CHAPTER 1

1 INTRODUCTION

CHROMOSOMAL VARIABILITY IN APHIDS, EVIDENCE AND ADAPTATION MECHANISMS

1.1 WHAT ARE APHIDS?

1.1.1 Aphids - A Major Crop And Garden Pest

Plant-sucking aphids are the bane of a crop existence. They are common pests of nearly all indoor and out door ornamental plants, as well as vegetables, field crops, and fruit trees. In spring, aphids appear as if by magic and begin draining the life out of tender plants. Their ability to reproduce, both sexually and asexually, allow hig prolificity since each adult aphid can produce up to 80 offspring in a matter of a week and aphid populations can increase with great speed. 4.000 species of aphids are known: of these, around 250 species are serious pests for agriculture and forestry. Aphids show a intraspecific polymorphism (wings and apterous forms), which is a recurring phenomenon and an important feature because it is associated with an adaptation to a specific phase of their life cycle (Baccetti et.al., 1994).

Aphids are small, soft-bodied insects with long, slender mouthparts with which they pierce stems, leaves, and other tender plant parts to suck out plant fluids. Almost every plant has one or more aphid species which occasionally feed on it. Many aphid species are difficult to distinguish from one another; however, identification to species is not necessary to control aphids in most situations. You can find them on the underside of leaves, and also clustering around buds and flowers (Fig.1.1).

Fig.1.1. Aphids are tipically scattered on underside of leaves of different host plants (from Queen’s printer for Ontorario, 2009)

Aphids are generally greenish but they can also be black, orange, or shades of brown.

The green aphids typically found on roses are usually called "greenfly", and the fat black aphids often found on broad beans are called "blackfly". Few aphids measure more than a couple of millimeters.

A few species appear waxy or woolly due to the secretion of a waxy white or gray substance over their body surface. All are small, pear-shaped insects with long legs and antennae. Up close, aphids resemble little “muscle cars” (Hadley 2012) with a pair of tailpipes called cornicles projecting backwards out of the hind end of their body.

The presence of cornicles distinguishes aphids from all other insects. Entomologists believe that these abdominal appendages secrete waxy lipids or alarm pheromones when the aphid senses a threat. Antennae may have five or six segments, with the final segment ending in a thin flagellum. At their other end, aphids possess a cauda, a short, tail-like appendage centered between the cornicles.

Adult aphids are generally wingless, but most species also occur in winged forms, especially when populations are high or during spring and fall. The ability to produce winged individuals provides the pest with a way to disperse to other plants when the food source gets scarce. Although they may be found singly, aphids often feed in dense groups on leaves or stems. Unlike leafhoppers, plant bugs, and certain other insects that might be confused with them, most aphids do not disperse rapidly when disturbed. Aphids feed on plant phloem tissues, sucking the sugary liquids from the host plant's vascular system. Reaching the phloem is not an easy task. Aphids feed using a straw-like proboscis that contains thin, delicate stylets for piercing plant tissues. In order to protect the stylets from damage, the aphid secretes a special fluid from them, which hardens into a protective sheath. Only then can the aphid begin feeding.

Aphids need nitrogen, but phloem juices contain mostly sugars. To get adequate nutrition, aphids must consume an enormous quantity of phloem liquids. They excrete the excess sugars in the form of honeydew, a sweet residue left behind on plant surfaces (Dixon and Glan 1971). Other insects, such as ants and wasps, follow behind the aphids, licking up the honeydew. Aphids are tiny and slow-moving – in other words, easy targets, for this motivations they are able to adopted a system for their defences. They're far from defenseless, however, aphids use both fight and flight, and everything in between, to protect themselves.

If a predator or parasitoid approaches an aphid, it can react in a number of ways. Aphids will literally kick their attackers, with some serious aggression. In other cases, the aphid may just walk away, hoping to elude the trouble. Sometimes, the

aphid does a stop, drop, and roll, and simply falls to the ground. Some aphid species employ soldier aphids to stand guard. Aphids also arm themselves with defensive weaponry. When a pursuing predator attempts to take a bite from behind, they can excrete a waxy lipid from their cornicles to fill the attacker's mouth. Alarm pheromones broadcast the threat to other aphids, or may summon protection from bodyguards of other species. If a "lady beetle" attempts to feed on it, a cabbage aphid will mix toxic chemicals within its abdomen to "bomb" the offender.
Aphids also use bodyguard ants, which they pay with sweet honeydew excretions.

1.1.2 Aphids And Their Damages

Low to moderate numbers of leaf-feeding aphids are usually not damaging in gardens or on trees. However, large populations cause curling, yellowing, and distortion of leaves and stunting of shoots; they can also produce large quantities of a sticky substance known as honeydew, which often turns black with the growth of a sooty mold fungus (Fig 1.2 a, b e c). Some aphid species inject a toxin into plants, which further distorts growth. A few species cause gall formations.
Aphids transmit viruses from plant to plant on certain vegetable and ornamental plants. Squash, cucumber, pumpkins, melons, beans, potatoes, lettuce, beets, chard and bok choy are all common hosts of aphid-transmitted viruses. The viruses cause mottling, yellowing or curling of leaves and stunting of plant growth. Although losses can be great, they are difficult to prevent through the control of aphids because infection occurs even when aphid numbers are very low; it only takes a few minutes for the aphid to transmit the virus while it takes a much longer time to kill the aphid with an insecticide.
A few aphid species attack parts of plants other than leaves and shoots. The woolly apple aphid, for example, infests woody parts of apple roots and limbs, often near pruning wounds, and can cause overall tree decline if roots are infested for several years.
These data as a whole emphasized the interest to use aphids as a biological models for agricultural interest considering that knowledge regarding aphid genome organization may be useful to improve biological/ chemical control strategies.

Fig. 1.2. (a) Typical distortion of leaf (b) sticky substance known as honeydew, which turns black with the growth of a sooty mold fungus (c) yellowing of the leaf typical plant disease (Image adjust by Google Image).

1.1.3 The Association Between Aphids And Their Host Plants

The known world fauna of aphids (Aphidoidea) consists of 4358 species, placed in 510 currently accepted genera (Stadler et.al., 2005). Of these, 2214 species in 284 genera spend all or part of their life feeding on trees. Thus, 50.7% of aphid species but members of 55.7% of aphid genera occur on trees. The discrepancy is mainly due to some large genera in the largest subfamily Aphidinae, whose members feed on the evolutionarily more recent families of herbaceous flowering

plants. It will be seen that all the other major groups of aphids are mostly or even entirely associated with trees.

The proportion of tree-living aphid species is probably even higher than indicated, as the unknown hosts of many species are likely to be trees. The trees most favored as hosts tend to be the older evolutionary groups such as Coniferae, Lauraceae, Fagaceae, Betulaceae, Hamamelidaceae, Ulmaceae and Juglandaceae, and it seems likely that the major groups of aphids differentiated before the appearance of herbaceous plants. Only three groups at or above the tribal level live only on herbs; the Saltusaphidinae which live on Cyperaceae and Juncaceae, the Siphini (subfamily Chaitophorinae) living on Gramineae, and the Tramini (subfamily Lachninae) living mostly on roots of Compositae (Grimaldi et.al., 2005) .

Aphids are predominantly a northern temperate group, with remarkably few species in the tropics. Dixon et al. (1987) postulated that the great diversity of the tropical forest fauna mitigates against short-lived host-specific insects such as aphids. Certainly the absence of aphids from many tropical forest trees is striking, with whole families (e.g. Dipterocarpaceae) seemingly almost immune from attack. The absence of records of aphids from economically important tropical forest trees such as mahogany (*Swietenia mahogoni*, Meliaceae) and rosewood (*Dalbergia nigra*, Leguminosae) can hardly be due to negligence by collectors, and suggests that aphids really do not occur on such trees, or at least do little damage. Aphidologists, however, think that the explanation for this can be found in the evolutionary history of aphids rather than in their present-day host relations or ecology. Psyllids have similar ecology and host relations to aphids, yet many tropical trees with few aphids bear a large psyllid fauna. It seems likely that aphids have failed to diversify in the tropics because of one particular, primitive feature of aphid biology, their cyclical parthenogenesis.

Cyclical parthenogenesis is a very successful way of exploiting the short-lived growth flushes of temperate plants, and aphids are thus a very successful group in temperate climates, using seasonal clues to time the alternation of the sexual and parthenogenetic phases of their life cycles (Harrington and Cheng, 1984; Williams et.al., 2000). Such life cycles cannot however be readily adapted to tropical conditions. Aphids moving from temperate zones into the tropics simply lose the sexual phase of the life cycle, and in doing so they lose the potential to evolve and diversify that is dependent on the recombination of genes. The tropics may also have acted in this way as a barrier to aphid colonization of southern temperate regions, which also have very small indigenous aphid faunas.

The occurrence of Neophyllaphis on Podocarpus, Araucaria and related conifers throughout the southern continents testifies to the age of aphid-tree relationships,

but very little is known about such evolutionarily ancient associations. Damage to the more recently introduced *Picea sitchensis* is particularly severe, heavy infestations resulting in complete needle loss. Aphid infestations have been shown to reduce the accretion of wood (Dixon, 1971a), and have deleterious effects on tree root growth (Dixon, 1971b).

Planted forests of exotic trees cover enormous areas of the globe. There are more than 5.5 million hectares of planted forests in Brazil, of which at least 40 are Eucalyptus spp. (Anon, 1985). *Pinus radiata* occupies only a small area in its native California but has been widely planted in New Zealand and elsewhere. During this century many European, oriental and American species of *Pinus* were introduced to various parts of Africa and grew aphid-free for many years. In recent times three aphid species, *Eulachnus rileyi* from Europe, *Cinara cronartii* from North America and *Pineus boerneri* of uncertain origin, have appeared on pines in Africa and caused far greater damage than they do in Europe or America. Similarly, *Cinara cupressi* is much more damaging to Cupressaceae in Africa than in Europe. These exotic conifers may be growing under stress, and the aphids are certainly without the complex of natural enemies associated with them in their countries of origin (Powell and Hardie, 2001).

Most aphid damage to trees seems to result directly from feeding, either by removal of sap or wounding of tissue, or in at least some cases by the toxic effect of saliva. Aphids are rarely recorded as vectors of viruses infecting trees (Biddle and Tinsley, 1967). Given the astronomical numbers of aphids in the air and the length of life of trees, there must be strong selection among trees for resistance to aphid-transmitted viruses. It would be interesting to know the mechanism of this resistance, and whether it could be transferred to shorter-lived crop plants. Perhaps the energy required to maintain such defences would be uneconomic for annual or biennial plants (Whitham et.al., 1981).

It is not known, instead, whether any aphids develop such long-term natural associations. Tree-dwelling aphids, especially those of the large subfamily Calaphidinae, tend to be rather more active insects than the aphids which colonize herbaceous plants, and may frequently move between trees - although the extent of movement by individual aphids is still largely unknown. Most aphid species in several other subfamilies alternate annually or biennially between their tree host and a herbaceous host, and therefore cannot develop genotype-specific associations, unless of course they were to return to the same tree year after year.

1.2 APHIDS- LIFE CYCLE

Aphids display a diverse range of relatively complicated life cycle indeed, they have many generations each year. Each life cycle is divided into a number of stages, with each stage characterized by one or more specialist morphs (Williams et.al., 2000). Each of this morphs has a specific function that is necessary for the completion of each stage of the life cycle. Typical aphid life cycle have morphs that specialize in reproduction, dispersal, and surviving severe or less favourable climatic or nutritional conditions.

Most pest aphids reproduce asexually throughout much of the year with adult females giving birth to live offspring (often as many as 12 a day) without mating or laying eggs (Blackman, 1981). The life cycle of aphids is an interesting aspect that highlights the high degree of specialization of aphids and adaptation to specific environmental conditions.

The result is a high biological potential, which uses on the one hand anphigonia and on the other hand thelytokous parthenogenesis (only females are generated). Thelitokous parthenogensis in animals are thought to be a relatively recent evolutionary phenomenon which have not had to stand the test of time (Balckman, 1981).

In general aphids are distinguished according to their complex life cycle, which can be of type holocycly or anholocycly (Fig 1.3). The first presents an alternation of parthenogenetic and anphigonic generations, whereas the second lacks of anphigonic generations therefore, absence of oviparus male and female. The holocycly strategy usually has annual development (only in a few groups, such as Adelgidi and Fordini, is biyearly) and consists of several parthenogenetic generations followed by one anphigonic generation (males and oviparae female) which concludes the seasonal activity with deposition over wintering egg (Williams et.al., 2000).

Conversely when the environmental conditions during the winter are compatible with the survival and the food resources are available, aphids reproduce parthenogentically all year and the cycle is called anholocyclic (in this specific case aphids loss the ability to produce sexual generation).

The same species of aphid can present both holocycly that anholocycly behavior (Blackman, 1974; Simon et.al., 1991; Helden and Dixon, 2002), according to environmental conditions and specific circumstances in which they develop. Aphids in some cases can even permanently lose the ability to originate the anphigonic generation leading to anholocycly obliged species.

Another interesting feature of the aphids, besides the presence of a biological plastic cycle, is given by the ability to contribute to the rapid population growth of aphids.

This typical reproduction is due to:

1. the cyclic parthenogenesis,
2. viviparous,
3. telescoping generation (each parthenogenetic female already contains her own granddaughters in embryonic state)
4. short time to give a generations (10 day for one generation)

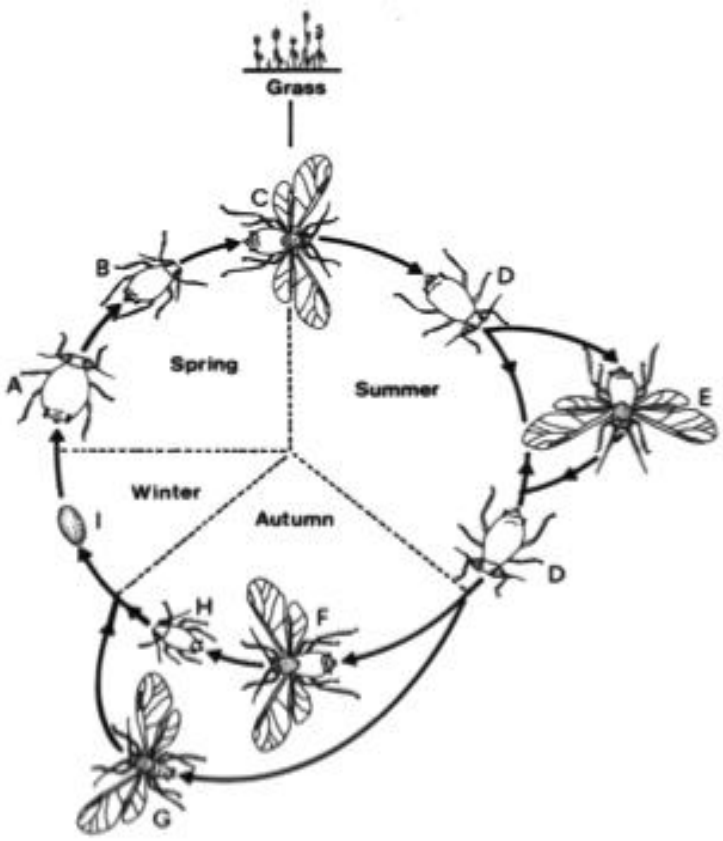

Fig.1.3. Schematic representation of biological cycle in aphids species (Jones et.al.,1977).

Regarding holocycly, both monoic and dioic life-cycle are known. About the first all aphid generations use for they life cycle only one host viceversa during the dioic cycle aphids pass from the primary host to another (Fig 1.4).

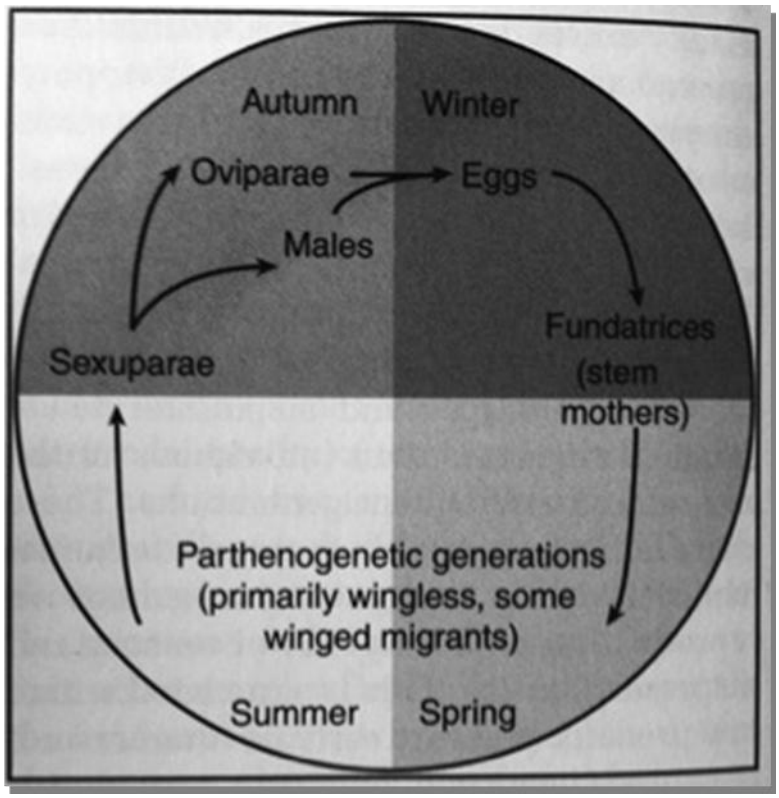

Fig.1.4. A generalized life cycle of a host-alternating (heteroecious) aphid (Image capture from the books "Aphids as crop pest" Edited by H.F.van Emden and Harrington).

In conclusion, it is important to highlight the high speed of reproduction associated with the plasticity of the biological cycle and the presence of winged forms involved to the diffusion, permit to the aphid species to colonize quickly new host thus become the most important pest crop.

1.3 THE MASTERS OF CLONING

Aphids are the cloning experts. A few yards from our door there may be hundreds of these tiny sucking- insects and all busy making identical copies of themselves. They have, moreover, hundreds of millions of years' experience of cloning, hundreds of millions of years in which evolution and natural selection have enabled them to overcome the problems and exploit all the advantages of clonal

reproduction (Harrington., 1994). Parthenogenesis in aphids is of the apomictic kind, without meiosis and hence without genetic recombination (meiosis is comparable at mitosis). Thus the descendants of a single foundress constitute a "clone" and are genotipically identical, although a number of alternative phenotypes may be expressed. In response to predictive environmental conditions, presexual and sexual morphs may be produced. These appears to be an "interval timer" (Lees, 1960) which requires a certain period of time to elapse before the offspring of a foundress are able to respond to conditions inducing sexual reproduction. In some aphid clones, the sexual generation can no longer be induced, and the clone will gradually diverge from others by accumulation of mutations. Parthenogenetic populations develop as a mixture of clones, with the most favored ones as potential dominators of the population. Populations that reproduce clonally can more readily evolve mechanisms for restricting their own population growth, because it is the survival of the clone that matters, not that of any one individual. In the world of aphids, natural selection acts on clones, and the individual is of little consequence, so thousands of individuals can be sacrificed as long as the clone survives to perpetuate its particular genetic constitution (its genotype). An aphid clone is in this sense a "super-individual", with all its parts – the individual aphids – acting in support of the whole. During the course of its life a successful clone may disperse over a large area, so that the "super-individual" becomes a diffuse organism that mortality factors acting at any one time or place are unlikely to kill.

However it is important to highlight that the term "clone", was coined over a hundred years ago but it is still in common parlance and widely used throughout the world, but its usage depends on definition, which is still obscure, especially if this involves the concept of genetic fidelity between clone mates rather than just the offspring from an asexual female founder (more correctly, an asexual lineage). To date, there have been no DNA sequencing studies proving such fidelity; on the contrary, the various DNA molecular marker studies performed on aphids display widespread genetic variation within and between different clonal lineages, as expected since mutation is a fundamental property of the DNA and hence the genome itself. In this overview, I will use aphids as a model system to show that, rather than being an unchanging evolutionary "dead end", asexual aphid lineages show rapid and widespread adaptive changes to changing ecological conditions in the field, including in relation to geography, host plant factors, and to insecticide applications. This being so, the so-called clone cannot be a fixed entity in time and space, but like all other living organisms in the real world, is evolving in response to its environment (Holman 2009).

Some modern molecular evidence gives credence to clonal fidelity, other evidence does not. For example the use of microsattelite markers has shown that aphids can have multilocus genotype which remain consistent over range of loci this suggest that such have descended from a common stem mother (Goldstein and Schlotterer, 1999). Instead cytogenetic evidence have suggested that aphids asexual lineages are not true clones because they show karyotype variants (Monti et.al., 2012). Therefore the meaning of clones don't supported the "clone" concept that generally has been attributed to the female offspring of aphids, but has only a semantic nature.

1.4 CYTOGENETICS KNOWLEDGE

1.4.1 Deficiency In The Genetic Knowledge Of Aphids

The genetics of aphids has been strangely neglected, considering their economic importance leading to a general and remarkable deficiency in our genetic knowledge of one of the worst pest for agriculture. At this regard there are several reasons: firstly, the animal's genetic informations has tended to advance on a rather narrow front; secondly, the extension of genetical studies in insects, in more recent years, has tended to encompass the interest of medical group rather than agricultural group. This situation could be explained in part because the schools of genetic research have the experience and interest for the medical and non-agricultural field, and in part because insects as Diptera-Culicidae-Simuliidae-Chironomidae, have giant salivary-gland or nurse-cell and polytene chromosomes facilitating the mapping of genetic markers and chromosomes rearrangements(Blackman,1981). Thirdly, many important crop-insects have a reputations, not necessarily justified, of being intractable subjects for genetic study for different reasons: 1) there are pratical difficulties in rearing or breeding them; 2) there are difficulties to obtain experimental material for studies of classical Mendelian genetics and cytogenetic; 3) finally a major problem is the observation at the microscope of their small chromosomes (Blackman,1981) .
With aphids, the difficulties of breeding them and undertaking any programme of Mendelian genetics are certainly real enough. No one has succeeded to reduce the time interval between sexual generations. However, there seems no reason why the problems of breeding aphids should be insuperable, and probably lack of success is in some degree a reflection of the few significant research efforts in this direction. If the difficulties could be overcome, aphids do have some significant compensating advantages for genetic work.

In the next paragraph I will analyze the the major motivations to use aphids as a biological model despite the few knowledge on the genetics of this taxon available to date.

1.4.2 Major Motivations To Examine In Depth The Cytogenetics of Aphids

The presence of many information on the biology of aphids is unfortunately not accompanied by a sound knowledge on the cytogenetics of this taxon. Indeed, in many cases the informations are limited to the description of the shape, the size and number of chromosomes. In the previous paragraph the difficulties to undertake any programme of mendelian genetics were described, but if the difficulties could be overcome, aphids do have a significant advantages (Blackman, 1981). The major ones are that 1) there is no theoretical limit to the number of eggs that can be obtained from one cross, as any number of oviparae or males can be obtained from one clone; 2) the parental genotypes can be maintained parthenogenetically under appropriate conditions for an indefinite length of time facilitating backcross experiments and making it possible to repeat crosses or backcrosses which are not initially successful; 3) any genotype can be maintained as a clone, so studies of the inheritance of characters which can only be observed by destructive methods are possible; for example, it is possible to study characteristics of morfology- cytology and enzyme biochemistry of one and the same genotype (Blackman,1981).
In the field of cytogenetics, aphids because of the easiness with which mitotic chromosomes can be obtained from embryonic tissues, represent a very useful model for a better understanding the nature of their chromosomes (Manicardi et al., 2002). Although most organisms possess chromosomes with localised centromeres (monocentric chromosomes), chromosomes with non- localized centromeres such as aphids, have been found in different taxa of the protist, vegetal and animal kingdom. Holokinetic chromosomes have been mainly described in animals -for example- they occur in all Nematoda (round worms) (Goday and Pimpinelli, 1986,1989): *Caenorhabditis elegans* is by far the most well-studied with holocentric chromosomes (Dernburg 2001; Maddox et al., 2004); Instead, among arthropods these chromosomes are typical of insects such as Dermaptera (earwigs), Heteroptera (stink bugs), Homopterans Sternorrhyncha (aphids mealyhugs, white flies), and Auchcnorrhyncha (cicadas, spittle bugs), Lepidoptera (butterflies and moths).
Because holocentric chromosomes lack a localized centromere, some researchers favor the term holokinetic rather holocentric. This specific chromosomes are

commonly referred to as having a diffuse centromere or kinetochore. Microtubule attachment during mitosis, is distributed along the whole length of the holocentric chromosome, in contrast to monocentric chromosomes, in which the kinetochore and hence microtubule attachment is localized to one region. Thus, chromosomes migrate broadside toward the poles in mitosis. Meiotic holokinetic chromosomes move end-on toward the spindle poles, as the chromosome ends assume the centromeric role (Fig.1.5.A) (Chung, et al. 2013). Ideally, they are the main criteria for the recognition of holokinetic chromosomes.

