Molecular Ecology (2001) **10**, 2833–2847

Extensive trans-species mitochondrial polymorphisms in the carabid beetles *Carabus* subgenus *Ohomopterus* caused by repeated introgressive hybridization

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Abstract

To study the potential importance of introgressive hybridization to the evolutionary diversification of a carabid beetle lineage, we studied intraspecific and trans-species polymorphisms in the mitochondrial NADH dehydrogenase subunit 5 (*ND5*) gene sequence (1083 bp) in four species of the subgenus *Ohomopterus* (genus *Carabus*) in central and eastern Honshu, Japan. Of the four species, *C. insulicola* is parapatric with the other three, and can hybridize naturally with at least two. This species possesses two haplotypes of remote lineages. We classified *ND5* haplotypes using polymerase chain reaction–restriction fragment length polymorphism with *Taq*I endonuclease for 524 specimens, and sequenced 143 samples. Analysis revealed that each species was polyphyletic in its mitochondrial DNA phylogeny, representing a marked case of trans-species polymorphism. Recent one-way introgression of mitochondria from *C. arrowianus nakamurai* to *C. insulicola*, and from *C. insulicola* to *C. esakii*, was inferred from the frequency of identical sequences between these species and from direct evidence of hybridization in their contact zones. Other intraspecific polymorphisms in the four species may be due to undetected introgressive hybridization (e.g. *C. insulicola* to *C. maiyasanus*) or from stochastic lineage sorting of ancestral polymorphisms. This beetle group has a genital lock-and-key system, with species-specific or subspecies-specific genital morphology that may act as a barrier to hybridization. However, our results demonstrate that introgressive hybridization has occurred multiple times, at least for mitochondria, despite differences among, and stability within, morphological characters that distinguish local populations. Thus, hybridization and introgression could have been key processes in the evolutionary diversification of *Ohomopterus*.

Keywords: *Carabus*, hybridization, introgression, mitochondria, phylogeny, polymorphism

Received 3 May 2001; revision received 15 August 2001; accepted 15 August 2001

Introduction

The role of hybridization and introgression in the evolutionary diversification of animals remains unclear, unlike that of plants (Dowling & Secor 1997). In diploid, sexually reproducing animal taxa, hybridization and subsequent selection may produce novel taxa that are distinguishable from their progenitors (Arnold 1997; Dowling & Secor 1997), whereas introgression of heterospecific genes may have only minor or no visible effect on the introgressant phenotype. A common consequence of interspecific hybridization in animals may be the introgression of mitochondria. Like chloroplast DNA, cytoplasmic DNA may be more readily introgressed than nuclear DNA by interspecific hybridization (Rieseberg & Soltis 1991; Avise 1994). Although mitochondrial DNA (mtDNA) has been considered a useful marker in the study of population genetics and phylogeny, introgression of mtDNA usually causes significant incongruence between mitochondrial gene genealogy and phylogeny based on morphological markers and nuclear DNA markers.

Theoretically, incongruence between gene genealogy and species phylogeny can result from a variety of processes, such as homoplasy, stochastic lineage sorting of ancestral

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polymorphisms, or introgressive hybridization (Brower *et al*. 1996; Avise 2000). However, introgression of mitochondria can be distinguished from other processes by detailed analysis of the incongruence pattern, and/or circumstantial evidence for the occurrence of hybridization (but note the difficulty in discriminating hybridization from lineage sorting when comparing gene tree topology; e.g. Sang & Zhong 2000). Detecting introgressive hybridization events among closely related species by using mtDNA plus nuclear markers may provide useful information about the evolutionary history of the group (Avise 1994, 2000; Arnold 1997; Dowling & Secor 1997).

Although hybridization and introgression may have been common in the history of species-rich animal groups, empirical evidence has mostly been limited to cases between two species (see e.g. Arnold 1997; Dowling & Secor 1997). If multiple events of introgressive hybridization of mitochondria occurred in a group of species, a complex pattern of trans-species polymorphism (sharing of allelic lineages across species; Klein *et al*. 1998) might emerge. Here we tested for extensive trans-species polymorphisms in flightless carabid beetles of the Japanese subgenus *Ohomopterus* (genus *Carabus*, Coleoptera). This beetle group consists of 15 species and > 50 subspecies, the result of a marked radiation of genital morphology and body size, with sympatric assemblages of two to five species in different locales throughout the Japanese archipelago (Kubota & Sota 1998; Sota *et al*. 2000b). Parapatric taxa with differentiated genital characters sometimes form hybrid zones, or produce occasional natural hybrids without recognizable hybrid zones (Kubota & Sota 1998). In this subgenus, the genital lock-and-key system can discourage hybridization (when the interspecific difference is large), effectively maintain the parapatric condition, and perhaps reinforce divergence of reproductive characters (Sota & Kubota 1998). Where the interspecific difference is small, hybridization has formed a hybrid swarm between the ranges of parental species (Sota *et al*. 2000a). Thus, hybridization may have played a role in the diversification of *Ohomopterus* (Kubota & Sota 1998). The potential importance of hybridization in the evolution of carabids has been reported for related groups such as *Leptocarabus* and *Chrysocarabus* (Mossakowski & Weber 1976; Mossakowski *et al*. 1986, 1990; Kubota 1991).

The potential importance of hybridization in *Ohomopterus* has also been suggested by molecular phylogenetic analysis. Su *et al*. (1996b) found that morphologically discernible *Ohomopterus* species and their mitochondrial lineages were significantly contradictory. In the mtDNA gene tree, one morphological species is often separated into two distant clades, whereas species from the same region tend to form a single clade. This observation led Su *et al*. (1996b) to hypothesize that several morphological types in different regions evolved in parallel. However, Sota & Vogler (2001) analysed the gene genealogies of two mitochondrial and three nuclear DNA loci, and found that the nuclear gene lineages generally conformed to traditional morphological species boundaries, despite the significant mtDNA–morphology incongruence. They suggested that introgression of mitochondria via hybridization and subsequent backcrossing was the principal cause of the incongruence.

Because assessment of the mitochondrial diversity in *Ohomopterus* to date was limited to a small number of samples, we undertook an extensive survey of mitochondrial diversity, to document both intraspecific and trans-species polymorphisms. In this study, we focused on *Carabus insulicola*, which possesses two distinct mitochondrial haplotypes, and studied variation in mitochondrial type for *C. insulicola* and three related species that are in contact with *C. insulicola* in central and eastern Honshu, Japan. We demonstrated the presence of extensive intraspecific polymorphisms and trans-species polymorphisms, and suggest that past and present interaction and occasional introgressive hybridization could have played an important role in the evolution of *Ohomopterus*.

Materials and methods

Study organisms

The four species of the subgenus *Ohomopterus* studied belong to the *insulicola* species group (*sensu* Ishikawa 1985, 1991): *Carabus* (*Ohomopterus*) *insulicola* Chaudoir, *C.* (*O.*) *esakii* Ciski, *C.* (*O.*) *arrowianus* (Breuning) and *C.* (*O.*) *maiyasanus* Bates. These species exhibit more or less similar external morphology; the key diagnostic characters for classification are those of the genitalia, especially the shape of the copulatory piece. The copulatory piece is an apophysis on the endophallus of the male genitalia, which is inserted into the vaginal appendix, a pocket attached to the vaginal apophysis of the female genitalia, to lock the male and female genitalia together (Ishikawa 1987). The size and shape of the vaginal appendix correspond to those of the copulatory piece. The *insulicola* group is characterized by various types of elongated hook-like copulatory pieces (Fig. 1; see also Figs 2 and 3 for their distribution ranges).

