

Morphological, genetic and behavioural analyses of a hybrid zone between the ground beetles *Carabus lewisianus* and *C. albrechti* (Coleoptera, Carabidae): asymmetrical introgression caused by movement of the zone?

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We analysed the putative hybrid zone between the ground beetles *Carabus lewisianus* Breuning and *C. albrechti* Morawitz from the Kanto and Tanzawa Mountains in central Honshu, Japan, using morphological, genetic, and behavioural data. Canonical discriminant analysis of three external and five genital morphological characters revealed an apparent morphological gap, suggesting restricted sympatry in the contact zone. RFLP and molecular phylogenetic analyses of the mitochondrial gene ND5 revealed that haplotypes originating from *C. lewisianus* introgressed extensively into *C. albrechti*. Fitting tanh curves to morphological (external and genital) and genetic characters showed differential cline widths and centres, suggesting stronger selection for genital morphology with narrower cline widths, and extensive introgression of the nuclear genes responsible for external characters relative to those controlling genital morphology into the range of *C. albrechti*. Linkage disequilibria between the morphological characters did not differ from zero, suggesting sufficient recombination occurred in the zone but the effect of small sample sizes was not negligible. Mate choice trials demonstrated that males showed a weak but not significant preference for conspecific mates. We interpret these patterns as evidence for (1) partial reproductive isolation presumably by postmating processes; (2) selection against intermediate genital morphologies, and (3) independent responses of the characters to different evolutionary forces that may cause movement of genital clines and introgression of the mitochondrial gene and external morphology. The movement of the boundaries may have resulted in the particular species distribution patterns in the study area. © 2005 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2005, 86, 79–94.

ADDITIONAL KEYWORDS: cline – genitalia – hybridization – *Ohomopterus* – reproductive isolation.

INTRODUCTION

Hybrid zones have been used to study evolutionary processes (Harrison, 1993), because they provide insights into the patterns of species distribution and the processes that maintain species identities. On the other hand, hybridization and introgression can increase taxonomic and genetic diversity, both in

plants and in animals (Arnold, 1997; Dowling & Secor, 1997), thereby affecting the evolution and speciation of organisms (e.g. Grant & Grant, 1994). Therefore, clarifying the structure of hybrid zones is important for understanding the mechanisms that maintain these zones, as well as the level of reproductive isolation between species, and the processes underlying speciation (Barton & Hewitt, 1985, 1989).

Analysing multilocus clines provides inferences that show how the hybrid zone is structured by various factors, such as migration into the zone, selection

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against hybrids, selection for environmental adaptation and random genetic drift (Kruuk *et al.*, 1999; Marshall & Sites, 2001). Strict concordance and coincidence of individual clines of unlinked loci suggest strong selection against hybrid genotypes, which are due to incompatibility of genes from genetically diverged taxa (endogenous selection), or hybrid genotypes that are less adaptive within the habitats of the parental taxa (exogenous selection). An example of the former is tension zones seen in *Podisma* and *Chorthippus* grasshopper hybrid zones in Europe (Barton & Hewitt, 1985; Butlin, 1998), while an example of the latter is mosaic hybrid zones seen in *Bombina* toads and *Chorthippus* grasshoppers in Europe (Bridle, Baird & Butlin, 2001; Bridle & Butlin, 2002; Vines *et al.*, 2003). These two mechanisms maintaining narrow hybrid zones are not mutually exclusive, together affecting the dynamics of the zones (Bridle *et al.*, 2001).

Differential cline centres and widths suggest that very weak selection may act on the whole set of genes, with individual genes responding independently to evolutionary forces. Differential cline centres are often observed between mitochondrial and nuclear markers, in which the former are assumed to be selectively neutral but the latter are affected by selection (e.g. Marshall & Sites, 2001; Babik, Szymura & Rafiski, 2003; Cianchi *et al.*, 2003). Differences in cline widths indicate that selection acting on the loci differs in strength, with narrower and steeper clines resulting from stronger selection (Barton & Hewitt, 1985).

The ground beetle subgenus *Ohomopterus* Reitter (genus *Carabus* Linnaeus) is endemic to Japan; it comprises 15 species, many subspecies, and shows extensive interspecific hybridization (Sota & Vogler, 2001; Sota, 2002). Male beetles have a strongly sclerotized copulatory piece in the intromittent organ; its counterpart in females is the membranous vaginal appendix (Ishikawa, 1987). Morphological mismatch between heterospecific genitalia, albeit incomplete, is a reproductive barrier between species (Kubota, 1988; Sota & Kubota, 1998).

In *Ohomopterus*, some species form hybrid zones where their distributions come into contact. The mode of hybridization varies and can be classified into three types (Kubota & Sota, 1998, 2001). Type 1 is represented by *Carabus maiyasanus* (with a long-hooked copulatory piece) and *C. iwawakianus* (with a short, pentagonal one), which form a narrow hybrid zone in Mie Prefecture (Kubota, 1988). Within this zone, various stages of hybrids (F_1 and backcrossed generations defined by genital morphology) are found with the parental types, and their frequency changes clinally along a transect (Kubota & Sota, 1998). This narrow zone is maintained by strong selection against hybridizing individuals, which suffer genital injury caused

by the mismatched genital morphology (Sota & Kubota, 1998).

Type 2 is represented by *C. insulicola* and *C. arrowianus*, both with long-hooked copulatory pieces, which form hybrid swarms in Nagano Prefecture (Sota, Kusumoto & Kubota, 2000a). These swarms contain hybrid individuals without parental types and are assumed to be established by selection for fertile hybrid individuals and restricted migration from parental populations within segmented river basins (Sota *et al.*, 2000a). Genital mismatch may occur in interspecific mating between these two species during experimental hybridization, resulting in genital injury (Sota *et al.*, 2000a). However, the mismatch is smaller than that between *C. maiyasanus* and *C. iwawakianus*, suggesting potential but weak effects on hybridization. Morphological characters also change clinally along the transect, and the mitochondria of *C. arrowianus* introgress extensively into *C. insulicola* through the hybrid swarms, but not in the other direction (Sota *et al.*, 2001). There is no evidence for asymmetry in male mate choice and interspecific hybridization success (Sota *et al.*, 2000a).

Type 3 is represented by *C. insulicola* (with a long-hooked copulatory piece) and *C. esakii* (with a short-hooked copulatory piece), which form broad sympatric zones (up to 10 km wide) in Shizuoka and Yamanashi Prefectures (Kubota & Sota, 2001). Difference in genital morphology between these species is considerable and comparable to that between *C. maiyasanus* and *C. iwawakianus*. Possible F_1 hybrids between these species with intermediate genital morphology are very rare, but mitochondrial introgression, variation in genital morphology in the sympatric zones, and the absence of assortative mate choice suggest hybridization between them.

In all three types, species have variously elaborate genitalia, the mismatch of which may have to a greater or lesser extent affected hybridization events. In contrast, hybridization among species with relatively simple genitalia has never been investigated in *Ohomopterus*.

Carabus lewisianus Breuning and *C. albrechti* Morawitz are small ground beetles that belong to the subgenus *Ohomopterus*. Males of both species possess simple triangular copulatory pieces, suggesting very little genital mismatch during interspecific copulation. The distributional ranges of the two species come into contact in the Kanto and Tanzawa Mountains (Ishikawa *et al.*, 1987; Takami & Ishikawa, 1997). However, little is known about the nature of hybridization at the boundaries. In this study, we analysed the structure of a putative hybrid zone between *C. lewisianus* and *C. albrechti* to examine how morphological (external and genital) characters and mitochondrial haplotypes change across this zone, and how

the contact zones are maintained. We show that an apparent morphological gap and restricted sympatry in the contact zone, and very weak assortativeness in male mate choice, suggest partial but incomplete reproductive isolation between the species. Incongruence in the cline widths and centres between the genital morphology and other characters, and no significant linkage disequilibria between the characters, suggest strong selection against intermediate genital morphology and independent responses of the characters to different evolutionary forces that may cause movement of genital clines and asymmetrical introgression of the mitochondrial gene and external morphology. We infer that the movement of the boundaries may have caused the particular species distribution patterns found in the study area.

MATERIAL AND METHODS

ORGANISMS AND STUDY AREA

At the western margin of the Kanto district of Japan, the Kanto Mountains are connected with the Tanzawa Mountains along a north–south gradient. In the val-

leys between these mountain ranges, the Tamagawa, Sagami-gawa and Dôshigawa Rivers run from west to east (Fig. 1). *C. albrechti* and *C. lewisianus* inhabit the northern and southern parts of this area, respectively (Fig. 1). Ishikawa *et al.* (1987) revealed that these species are parapatrically distributed. Our subsequent investigation confirmed that their parapatric contact zones, defined by genital morphology, are also present across the Dôshigawa River (Fig. 1; Takami & Ishikawa, 1997). The western part of this area is mountainous and mostly covered with coniferous and broad-leaved deciduous forests. In the eastern part, altitudes are lower and the forests are gradually displaced by disturbed areas, such as cultivated fields and cities. This environmental gradient results in *C. lewisianus* inhabiting better conserved forests than *C. albrechti* in this area. However, it is unknown whether this distributional pattern is formed by the difference in ecological requirements of these species, because both *C. lewisianus* and *C. albrechti* inhabit forest floors and margins.

