Genet. Res., Camb. (2006), **87**, *pp.* 175–185. © 2006 Cambridge University Press
doi:10.1017/S0016672306008202 Printed in the United Kingdom

The genetic outcomes of sex and recombination in long-term functionally parthenogenetic lineages of Australian *Sitobion* aphids

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(*Received 19 December 2005 and in revised form 2 March 2006*)

Summary

The typical life cycle of an aphid is cyclical parthenogenesis which involves the alternation of sexual and asexual reproduction. However, aphid life cycles, even within a species, can encompass everything on a continuum from obligate sexuality, through facultative sexuality to obligate asexuality. Loss of the sexual cycle in aphids is frequently associated with the introduction of a new pest and can occur for a number of environmental and genetic reasons. Here we investigate loss of sexual function in *Sitobion* aphids in Australia. Specifically, we aimed to determine whether an absence of sexual reproduction in Australian *Sitobion* results from genetic loss of sexual function or environmental constraints in the introduced range. We addressed our aims by performing a series of breeding experiments. We found that some lineages have genetically lost sexual function while others retain sexual function and appear environmentally constrained to asexuality. Further, in our crosses, using autosomal and X-linked microsatellite markers, we identified processes deviating from normal Mendelian segregation. We observed strong deviations in X chromosome transmission through the sexual cycle. Additionally, when progeny genotypes were examined across multiple loci simultaneously we found that some multilocus genotypes are significantly over-represented in the sample and that levels of heterozygosity were much higher than expected at almost all loci. This study demonstrates that strong biases in the transmission of X chromosomes through the sexual cycle are likely to be widespread in aphids. The mechanisms underlying these patterns are not clear. We discuss several possible alternatives, including mutation accumulation during periods of functional asexuality and genetic imprinting.

1. Introduction

Extensive population genetic studies have demonstrated that grass- and cereal-feeding *Sitobion* aphids, since their arrival in Australia and New Zealand, reproduce in the absence of sexual reproduction (Sunnucks *et al.*, 1996; Wilson *et al.*, 1999). This observation strongly contrasts with the predominant reproductive strategy of closely related *Sitobion* species from the United Kingdom (Sunnucks *et al.*, 1997), Europe (Simon *et al.*, 1999; Papura *et al.*, 2003) and Taiwan (Wilson *et al.*, 1999) but is similar to the population structure of *Sitobion avenae* following its introduction into Chile (Figueroa *et al.*, 2005).

The typical life cycle of an aphid is that of cyclical parthenogenesis which involves the alternation of sexual and asexual reproduction (Blackman & Eastop, 2000). However, aphids exhibit a tremendous amount of phenotypic and life cycle plasticity (Moran, 1992). While no aphid species are known to have lost the parthenogenetic phase of their life cycle, there are many species or lineages within species that have secondarily lost the sexual phase, either entirely or partially (Blackman & Eastop, 2000). With complete loss of the ability to reproduce sexually, an aphid lineage reproduces by continual asexual reproduction throughout the year, failing to respond to scotoperiodic changes that would normally elicit a switch to the production of sexual forms. In addition

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to the possible alternation between phases of asexual and sexual reproduction, the life cycle of some aphid species involves an obligate seasonal switching between two, often distantly related, host plants where one host plant (the primary host) is used during the sexual phase and the other (the secondary host) during the asexual phase (Blackman & Eastop, 2000).

The fact that many aphids are able to abandon the sexual part of their life cycle can result in the rapid establishment of an introduced aphid from a single individual. In the case of the tobacco-feeding race of *Myzus persicae* in Chile, the invasion of a single genotype was responsible in 1998 for a severe aphid outbreak on crop tobacco in central Chile (Fuentes-Contreras *et al*., 2004). Many aphid invaders will be environmentally constrained to asexuality not because they have lost the ability to reproduce sexually but rather because the environmental conditions of their introduced range differ significantly from those of their native range either in terms of seasonal scotoperiod and temperature or in the case of host alternating aphids, the presence of their primary host plant (Blackman, 1974; Fuentes-Contreras *et al*., 2004). However, once an aphid invader has been environmentally constrained to asexuality, it will, in the absence of natural selection to maintain sexual function, eventually lose the ability to reproduce sexually because of the erosion of complex physiological and chromosomal functions (Blackman, 1979; Innes & Hebert, 1988).

Studies of the population and reproductive biology of the aphid *Rhopalosiphum padi* have demonstrated that there are at least three routes to asexuality in aphids: (i) mutation of gene(s) essential to sexual function, (ii) hybridization and (iii) contagious parthenogenesis (Delmotte, 2002). Whilst Delmotte (2002) discusses mutation of gene(s) associated with sexuality resulting in an immediate and spontaneous loss of sex, there is another sort of genetic change that could also contribute to loss of sexual function: chromosomal change. Chromosomal change is very common in aphids and can potentially hinder sexual reproduction by creating a meiotic barrier (although this is not always the case, *vide Myzus persicae*; Blackman & Takada, 1977). Hybridization between closely related aphid species has been shown to result in unaltered parthenogenetic reproduction but greatly reduced sexual function (Rakauskas, 2000) where contagious parthenogenesis results from males from male-producing, asexual (androcyclic) lineages mating with sexual females and transmitting alleles conferring asexuality to their daughters.

In this study we aimed to determine whether the absence of sexual reproduction in Australian *Sitobion* results from genetic loss of the ability to reproduce sexually or whether it is attributable to failure of the synchrony of sexual induction cues in the introduced range so that any investment in sexual reproduction fails. This sort of failure of sexual reproduction has been reported in *Myzus persicae* in places including Cairo, Egypt (Willcocks & Bahgat, 1937), Israel (Zimmerman-Gries & Swirski, 1956) and Lahore, Pakistan (Das, 1918) where sexual morphs are produced but at the wrong time of year to result in successful sexual reproduction (Blackman, 1972). We addressed our aims by performing a series of breeding experiments in Australian lines of *Sitobion miscanthi* and *Sitobion* near *fragariae* and asked the following questions: can Australian lines of *S. miscanthi* and *S.* near *fragariae* produce sexual forms and mate successfully and do genetic markers in the progeny conform to Mendelian expectations?

