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Biological and molecular characterization of the post-invasion immature stages of *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae)

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Abstract

We performed molecular and biological characterizations of immature stages of *Helicoverpa armigera* Hübner (Lepidoptera: Noctuidae) using insects from different states in Brazil, and different host plants. Three laboratory colonies of *H. armigera* were established with insects from the states of São Paulo, Bahia, and Distrito Federal, that were collected from citrus, cotton, and corn, respectively. For each colony, microsatellites were used to assess genetic similarity among insects. The biology of the immature stages from each colony also was compared under laboratory conditions (25 ± 1 °C, 70 ± 10% RH, 14:10 h [L:D] photoperiod). Microsatellite analysis revealed genetic variability in sampled specimens and that the grouping of individuals was independent of the geographic origin. The utility of an artificial diet was validated during the comparative biology experiment, and the ingredients of the artificial diet were analyzed for purity, particle size, and the presence and quantity of aggregates. The mean development times of the larvae and pre-pupae from the 3 locations were similar, and mean total development time (egg to adult) was not significantly different.

Key Words: Artificial diet; larval development; old world bollworm; standardization of breeding; molecular characterization

Resumo

Neste estudo foram realizadas a caracterização molecular e a comparação da biologia de estádios imaturos de *Helicoverpa armigera* Hübner (Lepidoptera: Noctuidae). Foram estabelecidas três colônias desta espécie, a partir da coleta de insetos oriundos dos estados de São Paulo, Bahia, e Distrito Federal, em plantas de citros, algodão e milho, respectivamente. Análises genética foram conduzidas usando microssatélites de forma a verificar a similaridade genética dos insetos de cada colônia. A biologia de imaturos foi estudada sob condições de laboratório (T = 25 ± 1 °C, UR = 70 ± 10%, fotoperíodo = 14:10 h L:D). A análise de microssatélites revelou variabilidade genética nos espécimes amostrados e que o agrupamento dos indivíduos foi independente da origem geográfica. Uma dieta artificial foi utilizada e validada durante o estudo de biologia comparada. Amostras de cada um dos ingredientes da dieta artificial foram analisadas quanto à pureza, tamanho de partícula e presença e quantidade de agregados. As viabilidades médias das larvas e pré-pupas nas três populações foram semelhantes. E o tempo total de desenvolvimento total (ovo a adulto) não foi significativamente diferente.

Palavras Chave: Dieta artificial; desenvolvimento larval; padronização de criação; caracterização molecular

The first record of occurrence of *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae: Heliothinae) in Brazil was in 2013 (Embrapa 2013; Czepak et al. 2013; Specht et al. 2013), although introduction likely occurred at least 5 years earlier (Sosa-Gómez et al. 2016). At the time of detection (2012–2013 crop season), a population outbreak of the species had occurred, with remarkable levels in several crops including cotton, citrus, corn, soybeans, and tomatoes in different regions of the country (Embrapa 2013; Czepak et al. 2013; Specht et al. 2013; Bueno et al. 2014; Pratissoli et al. 2015).

The initial records of occurrence were based on samples obtained in the states of Goiás, Distrito Federal, Mato Grosso, and Paraná (Embrapa 2013; Czepak et al. 2013; Specht et al. 2013). However, subsequently it was determined that specimens had been collected in southern Brazil (Rio Grande do Sul) in 2011 and in the extreme North (Amapá) (Sosa-Gómez et al. 2016), and São Paulo (Bueno & Sosa-Gómez, 2014) in 2012. Late detection allowed this species to become abundant, and dispersed throughout South America. Indeed, recovery in Brazil, the insect was detected in Argentina (Murúa et al. 2014), Paraguay (SENAVE 2014), and Uruguay (Arnemann et al. 2016).

Several studies since the detection of *H. armigera* in Brazil have reported infestations as "populations" collected in different regions and from various host plants, highlighting the occurrence of several

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haplotypes in Midwest, North and Northeast Brazil (Leite et al. 2014; Mastrangelo et al. 2014; Tay et al. 2013). However, it remains unknown whether the dispersion was the result of multiple recent incursions or a single invasion by different populations of *H. armigera* (Mastrangelo et al. 2014).

Although several laboratories in Brazil have attempted to maintain populations of *H. armigera*, only a few generations have been reported, apparently due to loss in genetic quality. The causes of failure could be due to inbreeding depression, but also may be due to the lack of a suitable diet. The principal purpose of mass rearing using an artificial diet is to provide high-quality insects and in desirable quantities while retaining the natural characteristics of the species (Parra 2009; Schneider 2009).

The objectives of this study were to establish 3 colonies of *H. armigera* with insects collected from 3 different states in Brazil (São Paulo, Bahia, and Distrito Federal), from citrus, cotton, and corn, respectively, and to perform genetic analyses and characterization of the biology of the immature stages. The genetic analysis was performed using microsatellites to verify genetic similarity among insects from the 3 colonies. The biological study of the immatures compared the developmental period, survival, and pupal weights among the 3 colonies. The suitability and nutritional characteristics of a suitable synthetic diet also are presented. This information will serve as a basis for future comparisons based on natural and artificial diets.

