The Developmental Characteristics for the Head Capsule Width of *Monochamus alternatus* (Coleoptera: Cerambycidae) Larvae and Determination of the Number of Instars

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Abstract

The objective of this study was to determine the number of instars of *Monochamus alternatus* Hope (Coleoptera: Cerambycidae) larvae by comparing their head capsule widths (HCW) published in previous studies, as well as additional laboratory experiments. Larvae of *M. alternatus* showed repeated molting in the laboratory. Most larvae ceased their development at the 10th instar stage. Frequency distributions of HCW for the first, second, and third instar larvae were clearly separated while those of the fourth through 11th instar larvae largely overlapped between successive instars in our results. The HCW values for the first, second, and third instar larvae directly measured for each instar in our study indicated that they were more precise than those of previous reports based on field-collected HCW which might have missed HCW of the first instar larvae or wrongly determined HCW for some instars. Unlike the reports of four instars of previous studies, *M. alternatus* larvae passed five instars in the field, which was confirmed by the discovery of five pairs of mandibles in the feeding gallery and pupal chamber. Also, the comparative study for the frequency distributions of HCW revealed that most *M. alternatus* larvae passed five instars. Consequently, the average sizes of HCW for their first, second, and third instar larvae are newly suggested to be 0.896 ± 0.069, 1.291 ± 0.131, and 1.707 ± 0.165 mm (mean ± SD).

Key words: Japanese pine sawyer, HCW, pine wilt disease, frequency distribution, diapausing larvae

The Japanese pine sawyer,*Monochamus alternatus* Hope (Coleoptera: Cerambycidae), is a major vector of pine wilt disease (PWD) caused by pine wood nematode (PWN), *Bursaphelenchus xylophilus* (Steiner and Buhrer) (Nematoda: Aphelenchoididae), in Asia (Kiyohara and Tokushige 1971, Mota et al. 1999). This beetle is a native species in Korea (KSPP 1986). It was not an important pest in Korean pine forests until the invasion of PWN. The PWN is native to North America (Knowles et al. 1983). It was introduced to Japan in 1905 (Kiyohara and Tokushige 1971). PWD was first found in Busan in the southern region of Korea in 1988, probably caused by the introduction of *M. alternatus* carrying PWN from Japan (Yi et al. 1989). Since its invasion, approximately 12.3 million pine trees (*Pinus thunbergii* Parl. or *P. densiflora* Siebold & Zucc.) (Pinales: Pinaceae) in Korea have been removed due to PWD as of 2017 (MKFS 2018).

PWD is now causing serious damage to forest ecosystems in the European Union (EU) such as Portugal (Mota et al. 1999), as well as in Asian countries. It has damaged forests of 642 and 700 million ha in Japan (Kiyohara and Tokushige 1971, Mamiya 1988) and China (Shi et al. 2008), respectively. The key for the control of PWD is primarily focused on vector management. Thus, understanding the exact biology of *M. alternatus* is required.

Female *M. alternatus* gnaws at the bark surface of host plants (mostly pine trees) with their mandibles to make a wound (Ko and Kim 2017). They lay eggs singly in the inner bark through the wound, which later becomes an oviposition scar. The larvae feed on the sapwood and phloem tissues of pine trees. The final instars (mature larvae) construct a pupal chamber in the wood and overwinter in the space, plugging the opening with wood debris. In spring of the following year, larvae pupate in the pupal chamber and emerge as adults through an exit hole.

Under the assumption that *M. alternatus* larvae have four instars in the field, Togashi (1990) has suggested that this beetle has a
life cycle of 1 to 2 yr. *M. alternatus* can overwinter in all instars of larvae. After overwintering in the third and fourth instar, these larvae can emerge as adults after resumption of activity followed by pupation in the following year without diapause (Togashi 1990). However, the first and second instar larvae have to resume feeding after overwintering and then enter obligate diapause when reach the fourth instar to emerge in the next year, resulting in a 2-yr life cycle (Togashi 1989, 1990).

The number of larval instars of *M. alternatus* occurring in the field has been controversial. Kojima and Katagiri (1964) reported a total of five instars, based on namely five peaks in a frequency distribution of head capsule width (HCW) data in Japan. This was later confirmed by Ochi and Katagiri (1974). However, Morimoto and Iwasaki (1974) and Yamane (1974) asserted that there were four instars based on their field observations of HCW. Furthermore, Ochi (1975) found that larger eggs hatched to larvae with larger HCW than those produced by small eggs, and, therefore, combined 1st and second instars reported by Kojima and Katagiri (1964), consequently resulting in four instars. This idea has been widely accepted by many entomologists (Kobayashi et al. 1984; Togashi 1989, 1990, 1991; CABI 2017). Meanwhile, Liu et al. (2008) have reported five instars based on a field experiment in China.

