Diversification in a fluctuating island setting: rapid radiation of *Ohomopterus* ground beetles in the Japanese Islands

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The Japanese Islands have been largely isolated from the East Asian mainland since the Early Pleistocene, allowing the diversification of endemic lineages. Here, we explore speciation rates and historical biogeography of the ground beetles of the subgenus *Ohomopterus* (genus *Carabus*) based on nuclear and mitochondrial gene sequences. *Ohomopterus* diverged into 15 species during the Pleistocene. The speciation rate was 1.92 Ma^{-1} and was particularly fast (2.37 Ma^{-1}) in a group with highly divergent genitalia. Speciation occurred almost solely within Honshu, the largest island with complex geography. Species diversity is highest in central Honshu, where closely related species occur parapatrically and different-sized species co-occur. Range expansion of some species in the past has resulted in such species assemblages. Introgressive hybridization, at least for mitochondrial DNA, has occurred repeatedly between species in contact, but has not greatly disturbed species distinctness. Small-island populations of some species were separated from main-island populations only after the last glacial (or the last interglacial) period, indicating that island isolation had little role in speciation. Thus, the speciation and formation of the *Ohomopterus* assemblage occurred despite frequent opportunities for secondary contact and hybridization and the lack of persistent isolation. This radiation was achieved without substantial ecological differentiation, but with marked differentiation in mechanical agents of reproductive isolation (body size and genital morphology).

Keywords: beetles; Japan; divergence time; genital evolution; molecular phylogeny; speciation rate

1. INTRODUCTION

The Japanese Islands constitute a distinct system differing from highly isolated oceanic islands. Located close to the East Asian mainland, a rich biota has colonized the Japanese Islands, but many endemic lineages have evolved on the islands owing to the geographical complexity and wide range of climatic conditions along latitudinal and elevational gradients. The Japanese archipelago is based on an accretionary prism that formed at the edge of the East Asian mainland and existed as many fragmented islands in the Middle Miocene (Iijima & Tada 1990; Tada 1994; Yonekura *et al.* 2001). A land bridge connected eastern China and western Japan continuously from the Late Miocene to the end of the Pliocene (10–1.7 Ma). Thereafter, only a narrow land bridge between the Korean peninsula and western Japan occurred temporarily at glacial maxima during the Pleistocene (Tada 1994; Kitamura *et al.* 2001; Kitamura & Kimoto 2006). Thus, the current terrestrial fauna of the Japanese Islands originated from colonization events via the western connection at various times from the Late Miocene to the end of the Pleistocene, as evidenced by the fossil records of insects and mammals (Dobson & Kawamura 1998; Hayashi 2004). A large part of northern Japan (Hokkaido and northern Honshu)

emerged after the Late Miocene, and Hokkaido and Honshu were separated by the deep Tsugaru Strait except during the Late Pleistocene glacial periods (Tada 1994; Yonekura *et al.* 2001). During the glacial period, most of Hokkaido and parts of northern Honshu were covered with boreal conifer forest and glaciers (Tsukada 1984), and the terrestrial biota was dominated by cool adapted immigrants from the north. In these areas, colonization by organisms adapted to a temperate climate might have occurred after the last glacial period.

Among endemic insects in the temperate zone of Japan, flightless ground beetles of the subgenus *Ohomopterus* of the genus *Carabus* exhibit marked diversity in body size and genital morphology (Ishikawa 1985, 1991; Takami 2000), as well as a typical assemblage pattern consisting of two or more different-sized species (Sota *et al.* 2000a). They are an intriguing group for evolutionary study. Since the 1980s, hybrid zones of various species pairs have been explored and the consequences of interspecific hybridization between parapatric species with divergent genitalia have been investigated (Kubota 1988; Kubota & Sota 1998; Sota *et al.* 2000c; Takami & Suzuki 2005; Ujiie *et al.* 2005). Interspecific body size differences in local species assemblages have been described and their possible effects on species coexistence investigated (Sota *et al.* 2000a). The role of genitalic divergence in mechanical reproductive isolation has also been documented (Sota & Kubota 1998; Usami *et al.* 2006; Takami *et al.* 2007).

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Since the 1990s, molecular phylogenetic approaches have promoted further evolutionary studies of this group. The contradiction between a mitochondrial gene tree and morphological species (Su *et al.* 1996) prompted a test of incongruence between mitochondrial and nuclear gene trees, and morphological classification (Sota & Vogler 2001), phylogenetic reconstruction with multiple nuclear genes (Sota & Vogler 2003), and studies of interspecific mitochondrial introgression and intraspecific phylogeography based on mitochondrial data (Sota *et al.* 2001; Sota 2002; Nagata *et al.* 2007a,b), as well as allelic sequence diversity of nuclear genes (Sota & Sasabe 2006). Also, the function and evolution of the genital lock and key system have been explored in terms of sexual selection (Takami 2002, 2003, 2007; Takami & Sota 2007) and the genetic basis of exaggerated genital morphology has recently been investigated (Sasabe *et al.* 2007).

