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Reproductive isolation via divergent genital morphology due to cascade reinforcement in *Ohomopterus* ground beetles

Short title: Cascade reinforcement in ground beetles

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Abstract

Secondary contact between incipient species and selection against maladaptive hybridization can drive reinforcement between populations in contact and result in reproductive character displacement (RCD). Resultant divergence in mating traits within a species may generate downstream reproductive isolation between populations with displaced and non-displaced traits, referred to as the cascade reinforcement hypothesis. We examined this hypothesis using three allopatric populations of the ground beetle *Carabus maiyasanus* with a genital lock-and-key system. This species shows RCD in male and female genital morphologies in populations in contact with the sister species *C. iwawakianus*. In a reciprocal mating experiment using three allopatric populations with differences in male and female genital sizes, insemination failure increased as the difference in genital size increased. Based on the reproductive isolation index, insemination failure was the major postmating-prezygotic isolation barrier, at least in one population pair with comparable total isolation to those of other species pairs. By contrast, there was only incomplete premating isolation among populations. These results suggest that RCD in genital morphologies drives incipient allopatric speciation, supporting the cascade reinforcement hypothesis. These findings provide insight into the roles of interspecific interactions and subsequent trait diversification in speciation processes.

Keywords: *Carabus maiyasanus*, genitalia, lock-and-key, reproductive character displacement, reproductive interference

Introduction

Diversification of mating traits and its effect on species diversification are long-standing but not yet fully resolved problems in evolutionary biology (West-Eberhard 1983; Parker and Partridge 1998; Questiau 1999; Panhuis et al. 2001; Ritchie 2007; Kraaijeveld et al. 2011; Gavrilets 2014; Servedio and Boughman 2017; Tinghitella et al. 2018; Mendelson and Safran 2021). Research on these processes has focused on the key roles of sexual selection and sexual conflict (Barraclough et al. 1995; Arnqvist et al. 2000; Gray and Cade 2000; Boughman 2001; Masta and Maddison 2002; Martin and Hosken 2003; Boul et al. 2007; Seddon et al. 2008; Janicke et al. 2018) as well as the contribution of natural selection due to variation in ecological conditions (Krüger 2008; Seehausen et al. 2008; Mann and Seehausen 2011; Wagner et al. 2012; Langerhans and Riesch 2013; Safran et al. 2013; Svensson and Waller 2013; Scordato et al. 2014; Lackey and Boughman 2014). Many recent studies have focused on male traits and female preferences in premating stages; however, relatively few studies have evaluated processes in postmating-prezygotic stages, despite their potential effects on reproductive isolation (e.g., Poikela et al. 2019). To understand the process of mating trait diversification and speciation, further studies of postmating-prezygotic isolation barriers are needed.

Morphological incompatibility between the male and female genitalia is hypothesized to cause postmating-prezygotic reproductive isolation between divergent lineages (mechanical reproductive isolation, Dufour 1844, Masly 2012). Genital morphology in animals with internal fertilization diverges rapidly, and this process is driven mainly by postmating sexual selection and sexual conflict (Eberhard 1985, 2010, Arnqvist 1998, Hosken and Stockley 2004, Simmons 2014, Sloan and Simmons 2019), occasionally resulting in species-specific correspondence between male and female genitalia (Brennan and Prum 2015). Natural selection against maladaptive interspecific interactions and due to external environmental variation can also result in species-specific correspondence in genital morphologies (Dufour 1844, Masly 2012, Langerhans et al. 2016). Consequently, physical incompatibilities between divergent genital morphologies can prevent their proper coupling and insemination and/or can damage reproductive organs, resulting in fitness costs to both sexes (structural isolation, Coyne and Orr 2004). Incompatibilities in genitalia may also be detected by sensory organs on the genital parts, thereby preventing copulation between individuals with divergent genital morphologies via mate choice processes (tactile isolation, Coyne and Orr 2004), as in the female damselfly showing greater rejection to mate with heterospecific or hybrid males (Barnard et al. 2017). For the past several decades, mechanical reproductive isolation has been repeatedly criticized owing to a lack of evidence (Shapiro and Porter 1989) and research has largely focused on other mechanisms of reproductive isolation (Masly 2012). Recently, however, evidence for mechanical reproductive isolation is growing (Sota and Kubota 1998; Nagata et al. 2007; Takami et al. 2007; Mikkola 2008; Tanabe and Sota 2008; Sota and Tanabe 2010; Kamimura and Mitsumoto, 2012; Kubota et al. 2013; Wojcieszek and Simmons 2013; Richmond 2014, Anderson and Langerhans 2015; Barnard et al. 2017, Tanaka et al. 2018, Frazee et al. 2021).

When incipient species come into secondary contact, natural selection against maladaptive hybridization or costly interspecific interactions may favor divergence in mating traits that promote mate discrimination and reinforce reproductive isolation (Dobzhansky 1937), resulting in reproductive character displacement (RCD) (Brown and Wilson 1956; Howard 1993; Butlin 1995; Grether et al. 2017). As a downstream effect, this process also increases the geographical differentiation of traits between sympatric populations with displaced traits and allopatric populations with non-displaced traits within a species. Incompatibility in divergent mating traits between these allopatric populations may serve as a reproductive barrier, possibly driving speciation (Howard 1993; Hoskin et al. 2005; Pfennig and Ryan 2006; Lemmon 2009, Ortiz-Barrientos et al. 2009, Abbott et al. 2013, Pfennig and

Rice 2014; Poikela et al. 2019), referred to as the cascade reinforcement hypothesis (Hoskin and Higgie 2010, Comeault and Matute 2016, Carabrese and Pfennig 2020). RCD in genital morphology has been reported in several taxa (Kawano 2002, 2004; Kameda et al. 2009; Hollandar et al. 2010; Kawakami and Tatsuta 2010; Anderson and Langerhans 2015; Kosuda et al. 2016). However, evidence for reproductive isolation via divergent genital morphologies in relation to RCD between allopatric populations within a species is lacking.

Ohomopterus ground beetles, a subgenus of *Carabus* (Coleoptera, Carabidae), are a model system for studies of the evolution of genital morphology and speciation (Ishikawa 1987, 1991; Sota and Kubota 1998; Usami et al. 2005; Nagata et al. 2007; Takami et al. 2007; Kubota et al. 2013; Fujisawa et al. 2019; Nishimura et al. 2022; Sota 2022). This group of insects is endemic to the Japanese archipelago and comprises approximately 15 species. Their dispersal ability is limited due to degenerate hindwings, resulting in marked geographic diversification at the species and population levels (Ishikawa 1991; Sota and Nagata 2008). Males possess a sclerotized projection on the endophallus of the intromittent organ, called a copulatory piece (CP). During copulation, the CP is inserted into the counterpart in females, called the vaginal appendix (VA) (Fig. 1A, Ishikawa 1987). The CP and VA show species-specific morphological matches (Sasabe et al. 2010, Fujisawa et al. 2019). Therefore, interspecific hybridization was assumed to be hindered by incompatibility between heterospecific genitalia (Ishikawa 1987, 1991). *Ohomopterus* species have also diverged in body sizes as a result of climatic adaptation and interspecific interaction (Sota et al. 2000b). Body size difference between species also acts as a reproductive isolating barrier (Okuzaki et al. 2010). Reproductive isolation has been quantified by experimental crosses in five parapatric or sympatric species pairs in *Ohomopterus* (*C. maiyasanus* and *C. iwawakianus*, Sota and Kubota 1998; *C. arrowianus* and *C. insulicola*, Sota et al. 2000a; *C. uenoi* and *C. iwawakianus*, Usami et al. 2006; *C. yamato* and *C. albrechti*, Takami et al. 2007; *C. insulicola* and *C. esakii*, Kubota et al. 2013), providing clear evidence for postmating-prezygotic isolation due to genital incompatibility between species. Although relatively young allopatric populations within a species are good candidates for studying early stages of speciation, little is known about reproductive isolation between conspecific allopatric populations.

