Doctoral Dissertation (Censored)

博士論文（要約）

The studies on the reproductive barrier between *Kulikovia alborostrata* and its cryptic species

(タカクラヒモムシおよびその隠蔽種間の生殖隔離に関する研究)

A Dissertation Submitted for the Degree of Doctor of Philosophy
December 2021

令和３年１２月博士（理学）申請

Department of biological Science, Graduate School of Science,
The University of Tokyo

東京大学大学院理学系研究科生物科学専攻

Jumpei Ikenaga

池永 潤平

Abstract

Fertilization is indispensable for maintaining species. The gametes must meet conspecific counterparts, but this is a challenge for animals living in marine environment where gametes of many species coexist. To overcome the challenge, gametes are often equipped with the mechanism for recognizing the conspecific counterparts. This recognition is mainly due to species-specific protein—protein interaction. Although such proteins are identified in mammals, teleost, sea urchins and abalones, there have been no studies in other animals. In this dissertation, I have studied the mechanism by which the gametes recognize its conspecific counterparts in the nemertean species.

In the part I, I examined the identity of the two color variants (purple type and yellow type) in *Kulikovia alborostrata* (Takakura, 1898). I revealed that there is reproductive isolation between the variants by cross-fertilization assay. Genetic analyses also showed that there is no gene flow between the variants even though they are closely related. Finally, I concluded that they are different species, and I described the yellow-type variant as *Kulikovia fulva* comb. nov.

In the part II, I examined the species-recognition during fertilization of *K. alborostrata* and *K. fulva*. I found that pre-zygotic reproductive barrier is present between the species and suggested that the egg surface seems to have an important role for the species-recognition between them.

In the part III, I explored genes for species-recognition in *Kulikovia* species and found six ZP domain-containing genes (named as NeZPL1~6) as the candidate. Comparison of the orthologs between the species revealed a three-amino-acid difference located in the predicted loop in EGF-like

domain of NeZPL6. Thus, NeZPL6 may be involved in the species-recognition in the gametes of *K. alborostrata* and *K. fulva*.

In the series of studies, I have unveiled the basis of species-recognition among nemertean gametes, and finally proposed the hypothesis that newly identified NeZPL genes look to be essential for the reproductive barrier in nemerteans.

Contents

General Introduction

Fertilization is the fundamental event for sexual reproduction and is the most important matter for animals to maintain their own species. Generally, the process of fertilization is roughly classified into two styles; One is the internal fertilization known in most of terrestrial animals and in some aquatic animals. In internal fertilizers, sperm are usually ejaculated to the female body. On the other hand, in some aquatic internal fertilizers, sperm are spawned into surrounding water before entering the female body. The other is the external fertilization, in which sperm are spawned into the surrounding water and fertilized in the extracorporeal environment. This style is often observed in marine organisms. Because the sea contains gametes from so many species that they have a hard time finding their way to conspecific counterparts. To overcome their challenge, some mechanism for searching conspecific counterparts is equipped in the gametes in the most of marine organisms.

Generally, fertilization is subdivided into some processes such as activation of sperm motility, sperm chemotaxis, acrosome reaction and sperm–egg fusion (Fig. I-1). Sperm is usually kept immotile in testis and start moving when they are spawned into outer environment (activation). In some animals, the motile spermatozoa swim up to the egg with tracing the concentration of the substance secreted from the conspecific egg (sperm chemotaxis). When sperm reaching the egg, the sperm contact with the vitelline membrane, outer structure surrounding the eggs, and inner acrosomal membrane of the sperm is exposed (acrosome reaction). Then, the sperm pass through the vitelline

membrane, bind to the plasma-membrane of the egg, and finally fuse to the egg (sperm−egg fusion). The way and process by which the gametes detect the conspecific counterparts vary from species to species. For example, the sperm of *Arbasia punctulata*, one species of sea urchin, show chemotaxis towards the conspecific eggs (Ward et al., 1985), but another sea urchin species *Strongylocentrotus purpuratus* do not show it (Guerrero et al., 2010a). In both species, however, the binding between the sperm and the vitelline membrane of the egg occurs in a species-specific manner (Glabe and Lennarz, 1979).

To date, several molecules concerning the species-recognition between sperm and egg have been revealed in some animals. In corals, some fatty alcohols were identified as the sperm attractant (Coll et al., 1994; Coll et al., 2015). In cuttlefish *Sepia officinalis*, sep-SAP, the six-amino-acid peptide, is the sperm-attractant (Zatylny, 2002). Particularly, in abalones, the species-specific interaction between sperm and egg vitelline membrane have been studied in detail. The interaction is mediated by two proteins, lysin in sperm and VERL (vitelline envelope receptor of lysin) which composes the vitelline membrane of abalones. The lysin binds to the VERL and lyses the vitelline membrane by disrupting VERL complex in a species-specific manner, allowing conspecific sperm to approach egg cell. (Rewis, 1982, Raj et al., 2017). The amino-acid sequences of lysin and VERL varies from species to species (Lee and Vacquier, 1992; Galindo et al., 2003) and these variations are shown to be essential

for the species-specific interaction (Raj et al., 2017).

In sea urchins, another species-specific inter-protein interactions have been studied. Bindin, the protein on acrosomal process of the sperm, binds to SBP (sperm binding protein) and EBR1 (egg receptor for bindin) on vitelline membrane of the conspecific eggs (Glabe and Vacquier, 1978; Foltz and Lennarz, 1990; Ohlendieck et al., 1993). The amino-acid sequences of these proteins also differ depending on species (Metz and Palumbi, 1996; Biermann, 1998; Kamei and Glabe, 2003; Calderón et al., 2009). Other species-specific interaction in sea urchins is known between REJ (receptor for egg jelly) on the sperm and FSP (fucose sulfate polymer) on the jelly layer, which is also necessary for species-recognition between gametes (SeGall and Lennarz, 1979; Vacquier and Moy, 1997; Vilela-Silva et al., 2002). Moreover, the oligopeptides called SAPs (sperm-activating peptides) contained in the jelly layer possibly activate the sperm in an order-specific manner (Suzuki, 1995).

In ascidians, sperm chemotaxis towards the conspecific eggs is known (Miller, 1982; Yoshida et al., 2013). This chemotaxis is mediated by the sulfated steroid called SAAF (sperm -activating and -attracting factor) released from the eggs and its possible receptor PMCA (plasma membrane Ca^{2+}/ATPase) on sperm membrane (Yoshida et al., 2018). The structures of SAAF in *Ciona intestinalis* and *Ascidia sydneiensis* are different at the position of sulfate group and the position of double bonds (Yoshida et al., 2002; Matsumori et al., 2013). These slight differences are thought to originate species-specific interaction between SAAF and PMCA.

In teleost, the GPI-anchored protein on egg plasma membrane named Bouncer is responsible for the recognition of conspecific sperm (Herberg et al., 2018). Bouncer has a role of binding sperm to egg plasma membrane, but its counterparts are still unknown.

Some species-specific interactions have been known even in mammalian fertilization, although the sperm and the egg meet internally. The proteins of zona pellucida, ZAN and ZP2 interact with the conspecific sperm, although the counterparts of ZAN and ZP2 have not been known. (Tardif et al., 2010; Avella et al., 2014). On the other hand, IZUMO1, that is located at inner acrosomal membrane of the sperm, and JUNO, the protein GPI-anchored on the egg plasma membrane, are necessary for fusion of gametes (Bianchi et al., 2014). The interaction also seems species-specific (Bianchi and Wright, 2015).

In addition to these studies, species-specific interaction between gametes have been tested in many animals from cnidarians to chordates (Dan, 1950; Miller, 1966; Miller, 1977; Miller, 1982, Miller, 1985a; Miller, 1985b; Yoshida et al., 2013), but the substances responsible for the species-specific interactions are not identified yet in these studies.

Nemerteans are the animals belonging to the phylum Nemertea and about 1,300 species have been known for now (Kajihara et al., 2008). Habitat of most nemerteans is marine environment, while some nemerteans live in freshwater and terrestrial environment (Moore et al., 2001; Strand and

Sundberg, 2015). Nemerteans are gonochoristic and the major style of fertilization is external fertilization, albeit some species showing mating and internal fertilization (Thiel and Junoy, 2006). The morphology of nemertean gametes has been studied in some species. The eggs of some species are enclosed by vitelline membrane, but the vitelline membrane is not seen in other species probably because it is integrated with plasma membrane. (Stricker et al., 2002; Stricker et al., 2013; Hiebert and Maslakova, 2015a; Hiebert and Maslakova, 2015b). Nemertean sperm has the typical morphology, consisting of an acrosome, a nucleus in the head region, mitochondrion in the middle piece and a single flagellum (Döhren and Bartolomaeus, 2006; Döhren et al., 2010; Chernyshev et al., 2020).

Phylogenetically, the phylum Nemertea is suggested to belong to Kryptrochozoa, a subgroup of Lophotrochozoa, by whole-genome analysis containing the phyla Phoronida and Brachiopoda (Fig. I-2) (Luo et al., 2018). Phoronids and brachiopods are sessile animals and fertilization occurs externally. Therefore, although it seems that species-recognition have important roles on maintaining Kryptrochozoan species, how their gametes recognize the counterparts have been completely unknown. Moreover, in nemerteans, some cryptic species have been reported which have similar morphologies but genetically isolated (Tulchinsky et al., 2012; Hiebert and Maslakova, 2015; Krämer et al., 2017; Chernyshev et al., 2018; Verdes et al., 2021). These cryptic species often live sympatrically without crossbreeding. Hence, it also seems that the veiled species-recognition system is indispensable for maintaining the genetic diversity among nemerteans.

In my doctoral dissertation, I tried to understand the mechanism involved in the species-recognition among nemerteans. In the part I, I described the two nemerteans, *Kulikovia alborostrata* and *K. fulva*. I also showed that these two are closely related species but reproductively isolated, suggesting these can be good nemertean models for studying the species-recognition during fertilization. In the part II, I examined that the mechanism for species-recognition works during fertilization and showed it possibly works on the egg surface in those species. In the part III, I tried to identify the molecules responsible for species-recognition between gametes of *Kulikovia* species. Finally, I proposed a novel protein named NeZPL6 (nemertean ZP-domain containing protein like 6) as a candidate for a molecule concerning the species-recognition between gametes of *Kulikovia* species.