The *Ohomopterus* radiation lacks notable differentiation in food habits and associated morphology, a major aspect of adaptive radiation in other groups such as Galápagos finches and African lake cichlids (Schluter 2000). Instead, *Ohomopterus* represents a radiation driven by differentiation in mating traits (genital morphology and body size) due to local adaptation and sexual selection. Thus, studies of *Ohomopterus* will contribute to our understanding of various adaptive radiation pathways. In this paper, we give an overview of *Ohomopterus* speciation in the Japanese Islands and estimate speciation rates. We then describe genetic differentiation, diversity and introgression between species and among geographical populations within species using a large mitochondrial gene sequence dataset. We estimate divergence times for small-island from main-island populations. In doing so, we outline the historical processes of *Ohomopterus* diversification in the Japanese Islands.

2. BIOLOGY AND BIOGEOGRAPHY OF OHOMOPTERUS

Ohomopterus is a subgenus of the genus *Carabus* (*sensu stricto*) of the holarctic subtribe Carabina (=genus *Carabus sensu lato*) and consists of 15 species (Ishikawa 1985). Adult beetles are 18–35 mm in body length, flightless with degenerate hind wings and inhabit the forest floor and grasslands. They reproduce in spring and summer. The larvae develop during the summer and eclose as adults by the autumn, overwintering without reproduction (Sota 1985a,b). The adults are polyphagous, mainly feeding on earthworms, whereas the larvae are specialized predators of megascolecid earthworms (Sota 1985a,b). Among Megascolecidae in Japan, the genus *Pheretima* is the most abundant and diversified, with at least 124 described species (Ishizuka 2001). Thus, *Ohomopterus* populations depend on the presence of *Pheretima*.

Ohomopterus ranges from southern Kyushu to southern Hokkaido (31–44° N, 129–145° E; Sota *et al.* 2000a; figure 1a). The altitudinal range is 0–1750 m (occasionally reaching 2000 m, i.e. *Carabus albrechti* in central Honshu), with an annual mean temperature range of 5–15°C (occasionally 3°C for *C. albrechti*). Thus, *Ohomopterus* occurs widely in the

temperate zones of Japan but cannot colonize subalpine coniferous forests and alpine zones. Among the four main islands, Honshu is the centre of diversity (14 species). Shikoku and Kyushu harbour four and two species, respectively, and only one species (*C. albrechti*) occurs in Hokkaido. *Ohomopterus* also occurs on other small islands (one to three species per island) but no species is endemic to such islands. Overall, species diversity is highest in central Honshu and low in both the southwest and northeast regions (figure 1b).

In most of its range, two or more species with different body sizes co-occur (figure 1b; Sota *et al.* 2000a). The most species-rich assemblage, with five species, exists in a small mountain area of central Honshu. *Carabus dehaanii* is the largest species in an assemblage whenever it occurs, and *Carabus japonicus*, *Carabus daisen* and the species of the *albrechti* group are the smallest in assemblages with two or more species. *Carabus yaconinus*, *Carabus tosanus* and species of the *iwwakianus-insulicola* group are intermediate or the largest in an assemblage.

3. ORIGIN AND PHYLOGENY OF OHOMOPTERUS

A molecular phylogeny of the subtribe Carabina based on nuclear gene sequences indicates that within *Carabus* (*sensu stricto*), *Ohomopterus* is sister to the subgenus *Isiocarabus*, which occurs in warm temperate regions of China and Korea (Sota & Ishikawa 2004). The ancestral *Ohomopterus* might have colonized Japan during the Late Miocene through to the Pliocene, when western Japan was connected to the East Asian mainland. A few fossilized elytra resembling *Ohomopterus* have been discovered from Late Miocene or Early Pliocene strata (8–6 Ma old; Hiura 1971; Hayashi 2001), but cannot be identified as extant *Ohomopterus* because their elytral sculpture is one of the common patterns of the present Carabina (i.e. triploid homodynamic). Fossils directly related to the present species have been discovered, mostly from Late Pleistocene strata (Hayashi 2001). After the beginning of the Pleistocene (1.7 Ma), the connection between Japan and the mainland was broken when the Korean (Tsushima) Strait opened (Kitamura *et al.* 2001; Kitamura & Kimoto 2006), so *Ohomopterus* probably diversified during the Pleistocene.

Reconstructing species relationships based on phylogenetic analyses of mitochondrial genes is difficult for *Ohomopterus* because mitochondrial genes show extensive trans-species polymorphisms. Nuclear genes provide phylogenetic information largely concordant with morphological species boundaries (Sota & Vogler 2001, 2003; Sota & Sasabe 2006). Here, we have expanded the nuclear data of Sota & Vogler (2003) to include 4164 base pairs (bp) from six genes (see table 1 for gene regions and primers). The specimens used were the same as those in table 1 of Sota & Vogler (2003) except for exclusion of a putative hybrid (*Carabus insulicola pseudinsulicola*) and inclusion of three *Carabus maiyasanus* specimens (sample codes MAI330, MAI332 and MAI349). For GenBank accession numbers of nuclear sequences see Sota & Vogler (2001, 2003); numbers for additional sequences are EU435018–EU435132.

Figure 1. (a) The Japanese Islands and the distribution of *Ohomopterus* ground beetles. The coastline 20 000 years ago (the last glacial period) is based on Ohshima (1990) and Yonekura *et al.* (2001). (b) Number of species in each prefecture or island. The green line encloses localities where two species (large and small) can co-occur, and the red line where three (occasionally four or five) species can co-occur.

