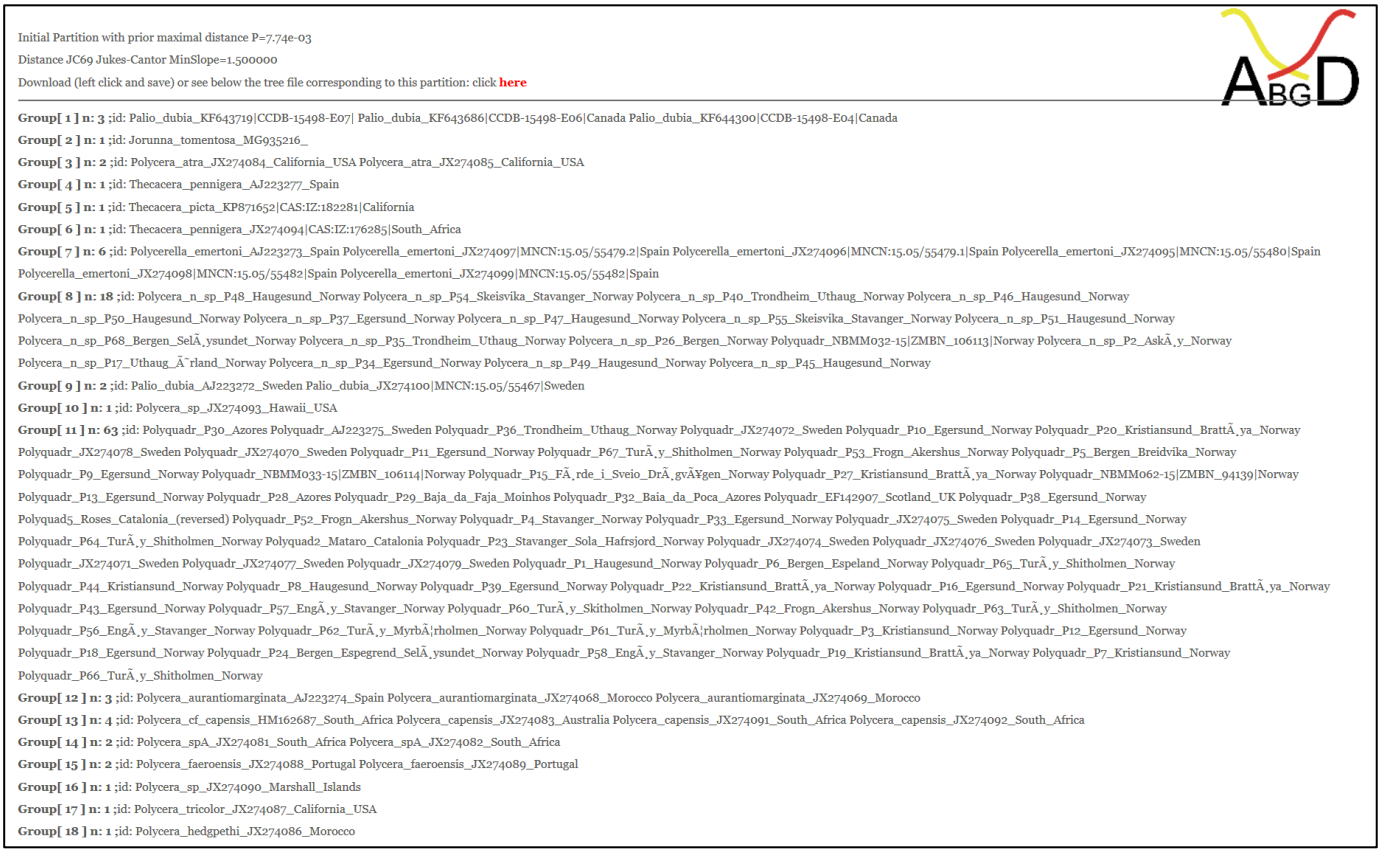


Figure 5 – Schematic illustration of the Automatic Barcoding Gap Discovery (ABGD) using 'Jukes-Cantor (JC69)', analysis. **A.** distribution of pairwise differences with count-values in the vertical direction and distance values in the horizontal direction

