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Egg morphology of two sibling species of the *Bactrocera dorsalis* complex Hendel (Diptera: Tephritidae)

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Abstract
In the present study, the eggs of *Bactrocera carambolae* (Drew and Hancock) and *Bactrocera papayae* (Drew and Hancock) were described for the first time. Eggs were collected from 10 F_1 females of each species emanating from infested guava fruits sampled from guava orchards in Songkhla province of southern Thailand. Eggs were examined by using Olympus microscope with inbuilt ocular micrometer for morphometric study, Olympus DP72 Universal Camera microscope to capture the image of the eggs and Scanning Electron Microscope (SEM) was used for the detailed morphological studies. At least 30 eggs of each species were used for morphometric and image capturing, and additional 30 eggs of each species were submitted for SEM analysis. None of the eggs of the two species had conspicuous respiratory appendage. The eggs were similar in gross morphology, and tapering towards the anterior and posterior ends. Presence of papillae, mycropyles and aeropyles are peculiar to both species but with some variations. The papilla and micropyle with clumsy woolly structure was common to *B. carambolae,* but aeropyles on the chorion of *B. papayae* were numerous and in variable diameters. The diagnostic characters to differentiate between these two species include the chorion ornamentation, location of aeropyles, and rim of chorion.

Keywords: *Bactrocera carambolae*, *Bactrocera papayae*, egg, micropyle, aeropyle.

1. Introduction

Bactrocera dorsalis (Hendel) has been thought to be responsible for causing enormous losses to horticultural crops throughout Asia / South-east Asia. But it is now clear that a complex of sibling species exist in the region that are of serious economic importance. In the *B. dorsalis* complex, are certainly the most significant fruit fly pest species in Asia / South-east Asia [1]. Greater difficulty has been encountered in identifying *B. dorsalis* and its related sibling species than any other group of Dacinae. The history of confusion in the nomenclature of the *B. dorsalis* complex was documented by [2]. For every complex member, accurate identification is essential for appropriate ecological study and application of quarantine restrictions law placed on fruits and fruit fly from one country to another.

The major focus of this study is on *Bactrocera carambolae* (Drew and Hancock) and *Bactrocera papayae* (Drew and Hancock), which are sibling species belonging to the *B. dorsalis* complex [1, 3, 4]. These two species have been found to be well established and distributed in Southern Thailand, and affecting different kinds of fruits and vegetables [5]. Drew and Hancock [1] and Allwood *et al.* [6] had worked extensively on the host plant records for fruit flies (Diptera: Tephritidae) in Southeast Asia. It was observed from the list of the hosts available that the two species share common ecological niche overlaps via host plants in peninsula Thailand [5]. Accurate identification of these species is a prerequisite for control and regulatory measures. However, identification has been difficult between these sibling species [1, 7, 8]. The black band on the abdomen, colour of the occiput and the coastal band of the wing were used to distinguish these species [1]. However, some of the distinguishing characters were not reliable because their intermediate states are frequently found in both species [7, 8]. Furthermore, Iwaizumi *et al.* [7] and Iwahashi [8] did an extensive morphometric study on the male aedeagus and the female aculeus, and observed that differences in the length of the aedeagus and aculeus were also used to distinguish the males and females of these species, respectively. Ebina and Ohto [9] studied the morphological characters and PCR-RFLP Markers in the interspecific hybrids and concluded that in both species, the inconsistency between the morphological characters and the DNA markers, as well as the continuous variation of the aculeus length, were mainly caused by interspecific hybridization in the distribution area. The

blending of traits observed between the sibling species suggested that heterospecific crosses between the two species might be occurring under natural conditions, and if this is true, it would be interesting to examine the length of the terminalia of hybrid males and females resulting from these crosses [7]. This was confirmed recently by Schutze *et al.* [10] that these species demonstrated significant deviation from random mating towards assortative mating.
The need to explore additional morphological characters is pertinent at this juncture in order to generate other characters that may be used to separate these species. In our previous study on the effect of constant temperatures on the survival and developmental stages of *B. carambolae* and *B. papayae*, the range of constant temperatures verified revealed that *B. papayae* was faster than *B. carambolae* at each developmental stage [11]. The disparity observed for egg developmental time for both fly species triggered a thought that lead to the screening of the morphology of the eggs, with the aid of a Scanning Electron Microscope (SEM), because the eggs of insect species present morphological peculiarities which are related to their life strategies [12]. The relationships of these morphological adaptations to evolutionary patterns were clearly demonstrated for some group of insects [13, 14, 15]. Among the tephritid fruit flies of the genus *Anastrepha* Schiner, the importance of egg morphology for taxonomy and phylogenetic inferences was predicted by Norrbom *et al.* [16]. Hence, the objective of this study was to improve on the ability to identify the egg stage of *B. carambolae* and *B. papayae* that will better enhance the understanding of the disparity of their developmental time, and to serve as distinctive taxonomic characters between the two species. This study is paramount, as it will lead to improvement in the rearing of the flies in the laboratory, and for mass production of the flies for control programs.

