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Diagnostic characters within ITS2 DNA support molecular identification of *Anastrepha suspensa* (Diptera: Tephritidae)

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The Caribbean fruit fly, *Anastrepha suspensa* (Loew) (Diptera: Tephritidae), is a pest of loquat, Surinam cherry, tropical almond, guava, and rose apple in Florida and capable of developing on a wide range of less preferred hosts (White & Elson-Harris 1992; Weems et al. 2001). It is the only major *Anastrepha* pest species that is established in Florida (Sutton & Steck 2005). Other exotic *Anastrepha* species are capable of using similar hosts and are attracted to the same trapping lures. Consequently, accurate identification of flies captured in Florida as *A. suspensa*, or as not being *A. suspensa*, is important in detecting new invasive species and facilitating safe trade.

Expert identification of *A. suspensa* is performed using adult morphology, and confirmation can require examination of the female aculeus (White & Elson-Harris 1992; Norrbom et al. 2012). Adult males and especially the immature life stages are more difficult to identify reliably. For example, *A. suspensa* can be confused with other pests in the *fraterculus* species group such as *A. ludens* (Loew), *A. obliqua* (Macquart), and *A. fraterculus* (Wiedemann) (Norrbom et al. 1999, 2012). These 4 species are included in the internationally adopted diagnostic protocol for *Anastrepha* pests (International Plant Protection Convention ISPM 27 Annex 9, https://www.ippc.int/en/core-activities/standards-setting/ispms/). Only 3 other species of major economic importance are included in that protocol: *A. grandis* (Macquart), *A. striata* Schiner, and *A. serpentina* (Wiedemann). These 3 pest species belong to 3 different species groups in the genus (Norrbom et al. 1999).

Molecular diagnostic methods for *Anastrepha* species have been explored as a way to supplement morphological characters (Armstrong et al. 1997; Armstrong & Ball 2005). No study has yet demonstrated diagnostic specificity for *A. suspensa* based on good sampling of both species and populations. Variation in the mitochondrial cytochrome oxidase I (*COI*) gene shows that DNA barcoding will not reliably distinguish all flies in the *fraterculus* species group (Frey et al. 2013). Our *COI* data support this observation for *A. suspensa* (Barr unpublished; GenBank accession numbers KU511143–KU511157).

In this study, we tested whether a portion of the nuclear encoded internal transcribed spacer 2 (ITS2) between the *5.8S rRNA* and *28S rRNA* genes can aid in diagnosis of *A. suspensa*. The ITS regions have been useful in discriminating closely related *Bactrocera* species (Diptera: Tephritidae) (Boykin et al. 2014) and a diversity of other organisms (Coleman et al 2009). We generated DNA sequences of ITS2 of flies from populations of 5 pest species in the *fraterculus* group (i.e., *A. suspensa*, *A. ludens*, *A. obliqua*, *A. fraterculus*, and *A. distincta* Greene) to identify characters useful for species identification (Table 1). Representatives of the species *A. grandis*, *A. serpentina*, and *A. striata* were also included in the study. DNA samples for a subset of the specimens in our study were included in prior genetic studies that analyzed mitochondrial and microsatellite DNA (Boykin et al. 2010; Ruiz-Arce et al. 2012, 2015). For new samples, DNA was extracted from a leg of each specimen using the DNeasy® Blood & Tissue Kit for animal tissue (Qiagen, Valencia, California). The rest of the fly body is maintained as a voucher at the United States Department of Agriculture facility in Texas. Morphological identifications of fly specimens were performed by A. Norrbom, D. Thomas, or G. Steck (Florida Department of Agriculture and Consumer Services, Division of Plant Industry, Florida).

Polymerase chain reaction (PCR) was performed using a forward primer of Ji et al. (2003), CAS5p8Ft (5'-TGAACATCGACATTTYGAACGCATAT), and a reverse primer, AsusR1 (5'-TTTTCATTTCATTTTATTTGAGAGG), that was selected using Primer3 (Untergrasser et al. 2012) and an *A. suspensa* sequence (GenBank accession number KT594196). The targeted ITS2 region for the primer set is approximately 220 bp. Cycling conditions for the reactions were 94 °C for 3 min; 39 cycles of 94 °C for 20 s, 50 °C for 40 s, and 72 °C for 30 s; and 72 °C for 5 min. Reactions were performed in 25 µL volumes and final concentrations of 1× buffer, 2 mM $MgCl_2$, 0.2 mM each dNTP, 0.4 µM each primer, and 0.625 Units TaKaRa Ex Taq® DNA Polymerase (TaKaRa, Mountain View, California) per reaction. Each reaction included 2 µL of template or water as a negative control. PCR products were visualized on 1% agarose gels stained with ethidium bromide, purified using ExoSAP-IT® PCR Product Cleanup (Affymetrix USB, Santa Clara, California), and sequenced by GeneWiz LLC (South Plainfield, New Jersey) in both directions. The raw trace files were edited and consensus sequences generated using Sequencher® v5 (GeneCodes, Ann Arbor, Michigan). The edited sequences were aligned in MEGA5 (Tamura et al. 2011), and unique DNA sequences were identified by visual inspection. Each nucleotide site