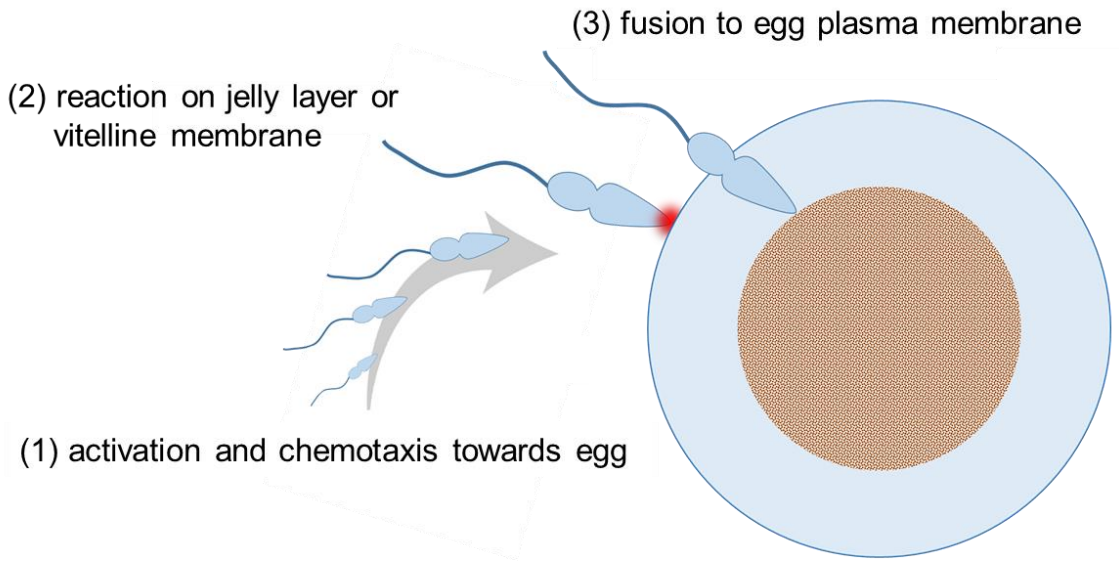

Fig. I-1. The diagram of the processes involving the species-recognition during fertilization.

.

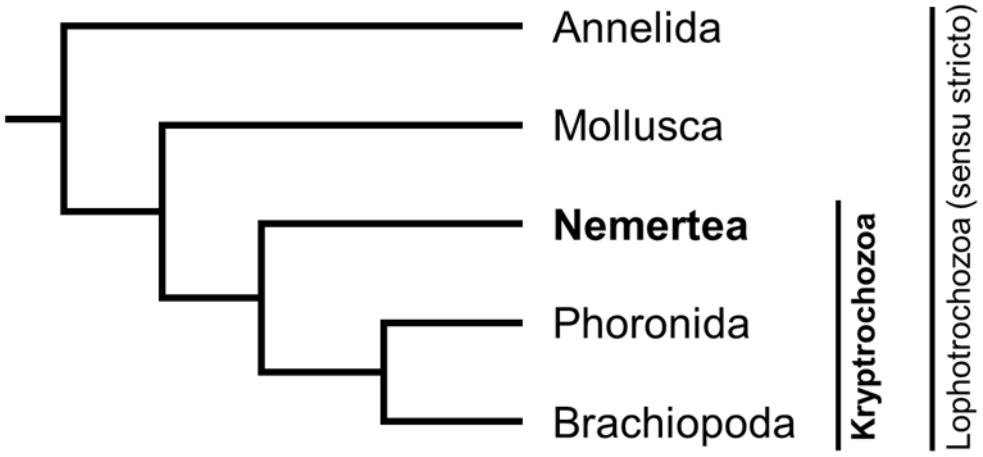

Fig. I-2. The phylogeny among Lophotrochozoan animals. [modified from the Fig. 1e in Luo et al., 2018]

Part I

The cross-fertilization assay and genetic analyses of *K. alborostrata* and *K. fulva*

Introduction

There have been 1,300 nemertean species described so far (Kajihara et al., 2008) based on external and internal morphologies. Recently, their external morphologies are considered to be more informative than internal morphologies when it comes to distinguish the closely related species. This is because there are often no clear differences in internal morphologies among closely related species (Strand and Sundberg, 2011). On the other hand, external morphology alone is not sufficient for identifying species, since similar characters are seen among some different species. For example, *Lineus ruber* (Müller 1774) and *L. viridis* (Müller 1774) had been recognized as the same species for a while since they were almost indistinguishable in external morphologies (Gibson, 1995), although the species were originally described as different species in 1774. Recently, however, the molecular analysis revealed that these are clearly different species (Krämer et al., 2017). Therefore, nowadays, at least both external morphologies and molecular data are indispensable for description of nemertean species (Sundberg, 2015).

Kulikovia alborostrata (Takakura, 1898) is the nemertean species described with the materials specimens collected at Misaki, Kanagawa, Japan. The species is distinguished by a white band at the tip of the head and the spatula-shaped head divided from the trunk by a small constraint. In the original description, variations of body color were described, ranging from purple-tinged dark yellow (yellow type) to pale purple (purple type) (Takakura, 1898). These variants are found in Misaki

even now. On the other hand, other two color variants of *K. alborostrata* have been found in The Vostok Bay, Russia: one of the variants is reddish and another is brown-violet, which are classified as the same species by the phylogenic analysis (Chernyshev et al., 2018). Although the brown-violet variant in Russia appears to have the same body color with the purple-type one in Misaki, its taxonomic identity has never been tested based on molecular analysis. On the other hand, the yellow-type variants have been described only in Misaki and its taxonomic identity also has not tested with molecular analysis.

In this part, for revealing the taxonomic identities of these variants in Misaki, I conducted cross-fertilization assay, species delimitation analyses and phylogenic analyses.

Methods

Specimens Collection

The specimens were collected among the calcareous algae growing around the Moroiso and Araihama, Kanagawa, Japan. Sampling were conducted during spring tide from March 2019 to May 2019. Specimens were kept at 18℃ in the 1.5 L plastic containers filled with seawater and at most 5 specimens were kept in each container. Body size of the specimens was measured by imaging analysis of the photographs taken by the digital camera (Nikon 1 V3; Nikon) equipped on the stereomicroscope (SZX7; Olympus) with ImageJ (NIH). The photographs were taken after anesthesia of specimens in 73.5 g/L $MgCl_2$.

Gametes Collection and Fertilization

To collect gamete, the posterior end of specimens was cut (about 3 mm in length) with a razor, and the cut fragment was transferred to a glass dish containing filtrated sea water (FSW). The remaining individual was put back into the rearing container so that gamete was collected again from the individual in the same way. Sperm leaked out from the cut fragment containing testis were transferred into a microtube with a pipette. Oocytes leaked out from the fragment containing ovary were transferred into another glass dish filled with FSW. The transferred oocytes were incubated for approximately an hour at 18℃ to induce maturation. The concentration of sperm was calculated with

a hemocytometer. Eggs were inseminated by the sperm suspension in 3.5 mm dishes filled with 2 ml FSW. The fertilization rate was examined 2 hours after insemination by counting the eggs in cleavage. The photographs of gametes were taken under the light microscopy (BX51, Olympus).

DNA extraction, PCR amplification and Sequencing Analysis

Genomic DNA was extracted from the cut fragment prepared in the same way as the collection of gametes. The cut fragment was processed in KPE buffer (0.1 M Tris-HCl (pH 9.5), 10 mM EDTA, 1 M KCl) with incubation at 95℃ for 20 min and -20℃ until frozen. This treatment of heating and freezing was repeated one more time. The suspeghg after the treatment was collected and purified DNA was obtained from it with phenol/chloroform extraction and ethanol precipitation. The purified DNA was dissolved in distilled water (DW) and used for a template of following PCR.

Partial sequence of 16S (primers: ar-L and br-H) (Palumbi et al., 1991), 18S (primers: forward 5′-CCGGAGAGGGAGCCTKA-3′ and reverse 5′-GACGGGCGGTGTGTRC-3′), 28S (primers: C1′ and C2) (Le et al., 1993), histone H3 (H3) (primers: LCO1490 and HCO2198) (Folmer et al., 1994), cytochrome oxidase subunit I (COI) (primers: aF and aR) (Colgan et al., 1998) genes in the template DNA were amplified by PCR with KOD FX NEO (TOYOBO) was amplified. PCR was performed under 94℃ for 1 min, 40 cycles of 94℃ for 15 sec , 48℃ for 15 sec, 68℃ for 1 min, followed by 68℃ for 2 min.

To prepare templates for sequencing reaction, exonuclease I (NEB) and rAPid alkaline phosphatase (Roche) were added to the PCR product and incubated at 37℃ for 30 min and at 80℃ for 15 min. Following cycle-sequencing reaction was performed with BigDye™ Terminator v3.1 (Applied Biosystems). Cycle-sequencing reaction was performed under 94℃ for 1 min, 25 cycles of 94℃ for 10 sec, 46℃ for 5 sec, 60℃ for 1 min 30sec. Primer for the cycle-sequencing reaction was the same as that for the PCR amplification. The product of cycle-sequencing reaction was purified by ethanol precipitation and dissolved in Hi-Di™ Formamide (Applied Biosystems) before sequencing with ABI3130 (Applied Biosystems).

Species delimitation analysis, phylogenic analysis and Genetic distance

Species delimitation analyses were performed by ABGD (Puillandre et al., 2012), bPTP (Zhang et al., 2013) and statistical parsimony (Templeton et al., 1992). The obtained 16S and COI sequences were aligned with MAFFT 7.0 (Katoh and Standley, 2013). ABGD analysis was performed on the ABGD server with default parameters. bPTP analysis was performed on the bPTP web server with default parameters based on the phylogenic tree generated by GTR+CAT+I model on RaxML ver. 8 (Stamatakis, 2014). The statistical parsimony analysis and drawing haplotype networks were performed by TCS ver. 1.2.1 (Clement et al., 2000) with default parameters except for the connection limit set as 90 %.

The phylogenic tree among heteronemerteans was drawn with the sequence of 16S, 18S, 28S, H3, COI genes derived from both purple- and yellow-type variants captured in Misaki and other 23 species acquired from GenBank. Accession number of each sequence was listed in Table 1-1. The sequences were aligned by MAFFT 7.0 (Katoh and Standley, 2013) and trimmed on Gblocks Server ver. 0.91b (Castresana, 2000). The parameters in the series of treatment were the same as Chernyshev et al., 2018 except for allowing many contiguous nonconserved positions in Gblocks. Trimmed sequences were concatenated on SeaView (Gouy et al., 2010) and 2, 707 bp sequences were yielded. The optimized model for phylogenic analysis was obtained by PartitionFinder ver. 2 (Lanfear et al., 2017). Maximum likelihood (ML) analysis was performed by IQ-TREE (Nguyen et al., 2014) with 1,000 bootstrap replicates. Bayesian inference (BI) analysis was performed by MrBayes (Ronquist et al., 2012) with four Markov chains for 1,000,000 generations, sampling every 1,000 generations.

