Original Article

LRH: Y. Okuzaki *et al*.

RRH: Body size evolution in *Carabus* beetles

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

44 also involved. Geographical differentiation in body size and associated genital
45 dimensions may result in prezygotic reproductive isolation among populations and
46 promote allopatric differentiation leading to speciation.
47
48 **Keywords**
49 **Character displacement, converse Bergmann's rule, genital evolution, introgressive**
50 **hybridization, Japan, molecular phylogenetic analyses, reproductive interference.**
51
52

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.

77 To examine whether the evolutionary consequences of character release can be
78 variable, we studied geographical body size variation in the carabid beetle *Carabus*
79 *japonicus* Motschulsky, 1857, a species in the subgenus *Ohomopterus*, which is endemic
80 to the Japanese islands. This species occurs widely in western Japan and is sympatric with
81 the larger *Carabus dehaanii* Chaudoir, 1848 in most of its range, although it occurs singly
82 in some coastal areas and on small islands adjacent to Kyushu, the southernmost main
83 island of Japan (hereafter we treat Kyushu as the mainland). The body size of
84 *Ohomopterus* species is positively correlated with habitat temperature, exhibiting a
85 converse Bergmann cline (Sota *et al*., 2000). However, *C. japonicus* is much smaller than
86 *C. dehaanii* in sympatry, and on an island where *C. japonicus* occurs singly, it is much
87 larger than expected from temperature conditions alone, suggesting that character release
88 is taking place (Sota *et al.*, 2000). According to the most reliable molecular phylogeny,
89 the two species are not sister species (Takahashi *et al.*, 2014), and although they do not
90 now form hybrid zones, they do share mitochondrial gene sequences extensively,
91 probably because of recent introgressive hybridization (Sota & Nagata, 2008). Thus, these
92 species have undergone reproductive interference—maladaptive interspecific interactions
93 during the process of mate acquisition (Gröning & Hochkirch 2008)—and the body size
94 enlargement in allopatric *C. japonicus* is hypothesized to be a case of character release in
95 the absence of reproductive interference with *C. dehaanii*.
96 We examine the patterns and causes of geographical variation in the body size of
97 *C. japonicus* to determine whether character release has occurred in this species. First,
98 we demonstrate that the effect of sympatry/allopatry on phenotypic values is significant
99 after controlling for annual mean temperature, which is a major abiotic influence on body
100 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*

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

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

136

137 **Statistical analysis of variation in body and genital size**

138 Using the statistical package JMP version 11 (SAS Institute Inc., Cary, NC), we conducted
139 a generalized linear model (GLM) analysis with a normal distribution and identity link
140 function to study variation in body and genital dimensions (not transformed values) at
141 localities where one or more individuals of both sexes were collected. For *C. japonicus*,
142 GLM was conducted for the effects of sex, sympatry/allopatry with *C. dehaanii*, and
143 annual mean temperature on mean body length at each site. For *C. dehaanii*, GLM was
144 conducted for the effects of sex and annual mean temperature on mean body length. We
145 did not consider the effect of sympatry/allopatry with *C. japonicus* because *C. dehaanii*
146 is always sympatric with *C. japonicus* except on a few small islands (Imura & Mizusawa,
147 2013) that we did not sample. The annual mean temperature of each sampling site was
148 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)

197 analysis using JMP version 11. Maternal and paternal body lengths were separately
198 considered as explanatory variables to distinguish the case in which maternal effects
199 predominantly determine offspring body length (Falconer, 1989; Mousseau & Fox, 1998).
200 We also considered offspring sex an explanatory variable and parental pair a random
201 variable. We used body weight of first instar larva as a proxy for egg weight because eggs
202 are extremely fragile and difficult to weigh. The correlation between body length and the
203 development time of offspring was also calculated. We also examined the effect of
204 parental body length and offspring sex on the growth rate of offspring as measured by
205 body size increase (g/day) from first instar to emerging adult using GLMM with parental
206 pair as a random variable.
207
208 **Phylogeography**
209 To study the genetic composition of local populations and introgressive hybridization
210 between *C. japonicus* and *C. dehaanii*, we obtained partial sequences of the mitochondrial
211 NADH dehydrogenase gene subunit 5 (*ND5*). *ND5* sequences have been used extensively
212 in phylogeographical studies of *Ohomopterus* because of their high sequence diversity,
213 and because they are useful for detecting introgressive hybridization (Nagata *et al.*, 2007;
214 Sota & Nagata, 2008). The possession of identical haplotypes of the rapidly evolving
215 *ND5* sequence may be interpreted as evidence of recent hybridization even when no
216 hybrid swarm is observed. For comparison with the mitochondrial gene data, we also
217 sequenced a nuclear *Carab1* gene (Sota & Vogler, 2003), whose genealogy is generally
218 consistent with phylogenies established based on morphology. Mitochondrial genealogies
219 may not reflect true species phylogeny when hybridization and backcrossing lead to gene
220 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

245 analysis with 1000 replications was conducted for each gene. We also conducted an
246 analysis of molecular variances (AMOVA) for each gene using ARLEQUIN 3.0 (Excoffier
247 *et al.*, 2005) to assess gene flow due to introgressive hybridization between *C. japonicus*
248 and *C. dehaanii*. Because the sample size for each site was sometimes small, we used
249 seven and four regions for *C. japonicus* and *C. dehaanii*, respectively, by combined site
250 data (Tsushima Islands, Northern Kyushu Islands, Northern Kyushu, Goto Islands,
251 Central-southern Kyushu, Amakusa Islands, and Koshiki Islands in Fig. 1). The
252 relationships among regional populations were presented as an unrooted population tree
253 generated using PHYLIP 3.69 (Felsenstein, 2004) based on the average sequence
254 difference between populations obtained in the ARLEQUIN analysis.
255
256 **RESULTS**
257 **Geographical patterns in body and genital size**
258 Body length differed between the sexes and increased with annual mean temperature in
259 both *C. japonicus* and *C. dehaanii* (Table 1a, Fig. 2a,b). In sympatric areas, a constant
260 difference was observed in body length between the two species for each sex (Fig. 2a,b):
261 7.4 ± 1.5 mm (mean ± SD) for males (15 sites) and 8.1 ± 1.2 mm for females (22 sites).
262 The body length of *C. japonicus* was affected by sympatry/allopatry with *C. dehaanii*
263 (Table 1a; Fig. 2a, b): 22.6 ± 1.2 mm for males and 23.7 ± 1.5 mm for females in sympatric
264 populations (25 sites), and 24.7 ± 1.9 mm for males and 26.3 ± 1.9 mm for females in
265 allopatric populations (17 sites). In addition, the significant interaction effect of sympatry
266 and temperature resulted in body lengths that were longer in allopatric areas with higher
267 annual mean temperatures (Table 1a, Fig. 2a,b): 23.8 ± 0.6 mm for males and 25.3 ± 0.7
268 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

