Comprehensive understanding of the origin and function of the "female penis" in cave insects

(雌がペニスを持つ昆虫の交尾器の起源と機能の総合的理解)

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Comprehensive understanding of the origin and function of the "female penis" in cave

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TABLE OF CONTENTS

Acknowledgements

Table of contents

Chapter 1   **Exploration of the homology among the muscles associated with the female genitalia of the three suborders of Psocodea (Insecta)**

Chapter 2   **Acquisition of novel muscles enabled protruding and retracting mechanisms of female penis in sex-role reversed cave insects**

**Chapter 3   Are males completely passive? Coupling mechanism
            of the cave insects with reversed genitalia**

General Introduction

Coevolution between females and males is like an endless arms race that has attracted attention of many biologists. As the most interactive structures between the sexes, genitalia have been exposed to strong sexual selection, that makes it being the most variable and divergent of all morphological structures (Simmons, 2014). Generally, morphology of male genitalia is more diverse than that of female. This is because that male usually has faster replenishment rate of gametes and can be benefit from multiple matings. In contrast, reproductive cost of females is much higher than that of males, and number of offspring is generally constraint by the number of gametes which can be produced by a female. Therefore, superfluous mating is not cost-effective for females, so they are more resistant to mating (Bateman, 1948; Eberhard,1985). Higher potential reproductive and optimal mating rates for males makes sexual selection acts strongly on males (Trivers, 1972), leading to the rapid evolution and diversification of male genitalia (Eberhard, 1985; Yoshizawa *et al.*, 2014).

Male genitalia of insects are extremely diverse and also an important diagnostic trait in insect systematics (Tuxen, 1970; Simmons, 2014). To increase the mating rate, aggressive structures have frequently evolved in male insect genitalia, such as grasping structures that control the female during mating (Arnqvist & Rowe, 2002; Lange *et al.*, 2013), intromittent organs that can remove rival sperm from female sperm storage organ (sperm competition) (Waage, 1979; Cordoba-Aguilar,1999) and

some species even have spines on the surface of the genitals (Edvardsson & Tregenza, 2005). Females also have some countermeasures, such as anti-grasping structures (Lange *et al.*, 2013), thick membranes (Rönn *et al.*, 2007), etc. to resist male's aggressions. However, in general, the structure of female genitalia is generally simpler than that of males.

A cave insects group having reverseoiuud genital structures, *Neotrogla* (Psocodea: Trogiomorpha: Prionoglarididae), was recently discovered in Brazil (Yoshizawa *et al.*, 2014). The female has a highly specific penis-like intromittent organ, called gynosome, which penetrates deeply into the male vagina-like cavity during copulation to receive voluminous and nutritious semen (Yoshizawa *et al.*, 2014). The central part of the gynosome is membranous with many spiny areas. When the gynosome is inserted into the male body, the spiny areas will unfold and internally anchor the male. During copulation, the male sternum is gripped by the gynosome and paraproct of female (Yoshizawa *et al.*, 2014). As the only group of species that has been found to have reversed genitalia in nature, its female penis is fully equipped with the aggressive structures that normal male genitalia may have, to control the mating and damage the female.

Recent studies also suggested that the character of gynosome was evolved independently twice in the small tribe Sensitibillini (the genera *Neotrogla* and *Afrotrogla*) (Yoshizawa *et al.*, 2018a). Some particular factors have been found to be the reason why female penises are so readily formed in this tribe. The oligotrophic cave environment (Lienhard & Ferreira, 2013; Yoshizawa *et al.*, 2018a) makes

females compete for mating opportunities for obtaining nutritious semen (Wearing-Wilde, 1996). In addition, the female also has a novel feature that allows them receiving more semen from multiple matings: two slots have been found on the spermathecal plate which means double the sperm storage, and a switching valve controlling the seminal flow toward one of the two slots (Yoshizawa *et al.*, 2018b). Those factors make reversed sexual selection in *Neotrogla* more intense.

The reversal of sexual selection induces *Neotrogla* females to evolve the very species-specific penis-like structure. The female genitalia of insects are composed of multiple structures, such as subgenital plate, spermapore plate and gonapophyses. Does the species-specific evolution occur when the reversed sexual selection acts on these structures? What structure did the gynosome evolve from? How is an intromittent structure formed from a non-intromittent structure? To investigate these problems, further morphological studies of external and internal genital structure in Psocodea, including sexually reversed/non-reversed species are needed.

In the past studies about the genital evolution of Psocodea, there are two points that have been overlooked. Female genital structures have more membranous parts than males, which is not easy to observe, and their importance is mostly ignored. The morphological description of the female genitalia is far less detailed than that of the male genitalia. In addition, studies on the function of the genital structures and associated muscles of Psocodea are sparse in both sexes. However, any study of selection acting on morphological traits requires an understanding of the functional morphology of those traits (Fairbairn *et al*., 2003; Jagadeeshan & Singh, 2006;

Simmons, 2014). Therefore, it is imperative to study the function and morphology of the female genital structures and associated muscles of Psocodea.

Recently, microcomputed tomography (μCT) has been widely used to study insect morphology (Friedrich et al., 2014). It can reconstruct the detailed configuration and interaction of the genital structures during copulation. Furthermore, this technology allows us to reconstruct the muscles of the genitalia more clearly than ever before, which is valuable for estimating the movements of genital structures (Cheng & Yoshizawa, 2019). By using μCT technology, we can restore even the membranous part or fine muscles of the tiny organism into a clear 3D model now. The present study took advantage of this technique by extensively modeling the genitalia of various species of Psocodea to obtain clear 3D images of the genital structures and associated muscles. In our previous study, function of various genital structures including muscles of a species of non-genital reversed Psocodea, and detailed coupling process were estimated by using μCT technique (Cheng & Yoshizawa, 2019). The present article will be divided into three chapters: (1) constructing the musculature of female genitalia and, using these 3D images, determining the homology of female genital structures and muscles among the wide variety of taxa of Psocodea, which put an important bases for the evolutionary study of the female genitalia in this insect group; (2) exploring the novelties involved in the acquisition of the intromittent function of the gynosome by comparing the species with well developed female penis and with intermediate pre-penis, which elucidate the evolutionary step toward the functional formation of the gynosome; and (3) the final

goal is to explore the morphology and function of the genital structures and associated muscles in genital reversed insects, restoring its specific copulating process and analyzing the sexual selection of *Neotrogla*. This thesis also provides a large number of detailed 3D models of female genitalia of Psocodea, which fills the gap of insufficient morphological description of female genitalia in this insect group.

Chapter 1

Exploration of the homology among the muscles associated with the female genitalia of the three suborders of Psocodea (Insecta)

Content

1.1 Abstract

1.2 Introduction

1.3 Materials and Methods

1.4 Results

1.5 Discussion

Chapter 1

1.1 Abstract

By using μCT technology, I reconstructed 3D models of the female genital structures and associated muscles of seven species from three suborders of Psocodea (free-living species only, formerly known as independent insect order "Psocoptera"). The homology of the female genital structures and associated muscles of different species is discussed. A total of 21 muscle groups were observed, and except for one muscle, all were homologized throughout the order. Moreover, some of the homologous muscles could be identified confidently in holometabolan insects. Using the muscles as landmarks, I discuss the homology of the ovipositor valves between Psocodea and other neopteran insects. Most importantly, the ovipositor of the suborder Trogiomorpha was identified to consist of the well-developed external valve (V3) plus a remnant of the dorsal valve (V2). I also examined the phylogenetic information included in the female genital muscles and found that certain muscles provide useful information and support deeper nodes (e.g., monophyly of the suborder Psocomorpha). The present study of female genital muscles not only helps us to better understand the phylogeny of Psocodea but also provides a solid foundation for research on muscle evolution.

1.2 Introduction

Insect genitalia are extremely diversified structures, and their morphology and evolution have received great interest from variable perspectives (Boudinot, 2019), such as taxonomic species recognition (e.g., Tuxen, 1970), lower- to higher-level phylogenetic estimation (e.g., Yoshizawa *et al.*, 2006), selection forces acting on these structures (e.g., Eberhard, 1985), or biomechanics (e.g., Matsumura *et al.*, 2017). In most cases, male genital structures are the subject of these studies because male genitalia are more strongly sclerotized than female genitalia, and their shapes and variations are more easily observable and detectable. In contrast, insect female genitalia, especially the structures having direct contact with male intromittent organ, are largely membranous, and their importance has long been overlooked. However, recent studies have shown that an accurate understanding of female genitalia, including the membranous parts, is very important for elucidating genital evolution (Kamimura *et al.*, 2016; Muto *et al.*, 2018; Simmons & Fitzpatrick, 2019; Sloan & Simmons, 2019; Genevcius *et al.*, 2020; Onuma *et al.*, 2021) and coupling mechanisms (Matsumura *et al.*, 2021).

Psocodea is one of the hemimetabolous insect orders composed of nonparasitic barklice and booklice (formerly known as Psocoptera, hereafter called psocopterans) and parasitic lice (Phthiraptera) (de Moya *et al.*, 2021). The order is subdivided into three monophyletic suborders, Trogiomorpha, Troctomorpha (in which parasitic lice are classified), and Psocomorpha. Over 6500 species in 41 families of psocopterans

are known to date (Lienhard & Smithers, 2002; Lienhard, 2016, 2021). As in the other insect groups, male genitalia are the most important characters for diagnosing species and recognizing some higher taxa (e.g., Smithers, 1972). Female genital characters are also important taxonomically and phylogenetically. In particular, this character system is known to be less homoplasious than male genital characters in estimating the phylogenetic relationships of the suborder Psocomorpha (Yoshizawa & Johnson, 2014). Moreover, two female genital apomorphies are the only morphological characters that support the monophyly of the suborder Trogiomorpha (Yoshizawa & Lienhard, 2020). These results suggest the potential importance of this character system for phylogenetic estimations. However, ambiguities remain in the interpretation of homology among certain key female genital structures; for example, simplified ovipositors of Trogiomorpha have usually been interpreted as composed of external valves (Lienhard, 1998) but alternatively of ventral valves (Azar *et al*., 2017).

In addition, female genitalia of Psocodea are of special interest. Recently, a penis-like genital structure (termed gynosome) was discovered in females of the Brazilian cave-dwelling psocopteran genus *Neotrogla* (Lienhard *et al*., 2010a). Subsequent studies showed that the gynosome is inserted into the male genital chamber during copulation, i.e., copulatory functions are completely reversed in *Neotrogla* (Yoshizawa *et al*., 2014; 2019). This is a very prominent novel structure that has only been identified thus far in this group of insects in the animal kingdom. However, the morphological origin and the functional background of the coupling role reversals in *Neotrogla* are completely unknown.

To elucidate the origin of the novel coupling structures and improve the utility of the female genital character system for taxonomic, phylogenetic, evolutionary and biomechanical studies, providing a detailed morphology and establishing a stable homology interpretation of female genitalia, including both external and internal structures, are essential. However, the detailed musculature related to female genital structures has only been examined for a very limited number of species (Badonnel, 1934).

In this study, I examined the skeletal morphology and musculature of a wide variety of psocopterans using the synchrotron micro-computed tomography (μCT) technique. Seven species from all three suborders were selected for examination. Based on the examinations, I established a homology scheme of female genitalia throughout psocopterans.

1.3 Materials and Mothods

1.3.1 Micro-CT imaging

Seven species of psocopterans, *Prionoglaris stygia* Enderlein, 1909, *Psyllipsocus clunjunctus* Lienhard, 2013 (Lienhard & Ferreira, 2013), *Lepinotus reticulatus* Enderlein, 1904, Amphientomidae Gen. sp., *Archipsocus* sp*., Valenzuela badiostigma* Okamoto, 1910 and *Trichadenotecnum pseudomedium* Yoshizawa, 2001 were examined (Table 1). All voucher specimens are stored in the Hokkaido University

Insect Collection. An uncopulated female of each species was used for μCT examination. Samples were fixed with FAA solution (formalin:alcohol:acetic acid = 6:16:1) or 80% ethanol and then preserved in 80% ethanol. Dehydration was conducted in ascending order with 80–100% ethanol before drying them at the critical point (EM CPD300, Leica, Wetzlar, Germany) to remove water without serious organ shrinkage. Samples were then scanned using the synchrotron μCT at the BL47XU (Uesugi *et al*., 2012) beamline of the Super Photon ring-8 GeV (SPring-8; Hyogo, Japan) using a stable beam energy of 8 keV in absorption-contrast mode. The tomography system consists of a full-field X-ray microscope with Fresnel zone plate optics (Uesugi *et al*., 2017). I used semiautomatic segmentation algorithms based on grey-value differences in the software ITK-SNAP (Yushkevich *et al*., 2006) to obtain 3D representations of the terminalia of all six species.

1.3.2 Examination of phylogenetic signals

I examined the phylogenetic significance of the female genital musculature by reconstructing the character information on the phylogenetic tree that was previously estimated using molecular and phylogenomic data (Yoshizawa *et al*., 2006; Yoshizawa & Johnson, 2014; Johnson *et al*., 2018; de Moya *et al*., 2021). The presence (coded as 1) or absence (0) of each muscle was used as the character state (Table 1.2), and this dataset was parsimoniously reconstructed on the tree using MacClade version 4 (Maddison & Maddison, 2001). All characters were treated as unordered. Because the homology of many muscles between psocopterans and outgroups was ambiguous (see

Discussion), no outgroup taxa were included in the analysis. Therefore, the character changes at the deepest node (between Trogiomorpha and Troctomorpha + Psocomorpha) could not be reconstructed.

1.4 Results

1.4.1 Skeletal and muscle structures of female terminalia.

I describe the basic structure of the female terminalia and muscles related to them. I grouped those muscles according to their origin as follows: muscles of the epiproct [ep]; paraproct [pa]; subgenital plate [sg]; ventral valve [ve]; dorsal valve [do]; external valve [ex]; spermapore plate or the membrane surrounding it [sp]. The distribution of the terminal muscles is summarized in Table 2.

  Abbreviations: O – origin; I – insertion; and F – assumed function (based on morphological conditions).

1.4.1.1 Clunium

The clunium is composed of the fused tergites of segments IX and X and houses many attachments of muscles originating from the epiproct, paraproct, gonapophyses, subgenital plate, and spermapore plate (e.g. Fig.1.7). Three valves of gonapophyses arise from the ventral end of the clunium and articulate posteriorly with the epiproct and paraproct.

Chapter 1

1.4.1.2 Epiproct and paraprcot

The epiproct is a structure located dorsal to the anus. The paraproct is a paired structure located ventrolateral to the epiproct and involves a reduced cercus (trichobothrial field). These two structures are thought to represent segment XI and together surround the anus. Males also have these two structures, usually with some characteristic protrusions on them, which play an important role in the copulation process. The paraproct and epiproct of females is usually simpler and less variable, with sparse bristles and no protrusions (Fig. 1.7C, D).

Muscle originating from the epiproct

01 epX01 (Fig. 1.2B); O: posterior end of the epiproct; I: mid-dorsal site of clunium (segment IX); and F: closure and/or flipping of the epiproct.

This is the only muscle originating from the epiproct observed throughout Psocopterans. However, this muscle is absent in *Prionoglaris* (Fig. 1.1B). In *Lepinotus*, additional paired muscles (paX02: see below; Fig. 1.3B) are also inserted into the epiproct.

Muscles originating from the paraproct

02 paX01 (Fig. 1.8); O: anterodorsal end of the paraproct, very close to the posterolateral margin of epiproct; I: anterolateral region of clunium (segment X); and F: involved in opening the paraproct.

03 paX02 (Fig. 1.8); O: anterodorsal end of the paraproct, very close to the anterolateral corner of epiproct; I: mediodorsal region of clunium (segment X); and F: involved in opening the paraproct.

04 paX03 (Fig. 1.9); O: internal margin of the paraproct near the anal opening; I: mediolateral region of clunium (segment X); and F: involved in opening the paraproct.

05 paX04 (Fig. 1.9); O: anterolateral margin of the paraproct; I: mediolateral region of clunium (segment IX); and F: involved in restoring the paraproct.

06 paX05 (Fig. 1.10); O: anteroventral end of the paraproct; I: anteroventral margin of clunium (segment IX), near the base of the dorsal valve; and F: involved in opening the paraproct.

The paX01–05 muscles are widely observed throughout psocopterans, although paX03 is absent in *Prionoglaris*, *Lepinotus*, *Amphientomidae*, *Archipsocus* and *Valenzuela* (Fig. 1.9), while paX05 is absent in *Psyllipsocus* (Fig. 1.2B).

07 papa01 (Fig. 1.9); O: internal margin of the paraproct near the anal opening; I: anterolateral margin of the paraproct; and F: involved in opening the anus.

08 papa02 (Fig. 1.9); O: internal margin of the paraproct near the junction of the two paraprocts; I: internal margin of the anteroventral region of the paraproct; and F: involved in opening the anus.

The papa01 muscle is widely observed throughout psocopterans but is absent in *Trichadenotecnum*. The papa02 muscle is only observed in *Prionoglaris* and coexists

with papa01 (Fig. 1.9A).

1.3.1.3 Gonapophyses

The female gonapophyses arise from sternum VIII (ventral valve) or the IX segment (dorsal and external valves), and the following three pairs of valves are tightly associated to each other: the most external valve on the outside, the dorsal valve at the middle and the most internal ventral valve on the inside. The shape and degree of development vary greatly among different taxa. A reduction of the gonapophyses is especially distinct in Trogiomorpha: in most members, the ventral and dorsal valves are highly reduced or completely absent. In contrast, in *Valenzuela* (Psocomorpha), the external valve is greatly reduced, although the ventral and dorsal valves are well developed (Fig. 1.6A). In *Archipsocus* (Psocomorpha), all gonapophyses are greatly reduced (Fig. 1.5A).

　　Muscle originating from the ventral valve

09 veVII01 (Fig. 1.11A, B); O: base of the ventral valve; I: ventral region of segment VII; and F: involved in opening the ventral valve.

This is the only muscle in psocopterans that originates from the ventral valve. The muscle is observed in *Amphientomidae* and *Valenzuella* but is absent in all other taxa examined here, which is partly due to the reduction of the ventral valve (Trogiomorpha and *Archipsocus*). However, in *Trichadenotecnum*, this muscle is absent, despite the species possessing a well-developed ventral valve.

Chapter 1

Muscles originating from the dorsal valves

10 dosp01 (Fig. 1.11C, D); O: base of the dorsal valve; I: on the membrane connected to the spermapore plate, near the posterior tip of the spermapore plate; and F: involved in restoring the position of the spermapore plate.

11 doIX01 (Fig. 1.11C, D); O: anterior end of the dorsal valve; I: posterolateral margin of segment IX; and F: involved in opening the dorsal valve.

These two muscles originate from the dorsal valve and are inserted into segment IX at either the spermapore plate (dosp01) or the clunium (doIX01). They are only observed in *Valenzuela* and *Trichadenotecnum* (Psocomorpha) but are absent in all other taxa examined.

Muscles originating from the external valves

12 exsp01 (Fig. 1.12); O: base of the external valve; I: on the membrane connected to the spermapore plate, near the posterior tip of the spermapore plate, and partially overlapping dosp01; and F: involved in restoring the position of the spermapore plate.

13 exIX01 (Fig. 1.12); O: lateral margin of the external valve; I: posterolateral margin of segment IX, and partially overlapping doIX01; and F: involved in opening the external valve.

14 exIX02 (Fig. 1.12); O: base of the external valve, near the middle of the junction with the clunium; I: anterolateral margin of the clunium; and F: involved in opening the external valve.

These three muscles originate from the external valve and are inserted on

segment IX at either the spermapore plate (exsp01) or the clunium (exIX01–02).

These muscles are widely observed throughout psocopterans but completely absent in

Archipsocus and *Valenzuela*. The exIX01 muscle is absent in *Prionoglaris,*

Archipsocus and *Valenzuela*. In addition, the exIX02 muscle is absent in Psocomorpha

(Fig. 1.12).

15 doex01 (Fig. 1.11E, F); O: base of the dorsal valve; I: base of the external valve;

and F: involved in opening the dorsal valve.

This is the only muscle connecting two gonapophyses (dorsal and external)

observed in psocopterans. The muscle is only observed in *Lepinotus* (Trogiomorpha)

and Amphientomidae (Troctomorpha).

1.3.1.4 Subgenital plate

The subgenital plate is formed by sternum VIII and covers the gonapophyses ventrally.

It is variable in shape between taxa, and it frequently bears a posterior extension (egg

guide). In contrast, the subgenital plate is greatly reduced in Trogiomorpha and only

covers the anterior part of the gonapophyses.

16 sgVIII01 (Fig. 1.13); O: middle of the dorsal surface of the subgenital plate; I:

middle of the ventral surface of the egg guide; and F: involved in closing the

subgenital plate

17 sgVIII02 (Fig. 1.13); O: middle of the dorsal surface of the subgenital plate; I:

anterior margin of sternum VIII, and partially overlapping sgVIII01; and F: involved in opening the subgenital plate.

These two groups of muscles originate from the subgenital plate and are only observed in Psocomorpha. No muscles are attached on the subgenital plate in the suborders Trogiomorpha and Troctomorpha. The subgenital plate of Trogiomorpha is greatly reduced and the absence of the subgenital muscles may be partly linked to this condition. The sgVIII02 muscle was present in all Psocomorpha taxa examined, whereas sgVIII01 was absent in *Archipsocus* (Fig. 1.13).

