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SPECIAL FEATURE: REVIEW

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Radiation and reticulation: extensive introgressive hybridization in the carabid beetles *Ohomopterus* inferred from mitochondrial gene genealogy

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Abstract In the evolutionary process of an animal lineage, interactions in secondary contacts of differentiated populations and introgressive hybridization may play an important role. In the Japanese islands, the carabid subgenus *Ohomopterus* (genus *Carabus*) exhibits a marked differentiation in body size and genital morphology. Although geographical differentiation is apparent, two or three species usually coexist at many localities. Their reproductive isolation relies on body size differences, chemical cues for mate recognition, and a species-specific genital lock-and-key system. However, these isolation mechanisms are not always effective enough to prevent interspecific hybridization. An initial assessment of the species-level phylogeny with mitochondrial gene sequences revealed that the gene genealogy is highly inconsistent with the morphology-based taxonomy. A comparison of mitochondrial and nuclear gene genealogies showed that these are strongly incongruent with each other, while the nuclear gene genealogy is more consistent with traditional taxonomy, indicating the repeated occurrence of introgression of mitochondria across species. Here, two different cases of mitochondrial introgression among *Ohomopterus* species are described in detail, one for parapatric species and the other for sympatric species. First, mitochondrial haplotypes and sequences were studied in *Carabus insulicola* and three taxa parapatric with *C. insulicola*, at least two of which hybridize with *C. insulicola* naturally. Among the four species studied, directional introgressions of mitochondria across boundary zones were detected. Second, in the Mt. Kongo area in central Honshu, which harbors five species, introgression of mitochondria among four out of the five species was detected, despite the apparent absence of on-going natural hybridization. These inferred cases of mitochondrial introgression indicate that species interactions through hybridization could have played an important role at various stages in the evolution of *Ohomopterus*.

Key words Hybridization · Introgression · Mitochondrial DNA · Molecular phylogeny

Introduction

Natural hybridization between species is not necessarily rare in an animal lineage, and could contribute to evolutionary diversity through the creation of novel lineages which are independent of their progenitors, or the reinforcement of phenotypic difference between populations which are subject to unfit hybridization (e.g., Harrison 1990, 1993; Arnold 1997; Dowling and Secor 1997). Gene flow or the introgression of genes across species may occur to a variable extent through hybridization. In some cases, the introgression of heterospecific genes may be absent, or restricted within the hybrid zone, but in other cases introgressed genes may have spread outside the hybrid zone. Thus, evaluating the extent of introgressive hybridization in a lineage would give a new insight into the importance of reticulate evolution in animals.

In molecular phylogenetic studies, introgressive hybridization is detected based on an incongruence between gene trees, or between a gene tree and a species tree based on other evidence such as morphology (Avise 1994, 2000; Brower et al. 1996). Different genes sampled from the same set of organisms may exhibit different phylogenetic relationships of the organisms due to common processes such as stochastic sorting of ancestral polymorphism (deep coalescence) and introgressive hybridization. Although distinguishing these different causes for the incongruence based solely on gene trees per se is difficult or even impossible (Sang and Zhong 2000; Holder et al. 2001), additional information such as the spatial distribution of different haplotypes and direct evidence of natural hybridization can be used to determine the factor responsible for the incongru-

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ence. Detecting introgressive hybridization for a portion of the vast genome by no means provides a sufficient answer to questions about the impact of reticulation on evolutionary diversification. Nevertheless, to uncover the extent of interactions accompanied by introgressive hybridization in a lineage is a reasonable starting point.

Mitochondrial DNA has been widely used in phylogenetic and population studies of animals because the abundance of mitochondria and the haploid nature of the genome make analysis of mitochondrial DNA easier than analysis of nuclear DNA (Avise 1994, 2000). With an increasing number of studies making use of mitochondrial markers, the introgression of mitochondria across species (mitochondrial capture) has been found in various animal taxa (e.g., Harrison 1990; Avise 1994, 2000; Arnold 1997). Mitochondria, like chloroplasts in plants, may be subject to introgression more frequently than nuclear DNA (Riesberg and Soltis 1991; Avise 1994). Thus, the screening of mitochondrial haplotypes can be an efficient initial approach to determine the extent of hybridization among closely related species, although there is the limitation that the mitochondrial markers track only the maternal lineage for a descent subject to introgressive hybridization.

This article introduces a recent study of a carabid beetle group, the subgenus *Ohomopterus* (genus *Carabus*, Carabidae), which is endemic to Japan and consists of 15 species. Although their external morphology is similar, the species exhibit marked differentiation in body size and genital morphology. In local assemblages, two or three species of different sizes frequently coexist. An initial molecular phylogenetic analysis of this group using a mitochondrial gene revealed an unexpected level of incongruence between mitochondrial gene genealogy and traditional taxonomy (Su et al. 1996). A subsequent analysis of mitochondrial and nuclear gene genealogies suggested that this might be the result of the repeated introgression of mitochondria through hybridization (Sota and Vogler 2001). In fact, there are hybrid zones between parapatric species, and some hybrid swarms exist (Kubota and Sota 1998, 2001). Therefore, mitochondrial introgression may have occurred repeatedly through such hybrid zones. Here, I summarize the comparative analysis of mitochondrial and nuclear gene genealogies, and describe intra- and interspecific variations of mitochondrial haplotypes that indicate the unusual extent of mitochondrial introgression in *Ohomopterus*.

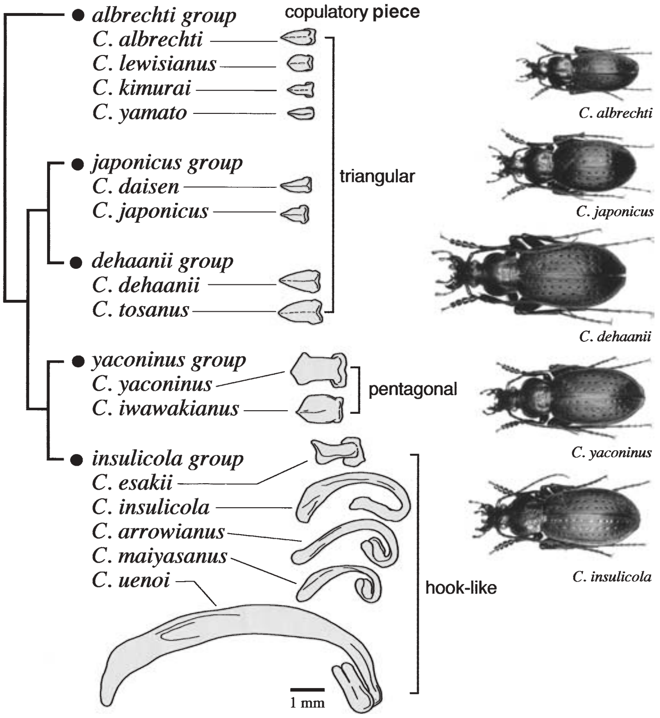

Fig. 1. Species, species groups, and morphological characters in *Ohomopterus*. The species and inferred cladistic relationship among species groups follow Ishikawa (1991). Drawings are male copulatory pieces, sclerotized parts of the endophallus which play a key role in genital coupling (dorsal or lateral view)

Radiation of *Ohomopterus*

The ancestral stock of *Ohomopterus* might have existed in the Japanese Archipelago as early as the late Miocene (6–8 MYA) or the early Pleistocene (1.6 MYA), from which strata a few fossils dubiously assumed to be *Ohomopterus* were discovered. However, convincing fossil records date back only to the late Pleistocene (Hayashi 2001; Sota 2002). Osawa et al. (1999) stated that the diversification of *Ohomopterus* might have started shortly after the formation of the Japanese Archipelago (15 MYA), but this estimate was based on an unacceptably slow mitochondrial molecular clock (see Sota 2002). Although further discoveries of fossils and a molecular-clock analysis of the extant taxa are needed, it is likely that the differentiation of the subgenus *Ohomopterus* occurred during the Pleistocene, when the repeated glacial/interglacial cycles probably caused frequent isolation, range shift, and secondary contacts in diverging local populations.