The genetic *p*-distances of 16S and COI sequences derived from both purple- and yellow-type variants were calculated by MEGA X (Kumar et al., 2018) with default parameters.

Results

Cross-fertility assay between two variants of Kulikovia alborostrata

First, I examined the cross-fertilization assay between the purple- and yellow-type variants in *K. alborostrata*. Eggs were inseminated with 1.0 x 10^4, 1.0 x 10^3 and 1.0 x 10^2 cells /ml sperm of the same type or the other type. The rate of fertilization is significantly different between the same-type gametes and different-type gametes (N=3, two-way ANOVA, $P < 0.001$) (Fig. 1-2). Almost all eggs inseminated with the same-type sperm developed to the two-cell stage, but the eggs inseminated with the another-type sperm developed to the two-cell stage in the lower rate. As the concentration of sperm was low, the rate eggs developed to the two-cell stage was low. In the natural condition, the concentration of sperm are probably kept under 1.0 $\times 10^2$ cells / ml, because sperm will be immediately dispersed in surrounding seawater after spawned. Thus, these results suggest that there is the reproductive isolation between the purple- and yellow-type variants in *K. alborostrata* at least in the natural environment.

Species delimitation analysis

To test the possibility of gene flow between the purple- and yellow-type variants of *K. alborostrata* in Misaki, species delimitation analyses were performed based on 16S and COI genes.

In these analyses, the 16S and COI sequences of *K. alborostrata* from Vostok Bay, Russia and the 16S sequence of the specimen formerly identified as *Lineus fulvus* from Oshoro, Hokkaido, Japan (Schwartz et al., 2009) was also contained. The all three ways of species delimitation analyses, ABGD, bPTP and TCS, indicated that there are genetic gaps between the variants (Fig. 1-3). This result denied the possibility of gene flow between the variants in the natural condition. The analyses also indicated that purple-type in Misaki is genetically identical with *K. alborostrata* in Vostok Bay and the yellow-type in Misaki is genetically identical with the specimen formerly identified as *L. fulvus* described in Hokkaido. Moreover, the haplotype networks were drawn based on the result of TCS analysis. Although all specimens fell within 5% difference in 16S sequences, at most 10% difference was detected among COI sequences of the yellow-type variant in Misaki (Fig. 1-4). Hence, it is suggested that the purple- and yellow-type variant is the separated species and I described them as *K. alborostrata* and *K. fulva*, respectively, in the section of taxonomy.

Gametes and External Morphology

I examined whether there are differences between the morphologies of the purple- and yellow-type variants. First, the morphologies of their gametes were compared, and no obvious difference was detected (Fig. 1 C-H); the diameter of both eggs was approximately 120 μm, the

shape and the size of sperm head were the same and the length of both sperm flagella was approximately 50 μm. On the other hand, the difference was seen in the size of the mouth; the length of the mouth along the anterior-posterior axis in the purple-type variant was shorter than that in the yellow-type variants (Fig. 1-5). The mouth length of the purple-type variant is less than approximately 0. 3 times as long as the body width at the constriction between the head and trunk (‘neck’ hereafter). On the other hand, that of the yellow-type variant is more than approximately 0.3 times as long as the body width at the neck.

Taxonomy

***Kulikovia alborostrata* (Takakura, 1898)**

(Figs. 1-1 A, 1-2 A)

Lineus alborostratus Takakura, 1898, p. 332, fig. 2 [in part]; Yamaoka (1940), p. 220, pl. 15, figs. 1–5; Xu et al. (2012), p. 85.

Kulikovia alborostrata: Chernyshev et al. (2018), p. 60, figs. 2–4, 17.

Material examined. Nine adult individuals collected in the Moroiso and the Araihama, Kanagawa,

Japan.

Description. Body 5–30 cm in length, dark slate blue on dorsal side and pale blue on ventral side with a white band on dorsal side at the tip of head (Fig. 1-1 A). Neck 0.6–1.1 mm in width. Mouth 0.1–0.3 mm in length along the antero-posterior axis; mouth length less than about approximately 0.3 times as long as neck width (Fig. 1-5 A, C). Reproductive season from the middle of February to late March in Misaki.

Remarks. Although *Lineus alborostratus* in the original description (Takakura, 1898) contains both the purple- and yellow-type variant, they turned out to be separate species (see the results above). To date, there are several studies on the species with the name of *Lineus alborostratus* or *Kulikovia alborostrata*, and these all have been consistent in pointing to the purple-type variant. Therefore, in accordance with those reports, I regarded the purple-type variant in Misaki as representing *Kulikovia alborostrata*, although the type material of *L. alborostratus* is not extant (Kajihara, 2004). The public sequences tagged with the names *L. alborostratus* or *K. alborostrata* in the GenBank can be regarded to be homologous to that of the purple-type variant in Misaki (Chernyshev et al., 2018) (Fig. 1-3).

***Kulikovia fulva* (Iwata, 1954) comb. nov.**

(Figs. 1-1 B, 1-2 B)

Lineus alborostratus Takakura, 1898, p. 332, fig. 2 [in part].

Lineus fulvus Iwata, 1954: 13, fig. 2C; Schwartz (2009), p. 273.

Materials examined. Ten adult individuals collected in the Moroiso and the Araihama, Kanagawa, Japan.

Description. Body 5–30 cm in length, yellowish brown on dorsal side and beige on ventral side with a white band on dorsal side at the tip of head (Fig. 1-1 B). Neck 0.5–1.2 mm in width. Mouth 0.2–0.5 mm in length along the antero-posterior axis; mouth length more than about approximately 0.3 times as long as neck width (Fig. 1-5 B, C). Reproductive season from the middle of February to late March in Misaki.

Remarks. The morphology of the yellow-type variant well conformed to the description of *Lineus fulvus* by Iwata (1954) pointing “anteriorly brownish yellow and posteriorly yellow” and “a white

band is clearly found on the tip of the head." The appearnce of mouth in the illustration in the original description also conformed to that in yellow-type variant. On the other hand, although black pigmentations were found at the tip of head in the yellow-type variant, it has not been proven that the pigmentation is identical to the 15 occelli pointed in the original description of *Lineus fulvus* because the type material is currently not available to us. Molecularly tested, the yellow-type variants in Misaki were identical to one individual identified as *Lineus fulvus* collected in Oshoro, Hokkaido (Schwartz et al., 2009) (Fig. 1-3). In addition, the yellow-type variants were firmly placed within the *Kulikovia* clade by the phylogenic analyses. Thus, I proposed a new combination *Kulukovia fulva* comb. nov.

The phylogenic analysis

To examine the phylogenic relationships between *K. alborostrata* and *K. fulva*, I performed the phylogenic analyses with the nucleotide sequences of 16S, 18S, 28S, H3 and COI. Both the ML and the BI analyses showed that these two species were in a sister-taxon relationship with 96% bootstrap value and 1.00 Bayesian posterior probability (Fig. 1-6). The phylogenic trees also showed that both two species were incorporated into the genus *Kulikovia* and formed a clade with *K. torquata* with 78% bootstrap value and 1.00 Bayesian posterior probability.

The genetic distance

The uncorrelated *p*-distance between *K. alborostrata* and *K. fulva* were calculated as 2.8—5.4 % and 14.4—17.3% in 16S and COI, respectively (Table 1-2). On the other hand, intraspecific uncorrelated *p*-distance were calculated as 0.0—0.5% (16S) and 0.1—1.1% (COI) in *K. alborostrata* and 0.5—1.5% (16S) and 1.6—2.4% (COI) in *K. fulva*.

Discussion

In this part, I examined the identities of the purple- and yellow-type variants of *K. alborostrata* in the Takakura's original description with the cross-fertilization assay and the molecular phylogenetics. I found that there is reproductive isolation between the two types and identified the purple-type variant in Misaki as *K. alborostrata* and the yellow-type variant in Misaki as *K. fulva* comb. nov. I showed that the fertilization rate between different species was significantly lower than that between the same species, even though the cross-fertilization occurred at very low rate in the experimental condition of $1.0^2 \times 10$ sperm cells/ml. Probably, the cross-fertilization will not occur in the natural environment because this density of sperm cell in the experimental condition is much higher than that in the natural environment where sperm are dispersed into seawater immediately after spawned. Moreover, in the natural condition, both *K. alborostrata* and *K. fulva* lives sympatrically and become mature at the same season. Therefore, although it is considered that the sperm from both species are present concurrently in the same area, eggs will be fertilized preferentially with conspecific sperm even when heterospecific sperm reach the eggs. The results of the genetic analyses indicated that there is no gene flow between the two species and supported this hypothesis. Although it is unknown whether these two are pre-zygotically or post-zygotically isolated, previous studies on other marine animals suggested that pre-zygotic isolation seems to be major among external fertilizers. In abalones, the vitelline membrane around an egg is important for selecting the conspecific sperm

(Swanson and Vacquire, 1997; Raj et al., 2017). In sea urchins, the acrosomal reaction occurring in sperm passing through the jelly layer is shown to be important for species-specific fertilization (Summers and Hylander, 1975). Although the mechanism for reproductive isolation in nemerteans have never been studied, the reproductive isolation between *K. alborostrata* and *K. fulva* may also be pre-zygotic, thus I tried to examine this point in the part II.

I also revealed that *K. alborostrata* and *K. fulva* are closely related species by phylogenic analyses and calculating the genetic distances. Between these species, the genetic distance of 16S gene was calculated as 2.8—5.4% in this study. The formerly reported minimum value of the genetic distance of 16S gene was 4.0% which is between other nemertean species *Maculaura alaskensis* and *M. oregonensis* (Hiebert and Maslakova, 2015). The genetic distance between *K. alborostrata* and *K. fulva* is comparable to this and it can be said that these two are one of the closest species among nemerteans. Remarkably, even though these two are closely related species, their gametes are already equipped with some systems for recognizing the conspecific counterparts. This fact highlights the possibility that *K. alborostrata* and *K. fulva* are good models for studying the reproductive isolation in nemerteans.