293 development time (n = 84, r = 0.58, P < 0.0001 for males; n = 90, r = 0.27, P = 0.0114
294 for females; Fig. 4c). The growth rate from first instar larva to emerging adult (g/day) was
295 larger when parental body lengths were larger (GLMM: n = 174; maternal body length,
296 d.f. = 1, F = 18.6, P = 0.0005; paternal body length, d.f. = 1, F = 12.2, P = 0.0022;
297 offspring sex, d.f. = 1, F = 39.3, P < 0.0001). Thus, enlargement of body size can be
298 attributed to increases in egg size, larval development time, and larval growth rate.
299
300 **Phylogenetic analysis in mitochondrial and nuclear DNA**
301 Individuals of *Carabus japonicus* and *C. dehaanii* were mixed in the phylogenetic tree
302 based on mitochondrial *ND5* gene sequences (Fig. 5a) but were largely separated from
303 each other in the phylogenetic tree based on nuclear *Carab1* gene sequences (Fig. 5b).
304 AMOVA showed that differentiation between species was not significant for *ND5*
305 sequences but was significant for *Carab1* sequences (see F_{CT} in Table 2). In both gene
306 sequences, the genetic differentiation among geographical regions was significant (see
307 F_{SC} in Table 2). In the trees showing the relationships among geographical regions (Fig.
308 5c), each species was monophyletic on the *Carab1* tree but not on the *ND5* tree.
309 *ND5* haplotypes were categorized into five groups, M1–M5 (Fig. 5a). Of these, M5,
310 comprising *C. japonicus* on the Tsushima Islands, was highly divergent from the M1–M4
311 haplotypes. In and around the Kyushu mainland, M3 and M4 exclusively comprised *C.*
312 *dehaanii* and *C. japonicus*, respectively, whereas M1 and M2 included members of both
313 species in Northern Kyushu and the Goto Islands (Table 3, Fig. 5a). *Carab1* sequences
314 were also divided into five groups, N1–N5 (Fig. 5b). *Carabus japonicus* had N1–N4
315 sequences, whereas most *C. dehaanii* individuals had N5 sequences. N2 sequences were
316 shared by the two species in Northern Kyushu, the Goto Islands, and Central-southern

317 Kyushu, and N5 sequences were shared in Northern Kyushu and Central-southern
318 Kyushu (Table 3, Fig. 5b).
319
320 **DISCUSSION**
321 **Character release in allopatric *C. japonicus* populations**
322 Our results are consistent with the hypothesis of character release for allopatric
323 populations of *C. japonicus* and with the expectation that evolution under character
324 release can be variable. Most island *C. japonicus* populations were derived from mainland
325 populations (Fig. 5), and body size enlargement was confirmed in some allopatric island
326 populations after controlling for the effect of habitat temperature (Table 1a, Fig. 2).
327 Further, the differences in body size between populations were heritable (Fig. 4).
328 Divergence time estimation suggests that the *C. japonicus* population on Iki Island
329 diverged from Kyushu mainland populations 15,000 years ago, the *C. japonicus* and *C.*
330 *dehaanii* populations on Fukue Island of the Goto Islands 15,000–8,000 years ago, and
331 the *C. japonicus* population on Tsushima 75,000 years ago (Sota & Nagata, 2008). Except
332 for Tsushima, the other satellite islands of Kyushu were connected to the Kyushu
333 mainland until recently, before the rising sea levels after the last glacial period, and this
334 allowed colonization of those islands by the two species. The sequence divergence of both
335 the *ND5* and *Carab1* genes occurred sequentially from the Central-southern to Northern
336 Kyushu mainland, and then to the three island regions to the north and west of the Kyushu
337 mainland (Northern Kyusyu Islands, Goto Islands, and Koshiki Islands) (Fig. 5). However,
338 the degree of sequence divergence is much smaller in the *Carab1* gene than in the *ND5*
339 gene owing to the lower divergence rate of the nuclear gene sequence. This pattern of
340 divergence may reflect the dispersal pattern of the species during the last glacial period

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

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

356

357 **Selective forces involved in body size evolution**

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

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

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

388 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.

ACKNOWLEDGEMENTS

We thank M. Hori, Y. Takami, N. Nagata and the members of Laboratory of Animal Ecology, Kyoto University for advice and discussion, T. Saitoh, C. Terada and the members of Field Science Center, Hokkaido University for rearing, and A. Ueda and people in Kyushu for sampling. We also thank K. Parr, W. Blanckenhorn, A. Giesen and two anonymous reviewers for their comments on our manuscript. This study was supported by JSPS KAHENHI grant no. 21418 and 253158 to Y.O. and 23370011 to T.S.,

and Fujiwara Natural History Foundation grant no. 19 (2011) to Y.O..

REFERENCES

Andújar, C., Serrano, J. & Gómez-Zurita, J. (2012) Winding up the molecular clock in the genus *Carabus* (Coleoptera: Carabidae): assessment of methodological decisions on rate and node age estimation. *BMC Evolutionary Biology*, **12,** 40.

Aubret, F. (2012) Body-size evolution on islands: are adult size variations in tiger snakes a nonadaptive consequence of selection on birth size? *The American Naturalist*, **179,** 756–767.

Ballard, J.W.O. & Whitlock, M.C. (2004) The incomplete natural history of mitochondria. *Molecular Ecology*, **13,** 729–744.

Blanckenhorn, W.U. & Demont, M. (2004) Bergmann and converse Bergmann latitudinal clines in arthropods: two ends of a continuum? *Integrative and Comparative Biology*, **44,** 413–424.

Boughman, J.W., Rundle, H.D. & Schluter, D. (2005) Parallel evolution of sexual isolation in sticklebacks. *Evolution*, **59,** 361–373.

Brown, W.L. & Wilson, E.O. (1956) Character displacement. *Systematic Zoology*, **5,** 49–64.

Dayan, T. & Simberloff, D. (1998) Size patterns among competitors: ecological character displacement and character release in mammals, with special reference to island populations. *Mammal Review*, **28,** 99–124.

Eberhard, W.G., Huber, B.A., Rodriguez, S.R.L., Briceño, R.D., Salas, I. & Rodriguez, V. (1998) One size fits all? Relationships between the size and degree of variation in genitalia and other body parts in twenty species of insects and spiders. *Evolution*,

52, 415–431.

