Re-examination of morphological variations in the female internal genitalia of *Helicoverpa armigera* and *Helicoverpa zea* (Lepidoptera: Noctuidae) for identification and pest management

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Abstract

Two of the most serious agricultural pests, *Helicoverpa armigera* (Hübner) and *Helicoverpa zea* (Boddie) (both Lepidoptera: Noctuidae), are similar in their appearances. To distinguish them, morphological characters of female internal genitalia were re-examined. Specimens were collected from 7 regions in 17 countries. All specimens were identified in advance by molecular methods. Significant differences were observed in the length of the bursa copulatrix, ductus bursa, and pigmented area on the base of appendix bursa. *Helicoverpa armigera* had dense spicules on the luminal surface of the appendix bursa which were absent on the corresponding surface in *H. zea*. Additionally, differences of length on these parts in female genitalia will be helpful for identification. We are confident these morphological characters will advance resolution of noctuid speciation by taxonomists to properly identify these species based on distinguishing features of the female genitalia.

Key Words: morphological variations; noctuids; invasive pest; bursa copulatrix; biological invasion

Resumen

Dos de las plagas agrícolas más graves, *Helicoverpa armigera* (Hübner) y *Helicoverpa zea* (Boddie) (ambas Lepidoptera: Noctuidae), son similares en su apariencia. Para distinguirlas, se reexaminaron los caracteres morfológicos de las genitalia de las hembras. Se recolectaron muestras de 7 regiones (17 países). Todas las muestras se identificaron de antemano mediante métodos moleculares. Se observaron diferencias significativas en la longitud de la bursa copulatrix, ductus bursa y el área pigmentada en la base de la bursa del apéndice. *Helicoverpa armigera* tenía densas espículas en la superficie luminal de la bolsa del apéndice que estaban ausentes en la superficie correspondiente en *H. zea*. Además, las diferencias de longitud en estas partes de las genitalia de las hembras serán útiles para la identificación. Estamos seguros de que estos caracteres morfológicos avanzarán en la resolución de la especiación de noctuidos por parte de los taxónomos para identificar adecuadamente estas especies en base a las características distintivas de la genitalia de la hembra.

Palabras Clave: variaciones morfológicas; noctuidos; plaga invasora; bursa copulatrix; invasión biológica

The genus *Helicoverpa* (Lepidoptera: Noctuidae) contains several important agricultural pests, especially, *Helicoverpa armigera* (Hübner) and *Helicoverpa zea* (Boddie) are recognized as 2 of the most serious pest species within the genus. Both species severely damage a wide range of important crops (Fitt 1989; Cunningham & Zalucki 2014). In the past, both species were geographically isolated from each other. However, the invasion of *H. armigera* into South America has changed this situation. The first report of *H. armigera* in the New World came from Brazil in 2013 (Czepak et al. 2013), and thereafter, distribution of this species rapidly spread in South and Central America (SENAVE 2013; Tay et al. 2013; Murúa et al. 2014; NAPPO 2014; Gilligan et al. 2015, 2019; Castiglioni et al. 2016).

Rapid and proper identification of noctuids is critical for pest detection, monitoring, and timely management. However, similarity in appearance of these 2 species makes it difficult to distinguish them using external morphological characters. Recently, several molecular-based methods have been developed to distinguish these 2 species (Behere et al. 2008; Gilligan et al. 2015; Perera et al. 2015; Zink et al. 2017; Amano & Nomura 2020). Molecular-based identification has several advantages: the applicability for any life stage, the efficiency for multiple and bulk samples, and the uniformity of techniques. However, these methods usually need expensive equipment and reagents, and sometimes can be more time consuming than morphological identification by experienced entomologists. Therefore, it is necessary to use molecular and morphological approaches to speed up the proper identification and diagnosis of these species. In terms of morphological identification, comprehensive keys on the genus *Helicoverpa* based on male and female internal genitalia were assembled by Hardwick (1965). Detailed morphological differences in male genitalia were provided by Pogue (2004). Morphological features in female genitalia were investigated by Yoshida and Takahashi (2005). *Helicoverpa armigera* samples used in their study were restricted to specimens from Japan (Yoshida & Takahashi 2005). In more recent studies, Perini et al. (2019) provided a morphological key to identification

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of *Helicoverpa* species based on male prothoracic leg characters, but these characters are not applicable to female moths.

In this study, we re-examined the morphological characters of female internal genitalia to identify *H. zea* and *H. armigera* using specimens collected from 7 regions and 17 countries. All samples were identified using molecular-based methods as a cross-check.

Materials and Methods

INSECT COLLECTIONS

Pinned dry specimens of adult individuals were obtained from the Japanese Plant Protection Station, Yokohama, Kanagawa, Japan (Table 1), and identified by macroscopic features and molecular-based methods.

MOLECULAR-BASED IDENTIFICATION

Multiplex Polymerase Chain Reaction (PCR) was employed as a molecular-based identification method with a specific primer set provided by Perera et al. (2015). DNA extraction and PCR assay were performed with the same procedure described in Amano and Nomura (2020).