2. Materials and methods

(i) *Study species*

Two species of grass- and cereal-feeding *Sitobion* aphids are known in Australia: *Sitobion miscanthi* and *Sitobion* near *fragariae*. *Sitobion miscanthi* (Takahashi, 1921) is widely distributed in Asiatic and Western Pacific rim countries (Blackman & Eastop, 2000). According to Blackman & Eastop (2000) this species is probably obligately parthenogenetic almost everywhere; however, museum collections of *S. miscanthi* from India include a sexual female and several males (Blackman & Eastop, 2000) and molecular and morphological data suggest that *S. miscanthi* may be extremely closely related to or even synonymous with the host-alternating, cyclically parthenogenetic species *S. akebiae* (Shinji) from Japan and Korea (Sunnucks *et al*., 1996; Blackman & Eastop, 2000). Genetic studies have confirmed that *S. miscanthi* is functionally parthenogenetic in Australia and New Zealand (Sunnucks *et al*., 1996; Wilson *et al*., 1999) but provide evidence of recent sexual reproduction in *S. miscanthi*-like aphids in Taiwan (Wilson *et al*., 1999). Four chromosomal races of *S. miscanthi* are known in Australia (2n = 17, 18, 20 and 21) (Hales *et al*., 1990; Sunnucks *et al*., 1996). These races can be distinguished using a single polymorphic microsatellite locus (Sunnucks *et al*., 1996). Little genotypic diversity has been detected within chromosomal races despite the application of up to 14 polymorphic microsatellite loci (Sunnucks *et al*., 1996; Wilson *et al*., 1999).

Sitobion near *fragariae* was first referred to as such by Hales *et al*. (1990). This species was originally identified by V. F. Eastop as *S. fragariae* (Walker) but was considered by M. Carver on the basis of morphometric analysis to be very similar to but probably distinct from *S. fragariae* (molecular data

support a very close affinity; Sunnucks & Hales, 1996; Sunnucks *et al*., 1996). It is now accepted by Blackman, Eastop and Carver (personal communications) to be distinct from *S. fragariae* and non-synonymous with any described *Sitobion* species. Records of *S. fragariae* from Australia and New Zealand are likely to represent specimens of this species. Highly resolving genetic studies of Australian *S.* near *fragariae* have found no evidence of sexual reproduction, or genotypic variation in this species (Sunnucks *et al*., 1996). However, there have been occasional field records of male *S.* near *fragariae* in both Australia and New Zealand (M. Carver and V. F. Eastop, personal communications; Sunnucks *et al*., 1996) and early laboratory sexual induction experiments in this species yielded males (Wilson *et al*., 1997). *Sitobion* near *fragariae* retains the ancestral karyotype of grass- and cereal-feeding *Sitobion* (2n = 18) (Hales *et al*., 1990).

(ii) *Choice of clones and details of induction experiments*

Representative clones (see Wilson *et al*., 2003 for discussion of the definition of the term clone) of all Australian chromosomal forms of *S. miscanthi* and of *S.* near *fragariae* were used in these experiments. Collection details of clones can be found in Supplemental Information Table S1. Nine separate induction experiments were undertaken between 1994 and 1999. Based on previous experimental work in Australian *Sitobion* lines (Turak, 1992; V. F. Eastop, personal communication to D. F. Hales) we used three sets of induction conditions over the period of these experiments: (1) 10 °C with 10 h of light followed by 14 h of darkness (10L : 14D); (2) 10 °C, 8L : 16D; and (3) 15 °C, 8L : 16D. A detailed description of the aphid culturing and handling materials and protocols used in these experiments can be found in the Supplemental Information.

(iii) *Breeding experiments*

We carried out breeding experiments using those clones that readily produced sexual morphs. These experiments can be divided into two parts that we executed in series. We carried out the experiments of Part I in large population pools of a single clone in cloth cages. Within the population of each clone, males could fall into two classes based on the X chromosome they carried. Sex determination in aphids is chromosomal: females are XX and males, which are produced by parthenogenesis, are XO. Thus, a given female X_1X_2 can produce two types of males: X_1 males and X_2 males. All the progeny of sexual reproduction are female. For a review of aphid sex determination see Wilson *et al*. (2003).

To complement these population-pool breeding experiments, and investigate further the results obtained from these experiments, in Part II we performed a series of individual-pair matings. In these we paired single virgin females and single males on barley seedlings in small Perspex cages for an average of 48 h. After this time, we removed the male from the cage and, if possible, moved him into another cage with a new virgin sexual female. In this way we allowed males to mate with multiple virgin females and the exact female and male parent of each batch of eggs were known. Following successive matings, we collected males into 100% ethanol for DNA extraction and microsatellite typing at X-linked loci. We chose males and sexual females randomly from *S.* near *fragariae* clone *Snf17* and *S. miscanthi* clone *Sm195*. Whilst the assignment of individuals to matings was random it was strongly dependent on the availability of adult males and adult virgin sexual females in the cultures. We attempted inter- as well as intraspecific matings because microsatellite analysis of English field collected populations detected *S. avenae*/*S. fragariae* hybrids (Sunnucks *et al*., 1997) and laboratory matings between these two species produced viable eggs (J. Hardie and R. Lilley, personal communication in Sunnucks *et al*., 1997).

(iv) *DNA extraction and microsatellite amplification*

We extracted DNA for microsatellite amplification from male aphids by salting-out (Sunnucks & Hales, 1996) and from fertilized eggs by chelex extraction (Sloane *et al*., 2001). We determined that eggs were fertilized on the basis of their colour: when eggs are laid they are green and if fertilized they turn shiny black over several days (Blackman, 1987). Unfertilized or inviable eggs do not turn black (Blackman, 1987).

