Reproductive Character Displacement in Genital Morphology in Ohomopterus Ground Beetles

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Abstract

Genital morphology reveals rapid diversification among species, and species-specific divergence in genital morphology may result in reproductive isolation and promote speciation. Natural selection against maladaptive hybridization may cause species-specific genital divergence. In this context, divergence in mating traits is expected to be greater between sympatric populations than between allopatric populations in a pair of species, known as reproductive character displacement (RCD). However, there are few examples of RCD in the genital morphology of closely related species. Additionally, processes leading to RCD have rarely been inferred. In this study, we examined RCD and its underlying mechanisms by focusing on species-specific genital morphologies of closely related *Ohomopterus* ground beetle species. A morphological analysis showed patterns of RCD in species-specific genital parts in both sexes. Interspecific hybridization was confirmed by a mate choice experiment and by a population genetic analysis indicating extensive interspecific gene flow, suggesting that reinforcement is the most plausible process underlying the observed RCD. We found variation in the degree of displacement in contact zones, which may correspond with the ongoing process of genital evolution and speciation. Our results provide support for the lock-and-key hypothesis of genital evolution in closely related *Ohomopterus* species.

Introduction

Rapid and species-specific divergence in genital morphology has been mainly explained by the sexual selection hypothesis, including sperm competition, cryptic female choice, and sexual conflict (Brennan and Prum 2015; Eberhard 1985, 2010; Hosken and Stockley 2004; Langerhans et al. 2016; Simmons 2014; Sloan and Simmons 2019). Natural selection against hybridization may also result in divergent genital morphologies between closely related species or populations (the lock-and-key hypothesis, Dufour 1844; Masly 2012). In the latter context, the morphology of the genitalia may diverge among species to increase reproductive isolation, and mating traits are expected to be more divergent between sympatric populations than between allopatric populations in a pair of species, known as reproductive character displacement (RCD) (Brown and Wilson 1958; Howard 1993). When species or populations diverge in allopatry and come into secondary contact, natural selection against costly interspecific mating (direct selection) and that against maladaptive hybrids (indirect selection) may favor character divergence that promotes mate discrimination (Brown and Wilson 1958; Howard 1993). Importantly, RCD is only predicted by the lock-and-key hypothesis. However, the lock-and-key hypothesis has been regarded as less important than the sexual selection hypothesis in genital diversification (Eberhard 1985; Arnqvist 1998, but see Langerhans et al. 2016), and RCD in genital morphology has been reported only in limited cases (Kawano 2004, 2004; Kameda et al. 2009; Kawakami and Tatsuta 2010; Hollander et al. 2013; Anderson and Langerhans 2015, Kosuda et al. 2016).

RCD in genital morphology can be resulted from multiple processes.

Firstly, when incipient species or populations diverge in allopatry and come into secondary contact, the mating traits that mechanically or sensorily hinder coupling with heterospecific mates or decrease costs of interspecific mating, including injury, energetic costs, gamete loss, and hybrid production, may be favored, resulting in further divergence in male and female mating traits, including genital morphologies (Masly 2012; Langerhans et al. 2016). This process is called reinforcement and may leads to full reproductive isolation between populations or species with incomplete postzygotic isolation (Dobzhansky 1940; Butlin 1987a,b, 1989, 1995; Rice and Hoster 1993; Rundle and Schluter 1998; Servedio and Noor 2003; Coyne and Orr 2004). Secondly, reproductive interference between species that are fully reproductively isolated may generate similar selection for divergence in mating traits, resulting in RCD (Butlin 1987a,b; Butlin and Ritchie 2009; Hollander et al. 2018). In addition, Templeton (1981) also suggested that RCD can result from the biased extinction and filtering of populations (Templeton effect), in which populations within each species exhibit variation in mating traits and only sufficiently differentiated populations can coexist (Paterson 1978; Templeton 1981; Coyne and Orr 1989, 2004). Furthermore, ecological factors, such as resource competition and resultant body size divergence, may result in character displacement in mating traits, including genital morphology (Noor 1999; Okuzaki et al. 2015). Most previous studies reporting RCD in genital morphology, however, do not distinguish between these processes (Kawano 2002, 2004; Kameda et al. 2009; Kawakami and Tatsuta 2010; Hollander et al. 2013; but see Kosuda et al. 2016) or support the reproductive interference hypothesis (Hollander et al. 2018).

The ground beetle subgenus *Ohomopterus* (Coleoptera, Carabidae, genus

Carabus) is one of the most well-studied taxa with respect to genital diversification and speciation to date (Ishikawa 1987, 1991; Sota and Kubota 1998; Takami and Sota 2007; Nagata et al. 2007; Sasabe et al. 2010; Kubota et al. 2013; Fujisawa et al. 2019). *Ohomopterus* includes approximately 15 flightless species with marked geographical diversification in body size and genital morphology at the species and subspecies levels in the Japanese Archipelago (Ishikawa 1991; Sota et al., 2000; Sota and Nagata, 2008). Differentiation in body size may be an adaptation to environmental conditions and species interactions (Sota et al. 2000; Okuzaki and Sota 2018), and can act as a premating reproductive barrier (Okuzaki et al. 2010). Males possess a sclerotized projection on the endophallus of the intromittent organ, called a copulatory piece (CP). During copulation, the CP is inserted into the counterpart in the female, called the vaginal appendix (VA) (Ishikawa 1987, Takami 2002). The CP and VA show species-specific morphological matches (Ishikawa 1987, 1991, Sasabe et al. 2010, Fujisawa et al. 2019). Interspecific hybridization is hindered by morphological incompatibility, which results in insemination and fertilization failure (Sota and Kubota 1998, Kubota et al. 2013). Additionally, during heterospecific mating, the CP is sometimes broken (Sota and Kubota 1998), preventing males from mating effectively (Takami 2003). Genital injury in females can also occur, thereby decreasing longevity and fecundity (Sota and Kubota 1998, Kubota et al. 2013). Note that these costs are agents of direct selection acting on isolation traits. Interspecific hybrids are often fertile and have intermediate genital morphologies due to their polygenic basis (Sasabe et al. 2007, 2010, Fujisawa et al. 2019), suggesting the potential for admixture. However, hybrids with varied intermediate genital morphologies, probably consisting of various intercross and backcross generations, are confined to narrow hybrid zones in the wild (Kubota 1988, Kubota and Sota 1998) as in the tension zone (Barton and Hewitt 1985). This suggests that hybrids have relatively lower fertility and viability than that of parental species, in which indirect selection may act on genital forms that favors increased divergence. Thus, there are substantial fitness costs to heterospecific mating, mostly due to genitalic incompatibility in *Ohomopterus*. The high cost of interspecific mating observed in *Ohomopterus* suggests that selection will favor traits that limit interspecific mating. In addition, sexual selection via sperm competition and sexual conflict are drivers of diversification and covariation in male and female genital morphologies in the subgenus (Takami & Sota 2007, Takami et al. 2018), suggesting that mechanical reproductive isolation is a byproduct of diversification of genital morphologies by sexual selection. Furthermore, sexual selection may operate within populations in contact with closely related species, where reinforcing selection is also expected to operate. Since sexual selection acting on genital morphology frequently includes complex nonlinear and correlational components (e.g., Simmons et al. 2009, Wojcieszek and Simmons 2011), it may also stabilize or oppose divergence. Here, we hypothesize that reinforcing selection against mating between closely related species may favor reproductive isolation and accelerate differentiation in body size and/or genital morphology in contact areas, resulting in RCD and its evolutionary causes.

To examine RCD and its evolutionary process in *Ohomopterus*, we focused on closely related species with divergent genital morphologies, *Carabus* (*Ohomopterus*) *maiyasanus*, *C.* (*O.*) *iwawakianus,* and *C.* (*O.*) *uenoi* (fig. 1). *Carabus maiyasanus* and *C. iwawakianus* have long, hook-shaped and short, broad CPs and corresponding VAs, respectively. These species are distributed in the northern and southern parts of Kinki District in central Honshu mainland, Japan, respectively, and the body and genital sizes show remarkable geographical differentiation (Ishikawa and Kubota 1994, 1995). *C. uenoi* is derived from a lineage of *C. maiyasanus* with an extremely elongated CP and VA (Fujisawa et al. 2019) and is confined to a single small population in sympatry with *C. iwawakianus* (Usami et al. 2005). The former two species, *C. maiyasanus* and *C. iwawakianus*, are parapatric and occasionally form a narrow hybrid zone at their boundary (Kubota 1988, Kubota and Sota 1998), where mitochondrial haplotypes are shared between the species, indicating bidirectional mitochondrial introgression (Nagata et al. 2007). These two species cannot coexist in sympatry due to interbreeding and strong reproductive interference due to genital incompatibility (Sota and Kubota 1998, see also Okuzaki et al. 2010). The boundary is complex, and several local populations of one species are isolated within the range of the other species (fig. 1), probably resulting from the reciprocal movement of the boundary due to interspecific interactions as well as climatic factors (Takami and Osawa 2016). Thus, these isolated populations (including *C. uenoi*) as well as populations close to the boundary are expected to be more strongly influenced by interspecific interactions than are populations that are more distant from the boundary.

