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Phylogeography and Introgressive Hybridization of the Ground Beetle *Carabus yamato* in Japan Based on Mitochondrial Gene Sequences

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To study the phylogeography of the ground beetle *Carabus yamato* in Japan, we compared 1,020-bp sequences of the mitochondrial NADH dehydrogenase subunit 5 (ND5) gene from 373 specimens from 37 localities with those of three parapatric species (*C. albrechti*, *C. kimurai*, and *C. japonicus*) that might share mitochondrial lineages with *C. yamato* through introgressive hybridization. We found 81 haplotypes from *C. yamato*. Of these, 17 haplotypes were considered to be of an introgressed lineage from *C. albrechti*, based on the phylogeny and geographic distribution. In addition, one haplotype of *C. kimurai* was likely an introgressant from *C. yamato*. Putative introgression events among the four species were restricted to these two directional cases. We analyzed the phylogeography of *C. yamato* using nested clade phylogeographical analysis and population genetic parameters. The mitochondrial lineages of *C. yamato* were estimated to have diverged no more than approximately 1.12 million years ago, implying that the estimated historical events occurred after the Early Pleistocene. *Carabus yamato* was inferred to have experienced a contraction of its distribution range, followed by recent range expansion. Populations in the western and eastern regions, segregated by Ise Bay and the Nobi and Okazaki Plains, diverged in the mitochondrial clades. The northern and most western populations possessed one clade only (except an introgressed lineage), whereas eastern and some southwestern populations possessed several diverged clades, which were considered to be ancestral; these populations may have been associated with refugia during glacial periods.

Key words: historical biogeography, molecular phylogeny, nested clade phylogeographical analysis, *Ohomopterus,* refugia

INTRODUCTION

The molecular phylogeographical approach is a powerful tool to estimate historical biogeographical events that have influenced the evolutionary history of species (Avise, 2000; Hewitt, 2000). To date, much attention has been devoted to fragmentation and confinement of populations to refugia with the development of glaciers, as well as postglacial range expansions, during the Pleistocene (1.8–0.01 million years ago) in North America (Knowles, 2001; Carstens *et al.*, 2004; Runck and Cook, 2005; Smith and Farrell, 2005) and Europe (Branco *et al.*, 2002; Pfenninger *et al.*, 2003; Hewitt, 2004; Emerson and Hewitt, 2005; Vila *et al.*, 2005), or range expansions of alpine species during glacial periods (DeChaine and Martin, 2004, 2005). However, few molecular phylogeographic studies have investigated the Pleistocene historical biogeography of insects in Japan and adjacent regions (Satoh *et al*., 2004; Sota *et al*., 2004).

Ground beetles of the subtribe Carabina (Coleoptera: Carabidae) are flightless and often show high morphological and genetic divergence, providing good material for phylogeographical studies (Sota *et al*., 2001; Garnier *et al*., 2004; Zhang *et al*., 2005). In Japan, the endemic subgenus *Ohomopterus* (genus *Carabus*) has differentiated into 15 or more species and numerous subspecies and exhibits remarkable variation in body size and genital morphology (Ishikawa, 1991; Imura and Mizusawa, 1996). The phylogeny of *Ohomopterus* has been studied with mitochondrial (Su *et al*., 1996a; Osawa *et al*., 2004) and nuclear DNA (Sota and Vogler, 2001, 2003). These studies revealed extensive trans-species polymorphisms in mitochondrial genes, probably caused by introgressive hybridization among species (Sota and Vogler, 2001, 2003). However, phylogeographical analysis, which is useful for studying evolutionary processes, has not been attempted for this subgenus, with the exception of one study that focused on mitochondrial introgression across species (Sota *et al*., 2001). A phylogeographic analysis of *Ohomopterus* species will contribute to our understanding of the historical biogeog-

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raphy of animals in Japan.

Here, we analyzed the phylogeography of *Carabus yamato* (Nakane), one of the small-bodied *Ohomopterus* species occurring in central Honshu, using mitochondrial gene sequences. This species is a member of the *albrechti* species group (Ishikawa, 1991), a monophyletic group of small-bodied species occurring in central to northeastern Japan (Sota and Vogler, 2001, 2003). In *Ohomopterus*, different-sized species often coexist in the same habitat (*e.g*., small and large, or small, medium, and large species), whereas similar-sized species occur parapatrically (Sota *et al*., 2000). Most cases of mitochondrial introgression have occurred in species of the same size class (Sota *et al*., 2001; Sota, 2002). *Carabus yamato* is parapatric with *C. albrechti* Morawitz and *C. kimurai* (Ishikawa), of the *albrechti* species group, and with *C. japonicus* Motschulsky, of the *japonicus* species group, but is of similar size. In previous molecular phylogenetic studies, the *albrechti* group showed trans-species polymorphism of mitochondria (Su *et al*., 1996b; Saito *et al.,* 2003).

Because of marked polymorphisms and their utility in molecular experimentation, mitochondrial genes have been used widely in phylogeographic studies, even though mitochondrial DNA is more likely to be affected by introgressive hybridization than nuclear DNA (Barton and Jones, 1983; Avise, 2004; Ballard and Whitlock, 2004; Gomez-Zurita and Vogler, 2006). To resolve the issue of mitochondrial introgression, some phylogeographic studies of animals have attempted to distinguish between the distributions of native and introgressed haplotypes (Sota *et al*., 2001; Masta *et al*., 2002; Redenbach and Taylor, 2002; Morando *et al*., 2004). Therefore, mitochondrial genes can still be useful for the phylogeographic analysis of *Ohomopterus*, which may have been affected by repeated introgressive hybridization.

The results from this study were threefold. We clarified the diversity of mitochondrial haplotypes in *C. yamato*, inferred the introgression of mitochondria between *C. yamato* and other parapatric species, and revealed the historical biogeography of *C. yamato*, with an estimate of divergence time.

