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Molecular Phylogeny of Japanese *Catocala* Moths Based on Nucleotide Sequences of the Mitochondrial ND5 Gene

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Phylogenetic relationships of 31 Japanese *Catocala* species were analyzed based on the partial nucleotide sequences of the mitochondrial NADH dehydrogenase subunit 5 (ND5) gene (762 bp). When several non-*Catocala* Noctuidae moths were designated as the outgroup, these *Catocala* species formed a monophyletic group. However, divergences between these *Catocala* species were very deep, and no close phylogenetic relationships were recognized among them except for that between the two recently separated species, *C. xarippe* and *C. fulminea*. The remote relationships implied for several pairs of species suggest that the color of the hindwings is a changeable characteristic, and does not reflect phylogenetic lineage. Continental specimens were analyzed in 20 of 31 *Catocala* species, and all of them showed a close relationship with their Japanese counterpart. However, the closeness of the nucleotide sequences between the Japanese and continental individuals of the same species varied from species to species, indicating that isolation between the Japanese and continental populations of these species occurred at many different times. The two analyzed species endemic to North America showed a close relationship with their morphologically inferred Japanese counterparts, indicating that the geographic separation and following speciation between these Eurasian and American species occurred much more recently compared with the speciation events among the *Catocala* species now found in Japan.

Key words: *Catocala*, ND5, molecular phylogeny, hindwing, evolution

INTRODUCTION

The genus *Catocala* (Noctuidae, Catocalinae) contains about 260 species distributed in the temperate to frigid zones of the Palearctic and Nearctic regions (Ishizuka, 2005a; Ishizuka, 2005b). *Catocala* moths are single-brooded and hibernate as eggs, and their larvae are monophagous to oligophagous, feeding on broadleaf trees such as Rosaceae, Fagaceae, Salicaceae, and Leguminosae. Therefore, many *Catocala* species localize to eastern Asia and eastern North America, where broadleaf forests flourish. *Catocala* moths were previously classified into four groups based on the positions of the spines on the fore-, middle-, or hind-tibia (Hampson, 1913). However, this classification is not widely accepted, as other morphological characteristics do not correlate with the spine pattern, suggesting that spine pattern does not reflect the phylogenetic relationship. The phylogenetic relationships among the *Catocala* species is mainly unresolved at present except for some North American groups comprising intimately related species. The larvae of these American species groups feed on Myricaceae, Juglandaceae, or Ericaceae, which are not host plants for Eurasian *Catocala* species, suggesting that acquisition of new food plants in North America promoted recent speciation of these species groups.

The forewings of *Catocala* moths, which usually cover the hindwings (underwings) when they are at rest, have camouflage colors and patterns similar to their background such as tree barks or rocks (Fig. 1). By contrast, their hindwings have spectacular color patterning in red, yellow, and white with black bands believed to startle predatory birds (Sargent, 1976); hence, their English name “underwings” or their Japanese name “shitaba”. Thus, colors and patterns of both the forewing and hindwing seem to have evolved through adaptive processes, although the lack of information about phylogenetic relationships among the *Catocala* species obstructs attempts to trace the actual evolutionary processes of the *Catocala* wing pattern.

Thirty-one *Catocala* species have been recorded in Japan so far, and all have identical or closely related counterpart species distributed in the Eurasian continent. Although it is tempting to speculate on the phylogenetic relationship among these 31 *Catocala* species based on colors of their hindwings or the food plant of their larvae, no apparent morphological characteristics support these grouping. In particular, comparison of male genitalia indicates that there is no close relationship among Japanese *Catocala* species

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Fig. 1. *Catocala* species analyzed in this paper. Thirty-one Japanese species and two American species are shown. All have forewings with camouflage colors and patterns and hindwings with spectacular color patterning in red, yellow, or white with black bands believed to have frightening effect to predatory birds.

except in two cases with clear similarity between *C. xarippe* and *C. fulminea* (Ishizuka, 2009), and between *C. duplicata* and *C. dissimilis* (Sugi, 1968). This suggests that the most speciation events among the Japanese *Catocala* species occurred a long time ago and that the phylogenetic distances between them are very large (Ishizuka, 2005b). Here we report the result of our molecular phylogenetic studies using the mitochondrial ND5 gene, which we performed to elucidate the phylogenetic relationship among Japanese *Catocala* species.