Materials and Methods

ORIGIN OF *HELICOVERPA ARMIGERA* COLONIES

The *H. armigera* used in this study were collected from citrus in São Paulo (Botucatu, 22.8858°S, 48.4450°W), corn in Distrito Federal (DF) (Planaltina, 16.0763°S, 47.6207°W), and cotton in Bahia (São Desiderio, 12.6628°S, 45.5093°W). The colonies were maintained separately in a climate-controlled room.

Identification to the species level was performed at the Entomology Laboratory of Embrapa Cerrados, Planaltina, Distrito Federal, Brazil, by comparison of the morphology of male genitalia (Pogue 2004).

MOLECULAR CHARACTERIZATION

The molecular characterization of each colony was performed at the Entomology Laboratory at Embrapa Soja (Londrina, Paraná, Brazil). DNA from males and females from each colony was extracted using the protocol of Rogers & Bendich (1988). Microsatellite markers (HaC14, HaB60, HaC87, HarSSR1, HarSSR2, HarSSR3, HarSSR4, and HarSSR5 loci), previously developed by Scott et al. (2004) and Ji et al. (2003), were used to characterize the sample of insects from each colony. The amplification products were analyzed by 12.5% polyacrylamide gel electrophoresis at 20 V/cm for 8 h in Tris-glycine buffer. The gels were treated with ethidium bromide (10 mg per mL^{-1}); bands were visualized under UV light and photographed and scanned using a transilluminator (model L-Pix Ex, Loccus Biotecnologia®, Cotia, São Paulo, Brazil) coupled to a personal computer. The sizes of the fragments were estimated by comparison with a 100 bp DNA marker (GeneRuler, Thermoscientific®, São Paulo, São Paulo, Brazil). The bands produced were coded and arranged in an array that was used to construct a dendrogram of genetic distance using the Neighbor Joining procedure (Saitou and Nei 1987) with the software Darwin 5, version 5.5.155 . The distances between the sampling points were calculated at http://www.movable-type.co.uk/scripts/latlong.html site.

BIOLOGICAL CHARACTERIZATION OF THE IMMATURES

Comparative biological tests were performed with immature *H. armigera* inside climate-controlled growth chambers (25 ± 1 °C, 70 ± 10% RH, 14:10 h [L:D] photoperiod) at the Plant Resistance to Insects Laboratory and Insecticide Plants (LARESPI), Department of Plant Protection of the FCA-UNESP, Botucatu, São Paulo, Brazil.

Eggs obtained from matings in the 3 different colonies were maintained in cylindrical polyvinyl chloride (PVC) cages (10 cm diam × 20 cm ht). These cages were lined with brown kraft paper, which served as a substrate for oviposition, and with 1 end covered with voile fabric for ventilation. The cages were supported on cylindrical plastic dishes (28 cm diam). The paper and voile containing eggs were removed daily. Portions of the substrate with eggs were cut and placed in plastic cups (500 mL) containing an artificial diet (see below) and placed in the rearing chamber until hatching of the larvae.

The diet for adults was supplied via capillary action using absorbant cotton soaked in a solution of water and 10% honey (Wild Flowers, Alvorada®, Botucatu, São Paulo, Brazil), placed on voile.

One hundred neonates from each colony were placed individually in Petri dishes (TPP®, Trasadingen, Switzerland) (9.6 cm diam × 2.1 cm ht) with approximately 1 cm^3 of an artificial diet. The diet was replenished as needed. The plates were cleaned daily to avoid possible contamination.

During larval development, the head capsules were removed and stored in Petri microplates (3.5 cm diam × 1.0 cm ht) (TPP®, Trasadingen, Switzerland) for subsequent measurement of the maximum width of the head capsule (Podoler & Klein 1978) and instar confirmation. The diet was removed when the larvae ceased feeding and decreased in size (a characteristic of the pre-pupal period). To provide moisture and facilitate preparation of the pupal chamber, 0.5 cm^3 of expanded vermiculite moistened with distilled water was added to each plate. Two days after metamorphosis, individuals were weighed using a balance (AL-500, Mars Cientifica®, São Paulo, São Paulo, Brazil) and sexed by comparing the genital opening of the last abdominal segments of the pupae (Kirkpatrick 1961). Because sex can be identified only in pupae, the identification number for each individual (plate) was maintained throughout development, which allowed comparisons between sexes, including at the larval stage. Daily maintenance of the pupae was restricted to keeping the vermiculite moist by applying a few drops of water until the adults emerged.

Observations of individuals from each colony were performed daily to determine the duration (d) of the egg, larva (including pre-pupae), pupal and total development time, as well as the total larval survival (%). Also, we obtained the weight (mg) of the pupae with the aid of a semi-analytical balance (AL-500, Mars Cientifica®, São Paulo, São Paulo, Brazil). Following a completely randomized design, each Petri dish containing a larva and the portion of the diet was considered a replicate (100 for each population).

