Original Article

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Body size evolution under character release in the ground beetle *Carabus japonicus*

Yutaka Okuzaki[1], Hisashi Sugawara[2] and Teiji Sota[2]

[1]*Field Science Center for Northern Biosphere, Hokkaido University, Kita, Sapporo, 060-0811, Japan,* [2]*Department of Zoology, Graduate School of Science, Kyoto University, Sakyo, Kyoto, 606-8502, Japan*

Correspondence: Y. Okuzaki, Field Science Center for Northen Biosphere, Hokkaido University, Kita, Sapporo, 060-0809, Japan.

E-mail: yutaka@fsc.hokudai.ac.jp

ABSTRACT

Aim We tested the hypothesis of character release in body size among allopatric populations of the carabid beetle *Carabus japonicus* by analysing geographical variation in body size in relation to habitat temperature and sympatry/allopatry with the larger congeneric species *Carabus dehaanii*.

Location The main and satellite islands of Kyushu in the south-western part of the Japanese archipelago.

Methods We studied geographical variation in body length and genital size of *C. japonicus* populations at different sites to examine the effects of both habitat temperature and sympatry/allopatry with *C. dehaanii*. To determine whether the conditions for character release were fulfilled, we then estimated heritable differences in body size by common garden rearing, and estimated phylogenetic relationships among populations by molecular phylogenetic analysis.

Results While body size was positively correlated with annual mean temperature, it was consistently small in sympatry but larger in some allopatric populations in warmer regions. The body size differences among populations were heritable. Allopatric *C. japonicus* populations on satellite islands were derived from the sympatric mainland populations. In a few sympatric areas, mitochondrial haplotypes were shared between the species because of introgressive hybridization, suggesting the occurrence of reproductive interference between the species. We also found that genital size was not affected by sympatry/allopatry but was positively correlated with body size.

Main conclusions We demonstrated that the increased body size of allopatric *C. japonicus* has evolved through a character release process. However, not all allopatric populations had enlarged body size, suggesting that additional environmental factors are

also involved. Geographical differentiation in body size and associated genital dimensions may result in prezygotic reproductive isolation among populations and promote allopatric differentiation leading to speciation.

Keywords

Character displacement, converse Bergmann's rule, genital evolution, introgressive hybridization, Japan, molecular phylogenetic analyses, reproductive interference.

INTRODUCTION

Character displacement and character release, originally described by Brown & Wilson (1956), are evolutionary processes by which the phenotypic state of a species changes under natural selection as a result of the presence or absence of one or more ecologically and/or reproductively similar species (Grant, 1972). The occurrence of morphological character displacement has been convincingly demonstrated in some cases (e.g., Schluter & McPhail, 1992; Radtkey *et al.*, 1997; Pfennig & Murphy, 2000; Taylor & McPhail, 2000; Grant & Grant, 2006; Rice & Pfennig, 2008), but there are few convincing cases of morphological character shifts under character release (Grant, 1972; Simberloff *et al.*, 2000; Meiri *et al.*, 2007; Meiri *et al.*, 2011). This may be because a character state, in the absence of interacting species, can vary among populations owing to environmental differences (Robinson *et al*., 2000), and because the same character state can be equally adaptive in both sympatric and allopatric habitats. Therefore, to understand character evolution under character release, it is important to examine the effects of environmental factors other than the presence/absence of interacting species on the character state in question (Meiri *et al.*, 2011).

Body size is a key adaptive trait that has often been examined in studies of character displacement and character release in animals (Dayan & Simberloff, 1998; Schluter, 2000; Rice & Pfennig, 2007; Grether, *et al.*, 2009; Pfennig & Pfennig, 2009). However, geographical variation in animal body size is affected by multiple factors, including climatic factors that cause clinal variation according to Bergmann's rule or its converse (Blanckenhorn & Demont, 2004). Therefore, it is essential to take into account the effects of climate on body size evolution as well as the presence/absence of interacting species when studying character displacement and release.

To examine whether the evolutionary consequences of character release can be variable, we studied geographical body size variation in the carabid beetle *Carabus japonicus* Motschulsky, 1857, a species in the subgenus *Ohomopterus*, which is endemic to the Japanese islands. This species occurs widely in western Japan and is sympatric with the larger *Carabus dehaanii* Chaudoir, 1848 in most of its range, although it occurs singly in some coastal areas and on small islands adjacent to Kyushu, the southernmost main island of Japan (hereafter we treat Kyushu as the mainland). The body size of *Ohomopterus* species is positively correlated with habitat temperature, exhibiting a converse Bergmann cline (Sota *et al*., 2000). However, *C. japonicus* is much smaller than *C. dehaanii* in sympatry, and on an island where *C. japonicus* occurs singly, it is much larger than expected from temperature conditions alone, suggesting that character release is taking place (Sota *et al.*, 2000). According to the most reliable molecular phylogeny, the two species are not sister species (Takahashi *et al.*, 2014), and although they do not now form hybrid zones, they do share mitochondrial gene sequences extensively, probably because of recent introgressive hybridization (Sota & Nagata, 2008). Thus, these species have undergone reproductive interference—maladaptive interspecific interactions during the process of mate acquisition (Gröning & Hochkirch 2008)—and the body size enlargement in allopatric *C. japonicus* is hypothesized to be a case of character release in the absence of reproductive interference with *C. dehaanii*.

We examine the patterns and causes of geographical variation in the body size of *C. japonicus* to determine whether character release has occurred in this species. First, we demonstrate that the effect of sympatry/allopatry on phenotypic values is significant after controlling for annual mean temperature, which is a major abiotic influence on body size variation in *Ohomopterus* (Sota *et al.* 2000). Second, we demonstrate that the

differences in phenotypic values between sympatric and allopatric populations are heritable, by performing a common garden experiment in the laboratory. Third, we demonstrate that the allopatric populations exhibiting character shift are derived from the sympatric populations, by performing phylogeographical analyses using mitochondrial and nuclear gene sequences. These molecular markers also suggest the occurrence of introgressive hybridization (Nagata *et al.*, 2007; Sota & Nagata, 2008). Although we are primarily concerned with body size (whole body length) as it affects precopulatory reproductive isolation, we also analysed variation in genital dimensions because they can contribute to prezygotic reproductive isolation in *Ohomopterus* (Nagata *et al.*, 2007; Kubota *et al.*, 2013).

MATERIALS AND METHODS

Sampling and measurement of morphology

The endemic ground beetles of the subgenus *Ohomopterus* in Japan (Coleoptera, Carabidae, genus *Carabus*) comprise 15 or more species and show marked variation in body size and genital morphology (Sota *et al.*, 2000; Sota & Nagata, 2008). These species are ecologically equivalent, having the same diet and seasonal life cycle (i.e., larvae are specialized earthworm eaters and spring breeders with a univoltine life cycle; Sota, 1985a). To study body size variation, adult beetles of *C. japonicus* and *C. dehaanii* were collected using pitfall traps on the Kyushu mainland and adjacent islands from 2009 to 2012 (Fig. 1). *Carabus japonicus* were collected at 53 sites shown in Fig. 1 (see Appendix S1 in Supporting Information for details). Of these sites, we defined 34 and 19 sites as sympatric and allopatric sites, respectively, based on the distribution of *C. dehaanii* described in Imura & Mizusawa (2013), although we were unable to locate *C. dehaanii*

in some presumed sympatric sites. The collected beetles were killed by ethyl acetate, and the gonads (testes or ovaries) were extracted and preserved in absolute ethanol at –30°C until DNA extraction. The bodies were stored as dry specimens for measurement of body length.

We defined body length as the distance from the front margin of the labrum to the apical part of the elytra, measured using a digital calliper to 0.01 mm. We also measured the lengths of the aedeagus and copulatory piece in males and the vagina and vaginal appendix in females using stereomicroscopes (Appendix S2). The copulatory piece is a hook-like chitinized piece on the endophallus that is stored in the aedeagus and inserted into the vaginal appendix, a pocket attached to the vagina, to secure genital coupling during copulation (Ishikawa, 1987; Takami, 2002).

Statistical analysis of variation in body and genital size

Using the statistical package JMP version 11 (SAS Institute Inc., Cary, NC), we conducted a generalized linear model (GLM) analysis with a normal distribution and identity link function to study variation in body and genital dimensions (not transformed values) at localities where one or more individuals of both sexes were collected. For *C. japonicus*, GLM was conducted for the effects of sex, sympatry/allopatry with *C. dehaanii*, and annual mean temperature on mean body length at each site. For *C. dehaanii*, GLM was conducted for the effects of sex and annual mean temperature on mean body length. We did not consider the effect of sympatry/allopatry with *C. japonicus* because *C. dehaanii* is always sympatric with *C. japonicus* except on a few small islands (Imura & Mizusawa, 2013) that we did not sample. The annual mean temperature of each sampling site was obtained from 1 km mesh climatic data for the Japanese archipelago based on

meteorological data collected from 1971 to 2000 (Mesh Climatic Data 2000; Japan Meteorological Business Support Center). For *C. japonicus*, GLM was also conducted for the effects of male body length and sympatry/allopatry with *C. dehaanii* on the mean lengths of the aedeagus and the copulatory piece, and for the effects of female body length and sympatry/allopatry with *C. dehaanii* on the mean length of the vagina and the vaginal appendix. The correlation between body length and genital dimensions was also calculated in each sex of both species. To account for the different sample sizes, the response variables (body and genital dimensions) were weighted by the number of samples at each site.

Common garden experiment in the laboratory

To determine the extent to which the geographical variation in body size in *C. japonicus* is heritable, we conducted a common garden experiment by bringing field-caught beetles into the laboratory and raising their offspring from eggs to adults during 2010–2012. We assumed that populations geographically and genetically proximate to one another are appropriate to detect genetic differences in body size. We used beetles from four sites in northern Kyushu representing a wide range of body sizes: site 6 (Mt. Sefuri, altitude 950–1030 m, annual mean temperature 10.2°C); site 7 (Mt. Abura, 550–597 m, 13.0°C); site 5 (Mt. Ishidaka, 250–284 m, 14.2°C); and site 3 (Kabe Island, 20–90 m, 15.5°C) (see Fig. 1 for site numbers). *Carabus japonicus* was sympatric with *C. dehaanii* at sites 6 and 7, and allopatric at sites 5 and 3.

