

Research Article

Terrance N. Todd*, Gary J. Steck and Norman B. Barr

New invasion or expansion: evaluating the genetic relationships of *Bactrocera dorsalis* (Diptera: Tephritidae) among detections in Florida

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Abstract: *Bactrocera dorsalis* (Hendel) (Diptera: Tephritidae), the oriental fruit fly, is a highly invasive species that has occasionally been detected in Florida, where conditions are highly favorable for the establishment of this pest. In 2015, a major infestation of *B. dorsalis* was detected in the Redland agricultural area of Miami-Dade county. Other detections of this pest have occurred in various locations in Florida, including the Redland area, before and after 2015. Questions arose whether any of these detections were related to the major infestation in Redland in 2015, possibly as one or more small, scattered populations that went undetected over time only to resurge as better conditions arose. To address this question, the genetic variation of the 2015 Redland outbreak was characterized using microsatellite DNA and by sequencing a portion of the mitochondrial cytochrome oxidase I (COI) gene. Population structure analyses and exclusion testing was performed on flies captured in years immediately preceding and after 2015 using microsatellites to determine (1) if flies were inconsistent with a shared source and (2) if the 2015 population could be excluded as source of subsequent captures. The COI sequences of the flies also were compared to determine if the 2015 outbreaks could explain variation in subsequent years. Results from this study found that flies captured before and after 2015 were not related to the population eradicated in Redland in 2015. Therefore, we concluded that flies captured in Florida between 2014 and

2021 were not part of any single, continuous breeding population that persisted over months.

Keywords: oriental fruit fly; eradication; invasive pest; SSR; microsatellite; pest establishment

Resumen: *Bactrocera dorsalis* (Hendel) (Diptera: Tephritidae), la mosca oriental de la fruta, es una especie altamente invasora que ocasionalmente se ha detectado en Florida, donde las condiciones son muy favorables para el establecimiento de esta plaga. En 2015, se detectó una infestación importante de *B. dorsalis* en el área agrícola de Redland en el condado de Miami-Dade. Se han detectado otros casos de esta plaga, antes y después del 2015, en varios lugares de Florida, incluido el área de Redland. Existen dudas de si alguna de estas detecciones estaba relacionada con la infestación principal en Redland en 2015, posiblemente producto de una o más poblaciones pequeñas y dispersas que pasaron desapercibidas con el tiempo, sólo para resurgir a medida que se presentaron mejores condiciones. Para abordar esta duda, la variación genética del brote de Redland del 2015 se caracterizó utilizando microsatélites de ADN y secuenciando una porción de la subunidad I del gen citocromo oxidase (COI), que está localizada en el ADN mitocondrial. Se realizaron análisis de estructura poblacional y pruebas de exclusión en moscas capturadas en años inmediatamente anteriores y posteriores a 2015 utilizando microsatélites para determinar: (1) si las moscas eran inconsistentes con un origen común y (2) si la población del 2015 podría excluirse como origen de capturas posteriores. También se compararon las secuencias COI de las moscas para determinar si los brotes del 2015 podrían explicar la variación en los años posteriores. Los resultados de este estudio encontraron que las moscas capturadas antes y después de 2015 no estaban relacionadas con la población erradicada en Redland en 2015. Por lo tanto, concluimos que las moscas capturadas en Florida entre 2014 y 2021 no forman parte de ninguna población continua reproductora que se mantuvo durante muchos meses.

Palabras Claves: mosca oriental de la fruta; erradicación; plaga invasora; SSR; microsatélite; establecimiento de plagas

*Corresponding author: Terrance N. Todd, USDA APHIS PPQ S&T Insect Management and Molecular Diagnostics Laboratory, 22675 North Moorefield Road, Edinburg, TX 78541, USA, E-mail: Terrance.N.Todd@usda.gov

Gary J. Steck, Division of Plant Industry (FDACS/DPI), Florida Department of Agriculture and Consumer Services, Gainesville, FL, USA, E-mail: G.Steck@fdacs.gov

Norman B. Barr, USDA APHIS PPQ S&T Insect Management and Molecular Diagnostics Laboratory, 22675 North Moorefield Road, Edinburg, TX 78541, USA, E-mail: Norman.B.Barr@usda.gov

1 Introduction

The oriental fruit fly, *Bactrocera dorsalis* (Hendel) (Diptera: Tephritidae), is a highly polyphagous pest native to Asia that can invade many agricultural areas around the world (Aketarawong et al. 2007; Peterson and Denno 1998; Stephens et al. 2007; Zeng et al. 2019). Accidental introduction of this species can have a devastating effect on economically important crops in areas with climates similar to those in its natural range (Drew and Hancock 1994; Ekesi et al. 2016; Stephens et al. 2007). With the continuous, widespread movement of fruits and vegetables that serve as hosts for this insect, commercial trade and international travel have been suggested as contributing mechanisms for its spread (Aketarawong et al. 2007, 2014; Malacrida et al. 2007; Qin et al. 2018; Turner et al. 2021). *B. dorsalis* has been observed throughout the Asia-Pacific region over the last century, from Pakistan to China, and across the Pacific being established in Hawaii as early as 1946 (Clarke et al. 2019; Stephens et al. 2007; Wan et al. 2012). This pest also has become established in Africa, being reported in Kenya in 2003 and the northern part of South Africa in 2004 (DeVillers et al. 2016; Manrankhan et al. 2015). It is now reported from approximately 40 African countries (DeVillers et al. 2016).

