SYSTEMATICS, MORPHOLOGY AND PHYSIOLOGY

Morphological and Molecular Characterization of Three Species of *Anastrepha* Schiner and of *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae)

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Neotropical Entomology 34(6):917-925 (2005)

Caracterização Morfológica e Molecular de Três Espécies do Gênero *Anastrepha* Schiner e de *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae)

RESUMO - Foram comparadas com base na morfologia do pupário e na técnica de HMA (Heteroduplex Mobility Assay)) quatro espécies de Tephritoidea, três delas pertencentes ao gênero *Anastrepha*, *A. obliqua* (Macquart), *A. sororcula* Zucchi e *A. serpentina* (Wiedemann) e uma do gênero *Ceratitis*, *C. capitata* (Wiedemann). Os pupários foram caracterizados pela primeira vez com base na morfologia da placa espiracular posterior. A aplicação da técnica de HMA possibilitou a detecção de variabilidade no segmento D2 do gene 28S rRNA nas quatro espécies (confirmada por seqüenciamento). Esta é uma técnica rápida, simples, sensível e barata que é aplicada pela primeira vez na análise de dois gêneros de Tephritoidea. A variabilidade observada sugere grande potencial da técnica no caso da determinação da infestação por duas ou mais espécies num mesmo fruto hospedeiro.

PALAVRAS-CHAVE: Mosca-das-frutas, Tephritoidea, pupário, HMA

ABSTRACT - Four species of Tephritoidea, three from genus *Anastrepha*: *A. obliqua* (Macquart), *A. sororcula* Zucchi and *A. serpentina* (Wiedemann), and one from genus *Ceratitis, C. capitata* (Wiedemann) were compared based on puparium morphology and application of the Heteroduplex Mobility Assay (HMA). Puparia were characterized for the first time using the spiracular posterior plate morphology. Application of HMA allowed the detection of variability in the D2 domain from 28S rRNA gene in all four species (confirmed by sequencing). This is a fast, simple, sensitive and inexpensive assay that was used for the first time in the analysis of two Tephritoidea genera. The detected variability suggests that the tecnique has great potential for rapid determination of infestations with two or more species in the same host fruit.

KEY WORDS: Fruit fly, Tephritoidea, puparium, HMA

The true fruit flies, family Tephritidae, have significant quarantine importance. In this group the species diversity is remarkable, including 4,448 recognized species arranged in 484 genera (Norrbom 2004). About 250 species of Tephritidae are considered pests (White & Elson-Harris 1992, Armstrong *et al*. 1997). Fruit flies of genus *Anastrepha* and *Ceratitis capitata* (Wiedmann) (Tephritidae) are the most important pests to the Brazilian pomiculture (Zucchi 2000).

The genus *Anastrepha* is represented by 94 species in Brazil (Zucchi 2000). Seven species are economically important: *A. grandis* (Macquart), *A. fraterculus* (Wiedmann), *A. obliqua* (Macquart), *A. pseudoparallela* (Loew), *A. sororcula* Zucchi, *A. striata* Schiner and *A. zenildae* Zucchi. Other species such as *A. bistrigata* Bezzi, *A. distincta* Greene and *A. serpentina* (Wiedmann) have potential economic importance (Zucchi 2000).

The genus *Ceratitis* is composed of approximately 65 species (White & Elson-Harris 1992). The Mediterranean fruit fly *C. capitata* is the most serious pest of fruit in the world, due to its high colonization potential (Fletcher 1989). Ihering (1901) reported the first record in Brazil, where this species infests native as well as introduced fruit cultures (Malavasi *et al*.1980, Uchôa-Fernandes *et al*. 2002).

Precise identification of the species of fruit flies is clearly essential to impose quarantine restrictions to fruit exporter

countries, as well as in monitoring programs. The larva and pupa stages are typically the first ones to be intercepted during an infestation, but the identification is frequently verified only after adult's emergency. However, heretofore, few pest species have useful larval or pupal characters for identification. The arrangement of the posterior spiracular processes and of the mouth hooks of larval instars, as well as the structure of the spiracular plate of the puparium have been used as a basis to distinguish fruit fly species (Greene 1929, Steck & Malavasi 1988, Steck & Wharton 1988, Carroll & Wharton 1989). Yamada *et al.* (1963) utilized the characters on the anterior ends of the puparia for the identification of *C. capitata* and the oriental fruit fly *Bactrocera dorsalis* Hendel.

Biochemical and molecular techniques can be useful in the cases that request ready identification of the immature instars. Dadour *et al*. (1992) developed a test that allows distinguishing *C. capitata* from *B. tryoni* in the larval instar using the alcoholic dehydrogenase enzyme (ADH) as a marker. In New Zealand, a method for quarantine purposes, for identification of tephritids larvae to the specific level, using a molecular technique based on the analysis of RFLPs of ribosomic DNA amplified by PCR was developed (Armstrong *et al.* 1997). Using the technique of RAPD-PCR with DNA isolated from larvae, Sonvico *et al*. (1996) demonstrated the existence of polymorphism in *C. capitata* and *A. fraterculus*. This technique also allowed the separation of two populations of *A. fraterculus* originated from Argentina.

We apply the Heteroduplex Mobility Assay (HMA) method to the D2 domain of the 28S rRNA gene to get indications of multiple infestation by tephritid species of the genera *Anastrepha* and *Ceratitis*. HMA (Delwart *et al.* 1993, Gross & Nilsson, 1995, Fuller *et al.* 1996, Oldach *et al.* 2000) is one of the simplest and fastest PCR-based methods that can be used to detect genetic variability between different samples. HMA is based on the denaturing and reannealing mixtures of homologous PCR products. When two identical DNA fragments are mixed only the homoduplexes and single strand molecules are visualized in polyacrylamide gels. When slightly different DNA fragments are mixed, then homoduplexes, heteroduplexes and single strand molecules can be observed (Ramos *et al*. 2001).

