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

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20   **ABSTRACT**

21   **Aim** We tested the hypothesis of character release in body size among allopatric

22   populations of the carabid beetle *Carabus japonicus* by analysing geographical variation

23   in body size in relation to habitat temperature and sympatry/allopatry with the larger

24   congeneric species *Carabus dehaanii*.

25   **Location** The main and satellite islands of Kyushu in the south-western part of the

26   Japanese archipelago.

27   **Methods** We studied geographical variation in body length and genital size of *C.

28   japonicus* populations at different sites to examine the effects of both habitat temperature

29   and sympatry/allopatry with *C. dehaanii*. To determine whether the conditions for

30   character release were fulfilled, we then estimated heritable differences in body size by

31   common garden rearing, and estimated phylogenetic relationships among populations by

32   molecular phylogenetic analysis.

33   **Results** While body size was positively correlated with annual mean temperature, it was

34   consistently small in sympatry but larger in some allopatric populations in warmer regions.

35   The body size differences among populations were heritable. Allopatric *C. japonicus*

36   populations on satellite islands were derived from the sympatric mainland populations.

37   In a few sympatric areas, mitochondrial haplotypes were shared between the species

38   because of introgressive hybridization, suggesting the occurrence of reproductive

39   interference between the species. We also found that genital size was not affected by

40   sympatry/allopatry but was positively correlated with body size.

41   **Main conclusions** We demonstrated that the increased body size of allopatric *C.

42   japonicus* has evolved through a character release process. However, not all allopatric

43   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

101 differences in phenotypic values between sympatric and allopatric populations are

102 heritable, by performing a common garden experiment in the laboratory. Third, we

103 demonstrate that the allopatric populations exhibiting character shift are derived from the

104 sympatric populations, by performing phylogeographical analyses using mitochondrial

105 and nuclear gene sequences. These molecular markers also suggest the occurrence of

106 introgressive hybridization (Nagata *et al.*, 2007; Sota & Nagata, 2008). Although we are

107 primarily concerned with body size (whole body length) as it affects precopulatory

108 reproductive isolation, we also analysed variation in genital dimensions because they can

109 contribute to prezygotic reproductive isolation in *Ohomopterus* (Nagata *et al.*, 2007;

110 Kubota *et al.*, 2013).

111

112 **MATERIALS AND METHODS**

113 **Sampling and measurement of morphology**

114 The endemic ground beetles of the subgenus *Ohomopterus* in Japan (Coleoptera,

115 Carabidae, genus *Carabus*) comprise 15 or more species and show marked variation in

116 body size and genital morphology (Sota *et al.*, 2000; Sota & Nagata, 2008). These species

117 are ecologically equivalent, having the same diet and seasonal life cycle (i.e., larvae are

118 specialized earthworm eaters and spring breeders with a univoltine life cycle; Sota,

119 1985a). To study body size variation, adult beetles of *C. japonicus* and *C. dehaanii* were

120 collected using pitfall traps on the Kyushu mainland and adjacent islands from 2009 to

121 2012 (Fig. 1). *Carabus japonicus* were collected at 53 sites shown in Fig. 1 (see Appendix

122 S1 in Supporting Information for details). Of these sites, we defined 34 and 19 sites as

123 sympatric and allopatric sites, respectively, based on the distribution of *C. dehaanii*

124 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

149 meteorological data collected from 1971 to 2000 (Mesh Climatic Data 2000; Japan

150 Meteorological Business Support Center). For *C. japonicus*, GLM was also conducted

151 for the effects of male body length and sympatry/allopatry with *C. dehaanii* on the mean

152 lengths of the aedeagus and the copulatory piece, and for the effects of female body length

153 and sympatry/allopatry with *C. dehaanii* on the mean length of the vagina and the vaginal

154 appendix. The correlation between body length and genital dimensions was also

155 calculated in each sex of both species. To account for the different sample sizes, the

156 response variables (body and genital dimensions) were weighted by the number of

157 samples at each site.

