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The Spatial Distribution of mtDNA and Phylogeographic Analysis of the Ant *Cardiocondyla kagutsuchi* (Hymenoptera: Formicidae) in Japan

I Okita[1], K Murase[2, 3], T Sato[3], K Kato[4], A Hosoda[5], M Terayama[2], K Masuko[6]

1 - Gifu University, Gifu, Gifu, Japan
2 - The University of Tokyo, Bunkyo-ku, Tokyo, Japan
3 - Tokyo University of Agriculture and Technology, Fuchu, Tokyo, Japan
4 - Shizuoka Prefectural Research and Coordination Office, Shizuoka, Shizuoka, Japan
5 - Hamamatsu Gakuin University Junior College, Hamamatsu, Shizuoka, Japan
6 - Senshu University, Kawasaki, Kanagawa, Japan

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Corresponding author
Kaori Murase
Graduate School of Natural Sciences
Nagoya City Univesity
Yamanohata 1, Mizuho-Cho, Mizuho-Ku
Nagoya, Aichi 467-8501, Japan
E-Mail: kmurase@nsc.nagoya-cu.ac.jp

Abstract

In this study, we investigated the geographical distribution of haplotypes of *Cardiocondyla kagutsuchi* Terayama in Japan using COI/II mitochondrial DNA. We also examined their genealogy with *C. kagutsuchi* in other areas and their close relative species. Four haplotypes were found. While two of them were found in a limited area (Ishigaki and Okinawa Islands) separately, the others were distributed widely across Honsyu, Shikoku, and Kyusyu areas in Japan. The newly invaded area by *C. kagutsuchi* in Japan was Shizuoka prefecture. Their haplotype of Shizuoka were the same as the two haplotypes of the Honsyu, Shikoku, and Kyusyu areas. The haplotype network showed that the two haplotypes were distant from each other. The distance between them was 33, even though the two haplotypes are distributed in the same area. From the phylogenetic tree that we constructed, we found that *C. strigifrons* was in the same clade as *C. kagutsuchi*.

Introduction

Many invasive ant species, such as the Argentine ant or the red imported fire ant, are not desirable because they outcompete and eliminate native ants (Suarez et al., 2008), as well as they cause serious agricultural damage and are harmful to humans (Heinze et al., 2006, Suarez et al., 2008). For such species, early detection and monitoring are important to the management of new invasions. However, the situation is not always simple. There are many invasive species that have been transferred without being noticed and have spread their distribution in new land. In such cases, to reveal their current distribution is important for the conservational actions regarding native species.

Cardiocondyla kagutsuchi Terayama (*C. kagutsuchi*) is one of such species. The genus *Cardiocondyla* is a common ant genus that belongs to the subfamily Myrmicinae. It is an invasive ant group, and is commonly known as "stealthy invaders" (Heinze et al., 2006). Approximately 50 species are currently recognized as belonging to this genus, most of which are distributed in the Old World tropics and subtropics, but a few of which occur in the temperate zone. Some species are also found widely separated in North America and the Pacific Islands, as a result of human introduction. Several species of this genus have a striking male polymorphism, with both winged and wingless forms (Terayama, 1999; Seifert, 2003; Heinze et al., 2005; Yamauchi et al., 2005). These males differ not only in morphology, but also in reproductive tactics (Yamauchi & Kawase, 1992; Yamauchi & Kinomura, 1993; Heinze et al., 1998; Anderson et al., 2003).

Recently, Seifert (2003, 2008) revised the taxonomy of this genus as a result of a morphological study that used the morphometrical method. However, questions remain around

the status of *Cardiocondyla kagutsuchi* Terayama, 1999. This ant is known to have three morphologically different colony types: type 1 - produces both winged and wingless (ergatoid) males in each colony; type 2 - produces both short-winged (brachypterous) and wingless males; and type 3 - produces only wingless males (Yamauchi et al., 2005). Consequently, Yamauchi et al. (2005) renamed *C. kagutsuchi* sensu Seifert (2003) as "*C. kagutsuchi* - complex."

