Distinction of Grapholita molesta Busck and Grapholita dimorpha Komai larvae based on morphological feature of anal prolegs

Lee, Seung-Yeol
College of Agriculture and Life Sciences, Kyungpook National University

Choi, Kwang-Shik
College of Natural Sciences, Kyungpook National University

Back, Chang-Gi
National Institute of Horticultural and Herbal Science, Rural Development Administration

Choi, Kyung-Hee
National Institute of Horticultural and Herbal Science, Rural Development Administration

他

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Distinction of *Grapholita molesta* Busck and *Grapholita dimorpha* Komai larvae based on morphological feature of anal prolegs

Seung–Yeol LEE[1], Kwang–Shik CHOI[2], Chang–Gi BACK[3], Kyung–Hee CHOI[3], In–Kyu KANG[1], Hee–Young JUNG[1]* and Shoji OHGA*

Laboratory of Forest Resources Management, Division of Forest Environmental Sciences,
Department of Agro–environmental Sciences, Faculty of Agriculture,
Kyushu University, Sasaguri, Fukuoka 811–2415, Japan
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Larvae of *Grapholita molesta* Busck and *Grapholita dimorpha* Komai, which are major moth pests that affect apples in Korea, are very difficult to identify because of their morphological similarities. In this study, we investigated how to distinguish the larvae of these two species by using specific morphological features. Between 2013 and 2014, a total of 84 specimens were collected from apples suspected of infestation in Gunwi–gun and Cheongsong–gun, Gyeongsangbuk–do, Korea, and they were observed using a stereo microscope, optical microscope, and scanning electron microscope. We found that the number of anal proleg crochets of the two species was different. PCR–RFLP results for the two species showed that *G. molesta* and *G. dimorpha* have 18–28 and 12–17 anal proleg crochets, respectively. Our findings suggest that the number of anal proleg crochets could be an excellent morphological character that could be used to identify the larvae of the two species without the need for any further experiments.

Key words: *Grapholita molesta*, *Grapholita dimorpha*, Morphological difference, Number of crochets, PCR–RFLP

INTRODUCTION

The major insect pests found in apple orchards belong to the order Lepidoptera, for example, *Carposina sasakii* Matsumura belongs to the family Carposinidae and *Grapholita molesta* Busck and *Grapholita dimorpha* Komai belong to the family Tortricidae. *Grapholita molesta* is distributed worldwide and appears to favor drupe and pome fruits (Il'ichev *et al*., 2003; Natale *et al*., 2003; Kovanci *et al*., 2004). *Grapholita dimorpha* has hosts similar to those of *G. molesta* (Rothschild and Vicker, 1991), and its distribution is limited to mainly Northeast Asia, including Korea, Japan, China and Russia (Park and Kim, 1986; Komai, 1999; Yan *et al*., 1999; Beljaev and Ponomarenko, 2005). In Korea, *G. dimorpha* was reported in apple orchards in 2009 (Choi *et al*., 2009), also, it has been reported in pear, peach, and plum orchards (Jung *et al*., 2012; Ahn *et al*., 2013).

Choi *et al*. (2009) reported that *G. molesta* and *G. dimorpha* emerge 4 times a year, which is also similar to the periods of adult emergence; however, the population density of *G. dimorpha* tends to increase after July. Choi *et al*. (2008) indicated that *G. molesta* has infested more apples than C. sasakii since 1997; however, it was suggested that *G. dimorpha* might have been mistakenly identified as *G. molesta* (Choi *et al*., 2009). Because larvae of both *G. molesta* and *G. dimorpha* infest apples, it is very important to accurate identify them to better understand the situation and use effective control measures. However, it is impossible to identify the larvae of these two species on the basis of morphology, even though the adults of these species can be differentiated on the basis of wing width and patterns of the male hind wings (Komai, 1979).

Currently, species–specific polymerase chain reaction (PCR) primers and PCR–restriction fragment length polymorphism (RFLP) have been mainly used to identify *G. molesta* and *G. dimorpha* (Hada and Sekine, 2011; Jung *et al*., 2013; Jung *et al*., 2012; Ahn *et al*., 2013). However, morphological keys for the larvae of these two species are not available. In a recent study (Lee *et al*., 2013), *G. molesta* and *C. sasakii* larvae collected from apple orchards were identified using specific morphological features. Therefore, we investigated specific morphological differences between the larvae of *G. molesta* and *G. dimorpha* to identify them effectively.

