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Research Article

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

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Species of the aphid genus *Neothoracaphis* (Hormaphidinae, Nipponaphidini) produce tiny, sessile, sclerotized apterous adults on leaves of oaks. Among Japanese species, "*N. glaucae*" has been known to have the largest, ovate apterae, while "*N. saramaoensis*" has smaller, elongated oval apterae on *Quercus glauca*. Through examining mitochondrial DNA sequences of Japanese *Neothoracaphis* species, we found that the two are the same species with a clear dimorphism. *Neothoracaphis glaucae* (Takahashi) was adopted as the valid name for the species. In Tokyo, Japan, apterae of the smaller type are abundantly seen throughout the year, and those of the larger type are generally few in number from summer to autumn. Alates, which are supposed to be sexuparae, appear from November to January. Nymphs developing into the alates are covered with long, semitransparent, bristle-like wax filaments. We conclude that *N. querciphaga*, *N. elongata*, and *N. yanonis* are distinct species and that both the genus *Neothoracaphis* and the three *Neothoracaphis* species other than *N. yanonis* form monophyletic groups among Japanese Nipponaphidini species we have examined.

1. Introduction

The aphid genus *Neothoracaphis* belongs to the tribe Nipponaphidini (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 apterous 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. querciphaga* 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 apterous 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 Apterae 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 Apterae 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 sexuals or virginoparae (i.e., whether their mothers were sexuparae or secondary migrants).

The nymphs of the focal species that would develop into alates (Figures1(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 sexuals from *Q. serrata* or

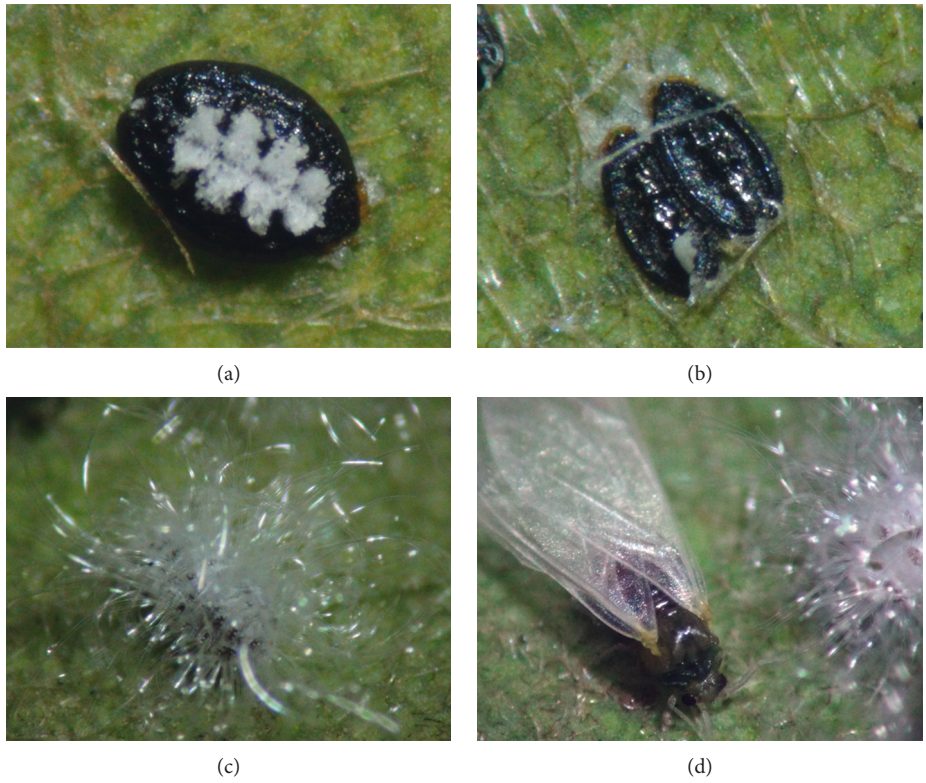

Figure 1: *Neothoracaphis glaucae* on the undersides of leaves of *Quercus glauca* found in Shinkiba, Tokyo, Japan: (a) an L-type apterous adult (20 January 2014); (b) two S-type apterous adults (20 January 2014); (c) a fourth-instar nymph to be alate (20 November 2013); (d) a teneral alate sexupara with a cast-off skin (20 November 2013).

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. They 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 *Neothoracaphis* 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 tortuousness on the slide-mounted specimens, the length was estimated by approximating curves with a number of straight lines. The approximate length of

Figure 2: Apterous adults of *Neothoracaphis*: (a) L-type of *N. glaucae* (collected from *Quercus glauca* in Shinkiba, Tokyo, Japan, on 4 June 2014); (b) S-type of *N. glaucae* (collected from *Q. glauca* in Shinkiba, Tokyo, Japan, on 4 June 2014); (c) a large individual of *N. querciphaga* (collected from *Q. myrsinifolia* in Ome, Tokyo, Japan, on 6 May 2013); (d) a small individual of *N. querciphaga* (collected from *Q. myrsinifolia* in Ome, Tokyo, Japan, on 2 July 2014). Scale bars: 100 μm.

