

Improving the Sterile Sperm Identification Method for Its Implementation in the Area-Wide Sterile Insect Technique Program Against *Ceratitis capitata* (Diptera: Tephritidae) in Spain

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ABSTRACT The success of sterile males in area-wide sterile insect technique (aw-SIT) programs against *Ceratitis capitata* (Wiedemann) is currently measured by using indirect methods as the wild:sterile male ratio captured in monitoring traps. In the past decade, molecular techniques have been used to improve these methods. The development of a polymerase chain reaction-restriction fragment-length polymorphism-based method to identify the transfer of sterile sperm to wild females, the target of SIT, was considered a significant step in this direction. This method relies on identification of sperm by detecting the presence of Y chromosomes in spermathecae DNA extract complemented by the identification of the genetic origin of this sperm: Vienna-8 males or wild haplotype. However, the application of this protocol to aw-SIT programs is limited by handling time and personnel cost. The objective of this work was to obtain a high-throughput protocol to facilitate the routine measurement in a pest population of sterile sperm presence in wild females. The polymerase chain reaction-restriction fragment-length polymorphism markers previously developed were validated in Mediterranean fruit fly samples collected from various locations worldwide. A laboratory protocol previously published was modified to allow for the analysis of more samples at the same time. Preservation methods and preservation times commonly used for Mediterranean fruit fly female samples were assessed for their influence on the correct molecular detection of sterile sperm. This high-throughput methodology, as well as the results of sample management presented here, provide a robust, efficient, fast, and economical sterile sperm identification method ready to be used in all Mediterranean fruit fly SIT programs.

KEY WORDS mating assessment, Mediterranean fruit fly, stored sperm identification, PCR-restriction fragment-length polymorphism

The Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae), is a key destructive pest in citrus around the world. Native of sub-Saharan Africa, it is now established in almost all temperate regions and has the potential to spread further still because of its large host range, its ability to survive unfavorable conditions, and even its ability to spread into new territories after unplanned human-caused introductions (White and Elson-Harris, 1992; De Meyer et al. 2004, 2008; Malacrida et al. 2007).

The sterile insect technique (SIT) is the basis of environmentally safe Mediterranean fruit fly management procedures in many citrus-producing areas and the key in eradication programs in countries where this species has a quarantine status (such as the United

States, Guatemala, Japan, or Australia) (Hendrichs et al. 2002). Currently, the evaluation of area-wide SIT programs (aw-SIT) against fruit flies relies on the recapture ratio of sterile vs. fertile males without taking into consideration the real target of SIT, which are the wild females (Dyck et al. 2005). Moreover, the current monitoring system based on fluorescent dye presence (to establish the recapture male type ratio) is being questioned because of the dye cost, its implication in human health, and its intrinsic error rate (dye can be lost because of time and environmental conditions, and can be transferred to wild-type flies) (Hagler and Jackson 2001). Other methods such as egg hatchability or sperm head size measurement (McInnis 1993, McInnis et al. 1994, Katsouyannos et al. 1999, Rendon et al. 2004) are direct methods to assess the mating success of released sterile males, but both are tedious and time consuming and require large laboratory spaces and personnel to keep the captured females and their progeny alive.

The application of molecular genetic methods to pest control programs has been of interest since the early 1990s (Hoy 2000). However, its application to

