Establishment of hybrid-derived offspring populations in the *Ohomopterus* ground beetles through unidirectional hybridization

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Abstract: An approach to deduce the mechanism of stabilization of the hybrid-derived populations in the *Ohomopterus* ground beetles has been made by comparative studies on the phylogenetic trees of the mitochondrial and nuclear DNA. A phylogenetic tree based on the internal transcribed spacer (ITS) of nuclear ribosomal gene roughly reflects the relations of morphological species group, while mitochondrial NADH dehydrogenase subunit 5 (ND5) gene shows a considerable different topology on the tree; there exist several geographically-linked lineages, most of which consist of more than one species. These results suggest that the replacement of mitochondria has occurred widely in the *Ohomopterus* species. In most cases, hybridization is unidirectional, i.e., the species A (♀) hybridized with another species B (♂) and not vice versa, with accompanied replacement of mitochondria of A by those of B. The results also suggest that partial or complete occupation of the distribution territory by a hybrid-derived morphological species. The morphological appearance of the resultant hybrid-derivatives are recognized as that of the original species A. Emergence of a morphological new species from a hybrid-derived population has been exemplified.

Key words: *Ohomopterus* ground beetles; phylogenetic trees; ND5 gene; ITS I; unidirectional hybridization; mitochondrial flow.

Introduction. The number of known Carabinae species is 1,000 to 1,500, among which only about 40 species have been known from the Japanese Islands. Most of the Carabinae ground beetles are hind-wingless and cannot fly and move only by walking. The Japanese species may be classified into two categories from the viewpoint of their origin.1) The first category includes species that were directly derived from ancestors that inhabited the ancient Japan area when it was attached to the eastern periphery of the Eurasian Continent ca. 15 million years ago (the autochthons). The second category contains species that invaded from the Eurasian Continent through Sakhalin and/or the Kurile Islands or from the Korean Peninsula during the glacial era (< 2 million years; the invaders). *Ohomopterus* is a genus belonging to the first category, i.e., one of the autochthons. The second category contains species that invaded from the Eurasian Continent through Sakhalin and/or the Kurile Islands or from the Korean Peninsula during the glacial era (< 2 million years; the invaders).

The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL and GenBank nucleotide sequence databases with the accession Nos. AB271970-AB272060 and AB272237-272316 as shown in Supplementary data.

The Supplementary data associated with this article can be found in the online version at http://www.jstage.jst.go.jp/browse/pjab.
ological properties such as phylogenetic features remains unsolved. Adopting only morphological properties that are often prepossessed for nature of the biological taxa has mostly brought about this situation. To solve this problem a little further, we introduced to clarify the phylogenetic properties of the *Ohomopterus* species using mitochondrial ND5 gene sequence. In some (but not all) cases, nuclear genes may be more appropriate than mitochondrial genes to deduce the species history, because mitochondria are inherited maternally. We first planned to study the Carabinae phylogeny using nuclear DNA such as the genes for elongation factors or nuclear 28S ribosomal RNA to study. However, it was soon found that these nuclear genes were not suited for research because of slow rate of nucleotide substitution. We therefore chose the mitochondrial ND5 gene, which shows much more rapid nucleotide substitution rate than nuclear genes. To our knowledge, almost no molecular phylogenetic analyses of any particular insect group had been undertaken at the time we started this work in 1993. Surprisingly, the phylogenetic tree constructed from the ND5 gene was quite different from the phylogeny predicted from morphological knowledge. The tree seemed to counter-intuitive when look at from the viewpoint of phylogeny, but we had to learn it objectively from the viewpoint of molecular phylogeny. The tree showed that morphologically similar (apparently conspecific) individuals fall out in many different places. We considered three possibilities to account for this phenomenon. Taking, for example, *O. dehaanii* and *O. japonicus*, these two species appear in a pair-wise way in three geographically independent lineages. The first possibility would be that a protoform of *O. japonicus* (or *O. dehaanii*) that was isolated geographically into the three regions so as to have formed three distinct lineages. During independent accumulation of nucleotide substitutions in mitochondrial DNA in the respective lineages, a type-switching (i.e., morphological transformation) from *O. dehaanii* to *O. japonicus* (or vice versa) took place in parallel within each lineage. Such a possibility is not unlikely, because parallel morphological evolution has been known not only among the species in the same genus but even among the different genera or tribes. Thus, Su *et al.* adopted the possibility of parallel morphological evolution. The second possibility to explain this phenomenon would be that the speciation may have occurred only once in each case, meaning that the appearance of two species in one lineage may be explained as the result of random linear sorting among polymorphic mitochondrial haplotypes into the ancestral population. In this genus, however, the distribution of the “same species”, as represented by mitochondrial sequences, is not random. It is difficult, moreover, to imagine that the protoform of the present-day species had a mitochondrial polymorphism that would affect the appearance on the tree. The third possibility is that the pair-wise occurrence of two species in the tree is the result of horizontal transfer of mitochondria resulting from hybridization of the two species (mitochondrial introgression). If the hybrid hypothesis is correct, then the *O. japonicus* and the *O. dehaanii*-type mitochondria should be identifiable within one region. This, however, is not the case. Thus, the introgression, if it is the case, must be accompanied by some other special mechanism(s), for example, such that mitochondria in one species, *O. japonicus* (or *O. dehaanii*) were replaced by those of *O. dehaanii* (or *O. japonicus*) in all the regions. In this case, hybridization should have taken place between *O. dehaanii* (♂) and *O. japonicus* (♀) (or vice versa) and the authentic *O. japonicus* (or *O. dehaanii*) should have become extinct. This may be proved or disproved, at least partly, by analysis of nuclear DNA (see below).

Incongruence between the mitochondrial and nuclear DNA phylogenetic trees has in fact been reported in several organismic groups including *Ohomopterus*, insisting that the incongruence is due to the mitochondrial introgression, albeit without giving any convincing evidence or explanation of the mechanism involved.

Here, to approach more reasonable *Ohomopterus* phylogeny, we have constructed phylogenetic trees of mitochondrial ND5 gene and the nuclear ITS 1 from all the species and many “subspecies”, that reveal a series of little known phenomena to be described in this article. The authentic species that have retained both the original nuclear and mitochondrial lineages are assumed to be only five, the remainders having passed hybridization with another species at least once. The hybrid-derived “species” almost always carries mitochondrial type of one species (A), while morphologies of the offspring reveal those of the other species (B) that participated in the ancestral hybridization with the species A. This would imply that hybridization is
unidirectional as has been assumed as the third possibility (see above), i.e., \( A(\,+) \times B(\,\)\:\( -)\) and not vice versa, resulting in replacement of mitochondria of the species B by that of the species A. In some cases, such hybrid-derivatives, without hybrid breakdown, expanded their distribution range where the species involved in the hybridization did not inhabit. As the same token, in considerable number of cases, the flow of mitochondria of a certain ancestral species occurred consecutively from one species to a second,
which then became occupied the whole distribution range of the second species. In these cases, the original mitochondrial lineage of the species that directly branched off from the ancestral *Ohomopterus* mitochondrial lineage became completely replaced and disappeared. An example of the emergence of new species from a hybrid-derivative has been also presented.