The ribosomal DNA (rDNA) is a multigene family composed of a number of repeating units (RU) disposed in a tandem array. Each RU contains three genes: 18S, 5.8S and 28S. Transcribed spacers separate genes while intergenic spacers separate RU. Intragenomic homogeneity between RU was observed, and it seems that RU evolves as a unit (Arnheim 1983).

This phenomenon is called concerted evolution and suggests the existence of molecular mechanisms of homogenization (Coen *et al*. 1982). Dover (1982) proposed the molecular drive process to account for homogenization. This dynamic process includes gene conversion, unequal crossing-over and transposition and causes the continual turnover of RU in rDNA locus.

Expansion segments derive their names from their absence in prokaryotic ribosomal genes. They are variable in length and sequence between different eukaryotic species, and were studied especially in the genus *Drosophila* (Ruiz Linares *et al.*1991, Willians & Robbins 1992, Thompson *et al*. 1997). D2 is the largest expansion segment in *Drosophila*, and most of its variability is based on single base changes (Hancock *et al*. 1988). Strong selection pressure on expansion segments accounts for their slow evolutionary rate when compared to other regions in the RU (Ruiz Linares *et al.* 1991). Hence, the level of D2 expected polymorphism between individuals and different RU from the same species is very low (Ruiz Linares *et al*. 1991). In this work we chose the expansion segment D2 from 28S rRNA gene.

Additional morphological and molecular characters need to be explored to improve our knowledge of the relationships among the species within the various *Anastrepha* species groups (MacPheron *et al*. 1999, Norrbom *et al*. 1999). Thus, in this paper, we outline two morphological and molecular techniques for species of fruit flies of the genera *Anastrepha* and *Ceratitis*. The morphological technique can be used to separate species of fruit flies while for the molecular technique it is possible to demonstrate the presence of multiple infestation in a host fruit.

Table 1. Collection data and information on species sequenced and analyzed morphologically in this study

Species/ (group codes)	Collection data	Collection sites	Location	Altitude (m)	Color
A. obliqua (Ca1)	*Averrhoa carambola*, Oxalidaceae	Rio de Janeiro, Ilha do Governador,RJ,Brazil	22°49'03" S; 43°12'36" W	0 m	dark
A. sororcula (Mo5, Mo6)	*Psidium guajava*, Myrtaceae	Rio de Janeiro, Campo Grande, RJ, Brazil	22°54'12" S; 43°33'42" W	30 m	clear
A. serpentina(SNA9)	*Pouteria caimito*, Sapotaceae	Rio de Janeiro, Penha, RJ, Brazil	22°50'37" S; 43°16'28" W	111 m	dark,clear
A. serpentina(Mo10,Mo12)	*Pouteria caimito*, Sapotaceae	Rio de Janeiro, Campo Grande, RJ, Brazil	22°54'12" S; 43°33'42" W	30 m	dark,clear
C. capitata (Ca3, SNA8)	*Averrhoa carambola*, Oxalidaceae	Rio de Janeiro, Ilha do Governador, RJ,Brazil	22°49'03" S; 43°12'36" W	0 m	clear

Material and Methods

Fruit Collection. Fruit samples were collected from August 1999 to January 2000, in three urban areas in the municipality of Rio de Janeiro, RJ (Table 1). Infested fruits were collected from the ground underneath the tree canopy and transported to the laboratory. For each sample, infested fruits were placed in plastic trays over a sand (5-cm) layer to provide a pupation site for the larvae exiting the fruit.

Pupa Collection. The total of 1,076 puparia of frugivorous tephritids was obtained from five host fruit species sampled in four families of host plants. Pupae were sieved from sand under the fruit samples and selected in-groups in accordance to their colors and size 72h after the formation of puparium. These puparia were discriminated by host plant and sampling site, and were distributed in 26 groups identified by codes (data not shown). Groups of puparia with doubtful data (lack of emergence, hatching or/and small number of puparia in each group) were not used in this paper. The pattern of colors used for the separation of the groups of puparia was based on the table of colors described by Rocha & Amarante (1985) as follows: clear (yellow-ochre) and dark (reddish to brown). The size was established in agreement with a scale of three intervals for the length of the puparium according to Greene (1929): small (3 - 4.9 mm), medium (5 - 6.9 mm) and large (7 - 10 mm). Half of the selected puparia of each group were transferred individually to glass tubes with a layer of sand, which were discriminated with codes. The tubes were maintained at 25-27ºC, 65-70% UR and 14h photophase until the adults' emergence. The other half was preserved in freezer following the same separation methodology described above until morphologic puparia studies.

Species Identification. After the emergencies, the adults of each tube were transferred to maintenance cages. The flies were maintained in cages until the oviposition phase and fed with water and hydrolyzed corn protein. Eggs were collected in illuminated recipients (Bressan-Nascimento, unpublish.) and preserved in ethanol 70° until identification to species level. Eggs obtained from each cage were analyzed in conformity with the external characteristics described by Sein (1932) and Selivon & Perondini (2000) as a specific character. All females producing the analyzed eggs were individually identified. Female's specific identification was made following Steyskal (1977), White & Elson-Harris (1992) and Zucchi (2000). All identified specimens were preserved in ethanol 70°. Voucher specimens have been deposited in the Entomological Collection of the Instituto Oswaldo Cruz and in the Laboratory of Medical Entomology of the Instituto de Biofísica Carlos Chagas Filho/UFRJ (Rio de Janeiro State, Brazil).