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′-AAWAAACTAGGATTAGATACCCTA-3′) and

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

Species	Sample code	Collection date	Collection locality[†]	Host species of *Quercus*	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. myrsinifolia*	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[§]	175

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

Table 2: Aphid samples subjected to DNA sequencing.

Insect sample	Collection locality[†]	Collection date	Host plant	Accession no.[‡]
Neothoracaphis glaucae: L-type (#13040)	Ome, Tokyo	6.5.2013	*Quercus glauca*	LC487692[§]
N. glaucae: S-type (#13041)	Ome, Tokyo	6.5.2013	*Quercus glauca*	LC487693[§]
N. glaucae: S-type (#13010)	Shinkiba, Tokyo	8.2.2013	*Quercus glauca*	LC487694[§]
N. glaucae: wingpadded nymph (#13154)	Shinkiba, Tokyo	1.11.2013	*Quercus glauca*	LC487695[§]
N. glaucae: wingpadded nymph (#13187)	Shinkiba, Tokyo	14.11.2013	*Quercus glauca*	LC487696[§]
N. glaucae: S-type (#18078)	Takao, Tokyo	16.5.2018	*Quercus glauca*	LC487697[§]
N. glaucae: L-type (#18079)	Takao, Tokyo	16.5.2018	*Quercus glauca*	LC487698[§]
N. glaucae: S-type (#18082)	Kitakyushu, Fukuoka Prefecture	16.6.2018	*Quercus myrsinifolia*	LC487699[§]
N. glaucae: S-type (#18098)	Yugawara, Kanagawa Prefecture	29.7.2018	*Quercus myrsinifolia*	LC487700[§]
N. elongata (#17093)	Tanegashima Island, Kagoshima Prefecture	5.11.2017	*Quercus acuta*	LC487701[§]
N. elongata (#19005)	Shiba, Tokyo	8.1.2019	*Quercus acuta*	LC487702[§]
N. querciphaga: L-type (#13035)	Ome, Tokyo	6.5.2013	*Quercus myrsinifolia*	LC487703[§]
N. querciphaga: S-type (#13037)	Ome, Tokyo	6.5.2013	*Quercus myrsinifolia*	LC487704[§]
N. querciphaga: L-type (#18047)	Hachioji, Tokyo	26.3.2018	*Quercus myrsinifolia*	LC487705[§]
N. querciphaga: S-type (#18048)	Hachioji, Tokyo	26.3.2018	*Quercus myrsinifolia*	LC487706[§]
N. yanonis (#17084)	Tanegashima Island, Kagoshima Prefecture	3.11.2017	*Quercus dentata*	LC487691[§]
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.

MtrB1 (5′-TCTTAATYCAACATCGAGGTCGCAA-3′), 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. The 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 (Thermo Fisher Scientific, Massachusetts, USA). In addition to MtrA1 and MtrB1 primers, the following internal primers were used for sequencing: MtrA2 (5′-ACAAAGTAARTGTACTGGAAAGTGT-3′), MtrA3 (5′-ATTTTYATCTGTTTAACAAAAACAT-3′), MtrA4 (5′-AGAYAAGTCGTAACAWAGTA-3′), MtrA5 (5′-AATAGCTGCAGTATTTTRACTGT-3′), MtrB2 (5′-TTAATACAATGTTTTTGTTAAACAG-3′), MtrB3 (5′-ACACTTTCCAGTACAYTTACTTTGT-3′), MtrB4 (5′-TACTWTGTTACGACTTRTCT-3′), and MtrB5 (5′-ACAGTYAAAATACTGCAGCTATT-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.

(a) (b) (c)

Figure 3: Apterous adults of *Neothoracaphis*: (a) a large individual of *N. elongata* (collected from *Quercus acuta* in Tanegashima Island, Kagoshima Prefecture, Japan, on 5 November 2017); (b) a small individual of *N. elongata* (collected from *Q. acuta* in Shiba, Tokyo, Japan, on 8 December 2018); (c) *N. yanonis* (collected from *Q. serrata* in Tama, Tokyo, Japan, on 9 October 2013). Scale bar: 100 μm.