In this study, we first examined whether patterns of variation in body and genital sizes in *C. maiyasanus, C. iwawakianus,* and *C. uenoi* are consistent with RCD. Morphological variation in male and female body and genital sizes were examined by accounting for confounding effects (scaling relationships with body size, climatic factors, and geographical and genetic distances). Then, to discriminate reinforcement from other possible causes of RCD, we performed a mate choice experiment and population genetic analyses for evaluating the possibility of hybridization. These results provide insight into the evolutionary processes resulting in RCD in genital morphology between closely related species.

Material and methods

Sampling localities

Twenty-six populations were chosen from the distributions of *C. maiyasanus* (N = 12), *C. iwawakianus* (N = 13), and *C. uenoi* (N = 1) (fig. 1). These populations included six of the seven subspecies of *C. maiyasanus* and all five subspecies of *C. iwawakianus*, covering most of the geographical range of these species. All individuals were identified based on male and female genital morphologies, and no possible hybrids with intermediate phenotypes were found. Populations isolated within the range of other species and close to the boundary (within ca. 15 km) were defined as populations in contact, while populations distant from the boundary were treated as remote populations (Takami and Osawa 2016). The estimated dispersal rates of adult beetles were 13.8 m/day for *C. maiyasanus* and 11.3 m/day for *C. iwawakianus*, although these may be underestimates owing to the limited survey area (Kubota 1996). Given the univoltine life cycles with 2 or 3 months of adult activity per year as well as continuous habitats, individuals of two species separated by 15 km are expected to meet within less than 10 generations. However, the populations of the two species are in contact in the boundary zone and are separated only by rivers or narrow hybrid zones in many cases; accordingly, dispersal across the boundary and interspecific interactions may be more frequent than the above estimates.

Only a single population of *C. uenoi* (ue1) was evaluated; this population was in sympatry with *C. iwawakianus* (iw6, fig. 1).

Because *Ohomopterus* beetles have univoltine life cycles with reproduction in the spring and growth in the summer (Sota 1985), the annual mean temperature (AMT) is a simple index of the available heat for larval development and an important environmental predictor of variation in body and genital sizes (Sota et al. 2000, Okuzaki et al. 2015). To examine the effect of climate on morphological variation, the AMT at each sampling locality was obtained from 1 km mesh climatic data for the Japanese Archipelago collected from 1981 to 2010 (Japan Meteorological Agency 2010).

Analysis of character displacement

To quantify phenotypic variation in genital morphology as well as adult body sizes, the male and female body lengths (MBL and FBL, respectively), male aedeagus length (ADL), male CP length (CPL), and female VA length (VAL) were measured. Adults of *C. maiyasanus*, *C. iwawakianus* and *C. uenoi* were collected in 2016–2020 using pitfall traps from nine (ma1, 3, 5, 6, 8, 9, 10, 11, and 12), nine (iw2, 3, 5, 6, 7, 8, 9, 11, and 13) and one (ue1) populations, respectively (Table S1, fig. 1). Body length was defined as the distance from the anterior margin of the labrum to the apices of the elytra and was measured with digital calipers (0.01 mm increments). The other three morphological traits were defined as their maximum lengths (fig. 3) and were measured on images obtained with a digital camera attached to a microscope (Leica EZ4HD) using ImageJ to the nearest 0.001 mm. A total of 331 males and 304 females of *C. maiyasanus*, 230 males and 257 females of *C. iwawakianus*, and 30 males and 30 females of *C. uenoi* were measured (Table

S1). Because *C. uenoi* had much larger genital parts than those of other species and could be identified as an outlier (fig. 1), the measurements are shown for comparison (fig. 3) but were excluded from this analysis. Given that *C. uenoi* is closely related to *C. maiyasanus* and is classified as a population in contact, the exclusion of *C. uenoi* was conservative for the detection of RCD in genital parts.

To evaluate RCD, geographical differentiation in body and genital sizes within species was evaluated using individual-based measurements. We constructed generalized linear models (GLMs) with the identity link function and a normal distribution including one of the morphological traits as the dependent variable and distribution (remote or contact), population (nested within distribution), body size (except for the GLM for body size), and the interaction between body size and distribution as independent variables. The effect of distribution was tested against the nested effect of population because there was clear evidence for variation among populations. Body size and its interaction with distribution were evaluated to detect allometric scaling of genitalia. Non-significant interactions were excluded from the final models. Then, Tukey–Kramer's HSD tests were used to examine pairwise differences between populations.

In addition, to examine the effects of climatic condition and geographical distance on geographical variation in genital morphology and body size, we modified the above models by replacing population with AMT and geographical variables (see below). Genetic differentiation between populations was estimated by D_{est} (Jost 2008) based on microsatellite genotypes (see below) using GenAlEx ver. 6.5 (Peakall and Smouse 2006), but was not used in these analyses because of significant correlations with geographical distance (*C. maiyasanus*; $r = 0.46$, $P =$

0.0013, *C. iwawakianus*; $r = 0.55$, $P = 0.033$). To evaluate whether geographically closer specimens have similar phenotypes, we used distance-based Moran's eigenvector map (dbMEM) analysis (Legendre et al. 2015). We calculated principal coordinates of a geographic neighborhood matrix (PCNM) using the function *vegan* version 2.4-5 in R (Oksanen et al. 2017), and all eigenfunctions showing positive spatial correlation were included to the GLMs as geographic variables (PCNMs) (Borcard and Legendtre 2002; Dray et al., 2006). In these models, RCD was detected when a trait was consistently larger (or smaller) in populations in contact than in remote populations.

We also assessed whether the interspecific trait difference was greater in pairs of populations in contact than in other pairs of populations. Interspecific population pairs were assigned to two categories: (1) pairs of populations in contact (*maiyasanus* contact (maC) vs. *iwawakianus* contact (iwC), N = 16) and (2) other pairs (*maiyasanus* remote (maR) vs. *iwawakianus* remote (iwR), maR vs. iwC, and maC vs. iwR, N = 56). The iw6 population in sympatry with *C. uenoi* (ue1) was excluded from this analysis. In this analysis, the effects of allometry and climatic factors on trait differences were evaluated. GLMs were constructed using differences in one trait between species as the dependent variable and distributional pair (maC vs. iwC or other pairs), difference in mean body size (except for the model for body size), and difference in AMT as independent variables. Trait differences were consistently calculated as values for *C. maiyasanus* minus *C. iwawakianus*. Statistical significance was calculated based on the Mantel permutation test with 10,000 pseudoreplications using *multi.mantel* in the R package *Phytools* version 0.7-70 (Revell 2012). All statistical analyses were performed using the statistical package JMP ver. 8 (SAS Institute 2009), unless otherwise indicated.

Mate choice experiment

Among the possible processes of RCD, reinforcement assumes incomplete reproductive isolation between populations, while reproductive interference assumes complete isolation without gene flow. In order to assess whether premating isolation is currently complete, we conducted mate choice experiments. For evaluating premating reproductive isolation by male mate choice, adults of *C. maiyasanus* and *C. iwawakianus* were collected in the reproductive season (April to June) in 2016–2020 using pitfall traps from nine (ma1, 3, 5, 6, 8, 9, 10, 11, and 12) and seven (iw2, 3, 7, 8, 9, 11, and 13) populations, respectively (Table S1, fig. 1). These included four populations in contact (ma5, 6, 11, and 12) and five remote populations (ma1, 3, 8, 9, and 10) of *C. maiyasanus*, and four populations in contact (iw2, 3, 11, and 13) and three remote populations (iw7, 8, and 9) of *C. iwawakianus* (Table S1, fig. 1). Collected beetles were kept in an incubator at 20˚C and long-daylight conditions (16L: 8D) to maintain sexual maturity.

The ability of males to discriminate between conspecific and heterospecific females was examined using a mate choice experiment from May to June in 2016–2020. Male mate choice is an important determinant of heterospecific mating because a male initiates mating by attempting to mount females and female rejection is often ineffective (Takami 2002). In each trial, one male was released in a plastic box (13.5 × 13.5 × 8.5 cm) with one conspecific and one heterospecific female. The male was allowed to search for a mate and was assumed to have “made a choice” when it mounted a female and attempted to copulate. Males of remote populations of *C. maiyasanus* (ma1, 8, 9, and 10) were paired with *C. iwawakianus* females from the iw3 population. Males of populations in contact (ma6, ma11, and ma12) were paired with those from their closest heterospecific populations (iw3, iw11, and iw13, respectively). Males of the population in contact ma5 were engaged with *C. iwawakianus* females from two populations in contact (iw2 and iw3), and results were pooled owing to the non-significant difference in the rate of conspecific choice (Fisher's exact test, $P = 0.62$). Individuals were used only once. A mate choice experiment was conducted only for *C. maiyasanus* males to complement previous data for male mate choice in *C. iwawakianus* and *C. uenoi* (Sota and Kubota 1998, Usami et al. 2005). Departure from random choice (i.e., 50% conspecific mating) was evaluated using a binominal test.

Although our main purpose was to examine whether male mate discrimination was complete or not, RCD in male mate choice is also of interest. To assess whether mate discrimination in *C. maiyasanus* males differs between populations classified as remote or in contact, we constructed a generalized linear mixed model (GLMM) with the logit link function and a binominal distribution, including the frequency of conspecific mate choice as the dependent variable, distribution type (remote or contact) as an independent variable, and the male and female populations as random factors. Data from the present and previous studies were combined (13 experiments for *C. maiyasanus* males, treating ma5 vs. iw2 or iw3 as different experiments; fig. S1). This analysis could be influenced by variation among studies and years. However, most populations included in previous studies were also examined in this study; therefore, variation among studies and years is taken into account by our mixed effects models with populations as random terms. The model was fitted by restricted error maximum likelihood (REML) using the *glmer* function in the R package *lme4* (Bates et al.