158

159 **Common garden experiment in the laboratory**

160 To determine the extent to which the geographical variation in body size in *C. japonicus*

161 is heritable, we conducted a common garden experiment by bringing field-caught beetles

162 into the laboratory and raising their offspring from eggs to adults during 2010–2012. We

163 assumed that populations geographically and genetically proximate to one another are

164 appropriate to detect genetic differences in body size. We used beetles from four sites in

165 northern Kyushu representing a wide range of body sizes: site 6 (Mt. Sefuri, altitude 950–

166 1030 m, annual mean temperature 10.2°C); site 7 (Mt. Abura, 550–597 m, 13.0°C); site

167 5 (Mt. Ishidaka, 250–284 m, 14.2°C); and site 3 (Kabe Island, 20–90 m, 15.5°C) (see Fig.

168 1 for site numbers). *Carabus japonicus* was sympatric with *C. dehaanii* at sites 6 and 7,

169 and allopatric at sites 5 and 3.

170 Parental adult beetles used in the experiment were collected using pitfall traps and

171 were transported to our laboratory at Kyoto University within 3 days. The beetles were

172 housed individually in plastic cups (9 cm diameter, 4 cm depth) with moistened moss,

173 and the cups were stored in an incubator set at a long-day photoperiod [light:dark (LD) h,

174 16:8] and at 20°C from the time of their capture until November. The beetles were fed

175 with minced beef every 2 days. Thereafter, temperature and day length were gradually

176 decreased toward an overwintering condition of 5°C and complete darkness from

177 December through April. In May, the photoperiod, temperature, and feeding regimes were

178 gradually restored to their initial states over the course of the month. Subsequently, pairs

179 of sexually mature males and females from the same site were transferred to plastic cups

180 (12 cm diameter, 5 cm depth) with humic soil and moistened moss and were allowed to

181 copulate and deposit eggs in the humic soil.

182 The common garden experiment was performed in an incubator at LD 16:8 and at

183 a temperature (20°C) close to the monthly mean temperature in July (20.8°C) at site 6

184 where *C. japonicus* larvae occurred (Y.O., unpublished data). The eggs deposited in the

185 soil were collected and kept in plastic cups with humic soil until hatching. Hatched larvae

186 were reared individually in plastic cups (7.5 cm diameter, 3.5 cm depth) with humic soil

187 and were supplied with a sufficient number of earthworms. The larvae had three instars

188 and pupated in the soil. After eclosion, adults appeared on the soil surface after several

189 days. The development of each individual was observed once a day from first instar

190 through emerging adult, and its body weight was measured within 1 day after hatching

191 (first instar), after moulting (second and third instar), and after appearing on the soil

192 surface as an adult. We defined the development time as the number of days from

193 oviposition to the appearance of the adult. The body length of adults was measured

194 approximately 30 days after emergence when their exoskeletons hardened.

195 The effect of parental body length on the weight of first instar larvae and on adult

196 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

221 relationships among species due to the dilution of the effects of introgressive

222 hybridization by recombination and slow mutation accumulation (Funk & Omland, 2003;

223 Ballard & Whitlock, 2004). Phylogenetic analyses based on both mitochondrial and

224 nuclear genes should be useful for elucidating the evolutionary history of closely related

225 species involved in reproductive character displacement and release.

226   Total DNA was extracted from the gonads of individual beetles using a Wizard

227 Genomic DNA Purification Kit (Promega, Madison, WI). For PCR and dye terminator

228 cycle sequencing reactions, we used the following primer sets: *ND5*, forward (6-1): 5′-

229 CCT GTT TCT GCT TTA GTT CA-3′; reverse (4-1): 5′-GCT ATA CTC TAA ATA

230 TAA GCT A-3′ (Su *et al.*, 1996); *Carab1*, forward (gwnck1): 5′-GTG ACG AAC AAG

231 AAG ATA TGG-3′ (Andújar *et al.*, 2012); reverse (CARCK2): 5′-GTG GTT CGC ATC

232 TCA ACA GA-3′ (Sota & Vogler, 2001). PCR was performed using the following

233 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

234 7 min at 72°C. The PCR products were treated with ExoSap-IT and subjected to a dye

235 terminator cycle sequencing reaction with BigDye version 3.1. The products were

236 electrophoresed on an ABI3130XL sequencer (Applied Biosystems, Foster City, CA).