Parental adult beetles used in the experiment were collected using pitfall traps and were transported to our laboratory at Kyoto University within 3 days. The beetles were housed individually in plastic cups (9 cm diameter, 4 cm depth) with moistened moss,

and the cups were stored in an incubator set at a long-day photoperiod [light:dark (LD) h, 16:8] and at 20°C from the time of their capture until November. The beetles were fed with minced beef every 2 days. Thereafter, temperature and day length were gradually decreased toward an overwintering condition of 5°C and complete darkness from December through April. In May, the photoperiod, temperature, and feeding regimes were gradually restored to their initial states over the course of the month. Subsequently, pairs of sexually mature males and females from the same site were transferred to plastic cups (12 cm diameter, 5 cm depth) with humic soil and moistened moss and were allowed to copulate and deposit eggs in the humic soil.

The common garden experiment was performed in an incubator at LD 16:8 and at a temperature (20°C) close to the monthly mean temperature in July (20.8°C) at site 6 where *C. japonicus* larvae occurred (Y.O., unpublished data). The eggs deposited in the soil were collected and kept in plastic cups with humic soil until hatching. Hatched larvae were reared individually in plastic cups (7.5 cm diameter, 3.5 cm depth) with humic soil and were supplied with a sufficient number of earthworms. The larvae had three instars and pupated in the soil. After eclosion, adults appeared on the soil surface after several days. The development of each individual was observed once a day from first instar through emerging adult, and its body weight was measured within 1 day after hatching (first instar), after moulting (second and third instar), and after appearing on the soil surface as an adult. We defined the development time as the number of days from oviposition to the appearance of the adult. The body length of adults was measured approximately 30 days after emergence when their exoskeletons hardened.

The effect of parental body length on the weight of first instar larvae and on adult body length of offspring was examined by generalized linear mixed model (GLMM)

analysis using JMP version 11. Maternal and paternal body lengths were separately considered as explanatory variables to distinguish the case in which maternal effects predominantly determine offspring body length (Falconer, 1989; Mousseau & Fox, 1998). We also considered offspring sex an explanatory variable and parental pair a random variable. We used body weight of first instar larva as a proxy for egg weight because eggs are extremely fragile and difficult to weigh. The correlation between body length and the development time of offspring was also calculated. We also examined the effect of parental body length and offspring sex on the growth rate of offspring as measured by body size increase (g/day) from first instar to emerging adult using GLMM with parental pair as a random variable.

Phylogeography

To study the genetic composition of local populations and introgressive hybridization between *C. japonicus* and *C. dehaanii*, we obtained partial sequences of the mitochondrial NADH dehydrogenase gene subunit 5 (*ND5*). *ND5* sequences have been used extensively in phylogeographical studies of *Ohomopterus* because of their high sequence diversity, and because they are useful for detecting introgressive hybridization (Nagata *et al.*, 2007; Sota & Nagata, 2008). The possession of identical haplotypes of the rapidly evolving *ND5* sequence may be interpreted as evidence of recent hybridization even when no hybrid swarm is observed. For comparison with the mitochondrial gene data, we also sequenced a nuclear *Carab1* gene (Sota & Vogler, 2003), whose genealogy is generally consistent with phylogenies established based on morphology. Mitochondrial genealogies may not reflect true species phylogeny when hybridization and backcrossing lead to gene introgression between closely related species, whereas nuclear genealogies show true

relationships among species due to the dilution of the effects of introgressive hybridization by recombination and slow mutation accumulation (Funk & Omland, 2003; Ballard & Whitlock, 2004). Phylogenetic analyses based on both mitochondrial and nuclear genes should be useful for elucidating the evolutionary history of closely related species involved in reproductive character displacement and release.

Total DNA was extracted from the gonads of individual beetles using a Wizard Genomic DNA Purification Kit (Promega, Madison, WI). For PCR and dye terminator cycle sequencing reactions, we used the following primer sets: *ND5*, forward (6-1): 5′-CCT GTT TCT GCT TTA GTT CA-3′; reverse (4-1): 5′-GCT ATA CTC TAA ATA TAA GCT A-3′ (Su *et al.*, 1996); *Carab1*, forward (gwnck1): 5′-GTG ACG AAC AAG AAG ATA TGG-3′ (Andújar *et al.*, 2012); reverse (CARCK2): 5′-GTG GTT CGC ATC TCA ACA GA-3′ (Sota & Vogler, 2001). PCR was performed using the following conditions: 2 min at 94°C; 30 cycles of 20 s at 94°C, 20 s at 50°C, and 45 s at 72°C; and 7 min at 72°C. The PCR products were treated with ExoSap-IT and subjected to a dye terminator cycle sequencing reaction with BigDye version 3.1. The products were electrophoresed on an ABI3130XL sequencer (Applied Biosystems, Foster City, CA). Alignment was performed with MEGA 5 (Tamura *et al.*, 2011). We determined 1024 bp for *ND5* and 548 bp, excluding an alignment ambiguous region, for *Carab1*. Sequence data have been deposited at the DNA Data Bank of Japan (DDBJ; accession numbers LC008547–LC008720 for *ND5*; LC008721–LC009001 for *Carab1*).

For both the *ND5* and *Carab1* gene sequences, we conducted a maximum-likelihood (ML) analysis using RAxML 8.0.20 (Stamatakis, 2014). Each gene sequence was partitioned according to three codon positions, and a general time-reversible (GTR)+gamma substitution model was applied to each partition. A rapid bootstrap

analysis with 1000 replications was conducted for each gene. We also conducted an analysis of molecular variances (AMOVA) for each gene using ARLEQUIN 3.0 (Excoffier *et al.*, 2005) to assess gene flow due to introgressive hybridization between *C. japonicus* and *C. dehaanii*. Because the sample size for each site was sometimes small, we used seven and four regions for *C. japonicus* and *C. dehaanii*, respectively, by combined site data (Tsushima Islands, Northern Kyushu Islands, Northern Kyushu, Goto Islands, Central-southern Kyushu, Amakusa Islands, and Koshiki Islands in Fig. 1). The relationships among regional populations were presented as an unrooted population tree generated using PHYLIP 3.69 (Felsenstein, 2004) based on the average sequence difference between populations obtained in the ARLEQUIN analysis.

RESULTS

Geographical patterns in body and genital size

Body length differed between the sexes and increased with annual mean temperature in both *C. japonicus* and *C. dehaanii* (Table 1a, Fig. 2a,b). In sympatric areas, a constant difference was observed in body length between the two species for each sex (Fig. 2a,b): 7.4 ± 1.5 mm (mean ± SD) for males (15 sites) and 8.1 ± 1.2 mm for females (22 sites). The body length of *C. japonicus* was affected by sympatry/allopatry with *C. dehaanii* (Table 1a; Fig. 2a, b): 22.6 ± 1.2 mm for males and 23.7 ± 1.5 mm for females in sympatric populations (25 sites), and 24.7 ± 1.9 mm for males and 26.3 ± 1.9 mm for females in allopatric populations (17 sites). In addition, the significant interaction effect of sympatry and temperature resulted in body lengths that were longer in allopatric areas with higher annual mean temperatures (Table 1a, Fig. 2a,b): 23.8 ± 0.6 mm for males and 25.3 ± 0.7 mm for females in allopatric populations on the Kyusyu mainland (4 sites), including cool

habitats at high altitudes, and 25.3 ± 0.7 mm for males and 26.6 ± 2.0 mm for females on the satellite islands (13 sites), consisting of warm habitats at low altitudes. Thus, some allopatric populations on the Northern Kyushu Islands and Goto Islands had longer body lengths than sympatric populations, whereas other allopatric populations had similar body lengths to sympatric populations (e.g., Tsushima and Amakusa Islands) (Fig. 1, Fig. 2a,b). The ratio of the maximum to minimum mean body length of *C. japonicus* populations was 1.32 for males and 1.36 for females across all the study sites. The genital dimensions of *C. japonicus* were positively correlated with body length irrespective of sympatry/allopatry with *C. dehaanii* (Table 1b, Fig. 2c–f, Fig. 3). Thus, like body length, genital dimensions showed a notable divergence among populations.

Heritable differences in body size

Larger female *C. japonicus* oviposited larger eggs leading to larger first instar larvae at hatching (GLMM: $n = 174$; maternal body length, d.f. = 1, $F = 7.0$, $P = 0.0187$; paternal body length, d.f. = 1, $F = 2.5$, $P = 0.1339$; offspring sex, d.f. = 1, $F = 0.6$, $P = 0.4459$; Fig. 4a, Appendix S3). Both maternal and paternal body lengths significantly affected offspring adult body length (GLMM: $n = 174$; maternal body length, d.f. = 1, $F = 29.8$, $P < 0.0001$; paternal body length, d.f. = 1, $F = 16.4$, $P = 0.0007$; offspring sex, d.f. = 1, $F = 318.3$, $P < 0.0001$; Fig. 4b, Appendix S3), suggesting that there is genetic variation in body length, although maternal effects may also be involved. The regression of mean offspring body length against mid-parent body length (mean of parental body lengths) had slopes close to unity for both sexes ($n = 18$, $b = 0.84$, $t = 16.7$, $P < 0.0001$ for males; $n = 19$, $b = 0.76$, $t = 14.2$, $P < 0.0001$ for females), indicating that body size differences in the field are highly heritable. There was a positive correlation between body length and

development time (n = 84, r = 0.58, P < 0.0001 for males; n = 90, r = 0.27, P = 0.0114 for females; Fig. 4c). The growth rate from first instar larva to emerging adult (g/day) was larger when parental body lengths were larger (GLMM: n = 174; maternal body length, d.f. = 1, F = 18.6, P = 0.0005; paternal body length, d.f. = 1, F = 12.2, P = 0.0022; offspring sex, d.f. = 1, F = 39.3, P < 0.0001). Thus, enlargement of body size can be attributed to increases in egg size, larval development time, and larval growth rate.

Phylogenetic analysis in mitochondrial and nuclear DNA

Individuals of *Carabus japonicus* and *C. dehaanii* were mixed in the phylogenetic tree based on mitochondrial *ND5* gene sequences (Fig. 5a) but were largely separated from each other in the phylogenetic tree based on nuclear *Carab1* gene sequences (Fig. 5b). AMOVA showed that differentiation between species was not significant for *ND5* sequences but was significant for *Carab1* sequences (see F_{CT} in Table 2). In both gene sequences, the genetic differentiation among geographical regions was significant (see F_{SC} in Table 2). In the trees showing the relationships among geographical regions (Fig. 5c), each species was monophyletic on the *Carab1* tree but not on the *ND5* tree.

