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Influence of remating on sterile insect technique in *Ceratitis capitata* (Diptera: Tephritidae): a molecular approach

Influência da recópula na técnica do inseto estéril em *Ceratitis capitata* (Diptera: Tephritidae): uma abordagem molecular

Vilmara P. Barboza[1]*, Beatriz A. J. Paranhos[2], Juvenal C. Silva Junior[1]

[1]Department of Biological Sciences, Universidade Estadual do Sudoeste da Bahia, Jequié, BA, Brazil. [2]Laboratory of Entomology, Embrapa Semiárido, Petrolina, PE, Brazil.

ABSTRACT - The Mediterranean fruit fly, *Ceratitis capitata* (Diptera: Tephritidae), is one of the most harmful species to world horticulture, thus requiring suppression or eradication programs. The sterile insect technique is widely used for autocidal control of nuisance insects because it neither releases exotic agents into the environment nor introduces new genetic material into existing populations. In 2005, the Moscamed Brasil biofactory imported the *tsl* Vienna 8 *C. capitata* strain from the International Atomic Energy Agency (IAEA); this action required genetic testing of the strain's biological characteristics. The objective of this study was to discriminate males of wild populations from *tsl* Vienna 8 using molecular markers, and to conclude which male line dominated in a remating condition. Four crosses, each using 100 males and 100 females, were performed: wild female × wild male; wild female × *tsl* Vienna 8 male; wild female × wild male, followed by remating with *tsl* Vienna 8 males after 48 h, and wild female × *tsl* Vienna 8 male, followed by remating with wild males after 48 h. The results showed that the *tsl* Vienna 8 strain is compatible with wild females from the São Francisco Valley region and that these males can successfully transfer their sperm to the female spermathecae. Regarding remating, the sperm of the second male dominated over that of the first one. Based on these findings, the sterile insect technique success may be reduced, since the progeny of a female that remates with a wild male could be viable.

Keywords: Genetic markers. Fruitgrowing. Mass rearing. Medfly. Spermathecae.

RESUMO - A mosca-das-frutas do Mediterrâneo, *Ceratitis capitata* (Diptera: Tephritidae), é uma das espécies mais prejudiciais à horticultura mundial, portanto requer programas de supressão ou erradicação. A técnica do inseto estéril é amplamente utilizada para o controle autocida de insetos nocivos, pois não libera agentes exóticos no meio ambiente nem introduz novos materiais genéticos nas populações existentes. Em 2005, a biofábrica Moscamed Brasil importou a cepa Vienna 8 *C. capitata* da Agência Internacional de Energia Atômica (IAEA); essa ação exigiu testes genéticos das características biológicas da linhagem. O objetivo deste estudo foi discriminar machos de populações silvestres de *tsl* Viena 8 usando marcadores moleculares, e inferir sobre qual linhagem masculina era dominante em condição de recópula. Quatro cruzamentos, cada um com 100 machos e 100 fêmeas, foram realizados: fêmea selvagem × macho selvagem; fêmea selvagem × macho *tsl* Viena 8; fêmea selvagem × macho selvagem, seguido por recópula com machos *tsl* Vienna 8 após 48 h, e fêmea selvagem × machos *tsl* Vienna 8, seguido por recópula com machos selvagens após 48 h. Os resultados mostraram que a cepa *tsl* Vienna 8 é compatível com fêmeas selvagens da região do Vale do São Francisco e que esses machos podem transferir com sucesso seus espermatozóides para a espermateca das fêmeas. Quanto à recópula, o esperma do segundo macho precedeu o primeiro. Com base nesses achados, o sucesso da técnica de insetos estéril pode ser reduzido, já que a progênie de uma fêmea que recopulou com um macho selvagem pode ser viável.

Palavras-chave: Criação massal. Espermateca. Fruticultura. Marcadores genéticos. Moscamed.