MATERIALS AND METHODS

Sample collection

Between July and October in 2013 and 2014, larvae were collected from approximately 200 apples suspected of infestation in apple orchards in Gunwi–gun and Cheongsong–gun, Gyeongsangbuk–do, Korea. After the fruits were cut, larvae greater than 10 mm in length were collected. The specimens were used after they were rinsed 2 to 3 times in distilled water to remove debris.

Species Identification

After the larvae were prepared for microscopy by using ethyl acetate to ensure that the larvae were stationary (Steiner *et al*., 2010), arrangement of adfrontal setae

[1] College of Agriculture and Life Sciences, Kyungpook National University, Daegu 702–701, Korea
[2] College of Natural Sciences, Kyungpook National University, Daegu 702–701, Korea
[3] National Institute of Horticultural and Herbal Science, Rural Development Administration, Suwon 440–760, Korea
* Corresponding author (E–mail: ohga@forest.kyushu–u.ac.jp, heeyoung@knu.ac.kr)

and other morphological features were observed using a stereo microscope (DIMIS–M, Siwon Optical Technology Co., Ltd., Anyang, Korea) and optical microscope (Olympus BX–50, Japan). Initially, the specimens were identified morphologically to separate the larvae of *C. sasakii* and *G. molesta* by using the keys given by Lee *et al*. (2013). After morphological identification, only larvae belonging to the family Tortricidae were used for the study.

Microscopic observation

Arrangement of adfrontal setae, characters of the head and tail, and the number of anal proleg crochets were observed using a stereo microscope and optical microscope. Also, the width of head capsules were measured to check the each of larval instar stage. Six specimens showed morphological differences, and they were prepared and examined using a scanning electron microscope (SEM; magnification, 350×; S–3500N, Hitachi, Japan), according to the method described by Moon *et al*. (2009).

Identification of *G. molesta* and *G. dimorpha* larvae by using PCR–RFLP

After morphological examination, the specimens were identified using PCR–RFLP (Jung *et al*., 2013). Initially, genomic DNA samples were extracted using the cetyl trimethyl ammonium bromide method (Martinelli *et al*., 2007). A partial sequence of the NADH dehydrogenase 4 region was amplified using ND4–J–89441/ND4–RP primers (Simon *et al*., 1994). A total volume of 20 μL for each reaction contained 0.5 μL of the genomic DNA, 2 μL of 10× Taq buffer, 1 μL of 10 mM dNTP, 10 pM of each primer, and 0.2 μL of *Taq* DNA polymerase (Solgent, Daejeon, Korea). PCR cycling conditions were as follows: a 2–min 94°C denaturation step, followed by 35 cycles at 94°C for 60 s, 45°C for 45 s, and 72°C for 90 s; there was a final extension step at 72°C for 10 min (Jung *et al*., 2013). PCR product was electrophoresed through a 1% agarose gel, which was stained with ethidium bromide, and observed under ultraviolet light by using a UV illuminator. After PCR, 0.2 U of Swa I (5'–ATTT^AAAT–3'; Takara, Japan) was added to the PCR products, and digestion was performed at 30°C for 2 h. Digested PCR products was electrophoresed through a 1% agarose gel, stained with ethidium bromide, observed under ultraviolet light by using a UV illuminator.

