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Variation in sex ratio of the leafminer *Phytomyza plantaginis* Goureau (Diptera: Agromyzidae) from Australia

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Running Title
Reproduction in *Phytomyza plantaginis* in Australia

This is the author manuscript accepted for publication and has undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/aen.12557

ABSTRACT

Parthenogenetic reproduction has only previously been demonstrated in two species of agromyzid leafminer flies (Diptera: Agromyzidae), both from the genus *Phytomyza*. The plantain leafminer, *Phytomyza plantaginis*, is Palaearctic in origin and bisexual populations have been observed in this region. However, historically only females had been collected in the Australasian, Nearctic, Neotropical and Oceanian regions. Here we show that southern Australian samples of this species from *Plantago* spp. can be comprised of both parthenogenic and bisexual populations. In sites around Melbourne, males were present although the sex ratio was female-biased, with females comprising 75% of sampled individuals. In contrast, males were absent from collections in western and northern Victoria, Australian Capital Territory, New South Wales and Western Australia. Female *P. plantaginis* flies were reared from three *Plantago* host plants (*Pl. lanceolata*, *Pl. major* and *Pl. coronopus*), although females were particularly common from *Pl. lanceolata*. Insect cages set up with leafminers from sites where only females occurred produced only females, while cages with leafminers from sites where males occurred produced both sexes and mating was observed. Individuals from all populations were infected by *Wolbachia* endosymbionts, suggesting that the presence/absence of *Wolbachia* is not directly responsible for the parthenogenesis. However, there was an association between a mtDNA variant (PP.02) and sexual reproduction, in that this variant was absent in areas where only females were collected but present in all males and some females from areas with sexual populations. The mechanism responsible for parthenogenesis in *P. plantaginis* remains unknown but is linked to maternal factors.

Key words
Unisexual reproduction, female-bias, distribution, colonization, parthenogenesis, thelytoky, *Wolbachia*

INTRODUCTION

Several modes of reproduction can be found in insects, ranging from sexual to unisexual, and either obligate, facultative or cyclical (Normark 2003, 2014; van der Kooi *et al.* 2017), but they are not equally distributed between orders. While parthenogenesis, or the production of offspring without egg fertilization, is more commonly found in some orders like Hemiptera and Coleoptera, it has a lower incidence in others like Diptera (Hoffmann *et al.* 2008). At least 11 dipteran families have unisexual species (Gokhman & Kuznetsova 2018). However, within the Agromyzidae, only *Phytomyza crassiseta* Zetterstedt (Hering 1926, Block 1969) and *Phytomyza plantaginis* Goureau (Frick 1951) have been shown experimentally to have parthenogenetic populations. (Note: nearly all scientific papers give Robineau-Desvoidy as the author for *P. plantaginis*, however this is a junior homonym and does not comply with Article 50.1 in the Code of the International Commission of Zoological Nomenclature (Evenhuis 2016)). Two other species of Agromyzidae, *Pseudonapomyza atra* (Meigen) and *Pseudonapomyza lacteipennis* (Malloch), are also suspected of reproducing parthenogenetically due to extremely low numbers of males collected from some populations in North America (Boucher 2004). By studying the conditions in which sexual and unisexual populations of a species exist, we can attempt to understand the evolution of parthenogenesis as a mode of reproduction.

Multiple drivers have been linked to the appearance of unisexuality as a reproduction mode in mostly bisexual species of insects and other species. Unisexual populations are often characterised as having broad ecological niches (van der Kooi *et al.* 2017). They tend to occupy higher altitudes or latitudes than their species' bisexual populations and can be common at the boundaries of species ranges as in the case of geographic parthenogenesis (Glesener & Tilman 1978; Tilquin & Kokko 2016). Unisexual populations may be common in areas where there is a limited exposure to pests and pathogens (Hamilton *et al.* 1990) and in more stable environments, where there are fewer benefits of new genetic variants provided through sex (Toman & Flegr 2018). Additionally, unisexual individuals may have an advantage during long-distance dispersal as they are better able to colonize and immediately increase in numbers in a new environment. Stable conditions and long-distance dispersal may

contribute to the relatively high incidence of unisexual species in agricultural pests (Hoffmann *et al*. 2008). Infectious endosymbionts such as *Wolbachia* may also influence the incidence of parthenogenesis in insects because unisexual reproduction favours the transmission of the endosymbiont (Simon *et al.* 2003).

Phytomyza plantaginis has been recorded from the Holarctic (Hering 1926; Spencer 1963b, Nearctic (Spencer 1969, 1981; Sehgal 1971), Neotropical (Černý *et al.* 2018), Afrotropical (Deeming 2006; Černý 2018), Macronesian (Černý *et al.* 2018), Oriental (Sasakawa 1972), Oceanian (Sasakawa 1964) and Australasian regions (Spencer 1963a, 1976, 1977). In Europe it has been recorded from Portugal to Russia and from Italy to Finland (Martinez 2011; Černý *et al.* 2018; Papp & Černý 2019). Agromyzid flies are considered 'moderate fliers' (Yoshimoto & Gressitt 1964) but do have the capacity to move longer distances by wind dispersal. Wind-assisted movement of agromyzids between islands is believed to have occurred in the Florida area (Spencer & Stegmaier 1973). *Chromatomyia horticola* (Goreau) has been trapped at sea as far as 150 km from the coast near Korea (Yoshimoto & Gressitt 1964). However, in a series of trapping studies in Antarctic areas and the Western Pacific (Yoshimoto & Gressitt 1964), only one ♀specimen of *P. plantaginis* was trapped: in the East China Sea (90 km WNW of Tokara Shima) in a 2.5 m suction trap on the deck of a ship (Yoshimoto *et al.* 1962). No specimens were trapped near New Zealand and sub-Antarctic waters. It is most likely *P. plantaginis* was inadvertently introduced into Australia and New Zealand from Europe by colonists (Spencer 1963a, 1976), possibly on infested *Pl. lanceolata* discarded from ship soil ballast. Worldwide, its main host plants are *Plantago lanceolata* L. and *Plantago major* L. (Spencer 1972, 1990), which are associated with human settlement, and seeds were likely introduced as contaminants (Smith *et al.* 2020).