2016).

Population genetic analysis

For evaluating the degree of interspecific gene flow, we conducted the population genetic analysis. Adults were collected from nine (ma2, 3, 4, 6, 7, 9, 10, 11, and 12; N = 149 in total), ten (iw1, 2, 3, 4, 5, 6, 9, 10, 11, and 12; N = 150), and one population (ue1, consisting of two subpopulations from Mts. Kongosan and Katsuragisan; N = 43) of *C. maiyasanus*, *C. iwawakianus,* and *C. uenoi*, respectively, in 2003–2008 (Table S1, fig. 1). Collected beetles were dissected to remove tissues for DNA extraction.

Population genetic analyses were performed based on multilocus microsatellite genotypes. Total DNA was extracted from gonads or muscles using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA). A total of 12 microsatellite loci (OMS33, OMS65, OMS102, OMS132, OMS317, OMS384, OMS413, OMS446, OMS552, OMS585, OMS598, and OMS665; Sasabe et al. 2010) were genotyped. PCR amplification with fluorescent-labeled primers were performed with appropriate annealing temperatures (Sasabe et al. 2010) in 10 μl volumes using AmpliTaq Gold 360 Master Mix (Applied Biosystems, Foster City, CA, USA). Amplified fluorescent fragments were analyzed using an ABI Prism 3130 Genetic Analyser with the GeneScan HD 400 ROX Size Standard (Applied Biosystems). Band size was determined using GeneMapper (Applied Biosystems).

Genetic differentiation and admixture were examined by a Bayesian clustering analysis using Structure ver. 2.32 (Pritchard et al. 2000), under assumptions of minimal Hardy–Weinberg disequilibrium and linkage disequilibrium within clusters. The number of clusters (K) was varied from 1 to 10, and the optimal number was determined based on the highest likelihood value and the highest rate of change in likelihood values (Evanno et al. 2005) using STRUCTURE HARVESTER (Earl and vonHoldt 2012). Ten independent runs were performed to ensure consistency, with a burn-in period of 10,000 steps followed by 1,000,000 Markov chain Monte Carlo iterations. We assumed correlated allele frequencies and an admixture model.

To further analyze admixture from interspecific hybridization between *C. maiyasanus* and *C. iwawakianus*, NewHybrids was used (Anderson and Thompson 2002). There were six possible genotype classes: two parental species, F1 and F2 hybrids, and first-generation hybrid backcrosses. A burn-in of 10,000 steps was used for the MCMC, followed by 100,000 subsequent steps under the assumption of Jefferys-like priors for the mixing proportion and allele frequencies.

Results

Character displacement in morphological traits

In *C. maiyasanus*, consistent with RCD in species-specific genitalia, individual measurements of genital traits (male CP length [CPL] and female VA length [VAL]) were significantly greater in populations in contact than in remote populations in the nested-population models even after adjusting for covariates (Table 1, fig. 2). In the models including annual mean temperature (AMT) and geographical variables, CPL, VAL as well as male aedeagus length (ADL) were significantly greater in populations in contact than in remote populations (CPL, $P < 0.0001$; VAL, $P < 0.0001$; ADL, $P < 0.0001$), while male body length (MBL) and female body length (FBL) were significantly smaller after adjusting for covariates (MBL, $P < 0.0001$; FBL, $P < 0.0001$) (Table S2). MBL and genital sizes (ADL, CPL and VAL) were negatively and positively associated with AMT, respectively (Table S2). ADL was positively associated with male body length in both models (Tables 1, S2).

In *C. iwawakianus*, individual measurements in MBL, FBL, and ADL were significantly greater in populations in contact than in remote populations in the nested-population models even after adjusting for covariates (Table 1, fig. 2). In the models including AMT and geographical variables, MBL, ADL and CPL were significantly greater in populations in contact than in remote populations even after adjusting for covariates (MBL, $P = 0.0076$; ADL, $P < 0.0001$; CPL, $P = 0.0029$) (Table S2). Note that these differences were directed toward decreasing interspecific differences in populations in contact (fig. 2), different from RCD. In both models, all genital traits were positively associated with body size, with a significant interaction between distribution type and MBL for CPL (Tables 1, S2). VAL was positively associated with AMT (Table S2).

Consistent with RCD in species-specific genitalia, interspecific differences in mean CPL and VAL were significantly greater in the pairs of populations in contact than in other pairs even after adjusting for covariates (Table S3, fig. 3). Note that these interspecific differences mostly resulted from differences between populations in contact and remote populations of *C. maiyasanus* rather than variation within *C. iwawakianus* (Tables 1, S2 and fig. 2B, E). Interspecific differences in MBL and FBL were positively correlated with differences in AMT and differences in mean genital sizes were positively correlated with differences in mean body size (Table S3).

Mate choice experiment

C. maiyasanus males showed incomplete mate discrimination between conspecific and *C. iwawakianus* females. Males from three of four remote populations (ma1, ma8, and ma9) were likely to mount conspecific females first, showing a significant departure from the random expectation (fig. 4; Binomial test, $P < 0.05$); however, this pattern was not detected for one remote population (ma10) (fig. 4; $P = 0.093$). Males from all four populations in contact (ma5, ma6, ma11, and ma12) tended to mount conspecific females; however, there was no significant departure from random choice (fig. 4). In the analysis including all present and previous data (fig. S1), as opposed to RCD in male mate choice, the frequency of conspecific mate choice was lower for males from populations in contact than for males from remote populations (GLMM, $\beta = -0.428 \pm 0.188$, $\chi^2_1 = 5.15$, $P = 0.023$), suggesting that interspecific mating is common between *C. maiyasanus* and *C. iwawakianus* populations in contact.

Population genetic analysis

The Bayesian clustering analysis using Structure indicated that the optimal number of clusters (K) was two based on the Evanno method, mostly corresponding to *C. uenoi* and others, with some evidence for introgression from *C. uenoi* to *C. iwawakianus* (especially iw6 in sympatry with ue1, fig. S2). This may be because *C. uenoi* with huge genital parts is strongly isolated from sympatric *C. iwawakianus* (Usami et al. 2005) and confined to a small population, having distinct genetic composition from other two species. By contrast, *C. iwawakianus* and *C. maiyasanus* undergo continuous interspecific hybridization, probably sharing alleles at least in neutral loci (Nagata et al. 2007; Takami and Osawa 2016; Fujisawa et al. 2019). Since our main purpose was to detect genetic differentiation and admixture between *C. maiyasanus* and *C. iwawakianus*, we reanalyzed the dataset excluding these populations (i.e., ue1 and iw6). Structure analysis based on the dataset excluding iw6 and ue1 showed that K was two. These two clusters corresponded to the two species, as indicated by low levels of admixture in populations distant from the boundary (e.g., ma2, ma3, ma4, ma7, ma9, ma10, iw5, iw9, and iw10) (fig. 5). By contrast, populations in contact for both *C. maiyasanus* (ma6, ma11, and ma12) and *C. iwawakianus* (iw1, iw2, iw3, iw4, iw11, and iw12) showed extensive admixture, with two genetic clusters or occasional alleles of the other species, especially for populations isolated within the range of other species. This result indicated a mismatch between morphospecies and genetic composition in possibly neutral loci, although no possible hybrids were found in these populations with respect to genital morphologies.

The results of a NewHybrids analysis were mostly concordant with those of the Structure analysis. No individuals were classified as F1 or first-generation backcrosses, while some individuals were classified as F2 hybrids, especially in populations in contact (fig. 5). Note that it was difficult to distinguish between F2 hybrids and later hybrid generations without larger number of markers in Newhybrids analysis. Since F1 hybrids were not found, individuals classified as F2 hybrids may consist of later hybrid generations. These results suggested that there is little ongoing but substantial past gene flow between species.

Discussion

We observed clear patterns of RCD in the species-specific genital parts (male CP and female VA) of closely related *Ohomopterus* species, especially in *C. maiyasanus*. The sizes of genital parts differed between populations in contact and remote populations of *C. maiyasanus*, and interspecific differences of the genital parts were elevated in contact areas. The extremely elongated genital parts in *C. uenoi* corroborated this result, although this species was not included in the morphological analysis. By contrast, body and aedeagus sizes showed no consistent patterns of geographical variation with respect to RCD. Our results accounted for confounding effects, including scaling relationships with body size, climatic factors, and geographical proximity that was significantly correlated with genetic differentiation. Recent phylogenomic analysis revealed that populations of *C. maiyasanus* in contact with *C. iwawakianus* were not monophyletic, suggesting that the elongation of species-specific genital parts occurred multiple times (Fujisawa et al. 2019). Overall, the observed RCD in the lengths of CP and VA is unlikely to be explained by divergence in other factors.

