Research Article

Dimorphic Sessile Apterai of the Aphid Neothoracaphis glaucae (Hemiptera) on the Evergreen Oak Quercus glauca

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1.Introduction

The aphid genus Neothoracaphis belongs to the tribe Nipponaphidinae (Hormaphidinae, Aphididae), and about ten nominal species have been reported from East Asia [1–3]. Except for one species, Neothoracaphis yanonis, which migrates between Distylium spp. (the primary host) and deciduous Quercus spp. (the secondary host) [4–7], all the other species have been recorded only from leaves of their secondary hosts, deciduous or evergreen Quercus trees (some from Lithocarpus trees [1]) and therefore have been thought to be anholocyclic [2, 8]. The aporterous adults (apterae) on the secondary hosts are sessile, heavily sclerotized with reduced tarsi and no cornicles, and very small, less than 1 mm and at times around 0.5 mm in length [1, 2]. In Japan, five nominal species have been recorded: N. yanonis on Q. serrata, Q. dentata, and Q. crispula (and also on Distylium racemosum) [5, 8], N. elongata on Q. myrsinifolia, Q. sessilifolia, and Q. acuta [8, 9], N. querципhaga on Q. myrsinifolia [8], N. saramaoensis on Q. glauca [8], and N. glaucae on Q. glauca [8]. Of them, N. glaucae and N. saramaoensis are rather commonly found on the undersides of leaves of Q. glauca. Neothoracaphis glaucae produces aporterous adults that are the largest among the Japanese species and ovate in shape (Figure 1(a)), while N. saramaoensis produces apterae which are smaller and elongated oval in shape (Figure 1(b)); the two seem to be very different from each other. However, we have often found the two kinds of apterae on the same leaf of Q. glauca. This led us to suspect that the two might be different phenotypes of a single species. The prime issue we address in this paper is whether the two nominal species on Q. glauca are the same species or not. In the course of the present study, we found, in some colonies of the focal species N. glaucae/saramaoensis, bizarre wingpadded nymphs
(nymphs that develop into alates), which were larger than the apterous adults of *N. glaucae* and covered with many, long, semitransparent, bristle-like wax filaments (Figure 1(c)). Alates of *Neothoracaphis* have hitherto been unknown from species on evergreen oaks. We wondered whether these nymphs were really of the focal species. In the present paper, we settle the two issues by examining their mitochondrial DNA sequences, describe size dimorphism found in the apterous adults, and discuss the life cycle of the focal species. In addition, by examining mitochondrial DNA sequences of three congeners, *N. querciphaga*, *N. elongata*, and *N. yanonis* (and some other Nipponaphidini species), we determine whether each nominal species is really a distinct species and whether the *Neothoracaphis* species constitute a monophyletic group among nipponaphidines.

2. Materials and Methods

2.1. Sampling of Apterous of the Focal Species. Apterous generations of the focal species *N. glaucae/saramaoensis*, as well as those of other species of *Neothoracaphis*, form sparse colonies on the undersides of leaves of the host oaks [1]. Aphids (apterous adults and nymphs) of the focal species were sampled mainly from a few trees of *Quercus glauca* in Shinkiba, Tokyo, Japan, in 2013 and 2014. Leaves of some trees were infested with both the larger, *glaucae*-type (“L-type”) apterae (Figures 1(a) and 2(a)) and the smaller, *saramaoensis*-type (“S-type”) apterae (Figures 1(b) and 2(b)). Several infested leaves were cut off the trees and preserved in bottles of 80% ethanol on 20 January 2014, 28 March 2014, 4 June 2014, 17 September 2013, and 5 November 2014 (Table 1). In and around Tokyo, trees of *Q. glauca* produce new shoots from April onward. The mean longevity of the leaves is estimated to be two or three years [10, 11]. New leaves were clearly discriminated from old ones from April to September. Two samples collected in June (#14117) and September (#13117) were from old (at least one-year-old) leaves. These five samples each contained approximately 150–240 apterous adults, which were later slide-mounted and used for morphometric analyses (see later sections). We noticed that only S-type apterae were seen on new leaves and, to confirm whether this is the case, sampled a few new leaves of *Q. glauca* infested with apterae on 22 May 2013, 4 June 2014, 15 July 2013, and 17 September 2013. These four samples from new leaves contained 222 apterous adults in total, and 148 apterae from one of them (#14115 collected on 4th June) were slide-mounted for morphometry.

Materials for DNA extraction were collected from leaves of *Q. glauca* in Shinkiba, Ome (Tokyo), and Takao (Tokyo) and from leaves of *Q. myrsinifolia* in Yugawara (Kanagawa Prefecture) and Kitakyushu (Fukuoka Prefecture), Japan, between 2013 and 2018 (Table 2). Some L-type and S-type apterous adults found in Ome and Takao were separately deposited in vials of 99% ethanol to determine whether they were of the same species by comparing their mitochondrial DNA sequences.

2.2. Sampling of Congener Apteræ for Comparison. Apterous adults of three other species of *Neothoracaphis* were also sampled for morphometry and DNA extraction. They were *N. querciphaga*, *N. elongata*, and *N. yanonis* (Figures 2(c), 2(d), and 3). The collection data are presented in Tables 1 and 2. Because there was a considerable amount of variation in the size and shape of apterous adults in *N. querciphaga*, large apterae and small apterae (indicated as “L-type” and “S-type” in Table 2) were collected from a single tree in Ome and Hachioji (Tokyo), and their mitochondrial DNA sequences were examined to confirm that they were of the same species.