MATERIALS AND METHODS

Sampling

We collected 373 individuals of *C. yamato* from 37 localities covering almost the entire geographic range of the species (Imura and Mizusawa, 2002; Imura, 2004) and 201 individuals of parapatric species (34 *C. kimurai*, 35 *C. japonicus*, and 132 *C. albrechti*) for mitochondrial DNA analysis (Table 1; Fig. 1). After identification by morphological characters, the collected beetles were stored in 99% ethanol or at –30°C. In the phylogenetic analysis, we included four sequences from three other *Ohomopterus* species: *C. daisen* (Nakane) from Kurayoshi, Tottori (present study); *C. lewisianus* Breuning from Hakone, Kanagawa (present study) and Nirayama, Shizuoka (GenBank, AF219432; Sota and Vogler, 2001); and *C. arrowianus* Breuning from Iida, Nagano (GenBank, AF219465; Sota and Vogler, 2001). As outgroups, we used five sequences from the *Carabus* subgenus *Isiocarabus* (two *C. miaorum* Lassalle et Prunier and three *C. fiduciarius* Thomson sequences), which is distributed in China and Korea, and one from *Apotomopterus porrecticollis* (Bates), which is distributed across Japan. *Isiocarabus* is the sister group of *Ohomopterus* (Sota and Ishikawa, 2004). The outgroup sequences were taken from GenBank/DDBJ (accession nos. AB041052, AB041053, and AB093147; Su *et al.,* 2004), except for

Table 1. Localities of the populations sampled, with corresponding locality codes, number of specimens (*n*), and haplotypes of ND5 sequences.

Species	Population	*n*	haplotype code (number of individuals when≥2)
C. yamato		373	
Y1	Agematsu	6	340, 1011 (2), 1012, 1013, 1014
Y2	Ano	1	685
Y3	Daian	21	035, 512, 513 (8), 514, 515, 518, 519 (4), 520 (3), 837
Y4	Fuchu	13	493 (7), 521 (6)
Y5	Fukuda	2	708 (2)
Y6	Hachiman	7	035 (3), 036 (4)
Y7	Hiei	26	373 (21), 480 (2), 900, 910, 911
Y8	Hino	2	036, 373
Y9	Inabu	4	035 (3), 717
Y10	Iwakura	1	1130
Y11	Kaga	12	598 (10), 599 (2)
Y12	Kameyama	24	175 (24)
Y13	Kamiichi	2	035, 355
Y14	Kamioka	2	035 (2)
Y15	Katsuragi	10	372, 373 (9)
Y16	Kimi	1	373
Y17	Kongo	27	372 (10), 373 (15), 488, 548
Y18	Kumano	47	1067 (43), 1091 (3), 1092
Y19	Mikawa	10	1040, 1041 (5), 1042 (3), 1120
Y20	Misakubo	8	1050 (6), 526 (2)
Y21	Miya	5	035 (2), 110 (2), 158
Y22	Mizusawa	6	175 (2), 674 (3), 686
Y23	Nabari	4	662 (3), 664
Y24	Nakatsugawa	2	615, 616
Y25	Sakuma	17	1030 (8), 1046 (3), 526 (5), 717
Y26	Sakyo	23	183, 373 (19), 374, 888, 1055
Y27	Sanage	20	184 (10), 185 (5), 186 (2), 187, 188, 607
Y28	Shinano	4	612, 613, 614, 617
Y29	Suzuka	24	175 (23), 570
Y30	Taga	2	183, 514
Y31	Takayama	6	035, 110
Y32	Tarao	8	373, 480 (6), 819
Y33	Tsugu	9	717 (7), 718, 719
Y34	Unazuki	6	562, 563 (2), 567, 568, 569
Y35	Yamazumi	8	526, 615, 706, 707, 709, 717 (2), 748
Y36	Yatsuo	2	492, 493
Y37	Yoro	1	543
C. albrechti		132	
A1	Itoigawa	6	350, 351, 352 (2), 353, 354
A2	Nagaoka	2	677, 938
A3	Naoetsu	6	332, 937 (4), 938
A4	Nou	15	110 (2), 339 (4), 352, 354, 510, 703, 725 (3), 726 (2)
A5	Oumi	8	332 (2), 350 (4), 355 (2)
A6	Unazuki	4	565 (4)
A7	Fujimi	9	340 (7), 530 (2)
A8	Matsumoto	8	907 (4), 908 (3), 909
A9	Minowa	2	974 (2)
A10	Nirasaki	3	323, 347, 348
A11	Otari	4	343, 352 (2), 358
A12	Tatsuno	15	322 (2), 530 (11), 532, 704
A13	Syariki	7	325 (5), 326, 327
A14	Tsuga	3	346, 1020 (2)
A15	Awashima	5	332 (4), 853
A16	Niiharu	10	342 (2), 346 (6), 929 (2)
A17	Tsukui	4	335, 336, 356, 357
A18	Azuma	9	344 (3), 345 (3), 346 (2), 752
A19	Sado	4	330, 331 (3)
A20	Asahi	2	609, 854
A21	Hidaka	4	338 (4)
A22	Morioka	2	338 (2)
C. japonicus		35	
J1	Sasari	25	576 (15), 579, 580 (2), 581 (3), 820 (3), 821
J2	Seppiko	2	459 (2)
J3	Togura	3	517 (2), 529
J4	Ohda	1	272
J5	Aya	1	847
J6	Nomura	1	254
J7	Kashima	1	1154
J8	Tosa	1	249
C. kimurai		34	
K1	Shizuoka	1	022
K2	Akiba	2	453, 744
K3	Haruno	12	453 (11), 739
K4	Kawane	9	022 (4), 369 (2), 370, 371 (2)
K5	Mineyama	10	022 (4), 044, 045 (4), 046