Phytomyza plantaginis is a specialist leafminer, known to feed only on *Plantago* spp. As with all other leaf-mining agromyzids, the female fly uses her ovipositor to pierce the epidermis of plant leaves and both females and males feed on the exudate. The female lays individual eggs under the leaf epidermis and, after hatching, the larva tunnels into the mesophyll. The larva can feed on either side of the leaf and sometimes tunnels into the stems. It pupariates within the leaf, forming an oval-shaped, slightly flattened puparium with some

colour variation, ranging from off-white to shiny black. These feeding patterns have been observed and illustrated in Europe (Ellis 2020), in the United States (Eiseman 2019), and in New Zealand (Martin 2019). The anterior spiracles of the puparium protrude from the epidermis and anchor it to the top layer of the mine (Eiseman 2019). A colour image of an adult *P. plantaginis* is available on-line (Černý et al. 2018). In a seven-month field study in Syria, Ghdifan *et al.* (2011) found populations of *P. plantaginis* on *P. lanceolata* and *P. major* peaked in July and August. A *Chrysocharis* sp. was reared from *P. plantaginis* and again numbers peaked in August.

Across its range, *P. plantaginis* exhibits variation in its mode of reproduction, with female-only populations in some places and bisexual populations in others. Spencer (1981) noted that both sexes of *P. plantaginis* are found in Europe, while parthenogenesis in an American population was demonstrated experimentally in California (Frick 1951). *Phytomyza plantaginis* specimens from New York and Pennsylvania were noted as having both sexes, but no information was provided to confirm the identification of the males (Frost 1924). Collections from Argentina, Chile, New Zealand and Australia (before this study) have yielded only females (Spencer 1963a, 1976, 1977; Lambkin *et al.* 2008; Černý *et al.* 2018) (Table S1). The first case of parthenogenetic reproduction in the Agromyzidae was demonstrated by Hering (1926) when he isolated unmated females of *P. crassiseta* in an enclosure containing the host plant *Veronica* sp. and obtained mines and later an all-female generation of offspring. In Scandinavia, females in parthenogenetic populations of *P. crassiseta* were found to be either diploid or triploid (Block 1969). *Phytomyza crassiseta* is closely related to *P. plantaginis* (Spencer 1990; Winkler *et al.* 2009) but it is not known whether polyploidy is involved in parthenogenesis of populations of *P. plantaginis*.

Phytomyza griffithsi Spencer is very similar in appearance to *P. plantaginis*, but Spencer (1963a, 1977) did not record *P. griffithsi* in Australia or New Zealand. It is widespread in southern and Central Europe (Papp & Černý 2019) and is primarily reared from *Plantago media* L. (Spencer 1963b), a species not found in Australia or New Zealand (Atlas of Living Australia 2020a).

Although *P. plantaginis* is widespread in Australia, relatively little is known about its ecology there. The extent of its range is unknown, along with its mode of reproduction. In this paper, we investigated the sex ratio and distribution of *P. plantaginis* in Australia. To do this, we: (1) verified the identity of *P. plantaginis* using morphological and molecular techniques, (2) assessed the relative abundance of *P. plantaginis* on *Plantago* spp. in urban settings around Melbourne, Victoria, (3) collected *P. plantaginis* from a range of locations around Australia, (4) compared the sex-ratio of populations collected in different localities, and (5) attempted to link geographic variation in sex ratio with the occurrence of *Wolbachia* endosymbionts.

MATERIALS AND METHODS

Sampling

Three types of sampling were undertaken at sites between 2018 and 2020: opportunistic, semi-quantitative and quantitative. Quantitative sampling was performed at six sites in the Melbourne area (Fitzroy North, Flemington Bridge, Syndal-Mount Waverley, Mount Waverley-Jordanville, Werribee, Werribee South) for flies reared from *Pl. lanceolata* over a period of 10 to 18 months, depending on the site. At each site, 50 *Pl. lanceolata* host plants were surveyed during each sampling event and the total number of plants found mined was recorded. A single mined leaf from each plant was removed and placed into a Ziploc® bag, labelled with the host plant, location, date, collector and sampling type. These were transferred to the laboratory for rearing and identification (see below). Additional leaves from each *Pl. lanceolata* plant found mined were collected, especially when the plants were heavily infested, as part of the semi-quantitative sampling to obtain additional samples for other assessments when flies were abundant. At two sites, Flemington Bridge and Fitzroy North, *Pl. major* was also present, and these plants were sampled following the same methodology. The maximum time spent sampling each site was 30 min.

Opportunistic sampling was undertaken in Victoria (Vic.) outside of the main Melbourne sites, as well as in Canberra in the Australian Capital Territory (ACT), in Lismore,

Ballina and Bangalow in New South Wales (NSW) and Bunbury in Western Australia (WA) (Table 1). Plants were collected from urban and peri-urban areas, such as roadside verges and along parks and green spaces. *Plantago lanceolata* was the sole host plant at these sites, with the exception of Forrest (Vic.) where *Pl. major* was the only host plant, Colac (Vic.) where *Pl. coronopus* was the only host plant and Sunbury (Vic.) where *Pl. coronopus* was found mined alongside *Pl. lanceolata*. Sampling duration was variable and dependent on the level of infestation (see Table 1).