ND5 haplotypes were categorized into five groups, M1–M5 (Fig. 5a). Of these, M5, comprising *C. japonicus* on the Tsushima Islands, was highly divergent from the M1–M4 haplotypes. In and around the Kyushu mainland, M3 and M4 exclusively comprised *C. dehaanii* and *C. japonicus*, respectively, whereas M1 and M2 included members of both species in Northern Kyushu and the Goto Islands (Table 3, Fig. 5a). *Carab1* sequences were also divided into five groups, N1–N5 (Fig. 5b). *Carabus japonicus* had N1–N4 sequences, whereas most *C. dehaanii* individuals had N5 sequences. N2 sequences were shared by the two species in Northern Kyushu, the Goto Islands, and Central-southern

Kyushu, and N5 sequences were shared in Northern Kyushu and Central-southern Kyushu (Table 3, Fig. 5b).

DISCUSSION

Character release in allopatric *C. japonicus* populations

Our results are consistent with the hypothesis of character release for allopatric populations of *C. japonicus* and with the expectation that evolution under character release can be variable. Most island *C. japonicus* populations were derived from mainland populations (Fig. 5), and body size enlargement was confirmed in some allopatric island populations after controlling for the effect of habitat temperature (Table 1a, Fig. 2). Further, the differences in body size between populations were heritable (Fig. 4). Divergence time estimation suggests that the *C. japonicus* population on Iki Island diverged from Kyushu mainland populations 15,000 years ago, the *C. japonicus* and *C. dehaanii* populations on Fukue Island of the Goto Islands 15,000–8,000 years ago, and the *C. japonicus* population on Tsushima 75,000 years ago (Sota & Nagata, 2008). Except for Tsushima, the other satellite islands of Kyushu were connected to the Kyushu mainland until recently, before the rising sea levels after the last glacial period, and this allowed colonization of those islands by the two species. The sequence divergence of both the *ND5* and *Carab1* genes occurred sequentially from the Central-southern to Northern Kyushu mainland, and then to the three island regions to the north and west of the Kyushu mainland (Northern Kyusyu Islands, Goto Islands, and Koshiki Islands) (Fig. 5). However, the degree of sequence divergence is much smaller in the *Carab1* gene than in the *ND5* gene owing to the lower divergence rate of the nuclear gene sequence. This pattern of divergence may reflect the dispersal pattern of the species during the last glacial period

when sea levels were lower. Thus, the enlargement of body size in allopatric *C. japonicus* populations is considered to have occurred following range expansion from the Kyushu mainland (sympatric areas). This scenario is amenable to the hypothesis of character release.

We also found identical mitochondrial haplotypes between the species in populations of the Northern Kyushu and Goto Islands (Table 3, Fig. 5a), which probably resulted from recent introgressive hybridization. This indicates that reproductive interference through interspecific mating has occurred between the two species and occasionally resulted in the production of hybrids, although no hybrid zone has been discovered for these species. In the nuclear *Carab1* sequences, which should have a lower evolutionary rate than mitochondrial genes, two groups of sequences were shared between the species on the Kyushu mainland and the Goto Islands (Table 3, Fig. 5b). The sharing of identical *Carab1* sequences may have originated from past hybridization events during the initial stage of speciation or may represent the retention of ancestral polymorphisms due to incomplete lineage sorting.

Selective forces involved in body size evolution

The mean body length of the study species shows a positive correlation with the mean annual temperature of their habitats (Fig. 2a,b). This clinal body size variation is interpreted to be a result of adaptation to the climatic gradient, in which the optimal adult body size to maximize reproductive output rises with a greater period available for larval development and growth (Masaki, 1967; Roff, 1980; Sota *et al.*, 2000). To attain a larger body size, a longer developmental period is required (Fig. 4c; see also Sota, 1985b; Tsuchiya *et al.*, 2012). However, the effect of the developmental period on enlargement

is far less than expected from the body size increase in *Ohomopterus* (a 60% increase in body length is associated with an 18% increase in development time; Sota *et al.*, 2002). We found here that the body size enlargement is achieved by the enlargement of egg size, as estimated by the body weight of hatching larvae (Fig. 4a,c), and by the increased growth rate, as well as by elongation of larval development time. Therefore, body size enlargement may be constrained by available time for larval development only in cool habitats, whereas in warm habitats, *Ohomopterus* beetles of various sizes may be produced with small variations in development time.

Our study suggests that the presence/absence of *C. dehaanii* is one of the main influences on body size in *C. japonicus*. Natural selection promotes character evolution that reduces the chance of maladaptive interspecific interactions including resource competition and reproductive interference between closely related species in sympatry (Schluter & McPhail, 1992; Pfennig & Murphy, 2000; Grant & Grant, 2006). In *Ohomopterus*, body size differences among species do not contribute to partitioning different sizes of prey in the larval stage, but they do contribute to the decreased frequency of maladaptive interspecific copulation in the adult stage (Okuzaki *et al.*, 2010). Indeed, interspecific copulation associated with the loss of gametes and injury to genitalia occurs frequently between species with small body size differences (Sota & Kubota, 1998; Nagata *et al.*, 2007). The large body size difference between *C. japonicus* and *C. dehaanii* in sympatric areas would have been sufficient to reduce the frequency of interspecific copulation; in fact, introgressive hybridization as indicated by the sharing of identical *ND5* sequences for sympatric populations was absent on the Kyushu mainland (Table 3, Fig. 5a).

Importantly, not all allopatric *C. japonicus* populations had a large body size (Fig.

2a,b), implying that factors other than habitat temperature and the absence of *C. dehaanii* affect the evolution of body size. For solitary carnivores, prey size would influence predation success and survival rate, especially in early developmental stages with small body sizes, and body size variation in adults may be the result of natural selection during the early developmental stages (Aubret, 2012). Larvae of *Ohomopterus* are specialized predators of earthworms (Sota, 1985a), and the predation success of the first instar larvae decreases with an increase in earthworm size in small-sized species (Okuzaki *et al.*, 2010). Because larger *C. japonicus* females produce larger eggs which result in larger first instar larvae (Fig. 4a), geographical variation in earthworm size may account for the body size variation among allopatric populations (Sota *et al.*, 2000; Y.O., unpublished data). An alternative hypothesis for the lack of body size enlargement in some allopatric *C. japonicus* populations is that the allopatry may be very new, as it would be if a local sympatric population of *C. dehaanii* had recently become extinct.

Consequences of body size divergence

Body size is a key trait for reproductive isolation in some animal species (e.g., Schluter & Nagel, 1995; Boughman *et al.*, 2005; Funk *et al.*, 2006), and this is particularly true for *Ohomopterus* species (Sota & Nagata, 2008). Over most of the range of *Ohomopterus*, two or three (rarely four or five) different-sized species co-occur sympatrically (Sota *et al.*, 2000), whereas similar-sized species are parapatric due to reproductive interference, sometimes forming narrow hybrid zones (Kubota & Sota, 1998). Interspecific body size ratio in two-species assemblages of *Ohomopterus* ranges from 1.15 to 1.28 for female body length (Sota *et al.*, 2000), which is generally large enough to prevent insemination in the laboratory (Okuzaki *et al.*, 2010). The ratio of body length difference between *C.*

japonicus populations in this study was as high as 1.36 for females. Therefore, the divergence in body size among populations of *C. japonicus* may result in allopatric speciation within this species in such a way that gene flow between populations during occasional secondary contacts is restricted.

In addition, we have shown that the mean genital dimensions were correlated with mean body length among *C. japonicus* populations (Table 1b, Fig. 3). In general, stabilizing selection acts on genital dimensions within populations and leads to low allometry of genital dimensions on body size (Eberhard *et al.*, 1998), but this does not apply to body size divergence among populations. Divergence in genital dimensions can also promote speciation via the enhancement of mechanical isolation in some arthropod groups including *Ohomopterus* (Sota & Kubota, 1998; Tanabe & Sota, 2008; Wojcieszek & Simmons, 2013; Kubota *et al.*, 2013). Interestingly, genital diversification and speciation in *Ohomopterus* have occurred most markedly in a lineage including species with medium to large body sizes (Sota & Nagata, 2008). Thus, body size divergence in *Ohomopterus* may be associated with divergence in genital dimensions and may promote speciation.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article:

Appendix S1 Information about sampling sites, sample sizes and morphological dimensions.

Appendix S2 Genital morphology of *Carabus japonicus*.

Appendix S3 Body weight of laboratory reared *Carabus japonicus* larvae and adults.

BIOSKETCHES

Yutaka Okuzaki is a postdoctoral research fellow of the Japan Society for the Promotion of Science at Hokkaido University and is interested in trait evolution caused by interspecific interactions and its involvement in species coexistence and speciation.

Hisashi Sugawara is a doctoral student at Kyoto University and studies physiological ecology of carabid beetles.

Teiji Sota is a professor at Kyoto University and is interested in phylogeography, diversification and speciation of carabid beetles and other invertebrates.

Editor: Kate Parr

Table 1. (a) Effects of environmental annual mean temperature, sex and sympatry/allopatry on the mean body length in *Carabus japonicus* and *C. dehaanii*. (b) Effects of body length and sympatry/allopatry on the genital size in *C. japonicus*.

(a)

Species	Factor	d.f.	χ^2	*P*
C. japonicus	Temperature	1	42.2	<0.0001
	Sex	1	31.7	<0.0001
	Sympatry	1	9.8	0.0018
	Temperature*Sympatry	1	5.3	0.0217
C. dehaanii	Temperature	1	16.3	<0.0001
	Sex	1	14.8	0.0001

(b)

Trait	Factor	d.f.	χ^2	*P*
Aedeagus length	Sympatry	1	0.3	0.5732
	Male body length	1	64.4	<0.0001
	Sympatry*Male body length	1	2.2	0.1367
Copulatory piece length	Sympatry	1	0.2	0.6480
	Male body length	1	36.3	<0.0001
	Sympatry*Male body length	1	0.4	0.5180
Vagina length	Sympatry	1	0.3	0.5934
	Female body length	1	43.7	<0.0001
	Sympatry*Female body length	1	0.7	0.4032
Vaginal appendix length	Sympatry	1	0.1	0.7259
	Female body length	1	11.4	0.0007
	Sympatry*Female body length	1	0.1	0.7605

Table 2. Analysis of molecular variance for the genetic differentiation between *Carabus japonicus* and *C. dehaanii* in two gene sequences. Regions and populations were represented in Fig. 1. Note that *C. japonicus* and *C. dehaanii* occurred in seven and four regions, respectively.