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***Corresponding author:**
<vilmara.barboza@gmail.com>

INTRODUCTION

The Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae), originated in Africa (DE MEYER, 2001). It has become one of the most important economic pests worldwide, because it is highly adaptable and invasive (VERA et al., 2002). The species has 317 hosts among fruits, legumes, and nuts (LIQUIDO; MCQUATE; SUITER, 2015). In Brazil, *C. capitata* explores 115 plant species (ZUCCHI; MORAES, 2021). In Brazil's northeastern region (São Francisco Valley, Bahia), there has been a large investment in research that aims to minimize losses and increase yield in fruticulture by controlling pest insects. *Ceratitis capitata* is the greatest cause of economic loss in this sector, because the species renders fruits unusable for commerce given the damage caused by its larvae (HAJI et al., 2005).

The sterile insect technique has been used in many countries to control fruit fly populations, because the indiscriminate use of agrochemicals harms both humans and the environment (HENDRICHS et al., 2002). This technique relies on mass rearing of the target pest followed by X-ray or gamma-ray sterilization and subsequent release (KLASSEN; CURTIS, 2005). The goal of the sterile insect

technique (SIT) is to promote mating between sterile males and wild females, because these crosses yield non-viable progeny, and would thus reduce the wild population (KLASSEN; CURTIS, 2005). The sterile insect technique is an autocidal technique, because the pest species itself controls its wild populations (WALDER, 2000). To improve the sterile insect technique efficacy, genetic sexing strains have been developed for *C. capitata* such that only adult males are reared and then released in the target area (IAEA, 1990; CÁCERES, 2002). In Brazil, this technique was first adopted in 2005 with the aim of suppressing *C. capitata* populations in the large irrigated fruticulture center in the northeastern Brazilian semiarid region (HAJI et al., 2005).

Several studies that used different molecular markers have aimed at detecting the presence of sperm from different males in female fruit fly spermathecae (SAN ANDRÉS et al., 2007; DHAKAL et al., 2010; JUAN-BLASCO et al., 2013a). From these studies, one can evaluate the percentage of sterile males that mate with wild females under laboratory and field conditions. Thus, it is possible to infer the success of the sterile insect technique (SAN ANDRÉS et al., 2007; JUAN-BLASCO et al., 2013a). Another application is to observe multiple paternity in fruit fly species, because 2 or more samples of male DNA may be observed in the spermatheca (SONG; DREW; HUGHES, 2007; DHAKAL et al., 2010).

For the sterile insect technique to be effective, the success of male copulation is directly influenced by female choice (MOSSINSON; YUVAL, 2003). Thus, the sterile insect technique is totally dependent on the species mating system (KNIPLING, 1959). This system represents lek mating (BRICEÑO; EBERHARD; SHELLY, 2007; ANJOS-DUARTE; COSTA; JOACHIM-BRAVO, 2011), and it requires high energy expenditure for pheromone release and territory demarcation (YUVAL et al., 1998). For a successful sterile insect technique, sterile males must have the appropriate physiological conditions to survive in the wild, exhibit mating behavior similar to that of wild males, and inhibit female remating (YUVAL et al., 2007). However, the courtship behavior of sterile males is not fully accepted by wild females due to mass rearing factors (CAYOL, 2000; LANCE et al., 2000).

In fruit fly species, reproductive success is related to several factors, including sperm competition (PARKER, 1970), male nutrition (JANG et al., 1998), and regulation mechanisms after intercourse (EBERHARD, 1991). A mating female seeks fruit odors, rather than male mating odors, to determine her oviposition (JANG, 2002). However, since reproductive factors influence female refractoriness, remating may occur (YUVAL et al., 2002). For effective sterile insect technique, it is unfavorable for *C. capitata* to mate more than once, because remating could result in a wild male copulating with a female after a sterile male (BLOEM et al., 1993). Many studies have shown that under natural conditions, *C. capitata* populations are polyandric; i.e., females can mate with more than 1 male during their life cycle (BONIZZONI et al., 2002; MOSSINSON; YUVAL, 2003; KRAAIJEVELD; CHAPMAN, 2004; BONIZZONI et al., 2006). As a result, the percentage of viable or non-viable sterile insect technique progenies changes if females choose a wild or sterile male for remating. Thus, the objective of this study was 2-fold: to discriminate between wild and *tsl* Vienna 8 *C. capitata* males using molecular markers, and to conclude about the predominance of the male lineage when females have remated.