Based on climatic models, Florida is vulnerable to the establishment of this pest with preferred habitat and optimal climatic conditions similar to those in the pest's natural range (Stephens et al. 2007). To mitigate the risk of accidental introductions, in addition to the safeguards at ports of entries, surveillance programs are implemented to detect incursions early. The Florida Department of Agriculture and Consumer Services (FDACS) and the United States Department of Agriculture, Animal and Plant Health Inspection Service, Plant Protection and Quarantine (USDA-APHIS-PPQ) collaborate on a fruit fly surveillance program. *B. dorsalis* has been detected in Florida 16 times between 1964 and 2014 (Clark et al. 1996; Steck et al. 2019). Thirty-six flies were detected during this period, and as only a few flies were captured during most detection events, the trapped numbers usually did not meet the threshold requirement to trigger an eradication program (Steck et al. 2019). However, in 2015, 45 male *B. dorsalis* were captured in a single trap located in the Redland Agricultural area of Miami-Dade county (Steck et al. 2019). The following day, larvae were discovered in mango, *Mangifera indica* L. (Anacardiaceae), at the same site indicating a breeding population existed. This triggered a major eradication program conducted in 2015–2016 to prevent the establishment of this pest in Florida (Steck et al. 2019). A total of 158 flies were reported from that 2015 to 2016 outbreak (Steck et al. 2019). The pest was declared eradicated in Florida on 13 February

2016. Subsequently, after the eradication program ended, nine additional detections of *B. dorsalis* occurred in Florida in the years 2016–2021.

Although *B. dorsalis* was declared eradicated after the Redland infestation, the source of the infestation was unknown, as were the sources for each of the subsequent detection events. Were each of the detection events an independent invasion or part of a nascent population of fruit flies that may have established at undetectable levels? To date, a formal comparison of genetic diversity of this pest in Florida has not been published. Population genetics of the outbreak could be used to test source estimation hypotheses for flies captured subsequent to eradication (Barr et al. 2014a,b; San Jose et al. 2018; Shi et al. 2012). Steck et al. (2019) suggested that these post-eradication captures most likely represented multiple independent invasions that were independent of the earlier 2015 outbreak based on unpublished cytochrome *c* oxidase I (COI) sequence data. In this paper we report those and additional data to address the question.

In this study, we characterized the genetic diversity of *B. dorsalis* in Florida based on flies captured in the years 2014–2021. DNA sequences of the COI gene were used to measure variation in the insect's mitochondrial genome, and nine simple sequence repeat (SSR) loci were used to estimate variation in the insect's nuclear genome. The specific goals of the project were to estimate genetic variability of the 2015 outbreak population and to test possible sources of the flies captured after eradication. In addition to the outbreak population, genotypes of one fly captured in Florida in 2014 and 10 flies captured in Florida in the years 2016–2021 were compared to the Redland population genetic structure to test if these flies shared a common source. Exclusion tests were performed on the post-eradication detections to examine the Florida 2015 outbreak as a source for subsequent detections. The flies also were compared to *B. dorsalis* populations from Hawaii to determine if that could be the source for the Florida detections.

2 Materials and methods

2.1 Specimens and DNA extractions

A total of 127 *B. dorsalis* captured in traps in Florida between 25 August 2014 and 16 August 2021 representing 10 different detection events (Table 1, Figure 1 [map]) were included in the study. The 115 *B. dorsalis* captured in Redland, Miami-Dade county between 27 August 2015 and 10 October 2015, were classified as the Redland outbreak population. Two additional flies trapped in Miami-Dade county (Palmetto Bay and Miami) in 2015 were treated as separate detection events from the

Redland population for initial analyses. The study also included additional flies from Florida captured in 2014 ($N = 1$), 2016 ($N = 1$), 2017 ($N = 3$), 2018 ($N = 4$), and 2021 ($N = 1$). The flies from Florida were morphologically identified as *B. dorsalis* by Gary Steck. Legs from each fly were removed and used for DNA extraction using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, California, USA) as described in Barr et al. (2012). To support morphological identification, flies were analyzed using internal transcribed spacer 1 (ITS1) region sequences as reported in Barr et al. (2021) (data not shown). ITS1 is used for distinguishing *B. dorsalis* from *Bactrocera carambolae* Drew & Hancock (Boykin et al. 2014; Drew and Hancock 1994).

To compare the Florida 2015 outbreak population to other fly populations, 352 *B. dorsalis* collected from four Hawaiian islands and 24 fruit flies originating from southeast Asia were selected as reference populations (Table 2). The Hawaiian populations were selected to provide an estimate of SSR diversity using an introduced population and source exclusion. The flies from southeast Asia were included to provide an independent measure of SSR diversity of individual flies from more diverse potential source populations. Each of these flies had DNA extracted from the whole body. However, only legs were provided for the flies captured in Florida. The legless bodies were kept as vouchers by the Florida Department of Agriculture and Consumer Services.

2.2 COI analysis

DNA samples of flies from Florida were analyzed for the COI gene using a protocol reported in Barr et al. (2014a). Reactions were performed in 25 μ l volumes containing 1 μ l of template (or water for the negative control), 2.5 μ l of 10X Ex Taq buffer (Takara Bio Inc., Kyoto, Japan), 2 μ l of dNTP (2.5 mM each, Takara Bio Inc.), 0.125 μ l of Ex TaqHS DNA polymerase (5 U/ μ l, Takara Bio Inc.), and 1 μ l of each primer (at 10 μ M working concentration). Polymerase chain reactions (PCR) were performed using cycling conditions of 3 min at 94 °C followed by 39 cycles of 20 s at 94 °C, 20 s at 53 °C, and 30 s at 72 °C, and a final extension of 5 min at 72 °C. The amplified fragment is referred to as the C3p790 fragment in Barr et al. (2014a) and includes a 790 bp region of the 3' portion of the gene. The primers for the PCR were HCO-2198RC [5'-TGATTTTGGTCCACCTGAAGTTTA-3'] and PAT-K508 [5'-TCCAATGCACTAATCTGCCATATTA-3'].