**Materials and methods.** *Sampling.* We collected specimens from various localities covering the distribution ranges of all the *Ohomopterus* species. The sampling list and locality map where the samples were collected are shown in Supplementary data and Fig. 1, respectively. Fig. 2 includes geographic names that will appear in this article. Many more samples of *O. albrechti* (s. lat.) and *O. yamato* were analyzed for the ND5 gene, which are not included in this paper, because the discussion in this paper is not at all affected by them. The specimens were preserved in 99% ethanol until use for DNA extraction.

**DNA extraction, PCR and sequencing.** Total DNA was prepared from thorax muscle of a single individual using a QIAamp DNA Mini Kit (QIAGEN, Germany). DNA for each specimen was finally dissolved in 200 µl elution buffer, and 2 µl of the DNA solution were used as a template for amplification of DNA fragments by polymerase chain reaction (PCR). The fragment of the mitochondrial DNA containing 1069 bp 3’-region of the NADH dehydrogenase subunit 5 (ND5) gene was amplified using a primer pair shown in our previous paper. For amplifying the internal transcribed spacer (ITS) region of the nuclear ribosomal gene, a primer set (ITS-1: 5’-AAGTCGTAACAAGGTTCGC-3’ ITS-4: 5’-TCCTTGGTTAGTTTCTTTCTTC-3’) was used. The thermal conditions for amplification were as follows: denaturation at 94°C for 5 min; 35 cycles of denaturation at 94°C (30 sec), annealing at 50°C (30 sec), extension at 72°C (2 min); and final extension at 72°C for 7 min. Direct sequencing was performed with an automated ABI PRISM 3100 Genetic Analyzer (Applied Biosystems Inc., HITACHI). Some samples, which were not possible for direct sequencing, were subjected to cloning, and several clones from one individual were sequenced.

**Phylogenetic Analyses.** Multiple alignments of DNA sequences of ITS I region and the ND5 gene were carried out by MAFFT, a multiple sequence alignment program developed by Katoh et al. and manually inspected on the XCED sequence alignment editor with default settings. A NEXUS format file of the complete alignment was used for all phylogenetic analyses with a beta version of PAUP 4.0b. The neighbor-joining (NJ) analysis was adopted and construction of the trees was performed using evolutionary distance computed by Kimura’s two-parameter method. All the trees were evaluated using the bootstrap test based on 1000 replicates.

**Morphological classification used in this study.** As mentioned in Introduction, the classification of the *Ohomopterus* species is complicated. Nakane recognizes 5 to 6 species with several subspecies under each species, while some others classified them into 16-17 species with many subspecies. In the present paper, the number of species is kept minimum for convenience (Table I). Fig. 3 shows the distribution ranges of the morphological species used in this study.

**Results and discussion.** Brief account of *Ohomopterus* phylogeny inferred from nuclear ITS I and mitochondrial ND5 gene sequences. A phylogenetic tree of the nuclear internal transcribed spacer I (ITS I) (Fig. 4) revealed that the *Ohomopterus* ancestor first separated into two lineages, one containing *O. yamato* and *O. albrechti*, and the other con-
Table I. Classification used in this study

| Species group | Species and subspecies in the species group | Symbol in distribution map and phylogenetic trees |
|---------------|--------------------------------------------|--------------------------------------------------|
| kiiensis      | kiiensis                                   | ▲                                                 |
|               | iwawakianus                                | ▲                                                 |
|               | i. muro                                    | ▲                                                 |
| yaconinus     | yaconinus                                  | ▼                                                 |
|               | y. blairi                                  | ▼                                                 |
| japonicus     | japonicus                                  | ●                                                 |
|               | j. tsushimae                               | ●                                                 |
|               | chugokuensis                               | ●                                                 |
|               | daisen                                    | ●                                                 |
|               | d. okianus                                 | ●                                                 |
| dehaanii      | dehaanii                                  | ○                                                 |
|               | tosanus                                   | ○                                                 |
| arrowianus    | arrowianus                                 | ●                                                 |
|               | a. murakii                                 | ●                                                 |
|               | a. kirimurai                               | ●                                                 |
| maiyasanus    | maiyasanus                                 | ◊                                                 |
|               | m. shigaraki                               | ◊                                                 |
|               | m. yoroensis                              | ◊                                                 |
| insulicola    | insulicola                                 | △                                                 |
|               | i. shinano                                 | △                                                 |
|               | i. sado                                   | △                                                 |
|               | i. kiso                                   | △                                                 |
|               | esakii                                    | △                                                 |
| yamato        | yamato                                    | □                                                 |
| albrechti     | albrechti                                 | ■                                                 |
|               | lewisianus                                 | ■                                                 |
|               | kirimurai                                 | ■                                                 |
| uenoi         | uenoi                                     | ★                                                 |

Classification is based on morphology. Only the species and subspecies relevant to this paper are shown in this table among many more subspecies that have been so far described. Based mainly upon morphology of male genital organ, Nakane\(^{13}\) classified the *Odomopterus* species into 6 species, i.e., *dehaanii* (including subspp. *tosanus* and 4 more subspecies or a variety), *insulicola* (including subspp. *arrowianus esakii*, and *maiyasanus*), *uenoi*, *yaconinus* (including subspp. *blairi*, *iwawakianus* and *kiiensis*), *japonicus* (including subspp. *tsushimae*, *chugokuensis*, *daisen* and *daisen okianus*) and *albrechti* (including *yamato*, *lewisianus*, *esakii* and *freyi*). Later, Nakane\(^{14}\) combined *albrechti* and *yamato* and other subspecies in the *albrechti* group with *japonicus*. Ishikawa\(^{15}\) and his co-workers\(^{26},27\) recognized *yamato*, *lewisianus*, *albrechti*, *daisen*, *iwawakianus*, *esakii*, *arrowianus* and *maiyasanus* as independent species with descriptions of one more species *kirimurai* and many subspecies. Imura\(^{28}\) separated *chugokuensis* as an independent species from *japonicus* and described a number of subspecies of *japonicus*, *dehaanii* and *yamato*. Many subspecific names other than those included in this table are not adopted for the phylogenetic trees and the text except those of *O. maiyasanus*, because they do not affect the discussions in this paper. The subspecies of *O. maiyasanus*, which are not included above table, are enumerated in Table IV to discuss their complex genetic structure, which is relevant to other parts of this paper.