MATERIALS AND METHODS

Rearing and maintenance of *Ceratitis capitata* lineages

Wild adults were obtained from Juazeiro City, Bahia State, Brazil, by collecting large quantities of larvae-infested fruits (mangos and guavas) to obtain pupae. Sampled fruits were transported to the Moscamed Brasil biofactory, and placed on vermiculite in plastic trays (60 × 30 × 15 cm). Trays were closed with cotton tissue to prevent contamination by *Drosophila*, and kept on shelves protected from ants. This step was carried out under uncontrolled conditions of temperature and humidity. After 7 and 15 d, pupae were sorted; on the fifteenth day fruits were discarded. Pupae were placed into cages with water and food (1 part yeast hydrolyzate enzymatic and 3 parts sugar) (SILVA NETO et al., 2012). Upon emergence, males and females were separated, and cages were identified by sex and emergence date, aiming to avoid possible effects of pheromone and for the flies to reach sexual maturity.

Females of the lineage Vienna 8 imported from the IAEA by Moscamed Brasil biofactory have mutations that render their pupae white (wild type pupae are brown), and their eggs lethally sensitive to temperatures over 34 °C (*tsl*). This lineage is currently reared by the Moscamed Brasil biofactory, from which the insects were obtained. Routine tests were carried out to determine the quality of sterile males (pupae weight, emergence rate, flight ability, etc.). One to 2 d before adult emergence, pupae were coated with fluorescent powder dye, bagged, and irradiated with X-ray radiation (115 Gy).

Crossbreeding experiments

Four types of crosses were performed: (1) 100 wild females (10 d old) × 100 wild males (10 d old); (2) 100 wild females (10 d old) × 100 *tsl* Vienna 8 males (5 d old); (3) 100 wild females (10 d old) × 100 wild males (10 d old), followed by remating of females after 48 h with 100 *tsl* Vienna 8 males (5 d old); (4) 100 wild females (10 d old) × 100 *tsl* Vienna 8 males (5 d old), followed by remating of females after 48 h with 100 wild males (10 d old). Wild insects take longer time to reach sexual maturity (MOSSINSON; YUVAL, 2003) than laboratory ones (Vienna 8 strain) (PAPADOPOULOS et al., 1998), so to guarantee that all males and females used in these experiments were sexually mature, we used wild ones at 10 d old and lab ones at 5 d of age.

For each cross (treatment), males were released into acrylic cages (30 × 30 × 30 cm) for approximately 30 min prior to the females, allowing males time to establish

territories and begin pheromone calling to attract females. Mating pairs were collected from 7:30 AM to 12:00 AM, by gently coaxing couples into acrylic vials. Wild male copulation durations (about 130 min) are longer than sterile male copulations (about 90 min) (PARANHOS et al., 2013), and can be a consequence of sterilization, since Paranhos et al. (2008) have found longer mating duration for fertile Vienna 8-*tsl* males than for sterile ones. At the end of mating, all females of single-mating pairs, regardless of copulation duration, were preserved in 98% alcohol and kept in a refrigerator at 4 °C for further molecular analysis. Females assigned for remating were placed in cages with water and food in the absence of males for 48 h, and then subjected to remating according to treatments. All preserved females were rehydrated with distilled water for 2 to 24 h for extraction of the two spermathecae, which were put into Eppendorf® tubes for DNA extraction to determine the source (s) of the sperm.

DNA extraction, Detection of specific markers of the Y chromosome and Discrimination between wild population and Vienna 8 strain

DNA of spermathecae of *C. capitata* females was extracted with the Wizard Genomic DNA Purification Kit (PROMEGA - Madison, Wisconsin, EUA) according to the manufacturer instructions. This included the first step of 1% dithiothreitol, because this increases the efficiency of isolation of sperm DNA (GILL; JEFFREYS; WERRET, 1985).