Among the four species studied, *C. insulicola* is known to naturally hybridize with both *C. arrowianus* and *C. esakii* (Ishikawa 1991; Kubota & Sota 1998; Sota *et al*. 2000a). In the field, hybrids can be identified as those individuals at interspecific contact zones that possess intermediate characters, primarily of male genitalia (Kubota 1988; Kubota & Sota 1998). In addition, hybrid specimens obtained in the laboratory are available for reference (Kubota & Sota 1998; Sota *et al*. 2000a; Kubota, unpublished data). Hybrid swarms have been found between *C. insulicola* and *C. arrowianus* contact zones in Chubu district, central Honshu. One of these hybrid swarms was termed *C. insulicola* ssp. *pseudinsulicola*. *C. insulicola* and *C. esakii* have a wide (≈ 100 km) and

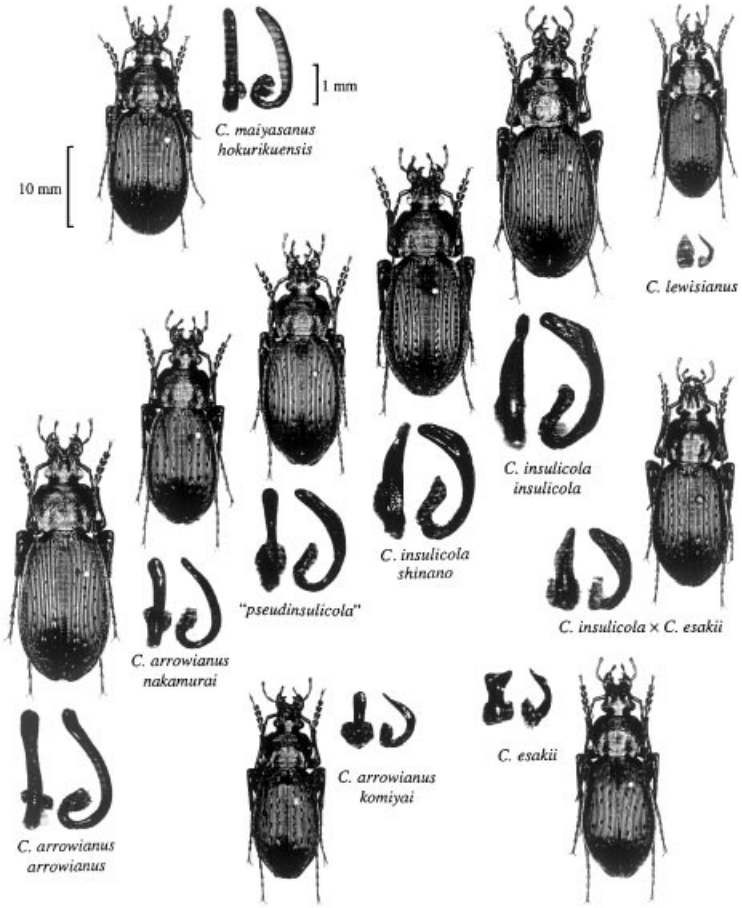

Fig. 1 Male beetles and copulatory pieces (a genital part playing a role of key in the lock-and-key system) of the *insulicola* species group of *Carabus* (*Ohomopterus*) used in this study. All beetles are field-collected, including a hybrid swarm '*pseudinsulicola*' between *C. insulicola* and *C. arrowianus nakamurai*, and probable F_1 between *C. insulicola* and *C. esakii*. One of the *albrechti* species group, *C. lewisianus*, is also shown; this group possesses triangle copulatory piece and is often sympatric with the *insulicola* species group. Each copulatory piece was cut from the endophallus; dorsal view (left) and lateral view (right) are shown; apex is upper with the basal plate at the bottom.

discontinuous contact zone, and putative hybrids have been collected occasionally at two localities in Yamanashi Prefecture (Mt. Ashitaka, Shizuoka and Nenba, Yamanashi; SZ* and YN5 in Fig. 3). Field-collected probable hybrids have copulatory pieces (see Fig. 1) similar in shape to those of F_1 males bred in the laboratory (K. Kubota, personal communication).

In this analysis, we distinguished seven taxa: *C. esakii*, *C. insulicola*, '*pseudinsulicola*' (the hybrid swarm between *C. insulicola* and *C. arrowianus* previously called *C. insulicola pseudinsulicola*), *C. arrowianus arrowianus*, *C. arrowianus nakamurai*, *C. arrowianus komiyai* and *C. maiyasanus hokurikuensis*. Recently, Ishikawa & Ujiie (2000) recognized nine *C. insulicola* subspecies based on morphological analysis; here, we also used their major subspecies groupings: *insulicola*, *shinano*, and the transient populations between these two groups.

Sampling and analysis of mitochondrial DNA

Beetles collected in the field for DNA analysis were preserved in 99% ethanol (see Appendix I and Figs 2 and 3 for localities and sample sizes). Thoracic muscles (and occasionally testes or leg muscles) were digested with protein kinase, and total DNA was extracted using the phenol–chloroform method. The polymerase chain reaction (PCR) was used to amplify a 1083-bp sequence of the mitochondrial NADH dehydrogenase gene (*ND5*) from a total DNA template using the primers 5′-CCTGTTTCTGCTTTAGTTCA-3′ and 5′-GTCATACTCTAAATATAAGCTA-3′ (Su *et al*. 1996a).

After scanning previous ND5 sequence data (Su *et al*. 1996b; Sota & Vogler 2001) for a restriction enzyme that would help distinguish two major lineages of the subgenus *Ohomopterus* containing two very different *C. insulicola* haplotypes, we chose *Taq*I. PCR products of the ND5 region were digested with *Taq*I, and fragment lengths were determined electrophoretically in a 1.5% agarose gel. The exact position of restriction sites was determined by sequencing selected specimens. We used analysis of molecular variance (AMOVA) as provided in ARLEQUIN Version 2000 (Schneider *et al*. 2000) to determine the haplotype distribution within polymorphic taxa.

To determine phylogenetic relationships among mitochondrial haplotypes, we sequenced the PCR-amplified *ND5* region for some of the specimens of different haplotypes from different localities (Table 1). We purified PCR products using a QIAquick PCR Purification Kit (Qiagen),

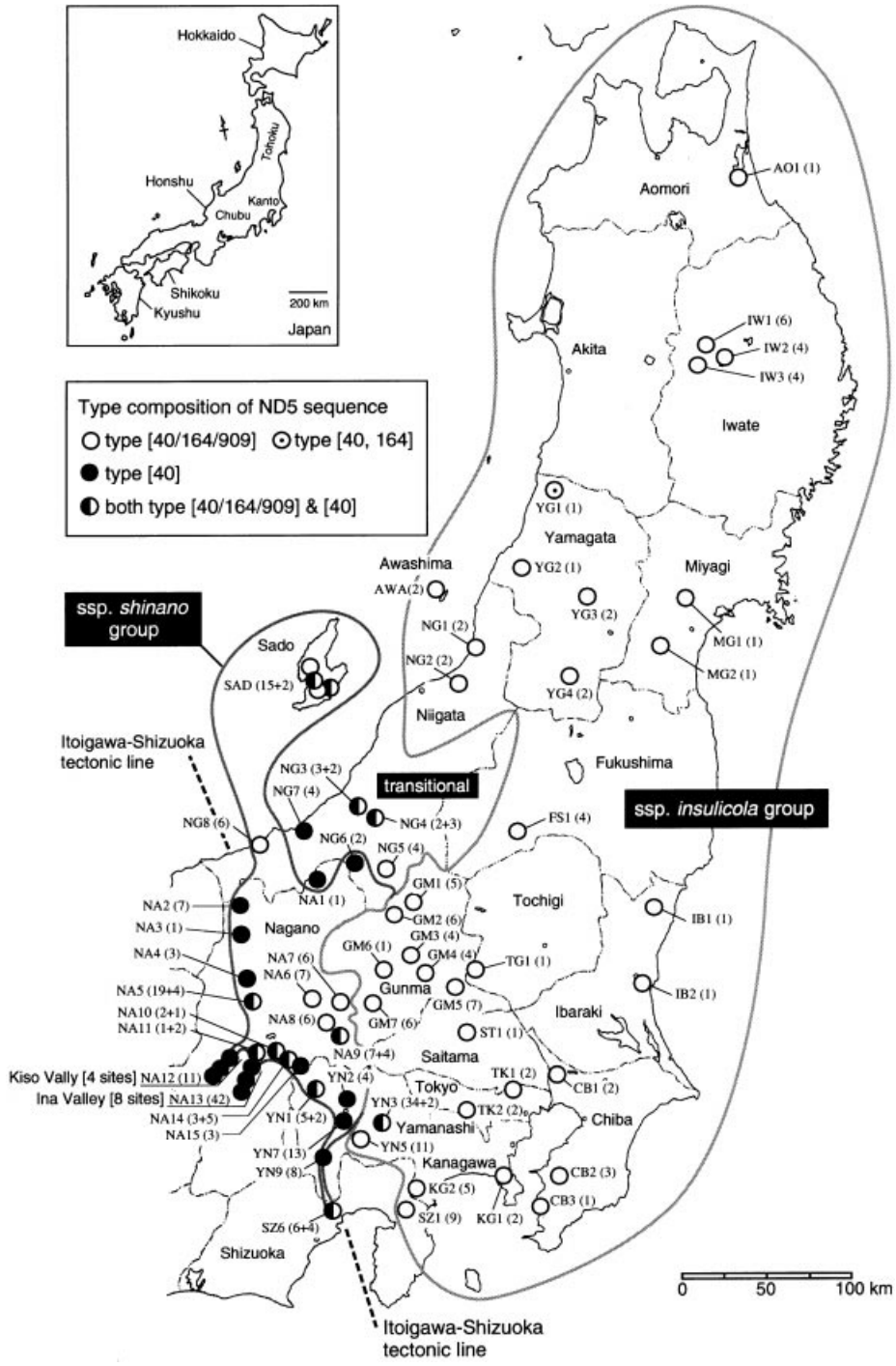

Fig. 2 Distribution of mitochondrial haplotypes detected by restriction site of *Taq*I endonuclease on ND5 region in *Carabus insulicola*. Sample sizes are shown in parentheses after the locality codes (see also Appendix I); for polymorphic localities, the first and second numbers are sample sizes for type [40/164/909] and [40], respectively.

and sequenced them using an ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer). Automated sequencing was performed with ABI 377 and ABI 373 sequencers (Perkin-Elmer). (The sequences are available from GenBank; Accession nos AF219429–AF219473 and AF227325–AF227417. The compiled sequence data, Nexus format, is available from the senior author.)