Rearing of agromyzids and morphological identification

Once in the laboratory, sheets of paper towel were laid between each layer of mined *Plantago* leaves to prevent condensation; these were replaced every week. All Ziploc® bags were inflated before being sealed to avoid crushing the leaves, and bags were kept upright in boxes for ease of storage. All Ziploc® bags were kept in a controlled temperature (CT) cabinet at 20°C, with a 16L:8D photoperiod. Ziploc® bags were inspected twice a week for the presence of *P. plantaginis* flies.

All emerged flies were carefully removed using a paintbrush moistened with ethanol and placed into a petri-dish containing absolute ethanol. Flies were sexed under a dissecting microscope based on external genitalia before being transferred to 2 mL vials with absolute ethanol and stored at -20°C. A sub-set of the samples were placed in vials of 80% ethanol and kept at 4°C for future dissection. All vials were labelled and assigned a numbered tag. Morphological identification of flies was performed using the keys in Spencer (1963b, 1977). The dissection of the genitalia to confirm the identity of male specimens was undertaken by Dr M. Malipatil (AgriBio, Agriculture Victoria Research, Bundoora, Vic.). He closely followed the methods described for polyphagous agromyzid leafminers in EPPO (2005) and Spencer (1981), except samples were soaked in 10% potassium hydroxide (KOH) at room temperature for 15 min instead of boiling KOH and soaking samples for 2 to 3 min.

Molecular identification and Wolbachia *detection*

DNA barcoding was applied to confirm the morphological identification of the *P. plantaginis* specimens using the 3' region of the mitochondrial CO1 (cytochrome oxidase 1) gene. DNA was extracted from single adult specimens crushed with two 3 mm glass beads in 200 µl of 5% Chelex-100, in sterilised water. The solution was incubated with 3 µl of proteinase K (20 mg/ml) at 56°C for one hour. The suspended cell mixture was then boiled for 10 minutes and used as DNA template. Polymerase chain reactions (PCR) were undertaken in 30 µl reactions, containing 5 µl of DNA template, 3 µl (10x) PCR buffer with 20 mm MgCl2, 2.4 µl dNTPs (2.5 mM), 1.5 µl of both forward and reverse primers (10 µm), 0.3 µl BSA and 0.2 µl NEB Taq polymerase (1 Unit). The PCR procedures for amplification and primer set followed those used by Parish *et al.* (2017). The PCR products were directly sequenced at Macrogen (Seoul, Korea) with an ABI 3730XL DNA sequencer (Applied Biosystems).

We screened *P. plantaginis* specimens of both sexes (total of 29 males, 144 females) from Victoria, NSW and ACT populations for the presence of the endosymbiont *Wolbachia*. Universal primers for the *Wolbachia* surface protein (*wsp*) gene were initially used but resulted in multiple PCR bands and unreliable detection. We therefore designed specific primers targeting a fragment of the *wsp* gene from our *Phytomyza* samples. The primers consisted of NP-*WSP*-F1: CTAGCTACTACGTTCGTTTACAA and NP-*WSP*-R1: AAAACTAGCACCATAAGAACC. The thermal conditions used for PCR were based on Baldo *et al.* (2006). Results were cross-referenced to the Barcode of Life Data System (BOLD) database and NCBI GenBank using the Basic Local Alignment Tool (BLAST).

[INSERT Table 1]

Population cultures and reproduction mode determination

Colonies of *P. plantaginis* were established using seed-grown *Pl. lanceolata* in a CT room at 25°C, with a 16L:8D photoperiod. Seven *P. plantaginis* populations, six from Victoria (Stanhope, Glenrowan, Romsey, Flemington Bridge, Sunbury, Venus Bay) and one from ACT (Canberra), were established using field collected material (see Table 1). Mined *Pl. lanceolata* leaves were collected from each location and emerged flies (F0) were identified,

counted and sexed before >30 flies from each location were introduced into separate fine mesh BugDorm® (475x475x930 mm, Australian Entomological Supplies, Murwillumbah) cages. The first generation (F1) was extracted by collecting all leaves once newly formed mines were observed, with adults collected immediately after emergence. Additionally, a portion of the pupae from each colony was excised from *Pl. lanceolata* leaves and isolated to allow for the separation of unmated females. These were then sexed and reintroduced into their respective BugDorm® with fresh host plants, either as an all-female colony, or mixed colony if males were present. For three of the mixed populations, a subsample of the unmated females was placed into a separate BugDorm® to check for parthenogenesis.

This rearing process was continued to collect the F2 offspring, resulting in two successive generations reared in the laboratory, although the Flemington Bridge populations was kept for five generations.

Statistical analyses

Contingency tests to compare male and female numbers were performed using IBM SPSS Statistics version 26.

RESULTS

Morphological and molecular identification

Dr M. Malipatil confirmed the male genitalia of flies collected from the field matched the male genitalia description by Spencer (1977, 1981, 1990) (Fig. 1). Voucher specimens of these *P. plantaginis* flies have been lodged in the Victorian Agricultural Insect Collection (VIAC), Melbourne, Victoria.

[INSERT Fig. 1]

DNA barcoding of additional specimens (male and female) further confirmed our field samples as *P. plantaginis* and excluded the possibility that *P. griffithsi* was present in Australia. We then compared the frequency and geographic distribution of haplotypes with

previously published data on this leafminer species. Two distinct CO1 haplotypes were identified, that we refer to as PP.01 and PP.02 (Table 2). PP.01 corresponds to the haplotype identified in the USA (100% sequence match), where only females have been recorded (Scheffer & Weigmann 2000; Scheffer *et al.* 2007). In Australia, we also only found this haplotype in populations of *P. plantaginis* where only females were reared (Table 2). A new haplotype, PP.02, was identified in our study and was restricted to sexual populations in Victoria (Table 2: Werribee, Flemington Bridge, Sunbury, Venus Bay) where it occurred in the majority of females and in all males. PP.02 had 98.3% sequence similarity to PP.01, representing a 24 base difference out of a 1404 bp CO1 sequence, and within the species genetic diversity boundary. There was no variation within PP.02. We detected female *P. plantaginis* of both haplotypes at Venus Bay, Werribee and Flemington Bridge.