The 15 species of *Ohomopterus* are traditionally classified into five species groups (Ishikawa 1991), characterized mainly by genital morphology and body size (Figs. 1, 2). The genital characteristics, especially the copulatory piece of the male, have been the key characteristics used to classify the species and subspecies of *Ohomopterus*. The shape of the copulatory piece is either triangular, pentagonal, or hook-like (Fig. 1). The copulatory piece is an apophysis on the endophallus of the male genitalia and is inserted into the vaginal appendix of the female, which is a pocket matching the shape and size of the copulatory piece (Ishikawa 1987). This "lock and key" system maintains the coupling of female and male genitalia.

The evolution of the genital lock-and-key system in *Ohomopterus* is an intriguing subject for study. There are several possible factors promoting the evolution of genitalia, which are not necessarily exclusive. First, sexual selection, either of male–male competition, or male–female interaction, or both can be a principal factor in genital evolution (Eberhard 1985; Arnqvist 1998). An apparent

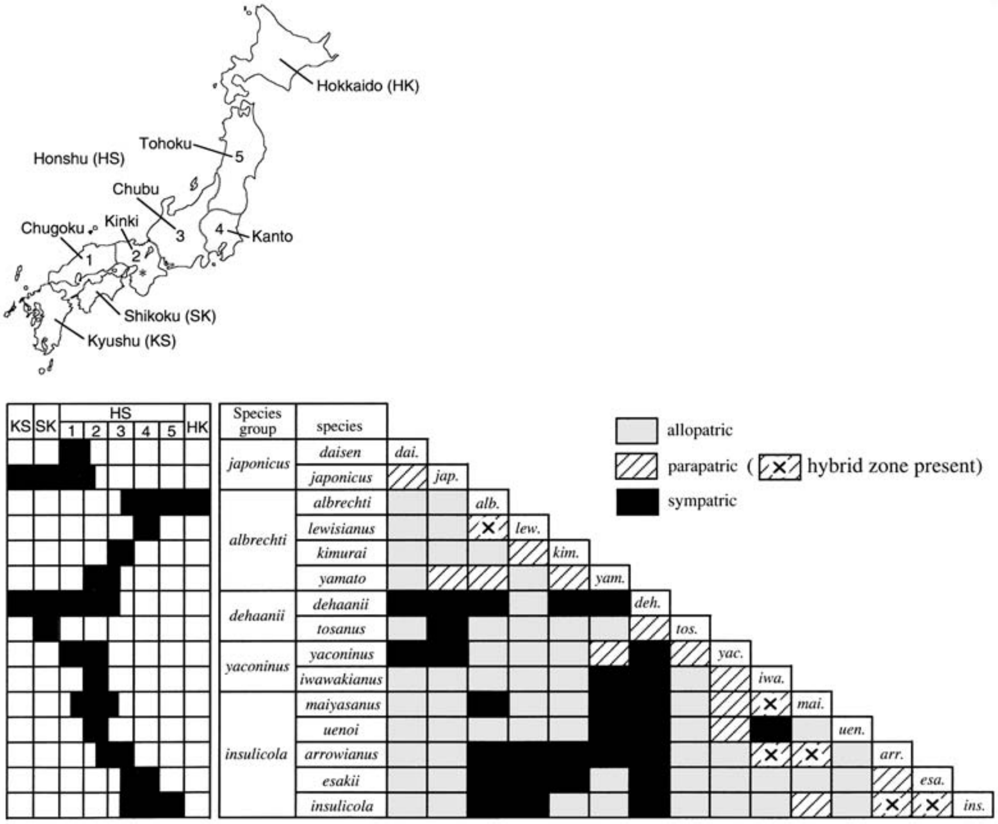

Fig. 2. Distribution pattern and natural hybridization of the subgenus *Ohomopterus*. For the distribution (*left*), regions of the Japanese mainland are shown in the inset map, and partially filled cells indicate that the species occurs only in the margins of the region. The asterisk in the map indicates the location of Mt. Kongo. The presence of the hybrid zone follows Kubota and Sota (1998) (single observations excluded)

function of the male copulatory organ and the female vaginal appendix is to fixate genital coupling (Ishikawa 1987). If a larger copulatory organ is a more effective anchor, and hence will be selected, a corresponding evolution of the vaginal appendix may occur to avoid physical damage to the vagina by the copulatory piece. Thus, a "chase-away" evolution may occur in these genital parts.

Takami (2000) suggested that the male copulatory piece may be used to remove the spermatophore deposited by the preceding male. Since a larger copulatory piece may be advantageous for this purpose, again chase-away evolution may occur. An additional factor in diversification may be sexual conflict. Since tight genital coupling due to morphological matching would reduce chances of female choice (rejection), there may be a conflict over the matching of genitalia between the sexes. Gavrilets (2000) suggested that sexual conflict may promote a divergence of mating traits among populations (see Arnqvist and Rowe 2002 for empirical evidence in an insect group). Finally, interspecific differentiation of genitalia may be promoted by selection acting through costly interspecific hybridization in order to reduce the incidence of interspecific mating. This is related to the classical lock-and-key hypothesis that has been rejected by some researchers (e.g., Eberhard 1985; Shapiro and Porter 1989). The fitness cost of mismatched genital coupling in heterospecific mating can be so great that it can be a strong selective force on precopulatory isolation mechanisms, or on genital morphology itself (Sota and Kubota 1998). As a reproductive isolation mechanism, however, the genital lock-and-key system always works following precopulatory factors such as differences in body size and sexual pheromones. In addition, increased interspecific differences in genital morphology that may prevent insemination may simultaneously cause more physical damage at copulation. Therefore, it is unlikely that differentiation of genital morphology, rather than the traits for precopulatory isolation, occurs following secondary contacts. Thus, interspecific genital differences in general are likely to have existed prior to secondary contacts.

Another variable characteristic in *Ohomopterus* is body size, which shows recognizable patterns of intra- (geographical) and interspecific variation (Sota et al. 2000b). Body size shows clinal variation along the gradient of habitat temperature (i.e., altitudinal and latitudinal decrease), but this variation is less conspicuous than marked interspecific differences at sympatry. Local species assemblages usually consist of two or three species (rarely four or five in one area) with different body sizes. The segregation of body sizes between sympatric species is usually clear. Species of the same species group with similar body sizes are parapatric, and sometimes form hybrid zones at the boundary. Because there are some cases of increased intraspecific variation in body size associated with the presence/absence of sympatric species, character displacement may have been involved in the evolution of body size (Sota et al. 2000b).

The matching of body size and genital morphology between sexes may greatly affect the success of copulation, and hence the chance of interspecific hybridization (Kubota and Sota 1998). Therefore, the evolution of these characters may be directly related to the historical occurrence of hybridization in *Ohomopterus*.

