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Rapid Communication

First record of the cedar bark aphid, *Cinara cedri cedri* Mimeur, 1936 (Hemiptera: Aphidoidea) in Japan, and identification of infecting *Wolbachia* strains

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Abstract

Aphids are phloem sap-feeding insects distributed worldwide that can cause serious crop production losses, especially in temperate regions. Biological invasion by aphids is common, and the accumulation of case reports is important for their control. Here, we provide the first report of the cedar bark aphid *Cinara cedri cedri* (Hemiptera: Aphidoidea) in Japan. In Okazaki City, Aichi Prefecture, and Nishinomiya City, Hyogo Prefecture, we found this species on twigs of ornamental trees, *Cedrus deodara*. These two collection localities are clearly distant from the region of known distribution of this species. *Cinara cedri cedri* is native to the East Mediterranean region and has been introduced into several European countries, the USA, Canada, and Korea. The mitochondrial COI sequences of the Japanese populations matched the identity of those in the USA and Canada, recently introduced populations, and some European populations. *Wolbachia* is an endosymbiont of this species, and we found that those infected in the Japanese population of *C. cedri* showed no variation in 16S rDNA sequences. The sequences were identical to those of one of the two *Wolbachia* strains detected in the native ranges of *C. cedri*. We observed *C. cedri* populations from early summer to late winter in 2021 and early summer in 2022 in both localities, indicating that these aphids are able to overwinter in Japan.

Key words: biological invasion, giant conifer aphids, Himalayan cedar, endosymbiont

Introduction

Phloem sap-sucking insects, such as those of the orders Hemiptera and Thysanoptera, are among the major feeding guilds of invasive insect species affecting both agricultural plants and forest trees (Kenis et al. 2007; Aukema et al. 2011; Mendel et al. 2016). Biological invasions by aphids (Hemiptera: Sternorrhyncha: Aphidinea) are common (Coeur d'acier et al. 2010) and can often cause serious damage to plants, not only directly by the sucking of plant phloem sap, but also indirectly as they act as secretors of honeydew on which sooty molds grow, and as vectors of viral diseases (Guerrieria and Digilio 2008). Their economic and ecological impacts are

difficult to predict, especially at the time of discovery; therefore, accumulating case reports of these insects is important.

The genus *Cinara* Curtis, 1835 (Hemiptera: Aphididae: Lachninae) is a large aphid genus comprising 256 known species worldwide (Blackman and Eastop 2018). All species of this genus are associated with coniferous trees (Cupressaceae and Pinaceae families) (Eastop 1972; Eastop et al. 1998). *Cinara* consists of four subgenera: *Cedrobium* Remaudière, 1954; *Cinara* Curtis, 1835; *Cupressobium* Börner, 1940; and *Schizolachnus* Mordvilko, 1909 (Blackman and Eastop 2018). These conifer aphids are recognized as pests in plantations of coniferous trees (Inouye 1970; Obiri 1994). For example, in the Mediterranean region, *C.* (*Cupressobium*) *cupressi* (Buckton, 1881), which is native to North America (Watson et al. 1999) can cause rapid dieback of the canopy of *Cupressus* spp. in spring (Mendel et al. 2016). Even though Mendel et al. (2016) argued that none of the alien *Cinara* spp. in the Mediterranean region, except *C. cupressi*, cause serious damage to native trees, newly recorded alien *Cinara* species have the potential to become devastating pests.

The cedar bark aphid *Cinara* (*Cinara*) *cedri* Mimeur, 1936 is a pest of *Cedrus* spp. that damages young branches and shoots (Binazzi et al. 2015). The species was first identified in the Moroccan Middle Atlas (Mimeur 1935), but the probable origin is the eastern Mediterranean, due to the presence of parasitoid wasps (Michelena et al. 2005). Recently, *C. cedri* was subdivided into two subspecies: *C. cedri cedri* and *C. cedri brevifoliae* (Binazzi et al. 2017). The species has been recorded in many countries, including France, Italy, Spain, and the UK in Europe; Morocco and Algeria in North Africa; Canada, and the United States in North America; Argentina in South America; Cyprus, Iran, Iraq, Israel, Lebanon, Syria, and Turkey in the Middle East; and China and Korea in East Asia (Delfino and Binazzi 2002; Yu and Wang 2014; Binazzi et al. 2015; Blackman and Eastop 2018; Lee et al. 2020); however, the phylogenetic relationships and the introduced subspecies are not well understood.