(Puillandre et al., 2012). Low divergence presumes intra-specific divergence, whereas higher divergence represents inter-specific divergence (Puillandre et al., 2012). **B.** ranked pairwise differences. Has the same representative principle as the latter one. **C**. slope of ranked pairwise differences. Here the method automatically finds the first statistical significant slope peak. A barcoding gap will appear where there is a sudden increase in slope region. A clear barcoding gap can be seen in **A–C**, illustrated by the dashed line, supporting the hypothesis about separate species. Screen-shot taken from the abgd interphase webpage at http://wwwabi.snv.jussieu.fr/public/abgd/abgdweb.html.



Initial Partition with prior maximal distance P=7.74e-03
Distance JC69 Jukes-Cantor MinSlope=1.500000
Download (left click and save) or see below the tree file corresponding to this partition: click **here**

Group[1] n: 3 ;id: Palio_dubia_KF643719|CCDB-15498-E07| Palio_dubia_KF643686|CCDB-15498-E06|Canada Palio_dubia_KF644300|CCDB-15498-E04|Canada
Group[2] n: 1 ;id: Jorunna_tomentosa_MG935216_
Group[3] n: 2 ;id: Polycera_atra_JX274084_California_USA Polycera_atra_JX274085_California_USA
Group[4] n: 1 ;id: Thecacera_pennigera_AJ223277_Spain
Group[5] n: 1 ;id: Thecacera_picta_KP871652|CAS:IZ:182281|California
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Group[11] n: 63 ;id: Polyquadr_P30_Azores Polyquadr_AJ223275_Sweden Polyquadr_P36_Trondheim_Uthaug_Norway Polyquadr_JX274072_Sweden Polyquadr_P10_Egersund_Norway Polyquadr_P20_Kristiansund_BrattÃ¸ya_Norway Polyquadr_JX274078_Sweden Polyquadr_JX274070_Sweden Polyquadr_P11_Egersund_Norway Polyquadr_P67_TurÃ¸y_Shitholmen_Norway Polyquadr_P53_Frogn_Akershus_Norway Polyquadr_P5_Bergen_Breidvika_Norway Polyquadr_P9_Egersund_Norway Polyquadr_NBMM033-15|ZMBN_106114|Norway Polyquadr_P15_FÃ¸rde_i_Sveio_DrÃ¸gvÃ¥gen_Norway Polyquadr_P27_Kristiansund_BrattÃ¸ya_Norway Polyquadr_NBMM062-15|ZMBN_94139|Norway Polyquadr_P13_Egersund_Norway Polyquadr_P28_Azores Polyquadr_P29_Baja_da_Faja_Moinhos Polyquadr_P32_Baia_da_Poca_Azores Polyquadr_EF142907_Scotland_UK