Identification of the Larvae of *Helicoverpa assulta* and *H. armigera* (Lepidoptera: Noctuidae) Through Morphological Difference and DNA Barcoding

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ABSTRACT: In present study, the real biological pictures, vivid and straightforward, were provided of the larval head and skin of *Helicoverpa assulta* and *H. armigera*. These visual pictures can be as the necessary and useful supplement on certain characters of these two larvae. In addition, we tested and verified the morphological result by DNA barcode based on our preliminary studies. Furthermore, we also compared and discussed the advantages and disadvantages between morphological and molecular data.

KEYWORDS: *Helicoverpa assulta*; *H. Armigera*; Morphology; DNA barcoding.

1 INTRODUCTION

The Helicoverpa is a genus of about 18 species of noctuid moths (Fitt, 1989). Most of members in this genus are important, cosmopolitan agricultural lepidopteran pest species, of which two closely related sister species, the oriental tobacco budworm, *Helicoverpa assulta*, and the cotton bollworm, *H. armigera*, are sympatric and damaging crop pests in China and many other countries (Fitt, 1989; Wang & Dong, 2000; Jin et al., 2001; Zhao et al., 2006; Teng et al., 2009). These two closely related sister species are very difficult to identify with certainty and mixed populations are often been come across on tobacco and on wild hosts in the family Solanaceae, especially at the larvae stage (Wang & Dong, 2000; Jin et al., 2001; Gao & Wang, 2005; Xin et al., 2005; Zhang, 2007; Behere et al., 2008; Li et al., 2011). *H. assulta* has a more restricted geographic distribution or host range than *H. armigera*. *H. armigera* is highly polyphagous and characteristically of the widest geographical distribution in the genus.

Many previous studies about the morphological differences between the larvae of *H. assulta* and *H. armigera* have been reported. However, in these studies, all they have done was just schematic diagram, not real biological picture (Wan & Wan, 1982; Huang et al., 1984). Prompt and accurate species identification with important roles within agricultural, medical, ecological and economic settings is critical in forming the basis for appropriate control and eradication measures, framing the correct response to any incursion (Li et al., 2010; Li et al., 2011). Therefore, we carried out this research work during 2008-2013 and focused on identifying the two sibling species larvae to gain some important supplement characters of these two larvae by Nikon SMZ1500 anatomical lens.
addition, we tested and verified the morphological result by DNA barcode based on our preliminary studies (Li et al., 2010; Li et al., 2011; Duan et al., 2013). Furthermore, we also compared and discussed the advantages and disadvantages between morphological and molecular data.

2 MATERIALS AND METHODS

2.1 Materials

A large proportion of the samples were obtained from tobacco and hot pepper fields as larvae in various localities of Yunnan province of China during the autumn of from June 2008 and September 2013 (Table 1). After roughly species detection, the larvae were directly into absolute ethanol and maintained at -20°C in the genetic lab of Life Science College of Southwest Forestry University University until test and DNA extraction. A total of 92 larvae of *H. assulta* and 96 larvae of *H. armigera* were used in this experiment. Their collection locations are listed in Table 1.

Table 1. Samples locations, number of samples, the number of samples tested by DNA barcoding and the rate of accuracy of two species considered in this study.

<table>
<thead>
<tr>
<th>Species</th>
<th>Location</th>
<th>No. of samples</th>
<th>No. of samples tested by DNA barcoding</th>
<th>Accuracy (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>H. assulta</em></td>
<td>Kunming</td>
<td>23</td>
<td>23</td>
<td>100.0</td>
</tr>
<tr>
<td></td>
<td>Dali</td>
<td>18</td>
<td>18</td>
<td>100.0</td>
</tr>
<tr>
<td></td>
<td>Qujing</td>
<td>30</td>
<td>29</td>
<td>96.7</td>
</tr>
<tr>
<td></td>
<td>Zhaotong</td>
<td>21</td>
<td>19</td>
<td>90.5</td>
</tr>
<tr>
<td><em>H. armigera</em></td>
<td>Kunming</td>
<td>30</td>
<td>29</td>
<td>96.7</td>
</tr>
<tr>
<td></td>
<td>Dali</td>
<td>24</td>
<td>22</td>
<td>91.7</td>
</tr>
<tr>
<td></td>
<td>Qujing</td>
<td>20</td>
<td>20</td>
<td>100.0</td>
</tr>
<tr>
<td></td>
<td>Zhaotong</td>
<td>22</td>
<td>21</td>
<td>95.5</td>
</tr>
</tbody>
</table>

2.2 Morphological test

Nikon SMZ1500 anatomical lens was used to observe and take pictures for all the samples.

2.3 Molecular data

2.3.1 DNA extraction

To avoid contamination by fungi and nematodes, after removing the abdomen, elytras and antennae, there are only left head, thorax and leg to further test. Total genomic DNA extractions of insect specimens were carried out using the standard phenol/chloroform protocols or using the DNA Mini Kit (Watson Biotechnologies, Shanghai), according to manufacturer’s instructions.