Cinara cedri harbors at most three endosymbionts: *Buchnera aphidicola* and *Serratia symbiotica* as obligate symbionts (Lamelas et al. 2011), and *Wolbachia* as a facultative symbiont (Gómez-Valero et al. 2004; Augustinos et al. 2011). *Cinara cedri* exhibits a high frequency of infection with *Wolbachia*, and two strains exist in *C. cedri*: one of which belongs to Supergroup M, the aphid-specific clade, and the other is included in Supergroup B, the clade frequently detected from wasps and spider mites (Gómez-Valero et al. 2004; Augustinos et al. 2011). *Wolbachia* is one of the most widespread maternally transmitted symbionts of arthropods and nematodes (Kaur et al. 2021), and the comparison of their phylogenetic relationships and genetic diversity in native and non-native ranges can provide a unique insight into the invasion history and introduction pathway of alien species (Tsutsui et al. 2003; Bouwma et al. 2006; Tseng et al. 2019).

Table 1. Sample information of *Cinara cedri* collected in the study.

#	Sample code	Latitude	Longitude	Collection date	Attending ants*	GeneBank accession no.	
						COI of aphid	16S rDNA†
1	NIBB (in NINS)	34.95023	137.16495	3 July 2021	Cj	LC700312	LC700320
2	NIPS (in NINS)	34.94862	137.16494	3 July 2021	-	LC700313	LC700321
3	IMS (in NINS)	34.94709	137.16705	10 July 2021	Pp	LC700314	LC700322
4	ADM (in NINS)	34.94771	137.16590	14 Dec. 2021	-	LC700315	NA‡
5	Minami-Koen	34.91601	137.16377	3 July 2021	Cj	LC700316	LC700323
6	Okazaki Castle	34.95741	137.15968	6 July 2021	Cj & Pp	LC700317	LC700324
7	Kota	34.88247	137.16243	10 July 2021	Pp	LC700318	LC700325
8	Nihsinomiya	34.72121	135.36598	5 Aug. 2021	–	LC700319	LC700326

* Abbreviation; Cj, *Camponotus japonicus* and Pp, *Pristomyrmex punctatus.*
† 16S rDNA sequences of infecting *Wolbachia*.
‡ NA; PCR amplification using α-proteobacteria specific primers was undetectable.

In this study, we found a population of aphids on twigs of *C. deodara* (Roxb.) G. Don in Okazaki City, Aichi Prefecture, and in Nishinomiya City, Hyogo Prefecture, Japan. The aphids were morphologically and genetically identified as *C. cedri*, and their phylogenetic relationships were estimated based on COI sequences between Japanese *C. cedri* and other known populations. We also investigated the phylogenetic location of *Wolbachia* symbionts in Japanese *C. cedri* using 16S rDNA sequences.

Materials and methods

Aphid collection and morphological identification

All sampling sites were located in urban areas where host *C. deodara* trees were grown as ornamental plants. When aphids were observed on twigs, they were carefully collected using aspirators or forceps. We treated the individuals found on the same twigs as derived from the same colony. Aphid samples were preserved in 99.5% ethanol, which was renewed multiple times. Slide glass specimens were prepared as follows: samples were treated with 5% potassium hydroxide overnight, washed, rinsed with distilled water, mounted on a slide glass with Hoyer's solution (Kenis, Japan), and observed under a light microscope (Leica DM IL LED, Leica, Germany). Samples were deposited in the Laboratory of Evolutionary Genomics, National Institute for Basic Biology (NIBB).