1.3.1.5 Spermapore plate

The spermapore plate is a sclerite bearing the spermapore (e.g., Figs. 1.1B and 1.12). According to Bitsch (1979) the spermapore plate is situated on the posterior part of sternum VIII (see Lienhard *et al.*, 2010). However, by observing the 3D modeling, in most cases, the spermapore plate is located behind the subgenital plate, but is not connected to it. The skeletal part of the spermapore plate is not connected to the surrounding structures directly but is connected to the genital structures of the segment IX through the spermapore membrane, so I judged it to be a part of the sternum IX. Its degree of sclerotization is highly variable, and it is almost completely membranous in some taxa (*Archipsocus* and *Valenzuela*; Figs. 1.5B and 1.6B). Its lateral margins loosely articulate with surrounding structures (paraproct, gonapophyses, and subgenital plate) via the spermapore membrane.

In addition to the muscles described below, the muscles dosp01 and exsp01, both originating on the gonapophyses, are also associated with the membranous part of the spermapore plate in many taxa examined (see above).

18 spVIII01 (Fig. 1.14C, E); O: on the spermapore membrane; I: anteroventral margin of segment VIII, near the junction of the clunium; and F: involved in restoring the position of the spermapore plate.

This is the only muscle that originates from the spermapore plate; it is inserted onto sternum VIII the examined if present. It is present in *Lepinotus* (Trogiomorpha) and *Valenzuela* (Psocomorpha).

19 spIX01 (Fig. 1.14); O: on the spermapore membrane; I: mediolateral region of the clunium; and F: involved in restoring the position of the spermapore plate.

20 spIX02 (Fig. 1.14); O: on the spermapore membrane; I: mediolateral region of the clunium, and partially overlapping with exIX01; and F: involved in restoring the position of the spermapore plate.

21 spIX03 (Fig. 1.14); O: the membranous part of the spermapore plate, near the internal part of the dorsal valve; I: anterolateral margin of segment IX, and partially overlapping with doVIII02 and exIX02; and F: involve in stretching the spermapore plate.

The spIX03 muscle is often present but missing in *Prionoglaris* and Amphientomidae. In contrast, muscle spIX02 is restricted to Trogiomorpha and spIX01 was only found in *Psyllipsocus* and *Lepinotus*.

1.5 Discussion

1.5.1 Homology of the muscles

Apart from the ovipositor valves (discussed below), the homology of the female terminal sclerites (i.e., clunium, epiproct, paraproct, subgenital plate and spermapore plate) is almost unambiguously identifiable throughout psocopterans (Figs. 1.1–7). Therefore, based on the origin and insertion points of each muscle, the homology of the female genital muscles of psocopterans can be established confidently throughout the order (Table 1.2). Badonnel (1934) also examined the female genital musculature of *Stenopsocus stigmaticus*, a close relative of *Valenzuela* examined here, and the muscles that he observed can be homologized with the muscles identified here (see also the ovipositor section below). However, the homology of several muscles is debatable.

For example, paX02 (Fig. 1.8) was consistently observed throughout psocopterans and almost always originates from the dorsal margin of the paraproct, very close to the lateral margin of the epiproct. However, in *Lepinotus*, the site of origin of the corresponding muscle is changed to the epiproct (Fig. 1.3B). Therefore, judging only from origin, this muscle may be regarded as an epiproctal muscle. However, in *Lepinotus*, no other muscle corresponding to paX02 could be detected. In addition, its site of origin is very close to that of paX02 observed in other

psocopterans, and its insertion site is consistent with that of paX02. Therefore, although its origin and probably also its function are different from that of paX02 in other psocopterans, the muscle originating from the lateral margin of the epiproct of *Lepinotus* was identified here as paX02 (Table 1.2).

A similar situation was observed for spIX02 (Fig. 1.14A–C), which only occurs in Trogiomorpha. The insertion site of the muscle in *Psyllipsocus* and *Lepinotus* is the mediolateral region of the clunium. The spIX02 muscle of *Lepinotus* consists of a pair of bundles, whereas spIX02 of *Prionoglaris* and *Psyllipsocus* consists of a single bundle. Moreover, the insertion site of spIX02 of *Prionoglaris* is the middle region of the external valve. Except for spIX02, no other muscles of *Prionoglaris* are connected to the spermapore membrane, and no muscles similar to those on the external valve are found in other psocopterans. In *Prionoglaris,* the external valve is significantly enlarged and thus expands dorsally in comparison to other trogiomorphan genera. The orientation of the muscles (horizontally directed towards its origin on the spermapore) is similar in *Prionoglaris* and *Psyllipsocus/Lepinotus*. Therefore, the muscle originating from the spermapore plate and inserted on the middle region of the external valve of *Prionoglaris* was identified as spIX02 (Table 1.2).

In addition, some muscles are confirmed only in few distantly related taxa. For example, veVII01 (Fig. 1.11A, B) was only observed in Amphientomidae (Troctomorpha) and *Valenzuela* (Psocomorpha), and doex01 (Fig. 1.11E, F) was only found in *Lepinotus* (Trogiomorpha) and Amphientomidae (Table 1.2). As discussed

below (see the phylogenetic significance section), independent gains of these muscles are the most parsimonious interpretation (i.e., Fig. 1.15), but corresponding muscles are also observed in some holometabolans (Table 1.2: Hünefeld *et al.*, 2012). It is generally regarded that the loss of a character occurs much more frequently than the independent gain of a novel feature (e.g., Trueman *et al.*, 2004). Therefore, although this remains ambiguous, I tentatively recognized the muscles only observed in distantly related psocopteran taxa and holometabolans as homologous (Table 1.2), suggesting multiple loss rather than more parsimonious independent gain.

Homology identification of the female genital muscles between psocopterans and other insects is complicated. Psocopterans are traditionally included in the superorder Paraneoptera (this view is still most strongly supported morphologically and also could not be rejected also by phylogenomics: Hennig, 1969; Yoshizawa & Lienhard 2016; Johnson *et al.*, 2018), but the order Psocodea was interpreted as the sister taxon of the Holometabola by phylogenomics (Misof *et al.*, 2014; Johnson *et al.*, 2018). The ground plan condition of the female genital musculature in Holometabola has been estimated by Hünefeld *et al.* (2012). Therefore, I tried to homologize the psocopteran female genital muscles according to the system of Hünefeld *et al.* (2012). As shown in Table 2, homology of only a few muscles (e.g., epiproctal epX01 muscle) can be identified confidently between psocopterans and Holometabola. In contrast, although homologous muscles are probably present in Holometabola (e.g., paraproctal muscles paX01–05), the homology of many psocopteran muscles cannot be specified with reasonable certainty, for which more than one candidate homologous muscle is

indicated in Table 1.2. Examination of the other paraneopteran and holometabolan taxa may improve these ambiguities.

1.5.2 Homology of the psocopteran ovipositor valves

In the neopteran ground plan condition, the ovipositor is composed of three sets of elongated valves derived from postabdominal appendages, namely, V1 (segment VIII), V2 and V3 (both segment IX). Frequently, a short valvifer (or gonocoxite) is also present basal to the valves of each segment (VIII and IX) (Beutel *et al*., 2014).

The psocopteran ovipositor valves are also usually composed of three valves, namely, ventral (segment VIII), dorsal and external valves (both segment IX), which are generally considered to be homologous to V1–V3 of the neopterans groundplan. When all valves are well developed, they can be addressed very clearly. Even though the dorsal and external valves are fused with each other, they can be clearly identified as elongated dorsal valve and basally bulged external valve (e.g., Yoshizawa, 2005). In contrast, the valvifer is never developed as a separated sclerite in psocopterans.

Hünefeld *et al*. (2012) examined the musculature of the female postabdomen of a wide variety of taxa and estimated the ground plan of the abdominal musculature of the Holometabola. According to the interpretation presented in Hünefeld *et al*. (2012), all intersegmental and dorsoventral muscles associated with the ovipositor valves are inserted on the valvifer (i.e., the coxal segment of the appendage).

In psocopterans, one intersegmental muscle (veVII01: homologous to the muscle

"c" of Badonnel, 1934: fig. 69) is inserted to the base of the ventral valve (segment VIII: Fig. 11A, B). This muscle is apparently homologous to either muscle 08 or 09 (intersegment VII) of Hünefeld *et al.* (2012) (Table 1.2), suggesting that the psocopteran ventral valve is composed of fused valvifer VIII and V1. In psocopteran segment IX, three dorsoventral muscles are observed (Figs 1.11C, D and 1.12): one is inserted on the dorsal valve (doIX01: muscle "e" of Badonnel, 1934) and the other two on the base of the external valve (exIX01 and 02: muscle "f" of Badonnel, 1934). They very likely correspond to either the muscles 50, 51, 52 or 53 (dorsoventral IX) of Hünefeld *et al.* (2012), which are usually inserted on valvifer IX in neopterans. Therefore, it is also likely that the psocopteran dorsal and external valves are possibly a composite structure formed by fusion of V2, V3, and valvifer IX.

In addition, a muscle named doex01 was observed in *Lepinotus* and Amphientomidae, which connect the external valve and dorsal valve (Fig. 1.11E, F). In the holometabolan ground plan condition, two muscles connecting valvifers IX and V2 (57 and 58) are present (Hünefeld *et al.*, 2012), and doex01 is very likely homologous to either 57 or 58 of Hünefeld *et al.* (2012) (Table 2). In Amphientomidae (with three well-developed valves), this muscle originates from the base of the dorsal valve and is inserted to the external margin of the external valve (Fig. 1.11F). It also provides additional support that valvifer IX is fused to V3 in psocopterans, together forming the external valve.

The presence of doex01 also provides interesting insights for the morphological

interpretation of ovipositor valves in psocopterans. In the suborder Trogiomorpha, they are greatly reduced and are usually represented by a single pair of valves (Figs 1.1–3). It is generally assumed that these single pair of valves are homologous to the external ones. However, the presence of the doex01 muscle (= either 57 or 58 of Hünefeld *et al*., 2012) strongly suggests that the well-developed valve of *Lepinotus* involves at least a vestigial element of the dorsal valve as the insertion site of the muscle doex01 (Fig. 1.11E).

Different morphological interpretations for the female ovipositor valves were also proposed for the mid-Cretaceous trogiomorphan genus *Palaeosiamoglaris* by Azar *et al*. (2017). *Palaeosiamoglaris* is thought to be most closely related to the extant genus *Siamoglaris* from Thailand, and they together form a monophyletic subfamily *Prionoglaridinae* with *Prionoglaris*. The basic female genital structures of *Siamoglaris* and *Prionoglaris* (examined here) are very similar. Azar *et al*. (2017) identified the most well-developed ovipositor valve of *Palaeosiamoglaris* as the ventral valve, and opaque structures presented next and dorsal to these structures were identified as external and dorsal valves, respectively (Azar *et al*., 2017: figs 1.5B, 1.15B). However, as discussed above, the most distinct ovipositor valve of *Prionoglaris* and other trogiomorphan taxa should be homologized with the external valve (with some vestigial element of the dorsal valve), and reduction of the ventral and dorsal valves should be considered as an autapomorphy of Trogiomorpha (Yoshizawa & Lienhard, 2020). As mentioned above, *Palaeosiamoglaris* is placed "within" a subfamily of a trogiomorphan family and is very closely related to

25

Prionoglaris. Under such conditions, it is less likely that a well-developed ventral valve is retained in *Palaeosiamoglaris*; thus, the structure is interpreted here as the external valve (plus some vestigial element of a dorsal valve), as also seen in *Prionoglaris*.

1.5.3 Phylogenetic significance of the musculature

The data presented in Table 1.2 were treated as a character state matrix and reconstructed on the phylogenetic tree of psocopterans estimated based on multiple genes (Yoshizawa *et al.*, 2006; Yoshizawa & Johnson, 2014) or transcriptomes (Johnson *et al.*, 2018; de Moya *et al*., 2021) to visualize the phylogenetic signal contained in the female genital musculature (Fig. 1.15). The consistency index (CI) was calculated as 0.69, and the retention index (RI) was 0.53. This CI value is relatively high compared to other morphological character system used for the phylogenetic reconstruction of Psocomorpha, e.g., 0.41 for the total morphology, 0.36 for male external genitalia, and 0.45 for female external genitalia (Yoshizawa & Johnson, 2014), suggesting that the characteristic system includes useful phylogenetic signals with relatively low levels of homoplasies.

The most parsimonious reconstruction of the character matrix on the tree yielded some useful characters supporting deep psocopteran phylogenetic splits (Fig. 1.15): the absence of exIX01 (14) and the presence of sgVIII01 (16) support the monophyly of the suborder Psocomorpha, and the presence of dosp01 (10), doIX01 (11) and

sgVIII02 (17) (all non-homoplasious) support the monophyly of Psocomorpha excluding Archipsocetae. In addition, the condition of spIX02 (20) was consistently different between Trogiomorpha (present) and Troctomorpha + Psocomorpha (absent) (Table 1.2). Therefore, this muscle likely contains a signal to support either of these clades (depending on the condition of the outgroup, but if the reduction of muscles is a general tendency, this may rather support Troctomorpha + Psocomorpha: Table 1.2). Some homoplasious and nonhomoplasious character states are only identified at the terminal branches (i.e., phylogenetically noninformative) (Fig. 1.15). However, with denser taxonomic sampling, these characters may be phylogenetically informative on a lower taxonomic level (i.e., infraorder, family, or genus).

It has to be noted that the female genital musculature may contain more homoplasies than discussed above. As already mentioned, independent losses of a character are generally considered to be much more frequent than independent gains of a novel feature. The present results suggested several independent gains of new muscles (Fig. 1.15), for which the alternative interpretation (multiple loss of the muscles) should also be considered. For example, exIX01 is absent in *Valenzuela* but was observed in *Stenopsocus* (a close relative of *Valenzuela*) by Badonnel (1934: fig. 69). Although the ancestral condition of this muscle is ambiguous for *Valenzuela* (Fig. 1.15), the presence of exIX01 in *Stenopsocus* strongly suggests that the absence of this muscle is an apomorphic condition independently derived in *Prionoglaris*, *Archipsocus*, and *Valenzuela*. Much denser sampling of psocopterans is needed to accurately identify the evolutionary trend of the female genital muscles. The present