By contrast, a tree of mitochondrial NADH dehydrogenase subunit 5 (ND5) gene (Fig. 5) differed from that of ITS I in many respects. There existed 8 geographically linked lineages on the tree (Fig. 5), i.e. the lineages of the Kinki District (abbreviated as KNK), the Central Japan (= the Chubu District) (CJP), the Kii Peninsula (KII), Kyushu/San-in (KSI), San-yo (SYO), Shikoku (SHK), the Japan Sea Is/E. Japan (JSE) and the Western Japan (WJP).
Fig. 3. Distribution ranges of the *Ohomopterus* morphological species. (a) *O. yaconinus* (Y), *O. kiensis* (Ki) and *O. iwawakianus* (Iw). Wy-Ey Line indicates the distribution boundary of *O. yaconinus* (W) and *O. yaconinus* (E). (b) *O. dehaanii* (s. lat.; D). Wd-Ed Line indicates the distribution boundary of *O. dehaanii* (W) and *O. dehaanii* (E). *O. dehaanii* (s. lat.) includes *tosanus* and *kawanoi* (t & k) in Shikoku. (c) *O. japonicus* (s. lat.; J), *O. yamato* (Ya) and *O. albrechti* (Al). *O. japonicus* (s. lat.) includes *daisen* (Da) and *chugokuensis* (C). *O. albrechti* (s. lat.) includes *lewissianus* (lw) and *kimurai* (km). (d) *O. maiyasanus* (M), *O. arrowianus* (A), *O. insulicola* (I) and *O. uenoi* (U). *O. insulicola* (s. lat.) includes *esakii* (e). *O. insulicola* is distributed in the Kanto District, the Tohoku District and the western tip of Hokkaido. It is also the case for *O. albrechti*, except that it is distributed in almost entire region of Hokkaido. For morphological species and subspecies (s. str.), see the second column of Table I. For W and E in (a) and (b), see the legend of Table II.

[In our previous paper,2] these lineages were treated as sublineages, some belonging to the western Japan lineage, and some others to the eastern Japan lineage. To avoid complexity, no distinction was made between the previously used two lineages. These lineages were not necessarily associated with the morphological species in such a way that most of the lineages contain two or more morphological species. For example, three lineages are distributed respectively in Kyushu/San-in (KSI), Shikoku (SHK), and San-ya (SYO), and all consist of two species, *O. japonicus* and *O. dehaanii*, although the lineage WJP contained solely *O. yaconinus*. *O. dehaanii* was also found as a member of the lineage KNK clustering with several other species. The ND5 gene of the species in the lineage KNK seems to share the common ancestry originated from that of *O. maiyasanus* that emerged first within KNK as shown in the phylogenetic tree of Fig. 5 (see also Fig. 2 in Ref. 2). The lineage JSE contained *O. japonicus*, *O. yamato*, *O. albrechti* and *O. insulicola*, the distribution range of which may be arranged in the above order from south-western Japan Sea Islands to northern Japan. *O. yaconinus* appeared in two independent lineages, one in western Japan (WJP) without contamination of other species, and the other in KNK, which was clustered with several other species. Two more lineages, the KII and the CJP were primarily
Fig. 4. Continued to the next page.
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Fig. 4. NJ-phylogenetic tree of the *Ohomopterus* species based on the nuclear internal transcribed spacer I (ITS I) sequences. The ITS I sequences of several species of *Coptolabrus* (a genus belonging to the Carabinae) were used as outgroup to construct the tree. The number in each branching point indicates the bootstrap percentage (those less than 50% are not shown). Symbols and specimen numbers before scientific names correspond to those of Table I and Figs. 1, 4 and 5. The localities where the specimens were collected are shown in the parentheses after the scientific names with their names of their prefectures. The ITS I sequences were analyzed only for the individuals which we considered to be meaningful for discussion, and therefore not all the individuals analyzed for ND5 sequences were included in the ITS I tree. Many samples identified as *O. maiyasanus* and its subspecies were purposely omitted from the phylogenetic trees of Figs. 4 and 5 to avoid much confusion. For the details of them, see Table IV and Discussion.

- 55-arrowianus kirimurai (Mihama, Mie)
- 47-arrowianus (Neba, Nagano)
- 54-arrowianus (Gifu, Gifu)
- 42-arrowianus (Sakumaharuno, Shizuoka)
- 46-insulicola sado (Sado, Niigata)
- 49-insulicola kiso (Kisofukushima, Nagano)
- 21-arrowianus muraki (Seiwa, Mie)
- 22-arrowianus muraki (Toba, Mie)
- 18-arrowianus muraki (Futami, Mie)
- 56-arrowianus (Hase, Nagano)
- 33-maiyasanus (Aogaki, Hyogo)
- 34-maiyasanus (Omiva, Kyoto)
- 48-arrowianus (Toyota, Aichi)
- 53-arrowianus (Nagiso, Nagano)
- 37-maiyasanus (Wajima, Ishikawa)
- 140-daisen okianus (Oki, Shimane)
- 139-daisen okianus (Oki, Shimane)
- 141-daisen okianus (Oki, Shimane)
- 100-insulicola (Hiraizumi, Iwate)
- 153-insulicola (Kimitsu, Chiba)
- 49-insulicola kiso (Kisofukushima, Nagano)
- 157-insulicola (Hatano, Kanagawa)
- 156-insulicola (Otsuki, Yamanashi)

- 132-yamato (Asuka, Nara)
- 131-yamato (Mt. Kongo, Osaka)
- 133-yamato (Minamikawachi, Osaka)
- 146-albrechtii (Samani, Hokkaido)
- 144-albrechtii (Oirase, Aomori)
- 145-albrechtii (Hara, Fukushima)
- 143-albrechtii (Sado, Niigata)
- 134-albrechtii (Obuchi, Yamanashi)

Fig. 4. NJ-phylogenetic tree of the *Ohomopterus* species based on the nuclear internal transcribed spacer I (ITS I) sequences. The ITS I sequences of several species of *Coptolabrus* (a genus belonging to the Carabinae) were used as outgroup to construct the tree. The number in each branching point indicates the bootstrap percentage (those less than 50% are not shown). Symbols and specimen numbers before scientific names correspond to those of Table I and Figs. 1, 4 and 5. The localities where the specimens were collected are shown in the parentheses after the scientific names with their names of their prefectures. The ITS I sequences were analyzed only for the individuals which we considered to be meaningful for discussion, and therefore not all the individuals analyzed for ND5 sequences were included in the ITS I tree. Many samples identified as *O. maiyasanus* and its subspecies were purposely omitted from the phylogenetic trees of Figs. 4 and 5 to avoid much confusion. For the details of them, see Table IV and Discussion.

composed of *O. kiiensis* and *O. arrowianus*, respectively (Fig. 5).