Excoffier, L., Laval, G. & Schneider, S. (2005) Arlequin (version 3.0): an integrated software package for population genetics data analysis. *Evolutionary Bioinformatics Online*, **1,** 47–50.

Falconer, D.S. (1989) *Introduction to quantitative genetics*, 3rd edn. Longman Scientific & Technical, Harlow, UK.

Felsenstein, J. (2004) *PHYLIP (phylogeny inference package) version 3.69*. Distributed by the author. Department of Genome Sciences, University of Washington, Seattle, WA.

Funk, D.J. & Omland, K.E. (2003) Species-level paraphyly and polyphyly: frequency, causes, and consequences, with insights from animal mitochondrial DNA. *Annual Review of Ecology, Evolution, and Systematics*, **34,** 397–423.

Funk, D.J., Nosil, P. & Esges, W.J. (2006) Ecological divergence exhibits consistently positive associations with reproductive isolation across disparate taxa. *Proceedings of the National Academy of Sciences USA*, **28,** 3209–3213.

Grant, P.R. (1972) Convergent and divergent character displacement. *Biological Journal of the Linnean Society*, **4,** 39–68.

Grant, P.R. & Grant, B.R. (2006) Evolution of character displacement in Darwin's finches. *Science*, **313,** 224–226.

Grether, G.F., Losin, N., Anderson, C.A. & Okamoto, K. (2009) The role of interspecific interference competition in character displacement and the evolution of competitor recognition. *Biological Reviews*, **84,** 617–635.

Gröning, J. & Hochkirch, A. (2008) Reproductive interference between animal species. *The Quarterly Review of Biology*, **83,** 257–282.

Imura, Y. & Mizusawa, K. (2013) *The* Carabus *of Japan*. Roppon-ashi, Tokyo, Japan.

Ishikawa, R. (1987) On the function of copulatory organs of *Ohomopterus* (Coleoptera, Carabidae, genus *Carabus*). *Kontyu*, **55,** 202–206.

Kubota, K. & Sota, T. (1998) Hybridization and speciation in the carabid beetles of the subgenus *Ohomopterus* (Coleoptera, Carabidae, genus *Carabus*). *Researches on Population Ecology*, **40,** 213–222.

Kubota, K., Miyazaki, K., Ebihara, S. & Takami Y. (2013) Mechanical reproductive isolation via divergent genital morphology between *Carabus insulicola* and *C. esakii* with implications in species coexistence. *Population Ecology*, **55,** 35–42.

Masaki, S. (1967) Geographic variation and climatic adaptation in a field cricket (Orthoptera: Gryllidae). *Evolution*, **21,** 725–741.

Meiri, S., Dayan, T. & Simberloff, D. (2007) Guild composition and mustelid morphology – character displacement but not character release. *Journal of Biogeography*, **34,** 2148–2158.

Meiri, S., Simberloff, D. & Dayan, T. (2011) Community-wide character displacement in the presence of clines: a test of Holarctic weasel guilds. *Journal of Animal Ecology*, **80,** 824–834.

Mousseau, T. A. & Fox, C. W. (1998) The adaptive significance of maternal effects. *Trends in Ecology & Evolution*, **13,** 403–407.

Nagata, N., Kubota, K., Yahiro, K. & Sota, T. (2007) Mechanical barriers to introgressive hybridization revealed by mitochondrial introgression patterns in *Ohomopterus* ground beetle assemblages. *Molecular Ecology*, **16,** 4822–4836.

Okuzaki, Y., Takami, Y. & Sota, T. (2010) Resource partitioning or reproductive isolation: the ecological role of body size differences among closely related species in

510 sympatry. *Journal of Animal Ecology*, **79,** 383–392.
511 Pfennig, D.W. & Murphy, P.J. (2000) Character displacement in polyphenic tadpoles.
512 *Evolution*, **54,** 1738–1749.
513 Pfennig, K.S. & Pfennig, D.W. (2009) Character displacement: ecological and
514 reproductive responses to a common evolutionary problem. *Quarterly Review of*
515 *Biology*, **84,** 253–276.
516 Radtkey, R.R., Fallon, S.M. & Case, T.J. (1997) Character displacement in some
517 *Cnemidophorus* lizards revisited: a phylogenetic analysis. *Proceedings of the*
518 *National Academy of Sciences USA*, **94,** 9740–9745.
519 Rice, A.M. & Pfennig, D.F. (2007) Character displacement: *in situ* evolution of novel
520 phenotypes or sorting of pre-existing variation? *Journal of Evolutionary Biology*,
521 **20,** 448–459.
522 Rice, A.M. & Pfennig, D.F. (2008) Analysis of range expansion in two species
523 undergoing character displacement: why might invaders generally 'win' during
524 character displacement? *Journal of Evolutionary Biology*, **21,** 696–704.
525 Robinson, B.W., Wilson, D.S. & Margosian, A.S. (2000) A pluralistic analysis of
526 character release in pumpkinseed sunfish (*Lepomis gibbosus*). *Ecology*, **81,** 2799–
527 2812.
528 Roff, D. (1980) Optimizing development time in a seasonal environment: the 'ups and
529 downs' of clinal variation. *Oecologia*, **45,** 202–208.
530 Schluter, D. (2000) *The ecology of adaptive radiation*. Oxford University Press, Oxford.
531 Schluter, D. & McPhail, J.D. (1992) Ecological character displacement and speciation in
532 sticklebacks. *The American Naturalist*, **140,** 85–108.
533 Schluter, D. & Nagel, L.M. (1995) Parallel speciation by natural selection. *The American*

534 *Naturalist*, **146,** 292–301.

535 Simberloff, D., Dayan, T., Jones, C. & Ogura, G. (2000) Character displacement and
536 release in the small Indian mongoose, *Herpestes javanicus*. *Ecology*, **81,** 2086–
537 2099.

538 Sota, T. (1985a) Activity patterns, diets and interspecific interactions of coexisting spring
539 and autumn breeding carabids: *Carabus yaconinus* and *Leptocarabus kumagaii*
540 (Coleoptera, Carabidae). *Ecological Entomology*, **10,** 315–324.

541 Sota, T. (1985b) Life history patterns of carabid beetles belonging to the subtribe Carabina
542 (Coleoptera, Carabidae) in the Kinki district, western Japan. *Kontyu*, **53,** 370–378.

543 Sota, T. & Kubota, K. (1998) Genital lock-and-key as a selective agent against
544 hybridization. *Evolution*, **52,** 1507–1513.