To examine morphological and genetic variation between the two species, we established a transect across the boundary, along the Dôshigawa River,

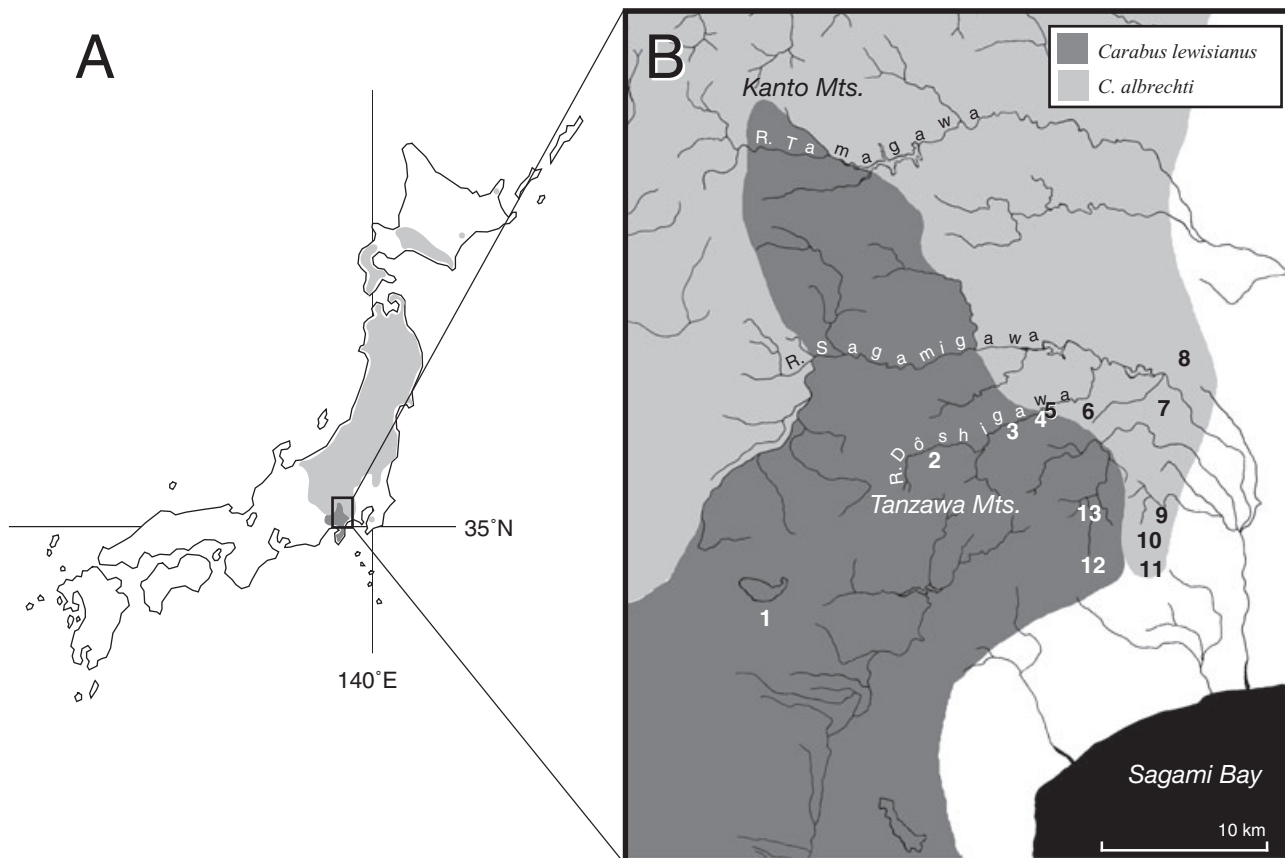


Figure 1. Distribution of *Carabus lewisianus* and *C. albrechti*: (A) in Japan, (B) in the Kanto and Tanzawa Mountains. White and black numerals (1–13) refer to the populations of *C. lewisianus* and *C. albrechti*, respectively.

Table 1. List of localities where *Carabus lewisianus* (1–4, 12 and 13) and *C. albrechti* (5–11) were collected. Annual mean temperatures are estimated from the longitude, latitude and altitude of the sites (Sota *et al.*, 2000a). Numbers of individuals used for morphological (N_m) and genetic (N_g) analyses are shown. AMT, annual mean temperature (°C)

Site	Locality	Longitude	Latitude	Altitude	AMT	N_m (male, female), N_g
1	Kagosaka-toge, Yamanakako-mura, Yamanashi	138.87	35.39	1200	7.89	69 (29, 40), 10
2	Kotsubaki, Doshi-mura, Yamanashi	139.06	35.53	560	11.52	17 (7, 10), 10
3	Hashitsubara, Tsukui-machi, Kanagawa	139.13	35.54	460	12.10	18 (4, 14), 10
4	Nishinono, Tsukui-machi, Kanagawa	139.18	35.56	290	13.08	52 (19, 33), 11
5	Nagano, Tsukui-machi, Kanagawa	139.18	35.56	290	13.08	39 (14, 25), 23
6	Yato, Tsukui-machi, Kanagawa	139.21	35.56	310	12.97	30 (19, 11), 11
7	Shida-toge, Tsukui-machi, Kanagawa	139.27	35.55	270	13.22	34 (12, 22), 14
8	Shichikoku-toge, Machida-shi, Tokyo	139.32	35.61	180	13.69	70 (26, 44), 10
9	Shimizugaoka, Kiyokawa-mura, Kanagawa	139.29	35.47	135	14.08	8 (3, 5), 9
10	Hinata, Isehara-shi, Kanagawa	139.32	35.42	230	13.57	16 (9, 7), 9
11	Ooyama, Isehara-shi, Kanagawa	139.26	35.42	180	13.86	10 (6, 4), 10
12	Yabitsu-toge, Hadano-shi, Kanagawa	139.22	35.43	800	10.22	10 (3, 7), 10
13	Susugaya, Kiyokawa-mura, Kanagawa	139.22	35.47	460	12.17	6 (4, 2), 6

where sites 1–4 and 5–8 were selected, in the ranges of morphologically defined *C. lewisianus* and *C. albrechti*, respectively (Fig. 1). Sites 4 and 5 were located on the banks of a small river, approximately 5 m wide, in a steep valley about 20 m deep that has the potential of being a barrier (Fig. 1). Five additional sites were chosen in the southern part of the transect, such that sites 9–11 were in the southern extension of the *C. albrechti* range, and sites 12 and 13 represented their counterparts in the range of *C. lewisianus* (Fig. 1). Populations at sites 1 and 8 were considered to be unaffected by contact between the species. The longitude, latitude and altitude of each site were recorded and used to calculate geographical distances along the transect from sites 1–8 (Table 1).

Adult beetles were caught by pitfall traps between May and July, 1996–2003. For morphological analysis, male genitalia were removed, and the endophallus was everted by injection of 80% ethanol and then dried. For genetic analysis, testes and muscles surrounding the vagina were removed from males and females, respectively, and stored in absolute ethanol. For behavioural analysis, adults were housed in an incubator at 20 °C with long daylight conditions (16L : 8D) to maintain sexual activity; the animals were fed minced beef every other day.

MORPHOLOGICAL ANALYSIS

To investigate morphological variation near the contact zone, we measured the following characteristics: body length from the anterior margin of the labrum to the apices of the elytra (BL), pronotal width (PW) and number of pronotal setae (PS, averaged for left and right margins); in males we measured length of the aedeagus (ADL), length of the apical portion of the

aedeagus (APL), length of the left basal lobe of the endophallus (LBL), and length and width of the copulatory piece on the endophallus (CPL and CPW, respectively; Fig. 2). BL was measured to the nearest 0.01 mm using digital calipers, PS was counted under a binocular microscope, and the remaining characters were measured to the nearest 0.0025 mm using a binocular microscope with an ocular micrometer.

Within one species of ground beetle, BL is correlated with the annual mean temperature of the habitat (Sota *et al.*, 2000b, c). In this study, beetle habitats ranged from 135 m to 1200 m a.s.l.; thus, BL was potentially affected by climatic differences (Table 1). To remove this effect, we corrected the original measurements of BL as follows.

First, we constructed a model in which BL was the dependent variable, and annual mean temperature, species, sex, and their interactions were the independent variables. The annual mean temperature (T) was estimated from the longitude, latitude and altitude of the site by the formula: T (°C) = 38.17 – 0.882 latitude (°N) + 0.0573 longitude (°E) – 0.00585 altitude (m a.s.l.) (Sota *et al.*, 2000b, c). Populations of *C. lewisianus* ($N = 50$) and *C. albrechti* ($N = 33$) distributed in the Kanto District were chosen from the data of Sota *et al.* (2000c), and the effects of three variables on mean BL were analysed. We found no interactions among temperature, species, and sex, indicating that the slopes of BL on temperature are common across species and sexes (species \times sex: d.f. = 1, $F = 0.302$, $P = 0.5844$; species \times temperature: d.f. = 1, $F = 0.393$, $P = 0.5325$; sex \times temperature: d.f. = 1, $F = 1.235$, $P = 0.2703$; species \times sex \times temperature: d.f. = 1, $F = 0.149$, $P = 0.7010$). We then performed an ANCOVA excluding these interactions (temperature: regression coefficient = 0.090, d.f. = 1,

