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Ann H. Fritz, *Eastern Illinois University*

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Department of Biological Sciences, Eastern Illinois University, 600 Lincoln Avenue, Charleston, IL 61920

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ABSTRACT Ribosomal DNA sequence data has been important in elucidating molecular evolution, discerning phylogenetic relationships, and in developing species diagnostics. Species in the genus *Anastrepha* Schiner, particularly members of the Fraterculus Species Group, are considered the most important native pests of fruit in the New World. This study presents the first complete rDNA sequence data spanning the 5.8S to the internal transcribed spacer ITS2 of a species in this genus, *Anastrepha suspensa* (Loew). The GC content of the rDNA genes in *A. suspensa* was 50.8 to 40% for the 5.8S and 2S genes, respectively, but spacers were >84% AT rich. Spacer and gene lengths were of similar size to those found in other tephritids. Except for *A. fraterculus* s.l. (Wiedemann), spacers of other tephritid taxa were AT rich and contained numerous simple repeats. Sequence alignment with *A. fraterculus* s.l. indicated minor differences in the 5.8S rRNA gene, but the ITS2a was identical and the 2S gene differed by a single transition. The ITS2 of *A. fraterculus* s. l. was unusual in having a 41.7% GC content with three regions of C repeats. The ITS2 of *A. suspensa* had higher percent identity values with five species in other genera than it did with *A. fraterculus* s.l., suggesting an unusually high divergence rate in the latter.

KEY WORDS *Anastrepha suspensa*, rDNA, ITS2, 5.8S, Tephritidae

THE NEW WORLD GENUS *Anastrepha* (Diptera: Tephritidae) includes >200 species, many of which are considered the most economically important native pests of fruiticulture in the Americas (Norrbom et al. 2000). The Caribbean fruit fly, *Anastrepha suspensa* (Loew), is endemic to the Greater Antilles (Norrbom and Foote 1989), including the islands of Cuba, Jamaica, Hispaniola, and Puerto Rico. *A. suspensa* was accidentally introduced into Florida in 1965 and quickly spread throughout most of the southern and central regions of the state (Weems 1965) where it infests a variety of tropical and subtropical fruit, such as guava, Surinam cherry, loquat, and ripe citrus. The rapid spread of *A. suspensa* in Florida prompted establishment of a biological control and mass rearing facility at the Florida State Division of Plant Industry, Gainesville, to develop sterile insect technique for this species and to provide flies for research purposes. Infestation of citrus, an important cash crop in Florida, led to export restrictions of oranges and grapefruit to Japan and certain regions of the United States, and implementation of the Caribbean Fruit Fly Pest Management System establishing protocols for fly-free zones for certifying citrus for export (Riherd et al. 1994, Clark et al. 1996).

Over the past three decades, sequence data on rDNA has been shown to be particularly informative for a variety of studies. For example, rDNA has been useful for establishing phylogenetic relationships at various levels of relatedness, because spacers evolve rapidly and the rRNA genes are relatively conserved (Tautz et al. 1987, 1988; Miller et al. 1996). In the tephritid fruit fly *Ceratitis capitata* (Wiedemann), polymerase chain reaction (PCR) amplification of the internal transcribed spacer (ITS) 1, and its subsequent digestion with a restriction enzyme, provide a molecular means of sexing flies (Douglas et al. 2004). Ribosomal DNA sequence data also has elucidated processes in molecular evolution (Glover 1977, Tautz et al. 1987, Hancock et al. 1988), including sequence diversification (Amako et al. 1996), homogenization, molecular drive, and concerted evolution (Dover 1982, Arnheim 1983, Gerbi 1985, Williams et al. 1985), male sterility, and meiotic drive (McKee et al. 1998, Robbins 1999). Because of the relatively rapid rate at which new mutants are fixed in the rDNA spacers, these regions also have been useful for distinguishing closely related species that otherwise show little genetic divergence (Brown et al. 1972, Furlong and Madden 1983, Tautz et al. 1987, Porter and Collins 1991) and may even distinguish populations (Williams et al. 1985). Because rDNA units occur in tandem-repeat arrays and undergo homogenization and concerted evolution (Arnheim 1983), sensitive species-specific probes and PCR primers have been developed from rDNA spacer sequences for a large number of taxa (Gallego and Galian 2001, Fritz et al. 2004, Wilkerson et al. 2004).

