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Morphological and Molecular Characterization of the Eggs of Some Noctuid Species Associated With Soybean in Brazil

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ABSTRACT The most important species of Lepidoptera that attack soybean, in descending order of importance, are *Chrysodeixis includens* (Walker), *Anticarsia gemmatalis* Hübner, *Spodoptera cosmioides* (Walker), *Spodoptera eridania* (Stoll), and *Spodoptera albula* (Walker). In addition, *Rachiplusia nu* (Guenée) is one of the most common Plusiinae in the southern region of Brazil, encompassing the states of Paraná, Santa Catarina, and Rio Grande do Sul. The correct identification of these species is essential for choosing the appropriate control measures, as they differ in their susceptibility to insecticides and biological control agents. To distinguish Lepidoptera species commonly found in Brazil, the eggshells of all these species were morphologically characterized by scanning electron microscopy. Diagnostic characters for differentiating species include shape of rosette petals, number of primary cells, number of secondary cells, number of ribs, and rosette diameter. Also, a molecular diagnostic method using polymerase chain reaction and restriction fragment length polymorphism (PCR–RFLP) analysis was developed to distinguish noctuid species commonly found in Brazil. A 658-bp region of the mitochondrial DNA cytochrome oxidase subunit I gene was amplified using PCR and then sequenced. The five Lepidoptera species were distinguished by restriction enzymes Bpm I and MboI. RFLPs produced by Bpm I endonuclease were useful to discriminate species from within *Spodoptera* (*S. cosmioides*, *S. eridania*, and *S. albula*) and *R. nu* from *C. includens*. However, Bpm I did not digest the amplicons from *S. eridania* and *C. includens*, which were discriminated by RFLP patterns produced by the restriction enzyme MboI. PCR–RFLP can be performed in a short period, and it is useful to distinguish the most important Brazilian Lepidoptera soybean pests.

KEY WORDS *Chrysodeixis includens*, *Rachiplusia nu*, *Spodoptera cosmioides*, *Spodoptera eridania*, *Spodoptera albula*

The most important species of Lepidoptera that attack soybean, in descending order of importance, are *Chrysodeixis includens* (Walker), *Anticarsia gemmatalis* Hübner, *Spodoptera cosmioides* (Walker), *Spodoptera eridania* (Stoll), and *Spodoptera albula* (Walker). In addition, *Rachiplusia nu* (Guenée) is one of the most common Plusiinae in the southern region of Brazil, encompassing the states of Paraná, Santa Catarina, and Rio Grande do Sul (Sosa-Gómez et al. 2010). The correct identification of these species is essential for choosing the appropriate control measures, as they

differ in their susceptibility to insecticides and biological control agents (Braga et al. 2011).

The identification of these species may be difficult under certain circumstances, such as at the egg stage, as small larvae, or when adult wings have no scales, especially when they are very close morphologically, such as in the cases of *S. albula* and *S. eridania* or *C. includens* and *R. nu*. However, adult specimens can be recognized, with some expertise, through the dissection of their genitalia, following the method described by Pogue (2002) and Eichlin and Cunningham (1978).

To the best of our knowledge, the eggs of just a few Lepidoptera species associated with soybean have already been described in detail. The first studies briefly described the eggs of *A. gemmatalis*, *Agrotis ipsilon* (Hufnagel), *Heliothis virescens* (F.), *S. albula* [mentioned as *Prodenia sunia* (Guenée)], *Spodoptera frugiperda* (J. E. Smith), and *C. includens*, and their descriptions did not allow differentiation among species (Peterson 1961, 1964). Cónsoli et al. (1999) described and illustrated the microstructure of the eggs of *A.*

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gemmatalis, *H. virescens*, and *S. frugiperda*. Furthermore, the morphological characterization of the eggs of *S. eridania* was performed by Valverde (2007). However, when morphological identification is impossible, molecular diagnostic protocols have been recommended to distinguish among species (Lewter and Szalanski 2007). Currently, DNA extraction can be performed within a few minutes, followed by amplification and restriction analysis to obtain diagnostic DNA profiles in one or two working days. The purpose of this study was to distinguish the eggs of the most important soybean Lepidoptera pests in Brazil, using both morphological and molecular techniques. Identification of eggs among species will distinguish them so that chemical and biological control strategies can be implemented.

Materials and Methods

Insects. Specimens of *C. includens* were collected in Rondonópolis, MT (16° 28' 15" S, 54° 38' 15" W), during the 2008 and 2009 soybean growing season; *R. nu* was collected in Vacaria, RS (28° 30' 44" S, 50° 56' 02" W), and in Monte Redondo, Tucumán, Argentina (27° 48' 2" S, 65° 26' 08" W), from soybean plants; and *S. albula* was collected in Jaboticabal, SP (21° 15' 22" S, 48° 18' 58" W). *S. eridania* and *S. cosmioides* adult specimens and eggs were obtained from larvae reared in the laboratory of Embrapa Soja in Londrina, PR, Brazil. Field populations of *S. cosmioides* were collected in Pelotas, RS (31° 0' 7" S, 52° 26' 09" W), from soybean and *Ricinus communis* L. plants; *S. eridania* was collected in Santa Helena de Goiás, GO (17° 50' 11.5" S, 49° 39' 12" W), from cotton plants; and *A. gemmatalis* in Sertanópolis, PR (23° 03' 44" S, 51° 00' 50" W), from soybean plants. The identification of the species was confirmed in adult specimens based on their genitalia morphology according to Eichlin and Cunningham (1978), Pogue (2002), and Barbut (2008).