545 Sota, T. & Vogler, A.P. (2001) Incongruence of mitochondrial and nuclear gene trees in
546 the carabid beetles *Ohomopterus*. *Systematic Biology*, **50,** 39–59.

547 Sota, T. & Vogler, A.P. (2003) Reconstructing species phylogeny of the carabid beetles
548 *Ohomopterus* using multiple nuclear DNA sequences: heterogeneous information
549 content and the performance of simultaneous analyses. *Molecular Phylogenetics*
550 *and Evolution*, **26,** 139–154.

551 Sota, T. & Nagata, N. (2008) Diversification in a fluctuating island setting: rapid radiation
552 of *Ohomopterus* ground beetles in the Japanese islands. *Philosophical Transactions*
553 *of the Royal Society B: Biological Sciences*, **363,** 3377–3390.

554 Sota, T., Takami, Y., Kubota, K., Ujiie, M. & Ishikawa, R. (2000) Interspecific body size
555 differentiation in species assemblages of the carabid subgenus *Ohomopterus* in
556 Japan. *Population Ecology*, **42,** 279–291.

557 Sota, T., Takami, Y., Kubota, K. & Ishikawa, R. (2002) Geographic variation in body size

of carabid beetles: patterns resulting from climatic adaptation and interspecific interaction. *Japanese Journal of Entomology*, **5,** 88–97.

Stamatakis, A. (2014) RAxML version 8: a tool for phylogeneic analysis and post-analysis of large phylogenies. *Bioinformatics*, **30,** 1312–1313.

Su, Z.H., Ohama, T., Okada, T.S., Nakamura, K., Ishikawa, R. & Osawa, S. (1996) Phylogenetic relationships and evolution of the Japanese Carabinae ground beetles based on mitochondrial ND5 gene sequences. *Journal of Molecular Evolution*, **42,** 124–129.

Takahashi, T., Nagata, N. & Sota, T. (2014) Application of RAD-based phylogenetics to complex relationship among variously related taxa in a species flock. *Molecular Phylogenetics and Evolution*, **80,** 137–144.

Takami, Y. (2002) Mating behavior, insemination and sperm transfer in the ground beetle *Carabus insulicola*. *Zoological Science*, **19,** 1067–1073.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. & Kumar, S. (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution*, **28,** 2731–2739.

Tanabe, T. & Sota, T. (2008) Complex copulatory behavior and the proximate effect of genital and body size differences on mechanical reproductive isolation in the millipede genus *Parafontaria*. *The American Naturalist*, **171,** 692–699.

Taylor, E.B. & McPhail, J.D. (2000) Historical contingency and ecological determinism interact to prime speciation in sticklebacks, *Gasterosteus*. *Proceedings of the Royal Society B: Biological Sciences*, **267,** 2375–2384.

Tsuchiya, Y., Takami, Y., Okuzaki, Y. & Sota, T. (2012) Genetic differences and

phenotypic plasticity in body size between high- and low-altitude populations of the ground beetle *Carabus tosanus*. *Journal of Evolutionary Biology*, **25,** 1835–1842.

Wojcieszek, J.M. & Simmons, L.W. (2013) Divergence in genital morphology may contribute to mechanical reproductive isolation in a millipede. *Ecology and Evolution*, **3,** 334–343.

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.

606

607 Editor: Kate Parr

608

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

690 **FIGURE LEGENDS**

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

698

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

706

707 Figure 3. Correlations between body length and genital dimensions in males (a, c, e) and
708 females (b, d, f) of *Carabus japonicus* and *C. dehaanii*. (a) Male body length and
709 aedeagus length (n = 42, r = 0.94, P < 0.0001 for *C. japonicus*, n = 17, r = 0.97, P <
710 0.0001 for *C. dehaanii*), (b) female body length and vaginal length (n = 42, r = 0.89, P <
711 0.0001 for *C. japonicus*, n = *17*, r = 0.57, P = 0.0175 for *C. dehaanii*), (c) male body
712 length and copulatory piece length (r = 0.88, P < 0.0001 for *C. japonicus*, r = 0.53, P =
713 0.0278 for *C. dehaanii*), (d) female body length and vaginal appendix length (r = 0.61, P

714 < 0.0001 for *C. japonicus*, $r = 0.07$, $P = 0.7895$ for *C. dehaanii*), (e) aedeagus length and
715 copulatory piece length ($r = 0.87$, $P < 0.0001$ for *C. japonicus*, $r = 0.61$, $P = 0.0090$ for
716 *C. dehaanii*) and (f) vaginal length and vaginal appendix length ($r = 0.56$, $P = 0.0001$ for
717 *C. japonicus*, $r = –0.06$, $P = 0.8239$ for *C. dehaanii*). Red, blue and black circles represent
718 *C. japonicus* in sympatry, *C. japonicus* in allopatry and *C. dehaanii*, respectively. Filled
719 and open circles represent populations on Kyushu mainland and satellite islands,
720 respectively.
721
722 Figure 4. Body size and development time of laboratory-reared *Carabus japonicus* in a
723 common garden experiment. (a) Body weight of first-instar larva in relation to body
724 length of female-parent; (b) body length of adult offspring in relation to body length of
725 mid-parent; and (c) body length of adult offspring in relation to the development time.
726 Diamonds, triangles, squares and circles represent original sites 6, 7, 5 and 3, respectively,
727 and filled and open symbols represent male and female offspring, respectively. Error bars
728 represent standard deviation.
729
730 Figure 5. Molecular phylogeny of *Carabus japonicus* and *C. dehaanii* in Kyusyu. (a)
731 Phylogeny of mitochondrial *ND5* and geographical frequencies of haplotype groups, (b)
732 phylogeny of nuclear *Carab1* and geographical frequencies of haplotype groups.
733 Operational taxonomic unit (OUT) represented by grey and black bars are *C. japonicus*
734 and *C. dehaanii*, respectively. *Carabus daisen* is the outgroup taxon. Numerals on
735 branches are bootstrap percentages (shown when >50%). (c) Unrooted population tree
736 based on average sequence difference of mitochondrial *ND5* and nuclear *Carab1* between
737 populations of seven regions in Fig. 1. [jap] and [deh] represents *C. japonicus* and *C.*

738 *dehaanii*, respectively.