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 *Quadrartus yoshinomiyai*, 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

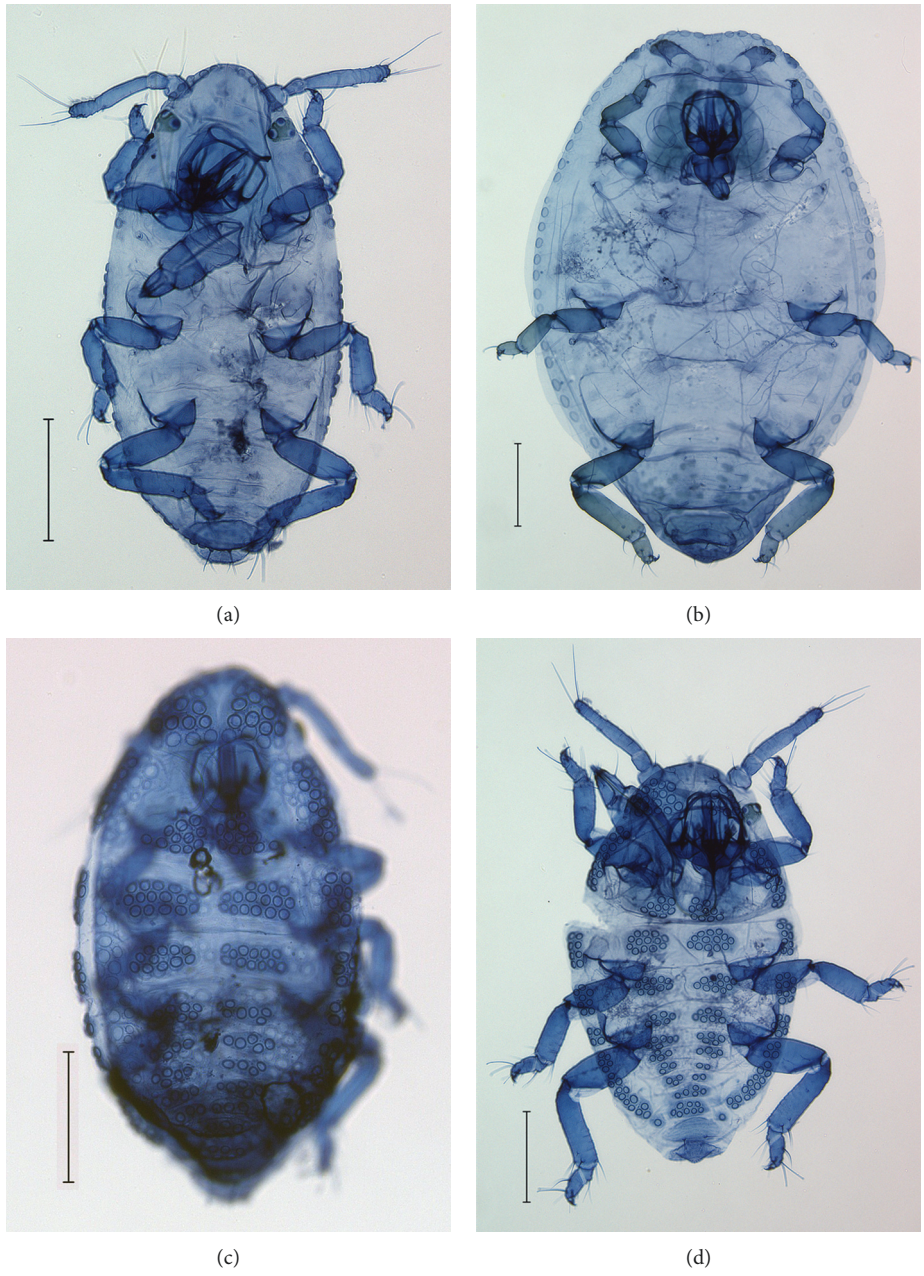

Figure 4: Nymphs of *Neothoracaphis glaucae*: (a) first-instar nymph to be aptera (collected on 5 November 2014); (b) nonfirst (probably third) instar nymph to be aptera (collected on 8 February 2013); (c) first-instar nymph to be alate (collected on 1 November 2013); (d) first-instar nymph produced by the alate (fixed on 1 December 2013). All aphids were collected from *Quercus glauca* in Shinkiba, Tokyo, Japan. Scale bars: 100 μm.