MATERIALS AND METHODS

Materials and DNA extraction

Moths collected in Japan, China, Russia, Canada, and the USA (listed in Table 1) were used as fresh, frozen, or dried specimens. DNA was extracted from one to six legs, depending on the size of the moths, using the DNeasy Tissue Kit (Qiagen KK, Tokyo, Japan), as described previously (Makita et al., 2003).

PCR amplification of the ND5 gene and sequence analysis

A part of the mitochondrial ND5 gene was amplified by polymerase chain reaction (PCR) using the primer pair, S1 (TACWCCTGTTTCTGCTTTAGTTCA) and AS1 (CCATAGGTTKATAAWGTTGGWATAAAT) designed to amplify an 836 bp fragment from most lepidopteran species. In samples where the quality of DNA was insufficient to amplify ~800 bp-long fragments, the corresponding sequence was amplified as two overlapping fragments: a ~450 bp fragment was amplified using S1 and AS2 (CTAAAATTAWATCYTTAGARTAGAAYCC) primers, and a ~450 bp fragment was amplified using the S2 (ATAWTTCTAATTTAKCTWTATGTGG) and AS1 primers. The parameters for the PCR reaction were: 95°C for 5 min; 40 cycles at 95°C for 0.5 min, 42°C for 0.5 min, and 72°C for 1 min; and 72°C for 5 min. The amplified fragments were analyzed for nucleotide sequences directly after treatment with alkaline phosphatase and exonuclease I. Sequencing reaction was performed with BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) using a 3100/3130xl Genetic Analyzer (Applied Biosystems).

Phylogenetic analysis

The nucleotide sequences were aligned using Clustal X 1.83.1 (Thompson et al., 1997) with the default setting. Phylogenetic and molecular evolutionary analyses were conducted using MEGA5 (Tamura et al., 2011). For the maximum-likelihood (ML) tree (Felsenstein, 1981), the GTR+G+I model was selected as the best-fit model according to the Bayesian Information Criterion. The ML tree was inferred using all three codon positions by Nearest-Neighbor-Interchange search based on this model. The neighbor-joining (NJ) tree (Saitou and Nei, 1987) was constructed based on Kimura's two-parameter distance (Kimura, 1980). The trees were tested by 1000 bootstrap replications.

RESULTS AND DISCUSSION

Thirty-one *Catocala* species have been recorded from Japan to date, including *C. macula*, which is sometimes treated as *Ultrichopus macula*. Specimens of these 31

Catocala species collected in Japan were analyzed together with continental specimens for 20 of these species collected in China and Russia (Table 1). In addition, two North American species, *C. relicta* and *C. cerogama*, considered to be related closely to *C. fraxini* and *C. lara,* respectively (Fig. 1), were also analyzed. Thus, in total 53 sequences of 33 *Catocala* species were analyzed here. There were no insertions or deletions among the ND5 sequences of these species, and 762 bp sequences were used for phylogenetic analysis. There were 253 variable sites and 205 parsimony informative sites. Average GC-contents at the first, second and third codon positions, and as a whole were, 23.4, 26.1, 5.5 and 18.3%, respectively. The number of base differences per site between 31 Japanese *Catocala* species were 0.037–0.083, except for that between *C. xarippe* and *C. fulminea*, which was only 0.014. As outgroups, six non-*Catocala* species of Catocalinae, Noctuidae, *Mocis annetta*, *Ercheia umbrosa*, *E. niveostrigata*, *Spirama retorta*, *Thyas juno*, and *Artena dotata* from Japan were used. Two phylogenetic trees based on these nucleotide sequences were constructed by the ML method or the NJ method (Figs. 2, 3).