Although partly unresolved, the maximum-likelihood tree resulting from simultaneous analysis of these genes (figure 2; see legend for details of the analysis) reveals that *Ohomopterus* consists of three clades, the *albrechti* group (Ishikawa 1991), the *iivavakianus-insulicola* group (Sota & Vogler 2003) and a third group, named here the *daisen* group.

The *albrechti* group consists of four small species distributed in central to northern Honshu and Hokkaido as well as Sado, Awa and Okushiri islands. The *daisen* group consists of five species distributed in Kyushu, Shikoku to central Honshu and on small islands in the western region. Body size is generally small in *C. daisen* and *C. japonicus*, intermediate in

Table 1. Nuclear genes used for phylogenetic analysis of *Ohomopterus*, with sequence length used in phylogenetic analyses and primers.

gene name; data length, bp primers	references
elongation factor 1- α (EF 1- α); 619 bp ^a F (CAREFA1): 5'-GAC AAC ATG TTG GAA CCA TC-3' R (EF1aA692NMF): 5'-GGT GGG AGR ATR GCR TCM ARA G-3'	Sota & Vogler (2003) Normark <i>et al.</i> (1999) Morse & Farrell (2005)
wingless (Wg); 440 bp F (CARWL1): 5'-ATG TCT GGC ACC TGC ACC GT-3' R (CARWL2): 5'-CAA GCG CAC CGT TCC ACA ACG A-3'	Sota & Vogler (2001) Sota & Vogler (2001)
phosphoenolpyruvate carboxykinase (PepCK); 630 bp F (CARPEK1): 5'-GCC ATG ATG ACA CCA ACA CT-3' R (CARPEK3): 5'-GAC GTG GAA GAT CTT GGG CA-3'	Sota & Vogler (2001) Sota & Vogler (2001)
cytochrome <i>c</i> (Cyt _c); 306 bp ^a F (CARCYC1): 5'-TGC CAC ACT GTT GAA AAA GG-3' R (CARCYC2): 5'-GA TGT ATT TCT TCG GGT TTT CGA-3'	Sota & Vogler (2003) Sota & Vogler (2003)
carab1; 600 bp ^a F (CARCK1): 5'-ATG TCA CTC ATC AAC ACT GC-3' R (CARCK2): 5'-GTG GTT CGC ATC TCA ACA GA-3'	Sota & Vogler (2001) Sota & Vogler (2001)
28S rDNA (28S) part 1; 790 bp F (28S-01): 5'-GAC TAC CCC CTG AAT TTA AGC AT-3' R (28S-R01): 5'-GAC TCC TTG GTC CGT GTT TCA AG-3'	Kim <i>et al.</i> (2000) Kim <i>et al.</i> (2000)
part 2; 779 bp F (28SFv5'): 5'-AAG GTA GCC AAA TGC CTC GTC-3' R (28SRjj3'): 5'-AGT AGG GTA AAA CTA ACC T-3'	Palumbi (1996) Palumbi (1996)

^aData length excluding ambiguously aligned region.

C. yaconinus and *C. tosanus* and the largest in *C. dehaanii*. The *iwwakianus-insulicola* group consists of six medium-sized species in central to northern Honshu. *Ohomopterus* exhibits high diversity in the morphologies of functional parts of the genitalia, especially the copulatory piece (a chitinized part of the endophallus) and its corresponding vaginal appendix. All the *albrechti* and *daisen* group species possess a small triangular copulatory piece (except *C. yaconinus* which has a pentagonal copulatory piece) and a correspondingly short vaginal appendix. The *iwwakianus-insulicola* group exhibits a small to highly elongate (hook like) copulatory piece with corresponding shapes of the vaginal appendix.

Of 15 species, eight are confined to the main island of Honshu and one is endemic to Shikoku. Therefore, allopatric differentiation on different islands was not the major speciation process. Speciation within Honshu is likely for *C. yaconinus*, the four species of the *albrechti* group and the six species of the *iwwakianus-insulicola* group. These speciation events might have been allopatric or parapatric; speciation would have been facilitated by population fragmentation, with rivers and mountains acting as dispersal barriers. Climatic fluctuation during the Pleistocene might also have affected the differentiation because populations of *Ohomopterus* might have been restricted to coastal refugia during glacial periods.

4. SPECIATION RATE

Speciation rates in *Ohomopterus* can be estimated by converting the phylogenetic tree to an ultrametric tree with a calibration for absolute time. In our previous study, the divergence between *Isiocarabus* and

Ohomopterus was estimated to have occurred 2.14 Ma based on mitochondrial NADH dehydrogenase subunit 5 (ND5) sequences, assuming that these sister groups diverged following the separation between the East Asian mainland and the western region of Japan 3.5–1.7 Ma (Nagata *et al.* 2007a). We used this age to calibrate the nodes of the nuclear gene tree (figure 2).

