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The cytogenetic architecture of the aphid genome

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ABSTRACT

In recent years aphids, with their well-defined polyphenism, have become favoured as model organisms for the study of epigenetic processes. The availability of the pea aphid (*Acyrthosiphon pisum*) genome sequence has engendered much research aimed at elucidating the mechanisms by which the phenotypic plasticity of aphids is inherited and controlled. Yet so far this research effort has paid little attention to the cytogenetic processes that play a vital part in the organisation, expression and inheritance of the aphid genome. Aphids have holocentric chromosomes, which have very different properties from the chromosomes with localised centromeres that are found in most other organisms. Here we review the diverse forms of aphid chromosome behaviour that occur during sex determination and male and female meiosis, often in response to environmental changes and mediated by endocrine factors. Remarkable differences occur, even between related species, that could have significant effects on the inheritance of all or parts of the genome. In relation to this, we review the particular features of the distribution of heterochromatin, rDNA genes and other repetitive DNA in aphid chromosomes, and discuss the part that these may play in the epigenetic modification of chromatin structure and function.

Key words: holocentric chromosomes, meiosis, parthenogenesis, heterochromatin, aphids.

CONTENTS

I. INTRODUCTION

Aphids are small plant-sucking insects, many of which are very important as agricultural pests, not only in view of their direct parasitic action are active vectors of crop viruses (Blackman & Eastop, 2000, 2006, 2007; van Emden & Harrington, 2007). Their unique biology, involving cyclical parthenogenesis, complex life cycles and environmentally determined polymorphism (= polyphenism), has for a long time attracted much interest and intensive research, but progress in genetic studies of aphids was impeded for many years due to difficulties in breeding them through the sexual phase. The rapid advances in the acquisition and analysis of genetic data in recent years have meant that many of the problems of studying the population genetics and quantitative genetics of aphids can now be resolved or circumvented, and the sequencing of the pea aphid *Acyrthosiphon pisum* (Harris) genome (International Aphid

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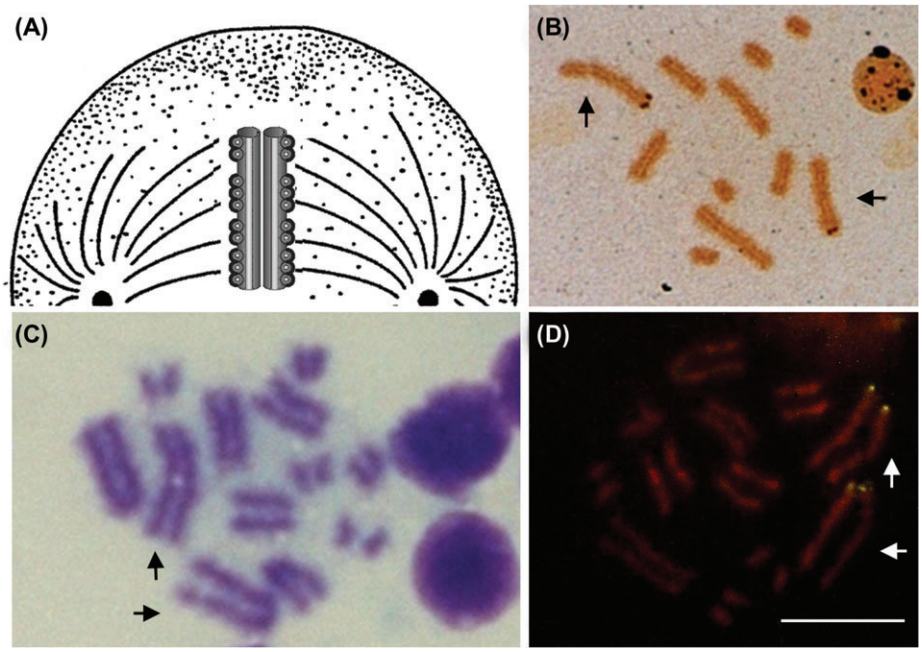

Fig. 1. Schematic representation (A) and preparations of aphid chromosomes stained with silver (B) and Giemsa (C), and hybridised *in situ* to a 28S rDNA probe (D). Aphid chromosomes do not have a localized centromere, but centromeric proteins (represented by circles in A) are localized in the outer portion of chromatids that run parallel without any point of intersection. Arrows indicate the X chromosomes. Scale bar, 10 μm.

Genomics Consortium, 2010) has brought us closer to an understanding of some of the unique functional aspects of aphid genetics, such as the genetic basis of phenotypic plasticity and interactions with endosymbionts (Srinivasan & Brisson, 2012).

For cytogeneticists, aphids also provide an interesting model because they possess holocentric chromosomes, showing centromeric activity along the whole chromosomal axis (Hughes-Schrader & Schrader, 1961; Blackman, 1987*b*). These chromosomes are also termed holokinetic because, during mitotic anaphase, they behave as if the spindle attachment is not localized, so that chromatids move apart in parallel and do not form the classical V-shaped figures usually observed during the movement of monocentric chromosomes (Fig. 1). Groups of organisms with holocentric chromosomes are scattered among the plant and animal kingdoms and are therefore thought to result from convergent evolution (Dernburg, 2001; Mandrioli & Manicardi, 2012; Melters *et al.*, 2012; Heckmann & Houben, 2013). Despite their radically different structure and frequent occurrence in animals and plants, holocentric chromosomes have not been intensively studied, with the exception of the nematode *Caenorhabditis elegans* (Maupas) (for a review see Maddox *et al.*, 2004). The paucity of studies has led to some misleading generalisations in the earlier literature about the structure, function and behaviour of holocentric chromosomes based on very little work on very few species. There is indeed a tendency to assume that basic cytogenetic processes that are fundamental to all eukaryotes, such as those involved in sex determination, spermatogenesis and oogenesis, must be evolutionarily conserved. On the contrary, organisms with holocentric chromosomes exhibit a remarkable diversity of cytogenetic processes. Even sister groups of Hemiptera (such as aphids and scale insects) have a variety of different systems, with variation both within and between these groups (Nur, 1980; Blackman, 1987*b*).

Mitotic chromosomes can easily be obtained from aphid embryonic tissues, so that they are a suitable model for understanding the structure and evolution of holocentric chromosomes and for disclosing differences and similarities between the architecture of holocentric and monocentric chromosomes (Blackman, 1987*b*; Mandrioli & Manicardi, 2012). However, holocentrism is also a great drawback for cytogenetic studies and karyotype comparisons, because holocentric chromosomes lack primary and/or secondary constrictions, so that in conventionally stained preparations homologues can only be recognised on the basis of their size (Fig. 1) (Blackman, 1985*a*, 1987*b*; Hales *et al.*, 1997; Manicardi *et al.*, 2002). The only readily detectable changes are those that affect the number of chromosomes or produce a karyotype with measurable structural heterozygosity. Other types of chromosome rearrangements, such as inversions that are common in many animal groups, almost certainly occur, but their presence cannot be detected in conventionally stained preparations because they do not alter the gross chromosome morphology. Despite this limitation, chromosomal rearrangements have frequently been reported in aphids, and autosomal fusions and dissociations, particularly the latter, seem to play a large part in aphid karyotype evolution (Blackman, 1980;