COMPOSITION AND PREPARATION OF THE ARTIFICIAL DIET

The artificial diet (modified from Greene et al. 1976) was composed of 1.20 L distilled water; 75.0 g white beans (Camil®, Itaqui, Rio Grande do Sul, Brazil); 60.0 g wheat germ (Natural life, Kodilar®, São José do Rio Preto, São Paulo, Brazil); 30.0 g pure casein (Synth®, Diadema, São Paulo, Brazil); 37.50 g brewer's yeast (Jasmine®, Curitiba, Paraná, Brazil); 30.0 g textured soy protein (Jasmine®, Curitiba, Paraná, Brazil); 3.60 g ascorbic acid ($C_6H_8O_6$) (Synth®, Diadema, São Paulo, Brazil); 114.0 mg tetracycline hydrochloride (Medquímica®, Juiz de Fora, Minas Gerais, Brazil); 3.40 ml 40% formaldehyde (CH_2O)

(Chemco®, Hortolândia, São Paulo, Brazil); 3.60 g methyl parahydroxybenzoate (nipagin) ($C_8H_8O_3$) (Synth®, Diadema, São Paulo, Brazil); 1.80 g sorbic acid ($C_6H_8O_2$) (Dynamic Chemical Contemporânea®, Diadema, São Paulo, Brazil); 9.0 mL vitamin solution (Vita Jr., Union Química®, São Paulo, São Paulo, Brazil); and 23.0 g agar-agar (Synth®, Diadema, São Paulo, Brazil). The composition of the vitamin solution had the following components per mL: vitamin A (retinol palmitate) 0.69 mg; vitamin B1 (thiamine hydrochloride) 0.40 mg; vitamin B2 0.50 mg; vitamin B6 (pyridoxine hydrochloride) 0.60 mg; vitamin B12 (cyanocobalamin) 0.50 µg (microgram); vitamin C (ascorbic acid) 35.0 mg; vitamin D3 (cholecalciferol) 0.01 mg; vitamin E (tocopherol acetate) 4.0 mg UI; folic acid 35.0 µg; nicotinamide 6.0 mg; panthenol 3.0 mg.

The beans were cooked for 40 min in a pressure cooker (4.5 L) with 500 mL of tap water, drained, and ground in a blender (18,000 rpm) for 10 min with 600 mL of distilled water. Next, the wheat germ, casein, brewer's yeast and soy protein were added gradually until a homogeneous mass formed. In a stainless steel vessel, the agar was dissolved in 500 mL of hot (approx. the boiling point) distilled water to obtain a gelatinous consistency. This bean mixture was transferred to the vessel containing the agar and mixed for 5 min.

The remaining ingredients (ascorbic acid, sorbic acid, nipagin, formaldehyde, tetracycline hydrochloride, and vitamin solution) together with 100 mL remaining distilled water were mixed in a blender for approximately 2 min (18,000 rpm) when the bean mixture cooled to 60 °C. Subsequently, when the formulation reached approximately 40 °C, it was packed into plastic boxes (11 cm × 11 cm × 3.5 cm), which were immediately placed under UV light in a laminar flow hood until a temperature of 25 °C was achieved. The boxes were then sealed, identified and refrigerated (5 °C) until used. Using a stainless steel spatula, the diet was cut into cubes of approximately 1 cm^3 immediately before being offered to the larva of *H. armigera*.

ANALYSIS OF THE COMPONENTS OF THE ARTIFICIAL DIET

Analysis of the artificial diet was performed by Analytical Quality Center (CQA®) in Campinas, São Paulo, Brazil. Physical and chemical analyses were conducted for detailed information of the major macro- and micronutrients in the artificial diet. The methods proposed by the AOAC (2010) and FDA (2010), following the RCD Resolution number 360 (RCD 360/2003-Brazil 2003) were used.

STATISTICAL ANALYSIS

The statistical analyses were performed using the statistical package Proc GLIMMIX (SAS Institute 2001). The means of the biological parameters (eggs, larvae, pre-pupae and pupae, and pupal weight) obtained for each population were tested and when the F test was significant ($P \leq 0.05$) the means were compared using Tukey's test.

Results

MICROSATELLITE MARKER COMPARISON

The genetic variability of the 3 colonies, as determined by PCR amplification using 7 microsatellite loci applying the Neighbor Joining procedure, based on molecular weight of SSR bands, revealed coefficients of dissimilarity varying between 0.07 and 0.5, which is an indication of genetic variability. The Neighbor Joining tree shows a separation between the lower and upper clusters of individuals from São Desiderio, Bahia State, and Botucatu, São Paulo State, with insects originally from Distrito Federal State spread in the 2 groups (Fig. 1).

BIOLOGICAL PARAMETER COMPARISON

No differences were observed ($F = 12.63$; df = 2; $P = 0.293$) in the period of egg incubation for the 3 colonies established from different states in Brazil, and the egg viability ranged from 61.9 to 72.6% (Table 1). There was a statistically significant difference in mean stadium length observed among the colonies from São Paulo, Distrito Federal, and Bahia ($F = 7.33$; df = 2; $P = 0.001$). Mean stadium length from São Paulo was greater than Distrito Federal, and Bahia was intermediate. The highest survival rate was observed in the colony from Distrito Federal (83.0%).