Morphological Study. Adults were fed a 10% honey solution. Eggs from each species ($n = 30$) were dipped in KAAD solution (7.1% kerosene, 14.3% glacial acetic acid, 71.5% ethanol, and 7.1% dioxane) and kept at -20°C for 24 h. After this, the eggs were transferred to 70% ethanol; the silk at the top of the egg was removed, and they were cut above the equatorial line with a surgical scalpel. The top of the eggs was embedded in Entellan (Merck KGaA, Darmstadt, Germany) and mounted on microscope slides. The characteristics of the eggs were observed under a phase contrast microscope (400×) (Olympus BX50 F-3, Tokyo, Japan). The eggs were photographed using a digital camera Moticam 2300 3.0M (Motic China Group Co. Ltd., Xiamen, China) to observe possible specific morphological differences, and the measures of the eggs were performed using the Motic Image version 2.0 software (Motic China Group Co. Ltd.). The morphology of the eggshells as well as the silk covering them were characterized using a scanning electron microscope (SEM) (FEI Quanta 200 FEG FEI Co., Eindhoven, The Netherlands) and gold coating. Features such as egg shape, diameter, height,

Table 1. Measurements (micrometers) of surface structures of the eggshell of two Plusiinae species; mean (range) of 30 observations

Surface structure	<i>C. includens</i>	<i>R. nu</i>
Egg ht	301 (274–340)	324 (300–350)
Egg diam	530.1 (490–544)	560.0 (544–620)
No. primary cells	8.4 (6–10)	7.7 (6–9)
No. secondary cells	12.2 (8–15)	12.1 (11–13)
No. micropyles	3.9 (3–6)	3.7 (3–4)
No. ribs	33.8 (32–37)	36.2 (33–41)
Rosette diam	36.7 (30.4–41.9)	43.2 (34.8–51.0)

micropylar area, number of micropyles, number of primary cells, and number of ribs were observed. Measurements are given in μm as means and standard error of the mean. Voucher specimens were deposited at the Coleção de Insetos, Embrapa Soja.

Molecular Study. Two methods were used for DNA extraction—the CTAB protocol for moth legs (Rogers and Bendich 1988) and the Chelex 100 method for individual eggs (Hoy 1994).

Samples of all the legs of 10–29 adult individuals from each species were submitted to sequencing analysis, and polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) was performed in at least 10 adult and 5 egg samples. DNA was amplified successfully, including the DNA extracted from single eggs, using the Chelex 100 protocol (Hoy 1994). PCR was performed using primers LCO 1490-J-1514 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO 2198-N-2175 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') (Integrated DNA Technology, Inc., Coralville, IA), designed by Folmer et al. (1994) and described by Simon et al. (1994). The expected size of the product was 680 bp, corresponding to the initial portion of the cytochrome oxidase subunit 1 (COI) gene. The PCR reaction had a total volume of 25 μl , containing 10 ng of noctuid DNA and 10 μM of each primer. The PCR reaction was performed using a PTC 200 thermal cycler (MJ Research Inc., Watertown, MA), programmed for 34 cycles of 94°C for 1 min, 48°C for 1 min, and 72°C for 1.5 min, with a final extension at 72°C for 5 min. PCR amplicons were separated by electrophoresis on a 1.5% agarose gel run at 120 V for 2.5 h in TBE buffer. Photos are images of ethidium bromide-treated gels. PCR amplification products were cloned directly into the TOPO vector system (Invitrogen, Carlsbad, CA) using the manufacturer's protocol. Both strands of positive clones were sequenced using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). Chromatograms were visualized and edited using Sequencher 4.1.4 software (Gene Codes Corporation, Ann Arbor, MI; <http://www.genecodes.com>) to measure the reliability of the data, and good quality sequences were used to build a consensus sequence for each sample. Consensus sequences of the COI partial gene were multiple aligned using ClustalW (Thompson et al. 1994) with default parameters. The biological sequence alignment editor Bioedit (Ibis Biosciences, Carlsbad,