The confusion described earlier might be due to differences in experimental methods or errors in sampling depending on researchers. In any case, determining the correct number of instars of *M. alternatus* in the field may be very important for population modelling or potential distribution studies to improve its management. Therefore, the objective of this study was to clarify the number of instars of *M. alternatus* larvae. This was achieved using two approaches: 1) Measuring HCW in laboratory experiments, and comparing it with the previously published literature, and 2) Investigating the mandible exuviae of larvae in the feeding gallery.

**Materials and Methods**

**Experiments for Measuring HCW in the Laboratory**

**The Procedure for Egg Collection**

Black pine trees, *P. thunbergii*, infested with larvae of *M. alternatus* were obtained from a forest in Jeju City, South Korea, in January to February 2016. These pine trees were cut into 1-m-long logs (diameter >3 cm), and a total of 82 pine logs were placed in two field cages (diameter 1.6 m, height 2.5 m) covered with a fine wire mesh (3 mm) at Mt. Halla-san, Jeju. *M. alternatus* adults started to emerge from late May, and they were introduced into an acrylic oviposition cage (30 × 60 × 30 cm) after washing out dirt and parasitic mites from their body surface in bottles filled with distilled water. A total of five oviposition cages were held in the laboratory at temperature of 26.0 ± 1.0°C, a photoperiod of 16:8 (L:D) h, and relative humidity (RH) >40%. Pine shoots (a length of 20 cm) from new growth were used as food supply of *M. alternatus* adults, by providing two to five shoots every 2–3 d depending on the consumption. In each cage, a minimum of seven adults pairs were kept during egg collection.

We used 5–8-yr-old *P. thunbergii* trees with a diameter of 5–8 cm collected from Jeju-city, Korea as oviposition substrates. Their stems were cut into 15- to 20-cm-long bolts. These pine bolts were left for 1 d at room temperature and submerged in water for 3–5 d to prevent secretion of resin. Pine bolts were introduced into oviposition cages after drying in the shade for 1 d. Pine bolts were replaced 3 times a week in oviposition cage, and collected pine bolts were held in a refrigerator (4°C) until used for various experiments as well as this study.

**Rearing Procedure of Larvae**

Collected eggs of *M. alternatus* were hatched on filter paper placed in a small petri-dish (diameter 5 cm, depth of 1 cm) held at 30°C (RH > 70%, 10:14 (L:D) h). The small petri-dish was also placed in a larger petri-dish (diameter 10 cm, depth 4 cm) lined with moistened cotton on the bottom. A group of 30–50 eggs was placed in one small petri-dish, preparing more than total 200 eggs for this experiment.

Hatched larvae (<12 h old, total 68 larvae) were singly introduced into pieces of pine bark inserted between two acrylic plates (4 × 5 cm; thickness 3 mm) and fitted by a clip. In pieces of pine bark, a slit was previously made for inoculation of larvae. These acrylic devises were placed individually in a petri-dish (diameter 10 cm, depth 4 cm) with mesh holes (diameter 3 cm) on the lid, and lined by moistened filter paper. Larvae in these experimental devices were reared in a growth chamber (Model = DS-14MCLP, Dasol Ltd, Korea) set at 30°C (RH ≥ 70%, 10:14 (L:D) h) until they reached the third instar, since the survival rate of early instars was high at the temperature in our preliminary study. These third instar larvae were then inoculated into pine bolts, because the thickness of pine bark between acrylic plates could not cope with the larger larvae. Also, the third instar larvae were moved to one of four constant temperatures (18, 22, 26, and 30 ± 0.5°C; RH ≥ 70%; 10:14 (L:D) h) to examine the possible effect of temperature on the development of HCW. Pine bolts with a length of 15–20 cm and a diameter of approximately 5 cm were prepared as described earlier. Cut ends of these bolts were sealed with vinyl tape to prevent drying. To make a space for inoculation of *M. alternatus* larvae, a small square of bark (10 × 10 cm) was removed from pine bolts in the mid-point. Then a pit was made on the exposed xylem with a medical knife. Larvae were introduced singly into the pit, covered again with the bark square, and fastened with a rubber band. These pine bolts with larvae were placed in a plastic bottle (diameter 15.6 cm, height 20 cm, volume 3 liter) in groups of 4–5 (total 15, 21, 14, and 18 larvae at 18, 22, 26, and 30°C, respectively). Wet cotton pads were placed inside the bottle and five holes with diameter of 5 mm were made on the lid.

We checked the development time of each instar until they showed diapause symptoms (i.e., completion of larval development). Diapausing larvae were determined based on the following criteria recommended by Kimura (1974) and Togashi (1991): body is yellowish white, whitish yellow, or yellow color; no food in the gut.