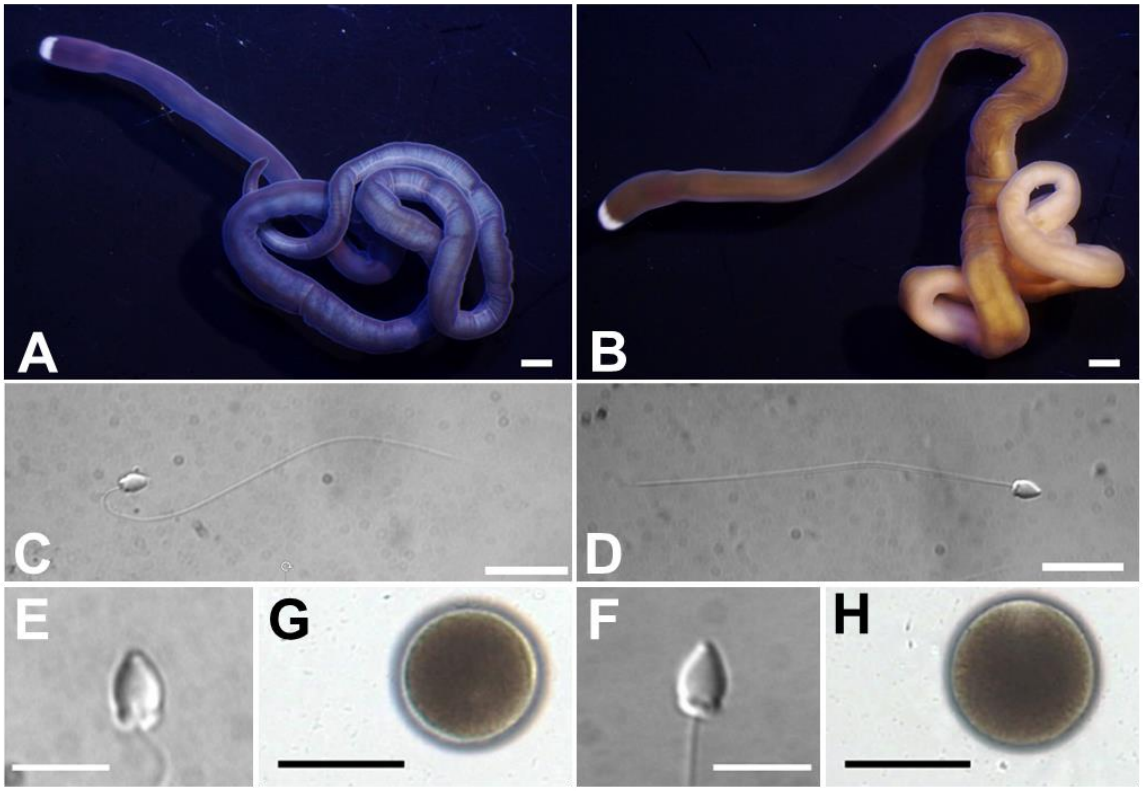

Fig. 1-1. (A, C, E, G) Purple-type variant of *Kulikovia alborostrata* in Misaki, Japan (Takakura, 1898). **(B, D, F, H)** Yellow-type variant in Misaki. **(A, B)** Living specimens captured in Misaki. **(C–F)** Spermatozoa. **(G, H)** Mature eggs. Scale bars: **(A, B)** 1 mm; **(C, D)** 10 µm; **(E, F)** 5 µm; **(G, H)** 100 µm.

Fig. 1-2.

本図は 5 年以内に雑誌等で刊行予定のため、非公開

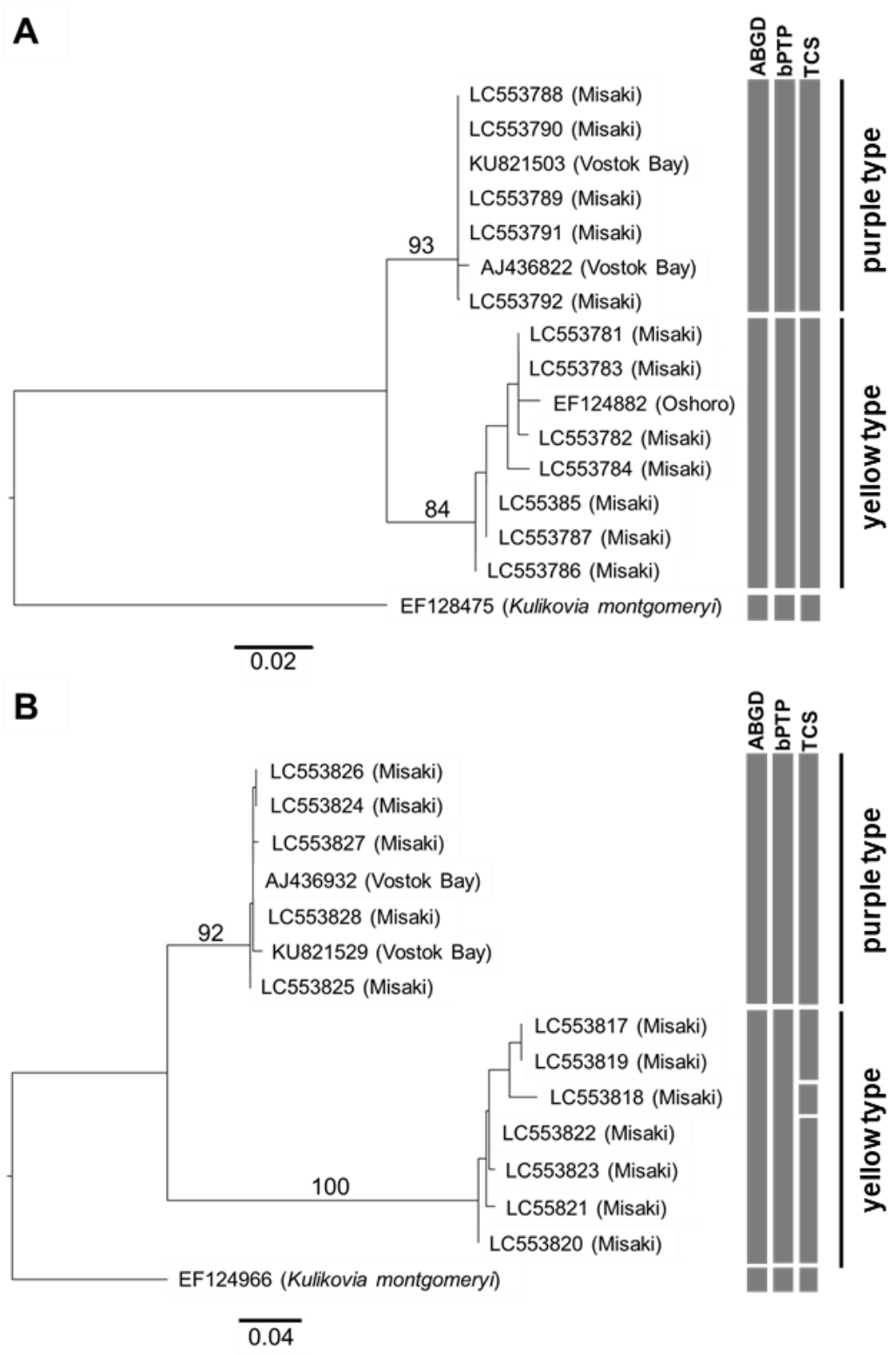

Fig. 1-3. Results of ABGD, bPTP, and TCS analyses shown on maximum-likelihood trees based on 16S **(A)** and COI **(B)** datasets, with numbers near nodes showing bootstrap values.

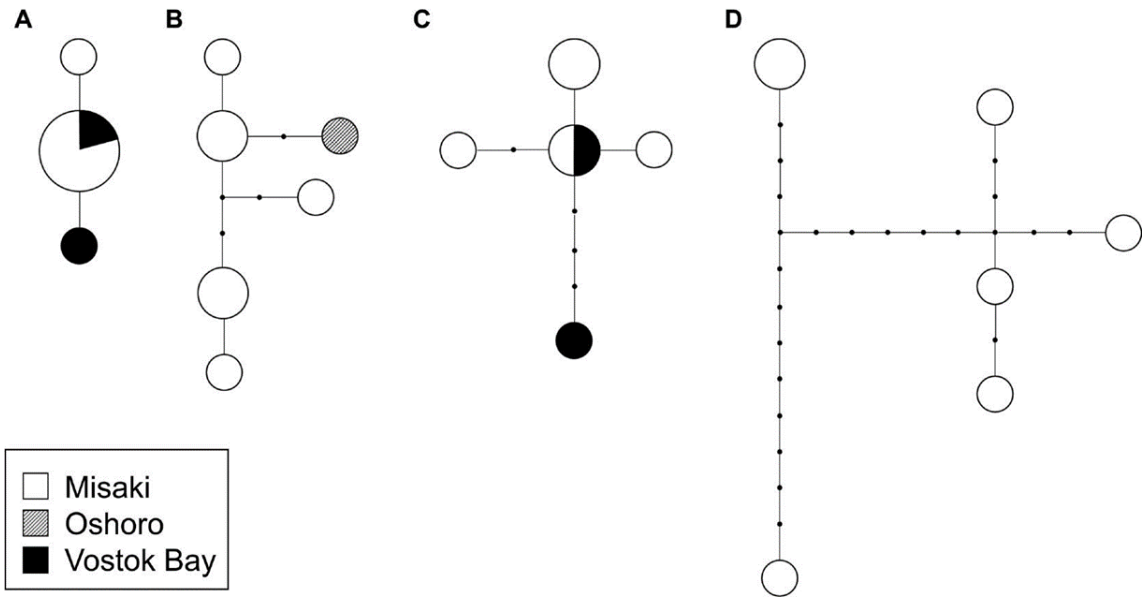

Fig. 1-4. Haplotype networks of the purple-type variant in Misaki (white), *K. alborostrata* in Vostok Bay (black) **(A, C)** and yellow-type variant in Misaki (white) and *L. fulva* in Oshoro (diagonal stripe) **(B, D)**, constructed by statistical parsimony analysis with the 16S **(A, B)** and COI **(C, D)** datasets; the size of pie chart reflects sample size. Each edge represents a single nucleotide mutation and black dots denote missing haplotypes.