At Flemington Bridge (Table 2), haplotypes were present in females at frequencies of 6% (PP.01) and 94% (PP.02). However, this changed in a *P. plantaginis* colony established from leafmines collected from Flemington Bridge; after having been reared for five successive generations in the laboratory, the frequency of the PP.01 haplotype increased markedly (79.2%; n=24) (Table 2). Although these flies were not individually sexed, we did notice a predominance of females in the colony.

[INSERT Table 2]

Wolbachia detections

All *P. plantaginis* individuals screened were found to be infected with *Wolbachia*, regardless of their sex. Based on the *Wolbachia* supergroups defined by *wsp* sequences (Baldo *et al*. 2006), the *wsp* allele wLsatA detected in *P. plantaginis* belonged to *Wolbachia* supergroup B (Table S2).

Distribution of unisexual populations

Samples across all three host plants from NSW (n=153), ACT (n=140) and WA (n=60) yielded only females, but in Victoria (n=2,942), 25% of the *P. plantaginis* flies were males. The lowest proportion of females (near 50%) among the Victorian sites was from Colac (Fig. 2). In a contingency test comparing male to female numbers across all Victorian sites where at least 10 individuals were collected, a significant difference in sex ratio was observed (χ^2 = 460.44, df = 13, $P < 0.001$).

[INSERT Fig. 2]

For those flies reared from *Pl. lanceolata*, no males were reared from Victorian locations with latitudes above Kilmore South (Table 1). Males were also absent from *Pl. lanceolata* at Inverleigh, one of the most southern locations sampled in our study (Fig. 3). Additionally, there were no male flies reared from *Pl. major* at Forrest, which is the most southern location sampled. This suggests there is no clear latitudinal cline in the occurrence of sexual populations. Three Victorian populations of *P. plantaginis* (Apollo Bay – 1 ♀ collected 01/01/1967; Melton – 1 ♀ collected 27/11/1948; and Wyperfeld National Park (51 km NW of Hopetoun) – 1 ♀ collected 04/11/1974) were examined by Spencer (1977). These are plotted in Fig. 3 along with records from this study.

[INSERT Fig. 3]

Sex-ratio biases associated with host species

Plantago lanceolata was the only plant sampled outside of Victoria to host *P. plantaginis*. Within Victoria however, three *Plantago* spp. were sampled (Table 3), so the influence of host plant on sex-ratio could be examined. A contingency test showed a significantly different distribution of males to females across host species (excluding unsexed flies) (χ^2 = 250.2, df = 2, $P < 0.001$). This reflected a higher incidence of females collected from *Pl. lanceolata*, although both sexes were collected from all three host plants and host plant comparisons are to some extent confounded by collection location and the time of collection.

[INSERT Table 3]

To investigate plant host effects more directly, the male to female ratios of flies sourced from *Pl. major* (466♀; 455♂) and *Pl. lanceolata* (268♀; 163♂) from the same collection dates at Flemington Bridge were compared. A contingency test showed a significant difference between plant types (χ^2 =15.9, df = 1, P <0.001), suggesting the sex ratio of *P. plantaginis* may depend on the host, with relatively higher numbers of males from *Pl. major* (Table 4). Sex ratio differences were also evident for some samples taken on a single day (e.g., 09/10/2018), suggesting an absence of a temporal pattern of male presence at the site with the highest sample size.

[INSERT Table 4]

Temporal sampling

The Flemington Bridge site was repeatedly sampled due to the high abundance of flies. The seasonal abundance of *P. plantaginis* mining *Pl. lanceolata* and *Pl. major* at this site fluctuated, with sporadic peaks in spring and summer (Table 4). *Phytomyza plantaginis* was present on *Pl. lanceolata* throughout the year, and both sexes were found across seasons. *Plantago major* was a common secondary host plant present at this site; *Pl. major* was abundantly mined by *P. plantaginis* from September to November 2018 (345♀; 335♂) and from January to May 2019 (67♀; 70♂).

Observations of laboratory P. plantaginis *cultures*

Three *P. plantaginis* populations (Canberra, Stanhope and Glenrowan), established from females, produced all-female offspring (F1) when cultured in the laboratory. These F1 flies further went on to produce all-female offspring (i.e., only female F2 flies) (Table 5). The remaining four populations contained males in the field. All four populations produced both sexes at the F1 generation when cultured in the laboratory, suggesting at least some sexual reproduction. Furthermore, male flies in these populations were observed copulating with females. F1 females were isolated at the pupal stage in three of these populations (Romsey,

Flemington Bridge and Sunbury) to see if they could produce offspring at the F2 generation. Only the isolated Romsey females produced an all-female F2 generation (n=26) from the 4 isolated F1 females (Table 5). Interestingly, while the Romsey F1 sample included male offspring (2♀; 2♂), no F2 males were reared in the F2 generation from these flies (24♀), whereas colonies from Flemington Bridge and Sunbury continued to produce both sexes in a similar ratio (Table 5).

[INSERT Table 5]

DISCUSSION

In this study, male *P. plantaginis* were confirmed for the first time in Victoria and were reared, primarily from *Pl. lanceolata,* at six urban and peri-urban sites in Melbourne surveyed between June 2018 and January 2020. Additional sampling in other locations (including other states and territories) showed males were only present in populations near Melbourne, and a reduction in their numbers was generally observed as distance increased from the city. A reduction in the numbers of males of *P. crassiseta* mostly in a northerly (colder) direction was noted in populations from Germany and Sweden (Hering 1926; Block 1969). Historical samples from Tasmania suggest that males may also be absent there, while males have also never been recorded from New Zealand (Spencer 1976). However, female-only populations of *P. plantaginis* north and south of Melbourne suggest that the absence of males is not solely driven by latitude.