C. maiyasanus is parapatrically distributed with its sister species *C. iwawakianus* with a shorter CP and VA (Fig. 2) with narrow hybrid zones at their boundary (Kubota 1988, Kubota and Sota 1998). Recently, RCD has been discovered in *C. maiyasanus*, in which the lengths of the CP and VA as well as interspecific genital differences from parapatric *C. iwawakianus* are increased in contact zones (Fig. 2) (Nishimura et al., 2022). The two species cannot stably coexist in sympatry due to interbreeding and sexual exclusion via strong reproductive interference (Sota and Kubota 1998, see also Okuzaki et al. 2010). Thus, hereafter we use "remote" and "contact" instead of "allopatric" and "sympatric" to represent the possibility of interspecific interaction, respectively (Takami & Osawa 2016, Nishimura et al. 2022). The boundary is complex and several local populations of one species are isolated within the range of the other species (Fig. 2), probably resulting from the reciprocal movement of the boundary due to interspecific interactions and climatic conditions (Takami & Osawa 2016). These isolated populations in contact with *C. iwawakianus* are most strongly influenced by interspecific interactions and have the longest CP and VA; other contact populations close to the boundary are also influenced by interspecific interactions and have the intermediate lengths of CP and VA; while populations remote from the boundary have no influence of interspecific interactions and relatively short CP and VA (Fig. 2). These geographical variation in genital morphologies are consistent with RCD (Nishimura et al., 2022). A mate choice experiment and population genetic analysis indicated that these two species have hybridized across contact zones, suggesting that the observed RCD resulted from the ongoing process of reinforcement (Nishimura et al., 2022). Unlike the typical case of reinforcement in which postzygotic isolation (e.g., hybrid inviability) generate indirect selection for prezygotic mate recognition traits (Dobzhansky 1937, Ortiz-Barrientos et al.

2009), direct selection for genital morphologies as a result of genital injury may drive the evolution of the CP and VA (Sota and Kubota 1998). Reinforcement via indirect selection is often difficult to proceed because it depends on a genetic link between prezygotic and postzygotic traits, which is likely to be impaired by interspecific gene flow and subsequent recombination, but reinforcement via direct selection is not (Shaw and Mendelson 2013). The biased extinction and filtering of populations with differences in mating traits also results in RCD (Templeton effect, Templeton 1981). The Templeton effect may be difficult to exclude, but *C. maiyasanus* populations with elongated CP and VA are only found near the boundary with *C. iwawakianus* (Fig. 2, Ishikawa and Kubota 1994), different from the prerequisite of the Templeton effect hypothesis.

The above-mentioned geographical variation in genital morphologies in *C. maiyasanus* allowed us to examine the cascade reinforcement hypothesis with respect to postmating-prezygotic isolation via divergent genital morphologies. To this end, we quantified premating and premating-postzygotic reproductive isolation between three allopatric populations of the ground beetle *C. maiyasanus* with divergence in genital sizes. We confirmed significant reproductive isolation at the postmating-prezygotic stage and its relation to genital incompatibility. We discuss how species interactions and resultant trait diversification drive speciation.

Materials and methods

Study organism

C. maiyasanus is distributed in central Honshu, Japan and consists of seven geographical lineages (i.e., subspecies) showing differentiation in CP and VA lengths (Ishikawa and Kubota, 1994). In this study, three populations (Kobe, Suzuka, and Takihara populations) covering the range of variation in CP and VA were included (Fig. 2). The Kobe population is remote from the *C. iwawakianus* population, the Suzuka population is in contact with the range of *C. iwawakianus*, and the Takihara population is isolated within the range of *C. iwawakianus*. Since these beetles are flightless and the three focal populations of *C. maiyasanus* were separated (Fig. 2), there is no current migration between these allopatric populations.

Beetles were collected at the onset of the reproductive season (April to May in 2013–2020) using pitfall traps. Male and female beetles were immediately separated and transferred to the laboratory. Beetles were kept in an incubator set at 20°C and long-daylight conditions (16L:8D) and were fed minced beef *ad libitum* to induce and maintain reproductive activity. Although their mating status was unknown, it is unlikely to affect our examination because males and females mate multiple times (Takami 2002). After behavioral experiments, beetles were dissected to measure genitalia and to remove tissues for DNA extraction. Bodies were pinned and dried.

Morphometric analysis

RCD is detected as a difference in mating traits between populations with and without reproductive interactions with other species. Male and female body length (BL), male aedeagus length (ADL), male CP length (CPL), and female VA length (VAL) were measured (Fig. 1A) in the three populations. BL was defined as the distance from the anterior margin of the labrum to the apices of the elytra and was measured using digital calipers to the nearest 0.01 mm. The other three morphological traits were defined as their maximal length and were measured on images obtained with a digital camera mounted on a microscope (Leica EZ4HD) using ImageJ to the nearest 0.01 mm. The lengths of the genital parts, especially CPL and VAL, were chosen because their functional (Okuzaki and Sota 2014) and genetic (Sasabe et al. 2010, Fujisawa et al. 2019) bases have been revealed. In total, 127 individuals (66 males and 61 females) from the Kobe population, 134 (72 and 62) from the Suzuka population, and 99 (43 and 56) from the Takihara population were measured.

To confirm geographical differentiation in body and genital sizes, morphometric data were compared among populations by general linear models (GLMs). Morphometric data were used as a dependent variable and population was used as an independent variable. To compare relative genital sizes, additional GLMs for ADL, CPL, and VAL were constructed in which population, BL, and the interaction between population and BL were included as independent variables. Interaction terms were not significant ($P > 0.05$) and were eliminated from the final models.

Genetic differentiation

To quantify genetic differentiation, 31, 20, and 25 individuals from the Kobe, Suzuka, and Takihara populations, respectively, were analyzed by multilocus microsatellite genotyping. Data for the latter two populations were reported in Nishimura et al. (2022). Total DNA was extracted from the gonads or muscles using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA). Eleven microsatellite loci (OMS33, OMS65, OMS102, OMS132, OMS317, OMS384, OMS446, OMS552, OMS585, OMS598, and OMS665; Sasabe et al. 2010, Nishimura et al. 2022) were amplified with fluorescent-labeled primers and appropriate annealing temperatures (Sasabe et al. 2010) in 10-mL volumes using AmpliTaq Gold 360 master mix (Life Technologies, Carlsbad, CA, USA). Amplified fluorescent fragments were analyzed using an ABI Prism 3130xl genetic analyzer with the GeneScan HD 400 ROX size standard (Life Technologies, Carlsbad, CA, USA). Band size was determined using GeneMapper (Life Technologies, Carlsbad, CA, USA).

Genetic differentiation between populations was evaluated by D_{EST} (Jost 2008) because conventional F_{ST} tends to underestimate genetic differentiation when using highly polymorphic loci. The statistical significance of D_{EST} was calculated based on 999 randomizations using GenAlEx ver. 6.5 (Peakall and Smouse 2012). Genetic differentiation among populations was also depicted by principal coordinate analysis using GenAlEx. Note that the Suzuka and Takihara populations show introgression from parapatric *C. iwawakianus* populations, as revealed by Structure (Pritchard et al. 2000) and NewHybrids (Anderson and Thompson 2002) analyses (Nishimura et al. 2022), and this is reflected in the estimated genetic differentiation. Other summary statistics within populations including allele richness, observed and unbiased expected heterozygosity, inbreeding coefficient, and linkage disequilibrium were also calculated using GenAlEx.

Premating isolation

To quantify premating isolation between the three allopatric populations of *C. maiyasanus*, male mate choice was examined by a two-choice experiment. In ground beetles, mating is initiated by the male, who mounts the female and attempts to insert his intromittent organ coercively (Takami 2002). Therefore, male mate choice is a principal process in premating isolation in this group of insects. One female from the same population of the male and one female from a different population were introduced to an experimental arena (plastic box, 16 cm × 12 cm × 6 cm). After the females settled, the male was gently released into the arena. The experiment was performed at 23 ± 2°C under room light. When a female was mounted by the male, the population was recorded as chosen. All six combinations of two females and one male for the three populations were examined (male vs. female of different population [N]: Kobe vs. Suzuka [20], Kobe vs. Takihara [33], Suzuka vs. Kobe [25], Suzuka vs. Takihara [21], Takihara vs. Kobe [20], and Takihara vs. Suzuka [20]). The rate of within-population mate choice was evaluated by binomial tests, in which the null hypothesis was 50% (i.e., no preference).

