Current Research in Insect Science 2 (2022) 100040

Contents lists available at ScienceDirect

Current Research in Insect Science

journal homepage: www.elsevier.com/locate/cris

Multi-locus genotyping of stored sperm reveals female remating rates in wild populations of the Queensland fruit fly

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ARTICLE INFO

Keywords:
Polyandry
sperm genotyping
microsatellites
sterile insect technique
Bactrocera tryoni

ABSTRACT

Female insects commonly have more than one mate during a breeding period ('polyandry'), storing and using sperm from multiple males. In addition to its evolutionary significance, insect polyandry has practical implications for pest management that relies on the sterile insect technique (SIT). The Queensland fruit fly, *Bactrocera tryoni* (Froggatt), is a major horticultural pest in Australia, and outbreaks are managed by SIT in some regions. The present study provides the first evidence for polyandry in female *B. tryoni* from field populations from New South Wales (NSW) and Queensland (QLD) through multi-locus genotyping (ten microsatellite markers in four fluorescent multiplexes) of the stored sperm in ovipositing females. Polyandry level was significantly higher in the NSW collection (80.0 %) than the QLD collection (26.1 %), suggesting substantial regional and/or temporal variation. These findings have important implications for the use of SIT to suppress *B. tryoni* populations and to eradicate outbreaks.

1. Introduction

Female mating decisions have substantial effects on fitness of both sexes; in many species males actively compete for access to mates while females are often selective about which males they will accept (Shuker, 2014). In some cases, females may accept more than one mate within a reproductive cycle ('polyandry'), and in these cases the processes of male-male competition and female choice can extend to mediate which male's sperm is used to fertilise ova (Firman et al., 2017; Parker, 1970; Parker and Pizzari, 2010). The evolutionary and ecological aspects of polyandry have come into focus through multi-disciplinary research in areas such as adaptive significance (Gowaty, 2012; Hosken and Stockley, 2003), strategic ejaculate allocation in response to rival males (Parker et al., 1997; Parker and Pizzari, 2010), sex allocation (Schärer and Pen, 2013), altruism and cooperation (Díaz-Muñoz et al., 2014), sex-biased gene expression (Hollis et al., 2014), selfish genetic elements (Price et al., 2008), sexually transmitted infections (Roberts et al., 2015), and population extinction and management (Price et al., 2010).

Understanding the extent and mediators of polyandry can have practical implications in management of some insect pests (Taylor et al., 2014). This includes the sterile insect technique (SIT), an environmentally sustainable, species-specific, pest management tool whereby mass-reared sterile males are released into the field to disrupt reproduction of wild females (Knipling, 1955). Some of the most economically important tephritid fruit flies have been extensively targeted by SIT (Shelly and McInnis, 2016). However, polyandry may diminish the effectiveness of SIT, particularly if sterile males and their sperm are less competitive than their wild counterparts (Barclay, 2005). Whilst female remating propensity has been studied in major pest lineages of tephritid fruit flies (genera *Ceratitis, Neoceratitis, Bactrocera, Dacus, Zeugodacus, Anastrepha* and *Rhagoletis*) under laboratory or field cage conditions (see Collins et al., 2012, Shelly 2020, Abraham et al., 2016 and references therein), direct field estimates of female remating are limited to *Ceratitis capitata* (Bonizzoni et al., 2002; Kraaijeveld et al., 2005), *Bactrocera cacuminata* (Song et al., 2007) and *B. oleae* (Zouros and Krimbas, 1970). These studies have collectively shown that female remating is common in tephritid fruit flies, and that remating frequencies are generally higher in laboratory or field cage conditions than in the wild.

The Queensland fruit fly (Qfly), *Bactrocera tryoni* (Froggatt), is Australia's most economically important pest of horticulture (Hancock, 2000; Sutherst et al., 2000) and has been a target of SIT (Andrewartha et al., 1967; Fisher, 1996; Klassen and Curtis, 2005). With increasing restrictions on the use of synthetic insecticides (Dominiak and Ekman, 2013), SIT is increasingly important as a sustainable management tool. However, success of large-scale SIT operations requires understanding of the species' reproductive behaviour, including polyandry rates in the field. Such knowledge can inform decisions of release rate and release intervals of sterile males. Female remating in Qfly under laboratory conditions has been reported (Ahmed et al., 2022; Harmer et al.,

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https://doi.org/10.1016/j.cris.2022.100040
Received 28 March 2022; Received in revised form 25 May 2022; Accepted 27 May 2022

2006; Radhakrishnan and Taylor, 2008) but the occurrence and rate of remating in wild populations remain unknown.

