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ORIGINAL ARTICLE

Functional, genetic, and structural constraints on the exaggeration and diversification of male genital morphology in *Ohomopterus* ground beetles

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Abstract

The evolution of exaggerated sexual traits may be possible by the relaxation of various constraints on exaggeration. Functional constraints refer to the reduced performance of exaggerated traits *per se* or increased survival costs by holding the exaggerated traits. Genetic constraints, such as genetic correlations or pleiotropy, may hinder the independent evolution and exaggeration of traits. Structural constraints, such as competition for space and resources among traits, may require the coordination of the exaggerated trait with surrounding structures. The remarkable diversity of male genital morphology provides an ideal opportunity for examining constraints on sexual trait exaggeration. In this study, we addressed the constraints on the evolution of exaggerated male genital morphology based on a comparative analysis of phenotypic covariation between the genitalia and other body parts using *Ohomopterus* ground beetles. We found that exaggerated male genitalia is related to a relaxation of functional constraint, as revealed by a steeper allometric slope in the species with exaggerated male genitalia. By contrast, genetic constraint based on a shared genetic basis for the male genitalia and other appendages may have little effect on diversification in male genitalia. Structural constraints were strongest in the species with the most exaggerated male genitalia, suggesting that the observed constraint was a result of exaggeration. These findings improve our understanding of sexual trait exaggeration and underlying constraints.

Key words: allometry, *Carabus*, canonical correlation analysis, genitalia, sexual selection, sexual trait

INTRODUCTION

Male ornaments subjected to female choice and male weapons used in male–male combat are typical examples of exaggerated sexual traits (Darwin 1871; Andersson 1994; Shuster & Wade 2003; Emlen 2008). The evolution of exaggerated sexual traits may involve the relaxation of various constraints. Functional constraints affect the utility of exaggerated traits *per se* or decrease individual survival by holding the exaggerated traits. For example, exaggerated sexual traits may attract the attention of potential predators and hinder antipredator behavior (Jennions *et al*. 2001; Godin *et al*. 2003; Langerhans *et al*. 2005). Genetic constraints, such as genetic correlations or pleiotropy, may hinder the independent evolution and exaggeration of a trait (Hansen 2003). For example, a combination of pleiotropy and linkage mediates genetic regulatory variation in the development of male ornaments (comb and bone) in the chicken, implying that there are constraints on comb mass (Jhonsson *et al*. 2014). Structural constraints, such as competition for space and resources among traits, may necessitate coordination between the exaggerated trait and surrounding structures. For example, the production of horns reduces the size of neighboring morphological structures in beetles (Emlen 2001), suggesting that horn development is constrained by competition among structures in the head. These constraints can be detected by phenotypic covariation between traits (Armbruster *et al*. 2013). However, few studies have comprehensively investigated the effects of these constraints on the evolution of exaggerated sexual traits. Comparative analyses of related species with and without exaggerated sexual traits are expected to be useful to evaluate the tradeoff between constraints and exaggeration (e.g., Satomi *et al*. 2021, Fasanelli *et al*. 2022).

Male genital morphology diverges more rapidly than other phenotypic traits among groups of animals with internal fertilization (Eberhard 1985). Postcopulatory sexual selection via sperm competition, cryptic female choice, and sexual conflict predominantly underlie the evolution and diversification of genitalia in most taxa (Eberhard 1985, 2010; Hosken & Stockley 2004; Simmons 2014; Brennan & Prum, 2015; Langerhans *et al*. 2016; Sloan & Simmons 2019). Natural selection against hybridization (Kawano 2004, 2006; Kameda *et al*. 2009; Kawakami & Tatsuta 2010; Hollander *et al*. 2013; Kosuda *et al*. 2016; Nishimura *et al*. 2022) and in response to ecological variation (Langerhans *et al*. 2016) may also result in divergent genital morphologies between closely related species or populations. The remarkable diversity of male genital morphology, determined by multiple selective contexts as well as structural (Gack & Peschke 2005, Matsumura *et al*. 2013) and genetic (Aspiras *et al*.

2011, Genevcius *et al*. 2020, Hagen *et al*. 2020) factors, provides an ideal opportunity for examining constraints on sexual trait exaggeration.

Extreme variation in male genital morphology is likely to be stabilized due to functional constraints. Since male genital organs are usually stored within the male body, natural selection via predation may operate infrequently (cf. Langerhans *et al*. 2005). However, exaggerated male genitalia may have various fitness consequences; for example, it may result in mismatches with female copulatory organs (the one-size-fits-all hypothesis, Eberhard *et al*. 1998). Extreme-sized male genitalia may lead to a physical mismatch with the female genitalia, or may be subjected to cryptic female choice, possibly resulting in reduced fertilization success. Under this type of functional constraint, reduced variation in genital size and resultant weak scaling relationships between genital and body sizes (i.e., negative allometry) are expected (Eberhard *et al*. 1998). On the other hand, some organisms have acquired extremely exaggerated male copulatory organs as a result of sexually antagonistic coevolution. In a species of waterfowl, the male phallus is almost half a meter long (McCracken *et al*. 2001). In waterfowls, vaginal structures function as a barrier to phallus penetration, suggesting that directional selection for male phenotypes able to penetrate the female barrier is likely responsible for the exaggeration of the male genitalia (Brennan *et al*. 2007, 2015). Such directional selection is expected to result in increase in genital size variation by increaseing exaggerated phenotypes, leading to steep scaling relationships, as in male ornamental and weapon traits (Kodric-Brown *et al*. 2006). Thus, a change in the selective regime from stabilizing selection (or selection against exaggeration) to directional selection (or selection for exaggeration) may be seen as a relaxation of functional constraint against exaggeration.