Postmating-prezygotic isolation

To examine postmating-prezygotic reproductive isolation, mating experiments including all nine cross types were conducted using the three populations: six reciprocal crosses between different populations and three crosses within same population as controls (232 pairs in total; male vs. female: Kobe vs. Kobe [40 pairs], Kobe vs. Suzuka [25], Kobe vs. Takihara [21], Suzuka vs. Kobe [25], Suzuka vs. Suzuka [48], Suzuka vs. Takihara [20], Takihara vs. Kobe [18], Takihara vs. Suzuka [10], and Takihara vs. Takihara [25]). A male and a female were arbitrarily chosen from the same or different populations, introduced into the experimental arena, and allowed to mate. The experiment was performed at 23 ± 2°C under room light. Females sometimes reject males attempting to mate, and genital insertion failure could be relevant to premating isolation. However, female rejection depends mostly on the female reproductive condition and mating history, which vary even within a population (Takami 2002, Hayashi and Takami 2014). We did not observe genital insertion failure due to physical incompatibility (i.e., an inability to insert the aedeagus, even when the female does not reject the male). Thus, we did not account for genital insertion failure as a component of reproductive isolation between populations. Only mated pairs were used in the following analysis.

After mating, pairs were frozen at -20°C and dissected to record damage in the male and female genitalia, evaluate the presence and site of a spermatophore within the female (Fig. 1B), and measure genitalia as described above. We assumed that field-caught beetles collected at the onset of the reproductive season show no genital damage. There were no cases of male genital injury after the experiment. The spermatophore is usually attached to the innermost part of the bursa copulatrix, and the sperm is transferred into the spermatheca from this site only (i.e., the proper site, Takami 2003) (Fig. 1B). Cases in which no spermatophores formed were recorded as spermatophore formation failures (Fig. 1B). Cases in which the spermatophore attached to other sites (mostly shallow sites in the bursa copulatrix) were recorded as spermatophore deposition failure (Fig. 1B). Failures in spermatophore formation and deposition as in Fig. 1B occur when the CP is experimentally shortened (Takami 2003). Additionally, spermatophore is not deposited properly when the CP is (even naturally) longer than the VA (Takami 2003). Therefore, these types of insemination failure in postmating-prezygotic stages are regarded as the result of incompatibility between male and female genitalia. In addition, at the end of copulation, difficulty in withdrawing the aedeagus from the female can contribute to reproductive isolation (Kamimura and Mitsumoto 2012; Richmond 2014). In ground beetles, this can also result in genital injury (Ishikawa 1991). Therefore, pairs connected to each other for a long time (about 24 hours; mean copulation duration of *C. maiyasanus* is 129 to 194 min, Takami and Sota 2007) were recorded as genital disengagement failure. The success frequency in postmating-prezygotic stages was compared between crosses within and between populations by Fisher's exact test.

Reproductive isolation index

If postmating-prezygotic isolation due to genital incompatibility is a major driver of incipient speciation within *C. maiyasanus*, its relative contribution to total isolation is expected to be large. To evaluate the relative strengths of isolation barriers, a reproductive isolation index (*RI*) was determined for each mating stage based on the decrease in female fitness due to inter-population mating. Five mating stages were evaluated: (1) male mate choice, (2) spermatophore formation, (3) spermatophore deposition at proper site, (4) female genital injury, and (5) genital disengagement. *RI* values can range from 0 to 1, corresponding to no barrier to gene flow and complete isolation between species, respectively.

For premating isolation, under the assumptions of a single mating opportunity and equal numbers of individuals in the two species, *RI* for male mate choice was calculated as follows:

$$RI_{premating} = 1 - 2 \times \frac{\text{Freq. of mating with male of different population}}{\text{Freq. mating with male of different pop.} + \text{Freq. mating with male of same pop.}}$$

where the frequencies of mating with a male from a same or different population were quantified by the two-choice experiment. Note that $RI_{\text{premating}}$ can be negative if mates from different population are preferred over those from same population; however, this may occur by chance in many cases.

For postmating-prezygotic isolation, *RI* was estimated as a decrease in female fitness in mating with a male from a different population compared to mating with a male of the same population as follows:

$$RI_{postmating} = 1 - \frac{\text{Frequency of success in mating with male of different population}}{\text{Frequency of sucess in mating with male of same population}}$$

where success was separately evaluated as spermatophore formation success, spermatophore deposition success at the proper site, genital injury, and genital disengagement success (Takami et al. 2007; Kubota et al. 2013).

To compute the total reproductive isolation between populations, a multiplicative function of the individual components of isolation at the premating and postmating-prezygotic stages was used (Ramsey et al. 2003). The *RI* index at a single stage indicates the strength of isolation at that stage, irrespective of other components. These indices of reproductive isolation were used to obtain the absolute contribution (*AC*) to total reproductive isolation as follows:

$AC_1 = RI_1$, $AC_2 = RI_2(1 - AC_1)$, and $AC_3 = RI_3[1 - (AC_1 + AC_2)]$; therefore,

$$AC_n = RI_n(1 - \sum_{i=1}^{n-1} ACi),$$

where RI_n indicates reproductive isolation at the *n*th stage. Total reproductive isolation (*T*) for *m* components was computed as follows:

$$T = \sum_{i=1}^{m} ACi.$$

This index for total reproductive isolation can range from 0 to 1 in principle. *T* was calculated separately for each reciprocal cross to assess the strength of reproductive isolation in each cross direction.

Factors influencing reproductive isolation

We evaluated whether differentiation in genital morphologies corresponding to RCD is responsible for postmating-prezygotic reproductive isolation between populations. Postmating-prezygotic isolation is expected to increase as the difference between male and female genital morphologies increases. To examine this, we constructed generalized linear mixed models (GLMMs, binomial distribution and logit link) to explain postmating-prezygotic isolation among individuals (171 pairs in total; male vs. female [N]: Kobe vs. Kobe [20], Kobe vs. Suzuka [23], Kobe vs. Takihara [21], Suzuka vs. Kobe [20], Suzuka vs. Suzuka [27], Suzuka vs. Takihara [18], Takihara vs. Kobe [17], Takihara vs. Suzuka [10], and Takihara vs. Takihara [15]). Each of spermatophore formation success and spermatophore deposition success at the proper site (Fig. 1B) was used as the dependent variable, in which success and failure were scored 1 and 0, respectively. Spermatophore deposition success at the proper site was analyzed for only the cases of spermatophore formation success (145 pairs in total) because the former was the subset of the latter (Fig. 1B). Female genital injury and genital disengagement success were not analyzed because of few failures. The body size difference between paired males and females (male BL - female BL) and its quadratic term as well as the genital size difference between paired males and females (CPL - VAL) and its quadratic term were used as independent variables. Quadratic terms were included to determine whether size matching between sexes influenced reproductive isolation. Male and female population IDs were included as random terms. These models were fitted using the *glmer* function in the *lme4* package ver. 1.1-29 (Bates *et al*. 2015) in R (R development core team 2022).

To examine the effect of genetic differentiation on postmating-prezygotic reproductive isolation, we modified the above models by removing the random terms (male and female population IDs) and including genetic differentiation between populations (D_{EST}) as an independent variable (generalized linear models [GLM]). Note that inclusion of both population IDs and genetic differentiation is redundant. These models were fitted using the *glm* function in R.

Results

Morphological and genetic differentiation

We confirmed that there is substantial morphological differentiation among the three populations. Male and female BL were largest in the Suzuka population (contact), followed by the Takihara (contact) and Kobe (remote) populations (Fig. 3, Table S1). CPL was largest in the Takihara population, followed by the Suzuka and Kobe populations (Figs. 2, 3, Table S1). ADL and VAL were significantly shorter in Kobe than in the other two populations (Tukey–Kramer HSD test, $P < 0.05$) but did not differ between the Suzuka and Takihara populations ($P > 0.05$) (Fig. 3, Table S1). By comparing least square means after controlling for BL, the results for CPL and VAL remained unchanged, while ADL was significantly longer in the Takihara population than in the Suzuka population (Tukey–Kramer HSD test, $P < 0.05$) (Table S1).

The values of D_{EST} based on 11 microsatellite markers were 0.439, 0.458, and 0.519 between the Kobe and Suzuka populations, Kobe and Takihara populations, and Suzuka and Takihara populations, respectively. These genetic differentiation values were all statistically significant ($P < 0.001$). Genetic differentiation among populations was clear as shown by principal coordinate analysis (Fig. S1). Measures of genetic diversity and inbreeding coefficient were shown in Table S2. There were no loci showing significant departure from Hardy-Weinberg equilibrium consistently in the three populations ($P > 0.05$). There were no pairs of loci showing significant linkage disequilibrium consistently in the three populations

($P > 0.05$).