A. suspensa is a member of the Fraterculus Species Group (McPheron et al. 2000), which includes the most important fruit pests in the genus *Anastrepha.* Within this group is the Fraterculus Cryptic Species

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Complex, which includes at least four species (Selivon et al. 1999, 2001, 2005). To date, there are no published sequences of the nuclear rDNA of any species in the genus *Anastrepha* except for the 18S gene of *A. suspensa* and *Anastrepha ludens* Loew (Han and McPheron 1994) and the ITS2 of *Anastrepha fraterculus* s.l. (Wiedemann) (Sonvico et al. 1999). This study presents the complete rDNA sequence of spacers and genes flanked by the ITS1 and the 28S gene of *A. suspensa.* The organization of genes and spacers, their lengths, and their nucleotide compositions, are compared with equivalent sequences in other tephritid flies currently available in the literature or genetic databases. Also for comparative purposes, the complete sequence of *A. suspensa* is aligned with that of its presumptive close relative *A. fraterculus* s.l. Finally, a sequence percent identity matrix for the ITS2, among species whose complete sequence is available, is provided to illustrate an unusually high divergence between *A. fraterculus* s.l. and all other taxa.

Materials and Methods

Collection of Specimens and Isolation of DNA. Specimens of *A. suspensa* sequenced included one sample each from Clewiston, Fort Pierce, and Homestead, FL, collected in 2001, and a specimen from the laboratory colony maintained at the Division of Plant Industry, Gainesville, FL. DNA was isolated using DNeasy kits (QIAGEN, Valencia, CA) following the instructions for the isolation of DNA from animal tissues.

PCR and Sequencing. A region of the nuclear rDNA, which included a section spanning the 3′ end of the ITS1 to the 5′ end of the 28S rRNA gene, was amplified using two sets of overlapping primers. One forward primer annealed to a conserved region in the 18S gene (5′-TAGGTGAACCTGCGGAAGGA-3′) and its reverse primer within a conserved region of the 5.8S gene (5′-GTACGTAACAGCATGGACTG-3′). The second pair of primers included one annealing to the 5.8S (5′-TGTGAACTGCAGGACACATG-3′) and overlapping the sequence of the first section mentioned above. The reverse primer annealed to a conserved region in the 28S gene (5′-ATGCTTAAATTCAGGGGGTAGTC-3′). All amplifications were done with the following touchdown temperature profile in a thermocycler: five cycles at 96°C for 20 s, at 60°C for 30 s, and at 72°C for 1 min; 21 cycles at 96°C for 30 s, at 60°C minus 0.5°C per cycle for 30 s, and at 72°C for 1 min; and last, 10 cycles at 96°C for 30 s, at 55°C for 30 s, and at 72°C for 1 min. Amplicons were subsequently ExoSAP-IT purified, quantified, and sequenced in forward and reverse reactions. Individual forward and reverse sequences were examined using Sequencher 3.0 (Gene Codes Corp., Ann Arbor, MI) and aligned with multiple sequences using the Clustal W alignment program (Thompson et al. 1994). Because of the conservation of nuclear rRNA genes and the ITS2a, it was possible to establish consistent gene and spacer delineations by sequence alignment with the complete rDNA of *Drosophila melanogaster* (Meigen) (Tautz et al. 1988). Thus, the delineations reported by Tautz et al. (1988) were used as a sequence standardization "key" adhered to for all sequence comparisons in this study.

Table 1. Size (and percentage of GC content) of four regions of the nuclear rDNA of *A. suspensa*, in representative tephritids, and in *D. melanogaster*

Species	5.8s	ITS2a	2S	ITS2
A. suspensa	124 (50.8)	30 (13.3)	20 (40.0)	450 (15.8)
A. fraterculus	125 (48.8)	30 (13.3)	20 (45.0)	384 (41.7)[a]
C. capitata	120 (48.3)	30 (20.0)	20 (40.0)	570 (20.0)
B. curcurbitae	123 (49.6)	28 (17.9)	20 (40.0)	457 (20.6)
R. completa	Incomplete	28 (21.4)	20 (40.0)	552 (19.2)
D. melanogaster	123 (50.4)	28 (17.9)	20 (40.0)	396 (20.2)

[a] May be a partial sequence.

