Inheritance of Fifteen Microsatellite Loci in Ceratitis capitata (Diptera: Tephritidae)

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Inheritance of fifteen microsatellite loci in *Ceratitis capitata* (Diptera: Tephritidae)

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Abstract

Molecular methods that rely on microsatellite markers have been developed for population genetic studies and diagnostics of tephritid pest species such as the Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae). Whereas many of these markers are tested to see if they are within the Hardy–Weinberg equilibrium, very few markers developed for pest species are tested to ensure the selected alleles behave according to the laws of Mendelian inheritance. Fifteen previously developed microsatellite markers were examined for Mendelian inheritance. Nine parental groups consisting of a laboratory reared parent and a wild type parent and their respective progeny were examined. In total, 174 flies, consisting of 90 males and 84 females, were analyzed. Seventy-seven segregation ratio tests were performed to determine if any departures from expected Mendelian inheritance occurred. Representatives from each of the observed alleles were cloned and sequenced. Troubleshooting was performed on loci that did not conform to expected Mendelian inheritance ratios to confirm the cause and improve laboratory procedures. Issues observed included incomplete adenylation at the 5' end in *Ccmic3*, the presence of artifactual bands leading to false calls in *Ccmic25*, and monomorphic alleles in *Ccmic7*. Only 1 locus, *Ccmic25*, deviated from Mendelian expectations after protocol optimization in the form of a detected transmission ratio distortion leading to excessive heterozygosity. Finally, 1 locus, *Ccmic9*, showed evidence of allelic homoplasy.

Key Words: allelic inheritance; Mediterranean fruit fly; medfly; Mendelian expectations; multiplex

Resumen

Se han desarrollado métodos moleculares que dependen de marcadores de microsatélites para los estudios genéticos de población y el diagnóstico de las especies de plagas tefritidos como la mosca de la fruta del Mediterráneo, *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae). Mientras que muchos de estos marcadores se prueban para ver si están dentro del equilibrio de Hardy Weinberg, muy pocos de los marcadores desarrollados para especies plaga se prueban para asegurar que los alelos seleccionados se comportan según las leyes de la herencia Mendeliana. Quince marcadores de microsatélites desarrollados anteriorment fueron examinados para la herencia Mendeliana. Se examinaron nueve grupos de parentales que consistían en un parental de crianza de laboratorio y un parental de mosca silvestre y su progenie respectiva. En total, se analizaron 174 moscas, compuestas de 90 machos y 84 hembras. Se realizaron 77 pruebas de relación de segregación para determinar si se produjo alguna desviación de la herencia Mendeliana esperada. Los representantes de cada uno de los alelos observados fueron clonados y secuenciados. Se realizó la solución de problemas en loci que no se ajustan a las proporciones esperadas de herencia Mendeliana para confirmar la causa y mejorar los procedimientos de laboratorio. Los problemas observados incluyeron adenilación incompleta en el extremo 5'en *Ccmic3*, la presencia de bandas artifactuales que conducen a llamadas falsas en *Ccmic25* y alelos monomórficos en *Ccmic7*. Sólo 1 locus, *Ccmic25*, se desvió de las expectativas Mendelianas después de la optimización del protocolo en forma de una distorsión de la relación de transmisión detectada que conduce a heterozigosidad excesiva. Finalmente, 1 locus, *Ccmic9*, mostró evidencia de homoplasia alélica.

Palabras Clave: herencia alélica; mosca mediterránea de la fruta; moscamed; expectativas Mendelianas; múltiplex

The increase in human travel and trade worldwide has facilitated the accidental introduction of non-native species and destructive pests such as the Mediterranean fruit fly (medfly), *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae). Accidental introduction of invasive insect species has impacted economies, habitat, diversity of native species, and has been responsible for the introduction of destructive diseases (Horsefall 1983; Vitousex et al. 1997; Cox 1999; Gandhi & Herms 2010). The spread of the medfly from its native Sub-Saharan Africa to established regions throughout the world, including countries in the Mediterranean, South America, and Central America, as well as Australia and Hawaii, has been well documented (White & Elson-Harris 1992; Malacrida et al. 2007; De Meyer et al. 2008; Barr 2009). This highly polyphagous pest has a broad geographic distribution and is capable of using more than 250 plants as hosts and thereby placing many economically important crops at risk should the pest become established in new areas with tropical to dry-summer subtropical and dry-summer temperate climates (White & Elson-Harris 1992; Copeland et al. 2002; De Meyer et al. 2002; Barr 2009). The use of molecular techniques can help identify the pathways of accidental introductions, which in turn can allow managers to develop programs to mitigate potential spread and establishment of pest species into non-native habitats (Barr 2009). Microsatellite DNA techniques have been used successfully to track medfly movement and diagnose geographic sources of invasive populations (Bonizzoni et al. 2000, 2001, 2004; Karsten et al. 2013).

Source estimations used in determining the origin of introduced organisms, such as medfly, are improved only after the estimated allele frequencies from potential source populations have been determined (Paetkau et al. 1995; Rannala & Mountain 1997; Pritchard et al. 2000).

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In order to achieve this objective, the loci chosen should be neutral, unlinked to other loci being used in the same study, and conform to Mendelian expectations (Kimura & Crow 1964; Ohta & Kimura 1971; Kimura 1979). These assumptions are commonly addressed during the development of the markers by testing loci for deviations from the Hardy–Weinberg equilibrium (HWE) (Detwiler & Criscione 2011). However, the cause of the deviations from HWE may not be easy to determine due to many factors at the molecular or the population level or both. At the molecular level, mutations resulting in null alleles, unrecognized duplicated loci, and unrecognized sex-chromosome loci can cause deviations from HWE (Callen et al. 1993; Jones et al. 1998; Guichoux et al. 2011; Detwiler & Criscione 2011). Inbreeding, selection, and the Wahlund effect can affect the HWE at the population level (Detwiler & Criscione 2011; Lee et al. 2012). Finally, the population just may not adhere to Mendelian expectations due to modifiers during meiosis allowing preferential associations that lead to greater fitness of an allele (Úbeda 2006). Testing for inheritance using Mendelian segregation analysis can address the HWE assumption that a population must conform to Mendelian expectations to be considered in equilibrium.

Mendelian segregation analysis is an accurate method for confirming the performance of microsatellite primer sets (McGoldrick et al. 2000; Reece et al. 2004; Guichoux et al. 2011; Detwiler & Criscione 2011). The use of family design provides advantages over unrelated samples as they can elucidate some of the individual errors via Mendelian inconsistencies, such as segregation distortion, and unlikely recombination patterns (Kirk & Cardon 2002; Úbeda 2006). Performing controlled crosses is ideal for nuclear DNA-based marker development and is common practice in the development of markers for plants (e.g., Smith & Devey 1994; Jakse et al. 2001; Tarazi et al. 2010; Carneiro et al. 2012; Lefèvre et al. 2012). For many insect groups, including pest tephritids such as medfly, this can be quite expensive and requires great care because it involves the rearing and the cross-mating of a reproductively viable agricultural pest species. This practice is difficult and resource intensive because such crosses would need to be performed in a specialized controlled environment. One research group was able to develop microsatellites for medfly by performing controlled crosses (Stratikopoulos et al. 2008). Using these and a few other previously published markers, Stratikopoulos et al. (2008) were able to estimate a genetic linkage map associating 67 microsatellite markers across 4 chromosomes. Additionally, these markers were tested for deviation from expected Mendelian ratios by backcrossing F2 progeny to F1 parents and most segregated in a 1:1 ratio (Stratikopoulos et al. 2008). However, information regarding Mendelian segregation observed for each specific microsatellite marker between family groups was not provided. The wild type strains used in initial crosses most likely exhibited low heterozygosity.

While microsatellite markers are readily available for medfly, the need to validate these markers is becoming increasingly important. This validation step is important when the markers have the potential to be used in making decisions that have large economic and ecological impacts. This is true for the medfly, which poses a significant risk to agricultural production and global trade (Barr 2009). The microsatellite methods developed to date for this invasive pest have been used to understand invasion patterns in fruit producing regions around the world (Bonizzoni et al. 2000, 2001, 2004; Gasperi et al. 2002), to estimate multiple paternity (Bonizzoni et al. 2002), and for the construction of cytogenetic maps (Straikopulos et al. 2008, 2009). Although verified using HWE tests with an initial examination to determine if these markers fall within Mendelian ratios, a more comprehensive examination of a select few markers is needed. We have selected 15 previously published microsatellite loci developed by Bonizzoni et al. (2000) and Stratikopoulos et al. (2009) for Mendelian segregation analysis. Initially 3 populations using 23 markers (data not shown) were analyzed during a pilot study to address the ease of interpreting the chromatographs when using these markers in a multiplex system. Linkages between each marker were measured using Fstat v2.9.3.2, and 15 loci were selected (unpublished data). Eight of the markers that we report here were developed in Bonizzoni et al. (2000) and were selected based on their historical use, application, and impact in decisions of regulatory importance. The 7 markers from Stratikopoulos et al. (2009) were selected based on their reported heterozygosity estimates, were within Hardy–Weinberg expectations, and estimates showed no linkage. Additionally, these same 15 loci are included in an ongoing study to examine population structure and genetic diversity of medfly to support United States Department of Agriculture (USDA) pathway analysis of the pest (Ruiz-Arce et al., unpublished).