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Table 1. Collection locations and ITS2 genotypes of *Anastrepha* species included in this study.

Taxon	ITS2 genotype	Location (*n*)	*n*
A. suspensa	Type 1	Dominican Republic (9); Jamaica (1); Puerto Rico (8); USA: Florida (18)	36
	Type 2	Cayman Islands (1); Puerto Rico (1); USA: Florida (12)	14
	Unknown	Puerto Rico (1); USA: Florida (4)	5
A. obliqua	Type 1	Barbados (1); Bolivia (2); Colombia (5); Costa Rica (1); Guatemala (5); Mexico (17); Panama (9); Peru (1)	41
	Type 2	Barbados (1); Belize (1); Costa Rica (3); Jamaica (1); Mexico (16); Panama (4); Peru (1)	27
	Type 3	Panama (1)	1
	Unknown	Colombia (1)	1
A. distincta	Type 1	Guatemala (1)	1
	Type 2	Guatemala (3)[a]; Mexico (1); Panama (9)[a]; Peru (7)	19
A. ludens	Type 1	Costa Rica (2); Guatemala (1); Mexcio (3); Panama (3); USA: Texas (3)	12
	Type 2	Costa Rica (1); Honduras (2); Panama (1)	4
	Type 3	Mexico (1)	1
A. fraterculus	Type 1	Guatemala (1); Mexico (7)[a]	8
	Type 2	Belize (3); Guatemala (4)	7
	Type 3	Guatemala (1); Peru (1)	2
	Type 4	Peru (2)	2
	Type 5	Peru (6)	6
	Type 6	Peru (1)	1
	Type 7	Bolivia (2)	2
	Type 8	Bolivia (2)	2
	Type 9	Bolivia (1)	1
	Type 10	Peru (6)	6
	Type 11	Bolivia (2)	2
	Type 12	Bolivia (2)	2
A. serpentina	Type 1	Belize (1); Mexico (1)	2
A. striata	Type 1	Bolivia (1)	1

[a]One fly from each of these populations generated a sequence <200 bp and was not submitted to GenBank.

was inspected for 5 possible character states: A, C, T, G, or a gap that is indicated with a dash ("–") in the alignment.

Two hundred and six flies were genotyped using this PCR protocol: 55 *A. suspensa*, 41 *A. fraterculus*, 70 *A. obliqua,* 17 *A. ludens*, 20 *A. distincta*, 2 *A. serpentina*, and 1 *A. striata*. Of these flies, 60 were previously analyzed using a different primer set (Scally et al. 2016) and those data are available from GenBank: *A. suspensa*, *n* = 2 (DQ279855, KT594196); *A. ludens*, *n* = 3 (KT594193–95); *A. obliqua*, *n* = 26 (KT594200–225); *A. fraterculus*, *n* = 18 (KT594187–92, KT594226–29, KT594238–45); and *A. distincta*, *n* = 11 (KT594183–86, KT594231–37). The new protocol failed to amplify ITS2 from multiple *A. grandis* specimens suggesting that it is not appropriate for analysis of all *Anastrepha* species. Consistent with prior work by Sutton et al. (2015) that had difficulty sequencing segments of fruit fly DNA containing nucleotide strands with multiple repeats of a single base (i.e., homopolymers), the reverse primer did not perform well as a sequencing primer for approximately 33% of the samples. These failures occurred across species. Bases were treated as ambiguous if (1) both sequenced strands called multiple bases at a site or (2) neither strand generated a high quality, but distinct, base call. The new ITS2 sequences >200 bp in length were submitted to GenBank with accession numbers KU510999–KU511142.

