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Morphological Differences between Larvae of the Oriental Fruit Moth (*Grapholita molesta* Busck) and the Peach Fruit Moth (*Carposina sasakii* Matsumura) in Korea

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The oriental fruit moth (*Grapholita molesta* Busck) and the peach fruit moth (*Carposina sasakii* Matsumura) are the most severe insect pests affecting apple orchards in Korea. To prevent an outbreak of these two species and to control these agricultural insect pests, it is important to identify them accurately. However, it is hard to classify them when they were in the larval stage since they tunnel into the apple fruit. In this study, surface structures of the two species of larvae were observed using stereo microscope and scanning electron microscope. Distinct differences between the two species of larvae were found. The prothorax spiracles of oriental fruit moth larvae were approximately twice as large as those of peach fruit moth larvae. The arrangements of subventral setae, located around the proleg, were different between oriental fruit moth and peach fruit moth larvae. Furthermore, subdorsal setae of oriental fruit moth were located next to the spiracle on the 8th abdominal segment, while that of peach fruit moth was located above the spiracle. The identification of the two species of larvae observed in this study was confirmed using polymerase chain reaction-restriction fragment length polymorphism method. Surface structural differences are intrinsic characteristics for each species of larvae and can easily be identified using stereo microscope. These specificities will be helpful where a large number of field-collected larvae need to be identified routinely in pest control research.

Key Words: Oriental fruit moth, Peach fruit moth, Morphological characteristics at larval stage

INTRODUCTION

The major moth pests in apple orchards in Korea are summer fruit tortrix (*Adoxophyes orana*), peach fruit moth (*Carposina sasakii*), oriental fruit moth (*Grapholita molesta*) and apple leaf miner (*Lyonetia prunifoliella*), and the larvae of these species result in economic losses and product damage due to larvae feeding directly on the fruit (Lee et al., 2007). Oriental fruit moth and peach fruit moth larvae attack not only apples but also pears, peaches, plums, apricots and quinces. These two species of larvae have to be controlled for fruit production (Lee et al., 1984; Park et al., 2008).

Oriental fruit moth (Lepidoptera: Tortricidae) is distributed worldwide, whereas peach fruit moth (Carposinidae) is limited in its distribution to northeastern Asia (Kim et al., 2000; Il'ichev et al., 2006). In Korea, oriental fruit moth and peach fruit moth are prevalent between June to August and between April to September respectively. The identification of the two species of larvae is very important for crop control and export of produce (Choi et al., 2010). However, these pests can only be identified after adult emergence since they exhibit similar larval morphology and outbreak periods (Choi et al.,

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2008). Although only oriental fruit moth leaves excreta on the surface of fruits (Choi et al., 2010), it is difficult to identify them in field due to the excreta falling off. Recent studies developed diagnostic methods for the two species of larvae using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and species-specific primers PCR (Song et al., 2007; Hada & Sekine, 2011). However, these methods require expensive facilities and equipment and are also time consuming. Furthermore, these assays might lead to misidentification with intraspecific variations (Song et al., 2007).

The aim of this study was to investigate morphological differences of surface structure between the two species of larvae using stereo microscope and scanning electron microscope (SEM). We present the observation of the differences between them from the prothorax spiracles, the arrangements of subventral setae (SV) and the 8th abdominal segment.

MATERIALS AND METHODS

Sample Collection

The two species of larvae were collected from approximately 100 apple fruits which were suspected of infestation. The fruit were damaged, showing signs of halls or excretia on the surface. Fruits were collected from Gyeongsan, Gunwi-gun and Sangju between 2010 and 2011 from June to September by the National Institute of Horticultural & Herbal Science, Rural Development Administration and Korea Fruit Pest Forecasting Research Center. The fruits were cut open and examined. Using cotton swabs, 60~70 larvae that were greater than 10 mm in length, were selected for the study. Each larva was placed in a 9 cm petri dish and kept at 10°C in an incubator to prevent pupation. Larvae were rinsed 2~3 times in distilled water before microscopic observation.