Potential role of hybridization in the population dynamics and evolution of *Ohomopterus*

Despite the development of complex genital systems in *Ohomopterus*, mechanical isolation during copulation is incomplete; although the probability of failed insemination, genital injury, or death due to injury is high, a fraction of the females that have interspecific mating produce offspring (Kubota and Sota 1998, 2001; Sota and Kubota 1998; Sota et al. 2000a). The premating reproductive isolation that probably relies on differences in body size and pheromones appears to be strong enough to prevent copulation among currently sympatric species. However, the differences in these traits between parapatric species appear to be small, often resulting in indiscriminate interspecific mating (Sota and Kubota 1998; Sota et al. 2000a).

A natural hybrid zone of *Ohomopterus* has been found in several pairs of species (see Fig. 2). Detailed analyses of hybrid zone revealed the existence of different types of hybrid zone (Kubota and Sota 1998, 2001). With relatively large differences in genital morphology and body size, *C. insulicola* and *C. esakii* produce occasional F_1 and subsequent progenies, but no hybrid swarm has been established (Kubota and Sota 2001). *Carabus insulicola* also has contact zones with *C. arrowianus* (ssp. *nakamurai*), which is closer to *C. insulicola* in body and genitalia size, and there are hybrid swarms with homogeneous intermediate morphological characters (Sota et al. 2000a). *Carabus iwawakianus* and *C. maiyasanus* possess quite different genitalia, but are otherwise very similar. Their hybrid zone is more or less like a tension zone (Barton and Hewitt 1985), with very strong selection against hybridization (Kubota 1988; Sota and Kubota 1998). However, despite the great reduction in reproductive success, there are hybrid swarms, with very heterogeneous genital morphology among individuals.

There are several ways that hybridization affects the populations concerned. First, when hybridization always results in the loss of gametes due to hybrid breakdown or the death of the hybridizing adults, two interactive populations would merely maintain parapatric distribution through sexual competitive exclusion (Rebeiro and Spielman 1986; Kuno 1992; Yoshimura and Clark 1994). Second, while resulting in great fitness reduction, secondary contact and hybrid breakdown may result in the reinforcement of reproductive isolation mechanisms (Liou and Price 1994; Butlin 1995; Noor 1999). Note that in *Ohomopterus*, the direct cost of heterospecific mating can be high because of occasional genital injury and female death (Sota and Kubota 1998). This may be a strong force promoting reinforcement selection. Third, hybridization may result in the introgression of DNA and reticulation of the genome. In some cases, hybridization may result in a fusion of two populations, or the establishment of a novel lineage which is independent of its progenitors (Arnold 1997). The different patterns of natural hybridization in *Ohomopterus* seem to be related to these possible patterns. As to the third process, there is now more evidence of introgressive hybridization for mitochondrial DNA, as is mentioned later, but the influence on nuclear DNA remains to be studied. A visible consequence of introgression may be revealed in the color of the upper body surface (Kubota and Sota 1998). The geographic pattern of these color characteristics is closely related to parapatry and/or the presence of natural hybridization.

Mitochondrial and nuclear gene genealogy

A molecular phylogenetic analysis of *Ohomopterus* was first conducted by Su et al. (1996) using the mitochondrial ND5 (NADH dehydrogenase subunit 5) gene sequence. Unexpectedly, the mitochondrial gene tree disagreed extensively with traditional taxonomy. One morphological species often possesses two distinct lineages of mitochondria, and two or more different species from the same region often share the same mitochondrial lineage. This finding led Su et al. (1996) to invoke a parallel speciation hypothesis based on a hypothetical "type-switching" mechanism which causes discontinuouos morphological transformation.

Sota and Vogler (2001) tested the parallel speciation hypothesis by comparing mitochondrial and nuclear gene trees. If parallel speciation had truly occurred, the nuclear gene tree in general should be consistent with the mitochondrial gene tree. Here, I recapitulate the results of Sota and Vogler (2001). The original analysis has been revised, but without altering the main conclusions. The major points of revision are (1) the addition of cytochrome oxydase subunit I (COI) data; (2) the use of *Carabus* (*Isiocarabus*) *fiduciarius* as the outgroup instead of *C.* (*Carabus*) *granulatus*, because the former is closer to *Ohomopterus* (T. Sota, unpublished data); (3) the addition of three *Ohomopterus* specimens, and (4) the correction of errors in the original sequence matrix. Table 1 gives the properties of the six loci used (three each from the mitchondrial and nuclear DNA). The mitochondrial loci were AT-rich, while the nuclear loci had more even base compositions. The average sequence divergence for each locus varied between 1.2% and 3.7%.

We tested for incongruence between loci using the incongruence difference length (ILD) of Farris et al. (1994, 1995). The statistical significance of ILD was assessed using a random partitioning procedure (the partition homogeneity test) as implemented in PAUP* ver. 4.0b8 (Swofford 2001) (Table 2). The mitochondrial loci were not incongruent with each other (ILD = 1.7%–1.9%), whereas the nuclear loci were moderately incongruent or not incongruent (ILD = 8.1%–11.4%). Notably, all pair-wise mitochondrial and nuclear loci showed significant incongruence (ILD = 7.7%–17.6%). Because the mitochondrial loci belong to the single-linkage group, incongruence among them

Table 1. Properties of the six loci used in the phylogenetic analysis of *Ohomopterus*

Locus	bp	% G + C	% divergence[a]	Tree length[b]	CI[c]	RI[d]
Mitochondrial						
16S	821	24.7 ± 0.3	1.2 ± 0.5	117	0.56	0.86
ND5	1020	20.5 ± 0.3	3.0 ± 1.0	405	0.48	0.80
COI	914	32.8 ± 0.7	3.7 ± 1.2	459	0.40	0.75
Nuclear						
Wg	440	48.7 ± 0.3	1.5 ± 0.6	103	0.44	0.71
PepCK	630	48.6 ± 0.4	1.3 ± 0.5	164	0.38	0.55
Carab1	577	47.6 ± 0.5	3.2 ± 1.2	257	0.40	0.73

16S, 16S ribosomal RNA; ND5, NADH dehydrogenase subunit 5; COI, cytochrome oxydase subunit I; Wg, wingless; PepCK, phosphoenolpyruvate carboxykinase; Carab1, unknown single copy locus named by Sota and Vogler (2003) (= anonymous in Sota and Vogler 2001)

[a] Mean ± SD of pair-wise sequence divergence within *Ohomopterus*

[b] Tree length is for the shortest trees in the maximum parsimony analysis

[c] CI, consistency index (excluding uninformative characters)

[d] RI, retention index

Table 2. Test for incongruence in phylogenetic information between loci based on incongruence length difference

Comparison	% ILD[a]	*P*-value[b]
Within mitochondrial		
16S vs. ND5	1.9	0.329
16S vs. COI	1.7	0.942
ND5 vs. COI	1.9	0.121
Three-mitochondrial	2.3	0.455
Within nuclear		
Wg vs. PepCK	11.4	0.151
Wg vs. Carab1	8.6	0.001
PepCK vs. Carab1	8.1	0.004
Three-nuclear	12.4	0.001
Between mitochondrial and nuclear		
16S vs. Wg	15.9	0.001
16S vs. PepCK	12.1	0.001
16S vs. Carab1	17.6	0.001
ND5 vs. Wg	7.7	0.001
ND5 vs. PepCK	9.4	0.001
ND5 vs. Carab1	15.6	0.001
COI vs. Wg	8.7	0.001
COI vs. PepCK	7.4	0.001
COI vs. Carab1	15.1	0.001
Mitochondrial vs. nuclear	9.0	0.001
All six loci	15.3	0.001

[a] ILD, incongruence length difference (Farris et al. 1994, 1995), i.e., length of combined data set minus the sum of the lengths of individual data sets. % ILD is the percentage of ILD within the sum of the lengths of individual data sets

[b] Significance level obtained using the partition homogeneity test in PAUP*. *P*-value is based on 999 random partitions of the original data set. For pair-wise comparisons of six loci (15 comparisons), the Bonferroni-corrected critical-level at $\alpha = 0.05$ is $P = 0.0033$

would not be expected unless the loci studied had very different evolutionary rates.