The pre-pupal period (Table 1) of the Bahia colony differed from the other 2 colonies ($P < 0.001$) and exhibited the lowest average (2.0 d). There was no difference among the colonies for pupal period (Table 1). Considering the total development period (egg to adult), there was no difference among the colonies ($P = 0.052$), and the mean development period was approximately 31 d. The survival rate from the Bahia colony averaged slightly higher (55.9%), followed by the Distrito Federal (52.4%) and São Paulo (40.2%) colonies (Table 1).

Considering the sex and number of instars in each population (Table 2), a longer duration of larval development was observed for the São Paulo colony ($P < 0.001$): 7-instar males and females. An exception was the 7-instar females of the Bahia colony. A longer pre-pupal period ($P < 0.0001$) was found for the females (with 6 and 7 instars) and males (with 7 instars) from São Paulo, males (with 6 and 7 instars) from Distrito Federal, and males (with 6 instars) from Bahia. The males (6 instars) from Bahia showed the lowest average (1.50 d) length of time at this stage.

For the pupal stage (Table 2), the 6- and 7-instar males of the Distrito Federal colony exhibited the longest average duration (13.5 and 13.3 d, respectively), differing ($P < 0.0001$) from the females (with 6 and 7 instars) and males (with 7 instars) of São Paulo colony, females (with 6 instars) of the Distrito Federal colony, and females (7 instars) of the Bahia colony. Regarding the total immature stage duration, the males (7 instars) from São Paulo colony displayed the longest average duration (33.6 d), differing ($P < 0.0001$), from the females (with 6 instars) of this population, the females from Distrito Federal and the females (with 6 instars) from Bahia colonies, respectively.

By considering the sex and number of instars within each colony (Table 3), the females (with 7 instars) from São Paulo had the highest average pupal weight (337.0 mg), differing ($P < 0.0001$) from the females (with 6 and 7 instars) and males (with 6 instars) from Distrito Federal, and the females (with 7 instars) from Bahia. Based on the overall averages, the pupae from the São Paulo colony showed a greater weight (324.0 mg), differing from the insects originating from Distrito Federal (282.0 mg) and from Bahia (296.0 mg).

Curves of head capsule measurements throughout the stadia (Fig. 2) showed similar exponential growth among insects originating from different states. The mean head capsule values ranged from 1.93, 1.90, and 1.95 mm during the first instar to 20.00, 19.80, and 20.20 mm in the last instar for individuals from São Paulo, Distrito Federal, and Bahia, respectively.

ANALYSIS OF THE COMPONENTS OF THE ARTIFICIAL DIET

Analysis of the artificial diet (Table 4) revealed the different amounts of components, especially macronutrients. The presence of adonitol sugars, arabinase, arabitol, cellobiose, fructose, galactose, glucose, inositol, lactitol, maltitol, maltose, maltotriose, mannitol, mannose, melezitose, meli-