PCR products were visualized using 1.2 % agarose gels of TAE buffer (BioRad, Hercules, California, USA) and purified with ExoSAP-IT (ThermoFisher, Applied Biosystems, Waltham, Massachusetts, USA) prior to Sanger DNA sequencing. The amplicons were sequenced using the two PCR primers and ABI BigDye® Terminator v.3.1 chemistry at commercial centers Functional Biosciences (Madison, Wisconsin, USA) or GeneWiz (South Plainfield, New Jersey, USA). The forward and reverse sequences were assembled into contigs using the program Sequencher v5 (Genecodes, Ann Arbor, Michigan, USA) and aligned using MEGA7 (Kumar et al. 2016). Templeton, Crandall, and Sing (TCS) haplotype networks of the COI data were generated using PopART v1.7 (Clement et al. 2002; Leigh and Bryant 2015; Templeton et al. 1992). Tajima's D statistic was estimated using Arlequin v3.5. Sequence data were submitted to NCBI GenBank (OP243539–OP243560).

2.3 SSR analysis

A total of nine microsatellites, 618A, BO-D48 (Li et al. 2007), BD1, BD19, BD39, BD42, BD76, BD85B (Aketarawong et al. 2006), and MS5 (Dai et al. 2004) were used, end-labeled with either 6-carboxyfluorescein (6-FAM) (ThermoFisher, Applied Biosystems, Waltham, Massachusetts, USA) or VIC® (ThermoFisher, Applied Biosystems, Waltham, Massachusetts, USA) dye set. Polymerase chain reactions were performed in 15 μ l reactions containing 1 μ l DNA template, 1.5 μ l of 10X buffer, 1.2 μ l 25 mM dNTP mix, 0.3 μ l of labeled 5' primer (10 nmol; Applied Biosystems), 0.3 μ l of unlabeled 3' primer (10 nmol Eurofins Genomics LLC, Louisville, Kentucky, USA), and 0.08 μ l *taq*DNA Polymerase (TaKaRa Ex Taq Hot Start Polymerase, Takara Bio USA, San Jose, California, USA). Amplification was performed on Applied Biosystem's Gene Amp 9700 thermocyclers. Cycling conditions were 94 °C for 5 min followed by 39 cycles of 1 min at 94 °C, 1 min at 55 °C, 1 min at 72 °C, and a final extension at 72 °C for 30 min. An aliquot (10 μ l) of PCR product was visualized on a 2.0 % TAE agarose gel pre-stained with ethidium bromide (0.4 μ g/ml final concentration). Documentation of gels was performed using the Gel-DocIt^{TS2} Imager (Analytik Jena GmbH, UVP, Beverly, Massachusetts, USA) and VisionWorksLS Imaging Software v 7.1 (Analytik Jena GmbH, UVP, Beverly, Massachusetts, USA). An aliquot (2 μ l) of the remaining PCR product was diluted 1:10 in water for fragment analysis. Fragment

Table 1: *Bactrocera dorsalis* detection events in Florida. Locations and number of oriental fruit flies found in the state of Florida between 2014 and 2021. A detection event was declared if one or more flies were detected in an 81-square mile delimitation zone and more than one life cycle had occurred between captured flies.

Capture site	Voucher code	Dates collected	No. flies	No. COI sequences	No. SSR genotypes
Broward county, Plantation	E2014-6069	25 Aug 2014	1	1	18
Miami-Dade county, Palmetto Bay	E2015-4798	17 Aug 2015	1	1	18
Miami-Dade county, Redland	See supplemental data	27 Aug 2015–10 Oct 2015	114	112	1,948
Miami-Dade county, Miami	E2015-5172	8 Sep 2015	1	1	18
Pinellas county, St. Petersburg	E2016-3335-1	1 Jul 2016	1	1	18
Lake county, Mount Dora	E2017-132-1	12 Jan 2017	1	1	18
Pinellas county, Clearwater	E2017-2251-1	6 Jun 2017	1	1	18
Broward county, Weston	E2017-2956	26 Jul 2017	1	1	16
Miami-Dade county, Redland	E2018-2988-1	1–26 Jun 2018	4	4	70
	E2018-3065-1				
	E2018-3066-1				
	E2018-3480-1				
Seminole county, Casselberry	E2021-4133-1	16 Aug 2021	1	1	16

All DNA extractions are maintained at the USDA-APHIS S&T IMMDL facility and all vouchered specimens are held with the FDACS/DPI.

