Elucidation of reproductive behaviors of inshore and oceanic squids using molecular and anatomical techniques

（分子生物学及び形態学手法を用いた沿岸性及び外洋性イカ類の繁殖行動の解明）

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Elucidation of reproductive behaviors of inshore and oceanic squids using molecular and anatomical techniques

by

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Author declaration

I, the undersigned **AZAD KAMRUN NAHER,** hereby declare that I am the sole author of this thesis. To the best of my knowledge, this thesis does not contain any previously published content except where due acknowledgment has been made. Any content, in English or any other language, which has been accepted as part of the requirements of any other academic degree or non-degree program is not included in this thesis.

Date: January 17, 2025

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Signature: _____

ABBREVIATIONS

ABI	Applied Biosystems Instrument
ACC	Accessory gland weight
ARM	Basal left IV arm
BM	Buccal membrane
BW	Body weight
COI	Cytochrome c oxidase subunit I
EPC	Extra pair copulation
EYE	Lateral head behind the left eye
FLA	Fragment Length Analysis
gDNA	Genomic DNA
GLM	Generalized Linear Model
GLMM	Generalized Linear Mixed Model
GSI	Gonadosomatic index
LMM	Linear Mixed Model
ML	Mantle length
NCBI	National Centre for Biotechnology Information
NIH	National Institute of Health
OSI	Ovarian somatic index
OW	Ovary weight
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
ROW	Relative ovary weight
RTW	Relative testis weight
SITE	Number of insemination sites used
SR	Seminal receptacle
SSO	Sperm storage organ
SSR	Simple sequence repeat
TSI	Testicular somatic index
TW	Testis weight
µg/l	Microgram per liter
µM	Micromolar

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CHAPTER 1

GENERAL INTRODUCTION

1.1. Squid

Squids are mollusks with an elongated soft body, large eyes, eight arms, and two long tentacles in the order Teuthoidea (or Teuthida) under Cephalopoda class and found in both inshore and oceanic waters. They are bilaterally symmetrical animals with a well-developed head and a body that consists of the mantle, the mantle cavity that houses the internal organs, and the external fins. The head bears an anterior circum-oral (surrounding the mouth) crown of mobile appendages (arms, tentacles). Arms and tentacles bear suckers with/without hooks, which are powerful tools for seizing prey (Fig. 1.1). The mouth, at the interior base of the arm crown, has a pair of chitinous jaws (the beaks) and, as in other mollusks, a chitinous tongue-like radula (band of teeth). The body of most squids is strengthened by a feathery-shaped internal shell composed of a horny material (Roper et al., 1984; Jereb and Roper, 2010). Squids are swift swimmers or part of the drifting sea life (Jabr, 2010).

The size of adult squid ranges from less than 10 mm mantle length (e.g. the smallest species are probably the benthic pygmy squids *Idiosepius*) to the giant squid *Architeuthis* sp. and the colossal squid *Mesonychoteuthis hamiltoni*, at well over 2 m mantle length (Xavier et al., 1999; O'Shea, 2003; Joe, 2017). The largest specimens may weigh over 500 kg. However, the average size of commercial species is 200 to 400 mm mantle length and about 0.1 to 2.0 kg total weight (Jereb and Roper, 2010).

Squids are used for human consumption with commercial fisheries in Japan, the Mediterranean, the southwestern Atlantic, the eastern Pacific and elsewhere. They are used in cuisines all over the world, known by many as calamari (Davidson, 2014).

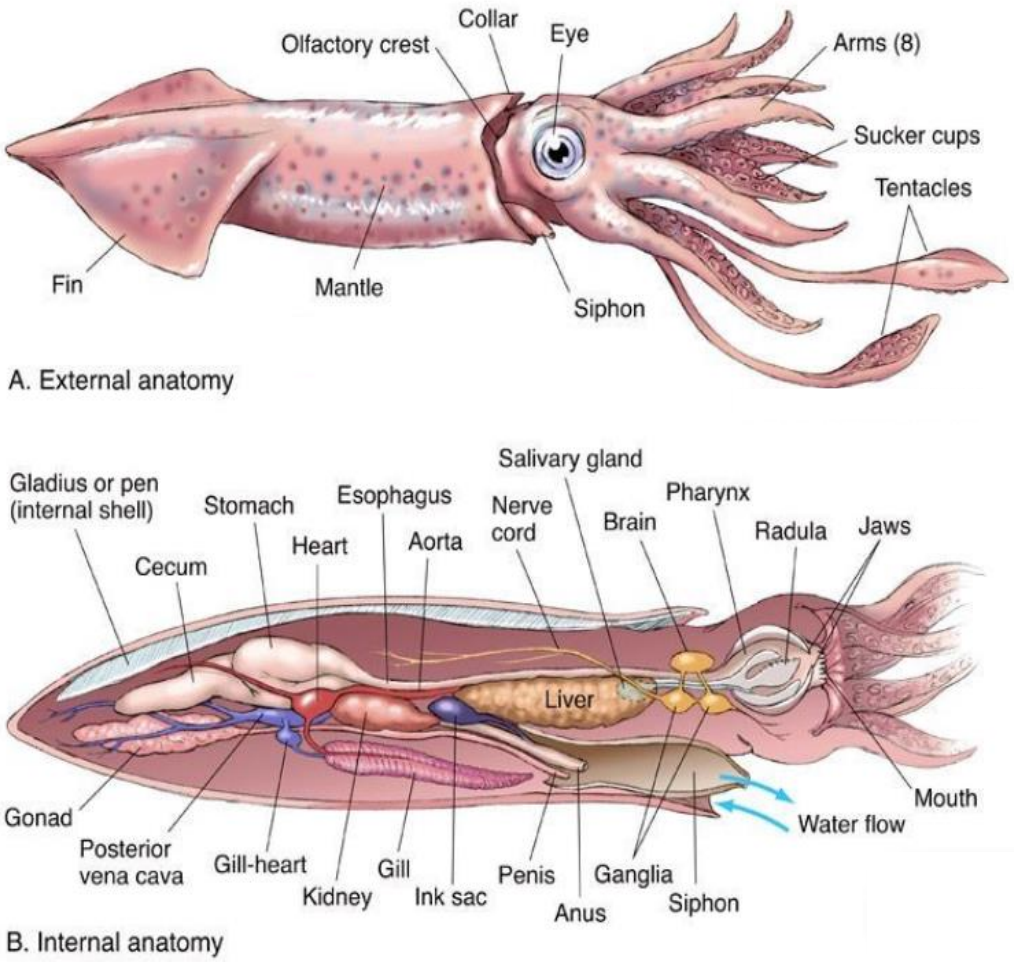

Fig. 1.1. External and internal anatomical views of a squid (RiNee, 2020).

1.2. Two major groups of squids

There are two main groups of squids, coming from two suborders of Teuthida order (squids). One is ‘Myopsid squid’, which are inshore, mostly demersal, covered-eyed squids, typically confined to shallower waters near shores or on continental shelves. The other one is ‘Oegopsid squid’, which are oceanic, mainly pelagic, open-eyed squids, generally found in deep oceanic waters, although these may come on to the continental shelves to spawn (Jan et al., 2009; Jereb and Roper, 2010).

1.2.1. Myopsid (inshore) squids

Members of Myopsida are distinguished from those of its sister group, Oegopsida, by a variety of morphological traits (Fig. 1.2). The shape of the eyes is one of the most evident differences: myopsid squids' eyes are covered by a transparent corneal membrane, the opening of which is typically limited to a minute anterior pore. They also lack a secondary eyelid. Simple suckers (without hooks) adorn the arms and tentacles, while additional suckers are typically carried on the buccal lappets. The tentacular club has a tentacle pocket on the head and no locking apparatus on the carpal (wrist) portion. There are no lateral adductor muscles in the funnel. A well-developed gladius, which is found dorsally within the mantle and extends for nearly its entire length, is an internalised shell. Unlike oegopsids, females have accessory nidamental glands in addition to the main nidamental glands and have a single oviduct at the left side. The sizes of myopsid squids vary from very small, dwarf-sized species (maximum recorded ML 20 to 22 mm) to rather large squid (over 900 mm ML) (Roper et al., 1984; Naef, 1916; Naef, 1923; Nesis, 1982; Sweeney and Vecchione, 1998; Okutani, 2005; Vecchione et al., 2005).

Myopsid squids consists of only two families: the monotypic Australiteuthidae (single small-sized species), and the diverse and commercially important Loliginidae (with 10 genera, 9 subgenera, ~50 species) (Jan et al., 2009; Jereb and Roper, 2010).

1.2.2. Oegopsid (oceanic) squids

Oegopsid squids are the only decapods that lack a pocket for the tentacles. No corneal membrane covers the eye. The buccal lappets do not have suckers. Funnel lacks the lateral adductor muscles. Some species have arms and clubs with suckers and/or hooks. Typically, tentacular clubs have carpal-locking devices. A gladius that stretches the entire length of the mantle is shell. Female gonoducts are paired. There are no accessory nidamental glands. Their size ranges from extremely small (dwarf) squids, such as some species of *Abralia* and *Abraliopsis* (Enoploteuthidae; maximum recorded size 20 mm ML), to the enormous *Architeuthis* (Architeuthidae), *Mesonychoteuthis* (Cranchiidae), *Moroteuthis*, and *Onychoteuthis* (Onychoteuthidae) squids, who frequently have mantle lengths exceeding 2 m (Roper et al., 1984; Sweeney and Roper, 1998; Norman, 2000; Young and Vecchione, 2004; Okutani, 2005).

Oegopsid squids comprise 24 families and 69 genera. Among them, squids of the family Ommastrephidae are the main contributors to the fisheries, where *Dosidicus gigas* (eastern Pacific Ocean), *Illex argentinus* (southwest Atlantic Ocean) and *Todarodes pacificus* (northwest Pacific) together accounted for about 95% of the total ommastrephid squid catch (Jan et al., 2009; Jereb and Roper, 2010).

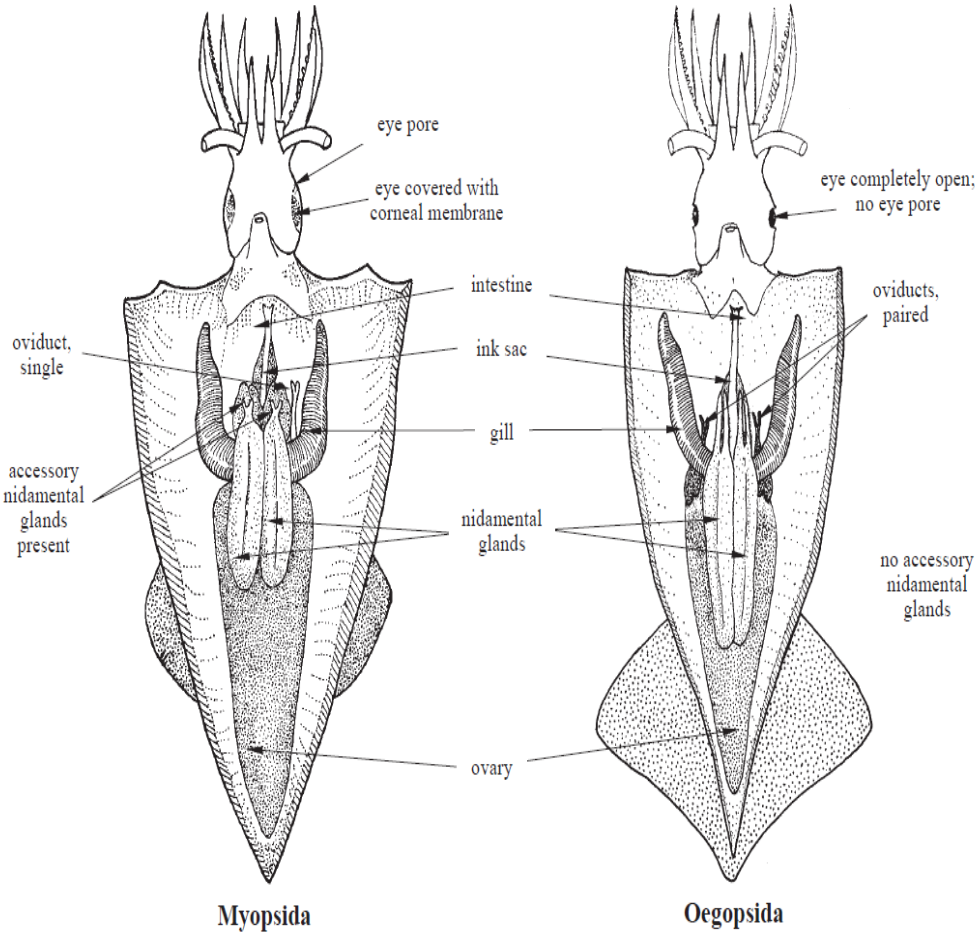

Fig. 1.2. Comparative illustration of myopsid and oegopsid squids (Jereb and Roper, 2010).

1.3. Reproductive behaviors of squids

1.3.1. Reproductive organs, sexual dimorphism and reproductive physiology

Squids are sexually reproducing organisms which have only one spawning season before dying. They are dioecious (separate sexes) with a single gonad in the posterior part of the body. The male has a testis from which sperm pass into a single gonoduct where they are rolled together into a long bundle, known as spermatophore. Spermatophores are expelled through the gonoduct, which is elongated into a penis that extends into the mantle cavity. The female has a large translucent ovary, located towards the posterior of the visceral mass. The reproductive systems are highly intricate structures with ducts, glands and storage organs (Ruppert et al., 2004; Jereb and Roper, 2010; Hirohashi et al., 2013; Guerra et al., 2014).

Many species, though not all, exhibit external sexual dimorphism. Females usually are larger than males and males of most species possess one, sometimes two, modified arm(s) (the hectocotylus) for passing spermatophores to females during mating. The hectocotylus can be simple or complex, with membranes, ridges and grooves, flaps, papillae, and modified suckers. The one or two nuptial limbs are used to transfer the spermatophores from the male's reproductive tract to an implantation site on the female. The spermatophores may be implanted around the mouth, inside the mantle cavity (where they may penetrate the ovary), into the oviducts themselves, around the mantle opening on the neck, on the head, in a pocket beneath the eye, or in other locations. Females of a few species also develop gender-specific structures (e.g. arm-tip photophores) when they reach maturity (Iwata et al., 2005; Jereb and Roper, 2010).

1.3.2. Courtship and mating

Mating is generally preceded or accompanied by courtship behavior that involves the male selecting a female and striking chromatophore patterns and display for attracting the female. However, before courtship, first males are to fight with other males (male-male competition). When the female responds to the male, copulation occurs. Copulatory behavior shows a greater variation, in terms of color and textural display, duration of display and spermatophore transfer, the location of implantation of the spermatophores on the female, and proximity of male and female (Arnold, 1965; Anderson, 1994; Hirohashi et al., 2013; Hanlon and Messenger, 2018).

During mating, the female receives the spermatophores transferred by the hectocotylized arm(s) from the male. The spermatophoric reaction starts when it comes into contact with seawater. Water seeping into the spermatophoric cavity, where the osmotic pressure is higher, causes the sperm packet to extrude as the spermatophores evert. The resulting extruded sperm packet is named spermatangium (Mann, 1984; Drew, 1911; Marian, 2012).

1.3.3. Egg laying and fertilization

Sperm can survive several months once stored in the female, at least in some species. With the exception of *Sepioteuthis*, neritic inshore squids generally lay relatively small (a few millimeters in diameter) eggs, which are often laid in finger-like pods containing a few to several hundred eggs each. Retained in multi-finger masses, these eggs are adhered to shells, rocks or other hard substrates on the bottom in shallow waters (Jereb and Roper, 2010). The female places her sperm-storage location over an egg held within her arm crown and inseminates the eggs one-by-one during adherence to the spawning

substratum. Sperm travel through a pathway within the jelly layers surrounding an egg. Such direct insemination behavior and the route through the egg jelly enables a female squid to fertilize her eggs with relatively few sperm (Iwata et al., 2019). Conversely, many oceanic squids lay their eggs into large spherical or sausage-shaped gelatinous masses carrying tens or even hundreds of thousands of eggs which drift submerged in the open sea. However, most squids die shortly after spawning (Hanlon and Messenger, 1998; Jereb and Roper, 2010).

1.4. Review of literatures related to reproductive behavioral strategies of inshore and oceanic squids

Squids are an extraordinary group for studies on sexual selection because of their complicated reproductive behavior, strong sexual dimorphism, polygamous behavior, as well as comparative evidence of pre- and postcopulatory male-male rivalry and female choice. Therefore, various studies had been carried out, those showed that most squids are polyandrous (a single female mates with multiple males) and semelparous (death after reproduction), which could lead to a situation where male-male competition for females become so intense that alternative male mating behavior could be introduced (Shaw and Saucer, 2004; Buresch et al., 2009; Iwata et al., 2011; Naud et al., 2016; Franklin and Stuart-Fox, 2017; Sato et al. 2017; Sato et al., 2023). Alternative reproductive techniques (ARTs) are the collective term for this phenomenon, where small males' extra-pair (or sneak) copulations could have some sort of reproductive fitness (Hirohashi et al., 2013). In several species of the commercially important squid family loliginidae inhabiting inshore waters, such as *L. pealeii* (Hanlon, 1996)*, L. reynaudii* (Hanlon et al., 2002), *H. bleekeri* (Iwata et al., 2005)*, S. lessoniana* (Wada et al., 2005a; Lin et al., 2017)*, S. australis* (Jantzen and Havenhand, 2003) and *U. edulis* (Hirohashi et al., 2016a), males

being dimorphic in size, are known to adopt this strategy that gives rise to insemination site dimorphism, i.e., large consort males inseminate within the mantle cavity (near oviduct) and small sneaker males do so outside the mantle cavity (at the buccal membrane around mouth) for fertilization, causing variations in fertilization success (Iwata and Hirohashi, 2020). In *Heterololigo bleekeri*, consorts have a 90% paternity rate, while sneakers have a 10% rate (Iwata et al., 2005). In addition, the number of spermatozoa in a single spermatophore are greater in consorts than in sneakers (Iwata et al., 2011). Moreover, these loliginid squids produce dimorphic sperm, which is closely associated with ART (Hirohashi et al., 2021). The smaller males produce longer spermatozoa and attempt sneaky (extra-pair) copulation, whereas the larger males have shorter spermatozoa and copulate via male-parallel (pair bonding) (Iwata et al., 2011; Apostolico and Marian, 2017; Hirohashi et al., 2021). The dimorphism in sperm size between sneakers and consorts occurs due to the variations in fertilization environment (external versus internal), because smaller and larger males inseminate at the outside and inside of the mantle cavity of females, respectively (Iwata et al., 2011).

However, flexibility in mating behavior by male individuals is common in some species (Hanlon et al., 1997; Wada et al., 2005b; Mather, 2016; Lin and Chiao, 2017; Apostolico and Marian, 2019) while it is either rare or non-existent in others (Iwata and Sakurai, 2007; Iwata et al., 2011). For instance, early adult males of the Caribbean reef squid *Sepioteuthis sepioidea* often practice sneaking but switch to pair bonding (consortship) when they mature (Mather, 2016). Intermediate-sized males of *Doryteuthis pleii* in Brazilian waters strategically choose each mating opportunity based on the reproductive context of the female; they sneak away from spawning and pair bond at egg laying (Apostolico and Marian, 2019). Male individuals in *Sepioteuthis lessoniana*

exhibit either sneaking (male-upturn) or pair bonding (male-parallel) based on the relative size of the female (Wada et al., 2005a), which is mostly dictated by the female's decision to accept or reject a particular male-mating posture (Lin and Chiao, 2017). On the contrary, although Japanese spear squid *Heterololigo bleekeri* showed behavioral flexibility in captive condition (Iwata et al., 2005), the speculation that individuals in the wild populations also display phenotypic plasticity in male mating behavior was not supported by the available data from field observations (Iwata and Sakurai, 2007) and anatomical studies of attached spermatangia on females (Iwata et al., 2011). Thus, squid ARTs in inshore loliginid squids display a wide range of adaptive traits including a composite repository of morphology, physiology, and behavior, as well as a varying degree of phenotypic plasticity (Marian et al., 2019).

On the other side, oceanic squids have variable reproductive strategies. The location where spermatangia are deposited during mating may be divided into several general types in deep-sea squids. For example, mature females of *Chiroteuthis* (Laptikhovsky et al., 2019), *Mastigoteuthis* (Laptikhovsky et al., 2019), *Megalocranchia* (Clarke, 1962), *Leachia* (Young, 1975), *Teuthowenia* (Voss, 1985), *Galiteuthis* (McSweeney, 1978; Nesis et al., 1998; Laptikhovsky and Arkhipkin, 2003), *Architeuthis* (Guerra et al., 2004) and *Bathothauma* (Voight, 2008) have spematangia implanted externally in the mantle, head, fins, sometimes arms. Females of *Liocranchia,* Ancistrocheiridae and Enoploteuthidae have specific modified areas for spermatangia reception inside of the mantle (Burgess 1998; Hoving and Lipinski, 2015; Laptikhovsky et al., 2019). On the contrary, in the commercially important oceanic squid *Todarodes pacificus*, male deposits spermatophores on the female's buccal membrane, and the female stores the spermatozoa in her seminal receptacles for a few weeks prior to

spawning (Sakurai et al., 2003; Sato et al., 2023). Moreover, synchronous ovulation is a prevailing type of gonad development with all eggs being spawned as a single batch, with or without brooding. Some species exhibit extended synchronous spawning, where this single batch is released in multiple subsequent portions rather than all at once (Muus, 1956; Voss, 1985; Laptikhovsky, 2001; Seibel et al., 2005; Laptikhovsky et al., 2007; Hoving and Lipinski, 2009; Laptikhovsky et al., 2019). However, data for deep-sea squids are scarce because they are very hard to obtain (Clark et al., 2016).

1.5. Scopes of study

Most loliginid squids show different varieties of ARTs, although that of many species is yet to be explored. Unlike most other loliginid species, *Loliolus sumatrensis* have three distinct insemination sites in a female body, which is very unusual among loliginids. However, how these sites are used by males, whether there is any dimorphism of male and sperm size, whether the condition or status of females have any impact on the usage pattern, how is the female promiscuity level are not known. On the other side, a few studies were centered on deep-sea squids. Among many families of oceanic squids (oegopsids), species of a few families had been studied, where most were done with anatomical methods. Comprehensive studies on their reproductive strategies are needed. Oceanic diamond squid of the monotypic Thysanoteuthidae family show a unique behavior, i.e. they are often found in pairs, consisting of one male and one female. It indicates behavioral monogamy; however, no genetic basis is available for their tight relationship. In addition, a handful of studies conducted on squids were molecular based. Therefore, there are potential scopes of research both on inshore loliginid squids as well as oceanic squids, especially using molecular techniques along with anatomical methods.

1.6. Objectives of the study

1.6.1. General objectives

The main objectives of the study were to explore alternative reproductive tactics (ART) of an inshore loliginid kobi squid *Loliolus sumatrensis* having three different insemination locations in a female body, along with the female promiscuity level, and to find out genetic basis of partnership in a pair-forming oceanic diamond squid *Thysanoteuthis major*, using molecular and anatomical techniques. Here, anatomical techniques include macroscopic examination or gross observation using naked eye, and microscopic examination. Molecular techniques include DNA extraction and purification, polymerase chain reaction (PCR), gel electrophoresis and fragment length analysis for performing DNA sequencing, microsatellite markers development and genotyping of sperm.

1.6.2. Specific objectives

The study has the following specific objectives-

1. To investigate the usage patterns of three distinct insemination sites in a female inshore kobi squid, *Loliolus sumatrensis*, in relation to seasonal variations, mating history, maturity, fecundity, and growth indices of female

2. To know whether there is any dimorphism in male body size and sperm size in *L. sumatrensis*

3. To determine the preferred one among the three locations in terms of initial use and number of spermatangia attached in the kobi squid female

4. To explore the female promiscuity level of loliginid kobi squid, along with the comparison of the paternity number among the three sperm deposition sites.

5. To find out genetic evidence of mating system in pair-forming oceanic diamond squid *Thysanoteuthis major*

6. To identify the distribution pattern of sperm among the sperm storage organs within a female diamond squid

CHAPTER 2

Three distinct insemination sites in a female inshore kobi squid are used by monomorphic males based on mating history and maturity status of female

2.1. Introduction

Animals with sexual reproduction adjust their physiology and behavior in response to their socio-sexual surroundings (Wilson et al., 2014), since the environments can have a substantial impact on their fitness (Mohorianu et al., 2017). As a consequence, they often use alternative reproductive tactics (ARTs) in which both males and females obtain fertilization in alternative ways. In typical ARTs, recessive males of physical, social, or reproductive status among same-sex competitors adopt different approaches for gaining access to mates or gametes. Thus, ARTs would have evolved through male-male competition for mating (Gross, 1996; Brockmann, 2001; Dijkstra and Border, 2018). ARTs are known to occur in a wide range of taxa, including mollusks, crustacea, insects, fishes, amphibians, reptiles, and birds (Oliveria et al., 2008).