237 Alignment was performed with MEGA 5 (Tamura *et al.*, 2011). We determined 1024 bp

238 for *ND5* and 548 bp, excluding an alignment ambiguous region, for *Carab1*. Sequence

239 data have been deposited at the DNA Data Bank of Japan (DDBJ; accession numbers

240 LC008547–LC008720 for *ND5*; LC008721–LC009001 for *Carab1*).

241   For both the *ND5* and *Carab1* gene sequences, we conducted a maximum-

242 likelihood (ML) analysis using RAxML 8.0.20 (Stamatakis, 2014). Each gene sequence

243 was partitioned according to three codon positions, and a general time-reversible

244 (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 $\pm$ 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

269 habitats at high altitudes, and 25.3 ± 0.7 mm for males and 26.6 ± 2.0 mm for females on

270 the satellite islands (13 sites), consisting of warm habitats at low altitudes. Thus, some

271 allopatric populations on the Northern Kyushu Islands and Goto Islands had longer body

272 lengths than sympatric populations, whereas other allopatric populations had similar body

273 lengths to sympatric populations (e.g., Tsushima and Amakusa Islands) (Fig. 1, Fig. 2a,b).

274 The ratio of the maximum to minimum mean body length of *C. japonicus* populations

275 was 1.32 for males and 1.36 for females across all the study sites. The genital dimensions

276 of *C. japonicus* were positively correlated with body length irrespective of

277 sympatry/allopatry with *C. dehaanii* (Table 1b, Fig. 2c–f, Fig. 3). Thus, like body length,

278 genital dimensions showed a notable divergence among populations.

279

280 **Heritable differences in body size**

281 Larger female *C. japonicus* oviposited larger eggs leading to larger first instar larvae at

282 hatching (GLMM: $n = 174$; maternal body length, d.f. = 1, $F = 7.0$, $P = 0.0187$; paternal

283 body length, d.f. = 1, $F = 2.5$, $P = 0.1339$; offspring sex, d.f. = 1, $F = 0.6$, $P = 0.4459$; Fig.

284 4a, Appendix S3). Both maternal and paternal body lengths significantly affected

285 offspring adult body length (GLMM: $n = 174$; maternal body length, d.f. = 1, $F = 29.8$, P

286 < 0.0001; paternal body length, d.f. = 1, $F = 16.4$, $P = 0.0007$; offspring sex, d.f. = 1, $F =$

287 318.3, $P < 0.0001$; Fig. 4b, Appendix S3), suggesting that there is genetic variation in

288 body length, although maternal effects may also be involved. The regression of mean

289 offspring body length against mid-parent body length (mean of parental body lengths)

290 had slopes close to unity for both sexes ($n = 18$, $b = 0.84$, $t = 16.7$, $P < 0.0001$ for males;

291 $n = 19$, $b = 0.76$, $t = 14.2$, $P < 0.0001$ for females), indicating that body size differences

292 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

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.

389   2a,b), implying that factors other than habitat temperature and the absence of *C. dehaanii*

390   affect the evolution of body size. For solitary carnivores, prey size would influence

391   predation success and survival rate, especially in early developmental stages with small

392   body sizes, and body size variation in adults may be the result of natural selection during

393   the early developmental stages (Aubret, 2012). Larvae of *Ohomopterus* are specialized

394   predators of earthworms (Sota, 1985a), and the predation success of the first instar larvae

395   decreases with an increase in earthworm size in small-sized species (Okuzaki *et al.*, 2010).