The presence of centromeric activity spread along the whole chromosomal axis permit to tolerate the chromosomal fragmentation; that situation not occur, obviously, in monocentric chromosomes, (Hughes-Schrader and Ris 1941). It has long been recognized that chromosome rearrangements and fragments that would be lost in monocentric chromosomes may be propagated and become fixed in organisms with holocentric chromosomes. Chromosomes resulting from the fusion of two holocentric chromosomes, for example, may align and segregate to a single spindle pole, whereas in organisms with monocentric chromosomes, the linkage of two chromosomes may result in the formation of dicentric chromosomes that fail to segregate properly (Dernburg, 2001). Conversely, fragments from fissions of holocentric chromosomes are retained and may be inherited in a Mendelian fashion, because they retain the capability to attach to the spindle apparatus. In addition to fission (which results as increases in chromosome number) and fusion (which results as decreases in chromosome number), which may be only weakly underdominant or nearly neutral in holocentric chromosomes, holocentric chromosome structure facilitates translocations and inversions. The ability to tolerate fragmentations makes the chromosomal karyotype of aphids more variable in chromosome number than is possible in organisms with monocentric chromosomes and explains the extraordinary heterogeneity of ploidy values found in some species of aphids. In the genre *Amphorophora*, for example, have been described diploidy values ranging from n= 2 up to n=36 (Blackman, 1980). The changes of the chromosomes number have been observed a lot of times even at the intraspecific level, and their origin is most likely related to the holocentric nature of these chromosomes (Blackman 1980). Similar intraspecific variation of the karyotype in aphids populations, are also possible due to the reproduction by parthenogenesis. Indeed, the presence of fragmented chromosomes do not allow a correct pairing of homologues in meiosis and could represent a serious obstacle to the reproduction as a consequence of absent or reduced production of gametes. The presence of numerous apomictic parthenogenetic generations allows the fragment move to the offspring without consequences. There are some species of

aphids including some strains of *Myzus persicae*, which have completely or partially lost sexual reproduction (anholocyclic populations) and the majority of the variation of the karyotype found in these populations seems to be explained by chromosomal changes that developed as a result of chromosomal fragmentation. After all considerations discussed above, the set up of a specific chromosomes painting probe followed by fluorescente in situ hybridization would allow a deeper and more accurate characterization of these rearrangements, providing not only to identify the specific chromosomes involved in the fragmentation, but also could be an alternative tool for the kariological study of insecticide resistant aphid strains.

Apomictic parthenogenesis is another important motivation that makes aphids interesting as biological model. This reproduction is a event without meiosis and hence, without genetic recombination, so meiosis is comparable to mitosis. Therefore offspring constitutes a "clone" that should be genotipically identical but cytogenetic evidence suggests that aphids asexual lineages are not true clones because they show karyotype variants (Loxdale 2003a,b). In the matter of mitotic behavior of holokinetic chromosomes, they have been important for recognizing holocentry in many organisms. Holocentric chromosomes (as restate more times) lack the primary constriction that was first recognized in 1880 as demarcating the centromere of monocentric chromosomes. The diffuse kinetochore becomes visible at the ultrastructural level in prophase. The kinetochore proteins extend longitudinally from isolated loci at interphase to form a continuous linear body oriented in the outside chromatid grooves at metaphase. At metaphase, the chromosomes align parallel to the equator of the metaphase spindle and lie entirely within the spindle. Microtubule attachments are distributed along the kinetochore, so that at anaphase the chromosomes move broadside on to the spindle poles (Meters et.al., 2012) (Fig. 1.5).

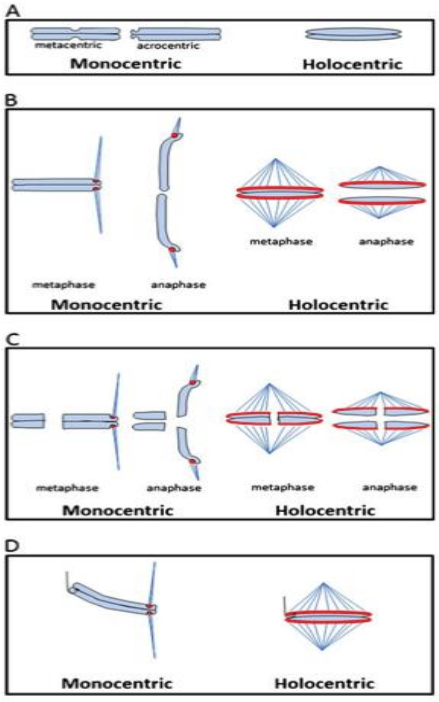

Fig.1.5. **A)** Monocentric chromosomes have a primary constriction. In contrast, holocentric chromosomes lack a primary constriction. **B)** During anaphase, monocentric chromosome are pulled by spindle microtubules from a single point where the kinetochore is assembled In holocentric chromosomes, the kinetochores are assembled along the length of the chromosome, and during anaphase, the two sister chromatids maintain their parallel relationship **C)** Upon chromosome fragmention, only two fragments of a monocentric chromosome will retain

kinetochore function and segregate to the spindle poles. In holocentric chromosomes, all fragments migrate to the poles. **D)** Micromanipulation of monocentric chromosome ends during metaphase results in the movement of just the chromosome end. In contrast, in holocentric chromosomes, where spindle microtubules display pulling forces over the length chromosome, the entire chromosome will be perturbed (Image from the article Meters et.al., 2012) .

Although the mitotic behavior of holokinetic chromosomes is unique in terms of orientation and pattern of chromatid migration, variations in their meiotic behavior determine the occurrence of the pre-reductional and post-reductional (inverted meiosis) types of meiosis (Nokkala et.al., 2002). The aim of meiosis was to reduce the chromosome number so haploid gametes are produced from a diploid parent cell. Reduction of chromosome number in meiosis happens because a single round of DNA replication is followed by two rounds of cell division. Correct chromosome segregation in meiosis requires changes in kinetochore geometry and differences in release of sister chromatid cohesion relative to mitosis. Holocentric chromosomes encounter many problems during meiosis that organisms with monocentric chromosomes do not face. We review several diverse mechanisms that have arisen in holocentric organisms to allow correct distribution of chromosomes during meiosis.

The way a chromosome divides is based on its geometry. Holocentric chromosomes can theoretically attach to the meiosis I spindle at many positions along their length. Therefore, if a holocentric bivalent has no modification to its chromosome structure or kinetochore positioning, its microtubule capture surfaces will face in all directions. Depending on how cohesion is released, chromosomes could segregate randomly or not at all. Obviously, holocentric organisms require special adaptations to allow correct segregation of one chromatid to each gamete. In other words in meiosis I, if there is no alteration of chromosome structure or attachment surface position, attachment sites can face in all directions, leading to problems in chromosome segregation. An alternative solution to the problem of holocentric chromosomes in meiosis is to invert the meiotic divisions, so that sister chromatids separate in meiosis I and homologues separate in meiosis II (Fig 1.6.C) (Mola et.al, 2006). In inverted meiosis, chromosomes align differently than in cases of restricted kinetochore activity. Sister chromatids face opposite poles and separate from one another in anaphase I. From a cytological perspective, there are differences between the pre and post reductional meiosis depending on the orientation of the bivalent (Hughes-Schrader 1955 and White 1973).

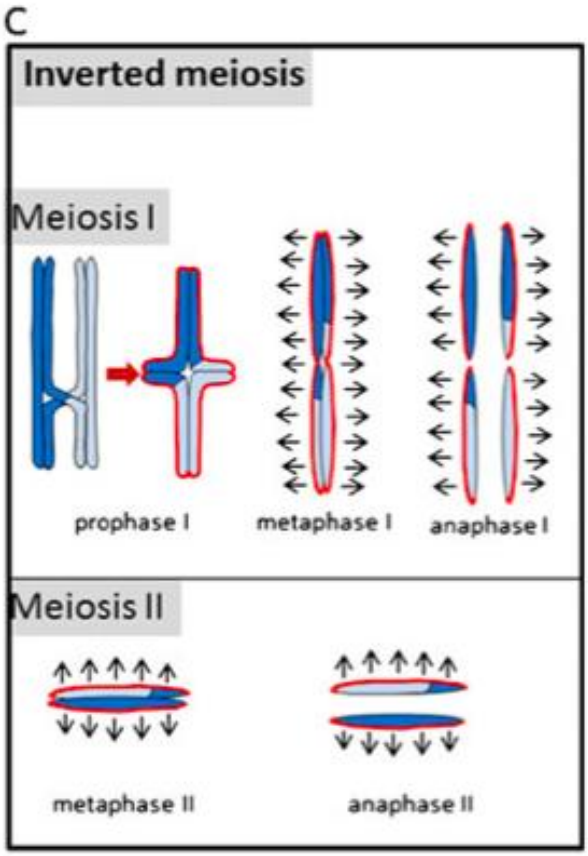

Fig 1.6.C In inverted meiosis, sister chromatids separate in the first meiotic division. It is speculated that this separation happens because chiasmata are terminalized in prophase of meiosis I, such that chromosomes are arranged in metaphase I with sister chromatids facing opposite poles. In anaphase I, all chromatids separate. Then, homologous chromatids re-pair prior to meiosis II (Image by Melters et.al.,2012).

In pre reductional meiosis, bivalents are oriented axial to the spindle, i.e. with their long axes parallel to the polar axis; while in post reductional meiosis, bivalents are oriented equatorial to the spindle, i.e. with their long axes perpendicular to the spindle (Fig 1.7) (Nokkala 1996, 2002).

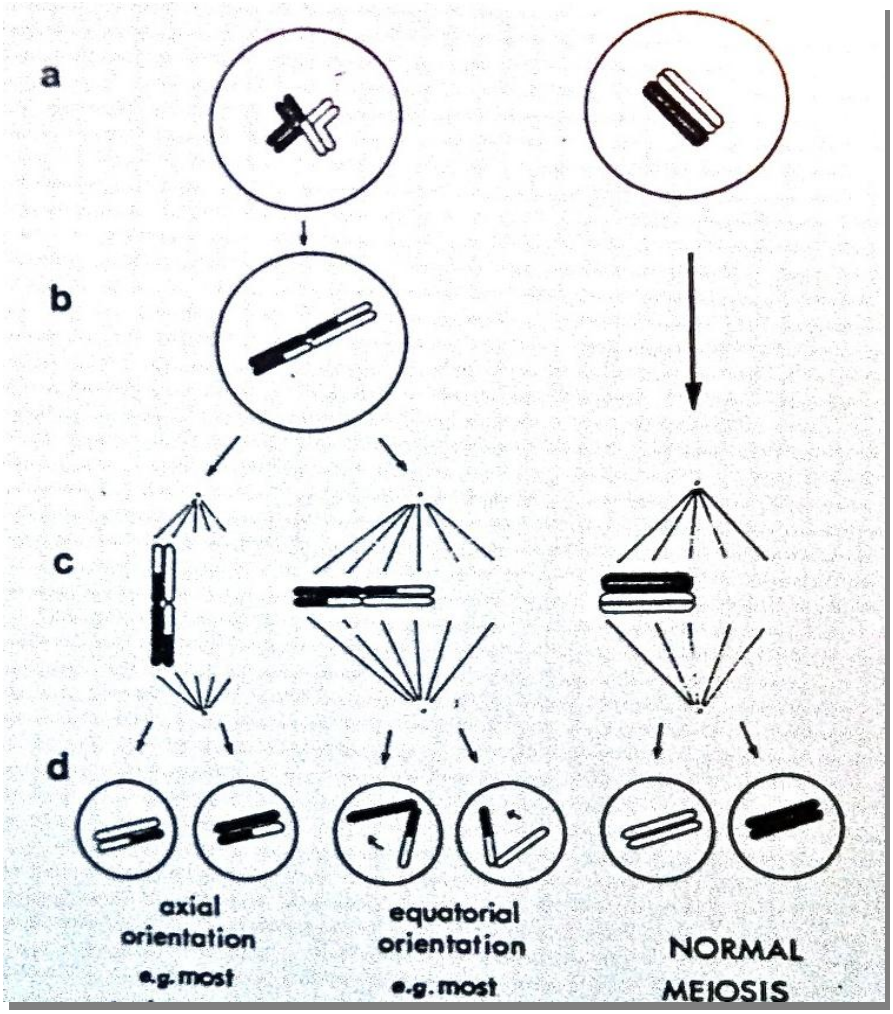

Fig.1.7. Types of first meiotic division illustrated diagrammatically by the behavior of a single holocentric bivalent. In black the paternal chromosome and maternal chromosome in white. After diakinesis the orientations at methaphase may be either (1) axial, in which case the first meiotic division is reductional for non crossover regions or (2) equatorial in which case the first division is equational for non crossover region (inverse meiosis). In (3) the simplest case of a normal first meiotic division with no crossing over is shown.

About the sex determination another interesting things regards the dosage compensation because as males and females differ in their optimal values for most phenotypic traits, selection often runs in opposite directions in the two sexes, a situation called sexual antagonism (Cox et.al., 2009). Since males and females share most of their genome, intra-locus conflicts appear when the same gene is selected for different optima in each sex. Because sex chromosomes have a sex-biased transmission pattern they are expected to accumulate different types of

sexually antagonistic mutations than autosomes, as originally shown by Rice (1984) and elaborated by further models (Jordan et.al., 2012). Aphids display an XX/X0 system and combine an unusual inheritance of the X chromosome with the alternation of sexual and asexual reproduction. Hence, in my study I compared male aphids with oviparous female in order to evaluated if the evolution of sex-chromosomes require gene dosage compensation.

Here-hence the rising question:X-linked genes expression are in balance between the sex or not? However, there are others features that make interesting the aphids as a model for example the easy availability in nature and facilities in their lab-rearing, because they do not require special needs, only temperature and photoperiod are necessary conditions. Marcovitch (1924) first demonstrated that photoperiod was important in the induction of sexual morphs, suggesting that a short photoperiod was associated with their production. Furthermore aphid ovarioles can contain up to two following generations of developing offspring, and this telescoping of generations may allow offspring to be influenced by the maternal investment into embryos. In aphid reproduction, the trade-off between number and size of offspring is partly determined by the size-distribution of the embryos in the aphid ovarioles (Blackman, 1981). A large number of small embryos results in a high rate of offspring production, whereas more varied sizes of the embryos, with a sharper increase in size towards the ovariole posterior, can result in fewer but larger offspring (Dixon and Dharma 1980a). Not only, these generations are produced in a very short of time and contributes to the rapid population growth thanking to: 1) presence of cyclic parthenogenesis, 2) a short generation time (average 10 days), 3) viviparity (excluding Philloxeridae and Adelgidae) and above all 4) each parthenogenetic female already contains her own granddaughters in embryonic state before she gives birth herself (telescopic generations). Furthermore Blackman (1981) has estimated that in two generations one anphigonyc female is capable of generating 50 daughters and could produce 1250 grandchildren while, each parthenogenetic female, in the same interval of time, will be able to produce 312 250. 000 descendants. The high speed of reproduction, associated to the plasticity of the biological cycle and the presence of winged forms delegated to diffusion, permit at aphids species to colonize extremely quickly new plants and make them one of the greatest dangers in the agricultural field. Therefore, the knowledge about the genetics of these organisms may be extremely useful for the study of the morphology of chromosome holocentric / holocinetic, in order to verify if the structure of the chromosome holocentric follow the rules of the acquisition of information about aphid's genetics could be a useful tool in order to:

1. establish the species that they belong. Aphids of different species, often, coexist on the same host plant and, given the scarcity of morphological characters specific to each species, it may be difficult to identify them. However it can be a problem when considering that aphids of different species have different capacities, for example to act viral vector. For example within the genus *Myzus*, many species live simultaneously on the peach tree, but few species are vectors of viral diseases. Regarding agrarian's point of view, it could be very useful to have genetic markers able to identify the aphids present in specific host. Such recognition can not be based on the kariotype analysis because the chromsomes number is a variable value within the same species, but may be based using species-specific chromosome markers (such as DNA satellites) or molecular analysis (as microsatellites analyses).
2. Develop new strategies. So far, the fight against this great scourge was conducted using pesticides (carbamates, organophosphours, ecc.) and such products are, however, relatively little specific and may cause damage, not only to other insects (including beneficial insects), but get also at the level of aquifers and, more in general to the enviromnel as a whole. Furthermore, it is increasingly evident that aphids have now developed pesticide resistance mechanisms making it necessary to use in higher and higher doses, with less and less satisfactory results. It therefore becomes increasingly pressing need to develop strategies to combat alternatives to those chemicals that are more selective and, as much as possible, eco-compatible.

1.5 EXPERIMENTAL MODEL: *Myzus persicae*

The green peach aphid, *Myzus persicae* (Sulzer) (Fig 1.8), is found throughout the world, including all areas of North America, where it is viewed as a pest principally due to its ability to transmit plant viruses. In addition to attacking plants in the field, green peach aphid readily infests vegetables and ornamental plants grown in greenhouses. This allows high levels of survival in areas with inclement weather, and favors ready transport on plant material.
When young plants are infested in the greenhouse and then transplanted into the field, farm lands will not only be infested by aphids but insecticide resistance may be introduced. These aphids also can be transported for long distances by wind and storms.

Fig.1.8. Image that show Myzus persicae species into the foreground (Image from Website).

Green peach aphid feeds on hundreds of host plants in over 40 plant families. However, it is only the viviparous in summer stages that feed so widely; the oviparous (egg producing) winter stages are much more restrictive in their diet choice. In temperate latitudes the primary or overwintering hosts for *Myzus persicae* are trees of the genus *Prunus*, particularly peach and peach hybrids, but also apricot and plum. During the summer months the aphids abandon their woody hosts for secondary or herbaceous hosts, including vegetable crops in the families Solanaceae, Chenopodiaceae,Compositae, Cruciferae, and Cucurbitaceae. Vegetables that are reported to support green peach aphid include artichoke, asparagus, bean, beets, broccoli, Brussels sprouts, cabbage, carrot, cauliflower, cantaloupe, celery, corn, cucumber, fennel, kale, kohlrabi, turnip, eggplant, lettuce, mustard, okra, parsley, parsnip, pea, pepper, potato, radish, spinach, squash, tomato, turnip, watercress, and watermelon. Field crops such as tobacco, sugar beet, and sunflower also are attacked (see on the left Figure 1.9.).

Fig.1.9. Aphids the major pest crop (Image from Website).

Numerous flower crops and other ornamental plants are suitable for green peach aphid development. Stone fruit crops such as peach are sometimes damaged before the aphids leave for summer hosts. Crops differ in their susceptibility to green peach aphid, but it is actively growing plants, or the youngest plant tissue, that most often harbors large aphid populations (Heathcote 1962). In warmer climates the aphids do not seek out overwintering hosts, but persist as active nymphs and adults on hardy crops and weeds throughout the winter months. Green peach aphids can attain very high densities on young plant tissue, causing water stress, wilting, and reduced growth rate of the plant. Prolonged aphid infestation can cause appreciable reduction in yield of root and foliage crops. Early season infestation is particularly damaging to potato, even if the aphids are subsequently removed (Petitt and Smilowitz, 1982).

The major damage caused by green peach aphid is through transmission of plant viruses. Indeed, this aphid is considered by many to be the most important vector of plant viruses throughout the world. Nymphs and adults are equally capable of virus transmission (Namba and Sylvester 1981), but adults, by virtue of being so mobile, probably have greater opportunity for transmission. Both persistent viruses, which move through the feeding secretions of the aphid, and non-persistent viruses, which are only temporary contaminants of aphid mouthparts, are effectively transmitted. Kennedy et al., (1962) listed over 100 viruses transmitted by this species. Some of the particularly damaging diseases include potato leafroll virus and potato virus Y to Solanaceae, beet western yellows and beet yellows viruses to Chenopodiaceae, lettuce mosaic virus to Compositae, cauliflower mosaic and turnip mosaic viruses to Cruciferae, and cucumber mosaic and watermelon mosaic viruses to Cucurbitaceae. A discoloration in potato tubers, called net necrosis, occurs in some potato varieties following transmission of potato leafroll.

In addition *Myzus persicae* represents an important cytogenetic model, because it shows the presence of genetic differences among clones, highlighting the extremely dynamic nature even at the level of karyotype. Moreover some *M. persicae* clones shows recurrent fissions of the same chromosomes in the same regions, thereby suggesting that the *M. persicae* genome has fragile sites that are at the basis of the observed changes in chromosome number. In other words this aphid species shows chromosome polymorphisms in term of number, sometimes extensive (Blackman & Eastop, 2007). In the peach potato aphid *Myzus persicae* (Sulzer), variations in the chromosome number and structure have been observed. These are mainly the result of chromosomal deletions, translocations and occasionally, fragmentations, that give rise to an increased chromosomes number (Blackman, 1980; Lauritzen, 1982). For example, several populations of *M.*

persicae were heterozygous for a translocation between autosomes 1 and 3, and this particular rearrangement has been shown to be involved in resistance to organophosphate and carbamate insecticides (Blackman, Takada & Kawakami, 1978; Spence & Blackman, 1998). The standard karyotype of this insect is 2n=12 but reccorent karyotipic variant was found among and even within lineages from 2n=13 to 2n=18. *Myzus persicae* populations with 13 chromosomes have also been identified in different countries as a result of autosome 3 fission (Blackman, 1980). Interestingly, at least two independent and diverse fragmentations of the autosome 3 were reported (Blackman, 1980; Lauritzen, 1982), suggesting that different naturally occurring rearrangements of the same chromosome may be observed in this aphid's karyotype (Blackman, 1980; Lauritzen, 1982). In some *M. persicae* populations, a further fission of autosome 2 give raise to karyotype consisting of 2n = 14 chromosomes (Blackman,1980; Lauritzen, 1982), making this species a good experimental model for the study of chromosome rearrangements in this taxa (Spence & Blackman, 1998).

The presence of genetic differences among clones is potentially very important in worldwide attempts to control aphid pests. This is because several studies have clearly shown that cryptic sympatric speciation occurs in a wide range of aphid species (Loxdale and Lushai 2007; Loxdale 2008a, 2010b), including evidence of rapid chromosomal changes affecting host adaptation-speciation events, as, for example, in the corn leaf aphid, *Rhopalosiphum maidis* (Fitch) and in the global insecticide resistant pest, the peach potato aphid, *Myzus persicae* (Sulzer) (Blackman 1987; Brown and Blackman 1988; Field and Blackman 2003).

In view of their impact in agriculture (in particular, in terms of plant pathogenic virus transmission), aphids need to be controlled by pesticides and/or using biological control agents (see Van Emden and Harrington 2007). However, in the absence of a thorough understating of the genetics of aphid populations/clones, it is difficult to properly evaluate the presence of transmissible and adaptive variations that may well make biological and chemical controls less effective.

Moreover the *Myzus persicae* model was used to evaluated the structure of the telomeric sequence (TTAGG) in order to study and discuss the presence of mechanisms involved in *de novo* telomere synthesis. The aim of telomeres analysis in a strain with a variable chromosome number lies in the fact that aphid telomerase can initiate the *de novo* synthesis of telomere sequences at internal breakpoints, resulting in the stabilization of chromosomal fragments.

It must be important to note that telomeres are specialized DNA–protein structures constituting the end 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 chromatin remodelling at cell division. Telomeres are partially lost at each replication cycle in most somatic cells due to incomplete replication of the DNA molecule end (Blackburn 1991). This loss can be avoided by telomere elongation mediated by reverse transcription due to telomerases—ribonucleoprotein enzymes, that are highly conserved from unicellular organisms to flowering plants and vertebrates (Krupp et al. 2000). Nevertheless, not all eukaryotes possess telomerase and some organisms compensate for telomere loss by different mechanisms, including the insertion of mobile elements and unequal recombination between long tandem repeats (Biessmann and Mason 2003; Pardue and DeBaryshe 2003). Therefore in this elaborate we also identified the telomerase (TERT) coding gene and analyzed its expression pattern in different tissues and developmental stages in order to verify if TERT transcription in aphids is highly regulated as reported in humans (Krupp et al., 2000), or diffuse as observed in some insects (Sasaki and Fujiwara 2000; Honey Bee Genome Sequencing Consortium, 2006).

If we come back to the headline "chromosomal variability", another concept at the basis of variability will come soon at the mind: retrotransposable elements. The study and deepening of retrotransposons is of great interest because they represent an important genetic component because it can lead to the onset of mutations, thus becoming a source of genetic variability. In aphids, the reproduction is based on the apomictic parthenogenesis (where meiosis is comparable at mitosis without recombination) so is interesting to search the mobile elements in their genome. In the past the only source of genetic variability sufficient to ensure the evolution and survival of the species, was caused by meiotic crossing over during autumn anphigony. The genetic variability that may be linked to mobile genetic elements leads to revise the concept of clone in aphids (and supporting its semantic nature), because mobile genetic elements could represent a source of variability that aphids could use during the parthenogenetic generations.

The high number of parthenogenetic generations that are present in each breeding season (about 40) make the transposition, although uncommon, an important event. Therefore, there are different point of view that permit to considered the research of mobile elements in the aphids very interesting :

1. regard to the type of playback implemented by these insects, the presence of transposons in their genome, can be a useful tool in order analyse the genetic variability during the parthenogenetic reproduction (since in these generations is completly absent the main source of genetic recombination);
2. these retrotransposable elements could be a useful tool as a chromosomes markers because the trasposons-cluster could be chromosome-specific;

3. Transposons, could become a genetic carriers for cells in metazoans alternative to viral vectors (Spradling et al. 1982; Engels 1983; Karess al. 1984) after their structure change. The development and the extreme spread of the technology of DNA, indicated that such techniques can represent a very useful tool for manipulating the genome of various organisms (we consider in this respect the results obtained with *Drosophila melanogaster*). They could therefore be potential vectors useful to modify the genome of insect pests or vectors of viral diseases of animals and plants obtaining control and opening thus the way to a fight targeted against these harmful agents (Kimura et al. 1993; Kidwell 1993; Shukle et al. 1995).

In conclusion the primary goal of this PhD thesis is a deep study of genetic and cytogenetic mechanisms underlying the intra-and inter individual variability, also verifying if genetic differences among clonal lineages could have an adaptative effect as host choice and speciation events.

At this regard, I investigated different strains of the aphid *M. persicae* analyzing in detail their karyotype by using different chromosomal marker (sub-telomeric probe, HIND200 satellite repeat X chromsomes specific probe, TRAS element probe) and evidencing cytogenetic changes that occurred both at an inter- and intra-individual level.

Therefore, this elaborate will try to answer to these questions: what is at the basis of the chromosomal variability? Which are the biological properties of chromosomal fragments stabilization? Which are the molecular tools to investigate at this end?

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2 MATERIAL AND METHODS

2.1 *Myzus persicae:* COLLECTING SITES AND REARING

Specimens of *M. persicae* used for these experiments were collected mainly from peach (*Prunus persica* (L.) Batsch) orchards, but also from herbaceous hosts like tobacco, tomato, potato and aubergine at various locations in different areas of Italy as shown Fig 2.1 and table 2.2, and maintained as parthenogenetic female colonies on pea-seedlings (*Pisum sativum* cv 'Meraviglia d'Italia') under constant environmental conditions: 21°C, 16 h light: 8 h dark photoperiod.

Fig 2.1. Geographic distribution of the sampling sites.