Among Agromyzidae, only *P. crassiseta* and *P. plantaginis* have been shown to have some parthenogenetic populations (Hering 1926; Frick 1951; Block 1969). Globally, the sex ratio of *P. crassiseta* and *P. plantaginis* populations are not distributed evenly. The European range of *P. crassiseta* overlaps with *P. plantaginis* (Martinez 2011). The German population of *P. crassiseta* tested by Hering (1926) was highly female-biased, however he noted that more southern populations in Europe had an equal male-to-female ratio. Female-biased populations have been reported in several localities in Finland, Sweden and Norway (Block 1969). In Argentina, only females of *P. crassiseta* have been collected (Valladares *et al.*

2002). For *P. plantaginis*, both sexes are present in the Holarctic Region (Hering 1926; Spencer 1963b), but only females have been confirmed from North America, South America and New Zealand.

Frick (1951) investigated the likelihood of parthenogenetic reproduction in Californian *P. plantaginis* by isolating unmated females with a *Plantago* host plant and rearing the subsequent female-only generation. This process was followed for a single additional generation and showed that new larvae were present in the plant. Interestingly, Frick (1951) reported that the females had functional spermathecae and suggested that they might still be able to reproduce sexually in the presence of male flies. In a similar vein, we established colonies from several field populations and were able to validate the existence of parthenogenetic lines in Australian *P. plantaginis*. We also showed that Australian *P. plantaginis* can exist in bisexual and unisexual populations, and that some populations possess both sexual reproduction and parthenogenesis, as was observed in Romsey. The mechanism(s) responsible for parthenogenesis in *P. plantaginis* warrants further investigation. A first step would be to rear colonies of both CO1 haplotypes to compare life histories, as well as endosymbiont status. Crossing experiments and spermathecal dissections could be used to examine the ability of parthenogenetic females to mate with males and if so, how their daily fecundity, fertility rate, egg and larval viability compares with unfertilized females and females from a bisexual line. The nature of the parthenogenesis could be examined using nuclear genetic markers and chromosome counts to test if parthenogenesis occurs without meiosis via apomixis (retaining heterozygosity) or with meiosis (leading to a high level of homozygosity), although intermediate forms are also possible particularly through polyploidy. Block (1969) previously showed that parthenogenesis in *P. crassiseta* results in both diploid forms that were likely to be of the meiotic type and triploid forms that retained heterozygosity in chromosome rearrangements and could not be easily classed as either meiotic or ameiotic.

Our CO1 haplotype analysis indicates haplotype PP.01 is related to parthenogenetic strains of *P. plantaginis*, given it was only found in females. Conversely, haplotype PP.02 dominated field populations which undergo bisexual reproduction. The increase in the proportion of the PP.01 haplotype in our laboratory colonies may indicate that individuals

with PP.01 are more competitive than haplotype PP.02. This might reflect the well-known two-fold cost of sex (Maynard Smith 1978) given that mitochondrial haplotypes are maternally transmitted and unisexual reproduction results in only females that pass on this haplotype to all offspring whereas haplotypes are not transmitted through male offspring. The cost of sex might also help to explain the production of the all-female F2 generation in the Romsey colony. However, we have not yet established that there is a fitness advantage of parthenogenic flies – it might also be the case that sex ratio distortion reflects maternally inherited male killers. Interestingly, individuals of both parthenogenetic populations and sexual populations were infected with *Wolbachia,* which suggests that the presence of *Wolbachia* is not responsible for parthenogenesis, as it is in several insect species (O'Neill *et al.* 1997). We did however find an association between the two CO1 haplotypes and parthenogenesis. Given that mtDNA haplotypes are often tightly associated with *Wolbachia* infections due to their common mode of maternal transmission (Hale & Hoffmann 1990; Ballard 2000), it is possible that there are two different *Wolbachia* strains present in *P. plantaginis* that are indistinguishable by the *wsp* sequencing we undertook here, with only one of these producing parthenogenesis. *Wolbachia* in Diptera can be responsible for sex ratio distortions by male-killing (Hurst *et al*. 2000; Jaenike 2007; Richardson *et al.* 2016), as well as cytoplasmic incompatibility (Bourtzis *et al.* 1996; Tagami *et al.* 2006). Parthenogenesis in Diptera has not been associated with *Wolbachia* previously (Werren *et al.* 2008).

Among the bisexual populations collected in Victoria, the sex-ratio of *P. plantaginis* reared from *Pl. lanceolata* was biased towards females, but the same bias was not observed in samples from the *Pl. coronopus* and *Pl. major* collections. Although the sample size for flies reared from *Pl. coronopus* was small, this was not the case for the *Pl. major* samples. *Plantago major* favours moist, disturbed habitats (VicFlora 2020) and in our study was restricted to two sites that bordered a creek: Fitzroy North and Flemington Bridge. At these sites, both *Pl. lanceolata* and *Pl. major* were present. Given this, host plants may affect the incidence of the parthenogenetic form observed in field populations of *P. plantaginis*. The mechanism(s) underlying the sex biases in *P. plantaginis* are unknown; it is uncertain if fertilised females prefer *Pl. major*, resulting in the significantly higher number of male

offspring reared from this host, or if host plant preferences are passed down through generations, occurring in lines reproducing only sexually. Our CO1 barcoding showed flies reared from *Pl. major* are haplotype PP.02 and undergo sexual reproduction. However, it is worth noting that there is a sequence difference of ~1.7% between the two haplotypes and it would be worth characterising the molecular differences between these haplotypes further as well as any quantitative differences among the females in morphological traits. While male genitalia are typically used to identify different species of *Phytomyza*, these are not available for the (female only) PP.01 haplotype, but perhaps male production could be triggered in PP.01 lines through antibiotic exposure.

