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Kumamoto University
Natural History Note
Complex Copulatory Behavior and the Proximate Effect of Genital and Body Size Differences on Mechanical Reproductive Isolation in the Millipede Genus Parafontaria

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Abstract: The role of species-specific genitalia in reproductive isolation is unclear. Males of the millipede genus Parafontaria use gonopods (modified eighth legs) charged with sperm from the genital openings of the second legs as intromittent organs. Males perform both preliminary and true intromission during mating. During preliminary intromission, a male attempts to insert his gonopods into the female genitalia before charging the gonopods with sperm. If this intromission is completed, it is followed by the ejaculation of sperm to the gonopods and true intromission for insemination. In two sympatric species of Parafontaria that lack effective precopulatory isolation, copulation was terminated without insemination because of preliminary intromission failure caused by mismatched genital and body sizes. Thus, mechanical isolation between these sympatric species resulted from morphological differentiation mediated by the obligatory preliminary intromission. These findings demonstrate the proximate importance of genital and body size differences for reproductive isolation within this genus of millipede.

Keywords: Diplopoda, genitalia, mate recognition, mating behavior, mechanical isolation.

Sexual selection can promote tremendous diversity in animal genitalia (Eberhard 1985; Hosken and Stockley 2004; Arnqvist and Rowe 2005). Although diversified genital morphology can be involved in prezygotic reproductive isolation between related species, the role of genitalia in reproductive isolation is not well understood (Coyne and Orr 2004). Species-specific differences in genital morphology among related species have been considered effective in preventing interspecific fertilization (Dufour 1844). Although there is little empirical evidence for this classic idea (Eberhard 1985, 1992; Shapiro and Porter 1989), the mechanical “lock and key” do function as a partial mechanism of reproductive isolation in parapatric species that lack effective premating isolation (Sota and Kubota 1998; reviewed in Coyne and Orr 2004). Genitalia can also be used as a mate recognition device through tactile recognition (De Wilde 1964; Eberhard 1985, 1992; Coyne 1993). However, sensory mate recognition during copulation has never been examined. Empirical studies of interspecific genital coupling are frequently difficult because of concealed and often microscopic genitalia in animals with internal fertilization. Furthermore, such studies are possible only when premating (precopulatory) isolation is incomplete (Coyne and Orr 2004).

Millipedes are suitable organisms for studying the role of species-specific genitalia in reproductive isolation. In males, the genital openings (openings of ejaculatory ducts) and the intromittent organs (gonopods) are separated (fig. 1). The genital opening is located at the top of the coxal projection of the second legs, and the gonopods are modified eighth legs. Males must charge their gonopods with sperm before insemination, and ejaculation is evident from changes in male behavior. Because it is thought that the male will not ejaculate sperm to his gonopods unless he recognizes the female as a mate, ejaculation may be a visible criterion for mate recognition (here, we follow Ryan and Rand’s [1993] definition of “mate recognition” as a behavioral response indicating that one individual considers another an appropriate mate, even if mistakenly).

The millipede genus Parafontaria (Xystodesmidae) is
Mechanical Isolation in Millipedes

Figure 1: Genital systems of male and female Parafontaria.

Mechanical isolation in millipedes is exemplified by the genital systems of male and female Parafontaria, an endemic species complex to Japan. The male gonopods are elongated and equipped with a receptacle to receive sperm, while the female genitalia are designed with a mechanism to couple with male gonopods. This co-ordinated mechanism ensures effective reproductive isolation.

The tomoninea species complex is a lineage within Parafontaria with marked geographical variation in genital morphology and body size. This variation likely contributes to the reproductive isolation of sympatric species. These isolated species exhibit clear morphological differences, offering an excellent opportunity to study the mechanism of reproductive isolation.

We collected specimens of Parafontaria from Rurikei, Sonobe, Kyoto Prefecture, Japan, on May 22 and 26, 2003 (Parafontaria sp. A: 16 males, 24 females; Parafontaria sp. B: 5 males, 8 females), and May 17, 2004 (Parafontaria sp. A: 10 males, 8 females; Parafontaria sp. B: 2 males, 2 females). These specimens were used for mating behavior and genital coupling examinations. An allopatric species (Parafontaria sp. C) from Sanagouchi, Tokushima Prefecture, was also collected for comparative studies.