Phylogenetic analysis of C. cedri

To confirm the identity of the aphids, which were morphologically identified as *C. cedri cedri* (see Results and discussion) and to clarify their relationship with other known populations of *C. cedri*, we performed phylogenetic analysis based on sequences of the mitochondrial cytochrome c oxidase subunit I (COI) gene. One randomly chosen aphid (late instar nymph or adult) from the seven colonies of Okazaki City and the colony from Nishinomiya City was used for the analysis (Table 1). Total DNA was extracted from the whole body of aphids using the DNeasy Prep kit (Qiagen, Japan) according to the manufacturer's protocol, and used as a

template for polymerase chain reaction (PCR). A fragment of COI was amplified by PCR using the primer set LepF (5′-ATTCAACCAATCATAA AGATATTGG-3′) and LepR (5′-TAAACTTCTGGATGTCCAAAAAA TCA-3′) (Monnin et al. 2020). PCR amplification was performed in a total reaction volume of 20 µL, containing 10 µL of PrimeSTAR HS DNA Polymerase (Takara, Japan), 0.6 µL of each primer (10 µM), 1 µL of template DNA, and 7.8 µL of high-purity water. PCR cycling conditions consisted of 35 cycles of denaturation (98 °C for 10 s), annealing (49 °C for 30 s) and extension (72 °C for 40 sec), followed by a final extension step (72 °C for 3 min). Amplified double-stranded DNA was purified using a QIAquick PCR Purification Kit (Qiagen, Japan) and sent to an outsourcing sequence service (FASMAC Inc., Japan). The sequences were investigated and corrected manually, and the resultant sequences were aligned using the ClustalX algorithm (Thompson et al. 2003). We obtained 533 bp of COI sequences. COI sequences of *C. cedri* in known distribution were also used in the analysis, including those from European countries such as Spain, Italy, France, Cyprus, and Croatia, as well as Canada, the USA, and China [GenBank accession numbers (GB#): Spain, LT600419; Italy, KF6493349–KF649351 and KU321599; France, LT600418, KF64997, and KF649387; Cyprus, KU321598; Croatia, KU754491 and KU754492; Canada, KR573470 and KR567761; the USA, KF649509; and China, KM501340, KM501341, and KJ433268]. Note that among these sequences, GB#KU321598 and KU321599 were of the subspecies *C. cedri brevifolinae*, which was collected from Cyprus and Tuscany, Italy. The COI sequences obtained in this study were deposited in the DDBJ/EMBL/GenBank nucleotide sequence databases (GB#LC700312–LC700319). As outgroups, sequences deposited from *C.* (*Cinara*) *tellenica* Binazzi & Strangi, 2020 (the closest sister species, GB#MN577067–MN577069); *C.* (*Cinara*) *puerca* Hottes, 1954 (KF649473); and *C.* (*Cupressobium*) *tujafilina* (Del Guercio, 1909) (KF649398 and KF649348) were also included in the phylogenetic analysis. The COI sequence of *C. tujafilina* collected on the NIBB campus (GB#LC709254), which was obtained using the method described above, was also analyzed. Phylogenetic analysis involving 32 nucleotide sequences was conducted using MEGA X (Kumar et al. 2018), and evolutionary history was inferred using the maximum likelihood method and the Tamura-Nei model. A discrete Gamma distribution was used to model the evolutionary rate differences among the sites [5 categories (+G, parameter = 0.1665)].

Phylogenetic analysis on Wolbachia *infecting the Japanese populations of* C. cedri

To determine the infection status of *Wolbachia* in the Japanese population of *C. cedri*, and identify the strain of the bacteria, we amplified the 16S rDNA region by PCR with primers specific to α-proteobacteria, sequenced the PCR product, and performed a phylogenetic analysis. We used the total

