

Research Article

Population dynamics of invasive Argentine ant *Linepithema humile* Mayr, 1868 (Hymenoptera: Formicidae) haplotypes in Kobe Port, Japan, and implications for the prediction of future dispersal and effective management

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Abstract

The Argentine ant *Linepithema humile* Mayr, native to South America, has been unintentionally introduced worldwide. Although *L. humile* usually forms an extremely large supercolony all sharing the same haplotype among members in its introduced ranges, four haplotypes (LH1, LH2, LH3, LH4) with different genetic structures have been discovered in Kobe Port (Port-Island and Maya Wharf), Japan. In 2019, a new *L. humile* population was discovered, the first since the initial invasion in 2006. Therefore, we identified the haplotypes among these newly invaded *L. humile* populations via mtDNA analysis and then compared the distributions among the haplotypes during these two time periods (2006 versus 2019). The haplotypes of all newly introduced *L. humile* populations corresponded to only the haplotypes previously detected in Kobe Port. Although the distribution ranges of haplotypes LH1 and LH4 changed little in Maya Wharf over approximately 15 years, major distribution expansions of LH2 and LH3 were found on Port-Island. Given the low natural dispersion ability of *L. humile*, it is likely that the range expansions of LH2 and LH3 on Port-Island were caused by the extensive transportation of construction materials within Kobe Port. In addition to strengthening the quarantine at existing trading ports, greater attention should be paid to the introduction of biological invaders in conjunction with the transportation of construction materials when new ports are developed and maintained.

Key words: biological invasions, urban ecology, uniclonal ant, mtDNA, population genetics, unintentional introduction

Introduction

Biological invasions have increased along with the increase in world tourism and trading. Among them, species that are introduced unintentionally in conjunction with the importation of commodities have been rapidly increasing (e.g., Suarez et al. 2001; Hulme 2009; Saul et al. 2017).



Figure 1. Worker of Argentine ant *Linepithema humile* Mayr, 1986. Photo by Hiro Kasaki.

In particular, many researchers have indicated that the trading ports and transit points of cargo ships are donor and/or recipient spots for unintentionally introduced invasive species (e.g., McGlynn 1999; Hulme 2009; Maebara et al. 2020).

The Argentine ant *Linepithema humile* Mayr, 1868 (Figure 1), which is native to South America, is a biological invasive species that has been unintentionally introduced worldwide (Suarez et al. 2001). Based on its high invasiveness, *L. humile* is described as one of the 100 most hazardous invasive species in the world due to the serious damage to human health, agricultural production and native arthropod communities it causes in its introduced regions (Lowe et al. 2000).

Invasive *L. humile* populations form a unique social structure called unicoloniality in which workers and reproductive castes (queens) can move freely among interconnected nests within the same very large colony (i.e., supercolony), but they show hostile behaviors toward individuals from different supercolonies (Suarez et al. 1999; Pedersen et al. 2006). Recent studies have shown that each *L. humile* supercolony shares a single unique mitochondrial haplotype, functioning as an independent reproductive unit (Sunamura et al. 2009b). As a result, the lack of hostility among *L. humile* workers within the same haplotype is maintained as a genetic boundary (Sunamura et al. 2009a).

In contrast, *L. humile* cannot disperse widely by themselves. One reason for this limitation is their behavior during the breeding period. In most ant species, virgin queens mate with males while flying during the breeding periods (i.e., the nuptial flight). Then, they form a new colony in new environments. Some researchers have reported that the annual expansion ranges of ants with nuptial flight can reach up to 5–16 km (Markin et al. 1971; Vogt et al. 2000). On the other hand, the annual expansion range of *L. humile*, which does not exhibit the nuptial flight behavior (Markin 1970),

is less than 15–180 m on average (maximum up to 340 m) (Suarez et al. 2001; Tatsuki 2014; Kido et al. 2017). However, *L. humile* can spread over 100 km per year through the transportation of commodities (Suarez et al. 2001).

