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SHORT PAPER

Random loss of X chromosome at male determination in an aphid, *Sitobion* near *fragariae*, detected using an X-linked polymorphic microsatellite marker

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Summary

This paper reports the first direct molecular evidence that X chromosome loss during determination of male aphids (XO) is random. Clonal and sexual females, and males, of the species *Sitobion* near *fragariae* were screened using three polymorphic microsatellite loci. Two loci, Sm10 and Sm17, showed the same heterozygous genotypes in all three aphid morphs. The third, Sm11, was heterozygous for the same two alleles in clonal and sexual females, but of the 25 males screened 11 showed the '160' allele and 14 showed the '156' allele. This result indicates X-linkage of locus Sm11, with random loss of the X chromosome during the formation of male embryos. The possible implications of this result are discussed with respect to aphid sex determination, recombination and chromosome evolution.

1. Introduction

Reproduction in Aphididae encompasses a large range of strategies, including obligate apomictic parthenogenesis, and a facultative yearly sexual generation with one to many intervening parthenogenetic generations (for review see Hales *et al.*, 1997). Sex determination in most aphids is XX/XO. All aphid zygotes are originally XX female, but some become male on receiving only one X chromosome during division of an oocyte in a parthenogenetic female (Orlando, 1974; Blackman & Hales, 1986). Multiple X chromosome systems are also known in aphids (Blackman, 1995). There are still many unanswered questions about chromosomal behaviour during male meiosis and male determination in aphids (see above references). Male meiosis in aphids is peculiar in that only those autosomes that associate with an X chromosome survive and the set of autosomes lacking an X chromosome receives little cytoplasm and does not form a viable spermatocyte (for review of early work see Blackman, 1987). Factors determining which autosomes become associated with an X chromosome are not known. Even during parthenogenetic reproduction, in which oogenesis is essentially mitotic, observations of chromosome behaviour suggest the possibility of X chromosome recombination (Blackman & Hales, 1986; Blackman & Spence, 1996), but this has not been tested with genetic data.

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The Coccoidea (scale insects, mealybugs) constitute the sister group to the aphids within the Suborder Sternorrhyncha of the Order Hemiptera (Moran *et al.*, 1994). There is a wide range of bizarre cytological events in different coccoid families, often including a form of imprinting of the paternal chromosomes such that they are inactive. For example, in lecanoid scale insects, the paternal chromosomes become heterochromatic in male embryos at the blastoderm stage, and are ultimately eliminated during spermatogenesis at the second meiotic division, so that males pass on only those chromosomes received from their mothers. In diaspidid scale insects, the whole paternal set of chromosomes is completely eliminated in male embryos at late cleavage (White, 1973).

Crema (1981) found an approximately equal ratio of aborted eggs to viable male eggs in *Megoura viciae* and suggested that it was attributable to a recessive lethal gene on one of the X chromosomes. Further, Smith & MacKay (1989) inferred that the ratio of 1:1 winged to wingless phenotype in males of *Acyrthosiphon pisum* resulted from a sex-linked gene. Their data suggested that X chromosome elimination was random with respect to chromosomal identity. However, wing phenotype is a highly labile feature in aphids, responding to numerous environmental variables acting in late embryonic or early larval development (reviewed by Hille Ris Lambers, 1966; Lees, 1966; Hales, 1976; Kawada, 1987), and it is thus desirable to test for equality of elimination of each X chromosome using appropriate molecular markers.

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It cannot be assumed that there is an equal probability of elimination of either of the X chromosomes at male determination in aphids, nor of the elimination of either set of autosomes at male meiosis. We have previously attempted to address the former issue using allozymes, but did not find a polymorphic sex-linked marker in any species studied (D. F. Hales, unpublished data). The discovery of an X-linked microsatellite has provided us with a molecular marker of suitable resolution to study the probability of elimination of X chromosomes in aphids at male determination.

Microsatellites are co-dominant, single-locus markers which exhibit a large range of genetic diversity and are relatively neutral to selection (Bruford & Wayne, 1993; Hughes & Queller, 1993; Estoup *et al.*, 1995; England *et al.*, 1996). They provide powerful tools for linkage and population studies. X-linked microsatellites have previously been reported in other insects including *Drosophila melanogaster* (Goldstein & Clark, 1995; England *et al.*, 1996) and a mosquito, *Anopheles gambiae* (Lanzaro *et al.*, 1995).