RESULTS AND DISCUSSION

A total of 116 larvae were observed the size of the prothorax spiracles, number of subventral setae next to the proleg, and arrangement of the 8th abdominal segment using the method described by Lee *et al*. (2013). As the results, 32 and 84 larvae were identical to that of larvae that belong to the families Carposinidae and Tortricidae, respectively (Data not shown). Among the classified Tortricidae larvae, we observed various characteristics such as head shape and width, arrangement of subventral setae, anal comb and number of anal proleg crochets using stereo and optical microscope. All the examined Tortricidae larvae were identified using PCR–RFLP method (Jung *et al*., 2013). PCR–RFLP results revealed that 13 of the 84 larvae identified as belonging to the family Tortricidae were *G. dimorpha*, and the other 71 larvae were *G. molesta* (Fig. 1). Based on PCR–RFLP results, we compared each of morphological characteristics that observed using stereo and optical micro-

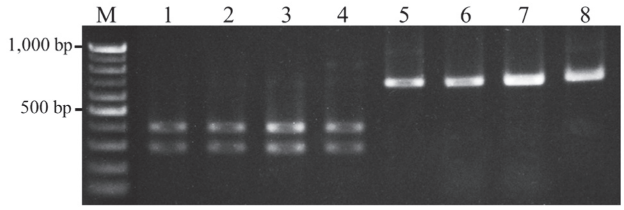

Fig. 1. Agarose gel (1%) stained with ethidium bromide and showing the amplicons after PCR–RFLP. M: 100–bp DNA ladder, 1–4 lanes: *G. dimorpha* (390 bp and 290 bp), 5–8 lanes: *G. molesta* (690 bp).

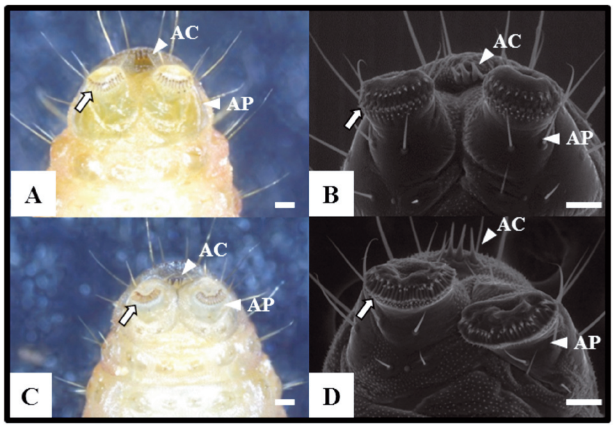

Fig. 2. Observed shape of anal proleg crochets from *G. molesta* and *G. dimorpha* larvae by using a stereo microscope and scanning electron microscope. A, B: *Grapholita dimorpha*. C, D: *Grapholita molesta*. AC: Anal comb, AP: Anal proleg, Arrow: Crochets. Scale bar = 100 μm.

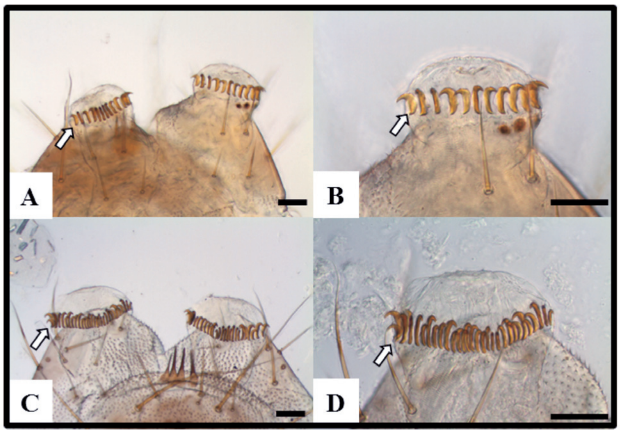

Fig. 3. Observed shape of the anal proleg crochets from *G. molesta* and *G. dimorpha* larvae by using an optical microscope. A, B: *Grapholita dimorpha*. C, D: *Grapholita molesta*. Arrow: Crochets. Scale bar = 100 μm.

scope. Although there were no differences in head shape, arrangement of subventral setae, and presence of an anal comb (data not shown), larvae of the two species showed different numbers of anal proleg crochets when observed using a stereo microscope and SEM (Fig. 2, Table 1). Also, number of crochets of two anal legs; both side showed same number of crochets (Data not shown). Observation using an optical microscope confirmed that

Table 1. Identification of larvae belonging to the family Tortricidae and their number of anal proleg crochets