396   Because larger *C. japonicus* females produce larger eggs which result in larger first instar

397   larvae (Fig. 4a), geographical variation in earthworm size may account for the body size

398   variation among allopatric populations (Sota *et al.*, 2000; Y.O., unpublished data). An

399   alternative hypothesis for the lack of body size enlargement in some allopatric *C.*

400   *japonicus* populations is that the allopatry may be very new, as it would be if a local

401   sympatric population of *C. dehaanii* had recently become extinct.

402

403   **Consequences of body size divergence**

404   Body size is a key trait for reproductive isolation in some animal species (e.g., Schluter

405   & Nagel, 1995; Boughman *et al.*, 2005; Funk *et al.*, 2006), and this is particularly true for

406   *Ohomopterus* species (Sota & Nagata, 2008). Over most of the range of *Ohomopterus*,

407   two or three (rarely four or five) different-sized species co-occur sympatrically (Sota *et*

408   *al.*, 2000), whereas similar-sized species are parapatric due to reproductive interference,

409   sometimes forming narrow hybrid zones (Kubota & Sota, 1998). Interspecific body size

410   ratio in two-species assemblages of *Ohomopterus* ranges from 1.15 to 1.28 for female

411   body length (Sota *et al.*, 2000), which is generally large enough to prevent insemination

412   in the laboratory (Okuzaki *et al.*, 2010). The ratio of body length difference between *C.*

413   *japonicus* populations in this study was as high as 1.36 for females. Therefore, the

414   divergence in body size among populations of *C. japonicus* may result in allopatric

415   speciation within this species in such a way that gene flow between populations during

416   occasional secondary contacts is restricted.

417   In addition, we have shown that the mean genital dimensions were correlated with

418   mean body length among *C. japonicus* populations (Table 1b, Fig. 3). In general,

419   stabilizing selection acts on genital dimensions within populations and leads to low

420   allometry of genital dimensions on body size (Eberhard *et al.*, 1998), but this does not

421   apply to body size divergence among populations. Divergence in genital dimensions can

422   also promote speciation via the enhancement of mechanical isolation in some arthropod

423   groups including *Ohomopterus* (Sota & Kubota, 1998; Tanabe & Sota, 2008; Wojcieszek

424   & Simmons, 2013; Kubota *et al.*, 2013). Interestingly, genital diversification and

425   speciation in *Ohomopterus* have occurred most markedly in a lineage including species

426   with medium to large body sizes (Sota & Nagata, 2008). Thus, body size divergence in

427   *Ohomopterus* may be associated with divergence in genital dimensions and may promote

428   speciation.

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589   **SUPPORTING INFORMATION**

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

591   **Appendix S1** Information about sampling sites, sample sizes and morphological

592   dimensions.

593   **Appendix S2** Genital morphology of *Carabus japonicus*.

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

595

596   **BIOSKETCHES**

597   **Yutaka Okuzaki** is a postdoctoral research fellow of the Japan Society for the Promotion

598   of Science at Hokkaido University and is interested in trait evolution caused by

599   interspecific interactions and its involvement in species coexistence and speciation.

600

601   **Hisashi Sugawara** is a doctoral student at Kyoto University and studies physiological

602   ecology of carabid beetles.

603

604   **Teiji Sota** is a professor at Kyoto University and is interested in phylogeography,

605   diversification and speciation of carabid beetles and other invertebrates.

606

607   Editor: Kate Parr

608

609   Table 1. (a) Effects of environmental annual mean temperature, sex and

610   sympatry/allopatry on the mean body length in *Carabus japonicus* and *C. dehaanii*. (b)

611   Effects of body length and sympatry/allopatry on the genital size in *C. japonicus*.

612

613   (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

614–621 (row labels)

622

623   (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

640

641 Table 2. Analysis of molecular variance for the genetic differentiation between *Carabus*

642 *japonicus* and *C. dehaanii* in two gene sequences. Regions and populations were

643 represented in Fig. 1. Note that *C. japonicus* and *C. dehaanii* occurred in seven and four

644 regions, respectively.