Source of variation	d.f.	Variance	Fixation index		*P*
Mitochondrial *ND5*					
Between species	1	0.19610	F_{CT}	0.03010	0.26197
Among regions within species	9	3.07025	F_{SC}	0.48589	0.00000
Within population	386	3.24854	F_{ST}	0.50137	0.00000
Nuclear *Carab1*					
Between species	1	0.86386	F_{CT}	0.22801	0.00782
Among regions within species	9	0.96766	F_{SC}	0.33085	0.00000
Within population	272	1.95714	F_{ST}	0.48342	0.00000

F_{CT}, between species; F_{SC}, among populations within species; F_{ST}, among populations. The *P*-value was obtained by 1000 permutations.

Table 3. Number of individuals belonging to each haplotype groups of mitochondrial gene *ND5* (M1-M5 in Fig. 5a) and nuclear gene *Carab1* (N1-N5 in Fig. 5b) for *Carabus japonicus* and *C. dehaanii* occurring in seven regions (Fig. 1). Bold numerals emphasize shared sequence groups in each region.

		ND5 group					*Carab1* group				
Region	Species	M1	M2	M3	M4	M5	N1	N2	N3	N4	N5
Tsushima Islands	*C. japonicus*	0	0	0	0	6	0	0	6	0	0
Northern Kyushu Islands	*C. japonicus*	11	29	0	0	0	1	2	20	2	1
Northern Kyushu	*C. japonicus*	**3**	**45**	0	44	0	0	**20**	37	4	**2**
	C. dehaanii	**23**	**2**	2	0	0	0	**5**	0	0	**16**
Goto Islands	*C. japonicus*	**55**	**19**	0	1	0	42	**5**	5	0	0
	C. dehaanii	**14**	**8**	0	0	0	0	**5**	0	0	12
Central-southern Kyushu	*C. japonicus*	1	0	0	73	0	0	**7**	2	30	**1**
	C. dehaanii	0	4	21	0	0	0	**12**	0	0	**10**
Amakusa Islands	*C. japonicus*	0	0	0	5	0	0	0	0	5	0
Koshiki Islands	*C. japonicus*	2	0	0	3	0	0	0	5	0	0
	C. dehaanii	0	26	0	0	0	0	0	0	0	26
All regions	*C. japonicus*	72	93	0	126	6	43	34	75	41	4
	C. dehaanii	37	40	23	0	0	0	22	0	0	64

FIGURE LEGENDS

Figure 1. Study sites and body size variations of *Carabus japonicus* and *C. dehaanii* in Kyushu. Filled circles represent symatric sites where both *C. japonicus* and *C. dehaanii* co-occurred, whereas open circles represents allopatric sites where only *C. japonicus* occurred, based on potential distribution areas of *C. dehaanii* summarized in Imura & Mizusawa (2013). We divided Kyushu into seven regions for descriptive purposes. *Carabus japonicus* distributes in all seven regions, whereas *C. dehaanii* distributes in four regions.

Figure 2. Geographical patterns of (a) male body length, (b) female body length, (c) length of the aedeagus, (d) length of the vagina, (e) length of the copulatory piece and (f) length of the vaginal appendix in relation to mean annual temperature in *Carabus japonicus* and *C. dehaanii*. Red, blue and black circles represent *C. japonicus* in sympatry, *C. japonicus* in allopatry and *C. dehaanii*, respectively. Filled and open circles represent populations on Kyushu mainland and satellite islands, respectively. In (a) and (b), numbers pointing to red or blue circles (*C. japonicus*) correspond to site numbers in Fig. 1.

Figure 3. Correlations between body length and genital dimensions in males (a, c, e) and females (b, d, f) of *Carabus japonicus* and *C. dehaanii*. (a) Male body length and aedeagus length (n = 42, r = 0.94, P < 0.0001 for *C. japonicus*, n = 17, r = 0.97, P < 0.0001 for *C. dehaanii*), (b) female body length and vaginal length (n = 42, r = 0.89, P < 0.0001 for *C. japonicus*, n = *17*, r = 0.57, P = 0.0175 for *C. dehaanii*), (c) male body length and copulatory piece length (r = 0.88, P < 0.0001 for *C. japonicus*, r = 0.53, P = 0.0278 for *C. dehaanii*), (d) female body length and vaginal appendix length (r = 0.61, P

< 0.0001 for *C. japonicus*, $r = 0.07$, $P = 0.7895$ for *C. dehaanii*), (e) aedeagus length and copulatory piece length ($r = 0.87$, $P < 0.0001$ for *C. japonicus*, $r = 0.61$, $P = 0.0090$ for *C. dehaanii*) and (f) vaginal length and vaginal appendix length ($r = 0.56$, $P = 0.0001$ for *C. japonicus*, $r = –0.06$, $P = 0.8239$ for *C. dehaanii*). Red, blue and black circles represent *C. japonicus* in sympatry, *C. japonicus* in allopatry and *C. dehaanii*, respectively. Filled and open circles represent populations on Kyushu mainland and satellite islands, respectively.

Figure 4. Body size and development time of laboratory-reared *Carabus japonicus* in a common garden experiment. (a) Body weight of first-instar larva in relation to body length of female-parent; (b) body length of adult offspring in relation to body length of mid-parent; and (c) body length of adult offspring in relation to the development time. Diamonds, triangles, squares and circles represent original sites 6, 7, 5 and 3, respectively, and filled and open symbols represent male and female offspring, respectively. Error bars represent standard deviation.

Figure 5. Molecular phylogeny of *Carabus japonicus* and *C. dehaanii* in Kyusyu. (a) Phylogeny of mitochondrial *ND5* and geographical frequencies of haplotype groups, (b) phylogeny of nuclear *Carab1* and geographical frequencies of haplotype groups. Operational taxonomic unit (OUT) represented by grey and black bars are *C. japonicus* and *C. dehaanii*, respectively. *Carabus daisen* is the outgroup taxon. Numerals on branches are bootstrap percentages (shown when >50%). (c) Unrooted population tree based on average sequence difference of mitochondrial *ND5* and nuclear *Carab1* between populations of seven regions in Fig. 1. [jap] and [deh] represents *C. japonicus* and *C.*

dehaanii, respectively.

Figure. 1

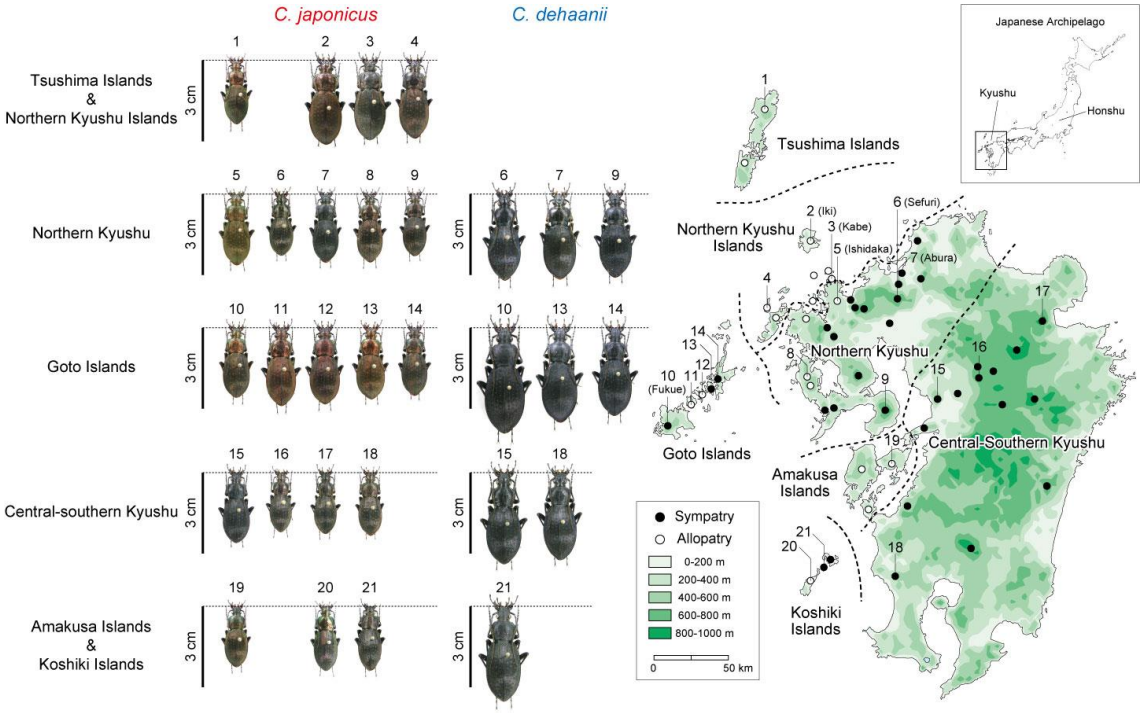

C. japonicus
C. dehaanii
Tsushima Islands & Northern Kyushu Islands
Northern Kyushu
Goto Islands
Central-southern Kyushu
Amakusa Islands & Koshiki Islands
3 cm
Japanese Archipelago
Kyushu
Honshu
Tsushima Islands
Northern Kyushu Islands
2 (Iki)
3 (Kabe)
5 (Ishidaka)
6 (Sefuri)
7 (Abura)
Northern Kyushu
Goto Islands
(Fukue)
Central-Southern Kyushu
Amakusa Islands
Koshiki Islands
Sympatry
Allopatry
0-200 m
200-400 m
400-600 m
600-800 m
800-1000 m
0
50 km

Figure 2.