Premating and postmating-prezygotic isolation

We found no or incomplete premating isolation among the three populations (Table 1). Kobe males tended to discriminate between females from their own population and those from other populations. Suzuka and Takihara males did not discriminate between females from their own population and other populations (binomial test, $P > 0.05$). Therefore, individuals from these populations will mate if they meet in secondary contact.

In support of the contribution of postmating-prezygotic isolation, we found that the rate of insemination success was lower in inter-population matings than in intra-population matings (Table 2). Females of the Kobe population (remote) with the shortest VA exhibited significantly lower rates of spermatophore formation ($P < 0.0001$) and spermatophore deposition at the proper site ($P = 0.0008$) when paired with males of the Takihara population (contact) with the longest CP. Females of the Takihara population (contact) with a relatively longer VA also showed significantly lower rates of spermatophore formation ($P = 0.0073$) when paired with the male of the Kobe population (remote) with the shortest CP.

Reproductive isolation index

As expected, *RI* indices showed that postmating-prezygotic isolation contributed substantially to the total isolation between populations of *C. maiyasanus* (Table S3, Fig. 4). The *RI* values for the stages of spermatophore formation and spermatophore deposition at the proper site were higher than those for other stages, especially when Takihara males with the longest CP mated with Kobe females with the shortest VA (Table S3). The *RI* values for these stages frequently showed asymmetric reproductive isolation between reciprocal crosses of the Kobe and Takihara populations and crosses of the Kobe and Suzuka populations. In addition, *RI* values were relatively high at the stage of male mate choice when Kobe males engaged with females of other populations.

Factors influencing reproductive isolation

As expected, the genital size difference between sexes was revealed to have significant effects on postmating-prezygotic reproductive isolation, even after controlling for male and female population IDs (GLMM in Table 3) and genetic differentiation between populations (GLM in Table 3). Quadratic terms for the genital size difference were consistently and significantly associated with spermatophore formation success and spermatophore deposition success at the proper site. The negative quadratic coefficients indicated that increasing the genital size difference decreased insemination successes (Fig. 5). Genetic differentiation was significantly and negatively associated with spermatophore formation success, indicating that postmating-prezygotic isolation increased with increasing genetic differentiation, while it was nonsignificant for spermatophore deposition success (Table 3).

Discussion

Postmating-prezygotic isolation and the cascade reinforcement hypothesis

Divergence in male signals and female preferences can serve as a premating reproductive barrier; however, this is difficult to validate in the wild because these traits are influenced by a wide range of ecological, demographic, and evolutionary factors (e.g., predation risk, population density, sensory environment, mating system, sex ratio, and sensory system) (Endler and Basolo 1998; Boughman 2002; Scordato et al. 2014). By contrast, postmating-prezygotic isolation via morphological incompatibility between male and female genitalia is mostly a physical process or a sensory process within the female, independent of environmental factors (cf. Langerhans et al. 2005, Anderson and Langerhans 2015). In this study, we showed that a mismatch between male and female genital sizes contributed to postmating-prezygotic isolation among allopatric populations of the ground beetle *C. maiyasanus*. Our evidence for mechanical reproductive isolation at the postmating-prezygotic stages provides a basis for predicting processes in the wild. Nevertheless, our estimates of reproductive isolation are based on single mating experiments. The mating frequency, population density, and relative frequency of heterospecifics should be considered for better estimates of postmating-prezygotic reproductive isolation in the wild (e.g., Sota and Kubota 1998). Postmating-prezygotic isolation could be a byproduct of premating isolation via a genetic correlation between male sexual ornamentation and fertilizing capacity (Polak et al. 2021). However, male sexual traits targeted by female preference in premating stages may be absent in *Ohomopterus* because the male coercively mates with the female (Takami 2002) even if the female is heterospecific (Okuzaki et al. 2010).

Since reinforcement is the most plausible explanation of the geographical differentiation in male and female genital morphologies (i.e., RCD, Fig. 2, Nishimura et al., 2022), our results of reproductive isolation among allopatric populations with divergent genital morphologies provide a support for the cascade reinforcement hypothesis (Hoskin and Higgie 2010, Comeault and Matute 2016, Carabrese and Pfennig 2020). Nevertheless, further evidence may be necessary to obtain the firm conclusion. It is warranted to detect reinforcing selection for genital morphologies, and to examine whether reproductive isolation between the species increases in contact populations with displaced genital morphologies. Replication of remote populations may reveal "baseline" isolation among allopatric populations without the effect of the secondary contact to the other species. The total isolation index between the Kobe (remote) and Takihara (contact) populations was high (0.609–0.808), similar to those between species forming hybrid zones at distributional boundaries (0.540–0.981; Sota and Kubota 1998, Takami et al. 2007, Kubota et al. 2013). These isolations between allopatric populations of *C. maiyasanus* were mostly explained by genital size difference between the sexes, even after controlling confounding effects: body size difference (including the effect of environment, see Introduction) and genetic difference (Table 3). If geographical or biological (i.e., distribution of *C. iwawakianus*, Fig. 2) barriers between allopatric populations of *C. maiyasanus* disappear and the populations come into contact, mechanical reproductive isolation via divergence in genital morphologies is expected to serve as a barrier to admixture. Therefore, mechanical reproductive isolation has the potential to drive speciation. Since the reproductive isolation between allopatric populations were occasionally asymmetric (Table 2, Fig. 4), the parental origin of hybrids at their secondary contact may be biased to one of two cross types and result in unidirectional introgression of mitochondrial DNA as frequently observed in this group of insects (Takami et al. 2007). RCD in genital morphology may drive two speciation processes: the completion of speciation between *C. maiyasanus* and *C. iwawakianus* (Nishimura et al., 2022) and incipient speciation among allopatric populations within *C. maiyasanus* (present study).

Previous studies of *Ohomopterus* species have mainly focused on genital injury as a product of genital mismatches between species (Sota and Kubota 1998; Usami et al. 2005; Takami et al. 2007; Kubota et al. 2013). However, our results indicated that the size mismatch between male and female genital parts generally causes a failure in insemination, rather than genital injury (Table 2). The difference in genital sizes between populations within a species is smaller than that between species and may not result in genital injury, as observed for species with relatively small genital differences (Sota et al. 2000a). In addition, the long CP in some populations (including the Takihara population in contact) is thinner and more elastic than the short CP in other populations (Fig. 2, Ishikawa and Kubota 1994); therefore, it is unlikely to break and injure the female. When the CP is slightly shorter or longer relative to the VA, it may be difficult for the male to hook the female genitalia properly, resulting in a failure to deposit a spermatophore at the proper site (Takami 2003). In addition, the mismatch between CP and VA could be detected sensorily by the male and/or female, thereby insemination and genital injury might be avoided (i.e., tactile isolation). Genital size variation among populations of *C. maiyasanus* also influences male fertilization success in the context of sperm competition through the displacement of rival spermatophores (Okuzaki and Sota 2014). These results suggest that postmating sexual selection also contributes to reproductive isolation between populations (Wojcieszek and Simmons 2013).

Other stages of reproductive isolation

The strength of premating isolation varied among pairs of populations (Tables 1 and S3), suggesting differentiation in male mate preference. Male mate preference in the Kobe population (remote) tended to be stronger than those in the Suzuka and Takihara populations (contact) (Table 1). Since the Suzuka and Takihara populations show extensive gene flow with the parapatric species *C. iwawakianus* (Nishimura et al. 2022), a preexisting male mate preference could be diminished due to decreased genomic integrity (e.g., Ostevik et al. 2020), providing evidence against reinforcement in male mate choice. Alternatively, populations that are widely distributed in the northern part of the species range (including the Kobe population, Fig. 1, Ishikawa and Kubota 1994) frequently coexist with closely related species of which distributions are largely overlapped with the northern populations of *C. maiyasanus* (*C. dehaanii*, *C. yaconinus,* and *C. yamato*, Okuzaki et al. 2010), whereas the other two populations (Suzuka and Takihara) share their habitats with few *Ohomopterus* species (*C. yamato* in limited localities). Since reproductive isolation is an important prerequisite for species coexistence (Okuzaki et al. 2010) and reinforcement of male mate choice can occur between sympatric species in *Ohomopterus* (Usami et al. 2005), it is hypothesized that the northern populations evolved strong male mate discrimination to effectively identify conspecific mates. Therefore, this mate preference may also allow greater mate discrimination among populations within species, as observed in the Kobe population. Although the cue for this male mate preference is unclear, body size is a candidate because it differs among coexisting species in *Ohomopterus* (Sota et al. 2000b, Okuzaki et al. 2010). This is congruent with the observation that Kobe males discriminated larger Suzuka females more strongly than smaller Takihara females (Tables 1 and S1). These hypotheses are warranted to be examined in the future study.