Morphological Technique. The puparia preserved in freezer were described for size, color and external aspects of the posterior ends. Specimens were first boiled in water with commercial detergent until the tissues were sufficiently soft for observation. The puparia were mounted on strips of softwood (inserted in drilled holes of 3-mm diameter and about 4-mm deep), with the posterior (wider) ends up and the ventral sides toward the observer. All illustrations were done with the aid of a camera lucida coupled to the stereomicroscope model Wild M5. The number of specimens analyzed depended on the puparia in each sample of fruits. The magnification was registered on the scales that accompany the drawings. The measurements were taken with a micrometric ocular attached to the stereomicroscope.

The morphological terms proposed by Greene (1929), with some modifications for the description of the pupal stage, are adopted in this paper. The structures that constitute some of the main characters were defined as follows:

The position of structures was established in relation to a line drawn through the center of the anterior diameter of the puparium and emerging at the center of the posterior diameter end thereof.

Anal plate (An): A chitinous rounded visible area with paired organ (anal lobe).

Spiracular plate (Sp): A sclerite is heavily chitinized containing the button (bu) and three openings for respiration called spiracular entrances (Se) limited by the peritreme (pe).

Peritreme (pe): The chitin's ridge or elevation, which directly surrounds and limits a spiracular entrance.

Button (bu): A chitinous area or scar marking the place where the trachea opens on the surface of the first-stage larva.

Scar (s): The place in the larval integument where muscles were attached.

Hexagonal depression (hd): Area containing the spiracular plates.

Projection (p): Dark projection in central position and above the spiracular plates.

Specimens Used in DNA Analysis. Specimens collection data are listed in Table 1. All specimens used in this work were fixed and preserved in ethanol/methanol (1:1).

DNA Extraction. Fixed adults were hydrated in 0.2 ml of lysis buffer (150 mM Tris, 50 mM EDTA pH 8.0, 10 mM NaCl) for 15 min at room temperature. Genomic high molecular weight DNA was isolated following Ashburner (1989). DNA isolation was carried out from a pool of five specimens for each species.

PCR Analysis. The polymerase chain reaction (PCR) was used to amplify a 400 bp DNA fragment within the 28S rRNA gene (D2 expansion segment). The primers used were D23, 5'ATG TTA GAC TCC TTG GTC3' and D25 5'ACG TGA AAC TGC TTA GAG GTT3' (Ruiz Linares *et al*. 1991).

Reactions were performed in 50 mM KCl, 10 mM Tris pH 8.3, 2 mM $MgCl_2$, 100 µM each of dNTPs, 50 ng of genomic DNA, five units of *Taq* DNA polymerase (Biotools Ltd.), 25 pmol of each primer, in a final volume of 100 µl.

Amplifications were carried out in a GeneAmpPCR System 2400 (Applied Biosystems). Reaction conditions were as follow: 95°C 3 min; 35 cycles of 94°C 30 s, 55°C 1 min and 72°C 30 s, and a final extension step at 72°C for 1 min. One negative control (absence of template DNA)

was included for each set of amplifications.

Heteroduplex Mobility Assay. For heteroduplex formation, 1 µl of each PCR products was mixed with 7 µl of distilled water and 1 µl of 10x annealing buffer (1 M NaCl; 100 mM Tris, pH 8.0; 20 mM EDTA). DNAs were heated at 94°C for 90 s and rapidly cooled in ice. DNAs were separated by electrophoresis on a 5% polyacrylamide gel (Life Tech Electrophoresis Apparatus Model V16, 1.5 mm gels thick) at constant 250 V for 2h 30 min. Gels were stained with ethidium bromide (0.5 µg/ml) and photographed. Gels were reproduced three times in the same conditions using fresh PCR products.

DNA Sequencing. For DNA sequencing we purified PCR products using GFXTM PCR DNA purification kit (Amersham Biosciences). Automated DNA sequencing was carried out at the Instituto Nacional do Câncer (INCA, RJ, Brazil) using cycle sequencing with dye terminator in a MegaBACE DNA Analysis System (Amersham Biosciences). Sequences from both strands were obtained for each sample. Sequencing was repeated three times using fresh independent PCR reactions.

Data Analysis. The sequences obtained in this study were edited using SeqMan II (DNASTAR Inc.) and deposited in EMBL Bank, under the accession numbers AJ634702-AJ634703-AJ634704. Alignments were carried out with CLUSTALX (Thompson *et al*. 1997) using default parameters. All alignments were confirmed and edited manually, when necessary. Phylogenetic analysis was made using MEGA software (Molecular Evolutionary Genetics Analysis, 2.0 Beta Built 3, Kumar, S., Tamura, K., Jakobsen I. and Nei, 1991-2000).

Results and Discussion

The puparia of Diptera Cyclorrhapha exhibit an enormous variety in form and size, as described by Greene (1921, 1929), Yamada *et al.* (1963), Prins (1982) and Dadour *et al.* (1992). However, some common structural characteristics can be recognized, allowing the comparison of puparia from different species. In the tephritids, as for the other Diptera Cyclorrhapha, the hardened cuticle of the last larval instar, which is called a puparium, covers the pupae.

Samples of fruit collected in the field and held in bulk yielded many lots of pupae from various species of fruit flies. To determine which species of flies had been reared from host fruits, a method of identifying the puparia, as part of monitoring an invasive pest, is necessary. The conventional approach in species identification is to use only the characters on the larval stage and posterior ends of puparia that remain intact when the flies or parasites emerge, though the anterior parts of the puparia are broken off (Greene 1921, 1929). Characters of the entire puparia were used to identify *Bactrocera curcubitae* (Coquillett), *B. dorsalis* and *C. capitata* (Hardy 1949, Yamada *et al.* 1963).