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

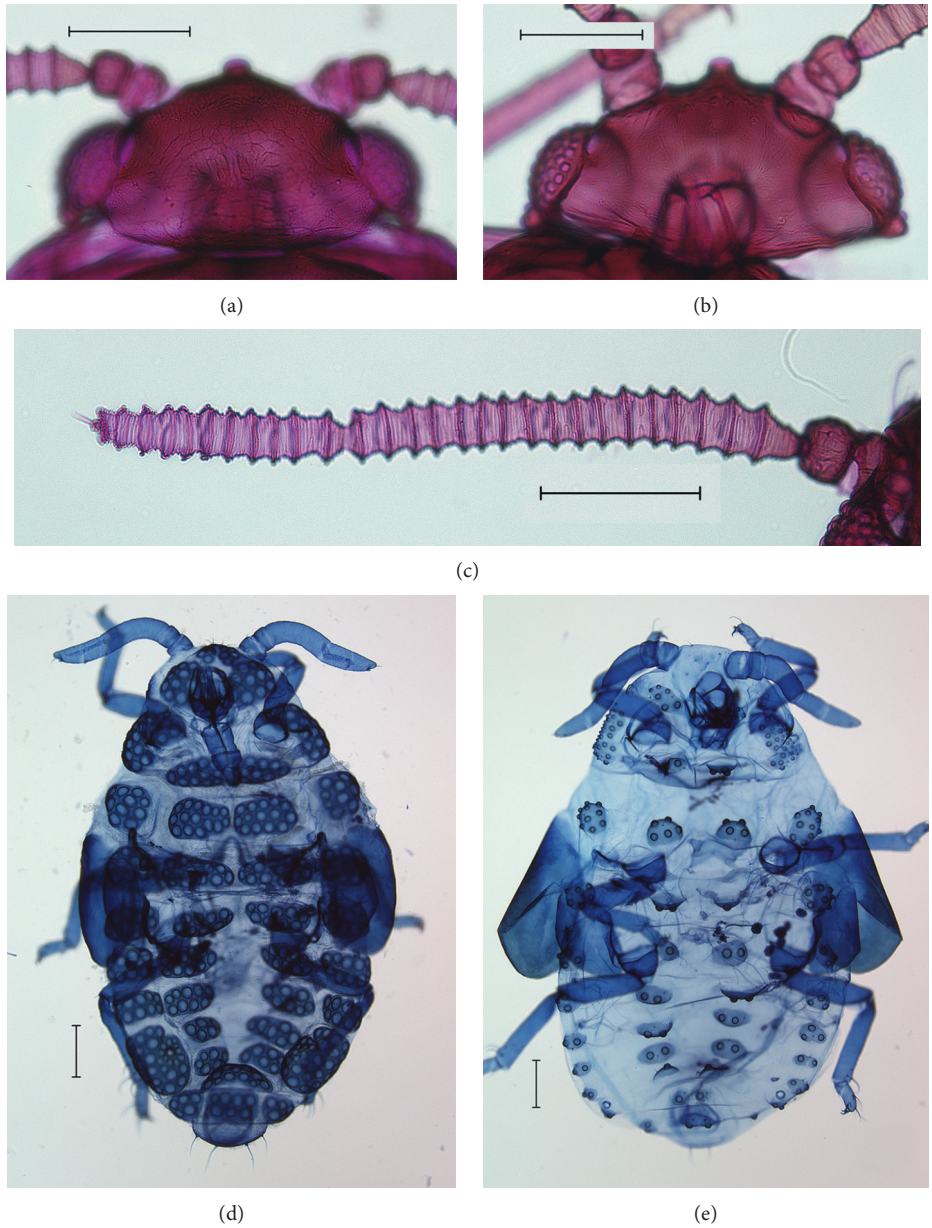

Figure 5: Alates and wingpadded nymphs of *Neothoracaphis glaucae* and *N. yanonis*: (a) head of the alate of *N. glaucae* (collected from *Q. glauca* as a nymph in Shinkiba, Tokyo, Japan, on 25 November 2013 and fixed on 8 December 2013); (b) head of the alate of *N. yanonis* (collected from *Distylium racemosum* in Tama, Tokyo, on 20 November 2013); (c) left antenna of the alate of *N. glaucae* (collected from *Q. glauca* as a nymph in Shinkiba on 14 November 2013 and fixed on 26 November 2013); (d) fourth-instar nymph of *N. glaucae* (collected from *Q. glauca* in Shinkiba on 14 November 2013); (e) fourth-instar nymph of *N. yanonis* (collected from *Q. serrata* in Tama on 1 December 2013). Scale bars: 100 μm.

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 ($p \ll 0.01$), 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; $p \ll 0.01$), 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; $p \ll 0.01$).

Although there was considerable variation in the body size and shape of *N. querciphaga* (Figures 2(c) and 2(d)) and