study provides the foundation for such a future project.
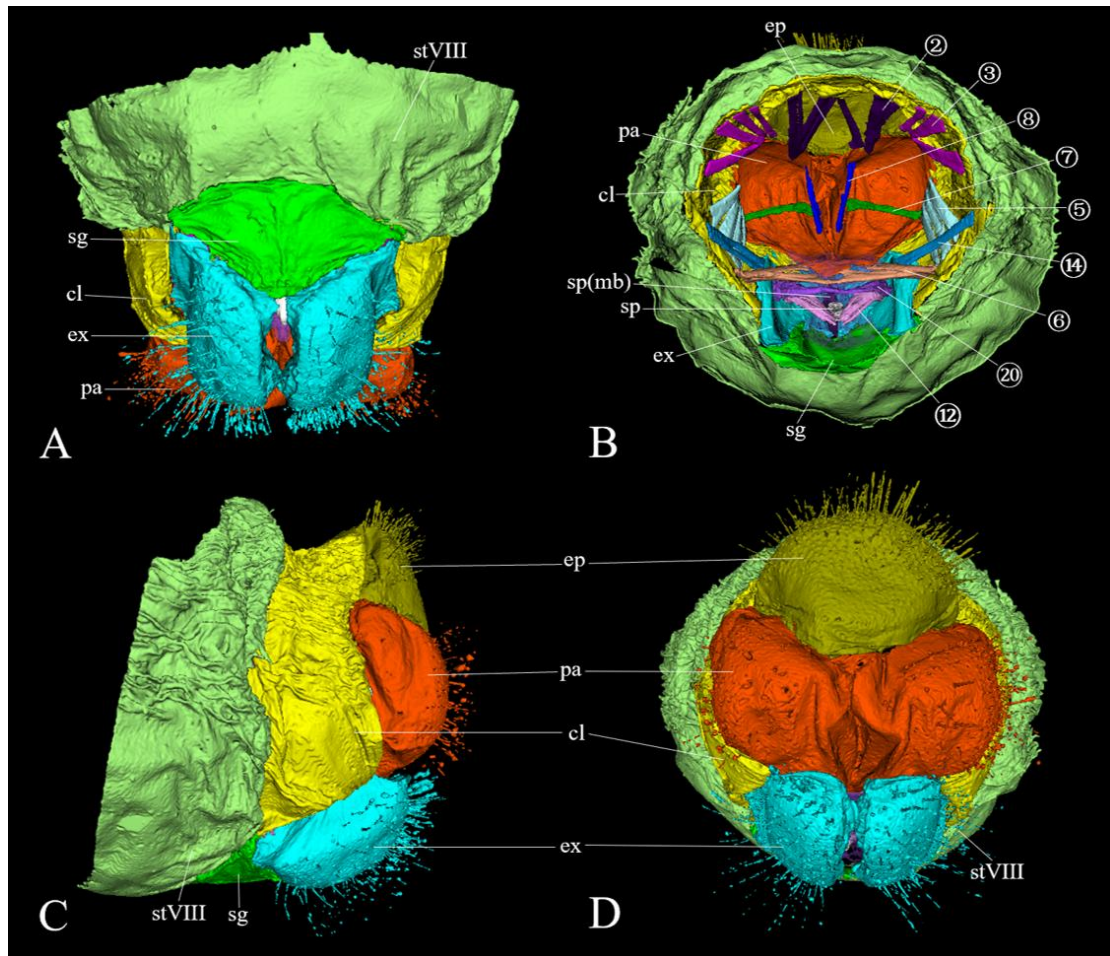
Figure 1.1 3D reconstruction of the female terminalia of *Prionoglaris stygia*: (A) Ventral view; (B) Internal view: (2) paX01; (3) paX02; (5) paX04; (6) paX05; (7) papa01; (8) papa02; (12) exsp01; (14) exIX02; (20) spIX02; (C) Lateral view; (D) Posterior view. Abbreviations: cl = clunium; ex = external valve; sg = subgenital plate; ep = epiproct; pa = paraproct; sp = spermpore plate; mb = membrane; st = sternum.
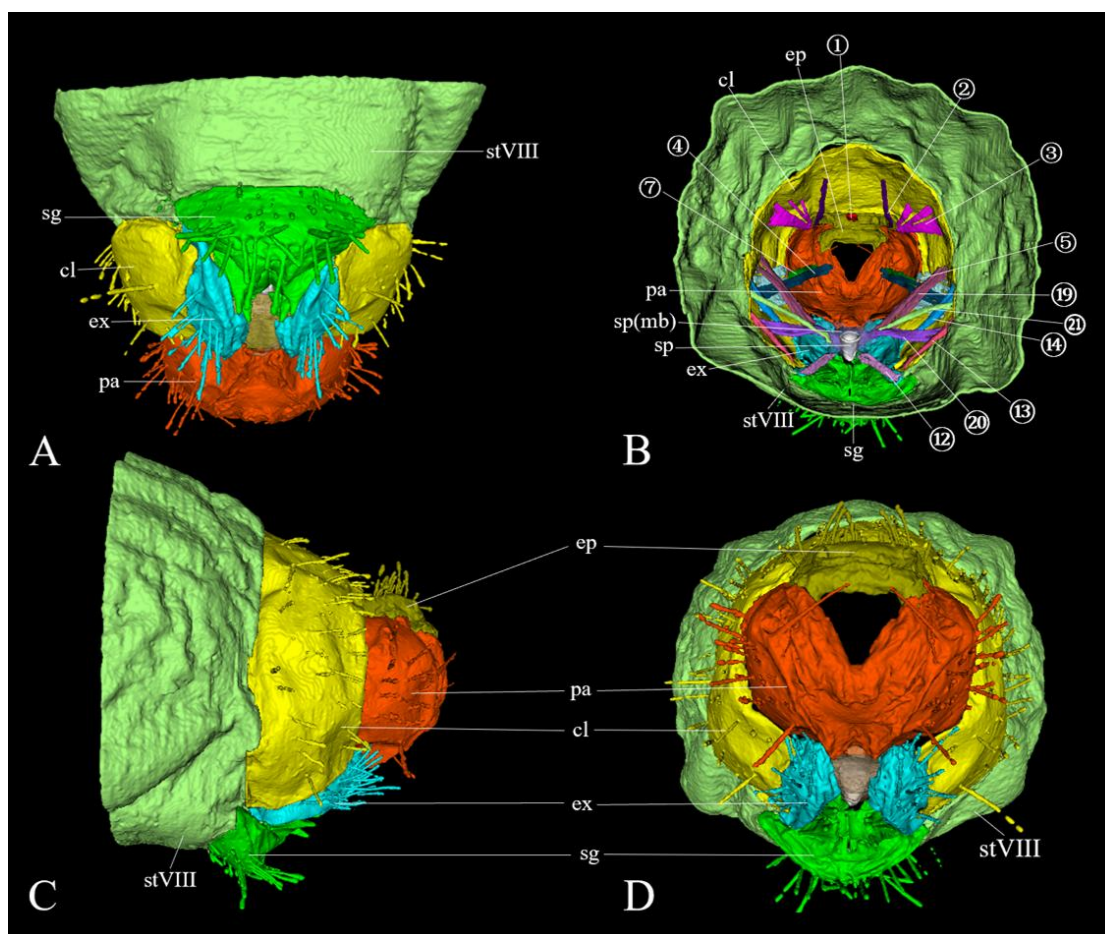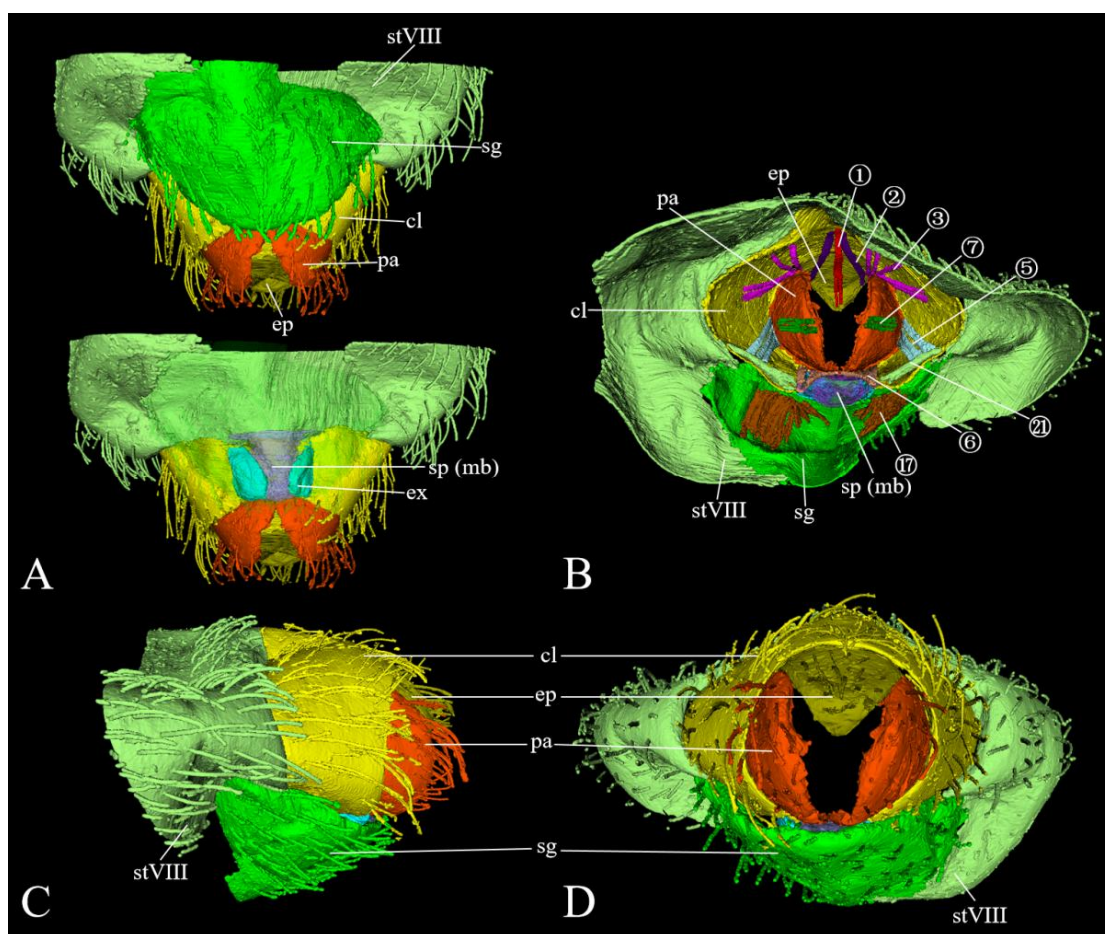
Figure 1.2 3D reconstruction of the female terminalia of *Psyllipsocus clunjunctus*: (A) Ventral view; (B) Internal view: (1) epX01; (2) paX01; (3) paX02; (4) paX03; (5) paX04; (7) papa01; (12) exsp01; (13) exIX01; (14) exIX02; (19) spIX01; (20) spIX02; (21) spIX03; (C) Lateral view; (D) Posterior view. See Figure 1.1 for abbreviations.
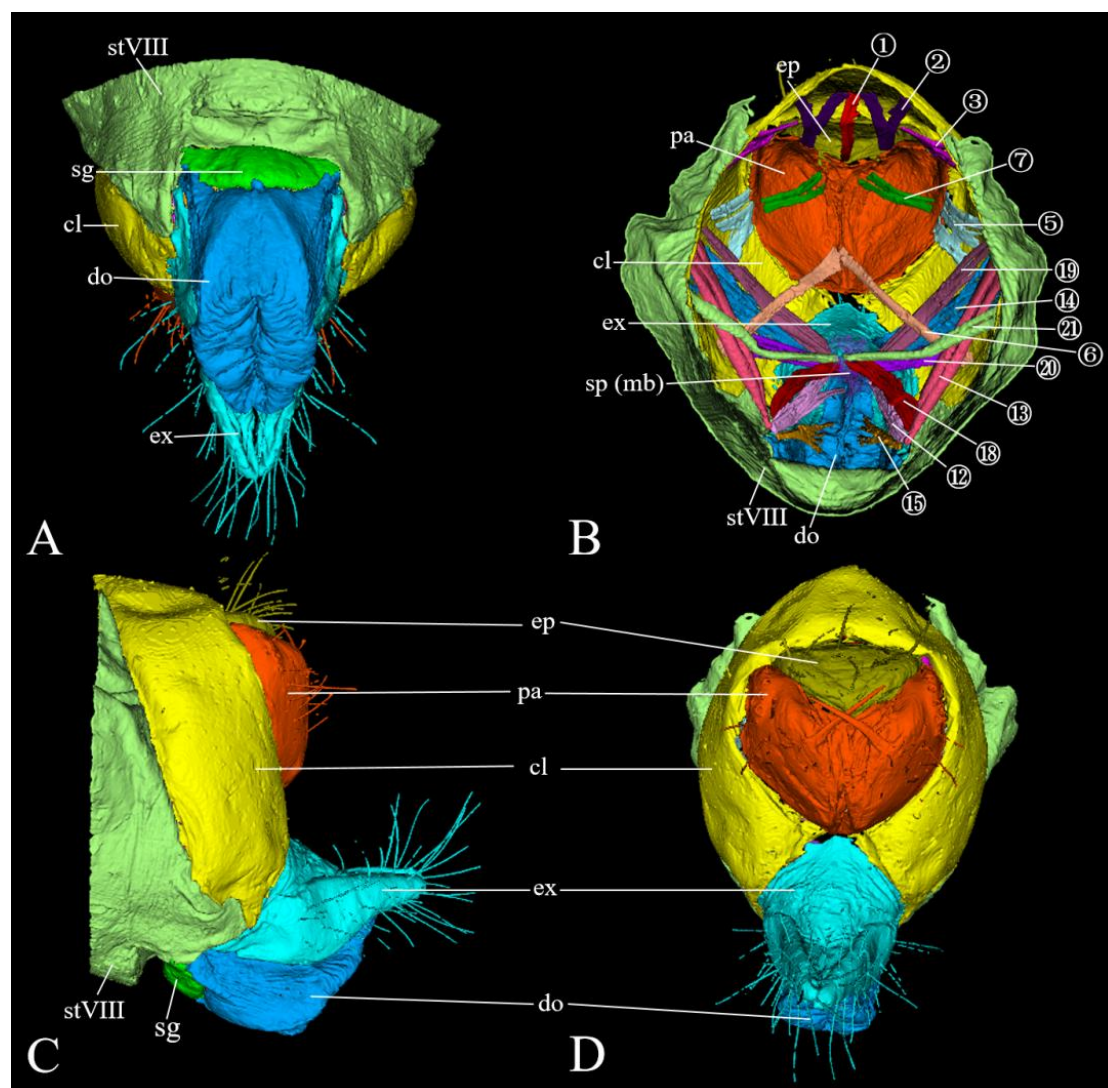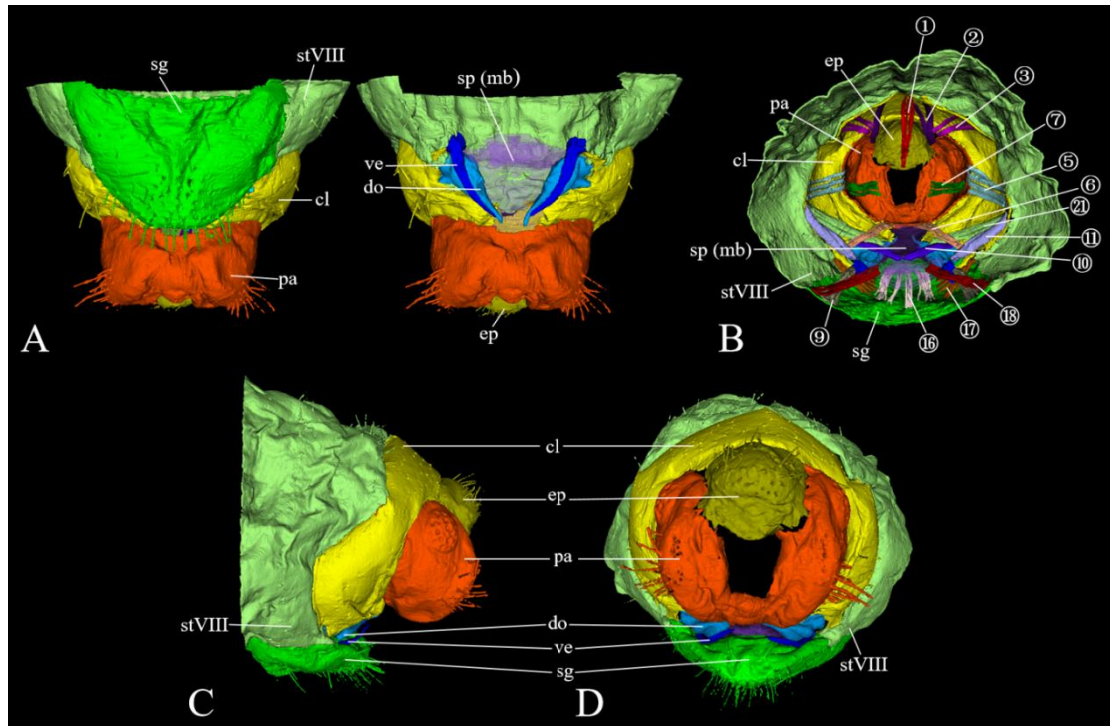
Figure 1.3 3D reconstruction of the female terminalia of *Lepinotus reticulatus*: (A) Ventral view; (B) Internal view: (1) epX01; (2) paX01; (3) paX02; (5) paX04; (6) paX05; (7) papa01; (12) exsp01; (13) exIX01; (14) exIX02; (15) doex01; (18) spVIII01; (19) spIX01; (20) spIX02; (21) spIX03; (C) Lateral view; (D) Posterior view. See Figure 1.1 for abbreviations.
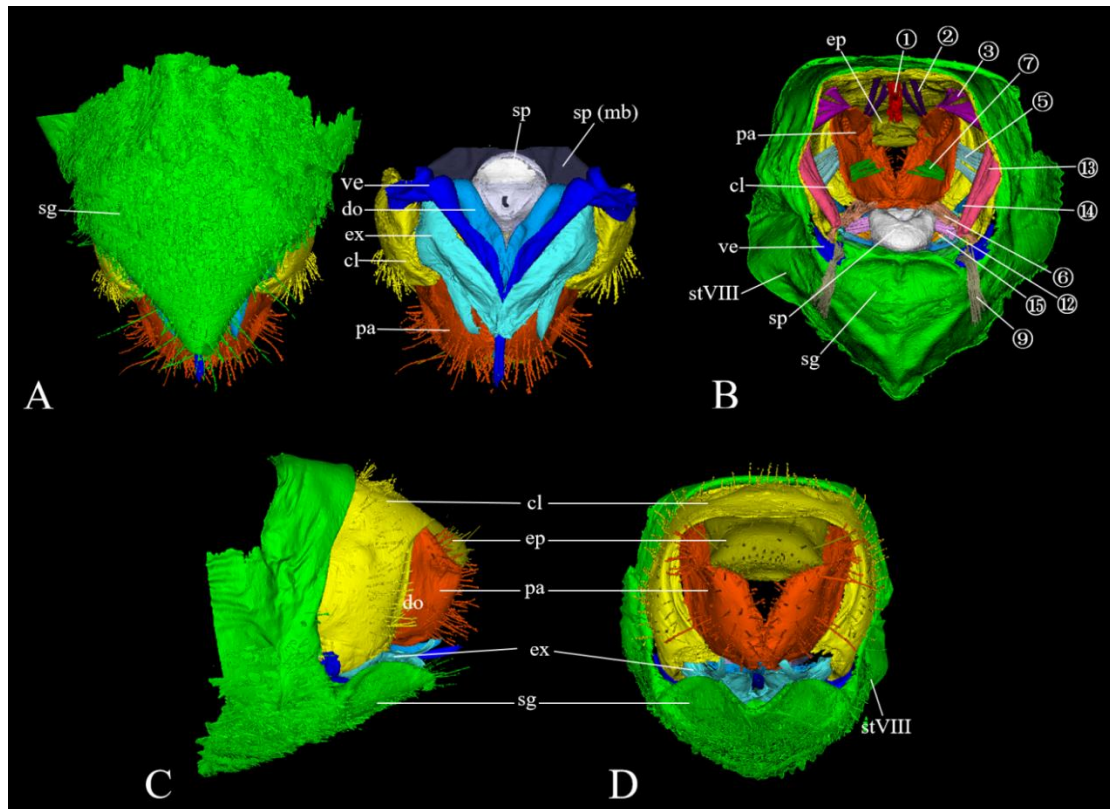
Figure 1.4 3D reconstruction of the female terminalia of Amphientomidae Gen. sp.: (A) Ventral view (right: the subgenital plate is invisible); (B) Internal view: (1) epX01; (2) paX01; (3) paX02; (5) paX04; (6) paX05; (7) papa01; (9) veVII01; (12) exsp01; (13) exIX01; (14) exIX02; (15) doex01; (C) Lateral view; (D) Posterior view. See Figure 1.1 for abbreviations.

Figure 1.5 3D reconstruction of the female terminalia of *Archipsocus* sp.: (A) Ventral view (below: the subgenital plate is invisible); (B) Internal view: (1) epX01; (2) paX01; (3) paX02; (5) paX04; (6) paX05; (10) dosp01; (11) doIX01; (12) exsp01; (13) exIX01; (17) sgVIII02; (21) spIX03; (C) Lateral view; (D) Posterior view. See Figure 1.1 for abbreviations.
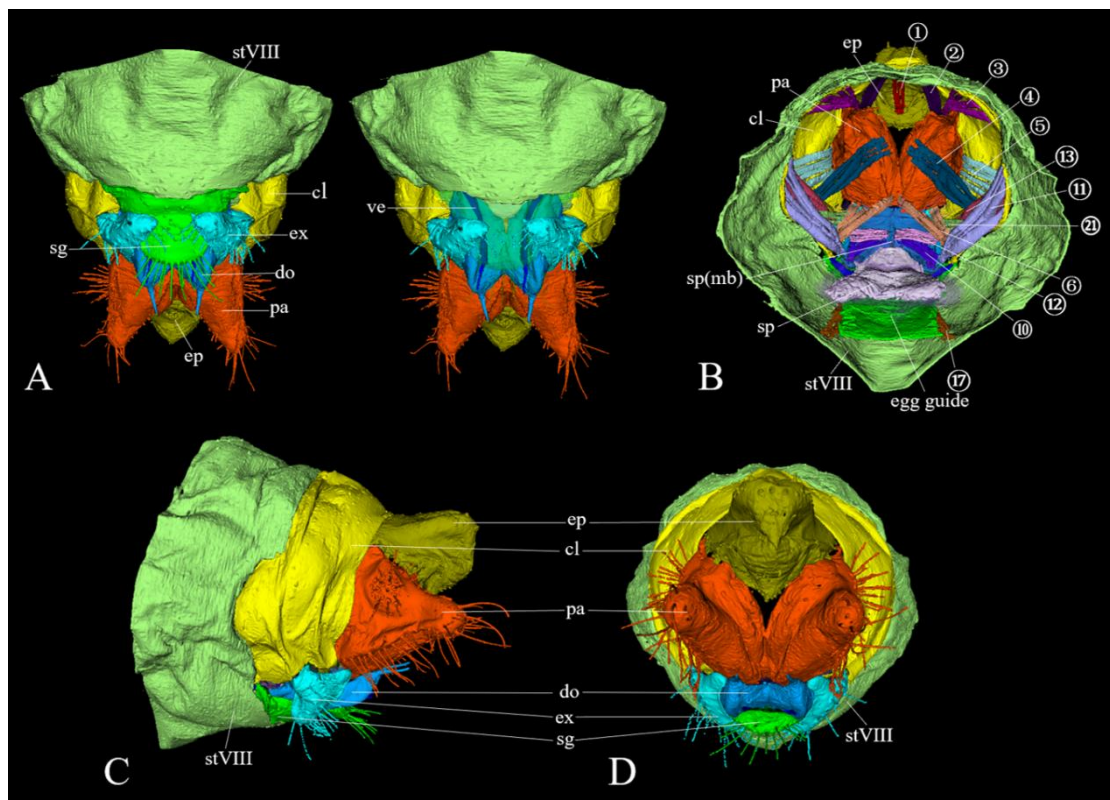
Figure 1.6 3D reconstruction of the female terminalia of *Valenzuela badiostigma*: (A) Ventral view (right: the subgenital plate is invisible); (B) Internal view: (1) epX01; (2) paX01; (3) paX02; (5) paX04; (6) paX05; (7) papa01; (9) veVII01; (10) dosp01; (11) doIX01; (16) sgVIII01; (17) sgVIII02; (18) spVIII01; (21) spIX03; (C) Lateral view; (D) Posterior view. See Figure 1.1 for abbreviations.

Chapter 1

Figure 1.7 3D reconstruction of the female terminalia of *Trichadenotecnum pseudomedium*: (A) Ventral view (right: subgenital plate is invisible); (B) Interal view: (1) epX01; (2) paX01; (3) paX02; (4) paX03; (5) paX04; (6) paX05; (10) dosp01; (11) doIX01; (12) exsp01; (13) exIX01; (17) sgVIII02; (21) spIX03; (C) Lateral view; (D) Posterior view. See Figure 1.1 for abbreviations.
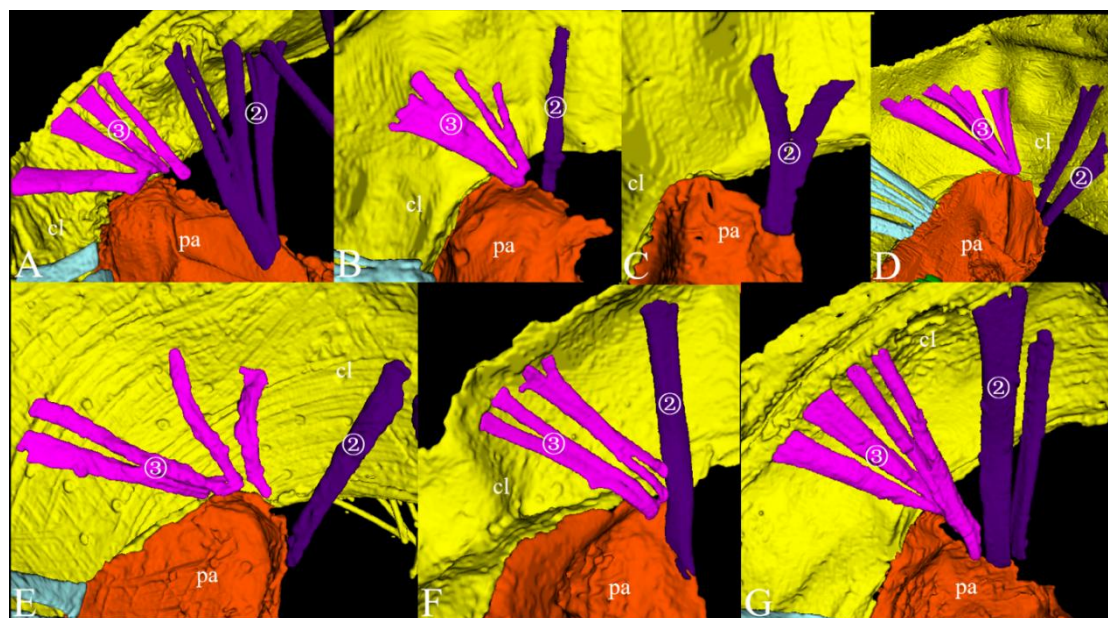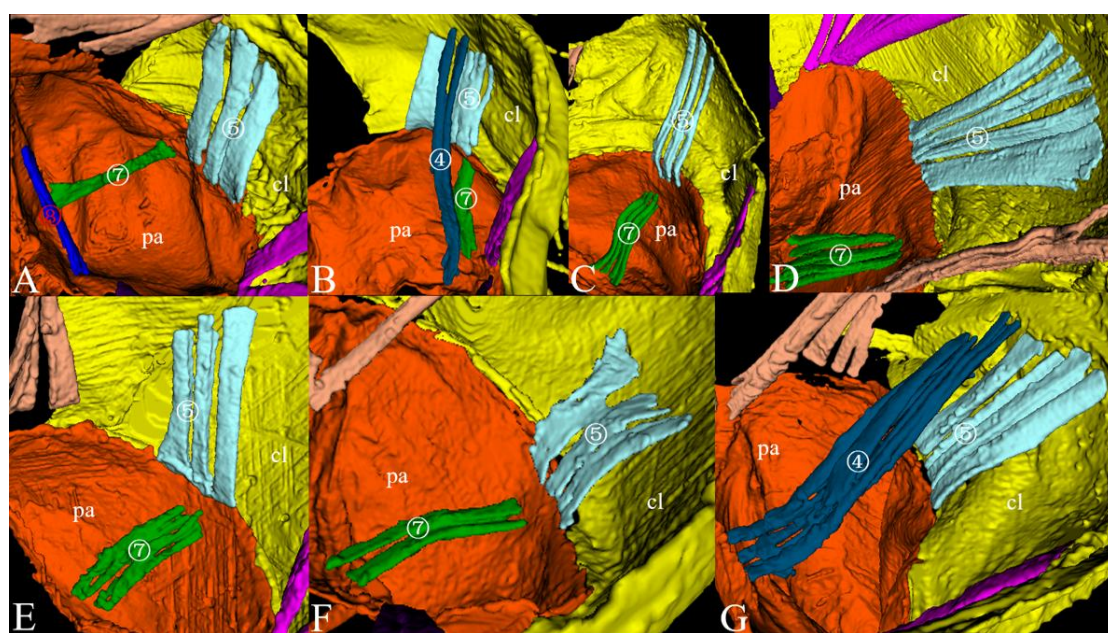

Figure 1.8 Close up of the paraproct (dorsal part) and clunium, internal view: (A) *Prionoglaris stygia*; (B) *Psyllipsocus clunjunctus*; (C) *Lepinotus reticulatus*; (D) Amphientomidae Gen. sp.; (E) *Archipsocus* sp.; (F) *Valenzuela badiostigma*; (G) *Trichadenotecnum pseudomedium*. (2) paX01; (3) paX02. See Figure 1.1 for abbreviations.