**Measurement of HCW**

HCW of treated larvae above was measured at every 2–3 d across the greatest width of the head capsule using a digital microscope (Dino-Lite, AD413TL-MA1(R4), ANMO Electronics Co.). The scale of the digital microscope was accurately calibrated with the micrometer provided by Dino-Lite. The ventral side of the head capsule was measured since the dorsal side of the head capsule of *M. alternatus* larva was always hidden by the pronotum, making it difficult to find the greatest width of the head capsule. In addition, larvae were made immobile by pressing their head slightly using the
bottom plate of petri-dish (diameter 50 mm, depth 10 mm) for convenience of measurement (Yamane 1974).

The larvae were put back to their original acrylic devises or pine bolts after measuring the HCW. In the experiment using pine bolts, larvae were tracked along the feeding gallery packed by feces mingled with white wood shreds under pine barks. We caught these larvae by using soft forces or by fishing with thin narrow strips of pine barks that larvae of M. alternatus would bite when inserted between their mandibles. We tried not to destroy the bark of pine bolts as possible. In most cases, pine bolts were strong enough to support the completion of larval development fully. When needed, old pine bolts were replaced with new ones (<5% of old pine bolts were changed).

We measured the HCW of the same instar several times to prevent error caused by capsule split after ecysis. Such error could occur when the exuviae of head capsule was measured.

Data Analysis
All data sets were tested for normality and subjected to analysis of variance (ANOVA) using SAS (SAS Institute 1999). The effect of temperature was evaluated on the development time and number of instars until diapause induction and the HCW of larvae in diapause. Also, the effect of temperature and instar were tested on the growth of HCW by two-way ANOVA. Because of significant interaction effects between temperature and instar on the growth of HCW ($F = 4.07$; $df = 27, 428$; $P < 0.0001$), these data sets were separated and one-way ANOVA was applied. Means were separated by Tukey’s range test at $P = 0.05$ in all cases.

It has been assumed that head capsule distribution of insect larvae is normally distributed (Caltagirone et al. 1983, Beaver and Sanderson 1989). The normal distribution model using sample mean ($\bar{X}$) and variance ($s^2$) has been successfully used to describe instar distribution pattern of larval head capsule data of insects (McClellan and Logan 1994). Accordingly, we calculated mean and variance for each instar to construct normal distribution curve (all data in different temperature were combined). Heights of normal distribution curve were scaled to sample sizes of actual frequencies for comparison (i.e., the height of frequency distribution; McClellan and Logan 1994). For the purpose of visualization, frequency distributions of HCW of each instar were made using the class interval of 0.02 and provided.

Our frequency distribution peaks of HCW were compared with previous reports by Kojima and Katagiri (1964), Ochi and Katagiri (1974), Morimoto and Iwasaki (1974), Yamane (1974), Ochi (1975), and Liu et al. (2008) (see Supp Information I [online only] for details). Also, the probability of misclassifying the $i$-th instar as other instar using was estimated based on the procedure of McClellan and Logan (1994) and analyzed (see Supp Information II [online only] for details).

Comparing HCW of the First Instar
In this study, we secured a total 145 HCWs (including already measured 68 samples in the development study and additionally 77 samples from hatched larvae in the previous rearing study) for the first instar for comparison with previously reported values. The data set was subjected to normality testing using PROC Univariate (SAS Institute 1999). Frequency distribution and the curve of normal distribution were also constructed as described earlier.

Analysis for HCW of Larvae in Diapause
Pine logs infested with M. alternatus were collected between 23 October and 6 November 2016 from felled pine trees in a forest in Jeju-city, Korea where felling practice was conducted for eradication. Pine logs were selected based on the presence of the oviposition scars on the bark of trunks, feeding traces under barks, and entrance holes of the tunnel into the wood (CABI 2017). These pine logs were collected and transferred to a plastic house in Jeju National University and split using a hand axe to collect larvae inside. By examining the diapause symptom as described earlier, we obtained 129 larvae in diapause and 2 larvae in non-diapause state. HCWs of diapausing larvae were measured using the same method as described earlier, and compared with previously reported final instar’s sizes using one sample $t$-test.

Investigating the Mandible Exuviae of M. alternatus Larvae in the Feeding Gallery
Hatched larvae (the first instar) of M. alternatus were singly inoculated into pine bolts prepared with the same procedure as described earlier (see Rearing Procedure of Larvae). In late May 2017, these pine bolts were placed in field cages (diameter 1.6 m, height 2.5 m) covered with fine wire mesh (3 mm) on Mt. Halla-san, Jeju-city. These pine bolts were taken back and placed into an acrylic cage (30 × 60 × 30 cm) in the laboratory ($26.0 \pm 1.0^\circ$C, a photoperiod of 16:8 (L:D) h, and RH > 40%) in the spring of 2018 (on 12 May). After emergence of adults, pine bolts were examined to find mandibles of M. alternatus ($n = 15$). Originally, the purpose of this field treatment of pine bolts with the introduction of M. alternatus larvae was to examine the voltinism, which was conducted independently with this study. But the samples of pine bolts left after adult emergence were best one to check the number of mandibles in the feeding gallery.