We amplified microsatellite loci $Sm10^A$, $Sm17^A$, $S16b^A$, $Sm11^X$, $S10^X$, $S17b^X$ and $S49^X$ from eggs (Simon *et al*., 1999; Wilson *et al*., 2004). Superscripts in microsatellite locus names indicate whether the locus is autosomal (e.g. $Sm10^A$) or X-linked (e.g. $S17b^X$). For the males from Part II of the breeding experiments we only amplified X-linked loci. Details of microsatellite amplification are described in Wilson *et al*. (2004). We ran PCR products on a 6% acrylamide sequencing gel (SequaGel-6, National Diagnostics) using the positive control M13 DNA of the Sequenase version 2.0 kit (United States Biochemicals) as a marker ladder for precise sizing of alleles.

(v) *Analysis*

We used the *G*-test for goodness of fit (Sokal & Rohlf, 1995) to test the observed genotypic frequencies in

https://doi.org/10.1017/S0016672306008202 Published online by Cambridge University Press

eggs at all loci against Mendelian expectations. Since all successful matings were intraclonal, the expected frequency of genotypes in a population where X_1 and X_2 males are represented in the ratio of 1 : 1 is 1 : 2 : 1 (homozygote allele 1 : heterozygote : homozygote allele 2) for both autosomal and X-linked loci.

After examining the egg genotypic data on a locus-by-locus basis we examined the multilocus genotype of each egg. We pooled all eggs from both breeding experiments into two data sets: (1) *Sm195* × *Sm195* eggs and (2) *Snf17* × *Snf17* eggs. We calculated the observed and expected frequencies of multilocus genotypes over two heterozygous autosomal and two heterozygous X-linked loci ($Sm10^A$, $Sm17^A$, $S10^X$ and $S49^X$), from hereon referred to as the 'quadrilocus genotype'. Assuming linkage equilibrium, the maximum number of genotypes possible for a cross where both parents are heterozygous for the same alleles at each locus is 3^n (where n is the number of loci comprising the composite genotype). Thus, for a four-locus genotype there are a maximum of 81 genotypes. In this study, where only one male type contributes to the offspring, the number of possible genotypes is reduced depending on the number of X-linked loci. The maximum number of quadrilocus genotypes at two autosomal and two X-linked loci is $3^2 \times 2^2 = 36$. Expected quadrilocus genotype frequencies are the product of allele frequencies, assuming Mendelian inheritance and taking recombination fraction into account between the two markers on the X chromosome (which are by definition known to be physically linked). In order to avoid circularity, we used the recombination fraction calculated from the *S. miscanthi* data set to calculate expected frequencies of quadrilocus genotypes in *S.* near *fragariae* eggs and vice versa. This approach assumes that physical linkage is similar in these closely related species and data from cross-species amplification of X-linked and autosomal microsatellite loci are consistent with this assumption (Wilson *et al.*, 2004). Given that sexual reproduction in these clones is likely to be an unusual event, there exists an *a priori* expectation that processes deviating from 'normal' recombination and segregation may be operating. While the use of recombination fractions calculated from these data to predict expected outcomes is not the most desirable approach it is the best available.

Our approach to examining the significance of the observed quadrilocus genotype frequencies of the eggs was to use resampling procedures and permutation tests (Zar, 1996). We computed the expected frequencies of progeny with different genotypes as described above and converted the expected proportion of each genotype in a population into integers by multiplying the expected proportion by a constant based on the frequency of the rarest genotype. A simulated population of 800 individuals for *S.* near *fragariae* and 1002 for *S. miscanthi* was thus computed. We then sampled members of the population at random with replacement (32 times for the *S. miscanthi* eggs and 63 times for the *S.* near *fragariae* eggs, i.e. the number of eggs actually assigned quadrilocus genotypes in each species) and recorded the number of times that each genotype was sampled. We repeated this procedure 10 000 times and plotted the frequency distribution of the number of times a genotype was selected (expected genotype frequency). We then compared the observed frequency of a genotype with this expected frequency to assess whether the observed values were greater or less than the expected values ($P < 0{\cdot}05$). We executed the permutation tests using the software package Resampling Stats for Macintosh computers (http://www.resample.com/content/about.shtml).

Considering only those loci that were heterozygous in *Snf17* and *Sm195*, we counted the number of heterozygous loci for each egg that had been typed at two or more of the seven loci (*S. miscanthi*, $n = 59$; *S.* near *fragariae*, $n = 93$). Therefore, in accordance with Hardy–Weinberg equilibrium (HWE), offspring were expected to be heterozygous at half of the loci for which they had been scored. We converted observed and expected numbers of heterozygotes to integer values (by multiplying through by a factor of 2) and performed a Wilcoxon paired signed-rank test to test for a deviation between observed and expected heterozygosities for each species (Sokal & Rohlf, 1995). To ensure that this analysis was not biased by using individuals with only a few loci scored, we repeated it in the *S. miscanthi* eggs using only those eggs scored at all five loci and in the *S.* near *fragariae* eggs using only eggs scored at six and seven loci.

Offspring genotypes resulting from a cross of individuals heterozygous for the same two alleles (e.g. Aa × Aa) are expected to be in HWE. We examined deviations from HWE using the exact tests of GENEPOP (version 3.1d) on *S. miscanthi* and *S.* near *fragariae* eggs typed at all seven loci (*S. miscanthi*, $n = 29$; *S.* near *fragariae*, $n = 25$). As a familiar measure of deviation from HWE, we computed F_{IS} using a subset of eggs typed at all seven loci in GENEPOP (version 3.1d).