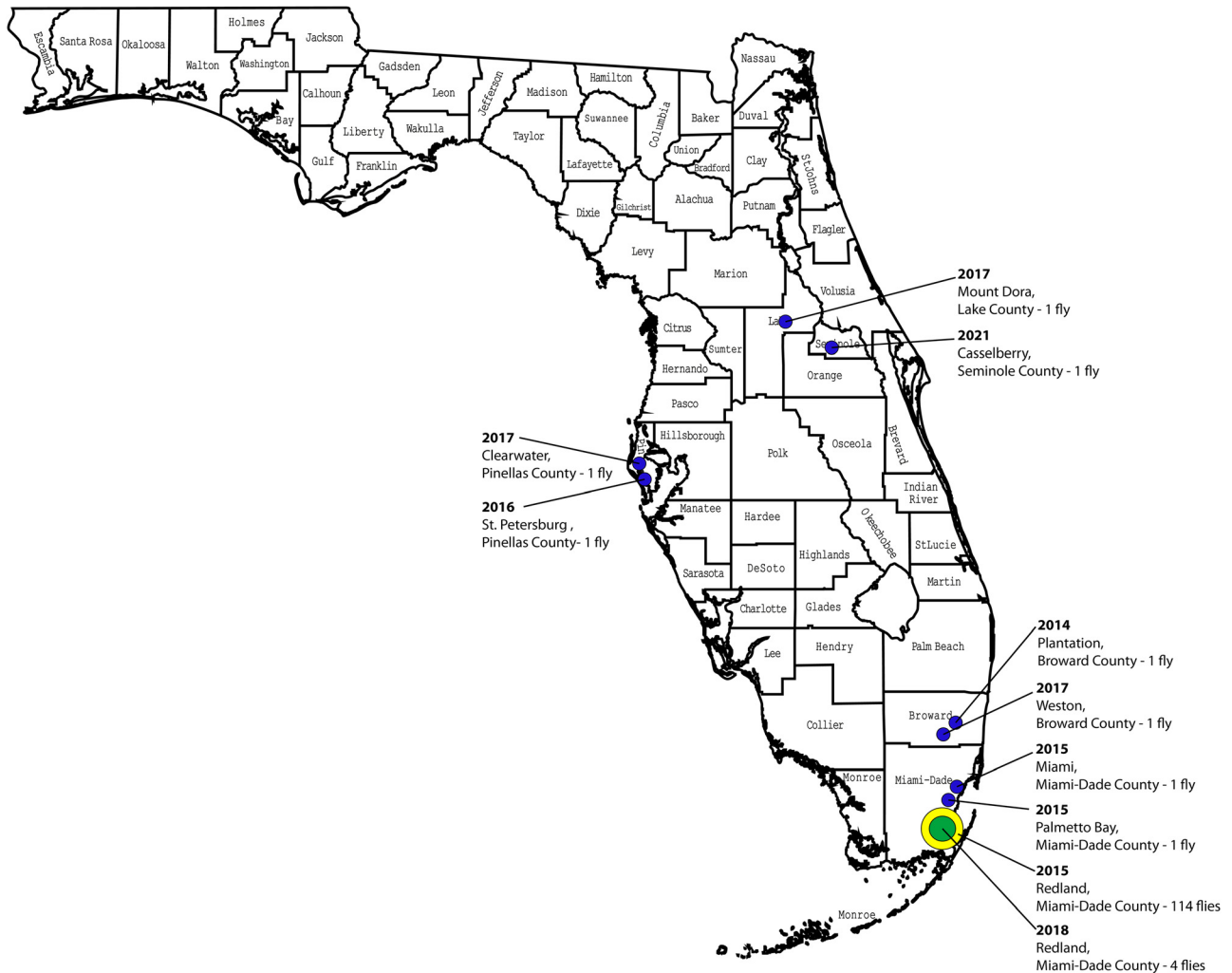


Figure 1: Collection locations of oriental fruit flies in Florida from 2014 to 2021. Location, year and number of flies detected is provided for each detection.

analysis was performed on an ABI 3730XL DNA Analyzer with the ABI Data Collection Program (v 2.0) provided by Functional Bioscience (Madison, Wisconsin, USA).

PeakScanner v2.0 (ThermoFisher, Applied Biosystems, Waltham, Massachusetts, USA) was used to determine fragment size, and binning was performed using Microsatellite Toolkit v3.1 (Park 2001) and Tandem (Ruby v1.08) (Matschiner and Salzburger 2009). Population differentiation was determined by F_{st} (Weir and Cockerham 1984) for all reference populations using Arlequin (Excoffier and Lischer 2010), and a Bayesian Markov Chain Monte Carlo (MCMC) based approach was implemented using STRUCTURE v2.3.4 (Pritchard et al. 2000) using 25 independent runs ($K = 2-5$) with 100,000 generation burn-in over 1,000,000 generations. Individual *B. dorsalis* flies detected before and after the 2015 Redland event and an outgroup of flies representing the southeast Asian population were then compared to the Hawaiian and the 2015 Redland outbreak collection using GeneClass2 (Piry et al. 2004). Assignment tests also were performed on a fly collected from the 2015 Redland outbreak (E2015-4798) as a reference point and the two flies collected in 2015 from Miami-Dade county in Palmetto Bay and Miami to determine if the Redland outbreak site could be excluded as a source.

To observe if changes occurred in allele and homozygosity values over the outbreak, the Redland population was divided into three

temporal lifecycles: f1 (17 August–7 September 2015), f2 (8–22 September 2015), and f3 (23 September–13 October 2015). This division of cycles was based on generational life cycles of *B. dorsalis* (Borchert et al. 2011). The allele frequency of each division was estimated using Arlequin and changes in heterozygosity were estimated.

3 Results

3.1 COI analysis

COI sequences were successfully generated from 124 of the 127 extraction samples. After editing, the sequence length of data records was 765 bp and comparison in an alignment detected no evidence of insertions or deletions or disruptions to reading frame of translated amino acids. These 124 records included nine distinct COI sequence haplotypes (Table 3). A TCS network of the nine haplotypes is presented in Figure 2. The greatest distance between any two sequences in the

Table 2: Reference populations for captured oriental fruit flies in Florida between 2014 and 2021.

Population	Sampling site	(n)
Hawai'i	South Kona	30
	Waimea Municipal Watershed Reservoir	31
	Kalōpā State Recreation Area	29
Maui		88
	Kahului	8
	'Iao Valley State Park	16
	Honoapi'ilani Hwy Mile Marker 12	16
	Ka'eleku Cave	16
	Kula Agriculture Park	16
	Kaupo General Store	16
O'ahu		91
	Kahuku Agricultural Park	23
	Mākaha Valley (Wai'anae Mountains)	16
	'Ewa Beach	20
	Haha'ione Valley	20
Kaua'i	Honolulu	12
		94
	CTAHR Agriculture Research Center, Kaua'i County	16
	Kuilau Ridge Trail	16
	Hulemalu Road, Lihue	22
	Common Ground (Guava Kai Plantation)	20
	Polihale State Park	20
Southeast Asia		24
	Cambodia; Krong Koh Kong	12
	Laos; Luang Namtha	4
Redland, Florida 2015 outbreak	Thailand; Chiang Mai	8
		113
	Captures 17 Aug–7 Sept 2015	78
	Captures 8–22 Sept 2015	29
	Captures 23 Sept–13 Oct 2015	6

A single fly was excluded from the Redland 2015 reference population (captures 17 Aug–7 Sep 2015) and was instead used for assignment testing.

network is 13 mutational steps and an uncorrected *p*-distance of 0.017.