The observed RCD in species-specific genital morphology was asymmetric. Both male CPL and female VAL of *C. maiyasanus* increased in populations in contact with *C. iwawakianus* (Table 1, S2 and fig. 4B, E); however, those of *C. iwawakianus* did not differ between remote populations and populations in contact with *C. maiyasanus*, or they increased in some populations in contact (decreasing the interspecific difference) (Table 1, S2 and fig. 4B, E). Such an asymmetry in RCD may result from selection for mechanical reproductive isolation, whereby selection against heterospecific mating is stronger in *C. maiyasanus*. In interspecific mating, the long CP in *C. maiyasanus* was more frequently broken than the short CP in *C. iwawakianus* (Sota and Kubota 1998), suggesting stronger selection against heterospecific mating in *C. maiyasanus* males. Additionally, interspecific gene flow between *C. maiyasanus* and *C. iwawakianus* can also explain elongation of the CP and VA of *C.*

iwawakianus in some contact areas because genital traits of hybrids were intermediate to those of the two species (Sasabe et al. 2007). The quantification of selection on genital morphologies and gene flow at candidate loci at the boundary is warranted to further understand the asymmetric RCD.

The reinforcement of reproductive isolation between incipient species with incomplete postzygotic reproductive isolation can lead to RCD after secondary contact (Dobzhansky 1940; Howard 1993; Coyne and Orr 2004). This process may proceed only with difficulty because gene flow between incipient species counteracts the diversification of mating traits. Additionally, recombination also opposes the reinforcement process, if selection is indirect and depends on hybrid fitness that is determined by traits other than those involved in mate choice. The results of the present and previous studies of *Ohomopterus* species are consistent with the predictions of reinforcement. Interspecific mating can occur, especially between populations in contact of *C. maiyasanus* and *C. iwawakianus,* as indicated by incomplete mate discrimination (fig. S1, Sota and Kubota 1998; Usami et al. 2005). Although the estimated degrees of mate choice in remote populations of *C. maiyasanus* could be biased because only females from the single population (iw3) was used owing to sample availability. Nevertheless, there was virtually no mate choice in males from populations in contact suggests that interspecific mating is likely to occur if they contact. Postzygotic isolation is also incomplete because viable hybrids can be generated (Sota and Kubota 1998; Sasabe et al. 2007, 2010). Although no possible hybrids with intermediate phenotypes were found in the wild, past gene flow has been detected by the introgression of mitochondrial (Nagata et al. 2007) and nuclear (fig. 5) genes at the species boundary. These suggested that the potential for gene flow has been reduced, as predicted by reinforcement, although some populations are still quite admixed (iw11 and iw12). Interestingly, a recent genome-wide analysis has revealed that interspecific gene flow is suppressed at loci that are tightly linked to male and female genital dimensions (Fujisawa et al. 2019).

The reproductive interference between species with complete postzygotic reproductive isolation can also result in RCD (Butlin 1987a,b; Butlin and Ritchie 2009). This process predicts that interspecific mating does not result in gene flow between species, different from our results. Patterns similar to RCD can also result from the biased extinction and filtering of populations with differences in mating traits (i.e., the Templeton effect). The Templeton effect may be difficult to exclude. Although *C. maiyasanus* populations with elongated CP and VA are only found near the boundary with *C. iwawakianus* (Ishikawa and Kubota 1994), CPL and VAL in *C. maiyasanus* also vary among populations remote from the boundary (figs. 1 and 3), suggesting that there was substantial variation in genital traits outside of the contact zone. Collectively, reinforcement is the most plausible process leading to RCD in the species-specific genital morphology of *C. maiyasanus* and *C. iwawakianus*, and the Templeton effect may also contribute to the observed patterns of RCD. These results provide novel evidence for the lock-and-key hypothesis of genital evolution. Unlike most previous studies that detected RCD in genital morphology, the observed RCD in this study is suggested to be driven by direct selection, because mismatch of heterospecific genitals inflicts direct costs to individuals engaging in interspecific mating (i.e., injury and subsequent fitness loss) (Sota and Kubota 1998, Kubota et al. 2013). Determining the type of selection is warranted in future studies of the lock-and-key hypothesis and the detection of RCD (Shaw and Mendelson 2013).

The observed variation in genital parts among *C. maiyasanus* populations and the coexistence of *C. uenoi* with *C. iwawakianus* suggests that reinforcing selection for genital morphologies drives toward the completion of reproductive isolation. The degree of RCD in genital parts varied among populations of *C. maiyasanus*; populations isolated within the range of *C. iwawakianus* (ma11 and 12) had the longest genitalia, followed by populations near the boundary (ma5 and 6), and remote populations had the shortest genitalia (figs. 1, 2B, E). *C. uenoi* may contribute to this pattern of variation because this species is a lineage derived from *C. maiyasanus* (Fujisawa et al. 2019). Unlike other populations of *C. maiyasanus* in contact zones, the extremely elongated genital parts (fig. 1) and strong male mate choice (fig. S1) of *C. uenoi* hinder interspecific mating with *C. iwawakianus* (Usami et al. 2005; Kubota et al. 2013). This association between genital evolution and the degree of interspecific interaction (i.e., allopatry to sympatry) allowed us to hypothesize that selection against maladaptive interspecific mating (i.e., reinforcing selection) promoted reproductive isolation between *C. maiyasanus* and *C. iwawakianus* by driving the elongation of genital parts in *C. maiyasanus* populations. Consequently, *C. uenoi* showed remarkable genital evolution as well as male mate choice, resulting in virtually complete reproductive isolation and its coexistence with *C. iwawakianus*.

Natural selection related to resource competition can also promote character evolution, reducing the probability of maladaptive interspecific interactions between closely related species in sympatry (ecological character displacement: Brown and Wilson 1956, Schluter and McPhail 1992; Pfennig and Murphy 2000; Grant and Grant 2006) and may result in mating trait differentiation as a byproduct. In *Ohomopterus*, differentiation in body size may be an adaptation to environmental conditions (Sota et al. 2000) and may contribute to the decreased frequency of maladaptive interspecific mating (Okuzaki et al. 2010, 2015). This would result in correlated evolution of genital sizes and character displacement (Okuzaki 2021). However, we did not detect patterns of RCD in body size. Additionally, there were no associations between genital sizes (male CP and female VA) and body sizes in *C. maiyasanus* (Tables 1 and S2). Therefore, the observed RCD in genital morphologies is not likely to be a byproduct of ecological character displacement in body size. Sexual selection also results in differentiation in genital morphology (Eberhard 1985, 2010, Arnqvist 1998, Hosken and Stockley 2003, Simmons 2014, Brennan and Prum 2015), along with natural selection (Langerhans et al. 2016). In *Ohomopterus*, sexual selection via sperm competition (Takami and Sota 2007, Okuzaki and Sota 2014) and sexual conflict (Takami et al. 2018) may be the principal processes contributing to differentiation in genital morphologies among populations and species. It is possible that sexual and reinforcing selections drive the evolution of genital morphologies toward different optima, constraining the diversification of genital morphologies. However, our results indicate that genital diversification can be boosted by reinforcing selection for reproductive isolation after secondary contact as observed in the extremely enlarged genitalia of *C. uenoi*.

Our results demonstrated RCD in species-specific male and female genitalia in *Ohomopterus* ground beetles. Incomplete premating isolation and extensive gene flow between species support the role of reinforcement. Unlike previous studies of RCD focusing on sympatric populations, we focused on parapatric species, which may be particularly well-suited for detecting reinforcement because they are likely to be young and experiencing ongoing speciation (i.e., the ideal stage for reinforcement). Thus, parapatric species pairs should be a focus of further studies of RCD. Additionally, further investigations of the effects of displacement in genital morphologies on the cost of hybridization are necessary to better understand the process of RCD in species-specific genital morphology.

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Table 1. General linear models explaining variation in body and genital sizes in individuals of *C. maiyasanus* and *C. iwawakianus*. Significance at the 5% level is shown in boldface.

	C. maiyasanus				*C. iwawakianus*			
	Coefficient ± SE	*F*	d.f.	*P*	Coefficient ± SE	*F*	d.f.	*P*
Model explaining male body length (MBL)		56.47	8, 321	**<0.0001**		69.87	8, 220	**<0.0001**
Distribution (remote/contact)	-0.410 ± 0.048	1.31	1, 7	0.29	-1.054 ± 0.057	36.41	1, 7	**0.0005**
Population [distribution]		55.12	7, 321	**<0.0001**		9.42	7, 201	**<0.0001**
Model explaining female body length (FBL)		63.99	8, 295	**<0.0001**		51.21	8, 247	**<0.0001**
Distribution (remote/contact)	-0.370 ± 0.053	0.72	1, 7	0.43	-0.881 ± 0.061	25.14	1, 7	**0.0015**
Population [distribution]		65.76	7, 295	**<0.0001**		8.26	7, 247	**<0.0001**
Model explaining male aedeagus length (ADL)		131.11	9, 317	**<0.0001**		120.21	9, 219	**<0.0001**
Distribution (remote/contact)	-0.130 ± 0.013	1.64	1, 7	0.24	-0.199 ± 0.023	10.61	1, 7	**0.014**
MBL	0.077 ± 0.010	31.97	1, 317	**<0.0001**	0.154 ± 0.017	84.36	1, 219	**<0.0001**
Population [distribution]		65.16	1, 317	**<0.0001**		7.24	7, 219	**<0.0001**
Model explaining male CP length (CPL)		370.82	9, 277	**<0.0001**		28.91	10, 212	**<0.0001**
Distribution (remote/contact)	-0.355 ± 0.009	11.06	1, 7	**0.013**	-0.033 ± 0.011	3.68	1, 7	0.97
MBL	-0.001 ± 0.010	0.02	1, 277	0.88	0.032 ± 0.007	21.04	1, 212	**<0.0001**
Population [distribution]		148.28	7, 277	**<0.0001**		2.68	7, 212	**0.009**
Distribution * MBL	–	–	–	**–**	0.024 ± 0.007	10.96	1, 212	**0.001**
Model explaining female VA length (VAL)		67.2	9, 293	**<0.0001**		24.19	9, 246	**<0.0001**
Distribution (remote/contact)	-0.232 ± 0.014	11.66	1, 7	**0.011**	-0.038 ± 0.018	0.26	1, 7	0.63
FBL	0.016 ± 0.014	1.21	1, 293	0.27	0.037 ± 0.014	7.1	1, 246	**0.008**
Population [distribution]		23.23	7, 293	**<0.0001**		17.27	7, 246	**<0.0001**

Table S1. Sampling localities and sample sizes for phenotypic and genetic analyses.