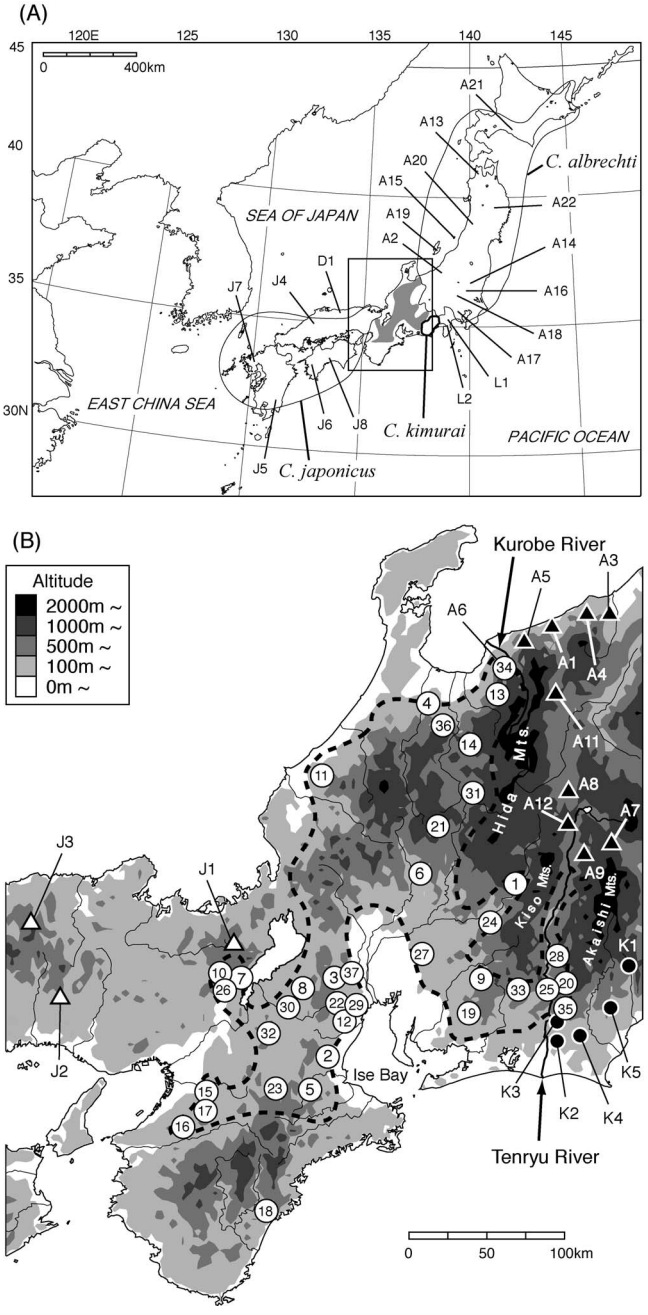

Fig. 1. Sampling localities for *C. yamato* and its related species. **(A)** The shaded area represents the geographic range of *C. yamato*. Sampling localities for *C. albrechti* (indicated by numbers with "A"), *C. lewisianus* (L), *C. japonicus* (J), and *C. daisen* (D) are also shown. **(B)** Sampling localities of *C. yamato* (open circles), with the range indicated by a dashed line. Solid circles represent localities for *C. kimurai*; open triangles, *C. japonicus*; solid triangles, *C. albrechti*. The locality numbers correspond to those in Table 1 (note that "Y" is omitted from the locality numbers of *C. yamato*).

those we obtained.

Sequencing

Total DNA was extracted from thoracic muscles or testes using standard phenol–chloroform method with ethanol precipitation. A 1,083-bp fragment of mitochondrial NADH dehydrogenase subunit 5 (ND5) was PCR-amplified using the primers 6-1 (5'-CCT GTT TCT GCT TTA GTT CA-3') and 4-4 (5'-GTC ATA CTC TAA ATA TAA GCT A-3'; Su *et al*., 1996b). PCR was carried out with the following conditions: 2 min at 94°C; 30 cycles of 30 s at 94°C, 30 s at 55°C, and 1 min at 72°C; and 10 min at 72°C. PCR products were checked by electrophoresis on a 1% agarose gel and purified using a QIA Quick PCR Purification Kit (Qiagen, Hilden, Germany) or polyethylene glycol (PEG) precipitation. Cycle sequencing reactions were carried out using Big Dye v.1.1 Kit and v. 3.1 Kits (Applied Biosystems, Foster City, CA, USA) with the same primers as in PCR. After purification by ethanol precipitation, electrophoresis was performed with an ABI 377 sequencer (Applied Biosystems). Sequences were aligned manually. We excluded the first 30 bp and the last 33 bp of the 1,083-bp data matrix because of sequence ambiguity in some specimens (Sota *et al*., 2001). Haplotypes in a 1,020-bp sequence matrix were detected using Collapse ver. 1.1 (D. Posada; http://darwin.uvigo.es/). The sequence data of all haplotypes were deposited in GenBank/DDBJ (accession numbers AB247658–AB247815).

Phylogeny and divergence time

We reconstructed the phylogeny among the ND5 haplotypes using the maximum likelihood (ML) and Bayesian inference methods. For tree searches, we selected the GTR+I+G model based on the Akaike information criterion (AIC) using Modeltest ver. 3.7 (Posada and Crandall, 1998). ML analysis was performed using Phyml ver. 2.4.4 (Guindon and Gascuel, 2003). The proportion of invariable sites and the gamma distribution parameters were estimated as part of the analysis. The confidence of nodes in the ML tree was estimated using 1,000 bootstrap pseudoreplicates.

Bayesian analysis was carried out using MrBayes ver. 3.1.1 (Ronquist and Huelsenbeck, 2003). We ran two Markov chain Monte Carlo (MCMC) analyses in parallel for two million generations, sampling trees every 100th generation. After discarding the first 6,000 trees as burn-in, we obtained a 50% majority rule consensus tree and the posterior probabilities of nodes in the tree. To confirm the tree topology, we repeated the MCMC analysis three times.

To estimate the times of phylogenetic events (nodes) on the ML tree, we employed a Bayesian method for divergence-time estimation that does not assume a constant molecular clock (Kishino *et al*., 2001; Thorne and Kishino, 2002), using the BASEML programs in PAML (Yang, 1997) and Multidivtime (Thorne and Kishino, 2002), available at http://statgen.ncsu.edu/thorne/multidivtime.html. For the calibration of node times, we used a constraint at the branching between the continental subgenus *Isiocarabus* and the Japanese subgenus *Ohomopterus*. The differentiation of these subgenera was likely facilitated by the geographical separation of Japan from the East Asian continent, associated with the opening of the southwest channel between the Sea of Japan and the East China Sea. Paleoceanographical studies of the Sea of Japan have revealed that southwestern Japan was connected to the continent from 10 to 3.5 or 3.0 million years ago (Mya; Koizumi, 1992; Tada, 1994). More recently, Kitamura *et al*. (2001) and Kitamura and Kimoto (2004) reported that the Japanese archipelago was connected to the continent until 1.7 Mya, except for a few temporary disconnections, based on more reliable fossil evidence. Thereafter, the continental connection was restricted to glacial maxima, and colonization of small terrestrial animals from the continent to Japan appears to have been greatly limited (see Suzuki *et al*., 1997, 2003; Tsuchiya *et al*., 2000; Shinohara *et al*., 2004, for the absence of Pleistocene colonization by most endemic Japanese small mammals). Therefore, we used 3 Mya and 1.7 Mya as the upper and lower limits of the divergence time between *Ohomopterus* and *Isiocarabus*.