Name	Mp_Strain	Crops	Region	Province
Paglieta	64H	Tobacco	Abruzzo	CH
Torino_99-01a	46	Peach	Piemonte	TO
Cuneo_97-01	14	Peach	Piemonte	CN
Como_03-01	83	Peach	Lombardia	CO
Benevento -Tabacco (A)	48	Tobacco	Campania	BN
Scafati - Tabacco (B)	49	Tobacco	Campania	SA
Padova_99-01b	55	Peach	Veneto	PD
SIPCAM	3	Peach	Emilia-Romagna	BO
Piacenza_03-01	81	Aubergine	Emilia-Romagna	PC
Piacenza_96-01x	17	Tomato	Emilia-Romagna	PC
Bologna_00-02	68	Peach	Emilia-Romagna	BO
Bologna_98-02	24	Peach	Emilia-Romagna	BO
FC11-01	102	Peach	Emilia-Romagna	FC
Bologna_99-02a	45	Peach	Emilia-Romagna	BO
Bologna_99-02b	59	Peach	Emilia-Romagna	BO
Mainardi 99	37	Peach	Emilia-Romagna	FE
Rivergaro	16	Peach	Emilia-Romagna	PC
Ferrara_01-01	71	Peach	Emilia-Romagna	FE
Patologia	31	Tomato	Emilia-Romagna	PC
PC-Serra	69	Aubergine	Emilia-Romagna	PC
Savorani 2	27H1	Peach	Emilia-Romagna	RA
Ferrara_97-02	12	Peach	Emilia-Romagna	FE
Ravenna_99-11dh	62H2	Peach	Emilia-Romagna	RA
Forlì_10-01h	92H5	Peach	Emilia-Romagna	FC
SIPCAM - Verde/Nero	63	Peach	Lombardia	LO
Pescara_99-01	56	Tobacco	Abruzzo	PE
Moresco	58	Peach	Marche	AP
Cerignola-Foggia	76	Peach	Puglia	FG
Pescara_99-02	57	Tobacco	Abruzzo	PE
Salerno_99-03	51	Tobacco	Campania	SA
Cavour 99 (NEEM)	53	Peach	Piemonte	TO
US1L-H	33H	Lab	Piacenza	RF
Sardegna	72	Peach	Sardegna	CA
Pisa	26	Lab	Toscana	PI
Danese (1)	54H2	Peach	Veneto	PD
Cosenza_97-01	22	Peach	Calabria	CS
Catanzaro_98-01	23	Potato	Calabria	CZ
Catanzaro_99-01	34	Peach	Calabria	CZ
San Prospero - Imola	109	Peach	Emilia-Romagna	BO
Martorano - Cesena	112	Peach	Emilia-Romagna	FC
S. Martino in Fiume - Cesena	113	Peach	Emilia-Romagna	FC
S. Salvo	114	Peach	Abruzzo	CH
Cesena	125	Peach	Emilia-Romagna	FC
Imola	127	Peach	Emilia-Romagna	BO
Bagnacavallo	133	Peach	Emilia-Romagna	RA
Imola	142	Peach	Emilia-Romagna	BO
Cerignola	145	Peach	Puglia	FG
Lanuvio	155	Peach	Lazio	Roma
Cesena	92	Peach	Emilia-Romagna	RM
Casalfiumane	68	Peach	Emilia-Romagna	BO
VR02-01	78	Peach	Veneto	VR
RA11-03	100H	Peach	Emilia-Romagna	RA
FE98-02	7	Peach	Emilia-Romagna	FE
Lodi03-01	84	Peach	Lombardia	MI
VR03-01	86	Peach	Veneto	VR
VR03-02	87	Peach	Veneto	VR
CZ04-01	90	Peach	Calbria	CZ
BO11-01	93	Peach	Emilia-Romagna	BO
BO11-03	95	Peach	Emilia-Romagna	BO
Calabria-Plenum	70H	Peach	Calabria	CZ

Table 2.2. List of the Italian populations of *M. persicae*

2.2 CYTOGENETIC TECHNIQUES

2.2.1 Chromosome Preparation By Spreading

In order to carry out cytogenetic investigations the chromosome preparations from parthenogenetic females were obtained by spreading embryo cells and by squash preparation of single embryos.
Adult wingless females were dissected in 1% sodium citrate hypotonic solution and embryos were kept in the same solution for about 30 min.
The embryos were then transferred to minitubes and centrifugated at 400 x g for 3 min.
Methanol-Acetic acid 3:1 was added to the pellet, which was made to flow up and down for 1 min through the needle of a 1 ml hypodermic syringe to disaggregate the material, followed by a further centrifugation at 1.000 x g for 3 min. This step was repeated with fresh fixative.
Finally the pellet was resuspended in new fixative and 20 µl of cellular suspension was dropped onto clean slides and stained with 5% Giemsa solution in Soerensen buffer pH 6.8 for 10 min.

2.2.2 *Chromosome Banding and Staining* (AgNO3- CMA3-DAPI)

AgNO3

To evidence the nucleolar organizer regions (NORs) two solutions were used and mixed together: the first solution is obtained dissolving 0.25 gr of AgNO3 in 500 µl of distilled water, the second solution called ‘’colloidal developer’’ is obtained dissolving 2 gr of gelatine in 100 ml of distilled water and to add 1 ml of formic acid to bring the pH to 3.
Adding 60 µl of AgNO3 and 30 µl of ‘’colloidal developer’’ on each slide until the color became yellow-brown. To stop the reaction it is necessary rinsing with distilled water. Finally the slides, completely dry, were mounted with DPX. Slides were examined using a Nikon Eclipse 80i microscope and photographs taken using Nikon digital sight DS-U1.

CMA3-DAPI

The study of the DNA base composition of heterochromatin was possible using specific fluorochromes such as chromomycin CMA3 (which has a specificity of staining for GC-rich sequences) (Schweizer, 1976; Donlon and Magenis, 1983) and DAPI which has a specific affinity for AT-rich sequences).
100 µl of CMA3 (0.125 mg / ml in PBS with the addition of 10 mM MgCl2) were put on each slide; each slide was covered with coverslips and put in a wet chamber in the dark to avoid the decay of the fluorescent signal for 15 minutes at room temperature; slides were rinsed with distilled water, was shaked off fron excess water and mounted with 40µl of DABCO.
Slide were preserved in the refrigerator at least 3 hours before observation.
For DAPI staining 100 ul of DAPI solution (100ng/ml) were put on each slide ,in a wet chamber in the dark for 10 minutes.

C-Banding

The constitutive heterochromatin could be highlighted using C banding exploiting the technique of Sumner (1972), which provides the transition in barium hydrate in order to facilitate the control of the denaturation.
The slide is placed in a coplin jar containing 0.2N HCl for 30 minutes at room temperature, rinseed in tap water and distilled water and dipped in a 5% solution of barium hydrate pre-warmed at 50° C for four min.
Slides are rinsed in tap and distilled water, transferred in 2 x SSC (0.3 M NaCl + 0.030 M sodium citrate) for 1 hour at 60 ° C and finally rinsed again under running water. Slides must be dry before proceeding to the staining.

2.2.3 Measurament Of Chromosomes Length

Morphometric analyses of mitotic plates were carried out on metaphases plates using the software MicroMeasure, freely available at the Biology Department at Colorado State University website (http://rydberg.biology. colostate. edu/ MicroMeasure).

2.2.4 Fluorescent In Situ Hybridization (FISH)

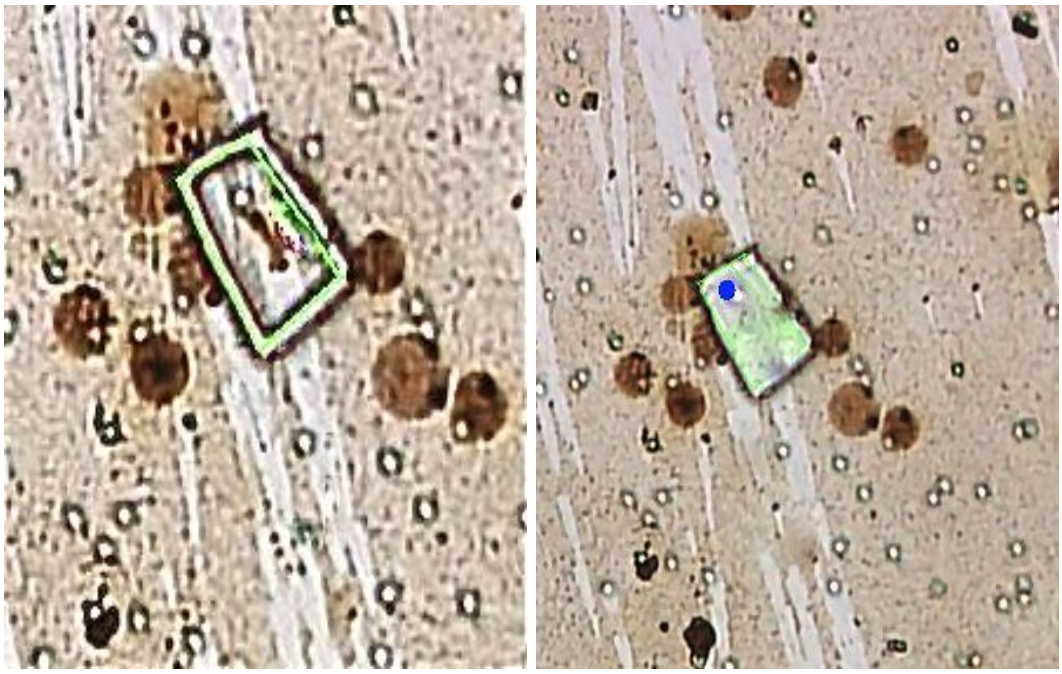
Chromosome preparations were pretreated with 200 μg/ml RNase diluted in PBS, in a humid chamber. the slides were treated with 100 μl of this solution for 30 min at 37°C and successively rinsed with distilled water; afterwards, the slides were treated with Pepsine 20 μg/ml in PBS, for 10 min a 37°C (100 μl for each slide), fixed in freshly depolymerized paraformaldehyde 4% for 15 min at room temperature and washed in tap water for 15 min in a coplin jar.
50 ml of denaturing solution (70%formamide, 2 X SSC) were prewarmed at 70/ 72°C, then slides were incubate for 5 min and 30s (be careful not always the denaturation's time is the same); immediatly after, the slides were immersed through a sequence of cold (-20 °C) ethanol washes in a coplin jar for 2 min (70%, 80, 90, 100%).
The probe cocktail containing 450 ng of the labeling, 50% formamide, 2 x SSC, 0,25% SDS, 10% dextran-sulfate in final volume of 30 μl was prepared .and the probes were denatured in a boiled water for 7 min and placed on ice;
30μl of the probe were added to each slide which is covered with coverslips. The hybridization was carried on for 15 hours or overnight at 37 °C.
The next day, slides were washed twice for 15 min, in 1 x SSC at room temperature, twice in 0.1 x SSC at 37°C. Finally in PBS pH 7.2 – 7.4 at room temperature for 5 min: It must be remembered that all the washing solutions should be carried out in stirred conditions.
Preincubation was carried out in *blocking reagent* 1x in PBS at 37°C for 30 min; detection was performed at 37°C with anti-digoxigenin antibody-antifluorescein isothiocyanate conjugate (FITC) for 45 min (1:400 in *blocking reagent* 1x). Slides were then washed in two washing steps with PBS for 10 min at room temperature.
After FISH, the chromosome were counterstained with 200 ng/ml (diluted in PBS) of Propidium Iodide for 15 min at room temperature and mounted in DABCO antifade mounting medium.
FISH slides were observed using a Zeiss Axioplan epi-fluorescent Microscope: Images were taken by means of a CCD camera (Spot; Digital Instrument, MA) using the Spot software supplied with the camera and processed with Adobe Photoshop (Adobe Systems, Mountain View, CA).

2.2.5 Fiber FISH

Slides with DNA fibers have been obtained from chromosomes fixed onto slides by spreading. Successively, as soon as an iridescent halo appeared on the slide drying surface, slides have been washed in 1× PBS pH 7.4 for 2 min before dropping onto the slides 60 μL of a NaOH/etha- nol solution (0.07 N NaOH/absolute ethanol 5:2) that has been smeared on the slide with a cover slip. Successively, 2 drops of methanol have been added and, after 20s, 2 further methanol drops have been added before placing the slide in vertical position for draining them. Successively, further 4 drops of methanol have been added.
When dried, slides have been examined by phase-contrast microscopy, dehydrated in an alcohol hydration series (alcohol 70%, alcohol 90%, ethyl alcohol
95%, alcohol 100%) before FISH experiments. Fiber FISH hybridization has been performed according to previously reported protocol, whereas slides have been observed using a laser-scanning confocal microscope SP2-AOBS (Leica).

2.2.6 Microdissection And Preparation Of Sex-Chromosomes for FISH Experiments using a Painting Probe

Laser capture microdissection (LCM) is a process to collect pure cell populations for subsequent DNA and RNA extraction. Although laser microdissection is generally used to isolate specific cells from fixed tissue sections, it has been also effective for isolation of individual chromosomes. The microdissected chromosomes could be used for chromosome paints (Kubalàkovà et.al., 1997) or FISH hybridization.
One challenge of microdissecting chromosomes is the minimal diameter of samples collected. Non-contact LCM transfers the dissected pure material directly into a collection device. This enables the fast procurement of a homogeneous specimen of just 0.5 μm in diameter without intrusion into the adjacent area.
The first step for the chromosome preparation requires the use of a specific slide called " MembraneSlide 0.17 PEN". This membrane is easily cut together with the sample and acts as a stabilizing backbone during lifting.
Therefore even large areas can be dissected by a single laser pulse without affecting the morphological integrity (see below in Fig 2.3).



Step1 ***Step2***

Fig 2.3. Action of Microdissector laser catapulting with aphid's chromosomes (these image were captured directly during the microdissection from Laboratorio di Patologia molecolare from Locarno, Swizerland).

Mitotic chromosomes were prepared from embryos of *M. persicae* parthenogenetic females, essentially as described in Bressa et al. (2009). The embryos were dissected out from the females, hypotonized for 20 min in 75 mM KCl, and fixed for 15–30 min in freshly prepared Carnoy fixative (ethanol/chloroform/acetic acid, 6:3:1). Then the embryonic cells were spread in a drop of 60% acetic acid on a glass slide coated with a polyethylene naphthalate membrane (PEN membrane slide) using a heating plate at 40-45°C. Shortly before spreading, the membrane was treated with UV light for 30 min to prevent DNA contamination. Then the preparation was passed through an ethanol series (70%, 80%, and 100%, 30 s each), air-dried, and stained with 5 % Giemsa for 5 min. Now the slides are ready to be catapulted by a single laser pulse into the cap (containing 3 µl of mineral oil) of a PCR tube using the PALM MicroBeam Carl Zeiss laser Microdissector (Fig.2.4) kindly offered by University of Veterinary and Parmaceutical Sciences in Brno - Czech Republic.

For laser microdissection, we used only well-spread mitotic metaphases. X chromosomes were identified in an inverted microscope according to their morphology and size (the X chromosome is the longest element of *M. persicae*

complement (Manicardi et.al., 1998) and microdissected with the help of a P.A.L.M. MicroLaser System (Carl Zeiss MicroImaging GmbH, Munich, Germany) as described in Kubickova et al. (2002). Each microdissected chromosome was catapulted by a single laser pulse into the cap (containing 3 μl of mineral oil) of a PCR tube. DNA of microdissected samples, each containing 8 X chromosomes, was used as a template for PCR amplification in a T-personal thermocycler (Biometra, Go¨ttingen, Germany) using a WGA4 GenomePlex Single Cell Whole Genome Amplification Kit (Sigma-Aldrich, St. Louis, MO, USA) that was succesfully used to prepare sex-chromosome painting probes in Drosopoulou et al., (2012). Shortly before the amplification, 9 μl of DNase-free ultrapure water were added to each sample and the sample was spun down in a microcentrifuge at 6,000rpm (2,000xg) for 3 min. The amplified products were purified using a Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA). If the amount of amplified DNA was measurable, about 20 ng of the DNA was used for PCR labelling with Cy3-dUTP (GE Healthcare, Milwaukee, WI, USA) using a WGA3 GenomePlex WGA Reamplification Kit (Sigma-Aldrich). If the amount of amplified DNA was too low, the product was first re-amplified DNA using the WGA3 Kit and then labelled in the same way. All amplification and purification steps were done according to protocols of the manufacturers.

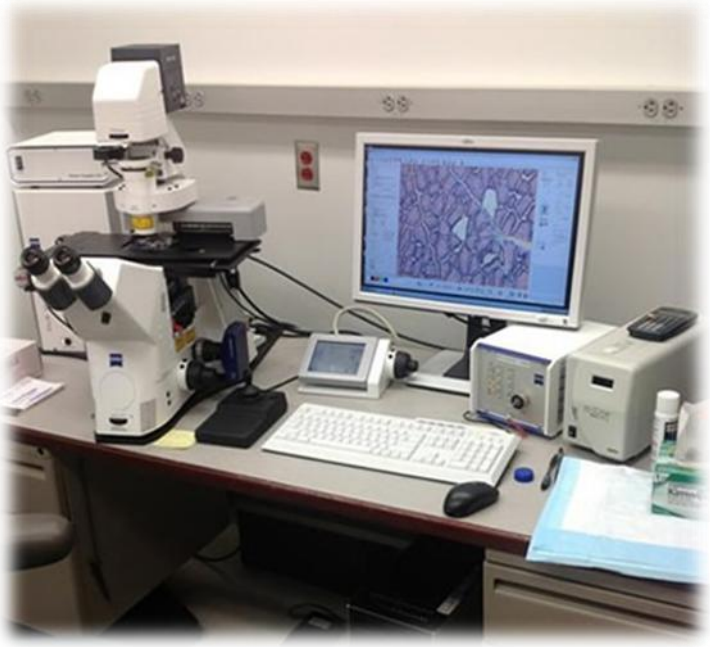


Fig 2.4. Zeiss PALM MicroBeam IV laser-capture microdissection system (Imafe from website Cal Zeiss Microscopy).

Finally FISH with X-chromosome painting probes experiments, were performed following the procedure of Traut et al. (1999) with several modifications. Mitotic chromosomes were denatured at 70°C for 3 min and 30 s in 70% deionized formamide in 2x SSC. After denaturation, slides were dehydrated in 70% (ice cold), 80% and 100% ethanol (30 s each) and air dried. For each slide the probe cocktail contained about 500–1,000 ng of the labelled sex-chromosome probe and 25 μg of sonicated salmon sperm DNA (Sigma-Aldrich) in 10 μl of 50% deionized formamide and 10% dextran sulfate in 2x SSC. Hybridization was carried out for 3 days at 37°C. Then the slides were washed for 5 min in 0.1x SSC containing 1% Triton X-100 (Sigma-Aldrich) at 62°C and for 2 min in 2x SSC containing 1% Triton X-100 at room temperature. Preparations were counterstained with 0.5 ug/ml DAPI (Sigma-Aldrich) in 1% Triton X-100/1x PBS for 15 min and mounted in antifade based on DABCO (Sigma-Aldrich; for composition, see Traut et al. 1999). The preparations were observed in an epifluorescence microscope Zeiss Axioplan 2 (Carl Zeiss Jena, Germany) equipped with a cooled F-View CCD camera and AnalySIS software, version 3.2 (Soft Imaging System GmbH, Mu¨nster, Germany). Black-and-white images of chromosomes were captured separately for each fluorescent dye. Images were pseudocoloured (light blue for DAPI, red for Cy3) and processed with Adobe Photoshop CS4, version 11.The painting probe protocol was kindly offered by Prof. Frantisek Marec from Laboratory of Molecular Cytogenetics Institute of Entomology Biology Centre in Ceske Budejovice - Czech Republic.

2.2.7 PRINS (Primed In Situ Hybridization)

PRINS method have some advantages: first of all it use a very small amount of probes which easily penetrate to almost any target. The probes are small DNA sequences (primer F and R) that are extended on chromosomes through a specific enzyme (Taq polymerase). During the extension, labeled nucleotides are incorporated. Moreover this method represents an alternative to fluorescence *in situ* hybridization because it is very fast, reproducible, and simple to perform.
Slides with the spreaded chromosomes must be prepared 24 hours before the PRINS treatment, dehydrated through an ethanol series air dried and stored at 4°C. No pretreatment is necessary.
Successively 30μl of reaction mix was prepared: 17μl of water, 3ul of buffer taq 10X, 2 μl Rhodamine PRINS labelling mix 10x, 5μl di primer mix (2,5 Primer forward and 2,5 primer reverse) and finally 2,5 μl of Taq polymerase for each

slide. Slides were placed on a preheated block at 94℃ for 5 min in direct contact with the hot surface.
The mix was put on each slide (this step must be fast) into a stove for 5 min. Then, slides sould be transferred into a stove at about 58°C for 30 min (this temperature is related to the T melting temperature of the primer) and successively washed in stop buffer for 5 min at 58°C and for 5 min at room temperature. Finally slides were dehydrated through an ethanol series and air dried.
1µl of the DAPI (used as a counterstaining) was diluted with 9µl of distilled water. 5µl of the diluted counterstain were added to 200µl of DAPCO mountant, well mixed and 40µl of this mixuter were put on slides which were overlaied with a coverslip and sealed with nail varnish. Slides should be stored in the dark at 4°C. Slides were analyzed with a Zeiss Axioplan epi-fluorescent microscope.

2.3 MOLECULAR TECHNIQUES

2.3.1 Extraction Of Genomic DNA

Total genomic DNA was isolated and purified from parthenogenetic adult females containing embryos, homogenized in 200µl of HB solution. The pestle was cleaned with other 300µl of HB solution. After this steps 30 µl of SDS (10%),with 10µl of proteinase K (10mg/ml) and 10µl of RNasi (10mg/ml) was added to the homogenate. and mixed by tapping . All of this was incubated at 55°C overnight (not over 12-16 h). A volume of 500µl phenol:chloroform (1:1) was prepared and added to each samples. The mixture was gently stirred and then centrifuged (10,000 g) for 5 min. The supernatant was treated twice as described above and the lower phase was removed. Finally 500 µl of CIA (chloroform: isoamyl alcohol 24:1) was added, mixed for some minutes and centrifuged at 10,000 g for 5 min.
For the last step: 1ml of cold ethanol 100% was added, (you could see a DNA floccule); the pellet was washed three times with 70% ethanol, air-dried and resuspended in 30µl of sterile bidistilled water. The complete DNA dissolution was obtained leaving the pellet at 37°C or 4°C for.
The next day it is possible to quantify the DNA by mean of a Nanodrops tool (spectrophotometer). Electrophoresis was carried out in 1.2% agarose gels in TRIS-borate buffer at 90 V for 45 min.

2.3.2 PCR (*Polymerase Chain Reaction*, PCR)

1) Telomere Repeat Probe (TTAGGG)n:

In order to test the presence of the telomeric (TTAGG)n repeat, the probe was generated by PCR amplification using the two primers F $(TTAGG)_5$ and R $(CCTAA)_5$ in the absence of template, as described by Ijdo et al. (1991). Random priming probe biotin-labeling was performed with the Biotin High Prime (Roche, Basel, Switzerland), whereas PCR digoxigenin labelling was performed using the Dig High Prime kit (Roche). Both types of labelling were done according to Roche protocols. Primer F- TEL $(TTAGG)_5$ Primer R- TEL $(CCTAA)_5$.. The amplification program was compound from these steps: 94°C for 90s, and a total of 30 cycles of 93°C for 30s, 60°C for 30s and 72°C for 90s.

1) H*ind* 200 X-Chromosomes Probe

The H*ind*200 probe was isolated by digesting *M. persicae* genomic DNA with the H*ind*III restriction enzyme at 37 °C for 16 h and eluting from the 1.2% agarose gel the band corresponding to the Hind200 satellite monomers (GenBank AF161255). The eluted DNA was labelled using a random priming DIG labelling kit in accordance with the manufacturer's instructions (Roche Diagnostics).

3) Sub-Telomeric Repeat Amplification

Polymerase chain reaction digoxigenin labelling of the subtelomeric repeat was performed with a PCR DIG labelling kit according with the manufacturer's instructions (Roche) using the specific oligonucleotide primers MpR-F (5′-TCAAAGTTCTCGTTCTCC-3′) and MpR-R (5′-GTTTTAACAGAGTGCTGG-3′), designed in accordance with the sub-telomeric repeat sequence available in literature (Spence et al., 1998). The reaction conditions were 94°C for 90s, and a total of 30 cycles of 93°C for 30s, 49°C for 30s, and 72°C for 30s.

4) Amplification Of The Telomerase Gene

Amplification of the telomerase gene was performed using a Hybaid thermocycler with the primers F-TERT1200 (5'-ACAACGTATGCCGGGTGT-3′) and R-TERT1200 (5′-AACCCCAAAAACTTGACCATC-3′) at an annealing temperature of 60°C for 1 min and an extension step at 72C for 1 min.

5) Amplification Of The Retrotrasposones TRAS

The presence of TRAS retrotransposons has been preliminary evaluated using the BLAST algorithm at *Aphidbase* (http://www.aphidbase.com/aphidbase/). Successively, 801-bp-long portion of the TRAS ORF coding for reverse transcriptase (RT) has been amplified using the primers F-TRAS (5-ATG AGT ACC CCC ACC ATC AA) and R-TRAS (5′-CCT CTC CGA GAT GAC CTG AA), designed according the *A. pisum* partial TRAS3 sequence available in GenBank (XM_001942587).
The amplification mix contains 100 ng of genomic DNA, 25 µM of each primer, 200 µM dNTPs and 2U of DyNAZyme II polymerase (Finnzymes Oy). Amplification was performed with a Hybaid thermalcycler at an annealing temperature of 56 °C for 1 min and making extension at 72 °C for 2 min.
Aphid TRAS sequence was completed by inverse PCR with the primers F-TRAS-i (5′-CACTCTCACCCACCCTTCAT) and R-TRAS-i (5′-AAAAGAGTGCCCGTAACCCT), according to Martin and Mohn (1999). Aphid's labelling probes was performed with the "PCR DIG labelling mix" according to the Roche protocol.