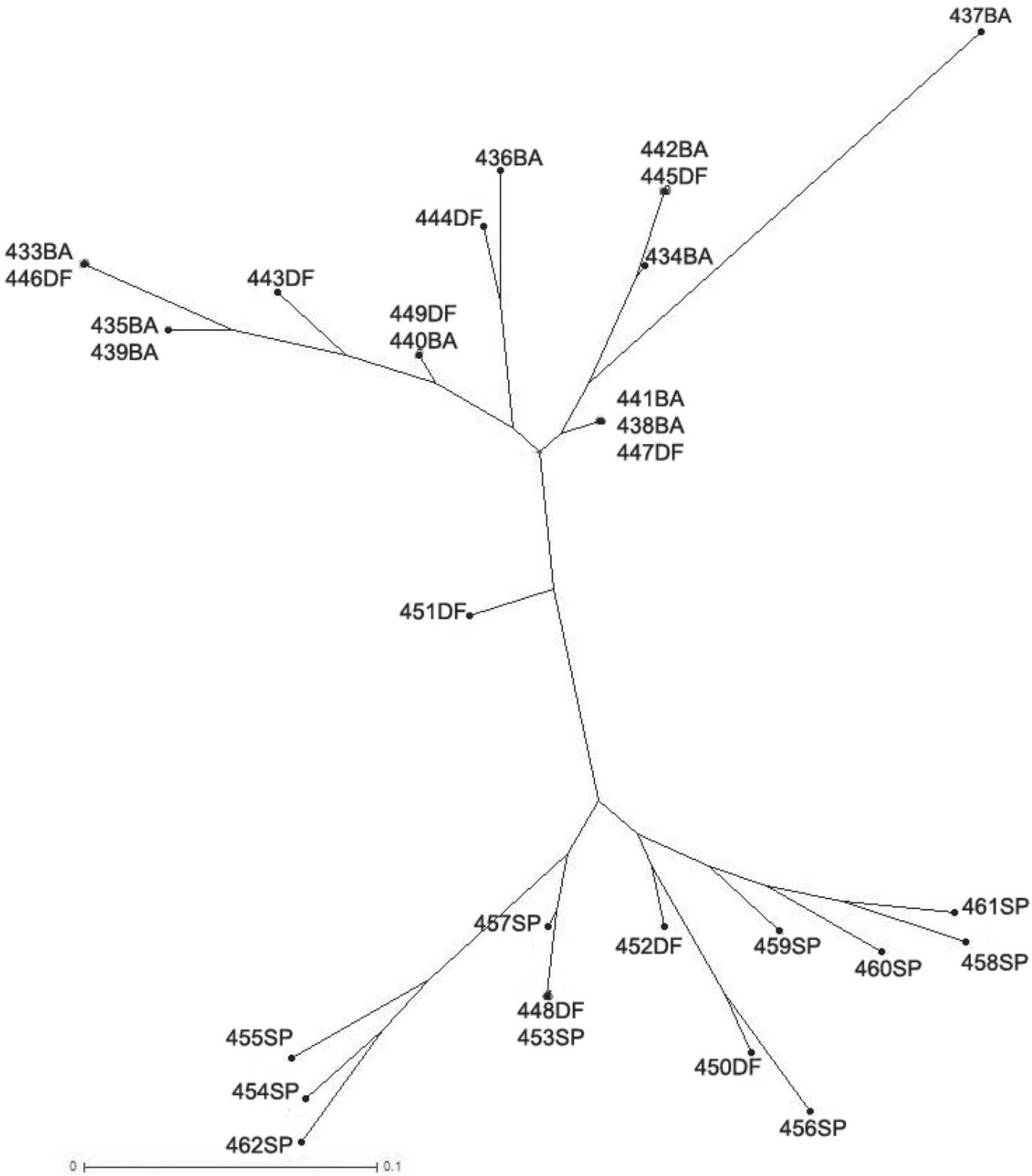

Fig. 1. Genetic relationships among 30 individuals of *Helicoverpa armigera* by Neighbor Joining (NJ) by using microsatellites. Samples are identified by their geographic origin, BA: Bahia; SP: São Paulo; and DF: Distrito Federal.

biose, raffinose, sorbitol, trehalose, turanose, xylitol, or xylose was not detected [AOAC/FQ-0093]. Among the prominent macronutrients were carbohydrates (8.1 g per 100 g sample), followed by total protein (5.3 g per 100 g sample) and total fat (0.72 g per 100 g sample) (Table 4). Additional analyses indicated an acidity of 5.61% [AOAC/FQ-0105] and volatile acidity of acetic acid of 3.37 g per 100 g [AOAC/FQ-0068] (AOAC 2010).

Table 1. Mean (± SE) duration (d) and survival (%) of immature-stage *Helicoverpa armigera* from 3 colonies (São Paulo, Distrito Federal, and Bahia) feeding on an artificial diet under controlled conditions (25 ± 1 °C, 70 ± 10% RH, and 14:12 h [L:D] photophase).

	São Paulo colony			Distrito Federal colony			Bahia colony		
Phase	N	Duration (d)[a]	Survival (%)	N	Duration (d)[a]	Survival (%)	N	Duration (d)[a]	Survival (%)	*P*
Egg		2.9 ± 0.41a	61.90		2.8 ± 0.52a	69.86		2.9 ± 0.24a	72.65	0.2934
Larvae	100–74	14.2 ± 1.68a	74.00	100–83	13.4 ± 0.98b	83.00	100–77	13.9 ± 0.56ab	77.00	0.0011
Prepupae	74–73	2.9 ± 0.99a	98.65	83–80	2.5 ± 1.04a	96.39	77–77	2.0 ± 0.92b	100.00	0.0003
Pupae	73–68	11.9 ± 1.36a	89.04	80–75	12.3 ± 1.23a	93.75	77–77	12.5 ± 1.11a	100.00	0.1374
Total		31.9 ± 4.52a	40.24		31.2 ± 3.45a	52.40		31.2 ± 3.23a	55.94	0.0523

[a]Means followed by the same letter in each row do not differ statistically by Tukey's test at 5% probability.

Discussion

Unlike the report by Subramanian & Mohankumar (2006), the primers amplifying the HarSSR2 locus generated polymorphic bands in insects originating from different states of Brazil. However, the primers amplifying HarSSR4 and HarSSR5 loci produced monomorphic bands in the 3 colonies of *H. armigera*.

Cluster analysis of genetic distance among individuals from the 3 colonies originating from different states (Fig. 1) indicated genetic variability and that the grouping of individuals was independent of the geographic origin. Although the colonies were composed of individuals collected from different host plants in remote states, low genetic distance was observed among genotypes from Bahia and Distrito Federal, or São Paulo and Distrito Federal, as also reported by Vassal et al. (2008). This low genetic distance can be explained by the dispersion capacity of this species (Feng et al. 2004), and the short period of demographic expansion in the Neotropical region, as reported by Sosa-Gómez et al. (2016) and Murúa et al. (2016).