Separation between male and female DNA, as well as determination of the molecular profile of sterile and wild males, was performed using the markers CcYsp5'dir (5'-CGA AGC CAG ACA TACACG AGG AG-3') and CcYsp-3'rev (5'-ACA CTT ACC GAC ATT GAT TCC TG-3') as described by San Andrés et al. (2007). These markers have 4 sequences specific to the Y chromosomes of *C. capitata* males, i.e., AF071418, AF115330, AF116531, and AF154063, which were used for PCR as described by San Andrés et al. (2007). These primers were designed for non-flanked regions of A-T repetitions so as to obtain a single fragment, as stressed by Zhou, Untalan and Haymer (2000) and San Andrés et al. (2007). Primers were tested using total DNA of males and females.

Discrimination of the source of the sperm present in spermathecae (wild males or Vienna 8 males) was performed using a RFLP-PCR marker based on the mitochondrial polymorphism found in the mutant lineage Vienna 8 (GASPARICH et al., 1995; SPANOS et al., 2000). Two primers were used: Ccmt5495 (AAA TCA CCA CTT TGG ATT TGA AGC) and Ccmt5827 (TGA AAA TGG TAA ACG TGA AGA GG). These primers flank the tRNA-Gly region that carry a polymorphic site for *Hae*III, generating a PCR product of 330 bp. After digestion by endonuclease *Hae*III, samples of the wild type remain undigested, whereas those of the Vienna 8 type generate 2 bands, 1 of 190 bp and another of 140 bp. Primers were tested using the total DNA of wild and Vienna 8 males.

PCR conditions were as follows: 300 nM of dNTPs, *Taq* buffer 1×, 2 mM of $MgCl_2$, 0.75 U of *Taq* polymerase, 10 pmol of each primer, and 10 ng of total DNA. The amplification profile was as follows: 1 denaturation cycle at 94 °C for 5 min; 30 cycles at 94 °C for 30 s, at 55 °C for 40 s, and at 72 °C for 1 min, followed by 1 final elongation cycle of 72 °C for 7 min. PCR products were run on agarose gel at 2%. Samples with positive amplification were analyzed regarding the restriction length polymorphism by digestion of PCR products with 5 U of nuclease *Hae*III for 2 h at 37 °C. PCR and PCR/*Hae* products of each sample were run side by side on 2% agarose gel.

Statistical analysis

To analyze the level of significance in crosses 3 and 4 (defined above), the program GENES version 2009.7.0 (CRUZ, 2008) was used to perform a chi-squared test ($P < 0.05$).

RESULTS AND DISCUSSION

Amplification by the 2 pairs of primers (CcYsp-5' dir. and CcYsp-3' rev./ Ccmt5495 and Ccmt5827) was successfully performed with total DNA of males and females. The molecular profiles of both wild and Vienna 8 males can be observed for the 2 markers (Figure 1). The molecular profile of wild males (Figure 1) differed from that found by San Andrés et al. (2007), because the former displayed only 2 DNA bands on the gel (a larger band of about 500 bp, and a smaller one of about 150 bp), whereas that in the literature had 4 bands of different sizes.

The observed difference in the wild male molecular profile compared to that reported in the literature suggests that there has been genetic differentiation within the species with regards to the CcYsp marker. This change possibly occurred because the wild lineage cited in the literature was from another continent (SAN ANDRÉS et al., 2007), or because over 100 yr have elapsed since *C. capitata* was introduced into Brazil (ARAUJO; ZUCCHI, 2003). However, this variation does not invalidate the sterile insect technique (SAN ANDRÉS et al., 2007). The observed Ccmt-*Hae*III band pattern correlated with that reported in the literature (SAN ANDRÉS et al., 2007) and allowed easy discrimination between the wild and *tsl* Vienna 8 lineages (Figure 1).