(vi) *Genotyping male* S. miscanthi *and* S. *near* fragariae

To investigate non-Mendelian patterns of inheritance in the eggs of intraclonal crosses, we genotyped 97 *S. miscanthi* clone *Sm195* males and 150 *S.* near *fragariae Snf17* males at multiple X-linked microsatellite loci to determine whether they were X_1 or X_2 males.

https://doi.org/10.1017/S0016672306008202 Published online by Cambridge University Press

Table 1. *Summary of sexual induction experiments* (*see Supplemental Information Table S2 for full details*)

Species	ID	2n	Genotype	No sexuals	Males	Sexual females	Fertilized eggs
S. miscanthi	*Sm67*	17	*Sm17.0*	⊙			
	Sm140	17	*Sm17.0*	⊙			
	SmBelA	20	*Sm20.0*	⊙			
	Sm57	20	*Sm20.0*	⊙			
	Sm99	20	*Sm20.0*	⊙			
	Sm177	20	*Sm20.0*	⊙			
	Sm180	21	*Sm21.0*	⊙			
	Sm192	21	*Sm21.0*	⊙			
	Sm195	18	*Sm18.1*		⊙	⊙	⊙
	Sm197	18	*Sm18.0*		⊙	⊙	⊙
	Sm4	19[a]	*Sm18.0*		⊙	⊙	⊙
S. near *fragariae*	*SnfSA2*	18	*Snf*		⊙	⊙	⊙
	Snf17	18	*Snf*		⊙	⊙	⊙

⊙, observation of indicated morph; 2n, diploid chromosome number (2n = 18 is the ancestral chromosome number for grass- and cereal-feeding *Sitobion*). Genotype refers to the multilocus microsatellite genotype of the clone. No sexuals means these clones are obligate parthenogens.
[a] This isofemale line was originally 2n = 18 when it was collected in 1986. Sometime during 1993 it mutated in culture and became fixed for an additional small extra chromosome fragment. For more details see Sunnucks *et al.* (1996).

Table 2. *Multilocus microsatellite genotypes of* S. miscanthi *isofemale line* Sm195 *and* S. *near* fragariae *isofemale line* Snf17

	Microsatellite locus						
	$Sm10^A$	$Sm17^A$	$S16b^A$	$Sm11^X$	$S10^X$	$S17b^X$	$S49^X$
Sm195	170:194	187:189	179:185	145:145	096:120	208:208	112:130
Snf17	157:161	174:178	202:206	156:160	092:102	232:267	163:173

Allele sizes are given in base pairs.

(vii) *X_1 and X_2 terminology*

It is not possible (and indeed probably meaningless) to designate homology between X_1 in *S.* near *fragariae* and X_1 in *S. miscanthi*. We use designation X_1 to refer to the empirically more favoured chromosome.

3. Results

(i) *Production of sexual forms*

The clones fell into two clear groups: those that produced sexual forms and those that did not (Table 1; for details see Supplemental Information Table S2). Clones that produced sexual morphs did so most rapidly and reliably at 15 °C, 8L : 16D. Under these conditions we found males in the cultures after approximately 4 weeks and sexual females and fertilized (black) eggs within 8–11 weeks. Both *S.* near *fragariae* isofemale lines, *Snf17* and *SnfSA2*, and all three isofemale lines of the *S. miscanthi* Sm18 complex (*Sm195*, *Sm197* and *Sm4*) produced males, sexual females and fertilized eggs (Table 1). The remaining isofemale lines, all *S. miscanthi* in the karyotypically derived Sm17/20/21 complex, never produced sexual forms (Table 1).

(ii) *Breeding experiments*

(a) *Part I: Population-pool matings*

Two of the five intraspecific, intraclonal population-pool matings yielded substantial numbers of black eggs. We genotyped a total of 65 *Snf17* × *Snf17* and 43 *Sm195* × *Sm195* eggs at between two and seven microsatellite loci (three autosomal and four X-linked). The multilocus genotypes of *S. miscanthi* clone *Sm195* and *S.* near *fragariae* clone *Snf17* are presented in Table 2. All X-linked loci in both species strongly and significantly deviated from Mendelian expectations ($P < 0{\cdot}001$; Fig. 1*A*, *B*). In addition, we observed a significant deviation from expectation in the *Snf17* × *Snf17* eggs at autosomal locus $S16b^A$ ($P < 0{\cdot}001$; Fig. 1*A*).

Only one of 72 *S.* near *fragariae* eggs (egg 008) inherited any paternal X_2 alleles: $Sm11^X$ X_2:X_2