6) Amplification Of Cytoplasmic Actin

Cytoplasmic actin (primersF 5′-AGCAGGAGATGGCCACC-3′ and primersR 5′-TCCACATCTGCTGGAAGG-3′) was amplified as a loading control in RT-PCR experiments. For the cytoplasmic actin PCR reactions, 25 cycles were performed, with annealing conditions of 40 s at 58 °C and elongation pamameters of 45 s at 72°C. Both the primer sets have been designed according to the orthologous genes identified in the pea aphid *Acyrthosiphon pisum* genome.

2.3.3 RNA Extraction

Around 60-120 apterous adult aphids were directly homogenized in 500 µl TRI-Reagent on ice, using a manual glass-Teflon homogenizer. Debris was eliminated through centrifugation at 12.000 g for 15 minutes at 4 °C. The surnatant was stored for 5 minutes at room temperature for a complete dissociation of nucleoprotein complexes from nucleic acids. Next, it was supplemented with 100 µl chloroform, shaked vigorously for 15 seconds and stored at room temperature for 10 minutes before centrifugation at 12.000 g for 15 minutes at 4 °C. After the

centrifugation step, the mixture separated into a lower red phenol-chloroform phase, interphase and the colorless upper aqueous phase. RNA remained almost exclusively in the aqueous phase whereas DNA and proteins were retained in the interphase and organic phase. The aqueous phase was then transferred to a fresh tube and total RNA precipitated by mixing with 250 µl of isopropanol. The samples were stored at room temperature for 10 minutes and centrifuged at 12.000 g for 8 minutes at 4 °C to allow RNA precipitation as gel-like pellet.
After supernatant removing the RNA pellet was washed (by vortexing) with 75% ethanol in DEPC water, centrifuged for 5 minutes at 7.500 g at 4 °C. This washing step was repeated twice. Once removed the ethanol, the RNA pellet was air-dryed for 3 - 5 min and finally dissolved in 20- 30 µl of DEPC water. The quality of the RNA extraction will evaluated at spectrophotometer: A260/A280 ratio should be > 1.7.

2.3.4 Reverse Transcriptase-Polimerase Chain Reaction (RT-PCR)

For measuring gene expression of cells or tissues several techniques were carried out.
One of the most sensitive and versatile involved reverse transcription (RT) associated with PCR amplification, actually called RT-PCR. This technique can be used for the amplification of specific RNA target, including the totality of RNA or mRNA. This system exploits the reverse transcriptase of the virus to the *avian myeloblastosis* (AMV RT) for the synthesis of the first DNA strand. Otherwise, the synthesis of the second strand and for subsequent amplification steps, the thermostable DNA polymerase derived from the bacterium *Thermus flavus* was used. The kit used, Promega, is called the Access RT-PCR System Introductory and contains, in addition to the already mentioned reverse transcriptase and DNA polymerase, the reaction buffer, a solution of 25mM MgSO4, the mixture of deoxynucleotide triphosphate, an RNA control, two control primers (upstream and downstream) and water free of nuclease activity. Two couple of primer are used: TERT-F primer (5'-ACA ACG TAT GCC GGG TGT) and TERT-R primer (5'-GCT TGG GAA CAC TAC GTG CC), F-TRAS-(5′-CAC TCT CAC CCA CCC TTC AT) and R-TRAS-i (5′-AAA AGA GTG CCC GTA ACC CT).
For the synthesis of the first cDNA strand, a cycle of reverse transcription at 48 º C for 45 minutes was carried on, followed by a cycle of two minutes at 94 º C for the inactivation transcriptase AMV and for the dissociation of the complex RNA / cDNA / primer. Subsequently 40 cycles were perfomed, each composed of three

phases: 30 seconds at 94 º C for denaturation, 1 minute at Tm º C for annealing of primers, 2 minutes at 68 º C for the synthesis of the new strand.
The final cycle, at 68 º C for 7 minutes, allows an optimal extension of the filament. The PCR product is then analyzed by electrophoresis in agarose gel.

2.3.5 Cloning and Sequencing

The cloning process allows to amplify a DNA fragment isolated, in order to have sufficient quantities for sequencing. All the amplified fragments (this method was applied to TRAS fragments) were cloned using the pGEM T-easy cloning kit (Promega) (Fig 2.5) according to the supplier's datasheet. The cloning protocol include three important steps for the fragment insertion into the vector: linearization, digestion and purification.

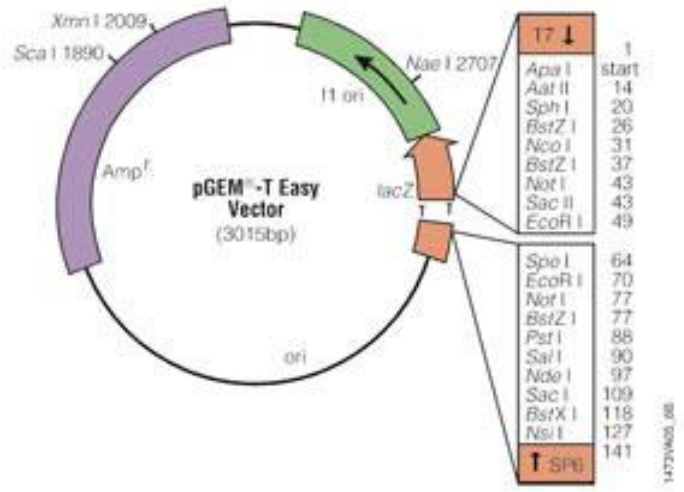


Fig 2.5. Schematic rappresentation of pGEM-T Easy Vector from WebSite.

After this, the plasmid was transferred into the competent bacteric cells and left at 39ºC overnight, the ideal temperature for the colonie's growth. The next day it was possible to analyse the plates and collected the plasmidic DNA containing the insert using GenElute Plasmid Miniprep (SIGMA) for extraction.
Amplified fragments have been sequenced at BMR Genomics, whereas sequence alignments and general sequence analysis were done using ClustalW, FASTA, BLAST software.

2.3.6 Southern Blot

Transfering DNA from agarose gel to nylon membrane (Hybond-N+, Amersham) was performed by submerging agarose gels first in 250 mM HCl for 5 min at room temperature with agitation, then in denaturation solution (0.5 N NaOH, 1.5 N NaCl) twice for 15 min and finally in neutralization solution (1.5 N NaCl, 0.5 M TRIS-HCl, pH 7.5) twice for 15 min. DNA was then blotted overnight by capillary transfer (Fig 2.6) onto the membrane using 20×SSC buffer and fixed on the membrane by exposing it to UV rays for 1 min (UV-crosslinking).

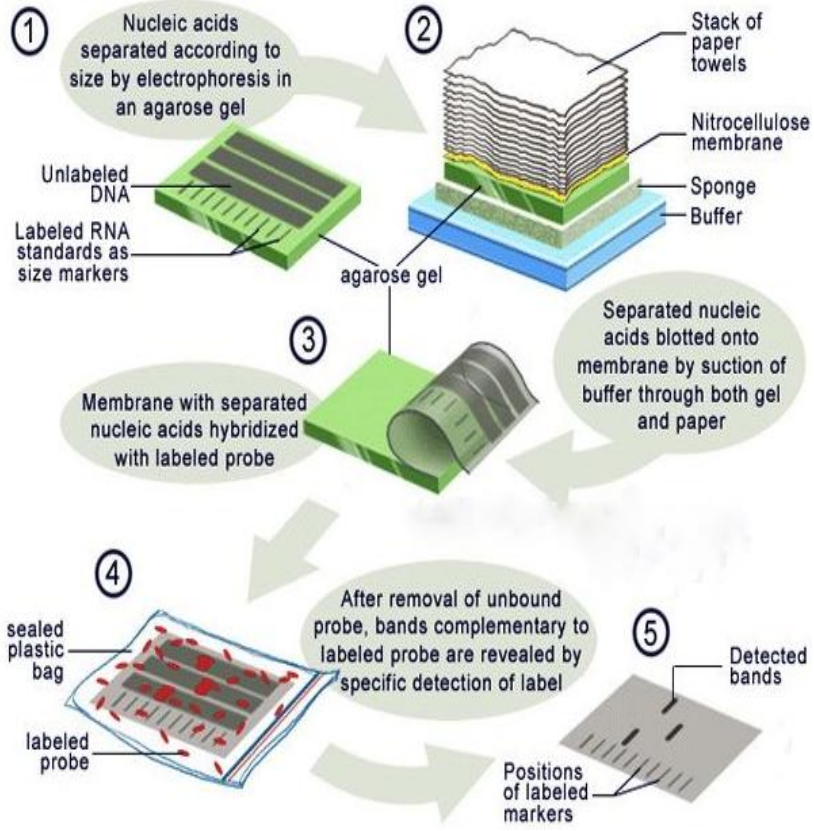


Fig 2.6. Schemtic rappresentation of Southern Blotting method (Image from BioMArt.cn)

The membrane was utilized immediately for prehybridization, which was performed in a rolling-bag containing 10 ml of DIG Easy Hyb solution (Boehringer) at 45°C for 1 h. Hybridization was performed in a rolling-bag containing 10 ml of hybridization solution (DIG Easy Hyb added with 40 ng/ml probe) at 45°C for 16h. The probe was denatured by boiling for 6 min. At the end of hybridization the membrane was washed twice in 2×SSC for 15 min at room temperature, in 0.1×SSC for 15 min at 68°C and once in 1×washing solution (0.1

M maleic acid, 0.15 M NaCl, 1%Triton X) at room temperature. Afterwards, the filter was ready for detection. It was incubated at room temperature for 5 minutes with CSPD (which represents the alkaline phosphatase substrate which becomes fluorescent only when it is metabolized). This is a chemiluminescent reaction; the filter was wrapped in a plastic wrap. The chemiluminescence reaction could now be detected by exposure of the filter to a photographic plate. Exposure times depends on type of signal, but it is convenient to start with short exposures and then switch to longer exposures;
The photographic plate was placed for 2 minutes in the developing solution, for 4 minutes in the fixing solution and then washed in running water and in distilled water.

2.3.7 DOT Blot

Dot blot is a semiquantitative analysis. This method was set up by hand, spotting small aliquots of each sample on a positively charged nylon membrane (Amersham) and waiting for the blot to dry.
The denaturation mix with 0.5N NaOH and 1,5 M NaC was spotted on each sample. The Membrane was put on the transilluminator with 60% of irradiation for the cross-link and after it was move into the hybridization mix (Roche) at 37°C for 1h. The hybridization and detection were performed as described for Southern blot hybridization. Quantitative Dot blot assays were evaluated using the Imagej 1.47 – RSB software.

2.3.8 Real Time PCR To Study The Compensation Dosage In Acyrthosiphon pisum

Real-Time PCR is identical to a simple traditional PCR except that the progress of the reaction is monitored by a camera or detector in “real-time”. There are a number of techniques that are used to allow the progress of a PCR to be monitored. Each technique uses some kind of fluorescent marker which binds to the DNA. Hence as the number of gene copies increases during the reaction so the fluorescence increases. This is advantageous because the efficiency and rate of the reaction can be seen, moreover there is also no need to run the PCR product out on a gel after the reaction.
It has been used the SYBR Green method, which exploits a double - stranded DNA dye added to the PCR reaction, able to bind newly synthesized double-

stranded DNA, for generating a progressive increase of a fluorescent signal proportional to the amplicone's concentration at each PCR cycle.
So SYBR Green provides the simplest and most economical format for detecting and quantitating PCR products in real-time reactions. SYBR Green binds double-stranded DNA, and upon excitation emits light. Thus, as a PCR product accumulates, fluorescence increases.
The present work take advantage of SYBR Green based qPCR technique in order to evaluate the compensation of dosage. The dosage compensation consists in a mechanism matching the amount of the gene products associated with the sex chromosomes in males and females in *Acyrthosiphon pisum*.
Aphid sequences are poorly represented in gene databases: this study begin in November 2003 and only 6,491 nucleotide sequences (including a majority of anonymous molecular markers) were found in GenBank for the whole Aphididae family.
Therefore, the dosage compensation was not realible since there were not genes mapped on X chromosome.
For this purpose, since the rDNA genes are among the few genes mapped to the chromosomal level, a BLAST search in Aphidbase had been performed to identify scaffolds containing those sequences (Personal Comunication with Prof. Mauro Mandrioli).
Thanks to this research the scaffold 13053 including putative rDNA genes (GenBank accession number EQ123825) was retrieved. The scaffold also contains the genes fibronectin (XM_001943850.2), RPP15 (XM_001950943.2) and RAD-50 (XM_001948524.2) which have been chosen as target genes (Fig. 2.7.).

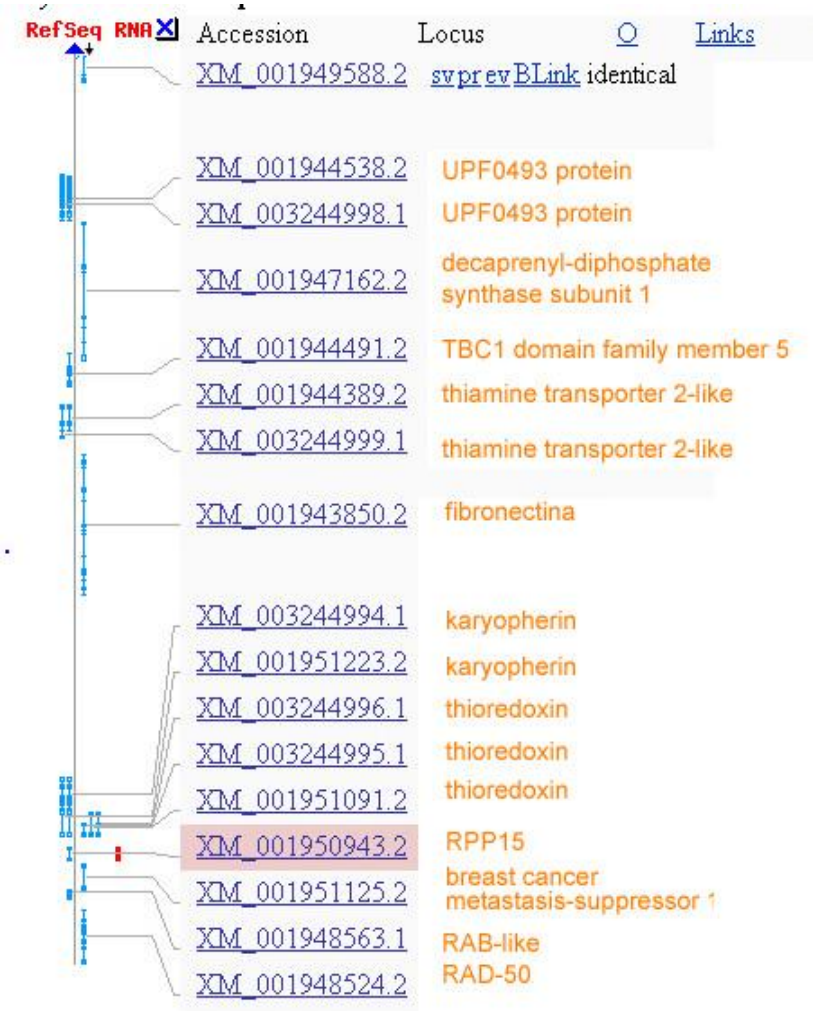


Fig 2.7. Schematic representation of genic content in the scaffold 13503 (Image from Aphidbase).

The actin gene was used as a reference gene to normalize the relative transcription level of the target genes among different RNA extracts, since its expression was expected unrelated to sex (Kwon et. al., 2009).

The primers were designed using IDT Integrated DNA Technologies SciTools qPCR (http//eu.idtdna.com/pages/scitools). Below are reported the primer sequences for X-linked and actin genes .

Primer Product 152 bp of **Actin** :

5'MpActin 5'CAAA TCATGTTTGAAACCTTCA
3'MpActin 5'AATGCATAACCTTCATAGATG

Primer Product Size 187 bp **Fibronectin**:

PrimerF_FIB- ATCGTTGGAGGCATAGTAGCTGGT
PrimerR_FIB- TCCATTCAGCGGTATTGAGTCCGT

Primer Product Size 187 bp **RPP 15**:

PrimerF_RPP 15- ACCAGACTTGGAACATTCAGAACC
PrimerR_RPP 15- CTTCTCTACAGTTCTTTGTTGCTGC

Primer Product Size 193 bp **RAD 50**:

PrimerF_RAD 50- CAACAAAGCTCTTGAATGGGC
PrimerR_RAD 50- AACTCTATAGTTGAAGACCCT

For real-time assays I have performed a total of three biological replications for each genes : 1 biological replicates, 1 RT and 1 Real Time with three technical replicates for each sex. The expression level has been determined following the standard curve by serial dilutions of a cDNA sample prepared from total female's RNA . The expression ratio for each target gene, as fold mean, was calculated between male and female samples after actin normalization. Then I calculated the interval of confidence 95% (Ci (95%)).

3 RESULTS

3.1 THE VANISHING CLONES : KARYOTYPIC EVIDENCE FOR EXTENSIVE INTRA AND INTER-CLONAL GENETIC VARIATIONS

The analysis of mitotic cells of embryos, obtained from parthenogenetic females, confirmed that 2n = 12 is the standard chromosome number in M. persicae comprising two homologous X chromosomes and five homologous sets of autosomes. Conversely, the cytogenetic study of mitotic metaphases of the M. persicae clone 33H (observed after both spreading and squashing) revealed the occurrence of different chromosome numbers ranging from 12 to 17 also within each analysed embryo, hence revealing the occurrence of an intra-individual chromosomal mosaicism (Fig.3.1 A-F) . About 18% of the observed plates showed the 2n=12 standard chromosome number, approximately 21% had 13 chromosomes, whereas 46% of the observed metaphases had 14 chromosomes. Metaphases with more than 15 chromosomes were scarcely represented and as a whole constituted about 14% of the observed plates (Fig. 3.1. G).

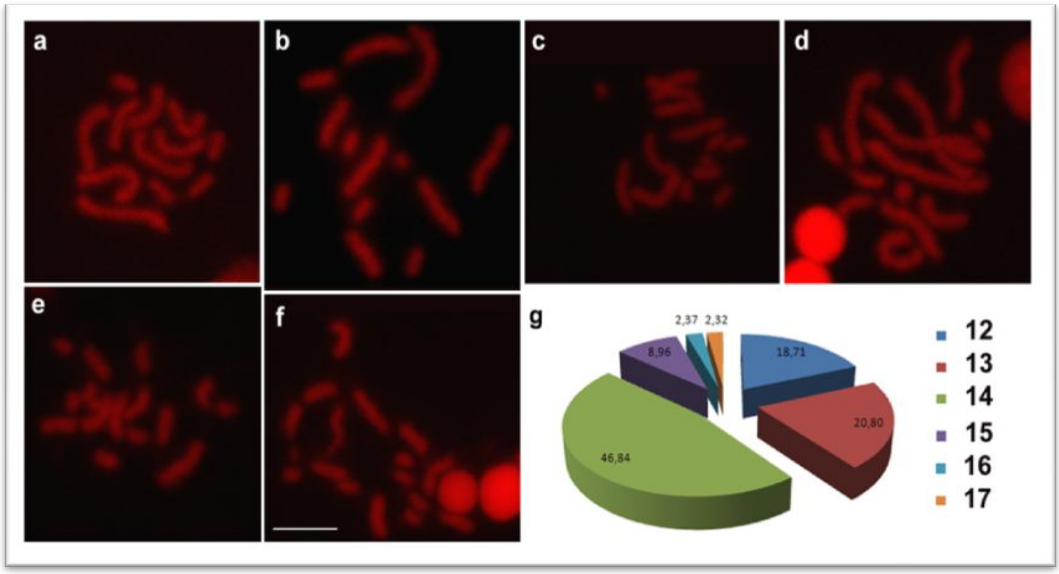


Fig 3.1 Propidium iodide staining of *Myzus persicae* 33H metaphases gave plates with 12 (a), 13(b), 14(c), 15(d), 16(e), and 17(f) chromosomes within each single aphid embryo. (g) are the frequencies of the different complements in the analyzed embryos. (Image from Monti et.al., 2012) Bar = 10 μm.

Analyzing the observed changes in the chromosome number, I focused my attention on the four most commonly observed karyotypes consisting of 12, 13,

14, and 15 chromosomes. To identify the chromosomes involved in the fissions, I performed FISH experiments with the subtelomeric satellite DNA that labelled all the *M. persicae* chromosome ends with the exception of the rDNA telomere in each X chromosome (Spence et. al., 1998) (Fig 3.2).

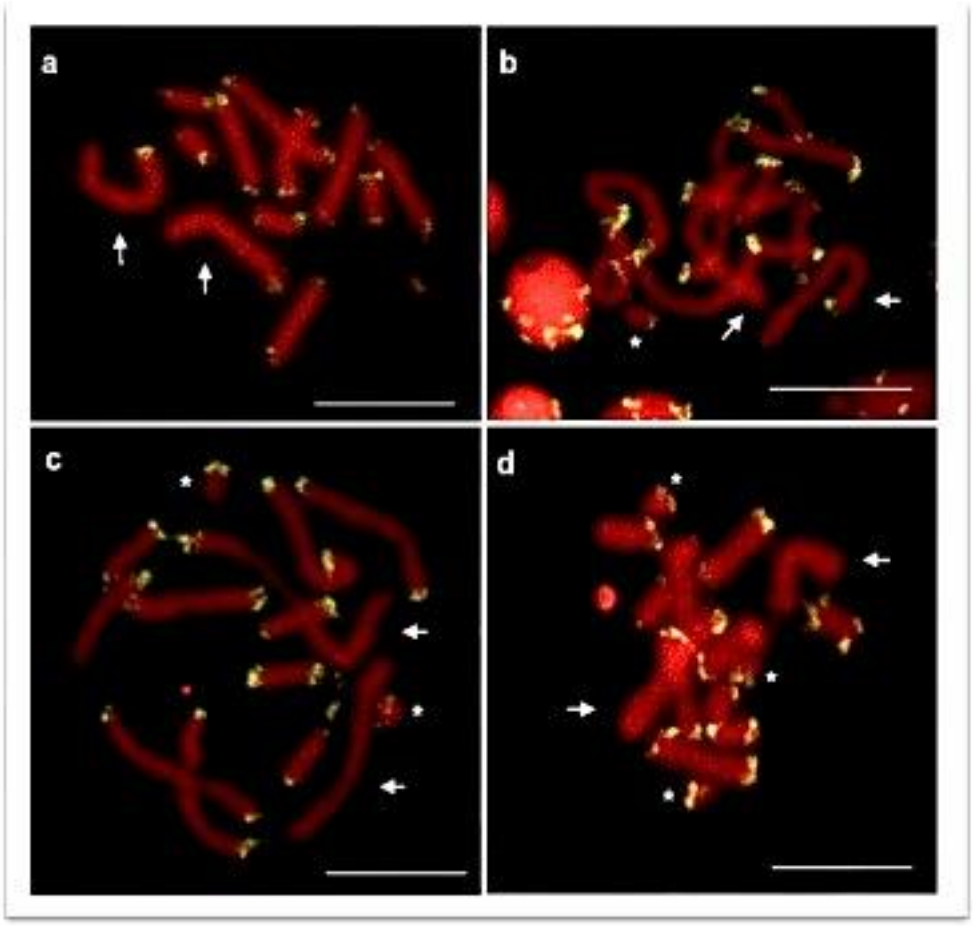


Fig 3.2. Fluorescent in situ hybridization with the subtelomeric probes allowed the identification of the chromosomes involved in the fragmentations resulting in changes in the chromosome number of *Myzus persicae* 33H metaphases with 12 (A), 13 (B), 14 (C), and 15 (D) chromosomes. Arrows indicate X chromosomes. Asterisks indicate chromosome fragments. Scale bars = 10 (Image from Monti et.al., 2012).

Measurements revealed that observed mosaicism is a result of fragmentations (Fig. 3.3). All the karyotypes with 12 chromosomes represented the standard complement consisting of five couples of autosomes with both the telomeres labelled with the subtelomeric probes and two X chromosomes with a single-labelled end (Figs 3.2a, 3.3A). Karyotypes consisting of 13 chromosomes were the result of a single fission involving autosome 1 or 3 (Figs 3.2 b and 3.3 b) but

more frequently, the X chromosome, which was fragmented in more than half of the observed plates (3.3.b,c and d).