With the age of the most recent common ancestor of *Ohomopterus* at 1.4 Ma, the speciation interval of *Ohomopterus* (i.e. millions of years divided by logarithm of species number) is 0.52 Ma (speciation rate, 1.92 Ma⁻¹). The values were 0.63 Ma (1.59 Ma⁻¹) for the *albrechti* group, 0.85 Ma (1.18 Ma⁻¹) for the *daisen* group and 0.42 Ma (2.37 Ma⁻¹) for the *iwwakianus-insulicola* group.

5. GENETIC DIVERSITY IN A MITOCHONDRIAL GENE

The mitochondrial gene ND5 has served as a marker to explore the evolution of *Ohomopterus*, including intraspecific phylogeography and interspecific introgression (Sota *et al.* 2001; Nagata *et al.* 2007a,b). For this study, we compiled a large ND5 dataset to investigate the overall genetic diversity and analyse the historical aspects of differentiation in *Ohomopterus*.

Figure 3 shows a ND5 gene tree of 1463 haplotypes detected from 6136 specimens (table 2), in which the major clades are named clades A–F and subclades A1, A2 and so on. There were 515 variable sites among the 1020 nucleotide sites, yielding 642 different amino acid sequences. The gene tree showed extensive trans-species polymorphisms. Clades A–C were mostly members of the *iwwakianus-insulicola* group, but *C. dehaanii* and *C. yaconinus* possessed many

Figure 2. Species relationships in *Ohomopterus* with the ranges of the three species groups, male copulatory piece and gross morphology of male (left) and female (right) beetles. The tree is a maximum-likelihood (ML) tree resulting from simultaneous analysis of six nuclear DNA sequences (4164 bp). The ML analysis with a genetic algorithm was performed in GARLI v. 0.951 (Zwickl 2006) with the GTR+I+G substitution model. Node support was obtained by 1000 bootstrap pseudoreplications (bootstrap percentages shown when greater than 50%). The Bayesian posterior probability of a node is shown following the bootstrap value, based on a partitioned analysis with MRBAYES v. 3.12 (Huelsenbeck & Ronquist 2001). Node ages were determined using PATHd8 (Britton *et al.* 2007) with 2.14 Ma to the divergence between *Isiocarabus* and *Ohomopterus*.

haplotypes from the A and B clades. Clades D and E were of the *daisen* group. Clade F consisted of distinct subclades of *C. japonicus* (F4) and *C. daisen* (F2); other subclades consisted of haplotypes of the *albrechti* group and of *Carabus insulicola* (with three other species of the same group). Clade G contained *C. tosanus*, *C. japonicus* and *C. dehaanii* within the *daisen* group. The pattern of sharing of haplotype lineages revealed introgressive hybridization and incomplete sorting of ancestral polymorphisms (Sota & Vogler 2001; Sota *et al.* 2001; Sota 2002; Nagata *et al.* 2007a,b).

Figure 4 shows the pattern of sharing of haplotype clades among species. For a mitochondrial gene with a fast evolutionary rate, sharing of a haplotype between species in sympatry or parapatry probably indicates recent introgression. Also, the geographical distribution of haplotype clades within a species (figure 5) suggests that an introgressed haplotype or lineage probably occurs close to the contact zone with other species that share the haplotype or lineage (Sota 2002). Haplotype sharing was common in clade A and B haplotypes that appear to have been extensively introgressed into *C. dehaanii* and *C. yaconinus*.

Although A4 is exclusively made up of *C. dehaanii*, it may have originated from an ancient introgression from the *iwawakianus*–*insulicola* group; A4, and A1 and A2 occurred only in eastern populations of *C. dehaanii* and are unlikely to be an original clade. F1a haplotypes, mostly from *C. insulicola* and occurring throughout its range, might have originated from ancient introgression from the *albrechti* group (see Sota *et al.* 2001). In fact, clade F1a includes a haplotype of *C. albrechti*. At the western margin of the *C. insulicola* range, F1a is replaced with another introgressant clade (B1) from *C. arrowianus* (Sota *et al.* 2001). Thus, *C. insulicola* may not have retained any original haplotypes.

6. DISPERSAL OF WIDELY DISTRIBUTED SPECIES

For species with wide ranges, the direction of range expansion may be inferred from the distribution of mitochondrial haplotypes using the framework of nested clade phylogeographic analysis (Templeton 1998). Here we simply examined the geographical distribution of interior (ancestral) and tip (derived) clades

Figure 3. Phylogeny of mitochondrial ND5 and distribution of species among lineages. A neighbour-joining tree was constructed using PAUP* v. 4.10b (Swofford 2002) with a substitution model (GTR+I+G) and parameters selected by MODELTEST v. 3.07 (Posada & Crandall 1998) for distance correction. Node supports resulting from 1000 bootstrap analyses are shown only for major nodes (when greater than 50%). Nh, number of haplotypes.

within haplotype networks constructed with statistical parsimony analysis (Templeton *et al.* 1992; Clement *et al.* 2000).