Figure 1.9 Close up of the paraproct (lateral part) and clunium, internal view: (A) *Prionoglaris stygia*; (B) *Psyllipsocus clunjunctus*; (C) *Lepinotus reticulatus*; (D) Amphientomidae Gen. sp.; (E) *Archipsocus* sp.; (F) *Valenzuela badiostigma*; (G) *Trichadenotecnum pseudomedium*. (4) paX03;

(5) paX04; (7) papa01; (8) papa02. See Figure 1.1 for abbreviations.
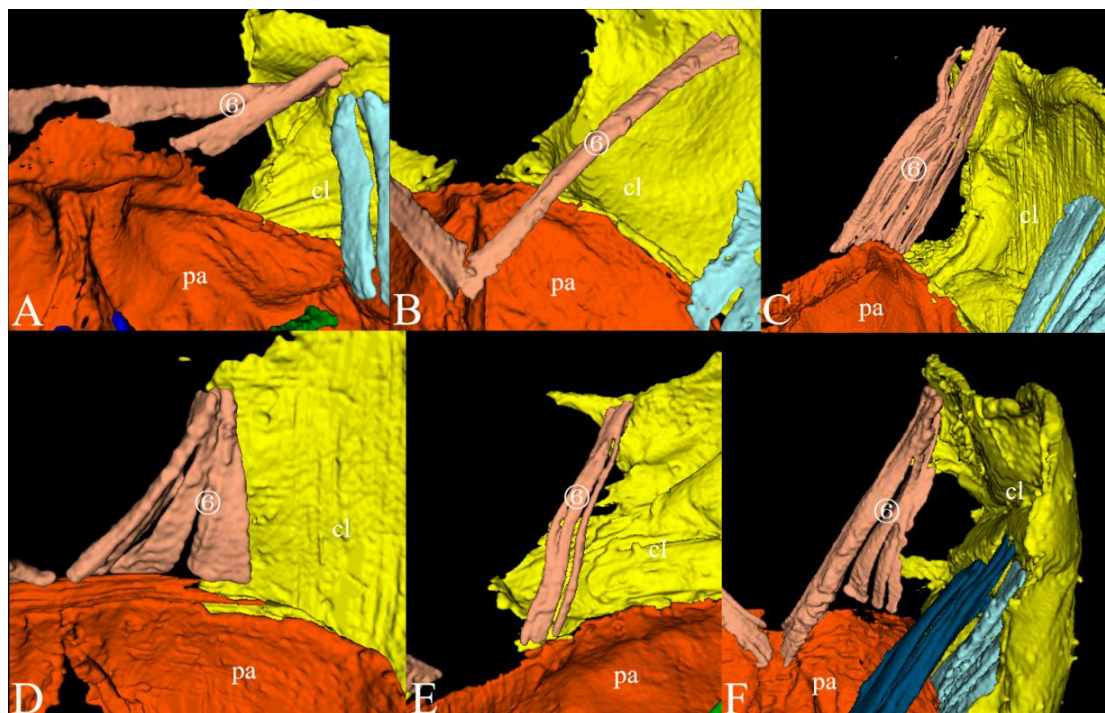
Figure 1.10 Close up of the paraproct (ventral part) and clunium, internal view: (A) *Prionoglaris*; (B) *Lepinotus reticulatus*; (C) Amphientomidae Gen. sp.; (D) *Archipsocus* sp.; (E) *Valenzuela badiostigma*; (F) *Trichadenotecnum pseudomedium*. (6) paX05. Abbreviations: cl = clunium; pa = paraproct.
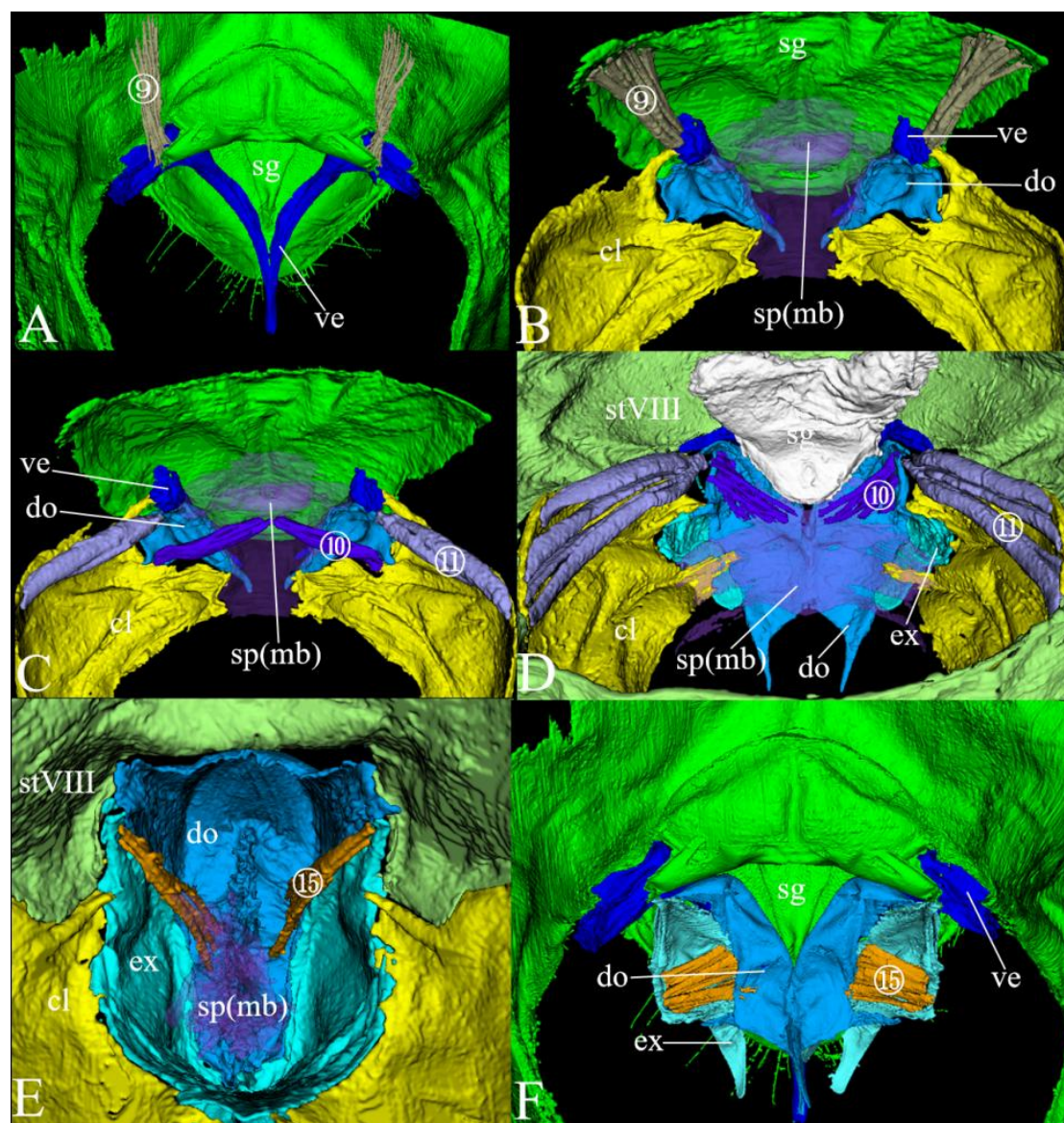
Figure 1.11 Close up of the gonapophyses and related structures, internal view: (A) Amphientomidae Gen. Sp.; (B) *Valenzuela badiostigma*; (C) *Valenzuela badiostigma*; (D) *Trichadenotecnum pseudomedium*; (E) *Lepinotus reticulatus*; (F) Amphientomidae Gen. sp. (9) veVII01; (10) dosp01; (11) doIX01; (15) doex01. See Figure 1.1 for abbreviations.
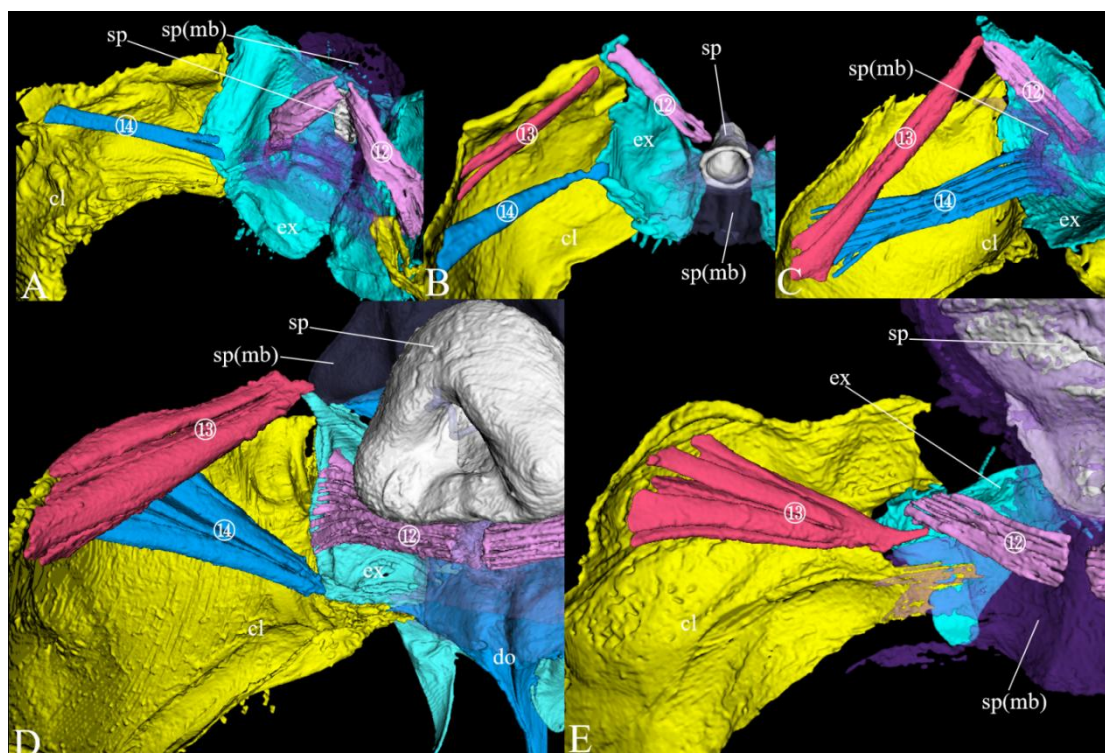
Figure 1.12 Close up of the gonapophyses and related structures, internal view: (A) *Prionoglaris stygia*; (B) *Psyllipsocus clunjunctus*; (C) *Lepinotus reticulatus*; (D) Amphientomidae Gen. sp; (E) *Trichadenotecnum pseudomedium*. (12) exsp01; (13) exIX01; (14) exIx02. See Figure 1.1 for abbreviations.
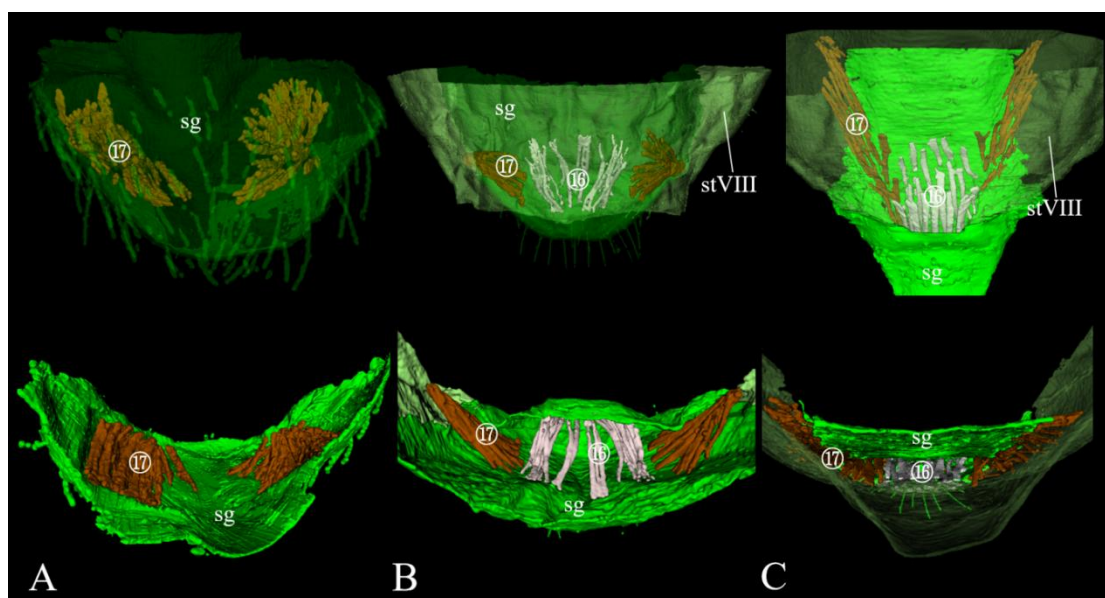
Figure 1.13 Subgenital plate, internal vew: (A) *Archipsocus* sp. (top: ventral view; below: internal view); (B) *Valenzuela badiostigma* (top: ventral view; below: internal view); (C) *Trichadenotecnum pseudomedium* (top: ventral view; below: internal view). (16) sgVIII01; (17) sgVIII02. See Figure 1.1 for abbreviations.
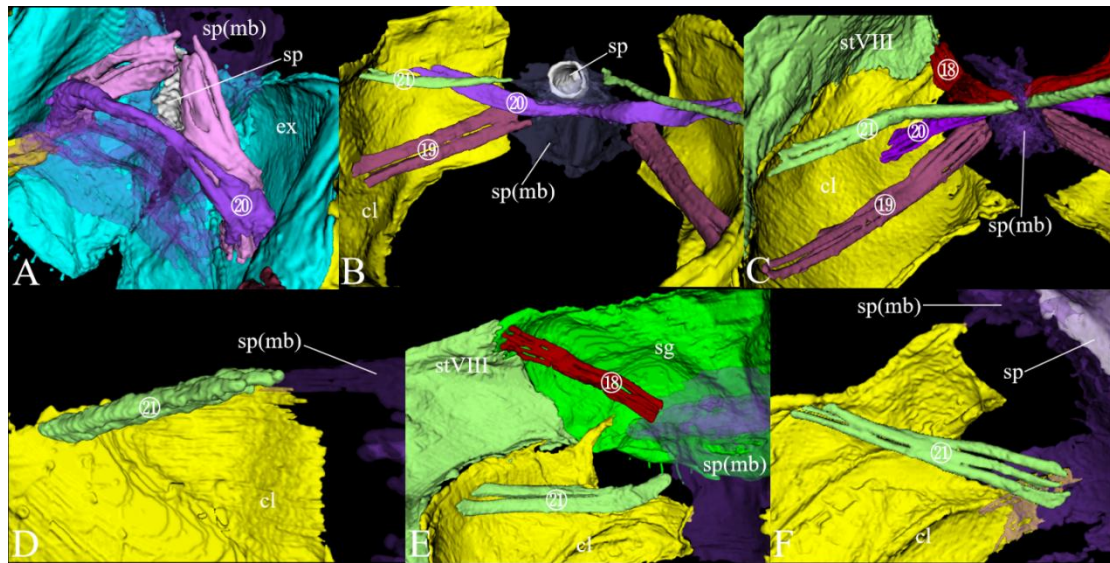
Figure 1.14 Muscles related to the spermapore membrane, internal view: (A) *Prionoglaris stygia*; (B) *Psyllipsocus clunjunctus*; (C) *Lepinotus reticulatus*; (D) *Archipsocus* sp.; (E) *Valenzuela badiostigma*; (F) *Trichadenotecnum pseudomedium*. (18) spVIII01; (19) spIX01; (20) spIX02; (21) spIX03. See Figure 1.1 for abbreviations.
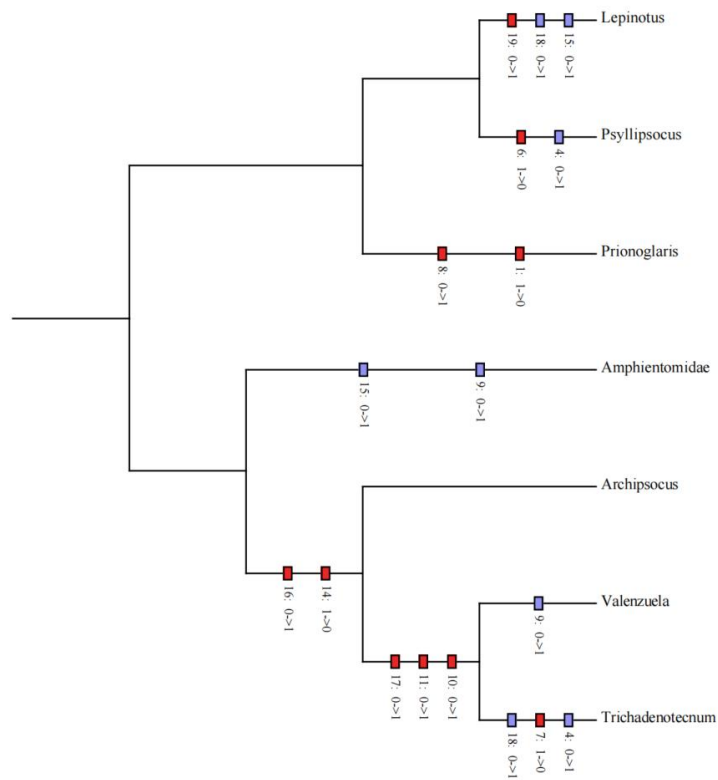
Chapter 1

Figure 1.15 Most parsimonious reconstruction of the presence (1) or absence (0) of the female genital muscles. Tree topology is from the phylogenomic tree estimated by de Moya et al. (2021). See Table 1.2 for character table. Red bars indicate non-homoplasious characters and blue bars indicate character including homoplasy.

Suborder	Infraorder	Family	Species	Locality	Specimen ID	Collector
Trogiomorpha	Prionoglaridetae	Prionogralididae	*Prionoglaris stygia*	France	S8KY37	B. Horser
	Psyllipsocetae	Psyllipsocidae	*Psyllipsocus clunjunctus*	Brazil	S8KY10	R. Ferreira
	Atropetae	Trogiidae	*Lepinotus reticulatus*	Japan	S8KY32	K. Yoshizawa
Troctomorpha	Amphientometae	Amphientomidae	Genus sp.	Malaysia	S8KY14	K. Yoshizawa
Psocomorpha	Archipsocetae	Archipsocidae	*Archipsocus* sp.	Mexico	S8KY75	K. Yoshizawa
	Caeciliusetae	Caeciliusidae	*Valenzuela badiostigma*	Japan	S8KY18	K. Yoshizawa
	Psocetae	Psocidae	*Trichadenotecnum pseudomedium*	Japan	S8KY28	K. Yoshizawa

Table 1.1 Taxa examined in this study.

Muscle #	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
	epX01	paX01	paX02	paX03	paX04	paX05	papa01	papa02	veVIII01	dosp01	doIX01	exsp01	exIX01	exIX02	doex01	sgVIII01	sgVIII02	spVIII01	spIX01	spIX02	spIX03
Prionoglaris	0	1	1	0	1	1	1	1	0	0	0	1	0	1	0	0	0	0	0	1	0
Psyllipsocus	1	1	1	1	1	0	1	0	0	0	0	1	1	1	0	0	0	0	0	1	1
Lepinotus	1	1	1	0	1	1	1	0	0	0	0	1	1	1	1	0	0	1	1	1	1
Amphientomidae	1	1	1	0	1	1	1	0	1	0	0	1	1	1	0	0	0	0	0	0	0
Archipsocus	1	1	1	0	1	1	1	0	0	0	0	0	0	0	0	1	0	0	0	0	1
Valenzuela	1	1	1	0	1	1	1	0	1	1	1	0	0	0	0	1	1	0	0	0	1
Trichadenotecnum	1	1	1	1	1	1	0	0	0	1	1	1	1	0	0	1	1	0	1	0	1
# in Hünefeld et al.	59			65, 67-69			66	64	08, 09		53		50-52		57,58	27, 33	30, 31	70		75-77	
Badonnel (1934)									c	d	e		f								

Table 1.2 List of the muscles identified in the psocopteran female terminalia and their distribution throughout the taxa examined (present 1 / absent 0). The muscle numbers corresponds to the character numbers shown in Figure 1.15. Homology with the holometabolan female genital muscles (as proposed in Hünefeld *et al.* 2012) and homology of the gonapophyses muscles observed by Badonnel (1934) are also shown at the bottom of the table (for holometabolan muscles, more than one candidate homologous muscles are listed for many cases).

Chapter 2

Acquisition of novel muscles enabled protruding and retracting mechanisms of female penis in sex-role reversed cave insects

Content

2.1 Abstract

2.2 Introduction

2.3 Materials and Methods

2.4 Results and Discussion

2.1 Abstract

Brazilian sex-role reversed cave insects (genus *Neotrogla*) have a striking structure called the gynosome (or female penis), which deeply penetrates male vagina-like genitalia during copulation to receive nutritious semen. However, the protruding and retracting mechanisms of the female penis, including their evolutionary origin, are poorly understood. By using the μCT technique, I compared the genital morphology and musculature between species with a gynosome and others lacking this structure. As a result, I discovered two groups of novel muscles related to the protrusion and retraction of gynosomes. These muscles were also observed in species having a primitive, nonprotrutable gynosome (prepenis). This strongly suggests that evolution of these muscles preceded the acquisition of the protruding function of the gynosome, originally having a putative stimulatory function to receive nutritious semen. This intermediate stage probably allowed for the reversal of genital functions.