While analyzing the laboratory crosses, we found that all female spermathecae in cross 1 showed the genetic profile of wild males, whereas all female spermathecae in cross 2 showed the genetic profile of *tsl* Vienna 8 males. Thus, it was shown that sterile, *tsl* Vienna 8 males are sexually compatible with wild females from the São Francisco Valley and that *tsl* Vienna 8 males can transfer their sperm into the spermathecae of wild females. Females did not show any type of band, which was expected given that they do not possess the Y chromosome. Crosses 1 and 2 showed the expected band pattern for the 2 male strains, and indicated that the markers presented by San Andrés et al. (2007) are robust and can be used in the different regions where *C. capitata* resides, corroborating the findings by Juan-Blasco et al. (2013b).

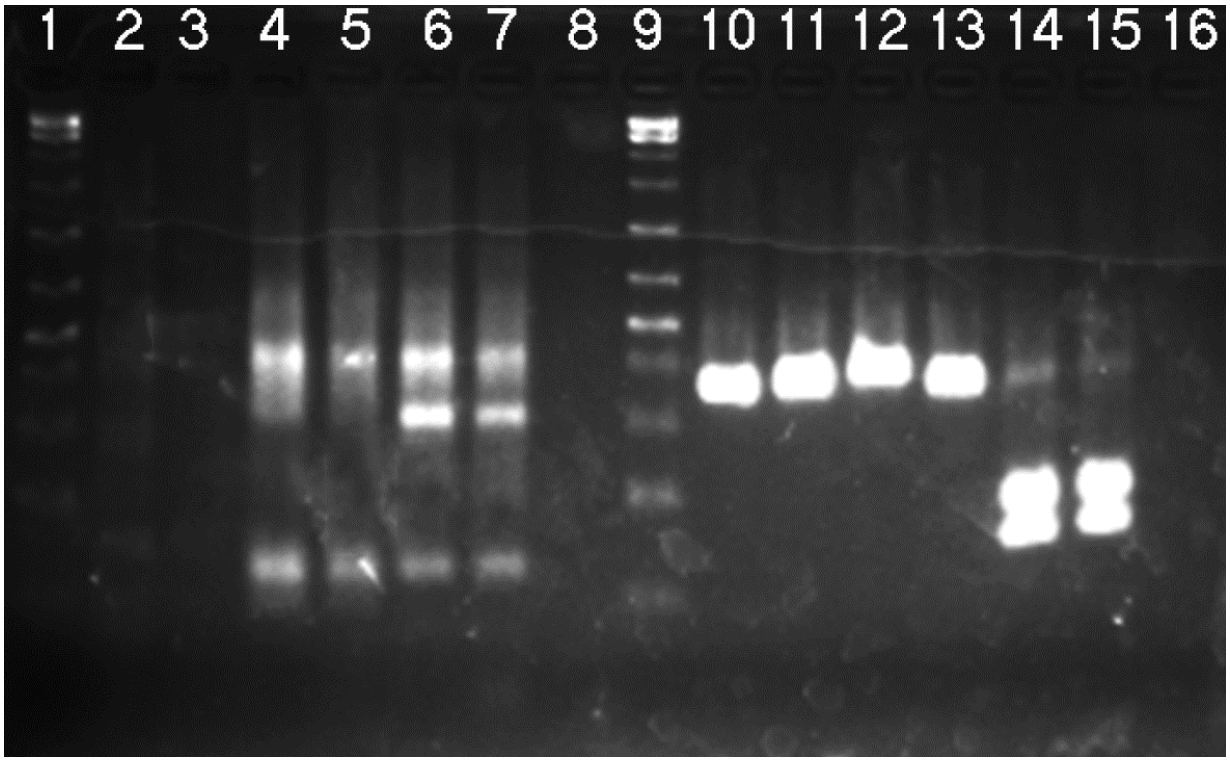

Figure 1. Agarose gel that shows PCR amplification using primer pairs CcYsp-5'dir and CcYsp-3'rev (lanes 2–7) and Ccmt5495 and Ccmt5827 (lanes 10–15) in males and females. Lane identities: wild females (lanes 2, 3, 10, and 11), wild males (lanes 4, 5, 12, and 13), *tsl* Vienna 8 males (lanes 6, 7, 14, and 15), and 100 bp Kasvi ladder (lanes 1, and 9). Negative control: lanes 8 and 16.