Microscopic Observation

By using ethyl acetate as an anesthetic (Steiner et al., 2010), collected larvae were anesthetized for stereo microscopy observation, to ensure that larvae were stationary. The arrangements of the SV were observed using stereo

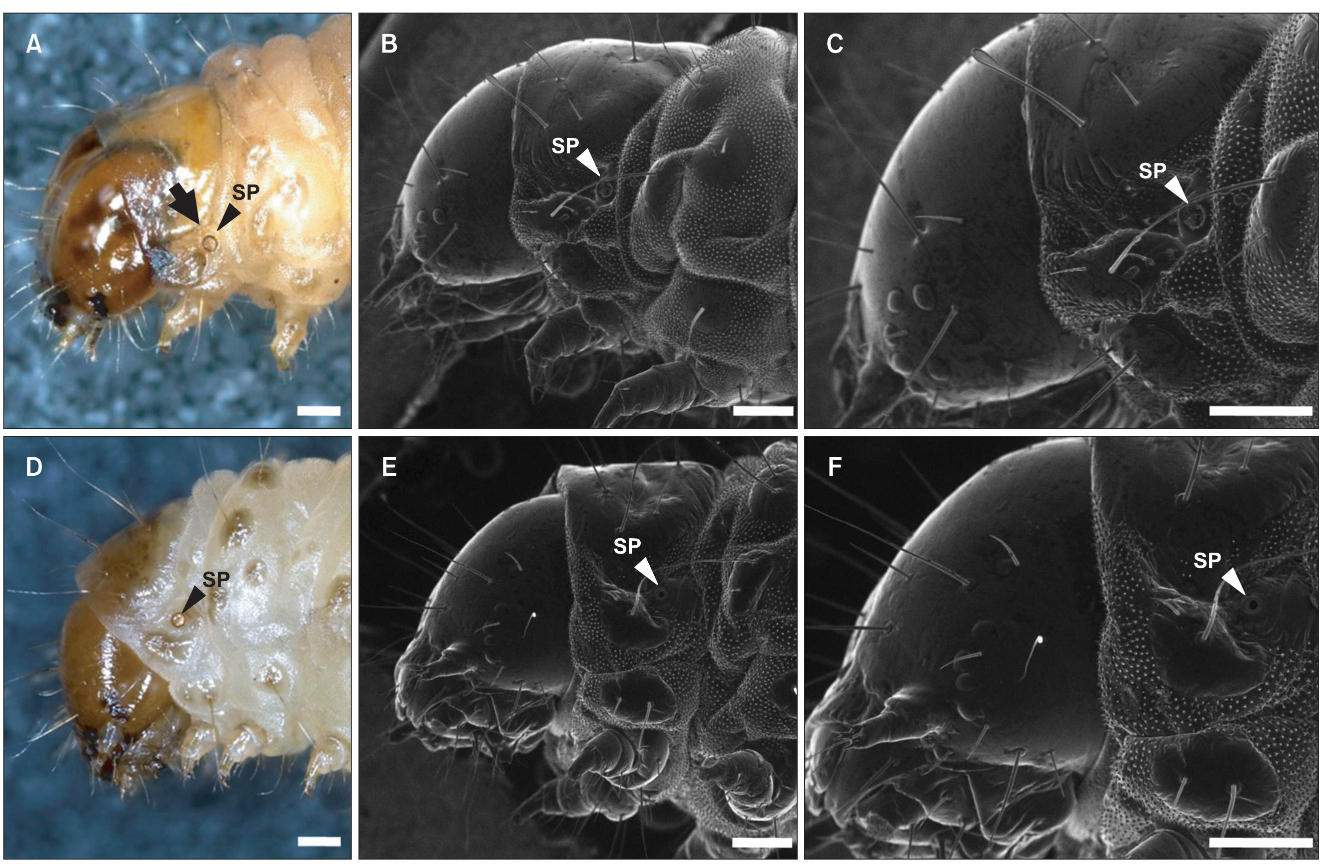

Fig. 1. Stereo micrograph and low vacuum scanning electron micrograph of oriental fruit moth (A-C) and peach fruit moth (D-F) at prothorax. (C) and (F) are enlarged images of (B) and (E), respectively. Scale bar=200 μm. SP, spiracle.