Postzygotic isolation due to genetic incompatibility is a component of total reproductive isolation among species or populations, although it was not investigated in this study. We assumed that postzygotic isolation between the three populations of *C. maiyasanus* contributed very little to total isolation. In the *Ohomopterus* species pairs, postzygotic isolation from hatching to adult eclosion was negligible (RI = -0.021 to 0.030 for three interspecific pairs, including *C. maiyasanus* vs. *C. iwawakianus*, Sota and Kubota 1998, Sota et al. 2000a, Takami et al. 2007), suggesting that interspecific hybrid larvae can survive as well as larvae from conspecific crosses. The hatching rate occasionally decreases in interspecific crosses (Takami et al. 2007, Kubota et al. 2013); however, it is difficult to discriminate between a failure in insemination and subsequent fertilization (i.e., postmating-prezygotic isolation) and a failure in embryonic development due to genetic incompatibility (postzygotic isolation). The hatching rate in *Ohomopterus* is sometimes low even for conspecific mating because the male may manipulate the female to dump unfertilized eggs (Takami et al. 2018). Note that a lack of postmating isolation is not evidence against the reinforcement hypothesis because direct selection via genital injury can contribute to the evolution of genital morphologies (Sota and Kubota 1998).

In conclusion, we demonstrated that RCD and resultant differentiation in genital morphology among allopatric populations of *C. maiyasanus* has the potential to drive the early stage of speciation, providing support for the cascade reinforcement hypothesis. Genital size incompatibility was a major determinant of postmating-prezygotic reproductive isolation, and male mate preference also contributed to premating reproductive isolation in some cases. Our findings provide insight into the process by which species interactions lead to diversification.

AUTHOR CONTRIBUTIONS

T.X. and Y.T. designed the study. T.X., T.N., N.N., K.K., T.S. and Y.T. performed field sampling. T.X., T.N. and N.N. performed laboratory works. T.X., N.N. and Y.T. analyzed the genetic data. T.X. and Y.T. analyzed the morphological data. T.X. and Y.T. wrote the first draft. All of the authors contributed to the final version.

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DATA AVAILABILITY STATEMENT

All data are publicly available on Dryad (https://doi.org/10.5061/dryad.8931zcrv0).

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Table 1. Male mate choice in three populations of *C. maiyasanus*. *P*-values were calculated by binomial tests against the null hypothesis of random pairing (50% chance of choosing a female from the male's population).

Population pair (male vs. female of a different population)	% Correct choice (*N*)	*P*
Kobe vs. Suzuka	75.0% (15/20)	0.041
Kobe vs. Takihara	66.7% (22/33)	0.080
Suzuka vs. Kobe	48.0% (12/25)	1
Suzuka vs. Takihara	52.4% (11/21)	1
Takihara vs. Kobe	50.0% (10/20)	1
Takihara vs. Suzuka	55.0% (11/20)	0.82

Table 2. Results of mating experiments between three populations of *Carabus maiyasanus*. A significantly lower value for inter-population mating than mating within a population is indicated by asterisks. Results of mating within a population were shown repeatedly for easier comparison.

	Cross type (male vs. female)			
	Kobe male vs. Kobe female	Suzuka male vs. Kobe female	Kobe male vs. Suzuka female	Suzuka male vs. Suzuka female
Number of pairs	40	25	25	48
%Spermatophore formation	97.5 (39/40)	96.0 (24/25)	88.0 (22/25)	95.8 (46/48)
%Spermatophore deposition at proper site	100.0 (39/39)	100.0 (24/24)	90.9 (20/22)	100.0 (46/46)
%Female not injured	97.5 (39/40)	100.0 (25/25)	100.0 (25/25)	100.0 (48/48)
%Genital disengagement success	100.0 (40/40)	100.0 (25/25)	96.0 (24/25)	100.0 (48/48)
	Kobe male vs. Kobe female	Takihara male vs. Kobe female	Kobe male vs. Takihara female	Takihara male vs. Takihara female
Number of pairs	40	18	21	24
%Spermatophore formation	97.5 (39/40)	27.8 (5/18)***	61.9 (13/21)**	95.8 (23/24)
%Spermatophore deposition at proper site	100.0 (39/39)	40.0 (2/5)***	92.3 (12/13)	100.0 (23/23)
%Female not injured	97.5 (39/40)	88.9 (16/18)	100.0 (21/21)	100.0 (24/24)
%Genital disengagement success	100.0 (40/40)	88.9 (16/18)	95.2 (20/21)	100.0 (24/24)
	Suzuka male vs. Suzuka female	Takihara male vs. Suzuka female	Suzuka male vs. Takihara female	Takihara male vs. Takihara female
Number of pairs	48	10	20	24

%Spermatophore formation	95.8 (46/48)	90.0 (9/10)	85.0 (17/20)	95.8 (23/24)
%Spermatophore deposition at proper site	100.0 (46/46)	100.0 (9/9)	100.0 (17/17)	100.0 (23/23)
%Female not injured	100.0 (48/48)	100.0 (10/10)	100.0 (20/20)	100.0 (24/24)
%Genital disengagement success	100.0 (48/48)	100.0 (10/10)	100.0 (20/20)	100.0 (24/24)

***$P < 0.001$, **$P < 0.01$, *$P < 0.05$

Table 3. Factors influencing reproductive isolation among three populations of *Carabus maiyasanus*. Statistical significance is indicated by boldface ($P < 0.05$).

	GLMM including male and female population IDs as random terms			GLM including genetic differentiation between populations		
	Coefficient ± SE	*z*	*P*	Coefficient ± SE	*z*	*P*
Spermatophore formation ($N = 171$)						
Body size difference	0.350 ± 0.284	1.236	0.22	0.376 ± 0.269	1.401	0.16
Body size difference^2	0.079 ± 0.089	0.893	0.37	0.124 ± 0.095	1.306	0.19
Genital size difference	0.855 ± 0.444	1.925	0.054	0.613 ± 0.453	1.354	0.18
Genital size difference^2	-1.573 ±0.345	–4.562	**<0.0001**	-1.260 ± 0.346	-3.637	**0.0003**
Genetic differentiation	–	–	–	-4.742 ± 2.044	-2.319	**0.020**
Spermatophore deposition at proper site ($N = 145$)						
Body size difference	0.334 ± 0.558	0.598	0.55	0.351 ± 0.540	0.651	0.51
Body size difference^2	0.048 ± 0.165	0.290	0.77	0.089 ± 0.186	0.479	0.63
Genital size difference	0.541 ± 0.771	0.702	0.48	0.326 ± 0.795	0.410	0.68
Genital size difference^2	-1.786 ± 0.648	–2.755	**0.0059**	-1.444 ± 0.663	-2.179	**0.029**
Genetic differentiation	–	–	–	-4.785 ± 4.235	-1.130	0.26

Table S1. Morphological traits in the three populations of *Carabus maiyasanus*. Means ± SE (N) are shown. Least square means (LSM) were calculated relative to body length. Different letters indicate a significant difference between populations by Tukey–Kramer HSD tests ($P < 0.05$).

	Kobe population	Suzuka population	Takihara population	*F*	d.f.	*P*
Male body length	22.77 ± 0.10 (66)^{a}	24.72 ± 0.07 (72)^{b}	23.78 ± 0.11 (43)^{c}	118.91	2, 178	<0.0001
Female body length	23.77 ± 0.12 (61)^{a}	25.40 ± 0.09 (62)^{b}	24.69 ± 0.11 (56)^{c}	59.84	2, 176	<0.0001
Aedeagus length	7.27 ± 0.03 (66)^{a}	8.08 ± 0.02 (72)^{b}	8.17 ± 0.04 (35)^{b}	340.85	2, 170	<0.0001
LSM aedeagus length	7.37 ± 0.03 (66)^{a}	7.98 ± 0.03 (72)^{b}	8.18 ± 0.03 (35)^{c}	164.29	2, 169	<0.0001
Copulatory piece length	2.29 ± 0.01 (66)^{a}	3.00 ± 0.02 (70)^{b}	3.72 ± 0.03 (43)^{c}	1399.39	2, 176	<0.0001
LSM copulatory piece length	2.31 ± 0.02 (66)^{a}	2.98 ± 0.02 (70)^{b}	3.72 ± 0.02 (43)^{c}	1131.44	2, 175	<0.0001
Vaginal appendix length	2.07 ± 0.03 (61)^{a}	2.71 ± 0.03 (62)^{b}	2.74 ± 0.04 (56)^{b}	121.86	2, 176	<0.0001
LSM vaginal appendix length	2.10 ± 0.04 (61)^{a}	2.68 ± 0.04 (62)^{b}	2.74 ± 0.04 (56)^{b}	72.87	2, 175	<0.0001

Table S2. Genetic diversity and inbreeding coefficient of the study populations of *Carabus maiyasanus*.