Twenty-four unique genotypes were observed in the data set (Table 1). Consensus sequences with ambiguous base calls were classified as unknown but were compared with other records to confirm diagnostic characters. An alignment of 22 unique genotypes from the *fraterculus* group species was 220 bp (Table 2). The aligned ITS2 region included the short ITS2a spacer region (sites 1–21), the *2S* gene (sites 22–41), and part of the ITS2 spacer region (sites 42–220) according to Fritz (2006).

We did not detect the presence of alternate intra-individual copies of ITS2 for *A. suspensa*, *A. obliqua*, *A. ludens*, or *A. fraterculus* using our protocol. However, when confirming the ITS2 sequences of specimens reported by Scally et al. (2016) with our new protocol, we did detect evidence of alternative copies within a single specimen of *A. distincta*. Scally et al.'s (2016) study generated the type 1 genotype (GenBank accession number KT594183) and our study generated the type 2 genotype (GenBank accession number KU522208). The 2 types differ only by the presence of the doublet "AA" at sites 207–208 in type 1. This finding suggests that at least 2 different copies of ITS2 are in that specimen. It is possible that the primer set in our study did not amplify the ITS2 copy reported by Scally et al. (2016). Alternative experiments using cloning or next generation sequencing will be explored to follow up on these results.

Intra-species variation of ITS2 was observed for each species in the *fraterculus* group. *Anastrepha fraterculus*, known to comprise a cryptic species complex (Hernández-Ortiz et al. 2012; Sutton et al. 2015), had the greatest number of types (*n* = 12). The counts for *A. ludens*, *A. obliqua*, and *A. distincta* each had between 2 and 3 types (Table 1).

The alignment of the 22 unique sequences demonstrated a high rate of invariant sites (96%) in this fragment. Diagnostic (apomorphic) states were observed for 3 species and are highlighted in Table 2: *A. obliqua* at site 8 (C versus A); *A. ludens* at sites 186 to 187 (TT versus "-" gap in other *fraterculus* group species); and *A. suspensa* at sites 95 (T versus gap) and 218 (T versus A). The unique sequences and diagnostic character states observed in our study were also confirmed by comparing them with ITS2 sequences of other *Anastrepha* species from GenBank (Scally et al. 2016: KT594179–82 [*A. canalis* Stone]; KT594197–99 [*A. zuelaniae* Stone]; KT594230 [*A. schultzi* Blanchard]).

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Table 2. Variable sites in alignment of *Anastrepha* ITS2 genotypes from 5 *fraterculus* group species.