2.2 Introduction

To date, the occurrence of a female penis has only been found in a small tribe of cave insects, Sensitibillini (Psocodea: Trogiomorpha: Prionoglarididae) (Yoshizawa *et al*, 2014; Yoshizawa *et al*, 2019). In the genus *Neotrogla*, females have a penis-like intromittent organ called the gynosome, which penetrates deeply into the male vagina-like cavity during copulation to receive voluminous and nutritious semen (Yoshizawa *et al*, 2014). The origin of the female penis is still an insufficiently understood phenomenon. Frequently, there is a significant gap between ancestral and derived morphology, and how the intermediate condition functioned is poorly understood (Müller &Wagner, 2003). The reversals of male and female genital function require complete correlation between the sexes, and the evolution of such anomalies must be strongly constrained (Yoshizawa *et al*, 2019). The oligotrophic cave environment (Lienhard & Ferreira, 2013) and seminal nuptial gift (Yoshizawa *et al*, 2014) are thought to be the key factors facilitating female competition and, as a consequence, the origin of the female penis (Gwynne, 2008; Kamimura *et al*., 2021). In addition, females of *Neotrogla* have multiple sperm storages (Yoshizawa *et al*, 2018a). This unique feature enables a female to receive twice the amount of nutritious semen, which renders female-female competition more intense, favouring the evolution of a female penis (Kamimura *et al*., 2021; Yoshizawa *et al*, 2018b; Yoshizawa *et al*, 2019).

There are also factors relaxing the constraints against genital reversal (Yoshizawa *et al*, 2019; Huber *et al*., 2007; Klier, 1956; Lienhard, 1998). Males of Psocodea usually do not have a penetrative penis, and seminal transfer is achieved by tight contact between the flat female spermapore plate (a plate surrounding the opening of the spermathecal duct, which leads to the sperm storage organ) and the nonbulging opening of the male seminal duct. The spermapore plate is hooked and pulled out from the female genital chamber towards the male seminal duct by using penile or nonpenile structures of the male terminalia (Yoshizawa *et al*, 2019; Klier,

1956; Cheng &Yoshizawa, 2019). In a close relative of Sensitibillini (Trogiidae: *Trogium*), the female's spermapore plate is modified into a small tubercle. During copulation, the male grasps the tubercle by using the parameres so that it shallowly penetrates the male genitalia (Yoshizawa *et al*, 2019; Klier, 1956). The female tubercle is widely observed in Trogiomorpha, including in a close relative of Sensitibillini, which is thought to exhibit a preadaptive condition, relaxing the constraint against the evolution of a deeply penetrating female penis (Lienhard, 1998; Lienhard & Ferreira, 2013a; Cheng &Yoshizawa, 2022).

However, the origins of the protrusion and retraction mechanisms of the gynosome, including its function in transitional evolutionary stages, are completely unknown to date. In this study, I reconstructed 3D models of female genital structures of *Neotrogla* using the μCT technique and compared musculature related to the gynosome with the muscles of female genitalia in Trogiomorpha to detect their homology and to identify novelties related to the mechanical function of the female penis.

2.3 Materials and Methods

A female not in copula (Voucher ID, Hokkaido University: S8KY01) and a copulating pair (S8KY05) of *Neotrogla curvata* Lienhard & Ferreira, 2013 (Lienhard & Ferreira, 2013) and a female not in copula (S8KY03) of *N. brasiliensis* Lienhard, 2010(a) were used for μCT examination. Samples were fixed with hot water (~80 °C). Dehydration was conducted in ascending order with 80–100% ethanol before drying the specimens at the critical point (EM CPD300, Leica, Wetzlar, Germany) avoiding serious organ shrinkage. Samples were scanned using the synchrotron μCT at the BL47XU (Uesugi *et al*., 2012) beamline of the SPring-8 (Hyogo, Japan) using a stable beam energy of 8 keV in absorption-contrast mode. The tomography system consists of a full-field X-ray microscope with Fresnel zone plate optics (Uesugi *et al*., 2012). I used semiautomatic segmentation algorithms based on grey-value differences in

ITK-SNAP software (Yushkevich *et al.*, 2006) to obtain 3D representations.

Morphological information of *Afrotrogla oryx* Lienhard, 2007, *Sensitibila etosha* Lienhard & Holusa, 2010 (b), and *Speleketor irwini* Mockford, 1984 were obtained by using a compound light microscopy observation (Zeiss Axiophot: Oberkochen, Germany). A photograph of *S. etosha* was taken with an Olympus E-M5 (Tokyo, Japan) attached to a Zeiss Axiophot.

The following terminology was adopted for the gynosomal structures (Yoshizawa *et al*, 2014): apical sclerite – well sclerotized structure bearing the opening of the spermathecal duct (Yoshizawa *et al*, 2014) (Figures 1*b* and 2*a*); spiny membrane – the membrane surrounding the apical sclerite and bearing many spines anchoring males during copulation; gynosome membrane – the additional membrane extending from the ventral region of the spiny membrane and bearing the muscle attachments (Figure 2*a*); basal shaft – the internal apodeme extending from the anteroventral margin of the apical sclerite: (Figure 2*a*).

The ancestral state of the genital characters were estimate based on a molecular phylogenetic tree of Prionoglarididae (Yoshizawa *et al*, 2018b) using the likelihood method as implemented in Mesquite Version 3.5 (Maddison & Maddison, 2018). Taxa lacking morphological information were trimmed from the tree. The morphological information of the outgroups were from the µCT-based character analyses conducted by Cheng & Yoshizawa (2022). However, for most outgroup species, µCT data were not available. Therefore, morphological information obtained from the same family member were adopted for the terminal taxa.

2.4 Results and Discussion

Among genital structures, the epiproct and paraproct (structures surrounding the anus) of *Neotrogla* showed little morphological differences from the other Psocodea, but there were deviations in gonapophyses, the spermapore plate, and the subgenital plate (Cheng & Yoshizawa, 2022; Yoshizawa, 2005). The gynosome may have been homologous to one of these structures.

Chapter 2

In *Neotrogla*, I found two structures that may be homologous to gonapophyses. One was a pair of well-developed valves forming the ovipositor (Figure 2.1*a*: light blue). Comparisons of the external morphology (positional congruence) and musculature (see below for the additional muscles supporting the interpretation) strongly suggested that the ovipositor valves of *Neotrogla* were homologous to the external valves, as in other trogiomorphan species (Cheng & Yoshizawa, 2022; Lienhard & Holusa, 2010b). The other structure was an undivided lobe situated between the bases of the external valve (Figure 2.1*a*: blue). In previous studies, this structure was interpreted as a part of the subgenital plate (Lienhard & Ferreira, 2013a, Lienhard & Holusa, 2010b). However, a muscle originating from the base of external valve was inserted into the anteroventral margin of this structure (Figure 2.1*g*: muscle 2.7). Since no muscles connecting the gonapophyses and the subgenital plate have been found in the other Psocodea (Cheng & Yoshizawa, 2022) this structure is not homologous to the subgenital plate. The only muscle that connects two structures in this way is doex01 (connecting dorsal and external valves) (Figure 2.1*h*) (Cheng & Yoshizawa, 2022). Therefore, it is most likely that this lobe-like structure corresponds to the medially fused pair of dorsal valves (Cheng & Yoshizawa, 2022). Thus, homology between the gynosome and the gonapophyses can be ruled out, and the subgenital plate of *Neotrogla* is considered to be reduced.

Instead, most of the muscles associated with the gynosome can be homologous with those associated with the spermapore plate of other psocodeans (Cheng & Yoshizawa, 2022): muscle 1 – dosp01 (connecting the dorsal valve and spermapore plate: Figure 2.1*c,i*), muscle 2 – exsp01 (external valve and sprmapore plate: Figure 2.1*c–f*), muscle 3 – spIX01 (spermapore plate and 9th tergum: Figure 2.1*c, e, f*), and muscle 4 – spIX02 (spermapore plate and 9th tergum: Figure 2.1*b–f*). Although no muscle corresponding to dosp01 (muscle 1) was found in other species of the suborder Trogiomorpha (probably related to the reduction of the dorsal valves in this suborder), most species in the other psocodean suborders have this muscle (Cheng &

Yoshizawa, 2022). Sharing of these muscles, including the possession of the opening of the spermathecal duct, strongly suggests homology between the spermapore plate and the gynosome. The muscles associated with the spermapore plate functions to restore the plate and the surrounding membrane after copulation in psocopterans without female penis (Cheng & Yoshizawa, 2022). The abovementioned gynosome muscles are not directly associated with the sclerotized part. Therefore, they most likely act to restore the gynosomal spiny membrane at the right position after copulation (Figure 2.2*a,b*). Of them, the muscles 3 and 4 of *Neotrogla* are especially thicker than those of the species lacking the gynosome (Figure 2.1*b–f*), suggesting that it needs more muscle power to restore the enlarged and complicated gynosomal spiny membrane.

In addition to the muscles mentioned above, I found two pairs of novel muscles directly attached to the gynosomal basal shaft in both species of *Neotrogla*. Comparisons of the muscles of individuals in copulating and uncopulating states showed that these muscles function as protractors and retractors of the gynosome and are key factors for its function. The muscle 5 (Figure 2.2*a–c*) is originated from the gynosome membrane very close to dorsal valves and is inserted on the anterior end of the basal shaft; they were contracted in the copulated state (i.e., gynosome protruded) and were relaxed in the uncopulated female. Therefore, the muscle pair is interpreted as a protractor of the gynosome. The muscle 6 (Figure 2.2*a–c*) is originated from an internal organ (specific insertion site not detected) and is inserted into the anterior end of the basal shaft. These muscles were relaxed in the copulated state but were contracted in the uncopulated female (i.e., gynosome stored in the abdomen). Therefore, the muscle pair is interpreted as a retractor of the gynosome. These observations clearly showed that the development of the basal shaft and two groups of novel muscles attached to it were key innovations to achieve the protruding and retracting movements of the gynosome.

Key questions remain regarding the origins of the shaft and these muscles and

their function in a primitive stage. The basal shaft have been observed in all genera of Sensitibillini (*Afrotrogla*, *Neotrogla* and *Sensitibilla*) (Yoshizawa *et al*., 2018b), and muscles homologous to muscle 5 have been observed in *Sensitibilla* (Figure 2.2*d*) (Yoshizawa *et al.*, 2019) (the presence/absence of this muscle unknown for *Afrotrogla*) (Table 2.1). The ancestral state reconstruction of these characters suggested that the origin of the shaft and muscle 5 can be traced back to the common ancestor of Sensitibillini (Yoshizawa *et al*., 2018b) (Figure 2.3 and Supplementary Figure). However, the previous morphological analyses strongly suggested that the spermapore plate of *Sensitibilla* (called prepenis) does not form a protruding structure, as observed in *Neotrogla* and *Afrotrogla* (Yoshizawa *et al*., 2018b). Therefore, the shaft and associated muscles in the nonprotruding prepenis of *Sensitibilla* are expected to have different preceding functions.

The spermapore plate is modified into a small tubercle in many trogiomorphan species, including *Sensitibilla* (Figure 2.3)*,* and during copulation, this tubercule slightly penetrates the male genitalia (Yoshizawa *et al*, 2019; Klier, 1956). In addition, active competition for seminal nutrition by females has also been observed in other trogiomorphans (Yoshizawa *et al*, 2019; Wearing-Wilde, 1996). The most likely explanation is that the modification of the spermapore plate towards the female penis was promoted by sexual selection to receive more seminal nutrition (Yoshizawa *et al*, 2019). The shaft and associated muscles in the nonprotruding prepenis of *Sensitibilla* are also expected to have functions increasing the female's benefit, such as stimulation of the male mate in order to receive more nutritious semen.

Our present examinations showed that many novelties are involved in the evolution of the functional female penis, including the modification of the pre-existing structures (e.g., from the tubercle to the gynosomal apical sclerite) or development of completely novel ones (e.g., the basal shaft and muscles 5/6). Most of these structures are thought to have different functions at intermediate conditions. To identify a possible trigger of the unique evolution of the female penis, mechanical analyses of the *Sensitibilla* preprenis will be a key for understanding the evolutionary history.
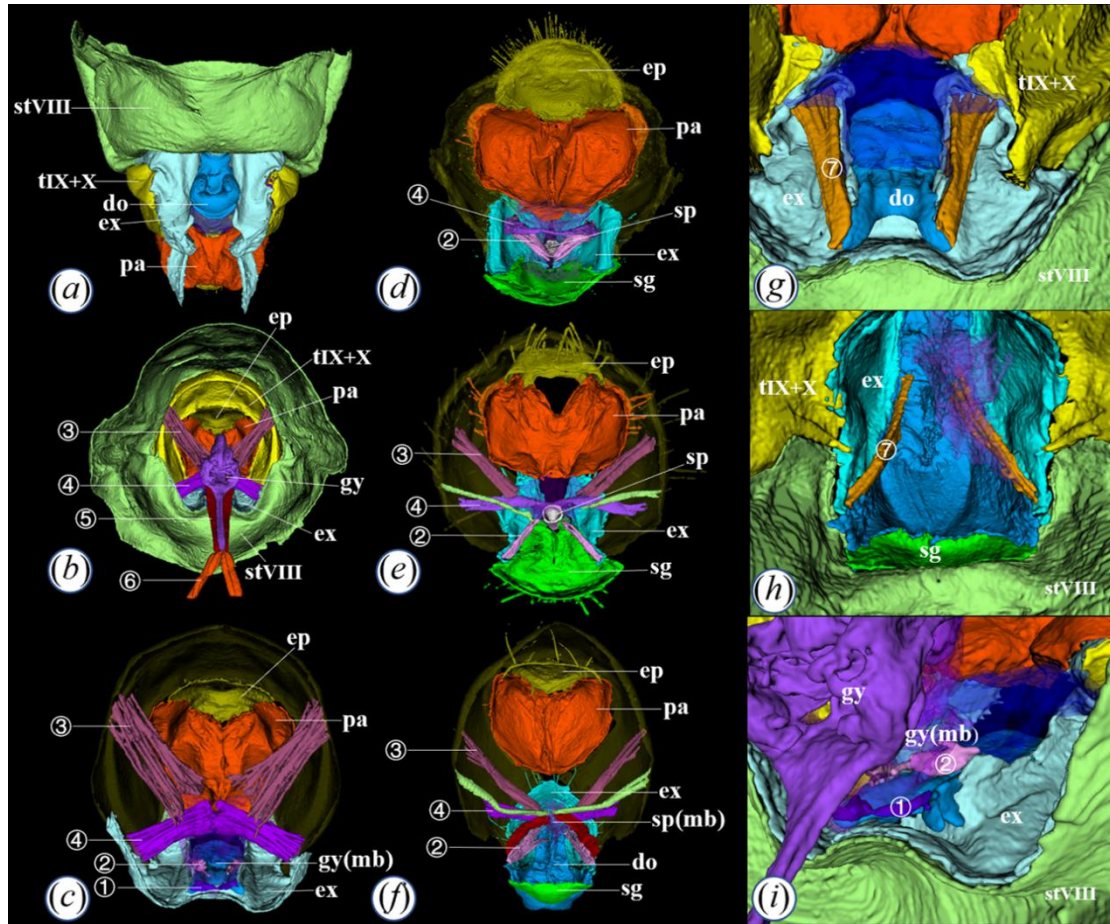
Figure 2.1 Female terminalia of the trogiomorphan species: *Neotrogla curvata* (*a–c*), *Prionoglaris stygia* (*d*), *Psyllipsocus clunjunctus* (*e*), *Lepinotus reticulatus* (*f*): (*a*) ventral external view; (*b–f*) internal view (*c*: gynosome omitted); (*g–i*) close-up of the gonapophyses and related structures, internal view: *Neotrogla curvata* (*g,i*); *Lepinotus reticulatus* (*h*). Muscle numbers (for terminology see [13]): (1) dosp01; (2) exsp01; (3) spIX01; (4) spIX02; (5) muscle 5; (6) muscle 6; (7) doex01. Abbreviations: do = dorsal valve; ep = epiproct; ex = external valve; gy = gynosome; mb = membrane; pa = paraproct; sg = subgenital plate; sp = spermapore plate; st = sternum; t = tergum.
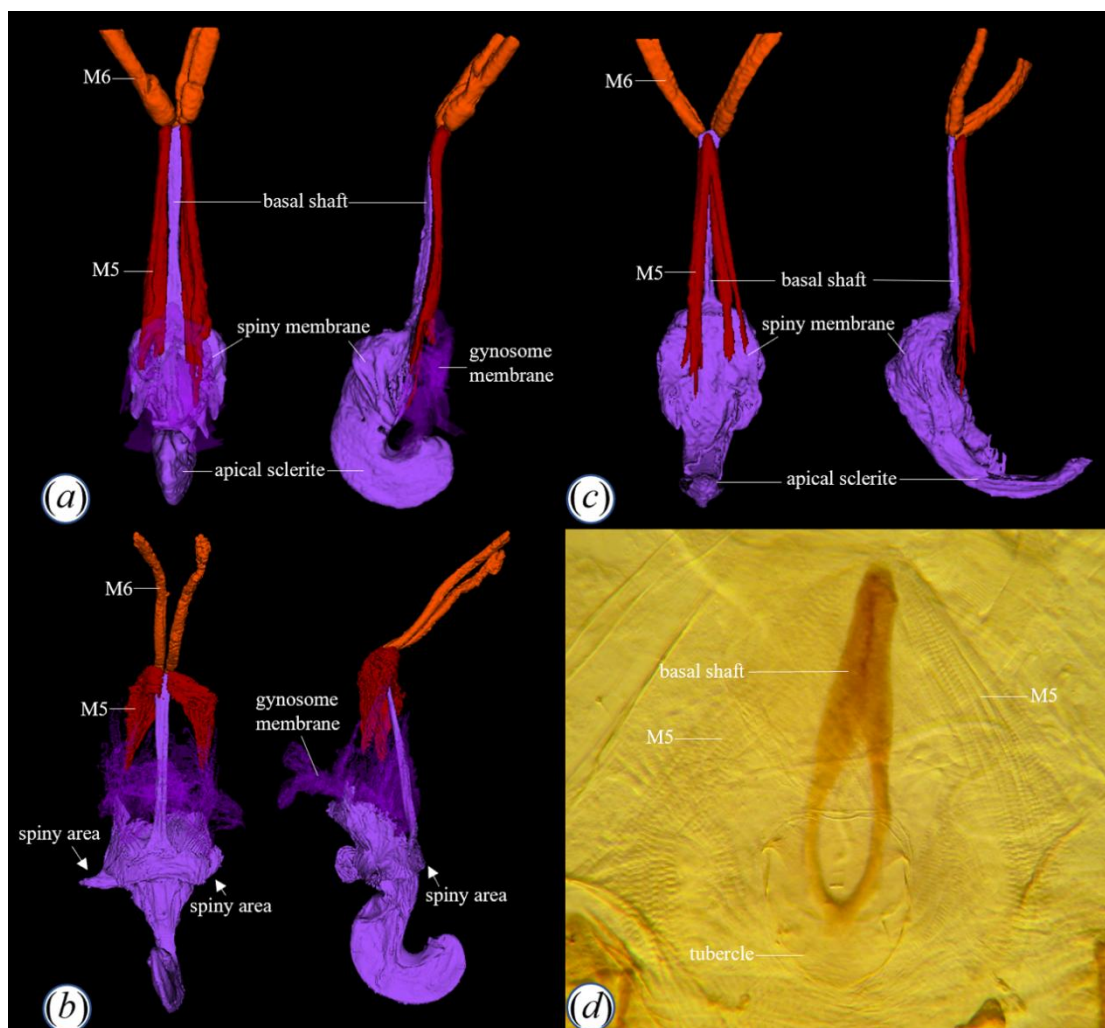
Figure 2.2 Figure 2. (*a*, *b*) Gynosome of *Neotrogla curvata* (left: ventral view; right: lateral view): (*a*) uncopulated state; (*b*) copulated state. (*c*) Gynosome of *Neotrogla brasiliensis* in uncopulated state (left: ventral view; right: lateral view); (*d*) Prepenis and its associated muscles of *Sensitibilla etosha* (ventral view). Abbreviations: M5 = muscle 5; M6 = muscle.
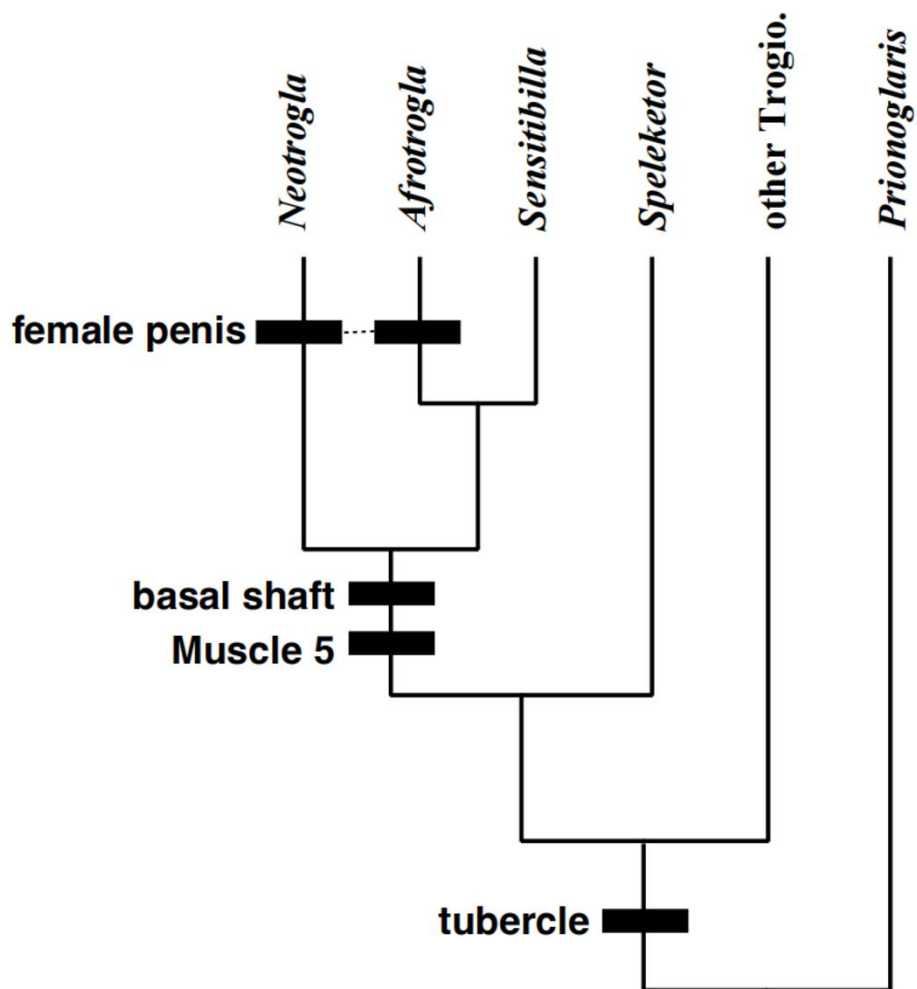
Figure 2.3 Phylogeny of Trogiomorpha, and the ancestral state reconstruction of characters related to the evolution of the gynosome. Black bar indicates the branch where the novel character evolved.
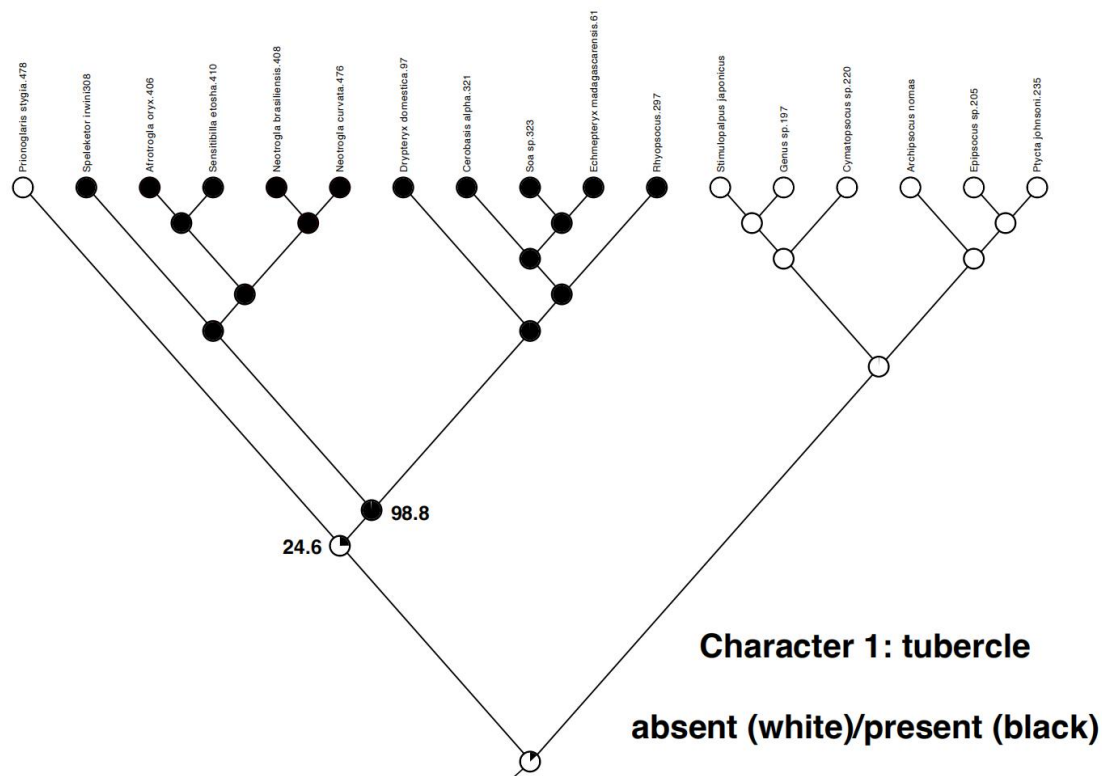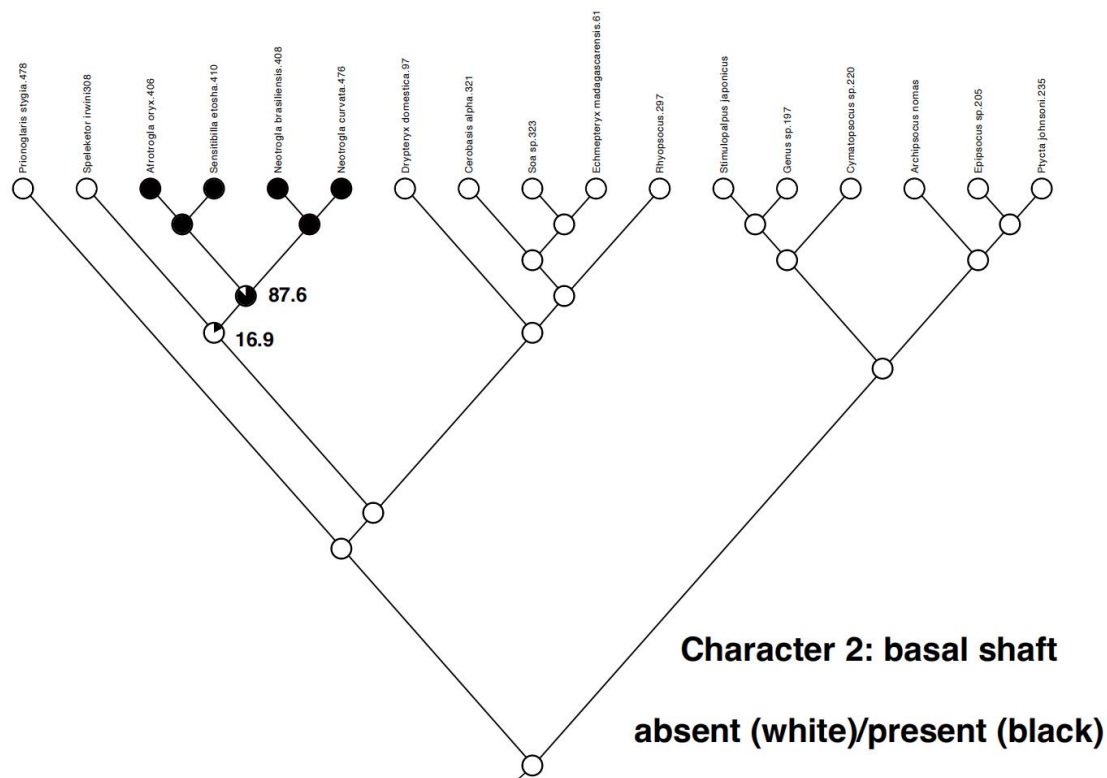
Figure 2.4-1