In this work we analyzed only those puparia from which adults emerged and were positively identified. Eight out of the 26 groups of puparia were analyzed. Collection data on host plant and puparia for *Anastrepha* species and *C. capitata* are presented in Table 1. Other unidentified genera were included in the families Drosophilidae and Lonchaeidae. Four species out of the eight groups of frugivorous Tephritoidea were identified using characteristics of the egg external morphology described in Selivon and Perondini (2000) and morphological adult parameters described by Zucchi (2000) in general key for *Anastrepha* species registered in Brazil (Table 1). The species of genus *Anastrepha*: *A. obliqua* (Macquart) (Ca1), *A. sorocula* Zucchi (Mo5 and Mo6) and *A. serpentina* (Wiedemann) (SNA9, Mo10 and Mo12) were the most frequent. The first two are included in the group *fraterculus* according to Norrbom *et al.* (1999) and Zucchi (2000). Those species are particularly important from an economic point of view. Although *A. serpentina* is not economically important, Zucchi (2000) considers that due to the kind of fruits it attacks and the frequency of these attacks, it can reverse this situation becoming an economically important species. *C. capitata* (Ca3 and SNA8) is the only species of this genus in Brazil and it is easily identified by the diagnosis discussed by White & Elson-Harris (1992).

Morphological Characterization. Frozen puparia of the groups Ca1, Ca3, Mo5, Mo6, Mo10, Mo12, SNA8 and SNA9 from which the adult fly emerged were used as a basis for morphological analysis in this work (Table 1). The cylindrical puparia form was observed in the species of frugivorous Tephritoidea that were analyzed. The puparium may vary in size as well as in color. Our studies revealed differences between length ($F = 64.4$, $P > 0.05$) and width ($F = 69.9$, $P > 0.05$) of the puparia of the four species of the fruit flies identified (Fig.1). Our measurements for *C. capitata* and *A. serpentina* were performed based on the works by

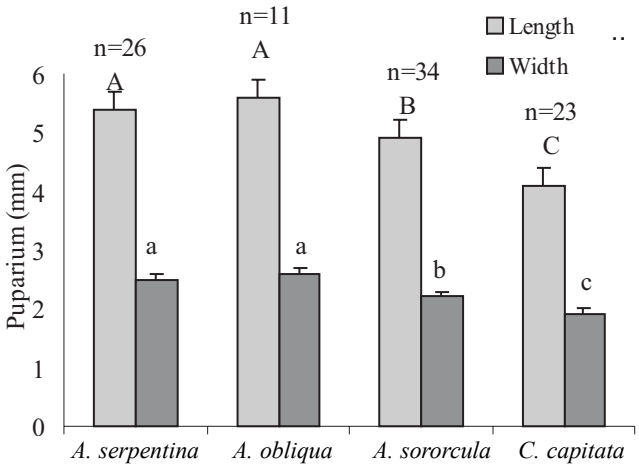

Figure 1. Measurements of the puparia of Tephritoidea (Diptera) from three species of *Anastrepha* and *C. capitata*, reared on host fruits from three urban areas in the municipality of Rio de Janeiro, Brazil. The averages followed by the same letters are not significantly different by Tukey test ($P > 0.05$).

Morgante (1991), Quesada-Allué *et al*. (1996). Meanwhile, the puparia variation in length as well as in width in all four species shows overlap, which makes the use of these characters unsuitable for species separation.

The color is an unequal character between the groups of puparia analyzed (Table 1). Puparium of *A. serpentina* and *A. obliqua* presents dark coloration, varying from the reddish to brown of several tonalities. *A. sororcula* and *C. capitata* present clear translucent puparia.

Frias (2001) observed morphologic differences among the egg, larva and pupa stages of two host races of *Rhagoletis conversa* (Bréthes) that live on *Solanum* plants. According to this author, in these host races the stability of some of the morphological characters in the immature stages contrasts with the existent high polymorphism in the adult stage.

The descriptive drawing of the posterior end of the puparium, presented in Fig.2, corroborates the expectation that the puparium of the fruit fly has taxonomic potential and can contribute to the understanding of phylogenetic and evolutionary aspects of the tephritids. The localization and shape of the spiracular plates and also of the anal opening in relation to the horizontal and vertical axis of the puparium constitute a group of characters that are not only easy of application but also, due to their constancy, renders them exceedingly reliable for diagnostic purposes. Further, in specific diagnosis of the puparia other characters can be used also as specific diagnosis, as follows: projection, depressed hexagonal and peritreme.

Descriptions of Puparia

Anastrepha serpentina Wiedemann, 1830
(Fig. 2)

Plant host: yellow star apple, *Pouteria caimito*, (R. & P.) Radlk (Sapotaceae)
Local of collection: Penha, RJ and Campo Grande, RJ
Group Codes: SNA9, Mo10 and Mo12

Cylindrical form, middle size, dark (yellow ocher to dark reddish). Posterior spiracle (Fig. 2,1), dark reddish, divided into two distinct areas. Each area is called a spiracular plate (Fig. 2,1,Sp) and is located in a very evident depressed hexagonal area which is on the central-horizontal line; each spiracular plate with three yellowish spiracular entrances, each on top of a well-defined ridge (peritreme), spiracular plates resembling a bean (Fig. 2,1,a). Just above the spiracular plates is a somewhat wide projection with a dark round spot on median depression. A small and weakly defined button. Just below the depressed hexagonal area are four scars. Large, rough and black anal plates. Length 6.4 mm to 4.9 mm and width 2.7 mm to 2.2 mm. Greene (1929) described the posterior spiracles for *A. serpentina* reared from nespero, star