Phenotypic variation in male genital morphology can be constrained, or modulated, by genetic and developmental factors. The male copulatory organs in insects are hypothesized to be homologous to appendages in other body segments, such as antennae, jaws, and legs, as evidenced by the regulatory roles of appendage-patterning genes in genitalia development (Aspiras *et al*. 2011, Macagno & Moczek 2015, Nomura et al. 2021). In addition, genetic correlations have been detected between the male genitalia and other body parts (Arnqvist & Thornhill 1998) and between the male and female genitalia (Simmons & Garcia-Gonzalez 2011). These genetic constraints are also expected to lead to significant phenotypic correlations between genitalia and other morphological traits. Exaggeration of external body parts may be limited by natural selection (e.g., by behavioral changes or predation), thereby indirectly constraining the exaggeration of internal organs, such as genitalia, via genetic correlation. Alternatively, appendages in insect body segments are morphologically so diverse among antennae, jaws, legs, and genitalia, and the genetic constraints on these appendages may have already been relaxed. If this is the case, no signs of constraints are expected to be detected. For example, the condition-dependent expression of shared genes may facilitate the exaggeration of genitalia, resulting in a decrease in phenotypic correlations and a relaxation of genetic constraint.

Structural constraints may limit the exaggeration of genital morphology. Males have to accommodate unwieldy structures, copulatory organs, in a limited space (i.e., the abdomen) and manipulate them acutely during copulation (Snodgrass 1935, Jałoszynski *et al*. 2014). Anatomical specializations and/or mechanisms to accommodate and efficiently manipulate the genitalia may be necessary prior to, or in concert with the evolution of exaggerated genitalia (Briceño *et al*. 2011; Eberhard 2005; Gack & Peschke 2005; Matsumura & Yoshizawa 2010, 2012). The flagellum of Criocerinae leaf beetles is an extremely elongated intromittent organ. These beetles have acquired a specialized structure to store the flagellum in the abdominal cavity, and this promotes effective insertion and retraction. The acquisition of the novel trait surrounding the intromittent organ may facilitate the evolution of highly elongated genitalia (Matsumura *et al*. 2014). The coevolution of genital parts and surrounding structures may result in covariation, indicating that the exaggeration of the genitalia requires the evolution of surrounding structures and is not free from structural constraints.

Ground beetles belonging to the subgenus *Ohomopterus* (Coleoptera, Carabidae, genus *Carabus*) have species-specific genitalia with evidence for coevolutionary divergence between the sexes (Ishikawa 1987, 1991, Sasabe *et al*. 2010, Fujisawa *et al*. 2019). Genital morphologies in this group are related to sexual selection (Takami & Sota 2007, Okuzaki & Sota 2014, Takami *et al*. 2018) and speciation (Sota & Kubota 1998, Fujisawa *et al*. 2019, Nishimura *et al*. 2022). The male has a sclerotized hook-like structure on the intromittent organ called the copulatory piece (CP, Fig. 1). The CP is a sclerotized projection in the wall of the endophallus (internal sac); it is everted from the aedeagus (AD) in copula (Fig. 1). The female has a membranous pocket attached to the ventral wall of the bursa copulatrix (BC) called the vaginal appendix (VA). In copula, the CP is inserted into the VA, and the male and female genitalia are rigidly coupled (Ishikawa 1987, Takami 2002). The CP plays roles in spermatophore deposition and sperm transfer (Takami 2003) as well as in the displacement of rival spermatophores (Takami 2007; Okuzaki & Sota 2014). A relatively long CP is more likely to manipulate fertilization and oviposition activity in females and to inflict fitness costs to the female, while a longer VA can mitigate these costs, consistent with sexually antagonistic coevolution of the CP and VA (Takami *et al*. 2018). Interspecific differences in the size of CP and VA are mediated by differences in developmental timing in males and in growth rate in females, respectively (Terada *et al*. 2021).

In *Ohomopterus*, *Carabus uenoi* has an extraordinarily enlarged CP, reaching a third of the male body length (Fig. 1). By contrast, the closely related species *C. maiyasanus* and *C. iwawakianus* have much smaller and broader hook-like CPs than those of *C. uenoi* (Fig. 1). This exaggeration and interspecific variation in genital morphology may be due to sexual selection via sperm competition and/or sexual conflict (Takami & Sota 2007, Takami *et al*. 2018) and natural selection for species isolation (Nishimura *et al*. 2022). Therefore, the genital exaggeration in *C. uenoi* is hypothesized to be a result of change in selective regimes, i.e., relaxation of functional constraint. The analysis of gene expression in the development of the CP and VA indicated that genes associated with appendage developement were enriched in a comparison between *C. uenoi* and other two species, suggesting contribution of appendage development genes in genital development and diversification (Nomura et al., 2021). Phylogenomic analysis revealed the relationships among the three species could be described as (*C. iwawakianus*, (C. *maiyasanus*, *C. uenoi*)), suggesting that the exaggeration of the male genitalia occurred after the divergence of *C. maiyasanus* and *C. uenoi* (Fig. 1, Fujisawa *et al*. 2019). These species are suitable materials for analyses of constraints and the exaggeration of the male genitalia.