Phylogeography and population genetics

To estimate genetic diversity in each species, we performed an analysis of molecular variance (AMOVA; Excoffier *et al*., 1992) and

calculated haplotype diversity (h) and nucleotide diversity (π) using Arlequin ver. 3.0 (Excoffier *et al*., 2005). For populations with five or more samples of *C. yamato*, we calculated haplotype diversity and nucleotide diversity, Fu's F_s (Fu, 1997), and the population growth rate g (Kuhner *et al*., 1998). Fu's F_s indicates demographic expansion when F_s is significantly negative (Fu, 1997). We employed Arlequin ver. 3.0 to calculate Fu's F_s, and its significance using a randomization procedure based on 1,000 replicates. The population growth rate g indicates population expansion or contraction when g is positive or negative, respectively. We calculated g for each population using Fluctuate ver. 1.4 (Kuhner *et al*., 1998), which implemented an MCMC simulation. For the simulation, the initial theta was obtained by Watterson's (1975) method and a transition:transversion ratio of 4.3687:1 was obtained using Modeltest ver. 3.7 (Posada and Crandall, 1998). We ran 20 short chains of 5,000 steps and 10 long chains of 100,000 steps, sampling every 20th generation.

To study the phylogeography of *C. yamato*, we used nested clade phylogeographical analysis (NCPA; Templeton *et al*., 1995; Templeton, 1998, 2004). A statistical parsimony network was constructed using TCS ver. 1.13 (Clement *et al*., 2000). To resolve ambiguous connections (loops), we applied three criteria, as suggested by Pfenniger and Posada (2002), based on predictions from coalescent theory (Crandall and Templeton, 1993). Cladogram nesting was performed manually, and tip and interior clades were determined following Templeton and Sing (1993) and Templeton *et al*. (1995). Using the program Geodis 2.0 (Posada *et al*., 2000), each clade was tested for the null hypothesis of random geographic distribution using a permutational contingency test. Two distance measures, clade distance (D_c) and nested clade distance (D_n), were obtained, and their significance was determined using 10,000 permutations. To infer phylogeographical events based on the output of Geodis, we used the latest inference key ver. 11 November 2005 (http://darwin.uvigo.es/software/geodis.html). In interpreting the direction of range expansion, an interior clade was considered to be older than its associated tip clade(s), based on coalescent theory (Templeton, 1998).

RESULTS

Phylogeny and divergence time

We found 273 variable sites in the 1,020-bp ND5 gene sequence. From 584 individuals, 163 haplotypes were detected, including 81 haplotypes from *C. yamato*, 14 from *C. japonicus*, 51 from *C. albrechti*, and 10 from *C. kimurai* (Table 1). All haplotypes were species-specific, except for three (110, 340, and 355) that were shared between *C. yamato* and *C. albrechti*.

A maximum likelihood (ML) tree was determined for the ND5 haplotypes (Fig. 2). The Bayesian analysis with three independent runs resulted in the identical topology in a 50% majority-rule consensus tree, which was similar to the topology of the ML tree. In these trees, none of *C. yamato*, *C.*

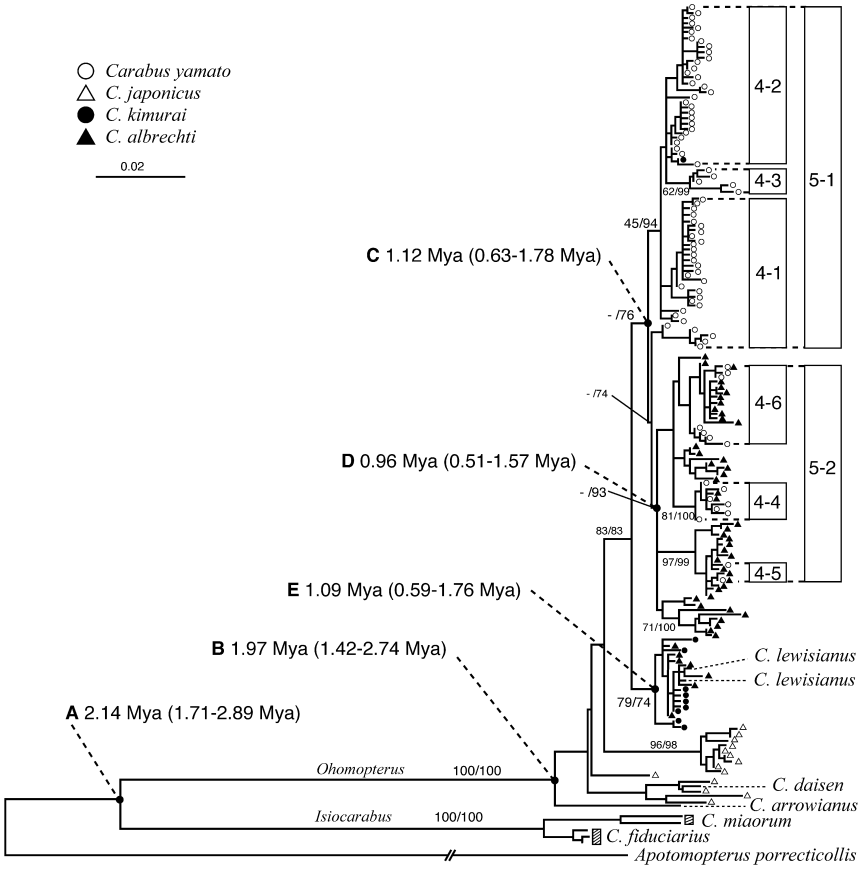

Fig. 2. Maximum likelihood tree of haplotypes possessed by *C. yamato* and related species. The divergence time with the 95% confidcnce interval is shown for each node (A to E). Node supports are bootstrap percentages for the maximum likelihood analysis, followed by Bayesian posterior probabilities (percentages; both shown only when>70%). At the ends of branches, open circles indicate haplotypes possessed by *C. yamato*; closed circles, *C. kimurai*; open triangles, *C. japonicus*; closed triangles, *C. albrechti*. The four-step and five-step clades indicated at the right correspond to those in Fig. 3.

