Fruitless mating with the exes: the irreversible parthenogenesis in a stick insect

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Abstract

Parthenogenetic lineages, common in many animals, have sparked debate about their evolutionary persistence. Even after the loss of male individuals, males can survive on the genomes; in fact, rarely occurring males were described in many asexual lineages. Theoretical predictions suggest that genes related to male sexual traits in parthenogenetic lineages will decay over long evolutionary times free from the pressure of stabilizing selection. However, in many cases, such rare males are potentially functional. In this study, we investigated the functionality of rare males in the Japanese parthenogenetic stick insect, *Ramulus mikado*. Rarely occurring males in the species exhibited the external and anatomical morphology typical in stick insect males, and vigorously and successfully performed mating behaviors with conspecific females; nevertheless, no paternal genotypes were detected in the offspring; all embryos were genetically identical to their mothers. Our histological observation demonstrated that females received a spermatophore without sperms in their copulatory pouch, and revealed that in a male, spermatogenesis was completely deformed. These results suggest the irreversible clonal reproduction of *R. mikado* due to compromised male function. Our present study provides the first case of evident dysfunction in rare males of stick insects, illuminating the degenerative evolution of sexual traits in asexual lineages.

Keywords

Sexual decay under asexuality, Rare males, Stick insects, Microsatellite markers

Introduction

Sexual reproduction is a widespread strategy among multicellular organisms; however, there are alternative reproductive modes, i.e., asexual modes (Bell, 1982, Avise, 2008). One of the representatives is parthenogenesis in which offspring are generally produced from female gametes without fusion with male gametes (Suomalainen, 1962, Bell, 1982). Parthenogenesis has repeatedly emerged and is pervasive among almost all metazoan groups (Judson & Normark, 1996, Butlin, 2002, Simon et al., 2003). In predominantly parthenogenetic species, females have substantially reduced opportunities for mating with males and have ultimately lost it during the evolution (van der Kooi & Schwander, 2014, Rayner et al., 2022). Under such a condition, sexual traits of females would become free from stabilizing selection, which leads to the degeneration of female specific traits due to the accumulation of neutral mutations or enhancement of the trade-off effect with other traits (Muller, 1949, Fong et al., 1995). In fact, a selection experiment revealed that female mating behavior in the fruit fly *Drosophila mercatorum* becomes deteriorated in a parthenogenetic strain (Carson et al., 1982). Similarly, many researchers have been reported decayed sexual traits in parthenogenetic lineages

(reviewed in van der Kooi & Schwander, 2014). However, the degenerative process of sexual traits in parthenogenetic lineages, especially in males, is still under investigation.

Studies on the decay of sexual traits in parthenogenetic species have mainly focused on female-specific traits, including mating attraction, mating acceptance, and morphology of female reproductive systems (van der Kooi & Schwander, 2014). On the other hand, reports on male traits are much fewer than female ones, since male individuals rarely occur in parthenogenetic lineages. Furthermore, many of these few studies show that the "rare males" are nearly normal ones, i.e., fertile ones, and that the mating failure with females would be attributed to the degeneration of female traits (Itow et al., 1984, van der Kooi & Schwander, 2014). For example, in Phasmida, stick and leaf insects, which is a well-studied group in terms of the evolution of parthenogenetic lineages (e.g., Pantel, 1917, Bedford, 1978, Brock 2000, Scali 2009, Morgan-Richards et al., 2010, Bast et al., 2018, Nakano et al., 2019), Schwander et al. (2013) reported the functional males in an ancient asexual species of *Timema*. These males can copulate with the females in sister sexual species and successfully produce offspring. Interestingly, in a parthenogenetic strain of the laboratory stick insect, *Carausius morosus*, rare males have normal sperms as well as abnormal spermatids in their testis (Pehani, 1925, Pijinacker, 1964). Similarly, partially deformed spermatogenesis, or the coexistence of both normal and abnormal spermatids has been observed in a few animal species, while most of the males exhibit normal external morphology and behavior (van der Kooi & Schwander, 2014, Jalinsky et al., 2019). Hence, one can reasonably predict that spermatogenesis is initially deformed in males when males eventually become fruitless. However, to the best of our knowledge, the completely fruitless males have not been discovered in predominantly parthenogenetic lineages yet, so this gradual loss of male fertility needs more consideration.