In this study, we evaluated the constraints on the evolution of exaggerated male genital morphology based on a comparative analysis of phenotypic covariation between traits using *Ohomopterus* ground beetles. We compared correlation coefficients for sizes and genital parts and other body parts among species. Functional constraints due to mismatches with female genitalia were examined by comparing allometric scaling relationships. Genetic constraints were examined based on correlation coefficients between sizes of genital parts and other appendage-derived organs. Structural constraints were examined based on correlation coefficients between measurements of abdominal and genital parts. We discuss the roles of functional, genetic, and structural constraints on the exaggeration of male genitalia.

MATERIALS AND METHODS

Sampling

Adult males were collected in Mt. Kongo-san, Osaka, Japan (*C. uenoi*), Kobe, Hyogo, Japan (*C. maiyasanus*), and Suzuka, Mie, Japan (*C. iwawakianus*) during the reproductive season (early May to early July, 2012) using pitfall traps. Collected beetles were immediately transferred to the laboratory and fixed with ethyl acetate. Males were dissected to remove the aedeagus, CP, and other body parts (see below) for morphological measurements. The carcasses of beetles were pinned and dried.

Measurement and correlation structure

To quantify phenotypic variation and covariation, five body, two appendage, and four genital dimensions were measured. Body dimensions were the prothorax length (TL), prothorax width (TW), elytra length (EL), abdomen width (ABW), and abdomen depth (ABD, defined as the maximal distance from the top of elytra to the bottom of abdominal sternum) and were measured by digital calipers (0.01 mm increments). The appendage parameters were the 5th antennal segment length (ATL) and right hind tibia length (HTL). Genital dimensions were the aedeagus length (ADL), aedeagus width (ADW), CP length (CPL), and CP width (CPW). These parts were separated from the body and stuck on drafting paper, and the maximal length or width was measured on images obtained by a digital camera attached to a microscope (Leica EZ4HD) using ImageJ to the nearest 0.01 mm. To minimize measurement error, each part was measured five times (10 times for relatively small parts, ATL and CPW), and the average value was used for analyses. These measurements were highly repeatable ($R^2 > 0.98$), except for measurements of ATL and HTL in *C. maiyasanus* ($R^2 = 0.60$ and 0.74, respectively) and CPW in *C. uenoi* ($R^2 = 0.89$). A total of 30 males of *C. uenoi*, 30 males of *C. maiyasanus*, and 30 males of *C. iwawakianus* were measured.

To capture and compare overall correlation structures across the traits among species, pairwise Pearson's correlation coefficients (r) and P-values were calculated across five body size, two appendage size, and four genital size parameters for *C. iwawakianus*, *C. maiyasanus,* and *C. uenoi*. The correlation matrix for each species included 55 off-diagonal elements, consisting of 21 correlation coefficients between external (body and appendage) parts, 6 between genital parts, and 28 between external and genital parts. Differences in correlation coefficients among species were evaluated using the package *psych* (Revelle 2022) in R ver. 4.1.2. False discovery rates for multiple comparisons (three comparisons among species) were corrected using the Benjamini and Hochberg (1995) method.

Functional constraint

The one-size-fits-all hypothesis, which proposes that exaggeration in the male genitalia is constrained by the morphological fit with the typical female genitalia, predicts negative allometry of male genital sizes (Eberhard *et al.* 1998). However, relatively steep allometric slopes are also predicted in species with exaggerated male genitalia as a result of the relaxation of functional constraint. To examine this, allometric slopes of four genital dimensions (ADL, ADW, CPL, and CPW) were evaluated in the three species by ordinary linear regression and major axis regression based on $\log_{10}$-transformed values. Differences in allometric slopes among species were tested by general linear models based on the pooled dataset across the three species, in which genital size was used as the dependent variable, and the proxy of body size, species, and their interaction term were included as independent variables. A significant interaction indicated a difference in allometric slopes among species.

C. maiyasanus and *C. iwawakianus* have similar body lengths, while *C. uenoi* is slightly larger (distance from the anterior margin of the labrum to the apices of the elytra in male, mean ± s.d.; *C. maiyasanus*: 23.01 ± 0.18 mm [N = 51], *C. iwawakianus*: 23.31 ± 0.74 mm [N = 82], *C. uenoi*: 26.34 ± 0.47 mm [N = 30]) (Takami & Sota 2007). Unlike other ground beetle species, *C. uenoi* males are longer than females (Sota *et al.* 2000). This interspecific variation in the sex difference in body lengths could be due to the elongation of the abdomen in *C. uenoi* as a direct consequence of the exaggeration of the male genitalia. Thus, rather than body length (including abdomen length), TL was used as a proxy of body size in the analysis of allometry.