albrechti, *C. kimurai*, or *C. japonicus* showed monophyly within the clade of *Ohomopterus* (clade B; Fig. 2). The haplotypes of *C. yamato* were included in clade C, which also contained haplotypes of *C. albrechti* and one *C. kimurai*. Most haplotypes of *C. albrechti* were included in clade D, which also contained some haplotypes of *C. yamato*. Most haplotypes of *C. kimurai* and some haplotypes of *C. albrechti* were included in clade E. All haplotypes of *C. japonicus* occurred in clades distinct from those of *C. yamato*, *C. albrechti*, and *C. kimurai*. The pattern of trans-species polymorphisms indicated that mitochondrial introgression occurred from *C. yamato* to *C. kimurai*, and from *C. albrechti* to *C. yamato*.

The divergence times of major nodes A–E were estimated using the Bayesian approach (Fig. 2). The estimated ages were 2.14 Mya for node A (differentiation between *Isiocarabus* and *Ohomopterus*), which was constrained to 3.0–1.7 Mya; 1.97 Mya for node B (most recent common ancestor of *Ohomopterus* mitochondria); 1.12 Mya for node C (all haplotypes of *C. yamato*); 0.96 Mya for node D (most haplotypes from *C. albrechti* and some from *C. yamato*); and 1.09 Mya for clade E, with most haplotypes from *C. kimurai* and some from *C. albrechti*.

Population genetics and phylogeography

All species showed high haplotype and nucleotide diversity (Table 2). All populations with three or more samples revealed two or more haplotypes, except for one population (Kameyama, Y12) of *C. yamato* and two populations (Unazuki, A6 and Hidaka, A21) of *C. albrechti* (Table 1). AMOVA indicated large genetic variance among populations in each species (Table 2).

The 23 populations of *C. yamato* with five or more samples showed haplotype and nucleotide diversities of 0.00–0.96 and 0.0000–0.0097, respectively (Table 3). The analysis of population growth rate *g* suggested the occurrence of demographic expansion in five populations (Y7, Y15, Y29, Y32, and Y33). Furthermore, demographic expansion in Y7 and Y33 was supported by Fu's F_S. In contrast, demographic contraction was suggested by a negative *g* for five populations (Y11, Y18, Y20, Y25, and Y31).

The phylogeographic structure and historical biogeography of *C. yamato* populations were further analyzed using NCPA (Table 4). In the statistical parsimony network, all 81 haplotypes of *C. yamato* were connected within the 95% connection limit of 13 steps, with all loops resolved (Fig. 3). Distributions of the higher clades were biased geographically (Fig. 4). When we analyzed a network containing all haplotypes of the four species (not shown), most haplotypes of *C. albrechti* were connected within clade 4-5, but two *C. albrechti* haplotypes were connected to clade 1-28 within

Table 2. Results of analysis of molecular variance for population differentiation of ND5 gene sequences in four species, and haplotype diversity (*h*) and nucleotide diversity (π) for each species.

species	F_{ST}	*h* (SD)	π (SD)
Carabus yamato	0.71**	0.93 (0.01)	0.0081 (0.0041)
C. albrechti	0.66**	0.97 (0.01)	0.0137 (0.0068)
C. japonicus	0.94**	0.80 (0.05)	0.0048 (0.0027)
C. kimurai	0.51**	0.81 (0.06)	0.0136 (0.0069)

Significance of population differentiation: $^{**}P<0.001$

Table 3. Results of population-genetic analyses for populations with five or more samples. *h*, haplotype diversity; π, nucleotide diversity; *Fs*, Fu's *Fs*; *g*, population growth rate.

Population		*n*	*h* (SD)	π (SD)	*Fs*	*p*	*g*	(SD)
Y1	Agematsu	6	0.93 (0.12)	0.0071 (0.0045)	0.27	0.48	17.39	(77.29)
Y3	Daian	21	0.82 (0.07)	0.0057 (0.0032)	0.62	0.64	47.48	(123.66)
Y4	Fuchu	13	0.54 (0.06)	0.0005 (0.0005)	1.23	0.69	–646.37	(2417.01)
Y6	Hachiman	7	0.57 (0.12)	0.0011 (0.0009)	2.05	0.82	–511.43	(874.47)
Y7	Hiei	23	0.17 (0.10)	0.0003 (0.0003)	–1.30	0.03**	10000.00	(n.a.)
Y11	Kaga	12	0.30 (0.15)	0.0012 (0.0009)	2.86	0.91	–680.12	(428.9)
Y12	Kameyama	24	0.00 (0.00)	0.0000 (0.0000)	n.a.	n.a.	n.a.	n.a.
Y15	Katsuragi	10	0.20 (0.15)	0.0002 (0.0003)	–0.34	0.15	10000.00	(8384.09)
Y17	Kongo	27	0.57 (0.06)	0.0008 (0.0007)	–0.07	0.45	224.70	(624.70)
Y18	Kumano	47	0.12 (0.06)	0.0013 (0.0009)	5.05	0.97	–563.37	(162.32)
Y19	Mikawa	10	0.71 (0.12)	0.0029 (0.0019)	1.55	0.81	–86.35	(205.81)
Y20	Misakubo	8	0.43 (0.17)	0.0092 (0.0054)	9.58	1.00	–236.99	(78.01)
Y21	Miya	5	0.80 (0.16)	0.0086 (0.0056)	3.51	0.94	–111.35	(103.28)
Y22	Mizusawa	6	0.73 (0.16)	0.0039 (0.0026)	2.40	0.87	–156.38	(148.22)
Y25	Sakuma	17	0.70 (0.08)	0.0073 (0.0040)	7.22	1.00	–286.94	(108.13)
Y26	Sakyo	23	0.32 (0.12)	0.0010 (0.0008)	–0.84	0.25	–184.09	(163.61)
Y27	Sanage	20	0.71 (0.09)	0.0027 (0.0017)	0.64	0.67	107.85	(329.17)
Y29	Suzuka	24	0.08 (0.07)	0.0001 (0.0002)	–1.03	0.07	10000.00	(n.a.)
Y31	Takayama	6	0.33 (0.22)	0.0046 (0.0030)	5.30	0.98	–228.69	(117.10)
Y32	Tarao	11	0.49 (0.18)	0.0010 (0.0008)	–0.70	0.19	2092.49	(1410.51)
Y33	Tsugu	9	0.42 (0.19)	0.0004 (0.0004)	–1.08	0.03**	10000.00	(6624.26)
Y34	Unazuki	6	0.93 (0.12)	0.0097 (0.0060)	0.80	0.59	131.89	(68.18)
Y35	Yamazumi	8	0.96 (0.08)	0.0078 (0.0046)	–0.89	0.26	176.78	(110.13)