Here, we were fortunate to have the opportunity to obtain rare males of the Japanese stick insect *Ramulus mikado* (Figure 1a), which is known as a predominantly parthenogenetic insect (Nagashima, 2001, Machida et al., 2016, Yano et al., 2021). Males of this species are extremely rare; in fact, there have only ever been about 10 observed cases of *R. mikado* males, despite its being the most common species of stick insects in Japan (Yano et al., 2021). Yano et al. (2021) described an outbreak of the species in the Akashina region, Nagano prefecture, Japan, and could discover only one male during the survey for 4 years wherein they counted almost thousand females per year (Yano et al., unpublished data). In this study, we examined whether the rare males were functional or not. First, we observed mating behavior and external morphology of the males. Second, to assess the paternity of the males, we genotyped parents (fathers and mothers) and embryos from the eggs that were oviposited before/after mating using microsatellite markers. Third, we performed morphological observation on the sexual traits of the males. We revealed no genetic contribution of males to offspring against their normal behaviors and external/internal reproductive systems. Furthermore, we found the completely fruitless spermatids in an *R. mikado* male.

Methods

Stick insects and their mating behavior

In this study, we collected individuals of *Ramulus mikado* (Rehn, 1904), which is a parthenogenetic and wingless stick insect in Japan (Okada, 1999, Machida et al., 2016). We used a total of 20 field collected individuals, including five males (Male#1-4, and 6). These individuals were collected in 2021 or earlier. In addition, one male that occurred in a breeding population was provided by Mr. Nawa Tetsuo as a dried specimen (Male#5, Table S1). Of the field-collected males, three males were displayed and mated in museums (Male#1, The Nawa Insect Museum, Gifu; #3, Ryuyo Town Insect observation park, Shizuoka; #4, Yokosuka City Museum, Kanagawa), one was an ethanol-preserved sample reported in Yano et al., 2021 (#6) and one was generously provided by E. Kato (#2, Chiba). Male#2 was employed for detailed observation of sexual behavior and internal morphology (details below). All collected individuals were in the late stage of nymph or adult. The legs of all-field collected samples were finally kept in 99.5% ethanol for genotypic analysis. To gain general insight into the sexual traits of stick insects, we also performed morphological observations on the bisexual stick insect, *Phraortes elongatus* (Thunberg, 1815) (details see SI Methods).

In order to investigate whether the rare males were able to mate with conspecific females, we observed the sexual behavior of *R. mikado*. The individuals used for the mating experiment were separately kept in plastic insect cages at room temperature (RT, approximately 24°C to 28°C) with the food plants until mating. The species is polyphagous, and the leaves of *Cerasus* sp., *Zelkova* sp., *Quercus* sp., or *Celtis* sp. were placed in plastic insect cages. Tap water was sprayed once every few days to provide humidity. We placed Male#2 in the plastic cage with a female. Using a smartphone camera (iPhone SE, Apple, USA), the mating behaviors of the male (approaching, mounting, and penis extension and insertion into the female gonopore) were recorded. This trial was performed three times with different females (Female#3, 4, 5) on alternate days. Male#1, 3, and 4, which were displayed in insect museums, were also placed in insect cages with conspecific females and observed the sexual behavior. We collected eggs that were oviposited before mating experiment (virgin eggs) and those that were oviposited after the experiment (mated eggs) to analyze the genetic contribution of males to the next generation.

External and internal morphology of males and females

To determine the normality of external morphology, we observed the males after they were killed or died. We especially focused on mating related structures, such as claspers and penis, according to previous papers that described *R. mikado* males (Nagashima, 2001, Yano et al., 2021). Then we dissected Male#2 in phosphate-buffered saline (PBS: 33 mM KH_2PO_4, 33 mM Na_2HPO_4, pH 6.8)