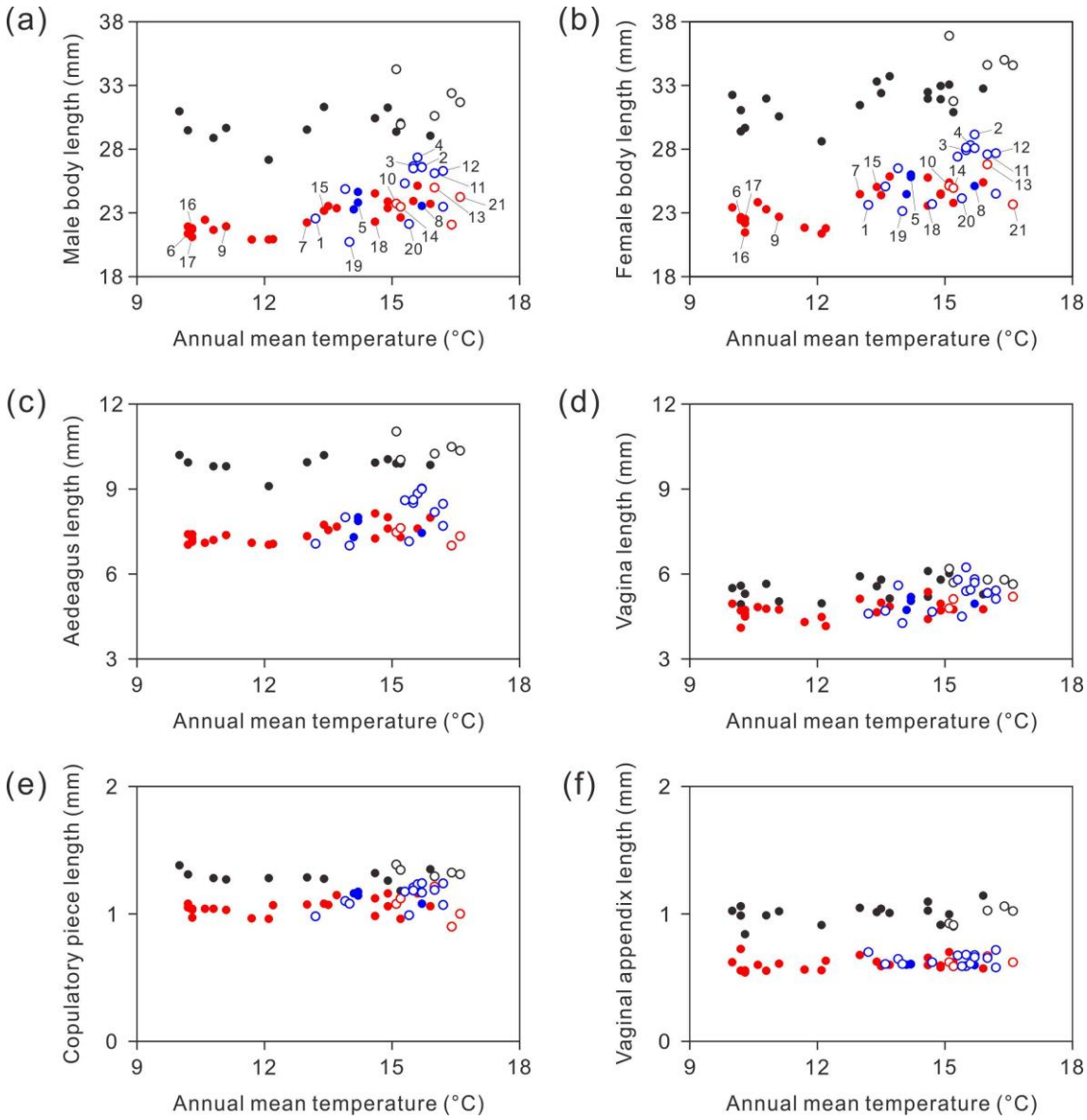

(a)
Male body length (mm)
Annual mean temperature (°C)
(b)
Female body length (mm)
Annual mean temperature (°C)
(c)
Aedeagus length (mm)
Annual mean temperature (°C)
(d)
Vagina length (mm)
Annual mean temperature (°C)
(e)
Copulatory piece length (mm)
Annual mean temperature (°C)
(f)
Vaginal appendix length (mm)
Annual mean temperature (°C)

Figure 3.

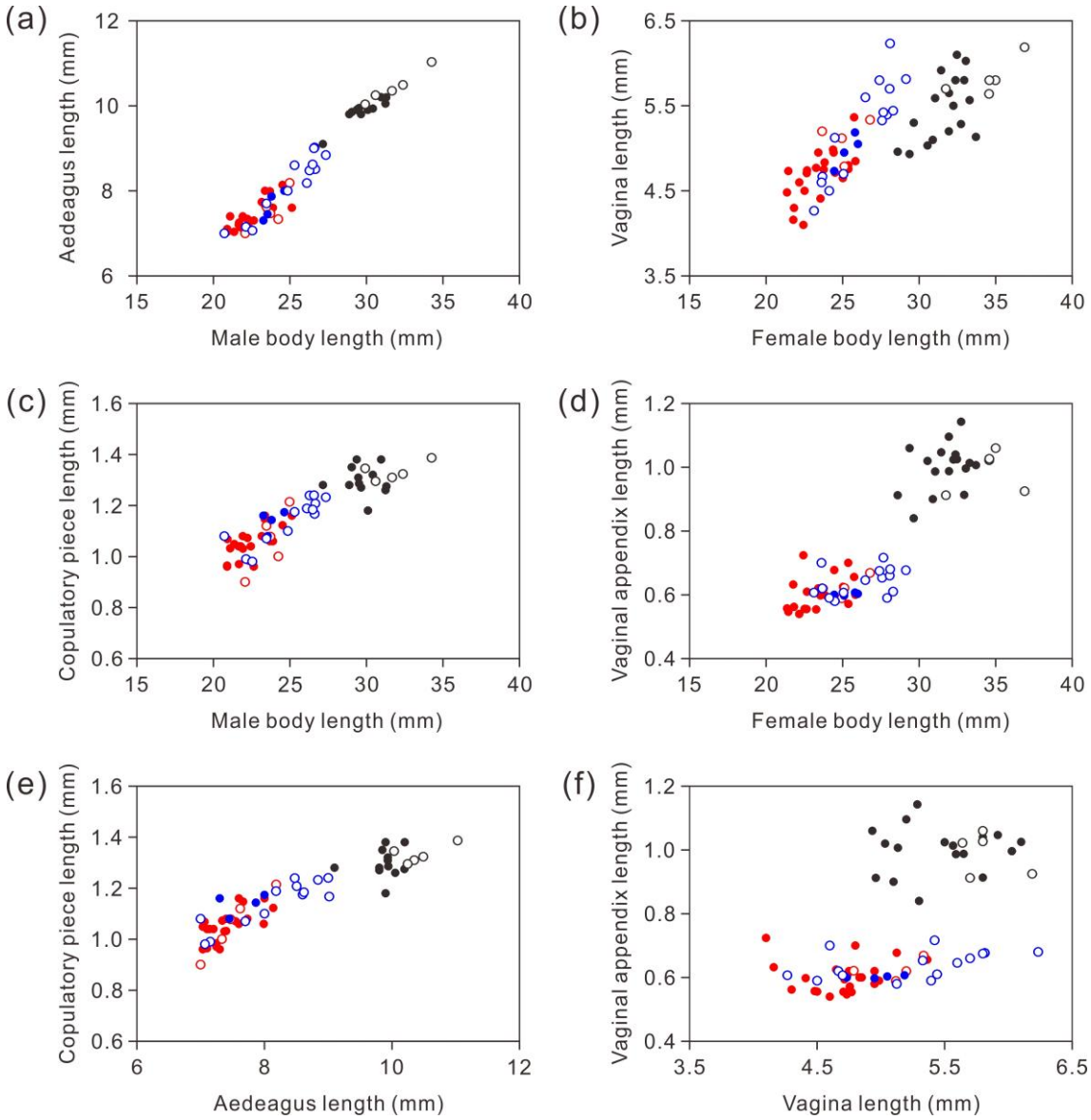

(a)
Aedeagus length (mm)
Male body length (mm)
(b)
Vagina length (mm)
Female body length (mm)
(c)
Copulatory piece length (mm)
Male body length (mm)
(d)
Vaginal appendix length (mm)
Female body length (mm)
(e)
Copulatory piece length (mm)
Aedeagus length (mm)
(f)
Vaginal appendix length (mm)
Vagina length (mm)

Figure 4.

