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＜**Original Article**＞

Ecological and Morphological Differentiation between Two Cryptic DNA Clades in the Red Ant *Myrmica kotokui* Forel 1911 (Formicidae, Myrmicinae)

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Abstract : Ecological and morphological differences were examined between two DNA clades within a myrmicine red ant, *Myrmica kotokui*. Twenty-four ant colonies were collected in the Japan Alps at elevations between about 1,000 and 2,200 m, and their DNA clades were determined from their mitochondrial *cytochrome oxidase I* gene sequences. Then, their several ecological and morphological characters, such as habitat and nesting preferences, the queen's head width, and the number of queens per colony were compared between clades. Two clades were identified. Colonies belonging to different clades were found to prefer different habitats and nesting microhabitats, and the head width of queens also differed between them. These differences suggest that each clade represents a cryptic species that is differentiated genetically, ecologically, and morphologically from the other. In contrast, the number of queens did not differ between the DNA clades, suggesting that it is more influenced by environmental than by genetic factors.

Key words : cryptic species, DNA identification, monogyny, morphological comparison, polygyny

Introduction

Advances in modern molecular phylogenetics have led to considerable attention being focused on cryptic species, that is, two or more reproductively isolated species that have been erroneously classified as a single morphological species (Bickford *et al*., 2007). The recognition of cryptic species would raise estimates of the biodiversity of a taxonomic group. Thus, for accurate evaluation of biodiversity, the identification of cryptic species is essential (Schlick-Steiner *et al*., 2010). However, Seppä *et al*. (2011) recently pointed out that a molecular phylogenetic analysis cannot be used alone to delimit cryptic species. Different genes have independent genealogical histories, and gene tree topologies may differ among genes and from the species tree topology, which reflects speciation history (Knowles and Carstens, 2007). Therefore, to identify cryptic species, we should integrate various types of information, including molecular,

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chemical, and morphological characters (Ross *et al*., 2010 ; Schlick-Steiner *et al*., 2010 ; Seppä *et al*., 2011). For instance, Schlick-Steiner *et al*. (2006) identified seven cryptic species in a *Tetramorium* ant species complex by using three independent analyses : mtDNA phylogeny, the morphologies of male genitalia, and cuticular hydrocarbons.

Myrmica kotokui (Myrmicinae), a common ant species in the Japanese Archipelago, is distributed from northern Hokkaido to southern Yakushima Island (it is also found in the Korean Penisula and on Sakhalin Island ; Japanese Ant Database Group, 2008). In the Japan Alps of central Honshu, the species is distributed over a broad elevational range from approximately 1,000 to 2,000 m (Togai *et al*., 2012). To evaluate the genetic diversity of this species over its geographical and elevational range, Ueda *et al*. (2012) collected *M. kotokui* specimens from six mountain ranges in the Japan Alps and reconstructed their molecular phylogenetic relationships by using DNA sequences of the mitochondrial *cytochrome oxidase I (COI)* and the nuclear *long-wavelength rodopsin (LwRh)* genes. They identified four highly differentiated clades, which did not show geographical segregation by mountain range but which did show distributional differences by elevation (Ueda *et al*., 2012). These results suggest that the single morphological species known as *M. kotokui* may be composed of several putative cryptic species. However, it is not known whether the detected clades are differentiated ecologically or morphologically from the others.

As the four clades of *M. kotokui* differ in their elevational distribution, each might be expected to show local adaptation to its elevational environment, which would be expressed as a habitat preference. Furthermore, we hypothesized that both the head width and number of queens per colony might differ among the clades, because populations of *M. kotokui* in Hokkaido exhibit either a monogynous or a polygynous social structure, which are differentiated by several morphological characters, including head width, the ratio of wing length and head width, and the ratio of thorax width and head width (Mizutani, 1981 ; Kikuchi *et al*., 1999 ; Kikuchi, 2002).

