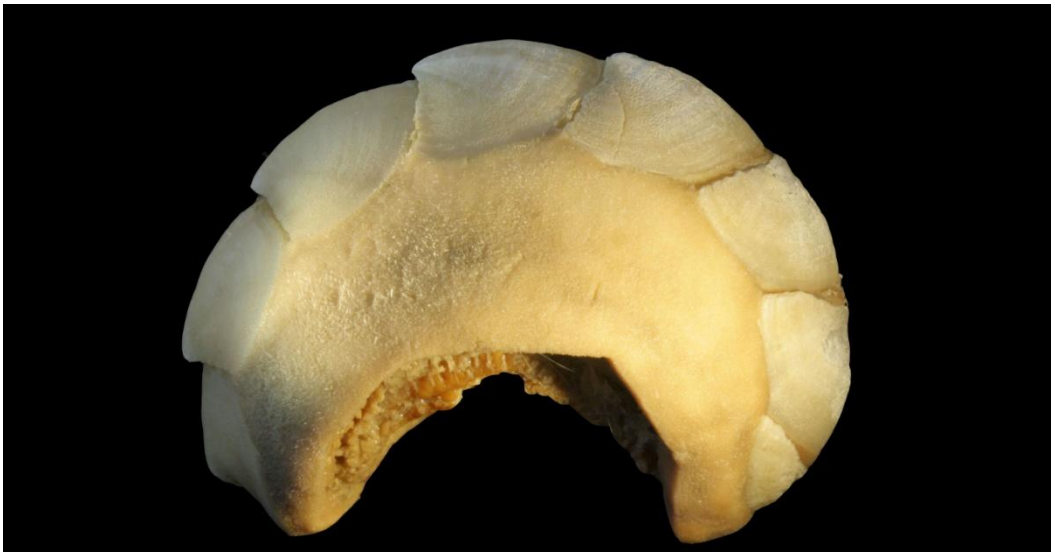
Morphological and genetic variation in *Hanleya nagelfar* and *Hanleya hanleyi* (Mollusca: Polyplacophora) in the Northern European Atlantic and the phylogenetic position of Hanleyidae based on nuclear and mitochondrial genes



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Photograph on front page of a large *Hanleya nagelfar*.

Photo: Audun Schrøder-Nielsen

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ABSTRACT

Large specimens of the spongivore *Hanleya nagelfar* (Mollusca: Polyplacophora) differ noticeably from the smaller congeneric *H. hanleyi*, but many scientists have reported that small specimens are very similar and perhaps identical. The debate of their relationship has been ongoing for over 140 years, without reaching a conclusion.

This thesis uses genetics combined with statistical analyses on morphometric characters previously used for separating the two species, as well as examination of girdle armature and radulae, in the attempt to resolve the relationship of the chitons.

Partial sequences of the genes 18S rRNA, 16S rRNA and cytochrome B are used to find differences within *Hanleya*. A Bayesian analysis is used to infer the molecular phylogenetic position of Hanleyidae among Polyplacophora for the first time.

Statistical analyses find substantial ontogenetic changes in all external diagnostic characters, and no distinct clustering indicating two species are found in a multivariate analysis including morphological characters from over 100 specimens of *Hanleya.* Radula and cytochrome b variations indicate presence of a hidden species within *Hanleya* in the Northern European Atlantic.

1. INTRODUCTION

1.1. Polyplacophora

Chitons (Polyplacophora) are exclusively marine epibenthic mollusks found from the intertidal to the hadal zone. They feed by using their long stereoglossate radula (rigid in the longitudinal direction) to scrape off detritus and macroalgae. A few species are carnivorous. The animals are dorsoventrally flattened with eight dorsal calcareous shell plates, also called valves. Surrounding the valves is a thick, sclerite-covered mantle, called the girdle. A broad ventral foot is separated from the girdle by a pallial groove where the gills, gonopores and excretory pores are situated. The ventral head lies in front of the foot, separated by a transverse groove. The chiton can adhere to the substrate by creating a vacuum using the girdle and the foot. Most species are dioecious, without sexual dimorphism, but hermaphroditism and even occasional hermaphroditism have been reported (Kaas and van Belle 1985, Eernisse 1988, Scarano and Ituarte 2008). Developed trocophore-larvae hatch from the eggs and after some days settle on the substrate. Duration of the larval stage is species-specific and settling can be affected by factors such as temperature and chemical stimuli (Leise 1984, Barnes and Gonor 1973). Most chiton species can easily be kept apart by studying external characters such as the shape of the valves, girdle armature and gill placement. However, not all species are easily distinguishable.

1.2. The genus *Hanleya*

Hanleya (Polyplacophora, Mollusca) is a relatively small genus described by John Edward Gray (1857). Members have a spiculose girdle and a well developed un-slit insertion plate in the head valve only. It currently comprises four valid species: *Hanleya hanleyi* (Bean *in* Thorpe, 1844); *H. nagelfar* (Lovén, 1846); *H. tropicalis* Dall, 1881 and *H. sinica* Xu, 1990. *Hanleya tropicalis* is only known from the type locality in Florida (USA), Sands Key. *Hanleya sinica* is only known from the holotype from the East China Sea. *Hanleya nagelfar* is known from the North East Atlantic, Skagerrak, Kattegat and the coast of Portugal while *H. hanleyi* has a much wider distribution and is known from the Bay of Fundy (Canada), Greenland,

Iceland, the Norwegian coast, Kattegat and Skagerrak, the Azores and Canary Islands and the Mediterranean Sea. Systematic relationships are listed in Table 1.

Phylum:	Mollusca
Class:	Polyplacophora Blainville, 1816
Subclass:	Neoloricata Bergenhayn, 1955
Order:	Lepidopleurida Thiele, 1909
Suborder:	Lepidopleurina Thiele, 1909
Family:	Hanleyidae Bergenhayn, 1955

Table 1 - Taxonomy of Hanleyidae following WoRMS (Schwabe and Gofas 2009).

This thesis takes a closer look at the species *H. hanleyi* and *H. nagelfar*, two of the thirteen species of chitons known along the Norwegian Coast (Høisæther et al. 1997). *Hanleya hanleyi* is a relatively rare species (Sars 1878, Jones and Baxter 1987, Schander 2005a), usually collected from *Lophelia*-reefs, but found on virtually any hard substrate (Warén and Klitgaard 1991, Jensen and Frederiksen 1992). Specimens of *H. hanleyi*, with a maximum size of 25 mm (usually < 13 mm) look very different from large *H. nagelfar*. The latter is the chiton with the largest body size in the European North Atlantic, reaching lengths exceeding 70 mm, and is characterized by having a broader girdle and longer valves than *H. hanleyi* (Warén and Klitgaard 1991). *Hanleya nagelfar* have been reported living on large choristid sponges (Jeffreys 1865, Sars 1878, Storm 1879, Grieg 1914, Dons 1944, Burden-Jones and Tambs-Lyche 1960, Jensen and Frederiksen 1992) where they feed on the pinacoderm and can rest partially enclosed by the sponge (Warén and Klitgaard 1991, Klitgaard 1995, Hoffmann et al. 2004, Todt et al. 2009). Although identifying large specimens of *H. nagelfar* is easy, several authors have noticed the general scarcity of small specimens of this species and raised the question that it might be the adult form or an ecotype of *H. hanleyi* (Jeffreys 1865, Sparre Schneider 1886, Tryon and Pilsbry 1892, Muus 1959, Warén and Klitgaard 1991). The discussion has been ongoing for over 140 years, but no rigid conclusion has been reached.

1.2.1. Taxonomic history of *Hanleya hanleyi* and *Hanleya nagelfar*

Chiton hanleyi (=*Hanleya hanleyi*) was described by William Bean in the book "British Marine Conchology" (1844) by Charles Thorpe. The short description was based upon two specimens found at the underside of rocks at the lowest spring tide in Scarborough (east coast of England). Bean described the shape of the animal (width/length = 0.5) and its color (brownish white). The carinated valves have granules increasing in size towards the margin which is covered by minute spines. The valves inner surface is pale green. The chiton was named after the conchologist Silvanus Charles Thorpe Hanley (1819 – 1899).

Two years later, Sven Ludvig Lovèn described the large *Chiton nagelfar* (=*Hanleya nagelfar*) in the book "Index molluscorum litora Scandinaviae occidentalia habitantum" (1846). He portrayed the animal as elongate (width/length = 20/48 = 0.41) with a purple color and a tail valve broader than the head valve. Median valves are almost kidney-shaped (length/width = 1/2.2) without a posterior beak and the second valve is more triangular (l/w = 1/1.8) than the others. The tail valve is almost rhomboid. Valves are sculptured with granules, small on jugal areas, longitudinally chain-like arranged on central areas, getting larger towards the lateral margins. In lateral areas the granules are large and randomly arranged. The head valve has a striate, but unslit insertion plate, which is not seen in the tail valve. Its girdle is thick and bears a uniformly dense cover of short spicules. The chiton got its name from "Naglfar", a Norse mythological ship made entirely of finger- and toenails of dead humans. Lovén also mentioned *Chiton hanleyi* and points out that it differs from *C. nagelfar* by having a smaller tail valve than head valve, a l/w ratio on the median valves of 0.37 and a l/w ratio in valve II of 0.45. The color is said to be whitish-brownish compared to the purple color of *C. nagelfar*.

Chiton abyssorum M. Sars, 1859 MS is worth mentioning here, because many authors refer to it frequently when discussing the difficulties of identifying *Hanleya* specimens. The species was first mentioned by Michael Sars in "Bidrag til en skildring af den arktiske Molluskfauna ved Norges nordlige kyster" (1859). The brief description is based on a 55 mm long specimen from Bergen, noting only that it is very similar to *C. nagelfar* except for the white color. The species is today treated as a synonym of *Hanleya nagelfar* (Kaas and van Belle 1985). Michael Sars (1859) is one of the few who mentions *C. hanleyi, C. nagelfar* and

C. abyssorum (*=H. nagelfar*) in the same publication, but they are not discussed apart of their varying distribution range.

A thorough description of *Chiton hanleyi* was published by John Gwyn Jeffreys in "British conchology" – volume III (1865). Jeffreys was of the opinion that *C. nagelfar* (and *C. abyssorum*) should be regarded as *C. hanleyi* of extraordinarly large size. He noted that the girdle is tough with whitish spicules and that longer spines cluster in the valve sutures. The moderately solid, opaque valves are wide and have a deep notch in front and small, moderately pointed beaks posteriorly. Jeffreys wrote that granules were arranged chain-like, gradually getting larger and more irregular towards the sides. The margin (insertion plate) is microscopically crenulated without any notches. Jeffreys was the first to look at the radula in *Hanleya* specimens, describing the teeth as arranged in rows, two of which are prominent and has black hooks.
A supplement to the description was published in volume V of "British Conchology" (Jeffreys 1869). Here, the animals were described as pale yellowish-white with a slight degree of pink. The head valve is shaped like a horseshoe with a narrow, pink edge. Jeffreys is the only one who has described the foot: being whitish, anteriorly truncated or rounded and dotted with microscopic white flakes. The ventral girdle is thick, grayish with black specks.

Georg Ossian Sars, in contrast, did distinguish between *H. hanleyi* and *H. nagelfar* and he mention catching a 1 mm long specimen of the latter species on an expedition to "Storeggen" (Sars 1872). He had seen larger specimens of *H. nagelfar* on sponges and *Lophelia*-corals.
Later, G. O. Sars published a description of *Chiton hanleyi* (1878) differing from Bean's in some characters. He describes the valves as bluntly carinated as opposed to carinated. Like Jeffreys (1869), Sars also states the animal to be pale yellowish and aggregations of long spines are seen in the valve sutures. Head and tail valves are of more or less equal size and semicircular. The median valves have a length/width ratio of 0.5 and are slightly emarginated (notched) before the apex. G. O. Sars was of the opinion that *Chiton abyssorum* is very similar to *C. hanleyi* except for the larger size, broader and thicker girdle and the less distinct sculpturation, and that it might even be a deep water form of the latter. At this time, he states that he has never found *Chiton nagelfar*, with its purple color and the broader tail

valve than head valve. Thus, he must have revised the specimens of *C. nagelfar* he caught in 1872 as *C. abyssorum* or *C. hanleyi*.

Sparre Schneider (1886) was of the opinion that *C. abyssorum* is the adult form of *C. hanleyi*, as he stated in "Undersøgelser af dyrelivet i de arktiske fjorde". He did not see the less distinct sculpturation in large (ca 30 mm total length) specimens, which G. O. Sars described. He stated that girdle width can increase with size in other species (examples are not given) and found it peculiar that almost all *C. abyssorum* caught were fully grown or near so.

George Washington Tryon (1892) mentioned *H. hanleyi*, *C. nagelfar* and *H. hanleyi* var. *abyssorum* in "Manual of Conchology" (edited by Henry Augustus Pilsbry). The description of *H. hanleyi* was mostly congruent with previous publications, except for the valves' carination, which was said to be "rather angular" (vs. Sars' bluntly carinated valves). The tail valve's mucro was said to be median. *Chiton nagelfar* was listed as a synonym of *H. hanleyi* and the third form was describes as *H. hanleyi* var*. abyssorum*. Tryon evidently was of the opinion that they all are one species. In the book, there are two illustrations of the "var*. abyssorum*", and one of them is remarkably similar to *H. nagelfar*: the specimen show a broad girdle, long intermediate valves and valve VIII is broader than valve I (which fits to Lovèn's description). Growth marks in the head valve and in lateral areas of the intermediate valves are drawn. The other illustration of *H. hanleyi* var. *abyssorum* shows a specimen with intermediate valves more similar to *H. hanleyi* (sensu Bean 1844), a girdle that is of moderate width and a tail valve that is almost semicircular and of more or less equal width as the head valve.

James Alexanderssön Grieg (1898) wrote in "Skrabninger i Vaagsfjorden og Ulvesund, ytre Nordfjord" that he did not follow Jeffreys' and Tryon's view that *H. abyssorum* should be treated as a variety of *H. hanleyi*, but that it is merely the adult form of the species, as Sparre Schneider (1886) also had suggested. Grieg also observed that girdle width and valve sculpturation varies with the size of animals. However, he did not dare to discard the *abyssorum*-name until more specimens were examined. Some years later (1913), he wrote about small specimens (10 mm) from Bergen Museum, presumably with *H. abyssorum*-characteristics and after discussion with G. O. Sars they concluded to keep the species separate. In "Bergens Museums Aarbok 1913", Grieg (1914) showed an illustration of a large

H. abyssorum in which he depicted it different from Sars' illustration of *C. abyssorum*. Grieg's drawing has width-measurements for all valves attached and by comparing his attached valve-measurements with the drawing, it appears he had mistaken the anterior part of the animal for the posterior. That is why he found it different from Sars' specimen. The illustration actually shows a specimen with a typical large *H. nagelfar*-shape. He also briefly mentions a 10 mm long specimen he caught some years before the publication with *H. hanleyi* characteristics, but he does not describe this specimen in detail.

Kiær and Wollebæk (1913) caught several *Hanleya*-specimens in the Kristianiafjord (=Oslofjord) on sponges and *Lophelia* corals. Two specimens, 22 mm long and caught in hauls with sponges and coral-branches, were different from each other. They identified one as *H. hanleyi*, the other as *H. abyssorum*. Larger specimens caught were all of *H. abyssorum*-form.

Thiele (1909) was of the opinion that girdle armature and radulae characters should be included to see if the species differ from each other. He found that large *H. abyssorum*' have 500 µm long, round spicules interspersed between smaller (ca. 250 µm long, 40-45 µm broad), flattened spicules. In smaller *H. hanleyi* specimens, he mainly saw small elongate, sharply pointed spicules with three ribs (at most) facing upwards. The downward facing side was smooth. Thiele also had a thorough look on radulae. In *H. abyssorum* the central tooth ("mittelplatte") is broadest at the anterior part and the anterior end is rounded, while in *H. hanleyi* the broadest point is more posterior than the former and the anterior end more or less straight with pronounced corners. On basis of the observed variations in girdle armature and radula characters he concluded that the species should be kept separate.

In the short publication "Om utbredelsen av *Hanleya nagelfar*", Dons (1933) questioned whether some of his older material was *H. nagelfar* or *H. abyssorum*. He sent the specimens he was unsure of to J. Bergenhayn (see below), who concluded that all specimens Dons doubted were *H. nagelfar*. An illustration of two large *H. nagelfar* and one *H. abyssorum* with measurements (total length, valve I and VIII width) is included in the publication. Dons separated the species based on the terminal valve, *H. abyssorum* having terminal valves of equal width to each other. If Bergenhayn was of the opinion that *H. abyssorum* is a separate

species from *H. nagelfar*, we do not know, but based on his identifications returned to Dons, it seems likely that he thought they were synonyms (as they are treated today).

The orders within Polyplacophora have been revised by Johan Richard Melin Bergenhayn in 1955. He noticed that the genera *Hemiarthrum* Carpenter and *Hanleya* did not belong in the family Lepidopleuridae (=Leptochitonidae) and he established the new family Hanleyidae. The family was characterized by having an insertion plate in only one, or both terminal valves; apophyses are very broad and reach, or nearly reach, the anterior apical part of the valve and there are aggregations of spicules in the valve sutures. Based on the valve characters he placed the group close to Leptochitonidae. Within Hanleyidae he stated that *Hanleya* most likely is a primitive taxon form compared to *Hemiarthrum*.

In "Danmarks Fauna", Bent Jørgen Muus (1959) provided a small illustration and a description of *H. hanleyi.* Nothing new was said about the species, except for the jugal area, which he described as very broad. Muus also mentioned *H. abyssorum*. In spite of its broader girdle and more rounded valves and the fact that it is frequently found on sponges and corals, Muus stated that the *abyssorum*-form hardly can be separated from *H. hanleyi*.

Detailed descriptions of *H. hanleyi* and *H. nagelfar* are found in "Monograph of Living Chitons" (Kaas and van Belle 1985). There are also drawings of the animals, valves, radulae and spicules. The moderately broad girdle of *H. hanleyi* is described to be densely covered by smooth, straight spicules (92x8 µm) randomly interspersed with larger spicules (122x18 – 256 x 20 µm). Weakly longitudinally ribbed spicules (92-96 µm) are found on the ventral girdle. *H. nagelfar* has a girdle densely covered by glassy, smooth spicules (150-200 µm) interspersed with large, relatively thicker spines up to 500 µm long. Valves of *H. hanleyi* are less elevated (dorsal elevation (DE) = valve IV height/ valve IV width = 0.30) than *H. nagelfar* (DE = 0.35). The former's intermediate valves are more or less rectangular and the tail valve is less than semicircular with a median mucro. *H. nagelfar* has rather long intermediate valves and a transversely elliptical tail valve with a somewhat posterior mucro. Kaas and van Belle were of the opinion that differences in girdle armature, valve morphology and way of life are evidence for the presence of two species.

In "Synopses of the British Fauna" (Jones and Baxter 1987) *H. hanleyi* is described as a chiton with robust valves, the head valve being disproportionately large. Shell width/total width =

0.8. Median valves are moderately tall, carinated and distinctly beaked. In contrast to several other descriptions, the tail valve is portrayed as having rounded shape. The sculpture is said to vary at the jugal, median and lateral areas of the valves. Jugal areas have randomly arranged granules, as opposed to the longitudinal arranged sculpturing in other descriptions. A central apical cap with a crescent-shaped ring of subsidiary caps around it can be seen on each granule. 50 µm long spicules, each with 2-3 longitudinal ridges, cover the girdle. Longer, smooth spines (200 µm) are especially abundant in the valve sutures but also randomly distributed over the girdle. The central radula tooth is longer than wide with rounded angles and a small notch in the posterior margin. First lateral teeth extend just beyond the anterior margin of the central tooth. Jones and Baxter (1987) do not mention other species in the genus.

The article "*Hanleya nagelfar*, a sponge-feeding ecotype of *H. hanleyi* or a distinct species of chiton?" (Warén and Klitgaard 1991) tried to put an end to the taxonomic discussions. The authors did not find any morphological differences except size between the congeneric chitons. However, they did not want to combine the species due to indirect evidence: *Hanleya nagelfar* had been found in areas without the usual sponges indicating that the species is not dependant on certain sponge species; *H. nagelfar* is not found in the Mediterranean Sea, although choristid sponges as well as *H. hanleyi* are found there; *Hanleya nagelfar* show a very high habitat preference compared to *H. hanleyi*; Specimens from different habitats (rocks and shells vs. corals vs. sponges) reach sexual maturity at various sizes. Characters helpful for classification are substrate (sponge vs rocks) and size (specimens larger than 25 mm are probably *H. nagelfar*). These characters are not very rigid and small specimens from unknown substrate cannot be classified at all.

Partially contradicting characters, i.e. carination and size of beaks, and the fact that several authors have stated that *H. hanleyi* and *H. nagelfar* are very similar or even identical illustrates the need for a clarification of the species-relationships within *Hanleya*.

1. 3. Solving the problem

When morphological variation parallels genetic variations, support for a certain grouping is higher. Thus, morphological and genetic characters should be compared whenever possible (Ward et al. 2005). The use of molecular and morphological methods combined have previously been successful in determining relationships between closely related, cryptic species (Järnegren et al. 2007, Svendsen 2009). Therefore, a similar combination has been followed here.

1.3.1. A morphological approach

Statistical tests can be executed on morphometric characters used for separating species. A diagnostic character should show a bimodal distribution when samples are collected from two species. For *Hanleya hanleyi* and *H. nagelfar*, the most obvious difference is perhaps the observed large difference in girdle width between the two species. Dorsal elevation is said to have a ratio of 0.30 for *H. hanleyi* and 0.35 for *H. nagelfar* (Kaas and van Belle 1985). Authors have also noticed a variation in the position of the mucro on the tail valve, which is said to be central for *H. hanleyi* (Tryon and Pilsbry 1892, Kaas and van Belle 1985, Jones and Baxter 1987) and "somewhat posterior" in *H. nagelfar* (Kaas and van Belle 1985). Bean described *H. hanleyi* as having a total width/total length ratio of 0.5 (Bean 1844), while Lovèn in his description of *H. nagelfar* noted the ratio to be 0.41 (Lovén 1846). Sars (1878) said that in *H. hanleyi* the head and tail valve was of equal width compared to the broader tail valve of *H. nagelfar*. The length to width ratio of intermediate valves and valve II was said to be 1/2.2 and 1/1.8 for *H. nagelfar*, respectively (Lovén 1846). For *H. hanleyi* the intermediate valve length to width ratio should be 0.5 (Sars 1878). Differences in spicules and radulae have also been recorded and their correlation with other variables have to be considered when trying to resolve the problems with the identification of *Hanleya hanleyi* and *Hanleya nagelfar*.

1.3.2. A molecular approach

Identifying a species can be difficult if a species is morphologically cryptic, show phenotypic plasticity (morphological variation in an individual based on environmental influence),

changing morphology during various life stages or if there is genetic variability in characters used for species determination can lead to misidentifications. Microgenomic identification systems such as genetic barcoding can be useful tools when dealing with species that are morphologically cryptic (Hebert et al. 2003, Schander and Willassen 2005, Järnegren et al. 2007). Phylogenetic analyses based on single genes have been carried out for over 30 years (Woese and Fox 1977) and the amount of genetic data easily available through the internet is ever increasing. In GenBank there are over 30 million genetic sequences deposited. This information can be downloaded by anyone who wants to perform a phylogenetic analysis.

Conservation rates vary between genes, making single gene analyses applicable to phylogenic analyses of both higher and lower taxa (Palumbi 1996). Genes such as Cytochrome B and 16S have fairly high mutation rates making them applicable to distinguish between lower taxa, i.e. species, genera or families. These little conserved genes are often not useful for all phylogenetic studies on higher taxa, since the genetic sequence can be saturated (too many mutations have occurred). In contrast, in more conserved genes, such as 18S rRNA, the number of informative sites varying between closely related species generally is low and they are mostly used in analyzing higher phylogenies (Woese and Fox 1977, Hasegawa et al. 1993, Winnepenninckx et al. 1996, Okusu et al. 2003).

Järnegren et. al (2007) found 0 – 1 % (uncorrected p-distance) intraspecific genetic variation in three bivalve species (*Acesta spp.),* while interspecific variation ranged from 6.2 – 11.9 % for the mitochondrial gene Cytochrome B. The slightly less conserved mitochondrial 16S rDNA had an intraspecific variation of 0 – 0.4 % and an interspecific variation of 3.9 – 6.8 %. The results show more similarity within one species than between species, indicating a logical grouping. Although 18S is considered one of the more conserved genes, differences can also be seen in congeneric species as Winnepenninckx et. al. (1998) found 0.22 – 2.31 % genetic diversity between five species of *Littorina* (Gastropoda, Mollusca) in 18S rRNA. No absolute general rules thus apply to how conservative genes are. The mitochondrial gene Cytochrome oxidase c subunit I (COI) has been chosen as a barcoding gene (Hebert et al. 2003) for its qualities in distinguishing between closely related species. There are also "universal" primers that can be used in several distantly related groups for this gene (i.e. Folmer et al. (1994)). Although enough point mutations occur for resolving lower taxa phylogenies, its amino acid sequence is quite preserved making it useful even for higher taxa

phylogenetic analyses (Palumbi 1996). Several authors discourage this method because of the possibility for overlap between intra- and interspecific differences (Meyer and Paulay 2005) as well as the problems with maternal inheritance of the mitochondrial genome when species hybridize (Ward et al. 2005).

1.4. Aims

Previous studies, based on morphology, have not come to any rigid conclusions to whether *H. hanleyi* and *H. nagelfar* should be regarded as separate species. In this thesis, genetic and morphological methods are combined in the attempt to resolve the question whether there are two (or perhaps more?) distinct species within the genus *Hanleya* in the European North Atlantic. Material consisting of over 100 specimens from the different geographical regions and earlier identified as *H. hanleyi* or *H. nagelfar* is examined. The morphological variation of characters previously used for separating the species, such as sclerites, radulae, valve and girdle morphology, is described and statistically tested for normality.

Members of Hanleyidae have previously not been studied genetically. In this thesis, partial sequences of mitochondrial as well as nuclear genes are used to place Hanleyidae in a molecular phylogenetic tree for the first time. Sequences from GenBank make it possible to analyze the sequences from *Hanleya* together with homologous sequences from twelve other polyplacophoran families. The co-occurance of variations in presumably species-specific morphological characters and molecular characters is assessed.

2. MATERIAL AND METHODS

2.1. Material

2.1.1. Studied material

Studied animals from the genus *Hanleya* (n=112) were provided by the University of Bergen, Bergen Museum - natural history department, the Museum of Natural History and Archaeology in Trondheim, University of Copenhagen – Zoological Museum, National Museum of Scotland, Göteborg Natural History Museum and National Museum of Natural History (Naturalis, Leiden, Holland). The material originated from Greenland, Iceland, the Nordic Margin (Sotbakken and Røst reef), the fjords surrounding Trondheim and Bergen (Norway), Kattegat and Skagerrak (Sweden), Oban (Scotland), Porcupine Bank (320 km west of Ireland), Murchison Field (190 km north east of Shetland Islands) and Fuerteventura (Canary Islands, Spain). Animals in the dataset were collected on various expiditions from 1846 to 2008. Figure 1 shows a distribution map of specimens included in this work.

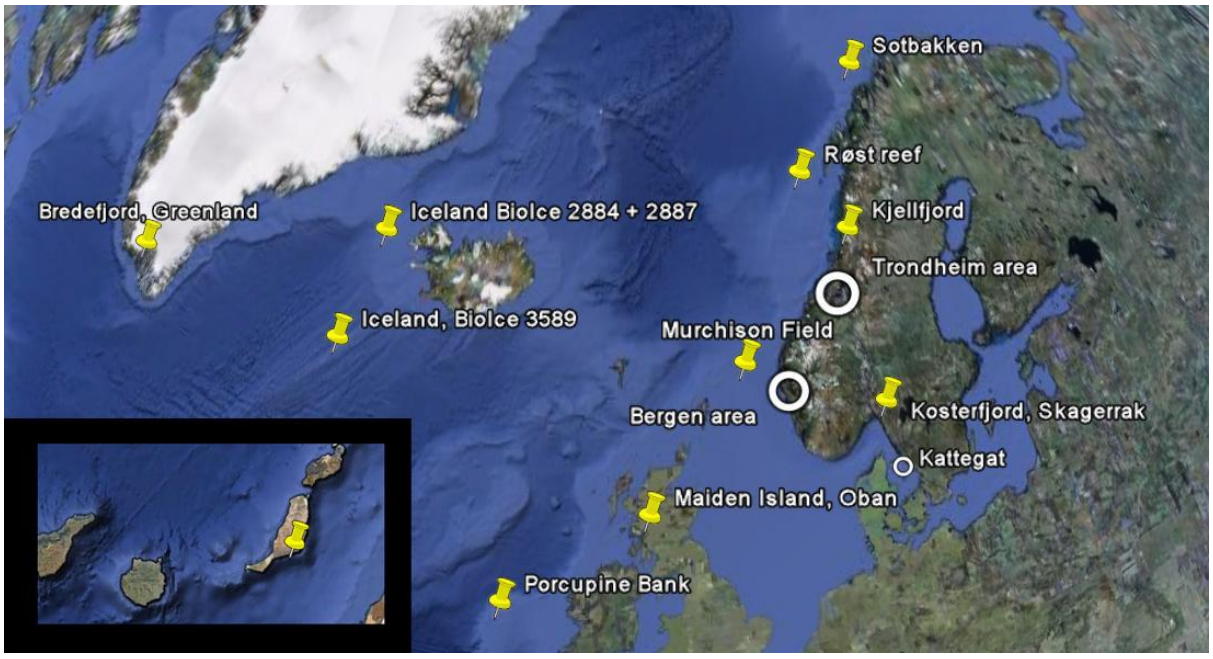


Figure 1 – Distibution map: Specimens examined in this study. Circles indicate several stations from the area; pins are from single stations (except BioIce 2884 + 2887). Bottom left frame is the Canary Islands, pinpointing Fuerteventura. Map from Google Earth version 5.0.