Name	Identification Results	Number of anal proleg crochets	Name	Identification Results	Number of anal proleg crochets
Tortri–1	*G. dimorpha*	13	Tortri–43	*G. molesta*	23
Tortri–2	*G. dimorpha*	13	Tortri–44	*G. molesta*	19
Tortri–3	*G. dimorpha*	17	Tortri–45	*G. molesta*	24
Tortri–4	*G. dimorpha*	13	Tortri–46	*G. molesta*	19
Tortri–5	*G. dimorpha*	12	Tortri–47	*G. molesta*	20
Tortri–6	*G. dimorpha*	13	Tortri–48	*G. molesta*	20
Tortri–7	*G. dimorpha*	13	Tortri–49	*G. molesta*	18
Tortri–8	*G. dimorpha*	15	Tortri–50	*G. molesta*	20
Tortri–9	*G. dimorpha*	13	Tortri–51	*G. molesta*	25
Tortri–10	*G. dimorpha*	16	Tortri–52	*G. molesta*	22
Tortri–11	*G. dimorpha*	15	Tortri–53	*G. molesta*	20
Tortri–12	*G. dimorpha*	13	Tortri–54	*G. molesta*	19
Tortri–13	*G. dimorpha*	16	Tortri–55	*G. molesta*	18
Tortri–14	*G. molesta*	20	Tortri–56	*G. molesta*	20
Tortri–15	*G. molesta*	20	Tortri–57	*G. molesta*	22
Tortri–16	*G. molesta*	20	Tortri–58	*G. molesta*	25
Tortri–17	*G. molesta*	25	Tortri–59	*G. molesta*	20
Tortri–18	*G. molesta*	18	Tortri–60	*G. molesta*	23
Tortri–19	*G. molesta*	21	Tortri–61	*G. molesta*	28
Tortri–20	*G. molesta*	23	Tortri–62	*G. molesta*	27
Tortri–21	*G. molesta*	22	Tortri–63	*G. molesta*	24
Tortri–22	*G. molesta*	20	Tortri–64	*G. molesta*	21
Tortri–23	*G. molesta*	20	Tortri–65	*G. molesta*	18
Tortri–24	*G. molesta*	21	Tortri–66	*G. molesta*	21
Tortri–25	*G. molesta*	22	Tortri–67	*G. molesta*	22
Tortri–26	*G. molesta*	21	Tortri–68	*G. molesta*	22
Tortri–27	*G. molesta*	25	Tortri–69	*G. molesta*	22
Tortri–28	*G. molesta*	22	Tortri–70	*G. molesta*	20
Tortri–29	*G. molesta*	24	Tortri–71	*G. molesta*	22
Tortri–30	*G. molesta*	24	Tortri–72	*G. molesta*	18
Tortri–31	*G. molesta*	26	Tortri–73	*G. molesta*	19
Tortri–32	*G. molesta*	21	Tortri–74	*G. molesta*	18
Tortri–33	*G. molesta*	23	Tortri–75	*G. molesta*	19
Tortri–34	*G. molesta*	24	Tortri–76	*G. molesta*	19
Tortri–35	*G. molesta*	23	Tortri–77	*G. molesta*	18
Tortri–36	*G. molesta*	19	Tortri–78	*G. molesta*	21
Tortri–37	*G. molesta*	24	Tortri–79	*G. molesta*	23
Tortri–38	*G. molesta*	24	Tortri–80	*G. molesta*	20
Tortri–39	*G. molesta*	18	Tortri–81	*G. molesta*	20
Tortri–40	*G. molesta*	24	Tortri–82	*G. molesta*	19
Tortri–41	*G. molesta*	23	Tortri–83	*G. molesta*	23
Tortri–42	*G. molesta*	24	Tortri–84	*G. molesta*	18