Genetic constraint

The male genitalia of beetles (the median lobe and parameres) are serially homologous to other appendages, which are commonly regulated by appendage-patterning genes in development (Macagno & Moczek 2015, Nomura et al. 2021). The median lobe corresponds to the aedeagus in *Ohomopterus* species. If trait exaggeration was enabled by the relaxation of genetic and developmental constraints based on a shared morphogenetic basis across appendages, the sizes of male genitals and other appendages are expected to show weaker covariances in species with more exaggerated male genitalia.

Pairwise correlation coefficients between genital (ADL, ADW, CPL and CPW) and appendage (ATL and HTL) sizes were compared among species using the *psych* package in R. Additionally, the canonical correlation coefficient (CCC) between the set of genital sizes and the set of appendage sizes in each species was calculated using the *CCA* package in R. CCC represent maximal correlation between two sets of variables, so that we can evaluate covariation between two body parts. The greater CCC indicates the stronger covariation. For comparisons among species, the 95% confidence interval of the CCC was calculated by bootstrapping using the *boot* package in R with 1000 replicates.

Structural constraint

If a body part is stored within an outer part, the exaggeration of the inner part is expected to be constrained by the size of the outer part. The male genitalia of *Ohomopterus* ground beetles exhibit this type of nested structure: the CP is stored within the AD, and the AD including the CP is stored within the abdomen (Fig. 1). This type of structural constraint predicts that the exaggeration of inner parts requires modification (i.e., enlargement) of the outer parts, resulting in size covariation between inner and outer parts.

Correlations between sizes of inner and outer parts were evaluated. ABW and ABD were used as indicators of abdominal size. EL was also used as a measure of the length of the abdomen because the elytra entirely covers the dorsal surface of the functional abdomen in beetles (including meso- and metathoraces). AD size was measured by ADL and ADW, and the size of the innermost part, CP, was determined by the CPL and CPW. Four types of nested relationships were evaluated: abdomen vs. AD and CP, abdomen vs. AD, abdomen vs. CP, and AD vs. CP. Pairwise correlation coefficients were compared. Additionally, the CCC between the sizes of the outer and inner structures and 95% confidence intervals were calculated as explained above and compared between the three species.

RESULTS

Correlation structure

The overall correlation structure among traits was similar in *C. iwawakianus* and *C. maiyasanus*; however, it differed between these two species and *C. uenoi,* especially for correlations involving genital traits (Table 1). Relatively strong and significant correlations were detected between body dimensions in *C. iwawakianus* and *C.*

maiyasanus; however, these correlations tended to be weak for *C. uenoi.* Among genital dimensions, ADL was moderately correlated with body dimensions in the three species; however, it was not significantly correlated with other genital dimensions in *C. iwawakianus* and *C. maiyasanus*. ADW, CPL, and CPW were only weakly correlated with each other in *C. iwawakianus* and *C. maiyasanus*. By contrast, we detected relatively strong correlations between CPL and other body and genital dimensions in *C. uenoi*. The correlation coefficients between CPL and TL were significantly greater in *C. uenoi* than in *C. iwawakianus* ($P = 0.015$) and in *C. maiyasanus* ($P < 0.001$), even after correction of false discovery rate ($P < 0.05$, Table 1). The correlation coefficients between ABD and EL and between HTL and EL were significantly lower in *C. uenoi* than in *C. maiyasanus* ($P = 0.030$ in both cases); however, significance was not retained after controlling for the false discovery rate ($P > 0.05$, Table 1). Parameter values are summarized in Table S1.

Functional constraint

The allometric slopes of ADL showed significant departures from zero in all three species, as revealed by OLS regression analyses (Table 2). The allometric slope of CPL was significantly non-zero only in *C. uenoi*. The remaining two genital dimensions (ADW and CPW) revealed non-significant slopes. All four significant allometric slopes were less than 1.0 in MA regression analyses, indicating negative allometry. However, the 95% confidence intervals of these MA slopes included 1.0 (Table 2).

Allometric slopes of CPL differed significantly among species (GLM, interaction between body size and species, $P = 0.0354$). *C. uenoi* with the most exaggerated male genitalia showed the steepest OLS slope, as predicted by the hypothesis that the relaxation of functional constraint facilitates exaggeration. There were no significant differences in allometric slopes in other three genital dimensions ($P > 0.14$).

Genetic constraint

Pairwise correlation coefficients between genital (ADL, ADW, CPL, and CPW) and other appendage (ATL and HTL) sizes were not significantly different from zero at the 5% level in the three species, except for those between ADL and ATL in the three species and that between ADL and HTL in *C. maiyasanus* (Table 1).

Inconsistent with the hypothesis that exaggeration in male genitalia was facilitated by release from the genetic constraint based on a common morphogenetic basis across appendages, correlations between genital and other appendage sizes tended to be weaker in species with the least exaggerated male genitalia. Although pairwise correlation coefficients between sizes of genitalia and other appendages did not differ between species (Table 1), the CCC between genital sizes and appendage sizes was significantly smaller in *C. iwawakianus*, and the CCCs of *C. maiyasanus* and *C. uenoi* were not within the 95% confidence intervals of the CCC for *C. iwawakianus* (Fig. 2).