Mating experiments were conducted in incubators at 20°C and cycles of 16L : 8D or 14L : 10D for millipedes from Rurikei, and at 20°C to 22°C and 14L : 16D for millipedes from Sanagouchi. In the laboratory, millipedes were maintained in incubators at 20°C and cycles of 16L : 8D and 15L : 9D for millipedes from Rurikei and at 20°C–21°C and 14L : 10D to 16L : 8D or at room temperature and approximately 11L : 13D for millipedes from Sanagouchi.

We examined the genitalia of these species and compared the body sizes of Parafontaria spp. A and B from Rurikei. The bodies of millipedes preserved in ethanol were often distorted, making body length difficult to measure. Thus, the tenth metatergal width, which is the distance between the left and the right ozopores (openings represent reproductively isolated species are sympatric in many localities (Tanabe et al. 2001); two sympatric forms differ from each other at other localities in both morphology (Tanabe et al. 2001; Tanabe 2002) and mitochondrial lineage (T. Tanabe and T. Sota, unpublished data). Thus, each pair of sympatric forms likely represents a locally unique set of species. Because these sympatric species feed on the same resources (leaf litter) and exhibit similar patterns of seasonal activity, an effective mechanism of reproductive isolation likely maintains their coexistence.

These morphologically diverse pairs of sympatric Parafontaria species provide an excellent system for the examination of the effects of morphological differences in reproductive isolation. Because the mating system of Parafontaria is poorly understood, we initially described its mating behavior and genital coupling. Subsequently, we conducted interspecific mating experiments between two sympatric species to elucidate the mechanism of reproductive isolation.

Methods

Millipedes

We collected specimens of a pair of sympatric species of the Parafontaria tomoninea species complex (hereafter referred to as Parafontaria sp. A and Parafontaria sp. B) in Rurikei, Sonobe, Kyoto Prefecture, Japan (35°02′26.7″N, 135°24′47.2″E) on May 22 and 26, 2003 (Parafontaria sp. A: 16 males, 24 females; Parafontaria sp. B: 5 males, 8 females), and May 17, 2004 (Parafontaria sp. A: 10 males, 8 females; Parafontaria sp. B: 2 males, 2 females). These specimens were used for observations of mating behavior and interspecific mating experiments. In addition, we used an allopatric species (Parafontaria sp. C) of the P. tomoninea species complex from Sanagouchi, Tokushima Prefecture (33°57′34.1″N, 134°5′46.8″E), collected in October and November 2002 (8 males, 8 females) and in April and June 2003 (12 males, 12 females) for complementary observations of mating posture and genital coupling. In the laboratory, millipedes were maintained in incubators at 20°C and cycles of 16L : 8D and 15L : 9D for millipedes from Rurikei and at 20°C–21°C and 14L : 10D to 16L : 8D or at room temperature and approximately 11L : 13D for millipedes from Sanagouchi.
Mating Behavior and Genital Coupling

Intraspecific mating behavior was videotaped for 18 pairs of Parafontaria sp. A and 11 pairs of Parafontaria sp. B. Mating posture and genital coupling were observed in copulating pairs of Parafontaria sp. C. Twenty copulating pairs engaged in different phases of mating were frozen in liquid nitrogen. The coupling pairs were frozen during preliminary intromission (n = 5), ejaculation (n = 2), the input movement phase (n = 2), the immobile phase (n = 3), and unknown phases (n = 8), for which we did not record the phase upon freezing. Detailed explanations of each phase are provided in “Results.” Frozen pairs were freeze-dried, and the coupling of body parts and genitalia was observed by dissecting the dried pairs. The body parts and genital structures of the dried pairs were embedded in paraffin for observation of genital coupling.

Interspecific Mating

We conducted interspecific mating experiments between Parafontaria spp. A and B from Rurikei to examine whether differences in genital morphology and body size were involved in the reproductive isolation of the two species. Male mate choice between conspecific and heterospecific females and mating behavior with heterospecific females were studied using Parafontaria spp. A and B from Rurikei (25 pairs with Parafontaria sp. A males; 23 pairs with Parafontaria sp. B males). One conspecific and one heterospecific female were placed with a male in a plastic cup (12 cm diameter, 10 cm deep). Millipedes were provided with Cryptomeria japonica leaf litter at the bottom of the cup. Each male was allowed to search for a female for 10 min, and the behavioral sequence was videotaped. If a male attempted to mate with one of the females, the mating behavior was observed until completed.