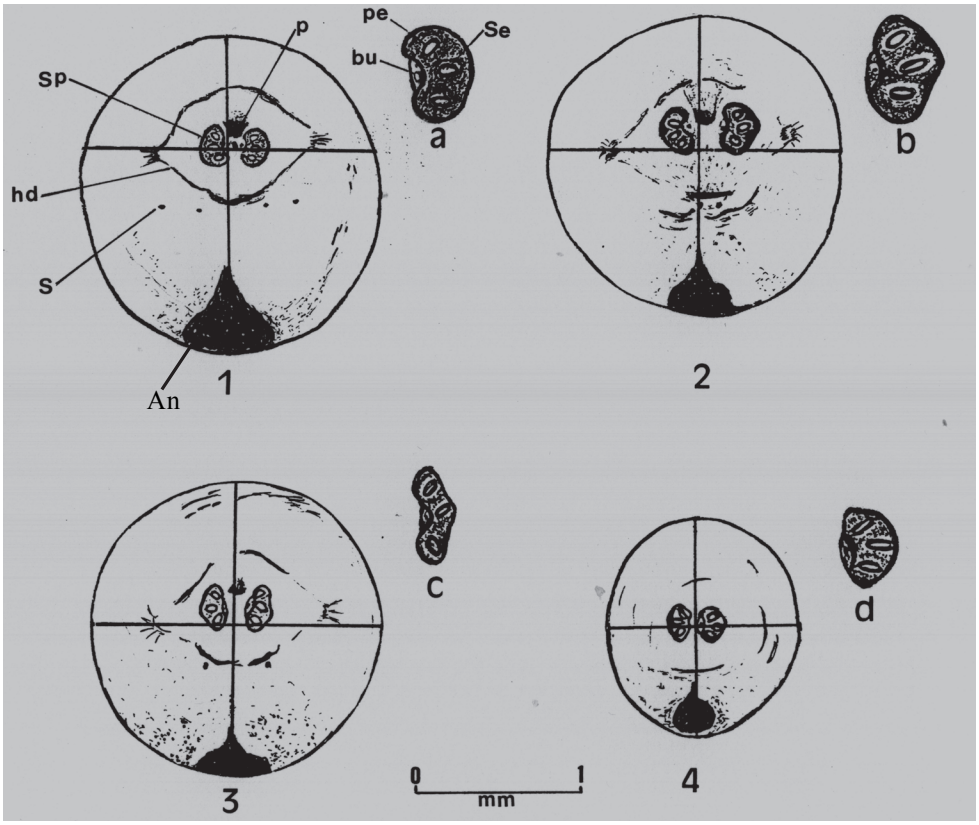

Figure 2. Frontal view of the posterior end of puparia. 1 - *Anastrepha serpentina*: a; spiracular plate; 2 - *A. obliqua*: b, spiracular plate; 3 - *A. sororcula*: c, spiracular plate; 4 - *Ceratitis capitata*: d, spiracular plate. An - anal plate; bu - button; hd - hexagonal depression; pe - peritreme; Se - spiracular entrances; p - projection.

apple, guava, and various Sapotaceae in Panama. Our specimens show difference in size of the puparium and details between spiracular plates (projection). Described from 26 specimens from the same source as the adult.

Anastrepha obliqua Macquart, 1835
(Fig. 2)

Plant host: star fruit, *Avehrroa carambola* L. (Oxalicaceae)
Local of collection: Ilha do Governador, RJ
Group Codes: Ca1

Cylindrical form, middle size, dark (yellow ocher to dark reddish), eleven segments lacking array of thorns. Posterior spiracle (Fig. 2,2), dark reddish, divided into two distinct areas, each area called a spiracular plate (Fig. 2,1,Sp), located in a faintly depressed hexagonal area which is on but mostly below the horizontal line; each spiracle with three narrow yellowish entrances on top of a well-defined ridge (peritreme). Spiracular plates resembling a triangular form (Fig. 2,2,b); a small and apparently wanting button. Just above the spiracular plates is a somewhat wide-triangular projection with a median depression. A not very large anal plate, forming an elliptical dark area. Length 5.9 mm to 5.3 mm; width 2.8 mm to 2.3 mm. Described from 11 specimens from the same source as the adult.

Anastrepha sororcula Zucchi, 1977
(Fig. 2)

Plant host: guava, *Psidium guajava* L. (Myrtaceae)
Local of collection: Campo Grande, RJ
Group Codes: Mo5 and Mo6

Cylindrical form, middle size, clear (yellow ocher), very translucent, eleven distinct segments, with band of thorns very evident. Posterior spiracles (Fig. 2,3), deep yellowish brown, divided into two distinct areas, each area called a spiracular plate (Fig. 2,1,Sp), located in a faintly depressed hexagonal area which is on but mostly below horizontal line; each spiracular plate having three fairly broad entrances each of which has the surrounding area paler on top of a well-defined ridge (peritreme), spiracular plates resembling an obtuse triangle (Fig. 2,3,c); button small and appearing to be separated from the plate. Just above the spiracular plates is a somewhat narrow projection with a median depression. Below the depressed hexagonal area are two scars. Large anal plate, forming an elliptical dark area. Length 5.0 mm to 2.1 mm; width 2.6 mm to 1.9 mm. Described from 34 specimens from the same source as the adult.

Ceratitis capitata Wiedmann, 1824
(Fig. 2)

Plant host: star fruit, *Averrhoa carambola*, L. (Oxalicaceae)
Local of collection: Ilha do Governador, RJ and Penha, RJ
Group: Ca3 and SNA8

Cylindrical form, small size, clear (yellow ocher), very translucent, eleven distinct segments. Spiracular plates (Fig. 2,4), reddish to dark red, divided into two distinct areas, each area called a spiracular plate (Fig. 2,**1**,Sp), located centrally on horizontal line. Each spiracle with three reddish brown entrances each of which has the surrounding area darker on top of a well-defined ridge (peritreme), spiracular plates resembling a semi-circle (Fig. 2,4,d); small and round button, and appearing to be separated from the plate. The hexagonal depression is absent. Small anal plate and surface with discreet roughness and dark red. Greene (1929) described the posterior spiracles for *C. capitata* reared from grape, peach, mango, apricot, pear, fig, orange passion flower, nectarine, guava and loquat in Panama. Our descriptions of puparia are in accordance to Greene (1929). Length 4.4 mm to 3.5 mm; width 2.1 mm to 1.5 mm. Described from 23 specimens from the same source as the adult.