Upon model testing, the GTR+G+I (general time reversible, gamma distributed with invariant sites) model gave the lowest Bayesian Information Criterion (BIC) scores. A phylogenetic tree was inferred by the maximum likelihood (ML) method using the GTR+G+I model (Fig. 2). All *Catocala* species formed a clade, supported by a bootstrap percentage of 88%. Monophyly of the Japanese and continental sequences of the same *Catocala* species was supported by the high bootstrap percentages of 93% to 100% in all cases. By contrast, none of the groupings of the Eurasian *Catocala* species was supported by a high bootstrap percentage, except for the clade comprising *C. xarippe* and *C. fulminea,* which was supported by a bootstrap percentage of 99%. These two very similar species were recognized as a single species, *C. fulminea,* for a long time and have been divided into two species recently (Ishizuka, 2009). Except for this clade, the only clade supported by a bootstrap percentage of > 50% was that comprising *C. patala* and *C. nagioides*. Fig. 3 shows a NJ tree of the same data set based on Kimura's two-parameter model (Kimura, 1980). In this tree, the grouping of 33 *Catocala* species (31 Eurasian and two American species) was supported by a higher bootstrap percentage of 93%. In both the ML and NJ trees, *C. macula* is located at the most basal position within the *Catocala* clade. However, the bootstrap percentages to support the *Catocala* clade excluding *C. macula* were less than 50% in both the ML and NJ trees. In addition, an extended phylogenetic analysis based on the ND5 sequences in more than 100 species located *C. macula* in the middle of the *Catocala* clade, not at the most basal position (Ishizuka et al. unpublished data). All these results support the inclusion of this species into the *Catocala* genus.

In the NJ tree, no interspecies relationship among the

Table 1. Moth samples used in this study.

Genus	Species	Locality	Accession No	Genus	Species	Locality	Accession No
Artena	*dotata*	Shizuoka, Japan	AB292106	*Catocala*	*kuangtungensis*	Anhui, China	AB291937
Catocala	*actaea*	Nagano, Japan	AB291938	*Catocala*	*lara*	Toyama, Japan	AB291923
Catocala	*actaea*	Gansu, China	AB292099	*Catocala*	*lara*	Gansu, China	AB292098
Catocala	*bella*	Nagano, Japan	AB291924	*Catocala*	*macula*	Kagoshima, Japan	AB605781
Catocala	*bella*	Shandong, China	AB291925	*Catocala*	*macula*	Hubei, China	AB292101
Catocala	*cerogama*	New York, USA	AB291940	*Catocala*	*mabella*	Nagano, Japan	AB291929
Catocala	*columbina*	Nagano, Japan	AB291926	*Catocala*	*mirifica*	Mie, Japan	AB291928
Catocala	*columbina*	Hubei, China	AB291927	*Catocala*	*nagioides*	Nagano, Japan	AB292085
Catocala	*connexa*	Toyama, Japan	AB292092	*Catocala*	*nivea*	Gifu, Japan	AB291934
Catocala	*deuteronympha*	Hokkaido, Japan	AB291942	*Catocala*	*nivea*	Hunan, China	AB291935
Catocala	*deuteronympha*	Liaoning, China	AB292100	*Catocala*	*nubila*	Nagano, Japan	AB292086
Catocala	*dissimilis*	Nagano, Japan	AB292095	*Catocala*	*nupta*	Nagano, Japan	AB291931
Catocala	*dula*	Nagano, Japan	AB292079	*Catocala*	*nupta*	Far East, Russia	AB291932
Catocala	*dula*	Shaanxi, China	AB292080	*Catocala*	*patala*	Nagano, Japan	AB291920
Catocala	*duplicata*	Yamanashi, Japan	AB291919	*Catocala*	*patala*	Gansu, China	AB291921
Catocala	*electa*	Gifu, Japan	AB292093	*Catocala*	*praegnax*	Nagano, Japan	AB291916
Catocala	*electa*	Shandong, China	AB292094	*Catocala*	*praegnax*	Far East, Russia	AB291917
Catocala	*ella*	Toyama, Japan	AB291918	*Catocala*	*relicta*	Saskatchewan, Canada	AB291939
Catocala	*fraxini*	Gunma, Japan	AB292081	*Catocala*	*separans*	Toyama, Japan	AB292087
Catocala	*fraxini*	Shandong, China	AB292082	*Catocala*	*streckeri*	Mie, Japan	AB291915
Catocala	*fulminea*	Hokkaido, Japan	AB605780	*Catocala*	*streckeri*	Far East, Russia	AB292096
Catocala	*fulminea*	Far East, Russia	AB605779	*Catocala*	*tokui*	Mie, Japan	AB291922
Catocala	*hyperconnexa*	Nagano, Japan	AB291913	*Catocala*	*tokui*	Fujian, China	AB303307
Catocala	*hyperconnexa*	Guizhou, China	AB291914	*Catocala*	*xarippe*	Nagano, Japan	AB292088
Catocala	*intacta*	Gifu, Japan	AB292090	*Ercheia*	*niveostrigata*	Nagano, Japan	AB292103
Catocala	*intacta*	Fujian, China	AB292091	*Ercheia*	*umbrosa*	Yamanashi, Japan	AB291933
Catocala	*jonasii*	Gifu, Japan	AB292083	*Lagoptera*	*juno*	Nagano, Japan	AB292104
Catocala	*jonasii*	Shaanxi, China	AB292084	*Spirama*	*retorta*	Nagano, Japan	AB292105
Catocala	*koreana*	Nagano, Japan	AB291941	*Sypnoides*	*hercules*	Nagano, Japan	AB292107
Catocala	*kuangtungensis*	Wakayama, Japan	AB291936				