Sitobion near *fragariae* is a grass aphid thought to have arrived in Australia within the last 50 years, and is now common in south-eastern Australia. It is morphologically similar, but not identical, to *S. fragariae* (Walker) from the Holarctic region. Molecular evidence also supports a close relationship of this species with *S. fragariae* (Sunnucks & Hales, 1996).

Males, but not sexual females, of *S.* near *fragariae* have been reported in field collections in Australia (M. Carver, personal communication). These aphids under field conditions appear to be behaving as obligate parthenogens: we have used microsatellites to demonstrate the absence of genetic recombination in over 150 Australian *S.* near *fragariae* from diverse collections (Sunnucks *et al.*, 1996, and unpublished data). However, under laboratory conditions we have induced a clone of *S.* near *fragariae* to produce males and sexual females (present study).

The cloning of microsatellites has given us the tools to investigate X-linkage and, further, we have analysed sufficient males to determine whether the elimination of the X chromosome is random at male determination. We are not aware of previous reports of X-linked microsatellites (or indeed of any highly variable X-linked single loci) in aphids. The relevance of our study to recently published work concerning rDNA (Blackman & Spence, 1996), and the observations of 'aborted' embryos during the transition to male production (Crema, 1981; Searle & Mittler, 1981) is discussed. We show that microsatellite markers should be valuable in elucidation of cytogenetic observations of aphid male meiosis, and of possible recombination between X chromosomes during parthenogenetic and sexual reproduction.

2. Materials and methods

A single clone (clone 17) of *Sitobion* near *fragariae* originating from Tasmania was raised on barley seedlings in pots in a temperature-controlled room at 15 °C and an 8 h light/16 h dark photoperiod. Under these conditions parthenogenetic winged and wingless females, sexual females and males were produced. Sexual females and males were stored in 70% ethanol. (Under the same conditions, clones of *S. miscanthi*, another aphid of grasses and cereals in Australia, did not produce sexual forms.)

DNA from individual aphids was extracted using a 'salting-out' protocol (Sunnucks & Hales, 1996) and dissolved in 20 μl of TE Buffer (1 mM EDTA, 10 mM Tris base pH 7·5). Microsatellites were cloned and developed from *Sitobion miscanthi* as described in Sunnucks *et al.* (1996). The sequence of primers for loci Sm10 and Sm17 will be published elsewhere, but in the meantime may be requested from the authors. The sequences of primers to amplify locus Sm11 were (5′ to 3′): Sm11F aac cct acg ggt aac gcc ($-T_m =$ 59·8 °C) and Sm11R ggt acc cct atg tta tta cgc g ($-T_m = 59·7$ °C).

Microsatellite polymerase chain reaction (PCR) was carried out in 10 μl reaction volumes with 0·1 μl of *Taq* polymerase (0·5 units, Promega), Mg^{2+}-free reaction buffer, 2 mM $MgCl_2$, 200 μM dGTP, dCTP and dTTP, 20 μM dATP, 5–10 pmoles of primers, 5–50 ng DNA (typically 1 μl as prepared above) and 0·05 μl of [α^{33}P]dATP (10 mCi/ml; Bresatec). The PCR cycling conditions for locus Sm11 were: 94 °C for 3 min, four cycles of 'touch-down' (annealing first at 55 °C for 30 s and decreasing for the next three cycles (53 °C, 51 °C, 49 °C), extending at 72 °C for 45 s, denaturing at 94 °C for 15 s) and then cycling for 29 cycles, annealing at 47 °C, with a final extension at 72 °C for 2 min. For Sm10 and Sm17, all annealing temperatures were increased by 8 °C. Four microlitres of each reaction were run on a 6% acrylamide sequencing gel using the positive control M13 DNA of the Sequenase version 2.0 kit (United States Biochemicals) as a marker ladder for the precise sizing of alleles. Reference DNA can be obtained from the authors where confirmation of allelic length identity is necessary.

Lengths of chromosomes in air-dried preparations (Hales & Lardner, 1988) from *Sitobion* clones were measured from photographic prints using vernier calipers, and X chromosome length as a percentage of haploid genome length in each chromosome set was calculated ($n = 8$; 3 *S.* near *fragariae* and 5 *S. miscanthi*).

The G-test for goodness of fit (Sokal & Rohlf, 1995) was used to test the observed allelic frequencies in males at locus Sm11 against equal hemizygous frequencies predicted if each maternal X chromosome has a 50% chance of being observed in a given male offspring.