Molecular Differentiation. After the DNA isolation from four tephritid species (five specimens for each species) we performed the molecular amplification of the D2 rDNA domain. Then we set combinations among the amplified samples to apply HMA. Finally we sequenced all amplified products (approximately 400 pb in length).

The HMA has been applied in a wide range of studies, such as estimating genetic divergence of phytoplasmas (Wang & Hiruki 2000), sequence similarity and bacterial diversity (Espejo *et al*. 1998), identification of mycobacterium (Waléria-Aleixo *et al*. 2000) and in the differentiation of *Saccharomyces* (Ramos *et al*. 2001). HMA is based on the denaturing and reannealing of mixtures of homologous PCR products. As a result, three kinds of bands can be observed on the polyacrylamide gel. First, perfectly matching nucleotide sequences result in reannealed homoduplexes bands (usually one) that are visualized at the bottom of the gel; imperfectly matching sequences result in heteroduplexes bands (usually two) that migrate more slowly than homoduplexes and finally single strands that migrate forming a diffuse band at the top.

All D2 HMA experiments between the species of the tested tephritids showed heteroduplex formation. When PCR products with similar D2 sequences were mixed, as observed in Fig. 3 (lane 6), the heteroduplexes appeared near the homoduplexes. Sequences with more nucleotide differences showed heteroduplexes distant from the homoduplexes (Fig. 3, lanes 1, 3 and 5).

Using the D2 rDNA domain it was possible to differentiate between species listed in Table 1. From the 29 species included in the *fraterculus* group, 17 of which occur in Brazil (Norrbom *et al.*1999, Zucchi 2000), we had specimens from the species *A. obliqua* and *A. sororcula*. We also had speciemens of *C. capitata* (external group), species introduced in Brazil in the beginning of the century, that infests both native and introduced fruits. Species of the *fraterculus* group infest a very diverse group of hosts, for example, *A. obliqua* infests 28 host species in Brazil (Zucchi 2000). The analysis of Fig. 4 shows the existence of a grouping formed by the species of the group *fraterculus*, *A. obliqua* and *A. sororcula*, being more related amongst themselves than to the others. These species are generalist; for example, *A. obliqua* has 70 reported hosts. Even these species have distinct preferences, however; for example, *A. obliqua* is the main species attacking

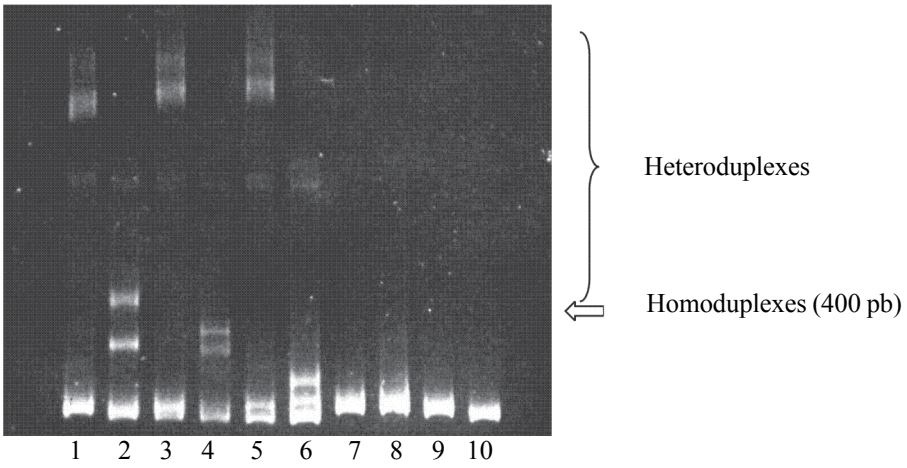

Figure 3. Heteroduplex mobility assay (HMA) patterns of D2 expansion segment region of the 28S rRNA gene. Lanes: 1 - *C. capitata* and *A. serpentina*; 2 - *A. sororcula* and *A. serpentina*; 3 - *A. sororcula* and *C. capitata*; 4 - *A. obliqua* and *A. serpentina*; 5 - *A. obliqua* and *C. capitata*; 6 - *A. obliqua* and *A. sororcula*; 7 - *A. serpentina*; 8 - *C. capitata*; 9 - *A. sororcula*; 10 - *A. obliqua*. (6% polyacrylamide gel stained with ethidium bromide).

Anacardiaceae, whereas *A. sororcula* is found mostly on Myrtaceae (Norrbom & Kim 1988). Another species of the genus *Anastrepha, A. serpentina*, was analyzed in this same work, showing that *A. serpentina* is generalist, attacks preferentially hosts including those belonging to the Sapotaceae family (Norrbom & Kim 1988). According to Norrbom (2004), the association of more primitive groups of species with fruits of this family suggests that these might have been the primitive hosts for *Anastrepha*.

Both NJ (neighbor-joining) and polyacrylamide gel analysis showed *A. obliqua* as more related to *A. sorocula* than *A. serpentina.*

The use of different host fruit by the same species makes it impossible to associate definitely puparial forms with specific fruits. Our study has shown that the identification of these four species of frugivorous tephritids is possible on the basis of puparia morphology. Yet, the results suggest that further attention should be directed to an examination of the puparium and HMA can only determine that more than one species is present in a fruit but fails to identify which one. The amplification and sequencing of D2 segments is necessary to make a species differentiation of the molecular level in host fruit. This paper suggests that it is very important to submit more gene sequences to data bank so that in the future we will have a complete set of gene sequences that will make possible the molecular identification in the genus *Anastrepha*.