Regarding cross 3, 31 wild females remated with sterile, *tsl* Vienna 8 males. In cross 4, 40 wild females remated with wild males. According to the statistical analysis, the difference between remating frequencies was not significant (P = 0.21). This result shows that females can remate with both wild and sterile males. Specifically, statistical analysis showed that both types of males had a similar capacity to inhibit remating, as the observed difference was statistically insignificant (P > 0.05). However, Kraaijeveld and Chapman (2004) found that when a female mated first with a sterile male, she was more likely to remate than if she first mated with a wild male. This behavior would reduce the sterile insect technique success (KRAAIJEVELD; CHAPMAN, 2004).

As for the genetic profile of the mitochondrial marker Ccmt-*Hae*III, the presence of DNA of both male lineages was detected in both crosses 3 and 4 (Figure 2). However, the second male's DNA predominated over that of the first one. This can be seen in cross 4, where the DNA bands of the sterile, *tsl* Vienna 8 males generated by PCR/RFLP were much less visible than those from cross 3, or even invisible due to the lower DNA content. This pattern was observed in all 40 sampled individuals. However, this pattern could not be seen in cross 3, where the female DNA present in spermathecae was not completely degraded, even using 1% dithiothreitol. The latter only increased the efficiency of extraction of male DNA from both lineages, so that it was successfully amplified by PCR (GILL; JEFFREYS; WERRET, 1985). Thus, the 330 bp band of the wild male appeared as intense as that from the sterile, *tsl* Vienna 8 male due to the presence of the wild female's DNA.

For crosses 3 and 4, it can be inferred that when a female *C. capitata* mates with 2 males, the DNA from both can be found in the spermatheca. However, the presence of sperm from different males makes it possible for sperm competition and selection to occur (BONIZZONI et al., 2006). Nevertheless, DNA from the last male to mate was more evident than that of the first; the second male's DNA dominates over that of the first. In the majority of insects in which this phenomenon occurs, the mechanism is not entirely clear (TSUBAKI; YAMAGISHI, 1991). Several authors suggest that upon remating, the last male tends to account for a larger proportion of the progeny compared to the first male; i.e., mating success depends on mating order (TSUBAKI, 1988; SAUL; MCCOMBS, 1993; LEE; MCCOMBS; SAUL, 2003; BONIZZONI et al., 2006).

Both wild and *tsl* Vienna 8 *C. capitata* males can inhibit remating (MOSSINSON; YUVAL, 2003). This phenomenon occurs because the sperm contains substances produced by accessory glands (CHAPMAN et al., 1998) that switch the female's behavior from mating to oviposition (JANG, 1995; 2002). However, when the first male does not effectively inhibit remating, the female can mate again. Morphological characteristics (e.g., male size) influence the female's decision to accept a new copulation (AQUINO; JOACHIM-BRAVO, 2013). Nevertheless, male origin (wild or laboratory) has a stronger effect on the decision of the female than male body size (AQUINO; JOACHIM-BRAVO,

2013). If the female's choice to remate is related to the sperm amount released by the male during mating (MOSSINSON; YUVAL, 2003), sterile males would be at a disadvantage given that they release less sperm than wild males (TAYLOR et al., 2001). Hence, ineffective transfer and storage of sperm could foster a female's receptivity after mating (MOSSINSON; YUVAL 2003).