The specimens were mainly caught by dredges, although grabs, Agassiz-trawls, RP-sleds and an ROV had also been used when collecting the animals. The lectotype of *Hanleya nagelfar* (Lovén, 1846) from Naturhistoriska Riksmuseet, Stockholm, catalogue number "SMNH-Type 1329", collected in Finnmark, Norway was also examined.

2.1.2. Fixation and preservation of animals

Most animals available were collected before 1973 and had been fixed in formalin, later replaced with ethanol. Fifty-five of the available specimens were collected from 1986-2008 and preserved in ethanol. Four living animals collected by Hans Tore Rapp (University of Bergen) during my thesis work were fixed in 96 % ethanol. Prior to fixation the specimens were positioned flat on a small wooden board or a glass and tied down using an elastic nylon stocking. This was done to prevent the animals from curling up during fixation. If a live animal had detached from the surface and curled up, it was placed in a solution of dissolved MgCl (7.7 g 98% MgCl, 100 ml tap water, 100 ml sea water). This relaxes the muscles of the chiton and the specimen could be mounted on the flat surface before fixation. Plenty of ethanol was used (approximately 4 dl for large specimens), so the body fluids would not dilute the ethanol to a large extent. After one day animals were placed in fresh 96 % ethanol to guarantee optimal preservation.

2.2. Morphological methods

2.2.1. Morphometrics

All measurements, (except those obtained from SEM and light microscopy) were taken using a vernier caliper. The accuracy of measurements is ±0.1 mm.
Most of the specimens available were in a curled state (n=82), which make it difficult to measure the total lengths accurately. Length and widths of the animals were therefore measured dorsally on the animal by stretching a fine thread longitudinally over the median axis of the animal, including the anterior and posterior dorsal parts of the girdle. Dorsal width was measured with the thread placed transversely over valve IV, including the lateral

dorsal girdle on each side. The thread was marked, straightened and measured with a vernier caliper. The following characters (except valve IV height) were measured directly on the animal using the vernier caliper, as shown in Figure 2. Girdle width is the distance from the outermost part of valve IV until the lateral margin of the girdle. All valve widths (Valve I width, valve II width, valve IV width and valve VIII width) are the distances between the tegmentum lateral area margins for each valve. All valve lengths (valve II length, valve IV length and valve VIII length) are the visible distances across the jugal area from the posterior to the anterior margin. Valve IV height is the height of the tegmentum for valve IV (Figure 2). Antemucronal length is the distance between the mucro and the most anterior visible point of valve VIII. Valve IV height was measured in a stereo microscope fitted with a corrected measuring grid. Abbreviations are listed in Table 2.

Character	Abbreviation
Dorsal length (corrected)	DL
Dorsal width	DW
Girdle width	GW
Valve I width	IW
Valve II width	IIW
Valve II length	IIL
Valve IV width	IVW
Valve IV length	IVL
Valve IV height	IVH
Valve VIII width	VIIIW
Valve VIII length	VIIIL
Antemucronal distance	AM
Mucronal position (% from anterior marging of valve VIII)	Mucro
Intermediate valve shape (rectangular vs almost rectangular vs long)	IM
Tail valve shape (Less than semicircular vs transversely elliptical/round)	TV

Table 2 - Abbreviations for the measured characters.

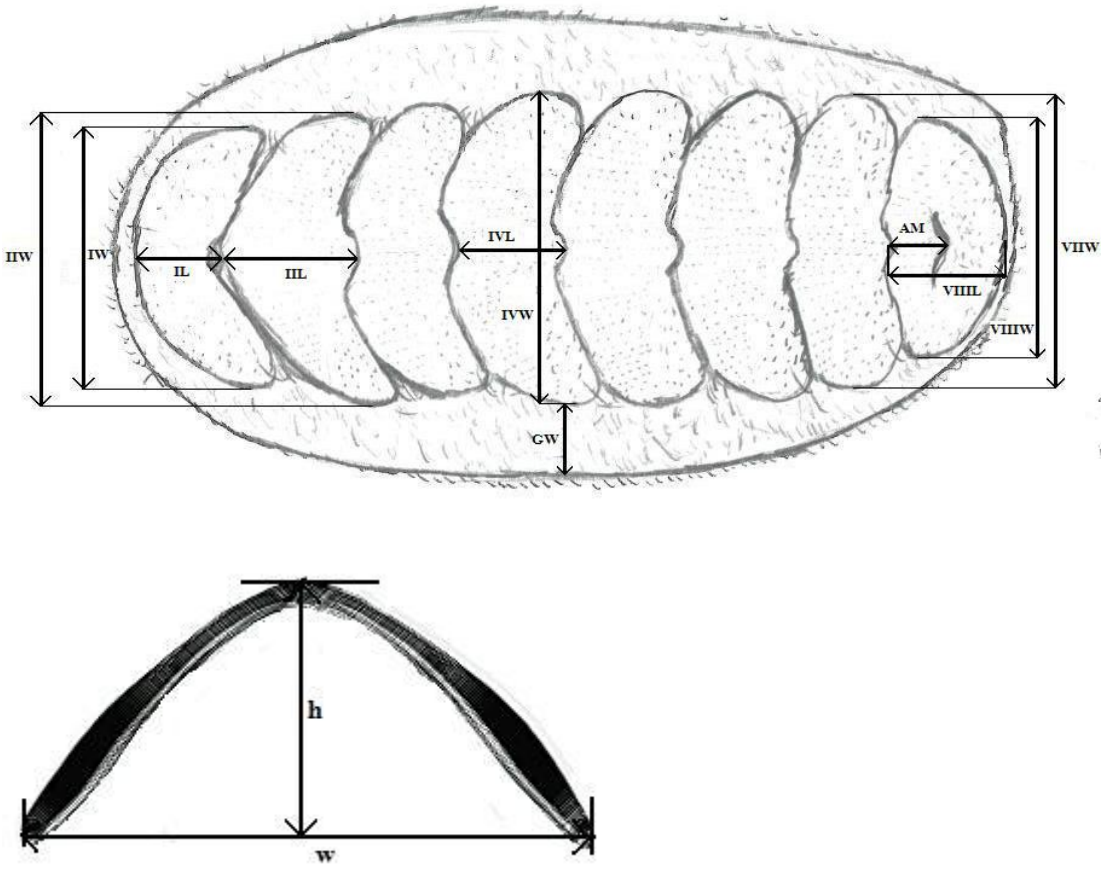


Figure 2 – Top: Measurements done with the vernier caliper. Bottom: Valve IV, posterior view. Dorsal elevation =h/w.

2.2.2. Correcting the dorsal length

The dorsal length of a strongly curled specimen is different than that of an animal of the same size that is almost straight. This was corrected for by measuring a living specimen in three different states, 1: straight; 2: partially curled; 3: strongly curled (Figure 3). Dorsal length measurements of the living animal in the respective states were 70.8 mm, 80.2 mm and 93 mm. All specimens were corrected to the second state by multiplying the uncorrected length of specimens in state 1 with the factor 1.1328. The uncorrected dorsal length of the specimens in state 3 were multiplied with 0.8624. All given dorsal lengths in this study are the corrected values, unless specified.

Figure 3 - Variable curling of specimens. Right to left: State 1 to state 3. Scale bars, right to left: 2 mm, 5 mm, 10 mm.

2.2.3. Light microscopy of girdle armature

Girdle spicule morphology was examined in a LEICA DIALUX 20 light microscope fitted with a measuring grid. A small piece of cuticle with spicules was cut from the girdle with a clean scalpel. There is a possibility that spicule size varies on the location of the girdle. Spicules were therefore always dissected from the middle area of the girdle. The tissue was placed in diluted chlorine (1 part commercial bleach/1 part dH_2O) until the organic tissue was dissolved and the spicules were loosened from the cuticle with a needle and a very fine brush. Chlorine was removed and the tissue was rinsed by adding and removing distilled water with a pipette 3-5 times. This was done under a dissection microscope. Spicules were then placed on a microscope slide using a pipette and set to air dry. A drop of glycerin and a cover slip was placed on the slide. Finally, the cover slips edges were sealed with nail polish.

2.2.4. Scanning electron microscopy

Scanning electron microscopy (SEM) images were taken of radulae, valves, cuticle with spicules and free spicules on selected specimens. Specimens were chosen based on the morphometrics (dorsal length, girdle width and dorsal elevation being most important), the molecular results and the substrate they were caught on to see if the valve ultrastructure, radulae and spicules vary with size and habitat of the specimens. Specimens previously classified as both *H. hanleyi* and *H. nagelfar* were included.

Cuticle with spicules was dissected next to the intermediate valves and from the median area of the girdle. The cuticle with spicules was placed into chlorine (1 part commercial bleach /1 part distilled water) until all organic tissue was dissolved (30-90 minutes). The cuticle with spicules was then placed in a small container with dH_2O using a pipette. To remove all of the chlorine, the water was replaced 4-5 times. Loose spicules in a drop of water were then placed on a SEM stub, fitted with a glass plate. The stub was then air dried before it was sputter coated with gold palladium (see below).

Radulae were dissected from the specimens and placed in numbered containers with diluted chlorine (1 part commercial bleach/9 parts dH_20) until all tissue surrounding the radula was dissolved (approximately 20 minutes). Chlorine was rinsed away with dH_20 before mounting

the radulae with a drop of water on stubs covered with a thin layer of water soluable glue. Before the water evaporated, the radulae were positioned with the dorsal side facing up.

Valves were dissected and remaining tissue was dissolved in chlorine (1 part commercial bleach /1 part distilled water). Chlorine was rinsed of with dH2O, before the valves were set to air dry. When dry, they were placed on stubs fitted with carbon tape or carbon glue. Equipment was thoroughly washed before moving from one specimen to another.

All preparations were sputter coated with gold-palladium for 90 seconds in 30 mA, under a vacuum of 6-8 x 10^{-2} mbar prior to the SEM. They were studied with a Zeiss Supra VP55 or a JEOL 6400 scanning electron microscope fitted with cameras.

2.2.5. Univariate morphometric analyses

Morphometric data were imported into R 2.9.1 (R:Development_Core_Team 2009) where total number, range, mean and standard deviation was found for each of the measured characters. Analyses were performed on the species determinant characters: Girdle width in percent of dorsal length (GW); dorsal elevation = valve IV height/valve IV width ratio (DE); Mucronal distance in % from anterior margin of tail valve (Mucro); dorsal width/dorsal length (DW/DL); Tail valve width in % of DL – head valve width in % of DL (VIIIW-IW); length/width ratio of valve II (IIL/IIW) and length/width ratio of valve IV (IVL/IVW). Analyses were also performed on each character used for the calculations. R 2.9.1 was used to make histograms, for visualization of the distribution of characters. A Shapiro-Wilk test (Shapiro and Wilk 1965) was executed to see if the characters were normally distributed. Regression plots of the characters in relation to the corrected dorsal length were made. An F-test was executed to see whether a linear regression would fit the plot better than the mean. The regression line indicates the character change trend from small to large specimens. The Shapiro-Wilk null hypothesis that character distribution originates from a normally distributed population, is discarded if the p-value is <0.05. The same critical value was used for the regression analyses. Commands used in the program are shown in Appendix II.

2.2.6. Multivariate morphometric analyses

Multivariate analyses were executed in the computer program CANOCO 4.5 (ter Braak and Šmilauer 2002) to see how characters previously used to separate *H. hanleyi* and *H. nagelfar* (DL, GW, DE, IM and TV) correlated with each other and to see if the specimens with certain characteristics would cluster in groups. The software does not allow NA's in the dataset, and specimens with lacking data were removed. Number of specimens included in the analysis was 101. A detrended correspondence analysis (DCA) was done to decide whether a linear or unimodal method was appropriate. A principal component analysis (PCA) was executed and visualized in an ordination diagram. Different measuring units in the analysis were accounted for by choosing the "center and standardize"-option in CANOCO (ter Braak and Šmilauer 2002).

Angles between the arrows in the ordination diagram indicate the character-correlation, whereas 90^0 indicate no correlation, 0^0 indicate positive correlation and 180^0 indicate negative correlation.

2.2.7. Photography

Pictures of small animals and animal parts were taken in a Leica MZ 16 A stereomicroscope with a Nikon Digital Sight DS-5M camera attached. Using the program Nikon ACT-2U (Excel Technology, Inc.) a series of images were taken with different focus points on the animal on each image. The image series was imported to the program Auto Montage (Syncroscopy), which aligns the image stack and makes a montage of focused areas in each image. The end result is a high quality image with every part of the animal in focus. The number of images (n=25-55) required for each montage varied with the height of the animal or animal part. Large specimens (> 20 mm DL) were photographed with a Canon 1000D fitted with a macro lens.

2.3. Molecular methods

2.3.1. Obtaining tissue

Tissue for molecular analysis was dissected from the girdle, foot or gills. This was done for 40 of the ethanol preserved animals.

Tissue was dissected from the girdle if the specimen was strongly curled or if the foot was covered with remnants of the substrate. When obtaining tissue from the girdle, a small piece (maximum 1 mm^3) was cut out. Cuticle with spicules was dissected away leaving only muscle tissue for the extraction. This was done as a precaution to reduce the risk of contamination by possible epifauna and to avoid spicules that could clogg the filters in the mini columns in the DNA extraction procedure. A scalpel was used to scrape off as much as possible of the spicules when the piece of girdle was very small.

Tissue was dissected from the foot if the specimen was very small and had a minute girdle. If any sediment was stuck to the foot, a scalpel was used to scrape away the sediment and then to dissect from the clean area.

After having problems in the lab with some of the tissue dissected from girdle or foot, DNA-extractions using gill tissue were also done on some specimens (see discussion). One of the most anterior gills from the right side of the animal was pinched off with a clean tweezer. Foreign material (detritus/sponge spicules etc.) was frequently observed in the pallial fold and on the gills. As much as possible of the particles was removed with a tweezer, before placing the gills in a Branson 2210 ultrasound cleaner for approximately ten minutes to remove possible contaminants.

All dissected tissue was always carefully examined for any foreign particles under a stereo microscope prior to DNA extraction.

Dissected tissue was put in numbered small containers filled with 96% ethanol until the DNA extractions were to be done. All equipment in contact with the organic tissue under the procedure, such as scalpel, tweezers and petri-dishes, was thoroughly cleaned between specimens to avoid contamination.

2.3.2. Genes and primers

Molecular work was done on the nuclear ribosomal gene 18S, the mitochondrial ribosomal genes 12S and 16S, and the mitochondrial protein coding genes cytochrome b (cyt b) and cytochrome oxidase subunit I (COI).

A total of 20 primers were used for five genes. Primers and primer sequences can be seen in Table 3.

Gene	Primer	Primer sequence	References
12S	F	AGA CAT GGA TTA GAT ACC C	(Barucca et al. 2003, Kocher et al. 1989)
	R	CCC TAC CTT GTT ACG ACT T	(Barucca et al. 2003)
16S	Sar	CGC CTG TTT ATC AAA AAC CAT	(Palumbi et al. 1991)
	Sbr	CCG GTC TGA ACT CAG ATC ACG T	"
	16RTHB*	ACG CCG GTT TGA ACT CAG ATC	(Koufopanou et al. 1999)
	16LRN13398*	CGC CTG TTT AAC AAA AAC AT	"
Cyt B	397F*	YWY TRC CTT ggR ggR CAR ATA TC	(Dahlgren et al. 2000)
	811R*	gCR WAY ARA AAR TAY CAY TCW gg	"
	UCYTB144F	TGA GSN CAR ATG TCN TWY TG	(Merritt and Shi 1998)
	UCYTB272R	GCR AAN AGR AAR TAC CAY TC	"
	UCYTB151F*	TGT GGR GCN ACY GTW ATY ACT AA	"
	UCYTB270R*	AAN AGG AAR TAY CAY TCN GGY TG	"
COI	LCO1490	GGT CAA CAA ATC ATA AAG ATA TTG G	(Folmer et al. 1994)
	HCO2198	TAA ACT TCA GGG TGA CCA AAA AAT CA	"
	dgLCO*	GGT CAA CAA ATC ATA AAG AYA TYG G	(Meyer et al. 2005)
	dgHCO*	TAA ACT TCA GGG TGA CCA AAR AAY CA	"
18S	18S5F	GCG AAA GCA TTT GCC AAG AA	(Norén and Jondelius 1999)
	1100R	GAT CGT CTT CGA ACC TCT G	"
	600F	GGT GCC AGC MGC CGC GGT	"
	600 r	CCG AGA TCC AAC TAC GAG CT	(Steiner and Dreyer 2003)

Table 3 - Primer sequences. Directions is from 5' to 3'. * = Mollusk-designed primers.

2.3.3. DNA extraction

The "Blood & Tissue Genomic DNA Extraction Miniprep System" (Viogene) was used when extracting DNA. The Tissue Protocol of this kit was followed with minor modifications:

- Dissected tissue was put in an Eppendorf tube (1.5 ml) with approximately 5 ml ddH_2O in 2.5-3 hours to dilute the alcohol in the tissue. The tissue was then put in the tubes open lid until it was completely dry and all remaining alcohol had evaporated.
- The small piece of tissue was then placed in a sterile Eppendorf tube using clean forceps. The forceps was cleaned with a piece of paper towel soaked in ethanol before picking up the next

piece of tissue.
200 µl LYS buffer was added and the tube was vortexed to start the lysis of the tissue.

- 20 µl Proteinase K was added to start the digestion of proteins. The sample was mixed immediately after the addition of Proteinase K by vortexing for 20 seconds.
- The sample was incubated for 16-24 hours in a block heater (60^0C), accelerating tissue lysis. The sample was mixed by turning or vortexing the tube at least 3 times during this step.
- The sample was incubated for 20 minutes at 70^0C in the block heater.
- If there still was some tissue left in the Eppendorf tube, all of the content except the solid tissue was moved to new sterile Eppendorf tubes.
  200 µl EX-buffer was added to the sample before vortexing and centrifuging. The sample was then incubated for another 10 minutes at 70^0C in the heater block.
- 100 % ethanol (210 µl) was added and the sample was mixed by vortexing. Drops on the lid or on the sides were centrifuged down.
- A B/T Genomic DNA Mini Column was placed in a collection tube and the mixture was placed in the columns using a pipette.
- The collection tube with the mini column was centrifuged for 2 minutes at 8000 rpm, before placing the mini column in a new collection tube.
- The column was washed twice with 500 µl of WS buffer, centrifuging for 2 minutes at 8000 rpm each time and removing the flow-through between the spins.
- To make sure all ethanol residues are removed, the sample was centrifuged at full speed (13000 rpm) for 2 minutes.
- The mini column was placed in a sterile Eppendorf tube and DNA was eluted with 200 µl of heated (70^0C) TE buffer.
- The column was set aside for 5 minutes before centrifuging for 2 minutes at 13000 rpm.
- The mini column was discarded and the eluted DNA in the Eppendorf tubes were stored at 4^0C.

2.3.4. Amplification using REPLI-g

Amplification of the total genome in DNA extracts that failed to yield results from the PCR-programs was done using the "REPLI-g UltraFast Mini Kit" (Qiagen). The kit's protocol was followed:

- DNA extract (1 µl) and D1 (denaturation) buffer (1 µl) was placed into a PCR-tube using a pipette. This was mixed by flicking and centrifuged briefly.
- The tube was set aside in a PCR tube rack in room temperature to incubate for three minutes.
- 2 µl N1 (neutralization) buffer was added. The tube was flicked and centrifuged briefly.
- 15 µl REPLI-g UltraFast Reaction Buffer and 1 µl REPLI-g UltraFast DNA Polymerase was added to the PCR tube, making a total volume of 20 µl.
- The tube was incubated at 30^0C for 1.5 h followed by an inactivation step at 65^0C for 3 minutes. The sample was held at 4^0C. This step was done in a thermal cycler (Peltier Thermal Cycler – DNA Engine DYAD™).

- The content that was not immediately used when taken out of the PCR machine was stored at -20^{0}C.
- When using the REPLI-g amplified extract for PCR, the DNA was diluted with ddH_2O at the ratio 1:24. The diluted DNA was mixed and vortexed before adding 2 µl to the PCR-sample mixture, (see "2.2.5.The Polymerase Chain Reaction (PCR)").

2.3.5. The Polymerase Chain Reaction (PCR)

When using the Takara (Takara Bio-Inc) set-up, each PCR-sample had a volume of 25 µl (2 µl DNA extract, 16.35 µl H_2O, 2.5 µl Takara 10X buffer, 2 µl Takara dNTPs, 1 µl forward primer (10 µM), 1 µl backward primer (10µM) and 0.15 µl Takara LA Taq HS).

To work more efficiently, a master mix with all of the ingredients above (except the DNA) was first made in a DNA-free environment. This was a small cabinet with all equipment needed for the making of the master mix. The cabinet had been UV-radiated for at least 10 minutes before the master mix was made. Twenty-three µl of the master mix were portioned into each of the PCR-tubes and then 2 µl of DNA extract was added. Positive and negative controls were set up with the other extractions to make sure that the PCR had run without problems and to be sure that the samples were uncontaminated. DNA-extract that had given an easily visible, clean band on previous PCR's was used in the positive control. DNA-extract was exchanged with ddH_2O in the negative controls. All pre-PCR work was done on ice to prevent the enzymes catalyzing the reactions earlier than wanted.

2.3.6. PCR-programs

For optimization of PCR-programs, see "2.3.10. Optimizing".
The optimal temperature profile for 16S using the 16Sar and 16Sbr primers was [94^{0}C for 2 min; (94^{0}C for 30 sec, 54^{0}C for 20 sec, 72^{0}C for 1 min) x 40; 72^{0}C for 7 min, 7^{0}C hold] step. The optimal temperature profile for 18S using the primers 18S5F, 18S1100R, 18S 600R and 18S 600F was [94^{0}C for 3 min; (94^{0}C for 45 sec, 50^{0}C for 45 sec, 72^{0}C for 2 min) x 40; 72^{0}C for 10 min; 4^{0}C hold]. The optimal temperature profile for 12S was [96^{0}C for 2 min; (93^{0}C for 30 sec, 55^{0}C for 30 sec, 72^{0}C for 1 min) x 40; 72^{0}C for 7 min, 4^{0}C hold].

The optimal temperature profile for Cyt B, using the primers 151F and 270R was [96^{0}C for 2 min; (93^{0}C for 30 sec, 50^{0}C for 25 sec, 72^{0}C for 1 min) x 40; 72^{0}C for 7 min; 4^{0}C hold].

2.3.7. Gel electrophoresis

Agarose (1 %) with either Ethidium Bromide (0.06 µl EtBr/ml agarose) or GelRed™ (Biotium) (0.04 µl/ml agarose) was used in this step. Ethidium Bromide or Gel Red™ was added to the agarose to make the visualization of DNA possible. The mixture was poured in a tray and combs were added. When the agarose had cooled and congealed, the combs were removed and TBE-buffer (0.5X) was added to the gel apparatus. 4 µl PCR-product was mixed with 1 µl loading dye (3-6X) and added to the wells in the agarose. A ΦX174 HAE III (Promega) ladder with DNA-fragments (n=11) of certain molecular weight (72-1353 bp) and amount was added in the last well. The electrophoresis was run at 80-90 V for 40-70 minutes depending on the size of the gel. Visualization of the DNA was done under ultra violet light and photographs were taken with GeneSnap v.7.7.1 (Syngene). Visualization of the PCR-product can be seen in Figure 5.

2.3.8. Preparing the PCR-product for sequencing

It is necessary to remove contaminants, unconsumed dNTP's and primers, in the amplified PCR-product before the sequencing. This was done by mixing 0.05 µl EXO1, 0.5 µl Shrimp alkaline phosphatase (SAP) and 1.45 µl ddH_2O with 8 µl of the PCR-product. Due to the heat-sensitivity of the enzymes, this step was done on ice before the samples were mixed by flicking, briefly centrifuged and put in a thermal cycler set for an incubation step of 37^{0}C for 30 min and an inactivation step of 85^{0}C for 15 min. During the incubation the enzymes will chew up the dNTPs and primers, the inactivation period destroys the enzymes, leaving only clean DNA sequences.

ExoSAP-IT® (Affymetrix Inc.) is a pre-mixed clean-up kit and was used on some of the samples. 1 µl of ExoSAP-IT® was added to 5 µl PCR-product when using this kit. The incubation and inactivation step were 15 min (37^{0}C) and 15 min (80^{0}C), respectively.

The amount of DNA in the samples was calculated using the light intensity from the ladder included in the gel electrophoresis. The computer program GeneTools v.4.1.2 (Syngene) was used for this calculation. A 10 µl sample was made with an amount comparable to 5-20 ng of the clean DNA (5-10 ng for 12S, due to its shorter length), 1 µl sequence primer, 1 µl buffer, 1 µl BigDye® Terminator v3.1 and ddH_2O. This sample was run in a sequencing program (94^0C for 30 sec; 96^0C for 10 sec; 50^0C for 5 sec; 60^0C for 4 min; cycle to step 2 * 39 times) in the thermal cycler and the product was sent to SeqLab for sequencing (Department of Molecular Biology, University of Bergen).

2.3.9. Editing and analyzing the sequences

The general quality of the sequence trace files was viewed in FinchTV (Geospiza). Trace files were also blasted to detect possible contaminations. Forward and backward sequences were assembled using the software Lasergene® SeqMan Pro (DNASTAR). The trace-file was thoroughly checked for base ambiguity in SeqMan and corrected in BioEdit 7.0 (Tom Hall, Ibis Biosciences). The optimal alignment for the edited sequences was found by using the program ClustalW (Larkin et al. 2007), implemented in BioEdit, using its default parameters. If a contig sequence did not match up with the others, the reverse complementary sequence was inserted and the alignment was run again. All sites showing nucleotide variation in the alignment were carefully examined in SeqMan. If there were double nucleotides in these sites, the specific site would be changed to the corresponding code (see Figure 4). For the protein coding genes, an invertebrate codon table (*Drosophila yakuba*) by Endre Willassen (Bergen Museum) was used to check for conspicuous stop codons in the sequences.

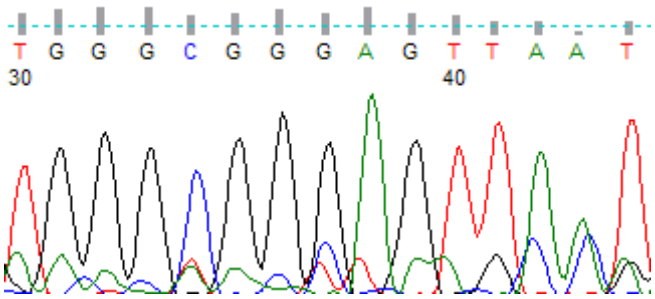


Figure 4 - One of the cytochrome B sequences. Position 43, by the program interpreted as an A, would be changed to an M (=A or C). From FinchTV (Geospiza).

The computer program MrBayes 3.1.2 (Huelsenbeck et al. 2001, Ronquist and Huelsenbeck 2003) was used to find the phylogenetic position of Hanleyidae, and to see if sequences obtained in the lab would group together. Twenty-six sequences from representatives of all available polyplacophoran families (n=12) on GenBank were downloaded. Two bivalves were included in the analyses as outgroups. Table 4 lists accession ID for the downloaded sequences. Cytochrome b sequences used in analyses were extracted from the complete mitochondrial genomes of *Argopecten irradians* (DC665851) and *Katharina tunicata* (NC001636). Prior to an analysis, the sequences were aligned in BioEdit and converted to nexus-files. Nucleotide sites with missing information were coded with "?" in the alignment. MrModeltest 2.2 (Nylander 2004) was used with the software PAUP* 4.0 beta 10 (Swofford 2003) to find the best fitting nucleotide substitution model for each gene and alignment. Analyses in MrBayes were run for 1.5 million generations with a sample and print frequency set to 100. Two parallel runs were done for each analysis. The computer program Tracer v1.4.1 (Rambaut and Drummon 2007) was used to check if number of generations was sufficient. Consensus trees were made using a burn-in of 10 %. An example batch-file for MrBayes is shown in Appendix II.

Species	18S	16S	Species	18S	16S
Argopecten irradians +	L11265	DQ665851*	*Schizochiton incisus*	AY377646	AY377600
Nucula proxima +	AF120526	AY377617	*Lorica volvox*	AY377647	AY377601
Lepidopleurus cajetanus	AF120502	AY377585	*Mopalia muscosa*	AY377648	AY377602
Acanthochitona crinata	AF120503	AY377609	*Plaxiphora albida*	AY377649	AY377603
Leptochiton asellus	AY377631	AY377586	*Katharina tunicata*	AY377650	AY377604
Callochiton septemvalvis	AY377632		*Chiton olivaceus*	AY377651	AY377605
Tonicella lineata	AY377635	AY377585	*Liolophura japonica*	AY377652	AY377606
Chaetopleura apiculata	AY377636	AY377590	*Sypharochiton pelliserpentis*	AY377653	AY377607
Chaetopleura angulata	AY377637	AY377591	*Cryptochiton stelleri*	AY377655	AY377610
Ischnochiton comptus	AY377639	AY377593	*Cryptoplax japonica*	AY377656	AY377611
Ischnochiton australis	AY377641	AY377596	*Nuttallochiton mirandus*	AY3777638	AY377592
Ischnochiton elongatus	AY377642	AY377595	*Acanthopleura granulata*	AY3777654	AY377608
Lepidozona mertensii	AY377643	AY377597	*Mopalia cirrata*	EU406876	EU407007
Callistochiton antiquus	AY377645	AY377599	*Mopalia ferreirai*	EU406884	EU407015

Table 4 - Accession ID for 18S and 16S sequences used in the phylogenetic analyses. + = bivalve species; * = sequence extracted from full mitochondrial genome.