Clade D (D1 and D2) haplotypes of *C. japonicus* dispersed from Kyushu to Honshu (Chugoku) and

Shikoku (figures 5 and 6a). This species also possessed clade G3 haplotypes in Honshu (Chugoku) and Awaji Island, and a unique clade F4 haplotype from Tsushima Island. Thus, *C. japonicus* seems to have retained highly diverged original mitochondrial lineages. Clade

Table 2. Sample sizes, regional and intra-population nucleotide diversity, calculated using ARLEQUIN v. 3.0 (Excoffier *et al.* 2005) of the mitochondrial ND5 gene sequence.

species	island (: district)	no. of populations	no. of sequenced individuals	nucleotide diversity (%)	
				regional ($\pm$ s.d.)	population (range)
<i>C. albrechti</i>	Hokkaido	6	58	0.28 ± 0.18	0.00–0.13
	Honshu: Tohoku	9	30	1.62 ± 0.85	0.10–1.45
	Awa I.	1	5	0.10 ± 0.14	—
	Sado I.	1	7	0.23 ± 0.19	—
	Honshu: Kanto	10	78	1.44 ± 0.74	0.07–1.16
<i>C. lewisianus</i>	Honshu: Chubu	17	120	1.12 ± 0.58	0.00–1.05
	Honshu: Kanto	2	58	0.49 ± 0.29	0.41–0.47
<i>C. kimurai</i>	Honshu: Chubu	4	40	1.15 ± 0.61	0.24–0.36
	Honshu: Chubu	7	44	0.63 ± 0.36	0.26–0.31
<i>C. yamato</i>	Honshu: Kinki	16	258	0.73 ± 0.39	0.00–0.57
<i>C. japonicus</i>	Honshu: Chubu	18	148	1.18 ± 0.60	0.04–1.12
	Honshu: Kinki	5	39	1.67 ± 0.91	0.00–0.18
<i>C. daisen</i>	Honshu: Chugoku	14	112	1.27 ± 0.65	0.00–2.11
	Awaji I.	1	8	0.39 ± 0.33	—
	Shikoku	11	56	2.52 ± 1.26	0.14–1.84
	Kyushu	16	167	1.38 ± 0.70	0.38–1.17
	Iki I.	1	14	0.23 ± 0.16	—
	Fukue I.	1	36	0.26 ± 0.17	—
	Tsushima I.	2	108	0.44 ± 0.25	0.20–0.35
	Honshu: Kinki	1	11	0.31 ± 0.24	—
<i>C. dehaanii</i>	Honshu: Chugoku	9	110	1.84 ± 0.97	0.00–0.38
	Oki I.	2	54	0.38 ± 0.22	0.17–0.22
<i>C. dehaanii</i>	Honshu: Chubu	15	131	0.46 ± 0.26	0.00–0.41
	Honshu: Kinki	21	254	0.92 ± 0.47	0.06–0.85
<i>C. tosanus</i>	Awaji I.	1	4	0.78 ± 0.83	—
	Shikoku	2	7	2.09 ± 1.60	0.00–0.95
	Honshu: Chugoku	10	49	1.89 ± 0.98	0.34–1.90
	Kyushu	9	51	0.96 ± 0.52	0.05–0.79
	Fukue I.	1	51	0.38 ± 0.23	—
<i>C. yaconinus</i>	Shikoku	11	34	0.56 ± 0.31	0.00–0.44
<i>C. yaconinus</i>	Honshu: Chubu	2	48	1.96 ± 1.07	0.34–1.24
	Honshu: Kinki	19	322	1.71 ± 0.86	0.00–1.88
<i>C. iwawakianus</i>	Awaji I.	1	3	0.29 ± 0.34	—
	Shikoku	3	19	0.44 ± 0.28	0.00–0.28
	Oki I.	1	15	0.13 ± 0.13	—
	Honshu: Chugoku	9	103	1.58 ± 0.81	0.20–0.92
	Honshu: Kinki	21	362	1.81 ± 0.90	0.20–1.93
<i>C. maiyasanus</i>	Honshu: Chubu	14	279	1.63 ± 0.82	0.19–1.45
	Honshu: Kinki	29	725	1.77 ± 0.87	0.00–1.88
<i>C. uenoi</i>	Honshu: Kinki	2	54	0.45 ± 0.31	0.04–0.06
<i>C. arrowianus</i>	Honshu: Chubu	48	987	0.93 ± 0.48	0.00–1.24
	Honshu: Kinki	5	78	1.52 ± 0.80	0.00–0.91
<i>C. esakii</i>	Honshu: Chubu	17	121	2.57 ± 1.28	0.10–2.54
<i>C. insulicola</i>	Hokkaido ^a	2	17	0.22 ± 0.14	0.00–0.20
	Honshu: Tohoku	10	66	0.49 ± 0.27	0.00–0.41
	Awa I.	1	1	—	—
	Sado I.	1	14	1.33 ± 0.78	—
	Honshu: Kanto	38	306	0.57 ± 0.30	0.02–0.56
	Honshu: Chubu	42	473	2.24 ± 1.10	0.00–2.73
	Honshu: Kinki ^a	1	1	—	—

^aProbable anthropogenic introduction.

D1 haplotypes of *C. dehaanii* have expanded eastwards from western Honshu or Kyushu, with clade D3 haplotypes confined to Kyushu (figures 5 and 6b); the abrupt replacement with clade A haplotypes in the east could have been caused by introgressive hybridization. The populations on Fukue Island and east Kyushu possess haplotypes of the internal clade within clade D, and these populations may retain ancestral haplotypes. *Carabus dehaanii* is the largest species in

this subgenus and its range expansion resulted in formation of species assemblages with small- and medium-sized species; this species co-occurs with all but one species.