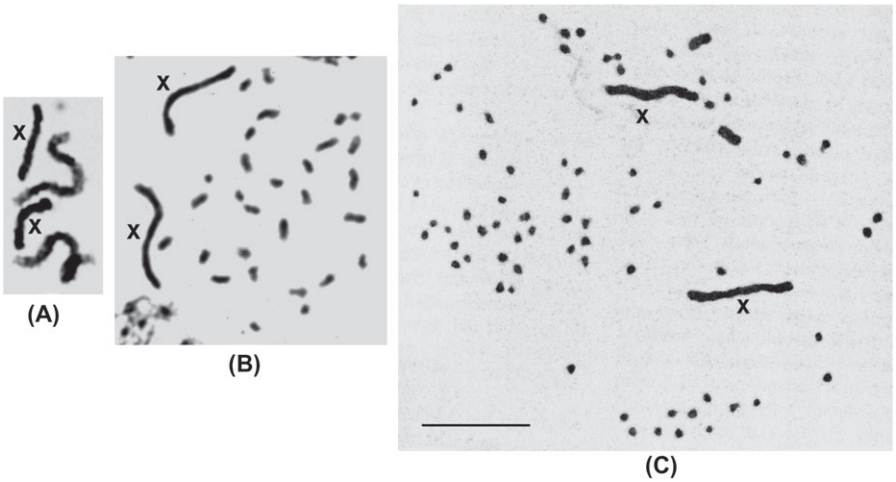

Fig. 2. Somatic metaphases from female embryos of three representative species of the genus *Amphorophora*: *A. tuberculata* Brown and Blackman, 2n = 4 (A); *A. rubitoxica* Knowlton, 2n = 30 (B); *A. sensoriata* Mason, 2n = 72 (C). The extreme variation in chromosome number is presumed to be due to successive autosomal fusions (A) or dissociations (B, C). The genera most closely related to *Amphorophora* (which include *Acyrthosiphon*) have chromosome numbers ranging from 2n = 8 to 2n = 18 (Blackman, 1980). Scale bar, 10 μm.

Monti *et al.*, 2012; Rivi *et al.*, 2012). Some aphid genera show great variation in chromosome number with little corresponding morphological change (Blackman, 1980; Blackman, Spence & Normark, 2000). In the genus *Amphorophora*, species with similar morphology and biology vary in female karyotype from 2n = 4 to 2n = 72 (Fig. 2). However, other genera have remarkably stable karyotypes. In the large genus *Dysaphis*, for example, all the species that have been karyotyped have 2n = 12. This raises questions of a fundamental nature about both evolutionary and functional aspects of the organisation of the genome.

One feature of aphid cytogenetics of particular interest is that chromosome behaviour is often under environmental control. Both sex determination and the switch from parthenogenetic to sexual reproduction are under the influence of external factors, such as temperature and photoperiod in many aphids. The endocrinological pathways involved are still little understood (Hales *et al.*, 1997), except that juvenile hormone titres have a definite influence on the behaviour of the chromosomes in sex determination (Hales & Mittler, 1987), and juvenile hormones are also implicated in deciding whether or not oocytes undergo meiosis (Le Trionnaire *et al.*, 2008). There is also often an innate interval timing mechanism that governs the length of the parthenogenetic phase of the aphid's life cycle, inhibiting the production of sexual females for a certain length of time. In *Megoura viciae* Buckton, this has been shown to measure the actual time (or number of light/dark cycles) that has elapsed, rather the number of parthenogenetic generations (Lees, 1966). Furthermore, the interval timing mechanism that governs sex determination may differ from that which inhibits the switch from parthenogenetic to sexual reproduction (Dixon, 1972).

Taking into account both the biological and economic interest in aphids, and current research activity in the field of aphid genomics, it is timely to review the main features of the structure and function of aphid chromosomes.

II. THE ROLE OF CHROMOSOMES IN THE TRANSITION BETWEEN PARTHENOGENESIS AND SEXUAL REPRODUCTION

The well-known alternation of parthenogenetic (thelytokous) and bisexual phases of reproduction in the life cycle of aphids involves a number of cytogenetic processes which are peculiar to this group. Some of these processes must be as old as the group itself, dating back more than 160 million years. This is worth stressing, because most instances of thelytoky in animals are thought to be relatively recent phenomena. The various phenomena of so-called cyclical parthenogenesis found in aphids and other groups are clearly highly successful adaptations that have been well tested and refined during the long course of their evolution. The cytogenetic mechanisms involved in the aphid life cycle should not, therefore, be regarded as aberrant processes, but as highly tuned and extremely functional systems. Developmental and physiological aspects of the transition process from parthenogenetic to sexual reproduction were reviewed by Le Trionnaire *et al.* (2008), so that here we will focus only on aspects related to chromosome behaviour.

Parthenogenesis in aphids is apomictic; that is, there is no regular mechanism for generating variation by genetic recombination during the oogenesis of the parthenogenetic egg (Blackman, 1987*b*; Hales *et al.*, 1997; Sloane *et al.*, 2002). During the embryological development of female aphids, irrespective of whether they are destined to become parthenogenetic or sexual, oogonial divisions result in germaria that contain 32 undifferentiated oogonial cells

(Büning, 1985). The chromatin within their nuclei at this time is partially condensed, although the number of separate chromosomes is not countable. The course of development then usually depends on environmental conditions, which in aphids adapted to temperate climates mainly involves seasonal variations in photoperiod and temperature. It is dangerous to generalise on the basis of the few species that have been studied in any detail, but the following summary applies at least to several members of the aphid tribe Macrosiphini, including *A. pisum* and *Myzus persicae* (Sulzer).

Sexual oogenesis is inhibited in spring and summer and as soon as a germarium is fully formed one of the posterior cells starts to develop as an oocyte. The nucleus of this first oocyte resembles that of all the other germarial cells, but as it grows and passes out of the germarium the chromosomes become more condensed and it is possible to count them; by this time any association between homologues has ceased, and they are present in diploid number. When the oocyte has grown to about the size of the germarium its nucleus moves to the periphery of the cytoplasm and enters metaphase. The single maturation division then occurs, throwing out one polar body. This division is mitosis-like, preserving the diploid number of chromosomes. The cleavage divisions then follow to initiate the rapid growth of the embryo as it passes down the ovariole.

Aphid parthenogenesis has evolved as a way of producing large numbers of progeny very rapidly indeed, and one particular feature of all species that have been studied to date is that no time is lost between formation of the germarium and maturation of the first oocyte, in order to start immediately with the development of the next generation (Blackman, 1978). Once this first oocyte has undergone its maturation division, however, there is a change in the nuclei of a group of cells in the posterior part of the germarium, which identifies them as presumptive oocytes. Their chromosomes enter a condensation phase, in which they remain until they leave the germarium, one at a time. The degree of condensation seems to vary among species (Blackman & Hales, 1986); in the genus *Forda*, and possibly in other Eriosomatinae, all the chromatin in each oocyte nucleus is compacted into a single, very dense body (Blackman, 1987*a*). In *Amphorophora tuberculata* Brown and Blackman, which has the lowest possible chromosome number for an aphid (n = 2), it has been possible to examine the behaviour of the chromosomes at this stage in some detail; the autosomes are separated, but the X chromosomes are much more condensed, and are joined end-to-end (Blackman & Hales, 1986). This end-to-end pairing happens at about the time that the oocytes pass out of the germarium one at a time and enter their growth phase, and seems to be a necessary precondition for sex determination, which involves the loss of one X chromosome, male aphids being XO. During the growth phase, the autosomes gradually condense until, at metaphase of the single maturation division, both X chromosomes and autosomes appear as very highly condensed, almost circular, bodies. In oocytes destined to be females the X chromosome pairing is lost prior to the maturation division. By contrast, the X chromosomes of oocytes destined to be males remain connected and undergo reduction in a rather remarkable way on the spindle at maturation division (Orlando, 1974; Blackman & Hales, 1986), so that only one of the Xs is inherited, the other being eliminated in the polar body. The inherited X chromosome seems to be selected randomly in some cases (Wilson, Sunnucks & Hales, 1997; Caillaud *et al.*, 2002), but not in others (Franz *et al.*, 2005; Monti, Manicardi & Mandrioli, 2011*b*).