The 112 samples that represent the Redland outbreak population in 2015 included three haplotypes. The most common haplotype was the C3pX326 (*N* = 98) followed by C3pX357 (*N* = 11), and C3pX359 (*N* = 3). The two additional flies detected in 2015 from Palmetto Bay and Miami shared the C3pX326 haplotype. Consequently, these 114 flies from Miami-Dade county in 2015 were treated as the 2015 outbreak population. The estimated mean diversity of the 2015 outbreak population was $d = 0.001$ (S.E. 0.001) using *p*-distance and 1,000 bootstraps. The data set had seven segregating sites, Theta of 0.00170, and nucleotide diversity of 0.00127. Estimates of Tajima's *D* statistic were not significant ($p > 0.10$).

Table 3: Observed cytochrome oxidase I haplotypes found in captured oriental fruit flies in Florida between 2014 and 2021. Accession number in GenBank provided for reference.

Haplotype	Florida populations	GenBank
C3p01	Broward county: Weston (2017, <i>N</i> = 1)	OP243550
	Seminole county: Casselberry (2021, <i>N</i> = 1)	
C3pX326	Miami-Dade county: Redland (2015, <i>N</i> = 98)	OP243540
	Miami-Dade county: Palmetto Bay (2015, <i>N</i> = 1)	
	Miami-Dade county: Miami (2015, <i>N</i> = 1)	
C3pX357	Miami-Dade county: Redland (2015, <i>N</i> = 11)	OP243541
C3pX358	Broward county: Plantation (2014, <i>N</i> = 1)	OP243539
C3pX359	Miami-Dade county: Redland (2015, <i>N</i> = 3)	OP243543
C3pX364	Pinellas county: St. Petersburg (2016, <i>N</i> = 1)	OP243547
C3pX369	Lake county: Mount Dora (2017, <i>N</i> = 1)	OP243548
C3pX371	Pinellas county: Clearwater (2017, <i>N</i> = 1)	OP243549
C3pX373	Miami-Dade county: Redland (2018, <i>N</i> = 4)	OP243551

The single fly collected in Broward county in 2014 had a haplotype sequence (C3pX358, Table 3) that was not previously reported but was only one mutation different from a haplotype (C3p02) frequently collected in Hawaiian populations of *B. dorsalis* (Barr et al. 2014b). The 2014 haplotype has not been reported in previous studies including amongst any flies captured in Florida. The flies trapped in Florida from 2016 to 2021 do not share haplotype sequences with the 2015 outbreak flies. The four flies detected in 2016 and 2017 had distinct haplotypes from each other. The St. Petersburg fly trapped in 2016 had a new haplotype (C3pX364) separated by at least four mutations from the other flies trapped in Florida. This haplotype has not been reported in previous studies. The three flies captured in 2017 each had unique haplotypes. The flies from Lake county and Pinellas county had new haplotypes. The flies from Broward county (2017) and Seminole county (2021) had the haplotype (C3p01), which is common to Hawaii (Barr et al. 2014b), found frequently in California (Barr et al. 2014b), and reported from China and Tahiti (San Jose et al. 2018). The four flies trapped in 2018 all shared the C3pX373 haplotype, which has not been previously reported.

Based on the Barr et al. (2014b) study, Hawaii can be excluded as a possible source for 122 (out of 124) of the flies in the study including all the flies collected in the years 2015, 2016, and 2018, but Asia and Africa are not excluded as possible sources of these flies. Only three flies had DNA consistent with the Hawaiian populations: the flies caught in 2014 and 2017 from Broward county and the 2021 fly from Seminole county. Failure to exclude Hawaii suggests a link with that source, but alternative regions also are possible, such as Tahiti and Asia where similar types have been reported. Prior studies have reported high COI genetic

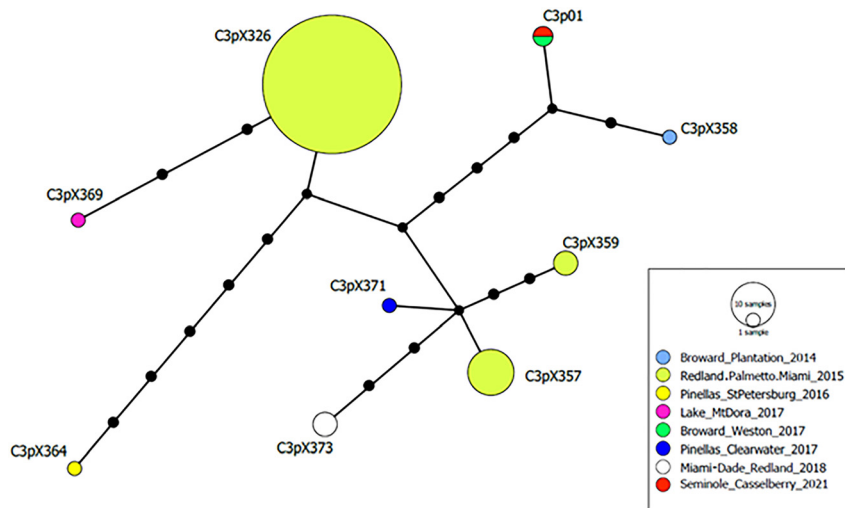


Figure 2: TCS haplotype network of the cytochrome oxidase I data generated from *Bactrocera dorsalis* collected in Florida in 2014–2021. The haplotypes are presented as circles and each circle size represents the number of flies with that haplotype. The black circles represent hypothetical haplotypes that connect the sampled haplotypes. Mutation (substitution) steps are represented by the lines in between haplotypes. The figure was generated using TCS method in PopArt (<http://popart.otago.ac.nz>).

variation for *B. dorsalis* and a lack of structure needed to reliably exclude or assign the origin of flies from Asia (Barr et al. 2014a; Garzón-Orduña et al. 2019; San Jose et al. 2018). Consistent with these prior results, the Florida flies include seven COI haplotypes that have not been previously reported.