Evaluation for these selected markers was conducted by testing and analyzing information from single-pair matings of medfly. Using a multiplex PCR system, our objective was to evaluate the performance of the aforementioned 15 loci. Segregation analysis between known parental-progeny strains will be conducted to detect any bias that may exist in the selected markers.

Materials and Methods

SAMPLES ANALYZED

Mediterranean fruit fly, *C. capitata*, families for this segregation analysis were produced at Planta El Pino Moscamed Guatemala. The laboratory strain used as the basis to perform these crosses is the currently mass reared genetic sexing strain (GSS) temperature sensitive lethal (TSL) known as Vienna 8 $^{(-invD53)}$/Toliman99, which lacks the inversion characteristic of Vienna 8 strains, which were developed at a later date. To mass rear this strain, huge numbers of individuals are maintained in the breeding colony, which favors the presence of heterozygosity. This strain was selected because it is the current choice for sterile insect technique releases to control outbreaks in areas where the medfly has not established. This laboratory strain was crossed to wild type flies that emerged from larvae recovered from ripe coffee beans *Coffea arabica* L. (Rubiaceae) collected in the field in Guatemala.

SCHEME OF CROSSES

Twenty single-pair matings of laboratory insects were set up to collect eggs to produce F1 progeny for the parental-progeny analysis. Ten single pairs were formed by laboratory reared males crossed to wild type females (identified as "A" families) for the reciprocal cross, 10 single pairs of laboratory reared females were crossed to wild type males were identified as "B" families. Eggs collected from the respective crosses were raised on an artificial diet, which consisted of a blend of ground corn cob, torula yeast, granular sucrose, water, and preservatives. The environmental conditions for insect rearing were 24 ± 1 °F (−4 °C) and 60 to 65% RH for 6 d followed by 68 ± 1 °F (20 °C) and 60 to 65% RH for 4 d to reach full larval development for collection. From these crosses, 9 families from cross A and 10 from cross B were collected. Nineteen glass test tubes containing a minimum of 25 F1 pairs of insects (tube A4 had only 14 F1 pairs and was not included) in 1,2-propylene glycol USP (BASF CORP., Florham Park, New Jersey) were shipped to the CPHST laboratory (Mission, Edinburg, Texas) for analysis. Upon arrival, offspring were sexed and family groups that yielded a sex ratio close to 1:1 (male to female) were chosen to be included in this study. Five of the groups were pooled from family A (i.e., families A1, A2, A3, A7, and A8), and 4 groups were selected from family B (i.e., families B1, B3, B5, and B8). In total, 174 flies, consisting of 90 males and 84 females, were analyzed (Table 1).

Table 1. Mating of *Ceratitis capitata* pairs performed at Planta El Pino Moscamed Guatemala. Families were chosen if sex ratio was close to 1:1 with at least 6 males and 6 females being analyzed per mated pair.

Family name	Female origin	Male origin	Progeny analyzed
A1	wild capture	laboratory reared	32
A2	wild capture	laboratory reared	20
A3	wild capture	laboratory reared	30
A7	wild capture	laboratory reared	24
A8	wild capture	laboratory reared	16
B1	laboratory reared	wild capture	12
B3	laboratory reared	wild capture	16
B5	laboratory reared	wild capture	12
B8	laboratory reared	wild capture	12

DNA ISOLATION

DNA was isolated from whole fly samples using a nondestructive high-throughput magnetic bead-based genomic DNA purification technology using the extraction kit InviMag® Tissue DNA Mini Kit/KF96 (STRATEC Biomedical AG, Birkenfeld, Germany), on an automated magnetic-particle nucleic acid purification system, KingFisher™ Flex (Model # 711, Thermo Scientific, Waltham, Massachusetts). Each whole fly was placed in an individual well containing 400 µL of the lysis buffer and 25 µL of proteinase K. The plate containing the reagents and specimen was then placed in an ultrasonic water bath (Lab Companion UC-10, Jeio Tech, Seoul, Korea) at 52 °C and sonicated at the medium setting for approximately 30 min to increase tissue disruption. The lysis plate was then placed on a rocking platform located in an incubator set to 52 °C and left to rock overnight. The lysate was then transferred to a new deep well plate containing a 200 µL of binding buffer (Binding Buffer T) and 20 µL of magnetic beads (MAP Solution A). The “binding plate,” containing DNA lysate, binding buffer, and magnetic beads, 3 wash plates (800 µL Wash Buffer per well), and an elution plate (200 µL Elution Buffer D per well) were loaded into the bead beater. The following program was used for isolating DNA: an initial binding for 5 min at fast speed setting at room temperature, 3 washes at 1.5 min on medium speed setting at room temperature, drying for 5 min at room temperature, and elution for 15 min at slow speed setting at 70 °C.

PCR MULTIPLEX AND FRAGMENT ANALYSIS

Fifteen microsatellites primer sets reported by Bonizzoni et al. (2000) and Stratikopoulos et al. (2009) to be in HWE were used in this study (Table 2). These primer sets were first tested in a singleplex method on 93 Mediterranean fruit flies representing 3 geographic areas (data not shown). They were then assigned to 1 of 6 panels to

Table 2. Multiplex PCR panels with respective microsatellite primers used in Mendelian segregation analysis with *Ceratitis capitata*. Each primer shows the primer sequence, fluorescent label used, and the expected allele size according to original published data.

Panel	Locus	Primers 5′ to 3′	Label	Expected allele size (bp)
1	*Medflymic30*	TACTGGACAACGGGTTAACAGC TTTTATGTTCAACGCTGCGAC	VIC	126–135
	Medflymic78	ATTTGCCCGTCATTCAAACAAC ATTTATACACCCAGTCATGCCC	6-FAM	153–157
	Medflymic43	TTTTCGAACGGCTGCATC TTAGAGGCAAGCCACCAGG	VIC	167–221
2	*Medflymic92*	AAATGACACAAAACCGTAACCC GCAACCGTTTACTGCTCAATG	VIC	138–143
	Medflymic67	AAAATCCCCTTGATGCCTG ACATAAGCGTGTACCTTGTGCC	6-FAM	155–170
	Medflymic74	TCAAAGAAACAAAGAGGCGTG	VIC	188–194
3	*Ccmic15*	GTTCGAAAGTGGGGTATGTACG CACAAGAGCCAAAGACGCAT	VIC	85–109
	Ccmic25	GCACATACACACAACCATTT CGCCACAACGCAACAAAG	6-FAM	142–172
4	*Ccmic14*	AATTCAGATACACGCTCACAAG TCGTATTGCTATGCGCATAT	VIC	75–111
	Ccmic9	GAAGTGACTCATATTTTTAGGAACGA TCTTTCTTTCATACTCACTCATTTC	6-FAM	107–167
	Ccmic32	ACCACCCAATAACTTCATA GCTTTCATCATCCGTTCC	VIC	174–195
5	*Ccmic6*	AAGGTAGCCAGCAGTGTCTACG ACGAATGGGAGTTATTCATACTGC	VIC	70–117
	Medflymic81	TTAACTACCCTCGGTGATGGC TTTGGTTCATATCGACGCTTG	6-FAM	130–161
6	*Ccmic3*[a]	ggTGCACATGTATTCGTTCTTA AATTACCTATAAACATGCATACTG	6-FAM	72–96
	Ccmic7	TGTAAGTGAGCAAGGGGCAT CATCAAAGGCAGAGAACTGCA	VIC	108–136

[a] Primer redesign. Original primer did not have the “g” guanine bases as indicated in lowercase.