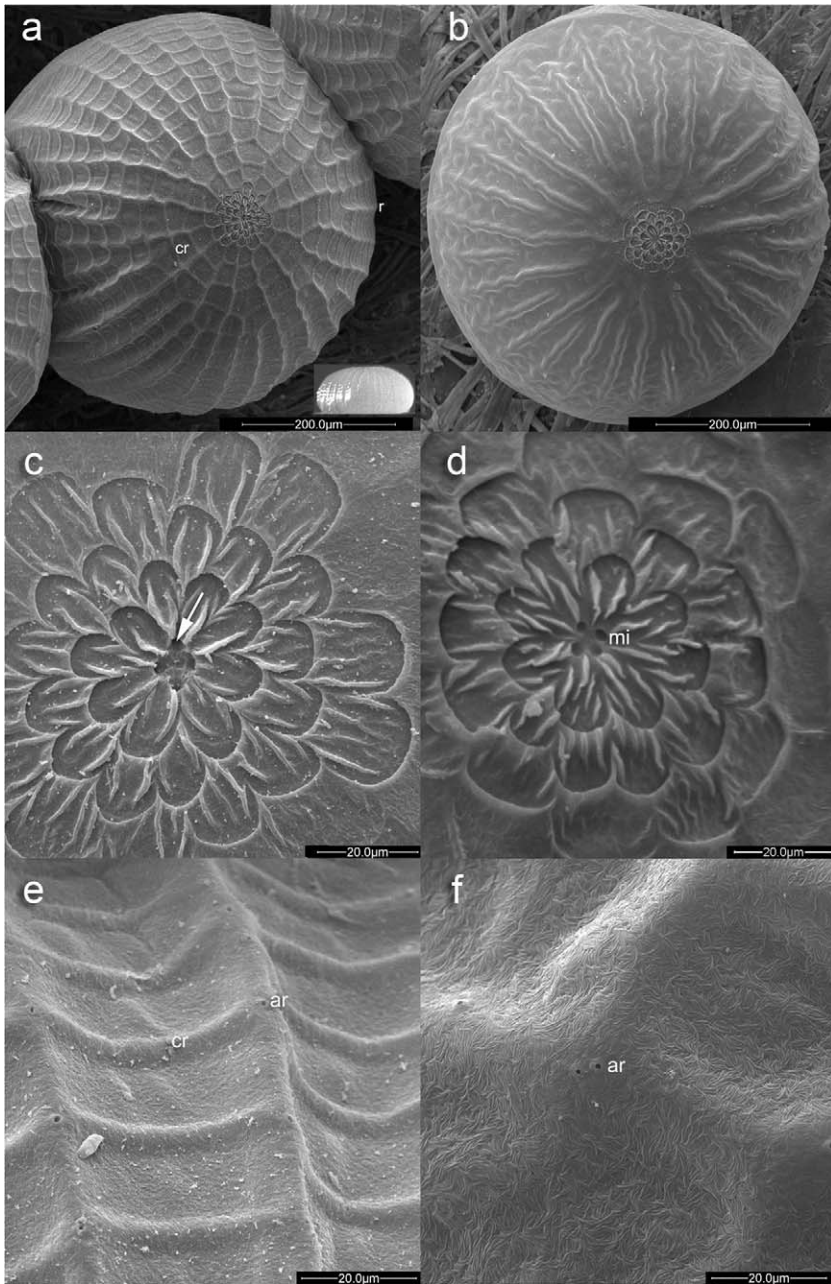


Fig. 1. (a) Eggshell surface topography of *C. includens* (inset, lateral view). (b) Eggshell surface topography of *R. nu.* (c) Micropylar rosette of *C. includens* (arrow, micropyle). (d) Micropylar rosette of *R. nu.* (e) Surface fine texture of *C. includens*. (f) Surface fine texture of *R. nu.* r, ribs; cr, crossribs; mi, micropyle; ar, aeropyle.

CA; <http://www.mbio.ncsu.edu/bioedit/bioedit.html>) was used to identify restriction sites. PCR amplification products of DNA from eggs and legs were digested at 30°C for 2 h with restriction enzyme Bpm I (Fermentas International Inc., Glen Burnie, MD) and at 37°C for 3 h with restriction enzyme MboI (Invitrogen, Life Technologies Corporation, Grand Island, NY), following the manufacturers' recommendations. Fragments were visualized using elec-

trophoresis on a 2% agarose gel stained with ethidium bromide and a 100-base pair (bp) DNA ladder (GeneRuler, Fermentas International Inc., Glen Burnie, MD). The agarose gels were photographed using a transilluminator (L-PIX, Loccus Biotecnologia, Cotia, SP, Brazil). Nucleotide sequences were submitted to the National Center for Biotechnology Information (NCBI; GenBank KC354733.1-KC354738.1 and KF261121-KF261200).

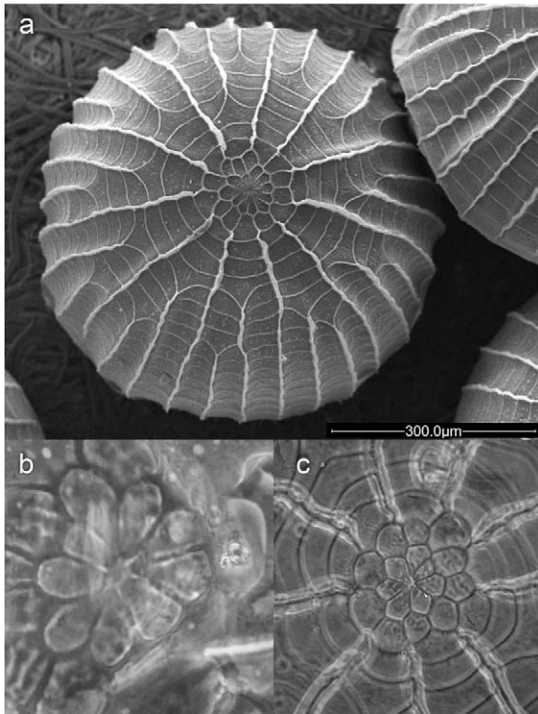


Fig. 2. (a) Eggshell surface topography of *A. gemmatalis*. (b) and (c) Micropylar rosette of *A. gemmatalis*.