In embryos destined to become sexual females because they are developing inside mothers that have experienced a certain number of long nights in autumn, the cells in the fully formed germaria undergo marked changes. These changes follow the normal course of events in sexually reproducing insects with telotrophic ovarioles (that is, ovarioles with terminal germaria). Most of the germarial cells undergo several rounds of endomitosis to become polyploid nurse cells (Blackman, 1987*b*; Le Trionnaire *et al.*, 2008), and the germaria in consequence become much larger – in fact about 500 times larger – than those of the parthenogenetic female. The presumptive oocytes all enter prophase of a normal meiotic division, with synapsis and formation of multiple chiasmata between homologous chromosomes (Blackman, 1987*b*). Although the contents of the oocyte nucleus are difficult to study after it has left the germarium and entered the growth phase, it is likely that the chromosomes remain paired and in a highly condensed state (diakinesis) until the oocyte is fully grown, and that meiosis then proceeds normally with a reductional first division and equational second, resulting in the two polar bodies first observed by Blochmann (1887).

In summary, chromosome behaviour during the development of aphids differs greatly according to whether they are destined to become parthenogenetic or sexual females, or males. The changes often occur as a response to environmental stimuli, and are under the influence of endocrine factors which have yet to be clearly defined. The transition between parthenogenetic and sexual development involves major changes in the chromosomes of the germarial cells, both in presumptive oocytes and in those destined to be nurse cells, and operates as soon as a germarium is fully formed. In many aphids this first occurs in embryos while the mother is still not adult, usually in the fourth instar. If parthenogenetic development is favoured, the first oocyte in each ovariole immediately starts to develop without delay and will be female, following which the chromosomes of subsequent oocytes condense and the X chromosomes remain paired end-to-end preparatory to sex determination, which occurs as each oocyte enters its growth phase.

III. CHROMOSOME BEHAVIOUR DURING APHID SPERMATOGENESIS

Male aphids are X0, but for cyclical parthenogenesis to occur all the progeny developing from fertilised eggs have

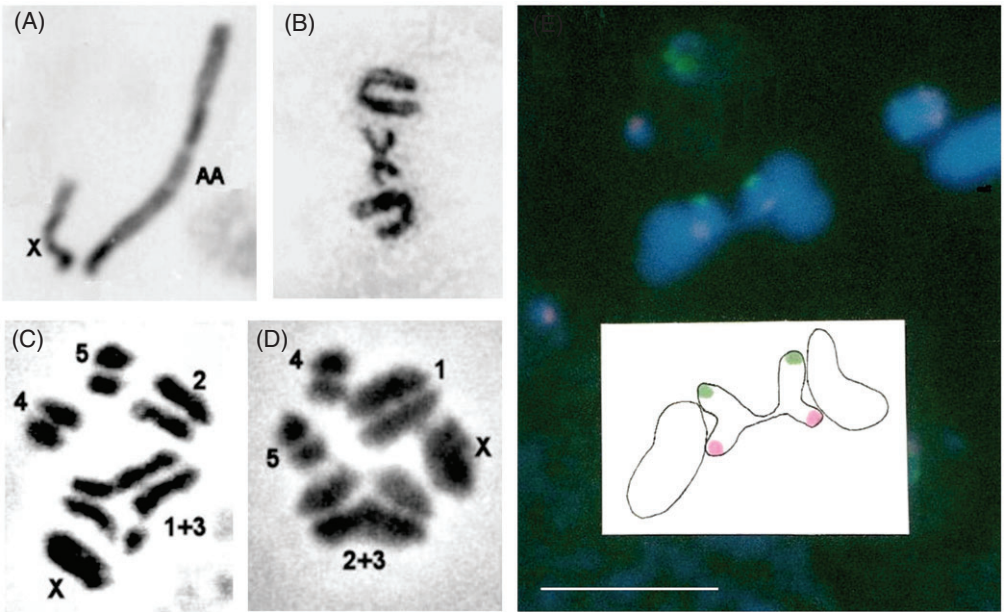

Fig. 3. Chromosome behaviour during spermatogenesis. (A) Prophase I of *A. tuberculata* (n = 2); the autosomal homologs are paired end-to-end (compare autosome/X chromosome relative lengths with Fig. 2A). (B) Doubling back of autosomal half-bivalents in prophase II of *A. tuberculata*, to align the non-sister chromatids side-by-side prior to the second meiotic division. The X chromosome has a metacentric appearance due to the persistence of the mid-way connection between the chromatids that held them together in anaphase I. (C) Prometaphase I in *M. persicae* heterozygous for a translocation between autosomes 1 and 3, showing parallel pairing of homologous sections of chromosomes. (D) Metaphase I in *M. persicae* heterozygous for a fusion between autosomes 2 and 3, showing parallel pairing of homologous chromosomes. (E) Anaphase I in *A. tuberculata*, hybridised to a mixture of two probes to reveal the orientation of the X chromosome, by labelling each end with a different dye; the rDNA probe pDm238 (labelled with fluorescein), and a repeat unit specific to *A. tuberculata*, At3 (labelled with Texas red). Counterstain is DAPI. (A, B) After Blackman (1985*b*), (E) after Spence & Blackman (1998). Scale bar, 10 μm.

to be female, so all sperm must have an X chromosome. Spermatogenesis in aphids is therefore a very unusual process in which autosome sets apparently 'compete' for a single X chromosome, followed by degeneration of the unsuccessful autosome set. Studies of spermatogenesis have involved relatively few species, but have nevertheless revealed a remarkable variety of chromosome behaviour, demonstrating that the holocentric chromosomes of aphids can be extremely labile in the ways in which they orientate, join and separate during this process.

In contrast to the female meiosis of aphids with its multiple chiasmata, spermatogenesis is essentially achiasmate (Sloane *et al.*, 2002). During prophase I of meiosis, homologous autosomes may either pair side-by-side, as in the birch aphid *Euceraphis betulae* (Koch) (Blackman, 1976), or end-to-end, as in *A. tuberculata* (Figs 3A and 4). At one time it was thought that the end-to-end connection was due to the persistence of a terminal chiasma, as known to occur in many Hemiptera, but this does not seem to be the case in aphids. The sequence of events is particularly clear in *A. tuberculata*, in which males have one pair of autosomes and a single X chromosome. In whole mounts of testis lobes cleared and stained with Feulgen one can see a distinct zonation, each zone containing hundreds of nuclei with chromosomes all behaving synchronously (Blackman, 1985*b*). The end-to-end pairing of the autosomes persists through anaphase I (Fig. 4A). This is remarkable because meiosis in this species is of the 'inverse' form, so that it is the sister chromatids which move apart at this division, the terminal bonding being maintained between non-sister telomeres. The terminal connection only breaks during prophase II, after each autosomal half-bivalent has doubled back on itself, so that non-sister chromatids are aligned side-by-side for the second meiotic division (Figs 3B and 4A). It is difficult to conceive how this doubling-back process is initiated and controlled.