3.2 SSR population structure of reference populations

PCR of the SSR markers for the reference populations was highly successful with 99.4 % of all 3,364 PCR reactions producing results for analysis (Table 4). A total of 65 alleles across nine loci was detected. A total of 487 *B. dorsalis* originating from the state of Hawaii ($N = 352$), southeast Asia ($N = 24$), and captured during the Redland 2015 infestation ($N = 111$) were successfully genotyped and was used as reference populations

for comparing diversity and performing tests of structure. The Hawaiian reference collection includes four sub-populations of the Big Island (Hawai'i), Maui, O'ahu, and Kaua'i (Tables 3 and 4). The Redland 2015 reference population did not include the two flies found in Palmetto Bay and Miami in 2015 (Table 1). The southeast Asia individuals were used to examine heterozygosity values but not to test structure based on *a priori* collection data because that reference population represents a composite of flies from multiple collections and likely not a single natural population. All individuals were included in Structure models to sort according to genetic similarity.

B. dorsalis individuals originating from Asia show the highest diversity of alleles ($x = 6.56$), followed by the fruit flies collected during the Redland outbreak in 2015 ($x = 4.57$). However, heterozygosity was low (0.12) in the flies captured in Redland in comparison to Hawaii ($\bar{x} = 0.453$) and SE Asia (0.505). F_{st} Pairwise distance analysis was then performed

Table 4: Observed genetic diversity seen across reference populations for oriental fruit flies in Florida, Hawaii and southeast Asia. Table provides breakdown of number of alleles and mean expected (Exp Het.) and observed (Obs Het.) heterozygosity in reference populations used to determine assignment for captured oriental fruit flies in Florida in 2015.

Locus	Hawai'i ($N = 87$)	Maui ($N = 91$)	O'ahu ($N = 92$)	Kaua'i ($N = 82$)	SE Asia ($N = 24$)	Redland ($N = 111$)	Mean alleles	S.D.	Total alleles
BD1	3	4	3	3	8	4	4.167	1.941	8
BD19	2	2	2	2	6	1	2.500	1.761	6
BD42	2	2	2	2	6	5	3.167	1.835	7
BD76	3	3	3	3	5	1	3.000	1.265	5
BoD48	4	3	4	3	8	4	4.333	1.862	8
MS5	3	3	3	4	9	5	4.500	2.345	9
618A	3	3	3	3	3	5	3.333	0.816	6
BD85B	4	3	3	3	6	5	4.000	1.265	7
BD39	3	3	3	3	8	4	4.000	2.000	9
Exp Het.	0.48617	0.48752	0.52507	0.55837	0.66085	0.09654			
Obs Het.	0.41238	0.44986	0.43631	0.51300	0.50451	0.12101			
S.D.	0.17852	0.15765	0.15420	0.09307	0.16244	0.27274			

Table 5: Population pairwise F_{st} values for reference populations for oriental fruit flies in Florida, Hawaii, and southeast Asia. Reference populations arranged by distance. All values were found to be significant ($p < 0.001$).

	Hawai'i	Maui	O'ahu	Kaua'i	SE Asia	Redland
Hawai'i	–					
Maui	0.01871	–				
O'ahu	0.02711	0.02682	–			
Kaua'i	0.03139	0.02775	0.01412	–		
SE Asia	0.17977	0.18191	0.16741	0.10831	–	
Redland	0.29530	0.27428	0.28191	0.23299	0.17692	–

on the reference populations (Table 5). The closest genetic relationships (smallest genetic differentiation) in the study were exhibited between populations collected from each of the sampled Hawaiian Islands. The highest genetic distance measured among the population of the Hawaiian Islands was seen between the furthest islands (Hawai'i and Kaua'i, $F_{st} = 0.0314$). However, all Hawaiian fly populations were more closely related to flies collected from southeast Asia than to the flies collected in Redland, Florida.

3.3 SSR Bayesian structure analysis of individuals from each detection event

In addition to the Redland reference flies, 11 flies captured in Florida from 2014 to 2018 were included in SSR analyses. Two of the four flies collected from Miami Dade county in June 2018 failed to produce adequate PCR fragments for analysis. There was also difficulty in producing SSR fragments of the BD1 locus with >40 % of the Florida flies failing. This could be