The aim of this study was to examine ecological and morphological differentiation between *M. kotokui* clades. To test our hypotheses, we collected 24 ant colonies in the Japan Alps from three different habitats (grassland, forest edge, or woodland) and two different nesting microhabitats (soil or decayed wood), and measured queen head width and the number of queens in each colony. We then compared these characters between the two identified clades.

Materials and Methods

Sampling

From May to August 2011, and from August to October 2012, we haphazardly collected 24 *Myrmica kotokui* colonies in six mountain ranges of Honshu (Sugadaira, Kita-Alps, Yatsugatake, Oku-chichibu, Chuo-Alps, and Minami-Alps ; Fig. 1) at several elevations ranging from about 1,000 to 2,200 m (Table 1). We used a hand-held GPS unit (Garmin, Olathe, KS, USA) to determine the sampling location (latitude, longitude, and elevation), and recorded the habitat environment (*i.e*., grassland, forest edge, or woodland) and the nesting microhabitat (soil or decayed wood). In the case of ant nests found on the ground underneath decayed wood, we recorded the nesting microhabitat as "decayed wood" because in all such nests the queen's chamber was in the decayed

Table 1 Specimens used for the ecological and morphological comparison.

Colony #	DNA clade	Mountain range	Location	Elevation (m a.s.l.)	Longitude	Latitude	Habitat environment	Nesting microhabitat	Number of queens	Queen head width (mm)	Collection date (yyyy.mm.dd)	GenBank #
AT02	L2	Yatsugatake	Sanjiro	1,492	N36°12'39.6"	E138°06'21.1"	forest edge	soil	4	–	2011.06.03	AB819125
AT04	L2	Yatsugatake	Utsukushigahara	1,966	N36°13'40.7"	E138°08'44.2"	grassland	soil	21	–	2011.07.19	AB819127
AT06	L2	Kita-Alps	Norikura	1,457	N36°07'19.9"	E137°37'46.1"	forest edge	soil	0	–	2011.08.04	AB819129
AT08	L2	Kita-Alps	Norikura	1,787	N36°06'47.6"	E137°35'56.3"	grassland	soil	1	1.14	2011.07.01	AB819131
AT09	L2	Kita-Alps	Norikura	2,041	N36°07'21.4"	E137°35'05.2"	forest edge	soil	0	–	2011.06.10	AB819132
AT20	L2	Yatsugatake	Nomugitouge	2,116	N36°03'29.8"	E138°21'11.9"	woodland	soil	48	1.12	2011.07.20	AB819141
AT25	L2	Kita-Alps	Norikura	1,160	N36°08'24.1"	E137°38'48.9"	grassland	soil	0	–	2011.06.10	AB819142
AT26	L2	Kita-Alps	Norikura	1,228	N36°07'56.5"	E137°39'48.0"	grassland	soil	16	1.16	2011.06.10	AB819143
AT33	L2	Kita-Alps	Norikura	1,332	N36°07'04.1"	E137°38'46.2"	forest edge	soil	0	–	2011.06.16	AB819145
AT35	L2	Kita-Alps	Norikura	1,350	N36°07'05.0"	E137°38'48.1"	forest edge	soil	1	1.13	2012.08.28	AB819146
AT36	L2	Kita-Alps	Norikura	1,353	N36°07'05.4"	E137°38'50.1"	forest edge	soil	5	1.17	2012.09.13	AB819147
AT39	L2	Oku-chichibu	Ono	*	*	*	grassland	soil	23	1.07	2012.10.04	AB819149
AT43	L2	Oku-chichibu	Ono	*	*	*	grassland	soil	2	0.98	2012.10.04	AB819152
AT44	L2	Oku-chichibu	Ono	*	*	*	grassland	soil	0	–	2012.10.04	AB819153
AT01	L3	Yatsugatake	Sanjiro	1,475	N36°12'39.3"	E138°06'19.0"	woodland	decayed wood	10	1.18	2011.06.03	AB819124
AT03	L3	Yatsugatake	Utsukushigahara	1,711	N36°10'13.0"	E138°07'57.4"	woodland	decayed wood	1	–	2011.08.22	AB819126
AT11	L3	Kita-Alps	Abotouge	1,511	N36°12'18.5"	E137°36'05.6"	forest edge	decayed wood	1	–	2011.07.29	AB819133
AT13	L3	Sugadaira	Sugadaira	1,222	N36°32'10.2"	E138°20'15.9"	woodland	decayed wood	0	–	2011.05.20	AB819134
AT14	L3	Sugadaira	Sugadaira	1,594	N36°31'09.8"	E138°23'54.1"	woodland	decayed wood	0	–	2011.05.20	AB819135
AT16	L3	Minami-Alps	Nyuukasa	1,771	N35°54'00.0"	E138°10'11.3"	woodland	decayed wood	3	1.27	2012.09.25	AB819137
AT17	L3	Minami-Alps	Nyuukasa	1,608	N35°55'22.7"	E138°09'49.1"	forest edge	soil	1	1.21	2112.09.28	AB819138
AT18	L3	Chuo-Alps	Nishikomagatake	1,321	N35°49'31.9"	E137°51'12.4"	forest edge	soil	1	1.17	2012.09.15	AB819139
AT19	L3	Chuo-Alps	Nishikomagatake	1,550	N35°49'16.8"	E137°51'08.7"	woodland	decayed wood	5	1.23	2012.09.25	AB819140
AT38	L3	Kita-Alps	Norikura	1,446	N36°07'19.3"	E137°37'46.8"	forest edge	soil	9	1.16	2012.09.13	AB819148