be used in a multiplex system. Three panels consisted of a combination of 3 primer sets, and the 3 additional panels consisted of 2 primer sets (Table 2). Each forward primer was end-labeled with either 6-carboxyfluorescein (6-FAM) or VIC® dye set (Life Technologies, Carlsbad, California). Pairing loci to panels was determined based on range in allele sizes, hybridization kinetics, and probability of primers forming "primer-dimers." Alternating labeled primers also was used to further allow for differentiating between primer sets, i.e., VIC – FAM – VIC (Table 2). Polymerase chain reactions (PCR) were performed in 15 µL reactions containing 1 µL DNA template, 1.5 µL of 10X buffer, 1.2 µL of 25 mM dNTP mix, 0.3 µL of each labeled 5' primer (10 nmol, Applied Biosystems, Foster City, California), 0.3 µL of each unlabeled 3' primer (10 nmol, Eurofins MWG Operon LLC, Huntsville, Alabama), and 0.08 µL *taq*DNA polymerase (TaKaRa Ex Taq™ Hot Start Version, Takara Bio Inc., Otsu, Japan). Adjusting to a final volume of 15 µL required a varying amount of water dependent on the number of primers used in a single reaction. Amplification was performed on a GeneAmp® PCR System 9700 thermocycler (Applied Biosystems, Foster City, California). 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 µL) of PCR product was visualized on a 2% TAE agarose gel prestained with ethidium bromide (0.4 µg/mL final concentration). Documentation of gels was performed using the GelDocIt® TS2 Imager (UVP LLC, Upland, California) and VisionWorks® LS Image Acquisition and Analysis Software v 7.1 (UVP LLC, Upland, California). A 2 µL portion of the PCR product was diluted 1:10 in water and submitted for fragment analysis. Fragment analysis was performed at the Genomics Core Facility, Huck Institute for the Life Sciences, Penn State University, using fluorescent-labeled primers and the GeneMapper® fragment analysis program (LifeTechnologies, Carlsbad, California). PCR products were analyzed on an Applied Biosystems 3730xl DNA Analyzer, using the Applied Biosystems Data Collection Software v 2.0 (Foster City, California). The resulting data was visualized with Applied Biosystems PeakScanner v1.0 (Foster City, California) to determine fragment size, and sorted using Microsatellite Toolkit v3.3.1 (University of California, Davis, California) in Microsoft® Excel 2013 (Microsoft, Redmond, Washington). Inheritance ratios were estimated from all 15 microsatellite loci. Goodness-of-fit G tests with William's correction for small sample size (Sokal & Rohlf 1995) and Bonferroni corrections (Rice 1989) were used to compare genotypic ratios in progeny to Mendelian expectations.

CLONING AND SEQUENCING

PCR was repeated using unlabeled primers for each observed allele selected as follows. For those loci that did not exhibit any departures from Mendelian expectations or other issues listed below, 1 parent and 1 progeny was chosen to represent the allele for cloning and subsequent sequencing. For those markers where departures from Mendelian expectation occurred, the family group exhibited a potential null allele or an unexpected band was observed, both parents and 6 progeny were selected and DNA amplified. PCR products for each allele were cloned into the TOPO 2.1 vector (Invitrogen, Life Technologies, Carlsbad, California) and grown on Luria Broth plates treated with 50 µg/mL of kanamycin. After the plates were incubated overnight at 37 °C, 6 colonies were chosen from each plate for screening. For family group plates exhibiting the potential issues listed previously, an additional 6 colonies were screened. Each colony was grown in a 5 mL Luria Broth containing 50 µg/mL of kanamycin. DNA was extracted from clones using the QIAprep Spin Miniprep Kit (QIAGEN, Hilden, Germany). Cycle sequencing reactions were performed at the Genomics Core Facility, Huck Institute for the Life Sciences, Penn State University, using 3' BigDye-labeled dideoxynucleotide triphosphates (v 3.1 dye terminators; Life Technologies, Carlsbad, California) and T3 or T7 universal primers. Reactions were run on an Applied Biosystems 3730xl DNA Analyzer following manufacturer's instructions (Applied Biosystems, Protocol #4303237), using Applied Biosystems Data Collection Software v 2.0 (Foster City, California). Sequencing trace files were then analyzed and aligned using Sequencher (v5.0, Gene Codes Corp., Ann Arbor, Michigan).

Results

In total, 1,128 multiplex PCRs and 94 singleplex PCRs were performed. Approximately 99.5% of all PCRs (multiplex and singleplex) produced results for analysis (Table 3). All of the parental samples and 98.3% of all loci tested on the progeny samples produced interpretable results. There were various characteristics used to improve the interpretation of multiplex marker data. This included wide gaps separating fragments for each marker, the use of alternating fluorescent labels, and the ability to use marker-specific shapes in order to increase allele call accuracy in chromatographs. All these facilitated and allowed for high confidence in making calls for the loci tested. The presence of double peaks was common in several panels, however, did not impede making accurate calls. We also observed a variation in mobility between sample plates resulting in a minute difference in allele size calls. This has the potential to lead to errors when rounding to the nearest whole number, which in turn may cause a 1 bp difference between fragment analysis plates. When a rounding error occurred, it was often consistent for all samples throughout the analyzed plate. In order to correct for this error, rounding rules were adjusted so that allele calls were similar between all plates; i.e. round down even if fragment size is 96.67. This method for normalizing results allowed for accurate comparisons between plates and thus reduced length bias. When running unknowns, internal controls from previous runs were used to help account for these variations and aided in determining the rounding rules.

We observed inconsistencies in 4 of 15 loci. The results of the analysis show that only 2 loci, *Ccmic3* and *Ccmic25*, deviated from expected segregation ratios after applying the Bonferroni correction (Table 4). Fragment analysis revealed that 1 locus, *Ccmic7*, was monomorphic for all the tested families precluding validation of the primer set using segregation analysis. Sequencing identified evidence of allelic homoplasy in locus *Ccmic9*. The other loci generated genotypes within families consistent with expected Mendelian segregation ratios (Table 4). Troubleshooting was performed to determine possible causes of departures from Mendelian expectations for *Ccmic3* and *Ccmic25*.

The departures in segregation pattern from the expected ratio, which was observed for the *Ccmic3* locus in Families A1 and A7, were initially thought to be caused by the presence of a null allele. Analysis of the progeny revealed 2 improbable Mendelian ratios for both these crosses (A3 expected 1:1, A3 observed 1:2:1; A7 expected 1, A7 observed 1:1; Table 4). Cloning and sequencing revealed the presence of 2 thymine bases at the 5' end of the PCR fragment, suggesting that the cause for departures was due to incomplete adenylation resulting in an extra allele (Fig. 1). If adenylation is incomplete, it may result in 2 products for 1 allele observed as double peaks: a peak for the non-adenylated fragment and an additional peak 1 bp longer corresponding to the adenylated fragment. Incomplete adenylation compromises peak recognition, particularly for heterozygote genotypes with adjacent alleles (Guichoux et al. 2011). Sequencing revealed that either an 11 or 12 dimer repetitive motif occurred at the *Ccmic3* locus (Fig. 1). In order to correct this issue, the forward primer was redesigned with the addition of 2 guanine bases and the 6-FAM dye was placed on the reverse primer. These adjustments minimized double peaks, increased resolution, and thereby improved interpretation (Fig. 2). These modi-

Table 3. Description and frequencies of microsatellite alleles in individuals from 9 *Ceratitis capitata* families sampled for this present study.

Locus	No. of alleles	Observed allele sizes (bp)	Fragment description and/or repeat motif	No. of alleles in parents	No. of alleles in progeny	Allele frequency in parents	Frequency in progeny	Overall allele frequency	H_E	H_O
Medflymic30	2	126	$(CA)_{12}$	6	75	0.167	0.218	0.213		
		130	$(CA)_{14}$	30	269	0.833	0.782	0.787		
									0.278	0.341
Medflymic78	2	153	$(AC)_{13}$	4	31	0.111	0.090	0.092		
		155	$(AC)_{14}$	32	315	0.889	0.910	0.908		
									0.198	0.163
Medflymic43	4	168	$(AC)_{10}$	2	9	0.056	0.026	0.029		
		180	$(AC)_{15}$ATAC	11	104	0.306	0.301	0.301		
		215	$(AC)_{30}$	22	214	0.611	0.618	0.618		
		221	$(AC)_{32}$	1	19	0.028	0.055	0.052		
									0.529	0.523
Medflymic92	3	138	$(AG)_{11}TG(AG)_2TG$	18	163	0.500	0.474	0.476		
		140	$(AG)_{12}TG(AG)_2TG$	5	44	0.139	0.128	0.129		
		143	$(AG)_7GG(AG)_5TG(AG)_2TG$	13	137	0.361	0.398	0.395		
									0.600	0.601
Medflymic67	3	156	$(GT)_4GGGG(GT)_3$ + internal deletion[d]	6	62	0.167	0.180	0.179		
		168	$(GT)_9$	27	253	0.750	0.735	0.737		
		170	$(GT)_{10}$	3	29	0.083	0.084	0.084		
									0.403	0.420
Medflymic74	2	188	$(GT)_8$-107_{bp}-$(CA)_9$	12	130	0.333	0.401	0.394		
		191	$(GT)_6$ATGT-107_{bp}-$(CA)_{11}$	24	194	0.667	0.599	0.606		
									0.444	0.470
Ccmic15	3	92	$(TG)_{10}$	20	177	0.556	0.509	0.513		
		101	$(TG)_{15}$	6	70	0.167	0.201	0.198		
		103	$(TG)_4TA(TG)_3A(GT)_7$	10	101	0.278	0.290	0.289		
									0.586	0.617
Ccmic25	3	141	$(TATG)_7$	28	275	0.778	0.790	0.789		
		146	$(TATG)_8$	1	9	0.028	0.026	0.026		
		159	$(TATG)_{11}$	7	64	0.194	0.184	0.185		
									0.356	0.341
Ccmic14	2	74	$(CA)_6CC(CA)_3$	25	237	0.694	0.685	0.686		
		82	$(CA)_{10}CCAA(CA)_2$	11	109	0.306	0.315	0.314		
									0.424	0.432
Ccmic9	2[c]	125	Type 1 - $GACA(GA)_9TA(GA)_6GT(GA)_2GGTA(GA)_5$	34	334	0.944	0.965	0.963		
		125	Type 2 - $GACA(GA)_{10}TA(GA)_6GT(GA)_2GGTA(GA)_4$							
		125	Type 3 - $GACA(GA)_{10}TA(GA)_5GT(GA)_2GGTA(GA)_5$							
		139	$GACA(GA)_6GG(GA)_3TAGAAA(GA)_4GT(GA)_2GGTA(GA)_5$	2	12	0.056	0.035	0.037		
									0.105	0.067