Types 1 and 3 have been found in Japan. Type 1 occurs only on Ishigaki Island, the Ryukyu Islands, whereas type 3 is distributed from Kanto District to Okinawa Island (Terayama et al., 1992; Terayama, 1999). Terayama (1999) considered both types to be different species due to the difference in morphology of the male caste and karyotype of the worker caste (Terayama, 1999). However, in the recent taxonomic revision of the genus, both types were considered conspecific due to the high morphological similarity between the worker castes (Seifert, 2003; Yoshimura et al., 2008).

As mentioned above, there have been many studies about its classification or male polymorphism. Such kind of studies is not good enough to understand all the aspects of the problems with invasive species. In the study of invasive species, it is important to estimate the invasion routes. When estimating the invasion routes, we need to know the haplotype distribution of the target species. Thus, we need to know the haplotype distribution of *C. kagutsuchi* in order to promote the conservation biology of the native ant diversity in Japan. However, there was no study about its haplotype distribution. The first purpose of this study is to collect this ant from across Japan and investigate their mtDNA sequences to reveal the distribution of their haplotypes in Japan. The second purpose is to construct genealogical trees and a haplotype network to clarify their position in the genus *Cardiocondyla*.

According to "Japanese Ant Image Database" (http://ant.edb.miyakyo-u.ac.jp/J/index.html), *C. kagutsuchi* is not distributed in the eastern Japan without Kanagawa prefecture. We, however, have collected a lot of colonies of *C. kagutsuchi* in Shizuoka prefecture, located in the eastern Japan, in the last few years. Where do they come from? There has been no report of their existence in the area. They may have come from foreign countries. The third purpose is to infer the invasion routes of *C. kagutsuchi* in Shizuoka prefecture, using haplotype distribution of mtDNA across Japan and *C. kagutsuchi* in foreign countries.

Materials and Methods

Sampling

We collected all samples during the species' reproductive season (June and July) in 2010. This ant is known to inhabit open areas and nest in the soil (Terayama, 1999); therefore, we searched for these ants in green areas, vegetable fields, parks, near the seashore, etc. In each locality, we spent at least one hour searching for the ant; if we did not find any ants within this time, we moved to a different place. Colonies or foraging workers of *C. kagutsuchi* were collected from 27 localities between Kanto and Ishigaki Islands in Japan. Nests were located by following foragers back to the nest entrance, and complete colonies and colony fragments were then collected by carefully digging up soil using a scoop. Some nests were found in gaps between tiles and under stones. All samples were collected near the seashore, with the exception of those collected from Shizuoka Prefecture and Ishigaki Island. Following collection, samples were stored in ethanol (99.5%). Only workers were used for DNA analysis. When we could sample male individuals in each locality, we examined their wing polymorphism.

DNA extraction, amplification, and purification

A 890-bp fragment of COI/II (including 58 base pairs of leucine tRNA), corresponding to positions 2284 to 3191 in the *Drosophila yakuba burla* mitochondrial genome (Clary & Wolstenholme, 1985), was used for phylogenetic analysis. DNA was extracted from individual workers using Landry's method (Cheung et al., 1993). The dried pellet was eluted in purified water and preserved at –80°C until further analysis.

We amplified COI/II using the primers C1-J-2195 and C2-N-3661 (Simon et al., 1994). Polymerase chain reactions (PCRs) were performed using SYBR Premix Ex Taq (TaKaRa) and conducted in a MyCycler (Bio-Rad) and MJ Mini Gradient Thermal Cycler (Bio-Rad). PCR consisted of 34 cycles of denaturing at 95°C for 1 min, annealing at 59°C for 1 min, and extension at 72°C for 2 min, with the exception of the initial denaturing step at 95°C for 4 min. Amplified DNA was purified using a PCR Purification Kit (Qiagen).