*The coordinates are not shown for the conservation of an ant-associated endangered insect distributed.

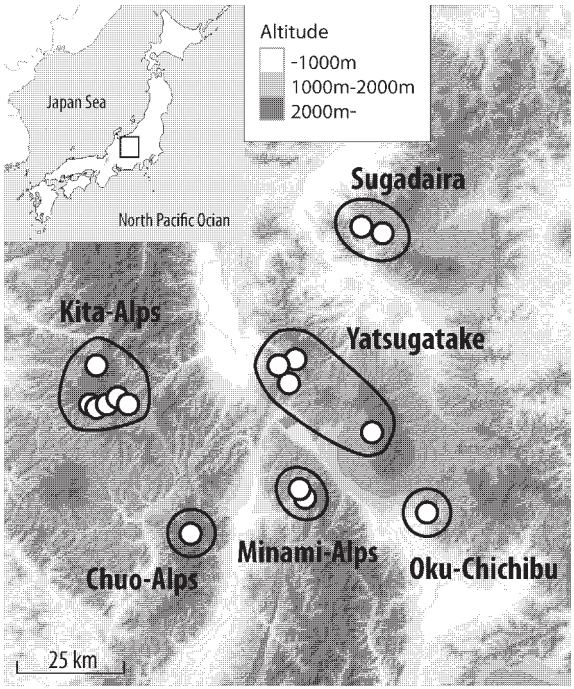

Fig. 1 Sampling locations of colonies of *Myrmica kotokui* in the Japan Alps.

wood. The nests of *M. kotokui* tend to be shallow and to extend horizontally in the soil, so to collect each colony we excavated all soil to a depth of 0.2 m and within a radius of 0.5 m of the nest entrance opening. We carried each collected colony (including the soil) to our laboratory, and counted the number of queens there. We preserved 10 workers and all queens in 100% ethanol from each colony for DNA analysis and for head-width measurement, respetively.