MORPHOLOGICAL STUDIES

The abdomen was removed from each dry specimen and treated with 10% KOH at 80 °C for 30 min, then the genitalia was dissected. Observation of genitalia was carried out under a stereomicroscope system SZX 16 (Olympus, Tokyo, Japan). The length of bursa copulatrix, ductus bursa, and pigmented area on the base of appendix bursa (Fig. 1) were measured under the stereomicroscope with an ocular micrometer U-OCMC 10/100XY (Olympus, Tokyo, Japan). In this study, the length of bursa copulatrix was defined as the length between the apex of ostium bursa and apex of appendix bursa, and bursa copulatrix was straightened for measurement using tweezers without stretching. The length of ductus bursa and pigmented area were measured according to Yoshida and Takahashi (2005).

The texture of the appendix bursa was observed under the stereomicroscope system. To confirm the dense spicules on the luminal surface, slides were examined using a biological microscope system BX 53 (Olympus, Tokyo, Japan). A small piece of tissue was cut off from the appendix bursa of *H. armigera* with a scalpel, and the slice was mounted on the slide with Hoyer's Medium (Anderson 1954) prepared in our laboratory.

Table 1. Details of specimens used in this study.

Species	Region	Country	Quantity
Helicoverpa armigera	Africa	Kenya	7
		South Africa	4
	Asia	India	8
		Israel	4
		Japan	5
		Korea	3
		Malaysia	3
		Philippine	3
		Taiwan	1
		Thailand	7
	Europe	Italy	3
	Middle East	Oman	4
	Oceania	Australia	40
	South America	Peru	7
		Colombia	1
Helicoverpa zea	North America	Mexico	67
		USA	28
	South America	Peru	5

Results

The lengths of bursa copulatrix, ductus bursa, and pigmented area were significantly longer in *H. zea* than in *H. armigera*. Overlap was not found (Table 2). The mean (± SD) measurements of bursa copulatrix, ductus bursa, and pigmented area were, respectively, 11.8 ± 0.6 mm, 1.7 ± 0.14 mm, and 2.0 ± 0.17 mm in *H. zea*, whereas the measurements were 8.7 ± 0.5 mm, 1.0 ± 0.12 mm, and 1.1 ± 0.18 mm in *H. armigera* (Table 2).

Figure 1B, E shows the outward appearances of the appendix bursa. A punctation-like texture was observed in all specimens of *H. armigera* but was absent in *H. zea* (Fig. 1B, E). Figure 1C shows the luminal surface texture on the appendix bursa of *H. armigera*. It indicates that dense spicules on the luminal surface look like punctations under stereomicroscopic observation (Fig. 1B, C).

Discussion

The bursa copulatrix was significantly longer in *H. zea* than in *H. armigera.* This result seems to be consistent with the difference in the male aedeagus because the bursa copulatrix receives the aedeagus and inflated vesica (sensu Pogue 2004; the 'extension' of Mallet 2018) during copulation (Callahan 1958; Mallet 2018), and the inflated vesica also is longer in *H. zea* than in *H. armigera* (Pogue 2004).

As in the previous study of Yoshida and Takahashi (2005), lengths of both ductus bursa and pigmented area also were significantly longer in *H. zea* than in *H. armigera*. However, these 2 characters are difficult to use because the observed length of the ductus bursa depends on the observation angle, and measuring the pigmented area sometimes is difficult due to its curved shape. Moreover, overlaps were observed in both lengths of the ductus bursa and in the pigmented areas of the 2 species in the previous study (Yoshida & Takahashi 2005).

In this study, we immersed abdomens of specimens in 10% KOH at high temperature to dissect genitalia from dried specimens. Use of hot or boiling caustic is a common procedure in preparation of slide mounts of small insects such as mealybugs or aphids (Williams & de Willink 1992; Blackman & Eastop 2000). If the specimens are treated under the same procedure, it appears that these specimens are usable for morphological comparison. On the other hand, Hardwick (1950) mentioned that the use of cold caustic generally gave more uniform results than short immersion in hot or boiling caustic. Thus, the influence of artifacts from hot caustics on the genitalia should be clarified by further studies.

Hardwick (1965) described the difference in the luminal surface texture of appendix bursa. In the same way, dense spicules on the luminal surface were observed in *H. armigera* but were absent in *H. zea*. Although it needs high magnification to observe the spicules, this feature is observed easily under relatively low magnification as a punctated texture.