2. Material and methods

The study was carried out for the period of 7 months (October 2012 – April 2013) at the Entomology Research Unit of the Department of Biology, Faculty of Science, and the Central Laboratory of the Prince of Songkla University (PSU), Hat Yai, Thailand.

2.1 Insect culture

This study was conducted on the first filial generation (F_1) laboratory reared fruit flies. The population was initiated from infested guava fruits sampled from PSU ($7^0$00'13.05"N and $100^0$29'57.11"E) guava orchard. The fruit fly colonies were reared and maintained at the Entomology Unit of the Department of Biology, PSU, Hat Yai, Thailand. Rearing conditions were maintained at $25 \pm 1\ ^0$C, $75 \pm 5\%$ relative humidity (RH) and Photoperiod of L12:D12.

2.2 Egg collection

Fruit fly egg collection method was adopted according to Danjuma *et al*. [5]. Eggs were collected from *B. carambolae* and *B. papayae* stock colony with the aid of an artificial egg-laying device offered to 10 F_1 females of both flies maintained separately in 27 x 27 x 27 cm cages. The egg-laying device consisted of a yellow ball which was cut into two equal halves to produce a dome-like structure. Each dome was pierced with an entomological pin number 4 (4 cm long and 0.3 mm in diameters) to make 150 tiny holes on each dome. Each dome was placed in a 9 cm diameter petri dish laced at the background with a black coloured filter paper. Before the domes were place in the petri dishes, they were spray with water to simulate the surface of fruits in order to facilitate oviposition. Eggs were collected within 4 hours of setting with the aid of a soft camel hair brush onto the black background.

2.3 Morphometric study

From each species egg dome, 60 eggs each were collected with the aid of a camel brush under a Stereo microscope. Eggs were carefully placed in a vial of 2 ml from where 30 eggs per species were randomly picked and morphometric data (length and width) were taken with the aid of an Olympus microscope with inbuilt ocular micrometer. Furthermore, the eggs were examined and the images of 5 eggs each were captured with the aid of an Olympus DP72 Universal Camera at the Department of Biology microscopy room, PSU, Hat Yai, Thailand. The remaining eggs were further held in vial of 2 ml containing 2.5% glutaraldehyde (CH_2 $(CH_2CHO)_2$) organic compound. These vials were immediately taken to the Central Laboratory, PSU for preparation for electron microscopy.

2.4 Electron microscopy process

Egg preparation followed the method of Selivon and Perondini [17]. Thirty eggs of each species were transferred into 0.1M cacodylate buffer (pH 7.4), washed, post-fixed in an aqueous solution of 1% Osmium tetroxide (OsO_4) for an hour. These eggs were dehydrated in an ethanol series then critical point dried in CO_2 for 3 hours and sputter-coated with gold layer [18, 19]. The eggs were then examined under scanning electron microscope (Quanta 400, FEI, Czech Republic) at high vacuum, 10.00kv. All scanning were done at the Central Laboratory of the PSU, Hat Yai. Thailand. SEM was used to examine the chorion in at least 10 eggs of each species. The anterior pole (the end of the egg that bears the pedicel or a slight projection with the micropyle and aeropyles) and the posterior pole (the end opposite of the pedicel which is usually smooth and bluntly rounded and bears no external opening or structure) were also examined. The convex side of the egg is referred to as the ventral side and the concave side as the dorsal side [19].

3. Result

The eggs of *B. carambolae* and *B. papayae* have similar characteristics in their gross morphology. Observations under the Olympus DP72 Universal Camera microscope revealed that the eggs of these species were white in colour and tapered towards the anterior and posterior ends. The anterior pole, possessing the micropyle, is more tapered than the posterior pole which ends bluntly and rounded (Fig. 1, a. and b.).

Diagnostic characters to differentiate between these two species eggs include chorion ornamentation, location of aeropyles and a pronounced rim of the chorion with a woolly appearance surrounding the micropyle. None of the eggs of these species had a conspicuous respiratory appendage.