645

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

656

657 F_{CT}, between species; F_{SC}, among populations within species; F_{ST}, among populations.

658 The *P*-value was obtained by 1000 permutations.

659

660

661 Table 3. Number of individuals belonging to each haplotype groups of mitochondrial gene

662 *ND5* (M1-M5 in Fig. 5a) and nuclear gene *Carab1* (N1-N5 in Fig. 5b) for *Carabus*

663 *japonicus* and *C. dehaanii* occurring in seven regions (Fig. 1). Bold numerals emphasize

664 shared sequence groups in each region.

665

Region	Species	*ND5* group					*Carab1* group				
		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

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689

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.

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762   Figure. 1

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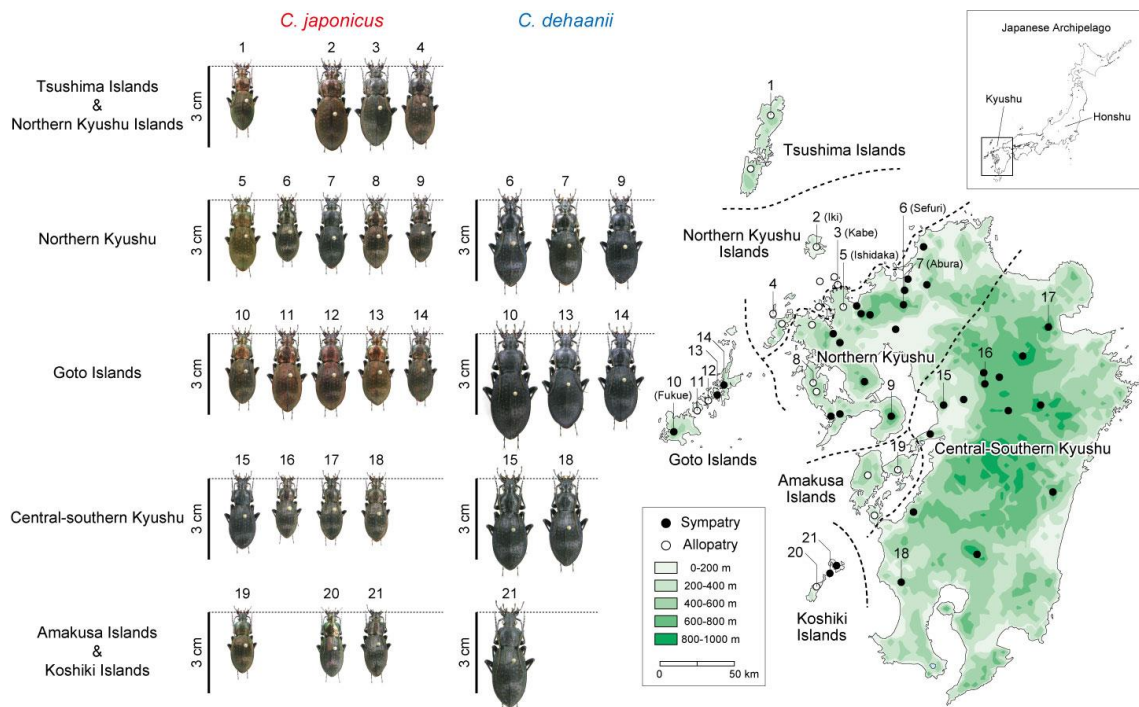
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777   Figure 2.