[a] The 156 bp PCR fragment had a non-repetitive deletion just upstream the repeat when compared to other alleles.
[b] Three different genotypes were observed for 125 bp PCR fragment characterizing *Ccmic9*.
[c] Original primer as published by Bonizzoni et al. (2000).
[d] Primer redesign with extra guanine bases and fluorescent label on reverse primer.

Table 3. (Continued) Description and frequencies of microsatellite alleles in individuals from 9 *Ceratitis capitata* families sampled for this present study.

Locus	No. of alleles	Observed allele sizes (bp)	Fragment description and/or repeat motif	No. of alleles in parents	No. of alleles in progeny	Allele frequency in parents	Frequency in progeny	Overall allele frequency	H_E	H_O
Ccmic32	4	174	TG(TTG)$_{7}$-57$_{bp}$-(TTG)$_{5}$(CTG)$_{3}$(TTG)$_{4}$	1	14	0.028	0.040	0.039		
		177	TG(TTG)$_{8}$-57$_{bp}$-(TTG)$_{5}$(CTG)$_{3}$(TTG)$_{4}$	12	107	0.333	0.309	0.312		
		180	TG(TTG)$_{9}$-57$_{bp}$-(TTG)$_{5}$(CTG)$_{3}$(TTG)$_{4}$	19	181	0.528	0.523	0.524		
		196	TG(TTG)$_{8}$-57$_{bp}$-(TTG)$_{5}$TGCTG(TTG)$_{4}$(CTG)$_{3}$(TTG)$_{4}$	4	44	0.111	0.127	0.126		
									0.597	0.613
Ccmic6	4	83	(TG)$_{12}$	10	102	0.278	0.311	0.308		
		85	(TG)$_{13}$	1	5	0.028	0.015	0.016		
		91	(TG)$_{16}$	8	70	0.222	0.213	0.214		
		96	(TG)$_{19}$	17	151	0.472	0.460	0.462		
									0.650	0.646
Medflymic81	2	130	GC-0$_{bp}$-TGTA(TG)$_{5}$TATGTA(TG)$_{3}$TATG	24	250	0.667	0.718	0.714		
		160	GC-29$_{bp}$-TGTA(TG)$_{4}$TA(TG)$_{2}$TA(TG)$_{5}$	12	98	0.333	0.282	0.286		
									0.444	0.405
Ccmic3[a]	2	74	(TG)$_{11}$	4	52	0.143	0.224	0.215		
		76	(TG)$_{12}$	24	180	0.857	0.776	0.785		
									0.245	0.348
Ccmic3[b]	2	72	(TG)$_{11}$	9	84	0.250	0.241	0.242		
		74	(TG)$_{12}$	27	264	0.750	0.759	0.758		
									0.375	0.366
Ccmic7	1	116	(TG)$_{8}$							
				32	252	1.000	1.000	1.000		
									0.000	0.000

[a] The 156 bp PCR fragment had a non-repetitive deletion just upstream the repeat when compared to other alleles.

[b] Three different genotypes were observed for 125 bp PCR fragment characterizing *Ccmic9*.

[c] Original primer as published by Bonizzoni et al. (2000).

[d] Primer redesign with extra guanine bases and fluorescent label on reverse primer.

Table 4. The results of the segregation analysis of microsatellite alleles in individuals from 9 *Ceratitis capitata* families sampled.

Locus	Multiplex panel	Female alleles	Male alleles	*N*	Alleles of progeny	Expected ratio	Observed ratio
Family A1							
Medflymic30	1	130/130	126/130	32	126/130:130/130	1:1	11:21
Medflymic78	1	155/155	155/155	32	155/155	1	32
Medflymic43	1	215/221	215/215	32	215/215:215/221	1:1	13:19
Medflymic92	2	140/143	143/143	31	143/140:143/143	1:1	14:17
Medflymic67	2	156/168	168/168	31	156/168:168/168	1:1	16:15
Medflymic74	2	188/188	191/191	31	188/191	1	31
Ccmic15	3	92/103	92/101	32	92/92:92/101:92/103:101/103	1:1:1:1	9:8:7:8
Ccmic25	3	141/146	141/141	32	141/141:141/146	1:1	23:9[b]
Ccmic14	4	74/82	74/82	32	74/74:74/82:82/82	1:2:1	2:22:8[b]
Ccmic9	4	125/125	125/125	32	125/125	1	32
Ccmic32	4	180/196	177/190	32	177/180:177/196:180/180:180/196	1:1:1:1	8:11:8:5
Ccmic6	5	83/83	83/96	32	83/83:83/96	1:1	17:15
Medflymic81	5	130/160	130/160	32	130/130:130/160:160/160	1:2:1	12:15:5
Ccmic3	6	74/76	76/76	32	74/74:74/76:76/76	1:1	5:16:11[d]
Ccmic7	6	116/116	116/116	32	116/116	1	32
*Ccmic3**[a]	6	72/74	74/74	32	72/74:74/74	1:1	17:15
Family A2							
Medflymic30	1	126/130	130/130	20	126/130:130/130	1:1	7:13
Medflymic78	1	153/155	153/155	20	153/153:153/155:155/155	1:2:1	5:11:4
Medflymic43	1	180/215	168/215	20	168/180:168/215:180/215:215/215	1:1:1:1	3:3:6:8
Medflymic92	2	138/143	143/143	20	138/143:143/143	1:1	10:10
Medflymic67	2	168/170	168/168	20	168/168:168/170	1:1	13:7
Medflymic74	2	188/188	191/191	20	188/191	1	20
Ccmic15	3	103/103	92/101	20	92/103:101/103	1:1	11:9
Ccmic25	3	141/159	141/159	20	141/141:141/159:159/159	1:2:1	1:13:6
Ccmic14	4	74/74	74/82	20	74/74:74/82	1:1	10:10
Ccmic9	4	125/125	125/125	20	125/125	1	20
Ccmic32	4	180/196	180/180	20	180/180:180/196	1:1	7:13
Ccmic6	5	83/96	83/96	20	83/83:83/96:96/96	1:2:1	5:11:4
Medflymic81	5	130/130	130/160	20	130/130:130/160	1:1	16:4[b]
Ccmic3	6	76/76	76/76	12	76/76	1	12
Ccmic7	6	116/116	116/116	12	116/116	1	12
*Ccmic3**[a]	6	74/74	74/74	20	74/74	1	20
Family A3							
Medflymic30	1	126/130	126/130	29	126/126:126/130:130/130	1:2:1	9:15:5
Medflymic78	1	155/155	155/155	30	155/155	1	30
Medflymic43	1	215/215	180/180	30	180/215	1	30
Medflymic92	2	138/138	138/138	30	138/138	1	30

[a] Primer redesign. Original primer did not have the guanine bases as indicated in Table 1.
[b] Indicates nominal significant deviation from expected Mendelian ratio ($P < 0.05$).
[c] Indicates significant deviation from expected Mendelian ratio after sequential Bonferroni correction ($P < 0.001$).
[d] Deviation from expected ratio suspected as a null allele later determined to be caused by incomplete adenylation.
[e] This family group was not tested with original *Ccmic3* primers so no data available.
[f] Original expected ratio when 136 bp artifact was treated as a true allele.
[g] Observed ratio when including 136 bp signal as true allele.