739

740

741

742

743

744

745

746

747

748

749

750

751

752

753

754

755

756

757

758

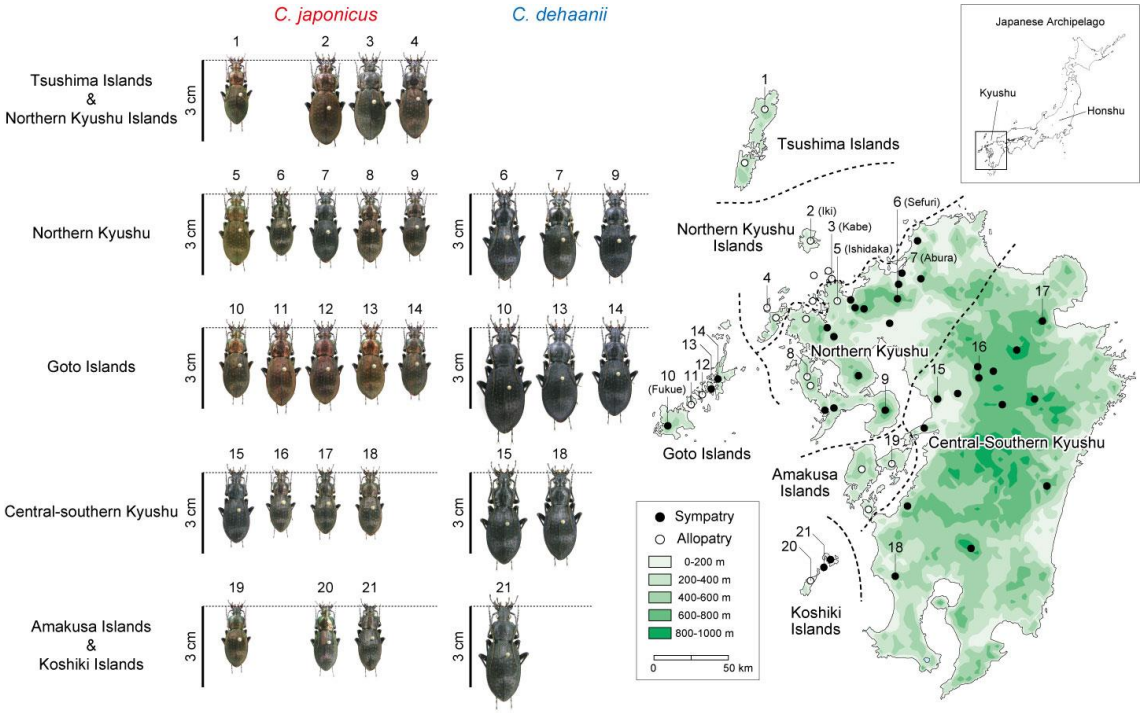
759

760

761

762 Figure. 1

763



764

765

766

767

768

769

770

771

772

773

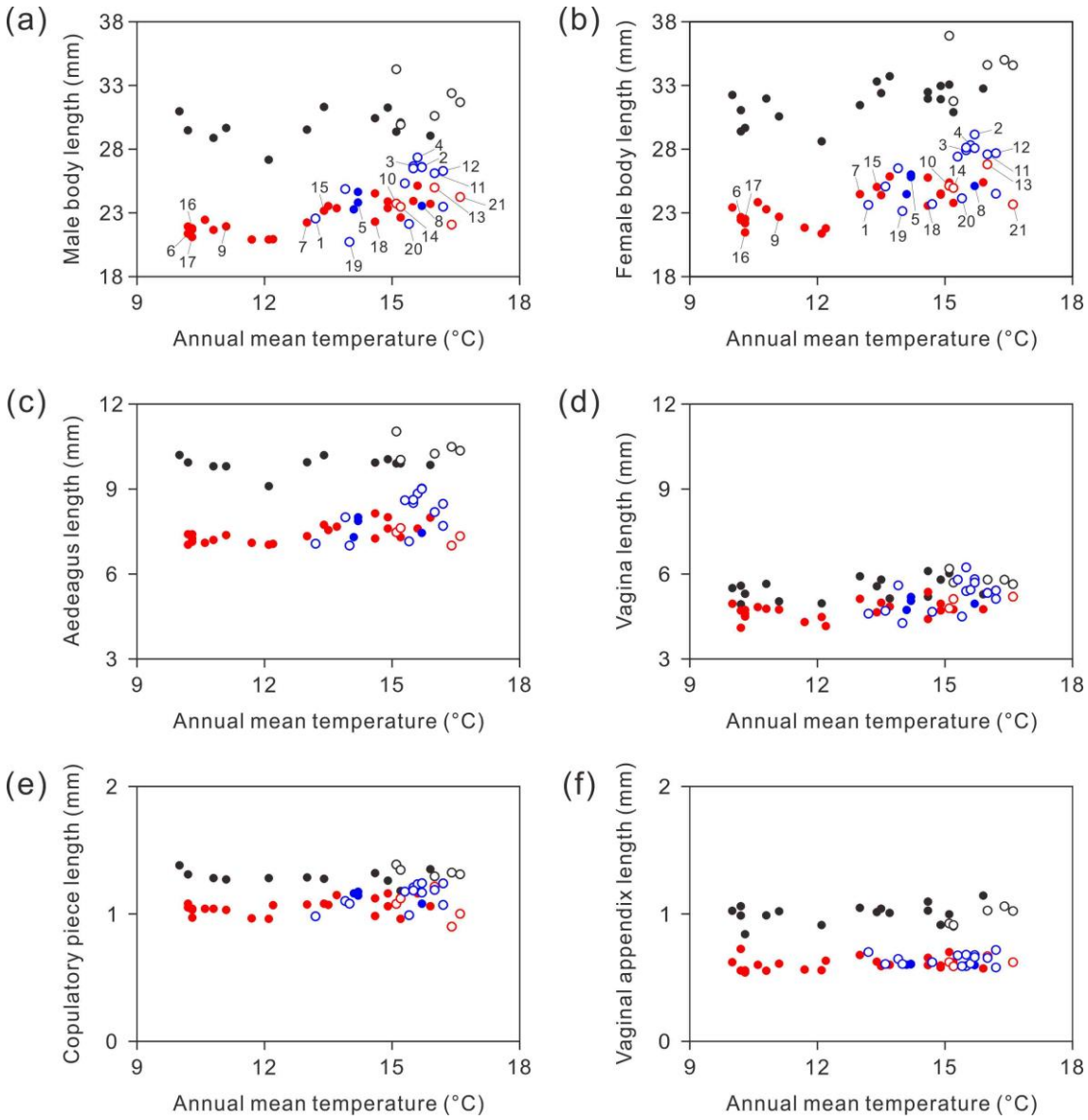
774

775

776

777 Figure 2.

778



779