Although the molecular comparisons detected genetic variability across regions and different host plants, assessment of the biological parameters of *H. armigera* in the 3 colonies shows biological proximity between the groups. This finding indicates that microsatellite alleles are shared among distant regions in Brazil, as has been found when the partial sequence of the cytochrome c oxidase subunit was studied (Leite et al. 2014; Tay et al. 2017), as well as in other studies with gene markers (Mastrangelo et al. 2014; Tay et al. 2017).

The egg incubation period was similar among the 3 colonies of *H. armigera*, as was previously reported when this species was reared on an artificial diet, sweet corn hybrids, and asparagus (Jha et al. 2014), rose buds (Patel et al. 2011), and beans (Naseri et al. 2014).

The average duration of the larval stage (from 13.4–14.2 d) in the 3 *H. armigera* colonies in the present study are close to those reported by other authors who also evaluated insect biology on an artificial diet (Amer & El-Sayed 2014). However, long duration of the larval period has been reported when larvae of *H. armigera* were fed sweet corn (18.3 d), artificial diet (19.5 d) (Jha et al. 2012), and beans (19.8 d) (Naseri et al. 2014). These variations are most likely related to differences in the nutritional values of the diet offered to the larvae (Awmack & Leather 2002), as well as the rearing environment (Zalucki et al. 2002).

The pre-pupal period (2.0–2.9 d) recorded for the colonies of *H. armigera* was similar to that obtained on rose buds (Patel et al. 2011) and bean cultivars (Naseri et al. 2014), at 2.3 and 2.4 d, respectively. However, the duration was longer than obtained on tomato (Singh & Singh 1975), which ranged from 1 to 2 d.

The pupal stage of *H. armigera* was stable among the 3 colonies, approximately 12 d in duration. These results are similar to those found

Table 2. Mean (± SE) duration (d) of immature-stage *Helicoverpa armigera* from 3 colonies (São Paulo, Distrito Federal, and Bahia), with sex discrimination, developing for 6 and 7 instars on an artificial diet under controlled conditions (25 ± 1 °C, 70 ± 10% RH, and 14:12 h [L:D] photophase).

Sex	Instars	N	Larva (d)[a]	Prepupa (d)[a]	Pupa (d)[a]	Total immature (d)[a]
			São Paulo colony			
Female	6	21	13.9 ± 1.17a	2.9 ± 0.66b	11.4 ± 1.12ab	31.0 ± 1.56ab
	7	9	15.6 ± 1.01b	3.0 ± 0.87b	10.8 ± 1.20a	32.3 ± 1.12bc
Male	6	22	13.4 ± 1.14a	2.7 ± 0.71ab	12.8 ± 0.96bcdef	31.9 ± 1.21abc
	7	9	15.7 ± 2.00b	3.2 ± 0.83b	11.8 ± 1.99abcd	33.6 ± 1.12c
			Distrito Federal colony			
Female	6	27	13.3 ± 0.67a	2.0 ± 0.86ab	11.6 ± 1.02abc	29.8 ± 0.27a
	7	8	13.5 ± 1.80a	2.6 ± 1.06ab	11.9 ± 2.25abcde	30.9 ± 0.35ab
Male	6	20	13.4 ± 0.80a	2.8 ± 1.06b	13.5 ± 1.26f	32.5 ± 0.32bc
	7	11	13.7 ± 1.35a	2.9 ± 0.95b	13.3 ± 2.12ef	32.8 ± 1.21bc
			Bahia colony			
Female	6	31	13.8 ± 0.63a	2.1 ± 1.01ab	12.0 ± 0.93abcdef	30.8 ± 1.73ab
	7	6	14.8 ± 2.56ab	2.7 ± 1.76ab	11.5 ± 1.64ab	32.0 ± 3.29abc
Male	6	26	13.6 ± 0.57a	1.5 ± 0.70a	13.2 ± 0.89cdef	31.2 ± 1.44ab
	7	4	14.0 ± 0.81a	2.8 ± 0.50ab	13.0 ± 0.81bcdef	32.7 ± 0.96bc
P	< 0.0001					

[a]Means followed by the same letter in each column do not differ statistically by Tukey's test at 5% probability.

Table 3. Mean (± SE) weight (mg) of pupae of *Helicoverpa armigera* from 3 colonies, showing differences by sex and total number of larval instars, plus the overall mean values, when fed artificial diet under controlled conditions (25 ± 1 °C, 70 ± 10% RH, and 14:12 h [L:D] photophase).

Sex	Instars	São Paulo colony	Distrito Federal colony	Bahia colony
Female	6	332.0 ± 34.00bc	275.0 ± 28.00a	299.0 ± 36.00abc
	7	337.0 ± 23.00c	285.0 ± 32.00ab	286.0 ± 68.00ab
Male	6	319.0 ± 36.00abc	285.0± 43.00ab	293.0 ± 36.00abc
	7	307.0 ± 45.00abc	293.0 ± 28.00abc	302.0 ± 30.00abc
Mean		324.0 ± 36.00b	282.0 ± 33.00a	296.0 ± 39.00a
P		< 0.0001		

[a]Means followed by the same letter do not differ statistically by Tukey's test at 5% probability. In each column for females and males throughout for 6 and 7 larval instars for each colony and in row considering both sexes of each colony.

with this insect on other hosts such as beans (Naseri et al. 2014) and maize (Arghand et al. 2011), with averages of 12 to 13 d, respectively.