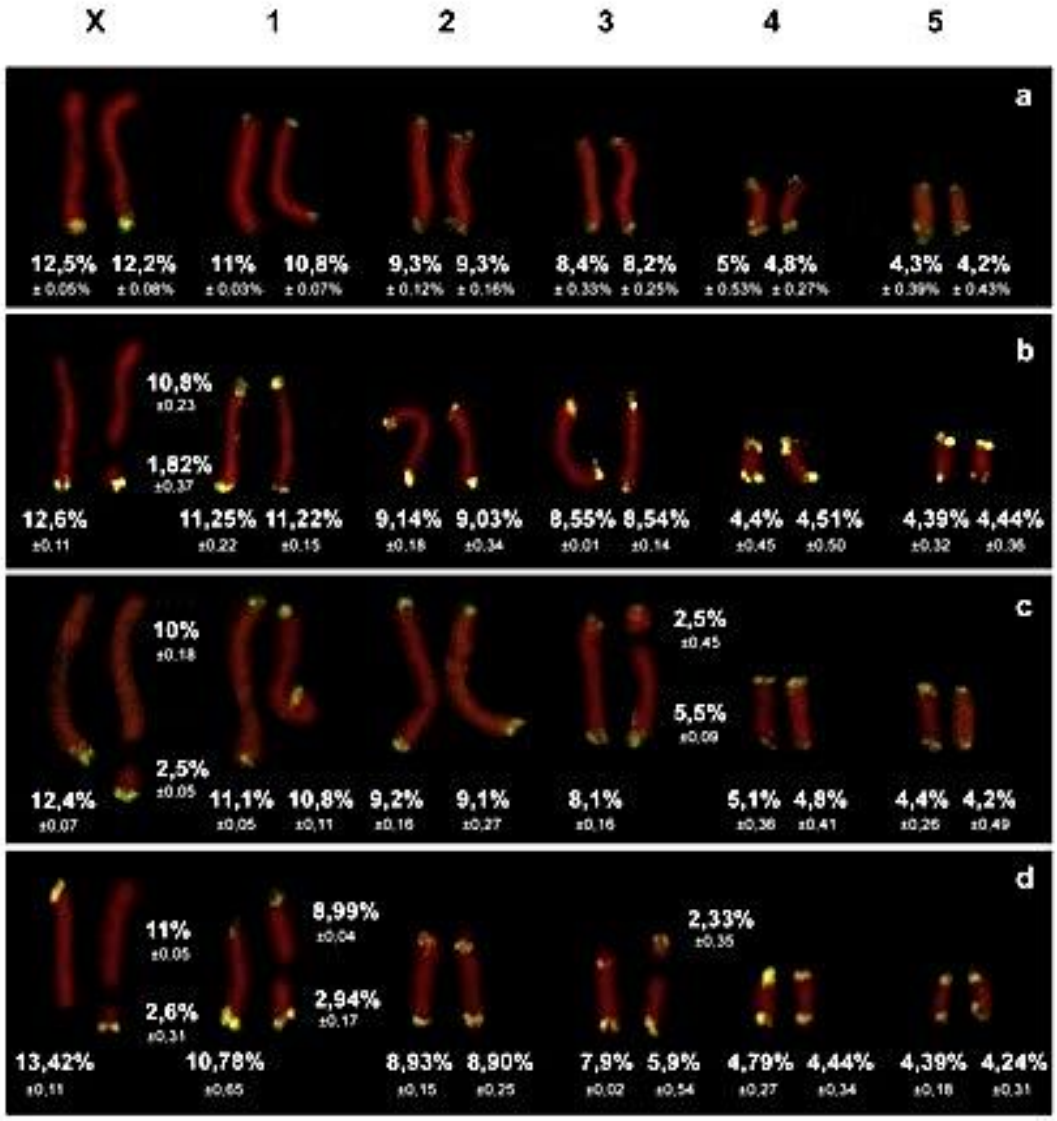


Fig 3.3. (A) Comparing standard karyotype with the variable ones, the most frequent fissions involved chromosome X in plates with 13 chromosomes (B), chromosomes X and 3 in metaphases with 14 chromosomes (C), and chromosomes X, 1, and 3 in plates with 15 chromosomes (D). The percentages near each chromosome indicate the relative length of each chromosome (i.e. expressed as a percentage of the total complement length) (Image from Monti et.al., 2012).

X chromosome identification was confirmed by *in situ* hybridization with the Hind200 satellite DNA, highlighting several intercalary bands on both the X chromosomes (Mandrioli et al., 1999a) (Fig. 3.4A). CMA3 staining (Fig. 3.4D)

further showed that the X chromosome fission involved the X telomere opposite to the rDNA-bearing one (Fig. 3.5E). Interestingly, the presence of heteromorphism in the size of the CMA3 stained-telomeres revealed that the fission always occurred in the X chromosome possessing a smaller CMA3-positive telomere (Fig. 3.4F).

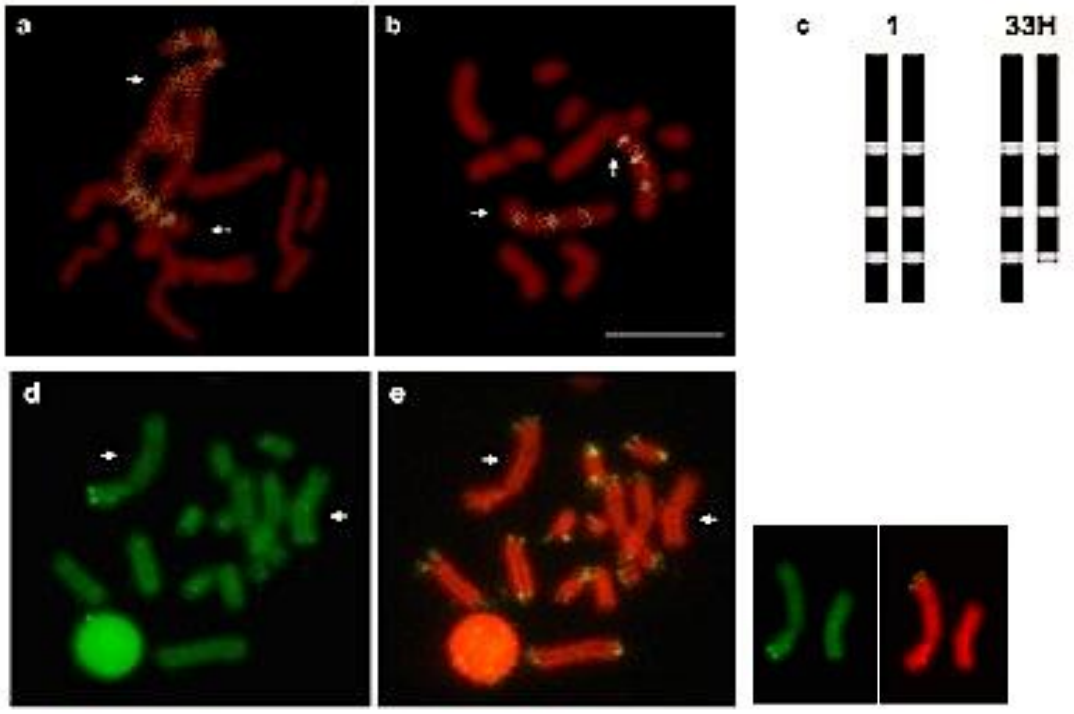


Fig 3.4. Fluorescent *in situ* hybridization (FISH) with the Hind200 satellite DNA allowed the identification of the two X chromosomes in the metaphases of *M. persicae* strain 1 (A) and strain 33H (B). Comparison of the hybridization signals allowed the reconstruction of karyograms (C) showing the fragmentation of an X chromosome telomere below a Hind200 band (in grey). CMA3 staining (D) identified the nucleolar organizer region-bearing X telomeres that were not involved in X chromosome fragmentation, as indicated by FISH of the same plate with the subtelomeric repeat (E). Comparison of X chromosomes after CMA3 staining and FISH with the subtelomeric repeat is shown in (F). Arrows indicate the X chromosomes (Image from Monti et.al., 2012) Bar = 10 μm.

Metaphase plates consisting of 14 chromosomes were the result of fissions involving chromosomes 1, 3, and X, although simultaneous fragmentations occurring on one X chromosome and one autosome 3 were most frequent (Figs 3.2C, 3.3C).

Instead, karyotypes consisting of 15 chromosomes, involved simultaneously the fission of chromosomes 1, 3, and X, thus producing three small chromosomes (Figs 3.2D, 3.3D). Male induction revealed that the *M. persicae* clone 33H is anholocyclic since it wasn't possible to induce the sexual generation, contrarily to what was obtained under the same experimental conditions with the holocyclic *M. persicae* strain 1 used as a control.The originality of the above mentioned data prompted us to extend the analysis to other *M. persicae* strains. Among them, the cytogenetic study of the *M. persicae* clones 50, 51 and 70 after propidium iodide staining, confirmed the presence of a strong mosaicism, both at the intra-individual and inter-individual level. In particular, 80% of the metaphase plates observed in clone 50 consisted of 13 chromosomes whilst just 20% showed the usual karyotype of 12 chromosomes. Analysis of clone 70 showed that 70% of the observed metaphases had 14 chromosomes, 21.5% of which possessed 13 chromosomes, whilst 8.5% had the standard karyotype of 2n=12.

Lastly, clone 51 showed 78.5% of the observed plates to have 13 chromosomes and 21.5% with the typical karyotype of 12 chromosomes.As previously carried out in M.p 33H, we combined FISH experiments, performed with a subtelomeric satellite DNA that labelled all the *M. persicae* chromosome ends with the exception of the rDNA telomere in each X chromosome (Spence et al. 1998), with the micro-measurements of each chromosome (Figs 3.5, 3.6 and 3.7).

This approach, previously revealed that the mosaicism observed in clones 50, 51 and 70 is due to chromosomal fragmentations, even if it was not possible to clearly identify (at least in some clones) the actual chromosomes involved in the various observed karyotypic changes. Specimens of *M. persicae* strain 50 showed metaphases with 12 and 13 chromosomes.

Interestingly, karyotypes with 13 chromosomes were due to the presence of a supernumerary chromosome with both the ends labelled after FISH (Figure 3.5).

Similar to clone 50, metaphase plates of aphids of clones 51 showed either 12 or 13 chromosomes. However, 50% of the plates with 13 chromosomes had a fission to have occurred at one X chromosome, of which 25% was due to fission of autosome 3 and 25% to the presence of a small chromosome with both the termini labelled after FISH with the subtelomeric probe (Figures 3.6a).

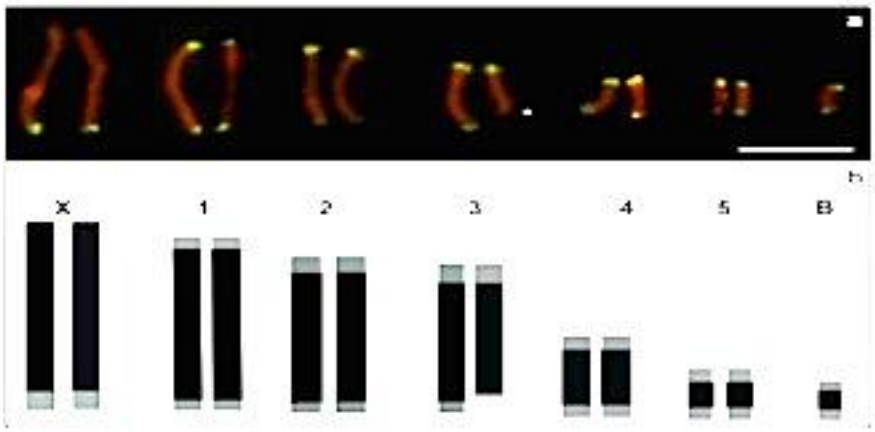


Fig 3.5 FISH with the FITC-labeled subtelomeric probe *in M. persicae* clone 50 presented unusual plates with 13 chromosomes due to the presence of a supernumerary chromosome, together with a deleted autosome 3 (asterisk) (a) as evident in the corresponding karyogram (b) (Image from Monti et.al. ,2012) Bar = 10 μm.

Unexpectedly, plates with fissions occurring at the X chromosome also showed a deletion of a subtelomeric region on autosome 3 (Figures 3.6a-c).

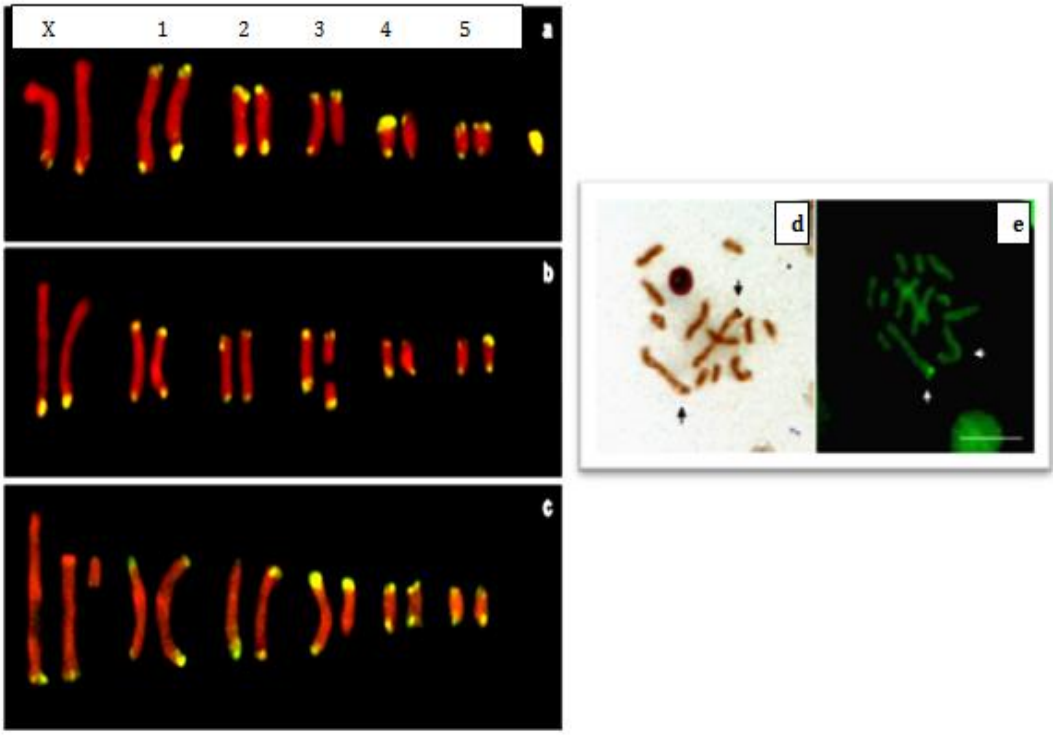


Fig 3.6 FISH with the FITC-labeled subtelomeric probe on propidium iodide-stained chromosomes showed three different 2n = 13 karyotype variants in M. persicae clone 51

consisting of plates with a small supernumerary chromosome with both the termini labelled after FISH with the subtelomeric probe (a), metaphases with a fission occurred at one autosome 3 (indicated by the asterisk) (b) and plates with afission of one X chromosome (c). Silver (d) and CMA3 staining (e) indicate the fission at the X chromosome did not involved the NOR bearing telomere of the X chromosomes but it is due to a deletion of an intercalary portion of the X chromosome.. Arrows indicate the X chromosomes (Image from Monti et.al., 2012).

Analysis of the deleted X chromosome after hybridization with the subtelomeric probe suggested that the deletion probably occurred at the NOR bearing telomere and therefore, I performed CMA3 and silver staining to confirm this hypothesis (Fig.3.6 d, e). Both staining approaches showed that the X fragment did not contain the ribosomal DNA genes so that the sighted fission of the X chromosome involved an interstitial region rather than the telomeric one (Fig 3.6 c). Furthermore, the presence of heteromorphism in terms of the size of the CMA3 stained-telomeres revealed that the fission always occurred in the X chromosome possessing the smaller CMA3-positive telomere. Specimens of clone 70 showed metaphases with chromosome number ranging from 12 to 14. About 70 % of karyotypes ranging from 13 chromosomes, fission was due to a single break involving the X chromosome (Figs. 3.7 a), whereas 30 % of the observed plates showed fissions occurring at autosome 3 (Fig 3.7b).

Metaphase plates comprising 14 chromosomes resulted from simultaneous fragmentations on chromosomes 1 and 3 (25 % of the observed varitypes) (Fig 3.7.c), or alternatively because of fissions involving one X chromosome and one autosome 3 (75 % of the observed kariotypes) (Fig 3.7 e).

I never observed plates with 13 chromosomes involving autosome1 alone, suggesting that autosome 1 was not frequently involved in such fissions. In order to confirm the presence of B chromosomes in *M. persicae* clones 50 and 51, a DAPI staining of C banded chromosomes was performed, thereby revealing that in each clone, a small supernumerary chromosome exists, highly stained by DAPI and enriched in heterochromatin (Fig 3.8).

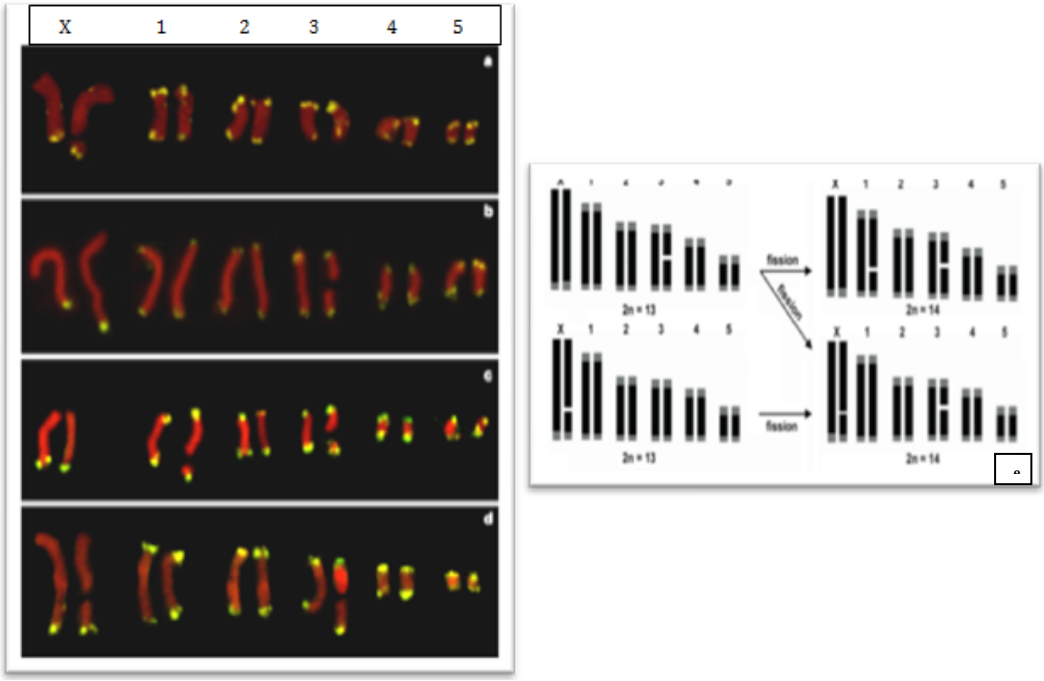


Fig 3.7 As showed by FISH with the FITC-labeled subtelomeric probe, M. persicae clone 70 presented plates with chromosome number ranging from 12 to 14. Karyotypes consisting of 13 chromosomes were due to a single fission involving a single X chromosome (a) or a fission occurring at autosome 3 (b). Metaphase plates consisting of 14 chromosomes resulted from simultaneous fragmentations of chromosomes 1 and 3 (c) or of fissions involving one X chromosome and one autosome 3 (d). (Image from Monti et.al., 2012) Bar = 10 µm.

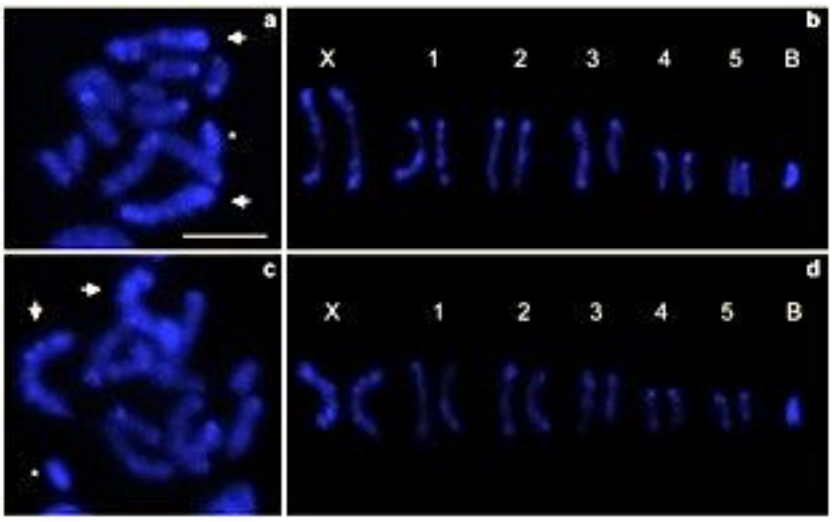


Fig 3.8 DAPI staining of C banded chromosomes of the *M. persicae* both the clones as typical for B chromosomes. Arrows indicate the X clone 50 (a, b) and 51 (c, d) evidenced that the small supernumerary chromosomes (Image from Monti et.al., 2012).

The comparison of the hybridization pattern of the subtelomeric satellite suggested a different amplification of this repeated DNA in the analyzed clones. After FISH experiments, some clones such as clone 70 (Fig. 3.9a) presented larger subtelomeric areas than others, as evident for clone 51 (Fig. 3.9b). Bar = 10 μm.

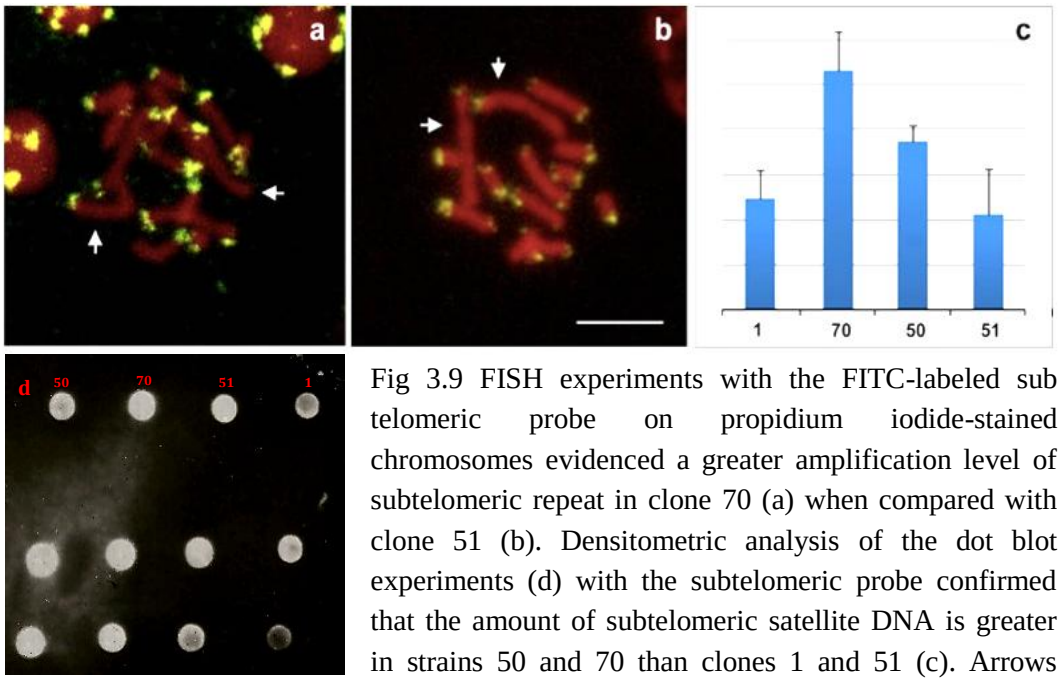


Fig 3.9 FISH experiments with the FITC-labeled sub telomeric probe on propidium iodide-stained chromosomes evidenced a greater amplification level of subtelomeric repeat in clone 70 (a) when compared with clone 51 (b). Densitometric analysis of the dot blot experiments (d) with the subtelomeric probe confirmed that the amount of subtelomeric satellite DNA is greater in strains 50 and 70 than clones 1 and 51 (c). Arrows indicate X chromosomes (Image from Monti et.al., 2012). Bar = 10 μm.

Dot-blot experiments with the subtelomeric probe confirmed this difference and showed that the amount of subtelomeric satellite DNA was greater in clones 50 and 70 than in clones 1 (used as control with standard karyotype) and 51, respectively, revealing the occurrence of clone-specific amplification of the subtelomeric regions of the chromosomes.

Differences among clones were not limited to the presence of variant karyotypes, since rearrangements were also observed upon comparing plates with the standard 2n= 12 chromosome number. Indeed, applying the FISH approach using the Hind200 probe I showed that the X chromosomes could have a different hybridization pattern (Fig 3.10). In particular, clones 33H (previously analysed) (Fig 3.10a), 51 (Fig 3.10c), 70 (Fig 3.10d) and 1 (Fig 3.10e) showed four bands on the X chromosomes after Hind200 hybridization, whereas clone 50 (Fig 3.10b) showed five bands, suggesting that a duplication of a Hind200 band occurred.

Interestingly, clones 1 and 33H presented a band in a different position on the X chromosomes than clones 51 and 70, clearly indicating that an inversion event had occurred (Fig 3.10f).

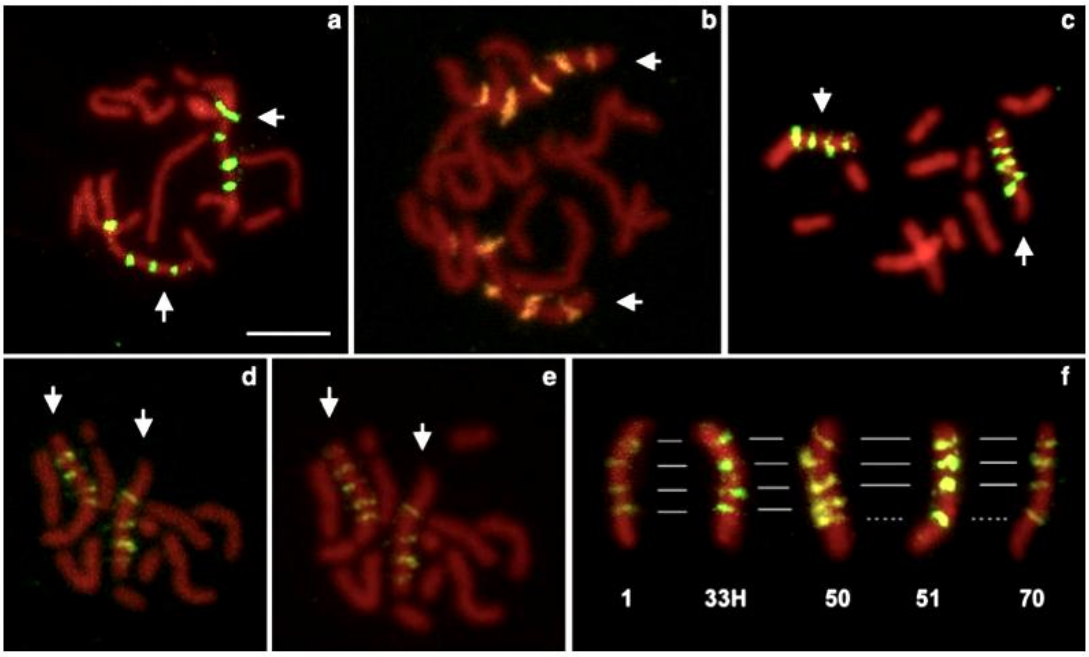


Fig 3.10 FISH with the FITC-labelled Hind200 probe showed four hybridization bands on the X chromosomes of clones 33H (a), 51 (c), 70 (d) and 1 (e) and five bands in clone 50 (b), as evident in the comparison of the X hybridization pattern (f). Arrows indicate the X chromosoemes (Image from Monti et.al., 2012) Bar = 10 μm.