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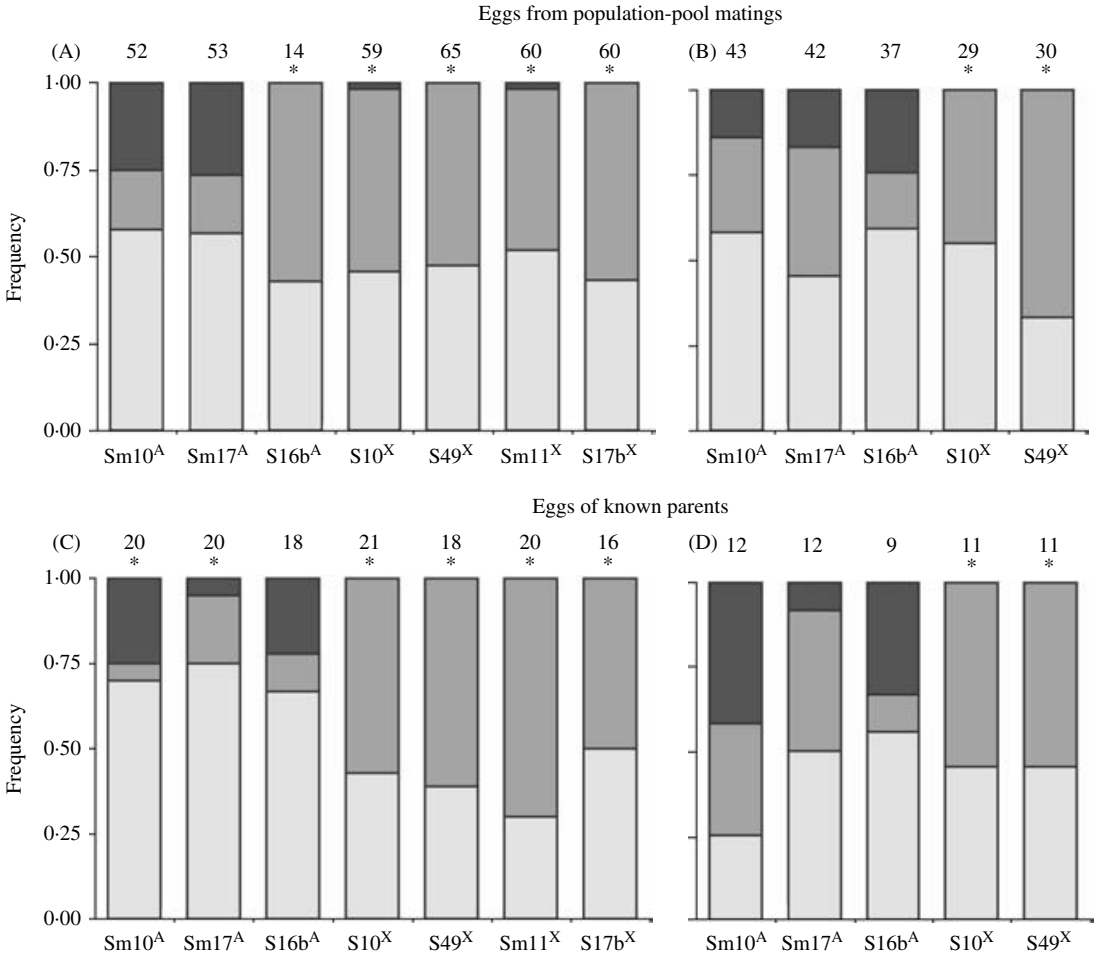

Fig. 1. Stack histograms of genotype frequency at individual loci. From bottom to top of the histogram bars: light grey, heterozygotes; mid-grey, homozygote allele 1; dark grey, homozygote allele 2. (*A*) Population-pool matings of *Snf17* × *Snf17* eggs. (*B*) Population-pool matings of *Sm195* × *Sm195* eggs. (*C*) Individual-pair matings *Snf17* × *Snf17* eggs. (*D*) Individual-pair matings of *Sm195* × *Sm195* eggs. Numbers above the stacks are the number of eggs typed at that locus. * indicates a significant deviation from expectation $P<0{\cdot}05$ or smaller. For: (*A*) *Snf17* × *Snf17* locus $Sm11^X$ $G=31{\cdot}57$, $S10^X$ $G=35{\cdot}89$, $S17b^X$ $G=48{\cdot}20$, $S49^X$ $G=47{\cdot}27$, $S16b^A$ $G=11{\cdot}38$; (*B*) $S10^X$ $G=18{\cdot}33$, $S49^X$ $G=31{\cdot}12$; (*C*) *Snf17* × *Snf17* locus $Sm11^X$ $G=22{\cdot}70$, $S10^X$ $G=17{\cdot}07$, $S17b^X$ $G=11{\cdot}09$, $S49^X$ $G=16{\cdot}15$, $Sm10^A$ $G=6{\cdot}20$, $Sm17^A$ $G=7{\cdot}16$; (*D*) $S10^X$ $G=8{\cdot}41$ and $S49^X$ $G=8{\cdot}41$ with two degrees of freedom.

(160bp:160bp), $S10^X$ X_2:X_2 (102bp:102bp), $S17b^X$ X_1:X_2 (232bp:267bp), $S49^X$ X_1:X_2 (173bp:163bp). None of the *S. miscanthi* eggs carried any paternal X_2 alleles.

(b) *Part II: Individual-pair matings*

We attempted 61 individual matings; 53% of them were interspecific matings, and the remainder intraspecific (Supplemental Information Table S3). Of the 29 intraspecific matings, 55% were within *S.* near *fragariae* isofemale line *Snf17* and 45% within *S. miscanthi* isofemale line *Sm195*. No interspecific pairing resulted in fertilized eggs. Of the 29 intraspecific attempted matings, 9 (31%) yielded black eggs, represented by 5 of 16 matings from clone *Snf17* and 4 of 13 from clone *Sm195*. We genotyped black eggs from all successful individual-pair matings at up to seven microsatellite loci. Again, we found strong and significant deviations from Mendelian expectations at all X-linked loci in both species ($P<0{\cdot}025$ to $P<0{\cdot}001$; Fig. 1*C*, *D*). Additionally, we found small but significant deviations from expectation in the *Snf17* × *Snf17* eggs at autosomal loci $Sm10^A$ and $Sm17^A$ ($P=0{\cdot}05$). In contrast to the result in population-pool eggs (above), we did not find deviations from Mendelian expectations at locus $S16b^A$.

We used 32 males in the 61 attempted matings. Of these males, 25 were *S.* near *fragariae* clone *Snf17* and seven were *S. miscanthi* clone *Sm195*. In our sample of *S.* near *fragariae* clone *Snf17* males, X_1 and X_2 males were equally represented (14 X_1 males and 9 X_2 males (2 unknown); $G=1{\cdot}09$, df = 1, $P>0{\cdot}05$). In contrast, all seven *S. miscanthi Sm195* males were X_1.