2.3. Sampling of Alate-Generation Aphids of the Focal Species. Alates of the focal species have hitherto been unknown. We found three alates (Figure 1(d)) and a number of nymphs that would develop into alates (Figure 1(c)) on the undersides of leaves of *Q. glauca* in Shinkiba from November to January. We sampled these aphids on 1, 14, and 25 November 2013, 20 January 2014, and 5 and 24 November 2014. Third- and fourth-instar wingpadded nymphs on leaves were kept in plastic containers in the laboratory at room temperature, and a total of 61 alates emerged later. To obtain first-instar nymphs from these alates, 37 alates were confined, together with a piece of paper, in 5 mL cotton-plugged glass vials to force their larviposition there. Two to eight days later, first-instar nymphs were seen walking in some of the vials, and 80% ethanol was poured into them. A total of 42 first-instar nymphs were obtained. These nymphs were slide-mounted, and their morphology was examined to determine whether the nymphs were sexually or virginooparae (i.e., whether their mothers were sexuparae or secondary migrants).

The nymphs of the focal species that would develop into alates (Figures 1(c), 4(c), and 5(d)) looked very different from nymphs developing into apterae (Figures 4(a) and 4(b)), and we wondered whether they were really of the same species. A few wingpadded nymphs were therefore deposited in vials of 99% ethanol, and from two of them (#13154 and #13187 in Table 2), mitochondrial DNA was extracted and sequenced. Some others, including one first-instar nymph (Figure 4(c)), were kept in 80% ethanol and later were mounted on glass slides for microscopic examination.

2.4. Sampling of Alates and Nymphs of *Neothoracaphis yanonis* for Comparison. *Neothoracaphis yanonis* is a very common nipponaphidine in Japan. Galls of the species are seen on almost all trees of *Distylium racemosum* in Honshu, Shikoku, and Kyushu. Aphids of its secondary-host generation are also commonly found on leaves of *Quercus serrata*. *Neothoracaphis yanonis* has been the only species of the genus whose secondary-host generation produces alate sexuparae in Japan [4–6, 8]. To determine whether the first-instar nymphs produced by alates of the focal species are of the sexual generation or not, it was necessary to compare their morphology with that of first-instar nymphs of *N. yanonis*. However, there has been no description of first-instar nymphs of *N. yanonis* in the literature. We therefore sampled, besides adults, first-instar (and later-instar) nymphs of *N. yanonis* that would develop into apterous adults, alate sexuparae, and sexuoparae from *Q. serrata* or
D. racemosum in Tama and Hachioji in 2013 and 2014 and examined slide-mounted specimens of these nymphs.

2.5. Slide Preparation. Aphids preserved in 80% ethanol were cleared in cool or heated 10% KOH solution. Nymphal aphids and alates were stained with Evans’ blue and acid fuchsin, respectively, in approximately 50% lactic acid. Almost all apterous adults were unstained. HU_hey were dehydrated in a mixture of glacial acetic acid and methyl salicylate for 1 day and mounted in balsam via a mixture of xylol-phenol and pure xylol.

2.6. Morphometry. Slide-mounted apterous adults of Neo-thoracaphis were examined and measured using a digital camera (FX630; Olympus, Tokyo, Japan) equipped with the image analysis software (FlvFs; Flovel, Tachikawa, Japan). The body length (from the head to the end of the eighth abdominal tergite) and the body width were measured for all specimens on collected leaves (Table 1) except for broken specimens without the eighth abdominal tergite and a few ill-mounted specimens. Since it was difficult to judge whether each aptera was alive at collection, broken aphids, which were certainly already dead, were not omitted so far as they could be measured. Approximately 140–220 apterous adults from six samples of the focal species, one sample of N. querciphaga, one sample of N. elongata, and one sample of N. yanonis were measured (Table 1). Because the sample of N. yanonis contained too many aphids, 191 apterae were randomly subsampled from it.

2.7. Data Analysis. The lengths and widths of apterous adults from the nine Neothoracaphis samples mentioned above were checked for normal distribution by the Kolmogorov–Smirnov test. When the null hypothesis of a normal distribution was rejected ($p < 0.05$), the data were further analyzed by Hartigans’ dip test [12] under the null hypothesis of a unimodal distribution. All statistical analyses were performed with the software R v3.2.3 [13].

2.8. Examination of Specimens of Other Morphs. Many slide-mounted specimens (including alates, apterous adults, first-instar nymphs produced by the alates, and nymphs to be apterae and alates) were examined under a light microscope. Photographing of mounted specimens and the measurements for description were made using the digital camera equipped with the image analysis software mentioned in Section 2.6.