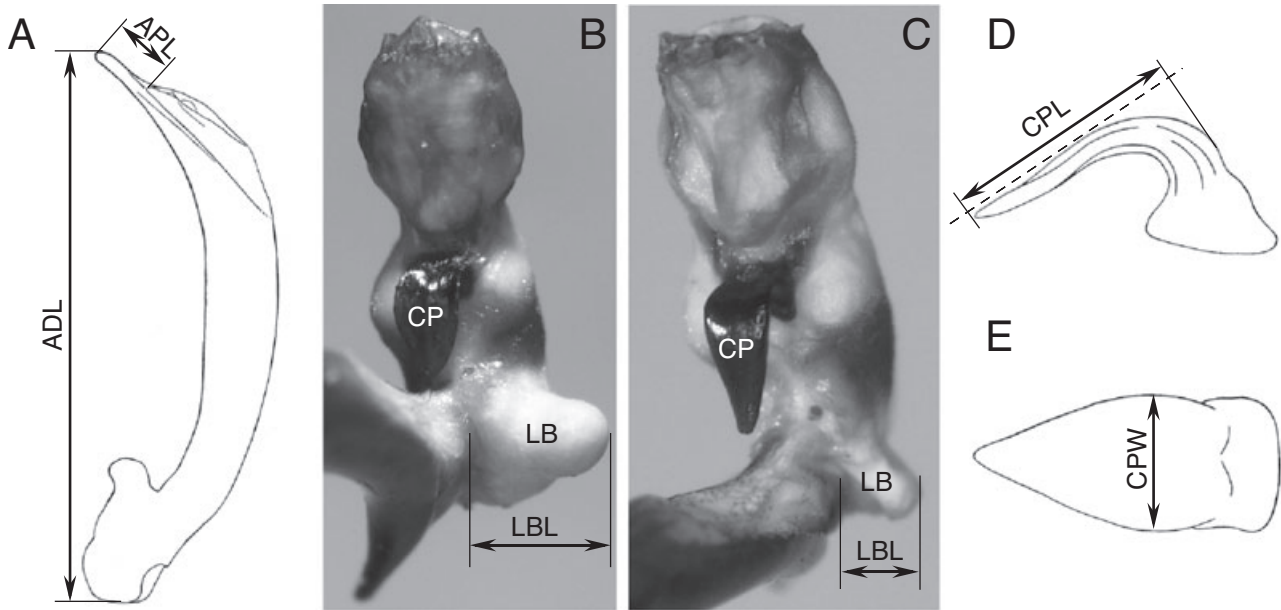


Figure 2. Male genitalia of *Carabus lewisianus* and *C. albrechti*. A, aedeagus of *C. albrechti*. B, C, endophalli of *C. lewisianus* and *C. albrechti*, respectively. D, copulatory piece of *C. albrechti*, lateral view. E, ditto, dorsal view. *Abbreviations:* ADL, length of aedeagus; APL, length of apical portion of aedeagus; CP, copulatory piece; CPL, length of copulatory piece; CPW, width of copulatory piece; LB, left basal lobe; LBL, length of left basal lobe.

$F = 19.231$, $P < 0.0001$; species: d.f. = 1, $F = 86.178$, $P < 0.0001$; sex: d.f. = 1, $F = 248.744$, $P < 0.0001$). BL in both species and both sexes increased with annual mean temperature at the rate of $0.090 \text{ mm}/^{\circ}\text{C}$. We then corrected the original measurements of BL, based on the formula: BL (controlled) = BL (original) + $0.090 \times (T - \text{annual mean temperature})$, where T is a constant (= 12.42), i.e. the mean annual temperature averaged for the 13 sites used in this study. These analyses were performed using StatView 5.0 (SAS Institute Inc., 1998).

To summarize morphological variation among populations, canonical discriminant analysis (CDA) was performed based on 13 populations of both species using STATISTICA (Statsoft, 1994), in which eight variables (BL, PW, PS, ADL, APL, LBL, CPL and CPW) were included for males, and three variables (BL, PW and PS) for females. BL was corrected for annual mean temperature, as described above. PW and ADL were standardized, based on the original BL. APL, LBL, CPL and CPW were standardized, based on ADL. All variables were log-transformed prior to analysis.

GENETIC ANALYSIS

To reveal the diversity and introgression of mitochondrial genes, we surveyed restriction fragment length polymorphisms (RFLPs) of the mitochondrial NADH dehydrogenase subunit 5 (ND5) gene, as described by

Suzuki, Sato & Ohba (2002). Between six and 23 individuals were selected from each population; total DNA of each individual was extracted using a Mag extractor genome nucleic acid purification kit (Toyobo), and stored in $100 \mu\text{L}$ of sterile water. For amplification of the ND5 gene (1083 bp) by the polymerase chain reaction (PCR), we used the primers 5'-CCTGTTTCTGCTTTAGTTCA-3' and 5'-GTCATACTCTAAATATAAGCTA-3' (Su *et al.*, 1996). PCR product ($5 \mu\text{L}$) was digested overnight with 2 U each of the restriction endonucleases *AseI*, *DraI*, *HhaI*, and *HinfI*, at 37°C . Digested fragments were electrophoresed on 4% polyacrylamide gels. Haplotypes were determined on the basis of the fragment lengths produced by the four enzymes.

We determined the DNA sequences of all discriminated haplotypes of the ND5 gene within each population. PCR products of each haplotype were cloned into the pCR 2.1 plasmid vector (Original TA Cloning Kit, Invitrogen) and transformed into *Escherichia coli* competent cells. Inserted DNA sequences were determined using a Thermo-sequence fluorescence-labelled primer cycle sequencing kit (Amersham Pharmacia Biotech) with a Li-Cor 4000 L DNA sequencer (Aloka), and using an ABI PRISM dye terminator cycle sequencing-ready reaction kit with an ABI PRISM 377 automated sequencer (PE Biosystems).

To reveal genealogical relationships among haplotypes, molecular phylogenetic analyses were conducted on the basis of the haplotype sequences.

Sequences of the ND5 gene of *C. lewisianus* (D50724), *C. albrechti* (D50359, D871311, D87132, D87133, D87134, D87174 and AF219429), and *C. yamato* (D50733, as an outgroup) that were retrieved from GenBank were also included. We used Modeltest 3.06 to choose the model of DNA substitution suitable for our data based on the results of hierarchical likelihood ratio tests (Posada & Crandall, 1998). We then constructed a maximum likelihood (ML) tree using PAUP* 4.0b10, given the chosen model (Swofford, 2002). Heuristic searches were performed using 100 replicates of random addition sequences with the tree bisection-reconnection branch-swapping method. To estimate the statistical reliability of the nodes, we performed bootstrap analyses with 100 replicates.

CLINE ANALYSIS

We attempted to fit ML tanh curves to the population means of the morphological and genetic characters in the transect using ANALYSE 1.3 (Barton & Baird, 2002). A tanh cline is defined as $y = (1 + \tanh[2(x-c)/w])/2$, where x and y are variables corresponding to a location along the transect and population means of the character, respectively, while c and w are constants for the position of cline centre and cline width, respectively (Szymura & Barton, 1986). This cline model was originally developed for one locus with two alleles of which frequencies changed from 0 to 1 along the transect. In order to apply the model to quantitative characters, the above model is modified as $y' = P_{\min} + y(P_{\max} - P_{\min})$, where y' is population mean of the character in the modified model, and P_{\min} and P_{\max} refer to the minimum and maximum character scores in the tails of the cline, instead of 0 and 1 in the original model (S. J. E. Baird, pers. comm.). Before fitting clines, we rescaled our data to range between 0 and 1.

In searching the ML cline with a Metropolis algorithm using ANALYSE, we fixed P_{\min} and P_{\max} , and gave a suboptimal score for c as a starting point. These three scores are obtained by fitting individual data to the sigmoid function: $y = s + p/[1 + q \exp(rx)]$, where p , q , r , and s were constant, using the non-linear fitting module in STATISTICA, in which P_{\min} , P_{\max} and c are given as s , $s + p$, and $-\ln(q)/r$, respectively (Sota *et al.*, 2000a). To control for the effect of small sample sizes (genetic drift) on cline fitting, which may cause a departure of the population mean score from the cline, we computed F_{ST} based on mitochondrial haplotype frequencies using TFPGA (Miller, 1997). The values of F_{ST} were relatively high: 0.1628 in *C. lewisianus* and 0.3167 in *C. albrechti*. We used the conservative value 0.1628 in cline fitting.

Statistical significance of cline parameters c and w was examined by computing likelihood profiles (Phillips, Baird & Moritz, 2004) using the cross-section

option of ANALYSE. The 95% confidence intervals were estimated at points with a two unit decrease of log likelihood below the maximum (Edwards, 1972).

The difference in the position of the cline centre (c) between two characters (i.e. coincidence) was examined by fitting a tanh curve to the data of the first with the cline centre fixed to that of the second, allowing only the cline width to vary (Dasmahapatra *et al.*, 2002). If the fitted cline showed significant decrease of log likelihood below the maximum, i.e. larger than two units, it was determined that these two characters had different cline positions. It was same in the case of cline width (w) (i.e. concordance), in which a tanh curve was fitted to the data of one character while keeping the cline width of the other and allowing the cline centre to vary.

It is expected that covariance between characters is increased in a hybrid zone due to selection against recombined hybrid genotypes (Nürnberger *et al.*, 1995). We computed linkage disequilibria (D^*) between morphological characters within each population using the formula $D^* = 2\text{cov}(z, z^*)/\Delta z \Delta z^*$, where z and z^* are two quantitative characters, and Δz and Δz^* are the differences in fitted character scores at each end of clines, i.e. $P_{\max} - P_{\min}$ (Bridle & Butlin, 2002). Significant increase of D^* from zero was tested by bootstrapping with 1000 replications for estimating 95% confidence intervals.

BEHAVIOURAL ANALYSIS

To clarify the magnitude of premating reproductive isolation between species, we examined assortative mate choice by males of each species. Ten females each of *C. lewisianus* and *C. albrechti* were introduced into a plastic box (38 cm × 26 cm × 15 cm), into which a randomly chosen male of one species was gently released. The behaviour of the males was observed for up to 3 min; the species of the females mounted by the males was recorded, and only males that attempted to insert the aedeagus were considered to have made a 'choice'. Males were gently removed from females prior to aedeagal insertion. To examine the effect of inter-specific contact on male mate choice, we performed two experiments using populations 4 and 5 (nearest to the contact zone) and populations 1 and 8 (probably no effect of contact).