Considering the total immature period for the insects from the 3 colonies, the duration (approximately 31 d) was also similar to that on beans (Naseri et al. 2014). However, the total immature period was shorter than on soybeans (42.7 d) (Fathipour & Naseri 2011), and tomato (45.3 d) (Hemati et al. 2012). Based on this information, it appears that the artificial diet used in this study is more suitable than some natural diets.

A morphometric assessment of the different colonies was included in this study. Measurement of head capsule was crucial for confirmation of the larval instars in the 3 colonies. Regardless of the origin of the colony, the values showed exponential growth during the instars; this was similar to what was reported by other authors, who measured immature head capsules of *H. armigera* for up to 10 generations (Mohammadi et al. 2010). According to Dyar (1890), the head capsule of

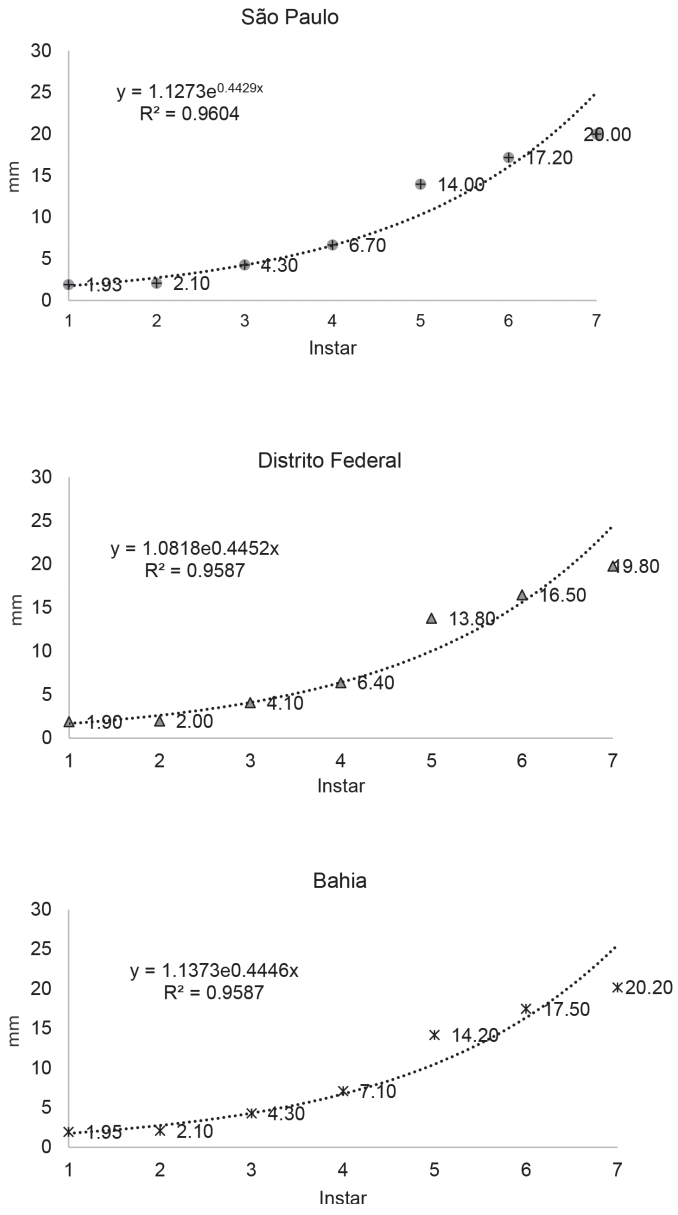

Fig. 2. Size of the head capsule (mm) of *Helicoverpa armigera* larvae from 3 colonies.

Table 4. Proximate composition of artificial diet provided to *Helicoverpa armigera* larvae from 3 colonies under controlled conditions (25 ± 1 °C, 70 ± 10% RH, and 14:12 h [L:D] photophase).

Constituent	Value	Unit
Carbohydrates(1)	8.1	g per 100 g
Sucrose(1)	0.824	g per 100 g
Reducing sugars(1)	3.04	g per 100 g
Lactose(1)	0.003042	g per 100 g
Insoluble dietary fiber(1)	1.5	g per 100 g
Soluble dietary fiber(1)	0.1	g per 100 g
Total dietary fiber(1)	1.6	g per 100 g
Unsaturated fats(1)	0.7	g per 100 g
Saturated fats(1)	0.02	g per 100 g
Trans fats(1)	0.47	g per 100 g
Free fatty acids(1)	5.61	g per 100 g
Linoleic fatty acids(1)	<LQ**	g per 100 g
Total fats(1)	0.72	g per 100 g
Total nitrogen(1)	0.85	g per 100 g
Total protein(1)	5.3	g per 100 g
Ashes to 550°c(1)	0.7	g per 100 g
Iron(1)	0.00161	g per 100 g
Phosphorus(1)	0.14503	g per 100 g
Magnesium(2)	0.0361	g per 100 g
Manganese(2)	0.00995	g per 100 g
Potassium(2)	0.1925	g per 100 g
Sodium(2)	0.0132	g per 100 g
Zinc(2)	0.00105	g per 100 g
Energy value(3)	0.6	kcal per g
Energy value(3)	2.5	kJ per g