Attempts at male induction revealed that *M. persicae* clones 33H, 50, 51 and 70 were anholocyclic, since it was not possible to produce the sexual morphs, contrary to the situation obtained under the same experimental conditions with the holocyclic *M. persicae* clone 1 used as a control.

3.2 STABILIZATION OF BROKEN CHROMOSOMES ENDS BY *TELOMERASE*

The structure of telomeres was evaluated in order to study if the chromosome fragmentation could be stabilized by "*de novo*" synthesis of telomeres. Hence, I performed some analysis comparing *M. persicae* clones with variable chromosome number with a stable one.

In addition, the presence of the (TTAGG)n repeat was evaluated also in the other aphid species *A. pisum, Megoura viciae, and R. padi* by Southern blotting and FISH. Southern blotting revealed a widespread smear of hybridization, together with several well recognizable electrophoretic bands in all lanes (Fig 3.11), whereas FISH experiments with the telomeric (TTAGG)n probe showed bright FITC-fluorescent spots at the ends of all chromosomes (Fig. 3.12 a–d).

In the interphase nuclei of *M. persicae*, telomeres clustered in few highly fluorescent foci (Fig 3.12 g).

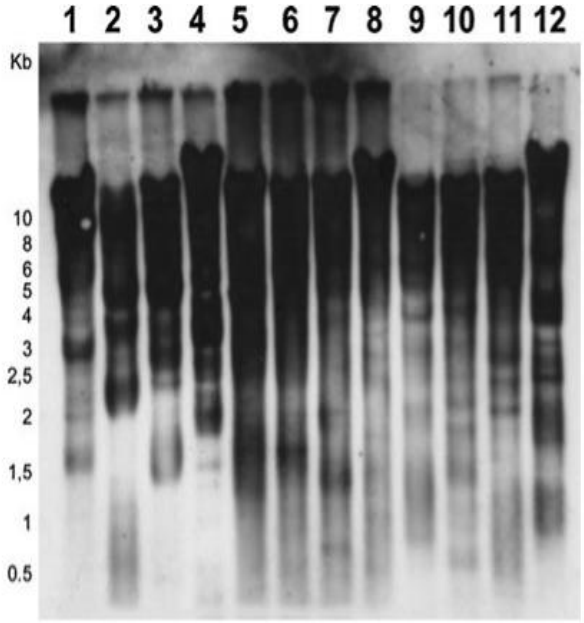


Fig 3.11 Genomic DNAs of *Megoura viciae* (lanes 1–4), *Myzus persicae* (lanes 5–8) and *Rhopalosiphum padi* (9–12) digested with HindIII (lanes 1, 5, 9), EcoRI (lanes 2, 6, 10), ScaI (lanes 3, 7, 11) and XhoI (4, 8, 12) and hybridized with the telomeric probe (TTAGG)n showing a diffuse and intense labelling of aphid DNAs (Image from Monti et.al., 2011).

The identification of telomeric TTAGG sequence at the ends of chromosomes implies that the aphid genomeshould encode a telomerase reverse transcriptase (TERT).

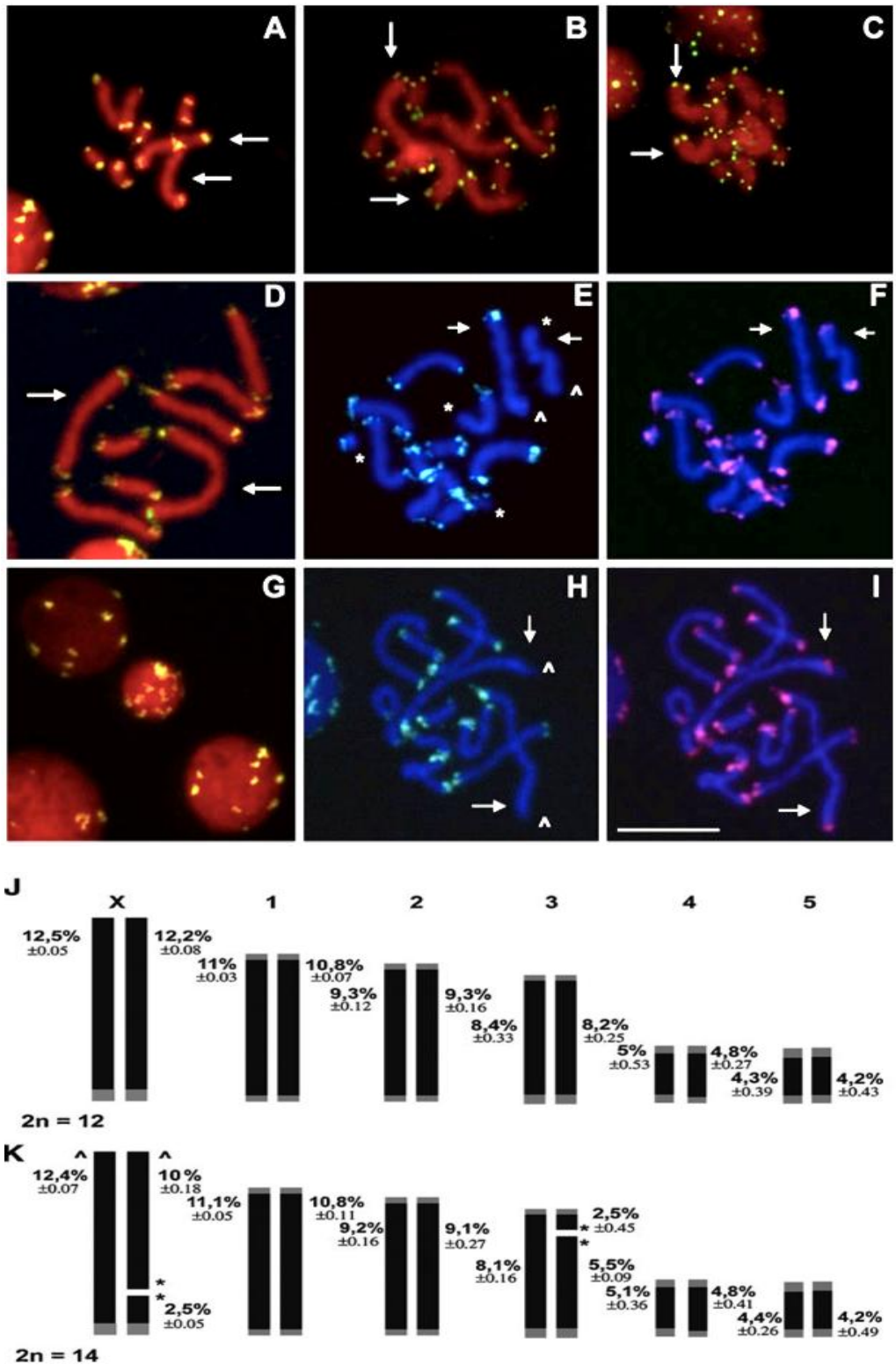


Fig 3.12 a–i Fluorescence *in situ* hybridization (FISH) analysis of *R. padi* (a) and *M. viciae* (b), *M. persicae* (c) and *A. pisum* (d) chromosomes showed bright FITC fluorescent signals at both ends of all chromosomes with telomeric (TTAGG)n probes.

Bright spots were also observed in several foci in *M. persicae* interphase nuclei (g). Signal comparison after hybridization with the FITC-labelled 169 bp subtelomeric probe (e, h) and the TRITC-labelled telomeric probe (f, i) on *M. persicae* chromosomes of strains 33 H (e–f) and 1(h, i) allowed identification of *de novo* synthesized telomeres. Comparison between the karyotypes of strains 1 and clone 33 H (lower panel: J and K) shows labelling after hybridization with a subtelomeric probe (gray) with the chromosomes (black). The percentages indicate the length of each chromosome expressed as a percentage of the total complement length. Arrows indicate X chromosomes, asterisks indicate chromosomal ends involved in *de novo* telomere synthesis (i.e., positive labelling with telomeric probe and absence of labelling with subtelomeric probe). The symbol (^) indicates the NOR-bearing telomeres of the X chromosomes (Image from Monti et.al., 2011) Bar = 10 μm.

Using sequences of other insects telomerases (isolated in the honey bee, *Apis mellifera* and in the flour beetle, *Tribolium castaneum)*, I identified a candidate gene encoding a putative telomerase (predicted protein XP_001946970) in the recently sequenced genome of the pea aphid (International Aphid Genomic Consortium 2010). The identified telomerase gene encodes a predicted protein with an N-terminal region typical of a telomerase containing the telomerase RNA binding domain (TRBD) made up of alpha helices and two short beta sheets, and a TERT domain corresponding to the catalytic subunit of all telomerase reverse transcriptases. Unusually for a telomerase, the C-terminal region of peptide XP_001946970 contains a ICMT domain identified in a number of bacterial and eukaryotic proteins of unknown function that are approximately 300 residues long. In order to clarify the real structure of pea aphid telomerase, a TERT cDNA was amplified by 5′ and 3′ RACE. The resultant sequence was 2,521 bp in length and encoded a 840 amino acid telomerase. At a structural level, aphid telomerase possesses the two main TERT functional domains reported previously in *A. mellifera* (Fig 3.13).

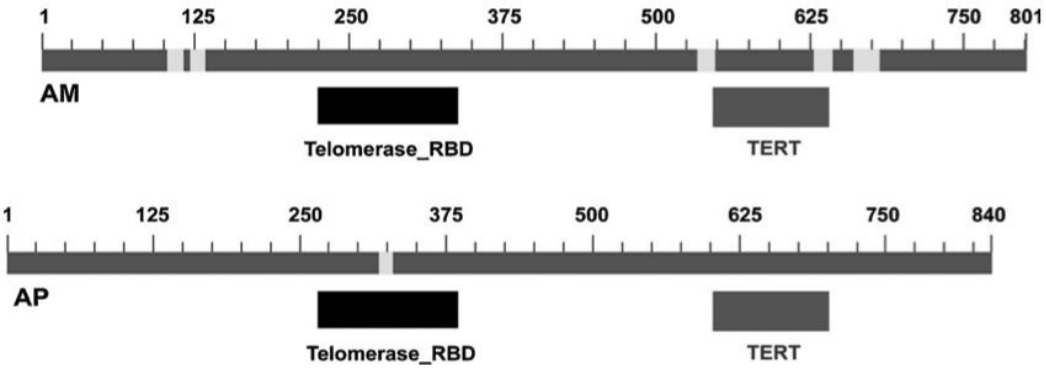


Fig 3.13 Comparison of the telomerase functional domains identified in *Apis mellifera* (AM) and *Acyrthosiphon pisum* (AP). The two main domains are both conserved and spaced in he same way in the two insect TERTs. Telomerase_RBD Telomerase ribonucleoprotein complex–RNA binding domain, TERT telomerase reverse transcriptase domain.

RT-PCR experiments performed using RNA samples extracted from embryos, head, gut and the whole aphid body (but without embryos, gut and head) showed that TERT expression was present both in adults and embryo tissues of all aphid species analyzed (Fig 3.14).

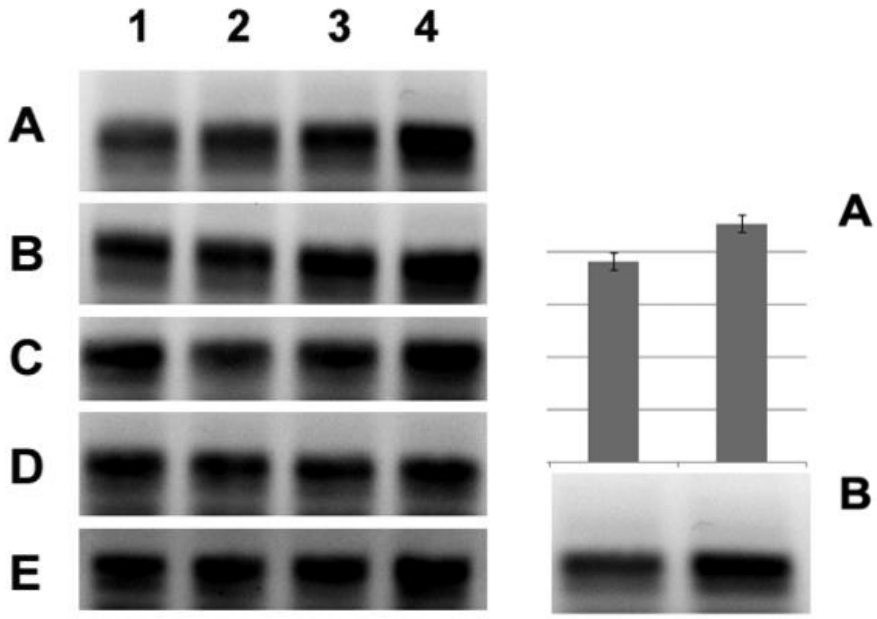


Fig 3.14 RT-PCR of RNA isolated from different tissues of *A. pisum* (a), *M. viciae* (b), *R. padi* (c), *M. persicae* strain 1 (d) and M. persicae strain 33 H (e) to detect telomerase expression. Lanes: 1 Embryos, 2 gut, 3 head, 4 aphid body devoid of embryos. (A) (B)

Graphic rappresentation of a greater telomerase's expression in a variable strain compared to stable strain (Image from Monti et.al., 2011).

Chromosomes of *M. persicae* strains 1 and 33H were hybridized simultaneously with a TRITC- telomeric (TTAGG)n probe and with an FITC- labelled subtelomeric 169 bp satellite DNA (Spence et.al., 1998). In strain 1, which has the standard *M. persicae* karyotype with 2n=12, the 169 bp satellite DNA probe labelled the subtelomeric regions of all chromosomes, with the exception of the NOR-bearing telomeres of the X chromosomes (Fig 3.12h), in agreement with published results (Spence et.al., 1998). Hybridization of the same plate with the telomeric (TTAGG)n repeat showed bright spots at the ends of all chromosomes (Fig 3.12 i). Analysing 33H, this strain exhibited intra-individual chromosome mosaicism so that several plates revealed 14 chromosomes in place of the standard 2n = 12 chromosome number. Accordingly to estimates of relative chromosome length, the observed variations in chromosome number were due to chromosomal fragmentations (Fig 3.12 j–k).

In view of the recurrent fragmentation observed in *M.persicae* strain 33H, plates with 2n = 14 were very interesting in order to evaluate if a new telomere has been synthesized at the broken end. After hybridization with the 169-bp satellite DNA probe, six telomeres were negative for labelling: two corresponding to the NOR-bearing X telomeres and four due to chromosomal fragmentations (Fig 3.12 f).

On the contrary, all the telomeres were labelled with the telomeric probe, including both termini of the two broken chromosomes, indicating that de novo (TTAGG)n telomere synthesis occurred after chromosomal fragmentation.

In this PhD thesis I reported a new molecular cytogenetic approach the Fiber FISH method (Fig 3.15), not easily available elsewhere, applied for the first time in the aphids and useful to show the chromatin fibers (a). This specific technique has been utilized in order to focus the localization and distribution of sub-telomeric and telomeric regions (b) in normal chromosome and in fragmented chromosomes.

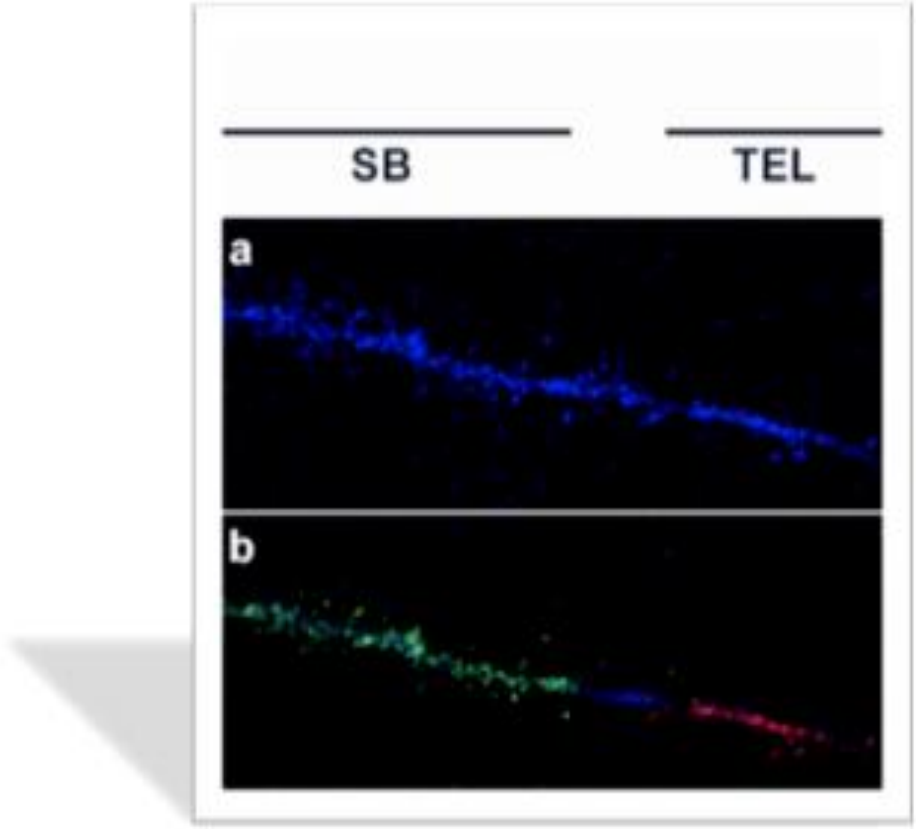


Fig 3.15 Chromatin fiber with FITC-labelled subtelomeric sDNA (b) and Texas red-labelled $(TTAGG)_n$ probe (b) (Image from Monti et.al., 2012-2013).

3.3 CHARACTERIZATION OF *NON*-LTR RETROTRANSPOSABLE *TRAS* ELEMENT

In order to better understand the structure of aphid sub- telomeric regions, I amplified, sequenced, and localized copies of the non-LTR retrotransposon TRAS in the aphids *A. pisum* and *M. persicae*, two of the most studied species at a cytogenetic level due to their relevance as experimental models and crop pests. Moreover, the characterization of TRAS element could also represent another marker useful to study more accurately the telomeres of sex chromosomes. According to the results of the *A. pisum* genome project, the sequence XM_001942587 has been annotated in GenBank as partial TRAS sequence similar to TRAS3.

This sequence corresponded to the typical RT domain of elements belonging to the TRAS families. Starting from this annotated sequence, I designed two couples of primers which allowed the amplification, by direct PCR, of a 800-bp-long

fragment of this ORF both in *M. persicae* (Fig 3.16, line 1) and in *A. pisum* (line 2). In both species, electrophoresis evidenced several other smaller PCR bands that sequencing identified as truncated copies of the TRAS elements.

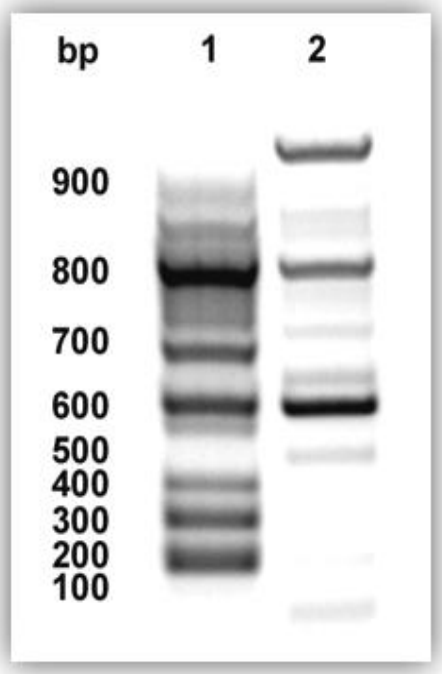


Fig 3.16 Amplification of the TRAS portions in *A. pisum* (1) and *M. persicae* (2) by direct PCR. Molecular weight has been evaluated using the markers 100-kb ladder (Image from Monti et.al., 2013).

In view of the unusual presence of truncated TRAS copies, we have verified the presence of deleted copies of TRAS elements in *Aphidbase* evidencing several scaffolds containing portion of this retrotransposon; all the deleted copies were not flanked by the canonical telomeric TTAGG sequence.
Interestingly, PCR evidenced several other smaller bands that sequencing identified as truncated copies of the TRAS elements in both species. Successively I complete the sequence of the *A. pisum* TRAS elements assembling a 7.762 bp TRAS element identified as TRASAp1 (GenBank ID JX875955).

TRASAp1 elements possessed the typical CA repeat in the 5' portion and two overlapping ORF regions with a *gag*-like ORF1 and a *pol*-ORF2 containing the RT and the endonuclase domains (Fig 3.17).

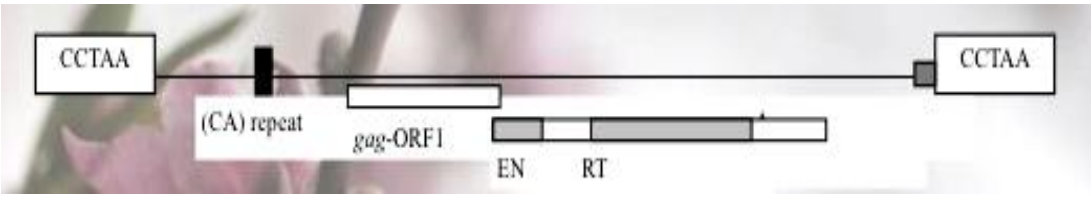


Fig 3.17 Schematic rapresentation of sequence of the *A. pisum* TRASAp1 element (Image from Monti et.al., 2013but readapted).

The investigation of the TRAS chromosomal insertion sites through standard hybridization, evidenced different bright spots at both the termini of all *A. pisum* (Fig. 3.18a) and *M. persicae* autosomes (Fig 3.18 b). Notably, the X chromosome telomeres (arrows) showed hybridization signals only at the telomere opposite to the NOR-bearing one, as evidenced by the CMA3 staining (Fig 3.18c, d).

The image 3.18 showed a several small discrete spots in interphase *M. persicae* (**e**) and *A. pisum* (**f**) nuclei after FISHwith TRAS probe. Morover the FISH experiments, carried out in *M. persicae* strain 33H possessing spontaneous fragmentations of the X chromosomes (arrow head) allowed us to assess that TRAS elements were not involved in the *de novo* healing of chromosomal ends (g).

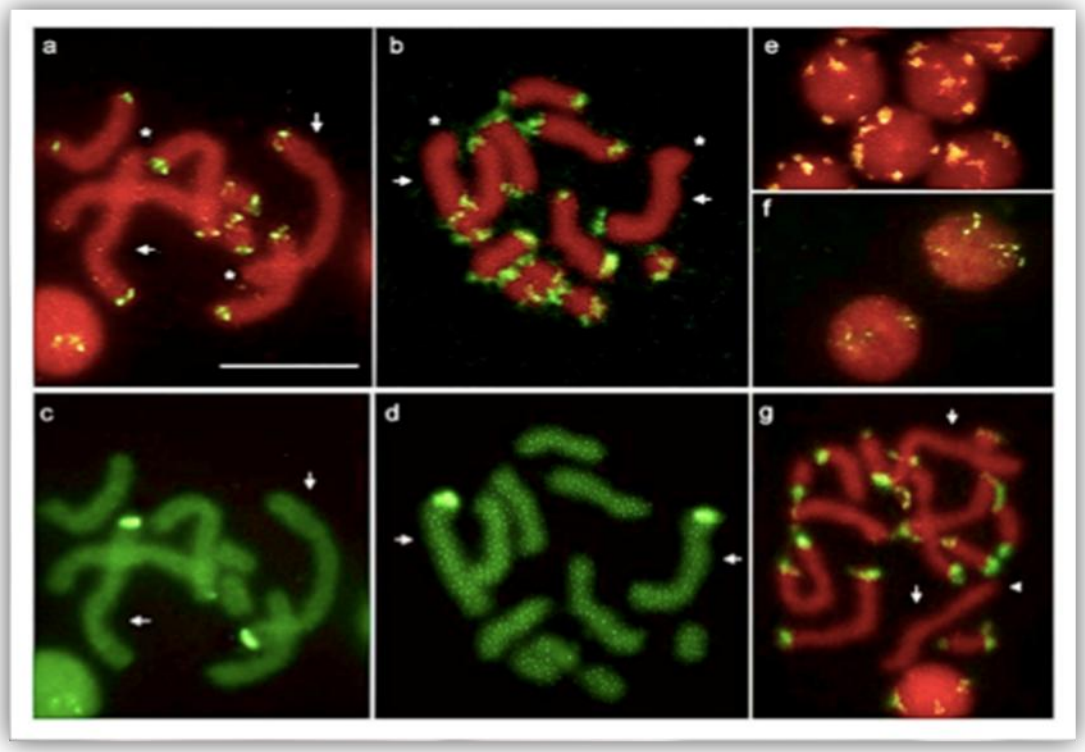


Fig 3.18 FISH with the *A. pisum* TRAS probe showed the TRAS elements location on metaphase chromosomes in each species of aphids. Asterisks indicate X telomere devoid of TRAS elements. Arrow head indicates fragmented X chromosome without any TRAS hybridization signal. Arrows indicate X chromosomes. Bar = 10 μm (Image from Monti et.al., 2013).

Finally, considering that literature data (Spence et al. 1998), attested the presence of a satellite DNA sequence in *M. persicae* subtelomeric regions, I successfully applied for the on aphid chromatin the fiber FISH technique (Fig. 3.19) in order to localize the precise distribution and insertion sites of TRAS retrostrasposons. According to the results obtained, TRAS elements were located between the inner portion of the telomeric (TTAGG)*n* array and the outer part of the subtelomeric satellite DNA cluster.