$^{**}P<0.5$; n.a., not applicable.

Table 4. Inferences from the nested clade phylogeographical analysis for clades of *C. yamato* with significant non-random geographic association.

Clade	χ^2 statistics	Inference chain	Inference[a]
1-1	86.3**	1-2-11-12-No	Contiguous range expansion (from haplotype 175 area to haplotype 685 area)
1-2	248.6*	1-2-3-5-6-7-Yes	Restricted gene flow/dispersal but with some long distance dispersal
1-14	47.9*	1-2-3-5-6-7-Yes	Restricted gene flow/dispersal but with some long distance dispersal
1-17	71.8*	1-2-3-5-6-7-Yes	Restricted gene flow/dispersal but with some long distance dispersal
2-1	239.0**	1-2-3-4-No	Restricted gene flow with isolation by distance
2-3	1.0*	1-19-No	Allopatric fragmentation
2-6	90.5**	1-2-3-4-No	Restricted gene flow with isolation by distance
2-8	10.0*	1-19-20-2-3-4-9-No	Allopatric fragmentation
3-1	166.0**	1-19-20-No	Inadequate geographical sampling
3-2	46.0*	1-19-2-11-12-No	Contiguous range expansion (from clade 2-3 area to clade 2-14 area)
3-4	50.0**	1-2-3-4-No	Restricted gene flow with isolation by distance
3-5	62.3**	1-2-3-4-No	Restricted gene flow with isolation by distance
4-1	448.0**	1-19-20-2-3-5-15-No	Past fragmentation and/or long distance colonization
4-2	96.8**	1-2-11-12-No	Contiguous range expansion (from clade 3-4 area to clade 3-5 area)
4-3	14.0**	1-19-20-2-11-12-13-Yes	Long distance colonization possibly coupled with subsequent fragmentation or Past fragmentaion followed by range expansion
4-4	23.0**	1-2-11-12-No	Contiguous range expansion (from clade 3-8 area to clade 3-9 area)
5-1	436.7**	1-2-11-12-No	Contiguous range expansion (from clade 4-2 area to clade 4-1 area)
5-2	34.3**	1-2-11-12-No	Contiguous range expansion (from clade 4-4 area to clade 4-5 and 4-6 area)
Total	343.2	1-2	Inconclusive outcome (tip/interior status cannot be determined)

*P<0.05, **P<0.001.

[a] Direction of range expansion in parentheses. See Fig. 4 for the ranges of 4-step and 5-step clades.