Additional *Plantago* species could be used to study the differences more thoroughly in reproductive modes across host plants. In the USA, *Plantago rugelii* Decne and *Plantago wrightiana* Decne are also recorded as hosts of *P. plantaginis* (Eiseman *et al.* 2019). In Australia, there are 34 *Plantago* spp. recorded, 10 of which are introduced, which potentially might be additional hosts for *P. plantaginis* (VicFlora 2020).

Phytomyza plantaginis is of increasing interest in horticulture as a source of generalist hymenopteran parasitoids that attack pest *Liriomyza* spp. (Lambkin *et al.* 2008; Ridland *et al.* 2020). A unisexual *P. plantaginis* population could act as an important reservoir for parasitoids like *Diglyphus isaea* by providing a stable source of host larvae. Though originating from a temperate climate, *Pl. lanceolata* has been recorded across large areas of southern Australia, as well as in locations around Alice Springs and as far north as Cairns (Atlas of Living Australia 2020b) (Fig. S1). Additional sampling will help delimit the distribution of *P. plantaginis* in Australia as well as the extent of bisexual and unisexual populations. *Plantago lanceolata* cultivars are already used in New Zealand for dairy pastures (Lee *et al.* 2015) and are being considered as a drought-tolerant component to south-eastern Australian dairy pastures (Langworthy *et al.* 2018; Raedts & Langworthy 2019). *Plantago lanceolata* plants are already common on field margins and an increased abundance in pastures could augment its potential as a source of agromyzid parasitoids.

ACKNOWLEDGEMENTS

We are very grateful for the technical expertise of Mallik Malipatil (Agriculture Victoria Research, Bundoora, Vic.) who performed male genitalia dissections and confirmed the identification of *P. plantaginis*. We are also grateful to two anonymous reviewers who provided very useful detailed comments. We thank Neal Evenhuis (Bishop Museum, Hawaii) for his detailed advice on the nomenclatural status of *P. plantaginis,* and we thank Qiong Yang, Nancy Endersby and Alex Gill for technical advice and assistance and Veronique Paris for preparing Fig. 3. The culture of *Pl. lanceolata* was made possible due to the generous donation of seeds by Rachel Burton (University of Adelaide). This project was supported by the RD&E program for control, eradication and preparedness for vegetable leafminer (MT16004), funded by Hort Innovation, as well as the Australian Research Council (DP120100916). The development of this manuscript was supported by a Writing-up Award from The University of Melbourne to MPC.

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SUPPORTING INFORMATION

Additional information can be found online in the supporting information tab for this article.

Table S1 Published records of *Phytomyza plantaginis* specimens reared and sexed from different locations outside of its endemic range.

Table S2 *Wolbachia* screening results of *Phytomyza plantaginis* specimens and their sex, sample location and host plant.

Fig. S1 Distribution records for *Plantago lanceolata* in Australia (Source: Atlas of Living Australia 2020).

Table 1 Details of the sampling *Phytomyza plantaginis* in Australia between 2018 and 2020.

Location	Latitude	Longitude	Date collected	Type of sampling	Host plant	No. flies ♀	♂
Victoria							
Wangaratta North	-36.342	146.301	16/06/2019	O	*Pl. lanceolata*	12	0
Wangaratta South	-36.374	146.316	16/06/2019	O	*Pl. lanceolata*	38	0
Stanhope	-36.462	144.791	15/06/2019	O	*Pl. lanceolata*	43	0
Glenrowan	-36.462	146.224	15/06/2019	O	*Pl. lanceolata*	230	0
Elmore	-36.493	144.608	14/06/2019	O	*Pl. lanceolata*	3	0
Benalla East	-36.558	145.999	16/06/2019	O	*Pl. lanceolata*	20	0
Kilmore South	-37.308	144.952	17/06/2019	O	*Pl. lanceolata*	17	2
Romsey	-37.354	144.747	14/06/2019	O	*Pl. lanceolata*	15	3
Ballarat	-37.570	143.135	31/12/2020	O	*Pl. lanceolata*	73	4
Sunbury	-37.572	144.729	27/09/2019	O	*Pl. lanceolata*	6	4
				O	*Pl. coronopus*	46	32
Northcote	-37.765	144.998	(multiple)	O	*Pl. lanceolata*	2	2
Fitzroy North	-37.778	144.989	(multiple)	Q+S	*Pl. lanceolata*	75	9

Location	Latitude	Longitude	Date collected	Type of sampling	Host plant	No. flies ♀	♂
			15/03/2019	O	*Pl. major*	2	1
Flemington Bridge	-37.787	144.940	(multiple)	Q+S	*Pl. lanceolata*	301	196
				O	*Pl. major*	254	276
North Melbourne	-37.797	144.948	(multiple)	O	*Pl. lanceolata*	2	2
Syndal–Mt Waverley	-37.876	145.147	(multiple)	Q+S	*Pl. lanceolata*	292	82
Mt Waverley-Jordanville	-37.875	145.122	(multiple)	Q+S	*Pl. lanceolata*	359	50
Glen Waverley	-37.881	145.151	(multiple)	O	*Pl. lanceolata*	26	3
Werribee	-37.915	144.669	(multiple)	Q+S	*Pl. lanceolata*	282	30
Werribee South	-37.966	144.686	(multiple)	Q	*Pl. lanceolata*	63	28
Inverleigh	-38.094	144.009	17/04/2019	O	*Pl. lanceolata*	12	0
Colac	-38.338	143.595	03/01/2021	O	*Pl. coronopus*	20	18
Shoreham	-38.431	145.043	10/02/2020	O	*Pl. lanceolata*	33	0
Venus Bay	-38.696	145.809	21/12/2019	O	*Pl. lanceolata*	22	16
Forrest	-38.550	143.745	11/01/2020	O	*Pl. major*	6	0
ACT							