Table 4. (Continued) The results of the segregation analysis of microsatellite alleles in individuals from 9 *Ceratitis capitata* families sampled.

Locus	Multiplex panel	Female alleles	Male alleles	*N*	Alleles of progeny	Expected ratio	Observed ratio
Medflymic67	2	156/170	156/168	30	156/156:156/168:156/170:168/170	1:1:1:1	9:5:9:7
Medflymic74	2	191/191	191/191	30	191/191	1	30
Ccmic15	3	92/103	92/101	30	92/92:92/101:92/103:101/103	1:1:1:1	9:7:6:8
Ccmic25	3	141/159	141/159	30	141/141:141/159:159/159	1:2:1	5:24:1[c]
Ccmic14	4	74/82	74/74	30	74/74:74:82	1:1	15:15
Ccmic9	4	125/125	125/125	30	125/125	1	30
Ccmic32	4	180/180	177/177	30	177/180	1	30
Ccmic6	5	91/96	91/96	20	91/91:91/96:96/96	1:2:1	5:10:5
Medflymic81	5	130/130	130/160	30	130/130:130/160	1:1	16:14
Ccmic3	6	76/76	74/76	12	74/76:76/76	1:1	9:3
Ccmic7	6	116/116	116/116	22	116/116	1	22
*Ccmic3**[a]	6	74/74	72/74	30	72/74:74/74	1:1	16:14
Family A7							
Medflymic30	1	126/130	126/130	23	126/126:126/130:130/130	1:2:1	6:12:5
Medflymic78	1	155/155	155/155	23	155/155	1	23
Medflymic43	1	215/215	180/215	23	180/215:215/215	1:1	10:13
Medflymic92	2	138/138	138/143	23	138/138:138/143	1:1	7:16
Medflymic67	2	168/168	168/168	23	168/168	1	23
Medflymic74	2	188/191	188/191	23	188/188:188/191:191/191	1:2:1	9:9:5
Ccmic15	3	103/103	92/101	24	92/103:101/103	1:1	9:15
Ccmic25	3	141/141	141/141	24	141/141	(1:2:1)[e] 1	(0:9:15)[f] 24
Ccmic14	4	74/74	74/74	24	74/74	1	24
Ccmic9	4	125/125	125/125	24	125/125	1	24
Ccmic32	4	174/196	177/180	24	174/177:174/180:177/196:180/196	1:1:1:1	4:10:3:7
Ccmic6	5	96/96	91/96	24	91/96:96/96	1:1	12:12
Medflymic81	5	130/130	130/130	24	130/130	1	24
Ccmic3	6	76/76	76/76	24	76/76	1	4:20[d]
Ccmic7	6	116/116	116/116	24	116/116	1	24
*Ccmic3**[a]	6	74/74	72/74	24	72/74:74/74	1:1	7:17
Family A8							
Medflymic30	1	130/130	130/130	16	130/130	1	16
Medflymic78	1	155/155	155/155	16	155/155	1	16
Medflymic43	1	215/215	180/215	16	180/215:215/215	1:1	5:11
Medflymic92	2	138/140	138/143	16	138/138:138/140:138/143:140/143	1:1:1:1	3:4:7:2
Medflymic67	2	156/168	168/168	16	156/168:168/168	1:1	5:11
Medflymic74	2	188/191	191/191	16	188/191:191/191	1:1	11:5
Ccmic15	3	101/103	92/101	16	92/101:92/103:101/101:101/103	1:1:1:1	4:5:4:3
Ccmic25	3	141/141	141/141	16	141/141	1	16
Ccmic14	4	74/74	82/82	16	74/82	1	16

[a] Primer redesign. Original primer did not have the guanine bases as indicated in Table 1.
[b] Indicates nominal significant deviation from expected Mendelian ratio ($P < 0.05$).
[c] Indicates significant deviation from expected Mendelian ratio after sequential Bonferroni correction ($P < 0.001$).
[d] Deviation from expected ratio suspected as a null allele later determined to be caused by incomplete adenylation.
[e] This family group was not tested with original *Ccmic3* primers so no data available.
[f] Original expected ratio when 136 bp artifact was treated as a true allele.
[g] Observed ratio when including 136 bp signal as true allele.

Table 4. (Continued) The results of the segregation analysis of microsatellite alleles in individuals from 9 *Ceratitis capitata* families sampled.

Locus	Multiplex panel	Female alleles	Male alleles	*N*	Alleles of progeny	Expected ratio	Observed ratio
Ccmic9	4	125/139	125/125	16	125/125:125/139	1:1	10:6
Ccmic32	4	180/180	177/177	16	177/180	1	16
Ccmic6	5	91/91	96/96	16	91/96	1	16
Medflymic81	5	130/130	160/160	16	130/160	1	16
Ccmic3	6	--/--[e]	--/--[e]		--/--[e]		
Ccmic7	6	116/116	116/116	16	116/116	1	16
*Ccmic3**[a]	6	72/74	72/74	16	72/72:72/74:74/74	1:2:1	5:10:1
Family B1							
Medflymic30	1	130/130	130/130	12	130/130	1	12
Medflymic78	1	155/155	155/155	12	155/155	1	12
Medflymic43	1	215/215	215/215	12	215/215	1	12
Medflymic92	2	138/143	138/140	12	138/138:138/140:138/143:140/143	1:1:1:1	0:4:5:3
Medflymic67	2	168/168	168/168	12	168/168	1	12
Medflymic74	2	191/191	188/191	12	188/191:191/191	1:1	3:9
Ccmic15	3	92/92	92/92	12	92/92	1	12
Ccmic25	3	141/159	141/159	12	141/141:141/159:159/159	(1:1:1:1)[f] 1:2:1	(4:2:4:2)[g] 6:4:2
Ccmic14	4	82/82	74/74	12	74/82	1	12
Ccmic9	4	125/125	125/125	12	125/125	1	12
Ccmic32	4	177/177	180/180	12	177/180	1	12
Ccmic6	5	83/85	83/96	12	83/83:83/85:83/96:85/96	1:1:1:1	3:4:4:1
Medflymic81	5	130/160	160/160	12	130/160:160/160	1:1	6:6
Ccmic3	6	76/76	76/76	12	76/76	1	12
Ccmic7	6	116/116	116/116	12	116/116	1	12
*Ccmic3**[a]	6	74/74	74/74	12	74/74	1	12
Family B3							
edflymic30	1	130/130	130/130	16	130/130	1	16
Medflymic78	1	155/155	153/155	16	153/155:155/155	1:1	5:11
Medflymic43	1	168/180	180/215	16	168/180:168/215:180/180:180/215	1:1:1:1	1:2:10:3[b]
Medflymic92	2	138/143	138/140	16	138/138:138/140:138/143:140/143	1:1:1:1	3:4:2:7
Medflymic67	2	168/168	156/168	16	156/168:168/168	1:1	3:13[b]
Medflymic74	2	188/191	188/188	16	188/188:188/191	1:1	11:5
Ccmic15	3	92/92	92/92	16	92/92	1	16
Ccmic25	3	141/141	141/141	16	141/141	1	16
Ccmic14	4	74/82	74/74	15	74/74:74/82	1:1	9:6
Ccmic9	4	125/125	125/125	15	125/125	1	15
Ccmic32	4	177/180	177/180	15	177/177:177/180:180/180	1:2:1	1:9:5
Ccmic6	5	91/91	96/96	16	91/96	1	16
Medflymic81	5	130/130	130/130	16	130/130	1	16
Ccmic3	6	--/--[e]	--/--[e]		--/--[e]		
Ccmic7	6	116/116	116/116	16	116/116	1	16

[a] Primer redesign. Original primer did not have the guanine bases as indicated in Table 1.

[b] Indicates nominal significant deviation from expected Mendelian ratio ($P < 0.05$).

[c] Indicates significant deviation from expected Mendelian ratio after sequential Bonferroni correction ($P < 0.001$).

[d] Deviation from expected ratio suspected as a null allele later determined to be caused by incomplete adenylation.

[e] This family group was not tested with original *Ccmic3* primers so no data available.

[f] Original expected ratio when 136 bp artifact was treated as a true allele.

[g] Observed ratio when including 136 bp signal as true allele.

Table 4. (Continued) The results of the segregation analysis of microsatellite alleles in individuals from 9 *Ceratitis capitata* families sampled.