Alignment was simple and required no gaps. However, we excluded the first 30 bp and the last 33 bp of the 1083-bp sequence, because of sequence ambiguity in some

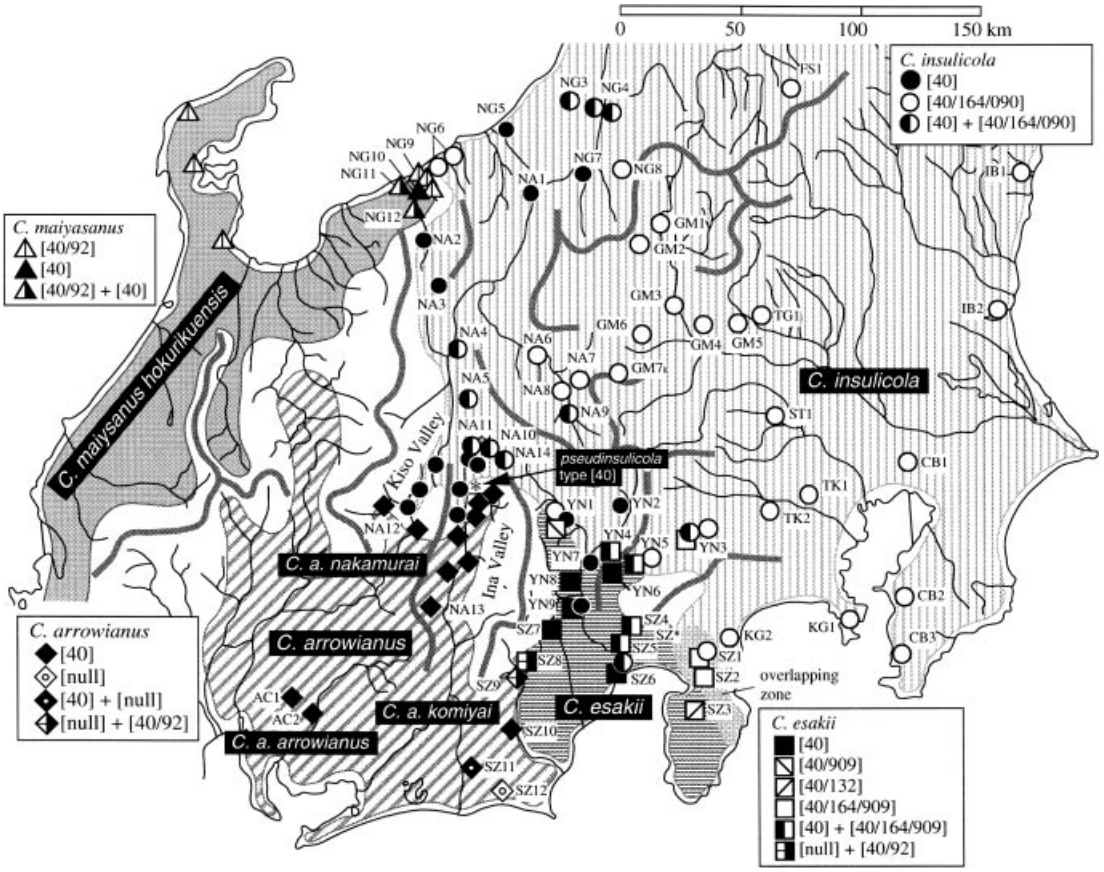

Fig. 3 Distribution of mitochondrial haplotypes detected by restriction site of *Taq*I endonuclease on *ND5* region in the *insulicola* species group in central Honshu. For *Carabus maiyasanus*, three samples in Su *et al*. (1996b) with type [40/92] from western localities are also shown.

Table 1 Distribution of haplotypes by taxa

		Haplotype by *Taq*I restriction sites						
Taxon	n_1 (n_2)	[null]	[40]	[40/92]	[40/132]	[40/909]	[40/164]	[40/164/909]
Carabus esakii	68 (19)	1 (1)	20 (8)	1 (1)	8 (1)	8 (2)	—	30 (6)
C. insulicola	362 (81)	—	130 (28)	—	—	—	1 (1)	231 (52)
pseudinsulicola	26 (10)	—	26 (10)	—	—	—	—	—
C. a. nakamurai	28 (17)	—	28 (17)	—	—	—	—	—
C. a. arrowianus	5 (3)	—	5 (3)	—	—	—	—	—
C. a. komiyai	16 (6)	7 (4)	7 (2)	2 (1)	—	—	—	—
C. m. hokurikuensis	19 (7)	—	7 (4)	—	—	12 (3)	—	—

n_1, number of PCR–RFLP samples; n_2, number of sequenced samples.

specimens. Data for the resulting 1020-bp *ND5* gene sequence were analysed with PAUP* Version 4.0b (Swofford 1999). We used the maximum parsimony method and the neighbour-joining (NJ) method for phylogenetic reconstruction. In the maximum parsimony analysis, an heuristic search of 100 random additions with tree-bisection-reconnection (TBR) branch-swapping was performed with MULPAR and steepest descent options. In tree reconstruction using the NJ method, the Kimura 2-parameter method (Kimura 1980) was used for distance correction. Node supports were provided by 1000 bootstrap replications. To examine relationships among closely related haplotypes, we constructed minimum-spanning trees based on the observed number of nucleotide differences using ARLEQUIN Version 2000 (Schneider *et al*. 2000). We used the Mantel test as provided in the R-package (Casgrain & Legendre 2000) to

determine the correlation between pairwise sequence divergence and geographical distance within *C. insulicola* clades.

Results

Mitochondrial types detected with PCR–RFLP

We detected seven *Taq*I restriction site haplotypes on the *ND5* 1083-bp fragment (Table 1; Appendix I). Types are described by the number of base pairs in between the cutting site and the 5′-terminal end of the 1083-bp PCR-amplified fragment. *Carabus insulicola* had three types: [40], [40/164] and [40/164/909]. *C. esakii* had six types: [null], [40], [40/92], [40/132], [40/909] and [40/164/909]. *C. arrowianus komiyai* had three types: [null], [40] and [40/92]. *C. arrowianus arrowianus* and *C. a. nakamurai* had only type [40], as did the hybrid swarm *pseudinsulicola* in the Ina Valley. *C. mayiasanus* had two types: [40] and [40/909]. The natural hybrid of *C. insulicola* and *C. esakii* had one type [40/164/909] (Appendix I).

The degree of population differentiation in the four taxa that exhibited polymorphism in the *Taq*I restriction site (*C. esakii*, *C. insulicola*, *C. a. komiyai*, *C. m. hokurikuensis*) was analysed with AMOVA using haplotype frequency data (Table 2). For each taxon except *C. a. komiyai*, there was large variance among populations, and significant and large fixation index (F_{ST}) values indicated differentiation among regional populations. In *C. insulicola*, type [40/164/909] was widely distributed, except along the western margin of the species range, where specimens had only type [40] or both type [40] and [40/164/909] (Figs 2 and 3). In the Ina and Kiso Valleys, where *C. insulicola* is in contact with *C. arrowianus*, *C. insulicola* populations had type [40] only, as did *C. a. nakamurai* and *pseudinsulicola*. Polymorphic populations of *C. insulicola* were found along the Itoigawa–Shizuoka tectonic line. In its eastern range, *C. esakii* populations were either monomorphic of type [40/164/909] (shared with *C. insulicola*) or polymorphic of types [40] and [40/164/909]. In the west, *C. esakii* shared two types, [null] and [40/92], with *C. a. komiyai*. Some *C. maiyasanus* populations were polymorphic of types [40] and [40/92]. In these polymorphic taxa, haplotypes shared with other species tended to occur at the boundary zones between species.