In Japan, *L. humile* was first detected in 1993 (Sugiyama 2000), and after that, this species was found to consist of four haplotypes (named LH1, LH2, LH3, and LH4) with different genetic structures that have been established in 12 prefectures and cities so far (Sunamura et al. 2007, 2009b; Inoue et al. 2013; Sakamoto et al. 2017; Nakahama et al. 2019). Although most *L. humile* populations share only one haplotype in each prefecture, populations in Kobe city, Hyogo Prefecture, share all of the haplotypes of Japan within a narrow range of several square kilometers (Sunamura et al. 2007, 2009b; Inoue et al. 2013). Given that Kobe Port is one of the major trading ports in Japan, its role as a recipient and/or donor spot of *L. humile* invasion and/or spread is of concern. In fact, we found evidence of range expansion of the invasive ant in Kobe Port in 2019. However, there is no information about what haplotype is shared within its newly detected populations. In addition, understanding the population dynamics of invasive species at the haplotype level is expected to lead to the detailed detection of their invasion routes (Maebara et al. 2020) and subsequent effective management of biological invaders (Hayasaka et al. 2015, 2018; Morrison et al. 2017; Seko et al. 2018), whether they are plants or animals. Furthermore, there is little information on the temporal changes in their genetic structure (i.e., within species) after invasion, leading to a better understanding of the biological invasion process despite many studies on initial/new invasion records of biological invaders and their genetic structures.

Here, we identified a new *L. humile* population with an unknown haplotype in Kobe Port in 2019. Then, the population dynamics among the four *L. humile* haplotypes were compared in this study area, the first since 2006 (Sunamura et al. 2007). Although a previous study detected the distribution of each *L. humile* haplotype (supercolony) in Kobe Port through the results of hostility tests (Sunamura et al. 2007), we applied the population genetic approach (mitochondrial DNA analysis) to detect their haplotypes.

Materials and methods

Study sites

This study was performed at Port-Island (34°39'56.2"N; 135°13'10.1"E) and the Maya Wharf (34°41'46.1"N; 135°13'40.3"E) of Kobe Port in Kobe city, Hyogo Prefecture, Japan (Figure 2).

Distribution survey and sampling of L. humile haplotypes

From September 2019 to February 2020, we conducted a visual survey to investigate the distribution of *L. humile* along roads at both study sites. The survey was also conducted at public facilities such as parks. Samples of

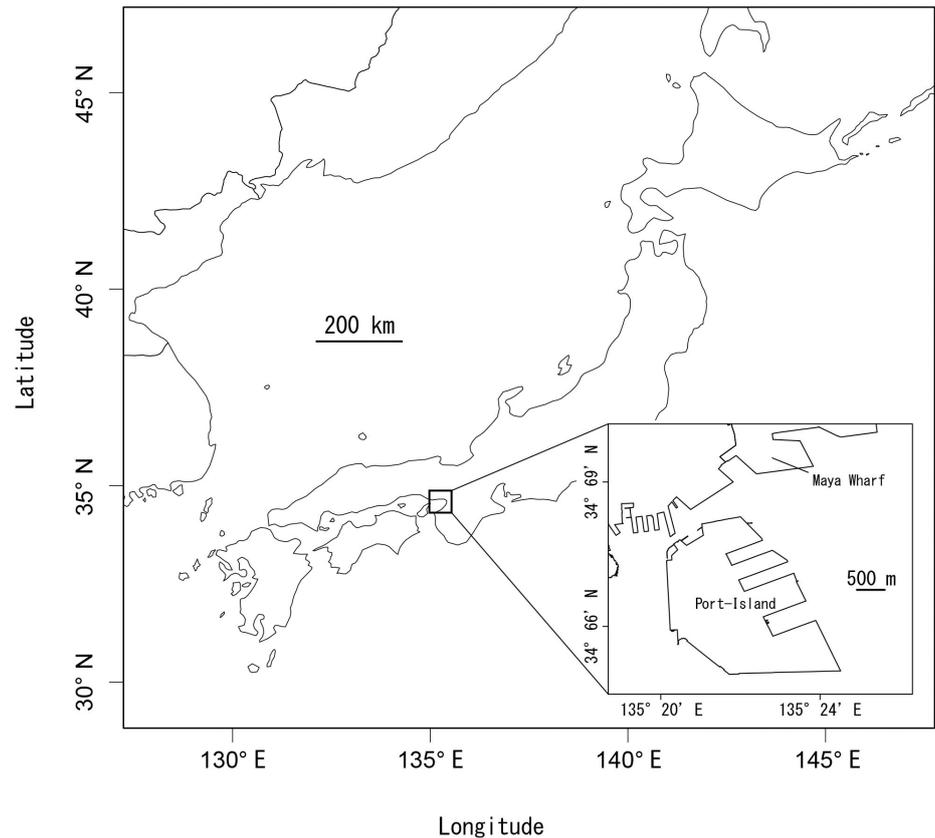


Figure 2. Locations of Port-Island and Maya Wharf (i.e., Kobe Port) and the sampling sites of *L. humile* workers in Kobe City, Hyogo Prefecture, Japan.