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fruit fly control started late in the 1990s and has been mainly focused on obtaining molecular genetic data to identify the geographic distribution and movement of the release strains, release strains in development, or to follow the dispersion of released flies of mixed sexes (Gasparich et al. 1997, Bonizzoni et al. 2002, 2004; Silva et al. 2003, Malacrida et al. 2007, Barr 2009, Isasawin et al. 2012). San Andrés et al. (2007) developed a polymerase chain reaction (PCR)-restriction fragment-length polymorphism diagnostic method to identify the sterile sperm in the female spermathecae. This study provided a novel approach for SIT against fruit flies, as it focuses on the real target of SIT, the wild females, which provide valuable information for the evaluation of the success of the released males. Fritz et al. (2010) added a new step to the procedure, which involved identifying and quantifying the sperm DNA by PCR (based on microsatellite analysis) in sperm storage organs, but was used in another tephritid species, *Anastrepha suspensa* (Loew) (Diptera: Tephritidae), the Caribbean fruit fly. In this case, microsatellites were chosen because of the lack of a characterized and selected strain for male-only production and release, which renders it difficult to identify released flies from natural populations. Several works have focused on sperm marking of Mediterranean fruit fly by transgenic technology with the aim of its use for effective monitoring of SIT programs (Schetelig et al. 2006, 2009; Scolari et al. 2008). The aim of releasing transgenic flies was to directly detect (under microscopy) the genetic nature of captured females and their stored sperm, reducing the cost of analysis (without dissection or rearing). Despite the advances in strain and sperm marking in the Mediterranean fruit fly, the regulatory governmental processes limit the release of transgenic animals and make this methodology unfeasible at the moment (Bossin et al. 2006, Knols et al. 2007, Mumford 2012). More recently, Isasawin et al. (2012) also used microsatellite DNA markers for the evaluation of aw-SIT against *Bactrocera dorsalis* (Hendel) (Diptera: Tephritidae), but focused on the dispersion and identification of released flies, not in the identification of sterile sperm in wild-fertile females. In both cases, cost and implementation of microsatellites remained the main drawback for its use in aw-SIT programs. Thus, the only method to determine the sperm identification in Mediterranean fruit fly females is the San Andrés et al. (2007) protocol that has been successfully applied in field trials (Juan-Blasco et al. 2013). However, its application to aw-SIT has two major drawbacks, the personnel cost and the analysis time gap. Therefore, it was advisable to develop a modification of the San Andrés protocol to establish a world-wide adapted protocol for the evaluation of aw-SIT programs against the Mediterranean fruit fly. In this way, the universality of the markers could be checked and updated if needed by testing Mediterranean fruit fly samples from around the globe. Furthermore, the sperm identification protocol should be simplified to allow the high-throughput analysis of samples from aw-SIT programs.

Table 1. Mediterranean fruit fly samples, collector, and no. individuals received

Country (locality)	Collector or supplier	No. individuals of each gender (wild-type and sterile strain)
Argentina (Medfly facility)	D. Segura	100
Australia (Perth, West Australia)	R. Magliani	100
Brazil (Medfly facility)	A. Malavasi	100
Brazil (Petrolina, Pernambuco)	B.J. Paranhos	100
Guatemala (Antigua)	C. Cáceres	100
Egypt (Giza)	IAEA Staff	35
Israel (Medfly facility)	Y. Gazit	50
Moroco (Agadir)	H. Aboussaid	150
Moroco (Marrakech)	H. Aboussaid	50
South Africa (Hex River)	B. Barnes	50
Tunisia (Bigerte, Taprelsa)	S. Elfekih	30
Portugal (Madeira)	L. Dantas	105
Greece (Magnisia)	N. Papadopoulos	48
Greece (Creta (Lab))	A. Economopoulos	100

Materials and Methods

Mediterranean Fruit Fly Samples to Test Universality of Sperm ID Markers. Samples were requested from worldwide SIT facilities or entomological laboratories working with *C. capitata* (Table 1). Each sample consisted of at least 30 individuals of each gender for the wild-type flies and of each sterile strain; almost all based in the Vienna temperature-sensitive lethal (*tsl*) series with the Egypt mitochondrial haplotype (International Atomic Energy Agency [IAEA] 2004, 2006).