Carabus albrechti and *C. insulicola* expanded northwards from central Honshu (figures 5 and 6c,d). In *C. albrechti*, clade F1 haplotypes expanded from Chubu to Tohoku from the Japan Sea side of Honshu, and from Chubu to Kanto and Tohoku

Figure 4. Haplotype clade composition of each species based on the number of ND5 haplotypes (large circles) and the pattern of sharing of clades and haplotypes between sympatric/parapatric species (lines connecting species: dashed lines, no clade shared; thin solid line, clade shared; thick solid line, haplotype shared). For each species, the number of sample beetles for ND5 sequences (n), the total number of haplotypes (Nh) and nucleotide diversity (d ; % $\pm$ s.d.) are indicated with the number of haplotypes within each clade. Clades with single asterisks are those with introgressed haplotypes, as evidenced by a haplotype sharing a pattern with another species. Clades with double asterisks are those putatively originating from introgression. Putative original haplotype clades (those not affected by introgression) are indicated by small circles.

from the Pacific side of Honshu. In *C. insulicola*, clade F (F1a) haplotypes spread from Chubu to central Kanto, and afterwards to Tohoku and the Japan Sea side of central Honshu. Clade B1, introgressed from *C. arrowianus* in central Chubu and spread northward and southeastward.

7. DIVERGENCE TIMES OF SMALL-ISLAND POPULATIONS

Ohomopterus populations inhabiting small islands adjacent to the main islands are often discriminated as subspecies. We estimated the divergence time from main-island populations of nine populations on six islands using ND5 (table 3). The time of the last connection between a small island and a main island is not always clear, based on the geological evidence, as is the case for Tsushima and Sado Islands and Hokkaido, which are separated by deep straits. However, Iki Island

was probably separated from Kyushu at the last postglacial transgression approximately 15 000 years ago (Ohshima 1990; Machida *et al.* 2001). Iki is a small volcanic island and the last eruption occurred 0.9–0.6 Ma; it is only 21 km from Kyushu and they were connected during the last glacial period. Therefore, it is reasonable to assume that the divergence of *C. japonicus* (the sole species on Iki) between Iki and Kyushu started 15 000 years ago.

In each of the nine cases, the divergence time between the small island and the main island was estimated by a coalescent simulation analysis using IMA (Hey & Nielsen 2007). Although this program allows migration between populations after the split, this was assumed not to have occurred between populations that are currently separated by the sea. The time calibration was performed assuming that the estimated divergence time in *C. japonicus* between Iki and Kyushu was 15 000 years. To avoid the effect of introgressed

Figure 5. Distribution of ND5 lineages by prefecture and island. Coloured areas indicate distribution ranges and small open circles indicate sampling localities. The pie graph for each prefecture/island shows the haplotype clade composition among individuals sampled, showing the geographical pattern of occurrence of different haplotype clades. Arrows on the distribution maps indicate the direction of dispersal for some haplotype clades.

haplotypes on the estimation, the main-island population used in the calculation consisted of populations adjacent to the small island and not including putative introgressed haplotypes.

Divergence times estimated by the IMA analysis fell in the Late Pleistocene to Holocene (75–1.4 ka ago;

table 3), suggesting that small-island (except Tsushima Island) populations separated after the Last Glacial Maximum. Around Kyushu, the Goto Islands, including Fukue Island, were last connected to Kyushu during the last glacial period; divergence of *C. japonicus* and *C. dehaanii* was 15 and 8 ka ago, respectively. Tsushima

Figure 6. Statistical parsimony networks of ND5 haplotypes showing the geographical relationships between internal and tip clades. We inferred directions of dispersal as from the geographical ranges of interior clades to those of tip clades. (a) *japonicus*, (b) *dehaanii*, (c) *albrechti* and (d) *insulicola*.

is far (88 km) from Kyushu and may have been separated 100 ka ago (Ohshima 1990); the divergence of *C. japonicus* 75 ka ago (the last interglacial) is congruent with geohistory. The Oki Islands were connected to Honshu (Chugoku district) during the last glacial period and divergence of *C. yaconinus* (9 ka ago) was consistent with this. However, divergence of another Oki Island inhabitant, *C. daisen*, is earlier (23 ka ago), the difference perhaps attributable to different habitat uses; *C. yaconinus* inhabits both floodplains and mountains and could probably cross the plain between Oki and Honshu, whereas *C. daisen* is confined to mountain areas and its dispersal was limited. Note that the haplotype lineages of Oki and Honshu populations of *C. daisen* differed completely and the haplotype composition of Honshu populations was highly

heterogeneous, possibly causing the uncertainty in divergence time estimates.

Sado Island has been separated from Honshu since the Middle Pleistocene according to geological inference (Ohshima 1990). However, divergence of *C. albrechti* dates approximately to the Last Glacial Maximum (18 ka ago), suggesting that gene flow occurred at the glacial maximum. Also, the estimated time of divergence for *C. insulicola* is quite recent at 1.4 ka ago. This suggests colonization as a result of flushing from the big river opposite to the island on Honshu. The occurrence of *C. insulicola* on Izu-Oshima Island off the Pacific coast of Honshu might result from a similar cause, but two recently discovered populations in Hokkaido are probably due to anthropogenic accidental transportation.