2.3.10. Optimizing

Several attempts were made trying to optimize the PCR-programs if the PCR product was insufficient or if several bands appeared in the visualization of the PCR product. Annealing time was adjusted to see if this resulted in more DNA in the PCR-product. If the primers were a poor match to the specimens DNA-strand, a longer annealing time makes it more likely

that primers bind to the template strands. Annealing temperature was adjusted to make the primers more or less specific. Visualization of PCR product before and after optimization can be seen in Figure 5. If none of these methods would help, the first five cycles were run with a lower annealing temperature than the last 35. This makes the amplification start at a low stringency, which in turn could result in a good starting-point for the last 35 cycles with a higher annealing temperature (Palumbi 1996).

Adjusting annealing time and temperature did not help for some of the DNA extracts. Other optimization methods used in this thesis included addition of more DNA-extract to the PCR-sample to see if there was too little DNA for the primers to attach to; adding more primer to the PCR-samples to see if this would increase the chance of primers binding to the DNA; diluting the DNA-extracts; PCR on PCR, using 2 µl diluted (1/100) PCR product when setting up the samples; amplifying the entire genome in the extracts using Repli-G (Qiagen); using different Taq-polymerases (*TaKaRa LA Taq*TM HS (Takara Bio-Inc), Qiagen (Qiagen) or AmpliTaq® (Applied Biosystems)); using mollusk-specific primers instead of the "universal primers"; PCR was performed on extracts from mitochondria rich organs (gills) to see if it was too small amounts of mtDNA in the original extractions.

When experimenting with different Taq polymerases, the set-up for the PCR-sample was slightly changed. A protocol known to work for the respective Taq polymerase used was followed since the ratios of the components should match as best as possible (e.g.: addition of $MgCl_2$ is needed if it is not included in the buffer from the kit).
The protocol for the Qiagen Taq was as followed: DNA extract (2 µl), Buffer (2,5 µl 10X), dNTP's (2.5 µl), Q-Solution (5 µl), $MgCl_2$ (3.5 µl), 10µM primer (0.5 µl forward; 0.5 µl backward), Qiagen Taq (0.25 µl), H_20 (8.25 µl). Making a total of 25 µl per reaction.
The protocol for the Amplitaq was: DNA extract (4 µl), dNTP's (4µl), buffer (5 µl 10X), MgCl2 (3 µl), 10 µMprimer (5 µl forward, 5 µl backward), AmpliTaq (0.5 µl), H_2O (23.5 µl). Making a total of 50 µl per reaction.

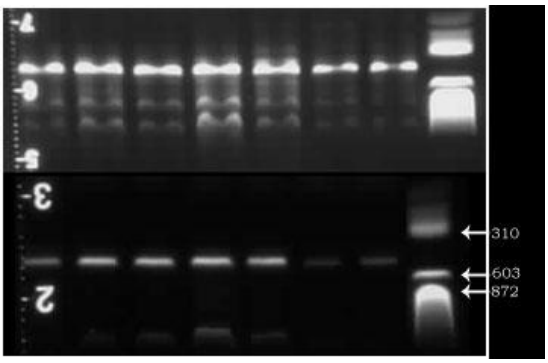


Figure 5 - Optimizing the PCR: Cytochrome b with 35 sec, 48^{0}C annealing (top) and 25 sec, 50^{0}C annealing (bottom). The increase in temperature and decrease in time removes multiple bands. Numbers to the right indicate length of bands in the ladder.

3. RESULTS

3.1. General morphology, radulae and girdle armature

3.1.1. General morphology

Animals included in the analyses (n=112) originate from several localities (see Figure 1) and has been caught on different substrate-types (rocks and/or shells, corals and sponges). The size range varied from small specimens (dorsal length = 2.04 mm), up to perhaps the largest specimen ever caught for this genus (138.84 mm dorsal length). Examined specimens had previosly been classified as *Hanleya sp., H. hanleyi*, *H. nagelfar* and *H. abyssorum*, but were analyzed together, not taking account of earlier identifications.

The girdle was highly variable. The narrowest girdle width measured was 4.08 % of the dorsal length (DL) while the widest measured 18.28 % of DL. Specimens with narrow or wide girdles are shown in Figure 6 and Figure 8. Intermediate valve shape was also variable and could be divided in three categories: rectangular valves with more or less evenly rounded side margins and straight anterior and posterior margin (i.e. Figure 6 b and d); long valves where the curve of the side margin did not even out until the anterior jugal margin (i.e Figure 8 f); intermediate state of the two former (i.e. Figure 8 c). The posterior valve shape also showed large variations. Very large animals (> 40 mm DL) often had an almost circular tail valve (i.e. Figure 9 e) while a transversely elliptical tail valve was more common in smaller animals (i.e. Figure 7 b). Tail valves were generally narrower than the head valve in small animals whereas larger animals (ca 25+ mm DL) showed a clear trend of having a broader tail valve than a head valve (see Figure 16 e).

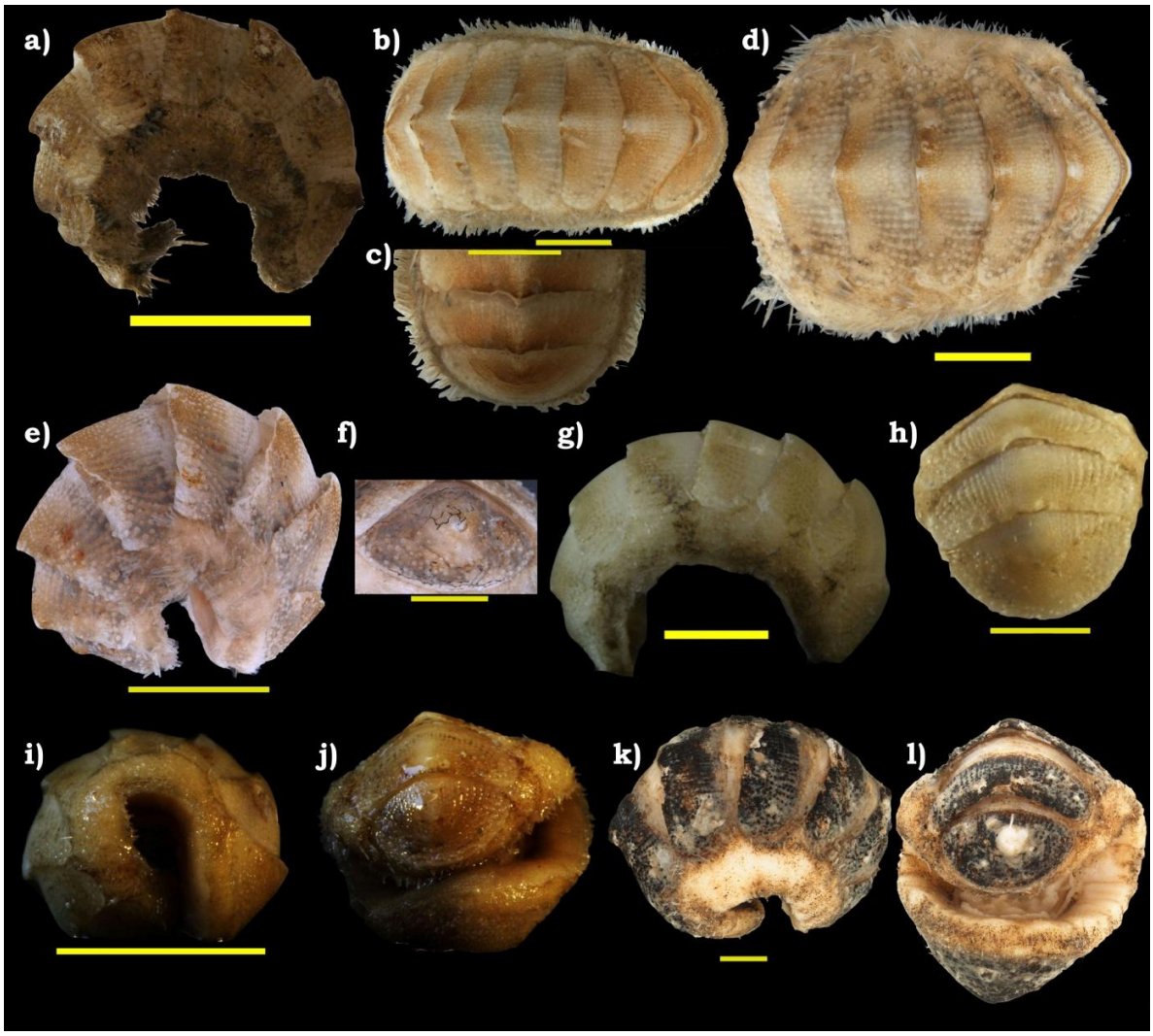


Figure 6- Showing morphological variation in small to medium sized *Hanleya*-specimens. Dorsal and lateral view.
a) Specimen 31 (2). Identified as *Hanleya sp.* Dorsal length (DL) = 7.3 mm. Scale bar = 2 mm.
b) and c) Specimen 29997. Identified as *H. hanleyi.* DL = 7.7 mm. Habitat = Shell gravel. Scale bar = 1 mm.
d) Specimen 55377. Identified as *H. hanleyi.* DL = 9.0 mm. Habitat = Shell gravel. Scale bar = 1 mm.
e) and f) Specimen 58016. Identified as *H. hanleyi. DL* = 9.4 mm. Habitat = Corals. Scale bar = 2 and 1 mm, respectively.
g) and h) Specimen a014. Identified as *H. nagelfar.* DL = 14.7 mm. Habitat = Sponge. Scale bar = 2 and 1mm, respectively.
i) and j) Specimen 30015. Identified as *H. hanleyi.* DL = 16.47 mm. Habitat = Shell gravel. Scale bar = 5 mm.
k) and l) Specimen Moll.Övr 7859. Identified as *H. hanleyi.* DL = 17.25. Habitat = Shell gravel. Scale bar = 1 mm. White areas on girdle is where spicule-samples were collected from.

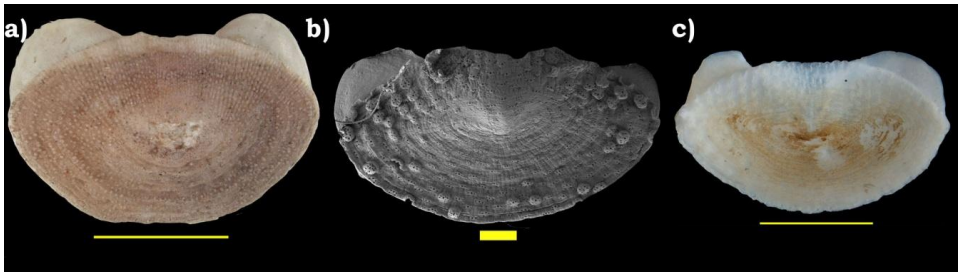


Figure 7 - Tail valve from *H. nagelfar* lectotype, from a small specimen *Hanleya* specimen found on on the sponge *Geodia baretti* and from a small specimen from shell gravel.
a) *H. nagelfar* Type 1329. DL = 58,9 mm. Scale bar = 5 mm.
b) Specimen a019. Identified as *H. nagelfar.* DL = ca 6 mm. Scale bar= 200 µm. SEM image.
c) Specimen 55376. Identified as *H. hanleyi.* DL = 9,8 mm. Scale bar = 1 mm.

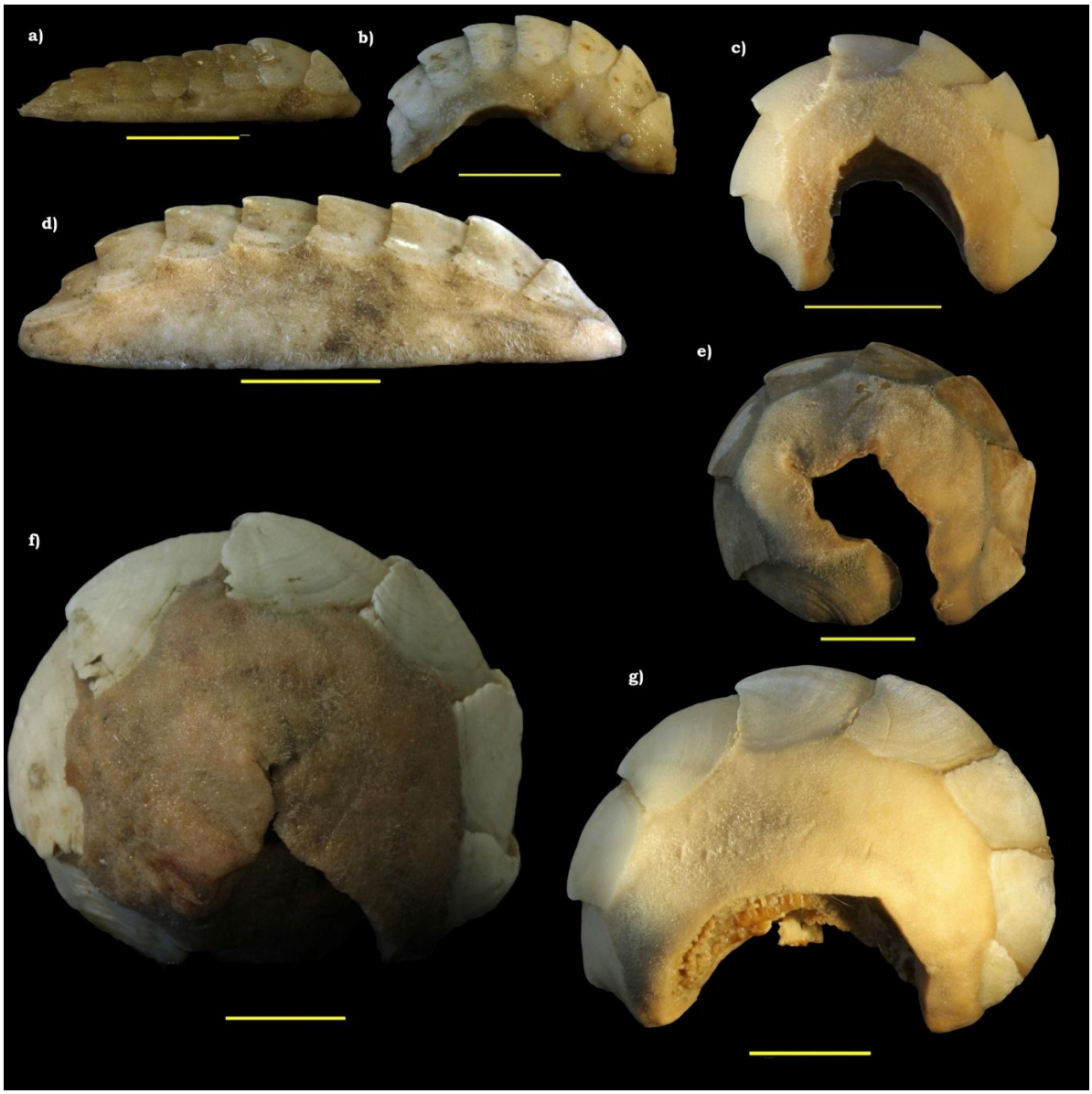


Figure 8 – Showing variation in medium to large specimens. Lateral view.
a) Specimen a023. DL = 19.94 mm; Identified as *H. nagelfar*. Scale bar = 5 mm.
b) Specimen 315. DL = 26.05 mm; Identified as *H. hanleyi.* Scale bar = 5 mm.
c) Specimen BioIce 3589 (13). DL = 33.40 mm; Identified as *H. nagelfar*. Scale bar= 5 mm
d) Specimen a021. DL = 61.06 mm; Identified as *H. nagelfar*. Scale bar = 10 mm, mirrored image
e) Specimen C1. DL = 82.10 mm; Identified as *H. hanleyi* var. *abyssorum*. Scale bar = 10 mm
f) Specimen a001. DL = 138.84 mm; Identified as *H. nagelfar.* Scale bar = 10 mm
g) Specimen BioIce 3589 (3). DL = 76.58 mm; Identified as *H. nagelfar*. Scale bar= 10 mm.
Caught on sponges: a-d, g. Habitat unknown: e, f.

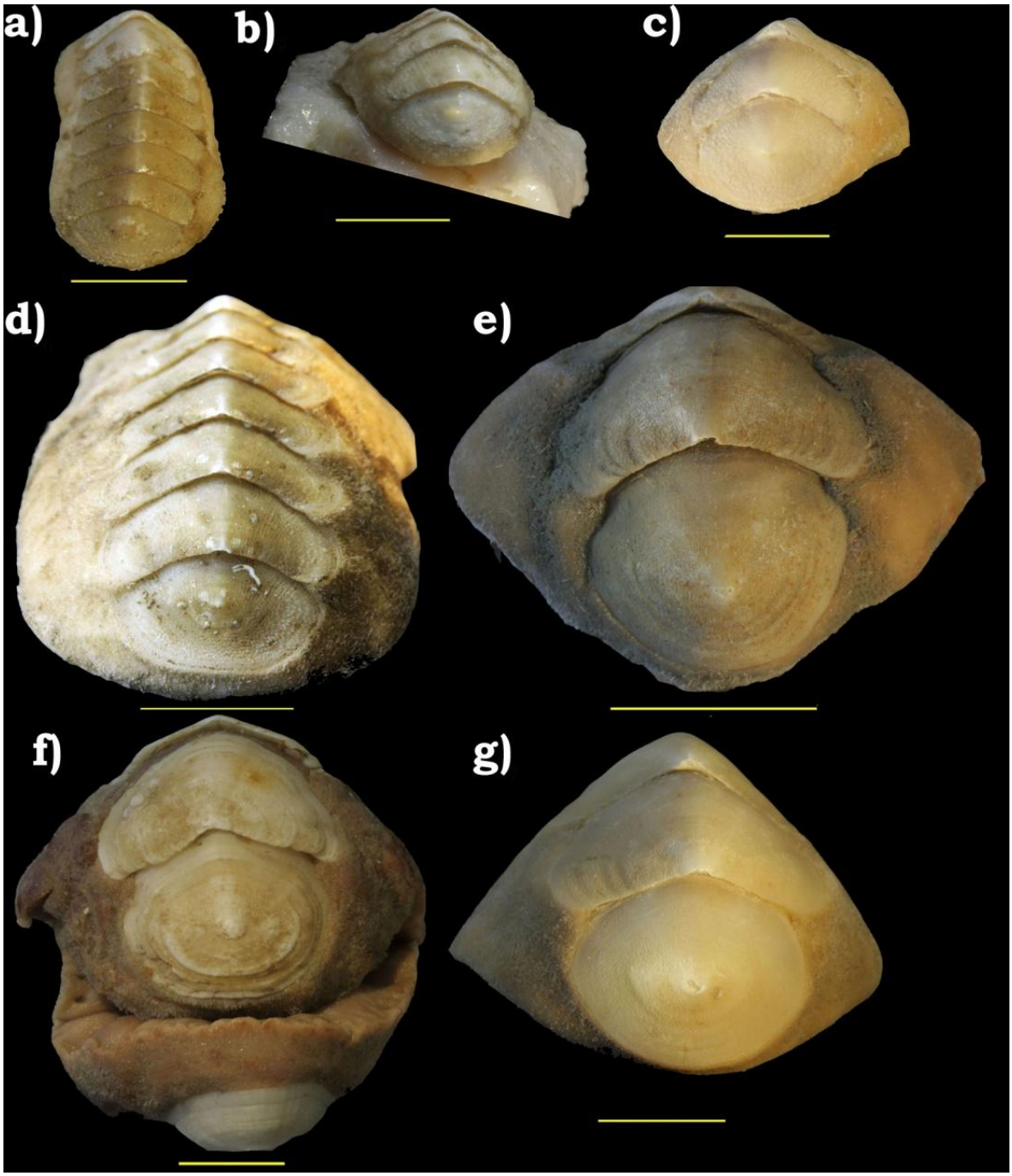


Figure 9 - Variation in tail valve shape in medium to large specimens. Dorso-posterior view. For specimen details, see label on Figure 8.

3.1.2. Radulae

The central teeth of the radulae are longer than wide. The blades are thin laterally and anteriorly and these parts may curl upwards. The teeth are broadest in the posterior 1/3. Ventrally, a broad medio-longitudinal ridge in the basal part (about 1/3 - 2/3 of the length) of the tooth can be seen. This ventral ridge is not visible in dorsal view in the SEM images,

but it can be seen in the light microscope due to the transparency of the teeth, as shown in the drawing in Figure 10. The first lateral teeth are embracing the central teeth ventrally and dorsally and seem to be supported by the ventral ridge. Tridentate cusps are seen on the major lateral teeth, with the median denticle larger than the two flanking. The two uncinal teeth was not easily observed and is not described herein. The spatulate uncinal teeth are much longer than wide (length to width ratio approximately 0.2) and almost as long as the major lateral teeth. Apically the teeth curl, forming a spoon-like structure. Three marginal teeth are seen on each side of the radula. These are longer than wide and pointy in the apical and basal end.

Two different radula types were found. In the first type (Figure 11 c and f), a thin ridge is running medio-longitudinally on the dorsal side of the central teeth. The dorsal ridge was observed two specimens from sponges, previously identified as *H. nagelfar* (a019 DL = ca 6 mm; a021, DL = 61.06 mm) and two specimens identified as *H. hanleyi* found on shell gravel (29997, DL = 7.7 mm) and on corals (30004, DL = 14.4). Images of these specimens (except 30004) can be seen in Figure 11.
In the second type, the central teeth did not have a dorsal ridge (Figure 12 c). This type was seen in two specimens from shell gravel and one from corals (Figure 12). They had previously been identified as *H. hanleyi* (55377, DL = 9 mm; Moll. Övr 7859, DL = 17.25 mm; 58016, DL = 9.4 mm).

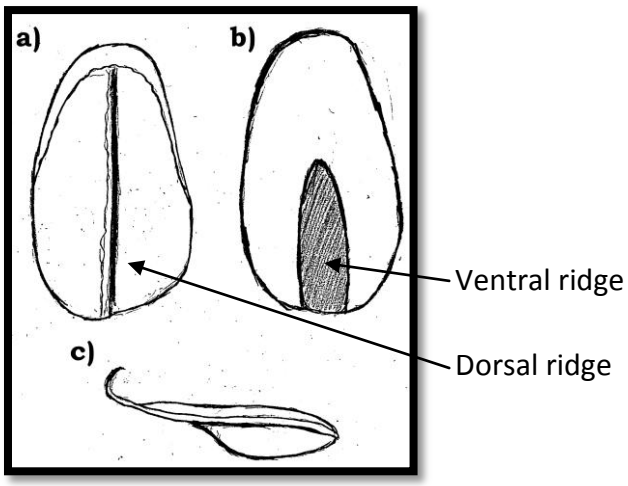


Figure 10 - Drawings of central tooth. Dorsal, ventral and lateral view.
a) Dorsal view. Showing the curling of the blade anteriorly.
b) Ventral view. Dark area indicating the broad ventral rib.
c) Lateral view. Ventral and dorsal rib is visible. Anterior curling of blade at the left side of the drawing.

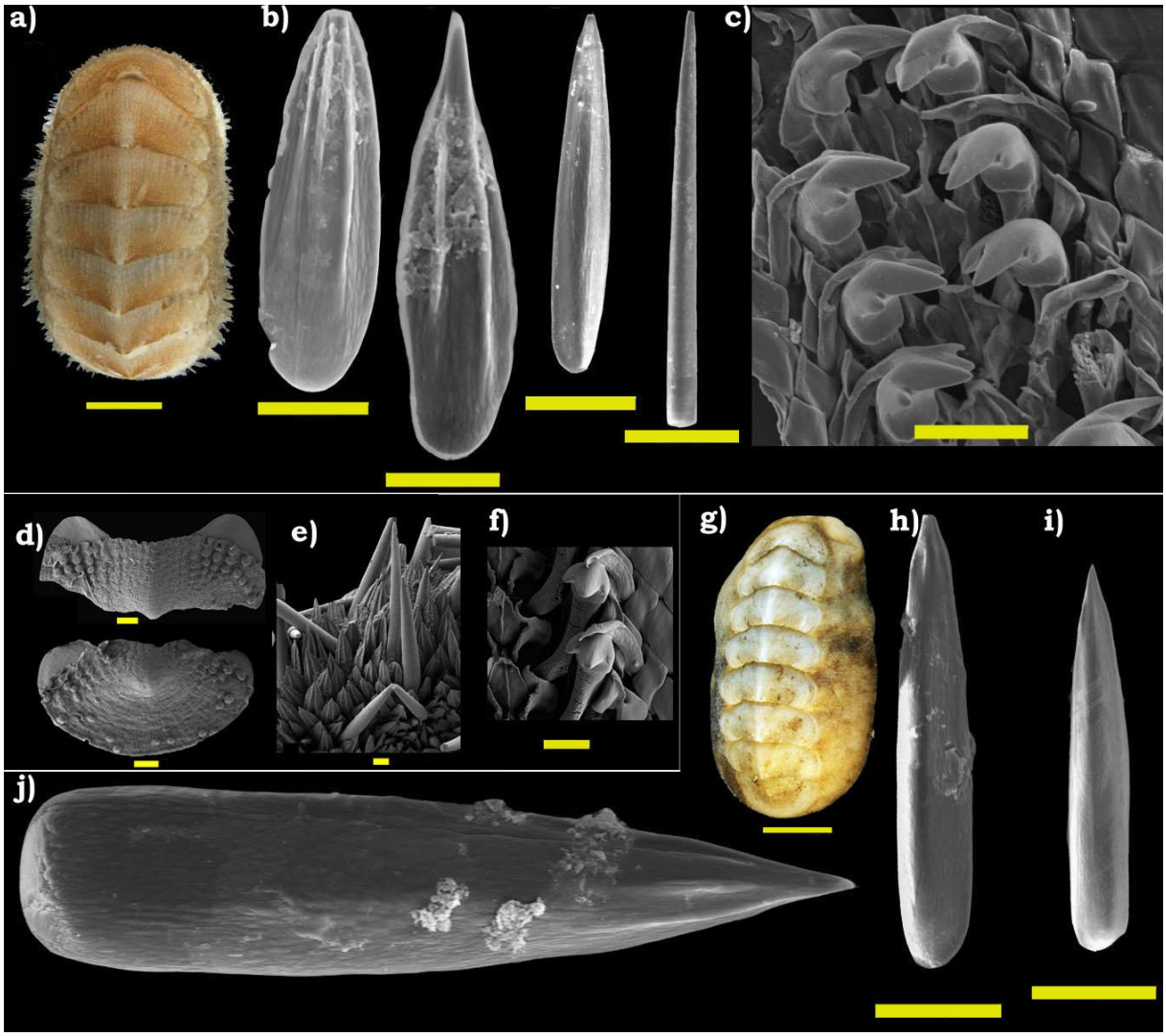


Figure 11 – Morphological variation in specimens with a dorsal ridge in the central radula tooth.
a) - c) Specimen 29997. Identified as *H. hanleyi*. DL = 7.70 mm. Overview of animal, spicule types and radula. Habitat = Shell gravel. Scale bars from left to right: 1 mm, 23 µm, 17 µm, 80 µm, 100 µm and 60 mm.
d) to f) Specimen a019. Identified as *H. nagelfar*. DL = ca 6 mm. Intermediate and tail valve. Spicules "in situ". Habitat = Sponge. Scale bar on d) = 200 µm. Scale bar on e) = 20 µm. Scale bar on f) = 30 µm.
g) to j) Specimen a021. Identified as *H. nagelfar.* DL = 61.06 mm. Overview of animal and spicule types. Habitat = Sponge. Scale bars from g-i = 10 mm, 60 µm and 80 µm. j = without scale.