136 under a stereomicroscope (SZ61, Olympus, Japan) with fine forceps. The presence/absence of
137 components of the reproductive system such as testes and seminal vesicle was recorded, with reference
138 to Matsuda 1976. One of the testes was then fixed in 4% paraformaldehyde in PBS for confocal-
139 microscopy. Another was fixed with Bouin's solution (saturated picric acid: formaldehyde: glacial
140 acetic acid=15:5:1) at 25°C overnight and washed with 90% ethanol plus Lithium Carbonate (Li_2CO_3)
141 for histological observation. To gain more detailed insight into mating of the species, we also observed
142 female morphology after they oviposited dozens of eggs. Female#3, 4, and 5 were observed externally
143 and their abdomen were dissected in PBS, to investigate the presence or absence of spermatheca, i.e.,
144 sperm storage organ, bursa copulatrix, i.e., copulatory pouch, where the penis reaches, and
145 spermatophore, which is a capsule or mass containing the spermatozoa and being formed in female
146 bursa copulatrix during mating (Matsuda, 1976, Chapman et al., 2013). Isolated spermatheca, bursa
147 copulatrix and spermatophores were fixed with FAA fixative (formaldehyde: ethanol: acetic
148 acid=15:5:1) at 25°C overnight and then preserved in 90% ethanol, for histological examination
149 (details below). As a negative control of mating, we dissected and observed bursa copulatrix of
150 additional two females (Female#14 and 15), which were field collected and assumed to be unmated.
151 External morphology and reproductive systems of both sexes were recorded using a digital camera
152 (FLOYD-4K, WRAYMER, Japan) attached to a stereomicroscope (SZ61, Olympus, Japan).
153
154 **Genotypic analysis on the rare males, females and their offspring**
155 To determine if the rare males originated from specific lineages, microsatellite marker analyses were
156 conducted. A total of 18 individuals, including the field-collected five males and one male from a
157 breeding population, were genotyped using 11 polymorphic microsatellite loci (Table S2, Suetsugu et
158 al., 2023). To assess the genetic involvement of rare males in the next generation, the genotypes of
159 seven parental pairs and a total of 97 embryos from eggs laid before and after mating were also
160 examined using microsatellite markers. Detection of male alleles in eggs laid after mating would
161 indicate the presence of the rare male's genes in the subsequent generation.
162 Genomic DNA was extracted from either one leg of the embryo or a portion of the leg from
163 adult individuals using the DNeasy Blood & Tissue Kit (QIAGEN, Hilden, Germany), following the
164 manufacturer's instructions. PCR was conducted in two sets using multiplex PCR (refer to Table S2).
165 The PCR amplification was carried out in 5 µl reactions using the QIAGEN Multiplex PCR Kit
166 (QIAGEN, Hilden, Germany). Each sample reaction contained 10–20 ng of genomic template DNA,
167 2.5 µl of Multiplex PCR Master Mix, 0.2 µM of fluorescently labeled forward primer, and 0.2 µM of
168 reverse primer (Table S2). The amplification conditions included an initial denaturation at 95 °C for
169 15 minutes, followed by 33 cycles of denaturation at 94 °C for 30 seconds, annealing at 57 °C for 1.5
170 minutes, and extension at 72 °C for 1 minute, with a final extension at 60 °C for 30 minutes. All
171 thermal cycling conditions were performed using the T100 thermal cycler (Bio-Rad Laboratories, Inc.,