For elucidating the function of first-instar nymphs produced by alates of the focal species, the length of their stylets may be used as a cue to infer their feeding site. Although it was difficult to measure the precise length of stylets because of their tortuosity on the slide-mounted specimens, the length was estimated by approximating curves with a number of straight lines. The approximate length of

Figure 1: Neothoracaphis glaucae on the undersides of leaves of Quercus glauca found in Shinkiba, Tokyo, Japan: (a) an L-type apterous adult (20 January 2014); (b) two S-type apterous adults (20 January 2014); (c) a fourth-instar nymph to be alate (20 November 2013); (d) a teneral alate sexupara with a cast-off skin (20 November 2013).
stylets was thus measured for 10 first-instar nymphs to be apterae of *N. yanonis* (feeding on leaves of *Quercus serrata*), 10 first- and five fourth-instar nymphs to be alate sexuparae of *N. yanonis* (feeding on leaves of *Q. serrata*), 10 first-instar females of *N. yanonis* (feeding on leaves of *Distylium racemosum*), nine first-instar males of *N. yanonis* (possibly nonfeeding on leaves of *D. racemosum*), 10 first-instar nymphs to be apterae of the focal species (feeding on leaves of *Q. glauca*), one first-instar nymph and 10 fourth-instar nymphs to be alates of the focal species (feeding on leaves of *Q. glauca*), and 40 first-instar nymphs produced by alates of the focal species whose feeding plants/sites were unknown.

2.9. DNA Sequencing. Total DNA was extracted from each of fresh or fixed insects using QIAamp Tissue Kit (Qiagen, Hilden, Germany). From the insect DNA, a 1.6 kB mitochondrial DNA fragment containing small subunit rRNA, tRNA-Val, and large subunit rRNA genes was amplified by PCR using two primers, MtrA1 (5′-AAWAAACTAGGATTAGATTACCCTA-3′) and
under the temperature profile of 95°C for 10 min followed by 40 cycles of 94°C for 1 min, 48°C for 1 min, and 65°C for 3 min. HU_he amplified DNA fragment was purified using exonuclease I (New England Biolabs, Massachusetts, USA) and alkaline phosphatase (shrimp) (Takara Bio, Shiga, Japan) at 37°C for 15 min followed by 80°C for 15 min and directly subjected to a sequencing reaction with BigDye Terminator v3.1 Cycle Sequencing Kit (HU_hermo Fisher Scientific, Massachusetts, USA). In addition to MtrA1 and MtrB1 primers, the following internal primers were used for sequencing: MtrA2 (5′-ACAAAGTAARTGTACTGGAAAATGTG-3′), MtrA3 (5′-ATTTTATCTGTITTTACAAAAAACAT-3′), MtrA4 (5′-AGAYAAGCTGTAACAWAGTA-3′), MtrA5 (5′-AATAGCITCGAGTATTTRCTGTG-3′), MtrB2 (5′-TTATACATGTTTTTGTTAAACAG-3′), MtrB3 (5′-ACACITTCCAC TACAYTTACTTTGT-3′), MtrB4 (5′-TACTWTGTITACGAC TTRTCT-3′), and MtrB5 (5′-ACAGTYAAAATACITTGGACT ATT-3′), under a temperature profile of 94°C for 2 min followed by 30 cycles of 94°C for 1 min, 48°C for 2 min, and 65°C for 3 min. The reaction products were analyzed with the Genetic Analyzer (3130xl; Applied Biosystems, Foster, CA, USA).

The accession numbers of the DNA sequences determined in this study are listed in Table 2.

### Table 1: Samples of *Neothoracaphis* species subjected to morphometric analysis.

| Species               | Sample code | Collection date | Collection locality | Host species | No. of leaves sampled | No. of mounted specimens | No. of specimens measured |
|-----------------------|-------------|-----------------|---------------------|--------------|----------------------|-------------------------|--------------------------|
| *N. glaucae*          | #14008      | 20.1.2014       | Shinkiba, Tokyo     | *Q. glauca*  | 2                    | 196                     | 160                      |
| *N. glaucae*          | #14071      | 28.3.2014       | Shinkiba, Tokyo     | *Q. glauca*  | 6                    | 208                     | 189                      |
| *N. glaucae*          | #14117      | 4.6.2014        | Shinkiba, Tokyo     | *Q. glauca*  | 3                    | 235                     | 219                      |
| *N. glaucae*          | #13117      | 17.9.2013       | Shinkiba, Tokyo     | *Q. glauca*  | 2                    | 198                     | 167                      |
| *N. glaucae*          | #14172      | 5.11.2014       | Shinkiba, Tokyo     | *Q. glauca*  | 3                    | 156                     | 146                      |
| *N. glaucae*          | #14115      | 4.6.2014        | Shinkiba, Tokyo     | *Q. glauca*  | 3                    | 148                     | 143                      |
| *N. querciphaga*      | #14122      | 2.7.2014        | Ome, Tokyo          | *Q. myrsinfolia* | 22               | 189                     | 154                      |
| *N. elongata*         | #17115      | 5.11.2017       | Tanegashima Island, Kagoshima Prefecture | *Q. acuta* | 2                    | 174                     | 123                      |
| *N. yanonis*          | #13127      | 9.10.2013       | Tama, Tokyo         | *Q. serrata* | 1                    | 191$^b$                 | 175                      |

†All collection localities are in Japan; ‡sampled from new leaves; ‡subsampled.