Population	Altitude (m)	Latitude (N)	Longitude (E)	AMT (°C)	$N_{\text{phenotypic}}$ male	$N_{\text{phenotypic}}$ female	N_{genetic}
Carabus maiyasanus							
ma1	380	34.8047	135.1483	12.90	39	41	-
ma2	400	35.0484	135.4114	11.70	-	-	10
ma3	150	35.0513	135.8026	14.10	18	14	30
ma4	180	34.9404	136.1433	12.80	-	-	11
ma5	410	34.9272	136.1317	12.00	51	45	-
ma6	110	34.9436	136.4817	14.10	51	41	20
ma7	50	35.0827	136.6280	14.50	-	-	10
ma8	100	35.1381	136.6306	13.80	41	42	-
ma9	10	35.2937	136.5490	14.10	22	20	20
ma10	100	35.4337	136.2613	13.50	21	21	16
ma11	120	34.3561	136.4067	14.10	41	35	25
ma12	50	34.3453	136.8264	15.60	47	45	7
C. iwawakianus							
iw1	180	34.9666	135.9850	13.30	-	-	12
iw2	320	34.9117	136.0158	11.70	25	21	18
iw3	100	34.9169	136.4583	14.10	64	83	17
iw4	50	34.7636	136.4283	14.50	-	-	16
iw5	380	34.5950	136.1102	12.60	20	18	15
iw6	1000	34.4110	135.6765	9.20	20	20	25
iw7	890	34.2145	135.5719	10.70	23	24	-
iw8	870	34.2669	135.8767	9.10	25	37	-
iw9	950	34.4243	136.0901	9.70	8	10	14
iw10	150	34.4374	136.3544	13.10	-	-	16
iw11	100	34.3717	136.4228	14.10	22	22	8
iw12	30	34.4284	136.5902	14.60	-	-	9
iw13	40	34.4711	136.7636	15.20	23	22	-
C. uenoi							
ue1	1000	34.4110	135.6765	9.20	30	30	43

Table S2. General linear models explaining variation in body and genital sizes in individuals of *C. maiyasanus* and *C. iwawakianus*, examining the effects of distribution (remote/contact), annual mean temperature (AMT), geographic variables (PCNM1-6), and body size (except for the GLM for body size). Significance at the 5% level is shown in boldface.

	C. maiyasanus				*C. iwawakianus*			
	Coefficient ± SE	*F*	d.f.	*P*	Coefficient ± SE	*F*	d.f.	*P*
Model explaining male body length (MBL)		56.47	8, 321	**<0.0001**		69.87	8, 220	**<0.0001**
Distribution (remote/contact)	1.749 ± 0.423	17.06	1, 321	**<0.0001**	-0.443 ± 0.164	7.25	1, 220	**0.0076**
AMT	-0.881 ± 0.399	4.87	1, 321	**0.028**	-0.073 ± 0.198	0.14	1, 220	0.71
PCNM1	-4.110 ± 1.032	15.88	1, 321	**<0.0001**	-2.075 ± 1.167	3.16	1, 220	0.0770
PCNM2	-5.828 ± 1.105	27.8	1, 321	**<0.0001**	-0.195 ± 0.164	1.42	1, 220	0.23
PCNM3	3.135 ± 0.916	11.7	1, 321	**0.0007**	1.309 ± 0.854	2.34	1, 220	0.13
PCNM4	1.549 ± 0.268	33.41	1, 321	**<0.0001**	0.604 ± 0.265	5.19	1, 220	**0.024**
PCNM5	1.813 ± 0.240	56.99	1, 321	**<0.0001**	0.070 ± 0.264	0.07	1, 220	0.79
PCNM6	1.535 ± 0.196	61.46	1, 321	**<0.0001**	0.709 ± 0.276	6.62	1, 220	**0.011**
Model explaining female body length (FBL)		63.99	8, 295	**<0.0001**		51.21	8, 247	**<0.0001**
Distribution (remote/contact)	1.942 ± 0.480	16.38	1, 285	**<0.0001**	-0.102 ± 0.171	0.35	1, 247	0.55
AMT	-0.830 ± 0.450	3.4	1, 285	0.066	-0.394± 0.214	3.38	1, 248	0.067
PCNM1	-3.893 ± 1.164	11.18	1, 285	**0.0009**	-3.911 ± 1.242	9.92	1, 249	**0.0018**
PCNM2	-6.518 ± 1.250	27.19	1, 285	**<0.0001**	0.126 ± 0.176	0.51	1, 250	0.47
PCNM3	3.445 ± 1.030	11,19	1, 285	**0.0009**	2.581 ± 0.920	7.88	1, 251	**0.0054**
PCNM4	1.688 ± 0.303	31.08	1, 285	**<0.0001**	1.014 ± 0.274	13.7	1, 252	**0.0003**
PCNM5	2.013 ± 0.271	55.36	1, 285	**<0.0001**	-0.091 ± 0.287	0.1	1, 253	0.75
PCNM6	1.396 ± 0.219	40.73	1, 285	**<0.0001**	1.105 ± 0.278	15.83	1, 254	**<0.0001**
Model explaining male aedeagus length (ADL)		131.11	9, 317	**<0.0001**		120.21	9, 219	**<0.0001**
Distribution (remote/contact)	-0.550 ± 0.106	26.92	1, 317	**<0.0001**	-0.175 ± 0.042	17.75	1, 219	**<0.0001**
AMT	0.605 ± 0.098	38.11	1, 317	**<0.0001**	0.090 ± 0.050	3.32	1, 219	0.07
MBL	0.077 ± 0.014	31.97	1, 317	**<0.0001**	0.154 ± 0.017	84.36	1, 219	**<0.0001**
PCNM1	1.239 ± 0.258	23.09	1, 317	**<0.0001**	0.393 ± 0.293	1.80	1, 219	0.18
PCNM2	0.706 ± 0.281	6.31	1, 317	**0.013**	0.001 ± 0.041	0.0004	1, 219	0.98

PCNM3	-1.310 ± 0.227	33.17	1, 317	**<0.0001**	-0.460 ± 0.214	4.63	1, 219	**0.033**
PCNM4	-0.339 ± 0.068	24.53	1, 317	**<0.0001**	0.120 ± 0.067	3.20	1, 219	0.075
PCNM5	-0.193 ± 0.064	9.2	1, 317	**0.0026**	0.233 ± 0.066	12.56	1, 219	**0.0005**
PCNM6	0.254 ± 0.052	23.72	1, 317	**<0.0001**	0.173 ± 0.070	6.17	1, 219	**0.0138**
Model explaining male CP length (CPL)		370.82	9, 277	**<0.0001**		28.35	9, 213	**<0.0001**
Distribution (remote/contact)	-0.516 ± 0.074	50.99	1, 277	**<0.0001**	-0.050 ± 0.017	9.06	1, 213	**0.0029**
AMT	0.458 ± 0.067	46.73	1, 277	**<0.0001**	0.004 ± 0.020	0.03	1, 213	0.86
MBL	-0.001 ± 0.009	0.02	1, 277	0.88	0.023 ± 0.007	11.87	1, 213	**0.0007**
PCNM1	0.498 ± 0.179	7.78	1, 277	**0.0057**	0.044 ± 0.117	0.14	1, 213	0.71
PCNM2	0.462 ± 0.193	5.72	1, 277	**0.017**	0.037 ± 0.016	4.98	1, 213	**0.027**
PCNM3	-0.825 ± 0.157	27.64	1, 277	**<0.0001**	0.008 ± 0.085	0.01	1, 213	0.92
PCNM4	-0.620 ± 0.046	180.17	1, 277	**<0.0001**	0.031 ± 0.027	1.38	1, 213	0.24
PCNM5	0.141 ± 0.043	10.53	1, 277	**0.0013**	-0.0004 ± 0.026	0.0002	1, 213	0.99
PCNM6	-0.017 ± 0.036	0.22	1, 277	0.64	0.020 ± 0.028	0.5	1, 213	0.48
Model explaining female VA length (VAL)		67.2	9, 293	**<0.0001**		24.19	9, 246	**<0.0001**
Distribution (remote/contact)	-0.482 ± 0.120	16.12	1, 293	**<0.0001**	-0.062 ± 0.037	2.8	1, 246	0.095
AMT	0.381 ± 0.111	11.82	1, 293	**0.0007**	0.152 ± 0.047	10.48	1, 246	**0.0014**
FBL	0.016 ± 0.014	1.21	1, 293	0.27	0.037 ± 0.014	7.12	1, 246	**0.0081**
PCNM1	0.682 ± 0.289	5.56	1, 293	**0.019**	0.901 ± 0.276	10.63	1, 246	**0.0013**
PCNM2	0.504 ± 0.320	2.59	1, 293	0.12	-0.095 ± 0.038	6.07	1, 246	**0.015**
PCNM3	-0.796 ± 0.256	9.7	1, 293	**0.002**	-0.596 ± 0.204	8.55	1, 246	**0.0038**
PCNM4	-0.396 ± 0.078	25.58	1, 293	**<0.0001**	0.156 ± 0.061	6.48	1, 246	**0.012**
PCNM5	-0.101 ± 0.072	1.97	1, 293	0.16	0.008 ± 0.063	0.02	1, 246	0.90
PCNM6	0.037 ± 0.057	0.42	1, 293	0.52	-0.011 ± 0.062	0.03	1, 246	0.86

Table S3. General linear models explaining interspecific differences in mean body and genital sizes between *C. maiyasanus* and *C. iwawakianus* populations. Significance at the 5% level is shown in boldface.