All products were sequenced with the 3730xl DNA Analyzer (Applied Biosystems) using the primers C1-J-2195 and C2-N-3661 (Simon et al., 1994). To obtain the complete sequences, some additional primers were also designed by Nukui and Okita (unpubl.):

- 3F [5′-CCTTTAATTAGAGGATACAC-3′] and 4F [5′-GGCAGATAAGTGCAAAGGAC-3′] (which were used with C1-J-2195).

- 1R [5′-TTCTATAGAGTGATTTTGGAGGAG-3′], 2R [5′-GGTATACCTCTGAGACC-3′] and 3R [5′-CAGCTCCTATAGAGAGAACATAG-3′] (which were used with C2-N-3661).

Phylogenetic analyses

We obtained 28 sequences and aligned these with a further 11 sequences obtained from GenBank using the CLUSTAL W algorithm (Thompson et al., 1994). The accession numbers of the 11 additional sequences were

DQ023083, DQ023084, DQ023085, DQ023086, DQ023087, DQ023088, DQ023091, DQ023094, DQ023102, DQ023108, and DQ023118 (Heinze et al., 2005). Six of the 11 sequences (DQ023083, DQ023084, DQ023085, DQ023086, DQ023087, and DQ023088) were sequences of *C. kagutsuchi* originating from Hawaii, Malaysia, and Indonesia; the other 5 sequences (DQ023091, DQ023094, DQ023102, DQ023108, and DQ023118) were sequences of *C. mauritanica*, *C. minutior*, *C. obscurior*, *C. strigifrons*, and *C. wroughtonii*, which were used as outgroups (Heinze et al., 2005). Phylogenetic relationships were inferred from the aligned COI/II using a distance and Bayesian analysis.

A neighbor-joining tree (Saitou & Nei, 1987) was constructed using the Kimura 2-parameter distance method (Kimura, 1980) in MEGA5, with bootstrap values estimated from 5000 replicates. In the Bayesian analysis, a single run consisted of 300,000 generations. We also constructed a haplotype network using TCS 1.21 (Clement et al., 2000) with the haplotypes identified by the sequences described above.

Results

We found four different haplotypes of *C. kagutsuchi* in Japan. The distribution of each of the four haplotypes is shown in Fig. 1. The number of sequences we obtained was 28, while the number of sampling locality was 27. This is because the two types of haplotype were found in sampling locality 5. The first was distributed in Kanagawa (1 locality), Shizuoka (4), Aichi (1), Wakayama (1), and Hyogo (1) (Honshu area); Kagawa (1) (Shikoku area); and Miyazaki (1) and Kagoshima (1) (Kyushu area). The second was distributed in Shizuoka (8 localities) (Honshu area); Tokushima (2), Kagawa (1), and Kochi (1) (Shikoku area); and Kumamoto (1) (Kyushu area). The third was found only on Okinawa Island (2 localities), and the fourth was found only on Ishigaki Island (2 localities). The first and second haplotypes were distributed widely across the temperate zone in Japan (Honsyu, Shikoku, and Kyusyu areas) and, in some cases, were found very close together. In contrast, the third and fourth haplotypes were found in a limited area (a single island).

We constructed a neighbor-joining tree and a Bayesian tree to analyze the relationship between haplotypes and male polymorphism (Figs. 2, 3). Both trees showed that *C. wroughtonii*, *C. obscurior*, and *C. minutior* were outgroups, but *C. strigifrons* was in the same group as *C. kagutsuchi*. *Cardiocondyla mauritanica* was also an outgroup according to the neighbor-joining tree, but was in the same group as *C. kagutsuchi* according to the Bayesian tree. The accession numbers of the 4 new sequences were showed in each Figure.

We also constructed a haplotype network (Fig. 4). It contained four haplotypes found in Japan in this study. The distance between the haplotype 1 and 2 was 33. The distance between the haplotype 1 and 3 was two, the number is the smallest among all haplotype pairs. The distance between the haplotype 1 and 4 was 10. The distance between the haplotype 2 and 3 was 31. The distance between the haplotype 2 and 4 was 41, the number is the highest. The distance between the haplotype 3 and 4 was 10.