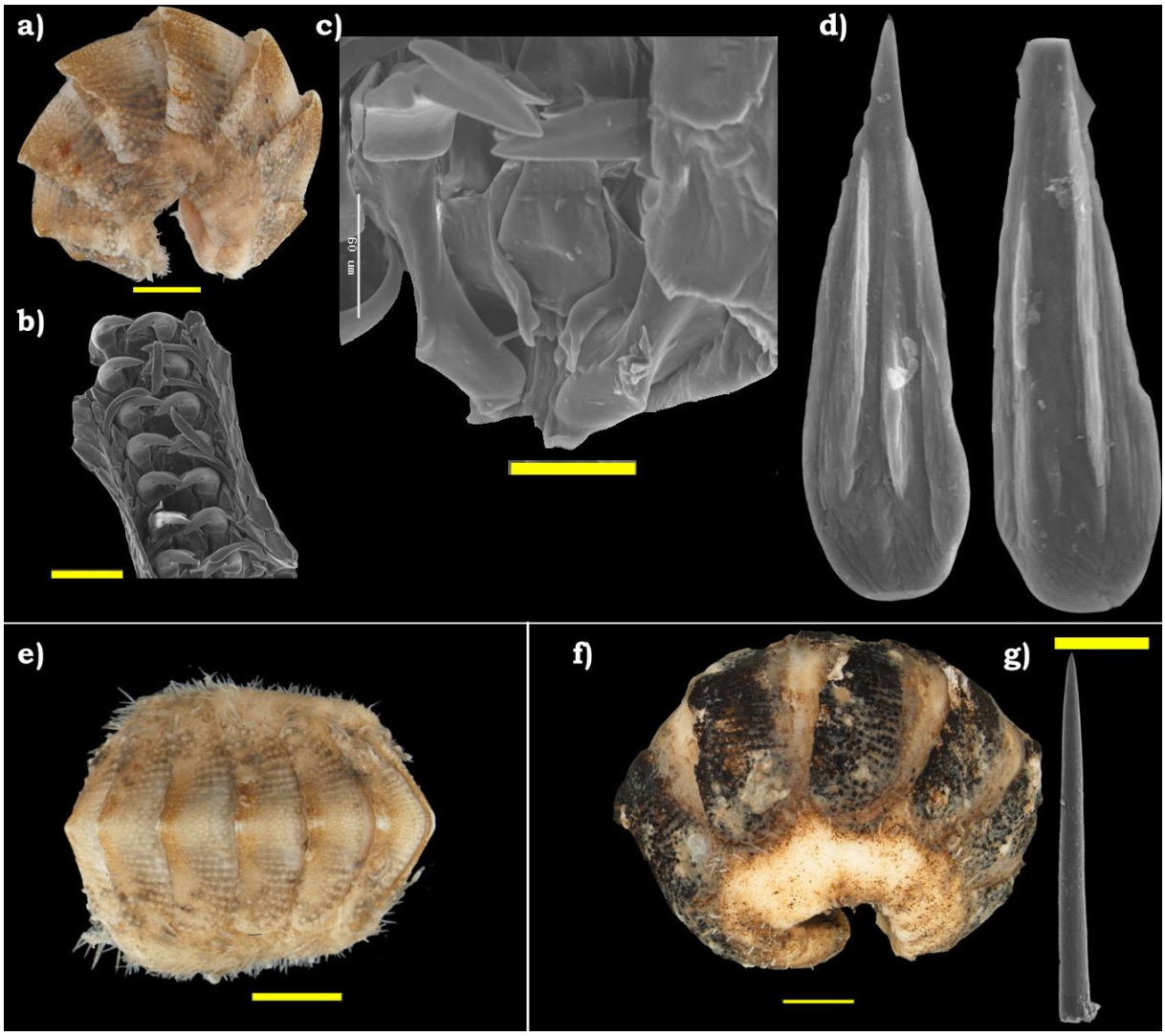


Figure 12 – Variation in specimens without a dorsal ridge in the central radula tooth. Identified as *H. hanleyi.*
a) to f) Specimen 58016. DL = 9.40 mm; Habitat = Corals. Overview of animal and radula, detailed radula and spicules. Scales a), b) and c) is 2 mm, 170 µm and 60 µm, respectively.
e) Specimen 55377. DL = 9.0 mm; Habitat = Shell gravel. Overview of animal. Scale = 1 mm.
f) and g) Specimen Moll.Övr 7859. Overview of animal and a large spicule. Scales are 1 mm and 120 µm.

3.1.3. Girdle armature

The dorsal girdle of small specimens (DL < 15 mm) is densely covered with flattened spicules, 60 – 110 µm long, each having 2-3 longitudinal ribs (i.e. Figure 11 b, e). The sides facing down towards the cuticle do not have ribs. They are pointy in the apical end, getting broader towards the base that is rounded. In the cuticle these are arranged like tiles on a roof. A very small specimen (a019, DL = ca 6 mm) caught on a sponge also had these ribbed spicules (Figure 11e). Specimens with both forms of radula have this spicule-type (Figure 11, Figure 12).

In somewhat larger specimens (DL ca 20 – 30 mm) the spicules are longer (100 - 200 µm) and the ribs do not extend as far towards the base (Figure 13). This was seen in specimens from shell gravel (i.e. Moll.Övr 7859, DL =17.3 mm from Kattegat) and specimens from sponges (i.e. BioIce 3589 (13), DL = 33.4 mm, from Iceland).

Larger needle-like spicules (250-300 µm) are situated randomly in between the small more triangular spicules. These spicules are found on specimens of all sizes (Figure 11 b and h). They are however dominating in larger specimens, but only occasionally seen amongst the more flattened, ribbed spicules in smaller specimens.

Two specimens with different sizes but very similar morphotypes (BioIce 3589: dorsal lengths of 33.40 mm and 76.60 mm), had different spicule types dominating the girdle. The largest had mostly the rounded, ± 200 µm long spicules, whilst the smallest had smaller flattened spicules (150 µm), with 2-3 ribs in the apical 1/3 of the spicule as the dominating type.

Very large smooth needles (i.e. Figure 12 g) are common in valve sutures and at the girdle margin and randomly dispersed in the dorsal girdle in all specimens. They vary in size from around 210 - 570 µm. 500 µm long spicules can even be found in small specimens (31 (2), DL = 7.3). These are all smooth, with no ribs or striata.

Ventral spicules (Figure 14) do not have ribs like the dorsal spicules. They are smooth, flattened and sharply pointed. Sides are more parallel than the small, ribbed spicule type seen dorsally on small specimens.

Figure 13 – A medium sized specimen (a023, DL = 19.94 mm) from a sponge.
a) Dorsal montage image, scale bar = 5 mm.
b) Scanning electron microscopy image of dorsal spicule, scale bar = 40 µm.
c) Dorsal spicule, scale bar = 80 µm.

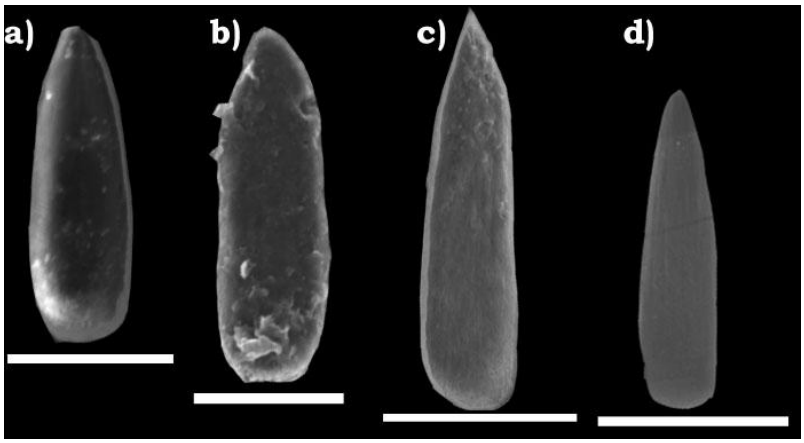


Figure 14 - Showing ventral spicules.
a) Spicule from specimen Moll.Övr 7859. Identified as *H. hanleyi.* Scale bar = 30 µm.
b) and c) Spicules from specimen 1935007.05. Identified as *H. hanleyi.* Scale bar b) = 23 µm c) = 40 µm
d) Spicule from specimen a021. Identified as *H. nagelfar*. Scale bar = 80 µm.

3.2. Morphometric results

3.2.1. Univariate analyses

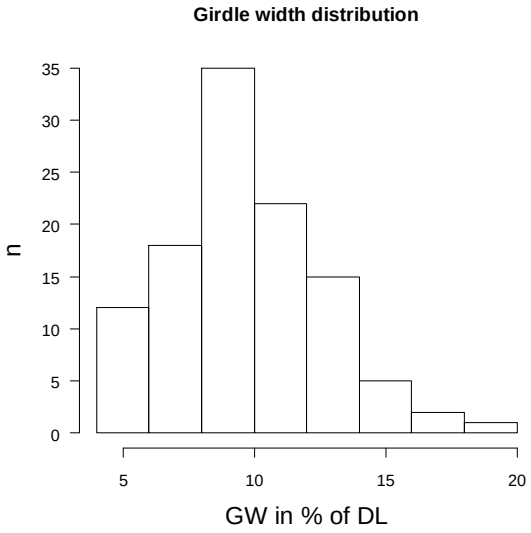
Univariate analyses were performed on characters that previously had been used for separating the species. The number of specimens measured for each character, morphometric range, mean values, standard deviation, the Shapiro-Wilk test results and the results from the regression analyses are listed in Table 5. Measurements for the *Hanleya nagelfar* lectotype are included in the table to see if the specimen is near the extremes in any of the measurements. Results for the previously used diagnostic characters for *H. hanleyi* and *H. nagelfar* are listed in the top frame of the table. Characters used for the calculations are presented in the lowest framed section of the table. Top frame characters, from top going down: Girdle width in % of dorsal length; dorsal elevation; mucronal distance from anterior margin in % of tail valve length; dorsal width/dorsal length ratio; tail valve width in % of DL– head valve width in % of DL; valve II length/width; valve IV length/width.

					Shapiro-Wilk test			Regression			
	Number	Range	Mean	Sd	W	p-value	Normal dist.	Regression	p-value	formula	Type 1329
GW	110	4,1-18,3	9,74	2,83	0,9859	0,3004	Yes	Yes	<0,0001	0,0463X+8,5	5,09
DE	107	0,13-0,47	0,33	0,05	0,9786	0,0821	Yes	Yes	0,0449	-0,0004+0,34	0,34
Mucro	107	28,57-63,13	47,17	8,28	0,9828	0,1826	Yes	Yes	<0,0001	40,8458X+0,23	54,2
DW/DL	112	0,37-0,72	0,52	0,07	0,9768	0,0485	No	Yes	0,0002	-0,0010X+0,6	0,4
VIIIW-IW	104	-6,79-3,44	-1,26	2,57	0,9585	0,0025	No	Yes	<0,0001	-3,4516X+0,8	1,7
IIL/IIW	109	0,25-0,82	0,58	0,09	0,9826	0,1655	Yes	Yes	<0,0001	0,0025X+0,5	0,66
IVL/IVW	107	0,13-0,71	0,44	0,1	0,9868	0,3759	Yes	Yes	<0,0001	0,0028X+0,4	0,49
IW	107	12,0-34-3	22,09	4,55	0,9747	0,0386	No	Yes	<0,0001	-0,1382X+25,9	18,17
IIW	109	12,6-39,2	23,1	4,78	0,9735	0,0286	No	Yes	<0,0001	-0,1441X+27,5	19,35
IIL	110	9,5-18,1	13,22	1,67	0,9868	0,3537	Yes	Yes	<0,0001	-0,0301X+14,0	12,73
IVW	107	14,1-39,2	25,33	4,54	0,9754	0,045	No	Yes	<0,0001	-0,1321X+29,0	21,73
IVH	107	3,8-13,8	8,45	1,99	0,9939	0,9174	Yes	Yes	<0,0001	-0,0520X+9,9	7,3
IVL	107	4,9-14,9	11	1,79	0,9689	0,013	No	No	0,59	0,59	10,7
VIIIL	108	8,3-17,4	12,94	1,75	0,9811	0,129	Yes	Yes	0,0241	0,0155X+12,5	12,22
VIIIW	109	13,6-29,4	20,72	2,92	0,9661	0,0071	No	Yes	<0,0001	-0,0541X+22,2	19,86
AM	107	2,0-9,9	6,15	1,57	0,9913	0,7325	Yes	Yes	<0,0001	0,0376X+5,1	6,62
DW	112	37,1-72,4	52,81	7,12	0,9769	0,0492	No	Yes	0,0002	-0,0979X+55,5	40,07

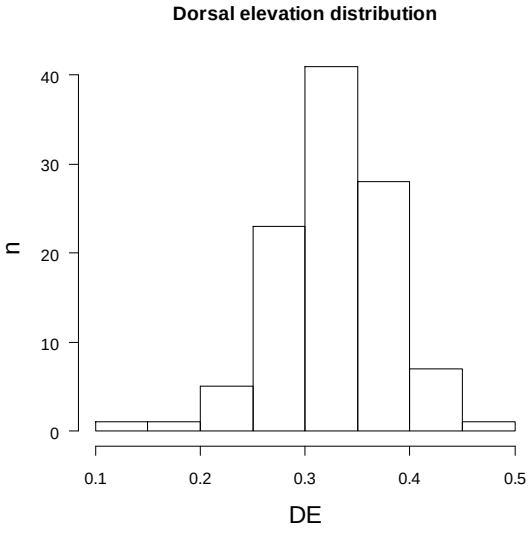
Table 5 - Results from analyses of characters used for separating *H. hanleyi* and *H. nagelfar* (upper framed section). Lower part of the table shows characters used in the calculations. Measurements for *H. nagelfar* lectotype 1329a are shown in the column to the right. Red numbers indicate values outside of the standard deviation. Abbreviations are listed in Table 2, Page 18.

Five of the seven characters (GW, DE, Mucro, IIL/IIW, and IVL/IWL) in Table 5 top frame are normally distributed according to the Shapiro-Wilk test. DW/DL and VIIIW-IW do not show a normal distribution. Histograms showing the distributions for these can be seen in Figure 15. A Gaussian (bell-shaped) distribution is expected when samples are gathered from one species. Four of the measurements used for the calculations (Table 5, bottom frame) show a normal distribution, while 6 show a non-normal distribution. Linear regression models fitted to the dot plots of characters used for separating the species are shown in Figure 16. As seen in Table 5, all characters show an ontogenetic variation in characters previously used as diagnostic characters in the examined specimens. Of the characters used for the calculations, only valve IV length is invariable with specimen size.

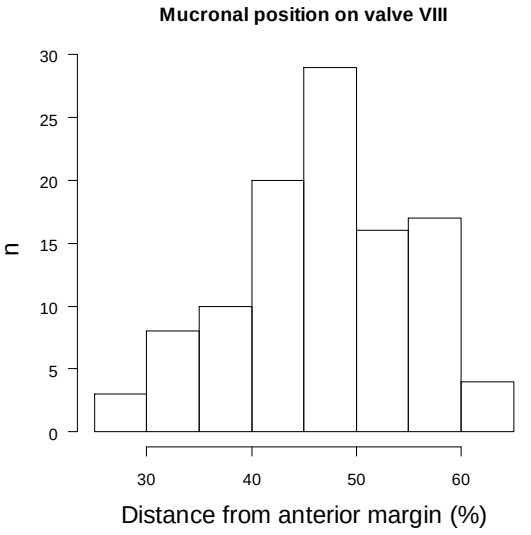
The *H. nagelfar* lectotype is within the standard deviation of all characters except GW, DW/DL, VIIIW-IW and DW.


Girdle width distribution
n
GW in % of DL

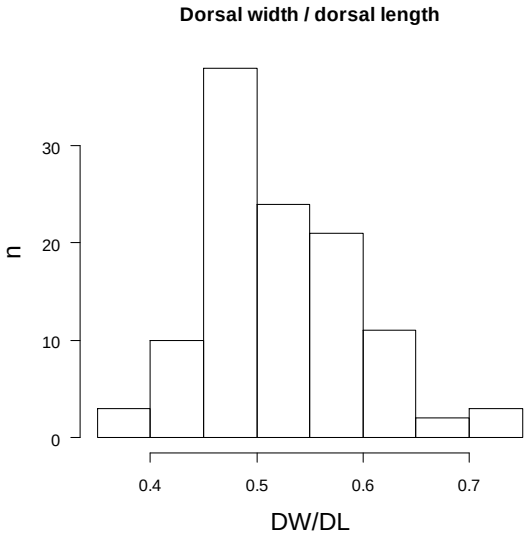
a)


Dorsal elevation distribution
n
DE

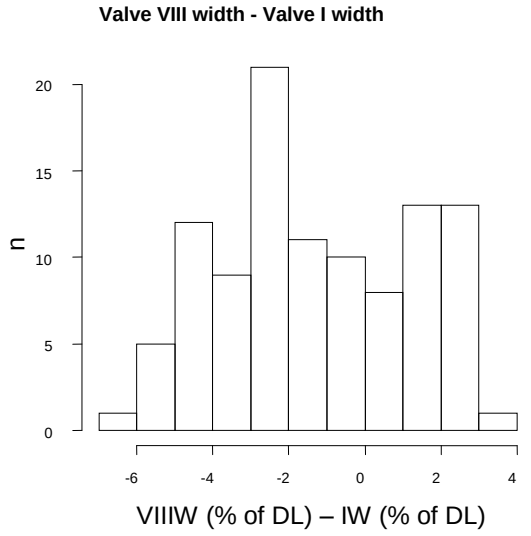
b)


Mucronal position on valve VIII
n
Distance from anterior margin (%)

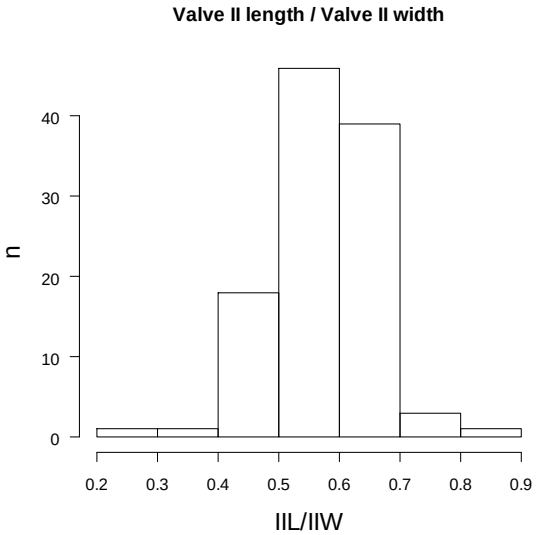
c)


Dorsal width / dorsal length
n
DW/DL

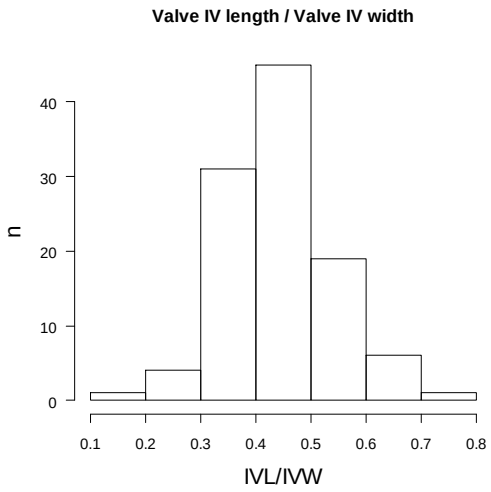
d)


Valve VIII width - Valve I width
n
VIIIW (% of DL) – IW (% of DL)

e)

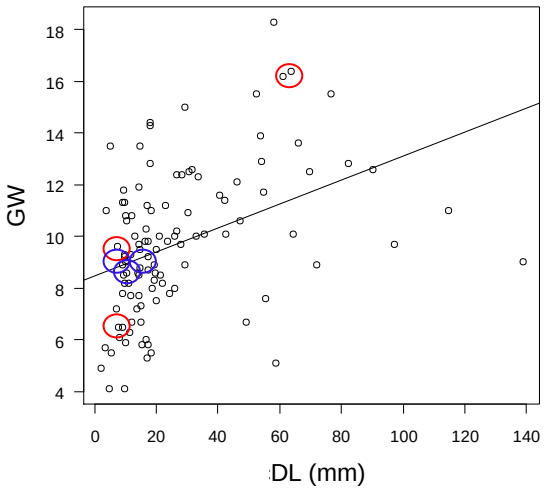

Valve II length / Valve II width
n
IIL/IIW

f)

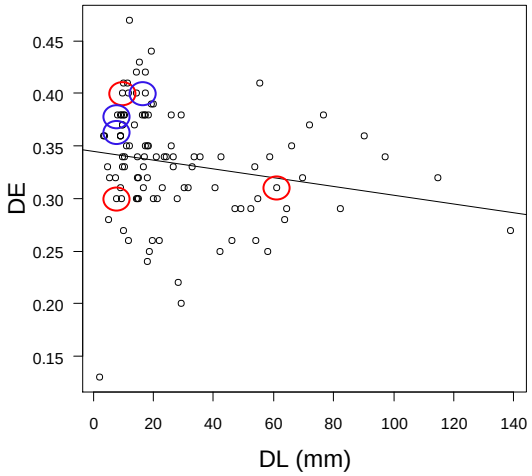


g)

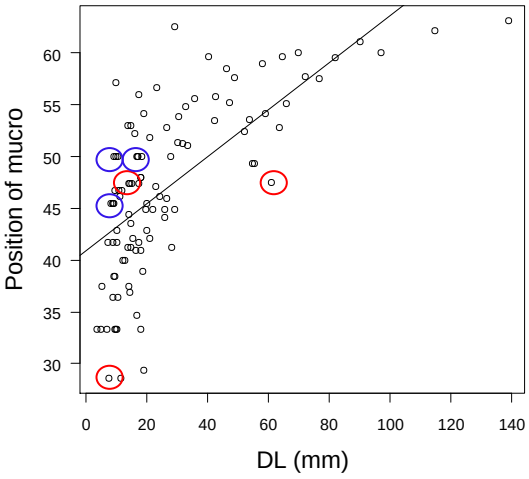
Figure 15 - Histograms showing distributions of the characters used for separating *H. hanleyi* and *H. nagelfar*. Values on the x-axis of a), c) and e) are in % of dorsal length.
Figure d) and e) is not normally distributed according to the Shapiro-Wilk test.


GW
DL (mm)

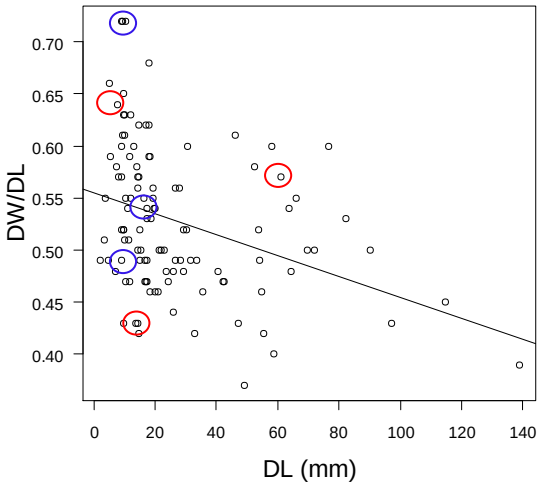
a)


DE
DL (mm)

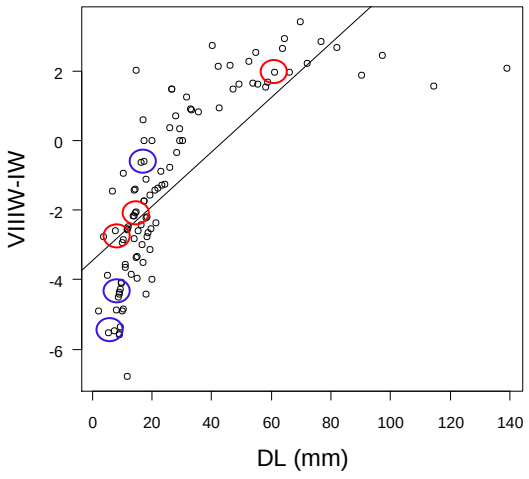
b)


Position of mucro
DL (mm)

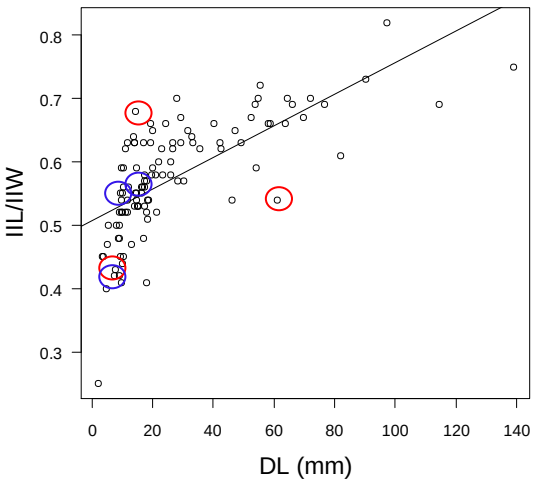
c)


DW/DL
DL (mm)

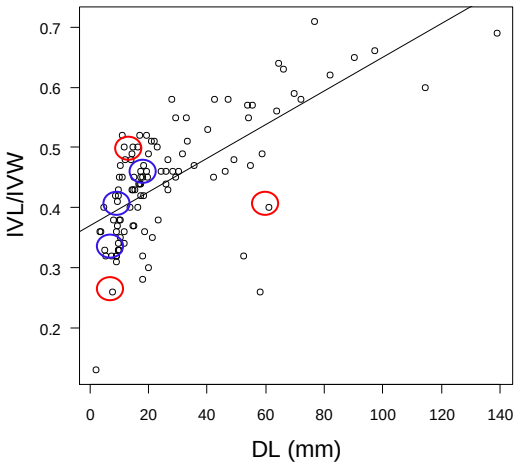
d)


VIIIW-IW
DL (mm)

e)


IIL/IIW
DL (mm)

f)



g)

Figure 16 - Dot plots showing change in characters (used for separating *H. hanleyi* and *H. nagelfar)* from small to large specimens. Best fitting linear regressions according to the Anova analyses are fitted to each plot.
Red circles indicate position of specimens with observed dorsal ridge in central radular teeth. Blue circles indicate position of specimens without the dorsal ridge in central radula teeth.
Values on the y-axis of a), b) and e) are in % of dorsal length. Values of f X-axes are in mm.

3.2.2. Multivariate analyses

Multivariate analysis was performed on 101 specimens. The detrended correspondence analysis resulted in a "longest gradient length" of 1.036. The low gradient length indicates that a principal component analysis (PCA) is a suitable method for the dataset (Lepš and Šmilauer 2003). Eigenvalues for axes one to four are 0.462, 0.220, 0.134 and 0,122, respectively. The results from the analysis are presented in ordination diagrams (Figure 17 - Figure 19). Correlation values, given in degrees are listed in Table 6. The characters dorsal length (DL), intermediate valve shape (IM) and tail valve shape (TV) show the strongest correlation of the characters. They show small degree of correlation with dorsal elevation (DE), but are partially correlated with girdle width (GW). Girdle width is more or less negatively correlated with dorsal elevation (Table 6). Specimens with smooth central radula tooth is marked with diamonds, specimens with a rib in the central radula-tooth is marked with squares in Figure 17. The different colored dots indicate the substrate-type the specimen was caught on. The specimens show a more or less uniform distribution with no easily distinguishable clusters. Specimens with a ridge on the radula tooth were found on sponges, corals and shell gravel, whereas the specimens with smooth radula were only found on shell gravel and corals. These specimens are widely separated from each other in

the diagram. Thus, the radula types do not correlate with the characters the analysis is based on.

Specimens with genetic data available are highlighted in Figure 18. They are found in most areas of the diagram. The two specimens from Kattegat is foun on the negative side of the x-axis only, but on positive and negative values of the y-axis.

	GW	DL	IM	TV
DE	142^0	104^0	93^0	79^0
GW		37^0	48^0	62^0
DL			11^0	25^0
IM				13^0

Table 6 - Correlations between characters in the multivariate analysis. 0^0 = 100 % positive correlation. 90^0 = 0 % correlation. 180^0 = 100 % negative correlation.

3.2.3 Biogeography

Sampling localities were plotted into the multivariate ordination diagram (Figure 19). Specimens from the Bergen area, marked with blue, is widely spread in the ordination diagram. Specimens from the Trondheim area are mostly found on the negative X-axis side, but are widely distributed over the Y-axis. This do also apply for the specimens from the Swedish west coast, although they are more constrained to the central regions of the Y-axis. The specimen from Porcupine Bank (West of Ireland) stand out from the others to some extent and due to its low Y-axis value.