L. humile workers, for which we obtained their location coordinates using a handheld GPS receiver (Garmin Oregon 300, Garmin Ltd., Kansas, USA), were collected with a fluke tube and then immediately stored in 100% ethanol until DNA analysis. A worker was randomly selected from nests every approximately 10 m since it was suggested that nests within a radius of 10 m had a possibility of sharing the same haplotype (supercolony) based on previous reports on the annual dispersal range of *L. humile* (Suarez et al. 2001; Tatsuki 2014; Kido et al. 2017). Of the 414 location coordinates of *L. humile*, 152 sampling points were used for genetic analysis (Supplementary material Table S1).

DNA analysis

Genomic DNA from the sampled *L. humile* individuals in this study was extracted from one leg of the individual ants using a standard phenol-chloroform method. Three primer sets (Lh1751, Lh2329b; Lh2195, Lh3014; and Lh2797, Lh3389b) were used to amplify three partially overlapping fragments that together provided the COI-COII sequence (approximately 1700 bp) (Inoue et al. 2013; Nakahama et al. 2019). A 524-bp sequence from the mtDNA cytochrome *b* (*Cyt b*) gene was also amplified with the L-Lhcb and R-Lhcb primer sets (Pedersen et al. 2006). All amplification reactions, except those using the Lh2195 and Lh3014 primer sets, were

performed in a 20 μ L volume containing 10–50 ng of template DNA, 0.1 μ L of BIOTAQ™ Polymerase (Nippon Genetics), 2.0 μ L of 10x NH4 reaction buffer (Nippon Genetics), 0.08 M dNTPs, 0.04 M MgCl₂ and 0.1 μ L of each primer at 20 μ M. The amplification reaction employing the Lh2195 and Lh3014 pairs was performed in a 20 μ L volume containing 10–50 ng of template DNA, 0.5 μ L of TaKaRa Ex Taq (Takara Bio), 2.0 μ L of 10x Ex Taq Buffer (Takara Bio), 2.0 μ L of dNTPs, 0.04 M MgCl₂ and 0.2 μ L of each primer at 100 μ M. The conditions of the PCR cycling for amplifying the COI-COII sequence were as follows: (1) template denaturation at 95°C for 10 min; followed by (2) 30 cycles of denaturation at 94 °C for 0.5 min, annealing at 46–47°C for 0.5 min, and extension at 72 °C for 2 min; and (3) a final extension at 72°C for 7 min. In contrast, the number of PCR cycles was changed to 40 cycles for the Lh2195 and Lh3014 pair. The conditions of the PCR cycling for amplifying the Cyt *b* sequence were as follows: (1) template denaturation at 94 °C for 3 min, followed by (2) 35 cycles of denaturation at 92 °C for 0.5 min, annealing at 60 °C for 0.5 min, and extension at 72 °C for 1 min. DNA sequencing was conducted by a commercial service of Macrogen Japan Corporation using a 3730xl DNA Analyzer (Applied Biosystems).

Data analysis

The *L. humile* mitochondrial sequence data were edited and aligned using an automatic multiple sequence alignment algorithm implemented in ClustalW. The base sequence data were compared with the existing 18 haplotypes (accession numbers FJ466647–FJ466664 for Cyt *b*, FJ466666–FJ466683 for COI and FJ535653–FJ535670 for COII) (Vogel et al. 2009, 2010) via ClustalW to identify its haplotype. These data analyses were implemented in the statistical software MEGA X ver. 10.1 (Kumar et al. 2018). The COI-COII and Cyt *b* sequences from the other *L. humile* haplotypes identified in Japan (accession numbers AB693876–AB693881 for Cyt *b*, AB568481–AB568484 and AB693875 for COI-COII) (Inoue et al. 2013) were obtained from the NCBI website (<http://www.ncbi.nlm.nih.gov>).

Results

We found that all *L. humile* corresponded to one of four haplotypes (LH1, LH2, LH3, and LH4), which were already identified at Kobe Port (Figure 3a, b), and no additional haplotypes were detected. Specifically, haplotypes LH1 and LH4 were found in Maya Wharf, and LH2 and LH3 were detected on Port-Island (Figure 3a, c).