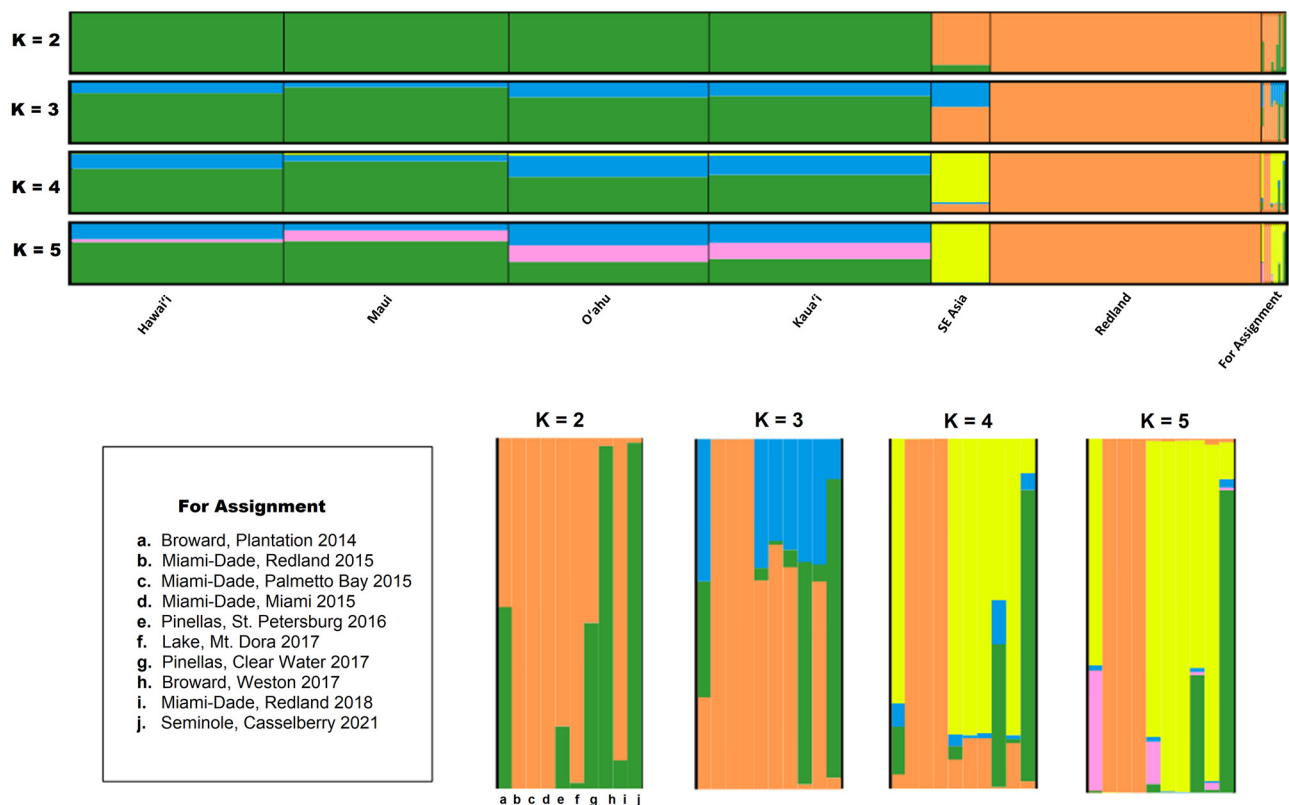


Figure 3: Bayesian clustering pattern for *Bactrocera dorsalis* reference population and flies captured between 2014 and 2021 in Florida. (Top) Assignment probabilities assuming an admixture ancestry model with independent allelic frequencies. Used a LocPrior distribution and organized data based on geographic distance and estimate ($K = 2-5$) in STRUCTURE v2.3.4 (Pritchard et al. 2002) for the Hawaiian Islands, southeast Asia and the Redland outbreak and each individual fly for population assignment. (Bottom) A blow up of the captured oriental fruit flies analyzed independent of reference population and compared to reference populations. Based on the observations seen in the assignment test, none of the other captured flies observed in the other capture events could possibly be a direct descendent of the flies detected during the Redland detection event of 2015.

due to low DNA yield from the extraction process. BD1 was able to amplify a PCR fragment from all the Hawaiian reference DNA samples and Asian outgroup, all of which had DNA extracted from whole bodies. PCR fragments were obtained for nine specimens using the remaining eight markers. This set of nine flies includes the Palmetto Bay and Miami flies trapped in 2015 within the same county as the Redland flies. Structure analysis of SSR profiles from a data set including the 487 reference individuals and the nine additional Florida collections is reported in Figure 3.

Bayesian analysis using STRUCTURE was performed including all individuals. Sample sites were divided into six groups (Hawai'i $n = 90$; Maui $n = 88$; O'ahu $n = 91$; Kaua'i $n = 94$; southeast Asia $n = 24$; and Redland $n = 113$). Population (K) was estimated to be between 2 and 5. In all estimates of K , Hawai'i strongly separated out as a single unique population with variations observed between the four islands. In contrast, at the lowest population estimates ($K = 2$) Redland and samples from southeast Asia had a structure consistent with a single population.

The genetic similarity observed between Redland and the small composite collection of specimens used to represent southeast Asia tends to indicate a shared heritage at lower K values. However, this might not have any biological significance as the Hawaiian population is strongly structured relative to the other flies in the dataset. At higher K values ($K > 3$), Redland begins to separate away from the representative southeast Asian flies and begins exhibiting a structure consistent with a single population. By using these higher K values, we can then compare individual capture events to Hawaiian Islands and Redland. The representative Redland 2015 fly (E2015-4996) and the two flies collected outside the Redland quarantine zone in 2015 (Palmetto Bay (E2015-4798) and Miami (E2015-5172)) were tested and found to 100 %

match to flies collected in Redland at $K = 1-4$ (Figure 3). Flies from the other seven detection events did not exhibit enough shared alleles to allow grouping with flies found during the Redland detection event. One fly (E2017-2956) had high similarity to flies found on the Hawaiian Islands when K was set at 2 and 3, but less similarity under higher values of K . Finally, the fly collected in 2021 (E2021-4133-1) was highly similar to the population observed on the Hawaiian Islands across all values of K indicating a shared relationship.

3.4 SSR assignment tests for captured flies

SSR first generation assignment tests (Paetkau et al. 2004) were performed for 10 fruit flies detected in Florida before, during, and after the 2015 Redland outbreak (Table 6). One fly from Broward county, Plantation (E2014-6069) was captured before the infestation (and could not be progeny of the outbreak), two flies were captured during the outbreak (but outside Redland area), and seven detections were made after the Redland infestation was declared eradicated. In 2018, four flies were captured within close proximity of the Redland infestation of 2015, but only one fly (E2018-3066-1) yielded high quality SSR results for all alleles, and was the only fly used in assignment testing.

As expected, a Redland source could not be rejected for the first 2015 Redland fly captured for first generation assignment tests using GeneClass2. A Redland 2015 source also was not rejected for the two flies collected in 2015 from Palmetto Bay and Miami. The other tested flies all had the Redland reference population rejected as the probable source (familial genetic relationship) with statistical significance ($p < 0.05$).

Table 6: Assignment test for oriental fruit flies captured in Florida between 2014 and 2021. SSR individual assignment tests were performed using Paetkau et al. (2004) probability computation using Monte-Carlo resampling with 10,000 simulated individuals; $p > 0.05$. Regions that cannot be excluded are in bold. However, the genetic diversity present throughout the natural range of *Bactrocera dorsalis* is not completely represented in these analyses to allow us to exclude southeast Asia as a source. There may be a population in the native range that matches the flies found in these detection events.