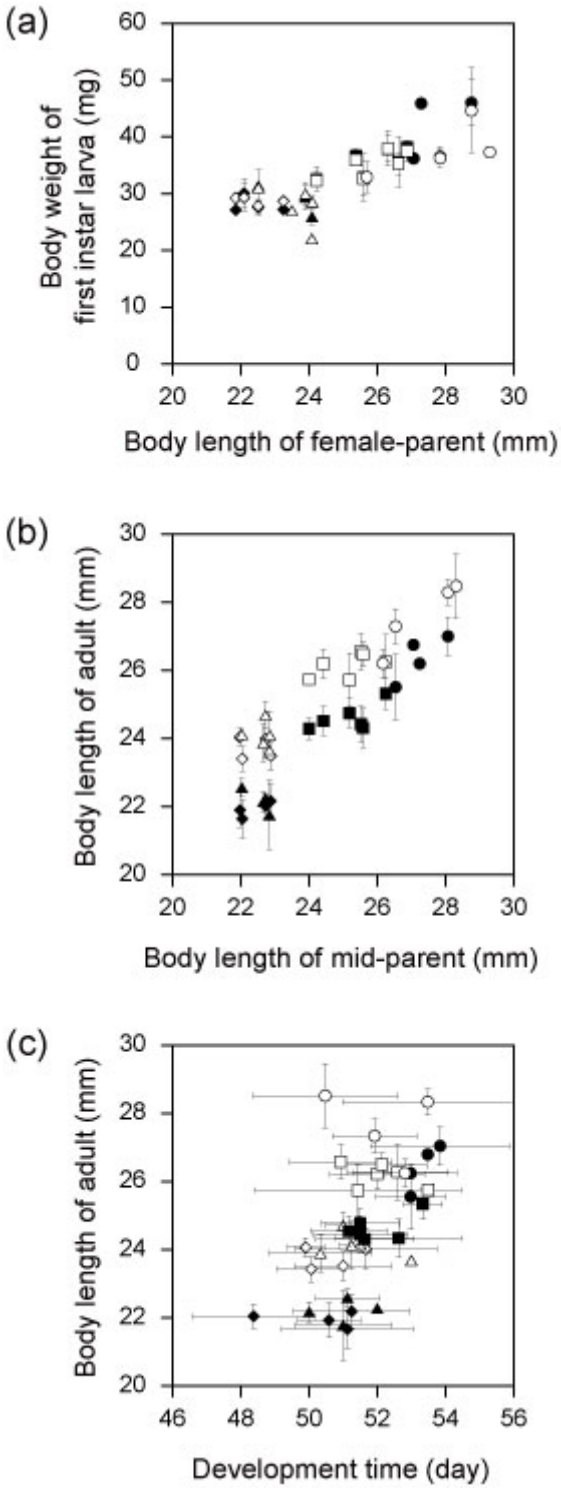
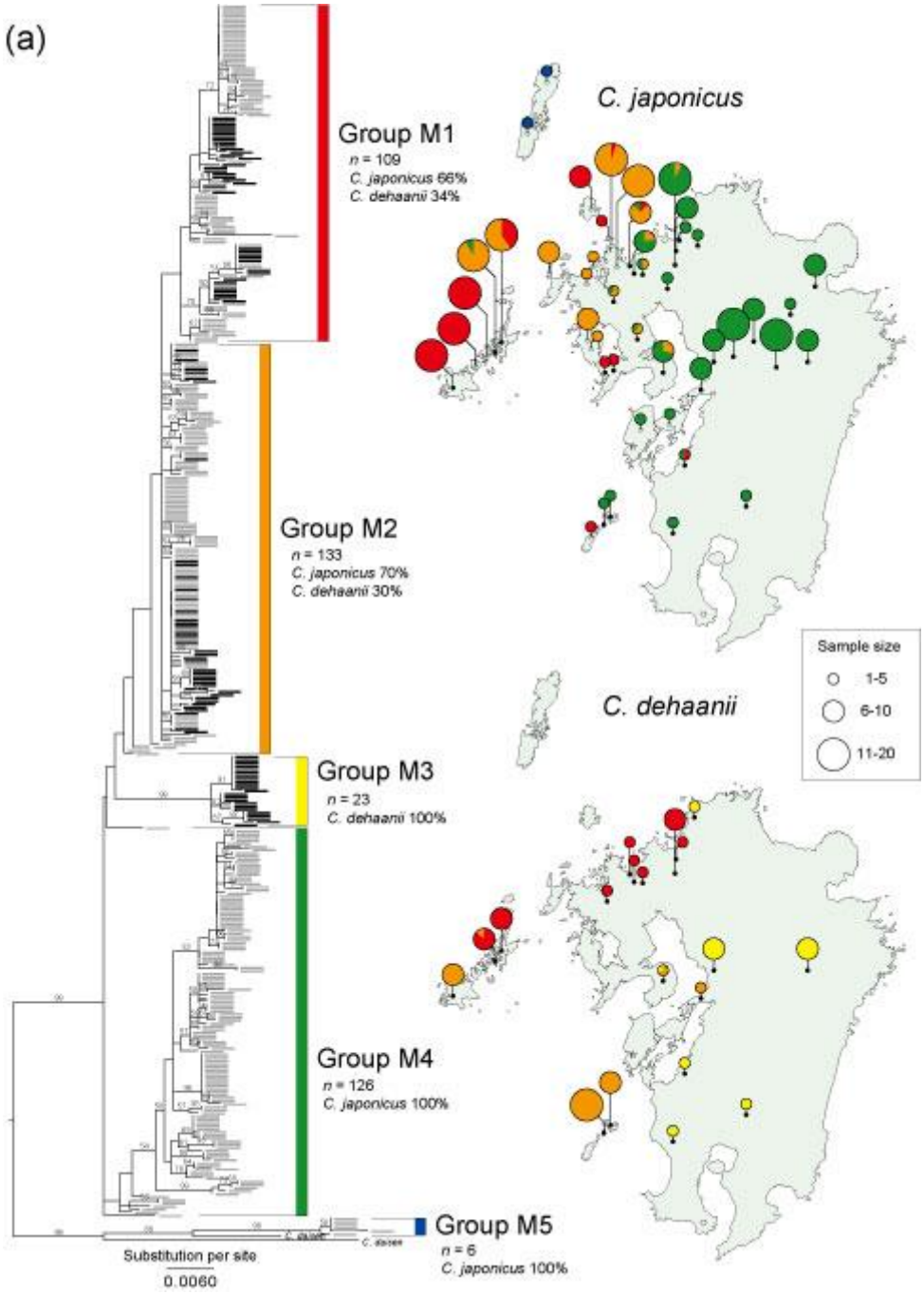
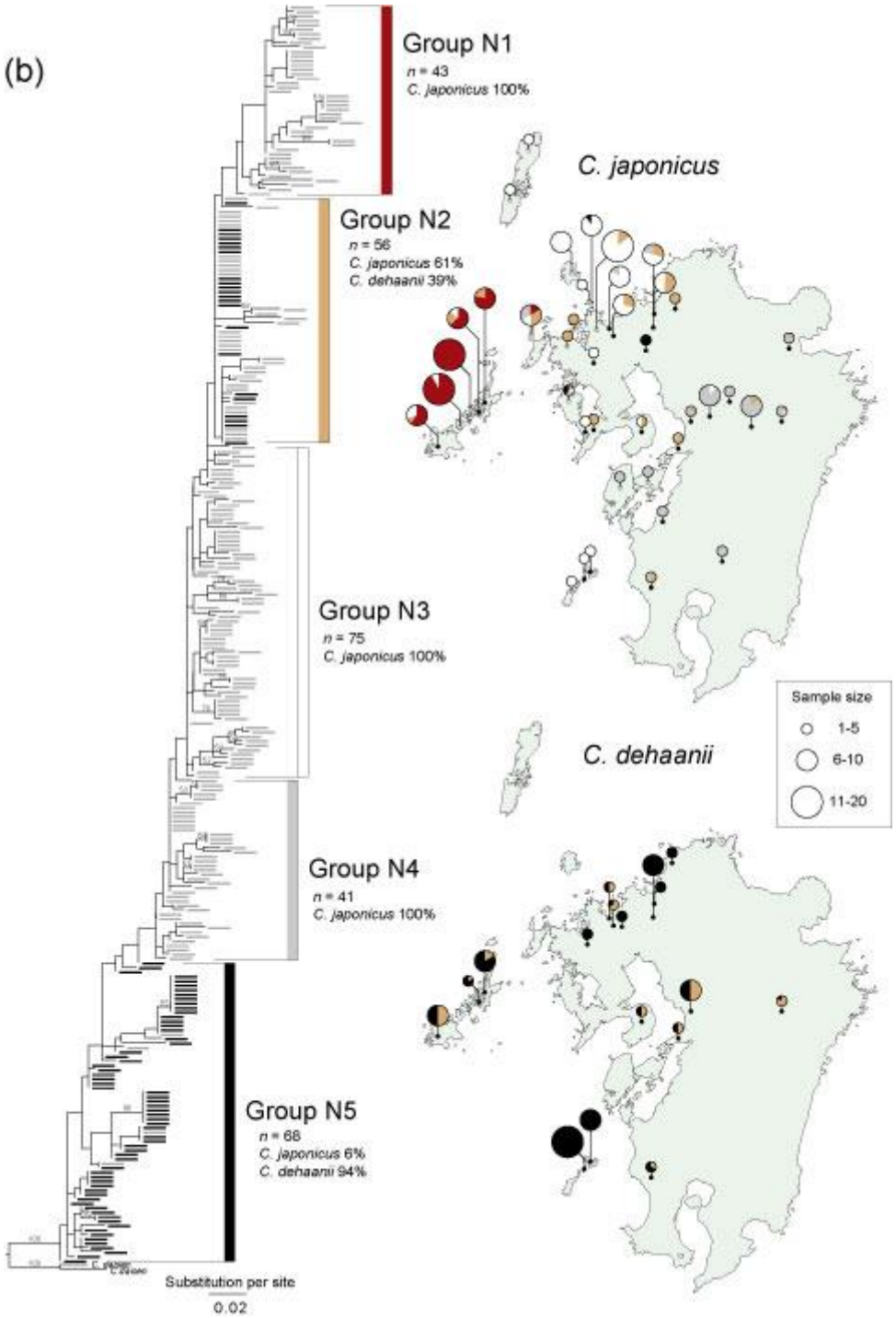

(a)
Body weight of first instar larva (mg)
0
10
20
30
40
50
60
20
22
24
26
28
30
Body length of female-parent (mm)
(b)
Body length of adult (mm)
20
22
24
26
28
30
20
22
24
26
28
30
Body length of mid-parent (mm)
(c)
Body length of adult (mm)
20
22
24
26
28
30
46
48
50
52
54
56
Development time (day)

Figure 5.


(a)
Group M1
n = 109
C. japonicus 66%
C. dehaanii 34%
Group M2
n = 133
C. japonicus 70%
C. dehaanii 30%
Group M3
n = 23
C. dehaanii 100%
Group M4
n = 126
C. japonicus 100%
Group M5
n = 6
C. japonicus 100%
Substitution per site
0.0060
C. japonicus
C. dehaanii
Sample size
1-5
6-10
11-20



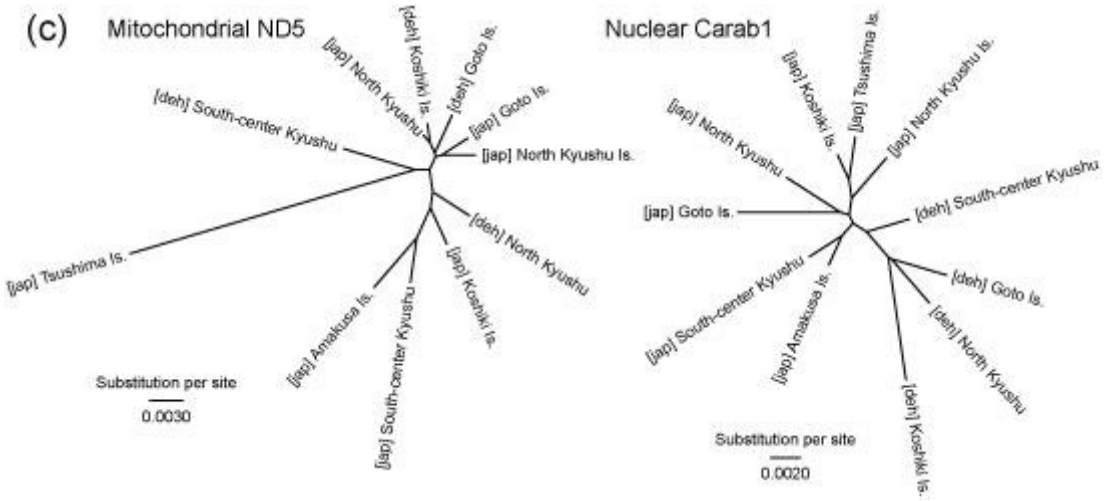
803

804

805

806

807


(c)
Mitochondrial ND5
[jap] North Kyushu
[deh] Koshiki Is.
[deh] Goto Is.
[jap] Goto Is.
[jap] North Kyushu Is.
[deh] South-center Kyushu
[deh] North Kyushu
[jap] Tsushima Is.
[jap] Koshiki Is.
[jap] Amakusa Is.
[jap] South-center Kyushu
Substitution per site
0.0030
Nuclear Carab1
[jap] Tsushima Is.
[jap] North Kyushu Is.
[jap] Koshiki Is.
[jap] North Kyushu
[jap] Goto Is.
[deh] South-center Kyushu
[deh] Goto Is.
[deh] North Kyushu
[jap] South-center Kyushu
[jap] Amakusa Is.
[deh] Koshiki Is.
Substitution per site
0.0020

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SUPPORTING INFORMATION

Body size evolution under character release in the ground beetle *Carabus japonicus*

Yutaka Okuzaki, Hisashi Sugawara and Teiji Sota

Appendix S1 (a) Information about sampling sites and sample sizes for *Carabus japonicus* and *C. dehaanii*. Temperature is shown by annual mean temperature. C, M, SN and SC indicate sample sizes for each sex in each species: C, collected individuals; M, measured for morphology; SN, sequenced for *ND5*; SC, sequenced for *Carab1*. (b) Morphological dimensions for *C. japonicus* and *C. dehaanii*: MBL, male body length; AL, aedeagus length; CPL, copulatory piece length; FBL, female body length; VL, vaginal length; VAL, vaginal appendix length.