The comparison of population dynamics among the four *L. humile* haplotypes between the present study and a previous report (Sunamura et al. 2007) showed that although haplotypes LH1 and LH4 in Maya Wharf varied little in regard to their colony size (LH4 slightly decreased) between

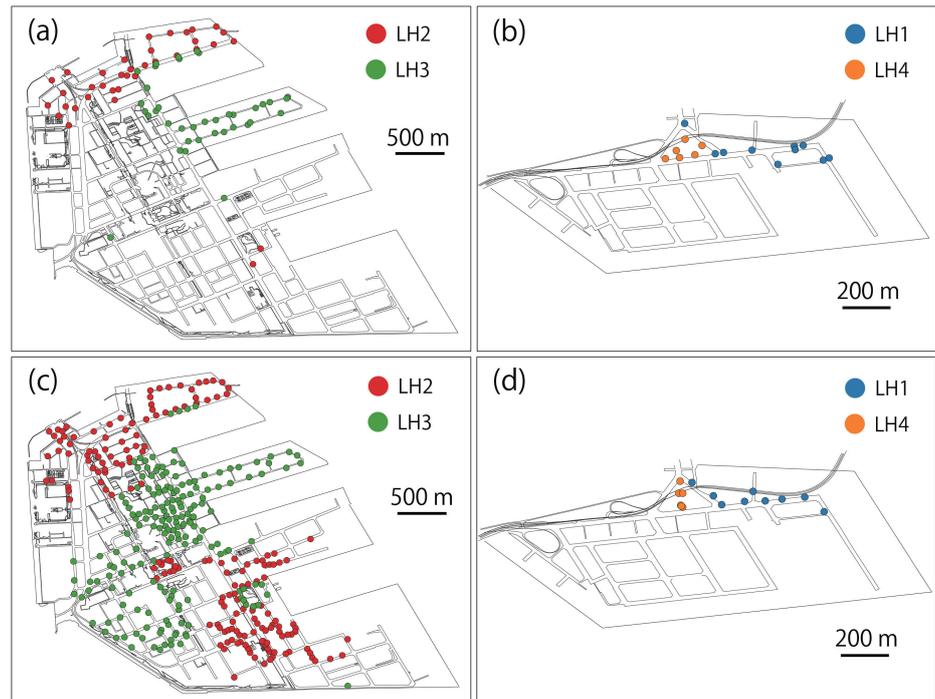


Figure 3. Distribution patterns of *L. humile* haplotypes (i.e., LH1, LH2, LH3, and LH4) (a, c) on Port-Island and (b, d) in Maya Wharf, Hyogo Prefecture, Japan. The spatial distributions of these haplotypes in 2006 (Sunamura et al. 2007) and 2019 (this study) are shown in (a, b) (partially modified from Sunamura et al. 2007) and (c, d), respectively.

the two time series, haplotypes LH2 and LH3 in Port-Island had significantly increased their colony size (1–2 km²). Additionally, among the points (nests) newly detected (LH2 and LH3) on Port-Island, mosaic distributions with some colonies of one haplotype interspersed with smaller (~ 200 m scale) colonies of another haplotype were found at three sites (34°39'47.0"N; 135°12'55.0"E, 34°39'36.2"N; 135°13'26.6"E, 34°39'02.9"N; 135°14'05.4"E).

Discussion

In this study, the introduction of *L. humile* haplotypes other than those previously detected in Kobe Port was not found. On the other hand, the comparisons of *L. humile* population dynamics between the two time series showed clear differences in the distribution expansion among the haplotypes, especially LH2 and LH3 (Figures 3a, c). In Maya Wharf, the distribution range (colony size) of haplotype LH4 slightly decreased compared with its range identified by Sunamura et al. (2007) (Figure 3b, d). In its native ranges (i.e., South America), it is known that the colony (supercolony) size of *L. humile* is variable within tens of meters in conjunction with changes in the surrounding environment, such as the season and the location of resources (Vogel et al. 2009), irrespective of the differences in its haplotypes. Given this information, there is a possibility that a slight decrease in haplotype LH4 was temporarily caused by landscape changes via roadside tree management regularly conducted in port areas

such as the present study sites. However, with the decrease in LH4, since roadside tree management had not been implemented at most sites with the introduction of LH1 (field observations), there is a possibility that they did not decrease their distribution range.