172 Hercules, CA, USA). Product sizes were determined using an ABI PRISM 3130 Genetic Analyzer and
173 analyzed with GeneMapper software (Applied Biosystems, USA).
174 To assess the kinship relations among genotypes, we constructed a neighbor-joining tree based
175 on Nei's genetic distance DA (Nei et al., 1983) using POPULATION 1.2.30 (Langella, 2007). The
176 genetic contribution of males to the next generation was assessed from two perspectives: 1) the
177 inheritance of male-specific alleles and 2) the presence of genotypic changes in eggs produced after
178 mating that could be attributed to recombination through Mendelian inheritance.
179
180 **Microscopic observation on the spermatogenesis of males and the sexual organs of females**
181 To investigate sperm production of rare males, we carefully observed the testes of a rare male (Male#2).
182 For confocal microscopic observation of spermatogenesis, a fixed testicular lobe was washed three
183 times in 0.3% Triton X-100 in PBS (PBS-X) for 15 min for permeabilization. It was then stained with
184 4,6-diamidino-2-phenylindole (DAPI) (1 μg/mL; Dojindo, Japan) for the nuclei and Alexa Fluor™
185 488 Phalloidin (66 nM; Thermo Fisher Scientific, USA) for the cytoskeleton (F-actin). After 1 h at
186 room temperature (from 20 to 25 °C), the testis was washed three times with PBS-X for 15 min and
187 mounted with VECTASHIELD antifade mounting medium (Vector Laboratories, USA). The
188 morphology of the testis and sperm cells was visualized using fluorescent staining and differential
189 interference contrast microscopy and observed by a confocal laser-scanning microscope (CLSM;
190 FV1000, Olympus, Japan). For histological observation, another testis fixed with FAA was dehydrated
191 and cleared using an ethanol-*n*-butanol series. The cleared samples were immersed and embedded in
192 paraffin at 60°C. The paraffin blocks were polymerized at 4°C and cut into 5-μm thick sections using
193 a microtome (RM2155, Leica, Germany). The sections were mounted on microscope slides coated
194 with egg white-glycerin and stained using Delafield's Hematoxylin and Eosin (EH) staining. The
195 stained slides were enclosed with Canada balsam. The slides were observed on an optical microscope
196 (Olympus) and photographs were taken using a digital single-lens reflex camera (Nikon, Japan). In
197 order to examine the decay of sexual organs in females, such as spermatheca, bursa copulatrix, and
198 spermatophore, samples from three females fixed with FAA were also processed for histological
199 observation. We also performed similar morphological observations for *P. elongatus*.
200
201
202 ## Results
203 **Possibility of sexual reproduction in *R. mikado***
204 Observation on the mating behavior of rare males in *R. mikado*
205 To investigate whether the rare male mates with females and whether he display some mating behavior,
206 we observed the mating of the male collected from Chiba (Male#2) in detail. Both active mating
207 behavior, i.e., approaching, touching his antennae, and mounting to a female, and the insertion of penis

into females genital opening were observed (Figure 1B, C, Figure S1). Other males (Male#1, 3 and 4) also successfully copulated with females.

Morphology, and anatomy of reproductive systems and eggs in *R. mikado*

We confirmed that males had an external morphology as same as the previous remarks such as a slender body, brown color and prominent white lines on the both sides of the thorax and abdomen (Figure S2, Nagashima 2001, Yano et al., 2021). The males also possessed an intromittent organ, i.e., penis, along the median of terminal. All males did not exhibit abnormalities such as characteristics of intersex as shown in *C. morosus* (Pehani 1925, Pijinacker 1964). We found that in a male there was a typical reproductive system including a pair of testes, accessory glands and seminal vesicles, as is typical in stick insects (Figure S1). We dissected three females mated with Male#2 after collection of eggs and two virgin females. Then we described female characteristics (Figure S3) and confirmed the formation of spermatophore (Figure S4). We also observed the external morphology of eggs (Figure S5) and found that the eggs were typical in stick insects. Micropyles, which are sperm gates; i.e., openings for sperm entry of insect egg chorion, were observed both in *R. mikado* and *P. elongatus* (Figure S6).

Male genotyping: their origin and genetic contribution in next generation

Our microsatellite analysis indicated the parthenogenetic origin of the rare males, the lack of kinship among them, and no genetic contribution of males in the next generation. All six males showed different genotypes to each other, indicating that they were not close relatives. Phylogenetic analysis revealed that the genotypes of three males were identical to those of females collected from nearby localities, suggesting parthenogenetic origin of rare males rather than sexual reproduction (Figure S7). We did not find any paternal alleles in eggs oviposited by females mated with males (mated eggs, Table S4). Both virgin and mated eggs consistently showed identical multilocus genotypes to their mother. For instance, in loci such as Rmika021 and Rmika091, where the mother exhibits heterozygosity for two alleles, the mated eggs also showed heterozygosity for the identical two alleles. The genotypic characteristics of the mated eggs were identical to those of the virgin eggs, indicating the parthenogenesis origin of mated eggs.