Location	Latitude	Longitude	Date collected	Type of sampling	Host plant	No. flies ♀	♂
Canberra	-35.238	149.085	(multiple)	O	*Pl. lanceolata*	140	0
NSW							
Lismore	-28.821	153.344	06/10/2018	O	*Pl. lanceolata*	64	0
	-28.811	153.280	06/10/2018	O	*Pl. lanceolata*	12	0
Ballina	-28.869	153.575	07/10/2018	O	*Pl. lanceolata*	31	0
Bangalow	-28.689	153.519	24/09/2018	O	*Pl. lanceolata*	19	0
WA							
Bunbury	-33.370	115.639	09-10/10/2019	O	*Pl. lanceolata*	60	0

O=Opportunistic sampling; S=Semi-quantitative; Q=Quantitative

Table 2 Mitochondrial haplotypes for *Phytomyza plantaginis* specimens based on the sex of sequenced individuals and their sampling location. The new haplotype, PP.02, is highlighted in bold. Numbers in parentheses (n) indicate the number of individuals.

Location		Host plant	Haplotype (n)		% PP.01 haplotype
			♀	♂	
USA					
NC Wake Co. NCSU, Method Road		*Pl. lanceolata*	PP.01 (1)	-	-
North Carolina, Raleigh		*Pl. lanceolata*	PP.01 (1)	-	-
Australia					
NSW	Lismore	*Pl. lanceolata*	PP.01 (24)		100.0
ACT	Canberra	*Pl. lanceolata*	PP.01 (32)		100.0
Vic.	Venus Bay	*Pl. lanceolata*	PP.01 (1)**, PP.02 (8)**	**PP.02 (9)**	5.5
	Werribee	*Pl. lanceolata*	PP.01 (8), **PP.02 (4)**	**PP.02 (12)**	33.3
	Flemington Bridge	*Pl. major*	**PP.02 (10)**	**PP.02 (11)**	0.0
	Flemington Bridge	*Pl. lanceolata*	PP.01 (3), **PP.02 (11)**	**PP.02 (12)**	11.3
	Flemington Bridge (colony after 5 generations)	*Pl. lanceolata*	PP.01 (19)[A]**, PP.02 (5)[A]**		79.2
	Sunbury	*Pl. lanceolata*	**PP.02 (12)**	**PP.02 (12)**	0.0
	Glenrowan	*Pl. lanceolata*	PP.01 (24)	-	100.0
	Stanhope	*Pl. lanceolata*	PP.01 (24)	-	100.0
	Elmore	*Pl. lanceolata*	PP.01 (4)	-	100.0
	Romsey	*Pl. lanceolata*	PP.01 (5)	-	100.0
	Ballarat	*Pl. lanceolata*	PP.01(10)	**PP.02 (2)**	83.3
	Colac	*Pl. coronopus*	**PP.02 (12)**	**PP.02 (12)**	50.0
	Shoreham	*Pl. lanceolata*	PP.01(12)		100.0

[A] Samples barcoded before sexing, no sex-ratio data available.

Table 3 Total number of female and male *Phytomyza plantaginis* across all sampling sites in Victoria reared from three *Plantago* species between 2018 and 2020.

P. plantaginis	**Host plant**		
	Pl. lanceolata	*Pl. major*	*Pl. coronopus*
♀	2,193	476	66
♂	630	456	50
Total	**2,823**	**932**	**116**

Table 4 Total number of *Phytomyza plantaginis* reared from *Plantago major* and *P. lanceolata* at Flemington Bridge between June 2018 and January 2020.

Sampling date	***Plantago lanceolata***		***Plantago major***	
	♀	♂	♀	♂
2018	**203**	**124**	**363**	**347**
25/06/2018	0	0		
20/08/2018	1	0		
24/08/2018	13	20		
12/09/2018	8	4	67	77
09/10/2018	144	79	257	234
01/11/2018	12	7	21	24
12/11/2018	2	1		
22/11/2018	2	3		
12/12/2018	1	0		
29/12/2018	5	1	8	5
31/12/2018	15	9	10	7
2019	**200**	**133**	**103**	**108**
20/02/2019	7	7	15	10
04/03/2019	8	4	19	27
25/03/2019	11	3	18	15
11/04/2019	5	5	13	13
24/04/2019	16	22	2	4
24/05/2019	20	18	0	1
24/06/2019	16	8		
26/08/2019	5	1	34	37
18/09/2019	48	39		
07/10/2019	12	8		
12/10/2019	4	3		
25/10/2019	7	6		
27/11/2019	12	3	2	1

12/12/2019	29	6		
2020	**32**	**16**		
13/01/2020	32	16		
Total	**435**	**273**	**466**	**455**

Table 5 Sex distribution in *Phytomyza plantaginis* colonies reared from different source populations (north-to-south gradient) for two generations in the laboratory. For three of the populations (Romsey, Flemington Bridge, Sunbury), an attempt to produce F2 flies from females isolated as puparia was also made.