The loliginid squid family inhabiting inshore waters shows sophisticated ARTs that are complex in terms of their time and place of occurrence. Larger males (consorts) copulate with females to deposit spermatangia immediately adjacent to the proximal end of the oviduct inside the mantle cavity, whereas smaller males (sneakers) copulate with females to deposit spermatangia on the female buccal membrane located apart from the oviduct (Drew, 1911; Sauer et al., 1992; Hanlon et al., 1997; Iwata et al., 2011).

Theoretically, the greater the distance between the insemination and egg deposition sites, the lower the fertilization success rate. Consequently, it is rational for large consorts to adopt favorable strategies to minimize such a distance and maximize reproductive success. In contrast, it is generally considered that sneakers must have no choice but to adopt the 'best of a bad job' strategy (Dawkins, 1980; Eberhard, 1982). However, male sneaker squids also deposit their spermatangia on the buccal membrane, irrespective of the presence or absence of consorts, at much earlier time points before egg spawning and then make their sperm be stored for longer periods in the female seminal receptacle located on the buccal membrane (Hirohashi et al., 2016a). Hence, the deposition of sperm onto the female buccal membrane is not always an alternative way to consort mating tactics; rather, it has independent reproductive benefits such as long-term sperm storage (Hirohashi et al., 2016a; Dhillon et al., 2020). Thus, two sperm deposition sites are used simultaneously within a female by different males with different fitness optima for sperm accessibility to eggs or the seminal receptacle.

Previous studies with a loliginid squid, *Heterololigo bleekeri* identified dimorphic euspermatozoa (both flagellum length and swimming performance) that are tightly linked to sperm deposition sites (Iwata et al., 2011; Hirohashi et al., 2013; Hirohashi et al., 2016b), suggesting that a tactical decision is established ontogenetically. Several following studies with number of loliginid species revealed this tight link to be prevalent, however, the extent of sperm dimorphism, particularly flagellum length dimorphism, differed among species (Lin and Chiao, 2017; Apostólico and Marian, 2018; Hirohashi et al., 2021). We assumed that dimorphism in sperm traits could be attenuated by behavioral plasticity during mating, as observed in some species in which males choose one of two sperm deposition sites (mating tactics) depending on their body size

relative to that of prospective mating partners (condition dependence) (Hanlon et al., 1997; Wada et al., 2005a; Mather, 2016; Apostolico and Marian, 2019). Nevertheless, in all loliginid species examined thus far, it is evident that there are two sperm deposition sites located discontinuously within a female: on the externally located buccal membrane and adjacent to the oviduct within the mantle cavity. The choice of insemination site depends largely, if not entirely, on male body size, either absolutely or relative to female body size.

However, the ARTs of another loliginid squid, *Loliolus sumatrensis* (d'Orbigny, 1835) is yet to be explored. *L. sumatrensis*, commonly known as Kobi squid, is a species of squid in the family Loliginidae under Myopsida order (Natsukari, 1983; Roper et al., 1984). They are small-sized inshore species, having maximum mantle length of 120 mm, thinner body and more reddish in color. They have an expanded tentacular club with 4 series of suckers. Sucker rings of arms II and III have 6-9 square-shaped plate-like teeth (Natsukari, 1984; Roper et al., 1984; Jereb and Roper, 2006). They are generally found in coastal waters of south-western Japan, the east and south China seas to the gulf of Thailand (Chotiyaputta, 1993; Okutani, 2015). Their spawning period extends year-round (Jereb and Roper, 2010). Males perform various displays to attract potential females for copulation (Ruppert et al., 2004). But how the intrasexual rivals approach the reproductive competitions is still unknown, which needs to be explored.

Therefore, we investigated into their insemination sites and found that *L. sumatrensis* females have three distinctive sperm deposition sites: buccal membranes (BM), basal areas of the left IV arm (ARM), and lateral head behind the left eye (EYE). Given the curiosity of such an atypical phenomenon in this family, we attempted to

identify rules and patterns of usage of these sites in relation to size differences in male individuals, and the mating history, maturity, fecundity, as well as growth indices of female individuals. We discuss the possible advantages of each site under circumstances in which sperm stored at all sites could be fertilized after females' egg spawning.

2.2. Materials and Methods

2.2.1. Animal collection

The squid, *Loliolus sumatrensi*s were purchased from a fisherman as dead animals around the Shodo Island in the Seto Inland Sea, Japan (Fig. 2.1) during the fishery season (July-September) of this species in 2021 and 2022 and transported as ice-cold (for sperm size measurements) or frozen specimens (for DNA analysis). During this period, 917 individuals (397 males and 520 females) were obtained on eight different fishing days (Appendix I & II). Fishing points were informed from a fisherman who caught these squids. Squids were killed by a fisherman as part of routine commercial food.

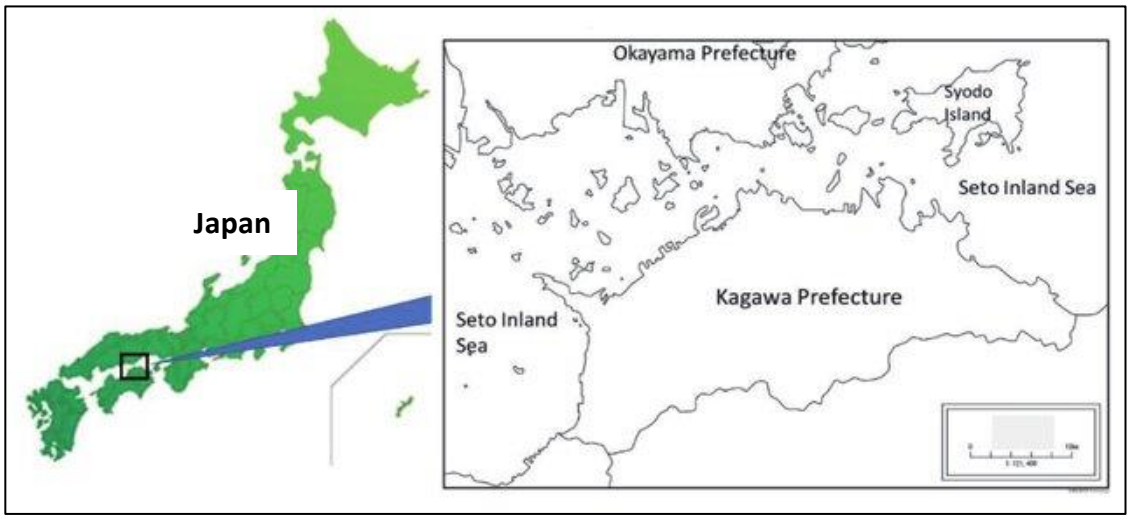

Fig. 2.1. Collection site of *Loliolus sumatrensis* squid samples (Source of map: Hidaka, 2020).

2.2.2. Species identification

First, species identification was carried out based on overall morphology and more specifically on largest sucker ring dentitions in the tentacles and the third arms (Jereb and Roper, 2005), allowing us to distinguish from closely related, morphologically similar species such as *L. japonica* or *L. beka.* However, the morphology of sucker rings in *L. sumatrensis* was undistinguishable from that of *L. uyii*, therefore cytochrome c oxidase I (*COI*) DNA sequencing was carried out with representative specimens to confirm the species. First, genomic DNAs were isolated from mantle tissue as well as testes and purified by DNA Purification Kit (QIAGEN Genomic-tip 20/G and TAKARA NucleoSpin® Tissue) following the manufacture protocol. The universal primers that potentially amplify *Loliolus* species were developed by aligning the mitochondrial *COI* sequences of *L. uyii, L. sumatrensis, L. beka and L. japonica* (four representative individuals per species) taken from the GenBank database (Fig. 2.2); *Loliolus* universal *COI* forward primer: 5'- CAATGTAGTAGTAACTGCTCACGG -3', *Loliolus* universal *COI* reverse primer: 5'- GCTCCTAAAATAGAAGAAATACCA -3'. Polymerase Chain Reaction (PCR) was carried out with a Kit (Platinum™ Direct PCR Universal Master Mix) and a thermal cycler (MiniAmp, Thermo Fisher Scientific) at optimized conditions: 20 ng of genomic DNA, 0.2 μM paired primers and PCR reaction consisting of an initial denaturing step of 94 °C for 2 min, then 40 cycles of 94 °C for 15 sec, 56 °C for 15 sec and 68 °C for 20 sec followed by a final extension of 68 °C for 5 min. After PCR, the SuperDye Direct Cycle Sequencing Kit (Thermo Fisher Scientific) system was used followed by Sanger sequencing with ABI PRISM 3130xl Genetic Analyzer. The obtained sequencing data ware analyzed using 4 Peaks v 1.8 and then blasted through NCBI database to identify the species.

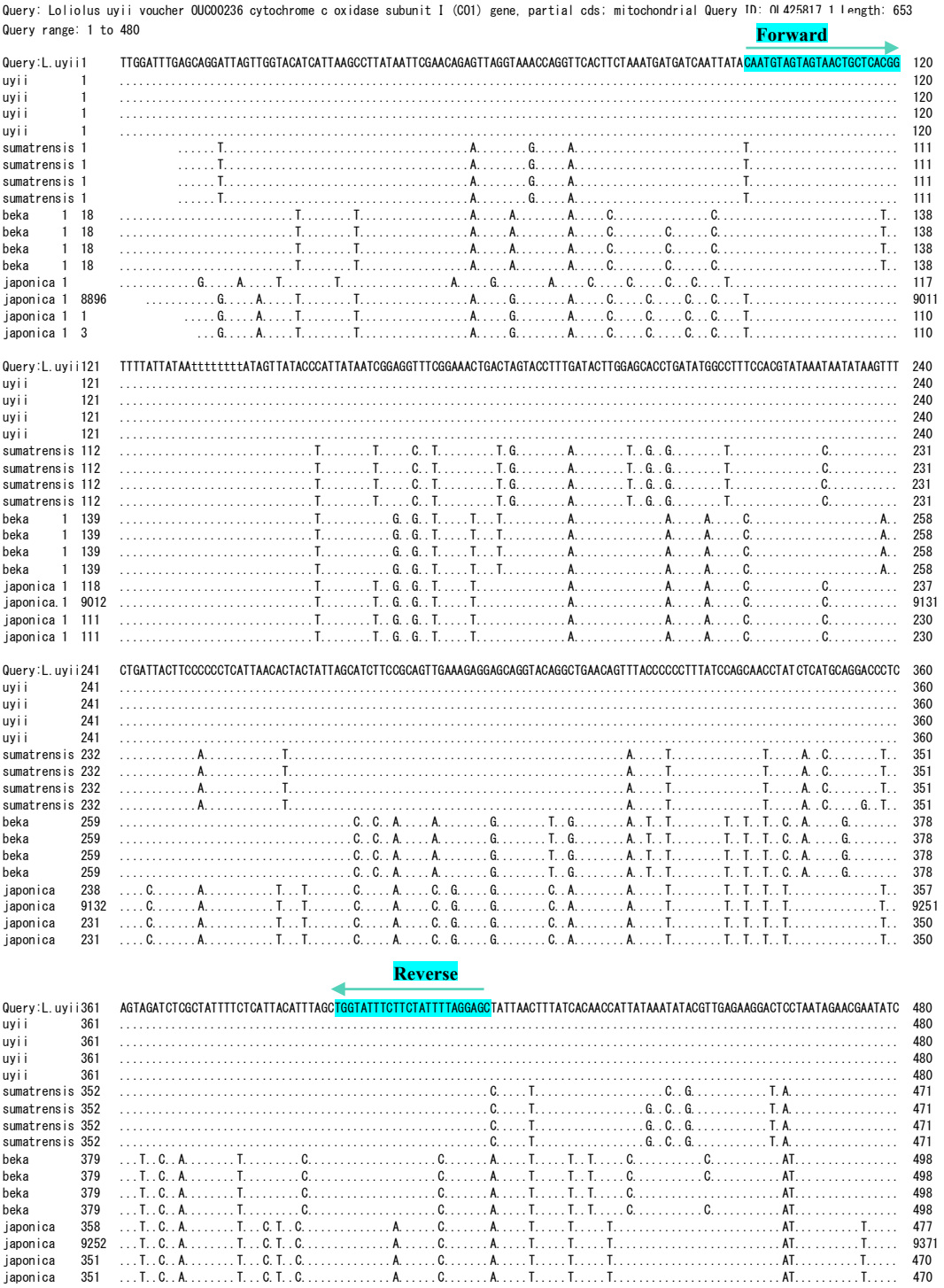

Fig. 2.2. Development of *Loliolus* universal primers for *COI* DNA sequencing. Here, the mitochondrial *COI* sequences of *L. uyii, L. sumatrensis, L. beka and L. japonica* (four representative individuals per species) taken from the GenBank database were aligned and thereby *Loliolus* universal forward and reverse primers (highlighted) were designed.

2.2.3. Quantitative analysis of reproductive anatomy

The squid specimens were measured (mostly within one day after fishing) for dorsal mantle length (ML), body weight (BW), accessory gland weight (ACC), testis weight (TW), relative testis weight (RTW), ovary weight (OW), relative ovary weight (ROW), number of insemination sites used (SITE). The mantle parts and reproductive organs of male and female are shown in Fig. 2.3. To measure somatic weight, gonad weight (OW + ACC) was subtracted from total body weight. ROW was calculated as 100 x OW x BW^{-1} and relative testis mass was calculated as 100 x TW x BW^{-1}. Sperm were retrieved from spermatophores of males and from the spermatangia attached to the females (unfrozen specimens), fixed with 10% formalin-containing seawater, photographed under a microscope (Nikon TE-2000) and thereafter sperm lengths were measured with Image J 1.52q (NIH, USA).

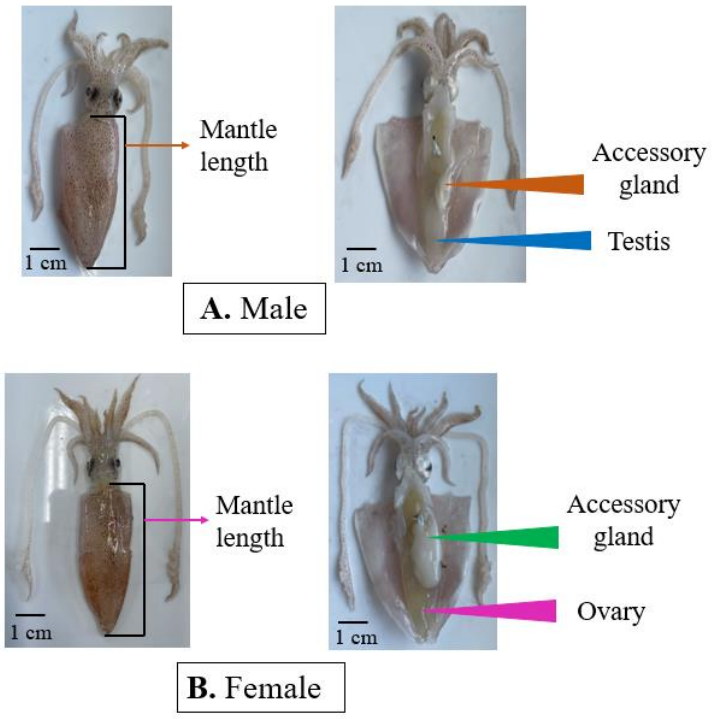

Fig. 2.3. Mantle lengths and reproductive organs in male and female of *L. sumatrensis*.

2.2.4. Counting and DNA barcoding of attached spermatangia in females

Under a stereomicroscope, the female tissues that contain attached spermatangia were dissected out and fixed in 70% ethanol for 1hr, thereafter every single attached spermatangium was removed with fine forceps from the tissue and counted, although the female buccal membranes were handled without fixation due to better visibility of attached spermatangia. In addition, to find out if there is any heterospecific cross insemination, the species of the attached spermatangia was required to identify. Consequently, each of 44 spermatangia from three insemination sites (16 from BM, 15 from ARM and 13 from EYE) from a female were used to purifiy genomic DNAs for DNA barcording. For this, every single spermatangium was first placed carefully into the bottom of each well of the 96-well plate. To each well, 10 µl of lysis buffer containing 0.1 mg/ml protease-K (Direct PCR Master mix kit, Thermo Fisher Scientific) was added, followed by a 30-min incubation at 52ºC and heat (95ºC) inactivation for 1 min. The lysates were used for mitochondrial genomic DNA sequencing with cephalopod-specific universal primers and the SuperDye Direct Cycle Sequencing Kit (Thermo Fisher Scientific) system followed by Sanger sequencing with ABI PRISM 3130xl Genetic Analyzer, where the PCR condition was same as described in the previous section of "Species identification".

2.2.5. Assessment of the costs/benefits of utilization of each insemination site

We assumed two hypothetical conditions:1) if the time until spawning was short (a common strategy adopted by consort squid), insemination sites proximal to the egg deposition site would have higher fertilization success; and 2) if the time until spawning was long (a common strategy adopted by sneakers), insemination sites proximal to the sperm storage site (the seminal receptacle) would have higher fertilization success.

Taking these into consideration, to evaluate the costs/benefits of utilization of each insemination site, we measured the subjects that could potentially influence fertilization success.

2.2.6. Statistical data analysis

The statistical data analyses were performed with JMP Pro software, version 17.0.0 and SPSS software, version 23.0. The parametric assumptions were met for the statistical analyses. Generalized Linear Mixed Models (GLMM) with poisson distribution and log link function, and Linear Mixed Models (LMM) were fit with sample ID as well as fishing days as random effects to analyze the attached spermatangium quantity per insemination sites, size variations of sperm among the different sites, and relative ovary weight of females using various number of sites for insemination. Additionally, Generalized Linear Model (GLM) was used to explore the variations of spermatangium numbers attached at each site during the fishing days. Tukey's Kramer test was also used for pairwise comparison of the means to determine their significant differences ($P < 0.05$). Furthermore, multiple regression models were used to investigate the effects of spermatangium quantity at one site on the insemination at other sites and to know the influence of female growth and maturity status on the number of spermatangia at each insemination site. The frequency distribution of mantle length of adult males and females was performed to determine the presence or absence of their size dimorphism.

2.3. Results

2.3.1. Species confirmation

The species was identified as *Loliolus sumatrensis* by the dentition morphology of the largest sucker rings in the tentacles and III arms (Jereb and Roper, 2005), and thereafter by mitochondrial *COI* genomic DNA sequencing. Since the DNA sequences in *L. uyii* and *L. sumatrensis* are varied at less extent, the nucleotides in the obtained genomic DNA sequences from representative specimens are shown at the specific positions where *L. uyii* and *L. sumatrensis* are different in Table 2.1.

2.3.2. Insemination at three different female locations by monomorphic males

We found that spermatangia were attached to *L. sumatrensis* females at three different sites: the buccal membrane (Figs. 2.4 A, 2.4 B; BM), basal left IV arm (Fig. 2.4 C; ARM), and lateral head behind the left eye (Fig. 2.4 D; EYE). However, no significant differences in sperm size (flagellum and head) were observed among the spermatangia at the three insemination sites (GLMM, $P > 0.05$; Fig. 2.5). The mature male individuals showed monomodal size distribution with similar relative testis mass (Fig. 2.6), suggesting the absence of male dimorphism in body size which is commonly observed in other squids with ARTs (Iwata and Sakurai, 2007).

Table 2.1. Species confirmation as *Loliolus sumatrensis* by showing the nucleotides in the obtained DNA sequences of representative specimens at the specific positions where *L. uyii* and *L. sumatrensis* are different

Nucleotide position	151	160	166	169	179	181	190	199	202	205	214	229	253	266
Loliolus uyii COI	**C**	**C**	**T**	**C**	**C**	**A**	**G**	**A**	**A**	**T**	**C**	**T**	**C**	**C**
Loliolus sumatrensis COI	**T**	**T**	**C**	**T**	**T**	**G**	**A**	**T**	**G**	**G**	**T**	**C**	**A**	**T**
Testis1	T	T	C	T	T	G	A	T	G	G	T	C	A	T
Testis2	T	T	C	N	T	G	A	T	G	G	T	C	A	T
Testis3	T	T	C	T	T	G	A	T	G	G	T	C	A	T
Testis4	T	T	C	N	T	G	A	T	G	G	T	C	A	T
Testis5	T	T	C	T	T	G	A	T	G	G	T	C	A	T
Testis6	T	T	C	T	T	G	A	T	G	G	T	C	A	T
Testis6	T	T	C	T	T	G	A	T	G	G	T	C	A	T
Testis8	T	N	C	T	T	G	A	T	G	G	T	C	A	T
Testis9	T	T	C	T	T	G	A	T	G	G	T	C	A	T
Testis10	T	T	C	T	T	G	A	T	G	G	T	C	A	T
Testis11	T	T	C	T	T	G	A	T	G	G	T	C	A	T
Testis12	T	T	C	T	T	G	A	T	G	G	T	C	A	T
Testis13	T	T	C	T	T	G	A	T	G	G	T	C	A	T
Testis14	T	T	C	T	T	G	A	T	G	G	T	C	A	T
Testis15	T	T	C	T	T	G	A	T	G	G	T	C	A	T
Testis16	T	T	C	T	T	G	A	T	G	G	T	C	A	T
Testis17	T	T	C	T	T	G	A	T	G	G	T	C	A	T
Testis18	T	T	C	T	T	G	A	T	G	G	T	C	A	T
Testis19	T	T	C	T	T	G	A	T	G	G	T	C	A	T
Testis20	T	T	C	T	T	G	A	T	G	G	T	C	A	T
Testis21	T	T	C	T	T	G	A	T	G	G	T	C	A	T
Testis22	T	T	C	T	T	G	A	T	G	G	T	C	A	T
Testis23	T	N	C	T	T	G	A	T	G	G	T	C	A	T
Testis24	T	N	C	T	T	G	A	T	G	G	T	C	A	T
Testis25	T	T	C	T	T	G	A	T	G	G	T	C	A	T
Testis26	T	T	C	T	T	G	A	T	G	G	T	C	A	T
Testis27	T	N	C	T	T	G	A	T	G	G	T	C	A	T
Testis28	T	T	C	T	T	G	A	T	G	G	T	C	A	T
Testis29	T	T	C	T	T	G	A	T	G	G	T	C	A	T
Testis30	T	T	C	T	T	G	A	T	G	G	T	C	A	T
Testis31	T	T	C	T	T	G	A	T	G	G	T	C	A	T
Testis32	T	T	C	N	T	G	A	T	G	G	T	C	A	T
Testis33	T	T	C	T	T	G	A	T	G	G	T	C	A	T
Testis34	T	T	C	T	T	G	A	T	G	G	T	C	A	T
Testis35	T	T	C	T	T	G	A	T	G	G	T	C	A	T
Testis36	T	T	C	T	T	G	A	T	G	G	T	C	A	T
Testis37	T	N	C	T	T	G	A	T	G	G	T	C	N	T
Testis38	T	T	C	T	T	G	A	T	G	G	T	C	A	T
Testis39	T	T	C	T	T	G	A	T	G	G	T	C	A	T
Testis40	T	T	C	T	T	G	A	T	G	G	T	C	A	T
Testis41	T	T	C	N	T	G	A	T	G	G	T	C	A	T
Testis42	T	T	C	T	T	G	A	T	G	G	T	C	A	T
Testis43	T	T	C	T	T	G	A	T	G	G	T	C	A	T
Testis44	T	T	C	T	T	G	A	T	G	G	T	C	A	T
Testis45	T	T	C	T	T	G	A	T	G	G	T	C	A	T

*N means not detected

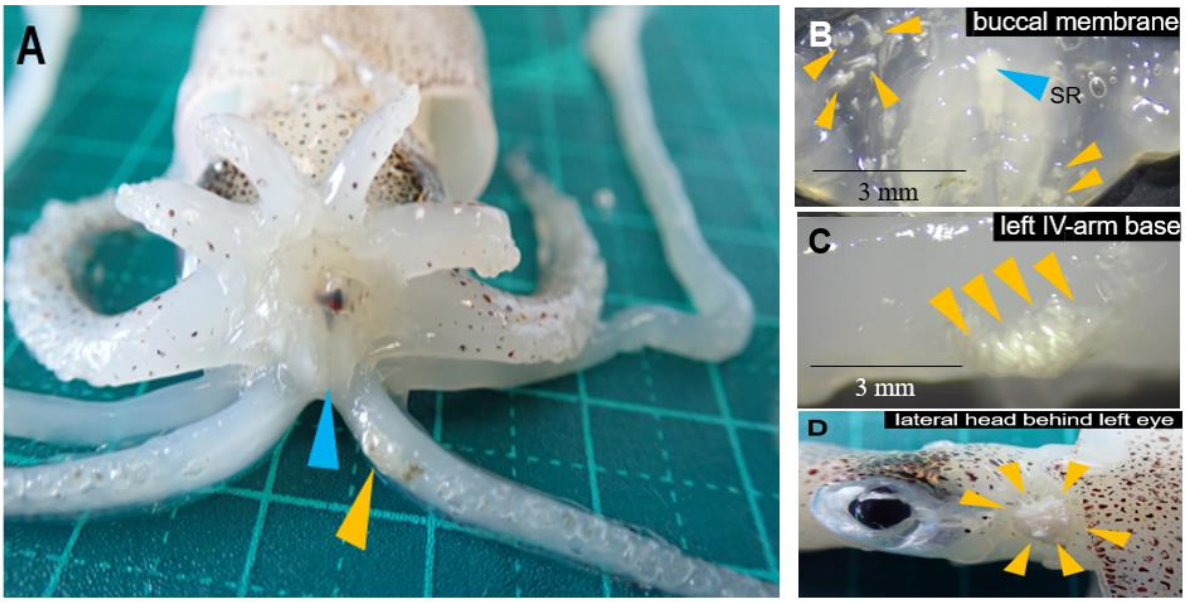

Fig 2.4. Three insemination sites within a female of *Loliolus sumatrensis*.