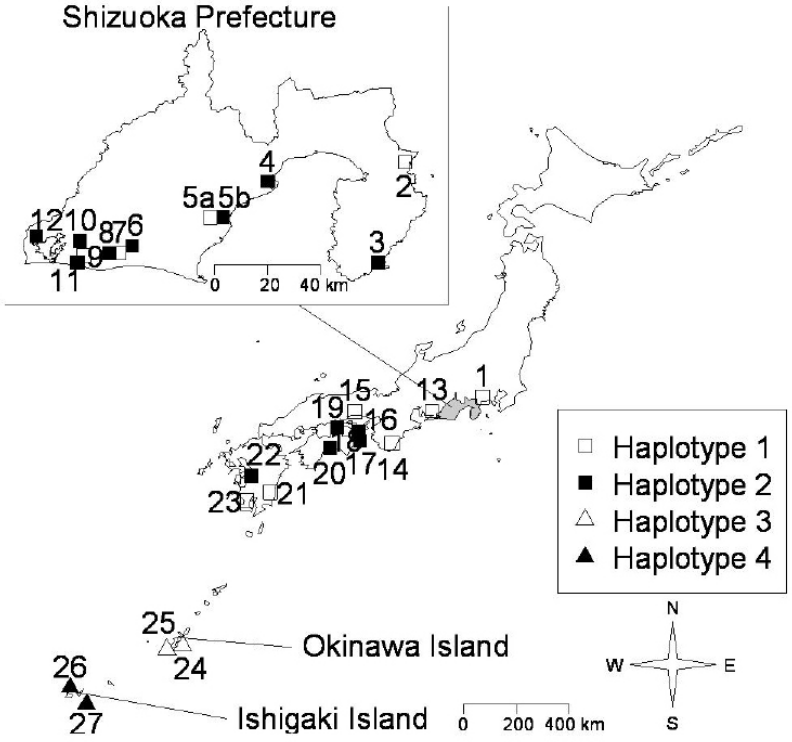

Fig. 1. Distribution of *C. kagutsuchi* in Japan. Sampling localities are numbered from 1 to 27. The haplotype of *C. kagutsuchi* in each locality is indicated by the corresponding symbol. Shizuoka Prefecture is enlarged because many sampling localities are included in it. Sampling locality 5 (in the enlarged part of the figure) is divided into 5a and 5b because the two types of haplotype were found.