Mediterranean Fruit Fly Strains and Rearing Conditions for Mating Assays. The wild-type strain adults were obtained from a laboratory colony (generations XIV to XVII, 2010–11) housed at the Generalitat Valenciana (GVA)-Instituto Valenciano de Investigaciones Agrarias (IVIA) emergence facility (Moncada, Spain). Sterile males of the Vienna-8 (*tsl*) Genetic Sexing Strain (GSS) mix 2002 (in which genetic material from the Portuguese and Spanish Mediterranean fruit fly's populations were introduced into the Vienna strain genetic background) strain were produced at the mass-rearing facility in Caudete de las Fuentes (Valencia, Spain) and transferred as pupae after irradiation to the GVA-IVIA emergence facility. After emergence, adult wild-type females, wild-type males (<24-h-old to ensure female virginity), or Vienna-8 males were separated by sex and strain into polymethyl methacrylate cages (20 by 20 by 20 cm), with 100 individuals per cage. Adults were kept in different rooms to prevent any pheromone effect before assay at $25 \pm 4^\circ\text{C}$, $65 \pm 10\%$ relative humidity (RH), with natural light until achieving the "release" age (10-d-old for the wild-type females and 7-d-old for the wild-type males). Vienna-8 males were maintained at $25 \pm 4^\circ\text{C}$, $75 \pm 5\%$ RH, and in complete darkness (no photoperiod) in a different environmental chamber until achieving the release age (3-d-old) to simulate pre-release conditions in the SIT facility. Wild-type adults

were fed with a mixture of sugar and hydrolyzed yeast (Biokar Diagnostics Co., Pantin, France) (4:1; wt:wt) (protein-enriched diet), whereas sterile males were fed with sugar (protein-deprived diet). Sugar is the prerelease diet of sterile males in the Spanish SIT facility. Water and diet were provided ad libitum. Vienna-8 males were subjected to aromatherapy treatment with ginger root oil (GRO, Guinama, Valencia, Spain) 24 h before release age, in the same room and at the same time as the sterile males used for release in the aw-SIT Valencia's program (Juan-Blasco et al. 2011, 2013).

Mating Assays. Mating assays were conducted to validate the PCR-restriction fragment-length polymorphism sperm identification method in females. The mating arena consisted of methacrylate cages (30 by 40 by 30 cm) with ventilation openings. Fifty males (wild-type or Vienna-8 depending on cross) were placed in the mating arena first (0800–0830 hours, photophase started at 0600 hours, including a 1-h dawn) and left to settle for 15 min, then 50 females were introduced, comprising the final ratio (1:1) tested (as for the sterile males quality control; FAO/IAEA/USDA 2003). In each arena, observations for mating pairs were carried out continuously for 3 h, removing each couple as formed into 50-ml plastic vials; after this time, the arena was supervised discontinuously (each 15–20 min) for an additional 3 h, after which any remaining uncoupled female was discarded. All vials were marked with copula starting time and supervised during 3 h to assess copula completion. Only females that mated for ≥ 3 h were used. After this time, all vials were frozen to ensure that all flies were dead. For each vial, the male type was confirmed by the presence (sterile) or absence (wild-type) of fluorescent dye (Dyck et al. 2005). Mating experiments were repeated several times to obtain a number of mated females for all the DNA extractions.

The validation of the sperm identification method on these females was analyzed at different days after death to assess performance of the method over time and thus to resemble the real scenario of capture in field, which would include different sampling times. Dead females were placed in a Tephri-trap (Sorygar S.L., Madrid, Spain) and kept in an environmental chamber (SANYO 560 MLR; Sanyo, Gumna, Japan) at $25 \pm 4^\circ\text{C}$, $75 \pm 5\%$ RH, and a photoperiod of 16:8 (L:D) h for 7, 28, or 56 d. After this "capturing" period, females were processed depending on DNA extraction protocol.

Female Storage for DNA Extraction Protocol. Mated "captured" females were stored for an additional 0, 7, 28, or 56 d either in 70% ethanol at room temperature (RT) or dry at -20°C , depending on spermathecae DNA extraction protocol. Two new methodologies were tested to improve sperm ID protocol: sonication and membrane spermathecae imprinting. For sonication, fresh (dissected immediately after copula completion) or 7-d-old mated captured female samples were used. Three different kinds of vials were tested to perform the sonication: 1) 1.5-ml centrifuge tubes (ref. 200400 Deltalab S.L., Barcelona,