RESULTS

MORPHOLOGICAL ANALYSIS

Variation in morphological characters was summarized by CDA, in which the first and second canonical components (CAN) significantly explained 87.1% and 6.75% of the total variation among males, and 94.2% and 4.60% of the total variation among females

Table 2. Parameters of canonical discriminant components on the male and female morphological characteristics of populations around the contact zone between *Carabus lewisianus* and *C. albrechti*

	Male		Female	
	CAN1	CAN2	CAN1	CAN2
Eigenvalue (%)	12.558 (87.1)	0.973 (6.75)	2.861 (94.2)	0.140 (4.60)
Wilks' λ	0.017	0.227	0.219	0.847
χ^2 (<i>P</i>)	586.69 (0.000)	212.59 (0.000)	326.07 (0.000)	35.635 (0.033)
Standardized canonical coefficient (row coefficient)				
BL	0.384 (12.205)	-0.948 (-30.156)	-0.395 (-11.897)	-0.797 (-23.987)
PW	0.047 (1.480)	-0.017 (-0.532)	-0.462 (-15.516)	0.627 (21.081)
PS	0.233 (1.080)	-0.447 (-2.069)	-0.812 (-4.098)	0.066 (0.332)
ADL	0.303 (9.326)	-0.541 (-16.652)	–	–
APL	0.202 (4.143)	0.651 (13.350)	–	–
LBL	-0.747 (-6.311)	-0.133 (-1.125)	–	–
CPL	0.621 (13.009)	-0.205 (-4.297)	–	–
CPW	-0.274 (-4.873)	-0.253 (-4.478)	–	–

ADL, length of aedeagus; APL, length of apical portion of aedeagus; BL, body length; CPL, copulatory piece length; CPW, copulatory piece width; LBL, length of the left basal lobe; PS, number of pronotal setae; PW, pronotal width.

(Table 2). Biplots of individuals from sites 1 and 8 on CAN1 and CAN2 showed that CAN1 explained interspecific differences both in males and females, and that males were separated better than females (Fig. 3). Populations around the contact zones (sites 2–7 and 9–13) were scattered between the two outer populations, such that males of both species were still separated by CAN1, whereas females largely overlapped with each other, probably because of the small number of measured characteristics (Fig. 3).

A frequency distribution of CAN1 scores showed the presence of an apparent gap between sites 4 and 5 along the transect (Fig. 4). The distributions of male scores did not overlap between sites 1–4 and 5–8, while those of females slightly overlapped, with an apparent shift of the peaks between sites 1–4 and 5–8. One male and one female with outlier scores were found at sites 5 and 4, respectively (arrowed in Fig. 4). In the southern part of the transect (sites 9–13), an apparent gap in male scores was observed between sites 9–11 and 12–13, whereas female scores slightly overlapped with an apparent shift of the peaks.

GENETIC ANALYSIS

RFLP analysis revealed seven haplotypes of the ND5 gene (A–G), in which *AseI*, *DraI*, *HhaI*, and *HinfI* produced 4, 2, 2, and 2 types of band patterns, respectively (Table 3). The ML tree showed that haplotype D belonged to a different lineage than the other haplotypes (Fig. 5). All haplotypes, except D, were clustered with *C. lewisianus* from Hakone and with *C. albrechti* ssp. *okumurai*, forming lineage I (Fig. 5). Within this lineage, haplotypes A and F were monophyletic, while

haplotypes B and C were not clustered with one another. Lineage I included haplotypes obtained from both *C. lewisianus* and *C. albrechti*, although they were not clustered specifically. On the other hand, haplotype D was clustered with the remaining *C. albrechti* sequences distributed in eastern Honshu and Hokkaido, forming lineage II. This lineage mostly comprised the haplotypes obtained from *C. albrechti*, except for one haplotype from site 4. Bootstrap probabilities at the nodes defining these lineages were moderate to high.

The geographical distribution of the haplotypes is shown in Figure 6. Haplotypes A and G were found only in the range of *C. lewisianus*, while haplotypes E and F were only found in the range of *C. albrechti*. Haplotype C was mainly found in the range of *C. lewisianus* but was shared at site 11, in the range of *C. albrechti*. In contrast, haplotype D was mainly found in the range of *C. albrechti* but was shared at site 4, in which the individual with haplotype D had *C. lewisianus* morphology. Haplotype B broadly occurred in all sites except site 8, which probably represented a pure population of *C. albrechti*.

CLINE ANALYSIS

Tanh curves were successfully fitted to six morphological (BL, PW, PS, LBL, CPL and CPW) characters and one genetic character (HAP) (Table 4). The cline centres of the external (BL, PW and PS) and genetic characters (HAP) were placed around 36–37 km points in the transect, which fell within the range of *C. albrechti* (Fig. 7). On the other hand, the cline centres of the genital characters (LBL, CPL and CPW) were placed

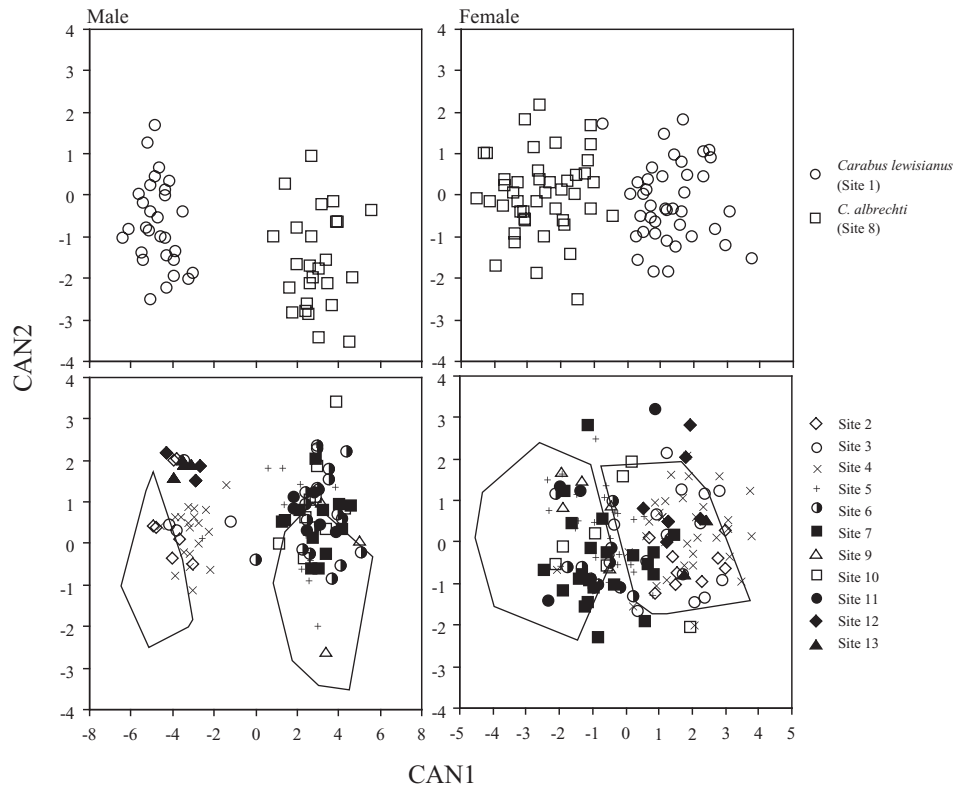


Figure 3. Plots of canonical discriminant scores of populations around the contact zone between *Carabus lewisianus* and *C. albrechti*. Upper panels show two distant populations (sites 1 and 8). Other populations are shown in the lower panels, with upper populations indicated by unbroken lines.

near the 33 km point in the transect, which nearly corresponded with the gap recognized by the CDA. These differences in the position of the cline centre were supported statistically (Table 5). No significant difference was observed within the external characters and between the external and genetic characters, while all pairs of the external and genital, and genetic and genital characters showed significant differences in the position of cline centre. Additionally, one pair of genital characters (LBL and CPW) also showed a significant difference in the position of the cline centre.

Cline widths were about ten times broader in the external and genetic characters than in the genital characters (Table 4). These differences were statistically supported by comparing log likelihood scores (Table 5), in which the widths between (1) the external and genital, and (2) the genetic and genital characters were all significantly different, while one pair of genital characters (LBL and CPL) also showed a significant difference in cline width.

Covariances between the morphological characters were evaluated by estimating linkage disequilibria between all pairs of the characters within all 13 populations. Ninety-five per cent confidence intervals of all estimated linkage disequilibria included zero, indi-

cating that increased linkage disequilibria in the hybrid zone could not be detected.

BEHAVIOURAL ANALYSIS

In the experiment using beetles from sites 4 and 5, males of *C. lewisianus* chose conspecific mates 56.9% of the time (37/65 trials), which was only marginally significant (Binomial test, $P = 0.0533$). Males of *C. albrechti* chose conspecific mates 55.6% of the time (25/45), which was not significant (Binomial test, $P = 0.0901$).

In the experiment using beetles from sites 1 and 8, males of *C. lewisianus* and *C. albrechti* chose conspecific mates 63.6% (7/11) and 54.5% of the time (18/33), respectively. These percentages did not differ from random choice (Binomial test, $P = 0.1611$ for *C. lewisianus* males, $P = 0.1207$ for *C. albrechti* males).