Figure 3 shows simultaneous analysis trees for mitochondrial and nuclear data. The mitochondrial tree shows that several species are markedly polyphyletic (appearing in widely separated clades), which conforms to the initial observation by Su et al. (1996). On the other hand, some of these traditional species are indeed monophyletic in the nuclear gene tree. Although the nuclear gene tree is not fully resolved, and remains ambiguous as to the species relationship, the topology is less incongruent with traditional taxonomy than is the mitochondrial gene tree (see Sota and Vogler 2001). The strong incongruence between the mitochondrial and nuclear data, together with the relative consistency of the nuclear data with traditional taxonomy, indicates that the parallel evolution hypothesis is not likely, and the discrepancy between the mitochondrial gene tree and the morphological taxonomy needs to be explained by other processes, such as introgression of mitochondria through hybridization, or the random sorting of ancestral polymorphisms.

The nuclear genes of *Ohomopterus* exhibited various levels of incongruence with each other, and this fact may imply that introgression hybridization has also affected the nuclear genome, although less extensively than in the mitochondrial DNA. Although population genetic analysis of nuclear gene sequences is still needed, we made an extended analysis of nuclear gene sequences for the same set of specimens as in Fig. 3 to study the heterogeneity in gene genealogies more thoroughly, and to test a simultaneous analysis of a combined data set for a reconstruction of species relationships (Sota and Vogler 2003). We used two additional nuclear loci (cytochrome *c* and elongation factor 1-α). Briefly, we found high levels of diversity and heterozygosity in alleles, especially in introns of three of the five loci that may have resulted from rapid radiation from polymorphic ancestors and/or frequent hybridization among recently diverged populations. In a phylogenetic reconstruction, the simultaneous analysis of five loci resulted in a well-resolved topology, despite the existence of heterogeneous, contradictory phylogenetic signals from different genes. According to the tree obtained, the basal clades of *Ohomopterus* consisted of beetles with triangular copulatory organs and small bodies (*albrechti* and *japonicus* species group), larger forms with triangular (*C. dehaanii* and *C. tosanus*) or pentagonal (*C. yaconinus*) copulatory organs appeared subsequently, and finally a derived group with *C. iwawakianus* and all taxa of the *insulicola* group differentiated with marked variations in genitalia (from pentagonal to hook-like copulatory pieces).

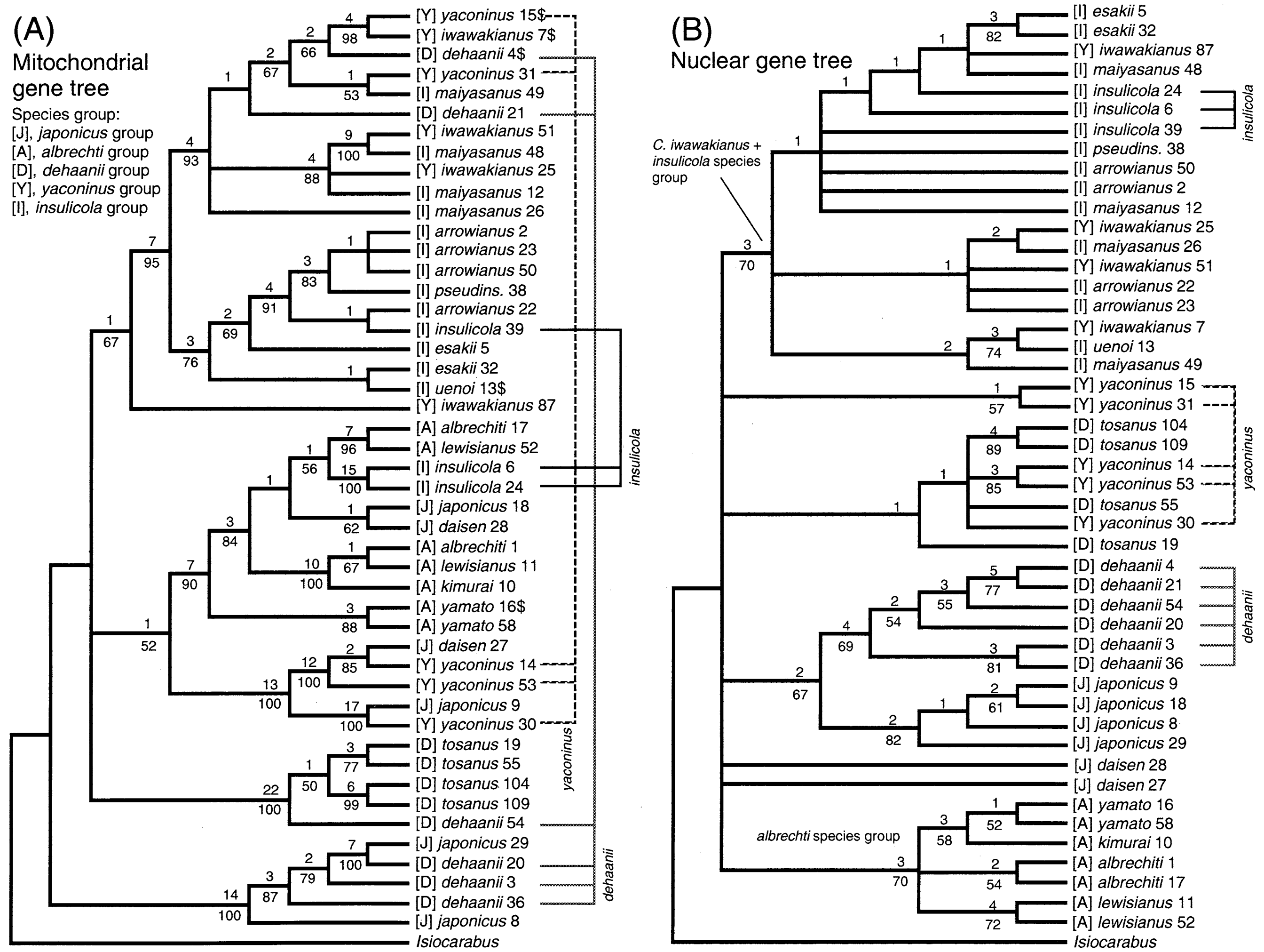

Fig. 3. Mitochondrial and nuclear gene trees resulting from a combined analysis of multiple loci. The mitochondrial gene trees (**A**) are a strict consensus of the 12 most parsimoneous trees of 1004 steps consistency index (CI) excluding uninformative sites = 0.43; retention index (RI) = 0.77; rescaled consistency index (RC) = 0.43. The nuclear gene tree (**B**) is a strict consensus of 116 most parsimoneous trees of 589 steps (CI excluding uninformative sites = 0.35; RI = 0.60; RC = 0.28). Numerals above and below the branches are bootstrap percentages (when greater than 50%) and Bremer support values, respectively. The numerals after the species name refer to sample numbers in Sota and Vogler (2001). "$" in (**A**) indicates samples from Mt. Kongo. The different patterns in the distribution of specimens from the same species between the two trees are indicated for three species