Population	N	A	H_O	Unbiased H_E	F
Kobe	31	6.636	0.624	0.656	0.025
Suzuka	20	7.273	0.573	0.686	0.134
Takihara	25	8.636	0.564	0.752	0.205

A, allele richness; H_o, observed heterozygosity, H_E, expected heterozygosity; F, inbreeding coefficient

Table S3. Reproductive isolation in premating and postmating-prezygotic stages between three populations of *Carabus maiyasanus*. Superscript numbers indicate the stage of reproductive isolation (see also Fig. 4).

Stages	Reproductive isolation index (*RI*)	Absolute contribution to total isolation (*AC*, %)	Reproductive isolation index (*RI*)	Absolute contribution to total isolation (*AC*, %)
	Kobe male vs. Suzuka female		Suzuka male vs. Kobe female	
Male mate choice^1	0.5*	0.500 (83.9)	-0.04	-0.040 (-129.0)
Spermatophore formation^2	0.082	0.041 (6.9)	0.015	0.016 (51.6)
Spermatophore deposition at proper site^3	0.165	0.038 (6.4)	0.015	0.000 (0.0)
Female genital injury^4	0	0.000 (0.0)	0.015	0.015 (48.4)
Genital disengagement^5	0.04	0.017 (2.9)	0.04	0.040 (129.0)
Total isolation$^{1-5}$		0.596 (100)		0.031 (100)
	Kobe male vs. Takihara female		Takihara male vs. Kobe female	
Male mate choice^1	0.33	0.330 (54.2)	0	0.000 (0.0)
Spermatophore formation^2	0.354**	0.237 (38.9)	0.715***	0.715 (88.5)
Spermatophore deposition at proper site^3	0.404	0.021 (3.4)	0.886***	0.049 (6.1)
Female genital injury^4	0	0.000 (0.0)	0.088	0.021 (2.6)
Genital disengagement^5	0.048	0.020 (3.3)	0.111	0.024 (3.0)
Total isolation$^{1-5}$		0.609 (100)		0.808 (100)
	Suzuka male vs. Takihara female		Takihara male vs. Suzuka female	
Male mate choice^1	0.05	0.050 (31.8)	0.1	0.100 (64.5)
Spermatophore formation^2	0.113	0.107 (68.2)	0.061	0.055 (35.5)

Spermatophore deposition at proper site[3]	0.113	0.000 (0.0)	0.061	0.000 (0.0)
Female genital injury[4]	0	0.000 (0.0)	0	0.000 (0.0)
Genital disengagement[5]	0	0.000 (0.0)	0	0.000 (0.0)
Total isolation[1-5]		0.157 (100)		0.155 (100)

***$P < 0.001$, **$P < 0.01$, *$P < 0.05$

Figure legends

Fig. 1. (A) Coupling of the male and female genitalia in *Ohomopterus* ground beetles (modified from Takami 2003). Simple and bold letters indicate female and male parts, respectively (copulatory piece length, CPL; vaginal appendix length, VAL). (B) Female genitalia after copulation showing the definitions of spermatophore formation success and spermatophore deposition success (spermatophore, SP).

Fig. 2. Distribution and geographical variation of *Carabus maiyasanus* (orange) and its sister species *C. iwawakianus* (blue). Circles and squares indicate remote populations and populations in contact with the other species, respectively. Upper left inset shows the study area (orange rectangle). Upper right inset shows character displacement in male and female genital sizes in *C. maiyasanus* (copulatory piece, CP, and vaginal appendix, VA, $^*P < 0.05$) (modified from Nishimura et al. 2022). Male body and male and female genitalia as well as geographical variation in the CP are shown. Three experimental populations (Kobe, Suzuka, and Takihara) are indicated by red rectangles surrounding the CP. Scale bar represents 10 mm and 2.5 mm for body and genital sizes.

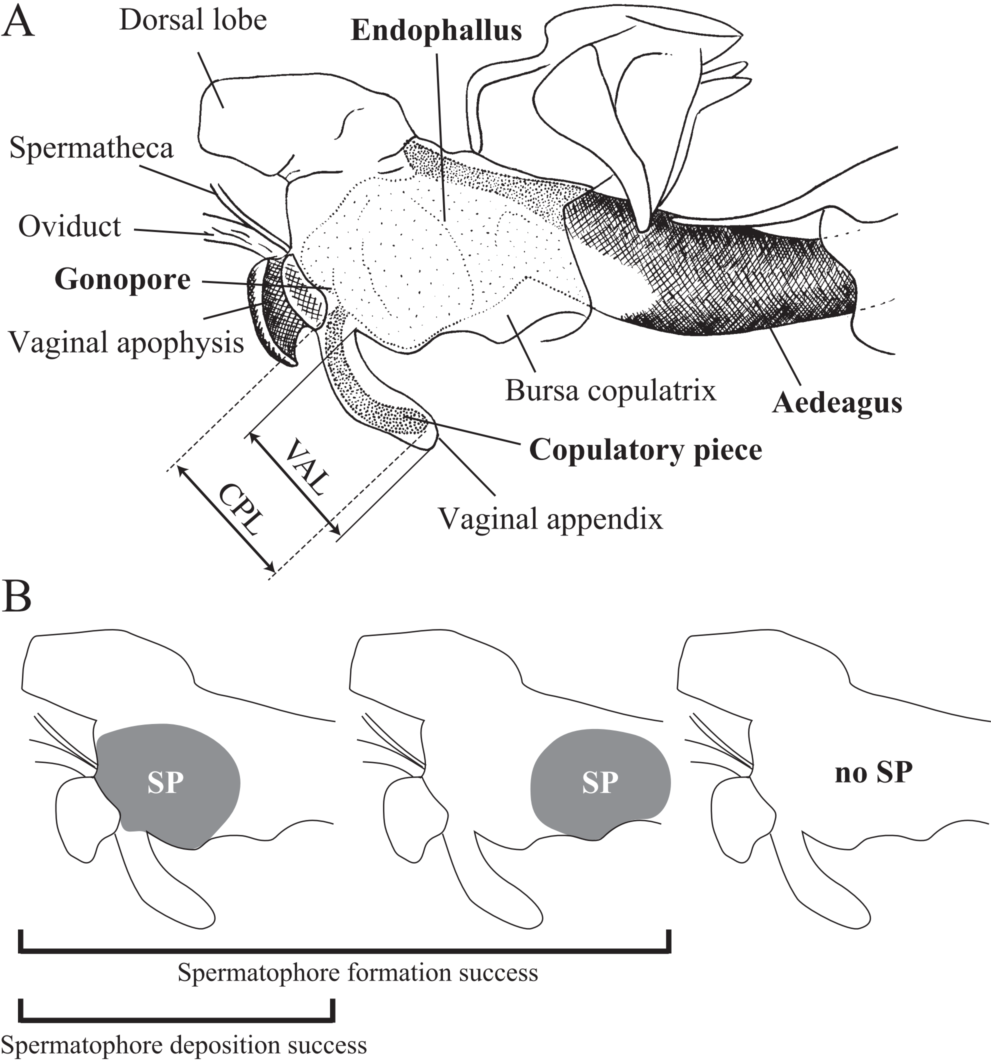
Fig. 3. Body and genital size variation among three populations of *C. maiyasanus*. Different letters (upper cases for the male, and lower cases for the female) indicate significant differences ($P < 0.05$).