Structural constraint

In *C. iwawakianus*, we detected significant pairwise correlation coefficients between inner genital parts and outer parts for ADL vs. three abdominal dimensions and for ADW vs. EL and ABD but not for CP sizes vs. others (Table 1). In *C. maiyasanus*, correlations were significant only for ADL vs. EL and were marginally nonsignificant for ADL vs. ABD and ABW (Table 1). In *C. uenoi*, correlations were significant in ADW vs. EL and in CPL vs. EL, ABW, ABD, and ADL and were marginally nonsignificant in ADL vs. EL and ABW (Table 1).

As opposed to the hypothesis that the exaggeration of the inner genital part is facilitated by a relaxation of structural constraint related to the size of the outer part, the inner genital parts were most strongly constrained by the outer parts in *C. uenoi* with the most exaggerated male genitalia (Fig. 3). Although there were no significant differences in pairwise correlation coefficients between abdominal and genital dimensions across species (Table 1), the CCCs between the AD and CP were significantly greater in *C. uenoi* than in other two species; the estimated CCC for *C. uenoi* fell outside of the 95% CI for the other species (Fig. 3). The CCCs between the abdomen and CP were also significantly greater in *C. uenoi* than in other two species for comparisons in both directions (Fig. 3). The CCCs between the abdomen and four dimensions of AD and CP were greater in *C. uenoi* than in *C. iwawakianus* in one direction (Fig. 3). The CCCs between the abdomen and AD measurements did not differ among species (Fig. 3).

DISCUSSION

We hypothesized that the exaggeration of sexual traits is possible by the relaxation of various constraints. To test this hypothesis, we examined three types of constraints: functional, genetic, and structural. We analyzed correlations between body and genital dimensions and compared these correlations among closely related *Ohomopterus* ground beetle species (*C. iwawakianus*, *C. maiyasanus,* and *C. uenoi*) with variation in the degree of exaggeration in male genital morphology. Our results indicated that the relaxation of functional constraints (but not the two other constraint types) may be responsible for the exaggeration of male genital morphology. Below, we discuss the effects of each type of constraint on the exaggeration of male genitalia in detail.

The one-size-fits-all hypothesis predicts that stabilizing selection favors intermediate male genital sizes and negative allometric scaling (Eberhard et al. 1998). Although the possibility of isometry was not statistically excluded, all four significant allometric slopes (three for ADL and one for CPL in *C. uenoi*) for genital dimensions tended to be negative, providing support for the one-size-fits-all-hypothesis. Interestingly, most genital dimensions other than above four were not significantly related to body size (Tables 1 and 2), suggesting that genital sizes vary almost independently from body sizes, especially in species with less exaggerated genitalia. For CPL, allometric slopes varied significantly among species, and the steepest OLS slope was detected in *C. uenoi* with the most exaggerated male genitalia. This result indicates that selection against relatively larger genitalia is weaker in *C. uenoi* than in the other two species, suggesting that the function of the exaggerated male CP in *C. uenoi* is relatively weakly constrained. Weak correlations observed in species with relatively small genital parts (*C. iwawakianus* and *C. maiyasanus*) could be explained by measurement errors given the small genital sizes; however, we obtained high repeatability in genital sizes for these species, suggesting that the effect of measurement errors was limited. In *C. insulicola*, a species in *Ohomopterus* ground beetles including the three species in question, sexual conflict between male manipulation of female oviposition activity and female resistance against male manipulation generates selection for a longer male CP and female VA (Takami *et al*. 2018). Secondary contact between closely related species, including *C. iwawakianus*, *C. maiyasanus* and *C. uenoi*, may also generate reinforcing selection favoring a longer CP (Nishimura *et al*. 2022). Note that the longer VA may also be favored in these two processes, as a counter adaptation in sexual conflict and for species isolation in reinforcement, thereby coevolution between the sexes may be promoted. These processes favoring a longer CP may facilitate release from functional constraints via stabilizing selection and drive the evolution of exaggerated male genitalia.

Since the appendages of the body segments of insects, including male genitalia, share a common genetic basis, we hypothesized that the exaggeration of male genitalia was constrained by genetic correlations among appendages across body segments. However, our results did not support this hypothesis; the pairwise correlation coefficients between genital and other appendage sizes were mostly non-significant and did not differ between species. Additionally, comparison of the CCCs between genital and other appendage sizes revealed that *C. iwawakianus* with the least exaggerated male genitalia was most weakly constrained among the species. Therefore, exaggerated male genitalia in *C. uenoi* cannot be explained by relaxed genetic and developmental constraints. As discussed above, weak correlations in *C. iwawakianus* also cannot be explained by measurement errors.

Nested configurations of body and genital parts (i.e., outer and inner parts) are often found in male insect genitalia, such as the aedeagus and its associated internal sac (Tuxen 1970), where exaggeration in the inner part may be constrained by the size of outer part (i.e., structural constraint). The CCC between the sizes of the outer and inner genital parts was greater in *C. uenoi* than in the other two species, suggesting that structural constraints on genital parts were strongest in the species with the most exaggerated male genitalia. This result was not consistent with the hypothesis that a relaxation of structural constraint facilitates the exaggeration of the male genitalia. In other words, the male genitalia of *C. iwawakianus* and *C. maiyasanus* are not exaggerated even under weak structural constraints. Interestingly, although males are generally smaller than females in ground beetles, including *Ohomopterus* species, males of *C. uenoi* are slightly larger than females (Sota *et al*. 2000). This may be a necessary compensation of the exaggeration of male genitalia in *C. uenoi*.