Locus	Multiplex panel	Female alleles	Male alleles	*N*	Alleles of progeny	Expected ratio	Observed ratio
*Ccmic3**[a]	6	72/74	72/74	16	72/72:72/74:74/74	1:2:1	0:11:5[b]
Family B5							
Medflymic30	1	130/130	130/130	12	130/130	1	12
Medflymic78	1	155/155	153/155	12	153/155:155/155	1:1	6:6
Medflymic43	1	180/180	180/215	12	180/180:180/215	1:1	6:6
Medflymic92	2	140/143	138/138	12	138/140:138/143	1:1	6:6
Medflymic67	2	168/168	156/170	12	156/168:170/168	1:1	6:6
Medflymic74	2	191/191	188/188	12	188/191	1	12
Ccmic15	3	92/92	103/103	12	92/103	1:1	12
Ccmic25	3	141/141	141/141	12	141/141	(1:2:1)[f] 1	(0:0:12)[g] 12
Ccmic14	4	74/82	74/74	12	74/74:74/82	1:1	6:6
Ccmic9	4	125/125	125/125	12	125/125	1	12
Ccmic32	4	177/180	180/196	12	177/180:177/196:180/180:180/196	1:1:1:1	4:1:3:4
Ccmic6	5	83/83	91/96	12	83/91:83/96	1:1	5:7
Medflymic81	5	130/160	130/160	12	130/130:130/160:160/160	1:2:1	2:4:6
Ccmic3	6	76/76	74/76	12	74/76:76/76	1:1	7:5
Ccmic7	6	116/116	116/116	12	116/116	1	12
*Ccmic3**[a]	6	74/74	72/74	12	72/74:74/74	1:1	6:6
Family B8							
Medflymic30	1	130/130	130/130	12	130/130	1	12
Medflymic78	1	155/155	155/155	12	155/155	1	12
Medflymic43	1	180/215	215/215	12	180/215:215/215	1:1	8:4
Medflymic92	2	143/143	138/138	12	138/143	1	12
Medflymic67	2	168/168	168/168	12	168/168	1	12
Medflymic74	2	191/191	191/191	12	191/191	1	12
Ccmic15	3	92/92	92/103	12	92/92:92/103	1:1	4:8
Ccmic25	3	141/159	141/141	12	141/141:141/159	(1:1:1:1)[f] 1:1	(2:0:5:5)[g] 7:5
Ccmic14	4	74/74	74/82	12	74/74:74/82	1:1	6:6
Ccmic9	4	125/125	125/139	12	125/125:125/139	1:1	6:6
Ccmic32	4	177/180	180/180	12	177/180:180/180	1:1	7:5
Ccmic6	5	83/96	96/96	12	83/96:96/96	1:1	6:6
Medflymic81	5	130/130	130/160	12	130/130:130/160	1:1	6:6
Ccmic3	6	76/76	74/76	12	74/76:76/76	1:1	6:6
Ccmic7	6	116/116	116/116	12	116/116	1	12
*Ccmic3**[a]	6	74/74	72/74	12	72/74:74/74	1:1	7:5

[a] Primer redesign. Original primer did not have the guanine bases as indicated in Table 1.
[b] Indicates nominal significant deviation from expected Mendelian ratio ($P < 0.05$).
[c] Indicates significant deviation from expected Mendelian ratio after sequential Bonferroni correction ($P < 0.001$).
[d] Deviation from expected ratio suspected as a null allele later determined to be caused by incomplete adenylation.
[e] This family group was not tested with original *Ccmic3* primers so no data available.
[f] Original expected ratio when 136 bp artifact was treated as a true allele.
[g] Observed ratio when including 136 bp signal as true allele.

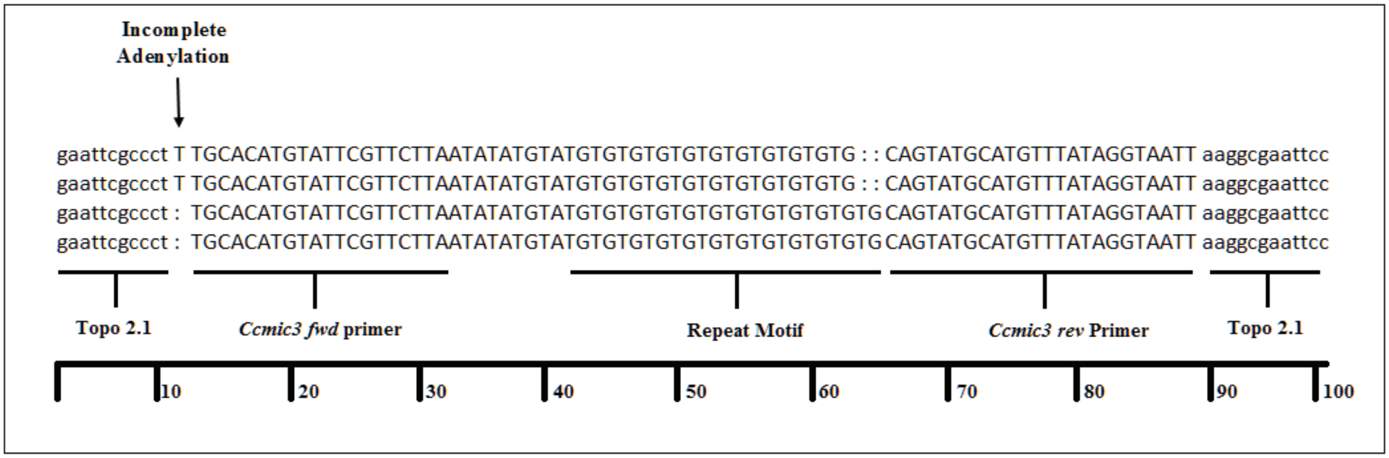

Fig. 1. Sequence alignment of *Ccmic3* with original primer design. Top two sequences are genotypes of 74 bp allele and bottom two sequences are genotypes of the 76 bp allele. One extra thymine residue on the 5′ end appeared in 8 of the 8 clones for this allele leading to difficulties in scoring this locus. Redesign of the forward primer by adding the extra guanines provided better resolution in scoring. Lower case sequence represents the Topo 2.1 vector just past the *EcoRI* in the multiple cloning site.

fications, however, resulted in a shift in the mobility causing the fragment size to appear 2 bp smaller as compared with fragments prior to modifications. After primer redesign, the interpretation of data generated with *Ccmic3* (called *Ccmic3**) was improved and provided more accurate results, as confirmed when retested for deviations from Mendelian expectations ratios (Table 4).

Modification to the locus (*Ccmic3**) improved assay performance and data interpretation. It could also eliminate potential issues because it is possible for the 74/76 allele described in our restricted sampling to occur in the wild. In a study by Bonizzoni et al. (2002), the authors reported the presence of a 74/78 male and a 78/78 male being present at other matings with a null allele being called (in Table 3 of Bonizzoni et al. 2002). Regardless of whether amplification of the region produced a PCR product, a null allele could have been called because of a potential cross from the presence of a 74/78 male and a 72/76 female. One potential result of this cross is a progeny with a 74/76 allele, the problematic allele observed in our study. If this allele was observed in the study, it would have appeared as a 76/76 allele (not possible based on potential parental genotypes) during fragment analysis resulting in a null call. However, utilizing the redesigned primers would have eliminated that possibility and reinforced the remating hypothesis (χ^2 = 11.923, *P* = 0.015), which is that female medflies have multiple male partners.