2.3.2 PCR amplification and sequencing

COI primers is the primers that once used by Hebert et al. (2004), namely, COIF: 5’ –ATT CAA CCA ATC ATA AAG ATA TTG G-3’ and COIR: 5’- TAA ACT TCT GGA TGT CCA AAA AAT CA-3’. PCR amplification was performed in a volume of 50 ll, which contains 10–25 ng of genomic DNA, 19 PCR buffer, 2.5 mM MgCl2, 1 mM mixed dNTP, 2 lg/ll BSA, 2 pM of each primer, 1 U Taq polymerase. The thermal profile included a 95 °C initial denaturing phase for 3 min, followed by 35 cycles of 94°C for 1 min, 50°C for 1 min and 72°C for 1 min, and 10 min extension at 72°C after the last cycle. According to the manufacturer recommended conditions, Sequencing using the BigDye Terminator kit (V2.0) on ABI 377 automatic sequencer (Applied Biosystems) by electrophoresis sequencing, and taken as forward and Reverse sequencing automatically. DNA sequences of CO1 coding region was edited and aligned using DNASTAR (DNASTAR Inc.), and further checked visually. Electrophoretogram analyzed by sequencer using DNASTAR Seqman combination of forward and Reverse chain stitching work using Clustal W (Thompson et al., 1994). Sequence variation analysis with MEGA 4.0 (Tamura et al., 2007).

3 RESULTS

3.1 Morphological differences

Larval head of *H. assulta* and *H. armigera* are shown as Figure 1. The small thorns on the outermost layer of the skin shown as Figure 2. Larvae samples of *H. assulta* in Figure 1 and Figure 2 collected from Dongtun village in Songming County of Kunming, Yunnan province On August 24, 2008. For *H. armigera*, the larvae samples were collected from West Kawamura of Xinjie town in Midu County of Dali on On August 2, 2009.

Figure 1. The side view of larval prothorax. The red circle shows the the relationship between line of two prothorax hairs and stigma. A: stigma; B: The line of two prothorax hairs. The line of two prothorax hairs is far away from the stigma in *H. assulta* (left). The line of two prothorax hairs intersects or tangent to the stigma in *H. armigera* (right).
3.2 Molecular identification

92 Larvae samples of *H. assulta* and 96 for *H. armigera* made a good PCR amplification, and a 658 bp fragment of COI was amplified. Based on our published paper and patent, the results and the accuracy after verification by DNA barcode are shown in Table 1.

4 DISCUSSION

Tobacco is one of the important pillars of finance in our country. *H. assulta* and *H. armigera* is one of the important pests on tobacco production. As two closely related sister species, the identification has been a difficult technical bottleneck in *H. assulta* and *H. armigera* research (Li et al., 2011). To distinguish the larvae of *H. assulta* and *H. armigera*, predecessors mainly according to either the ligature of the first two root hair base at larva’s prothoracic spiracles or spiracle are tangent. In fact, *H. assulta* is tangent, but *H. armigera* is not. The description of distinction as shown in the Table 2, Fig. 3 and Fig. 4 (Wan & Wan, 1982; Huang et al., 1984; Jin et al., 2001; Zhang, 2007). But reports of both at home and abroad, all images are simulated Figure, rare pictures (Jin et al., 2001; Zhang, 2007). The images of larvae that we provide in this paper, both the physical image (Fig. 1 and Fig. 2), visual image, and the color is rich, all of them has a strong visual impact, and given the sufficient and necessary complement for some important morphological structure of the larvae.

<table>
<thead>
<tr>
<th>Origin and varieties</th>
<th><em>H. assulta</em></th>
<th><em>H. armigera</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Whether the ligature of the two root hair at the side view of larval prothorax through the spiracles</td>
<td>Away from the spiracles’s offline</td>
<td>Through or With the spiracles’s offline tangent</td>
</tr>
<tr>
<td>Shape of the small thorns on the outermost layer of the skin</td>
<td>Conical</td>
<td>Long and sharp</td>
</tr>
</tbody>
</table>

In addition, some researchers also refer to the feeding preferences and host range of larvae to distinguish (Powell et al., 1998; Tang et al., 2006). *H. armigera* is polyphagous pest, they can harm more than 30 families and 200 many kinds of plants, such as malvaceae, solanaceae, leguminosae. *H. assulta* is a typical oligophagous insects, they main cause harm to solanaceae plant such as pepper. However. But both of they often appeared at the same time on tobacco (Tang et al., 2006; Zhao et al., 2006). Therefore, the host range and feeding preferences is helpless, this is confusing to distinguish them.

DNA barcode technology has opened up a new train of thought for the identification of closely related species (Li et al., 2010). With refer to our published articles and patents, all the larvae samples of *H. assulta* and *H. armigera* have identified through DNA barcode in this paper (Li et al., 2011; Duan et al., 2013). The results show that the number of *H. assulta* larvae which identified by morphological characteristics is 92, but the identification results by the DNA barcode show that the number is 89, with 97% accuracy; the number of *H. armigera* larvae which identified by morphological characteristics is 96, but the identification results by the DNA barcode show that the number is 92, with 96% accuracy. Compared the data from Morphological identification and the data from DNA barcode molecules, we have found their self-agreement were higher than 95%. It also means that, the validation and support from molecular data is also very important. Therefore, integrated a variety of different sources of data, such as morphological and molecular data, even Ecological data, and let them analysis together, complement each other, and work together, this will make the identification of the two related species, *H. assulta* and *H. armigera* more scientific and accurate.
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