Results

C. includens and *R. nu* frequently lay a single egg on a plant, but they can also lay them in small groups

of two to six. In both species, the color of the eggs ranges from pale yellow to cream. *R. nu* lays slightly larger eggs than *C. includens* in diameter as well as in height, and the primary cells forming the micropylar rosette in the former are fused (Olivares et al. 2005). Furthermore, the eggs of *R. nu* generally have bigger rosette diameters and numbers of radial ribs (Table 1). In both species, the micropylar region can be depressed, and its deep part is the outer border of the petals that constitute the rosette.

The eggs of *C. includens* are hemispherical in shape, with a slightly flattened top and a flattened base (Fig. 1a, inset). The micropylar rosette is composed of 6–10 petals (Fig. 1c), surrounded by two concentric rosettes, with gradually bigger petals on the outside border (Fig. 1c); the outer border of the petals in the third rosette is not always obvious. The flanks of the eggs have conspicuous ribs and cross-ribs (Fig. 1a and e). Aeropyles are present on the conjunction points of the ribs and cross-ribs, and not rarely, they can be found in pairs (Fig. 1e).

The eggs of *R. nu* sometimes have flattened hemispheres at both poles (Fig. 1b). The micropylar rosette is similar to *C. includens*, but the lateral ribs of the eggs are not as conspicuous as in *C. includens*, and their edges are usually smooth (Fig. 1b). Aeropyles are present on the conjunction points of the inconspicuous ribs and cross-ribs (Fig. 1f).

The eggs of *A. gemmatalis* have very conspicuous ribs (Fig. 2a) that are evident under optical microscopy (Fig. 2c). Cõnsoli et al. (1999) described these ribs as 9–10 well-defined ridges that reach the cells surrounding the micropyle.

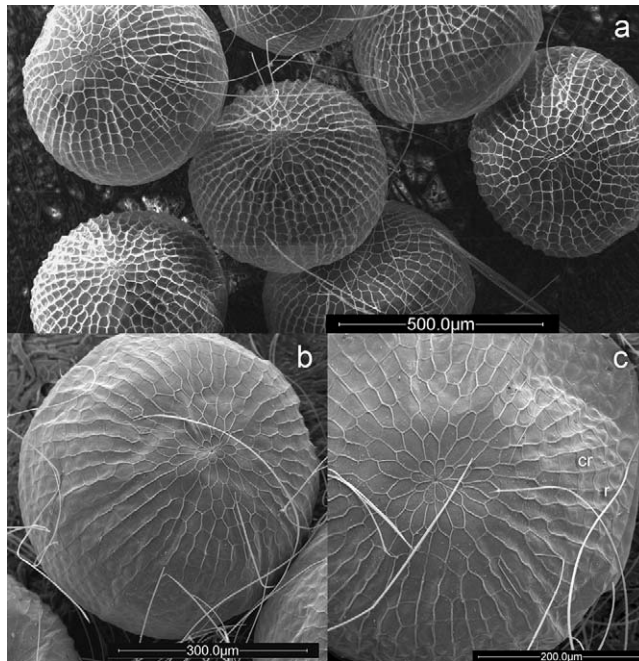


Fig. 3. (a) Eggshell surface topography of *S. albula*. (b) Eggshell surface topography of *S. eridania* and micropylar rosette. (c) Eggshell surface topography of *S. cosmioides* and hair silk on the egg surface. R, ribs; cr, crossribs; mi, micropyle.