### Table 2: Aphid samples subjected to DNA sequencing.

| Insect sample                  | Collection locality$^a$ | Collection date | Host plant   | Accession no.$^b$ |
|--------------------------------|-------------------------|-----------------|--------------|-------------------|
| *Neothoracaphis glaucae: L-type* (#13040) | Ome, Tokyo              | 6.5.2013        | *Quercus glauca* | LC487692$^a$     |
| *N. glaucae: S-type* (#13041)       | Ome, Tokyo              | 6.5.2013        | *Quercus glauca* | LC487693$^a$     |
| *N. glaucae: S-type* (#13010)       | Shinkiba, Tokyo         | 8.2.2013        | *Quercus glauca* | LC487694$^a$     |
| *N. glaucae: wingpadded nym (13154) | Shinkiba, Tokyo         | 1.11.2013       | *Quercus glauca* | LC487695$^a$     |
| *N. glaucae: wingpadded nymph (13187) | Shinkiba, Tokyo         | 14.11.2013      | *Quercus glauca* | LC487696$^a$     |
| *N. glaucae: S-type* (#18078)       | Takao, Tokyo            | 16.5.2018       | *Quercus glauca* | LC487697$^a$     |
| *N. glaucae: L-type* (#18079)       | Takao, Tokyo            | 16.5.2018       | *Quercus glauca* | LC487698$^a$     |
| *N. glaucae: S-type* (#18082)       | Kitakyushu, Fukuoka Prefecture | 16.6.2018       | *Quercus myrsinfolia* | LC487699$^a$    |
| *N. glaucae: S-type* (#18098)       | Yawarara, Kanagawa Prefecture | 29.7.2018       | *Quercus myrsinfolia* | LC487700$^a$   |
| *N. elongata* (#17093)             | Tanegashima Island, Kagoshima Prefecture | 5.11.2017 | *Quercus acuta* | LC487701$^a$     |
| *N. elongata* (#19005)             | Shiba, Tokyo            | 8.1.2019        | *Quercus acuta* | LC487702$^a$     |
| *N. querciphaga: L-type* (#13035)   | Ome, Tokyo              | 6.5.2013        | *Quercus myrsinfolia* | LC487703$^a$   |
| *N. querciphaga: S-type* (#13037)   | Ome, Tokyo              | 6.5.2013        | *Quercus myrsinfolia* | LC487704$^a$   |
| *N. querciphaga: L-type* (#18047)   | Hachioji, Tokyo         | 26.3.2018       | *Quercus myrsinfolia* | LC487705$^a$   |
| *N. querciphaga: S-type* (#18048)   | Hachioji, Tokyo         | 26.3.2018       | *Quercus myrsinfolia* | LC487706$^a$   |
| *N. yanonis* (#17084)              | Tanegashima Island, Kagoshima Prefecture | 3.11.2017 | *Quercus dentata* | LC487691$^a$     |
| *N. yanonis* (Gall_Mie204)          | Tsu, Mie Prefecture     | 6.5.1996        | *Distylium racemosum* | LC487689     |
| *N. yanonis* (Gall_Tokyo46)         | Shinkiba, Tokyo         | May 2003        | *Distylium racemosum* | LC487690     |

†All collection localities are in Japan; ‡DNA sequences are deposited in the DDBJ/EMBL/GenBank nucleotide sequence database; ‡slide-mounted aphids from the same colony are deposited in the collection of Systematic Entomology, Hokkaido University (Sapporo, Japan), as voucher specimens.
2.10. Molecular Phylogenetic Analysis. The DNA sequences of the *Neothoracaphis* species determined in this study (Table 2) and those we had already reported in the study of Kurosu et al. [14] were subjected to the molecular phylogenetic analyses. A multiple alignment of the nucleotide sequences was generated by using the program package Muscle implemented in MEGA version 7 [15, 16]. Aligned nucleotide sites containing gaps were removed from the dataset to generate a reliable alignment. The model selection and molecular phylogenetic analyses by maximum likelihood (ML) methods were also performed by MEGA version 7. The GTR + G + I model was selected as the nucleotide substitution model for the aligned sequences on the basis of the Bayesian information criterion [17]. Bootstrap tests were performed with 1,000 replications. Sixteen nipponaphidines, *Dermaphis coccidiformis*, *D. autumna*, *D. japonensis*, *D. crematogastri*, *Monzenia globuli*, *Metanipponaphis cuspidatae*, *M. rotunda*, *Nipponaphis distyliicola*, *N. monzeni*, *N. loochooensis*, *N. distychii*, *N. machilicola*, *Allothoracaphis piyananensis*, *Metathoracaphis isensis*, *Quernaphis tuberculata*, and *Quadrartusyoshinomiyai*, were also subjected to the analysis, and *Ceratovacuna nekoashi* (Hormaphidinae, Cerataphidini) was used as an outgroup. The nucleotide sequences of these species had already been deposited in the DNA Data Bank of Japan [18–21]; the accession numbers are indicated in Figure 6.