	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2	2	2	2	2	2	2	2	2
	9	0	1	1	5	5	5	5	5	7	7	7	7	8	8	8	8	8	9	9	9	0	0	0	0	0	0	1	1	1
	8	5	7	2	9	3	4	5	6	8	1	5	8	9	0	1	4	6	7	6	8	9	4	5	6	7	8	9	4	6	8
A. suspensa Type 1	A	T	A	G	—	—	—	—	—	A	—	T	T	G	A	A	T	—	—	T	—	—	C	T	T	—	—	A	A	A	T
A. suspensa Type 2	A	T	A	G	—	—	—	—	—	G	—	T	T	G	A	A	A	—	—	T	—	—	C	T	T	—	—	A	A	A	T
A. obliqua Type 1	C	—	A	G	—	—	—	—	A	A	—	T	T	G	A	A	T	—	—	T	—	—	C	T	T	T	—	—	A	A	A
A. obliqua Type 2	C	—	A	G	—	—	—	—	A	A	—	T	T	G	A	A	T	—	—	T	—	—	C	T	T	T	—	A	A	A	A
A. obliqua Type 3	C	—	A	G	—	—	—	—	A	A	—	A	T	G	A	A	T	—	—	T	—	—	C	T	T	T	—	—	A	A	A
A. distincta Type 1	A	—	T	G	—	—	—	—	—	A	—	T	T	G	A	A	T	—	—	T	—	—	C	T	T	A	A	A	A	A	A
A. distincta Type 2	A	—	T	G	—	—	—	—	—	A	—	T	T	G	A	A	T	—	—	T	—	—	C	T	T	—	—	A	A	A	A
A. ludens Type 1	A	—	A	G	—	—	—	—	—	A	T	T	T	G	A	A	T	T	T	T	T	A	C	T	T	A	A	A	A	A	A
A. ludens Type 2	A	—	A	G	—	—	—	—	—	A	—	T	T	G	A	A	T	T	T	—	T	A	C	T	T	—	A	A	A	A	A
A. ludens Type 3	A	—	A	G	—	—	—	—	—	A	T	T	T	G	A	A	T	T	T	T	T	A	C	T	T	—	A	A	A	A	A
A. fraterculus Type 1	A	—	A	—	—	—	—	—	—	A	—	T	T	A	A	A	T	—	—	T	—	—	C	T	T	A	A	A	A	A	A
A. fraterculus Type 2	A	—	A	—	T	—	—	—	—	A	—	T	T	G	A	A	T	—	—	T	A	—	C	T	T	—	—	A	A	A	A
A. fraterculus Type 3	A	—	T	G	—	—	—	—	—	A	—	T	T	G	A	A	T	—	—	T	—	—	C	T	T	—	—	A	A	A	A
A. fraterculus Type 4	A	—	T	G	—	—	—	—	A	A	—	T	T	G	A	A	T	—	—	T	—	—	T	—	—	—	A	A	A	A	A
A. fraterculus Type 5	A	—	T	G	—	—	A	A	A	A	—	T	T	G	A	A	T	—	—	T	—	—	T	—	—	—	A	A	A	A	A
A. fraterculus Type 6	A	—	T	G	—	A	A	A	A	A	—	T	T	G	A	A	T	—	—	T	—	—	T	—	—	—	A	A	A	A	A
A. fraterculus Type 7	A	—	A	G	—	—	—	—	A	A	—	T	T	G	A	A	T	—	—	T	—	—	C	T	T	A	A	A	A	A	A
A. fraterculus Type 8	A	—	A	G	—	—	—	—	A	A	—	T	T	G	A	A	T	—	—	T	—	—	C	T	T	—	A	A	A	A	A
A. fraterculus Type 9	A	—	A	G	—	—	—	—	A	A	—	T	T	G	A	A	T	—	—	T	—	—	C	T	T	—	—	A	A	A	A
A. fraterculus Type 10	A	—	T	G	—	—	—	—	—	A	—	T	—	—	—	—	T	—	—	T	T	A	C	T	T	—	—	A	A	A	A
A. fraterculus Type 11	A	—	A	G	—	—	—	—	—	A	—	T	T	G	A	A	T	—	—	T	—	—	C	T	T	T	—	A	C	T	A
A. fraterculus Type 12	A	—	T	G	—	—	—	—	—	A	—	T	T	G	A	A	T	—	—	T	—	—	C	T	T	—	—	A	A	A	A

The shading indicates diagnostic character states, and a dash ("—") indicates a gap in the alignment.

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Implementation of our ITS2 results for identification of pests may not be straightforward in all cases because species can have multiple genotypes. If the ITS2 sequence of a captured fly does not match perfectly with one of our genotypes, it is not possible to determine whether or not it is one of the studied species. In such cases, it is more conservative to report the mismatch as inconclusive.

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Summary

An approximately 220 bp fragment of the internal transcribed spacer 2 (ITS2) was screened as a diagnostic trait of *Anastrepha suspensa* (Loew) (Diptera: Tephritidae) and other pest fruit fly species in the genus *Anastrepha*. The majority (96%) of the sites in this fragment were invariant among the test species, but *A. suspensa* can be separated from other species by using 2 diagnostic characters. Similarly, *A. ludens* (Loew) and *A. obliqua* (Macquart) can be distinguished from other species based on 1 fixed character each. There is evidence of intraspecific ITS2 variation in 5 species tested, consistent with species complexes and incomplete homogenization through the process of concerted evolution.

Key Words: fruit fly; *Anastrepha ludens*; *Anastrepha obliqua*; concerted evolution

Sumario

Se examinó un fragmento de aproximadamente 220 pb del espaciador transcrito interno 2 (ITS2) como diagnóstico de *Anastrepha suspensa* (Loew) (Diptera: Tephritidae) y otras moscas de plagas en el género *Anastrepha*. La mayoría (96%) de los sitios en este fragmento fueron invariantes entre las especies de prueba, pero se puede separar *A. suspensa* de otras especies usando 2 caracteres diagnósticos. Similarmente, se pueden distinguir *A. ludens (*Loew) y *A. obliqua* (Macquart) de otras especies basadas en 1 carácter fijo cada una. Hay evidencia de variación intraespecífica ITS2 en 5 especies probadas, consistentes con complejos de especies y homogeneización incompleta a través del proceso de evolución concertada.

Palabras Clave: mosca de la fruta; *Anastrepha ludens*; *Anastrepha obliqua*; evolución

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