Gene genealogy and sequence divergence

Phylogenetic analysis of the *ND5* sequences was performed with 35 previously published sequences (Sota & Vogler 2001), in addition to the 143 sequences obtained in this study. The NJ tree with midpoint rooting (Fig. 4) revealed two major clades. Maximum parsimony (MP) analysis resulted in > 12 500 shortest trees (447 steps) (CI excluding uninformative sites = 0.45; RI = 0.91). Topology of the consensus MP tree was largely congruent with that of the NJ tree, and nodes appearing in both trees had similar bootstrap values. The distribution of *Taq*I haplotypes on the gene tree showed one major clade consisting mostly of type [40] and the other mostly of type [40/164/909]. Of the less frequent haplotypes, [null], [40/92], [40/32] and [40/909] were grouped in the type [40] major clade, whereas type [164/909] was found in the type [40/164/909] major clade. Each species was not monophyletic in the *ND5* genealogy. *C. insulicola* and *C. esakii* were split between the two major clades, and *C. esakii* was further split into a few clades within the type [40] major clade. In contrast, all *C. arrowianus* and *C. maiyasanus* sequences were grouped among a few clades within the type [40] major clade.

The two major clades of *ND5* sequence lineages in *C. insulicola*, *Taq*I types [40/164/909] and [40], are termed the east *insulicola* clade and west *insulicola* clade, respectively (Fig. 4). The east *insulicola* clade forms a unique branch of type [40/164/909] sequences, separated from other *Ohomopterus* sequences by > 1.3% (the closest is *C. lewisianus*, sample 61). Note that the type [40/164/909] major clade includes no *insulicola* species group sequences other than those of the east *insulicola* clade. In contrast, the major type [40] clade comprises mainly the *insulicola* species group, and clades involving *C. maiyasanus*, *C. esakii* and *C. a. komiyai* are basal to the west *insulicola* clade.

Sequence divergence within each of the two *insulicola* clades was examined using minimum spanning trees (MSTs) (Fig. 5), which revealed different patterns of sequence divergence. The east *insulicola* clade consisted of *C. insulicola*, *C. esakii* and their hybrid. Among *C. insulicola*

Table 2 Analysis of molecular variance for population differentiation in four taxa that show polymorphism of *Taq*I restriction site on *ND5* sequence

	Among populations		Within populations		
Taxon	d.f.	Variance (%)	d.f.	Variance (%)	F_{ST}
Carabus esakii	11	0.482 (69.8)	53	0.208 (30.2)	0.698***
C. insulicola	46	0.381 (75.0)	296	0.127 (25.0)	0.750***
C. a. komiyai	2	0.141 (32.3)	12	0.296 (67.7)	0.323 NS
C. m. hokurikuensis	3	0.268 (80.1)	15	0.067 (19.9)	0.801***

Significance of population differentiation: NS, $P > 0.05$; ***$P < 0.000001$.

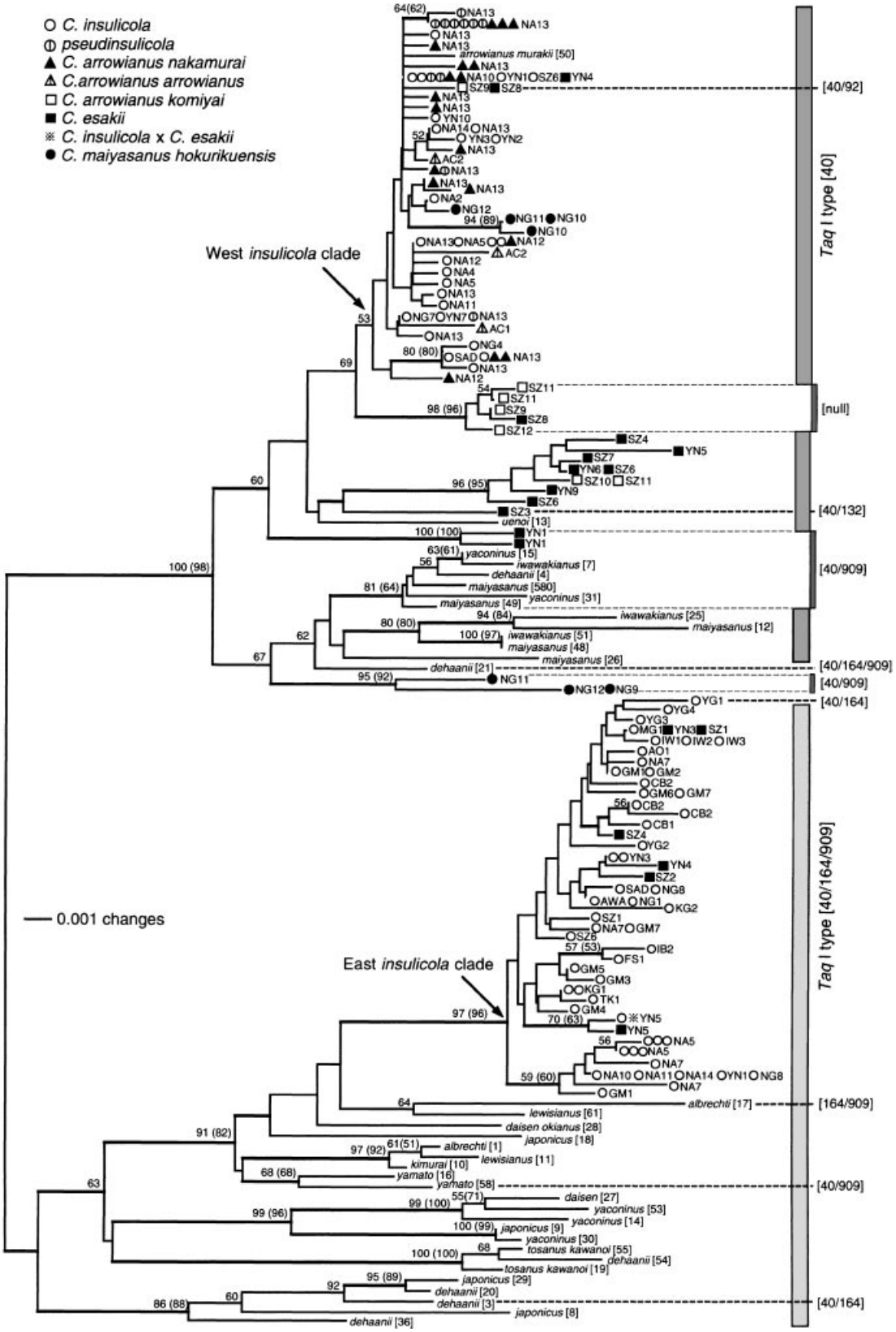

Fig. 4 Phylogram of *ND5* sequences obtained by the neighbour-joining method. Each symbol reveals one sequence and its location is indicated by locality code in Figs 2 and 3. Bootstrap per cents for the supports of nodes are given above the branch (only for > 50% support), with that by maximum parsimony analysis (in parentheses) when the node is also appeared and supported by > 50% in the MP analysis.