DNA samples extracted previously (in the COI analysis) as templates for PCR. The α-proteobacteria-specific primers used were as follows: 16SWup (5′-GCCTAACACATGCAAGTCGAA-3′) and 16SWlo (5′-AGCTTCGAG TGAAACCAATTCCC-3′) (Gomez-Valero et al. 2004). PCR amplification was performed as described for the COI analysis, with the PCR cycling conditions consisting of the first denaturation step (98 °C for 1 min), 35 cycles of denaturation (98 °C for 10 s), annealing (55 °C for 30 s), extension (72 °C for 1 min), and a final extension step (72 °C for 5 min). PCR products were purified when the amplification was recognized by gel electrophoresis, and they were then sequenced using the outsourced service. Both strands were sequenced using PCR primers (16SWup and 16SWlo) and sequence primers [515F (5′-GTGCCAGCMGCCGCGGTAA-3′, where M = A or G), 519R (5′-GWATTACCGCGGCKGCTG-3′, where W = A or T and K = G or T), and 1100R (5′-AGGGTTGCGCTCGTTG-3′) (Reysenbach et al. 2000; Reed et al. 2002)]. The sequences were reviewed and corrected manually. The resulting sequences were aligned using the ClustalX algorithm. Finally, 931 bp of 16S rDNA sequences were obtained. Based on the preliminary results of comparisons of the sequences with the GenBank non-redundant database using BLAST (available through the National Center for Biotechnology Information), we identified the α-proteobacteria in the Japanese population of *C. cedri* as that of a *Wolbachia* sp. The 16S rDNA sequences were deposited in the databases mentioned previously (GB#LC700320–LC700326). The 16S rDNA sequences of *Wolbachia* in *C. cedri* collected from Spain (Supergroup M, GB#AY620430, JN384064, and JN384065) and Israel (Supergroup M, JN384079, and B, JN384059 and JN384060) were deposited in previously mentioned databases. The 16S rDNA sequences of *Wolbachia* in other aphids (Supergroup M, GB#JN384075, JN384080, JN384082, JN384083, JN384085, and KC522606), a parasitoid wasp (Supergroup B, M84686), and spider mites (Supergroup B, EU499315, EU499317, and EU499319) were included in the analysis. As outgroups, the sequences of the 16S rDNA of *Wolbachia* of Supergroup A (aphid, GB#JN384066, JN384067, JN384072; fruit-fly, LC108848 and DQ412085; and parasitoid wasp, M84691) were also used. Phylogenetic analysis of the 29 nucleotide sequences was conducted in MEGA X. The evolutionary history was inferred using the maximum likelihood method and Kimura 2-parameter model, and a discrete Gamma distribution was used to model evolutionary rate differences among the sites [5 categories (+G, parameter = 0.1098)].

Results and discussion

On May 23, 2021, several aphid colonies were observed on the twigs of *C. deodara* planted on the campus of NIBB, Okazaki City, Aichi Prefecture, Japan. We conducted field surveys and collected aphids from seven *C. deodara* trees in Okazaki and Kota, Aichi Prefecture, and one in Nishinomiya, Hyogo