Collectively, the exaggeration of the male genitalia of *C. uenoi* may be facilitated by relaxed functional constraint, in which selective regimes may change from stabilizing selection to directional selection favoring a longer CP. However, this was not the case for AD. Genetic and developmental constraints based on a common genetic architecture across appendages may have little effects on the exaggeration and diversification of male genitalia in *Ohomopterus*. Constraints by the outer structures are also unlikely to limit exaggeration of the male genitalia. Alternatively, sexual selection and/or reinforcing natural selection may have led to the exaggeration of the CP of *C. uenoi*, until it reached the spatial capacity of the outer parts and became constrained. Our findings provide insight into sexual trait exaggeration and its underlying constraints.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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Table 1. Pearson's correlation coefficients (r) (upper triangle) and p-values (lower triangle, not corrected for false discovery rate) across five body, two appendage, and four genital dimensions of Carabus iwawakianus, C. maiyasanus, and C. uenoi. Black, dark grey, light grey, and white cells indicate 0.7 < *r*, 0.5 < *r* < 0.7, 0.3 < *r* < 0.5, and *r* < 0.3, respectively. For comparisons among species, different letters indicate significant differences between species after correction for the false discovery rate (*P* < 0.05).

C. iwawakianus	TL	TW	EL	ABW	ABD	ATL	HTL	ADL	ADW	CPL	CPW
TL		**0.808**	**0.788**	**0.647**	**0.765**	**0.498**	0.236	**0.465**	0.118	0.165^{a}	-0.002
TW	<.0001		**0.808**	**0.745**	**0.791**	**0.610**	**0.513**	**0.549**	0.249	0.168	-0.003
EL	<.0001	<.0001		**0.668**	**0.859**	**0.677**	**0.493**	**0.459**	**0.367**	0.019	0.176
ABW	<.0001	<.0001	<.0001		**0.702**	**0.492**	**0.310**	**0.615**	0.231	0.279	0.153
ABD	0.000	<.0001	<.0001	<.0001		**0.592**	**0.422**	**0.429**	**0.370**	0.224	0.077
ATL	0.005	0.000	<.0001	0.006	0.001		**0.584**	**0.405**	0.169	-0.019	0.162
HTL	0.209	0.004	0.006	0.095	0.020	0.001		0.286	0.174	0.114	-0.074
ADL	0.010	0.002	0.011	0.000	0.018	0.026	0.125		0.147	0.181	0.176
ADW	0.533	0.186	0.046	0.219	0.044	0.372	0.359	0.438		0.150	0.224
CPL	0.383	0.375	0.922	0.135	0.234	0.921	0.549	0.338	0.430		-0.055
CPW	0.991	0.987	0.353	0.421	0.687	0.393	0.699	0.354	0.235	0.772	
C. maiyasanus	TL	TW	EL	ABW	ABD	ATL	HTL	ADL	ADW	CPL	CPW
TL		**0.804**	**0.701**	**0.616**	**0.793**	**0.659**	**0.506**	**0.454**	**0.334**	0.024^{a}	0.004
TW	<.0001		**0.731**	**0.647**	**0.687**	**0.590**	**0.409**	**0.430**	**0.373**	0.223	-0.063
EL	<.0001	<.0001		**0.770**	**0.682**	**0.876**	**0.647**	**0.574**	0.236	0.193	-0.035
ABW	0.000	0.000	<.0001		**0.704**	**0.702**	**0.445**	**0.324**	0.165	0.229	0.096

ABD	<.0001	<.0001	<.0001	<.0001		**0.682**	**0.437**	**0.350**	0.070	0.246	0.003
ATL	<.0001	0.001	<.0001	<.0001	<.0001		**0.661**	**0.539**	0.181	0.131	0.116
HTL	0.004	0.025	0.000	0.014	0.016	<.0001		**0.428**	0.130	0.206	-0.056
ADL	0.012	0.018	0.001	0.081	0.058	0.002	0.018		-0.008	0.284	0.081
ADW	0.072	0.043	0.209	0.385	0.712	0.339	0.493	0.967		0.236	-0.006
CPL	0.901	0.237	0.308	0.223	0.190	0.489	0.276	0.128	0.734		-0.409
CPW	0.985	0.741	0.853	0.615	0.987	0.540	0.768	0.670	0.977	0.025	
C. uenoi	TL	TW	EL	ABW	ABD	ATL	HTL	ADL	ADW	CPL	CPW
TL		**0.621**	**0.566**	**0.471**	**0.480**	**0.540**	0.292	**0.608**	0.208	**0.696**[b]	-0.061
TW	0.000		**0.660**	**0.582**	**0.751**	**0.521**	0.244	**0.348**	0.108	**0.632**	-0.101
EL	0.001	<.0001		**0.322**	**0.722**	**0.581**	**0.436**	**0.350**	0.022	**0.417**	-0.114
ABW	0.009	0.001	0.082		**0.451**	0.269	0.093	**0.361**	**0.497**	**0.594**	0.074
ABD	0.001	<.0001	<.0001	0.012		**0.507**	0.229	0.296	-0.047	**0.487**	-0.066
ATL	0.002	0.003	0.001	0.152	0.004		**0.595**	**0.464**	-0.079	**0.356**	-0.006
HTL	0.117	0.194	0.016	0.624	0.224	0.001		0.056	0.036	0.217	-0.269
ADL	0.000	0.060	0.058	0.050	0.113	0.010	0.771		0.177	**0.501**	0.218
ADW	0.571	0.908	0.005	0.804	0.679	0.851	0.360	<.0001		0.254	0.092
CPL	<.0001	0.000	0.022	0.001	0.006	0.054	0.249	0.005	0.704		0.114
CPW	0.748	0.594	0.551	0.698	0.728	0.976	0.150	0.247	0.629	0.550	