DISCUSSION

PARAPATRY AND REPRODUCTIVE ISOLATION BETWEEN *C. LEWISIANUS* AND *C. ALBRECHTI*

CDA of the populations around the contact zone between *C. lewisianus* and *C. albrechti* showed that these two species are morphologically differentiated

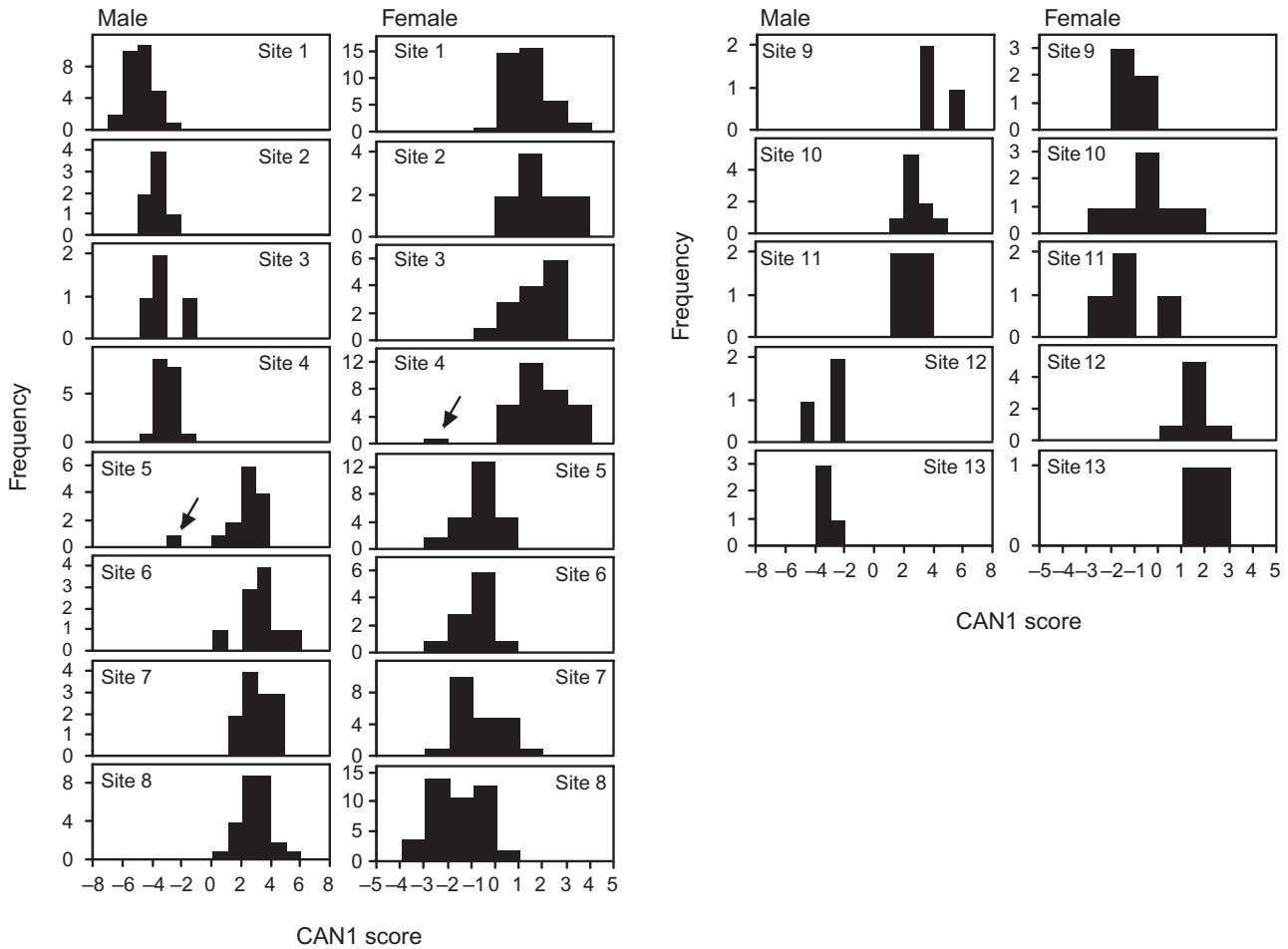


Figure 4. Changes in frequency distributions of CAN1 scores among populations around the contact zone between *Carabus lewisianus* and *C. albrechti*. Arrows indicate individuals with an outlier CAN1 score, suggesting sympatry.

Table 3. Variations of restriction fragment lengths (bp) produced from the mitochondrial ND5 gene (1083 bp) of *Carabus lewisianus* and *C. albrechti*. Variation in fragment lengths defines haplotypes (A–G). *AseI* and *DraI* are expected to produce fragments shorter than about 100 bp, but they are undetectable

Haplotype	Restriction enzyme			
	<i>AseI</i>	<i>DraI</i>	<i>HhaI</i>	<i>HinfI</i>
A	537/422	495	957/126	910/173
B	537/422	279/216	957/126	910/173
C	537/422	279/216	1083	910/173
D	438/324/105/99	279/216	1083	910/173
E	323/216/142/120/112/106	279/216	957/126	910/173
F	537/422	279/216	957/126	1083
G	567/422	279/216	957/126	910/173

with an apparent gap in their boundaries, forming parapatric distributions. We found individuals with heterospecific morphology in populations near the gap (sites 4 and 5), indicating that sympatry occurred in

these areas. Since the frequencies of these heterospecific individuals were low, and they occurred in limited areas, sympatry may be caused by migration across borders.

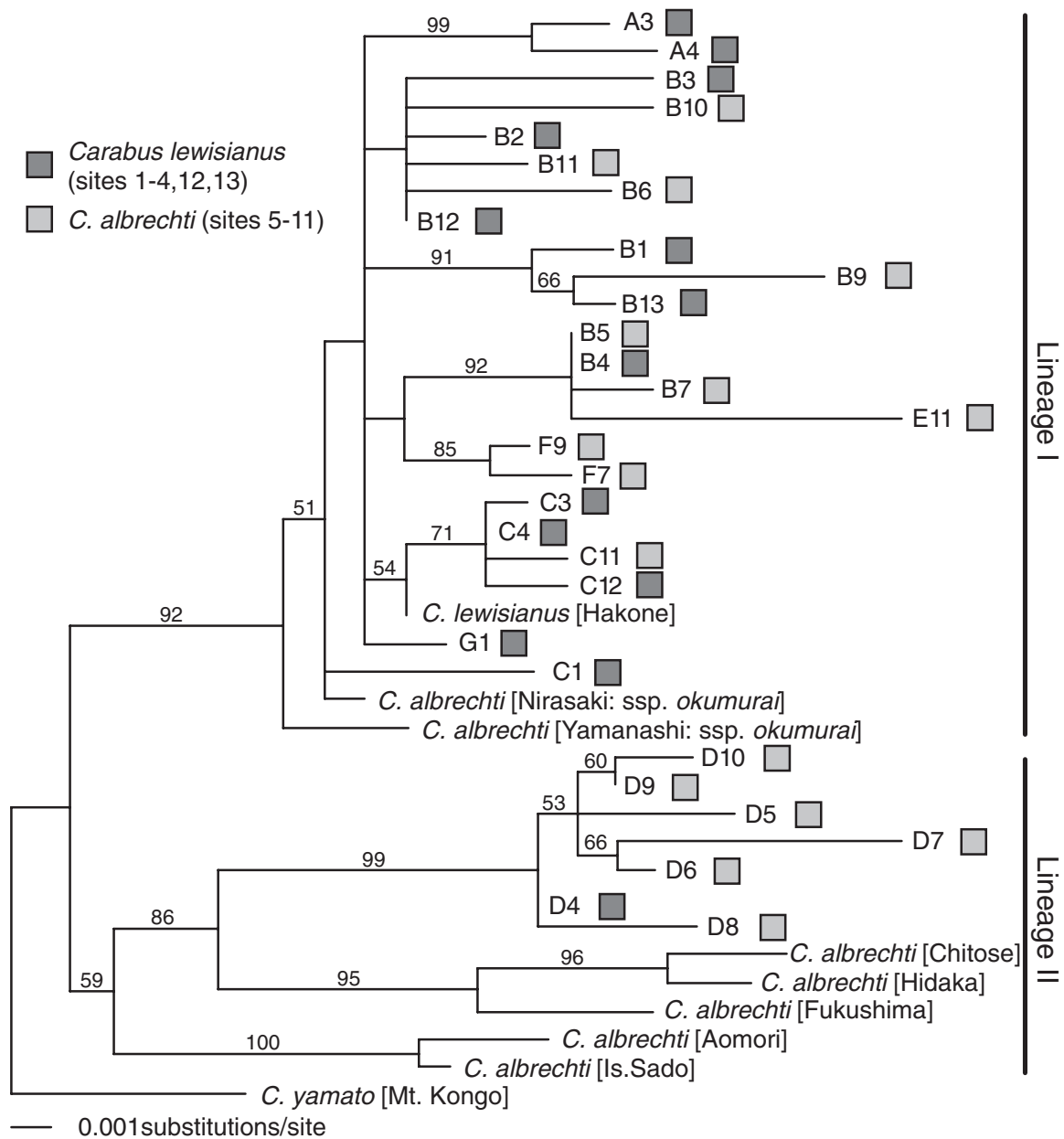


Figure 5. Maximum likelihood tree among haplotypes of the mitochondrial ND5 gene (1083 bp) of *Carabus lewisianus* and *C. albrechtii*. Samples are represented by haplotypes (A–G) and sites (1–13). Bootstrap probabilities (> 50%) are shown on branches.

RFLP analysis revealed that the predominant haplotypes (B, C and D) were shared by both species. Genealogical relationships among the haplotypes indicated that D originated from *C. albrechtii*, whereas the remainder originated from *C. lewisianus*. Their geographical distribution suggests that haplotypes from each species introgressed into the other, and the directionality was apparently asymmetrical and skewed toward *C. albrechtii* in this region. Since the haplotypes originating from *C. lewisianus* that

introgressed into the range of *C. albrechtii* were not clustered monophyletically, introgressions may have occurred repeatedly. Additionally, the sequences of *C. albrechtii* ssp. *okumurai* that were clustered within lineage I may be other introgressants from *C. lewisianus*.