	Coefficient ± SE	*t*	*P*
Model explaining male body length (MBL) difference			
Distributional pair (contact/others)	0.133 ± 0.311	0.43	0.77
AMT difference	0.536 ± 0.057	9.45	**0.0003**
Model explaining female body length (FBL) difference			
Distributional pair (contact/others)	0.224 ± 0.347	0.65	0.65
AMT difference	0.497 ± 0.063	7.86	**0.0006**
Model explaining male aedeagus length (ADL) difference			
Distributional pair (contact/others)	0.061 ± 0.063	0.97	0.52
MBL difference	0.333 ± 0.024	13.65	**0.0001**
AMT difference	-0.001 ± 0.017	-0.07	0.97
Model explaining male CP length (CPL) difference			
Distributional pair (contact/others)	0.571 ± 0.095	6.03	**0.0015**
MBL difference	0.159 ± 0.037	4.35	**0.024**
AMT difference	0.013 ± 0.026	0.48	0.79
Model explaining female VA length (VAL) difference			
Distributional pair (contact/others)	0.386 ± 0.070	5.48	**0.0010**
FBL difference	0.101 ± 0.024	4.13	**0.032**
AMT difference	0.030 ± 0.018	1.68	0.36

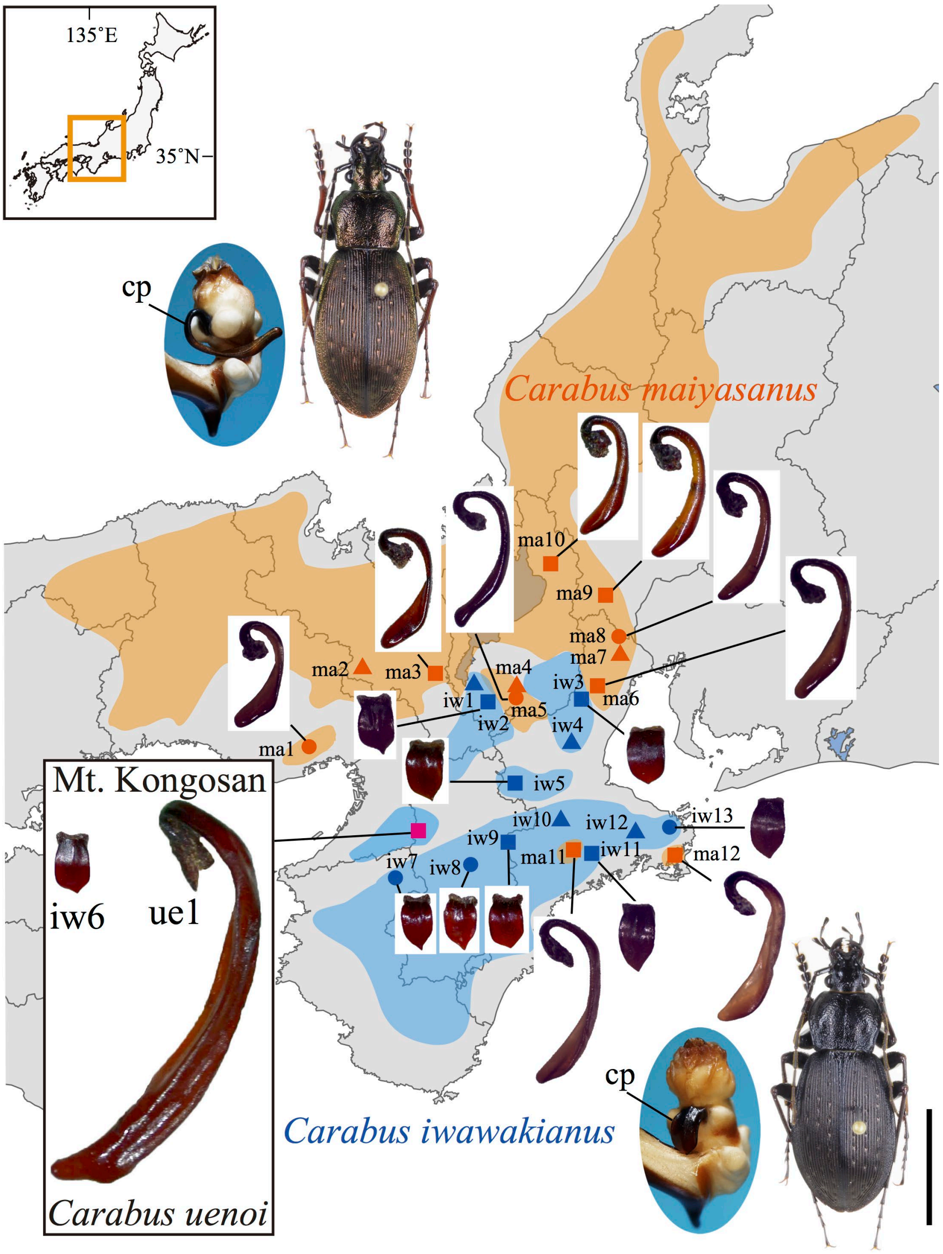

Fig. 1. Distribution and geographical variation of *Carabus maiyasanus* (orange area and plots), *C. iwawakianus* (blue area and plots), and *C. uenoi* (magenta plot, in sympatry with *C. iwawakianus*, iw6). Upper left inset shows a map of Japan showing the study area (orange rectangle). Circle, triangle, and square marks indicate populations used for phenotypic (morphological and behavioral), genetic, and both analyses, respectively. Male body and genitalia as well as geographical variation in the copulatory piece (cp) are shown. Scale bar represents 10 mm for the whole body and 2.5 mm for genitalia.