Introgression of mitochondria: evidence for hybridization

In general, each species may have multiple alleles or haplotypes at a locus, and some of these may be shared across related species. The sharing of lineages of alleles (or haplotypes) is called trans-species polymorphism (Klein et al. 1998). Trans-species polymorphism often constitutes a conflicting pattern between gene trees and the species tree. In *Ohomopterus*, several species shared very distinct mitochondrial lineages (Fig. 3A). In principle, such trans-species polymorphism can result from introgressive hybridization as well as stochastic sorting of ancestral polymorphism, as is illustrated in Fig. 4, where one species possesses two haplotypes, and one of these is sister to the haplotype of the other species. Distinguishing between these cases based on gene trees is generally difficult (e.g., Sang and Zhong 2000; Holder et al. 2001). However, population genetic analysis across geographical populations may enable us to infer the historical aspects of the trans-species polymorphisms. For example, a shared haplotype with an undifferentiated sequence may be found in the vicinity of a contact zone (or sympatric zone) of two species, but not in allopatric zones (Fig. 4, left). This pattern is not usually expected when a common ancestral lineage has been shared by both species, because if one species possess two lineages derived from ancestral polymorphism, the two haplotypes must show some divergence from each other (deep coalescence), and the occurrence of the type shared with other species is not expected to be restricted to near the boundary zones (Fig. 4, left). Thus, unless the introgression event is very ancient, these cases will be distinguishable from each other based on the pattern of coalescence of haplotypes (shallow vs deep)

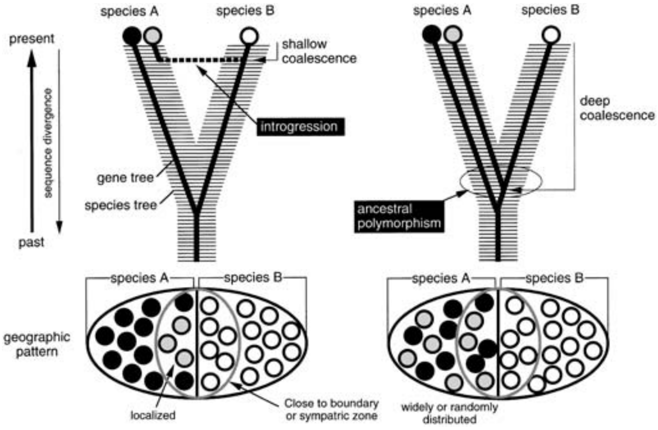

Fig. 4. Gene tree and geographic pattern of mitochondrial haplotypes in hypothetical cases of introgressive hybridization (*left*) and stochastic sorting of ancestral polymorphism (*right*). One of two haplotypes possessed by species A that is sister to a species B haplotype may be derived from introgression (*left*) or ancestral polymorphism (*right*). The two cases may differ in the coalescence pattern and the geographical distribution of the sister haplotypes

and the spatial distribution of individuals carrying different haplotypes (localized vs widely or randomly distributed).

A phylogeographical investigation of mitochondrial genes may thus be an efficient way to access the occurrence of introgressive hybridization in an animal lineage. We then extended the analysis of the mitochondrial ND5 sequence in *Ohomopterus* to include several hundred specimens (Sota et al. 2001; Sota, unpublished data). This analysis has revealed that sharing the same mitochondrial lineages is common between parapatric and sympatric species, indicating introgression through hybridization. Two analyses of mitochondrial introgression for parapatric and sympatric taxa are described in detail below.

Introgression between parapatric taxa: *Carabus insulicola* and allied species

Carabus insulicola possesses two widely separated types of mitochondria (see Fig. 3A). One of these is close to haplotypes of the *albrechti* group species that are sympatric with *C. insulicola* in many localities, and hence it may have been an introgressed lineage in a past hybridization (Sota and Vogler 2001). *Carabus insulicola* is paratric with three species of the *insulicola* group, *C. esakii*, *C. arrowianus* (ssp. *nakamurai*), and *C. maiyasanus* (ssp. *hokurikuensis*) (Fig. 5), and hybridizes naturally with at least *C. arrowianus* and *C. esakii* (Kubota and Sota 1998, 2001; Sota et al. 2000a). Therefore, the introgression of mitochondria was expected between these parapatric taxa. To study the distribution of mitochondrial haplotypes in these taxa, we used the polymerase chain reaction (PCR)-amplified ND5 region as a marker. For the initial screening of haplotypes, we used RFLP (restriction fragment length polymorphism) analysis with *Taq*I endonuclease to distinguish major haplotypes, and thereafter performed direct sequencing for representative samples.

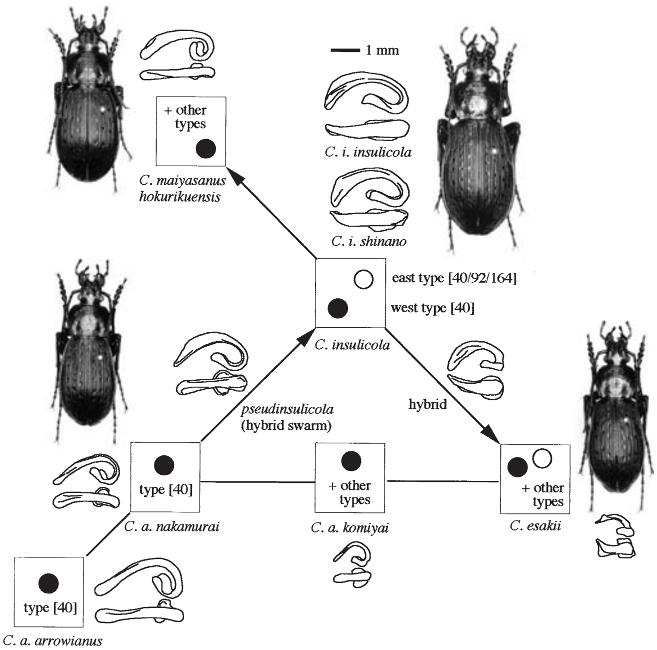

Fig. 5. *Carabus insulicola* and three allied species, *C. esakii*, *C. arrowianus*, and *C. maiyasanus*, which are parapatric with *C. insulicola*, and the inferred mitochondrial introgression between these species. Male copulatory pieces are illustrated for seven taxa of the four species and two natural hybrids. For haplotypes of the mitochondrial ND5 gene sequence, see text and Fig. 6