The larvae of M. alternatus make their feeding gallery by packing feces mingled with white wood shreds under the pine bark. Thus, we dug up the gallery carefully to find mandible exuviae using a knife and sharp forces after getting rid of the pine bark. According to Morimoto and Iwasaki (1974), the head capsule of first instar is commonly found within 10 mm from the oviposition scar. Thus, we started to dig up from the inoculation slit. The head capsules were recovered broken severely in most cases. Therefore, our strategy to find mandibles was useful for determining the number of instars.

We assumed that M. alternatus larvae passed five instars in the field if four classes of mandible in size were found in the feeding gallery because the final fifth instar was present in the pupal chamber. It was very difficult to find all four classes of mandible in a pine bolt since some mandibles were probably destroyed and lost during the feeding process. Therefore, we determined missing instar by comparing relative sizes of mandibles found in different pine bolts (see Supp Figs. 1 and 3 [online only]). The final instars were collected in the pupal chamber through the exit hole. Finally, we measured the length of mandible of the condyle center to incisor cusp using a digital microscope (Dino-Lite) (see Supp Fig. 3 [online only]). The data were subjected to one-way GLM after testing normality (SAS Institute 1999). Means were separated by Tukey’s range test at $P = 0.05$.

Results
Growth of HCW in the Laboratory
The larvae of M. alternatus showed repeated molting in the laboratory environment, although natural food sources were provided for rearing (Table 1). Most larvae ceased their development at the 10th instar stage (namely entering diapause state). A maximum of 12th instar occurred at 26°C. At the final instar, the HCW ($F = 4.82$;
Table 1. Changes of head capsule width (mm, Mean ± SE) of *M. alternatus* instars at constant temperature in the laboratory; this experiment was conducted using Jeju population of *M. alternatus* in 2016, Korea

| Instar | Temperature (°C) | n | 18°C | 22°C | 26°C | 30°C |
|--------|-----------------|---|------|------|------|------|
| 1st   |                 | 15| 0.85 ± 0.027aB | 0.86 ± 0.021aB | 0.90 ± 0.006aB | 0.93 ± 0.009aA |
| 2nd   |                 | 15| 1.22 ± 0.044aB | 1.29 ± 0.034aB | 1.31 ± 0.020aB | 1.34 ± 0.012aB |
| 3rd   |                 | 14| 1.61 ± 0.059aB | 1.72 ± 0.039aB | 1.70 ± 0.031aB | 1.78 ± 0.026aC |
| 4th   |                 | 12| 2.07 ± 0.083aB | 2.30 ± 0.061aB | 2.34 ± 0.094aB | 2.35 ± 0.044aD |
| 5th   |                 | 11| 2.43 ± 0.072aB | 2.63 ± 0.073aB | 2.64 ± 0.111aB | 2.73 ± 0.047aC |
| 6th   |                 | 10| 2.76 ± 0.072aB | 2.91 ± 0.080aA | 3.01 ± 0.120aA | 2.99 ± 0.046aA |
| 7th   |                 | 10| 3.12 ± 0.075aB | 3.12 ± 0.088aG | 3.30 ± 0.115aG | 3.22 ± 0.058aG |
| 8th   |                 | 10| 3.52 ± 0.120aA | 3.32 ± 0.097aG | 3.46 ± 0.131aG | 3.52 ± 0.055aH |
| 9th   |                 | 8 | 3.90 ± 0.134aB | 3.70 ± 0.108aH | 3.69 ± 0.139aG | 3.70 ± 0.057aH |
| 10th  |                 | 3 | 4.06 ± 0.102aH | 3.90 ± 0.057a | 3.74 ± 0.169aG | 3.91 ± 0.066aJ |
| 11th  |                 | – | – | – | 3.76 ± 0.121a | 4.16 ± 0.039a |
| 12th  |                 | – | – | – | 4.25 | – |

*All larvae of first and second instars were reared at 30°C and thereafter reared at each corresponding temperature.

Means with same lower case letter in a column or means with same upper case letter in a row are not significantly different by Tukey’s test at *P* = 0.05.