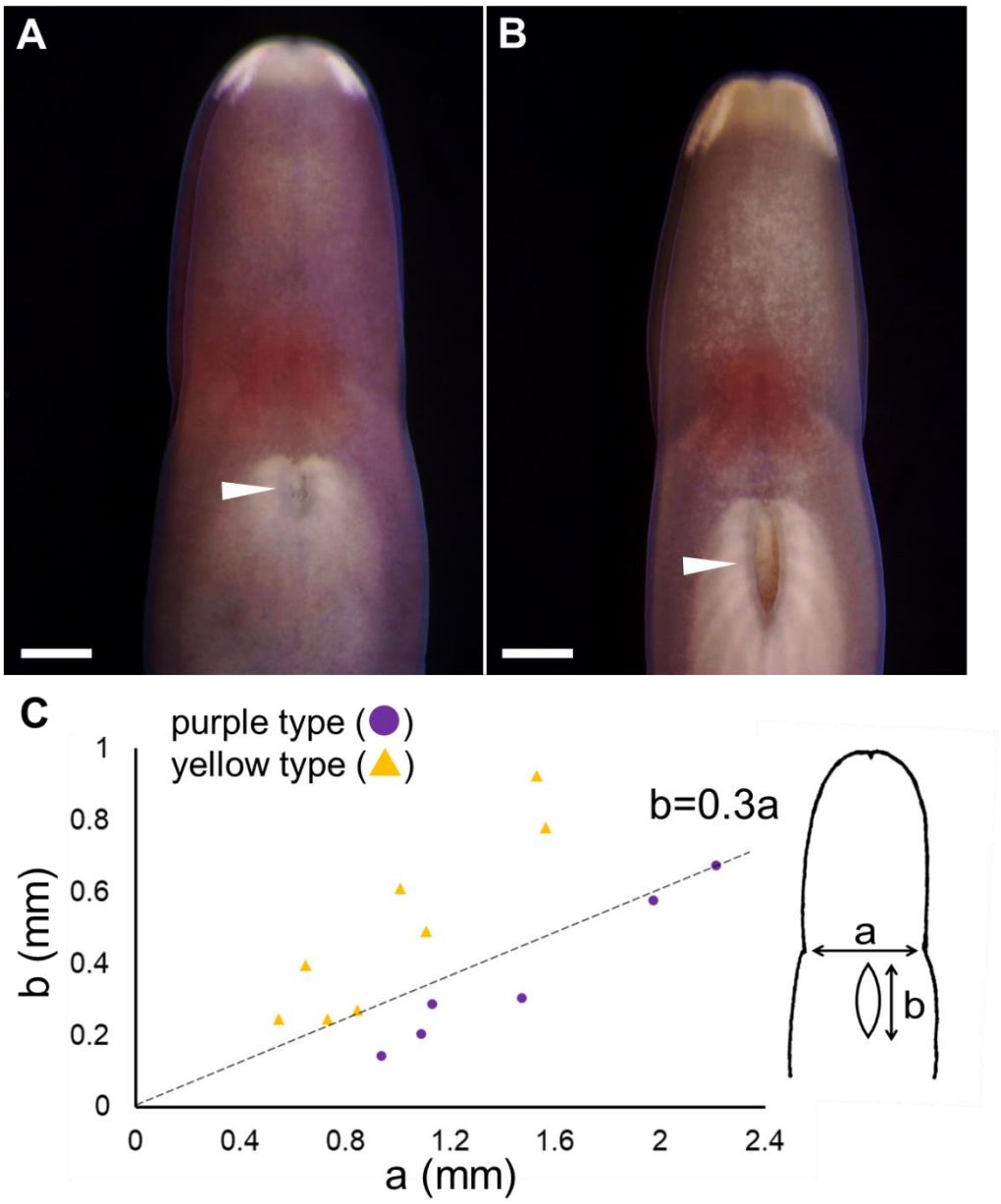

Fig. 1-5. (A, B) Head, ventral view, showing difference in size and shape of mouth (indicated by arrowheads) between the purple-type **(A)** and the yellow-type variant **(B)**. **(C)** each plot showing the length of neck width (a, vertical axis) and antero-posterior mouth length (b, horizontal axis) of each individual of the purple-type (circles, n = 6) and the yellow-type variant (triangles, n = 8). Scale bars: **(A, B)** 0.5 mm.

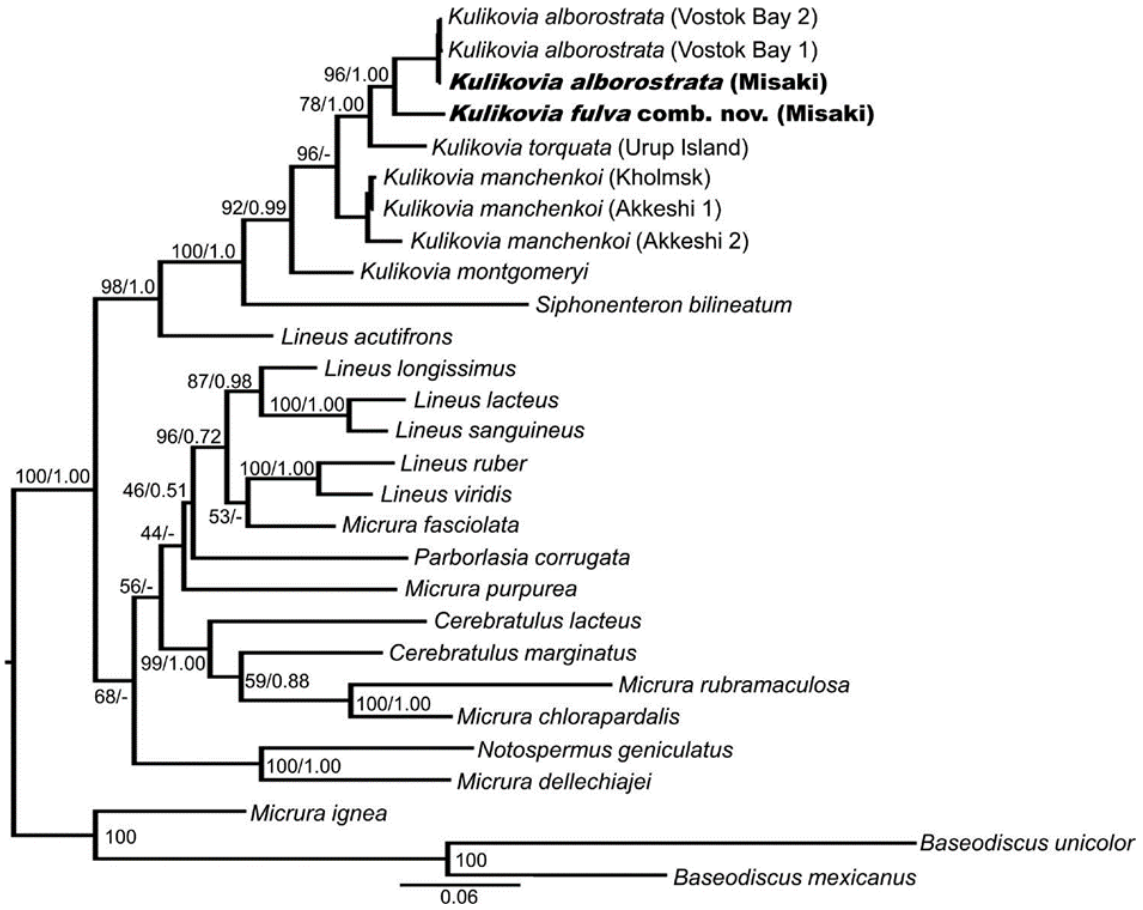

Fig. 1-6. Maximum-likelihood (ML) tree based on the concatenated sequence of 16S, 18S, 28S, COI, and H3 among heteronemerteans. Two *Baseodiscus* species were used as outgroup. Numbers on each node show ML bootstrap value /Bayesian posterior probability.

Species	16S	18S	28S	COI	H3	Reference
Baseodiscus mexicanus	KF935449	KF935281	KF935337	KF935503	KF935393	Kvist et al. (2014)
Baseodiscus unicolor	KF935451	KF935284	KF935340	KF935505	KF935396	Kvist et al. (2014)
Cerebratulus lacteus	JF277575	JF293044	HQ856857	HQ848576	JF277728	Andrade et al. (2012)
Cerebratulus marginatus	JF277576	JF293042	HQ856858	HQ848575	JF277729	Andrade et al. (2012)
Kulkovia alborostrata (Vostok Bay 1)	KU821503	—	KU856679	KU821529	KU821552	Chernyshev et al. (2018)
Kulkovia alborostrata (Vostok Bay 2)	AJ436822	—	AJ436877	AJ436932	AJ436979	Thollesson et al. (2003)
Kulkovia alborostrata (Misaki 1)	LC553788	LC553800	LC553812	LC553824	LC553836	present study
Kulkovia alborostrata (Misaki 2)	LC553789	LC553801	LC553813	LC553825	LC553837	present study
Kulkovia alborostrata (Misaki 3)	LC553790	LC553802	LC553814	LC553826	LC553838	present study
Kulkovia alborostrata (Misaki 4)	LC553791	LC553803	LC553815	LC553827	LC553839	present study
Kulkovia alborostrata (Misaki 5)	LC553792	LC553804	LC553816	LC553828	LC553840	present study
Kulkovia fulva (Oshoro)	EF124882	—	—	—	—	Schwartz and Norenburg (unpubl.)
Kulkovia fulva (Misaki 1)	LC553781	LC553893	LC553805	LC553817	LC553829	present study
Kulkovia fulva (Misaki 2)	LC553782	LC553894	LC553806	LC553818	LC553830	present study
Kulkovia fulva (Misaki 3)	LC553783	LC553895	LC553807	LC553819	LC553831	present study
Kulkovia fulva (Misaki 4)	LC553784	LC553896	LC553808	LC553820	LC553832	present study
Kulkovia fulva (Misaki 5)	LC553785	LC553897	LC553809	LC553821	LC553833	present study
Kulkovia fulva (Misaki 6)	LC553786	LC553898	LC553810	LC553822	LC553834	present study
Kulkovia fulva (Misaki 7)	LC553787	LC553899	LC553811	LC553823	LC553835	present study
Kulkovia manchenkoi (Kholmsk)	KU821497	KY468934	KU856671	KU821523	KU821546	Chernyshev et al. (2018)
Kulkovia manchenkoi (Akkeshi 1)	KU821492	KY468934	KU856678	KU821518	KU821541	Chernyshev et al. (2018)
Kulkovia manchenkoi (Akkeshi 2)	JF277572	—	—	HQ848574	—	Andrade et al. (2012)
Kulkovia montgomeryi (San Juan Island)	EF128475	—	EF178489	EF124966	—	Schwartz and Norenburg (unpubl.)
Kulkovia torquata (Urup Island)	KU821486	KY468935	KU856673	KU821511	KU821534	Chernyshev et al. (2018)
Lineus acutifrons	JF277573	JF304778J	HQ856855	GU590937	JF277727	Andrade et al. (2012)
Lineus lacteus	JF277584	JF293065	HQ856850	HQ848583	JF277725	Andrade et al. (2012)
Lineus logissimus	[1]AJ436825	[2]KY468932	[2]AJ436880	[2]AJ436935	[2]KY606234	[1]Tholleson and Norenburg (2003); [2]Chernyshev et al. (2018)
Lineus ruber	[1]KM878509	[2]KY468933	[2]KY468929	[2]KM878473	[2]KY606235	[1]Krämer et al. (2017); [2]Chernyshev et al. (2018)
Lineus sanguineus	KF935468	KF935301	HQ856854	KF935518	KF935413	Kvist et al. (2014)
Lineus viridis	JF277582	JF293032	—	HQ848579	JF277719	Andrade et al. (2012)
Micrura chlorapardalis	KF935459	KF935292	KF935348	KF935459	KF935404	Kvist et al. (2014)
Micrura dellechiajei	KF935461	KF935294	KF935350	KF935514	KF935406	Kvist et al. (2014)
Micrura fasciolata	JF277585	JF293038	HQ856846	HQ848578	JF277721	Andrade et al. (2012)
Micrura ignea	JF277588	JF293043	HQ856859	HQ848587	JF277734	Andrade et al. (2012)
Micrura purprea	JF277577	JF293036	HQ856845	HQ848586	JF277726	Andrade et al. (2012)
Micrura rubramaculosa	KF935460	KF935293	KF935349	KF935513	KF935405	Kvist et al. (2014)
Notospermus geniculatus	[1]KF935462	[1]KF935295	[1]KF935351	[2]AJ436934	[1]KF935407	[1]Kvist et al. (2014); [2]Tholleson and Norenburg (2003)
Parborlasia corrugata	[1]JF277578	[1]JF293037	[1]HQ856851	[2]EU194826	[1]JF277732	[1]Andrade et al. (2012); [2]Thornhill et al. (2008)
Siphonenteron bilineatum	[1]KX434772	[2]JF293041	[1]KY468931	[1]KY561816	[2]JF277731	[1]Chernyshev et al. (2018); [2]Andrade et al. (2012)

Table 1-1. List of GenBank accession numbers for sequences used in our phylogenetic analysis.