The inverse form of male meiosis in *A. tuberculata* means that it is the second meiotic division that separates the products of pairing, i.e. the maternal and paternal autosomes (Fig. 4A). In birch aphids, on the other hand, homologous autosomes are aligned in parallel during prophase I of male meiosis, and are separated in anaphase I; i.e. the first meiotic division is the reductional one, as is typical of most organisms. The difference is not merely of academic interest, but has significant genetic consequences, because only one autosome set – the one that 'captures' the two unpaired X chromosomes (*E. betulae* having X_1X_20 males) – survives the meiotic process. In *A. tuberculata*, the autosomes enter the second meiotic division with non-sister chromatids derived from different parents, whereas in *E. betulae* the 'selfish' autosomes– the ones which capture the X chromosomes – are at least potentially derived from

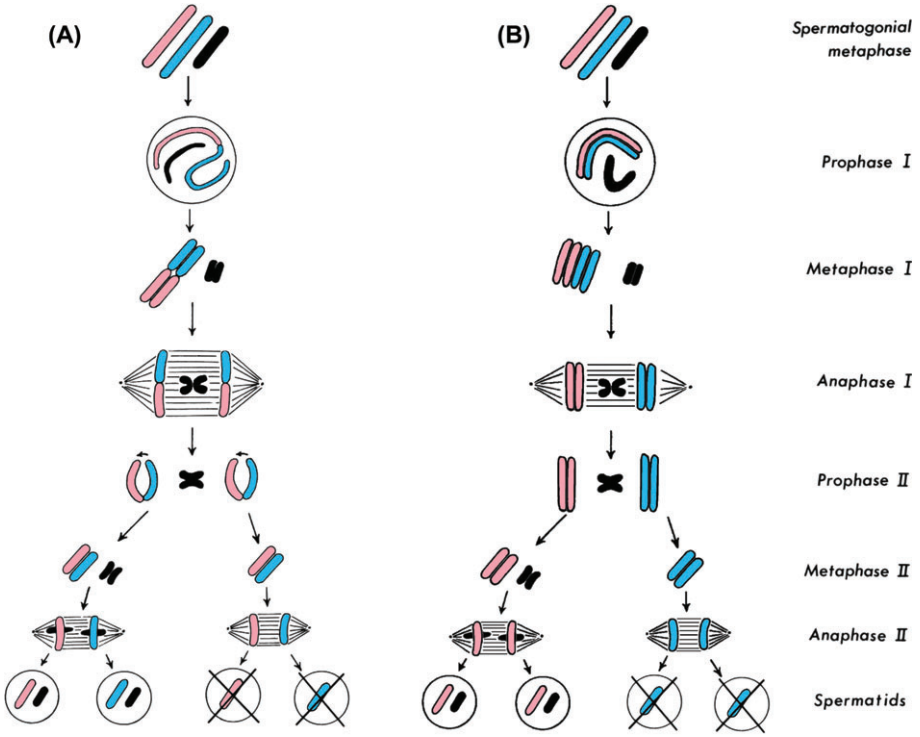

Fig. 4. Diagrammatic representation of two modes of spermatogenesis in aphids. Maternal autosomes are pink, paternal autosomes are blue, X chromosomes are black. (A) 'Inverse' meiosis in males of *Amphorophora tuberculata* (n = 2A + X). The two autosomal homologs are paired end-to-end when they enter prophase I, and the terminal bond between the non-sister chromatids is maintained through anaphase I. After realignment of the autosomal elements in prophase II the X chromosome becomes associated with one of the autosomal half-bivalents, so that after anaphase II there are two viable spermatids, each inheriting one of the parental autosomes. (B) 'Normal' meiosis as in males of *Euceraphis betulae*, except that for a simple comparison only one autosome pair and one X chromosome are shown. The two autosomal homologs are paired side-by-side when they enter prophase I. Anaphase I separates the products of pairing so is reductional, as in most other organisms. In metapase II the X chromosome becomes associated with either the maternal or the paternal autosome, and thus the two viable spermatids both inherit either the maternal or the paternal autosome. In the example here it is the maternal autosome that survives and the paternal autosome that is eliminated.

a single parent (Fig. 4B). In the latter case there is some similarity to the spermatogenesis of lecanoid scale insects, where it is the paternal autosome set that is always eliminated (Nur, 1980). However it is also possible that there is a random allocation of autosomes into the viable and inviable sets (Hales *et al.*, 1997).

Information is available from very few species, yet already there seems to be no phylogenetic pattern to the various forms of spermatogenesis in aphids. *E. betulae* is in subfamily Callaphidinae, whereas *A. tuberculata* with its end-to-end pairing and inverse meiosis is in subfamily Aphidinae. However, *M. persicae* is also in Aphidinae yet has side-by-side pairing of homologs during prophase I, as is evident in the quadrivalent figures formed by translocation heterozygotes, and the trivalents formed by dissociation and fusion heterozygotes (Spence & Blackman, 2000), where the homologous parts of the chromosomes involved in the rearrangements are aligned in parallel (Fig. 3C, D). It is still unknown whether, for example, *Amphorophora rubi* with n = 10, or *A. sensoriata* with n = 36, have the same form of meiosis as *A. tuberculata* with n = 2. The extreme lability of the spermatogenetic process is demonstrated most vividly by *Euceraphis punctipennis* (Zetterstedt), which is closely related to *E. betulae* yet has a form of male meiosis that dispenses completely with the first meiotic division (Blackman, 1976, and see below).

An even stranger variant of aphid spermatogenesis is the interaction between autosomes and X chromosomes found in *Schoutedenia ralumensis* Rübsaamen (= *lutea* van der Goot; subfamily Greenideinae), which has two pairs of X chromosomes (X_1 and X_2). In somatic cells, however, each of these four X chromosomes appears to be of different length. Studies of male meiosis (Hales, 1989), where homologues are paired in parallel during prophase I, showed that this anomaly is due to each one of a pair of autosomes being attached to an X chromosome, one to an X_1 and one to an X_2. During meiosis, either X_1 or X_2, apparently at random, gives up its attached autosome, which then passes at anaphase I into the inviable (X chromosome-less) spermatocyte. It may be a long time before we understand the adaptive value of such a bizarre process.

It is strange that, with the diversity of behaviour of the autosomes in aphid spermatogenesis, the peculiar behaviour of the univalent X chromosome is much more conservative,

its stretched configuration at anaphase I having been observed in aphids of at least six families of Aphidoidea, and occurring irrespective of the type of meiosis. The long-standing controversy about the orientation of the stretched X chromosome was resolved by *in situ* labelling of both ends of the X chromosome of *A. tuberculata* (Spence & Blackman, 1998), which produced the surprising conclusion that both ends of each X chromatid separate and move towards opposite poles, retaining a midway connection which is never broken, as the whole X chromosome is eventually 'captured' by one of the autosomes (Fig. 3E). This explains the quadripartite appearance of the stretched X chromosome observed by many authors, and the form of the X (Fig. 3B). The nature of this midway connection still remains unclear, and even more enigmatic is the reason for the persistence of this apparently abortive process for about 200 million years.