Two of the most common methods of polyandry estimation in wild insect populations involve capturing and maintaining wild females in controlled conditions and genotyping their progeny (Kraaijeveld et al., 2005; Richardson et al., 2015; Song et al., 2007) or genotyping the sperm in spermathecae (Bonomi et al., 2011; Demont et al., 2011; Tripet et al., 2003). To date, only the former method has been used to estimate polyandry rates of wild fruit fly populations. In the present study, we use ten polymorphic microsatellite markers to genotype the stored sperm in the spermathecae of female Qfly from two populations, to estimate the natural rate of polyandry. We discuss the implications of our findings in the context of Qfly reproductive biology and SIT.

2. Material and methods

2.1. Field sampling of males and ovipositing females

Female Qflies were collected from two locations, one in warm temperate New South Wales (Marsfield, NSW, 33°46′06"S, 151°05′56"E on 2nd October 2019) and one in subtropical Queensland (Maroochydore, QLD, 26°38′35"S, 152°56′23"E on 10th December 2019). Gravid females (N = 25 in NSW; N = 23 in QLD) attempting to oviposit into fruits (loquats in NSW; mangoes in QLD) were individually captured and stored at -20°C until dissection. Cuelure traps were deployed at both sites to catch males (N = 22 in NSW; N = 22 in QLD). A cuelure solution was prepared by dissolving one part of cuelure (Sigma-Aldrich, MO, USA) in two parts of acetone (Sigma-Aldrich, MO, USA). One millilitre of this solution was added to a cotton wick, which was then placed inside a Bio-Trap V2 X (BioTrap, Victoria, Australia) hung from a tree. Traps were checked daily for three days. Approximately 30 flies (males and females) were caught daily. Field-caught flies were transported to the laboratory in vials in an icebox (~4°C) and stored at -20°C until DNA extraction.

2.2. Extraction of DNA from stored sperm in gravid females, legs of females and legs of lure-trapped males for microsatellite genotyping

Sperm DNA was extracted from spermathecae (sperm storage organs) dissected from gravid females after complete removal of maternal tissues. Spermathecae were soaked for 8-10 minutes in a 10% W/V potassium hydroxide (KOH) (Sigma-Aldrich, MO, USA) solution in a Petri dish and briefly rinsed in Milli-Q water in a new Petri dish. The rinsed spermathecae were placed on a microscope slide and residual maternal tissues were removed under a stereomicroscope (Leica, Wetzlar, Germany) at 4X magnification. DNA from both spermathecae of each female was extracted in a single extraction tube following Shadmany et al. (2021b) and genotyped for ten microsatellites markers (Table 1). The genotype of the female was also determined by analysing the same ten microsatellite markers using DNA from her legs. The sperm and female genotypes were compared to check for maternal DNA contamination in the sperm DNA. The absence of female alleles in the spermathecal samples was taken as evidence for sperm DNA purity. To evaluate variability of the microsatellite markers in the sampled populations, additional wild male flies were also genotyped (leg DNA) using a modified Chelex-based method following Shadmany et al. (2021b).

2.3. Genotyping and assay validation

Ten microsatellite markers were selected for genotyping (Table 1). These markers are dispersed across all five autosomes (Chromosomes 2, 3, 4, 5, 6) and appeared single copy in the Qfly reference genome (NCBI accession number: GCA_016617805.2). The forward primer of each locus was fluorescently labelled using an ABI proprietary dye (Thermo Fisher Scientific, Waltham, MA, USA) and the ten microsatellite markers were grouped into four multiplex reactions (see multiplex groupings in Table 1). The PCR master mix was made using the Taq PCR Core Kit (Qiagen, Venlo, Netherlands). The 10 μl PCR reaction contained 1.5 μl DNA template, molecular biology grade water (Sigma-Aldrich, St. Louis, MO, USA), 0.5 units of Taq DNA polymerase, 1X reaction buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs, and 0.2 mM of each primer. PCR conditions included an initial denaturation at 94°C for 3 min, followed by 45 cycles of 94°C for 30 sec, 55°C for 45 sec, 72°C for 50 sec and a final extension at 72°C for 5 min. PCR products were diluted in water (1 μl in 79 μl) for fragment analysis using capillary electrophoresis in a 3730 DNA Analyzer (Thermo Fisher Scientific, Massachusetts, USA) at the Australian Genome Research Facility (AGRF). Electropherograms were analysed using GeneMapper™ 4 (Thermo Fisher Scientific, Massachusetts, USA) to produce the genotypes (see Supplementary File for genotypes of all individuals). Samples that yielded outlier alleles (i.e., amplicons that fell outside of the expected size ranges) were re-genotyped by fragment analysis on the capillary electrophoresis system. The confirmed outlier alleles were cloned and Sanger sequenced using the Macrogen TA cloning service to validate their identities. Outlier alleles (*1.6A*_112 bp, *Bt1.7A*_71 bp, *8.5A*_122 bp) confirmed by sequencing were retained, whereas amplicons (*8.5A*_76 bp, *Bp78*_83 bp) rejected by sequencing were excluded and the genotypes of the corresponding individuals were adjusted to homozygous for the other allele.