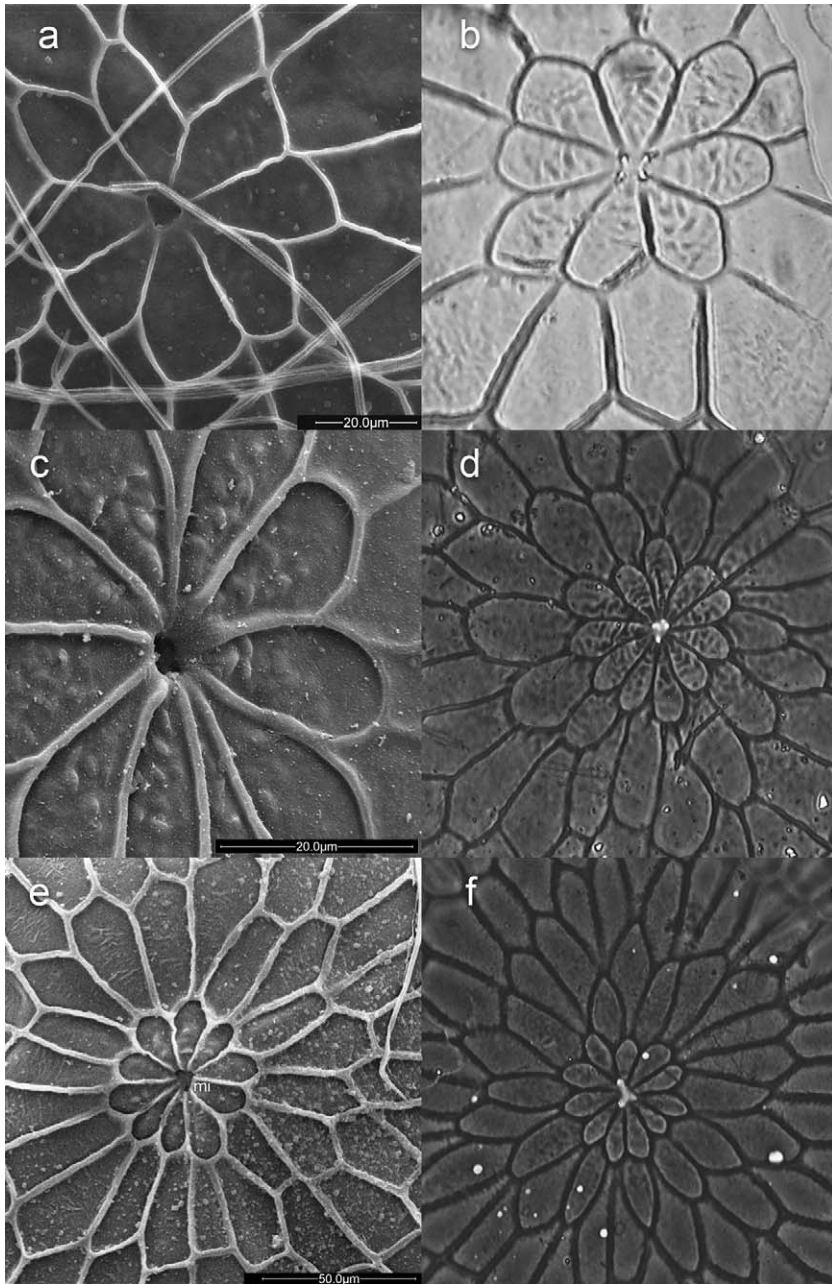


Fig. 4. Micropylar rosette of (a, b) *S. albula*, (c, d) *S. eridania*, and (e, f) *S. cosmioides*.

Spodoptera species lay egg masses, usually in layers, covered by abundant silk, normally spherical in shape and flattened at the base. Radial and cross ribs are conspicuous, and primary cells are not fused.

The egg masses of *S. albula* are pale green with gray hues and laid in one, two, or eventually three layers. They are usually spherical in shape and flattened at the base. In this study, the mean diameter of *S. albula* eggs was 505.4 μm (475–540) and the mean height was 411.1 μm (357–477). The mean numbers of ribs and micropyles were 52.3 (46–57) and 3.5 (3–4), respec-

Table 2. Measurements (micrometers) of surface structures of the eggshell of three *Spodoptera* species; mean (range) of 30 observations

Surface structure	<i>S. albula</i>	<i>S. eridania</i>	<i>S. cosmioides</i>
Egg ht	411.1 (357–477)	370.2 (292–420)	525.8 (476–573)
Egg diam	505.4 (475–540)	480.3 (430–521)	463.3 (396–517)
No. primary cells	9.1 (6–11)	10.6 (8–14)	10.2 (8–12)
No. secondary cells	15.7 (12–19)	20.3 (17–26)	21.2 (17–26)
No. micropyles	3.5 (3–4)	3.9 (2–5)	3.4 (3–7)
No. ribs	52.3 (46–57)	49.9 (46–54)	48.3 (46–54)
Rosette diam	66.2 (49.7–99.1)	55.33 (36.3–78.7)	41.81 (29.7–53.0)

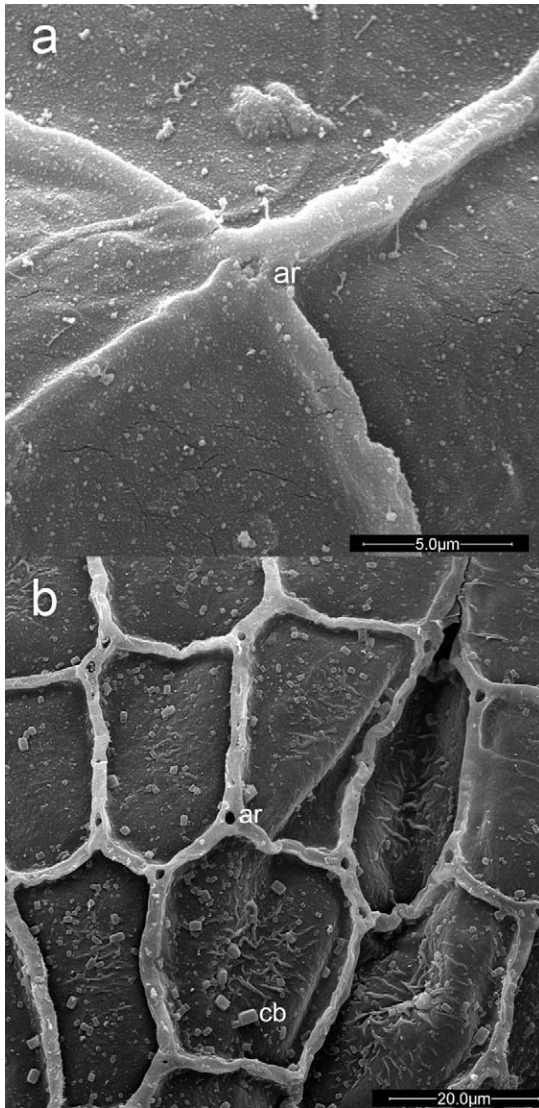


Fig. 5. Lateral surface topography of (a) *S. eridania* and (b) *S. cosmioides*. r, ribs; cr, crossribs; ar, aeropyles; cb, cuboidal elements.