A-D, representative photographs showing female spermatangia-attachment sites: the buccal area around the mouth (A) containing the sperm storage organ, seminal receptacle (*bule arrowheads* in A and B); spermatangia (*yellow arrowheads*) attached to buccal membrane (B), left IV arm (C) and lateral head behind the left eye (D).

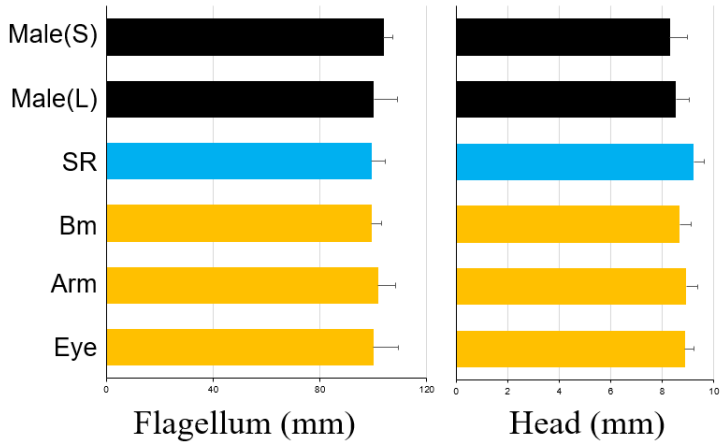

Fig. 2.5. Sperm flagellum and head lengths in *L. sumatrensis*. Sperm were collected from small males (*Male(S)*), large males (*Male(L)*), female seminal receptacles (*SR*), buccal membrane (*Bm*), left IV arm (*Arm*) and lateral head behind the left eye (*Eye*). The mantle length ranges for smaller males (*Male(S)*) and larger males (*Male(L)*) were 55-65 mm and 85-95 mm, respectively.

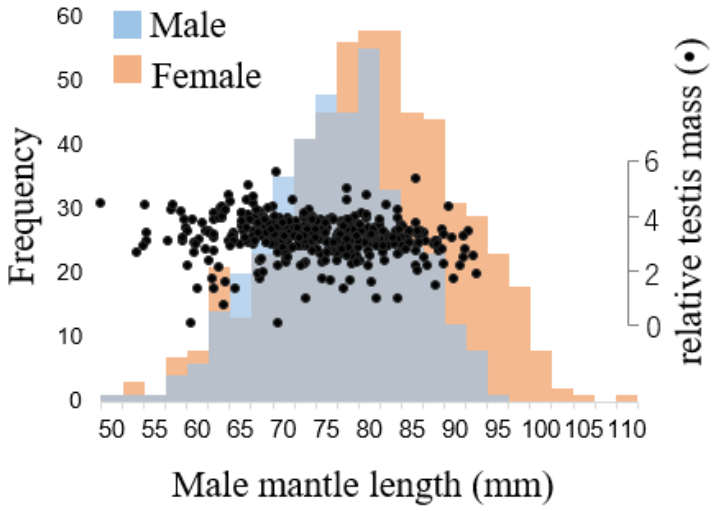

Fig. 2.6. The mantle length distributions of adult males and females of inshore kobi squid collected during the fishery season and relative testis mass across variance in body size.

2.3.3. No evidence of heterospecific cross insemination

With DNA barcoding for the spermatangia at the three insemination sites (BM, ARM and EYE), we found that males contributing sperm to all spermatangia attached at each site in females were of same species as female, *L. sumatrensis*. It suggests that there was no heterospecific cross-insemination in this species of squid. Table 2.2 shows nearly100% similarity of the acquired DNA sequences of the attached spermatangia with that of *L. sumatrensis COI* sequences stored in GenBank, especially by comparing the nucleotides at the particular differing positions between two morphologically similar, very closely related species, *L. uyii* and *L. sumatrensis*.

2.3.4. Seasonal dynamics of insemination patterns

The usage patterns of the insemination sites were classified where the three sites were used differently, and the proportion of females with these patterns was presented on each fishing day (Fig. 2.7). Six different patterns were observed during the early fishing season (early July), including the absence of spermatangia at any insemination site (unmated females). However, at the end of the fishery season (mid-September 2021), the pattern in which all three sites were simultaneously used was dominant because 91.8% females had spermatangia at all sites (Fig. 2.7).

Table 2.2. Conspecific insemination in loliginid kobi squid female evinced by DNA-based species confirmation of the male-delivered sperm from spermatangia attached at three sites of female

Nucleotide position	151	160	166	169	179	181	190	199	202	205	214	229	253	266
Loliolus uyii COI	**C**	**C**	**T**	**C**	**C**	**A**	**G**	**A**	**A**	**T**	**C**	**T**	**C**	**C**
Loliolus sumatrensis COI	**T**	**T**	**C**	**T**	**T**	**G**	**A**	**T**	**G**	**G**	**T**	**C**	**A**	**T**
Bm1	T	T	C	T	T	G	A	T	G	G	T	C	A	T
Bm2	T	N	C	T	T	G	A	T	G	G	T	C	A	T
Bm3	T	T	C	T	T	G	A	T	G	G	T	C	A	T
Bm4	T	T	C	T	T	G	A	T	G	G	T	C	A	T
Bm5	T	T	C	T	T	G	A	T	G	G	T	C	A	T
Bm6	T	T	C	T	T	G	A	T	G	G	T	C	A	T
Bm7	T	T	C	T	T	G	A	T	G	G	T	C	A	T
Bm8	T	T	C	T	T	G	A	T	G	G	T	C	A	T
Bm9	T	T	C	T	T	G	A	T	G	G	T	C	A	T
Bm10	N	T	C	T	T	G	A	T	G	G	T	C	A	T
Bm11	T	T	C	T	T	G	A	T	G	G	T	C	A	T
Bm12	T	T	C	T	T	G	A	T	G	G	T	C	A	T
Bm13	T	T	C	T	T	G	A	T	G	G	T	C	A	T
Bm14	T	T	C	T	T	G	A	T	G	G	T	C	A	T
Bm15	T	T	C	T	T	G	A	T	G	G	T	C	A	T
Bm16	T	T	C	T	T	G	A	T	G	G	T	C	A	T
Arm1	T	T	C	T	T	G	A	T	G	G	T	C	A	T
Arm2	T	T	C	T	T	G	A	T	G	G	T	C	A	T
Arm3	T	T	C	T	T	G	A	T	G	G	T	C	A	T
Arm4	T	T	C	T	T	G	A	T	G	G	T	C	A	T
Arm5	T	T	C	T	T	G	A	T	G	G	T	C	A	T
Arm6	T	T	C	T	T	G	A	T	G	G	T	C	A	T
Arm7	T	T	C	T	T	G	A	T	G	G	T	C	A	T
Arm8	T	T	C	T	T	G	A	T	G	G	T	C	A	T
Arm9	T	T	C	T	T	G	A	T	G	G	T	C	A	T
Arm10	T	T	C	T	T	G	A	T	G	G	T	C	A	T
Arm11	T	T	C	T	T	G	A	T	G	G	T	C	A	T
Arm12	T	T	C	T	T	G	A	T	G	G	T	C	A	T
Arm13	T	T	C	T	T	G	A	T	G	G	T	C	A	T
Arm14	T	T	C	T	T	G	A	T	G	G	T	C	A	T
Arm15	T	T	C	T	T	G	A	T	G	G	T	C	A	T
Eye1	T	T	C	T	T	G	A	T	G	G	T	C	A	T
Eye2	T	T	C	T	T	G	A	T	G	G	T	C	A	T
Eye3	T	T	C	T	T	G	A	T	G	G	T	C	A	T
Eye4	T	T	C	T	T	G	A	T	G	G	T	C	A	T
Eye5	T	T	C	T	T	G	A	T	G	G	T	C	A	T
Eye6	T	T	C	T	T	G	A	T	G	G	T	C	A	T
Eye7	T	T	C	T	T	G	A	T	G	G	T	C	A	T
Eye8	T	T	C	T	T	G	A	T	G	G	T	C	A	T
Eye9	T	T	C	T	T	G	A	T	G	G	T	C	A	T
Eye10	N	N	N	N	T	G	A	T	G	G	N	C	A	T
Eye11	T	T	C	T	T	G	A	T	G	G	T	C	A	T
Eye12	T	T	C	T	T	G	A	T	G	G	T	C	A	T
Eye13	T	T	C	T	T	G	A	T	G	G	T	C	A	T

*N means not detected; BM, buccal membrane; ARM, basal left IV arm; EYE, lateral head behind left eye

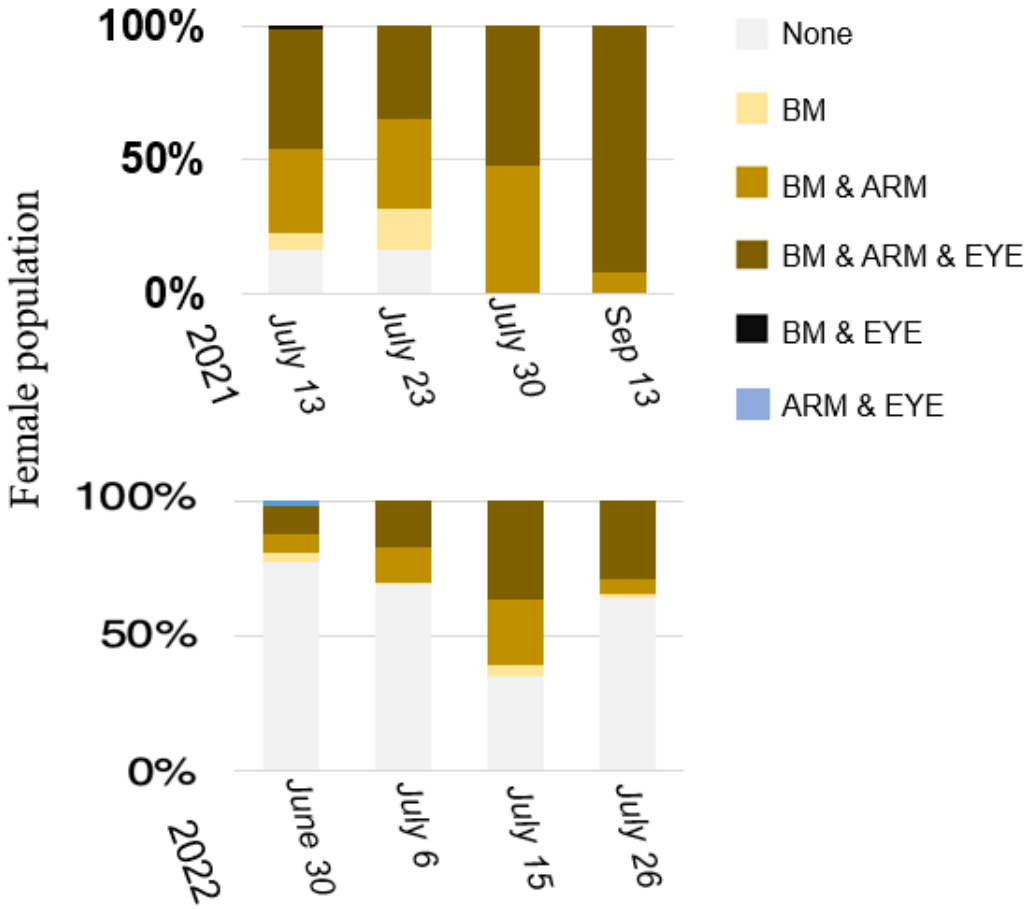

Fig. 2.7. Six different insemination patterns identified in *L. sumatrensis* females during the fishery seasons of two consecutive years (2021-2022).

2.3.5. A set priority for initial use of insemination sites within a female

The analysis of usage patterns allowed us to speculate the sequence of the initial use of insemination sites on a female. Among all mated females, 21 females had spermatangia at only BM, 98 females contained spermatangia both at BM and ARM, and 183 females used all three sites simultaneously; No female carried spermatangia at only ARM or only EYE. (Fig. 2.8). It suggests that the first appearance of spermatangia occurs at the BM and then ARM followed by EYE (Appendix III), which was further supported by the statistical analysis of rank cases (Appendix IV).

2.3.6. Sex ratio

From the seasonal changes of insemination patterns, it was assumed that the operational sex ratio becomes more male-biased as the season progresses; therefore, males use all three sites in response to increased male-male competition. However, the sex ratio was almost unbiased throughout the fishery seasons, except the late fishery season where female-biased sex ratio (%male on September 13 = 25.3; Fig. 2.9) was found, suggesting that the speculation was not exact.

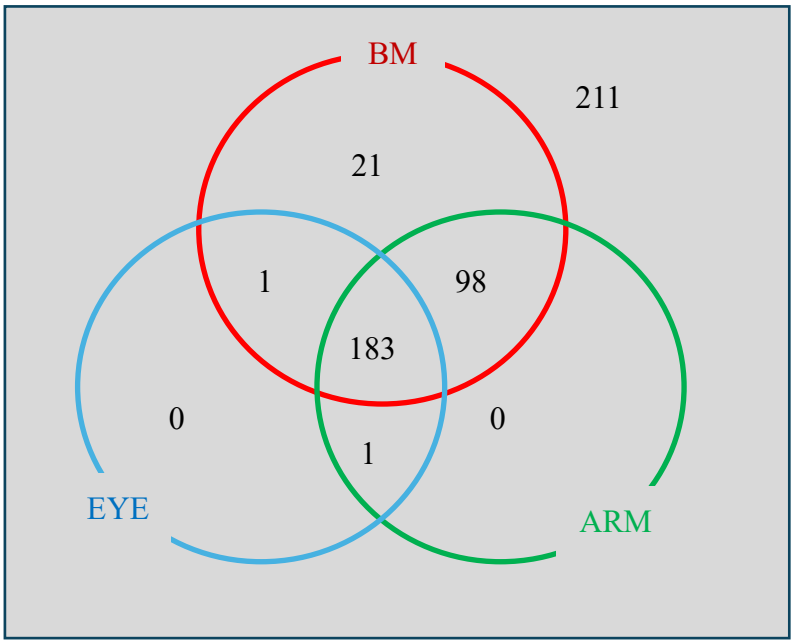

Fig. 2.8. A venn diagram showing the number of females with different insemination patterns in kobi squid. BM, buccal membrane; ARM, left IV arm; EYE, lateral head behind the left eye.

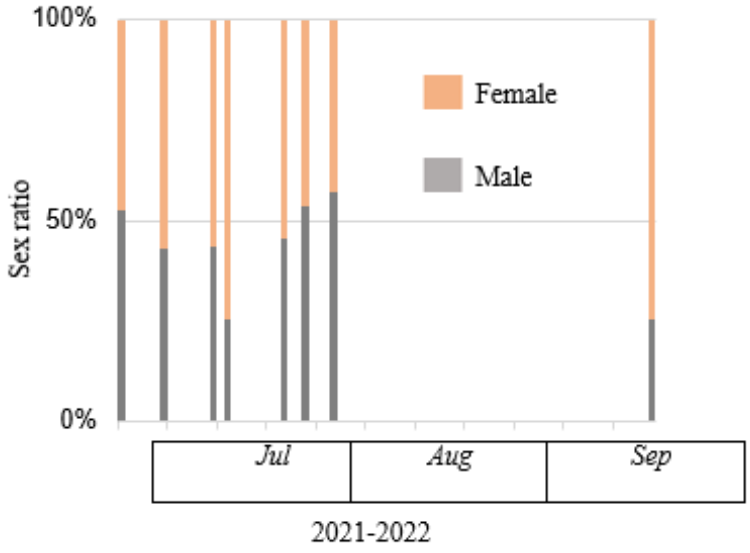

Fig. 2.9. Almost unbiased sex ratio of *L. sumatrensis* throughout the fishing seasons except at the end.

2.3.7. Spatio-temporal variations of attached spermatangia

In a population collected throughout the two seasons, the number of spermatangia attached to the BM was significantly smaller than that in the other two sites (GLMM, Tukey-Kramer test, $P < 0.0001$, BM, 46.2 ± 33.1; ARM, 126.4 ± 94.9; EYE, 116.3 ± 111.3; Fig. 2.10). However, among these three sites, the maximum number of stored spermatangia was found greater at EYE. At each insemination site, the number of spermatangia were substantially high in individual variance (Fig. 2.11; Fig. 2.13 A) and significantly different among fishing days (GLM; BM, $P = 0.0217$; ARM, $P = 0.0014$; EYE, $P = 0.0022$), however, we found no consistent trends (increasing or decreasing) of the spermatangium quantity throughout the season (Fig. 2.10). Furthermore, the multiple regression model showed that the spermatangium number at any of the insemination sites was not much affected by the spermatangium number at other sites (BM: $R^2 = 0.15, F_{2,241} = 22.66, P < 0.0001$; ARM: $R^2 = 0.24, F_{2,242} = 38.61, P < 0.0001$; EYE: $R^2 = 0.12, F_{2,241} = 17.73, P < 0.0001$), indicating that the observed set priority for the initial insemination (BM→ARM→EYE) was not due to the full occupancy of preferred insemination sites.

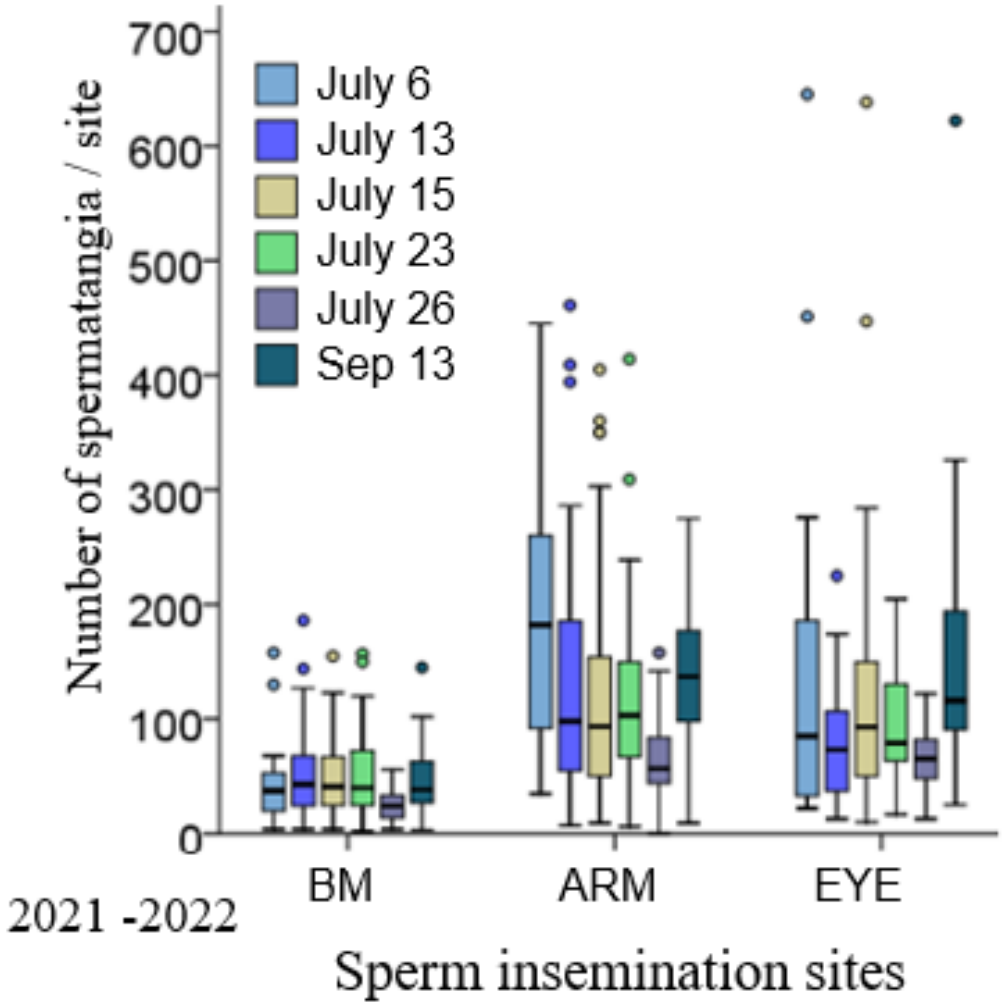

Fig. 2.10. The number of spermatangia attached to each insemination site within a *L. sumatrensis* female in different fishing days. BM, buccal membrane; ARM, left IV arm; EYE, lateral head behind the left eye.

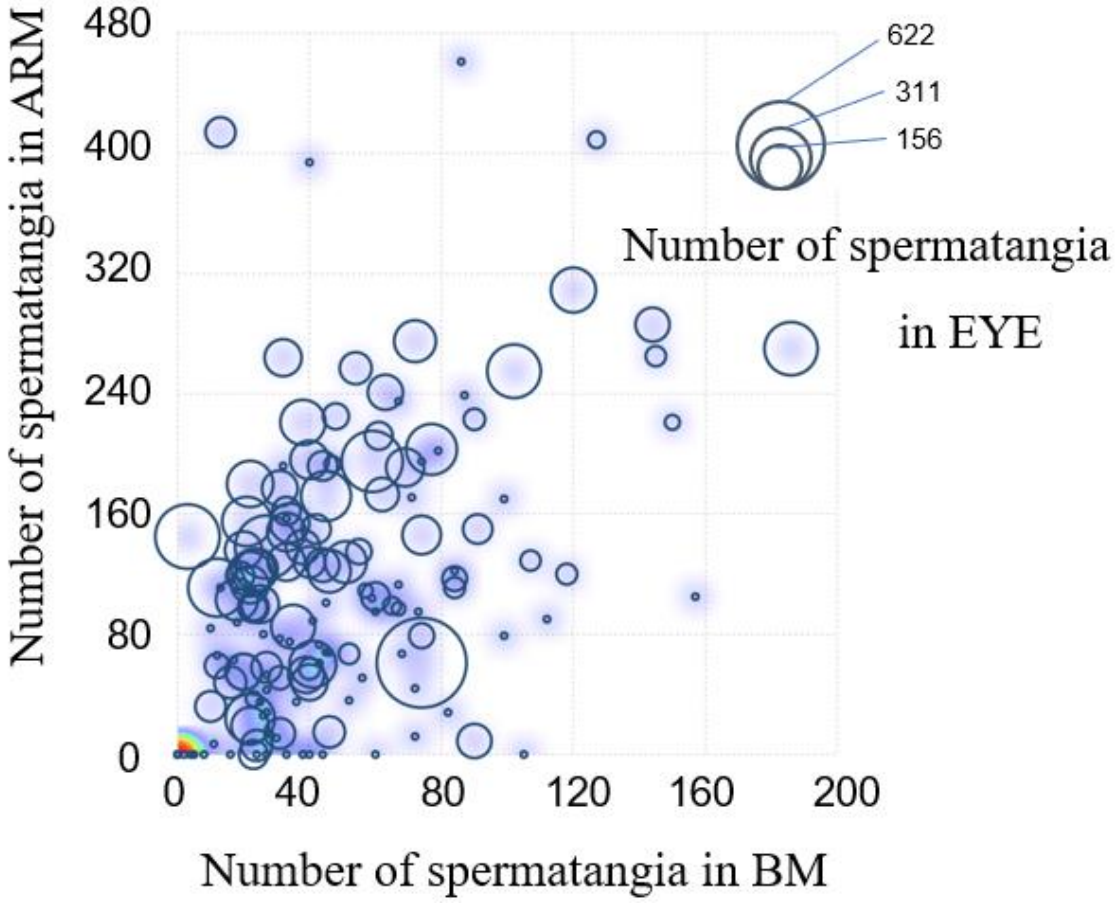

Fig. 2.11. Numerical distributions of attached spermatangia per site within a female kobi squid. Darkened *blue* indicates a higher plot density (number of individuals). BM, buccal membrane; ARM, left IV arm; EYE, lateral head behind the left eye.