Reproductive isolation between *C. lewisianus* and *C. albrechtii* may be incomplete, as indicated by introgressive hybridization of mitochondria and variation in morphological characters. However, the presence of

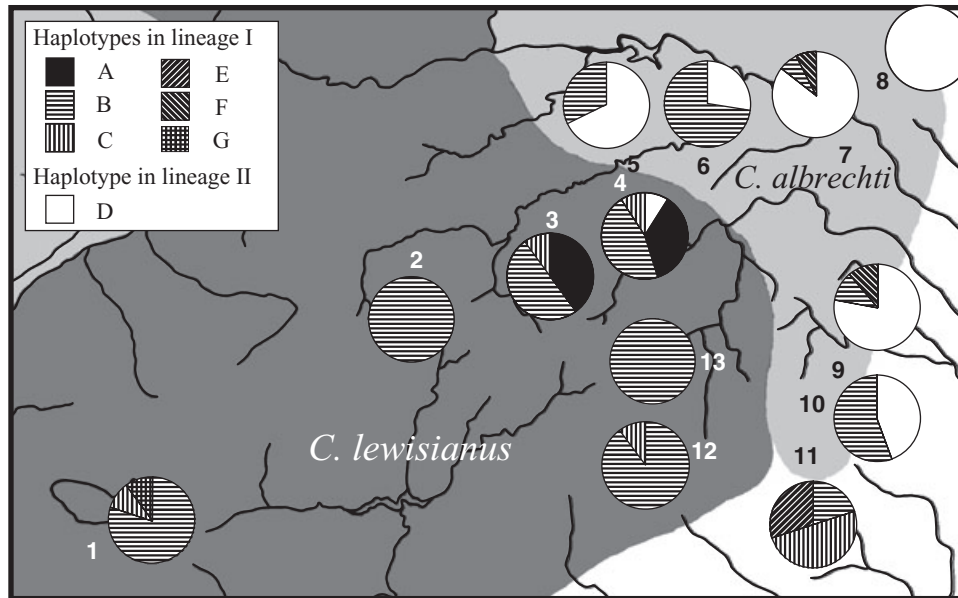


Figure 6. Distribution and frequency of mitochondrial ND5 gene haplotypes (A–G) in the populations of *Carabus lewisianus* (1–4, 12 and 13) and *C. albrechti* (5–11).

the apparent morphological gap between species suggests that these two species are more reproductively isolated than other congeneric species pairs, such as *C. maiyasanus* and *C. iwawakianus*, which form a narrow hybrid zone (Kubota, 1988; Sota & Kubota, 1998), and *C. insulicola* and *C. arrowianus*, which form hybrid swarms (Sota *et al.*, 2000a). The lack of male mate choice suggests that premating isolation mechanisms may be weak and that interspecific mating can easily occur. Factors that could maintain isolation between species may be in postmating processes; examples include a morphological mismatch between male and female genitalia, sperm precedence and female choice for conspecific males, and sterility and inviability of hybrid individuals. Artificial hybridization experiments remain to be performed.

The results of this study show that the hybrid zone between *C. lewisianus* and *C. albrechti* is similar to type 3 hybridization in the subgenus *Ohomopterus* (see Introduction). However, it differs from this type in that sympatric zones are restricted to areas adjacent to the borders. A possible explanation for this difference may be habitat preference of species. Habitats favoured by *C. insulicola* and *C. esakii* are clearly different; the former inhabits open areas whereas the latter prefers forested environments, thus allowing these species to coexist easily (Kubota & Sota, 2001). However, both *C. lewisianus* and *C. albrechti* prefer forests; therefore, as they are likely to compete ecologically, the coexistence of these two species would be more difficult (Bull, 1991).

SELECTIVE AGENTS OF DIFFERENTIAL CLINE WIDTHS

Fitting tanh clines to the external, genital and genetic characters revealed significant differences in cline widths and positions between the genital and other characters (Table 5). These results address two questions concerning how the difference in cline width between the genital and other characters is caused, and how the positions of the cline centres are differentiated.

Narrow and steep clines found in the genital characters suggest strong selection against intermediate genital morphology, in which two types of selective force, natural and sexual selection, are considerable. Natural selection for the genital morphologies of ground beetles is due to the particular morphological correspondence between the sexes, the so-called genital lock-and-key (Sota & Kubota, 1998). In the hybrid zone between *C. maiyasanus* and *C. iwawakianus*, a morphological mismatch of the heterospecific genitalia induces strong costs to the male and female through genital injury and subsequent reproductive failure. Such costs arising from heterospecific mating could maintain parapatric distribution, known as reproductive interference or the satyr effect (Rebeiro & Spielman, 1986; Kuno, 1992; Yoshimura & Clark, 1994). However, it is not known whether such an effect obtains in the contact zone between *C. lewisianus* and *C. albrechti*, with a smaller mismatch in genital morphologies.

Sexual selection for the genital morphologies of ground beetles is suggested in the context of sperm competition and sexual conflict, in which the male

Table 4. Parameters of tanh clines fitted on six morphological (three external and three genital) and one genetic characters in a transect across a *Carabus lewisianus* and *C. albrectii* hybrid zone. 95% confidence intervals are shown in parentheses

Character	Width	Centre	Pmin	Pmax	log likelihood
External morphology					
Male body length (MBL)	10.934 (0, 32.972)	37.558 (33.777, 51.312)	0.5523675	0.7357165	-1.708
Male pronotal width (MPW)	17.420 (0, 107.154)	36.731 (31.044, 48.036)	0.4063782	0.6179052	-2.216
Number of male pronotal setae (MPS)	16.768 (0.008, 70.138)	37.003 (32.761, 45.257)	0.3139154	0.6883954	-2.849
Genital morphology					
Left basal lobe length (LBL)	1.284 (0.837, 2.030)	33.440 (33.272, 35.614)	0.2268718	0.8761354	-5.096
Copulatory piece length (CPL)	1.429 (0.005, 7.344)	33.382 (32.620, 34.179)	0.3232034	0.7455084	-0.499
Copulatory piece width (CPW)	0.749 (0.010, 2.629)	33.182 (32.938, 33.526)	0.4693751	0.790658	-1.494
Genetic character					
Mitochondrial haplotype frequency (HAP)	9.213 (4.281, 24.427)	35.766 (33.243, 39.391)	0	1	-2.603

Table 5. Differences in log likelihood scores between the models with differential cline centres (upper diagonal) and widths (lower diagonal) on seven characters in a hybrid zone of *Carabus lewisianus* and *C. albrectii*. The first and second scores within cells are based on the data indicated on their law and column, respectively. Bold scores are statistically significant ($P < 0.05$)

	External morphology			Genital morphology			Genetic character	
	MBL	MPW	MPS	LBL	CPL	CPW	HAP	HAP
External morphology								
MBL	-	0.063, 0.048	0.030, 0.022	2.341, 38.399	2.399, 12.269	2.598, 17.247	0.284, 0.668	0.284, 0.668
MPW	0.256, 0.138	-	0.006, 0.006	0.978, 34.761	1.006, 10.539	1.104, 16.282	0.070, 0.238	0.070, 0.238
MPS	0.069, 0.113	0.001, 0.002	-	1.414, 36.009	1.568, 11.132	1.104, 16.620	0.110, 0.370	0.110, 0.370
Genital morphology								
LBL	14.695 , 0.659	17.784 , 0.187	17.525 , 1.005	-	0.231, 0.024	4.136 , 1.134	29.890 , 1.748	29.890 , 1.748
CPL	3.218 , 0.655	5.005 , 0.191	4.841 , 0.973	0.012, 0.129	-	0.339, 0.682	8.488 , 1.822	8.488 , 1.822
CPW	7.579 , 0.664	9.072 , 0.221	8.966 , 1.249	0.258, 2.713	0.420, 0.273	-	14.879 , 2.079	14.879 , 2.079
Genetic character								
HAP	0.077, 0.008	0.942, 0.435	0.841, 0.118	17.398 , 13.508	17.423 , 2.654	31.143 , 6.797	-	-

CPL, copulatory piece length; CPW, copulatory piece width; HAP, mitochondrial haplotype frequency; LBL, left basal lobe length; MBL, male body length; MPS, number of male pronotal setae; MPW, male pronotal width.

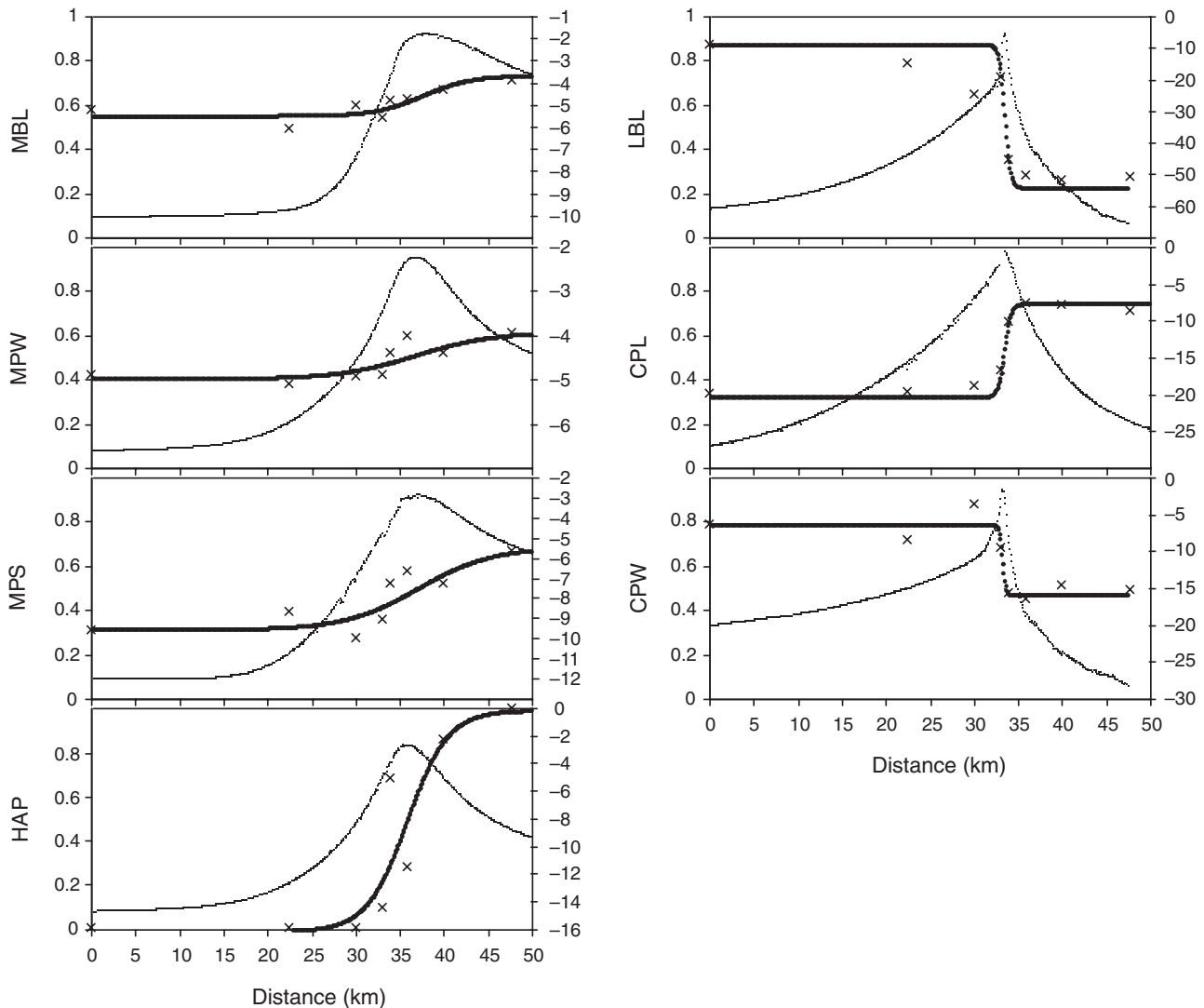


Figure 7. Changes in morphological (external: MBL, MPW and MPS; genital: LBL, CPL and CPW) and genetic (HAP) characters along the transect between *Carabus lewisianus* and *C. albrechti* (sites 1 [left] to 8 [right]). Bold curves are tanh clines fitted to population means (crosses). Simple curves are likelihood profiles for cline centres (log likelihood shown in right vertical axes); the highest points indicate the position of the centre.