**Reflection of ITS I lineages in the morphological species groups.** The phylogenetic tree of ITS I reveals that the lineages correspond to those of species groups [*morphological species (s. lat.)*] defined in Table I and the lineages largely reflected their history (Fig. 4)[17]–[19] with exceptions of *O. uenoii* (see later) and *O. daisen okianus*. One possibility would be that *O. daisen okianus* emerged earlier than the other *O. japonicus* (s. lat.). Another possibility would be that the ITS I of *O. daisen okianus* is a recombinant of those of *O. japonicus* and *O. yamato* or *O. albrechtii* formed upon hybridization of these two species (see below), and the recombinant ITS I has retained until now without replacement of the authentic *O. japonicus* ITS I, presumably because the islands would have been geographically isolated as a small restricted area from the mainland. There is no evidence to decide which possibility is the case at present. The fine resolution of the species and subspecies in the second column of Table I is not possible because of the sequence differences are not enough to distinguish them. As mentioned in Introduction and will be discussed below, incongruence of the ITS tree and the ND5 tree suggests not uncommon occurrence of hybridization between two species, showing an approximate correspondence of the ITS I lineages to the morphological species groups. This suggests that the genetic structures of the hybrid derived population approaches those of the original authentic morphological species. Thus, their ITS I tree largely repre-
Fig. 5. NJ-phylogenetic tree of the Ohomopterus species based on the mitochondrial ND5 gene sequences. The sample numbers are given to the specimens in the order from top to the bottom in the tree. The three-letter abbreviations indicate the phylogenetic lineages, below which the probable origin of mitochondrial species in each lineage is indicated (see text). For other explanations such as bootstrap values, symbols and locality names, see the legends of Fig. 4.

Sents the species history. There are, however, some exceptions in that the ITS I of some hybrid offspring deviates from the expected one. For example, some KNK yaconinus carries mostly yaconinus-type ITS I (Nos. 3, 7, and 14), but ITS I of some of them is of the kiiensis-type (No. 1; for the detailed process of this, see below). This is probably because almost all genes including the gene group responsible for the morphological characters, but not ITS I, became to be included in the offspring in these exceptional cases, because the genetic architecture of the hybrid offspring is unlikely to have been recovered to the one having exactly the same composition as the original one, even with the occurrence of repeated backcrosses.

Mitochondrial flow and the fate of hybrid-derived population. As described above, most of the lineages in the ND5 tree consist of more than one species. The situation is largely inconsistent with that of the nuclear ITS I tree, suggesting the occurrence of extensive mitochondrial introgression among the Ohomopterus species. Based on the compari-
son between the ND5 and the ITS phylogenetic trees (Figs. 4 & 5), together with the profile of the distribution range of each species, the origin and the flow of the ND5 gene have been tentatively assumed as shown in Fig. 6, respectively. Taking altogether, the authentic species that have maintained both the original nuclear and mitochondrial lineages could be only five, *O. japonicus* (part), *O. kiiensis*, *O. arrowianus* (part) and *O. yaconinus* (part) and *O. maiyasanus* (part), while the remainders such as *O. dehaanii*, *O. yamato*, *O. albrechti*, *O. insulicola*, *O. iwawakianus*, *O. uenoi* and many subspecies (*O. arrowianus* murakii, *O. insulicola* kiso, etc.) have passed hybridization with another species at least once as judged by
Table II. Mitochondrial ND5 gene and nuclear ITS I of all *Ohomopterus* species and a part of subspecies

| Morphological species | Type of ND5 | Type of ITS I |
|-----------------------|-------------|---------------|
| *japonicus*           | *japonicus* | *japonicus*   |
| *kiiensis*            | *kiiensis*  | *kiiensis*    |
| *arrowianus*          | *arrowianus*| *arrowianus*  |
| *maiyasanus*          | *maiyasanus*| *maiyasanus*  |
| *yaconinus* (W)       | *yaconinus* | *yaconinus*   |
| *albrechti*           | *japonicus* | *albrechti*   |
| *yamato*              | *japonicus* | *yamato*      |
| *dehaanii* (W)        | *japonicus* | *dehaanii*    |
| *dehaanii* (E)        | *maiyasanus*| *dehaanii*    |
| *yaconinus* (E)       | *iwawakianus*| *yaconinus/insulicola* |
| *iwawakianus*         | *maiyasanus*| *kiiensis*    |
| *insulicola*          | *arrowianus*| *arrowianus/insulicola* |
| *arrowianus* murakii  | *iwawakianus*| *arrowianus*  |
| *maiyasanus*          | *maiyasanus*| *iwawakianus* |
| *insulicola*          | *japonicus* | *insulicola*  |
| *uenoi*               | *arrowianus*| *kiiensis*    |

*1) *iwawakianus* is the offspring of *O. kiiensis* × *O. maiyasanus*.17*

*2) *maiyasanus* is a mixture of intrinsic *maiyasanus* plus offspring of hybrids such as with *iwawakianus*, *kiiensis* or *arrowianus*.17*

*3) *insulicola* includes esakii. Most of the “subspecies” of *insulicola* distributed along the Itoigawa-Shizuoka Tectonic Line are the hybrids or hybrid-derivatives with *arrowianus*.45 (W) and (E) denote western Japan and eastern Japan populations for both *yaconinus* and *dehaanii*. The ITS I of *yaconinus* is either of the *yaconinus*-type or of the *iwawakianus*-type (for possible explanation of the latter, see the text).*17–19*

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Fig. 6. Flow of mitochondria predicted from the phylogenetic trees. Arrow indicates the direction of mitochondrial flow.

1) *iwawakianus* is derived from the hybrid of the female of *maiyasanus* and the male of *kiiensis*, except *iwawakianus muro* which is equivalent to *O. kiiensis*, because it has the *kiiensis*-type ND5 gene.17

2) *uenoi* is derived from the hybrid of the female of *arrowianus* and the male of *kiiensis*.

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The incongruence of profile between the ND5 gene and ITS I or the presence of the heterozygous individuals containing the ITS I of two species (Table II).17–19 The mode of the mitochondrial flow and the fate of hybrid-derivatives may be classified into the following four categories.

1) **Total replacement of authentic mitochondria of a species by those of another species.** As seen in Fig. 5, *O. japonicus* (*O. j. tsushimae* and *O. daisen okianus*) in the Japan Sea Islands, *O. yamato*, *O. albrechti* and *O. insulicola* form a single cluster JSE on the ND5 tree. This strongly suggests that their ND5 genes are derived from the common ancestor, while in the ITS I tree (Fig. 4) *O. japonicus*, *O. daisen okianus*, *O. yamato/O. albrechti* and *O. insulicola* form the independent lineages, respectively. *O. yamato/O. albrechti* is the more ancient lineage as compared with the rest of the species, while on the ND5 tree the branching point of *O. yamato/O. albrechti* is by no means older than that of the other *Ohomopterus* species. These facts suggest that the ND5
gene of *O. yamato/O. albrechti* was replaced by that of the common ancestry of JSE. The direction of the flow of the ND5 gene is not decisively determined because of a poor resolution of each species in the JSE ND5 tree. However, it would be reasonable to assume that the flow took place beginning from *O. daisen okianus* and then to *O. yamato* and *O. albrechti* (or the ancestor of these species) and finally to *O. insulicola*, since at the time of upheaval of the Japanese Archipelago including their coastal area of the Japan Sea Island proceeded from west to east and a simple maximum parsimony (MP)-tree of the ND5 gene has shown the branching order of *yamato, albrechti* and then *insulicola*. Thus, the ND5 gene of the cluster JSE would have originated from *O. daisen okianus* in the Japan Sea Islands, and the intrinsic ND5 gene of the other species in this cluster would have been totally replaced by that of *O. daisen okianus* through unidirectional hybridization (Fig. 7a). An alternative though less likely possibility would be that the ND5 gene of the JSE cluster originated from *O. insulicola*, and that the intrinsic ND5 gene of the other species in this cluster has been totally replaced by that of *O. insulicola*.