Sexual dysfunction in *R. mikado*

We demonstrated that the normally mating male did *not* become father of his partners' offspring. Why did he lack the paternity? To look into the cause of the fruitlessness, we focus on the gametogenesis of the males. First, we histologically observed testis of a bisexual stick insect *P. elongatus* as a representative of typical gametes. The nuclear and F-actin staining showed that there were a lot of mature sperms with flagella, and they form sperm bundles (Figure 2). We could recognize the sperm cysts containing male germ cells, and some of them contained spermatids (acrosome was still not formed). We observed similar features in the paraffin sectioning with HE staining as well (Figure 3).

On the other hand, we observed no mature sperms and spermatids in the testis of *R. mikado* Male#2. While there were spermatogonia and some spermatocytes in the spermatogenic cysts, the nuclei morphology was strongly deformed. We could not observe any sperm flagella structure. In histological observation, some of nuclei were deformed/condensed, and strongly stained hematoxylin (Figure 3), which are typical characteristics in apoptotic cells (Nozaki & Matsuura 2021). These observations were summarized in Table S5. Next, we assessed spermatophores isolated from mated females. In *P. elongatus*, a lot of sperms were observed. In contrast, we could not find any sperm-like structure in the *R. mikado* spermatophore (Figure S8), suggesting not to pack up sperms during the ejaculation.

Finally, we observed the reproductive system of females: spermatheca and bursa copulatrix of *P. elongatus* (sexual species) and *R. mikado* were histologically observed (Figure S9, results were summarized in Table S5). *Phraortes elongatus* females exhibit spermatheca typical in insects; active secretion, thickened cuticle and eosinophilic cytoplasm (Matsuda 1976). Bursa copulatrix of them had thickened cuticle as is typical in insects. On the other hand, the cuticle of spermatheca and bursa copulatrix of *R. mikado* females was not thickened, and in spermatheca there were many degenerative nuclei.

Discussion

Our study revealed that the "rare males" in the parthenogenetic species *Ramulus mikado* are not functional for reproduction, i.e., sterile, and do not contribute to the next generations. We found that the rare males of *R. mikado* have seemingly complete genital organs such as a penis and show the usual mating with conspecific females (Figure 1, S1, S5). Nevertheless, we cannot detect any paternity of them for offspring laid by their mating partners (Table S4). Furthermore, the male used for histological observation did not have any normally matured sperms and spermatids, and only showed abnormal spermatocytes (Figure 2, 3). So far, the decay of spermatogenesis in "rare males" in parthenogenetic species has been assessed among a few animals including stick insects (Vandel 1931, 1934, Pijinacker 1964, Oliver, 1971, Oliver et al., 1973, Pijnacker et al., 1981, Itow et al., 1984, MacDonald & Browne 1987, Zchori-Fein et al., 1992, Kajita 1993, Hunter 1999, Giorgini 2001, Adachi-Hagimori et al., 2011, Schwander et al., 2013, Jalinsky et al., 2020, Kampfraath et al., 2020). However, such males were potentially functional: they have at least some normally mature sperms, suggesting that the spermatogenesis is *not entirely* degenerated. Therefore, to our knowledge, this is the first instance of the complete decay of spermatogenesis: the production of abnormal spermatids only and no genetic contribution to sexual reproduction. Histological observations were conducted on a single male, implying that the extent of dysfunction might differ among individual males, each being independently produced through parthenogenesis (Figure S7). Nonetheless, since all males investigated did not have paternity, it would be reasonable that a similar degree of functional

impairment generally occurs in the males of the predominantly parthenogenetic species, *R. mikado*. Our finding on the fruitlessness of rare males in *R. mikado* would provide crucial progress in the studies on the decay of male sexual traits after transitioning to asexuality.