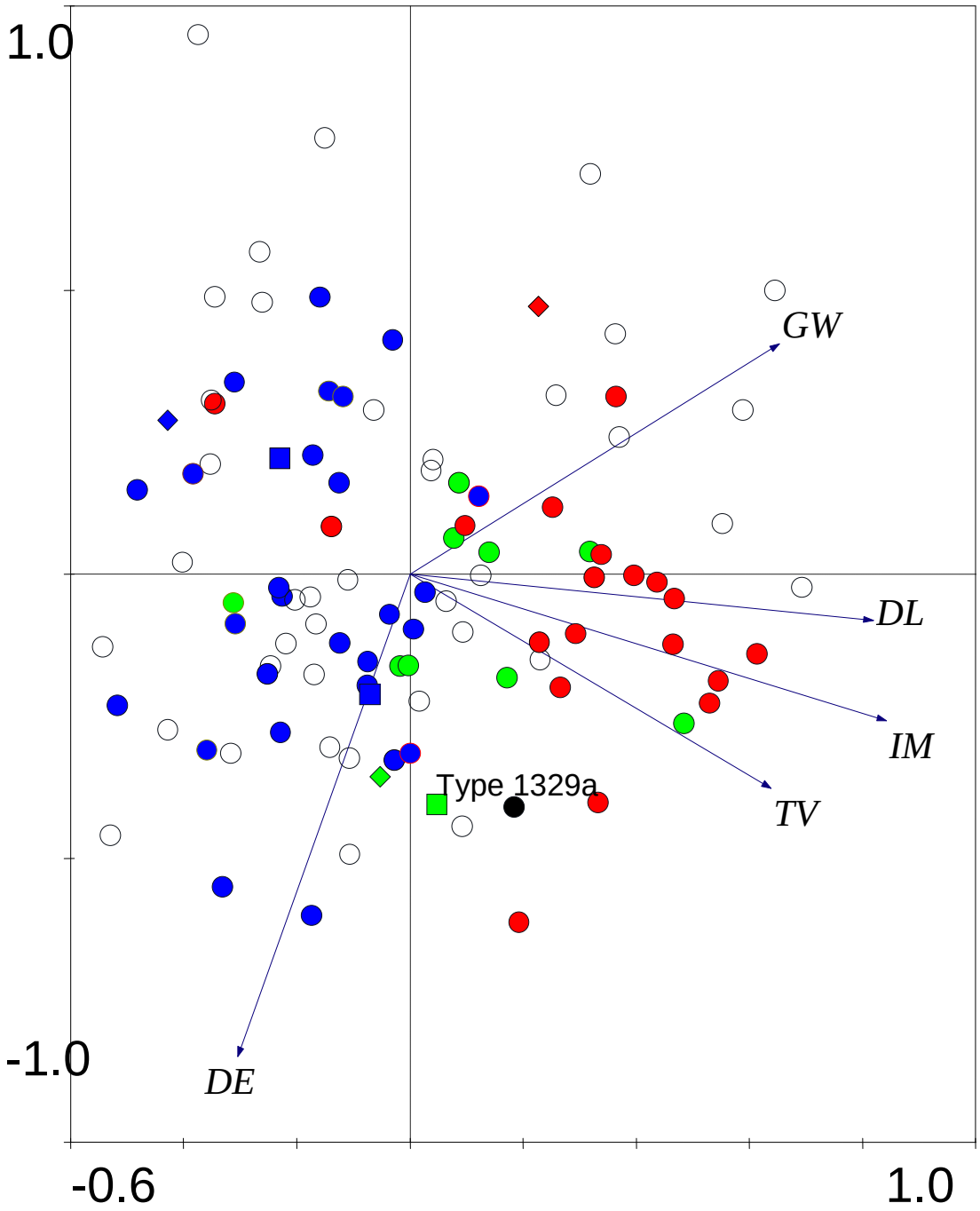


Figure 17 - Characters used for separating *H. hanleyi* and *H. nagelfar* analyzed in a PCA and presented in an ordination diagram. The 1st axis accounts for 46.2 %, 2nd axis for 22.0 % of total variance.
Square shapes (n=3) indicate specimens with a smooth central radula-tooth. Diamond shapes (n=3) are specimens with a rib in the central tooth of radula.
Colors indicate:
• = From sponge; • = From hard bottom (e.g. shell gravel, clay with stones); • = From corals; • = *H. nagelfar* lectotype;
O = Habitat unknown

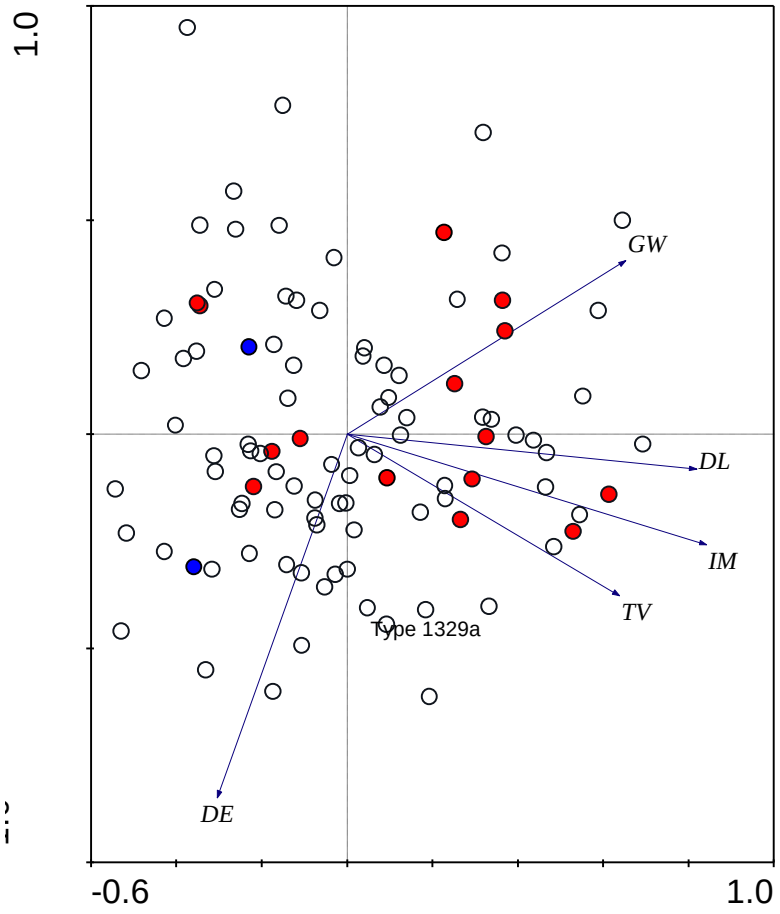


Figure 18 - An ordination diagram highlighting specimens with available sequences. Colors indicate:
• = Sequenced specimens; • = Sequenced specimens from Kattegat;
O = Habitat unknown

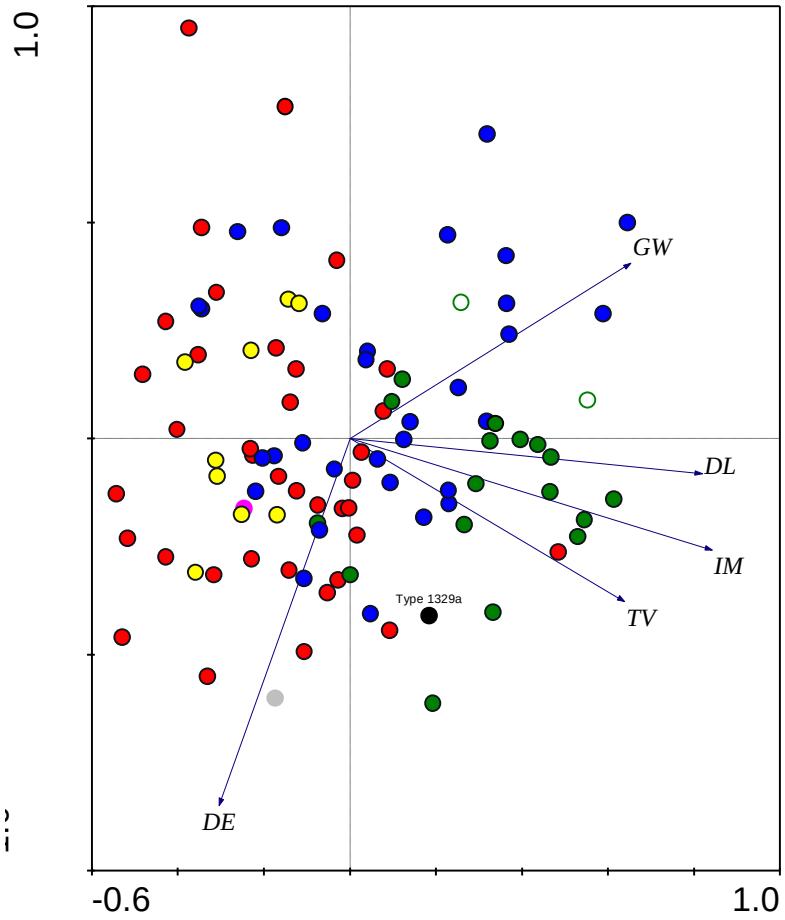


Figure 19 – Ordination diagram with biogeographic regions highlighted. Colors indicate:
• = Trondheim area; • = Bergen area; • Iceland; O = Greenland; • = Swedish west coast; • = Murchison field; • = Porcupine bank • = *H. nagelfar* lectotype

3.3. Molecular results

Sequences from one or several genes could be amplified from 20 of the 40 extractions made. Amplification of the genes cytochrome oxidase subunit I and mitochondrial 12S rRNA was not successful. Sequences were obtained from 18S rRNA (n=14), 16S rRNA (n=14) and Cytochrome B (n=12) with the primers, 18S5F, 1100R, 600F (Norén and Jondelius 1999) and 600r (Steiner and Dreyer 2003), 16Sar-L and 16Sbr-H (Palumbi et al. 1991) and UCYTB 251F and UCYTB270R (Merritt and Shi 1998) for the respective genes. Other primers listed in Table 3 did not yield any result. A table listing morphometric values for characters previously used for separation of *H. hanleyi* and *H. nagelfar,* for each successfully sequenced specimen, can be seen in Appendix III.

A partial region of the 18S gene ranging from 897-970 bp in length was amplified from 14 specimens. Nine sequences had identical haplotypes, while the five remaining had ambiguities in 1-9 sites that were replaced with DNA-codes as explained in "2.2.9. Editing and analyzing the sequences". Specimen BioIce 3589 (12) could be sequenced in only one direction and is shorter than the rest (599bp). The opposite direction did not yield a sequence of good quality and was discarded.

A partial region of 415-544 bp was amplified for the gene 16S. Identical haplotypes were found in 11 specimens. The remaining three had 2-22 sites that could not be assigned to specific nucleotides due to ambiguities in the sequence. DNA-codes were inserted in these sites. The specimens a021 (244 bp) and 31 (2) (479 bp) could only ble sequenced in one direction and the sequences are thus shorter than the others.

The partial sequences of the gene Cyt B ranged from 364-405 bp in length. Two distinct haplotypes of this gene were found. Within these two haplotypes, some sequences had sites showing ambiguities and DNA codes were used. Haplotype 1 was obtained from specimens from Iceland (n=1), Bergen area (n=8) and the "Røst reef" locality (n=1). The second haplotype was obtained from two specimens from Kattegat. The two distinct haplotypes had a genetic variation in 36 sites (9.97 %).

The blasting of uncontaminated 16S and 18S sequences with blastn 2.2.21 (Altschul et al. 1997) always resulted in *Leptochiton asellus* (Gmelin, 1791) as the most similar sequence.

When the cytochrome b sequences were blasted, birds, polychaetes and fishes were found in the top three positions.

Haplotypes are shown in alignments for each gene in Appendix IV. Sequences with ambiguous sites and sequences yielded from one direction are also included in the alignment.

3.3.1. Phylogenetic placement of Hanleyidae

Three Bayesian analyses were performed on the obtained sequences, one for each gene. The 18S consensus tree (Figure 20 a) clusters all Hanleyidae sequences as sister group to Leptochitonidae (= *Lepidopleuerus cajetanus* and *Leptochiton asellus*). This is very well supported by the posterior probability of 1. Posterior probabilities within the Hanleyidae clade are very low. The two Kattegat specimens, “Moll Övr 7858” and “Moll Övr 7859”, are placed in different clades in the tree. When studying the alignment used in this analysis, the only difference between these two sequences is two ambiguous sites.
The Hanleyidae 16S-sequences also form one clade with a posterior probability of 1 in the consensus tree (Figure 20 b). Leptochitonidae is paraphyletic, but this is only supported by a posterior probability of 0.63 and should be viewed with caution.
Hanleyidae forms one clade in the Cytochrome B tree as well (Figure 20 c). The two specimens from Kattegat represent a sister clade to the Iceland, Bergen and Sotbakken specimens, but this is not well supported with its posterior probability of 0.52. The two individual clades are supported by high posterior probabilities (0.94 and 0.99).

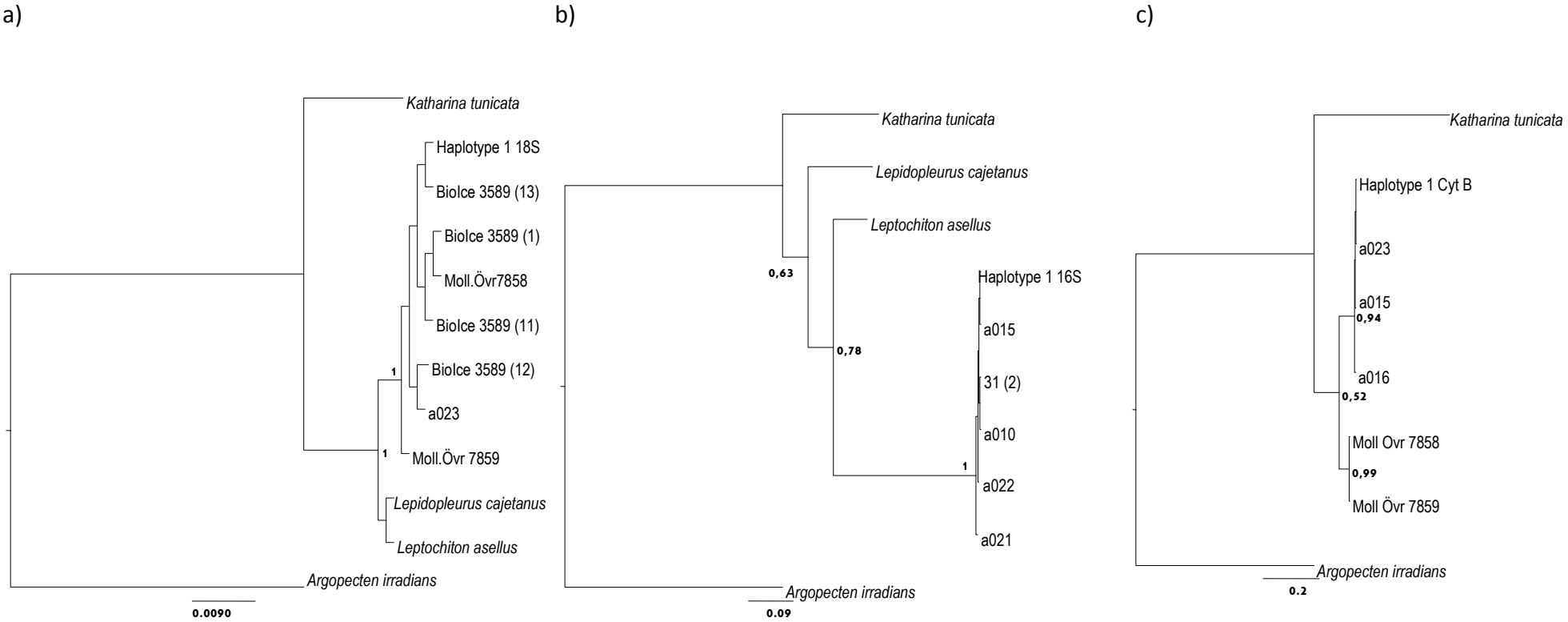


Figure 20 - 50 % majority rule consensus trees (phylograms) from Bayesian analyses showing clustering of the Hanleyidae sequences. Posterior probabilities (> 50%) are given for each node. Selected parameter model based on MrModeltest results: a) K80 + G; b) GTR + G; c) GTR.

The phylogram using species from all chiton families available in GenBank is shown below (Figure 21).

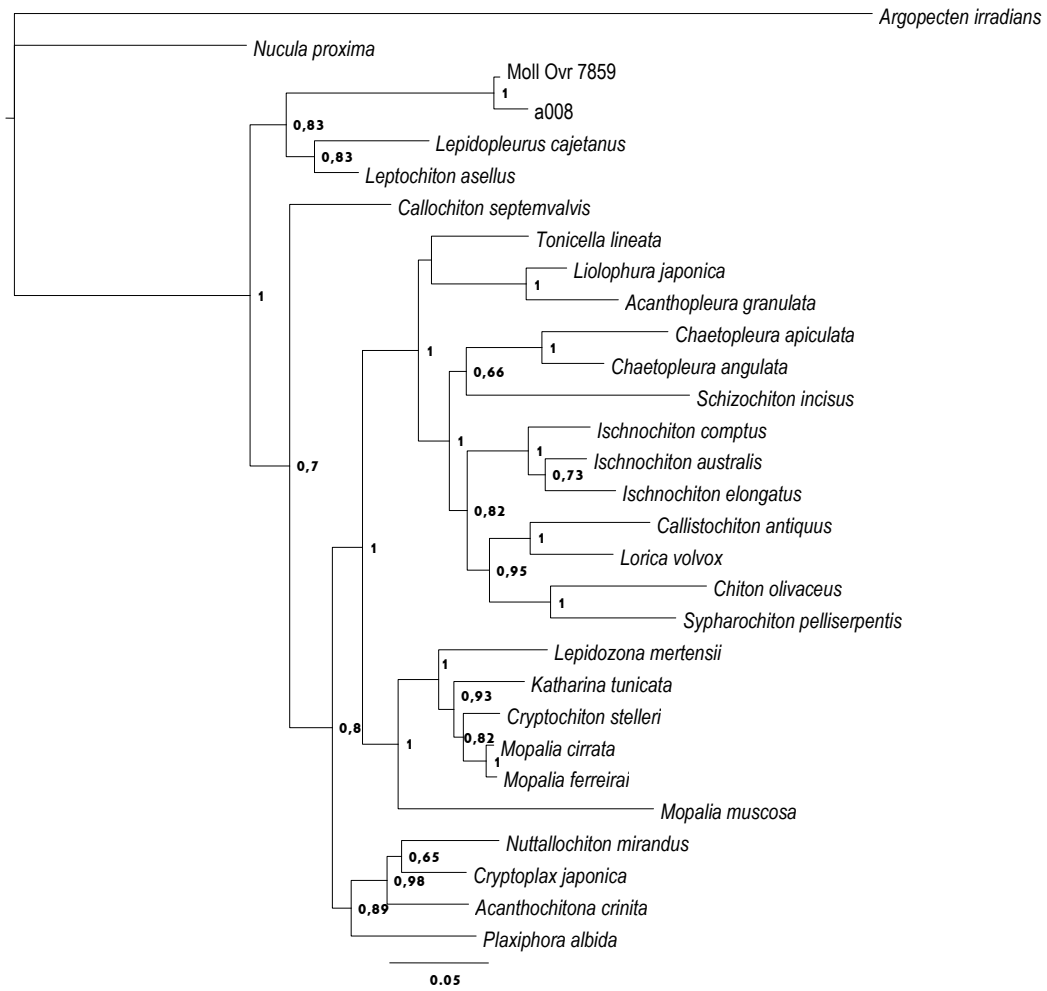


Figure 21 – 50 % majority rule consensus phylogram of combined 18S + 16S analysis showing the position of Hanleyidae within Polyplacophora. Posterior probabilities (> 50 %) are given for each node. Selected parameter models: 18S = Sym+I+G; 16S = GTR+I+G

In the consensus tree from the combined alignment of 18S and 16S, polyplacophorans constitute a monophyletic clade. Hanleyidae and Leptochitonidae are sister groups basal in the tree, supported by high posterior probabilities. They constitute the monophyletic Lepidopleurida.

When following the taxonomy of Sirenko (1997) (after Okusu et al. 2003) the following families are polyphyletic according to the Bayesian analysis herein: Chitonidae; Ischnochitonidae; Mopaliidae and Acanthochitonidae.

A Bayesian analysis was also run with the addition of Cytochrome B for the Hanleyidae species, *K. tunicata* and *A. irradians*. Sequences were unavailable for other species and thus the alignment had a lot of missing data, interpreted as "?". The resulting tree can be seen in Appendix V. The tree is mostly congruent with the 18S and 16S tree, but Leptochitonidae is paraphyletic, with *Leptochiton asellus* and a monophyletic Hanleyidae branching of *Lepidopleurus cajetanus*. The overall posterior probabilities are also lower than the tree in Figure 21.

4. DISCUSSION

4.1. Discussion of materials and methods

4.1.1. Studied material

The focus area for this study was the Scandinavian North Atlantic and thus the studied specimens cover the entire geographical distribution of *Hanleya nagelfar* (Figure 1). *Hanleya hanleyi* has a much larger geographic range and some areas where *H. hanleyi* is known from were not covered in this thesis. More specimens should be included, especially from the British Isles and close to the type-locality for *H. hanleyi* (Scarborough), as well as from the southwestern European coast and the Mediterranean Sea and from Canada. *H. hanleyi* is rare in these areas (Monterosato 1878, McMillan 1968, Jones and Baxter 1987) and although several institutions were contacted, only three specimens from the British Isles (including Porcupine Bank) plus one specimen from Fuerteventura could be obtained from these areas. The type specimen of *H. hanleyi* could not be included in this study because the institution hosting it (Wood End Museum, Scarborough, England) did not want to send it by mail and could not provide working space at the museum.

4.1.2. Morphological methods

Total length and total width has to be measured on animals with their body expanded as if attached to a flat surface. When a chiton is removed from the substrate or is submersed in fixative it usually curls up, similar to a woodlouse, with its valves facing out. Specimens that are preserved in this state are almost impossible to straighten after they have been fixed, making it difficult to get the exact total length and total width. Since most of the chitons available were curled, I chose to measure lengths and widths on the dorsal side as described in “2.2.1. Morphometrics”. Dorsal width and dorsal length are thus not comparable to total length and total width in other studies on chitons. Of the three states (stretched, slightly curved, fully curled), most specimens were in state 2. To make as few corrections as possible, specimens of state 1 and 3 were corrected to state 2. The degree of curling is

partially subjective since there is no clear definition between the states. This could to some extent affect the results. However, if the large variation between strongly curled and flat specimens was to be ignored, this would influence the results even more.

Animal proportions can vary greatly depending on the mode of preservation. As seen on the dry specimen, where the girdle on one side of the animal is twice as broad as the other Figure 23. The girdle can also be almost twice as broad when the animal is preserved anaesthetized under pressure on a flat surface compared to when it is just immersed in formalin (Warén and Klitgaard 1991). This could not be taken account of since it is unusual to anesthetize specimens prior to fixation (Warén and Klitgaard 1991) and probably this was not done in any of the museum specimens studied here.

Number of gills is species dependent, but increases with specimen size (Hunter and Brown 1965) thus being a poor species diagnostic in this case. This was also examined by Warén and Klitgaard (1991), who did not find differences in gill number between the two species of *Hanleya*.

Valves of a few specimens were to some extent hidden under the valve anterior to them or by the perinotum. Their lengths or widths were measured by manipulating the animals until the valves margins were visible. Dissection often was not an option since most of the material examined was museum material borrowed from other institutes.

The valves of the *H. nagelfar* lectotype (Type 1329) follow the specimen in a small box and each valve's original position is numbered. Valves were carefully placed back onto the animal before measuring of dorsal length and dorsal width (Figure 22). Dorsal length and dorsal width of the lectotype is therefore not as accurate as the rest of the dataset, but this was the best method for measuring this specimen. Indentations in the girdle are seen in the lectotype (Figure 22 a), which could imply that it was broader when alive. A possible explanation is that the animal has been dry/partially dry or the preservative has been suboptimal, thus affecting girdle width of this 163 year old specimen.

As mentioned above, the type specimen of *H. hanleyi* could not be studied. Two specimens were however obtained from the British Isles and one from west for Ireland. The two specimens (1935007.05 from Oban, Scotland) from the British Isles were of typical *H. hanleyi* morphology (Figure 23) and were probably the best substitutions for the *H. hanleyi* type. The specimens were however dry, effecting the state of the girdle to a large extent. Girdle with

could thus not be correctly measured and the specimens are not included in analyses including this character (the multivariate analysis and univariate DW/DL and GW). The spicules of these specimens did not differ from similar sized specimens from other localities.

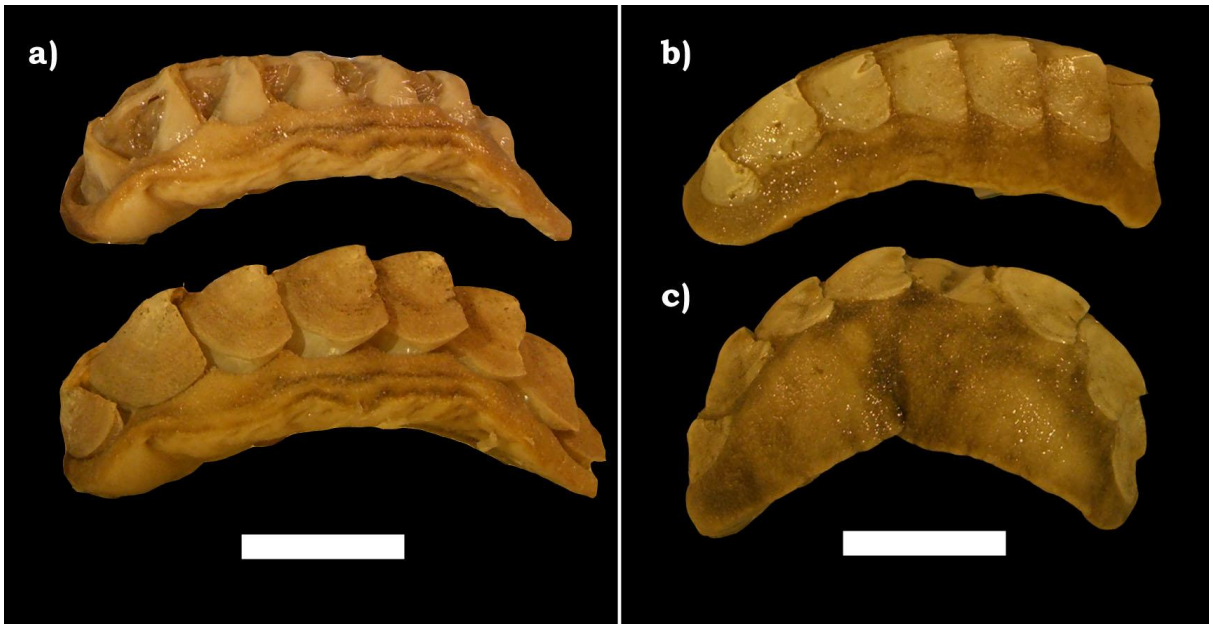


Figure 22 – Three specimens of *H. nagelfar* of comparable sizes, with variations in girdle width. Scale bars = 10 mm.
a) Type 1329. Indentations in girdle indicate suboptimal preservation. Top: Without valves; Bottom: Valves carefully inserted in their original positions.
b) Specimen 13395 with a narrow girdle.
c) Specimen 10571 with a wide girdle. Valve V has been removed.

Figure 23 – Dorsal montage image of the two dry specimens from the British Isles (Collection number 1935007.05). Scale bar = 1 mm.

The size of beaks and degree of carination has been used in several species descriptions for *Hanleya spp.* (Lovén 1846, Jeffreys 1865, Kaas and van Belle 1985). The present study, including more than 100 specimens, in contrast renders beak size and degree of carination not very trustworthy characters for the species. Especially the beaks tend to wear and break, thus being inferior as a character to be used for identification. Warén and Klitgaard (1991) also noticed that the dorsal keel of the shell plates often was worn in many specimens. This could lead to misinterpretation of the actual degree of carination. In specimens that seemed less affected by wear and tear, I often noticed a more rounded dorsal keel in the anterior part of jugal areas compared to the posterior part on the intermediate valves. This was seen on small as well as large animals.

4.1.3. Molecular methods

Mitochondrial genes were chosen based on their previous use for discriminating closely related species in various taxa (e.g. Merritt and Shi 1998, Pfenninger and Magnin 2001, Okusu et al. 2003, Järnegren et al. 2007, Yuan 2009). The nuclear 18S gene was chosen because of its usability for resolving higher phylogeny and because of a large amount of polyplacophoran 18S sequences was available in GenBank.

COI, the "optimal" mitochondrial gene based on the high number of polyplacophoran sequences available and on its suitability in resolving relationships on species-level did not yield any sequences. In the molecular work of Okusu et al. (2003), species from only 10 of 13 families yielded sequences using the Folmer (1994) primer. Thus, there appear to be groups/species where this primer does not work well. Similar problems have been experienced by one of my supervisors (C. Schander, personal comment) on other molluscan taxa. The difficulties with obtaining sequences from this gene, and the fact that 16S did not show any variation between highly morphologically variable specimens, made it important to try other variable genes. In bivalve mollusks Cytochrome b and 12S showed more variation than 16S (Järnegren et al. 2007).

Sequence results could only be obtained from twenty of the forty *Hanleya*-specimens fixed in ethanol and thus initially thought to be suitable for molecular work. A possible explanation could be the way the animals had been fixed and preserved. Thirteen of the

animals not yielding sequences were preserved in collection jars with several other specimens. It is possible that the ethanol in these jars never was replaced (J. A. Sneli, personal comment), making the water from the animals dilute the ethanol so that DNA was not optimally fixed and preserved and degradation could occur. Other animals were old and/or dry, or information of whether they had been in contact with formalin was not available. Four of the twenty specimens who did not amplify DNA (RMNH.MOL.HLS.0528; RMNH.MOL.114973; a009 and 31 (1)) were however recently caught (1977-2007) and should not have been in contact with other preservatives than ethanol. Unknown events during their preservation, or technical problems during the extraction to PCR procedure might be the reason why these four did not yield any sequences.
The initial primers used were so-called “universal primers”, having primer sequences that are conservative and thus work on several higher taxa. Primers designed for mollusks were also used (see Table 3), but only UCYTB151F and UCYTB270R yielded sequences on the *Hanleya*-specimens.

18S sequences were obtained from five specimens from Iceland, but mtDNA was only obtained from one of these animals. The fact that nuclear DNA was obtained indicates that the DNA is not destroyed and in fact it should be easier to sequence mtDNA due to the higher concentration per cell (Järnegren et al. 2007). To see if the DNA extracts contained too little mtDNA, new extracts from mitochondria rich tissue (gills) were made from the five Iceland specimens. Only one of the new extracts yielded a PCR product and a sequence.

The 18S sequences from the primer 1100R yielded good sequences from its 5’ binding site up until ± 290 bp, after this they show multiple nucleotides at each site (Figure 24). This abrupt change in quality was apparent in all sequences using this primer. A possible explanation for this might be the secondary structure of the gene. The transcription might be hampered by a hairpin-loop in this region. Alternatively, a pseudogene similar to the 18S-sequence in the first ± 290 nucleotide sites may be present in the specimens and may be amplified together with the real 18S-sequence. Pseudogenes have the potential of accumulating mutations and reducing the quality of sequences (Buhay 2009). The sequences from the primer 18S5F worked well, but assembling of the forward and reverse sequences only worked on four out of nine specimens due to the short sequence from the 1100R primer. The overlapping of the forward and reverse is in the area where the 1100R is losing

its quality. Including sequences from the primers 600F and 600R (starting point between 18S5F and 1100R) made assembling possible for five additional specimens.