780

781

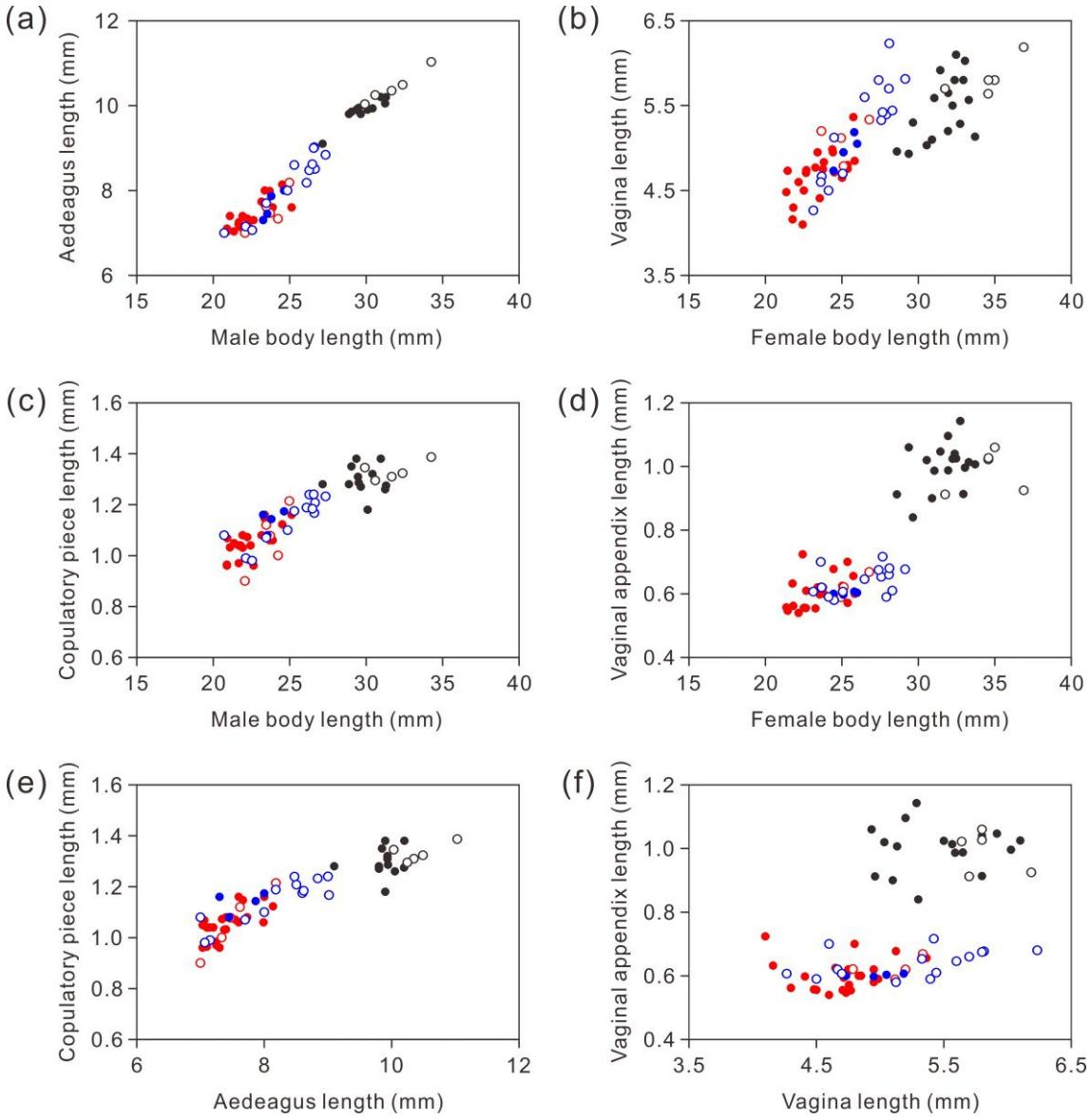
782

783

784

785 Figure 3.

786



787

788

789

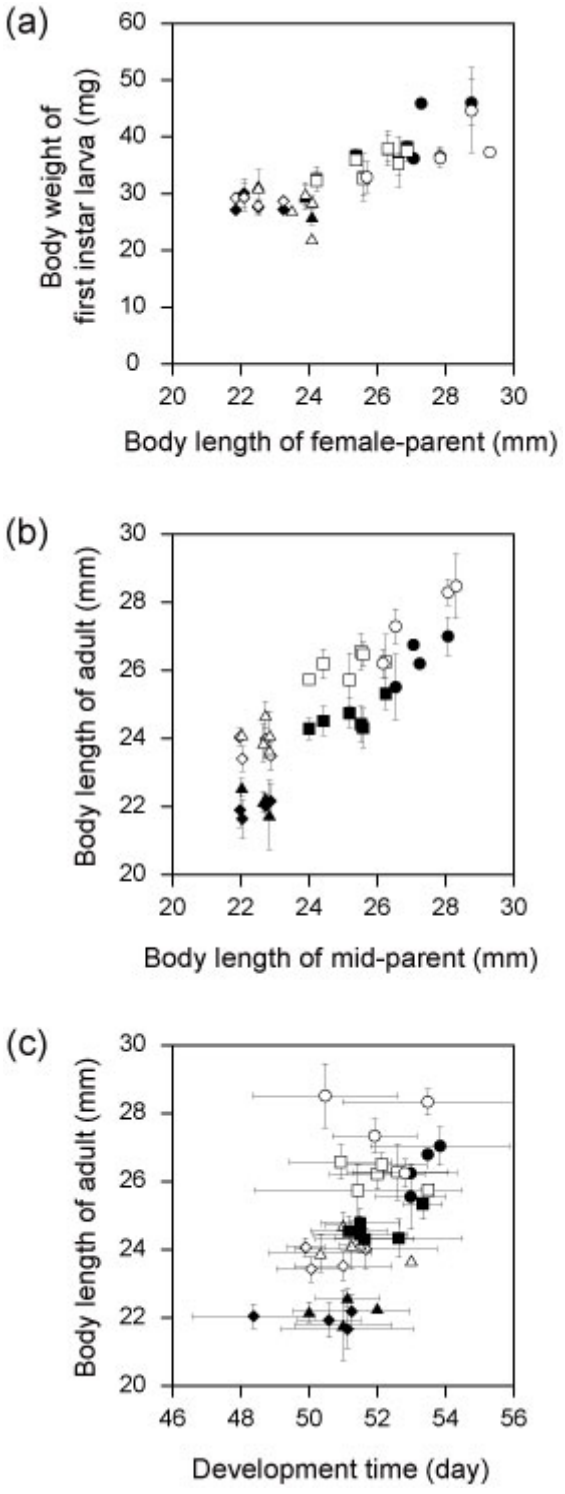
790

791

792

793 Figure 4.

794

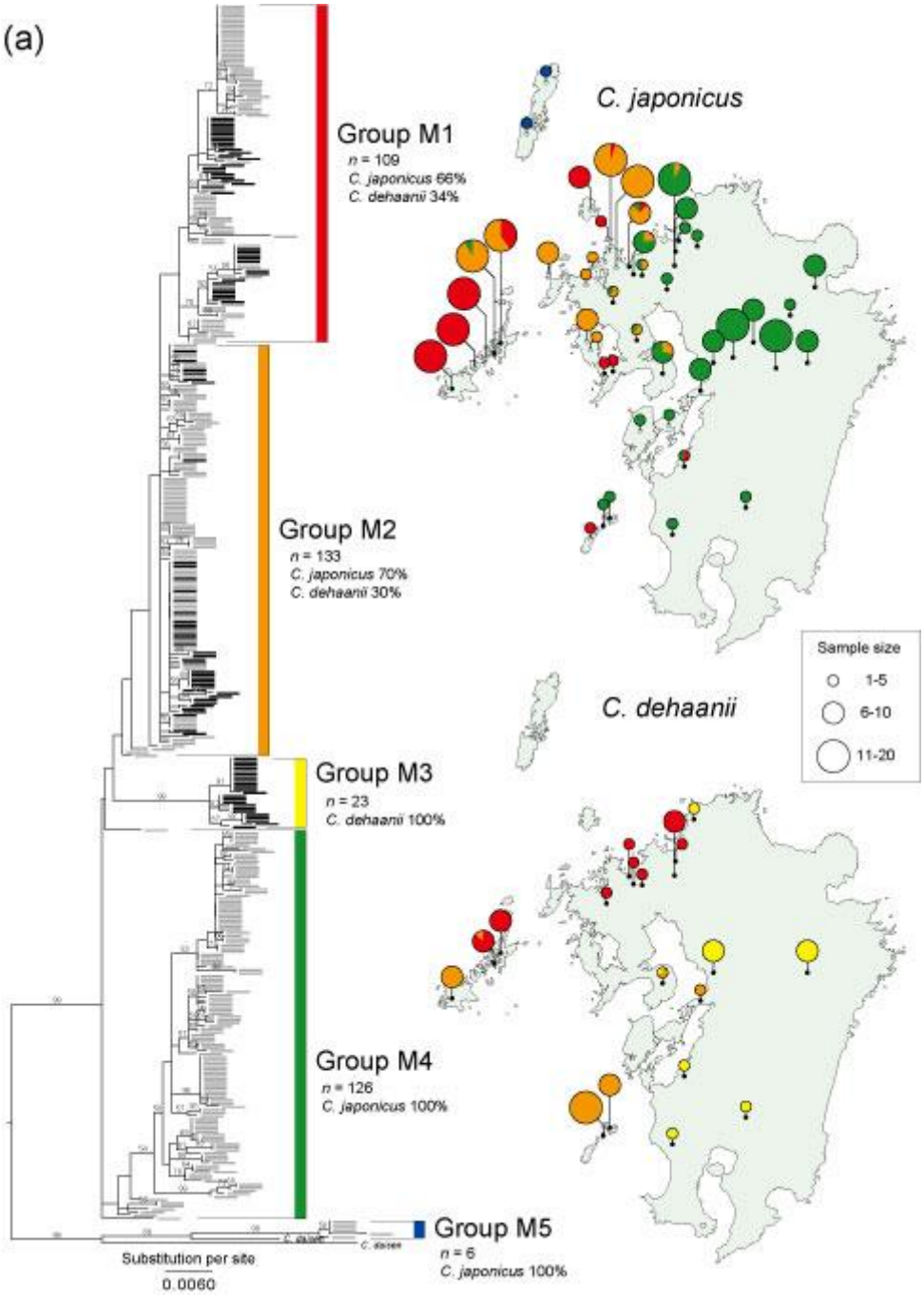


795

796

797 Figure 5.

798

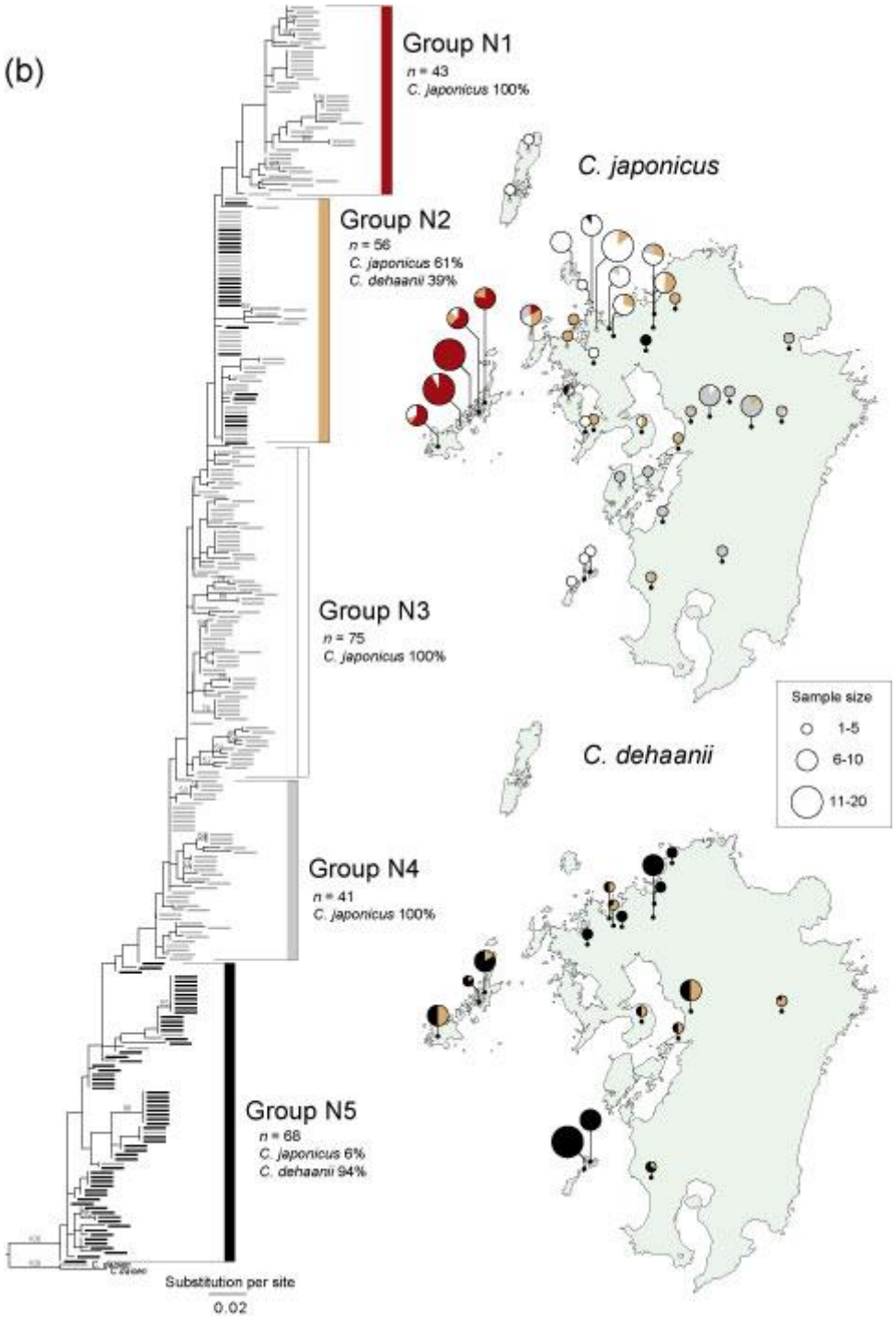


799

800

801

802