3. Results

3.1. Molecular Phylogenetic Analyses. The result of our molecular phylogenetic analysis based on mitochondrial ribosomal DNA sequences is summarized as the maximum likelihood tree (Figure 6). We found that all samples of *Neothoracaphis* species formed a monophyletic group with 87% bootstrap support and that both four samples of *N. yanonis* and the remaining 15 samples of the other *Neothoracaphis* species formed monophyletic groups with 100% bootstrap support. Two samples, #13040 and #13041, S-type and L-type apterae of the focal species collected from a single tree of *Quercus glauca*, had completely identical nucleotide sequences. Another two samples, #18078 (S-type) and #18079 (L-type), from a single tree of *Q. glauca* had almost identical nucleotide sequences except for a 6-bp insertion in #18078, which were removed from the dataset for the molecular phylogenetic analyses as described in Section 2.10. This indicated that the two types of apterous adults, or what have been called “*N. saramaoensis*” and “*N. glaucae*,” are different phenotypes of one and the same species. For the reason discussed later (Section 4.1), we hereafter use the name *N. glaucae* for this species. Eight samples of the focal species, including one sample from *Q. myrsinifolia* (#18082) and two wingpadded nymphs (#13154 and #13187), had almost identical nucleotide sequences and formed a monophyletic group with 100% bootstrap support. The remaining one sample collected from *Q. myrsinifolia* (#18098) had a slightly different sequence from those of the eight samples, but they together formed a monophyletic group with 68% bootstrap support. No reliable morphological differences were found between them.

The samples of *N. elongata* and *N. querciphaga* each formed a monophyletic group with 100% bootstrap support. Also in *N. querciphaga*, the nucleotide sequences of small
and large individuals (#18047 and #18048; #13035 and #13037) were almost identical, except for a 2-bp insertion in #18047 and #13037. Among the three species, however, it was not conclusively determined which two are more closely related to each other.

3.2. Morphometric Analyses. The body length and width of apterous adults in six samples of the focal species, _N. glaucae_, which were collected from _Q. glauca_ in Shinkiba, Tokyo, in January, March, June (from old and new leaves), September, and November (Table 1), are shown as scatter diagrams in Figure 7 (Figures S1–S5). There was large variation in the sizes of apterous adults of the January, March, and June (from old leaves) samples, in comparison with those of _N. yanonis_, _N. elongata_, and _N. querciphaga_ (Figure 8). This is because these samples contained many L-type (large) apterous adults in addition to S-type (small) apterous adults. While S-type apterous adults were abundantly contained in all six samples, or were abundantly found throughout the year, L-type apterous adults were few in the September and November samples (#13117 and #14172). The tendency is
manifested in the smaller mean and CV of body lengths in the September and November samples (Table 3). If we operationally define L-type apterae as those with the body width being larger than 390 μm, the percentage of L-type apterae in the latter two samples is less than 1.5 (Table 3). All 222 apterous adults sampled from new leaves, including 143 apterae of sample #14115 (Table 3; Figure S5), were of S-type.

In the three samples collected in January, March, and June (#14008, #14071, and #14117), the null hypothesis of a normal distribution was rejected for both the body lengths and body widths (p ≪ 0.01), while it was not for either in the remaining three samples of *N. glaucae*. The frequency distribution diagrams for body lengths and widths of the March sample (#14071) exhibit bimodal distributions (Figure 7). The null hypothesis of a unimodal distribution was rejected for the body widths (Hartigans’ dip test; p ≪ 0.01), but not for the body lengths (p = 0.059). In the March sample, the frequency distribution for the ratio of the body width to the body length also exhibits a clear bimodal pattern (Hartigans’ dip test; p ≪ 0.01).

Although there was considerable variation in the body size and shape of *N. querciphaga* (Figures 2(c) and 2(d)) and
N. elongata (Figures 3(a) and 3(b)), the samples did not show clear bimodal distributions (Figure 8). The null hypothesis of a normal distribution was rejected neither for the body lengths nor for the body widths in the sample of N. yanonis, only for the body lengths ($p = 0.023$) in the sample of N. querciphaga, and only for body widths ($p = 0.006$) in the sample of N. elongata. The null hypothesis of a unimodal distribution was not rejected for any data of the three species.

3.3. Morphology of Other Morphs. Photos of slide-mounted specimens of the following phenotypes of N. glaucae are shown as figures: first-instar nymph that develops into aptera (Figure 4(a)), nonfirst (probably third) instar nymph that develops into aptera (Figure 4(b)), first- and fourth-instar nymphs that develop into alates (Figures 4(c) and 5(d)), and first-instar nymph produced by the alate (Figure 4(d)). Also, those of the following phenotypes of N. yanonis are shown as figures: first-instar nymph that develops into aptera (Figure 9(a)), first- and fourth-instar nymphs that develop into alates (Figures 9(b) and 5(e)), first-instar male (Figure 9(c)), and first-instar (sexual) female (Figure 9(d)). In either species, first-instar nymphs to be apterae had only marginal wax plates and no spinal row of

![Figure 6: HU_he maximum likelihood (ML) phylogeny of nipponaphidines including four Neothoracaphis species inferred from unambiguously aligned 1,520 nucleotide sites of their mitochondrial rRNA gene sequences. Bootstrap values higher than 50% are indicated on the nodes.](image-url)
wax plates, whereas first-instar nymphs to be alates had spinal rows of wax plates.

First-instar nymphs to be apterae of *N. glaucae* (Figure 4(a)) had well-developed setae on the tips of antennae and the tarsi, whereas later-instar nymphs to be apterae had reduced antennae and reduced tarsi with shorter setae (Figure 4(b)). This suggests that the first-instar nymphs are likely to be dispersers within a tree, or even between trees.