However, one thing that has been subject to evolutionary change is the time when the autosome set that fails to capture the X chromosome degenerates. Its degeneration may commence while anaphase I is in progress, or after the secondary spermatocyte nucleus has reformed and the autosomes have undergone an X chromosome-less second meiotic division. In the extreme case, found in *E. punctipennis* which has a single autosome pair, the autosome that fails to 'capture' the X chromosomes is heterochromatinised and apparently ejected from the prophase nucleus, dispensing entirely with the need for the first meiotic division.

In female somatic cells of *Euceraphis* spp. there is a group of elements comprising two pairs of X chromosomes and one or two mainly or entirely heterochromatic elements that may be classified as B chromosomes. In other organisms B chromosomes are typically supernumerary to the normal chromosome complement, and may or may not be present, yet all species of *Euceraphis*, as well as some related genera, have one or more of these elements (Blackman, 1988). Males have the same number of B chromosomes as females, but nevertheless the B chromosomes are perhaps of X chromosomal origin, as during spermatogenesis they behave like the X chromosomes, undergoing anaphase I stretching before passing into the same daughter spermatocyte as the X chromosomes. Interestingly, this means that they are inherited without reduction through the male line. The number of these B chromosomes remains the same in successive generations, so in order for the zygote to retain the parental complement there must be some compensatory mechanism, as yet unknown, whereby they are eliminated during oogenesis. If there are any coding sequences on these elements then they will therefore be inherited exclusively through the male.

A similar phenomenon may occur in the eriosomatine aphid genus *Forda*, where germ line and soma have different numbers of chromosomes, several chromosomes being eliminated from somatic cells at an early stage in embryogenesis (Blackman, 1987*a*). The male karyotype, sex determination and spermatogenesis have not yet been studied in these aphids, so the functional significance of the elimination process is still a mystery.

IV. DISTRIBUTION OF REPEATED DNA IN THE APHID CHROMOSOMES

Differential staining of aphid chromosomes to identify repeated DNA sequences was first applied by Blackman (1976, 1980, 1985*a*), in order to identify cytogenetic markers which could be useful for the taxonomic identification of aphids, as well as for the analysis of karyotype evolution. Preparations made with a cell suspension technique after treatment with trypsin and staining with Giemsa produced results analogous to mammalian G-banding in prophase chromosomes of several species, but the results were not clear or consistent enough for use in species comparisons (Blackman, 1985*a*).

During the last three decades the distribution of constitutive (C-) heterochromatin has been evaluated in several aphid species, showing a tendency to be concentrated on the X chromosomes (Kuznetzova & Sapunov, 1987; Manicardi *et al.*, 1991, 1996, 1998, 2002; Criniti *et al.*, 2005; Mandrioli *et al.*, 2011). In particular, an exclusive localization of heterochromatin on the X chromosomes has been found in three species of the genus *Aphis*: *Aphis sambuci* Linnaeus (Manicardi *et al.*, 1998), *A. pomi* de Geer (Criniti *et al.*, 2005) and *A. nerii* Boyer de Fonscolombe (Mandrioli *et al.*, 2011) (Fig. 5).

The amount of heterochromatin on autosomes can vary significantly since it may be located in different intercalary positions, as reported for *Myzus varians* Davidson (Bizzaro *et al.*, 1999) and *Euceraphis betulae* (Blackman, 1976), or restricted to telomeres, as observed in *M. persicae* (Mandrioli *et al.*, 1999*a*). In *Trama troglodytes* and related species, where sexual reproduction is very rare (Blackman, De Boise & Czylok, 2001) and the X chromosomes have not been identified, there are large blocks of C-heterochromatin varying greatly in size, number and position both among and within species, a varying number of chromosomes being mainly or entirely heterochromatic (Blackman *et al.*, 2000).

These results differ substantially from those observed in monocentric chromosomes, in which C-heterochromatin typically occupies specific regions of all chromosomes, corresponding to centromeres and sometimes telomeres (Schweizer & Loidl, 1987). Research carried out in order to analyse heterochromatin localization in other taxa with holocentric chromosomes was in some cases unsuccessful (Collet & Westerman, 1987), but generally a telomeric and sometimes intercalary localization of C-positive bands on the whole chromosome complement has been described (Kuznetzova & Sapunov, 1987; Papeschi, 1988; Grozeva & Nokkala, 2003; Haizel *et al.*, 2005; Morielle-Souza & Azeredo-Oliveira, 2007; Hill *et al.*, 2009). These data as a whole suggest that the preferential, and in some case the exclusive, localization of C-heterochromatin on the X chromosome in aphids is not a consequence of their holocentrism, but must be considered a peculiar feature of aphid chromatin.

Vig (1987) presented evidence suggesting that the size of the heterochromatic blocks may influence the order of