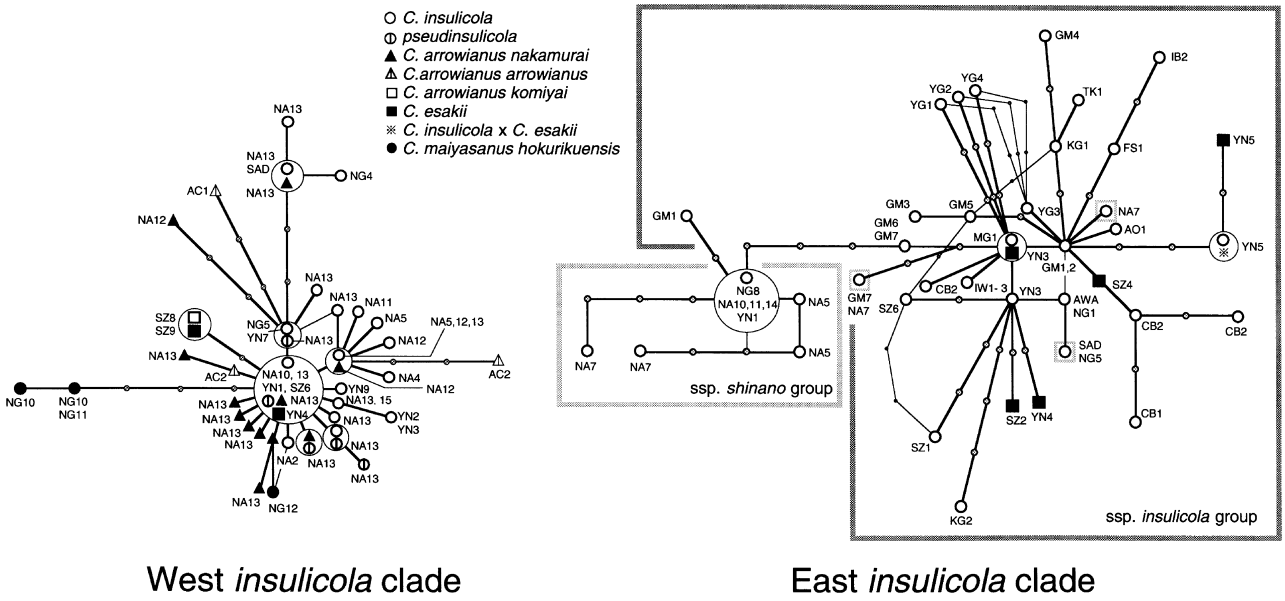

Fig. 5 Minimum spanning trees for the east and west *insulicola* clade sequences. Different sequences are connected by one-step branch. Symbols represent taxa and the localities are indicated besides. Small circles represent sequences not appeared in the sample. Some circles shared in multiple localities and taxa are enlarged. Thin lines represent alternative connections between sequences. Note that all the sequences of *Carabus insulicola* in the west *insulicola* clade are from the ssp. *shinano* group except the sequences from YN3 and SZ6 (ssp. *insulicola* group).

in the east clade, the sequences of the ssp. *shinano* group in the western range of this species were segregated from those of the ssp. *insulicola* group in the east to north range, although a few haplotypes from peripheral localities (NA7, SAD) were identical or similar to the ssp. *insulicola* sequences. Sequence divergence of *C. insulicola* within the east *insulicola* clade was < 1.3%, and there was no significant correlation between pairwise geographical distance and sequence difference (Mantel test, $P > 0.05$; Fig. 6) despite the wide range (650 km NE to SW) of *C. insulicola* with this mitochondrial type (Fig. 2). The sequences of *C. esakii* in the east *insulicola* clade were identical or similar to those of *C. insulicola* from adjacent localities. The sequence of a *C. insulicola*–*C. esakii* hybrid at YN5 was identical to that of *C. insulicola* from an adjacent site at YN5, and very similar to a *C. esakii* sequence from YN5.

The pairwise sequence divergence among *C. insulicola* of the west *insulicola* clade was < 0.7% (Fig. 6). Mitochondria of this clade have been distributed over 300 km longitudinally, from Shizuoka in the south to Sado Island in the north. Despite its low divergence value, the pairwise sequence divergence within this clade was significantly correlated with geographical distance (Mantel test, $P < 0.05$; Fig. 6). Some sequences of the west *C. insulicola* clade were shared among *C. insulicola*, *C. arrowianus nakamurai*, and a hybrid *pseudinsulicola* swarm. Also, one sequence was shared by *C. esakii* and *C. insulicola*.

Within other taxa, the sequence divergence was small for *C. a. nakamurai* (maximum 0.6%) and *C. a. arrowianus*

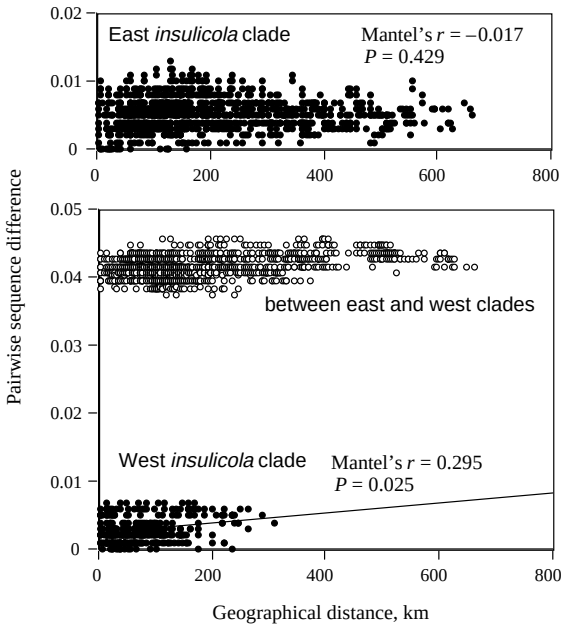

Fig. 6 Correlation between pairwise sequence difference (Kimura 2-parameter distance) and geographical distance within the east and west *insulicola* clade. Mantel test was applied to calculated the significance of the correlation by permutation.

(maximum 0.5%) (Table 3). *C. a. komiyai* exhibited divergent sequences (maximum 1.9%) that were distinct from those of *C. a. nakamurai* (except one from SZ9) (Fig. 4). However, all of the *C. a. komiyai* sequences were somewhat related

Table 3 Sequence divergence (Kimura 2-parameter distance) within and between taxa

Taxon	*esakii*	*insulicola*	*pseudins.*	*nakamurai.*	*arrowianus*	*komiyai*	*maiyasan.*
Carabus esakii	0.049	*0.000*	*0.000*	*0.000*	0.001	*0.000*	0.002
C. insulicola	0.050	0.046	*0.000*	*0.000*	0.001	0.002	0.001
pseudinsulicola	0.045	0.046	0.003	*0.000*	0.001	0.002	0.002
C. a. nakamurai	0.045	0.046	0.006	0.006	0.001	0.007	0.001
C. a. arrowianus	0.046	0.047	0.006	0.008	0.005	0.003	0.003
C. a. komiyai	0.045	0.046	0.016	0.016	0.018	0.019	0.004
C. m. hokurikuensis	0.048	0.050	0.024	0.024	0.026	0.025	0.024

Above diagonal: minimum divergence between taxa (sharing of identical sequence in italics). Below diagonal: maximum divergence between taxa. Diagonal elements: maximum divergence within taxa (underlined).

to those of *C. esakii*, which were highly diverse also (maximum divergence 2.5%). In *C. maiyasanus*, sequences from the same population showed a high divergence (1.7–2.2%); one was closely related to the west *insulicola* clade sequences.

The distribution of pairwise sequence divergence within *C. insulicola* was discretely bimodal (Fig. 7), owing to the possession of two distinct lineages of mitochondria. In other taxa, *pseudinsulicola* and *C. a. nakamurai* had unimodal distributions, whereas *C. esakii*, *C. a. komiyai* and *C. maiyasanus* showed bimodal or polymodal patterns, reflecting the possession of multiple mitochondrial lineages.

Discussion

We found complex trans-species polymorphisms as well as intraspecific, within-population polymorphisms in mitochondrial *ND5* sequences of the subgenus *Ohomopterus*. The geographical distribution pattern of shared mitochondrial haplotypes was related to the location of distribution ranges, the presence of contact zones, and the evidence of natural hybridizations. Sequences were frequently shared between parapatric species.

Stochastic lineage sorting of ancestral polymorphisms could cause trans-species mitochondrial polymorphism. This mechanism seems to be responsible for some aspects of the complex incongruence between mitochondrial genealogy and morphological species in *Ohomopterus* (Sota & Vogler 2001). However, the four study species may share mitochondrial lineages because of introgressive hybridization rather than stochastic lineage sorting, as the shared haplotypes are frequently identical in sequence and their distribution is localized to the boundaries between species ranges. Because mitochondrial haplotypes from any one ancestral lineage inherited by two species would have been diverging since the speciation event, the haplotypes from the two species would exhibit deeper coalescence than haplotypes shared following recent introgressive hybridization, unless the speciation event occurred very recently. In the case of stochastic lineage sorting, the distribution of two related haplotypes among others that are not shared is unlikely to be restricted to the boundary zones of two species, because the acquisition of common haplotypes is a prerequisite of population division that leads to a speciation event (except in the case of sympatric speciation).