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Table 3. *Multilocus genotypes that were significantly over-represented in eggs of the intraclonal matings*

Species	Genotype	No. of individuals	$Sm10^A$	$Sm17^A$	$S10^X$	$S49^X$
S. near *fragariae*	SNF1	5	161:161	174:178	092:102	173:173
	SNF2	10	157:161	174:178	092:102	173:173
	SNF3	8	157:161	174:178	092:092	173:173
S. miscanthi	SM1	3	194:194	187:189	096:120	130:130

In *S.* near *fragariae*, X_1 and X_2 males were similarly represented in all attempted matings, yet no X_2 male yielded any fertilized eggs. This result is consistent with the population-pool matings. However, it is possibly premature to conclude that X_2 males were inviable, because they represented only 2 of 16 of the male parents of intraspecific attempted matings (this was the only successful type of mating; see above). Both these X_2 males had only one opportunity to mate since both were dead when we checked them 48 h after establishment in their first pairings. Over all attempted matings, X_1 and X_2 *S.* near *fragariae* males were equally represented, yet we found significant under-representation of X_2 males in attempted paired intraspecific matings ($G = 17{\cdot}16$, $P < 0{\cdot}001$). We could not correct for this disparity at the time of the experiments because the X type of males could not be known until after all matings were complete. This under-representation may be stochastic variation coincidentally in the direction that X_2 males are inviable, or it may reflect lower fitness, vigour or longevity of X_2 males. Thirty-six per cent of the 13 *S.* near *fragariae* intraclonal attempted matings we established with an X_1 male were successful.

(iii) *Common patterns between population-pool and individual-pair experiments*

When all breeding experiments are considered, only one of the 143 eggs genotyped was sired by an X_2 male (*S.* near *fragariae*, egg 008). We observed deviations from Hardy–Weinberg expectations at some autosomal loci in *S.* near *fragariae* but found no common pattern of these deviations between the two experiments.

(iv) *Genotypic analysis*

Thirty-six quadrilocus genotypes are expected for each of the two crosses (see Section 2 for assumptions). In a sample of 64 *Snf17* × *Snf17* eggs typed over four loci ($Sm10^A$, $Sm17^A$, $S10^X$ and $S49^X$), we observed only 24 different quadrilocus genotypes: 12 were missing. Of the 24 seen, we sampled 14 more than once, and one 10 times. In 32 *Sm195* × *Sm195* eggs, we observed 20 different quadrilocus genotypes, seven of these more than once.

In the *Snf17* × *Snf17* eggs we found three genotypes, 36% of the 64 eggs sampled, to be significantly over-represented. These three multilocus genotypes, SNF1, SNF2 and SNF3, are listed in Table 3. All three genotypes are heterozygous at $Sm17^A$, homozygous for the X_1 allele at $S49^X$ and are missing part (genotypes SNF1 and SNF2) or all (genotype SNF3) of the X_2 parental chromosome. This pattern of tendency to homozygosity at the apparently favoured X chromosome is mirrored in *Sm195* × *Sm195* eggs where all four genotypes represented three or more times are X_1 homozygotes at locus $S49^X$ and three of the four are also X_1 homozygotes at locus $S10^X$. Of the 83 *Snf17* × *Snf17* eggs we typed at locus $S49^X$ (not all these eggs are represented in the quadrilocus set because some egg genotypes had been incompletely characterized) 38 are 163:173 heterozygotes and 45 are 173:173 homozygotes. This result does not represent a deviation from the null 1:1 expectation ($G = 0{\cdot}59$, $P > 0{\cdot}5$). However, all 23 eggs of the three over-represented genotypes are 173:173 homozygotes ($G = 31{\cdot}88$, $P < 0{\cdot}001$). We do not find similar deviations from 1:1 expectations at the other three heterozygous parental X-linked loci in these 23 *Snf17* × *Snf17* eggs.

Only one of the four *Sm195* × *Sm195* egg multilocus genotypes represented three or more times is significantly over-represented in the sample (SM1, seen four times, $P = 0{\cdot}048$; Table 3). Testing for a deviation from expectations in this sample of *Sm195* × *Sm195* eggs is hampered by the small sample size compared with the number of genotypic classes. However, as for the over-represented *S.* near *fragariae* genotypes, all four genotypes represented three or more times in the *S. miscanthi* sample are missing all or part of the X_2 chromosome and three of the four, including the significantly over-represented SM1, are heterozygous at locus $Sm17^A$.

(v) *Hardy–Weinberg equilibrium and heterozygosity*

We did not find any significant deviations from Hardy–Weinberg expectations in either the *Snf17* × *Snf17* eggs or the *Sm195* × *Sm195* eggs. Additionally, we found no significant difference between observed and expected heterozygosities of individual egg genotypes over the five loci for which *S. miscanthi*

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isofemale line *Sm195* is heterozygous ($P=0{\cdot}95$) or over the seven loci for which *S.* near *fragariae* isofemale line *Snf17* is heterozygous ($P=0{\cdot}82$). The exclusion of eggs scored at a low number of loci in this test did not alter the result (*Sm195* eggs $P=0{\cdot}63$, *Snf17* eggs $P=0{\cdot}34$).

Whilst all F_{IS} values were non-significant, with the exception of the *Sm195* × *Sm195* eggs at locus Sm17 ($F_{IS}=+0{\cdot}138$), all loci in both species had negative F_{IS}, indicative of higher than expected levels of heterozygosity. This result (11 negative F_{IS}, 1 positive F_{IS}, against an expectation of equality) indicates a highly significant ($G=9{\cdot}75$, $P<0{\cdot}005$) trend towards higher than expected levels of heterozygosity.

(vi) *X chromosome elimination at male production in clones* Sm195 *and* Snf17

Consistent with an earlier study, we found no significant deviations from equal representation of X_1 and X_2 males in *S.* near *fragariae* clone *Snf17* (Wilson *et al.*, 1997). Of the 150 males we genotyped, 81 were X_1 males and 69 were X_2 males ($G=0{\cdot}961$, $P>0{\cdot}05$). However, all 97 *S. miscanthi* clone *Sm195* males genotyped were X_1. We have never found an X_2 *Sm195* male.