Data not available.
Table 2. The head capsule width (mm) of *M. alternatus* larvae in diapause, development time (days), and number of instars until the state (mean ± SE) (larvae were regarded as diapausing larvae that showed the body color in yellowish white, whitish yellow, or yellow color and no food in the gut as recommended by *Togashi* (1991)); the samples were collected between 23 October and 6 November 2016 from felled pine trees in a forest in Jeju-city, Korea.

| Temperature (°C) | n  | Head capsule width | Development time | No. of instar |
|------------------|----|--------------------|------------------|---------------|
| 18               | 10 | 3.99 ± 0.092a       | 227.0 ± 24.94a   | 9.1 ± 0.233ab |
| 22               | 14 | 3.51 ± 0.094b       | 197.5 ± 16.12a   | 7.9 ± 0.366b  |
| 26               | 8  | 3.98 ± 0.081a       | 215.9 ± 14.51a   | 9.5 ± 0.567ab |
| 30               | 8  | 3.88 ± 0.100ab      | 199.3 ± 11.47a   | 9.9 ± 0.398a  |
| Average          | 40 | 3.82 ± 0.055        | 215.9 ± 9.07     | 9.0 ± 0.21    |

*Means followed by same letter in a column are not significantly different by Tukey test (*P* < 0.05).

Fig. 1. Frequency distribution of head capsule width (HCW) of *M. alternatus* larvae in the laboratory in the present study. Curves on the figures were generated by the normal distribution equation with means and standard deviations. The means of HCW for each instar or the HCW range (values in parenthesis) in published literatures were provided on the corresponding peaks for the purpose of comparison. Also, vertical solid and dotted lines at peaks of *Kojima and Katagiri* (1964) and *Liu et al.* (2008), respectively, were provided for the convenience of comparison (see Supp. I Fig. 1 [online only] for the details).

(3.753 mm) from *Liu et al.* (2008) (*t* = 1.2124; *df* = 38; not significant at *P* = 0.2328).

Probability of Misclassification

Boundary points and probabilities of misclassification for each instar according to researchers are summarized in Table 3. The frequency distribution of *Kojima and Katagiri* (1964) showed distinct separation of peaks with low misclassifications among all instars. The probability of misclassification by *Liu et al.* (2008) was especially large, showing a maximum in the third instar and a minimum in the fourth instar. In our results, there was a 3.58% of misclassification between the first and the second instar. Probabilities for misclassification of the second instar as first instar and third instar were 6.75 and 12.81%, respectively. The probability of misclassifying a third instar to another instar (namely the second or fourth instars) was 28.61%. Probabilities of misclassification for the fourth and fifth instars were much higher than those for previous instars.

Length of Mandible Found in the Feeding Gallery

An inconsistency was observed in the increase of mandible size among individuals when the mandibles were arbitrarily arranged by the order of size in each feeding gallery (Supp Fig. 2 [online only]). But the rearranged mandibles by the relative size showed apparently four classes (Supp Fig. 3 [online only]). The completed sample with four pairs (classes) of mandibles supported firmly the present of four instars in the feeding gallery (see #8 in Supp Fig. 1 [online only]). For the mandible found in the feeding gallery, the average size that measured from the center of condyle to incisor cups was 0.37, 0.66, 1.14, and 1.66 mm for the first, second, third, and fourth class (namely instar), respectively (Table 4). The mandible length of the final instar in the pupal chamber was 1.99 mm.

Discussion

The number of *M. alternatus* instars is known to be 4 or 5 in the field (reviewed in *Kobayashi et al.* 1984). However, *M. alternatus* larvae showed repeated molting and finished their development at an average the ninth or 10th instar depending on temperature in our laboratory rearing environment. Such prolonged larval development has been reported earlier by *Ishikubo* (1967) when reared with fresh inner bark in test tubes. For many insect species, variation in the number of instars occurs frequently due to shortage of food quality (Jobin et al. 1992, Esperk et al. 2007, Calvo and Molina 2008). *Calvo and Molina* (2008) have reported that the number of instars of moth *Streblote panda* Hubner (Lepidoptera: Lasiocampidae) varies from 5 to 8 depending on host plants. Unlike those under natural condition, *M. alternatus* larvae seem to show repeat molting probably to reach a threshold weight for pupation since artificial rearing may not provide sufficient nutrients for their growth. For insect species, *Slansky and Scriber* (1985) have established a theory that ‘larval critical weight is the minimal weight for pupation which allows for production of a functional adult.’ Thus, a relatively constant size is made at the critical stage (Nijhout and Williams 1974, Saha et al. 2009). Since the HCW of *M. alternatus* larvae is closely related to the weight of larvae (Kojima and Katagiri 1964), the critical threshold may be expressed by the HCW of the final instar. The value was
3.597 mm in the present study, 3.615 mm in the study of Kojima and Katagiri (1964), and 3.753 mm in the study of Liu et al. (2008). It seems to have a wide range in the field.