hook-like copulatory piece of *C. insulicola* is assumed to be used to remove a rival spermatophore and to fix the female genitalia to place his own spermatophore effectively (Takami, 2002, 2003). If these functions of the genitalia have been evolved within species through interactions between males and between sexes, hybrid phenotypes may be disadvantageous in these competitions. Hybrid individuals with poor mating success are known in flycatchers (Saetre, Kral & Bures, 1997), sticklebacks (Vamosi & Schluter, 1999) and newts (Babik *et al.*, 2003). This process may help establish and maintain species identities as well as steep clines between species (Gavrilets & Waxman, 2002). However, again, little is known about sexual selection in *C. lewisianus* and *C. albrechti*.

Wider clines in the external and genetic characters than in the genitals indicate that these are rather selectively neutral. Difference in the body sizes of *Ohomopterus* ground beetles is potentially to work as a agent of premating reproductive isolation, and to facilitate the coexistence of species (Sota *et al.*, 2000c). However, very weak male mating preferences indicated that the difference in body size and other external characters between *C. lewisianus* and *C. albrechti* may be too small to work as an isolation mechanism, suggesting these traits are not the targets of strong selection in the hybrid zone. Mitochondrial genes have been believed to be selectively neutral, but recent studies have elucidated that mitochondrial haplotypes are directly and indirectly (through the effect by

nuclear genes) selected (Ballard & Whitlock, 2004). Although no evidence is given about selection for mitochondrial haplotypes in ground beetles, one possibility is discussed below in relation to the differential cline centres between the genetal and other characters.

POSSIBLE CAUSES OF DIFFERENTIAL CLINE POSITIONS AND ASYMMETRICAL INTROGRESSION

The presence of significantly displaced clines between the genetal and other characters suggests that individual characters respond independently to different evolutionary forces. This is concordant with no linkage disequilibria differing significantly from zero, suggesting that sufficient recombination occurs in the zone. However, the results may be due to small sample sizes because significance was not detected even within the genetal characters with narrow and steep clines. The results of cline fitting indicated that the genetal characters under strong selection (evidenced by narrow clines) are placed westward, and the external and genetic characters that were selectively neutral (wide clines) are eastward.

Differential cline positions and asymmetrical introgression have often occurred in hybrid zones by asymmetry in mating (mating asymmetry hypothesis), by difference in selective advantage (selective advantage hypothesis), and by chance through drift (drift hypothesis).

The mating asymmetry hypothesis is widely supported in a range of organisms. For example, in the hermit and Townsend's warblers hybrid zone in North America, aggressive mating behaviour by Townsend's males is advantageous while mating in the hybrid zone. As a result, the clines of the plumage characters have moved from the Townsend's to the hermit ranges (Rohwer, Bermingham & Wood, 2001). This process caused major discordance between the plumage and mtDNA haplotype clines, in which the haplotypes originating from the Townsend's warblers remained in the range of the hermit warblers far from the present contact zone. A similar process of introgression due to asymmetrical mating patterns is also assumed to have taken place in frogs (Lamb & Avise, 1986), hares (Thulin, Jaarola & Tegelström, 1997), newts (Babik *et al.*, 2003), and spiders (Croucher, Oxford & Searle, 2004). Asymmetry in mating is, however, not only mediated behaviourally but genetically, as in grasshoppers (Butlin, 1998) and butterflies (Cianchi *et al.*, 2003).

The selective advantage hypothesis may be supported by the introgression of a certain character that is selectively advantageous within the environments of the recipient species. It is known that the introgression of mitochondria has resulted from fitness differences among distinct mtDNA haplotypes in *Drosophila* (Ballard, 2000). In this case, it is assumed

that temperature plays an important role in selection for certain mtDNA haplotypes introgressed from locally better-adapted species (Ballard & Whitlock, 2004).

The drift hypothesis is supported by one of the most famous examples of mtDNA introgression from *Mus domesticus* into *M. musculus* in Europe (Ferris *et al.*, 1983). Phylogenetic analysis of mtDNA suggests that the haplotype of *M. domesticus* found in the *M. musculus* range originated from one female during a single colonization event.

These three hypotheses are evaluated in light of the results of the present study. The mating asymmetry hypothesis may not be relevant to premating processes because no apparent asymmetry was found in assortative mate choice by males. However, we have not examined asymmetries in postmating (i.e. sperm transfer and selective fertilization) and genetic processes. One possible scenario is that differences in male genetal morphologies may cause asymmetry in cross-specific mating, in which the males of *C. albrechti* could mate with the females of *C. lewisianus* more successfully than the males of *C. lewisianus*. If so, the clines of the genetal characters would move from the range of *C. albrechti* to that of *C. lewisianus*, and the remaining characters assumed to be neutral would remain as footprints in the moving cline. It is important that the difference in genetal morphology might be too small to prevent hybridization mechanically (i.e. lock-and-key) but may be large enough to work in the context of sexual selection (Eberhard, 1985). To test this scenario, postmating mechanisms in cross-specific mating between *C. lewisianus* and *C. albrechti* should be investigated.

The selective advantage hypothesis may also explain the asymmetrical introgression of the mitochondrial haplotypes of *C. lewisianus* into *C. albrechti*. This mechanism is possibly important as an explanation of extensive directional introgression of mtDNA found in the *Ohomopterus* ground beetles (Sota, 2002). However, little is known about the selective agents of mtDNA in this group of organisms.

The drift hypothesis may be rejected because introgression of mtDNA occurred repeatedly, both along the transect and in its southern part, and introgression also occurred inversely within *C. lewisianus*, even at a low frequency.

The particular geographical distribution pattern of the two species may have resulted from the movement of the boundaries of the genetal characters from *C. albrechti* to *C. lewisianus*. In the Kanto and Tanzawa Mountains, a narrow range of *C. lewisianus* is sandwiched between that of *C. albrechti* to the east and west (Fig. 1). The formation of this pattern is hypothesized to have occurred as follows. Originally, *C. lewisianus* may have been distributed farther north

than its present range, while *C. albrechti* probably invaded the eastern foothills of the Tanzawa Mountains across the Sagami-gawa River. During the course of the invasion, the two species hybridized, and the mitochondria of *C. lewisianus* remained in the invaded area. This invasion may have resulted in clinal changes in haplotype frequency in the southern range of *C. albrechti* (sites 9–11), in which introgressants increase from north to south, although these sites are situated in a row parallel (not perpendicular) to the zone. The southernmost population (site 11), which may have been affected most strongly by hybridization during invasion, has completely replaced its haplotypes (Fig. 6). A similar invasion may also have occurred in the western range of *C. lewisianus*, and may have resulted in the other introgressants found in *C. albrechti okumurai*, which is distributed on the western side of the range.

The other scenario that might constitute an explanation for the establishment of the present distribution is that *C. lewisianus* invaded the range of *C. albrechti*. However, it is thought to be less plausible that *C. lewisianus* crossed two large rivers (Sagami-gawa and Tamagawa) while maintaining its narrow sandwiched range. To test this hypothesis, the distribution of other nuclear genetic markers, such as microsatellites (Takami & Katada, 2001), should be investigated in this and other areas.