	Kulikovia alborostrata		*Kulikovia fulva*	
	Misaki	Vostok Bay	Misaki	Oshoro
	1	2	3	4
1	0.0–0.2 0.1–0.5	–	–	–
2	0.0–0.3 0.4–0.7	0.0–0.5 0.4–1.1	–	–
3	2.8–4.7 14.4–17.2	2.9–4.8 14.4–17.3	0.5–1.0 1.6–2.4	–
4	3.1–5.3 NA	3.1–5.4 NA	0.6–1.5 NA	NA NA

Table 1-2. Uncorrected p-distance (%) for 16S (upper) and COI (lower) among and between *K. alborostrata* from Misaki (n = 5) and Vostok Bay (n = 2); *K. fulva* from Misaki (n = 7) and Oshoro (n = 1).

Part II

The species-recognition system in gametes of *K. alborostrata* and *K. fulva*

本章は 5 年以内に雑誌等で刊行予定のため、非公表

Part III

Exploration for the genes for the species-recognition among *Kulikovia* species

本章は 5 年以内に雑誌等で刊行予定のため、非公表

General Discussion

本章は 5 年以内に雑誌等で刊行予定の内容と

関連しているため、非公表

Conclusion and Perspectives

本章は 5 年以内に雑誌等で刊行予定の内容と

関連しているため、非公表

Acknowledgement

I am very grateful to my supervisor Dr. Manabu Yoshida (The University of Tokyo) and Dr. Kaoru Yoshida (Toin University of Yokohama) for teaching and supporting me throughout my doctoral course. I am also grateful to Dr. Hiroyuki Takeda (The University of Tokyo), Dr. Takema Fukatsu (National Institute of Advanced Industrial Science and Technology; AIST) and Dr. Tetsuya Higashiyama (The university of Tokyo) for reviewing my doctoral dissertation. I express my gratitude to Dr. Hiroshi Kajihara (Hokkaido University) for joining the study and helping me beside reviewing my doctoral dissertation.

I also express my gratitude to Dr. Toru Miura (The University of Tokyo), Dr. Daisuke Kurokawa (The University of Tokyo) and Dr. Masanori Okanishi (The University of Tokyo) for advising me throughout my doctoral course and supporting me in my experiments; to Mr. Mamoru Sekifuji, Mr. Hisanori Kohtsuka, Ms. Michiyo Kawabata and Miho Kyokuwa, the technical staffs in Misaki Marine Biological Station (MMBS), for helping me collect the samples; to Mr. Hisashi Maruya, Ms. Izumi Komori, Mr. Hiroshi Ishino and Ms. Kiyoko Ohtake in the office of MMBS for helping me live there; to Dr. Satoe Aratake, Mr. Makoto Nozawa and Ms. Megumi Kohtsuka for helping me study in MMBS; to Ms. Takako Miyagawa, Ms. Akiko Goto, Mr. Shinobu Iijima, Mr. Shinji Kiyohashi, Mr. Yusuke Ohtsu, Mr. Makoto Ohkubo for helping me live in MMBS; to Mr. Ryohei Taki for discussing with me; to Dr. Jumpei Shinji (MMBS), Dr. Kohei Oguchi (AIST), Dr. Yumiko Togawa (AIST), Dr.

Akari Okada, Mr. Sumio Udagawa, Ms. Mayuko Nakamura, Ms. Natsumi Hookabe, Mr. Ryosuke Kimbara, Ms. Haruka Yamaguchi, Mr. Yohei Ohtomo, Mr. Naoto Inui, Mr. Akifumi Yao, Ms. Hana Tamura, Mr. Daisuke Sato, Mr. Kento Watanabe, Mr. Kenta Kobayashi, Mr. Scott Walker and Ms. Rupandey Parekh for helping me in MMBS.

Lastly, I deeply express my gratitude to my grandparents and parents for supporting me all the time.

References

Aagaard, J. E., Vacquier, V. D., MacCoss, M. J., & Swanson, W. J. (2010). ZP domain proteins in the abalone egg coat include a paralog of VERL under positive selection that binds lysin and 18-kDa sperm proteins. *Molecular biology and evolution*, 27(1), 193-203.

Acevedo-Duncan, M., & Carroll Jr, E. J. (1986). Immunological evidence that a 305-kilodalton vitelline envelope polypeptide isolated from sea urchin eggs is a sperm receptor. *Gamete research*, 15(4), 337-359.

Andrade, S. C., Strand, M., Schwartz, M., Chen, H., Kajihara, H., von Doehren, J., ... & Sundberg, P. (2012). Disentangling ribbon worm relationships: multi-locus analysis supports traditional classification of the phylum Nemertea. *Cladistics*, 28(2), 141-159.

Avella, M. A., Xiong, B., & Dean, J. (2013). The molecular basis of gamete recognition in mice and humans. MHR: *Basic science of reproductive medicine*, 19(5), 279-289.

Avella, M. A., Baibakov, B., & Dean, J. (2014). A single domain of the ZP2 zona pellucida protein mediates gamete recognition in mice and humans. *Journal of Cell Biology*, 205(6), 801-809.

Blanc, G., Font, B., Eichenberger, D., Moreau, C., Ricard-Blum, S., Hulmes, D. J., & Moali, C. (2007). Insights into how CUB domains can exert specific functions while sharing a common fold: conserved and specific features of the CUB1 domain contribute to the molecular basis of procollagen C-proteinase enhancer-1 activity. *Journal of Biological Chemistry*, 282(23), 16924-16933.

Ban, S., Harada, Y., Yokosawa, H., & Sawada, H. (2005). Highly polymorphic vitelline-coat protein HaVC80 from the ascidian, *Halocynthia aurantium*: structural analysis and involvement in self/nonself recognition during fertilization. *Developmental biology*, 286(2), 440-451.

Baibakov, B., Boggs, N. A., Yauger, B., Baibakov, G., & Dean, J. (2012). Human sperm bind to the N-terminal domain of ZP2 in humanized zonae pellucidae in transgenic mice. *Journal of Cell Biology*, 197(7), 897-905.

Bianchi, E., & Wright, G. J. (2015). Cross-species fertilization: the hamster egg receptor, Juno, binds the human sperm ligand, Izumo1. *Philosophical transactions of the Royal Society B: biological sciences*, 370(1661), 20140101.

Biermann, C. H. (1998). The molecular evolution of sperm bindin in six species of sea urchins

(Echinoida: Strongylocentrotidae). *Molecular Biology and Evolution*, 15(12), 1761-1771.

Calderón, I., Turon, X., & Lessios, H. A. (2009). Characterization of the sperm molecule bindin in the sea urchin genus Paracentrotus. *Journal of molecular evolution*, 68(4), 366-376.

Castresana, J. (2000). Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. *Molecular biology and evolution*, 17(4), 540-552.

Chandler, D. E., & Heuser, J. (1980). The vitelline layer of the sea urchin egg and its modification during fertilization. A freeze-fracture study using quick-freezing and deep-etching. *The Journal of cell biology*, 84(3), 618-632.

Chernyshev, A. V., Polyakova, N. E., Turanov, S. V., & Kajihara, H. (2018). Taxonomy and phylogeny of Lineus torquatus and allies (Nemertea, Lineidae) with descriptions of a new genus and a new cryptic species. *Systematics and Biodiversity*, 16(1), 55-68.

Chernyshev, A. V., Neznanova, S. Y., & Yurchenko, O. V. (2020). Spermatozoa ultrastructure of two basal pilidiophoran nemerteans, *Hubrechtella juliae* and *Sonnenemertes cantelli* (Nemertea,

Pilidiophora). *Micron*, 133, 102853.

Coll, J. C., Bowden, B. F., Meehan, G. V., Konig, G. M., Carroll, A. R., Tapiolas, D. M., ... & Miller, R. L. (1994). Chemical aspects of mass spawning in corals. I. Sperm-attractant molecules in the eggs of the scleractinian coral *Montipora digitata*. *Marine Biology*, 118(2), 177-182.

Coll, J. C., Leone, P. A., Bowden, B. F., Carroll, A. R., König, G. M., Heaton, A., ... & Alderslade, P. N. (1995). Chemical aspects of mass spawning in corals. II. (-)-Epi-thunbergol, the sperm attractant in the eggs of the soft coral *Lobophytum crassum* (Cnidaria: Octocorallia). *Marine Biology*, 123(1), 137-143.

Colgan, D. J., McLauchlan, A., Wilson, G. D. F., Livingston, S. P., Edgecombe, G. D., Macaranas, J., ... & Gray, M. R. (1998). Histone H3 and U2 snRNA DNA sequences and arthropod molecular evolution. *Australian Journal of Zoology*, 46(5), 419-437.

Crandall, M. C. D. P. K., Clement, M., & Posada, D. (2000). TCS: a computer program to estimate gene genealogies. *Molecular ecology*, 9, 1657-1660.

Dan, J. C. 1950. Fertilization in the medusan, *Spirocodon saltatrix*. *The Biological Bulletin*. 99: 412–

415.

von Döhren, J., & Bartolomaeus, T. (2006). Ultrastructure of sperm and male reproductive system in *Lineus viridis* (Heteronemertea, Nemertea). *Zoomorphology*, 125(4), 175-185.

von Döhren, J., Beckers, P., Vogeler, R., & Bartolomaeus, T. (2010). Comparative sperm ultrastructure in Nemertea. *Journal of morphology*, 271(7), 793-813.