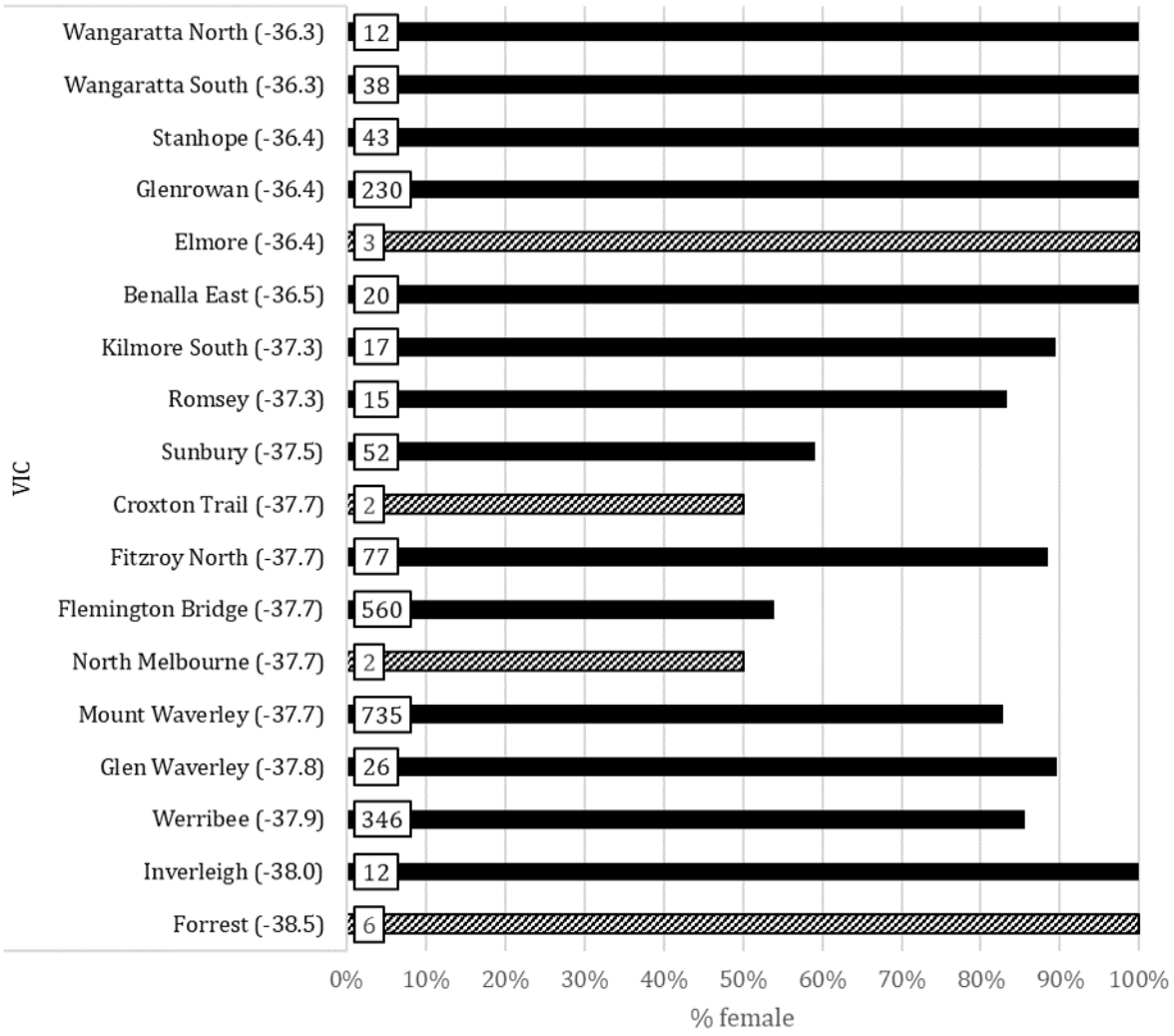
Location	Colony establishment	F1	F2	Reproductive mode
ACT				
Canberra	7♀	24♀	69♀	Unisexual
Vic.				
Stanhope	5♀	13♀	48♀	Unisexual
Glenrowan	6♀	12♀	52♀	Unisexual
Romsey	9♀ x 3♂	2♀ x 2♂	24♀	Bisexual +
		4♀ (isolated puparia)	26♀	Unisexual
Flemington Bridge	8♀ x 8♂	19♀ x 14♂	78♀ + 66♂	Bisexual
		15♀ (isolated puparia)	0	
Sunbury	8♀ x 8♂	21♀ x 20♂	84♀ + 76♂	Bisexual
		15♀ (isolated puparia)	0	
Venus Bay	8♀ x 8♂	23♀ x 16♂	71♀ + 59♂	Bisexual

Figure Legends

Fig. 1 (a) *Phytomyza plantaginis* male genitalia dissection, aedeagus, side view, photographed with compound microscope, magnification (x400); (b) Spencer (1990) European specimen *P. plantaginis* male genitalia, aedeagus, side view, illustrated.

Fig. 2 Proportion of female *Phytomyza plantaginis* from all Victorian locations (south to north distribution, latitude is indicated next to each location) collected across all host plants. The number on each bar is the total number of flies sexed at each site. Note: only locations where at least 10 individuals were sampled are included.

Fig. 3 Map of sampling locations for *Phytomyza plantaginis* in Victoria. Triangles indicate sites were both sexes were found, while circles indicate sites where only females were collected. Note: three historical sites from Spencer (1977) are included (marked by arrows).



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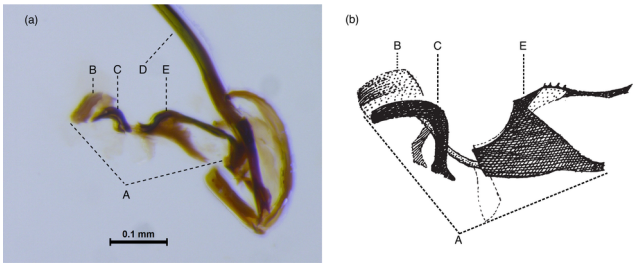

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