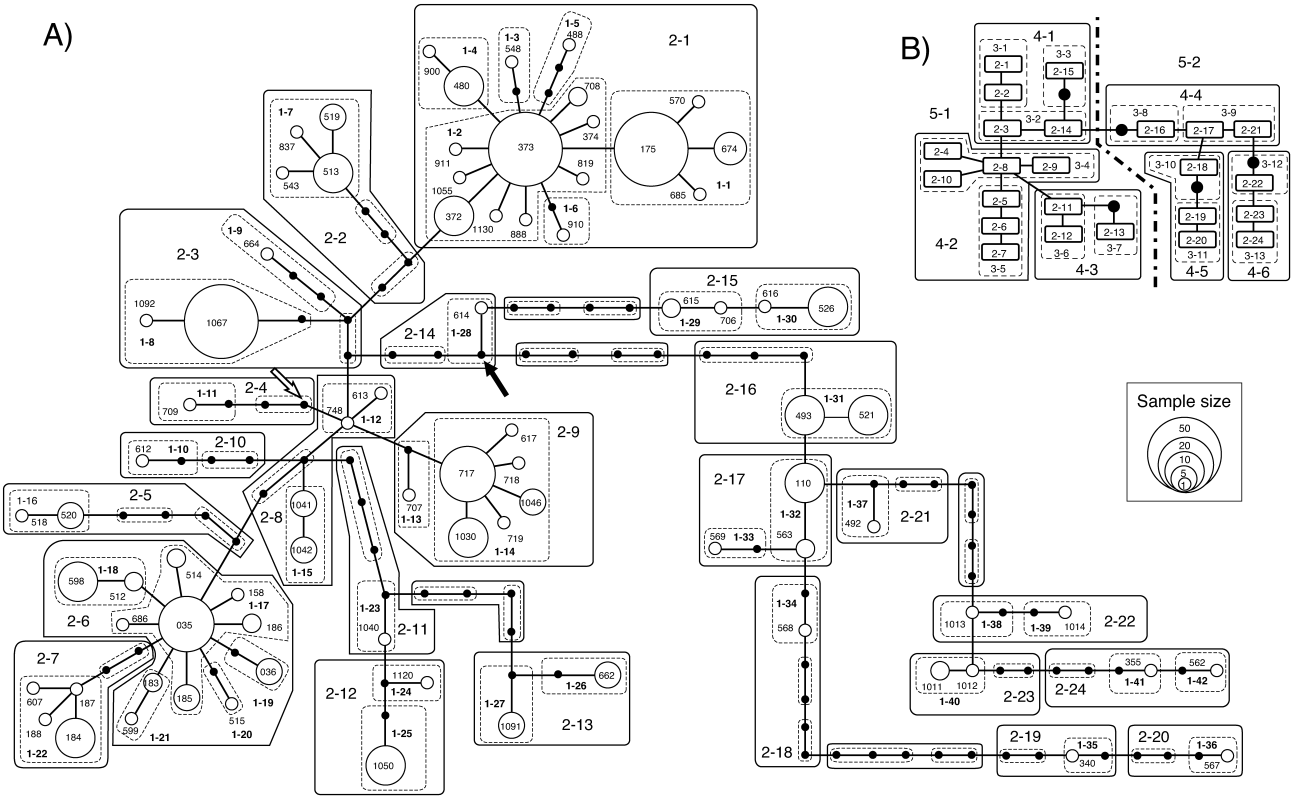

Fig. 3. Statistical parsimony network of haplotypes possessed by *C. yamato*. **(A)** Network with one-step and two-step clades. The size of the circles is proportional to sample size, and the numbers in the circles indicate haplotype codes (see Table 1). Small, filled circles indicate missing haplotypes. The white arrow indicates the position of haplotypes possessed by *C. kimurai*; the black arrow indicates the connection point of some *C. albrechti* haplotypes when haplotypes of these species were included in this network. **(B)** Nesting design for higher clades.

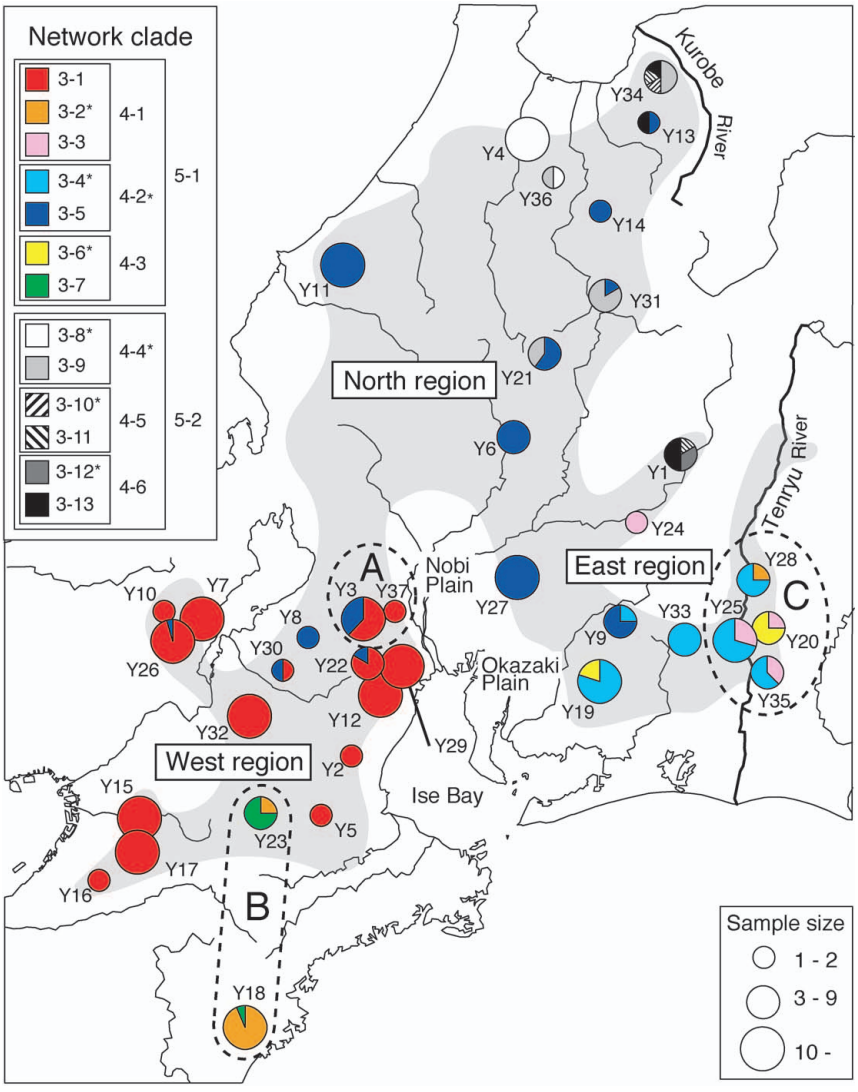

Fig. 4. Frequency distribution of mitochondrial clades (indicated by different colors) in each population of *C. yamato*. The shaded area indicates the geographic distribution of *C. yamato*, and the locality numbers (Y-numbers) are those given in Table 1. The size of the circles is proportional to sample size. In the legend, asterisks (*) indicate interior clades in the network (Fig. 3).

clade 4-1 (black arrow, Fig. 3) via seven missing haplotypes. One haplotype of *C. kimurai* was contained in clade 2-14 (white arrow, Fig. 3), but all other haplotypes were beyond the connection limits (clade E in Fig. 2).

NCPA revealed that 19 of 85 clades showed a nonrandom association between clade and geographic location (Table 4). Contiguous range expansion was inferred for each of the 5-step clades, two 4-step clades (4-2 and 4-4), one 3-step clade (3-2), and one 1-step clade (1-1). The inferred directions of these range expansions are described in Table 4. In two 4-step clades with wide ranges (clade 4-1 and 4-3), long-distance colonization or past fragmentation was inferred. This inference can be further resolved based on the dispersal ability of the study organisms (Masta *et al*., 2003). Because *C. yamato* does not fly and migrates contiguously, the most likely inference is past fragmentation following dispersal over the presently segregated areas. For the 2- and 1-step clades, restricted gene flow and allopatric fragmentation at local scales were often detected.

DISCUSSION

Mitochondrial introgression

Our results revealed that *Carabus yamato* possesses high diversity in mitochondrial ND5 sequences within and among populations. Such diversity among local populations has also been found in other species of the subtribe Carabina, including *Ohomopterus* (Sota *et al*., 2001; Garnier *et al*., 2004; Zhang *et al*., 2005). The diverse mitochondrial haplotypes in these carabid beetles may comprise those originating from intraspecific historical biogeography and introgression of heterospecific mitochondria through interspecific hybridization. Therefore, before considering the historical biogeography of *C. yamato*, we will discuss the introgression of heterospecific mitochondria.