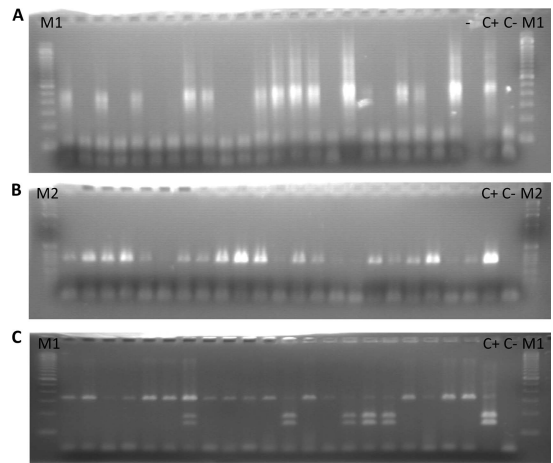


Fig. 1. Amplification success of sterile sperm markers CcYsp (A), Ccmt-HaeIII (B), and Ccmt-HaeIII-restriction fragment-length polymorphism (C) in a case study using "membrane imprinting" extraction protocol to obtain source-DNA. In all cases, C- corresponds to the PCR negative control (water), whereas C+ corresponds to positive PCR control (either Vienna-8 male DNA or wild-type female spermathecae of a recently Vienna-8 mated female). M1, 100-bp molecular ladder (Invitrogen). M2, 1-kb ladder (Invitrogen).

Spain), 2) 0.2-ml thin-wall PCR strip-tubes (ref. 4095.2N Deltalab S.L.), and 3) 96-well PCR plates (ref. 72.1978.202 Sarstedt AG & Co., Nümbrecht, Germany). For the spermathecae membrane imprinting method, 0-, 7-, 28-, and 56-d-old mated captured females were used (in 96-well PCR plates). For each female batch, two DNA preservation methods were tested: 1) females preserved in 70% ethanol at RT ($25 \pm 5^\circ\text{C}$), and 2) dry females at -20°C ; in both cases, for additional 0, 7, 28, and 56 d.

DNA Extraction Protocols and PCR Conditions. For mitochondrial *HaeIII* genotyping, DNA extraction was performed by using only the head or thorax of each fly by the "Salting out" method (Sunnucks and Hales 1996). Each sample was tested with the Ccmt-HaeIII marker to obtain the restriction fragment-length polymorphism pattern as previously described (San Andrés et al. 2007; Fig. 1).

For molecular sperm identification, the mated captured females were dissected and spermathecae were subjected to DNA extraction following these three different methods:

1) The San Andrés et al. (2007) method based on the Salting out method to individually extract the DNA of a spermathecae in a 1.5-ml centrifuge tube.

2) The *sonication DNA extraction method* consisting of sonication application of 10 pulse per second during 1 min followed by a second round of 1 min with a 30 s ice bath rest in between rounds, to a vial (1.5-ml tube, 0.2-ml PCR strip or 96-well plate) containing the female spermathecae in 100 μl of sonication buffer (10 mM Tris-HCl pH 7.5, 0.1 mM EDTA pH 8.0, 0.1% SDS). Sonicated vials were immediately frozen till use for

PCR. Sonication was performed in an ultrasonic water bath of 0.9 liters (Fungilab SA, Barcelona, Spain; HF-frequency 35 kHz).

3) *Spermathecae membrane imprinting*. The dissected spermathecae were crushed in 0.25-mm² nylon membrane (Hybond N+, Amersham-GE Healthcare, Buckinghamshire, United Kingdom) with the aid of a plastic pestle (ref. Z359947 Sigma-Aldrich Co., St. Louis, MO). Each imprinted membrane was deposited in a 96-well plate and covered with 100 μ l of extraction buffer (0.1 M Tris-HCl, 50 mM NaCl, 0.1% Triton, 1 M glycine, 1 mM EDTA pH 8.0, 1% DTT, and 0.1 mg/ml Proteinase K). DNA extraction was performed in a thermocycler (Eppendorf Mastercycler gradient; Eppendorf, Hamburg, Germany) at 60°C during 60 min followed by 95°C for 15 min. After this, the plates were spun down and frozen (-20°C) till amplification. DNA concentration and purity were quantified by using a Nanodrop 2000 spectrophotometer (Thermo Scientific Inc., Wilmington, DE).