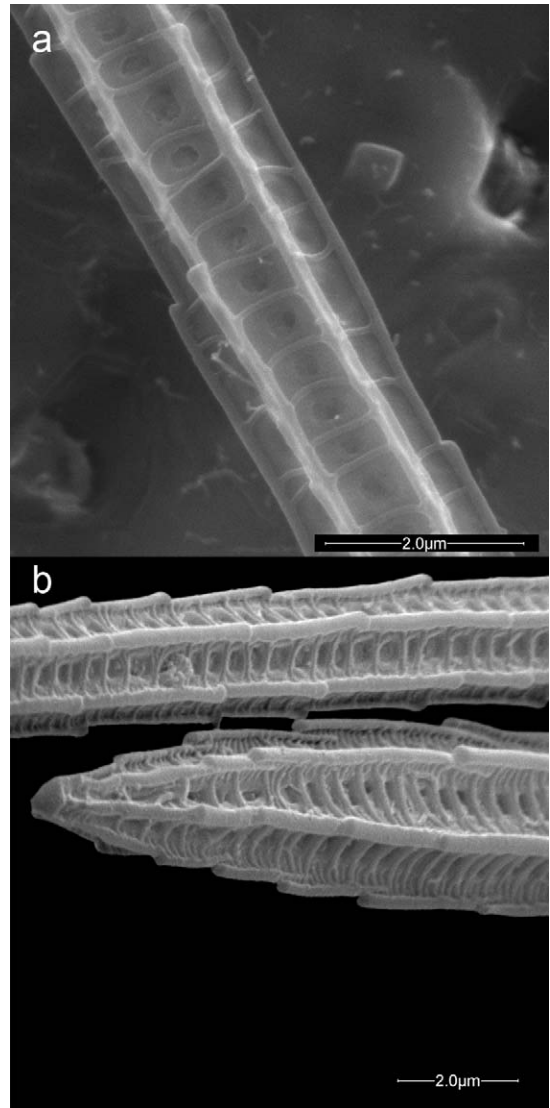


Fig. 6. (a) Silk (modified scales) of *S. albula*. (b) Tip of the silk of *S. eridania*, used to cover the egg mass.

tively, and the micropyle was surrounded by a rosette composed of 9.1 (6–11) primary cells (Figs. 3a and 4a and b; Table 2). The distal extremes of the rosette petals were angular in shape (Fig. 4a and b).

The egg masses of *S. eridania* are pale green, somewhat bright, and laid in one, two, or eventually three layers. They are spherical in shape and flattened at the base. In our study, the mean diameter of *S. eridania* eggs was 480.3 μm (430–521) and the mean height was 370.2 μm (292–420). The mean number of micropyles was 3.9 (2–5), and they were surrounded by a rosette composed of 10.6 (8–14) primary cells (Fig. 4c and d; Table 2). The distal extremes of the rosette petals were round in shape (Fig. 4c and d). In this species, the mean numbers of ribs was 49.9 (46–54),

within the range of *S. albula* and *S. cosmioides* (Table 2).

The egg masses of *S. cosmioides* present variable colors, ranging from green, gray, and brown to dull pink brownish, and they are laid in two or three layers. They are spherical in shape and flattened at the base. In the current study, the mean diameter of *S. cosmioides* eggs was 463 μm (396–517) and the mean height was 525.8 μm (476–573). The mean numbers of ribs and micropyles were 48.3 (46–54) and 3.4 (3–7), respectively, and the micropyle was surrounded by a rosette composed of 10.2 (8–12) primary cells (Fig. 4e and f; Table 2). The distal extremes of the rosette petals were round in shape (Fig. 4e and f). Aeropyles are present on the conjunction points of the conspicuous ribs and cross-ribs (Fig. 5a and b). Masses of eggs

Table 3. Sample list, location, host plant, collection dates, and haplotypes

Species	Location	Host	Collection data	Haplotypes
<i>S. albula</i>	Jaboticabal, SP	Peanuts	2008	1
<i>S. cosmioides</i>	Pelotas, RS	Laboratory	NA	1
		Castor Oil	Feb. 2009	1 & 2
<i>S. eridania</i>	Santa Helena, GO	Cotton	Mar. 2010	1, 2, & 3
		Laboratory	NA	4
<i>C. includens</i>	Rondonópolis, MT	Soybean	Dec. 2008	1, 2, 3, & 4
<i>R. nu</i>	Vacaria, RS	Soybean	NA	1, 2, 4, & 6
	Monte Redondo, Argentina	Soybean	Feb. 2012	1, 3, & 5

of *Spodoptera* species are covered by modified scales that are morphologically similar among species (Fig. 6a and b).