Seven haplotypes could be identified by the cutting sites of *Taq*I, among which types [40] and [40/164/909] were two major haplotypes of *C. insulicola* (types are described by the number of base pairs in between the cutting sites and the 5′-terminal end of the PCR-amplified fragment of 1083 bp). In most of its range, *C. insulicola* possessed type [40/164/909], which is referred to as the “east *insulicola* clade” (Sota et al. 2001). The other type [40] was found only in the western periphery (“west *insulicola* clade”), where many populations were polymorphic of the two types. The mitochondria of the east *insulicola* clade (type [40/164/909]) was shared by *C. esakii* and natural hybrids of *C. insulicola* and *C. esakii* (Fig. 6). Although *Carabus esakii* possessed several mitochondrial lineages, the type [40/164/909] was confined at the boundary zones with *C. insulicola*. Thus, this type of mitochondria was probably introgressed from *C. insulicola* to *C. esakii*. The west *insulicola* clade (one lineage of type [40]) shows a lower level of differentiation of the gene sequence than the east *insulicola* clade, and was shared by *C. arrowianus*, *C. insulicola pseudinsulicola* (putative hybrid swarm of *C. insulicola* and *C. arrowianus nakamurai*), *C. esakii*, and *C. maiyasanus hokurikuensis*. Because *C. insulicola* possessed mitochondria of type [40] only in the western periphery of its range, whereas *C. arrowianus nakamurai* and *C. a. arrowianus* possessed only this type, this type might be introgressed from *C. arrowianus* to *C. insulicola*. Similarly, the phylogeographic pattern of mitochondria suggests that the west *insulicola* clade was introgressed from *C. insulicola* to *C. esakii* and also to *C.*

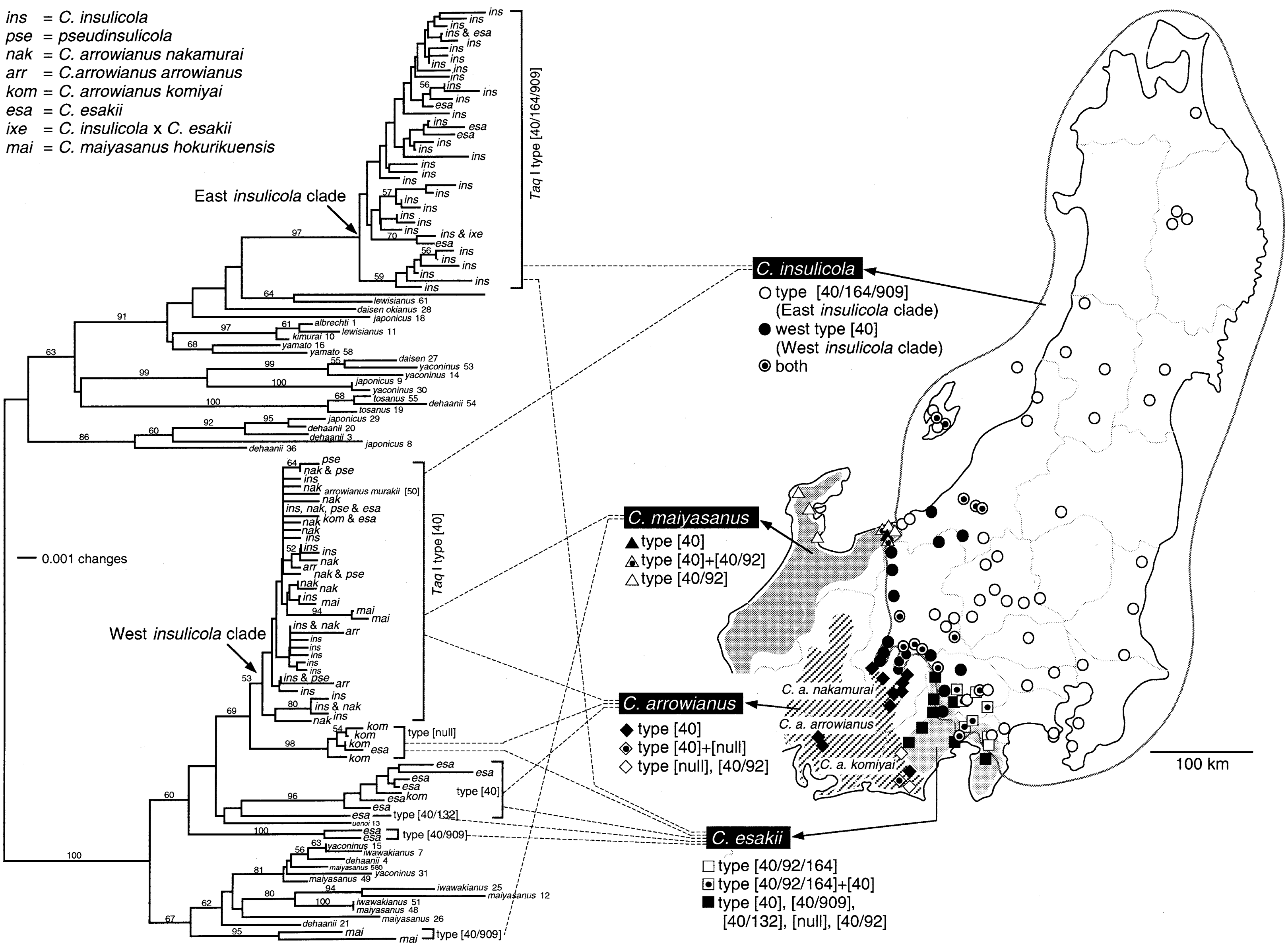

Fig. 6. Distribution of *Taq*I haplotypes of the polymerase chain reaction (PCR)-amplified mitochondrial ND5 region in *Carabus insulicola* and its parapatric taxa, and the genealogy of the ND5 sequences (modified from Sota et al. 2001). *Left*, phylogram of ND5 by the neighbor-joining method (distance corrected by the Kimura two-parameter method), with *Taq*I haplotypes indicated for the *insulicola* species group studied. Numerals on the branches are bootstrap percentages (shown when greater than 50%). OTUs other than the four *insulicola* group species are the same with those in Fig. 3 (A). *Right*, geographical distribution of different haplotypes of ND5 sequence identified by PCR-RFLP with *Taq*I endonuclease

maiyasanus hokurikuensis through their contact zones. In addition, *C. esakii* and *C. arrowianus komiyai*, which are parapatric with each other, shared a few mitochondrial lineages, and there may also have been introgressive hybridization between them.

Thus, the introgression of mitochondria may have occurred repeatedly between *C. insulicola* and its parapatric allied taxa (see Fig. 5). The direction of introgression may be affected by asymmetric success in interspecific copulation. *Carabus insulicola* females can copulate with *C. esakii* males, who have a short copulatory piece, whereas *C. esakii* females cannot copulate with *C. insulicola* males, who have an elongated copulatory piece (Kubota and Sota 2001). Therefore, the mitochondria is more likely to have introgressed from *C. insulicola* to *C. esakii*. However, between *C. insulicola* and *C. a. nakamurai* there is no strong asymmetry (Sota et al. 2000a), and the directional introgression (from *C. a. nakamurai* to *C. insulicola*) seems to have been affected by other factors or by chance.

Introgression among sympatric species: five species in the Mt. Kongo area

The area of Mt. Kongo, at the boundary of Osaka and Nara Prefectures, harbors five species (Fig. 7). They have different sizes and genital characteristics, and natural hybrids of these species have not been found. The smallest and largest species occur widely in the mountain area, whereas the three medium-sized species have different altitudinal ranges (i.e., *C. uenoi* in the higher, *C. iwawakianus* in the middle, and *C. yaconinus* in the lower altitudes) (Sota 1985). In the genealogy of mitochondria (see Fig. 3A), *C. dehaanii* and *C. yaconinus* from this area (*dehaanii* 4 and *yaconinus* 15 in Fig. 3A) are distantly related to other specimens from western regions, and are included in the clade consisting of *C. iwawakianus* and species of the *insulicola* group of the central Honshu area. This pattern might result from the introgression of mitochondria from the *iwawakianus–insulicola* group (see Fig. 3B) to *C. dehaanii* and *C. yaconinus* in the past. To analyze the diversity of mitochondria and detect mitochondrial introgression in the species assemblage at Mt. Kongo, a partial sequence of the ND5 gene (1020 bp) was analyzed in many specimens collected from this mountain area.