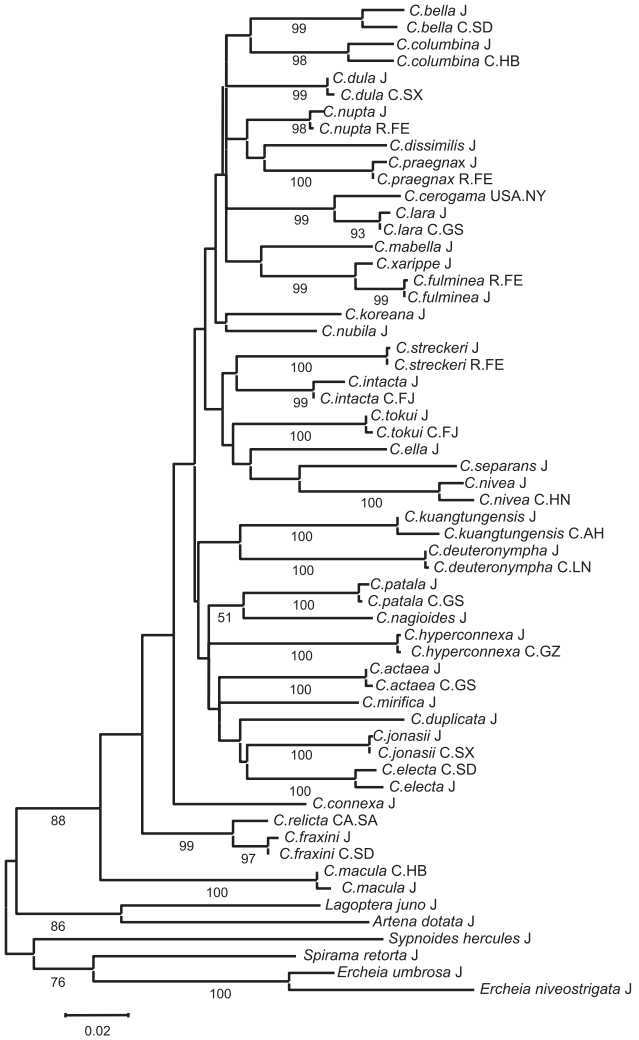

Fig. 2. ML tree of Japanese *Catocala* species based on mitochondrial ND5 gene sequences. Maximum-likelihood (ML) tree constructed based on the GTR+G+I model. Six non-*Catocala* Catocalinae species were used as outgroups. Bootstrap percentages higher than 50, based on 1000 replicates, are shown. Abbreviations for sampling locations are; J, Japan; R.FE, Russia far east; USA.NY, New York state of USA; CA.SA, Canada Saskatchewan; C, China. Abbreviations for provinces in China are; GZ, Guizhou; SX, Shaanxi; SD, Shandong; GS, Gansu; AH; Anhui; LN, Liaoning; FJ, Fujian; HN, Hunan; HB, Hubei.