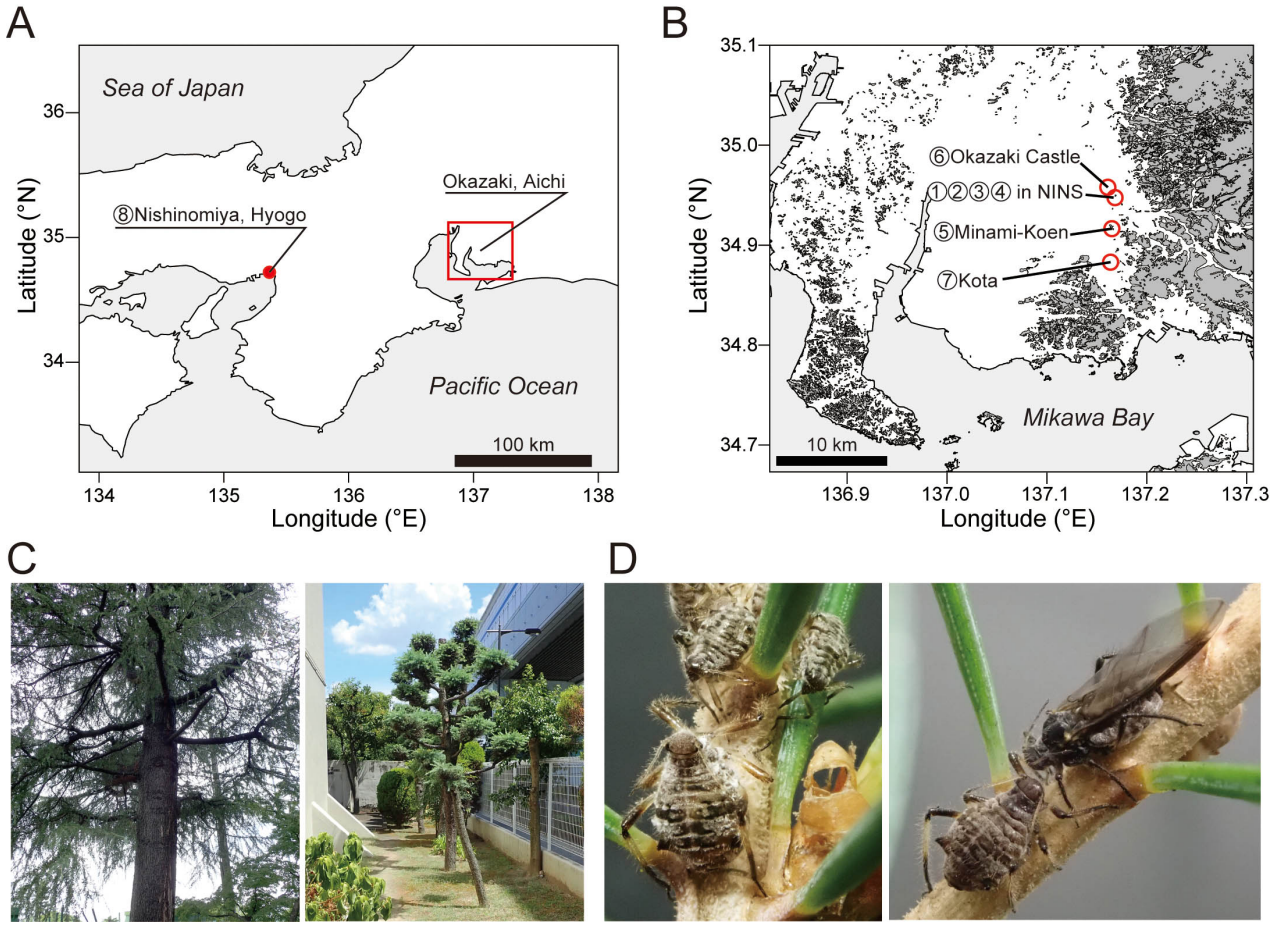

Figure 1. Collection sites and photographs of host trees and *C. cedri cedri* in Japan. A, B: Maps of collection sites. The numbers of the localities corresponds to the sample numbers in Table 1. The map was created using the library maptools of the R software (version 3.1.0; http://cran.r-project.org/) with the data provided by National Land Numerical Information, Ministry of Land, Infrastructure, Transport and Tourism (http://nlftp.mlit.go.jp/ksj-e/gml/datalist). C: Photographs of the host tree, *C. deodara*. During field sampling, *C. cedri* was collected from not only large trees (left), but also small garden trees (right). D: Photographs of the cedar bark aphid, *C. cedri cedri* on the twigs of *C. deodara*. Almost all individuals collected were apterous viviparous females, while two individuals with wings were observed. All photographs were taken by T. Nozaki.

Prefecture (Figure 1; Table 1) in 2021. We confirmed that they overwintered in Japan because they were observed on the same tree in May and June 2022. The aphids were identified as *C. cedri cedri* both morphologically and genetically (see below).

In total, five apterous adults (viviparous females) were selected from the Okazaki population, processed as slide glass specimens, and morphologically observed (Figure 2). Specifically, the following six morphological characteristics of viviparous adults were microscopically measured: antennal segment number, body length, presence/absence of sclerotization in the abdominal tergites, secondary rhinaria number on the third segment of the antennae, siphuncular cone diameter, and hind tibiae length. These are used in keys to *Cinara* species on *Cedrus* spp. worldwide (Binazzi et al. 2017). Six segmented antennae were noted and body length was less than 4.0 mm [3.17 ± 0.25 (mean ± SD)]. Their abdominal tergites lacked sclerotization, and the diameter of the siphuncular cones was less than 0.40 mm [0.30 ± 0.06 (mean ± SD)]. According to Binazzi et al. (2017), *C. cedri* is the only