DNA analysis

DNA was extracted from the whole body of each ant using a DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's protocols. The mitochondrial *COI* gene was amplified by polymerase chain reaction (PCR) using Takara Ex Taq (Takara Bio, Shiga, Japan) and the primers MyrCOI-F1 (5'-TA GGR TCR CCT GAT ATA GC-3') and MyrCOI-R1 (5'-CC AGG TAY YAT TAA AAT ATA AAC TTC-3'), which are at positions 1748 and 2191, respectively, in the *Drosophila yakuba* mtDNA genome. The amplification was carried out for 30 cycles of 95 ℃ for 30 s, 50 ℃ for 30 s, and 72 ℃ for 40 s. After amplification, the PCR products were purified with ExoSap-IT (USB, Cleveland, Ohio, USA). Sequencing of both strands was performed with a BigDye Terminator v1.1 Cycle Sequencing Kit (ABI, Weiterstadt, Germany) on an ABI 3130 Genetic Analyzer.

We imported the obtained *COI* dataset into the *COI* dataset of Ueda *et al*. (2012), and then determined the clade of each colony by neighbor-joining (NJ) analysis, performed with MEGA5

software (Tamura *et al*., 2011). Although Ueda *et al*. (2012) inferred the molecular phylogeny by using both the *COI* and *LwRh* sequences, in this study we analyzed only the *COI* sequences because the mutation rate of the *LwRh* gene is slow and it is possible to determine the clade by using only the *COI* gene data. The GenBank accession numbers of the *COI* gene sequences are listed in Table 1.

Morphological comparison and statistical analyses

We randomly picked out one queen from each colony for head-width measurement; thus, we measured the head widths of 13 queens, because seven colonies had no queen. Furthermore, in four colonies (AT02, AT03, AT04 and AT11), the queen's bodies were damaged to the extent that we could not measure their head width (Table 1). We assumed that the colonies without queens represent orphaned colonies, because we collected each colony carefully and the frequency of the colonies without queens in this study (29%) is congruent with that in Kikuchi *et al*. (2000) (31%), which investigated the frequency of queen-right and orphaned colonies of *M. kotokui* in Hokkaido. For this measurement, the queen's head was separated from her body, and a digitized image was captured at 50 times magnification by using a stereoscopic microscope (SMZ1500 M001, Nikon, Tokyo, Japan) and a digital camera (EOS Kiss X3, Canon, Tokyo, Japan). The head width was then measured with Photo Measure software (Kenis, Osaka, Japan).

The relation between the DNA clade and habitat was examined by using a χ^2-test of independence, and alternative habitat preferences (grassland or not-grassland and woodland or not-woodland) and nesting preferences between the two clades were determined by using Fischer's exact test. The elevational distribution, queen head width, and the number of queens were compared between clades by t-test. All statistical analyses were conducted at P-value threshold of 0.05 with JMP software (SAS, Cary, North Carolina, USA).

Results

Geographical and elevational distribution of DNA clades

The DNA clade of each ant colony was determined by NJ analysis of the 470-bp sequences of the mitochondrial *COI* gene. Of the 24 colonies, 14 belonged to the L2 clade and 10 to the L3 clade (Table 1). In this study, we examined the ecological and morphological differentiation between the DNA clades of *Myrmica kotokui* by using only ant colonies belonging to the L2 and L3 clades, because only colonies belonging to these clades were found.

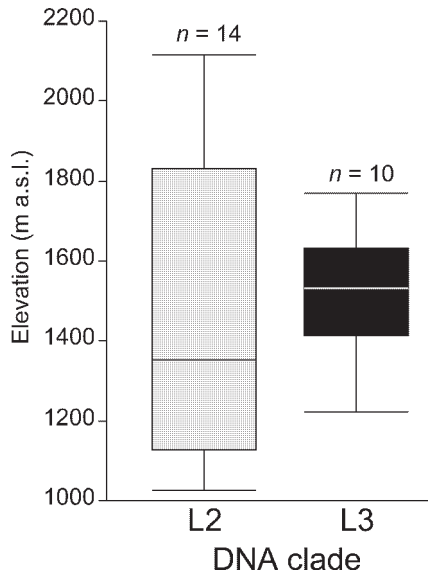

Fig. 2 Distributions of the DNA clades of *Myrmica kotokui* according to elevation. The short horizontal line within each box denotes the median, the bottom and the top of each box denote the first and third quartiles, respectively, and the whiskers show the 5% and 95% limits.