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3. Results

Six sexual females of *S.* near *fragariae* clone 17 screened using the microsatellite loci all had, as expected, genotypes identical to those found in 121 parthenogenetic females of the same species screened during a previous study (Sunnucks *et al.*, 1996) (Sm10 alleles 157/161; Sm11 156/160; Sm17 174/178). Twenty-five males of Clone 17 showed genotypes at loci Sm10 and Sm 17 identical to those of the parthenogenetic and sexual females. At locus Sm11 each male showed either allele 160 or allele 156 (Fig. 1). Of the 25 males screened, 11 had only allele 160 and 14 had only allele 156 (no significant deviation from a 1:1 ratio of alleles 160 and 156; $G = 0{\cdot}362$, $P > 0{\cdot}5$).

The X chromosome in *Sitobion* aphids represents 28% (± 3%, $n = 8$) of the total length of the haploid genome.

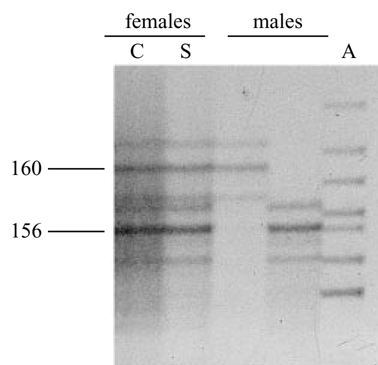

Fig. 1. Autoradiograph showing phenotypes at locus Sm11 in clonal ('C'), sexual ('S') females and males. Allele sizes are marked in base pairs; M13 DNA in lane A was the size marker ('A').

4. Discussion

The present data indicate that microsatellite locus Sm11 is sex-linked in *S.* near *fragariae*, and that, during the formation of an oocyte that will give rise to a male, the two X chromosomes have an equal chance of being retained. All parthenogenetic and sexual females (XX) were found to be heterozygous for alleles 156/160 of locus Sm11, and all males (XO) were found to carry only a single allele, 156 or 160, in proportions that did not differ significantly from equality. It is unlikely that this result could be due to recombination of autosomes during the formation of males, because we would then expect to find some heterozygous males and did not. Also there is no credible cytological evidence that such recombination occurs (references in Hales *et al.*, 1997).

We found no evidence of imprinting of chromosomes in aphids at male determination, inasmuch as neither the maternal nor the paternal X chromosome was selectively eliminated. In contrast, in the closely related scale insects, paternal chromosomes are inactivated or eliminated in early embryonic development of males: a clear indication of imprinting. This difference in imprinting may be related to differences in the reproductive modes of the two groups. Sexually reproducing scale insects have males in every generation, while many aphids, including *S.* near *fragariae*, may have many parthenogenetic generations between the zygote and the next production of males and sexual females. Possibly imprinting does not efficiently penetrate through many asexual generations. However, random elimination of X chromosomes at sex determination in aphids does not preclude some form of expressional imprinting of at least some genes on X chromosomes or autosomes. The possibility of paternal imprinting having effects several generations later could provide an interesting field for further research.

Recently, Blackman & Spence (1996) observed that aphids which have lost the sexual phase of their annual cycle frequently have multiple rDNA copies concentrated on one X chromosome; they suggested that such a situation could result from recombination between the X chromosomes during parthenogenesis. Further, they investigated the ability of these aphids to produce males. They found that the loss of rDNA arrays from one of the X chromosomes did not prevent the formation of males in a clone of *Acyrthosiphon pisum*. However, if an X chromosome passes into males independently of its ancestry, 50% of male embryos will be deficient in rDNA and should therefore be non-viable. Male-producing aphids are frequently observed to carry 'aborted' embryos amongst their developing embryos (reviewed in Blackman, 1987). Crema (1981; cited in Blackman, 1987) observed in a strain of *Megoura viciae* that the numbers of aborted eggs and viable male eggs were in equal proportion. Given the 1:1 association of aborted and viable male embryos we propose that these aborted embryos may have inherited an X chromosome with insufficient rDNA. Initial development of these embryos may occur by use of maternal ribosomes: aphid ovaries are telotrophic, allowing transport of maternal materials down a nutritive cord to embryos in the second or third position from the germarium.

Being XO, aphid males provide the opportunity to investigate chromosomal haplotypes (linkages) at multiple loci. We found one X-linked microsatellite out of three; since X chromosomes represent 28% of the genome in our sample of *Sitobion* aphids, it seems likely that more X-linked microsatellites can be found at about the same rate. It will then be possible to test the important and outstanding issues of X chromosome recombination in sexual and asexual aphids (see Blackman, 1987; and review in Hales *et al.*, 1997).

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