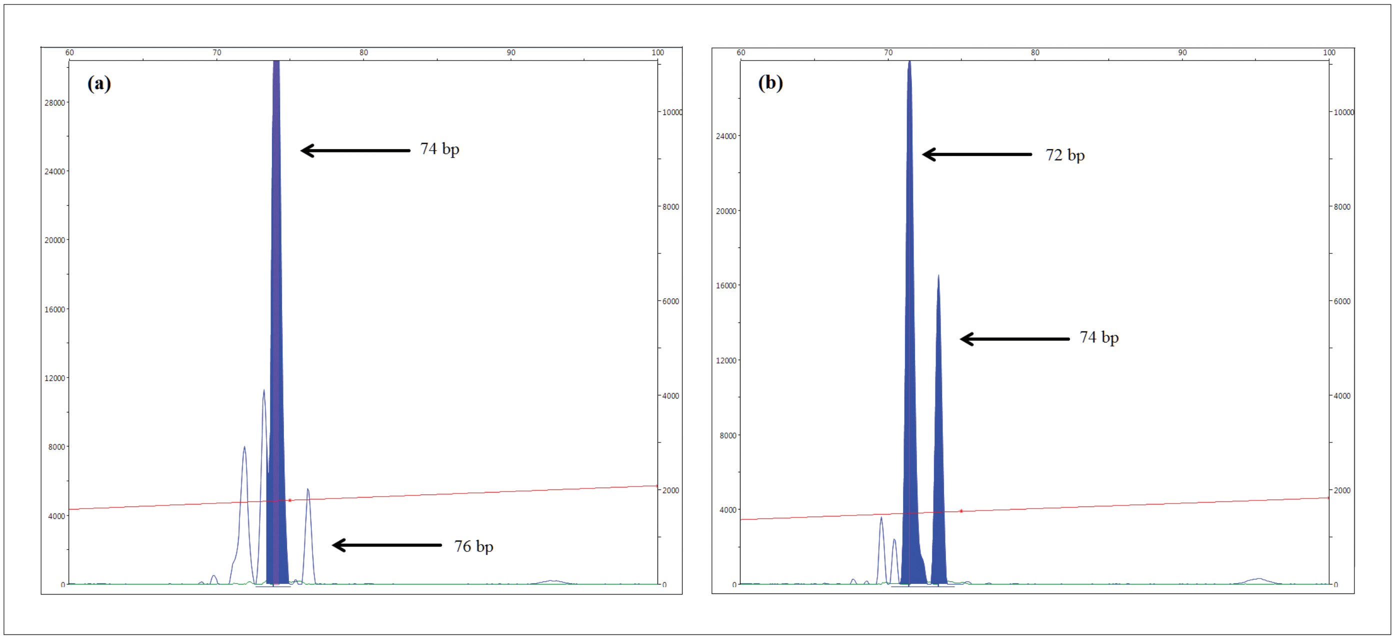

Fig. 2. Chromatograph comparing original and redesigned primers for *Ccmic3* on sample *A1-F1-07*, Family A1. Both reactions were run simultaneously on the same fragment analysis plate using the same PCR conditions, DNA concentrations, and dilution factor. a) Chromatograph of progeny exhibiting an allele call of 74/74. Parents are 74/76 and 76/76. The observed 76 bp peak was considered to be weak. Cloning and sequencing confirmed the existence of this 76 bp fragment. b) Chromatograph of the same progeny as in Fig. 2a now exhibiting an allele call of 72/74 after primer modification. Parents are now 72/74 and 74/74. The observed 74 bp peak is more pronounced compared to the previous 76 bp call. The intensity of the 74 bp peak also increased while the other 3 visible peaks decreased. This suggests an increase in adenylation has occurred.

Departures from expected ratios at the *Ccmic25* locus were detected for several families, but for 2 different causes. One departure was determined to be caused by the inclusion of an artifactual band of approximately 136 bp in Families A7, B1, and B5 (Table 4). Twelve attempts to isolate the 136 bp fragment through gel excision and/or direct cloning from the PCR product were unsuccessful. All sequences of screened clones corresponded with all the other observed genotypes. The cause of this anomaly has not been determined. It was noted that the shape of the peak of the 136 bp "allele" (Fig. 3a) was distinctly different and not characteristic of the other peak shapes from confirmed 137 bp alleles (Fig. 3b) that we observed and sequenced during panel assignment of medflies collected from South America (unpublished data). There was no significant departure from Mendelian ratios indicated when 136 bp fragment calls were not included in estimates (Table 4).

Evidence of non-Mendelian inheritance patterns was observed at the *Ccmic25* locus in Family A3 (Table 4). This family exhibited a transmission ratio distortion in the form of heterozygote excess. This is a common occurrence in inheritance studies (Reece et al. 2004; Karlsson et al. 2007; Li et al. 2007; Guzinski et al. 2008). This could be an incident of segregation distortion, a ubiquitous phenomenon, which is characterized by a deviation from expected Mendelian ratios due to high heterozygosity (Aparicio et al. 2010; Liu et al. 2010). It is possible that this marker is located on a portion of a chromosome affected by a segregation distorter system, a powerful evolutionary force that can affect the frequency of certain genotypes, leading to the observed transmission ratio distortion (Lyttle 1993; Aparicio et al. 2010; Liu et al. 2010). Using markers that exhibit transmission ratio distortion does pose difficulties when mapping chromosomes (Hacket & Broadfoot 2003). However, the impact on assignment testing due to the segregation distortion at one locus should be minimal due to the robust nature of the test (Pritchard, Stanford University, personal communication). The number of loci and degree of genetic differentiation has more impact on the accuracy of assignment testing (Carlsson 2008). Performing assignment tests using and then excluding this marker should be similar unless the segregation distortion is creating large regions of linkage disequilibrium in the data (Pritchard, Stanford University, personal communication).

Although the *Ccmic9* locus did not generate segregation ratios that deviate from Mendelian expectations, sequencing revealed a higher level of heterozygosity within this locus than expected based on allele numbers. The progeny from Families A8 and B8 revealed that 2 diplotypes occurred for this locus, 125/125 and 125/139, respectively (Table 4). However, sequencing of the clones revealed 2 distinct genotypes for the 125 bp allele. Additional cloning and sequencing of flies from other families for this locus revealed that 3 distinct genotypes were being represented by the 125 bp haplotype with a high number of indels being observed within the 4 observed genotypes. This is clear evidence of allelic homoplasy in the marker that could confound interpretation of data sets. Substantial difference between rates of indel mutations within the repeat sequence and during recombination could lead to series of alleles evolving essentially independently from other series of the locus, which in turn could represent a separate evolutionary process (Lehmann et al. 1996). The variation seen in this 1 haplotype could be associated to ecological or geographic trends, but additional data are needed to confirm this hypothesis. Sequencing this locus and other loci may provide insight on the application of this method for phylogenetic and phylogeographic studies.

Finally, *Ccmic7* was revealed to be monomorphic (*Ccmic7* x_{116}) for our sampling (Table 3). Cloning confirmed the sequencing results. The monomorphic nature of *Ccmic7* prevented us from determining if this locus was within Mendelian expectations. Testing with additional geographic collections is suggested for *Ccmic7* because this marker has historical significance among medfly captures gathered in California as reported by Bonizzoni et al. (2001) and Gasperi et al. (2002).

Discussion

The work in the present study may provide useful information in differentiating wild flies from strains used for sterile insect technique

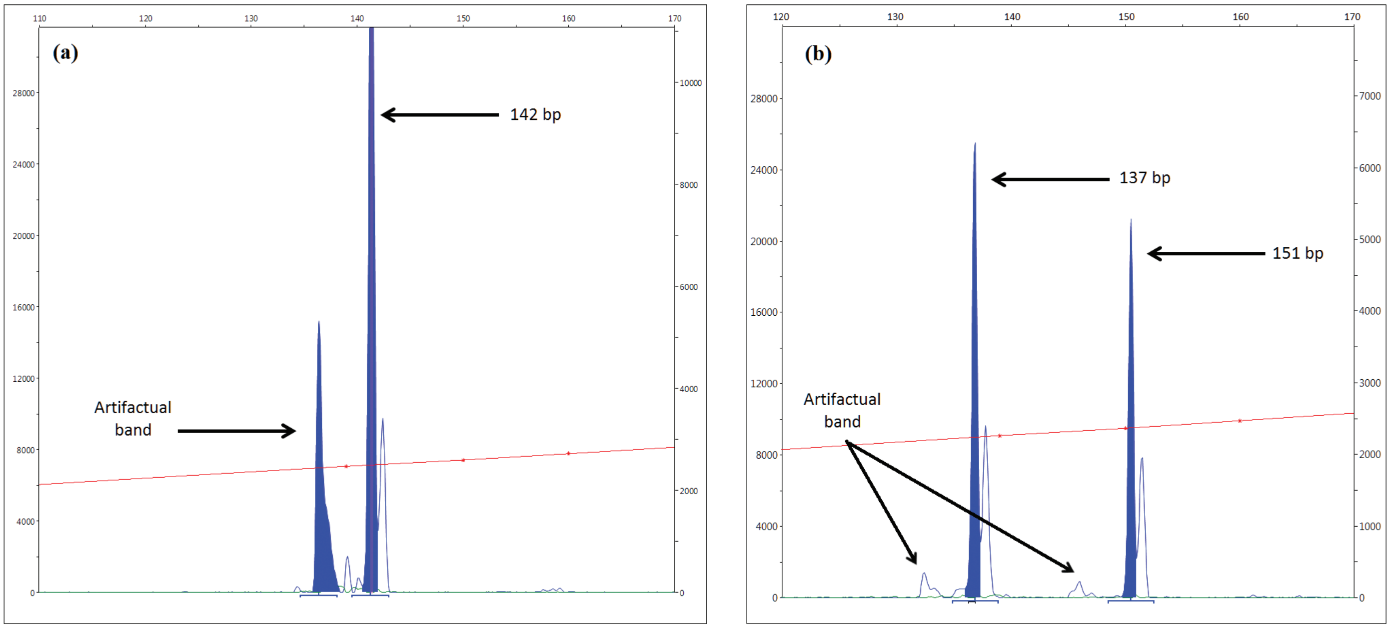

Fig. 3. Comparing artifactual bands to actual bands to determine allele as observed at the *Ccmic25* locus. a) Chromatograph of sample exhibiting an artifactual band can be observed at the 136 bp peak. The geometric shape and configuration is not consistent with those of microsatellites. b) This chromatograph shows the characteristic high peak followed by a low peak expected when making a call around the 137 bp peak. To the left of both peaks are very low broad bands, commonly observed during analysis. In homozygote individuals, these peaks may extend higher and resemble an artifactual band similar to what was observed in Fig. 3a.