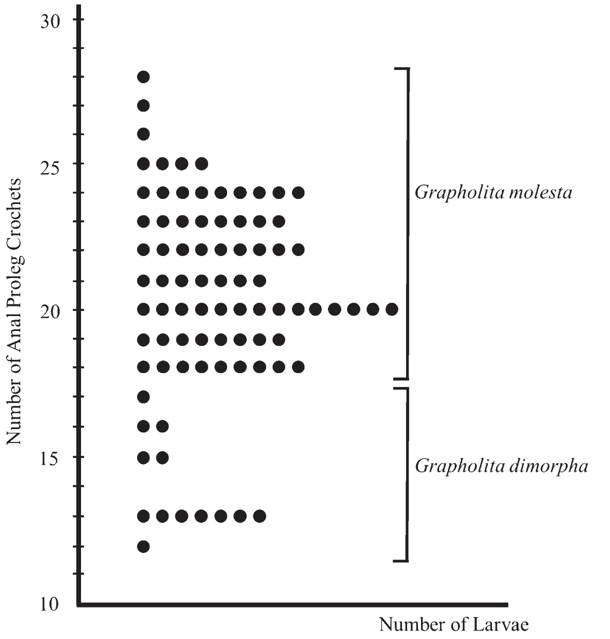

Fig. 4. Observed number of anal proleg crochets in *G. molesta* and *G. dimorpha* larvae.

the shape of the crochets of larvae of both species was semi–circular and uniordinal or biordinal (Fig. 3). Microscopic analysis showed that the number ranges of the anal proleg crochets for larvae of the two species were different (Table 1). Furthermore, width of the head capsule of examined larvae were shown as 1072.7± 143.2 μm (Data not shown). This range was almost matched with head capsule width of 5th instar of *G. molesta* (Yokoyama, 1987). Although all the examined larvae were equally 5th mature stage, the number ranges for the anal proleg crochets were 12–17 for *G. dimorpha* and 18–28 for *G. molesta* (Fig. 4). On the basis of the results of microscopic observation and molecular identification, different numbers of anal proleg crochets between *G. molesta* and *G. dimorpha* larvae can be considered a good diagnostic criterion for the identification of the two species.

The shape of the crochets on the prolegs of larvae is a species–specific morphological character (Nasu *et al.*, 2004; Matthews *et al.*, 1994). Moreover, this feature is used for the identification of larval species (Brown and Komai, 2008; Wearing *et al.*, 2001; Rentel, 2013). Nasu *et al.* (2004) reported that the number of anal proleg crochets in *Copromorpha kijimuna* (Copromorphidae) larvae was 11 or 12. The numbers of anal proleg crochets in *Exelastis cervinicolor* (Platyptiliinae) and *Exelastis pumilio* larvae were 4 and 11, respectively (Matthews *et al.*, 1994). Furthermore, cherry tree borer (*Synanthedon bicingulata* Staudinger, Lepidoptera: Sessidae) larvae, a major fruit pest in Korea, have approximately 11 anal proleg crochets (Lee *et al.*, 2004). *Cydia pomonella* (Lepidoptera; Tortricidae), a pest of sweet cherry, has 25–35 anal proleg crochets, whereas a closely related species, *Cydia splendana*, has 15–20 anal proleg crochets (Wearing *et al.*, 2001). Brown and Komai (2008) identified specimens as *C. splendana* if they had less than 18 anal proleg crochets and as *Cydia fagiglandana* if they had more than 18 anal proleg crochets, after taking body color and distance of the proleg on the 9th abdominal segment into account. Rentel (2013) reported that the number of anal proleg crochets was different for 6 agricultural pest species belonging to Tortricidae in South Africa: *Thaumatotibia leucotreta* (False codling moth), *C. pomonella* (Codling moth), *G. molesta* (Oriental fruit moth), *Thaumatotibia batrachopa* (Macadamia nut borer), *Cryptophlebia peltastica* (Litchi moth), and *Epichoristodes acerbella* (Pear leaf roller/Carnation worm). He also reported that the number of anal proleg crochets in *G. molesta* larvae was 19–25, similar to the findings of this study.

To date, the morphologies of *G. molesta* and *G. dimorpha* larvae, which are major apple pests in Korea, have not been differentiated (Komai, 1979). However, we found differences in the number of anal proleg crochets between *G. molesta* and *G. dimorpha* larvae by using a stereo microscope, optical microscope, and SEM. These results were confirmed using PCR–RFLP. Thus, the number of anal proleg crochets would be a good morphological criterion for the identification of *G. molesta* and *G. dimorpha* larvae. Further investigation should be performed by collecting the two species from different localities and hosts such as plum, pear, and peach.

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