Origin of mitochondrial polymorphism in Carabus insulicola

Carabus insulicola exhibits the most serious incongruence between mitochondrial genealogy and phylogeny based on morphology and nuclear genes. The two distinct *ND5* lineages of *C. insulicola* were first found by Su *et al*. (1996b), who classified specimens with west-*insulicola*-type mitochondria (lineage II in Su *et al*. 1996b) as *C. arrowianus*, despite the presence of distinct morphological markers. We demonstrated that the two distinct types of mitochondria coexist within local populations in central Honshu. There is no evidence that beetles with different mitochondria are isolated reproductively.

The eastern populations of *C. insulicola* possess *ND5* sequences of type [40/164/909] defined by the restriction sites of *Taq*I. This mitochondrial type is not directly related to those of other *insulicola* species groups. Because the genealogy of mitochondrial 16*S* rRNA and *COI* genes shows the same grouping for sequences from *C. insulicola* among *Ohomopterus* samples (Sota & Vogler 2001 and unpublished data), the observed pattern probably does not reflect homoplasy in the *ND5* gene. The east and west *insulicola* clades are the most deeply coalescent of the *ND5 Ohomopterus* phylogeny; the differentiation of the east *insulicola* clade sequences from those of type [40/164/909] is much shallower (Fig. 4). This topology indicates that stochastic sorting of type [40/164/909] only to *C. insulicola* or *C. esakii* but not to any other *insulicola* group (such as *C. arrowianus* and *C. maiyasanus*) is unlikely. The type [40/164/909] *C. insulicola* sequence is closest to haplotypes of the *albrechti* species group, which is sympatric with *C. insulicola* in eastern Honshu. It is possible that the original *C. insulicola* mitochondria (probably any of the major type [40] clade sequences) were replaced with *albrechti*

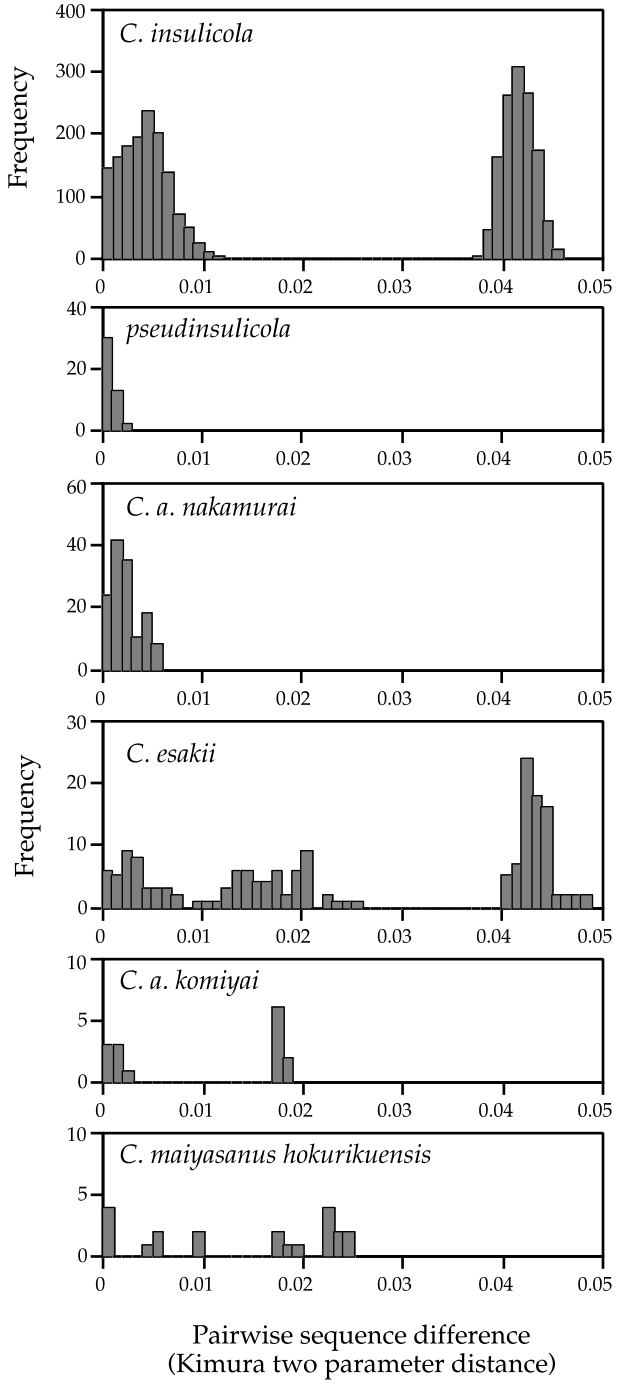

Fig. 7 The distribution of pairwise sequence difference (Kimura 2-parameter distance) in six taxa.

species group mitochondria via past hybridization (see Sota & Vogler 2001).

West *insulicola* mitochondria (type [40]) are confined to the western periphery of the *C. insulicola* distribution range, and are shared with *C. arrowianus nakamurai*. In the Ina and Kiso Valleys, the hybrid zone of these two species, only type [40] has been found; it is shared by the two species, as well as by the hybrid swarm *pseudinsulicola*. In *C. insulicola*, the divergence among type [40] sequences is small compared with that in the east *insulicola* clade. These results suggest that *C. insulicola* type [40] originated recently, probably as a result of introgressive hybridization from *C. a. nakamurai*.

Geographical differentiation of Carabus insulicola *and mitochondrial diversity*

Ishikawa & Ujiie (2000) analysed geographical variation in *C. insulicola* according to morphological characters, and recognized nine subspecies in two groups, the ssp. *shinano* group and the ssp. *insulicola* group. Because all the diagnostic characters of the ssp. *insulicola* group were thought to derive from those of the ssp. *shinano* group, which had no derived characters itself, the researchers assumed that the ssp. *insulicola* group had evolved directly from a population of the ssp. *shinano* group. Thus, they hypothesized that *C. insulicola* originated from an ancestral stock established in central Honshu (Chubu district), and subsequently differentiated into four subspecies of the ssp. *shinano* group. According to the researchers, *C. insulicola* then invaded the Kanto Plain and established an ancestral stock of the *insulicola* group, which differentiated during the Quaternary, when the Kanto plain experienced repeated transgression and regression. A large part of the present *insulicola* group range in north-east Honshu is occupied by a single subspecies (*kita*) that exhibits the most derived character state. It was suggested that this subspecies might have invaded the vast north-east Honshu area, probably after the last glacial period.

Our analysis of the *C. insulicola* mitochondrial lineage suggests that there are two major geographical divisions in this species. First, the east *insulicola* mitochondria clade consists of two subclades, one mostly from the ssp. *shinano* group and the other mostly from the ssp. *insulicola* group. A few of the haplotypes found in the ssp. *shinano* group are identical or similar to haplotypes of the ssp. *insulicola* group. These may have been transferred between the two groups by migration and introgressive hybridization, since such haplotypes from the ssp. *shinano* group occurred near the edge of the ssp. *insulicola* group range. The differentiation within the two east subclades is not obviously related to geographical division and the proposed subspecies. Second, the range of the west mitochondrial clade almost coincides with the proposed range of the ssp. *shinano* group. Because the mitochondria of the west clade are probably of recent origin, the range may reflect the range of dispersal of recently introgressed mitochondria, starting from the Ina and Kiso Valleys. This dispersal probably occurred longitudinally through basins along the Itoigawa–Shizuoka tectonic line (Fig. 2).

Mitochondrial polymorphism in other species

The *ND5* sequences of *C. esakii* are highly diverged. Two groups of sequences are unique to *C. esakii* (Fig. 4; localities SZ3 and YN1). However, some sequences are included in the east *insulicola* clade and are almost identical to *C. insulicola* sequences. *C. insulicola* and *C. esakii* are parapatric, and occasional hybridization seems to produce F_1 adults (Fig. 1). One hybrid individual collected at YN5 (Nenba) had a sequence identical to *C. insulicola* (type [40/164/909]) from an adjacent site (Kuwarubi, YN5). Other *C. esakii* at Nenba had type [40/164/909] or [40]. That the type [40/164/909] sequence is closest to those of *C. insulicola* and a natural hybrid (Figs 4 and 5) provides evidence that introgression of mitochondria occurs from *C. insulicola* to *C. esakii*. In addition, a mitochondrial type [40] sequence from YN4 is identical to the most dominant type in the west *insulicola* clade (Fig. 5). *Carabus esakii* from YN4 have both type [40] and type [40/164/909], which could have originated from introgressive hybridization with *C. insulicola*. Thus, mitochondria of both types seem to have introgressed from *C. insulicola* to *C. esakii*.