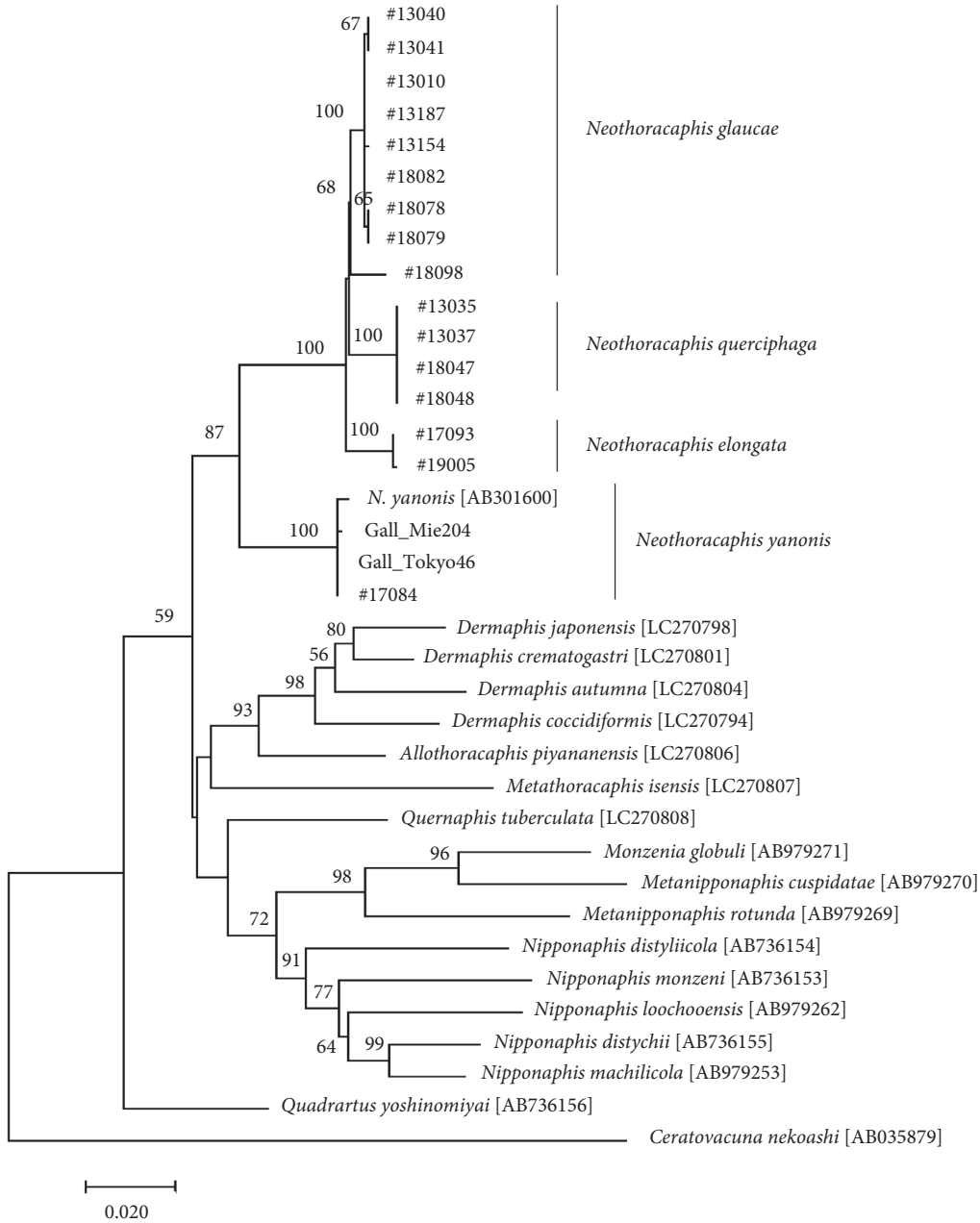

Figure 6: The maximum likelihood (ML) phylogeny of nipponaphidines including four *Neothoracaphis* species inferred from unambiguously aligned 1,520 nucleotide sites of their mitochondrial rRNA gene sequences. Bootstrap values higher than 50% are indicated on the nodes.

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

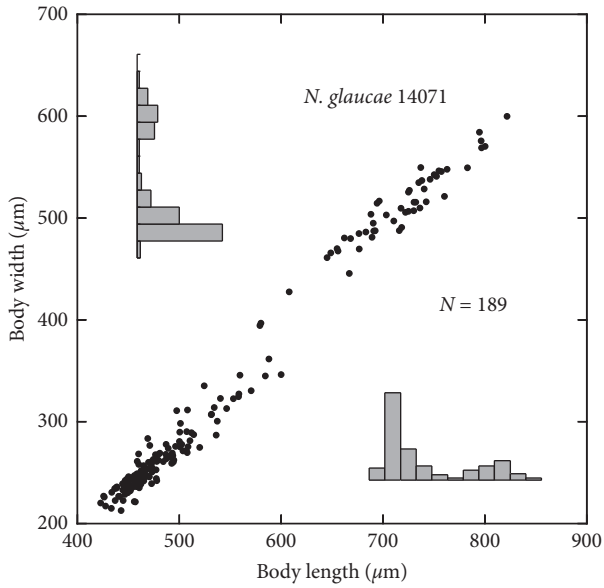

Figure 7: Scatter diagram of body length versus body width for apterous adults of *Neothoracaphis glaucae* (sample #14071), with frequency distribution diagrams of the body lengths and widths (40 μm for 1 unit of the axis).