Interestingly, the HCW of the final instar and the number of instars to reach the final instar were significantly different among temperatures (Table 2). Significantly smaller HCW size and fewer numbers of instars were also observed at 22°C. It is difficult to discuss further because we do not have available data for comparison yet. Aloo and Katagiri (1994) have reported that temperature alone does not have any influence on HCW development of *M. alternatus*. 
However, its HCW was significantly different among diets. They tested 11 diets at 25°C and room temperature with RH 50–60% and 16:8 (L:D) h for chemically defined diets, plant tissue diets, natural diets, and silk worm diet. However, their development times of larvae were very different from our results. All larvae entered diapause state after 95 d in their experiments.

In our laboratory study, all the first and second instars were held at the same temperature so that they could be combined for the analysis of frequency distribution. Especially, our data were obtained at the exact stage of instars, differently from other previous reports that were based on field collections of unknown stages in most cases. Therefore, our data sets basically did not have an error mixed by different instars that would appear in field collections. As seen in Fig. 2, HCWs of the first instar in previous reports were largely deviated from our values. Kojima and Katagiri (1964) likely have determined the first peak in their frequency distribution as the first instar. However, their first peak might be a minor part of the actual first instar when compared with the characteristic of our frequency distribution, although a sexual dimorphism in HCW size might be involved. Also, their second HCW might be underestimated because a large group of the first instar could be incorporated into the frequency of the second instar. Therefore, our data sets basically did not have an error mixed by different instars that would appear in field collections.

Table 3. Estimates of head capsule width limits and probabilities of misclassifying instars of *M. alternatus* (see Supp II [online only] for details)

| Instar, i  | n  | Mean ± SD | Intersection of HCW, mm | Probability of misclassifying |
|------------|----|-----------|---------------------------|------------------------------|
|            |    |           | Lower                      | Upper                        | i as i-1 | i as i+1 | i as another instar |
| Kojima and Katagiri (1964) |    |           |                           |                              |          |          |                      |
| 1          | 14 | 0.730 ± 0.062 | –                         | 0.8546                      | –        | 0.0510   | 0.0510                      |
| 2          | 28 | 1.013 ± 0.085 | 0.8546                    | 1.2505                      | 0.0700   | 0.0077   | 0.0777                      |
| 3          | 137 | 1.602 ± 0.133 | 1.2505                    | 1.9731                      | 0.0125   | 0.0083   | 0.0208                      |
| 4          | 260 | 2.643 ± 0.266 | 1.9731                    | 3.1430                      | 0.0166   | 0.0668   | 0.0834                      |
| 5          | 190 | 3.629 ± 0.253 | 3.1430                    | –                           | 0.0636   | –        | 0.0636                      |
| Liu et al. (2008) |    |           |                           |                              |          |          |                      |
| 1          | 110 | 1.218 ± 0.315 | –                         | 1.471                       | –        | 0.289    | 0.2888                      |
| 2          | 162 | 1.729 ± 0.223 | 1.471                     | 2.066                       | 0.204    | 0.128    | 0.3321                      |
| 3          | 160 | 2.483 ± 0.379 | 2.066                     | 2.897                       | 0.218    | 0.220    | 0.4375                      |
| 4          | 241 | 3.171 ± 0.161 | 2.897                     | 3.430                       | 0.093    | 0.109    | 0.2024                      |
| 5          | 345 | 3.753 ± 0.461 | 3.430                     | –                           | 0.312    | –        | 0.3123                      |
| Present study |    |           |                           |                              |          |          |                      |
| 1          | 145 | 0.896 ± 0.069 | –                         | 1.0469                      | –        | 0.0358   | 0.0358                      |
| 2          | 68  | 1.291 ± 0.130 | 1.0562                    | 1.4866                      | 0.0675   | 0.1281   | 0.1957                      |
| 3          | 68  | 1.707 ± 0.165 | 1.4866                    | 1.9591                      | 0.1628   | 0.1233   | 0.2861                      |
| 4          | 59  | 2.276 ± 0.278 | 1.9591                    | 2.4500                      | 0.2084   | 0.3282   | 0.5366                      |
| 5          | 52  | 2.620 ± 0.282 | 2.4500                    | –                           | 0.3329   | –        | 0.3329                      |

*aLiu et al (2008) noted it as SE in their report, but was regarded as SD considering the sample size.*

Table 4. The mandible length of *M. alternatus* larvae found in the feeding gallery; mandible length was measured from the center of condyle to incisor cups

| Location discovered | Instar | n  | Mean ± SE | Minimum | Maximum |
|---------------------|--------|----|-----------|---------|---------|
| Feeding gallery     | 1st    | 7  | 0.37 ± 0.011 | 0.34    | 0.43    |
|                     | 2nd    | 7  | 0.66 ± 0.009 | 0.62    | 0.69    |
|                     | 3rd    | 8  | 1.14 ± 0.018 | 1.07    | 1.22    |
|                     | 4th    | 9  | 1.66 ± 0.019 | 1.32    | 1.71    |
| Pupal chamber       | 5th    | 4  | 1.99 ± 0.028 | 1.92    | 2.05    |

*Means with same lowers case letter in a column are not significantly different by Tukey’s test at *P* = 0.05 (*F* = 1,357.69; df = 4, 30; *P* < 0.0001).