4. Discussion

(i) *Permanently parthenogenetic lineages of Australian Sitobion have derived karyotypes where lineages that retain the ability to reproduce sexually have normal karyotypes*

In earlier studies we have reported an absence of sexual reproduction in Australian and New Zealand *Sitobion* aphids (Sunnucks *et al.*, 1996; Wilson *et al.*, 1999). In Wilson *et al.* (1999) we proposed three (non-exclusive) explanations to account for the observed absence of sex in New Zealand *Sitobion*: (1) that New Zealand *Sitobion* aphids are obligately parthenogenetic, (2) that local conditions are not sufficiently extreme to produce sexual forms and (3) that sexually recombined genotypes are produced but fail to persist. The first explanation, that the clones are obligately parthenogenetic having genetically lost the ability to reproduce sexually, reasonably accounts for the absence of sexual reproduction in the karyotypically derived lineages of *S. miscanthi* in Australia. This result is not surprising: obligate asexuality is frequently associated with karyotypic rearrangements in aphids (Blackman, 1980; Normark, 1999). However, while the karyotypically derived lineages failed to produce sexuals under a range of conditions, including conditions that are more rigorous (at least with respect to scotoperiod) than those experienced by aphids under field conditions in Australia, the karyotypically normal lineages of both *S. miscanthi* and *S.* near *fragariae* were able to produce sexual morphs and mate successfully. Thus, in karyotypically normal *Sitobion* aphids in Australia, the absence of sexual reproduction results either from failure of the synchrony of sexual induction cues so that any investment in sexual reproduction fails, or from an absence of sufficiently extreme thermal and scotoperiodic conditions.

(ii) *Genetic markers show strong and significant deviations from Mendelian expectations in sexually produced eggs*

The most extreme patterns of deviation from Mendelian expectations in the sexually produced eggs of *S. miscanthi* and *S.* near *fragariae* intraclonal crosses result from only one type of male siring progeny in each of the isofemale lines used in these experiments. Sex determination in aphids is chromosomal; females are XX and males, which are produced by parthenogenesis, are XO. Thus, a given female (or isofemale line) can produce two types of males: X_1 and X_2. With one exception (*S.* near *fragariae* egg 008), all eggs were sired by only one of the two possible male types within each of the two intraclonal crosses. Both X_1 and X_2 males are equally represented in *S.* near *fragariae* lines (Wilson *et al.*, 1997 and these data), whereas no X_2 *S. miscanthi* clone *Sm195* male has ever been identified.

(iii) *Sexual reproduction results in an excess of heterozygotes at almost all loci in both functionally parthenogenetic species*

With the exception of locus *Sm17* in the *Sm195* × *Sm195* eggs, all loci showed an excess of heterozygotes. This observation is counter to expectation. Spermatogenesis in aphids is achiasmate (Sloane *et al.*, 2001; Hales *et al.*, 2002*a*) and therefore males pass on unrecombined chromosomes. Sexual oogenesis in aphids results in high levels of recombination (Sloane *et al.*, 2001). Thus in the case of these intraclonal crosses, depending on which chromosome homologues separate into a given sperm and the frequency of recombination at oogenesis, potentially large portions of the genome of a sexually produced egg will be homozygous. In this way, homozygosity is expected to increase in the sexually generated progeny of an intraclonal cross. But this is not what we observed, suggesting that either segregation is non-random in the aphid lineages studied or that there are fitness benefits to heterozygosity for these lineages, presumably through overdominance at one or more loci or selection against accumulated deleterious mutations.

(iv) *Over-representation of certain multilocus genotypes*

Whilst it is important to look at these data on a locus-by-locus basis, much can be gained by examining the multilocus genotypes of individual eggs. For example, there is clear evidence in these data of over-representation of certain genotypes. That some of these results represent highly significant deviations from expectations suggests that some multilocus genotypes may be fitter than others. Additionally by looking at multilocus genotypes over markers that are known to be physically linked, for example the X-linked loci, it is possible to examine the type and frequency of recombination between loci and see whether processes deviating from normal are detected (although the effect of epistatic selection is difficult to quantify). Female aphids experience high levels of recombination during meiosis, whereas male meiosis is achiasmate (Sloane *et al*., 2001).

The data indicate that the most important factor determining viability is the absence of the X_2 chromosome, or at least a section of it carrying $S49^X$. All four significantly over-represented quadrilocus genotypes (Table 3) are X_1X_1 homozygotes for $S49^X$ and none of the over-represented genotypes are the maximally heterozygous parental genotypes.

(v) *Interpreting non-Mendelian patterns of inheritance following sexual reproduction in a functional parthenogen*

This study has revealed several quite unexpected patterns of microsatellite marker inheritance in a functional parthenogen. First, progeny in each of the two intraclonal crosses examined were sired by only one of the two possible male X chromosome types. In the case of *S. miscanthi* line *Sm195* this result was explained by the fact that only one type of male was produced by this line. In earlier work, we had addressed the question of whether X chromosome loss at male production is random with respect to the identity of the X chromosome (Wilson *et al*., 1997). That study examined 25 males of the same *S.* near *fragariae* isofemale line used in these experiments, *Snf17*, and found that the two X chromosomes have an equal chance of being retained during male oocyte formation. In this study we screened another 150 males from *Snf17* and found, consistent with our earlier result, equal representation of both X chromosomes in those males. If *Snf17* males are equally represented, why does only one type of male sire all the progeny? The outcome is the same in both species: only one type of male sires the progeny. However, it is unclear whether this unexpected outcome of sexual reproduction results from the same mechanism in both species. The second type of unexpected pattern of microsatellite marker inheritance in these crosses was revealed most clearly by studying the over-representation of some genotypes in the progeny of the crosses. In both species, alleles associated with the X_2 chromosome are found at lower frequency than expected in over-represented progeny and, in particular, locus $S49^X$ appears to be closely linked to a region of the X_2 chromosome that has been strongly selected against. These were the most striking and consistent patterns of non-Mendelian inheritance in the crosses examined in this study but not the only ones. For example, non-Mendelian patterns of inheritance were also observed at autosomal loci S16b, Sm10 and Sm17 in some *Snf17* × *Snf17* eggs.