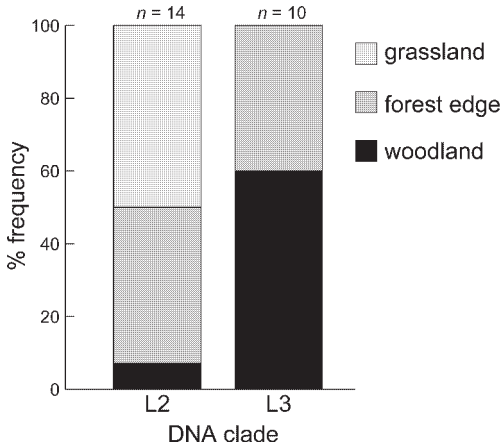

Fig. 3 Habitat preferences of two DNA clades of *Myrmica kotokui*.

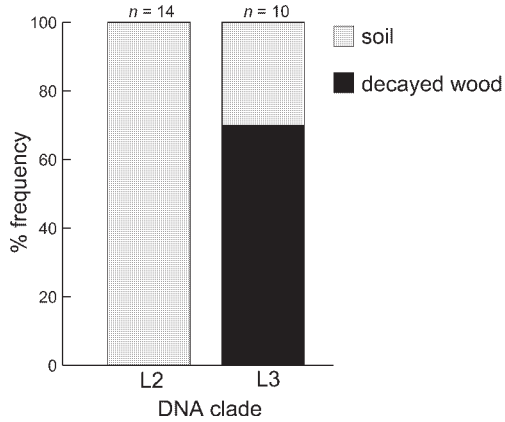

Fig. 4 Nesting microhabitat preferences of two DNA clades of *Myrmica kotokui*.

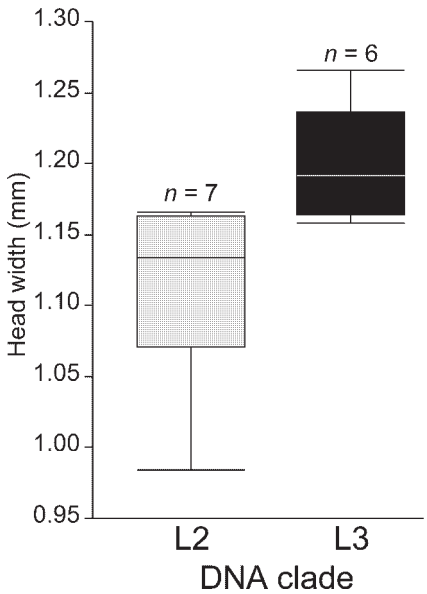

Fig. 5 Comparison of queen head width between two DNA clades of *Myrmica kotokui*. The short horizontal line within each box denotes the median, the bottom and the top of each box denote the first and third quartiles, respectively, and the whiskers show the 5% and 95% limits.

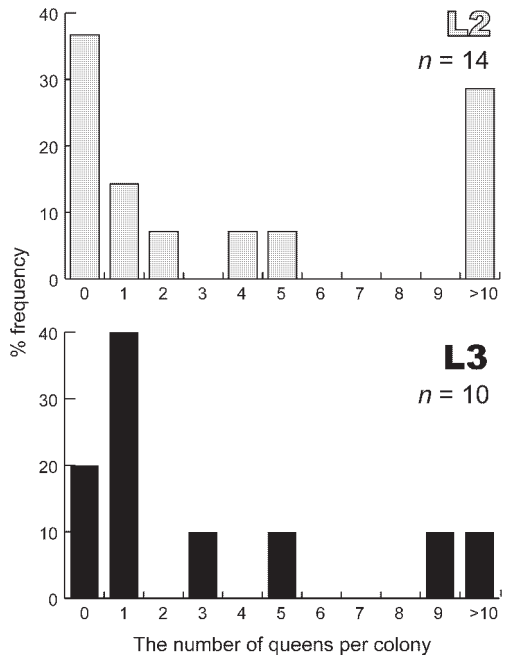

Fig. 6 Frequency distribution of the number of queens per colony in DNA clades L2 and L3.