microscopy (DIMIS-M, Siwon Optical Technology Co., Ltd., Anyang, Korea). Larvae were separated into two groups based on the arrangements of the SV. Three larvae from each of the two groups were selected and treated for SEM examination as follows. Samples were prefixed with 2% paraformaldehyde and 2.5% glutaraldehyde in 0.05 M sodium cacodylate buffer, pH 7.2, for 2 hours at 4°C. The samples were then washed in 0.05 M sodium cacodylate buffer solution three times for 15 minutes, and postfixed in 1% osmium tetroxide (O_SO_4). After that, samples were rewashed with the same buffer. The fixed samples were dehydrated in a graded ethanol series; 30%, 50%, 70%, 90%, absolute ethanol for 20 minutes in each concentration, and were dried with hexamethyldisilsazane for 15 minutes (Moon & Park, 2009). Thereafter treated specimens were observed at different magnification (×100, ×150 and ×200) using SEM (S-3500N, Hitachi, Tokyo, Japan) in low vacuum mode focusing on the prothorax and abdomen of the larvae.

Identification of Oriental Fruit Moth and Peach Fruit Moth Larvae Using PCR-RFLP

The identity of the two species of larvae classified by stereo microscopy was confirmed using the PCR-RFLP method (Song et al., 2007). Genomic DNA samples were extracted using the cetyl trimethyl ammonium bromide method (Martinelli et al., 2007). The *cytochrome b* (*CYT-B*) region was amplified using primers CB-J10933 (5'-TAT GTT TTA CCA TGA GGA CAA ATA TC-3') and CB-N11367 (5'-ATA ACT CCT CCT AAT TTA TTA GGA AT-3') (Simon et al., 1994). PCR cycling conditions for *CYT-B* were as follows: a 1 minute 94°C denaturation step followed by 35 cycles of 30 seconds at 94°C, 60 seconds at 56°C and 90 seconds at 72°C. A final extension step of 10 minutes at 72°C completed the cycling conditions. After amplification, 1 U of *Sau*3A1 in 1×buffer *Sau*3A1 (5'-^GATC-3', Promega, Madison, WI, USA) was added to the PCR reactions. Digestions were carried out at 37°C for two hours. Digested fragments were electrophoresed through an ethidium bromide 1% agarose gel