Table 3. Estimated times of the last separation of small-island populations from main-island populations. Geographical distance and geological separation time (Ohshima 1990) are given in square brackets.

islands species (small island–main island)	divergence time, ka ago ^a Hi (HPD90Lo–90Hi)
Iki–Kyushu [21 km; 15 ka] <i>C. japonicus</i>	15 ^b (8–21)
Tsushima–Kyushu [88 km; 0.1 Ma] <i>C. japonicus</i>	75 (36–105)
Fukue–Kyushu [78 km; 8.5 ka] <i>C. japonicus</i>	15 (7–20)
<i>C. dehaanii</i>	8 (5–13)
Oki–Honshu (Chugoku) [42 km; 16 ka] <i>C. daisen</i>	23 (10–69)
<i>C. yaconinus</i>	9 (4–15)
Sado–Honshu (Niigata) [32 km Mid-Pleistocene] <i>C. insulicola</i>	1.4 (0.6–3)
<i>C. albrechti</i>	18 (8–30)
Hokkaido–Honshu (Tohoku, Kanto) [18 km; 0.1 Ma] <i>C. albrechti</i>	6 (4–10)

^aResults of IMA analysis (Hey & Nielsen 2007); Hi, the time with the highest posterior probability after smoothing; HPD90Lo–90Hi, the lower and upper bound of the 90 per cent highest posterior density (HPD) interval. Each IMA analysis used a Markov chain Monte Carlo procedure with four chains for 5–10 million steps following 0.1 million steps of burn-in and repeated two or more times to judge the reliability of estimates.

^bThe divergence time used for calibration.

The divergence time for *C. albrechti* between Honshu and Hokkaido was also recent (6 ka ago; in the Holocene). Although a land bridge might not have appeared over the deep Tsugaru Strait during the last glacial period (Ohshima 1990; Koaze *et al.* 2003), the valley between Honshu and Hokkaido was narrow and might have allowed exchange of terrestrial fauna. Our estimate suggests that the Hokkaido population was established at or after the last glacial period. The *C. albrechti* population in Hokkaido possesses a common haplotype that also occurs in northern Honshu, and from which all other haplotypes are derived. Hokkaido was mostly covered with boreal conifer forest (otherwise tundra and glacier) during the last glacial period (Tsukada 1984), a unsuitable habitat for *Ohomopterus*. Therefore, the present Hokkaido population probably originated from a small refugium in southern Hokkaido or northern Honshu.

Overall, gene flow between small islands and main islands occurred until recently (later than the early Late Pleistocene). However, the single species populations on Iki Island and Hokkaido exhibit larger body sizes than main-island populations coexisting with larger species, possibly related to competitive release (Sota *et al.* 2000a). The short duration of segregation was therefore sufficient for ecologically significant evolution.

8. DISCUSSION

We examined a large dataset of mitochondrial ND5 gene sequences to determine the pattern of diversification of *Ohomopterus* ground beetles endemic to the Japanese Islands. Haplotype diversity was high and there was extensive trans-species polymorphism. Our study sets out an entire scheme of mitochondrial diversification in *Ohomopterus* that was partially reported previously (Sota *et al.* 2001; Sota 2002; Nagata *et al.* 2007a,b). Initial diversification of *Ohomopterus* occurred in the central region of Honshu, which harbours all but one of the 15 species. Shikoku

and Kyushu are separated from Honshu only by narrow, shallow channels. Separation of the Shikoku *Ohomopterus* population resulted in the origin of *C. tosanus*, and the populations of *C. dehaanii* and *C. japonicus* on Kyushu may be ancestral to those on Honshu because eastward range expansion was inferred for these species. Except for these species, speciation probably occurred only in central Honshu. Our divergence time estimates suggest that differentiation of small-island populations occurred recently, from the Late Pleistocene through the Holocene, resulting in minor morphological differentiation at the most. Thus, isolation on small islands or peripheral differentiation seems to have played only a minor role in speciation in this subgenus.

In central Honshu, large populations of *Ohomopterus* have lived in favourable temperate habitats (warm and wet) separated by rivers and mountains; allopatric or parapatric differentiation has occurred repeatedly, resulting in parapatry of several species. The parapatric species can hybridize at their contact zones, sometimes resulting in the formation of hybrid swarms, and mitochondrial introgression has occurred repeatedly. Nevertheless, interspecific gene flow is limited, and species boundaries are maintained by geographical barriers. Introgressive hybridization has also occurred between sympatric species but is largely prevented by differences in the genital and body sizes (Nagata *et al.* 2007b). By contrast, divergence among populations within species is ongoing, for instance, in polytypic *C. arrowianus* in central Honshu (N. Nagata, K. Kubota, Y. Takami & T. Sota 2008, unpublished data). In this species, allopatric divergence of both body and genital length occurred in the latest Middle Pleistocene with limited gene flow between separate populations. Thus, diversification and speciation of *Ohomopterus* has occurred without persistent isolation and despite frequent opportunities for secondary contact and hybridization under the fluctuating geographical conditions during the Pleistocene.