TL, prothorax length, TW, prothorax width, EL, elytra length, ABW, abdomen width, ABD, abdomen depth, ATL, 5th antennal segment length, HTL, right hind tibia length, ADL, aedeagus length, ADW, aedeagus width, CPL, copulatory piece length, CPW, copulatory piece width.

Table 2. Allometric slopes of four genital dimensions in the three species. Estimates based on ordinary least squares (OLS) and major axis (MA) regressions are shown. Confidence intervals for the MA slope are indicated only when the slope of the scaling relationship determind by OLS regression is significant.

Species	Trait	Allometric slope (OLS)	*P*	Allometric slope (MA)	95% Confidence interval
C. iwawakianus	ADL	0.342	0.0079	0.720	0.293/1.771
	ADW	0.155	0.58	1.470	
	CPL	0.224	0.41	1.436	
	CPW	-0.004	0.99	-1.402	
C. maiyasanus	ADL	0.178	0.012	0.392	0.144/1.068
	ADW	0.360	0.074	1.090	
	CPL	0.018	0.89	0.697	
	CPW	0.034	0.93	2.103	
C. uenoi	ADL	0.415	0.0003	0.673	0.392/1.158
	ADW	0.299	0.24	1.360	
	CPL	0.687	<.0001	0.989	0.647/1.512
	CPW	-0.123	0.83	-2.937	

ADL, aedeagus length, ADW, aedeagus width, CPL, copulatory piece length, CPW, copulatory piece width

Table S1. Summary of all measurements.

C. iwawakianus	TL	TW	EL	ABW	ABD	ATL	HTL	ADL	ADW	CPL	CPW
Average	4.94	6.48	15.76	6.45	8.88	7.73	6.41	7.56	1.35	1.31	0.73
Standard deviation	0.18	0.30	0.45	0.20	0.36	0.29	0.26	0.20	0.07	0.07	0.04
C. maiyasanus	TL	TW	EL	ABW	ABD	ATL	HTL	ADL	ADW	CPL	CPW
Average	4.68	6.05	15.47	6.27	8.48	7.49	6.10	7.29	1.37	2.31	0.32
Standard deviation	0.23	0.32	0.54	0.27	0.38	0.31	0.44	0.14	0.07	0.08	0.03
C. uenoi	TL	TW	EL	ABW	ABD	ATL	HTL	ADL	ADW	CPL	CPW
Average	5.36	7.09	17.97	7.19	10.00	8.72	6.79	9.57	2.25	8.53	0.64
Standard deviation	0.18	0.23	0.40	0.22	0.30	0.29	0.20	0.21	0.10	0.27	0.06