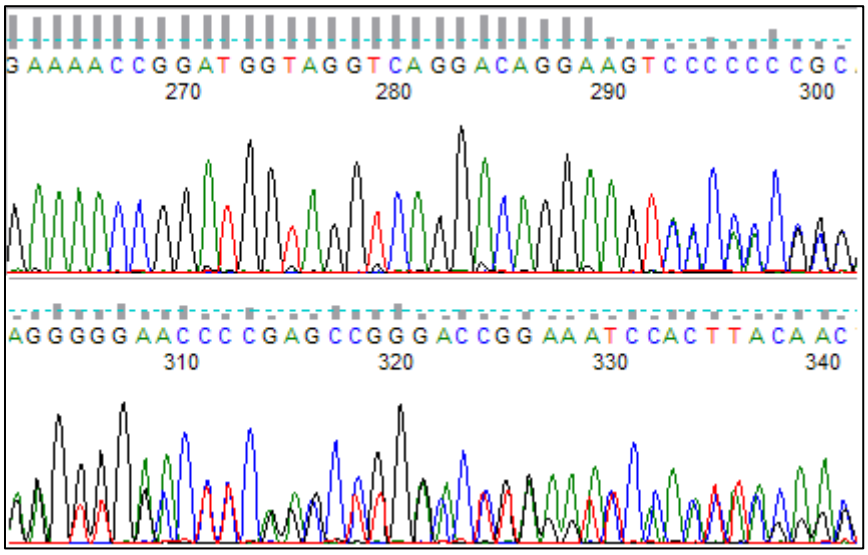


Figure 24 - Showing the abrupt change of quality in an 18S from the primer 1100R at around 290 bp. Image from FinchTV (Geospiza).

Since no sequences from the family *Hanleyidae* had been published in GenBank before, the top blast results from 18S and 16S (*Leptochiton asellus*) indicates that representatives of the correct taxon (Polyplacophora) had been sequenced. However, the blast results from the cyt b-sequences could have indicated contamination. The top ten blastn-results were dominated by birds, but polychaetes and fishes were found as high as the 1st and 2nd position. "Maximum identity" was always less than 72% in these results, indicating poorly matched top results. One weakness of the blast searching is that the top blast results might show wrong taxa if few sequences from closely related species are available in GenBank (Ekrem et al. 2007, Järnegren et al. 2007). Polyplacophoran cyt b is found only in the complete mitochondrial genome from the chiton *Katharina tunicata* (Wood, 1815) in the database. Aligning the *Hanleya*-sequences to the top results from the blast search showed a poor match compared to aligning with *K. tunicata* complete mitochondrial genome (GenBank accession number: NC_001636), indicating that Blast results from GenBank could not be trusted in this case and the obtained sequences in fact originate from *Hanleya* specimens and not from some kind of contamination. It will probably take long time until this no longer

is a problem, since sequences from less than five percent of the valid mollusk species to date are deposited in GenBank (Puillandre et al. 2009b).

Bayesian analysis uses Bayes theorem when inferring phylogeny. Incorporating the Monte Carlo Markov Chain-algorithm in the analysis, a tree roughly equivalent to the powerful maximum likelihood-method with bootstrapping can be built, but much faster (Huelsenbeck et al. 2001, Holder and Lewis 2003).

When constructing phylogenetic trees, the closest related taxa should be used as outgroups to reduce phylogenetic disturbance such as "long branch attraction" (Smith 1994, Moreira and Philippe 2000). Chitons and aplacophoran taxa (Solenogastres and Caudofoveata) are generally considered the most basal mollusks (Runnegar et al. 1979, Todt et al. 2008). Here, a bivalve was chosen as out outgoup taxon because published solenogaster 18S sequences show exogenous DNA-contaminations (Okusu and Giribet 2003, Wilson et al. 2009) and because substitution rates in solenogaster 18S are high, causing artifacts in phylogenetic reconstructions (C. Todt, personal comment).

4.2. Discussion of morphological results

4.2.1. Variation in girdle armature and radula

Girdle armature is not a species delimiting character for the specimens examined. The observed variation in spicules follows the size of specimens and no spicule-variations between similar sized specimens previously identified as *H. hanleyi* or *H. nagelfar* found in different habitats or with varying morphology in other characters were observed. Although the spicules of small animals (< ca. 20 mm dorsal length) fit Jones and Baxter's (1987) and Thiele's (1909) descriptions of *H. hanleyi* girdle armature, they are not in accordance with Kaas and van Belle (1985) who said the dorsal girdle of *H. hanleyi* was densely covered by "straight, smooth, needle-like spicules" and ventrally covered by "elongate, sharply pointed, longitudinally ribbed spicules". The small needle like spicules on the dorsal side could not be seen in any of the examined specimens in the present study and the ventral spicules were not ribbed. *H. nagelfar* is said to have smooth, needle-like spicules densely covering the dorsal girdle (Kaas and van Belle 1985). This is in accordance to my observation in large

animals (> ca 30 mm), but not in smaller animals. Kaas and van Belle's drawings are of a 60 mm (total length) specimen of *H. nagelfar,* and it is not written whether they examined girdle armature of smaller animals, too. The observed variation in small and large animals was not reflected in genetics since similar sized specimens with the two different cyt b haplotypes have the same girdle armature type. In contrast, there were differences in the spicules from large (ca. 60 mm DL) and from small (ca. 10 mm DL) specimens with the same haplotypes in all investigated genes, at least from Bergen. In Icelandic specimens, the "intermediate" spicules was seen on specimens of ca 30 mm, while larger specimens had the typical needle-like spicules dominating the dorsal girdle. If there was local variations in this character, only one spicule type would have been seen in specimens from Bergen and in specimens from Iceland. Genetic drift might also accumulate one species character in a population, but this is probably not true in this case, at least for Bergen and Iceland. It can, however be an indication of phenotypic plasticity or ontogenetic variation.

The straightness of the anterior end of the central tooth was partially used as a species diagnostic by Thiele (1909). In the animals examined herein, the degree of upwards curling in the anterior and lateral part could vary within each specimen, thus affecting the form of the blade when seen dorsally, and making the character sub-optimal for identification.

The only morphological character that could be related to genetic differences was the surface structure of the central teeth of the radula. The smooth central teeth were seen in three specimens and the ridged central teeth were seen in four, but molecular data could only be obtained from one of each, with an uncorrected p-distance of 9.97 % of the two haplotypes. There is a possibility that the smooth radula indicates *H. hanleyi*, as no longitudinal ridge was drawn or mentioned in the radula-descriptions of Thiele (1909), Kaas and van Belle (1985), Jones and Baxter (1987) or Warén and Klitgaard (1991). However, neither for *H. nagelfar* has the dorsal ridge been described before. In the herein studied specimens, the ridge was distinctly visible in SEM images (Figure 11), but very difficult to see in the light microscope, especially in the smallest specimens. It was most easily seen in the short time frame when the radula was placed outside of the ethanol/water and was just about to dry. Therefore, the ridge possibly has been overlooked by previous authors.

4.2.2. Univariate analyses

Girdle width is supposed to be one of the most distinctive differences between *H. hanleyi* and *H. nagelfar* (Sars 1878, Warén and Klitgaard 1991). The unimodal and normal distribution of this character indicates that it does not delimit the species in the specimens analyzed. Specimens 55377, 58016 and Moll.Övr 7859 (with the smooth central radula-tooth) have a girdle width close to the mean value while the girdle widths in animals with a ridged central tooth range considerably (6.5 – 16.2 % of dorsal length), thus making separation of *H. hanleyi* and *H. nagelfar* based on girdle width impossible due to the overlap. Although there is a large variation in small as well as in large animals, the character shows ontogenetic variation (Figure 16 a) with a tendency to broader girdles in large specimens. Ontogenetic variation further complicates the use of this character as species diagnostic, since size also has to be taken into consideration in the identification.

Dorsal elevation is used as a descriptive character by Sars (1878), Kaas and van Belle (1985) and Waren and Klitgaard (1991), but is normally distributed for the specimens examined in this study. Sars (1878) wrote that *H. nagelfar* had more blunt valves than *H. hanleyi,* which makes sense if the majority of *H. nagelfar* studied were large, as was pointed out by Sparre Schneider (1886) and Grieg (1898). Interestingly, Kaas and van Belle (1985) wrote the opposite, describing the valves of *H. nagelfar* as higher than *H. hanleyi*. The linear model fitted to the scatter plot (Figure 16) indicates that dorsal elevation decreases with size of specimens indicating that Sars might have studied mainly large animals of *H. nagelfar*. When examining the position of specimens with ridged central teeth, they are found both above and below the smooth-teethed specimens in the dot plot. The overlap makes it difficult to use dorsal elevation as a diagnostic character for *H. hanleyi* and *H. nagelfar*. The specimen with extremely low value for dorsal elevation (0.13) is the smallest specimen examined and had a valve height of 0.1 mm. This could be an example of one of the difficulties trusting certain morphological characters that might change during life stages (Buhay 2009).

Mucronal position, said to be median for *H. hanleyi* (Tryon and Pilsbry 1892, Kaas and van Belle 1985, Jones and Baxter 1987) and posterior for *H. nagelfar* (Kaas and van Belle 1985) but the univariate analysis indicate that the dataset originates from a normally distributed population. There is however a large degree of variation in small specimens under 25 mm

dorsal length (Figure 16 c) which is not as profound in larger specimens, thus it could seem to be two clusters in the figure. One for specimens smaller than 30 mm DL and with a mucronal position in the anterior part of the tail valve, and one cluster of animals with a larger size range and a posterior mucronal position. This is probably no "true" clustering, as specimens smaller than 30 mm and with a posterior girdle (mucro > 50 % from anterior margin) have both wide and narrow girdles, and are caught on various substrates, thus being both *H. hanleyi* and *H. nagelfar* according to previous descriptions and suggestions for the use of substrate as a species indicator. Specimens with smooth central teeth cannot be separated from the specimens where a dorsal ridge was seen as they overlap in this character as well.

Total width to total length ratio is different for *H. nagelfar* and *H. hanleyi* according to Lovén (1846) and Schander (2005b), *H. nagelfar* being longer. Several authors (Tryon and Pilsbry 1892, Jones and Baxter 1987) have used the same ratio as Bean (1844) when describing *H. hanleyi*. As this character show a non-normal distribution according to the Shapiro-Wilk test, it might be useful for separating the two species. Studying the histogram, there is no clear bimodal distribution except for a very little peak at values higher than 0.70 clustering three specimens together. One of these specimens stands out by its smooth central radula teeth, but this character do not follow the dorsal width to length ratio as the other specimens where this type of central tooth was seen overlap broadly with the specimens with ridged central teeth. If Lovén described *H. nagelfar* mainly looking at large specimens, the trend line indicates that most of these will have a ratio lower than *H. hanleyi.*

Tail valve width compared to head valve width was one of the diagnostic characters used for *H. hanleyi* and *H. nagelfar* (Lovén 1846). After Lovén's description other scientists have also used this character for identification (Sars 1878, Dons 1933, Jones and Baxter 1987). A normal distribution is not seen and based on the three distinct peaks in the histograms (Figure 15 e) there should be three clusters of this character, as a multimodal distribution might indicate several species. Comparing the only other observed character variation, the radula types, there are no correlations to the different peaks in the histogram, but the valve width variation follows size rigorously. All observed factors thus indicate that the tail valve gets broader with size, and is not a useful character for separating species.

According to Lovèn (1846) the second valves length to width ratio of *H. hanleyi* and *H. nagelfar* is different (0.45 vs. 0.56, respectively). The regression analysis indicates ontogenetic change, where a longer second valve is found in large specimens. Once again, no clustering of the radula morphotypes is observed in the dot plot, indicating that we are dealing with a hidden species separable by radula-type or cyt b variation.

Lovén (1846) also found a difference in length to width ratio in the intermediate valves between the two species. This is not a good species delimiting character for *H. hanleyi* and *H. nagelfar* as well as it is normally distributed and the radula morphotypes shows almost perfect overlap. Studying the scatter plot, the ratios of three ca. 60 mm long specimens seems to be too low (for their size). These are a021, a020 and 10571 (2) (ratios are 0.4, 0.32 and 0.26, respectively). No genetical differences were found between a020, a021 and BioIce 3589 (3), the latter having the highest ratio (0.71) of all specimens examined. This trait is thus not expressed in the analyzed gene sequences of the large specimens.

The type specimen is within the standard deviation of the dataset in all but three characters (Table 5) previously used for separating the *H. hanleyi* and *H. nagelfar*. The fact that the animal stands out in analyses of girdle width and dorsal width to length-ratio is likely caused by the poor state of its girdle and can not be heavily emphasized. For the tail valve width compared to head valve width the lectotype does not stand out compared to specimens of its own size, and since the character seem to show a profound ontogenetic change the type-specimens value is not representable for smaller specimens. If there are two species, the *H. nagelfar* specimens should cluster around the type specimen. This is difficult to observe in the analyses herein, since all characters previously used for delimiting the species show a clear ontogenetic change.

Kiær and Wollebæk (1913) wrote about several specimens found in the Oslo-fjord. All specimens larger than 30 mm total length were identified as *H. abyssorum* (=*H. nagelfar*), but two specimens (22 mm) were differentiated as *H. hanleyi* and *H. nagelfar*. The authors do not specify which characters the identifications was based upon. Looking at the dot plots in Figure 16 (especially c, e, f and g) the characters seem to have a sigmoid-like distribution where the flattening towards the upper asymptote starts around 20 – 30 mm dorsal length. The two 22 mm long specimens of Kiær and Wollebæk would be at the interface where both

the typical *H. hanleyi* and *H. nagelfar* morphologies are found. This might also be the reason why several authors (Sparre Schneider 1886, Kaas and van Belle 1985) have noticed the general scarcity of small *H. nagelfar* specimens.

4.2.3. Multivariate analysis

Specimens found on sponges (marked red) are mostly found on the positive side of the X-axis in Figure 17. As the arrows indicate, X-axis variation is mostly caused by dorsal length and the shape of intermediate and tail valve. Most specimens from sponges are large, and since the dorsal length is correlated with both intermediate valve shape and tail valve-shape it is logical to find these at positive X-axis values of the ordination diagram. Two of the examined sponge specimens were smaller than the rest (a023 and 315), both with a rather low dorsal length and girdle width compared to larger specimens found on sponges. More small specimens from sponges should be included to see if they cluster in the top left frame of the ordination diagram, or show a similar distribution as the ones from shell gravel and corals. The two small specimens from sponges are located among specimens from shell gravel, indicating that morphological characters previously used for separation are not applicable and habitat must be used as a determining character, if they in fact are two different species, as was suggested by Warén and Klitgaard (1991). Previous studies using morphometrics have shown cryptic species with a uniform distribution in such ordination diagrams (Pfenninger and Magnin 2001), so based on this result only one should not conclude that only one species is present.

4.3. Discussion of molecular results

Genetic variation is often reflected in morphology and it is thus important to include specimens from the whole range of morphological variability (e.g. specimens with a narrow girdle and specimens with a wide girdle should be included in molecular analyses). As shown in Table 7, some of the specimens with the most extreme measurements are not included in the molecular analyses. If *H. hanleyi* or *H. nagelfar* is limited to one of these extremes, the species is thus not included in the genetic analyses.

	Morphological range for all specimans examined		Morphological range covered by sequences used in genetic analyses					
			18S		16S		Cyt B	
	Min	Max	Min	Max	Min	Max	Min	Max
cDL	2,04	138,84	15,10	114,60	7,30	114,60	14,70	76,58
GW	4,08	18,28	7,28	16,21	7,52	16,21	7,28	16,21
DE	0,13	0,47	0,29	0,40	0,25	0,38	0,25	0,40
Mucro	28,57	63,13	42,86	62,09	41,67	62,09	42,86	57,48
DW/DL	0,37	0,72	0,44	0,60	0,42	0,60	0,42	0,60
VIIIW-IW	-6,79	3,44	-3,97	2,87	-5,48	2,87	-3,97	2,87
IIL/IIW	0,25	0,82	0,53	0,70	0,42	0,70	0,53	0,69
IVL/IVW	0,13	0,71	0,30	0,71	0,30	0,60	0,30	0,47

Table 7 – Morphological range for all specimens examined compared to the morphological range in the specimens with genetic results from the three genes. Girdle width values are in % of dorsal length. Abbreviations can be seen in Table 2, Page 18

4.3.1. Congeneric relationships

All haplotypes (or sequence-varieties) grouped together in the trees with other polyplacophorans (Figure 20), indicating the monophyly of *Hanleya*.

The weak support of the *Hanleya* clade in the cytochrome b consensus tree (Figure 21 c) might be due to the tree, with its few taxa, being suboptimal. It would be interesting to see how the addition of several, closely related species (i.e. *Hemiarthrum* spp., *Leptochiton* spp.) would affect the tree and its posterior probabilities, as this might improve the phylogenetic signal (Zweckl and Hillis 2002). The only groups in the cyt b tree, other than *Hanleya* is *Katharina tunicata* (Mopaliiade) and *Argopecten irradians* (Bivalvia) and these cannot be considered as closely related in this case.

The degree of haplotype similarity often reflects the degree of relationships and is used by many authors to delimit taxonomic groups (Puillandre et al. 2009a, Puillandre et al. 2009b). By comparing haplotype similarity with other groups, an indication of the relationship between the two *Hanleya* cytochrome b haplotypes can be found. In a study on atlantic cod

(*Gadus morhua*), Carr and Marshall (1991) found 3.69 % intraspecific variation in a partial cytochrome B region. For three species of weasels (Mammalia, *Mustela* spp.), Masuda and Yoshida (1994) found 4.0 – 6.9 % interspecific variation and 0 – 0.8 % intraspecific variation. A phylogeographic study on the bivalve *Arctica islandica* found 0.26 – 8.1 % intraspecific variation (Dahlgren et al. 2000). Worth mentioning is that one haplotype accounted for the majority (6.8 %) of this variation. As described in the introduction, intra vs. interspecific variation in the bivalve genus *Acesta* was 0 – 1 % and 6.2 – 11.9 %, respectively (Järnegren et al. 2007). The uncorrected p-distance between the *Hanleya* haplotypes is 9.97 %, which is higher than intraspecific variation in all of the above studies, indicating two different species. It is however very important to investigate more polyplacophoran cyt b sequences of other species before a conclusion can be made and to see if the genotype follows the radula characteristics.

Variation in the mitochondrial 16S rRNA-gene is caused by ambiguous sites and clustering within *Hanleya* should not be trusted (Figure 20 b). This is also reflected in the very low posterior probabilities. It would have been very interesting to see if the Kattegat-specimens had a distinctly different haplotype, but no 16S sequences could be obtained from these.

No variation, except for ambiguous sites were found in the specimens here analysed. Only two nucleotide sites were different between a Hanleya-sequences of good quality (Haplotype1_18S, Appendix IV) and both members of Leptochitonidae (Leptochiton asellus and Lepidopleurus cajetanus). The low degree of variation gives little information in the phylogenetic analysis and is one of the reasons this gene is mostly used at higher level phylogenies, where more variation is present. It would not be wise to use the 18S results for inferring the congeneric relationships (Puillandre et al. 2009b) in *Hanleya* because of this.

4.3.2. Position of Hanleyidae and insertion plates

Bergenhayn (1955) separated the genera *Hanleya* and *Hemiarthrum* as Hanleyidae from Lepidopleuridae (=Leptochitonidae) by establishing the family Hanleyidae partially based on the presence of un-slitted insertion plates in one or both of the terminal valves. For the first time now the relationship of Hanleyidae with Leptochitonidae has been examined using

molecular methods and their sister grouping gives further support to Bergenhayn's hypothesis.

The lack of insertion plates is often considered as a plesiomorphic character within chitons because it is the character state found in fossil taxa (Paleoloricata) and in Leptochitonidae (Stinchcomb and Darrough 1995); (Okusu et al. 2003) after (Sirenko 1997). In the consensus tree in Figure 21, Hanleyidae and Leptochitonidae are sister groups. Two possibilities regarding insertion plate evolution thus are possible. 1: The lack of insertion plates is the plesiomorphic state and the unslitted insertion plates present in Hanleyidae are an autapomorphic character (not homologous to the insertion plates of other polyplacophoran families). 2: Unslitted insertion plates were present in the ancestor of "modern" chitons, thus being the primitive character state. They were however reduced in Leptochitonidae. Slitted insertion plates are thus the derived character state.

4.4. Conclusion

None of the previously used diagnostic characters can be used effectively for separating *H. hanleyi* and *H. nagelfar* in Scandinavian waters. All external characters show a large degree of variation, especially in animals with a dorsal length shorter than 30 mm, but no clusters indicating two species are observed in the univariate or the multivariate analyses. All measured morphometric characters indicate ontogenetic variation, which is important to account for when arbitrating the published species descriptions for *Hanleya* species. If for example Lovén's description of *H. nagelfar* was primarily based on large specimens, it would be natural for somebody else (or himself) to identify small specimens as a different species, e.g. *H. hanleyi*.

The presence of two species within the investigated material was however indicated by variation in the protein coding mitochondrial gene cytochrome b. This genetic variation was also reflected in differences in radula morphology. Because of practical restrictions and technical difficulties encountered in this study, this could only be verified in a limited number of specimens and thus need further support. My results show that examination of radula or sequencing is necessary to find any differences in *Hanleya* in the North Atlantic.

In a Bayesian inferred phylogenetic consensus tree based on combined nuclear partial 18S rRNA data and mitochondrial 16S rRNA, Hanleyidae is placed as a sister group to Leptochitonidae, thus constituting monophyletic Lepidopleurida. The Lepidopleurida clade is placed as a sister group to all other polyplacophorans and thus its previously proposed basal position within Polyplacophora is supported.

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Appendix I

Raw data and calculations

Raw data.

Abbreviations: DL = Dorsal length (uncorrected); C = Curvature state; cDL = Dorsal length (corrected); GW = Girdle width; IW = Valve I width; IIW; Valve II width; IIL = Valve II length; IVW = Valve IV width; IVH = Valve IV height; IVL = Valve IV length; VIIIL = Valve VIII length; VIIIW = Valve VIII width; AM = Antemucronal distance; DW = Dorsal width.

All measurements in mm, except C. cDL is calculated from DL and C.

Specimen number	Identified as	DL	C	cDL	GW	IW	IIW	IIL	IVW	IVH	IVL	VIIIL	VIIIW	AM	DW
30000 (3)	*H. hanleyi*	1,8	1	2,04	0,1	0,7	0,8	0,2	0,8	0,1	0,1	na	0,6	na	1
30022	*H. hanleyi*	3,5	2	3,50	0,2	1,1	1,1	0,5	1,1	0,4	0,4	na	na	na	1,8
30000 (2)	*H. hanleyi*	4,2	3	3,62	0,4	1	1,1	0,5	1,1	0,4	0,4	0,3	0,9	0,1	2
30013	*H. hanleyi*	4,9	2	4,90	0,2	1,4	1,5	0,6	1,5	0,5	0,6	na	na	na	2,4
30020	*H. hanleyi*	6,0	3	5,17	0,7	1,7	1,7	0,8	1,8	0,5	0,6	0,9	1,5	0,3	3,4
30021	*H. hanleyi*	4,8	1	5,44	0,3	1,7	1,8	0,9	1,9	0,6	0,6	0,8	1,4	0,3	3,2
29996 (3)	*H. hanleyi*	6,1	1	6,91	0,5	1,9	na	na	na	na	na	0,9	1,8	0,3	3,3
31 (2)	*Hanleya sp.*	7,3	2	7,30	0,7	2,5	2,6	1,1	2,8	0,9	0,9	1,2	2,1	0,5	4,2
29997	*H. hanleyi*	6,8	1	7,70	0,5	2,1	2,3	1	2,3	0,7	0,6	0,7	1,9	0,2	4,9
30012	*H. hanleyi*	8,2	2	8,20	0,5	2,2	2,2	1,1	2,4	0,9	0,9	1,1	1,8	0,5	4,7
Moll. Övr 256	*H. hanleyi*	10,3	3	8,88	1	2,4	2,5	1,2	2,6	0,8	1,1	1,1	2	0,5	6,4
1998181.006	*H. hanleyi*	7,9	1	8,95	0,7	2,2	2,3	1,1	2,5	0,9	0,8	1,1	1,7	0,4	5,4
55377	*H. hanleyi*	9,0	2	9,00	0,8	2,3	2,4	1	2,5	0,9	0,8	1,1	1,8	0,5	4,4
1935007.05 (2)	*H. hanleyi*	8,0	1	9,06	dry	2,4	2,4	1,2	2,6	1	0,8	1,2	2	0,5	4,7
29999	*H. hanleyi*	9,2	2	9,20	0,6	2,2	2,3	1,2	2,5	0,9	0,9	1,1	1,8	0,5	5,2
31 (1)	*Hanleya sp.*	10,8	3	9,31	1,1	2,8	3,3	1,5	3	0,9	1,1	1,3	2,3	0,5	5,7
58016	*H. hanleyi*	10,9	3	9,40	0,8	3	3,1	1,7	3,4	1,3	1,4	1,6	2,6	0,8	6,8
30007 (3)	*H. hanleyi*	9,7	2	9,70	0,9	na	2,9	1,2	3	1,2	1	1,3	2,3	0,5	6,3
29994	*H. hanleyi*	8,6	1	9,74	0,4	na	na	1,4	3	1,1	1	1,2	2,1	0,4	5,1
30004 (3)	*H. hanleyi*	11,3	3	9,74	1,1	2,9	2,9	1,7	3	1,1	1,3	1,5	2,5	0,7	6,1
29995	*H. hanleyi*	9,8	2	9,80	0,8	na	2,6	1,4	2,9	1	1	1,2	2,1	0,4	5,1
55376	*H. hanleyi*	9,8	2	9,80	0,9	3	3,1	1,6	3,3	1,1	1,4	1,4	2,6	0,8	4,2
30001	*H. hanleyi*	11,6	3	10,00	0,9	na	3,1	1,6	3,2	1,2	1,2	1,4	2,8	0,7	6,3
30007 (2)	*H. hanleyi*	9,0	1	10,19	0,6	2,5	2,7	1,2	2,7	1,1	0,9	1,2	2,2	0,2	5,2
RMNH.MOL.114973	*H. hanleyi*	10,2	2	10,20	1,1	2,1	2,2	1,2	2,2	0,6	1	1,2	1,6	0,5	6,2
29998 (2)	*H. hanleyi*	12,0	3	10,35	1,1	2,9	2,9	1,7	3	1	1,4	1,4	2,4	0,6	5,7
Moll. Övr 258	*H. hanleyi*	10,5	2	10,50	0,9	2,8	2,9	1,3	3,2	1,1	1,2	1,4	2,5	0,7	7,6
1935007.05	*H. hanleyi*	9,3	1	10,53	dry	2,4	2,5	1,4	2,6	1	0,9	1,1	2,3	0,4	4,9
30015 (2)	*H. hanleyi*	12,7	3	10,95	0,9	2,7	2,9	1,8	3,1	1,1	1,6	1,5	2,3	0,7	5,9
Moll. Övr 246(2)	*H. hanleyi*	11,2	2	11,20	0,7	2,6	2,7	1,4	2,9	1,2	1,3	1,3	2,2	0,6	5,7
Moll. Övr 263	*H. hanleyi*	10,3	1	11,67	0,9	3,2	3,3	1,7	3,8	1	1,3	1,4	2,9	0,4	6,9
55375	*H. hanleyi*	10,4	1	11,78	1,1	2,4	2,4	1,3	2,5	1	0,9	1,2	1,6	na	5,5
30014 (3)	*H. hanleyi*	11,9	2	11,90	0,8	2,8	3	1,9	3	1,4	1,5	1,5	2,5	0,7	7,5
30011	*H. hanleyi*	14,0	3	12,07	1,3	2,5	2,7	1,5	3,1	1,1	1,5	1,5	2,2	0,6	6,6