Duckert, P., Brunak, S., & Blom, N. (2004). Prediction of proprotein convertase cleavage sites. *Protein Engineering Design and Selection*, 17(1), 107-112.

Folmer, O., Black, M., Hoeh, W., Lutz, R., Vrijenhoek, R. (1994). DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Molecular marine biology and biotechnology,* 3: 294–299

Foltz, K. R., & Lennarz, W. J. (1990). Purification and characterization of an extracellular fragment of the sea urchin egg receptor for sperm. *Journal of Cell Biology*, 111(6), 2951-2959.

Galindo, B. E., Moy, G. W., Swanson, W. J., & Vacquier, V. D. (2002). Full-length sequence of VERL,

the egg vitelline envelope receptor for abalone sperm lysin. *Gene*, 288(1-2), 111-117.

Galindo, B. E., Vacquier, V. D., & Swanson, W. J. (2003). Positive selection in the egg receptor for abalone sperm lysin. *Proceedings of the National Academy of Sciences*, 100(8), 4639-4643.

Gibson, R. (1995). Nemertean genera and species of the world: an annotated checklist of original names and description citations, synonyms, current taxonomic status, habitats and recorded zoogeographic distribution. *Journal of Natural History*, 29(2), 271-561.

Glabe, C. G., & Vacquier, V. D. (1978). Egg surface glycoprotein receptor for sea urchin sperm bindin. *Proceedings of the National Academy of Sciences*, 75(2), 881-885.

Glabe, C. G., & Lennarz, W. J. (1979). Species-specific sperm adhesion in sea urchins. A quantitative investigation of bindin-mediated egg agglutination. *The Journal of cell biology*, 83(3), 595-604.

Gouy, M., Guindon, S., & Gascuel, O. (2010). SeaView version 4: a multiplatform graphical user interface for sequence alignment and phylogenetic tree building. *Molecular biology and evolution*, 27(2), 221-224.

Gregory, L. A., Thielens, N. M., Matsushita, M., Sorensen, R., Arlaud, G. J., Fontecilla-Camps, J. C., & Gaboriaud, C. (2004). The X-ray structure of human mannan-binding lectin-associated protein 19 (MAp19) and its interaction site with mannan-binding lectin and L-ficolin. *Journal of Biological Chemistry*, 279(28), 29391-29397.

Guerrero, A., Nishigaki, T., Carneiro, J., Tatsu, Y., Wood, C. D., & Darszon, A. (2010). Tuning sperm chemotaxis by calcium burst timing. *Developmental biology*, 344(1), 52-65.

Han, L., Monné, M., Okumura, H., Schwend, T., Cherry, A. L., Flot, D., ... & Jovine, L. (2010). Insights into egg coat assembly and egg-sperm interaction from the X-ray structure of full-length ZP3. *Cell*, 143(3), 404-415.

Herberg, S., Gert, K. R., Schleiffer, A., & Pauli, A. (2018). The Ly6/uPAR protein Bouncer is necessary and sufficient for species-specific fertilization. *Science*, 361(6406), 1029-1033.

Hiebert, T. C., & Maslakova, S. (2015). Integrative taxonomy of the *Micrura alaskensis* Coe, 1901 species complex (Nemertea: Heteronemertea), with descriptions of a new genus Maculaura gen. nov. and four new species from the NE Pacific. *Zoological Science*, 32(6), 615-637.

Hiebert, T. C., & Maslakova, S. A. (2015). Larval Development of Two NE Pacific Pilidiophoran Nemerteans (Heteronemertea; Lineidae). *The Biological Bulletin*, 229(3), 265-275.

Hubank, M., & Schatz, D. G. (1994). Identifying differences in mRNA expression by representational difference analysis of cDNA. *Nucleic acids research*, 22(25), 5640-5648.

Ikenaga, J, Yoshida, M. (2020). Sperm activation and chemotaxis of invertebrates. in "Reproduction in Aquatic Animals: From Basic Biology to Aquaculture Technology" (Eds. Yoshida, M. and Asturiano, J.), *Springer-Nature*, pp. 31-46.

Inoue, N., Ikawa, M., Isotani, A., & Okabe, M. (2005). The immunoglobulin superfamily protein Izumo is required for sperm to fuse with eggs. *Nature*, 434(7030), 234-238.

Inoue, T. (2018). TI Workbench, an integrated software package for electrophysiology and imaging. *Microscopy*, 67(3), 129-143.

Iwata, F. (1954) The fauna of Akkeshi Bay: XX. Nemertini in Hokkaido. *Journal of the Faculty of Science, Hokkaido University*, Series VI Zool 12: 1–39

Iwata F (1965) *Lineus alborostratus*. In “New Illustrated Encyclopedia of the Fauna of Japan, Vol 1” Ed by Y Okada, S Uchida, T Uchida, Hokuryu-kan, Tokyo, p 393

Kajihara, H. (2004). Usamaro Takakura (1867–1944), Japanese pioneer nemertean researcher. *Archives of natural history*, 31(2), 208-213.

Kajihara, H., Chernyshev, A. V., Sun, S. C., Sundberg, P., & Crandall, F. B. (2008). Checklist of nemertean genera and species published between 1995 and 2007. *Species Diversity*, 13(4), 245-274.

Kamei, N., & Glabe, C. G. (2003). The species-specific egg receptor for sea urchin sperm adhesion is EBR1, a novel ADAMTS protein. *Genes & Development*, 17(20), 2502-2507.

Kato, K., Satouh, Y., Nishimasu, H., Kurabayashi, A., Morita, J., Fujihara, Y., ... & Nureki, O. (2016). Structural and functional insights into IZUMO1 recognition by JUNO in mammalian fertilization. *Nature communications*, 7(1), 1-9.

Katoh, K., & Standley, D. M. (2013). MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Molecular biology and evolution*, 30(4), 772-780.

Kiefer, S. M., & Saling, P. (2002). Proteolytic processing of human zona pellucida proteins. *Biology of reproduction*, 66(2), 407-414.

Krämer, D., Schmidt, C., Podsiadlowski, L., Beckers, P., Horn, L., & von Döhren, J. (2017). Unravelling the *Lineus ruber/viridis* species complex (Nemertea, Heteronemertea). *Zoologica Scripta*, 46(1), 111-126.

Kresge, N., Vacquier, V. D., & Stout, C. D. (2000). The high resolution crystal structure of green abalone sperm lysin: implications for species-specific binding of the egg receptor. *Journal of molecular biology*, 296(5), 1225-1234.

Kumar, S., Stecher, G., Li, M., Knyaz, C., & Tamura, K. (2018). MEGA X: molecular evolutionary genetics analysis across computing platforms. *Molecular biology and evolution*, 35(6), 1547.

Kvist, S., Laumer, C. E., Junoy, J., & Giribet, G. (2014). New insights into the phylogeny, systematics and DNA barcoding of Nemertea. *Invertebrate systematics*, 28(3), 287-308.

Litscher, E.S. and Wassarman, P. M. (2007) Egg extracellular coat proteins: from fish to mammals.

Histology and Histopathology, 22, 337-347.

Litscher, E. S., Williams, Z., & Wassarman, P. M. (2009). Zona pellucida glycoprotein ZP3 and fertilization in mammals. *Molecular Reproduction and Development*: Incorporating Gamete Research, 76(10), 933-941.

Lanfear, R., Frandsen, P. B., Wright, A. M., Senfeld, T., & Calcott, B. (2017). PartitionFinder 2: new methods for selecting partitioned models of evolution for molecular and morphological phylogenetic analyses. *Molecular biology and evolution*, 34(3), 772-773.

Langhans, S.A. (2018) Epidermal Growth Factor (EGF). In Encyclopedia of Signaling Molecules. Springer (Eds. Choi, S.), Cham. https://doi.org/10.1007/978-3-319-67199-4_101919

Le, H. L. V., Lecointre, G., & Perasso, R. (1993). A 28s based phylogeny of the Gnathostomes: first steps in the analysis of conflict and congruence with morphologically based cladograms. *Molecular Phylogenetics and Evolution*, 2, 31-51.

Lee, Y. H., & Vacquier, V. D. (1992). The divergence of species-specific abalone sperm lysins is

promoted by positive Darwinian selection. *The Biological Bulletin*, 182(1), 97-104.

Letunic, I., Khedkar, S., & Bork, P. (2021). SMART: recent updates, new developments and status in 2020. *Nucleic acids research*, 49(D1), D458-D460.

Luo, Y. J., Kanda, M., Koyanagi, R., Hisata, K., Akiyama, T., Sakamoto, H., ... & Satoh, N. (2018). Nemertean and phoronid genomes reveal lophotrochozoan evolution and the origin of bilaterian heads. *Nature ecology & evolution*, 2(1), 141-151.

Matsumori, N., Y. Hiradate, H. Shibata, T. Oishi, S. Simma, M. Toyoda, F. Hayashi, M. Yoshida, M. Murata, & M. Morisawa. 2013. A novel sperm-activating and attracting factor from the ascidian *Ascidia sydneiensis*. *Organic Letters* 15: 294–297.

Metz, E. C., & Palumbi, S. R. (1996). Positive selection and sequence rearrangements generate extensive polymorphism in the gamete recognition protein bindin. *Molecular Biology and Evolution*, 13(2), 397-406.

Miller, R. L. (1966). Chemotaxis during fertilization in the hydroid Campanularia. *Journal of*

Experimental Zoology, 162(1), 23-44.

Miller, R. L. (1977). Chemotactic behavior of the sperm of chitons (Mollusca: Polyplacophora). *Journal of Experimental Zoology*, 202(2), 203-211.

Miller, R. L. (1982). Sperm chemotaxis in ascidians. *American Zoologist*, 22(4), 827-840.

Miller, R. L. (1985). Sperm chemo-orientation in the metazoa. *Biology of fertilization*, 2, 275-337.

Miller, R. L. (1985). Demonstration of sperm chemotaxis in echinodermata: Asteroidea, Holothuroidea, Ophiuroidea. *Journal of Experimental Zoology*, 234(3), 383-414.

Moore, J., Gibson, R., & Jones, H. D. (2001). Terrestrial nemerteans thirty years on. *Hydrobiologia*, 456(1), 1-6.

Nguyen, L. T., Schmidt, H. A., Von Haeseler, A., & Minh, B. Q. (2015). IQ-TREE: a fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. *Molecular biology and evolution*, 32(1), 268-274.

Ohlendieck, K., Dhume, S. T., Partin, J. S., & Lennarz, W. J. (1993). The sea urchin egg receptor for sperm: isolation and characterization of the intact, biologically active receptor. *The Journal of cell biology*, 122(4), 887-895.