2.4. Estimation of remating rate

To infer if an ovipositing female had remated, we examined the number of alleles for each of the ten polymorphic microsatellite markers in stored sperm. A female was considered mated once if no more than two alleles were consistently detected across all ten markers. The presence of three or more alleles in any of the ten markers was taken as evidence that the female had remated.

2.5. Evaluation of sensitivity to detect alleles of low relative titres in sperm mixtures

It is expected that in wild remated females the relative titres of stored sperm from their mates may vary greatly. We assessed the robustness of the multi-locus PCR assay in detecting alleles of low relative quantities in artificially constructed sperm mixtures of known allelic ratios using three Qfly lines homozygous for *Bt32-90, Bt32-92* and *Bt32-105* of the microsatellite marker *Bt32* (Shadmany et al., 2021a). Separate sperm solutions were prepared from male *Bt32-90* and *Bt32-105* genotypes by first crossing these males individually with *Bt32-92* females. Following mating, spermathecae were dissected and the sperm DNA extracted and quantified following (Shadmany et al., 2021b). Four sperm mixtures that differed in the relative allele abundance of *Bt32-105* versus *Bt32-90* (3:1, 5:1, 9:1, 15:1) were constructed and served as templates for a 3-plex (*Bt1.7A- Bt8.6A-Bt32*) assay, to confirm that identical genotypes could be recovered from all four mixtures.

3. Results and Discussion

The ten microsatellite markers were polymorphic in the two populations sampled in 2019 (Table 1), providing a molecular basis for detecting multiple matings in the field and for retrospectively verifying the purity of the sperm DNA. All 48 spermathecal extracts from gravid wild females in the two populations were sperm-positive, while 26 of them (54.2 %) had remated. Of the 26 females that had remated, 25 were supported by genotypes of at least two markers (Figure 1). The maximum number of alleles per marker per sperm sample was four (Figure 1), suggesting that although remating is common in Qfly, these remating events could be explained by females having two mates carrying non-overlapping alleles for a subset of the markers.

As the allele-counting approach does not consider allele frequencies in the populations, the number of mates for a given female could be underestimated due to shared genotypes between mates. Bretman and Tregenza (2005) proposed a quasi-maximum likelihood approach using

Table 1
Microsatellite markers used in this study.