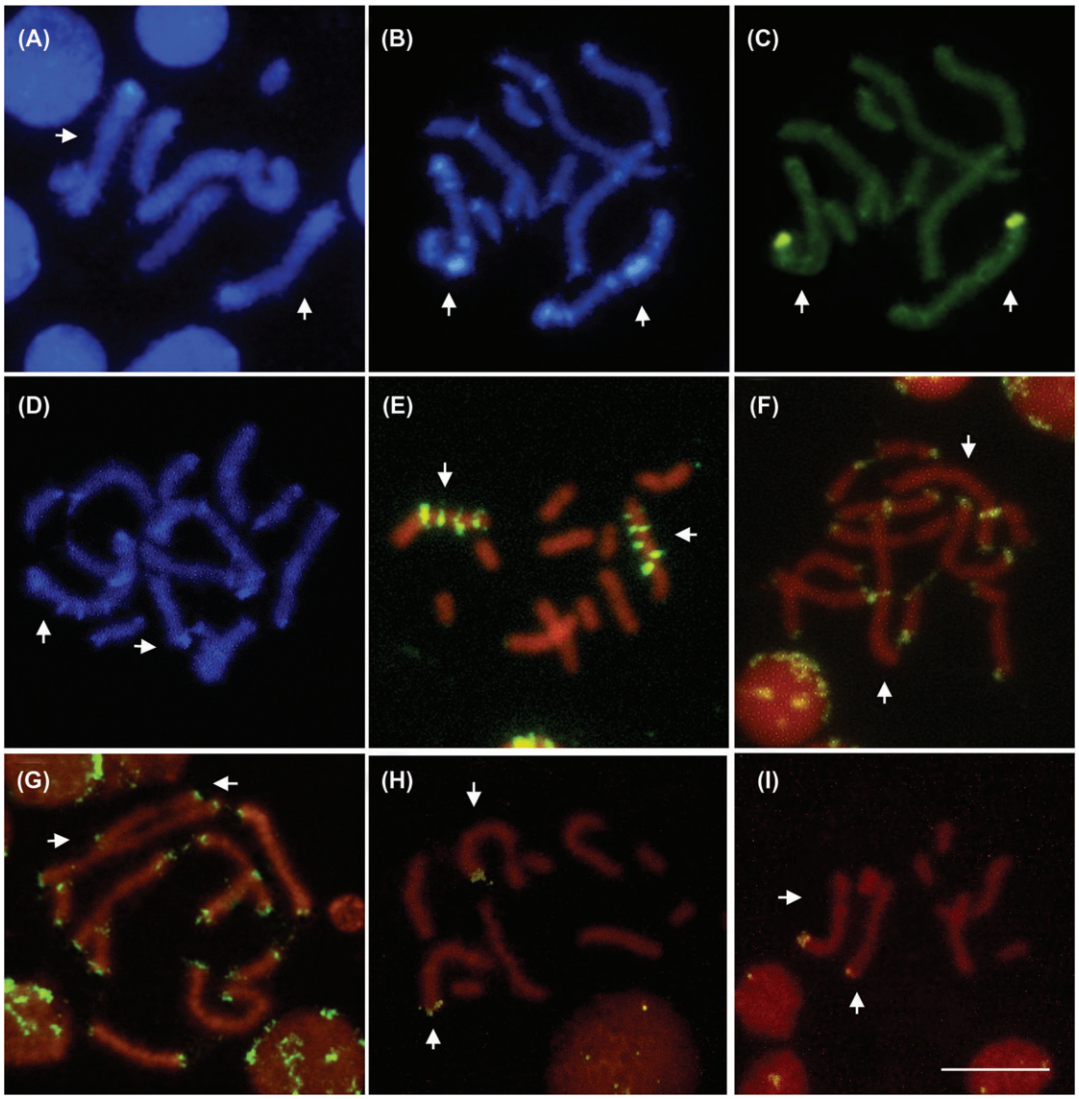

Fig. 5. (A–D) C-banded preparations of metaphase chromosomes of the aphids *A. pisum* (A), *M. viciae* (B, C) and *M. persicae* (D) after staining with DAPI (A, B, D) and CMA_3 (C). These show different amounts of heterochromatin on autosomes and X chromosomes, and also demonstrate heterogeneity in the composition of the heterochromatin (compare B and C), since GC-rich heterochromatin, brightly stained with CMA_3 in (C), is limited to a single telomere of each X chromosome, whereas all the bands in (A, B, D) are AT-rich regions revealed by DAPI staining. (E, F) Fluorescence *in situ* hybridisation (FISH) carried out on *M. persicae* chromosomes; (E) probing with Hind200 revealed the occurrence of X-specific satellite DNA; (F), probing with the 169 bp subtelometric repeat revealed highly repetitive sequences in heterochromatic regions of the whole chromosome complement and (G) probing with the $(TTAGG)_n$ repeat showed that this constitutes the telomeric end of each chromosome. (H, I) FISH with a 28S rDNA probe, showing heteromorphism between the size of the arrays of the rDNA genes on the X chromosomes of both (H) *M. viciae* and (I) *A. pisum*. Arrows indicate the X chromosomes. Scale bar, 10 µm.

separation of sister chromatids in different chromosomes at anaphase. This observation fits the hypothesis that the large heterochromatic blocks occurring on aphid X chromosomes could be involved in the delay in separation of the X chromosomes that occurs during the maturation of parthenogenetic oocytes, which is considered to be the basis of male sex determination (Orlando, 1974; Blackman, 1987*b*).

Satellite DNAs (satDNAs) constitute a considerable portion of the eukaryotic genome and represent the major components of heterochromatin (John & Miklos, 1979; John, 1988; Charlesworth, Sniegowski & Stephan, 1994; Cesari *et al.*, 2003; Palomeque & Lorite, 2008). They generally form long tandem arrays in which monomer sequences are repeated in a head-to-tail fashion. In view of this organisation, the digestion of the satDNA arrays with the appropriate restriction enzyme gives rise to a characteristic ladder of bands (multimers of the basic satellite DNA repeat unit) after agarose gel electrophoresis (e.g. Spence *et al.*, 1998; Mandrioli *et al.*, 1999*a*).

The few studies carried out to date on satDNA sequences in organisms possessing holocentric chromosomes have mostly focused on nematodes (Roth, 1979; Collet & Westerman, 1987; Naclerio *et al.*, 1992; Castagnone-Sereno *et al.*, 1998*a*,*b*). Among aphids, satellite DNAs have been isolated in five aphid species only: one in *Megoura viciae* (Bizzaro, Manicardi & Bianchi, 1996), one in *Rhopalosiphon padi* (Linnaeus) (Monti, Manicardi & Mandrioli, 2010), two in *M. persicae* (Spence *et al.*, 1998; Mandrioli *et al.*, 1999*a*), two in *Amphorophora tuberculata* (Spence & Blackman, 1998) and four in *Aphis nerii* (Mandrioli *et al.*, 2011)

The chromosomal localization of these satellites, investigated by fluorescent *in situ* hybridization (FISH), mostly corresponded to C-positive heterochromatic areas on the X chromosome (Fig. 5E). However, a 169 base pair (bp) tandem repeat DNA marker for subtelomeric heterochromatin on all chromosomes has been described in aphids of the *M. persicae* group by Spence *et al.* (1998) (Fig. 5F). These data as a whole indicate that aphid satDNAs are localized in heterochromatic areas of the chromosome, suggesting that they represent the principal components of C-heterochromatin, in agreement with results repeatedly described in monocentric chromosomes (John & Miklos, 1979; John, 1988).

Molecular analyses of these aphid satDNAs have shown that their consensus sequences are variable in length from 100 to 600 bp, and that none of them possesses significant direct or inverted repeats (Bizzaro *et al.*, 1996; Spence *et al.*, 1998; Monti *et al.*, 2010; Mandrioli *et al.*, 2011).

Bioinformatic analyses have shown that an internal portion of several aphid satDNAs (such as *Dra*I satDNA in *R. padi* and Hind200 in *M. persicae*) possesses a high curvature propensity, suggesting that aphid satDNAs could be involved in the tight winding of DNA in heterochromatin, as previously suggested for other insect satDNAs (Palomeque & Lorite, 2008). This hypothesis is strengthened by the presence, in all the aphid satDNAs, of short stretches of adenines and thymines that have been found to be implicated in the sequence-induced DNA curvature that is able to link particular heterochromatic proteins (Koo, Wu & Crothers, 1986; Martinez-Balbas *et al.*, 1990). A search for homology with other DNA sequences within GenBank and EMBL databases yielded no significant results.

Aphid satDNAs display other features typical of satellite sequences isolated in organisms possessing monocentric chromosomes, such as their coincidence with heterochromatic bands, a high A + T content (from 63 to 79%) and a high degree of sequence similarity between monomers (from 84 to 94%). In particular, the presence of few mutations, mainly nucleotide substitutions rather than insertions and deletions, could be due either to a process of 'concerted evolution' determined by molecular drive (Dover *et al.*, 1982; Dover & Tautz, 1986) or to a strong functional constraint related to the interaction of satDNAs with specific proteins involved in heterochromatin formation through direct binding or *via* RNA interference (as previously reported by Talbert, Bryson & Henikoff, 2004). However, with the exception of the subtelomeric DNA satellite isolated in *M. persicae* that is present in two other closely related species (Spence *et al.*, 1998), the species-specificity of the satDNAs found in aphids supports the view of their recent origin so that the high levels of sequence similarity could also be related to the recent origin of aphid satDNAs.