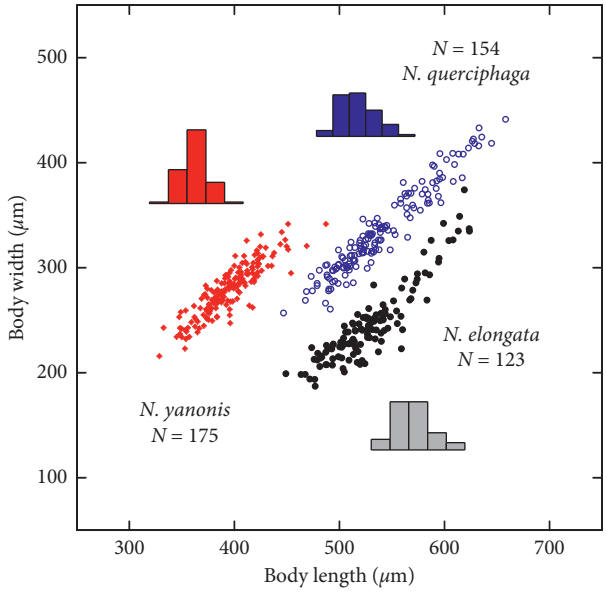

Figure 8: Scatter diagram of body length versus body width for apterous adults of *Neothoracaphis yanonis* (red squares), *N. querciphaga* (blue open circles), and *N. elongata* (black closed circles), each with a frequency distribution diagram of the body lengths (40 μm for 1 unit of the axis).

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 *Distylium 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 fore tibia, 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

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

Species	Sample code	Length of the body in μm					L-type apterae[†], *N* (%)
		N	Min.	Max.	Mean	CV	
N. glaucae	#14008[‡,§]	160	410	844	543	0.16	22 (13.8)
N. glaucae	#14071[‡,§,¶]	189	423	822	541	0.21	51 (27.0)
N. glaucae	#14117[‡,§]	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[‡]	154	447	658	540	0.08	18 (11.7)
N. elongata	#17115[§]	123	449	624	529	0.07	0
N. yanonis	#13127	175	329	487	395	0.07	0

[†]L-type apterae are operationally defined as those with the body width larger than 390 μm. [‡]The null hypothesis of a normal distribution is rejected for the body lengths. [§]The null hypothesis of a normal distribution is rejected for the body widths. [¶]The null hypothesis of a unimodal distribution is rejected for the body widths.

primary rhinarium on the segment V is ciliated, often united with the distal secondary rhinarium. The ultimate rostral segment is 41–50 (46) μm long ($n = 8$), without secondary setae. The legs are slender; the fore tibia is 206–255 (232) μm long, and the hind femorotrochanter is 202–235 (217) μm long. Tarsi are two-segmented: the segment I is with a pair of long, spatulate setae and one shorter seta on fore- and midlegs, and the segment II is 58–67 (63) μm ($n = 9$) on the hind leg, middorsally with a pair of pointed setae and apically with three pairs of setae, of which the dorsoapical and lateroapical setae are long and widened at the apex and the ventroapical setae are short and pointed; empodial setae are spatulate at the apex, reaching the apices of the claws. The forewing is with two branches of the media which usually are not connected, and the basal vein of the media is indistinct. The abdomen is with two pairs of sclerotized spiracles, and with a pair of ring-like cornicles which are 18–22 (20) μm in outer diameter. The abdominal tergite VII is membranous, mesially with two setae; the tergite VIII is weakly sclerotized, with four rather long setae. The cauda is small, 28–36 (32) μm wide, with 7–9 (8) setae. The anal plate is bilobed, with a total of 10–14 (12) setae. The genital plate is with 2–5 (4) setae anteriorly and 8–12 (10) setae along the posterior margin ($n = 9$).

The alates of *N. glaucae* were, in morphology, similar to those of *N. yanonis*, a description of which is given by Takahashi [8]. The former were discriminated from the latter in having the head with reticulated sculptures (Figure 5(a)); the head of *N. yanonis* was smooth on the dorsum (Figure 5(b)). Fourth-instar nymphs (to be alates) of the two species were easily discriminated from each other by their wax plates on the tergites (Figures 5(d) and 5(e)).

4. Discussion

4.1. Identity of Neothoracaphis glaucae and N. saramaoensis. Our molecular phylogenetic analyses clearly indicated that what have been called "*Neothoracaphis saramaoensis*" and "*N. glaucae*" are in reality two different phenotypes of a single species. The former name was given by Takahashi [22] in 1935 (as *Thoracaphis saramaoensis*) and the latter by Takahashi [8] in 1958 (as *Microthoracaphis glaucae*). Taking the present result in advance, Miyazaki et al. [23] adopted *N. saramaoensis* as the valid name for this species and regarded the name *N. glaucae* as a junior synonym. However, Takahashi [22] described "*Thoracaphis saramaoensis*" based on apterae collected from the deciduous *Quercus variabilis* at "Kunugigaoka near Saramao (altitude about 2061 m)," Taiwan, and he [8] applied this name to the Japanese material "with some hesitation." In Japan, *Quercus variabilis* is a rather common tree, but no *Neothoracaphis* species have been recorded from it up to now. In addition, there have been known no other species of Nipponaphidini that utilize *Q. variabilis* and evergreen oaks such as *Q. glauca* as host plants. It is therefore likely that Takahashi's [8] "*T. saramaoensis*" is a different species from the focal species of the present paper. For this reason, we adopt the name *Neothoracaphis glaucae* (Takahashi, 1958) for the focal species.