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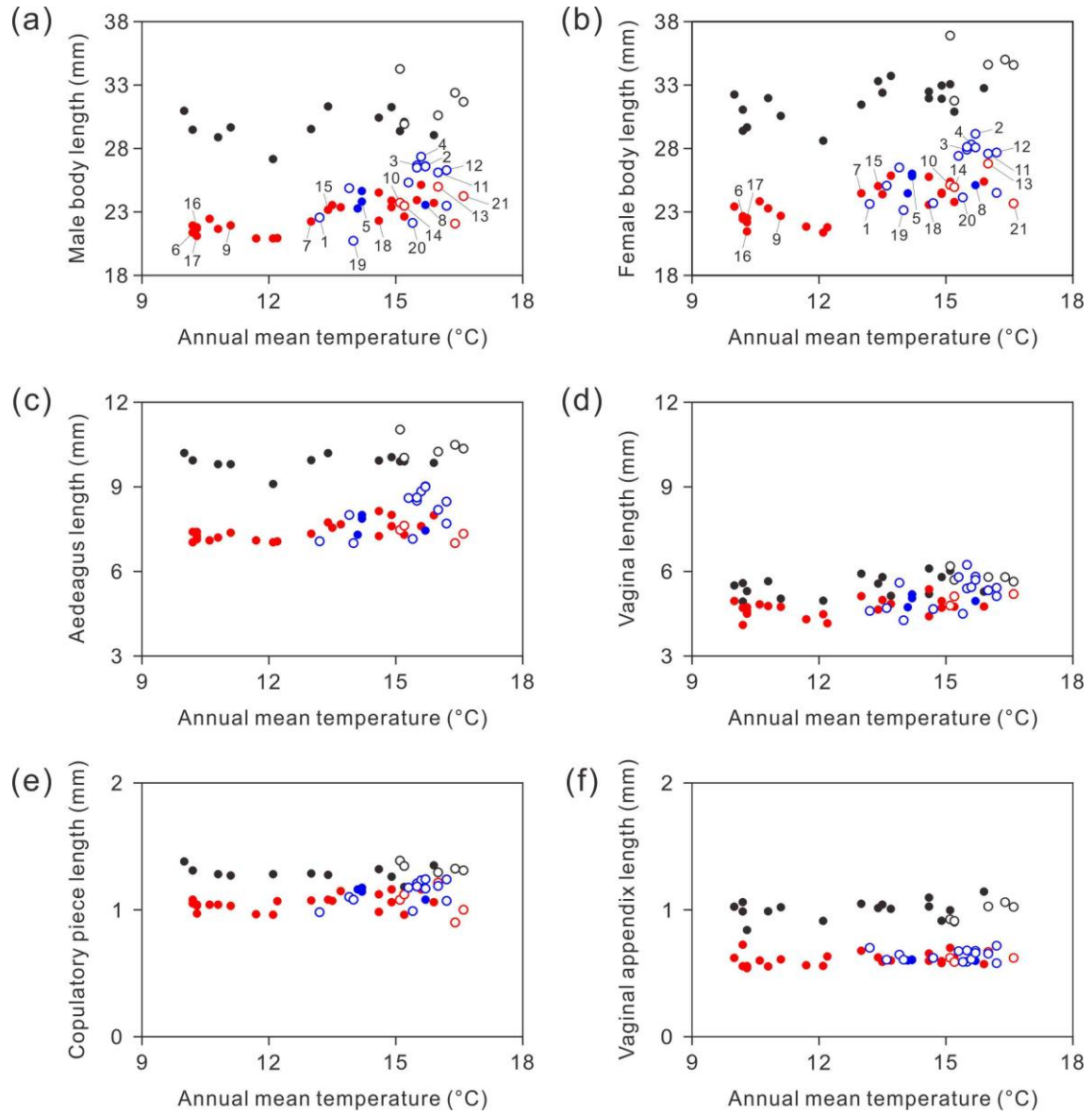
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785   Figure 3.

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793   Figure 4.

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797   Figure 5.