Fly collection location (year)	Figure 3. code	Hawai'i	Maui	O'ahu	Kaua'i	SE Asia	Redland
Broward county, Plantation (2014)	a	0.0000	0.0015	0.0172	0.0014	0.0018	0.0000
Miami-Dade county, Redland (2015)	b	0.0000	0.0001	0.0000	0.0005	0.0257	0.0725
Miami-Dade county, Palmetto Bay (2015)	c	0.0000	0.0006	0.0016	0.0076	0.0781	0.0792
Miami-Dade county, Miami (2015)	d	0.0000	0.0001	0.0001	0.0007	0.0082	0.8939
Pinellas county, St. Petersburg (2016)	e	0.0000	0.0000	0.0000	0.0011	0.0754	0.0000
Lake county, Mount Dora (2017)	f	0.0000	0.0000	0.0000	0.0001	0.0173	0.0000
Pinellas county, Clearwater (2017)	g	0.0000	0.0001	0.0001	0.0145	0.1127	0.0007
Broward county, Weston (2017)	h	0.3875	0.4781	0.2412	0.2159	0.0060	0.0011
Miami-Dade county, Redland (2018)	i	0.0008	0.0040	0.0071	0.0271	0.4894	0.0163
Seminole county, Casselberry (2021)	j	0.0680	0.0738	0.0849	0.0780	0.0049	0.0001

The Broward county fly captured in 2014 (E2014-6069) predates the Redland outbreak and would be excluded as possible progeny of that outbreak. The assignment tests demonstrated that the four Hawaiian populations also are excluded as a source of this fly. Although the analysis rejects southeast Asia as a source for this fly, that Asian reference data set is not representative of the true variability of southeast Asia and cannot be used to exclude it as a source. Therefore, a southeast Asian source is possible for this fly. Although provided in Table 6, the rejection or failure to reject the southeast Asia population as the source is not based on sufficient sampling. Therefore, this value is not reported or interpreted for other flies in this section.

The Broward county fly from 2017 (E2017-2956) did not have Hawaii excluded as a source. This is consistent with the mitochondrial data that was a match to a Hawaiian source. The exclusion tests performed here do not include adequate material to exclude other sources such as Asia, Africa, and the Pacific islands for this fly.

Finally, four detections of *B. dorsalis* occurred north of Miami-Dade county and were genetically different from the 2015 outbreak. For the two flies from Pinellas county (St. Petersburg 2016, Clearwater 2017) and the fly from Lake county (Mount Dora 2017), Hawaii was rejected as a source. For the most recent detection, the 2021 detection in Seminole county (E2021-4133-1), Hawaii was not rejected as a source. This fly had a COI sequence that is commonly observed in Hawaii.

4 Discussion

In this paper, we provide the first genetic analysis of the *B. dorsalis* from Florida describing the 2015 outbreak in Redland plus previous and subsequent detection events. We demonstrated that the outbreak population was relatively diverse compared to Hawaiian *B. dorsalis* populations when using COI and SSR genetic data sets. Three COI haplotypes were observed in the Redland 2015 outbreak flies, suggesting that at least three females were present to contribute to the genetic variation observed in the outbreak. The predominant haplotype, C3pX324, has been observed in *B. dorsalis* larvae intercepted from passenger baggage at several ports of entry throughout the United States, seen 29 times in a total of 10 ports between 2012 and 2018 (unpublished data). This genetic type also has been reported from flies collected in Asia (i.e., Cambodia, China, Thailand, and Bangladesh) (San Jose et al. 2018). It is not possible to determine what geographic area in Asia might be the most likely source using these data if the flies originated from this region. Collections from Africa were also not available, so Africa cannot be

excluded either, as it not known if this haplotype exists there. The COI genetic types reported from the Redland outbreak were distinct from diversity in Hawaii. Consequently, Hawaii was excluded as a source for this outbreak based on methods of Barr et al. (2014b). The SSR data also detected a genetic separation between the 2015 outbreak and Hawaiian populations of *B. dorsalis*. The STRUCTURE analysis of SSR data clustered all flies from the 2015 outbreak into a single population, supporting a single source hypothesis for the introduction.

The molecular data support the hypothesis that the 2015 outbreak was from one introduction event and that previous and subsequent detections in Florida have been the result of independent incursions. Reintroduction of the pest into California also has been reported using DNA methods (Barr et al. 2014a). The observed COI haplotypes found in these captures demonstrated non-overlapping genetic diversity between the flies captured in 2015 in Redland versus flies captured in other years. At least three detection events could not exclude Hawaii as a potential source using COI data, with one occurring pre-invasion (2014) and two post-invasion (2017 and 2021). The flies from 2014 to 2017 were captured in the same county, but were separated by several years, with the later (2017) capture more resembling *B. dorsalis* from Hawaii than the 2014 fly based on Bayesian clustering of the SSR data. Hawaii was not excluded as a potential source of the 2017 fly using exclusion testing of SSR data. The fly collected in Broward county in 2014 did resemble flies collected from Hawaii using pairwise assignment testing, but Hawaii was excluded as a source of the fly based on an exclusion test. The third fly with COI data matching Hawaiian populations was from Seminole county (2021) but it did not have SSR data generated for comparison. Hawaii is not the only potential source of flies with the C3p01 COI type, and as noted in our current study additional analysis of other geographic sources using genomic markers will be needed to refine source estimation methods. Failure to exclude Hawaii does not mean that it is the source of these three detections.

The other detection events of *B. dorsalis* in 2016, 2017, and 2018 have Hawaii excluded as a source. These events also appear to represent independent introductions based on COI and SSR data. The only flies that share genetic similarity are the four flies from the Miami-Dade county Redland collections in June 2018. These four flies represent one detection event.

Additional support for a reintroduction hypothesis of *B. dorsalis* into Florida is from trapping methods and records. While conditions in Florida are favorable for rapid expansion of invading populations (Stephens et al. 2007), new detections were limited to single or two captures per event. Since the 2015 Redland outbreak, there has not been a

detection event involving more than four flies being detected over multiple life cycles within a surveillance or delimitation zone.

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