van der Kooi and Schwander (2014) proposed that the male traits to initially degenerate in the asexual lineages may be involved in spermatogenesis. Our findings indicate that spermatogenesis proceeds to degeneration faster than other sexual traits such as male genitalia and mating behavior, in *R. mikado*. This results strongly support the hypothesis on the decay process of male traits under parthenogenesis. Previous studies also showed the partially abnormal spermatogenesis in rare males with normal external morphology and behavior in some parthenogenetic animals (MacDonald & Browne, 1987, Pijinacker 1956). It has been suggested that spermatogenesis is slowly degenerated in parthenogenetic lineages due to the sole effect of genetic drift after the relaxation of stabilizing selection (van der Kooi & Schwander 2014). Based on this assumption, it is plausible that the sperm production in the predominantly parthenogenetic lineages degenerates through the following process: 1) the normal spermatogenesis is retained, 2) both the abnormal and normal sperms coexist (the partial decay of spermatogenesis), and 3) the spermatogenesis completely disrupts, and then, males eventually become fruitless. Hence, the fruitless males in *R. mikado* might represent the final stage of the spermatogenesis decay, indicating that the parthenogenesis in the species has a long history as an asexual lineage. This infer aligns with the recent phylogeographic analysis on the species throughout Japan (Suetsugu et al., 2023). Together with the partial degeneration of female sexual traits (Figure S9, Table S5), *R. mikado* could no longer return to sexual reproduction.

Overall, this study provides a novel insight into the evolutionary fate of sexual traits in both sexes under asexuality. While lineages that entirely lack males are frequently regarded as synonymous with obligate parthenogenesis, our investigation uncovered a lineage characterized by rare males, which, contrary to expectations, is not facultative but rather exhibits obligate parthenogenesis. Despite the rare occurrence of males in this species, which display not only characteristic external and anatomical morphology but also robust mating behavior, the male sex is non-functional and serves as a mere vestige. Although completely decayed spermatogenesis in rare males has been predicted, this is the first report of such instances. Obtaining males in predominantly or obligate parthenogenetic lineages is challenging; nevertheless, the accumulation of case reports can further improve our understanding of the consequences of the loss of sex.

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Author contributions

T. N., K. S. and S. K. designed the research; T. N., Y. C. and K. Y. performed the morphological, anatomical and histological observation; R. S. and S. K. performed the microsatellite analysis. T. N., R. S., and S. K. analyzed the data; T. N. and S. K. wrote the original draft of the paper with the input from Y. C., K. Y., K. S and all authors approved the final version.