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REFERENCES

- Arnold ML. 1997.** *Natural hybridization and evolution*. Oxford: Oxford University Press.
- Babik W, Szymura JM, Rafiski J. 2003.** Nuclear markers, mitochondrial DNA and male secondary sexual traits variation in a newt hybrid zone (*Triturus vulgaris* × *T. montandoni*). *Molecular Ecology* **12**: 1913–1930.
- Ballard JWO. 2000.** When one is not enough: introgression of mitochondrial DNA in *Drosophila*. *Molecular Biology and Evolution* **17**: 1126–1130.
- Ballard JWO, Whitlock MC. 2004.** The incomplete natural history of mitochondria. *Molecular Ecology* **13**: 729–744.
- Barton NH, Baird SJE. 2002.** ANALYSE, Version 1.3: Free-ware: available from <http://helios.bto.edsac.uk/evolgen/Mac/Analyse>
- Barton NH, Hewitt GM. 1985.** Analysis of hybrid zones. *Annual Review of Ecology and Systematics* **16**: 113–148.
- Barton NH, Hewitt GM. 1989.** Adaptation, speciation and hybrid zones. *Nature* **341**: 497–502.
- Bridle JR, Baird SJE, Butlin RK. 2001.** Spatial structure and habitat variation in a grasshopper hybrid zone. *Evolution* **55**: 1832–1843.
- Bridle JR, Butlin RK. 2002.** Mating signal variation and bimodality in a mosaic hybrid zone between *Chorthippus* grasshopper species. *Evolution* **56**: 1184–1198.
- Bull CM. 1991.** Ecology of parapatric distributions. *Annual Review of Ecology and Systematics* **22**: 19–36.
- Butlin R. 1998.** What do hybrid zones in general, and the *Chorthippus parallelus* zone in particular, tell us about speciation? In: Howard DJ, Berlocher SH, eds. *Endless forms: species and speciation*. New York: Oxford University Press, 367–378.
- Cianchi R, Ungaro A, Marini M, Bullini L. 2003.** Differential patterns of hybridization and introgression between the swallowtails *Papilio machaon* and *P. hospiton* from Sardinia and Corsica islands (Lepidoptera, Papilionidae). *Molecular Ecology* **12**: 1461–1471.
- Croucher PJP, Oxford GS, Searle JB. 2004.** Mitochondrial differentiation, introgression and phylogeny of species in the *Tegenaria atrica* group (Araneae: Agelenidae). *Biological Journal of the Linnean Society* **81**: 79–89.
- Dasmahapatra KK, Blum MJ, Aiello A, Hackwell S, Davies N, Bermingham EP, Mallet J. 2002.** Inferences from a rapidly moving hybrid zone. *Evolution* **56**: 741–753.
- Dowling TE, Secor CL. 1997.** The role of hybridization and introgression in the diversification of animals. *Annual Review of Ecology and Systematics* **28**: 593–619.
- Eberhard WG. 1985.** *Sexual selection and animal genitalia*. Cambridge, MA: Harvard University Press.
- Edwards AWF. 1972.** *Likelihood*. Baltimore: Johns Hopkins University Press.
- Ferris SD, Sage RD, Huang CM, Nielsen JT, Ritte U, Wilson AC. 1983.** Flow of mitochondrial DNA across a species boundary. *Proceedings of the National Academy of Sciences, USA* **80**: 2290–2294.
- Gavrilets S, Waxman D. 2002.** Sympatric speciation by sexual conflict. *Proceedings of the National Academy of Sciences, USA* **99**: 10533–10538.
- Grant PR, Grant BR. 1994.** Phenotypic and genetic effects of hybridization in Darwin's finches. *Evolution* **48**: 297–316.
- Harrison RG. 1993.** *Hybrid zones and the evolutionary process*. Oxford: Oxford University Press.
- Ishikawa R. 1987.** On the function of the copulatory organs of *Ohomopterus* (Coleoptera, Carabidae, genus *Carabus*). *Kontyû* **55**: 202–206.
- Ishikawa R, Yamasaki T, Kojima J, Uchida S. 1987.** *Analytic studies on the distribution of some insect-groups in the Tamagawa River system and its upper reaches*. Tokyo: Tokyo Foundation for Better Environment. [In Japanese].
- Kruuk LEB, Baird SJE, Gale KS, Barton NH. 1999.** A comparison of multilocus clines maintained by environmental adaptation or by selection against hybrids. *Genetics* **153**: 1959–1971.

- Kubota K. 1988.** Natural hybridization between *Carabus* (*Ohomopterus*) *maiwasanus* and *C. (O.) iwawakianus* (Coleoptera, Carabidae). *Kontyû* **56**: 233–240.
- Kubota K, Sota T. 1998.** Hybridization and speciation in the carabid beetles of the subgenus *Ohomopterus* (Coleoptera, Carabidae, genus *Carabus*). *Researches on Population Ecology* **40**: 213–222.
- Kubota K, Sota T. 2001.** Genital lock-and-key of subgenus *Ohomopterus* (Coleoptera, Carabidae, genus *Carabus*). *Nature & Insects, Tokyo* **36** (2): 20–23. [In Japanese].
- Kuno E. 1992.** Competitive exclusion through reproductive interference. *Researches on Population Ecology* **34**: 275–284.
- Lamb T, Avise JC. 1986.** Directional introgression of mitochondrial DNA in a hybrid population of tree frogs: The influence of mating behavior. *Proceedings of the National Academy of Sciences, USA* **83**: 2526–2530.
- Marshall JC, Sites JW Jr. 2001.** A comparison of nuclear and mitochondrial cline shapes in a hybrid zone in the *Sceloporus grammicus* complex (Squamata; Phrynosomatidae). *Molecular Ecology* **10**: 435–449.
- Miller MP. 1997.** *TFGPA: tools for population genetic analyses*, Version 1.3. Distributed by the author.
- Nürnberg B, Barton N, MacCallum C, Gilchrist J, Appleby M. 1995.** Natural selection on quantitative traits in the *Bombina* hybrid zone. *Evolution* **49**: 1224–1238.
- Phillips BL, Baird SJE, Moritz C. 2004.** When vicars meet: a narrow contact zone between morphologically cryptic phylogeographic lineages of the rainforest skink, *Carlia rubrigularis*. *Evolution* **58**: 1536–1548.
- Posada D, Crandall KA. 1998.** Modeltest: testing the model of DNA substitution. *Bioinformatics* **14**: 817–818.
- Rebeiro JMC, Spielman A. 1986.** The satyr effect: a model predicting parapatry and species extinction. *American Naturalist* **128**: 513–528.
- Rohwer S, Bermingham E, Wood C. 2001.** Plumage and mitochondrial DNA haplotype variation across a moving hybrid zone. *Evolution* **55**: 405–422.
- Saetre GP, Kral M, Bures S. 1997.** Differential species recognition abilities of males and females in a flycatcher hybrid zone. *Journal of Avian Biology* **28**: 259–263.
- SAS Institute Inc. 1998.** *StatView 5.0 for Macintosh*. Cary: SAS Institute.
- Sota T. 2002.** Radiation and reticulation: extensive introgressive hybridization in the carabid beetles *Ohomopterus* inferred from mitochondrial gene genealogy. *Population Ecology* **44**: 145–156.
- Sota T, Ishikawa R, Ujiie M, Kusumoto F, Vogler AP. 2001.** Extensive trans-species mitochondrial polymorphisms in the carabid beetles *Carabus* subgenus *Ohomopterus* caused by repeated introgressive hybridization. *Molecular Ecology* **10**: 2833–2847.
- Sota T, Kubota K. 1998.** Genital lock-and-key as a selective agent against hybridization. *Evolution* **52**: 1507–1513.
- Sota T, Kusumoto F, Kubota K. 2000a.** Consequences of hybridization between *Ohomopterus insulicola* and *O. arrowianus* (Coleoptera, Carabidae) in a segmented river basin: parallel formation of hybrid swarms. *Biological Journal of the Linnean Society* **71**: 297–313.
- Sota T, Takami Y, Kubota K, Ishikawa R. 2000b.** Geographic variation in the body size of some Japanese *Leptocarabus* species (Coleoptera, Carabidae): the ‘toppled-domino pattern’ in species along a geographic cline. *Entomological Science* **3**: 309–320.
- Sota T, Takami Y, Kubota K, Ujiie M, Ishikawa R. 2000c.** Interspecific body size differentiation in species assemblages of the carabid subgenus *Ohomopterus* in Japan. *Population Ecology* **42**: 279–291.
- Sota T, Vogler AP. 2001.** Incongruence of mitochondrial and nuclear gene trees in the carabid beetles *Ohomopterus*. *Systematic Biology* **50**: 39–59.
- Statsoft. 1994.** *STATISTICA*. Tulsa, Oklahoma: Statsoft.
- Su ZH, Ohama T, Okada TS, Nakamura K, Ishikawa R, Osawa S. 1996.** Phylogenetic relationships and evolution of the Japanese Carabinae ground beetles based on mitochondrial ND5 gene sequences. *Journal of Molecular Evolution* **42**: 124–129.
- Suzuki H, Sato Y, Ohba N. 2002.** Gene diversity and geographic differentiation in mitochondrial DNA of the Genji firefly, *Luciola cruciata* (Coleoptera: Lampyridae). *Molecular Phylogenetics and Evolution* **22**: 193–205.
- Swofford DL. 2002.** *PAUP*: phylogenetic analysis using parsimony (*and other methods)*, Version 4. Sunderland, MA: Sinauer Associates.
- Szymura JM, Barton NH. 1986.** Genetic analysis of a hybrid zone between the fire-bellied toads, *Bombina bombina* and *B. variegata*, near Cracow in southern Poland. *Evolution* **40**: 1141–1159.
- Takami Y. 2002.** Mating behavior, insemination and sperm transfer in the ground beetle *Carabus insulicola*. *Zoological Science* **19**: 1067–1073.
- Takami Y. 2003.** Experimental analysis of the effect of genital morphology on insemination success in the ground beetle *Carabus insulicola* (Coleoptera: Carabidae). *Ethology, Ecology and Evolution* **15**: 51–61.
- Takami Y, Ishikawa R. 1997.** Subspeciation and distribution pattern of *Carabus albrechti* Morawitz in Japan (Coleoptera, Carabidae). *Tokyo Metropolitan University Bulletin of Natural History* **3**: 55–99.
- Takami Y, Katada S. 2001.** Microsatellite DNA markers for the ground beetle *Carabus insulicola*. *Molecular Ecology Notes* **1**: 128–130.
- Thulin CG, Jaarola M, Tegelström H. 1997.** The occurrence of mountain hare mitochondrial DNA in wild brown hares. *Molecular Ecology* **6**: 463–467.
- Vamosi SM, Schluter D. 1999.** Sexual selection against hybrids between sympatric stickleback species: evidence from a field experiment. *Evolution* **53**: 874–879.
- Vines TH, Köhler SC, Thiel M, Ghira I, Sands TR, MacCallum CJ, Barton N, Nürnberg B. 2003.** The maintenance of reproductive isolation in a mosaic hybrid zone between the fire-bellied toads *Bombina bombina* and *B. variegata*. *Evolution* **57**: 1876–1888.
- Yoshimura J, Clark CW. 1994.** Population dynamics of sexual and resource competitions. *Theoretical Population Biology* **45**: 121–131.