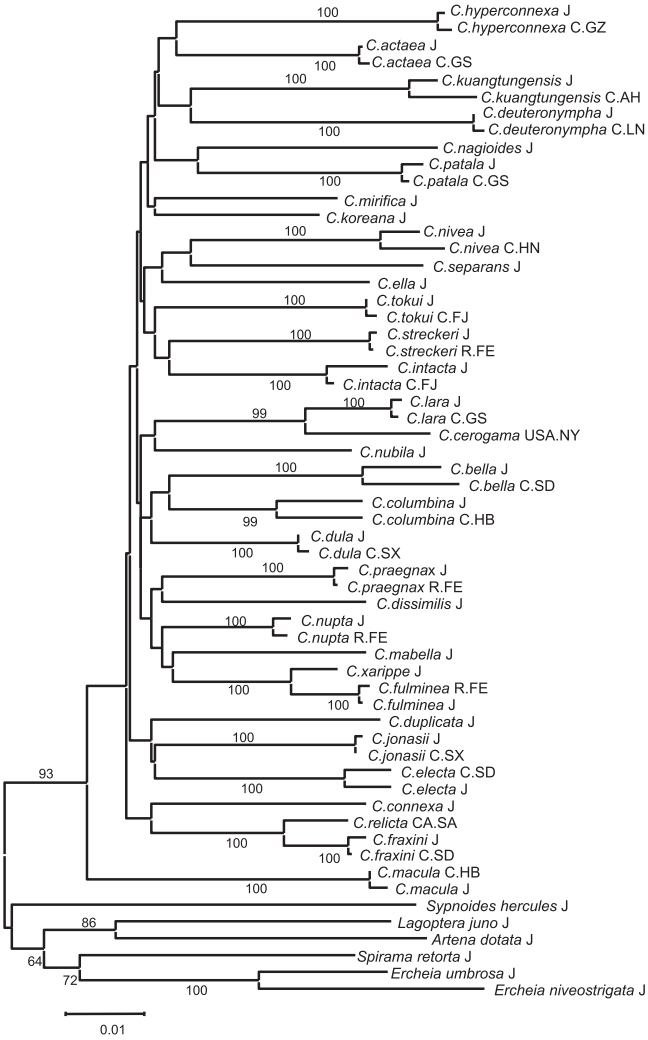

Fig. 3. NJ tree of Japanese *Catocala* species based on mitochondrial ND5 gene sequences. Other than that the tree was drawn using the neighbor-joining (NJ) method based on Kimura's two-parameter distance, all other conditions were the same as described in the legend to Fig. 2.

Eurasian *Catocala* species, except for that between *C. xarippe* and *C. fulminea*, was supported by a bootstrap percentage of more than 50%. These results indicate that the 31 Japanese *Catocala* species analyzed here diverged a long time ago and that their phylogenetic relationship is difficult to elucidate, except for an intimate relationship between *C. xarippe* and *C. fulminea*. This conclusion is not surprising because comparison of male genitalia indicated little similarity among Japanese *Catocala* species despite the apparent similarity in wing pattern recognized in some pair of species. However, the male genitalia of *C. duplicata* and *C. dissimilis* show a close similarity (Sugi, 1968), and it remains to be clarified by further phylogenetic analysis whether they are related closely or whether their male genitalia show coincidental resemblance.