Figures and captions

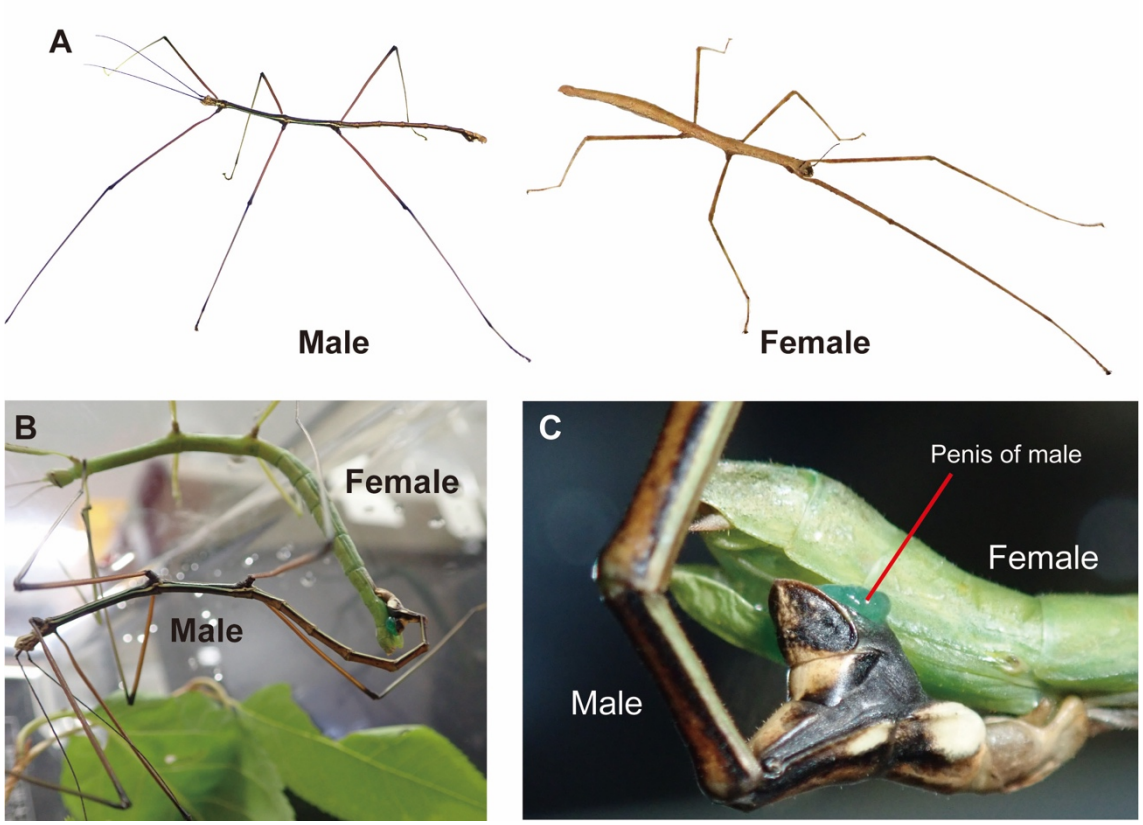

Figure 1. Japanese parthenogenetic stick insect *Ramulus mikado*. **A** photo of a rare male and a female (both adults). **B** Successful mating. A male (Male#2) mounts on a female (Female#3), grips the female abdominal tips using "claspers" and inserts their penis into the female gonopores. **C** A more magnified image of abdominal tips during the mating. Transparent greenish penis (external genitalia) is inserted into the female gonopore (For detailed information on morphology and anatomy, see Figure S2 and S3).