2.3.8. Effects of female growth and maturity status on insemination patterns and spermatangium quantity

The mated females were significantly different from the unmated females in their relative ovary weight (GLMM, Tukey-Kramer test, $P < 0.0001$, Fig. 2.12). Moreover, the relative ovary weight was significantly higher in females inseminated at all three sites than two or fewer sites (LMM, Tukey Kramer test, $P = 0.0002$, Fig. 3F). It indicates that if females become more mature and fecund, she becomes more attractive to males for mating. Furthermore, the multiple regression models incorporating ML, BW, ACC, ovary weight (OW) and SITE to explain variation in the total number of attached spermatangia/female (TOTAL) were statistically significant ($R^2 = 0.34$, $F_{5,238} = 26.23$, $P < 0.0001$, Table 2.3). Notably, however, the total and site-dependent number of attached spermatangia were not correlated with the growth and maturity status of female (Table 2.3; Fig. 2.13).

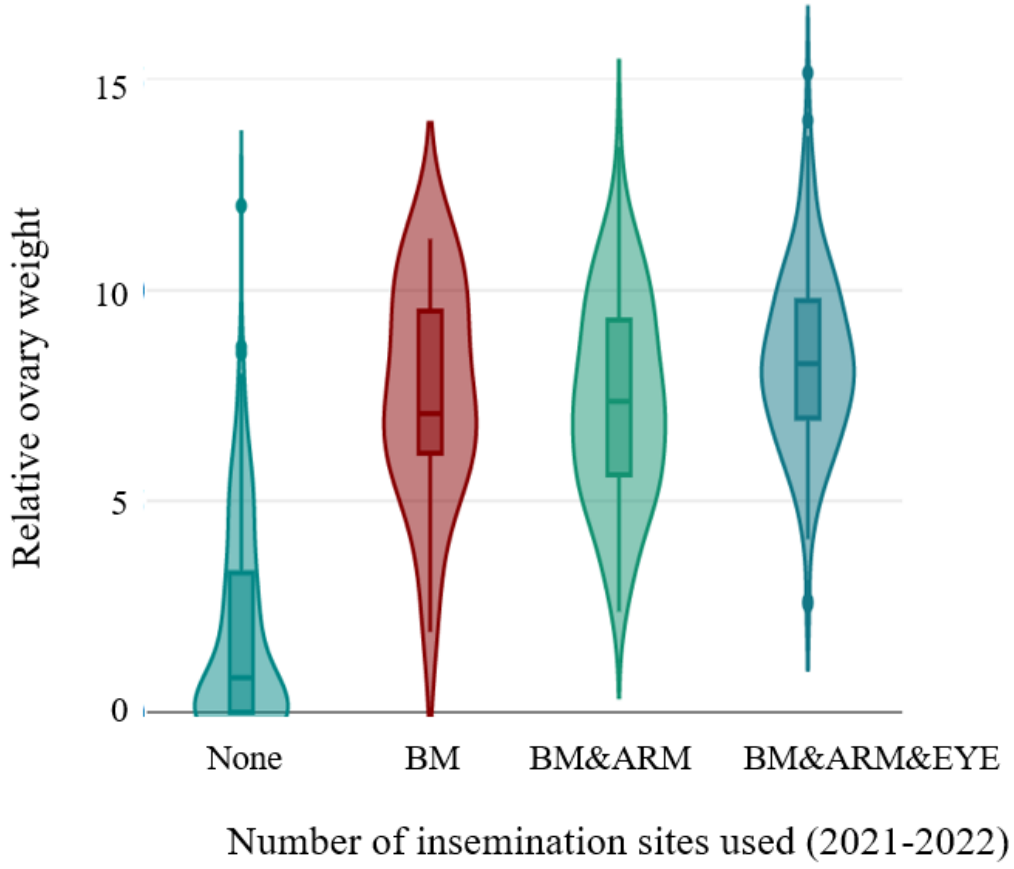

Fig. 2.12. Comparison of relative ovary weight among *L. sumatrensis* female individuals having different insemination patterns (BM, BM&ARM and BM&ARM&EYE). Darkened *blue* indicates a higher plot density (number of individuals). The graph represents boxplots (quartiles) merged with violin plots. BM, buccal membrane; ARM, left IV arm; EYE, lateral head behind the left eye.

Table 2.3. Effects of female growth/maturity indices and site usage on the number of spermatangia attached to each site or all sites in kobi squid

Site	ML		ACC		OW		BW		SITE		
	β_{ML}	*P*	β_{ACC}	*P*	β_{OW}	*P*	β_{BW}	*P*	β_{SITE}	*P*	R^2
TOTAL	0.076	*0.381*	0	*0.997*	**0.340**	*0.000*	**-0.293**	*0.011*	**0.529**	*0.000*	**0.34**
BM	-0.056	*0.600*	-0.025	*0.741*	0.174	*0.109*	0.077	*0.585*	0.199	*0.842*	0.02
ARM	0.035	*0.737*	0.026	*0.725*	**0.452**	*0.000*	**-0.320**	*0.022*	**0.188**	*0.004*	**0.13**
EYE	0.234	*0.195*	0.01	*0.104*	**0.283**	*0.040*	**-0.547**	*0.016*	0.038	*0.660*	0.02

Multiple regression of female growth (ML, mantle length; BW, body weight) and maturity (ACC, accessory gland weight; OW, ovary weight; site usage (SITE, Number of insemination sites used)) indices on the number of spermatangia at each site (BM, ARM and EYE) or all sites (TOTAL). Significant regression coefficients are indicated in bold.

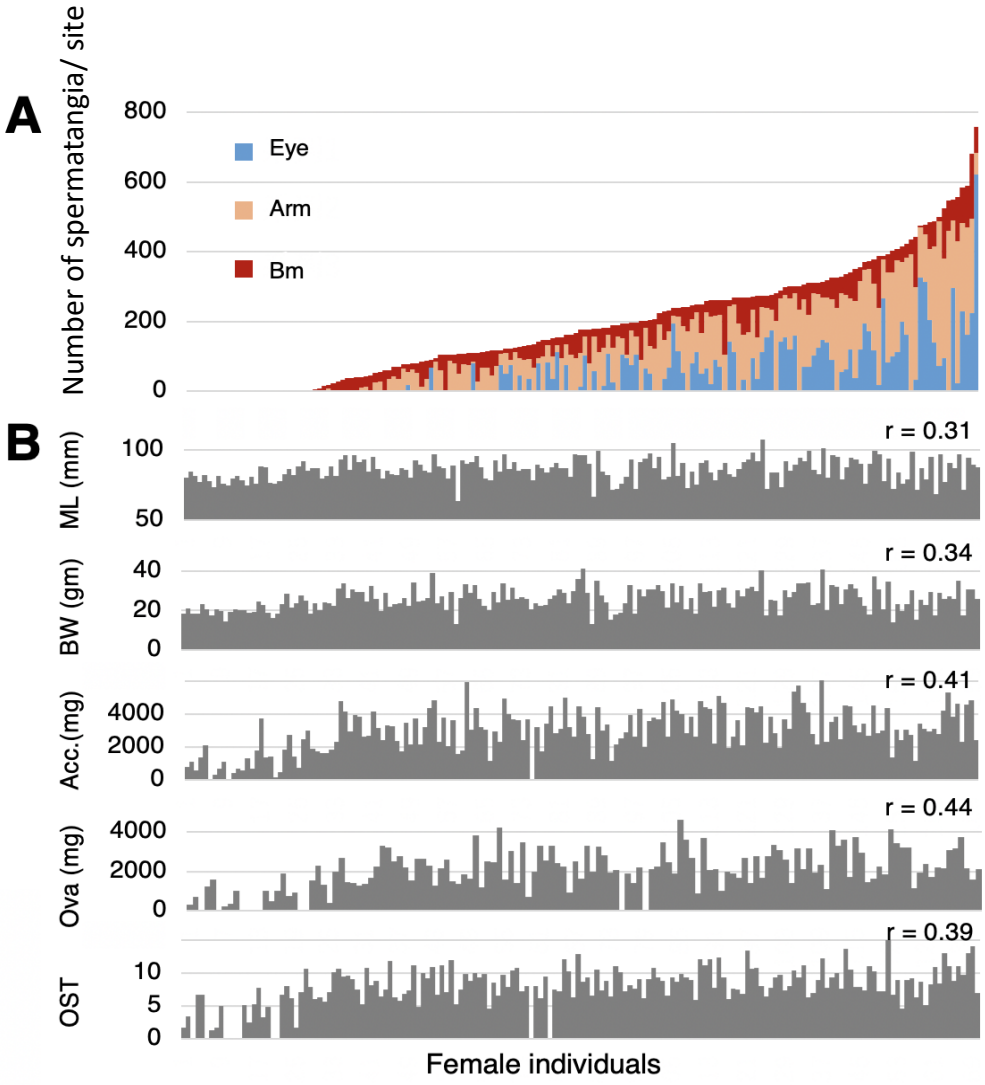

Fig. 2.13. Individual variableness in total and site-dependent numbers of attached spermatangia and their least correlations with maturation and growth status of *L. sumatrensis* females. A, B; The stacked bars (A) showing the total number of spermatangia attached to three insemination sites per female, arranged in ascending order, in relation to individual growth and mature indices (B). Shown in *top right* of each graph represents the correlation coefficient (r) between total number of spermatangia/female and each index. ML: mantle length, BW: body weight, Acc: accessory gland weight, Ova: ovary weight, OST: relative ovary weight.

2.3.9. Evaluation of costs/benefits of utilization of three insemination sites

We evaluated the costs/benefits of utilization of each insemination site. Based on hypothetical conditions (see *Materials and Methods*), we assessed and ranked the subjects that could potentially influence fertilization success of the deposited spermatangia (Table 2.4).

Table 2.4. Measurements, estimation and ranking of subjects that potentially influence fertilization success

Subjects	Site of insemination		
	BM	**ARM**	**EYE**
Distance from egg deposition site	Proximal	Sub-proximal	Distal
Distance from seminal receptacle	Proximal	Sub-proximal	Distal
Order in the first use	First	Second	Third
Mean (maximum) number of spermatangia attached	46.2±33.1 (186)	126.4±94.9 (461)	116.3±111.3 (645)
Estimated placement size for spermatangia attachment	Smaller	Intermediate	Larger
Estimated lifetime of spermatangia to be attached	Shorter	Longer	Longer

2.4. Discussion

Most squid species have a fast growth rate and 2 years of lifespan (Hanlon and Messenger, 2018). As a consequence, under a high-competition regime, reproduction generally occurs only once. Nevertheless, extremely high degrees of variation in growth rates and body sizes within a population let individuals to select different strategies for maximizing their mating opportunities, possibly resulting in two different evolutionary trajectories: early ontogenetic decision and phenotypic plasticity (Hirohashi et al., 2021). It is common in loliginid squid populations for mature males to exhibit two alternative forms of mating strategy dependent on body size (Hanlon and Messenger, 1996).

In the loliginid squid *L. sumatrensis,* surprisingly we found three insemination sites (BM, ARM, and EYE) located discontinuously on a female. This differs from the cases in other species of this family, where two separate locations—the buccal membrane and oviduct—are alternatively used by males along with different mating strategies (Peakall and Smouse, 2012; Marian et al., 2019). A remarkable feature that is common in most squid ARTs is the linkage between the insemination sites and sperm traits: sperm inseminated at BM has longer flagella, whereas sperm inseminated near the oviduct has shorter flagella (Morse, 2019). Thus, in squid, the morphological traits of sperm are generally considered to be adaptive to the insemination environments and their associated sperm storage modes (Squires et al., 2015).

However, some loliginid squid species also show context-dependent ARTs (Gage and Barnard, 1996; Parker et al., 1996; Wada et al., 2005a; Sato et al., 2017; Sato et al., 2020), where males flexibly change mating tactics in response to relative size differences between mating pairs (Parker et al., 1996; Sato et al., 2020), which resulted

in attenuated sperm dimorphism (Gage and Barnard, 1996). In any case, males must choose the designated areas of female body locations, because the insemination site greatly influences the fertilization success (but see Hoving et al., 2010b; Hoving et al., 2012; Murai et al., 2021; Sato, 2021).

The current study presents a sharp contrast to well-known squid ARTs. First, both male body size and sperm flagellum length showed monomodal distributions (Figs. 2.5; 2.6). Second, there was a set order for the first use of insemination sites (BM→ARM→EYE). Third, the insemination pattern was led by female maturity status.

We wondered what factors make a change in male mating behaviors (insemination sites) even during copulation. First, we considered the possibility that because male-male competition at mating is so intense, males must use other insemination sites in favor of reducing the sperm competition risk. We considered and measured some factors that might have impacts on fertilization of the deposited sperm at each insemination site (Table 2.4). Taking these conditions into account, we speculated that BM is the most favorable site for insemination by *L. sumatrensis* males owing to its proximity to both the egg deposition site and the seminal receptacle (Table 2.4). In agreement with this, BM was chosen as the first among the three sites (Fig. 2.8). However, the mean and maximum numbers of spermatangia attached to BM were smaller than those attached to the other two sites (Figs. 2.10; 2.11), despite having some vacant space for insemination at BM. The lower number of spermatangia at the BM might be associated with the seminal receptacle being progressively enriched with sperm, although the dynamics of sperm storage in the seminal receptacle is unknown. It is interesting to hypothesize that males can sense the vacant status of the seminal receptacle either directly

or indirectly. In fact, cephalopods have more neurons in their arms than in their brains and perceive chemotactile sensation through their arms, suggesting that the arms play more perceptive roles than just being used as flexible actuators. Notably, the spermatangium remnants were frequently observed at BM (Appendix V) but not at the other sites, suggesting the occurrence of rapid attachment-detachment turnover of the spermatangia. Thus, sperm at BM might be used immediately for fertilization (proximal time points to egg spawning) or translocated to the seminal receptacle for longer storage. The latter case can explain the occurrence of BM utilization in the first order because the vacant seminal receptacles (virgin females) are the most favorable for first-mating males to use (Wedell et al., 2002).

We assumed that because the area of EYE is larger and nearer to the oviduct opening than those of BM or ARM, EYE is preferred by males who can invest more sperm resources to females with higher fecundity (greater in relative OW, Fig. 2.12). In accordance with this, maximum number of attached spermatangia was found greater at EYE than at other sites. It indicates that males can easily attach sperm at EYE in the sea, although this place seemed to be obscured. However, it is difficult to envisage how the sperm located at EYE could reach fertilization. One speculation would be that because EYE site lies at the region where seawater enters the mantle cavity and the oviduct lies on the left side of the female, the influx of water might bring the sperm attached at the left side of EYE and results in fertilization. In contrast, ARM is located in the area capable of flexible movements around the mouth, which may allow the ARM-deposited spermatangia to become proximal to eggs or the seminal receptacle during egg capsule manipulation between the arms before deposition on the substrate. Thus, ARM is preferred by males with limited sperm expenditure.

Given that BM, ARM and EYE have mutually distinct sperm-storing characteristics (Table 2.4), this can be explained by the concept of a polymorphic fitness equilibrium (Pizzari, 2002) in which reproductive success of sperm at each sperm-deposition site changes dynamically depending on the current overall utilization state. In other words, at the individual level, once all insemination sites within a female have begun to be used, forthcoming inseminations by other males could occur anywhere based on the most favorable site under the current circumstances.

It was assumed that sperm-storing capacity in each site is limited, but the mating season continues; therefore, late-coming males must choose other sites. However, the observed set priority for the initial insemination (BM→ARM→EYE) and irrelevance of spermatangium number among the sites indicates as well that the choice of site to be inseminated is not dependent on the full occupancy of preferred insemination sites, but the mating history of the female.

Theory states that males make decision on their sperm insemination to females considering the reproductive conditions of the females (Wedell et al., 2002), for instance fecundity (Simmons et al., 1993; Reinhold et al., 2002). It suggests that males are more attracted to the more mature and fecund females, as males can assess the females' sexual maturity by their responses to some visual, chemical and auditory cues (Tuni and Berger-Tal, 2012). In agreement with this, female maturity status had impact on the insemination pattern in inshore kobi squid.

Fertilization requires several successful interactions between oocytes and sperm within a receptive female reproductive tract. These interactions are usually assumed to be species-specific (Hill and L'Hernault, 2001). Nevertheless, mating and insemination between a male individual and a female individual of two different species sometimes occur (Gregory and Howard, 1994; Dean and Nachman, 2009). Considering this phenomenon and monomodal male size distribution into account, preliminarily we speculated that three insemination sites are used by three different species of males. However, the loliginid *L. sumatrensis* showed no herespecific cross insemination at any site, suggesting that each site in female was used by male of same species.

The sex ratio at the late fishing season (September) shifted from unbiased to female-biased, which is probably associated with the semelparity of squids (Hoving et al., 2010a). Generally, male squids release sperm just after mating and die shortly after their spawning. On the other side, female squids store male-delivered sperm and spawn later. They also die shortly after spawning (Iwata et al., 2019). In agreement with this, the sex ratio turned to female-biased at the end of fishery season. Besides, seasonal movement or migration of males might be other possible causes (Gulczyński, 2023).

We hereto disregarded the likelihood of female active involvement in the process of male behavioral decisions as they lack conspicuous sexual dimorphism. However, we cannot rule out the possibility that male inseminations are under female control. In addition, we cannot rule out the possibility that seasonal changes in insemination patterns may reflect seasonal movements of females with different reproductive behaviors. To resolve these problems, future studies require captive experimental settings in which male mating behaviors to females with different mating

histories can be observed. In conclusion, we found that female sexual experiences and the resulting sperm storing status at insemination sites might provide cues for subsequent males to choose their insemination site(s). Hence, we propose that alternative reproductive tactics can arise even in species that lack both direct male-male combat for mating and male body size polymorphism.

2.5. Summary

The current study showed a sharp contrast from previously known general views on squid reproduction, especially on alternative insemination strategies. In an inshore species of squid in loliginidae family, *Loliolus sumatrensis*, three distinguishing sperm insemination sites were found on buccal membrane (BM), left IV arm (ARM) and lateral head behind left eye (EYE) in a female body. Six different usage patterns were observed during the early fishing season, whereas the pattern of concurrent use of all three sites was dominant at the end of fishing season. The seasonal dynamics of the female populations in seto inland sea identified a set priority for initial use of insemination sites as BM, followed by ARM and then EYE. At the individual level, however, once all insemination sites of a female have started to be used, subsequent inseminations by other males could take place anywhere depending on the most suitable site given the current situation. Thus, the choice of site to be inseminated was not dependent on full occupancy of preferred insemination sites, but the mating history of the female. However, the number of spermatangia attached to BM was lower than those attached to other sites, whereas the maximum number of stored spermatangia was found greater in EYE among the sites. Female maturity status influenced the insemination patterns, but not the number of stored spermatangia at any insemination site. These results suggest that males inseminate at different locations in a female according to the mating history and maturity status of the female.

CHAPTER 3

Same male inseminates at multiple locations within a female kobi squid regardless of polyandry

3.1. Introduction

Well over a century ago the theory of natural selection was introduced where all individuals compete for survival and reproduction (Schulpp, 2021). Another important force in evolution is sexual selection, where members of the same sex compete with each other for partners of the opposite sex, resulting in unequal success among individuals of the same species (Anderson, 1994). Thus, this form of selection favors the maintenance of traits that enhance reproductive success, even if they are costly (Anderson, 1994; Shuster and Wade, 2003). There are both intersexual mechanisms (*e.g.*, selective mate choice by females) and intrasexual mechanisms of sexual selection (*e.g.*, competition between males for access to females) (Anderson, 1994; Birkhead and Moller, 1998; Shuster and Wade, 2003).

As a prerequisite for postcopulatory sexual selection, and as a prevalent mating pattern across animal taxa, polyandry (a single female mating with multiple males during a single breeding/spawning cycle) has been identified based on research on behavioral and molecular ecology (Brockman et al., 1994; Jones and Avise, 1997; Birkhead and Pizzari, 2002; Grifth et al., 2002; Parker and Birkhead, 2013). Females are benefited directly or indirectly at greater extent by mating with multiple males than with a single one, resulting in offspring with “good genes” or higher genetic diversity (Fisher

et al., 2006; Boulton and Shuker, 2015). In addition, the costs of polyandry (or polygamy) are borne by both sexes through increased predation risks, disease, and coercion in courtship or copulation (Magnhagen, 1991; Shuker and Day, 2001; Roberts et al., 2015). Conversely, monogamy (each individual mating with only one partner during a single breeding/spawning cycle) as a whole, offers "mutual benefits" for both sexes (Boulton and Shuker, 2015; Laubu et al., 2016; Snekser and Itzkowitz, 2019) or "unilateral benefit" to one sex over the other (e.g., postcopulatory mate guarding). Monogamy is preferred only in environments where there is not any opportunity or advantage to monopolize mates (Emlen and Oring, 1977).

Moreover, to achieve reproductive success, males often alter their mating behaviors in response to environmental, intersexual, and intrasexual conditions. Such behavioral plasticity during mating by males is conceptualized by the theory of evolutionary stable strategy under promiscuous circumstances. For example, sperm allocation, a type of male-oriented mate choice, involves cost-effective distribution of reproductive resources. Because even though a substantial number of sperm are produced by males, sperm are regarded as a limited reproductive resource (Squires et al., 1979; Parker et al., 1996; Parker et al., 1997). Thus, the theory predicts that males allocate their ejaculate expenditure to females in response to future male mating opportunities (Pitnick and Markow, 1994) or the reproductive conditions of the focal females (Wedell et al., 2002) such as fecundity (Simmons et al., 1993; Reinhold et al., 2002) or promiscuity (Gage and Barnard, 1996; Simmons and Kvarnemo, 1997; Pilastro et al., 2002; Evans et al., 2003; Pizzari et al., 2003), in favor of a cost-benefit trade-off within the context of promiscuous mating. If so, males should immediately evaluate individual females and decide the extent of sperm expenditure to invest in each female, in accordance with their

estimated reproductive value. Sperm allocation is known to occur in a wide range of taxa, including insects (Pitnick and Markow, 1994; Gage and Barnard, 1996; Simmons and Kvarnemo, 1997), crustaceans (Yu et al., 2022), fish (Evans et al., 2003; Kondo et al., 2020) and birds (Pizzari et al., 2003; Alvarez-Fernandez et al., 2019).

Cephalopods exhibit some of the most complex behavioral adaptations amongst marine invertebrates, especially with respect to their mating strategies (Hanlon and Messenger, 1996). They are mostly highly promiscuous, and females of most species store sperm from multiple males and for long periods of time (Naud and Havenhand, 2006). In conformity with this, most loliginid squids engage in complex mating behaviors (Hanlon et al., 1997; Sauer et al., 1997; Hanlon et al., 2002; Hanlon et al., 2004; Iwata et al., 2005), where the females copulate with multiple males over short time periods (minutes to hours) and store sperm in two separate locations in/on their bodies. Larger males (consorts) transfer spermatophores adjacent to the proximal end of the oviduct inside the mantle cavity, whereas smaller males (sneakers) deliver sperm on the female buccal membrane located apart from the oviduct (Iwata et al., 2011; Shashar and Hanlon, 2013). Their polyandrous mating regime is also indicated by insemination of a female by dimorphic sized males (Van Camp et al., 2004; Buresch et al., 2009; Naud et al., 2016).

On the contrary, in inshore kobi squid *Loliolus. sumatrensis,* a species of loliginidae family under myopsida order, females possess three distinctive sperm deposition sites: buccal membranes (BM), basal areas of the left IV arm (ARM), and lateral head behind the left eye (EYE), where sperm are transferred from monomorphic sized males. The occurrence of inseminations at three different locations in a female, but absence of male size dimorphism or trimorphism prompted us to investigate into the

paternity levels of females to know whether they are polyandrous or monogamous. Therefore, microsatellite loci were identified and used to measure the level of paternity of the deposited spermatangia. In addition, parentage analyses were compared among three sperm-deposition sites to explore the site with higher paternity number. However, we hypothesized that they are polyandrous, and three sites are used by different males. Here we show genetic evidence for a unique behavior of sperm allocation in squid.

3.2. Materials and Methods

3.2.1. Sample source and species verification

The kobi squids, *Loliolus sumatrensis* in seto inland sea were obtained from a fishery at the Shodo Island, Kagawa, Japan during the fishery season (July-September) of this species in 2021 and 2022 and transported as dead specimens at either 4 ^{o}C or -20 ^{o}C. The resembling species, *Loliolus japonica* were obtained from the same fishery in May 2022.