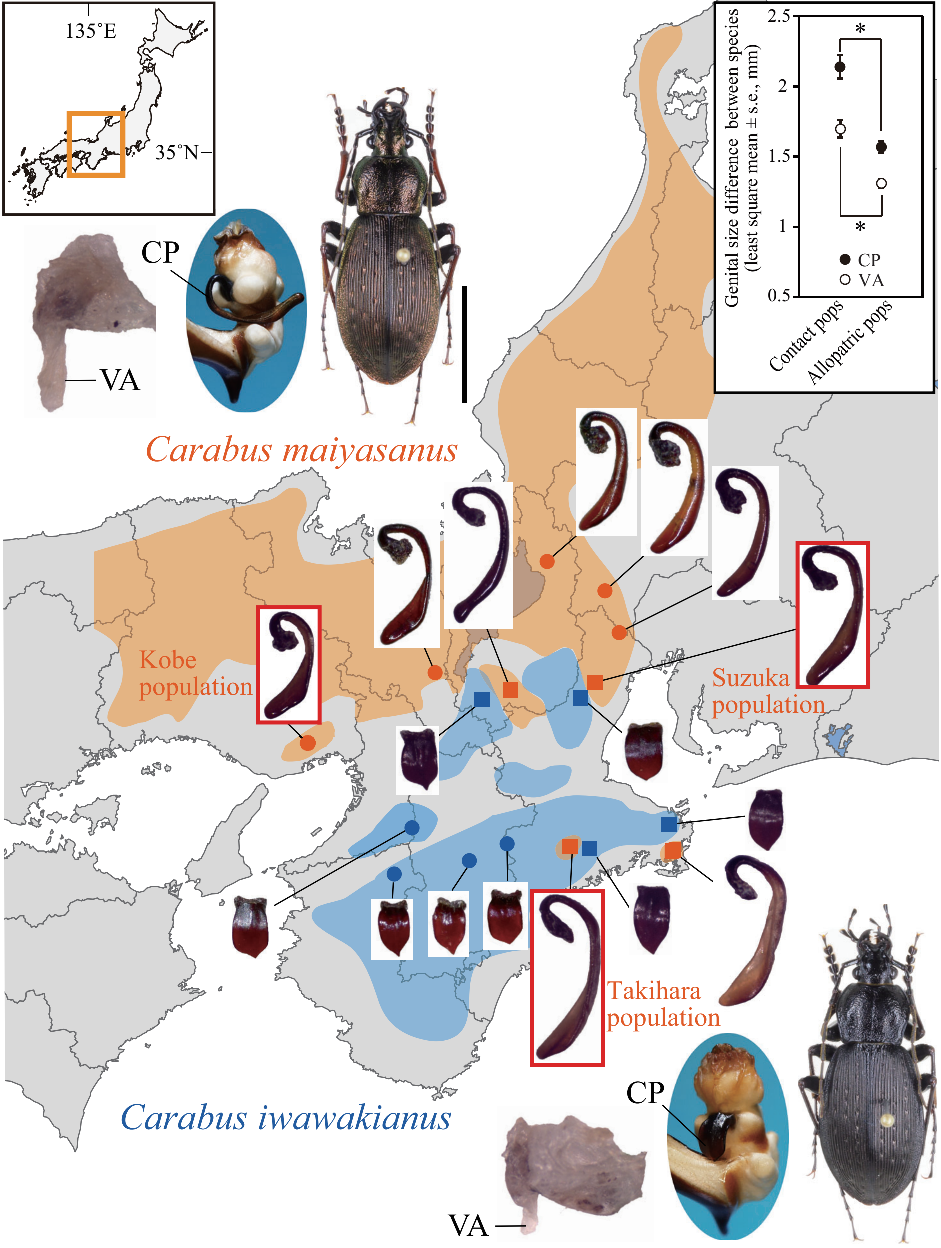
Fig. 4. Profiles of cumulative isolation indices in reciprocal crosses between (A) Kobe and Suzuka populations, (B) Kobe and Takihara populations, and (C) Suzuka and Takihara populations. Stages of isolation were as follows: (1) male mate choice, (2) spermatophore formation, (3) spermatophore deposition at the proper site, (4) female genital injury, and (5) genital disengagement. Bold line indicates a significant contribution to isolation (Table S3).

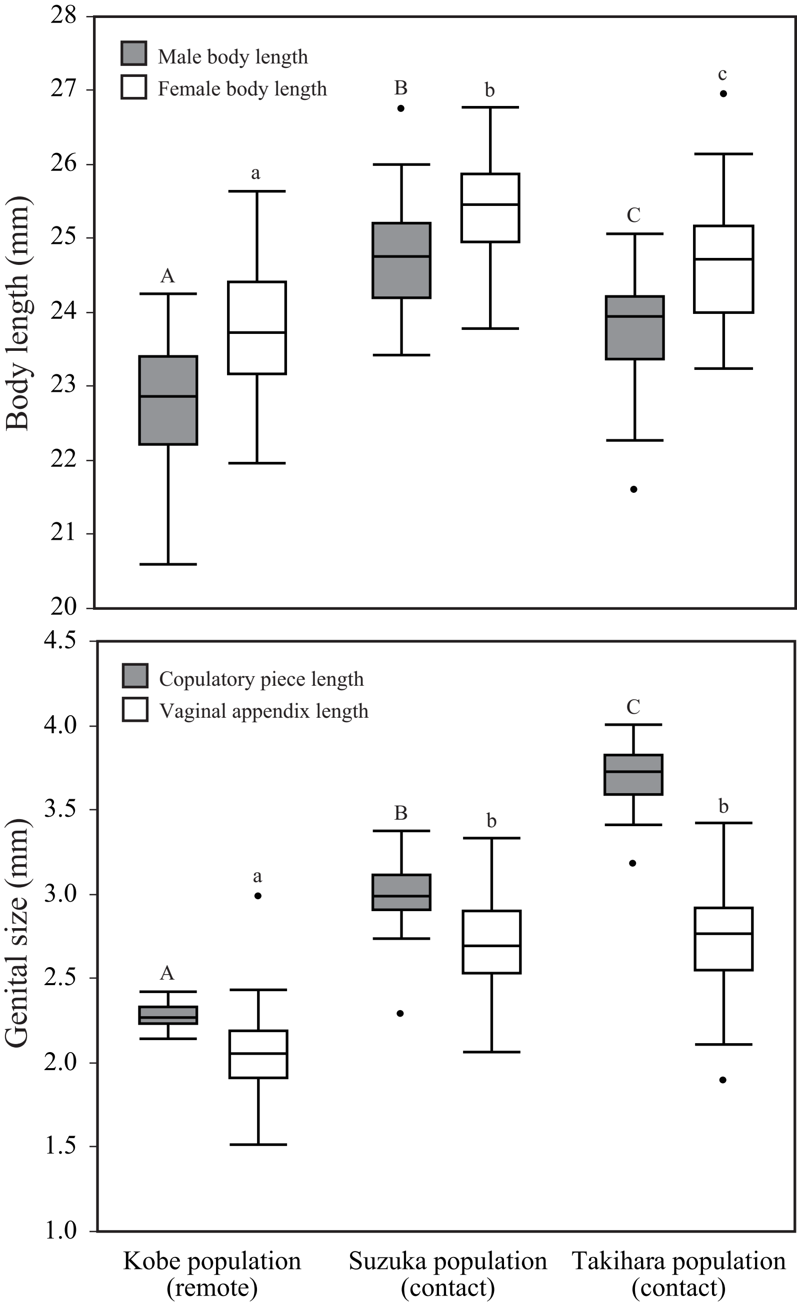
Fig. 5. Effect of genital size difference between the sexes on (A) spermatophore