Non-Mendelian patterns of inheritance can result from a variety of intrinsic and extrinsic genetic processes. Intrinsic genetic processes are those influenced by the genome of the organism in question and include processes such as genetic imprinting, mutation (both karyotypic and genic) and meiotic drive. Extrinsic genetic processes, for example cytoplasmic incompatibility, result from an antagonistic interaction between the genome of the study organism and the genomes of other organisms; in the case of aphids these would include bacterial endosymbionts and pathogens. Strong biases in the transmission of X chromosomes have been described in only one other aphid species, *Rhopalosiphum padi* (Frantz *et al*., 2005). Reciprocal crosses between two cyclically parthenogenetic lineages and two X-linked microsatellite markers were used to examine the transmission of X chromosomes at all life stages through a complete reproductive cycle (including both sexual and asexual phases). Patterns of non-Mendelian inheritance of X chromosomes in males were detected. One parental line (Lineage B in Frantz *et al*., 2005), like *S. miscanthi* lineage *Sm195*, produced only one type of male, where the other parental line, like *S.* near *fragariae* lineage *Snf17*, produced two types of male in equal proportions. In the case of the line that produced both types of males, one of the two male types sired only 9% of the total progeny of that cross (a highly significant deviation from equal representation). Frantz *et al*. (2005) also examined X chromosome elimination at male production in the new hybrid lineages they generated from the lineage A × lineage B crosses and they found under-representation of three of 10 X chromosomes in those lines. It is noteworthy that all three of these X chromosomes were of maternal origin and so had been through a recent bout of sex and recombination (Sloane *et al*., 2001).

(vi) *Low mating success: the result of inbreeding depression?*

The low mating success of each of the two attempted intraspecific matings is consistent with observations

https://doi.org/10.1017/S0016672306008202 Published online by Cambridge University Press

in *Sitobion avenae*, *Schizaphis graminum* and *Acyrthosiphon pisum* of inbreeding depression in intraclonal crosses (Puterka & Peters, 1989; Via, 1992; Helden & Dixon, 1997; Dedryver *et al*., 1998). Dedryver *et al*. (1998) report strong inbreeding depression in *S. avenae* self-crosses. Their experimental design did not allow for assessment of individual mating success, but they report lower fertility in sexual females, lower egg hatching success and lower survival and fecundity of the foundresses. Similarly, high levels of egg mortality dependent on reciprocal cross directionality was also reported by Frantz *et al*. (2005) in *R. padi*. Frantz *et al*. (2005) suggested that asymmetry in egg hatching rates of reciprocal crosses resulted from differential hybrid viability resulting from nuclear–cytoplasmic incompatibilities. We note that nuclear–cytoplasmic incompatibilities could not affect our crosses because they were intra-clonal and thus did not involve different cytoplasmic genomes.

(vii) *In summary*

Our unexpected observations of strong biases in X chromosome transmission through the sexual phase of the life cycle in facultatively parthenogenetic lineages of two species of *Sitobion* aphids is very similar to the patterns observed in cyclically parthenogenetic lines of *R. padi* (Frantz *et al*., 2005). It is becoming increasingly clear that these patterns are likely to be very widespread in aphids. The mechanism(s) underlying them is not clear. Frantz *et al*. (2005) posed two alternative hypotheses to explain biases in X chromosome transmission in *R. padi*. The first, that strong biases in X chromosome transmission are the result of mutation accumulation during bouts of asexual reproduction, seems the more plausible and results in a testable prediction: X chromosome transmission bias will increase with time spent in the asexual phase owing to the cumulative probability of mutations that are severely deleterious or lethal to their hemizygous male bearers occurring on one of the X chromosomes. (Note that this expectation may be undermined after such time when severely deleterious or lethal mutations accumulate on both X chromosomes, at which point the clone would cease producing males: see Section 1.) Given the natural variation in life cycle strategy that occurs within aphid species, these insects provide an ideal system in which to test this hypothesis. Under the mutation accumulation hypothesis, deviations from equal representation in male aphids should be most extreme in the least sexual aphid life cycle classes (male-producing obligate parthenogens) and absent in those lines that experience regular bouts of sexual reproduction (cyclical parthenogens).

The second hypothesis proposed by Frantz *et al*. (2005), genetic imprinting of paternal X chromosomes, results in the accumulation of genes favouring male function. Since females experience genetic recombination at oogenesis but not at parthenogenetic egg production (Sloane *et al*., 2001; Hales *et al*., 2002*b*) and males are produced exclusively by parthenogenesis, long-term survival of a given X chromosome is possible. Furthermore, paternal chromosomes passed to sons do not experience recombination, and thus male-favouring X chromosome lineages become possible (Frantz *et al*., 2005). Thus X chromosomes acquiring mutations favourable to sons may be inherited disproportionately commonly, and any such chromosomal lineages would experience frequent purifying selection in the hemizygous males. Another possibility, not raised by Frantz *et al*. (2005), is that these patterns, which have now been reported in two aphid species, result from the action of meiotic drive and modifier genes.

The data presented here add to those of Frantz *et al*. (2005) in suggesting that the phenomenon of biased X chromosome inheritance in male aphids may be quite general and that the somewhat peculiar genetic system of aphids may have predisposed them to the possibility of evolving specialized male-adapted X chromosomal lineages, or that the aphid genetic system may be an end point in the evolution of a complex system of drive and modifier genes. The puzzle is far from solved; further studies are required to determine the mechanism(s) underlying biases in X chromosome transmission in aphid populations and their evolutionary significance.

We are very grateful to Mike Ramsey for running the simulations. Wirt Atmar, Jean-Christophe Simon and Fabien Halkett have contributed significantly to our thinking on this subject. This research was supported by Australian Research Council and Macquarie University Research Grants to P.S. and D. F. Hales. During writing A.C.C.W. was funded by the Center for Insect Science (NIH Training Grant #1 K12 Gm00708).

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