Sperm identification markers CcYsp (dir: 5'-CgA AgC ACg ACA TAC ACg Agg Ag-3' and rev: 5'-ACA CTT ACC gAC ATT gAT TCC Tg-3') (Fig. 1A) and Ccmt-*HaeIII* (dir: 5'-AAA TCA CCA CTT Tgg ATT TgA AGgC-3' and rev: 5'-TgA AAA Tgg TAA ACg TgA AgA gg-3') (Fig. 1B) were tested by PCR by using 1, 3, 5, or 10 μ l of DNA extract depending on the extraction method (1, 2, or 3) and marker. PCR conditions were: 300 nM dNTPs, 1 \times *Taq* pol buffer (containing 2 mM MgCl₂; Biotools Ag, Madrid, Spain), 10 pmol each primer, and 0.75 U DNA polymerase (Biotools). PCR was performed in a Mastercycler EpS gradient thermal cycler (Eppendorf, Germany) with the following amplification conditions: one denaturation step at 94°C for 5 min, followed by 35 cycles at 94°C for 30 s, 52°C, or 55°C for 30 s (for CcYsp or Ccmt-*HaeIII*, respectively), 72°C for 45 s, followed by a final extension at 72°C for 3 min. After amplification, Ccmt-*HaeIII* PCR products were subjected to restriction fragment-length polymorphism with 5 U of *HaeIII* nuclease (Takara Bio Inc., Shiga, Japan) during 2–14 h at 37°C. Negative (PCR-grade water) and positive (Vienna-8 male DNA extraction) controls were included in each set of PCR reactions to test for cross-contamination (negative control) or for amplification success or failure (positive control), respectively. All PCR products were electrophoresed in 2% agarose gel, and visualized under ultraviolet light after staining with ethidium bromide, whereas the PCR-restriction fragment-length polymorphism with *HaeIII* was analyzed in 2.5% agarose gel (Fig. 1).

Statistical Analysis. Sperm identification was coded as 1 (positive) or 0 (negative) for detection or absence of sterile sperm (CcYsp marker and Ccmt-*HaeIII*) and was considered as an indicator variable. A Generalized Linear Mixed Model (GLMM, Breslow and Clayton 1993) was used to analyze the effect of extraction protocol on sterile sperm detection (fixed effect). The random effects were the sample processing before DNA extraction (the preservation time and preservative method). For the membrane imprinting, fitted values of expected sterile sperm detection were ob-

tained with a Generalized Linear Model (GLM, McCullagh and Nelder 1989) on which preservation times and preservative methods were included as fixed factors. Considering that trap servicing and rebaiting intervals for monitoring surveys are specific to each trap system in aw-fruit fly programs (IAEA 2003), the next analysis was a GLMM used to analyze the effect of preservation time on membrane imprinting as a fixed-effect parameter. Preservative methods were included as random-effect parameters in this analysis. Selection of the best model was based on the Akaike Information Criterion. This criterion revealed that the binomial distribution was appropriate to analyze sterile sperm identification data, with log-link in the case of the GLMM analysis. Bonferroni method was applied in all analyses to adjust significance levels after pairwise multiple comparisons.

Results

Universality of Ccmt-*HaeIII* Marker. All the tested Vienna *tsl* strains in use in the several aw-SIT programs (Table 1) gave a positive *HaeIII*-restriction fragment-length polymorphism pattern, confirming that all the tested strains (Vienna-6, Vienna-7, Vienna-7 Madeira, Vienna-8, and Vienna-8 mix 2002) maintain the Egypt mitochondrial haplotype (Fig. 1). None of the tested Mediterranean fruit fly wild-type samples gave a positive restriction fragment-length polymorphism pattern, with the exception of the Creta laboratory strain, which in fact corresponds to the original Egypt *wp* (white pupa) strain (A. Economopoulos, personal communication).

Improvement of the Sterile Sperm Identification Protocol. The San Andrés et al. (2007) sperm extraction method was followed as control and as a source of positive controls for PCR.