The rDNA sequence of *A. suspensa* was aligned with the only other comparable sequence data available for a species in the same genus, *A. fraterculus* s.l. from Argentina. The complete region spanning the 5.8S to the ITS2 has not been published for *A. fraterculus* s.l., but it can be obtained from three different, but overlapping sequence entries, to GenBank (accession nos. AF210891, AY693651, and AY775552). However, the entry for the ITS2 of *A. fraterculus* s.l. (AY693651), published by Sonvico et al. (1999), may be incomplete, because the 3′ end does not seem to include a region that is conserved in all other tephritid flies sequenced to date. Summary sequence statistics of *A. suspensa*, including length and percentage of GC for spacers and genes, were compared with representative species of other genera in the family Tephritidae for which complete and comparable sequence data were available (accession nos. AF189691, AF276515, AY766112, AF276516, M21017, AY209010, and AF332590; Table 1). Because the ITS2 of *A. suspensa* aligned poorly with that of *A. fraterculus* s.l., this spacer was further examined comparatively by generating a pairwise percent identity matrix (using the ALIGN program by Myers and Miller 1988) for a number of species in other tephritid genera for which complete ITS2 data were available. This comparison included *Rhagoletis completa* Cresson, *C. capitata*, and four species within the genus *Bactrocera*, which provided a comparative example of intra-generic percent identity values: *Bactrocera dorsalis* (Hendel), *Bactrocera cucurbitae* (Coquillett), *Bactrocera tau* (Walker), and *Bactrocera pyrifoliae* Drew & Hancock. The rDNA sequence of *A. suspensa* reported in this article has been deposited in the GenBank database (accession no. DQ279855).

Results and Discussion

The rDNA of tephritid fruit flies is organized in a similar manner as that in the Drosophilidae as described by Tautz et al. (1988): the 18S gene is separated from the 5.8S by the ITS1, and there are two spacers that separate the 5.8S from the 28S rRNA genes, the ITS2a and the ITS2. The latter two spacers flank the 2S ribosomal unit (Jordan 1974, Jordan and Glover 1977). The rDNA region of *A. suspensa* se-

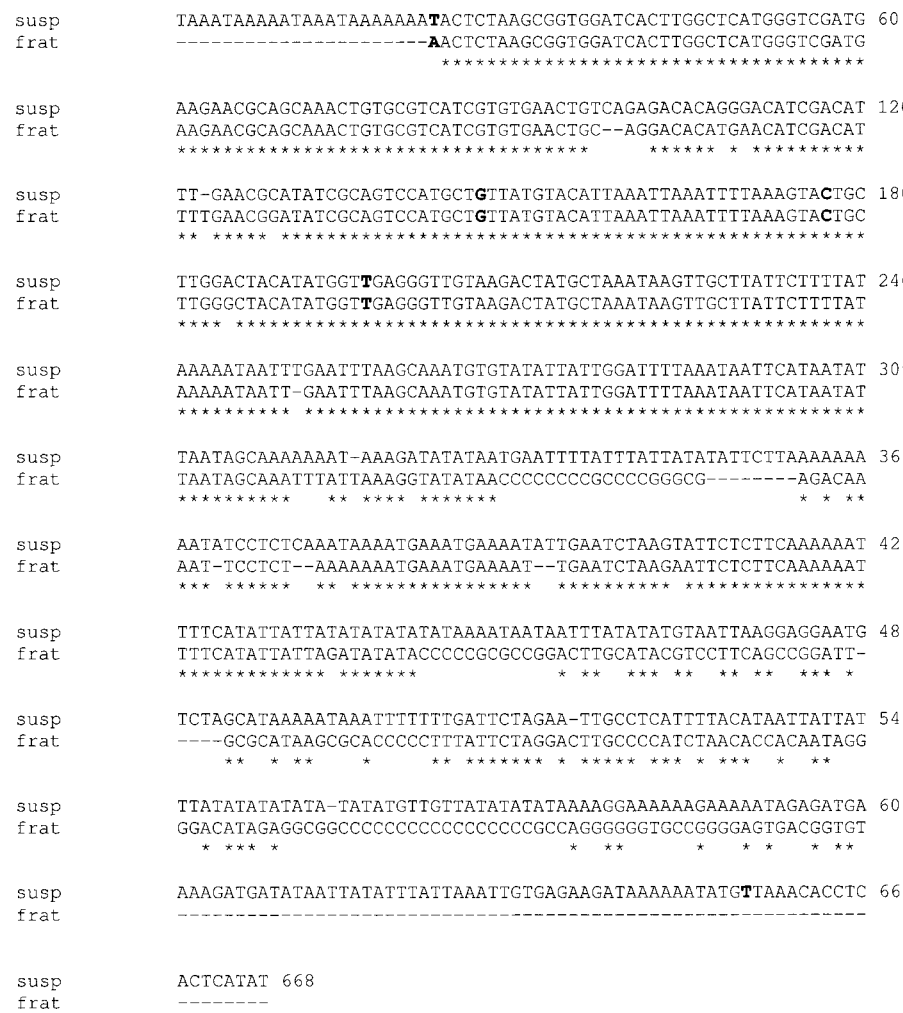

Fig. 1. Sequence alignment of the nuclear rDNA of *A. suspensa* and *A. fraterculus* s.l. The beginning of the 5.8S, the ITS2a, 2S, ITS2, and 28S are at nucleotides 23, 147, 177, 197, and 650, respectively, and are shown in bold letters. Asterisks indicate sequence similarity.

quenced in this study included a short section of the ITS1, the complete 5.8S, ITS2a, 2S, ITS2, and the beginning of the 28S gene (Fig. 1). Sequences of specimens from all three collection sites and the laboratory colony from the Division of Plant Industry had identical sequences. Because this study used PCR sequencing, it should be noted that these are consensus sequences and do not discount possible intraindividual sequence polymorphisms.