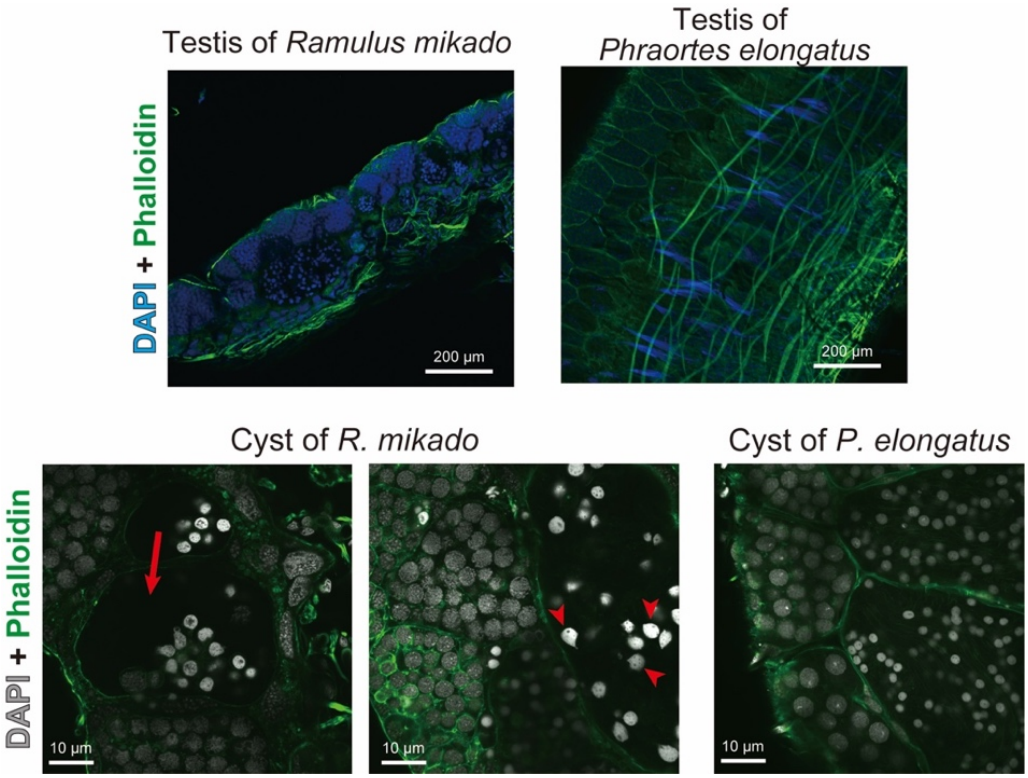

Figure 2. Confocal images of testes and spermatogenic cysts in a rare male in parthenogenetic stick insect *R. mikado* and a male in sexual species *P. elongatus*. Morphology of testis, cysts, and spermatids was visualized by DAPI-Phalloidin staining. DNA and F-actin were stained by DAPI (blue or gray) and Phalloidin (green), respectively. In a rare male testis, any mature sperms forming bundles did not observe, while there were a lot of sperm bundles in the testis of *P. elongatus* male. Spermatogenesis seemed to be deformed in a rare male; in spermatogenic cysts, there were empty spaces (the red arrow), and the nuclei exhibited irregular shapes and still large (red arrowheads). In the spermatogenic cysts of *P. elongatus*, small (probably due to meiosis) and spherical nuclei were observed.

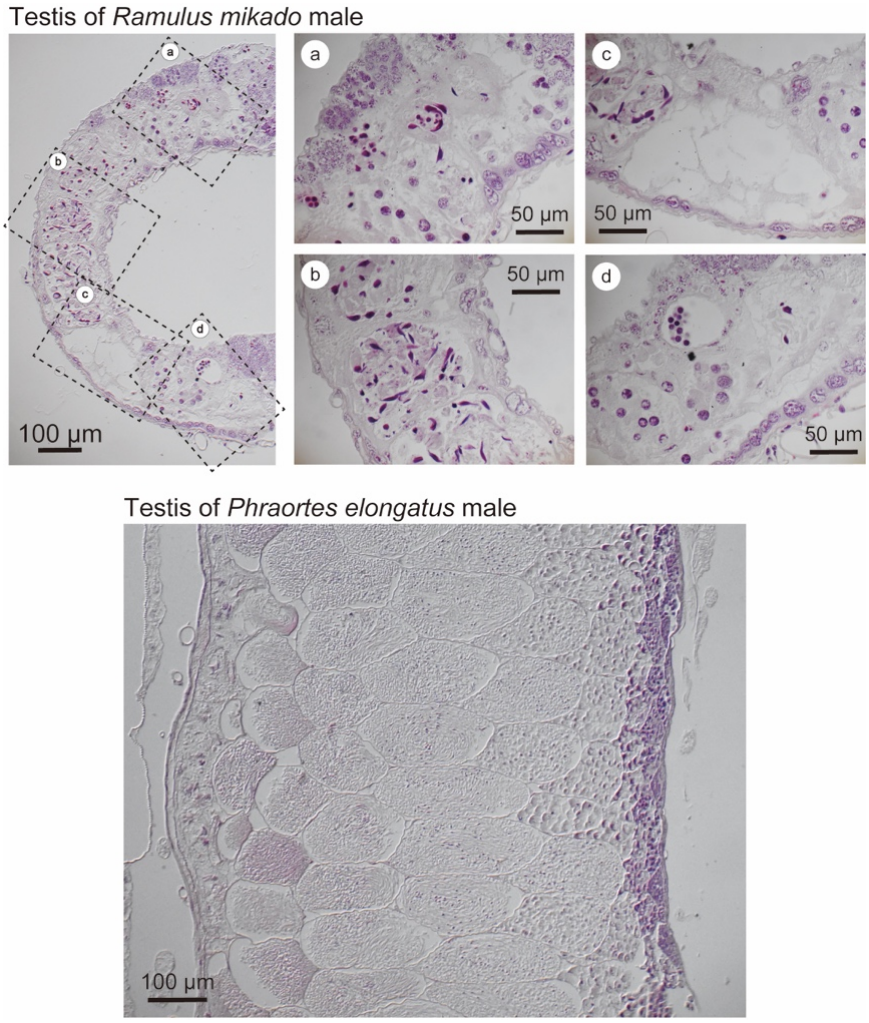

Figure 3. Histological images of male testis and spermatogenic cysts in *R. mikado* and *P. elongatus*. Morphology of testis, cysts, and spermatids was visualized by EH staining. In a testis of a *R. mikado* male, almost all nuclei in the spermatogenic cysts were strongly stained and showed an irregular shape, on the other hand, in *P. elongatus* there were both young and mature sperms that are typical in insects. In mature cysts of *P. elongatus*, sperm flagella were observable, while there was no such structure in the testis of a male in *R. mikado*.