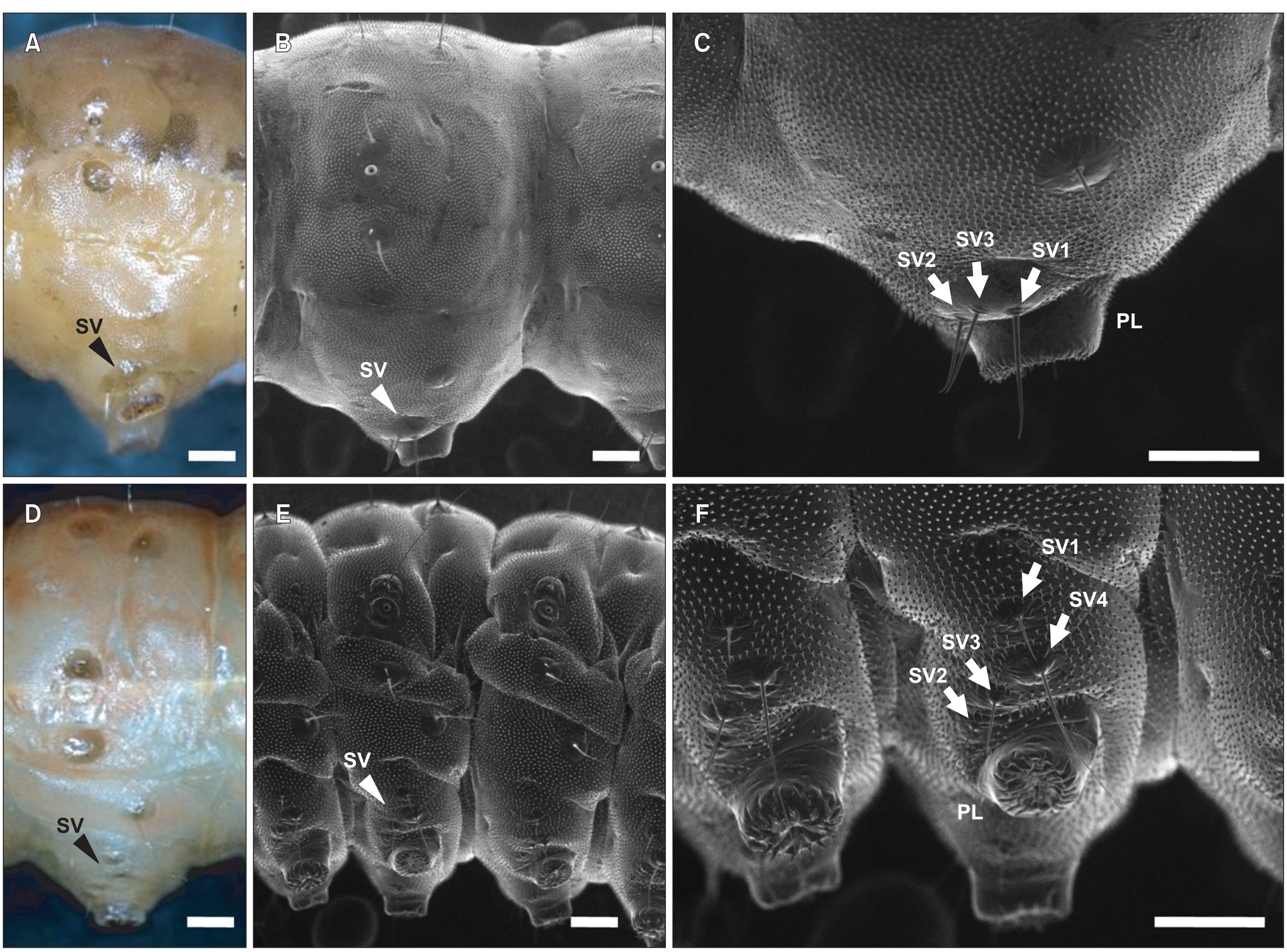

Fig. 2. Stereo micrograph and scanning electron micrograph of oriental fruit moth (A-C) and peach fruit moth (D-F) at abdominal segments A3-A6. (C) and (F) are enlarged images of (B) and (E), respectively. Scale bar=200 μm. SV, subventral setae; PL, proleg.

and photographed under ultraviolet light illumination using a gel imaging system.

RESULTS AND DISCUSSION

Initially oriental fruit moth and peach fruit moth larvae were observed using microscopy for size, shape, location of the prothorax spiracles and the arrangements of SV. The differences in the arrangements of SV were then compared using SEM. The size of the prothorax spiracles of oriental fruit moth larvae were approximately twice as large (65~75 μm) as those of peach fruit moth larvae (30~35 μm) (Fig. 1). Oriental fruit moth larvae have three SV next to the proleg (Fig. 2A-C) and subdorsal setae located next to the spiracle on the 8th abdominal segment (Fig. 3A-C). Peach fruit moth have four SV next to the proleg (Fig. 2D-F) and subdorsal setae located above the spiracle on the 8th abdominal segment (Fig. 3D-F). The arrangements of SV of larvae, reflecting the species specificity through evolution, have been used as the morphological key identifying these insect species (Gilligan et al., 2011a). Gilligan et al. (2011b) reported that *Cryptaspasma perseana* larvae (Lepidoptera: Tortricidae) has three SV from 3rd to 6th abdominal segments around the proleg and subdorsal setae are located next to the spiracle on the 8th abdominal segment. Solis (1999) reported that most Carposinidae larvae have four SV around the proleg. Although the morphological traits of oriental fruit moth and peach fruit moth larvae in this study may overlap with characteristics of other moth species, only these two species directly attack and make halls into the fruit in apple orchards in Korea (Choi et al., 2010).