Species’ Genetic Relationships

To determine the level of genetic differentiation and possibility of gene flow between the species of Parafontaria used in our experiments, we sequenced a partial mitochondrial cytochrome oxidase subunit I (COI) gene. We sequenced 36 samples for Parafontaria sp. A, 17 for Parafontaria sp. B, and six for Parafontaria sp. C. Total genomic DNA was extracted from millipede legs preserved in 80% or 99% ethanol using an Aquapure Genomic DNA Isolation Kit (Bio-Rad). Primers COS2183 (5’T-CAA CAT TTA TTT TGA TTT TTT GG-3’) and COA2745Y (5’T YAA MCC YAA RAA ATG YTG AG-3’) were used for polymerase chain reaction amplification and sequencing of 540 bp of the COI gene region. The sequence data were deposited in GenBank (DQ886427–DQ886439).

A population genetic analysis using the COI sequence data was conducted using Arlequin, version 2000 (Schneider et al. 2000). A minimum spanning network was constructed on the basis of uncorrected sequence differences. The population pairwise fixation index (Fst) and mean pairwise sequence differences (uncorrected) were estimated to determine population differentiation.

Results

General Mating Behavior

The sequence of behaviors that occurred during mating was identical for the three species studied (fig. 2; see also video 1 [3.34 MB] in the online edition of the American Naturalist, which shows copulation of a Parafontaria sp. A male and female from Rurikei). A description of mating behavior follows.

1. Antennal contact. The male touches the surface of the female body with his antennae.
2. Holding. The male walks along the female’s dorsum and curves the front of his body until the ventral surfaces of both sexes are contiguous. The male then holds the female with his legs (fig. 2B, 2). The female’s head is positioned just under the male’s head so that the male gonopods and female genitalia are aligned. The female does not hold the male and appears passive toward the male during mating.
3. Preliminary intromission. The male inserts his gonopods without sperm into the sclerotized entrance cavity of the female genitalia (preliminary intromission; table 1; fig. 2B, 3; video 1). This stage lasts for 1–4 min. The male gonopods are folded and do not contact the female valvae or receptacle (fig. 2B, 3). When preliminary intromission is not accomplished, the male terminates the mating behavior.
4. Ejaculation. When preliminary intromission is accomplished, the male withdraws his gonopods and ejaculates sperm from genital openings on his second legs onto the gonopods by curving his body, with his legs still holding the female (fig. 2B, 4; video 1). The precise portion of the male gonopods onto which sperm is ejaculated is unknown.
5. True intromission. The male reinserts his gonopods into the female genitalia for insemination (video 1). The male gonopods are inserted deep into the female genitalia, with the apical portion of the tibiotarsus and tibiotarsal process of the male gonopods coupled with the female valvae and receptacle. The tip of the tibiotarsus is inserted...
Figure 2: Genital structure (A) and mating behavior (B) of Parafontaria, with schematics of vertical cross sections of coupled genitalia based on samples frozen during copulation.
into a slit in the valvae and receptacle, which is the female genital opening (fig. 1; fig. 2B, 5' and 5'). Sperm appear to be moved through a prostatic groove and transferred from the tip of the tibiotarsus to the female genital opening (fig. 1; fig. 2B, 5' and 5').

5'. In-out movement phase. Early in true intromission, the male gonopods exhibit a typical in-out movement, which is repeated 10–77 times (table 1; fig. 2B, 5') and lasts for 6–69 min (table 1).

5'. Immobile phase. Both sexes are immobile, with male gonopods and female genitalia coupled for 29–215 min (table 1; fig. 2B, 5').

6. Withdrawal of gonopods. The male withdraws his gonopods to complete mating. The withdrawal of gonopods takes 19–183 s (table 1).

The entire behavioral mating sequence requires 57–265 min (table 1).

**Interspecific Mating**

Of the two sympatric species from Rurikei, the proportion of males holding a conspecific female after antennal contact was 0.667 ± 0.091 (SE) for *Parafontaria* sp. A (n = 27) and 0.478 ± 0.104 (SE) for *Parafontaria* sp. B (n = 23), neither of which were significantly different from random choice ($H_0$: proportion of conspecific choice = 0.5; binomial test, $P > .05$). Thus, premating isolation at antennal contact by the male was incomplete between the two species.