The mitochondrial sequences from *C. yamato*, *C. albrechti*, and *C. kimurai* showed numerous trans-species polymorphisms, which can be caused by introgressive hybridization and/or be ancestral polymorphisms. Discrimi-

nating the causes of trans-species polymorphisms is not always easy. However, considering both the phylogeny and the geographic distribution of haplotypes, these two processes can be distinguished, unless an introgression event happened a very long time ago (Masta *et al*., 2002; Sota, 2002; Morando *et al*., 2004). Mitochondrial introgression due to interspecific hybridization can be inferred when unique haplotypes or lineages shared by parapatric species occur around a shared range boundary, but not in areas away from the boundary. In contrast, incomplete lineage sorting of ancestral polymorphisms since speciation will not result in such a biased distribution of shared lineages, which should show deep coalescence with other species-specific lineages (Gomez-Zurita and Vogler, 2006).

Among the haplotypes of *C. yamato*, haplotypes in clade 5-2 are shared with *C. albrechti*, which possesses haplotypes of clade 5-2 widely in its range from central to northern Honshu and Hokkaido (Figs. 1A, 2). In *C. yamato*, clade 5-2 occurred only in the northeastern area adjacent to the western margin of the range of *C. albrechti* (Fig. 4). This distribution pattern of clade 5-2 suggests that introgression of mitochondria from *C. albrechti* to *C. yamato* occurred in the northeast around the Kurobe River, which is a boundary between these species (Takami and Ishikawa, 1997; Imura and Mizusawa, 2002). In addition, there may have been another introgression event south of the Kurobe River around Agematsu (Y1), because one haplotype (340) was shared with *C. albrechti* in Fujimi (A7), which is close to Agematsu.

Mitochondrial introgression in the reverse direction (from *C. yamato* to *C. albrechti*) does not appear to have occurred, because clades 4-1 to 4-3 contained no haplotypes from *C. albrechti*. Instead, a *C. yamato* haplotype may have introgressed into *C. kimurai* (one haplotype in clade 2-4). No hybrid swarm has been found for these three species; hence, introgressive hybridizations seem to have been rare events during occasional secondary contacts. Directional introgression of mitochondria has often been found in *Ohomopterus* and may be attributable to morphological factors (genital morphology and body size) that determine the feasibility of interspecific mating (Sota *et al*., 2001; Sota, 2002; Takami and Suzuki, 2005; Ujiie *et al*., 2005).

Because the *C. yamato* haplotypes of clade 5-2 are diverse and consist both of those that are identical to *C. albrechti* haplotypes as well as well-differentiated haplotypes, there would have been repeated introgressive hybridizations following the differentiation of *C. yamato*. The age of clade 5-2 was dated to 0.96 Mya (node D; Fig. 2). Thus, repeated introgressive hybridizations would have originated from repeated secondary contacts after the latest Early Pleistocene. We did not analyze the phylogeography of *C. albrechti* because of insufficient samples. Future analysis of *C. albrechti* may clarify these repeated mitochondrial introgressions from *C. albrechti* to *C. yamato.*

Phylogeography of *C. yamato*

Of the two major clades of ND5 haplotypes, clade 5-1 likely consisted of original *C. yamato* haplotypes, whereas clade 5-2 was likely a lineage of introgressed haplotypes, as discussed above. Many clade 5-1 haplotypes were located at the tips of the network, whereas many missing haplotypes occurred in the interior of the network (Fig. 3). In 4-step clades, two major clades, 4-1 and 4-3, occurred mainly in the eastern (around the Tenryu River) and western regions, which are segregated by Ise Bay and the Nobi and Okazaki plains. *Carabus yamato* inhabits mountains below the subalpine zone, but does not colonize floodplains. Therefore, the Nobi and Okazaki plains may have separated the eastern and western populations by providing unfavorable habitat. Our age estimation suggests that the present mitochondrial haplotypes of *C. yamato* started to diverge in the late Early Pleistocene (1.12 Mya); hence, differentiation of clades 4-1 and 4-3 may have been caused by transgression during the inter-glacial period, during which Ise Bay probably extended northward and provided a strong barrier to beetle dispersal.

The western- and northernmost populations possessed only clade 3-1 and clade 3-5, respectively, and the populations in the region between the western and northern regions possessed both of these clades (Fig. 4). The interior clades of clades 3-1 and 3-5 (clades 2-2 and 2-5, respectively) were distributed only in adjacent Daian (Y3) and Yoro (Y37; region A in Fig. 4). This suggests that clades 3-1 and 3-5 expanded from region A northward and westward. Demographic expansion, which likely followed the westward range expansion, was inferred from population-genetic parameters for some western populations (Table 3).

The Kameyama (Y12) and Suzuka (Y29) populations had very low genetic diversity (Tables 1 and 3), although they were located close to the highly diverse Daian population (Y3). These populations possessed only two haplotypes of clade 1-1, which was a derived (tip) clade within clade 2-1. Clade 2-1 was distributed mainly in the western region, and the clade 1-1 haplotypes may have originated from migrants from the west. These two populations may have been affected by a bottleneck or genetic drift, although we could not resolve their history (see Table 3).

The haplotype compositions of the southern populations were diverse and unique. In the southwest, the Kumano (Y18) and Nabari (Y23) populations (region B in Fig. 4) possessed haplotypes in the same 3-step clades (3-2 and 3-7), which were not found in other western populations. In the southeast, populations around the Tenryu River possessed haplotypes in all 4-step clades and interior (ancestral) 3-step clades (region C in Fig. 4). Populations in these regions may have persisted over a long period. It has been suggested that temperate forest refugia occurred near the Pacific coast during glacial periods, as revealed by the palynology (Kamei, 1981; Tsukada, 1982; Hattori *et al*., 1987) and phylogeography of evergreen trees (Aoki *et al*., 2004). Because *C. yamato* inhabits temperate forests, populations of *C. yamato* were likely restricted to low mountains along the Pacific coast during glacial maxima, and locations higher in altitude may have been near the refugia during the glacial periods.

In summary, we found that *C. yamato* populations diverged after the late Early Pleistocene and underwent changes in their ranges, including fragmentation by Ise Bay and the Nobi and Okazaki plains and the formation of refugia in the southern part of the current range, followed by range expansion. In addition, directional introgression of mitochondria between parapatric species occurred from *C.*

albrechti to *C. yamato* and from *C. yamato* to *C. kimurai*.

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