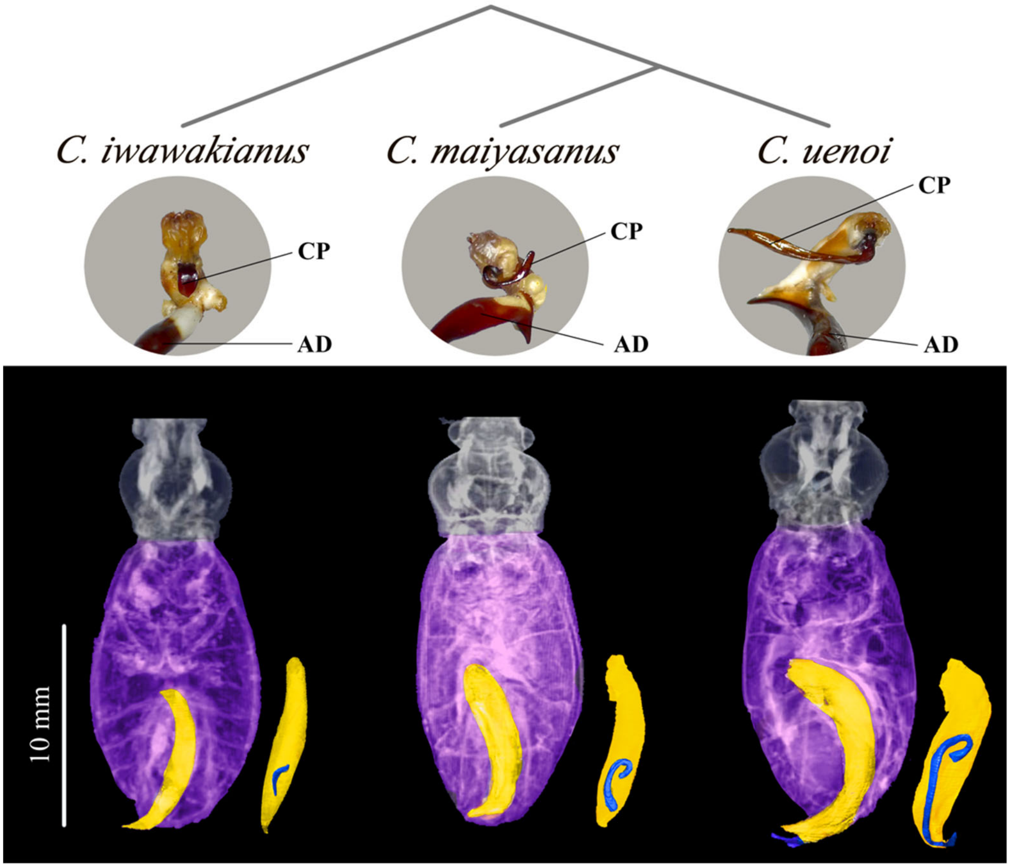

Fig. 1. Phylogenetic relationships among *Carabus iwawakianus, C. maiyasanus,* and *C. uenoi* and configurations of body and genital parts. Male genitalia are shown in circles; the endophallus and copulatory piece (CP) are everted from the aedeagus (AD). Nested configuration consisting of the functional abdomen including the meso- and metathoraces (purple), aedeagus (yellow), and copulatory piece (blue) are depicted in dorsal view based on µCT scan.

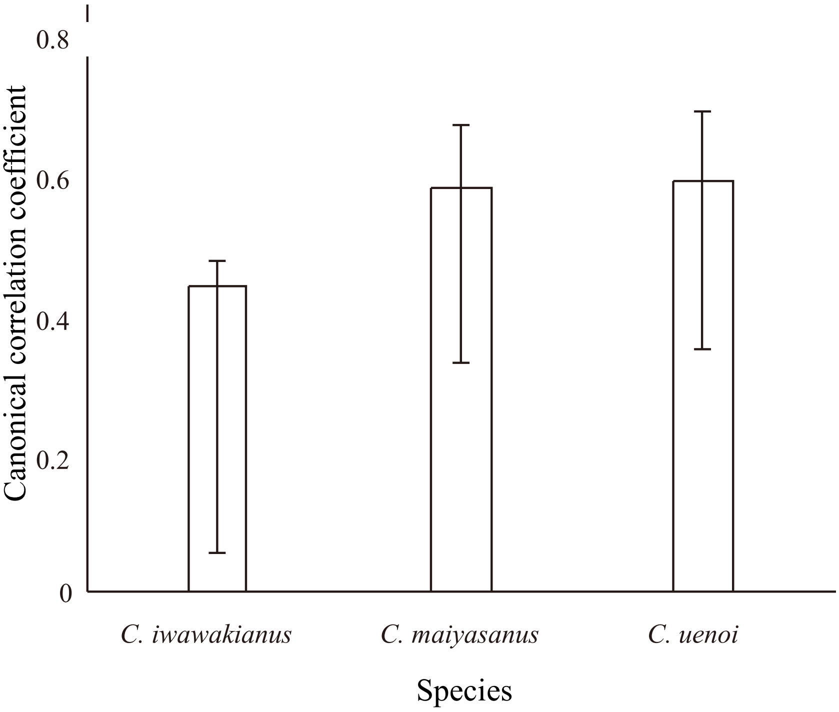

Fig. 2. Canonical correlation coefficients between genital and appendage sizes and 95% confidence intervals in *C. iwawakianus*, *C. maiyasanus,* and *C. uenoi*.

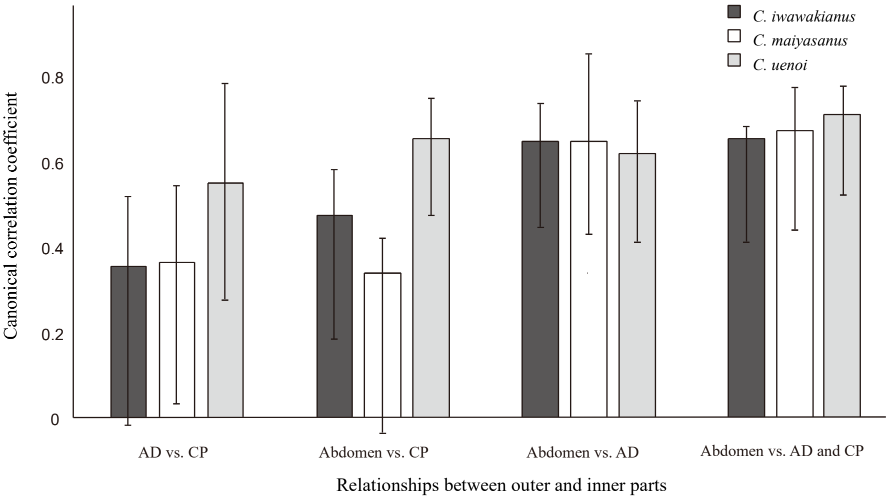

Fig. 3. Canonical correlation coefficients between the sizes of the inner genital parts and outer parts and 95% confidence intervals in *C. iwawakianus*, *C. maiyasanus,* and *C. uenoi*. AD, aedeagus; CP, copulatory piece.