First, species verification was conducted based on morphology, especially the third arms and tentacles with largest sucker rings (Jereb and Roper, 2005), led us to differentiate from closely related, phenotypically alike species like *L. japonica* and *L. beka*. However, the morphology of sucker rings in *L. sumatrensis* and *L. uyii* were identical, therefore species was validated through a rapid PCR based diagnosis assay. For high fidelity PCR validation, we developed species-specific primers for *L. sumatrensis* and *L. uyii*, and universal primers those likely amplify *Loliolus* species, by using their mitochondrial genomic cytochrome c oxidase (*COI*) sequences (Fig. 3.1). The primer sequences are: *L. sumatrensis*-specific *COI* forward primer: 5'-

CCTATTATAATTGGAGGCTTT -3', *L. sumatrensis*-specific *COI* reverse primer: 5'-CTACTGAAGGTCCTGCGTGT -3', *L. uyii*-specific *COI* forward primer: 5'-CCCATTATAATCGGAGGTTTC -3', *L. uyii*-specific *COI* reverse primer: 5'-CTACTGAGGGTCCTGCATGA -3'. *Loliolus* universal *COI* forward primer: 5'-CAATGTAGTAGTAACTGCTCACGG -3', *Loliolus* universal *COI* reverse primer: 5'-GCTCCTAAAATAGAAGAAATACCA -3'. The PCR based diagnosis was carried out for representative specimens with genomic DNAs purified from testes with kits (QIAGEN Genomic-tip 20/G and TAKARA NucleoSpin® Tissue), and the developed species specific as well as universal primer sets. PCR was run with 20 ng genomic DNA, 0.5 μM primers and KAPA2G Robust PCR Kit (Kapa Biosystems) according to a kit-provided standard protocol with annealing temperature at 58°C and 35 cycles, followed by 3% agarose-gel electrophoresis.

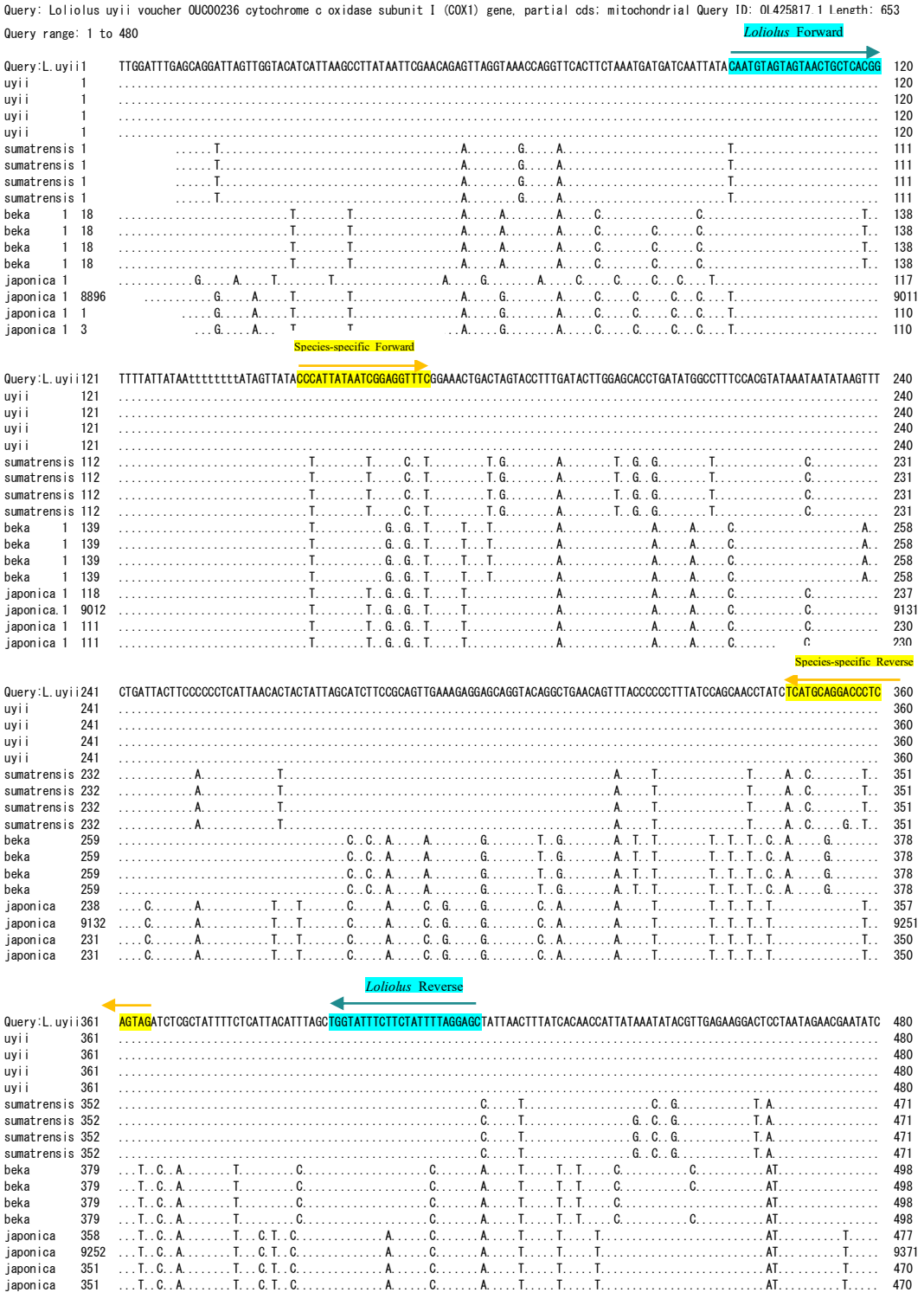

Fig. 3.1. Designed species-specific primers and universal primers for high fidelity PCR validation of loliginid kobi squid. Here, *L. uyii* and *L. sumatrensis* species-specific primers (*yellow*) and *Loliolus* universal primers (*cyan*) were developed from alignment of mitochondrial *COI* sequences of *L. uyii, L. sumatrensis, L. beka and L. japonica* (four representative individuals per species) taken from the GenBank database.

3.2.2. Development of SSR markers

Genomic DNAs were purified from testes (wet weight of ~20 mg) of 47 representative mature males with kits; (QIAGEN Genomic-tip 20/G and TAKARA NucleoSpin® Tissue) according to manufacture protocols, verified their degradation levels with 0.8% agarose gel electrophoresis followed by visualization with ethidium bromide, and quantified their yield and quality with a micro-volume spectrophotometer and stored at -80ºC. Short-read whole genome sequencing (150 bp paired-end, Novaseq6000/PE150, Novogene) was carried out, which yielded a total of 25.7 million clean reads (97.5% of raw reads) that were thereafter merged with PEAR, resulting in 3.19 million overlapped paired-end reads. The row reads were registered in the DNA Data Bank of Japan (DDBJ) Sequence Read Archive under accession number: DRA015757 (Submission), PRJDB15292 (BioProject), SAMD00579664 (BioSample) and DRX430961 (Experiment). Next, the sequence data were uploaded to Galaxy/NAAC to search for simple sequence repeat (SSR) with a MISA + Primer 3 pipeline, which detected a total of 22,298 SSRs. For an initial PCR test, 20 SSRs were selected at random and non-labelled primers synthesized. PCR was carried out with a kit (Platinum™ Direct PCR Universal Master Mix) and a thermal cycler (MiniAmp, Thermo Fisher Scientific) at optimized conditions: 20 ng of genomic DNA, 0.2 µM paired primers and PCR reaction consisting of an initial denaturing step of 94 °C for 2 min, then 40 cycles of 94 °C for 15 sec, 56 °C for 15 sec and 68 °C for 20 sec followed by a final extension of 68 °C for 5 min. Amplicons with genomic DNAs from 10 male individuals were subjected to run on 8% mini-slab polyacrylamide gel electrophoresis to verify apparent variability in size. Thereafter, four primer sets were validated temporally and fluorescence (Hex, Fam, Cy3 and Ros)-tagged forward primers synthesized. Fragment length analysis (ABI PRISM 3130xl Genetic Analyzer) was performed with GeneScan™ 600 LIZ dye Size Standard (Thermo Fisher Scientific).

Subsequently, the four selected microsatellite loci were fully characterized by open-source tools, OSIRIS (National Institutes of Health) v. 2.16 and GenAlEx v.6.5.1 (Peakall and Smouse, 2012).

3.2.3. Paternity analysis

Under a stereomicroscope, the female tissues (from three insemination sites- BM, ARM and EYE) that contain attached spermatangia were dissected out and fixed in 70% ethanol for 1hr, thereafter every single spermatangium removed with fine forceps from the tissue was placed carefully into the bottom of each well of the 96-well plate. To each well, 10 µl of lysis buffer containing 0.1 mg/ml protease-K (Direct PCR Master mix kit, Thermo Fisher Scientific) was added, followed by a 30-min incubation at 52ºC and heat (95ºC) inactivation for 1 min. The plates were kept frozen at -20ºC until use. Occasionally, the lysates in plates were spun down to precipitate undigested debris immediately prior to use. The fragment length analysis was carried out to genotype the spermatangia, where the amplification of newly developed microsatellite markers (SSRs) was performed with polymerase chain reaction with fluorescently tagged forward primer and non-labeled reverse primer, and the PCR condition was same as described in the previous section of "SSR development" except that multiplex PCR was carried out.

3.2.4. Statistical data analysis

The statistical data analyses were performed with JMP Pro software, version 17.0.0 and SPSS software, version 23.0. The parametric assumptions were met for the statistical analysis. Linear Mixed Models (LMMs) were fitted with sample ID as random effect to

analyze the site-dependent multiple paternity. The paternity share in each site of a female was analyzed through Microsoft excel.

3.3. Results

3.3.1. Validation of species as *Loliolus sumatrensis*

The species was validated as *Loliolus sumatrensis* by the dentition morphology of the largest sucker rings in the tentacles and III arms (Jereb and Roper, 2005), and thereafter by PCR based diagnosis using species-specific primer sets and *Loliolus*- universal primer sets (Fig. 3.2). Here, the morphologically similar *L. sumatrensis* and *L. uyii* were found distinguishable by the species-specific primers developed from their mitochondrial *COI* genomic DNA sequences. The *Loliolus*- universal primers were verified by using gDNA isolated from *L. japonica* (Fig. 3.2).

3.3.2. Development and validation of polymorphic SSR markers

Through the first and second rounds of screening (see Materials and methods) with genomic DNAs isolated from 47 individual *L. sumatrensis* specimens, four highly polymorphic SSR markers (luy1738, luy0529, luy4288, luy3099) were successfully developed. Across the four SSR loci, the number of alleles ranged from 14 to 30 among the 43 individuals (Table 3.1; Appendix VI) and the expected heterozygosity was found to be from 0.92 to 0.94. Two SSR loci (luy1738, luy3099) did not deviate significantly from Hardy-Weinberg equilibrium (p value >0.05), while other two (luy0529, luy4288) showed significant deviation. The multi-locus probability of identity (Pid) value of the SSRs was 1.6×10^{-8} which is far below the threshold value of 1.0×10^{-4} required to reliably distinguish between individual genotypes (Waits et al., 2001). Nucleotide sequences of SSR markers and their characteristics are shown in Table 3.1.

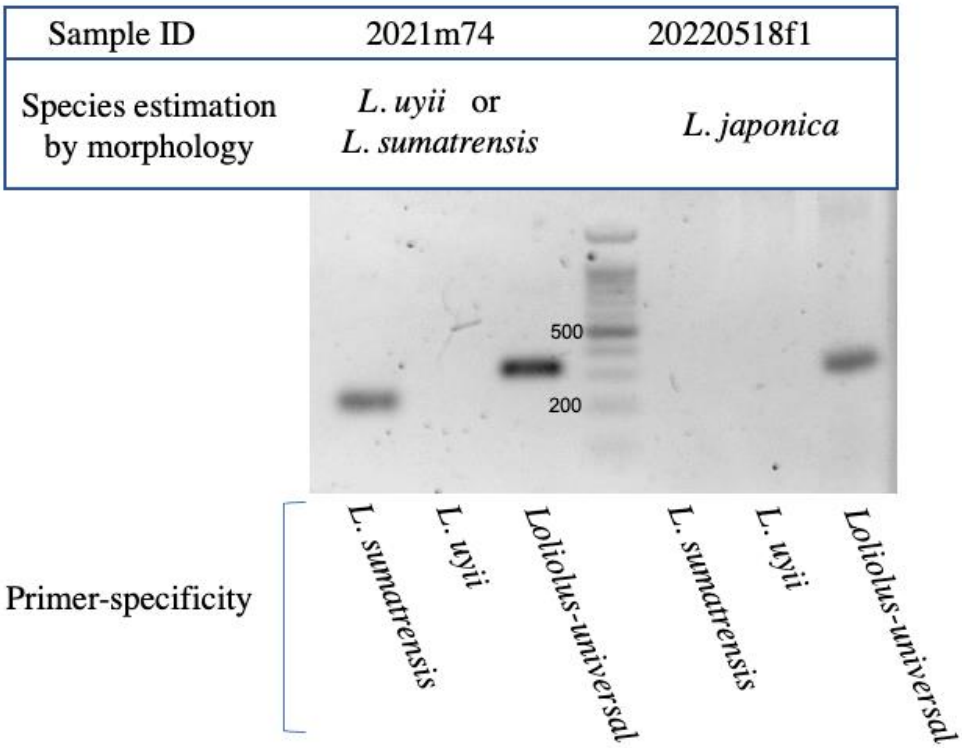

Fig. 3.2. Validation of species as *L. sumatrensis* through a rapid PCR-based diagnosis assay. This assay was performed with use of species-specific primer sets, by which morphologically similar *L. uyii* and *L. sumatrensis* were found distinguishable. Shown were representative results with genomic DNAs from *Loliolus s*quids collected in mid-summer (individual ID: 2021m74) and in early summer (individual ID: 20220518f1). Species collected in May were assingned morphologically as *L. japonica.*

Table 3.1. Information and characterization of newly developed SSR markers for *Loliolus sumatrensis*

Locus	Repeat	Forward sequence	Reverse sequence	N	Na	Ho	He	Fis	Pid
luy1738	(AGA)24	ATGCGGAAAGGTGTGATTGT	TTTATGCCCCTCTTCCTCCT	46	22	0.826	0.922	0.153	0.014
luy0529	(CTT)25/(TTA)17	TAACTGCAATGCCCAATCTG	CAAACACGCTGGCGATATAA	43	30	0.628	0.939	0.342*	0.007
Luy4288	(ATA)18	AAGACTCCAATGAAAGACCACT	CAGAAGCCACAAATCGCCTA	47	22	0.766	0.932	0.188*	0.009
Luy3099	(TTA)21	CCATTTAAACACGAGATGCAA	CCAGTTAACGTTGGTGTGAAAA	44	14	0.905	0.942	0.001	0.019

N, sample size; Na, number of alleles; Ho, observed heterozygosity; He, expected heterozygosity; Fis, fixation index; *, significance of departure from Hardy-Weinberg Equilibrium ($P<0.01$); Pid, probability of genetic identity

3.3.3. Genotyping-based documentation for the site-dependent promiscuity

To address the level of paternity at each insemination site, we developed microsatellite or SSR markers (Table 3.1), by which every spermatangium attached to each site (BM, ARM and EYE) was genotyped. Genotyping of attached spermatangia was carried out from three females, whereas female #01 possessed 252 spermatangia (12 at BM, 111 at ARM, 129 at Eye); female #02 had 313 spermatangia (28 at BM, 80 at ARM, 205 at EYE), and female #03 carried 535 spermatangia (97 at BM, 238 at ARM, 200 at EYE) (Table 3.2). Each female mated with 7 to 9 males, suggesting that *L. sumatrensis* are polyandrous. Moreover, each insemination site showed multiple paternity (Fig. 3.3). We found that the actual number of sires per site was higher at ARM (6.3 ± 2.5, $n = 3$) than at the other two sites (BM, 2.3 ± 0.5; EYE, 4.0 ± 2.6, $n = 3$), but there were no significant differences in number of sires among the three sites (GLMM, $P > 0.05$).

3.3.4. Multiple-site usage by single males

Surprisingly, we found that a few males inseminated simultaneously at multiple (two or three) sites on the same female. As summarized in Table 3.2, three males in female #01, two males in female #02 and four males in female #03 used multiple sites together (indicated by *,**). In addition, these small numbers of sires were found to have a major part of the paternity share ($95.4\% \pm 2.7\%$) in the total attached spermatangia (Table 3.2; Fig. 3.4), suggesting their dominancy in sperm contribution in the respective female.

Table 3.2. Paternity identification and paternity sharing of the spermatangia stored at three different insemination sites within a female kobi squid

female #01							
				Number of spermatangia			
Insemination site				BM	ARM	EYE	Combined (%)
Total spermatangia attached				12	111	129	252 (100)
Genotyping unexamined or failed				5	63	50	118 (46.8)
Genotyping succeded				7	48	79	134 (53.2)
	SSR locus						
Genotype	Luy1738	Luy3099	Luy4288	5	63	50	
# 1 sire**	137/140	148/157	166/175	6	16	60	82
# 2 sire*	161/173	136/145	169/175	0	24	14	38
# 3 sire	119/119	115/145	166/175	0	5	0	5
# 4 sire	149/164	148/157	154/178	0	0	5	5
# 5 sire*	161/164	154/160	172/171	1	1	0	2
# 6 sire	167/173	136/145	169/175	0	1	0	1
# 7 sire	158/167	139/157	154/166	0	1	0	1
Number of sires				2	6	3	7

female #02							
				Number of spermatangia			
Insemination site				BM	ARM	EYE	Combined (%)
Total spermatangia attached				28	80	205	313 (100)
Genotyping unexamined or failed				22	10	106	138 (33.8)
Genotyping succeded				6	70	99	175 (66.2)
	SSR locus						
Genotype	Luy1738	Luy3099	Luy4288	22	10	106	
# 1 sire**	143/143	136/142	184/184	5	35	73	113
# 2 sire**	140/152	145/154	163/178	1	33	18	52
# 3 sire	161/173	136/145	169/175	0	0	2	2
# 4 sire	140/140	157/157	166/175	0	0	2	2
# 5 sire	116/119	145/145	169/214	0	1	0	1
# 6 sire	158/158	139/142	166/190	0	1	0	1
# 7 sire	149/164	139/142	154/178	0	0	1	1
# 8 sire	137/155	145/148	175/184	0	0	1	1
# 9 sire	140/152	142/157	160/169	0	0	1	1
Number of sires				2	4	7	9

Table 3.2. (Contd.)

female #03							
				Number of spermatangia			
Insemination site				BM	ARM	EYE	Combined (%)
Total spermatangia attached				97	238	200	535 (100)
Genotyping unexamined or failed				52	155	120	327 (61.1)
Genotyping succeded				47	83	80	210 (38.9)
	SSR locus						
Genotype	Luy1738	Luy3099	Luy4288	52	155	120	
# 1 sire*	143/167	148/163	172/175	0	32	64	96
# 2 sire*	167/167	151/157	172/181	36	27	0	63
# 3 sire**	143/143	136/160	172/175	10	16	16	42
# 4 sire*	167/167	145/151	172/181	1	1	0	2
# 5 sire	158/158	142/148	175/202	0	2	0	2
# 6 sire	116/116	142/145	166/166	0	2	0	2
# 7 sire	137/155	145/148	187/187	0	1	0	1
# 8 sire	143/143	136/136	172/214	0	1	0	1
# 9 sire	164/164	142/151	166/172	0	1	0	1
Number of sires				3	9	2	9

**indicates the sires those inseminated at three sites of a female;

* indicates the sires those inseminated at two sites of a female

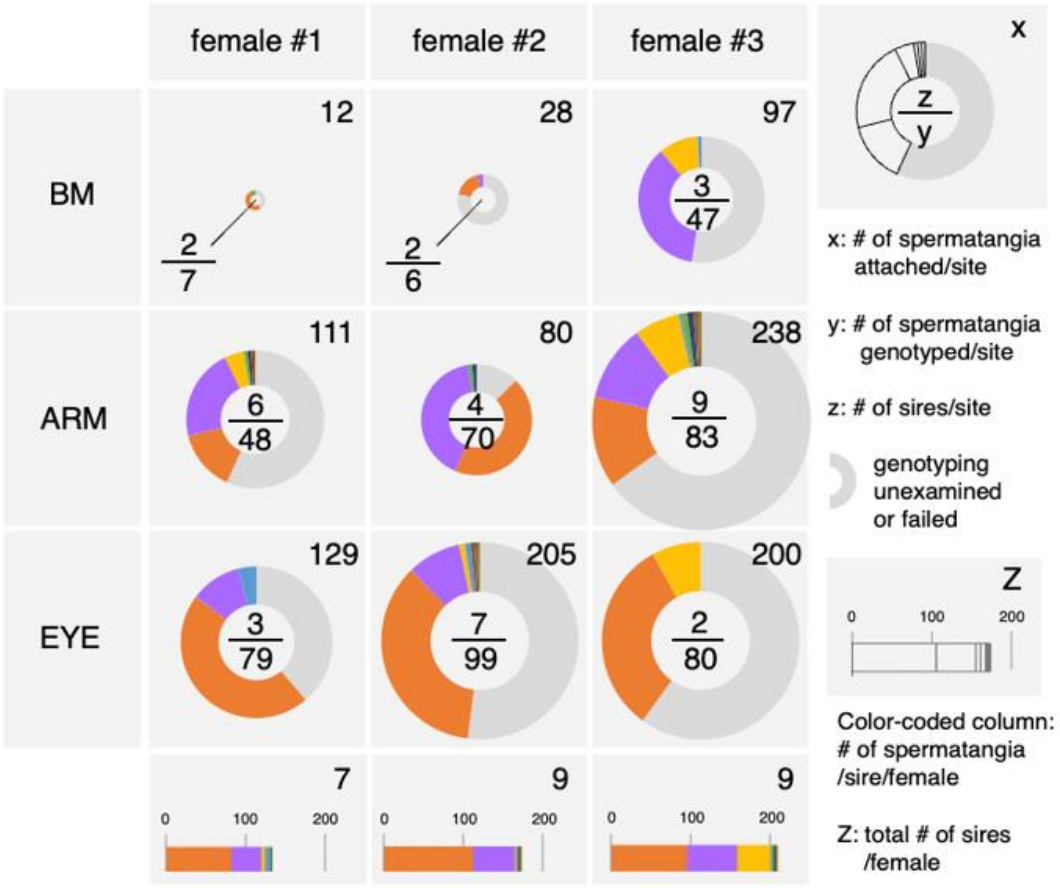

Figure 3.3. The insemination site-dependent spermatangium number and sire number in *L. sumatrensis*. Genotyping of attached spermatangia was carried out from three females. The number of spermatangia attached to each insemination site was shown (x, *top-right in each panel with a pie chart*), of which the number of spermatangia successfully isolated/genotyped was indicated as the denominator (y) and sire number was indicated as the numerator (z). The lower panels represent the total number of sires (Z, *top-right*) and total number of spermatangia/sire/female which was color-coded in the stacked columns. The size of each pie chart corresponds with the total number of attached spermatangia/site.

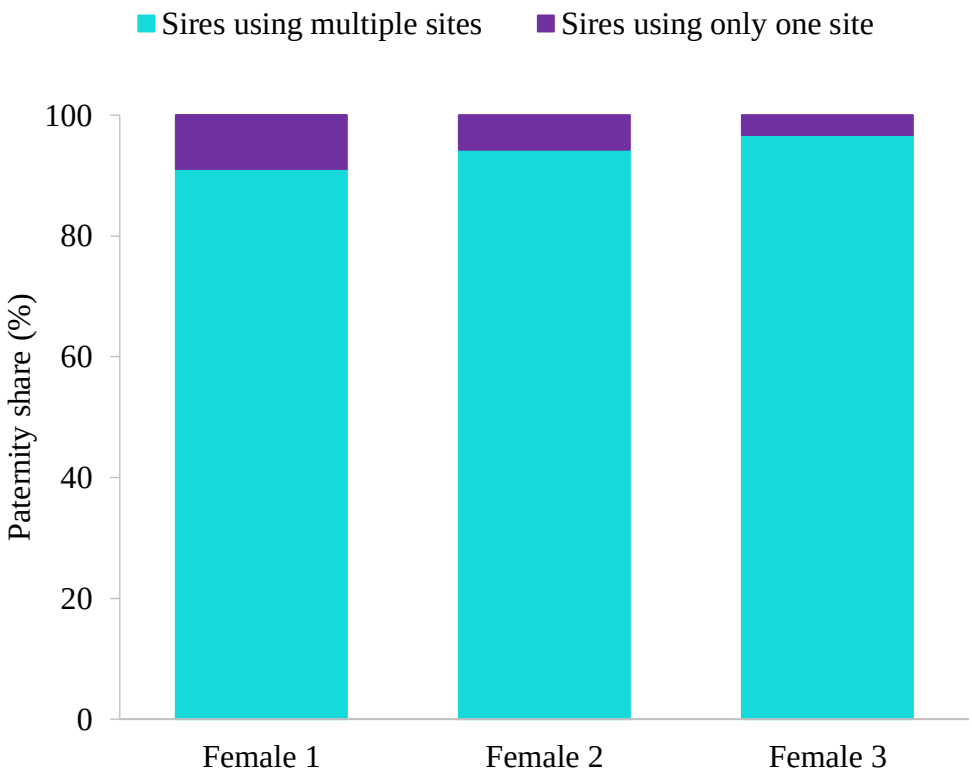

Fig. 3.4. A major part of the paternity shared by the sires using multiple sites simultaneously on a female kobi squid.