After observation using stereo microscopy and SEM, the identification of oriental fruit moth and peach fruit moth larvae were confirmed using the PCR-RFLP method (Song et al., 2007). A single *CYT-B* fragment (approx. 460 bp) was amplified for both species of larvae. Following this, restriction enzyme digestion profiles for oriental fruit moth and peach fruit moth larvae were typified by two fragments, 360 bp

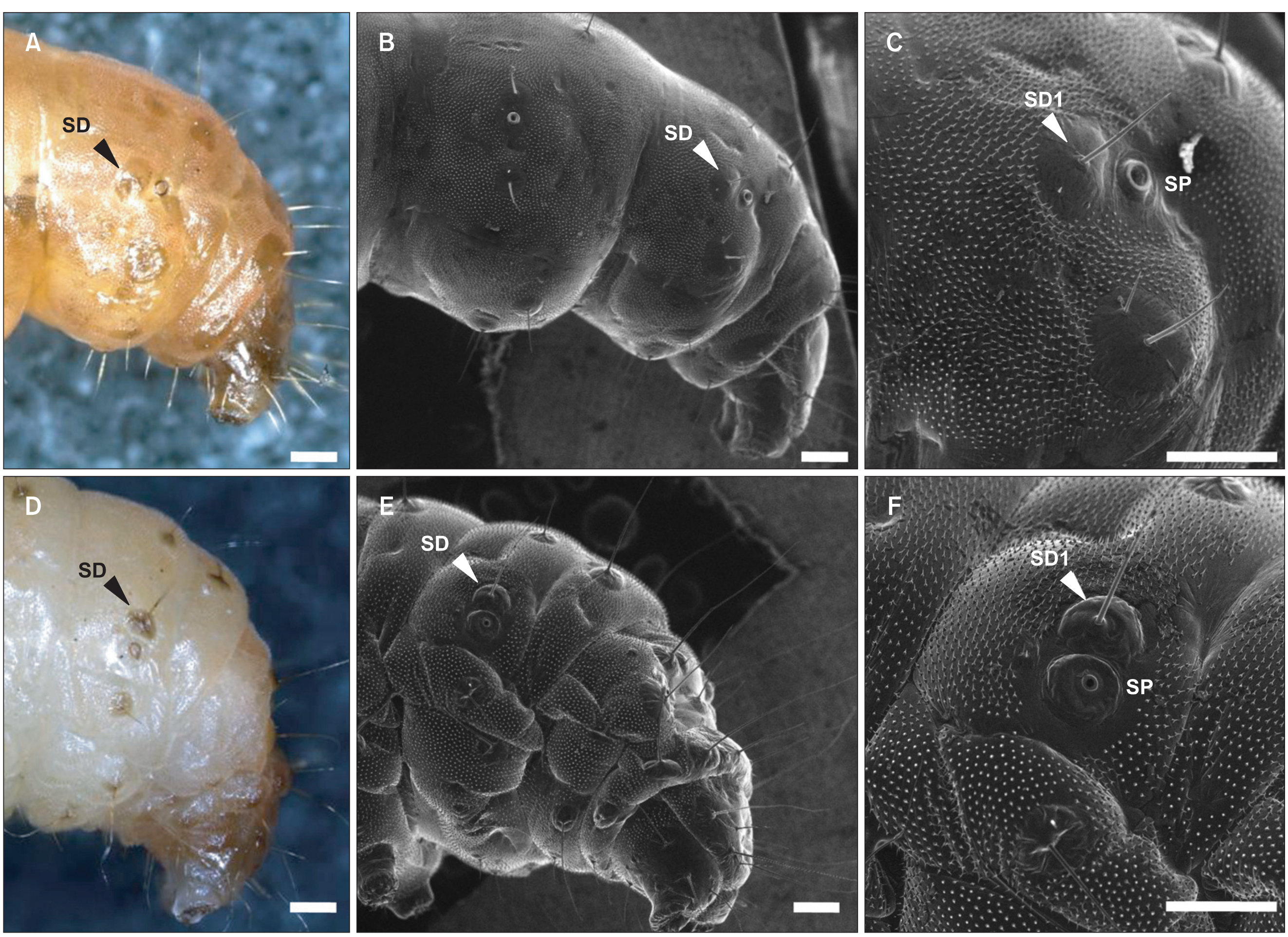

Fig. 3. Stereo micrograph and low vacuum scanning electron micrograph of oriental fruit moth (A-C) and peach fruit moth (D-F) at the 8th abdominal segment. (C) and (F) are enlarged images of (B) and (E), respectively. Scale bar=200 μm. SD, subdorsal setae; SP, spiracle.

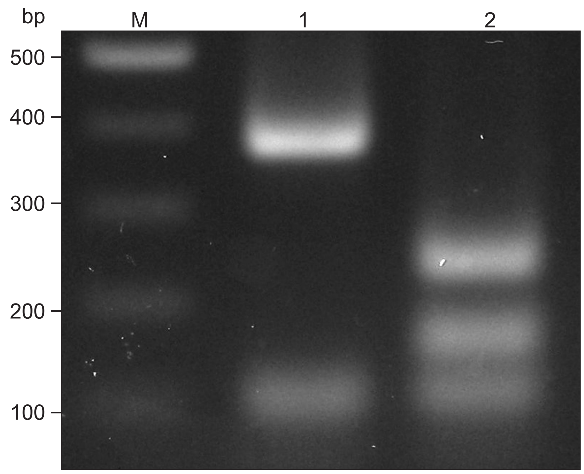

Fig. 4. Electrophoresis of the PCR products amplified using the CB-J10933/CB-N11367 and digested using the *Sau*3A1 (Song et al., 2007). Lane M: 100 bp DNA ladder, Lane 1: DNA fragment pattern of oriental fruit moth, Lane 2: DNA fragment pattern of peach fruit moth.

and 100 bp and three fragments, 220 bp, 140 bp and 100 bp respectively (Fig. 4). The result was consistent with that of Song et al. (2007). In addition, reproducibility test was conducted to reconfirm the method. Regardless of harvesting periods and regions, peach fruit moth larvae were easily differentiated with oriental fruit moth larvae.