On the other hand, on Port-Island, haplotypes LH2 and LH3 have expanded their distribution range over the last 15 years (Sunamura et al. 2007) (Figures 3a, c). One possibility for the differences in the dynamics of *L. humile* haplotypes between Port-Island and Maya Wharf may be related to the different timing of construction completion at both sites. The construction of Maya Wharf was completed prior to 1999 (circa 1991), when *L. humile* was first introduced unintentionally, and no notable port improvement has been made since (Kobe City 2019). In contrast, port improvement on Port-Island was conducted until 2009 (Kobe City 2019), three years after the first survey of *L. humile* by Sunamura et al. (2007), and the construction of large facilities such as factories and hospitals continued until the 2010s (Kobe City Medical Center General Hospital 2020). Given the transportation of a massive amount of construction materials associated with port improvement, it seems that haplotypes LH2 and LH3 were transported with the materials (i.e., jump dispersal) (McGlynn 1999; Suarez et al. 2001), which led to their subsequent range expansion. On the other hand, given that the expansion of haplotypes LH2 and LH3 was approximately 1–2 km over approximately 15 years (Figure 3a, c), we cannot rule out the possibility that they expanded their distribution range by themselves (Suarez et al. 2001; Tatsuki 2014; Kido et al. 2017). However, since there were mosaic distributions between LH2 and LH3 on Port-Island (Figure 3c), the probability of their expansion via transportation with construction materials is more likely. Given these findings, the expansion patterns of the two haplotypes might have been brought about by the interaction of natural and jump dispersal. As mentioned above, the dispersion of *L. humile* without nuptial flights is limited to only horizontal movement (Markin 1970). Therefore, haplotypes LH2 and LH3 would have little possibility of dispersion by themselves, at least in ranges with mosaic distributions (Figure 3c). Based on the above, it is suggested that the differences in the population dynamics of the four *L. humile* haplotypes in Kobe Port would be caused not only by their own dispersion (natural dispersal) but also by the transportation of construction materials, i.e., jump dispersal (McGlynn 1999; Suarez et al. 2001) and landscape changes, including vegetation management. However, although the present study identified haplotypes LH2 and LH3 in a newly invaded area, their derivations, which include the possibility of introductions from other countries, regions, or prefectures, were not clarified because only a few mitochondrial regions were used for sequencing. Therefore, individual-based data sampling/analyses, such as microsatellite analysis or MIG-seq (Suyama and Matsuki 2015), that correspond to temporal changes in genetic structure and distribution should be conducted to solve the mystery.

Recently, in Japan, which actively trades with many other countries, various invasive alien ants, such as the red imported fire ant *Solenopsis invicta* Buren (Murakami 2018), *Iridomyrmex anceps* Roger (Terayama and Sunamura 2019), and browsing ant *Lepisiota frauenfeldi* Mayr, have often been introduced unintentionally, especially in port areas. There is no doubt that international trade is one of the major driving factors promoting the introduction of biological invaders, both plants and animals (Hulme 2009; Maebara et al. 2020). To address these problems, for example, the quarantine system (Sistema de Inspección y Cuarentena para las Islas Galápagos: SICGAL) in the Galapagos Islands, Ecuador, was consolidated early to prevent the invasion of alien arthropods (e.g., Causton et al. 2000, 2006). Additionally, weed risk assessment (WRA) systems based on weed status as a biosecurity tool have been adopted in many countries and regions, including Hawaii and Australia, when introducing plants (e.g., Pheloung et al. 1999; Groves et al. 2001; Daehler et al. 2004). However, there is no end of the invasion of biological invaders. Two measures against invasive species, such as *L. humile*, can be taken: prediction of invasion routes (preinvasion phase) and management/eradication (post-invasion phase). Maebara et al. (2020) mentioned that it is essential to strengthen the quarantine system and establish an information-sharing network in port areas when conducting border control predictions of their introduction. Additionally, population genetic information is essential to predict the introduction of alien species and to understand the subsequent process of distribution expansion. There is no doubt that the integration of the above two approaches is necessary for future invasive species management. Unfortunately, if *L. humile* had newly invaded, rapid execution of chemical control (Hayasaka et al. 2015), taking susceptibility among haplotypes to toxics into consideration, within narrow invaded areas or population sizes, would lead to effective control/eradication of the invasive ant.

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Supplementary material

The following supplementary material is available for this article:

Table S1. Primary georeferenced data on *Linepithema humile* in the present study. *indicates the samples for genetic analysis.

This material is available as part of online article from:

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