We found many discrepancies in the branching patterns of the 31 Eurasian species between the ML and NJ trees, indicating poor reliability of the branching patterns shown in Figs. 2 and 3. However, sister grouping of the pairs of species such as *C. kuangtungensis* and *C. deuteronympha*, *C. patala* and *C. nagioides*, *C. streckeri* and *C. intacta*, *C. nivea* and *C. separans*, *C. praegnax* and *C. dissimilis*, and *C. bella* and *C. columbina,* was conserved between the ML and NJ trees, suggesting a remote relationship between these paired species. Although it remains to be clarified by further analysis of sequences and species whether these observed remote relationships reflect actual phylogenetic relatedness; we note that four species with a white pattern in the hindwings, *C. nivea*, *C. dissimilis*, *C. nagioides*, and *C. actaea* did not show kinship to each other and showed a possible kinship to four separate yellow-hindwing species. Similarly, no close relatedness was suggested between three red-hindwing species, *C. electa*, *C. nupta*, and *C.*

dula. These results suggest that the hindwing color has changed frequently during the speciation of the *Catocala* moths, and that hindwing color does not reflect phylogeny.

In contrast to the mostly inconclusive phylogenetic relationships between the analyzed Eurasian *Catocala* species, the two American species analyzed, *C. relicta* and *C. cerogama*, showed a clear and close relatedness to the Eurasian species, *C. fraxini* and *C. lara*, respectively, which was supported by high bootstrap percentages of 99% or 100% in both the ML and NJ trees (Figs. 2, 3). As shown in Fig. 1, *C. relicta* and *C. fraxini* show a close morphological similarity, and the larvae of both of these species feed on Salicaceae. Similarly, *C. cerogama* and *C. lara* show a close morphological similarity and are the only *Catocala* species known to feed on Tiliaceae. However, the colors of the hindwing band differ between these closely related Eurasian and American species: purple in *C. fraxini* versus white in *C. relicta*, and white in *C. lara* versus yellow in *C. cerogama*. Again, this suggests that the color of hindwings is a changeable characteristic. Some North American *Catocala* species feed on Juglandaceae. About 25 species show morphological similarity, suggesting recent speciation events, and have either yellow or black hindwings. Our preliminary nucleotide sequence analysis of the ND5 gene in these species produced a phylogenetic tree in which the yellow- and black-hindwing species intermingle (Ishizuka et al. unpublished data), indicating parallel evolution either from yellow to black or from black to yellow.

To estimate the divergence time between Japanese *Catocala* species, we constructed a linearized tree (Fig. 4) based on the NJ tree shown in Fig. 3 (Takezaki et al., 1995). Divergence times among Japanese *Catocala* species corresponded to 0.02 to 0.03 Kimura's two-parameter distance (Kimura, 1980). The evolutionary rate of the insect ND5 gene has been estimated using Lepidoptera butterfly groups, *Parnassius* (Yagi et al., 2001) and *Parides* (Kato and Yagi, 2004), and a *Coleoptera* Carabinae ground beetle, *Euleptocarabus porrecticollis* (Su et al., 1998), and the 0.01 Kimura's two-parameter distances were estimated at 0.75 MY, 1.8 MY, and 4.0 MY, respectively. If all three estimations are correct, the evolutionary rate of the ND5 gene is not constant in various insect groups. However, it is difficult to find a rigid correspondence between the speciation and paleogeological events used for age estimation, such as river, island, or mountain formations. Thus, these values, 0.75 to 4.0 MY/0.01 Kimura's two-parameter distances may contain errors, although they may be useful for tentative estimation of the ages of *Catocala* speciation. Based on these estimations, 0.02 to 0.03 Kimura's two-parameter distance between Japanese *Catocala* species (Fig. 4) corresponds to 1.5–12 MY. Because the islands of ancient Japan split from the continent about 15 MYA (Otofuji et al., 1994), these speciation events would have occurred after the Japan–continent split. The closely related Eurasian and American species analyzed here showed much shorter divergence times of 0.6–3 and 0.9–5 MY for *C. fraxini* and *C. relicta* and for *C. lara* and *C. cerogama*, respectively. These results indicate that *Catocala* moths migrated nearly freely between Japan and the Eurasian continent and between the Eurasian and American continents after the Japan–continent split.