Figure 2. Slide specimens of *Cinara cedri cedri* in Japan. (Left) an adult female of aptera vivipara (apterous viviparous female). (Right) an adult female of alate vivipara (winged viviparous female). Photographs were taken by T. Nozaki.

species with these morphological features among the genus *Cinara* feeding on *Cedrus* spp. Furthermore, the individuals had hind tibiae longer than 1.6 mm [1.73 ± 0.10 (mean ± SD)]; therefore, they were identified as *C. cedri cedri*, and not *C. cedri brevifoliae* (Binazzi et al. 2017). We observed one winged adult, which was confirmed to be *C. cedri* (data not shown; Figure 2). We genetically identified aphids collected from twigs of *C. deodara* as *C. cedri cedri*. The mitochondrial COI sequences of aphids from eight colonies collected in Okazaki, Kota and Nishinomiya (GB#LC00312–LC00319) matched exactly those from *C. cedri* in Canada (KR567761 and KR573470) and the USA (KF649509), the recently introduced populations, and those in four of seven localities in Europe (KF649349–KF649351, KF649387, KF649397, LT600418, and LT600419, Figure 3). All aphid sequences from Japan were identical. Our phylogenetical analysis also revealed that the *C. cedri* in China and Croatia were not that of *C. cedri cedri* but *C. cedri brevifoliae* (Figure 3).

We successfully detected *Wolbachia* infection in seven of eight individuals and identified the strain of the bacteria in Japanese populations as belonging to Supergroup M (Figure 4). All *Wolbachia* 16S rDNA sequences obtained from the seven individuals collected in Okazaki, Kota, and Nishinomiya were identical (GB#LC700320–LC700326). The sequences also completely matched those from one of the strains of *Wolbachia* among the *Cinara* aphids [*C. cedri*, GB#JN384065 and JN384079, *C. pinea* (Mordvilko, 1895), JN384075 and JN384082] (Figure 4). Due to limited information on *Wolbachia* strains, we cannot determine the source population of Japanese *C. cedri*; however, our data will aid in detecting the possible introduction pathways in the future, as similar data has been effectively utilized in studies on invasive ant species (Tsutsui et al. 2003; Bouwma et al. 2006; Tseng et al. 2019).

The host, *Cedrus deodara*, which is now grown as an ornamental tree globally, was first introduced into Japan approximately 150 years ago. Higo