3.1 *Bactrocera carambolae*

General features: Length, 0.88-1.23 mm and width, 0.21-0.23 mm. Eggs were white in colour, elongated, elliptical and strongly curved. Broader from the middle towards the anterior pole, tapering gradually towards both ends (Figs. 1a and 2. a1, b1).

Chorionic Sculpturing: The apex of the anterior pole bears a micropyle, surrounded by a conspicuous ring-shaped rim (papilla) of chorion which is strongly ornamented with woolly substance (Fig. 2. a1 arrow head y.). Chorion reticulation was less conspicuous and present well-defined threadlike-lines which presents the shell with firmed, solid and rigid polygonal wall (Fig. 2, c1). The irregular polygonal patterns on the chorion covered all the egg surfaces and present a fine grainy texture within the polygon formed by the chorion reticulation (Fig. 2, a1, b1 and c1). Aeropyles were rarely seen on the surface of the eggs. Minute openings were observed between 1-5 aeropyles in the egg of this species (Fig. 2. c1, arrow head x).

Egg morphology of two sibling species of *Bactrocera dorsalis* complex Hendel (Diptera: Tephritidae).

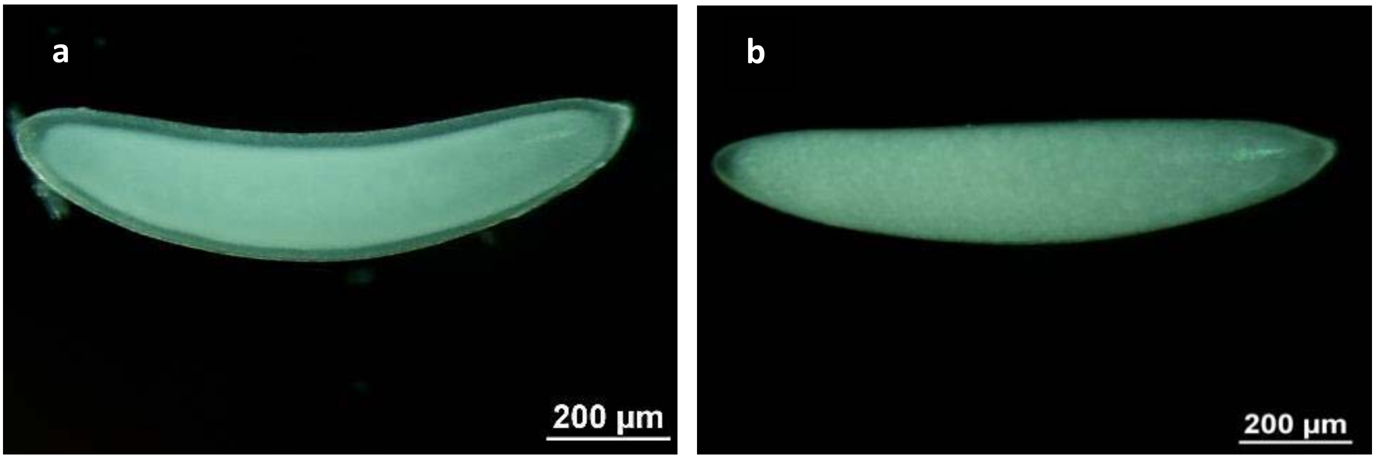
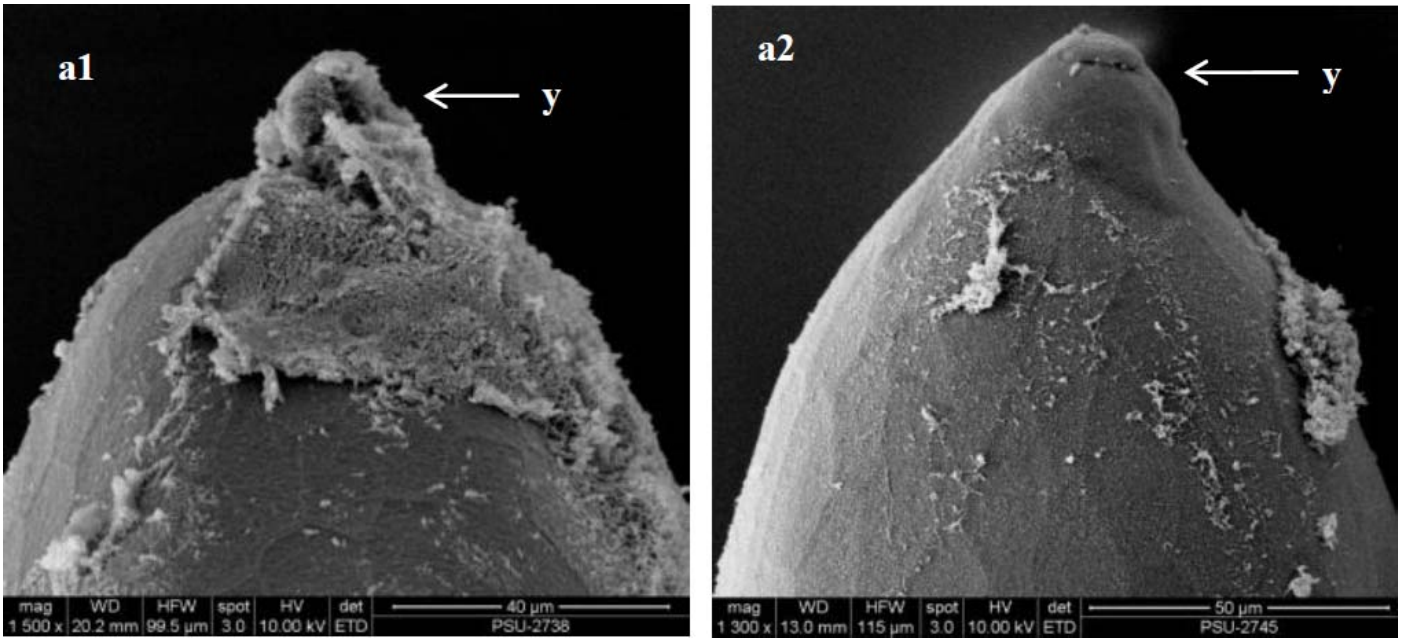