Figure 2.4-2
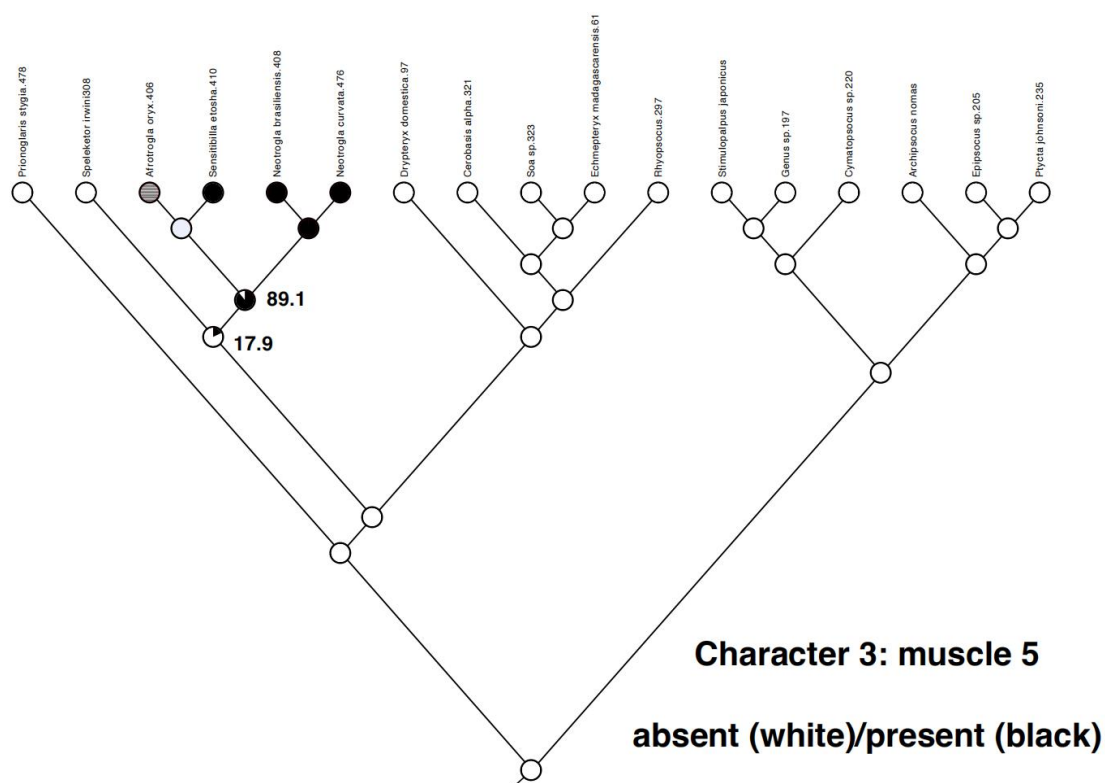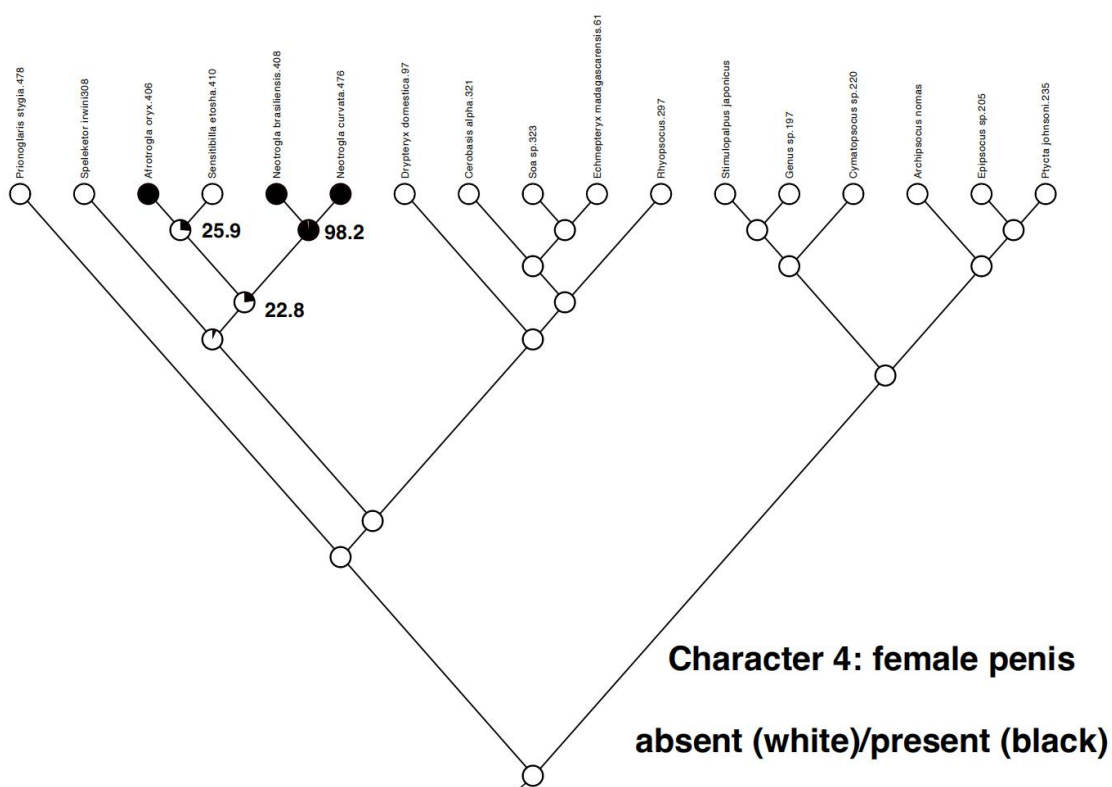
Figure 2.4-3

Figure 2.4-4
Likelihood-based ancestral state reconstruction of characters related to the evolution of the
gynosome. Likelihood of character state for the hypothesised ancestors is shown by pie chart and

percentage (for key ancestors only).

Speleketor	Yes	No	No	No
Sensitibilla	Yes	Yes	Yes	No
Afrotrogla	Yes	Yes	?	Yes
Neotrogla	Yes	Yes	Yes	Yes

Table 2.1 Distribution of the character related to the evolution of the gynosome among the subfamily Speleketorinae.

Chapter 3

Are males completely passive? Coupling mechanism of the cave insects with reversed genitalia

Content

3.1 Abstract

3.2 Introduction

3.3 Materials and Methods

3.4 Results

3.5 Discussion

Chapter 3

3.1 Abstract

By using μCT technique, I present 3D-models of genital structure and connected muscles of the male and female genitalia of *Neotrogla curvata*. By comparing the morphological changes of those structures and muscles before and during copulating, I clarified the functions of the male and female genital structures and analyzed the complete copulating process in *Neotrogla*. In addition, based on observations of the current 3D models, it is probably that a perfectly connected copulating state and step-by-step releases of each holding mechanism achieved by the cooperation of both sexes are beneficial for both males and females. And the co-evolution between the male and female genital structure in *Neotrogla* may provide a new example for the evolution of the tolerate traits derived by the sexual selection under the reversed sex-role.

3.2 Introduction

The Brazilian cave insect genus *Neotrogla* (Psocodea: Trogiomorpha: Prionoglarididae: Sensitibillini) have been receiving a lot of attention from biologists since they were discovered to have a penis-like intromittent structures, called gynosome (Yoshizawa *et al.*, 2014). The gynosome is inserted into the vagina-like male structure during copulation to obtain the nutritious semen (Yoshizawa *et al.*, 2014). "Female penis" has also been found in African genus *Afrotrogla* (Lienhard, 2007). Both *Neotrogla* and *Afrotrogla* belong to the small tribe Sensitibillini containing eleven named species only, and the well-developed female penis is considered to evolve independently in these two genera (Yoshizawa *et al.*, 2018). In recent years, several in-depth studies have been conducted on the factors affecting the formation of female intromittent structures, such as oligotrophic environment, male nuptial gifts, female multiple sperm storages, female-above copulating position, elongated spermathecal duct, absence of male penetrative organ and so on (Yoshizawa *et al.*, 2014; Lienhard & Ferreira, 2013; Gwynne, 2008; Kamimura *et al.*, 2021; Yoshizawa *et al.*, 2018; Yoshizawa *et al.*, 2019; Klier, 1956; Lienhard, 1998).

All these studies focused on the intromittent female penis, but the morphology and function of other genital structures in this sex-reversed insects are still poorly understood. By using homology of muscles in Chapter 2, I have determined that the female penis evolved from the spermapore plate, and its unique intromittent function is brought by two groups of novel muscles formed only within the Sensitibillini. Are

there new structures and muscles in the other genital structures? What functions do these structures have during copulation? Whether sexual conflict is strongly reflected in the copulating process? All these questions need to be answered under the overall grasp of the structure of the male and female genitalia and the mating process of the sex-reversed insects.

In this chapter, by using μCT technic, I present 3D-models of overall male and female genital structures and their associated muscles of *Neotrogla curvata*. By comparing the morphological changes of those genital structures and associated muscles before and during copulating, I clarified the functions of these structures and analyzing the complete copulating process. I also analyze the homology of muscles between the species of *Neotrogla* and the other non-genital-reversed Psocodea, and trace the origin of each structure and muscle of *Neotrogla*.

3.3 Materials and Mathods

Micro-CT imaging

A copulated pair and a non-copulated male and female of the coupling-role reversed cave psocid *Neotrogla curvata* Lienhard & Ferreira, 2013 (Trogiomorpha) were examined. *Trichadenotecnum pseudomedium* Yoshizawa, 2001 (Psocomorpha: copulated and non-copulated specimens), both have normal genital structures, were also examined for comparisons. All samples were subjected for μCT examination, and voucher specimens are stored in the Hokkaido University Insect Collection. Samples

were fixed with FAA solution (formalin:alcohol:acetic acid = 6:16:1) or 80% ethanol and then preserved in 80% ethanol. Dehydration was conducted in ascending order with 80–100% ethanol before drying them at the critical point (EM CPD300, Leica, Wetzlar, Germany) to remove water without serious organ shrinkage. Samples were then scanned using the synchrotron μCT at the BL47XU (Uesugi *et al*., 2012) beamline of the Super Photon ring-8 GeV (SPring-8; Hyogo, Japan) using a stable beam energy of 8 keV in absorption-contrast mode. The tomography system consists of a full-field X-ray microscope with Fresnel zone plate optics (Uesugi *et al*., 2017). I used semiautomatic segmentation algorithms based on grey-value differences in the software ITK-SNAP (Yushkevich *et al*., 2006) to obtain 3D representations of the terminalia of all two species.

3.4 Results

I describe the basic structure of the terminalia and its associated muscles of *Neotrogla curvata*. I grouped those muscles according to their origin as follows: muscles of the epiproct [ep]; paraproct [pa]; subgenital plate [sg]; dorsal valve [do]; external valve [ex]; spermapore plate/gynosome or the membrane surrounding it [sp/gy]; hypandrium [hy]; phallosome [ph].

  Abbreviations: O – origin; I – insertion; and F – assumed function (based on morphological conditions).

Chapter 3

3.4.1 Skeletal and muscle structures of female terminalia.

Among the female terminal structures of *Neotrogla*, the morphology of clunium and epiproct is not much different from the other Psocodea. In contrast, a significant difference was detected in the paraproct between *Neotrogla* and other psocodeans, i.e., a concavity occurring on the ventrolateral surface of the paraproct observed in *Neotrogla* (Figure 3.1d). One group of muscles is associated with the epiproct and four groups of muscles are associated with the paraproct, all of which are originated from the clunium. All of these muscles can be homologized with those associated with the epiproct or paraproct of other psocodeans (Figure 3.3c, i and j; Cheng & Yoshizawa, 2022).

epX01 (Figure 3.3c, d: = 01 of Cheng & Yoshizawa, 2019); O: mid-dorsal site of clunium (segment IX); I: posterior end of the epiproct; and F: closure and/or flipping of the epiproct.

paX01 (Figure 3.3c, d: = 02 of Cheng & Yoshizawa, 2019); O: anterolateral region of clunium (segment X); I: anterodorsal end of the paraproct, very close to the posterolateral margin of epiproct; and F: involved in opening the paraproct.

paX02 (Figure 3.3c, d: = 02 of Cheng & Yoshizawa, 2019, not distinguished from paIX01 in Cheng & Yoshizawa, 2019); O: mediodorsal region of clunium (segment X); I: anterodorsal end of the paraproct, very close to the anterolateral corner of epiproct; and F: involved in opening the paraproct.

paX03 (Figure 3.3c, d: = 03 of Cheng & Yoshizawa, 2019); O: mediolateral region of

clunium (segment X); I: internal margin of the paraproct near the anal opening; and F: involved in retracting the paraproct.

paX04 (Figure 3.3c, d); O: mediolateral region of clunium (segment IX); I: anterolateral margin of the paraproct; and F: involved in retracting the paraproct.

The dorsal valve of the gonapophyses is located in the center of the base of the external valve and forms an independent structure anteriorly articulated with the transversal sclerite of the external valve (see below). It is like a seat in shape with a central depression (Figure 1a). Two groups of muscles are connected to the dorsal valve, and they can be homologized with those associated with the dorsal valve of other psocodeans (Figure 3c, e and j; Cheng & Yoshizawa, 2022).

dosp01 (Figure 3.3c, e); O: base of the dorsal valve; I: on the membrane connected to the spermapore plate/ gynosome, near the posterior tip of the spermapore plate; and F: involved in restoring the position of the gynosome (spermapore plate) and dorsal valve.

doex01 (Figure 3.3c, e); O: base of the dorsal valve; I: base of the external valve; and F: involved in restoring position of the dorsal valve and closure of the external valve.

The external valve of the gonapophyses is a pair of crab claw-like structures basally connected by a transversal sclerite. The transversal sclerite is laterally articulated with clunium and anteriorly connected with the sternum VIII (Figure 3.1a, c). There are four groups of muscles connected to the external valve (of them, doex01 is described above), and two groups of those muscles are homologous with those

associated with the external valve of other psocodeans (Figure 3e, i and j; Cheng & Yoshizawa, 2022). The exVIII01 muscle is unique to *Neotrogla*.

exsp01 (Figure 3.3e, f); O: base of the external valve; I: on the membrane connected to the spermapore plate/gynosome, near the posterior tip of the spermapore plate; and F: involved in restoring the position of the gynosome (spermapore plate) and closure of the external valve.

exIX02 (Figure 3.3e, f); O: anterolateral margin of the clunium; I: base of the external valve, near the middle of the junction with the clunium; and F: involved in opening the external valve.

exVIII01 (Figure 3.3e, f); O: medioventral region of sternum VIII; I: base of the external valve; and F: involved in closure of the external valve.