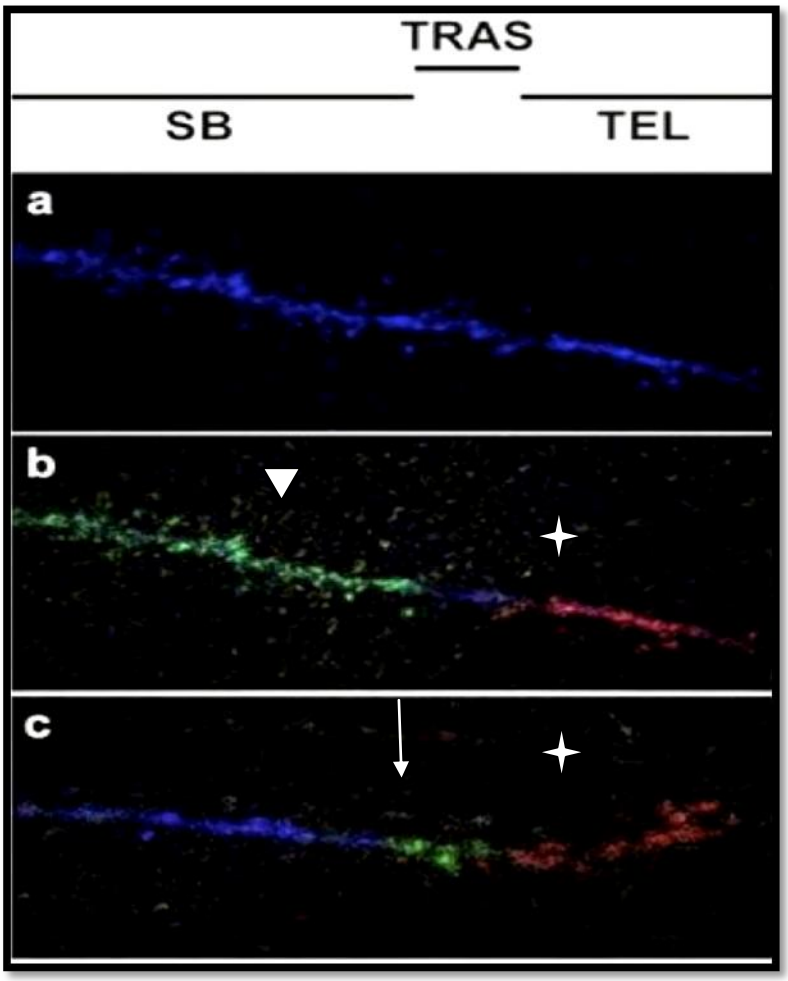


Fig. 3.19 FISH on *M. persicae* DNA fiber, stained with DAPI (**a**), showed that TRAS elements (indicated by the arrow in panel **c** after hybridization with the *A. pisum* TRAS probe) are interspersed in the inner portion of the telomeric TTAGG array (indicated by the star after the TTAGG*n* telomeric DNA probe labelling with TRITC, panels **b** and **c**) and not in the cluster of the subtelomeric satellite DNA (indicated by the arrow head in FISH experiments with the FITC-labelled subtelomeric DNA repeat as a probe, panel **b**) (Image from Monti et.al., 2012).

3.4 RELIABILITY OF PAINTING PROBES AS TOOLS FOR KARIOLOGICAL STUDIES IN *M.persicae*

Using laser microdissections two samples of X-chromosome DNA, each containing 8 chromosomes, were collected. DNA of both samples was amplified

by WGA4 kit and the products inspected by gel electrophoresis. The gel showed a pattern of smear consisting of of a heterogeneous population of DNA fragments. Their sizes ranged from less than 400 bp to more than 1650 bp (Fig 3.20).

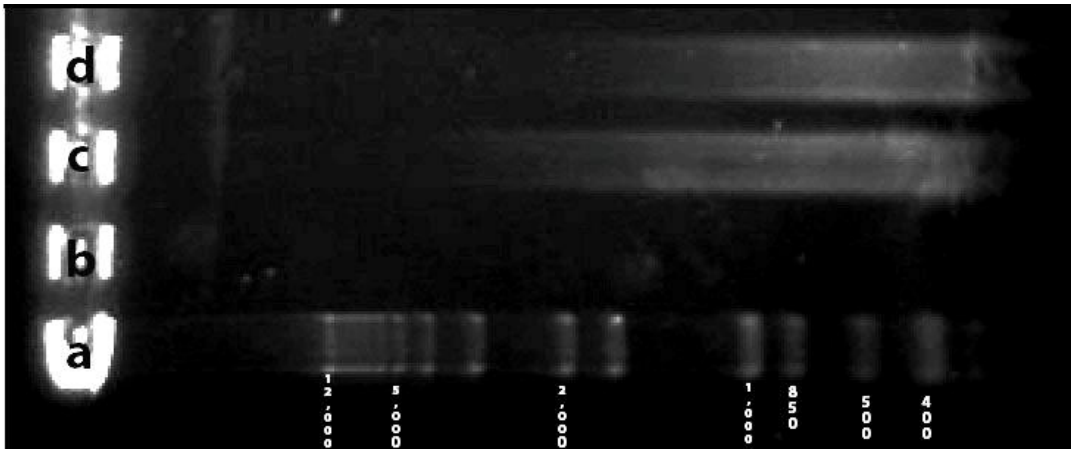


Fig 3.20. Electrophoresis of the microdissected samples of X-chromosome DNA, first amplified by WGA4 kit and then re-amplified by WGA3 kit. Amplification products were separated in a 1% agarose gel and stained with GelRed. Size specification (a) in bp was done with 1 Kb Plus DNA Ladder (Invitrogen Life Technologies, Carlsbad, CA, USA). Lane (b), blank control; lanes (c) and (d) show amplified DNA of the X chromosome samples obtained from *Acyrthosiphon pisum* and *Myzus persicae*, respectively.

In order to obtain chromosome-specific painting probes for aphids, I have used the P.A.L.M. MicroLaser System which works with a pulsed nitrogen laser. Laser microdissection allows dissection of chromosomes without any mechanical contact and collection of particular chromosomes by catapulting is rapid. Therefore, microdissection of chromosomes by this laser technique minimizes the hazard of contamination which is of great importance for PCR-based methods. A prerequisite for successful conventional and laser microdissection is a good supply of well-spread chromosomes. To identify the chromosome of interest, Giemsa staining, C-banding or G- banding may be used (Svatava Kubickova et.al., 2002). However, the banding quality of membrane-mounted chromosomes is always lower compared with chromosomes directly spread on glass slides. The Zeiss manual recommended the use of GIEMSA as a specific staining because it didn't interfere during microdissection (PALM User Protocols). Unfortunately, this staining is not able to allow a clear-cut identification of aphid chromosomes (Blackman et.al., 1981). Therefore, the only feature that can be used to differentiate the aphid X chromosome is their large size since other staining

techniques as CMA3 staining can't be use for microdisection.However, even by size it is not easy to identify *Myzus persicae* sex chromosomes because the autosome 1 is similar in size and also allocicly hampered a precise distinction of these chromosomes. FISH with the X-chromosome derived probes showed ambiguous results in *M. persicae*. In some metaphase complements, the X-probes exhibited notably stronger binding, though scattered, to the X chromosomes than to the other chromosomes (autosomes) (Fig 3.21 c-b). A cluster of strong hybridization signals was regularly found only in one subtelomeric region (most probably colocalized with the rDNA cluster; see Monti et.al., 2011) of the X-chromosomes, with few weaker signals scattered in the other parts of the X chromosome (Fig. 3.21 b-d). However, the probes also hybridized to telomeric regions in most autosomes (Fig. 3.21 e and Fig. 3.22c, d) which are known to consist of arrays of telomeric and subtelomeric repeats (Spence et al. 1998; Frydrychová et al. 2004; Monti et.al., 2012). The telomeric hybridization signals were often observed in all autosomes at either one or both terminal segments. In some metaphases, the X-probes preferentially labelled sex chromosomes and particularly a subtelomeric region, probably representing a cluster of rDNA sequences (nucleous organizer region, NOR). However, the X-probes hybridized less intensely to the other chromosomes as well (Fig. 3.22a, b). The X-chromosomes territory in the interphase nuclei was also painted (Fig. 3.21 f). However, in other chromosome complements and interphase nuclei, the X-chromosome derived probes showed various hybridization patterns (Fig 3.22 a-f). The scattered hybridization signals also in autosomes (Fig. 3.21 a, b, f) and the telomeric signals (Fig. 3.22c, d, f) suggest that the probes are not sufficiently specific to paint the X chromosomes regularly along their entire lenght. Instead the probes often highlighted common repetitive sequences such as telomeric and subtelomeric repeats. This was also obvious in many interphase nuclei that showed several clear spots of hybridization signals (Fig. 3.22e). However, the signals on interpahse nuclei could represent not only telomeric and subtelomeric repeats but also rDNA and possibly anonymous repeats common in the genome of *Myzus persicae*.

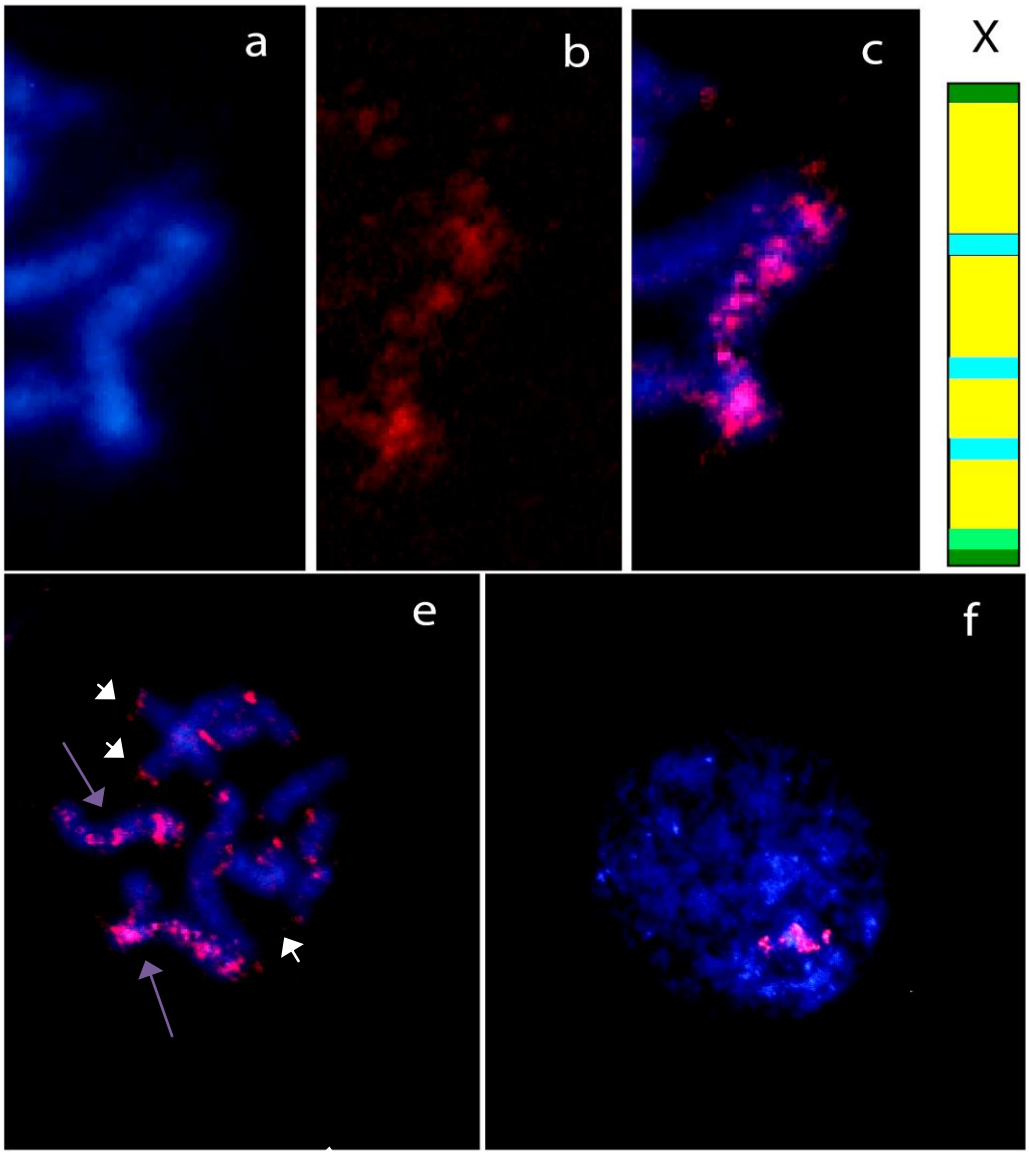


Fig 3.21. FISH patterns of the X-chromosome painting probe in spread preparations prepared from parthenogenetic female embryos of *Myzus persicae*. The probe was labeled with Cy3-dUTP (red), chromosomes were counterstained with DAPI (light blue). **a-c** - A detail of the X chromosome from metaphase in (**e**): a - DAPI image; **b** - hybridization signals; **c** - merged image showing hybridization signals of the X-probe scattered along the whole length of the X chromosome. **d** – A scheme of the painted X chromosome; blue indicates intercalary sequence repeats typical of the X-chromosomes (HIND satellite), yellow dispersed repeats and other unknown sequences, dark green telomeric sequences, and light green indicates strong hybridization signals in the subtelomeric region at one end only. **e** – A metaphase complement showing unequal pattern of hybrydization signals on two X chromosomes (arrows); note weaker hybridization signals at the telomeres of most autosomes (arrowheads). **f** - Interphase nucleus showing clustered hybridization signals, posibly indicating the X-chromosome territory. Bar = 10 μm.

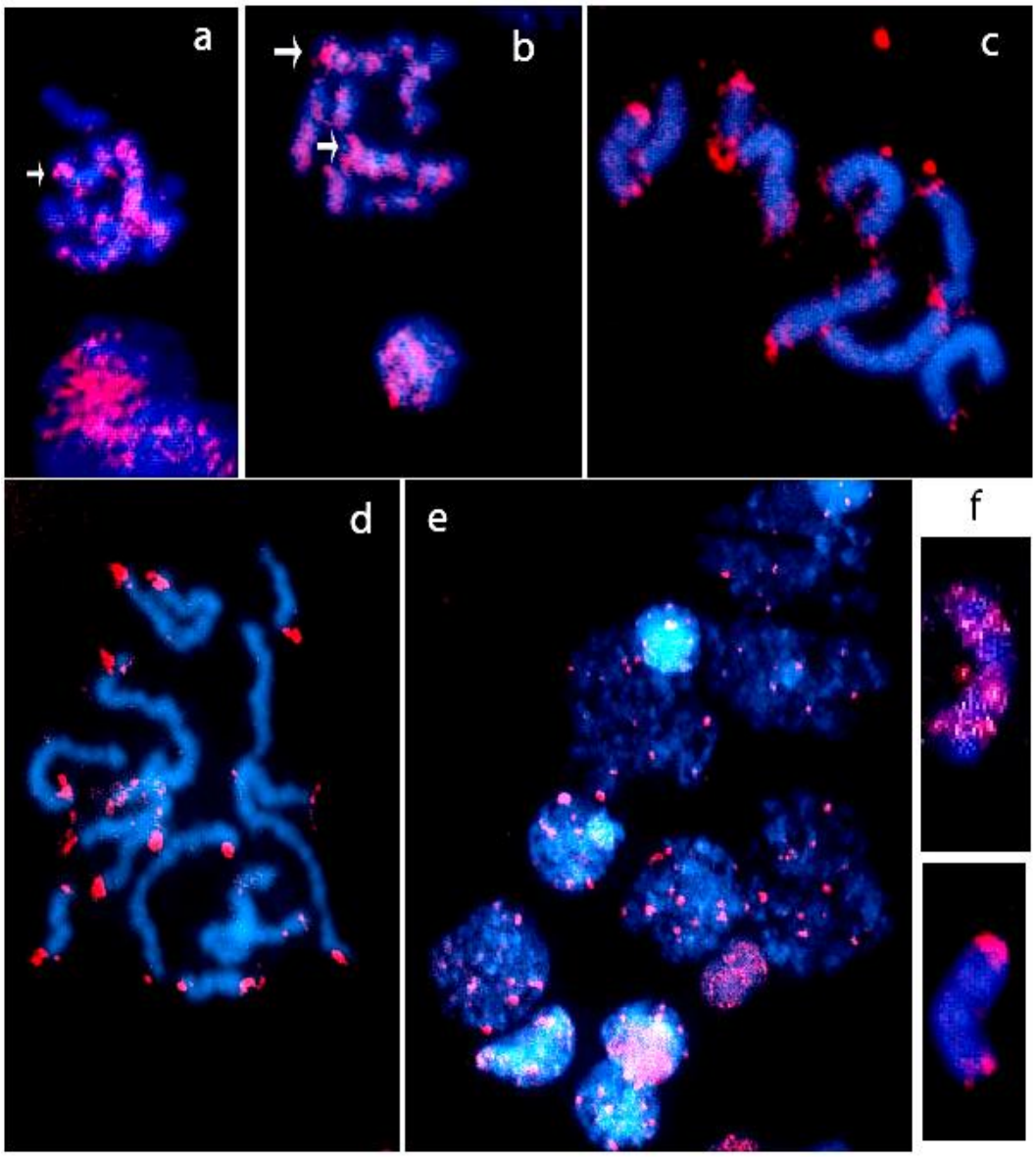


Fig 3.23. Further FISH patterns of the X-chromosome painting probe in spread preparations prepared from parthenogenetic female embryos of *Myzus persicae*. The probe was labeled with Cy3-dUTP (red), chromosomes were counterstained with DAPI (light blue). **a**, **b** – Metaphase complements showing scattered hybridization signals on the X chromosomes but to a lesser extent also on the autosomes indicating insufficient specificity of the probe; arrows point to strong hybridizations signals at one end of the X chromosomes, possibly highlighting the NOR region of the X chromosomes. **c** - Incomplete metaphase (10 chromosmes) showing telomeric hybridization signals at most chromosome ends. **d** - Late prophase complement with strong hybridization signals in most telomeres; note that the X chromosome is not highlighted with the probe. **e** - Interphase nuclei showing several scattered hybridization signals each, probably dut to the probe labelling of chromosome ends. f - a schematic rappresentation about scattered signal on X chromsome withput competitor and the telomere signal on autosome . Bar = 10 μm.

3.5 ANALYSIS OF DOSAGE COMPENSATION BY REAL TIME

Linear standard curves were obtained after serial dilution and amplification of six concentration points of cDNA ranging from 80 to 5 ng of retro-transcribed total RNA extract from female samples.

Genes	**slope**	**intercept**	**R2**
Fibronectin	-3.00	25.05	0.987
RAD 50	-3.22	27.59	0.998
RPP 15	-3.13	28.76	0.996
Actin	-3.35	21.40	0.995

Table 3.24 Standard curve parameters for reference (actin) an target genes

For dosage compensation assay, the level of each target genes expression (fibronectin, RAD 50 and RPP 15) were normalized on reference gene (actin) transcription rate. Twenty nanograms of cDNA was used for gene amplifcations in males.

Genes	Fold mean [a] ± SD [b]	CI (95%) [c]
Fibronectin	7.3 ± 3.21	3.00 ÷ 9.52
RAD 50	1.7 ± 0.48	1.00 ÷ 2.34
RPP 15	1.4 ± 0.09	1.31 ÷ 1.54

Table 3.25 Relative expression levels of target genes

a Relative level of gene expression represents the ratio of gene expression for each target gene in males compared with that in females

b Standard deviation of mean values

[c] 95% confidence interval for the mean

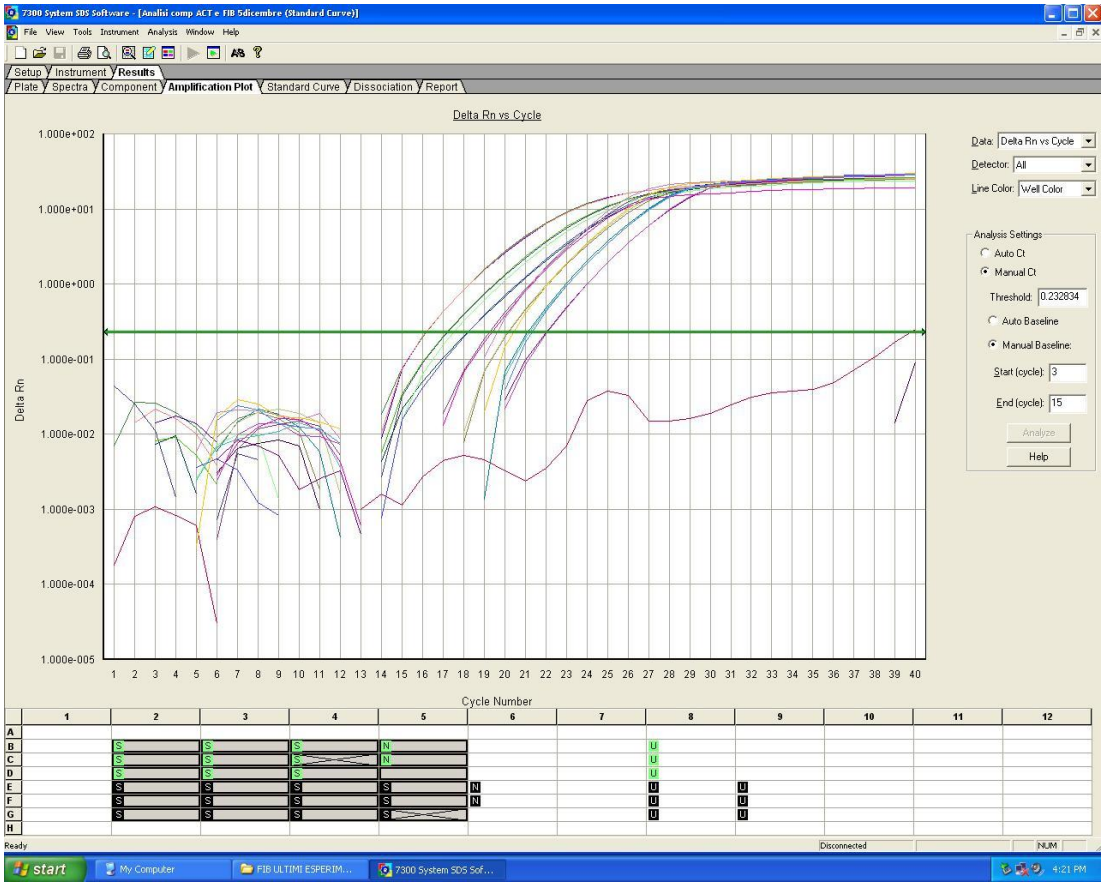


Fig 3.26 Representative amplification plot for target (fibronectin) and reference (actin) genes for standard curve.

4 DISCUSSION

Aphids (Insecta: Hemiptera: Aphididae), like all other living organisms, are not immune from the effects of mutation, selection/genetic drift, adaptation, and extinction (Thompson 1994). With this in mind, it is thus strange that the members of such a lineage are still assumed by some scientists to be "genetically identical" to their stem mother, and thereby, with little genetic/functional plasticity to be able to adapt in the face of changing ecological circumstances (Lushai et al. 2003). If the aphid "clone" *sensu stricto* really did exist a natural population of these organisms would be represented by a population mean with no variance for any given trait/s in question, a bizarre state of affairs and indeed, a Creationist's dream (H.D. Loxdale, 2009). This is surely an incorrect view of the real world that we know from studies of numerous other organisms (Loxdale and Lushai 2003b).

In reality, due to their fast rate of asexual reproduction (parthenogenetic, apomictic) and short generation time (10 days) involving "telescoping of generations" (Dixon 1998), a single virgin female of a given aphid species, e.g., the peach-potato aphid, *Myzus persicae* (Sulzer), can typically give rise to 30–90 offspring (Blackman 1971). Under ideal climatic conditions and with a dearth of predators, parasitoids and pathogens this can potentially result in billions of individuals derived from one individual in a single growing season (Dixon 1989; Harrington 1994). Even at typical mutation rates of 10^{-9} to 10^{-6} per gene per generation, a large number of mutant aphids are likely to be produced with such an astronomical rate of reproduction.

For example, in one large Alfalfa growing region in California alone, it has been calculated that some 1.7 × 1011 Spotted Alfalfa aphids, *Therioaphis trifolii* form *maculata* (Buckton) were produced in a couple of growing seasons (Dickson 1962). At a conservative mutation rate of 10^{-7}, this means that at any given locus, around 17,000 mutations might arise. The large majority of these mutations are likely to be mildly deleterious and at non-coding regions of the genome; however, others are probably important, that is to say, at coding regions and are thus positively or negatively selected for (Lambert and Moran 1998; Lynch and Blanchard 1998; Fry et al. 1999; Korona 2004; Begin and Schoen 2006; Paland and Lynch 2006; Barraclough et al. 2007; see also Vorwerk and Forneck 2007). In the case of the Spotted Alfalfa aphid, some mutations were shown to confer resistance to organo-phosphates insecticides, so that the insect became resistant to these pesticides within a relatively short time after its introduction into the USA in the early 1950s (Dickson 1962; Blackman and Eastop 2000).

In 1837, Charles Darwin (1809–1882) stated in his "B" notebook, relating to the phenotype of animals and plants (since genes had yet to be identified that "If all organisms merely replicated their kind by vegetative budding or splitting, history

would show a succession of identical individuals holding no potential for alterations of any kind" (Browne 1996). In relation to aphids, Dan Janzen argued in 1977 that because aphids have asexual phases during which the offspring produced are "genetically identical", the members of such a clone are in effect a super-organism, a single "evolutionary individual", and are thereby able to exploit a much larger geographic region and its resources.

In the present PhD thesis, I wish to show and discuss how the use of cytogenetic and molecular knowledges, have transformed our understanding about what constitutes a clone (asexual lineage) as well as higher levels of evolutionary divergence.

Recent use of new molecular markers suggests that the genome of aphids belonging to asexual lineages rapidly accumulate different kind of mutations (Loxdale 2008a, b, 2009, 2010b). In this respect, the presence of chromosome polymorphisms can be extensive in aphids, as reported in *Trama maritime* (Eastop), where specimens of the same species had different karyotypes (inter-individual polymorphisms) (Blackman et al. 2000). Chromosome instability within individuals is an almost unique finding in the animal kingdom at the level of population and species, except in malignant cells. Among insects, it has reported only in ants (Imai, Crozier & Taylor, 1977; Imai, Taylor & Crozier, 1994; Karnik et al., 2010). Until now, studies of different *M. persicae* clones have clearly demonstrated that this species not only comprises different populations with standard 2n = 12 karyotypes, but also several populations bearing different types of genome recombination and/or variant karyotypes (both involving chromosomal rearrangements and changes in chromosome number) making *M. persicae*, as a whole, a complex, but intriguing, aphid species (Lauritzen 1982; Blackman 1987; Fenton et al. 1998; Spence and Blackman 1998; Loxdale 2007; Mandrioli et al. 1999b; Monti et al. 2012).