Locus	Primer sequence 5'-3'	Label	Multiplex grouping	Chromosome co-ordinates	Number of alleles observed [size range]	Primary reference for locus name & primer sequences
1.6A	TGCATGTCTCGTTCTAAGGC TGAAGTGTTTGCGATAGCACAG	6FAM	A	Chr 2 (70,951,118 - 70,951,250) #	14 [112 - 140 bp]	(Wang et al., 2003)
1.7.7	CATACCATTGCGGAAATGCAC AAGAGCAAGCTGAATAACACG	6FAM*	C	Chr 3 (14,365,385 - 14,365,485)	8 [92 - 104 bp]	(Kinnear et al., 1998)
Bt1.7A	ACCAGGAAACTTTTGTTGAGTA ATCATTGCTACTGCCTCTGC	VIC	D	Chr 2 (50,033,701 - 50,033,788) #	13 [71 - 105 bp]	(Chen et al., 2016)
12.8.1B	TGTTGCCTGCTGACTCCTTG AAACGAATCCACTCACAACCG	6FAM*	C	Chr 2 (65,684,022 - 65,684,175)	4 [157 - 166 bp]	(Kinnear et al., 1998)
3.3.5	TTTTATGAAGCCGCAGTGGAG GCTGCAAATAAGTGTCGGTGG	PET	C	Chr 4 (84,274,586 - 84,274,740)	6 [154 - 164 bp]	(Kinnear et al., 1998)
8.5A	CAAGAAGAATAATAAAGCGCGG CGAACTATGCATGTATGCGTG	PET	B	Chr 5 (55,661,895 - 55,661,753) #	8 [122 - 147 bp]	(Wang et al., 2003)
8.6A	CCTTTGCCACTCTTGCCTAC ATTAGTGTCAGGGCAGGACAG	PET	D	Chr 6 (73,544,247 - 73,544,374) #	18 [104 - 147 bp]	(Wang et al., 2003)
Bp78	CTATGCCAGTTCATCTGAGAAC AAGCGGTTATTAGCATTGAAGTGC	NED	B	Chr 6 (3,391,379 - 3,391,489)	4 [97 - 110 bp]	(Chen et al., 2016)
Bt15	TGAAGTAGCACAAGCGTTTA TGTTTCCCTCACCTTAATGA	VIC	A	Chr 6 (77,001,787 - 77,001,909) #	11 [111 - 126 bp]	(Kinnear et al., 1998)
Bt32	AGTCACTCACCATGATGCCA TCAAAGCGAACATGGACAGGT	NED	D	Chr 2 (61,648,202 - 61,648,306) #	19 [86 - 126 bp]	(Kinnear et al., 1998)

Chromosome assignment also supported by genetic/cytological map by (Zhao et al., 2003). * Markers *1.7.7* and *12.8.1B* were 6FAM-labelled and distinguishable by their non-overlapping amplicon size ranges in multiplex group C.

sperm genotype data to estimate the number of mates. However, using the proposed likelihood function, if all the common alleles are found in a sperm sample, which is often the case in our dataset, the likelihood function will be unbounded, yielding an undefined and unrealistic solution for mate number estimates. Several markers fell into this category. Hence, we adopted the more conservative approach of direct allele counting to estimate the minimum number of mates for each female.

Polyandry level was significantly higher in the NSW collection than the QLD collection (NSW = 80.0 %, N = 25; QLD = 26.1 %, N = 23; χ^2 = 14.02; DF = 1; P < 0.001) (Figure 1). While field remating rate for the QLD collection was generally on par with laboratory estimates for three Qfly populations at their early stages of domestication (Ahmed et al., 2022) and field estimates from other tephritids including *C. capitata* (Bonizzoni *et al.*, 2002; Kraaijeveld *et al.*, 2005; Bonizzoni *et al.*, 2006) and *B. cacuminata* (Song *et al.*, 2007), remating rate (80.0 %) for the NSW collection was markedly higher. The > 50% difference between the NSW and QLD collections suggests that the polyandry rate in Qfly can vary substantially between populations and/or over time.

Natural variation in remating propensity is likely to be influenced by a myriad of genetic, physiological, and environmental factors. Genetic background, especially the genotype of the females, has been shown to influence remating frequencies between populations in Qfly (Ahmed et al., 2022) and *C. capitata* (Saul and McCombs, 1993). In addition, demography and season (including major weather events) may also contribute to remating propensity in the field. Before Qfly sampling from QLD, the sampling location had been affected by hailstorm and drought (Nichols, 2019). Many of the usual Qfly hosts were either unavailable or had reduced fruiting. Consequently, population density was likely to be atypically low at the time of sampling. On the other hand, the NSW population was collected from an urban area which was unaffected by any natural disasters immediately prior to sampling and more than half of the loquat fruits were already fully ripe. In many tephritid species, host availability can influence mating frequency or remating (Abraham et al. 2018; Aluja et al. 2009; Carsten and Papaj, 2005; Landeta-Escamilla et al. 2016; Landolt 1994; Sivinski and Heath, 1988). The availability of abundant suitable hosts for oviposition may lead to increased rate of sperm depletion due to higher rate of oviposition, which might trigger remating to replenish sperm, as seen in *Drosophila* (Turner, 1986; Wigby and Chapman, 2004) and the Mediterranean fruit fly (Whittier and Shelly 1993).