Miyazaki et al. [23] also regarded *N. querciphaga* and *N. elongata* as a single species. This turned out to be a mistake. Takahashi [8] illustrated a rather large individual of the apterous adult of *N. querciphaga*. Although our morphometric analyses showed that there is considerable variation in size among individual apterae of *N. querciphaga* (Figures 2(c), 2(d), and 8), the mitochondrial sequence data (Figure 6) clearly indicated that *N. elongata* is a distinct species.

Our molecular analyses also indicate that, among the Japanese Nipponaphidini species, *N. glaucae*, *N. querciphaga*, and *N. elongata* form a monophyletic group and that the genus *Neothoracaphis* (the above three species plus *N. yanonis*) is also monophyletic. Takahashi [8] originally placed the first three species in the genus *Microthoracaphis* and *yanonis* in *Neothoracaphis*, but Eastop and Hille Ris Lambers [24] later united the two into one genus and adopted *Neothoracaphis* as the valid genus name. The results of our analyses do not contradict either treatment. We follow Eastop and Hille Ris Lambers [24], as do Blackman and Eastop [2] and Favret [3].

4.2. 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

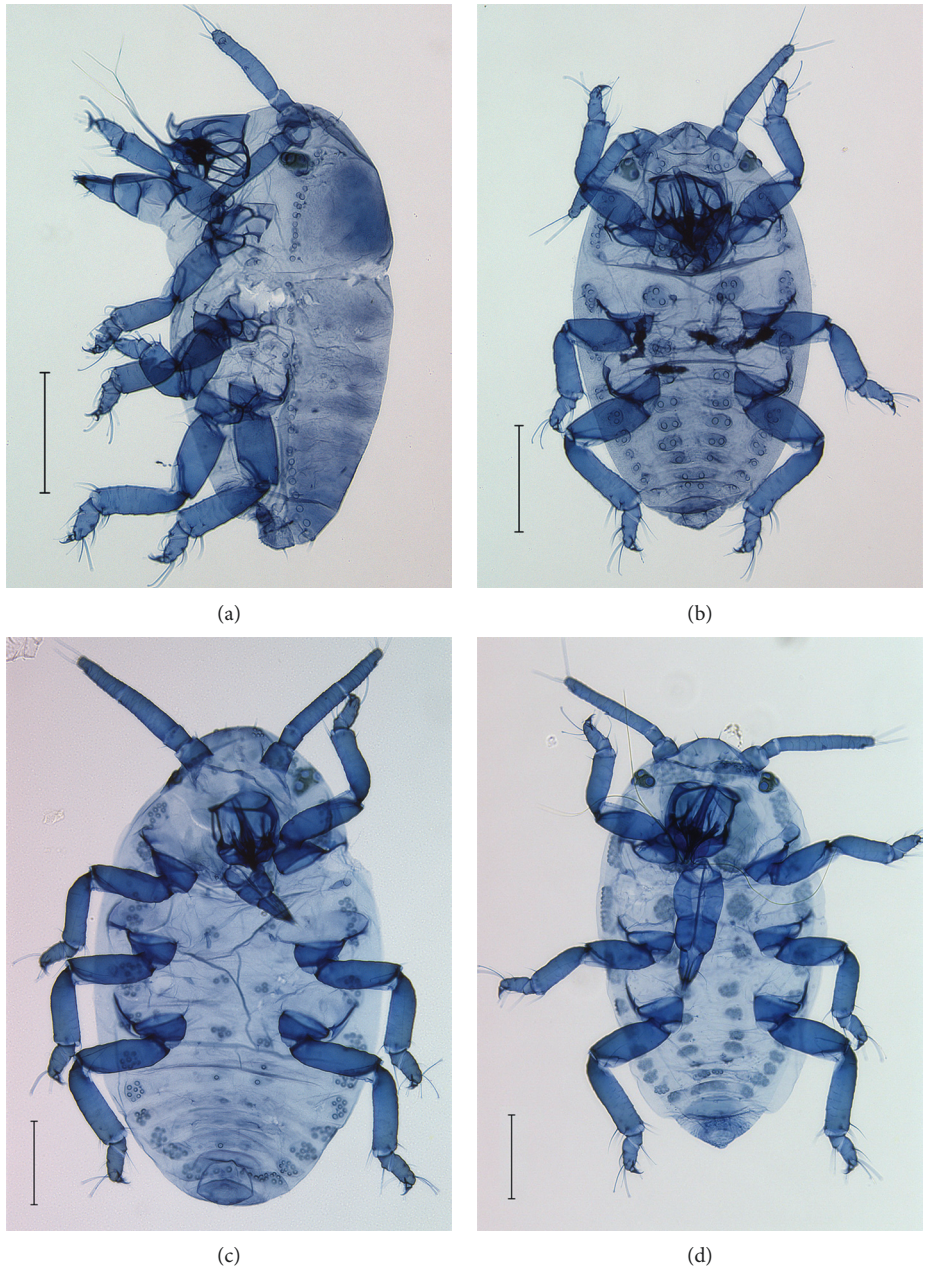

Figure 9: Nymphs of *Neothoracaphis yanonis*: (a) first-instar nymph to be aptera (collected from *Quercus serrata* on 8 October 2014); (b) first-instar nymph to be alate sexupara (collected from *Q. serrata* on 8 October 2014); (c) first-instar male produced by the alate sexupara (collected from *Q. serrata* on 13 November 2013 and fixed on 18 November 2013); (d) first-instar female (collected from *Distylium racemosum* on 23 November 2013). All aphids were collected in Tama, Tokyo, Japan. Scale bars: 100 μm.