In *N. yanonis*, first-instar females and males were clearly distinguished from each other. The main morphological differences found between sexes are summarized in Table 4. The adult females of *N. yanonis* had a pair of ring-like cornicles on the posterior abdominal tergites [8], while the adult males lacked them (Figure S6). First-instar sexuals lacked cornicles, but in the female, cornicles appeared from the second instar onward. We could therefore determine which of the two morphs were of the female by examining pharate first-instar sexuals (first-instar sexuals with the second-instar cuticle developing inside). Contrary to the sexual dimorphism in the adult, the males (Figure 9(c)) were larger than the females (Figure 9(d)) in the first instar (Table 4).

The approximate lengths of stylets of the nymphs of *N. yanonis* and *N. glaucae* are shown in Table 5. First-instar nymphs of *N. yanonis* to be apterae and first- and fourth-instar nymphs of *N. yanonis* to be alates, which were feeding on leaves of *Quercus serrata*, had stylets that were approximately 0.10–0.13 mm in length. First-instar (sexual) females of *N. yanonis*, which were feeding on leaves of *Distylus racemosum*, had stylets that were 0.21–0.23 mm long, longer than the stylets of the nymphs feeding on leaves of *Q. serrata*. First-instar males of *N. yanonis* on leaves of *D. racemosum* had short stylets that were 0.06–0.10 mm, suggesting that they could mature without taking food. First-instar nymphs of *N. glaucae* to be apterae and a first-instar nymph and fourth-instar nymphs of *N. glaucae* to be alates, which were feeding on leaves of *Q. glauca*, had stylets that were 0.14–0.18 mm long. First-instar nymphs produced by alates of *N. glaucae* in glass vials had stylets that were 0.25–0.29 mm long, which were clearly longer than the stylets of the nymphs feeding on leaves of *Q. glauca*.

Because alates of *N. glaucae* were found for the first time, they are described in the next section.

### 3.4. Description of Alates of *Neothoracaphis glaucae*.

Unless the sample size is indicated in parentheses, the following description is based on 10 specimens which were collected as nymphs from leaves of *Quercus glauca* in Shinkiba on 14 and 25 November 2013 and emerged in the laboratory between 26 November and 8 December 2013.

The body is 1.0–1.3 (mean 1.2) mm long. The head is 0.27–0.33 (0.30) mm wide across the compound eyes, weakly rugose, or reticulate on the dorsum (Figure 5(a)), ventrally with four minute setae between the bases of antennal sockets. The antenna (Figure 5(c)) is five-segmented: the segment III is 232–307 (269) μm long, longer than the femora, 1.7–2.4 (1.9) times as long as the length of the segments IV and V combined; the segment IV is 68–93 (82) μm long; and the segment V is shorter than IV, 49–68 (57) μm long. The processus terminalis is very short, with two apical setae which are about as long as the diameter of the segment at the base. Secondary rhinaria are narrow, often encircling the circumference of the segment; the segments III, IV, and V are with 13–18 (15), 5–7 (6), and 3–5 (4) secondary rhinaria, respectively. The primary rhinarium on the segment IV is indistinct, united with the distal secondary rhinarium; the...
primary rhinarium on the segment V is ciliated, often united with the distal secondary rhinarium. The ultimate rostral segment is 41–50 (46) \( \mu \text{m} \) long (n = 8), without secondary setae. The legs are slender; the fore tibia is 206–255 (232) \( \mu \text{m} \) long, and the hind femorotrochanter is 202–235 (217) \( \mu \text{m} \) long. Tarsi are two-segmented: the segment I is with a pair of setae along the posterior margin (\( n \)).

### Table 3: Body lengths of *Neothoracaphis* apterous adults.

| Species           | Sample code | Length of the body in \( \mu \text{m} \) | L-type apterae\(^{a}, N\ (%) \) |
|-------------------|-------------|------------------------------------------|----------------------------------|
| *N. glaucae*      | #14008\(^{b,c}\) | 160 | 410 | 844 | 543 | 0.16 | 22 (13.8) |
| *N. glaucae*      | #14071| 189 | 423 | 822 | 541 | 0.21 | 51 (27.0) |
| *N. glaucae*      | #14117\(^{b,c}\) | 219 | 425 | 775 | 536 | 0.13 | 23 (10.5) |
| *N. glaucae*      | #13117 | 167 | 420 | 677 | 526 | 0.08 | 2 (1.2) |
| *N. glaucae*      | #14172 | 146 | 411 | 686 | 503 | 0.10 | 2 (1.4) |
| *N. glaucae*      | #14115 | 143 | 428 | 543 | 480 | 0.05 | 0 |
| *N. querciphaga*  | #14122\(^{c}\) | 154 | 447 | 658 | 540 | 0.08 | 18 (11.7) |
| *N. elongata*     | #17115\(^{c}\) | 123 | 449 | 624 | 529 | 0.07 | 0 |
| *N. yanonis*      | #13127 | 175 | 329 | 487 | 395 | 0.07 | 0 |

\(^{a}\) L-type apterae are operationally defined as those with the body width larger than 390 \( \mu \text{m} \). \(^{b}\) The null hypothesis of a normal distribution is rejected for the body lengths. \(^{c}\) The null hypothesis of a normal distribution is rejected for the body widths. \(^{d}\) The null hypothesis of a unimodal distribution is rejected for the body widths.