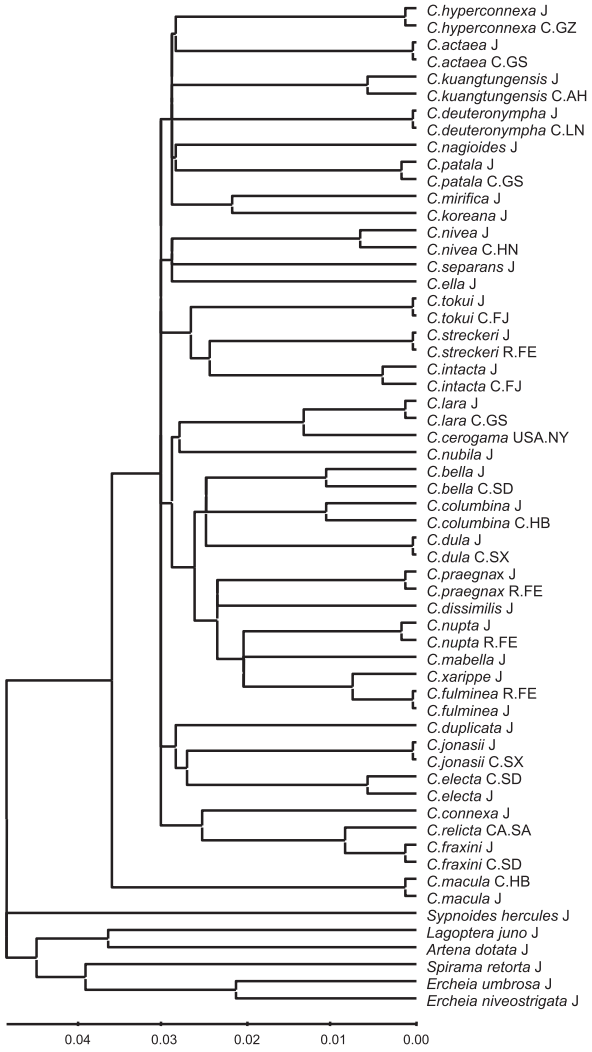

Fig. 4. Linealized tree of Fig. 3 constructed under the assumption of molecular clock. The linealized tree was constructed using Compute Linealized Tree in MEGA5 (Tamura et al., 2011) based on the tree shown in Fig. 3. The scales under the horizontal line indicate Kimura's two-parameter distance.

Evolutionary distances between the Japanese and continental specimens of the same species showed great variation depending on the species. The divergence times are about 0.75–4.0 MYA for *C. bella* and *C. columbina*; 0.3–2.4 MYA for *C. kuangtungensis*, *C. nivea*, *C. intact*, and *C. electa*; and less than 0.1–0.5 MYA for *C. hyperconnexa*, *C. actaea*, *C. deuteronympha*, *C. patala*, *C. tokui*, *C. streckeri*, *C. lara*, *C. dula*, *C. praegnax*, *C. nupta*, *C. fulminea*, *C. jonasii*, *C. fraxini*, and *C. macula.* This suggests that isolation of the Japanese from the continental populations of these species occurred at many different times, although the correspondence between these times and the paleogeological events has not been identified. Moreover, the genetic distances observed between the Japanese and continental populations of *C. bella* and *C. columbina* are larger than those between *C. xarippe* and *C. fulminea* or between *C. relicta* and *C. fraxini*, suggesting that taxonomic revaluation is required for *C. bella* and *C. columbina*.

Our phylogenetic analysis based on nucleotide sequences of the ND5 gene indicated that the 31 Japanese *Catocala* species including *C. macula* form a well-supported monophyletic group within Noctuidae. All the speciation events among the Japanese *Catocala* species, except for that between *C. xarippe* and *C. fulminea*, seemed to have occurred a long time ago, and there is no close phylogenetic relationship between these Japanese *Catocala* species. However, remote phylogenetic relationships were suggested between several pairs of species, indicating that hindwing color does not reflect the phylogenetic lineage, and seems to have changed frequently during the evolution of *Catocala* species. Further molecular phylogenetic analysis of *Catocala* species throughout the world will reveal the potentially adaptive evolutionary process of the spectacular wing colors and patterns of these moths.

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