The gynosome is composed of the apical sclerite, spiny membrane and basal shaft (Chapter 2). The gynosome is placed anterior to the gonapophyses in uncopulated state (Figure 3.3a). During copulation, all parts other than basal shaft protrude from the opening between paraproct and gonapophyses (Figure 3.1c, d). There are six groups of muscles connected to gynosome (of them, dosp01and exsp01 are mentioned above), and two groups of those muscles are homologous with those associated with the spermapore plate of other psocodeans (Figure 3.3g, i and j; Cheng & Yoshizawa, 2022). The gyIX01 and gy-01 muscles are unique to *Neotrogla*.

spIX01 (Figure 3.3g, h); O: mediolateral region of the clunium; I: on the spermapore membrane; and F: involved in restoring the position of the gynosome (spermapore

plate).

spIX02 (Figure 3.3g, h); O: mediolateral region of the clunium; I: on the spermapore membrane; and F: involved in restoring the position of the gynosome (spermapore plate).

gyIX01 (Figure 3.3g, h); O: gynosome membrane very close to dorsal valves (=ventral membrane of segment IX); I: anterior end of the basal shaft; and F: protractors of the gynosome.

gy-01 (Figure 3.3g, h); O: an internal organ (specific insertion site not detected); I: anterior end of the basal shaft; and F: retractors of the gynosome.

3.4.2 Skeletal and muscle structures of male terminalia.

Among the male terminal structures, no obvious specificity was detected for the clunium and epiproct. On the ventral side of paraproct, there is a pair of ridges, triangular in lateral view (Figure 3.2a, c). One group of muscles is associated with the epiproct and six groups of muscles are associated with the paraproct. Among them, five groups of muscles are originated from the clunium, and one group of muscle is originated from and inserted into the paraproct. Since the epiproct, paraproct and clunium exist in both sexes and have the similar function, five groups of muscles (epX01, paX01, paX02, paX03, paX04) can be homologized with those associated with the epiproct or paraproct of female (Figure 3.4c, i and j; Cheng & Yoshizawa, 2022). (see 3.4.1). The following two groups of muscles are unique to males, and the latter one is unique to *Neotrogla*.

papa01 (Figure 3.4c, d); O: internal margin of the paraproct near the anal opening; I:

anterolateral margin of the paraproct; and F: involved in opening the anus.

pahy01 (Figure 3.4c, d: = 04 of Cheng & Yoshizawa, 2019); O: anteroventral end of the paraproct; I: anterolateral region of hypandrium (segment IX); and F: involved in indirect opening the hypandrium and retracting the male paraproct (holding the female dorsal valve during copulation: see below).

The hypandrium represents the sternum IX and articulates laterally with the clunium. The hypandrium has no obvious protrusions in *Neotrogla* (Figure 3.2a, c). One group of muscles is connected to hypandrium which is homologous to that observed in *Trichadenotecnum* (Figure 3.5c).

hyVIII01 (Figure 3.4e, f: = 05 of Cheng & Yoshizawa, 2019); O: posterolateral region of segment VIII; I: mediolateral margin of the hypandrium; and F: involved in restoring the hypandrium.

The phallosome consists of two parts: a membranous part and a reversed U-shaped thin sclerite surrounding the membranous part (Figure 3.2g and 3.4a). The phallosome closely fits into the hypandrium via a closed genital chamber in an uncopulated state (Figure 3.2g). During copulation, the sclerotized part of the phallosome is raised upwards and its apex rests on the protrusions of the paraproct (Figure 3.2h). There are three groups of muscles connected to the phallosome. Among them, phIX01 and 02 were also observed in *Trichadenotecnum* (Figure 3.5d).

phIX01 (Figure 3.4g, h and 3.5e, f: = 07 of Cheng & Yoshizawa, 2019); O: end of the sclerite of phallosome; I: anterolateral region of hypandrium (segment IX); and F: involved in opening the hypandrium /protrusion of the phallosome.

phIX02 (Figure 3.4g, h and 3.5e, f: = 08 of Cheng & Yoshizawa, 2019); O: middle of the sclerite of phallosome; I: anterolateral region of hypandrium (segment IX), phIX01, phIX02 and the sclerite of phallosome present a triangle in position; and F: involved in opening the hypandrium/retraction of the phallosome.

phVIII01 (Figure 3.4g, h and 3.5e, f); O: posterolateral region of segment VIII; I: posterior of the sclerite of phallosome; and F: involved in protrusion of the phallsome (holding the female dorsal valve during copulation: see below).

3.4.3 Male–female genital interaction

In the copulated state, the male paraproct and epiproct are partly retracted into the male body towards the clunium. The ventral paraproctal ridges that were separated in an uncoplated condition, are closely associated during copulation, together forming a single projection (Figure 3.2b, d). The sclerite of the phallosome is protruded upward, and its apex fits with the anterior margin of the male paraprcotal projection. During copulation, these two male structures securely sandwich the female dorsal valve from both sides (Figure 3.2h and 3.6d: white arrow).

The hypandrium is opened in a copulated condition, exposing the male genital cavity formed between the pahllosome and the hypandrium. During copulation, the gynosome extends into the male's body except for the basal shaft. The apical sclerite of gynosome is deeply penetrate the membranous part of phallosome (Figure 3.2b and 3.6d: red arrow). The spiny membrane part of the gynosome expands to form internal anchor within male genital cavity. The female epiproct and paraproct are slightly retracted inwardly. The concave surface of the female paraproct and the swollen

gynosomal spiny area together anchor the male by sandwiching the hypandrium externally (paraproct) and internally (gynosomal spines) (Figure 3.6b, c: white arrow). The female external valve is opened and grasp the lateral sides of the hypandrium (Figure 3.1f and 3.6a).

3.5 Discussion

3.5.1 Homology of the gonapophyses valves

In Chapter 2, using the doex01 muscle as a landmark, the median flap occurred between the external valves of *Neotrogla* is homologized with the dorsal valve, not subgenital plate as speculated before (Lienhard et al., 2010). The reconstructed 3D model clarified that the structure forms an independent lobe (Figure 3.5a), anteriorly articulated with the external valve, which also supports its homology with the dorsal valve (the subgenital plate is formed by a direct extension from the posterior margin of the sternum VIII, not articulating with the external valve: Cheng & Yoshizawa, 2022).

The bases of the external valves are connected by a transversal sclerite, which has not been observed in other Psocodea. I confirmed by the presence of exVIII01 (Figure 3.5b) that this transversal sclerite belongs to the external valve rather than the sternum VIII or IX.

Homology of the other male and female genital structures can be assumed unambiguously based on the external and muscle structures. See also Chapter 2 for

the homology of the gynosome.

3.5.2 Copulation process and holding mechanisms

The present observation revealed that the following holding/locking systems are working to establish and stabilize the coupling condition: (1) the female dorsal valve is tightly sandwiched by the male paraproctal ridge and the phallosomal tip (Figure 3.6d, white arrow); (2–1) the gynosomal spiny membrane anchors the male internally by its inflation within the male genital cavity (Figure 3.6c, d); (2–2) the female paraproctal concavities support the ventral surface of the male hypandrium, and the hypandrium is sandwiched by the female paraproct (externally) and the gynosomal spines (internally) (Figure 3.6b, white arrow); (3) the female external valves grasp the lateral side of the male abdominal tip (Figure 3.6a). The estimated function and establishment process of each holding system are discussed below.

3.5.2.1 Fixation of the female dorsal valve by male

During copulation, the dorsal valve is almost fully inserted to the male and is tightly held by male's structures (the paraproctal ridge and the tip of phallosome; Figure 3.6d, white arrow). In the copulated state, the dosp01 muscle connected to the female dorsal valve is in a relaxed state (Figure 3.3f), and no muscles related to the opening of dorsal valve was found. Therefore, it is assumed that this muscle is related to restore the dorsal valve at the end of copulating.

Five groups of male muscles (paX01, paX02, paX03, and pahy01) connected to

the male paraproct contract together during copulation, which guide the male paraproct to partly retract into the male body (Figure 3.2e, f and 3.4d). The paired male paraproctal ridges are tightly associated during copulation, together forming a single process, due to the contraction of paX03 (Figure 3.2b, d and 3.4d). In the meantime, the contraction of the phVIII01 muscle, which is originated from the clunium and inserted to the dorsal part of the phallosomal sclerite, pulls up the phallosome toward the male paraproct (Figure 3.4h and 3.5f). The phIX01 muscle, originated from the hypandrium and inserted to the ventral tip of the phallosomal sclerite, may also have a function to protrude the phallosome but, even so, its role seems supplementary to phVIII01 judging from the size of the muscle. The merged male paraproctal ridges buckles the ventral grooves on the female dorsal valve, and the other side of the female dorsal valve is supported by the tip of the phallosomal sclerite (Figure 3.6d, white arrow). Furthermore, the contraction of the pahy01 muscle provides strong power to securely hook and pull inwardly the dorsal valve. The phIX01 and phIX02 muscles may also produce additional power to move the apex of the pahllosome inwardly (i.e., pull the hooked dorsal valve inwardly). These clearly show that the male *Neotrogla* actively hold the female during copulation.

Similar movement of the phallosome to hold and fix the female genital structure (the subgenital plate, in this case) is also known in *Trichadenotecnum* (Psocomorpha: Psocidae: Cheng & Yoshizawa, 2019). However, in the case of *Trichadenotecnum*, phIX01 and phIX02 have a function to restore the phallosome and the phVIII01 is absent. Therefore, the female holding mechanism by male is apparently

no-homologous between *Neotrogla* and *Trichadenotecnum*.

At the end of copulation, paX01 and 02 of the males probably contract to open the paraproct, and phIX01 contracts to restore the phallosome. Both movements (active movements by the male) function to release the grasp of the female dorsal valve.

3.5.2.2 The penetration of the gynosome and anchoring the male

During copulation, the male genital cavity is broadly opened (Figure 3.2b) The opening of the entrance of the male genital cavity is very likely a pre-requirement for the insertion of the gynosme by a female. This is probably caused by two factors: 1) retraction of the male paraprocts (see above), which are located above the genital cavity at non-copulated condition as if they block the male genital opening (Figure 3.2a, c); and 2) posterior movement of the posterior margin of the hypandrium. There is no muscle which causes direct posterior movement of the posterior margin of the hypandrium. However, as seen from Fig. 3.4b, the male terminalia are strongly compressed laterally during copulation, which is very likely to cause the indirect posterior movement of the posterior margin of the hypandrium, like a mouth of a spring-loaded coin purse. From the reconstructed 3D model of the copulated condition, two factors can be assumed to be associated with the lateral compression of the male terminalia: 1) contraction of the pahy01, phIX01 and phIX02 muscle (Figure 3.5j and 3.6f) causes the inward movement of the lateral side of the hypandrium (i.e., male's active movement); 2) grasping by the female external valve causes compression force

of the male terminalia (Figure 3.6a) (i.e., passive for the male: see below).

The phIX01 and phIX02 muscles sharing the same origination site on the hypandrium, together forming a triangular condition with the sclerite of the phallosome (Figure 3.5e, f). This state allows the hypandrium to move more effortlessly because the forces generated by the two muscles are in the same plane. If the intersection points of these two muscles on the hypandrium are inconsistent, it is difficult to ensure that all the forces are in the same plane, which will easily lead to conflicting forces. Similar arrangement of the muscles has been also observed in the female external valve (exIV01 and exIV02) of other Psocodea, such as *Lepinotes* and Amphientomidea, which is also considered to cause effective movement for the valve (Cheng & Yoshizawa, 2022).

In the copulated condition, the gy-01 muscle relaxes and gyIX01 contracts, and vice versa in the non-copulating condition. Therefore, the contraction of gyIX01 apparently has a function to stick out the gynosome, and gy-01 works to restore the stuck gynosome at the resting position (Figure 3.3g, h). Two stout muscles, spIX01 and spIX02 attached to the gynosomal membrane, relax during copulation, having the restoration function of the gynosome membrane (Figure 3.3g, h). The apical sclerite of the gynosome is deeply penetrate the membranous part of the phallosome during copulation, from where the semen is transported to the female. The spiny membrane of the gynosome also enters the male genital cavity (Figure 3.1c, d and 3.6c). Because there is no muscle acting to inflate the spiny membrane, the anchoring function by the spiny membrane is likely achieved by the increased body pressure. See also next

section for the additional female's active holding function working with the gynosomal spines.

At the end of copulation, reduction of the body pressure causes the deflation of the spiny membrane, and the contraction of the spIX01 and 02 muscles act to restore the gynosomal spiny membrane. The gynosome is retracted by the contraction of the gy-01 muscle. Finally, the relaxed pahy01, phIX01 and phIX02 muscles and release from the grasping by the female external valve cause the closure of the male genital cavity.

3.5.2.3 Ventral fixation of the hypandrium

The muscles paX01and paX02 connected to the female paraprcot contract during copulation, causing the paraproct to slightly indent in the direction of the female epiprcot (Figure 3.3d). The paX03 muscle contracts during copulation, directing the protrusions on the ventral surface of the female paraproct to inward direction (Figure 3.1d and 3.3d). By this movement, the ventrolateral paraproctal concavities form a continuous single circular concavity which fits perfectly to the ventral surface of the hypandrium (Figure 3.6b, white arrow). Such a close contact between the female paraproct and the male hypandrium is not observed in *Trichadenotecnum*.

The jointed female paraproctal cavity support the ventral surface of the male hypandrium externally during copulation, which sandwiches the hypandrium together with the internal anchor formed by the gynosomal spines (Figure 3.6b, white arrow). Therefore, it is apparent that the female actively holds the male during copulation by using the female paraproct and the gynosome. This paraproctal feature is the uniquely

observed in *Neotrogla*.

At the end of copulation, paX01 and 02 probably contract to open the paraproct to release the grasp of the male.

Function of the muscles related to the external valve is in common also in *Trichadenotecnum.* However, the external valve of *Trichadenotecnum* does not have function to grasp the male, and its closure muscles have a function to hold the subgenital plate, possibly to resist coercive mating by males.

5.2.3.4 Grasping the male by the female external valve

The exIX02 muscle, originated from the clunium and inserted to the anterolateral corner of the external valve, contracts during copulation and thus is associated with the active opening of the female external valve at the beginning of copulation (Figure 3.1c, f).

The female external valve grasps the lateral surface of the male hypandrium during copulation with the contraction of the stout doex01 and exVIII01 muscles (Figure 3.3e, f and 3.6a). As also mentioned above, this probably contains two functions: opening the male genital cavity at the beginning of the copulation and holding the male during copulation, both are the active movements by the female.

At the end of copulation, exIX02 probably contracts to open the external valve to release the grasp of the male.

3.5.3 Sexual selection

For the insects without sex-role reversals, the reproductive cost of females is much higher than that of males, so females are more resistant to mate and males are much

more active for mating. In the case of the coupling-role reversed *Neotrogla*, males give nutritious seminal gifts to female during copulation (Yoshizawa *et al.*, 2014, 2018b) Therefore, it is assumed that females gain more benefits from the copulating, while the mating is more costly for the males. Female's active holding of the males (probably partly coercive) is evident from the present examination: internal anchoring by the gynosomal spines and external grasping by the female paraproct and the external valves. Multiple matings actively controlled by *Neotrogla* female are also evident from the occurrence of multiple seminal capsules occurred in the female spermatheca (Yoshizawa *et al.*, 2014, 2018b). Female's aggressive demand for the seminal gifts is further suggested by the elongate copulation time (up to 72 hours: Yoshizawa *et al.*, 2014) and also by evolution of the twin insemination slots with switching valve to receive double amount of seminal gift at the same time (Yoshizawa *et al.*, 2018; Kamimura *et al.*, 2021).

It can also be assumed from the male's structures that the males probably have some ability to control the initiation of the coupling. The male paraproctal ridges and the phallsomal tip are tightly associated even in the non-copulated condition (Fig. 3.2a, c) and, without male's active upward movement of the paraproct (contraction of the paX01 and 02 muscles) and the downward movement of the phallosome (contraction of the phIX02 muscle), the female cannot insert the dorsal valve between them. The opening of the genital cavity is the most important pre-requirement for the gynosomal insertion and, although its opening may partly be achieved by the female's active movements (i.e., grasping of the terminalia using the external valve), some male's

muscles (pahy01, phIX01 and phIX02) seems to be related to the male's active opening of the genital cavity. These structures may act to resist the coercive copulation by females.

Because of the female's benefit from the mating, it has been assumed in the previous studies that the *Neotrogla* mating is mostly actively controlled by the females, and males are more passive (Yoshizawa *et al*., 2014, 2019). As also mentioned above, such factors can also be confirmed by the present examinations. However, it can be seen from the 3D model of the coupling pair that the male of *Neotrogla* is also actively holding a female during copulation. For example, the male securely fixes the female dorsal valve during copulation by using the male paraproctal ridge and the phallosomal sclerite. This may be interpreted as a remnant of the ancestral mating behavior (i.e., males are more active for mating) before the evolution of sex-role reversals. However, the well-developed male muscles related to this fixation function, including some muscles uniquely developed in *Neotrogla*, strongly suggest that the secure female fixation during copulation is crucial also for the male. The reasons for this may be attributed to the following factors.

Mismatching in genital coupling may cause leakage of seminal fluid, which may not only prolong the copulating process but also reduce the rate of sperm transfer. The leaked seminal fluid and sperm may also cause the female and male body to stick together. Not only does it take more time to separate from each other, but it also increases mortality (Matute & Coyne, 2010; Kamimura & Mitsumoto, 2012; Tanaka *et al.*, 2018; Polak & McEvey, 2022). Mismatching in genital coupling can also cause

copulatory wounds, as shown by the interspecific mating experiments of carabid beetles and *Drosophila* flies (Sota & Kubota, 1998; Kamimura & Mitsumoto, 2012). Especially, the copulating mechanism of *Neotrogla* is quite complicated, and females anchoring power is very strong (an experimental trial to separate apart a coupled specimen led to separation of the male abdomen from the thorax without breaking the genital coupling: Yoshizawa *et al.*, 2014). Therefore, a perfectly connected copulating state and step-by-step releases of each holding mechanism achieved by the cooperation of both sexes are probably beneficial for both males and females.

It is suggested that the unification of evolutionary interests between the sexes may provide "tolerance" traits rather than "resistance" traits in the face of sexual conflict (Michels *et al.*, 2015). In the case of *Neotrogla*, I did not find any aggressive structures on the male genital structures. The female also does not have an apparent structure for coercive copulating, and the achievement of the *Neotrogla* mating requires the male to open the paraproct-phallosome slot and the genital cavity. The anchoring system by the female spiny gynosome may involve some coercive factors, but no wounds have been detected in the male genital chamber, and males have some pockets to receive the spines. The co-evolution between the male and female genital structure in *Neotrogla* may provide a new example for the evolution of the tolerate traits driven by the sexual selection under the reversed sex-role.