Palumbi, S., Martin, A., Romano, S., McMillan, W. O., Stice, L., Grabowski, G. (1991). The simple fool's guide to PCR, version 2.0. *Department of Zoology and Kewalo Mar Lab, University of Hawaii*

Posch, S., Obser, T., König, G., Schneppenheim, R., Tampé, R., & Hinterdorfer, P. (2018). Interaction of von Willebrand factor domains with collagen investigated by single molecule force spectroscopy. *The Journal of chemical physics*, 148(12), 123310.

Puillandre, N., Lambert, A., Brouillet, S., & Achaz, G. J. M. E. (2012). ABGD, Automatic Barcode Gap Discovery for primary species delimitation. *Molecular ecology*, 21(8), 1864-1877.

Quinn, P. (1979). Failure of human spermatozoa to penetrate zona free mouse and rat ova in vitro. *Journal of Experimental Zoology*, 210(3), 497-505.

Raj, I., Al Hosseini, H. S., Dioguardi, E., Nishimura, K., Han, L., Villa, A., ... & Jovine, L. (2017).

Structural basis of egg coat-sperm recognition at fertilization. *Cell*, 169(7), 1315-1326.

Rebay, I., Fleming, R. J., Fehon, R. G., Cherbas, L., Cherbas, P., & Artavanis-Tsakonas, S. (1991). Specific EGF repeats of Notch mediate interactions with Delta and Serrate: implications for Notch as a multifunctional receptor. *Cell*, 67(4), 687-699.

Ronquist, F., Teslenko, M., Van Der Mark, P., Ayres, D. L., Darling, A., Höhna, S., ... & Huelsenbeck, J. P. (2012). MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. *Systematic biology*, 61(3), 539-542.

Sawada, H., Tanaka, E., Ban, S., Yamasaki, C., Fujino, J., Ooura, K., ... & Yokosawa, H. (2004). Self/nonself recognition in ascidian fertilization: vitelline coat protein HrVC70 is a candidate allorecognition molecule. *Proceedings of the National Academy of Sciences*, 101(44), 15615-15620.

Schwartz, M. (2009) Untying a Gordian knot of worms: Systematics and taxonomy of the Pilidiophora (phylum Nemertea) from multiple data sets. PhD thesis, The George Washington University, Washington DC

SeGall, G. K., & Lennarz, W. J. (1979). Chemical characterization of the component of the jelly coat from sea urchin eggs responsible for induction of the acrosome reaction. *Developmental biology*, 71(1), 33-48.

Stamatakis, A. (2014). RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics*, 30(9), 1312-1313.

Strand, M., & Sundberg, P. (2011). A DNA-based description of a new nemertean (phylum Nemertea) species. *Marine Biology Research*, 7(1), 63-70.

Strand, M., & Sundberg, P. (2015). Phylum Nemertea. In Thorp and Covich's Freshwater Invertebrates (pp. 205-209). Academic Press.

Stricker, S. A. (1996). Repetitive calcium waves induced by fertilization in the nemertean wormCerebratulus lacteus. *Developmental biology*, 176(2), 243-263.

Stricker, S. A. (1997). Intracellular injections of a soluble sperm factor trigger calcium oscillations and meiotic maturation in unfertilized oocytes of a marine worm. *Developmental biology*, 186(2), 185-

201.

Stricker, S. A., Smythe, T. L., Miller, L., & Norenburg, J. L. (2001). Comparative biology of oogenesis in nemertean worms. *Acta Zoologica*, 82(3), 213-230.

Stricker, S. A., Cline, C., & Goodrich, D. (2013). Oocyte maturation and fertilization in marine nemertean worms: using similar sorts of signaling pathways as in mammals, but often with differing results. *The Biological Bulletin*, 224(3), 137-155.

Stricker, S. A. (2014). Calcium signaling and endoplasmic reticulum dynamics during fertilization in marine protostome worms belonging to the phylum Nemertea. *Biochemical and biophysical research communications*, 450(3), 1182-1187.

Suda, K., Matsumoto, Y. (2015) Observation of Lineus alborostratus (Nemertea: Pilidiophora) in Oshoro Bay, Hokkaido, Japan. *Report of Systematic Zoology Lab Practicum* 6: e 15

Sundberg, P. (2015). Thirty-five years of nemertean (Nemertea) research—past, present, and future. *Zoological science*, 32(6), 501-506.

Suzuki, N. (1995). Structure, function and biosynthesis of sperm-activating peptides and fucose sulfate glycoconjugate in the extracellular coat of sea urchin eggs. *Zoological science*, 12(1), 13-27.

Swanson, W. J., & Vacquier, V. D. (1997). The abalone egg vitelline envelope receptor for sperm lysin is a giant multivalent molecule. *Proceedings of the National Academy of Sciences*, 94(13), 6724-6729.

Takakura, U. (1898). Classification of Nemertini in the vicinity of Misaki. *Zoological Magazine*, 10.

Tardif, S., Wilson, M. D., Wagner, R., Hunt, P., Gertsenstein, M., Nagy, A., ... & Hardy, D. M. (2010). Zonadhesin is essential for species specificity of sperm adhesion to the egg zona pellucida. *Journal of Biological Chemistry*, 285(32), 24863-24870.

Templeton, A. R., Crandall, K. A., & Sing, C. F. (1992). A cladistic analysis of phenotypic associations with haplotypes inferred from restriction endonuclease mapping and DNA sequence data. III. Cladogram estimation. *Genetics*, 132(2), 619-633.

Teufel, F., Almagro Armenteros, J. J., Johansen, A. R., Gíslason, M. H., Pihl, S. I., Tsirigos, K. D., ... & Nielsen, H. (2022). SignalP 6.0 predicts all five types of signal peptides using protein language

models. *Nature Biotechnology*, 1-3.

Thiel, M., & Junoy, J. (2006). Mating behavior of nemerteans: present knowledge and future directions. *Journal of Natural History*, 40(15-16), 1021-1034.

Thollesson, M., & Norenburg, J. L. (2003). Ribbon worm relationships: a phylogeny of the phylum Nemertea. Proceedings of the Royal Society of London. Series B: *Biological Sciences*, 270(1513), 407-415.

Thornhill, D. J., Mahon, A. R., Norenburg, J. L., & Halanych, K. M. (2008). Open-ocean barriers to dispersal: A test case with the Antarctic Polar Front and the ribbon worm *Parborlasia corrugatus* (Nemertea: Lineidae). *Molecular ecology*, 17(23), 5104-5117.

Tian, J., Gong, H., Thomsen, G. H., & Lennarz, W. J. (1997). Gamete interactions in *Xenopus laevis*: identification of sperm binding glycoproteins in the egg vitelline envelope. *The Journal of cell biology*, 136(5), 1099-1108.

Tulchinsky, A. Y., Norenburg, J. L., & Turbeville, J. M. (2012). Phylogeography of the marine

interstitial nemertean *Ototyphlonemertes parmula* (Nemertea, Hoplonemertea) reveals cryptic diversity and high dispersal potential. *Marine Biology*, 159(3), 661-674.

Vacquier, V. D., Carner, K. R., & Stout, C. D. (1990). Species-specific sequences of abalone lysin, the sperm protein that creates a hole in the egg envelope. *Proceedings of the National Academy of Sciences*, 87(15), 5792-5796.

Vacquier, V. D., Stout, C. D. and Swanson, W. J. (2006) The evolution of gamete-recognition proteins as a factor of speciation. In “Spermatology new edition” (Eds. Morisawa, M., Hoshi, K. and Okabe M.), University of Tokyo Press, pp. 272-295.

Vacquier, V. D., & Moy, G. W. (1997). The fucose sulfate polymer of egg jelly binds to sperm REJ and is the inducer of the sea urchin sperm acrosome reaction. *Developmental biology*, 192(1), 125-135.

Verdes, A., Arias, M. B., Junoy, J., Schwartz, M. L., & Kajihara, H. (2021). Species delimitation and phylogenetic analyses reveal cryptic diversity within *Cerebratulus marginatus* (Nemertea: Pilidiophora). *Systematics and Biodiversity*, 1-11.

Vilela-Silva, A. C. E., Castro, M. O., Valente, A. P., Biermann, C. H., & Mourão, P. A. (2002). Sulfated fucans from the egg jellies of the closely related sea urchins *Strongylocentrotus droebachiensis* and *Strongylocentrotus pallidus* ensure species-specific fertilization. *Journal of Biological Chemistry*, 277(1), 379-387.

Vo, L. H., & Hedrick, J. L. (2000). Independent and hetero-oligomeric-dependent sperm binding to egg envelope glycoprotein ZPC in *Xenopus laevis*. *Biology of reproduction*, 62(3), 766-774.

Ward, G. E., Brokaw, C. J., Garbers, D. L., & Vacquier, V. D. (1985). Chemotaxis of *Arbacia punctulata* spermatozoa to resact, a peptide from the egg jelly layer. *The Journal of cell biology*, 101(6), 2324-2329.

Wouters, M. A., Rigoutsos, I., Chu, C. K., Feng, L. L., Sparrow, D. B., & Dunwoodie, S. L. (2005). Evolution of distinct EGF domains with specific functions. *Protein Science*, 14(4), 1091-1103.

Yoshida, K., Shiba, K., Sakamoto, A., Ikenaga, J., Matsunaga, S., Inaba, K., & Yoshida, M. (2018). Ca^{2+} efflux via plasma membrane Ca^{2+}-ATPase mediates chemotaxis in ascidian sperm. *Scientific reports*, 8(1), 1-16.

Yoshida, M., Sensui, N., Inoue, T., Morisawa, M., & Mikoshiba, K. (1998). Role of two series of Ca^{2+} oscillations in activation of ascidian eggs. *Developmental biology*, 203(1), 122-133.

Yoshida, M., Murata, M., Inaba, K., & Morisawa, M. (2002). A chemoattractant for ascidian spermatozoa is a sulfated steroid. *Proceedings of the National Academy of Sciences*, 99(23), 14831-14836.

Yoshida, M., Hiradate, Y., Sensui, N., Cosson, J., & Morisawa, M. (2013). Species-specificity of sperm motility activation and chemotaxis: a study on ascidian species. *The Biological Bulletin*, 224(3), 156-165.

Yoshida, M., & Yoshida, K. (2011). Sperm chemotaxis and regulation of flagellar movement by Ca^{2+}. MHR: *Basic science of reproductive medicine*, 17(8), 457-465.

Zatylny, C., Marvin, L., Gagnon, J., & Henry, J. (2002). Fertilization in Sepia officinalis: the first mollusk sperm-attracting peptide. *Biochemical and biophysical research communications*, 296(5), 1186-1193.

Zhang, J., Kapli, P., Pavlidis, P., & Stamatakis, A. (2013). A general species delimitation method with applications to phylogenetic placements. *Bioinformatics*, 29(22), 2869-2876.