Among the six mountain ranges from which the collections were made, we collected L2 colonies from three (Kita-Alps, Yatsugatake, and Oku-chichibu), and L3 colonies from five (Sugadaira, Kita-Alps, Yatsugatake, Chuo-Alps, and Minami-Alps) (Table 1). The two clades did not differ significantly with regard to the elevational distribution of the colonies (mean ± SD : L2, 1,439 ± 95.8 m ; L3, 1,473 ± 60.5 m, *t*-test, $P > 0.05$, Fig. 2).

Ecological and morphological differentiation between the DNA clades

Comparison of habitat preferences between the two clades showed that 50.0%, 42.9%, and 7.1% of L2 colonies, and 0.0%, 40.0%, and 60.0% of L3 colonies preferred grassland, forest edge, and woodland, respectively (Fig. 3). In the alternative habitat preference analysis, the preferences of L2 and L3 colonies for grassland and not-grassland were 50.0%, 50.0%, 0.0% and 100.0%, respectively, and their preferences for not-woodland and woodland were 92.9%, 7.1%, 40.0% and 60.0%, respectively. The relation between DNA clade and habitat (grassland, forest edge, or woodland) was significant (χ^2-test of independence, df=2, $\chi^2=10.60$, $P < 0.005$). In the alternative preference test between grassland and not-grassland, L2 colonies did not show any preference (Fischer's exact test, $P > 0.05$), but L3 colonies significantly preferred not-grassland (Fischer's exact test, $P < 0.01$). In contrast, in the alternative preference test between woodland and not-woodland, L2 colonies significantly preferred not-woodland (Fischer's exact test, $P < 0.001$), but L3 colonies did not show any preference (Fischer's exact test, $P > 0.05$). With regard to nesting microhabitat, 100.0% and 0.0% of L2 colonies, and 30.0% and 70.0% of L3 colonies, preferred soil and decayed wood, respectively (Fig. 4). The L2 preference for soil was significant (Fischer's exact test, $P < 0.001$, Fig. 4), whereas the difference in L3 preferences was not significant. With regard to the queen head width and the number of queens per colony, although the head width (mean ± SD) in L3 colonies (1.20 ± 0.41 mm) was significantly larger than that in L2 colonies (1.11 ± 0.06 mm) (*t*-test, $P < 0.01$, Fig. 5), we detected no significant difference for the number of queens per colony between the two clades (mean ± SD : L2, 8.6 ± 3.7 ; L3, 3.1 ± 1.2, *t*-test, $P > 0.05$, Fig. 6).

Discussion

Ecological and morphological differentiation between DNA clades

The χ^2-test of independence showed that DNA clade was significantly related to habitat (Fig. 3). Moreover, Fischer's exact test showed that L2 colonies avoided woodland habitats, whereas L3 colonies avoided grassland habitats. In addition, L2 colonies preferred to nest in soil (Fig. 4). The preference of each clade for different environments suggests that theses two DNA clades of *Myrmica kotokui* are spatially segregated. Furthermore, the queen head width also differed between the L2 and L3 clades (Fig. 4). Taken together, these findings indicate that these two DNA clades may each be a cryptic species that is differentiated genetically, ecologically, and morphologically from the other. A similar pattern of genetic and ecological differentiation in the Japan Alps has been reported in the perennial herb *Cimicifuga simplex* (Pellmyr, 1987; Kuzume and Itino, 2013). It is likely that phylogenetic and ecological analyses of other mountain organisms distributed over a broad elevational range would uncover more cryptic diversification.