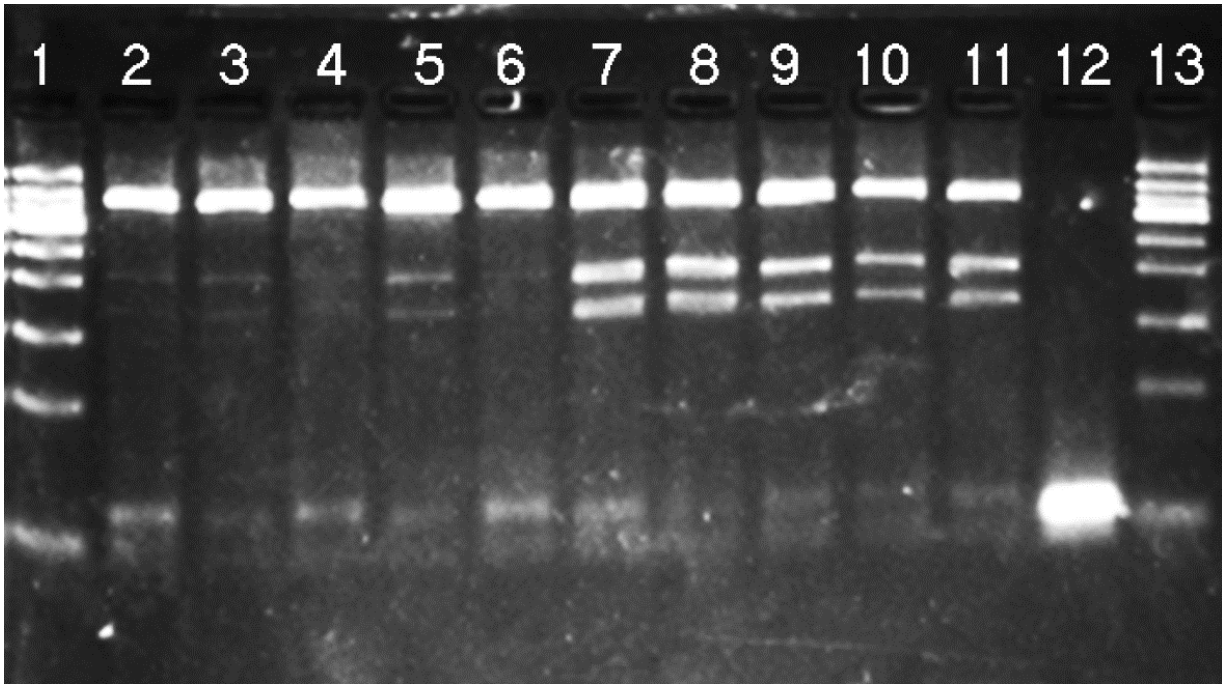

Figure 2. Agarose gel that shows restriction digestion of Ccmt-*Hae*III in spermathecae of females that remated with wild males (cross 4; lanes 2–6) or *tsl* Vienna 8 males (cross 3; lanes 7–11). Lanes 1 and 13 contain the Qiagen 50 bp ladder. Negative control: lane 12.

A study using aromatherapy with ginger root oil showed that this technique enhanced the efficacy of sterile males to inhibit female remating. It was found that when a sterile male was not subjected to aromatherapy, the remating rate by wild females (after copulating with the sterile male) reached 62.5%. However, when males were exposed to ginger root oil aroma, the female remating rate was much lower and was similar to that of when she originally mated with a wild male (32.2%) (MORELLI et al., 2010). Another study showed lower remating rates than observed in this study: 24.5% with wild males and 5.6% with *tsl* males (SHELLY, 2011). However, the rates can be differentiated in each population of this species (BONIZZONI et al., 2006), since it is a pest for world horticulture.

Polygamous copulations are advantageous for both the second male, due to sperm precedence, and the female, which obtains prolonged progeny (SAUL; MCCOMBS, 1993). Although remating is advantageous for the female (SAUL; MCCOMBS, 1993), it leads to problems for the sterile insect technique, because females that mated with sterile males can regain fertility if they copulate with a wild male (LEE; MCCOMBS; SAUL, 2003). Thus, although remating increases viable descendants, it consequently decreases sterile insect technique success.

CONCLUSION

Because the literature studies cited were carried out in different countries where this species is considered an agricultural pest, this finding may indicate genetic differentiation among *C. capitata* populations with regards to female remating. Studies combining aromatherapy and molecular markers should be performed so that both the enhancement of mating success and the inhibition of remating by sterile males can be validated. Further, it would be advisable to determine the precise amount of male DNA in the female spermathecae using more advanced techniques, such as quantitative real-time PCR.

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