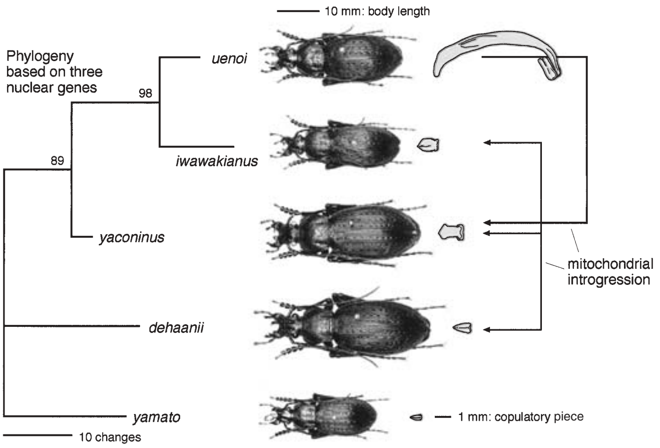

Fig. 7. Five species in the Mt. Kongo area. The phylogeny is a single maximum parsimony tree (phylogram) based on data from three nuclear genes, as in Fig. 3B (210 steps; CI excluding uninormative sites = 0.76; RI = 0.67; RC = 0.60). Node supports (bootstrap per centage) are indicated on the branch. Inferred mitochondrial introgression among species is indicated

The ND5 sequence was monotypic in *C. uenoi*, whereas *C. yamato* had two types, and the three remaining species had seven types each (Fig. 8). Identical sequences (haplotypes) were partially shared among species, except for *C. yamato*. *Carabus yaconinus* shared one haplotype with *C. uenoi*. *Carabus yaconinus*, *C. iwawakianus*, and *C. dehaanii* shared two haplotypes, and in addition *C. iwawakianus* and *C. yaconinus* shared another haplotype. Because the *C. uenoi* haplotype is not closely related to other haplotypes, and *C. yaconinus* possesses this type only in this region, hybridization between *C. uenoi* females and *C. yaconinus* males might result in mitochondrial introgression. Among *C. yaconinus*, *C. iwawakianus*, and *C. dehaanii*, the partial sharing of haplotypes suggests limited opportunities for introgressive hybridization. As I mentioned earlier, all the haplotypes possessed by *C. dehaanii* and *C. yaconinus* may be of introgressed lineages judging from their position in the whole mitochondrial genealogy of *Ohomopterus* (Fig. 3A). Therefore, shared identical sequences for these species may reflect relatively recent introgression, whereas slightly differentiated sequences reflect relatively ancient introgression. Note that since one nucleotide substitution for the 1020 bp of ND5 may require some 50 000 years with a standard mitochondrial DNA clock (2% per MYA) (Avise 2000), the introgression events for presently shared sequences could date back to well before the last glacial period at the most. Although an accurate estimation of the molecular clock is needed, the possible range of time since an introgression event implied by undifferentiated sequences may be great (e.g., zero to several tens of thousands of years ago).

With regard to the possibility of hybridization, laboratory experiments show that the males of the five species sometimes attempt to copulate with heterospecific females and genital coupling can occur for some pairs, but insertion of the aedeagus is prevented when the difference in body or genital size is large (T. Sota, unpublished data). Of the five species, *C. yamato* exhibited no sign of mitochondrial introgression. This species may have been free from introgessive hybridization for a long time because it is the smallest. Among the other four species, copulation is possible between species of similar sizes. However, the composition of mitochondrial lineages in each species is quite different from what would be expected from the nuclear gene tree (see Fig. 7). The probable sister taxa *C. uenoi* and *C. iwawakianus* have no shared a mitochondria, whereas each of these shared a mitochondrial haplotype with *C. yaconinus*. *Carabus yaconinus* occurs mainly at lower altitudes, while *C. iwawakianus* and *C. uenoi* occur at higher altitudes. Among these species, interspecific mating between *C. uenoi* males and females of other species is probably impossible

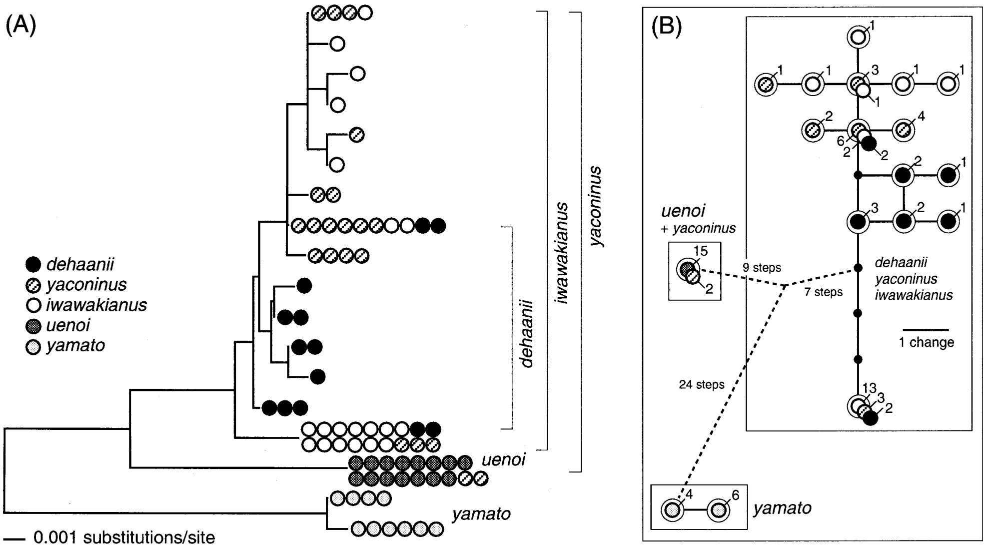

Fig. 8. Diversity in the sequences of mitochondrial ND5 gene among *Ohomopterus* species in the Mt. Kongo area. **A** Phylogram by the neighbor-joining method with distances corrected by the Kimura two-parameter method. **B** Parsimony network (Templeton et al. 1992) of the ND5 gene sequences. *Small closed circles* represent haplotypes which did not appear in the sample

because of the huge genitalia of *C. uenoi*. The sexual segregation between the related species *C. uenoi* and *C. iwawakianus* might have been established immediately after the initial secondary contact because of this difference in genital morphology, which would be especially harmful to *C. iwawakianus* females. *Carabus yaconinus* is usually found in habitats segregated from these species, and incomplete sexual isolation may have caused introgressive hybridization. The same may be true for the relationship between *C. yaconinus* and *C. dehaanii*, since the latter occurs at higher altitudes.

Carabus yaconinus was involved in every case of shared mitochondrial sequences, and is suspected to have played a central role in hybridization. This species is medium-sized, which may allow mating with both smaller and larger species, and has an ability to colonize disturbed areas on flat land as well as mountain habitats occupied by other species. Because of these characteristics, they would have had more chance to make contact with other species and hybridize.