On the contrary, low levels of sequence similarity (consistently lower than 50%) have been found when comparing sequences belonging to different satDNAs. This difference is present also when comparing the four satellite DNA sequences isolated from the *A. nerii* genome (Mandrioli *et al.*, 2011) suggesting that these repetitive sequences may be valuable as taxonomic tools for species determination.

The transcription of insect satDNAs has been studied in few species and just some of them present RNAs related to these repetitive DNAs (Palomeque & Lorite, 2008). Aphid satDNA transcripts have been searched by reverse-transcription polymerase chain reaction (RT-PCR) in *R. padi* only without any positive result (Monti *et al.*, 2010). However, transcription of insect satDNAs has been related to cell type, developmental stage and sex so that it may not be easy to detect any transcript (Renault *et al.*, 1999; Palomeque & Lorite, 2008). Moreover, satDNA-related transcripts may consist of small RNAs that cannot be detected by RT-PCR, as reported in *Drosophila melanogaster* (Aravin *et al.*, 2003; Usakin *et al.*, 2007), so that any assessment of satellite transcription in aphids requires further investigations.

V. EPIGENETIC MARKERS OF APHID HETEROCHROMATIN

According to current models, epigenetic modifications of chromatin affect its function by changing accessibility to regulatory complexes and transcription factors (Kouzarides, 2007). In particular, DNA methylation and a large set of different histone chemical modifications act in concert to create higher order structures that alter the chromatin shape (Kouzarides, 2007; van Steensel, 2011).

Data obtained from the *A. pisum* genome attested that the major chromatin remodelling complexes are present in aphids, as well as homologues to all the DNA methyltransferases found in vertebrates (Hunt *et al.*, 2010; Rider, Srinivasan & Hilgarth, 2010; Walsh *et al.*, 2010).

At present, only few epigenetic modifications have been studied at a chromosomal level in aphids and they solely concern the role of both DNA and H3 histone methylation in heterochromatin condensation (Mandrioli & Borsatti, 2007; Hunt *et al.*, 2010; Walsh *et al.*, 2010).

The involvement of DNA methylation at cytosine residues in heterochromatin tight winding is well documented in vertebrates (Adams, 1996), but there are few data regarding its occurrence in insects, and in particular in species with holocentric chromosomes. In contrast to the situation in vertebrates, aphid C-heterochromatin is poorly methylated, whereas methylated cytosine residues seem to be widespread in euchromatic chromosomal regions

(Mandrioli & Borsatti, 2007; Mandrioli *et al.*, 2011). This result has been strengthened by genome analyses showing that methylated sites are predominantly restricted to the gene body (including both exons and introns) at CpG sites (Walsh *et al.*, 2010), indicating that actively transcribed euchromatic regions in aphid chromosomes can be highly methylated, as previously reported in the hemipteran *Planococcus citri* (Risso) (Bongiorni & Prantera, 2003).

The absence of correspondence between heterochromatin and methylated chromosomal domains, also observed in *D. melanogaster* and *C. elegans* (Field *et al.*, 2004), supports the idea that invertebrate heterochromatin can be assembled and condensed without any involvement of DNA methylation (Field *et al.*, 2004; Mandrioli, 2004).

According to published data, the DNA methylation machinery interacts with histone proteins and other non-histonic proteins effectively to alter the chromatin structure. In particular, several studies have shown that H3 histone monomethylated at lysine 9 (methylated-K9 H3) residues are commonly present in heterochromatin, where they are a binding target for the heterochromatin protein 1 (HP1) (Lachner *et al.*, 2001). Thus, H3 histone monomethylated at lysine 9 and HP1 are common epigenetic markers for heterochromatin, both in vertebrates and invertebrates (e.g. Jacobs *et al.*, 2001; Lachner *et al.*, 2001; Cowell *et al.*, 2002; Borsatti & Mandrioli, 2005). Both in *A. pisum* (Mandrioli & Borsatti, 2007) and *A. nerii* (Mandrioli *et al.*, 2011), the only aphid species to date studied at the epigenetic level, Me9H3 and HP1 have been localized in C-positive areas indicating that aphid C-heterochromatin is enriched in both of these epigenetic markers.

VI. TELOMERES AND SUBTELOMERIC REGIONS

Telomeres are specialized DNA-protein structures constituting the ends of chromosomes (Blackburn, 1991). They are essential to protect chromosomal ends from erosion by exonucleases, to avoid chromosome stickiness and to mediate the attachment of chromosomes to the nuclear envelope before the chromatin remodelling that occurs at cell division (Blackburn, 1991; Zakian, 1995). Although telomeric sequences may vary in composition in eukaryotes, their conservation is strict in some taxa, and insects typically have a $(TTAGG)_n$ repeat unit (Okazaki *et al.*, 1993; Sahara, Marec & Traut, 1999; Bizzaro *et al.*, 2000; Frydrychová *et al.*, 2004; Vítkova *et al.*, 2005; Monti *et al.*, 2011*a*; Mandrioli, Monti & Manicardi, 2012).

At present, the occurrence of the $(TTAGG)_n$ repeat has been reported in a few aphid species (Spence *et al.*, 1998; Bizzaro *et al.*, 2000; Monti *et al.*, 2011*a*). FISH experiments clearly showed a hybridization signal on each telomere of all the aphid chromosomes, and there was no evidence of any interstitial labelling (Fig. 5G) (Monti *et al.*, 2011*a*). In interphase nuclei of most organisms the telomeres are situated in an ordered fashion with an association to the nuclear matrix and clustering at least at some stage of cell life (Palladino *et al.*, 1993; Luderus *et al.*, 1996). In aphid interphase nuclei (Monti *et al.*, 2011*a*), telomeres appear to be clustered into few foci and are not located mainly near the nuclear periphery, as reported in other insects such as *D. melanogaster* (Hochstrasser *et al.*, 1986) and the cabbage moth *Mamestra brassicae* (Linnaeus) (Mandrioli, 2002).

In contrast to the conservation of telomeres, subtelomeric regions are more polymorphic and variable in composition (Mandrioli *et al.*, 2012). Among aphids, a 169 bp subtelomeric satDNA (MpR) has been found in three closely related species of the genus *Myzus*: *M. persicae*, *M. antirrhinii* (Macchiati) and *M. certus* (Walker) (Spence *et al.*, 1998). This repeat occurs at both ends of all autosomes of the standard chromosome complement, and at one end of the X chromosome, but is absent from the end bearing the nucleolar organising region (NOR) (Fig. 5F). Recently a telomeric repeat-associated sequence (TRAS) retrotransposon (annotated as TRASAp1) has been identified in the aphids *A. pisum* and *M. persicae* (Monti *et al.*, 2013). The study of TRAS chromosomal insertion sites, performed by standard fluorescent *in situ* hybridization (FISH) and fiber FISH, showed that TRAS elements are located near the telomeric $(TTAGG)_n$ repeats of all autosomes. However, as in the case of MpR, the two X chromosome telomeres show a clear-cut structural difference (Monti *et al.*, 2013). Chromomycin A_3 (CMA_3) staining, together with FISH using a TRAS probe, revealed that TRASAp1 signals only occur at the X telomere opposite the NORs. The structural differences between the two telomeres of the X chromosome could be significant in relation to the peculiar ways that it behaves during aphid sex determination and spermatogenesis.