Polyquadr_P38_Egersund_Norway Polyquad5_Roses_Catalonia_(reversed) Polyquadr_P52_Frogn_Akershus_Norway Polyquadr_P4_Stavanger_Norway Polyquadr_P33_Egersund_Norway Polyquadr_JX274075_Sweden Polyquadr_P14_Egersund_Norway Polyquadr_P64_TurÃ¸y_Shitholmen_Norway Polyquad2_Mataro_Catalonia Polyquadr_P23_Stavanger_Sola_Hafrsjord_Norway Polyquadr_JX274074_Sweden Polyquadr_JX274076_Sweden Polyquadr_JX274073_Sweden Polyquadr_JX274071_Sweden Polyquadr_JX274077_Sweden Polyquadr_JX274079_Sweden Polyquadr_P1_Haugesund_Norway Polyquadr_P6_Bergen_Espeland_Norway Polyquadr_P65_TurÃ¸y_Shitholmen_Norway Polyquadr_P44_Kristiansund_Norway Polyquadr_P8_Haugesund_Norway Polyquadr_P39_Egersund_Norway Polyquadr_P22_Kristiansund_BrattÃ¸ya_Norway Polyquadr_P16_Egersund_Norway Polyquadr_P21_Kristiansund_BrattÃ¸ya_Norway Polyquadr_P43_Egersund_Norway Polyquadr_P57_EngÃ¸y_Stavanger_Norway Polyquadr_P60_TurÃ¸y_Skitholmen_Norway Polyquadr_P42_Frogn_Akershus_Norway Polyquadr_P63_TurÃ¸y_Shitholmen_Norway Polyquadr_P56_EngÃ¸y_Stavanger_Norway Polyquadr_P62_TurÃ¸y_MyrbÃ¦rholmen_Norway Polyquadr_P61_TurÃ¸y_MyrbÃ¦rholmen_Norway Polyquadr_P3_Kristiansund_Norway Polyquadr_P12_Egersund_Norway Polyquadr_P18_Egersund_Norway Polyquadr_P24_Bergen_Espegrend_SelÃ¸ysundet_Norway Polyquadr_P58_EngÃ¸y_Stavanger_Norway Polyquadr_P19_Kristiansund_BrattÃ¸ya_Norway Polyquadr_P7_Kristiansund_Norway Polyquadr_P66_TurÃ¸y_Shitholmen_Norway
Group[12] n: 3 ;id: Polycera_aurantiomarginata_AJ223274_Spain Polycera_aurantiomarginata_JX274068_Morocco Polycera_aurantiomarginata_JX274069_Morocco
Group[13] n: 4 ;id: Polycera_cf_capensis_HM162687_South_Africa Polycera_capensis_JX274083_Australia Polycera_capensis_JX274091_South_Africa Polycera_capensis_JX274092_South_Africa
Group[14] n: 2 ;id: Polycera_spA_JX274081_South_Africa Polycera_spA_JX274082_South_Africa
Group[15] n: 2 ;id: Polycera_faeroensis_JX274088_Portugal Polycera_faeroensis_JX274089_Portugal
Group[16] n: 1 ;id: Polycera_sp_JX274090_Marshall_Islands
Group[17] n: 1 ;id: Polycera_tricolor_JX274087_California_USA
Group[18] n: 1 ;id: Polycera_hedgpethi_JX274086_Morocco