Specimen number	*Identified as*	DL	C	cDL	GW	IW	IIW	IIL	IVW	IVH	IVL	VIIIL	VIIIW	AM	DW
29996 (2)	*H. hanleyi*	11,5	1	13,03	1,3	3,4	3,6	1,7	na	na	na	1,5	2,9	0,6	7,8
14343 (1)	*H. nagelfar*	13,8	2	13,80	1	3,1	3,3	2,1	3,5	1,3	1,4	1,7	2,8	0,7	5,9
30014 (1)	*H. hanleyi*	13,9	2	13,90	1,2	3	3,2	2	na	na	na	1,7	2,7	0,9	8
30018	*H. hanleyi*	14,2	2	14,20	1,1	2,7	3	1,6	na	na	na	1,6	2,5	0,6	7,1
30014 (2)	*H. hanleyi*	14,2	2	14,20	1,2	3	1,9	3	3,3	1,4	1,6	1,8	2,6	0,8	7,9
30024	*H. hanleyi*	16,6	3	14,32	1,7	3,6	3,8	2,1	4	1,2	1,7	1,9	3,4	0,9	8,2
30004 (2)	*H. hanleyi*	16,7	3	14,40	1,4	3,1	3,4	2,3	3,5	1,4	1,7	1,9	2,8	0,9	6,2
29996 (1)	*H. hanleyi*	17,0	3	14,66	1,4	3,3	3,7	2	4	1,2	1,7	1,9	3	0,7	8,4
a014	*H. nagelfar*	14,7	2	14,70	1,3	3,5	3,8	2,1	4,1	1,4	1,5	2,3	3,8	1	6,2
Bioice 2884 (2)	*Hanleya sp.*	17,2	3	14,83	2	3,5	3,7	2,2	3,8	1,2	1,9	1,7	3	0,7	9,2
30016	*H. hanleyi*	15,0	2	15,00	1	3,2	3,4	1,8	3,8	1,2	1,4	1,7	2,7	0,9	7,4
Moll. Övr 7858	*H. hanleyi*	15,1	2	15,10	1,1	3,3	3,6	1,9	4	1,2	1,8	1,9	2,7	0,9	7,8
30008	*H. hanleyi*	13,7	1	15,52	0,9	3,3	3,4	1,8	3,7	1,6	1,6	1,9	2,9	0,8	7,8
RMNH.MOL.HLS.0528	*H. hanleyi*	18,9	3	16,30	1,6	3,6	3,9	2,2	4,2	1,6	2,1	2,3	3,5	1,2	8,9
30015 (1)	*H. hanleyi*	19,1	3	16,47	1,7	3,7	4	2,2	4,2	1,4	1,7	2,2	3,3	0,9	8
30026	*H. hanleyi*	16,7	2	16,70	1	4	4,3	2,4	4,5	1,4	2	2,2	3,5	1,1	7,9
30017	*H. hanleyi*	16,9	2	16,90	1,9	4	4,4	2,1	4,8	1,8	2,1	2,6	4,1	0,9	10,5
Moll. Övr 261	*H. hanleyi*	19,8	3	17,07	0,9	4,3	4,3	2,7	4,4	1,5	2,3	2,2	3,7	1,1	8
Moll. Övr 7859	*H. hanleyi*	20,0	3	17,25	1,5	3,3	3,6	2	4	1,6	1,8	2,2	3,2	1,1	9,2
1981089.01101	*H. hanleyi*	17,3	2	17,30	1	3,7	4	2,3	4,3	1,8	1,9	2,5	3,4	1,4	8,5
9753	*H. nagelfar*	15,3	1	17,33	1,7	3,9	4,3	2,3	4,5	1,7	1,9	1,9	3,9	0,9	9,4
30004 (1)	*H. hanleyi*	20,1	3	17,33	1,6	4,1	4,4	2,5	4,8	1,7	2,2	2,4	3,8	1	8,2
58018	*H. hanleyi*	15,9	1	18,01	2,6	4,4	4,6	1,9	4,7	1,5	1,5	2,1	4	0,7	11,2
RMNH.MOL.K.4809	*H. hanleyi*	20,9	3	18,02	2,3	4,2	4,6	2,6	4,9	1,2	2,2	2,5	4	1,2	12,3
58017	*H. hanleyi*	16,0	1	18,12	2,6	4,4	4,4	2,3	4,6	1,6	1,3	2,2	3,6	0,9	10,7
30009 (1)	*H. hanleyi*	18,2	2	18,20	2	4,3	4,6	2,5	4,8	1,8	2	2,5	3,8	1,2	10,7
Moll. Övr 246(1)	*H. hanleyi*	18,3	2	18,30	1	3,7	3,9	2	4,3	1,5	2	2	3,3	1	8,4
Moll. Övr 259	*H. hanleyi*	16,6	1	18,80	1,5	3,9	4,1	2,2	4,4	1,1	1,6	1,8	3,4	0,7	9,9
30009 (2)	*H. hanleyi*	19,2	2	19,20	1,7	4	4,1	2,6	4,6	1,8	2,4	2,4	3,7	1,3	10,7
30010 (2)	*H. hanleyi*	17,0	1	19,26	1,6	4	4,1	2,7	4,1	1,8	1,9	1,7	3,4	0,5	10,6
a048 (1)	*Hanleya sp.*	19,7	2	19,70	1,7	4,8	5	2,9	5,3	1,4	2,4	2,9	4,3	1,3	10,7
a023	*H. nagelfar*	17,6	1	19,94	1,5	4,7	5,1	3	5,4	1,6	1,6	2,1	4,7	0,9	9,2
30023	*H. hanleyi*	23,2	3	20,01	1,9	4,5	4,6	3	4,9	1,9	2,4	2,2	3,7	1	10,8
55379	*H. nagelfar*	24,3	3	20,96	2,1	4,8	5,2	3	5,3	1,8	2,7	2,7	4,5	1,4	9,7
29998 (1)	*H. hanleyi*	18,7	1	21,18	1,8	4,5	4,8	2,5	5,4	1,6	1,9	1,9	4	0,8	10,5
a048 (2)	*Hanleya sp.*	25,5	3	21,99	1,8	4,9	5,3	3,2	5,7	1,5	2,9	2,9	4,6	1,3	11
Bioice 2887 (2)	*Hanleya sp.*	26,8	3	23,11	2,6	5,1	5,2	3,2	5,8	1,8	2,9	3,4	4,9	1,6	11,6
a015	*H. nagelfar*	27,2	3	23,46	2,3	5,3	5,5	3,2	6,1	2,1	2,3	3	5	1,7	11,3
30010 (1)	*H. hanleyi*	21,4	1	24,24	1,9	4,9	5,3	3,5	5,6	1,9	2,6	2,6	4,6	1,2	11,5
315	*H. hanleyi*	23,0	1	26,05	2,6	5,1	5,3	3,2	5,7	2	2,5	2,9	5,2	1,3	12,4
a016	*H. nagelfar*	26,1	2	26,10	2,1	5,5	6	3,5	6,3	2,4	2,9	3,4	5,3	1,5	11,4
a002	*H. nagelfar*	26,6	2	26,60	2,7	5	5,6	3,5	6,3	2,1	2,7	3,6	5,4	1,9	13,1
2212	*H. nagelfar*	23,5	1	26,62	3,3	5,6	6	3,7	6,7	2,3	3,2	3,7	6	1,7	14,9
30025	*H. hanleyi*	32,3	3	27,85	2,7	5,7	5,7	4	6	1,8	3,5	3,6	5,9	1,8	15,7
14343 (2)	*H. nagelfar*	28,3	2	28,30	3,5	5	5,1	2,9	5,4	1,2	2,5	3,4	4,9	1,4	13,9
Bioice 2884 (1)	*H. nagelfar*	29,2	2	29,20	2,6	5,3	5,7	3,8	6	2,3	3,3	2,9	5,4	1,3	14,1

Specimen number	*Identified as*	DL	C	cDL	GW	IW	IIW	IIL	IVW	IVH	IVL	VIIIL	VIIIW	AM	DW
27294	*H. nagelfar*	29,3	2	29,30	4,4	5,4	5,9	3,7	6,6	1,3	3	3,2	5,4	2	15,1
11291	*H. nagelfar*	30,3	2	30,30	3,3	6,1	6,8	3,9	7,2	2,2	3,3	3,7	6,1	1,9	15,8
a022	*H. nagelfar*	26,9	1	30,47	3,8	na	na	na	na	na	na	3,9	6,2	2,1	18,3
55378	*H. nagelfar*	36,8	3	31,74	4	6,1	6,2	4	6,8	2,1	3,3	4,1	6,5	2,1	15,7
10570	*H. nagelfar*	33,0	2	33,00	3,3	6,3	7	4,5	7,6	2,5	4,2	4,2	6,6	2,3	14
Bioice 3589 (13)	*H. nagelfar*	33,4	2	33,40	4,1	6,1	6,8	4,3	7,3	2,5	3,7	4,7	6,4	2,4	16,3
a017	*H. nagelfar*	35,7	2	35,70	3,6	7	7,4	4,6	8,23	2,8	3,9	4,5	7,3	2,5	16,4
Bioice 3589 (12)	*H. nagelfar*	46,8	3	40,36	4,7	7,2	7,6	5	8,8	2,7	4,7	5,7	8,3	3,4	19,5
a018	*H. nagelfar*	37,3	1	42,25	4,8	7,2	7,6	4,8	8,7	2,2	3,9	5,8	8,1	3,1	20
Bioice 3589 (11)	*H. nagelfar*	42,6	2	42,60	4,3	7,2	7,7	4,8	8,6	2,9	5	6,1	7,6	3,4	19,9
C2	*H. nagelfar*	53,7	3	46,31	5,6	10	10,9	5,9	12,4	3,2	5,7	7,7	11	4,5	28,4
Bioice 3589 (10)	*H. nagelfar*	47,1	2	47,10	5	7,9	8,5	5,5	9,5	2,8	5,5	6,7	8,6	3,7	20,4
13395	*H. nagelfar*	43,3	1	49,05	3,3	8	8,6	5,4	9,9	2,9	4,8	5,9	8,8	3,4	18,2
a020	*H. nagelfar*	46,2	1	52,33	8,1	10,9	11,1	7,4	13,1	3,8	4,2	8,4	12,1	4,4	30,4
Bioice 3589 (8)	*H. nagelfar*	53,8	2	53,80	7,5	8,7	9,6	6,6	10,9	3,6	6,2	7,1	9,6	3,8	28,1
Bioice 3589 (9)	*H. nagelfar*	54,2	2	54,20	7	9,2	10	5,9	11,4	3	6,3	na	na	na	26,7
a008	*H. nagelfar*	54,8	2	54,80	6,4	9,2	9,8	6,9	11,3	3,4	5,3	6,9	10,6	3,4	25,4
Bioice 2887 (1)	*Hanleya sp.*	64,4	3	55,54	4,2	9,9	10	7,2	10,8	4,4	6,2	7,5	10,8	3,7	23,5
10571 (2)	*H. nagelfar*	58,0	2	58,00	10,6	10	10,3	6,8	11,5	2,9	3	7,3	10,9	4,3	34,6
SMNH Type-1329	*H. nagelfar*	52,0	1	58,90	3	10,7	11,4	7,5	12,8	4,3	6,3	7,2	11,7	3,9	23,6
a021	*H. nagelfar*	53,9	1	61,06	9,9	11,9	13,1	7,1	14,2	4,4	5,7	8	13,1	3,8	35
10571 (1)	*H. nagelfar*	63,6	2	63,60	10,4	10,8	11,9	7,9	14,4	4	8	8,9	12,5	4,7	34,6
Bioice 3589 (6)	*H. nagelfar*	74,8	3	64,50	6,5	11,1	11,9	8,3	13,3	3,9	8,5	10,4	13	6,2	30,9
Bioice 3589 (7)	*H. nagelfar*	66,0	2	66,00	9	9,4	10,6	7,3	12,2	4,3	7,7	8,9	10,7	4,9	36
Bioice 3589 (5)	*H. nagelfar*	69,8	2	69,80	8,7	10,2	11,2	7,5	12,8	4,1	7,5	9,5	12,6	5,7	35
Bioice 3589 (4)	*H. nagelfar*	83,5	3	72,01	6,4	12,7	13,5	9,4	15,5	5,8	9	11,1	14,3	6,4	36
Bioice 3589 (3)	*H. nagelfar*	88,8	3	76,58	11,9	13,3	13,8	9,5	15,7	5,9	11,1	12,7	15,5	7,3	46,3
C1	*H. Hanleyi **	95,2	3	82,10	10,5	13,3	15,1	9,2	16,4	4,7	10,1	13,6	15,5	8,1	43,6
Bioice 3589 (2)	*H. nagelfar*	104,6	3	90,20	11,4	15	15,8	11,5	18,3	6,5	11,9	13,1	16,7	8	45
30037	*H. nagelfar*	97,1	2	97,10	9,4	14,1	15,2	12,4	17,2	5,9	11,3	12,5	16,5	7,5	42,1
Bioice 3589 (1)	*H. nagelfar*	114,6	2	114,60	12,6	16,8	17,8	12,2	21	6,7	12,7	15,3	18,6	9,5	51,5
a001	*H. nagelfar*	161,0	3	138,84	12,5	16,6	17,5	13,2	19,6	5,3	13,5	16	19,5	10,1	54,6

Next page: Table showing calculations used in analyses. Abbreviations: As for the table above. DE = dorsal elevation; Mucro = Antemucronal distance (in % from anterior margin of tail valve). All characters in % of corrected dorsal length, except DE, DW/cDL, IIL/IIW; IVL/IVW which are ratios.

Specimen number	Identified as	cDL	GW%cDL	DE	Mucro	DW/cDL	VIIIW-IW	IIL/IIW	IVL/IVW	IW%cDL	IIW%cDL	IIL%cDL	IVW%cDL	IVH%cDL	IVL%cDL	VIIIL%cDL	VIIIW%cDL	AM%cDL	DW%cDL
30000 (3)	*H. hanleyi*	2,04	4,90	0,13	NA	0,49	-4,90	0,25	0,13	34,33	39,24	9,81	39,24	4,90	4,90	na	29,43	na	49,04
30022	*H. hanleyi*	3,50	5,71	0,36	NA	0,51	NA	0,45	0,36	31,43	31,43	14,29	31,43	11,43	11,43	na	na	na	51,43
30000 (2)	*H. hanleyi*	3,62	11,04	0,36	0,33	0,55	-2,76	0,45	0,36	27,61	30,37	13,80	30,37	11,04	11,04	8,28	24,85	2,76	55,22
30013	*H. hanleyi*	4,90	4,08	0,33	NA	0,49	NA	0,40	0,40	28,57	30,61	12,24	30,61	10,20	12,24	na	na	na	48,98
30020	*H. hanleyi*	5,17	13,53	0,28	0,33	0,66	-3,87	0,47	0,33	32,86	32,86	15,46	34,79	9,66	11,60	17,39	28,99	5,80	65,71
30021	*H. hanleyi*	5,44	5,52	0,32	0,38	0,59	-5,52	0,50	0,32	31,27	33,10	16,55	34,94	11,03	11,03	14,71	25,75	5,52	58,85
29996 (3)	*H. hanleyi*	6,91	7,24	na	0,33	0,48	-1,45	NA	NA	27,50	na	na	na	na	na	13,02	26,05	4,34	47,76
31 (2)	*Hanleya sp.*	7,30	9,59	0,32	0,42	0,58	-5,48	0,42	0,32	34,25	35,62	15,07	38,36	12,33	12,33	16,44	28,77	6,85	57,53
29997	*H. hanleyi*	7,70	6,49	0,30	0,29	0,64	-2,60	0,43	0,26	27,26	29,86	12,98	29,86	9,09	7,79	9,09	24,67	2,60	63,61
30012	*H. hanleyi*	8,20	6,10	0,38	0,45	0,57	-4,88	0,50	0,38	26,83	26,83	13,41	29,27	10,98	10,98	13,41	21,95	6,10	57,32
Moll. Övr 256	*H. hanleyi*	8,88	11,26	0,31	0,45	0,72	-4,50	0,48	0,42	27,02	28,15	13,51	29,27	9,01	12,38	12,38	22,52	5,63	72,05
1998181.006	*H. hanleyi*	8,95	7,82	0,36	0,36	0,60	-5,59	0,48	0,32	24,58	25,70	12,29	27,94	10,06	8,94	12,29	19,00	4,47	60,34
55377	*H. hanleyi*	9,00	8,89	0,36	0,45	0,49	-5,56	0,42	0,32	25,56	26,67	11,11	27,78	10,00	8,89	12,22	20,00	5,56	48,89
1935007.05 (2)	*H. hanleyi*	9,06	na	0,38	0,42	0,52	-4,41	0,50	0,31	26,48	26,48	13,24	28,69	11,03	8,83	13,24	22,07	5,52	51,86
29999	*H. hanleyi*	9,20	6,52	0,36	0,45	0,57	-4,35	0,52	0,36	23,91	25,00	13,04	27,17	9,78	9,78	11,96	19,57	5,43	56,52
31 (1)	*Hanleya sp.*	9,31	11,81	0,30	0,38	0,61	-5,37	0,45	0,37	30,06	35,43	16,11	32,21	9,66	11,81	13,96	24,70	5,37	61,20
58016	*H. hanleyi*	9,40	8,51	0,38	0,50	0,72	-4,26	0,55	0,41	31,92	32,98	18,09	36,17	13,83	14,89	17,02	27,66	8,51	72,34
30007 (3)	*H. hanleyi*	9,70	9,28	0,40	0,38	0,65	NA	0,41	0,33	na	29,90	12,37	30,93	12,37	10,31	13,40	23,71	5,15	64,95
29994	*H. hanleyi*	9,74	4,11	0,37	0,33	0,52	NA	NA	0,33	na	na	14,37	30,80	11,29	10,27	12,32	21,56	4,11	52,35
30004 (3)	*H. hanleyi*	9,74	11,29	0,37	0,47	0,63	-4,10	0,59	0,43	29,76	29,76	17,45	30,79	11,29	13,34	15,39	25,65	7,18	62,60
29995	*H. hanleyi*	9,80	8,16	0,34	0,33	0,52	NA	0,54	0,34	na	26,53	14,29	29,59	10,20	10,20	12,24	21,43	4,08	52,04
55376	*H. hanleyi*	9,80	9,18	0,33	0,57	0,43	-4,08	0,52	0,42	30,61	31,63	16,33	33,67	11,22	14,29	14,29	26,53	8,16	42,86
30001	*H. hanleyi*	10,00	9,00	0,38	0,50	0,63	NA	0,52	0,38	na	30,99	15,99	31,99	12,00	12,00	14,00	27,99	7,00	62,98
30007 (2)	*H. hanleyi*	10,19	5,89	0,41	0,17	0,51	-2,94	0,44	0,33	24,52	26,48	11,77	26,48	10,79	8,83	11,77	21,58	1,96	51,01
RMNH.MOL.114973	*H. hanleyi*	10,20	10,78	0,27	0,42	0,61	-4,90	0,55	0,45	20,59	21,57	11,76	21,57	5,88	9,80	11,76	15,69	4,90	60,78
29998 (2)	*H. hanleyi*	10,35	10,63	0,33	0,43	0,55	-4,83	0,59	0,47	28,02	28,02	16,43	28,99	9,66	13,53	13,53	23,19	5,80	55,08
Moll. Övr 258	*H. hanleyi*	10,50	8,57	0,34	0,50	0,72	-2,86	0,45	0,38	26,67	27,62	12,38	30,48	10,48	11,43	13,33	23,81	6,67	72,38
1935007.05	*H. hanleyi*	10,53	na	0,38	0,36	0,47	-0,95	0,56	0,35	22,78	23,73	13,29	24,68	9,49	8,54	10,44	21,83	3,80	46,51
30015 (2)	*H. hanleyi*	10,95	8,22	0,35	0,47	0,54	-3,65	0,62	0,52	24,65	26,48	16,44	28,31	10,04	14,61	13,70	21,00	6,39	53,87
Moll. Övr 246(2)	*H. hanleyi*	11,20	6,25	0,41	0,46	0,51	-3,57	0,52	0,45	23,21	24,11	12,50	25,89	10,71	11,61	11,61	19,64	5,36	50,89

Specimen number	*Identified as*	cDL	GW%cDL	DE	Mucro	DW/cDL	VIIIW-IW	IIL/IIW	IVL/IVW	IW%cDL	IIW%cDL	IIL%cDL	IVW%cDL	IVH%cDL	IVL%cDL	VIIIL%cDL	VIIIW%cDL	AM%cDL	DW%cDL
Moll. Övr 263	*H. hanleyi*	11,67	7,71	0,26	0,29	0,59	-2,57	0,52	0,34	27,43	28,28	14,57	32,57	8,57	11,14	12,00	24,86	3,43	59,14
55375	*H. hanleyi*	11,78	9,34	0,40	NA	0,47	-6,79	0,54	0,36	20,37	20,37	11,03	21,22	8,49	7,64	10,19	13,58	na	46,69
30014 (3)	*H. hanleyi*	11,90	6,72	0,47	0,47	0,63	-2,52	0,63	0,50	23,53	25,21	15,97	25,21	11,76	12,61	12,61	21,01	5,88	63,03
30011	*H. hanleyi*	12,07	10,77	0,35	0,40	0,55	-2,48	0,56	0,48	20,71	22,36	12,42	25,68	9,11	12,42	12,42	18,22	4,97	54,67
29996 (2)	*H. hanleyi*	13,03	9,98	na	0,40	0,60	-3,84	0,47	NA	26,10	27,64	13,05	na	na	na	11,51	22,26	4,61	59,88
14343 (1)	*H. nagelfar*	13,80	7,25	0,37	0,41	0,43	-2,17	0,64	0,40	22,46	23,91	15,22	25,36	9,42	10,14	12,32	20,29	5,07	42,75
30014 (1)	*H. hanleyi*	13,90	8,63	na	0,53	0,58	-2,16	0,63	NA	21,58	23,02	14,39	na	na	na	12,23	19,42	6,47	57,55
30018	*H. hanleyi*	14,20	7,75	na	0,38	0,50	-1,41	0,53	NA	19,01	21,13	11,27	na	na	na	11,27	17,61	4,23	50,00
30014 (2)	*H. hanleyi*	14,20	8,45	0,42	0,44	0,56	-2,82	1,58	0,48	21,13	13,38	21,13	23,24	9,86	11,27	12,68	18,31	5,63	55,63
30024	*H. hanleyi*	14,32	11,88	0,30	0,47	0,57	-1,40	0,55	0,43	25,15	26,55	14,67	27,94	8,38	11,88	13,27	23,75	6,29	57,28
30004 (2)	*H. hanleyi*	14,40	9,72	0,40	0,47	0,43	-2,08	0,68	0,49	21,53	23,61	15,97	24,30	9,72	11,80	13,19	19,44	6,25	43,05
29996 (1)	*H. hanleyi*	14,66	9,55	0,30	0,37	0,57	-2,05	0,54	0,43	22,51	25,24	13,64	27,28	8,19	11,60	12,96	20,46	4,77	57,30
a014	*H. nagelfar*	14,70	8,84	0,34	0,43	0,42	2,04	0,55	0,37	23,81	25,85	14,29	27,89	9,52	10,20	15,65	25,85	6,80	42,18
Bioice 2884 (2)	*Hanleya sp.*	14,83	13,48	0,32	0,41	0,62	-3,37	0,59	0,50	23,60	24,94	14,83	25,62	8,09	12,81	11,46	20,23	4,72	62,03
30016	*H. hanleyi*	15,00	6,67	0,32	0,53	0,49	-3,33	0,53	0,37	21,33	22,67	12,00	25,33	8,00	9,33	11,33	18,00	6,00	49,33
Moll. Övr 7858	*H. hanleyi*	15,10	7,28	0,30	0,47	0,52	-3,97	0,53	0,45	21,85	23,84	12,58	26,49	7,95	11,92	12,58	17,88	5,96	51,66
30008	*H. hanleyi*	15,52	5,80	0,43	0,42	0,50	-2,58	0,53	0,43	21,26	21,91	11,60	23,84	10,31	10,31	12,24	18,69	5,15	50,26
RMNH.MOL.HLS.0528	*H. hanleyi*	16,30	9,82	0,38	0,52	0,55	-0,61	0,56	0,50	22,09	23,93	13,50	25,77	9,82	12,88	14,11	21,47	7,36	54,61
30015 (1)	*H. hanleyi*	16,47	10,32	0,33	0,41	0,49	-2,43	0,55	0,40	22,46	24,28	13,36	25,50	8,50	10,32	13,36	20,03	5,46	48,57
30026	*H. hanleyi*	16,70	5,99	0,31	0,50	0,47	-2,99	0,56	0,44	23,95	25,75	14,37	26,95	8,38	11,98	13,17	20,96	6,59	47,31
30017	*H. hanleyi*	16,90	11,24	0,38	0,35	0,62	0,59	0,48	0,44	23,67	26,04	12,43	28,40	10,65	12,43	15,38	24,26	5,33	62,13
Moll. Övr 261	*H. hanleyi*	17,07	5,27	0,34	0,50	0,47	-3,51	0,63	0,52	25,18	25,18	15,81	25,77	8,78	13,47	12,88	21,67	6,44	46,85
Moll. Övr 7859	*H. hanleyi*	17,25	8,70	0,40	0,50	0,53	-0,58	0,56	0,45	19,13	20,87	11,60	23,19	9,28	10,44	12,76	18,55	6,38	53,34
1981089.01101	*H. hanleyi*	17,30	5,78	0,42	0,56	0,49	-1,73	0,58	0,44	21,39	23,12	13,29	24,86	10,40	10,98	14,45	19,65	8,09	49,13
9753	*H. nagelfar*	17,33	9,81	0,38	0,47	0,54	0,00	0,53	0,42	22,50	24,81	13,27	25,96	9,81	10,96	10,96	22,50	5,19	54,24
30004 (1)	*H. hanleyi*	17,33	9,23	0,35	0,42	0,47	-1,73	0,57	0,46	23,65	25,38	14,42	27,69	9,81	12,69	13,85	21,92	5,77	47,31
58018	*H. hanleyi*	18,01	14,44	0,32	0,33	0,62	-2,22	0,41	0,32	24,43	25,54	10,55	26,10	8,33	8,33	11,66	22,21	3,89	62,18
RMNH.MOL.K.4809	*H. hanleyi*	18,02	12,76	0,24	0,48	0,68	-1,11	0,57	0,45	23,30	25,52	14,43	27,19	6,66	12,21	13,87	22,19	6,66	68,24
58017	*H. hanleyi*	18,12	14,35	0,35	0,41	0,59	-4,41	0,52	0,28	24,28	24,28	12,69	25,38	8,83	7,17	12,14	19,86	4,97	59,04
30009 (1)	*H. hanleyi*	18,20	10,99	0,38	0,48	0,59	-2,75	0,54	0,42	23,63	25,27	13,74	26,37	9,89	10,99	13,74	20,88	6,59	58,79

Specimen number	Identified as	cDL	GW%cDL	DE	Mucro	DW/cDL	VIIIW-IW	IIL/IIW	IVL/IVW	IW%cDL	IIW%cDL	IIL%cDL	IVW%cDL	IVH%cDL	IVL%cDL	VIIIL%cDL	VIIIW%cDL	AM%cDL	DW%cDL
Moll. Övr 246(1)	*H. hanleyi*	18,30	5,46	0,35	0,50	0,46	-2,19	0,51	0,47	20,22	21,31	10,93	23,50	8,20	10,93	10,93	18,03	5,46	45,90
Moll. Övr 259	*H. hanleyi*	18,80	7,98	0,25	0,39	0,53	-2,66	0,54	0,36	20,74	21,80	11,70	23,40	5,85	8,51	9,57	18,08	3,72	52,65
30009 (2)	*H. hanleyi*	19,20	8,85	0,39	0,54	0,56	-1,56	0,63	0,52	20,83	21,35	13,54	23,96	9,38	12,50	12,50	19,27	6,77	55,73
30010 (2)	*H. hanleyi*	19,26	8,31	0,44	0,29	0,55	-3,12	0,66	0,46	20,77	21,29	14,02	21,29	9,35	9,87	8,83	17,66	2,60	55,04
a048 (1)	*Hanleya sp.*	19,70	8,63	0,26	0,45	0,54	-2,54	0,58	0,45	24,37	25,38	14,72	26,90	7,11	12,18	14,72	21,83	6,60	54,31
a023	*H. nagelfar*	19,94	7,52	0,30	0,43	0,46	0,00	0,59	0,30	23,57	25,58	15,05	27,09	8,03	8,03	10,53	23,57	4,51	46,15
30023	*H. hanleyi*	20,01	9,50	0,39	0,45	0,54	-4,00	0,65	0,49	22,49	22,99	14,99	24,49	9,50	12,00	11,00	18,49	5,00	53,98
55379	*H. nagelfar*	20,96	10,02	0,34	0,52	0,46	-1,43	0,58	0,51	22,91	24,81	14,32	25,29	8,59	12,88	12,88	21,47	6,68	46,29
29998 (1)	*H. hanleyi*	21,18	8,50	0,30	0,42	0,50	-2,36	0,52	0,35	21,24	22,66	11,80	25,49	7,55	8,97	8,97	18,88	3,78	49,57
a048 (2)	*Hanleya sp.*	21,99	8,19	0,26	0,45	0,50	-1,36	0,60	0,51	22,28	24,10	14,55	25,92	6,82	13,19	13,19	20,92	5,91	50,02
Bioice 2887 (2)	*Hanleya sp.*	23,11	11,25	0,31	0,47	0,50	-0,87	0,62	0,50	22,07	22,50	13,85	25,10	7,79	12,55	14,71	21,20	6,92	50,19
a015	*H. nagelfar*	23,46	9,81	0,34	0,57	0,48	-1,28	0,58	0,38	22,60	23,45	13,64	26,01	8,95	9,81	12,79	21,32	7,25	48,17
30010 (1)	*H. hanleyi*	24,24	7,84	0,34	0,46	0,47	-1,24	0,66	0,46	20,21	21,86	14,44	23,10	7,84	10,73	10,73	18,98	4,95	47,44
315	*H. hanleyi*	26,05	9,98	0,35	0,45	0,48	0,38	0,60	0,44	19,57	20,34	12,28	21,88	7,68	9,60	11,13	19,96	4,99	47,59
a016	*H. nagelfar*	26,10	8,05	0,38	0,44	0,44	-0,77	0,58	0,46	21,07	22,99	13,41	24,14	9,20	11,11	13,03	20,31	5,75	43,68
a002	*H. nagelfar*	26,60	10,15	0,33	0,53	0,49	1,50	0,63	0,43	18,80	21,05	13,16	23,68	7,89	10,15	13,53	20,30	7,14	49,25
2212	*H. nagelfar*	26,62	12,40	0,34	0,46	0,56	1,50	0,62	0,48	21,04	22,54	13,90	25,17	8,64	12,02	13,90	22,54	6,39	55,97
30025	*H. hanleyi*	27,85	9,69	0,30	0,50	0,56	0,72	0,70	0,58	20,46	20,46	14,36	21,54	6,46	12,57	12,92	21,18	6,46	56,36
14343 (2)	*H. nagelfar*	28,30	12,37	0,22	0,41	0,49	-0,35	0,57	0,46	17,67	18,02	10,25	19,08	4,24	8,83	12,01	17,31	4,95	49,12
Bioice 2884 (1)	*H. nagelfar*	29,20	8,90	0,38	0,45	0,48	0,34	0,67	0,55	18,15	19,52	13,01	20,55	7,88	11,30	9,93	18,49	4,45	48,29
27294	*H. nagelfar*	29,30	15,02	0,20	0,63	0,52	0,00	0,63	0,45	18,43	20,14	12,63	22,53	4,44	10,24	10,92	18,43	6,83	51,54
11291	*H. nagelfar*	30,30	10,89	0,31	0,51	0,52	0,00	0,57	0,46	20,13	22,44	12,87	23,76	7,26	10,89	12,21	20,13	6,27	52,15
a022	*H. nagelfar*	30,47	12,47	NA	0,54	0,60	NA	NA	NA	na	na	na	na	na	na	12,80	20,35	6,89	60,06
55378	*H. nagelfar*	31,74	12,60	0,31	0,51	0,49	1,26	0,65	0,49	19,22	19,54	12,60	21,43	6,62	10,40	12,92	20,48	6,62	49,47
10570	*H. nagelfar*	33,00	10,00	0,33	0,55	0,42	0,91	0,64	0,55	19,09	21,21	13,64	23,03	7,58	12,73	12,73	20,00	6,97	42,42
Bioice 3589 (13)	*H. nagelfar*	33,40	12,28	0,34	0,51	0,49	0,90	0,63	0,51	18,26	20,36	12,87	21,86	7,49	11,08	14,07	19,16	7,19	48,80
a017	*H. nagelfar*	35,70	10,08	0,34	0,56	0,46	0,84	0,62	0,47	19,61	20,73	12,89	23,05	7,84	10,92	12,61	20,45	7,00	45,94
Bioice 3589 (12)	*H. nagelfar*	40,36	11,65	0,31	0,60	0,48	2,73	0,66	0,53	17,84	18,83	12,39	21,80	6,69	11,65	14,12	20,57	8,42	48,32
a018	*H. nagelfar*	42,25	11,36	0,25	0,53	0,47	2,13	0,63	0,45	17,04	17,99	11,36	20,59	5,21	9,23	13,73	19,17	7,34	47,33
Bioice 3589 (11)	*H. nagelfar*	42,60	10,09	0,34	0,56	0,47	0,94	0,62	0,58	16,90	18,08	11,27	20,19	6,81	11,74	14,32	17,84	7,98	46,71