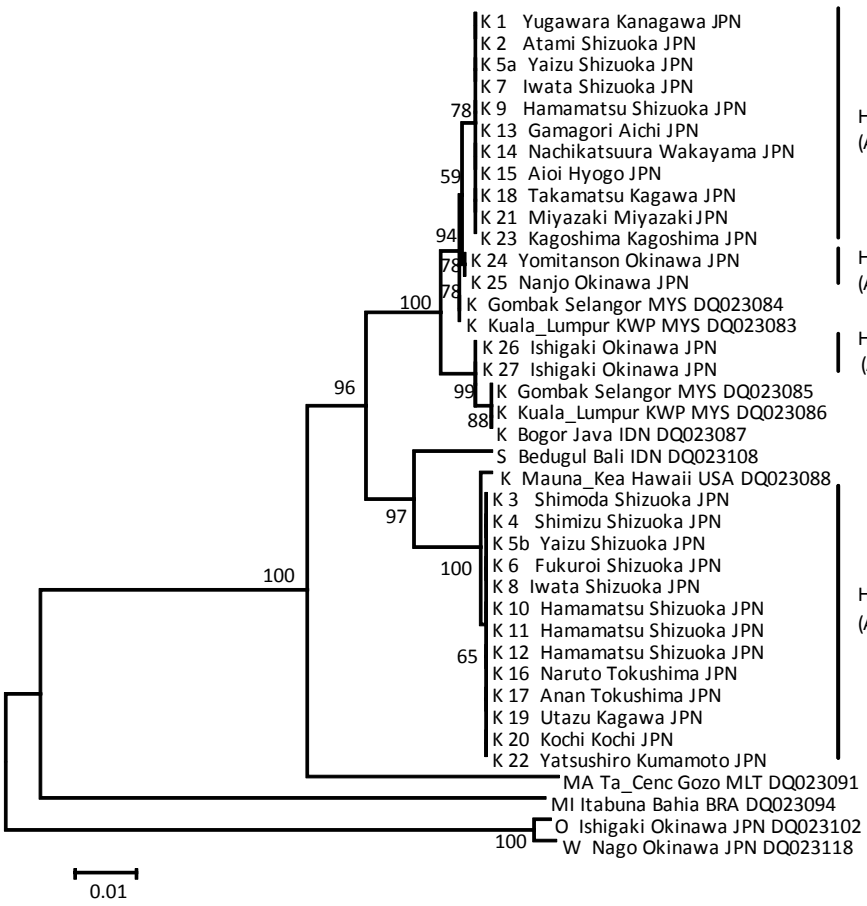

Fig. 2. Neighbor-joining tree based on the mitochondrial COI/II genes (890 bp) of the ant genus *Cardiocondyla*. Numbers indicate bootstrap values over 50% in 5000 pseudoreplications. Each sample name is given in the following order: (1) species name; (2); sampling locality number in Fig 1 if the sample is ours; (3) local area name; (4) prefecture or state name; (5) country name; and (6) accession number in GenBank if the sample is in the data of Heinze *et al.* (2005). The species names are as follows: K—*C. kagutsuchi*, S—*C. strigifrons*, MA—*C. mauritanica*, MI—*C. minutior*, O—*C. obscurior*, and W—*C. wroughtonii*. Sampling locality 5 is divided into 5a and 5b because the two types of haplotype were found. The haplotype numbers in Fig 1 are added in the right of the figure, with their accession numbers.

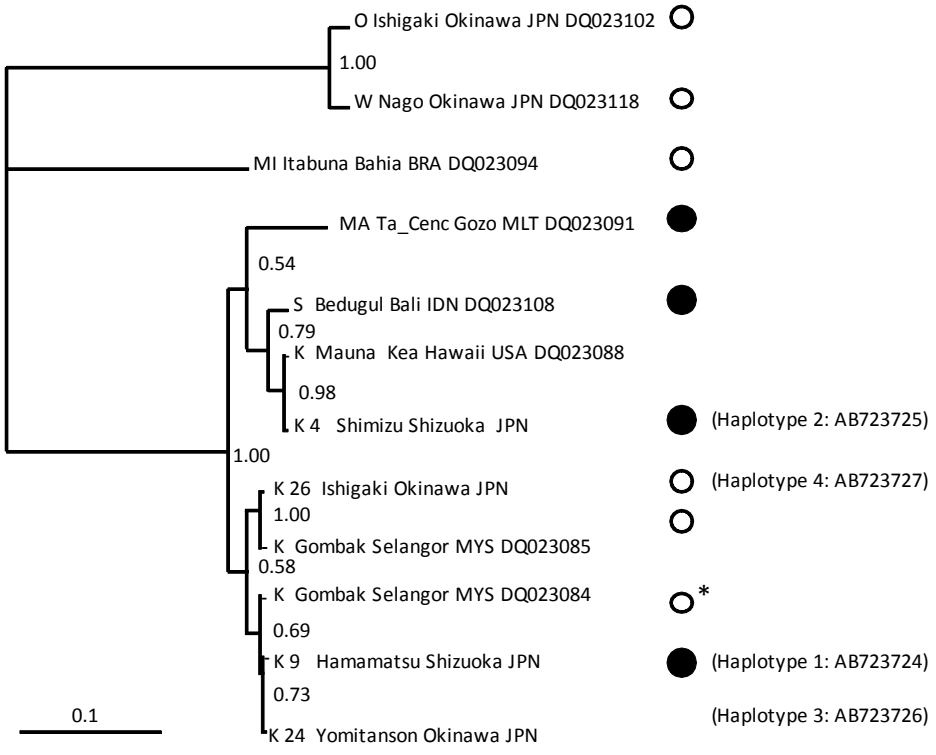

Fig. 3. Consensus tree of *Cardiocondyla* based on the mitochondrial COI/II using MrBayes (300,000 generations). Sample names are given in the same way as in Fig 2. White circles indicate that both winged and wingless males are present (type 1, dimorphic), a white circle with asterisk is for both short-winged and wingless males (type 2, dimorphic), and filled circles are for only wingless males (type 3, monomorphic), respectively. No males are known from *C. kagutsuchi* from Mauna Kea, Hawaii and Yomitanson, Okinawa. The haplotype numbers in Fig 1 are added in the right of the figure, with their accession numbers.