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(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

799

800

801

802

(b)

Group N1
n = 43
C. japonicus 100%

Group N2
n = 56
C. japonicus 61%
C. dehaanii 39%

Group N3
n = 75
C. japonicus 100%

Group N4
n = 41
C. japonicus 100%

Group N5
n = 68
C. japonicus 6%
C. dehaanii 94%

Substitution per site
0.02

C. japonicus

C. dehaanii

Sample size
1-5
6-10
11-20

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(c) Mitochondrial ND5   Nuclear Carab1

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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	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											
	MBL (mm)		AL (mm)		CPL (mm)		FBL (mm)		VL (mm)		VAL (mm)		MBL (mm)		AL (mm)		CPL (mm)		FBL (mm)		VL (mm)		VAL (mm)	
Region 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

No.																								
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		0.06					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	
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

No.																								
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												

ID	A1	A2	A3	A4	A5	A6	B1	B2	B3	B4	B5	B6	C1	C2	C3	C4	C5	C6	D1	D2	D3	D4	D5	D6
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

ID	A1	A2	A3	A4	A5	A6	B1	B2	B3	B4	B5	B6	C1	C2	C3	C4	C5	C6	D1	D2	D3	D4	D5	D6
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	0.09	31.67	0.75	10.35		0.18	1.31	0.04	34.57	0.67	5.64	0.35 1.02
53	22.07	0.68	7.00	0.14	0.90	0.00						0.09	32.39	1.05	10.49		0.22	1.32	0.03	34.99	1.16	5.80	0.27 1.06