The *sonication protocol* resulted in a fast but unreliable (low A260/280 values) protocol with an average DNA concentration of 2.70 ± 1.10 ng/ μ l (mean \pm SE) and A260/280 absorbance ratio of 0.61 ± 0.30 (mean \pm SE). When extracting more than one sample at a time, the percentage of sterile sperm positive detection decreased to $67 \pm 13\%$ (mean \pm SE) or as low as $43 \pm 13\%$ (mean \pm SE) when 7-d-old mated captured females were used as source of spermathecae tissue. These results were also affected by the vial material used to perform sonication (Table 2).

The *membrane imprinting* gave the best results with an average DNA concentration of 81.31 ± 7.46 ng/ μ l (mean \pm SE) and A260/280 absorbance ratio of 0.45 ± 0.10 (mean \pm SE). The small A260/280 ratio in this extraction protocol is mainly due to the presence of Proteinase-k in the extraction buffer, which is not removed before analysis, but is heat-inactivated. The A260 values were 1.54 ± 0.20 (mean \pm SE), indicating the presence of DNA and RNA in great quantities in the samples. CcYsp and Ccmt-*HaeIII* amplification with membrane imprinting and spermathecae as DNA source resulted in the 700–300-pb multiband or 350-pb band, respectively, as expected for each PCR marker (Fig. 1). Sterile sperm was detected even at 56 d after

Table 2. Quantity (percentage ± SE) of successful sperm identifications in spermathecae extracts of Vienna-8-mated females by sonication

Sonication performed in	Freshly dissected	7 d postmating
1.5-ml tubes (<i>n</i> = 10)	10 (100 ± 0)	9 (90 ± 10)
0.2-ml PCR tubes (<i>n</i> = 24)	18 (75 ± 15)	—
96-well PCR plate (<i>n</i> = 40)	27 (67 ± 13)	17 (43 ± 13)

—, not performed.

Sonication was performed in three kinds of vials (normal 1.5-ml centrifuge tubes, 0.2-ml thin-wall PCR tubes, and 96-well PCR plates).

Mated females were dissected immediately after copula completion or after a period of 7 d kept at 25°C to resemble the field monitoring system.

copula completion (100 ± 0.00%; Table 3). Overall, the molecular detection of sterile sperm by membrane imprinting showed no significant differences when compared with the San Andrés et al. (2007) method (GLMM binomial log-link: *F* = 0.465, *df* = 1,109, *P* = 0.497). Sterile sperm detection decreased greatly with longer preservation times and even more when 70% ethanol was used as preservative method (Table 3). When considering only the sterile sperm detection by the membrane imprinting method, no significant differences were observed (GLM binomial: $\chi^2 = 0.842$, *df* = 2, *P* = 0.657) between preservation time and preservation method. But when considering the same preservation time, the sterile sperm detection was significantly affected by the elapsed time from copula completion to storage (GLMM binomial log-link: *F* = 6.195, *df* = 6, 159, *P* < 0.0001; Bonferroni correction of significance level *P* = 0.017).

Discussion

Universality of *Ccmt-HaeIII* Marker. No partial restriction fragment-length polymorphism was obtained

Table 3. Percentage (mean ± SE) of successful sterile sperm identifications of sterile male-mated females subjected to different postmating timing (7, 28, and 56 d), with different preservation methods (ethanol or [minus]20°C), and with different conservation times (0, 7, 28, and 56 d) following San Andrés et al. (2007) dissection protocol or membrane imprinting

Female treatment (postmating days, conservation days at preservation method)	Membrane imprinting		San Andrés et al. (2007)	
	Mean ± SE	<i>n</i>	Mean ± SE	<i>n</i>
1 (7 d, 0 d)	100 ± 0.00	12	90 ± 10	10
2 (28 d, 0 d)	100 ± 0.00	12	75 ± 13	12
3 (56 d, 0 d)	100 ± 0.00	12	—	—
4 (7 d, 7 d at 70%)	100 ± 0.00	13	100 ± 0.00	10
5 (7 d, 7 d at -20°C)	60 ± 16	10	—	—
6 (7 d, 28 d at 70%)	69 ± 13	13	100 ± 0.00	9
7 (7 d, 28 d at -20°C)	69 ± 13	13	—	—
8 (7 d, 56 d at 70%)	64 ± 15	11	40 ± 16	10
9 (7 d, 56 d at -20°C)	82 ± 12	11	—	—
10 (28 d, 7 d at 70%)	91 ± 09	11	—	—
11 (28 d, 7 d at -20°C)	82 ± 12	11	—	—
12 (28 d, 28 d at 70%)	42 ± 15	12	—	—
13 (28 d, 28 d at -20°C)	67 ± 14	12	—	—
14 (28 d, 56 d at 70%)	56 ± 15	13	—	—