VII. rDNA GENES: FROM SEX DETERMINATION TO MITOTIC RECOMBINATION

NOR number and position have been frequently reported as highly variable in insects (Maryańska-Nadachowska, Warchalowska-Sliwa & Kuznetsova, 1992; Maryańska-Nadachowska & Grozeva, 2001; Nguyen *et al.*, 2010). For instance, rDNA genes have been found mainly, or only, on autosomes in Lepidoptera and Psylloidea (Maryańska-Nadachowska *et al.*, 1992; Maryańska-Nadachowska & Grozeva, 2001; Nguyen *et al.*, 2010), whereas several species with multiple NORs have been described in Coleoptera (e.g. Postiglioni & Brum-Zorrilla, 1988; Juan, Pons & PetitPierre, 1993). In this regard, aphids represent an interesting exception among insects since their 18S, 5.8S and 28S rDNA genes are usually arranged as tandemly repeated clusters at one telomere of each X chromosome, as revealed by silver staining (Fig. 1B) (Blackman & Hales, 1986; Hales, 1989; Manicardi *et al.*, 1992, 1996; Kuznetsova & Maryanska-Nadachowska, 1993; Mandrioli *et al.*, 2011), staining with the GC-specific fluorochrome CMA_3 (Manicardi *et al.*, 1992, 1996; Mandrioli *et al.*, 2011) and *in situ* hybridization with rDNA probes (Blackman & Spence, 1996; Mandrioli *et al.*,

2011) (Fig. 5H, I). Exceptions include the interstitial position of rDNA genes in *Amphorophora idaei* (Börner) (Fenton *et al.*, 1994) and *Glyphina* spp. (Blackman, 1989), and the autosomal localization of NORs in *Schoutedenia ralumensis* (Hales, 1989). Also, in *Trama troglodytes* and its relatives, where sex is rare and X chromosomes have not been identified, there is great variation in the number and locations of rDNA arrays (Blackman *et al.*, 2000).

A certain amount of heterogeneity between homologous NORs has been observed with all the staining techniques, indicating that there are a variable number of ribosomal genes clustered at each X telomere (Mandrioli *et al.*, 1999*a*,*b*; Monti *et al.*, 2011*a*) (Fig. 5I). Analysis of cells from single embryos showed that the observed heteromorphism is not only intra-clonal, but can also be intra-individual. These data, together with the finding that X chromosomes are connected by chromatin bridges between their NORs, suggest that mitotic unequal crossing over could be the main cause of this NOR heteromorphism. Mechanisms such as unequal crossing-over or unequal sister chromatid exchanges (SCEs) could be favoured by the X chromosome associations that are repeatedly observed in metaphase chromosome plates of somatic cells (Mandrioli *et al.*, 1999*a*,*b*; Monti *et al.*, 2011*a*).

It has been surmised that the conserved position of the rDNA genes in aphids could be due to their need to associate during sex determination (Orlando, 1974; Blackman & Hales, 1986). However, the absence of rDNA arrays from one X chromosome in a clone of *A. pisum* did not prevent it from producing males (Blackman & Spence, 1996). Interestingly, molecular analysis of rDNA intergenic spacers (IGSs) isolated from the *A. pisum* genome showed several 247 bp repeats containing short sequences having a high level of homology with the χ sequence of *Escherichia coli* and with the consensus core region of human hypervariable minisatellites (Mandrioli *et al.*, 1999*a*). These aphid repeats show structural homologies with a 240 bp repeat, which is considered to be responsible for sex chromosome pairing in *Drosophila melanogaster*, not only in view of their common presence within rDNA spacers but also in their length and structure.

The presence of χ sequences acting as potential hot spots of recombination in the IGSs of *A. pisum* within an open structure of chromatin resulting from the presence of active promoters, strongly supports the hypothesis that unequal crossing over between rDNA genes is the basis of the NOR heteromorphism described in various aphid species.

The loss of one X chromosome during male determination is reported to be random in some cases (Wilson *et al.*, 1997; Caillaud *et al.*, 2002), but not in others (Franz *et al.*, 2005; Monti *et al.*, 2011*b*). All males produced by a laboratory culture of *Macrosiphum euphorbiae* (Thomas) with a strong NOR heteromorphism had an X chromosome with a large NOR, indicating a strong selective bias favouring the X chromosome that had most rDNA genes (Monti *et al.*, 2011*b*). This could explain the varying reports of biased or random inheritance of X chromosomes, and if this is indeed the case then one may expect variation in the degree of bias within species.

The occurrence of somatic recombination between both homologous and non-homologous rDNA sites, resulting in asymmetric exchanges of these tandemly repeated DNA sequences is particularly widespread, having been described in other eukaryotes such as *D. melanogaster* (Tartof, 1974; Frankham, Briscoe & Nurthen, 1978; Lyckegaard & Clark, 1991). All these findings indicate that the genes for ribosomal RNA can represent active hotspots of mitotic recombination in aphids, even if this high frequency of recombination seems not to be a typical feature of the whole X chromosome, since Hales *et al.* (2002) demonstrated that no recombinant genotypes were observed in parthenogenetically produced males or females.

FISH experiments have shown that 5S rDNA genes are located in a single cluster on autosome 1 in *A. nerii* (Mandrioli *et al.*, 2011) and in two interstitial clusters on the X chromosomes of *A. pisum* (Bizzaro *et al.*, 2000), so 5S localization on aphid chromosomes may vary among species more than major rDNA genes. At the same time, no 5S heteromorphism has ever been observed in aphids, including *A. pisum*, further supporting the hypothesis that only part of the X chromosome has a high rate of recombination.

VIII. CONCLUSIONS

(1) Aphids were for many years on the sidelines of genetics, but because of their well-defined polyphenism they have recently become favoured model organisms for investigating the interactions between genome and environment. Much research has been aimed at elucidating the mechanisms by which the phenotypic plasticity of aphids is inherited and controlled. Yet so far this research effort has paid little attention to the cytogenetic processes that play a vital part in the organisation, expression and inheritance of the aphid genome. This is of some concern, because aphids have holocentric chromosomes that differ greatly in their structure and function from the chromosomes with localised centromeres found in most of the organisms familiar to geneticists.

(2) The karyotypic organisation of the aphid genome varies among species in ways that have little relation to phylogeny and are currently inexplicable. Chromosome numbers appear to be stabilised in some genera but may vary greatly in others, mostly due to successive autosomal dissociations with little or no accompanying morphological or biological change.

(3) The transition between parthenogenetic and sexual reproduction involves changes in chromosome behaviour that occur in response to environmental stimuli, mediated by endocrine factors. Remarkable differences occur, even between closely related species, in the ways in which aphid chromosomes pair and segregate during sex determination and meiosis, with consequences that can affect the inheritance of all or parts of the genome.

(4) Holocentric chromosomes are found in several groups of organisms, including all other Hemiptera, but there are

certain features of chromatin structure and organisation that seem to be peculiar to aphids. Constitutive heterochromatin tends to be concentrated mostly or even exclusively on the X chromosomes. Nucleolar organising regions are also almost invariably located in a subtelomeric position on the X chromosomes, and commonly display heteromorphism, with different numbers of rDNA genes clustered at each telomere, due probably to a high incidence of mitotic recombination at this location.

(5) We believe that these peculiarities of aphid cytogenetics need to be taken into account in studies of the aphid genome, as they could substantially influence the focus of research and the interpretation of results.

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