The estimated speciation rates of *Ohomopterus* are among the fastest of any animal group (Coyne & Orr 2004), with a speciation interval of less than 1 Ma. A high speciation rate was reported in the Hawaiian cricket genus *Laupala* (Mendelson & Shaw 2005) that diverged into 21 species in 3.7 Ma ($0.82 \text{ species Ma}^{-1}$). *Laupala* are dietary generalists and thus exhibit little ecological differentiation; they are differentiated primarily by secondary sexual traits, suggesting rapid speciation by sexual selection. The highest known speciation rate was in a monophyletic clade on the island of Hawaii (six species in 0.43 Ma; $4.17 \text{ species Ma}^{-1}$). The case of *Ohomopterus* parallels that of *Laupala*, in that the diversification of traits for reproductive isolation (genital morphology) due to local sexual selection might have resulted in rapid speciation. Speciation might be particularly fast in the *iwawakianus-insulicola* group on Honshu, as it exhibits marked divergence in genital morphology (figure 2). The *Ohomopterus* species used here are as defined morphologically by Ishikawa (1985, 1991). However, some consist of distinct populations (subspecies) with different body sizes or genital morphologies, which may be reproductively incompatible with each other. For instance, *C. tosanus* has been divided into two species by Imura & Mizusawa (1996) because there are contact zones between distinct populations with divergent body sizes. Thus, the current species may be further divided into additional biological species and our estimates of speciation rates are regarded as minimum rates.

Speciation of *Ohomopterus* was achieved without major ecological differentiation such as diet, but with divergence of body size and genital morphology, which facilitates mechanical isolation (Sota & Kubota 1998; Nagata *et al.* 2007b). It is tempting to conclude that body size differences among species would result in resource partitioning in terms of prey size and facilitate species coexistence, implying the possibility of ecological speciation as a by-product of resource partitioning. In fact, the body size of larvae, which is correlated with that of adults, is related to the efficiency of predation on different-sized earthworms (Y. Okuzaki & T. Sota 2008, unpublished data), and hence different-sized species may depend primarily on different prey size classes. However, owing to the wide, overlapping range of prey sizes that different-sized larvae can attack and consume, effective resource partitioning in prey size is not likely to occur. Compared to the density-dependent strength of resource competition, sexual competition (via interspecific mating) is frequency dependent and has a strong effect on demography (i.e. competitive exclusion; Ribeiro & Spielman 1986; Kuno 1992; Yoshimura & Clark 1994). Therefore, whereas vague resource partitioning might result from body size differences, sexual isolation due to body size differences will have more direct and hence ubiquitous effects on species coexistence.

The cause and process of divergence in body size and genital morphology need to be clarified. Because intraspecific body size variation in *Ohomopterus* is clinal on a temperature gradient (i.e. the converse of Bergmann's rule; Masaki 1967; Roff 1980), climatic adaptation must be involved in body size variation. However, the differences among sympatric species

cannot be explained by climatic adaptation. The fact that island individuals of *C. japonicus* and *C. albrechti* in the absence of co-occurring species are larger than those in populations with sympatric species suggests an effect of interspecific interactions (Sota *et al.* 2000a). A possible scenario is that the body size difference originated from local adaptation. Rapid dispersal of different-sized species during a glacial/interglacial cycle then resulted in secondary contact; and the difference has been maintained (or reinforced) by selection related to sexual (and resource) competition (i.e. this process implies parapatric speciation along a climatic gradient; see also Konuma & Chiba 2007). This explanation is similar to that of Sota *et al.* (2000b) for a geographical cline of interspecific body size of *Leptocarabus* on Japanese Islands, mostly on Honshu. This hypothetical process could be tested using intraspecific geographical variation in body size; species such as *C. tosanus* show steep altitudinal clines in body size and populations of different body size exist in an area. Selection on body size is not simple, and disentangling the components of adaptation to seasonality in temperature and food availability and intra- and interspecific sexual interactions would be necessary.

The evolution of genital morphology, however, is independent of environmental gradients and governed by within-population selection, most likely sexual selection (Eberhard 1985; Arnqvist 1998; Hosken & Stockley 2004). Notable changes in genital morphology have occurred mostly within the *iwawakianus-insulicola* group. A quantitative genetic analysis suggests that species differences in the dimensions of male and female genital parts (i.e. copulatory piece and vaginal appendix) are determined by a limited number of loci (Sasabe *et al.* 2007). The selective advantage of the genital lock and key system and that of the exaggerated genital parts is not fully understood. Strict matching of genital parts between sexes ensures insemination (Takami 2003) and hence is beneficial to males in particular. Elongation of male genital parts can improve a male's ability to remove a rival's spermatophore and be advantageous in sperm competition (Takami 2007), although other factors such as sexual conflict might explain the arms race of male and female genital evolution (Sota 2002).

In conclusion, the divergence of *Ohomopterus* beetles occurred with varying fragmentation of favourable habitats by geographical barriers, evolution of body size and genital morphology in local populations, and secondary contact and interactions between diverging populations. Resolving the details of the whole process and the genetic architecture of the key traits for reproductive isolation will greatly improve our understanding of the origin of species and local species richness.

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