The percentage of GC content of the rDNA genes in *A. suspensa* is 50.8 to 40% for the 5.8S and 2S genes, respectively. Alternatively, the two spacers are AT rich (>84%). Spacer and gene lengths are similar in size to those found in *A. fraterculus* s.l. and representative species in other genera of the family Tephritidae (Table 1). Except for *A. fraterculus* s.l., spacers of all tephritid taxa examined, and that of *D. melanogaster*, are similarly AT rich and contain numerous regions of simple repeats (Fig. 1; Table 1). Large repeats, however, were not discernible in any part of the rDNA sequenced for *A. suspensa.* The ITS2 of *A. fraterculus* s.l. was unusual in having a 41.7% GC content and three relatively long regions of C repeats. Even without these repeats, the ITS2 of *A. fraterculus* s.l. was >30% GC.

Sequence alignment of *A. suspensa* with *A. fraterculus* s.l. (Fig. 1) indicated minor sequence differences in the 5.8S rRNA gene. The ITS2a of both species was identical and the 2S gene differed by a single transition. Although spacers are commonly thought to be regions of the genome in which mutations are fixed at a high rate (because they do not code for an RNA), the ITS2a seems to be an exception. The size and sequence of the ITS2a were very similar in all tephritid species and differed from that of *D. melanogaster* by only four point mutations and one indel. This apparent conservation suggests sequence constraints that may have to do with posttranscriptional RNA processing.

Table 2. Pairwise matrix of sequence percent identity for the ITS2 of *A. suspensa*, *A. fraterculus*, *B. dorsalis*, *B. curcurbitae*, *B. tau*, *B. pyrifoliae*, *C. capitata*, and *R. completa*

Species	*A. sus*	*A. fra*	*B. dor*	*B. cur*	*B. tau*	*B. pyr*	*C. cap*	*R. com*
A. sus	100							
A. fra	60.6	100						
B. dor	67.2	50.0	100					
B. cur	66.2	54.0	77.0	100				
B. tau	67.2	54.2	78.6	96.2	100			
B. pyr	64.5	51.0	92.1	77.2	78.4	100		
C. cap	58.0	43.5	63.3	60.3	61.1	63.7	100	
R. com	62.6	46.6	66.8	64.5	65.4	65.7	65.9	100

Alternatively, the ITS2 of *A. suspensa* and *A. fraterculus* s.l. were very different and had only a 60.6% identity value (Table 2). This low value is unusual considering both species have been placed in the same Species Group (McPheron et al. 2000) as presumptive close relatives. In contrast, four species in the genus *Bactrocera* had percent identity values ranging from 77.0 to 96.2% (Table 2). Surprisingly, the ITS2 of *A. suspensa* had a greater percent identity with most species in other genera than it did with *A. fraterculus* s.l. (Table 2). Thus, the dissimilarity between both species seems primarily to be because of a high divergence rate in *A. fraterculus* s.l. If this level of divergence is indicative of a similar rate of sequence divergence for the other members of the Fraterculus Cryptic Species Complex, then the ITS2 should be a good candidate spacer for the development of species specific molecular diagnostics of this complex. Ribosomal DNA spacer sequences have commonly been used for the development of species-specific primers and probes, particularly for identifying cryptic insect species complexes and for identifying immature stages that are difficult to resolve with standard morphological keys (Porter and Collins 1991, Crabtree et al. 1995, Charlwood and Edoh 1996, Fritz et al. 2004, Wilkerson et al. 2004). Identification of tephritid fruit flies relies primarily on the rearing of larvae or eggs to adults. Because this procedure is lengthy and often unsuccessful, rDNA spacers have been proposed as a more efficient and accurate means of identifying species (Armstrong and Cameron 2000). For example, a method using PCR and restriction fragment length polymorphism of rDNA is currently used in New Zealand to identify fruit flies in infested fruit arriving at international ports (Armstrong and Cameron 2000). The results of this study indicate that the ITS2 of two species in the genus *Anastrepha* have diverged substantially. Additional sequence data of the ITS2 for other members of this genus will determine whether this apparent high rate of divergence is limited to *A. fraterculus* s.l. or is representative of the Fraterculus Group as a whole.

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