Partial sequencing of the mitochondrial (mt) COI amplicon from legs and eggs revealed an average size of 658 bp for *S. albula*, *S. eridania*, *S. cosmioides*, *C. includens*, and *R. nu*. The mtDNA sequencing for the studied *Spodoptera* species corresponded to positions 1 or 2 and 658 of the sequence encoding the COI gene, partial cds in *S. frugiperda* (GenBank GU090723), as well as in *C. includens* (GenBank GU090374.1), and in *A. gemmatalis* (GenBank JN401299). We observed nonvariable sites in *S. albula*, one variable site in *S. cosmioides*, two variable sites in *S. eridania* and *C. includens*, and three variable nucleotide sites in *R. nu* (Table 3). The recognition site for the restriction enzyme Bpm I (CTGGAGnnnnnnnnnnnnn nn') was absent in *C. includens* and *S. eridania*, and the unrestricted amplification product remained with 710 bp. Amplified COI products were digested with restricted endonuclease and resulted in a single visible band, with different molecular weights (MW), for *S. albula* (600 bp), *S. cosmioides* (550 bp), and *R. nu* (700 bp), but for *S. eridania* and *C. includens*, the restriction enzyme Bpm I did not digest the final product of amplification (Fig. 7; Table 4). Restrict digestion PCR from *S. albula*, *S. eridania*, and *C. includens* products with MboI resulted in three visible bands, with different MW, for *C. includens* and *R. nu* [280 bp, 120 bp

(possibly, an overlap of bands of similar MW), and a faint band close to 50 bp], and for *S. cosmioides* (290 bp, 120 bp, and faint band close to 100 bp) (Fig. 8). Restrict digestion PCR from *S. eridania* product with MboI resulted in three visible bands of 400 bp, a band close to 120 bp, and a faint band close to 50 bp (Fig. 8; Table 5). PCR-RFLP patterns (Tables 4 and 5) for Bmp I and MboI in *S. albula*, *S. cosmioides*, *S. eridania*, *C. includens*, and *R. nu* were—AA, BB, CA, CB, and CB, respectively. The presence of haplotypes in all the noctuid species had no influence on RFLP patterns. Agarose gel electrophoresis was not able to distinguish between *C. includens* and *R. nu*.

Discussion

Morphological differences between the Plusiinae (Noctuidae) species studied here and *A. gemmatalis* (Erebidae: Eulepidotinae) are easily distinguished by the rosette petals and the conspicuous ribs present in *A. gemmatalis* eggs (Cônsoi et al. 1999). Another feature that distinguishes *A. gemmatalis* from both Plusiinae species is the short radial threads in high relief that are present inside all rosette petals of *C. includens* and *R. nu* but are absent in *A. gemmatalis* (Fig. 1c and d). However, morphological differences between *C. includens* and *R. nu* eggs are almost indistinguishable. Angulo et al. (2006) described a dichotomic key based

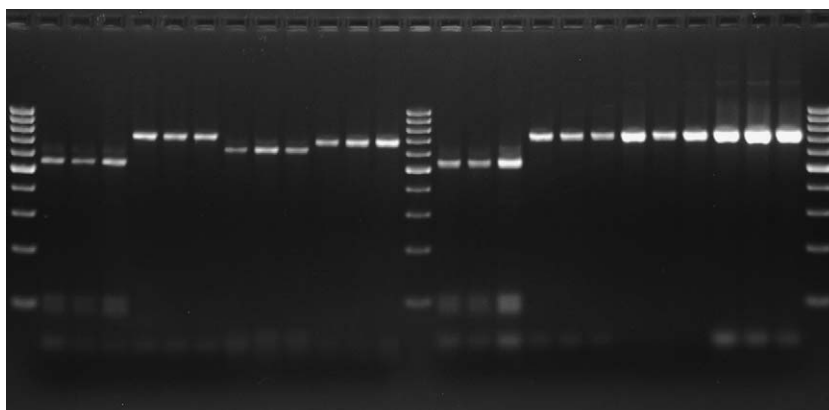


Fig. 7. PCR-RFLP agarose gel (2%) for COI amplification products using Bpm I endonuclease. Lane 1: 100 bp MW; lanes 2–4: digestion product of *S. cosmioides*; lanes 5–7: digestion product of *S. eridania*; lanes 8–10: digestion product of *S. albula*; lanes 11–13: digestion product of *R. nu*; lane 14: 100 bp MW; lanes 15–17: digestion product of *S. cosmioides*; lanes 18–20: digestion product of *S. eridania*; lanes 21–26: digestion product of *C. includens*; lane 27: 100 bp MW.

Table 4. Restriction sites and fragment size after restriction digestion of COI amplification products from Noctuidae species using the restriction enzyme Bpm I

Species	Restriction site	Fragment size (bp)	Pattern
<i>S. albula</i>	643, 583	15, 60, 583	A
<i>S. cosmioides</i>	583, 67	67, 75, 516	B
<i>S. eridania</i>	No digestion	658	C
<i>C. includens</i>	No digestion	658	C
<i>R. nu</i>	643	15, 643	C

on egg microstructure and reported that *R. nu* presents 25–46 lateral ribs, whereas *C. includens* has 31–33, according to Peterson (1964). Furthermore, the eggs of *C. includens* are hemispherical, with the basal part, at the opposite side of the micropylar region, flattened. Radial and transversal ribs are more conspicuous in *C. includens* than in *R. nu*.