(2) Sympatric distribution of two species, one authentic and the second of hybrid origin. The distribution range of *O. japonicus* overlaps with that of *O. dehaanii* in Kyushu, Shikoku and Chugoku District (Fig. 3b and c). The ND5 gene from these two species in the above overlapped distribution ranges are intermingled so as to form three different subclusters (KSI, SHK and SYO) (Fig. 5), while their ITS I's are not, forming the well-defined species-specific clusters (Fig. 4). These facts suggest that the mitochondrial gene flow took place from one species to another, presumably from *O. japonicus* to *O. dehaanii*, because the ITS I of *O. japonicus* began to diversify earlier than *O. dehaanii*. Thus, *O. japonicus* would have expanded its distribution throughout western mainland (Kyushu, Chugoku District and Shikoku) as well as the Japan Sea Islands before emergence of *O. dehaanii*. Therefore, *O. dehaanii* in western Japan have branched off from some population of *O. japonicus* (but not from the Japan Sea Islands population, because *O. dehaanii* does not inhabit there). *O. dehaanii* then expanded its distribution and its male hybridized unidirectionally with the female of *O. japonicus*, finally resulting in the complete replacement of mitochondria of *O. dehaanii* by those of *O. japonicus*. Thus, the intrinsic mitochondrial lineage of *O. dehaanii* does not exist at present, implying that the western *O. dehaanii* mitochondria had been derived totally from those of *O. japonicus* (Fig. 7c).

A series of observations described so far suggests strongly that the ND5 gene (and therefore mitochondria) of many *Ohomopterus* species was originated from those of *O. japonicus* group.

(3) Hybrid-derived offspring of a species invaded to new territory. When a species A immigrated to a new territory and hybridized with a species B at a limited area of their borderline, the resultant hybrid-derivatives carrying the mitochondria of the species B (not of the species A!), expands their distribution apparently keeping the original morphological characters of the species A (Fig. 7b). Two such examples are given below.

An extensive expansion of the distributional range by the hybrid-derived offspring population may be typically exemplified by *O. dehaanii* that is widely distributed in western Japan (Kyushu, Chugoku District, Shikoku). As described above, the mitochondria of *O. dehaanii* was replaced by those of *O. japonicus*. This species is also distributed in the
Kinki and Chubu Districts having the ND5 gene that clusters with other species in the KNK lineage (presumably derived from the O. maiyasanus ND5 gene). This KNK type O. dehaanii is clearly separated from that of the western Japanese type (KSI, SHK and SYO in Fig. 5), while the ITS I from the Kinki and Chubu Districts is included in the cluster to which the western population also belongs (the dehaanii-lineage in Fig. 4). Since distribution range of O. dehaanii is mainly the western Japan, it may not be unreasonable to suppose that a part of the western population would have invaded the Kinki District, and its male unidirectionally hybridized with the female of O. maiyasanus, followed by recent propagation of the hybrid-derived O. dehaanii within Kinki and Chubu Districts. In other words, the mitochondria of the KNK type O. dehaanii is replaced first by those of O. japonicus and then those of O. maiyasanus. The border of this species inhabiting western Japan and that in the Kinki-Chubu District lies across the east of Shikoku/Awajishima Island to the Sea of Japan through Hyogo/Kyoto Prefectures (Fig. 3b).

An expansion of the hybrid-derived population may also be seen in O. yaconinus. This species inhabiting western Japan (WJP in Fig. 5) is monophyletic with respect to both the ND5 gene and ITS I, while the ND5 gene of the same species in the Kinki District (KNK) is independent from WJP, clustering with that of several other species of KNK (presumably O. iwawakianus whose mitochondria were assumed to have originated from O. maiyasanus by hybridization of the male of O. kiiensis with the female of O. maiyasanus) (Fig. 5). By contrast, the ITS I of O. yaconinus forms a single cluster regardless having the WJP-type or KNK-type ND5 gene (with some exceptions; see below). A reasonable explanation from these observations would be that O. yaconinus in western Japan migrated eastwards, and the male of it unidirectionally hybridized somewhere in the Kinki District with the female of O. iwawakianus, thus having resulted in replacement of the authentic ND5 gene by that of O. iwawakianus. Involvement of O. iwawakianus in the hybridization may be not unreasonable because of the presence of some specimens (for example No. 1 in Fig. 4) of O. yaconinus in Kinki District, carrying the ITS I of the kiiensis/iwawakianus lineage which, by chance, would have been introduced in place of the yaconinus-type ITS I at a certain step of hybridization.

The unidirectionality is evidenced by the complete failure of detecting the WJP ND5 gene in the distribution range of the hybrid-derived O. yaconinus or in any other Ohomopterus species in the Kinki District (Fig. 5). Thus, only the hybrid-derived individuals, and not the authentic ones, expanded its distribution so as to have occupied their present distribution range. The border of the authentic W-O. yaconinus and the hybrid-derived offspring (E-yaconinus) lies a little west of the Biwako Lake-Yodogawa River Line (see Figs. 2 and 3a).

The subspecies O. arrowianus murakii inhabiting a part of the Kinki District (northeastern area of the Kii Peninsula) carries the O. arrowianus-type ITS I and yet its ND5 gene is of the O. kiiensis-type despite of only a small morphological difference from O. a. arrowianus. This suggests that O. a. arrowianus migrated from the Chubu District to a part of the Kii Peninsula and its male hybridized unidirectionally with the female of O. kiiensis (or O. iwawakianus), and thus O. arrowianus murakii would most probably be such a hybrid-derived offspring.

(4) Hybrids of two distinct species around their distribution border. Distribution boundary between O. arrowianus and O. insulicola lies roughly along the Itoigawa-Shizuoka Tectonic Line (see Fig. 2). Independent ITS I clones from a single specimen in some of the specimens obtained along the tectonic line include both O. insulicola- and O. arrowianus-type ITS I sequences, as exemplified by a sample from Fujimi-Cho and Otari-Mura (O. insulicola shinano) and that from Kiso-Fukushima (Sample No. 49; O. insulicola kiso). On the other hand, their ND5 gene is of the O. arrowianus-type with morphological appearance of the O. insulicola-type and no homozygous individuals having O. insulicola-type ND5 gene were found, so that they may be considered F1 or nearby heterozygous individuals. The results show that hybridization occurs between O. arrowianus × O. insulicola and not vice versa, because mitochondrial inheritance is female-mediated and all the hybrid-derivatives so far found in this section carry the O. arrowianus-type ND5 gene. In other words, the hybridization is unidirectional with morphologies of the hybrids always of the O. insulicola-type.