Figure 6 – Output data from the 'Jukes-Cantor (JC69)' ABGD analysis showing the total number of 18 groups, with n = number of sequences/specimens that exists within each group. An overview of the species included in the phylogenetic analysis. The output supports the hypothesis about having two separate species – *Polycera* n. sp. (n = 18) and *P. quadrilineata* (n = 63).

APPENDIX 7: HAPLOTYPE NETWORK ANALYSIS

7.1 Work conducted in the text editing program Notepad++

Notepad++ (v. npp.7.6.6) were used prior to PopArt (v. 1.7; Leigh & Bryant, 2015) to adjust and create separate alignment (phy.) and trait (txt.) files for each study species – one for *P. quadrilineata* (63) and one for *Polycera* n. sp. (17). The sample names had a maximum limit of 10 characters, each including the sample code (*i.e.* P1, P2, etc.) and its corresponding geographical initials (*e.g.* Az, Sw, NT, etc.). By using the files for *P. quadrilineata* as an example Figure 1 and 2 shows the setup of how the alignment –and trait files were created for each species. Note! For each screen-shot, only parts of the information is included due to large and long scripts.

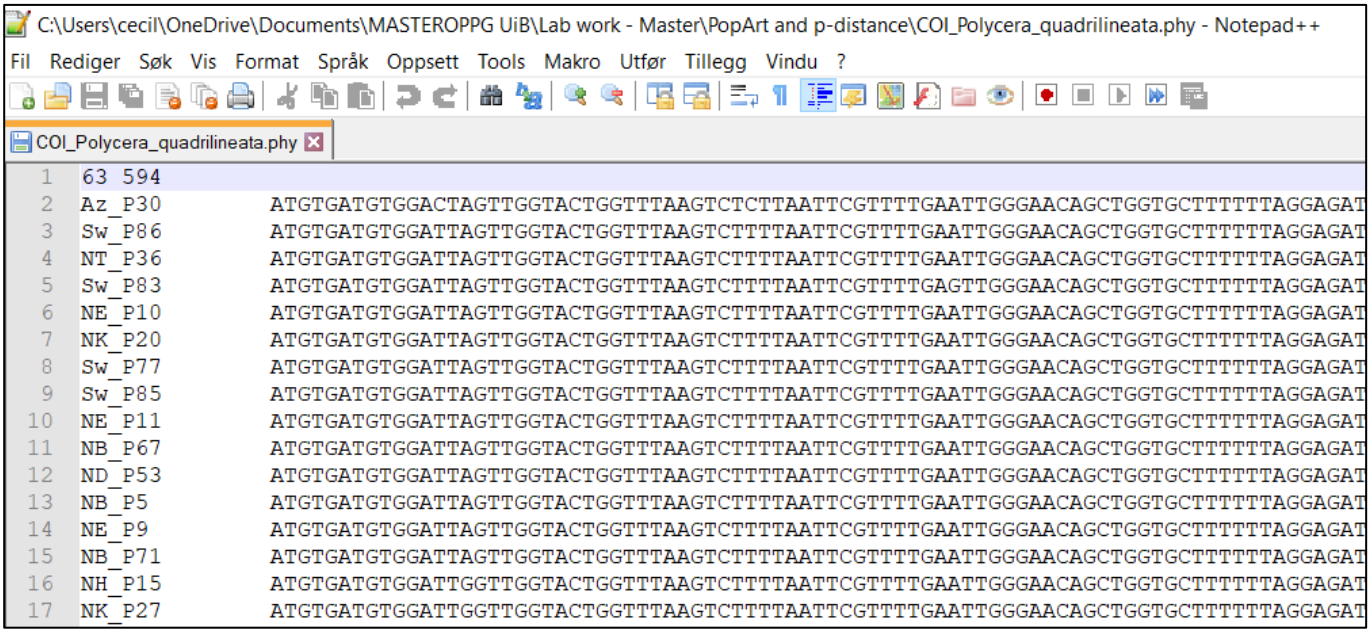

Figure 1 – Screen-shot example on how the COI sequence alignment file for *P. quadrilineata* was carried out using Notepad++ (v. npp.7.6.6). Shows only a fraction of the total alignment file containing all 63 COI sequences/samples (P). Total number of base pairs per sequence = 594 bp. Geographical acronyms; Az = Azores. Sw = Sweden. N = Norway. H = Haugesund. T = Trondheim. E = Egersund. B = Bergen. D = Drøbak. K = Kristiansund.

```
,Az,WM,Sc,Sw,NH,NS,NT,NE,NB,ND,NK
Az_P30,1,0,0,0,0,0,0,0,0,0,0
Sw_P86,0,0,0,1,0,0,0,0,0,0,0
NT_P36,0,0,0,0,0,0,1,0,0,0,0
Sw_P83,0,0,0,1,0,0,0,0,0,0,0
NE_P10,0,0,0,0,0,0,0,1,0,0,0
NK_P20,0,0,0,0,0,0,0,0,0,0,1
Sw_P77,0,0,0,1,0,0,0,0,0,0,0
Sw_P85,0,0,0,1,0,0,0,0,0,0,0
NE_P11,0,0,0,0,0,0,0,1,0,0,0
NB_P67,0,0,0,0,0,0,0,0,1,0,0
ND_P53,0,0,0,0,0,0,0,0,0,1,0
NB_P5,0,0,0,0,0,0,0,0,1,0,0
NE_P9,0,0,0,0,0,0,0,1,0,0,0
NB_P71,0,0,0,0,0,0,0,0,1,0,0
NH_P15,0,0,0,0,1,0,0,0,0,0,0
NK_P27,0,0,0,0,0,0,0,0,0,0,1
```