3.4. Discussion

Because sperm are a limited reproductive resource, males in polygamous species may be adapted to allocate their ejaculate expenditure to females effectively (Wedell et al., 2002). Thus, sperm allocation by an individual male occurs in response to sociosexual environments that could be influenced by the status of females, rivals, and that male's own condition (Wilson et al., 2014). To achieve maximum reproductive success, males must evaluate sociosexual orientations through the perception of visual, chemical, acoustic, and tactile cues (Xu and Wang, 2014; Esfandi et al., 2020; Cong and Wang, 2021). One of the key elements that could impact on male mating behavior regarding sperm allocation is the risk of sperm competition, which is a powerful evolutionary driver (Parker and Pizzari, 2010). Sperm competition also drives developmental, morphological, and behavioral plasticity in relation to sex.

In most loliginid squids, males are dimorphic in size, where larger males and smaller males alternatively use two separate locations in a female. Consequently, the females pursue polyandrous mating behaviors with dimorphic males (Naud et al., 2016; Lin and Chiao, 2017), where sperm produced by the smaller males are greater in number and swimming speed than bourgeois males (Dougherty et al., 2022).

Conversely, loliginid squid species *L. sumatrensis* females have three insemination sites (BM, ARM and EYE) located discontinuously in their body and males show no dimorphism in body size. Despite these differing characteristics from other species of this family, we found the *L. sumatresnsis* as polyandrous, with one female mating with seven to nine monomorphic males. This might be related to some other species of squid in other family or other taxa, including birds, mammals, insects, etc.,

where males are also not dimorphic (Birkhead and Moller, 1998; Hosken and Stockley, 2000; Sato et al., 2023). By mating with more than one male (female polyandry), the females gain material (direct) benefits derived from mating resources (e.g. nuptial gifts) and genetic benefits (indirect) from the males having better physical status (e.g. good genes) resulting in production of offspring with higher genetic diversity (Zeh and Zeh, 2001; Fedorka and Mousseau, 2002; Radwan, 2003; Slatyer et al., 2012). In addition, polyandrous females obtain over a two-fold greater hatching success and a 43% greater offspring survivorship, leading to a significantly higher relative fitness than the monoandrous strategy (Fedorka and Mousseau, 2002). Considering these, each *L. sumatrensis* female might allow more males to copulate with and deposit the male-delivered-sperm at three different locations in her body according to the current sperm storing status.

Since they were found to be polyandrous, we speculated that three insemination sites in a female are used by different males. But the present study presents a surprising finding that there were few males those inseminated at multiple sites simultaneously within a female and these handful number of males are dominant in paternity sharing in the respective females. Therefore, we considered the possibility of sperm allocation occurring within a female, which could be explained by theoretical models for sperm allocation.

Sperm allocation, defined as the total number of sperm allocated by a male to a particular female, is a consequence of sperm competition, whereas sperm competition is an important component of sexual selection occurring when two or more males copulate with a particular female during the same reproductive cycle, and their sperm

compete to reach the female's available eggs for fertilization (Parker, 1970; Birkhead & Møller, 1998). Two theoretical models predict how males adjust their sperm allocation in response to sperm competition: the risk and the intensity models (Parker et al., 1996; Parker et al., 1997; Parker, 1998; Parker and Pizzari, 2010). The sperm competition risk model involves two males competing for the same set of eggs (Parker et al., 1997), where the 'risk' is the probability that the ejaculate of one of the two males will compete against the ejaculate of the other male. This model predicts a lower sperm allocation when the risk of sperm competition is low and a higher sperm allocation when the risk is high (Parker et al., 1997; delBarco-Trillo, 2011). On the other hand, the sperm competition intensity model applies when females can mate with more than two males (Parker et al., 1996; Wedell et al., 2002). The intensity model predicts that as the intensity or number of male increases, a male should reduce his sperm allocation (Parker et al., 1996; Wedell et al., 2002) because with increasing the number of competing ejaculates, the benefit accrued by the increment of sperm allocation decreases (Parker et al., 1996; delBarco-Trillo, 2011). In agreement with these theoretical models, two or three sires found in each female examined contributed higher number of spermatangia due to high sperm competition risk (Table 3.2). However, with increasing the number of males, the late-coming males in each female started to contribute relatively lower amount of sperm (Table 3.2).

The males who contributed greater amount of sperm were found to use multiple sites (Fig 3.4). They might inseminate at multiple sites for maximizing the success rate of fertilization of their sperm with the mated females' eggs, although the routes for each site to reach the egg for fertilization are different. On the contrary, some other males chose only one site among three to invest less amount of sperm. It suggests

that males might adjust their ejaculate investment depending on the competition among rivals as well as the suitability of the insemination site within a female to utilize the sperm.

However, among the sites, although ARM exhibited the highest average paternity number (Fig. 3.3), further genotyping with more specimens is required to evaluate the site-dependent promiscuity. In conclusion, we found that males allocate sperm not only among the females, but also within a female.

3.3.5. Summary

The paternity level of inshore loliginid species of squid, *Loliolus sumatrensis* females, receiving sperm from monomorphic males at their three different body locations (BM, ARM and EYE), were explored by genotyping their spermatangia attached at each site. The parentage analysis of Seto inland sea population through using newly developed four polymorphic microsatellite markers showed that each female mated with 7 to 9 males, suggesting that *L. sumatrensis* are polyandrous. Moreover, each insemination site exhibited multiple paternity. The actual number of sires per site was found higher at ARM than at the other two sites. Surprisingly, a few males inseminated simultaneously at multiple (two or three) sites on a female and these small numbers of sires were found to have a major part of the paternity share in the total attached spermatangia. On the other side, a greater number of males those combinedly contributed a negligible portion in total paternity of the respective female used only one site. It suggests that besides allocating ejaculate investment based on the sperm competition risk and intensity, males might adjust their budget of ejaculation within a female depending on the suitability of insemination place for maximizing fertilization rate.

CHAPTER 4

Genetic evidence for mating system in pair forming oceanic squid, *Thysanoteuthis major*

4.1. Introduction

Molecular approaches in behavioral ecology over the recent decades have uncovered polyandry, a system in which one female mates with more than one male during a single breeding/spawning cycle, as a prevalent mating mode across animal taxa (Brockmann et al., 1994; Jones and Avise, 1997; Birkhead and Pizzari, 2002; Ohtani et al., 2022). Even in socially monogamous mammals and birds, females often mate outside their partner, known as extra pair copulation (EPC). EPC can be favored for both sexes, especially for females compared to adopting a single mating mode of either monogamy or polyandry, simply because females can receive collective benefits from both modes (Clutton-Brock, 2007; Trivers, 2017). On the contrary, genetic (true) monogamy, where one male and one female mate exclusively with each other, are very rare (Wittenberger, 1979; Petrie and Kempenaers, 1998; Isvaran and Clutton-Brock, 2006; NSF, 2013; Huck et al., 2014; Dolotovskaya et al., 2020). However, true monogamous or socially monogamous males often engage in pair-forming that is associated with mate guarding, food giving and paternal care for the young. On the other side, polyandrous females can receive direct and indirect benefits from multiple males such as nuptial gifts and different genetic elements, respectively (Zeh and Zeh, 2001; Whittingham and Dunn, 2010; Slatyer et al., 2012). In cephalopods, polyandry is prevalent but there are very few species reported to be monogamous (for example, 92-95% of females show genetic monogamy in *Watasenia*

scintillans) (Hanlon et al., 1999; Van camp et al., 2004; Franklin et al., 2012; Hoving et al., 2012; Ylitalo-Ward, 2014; Sato et al., 2020).

EPC has been documented in some squids and cuttlefish and may have developed as a counterstrategy against a consort tactic in which larger males can have priority to mate, while smaller males make sneaky copulations at the moments close to egg spawning (Gross, 1996; Dijkstra and Border, 2018). In cephalopods, it is uncommon that males regularly engage pair-boning before or after copulations. Exceptionally, the long-term pair-boning behavior has been reported to occur in the diamond squids, *Thysanoteuthis* spp. *Thysanoteuthis major* is the most abundant among the three allopatric species of the monotypic family Thysanoteuthidae under oegopsida order (Turgeon et al., 1998; Bower and Miyahara, 2005; Deville et al., 2024). They are large (>1m in adult body size and maximum 30 kg of body weight), semelparous, oceanic squids widely distributed in the eastern and western North Pacific Ocean, North Indian Ocean, and the limits of the warm current of the Indian Ocean close to the South Atlantic Ocean (Okutani, 1990; Miyahara et al., 2005; Hochberg and Camacho-García, 2009; Deville et al., 2024) and release planktonic egg masses. They are not active swimmers during most of its life cycle, rather, it propels itself slowly by gentle undulation of its long, broad, diamond-shaped fins; Nevertheless, is capable of a powerful reactive jet of short duration when threatened. Curiously, their social organization is unique among squids, because they are often observed in pairs of one male and one female with similar sizes. Anecdotal evidence suggests that the pairing begins at an immature stage and continues into adulthood. Their arm lengths and probably the anal photophores play the key role in pair formation in immature squids. Their such pairing lifestyle indicates behavioral monogamy (Nishimura, 1966; Nigmatullin et al., 1995; Jereb and Roper,

2010). Currently, however, there is no evidence supporting genetic monogamy, which led us to investigate the molecular basis of these relationships.

In this study, we aimed to determine the level of female promiscuity by genotyping stored sperm from multiple storage organs, seminal receptacles (SRs) within a female using newly developed polymorphic microsatellite markers. In this species, like many other squids, male and female mate in head-to-head position (Nigmatullin et al., 1995). The male transfers its spermatophores to the female via the hectocotylus, which attaches to the surface of the female's buccal membrane. The sperm released from the transferred spermatophores migrates, by unknown mechanism, into the SRs located at the ventral side of the buccal membrane. Here, we show genetic evidence for female promiscuity. Interestingly, similar sperm storage pattern was found in all the SRs within a female, where paternity share was biased to presumably a single dominant male. We discuss possible causes of their pair-bonding.

4.2. Material and Methods

4.2.1. Sample collection

The mature squid samples were collected from a fisherman as dead animals around East China sea off Okinawa, Japan during peak spawning season (April to July) in 2023. During this period, we obtained 37 individuals. In addition, three pairs of immature samples were collected from Sea of Japan off Hyogo, Japan in November 2023. All the mature and immature samples were transported in ice-cold or frozen conditions.

4.2.2. Species identification

First, species identification was carried out by their morphological observations, i.e. the presence or arms with two series of suckers and tentacular clubs with four series, and specifically by their diamond-shaped fins (Roper et al., 1984). However, there are three allopatric species of the monotypic squid family Thysanoteuthidae. Among them, *Thysanoteuthis major and Thysanoteuthis rhombus* are morphologically similar, closely related sister species, whereas *Thysanoteuthis* cf. *filiferum* is the most divergent species (Deville et al., 2024).

Therefore, species was confirmed through PCR based diagnosis. For high fidelity PCR validation, using the respective species' mitochondrial cytochrome c oxidase (*COI*) genomic sequences from the GenBank database, we developed species-specific reverse primers for *T. major* and *T. rhombus*, and a *Thysanoteuthis* common reverse primer that was used as positive control, whereas a forward primer was developed that worked in pairing for all primer sets (Fig. 4.1). The *Thysanoteuthis* forward *COI* primer is: 5'- GTTACCGCTCATGGGTTTATTATA -3'; The *T. major*- specific *COI* reverse primer: 5'- CTATATCTGGKGCCCCTAGT -3'; *T. rhombus*- specific *COI* reverse primer: 5'- CTATATCAGGTGCTCCCAGC -3'; *Thysanoteuthis* common *COI* reverse primer: 5'- TTCATYCGAGGGAAAGCTAT -3'.

All the collected specimens were performed PCR based diagnosis with genomic DNAs purified from testes with kits (QIAGEN Genomic-tip 20/G and TAKARA NucleoSpin® Tissue), and the developed species-specific as well as common primer sets. PCR was carried out with 20 ng genomic DNA, 0.5 μM primers and KAPA2G Robust PCR Kit (Kapa Biosystems) according to a kit-provided standard protocol with annealing temperature at 58ºC and 35 cycles, followed by 3% agarose-gel electrophoresis.

Query: *T. major* cytochrome c oxidase subunit I (*CO1*) gene, partial cds; mitochondrial (Deville et al., 2024)

Query range: 1 to 240

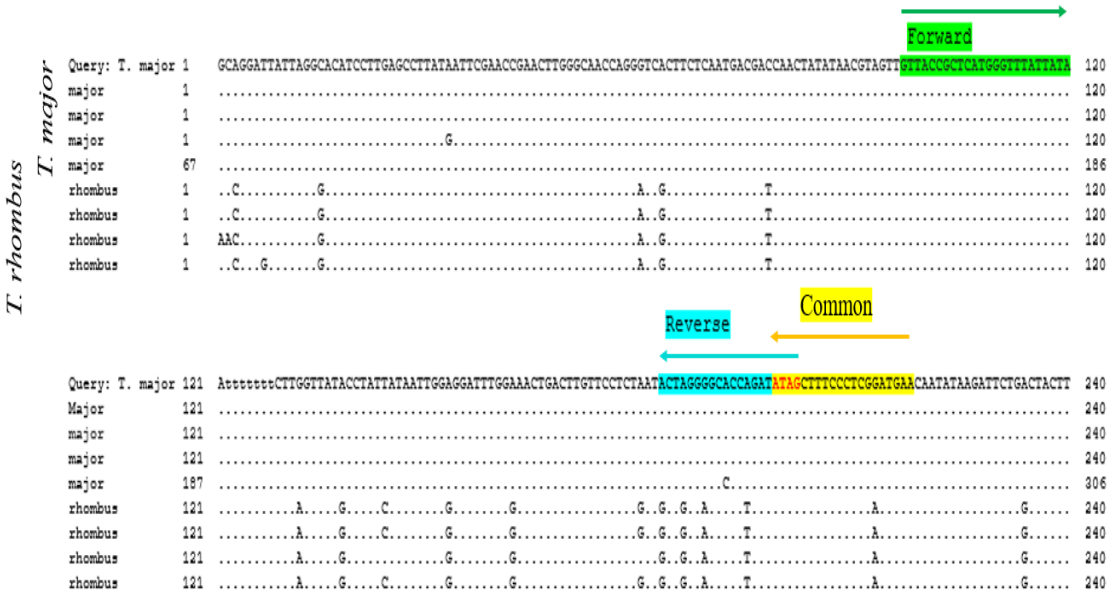

Fig. 4.1. Development of species-specific and common primers for PCR based diagnosis to confirm the species of diamond squid. Here, *Thysanoteuthis*- forward primer (*green*), *T. major* and *T. rhombus* species-specific reverse primers (*cyan*), and *Thysanoteuthis* common reverse primer acted as positive control (*yellow*) were designed from positional arrangement of mitochondrial *COI* sequences of *T. major and T. rhombus.* The sequences for four representative individuals per species were obtained from Deville et al. (2024) and GenBank database.

4.2.3. Development of polymorphic microsatellite markers (SSRs)

First, approximately 200 mg gDNA isolated from testis of a mature male was subjected to next generation sequencing (150 bp paired-end, Novaseq6000/PE150, Novogene), yielding 28,051,256 raw reads. More than three million paired-end reads were recovered after paired-end merger with PEAR. After filtering with a MISA + Primer 3 pipeline (Galaxy/NAAC, Japan) (Thiel et al., 2003), 46,934 SSR candidate sequences were obtained. Among them, 26 SSRs were selected randomly and Primer3 (Untergasser et al., 2012) was implemented to predict primer pairs targeting the flanking regions. PCR amplification was carried out with genomic DNAs isolated from five different individuals and validated by agarose gel electrophoresis, where PCR condition was same as described in previous section of 'species identification'. The primer pairs that gave no band or no obvious polymorphism in band size were eliminated. The remains were further analyzed by polyacrylamide gel electrophoresis with dozen of DNA samples obtained from different individuals. Subsequently, four primer sets were validated temporally and fluorescence (Cy3)-tagged forward primers were synthesized. Fragment length analysis (ABI PRISM 3130xl Genetic Analyzer) was performed with GeneScan™ 600 LIZ dye Size Standard (Thermo Fisher Scientific). Consequently, through open-source tools, OSIRIS (National Institutes of Health) v. 2.16 and GenAlEx v.6.5.1, the four selected microsatellite loci were fully characterized (Peakall and Smouse, 2012).

4.2.4. Microsatellite-based genotyping of sperm from sperm storage organs in female

Genotyping was carried out with sperm stored in sperm storage organs or seminal receptacles (SRs) in female. First, each SR of six females was collected and dissolved in 70% ethanol for 30 minutes. To account for possible errors due to contamination with DNA from host squid, arm-tip tissue samples were also taken. Then, DNA was purified using kits (QIAGEN Genomic-tip 20/G and TAKARA NucleoSpin® Tissue) following prescribed protocol. After validating the quality and quantity of genomic DNA with NanoDrop One Spectrophotometer (Thermo Scientific), singleplex PCR amplification of each of four newly developed microsatellite loci was performed using the respective primers and KAPA2GRobust PCR Kit (Kapa Biosystems), where the reaction mixture contained 100-300 ng DNA and a pair of 0.2 μM fluorescent-labeled and non-labeled primers. Conditions for PCR for all primer sets were initial denaturing of 94°C for 3 min; then 30 cycles of 94°C for 15 sec., 58°C for 15 sec. and 72°C for 15 sec.; and termination of 72°C for 5 min. The PCR amplicons were validated on polyacrylamide gel electrophoresis (PAGE), thereafter fragment lengths were analyzed using an ABI PRISM 3500xl Genetic Analyzer (Applied Biosystems) and sized with GeneScan™ 600 LIZ dye Size Standard (Thermo Fisher Scientific). Consequently, electropherograms were obtained, where the allele sizes were determined using OSIRIS v. 2.16 software (National Institutes of Health).

4.2.5. Estimating the number of alleles for exploring paternity level

For each female, the number of mating males was estimated from seminal receptacle's (SR) content. The number of mating males was estimated according to Naud et al. (2005) and Ohtani et al. (2022). First, the alleles found in the DNA of host squid (maternal

allele) were recorded. Then the electropherograms (with artefacts) of all the SRs were analyzed according to Anonymous (2014) and the number of paternal or nonmaternal alleles (i.e. after excluding those consistent with the female genotype, if present) were counted. However, most of the SRs were not contaminated with the maternal alleles (might be due to the muscle of buccal membrane not being rich in DNA). After counting, the primer with the maximum allele number was selected because the four newly developed primers were used for analyzing the SRs. We then calculated a conservative estimate of the minimal number of possible male mates through dividing the number of nonmaternal alleles by two, assuming that females copulated with only heterozygous males. In cases where the number of nonmaternal alleles were odd, the predicted minimum number of males was rounded up to the next whole number, (e.g. if the number of non-maternal allele was 7, the estimated least number of males was rounded up to 4). Thus, the paternity level, for each SR and thereby for each female, could be estimated.

4.2.6. Comparative analysis of allele peak patterns among the seminal receptacles within female

In terms of peak area, the proportion of each paternal allele were compared among all the seminal receptacles (SRs) within a female. Thereby, the similarity or dissimilarity in the peak patterns among the SRs within female could be identified. In addition, the rate of paternity share, in terms of peak area, for the alleles within the SRs were also estimated to investigate if there is any dominancy of allele in sperm contribution. Microsoft excel was used for these data analyses and graphical presentations.

4.3. Results

4.3.1. Species confirmed as *Thysanoteuthis major*

The species was confirmed as *Thysanoteuthis major* by high fidelity PCR validation using species-specific primer sets and a *Thysanoteuthis* common primer sets working as positive control. The agarose electrophoresis of the PCR products of gDNA for all samples showed band at *T. major*- specific primer and common primer, but not at *T. rhombus*- specific primer. It suggests that all the 43 specimens collected were *T. major* (Fig. 4.2).

4.3.2. Anatomical views of sperm storage organs in mature and immature females

The mature females possessed pink colored seminal receptacles (SRs), filled with sperm, at the ventral side of the buccal membrane (Fig. 4.3A). The number of SRs in each mature female was 18.8 ± 7.9 (n = 6) (Table 4.2). However, unlike most other squids, no spermatangia were found in the females. Interestingly, the immature females were also found to have fewer, smaller, white (similar color as muscle of Buccal membrane) SRs located ventrally at their buccal membrane (Fig. 4.3 B). However, the SRs of immature females were not found to carry any sperm, although the immature ones were also moving in pairing of a male and a female while fishing.

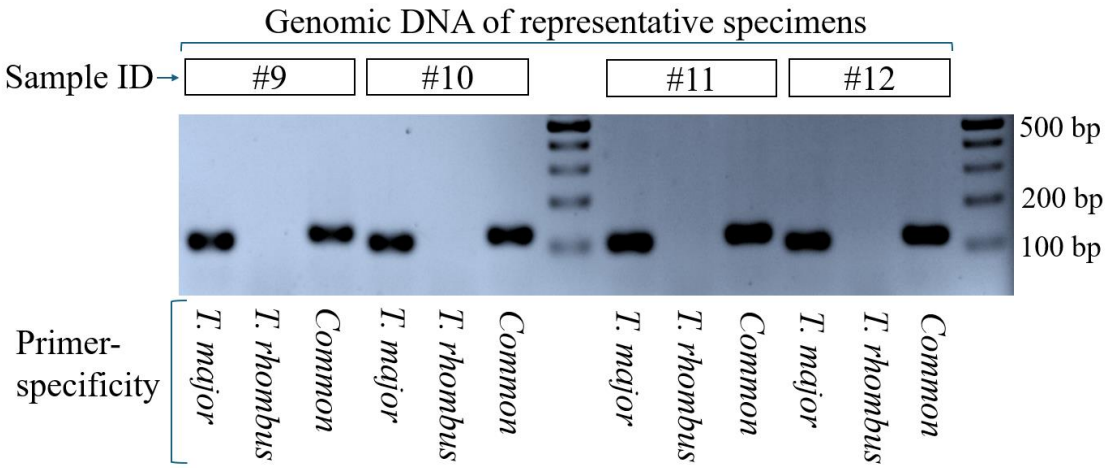

Fig. 4.2. Species validated as *Thysanoteuthis major* through PCR-based diagnosis assay. This assay was run with species-specific primer sets, by which sister species *T. major* and *T. rhombus* were found distinguishable. Shown were representative results with genomic DNAs from the pair-forming *Thysanoteuthis s*quids collected.

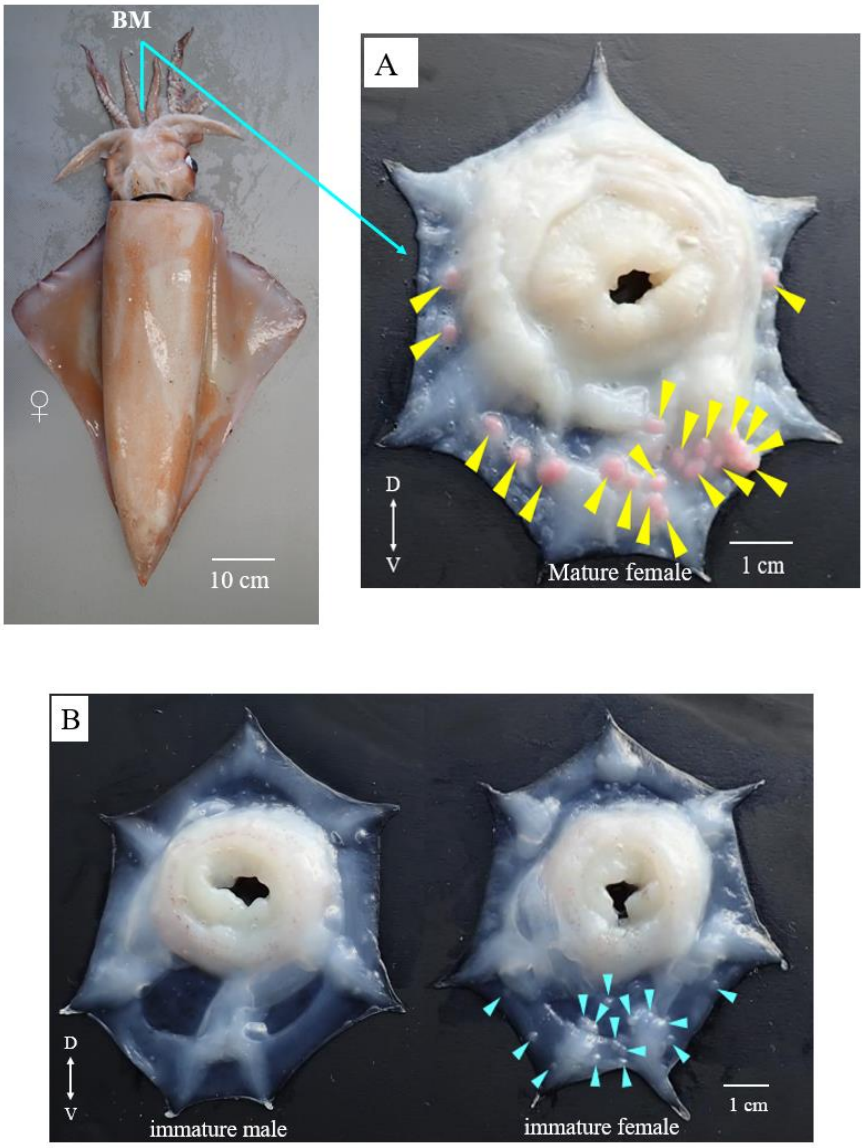

Fig. 4.3. Sperm storage organs (Seminal receptacles) in a mature female and an immature female of *T. major*. A, Pink colored seminal receptacles (*yellow arrowheads*), filled with sperm, in mature female, which are distributed widely at the ventral side of buccal membrane. B, Buccal membranes of immature paired male and female, where the females possessed smaller sized seminal receptacles (*cyan arrowheads*) with no sperm, located ventrally. BM, buccal membrane; D, dorsal side; V, ventral side.