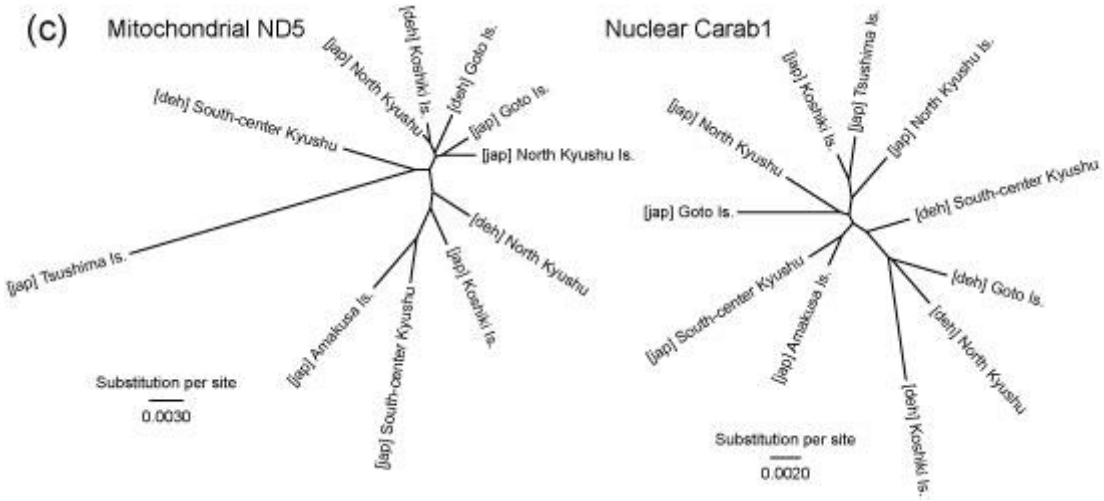
803

804

805

806

807


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

Journal of Biogeography

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
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)

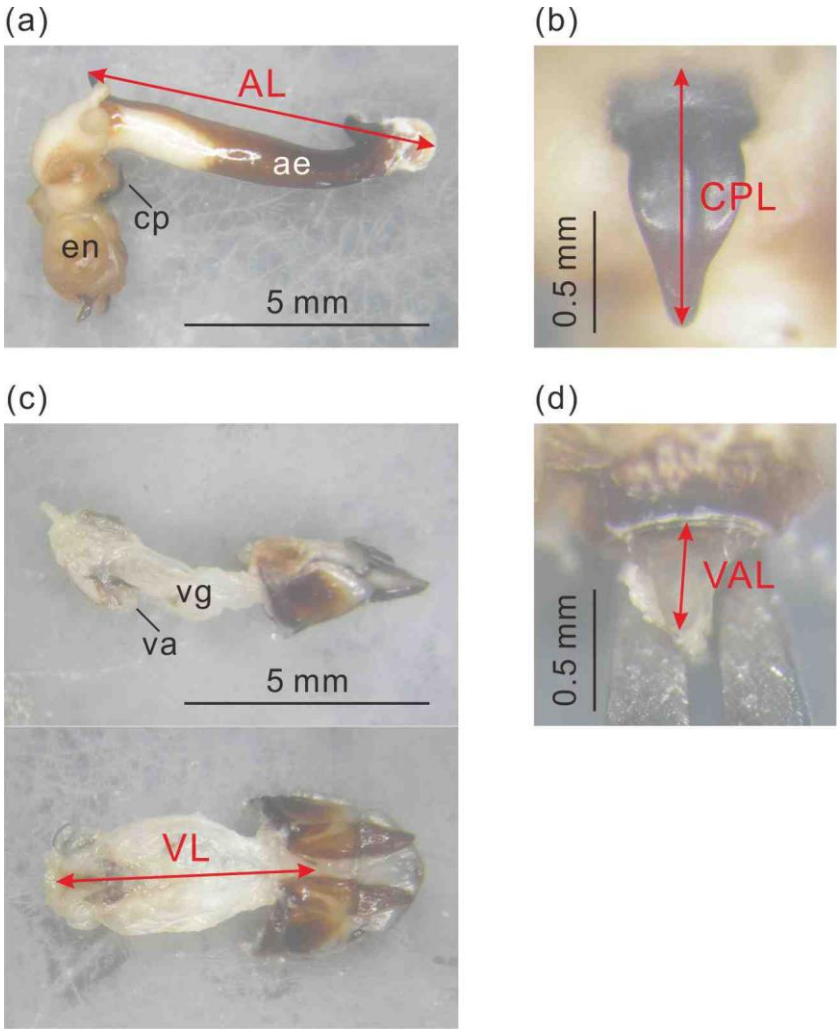
	C. japonicus												*C. dehaanii*											
Region	MBL (mm)		AL (mm)		CPL (mm)		FBL (mm)		VL (mm)		VAL (mm)		MBL (mm)		AL (mm)		CPL (mm)		FBL (mm)		VL (mm)		VAL (mm)	
No.	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Tsushima islands																								
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.