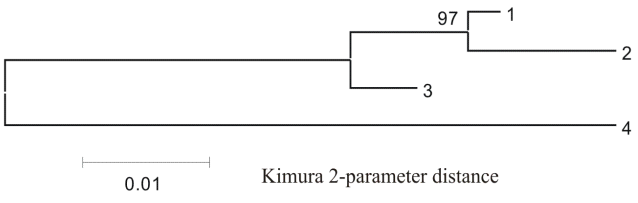

Figure 4. Unrooted tree showing Neighbor-Joining analysis based on D2 expansion segment sequences (Kimura 2-parameter distance, bootstrap test with 1000 replications). 1- *A. obliqua*, 2 - *A. sororcula*, 3 - *A. serpentina* and 4 - *C. capitata*.

Acknowledgments

We are very grateful to Dr. Miguel Angelo Martins Moreira (INCA, RJ, Brazil) for technical assistance in DNA sequencing. This work was supported by Fundação José Bonifácio (FUJB) and Universidade Federal do Rio de Janeiro (UFRJ).

Literature Cited

Armstrong, K.F., C.M. Cameron & E.R. Frampton. 1997. Fruit fly (Diptera: Tephritidae) species identification: A rapid molecular diagnostic technique for quaratine application. Bull. Entomol. Res. 87: 111-118.

Arnheim, N. 1983. Concerted evolution of multigene families, p.38-61. M. Nei & R.K. Koeh (eds.), Evolution of genes and proteins. Sunderland, MA, Sinauer Associates, 331p.

Ashburner, M. 1989. *Drosophila* a laboratory manual. Cold Spring Harbor Laboratory Press, 96p.

Carrol, L.E. & R.A. Wharton. 1989. Morphology of the immature stages of *Anastrepha ludens* (Diptera: Tephritidae). Ann. Entomol. Soc. Am. 82: 201-214.

Coen, E.S., J.M. Thoday & G. Dover. 1982. Rate of turnover of structural variants in the rDNA gene family of *Drosophila melanogaster*. Nature 295: 564-568.

Dadour, I.R., D.K. Yates & A. Postle. 1992. Two rapid diagnostic techniques for distinguishing Mediterranean

fruit fly from *Bractocera tryoni* (Froggat) (Diptera: Tephritidae). J. Econ. Entomol. 85: 208-211.

Delwart, E.L., E.G. Shpaer, J. Louwagie, B.B. MacCutchan, M. Grez, H. Rübsamen-Waigmann & J.L. Mullins. 1993. Genetic relationships determined by a DNA heteroduplex mobility assay: Analysis of HIV-1 *env* genes. Science 262:1257-1267.

Dover, G.A. 1982. Molecular drive: A cohesive mode of species evolution. Nature 299: 111-117.

Espejo, R.T., C.G. Feijóo, J. Romero & M. Vásquez. 1998. PAGE analysis of the heteroduplexes formed between PCR-amplified 16S rRNA genes: Estimation of sequence similarity and rDNA complexity. Microbiology 144: 1611-1617.

Fletcher, B.S. 1989. Life history strategies of Tephritid fruit flies, p.195-208. In A.S. Robinson & G. Hooper (eds.), Fruit flies their biology, natural enemies and control, v. 3B. Amsterdam, Elsevier, 447p.

Frías, D.A. 2001. Diferencias genéticas y morfológicas de los estados inmaduros de dos razas de *Rahgoletis conversa* (Bréthes) (Diptera: Tephritidae) asociadas a plantas *Solanum*: Distribuión geográfica y posible origen en simpatría de una nueva especie. Rev. Chil. Hist. Nat.74: 73-90.

Fuller, S.J., P.B. Mather & J.C. Wilson. 1996. Limited genetic differentiation among wild *Oructolagus cuniculus* (rabbit) populations in arid eastern Australia. Heridity 77: 138-145.

Greene, C.T. 1921. An illustrated synopsis of the puparia of 100 muscoid flies (Diptera). Proc. U. S. Nat. Mus. 60: 1-39.

Greene, C.T. 1929. Characters of the larvae and pupae of certain fruit flies. J. Agric. Res. 38: 489-504.

Gross, R. & J. Nilsson. 1995. Application of heteroduplex analysis for detecting variation within the growth hormone 2 gene in *Salmo trutta* L. (brown trout). Heredity 74: 286-295.

Hardy, D.E. 1949. Studies in Hawaiian fruit flies (Diptera, Tephritidae). Proc. Ent. Soc. Washington 51:181-205.

Hancock, J.M., D. Tautz & G.A. Dover. 1988. Evolution of the secondary structures and compensatory mutations of the ribosomal RNAs of *Drosophila melanogaster*. Mol. Biol. Evol. 5: 393-414.

Ihering, H. von. 1901. Laranjas bichadas. Rev. Agr. 6: 179-181.

MacPheron, B.A., H.Y. Han, J.G. Silva & A.L. Norrbom. 1999. Phylogeny of the genus *Anastrepha* and *Toxotrypana* (Trypetinae: Toxotrypanini) based upon 16SrRNA mitochondrial DNA sequences, p.343-361. In M. Aluja & A.L. Norrbom (eds.), Fruit flies (Tephritidae): Phylogeny and evolution behavior. Boca Raton, CRC Press, 944p.

Malavasi, A., J.S. Morgante & R.A. Zucchi. 1980. Biologia de "moscas-das-frutas (Diptera, Tephritidae). I - Lista de hospedeiros e ocorrência. Rev. Bras. Biol. 40: 9-16.

Morgante, J.S. 1991. Moscas-das-frutas (Tephritidae): Características biológicas, detecção e controle. Brasília, SENIR, Boletim Técnico. 2, 19p.