Although Ueda *et al*. (2012) found that L3 colonies were distributed at a significantly higher elevational range than L2 colonies, we detected no significant difference in elevational distribution between these clades (Fig. 1). This disagreement suggests that the distribution of each clade may be more affected by ecological factors such as habitat and nesting microhabitat than by elevation. Ueda *et al*. (2012) may have found L3 colonies distributed at higher elevations than L2 colonies because woodland habitats having many decayed wood nesting sites may be more frequently found at higher elevation, whereas grassland habitats lacking decayed wood nesting sites may occur more frequently at lower elevation. Thus, in the elevational range from about 1,000 to 2,200 m, L2 colonies might be able to survive at higher elevation when suitable grassland habitat is available, and L3 colonies might survive at lower elevation when suitable woodland habitat is available.

Differences in the number of queens per colony between ant lineages

Mizutani (1981) showed that polygynous colonies of *M. kotokui* prefer riverside habitats, whereas monogynous colonies prefer woodland. Comparison of Mizutani's result with the habitat preference results for the DNA clades in this study suggests that L2 colonies might be expected to be polygynous whereas L3 colonies should be monogynous. However, we detected no significant difference in the number of queens between the L2 and L3 clades: each clade was composed of orphaned, monogynous, and polygynous colonies (Fig. 5). We propose the following hypotheses to explain why the genetic grouping did not correspond to social structure (*i.e*., queen number) in *M. kotokui*.

First, the number of queens per *M. kotokui* colony may be strongly influenced by spatial and ecological factors such as habitat and nesting microhabitat, rather than by genetic factors. In Hokkaido, *M. kotokui* colonies with monogynous and polygynous social structures are allopatrically distributed, in riverside and woodland habitats, respectively (Mizutani 1981), suggesting that clear-cut differences in social structure are more likely to be found between dichotomous habitats. In addition, Elmes and Keller (1993) pointed out that polygyny is a general trait of *Myrmica* ants, and that the number of queens varies intraspecifically depending on environmental conditions.

Second, monogynous populations of *M. kotokui* may not occur in the Japan Alps. This possibility is supported by the fact that the mean head widths of both L3 (1.20 ± 0.41 mm) and L2 (1.11 ± 0.06 mm) queens in the Japan Alps are shorter than the mean head width of monogynous colonies (1.31 ± 0.04 mm) and polygynous colonies (1.23 ± 0.06 mm) in Hokkaido (Kikuchi, 2002). In *Myrmica*, egg production is strongly correlated with queen's body size (Elmes and Keller, 1993); thus, the relatively smaller head widths of both L2 and L3 queens implies that colonies of both clades might be polygynous. Nonetheless, in this study, 35.7% and 14.3% of L2 colonies, and 20.0% and 40.0% of L3 colonies were orphaned and single-queen colonies, respectively (Table 1, Fig. 5). Orphaned *Myrmica* colonies are often found (Elmes and Keller, 1993), and the percentage of orphaned colonies in this study is comparable to the percentage (>30%) in *M. kotokui* populations in Hokkaido (Kikuchi *et al*., 2000). Single-queen colonies may be newly budded, because in *M. kotokui*, polygyny, when it occurs, seems to be secondary, where mated

alate queens return to their mother nests after endogamous copulation (Mizutani 1981; Kasugai *et al*., 1983).

Third, the number of queens in *M. kotokui* colonies might be strongly influenced by temporal factors, such as colony age, season of the year, and interannual changes, rather than by genetic effects. In this study, even colonies in the same locality and environment and belonging to the same DNA clade differed with regard to the number of queens (e.g., AT39, AT43, and AT44 in Table 1). These differences might be attributed to colony age. In *Myrmica*, queens generally have a short life span (Elmes, 1973; Elmes and Petal, 1990; Elmes and Keller, 1993) and queen recruitment occurs at a high frequency (Elmes, 1980; Elmes and Keller 1993), and both of these might cause queen number variation.

In conclusion, this study showed that colonies in the Japan Alps belonging to two different DNA clades of *M. kotokui* were also differentiated both ecologically and morphologically, suggesting that each clade might represent a cryptic species. In future, we plan to investigate whether the clades are reproductively isolated and, if reproductive isolation is present, how it is maintained.

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