Mitochondrial flow also occurred from O. arrowianus to O. yaconinus blairi (part) (Nos. 50, 51) and to O. maiyasanus (part) (No. 52) as in the case of O. arrowianus and O. insulicola (see Figs. 4 and
Table III. Morphology of hybrid-derived offspring resulted by unidirectional hybridization

| Recipient (A♂) of mitochondria | Donor (B♀) of mitochondria | Morphology of hybrid-derived offspring (A × B) |
|--------------------------------|----------------------------|-----------------------------------------------|
| dehaanii                      | japonicus                  | dehaanii                                      |
| dehaanii                      | maiyasanus                 | dehaanii                                      |
| yaconinus                     | iwawakianus                | yaconinus                                     |
| yamato                        | japonicus                  | yamato                                        |
| albrechti                     | yamato                     | albrechti                                     |
| insulicola                    | albrechti                  | insulicola                                    |
| yaconinus                     | arrowianus                 | yaconinus (blairi) (part)*¹                   |
| kiiensis                      | arrowianus                 | yaconinus (nr. kiiensis)                      |
| arrowianus                    | arrowianus                 | uenoi (nr. arrowianus)*²                      |
| arrowianus                    | iwawakianus                | arrowianus (murakii)*²                        |

*¹ Part of subs. blairi belongs to the authentic lineage and some others distributed around the tip of Noto Peninsula are the hybrid-derived offspring as shown in the table.¹⁸
*² Hybridization between kiiensis and arrowianus occurred in either direction.

5). Whether these hybrids are established as stabilized populations or they are merely unstable hybrids as in the case of O. arrowianus and O. insulicola should await until more examples are analyzed.

**Unidirectional hybridization.** As discussed above, the replacement of mitochondria by hybridization of two species occurred quite frequently. The hybridization is unidirectional, i.e., the species A (♂) hybridized with another species B (♀) and not vice versa except two examples (Table III), which will be discussed later. One explanation would be that it happened by chance, and another would be the participation of so-called "lock- and key" mechanism. The *Ohomopterus* species may be classified into four groups by the shape of copulatory piece of the male genital organ, i.e., small-triangle type (O. japonicus, O. yamato and O. albrechti), a little longer triangle (O. dehaanii), pentagonal (O. yaconinus, O. kiiensis and O. iwawakianus) and long and large hook-like (O. arrowianus, O. insulicola and O. uenoi). It has been stressed that male having long hook-like copulatory piece cannot copulate with female of the other types, so that the species isolation may be ascribed to the "lock- and key" mechanism.¹⁵ However, as enumerated above, this rule is not always the case. For example, O. arrowianus (♂) × O. iwawakianus (♀) produced O. arrowianus murakii and mitochondrial flow occurred from O. albrechti (♀) to O. insulicola (♂) upon hybridization, etc. In all these examples, a huge and long copulatory piece of these male is said to be unacceptable by a female genital organ of the mitochondrial donor species. Furthermore, the lock- and key relationship does not play any role to prevent hybridization between the species with the same type of copulatory piece, i.e., O. japonicus × O. yamato, O. yamato × O. albrechti and O. yaconinus × O. iwawakianus/kinesis. Thus, at present the isolation mechanism of species remains unsolved.

As mentioned above and shown in Table III, most of the hybrid-derived offspring reveal morphologies of the mitochondrial recipient-species, and not the intermediate between the two species involved in hybridization. Then, it is of interesting to see the morphologies of the hybrid F1 by laboratory crossing experiments.

**Exceptional case against unidirectional hybridization.** O. arrowianus in the Chubu District seems to be one of the authentic species retaining the authentic ND5 gene and the ITS I, while its subspecies murakii inhabiting a part of the Kinki District (northeastern area of the Kii Peninsula) carries the O. arrowianus/O. maiyasanus-lineage ITS I and yet its ND5 gene is almost the same to that of O. iwawakianus in the KNK lineage (Nos. 18, 21 and 22) despite only a small morphological difference from O. a. arrowianus. This suggests that O. a. arrowianus migrated from the Chubu District to a part of the Kii Peninsula and its male hybridized with the female of O. iwawakianus, and thus O. arrowianus murakii would most probably be such a
hybrid-derived offspring. O. uenoi resembles O. arrowianus in external appearance however with an exceptionally huge male genital organ. This species is distributed in a restricted area west of the habitat of O. a. murakii and has the ND5 gene of the CJP (O. arrowianus) lineage (Fig. 5) and the ITS of the O. kiiensis/O. iwawakianus-lineage (Fig. 4). This means that O. uenoi is a hybrid-derived offspring from O. arrowianus (female) and O. kiensis (or O. iwawakianus) (male). Thus these two examples (O. arrowianus murakii and O. uenoi) represent an apparent exception of the above-described unidirectional hybridization, i.e., hybridization between O. arrowianus and O. kiiensis/iwawakianus is not unidirectional and could occur in either direction (Table III).¹⁹

*Mechanism for establishment of hybrid-derived population.* In most cases, hybridization between two animal species results in hybrid-breakdown after F1 or at least after several generations even when occasional backcrosses take place.²⁰ In contrast to this general rule, continuous reproduction of the hybrid derivatives and/or their expansion of distribution range are not rare in the *Odomopterus* ground beetles. How are mitochondria of a species replaced by those of another species upon hybridization and how can the species having the replaced mitochondria be stabilized without hybrid breakdown? Takahata and Slatkin²¹ proposed a model that extremely strong selection against heterozygotes may prevent the transmission of mitochondria between two species, but otherwise the transmission can easily occur over species boundaries. Their model may be account for the transmission of mitochondrial DNA between two species of *Drosophila*²² and between two subspecies of *Mus musculus.*²³ The Takahata-Slatkin’s model requires the occurrence of backcrosses of the hybrids in every generation until transmission of mitochondria ceases. This may be one explanation for the establishment of hybrid-derived populations accompanied by the mitochondrial flow, for example, from *O. japonicus* to *O. yamato/O. albrechti,* where the mitochondria of *O. yamato/O. albrechti* (hereafter called the “recipient” species) are replaced by those of *O. japonicus* (the “donor” species). This possibility that backcrossing of these hybrids occurred continuously as a result of the habitation of the mitochondrial recipient species throughout the distribution range of the recipient species suggests that this may have occurred easily (Fig. 7 a).