releases, and markers tested herein will be used in the future to assist in documenting the hypothetical consequences of marginal reproduction of wild and partially sterile insects. In our study, we demonstrated that 14 microsatellite loci for this international pest perform according to Mendelian expectations thereby supporting their use for identifying the source population of medfly interceptions or incursions. Several of these microsatellite markers are an important resource in source estimations for fruit fly captures and provide information useful in the management of medfly (Bonizzoni et al. 2000, 2001, 2004; Stratikopoulos et al. 2009; Kartsen 2013). Six of the evaluated loci (i.e., *Ccmic3*, *Ccmic6*, *Ccmic7*, *Ccmic9*, *Ccmic14*, and *Ccmic15*) were developed and used on medflies collected from tropical Africa, the Mediterranean basin and South America (Bonizzoni et al. 2000). The results were consistent with previous studies using alternative methods (Gomulski et al. 1998; Malacrida et al. 1998). *Ccmic3*, *Ccmic25*, and *Ccmic32* were used in characterizing Australian populations of medfly with fixed alleles being identified in the *Ccmic3* and *Ccmic32* loci (Bonizzoni et al. 2004). Additionally and very importantly for the diagnostics of medflies in California, at the *Ccmic7* locus, a unique genotype (*Ccmic7* x_{142}) was reported among medfly captures made in the Los Angeles basin (Bonizzoni et al. 2001; Gasperi et al. 2002). Finally, the *Ccmic3* locus has been used to infer paternity comparing wild caught medfly mothers and their offspring (Bonizzoni et al. 2002).

Table 5. GenBank accession numbers associated with observed alleles

Locus	Observed allele sizes (bp)	Fragment description and/or repeat Motif	Accession Number
Medflymic30	126	(CA)$_{12}$	KM410072
	130	(CA)$_{14}$	KM410073
Medflymic78	153	(AC)$_{13}$	KM410083
	155	(AC)$_{14}$	KM410084
Medflymic43	168	(AC)$_{10}$	KM410074
	180	(AC)$_{15}$ATAC	KM410075
	215	(AC)$_{30}$	KM410076
	221	(AC)$_{32}$	KM410077
Medflymic92	138	(AG)$_{11}$TG(AG)$_2$TG	KM410045
	140	(AG)$_{12}$TG(AG)$_2$TG	KM410046
	143	(AG)$_7$GG(AG)$_5$TG(AG)$_2$TG	KM410047
Medflymic67	156	(GT)$_4$GGGG(GT)$_3$ + internal deletion	KM410078
	168	(GT)$_9$	KM410079
	170	(GT)$_{10}$	KM410080
Medflymic74	188	(GT)$_8$-107$_{bp}$-(CA)$_9$	KM410081
	191	(GT)$_6$ATGT-107$_{bp}$-(CA)$_{11}$	KM410082
Ccmic15	92	(TG)$_{10}$	KM410063
	101	(TG)$_{15}$	KM410064
	103	(TG)$_4$TA(TG)$_3$A(GT)$_7$	KM410062
Ccmic25	141	(TATG)$_7$	KM410065
	146	(TATG)$_8$	KM410066
	159	(TATG)$_{11}$	KM410067
Ccmic14	74	(CA)$_6$CC(CA)$_3$	KM410060
	82	(CA)$_{10}$CCAA(CA)$_2$	KM410061
Ccmic9	125	Type 1 - GACA(GA)$_9$TA(GA)$_6$GT(GA)$_2$GGTA(GA)$_5$	KM410056
	125	Type 2 - GACA(GA)$_{10}$TA(GA)$_6$GT(GA)$_2$GGTA(GA)$_4$	KM410057
	125	Type 3 - GACA(GA)$_{10}$TA(GA)$_5$GT(GA)$_2$GGTA(GA)$_5$	KM410058
	139	GACA(GA)$_6$GG(GA)$_3$TAGAAA(GA)$_4$GT(GA)$_2$GGTA(GA)$_5$	KM410059
Ccmic32	174	TG(TTG)$_7$-57$_{bp}$-(TTG)$_{5(}$CTG)$_3$(TTG)$_4$	KM410068
	177	TG(TTG)$_8$-57$_{bp}$-(TTG)$_5$(CTG)$_3$(TTG)$_4$	KM410069
	180	TG(TTG)$_9$-57$_{bp}$-(TTG)$_5$(CTG)$_3$(TTG)$_4$	KM410070
	196	TG(TTG)$_8$-57$_{bp}$-(TTG)$_5$TGCTG(TTG)$_4$(CTG)$_3$(TTG)$_4$	KM410071
Ccmic6	83	(TG)$_{12}$	KM410051
	85	(TG)$_{13}$	KM410052
	91	(TG)$_{16}$	KM410053
	96	(TG)$_{19}$	KM410054
Medflymic81	130	GC-0$_{bp}$-TGTA(TG)$_5$TATGTA(TG)$_3$TATG	KM410085
	160	GC-29$_{bp}$-TGTA(TG)$_4$TA(TG)$_2$TA(TG)$_5$	KM410086
Ccmic3	74	(TG)$_{11}$ In Topo 2.1 w/ 2 Adenine bases	KM410048
	74	(TG)$_{11}$ In Topo 2.1 w/ 3 Adenine bases	KM410049
	76	(TG)$_{12}$	KM410050
Ccmic7	116	(TG)$_8$	KM410055

The inclusion of the markers developed by Stratikopoulous et al. (2009) should also provide more resolution. The reported high observed heterozygosity in the selected markers should prove to be informative (Stratikopoulous et al. 2008). Additionally, these chosen markers have the potential to identify other species from the genus *Ceratitis* (Stratikopoulous et al. 2009). The markers we selected are spread across at least 4 chromosomes. However, several markers were shown to share linkage groups (Stratikopoulous et al. 2008, 2009). In particular, 2 markers, *Medflymic67* and *Medflymic78*, were shown to be separated by 2 centimorgans (Stratikopoulous et al. 2008). However, a recent pilot study we performed showed that all markers for the present study did not exhibit linkage (unpublished data). This indicates the possibility that independent recombination still occurs even between *Medflymic67* and *Medflymic78* considered within the same linkage group. Alternatively, genomic architecture could vary among different evolutionary lineages among the medfly. Eight of the selected markers were not present on the linkage map and could not be identified to a particular chromosome.

Questions may arise should *Ccmic7* or *Ccmic9* be included in any future studies. However, their use in previous studies may preclude omitting them. Although the *Ccmic7* and *Ccmic9* loci were not rejected by the segregation test, our study does not validate these loci for diagnostic use. The *Ccmic7* locus was monomorphic in the samples tested precluding a biologically significant interpretation. This can be rectified by performing crosses on flies that exhibit variability at the *Ccmic7* locus. The *Ccmic9* locus exhibited evidence of a homoplasious allele state that could possibly provide misleading results when used to examine medfly invasions. A high presence of indels in the repeat region of the *Ccmic9* has been observed leading to multiple genotypes being identified. This variability is not readily observed when basing calls on fragment size. This could lead to the accidental grouping of unrelated populations, whereas genotyping might reveal a more substantial genetic distance between the populations. Additional genotyping studies are required to test the loci for variability and the relative impact of homoplasy based on more diverse populations than the El Pino strains included in our study.

In light of these results, it is likely that many of the markers examined here will prove useful to researchers performing population studies for medfly. We have identified 2 medfly loci that should be treated with caution in future analysis, with suggestions on how to fully validate them. Although not completely validated, the decisions to include loci that may violate Mendelian segregation ratios or HWE will depend on the intended application, number of markers, and variability in the populations characterized.

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Data Accessibility

GenBank accession numbers are listed in Table 5. For chromatograph files obtained during fragment analysis, please contact the corresponding author by email. Reference FA162-167 (Parents on these plates), FA179-184, and FA192-194. Reference FA196-197 to see comparison of primer redesign for *Ccmic3*. DNA from Family crosses may be available, please contact the corresponding author by email. Reference Plate Code: MED024, MED025, MED027.

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