(a)

							Sample size															
							C. japonicus								*C. dehaanii*							
Region							Male				Female				Male				Female			
No.	Site	Latitude	Longitude	Temp. (˚C)	Elevation (m)	Distribution	C	M	SN	SC	C	M	SN	SC	C	M	SN	SC	C	M	SN	SC
Tsushima Islands																						
1	Kamitsushima	34˚34´20´	129˚22´54´	13.2	306	Allopatry	3	3	3	3	1	1	1	1								
22	Shimotsushima	34˚15´50´	129˚14´59´	13.6	281	Allopatry	0	0	0	0	3	3	2	2								

Northern Kyushu Islands																						
2	Iki	33˚47´18´	129˚42´23´	15.7	63	Allopatry	8	7	2	2	21	13	6	6								
3	Kabe	33˚32´55´	129˚52´56´	15.5	39	Allopatry	57	16	10	9	68	20	9	1								
4	Ikitsuki	33˚22´02´	129˚24´50´	15.6	144	Allopatry	5	5	1	0	12	12	8	6								
23	Hirado	33˚19´49´	129˚28´01´	13.9	404	Allopatry	3	3	0	0	10	10	0	0								
24	Taka	33˚25´13´	129˚43´51´	15.5	44	Allopatry	5	5	0	0	3	3	2	2								
25	Madara	33˚34´28´	129˚45´00´	15.3	107	Allopatry	4	4	0	0	4	4	0	0								
26	Kakara	33˚35´27´	129˚51´39´	15.7	32	Allopatry	1	1	0	0	5	5	2	2								
Northern Kyushu																						
5	Ishidaka	33˚25´28´	129˚54´51´	14.2	198	Allopatry	69	6	9	7	95	14	8	7								
6	Sefuri	33˚26´04´	130˚22´12´	10.2	901	Sympatry	41	9	7	6	70	29	7	4	18	11	0	0	31	9	8	7
7	Abura	33˚30´43´	130˚21´52´	13	447	Sympatry	62	9	6	5	86	9	1	1	30	7	1	0	62	12	1	1
8	Yukinoura	32˚57´30´	129˚41´38´	15.7	104	Allopatry	2	2	1	1	8	8	7	4								
9	Unzen	32˚45´45´	130˚16´32´	11.1	908	Sympatry	11	11	2	2	16	16	5	2	2	2	0	0	3	3	3	2
27	Shiratake	33˚18´35´	129˚39´49´	14.2	263	Allopatry	3	3	0	0	6	6	3	2								
28	Koshitake	33˚14´07´	129˚52´38´	13.7	290	Sympatry	3	3	0	0	2	2	0	0	0	0	0	0	3	3	2	1
29	Kurokami	33˚12´51´	129˚54´06´	13.5	303	Sympatry	4	4	2	2	14	14	1	1	0	0	0	0	1	1	0	0
30	Kagami	33˚25´46´	130˚01´26´	14.6	102	Sympatry	10	10	4	4	20	14	4	2	4	4	1	1	9	9	3	3
31	Sakurei	33˚21´37´	130˚04´19´	10.8	739	Sympatry	14	9	2	2	26	10	7	5	1	1	1	1	8	8	3	2
32	Tennzann	33˚20´20´	130˚08´34´	10	898	Sympatry	0	0	0	0	2	2	2	0	1	1	1	1	5	5	2	1
33	Kinryu	33˚19´56´	130˚18´04´	14.9	106	Sympatry	4	4	0	0	11	11	2	1	0	0	0	0	3	3	0	0
34	Minami Park	33˚34´26´	130˚23´22´	16.3	30	Sympatry	0	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0

35	Houman	33˚32´23´	130˚34´08´	12	591	Sympatry	0	0	0	0	6	6	5	2	0	0	0	0	0	0	0	0
36	Miyajidake	33˚46´59´	130˚29´16´	15.1	62	Sympatry	0	0	0	0	1	1	0	0	1	1	0	0	12	12	1	1
37	Nagauratake	32˚54´11´	129˚42´48´	14.1	397	Allopatry	2	2	0	0	6	6	1	0								
38	Inasa	32˚45´33´	129˚50´51´	15.6	177	Sympatry	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0
39	Konpira	32˚45´54´	129˚52´57´	15.5	197	Sympatry	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0
40	Tara	32˚57´29´	130˚04´35´	10.6	873	Sympatry	1	1	0	0	6	6	3	0	0	0	0	0	0	0	0	0
Goto Islands																						
10	Fukue	32˚39´06´	128˚41´37´	15.1	269	Sympatry	16	16	7	7	19	17	13	3	12	8	2	2	11	9	5	4
11	Hisaka	32˚47´48´	128˚51´55´	16	57	Allopatry	14	14	12	12	23	23	4	0								
12	Naru	32˚49´56´	128˚56´26´	16.2	60	Allopatry	24	24	4	4	18	18	9	9								
13	Wakamatsu	32˚53´06´	129˚00´56´	16	97	Sympatry	7	7	5	4	16	16	6	4	6	6	3	2	8	8	4	3
14	Nakadoori	32˚55´54´	129˚02´59´	15.2	257	Sympatry	11	11	6	6	22	22	9	3	4	4	3	3	5	5	5	3
Central-southern Kyushu																						
15	Kinpou	32˚48´48´	130˚38´21´	13.4	479	Sympatry	6	6	3	2	4	4	3	1	4	4	4	4	6	6	6	6
16	Kuratake	32˚57´11´	130˚56´19´	10.3	930	Sympatry	5	5	4	4	6	6	5	0	0	0	0	0	0	0	0	0
17	Yufu	33˚17´22´	131˚24´31´	10.3	860	Sympatry	20	5	0	0	46	5	8	4	0	0	0	0	0	0	0	0
18	Kanmuridake	31˚44´56´	130˚19´51´	14.6	364	Sympatry	6	6	0	0	11	11	3	2	0	0	0	0	5	5	3	3
41	Kujyu	33˚05´47´	131˚12´29´	7.8	1296	Sympatry	0	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0
42	Sobo	32˚51´12´	131˚20´21´	12.1	676	Sympatry	3	3	1	1	16	16	9	4	1	1	1	1	5	5	5	4
43	Shirogatake	32˚48´11´	131˚05´46´	11.7	741	Sympatry	5	5	3	3	9	9	8	4	0	0	0	0	0	0	0	0
44	Nishiyuura	32˚59´05´	131˚00´04´	10.2	927	Sympatry	7	5	0	0	18	5	0	0	0	0	0	0	3	3	0	0

45	Kigo	33˚02´01´	130˚56´01´	12.2	573	Sympatry	25	5	0	0	109	5	0	0	0	0	0	0	0	0	0	0
46	Tatsuta	32˚49´37´	130˚43´55´	15.9	63	Sympatry	10	10	9	9	8	8	8	0	2	2	0	0	7	7	0	0
47	Ootake	32˚39´18´	130˚35´23´	14.9	264	Sympatry	1	1	1	1	7	7	5	2	2	2	2	2	3	3	2	2
48	Nakao	32˚11´30´	130˚25´14´	15.2	181	Sympatry	1	1	1	1	2	2	1	0	1	1	0	0	1	1	1	0
49	Nakadake	31˚53´12´	130˚53´41´	10.3	1049	Sympatry	2	2	1	1	1	1	0	0	0	0	0	0	1	1	1	0
50	Osuzu	32˚17´05´	131˚27´56´	13.5	488	Sympatry	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0
Amakusa Islands																						
19	Kamiamakusa	32˚25´40´	130˚19´37´	14	407	Allopatry	1	1	1	1	3	3	2	2								
51	Shimoamakusa	32˚23´04´	130˚05´33´	14.7	316	Allopatry	0	0	0	0	3	3	2	2								
52	Naga	32˚08´58´	130˚09´37´	16.2	166	Allopatry	2	2	0	0	4	4	0	0								
Koshiki Islands																						
20	Shimokoshiki	31˚43´24´	129˚44´22´	15.4	329	Allopatry	2	2	0	0	2	2	2	2								
21	Nakakoshiki	31˚48´03´	129˚49´48´	16.6	186	Sympatry	3	3	1	1	1	1	1	1	12	12	7	7	10	10	10	10
53	Kamikoshiki	31˚50´01´	129˚53´50´	16.4	227	Sympatry	2	2	1	1	0	0	0	0	6	6	3	3	9	9	6	6

(b)