Figure 2 – Screen-shot example on how the geographical trait-file for *P. quadrilineata* was carried out using Notepad++ (v. npp.7.6.6). Shows only a fraction of the total 63 samples (P). The 0–1 system shows where individuals were collected from; 0 = not found, 1 = found. Geographical acronyms; Az = Azores. Sw = Sweden. N = Norway. H = Haugesund. T = Trondheim. E = Egersund. B = Bergen. D = Drøbak. K = Kristiansund.

Table 1 – Geographical and specimens overview. Showing the total number of *P. quadrilineata* (63) and *Polycera* n. sp. (18) specimens collected at the different geographical sampling areas. **Note!** despite P48 being removed from the rest of the haplotype network analysis, it is included in this table. Table showing highest number of *P. quadrilineata* specimens was collected from Sweden, Egersund, and Bergen, whereas highest number of *Polycera* n. sp. specimens was collected from Haugesund. *Polycera* n. sp. only representative in Norway.

Geographical area	**No. of specimens**	
	Polycera quadrilineata	*Polycera* n. sp.
Azores	4	0
Western Mediterranean	2	0
Scotland, UK	1	0
Sweden	11	0
Haugesund, Norway	3	7
Stavanger, Norway	5	2
Trondheim, Norway	1	3
Egersund, Norway	12	2
Bergen, Norway	13	4
Drøbak, Norway	3	0
Kristiansund, Norway	8	0

Table 2 – Matching COI sequences of *P. quadrilineata* individuals. Showing the total number of individuals and geographical areas represented within a shared haplotype (= genotype). Sample (= individual) involved represented by its personal sample code.

Haplotype size (tot. no. individuals)	Geographical area	Sample code
2	Norway: Bergen & Trondheim	P66 ; P36
2	Norway: Bergen & Egersund	P72 ;P13
2	Norway: Bergen & Egersund	P71 ; P39
2	Norway: Bergen	P6 ; P65
3	Norway: Bergen & Egersund (n = 2)	P64 ; P14 ; P10
3	Norway: Bergen & Drøbak; Sweden	P63 ; P42 ; P84
4	Norway: Egersund (n = 2) & Kristiansund (n = 2)	P43 ; P16 ; P21 ; P22
5	Norway: Bergen, Drøbak & Egersund; Sweden (n = 2)	P5 ; P53 ; P11 ; P77 ; P85
11	Norway: Bergen (n = 3), Egersund (n = 2), Kristiansund (n = 2), & Stavanger (n = 2); Sweden (n = 2)	P24 ; P61 ; P62 ; P18 ; P12 ; P3 ; P44 ; P58 ; P56 ; P76 ; P78

7.2 Manual: How PopArt was used

Open PopArt → 'File' → 'Import alignment' (phy-file).

'File' → 'import traits' (txt-file)→ 'no' (when asked if you want to 'clean alignment data') → check if the right 'delimiter' is hooked of. Do not change any of the standard setting → 'ok'.

After all alignment and trait files had been imported into PopArt, the TCS network analysis were created by; 'Network' → 'TCS Network'.

The TCS Network is a standard haplotype network used in PopArt due to the program's fast creation time. After the networks had been created, re-organization of visual adjustments were done by dragging the haplotypes, or dots, around with the screen key.

Changing the layout of the mutations was done by; 'View' → 'show mutation' as '1-step edges' (only for personal illustration preferences). Adding and editing of colouration to the haplotypes was done by clicking on the square illustrated with small coloured circles.

Saving the Haplotype Network; 'File' → 'Export graphs' → save as PNG.