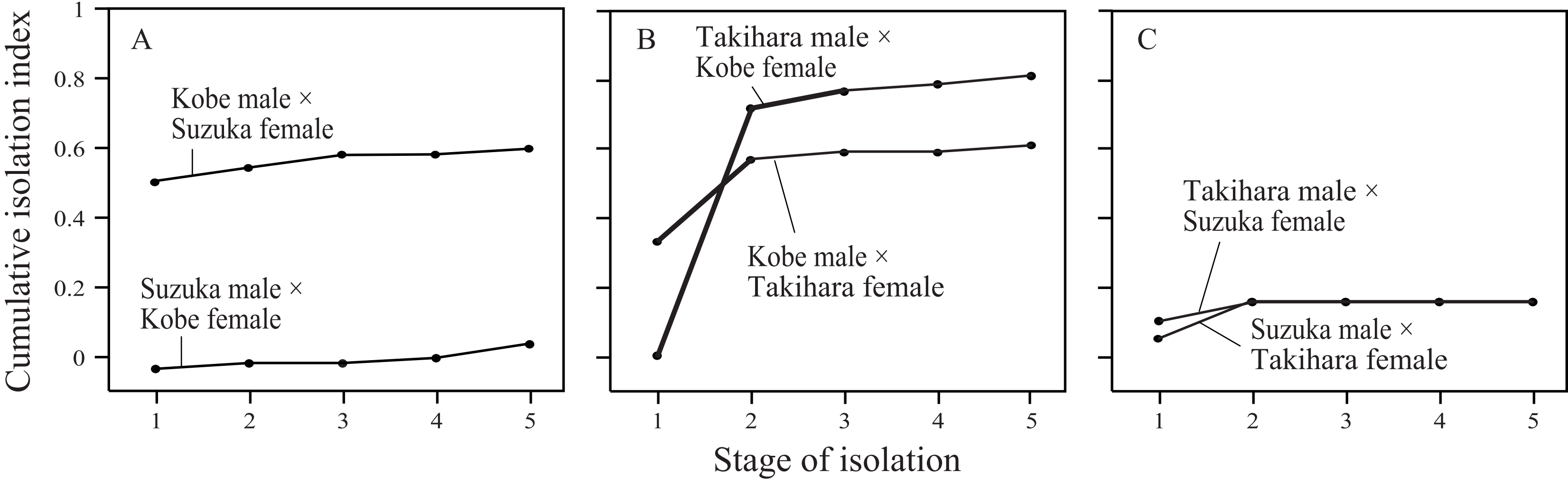
formation success and (B) spermatophore deposition success at the proper site in the bursa copulatrix. Solid and open points refer to intra- and inter-population pairs, respectively. Fitted curve is based on generalized linear models including genital size difference and its quadratic term as independent effects. See Table 3 for detailed interpretation based on full models.

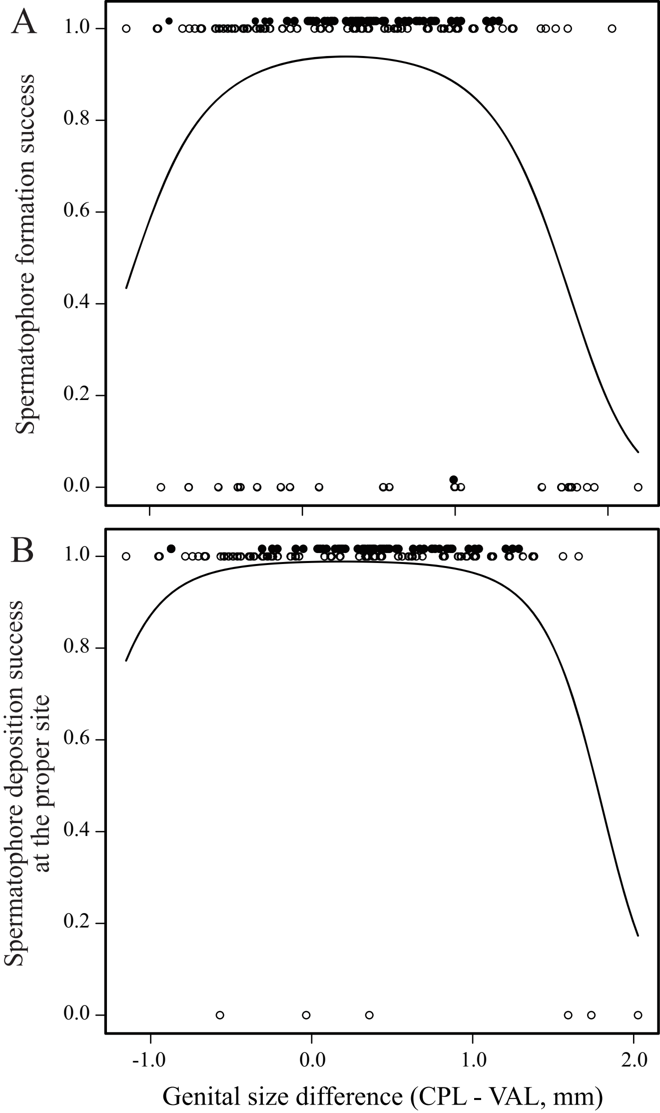
Fig. S1. Principal coordinate analysis of genetic difference among individuals from three populations of *C. maiyasanus*.

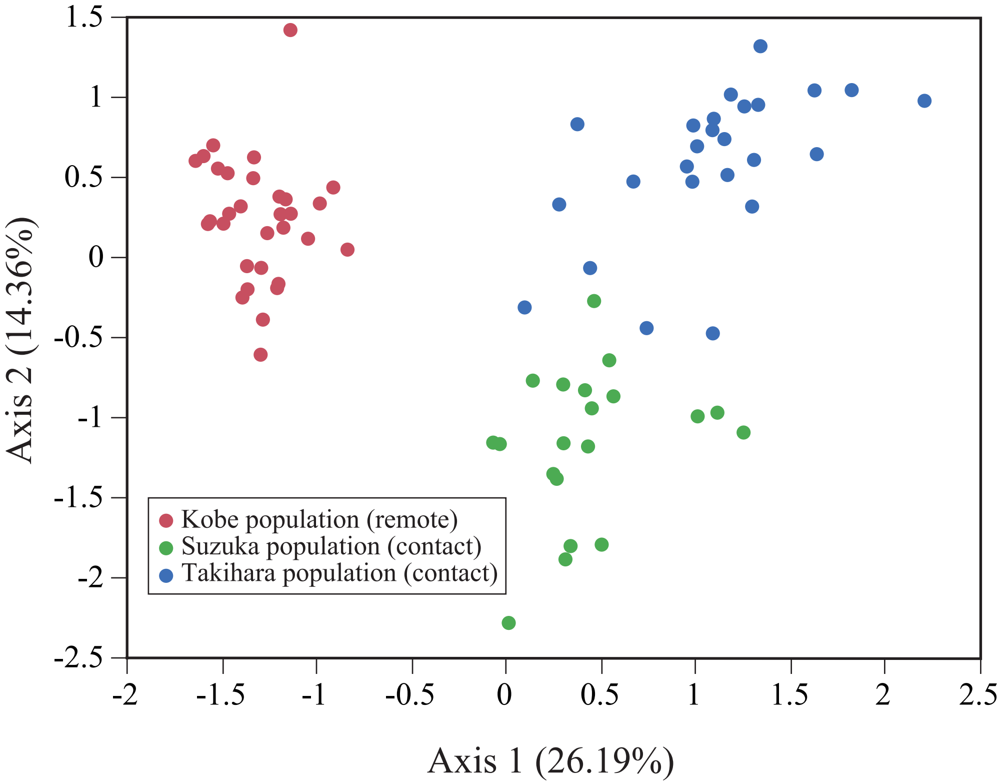

A
Dorsal lobe
Endophallus
Spermatheca
Oviduct
Gonopore
Vaginal apophysis
Bursa copulatrix
Aedeagus
Copulatory piece
VAL
CPL
Vaginal appendix
B
SP
SP
no SP
Spermatophore formation success
Spermatophore deposition success


135˚E
35˚N
CP
VA
Carabus maiyasanus
Kobe population
Suzuka population
Takihara population
Carabus iwawakianus
CP
VA
Genital size difference between species (least square mean ± s.e., mm)
2.5
2
1.5
1
0.5
*
*
● CP
○ VA
Contact pops
Allopatric pops


Male body length
Female body length
Body length (mm)
28
27
26
25
24
23
22
21
20
A
a
B
b
C
c
Copulatory piece length
Vaginal appendix length
Genital size (mm)
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
A
a
B
b
C
b
Kobe population (remote)
Suzuka population (contact)
Takihara population (contact)


Cumulative isolation index
1
0.8
0.6
0.4
0.2
0
A
Kobe male ×
Suzuka female
Suzuka male ×
Kobe female
B
Takihara male ×
Kobe female
Kobe male ×
Takihara female
C
Takihara male ×
Suzuka female
Suzuka male ×
Takihara female
1
2
3
4
5
Stage of isolation


A
Spermatophore formation success
1.0
0.8
0.6
0.4
0.2
0.0
B
Spermatophore deposition success at the proper site
1.0
0.8
0.6
0.4
0.2
0.0
-1.0
0.0
1.0
2.0
Genital size difference (CPL - VAL, mm)


1.5
1
0.5
0
-0.5
-1
-1.5
-2
-2.5
Axis 2 (14.36%)
-2
-1.5
-1
-0.5
0
0.5
1
1.5
2
2.5
Axis 1 (26.19%)
● Kobe population (remote)
● Suzuka population (contact)
● Takihara population (contact)