Seasonal fluctuations in population age structure is another important factor that could influence female remating rates. In Qfly, substantial seasonal variation in age distribution has been observed in Tweed Valley, NSW (28.17′S, 153.31′E): early spring populations (early-September 2017 and mid-September 2018) were predominantly old flies, whereas an early summer population (mid-November 2017) contained individuals from more heterogeneous age groups (Tasnin et al., 2021). In the present study, our NSW sampling was conducted in spring (early October) and the QLD sampling in summer (early December). It is likely that our NSW collection in spring comprised mainly of old female flies of the previous season and that our QLD collection in summer comprised flies of diverse age groups; differences in age structure between NSW and QLD collections might contribute to the detected population differences in remating rates. As in *C. capitata* (Costa et al., 2012), female Qflies are more likely to remate if their first mate is old (Adnan et al., 2020a; Akter and Taylor, 2018). Higher levels of polyandry might reflect that male Qflies in the NSW population were old, having overwintered as adults. It is also possible that some female flies in NSW had mated the previous season and had stored sperm over winter (e.g. Fletcher, 1975), whereas young female flies in QLD may not have had the opportunity to remate. Female flies in NSW might have higher remating propensity as a response to time since first mating or reduced viability of sperm from their first mates. In contrast to the spring collection in NSW, such potential overwintering effects on remating might be less pronounced in the summer collection in QLD, considering the likely differences in age structure between the two collections.

The ability to detect high remating rates in natural populations could also be attributable to the multi-locus PCR approach of sperm genotyping, which can buffer disparity in sperm titres between different mates and is unaffected by sperm use. In our laboratory evaluation of the PCR assay (see section 2.5), identical genotypes were recovered from sperm mixtures of different allele ratios (*Bt32* allele mixtures between 3:1 and 15:1). This suggests that the assay can tolerate substantial titre imbalance between sperm of different origins, which is expected to be common amongst field remated females with unknown mating intervals. Further, accuracy of polyandry estimation via progeny genotyping is typically constrained by finite offspring sample size (Chapuisat, 1998; Gertsch and Fjerdingstad, 1997). Genotyping spermathecal extracts from mated females, on the other hand, can directly reveal the constituent alleles in the stored sperm, irrespective of

NSW collection											
ID of stored sperm in gravid female	***1.6A***	***1.7.7***	***Bt1.7A***	***12.8.1B***	***3.3.5***	***8.5A***	***8.6A***	***Bp78***	***Bt15***	***Bt32***	**Inferred female mating history**
01_R02	2	2	2	1	3	2	3	3	3	3	**Remated**
02_R03	3	1	2	2	3	2	3	2	2	3	**Remated**
03_R04	3	1	2	3	2	1	3	3	2	2	**Remated**
04_R05	2	2	2	2	2	1	2	1	2	2	**Mated once**
05_R06	4	1	3	2	3	2	4	2	2	4	**Remated**
06_R36	2	2	1	1	1	2	2	2	2	2	**Mated once**
07_R37	2	1	1	2	1	2	2	2	2	3	**Remated**
08_R38	2	1	1	3	3	2	4	2	2	3	**Remated**
09_R39	4	1	3	2	2	2	2	2	2	3	**Remated**
10_R41	3	1	3	2	2	2	3	3	2	3	**Remated**
11_R42	1	1	1	1	2	1	2	1	2	2	**Mated once**
12_R44	3	1	2	2	3	2	4	2	2	3	**Remated**
13_R45	3	2	2	1	2	2	4	2	2	3	**Remated**
14_R46	3	1	3	2	3	2	3	2	2	2	**Remated**
15_R47	2	2	2	2	2	1	2	1	2	2	**Mated once**
16_R48	2	2	4	2	2	2	2	2	3	2	**Remated**
17_R49	4	1	3	2	1	3	2	3	2	3	**Remated**
18_R50	4	1	3	3	2	2	3	4	0	4	**Remated**
19_R51	2	1	1	2	1	1	2	2	2	2	**Mated once**
20_R52	3	1	3	2	2	2	3	2	2	3	**Remated**
21_R69	4	2	4	3	2	2	1	2	3	3	**Remated**
22_R70	2	1	2	1	2	2	2	2	3	3	**Remated**
23_R71	3	1	3	2	3	2	2	4	3	3	**Remated**
24_R72	2	1	2	4	2	2	3	1	2	2	**Remated**
25_R73	3	2	3	2	2	2	2	2	0	2	**Remated**
QLD collection											
01_Q02	1	1	2	2	1	2	1	2	1	2	**Mated once**
02_Q03	4	2	1	2	2	2	3	1	3	1	**Remated**
03_Q04	3	1	1	2	1	2	0	2	0	2	**Remated**
04_Q05	2	1	0	2	1	2	2	1	2	2	**Mated once**
05_Q06	2	1	1	2	1	2	1	2	1	2	**Mated once**
06_Q07	2	1	2	2	1	2	2	2	2	2	**Mated once**
07_Q08	2	1	2	2	2	1	1	2	1	2	**Mated once**
08_Q09	2	1	2	2	2	1	1	2	2	2	**Mated once**
09_Q10	1	1	2	1	2	2	1	2	1	2	**Mated once**
10_Q11	3	1	2	3	2	2	2	2	2	1	**Remated**
11_Q12	2	1	2	2	1	1	2	1	2	2	**Mated once**
12_Q14	2	1	2	3	1	1	2	2	2	2	**Remated**
13_Q15	2	1	2	1	1	1	2	1	2	2	**Mated once**
14_Q16	2	2	2	2	2	2	2	2	1	1	**Mated once**
15_Q17	2	2	2	1	2	2	2	2	1	2	**Mated once**
16_Q18	1	2	1	2	2	1	1	2	2	2	**Mated once**
17_Q19	3	1	1	1	2	3	2	2	1	2	**Remated**
18_Q20	2	1	1	2	2	2	2	2	1	2	**Mated once**
19_Q21	2	1	2	2	2	2	1	2	2	1	**Mated once**
20_Q22	0	0	0	1	0	2	0	1	1	0	**Mated once**
21_Q23	4	2	1	2	3	3	3	3	3	4	**Remated**
22_Q24	2	1	2	2	2	1	2	2	1	2	**Mated once**
23_Q26	1	1	1	2	1	1	2	1	2	1	**Mated once**