4.4. Production of Alates in *Neothoracaphis* glaucae. Colonies of *Neothoracaphis yanonis* on leaves of deciduous oaks (*Quercus serrata*, *Q. dentata*, and *Q. crispula*) produce many alate sexuparae in autumn [2, 5, 8]. *Neothoracaphis quercicola* is also known to produce alates on deciduous oaks (*Q. acutissima* and *Q. variabilis*) in Taiwan and China.
However, alates have hitherto been unknown from Neothoracaphis species on evergreen oaks [2, 8]. In this study, we found that N. glaucae produces alates from November to January in Tokyo, Japan. As has been reported in some Nipponaphidini species such as Reticulaphis sp. [26], Thoracaphis kashifolia [27], and Dermaphis coccidiformis [21], nymphs that develop into alates are different from those that develop into apterae in morphology from the first instar. Such clear morphological differentiation in the first instar between the alate and apterous generations on the secondary host seems to occur in a number of nipponaphidine species with small apterae but not in species with rather large apterae like Nipponaphis spp. [20, 28]. In N. glaucae, nymphs to be alates were covered with bristle-like semitransparent wax filaments (Figure 1(c)) and larger in the fourth instar than apterous adults. Well-developed wax plates were seen on the tergites of the slide-mounted specimens (Figures 4(c) and 5(d)). In N. yanonis, nymphs that develop into alates (Figures 9(b) and 5(e)) are also different from those that develop into apterae (Figure 9(a)), but they do not produce long bristle-like wax filaments but shorter, needle-shaped wax filaments (see a
HU_He sexuparae of morphologically the same as those produced by the apterae Neothoracaphis yanonis on secondary host. Secondary host are either sexuparae that fly to the primary nymphs to perceive predators approaching them. Long bristle-like filaments, just like bristles, might help the N. glaucae photo in [5]). It is unknown why the nymphs developing on leaves of Q. glauca. In comparison with N. yanonis, they are likely to be first-instar sexual females. However, among the 44 nymphs produced by alates, we found no first-instar nymphs which had shorter stylets and seemed to be first-instar males. To confirm this, we also examined the morphology of a total of 259 embryos remaining in the bodies of 58 slide-mounted alates. Except for 23 embryos which were heavily distorted and could not be identified, the remaining 236 were not different from the 44 nymphs in morphology. We here present two hypotheses to explain the situation: (1) The first-instar nymphs were of the sexual female, and no males were contained in the sample. Sexuparae of N. glaucae that produce males might occur elsewhere, in other clones. (2) The first-instar nymphs actually consisted of both sexes, and sexual dimorphism might have merely been indiscernible in the first instar of N. glaucae.

Table 4: Main morphological differences between sexes in the first instar of Neothoracaphis yanonis.

| Female                                      | Male                                      |
|---------------------------------------------|-------------------------------------------|
| Body: smaller than male, 0.42–0.49 (mean 0.46) mm in length (n = 10); hind femorotrochanter: 89–105 (99) μm long (n = 10) | Body: 0.56–0.60 (0.58) mm in length (n = 9); hind femorotrochanter: 104–113 (109) μm long (n = 9) |
| Antenna: 3-segmented; apical segment: 98–123 (114) μm long (n = 10) and 17–21 (18) μm wide (n = 10) | Antenna: 4-segmented; apical two segments combined: 130–145 (137) μm long (n = 9) and 20–33 (26) μm wide (n = 9) |
| Stylets: far longer than rostrum, approximately 0.21–0.23 (0.22) mm long (n = 10) | Stylets: short, approximately 0.06–0.10 (0.08) mm long (n = 9) |
| Spinal rows of wax plates: well developed, with 3–8 (5.4) cells on abdominal tergite VI (n = 10) | Spinal rows of wax plates: often reduced, with 0–5 (1.7) cells on abdominal tergite VI (n = 9) |

Table 5: Approximate length of the styles of Neothoracaphis glaucae and N. yanonis.

|                         | Stylet length in mm |
|-------------------------|---------------------|
| N. glaucae: morph       |                     |
| First-instar nymph to be aptera | 0.15 (mean 0.15) (n = 10) |
| First-instar nymph to be alate | 0.14 (n = 1) |
| Fourth-instar wingpadded nymph | 0.14–0.18 (0.16) (n = 10) |
| First-instar nymph produced by the alate | 0.25–0.29 (0.27) (n = 40) |
| N. yanonis: morph       |                     |
| First-instar nymph to be aptera | 0.10–0.12 (mean 0.11) (n = 10) |
| First-instar nymph to be alate | 0.11–0.13 (0.12) (n = 10) |
| Fourth-instar wingpadded nymph | 0.11–0.12 (0.11) (n = 5) |
| First-instar female | 0.21–0.23 (0.22) (n = 10) |
| First-instar male | 0.06–0.10 (0.08) (n = 9) |

In the tribe Nipponaphidini, alates produced on the secondary host are either sexuparvae that fly to the primary host or “secondary migrants” that fly to trees of the secondary host. Neothoracaphis yanonis produces sexuparvae [8], while Thoracaphis kashifolia is known to produce secondary migrants only [27]. The alates (secondary migrants) of T. kashifolia give birth to first-instar nymphs that are morphologically the same as those produced by the apterae [27]. The sexuparvae of N. yanonis produce dimorphic first-instar nymphs that develop into males and sexual females. The first-instar females (Figure 9(d)) are smaller than the first-instar males (Figure 9(c)), but the stylets of the former are much longer than those of the latter (Table 4). Both of these first-instar sexuals are distinct from the first-instar nymphs that develop into apterae (Figure 9(a)) in having rows of spinal wax plates on the tergites. In this respect, they are similar to the first-instar nymphs that develop into alate sexuparvae (Figure 9(b)). The stylets of the first-instar females are longer than the stylets of first-instar nymphs to be apterae and nymphs to be alate sexuparvae, which are longer than the stylets of first-instar males (Table 5).