4.3.3. Newly developed microsatellite markers

Based on the PCR-PAGE-fragment analysis workflow with genomic DNAs isolated from 43 individual *T. major* as described in 'Materials and Methods' section, four highly polymorphic microsatellite markers (Tm40370, Tm45293, Tm11117, and Tm8400) were successfully developed. Important characteristics of these four SSR markers were presented in Table 4.1. As summarized in the table, across the four SSR loci, 16 to 34 alleles were detected, while expected heterozygosity showed a higher value range of 0.908-0.959. Two SSR loci (Tm40370 and Tm45293) did not deviate significantly from Hardy-Weinberg equilibrium (HWE) ($P > 0.05$), whereas other two deviated (Table 4.1). The multilocus probability of identity (pid) for all SSR loci was found to be 2.3E-09, which is far better than the threshold value 1E-04. Thus, these are highly polymorphic and could reliably distinguish individual genotypes, thereby differentiating one individual from another. The allele frequency of these four SSR markers were shown in Appendix VII. We used these microsatellite markers for genotyping sperm stored in the seminal receptacles in females.

Table 4.1. Information and characterization of newly developed microsatellite markers for *Thysanoteuthis major*

SSR Locus	Motif/repeat	Forward Sequence	Reverse Sequence	Fluorescent dye
Tm40370	(GAA)21	CAACTGTCTGACCCGAAGGT	TTCGCAGCCCTTTTCTTCTA	Cy3 (Tail/C)
Tm45293	(TAT)28	GTGTGTATTGGGCCGTTCTT	GGATCCGAAATTTTCCTGGT	Cy3 (Tail/C)
Tm11117	(AAG)26	CGATTTCGAAGGGAAAGAGA	TACTCCCCACCCAGTCACTC	Cy3 (Tail/C)
Tm8400	(ATA)31	CAGTGACCACCGTGCAACT	TGTCAAAAACTCGATCCTCCT	Cy3 (Tail/C)

SSR Locus	N	Na	Ne	Ho	He	Fis	DF	Chisq	Signif	Pid
Tm 40370	42	34	24.671	0.929	0.959	0.032	561	543.495	ns	3.2E-03
Tm 45293	41	16	10.845	0.976	0.908	-0.075	120	120.203	ns	1.6E-02
Tm 11117	43	31	17.779	0.860	0.944	0.088	465	569.355	***	5.8E-03
Tm 8400	43	22	15.408	0.767	0.935	0.179	231	313.025	***	8.0E-03

N, sample size; Na, number of alleles; Ho, observed heterozygosity; He, expected heterozygosity; Fis, fixation index; DF, degree of freedom;

***, significance of departure from Hardy-Weinberg Equilibrium (P<0.01); Pid, probability of genetic identity

4.3.4. Genetic evidence for multiple paternity in a diamond squid

Paternity analysis was carried out on the sperm obtained from each seminal receptacle (SR) in six females. The microsatellite-based genotyping of sperm stored in SRs detected alleles for each female. The electropherograms obtained from fragment length analysis showed multi-allelic peaks for each SR. Fig. 4.4 shows the representative electropherograms of two SRs (SR-1 and SR-7) in 'Female 1'. In SR-1, 8 paternal alleles were detected, so the estimated minimum number of possible male mates was 4 (according to the paternity estimation equation in 'Materials and Methods' section). Similarly, SR-7 estimated at the minimum of 4 male mates, resulting from 7 paternal alleles detected, indicating the evidence for presence of homozygous male. The results of these SRs indicated that the 'Female 1' mated with at least 4 males. Similarly, all the females analyzed revealed the maximum number of paternal alleles for one female from 4 to 9 (7 ± 1.6, $N_{female} = 6$), indicating that the minimum number of possible males mating with one female ranged from 2 to 5 (3.6 ± 0.9, $N_{female} = 6$) (Table 4.2). Even each SR of all females also showed multiple paternity (Fig. 4.4; 4.5). These results suggest that this pair forming diamond squids are not monogamous, they pursue polyandrous mating behavior.

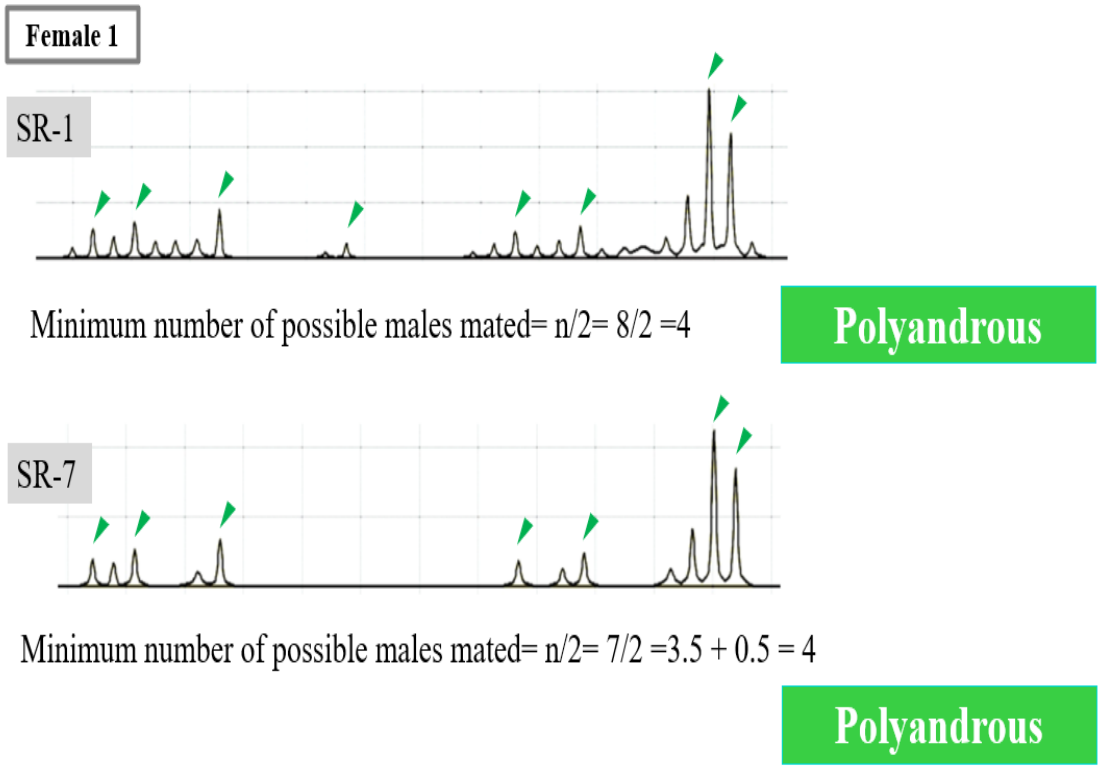

Fig. 4.4. Genetic evidence for multiple paternity in oceanic diamond squid. Here, representative electropherograms of two SRs (SR-1 and SR-7) of a female (Female 1) are presented. n, total number of paternal alleles (*green arrowheads*); SR, seminal receptacle.

Table 4.2. Seminal receptacles, maximal number of paternal alleles and least number of possible males that contributed sperm to each female of *T. major*

Female squid ID	Number of seminal receptacles	Maximal number of paternal alleles	Least number of possible males
Female 1	7	8	4
Female 2	25	4	2
Female 3	17	9	5
Female 4	31	7	4
Female 5	12	6	3
Female 6	21	8	4
Mean ± SD	18 ± 7.9	7 ± 1.6	3.6 ± 0.9

4.3.5. Similar pattern of sperm storage among seminal receptacles within female

Although the females are polyandrous, surprisingly, the peak patterns in all seminal receptacles (SRs) within each female were found similar in terms of number of peaks along with their peak height and peak area (Fig. 4.5). Accordingly, each SR in a female carried nearly the same number of paternal alleles with about similar proportion of peak area (Fig. 4.6). The peak patterns likely reflect the sperm storage patterns in female. Thus, male-delivered sperm were almost similarly distributed among all the SRs within the females.

4.3.6. Paternity share biased to presumably a dominant male

For each female, two alleles were found to share a greater proportion (40-80%) of peak area in totality than the other alleles found in its all the seminal receptacles (SRs) (Fig. 4.6). According to Zhang et al. (2012), the amount of gDNA is linearly correlated with the peak area of the electropherograms obtained from fragment length analysis, which suggests dominancy of these two alleles in paternity sharing. Assumption of these two alleles coming from a heterozygous male indicates that paternity share in each female was biased to possibly a dominant male.

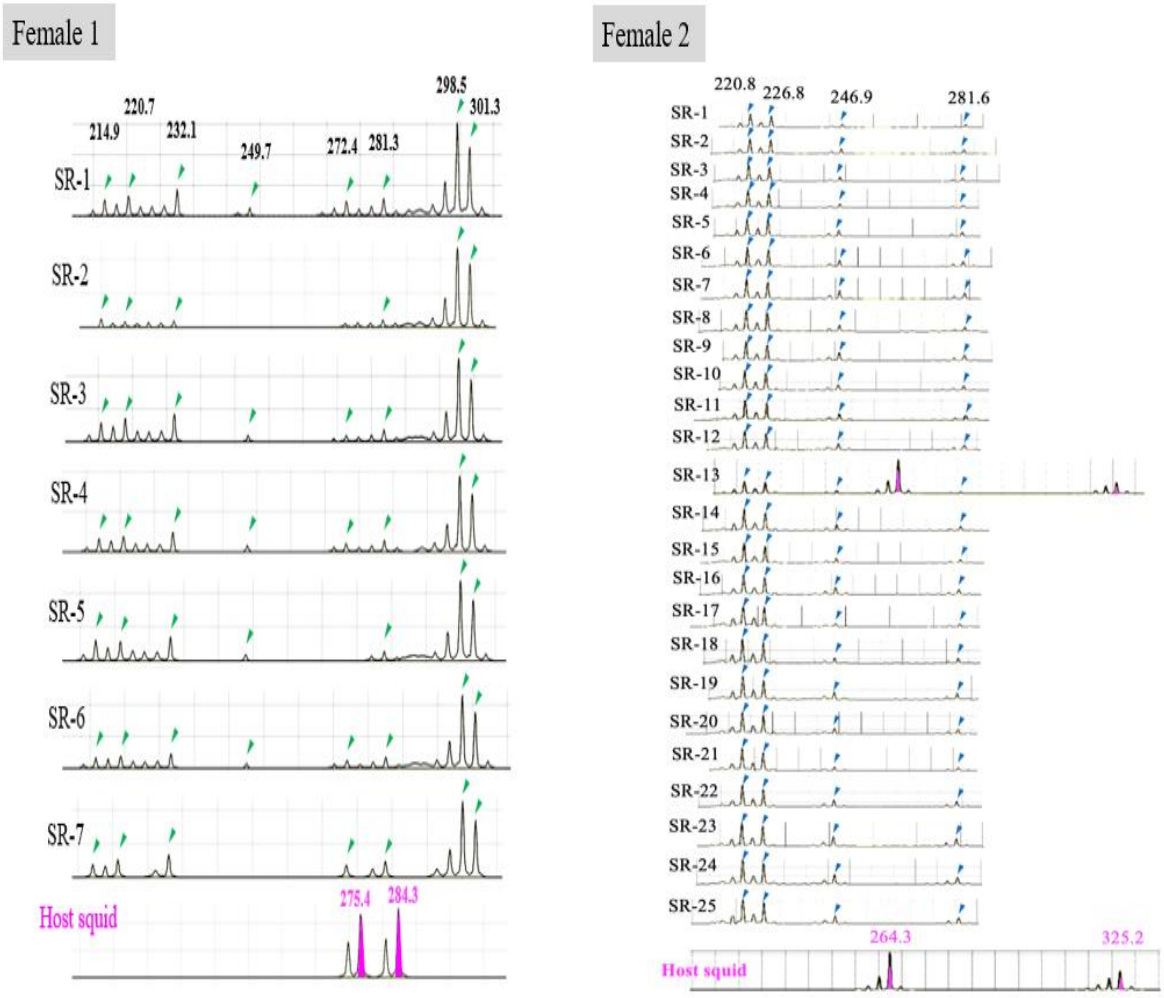

Fig. 4.5. Similar peak patterns in all seminal receptacles within female diamond squid. Here, the electropherogram plots of all SRs in two exemplary females (Female 1 with 7 SRs and Female 2 with 25 SRs) are shown, which are aligned here based on the allele sizes. The peak ornamentation for each SR in terms of number and height/area looks similar within female. The paternal alleles (*green arrowheads in Female 1* and *blue arrowheads in Female 2*) and maternal alleles (*pink-colored*) are also exhibited. No SRs were contaminated with maternal alleles except SR-13 in Female 2. SR, seminal receptacle.

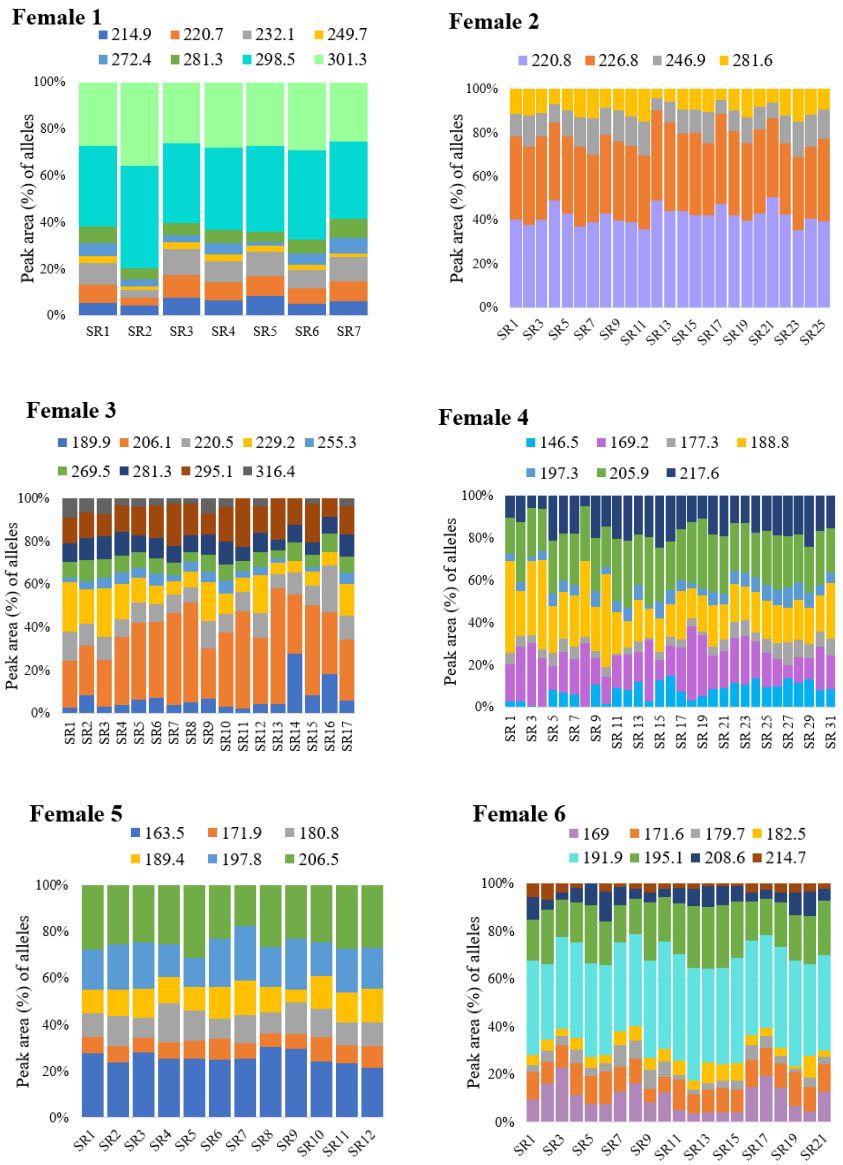

Fig. 4.6. Nearly uniform sperm distribution pattern among all seminal receptacles within a female with likely dominancy of a male in paternity sharing in diamond squid. Here, all the paternal alleles found in each SR are proportionately shown for each of six females in terms of peak area, where each color represents different alleles within female. Regarding the proportion, two alleles in each female shared relatively greater part in totality than other alleles found; *Female 1*, 298.5 and 301.3 bp; *Female 2*, 220.8 and 226.8 bp; *Female 3*, 206.1 and 295.1 bp; *Female 4*, 188.8 and 205.9 bp; *Female 5*, 163.5 and 206.5 bp; *Female 6*, 191.9 and 195.1 bp. SR, seminal receptacle.

4.4. Discussion

Animal mating behaviors are diverse and fascinating. Traditionally, it has been believed that monogamy is a common strategy among animals (Ahnesjo and Bussiere, 2021). Following studies, however, revealed that having a long-term pair bond and mating exclusively with each other to raise their offspring, are rare (Wittenberger, 1979; Petrie and Kempenaers, 1998; Isvaran and Clutton-Brock, 2006; NSF, 2013; Huck et al, 2014; Dolotovskaya et al., 2020). Instead, polygamous relationships have become very frequent in the animal kingdom (Lank et al., 2002; Quinteiro et al., 2011; Ribolli et al., 2020). Even in socially monogamous mammals which were initially thought to perform genetic monogamy (Grith et al., 2002; Grinkov et al., 2022), females may occasionally mate outside of their partner, known as extra pair copulation, to obtain benefits such as 'good genes', not provided from their pair-bonded partner (Clutton-Brock, 2007; Trivers, 2017). Currently the prevalent mating strategy across animal taxa is polyandry, which leads to increased male-male competition and production of offspring with higher genetic diversity (Brockmann et al., 1994; Jones and Avise, 1997; Birkhead and Pizzari, 2002; Ohtani et al., 2022). Likewise, most cephalopods are polyandrous, where a single female receive sperm from few competing males and store in sperm storage organs (SSOs) located at the buccal membrane (Hanlon et al., 1999; Eberhard 2009; Franklin et al., 2012; Firman et al. 2017). Inshore squids develop no more than two SSOs, whereas most oceanic squids have a higher number of SSOs in the form of specialized pockets, called seminal receptacles (SRs) (Sato, 2021; Sato et al., 2023).

However, in oceanic diamond squid, *Thysanoteuthis major*, we found about 18 SRs per female for sperm storage, which are located only at the ventral side of the buccal membrane. In addition, no individual spermatangia were found in the females. These differ from most other oceanic squids, where the individual females possess both

of spermatangia and SRs, and their SRs are widely distributed within their buccal membrane (Sato et al., 2023). Because sperm located at ventral side are little nearer to the oviduct opening than those at dorsal side, resulting in faster fertilization, the ventral side might be favored by the diamond squids. Beyond mature females having SRs, the immature females were also observed to possess smaller SRs without sperm, suggesting that the formation of SRs in diamond squid might start at the earlier stage of their life. Notably, few sperm mass appeared on the surface of buccal membrane, but outside the SRs in two of the mature females examined. Therefore, absence of spermatangia as well as presence of sperm mass outside SRs in mature females, and formation of SRs at immature stage together indicate that males transfer their spermatophores on the surface of buccal membrane, and then immediately sperm, by unknown mechanism, are stored in the developed SRs.

The diamond squid shows an unusual bahaviour among the squids, i.e. they often occur in pairs near the surface (Nishimura, 1966; Okutani, 1982; Yano et al., 1998), and longline catches during the day suggest that these pairs also occur at depth (Bower and Miyahara, 2005). Their pairs consist of a male and a female of the similar sizes and pairing start from the juvenile or immature stage and remains together until death (Nigmatullin et al., 1995). It was also evident from our collected three pairs of immature squids caught by longline catches, where each pair had one male and one female of almost similar sizes. According to Nigmatullin et al. (1995), immature squids recognize the opposite sex by the sexual dimorphism in length of the third pair of arms: in males they are twice as long as in female. People think that the main function of their paired lifestyle is reproduction. Accordingly, based on their such unique pairing behavior, it was believed to be monogamous without any genetic evidence (Nigmatullin et al., 1995). However,

this study showed genetic evidence for multiple paternity ranging from 2 to 5 males for each diamond squid female, suggesting that they are not monogamous.

Inspite of having multiple paternity, they form pairs probably due to some other factors besides reproduction. Their low population density results in difficulty to find a partner (Nigmatullin, 1987). Moreover, their short life span of about 1 year and faster growth rate might have impacts on their pairing behavior (Nigmatullin et al., 1995; Bower and Miyahara, 2005).

However, sexual maturity comes to males earlier than female in terms of mantle length. Males become sexually mature at about 47–52 cm ML, whereas the females become at about 59–61 cm ML (Nazumi, 1975; Takeda and Tanda, 1997; Takeda and Tanda, 1998). Consequently, they don't reach at sexual maturity at similar sizes although similar sized males and females move in pairs from their immature stage. Owing to being sexually mature at comparatively earlier stage, males might copulate with other females outside of their pair-bonded-partner. For the same reason, probably many males mated and transferred sperm to one female, resulting in multiple paternity.

Despite mating with multiple males, paternity share was found to bias towards presumably a single dominant male. This is possibly similar to extra pair copulation (EPC), where the female often mates outside of the paired partner to improve the genetic content of their offspring (Westneat et al., 1990; Birkhead and Møller, 1992; Birkhead, 1998; Jennions and Petrie, 2000). Unlike other pair bonded animals, hatchlings of diamond squids don't need any parental care as well (Watanabe and Segawa, 1998; Ando et al., 2004). Moreover, Nishimura (1966) and Bower and Miyahara (2005) reported that diamond squid occurs in very small groups, sometimes in only 1-2 squids, sometimes fewer than five squids. These might indicate that in diamond squid, the female frequently

mates with the paired male, but sometimes mate outside the pair, who are likely parts of their small groups occurring together. Usually, EPC occurs in the absence of the social mates; However, the paired male vigorously attacks intruding males if he witnesses his partner engaging in EPC (Lament, 2006).

In addition, this study showed a surprising finding regarding sperm storage pattern. Although the females mated with multiple males, the sperm of the mated males were similarly distributed among all the SRs within female. This differs from another oceanic squid, Japanese common squid, *Todarodes pacificus*, where each SR exhibits different pattern of paternity composition and wider distribution of paternity share (Sato et al., 2023). The similar sperm distribution pattern among SRs in diamond squid is unique and unusual. This might indicate the authority/control of female over sperm storage mechanism in SRs. After deposition of the male-delivered spermatophores on the surface of the female's buccal membrane, females might store the sperm evenly among the SRs so that sperm from different males could be chosen easily for fertilization and thereby offspring with higher genetic diversity could be produced.

However, the female diamond squids examined for paternity analysis were obtained during the peak spawning season (March – June), when the Gonadosomatic index (GSI) of both male and female were higher (Kawasaki and Kakuma, 1998). Consequently, during this time they were highly mature and many of the males might try to mate with one female so that their sperm could get the opportunity to be fertilized and contribute to offspring. Thus, the females were found to carry sperm from multiple males. We therefore cannot rule out the possibility that the mating behavior may change with seasons. To address this issue, future studies are required with the specimens collected in early spawning season. In addition, we hereto used few females for paternity analysis,

which could be carried out with a greater number of samples. In conclusion, we found that despite pairing behavior, diamond squid females showed multiple paternity due to extra pair copulations with other males outside the pair-bonded-partner for producing offspring with higher genetic diversity.