Specimen number	Identified as	cDL	GW%cDL	DE	Mucro	DW/cDL	VIIIW-IW	IIL/IIW	IVL/IVW	IW%cDL	IIW%cDL	IIL%cDL	IVW%cDL	IVH%cDL	IVL%cDL	VIIIL%cDL	VIIIW%cDL	AM%cDL	DW%cDL
C2	*H. nagelfar*	46,31	12,09	0,26	0,58	0,61	2,16	0,54	0,46	21,59	23,54	12,74	26,78	6,91	12,31	16,63	23,75	9,72	61,33
Bioice 3589 (10)	*H. nagelfar*	47,10	10,62	0,29	0,55	0,43	1,49	0,65	0,58	16,77	18,05	11,68	20,17	5,94	11,68	14,23	18,26	7,86	43,31
13395	*H. nagelfar*	49,05	6,73	0,29	0,58	0,37	1,63	0,63	0,48	16,31	17,53	11,01	20,18	5,91	9,79	12,03	17,94	6,93	37,11
a020	*H. nagelfar*	52,33	15,48	0,29	0,52	0,58	2,29	0,67	0,32	20,83	21,21	14,14	25,03	7,26	8,03	16,05	23,12	8,41	58,09
Bioice 3589 (8)	*H. nagelfar*	53,80	13,94	0,33	0,54	0,52	1,67	0,69	0,57	16,17	17,84	12,27	20,26	6,69	11,52	13,20	17,84	7,06	52,23
Bioice 3589 (9)	*H. nagelfar*	54,20	12,92	0,26	NA	0,49	NA	0,59	0,55	16,97	18,45	10,89	21,03	5,54	11,62	na	na	na	49,26
a008	*H. nagelfar*	54,80	11,68	0,30	0,49	0,46	2,55	0,70	0,47	16,79	17,88	12,59	20,62	6,20	9,67	12,59	19,34	6,20	46,35
Bioice 2887 (1)	*Hanleya sp.*	55,54	7,56	0,41	0,49	0,42	1,62	0,72	0,57	17,83	18,01	12,96	19,45	7,92	11,16	13,50	19,45	6,66	42,31
10571 (2)	*H. nagelfar*	58,00	18,28	0,25	0,59	0,60	1,55	0,66	0,26	17,24	17,76	11,72	19,83	5,00	5,17	12,59	18,79	7,41	59,66
SMNH Type-1329	*H. nagelfar*	58,90	5,09	0,34	0,54	0,40	1,70	0,66	0,49	18,17	19,35	12,73	21,73	7,30	10,70	12,22	19,86	6,62	40,07
a021	*H. nagelfar*	61,06	16,21	0,31	0,48	0,57	1,97	0,54	0,40	19,49	21,46	11,63	23,26	7,21	9,34	13,10	21,46	6,22	57,32
10571 (1)	*H. nagelfar*	63,60	16,35	0,28	0,53	0,54	2,67	0,66	0,56	16,98	18,71	12,42	22,64	6,29	12,58	13,99	19,65	7,39	54,40
Bioice 3589 (6)	*H. nagelfar*	64,50	10,08	0,29	0,60	0,48	2,95	0,70	0,64	17,21	18,45	12,87	20,62	6,05	13,18	16,12	20,15	9,61	47,90
Bioice 3589 (7)	*H. nagelfar*	66,00	13,64	0,35	0,55	0,55	1,97	0,69	0,63	14,24	16,06	11,06	18,48	6,52	11,67	13,48	16,21	7,42	54,55
Bioice 3589 (5)	*H. nagelfar*	69,80	12,46	0,32	0,60	0,50	3,44	0,67	0,59	14,61	16,05	10,74	18,34	5,87	10,74	13,61	18,05	8,17	50,14
Bioice 3589 (4)	*H. nagelfar*	72,01	8,89	0,37	0,58	0,50	2,22	0,70	0,58	17,64	18,75	13,05	21,53	8,05	12,50	15,42	19,86	8,89	49,99
Bioice 3589 (3)	*H. nagelfar*	76,58	15,54	0,38	0,57	0,60	2,87	0,69	0,71	17,37	18,02	12,41	20,50	7,70	14,50	16,58	20,24	9,53	60,46
C1	*H. Hanleyi* *	82,10	12,79	0,29	0,60	0,53	2,68	0,61	0,62	16,20	18,39	11,21	19,98	5,72	12,30	16,57	18,88	9,87	53,11
Bioice 3589 (2)	*H. nagelfar*	90,20	12,64	0,36	0,61	0,50	1,88	0,73	0,65	16,63	17,52	12,75	20,29	7,21	13,19	14,52	18,51	8,87	49,89
30037	*H. nagelfar*	97,10	9,68	0,34	0,60	0,43	2,47	0,82	0,66	14,52	15,65	12,77	17,71	6,08	11,64	12,87	16,99	7,72	43,36
Bioice 3589 (1)	*H. nagelfar*	114,60	10,99	0,32	0,62	0,45	1,57	0,69	0,60	14,66	15,53	10,65	18,32	5,85	11,08	13,35	16,23	8,29	44,94
a001	*H. nagelfar*	138,84	9,00	0,27	0,63	0,39	2,09	0,75	0,69	11,96	12,60	9,51	14,12	3,82	9,72	11,52	14,04	7,27	39,33

Appendix II

Commands in "R" and MrBayes

Commands used in the univariate analyses in R:
Example from girdle width:

```
> statGW<-read.table("clipboard",dec=",",header=T)
> attach(statGW)
> summary(GW)
  Min.   1st Qu.  Median  Mean   3rd Qu.  Max.
 4.100   7.850    9.650   9.739  11.380   18.300
> length(GW)
[1] 110
> sd(GW)
[1] 2.827262
> shapiro.test(GW)
        Shapiro-Wilk normality test
data:  GW
W = 0.9859, p-value = 0.3004
> hist(GW,main="Girdle width distribution",xlab="GW in % of DL",ylab="n",cex.lab=1.5,las=1)
> GWmod0<-lm(GW~1)
> GWmod1<-lm(GW~cDL)
> anova(GWmod0,GWmod1,test="F")
Analysis of Variance Table
Model 1: GW ~ 1
Model 2: GW ~ cDL
            Res.Df    RSS     Df    Sum of Sq    F       Pr(>F)
1           109       871.28
2           108       729.15  1     142.14       21.053  1.211e-05 ***
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
> GWmod1
Call:
lm(formula = GW ~ cDL)
Coefficients:
            (Intercept)          cDL
            8.46516              0.04628
> plot(cDL,GW,xlab="cDL (mm)",ylab="GW",cex.lab=1.5,las=1)
> abline(GWmod1)
```

Batch file used in MrBayes for the combined 18S+16S analysis:

```
#NEXUS
Begin MrBayes;
Execute C:\18S16S\matrix.nex;
End;
Begin MrBayes;
log start filename = 18S16SA_irr_utg_log replace;
[18S SYM+I+G, 16S = GTR+I+G]
partition 18S16S = 2: 18S, 16S;
set partition = 18S16S;
      Prset applyto=(1) statefreqpr=fixed(equal);
      Lset  applyto=(1) nst=6  rates=invgamma;

      Prset applyto=(2) statefreqpr=dirichlet(1,1,1,1);
      Lset  applyto=(2) nst=6  rates=invgamma;
mcmc ngen=1500000 nchains=4 samplefreq=100 printfreq=100 savebrlens=yes
Filename=18S16SA_irr_utg_trees;
sumt burnin=1500 nruns=2 filename=18S16SA_irr_utg_trees contype=allcompat
showtreeprobs=yes;
log stop;
end;
```

Appendix III

Morphological variation and localities for sequenced specimens.

Specimen	DL	GW	DE	DW/DL	Mucro	VIIIW-IW	IVL/IVW	IIL/IIW	Locality	18S	16S	CytB
MollÖvr7858	15,1	7,28	0,3	0,52	47,37	-3,97	0,45	0,53	Kattegatt	1	0	1
MollÖvr7859	17,3	8,70	0,4	0,53	50,00	-0,58	0,45	0,56	Kattegatt	1	0	1
BioIce3589(1)	114,6	10,99	0,32	0,45	62,09	1,57	0,60	0,69	Iceland	1	0	0
BioIce3589(3)	76,6	15,54	0,38	0,60	57,48	2,87	0,71	0,69	Iceland	1	1	1
BioIce3589(11)	42,6	10,09	0,31	0,47	55,74	0,94	0,58	0,62	Iceland	1	1	0
BioIce3589(12)	40,4	11,65	0,34	0,48	59,65	2,73	0,53	0,66	Iceland	1*	0	
BioIce3589(13)	33,4	12,28	0,34	0,49	51,06	0,90	0,51	0,63	Iceland	1	0	
a007	<15	na	na	na	na	na	na	na	Bergen		1	
a008	54,8	11,68	0,3	0,46	49,28	2,55	0,47	0,70	Bergen	1	1	
a010	< 20	na	na	na	na	na	na	na	Sotbakken		0	1
a014	14,7	8,84	0,34	0,42	43,48	2,04	0,37	0,55	Bergen		1	1
a015	23,5	9,81	0,34	0,48	56,67	-1,28	0,38	0,58	Bergen		1	1
a016	26,1	8,05	0,38	0,44	44,12	-0,77	0,46	0,58	Bergen	1	1	1
a017	35,7	10,08	0,34	0,46	55,56	0,84	0,47	0,62	Bergen	1	1	1
a018	42,3	11,36	0,25	0,47	53,45	2,13	0,45	0,63	Bergen		1	1
a020	52,3	15,48	0,29	0,58	52,38	2,29	0,32	0,67	Bergen	1	1	0
a021	61,1	16,21	0,31	0,57	47,50	1,97	0,40	0,54	Bergen	1	1*	1
a022	30,5	12,47	na	0,60	53,85	na	0,30	0,59	Bergen	1	1	1
a023	19,9	7,52	0,3	0,46	42,86	0,00	0,37	0,45	Bergen	1	1	1
31 (2)	7,3	9,59	0,32	0,58	41,67	-5,48	0,32	0,42	Bergen		1*	0

Table: Sequences obtained from specimens. 1 = specimen yielded a sequence; * = only one direction sequenced; 0 = No sequence yielded.

Appendix IV

Genetic sequences

DNA codes	Nucleotide
R	G or A
Y	C or T
K	G or T
W	A or T
S	C or G
M	A or C

Tabell 1 - DNA codes and the respectable nucleotides.

Group:	Sequences in group
Haplotype1_18S	BioIce 3589 (3), a008, a016, a017, a020, a021, a022
Haplotype1_16S	BioIce 3589 (3, 11), a007, a008, a014, a016, a017, a018, a023
Haplotype1_Cyt_B	BioIce 3589 (3), a008, a010, a014, a017, a018, a021, a022

Tabell 2 - Sequences in "Haplotype"-groups for each gene.

Specimens not included in the "Haplotype" for each gene has ambiguous base pairs present or was sequenced in only one direction.

18S rRNA:

```
                        10        20        30        40        50
                ....|....|....|....|....|....|....|....|....|....|
Haplotype1_18S  TGCATGTCTAAGTACAGACTTTCACATAGTGAAACCGCAAATGGCTCATT
3589(1)         ..................................................
3589(12)        ?????.............................................
A023            ..................................................
7858            ..................................................
7859            ..................................................
3589(11)        ..................................................
3589(13)        ..................................................

                        60        70        80        90       100
                ....|....|....|....|....|....|....|....|....|....|
Haplotype1_18S  AAATCAGTTATGATTTCTTAGATCGTACAATCCTACTTGGATAACTGTGG
3589(1)         ..................................................
3589(12)        ..................................................
A023            ..................................................
7858            ..................................................
7859            ..................................................
3589(11)        ..................................................
3589(13)        ..................................................

                       110       120       130       140       150
                ....|....|....|....|....|....|....|....|....|....|
Haplotype1_18S  TAATTCTAGAGCTAATACATGAAACTCCGCTCCGACCTCACGGGAAGAGC
3589(1)         ..................................................
3589(12)        ..................................................
A023            ..................................................
7858            ..................................................
7859            ..................................................
3589(11)        ..................................................
3589(13)        ..................................................
```

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                        160       170       180       190       200
                   ....|....|....|....|....|....|....|....|....|....|
Haplotype1_18S     GCTTTTATTAGATCAAGATCAATCGGGCTTGCCCGTCTATTGGTGATTCT
3589(1)            ..........................................................
3589(12)           ..........................................................
A023               ..........................................................
7858               ..........................................................
7859               ..........................................................
3589(11)           ..........................................................
3589(13)           ..........................................................

                        210       220       230       240       250
                   ....|....|....|....|....|....|....|....|....|....|
Haplotype1_18S     GAATAACTTTGGGCTGATCGCATGGCCACGAGCCGGCGACGTATCTTTCA
3589(1)            ..........................................................
3589(12)           ..........................................................
A023               ..........................................................
7858               ..........................................................
7859               ..........................................................
3589(11)           ..........................................................
3589(13)           ..........................................................

                        260       270       280       290       300
                   ....|....|....|....|....|....|....|....|....|....|
Haplotype1_18S     AGTGTCTGCCCTATCAACTTTCGATGGTACGTGATATGCCTACCATGGTT
3589(1)            ..........................................................
3589(12)           ..........................................................
A023               ..........................................................
7858               ..........................................................
7859               ..........................................................
3589(11)           ..........................................................
3589(13)           ..........................................................

                        310       320       330       340       350
                   ....|....|....|....|....|....|....|....|....|....|
Haplotype1_18S     GTAACGGGTAACGGAGAATCAGGGTTCGATTCCGGAGAGGGAGCATGAGA
3589(1)            ..........................................................
3589(12)           ..........................................................
A023               ..........................................................
7858               ..........................................................
7859               ..........................................................
3589(11)           ..........................................................
3589(13)           ..........................................................

                        360       370       380       390       400
                   ....|....|....|....|....|....|....|....|....|....|
Haplotype1_18S     AACGGCTACCACATCCAAGGAAGGCAGCAGGCGCGCAAATTACCCACTCC
3589(1)            ..........................................................
3589(12)           ..........................................................
A023               ..........................................................
7858               ..........................................................
7859               ..........................................................
3589(11)           ..........................................................
3589(13)           ..........................................................

                        410       420       430       440       450
                   ....|....|....|....|....|....|....|....|....|....|
Haplotype1_18S     TGGCACGGGGAGGTAGTGACGAAAAATAACAATACGGGATCTCTTCGAGG
3589(1)            ..........................................................
3589(12)           ..........................................................
A023               ..........................................................
7858               ........................M.................................
7859               ........................M..........Y......................
3589(11)           ..........................................................
3589(13)           ..........................................................

                        460       470       480       490       500
                   ....|....|....|....|....|....|....|....|....|....|
Haplotype1_18S     CCCCGTAATTGGAATGAGTACACTTTAAATCCTTTAACGAGGATCTATTG
3589(1)            ..........................................................
3589(12)           ..........................................................
A023               ..........................................................
7858               ................M.....................Y.....
7859               ..........................................................
3589(11)           ..........................................................
3589(13)           ..........................................................
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                          510        520        530        540        550
                 ....|....|....|....|....|....|....|....|....|....|
Haplotype1_18S   GAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGT
3589(1)          .....................................................
3589(12)         .....................................................
A023             .....................................................
7858             .....................................................
7859             .....................................................
3589(11)         .....................................................
3589(13)         .....................................................

                          560        570        580        590        600
                 ....|....|....|....|....|....|....|....|....|....|
Haplotype1_18S   ATATTAAAGTTGTTGCAGTTAAAAAGCTCGTAGTTGGATCTCAGGTCCAG
3589(1)          .....................................................
3589(12)         .....................................................
A023             .....................................................
7858             ..............S...........Y.S..........Y..........
7859             ..............S...........Y.S..........Y..........
3589(11)         .....................................................
3589(13)         .....................................................

                          610        620        630        640        650
                 ....|....|....|....|....|....|....|....|....|....|
Haplotype1_18S   GCTCGAGGTCCACCTCGCGGTGGKTACTTCCTGTCCTGACCTACCATCCG
3589(1)          ......................??.............................
3589(12)         ...??????????????????????????????????????????????
A023             .....................................................
7858             ...........M............-....Y.......................
7859             ...........M............-....Y.......................
3589(11)         ...S.....................-...........................
3589(13)         .....................................................

                          660        670        680        690        700
                 ....|....|....|....|....|....|....|....|....|....|
Haplotype1_18S   GTTTTCCCTTGGTGCTCTTGATTGAGTGTCTCGGGTGGCCAGAACGTTTA
3589(1)          .....................................................
3589(12)         ?????????????????????????????????????????????????
A023             .....................................................
7858             .....................................................
7859             .....................................................
3589(11)         .....................................................
3589(13)         .....................................................

                          710        720        730        740        750
                 ....|....|....|....|....|....|....|....|....|....|
Haplotype1_18S   CTTTGAAAAAATTAGAGTGTTCAAAGCAGGCGCATCGCCTGAATAATGGT
3589(1)          .....................................................
3589(12)         ?????????????????????????????????????????????????
A023             .....................................................
7858             .....................................................
7859             .....................................................
3589(11)         .....................................................
3589(13)         ..............R......................................

                          760        770        780        790        800
                 ....|....|....|....|....|....|....|....|....|....|
Haplotype1_18S   GCATGGAATAATGGAACAGGACCTCGGTTCTATTTTGTTGGTTTTCGGAA
3589(1)          .....................................................
3589(12)         ?????????????????????????????????????????????????
A023             .....................................................
7858             .....................................................
7859             .....................................................
3589(11)         .....................................................
3589(13)         .....................................................

                          810        820        830        840        850
                 ....|....|....|....|....|....|....|....|....|....|
Haplotype1_18S   CTCGAGGTAATGATTAAGAGGGACRGACGGGGGCATTCGTATTACGGTGT
3589(1)          .....................................................
3589(12)         ?????????????????????????????????????????????????
A023             .....................................................
7858             .....................................................
7859             .....................................................
3589(11)         .....................................................
3589(13)         ................R....................................
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                        860       870       880       890       900
                   ....|....|....|....|....|....|....|....|....|....|
Haplotype1_18S     TAGAGGTGAAATTCTTGGATCGCCGTAAGACGAACTACTGCGAAAGCATT
3589(1)            ..................................................
3589(12)           ??????????????????????????????????????????????????
A023               ..................................................???
7858               ..................................................
7859               ..................................................
3589(11)           ..................................................?
3589(13)           ..R.................R.................R.............
```

16S rRNA:

```
                         10        20        30        40        50
                   ....|....|....|....|....|....|....|....|....|....|
Haplotype1_16S     CACGTAAGAGTTGCGTGGTCGAACAGACCATCTTGTTTTAGCCGATACGC
A021               ??????????????????????????????????????????????????
31 (2)               ..................................................
A022               ..................................................
A010               ............M.....................................
A015               ................Y.................K.................

                         60        70        80        90       100
                   ....|....|....|....|....|....|....|....|....|....|
Haplotype1_16S     CAAACAGATCTCTTGATCCAACATCGAGGTCGCAAACCTTTTTTTTGATA
A021               ??????????????????????????????????????????????????
31 (2)               ..................................................
A022               ..................................................
A010               ..................................................
A015               ..........Y.......................................

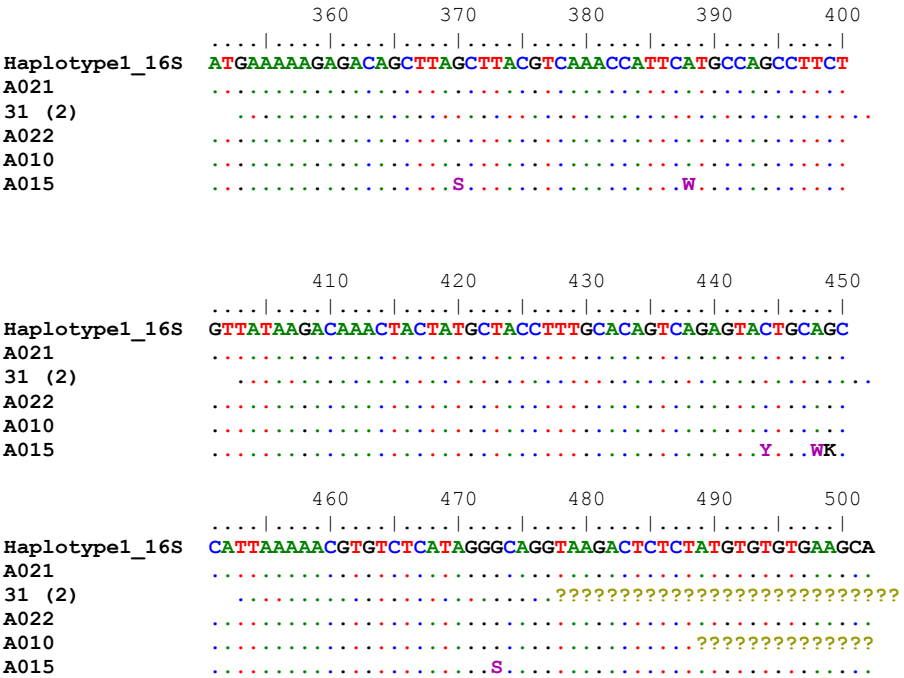
                        110       120       130       140       150
                   ....|....|....|....|....|....|....|....|....|....|
Haplotype1_16S     CGTGCTCTCCAAAAAGATTACGCTGTTATCCCTAGGGTAGCGGCTTGTTC
A021               ??????????????????????????????????????????????????
31 (2)               ..................................................
A022               ..................................................
A010               ..................................................
A015               ..................SY..............................

                        160       170       180       190       200
                   ....|....|....|....|....|....|....|....|....|....|
Haplotype1_16S     CCTTAAACATAATGGTATGGGTCTGGTTAACTGATTAGTTGGTGGGATAA
A021               ??????????????????????????????????????????????????
31 (2)               ..................................................
A022               ..................................................
A010               ..................................................
A015               ..............................Y...................

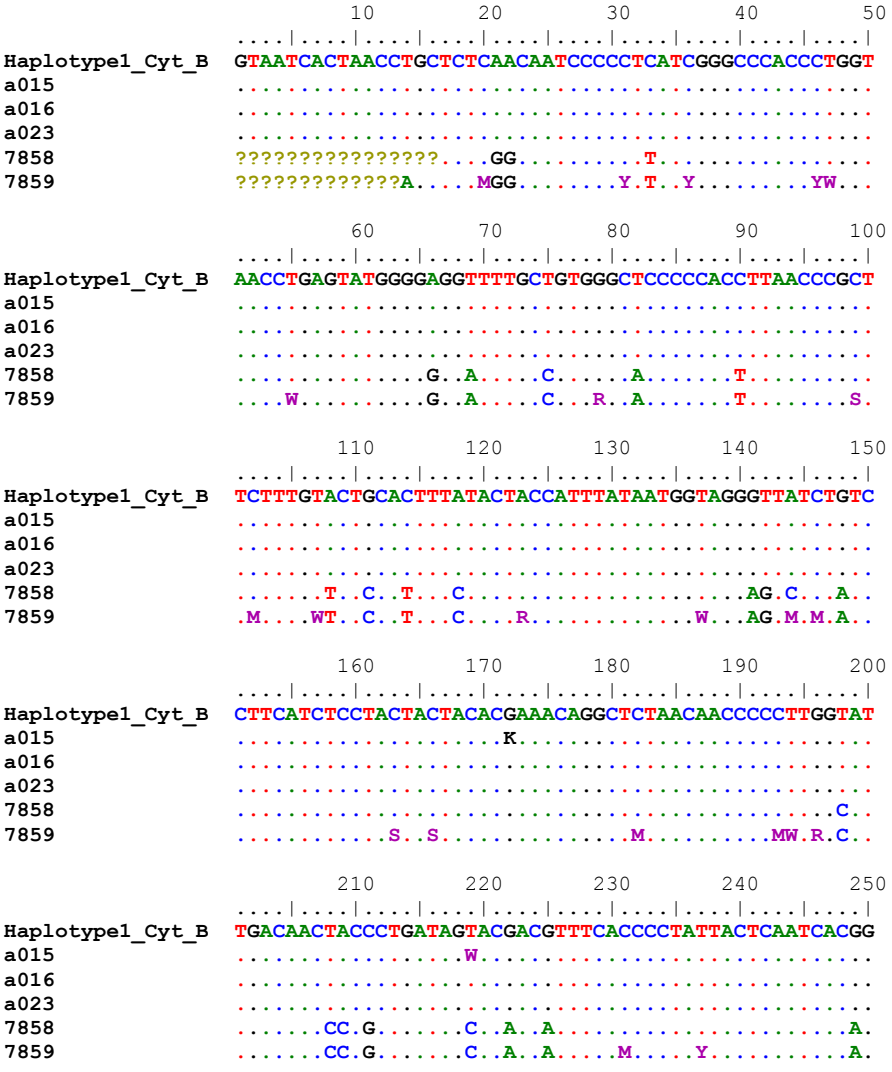
                        210       220       230       240       250
                   ....|....|....|....|....|....|....|....|....|....|
Haplotype1_16S     GAAGAAGCTTTGTGTGTTCTTTTGTTGCCCCAACAAAAGGGAATAAGAAA
A021               ??????????????????????????????????????????????????
31 (2)               ..................................................
A022               ..................................................
A010               ..................................................
A015               R.........................W..R.W.....................

                        260       270       280       290       300
                   ....|....|....|....|....|....|....|....|....|....|
Haplotype1_16S     ATTTGGTTGTTTTATATTTTCCAAGCCCTATAGCTCCATAGGGTCTTTTC
A021               ????????.........................................
31 (2)               ..................................................
A022               ..................................................
A010               ..................................................
A015               ......W...................M..........Y.....

                        310       320       330       340       350
                   ....|....|....|....|....|....|....|....|....|....|
Haplotype1_16S     GTCTTTCAGGGTTATTTAGGGTTCTTCACCTGAAGAATAATTTTTAGTTA
A021               ..................................................
31 (2)               ..................................................
A022               ..................................................
A010               ....................Y.............................
A015               ..Y................Y..........K.....................
```



Cytochrome B:



```
                        260       270       280       290       300
                   ....|....|....|....|....|....|....|....|....|....|
Haplotype1_Cyt_B   ACCTCCTAGGTTTTGCCGTCCTCCTTACCGTTCTCGCACTCCTTACAACC
a015               ...................................................
a016               ...................................................
a023               ...................................................
7858               ..........C.....T..A.....C...A.C..................
7859               ..........C.....T..A.....C...A.C................M.

                        310       320       330       340       350
                   ....|....|....|....|....|....|....|....|....|....|
Haplotype1_Cyt_B   CTCGCCCCCAACCTGCTAACTGACCCCCAAAACTATATCCCCGCTAACCC
a015               ...................................................
a016               ...................................................
a023               ...................................................
7858               ........................T...........C..T.....C..T..
7859               .........R.............T...........C..T.....C..T..

                        360       370
                   ....|....|....|....|....|..
Haplotype1_Cyt_B   CCTAGTAACTCCCGCCCACATCCAGCC
a015               ....K.....................
a016               ........................M..
a023               ....K.....................
7858               .........C................
7859               .........C............R...
```

Appendix V

Phylogenetic tree from 18S, 16S and Cytochrome B

Figure 25 – Phylogenetic tree based on a combined alignment of 18S, 16S and Cyt B sequences. Cyt B was only available for *A. irradians, Hanleyidae* and *K. tunicata*.