Korean apple orchards export more than 90% of their produce to Taiwan and the amount of apples has been increasing since 2000. Since Taiwan designates peach fruit moth as a quarantine pest, it is very important to control this species for agricultural export produce in Korea (Choi et al., 2010). However, mis-identification of pest species due to morphological confusion between oriental fruit moth and peach fruit moth can result in the rejection of exported apples (Song et al., 2007). Furthermore, Taiwan would stop importing apples if peach fruit moth larvae were identified in imported apples more than twice in a year (Choi et al., 2010). Hence, the accurate identification of oriental fruit moth and peach fruit moth larvae plays a crucial role in the Korean apple export industry.

Although distinct differences between the oriental fruit moth and peach fruit moth larvae have been identified, these specimens were taken from limited geographical localities. Furthermore, morphological traits were only investigated within these two species of agricultural pests. These methods might be useful for large numbers of field-collected larvae to be identified rapidly and accurately. However, further investigations are needed to confirm the results from a wider range of localities and species collections. Comparisons with species from other countries, possibly exhibiting morphological variations, also need to be carried out.

CONCLUSIONS

These results demonstrate that morphological differences were observed between oriental fruit moth and peach fruit moth larvae using stereo microscope and scanning electron microscope. These findings suggest that each of oriental fruit moth and peach fruit moth larvae could be easily differentiated with their own structures.

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