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.
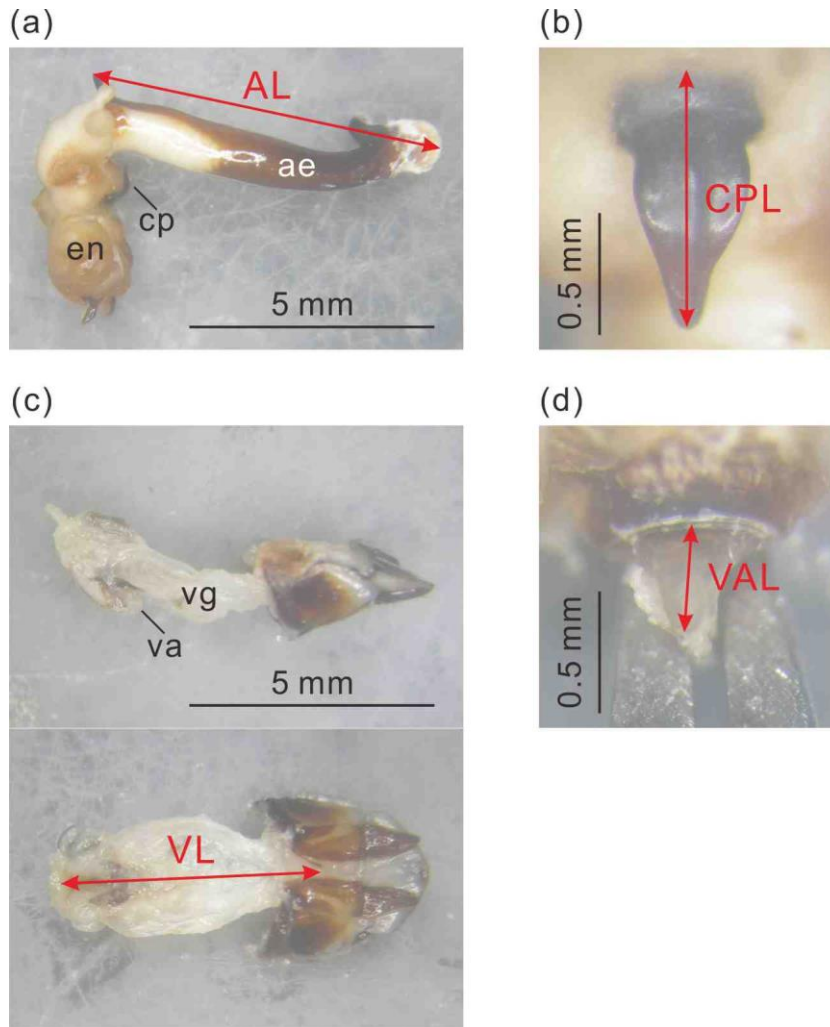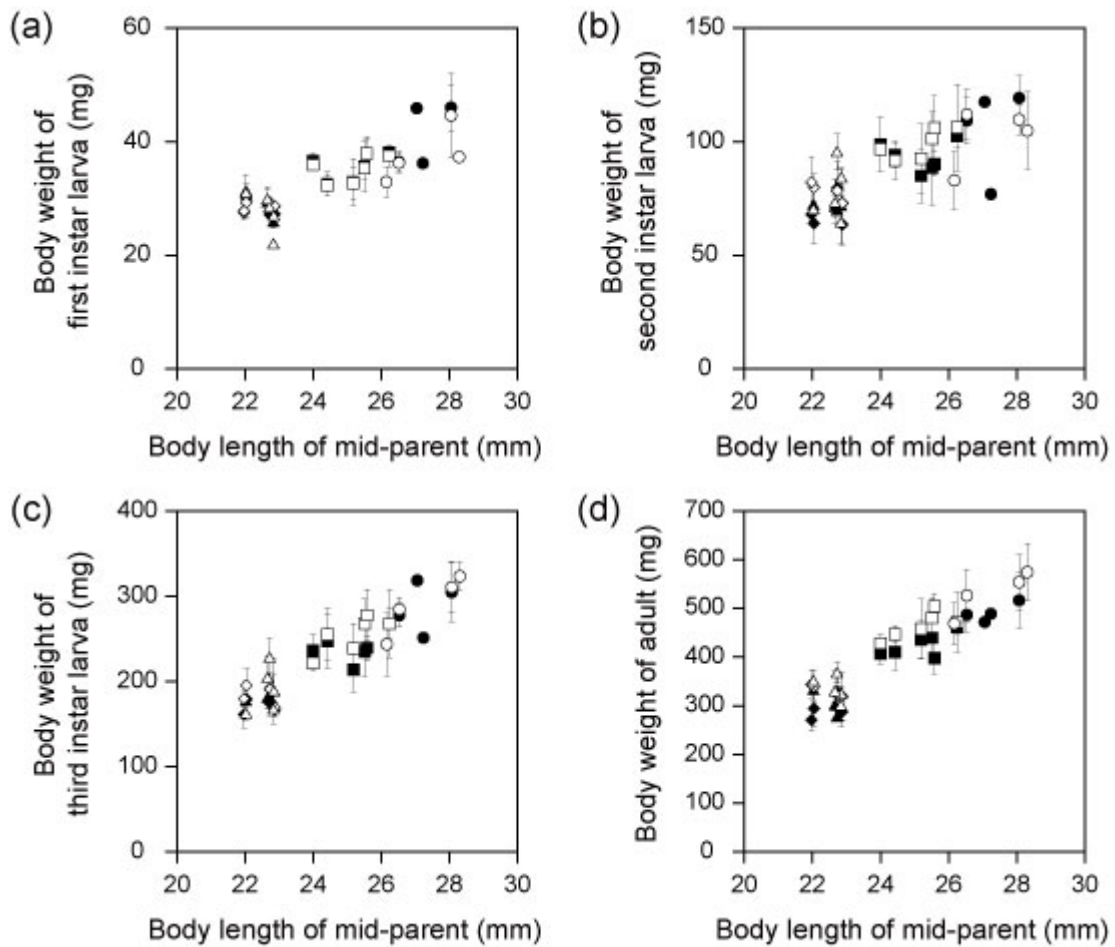
10 **Appendix S3** Body weight of laboratory reared *Carabus japonicus* larvae and adults in
11 relation to mid-parent body length. (a) first instar larva; (b) second instar larva; (c) third
12 instar larva; (d) adult offspring. Different symbols refer to source localities: diamond, site
13 6; triangle, site 7; square, site 5; circle, site 3 (see Fig. 1 for site number). Closed and open
14 symbols represent male and female offspring, respectively. Error bars represent standard
15 deviation.

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