4.5. Summary

The oceanic diamond squid, *Thysanoteuthis major* are often found in pairs consisting of one male and one female, indicating behavioural monogamy. A study was carried out to investigate whether these squids are truly monogamous, by genotyping the sperm stored in the female's multiple seminal receptacles (SRs) those are ventrally located on the female's buccal membrane. Four highly polymorphic microsatellite markers were newly developed for genotyping the sperm. The fragment length analysis (FLA) detected the maximum number of paternal alleles for one female from 4 to 9, indicating that one female mated with at least 2 to 5 males. Even each SR in all females also showed multiple paternity. Interestingly, the FLA electrograms showed multi-allelic peaks with significant similarity between the contents of different SRs from the same female. Despite mixed paternity being evident, we found that sperm from presumably one male dominated in all the SRs within a female. This contrasts with the case of the oceanic Japanese common squid, *Todarodes pacificus*, where each SR exhibits different pattern of paternity composition and wider distribution of paternity share. However, dominancy of a single male in paternity sharing supports their pairing behavior and is somewhat related to extra pair copulation. Therefore, regardless of pairing behavior, the female diamond squids pursue extra pair copulations outside the pair-bonded partner to have genetically diversified offspring.

CHAPTER 5

GENERAL SUMMARY

There are two major groups of squids, myopsid squids (also known as ‘inshore squids’) and oegopsid squids (also known as ‘oceanic squids’). The diversified and commercially important squid family loliginidae is under myopsid group. During copulation, in several species of Loliginidae family, females receive male-delivered sperm capsules, or spermatangia, from dimorphic sized males and store at two different body locations: the buccal membrane and the distal end of the oviduct. This dimorphism of insemination site is linked to alternative reproductive tactics (ART). However, most loliginid squids show different varieties of ARTs, although that of many species are yet to be investigated. On the other side, very limited research was focused on deep-sea squids as they are hard to catch. On top of that, species of only a few families among many families in oceanic squids had been studied, where most were done with anatomical methods and only a handful of studies were done with molecular techniques. Therefore, comprehensive studies both on inshore loliginid squids as well as oceanic squids, using molecular techniques along with anatomical methods, are very significant to ascertain their reproductive strategies. On that account, research was carried out to explore the alternative reproductive tactics (ART) of inshore loliginid kobi squid *Loliolus sumatrensis,* along with the female promiscuity level, and to find out genetic basis of partnership in pair-forming oceanic diamond squid *Thysanoteuthis major*, using molecular and anatomical techniques.

In inshore loliginid species of squid, *Loliolus sumatrensis*, three distinguishing sperm insemination sites were found on buccal membrane (BM), basal left IV arm (ARM) and lateral head behind the left eye (EYE) in a female body, which is unusual phenomenon in this family. Therefore, we attempted to identify rules and patterns of usage of these sites in relation to size differences in male individuals, and the mating history, maturity, fecundity, as well as growth indices of female individuals. Unlike most other loliginid squids, the males were found monomorphic in body size as well as their sperm size. Besides, no significant differences in sperm size (flagellum and head) were observed among spermatangia at the three insemination sites. However, the periodic data analysis of a population in seto inland sea revealed six different usage patterns of the insemination sites during the early fishing season (early July), whereas the pattern of simultaneous use of all three sites was dominant at the end of the fishery season. The seasonal dynamics of the female populations also identified a set priority for initial use of insemination sites as BM, followed by ARM and then EYE. At the individual level, however, once all insemination sites of a female have started to be used, subsequent inseminations by other males could take place anywhere depending on the most suitable site given the current situation. Thus, the choice of site to be inseminated was not dependent on full occupancy of preferred insemination sites, but the mating history of the female. However, the number of spermatangia attached to BM was lower than those attached to other sites, whereas the maximum number of stored spermatangia was found greater in EYE among the sites. Female maturity status was correlated with the insemination patterns, but not with the number of stored spermatangia at any insemination site. These results suggest that males inseminate at different locations in a female according to the mating history and maturity status of the female.

The inseminations at three different locations in a loliginid *L. sumatrensis* female occurred by monomorphic sized males. Their absence of male size dimorphism, but insemination site trimorphism prompted us to conduct the following study. In this study, we investigated into the female promiscuity level to know whether they are polyandrous or monogamous. Therefore, four highly polymorphic microsatellite markers were developed through PCR-PAGE-fragment analysis workflow and used to measure the level of paternity of the deposited spermatangia. In addition, parentage analysis at each sperm-deposition site were compared to explore the site with higher paternity number. The microsatellite-based genotyping showed that each female mated with 7 to 9 males, suggesting that *L. sumatrensis* are polyandrous. Moreover, each insemination site exhibited multiple paternity. We found that the actual number of sires per site was relatively higher at ARM than at the other two sites, but there were no significant differences in number of sires among the three sites. Surprisingly, a few males were found to inseminate simultaneously at multiple (two or three) sites on the same female, and these small numbers of sires were dominant in paternity sharing in the total attached spermatangia. It suggests that sperm allocation occurs within a female.

Besides exploring a unique ART along with the female promiscuity level of an inshore loliginid squid, an oceanic squid with mysterious behavior was also investigated. The oceanic diamond squid, *Thysanoteuthis major* occurring in deep sea are often found in pairs of one male and one female. This pairing behavior is unique among the squids. Interestingly, their pairing starts from an immature stage and continues into adulthood, indicating behavioral monogamy. However, there is no genetic evidence for their partnership. Therefore, my following study was carried out to find out the genetic evidence of mating system in pair forming diamond squid. In this squid, mature females

possessed several sperm storage organs, known as seminal receptacles (SRs), filled with male-delivered sperm, located ventrally at their buccal membrane. The paternity level was explored by genotyping the sperm stored in these SRs. Hence, four highly polymorphic microsatellite markers were newly developed for *T. major*.

The fragment length analysis (FLA) detected the maximum number of paternal alleles for one female from 4 to 9, indicating that one female mated with at least 2 to 5 males. Even each SR in all females also showed multiple paternity. Interestingly, the FLA electropherograms showed multi-allelic peaks with significant similarity between the contents of different SRs from the same female. Despite mixed paternity being evident, we found that sperm from presumably one male dominated in all the SRs within a female. This contrasts with the case of the oceanic Japanese common squid, *Todarodes pacificus*, where each SR exhibits different pattern of paternity composition and wider distribution of paternity share. However, dominancy of a single male in paternity sharing supports their pairing behavior and is somewhat related to extra pair copulation. Therefore, regardless of pairing behavior, the female diamond squids pursue extra pair copulations outside the pair-bonded partner to have genetically diversified offspring.

The anatomical and molecular analysis conducted for both inshore and oceanic squids for exploring their reproductive behaviors suggests that squids have a varying degree of behavioral strategies for copulation, sperm insemination, storage, and fertilization.

However, our study showed a sharp contrast from previously known general views on squid reproduction, especially on alternative insemination strategies. Males of inshore *Loliolus sumatrensis* conditionally choose the insemination sites from three possible locations on the female body. Moreover, it is widely accepted that in several animal groups, upon mating, males allocate their limited reproductive expenditure (e.g., sperm) to females in accordance with female promiscuity, fecundity, and quality as well as future opportunities to mate with other females. In all cases, 'sperm allocation' is expected to occur among different females. However, we found, in *L. sumatrensis*, that 'sperm allocation' occurs within a female. Our study revealed that males can choose one or more insemination sites within a female according to her mating history, possibly with a clue of previously implanted spermatangia, providing novelty in animal mating behavior, which we believe adds a new avenue to decipher the underlying mechanisms of sperm allocation and alternative reproductive tactics. In addition, based on the mysterious pairing lifestyle in oceanic diamond squid *Thysanoteuthis major*, people believed that they are monogamous, although there was no genetic evidence for their partnership. However, our study unveiled the genetic evidence, which refutes the general belief. Thus, the conducted studies have added advanced knowledge in animal reproductive behaviors and will open a new window for future studies.

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APPENDICES

Appendix I: Summary of measurements for growth and maturity of *Loliolus sumatrensis* individuals

Sex	year	dd-m	Number of individuals	%Male/ %Female	ML (mm)		BW(g)		Acc mg)		Testis/ Ovary (mg)		TSI/OSI	
					ave	std	ave	std	ave	std	ave	std	ave	std
Male	2021	13-Jul	51	42.15	78.8	6.14	20.5	10.7	335	124	634	110	3.1	0.76
	2021	23-Jul	55	45.45	81.3	4.9	18.9	2.46	360	88	610	134	3.23	0.71
	2021	30-Jul	24	55.81	80.8	6.42	19.6	5.58	373	150	614	89	3.13	0.42
	2021	13-Sep	17	25.37	76.2	8.51	15.7	3.85	253	68.4	402	89.1	2.57	0.59
	2022	30-Jun	62	51.67	66.3	6.96	11.6	2.73	213	129	407	138	3.51	0.85
	2022	6-Jul	75	42.86	72.9	5.21	15.2	2.62	261	86.4	526	105	3.46	0.98
	2022	15-Jul	29	25.44	76.4	8.87	17.2	3.91	282	114	545	156	3.17	0.66
	2022	26-Jul	84	53.85	73.5	7.11	17.3	3.82	297	128	478	126	2.76	0.67
		Total	**397**	**average**	**75.8**	**6.76**	**17**	**4.46**	**297**	**111**	**527**	**118**	**3.12**	**0.71**
Female	2021	13-Jul	70	57.85	86.3	6.68	26.7	5.79	3150	1487	2318	1197	8.69	4.49
	2021	23-Jul	66	54.55	88.6	10.1	27.5	5.28	3038	1262	2367	1007	8.59	3.66
	2021	30-Jul	19	44.19	87.8	10.1	27.4	6.72	3514	915	2287	644	8.34	2.35
	2021	13-Sep	50	74.63	78.1	6.48	21.1	3.77	2649	684	1712	470	8.12	2.23
	2022	30-Jun	58	48.33	67.8	8.31	13.2	4.36	601	776	462	639	3.51	4.85
	2022	6-Jul	100	57.14	75.3	6.45	18.6	3.82	1102	975	825	774	4.44	4.16
	2022	15-Jul	85	74.56	82.5	6.29	23.4	4.51	1965	1099	1417	940	6.06	4.02
	2022	26-Jul	72	46.15	77.6	7.62	21.4	4.88	1480	2593	742	863	3.47	4.03
		Total	**520**	**average**	**80.5**	**7.75**	**22.4**	**4.89**	**2187**	**1224**	**1516**	**817**	**6.4**	**3.72**
Male & Female		**Sum**	**917**											

dd, date; m, month; ML, mantle length; BW, body weight; ACC, accessory gland weight; TSI, testicular somatic index; OSI, ovarian somatic index; ave, average; std, standard deviation

Appendix II: Summary of *L. sumatrensis* female individuals with spermatangia at different sperm insemination sites

		Number of females having spermatangia at insemination sites					% females					
year	dd-m	none	BM	BM & ARM	BM & ARM & EYE	BM & EYE	total	none	BM	BM & ARM	BM & ARM & EYE	BM & EYE
2021	13-Jul	14	4	21	30	1	70	20	5.71	30	42.9	1.43
2021	23-Jul	11	10	22	23	0	66	16.7	15.2	33.3	34.8	0
2021	30-Jul	0	0	9	10	0	19	0	0	47.4	52.6	0
2021	13-Sep	0	0	4	46	0	50	0	0	8	92	0
2022	30-Jun	45	2	4	6	1	58	77.6	3.45	6.9	10.3	1.72
2022	6-Jul	69	1	13	17	0	100	69	1	13	17	0
2022	15-Jul	30	3	21	31	0	85	35.3	3.53	24.7	36.5	0
2022	26-Jul	46	1	4	21	0	72	63.9	1.39	5.56	29.2	0

dd, date; m, month; BM, buccal membrane; ARM, basal left IV arm; EYE, lateral head behind the left eye

Appendix III: The hypothetical rule of a set order in a male insemination preference in *L. sumatrensis*

Suppose there is a set order in which the insemination site is preferentially used first by males. Suppose further that males prefer to inseminate first at X followed by Y, then Z, which is defined as (X, Y, Z). In this rule, there are three possible cases of female status: only X is used; both X and Y are used; and all X, Y, and Z are used. In any other case, they are regarded as "against the rule."

For all possible combinations of (X, Y, Z), the frequency (% in total) of each sequential event that could occur in the collected specimens (n = 304) is as follows. "others" indicate the frequencies of exceptions (against the rule).

	inseminated at			
(X, Y, Z)	X	X, Y	X, Y, Z	others
(EYE, ARM, BM)	*0.000 %*	*0.329 %*	*60.20 %*	*39.47 %*
(EYE, BM, ARM)	*0.000 %*	*0.329 %*	*60.20 %*	*39.47 %*
(ARM, BM, EYE)	*0.000 %*	*0.329 %*	*60.20 %*	*39.47 %*
(ARM, EYE, BM)	*0.000 %*	*32.24 %*	*60.20 %*	*7.566 %*
(BM, EYE, ARM)	*6.908 %*	*0.329 %*	*60.20 %*	*32.57 %*
(BM, ARM, EYE)	*6.908 %*	*32.24 %*	*60.20 %*	*0.658 %*

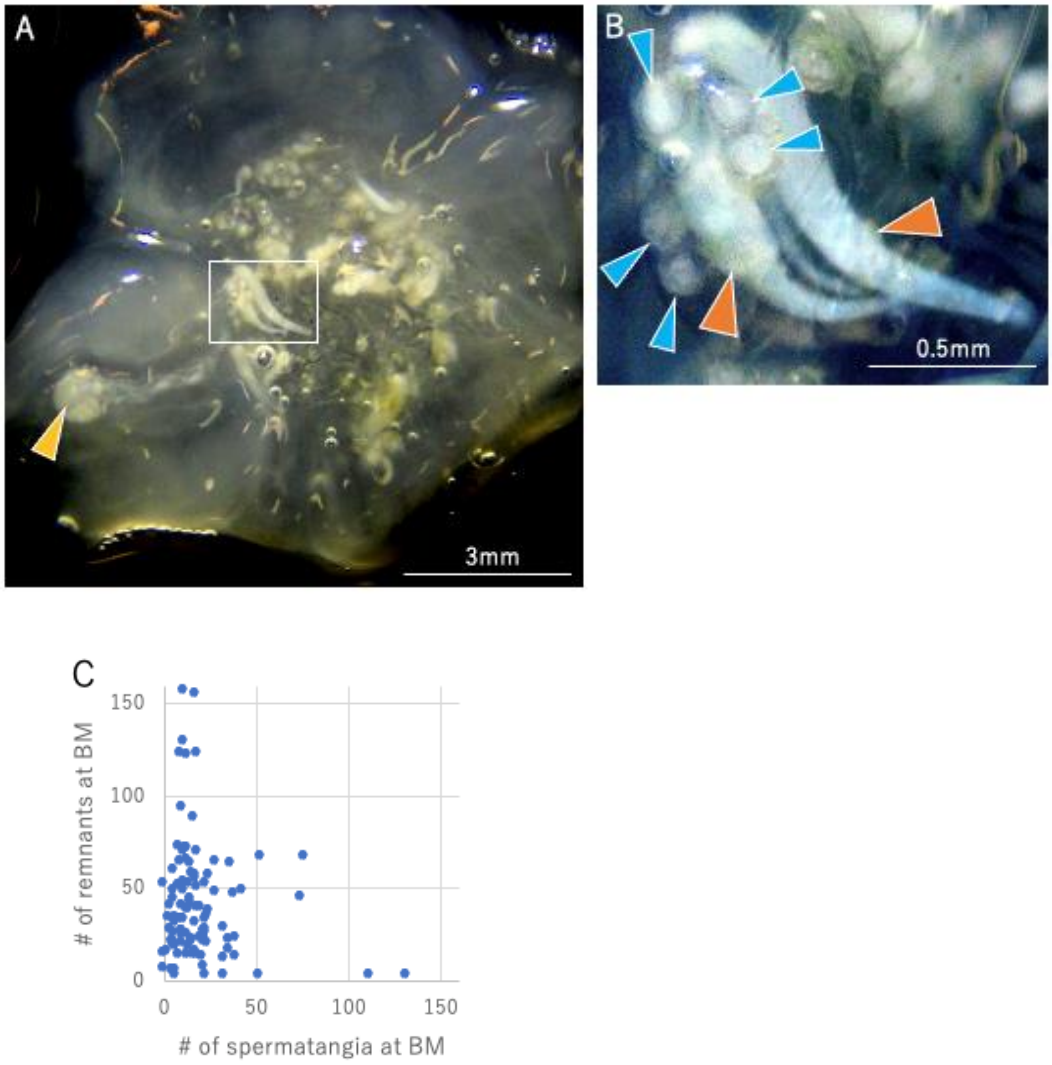
A low frequency (<1%) of other cases (frequency of exceptions) was observed only in (BM, ARM, EYE). If the rule is applied strictly, then it is most appropriate that a set order of initial use for insemination is BM→ARM→EYE.

Appendix IV: Statistical analysis by rank cases of spermatangia insemination at female body sites of *L. sumatrensis*

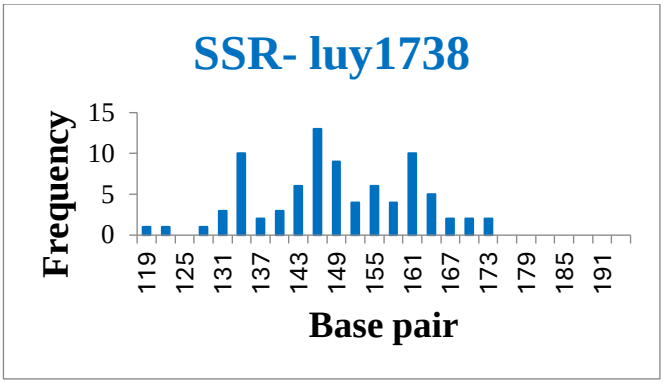
Insemination of spermatangia	R_Insemination	R_AN001
BM	13	1
BM & ARM	72	2
BM & ARM & EYE	214	3

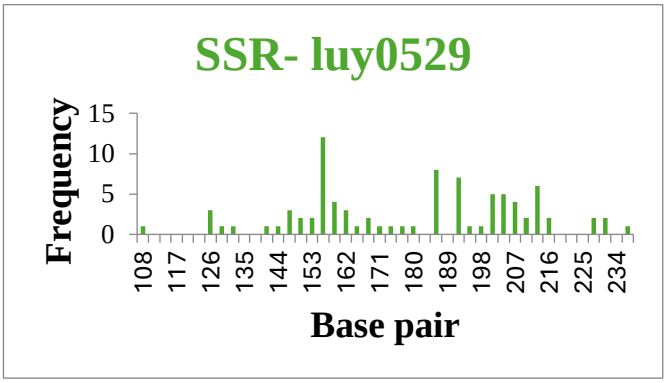
R_Insemination, Mean Rank of tied values; R_AN001, Consecutive Ranks of ties sharing the same value; BM, buccal membrane; ARM, basal left IV arm; EYE, lateral head behind the left eye

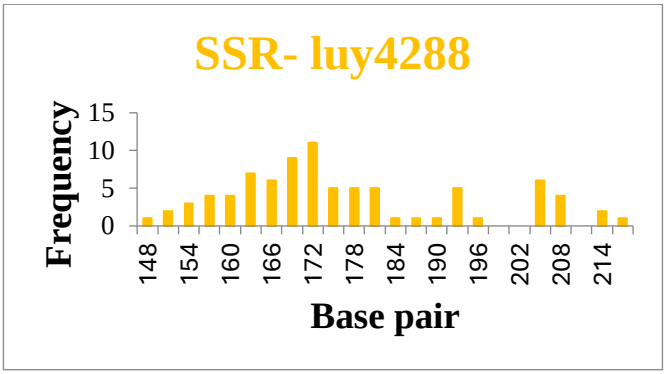
*Ranks are in ascending order.

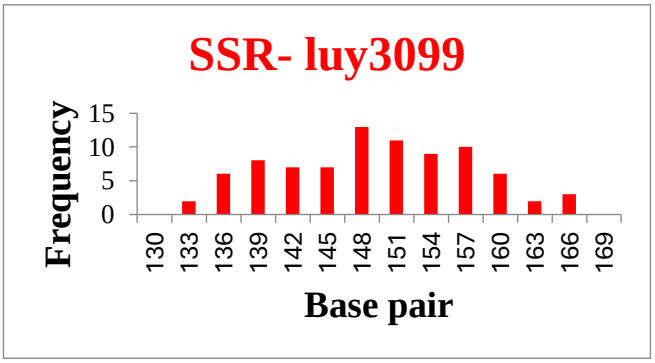


Appendix V: A representative photograph of the isolated female buccal membrane with attached spermatangia and their remnants *in L. sumatrensis* **A**, Viewing from the surface of a whole buccal membrane with the seminal receptacle (*yellow arrowhead*). **B**, *Inset* showing intact spermatangia (*orange arrowhead*) and the remnants of the spermatangia (*blue arrowhead*). **C**, Individual variability in holding spermatangium number and remnant number at the buccal membrane. Each dot represents an individual female.

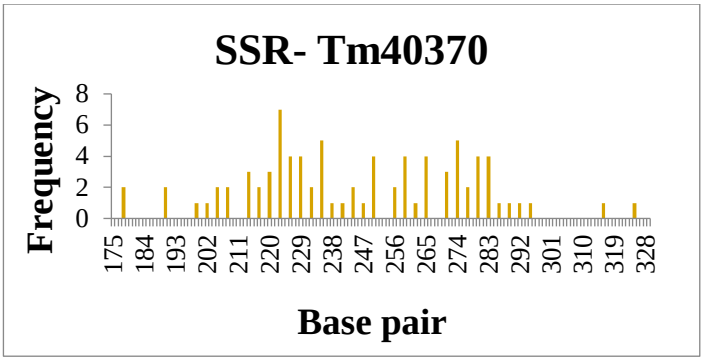


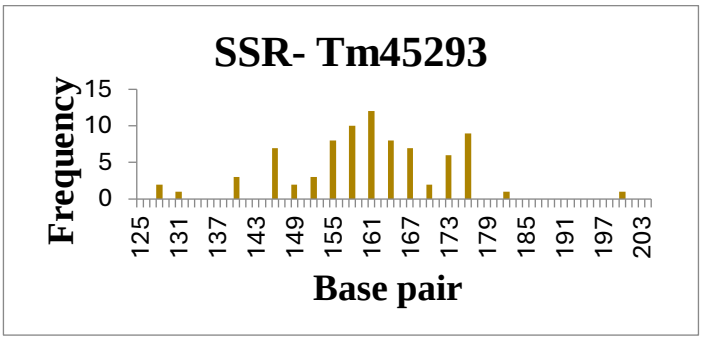


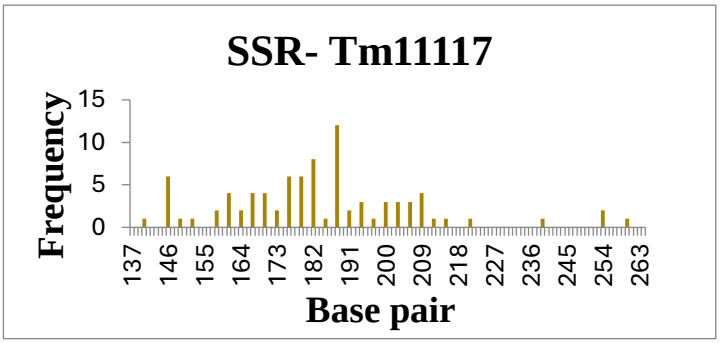


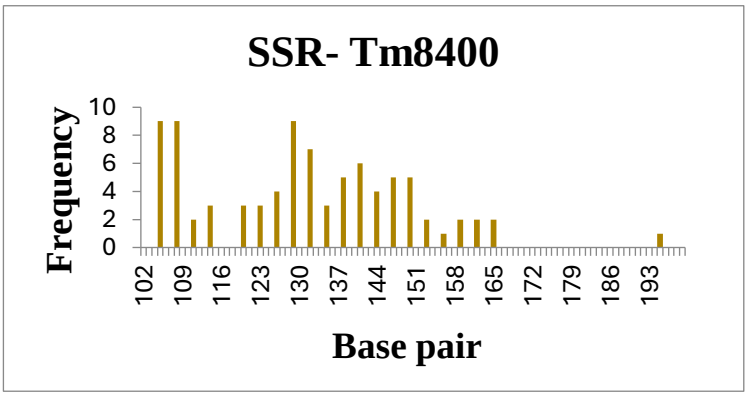


Appendix VI: Allele frequency of the four polymorphic SSR markers developed for paternity analysis of *L. sumatrensis.*









Appendix VII: Frequency of alleles for the newly developed four polymorphic microsatellite markers for genotyping sperm stored in female of *T. major*.

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