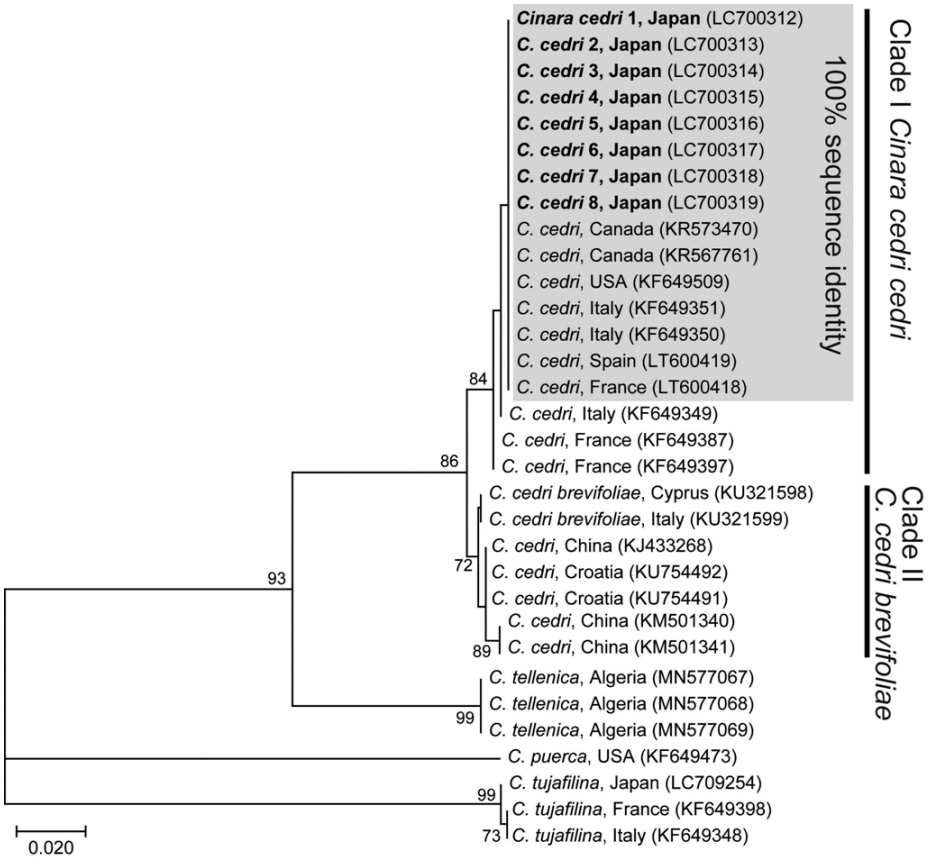

Figure 3. Phylogenetic tree of *Cinara cedri* and related species based on mitochondrial COI sequences, including aphids collected from the *Cedrus deodara* in Japan, which was identified as *C. cedri cedri* (presented in bold). The evolutionary history was estimated using the Maximum Likelihood method and Tamura-Nei model, and the tree with the highest log likelihood (−1243.53) is shown. Bootstrap support for the tree (≥ 70%, 1000 replicates) is shown next to the branches. GenBank accession numbers are presented in parentheses. Countries of collection were placed after the species names. In the Japanese populations, the numbers after the species name correspond to those in Figure 1 and Table 1. A discrete Gamma distribution was used to model evolutionary rate differences among the sites [5 categories (+G, parameter = 0.1665)]. The tree is drawn to scale, with branch lengths measured as the number of substitutions per site. This analysis involved 32 nucleotide sequences. All positions containing gaps and missing data were eliminated (complete deletion option). There were 533 positions in the final dataset.

(1961) reported that in 1879, one *Ce. deodara* tree from the USA was planted in a park in Tokyo by Ulysses S. Grant. However, considering that *C. cedri* was not listed in a previous revision of the conifer aphid fauna in Japan (Inouye 1970; Eastop et al. 1998), and both host COI and symbiont *Wolbachia* 16S rDNA sequences exhibited complete identity in Japan, *C. cedri cedri* in Japan may have been introduced recently from European countries or from other non-native populations in Canada, the USA, or Korea. In this study, we collected *C. cedri* from only two localities in Japan, and revealing the invasion history of *C. cedri* will require more extensive field surveys and detailed phylogenetic analysis.

During field observations, we found that viviparous females continuously produced larvae even in winter, when a small number of oviparous females emerged. No males were observed in this study. This raises the possibility that *C. cedri* in Japan is anholocyclic, that is, parthenogenetic diploid females