Norrbom, A.L. 2004. Fruit fly (Diptera: Tephritidae) classification & diversity. Available in http://www.sel.barc.usda.gov//diptera/tephriti/Tephclas.htm. Lat updated: November 10, 2004.

Norrbom, A.L. & K.C. Kim. 1988. A list of the reported host plants of the species of *Anastrepha* (Diptera: Tephritidae). Washington, USDA, APHIS 81-52.

Norrbom, A.L., R.A. Zucchi & V. Hernández-Ortiz. 1999. Phylogeny of the genus *Anastrepha* and *Toxotrypana* (Trypetinae: Toxotrypanini) based on morphology, p.299-342. In M. Aluja & A.L. Norrbom (eds.), Fruit flies (Tephritidae): Phylogeny and evolution behavior. Boca Raton, CRC Press, 944p.

Oldach, D., W., C.F. Delwiche, K.S. Jakobsen, T. Tengs, E.G. Brown, J.W. Kempton, E.F. Schaefer, H.A. Bowers, H.B. Glasgow Jr., J.M. Burkholder, K.A. Steidinger & P.A. Rublee. 2000. Heteroduplex mobility assay-guided sequence discovery: Elucidation of the small subunit (18S) rDNA sequences of *Pfiesteria piscicida* and related dinoflagellates from complex algal culture and environmental sample DNA pools. Proc. Natl. Acad. Sci. 97: 4303-4308.

Prins, A.J. 1982. Morphological and biological notes on six south African blow-flies (Diptera, Calliphoridae) and their immature stages. Ann. S. Afr. Mus. 90: 201-217.

Quesada-Allué, L.A., P. Wappner, G.L. Boccaccio & A. Rabossi. 1996. Metamorphosis in the Mediterranean Fruit Fly, *Ceratitis capitata*, p.445-457. In G.J. Steck & B.A. Macpheron (eds.), Fruit fly pest: A world assessment of their biology and management. Delray Beach, St. Lucie Press, 596p.

Ramos, J.P., P. Valente, R.A. de Souza, C.A. Rosa & O. Leoncini 2001. Heteroduplex mobility assay of the D1/D2 region of the 26S rDNA for differentiation of *Saccharomyces* species. Lett. Appl. Microbiol. 33: 206-210.

Rocha, C. & R. Amarante. 1985. Curso de desenho e pintura.v. I. São Paulo, Globo, 96p.

Ruiz Linares, A., Hancock, J.M. & Dover, G.A. 1991. Secondary structure constrains on the evolution of *Drosophila* 28S ribosomal RNA expansion segments. J. Mol. Biol. 219: 381-390.

Selivon, D. & A.L.P. Perondini. 2000. Morfologia dos ovos de moscas-das-frutas do gênero *Anastrepha*, p.49-54. In A. Malavasi & R.A. Zucchi (eds.), Moscas-das-frutas de importância econômica no Brasil: Conhecimento básico e aplicado. Ribeirão Preto, FAPESP-Holos, 327p.

Sein, F. 1932. *Anastrepha* (Trypetidae, Diptera) fruit flies in Puerto Rico. J. Puerto Rico. 17: 183-196.

Sonvico, A., F. Manso & L.A. Quesada-Allué. 1996. Discrimination between the immature stages of *Ceratitis capitata* and *Anastrepha fraterculus* (Diptera, Tephritidae) populations by random amplified polymorphic DNA polymerase chain reaction. J. Econ. Entomol. 89: 1208-1212.

Steck, G.J. & R.A. Wharton. 1988. Description of immature stages of *Anastrepha interrupta*, *A. limae*, and *A. grandis* (Diptera: Tephritidae). Ann. Entomol. Soc. Am. 81:994-1003.

Steck, G.J. & A. Malavasi. 1988. Description of immature stages of *Anastrepha bistrigata* (Diptera: Tephritidae). Ann. Entomol. Soc. Am. 81: 1004-1009.

Steyskal, G.C. 1977. Pictorial key to species of the genus *Anastrepha* (Dip.: Tephritidae). Entomol. Soc. Washington, 35p.

Thompson, J.D., Gibson, T.J. Plewniak, F. Jeanmougin & D.G. Higgins. 1997. The cClustal X windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 24: 4876-4882.

Uchôa-Fernandes, M., I. de Oliveira, R.M.S. Molina & R.A. Zucchi. 2002. Species diversity of frugivorous flies (Diptera: Tephritoidea) from hosts in the cerrado of the state of Mato Grosso do Sul, Brazil. Neotrop. Entomol. 31: 515-524.

Waléria-Aleixo, A., E.G. Kroon, M.A.S. Campos, M.E. Margutti-Pinto, C.A. Bonjardin & P.C.P. Ferreira. 2000. Heteroduplex mobility assay for rapid, sensitive and specific detection of mycobacteria. Micobacteriology 36: 225-235.

Wang, K. & C. Hiruki. 2000. Heteroduplex mobility assay detects DNA mutations for differentiation of closely related phytoplasma strains. J. Microbiol. Methods 41: 59-68.

Willians, S.M. & L.G. Robbins. 1992. Molecular genetic analysis of *Drosophila* rDNA arrays. TIG 8: 335-340.

White, I.M. & M.M. Elson-Harris. 1992. Fruit flies of economic significance: Their identification and bionomics. Wallingford, CAB, 601p.

Yamada, T., S. Nakagawa & H. Kamasaki. 1963. Identification of three species of reared Hawaiian fruit fly pupae. Proc. Hawaiian Entomol. Soc. 18: 319-321.

Zucchi, R.A. 2000. Taxonomia, p.13-24. In A. Malavasi & R.A. Zucchi (eds.), Moscas-das-frutas de importância econômica no Brasil: Conhecimento básico e aplicado. Ribeirão Preto, FAPESP-Holos, 327p.

Received 08/VII/04. Accepted 21/VII/05.