Both *C. arrowianus komiyai* and *C. esakii* have mitochondria of types [null], [40] and [40/92]. Although it is not known whether these two parapatric species can hybridize naturally, that they share mitochondrial types suggests that there has been occasional hybridization in the past. Type [null] forms a sister clade to the west *insulicola* clade, which includes all the *C. a. arrowianus* and *C. a. nakamurai* sequences. One type [null] mitochondria isolated from *C. esakii* at SZ8 may have been introgressed. However, most of the type [40] mitochondria from *C. esakii* form a clade that is different from the west *insulicola* clade. One *C. a. komiyai* haplotype in this clade may have been introgressed from *C. esakii*.

C. maiyasanus has a contact zone with *C. insulicola* at its eastern-most range (Ishikawa 1991). In the eastern periphery the two types of *C. maiyasanus* mitochondria differ greatly, so it is unlikely that they have diverged only within the *C. maiyasanus* lineage. Because type [40] is similar to that of *C. insulicola*, it is possible that it has been introgressed through past hybridization.

Mitochondrial genealogy and evolution of Ohomopterus

Ishikawa (1989) proposed that the *insulicola* species group possessed elongate, hook-like copulatory pieces, and assumed that *C. esakii* and *C. a. komiyai*, which possess relatively short copulatory pieces, are ancestral forms of this group. Later, Takami (2000) performed a cladistic analysis of *Ohomopterus* with 23 morphological characters and presented a phylogenetic hypothesis in which the *insulicola* species group is monophyletic, with *C. esakii* and *C. a. komiyai* at its base. He hypothesized that *C. iwawakianus* (the *yaconinus* species group with the pentagonal copulatory piece) was ancestral to the *insulicola* species group. This scenario is congruent with a phylogeny based on five nuclear DNA markers (Sota and Vogler, unpublished data), in which the *insulicola* species group containing *C. iwawakianus* is the most derived monophyletic group.

Despite considerable inconsistency between mtDNA- and morphology-based phylogenies, the mitochondrial genealogy provides some circumstantial evidence for part of the Takami (2000) morphological hypothesis. Although *C. esakii* is characterized by diverse mitochondrial haplotypes, the haplotypes specific to this species are basal to one of the two clades dominated by type [40] haplotypes. In the same clade, the *C. a. komiyai* type [null] occupies the next-basal position, ancestral to all haplotypes from *C. a. arrowianus* and *C. a. nakamurai*.

In contrast to the morphological hypothesis, the *C. maiyasanus* mitochondria (excluding those probably originating from *C. insulicola*) are similar to *C. iwawakianus* mitochondria and are not derived from *C. esakii* and *C. a. komiyai* mitochondria. Because *C. maiyasanus* and *C. iwawakianus* have hybrid zones in the Kinki district (Kubota 1988), their common mitochondrial lineage may have resulted from introgressive hybridization. Alternatively, *C. maiyasanus* may have evolved from *C. iwawakianus*, rather than from *C. esakii* and *C. a. komiyai*.

Our analysis suggests at least three unidirectional introgression events: from *C. insulicola* to *C. esakii*, *C. a. nakamurai* to *C. insulicola*, and *C. insulicola* to *C. maiyasanus*. These one-way transfers of mitochondria may be due to asymmetrical success of interspecific hybridization. Because *C. insulicola* and *C. esakii* vary greatly in body size and genital morphology (Fig. 1), the chance of interspecific genital coupling is limited. The large, elongated genitalia of the large *C. insulicola* prevent genital coupling with the small *C. esakii*, whereas the small male genitalia of *C. esakii* can be inserted into the large vagina of *C. insulicola*. Thus, F_1 offspring can be produced more easily between *C. insulicola* females and *C. esakii* males (K. Kubota, personal communication), explaining the one-way mitochondrial introgression from *C. insulicola* to *C. esakii*. In other species pairs, however, differences in body size and genital morphology are smaller (Fig. 1). When *C. insulicola* and *C. arrowianus nakamurai* were hybridized experimentally, F_1 and B_1 offspring were obtained from either parental combination of interspecific crossing, and no significant asymmetry in the possibility of introgression was detected (Sota *et al.* 2000a). Therefore, the one-sided introgression observed for these two species may have occurred by chance, assuming that effective introgressive hybridization has been very limited in the past.

Besides introgressive hybridization, the presence of ancestral polymorphism may also have contributed to the inconsistency between the mitochondrial gene tree and the species tree, although this does not easily account for the coexistence of very different mitochondrial lineages within the same populations. In the subgenus *Ohomopterus*, it is likely that distinct morphological clades based on genital characters emerged rapidly before mitochondrial lineages had diverged sufficiently, and that repeated hybridization events have formed intraspecific or intrapopulation polymorphisms, or both. This complex divergent-reticulate speciation pattern may have been facilitated by the topographical and geohistorical complexity of the Japanese Archipelago.

The effect of introgressive hybridization on phenotypic evolution remains a topic for future research. Intraspecific variation in *C. insulicola* body colour (upper body surface and legs) is possibly influenced by introgression (see also Kubota & Sota 1998). For example, *C. insulicola* with reddish tibiae (vs. the normal black) are found only near contact zones with *C. esakii* and *C. maiyasanus*, which usually possess reddish tibiae (R. Ishikawa and T. Sota, unpublished data).

Conclusions

The distribution of mitochondrial haplotypes in *Ohomopterus* represents one of the most extensive cases of polymorphism that involves contradictions between molecular and species phylogenies. The complex pattern of this subgenus could be the result of repeated introgressive hybridization events, combined with the presence of polymorphic mtDNA of ancestral taxa, which may have diverged geographically. Thus, *Ohomopterus* can be viewed as a complex of populations that are diverging locally but sometimes reticulate with each other. The extent of trans-species polymorphisms in these parapatric species, together with the evidence for natural hybridization at their contact zones, indicates that, at least within the *insulicola* species group, the morphological divergence in genital characters that is expected to reproductively isolate groups incompletely prevents gene flow at secondary contacts. Nevertheless, the morphological definitions of species or subspecies remain mostly intact, and nuclear DNA markers, unlike mitochondria, are mostly congruent with morphological species boundaries (Sota & Vogler 2001). These results indicate that the selective forces acting on phenotypes related to reproduction and survival are strong. To reveal the importance of hybridization in the diversification of *Ohomopterus*, the relative roles of internal (selection and drift) and external (introgression or reinforcement via hybridization) factors in phenotypic evolution need to be studied.

Acknowledgements

We are grateful to colleagues who kindly supplied specimens for the present study: K. Kubota, K. Kusakari, T. Miyagawa, Y. Nagahata, Y. Oka, Y. Takami and T. Tanabe; Takashi Okumura provided opportunity for examining a series of specimens from a hybrid zone. Supported by a Grants-in-Aid from the Ministry of Education, Science, Culture and Sports, Japan (Nos 09640748, 11304056).

References

Arnold ML (1997) *Natural Hybridization and Evolution*. Oxford University Press, New York.

Avise JC (1994) *Molecular Markers, Natural History and Evolution*. Chapman & Hall, New York.

Avise JC (2000) *Phylogeography. The History and Formation of Species*. Harvard University Press, Cambridge, MA.

Brower AVZ, DeSalle R, Vogler A (1996) Gene trees, species trees, and systematics: a cladistic perspective. *Annual Review of Ecology and Systematics*, **27**, 423–450.

Casgrain P, Legendre P (2000) *The R Package for Multivariate and Spatial Analysis, Version 4.0 (Development Release 3). User's Manual*. Université de Montréal, Montréal.

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.

Ishikawa R (1985) The subfamily Carabinae (Carabidae). In: *The Coleoptera of Japan in Color*, Vol. II (eds Ueno S, Kurosawa Y, Sato M), pp. 14–54. Hoikusha, Osaka.

Ishikawa R (1987) On the function of copulatory organs of *Ohomopterus* (Coleoptera, Carabidae, genus *Carabus*). *Kontyû, Tokyo*, **55**, 202–206.

Ishikawa R (1989) The Japanese Carabina: geographical distribution and speciation within an archipelago (Coleoptera, Carabidae). In: *Current Aspects of Biogeography in West Pacific and East Asian Regions. Nature and Culture, No. 1* (eds Ohba H, Hayami I, Mochizuki K), pp. 147–168. The University Museum, Tokyo.

Ishikawa R (1991) *The Evolution of Carabus*. Yasaka-shobo, Tokyo (in Japanese).

Ishikawa R, Ujiie M (2000) A revision of *Carabus* (*Ohomopterus*) *insulicola* Chaudoir, 1869 (Coleoptera, Carabidae) in Honshu, Japan. *Japanese Journal of Systematic Entomology*, **6**, 253–297.