*The final instars found in pupal chamber were regarded as the fifth instar.*

However, its HCW was significantly different among diets. They tested 11 diets at 25°C and room temperature with RH 50–60% and 16:8 (L:D) h for chemically defined diets, plant tissue diets, natural diets, and silk worm diet. However, their development times of larvae were very different from our results. All larvae entered diapause state after 95 d in their experiments.

In our laboratory study, all the first and second instars were held at the same temperature so that they could be combined for the analysis of frequency distribution. Especially, our data were obtained at the exact stage of instars, differently from other previous reports that were based on field collections of unknown stages in most cases. Therefore, our data sets basically did not have an error mixed by different instars that would appear in field collections. As seen in Fig. 2, HCWs of the first instar in previous reports were largely deviated from our values. Kojima and Katagiri (1964) likely have determined the first peak in their frequency distribution as the first instar. However, their first peak might be a minor part of the actual first instar when compared with the characteristic of our frequency distribution, although a sexual dimorphism in HCW size might be involved. Also, their second HCW might be underestimated because a large group of the first instar could be incorporated into the frequency of the second instar. Although the misclassification probability of Kojima and Katagiri (1964) was low, peaks of the first and second instars might have been wrongly established. The first HCW of Ochi (1975) was likely overestimated as a number of large eggs were included. Thus, strategies of Ochi (1975) and Togashi (1991) might not be robust since they combined the first and second instars of Kojima and Katagiri (1964) and regarded them as the group of first instar.

The range of first instar of Yamane (1974) overlapped about half with our distribution to the right (Fig. 2). This range was largely determined based on the median HCW of 1.1 mm for the just hatched first instars in the laboratory rearing using artificial diet. It might be somewhat overestimated since he showed that the HCW at 6 d after hatching ranged from 1.1 to 2.8 mm in the same rearing study. In other words, if the first instars were in the just hatched state, they would be difficult to develop into the range discussed earlier for 6 d because the development time for the first instar is longer than 6 d at below 26°C (Kwon 2017). The range was overlapped largely with the HCW range of second and third instars (1.5−1.9 and 2.1−2.9 mm, respectively, based on his study). Thus, if the HCW of Yamane (1974) is correct, it indicates that artificial rearing is largely different from natural food for the development of *M. alternatus* larvae.

Morimoto and Iwasaki (1974) found that their HCWs of hatched larvae were equivalent to those of second instar of Kojima and Katagiri (1964). Under this perception, they separated the first group (namely approximately the second size of Kojima and Katagiri) as
It may not be abnormal that the HCW of hatched larvae is similar to the second HCW of Kojima and Katagiri (1964). As seen in Fig. 2, a large number of second HCW in Kojima and Katagiri (1964) belonged to the first HCW in the frequency distribution of our study. Therefore, if the HCW sample had been collected accidently from a group of large eggs, the above results of Morimoto and Iwasaki (1974) could have been made.

The frequency distribution of Liu et al. (2008) was missing in the position of the first HCW of our results while their first HCW peak occurred near our second HCW peak. Liu et al. (2008) obtained eggs for 10 d by exposing pine logs in the forest field infested heavily with M. alternatus. They covered them with steel mesh in the site and collected the first sample at 10 d after field treatment, taking maximum 20 d in total. Because the development time of M. alternatus eggs and the first instar at 26°C are 6.7 and 7.1 d, respectively, according to Kwon (2017), the first sample of Liu et al. (2008) might have included lots of the second instars. Consequently, the second HCW presented by Liu et al. (2008) would be actually the third HCW, which well corresponded with the third HCW of Kojima and Katagiri (1964) and the present study. Ochi and Katagiri (1974) found that HCWs of M. alternatus larvae were consisted of the first and second instars equally (based on the HCW criterion of Kojima and Katagiri 1964) at 12 d after oviposition in the field during the period of late August to early September. Thus, results of Liu et al. (2008) might have largely overestimated the first HCW by missing a large number of actual first HCW.