—, not performed.

in anyone of the samples, despite the prediction by Barr (2009), which detected sequencing errors and mismatches in the mitochondrial DNA of the Mediterranean fruit fly from samples around the world. Therefore, the absence of positive *Ccmt-HaeIII* pattern in samples from all the Mediterranean fruit fly SIT regions tested validates the Sperm ID marker *Ccmt-HaeIII* for its implementation in the studied regions. The marker developed by San Andrés et al. (2007) for Vienna sperm identification is still reliable.

Improvement of the Sterile Sperm ID Protocol. To evaluate the success of the SIT program against *C. capitata* in Spain, it was advisable to test for the presence of sterile sperm in the captured wild-females irrespective of its location within the female’s body (Taylor et al. 2000). However, because of the high number of flies to be tested, an alternative or a modification to the San Andrés et al. (2007) extraction protocol was required to handle the large quantity of flies in a reasonable time frame.

The *sonication protocol* resulted in a simple, fast, and economical method, but unfortunately the results were unreliable. False-negatives increased with sample age (age determined as time elapsed from mating event to spermathecae dissection) and with type of vial used to perform the extraction (1.5-ml tube, 0.2-ml thin-wall PCR tube, or 96-well PCR plate). These results contrast with the sonication results obtained by Fritz et al. (2010), but in this case, sonication was used to remove maternal cells from spermathecae content to determine the origin of stored sperm (single or multiple mated females). Sonication was successfully used to extract high-quality DNA from museum-preserved vouchers, as a way of not destroying these valuable often-unique specimens (Hunter et al. 2008). Our results are in agreement with the overall 66% amplification efficiency of Hunter et al. (2008). However, we obtained variable results depending on vial used. As for the SIT program evaluation requirements, a high-throughput technology should be desirable to decrease sample processing time, as the current use of single tubes to perform DNA extraction is limiting its use (Juan-Blasco et al. 2013).

The *membrane imprinting method* simplifies the procedure and was determined to be a better method than the San Andrés et al. (2007) protocol to perform analysis of all aw-SIT monitoring traps samples, mainly due to the reduced handling time required. We tried to improve the San Andrés et al. (2007) method by performing DNA extraction on 96 deep-well plates to reduce handling time, but due to the size of spermathecae, the homogenization system, and centrifuge type, an increase in lost samples was observed, which rendered this method unreliable (data not shown). Further research should be done to improve the homogenization and extraction protocol with 96-well plates to overcome this limitation. The *membrane imprinting* protocol allowed for the extraction of 96 samples at the same time by using PCR plates in a hands-off system that make the spermathecae dissection process more efficient. When compared with the thirty 1.5-ml vials used per run (limited by the cen-

trifuge rotor size) in the San Andrés et al. (2007) protocol with a hands-on time requirement of 2 h, it becomes clear that the *membrane imprinting* extraction method is more efficient and economical for an aw-SIT evaluation system.

One key point that also was highlighted with this work was the DNA degradation or a decrease in amplification efficiency of samples preserved in 70% ethanol at RT for >28 d. This result was in agreement with the reduced DNA concentration and purity observed in dried samples that were field-collected and stored in 70% ethanol before sample manipulation for microsatellite analysis of tephritid flies (Maxwell et al. 2011). A reduced DNA yield may result in an increase of false-negative results, because of no amplification of sterile male DNA.

Overall, our results using the membrane imprinting method indicate that *C. capitata* females captured after being in monitoring traps for 7–56 d are useful samples to assess mating success of released sterile males and thus to assess the efficacy of the SIT program. However, special care should be taken to preserve sperm DNA while not processing the samples (Table 3). Future studies should examine a way to avoid spermathecae dissection, as it remains the most time-consuming step.

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