The invasion of W.-*O. dehaanii* or W.-*O. yakoninus* to the Kinki-Chubu Districts seems to require a model somewhat different from that of Takahata-Slatkin. There is no evidence whether the supply of the male of the “recipient” species enough to fill the requirement for the repeated backcrosses after invasion of the “recipient” species (for example W.-*O. dehaanii*) to a limited territory of the “donor” species (O. maiyasanus), between which unidirectional hybridization occurred (Fig. 7 b). Moreover, even if the occasional backcrosses occur, the offspring of the invaded W.-*O. dehaanii* and the hybrid individuals would have become to be the target of random drift within a limited size of population. If this is the case, the offspring of W.-*O. dehaanii* was either expelled by the hybrid so that the mitochondria of all the invaded W.-*O. dehaanii* (“recipient”) are completely replaced by those of the “donor” species, or the hybrid-derivatives (W.-*O. dehaanii*) would have been excluded. However, what we actually observed is only the former event, and no cases have been found for the occurrence of the latter event. A similar situation is also the case for the invasion of W.-*O. yakoninus* to the Kinki District to have formed K.-*O. yakoninus.* Therefore, there is some difficulty to explain these events by a simple random drift.

Another explanation would be that one of the heterozygote acquired a hybrid vigor that was caused by some introduced “donor” gene(s) and/or by some advantageous effect of the transmitted “donor” mitochondria. In fact, the effect of modified longevity by mitochondria was reported by Rand *et al.*²⁴ Theoretically, a possible existence of the hybrid-derived populations having the phenotype of the “donor” species (e.g., *O. maiyasanus*-phenotype) could not be excluded, because a certain zygote in F2 could have acquired a part of the “donor” DNA and became approached the authentic *O. maiyasanus* in its gene composition after repeated backcrosses by widely distributed authentic *O. maiyasanus* around the area where the hybridization between the two species occurred. Unless the whole genome, or at least some other nuclear genes, is analyzed, such individuals, even if they exist, cannot be detected, because they would carry the original “donor” mitochondria, and presumably reveal the morphologies that are hardly distinguishable from those of the authentic “donor” species.

As mentioned above, *O. japonicus* and *O. dehaanii* co-inhabit in western Japan, and the ND5
gene of *O. dehaanii* is assumed to have been replaced by that of *O. japonicus* by the unidirectional hybridization of these two species. In other words, the mitochondrial donor (*O. japonicus*) and the recipient (*O. dehaanii*) inhabit sympatrically. In this case, backcrosses took place in the mitochondrial recipients extensively to approach the original nuclear genome composition (Fig. 7c).

Species, subspecies and hybrid-derived populations. As described in this article, the examination of morphology alone cannot reveal the insight into the biological species as may be seen by an almost indistinguishable morphologies between *W.-O. dehaanii* with E-*O. dehaanii*, or *W.-O. yaconinus* with E-*O. yaconinus*, etc. In other words, the authentic species and the hybrid-derived species, even though their morphologies are quite similar to one another, can be distinguished only by examination of their molecular phylogenetic properties. Subspecies are generally considered as geographic races, but similarly to the morphological species, many subspecies so far described are of the hybrid-origins such as *O. arrowianus murakii* (Nos. 18, 21, 22), *O. maiyasanus takasharesis* (No. 25), *O. m. yoroensis* (No. 20), *O. yaconinus blairi* (Nos. 50, 51), *O. insulicola kiso* (No. 49), *O. i. shinano* (Nos. 45, 58, 59), etc. However, once the hybrid-derived population is stabilized and geographically isolated, the geographic races of the hybrid-origin may be established. How should the authentic species (or subspecies) and its hybrid-derived “species” (or “subspecies”) be treated in taxonomy? Another problem would be whether the subspecies is of preceded and indispensable step for evolution of the species or not. Mayr20 noted “in many cases the subspecies is an artifact and is not a unit of evolution”. In the genus *Ohomopterus*, there exist no clear cases in which the subspecies evolved to the species, except a remarkable example of an apparent emergence of a morphological species from the hybrid-derived “subspecies” as described below.

Emergence of a morphological new species from a hybrid-derived population. As has been described above, *O. uenoi* resembles *O. arrowianus* in external appearance with an exceptionally huge male genital organ (and yet inner plate of ligular apophysis in female genitalia is similar to *O. kiiensis*-type), thus recognizing as a distinct morphological species. This species is distributed in a restricted area in Kii peninsula and has the *O. arrowianus*-type ND5 gene and their ITS I of the *O. kiiensis/O. iwawakianus*-lineage.19) Presumably, the hybrids between the above two species were geographically isolated, and then evolved to the distinct species.

Unsolved problems. As may be seen above, there are a number of phenomena, the final answers of which should be awaited until more extensive works are done. One of them, which has not been discussed above, an apparent morphological convergence observed between *O. arrowianus* and *O. insulicola*. As shown in Fig. 3c and d, *Ohomopterus* species inhabiting Sado Islands have been believed to be *O. insulicola* and *O. albrechti*, and the distribution of *O. arrowianus* has been excluded from there. The sequences of the ND5 and ITS I from what morphologically looks like *O. insulicola* (subsp. *sado*) are both *O. arrowianus*-type so far as we have examined (No. 46; more samples were analyzed; data not shown), although Sota et al.25) claimed the existence of individuals having *O. insulicola*-type ND5 gene in addition to those having the *arrowianus*-type. The same situation is also true for a part of our samples of “*O. insulicola*” *shinano* (No. 45) and “*O. insulicola*” *kiso* (No. 49), namely the presence of heterozygous individuals having the *arrowianus*-type ND5 and both the *arrowianus* and *insulicola*-type ITS I (data not shown). Whether this represents the convergence of *O. arrowianus* morphology to *O. insulicola* remains to be solved.

Another problem is the sequence of events through which the quite complex *Ohomopterus* fauna in the Kinki District has been formed. *O. iwawakianus*, which is the offspring of the hybrid between the male of *O. kiiensis* and the female of *O. maiyasanus*, has extensively participated in this process.17) In addition to *O. iwawakianus*, *O. maiyasanus*, *O. arrowianus* and *O. kiiensis* are also involved in such a complexity, while it is rather strange that *O. yamato* is not included in this event. The most striking is the incredible complex molecular structure of *O. maiyasanus*, in which hybridizations with other species or the “subspecies” of the same species would often have occurred more than twice. Therefore, the process, including the directionality of hybridization, which has led to the present-day inhabitants, is hard to trace in many cases. Ishikawa and Kubota29) classified *O. maiyasanus* into 7 subspecies based on minor morphological differences in conjunction with their distribution ranges (Fig. 8 & Table IV). The “authentic” individuals of *O. maiyasanus*, presum-
Table IV. Morphological subspecies of *Ohomopterus maiyasanus* and their molecular structures