Preliminary intromission was not accomplished and mating was terminated in all heterospecific matings between *Parafontaria* sp. A males and *Parafontaria* sp. B females. The male attempted to insert his gonopods into the female genitalia, and his gonopods made contact with the entrance of the female genitalia. However, intromission was not accomplished because his gonopods were larger than the entrance of the female genitalia ($n = 10$; table 2; video 2 [9.78 MB] in the online edition of the *American Naturalist* shows unaccomplished preliminary intromission because of genital size differences between a *Parafontaria* sp. A male and a *Parafontaria* sp. B female; for the genital difference, see fig. 3).

In nine of 14 pairs composed of a *Parafontaria* sp. B male and a *Parafontaria* sp. A female and in two of 11 conspecific pairs of *Parafontaria* sp. B, preliminary intromission was not accomplished because the male was shorter than the female and could not align his gonopods with the female genitalia (table 2; video 3 [3.30 MB] in the online edition of the *American Naturalist* shows unaccomplished preliminary intromission because of body size differences between a *Parafontaria* sp. B male and a *Parafontaria* sp. A female). In these cases, the male attempted intromission repeatedly before he terminated the mating (video 3). The body size index (tenth metatertal width) was larger for *Parafontaria* sp. A (male: 8.47 ± 0.16 mm, $n = 10$; female: 8.51 ± 0.21 mm, $n = 10$) than for *Parafontaria* sp. B (male: 6.97 ± 0.29 mm, $n = 7$; female: 7.52 ± 0.21 mm, $n = 10$). In five heterospecific crosses between a *Parafontaria* sp. B male and a *Parafontaria* sp. A female, preliminary intromission was accomplished; that is, the male inserted his small gonopods into the large genital cavity of the female. Four of these five preliminary intromissions were followed by ejaculations. Thus, accomplished preliminary intromission was followed by ejaculation even in heterospecific pairs. Three of the four ejaculations were followed by true intromission. However, during these three intromissions, the males did not exhibit in-out movements and terminated true intromission prematurely. It is unknown whether insemination was completed during these premature intromissions.

**Species’ Genetic Relationships**

*Parafontaria* spp. A and B from Rurikei exhibited five and four COI haplotypes, respectively (fig. 3). One haplotype (Y173) was shared by four of 36 individuals of *Parafontaria* sp. B and two of 19 individuals of *Parafontaria* sp. A. Nevertheless, population differentiation among these species was high ($F_\text{ST} = 0.819; P < .001$).

**Discussion**

Male *Parafontaria* routinely performed preliminary intromission of the gonopods. The completion of this behavior was a prerequisite for ejaculation and true intromission (i.e., insemination). Thus, preliminary intromission is likely the primary mate recognition process by which male *Parafontaria* identify a female as an appropriate mate. During heterospecific matings between two sympatric species of the *Parafontaria tonominea* species complex, preliminary intromission was not accomplished in most cases because of a mismatch in genital morphology or position, and thus male mating behavior was terminated. Provided that precopulatory isolation was incomplete at antennal contact, preliminary intromission may serve as an effective mechanical agent of reproductive isolation between these two sympatric species. This mechanical isolation mechanism may be realized only in pairs of species that lack substantial precopulatory isolation (e.g., lack of differentiated sexual pheromones because of genetic closeness; fig. 3). In other pairs of genetically distant *Parafontaria* species, males never attempt copulation with heterospecific females after antennal contact (T. Tanabe and T. Sota, unpublished data), suggesting the presence of differentiated sexual pheromones.