The main characteristic that could be used to distinguish the eggs of *Spodoptera* from *A. gemmatalis* and Plusiinae is the “net” appearance of the surface with no conspicuous ribs in *Spodoptera* (Fig. 3a–c), together with the presence of modified scales (silk) covering the egg masses.

S. albula presented a lower number of primary cells than that observed in *S. eridania* and *S. cosmioides*, although the primary cells are bigger in *S. albula* (Fig. 4; Table 2). Observations with light microscopy suggest that all traits overlap (Table 2). The eggs of *Spodoptera* species are covered by modified scales (silk) as usually mentioned (Beserra et al. 2002, Valverde 2007). Variation in egg color is expected depending on the diet and the age of the eggs (Cónsoli et al. 1999, Valverde 2007).

The current study shows that *S. albula* presented a higher number of ribs (46–57) than that observed by Peterson (1964) (47–50), who referred to the species as *P. sunia*. Regional morphological variability in number of ribs has been found in other noctuid species, such as *Peridroma saucia*, which in the United States has 43–44 ribs (Peterson 1964) and in Chile, 62–70 ribs (Angulo et al. 2006).

Table 5. Restriction sites and fragment size after restriction digestion of COI amplification products from Noctuidae species using MboI

Species	Restriction site	Fragment size (bp)	Pattern
<i>S. albula</i>	87, 104, 488, 587, 614, 632	17, 18, 26, 27, 87, 99, 384	A
<i>S. cosmioides</i>	87, 104, 375, 488, 587, 614	17, 27, 44, 87, 99, 113, 271	B
<i>S. eridania</i>	87, 104, 488, 587, 632	17, 26, 45, 87, 99, 384	A
<i>C. includens</i>	87, 104, 375, 488, 587, 614, 632	17, 18, 26, 27, 87, 99, 113, 271	B
<i>R. nu</i>	87, 104, 375, 488, 587, 632	17, 26, 45, 87, 99, 113, 271	B

Insect species identification or differentiation based on morphological characteristics of undifferentiated phases, such as eggs or small larvae, can be inaccurate. Therefore, additional molecular characterization, based on the nucleotide sequence of the first portion of the mitochondrial COI gene, was used in this study. This region of the mtDNA genome has been widely used as DNA barcoding for species identification (Jinbo et al. 2011). Also, PCR–RFLP analyses of these gene fragments can be useful to determine differences among species. Double digestion with Bpm I and MboI may be confusing, considering the multiple bands generated after the process.

Although band separation is better in polyacrylamide gel, in this study, agarose gel was used because of the lower cost and nontoxic reagents. RFLP patterns produced by Bpm I endonuclease was useful to discriminate species within *Spodoptera* (*S. cosmioides*, *S. eridania*, and *S. albula*), but no differences between *R. nu* and *C. includens* were found. Bpm I did not digest the amplicons from *S. eridania* and *C. includens*, which were discriminated by RFLP patterns produced by MboI enzyme. PCR–RFLP methodology showed diagnostic utility to distinguish the most important Lepidoptera species associated with soybean production. Additional studies, with a more extensive sampling,

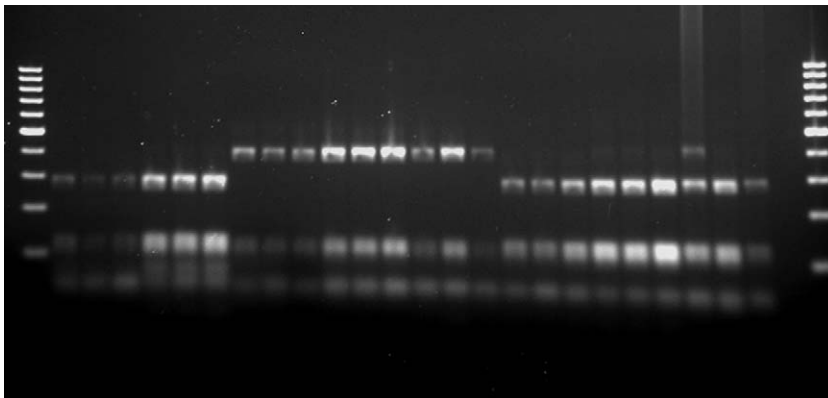


Fig. 8. PCR–RFLP agarose gel (2%) for COI amplification products using MboI endonuclease. Lane 1: 100 bp MW; lanes 2–7: digestion product of *S. cosmioides* (adults and eggs); lanes 8–13: digestion product of *S. eridania* (adults and eggs); lanes 14–16: digestion product of *S. albula*; lanes 17–22: digestion product of *C. includens* (adults and eggs); lanes 23–25: digestion product of *R. nu*; lane 26: digestion without DNA template; lane 27: 100 bp MW.

are necessary, as different haplotypes may occur in related species belonging to the same genus or family, such as *S. frugiperda* (Nagoshi et al. 2007) and *H. virescens* (Albernaz et al. 2012).

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