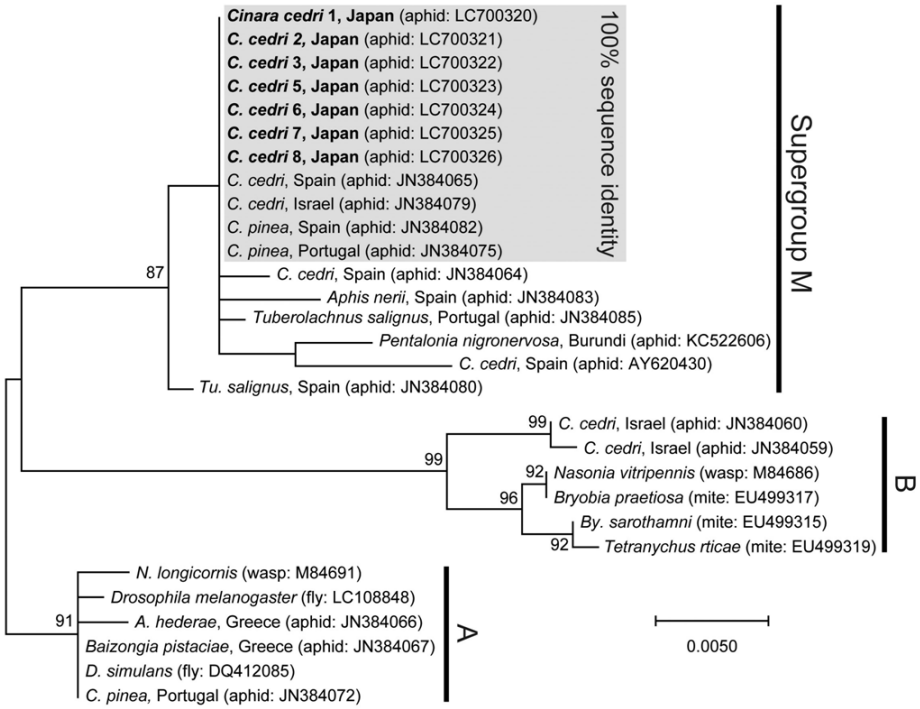

Figure 4. Phylogenetic tree of *Wolbachia* belonging to Supergroups A, B, and M based on 16Sr DNA sequences, including the α-proteobacteria detected in *Cinara cedri cedri* in Japan, which was identified as *Wolbachia* (presented in bold). The evolutionary history was inferred by using the Maximum Likelihood method with the Kimura 2-parameter model, and the tree with the highest log likelihood (−1679.07) is shown. The host of each *Wolbachia* strain was placed on the branches. Bootstrap support for the tree (≥ 70%, 1000 replicates) is shown next to the branches. The taxonomic names of hosts (aphid, fly, wasp, and mite) and GenBank accession numbers are shown in parentheses. For aphids, countries of collection were placed after the species names of host insects. In *C. cedri* from Japanese populations, the numbers correspond to those in Figure 1 and Table 1. A discrete Gamma distribution was used to model evolutionary rate differences among the sites [5 categories (+G, parameter = 0.1098)]. The tree is drawn to scale, with branch lengths measured as the number of substitutions per site. This analysis involved 29 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated (complete deletion option). There were 913 positions in the final dataset.

continuously reproduce only by viviparity, as is commonly reported in introduced populations of aphids (Fuentes-Contreras et al. 2004; Margaritopoulos et al. 2013; Mendel et al. 2016), although long-term observation would be required to confirm this. We did not find any aphid mummies (dead bodies of aphids parasitized by wasps), implying the absence of parasitoid wasps. Two ant species, *Camponotus japonicus* Mayr, 1866 and *Pristomyrmex punctatus* (Smith, F., 1860), were frequently observed with the aphid colonies during the summer. Both ants are common and abundant species in urban areas of Japan (Japanese Ant Database Group 2003). Attending ants indicate that the aphids excrete honeydew, and it has been reported that the secretion of large amounts of honeydew covering the foliage and bark of trees causes an outbreak of sooty mold (Mendel et al. 2016). We have not seen serious damage to the trees in Japan, yet we will need to monitor the influence of *C. cedri* on the *C. deodara* for landscape protection.

In this study, we present the first record, to our knowledge, of *C. cedri* in Japan. While the species is considered to be endemic to Mediterranean regions (Binazzi et al. 2015; Mendel et al. 2016), these aphids have been recognized worldwide, presumably because the host plant, *C. deodara* has also been introduced globally in gardens or as ornamental trees (Bisht et al. 2021). These results suggest that *C. cedri* is likely found in other Japanese urban areas; in fact, we recently observed the aphid in Tsukuba City, Ibaraki (May 15, 2022) and Mitaka City, Tokyo (May 30, 2022). A more comprehensive field survey and analysis of genetic diversity of *C. cedri* and their symbionts in native and non-native populations will promote the understanding of their introduction pathways.

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Authors' contributions

Research conceptualization: T.N., Y.K. and S.S.; sample design and methodology: T.N. investigation and data collection: T.N.: data analysis and interpretation; T.N., Y.K. and S.S.; funding provision: T.N.; writing – original draft, review and editing: T.N., Y.K. and S.S.

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