**<LQ: Less than the limit of quantification (1) AOAC - A.O.A.C. International, Official Methods of Analysis, 18th ed., Silver Spring, Maryland, USA, Current Through - Revision 3, 2010; (2) FDA - Food And Drug Administration. 2010. Elemental Analysis Manual. United States of America. Section 4.4 Inductively Coupled Plasma-Atomic Emission Spectrometric Determination of Elements in Food Using Microwave Assisted Digestion; (3) RDC360 / 2003 - Brazil. 2003. RCD Resolution No. 360 of 23 December 2003. Approves Technical Regulation on Nutritional Labeling of Packaged Food, nutrition labeling becoming mandatory, the National Health Surveillance Agency. official gazette, Brasilia, Distrito Federal, Brazil.

a larva grows in geometric progression, increasing in width with each molt, at a constant rate for a given species.

Helicoverpa armigera can pass through 7 instars, regardless of the origin of the colony. The same result was reported by Jha et al. (2014), who confined the insect to asparagus leaves. However, a predominance of larvae pupated after the sixth instar in all 3 colonies. Hardwick et al. (1965) described variations in 5 through 7 instars in the genus *Helicoverpa*, with the sixth instar most frequent. The prevalence of 6-instar *H. armigera* also has been reported in trials using pigeon pea (Borah & Dutta 2002), sweet corn (Arghand et al. 2011), artificial diet (Jha et al. 2012), white beans, and chickpeas (Razmjou et al. 2014).

The occurrence of only 5-instar *H. armigera* also has been documented in the literature (Hosseininejad et al. 2015), and temperature, photoperiod, humidity, quantity and quality of food offered are the principal factors responsible for such variations (Esperk et al. 2007). Razmjou et al. (2014) suggested that variation in the number of larval instars may be related to genetic inheritance and sex in *H. armigera* larvae.

The average values of pupal weight (approximately 300 mg) obtained in this study are similar to those described for *H. armigera* individuals fed on corn (355.6 mg), chickpea (322.6 mg), and artificial diet (305.9 mg) (Serate et al. 2012). However, these previous studies do not mention the difference between the weight of male and female pupae. Stillwell et al. (2010) described that lepidopteran females are usually larger than male.

In the present study, the diet ingredients were well detailed, with physical and chemical analyses conducted for detailed information of the major macro- and micronutrients. Therefore, it will be possible in future studies to nutritional value of other artificial and natural diets. The aspects regarding purity, particle size, and the presence and quantity of aggregates are relevant For example, most diet formulations include soy protein. However, soy protein can be found in texturized, concentrated, and isolated forms at approximate total protein proportions of 50, 70, and 90%, respectively. This variable can influence insect development when crude protein is the main source of nitrogen. Other variations may also occur with the sources of bean, casein, and wheat germ, among others. Other essential elements, such as ash and microelements, should also be evaluated for each insect to allow comparisons between natural and artificial diets. In the diet used in this study, total fiber accounted for 39.5% of total carbohydrates. Some insects, especially chewing insects, do not develop properly if the amount of fiber is low (less than 50% of the carbohydrate) (Cohen 2003; Parra 2009). In the diet used in this study, the proportion of total protein (5.3 g per 100 g sample) was higher than that of total nitrogen (0.85 g per 100 g sample), indicating the presence of protein for biological functions. It should be noted that many of the dietary ingredients (soy protein, bean, casein, wheat germ) present the type of proteins that were quantified together in the analysis. The lipid analysis of the diet focused primarily on the determination of total fats and free fatty acids, the latter of which are important for insects because they are small, easily metabolized molecules. In addition to the importance of lipids for insects is their influence on the stability of the diet, because lipids can oxidize and produce undesirable toxic substances. In this regard, the present amount of unsaturated fats in the diet (Table 4) is undesirable (Cohen 2003; Parra 2009).

In this study, *H. armigera* adapted well to the diet, which allowed comparison of the biological performance of the colonies with insects originating from São Paulo, Distrito Federal, and Bahia, for which the original host plants were citrus, corn, and cotton, respectively. This information allows for standardization of insect breeding, regardless of the origin of the insects. The small variations we observed for some parameters are commonly found in studies of Lepidoptera, and represent normal variation within a species. The molecular analyses indicated that the microsatellite allelic variation of *H. armigera* colonies from São Paulo, Distrito Federal, and Bahia are not clearly associated with their geographical origin. However, differences found between São Paulo and Bahia populations demand more studies with microsatellite markers.

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