Evolutionary history and mitochondrial introgression

The two cases presented above are only a part of the tangled mitochondrial gene genealogy of *Ohomopterus*. The trans-species polymorphism in the mitochondria is unusually complicated despite the distinct morphological differentiation in *Ohomopterus*. In a sympatric assemblage of closely related animals, the possibility of introgressive hybridization is usually very low because of reproductive isolation mechanisms that, in turn, allow the coexistence of closely related species. Therefore, introgressive hybridization may occur most frequently in the initial phase of secondary contacts between differentiated species, and during the period before they establish sympatry. Since the present local assemblage of *Ohomopterus* consists of two or three species, and occasionally four or five species within one area, there must have been many events of secondary contacts between species with various levels of differentiation. Repeated allopatric differentiation and range shift might have occurred as a result of geological and climatic changes in the Pleistocene. Thus, during the radiation process of *Ohomopterus*, there could have been many chances of reticulation through hybridization, where mitochondria might be subject to introgression more frequently than nuclear DNA. The hypothesized process leading to repeated introgressive hybridization is shown in Fig. 9, where the repetition of secondary contacts at different times could create complex patterns of trans-species polymorphism.

As is seen in the literature, mitochondrial introgression in animals is usually documented between two species and across a restricted contact zone. However, analyses of a wide range of populations and species within a lineage may reveal more complex patterns of mitochondrial introgression, as in the cases of *Ohomopterus*. For example, in the brackish-water gobiid genus *Tridentiger* in Japan, mitochondrial introgression between two species seems to have occurred in separate coastal regions around the Japanese Archipelago (Mukai et al. 1997). In a cyprinid fish genus *Leuciscus*, in Europe, mitochondrial introgression was inferred between the widely distributed chub *L. cephalus* and some local endemic species (Durand et al. 2000). In this *L. cephalus* complex, secondary contacts between the local species and the chub that invaded the area of previous colonizers have resulted in multiple introgression events. This situation is similar to that found between widely distributed *Carabus insulicola* and its parapatric species. In other groups of carabids, Kim et al. (2000) found extensive trans-species polymorphisms of mitochondrial gene sequences in the genus *Leptocarabus* in Japan. Although less diverse than *Ohomopterus*, this group exhibits parapatry and sympatry of locally differentiated species, as well as natural hybridization between parapatric species (Kubota 1991; Yahiro et al. 2001). Several introgression mitochondrial events in this group may also have occurred, because

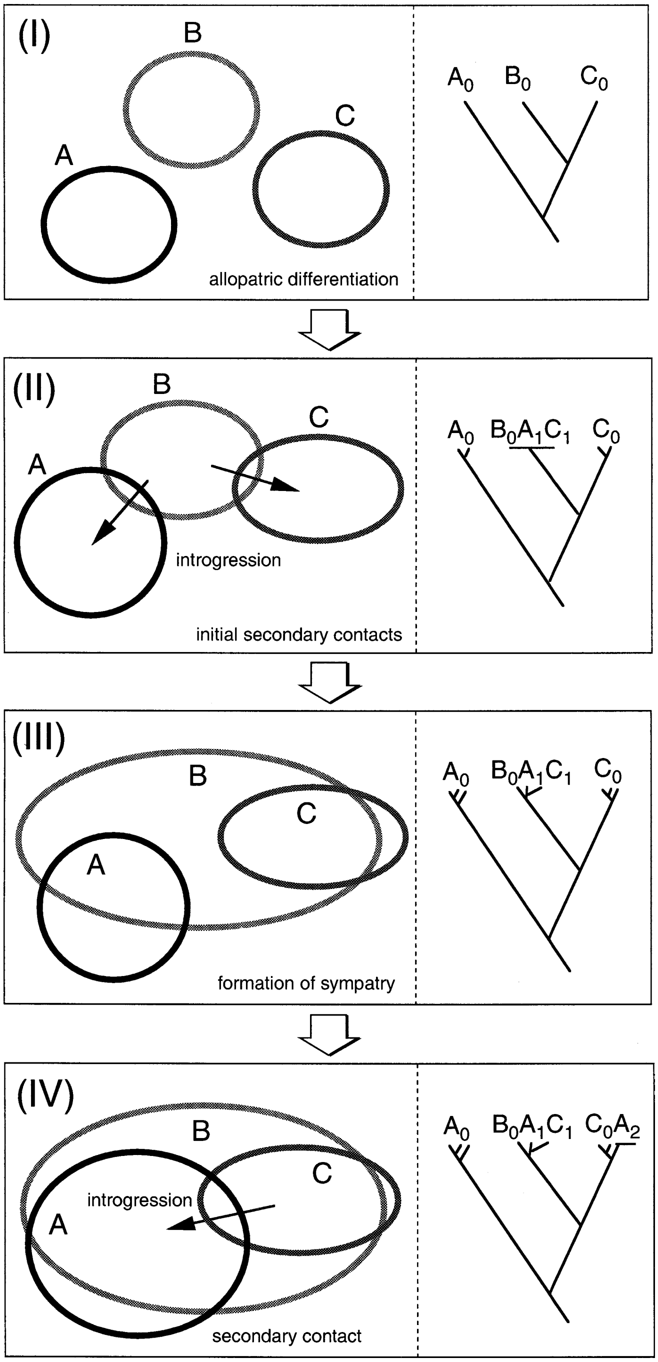

Fig. 9. A hypothetical development process of trans-species polymorphism of mitochondria. In each panel, *circles* represent the distribution ranges of species A, B, and C (*left*), and the phylogenetic tree represents the mitochondrial genealogy (*right*) with original haplotypes A_0, B_0, and C_0 for species A, B, and C, respectively. **I** Initial allopatric speciation following the colonization of an ancestral stock. **II** Initial secondary contacts where mitochondrial introgression occurred from B to A and C (the introgressants are A_1 and C_1. **III** Range expansion by species B, and the formation of sympatric areas. **IV** Secondary contact between species A and C with mitochondrial introgression from C to A (A_2 is introgressant). For simplicity, each species initially possessed monotypic mitochondria, and the introgressions are arbitrarily directional; also, there is no extinction for species and haplotype lineages

sharing mitochondrial lineages between species is associated with overlapping or adjacent distribution ranges. With the increasing number of phylogeographic studies of mitochondria in animal lineages, more cases of complex diversification–reticulation patterns may be revealed.

Conclusion

The sharing of the same mitochondrial lineages among *Ohomopterus* species, and the phylogeographic pattern of mitochondrial haplotypes, indicate repeated occurrences of mitochondrial introgression not only between parapatric species which hybridize naturally, but also between sympatric species for which natural hybridization is apparently absent. The pattern of trans-species polymorphisms in mitochondria observed among *Ohomopterus* species is unusually tangled, and implies that during the radiation of this group, interactions between once differentiated populations have been frequent and intensive. In this regard, it is surprising that different morphological entities (and probably biological species), recognizable across the wide range of *Ohomopterus*, have persisted despite the tendency of reticulation by natural hybridization that would obscure the species boundaries. The fact that phylogenetic analysis with several nuclear genes, unlike mitochondrial genes, could recover traditional species (Sota and Vogler 2003) suggests that genome parts that determine the key characters of each species may have been largely intact even with the occurrence of introgressive hybridization. However, there is still the possibility that the introgression of nuclear genes has directly affected phenotypic evolution in *Ohomopterus*. Because analyses of the marker genes that do not affect visible phenotypes have only a limited power to resolve the reticulate evolution, we need a direct genetic analysis for the characters of interest, such as genital and body size, in order to understand the consequences of these interactions among populations. It is vital to understand how and why morphological entities that are recognizable as biological species could have been present in spite of the reticulate nature of a lineage of closely related organisms.

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