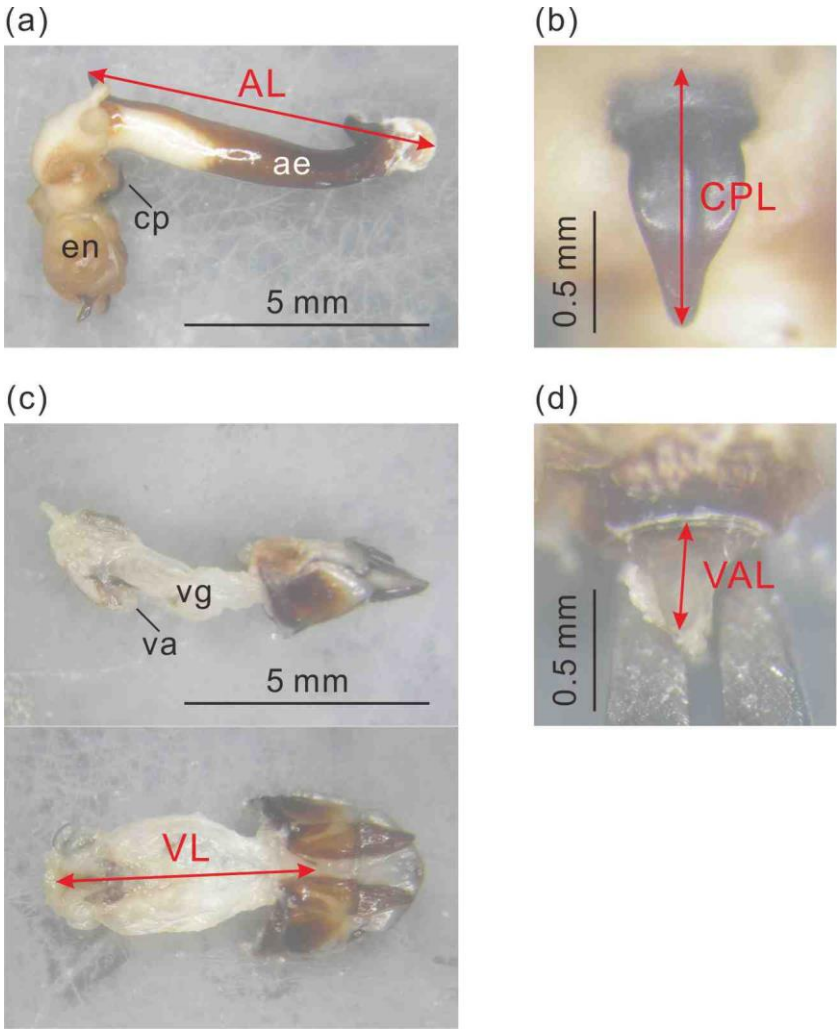
	C. japonicus												*C. dehaanii*											
Region	MBL (mm)		AL (mm)		CPL (mm)		FBL (mm)		VL (mm)		VAL (mm)		MBL (mm)		AL (mm)		CPL (mm)		FBL (mm)		VL (mm)		VAL (mm)	
No.	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Tsushima islands																								
1	22.54	0.29	7.07	0.06	0.98	0.02	23.62		4.60		0.70													
22							25.06	0.36	4.70	0.20	0.61	0.03												
Northern Kyushu islands																								
2	26.61	0.93	9.02	0.16	1.17	0.03	29.14	1.03	5.82	0.37	0.68	0.07												
3	26.66	0.76	8.51	0.17	1.21	0.04	27.90	1.15	5.40	0.41	0.59	0.06												
4	27.35	0.93	8.84	0.18	1.23	0.04	28.30	0.76	5.44	0.18	0.61	0.05												
23	24.86	0.93	8.00	0.14	1.10	0.03	26.49	0.98	5.60	0.33	0.65	0.04												
24	26.49	1.49	8.62	0.36	1.18	0.06	28.11	1.57	6.23	0.32	0.68	0.08												
25	25.31	0.77	8.60	0.22	1.18	0.03	27.41	1.17	5.80	0.32	0.68	0.06												
26	26.57		9.00		1.24		28.08	0.94	5.70	0.23	0.66	0.04												
Northern Kyushu																								
5	23.80	1.50	7.87	0.25	1.14	0.04	25.81	1.40	5.19	0.27	0.61	0.04												
6	21.36	0.59	7.03	0.21	1.05	0.05	22.66	0.85	4.71	0.35	0.56	0.06	29.47	0.80	9.93	0.29	1.31	0.04	31.05	0.70	5.59	0.63	0.99	0.09

7	22.23	0.68	7.34	0.20	1.07	0.05	24.46	0.50	5.13	0.38	0.68	0.08	29.52	1.06	9.94	0.21	1.29	0.02	31.44	1.09	5.92	0.50	1.05	0.05
8	23.54	0.16	7.45	0.21	1.08	0.03	25.10	0.50	4.95	0.21	0.60	0.07												
9	21.93	0.70	7.37	0.16	1.03	0.03	22.67	0.83	4.74	0.27	0.61	0.04	29.65	0.70	9.80	0.14	1.27	0.01	30.55	0.22	5.03	0.15	1.02	0.02
27	24.63	0.22	8.00	0.17	1.17	0.05	26.00	1.10	5.05	0.41	0.60	0.04												
28	23.35	0.46	7.67	0.15	1.15	0.04	25.84	0.96	4.85	0.49	0.60	0.00							33.71	0.67	5.13	0.12	1.01	0.07
29	23.53	0.95	7.55	0.07	1.07	0.01	24.38	1.04	4.99	0.22	0.59	0.05							32.38		5.80		1.04	
30	24.52	1.23	8.14	0.13	1.12	0.05	25.75	1.07	5.36	0.26	0.66	0.07	30.42	1.63	9.93	0.31	1.32	0.11	32.47	0.97	6.10	0.61	1.03	0.05
31	21.66	0.72	7.20	0.21	1.04	0.04	23.27	1.36	4.77	0.34	0.55	0.06	28.88		9.80		1.28		31.95	1.60	5.65	0.58	0.99	0.10
32							23.41	0.34	4.95	0.35	0.62	0.03	30.96		10.20			1.38		32.24	0.61	5.50	0.48	1.02
	0.06																							
33	23.37	1.57	8.00		1.16		24.42	0.46	4.95	0.25	0.58	0.05							31.90	2.30				
34							25.67																	
35							23.43	0.47	4.36	0.15	0.56	0.04												
36							25.37		4.80		0.70		29.36		9.90		1.38		33.05	1.20	6.03	0.39	1.00	0.06
37	23.27	0.34	7.30		1.16		24.46	0.90	4.73	0.33	0.60	0.06												
38	25.13		7.60		1.16																			
39	23.92																							
40	22.44		7.10		1.04		23.82	0.41	4.83	0.33	0.60	0.05												
Goto islands																								
10	23.72	0.58	7.47	0.14	1.08	0.03	25.12	0.66	4.79	0.34	0.62	0.05	34.26	1.02	11.03		0.21	1.39	0.07	36.89	1.22	6.19	0.27	0.93
	0.11																							
11	26.11	0.70	8.18	0.20	1.19	0.05	27.58	1.00	5.33	0.40	0.65	0.05												

12	26.28	0.94	8.48	0.20	1.24	0.05	27.67	0.89	5.42	0.31	0.72	0.04											
13	24.98	0.80	8.18	0.31	1.21	0.04	26.79	0.86	5.34	0.32	0.67	0.06	30.60	0.98	10.25		0.17	1.30	0.06	34.59	1.53	5.80	0.30	1.03
	0.07																							
14	23.47	0.58	7.62	0.13	1.12	0.03	24.95	0.98	5.12	0.36	0.59	0.04	29.92	1.10	10.03		0.32	1.35	0.04	31.75	1.37	5.70	0.33	0.91
	0.08																							
Central-southern Kyushu																								
15	23.16	0.86	7.73	0.18	1.08	0.04	25.02	1.03	4.65	0.21	0.63	0.10	31.30	0.74	10.20		0.14	1.28	0.03	33.30	0.98	5.57	0.30	1.01
	0.06																							
16	21.78	0.35	7.14	0.27	1.04	0.02	21.46	0.50	4.73	0.26	0.55	0.05												
17	21.09	0.56	7.40	0.12	1.03	0.05	22.52	1.16	4.50	0.23	0.56	0.04												
18	22.30	0.92	7.25	0.22	0.98	0.04	23.56	0.68	4.41	0.20	0.60	0.05							31.95	0.61	5.20	0.24	1.10	0.05
41							21.59		4.60		0.60													
42	20.90	0.58	7.03	0.06	0.96	0.05	21.37	0.84	4.48	0.19	0.56	0.05	27.16		9.10		1.28		28.60	1.17	4.96	0.30	0.91	0.09
43	20.90	0.64	7.10	0.10	0.96	0.02	21.83	0.71	4.30	0.25	0.56	0.04												
44	21.92	0.44	7.40	0.14	1.08	0.04	22.44	0.94	4.10	0.37	0.72	0.05							29.38	0.77	4.93	0.71	1.06	0.04
45	20.92	0.25	7.06	0.09	1.07	0.04	21.78	0.76	4.16	0.15	0.63	0.02												
46	23.69	0.73	7.99	0.23	1.06	0.04	25.38	0.89	4.76	0.33	0.57	0.04	29.04	0.41	9.85	0.07	1.35	0.07	32.74	0.67	5.29	0.44	1.14	0.06
47	23.89		7.60		1.06		24.50	0.50	4.71	0.25	0.59	0.03	31.25	0.61	10.05		0.07	1.26	0.03	32.95	0.61	5.80	0.10	0.91
	0.04																							
48	22.63		7.30		0.96		23.77	1.42	4.75	0.21	0.62	0.00	30.09		9.90		1.18		30.89		5.10		0.90	
49	21.67	0.91	7.25	0.35	0.97	0.01	22.17		4.60		0.54								29.65		5.30		0.84	
50							21.26		4.60		0.56													

Amakusa islands																							
19	20.72		7.00		1.08		23.13	0.53	4.27	0.32	0.61	0.09											
51							23.68	0.88	4.67	0.72	0.62	0.05											
52	23.47	0.59	7.70	0.00	1.07	0.04	24.49	0.69	5.13	0.17	0.58	0.03											
Koshiki islands																							
20	22.13	0.13	7.15	0.07	0.99	0.04	24.13	0.42	4.50	0.00	0.59	0.07											
21	24.24	0.55	7.33	0.06	1.00	0.04	23.66		5.20		0.62		31.67	0.75	10.35	0.18	1.31	0.04	34.57	0.67	5.64	0.35	1.02
	0.09																						
53	22.07	0.68	7.00	0.14	0.90	0.00							32.39	1.05	10.49	0.22	1.32	0.03	34.99	1.16	5.80	0.27	1.06
	0.09																						

Appendix S2 Genital morphology of *Carabus japonicus*. (a) Male genitalia; (b) copulatory piece; (c) female genitalia (upper, lateral view; lower, ventral view), (d) vaginal appendix. Abbreviations: ae, aedeagus; en, endophallus; cp, copulatory piece; vg, vagina; va, vaginal appendix. Definitions of aedeagus length (AL), copulatory piece length (CPL), vaginal length (VL) and vaginal appendix length (VAL) are also shown.



Appendix S3 Body weight of laboratory reared *Carabus japonicus* larvae and adults in relation to mid-parent body length. (a) first instar larva; (b) second instar larva; (c) third instar larva; (d) adult offspring. Different symbols refer to source localities: diamond, site 6; triangle, site 7; square, site 5; circle, site 3 (see Fig. 1 for site number). Closed and open symbols represent male and female offspring, respectively. Error bars represent standard deviation.