Fig 1: *Bactrocera* egg (a) *B. carambolae* egg (b) *B. papayae* egg

3.2 *Bactrocera papayae*

General features: Length, 0.91-1.16 mm and Width, 0.20-0.26mm. Eggs were white, elongated, elliptical and slightly curved (Fig. 1b). Broader from the middle towards the anterior pole, gradually tapering towards both ends, but bluntly rounded at the posterior pole (Fig. 2. a2 and b2).

Chorionic sculpturing: The apex of the anterior pole bears a papilla and a limited chorion ornamentation, with less pronounced reticulation in a polygonal arrangement (Fig. 2. c2), and a surface bearing roughly textured polygons. Located at the vertices of the polygons on the egg, were between 15-25 aeropyles, mostly pronounced at both the dorsal and ventral sides (Fig. 2. c2.). The aeropyle openings were of variable diameters (Fig. 2. c1 arrow head x.). The reticulation was poorly developed, forming into a rough bulge or protuberance which gets faint towards the posterior pole. The micropyle was located at the apex of the anterior pole and ornamented with a pronounced rim (papilla) of the chorion. This rim was ringed-shaped and has a smooth appearance devoid of woolly materials (Fig. 2. a2 arrow head y).



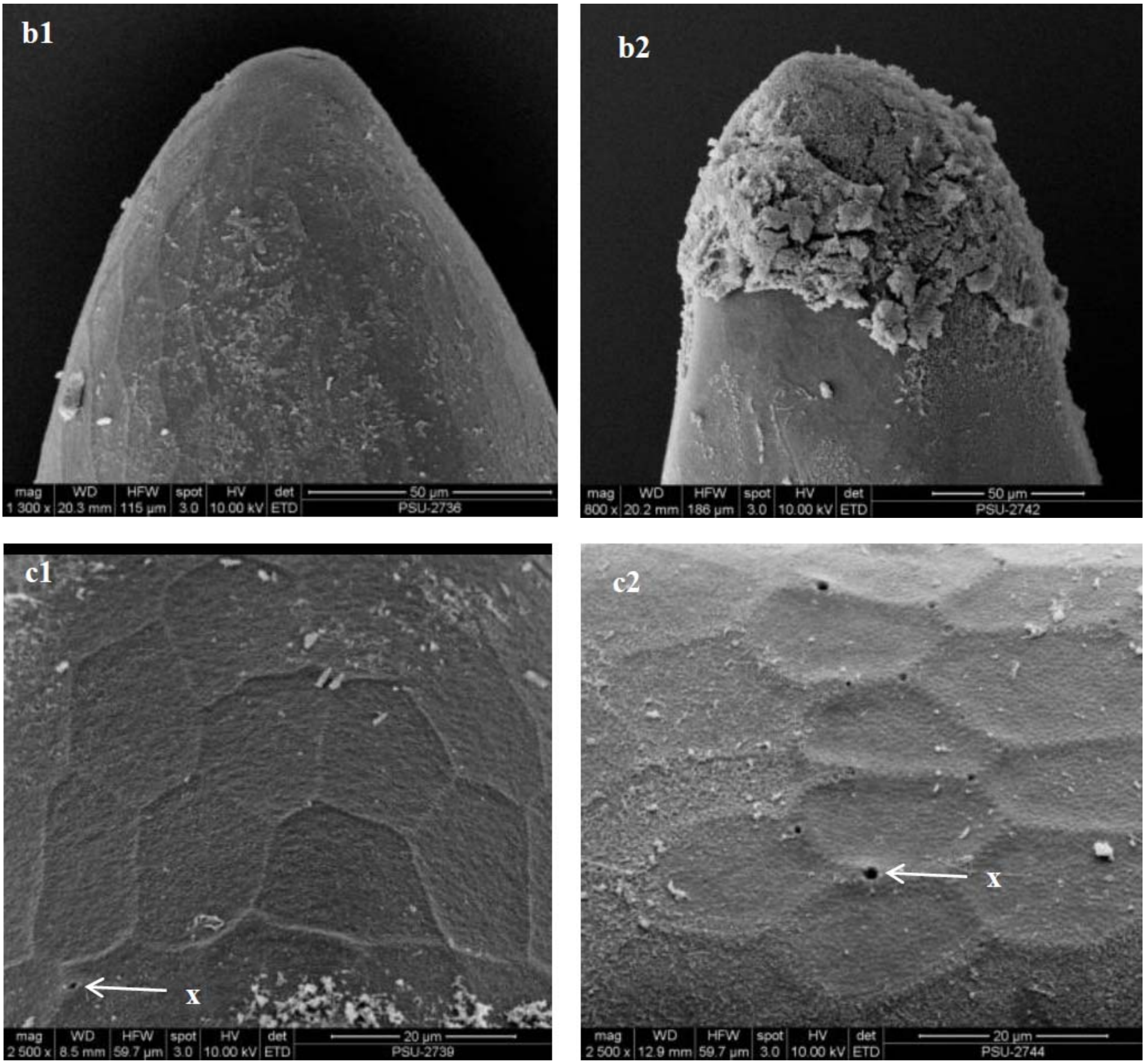


Fig 2: Electron microscope scanned eggs of *Bactrocera* species showing some characteristic features. a = anterior poles of *B. carambolae* (a1) and *B. papayae* (a2), b =) posterior poles of *B. carambolae* (1b) and *B. papayae* (b2), c = pre-anterior poles of *B. carambolae* (c1) and *B papayae* eggs (c2).

The main diagnostic characters of the eggs of the two species are shown in Table 1.

Table 1: Diagnosis of the eggs of the two *Bactrocera* sibling species

Species	Chorion		Micropyle		Aeropyle		
	Reticulation	Sculpturing in reticular	Location	Rim	Location	Number	Diameter
B. carambolae	Threadlike lines distinguishing each polygon	None, fine grained surface	Apex of the anterior	Clumsy and woolly	Anterior lateral side	1 – 5	Minute
B. papayae	Wide and poorly developed polygonal marking	Poor protuberance, rough surface	Apex of the anterior	Clear and smooth	All sides	15 – 25	variable

4. Discussion

The eggshell morphology of the two *Bactrocera* species examined in this study correspond to the general pattern of tephritid fly eggs, with reference to their gross morphology such as colour, shape and chorion [19, 20]. The eggshell chorion revealed some specific characters for the two species examined.