In the analysis for misclassification probability of instars in our study, the first and second instars could be separated from other instars with a low error. Also, the third instar could be classified with an error of 28.61%. However, errors in the classification for the fourth and fifth instars were much higher than those for previous instars. That is, HCWs after the third instar in the present study were largely underestimated probably because of poor nutritional quality in the laboratory environment. Dead pine trees left in natural condition may provide better condition nutritionally to larvae of M. alternatus because various microorganisms could grow in the inner barks of pine trees. Thus, larger larvae (the fourth and fifth instars) may be subjected to nutritional deficiency under laboratory condition since they would enter the sapwood deeper and require more food (Ochi and Katagiri 1974). However, small larvae feed mostly on nutritionally excellent phloem tissue and require a small amount of food source to complete their development (reviewed by Kobayashi et al. 1984). Thus, it seems that these larvae would grow successfully without nutritional limit in the laboratory environment. Dead pine trees in natural condition may provide better condition nutritionally to larvae of M. alternatus because various microorganisms could grow in the inner barks of pine trees. Thus, larger larvae (the fourth and fifth instars) may be subjected to nutritional deficiency under laboratory condition since they would enter the sapwood deeper and require more food (Ochi and Katagiri 1974). However, small larvae feed mostly on nutritionally excellent phloem tissue and require a small amount of food source to complete their development (reviewed by Kobayashi et al. 1984). Thus, it seems that these larvae would grow successfully without nutritional limit in the laboratory environment. In this respect, our HCW data for the first, second, and third instars may represent actual populations of M. alternatus better than previous reports.

Since our laboratory data sets might not be valid after the third instar, we re-analyzed previous reports (Kojima and Katagiri 1964, Liu et al. 2008) to specify the HCW of later instars. The peaks of Liu et al. (2008) largely overlapped with each other. Their misclassification errors were very high among instars. This is not normal because these instars are not separated readily by the frequency curve. Also, the fourth HCW peak of Liu et al. (2008) is somewhat abnormal (see Supp I Fig. 1 [online only]). The peak is very sharp with a small variation compared with peaks of other instars. It is difficult to justify that the preceding large variation has converged in the fourth instar (please see curves for the first, second, and third HCW in Supp I Fig. 1). This fourth peak is located between the fourth peak and the fifth peak of Kojima and Katagiri (1964). Therefore, we strongly suspect that the fourth peak of Liu et al. (2008) may be made by overestimating marginal groups of HCWs coming from actual fourth and fifth instars. Such abnormally skewed frequencies are found in the group of larvae in diapause (Fig. 3A). If the fourth peak is not valid in the results of Liu et al. (2008), the frequency distribution will have four peaks, which supports the appearance of five instars in the field as adding missed 1st instar.

The fact that M. alternatus pass five instars in the field was confirmed by the results from investigation of mandibles in the feeding gallery. The same experiment was conducted previously by Morimoto and Iwasaki (1974) in Japan. They found only three pairs of HCW in the feeding gallery of M. alternatus, resulting in the occurrence of four instars. It is not easy to find all HCWs or mandibles in branches of pine trees where M. alternatus has completed its life cycle. Especially, HCW is more fragile and putrescible than mandible. If previous researchers had tried to look for mandibles, they might have found four pairs of mandibles like our case. We have found the evidence that M. alternatus larvae pass five instars in the field (see sample #8 in Supp III Fig. 1 [online only], Table 4). This fact was confirmed successfully by identifying four pairs of mandibles by comparing the relative size among individuals. Unfortunately, we could not determine the relationship between the size of HCW and the mandible because the part of HCW is not preserved well in available samples. As a next step, we provided lengths of mandibles in overall averages: 0.37, 0.66, 1.14, 1.66, and 1.99 mm for the first, second, third, fourth, fifth instars, respectively.

Integrating all frequency distributions of HCW and our experiments, most M. alternatus larvae passed five instars in the field. According to our results, average sizes of HCW for the first, second, and third instars are newly suggested to be 0.896, 1.291, and 1.707 mm, respectively. Average sizes of HCW for fourth and fifth instars are 2.632 and 3.615 mm, respectively, based on the report of Kojima and Katagiri (1964). Results of Liu et al. (2008) need to be examined further since their peaks of HCW largely overlapped with each other with very high misclassification errors among instars. In Jeju area, the HCW of 3.597 mm for the final instar in diapause nearly fitted to the fifth HCW, indicating most larvae passed five instars. According to this criterion, previous records (Kobayashi et al. 1984; Togashi 1989, 1990, 1991; CABI 2017) which regarded HCW of >3.15 mm as the fourth instar or noted passing four instars in the field indicate that they were actually mostly the fifth instars. In this study, we challenge initial understanding that there are four instars of larvae of M. alternatus (Morimoto and Iwasaki 1974, Yamane 1974, Ochi 1973).

Since the life cycle of M. alternatus is complicated as described earlier, the number of instars is a decisive factor on the interpretation of its life history. By modifying the suggestion of Togashi (1989, 1990), M. alternatus larvae that have completed the fourth or fifth instar can emerge as adults in the following year. Thus, fitness of this beetle in regions where its larvae cannot reach the fourth instars within seasons will be greatly reduced since overwintered larvae will emerge in the next year. Thus, our results can be used for population study of M. alternatus in the future.

Supplementary Data

Supplementary data are available at Journal of Insect Science online.

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