| Morphological subspecies | Mitochondrial ND5 gene | Nuclear ITS I | Locality | Remarks |
|--------------------------|------------------------|---------------|----------|---------|
| *maiyanus*               | arr/arrowianus         | arr/maiya     | Azai, Shiga |         |
| *maiyanus*               | arr/arrowianus         | kii/miaya     | Hanzai, Gifu |         |
| *maiyanus*               | arr/arrowianus         | kii/miaya     | Mt. Hiei, Kyoto |         |
| *maiyanus*               | arr/arrowianus         | kii/miaya     | Naka, Hyogo | “authentic” |
| *maiyanus*               | kii/iwawaki +         | arr/maiya     | Ibaraki, Osaka | heterozygous |
| *maiyanus*               | kii/iwawaki +         | arr/maiya     | Minoo, Osaka |         |
| *maiyanus*               | kii/iwawaki +         | arr/maiya     | Nishiazai, Shiga |         |
| *maiyanus*               | arr/miaya              | kii/iwawaki   | Yoka, Hyogo | “authentic” |
| *maiyanus*               | arr/miaya              | kii/iwawaki   | Ono, Fukui |         |
| *maiyanus*               | arr/miaya              | kii/iwawaki   | Aogaki, Hyogo | “authentic” |
| *maiyanus*               | arr/miaya              | kii/iwawaki   | Omiya, Kyoto | “authentic” |
| *maiyanus*               | kii/iwawaki            | arr/maiya     | Fujiwara, Me |         |
| *kiiensis*               | arr/maiya              | NE            | Fujiiwa, Me |         |
| *hokurikuensis*          | arr/arrowianus         | arr/maiya     | Itoigawa, Niigata | heterozygous |
| *hokurikuensis*          | kii/iwawaki +         | kii/iwawaki   | Nagara, Gifu |         |
| *hokurikuensis*          | kii/iwawaki +         | kii/iwawaki   | Takefu, Fukui |         |
| *hokurikuensis*          | kii/iwawaki +         | arr/maiya     | Sueno, Fukui |         |
| *hokurikuensis*          | arr/arrowianus         | kii/iwawaki   | Shiratori, Gifu (2 exs.) |         |
| *hokurikuensis*          | arr/arrowianus         | kii/iwawaki   | Tsuruga, Fukui |         |
| *hokurikuensis*          | arr/arrowianus         | kii/iwawaki   | Shirakawa, Gifu |         |
| *hokurikuensis*          | arr/arrowianus         | kii/iwawaki   | Asahi, Toyama |         |
| *hokurikuensis*          | arr/arrowianus         | kii/iwawaki   | Unazuki, Toyama |         |
| *hokurikuensis*          | arr/arrowianus         | kii/iwawaki   | Himi, Toyama |         |
| *hokurikuensis*          | arr/arrowianus         | kii/iwawaki   | Wajima, Ishikawa | “authentic” |
| *hokurikuensis*          | kii/iwawaki            | kii/iwawaki   | Notojima, Ishikawa |         |
| *yoronsis*               | kii/iwawaki            | kii/iwawaki   | Hokusei, Me |         |
| *yoronsis*               | kii/iwawaki            | kii/iwawaki   | Hokusei, Me |         |
| *suzukanus*              | arr/arrowianus         | kii/iwawaki   | Komono, Me |         |
| *suzukanus*              | arr/arrowianus         | kii/iwawaki   | Yotkachi, Me |         |
| *suzukanus*              | arr/arrowianus         | kii/iwawaki   | Suzuki, Me |         |
| *suzukanus*              | kii/iwawaki            | kii/iwawaki   | Suzuki, Me |         |
| *suzukanus*              | kii/iwawaki            | kii/iwawaki   | Komono, Me |         |
| *suzukanus* ×             | kii/iwawaki            | kii/iwawaki   | Suzuki, Me |         |
| *iwawakanus*             | kii/iwawaki            | kii/iwawaki   | Kono, Shiga |         |
| *shigaraki*              | kii/iwawaki            | kii/iwawaki   | Odai, Me |         |
| *takiharensis*           | arr/arrowianus         | arr/maiya     | Odai, Me |         |
| *takiharensis*           | arr/arrowianus         | kii/iwawaki   | Miyagawa, Me |         |
| *okkawai*                | arr/arrowianus         | kii/iwawaki   | Ago, Me (2 exs.) |         |
| *okkawai*                | arr/arrowianus         | kii/iwawaki   | Ago, Me (2 exs.) |         |
| *okkawai*                | arr/arrowianus         | NE            | Isobe, Me |         |
| *okkawai*                | arr/arrowianus         | NE            |                |         |

In the ITS column, abbreviations, *arr/maiya* and *kii/iwawaki*, were used for the *arrowianus-maiyanus* and *kiiensis-iwawakanus* lineages, respectively. “Authentic” in the Remarks column denotes the probable authentic individual of *O. maiyanus* (the authenticity is however not decisive, because the ITS of *O. maiyanus* and *O. arrowianus* are indistinguishable in the phylogenetic tree, and the individual with the *maiyasanus* ND5-*arrow/maiya* ITS combination could appear in the hybrid between *O. maiyanus* and some other species after the F2 or the backcrossing of the hybrid with the authentic *O. maiyanus*). Note that the “authentic” *maiyasanus* specimens were mainly found along the coastal regions facing the Sea of Japan. Most of the morphological subspecies of *O. maiyanus* in this table are not included in Table I (see also the legend of Table I). NE: Not examined.
ably without having passed hybridization with other species (see legend of Table IV), are only rarely found in some nominotypical maiyas anus and subsp. hokurikuensis as judged by the congruency of the ND5 gene and the ITS I, while individuals of all other subspecies and most of maiyas anus and hokurikuensis do show the sufficient evidence of the past hybridization, i.e., incongruence of the ND5 gene and the ITS I. The ND5 gene of some individuals is of O. arrowianus and not O. maiyas anus in subsp. maiyas anus, hokurikuensis, suzukanus and takiharen sis. The most interesting fact would be that the ND5 gene of some individuals of subsp. yoroensis and suzukanus is of O. kiiensis (not O. iwawakianus)-type. These facts strongly suggest that O. arrowianus and O. kiiensis were once widely distributed in the Kinki District although their authentic species do not inhabit there at present. The ITS I of many individuals of the hybrid-derivatives is of O. iwawakianus-type as already reported by Imura et al.17) Another thing of worthwhile noted is that the each morphological subspecies defined by Ishikawa and Kubota20) by no means consists of a homogeneous population. So far as examined, maiyas anus, hokurikuensis, yoroensis, suzukanus and takiharen sis are respectively reveal 3, 3, 2 and 2 different genetic structures with respect to the ND5 gene and ITS I. Even in the same locality, more than two individuals with different genetic structure co-inhabit. Whether these phenomena represent the transient stage of convergence into a single population, or of a stage leading to the hybrid-breakdown are hard to decide by the phylogenetic analysis alone. The dominant occurrence of the hybrid-derived individuals throughout whole distribution regions might mean that the authentic O. maiyas anus will become extinct in the future.

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