The chorionic sculpturing of the two species fall under the broad category of eggs of Diptera Cyclorrhapha as designated by [21]. *B. carambolae* and *B. papayae* have their egg chorion faintly reticulated. The reticulation observed in *B. carambolae* was threadlike-lines devoid of protuberance and present the chorion as firm and solid fine-grained surface. Poor protuberance of reticulate was observed on the chorion of *B. papayae* and this presented the chorion as roughed surface. Faint reticulation was already observed in the eggs of *B. tryoni* [20] and *Anastrepha ludens* [22], *A. coroilli* and *A. distincta* [19]. On the contrary, well-developed chorionic reticulation is known to occur in some *Anastrepha* species such as *A.* sp.1 aff. *Fraterculus*, *A.* sp. 2 aff. *Fraterculus* [17], *A. obliqua* [24], and *A. sororcula* [24]. *B. carambolae* and *B. papayae* eggs presented chorion reticulation on all sides, and were found all over the chorion in *B. carambolae*. However, it become fainter towards the posterior pole in *B. papayae*. Studies have shown that sculpturing may be related to the differential activity of the follicle epithelium, and to an adaption to the fly habitat [25]. It is also believed that reticulation together with aeropyles, plays some significant role in embryo respiration [17], and also provides protection against desiccation [25].

The posterior poles of the eggs of the two species showed similarities in tapering and end bluntly. The anterior poles, though show similarities in ending pattern and possession of rim ring-shaped micropyle, differ with respect to the presence of the clumsy and woolly structure on the mycropyle in *B. carambolae* (which was absent in that of *B. papayae*). Aeropyles of a few (1-5) minute openings were observed on the lateral sides of the *B. carambolae* eggs, while many more aeropyles (15-25) of distinct variable diameters, were revealed on all sides of the chorion of eggs belonging to *B. papayae*. In both cases, all aeropyles were more to the anterior than to the posterior regions, and were sighted on the reticular ridges. Dutra *et al*. [19] reported aeropyle locations on *Anastrepha* eggs as, ventral in *A. antunesi*, *A. bahiensis* and *A. coronilli,* and observed them on both sides (dorsal and ventral) in large numbers in *A. turpiniae, A. distincta* and *A. zenildae.* Their work also revealed variations in aeropyle diameters and confirmed that in *A. distincta*, aeropyles with larger diameters were located on the ventral side, while those with smaller diameters were located on the dorsal side. In the present work, aeropyles of variable diameters were observed on both sides of *B. papayae* eggs. The disparity observed might be because the present flies studied belong to a different genus (*Bactrocera*) and occurring in a distinct region and habitat. No respiratory appendage was found on the eggs of both species. Dutra *et al.* [19] also reported a similar observation in six *Anastrepha* species (*A. antunesi*, *A. bahiensis*, *A. coronilli*, *A. distincta*, *A. turpiniae* and *A.zenildae*). It was only in *A. barbiellinii*, *A. manihoti*, *A.obliqua, A. nigrifacia* and *A.pittieri* that presence of respiratory appendage has been reported [23, 26, 27]. It has been suggested that the structure of chorion and the number of aeropyles are related to adaption strategies to the environment where the eggs were deposited [19]. The number of aeropyles was also reported by Cônsoli *et al.* [25] to be related to the species metabolic rate, need for gas exchange and control of water loss. Distinct papilla was observed in the two species, located at the anterior pole of the eggs analysed. However, it is more pronounced in *B. carambolae*, similar to what has been reported for *A. coronilli*, *A. distincta*, *A. turpiniae* and *A.zenildae* [19], *A. grandis* [28], *A. luden* [22], *A.* sp.1 aff. *Fraterculus* [17, 29] and *A. sorocula* [24].

The mycropyle of the eggs of the two species studied were located directly on the apex of the anterior poles. This is similar to the work of Dutra *et al.* [19] that reported mycropyle location on the anterior pole apex for *A. antunesi*, *A. bahiensis*, *A. coronilli*, *A. distincta* and *A. zenildae*. However, a slightly displaced mycropyle from the apex, was reported in *A. turpiniae* [19], and *A.* sp.1 aff. *Fraterculus* [17,23,29]. A dislocated mycropyle due to respiratory appendage has also been reported in *A. oblique* [23, f27].

The present study of the eggs of the two sibling species with the aid of SEM revealed some basic similarities between the species and among other *Anastrepha* species reported before as there was rarely such studies of eggs of *Bactrocera* species except for scanty documentation in few texts. The combination of the characteristics displayed by these eggs, such as chorion ornamentation, location, diameters of aeropylesand of mycropyles, are useful as taxonomic characters [19]. The results of this work will increase the understanding of eggshell morphology and the probable reasons behind the variations observed in the egg hatching time of these species and their life history strategies. This will enhance the rearing of these species for mass production with reference to Sterile Insect Technique (SIT) and for other control strategies. It is worth mentioning that further work on other *Bactrocera* species, especially members of the *B. dorsalis* complex, are required to increase the understanding of this monophyletic group with reference to their phylogenetic relationship.

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