The alates of N. glaucae are rather enigmatic. The 44 first-instar nymphs produced by alates had well-developed spinal rows of wax plates on their tergites (Figure 4(d)). In morphology, they are quite different from first-instar nymphs that develop into apterae (Figure 4(a)). They are rather similar to first-instar nymphs that develop into alates (Figure 4(c)), but their stylets are clearly longer than those of the latter (Table 5). It is therefore unlikely that they are nymphs that grow on leaves of Q. glauca. In comparison with N. yanonis, they are likely to be first-instar sexual females. However, among the 44 nymphs produced by alates, we found no first-instar nymphs which had shorter stylets and seemed to be first-instar males. To confirm this, we also examined the morphology of a total of 259 embryos remaining in the bodies of 58 slide-mounted alates. Except for 23 embryos which were heavily distorted and could not be identified, the remaining 236 were not different from the 44 nymphs in morphology. We here present two hypotheses to explain the situation: (1) The first-instar nymphs were of the sexual female, and no males were contained in the sample. Sexuparvae of N. glaucae that produce males might occur elsewhere, in other clones. (2) The first-instar nymphs actually consisted of both sexes, and sexual dimorphism might have merely been indiscernible in the first instar of N. glaucae.

4.3 Dimorphic Apterous Adults. Although we showed that N. glaucae produces dimorphic aperuous adults on leaves of the host oak Quercus glauca, it remains still unclear why such dimorphic apterae occur in this species. Both S-type and L-type aperuous adults produce first-instar nymphs that develop into apterae. In the tribe Nipponaphidini, because of their strongly sclerotized tergites, it is difficult to examine the morphology of embryos in the bodies of slide-mounted aperuous adults. However, we found four S-type adults and an L-type adult (collected in Shinkiba on 25 November 2013) which each were just giving birth to a first-instar nymph. Also, we observed four L-type adults (collected in Shinkiba) producing nymphs in the laboratory on 1 and 2 April 2013. All these newly born nymphs were those to be apterae. We did not determine which types of apterae produce nymphs that develop into alates, but the production of alates is likely to be irrelevant to the dimorphism. (In N. yanonis, monomorphic tiny apterae produce nymphs that develop into apterae and alates.) Some S-type apterae contained a single mature embryo which occupied the majority of the body cavity, while some L-type apterae contained more than
one developed embryo. This suggests that, under favorable conditions, L-type apterae are more productive than S-type apterae. L-type apterae were more abundant in January, March, and June than in September and November (Table 3). In addition, apterae collected from new leaves of *Q. glauca* from May to September were all of S-type, indicating that first-instar nymphs that have settled on new leaves become S-type apterous adults. We found fresh, undoubtedly live L-type apterae in Shinbiki from the end of November onward. Winter and spring, and not summer or early autumn, may be their favorable seasons for reproduction in and around Tokyo.

5. Conclusion

In this paper, we made it clear that the aphid *Neothoracaphis glaucae* produces dimorphic sessile apterous adults on leaves of the evergreen *Quercus glauca*. Apterae of the smaller type (S-type) are abundantly seen throughout the year, while those of the larger type (L-type) are few in number from summer to early autumn. We provisionally conclude that the latter may be produced in the favorable seasons for reproduction. Such clear dimorphism in size and shape has hitherto been unknown among aphids with sessile apterae. It will be interesting to know whether there are other species with similar dimorphism and, if not, why only *N. glaucae* maintains the dimorphism.

Data Availability

The DNA sequence data used to support the findings of this study have been deposited in the DDBJ/EMBL/GenBank nucleotide sequence database repository (http://www.insdc.org/).

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Supplementary Materials

Figure S1: scatter diagram of body length versus body width for apterous adults of *Neothoracaphis glaucae* (sample #14008), with a frequency distribution diagram of the body lengths (40 μm for 1 unit of the axis). Figure S2: scatter diagram of body length versus body width for apterous adults of *Neothoracaphis glaucae* (sample #14117), with a frequency distribution diagram of the body lengths (40 μm for 1 unit of the axis). Figure S3: scatter diagram of body length versus body width for apterous adults of *Neothoracaphis glaucae* (sample #14117), with a frequency distribution diagram of the body lengths (40 μm for 1 unit of the axis). Figure S4: scatter diagram of body length versus body width for apterous adults of *Neothoracaphis glaucae* (sample #14117), with a frequency distribution diagram of the body lengths (40 μm for 1 unit of the axis). Figure S5: scatter diagram of body length versus body width for apterous adults of *Neothoracaphis glaucae* (sample #14115), with a frequency distribution diagram of the body lengths (40 μm for 1 unit of the axis). Figure S6: adult male of *Neothoracaphis yanonis* (collected from *Distylus racemosum* in Tama, Tokyo, Japan, on 8 January 2014). Scale bar: 100 μm. (Supplementary Materials)

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