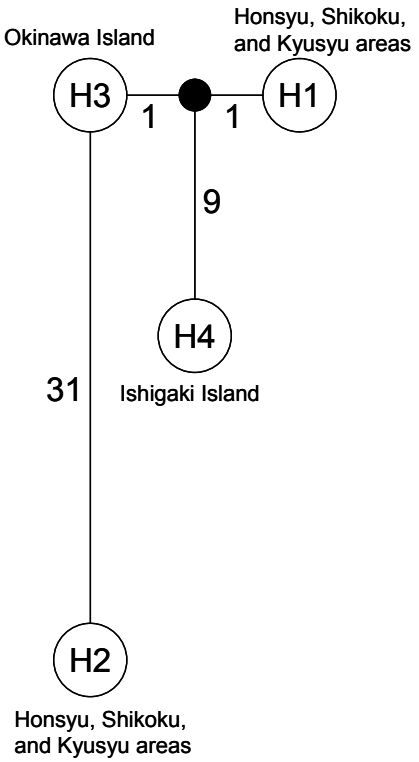

Fig. 4. Parsimonious network among four haplotypes. Open circles indicate identified haplotypes, and the filled circle indicates a hypothetical haplotype. "H1" means haplotype 1 in Fig 2, "H2" does haplotype 2, and so forth. The numbers between nodes indicate the number of nucleotide substitutions.

Discussion

At first, we want to discuss the first purpose. The distributions of the four different haplotypes are shown in Fig. 1. Haplotypes 1 and 2 were distributed widely across Japan, rather than being restricted to a single area, such as an island. This overlapped distribution pattern and this relatively low level of genetic variability across a wide area indicate that *C. kagutsuchi* is not native ant species in Japan and it may have extended its distribution quickly. What caused this distribution pattern and this low variability? Is it a result of this species being invasive, mating in its natal nest, or being a cryptic species, or is there some other reason? This would be a very interesting area for future study.

We want to discuss the second purpose. The haplotype 1 and 2 were placed in distant clades in phylogenetic trees (Figs. 2, 3) and they were considerably distant from each other in the haplotype network (Fig. 4). This suggests that the haplotype 1 and 2 are originated from other localities. The haplotype 1 is a near relation of DQ023083 and DQ023084, which were sampled in Malaysia, whereas the haplotype 2 is closely related to DQ023088, which was sampled in Hawaii. Further, the haplotype 2 is more closely related to DQ023108, *C. strigifrons*, than the other haplotypes of *C. kagutsuchi*. This might indicate that the two haplotypes are different species rather than the same species. For the topic whether the two haplotypes are different species or not, their nuclear DNA would be needed to be examined because of the possibility of their crossing.

We want to discuss the third purpose. *C. kagutsuchi* that had expanded its distribution to the eastern Japan was neither the foreign new genotype nor the one in isolated islands like Okinawa: it was the haplotype 1 and 2. This result indicates that there is a strong possibility of its distribution expansion in Honsyu area in Japan. We should keep an eye on the routes it will use for its future expansion.

Most of the colonies in this study were sampled in its breeding season. In consequence, as for male dimorphism, Type 1, which is regarded as *C. kagutsuchi* sensu Terayama, 1999 and in which there is male dimorphism, was only found on Ishigaki Island and appeared as an independent clade in the phylogenetic analysis. For future study, whether male wing polymorphism and other ecological differences are related to genetic variations is an interesting issue.

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