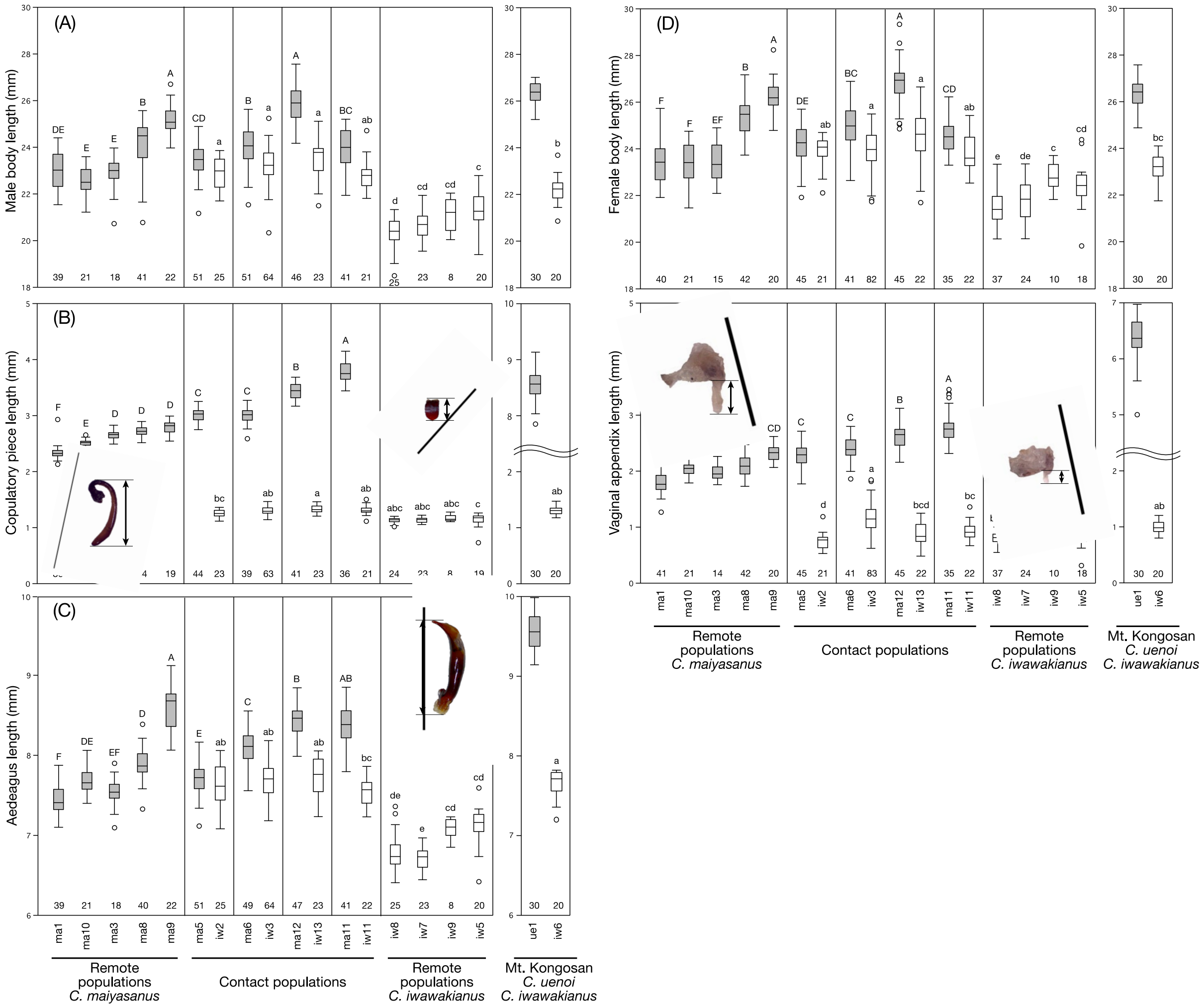

Fig. 2. Phenotypic variation in remote and contact populations of *Carabus maiyasanus* (gray plot), *C. iwawakianus* (white plot), and *C. uenoi* (in separate panels with the sympatric *C. iwawakianus* population): (A) male body length, (B) copulatory piece length, (C) aedeagus length, (D) female body length, and (E) vaginal appendix length. Insets show measurements of genital parts. Different letters indicate significant differences (Tukey–Kramer test, $P < 0.05$, upper and lower case letters indicate *C. maiyasanus* and *C. iwawakianus*, respectively). Data for *C. uenoi* are shown only for descriptive purposes and were not included in analyses.

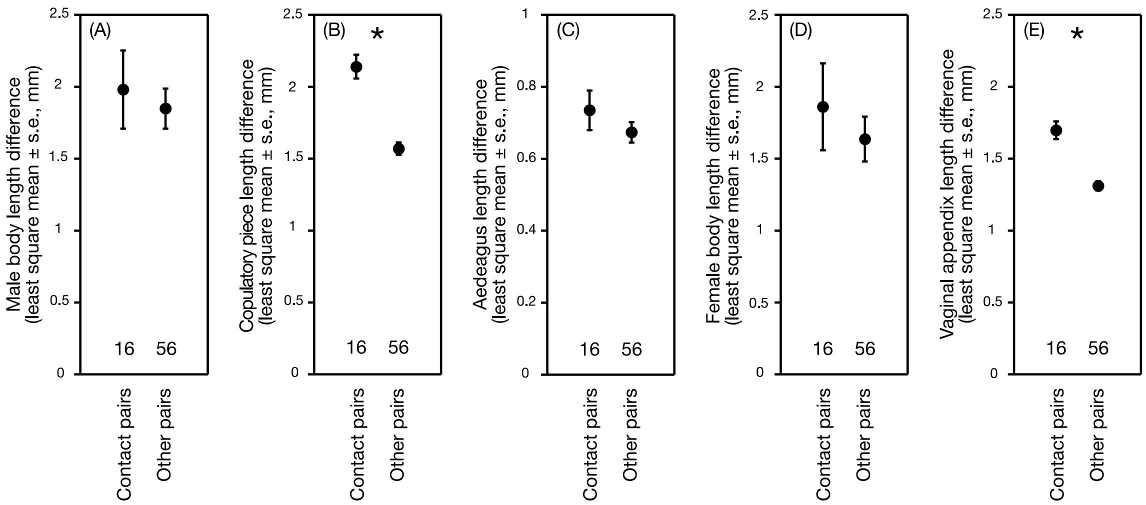

Fig. 3. Interspecific differences in mean body and genital sizes between *Carabus maiyasanus* and *C. iwawakianus* for pairs in contact zones and other pairs. The least squares means (± s.e.) of (A) male body length difference, (B) copulatory piece length difference, (C) aedeagus length difference, (D) female body length difference, and (E) vaginal appendix length difference are shown.*$P < 0.05$.

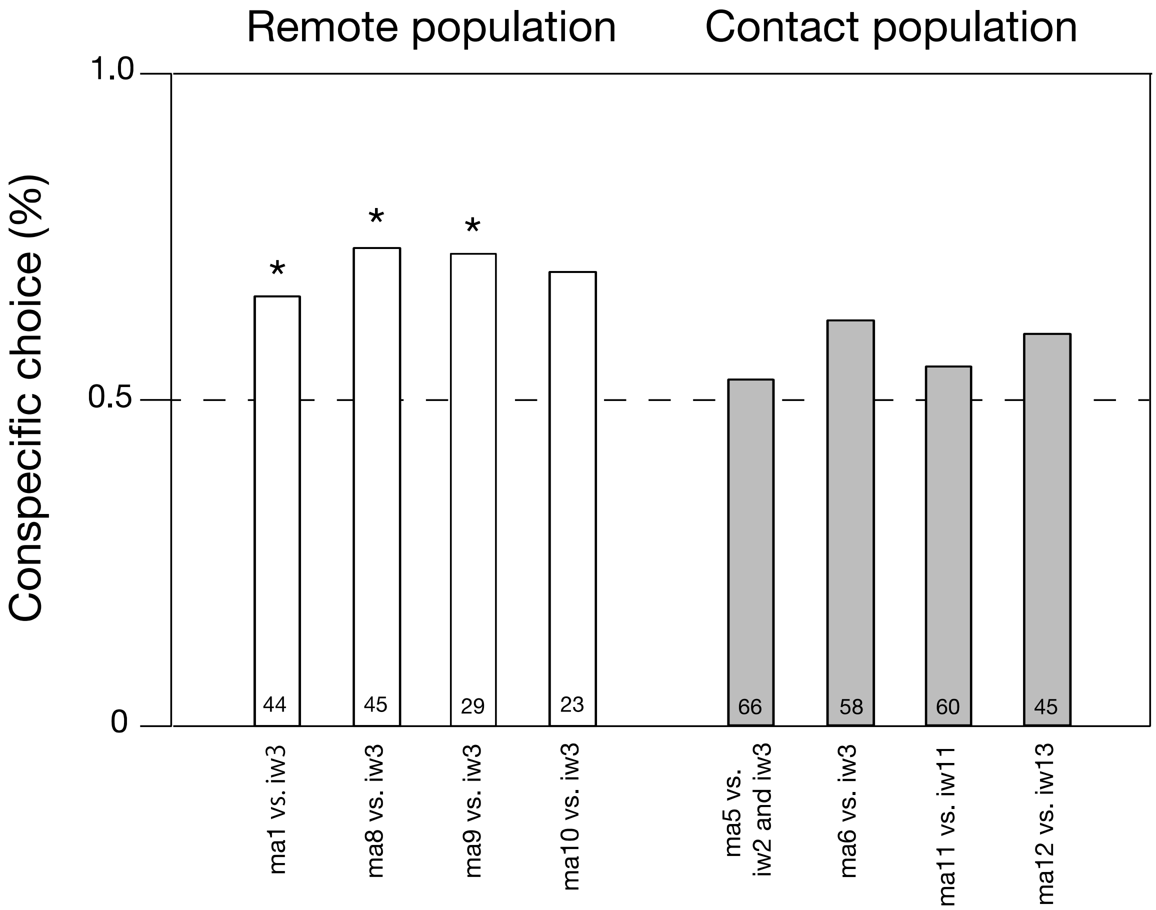

Fig. 4. Results of a mate choice experiment using *C. maiyasanus* males (male from remote population, white column,; that from contact population, grey column). Significant differences from random choice at the 5% level are shown.*$P < 0.05$.