The intromission of male genital organs without insem-
Table 1: Temporal courses and number of repetitions of mating behavior (mean ± SD) in conspecific matings in two species of *Parafontaria*

<table>
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<tr>
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<th><em>Parafontaria</em> sp. A</th>
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<th><em>Parafontaria</em> sp. B</th>
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<tr>
<td></td>
<td>Duration</td>
<td>Range</td>
<td>n</td>
<td>Duration</td>
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<tr>
<td>Preliminary intromission (min:s)</td>
<td>1:25 ± 0:34</td>
<td>0:43–2:27</td>
<td>9</td>
<td>2:07 ± 0:57</td>
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<tr>
<td>In-out movement (min:s)</td>
<td>31:15 ± 08:52</td>
<td>19:13–49:35</td>
<td>16</td>
<td>27:01 ± 24:06</td>
</tr>
<tr>
<td>Withdrawal of genitalia (min:s)</td>
<td>1:28 ± 0:41</td>
<td>0:32–3:03</td>
<td>16</td>
<td>0:40 ± 0:19</td>
</tr>
<tr>
<td>Whole mating (min:s)</td>
<td>161:03 ± 48:45</td>
<td>79:06–265:28</td>
<td>16</td>
<td>103:15 ± 32:30</td>
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<tr>
<td>No. repetitions of in-out movement</td>
<td>40.3 ± 13.9</td>
<td>24–77</td>
<td>16</td>
<td>38.4 ± 21.3</td>
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Note: Data that could be observed until the end of mating are provided.

Mechanical Isolation in Millipedes

Although preliminary intromission was the primary process by which reproductive isolation occurred, heterospecific mating continued to the stage of true intromission in several pairs composed of a *Parafontaria* sp. B male and a *Parafontaria* sp. A female (table 2). During these pairings, small male genitalia were inserted into large female genital cavities. During the three true intromissions, the *Parafontaria* sp. B males terminated true intromission without in-out movement behavior, although it is unknown whether insemination was achieved (table 2). Our genetic analysis (fig. 3) revealed the sharing of one mitochondrial haplotype, which may be a result of either introgressive hybridization or retention of an ancestral haplotype. However, if the shared haplotype had been subject to introgressive hybridization, it was likely introgressed because of genital size differences between the sexes. However, in pairs composed of a small *Parafontaria* sp. B male and a large *Parafontaria* sp. A female, the alignment of male and female genitalia was not possible in most cases because the male body was too short. Thus, the compatibility of both genital and body size is required for the completion of insemination. The evolution of body size may be affected by habitat conditions (e.g., climate, food), interspecific interactions, or sexual selection. Moreover, the evolution of body size may be (but is not always) accompanied by a correlated evolution of genital size, as in *Parafontaria* spp. A and B. Thus, several selective forces acting on body size could affect mechanical reproductive isolation in *Parafontaria*.
from a Parafontaria sp. B female to Parafontaria sp. A, given the maternal inheritance of mitochondria, and the haplotype originated from Parafontaria sp. B (fig. 3). This expected direction is opposite that of the above case of true intromission. Therefore, true intromission between a Parafontaria sp. B male and a Parafontaria sp. A female may not result in effective fertilization.

Given the manner of genital coupling during true intromission of Parafontaria (fig. 2B, 5′ and 5″), morphological compatibility between male gonopods and female genitalia appears to be essential for the accomplishment of true intromission, potentially providing an additional agent of mechanical isolation. The process and apical portion of the tibiotarsus of the male gonopods of Parafontaria form a forcepslike structure (fig. 2A) that may be used to move the female valvae and receptacle (fig. 1) to the proper position for insemination during the in-out movement and immobile phases of true intromission (fig. 2B, 5′ and 5″). Preliminary morphometric analyses of male gonopods of Parafontaria indicated that the forcepslike structure exhibits intra- and interspecific variation higher than that of other structures (T. Tanabe, unpublished data). These results suggest that sexual selection acts on the forcepslike structure more intensively than on other structures. Therefore, future studies should focus on the roles of male Parafontaria genital morphology in sexual selection during preliminary and true intromission.

In conclusion, we have demonstrated the proximate effect of morphological differences in genitalia and body size on reproductive isolation mediated by complex mating behavior with repeated genital contact. Our results help to elucidate the importance of genital intromission to reproductive isolation among animals with internal fertilization.
**Acknowledgments**

We thank J. Uoshima for preparing freeze-dried samples and Y. Takami for helpful comments. Support was provided by grants-in-aid from the Japan Society for the Promotion of Science (15918029 to T.T.; 15207004 to T.S.) and by the Global Center of Excellence Program “Formation of a Strategic Base for Biodiversity and Evolutionary Research: From Genome to Ecosystem” of the Ministry of Education, Culture, Sports, Science, and Technology, Japan.

**Literature Cited**


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