Fig. 1. Number of alleles detected in each of the ten microsatellite markers in stored sperm of gravid females and their inferred mating history. Rows represent individual stored sperm samples extracted from gravid females collected in NSW (N = 25) and QLD (N = 23). Integer in a cell indicates the observed number of alleles for a given microsatellite marker. Cells highlighted in red denote markers with three or more alleles detected, which serves as evidence for remating of the respective females.

progeny sample sizes, bypassing such post-insemination complications as cryptic female choice and the logistical challenges associated with insect rearing and large-scale progeny genotyping. This study for the first time showed that polyandry estimation via sperm genotyping is feasible in tephritids and can be used to routinely assess remating frequencies.

Our findings of high remating rate in Qfly has important implications for SIT. Polyandry can be compatible with SIT if mating is random and sterile males are fully competitive (Barclay, 2005). While some studies have reported no evidence of assortative mating in Qfly (Pérez-Staples et al., 2009; Weldon, 2005), others have reported competitive disadvantage of sterile males (Adnan et al., 2020b). Additionally, irradiation, dyeing, and packing negatively affect fly quality (Benelli et al., 2021; Dominiak et al., 2002; Dominiak et al., 2007) and may affect mating competitiveness. High remating rate can potentially jeopardise efficacy of Qfly SIT. Analysis of more populations at different times in the season would provide valuable insight to the prevalence and extent, and likely impact, of polyandry on the mating system generally and on SIT. Generating such information is more feasible than ever with the developed tools for sperm genotyping due to tremendous cost reduction, as well as increased flexibility, speed and accuracy. Under some conditions, increases in SIT release rate or frequency might be required to compensate for the impacts of polyandry.

4. Conclusion

The present study provides the first evidence of polyandry in Qfly field populations by multi-locus genotyping of stored sperm from ovipositing females. Significant difference in the prevalence of remating was detected between the NSW and QLD collections, suggesting spatial and/or temporal variation.

Declaration of Competing Interest

None.

Data accessibility

Raw microsatellite genotypes for all wild caught males, gravid females, and their stored sperm can be accessed in the Supplementary File, which contains two Microsoft Excel spreadsheets, one for each sampling site.

Authors contributions

JS, SFL, PT designed, analysed and wrote the manuscript. JS, SFL, HLY evaluated the probability approach for estimating polyandry rates based on population frequency data.

Acknowledgements

We thank Brendan Missenden, Nazma Akter Tithi and Vivek Kemparaju for their kind assistance in Qfly sampling. This research was conducted as part of the SITplus collaborative fruit fly program. Project *Raising Q-fly Sterile Insect Technique to World Standard* (HG14033) is funded by the Hort Frontiers Fruit Fly Fund, part of the Hort Frontiers strategic partnership initiative developed by Hort Innovation, with co-investment from Macquarie University and contributions from the Australian Government. JS was supported by a Macquarie University iRTP scholarship.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.cris.2022.100040.

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