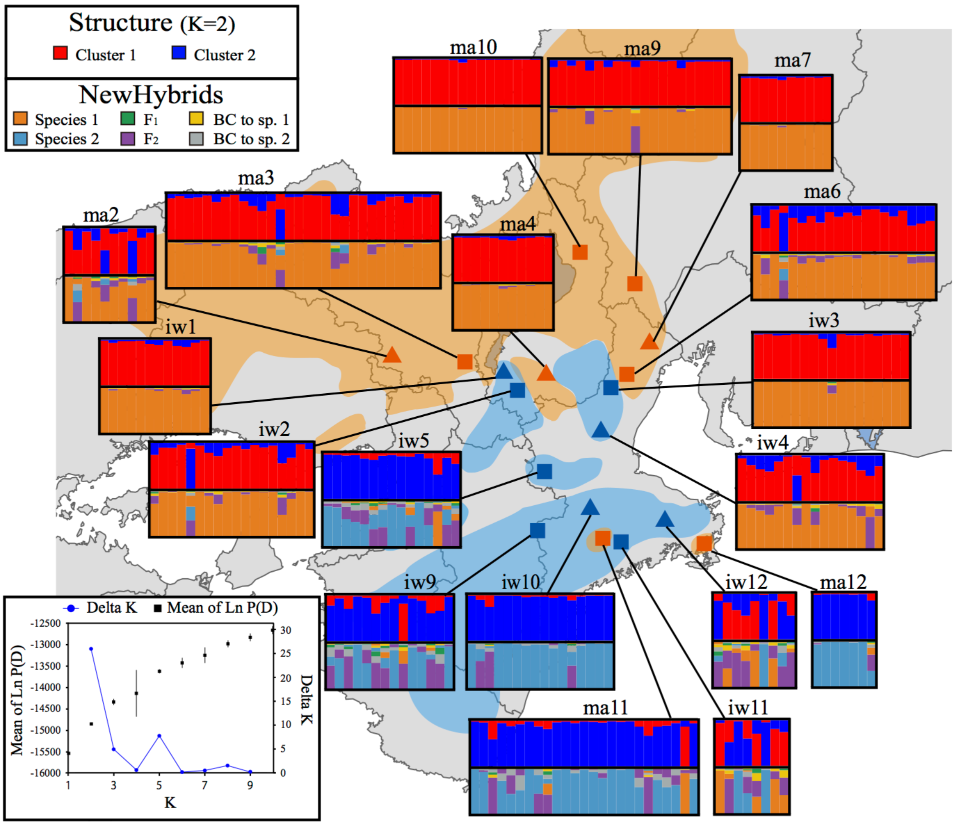

Fig. 5. Bar plots of population structure based on 12 microsatellite loci. Upper panels in the top box show two inferred clusters corresponding to *C. maiyasanus* and *C. iwawakianus* as obtained by Structure. Lower panels in the top box show six inferred hybrid classes between *C. maiyasanus* and *C. iwawakianus* as obtained by NewHybrids. Lower box shows the mean posterior probability values for the data (left axis) and delta K (right axis) in the Structure analysis.

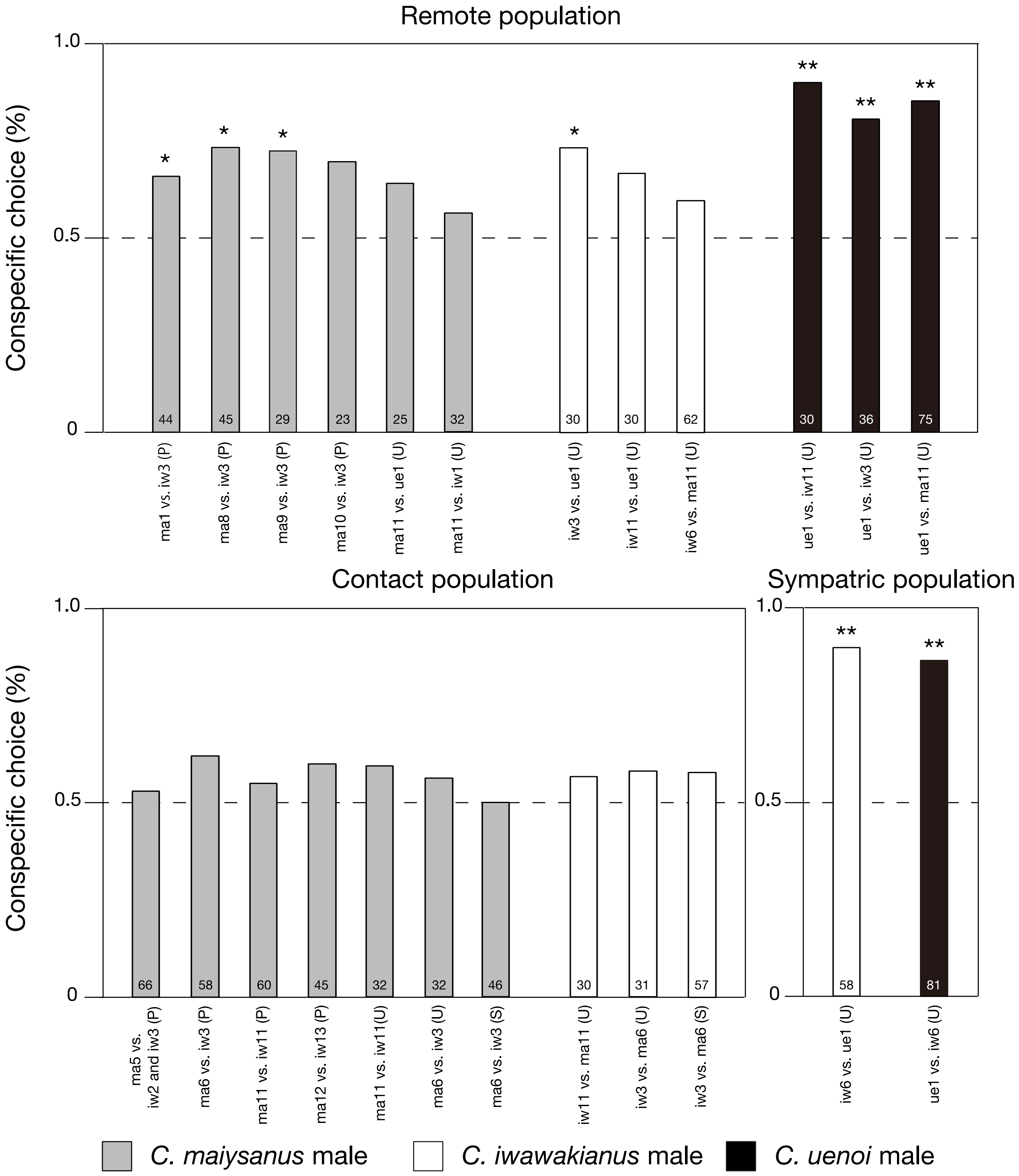

Fig. S1. Results of a mate choice experiment (male vs female) using *C. maiyasanus* (gray column), *C. iwawakianus* (white column), and *C. uenoi*. (black column) males. Letters within parentheses attached to each cross type indicate the source of the data (present study, P; Sota and Kubota 1998, S;, Usami et al. 2005, U). Significant differences from random choice at the 5% level are shown.**P*< 0.05, ** *P*< 0.001.

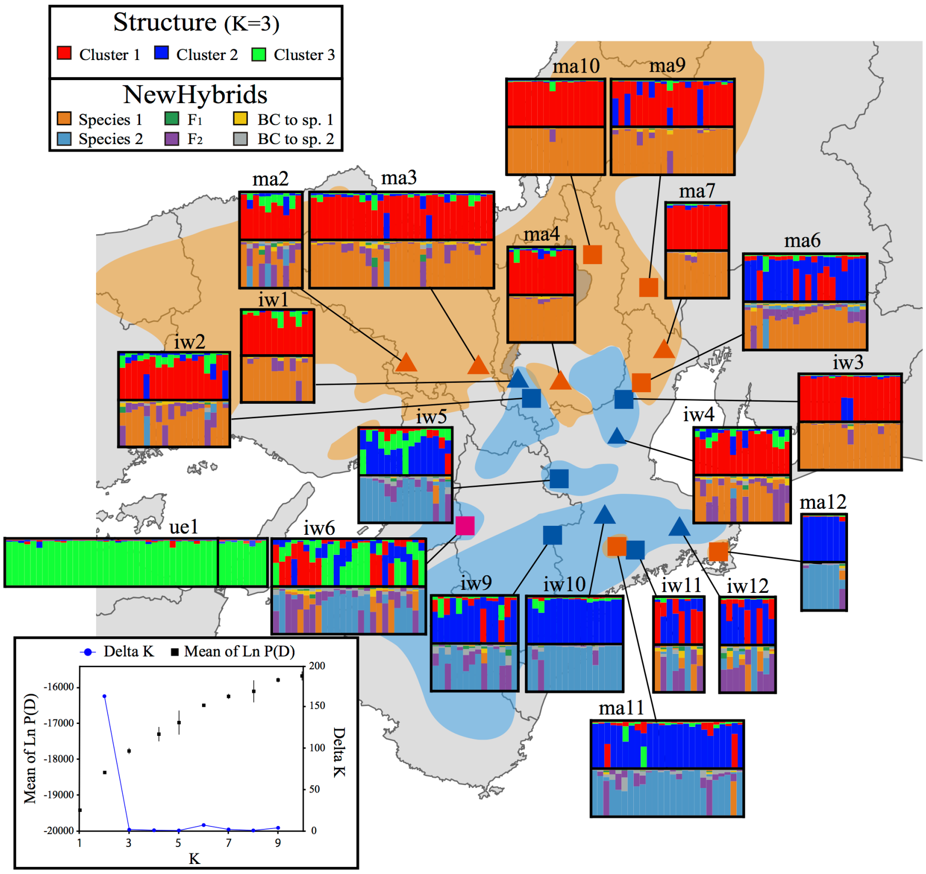

Fig S2. Bar plots of population structure based on 12 microsatellite loci. Upper panels in the top box show three inferred clusters corresponding to *C. maiyasanus*, *C. iwawakianus,* and *C. uenoi* as obtained by Structure. Lower panels in the top box show six inferred hybrid classes between *C. maiyasanus* and *C. iwawakianus* as obtained by NewHybrids. Lower box shows the mean posterior probability values for the data (left axis) and delta K (right axis) in the Structure analysis. The results with K = 3 are shown for three species, although K = 2 was identified as optimal based on delta K values.