Kimura M (1980) A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution*, **16**, 111–120.

Klein J, Sato A, Nagl S, O'hUgín C (1998) Molecular trans-species polymorphism. *Annual Review of Ecology and Systematics*, **29**, 1–21.

Kubota K (1988) Natural hybridization between *Carabus* (*Ohomopterus*) *maiyasanus* and *C.* (*C.*) *iwawakianus* (Coleoptera, Carabidae). *Kontyû, Tokyo*, **53**, 370–380.

Kubota K (1991) Natural hybridization between *Leptocarabus* (*L.*) *procerulus* and *L.* (*L.*) *kumagaii* (Coleoptera, Carabidae). *Japanese Journal of Entomology*, **59**, 323–329.

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.

Mossakowski D, Braun S, Roschen A (1990) Hybridization in natural populations of ground beetles (Coleoptera, Carabidae). *Canadian Journal of Zoology*, **68**, 1783–1789.

Mossakowski D, Roschen A, Vaje S (1986) Hybridization in *Chrysocarabus*. In: *Carabid Beetles: Their Adaptations and Dynamics* (eds Den Boer PJ, Luff ML, Mossakowski D, Weber F), pp. 281–295. Gustav Fischer-Verlag, Stuttgart.

Mossakowski D, Weber F (1976) Chromosomale und morphometrische divergenzen bei *Carabus lineatus* und *Carabus splendens* (Carabidae): I. Ein Vergleich sympatrischer und allopatrischer Populationen. *Zeitschrift Fur Zoologische Systematik und Evolutionsforschung*, **14**, 280–291.

Rieseberg LH, Soltis DE (1991) Phylogenetic consequences of cytoplasmic gene flow in plants. *Evolutionary Trends in Plants*, **5**, 65–84.

Sang T, Zhong Y (2000) Testing hybridization hypothesis based on incongruent gene trees. *Systematic Biology*, **49**, 422–434.

Schneider S, Roessli D, Excoffier L (2000) ARLEQUIN, *Version 2000 A Software for Population Genetics Data Analysis*. University of Geneva, Geneva.

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 *C. 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, Ujiie M, Ishikawa R (2000b) 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.

Su ZH, Ohama T, Okada TS, Nakamura K, Ishikawa R, Osawa S (1996a) Phylogenetic relationships and evolution of the Japanese Carabinae ground beetles based on mitochondrial *ND5* gene sequences. *Journal of Molecular Evolution*, **42**, 124–129.

Su ZH, Tominaga O, Ohama T *et al*. (1996b) Parallel evolution in radiation of *Ohomopterus* ground beetles inferred from mitochondrial *ND5* gene sequences. *Journal of Molecular Evolution*, **43**, 662–671.

Swofford DL (1999) PAUP*. *Phylogenetic Analysis using Parsimony (*And Other Methods), Version 4.0b*. Sinauer Associates, Sunderland, MA.

Takami Y (2000) Phylogeny of the subgenus *Ohomopterus* (Coleoptera, Carabidae, genus *Carabus*): a morphological aspect. *Tokyo Metropolitan University Bulletin of Natural History*, **4**, 1–32.

This work is part of our extended research in the evolutionary diversification and hybridization of the carabid subgenus *Ohomopterus* using morphological, ecological and molecular approaches. R. Ishikawa and M. Ujiie study morphological diversification in *Carabus*. T. Sota and A. P. Vogler study evolution of various of insects using mainly molecular phylogenetic approaches.

Appendix I Distribution of haplotypes of *Taq*I restriction sites on *ND5* gene region

Taxon Locality	No. of specimens for type:						
	[null]	[40]	[40/92]	[40/132]	[40/909]	[40/164]	[40/164/909]
Carabus insulicola							
Group of ssp. *insulicola*							
[AO1] Aomori	—	—	—	—	—	—	1
[IW1-3] Iwate	—	—	—	—	—	—	14
[MG1-2] Miyagi	—	—	—	—	—	—	2
[YG1-4] Yamagata	—	—	—	—	—	1	4
[FS1] Fukushima	—	—	—	—	—	—	4
[GM1-7] Gunma	—	—	—	—	—	—	33
[TG1] Tochigi	—	—	—	—	—	—	1
[IB1-2] Ibaraki	—	—	—	—	—	—	2
[ST1] Saitama	—	—	—	—	—	—	1
[CB1-3] Chiba	—	—	—	—	—	—	6
[TK1-2] Tokyo	—	—	—	—	—	—	4
[KG1-2] Kanagawa	—	—	—	—	—	—	7
[SZ1, 6] Shizuoka	—	4	—	—	—	—	15
[AWA] Awashima I.	—	—	—	—	—	—	2
[NG1] Nigata	—	—	—	—	—	—	2
[YN3, 5] Yamanashi	—	2	—	—	—	—	45
Transitional							
[NG2-5, 7] Niigata	—	9	—	—	—	—	11
Group of ssp. *shinano*							
[SAD, 4 sites] Sado I., Niigata	—	2	—	—	—	—	15
[NG6] Tsunan, Niigata	—	2	—	—	—	—	—
[NG8, 3 sites] Nou, Niigata	—	—	—	—	—	—	6
[NA1-4] North Nagano	—	12	—	—	—	—	—
[NA6-9] East Nagano	—	4	—	—	—	—	26
[NA5, 11–15] West and south Nagano	—	15	—	—	—	—	25
[NA12, 4 sites] Kiso Valley, Nagano	—	11	—	—	—	—	—
[NA13, 8 sites] Ina Valley, Nagano	—	41	—	—	—	—	—
[YN1-2, 7, 9] West Yamanashi	—	27	—	—	—	—	5
pseudinsulicola							
[NA13, 3 sites] Nagano	—	26	—	—	—	—	—
Carabus esakii							
[YN1] Nirasaki, Yamanashi	—	—	—	—	8	—	—
[YN 3] Ohtsuki, Yamanashi	—	—	—	—	—	—	1
[YN 4] Nakamichi, Yamanashi	—	1	—	—	—	—	5
[YN 5] Ashiwada, Yamanashi	—	4	—	—	—	—	3
[YN 6] Kamikuishiki, Yamanashi	—	3	—	—	—	—	—
[YN 9] Nakatomi, Yamanashi	—	1	—	—	—	—	—
[YN 10] Minobu, Yamanashi	—	2	—	—	—	—	—
[SZ1] Kannami, Shizuoka	—	—	—	—	—	—	6
[SZ2] Nirayama, Shizuoka	—	—	—	—	—	—	2
[SZ3] Shuzenji, Shizuoka	—	—	—	8	—	—	—
[SZ4] Ohbuchi, Fuji, Shizuoka	—	2	—	—	—	—	12
[SZ5] Iwamoto, Fuji, Shizuoka	—	3	—	—	—	—	1
[SZ6, 2 sites] Fujikawa, Shizuoka	—	3	—	—	—	—	—
[SZ7] Abe Pass, Shizuoka	—	1	—	—	—	—	—
[SZ8] Nakakawane, Shizuoka	1	—	1	—	—	—	—
esakii × *insulicola* [probable F_1 hybrid]							
[YN 5] Nenba, Ashiwada, Yamanashi	—	—	—	—	—	—	1
C. arrowianus arrowianus							
[AC1-2] Aichi	—	5	—	—	—	—	—
C. arrowianus nakamurai							
[NA12, 2 sites] Kiso Valley, Nagano	—	4	—	—	—	—	—
[NA13, 8 sites] Ina Valley, Nagano	—	24	—	—	—	—	—

Appendix I *Continued*

Taxon Locality	No. of specimens for type:						
	[null]	[40]	[40/92]	[40/132]	[40/909]	[40/164]	[40/164/909]
C. arrowianus komiyai							
[SZ9] Nakakawane, Shizuoka	1	—	2	—	—	—	—
[SZ10] Ichio, Kawane, Shizuoka	—	3	—	—	—	—	—
[SZ11, 2 sites] Tenryu, Shizuoka	5	4	—	—	—	—	—
[SZ12] Fukuroi, Shizuoka	1	—	—	—	—	—	—
C. maiyasanus hokurikuensis							
[NG9, 3 sites] Itoigawa, Niigata	—	—	—	—	10	—	—
[NG10, 2 sites] Itoigawa, Niigata	—	5	—	—	—	—	—
[NG11] Aono, Niigata	—	1	—	—	1	—	—
[NG12] Kotaki, Itoigawa, Niigata	—	1	—	—	1	—	—