The present examinations of the coupling and non-coupling genital conditions of Neotrogla strongly suggest that multiple factors (resistance by males, coercion by females, and cooperation between sexes) seem to work complicatedly for the genital

structures and mating processes of this insect with reversed coupling roles. However, the mating behavior of *Neotrogla* is very poorly understood to date, and the functional and evolutionary hypotheses discussed above are based only on the observations of the fixed specimens. The detailed understanding of the *Neotrogla* mating behavior is crucial for more exact understanding of the origin and evolution of the reversed genital structures in this genus.
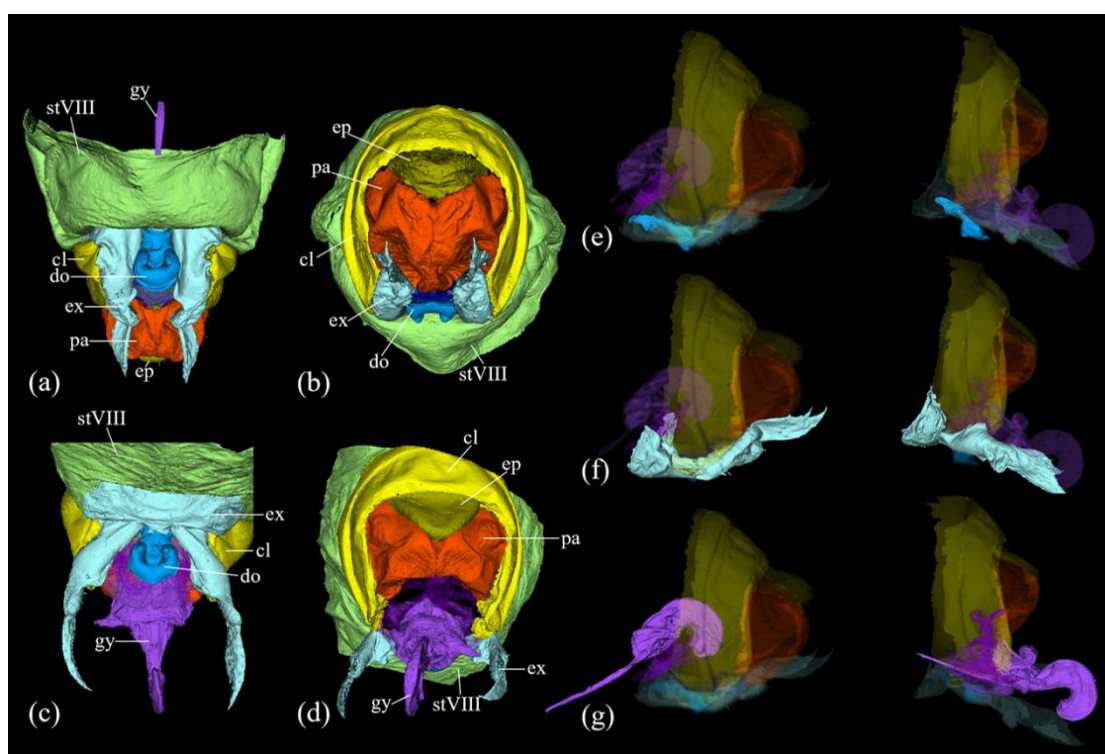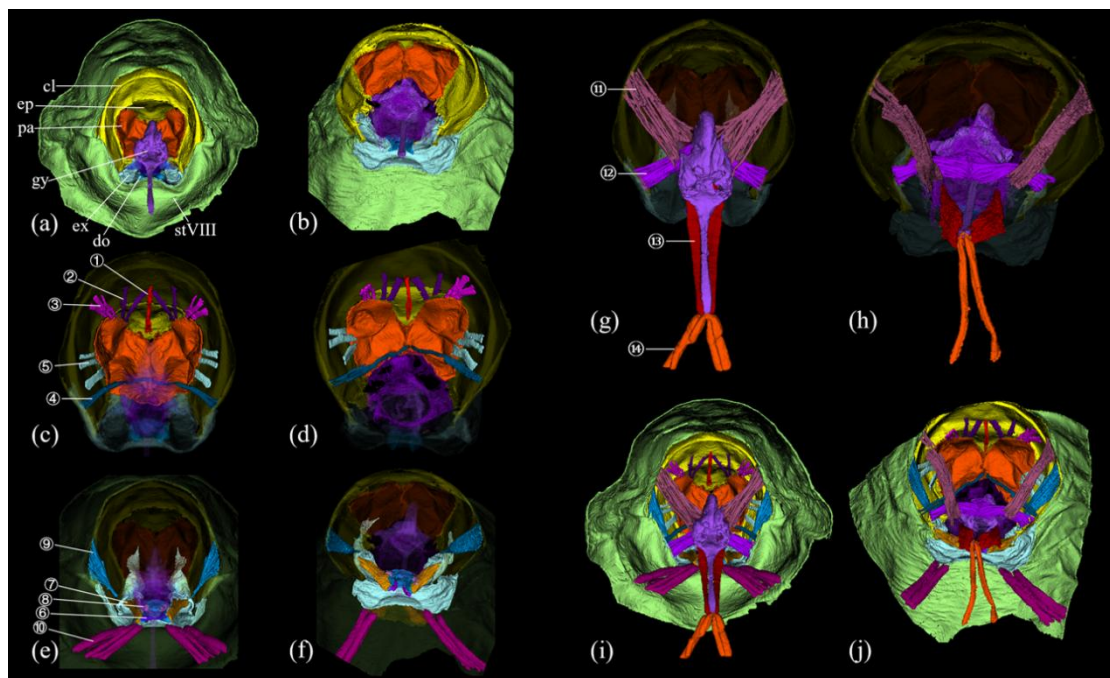

Figure 3.1. 3D reconstruction of the female terminalia of *Neotrogla curvata*; (left: uncopulated state; right: copulated state) (c-e): (a) ventral view, uncopulated state; (b) posterior view, uncopulated state; (c) ventral view, copulated state; (d) posterior view, copulated state; (e) lateral view (highlighting dorsal valve); (f) lateral view (highlighting external valve); (g) lateral view (highlighting gynosome).

Abbreviations: ep = epiproct; pa = paraproct; cl = clunium; st = sternum; gy = gynosome; do = dorsal valve; ex = external valve.
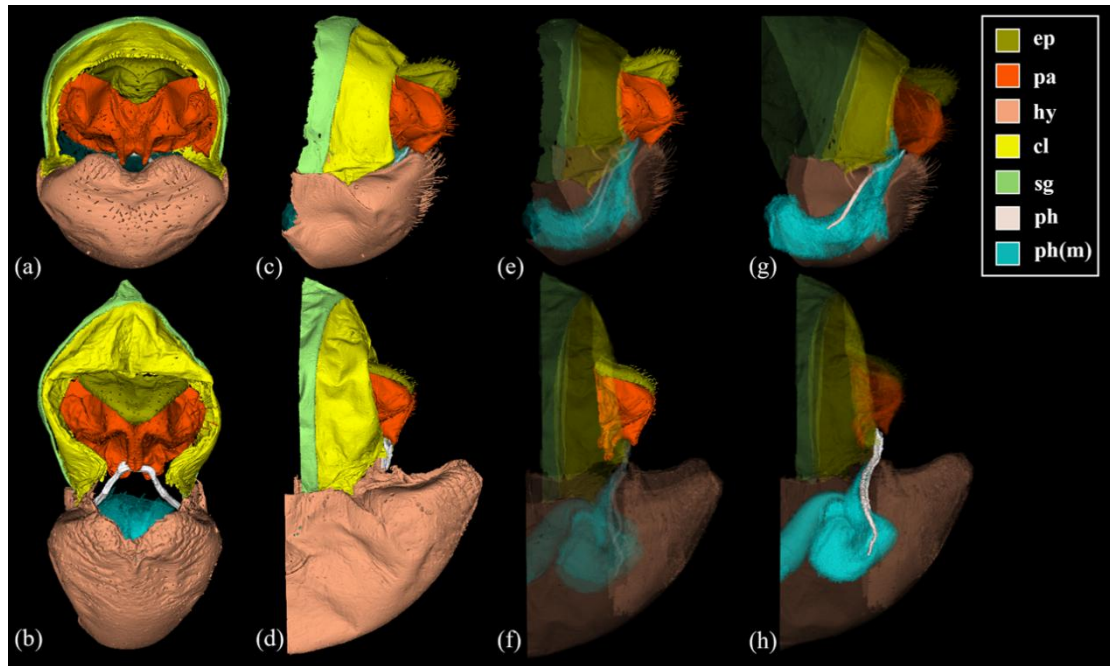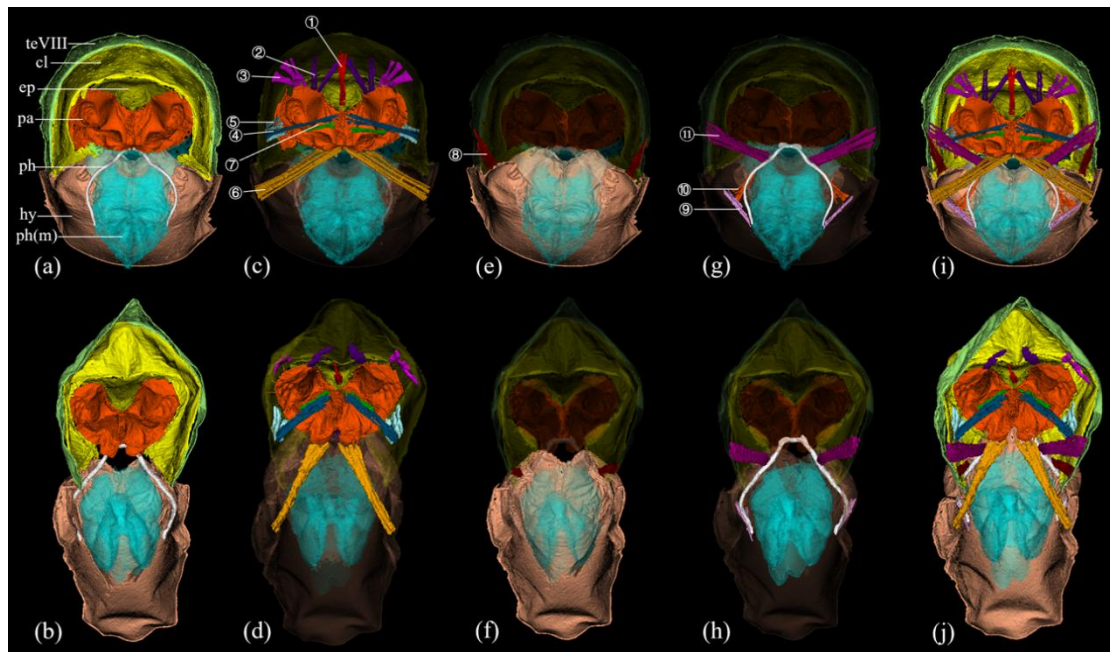
Figure 3.2. 3D reconstruction of the male terminalia of *Neotrogla curvata*, uncopulated state(a, c, e and g); copulated state (b, d, f and h): (a, b) posterior view; (c, d) lateral view; (e, f) lateral view (highlighting epiproct and paraprcot); (g, h) lateral view (highlighting phallosome)

Abbreviations: ep = epiproct; pa = paraproct; hy = hypandrium; cl = clunium; sg = segment VIII; ph = phallosome; m= membrane.

Figure 3.3. 3D reconstruction of the female terminalia of *Neotrogla curvata*, internal view; uncopulated state (a, c, e, g and i); copulated state (b, d, f, h and j); highlighting different structures and associated muscles(c-h): (1) epX01; (2) paX01; (3) paX02; (4) paX03; (5) paX04; (6) dosp01; (7) doex01; (8) exsp01; (9) exIX02; (10) exVIII01; (11) spIX01; (12) spIX02; (13) gygy01; (14) gy-01

Abbreviations: ep = epiproct; pa = paraproct; cl = clunium; st = sternum; gy = gynosome; do =
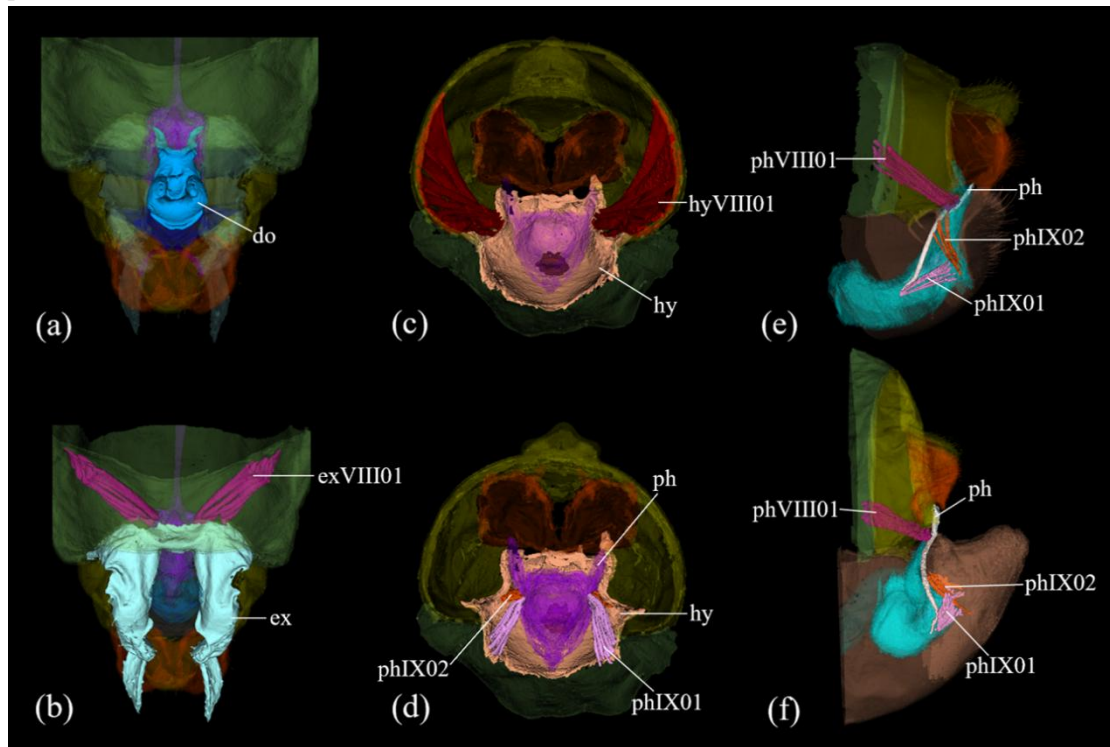
dorsal valve; ex = external valve.

Figure 3.4. 3D reconstruction of the male terminalia of *Neotrogla curvata*, internal view; uncopulated state (a, c, e, g and i); copulated state (b, d, f, h and j), highlighting different structures and associated muscles(d-h): (1) epX01; (2) paX01; (3) paX02; (4) paX03; (5) paX04; (6) pahy01; (7) papa01; (8) hyVIII01; (9) phIX01; (10) phIX02; (11) phVIII01

Abbreviations: ep = epiproct; pa = paraproct; hy = hypandrium; cl = clunium; te = tergum; ph = phallosome; m= membrane.

Figure 3.5. 3D reconstruction of the terminalia in uncopulated state, highlighting different structures and associated muscles: female of *Neotrogla curvata* (a,b), male of *Trichadenotecnum pseudomedium* (c,d); male of *Neotrogla curvata* (f,g). (a,b) ventral valve; (c, d) internal view; (e, f)

lateral view. See Fig. 1 and 2 for abbreviations.
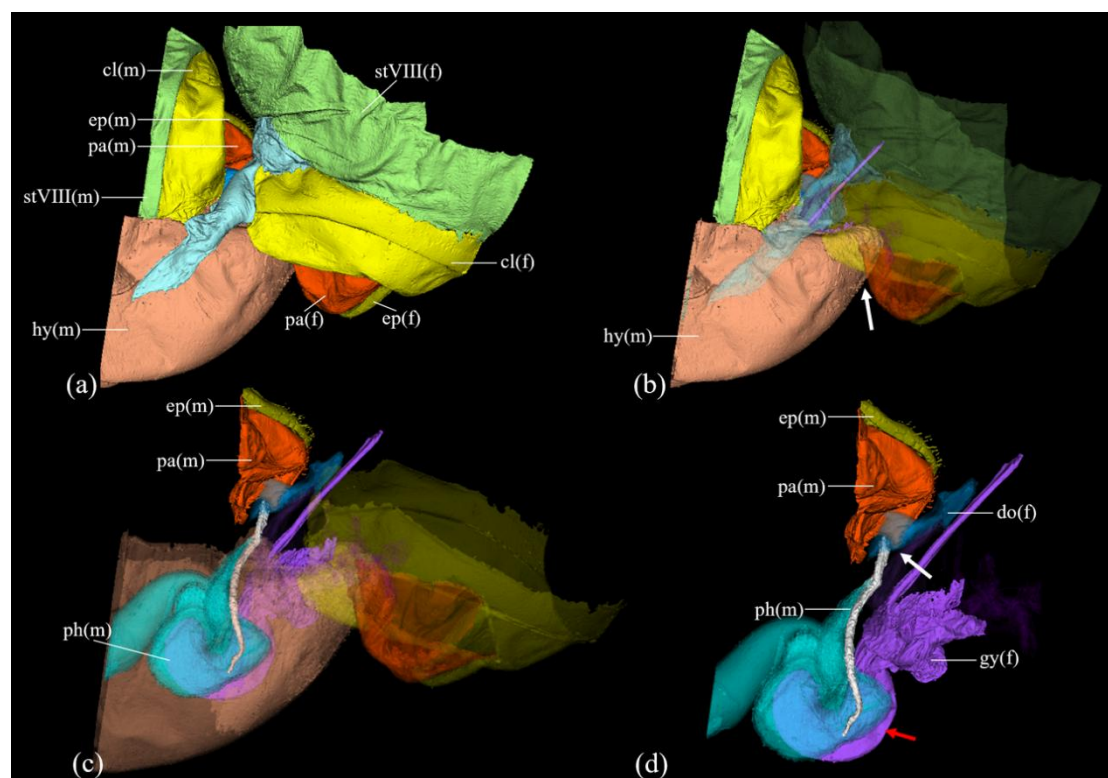
Figure 3.6. *Neotrogla curvata*, 3D reconstruction of terminalia of the copulating pair, lateral view. (b) highlighting male; (c, d) highlighting gynosome, phallosome, paraproct (m) and epiprcot (m) Abbreviations: ep = epiproct; pa = paraproct; cl = clunium; st = sternum; gy = gynosome; do = dorsal valve; ex = external valve; hy = hypandrium; ph = phallosome; m = male; f = female.

General Discussion

In this thesis, I discussed the differences and homogeneity in the female genital structures and associated muscles between the ordinary Psocodea and the Brazilian cave psocids with reversed genitalia. I also explored the function and evolutionary origins of the various genital structures on the reversed genitalia and analyzed the relationship between the novel muscles and the intromittent functions of the "female penis". The present study also clarified the functions of the male and female genital structures and associated muscles and revealed the copulating process in this group of insects.

In the previous studies, the morphological understanding of the female genitalia of Psocodea is far from sufficient, because insect female genitalia, especially the structures having direct contact with the male intromittent organ, are largely membranous, and their importance has long been overlooked. Therefore, in Chapter 1, I extensively studied seven species selected from three suborders of Psocodea to reconstruct the 3D models of the female genitalia, discussed the homology of associated muscles among these species, and constructed the musculature framework of the female genitalia of this order. It lays a solid foundation for the follow-up research on the sex-role reversed insects, *Neotrogla*.

In the previous studies on *Neotrogla*, although the morphology of the female penis was described in detail (Yoshizawa *et al.*, 2014), its evolutionary origin has not been determined, and there was no systematic description of the muscles associated to

the female genitalia. In Chapter 2, all the associated muscles of the genital structures of *Neotrogla* were described in detail for the first time. By comparing the homological muscles of the ordinary Psocodea, the origin of the gynosome and the homology of the gonapophyses in this group of insects were confirmed. Furthermore, I found two groups of gynosomal muscles uniquely developed in the insects with reversed genital structures. By comparing the state of these two groups of muscles before and during copulation, I confirmed that these muscles are related to the protrusion and retraction of the gynosome, giving the novel function of the gynosome that ordinary Psocodea do not have. These muscles were also observed in species of *Sensitibilla* having a primitive, non-protrudable gynosome (prepenis). This strongly suggests that evolution of these muscles preceded the acquisition of the protruding function of the gynosome.

The previous researches focused more on the intromittent gynosome and less on the other genital structures including the male ones. The mating action is often completed by the cooperation of multiple genital structures of males and females. Only focusing on the gynosome will not provide a comprehensive understanding of genital structures of the sexual reversed insects. In Chapter 3, by comparing the morphological changes of the genital structures and associated muscles of *Neotrogla* before and during copulation, the functions of the male and female genital structures and the copulating process in this group of insects with reversed genitalia were estimated.

(1) Lay the foundation for the morphological and phylogenetic studies of Psocodea and its close relatives

General Discussion

Based on the origin and insertion sites of each muscle, I discussed the homology of the 20 muscle groups associated with the female genital structures among the ordinary Psocodea. The large number of female genitalia data of Psocodea provided in this study have laid a solid foundation for future researches on the morphology and evolution of the genitalia of this order. It also provides important information on the evolution of the genital structures of the insect groups closely related to Psocodea. The order Psocodea was interpreted as the sister taxon of the Holometabola by phylogenomic studies (Misof *et a*l., 2014; Johnson *et al.*, 2018), but this result is known to be highly unstable. The ground plan condition of the female genital musculature in Holometabola has been estimated by Hünefeld *et al*. (2012). Some of the homologous muscles of Psocodea could be identified confidently in the holometabolan insects. Using the muscles as landmarks, the homology of the ovipositor valves between Psocodea and other neopteran insects could also be determined confidently. These will provide strong foundation for the future morphological and evolutionary studies, as well as for utilizing the female genital structures for phylogenetic estimations, of Psocodea and its related insect groups.

(2) Importance of *Neotrogla* for studying sexual selection

The genital evolution driven by sexual selection is one of the most actively studied areas in the evolutionary biology. Several different hypotheses have been proposed concerning the evolution of the genital structures and the sexual selection working on this character system (House & Simmons, 2006; Simmons, 2014; Michels *et al.*, 2015). In the present study, I found evidence of complicated co-existence of the

structures suggesting the male's resistance, female's coercion, and cooperation between both females and males of *Neotrogla* during copulation, which is very rare among animal gentialia. Furthermore, *Neotrogla* provides an extremely rare opportunity to test the previous hypotheses on the evolution of the genital structures under the reversed direction of the sexual selection. Absence of the aggressive structures in both male and female genital structures is also notable. While females have a gynosome, the only potentially aggressive structure, it does not have the function to injure the mate. The co-evolution between the male and female genital structure in *Neotrogla* may provide a new example for the evolution of the tolerate traits, newly proposed idea concerning the genital evolution (Michels *et al*., 2015), derived by the sexual selection under the reversed sex-role.

(3) Importance of *Neotrogla* for studying novelty

Evolution of the novel structures is also an active area of evolutionary biology. How to overcome the intermediate evolutionary step (i.e., less functional condition) toward the origin of novelties is one of the key questions regarding the origin of the novelties (Moczek, 2008). In many cases, novel structures are known to have originated at deep phylogenetic level (e.g., origin of the insect wings), and therefore intermediate condition between having and lacking such novel structures is usually absent.

For the case of the gynosome, it evolved within a genus, and intermediate pre-penis also exists in the close relatives, *Sensitibilla*. Therefore, *Neotrogla* and its relatives could be a model system to study the origin and evolution of novel

morphological characters. The genital evolution of *Neotrogla* is especially interesting because completely harmonious evolution of novel structures between males and females are required for the evolution of functional genitalia.

Ernst Mayr (1960) defined novelty as "any newly acquired structure or property that permits the assumption of a new function". In this thesis, the evolution of the associated muscles is tied to the emergence of the novelties. In Chapter 2, I discussed that the newly generated intromittent function of the gynosome is conferred by two groups of novel muscles. Can it be considered that these two groups of muscles are the key to the formation of the novelties? This new group of muscle was also observed in closely related genera (*Sensitibilla*) that has a primitive, non-protrudable prepenis. This is undoubtedly a very valuable research object to explore the evolution of muscles and the formation of its function.

For the studies of the sexual selection and evolution of novelties, detailed information of the mating behavior of *Neotrogla* and its relatives will be the key, which is very poorly understood to date. Accumulation of the basic behavioral information is highly desired for more accurate and detailed understanding for the evolution of the gynosome in *Neotrogla*.

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