When there are only a few samples to be identified, morphological identification is faster and easier than the molecular-based method. After 30 min of KOH treatment and dissection, careful observation of the appendix bursa is performed. If dense spicules are absent on the appendix bursa, the specimen is identified as *H. zea*. If dense spicules are observed on the appendix bursa, the specimen is identified as *H.*

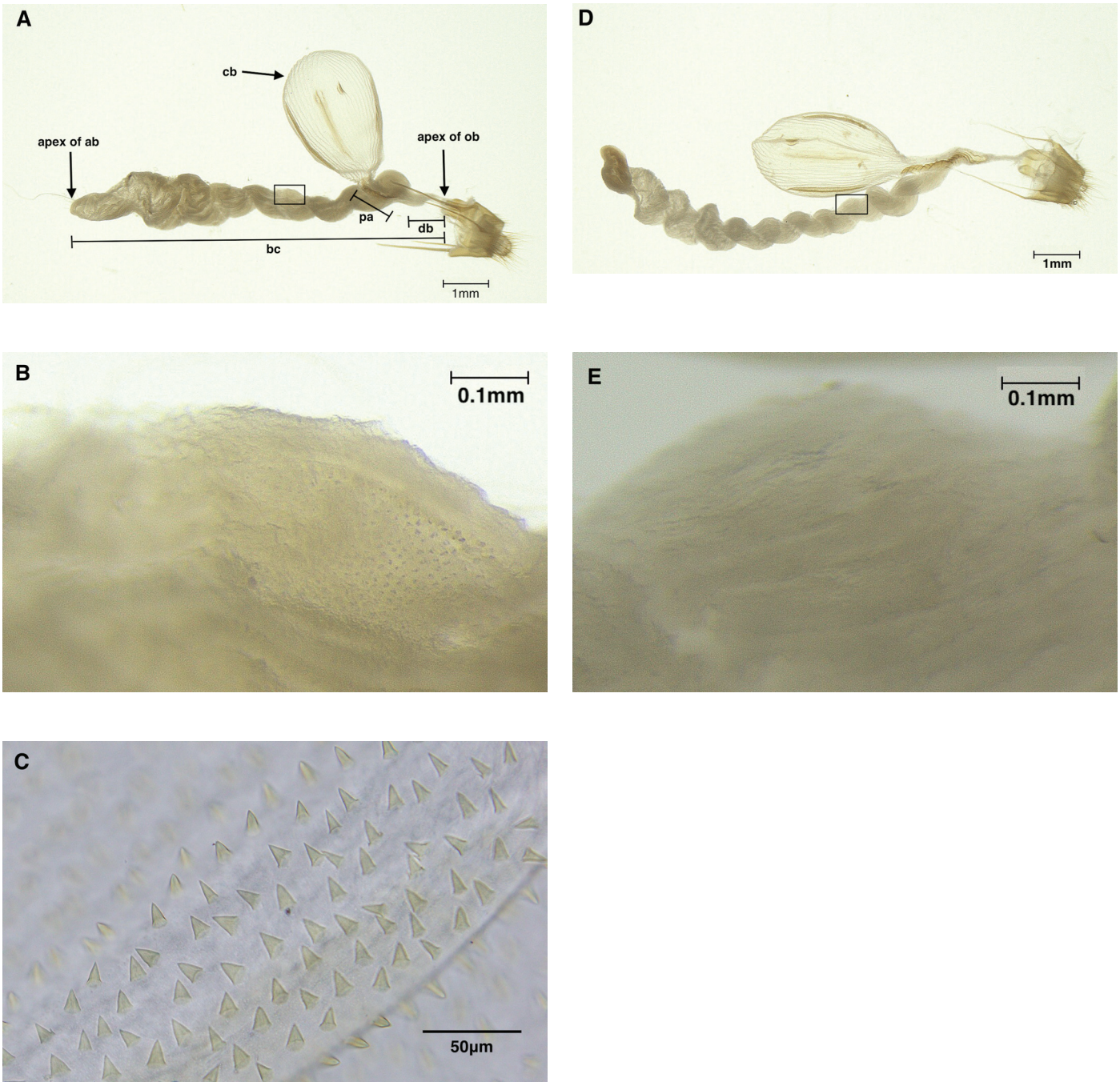

Fig. 1. (A) Female genitalia of *Helicoverpa armigera*. (B) Texture on appendix bursa of *H. armigera*. (C) Luminal surface on appendix bursa of *H. armigera*. (D) Female genitalia of *Helicoverpa zea*. (E) Texture on appendix bursa of *H. zea*. (ab) appendix bursa; (bc) bursa copulatrix; (cb) corpus bursa; (db) ductus bursa; (ob) ostium bursa; (pa) pigmented area.

Table 2. Comparison of the characteristics between *Helicoverpa armigera* and *Helicoverpa zea*.

	H. armigera (*n* = 100)		*H. zea* (*n* = 100)		*t*-test
	Mean ± SD	Range	Mean ± SD	Range	(*P* value)
Length of bursa copulatrix (mm)	8.7 ± 0.5	7.5–9.8	11.8 ± 0.6	11.0–13.1	$P < 0.0001$
Length of dactus bursa (mm)	1.0 ± 0.12	0.7–1.3	1.7 ± 0.14	1.5–2.0	$P < 0.0001$
Length of pigmented area (mm)	1.1 ± 0.18	0.8–1.5	2.0 ± 0.17	1.7–2.4	$P < 0.0001$

Student's *t*-test was used to compare each feature of *H. armigera* and *H. zea*.

armigera. Moreover, measurement of the bursa copulatrix also can be helpful for identification.

We believe that the morphological characters re-examined in the female internal genitalia could be useful to distinguish *H. armigera* from *H. zea*. We are confident that findings of this study will contribute toward the detection, monitoring, and management of these invasive pests.

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