[1, 2, 25]. 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

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 stylets 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) ($n = 9$)

photo in [5]). It is unknown why the nymphs developing into alates of *N. glaucae* have such long wax filaments. The long bristle-like filaments, just like bristles, might help the nymphs to perceive predators approaching them.

In the tribe Nipponaphidini, alates produced on the secondary host are either sexuparae that fly to the primary host or "secondary migrants" that fly to trees of the secondary host. *Neothoracaphis yanonis* produces sexuparae [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 sexuparae 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 sexuparae (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 sexuparae, 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. 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*.

4.3. Dimorphic Apterous Adults. Although we showed that *N. glaucae* produces dimorphic apterous 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 apterous 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 apterous 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 Shinkiba 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 #13117), 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 #14172), 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 *Distylium racemosum* in Tama, Tokyo, Japan, on 8 January 2014). Scale bar: 100 μm. (*Supplementary Materials*)

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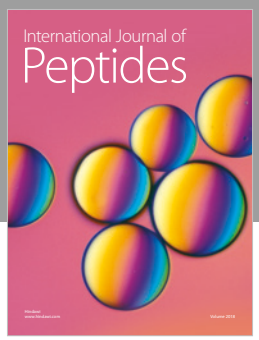

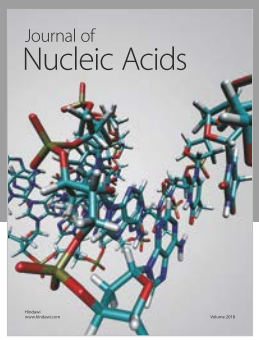

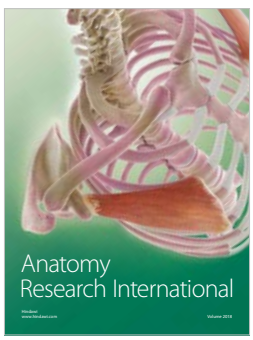

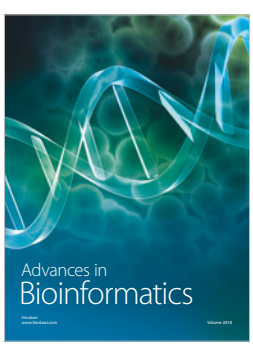

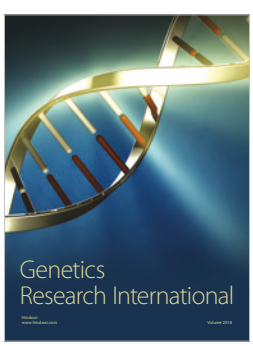

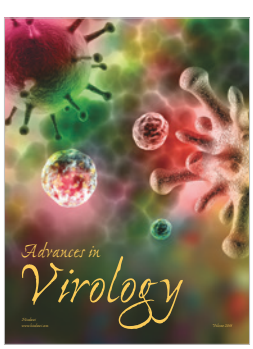

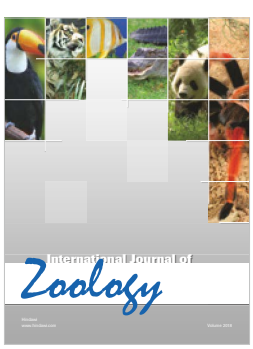

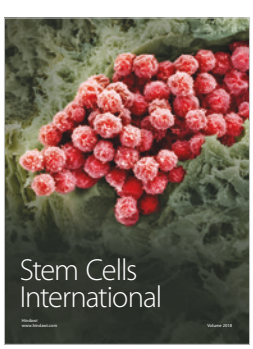

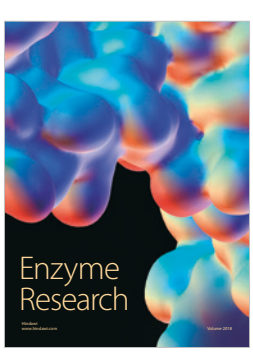

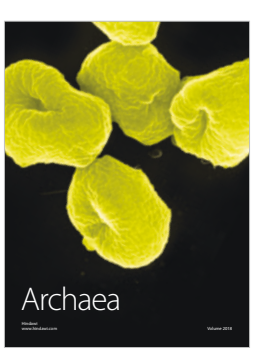


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