Analysis of mitotic metaphase chromosomes of *M. persicae* clones 33H, 50, 51 and 70 revealed different chromosome numbers and karyotypes, in contrast to the stan- dard karyotype of this species (2n = 12). In particular, we observed karyotype variations in embryos from different individuals within the same asexual lineage, between embryos from the same individual, and also within each embryo. Chromosome length measurements, combined with FISH experiments, revealed that variant karyotypes were due to recurrent fragmentations of the chromosomes X, 1 and 3, supporting earlier molecular studies reporting intraclonal genetic variations (Loxdale and Lushai et al. 2003). These results can be explained by considering that the holokinetic structure of aphid chromosomes, combined with the apomictic mode of parthenogenesis, facilitates the inheritance of chromosomal rearrangements, since each fragment can be attached to

microtubules so that they can be inherited without the constraint of homologous pairing typical of meiosis. Moreover, this data support the earlier molecular evidence indicating that intraclonal genetic variation occurs in nature and is a potentially important force for generating variation in asexual lines (Loxdale & Lushai, 2003; Lushai, Loxdale & Allen, 2003).

Previous data have shown that autosomes 3 and, more rarely, 1 are the chromosomes mostly involved in changes in the *M. persicae* karyotype (Spence & Blackman, 1998). By contrast, fragmentations associated with the X chromosomes till now have been observed very rarely in aphids not only in natural populations, where only one case has been reported in *Schoutedenia lutea* (van der Goot) (Hemiptera, Aphididae, Greenidinae) (Hales, 1989), but also in X-ray irradiated aphids (Khuda-Bukhsh & Pal, 1985). Irradiation of *Aphis gossypii* (Glover) and *Aphis nerii* (Boyer de Fonscolombe) specimens produced several breaks occurring mainly on autosome 1 and never on the X chromosome, suggesting that X fragmentations are less viable than autosome ones (Khuda-Bukhsh & Pal, 1985). Studies on natural populations of the same species confirmed these results, showing fragmentations of autosomes 1 and 4 but never at X chromosomes (Khuda-Bukhsh & Pal, 1985). The fission of the X chromosome observed in *M. persicae* 33H, 50, 51 and 70 clones using both the subtelomeric repeat and Hind200 probes, involved duplications and inversions occurring near (or within) a heterochromatic band enriched in satellite DNAs. In clone 70 inversion occurred near a subtelomeric heterochromatic band and this change is presumably unlikely to lead to any significant phenotypic change and therefore could perhaps be described as ‘selectively neutral’ (John 1983; Blackman et al. 2000). The presence of chromosome breakpoints occurring within constitutive heterochromatin is well established in the scientific literature and, for example, much of the evolution of mammals has involved peri-centromeric heterochromatin that is known to be particularly variable, as is also known in some insects, such as grasshoppers (Orthoptera: Acrididae) (John, 1983; Blackman, Spence & Normark, 2000).

In contrast, the fragmentation of the X chromosome observed in clone 51 involved euchromatic areas and may well have a phenotypic effect. Indeed these fragmentations that move genes from the proximity of heterochromatic areas of the X chromosomes to a new chromosome, could affect the expression of genes by position-effect variegation, making these changes not neutral at a selective level. For example, in *M. persicae*, translocation of the E4 esterase genes enhanced their expression so that aphids with this rearrangement produce a greater amount of the enzyme, thereby becoming resistant to a range of insecticides (i.e. organophosphates, carbamates and pyrethroids) (Blackman et al.,

1978; Foster & Devon- shire, 2007; Loxdale, 2009). This effect is very interesting considering that the E4 array is generally located near the sub-telomeric region of the *M. persicae* chromosome 3 that is made by the tandem repetition of the 169-bp satellite and that, as shown in *Drosophila melanogaster* Meigen (Eberl, Duyf & Hilliker, 1993), the presence of a gene near repetitive DNAs influences its expression through position-effect variegation. Translocations may therefore turn on and off the E4 genes modifying their expression and, consequently, the level of resistance of aphids to insecticides. Further analysis of this *M. persicae* karyotype variant could therefore be very interesting in view of analyzing the relationship between chromosomal rearrangements and the insecticide resistance level. In addition, fragmentation observed in metaphase plates of chromosomes 1 and 3, involved euchromatic areas and it has been hypothesized that these chromosomal rearrangements could affect some complex phenotypic traits, such as host choice (Blackman, 1987; Ffrench-Constant Margaritopoulos et al., 2000; Vargas et al., et al.,2005). Indeed, several papers clearly indicate that chromosomal changes may affect host preference, as reported in *Sitobion*, *Rhopalosiphum* and *Myzus* taxa (Brown and Blackman 1988; De Barro et al. 1995; Hales et al. 2000), and may well have effects in aphid speciation events (Loxdale and Lushai 2007; Loxdale 2010b). A peculiar example of host adaptation concerns *M. persicae* strains feeding on tobacco. Morphometric analyses of specific taxonomic markers revealed that they are distinguishable from those living on other host plant so that the tobacco- feeding form was elevated to the status of a separate species by Blackman (1987). Further molecular evidences failed to confirm the genetic isolation of the population living on tobacco (Field et al., 1994; Clements et al., 2000), although other data, as well as behavioural/pheromonal evidence, suggest that the two forms undergone some significant degree of ecological-evolutionary divergence (Kephalogianni et al., 2002; Margaritopolous et al., 2003; Blackman et al., 2007). Data presented in this PhD tehsis showed that all but one of the strains collected on tobacco plants highlight karyotype variations, whereas only four of the 56 population collected on other hosts (corresponding to about 7% of the total) displayed chromosomal rearrangements. A suggestive explanation for the observed relationships between chromosomal rearrangements and tobacco plants could rely in the clastogenic effect of nicotine.

Nicotine is a naturally occurring alkaloid found primarily in members of the solanaceous plant family, including *Nicotiana tabacum*. Several reports showed that nicotine, as a consequence of DNA replication fork stress (Richards, 2001; Freudenreich, 2005), produces genotoxic effects on Chinese hamster ovarian (CHO) cells (Trivedi et al., 1990, 1993) and sister chromatid exchanges and chromosome aberrations in bone marrow cells of mice (Sen et al., 1991).

Extensive chromosomal rearrangements have also been described in a mice population known as ‘tobacco mice’ since they live close to kiln for drying tobacco (Fraguedakis-Tsolis et al., 1997). In addition, DNA fragmentation by nicotine has been demonstrated both in peripheral lymphocytes (Sassen et al., 2005) and in human spermatozoa (Arabi, 2004). Nicotine, together with ultraviolet exposure, has also been considered an exogenous factor which can contribute to the generation of mutations which could be at the basis of chromosomal mosaicism (De, 2011), a very rare phenomenon we have observed in 51 clone , one of the strains collected on tobacco plants.

Even if there are no literature data analyzing nicotine effects on organisms possessing holocentric chromosomes, the previously reported data allow us to propose that its clastogenic effects together the peculair architecture of holocentric chromosome could have a pivotal role in aphid chromosome evolution and rearrangements.

During my analysis I evidenced that several chromosomal plates in both clones 50 and 51 possessed a small and heterochromatic chromosome resembling a B chromosome. As reviewed in Camacho (2005), B chromosomes are additional dispensable chromosomes originated by A chromosomes showing a remarkably higher heterochromatin storage compared with A ones. In view of the small length and large size of the subtelomeric repeat cluster at the telomeres, autosomes 3 and 4 are the most probable candidates for the origin of the observed B chromosome. Previous studies have identified supernumerary B chromosomes in aphids of the genus *Euceraphis*, derived from non-functional X chromosomes showing a greater within-species stability in size and number than typical B chromosomes (Blackman 1988). The results observed in this PhD thesis suggest that aphid B chromosomes could originate also from autosomes.

C banding, followed by DAPI staining, showed that in both clones 50 and 51, B chromosomes are rapidly heterochromatinized, being highly enriched in heterochromatin. B chromosomes are generally enriched in satellite DNA and TEs and the accumulation of repeated DNA seems to be a very common event in B chromosomes differentiation. However, the occurrence of B chromosomes at an intra-clonal level in aphids argues that accumulation of repetitive DNA is not the primary cause of B chromosome differentiation. In particular, the rapid process of heterochromatinization could primarily result from epigenetic changes of the B chromosomes that are subsequently followed by structural modifications, including TE invasion and repetitive DNA amplification. Perhaps the chromosomal fragmentations presently seen, as suggested for intraclonal, intramorph RAPD (genomic) polymorphisms in aphids (e.g. Loxdale 2008a, b), may be due to TE effects, transposon rich ‘hotspots’ being known to cause

rearrangements, i.e. inversion polymorphisms in other insects like *Drosophila* (Ca´ceres et al. 1999).

The random-breakage model of chromosome evolution has been the dominant paradigm for several years (Becker & Lenhard, 2007). Interestingly, several comparative mapping studies in a wide variety of closely-related eukaryotes showed a relationship between large-scale chromosomal rearrangement and repetitive DNA. The nature of the repetitive DNA within these breakpoint regions varies significantly, from clusters of rRNA and tRNA genes to various mobile elements (Caceres etal., 1999; Carlton et al., 2002; Coghlan & Wolfe, 2002; Kellis et al., 2003). Segmental duplications that distinguish the human and great-ape karyotypes have been, for example, related to breakpoints revealing that some human diseases may also be the result of chromosomal breakpoints because of hot spots of recombination (Bailey et al., 2004). In addition, computational analyses of breakpoints suggested that recurrent evolutionary breaks are found in fragile regions or hot spots, so that the random breakage model required substantial reassessment in favour of models that put the architecture of the chromosomes in a pivotal position for revealing the molecular basis of chromosomal evolution among species.

In view of the recurrent fission of the same chromosomes in the same region, the *M.persicae* genome appears therefore to have some fragile sites that could be the basis for the observed changes in the chromosome number. Hot spots of chromosomal recombination have already been identified in aphids within rDNA genes that contain specific sequences with high similarity to the consensus core region of human hypervariable minisatellites (Jef- freys, Wilson & Thein, 1985) and with the c sequence of *Escherichia coli* (Smith, 1983). They are involved in rDNA pairing (Mandrioli et al., 1999b), as also previously reported in *D. melanogaster* (Ault, Lin & Church, 1982; Park & Yamamoto, 1995). As a consequence of such hot spots, the occurrence of X chromosomes paired at nucleolar organizer regions (NORs) has been observed in several aphid species (Mandrioli et al., 1999b) together with the occurrence of intra- and inter-individual NOR heteromorphism as a result of unequal crossing over between the two X chromosomes (Mandrioli et al., 1999a).

The holokinetic structure of aphid chromosomes that ensures the attachment of spindle microtubules along most of the chromosome length is itself not sufficient to stabilize chromosome fragments because chromosomal breakpoints may be highly unstable, displaying a propensity to fuse with other broken ends. The breakpoints need therefore to be stabilized before the transmission of chromosomal fragments to the daughter cells (Vermeesch & Price, 1994; Hug & Lingner, 2006; Pennaneach, Putnam & Kolodner, 2006).

Thereby it is important to wonder which are the biological properties of chromosomal fragments stabilization?

First criteria underlying the stabilization of chromosomal fragment are known as "*healing of breakpoints*" or "*de novo telomere synthesis*", generally involving the addition of repetitive telomeric sequences at the breakpoints by telomerase. Indeed, the essential function of telomeres is to protect chromosome ends from nucleolytic degradation, chromosome fusion and the inappropriate engagement of checkpoint signaling (Lydall, 2003). Hence, the addition of telomere repeats results in the stabilization of the new chromosome end and allows resumption of cell cycling (Vermeesch and Price 1994; Hug and Lingner 2006; Pennaneach et al. 2006). In the absence of healing, irreparable double-strand breaks lead to programmed cell death, as reported in yeast (Sandell and Zakian 1993), or to the activation of proto-oncogenes, as described in mammals (Lee and Myung 2009).

On the basis of the results illustrated in the present study in *M. persicae* strain 33H, it has been showed that aphids are able to carry out the *de novo* synthesis of telomeric sequences and telomerase is expressed at higher level in strain 33H than in strain 1 (utilized as a control because it possesses a stable karyotype 2n = 12), suggesting an active role of telomerase in the stabilization of chromosomal fragments. The latter event by telomere sequence addition, has been observed in many organisms, from yeast to man (Vermeeschand Price1994; Hugand Lingner 2006; Pennaneach et al. 2006), but until now, in only two insect species (*D. melanogaster* and the coccid, *Planococcus lilacinus*) (Biessmann et al. 1990; Mohan et al. 2011). Considering that *Drosophila* exhibits a non-canonical telomere-telomerase system, aphids and coccids are therefore the first insect models to be shown experimentally to exhibit *de novo* telomere synthesis. Aphid TERT's expression has been evaluate in different body parts, such as gut and head, in full agreement with data published by Sasaki and Fujiwara (2000) reporting telomerase activity in different organs and tissues of crickets and cockroaches. Overall, we have different evidence suggesting that, in insects, robust telomerase expression is present also in somatic tissues and not only in germ and pluripotent stem cells as observed in human tissues (Krupp et al. 2000; Donate and Blasco 2011). TERT expression was also reported in *A. mellifera* and *B. mori*, where telomerase mRNAs have been found in different tissues albeit in low amounts (Honey Bee Genome Sequencing Consortium 2006; Osanai et al. 2006). Interestingly, weak telomerase activity was observed in different adult human tissues, where it is insufficient to prevent telomere shortening. It could therefore be intriguing to delve deeper into the study of TERT activity in insects, in order to better comprehend the role of telomerase expression in insect somatic tissues. To date telomerases have been identified in three insect species (*A.*

mellifera, *T. castaneum*, *B. mori*); these three telomerases share ~ 20% sequence identity among themselves and similar identity to their vertebrate homologues (Honeybee Genome Sequencing Consortium 2006; Osanai et al. 2006; Robertson and Gordon 2006). *A. pisum* TERT exhibits sequence identity to both invertebrate and vertebrate homologues ranging from 12% to 18% and possesses the two main domains involved in the telomerase activity, indicating that it is a functional enzyme. The presence of a telomerase in aphids is coupled to the presence of the (TTAGG)n repeats that are located in all the telomeres of the four aphid species studied here. Southern blot experiments with the (TTAGG)n probe showed both smears and bands in all four aphid species analysed, suggesting that aphid telomeres are composed of TTAGG repeats that are occasionally interrupted by other repeated sequences still not identified. Future experiments on the study of aphid telomeric regions could thus be of great interest as these regions seem to be very complex and enriched in repetitive sequences belonging to different families. Considering that *A. pisum*, *Megoura viciae* and *Myzus persicae* belong to the tribe Macrosiphini, whereas *R. padi* belongs to the tribe Aphidini, it may be inferred that aphids have telomeres with a conserved composition in the different subfamilies. FISH experiments showed a clear hybridization signal on each telomere of all aphid chromosomes and there was no evidence of any interstitial labelling. So data illustrated in this PhD thesis demonstrate that TTAGG repeats are restricted to the terminal regions of all aphid chromosomes, as previously hypothesized by Bizzaro et al. (2000). In the interphase nuclei of most organisms, the telomeric regions are arranged in an ordered fashion with an association to the nuclear matrix and clustering at least at some stages of cell life (Palladino et al. 1993; Luderus et al. 1996; Pryde et al. 1997). In aphid nuclei, telomeres appeared to be clustered into a few foci and were not located predominantly near the nuclear periphery as reported in other insects such as *D. melanogaster* (Hochstrasser et al. 1986) and the cabbage moth *Mamestra brassicae* (Mandrioli 2002). In view of such findings, it appears evident that, even if the telomeric structure and function are conserved in insects, the positioning of telomeres in interphase nuclei could, and probably does, vary among different species. Loxdale and Lushai suggested that, in aphids, sexual reproduction was related not only to genetic recombination and variability, but perhaps also to the resetting of telomere length (Loxdale and Lushai 2003; Lushai and Loxdale 2007). In the absence of sexual reproduction, asexual aphid generations will thus shorten telomere length consecutively, resulting in a short persistence of obligate parthenogenetic strains/generations (Lushai and Loxdale 2007). Whilst this suggestion is intriguing, the experimental data above illustrated do not support this hypothesis, since telomerase expression is not highly regulated as it has been shown to be in

vertebrates (particularly in mammals), but telomerase mRNAs have been observed in different tissues in both adults and embryos. Telomere length can therefore be seemingly regulated also during parthenogenetic generations. Moreover, FISH experiments performed in different aphid species showed bright telomeres also, for instance, in *R. padi* specimens reared in our laboratory for more than 40 years that reproduce themselves by clonal reproduction only. If the Lushai and Loxdale hypothesis is correct, strains reproduced in our laboratory from more than 40 years should have very short telomeres, but FISH experiments do not seem to support this assertion, and RT-PCR revealed telomerase activity in clonal generations, although no quantitative assays have yet been performed. Therefore, in aphids, telomere length seems to be regulated by telomerase not only during sexual generations, but also in parthenogenetic females, leaving open the question of the presence and purpose of sexual generations in aphids. Considering that aphids can stabilize chromosomal fragmentation by combining the holocentric nature of their chromosomes with the de novo synthesis of telomeres, it could be hypothesized that maintenance of meiosis is important not only for the production of variable offspring, but also for the stability of both karyotype and chromosome structure. Indeed, chromosomes not involved in pairing and crossing over can quickly differentiate so that, in the absence of sexual reproduction, chromosomal rearrangements could well become fixed in the aphid karyotype, facilitating one-way, potentially detrimental karyotypic changes and genomic instability. Telomeres are specialized nucleoprotein structures constituting the end of chromosomes (Blackburn 1991). Although eukaryotic telomeric sequences may vary in composition, they are strictly conserved in some taxonomic groups, so that the hexameric (TTAGGG)n repeat is typical of vertebrates (Meyne et al. 1989) and the (TTTAGGG)n sequence was found in plants (Fajkus and Zentgraf 2002), whereas the (TTAGG)n telomeric repeat was reported in many invertebrate species, including the main lineages of insects (Okazaki et al. 1993; Sahara et al. 1999; Bizzaro et al. 2000; Frydrychová and Marec 2002; Mandrioli 2002; Frydrychová et al. 2004; Vítková et al. 2005; Lukhtanov and Kuznetsova 2010; Monti et al. 2011). Differently from the conservation of telomeres, subtelomeric regions are more polymorphic and variable in composition (Mandrioli et al. 2013). In this regard, repetitive telomere-associated sequences have been commonly found in the subtelomeric region of various species, such as the 169-bp MpR satellite DNA sequence in the aphid Myzus persicae (Spence et al. 1998). The telomere-associated sequences identified up till now bear a structural resemblance to *Chironomus* TA repeats (Mason et al. 2011), which evolved from telomeric repeat sequences and truncated retrotransposons (Zhang et al. 1994; Martinez et al. 2001; Mason et al.

2011) suggesting that retrotransposons could be commonly present below telomeres shaping the structure of the subtelomeric regions (Mandrioli et al. 2013).In order to better understand the structure of aphid sub-telomeric regions, I amplified, sequenced, and localized copies of the non-LTR retrotransposon TRAS in the aphids *A. pisum* and *M. persicae*, two of the most studied species at a cytogenetic level due to their relevance as experimental models and crop pests. Highly conserved telomere-specific non-LTR retrotransposons have been found for the first time in the moth *B. mori* (Kubo et al. 2001; Fujiwara et al. 2005). Among them, the first identified telomere-associated mobile DNA is the TRAS1 element consisting of a stretch of 7.8 kb and encoding 2 overlapping ORFs that make this retrotransposon an autonomously transposing element (Kubo et al. 2001; Fujiwara et al. 2005). Further screening for telomeric-repeat associated retrotransposons revealed different TRAS families, so that in the silk- worm there are at least 8 TRAS families (TRAS1, TRAS3, TRAS4, TRAS5, TRAS6, TRASY, TRASZ, and TRASW), which have the same telomeric target sequence, but they can be distinguished into different groups on the basis of their sequences (Kubo et al. 2001; Fujiwara et al. 2005). Despite the presence of several insect genome projects, only partial copies of TRAS elements have been annotated outside lepidopteran species (Tribolium Genome Sequencing Consortium 2008; International Aphid Genomic Consortium 2010), making the pea aphid TRAS element here identified the first telomere-specific non-LTR element fully sequenced and studied in insects other than moths. *A. pisum* TRAS retrotransposons presents all the typical features of TRAS elements, suggesting that these retrotransposons are highly conserved not only among lepidopteran species (as previously suggested by Fujiwara et al. 2005) but also in other insect orders possessing (TTAGG)n telomeres. Bioinformatic analyses on *A. pisum* assembled scaffolds revealed that several truncated TRAS copies were not inserted near a telomeric TTAGG sequence, and we were unable to identify any consensus sequence for these unusual TRAS insertions. As recently revised by Mandrioli and Manicardi (2012), meiotic recombination seems to be much higher on the chromosome termini than in the central region of chromosomes in species possessing holocentric chromosomes. The presence of truncated TRAS copies could, therefore, be due to unequal recombination events occurring of the aphid telomeric regions. This suggestion is in accordance to Boissinot et al. (2001) that hypothesized that the high frequency of full-length L1 elements on the human Y chromosome could be due to the inability of the Y chromosome to recombine. The investigation of the chromosomal insertion sites showed that TRASAp1 retrotransposons have been integrated near the telomeric end of all the autosomes and in a single telomere of the 2 X chromosomes in both the aphid species. In

particular, fiber FISH in *M. persicae* revealed that TRAS copies are not located within the most external telomeric sequences, but between the subtelomeric region (constituted by the *M. persicae* subtelomeric satellite DNA) and the (TTAGG)n array. This localization is consistent with data obtained in *B. mori*, where the TRAS copies closest to the termini were found 6–8 kb away from the chromosome ends (Okazaki et al. 1995: Fujiwara et al. 2005), and strengthen the hypothesis that some telomere-binding proteins may protect telomeres preventing TRAS insertion in the most external portion of the chromosomal tip (Kubo et al. 2001; Fujiwara et al. 2005). In both the aphid species analyzed, TRAS elements were not present in the subtelomeric region near the NOR-bearing telomeres of the 2 X chromosomes. These subtelomeric regions in *M. persicae* also do not have any telomere-associated satellite DNA (Spence et al. 1998), suggesting that the X telomeres may face different functional constraints. Interestingly, the association of aphid X chromosomes at NORs has been frequently reported as occurring during the maturation division of the parthenogenetic oocytes (Schrader 1940; Orlando 1974; Hales and Mitler 1983; Blackman and Hales 1986). Indeed, male determination in aphids is based on the loss of one X chromosome because almost all aphid species present females with 2 X chromosomes (XX) and males with only one X chromosome (XO) (Blackman and Spence 1996) (Fig 4.1).

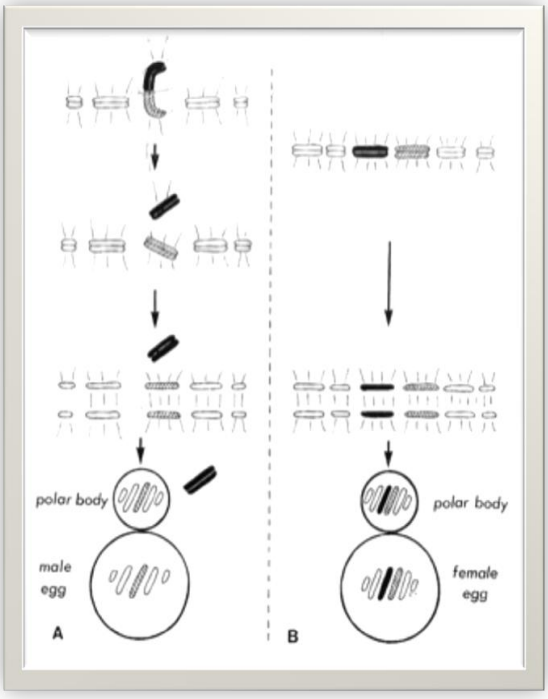


Fig 4.1 Diagram showing he behavior of X chromosomes during maturation (A) of male and (B) female parthenogenetic oocytes. One X-chromosomes is shown black the other cross-hatched:the autosomes are white.

All the parthenogenetic eggs during the prophase present two X chromosomes linked by NORs (Schrader 1940; Orlando 1974; Hales and Mitler 1983; Blackman and Hales 1986). However, in eggs developing as females, the connection is quickly lost, but in male-generating eggs, the X chromosomes remain attached by sticky NORs and undergo a sort of non-canonical reductional division (Blackman and Hales 1986). At the end of this peculiar division, the egg has one X chromosome only and it is determined as a male .
The absence of telomere-associated repeated sequences, such as satellite DNAs and transposable elements, at the NOR telomeres of the X chromosomes could be, therefore, due to the need of favouring rDNA genes pairing, which is at the basis of sex determination.
On the basis of literature data, TRAS seems to be not directly involved in healing chromosomal breakages in *B. mori* (Okazaki et al. 1995; Fujiwara et al. 2005). FISH experiments in the *M. persicae* laboratory clone 33H confirmed indeed, the absence of TRAS retrotransposons in the *de novo* telomeres, whereas previous experiments showed the presence of a *de novo* synthesised (TTAGG)*n* telomeric array (Monti et al. 2011). As a whole, we have, therefore, further evidence assessing that telomere-specific TRAS retrotransposons are not involved in the healing and stabilization of the broken chromosomal ends.

The large amount of chromosomal rearrangements observed in *M. persicae* strain analysed in this PhD thesis, prompt me to set up a chromosome specific library by laser microdissection gently offered by the equipe headed by Prof. Frantisek Marec of the Laboratory of Molecular Cytogenetics at the Institute of Entomology Biology Centre (Czèch Republic). Considering that the holocentic nature of aphid chromosomes hampered a clear-cut identification of the chromosome, the aim of this experimental approach was that of a precise identification of chromosome involved the karyotype variations repeatedly observed. In absence of feasible bandon technique able I focused my attention toward X chormosmes, considering that they are the longest of the complement so that they are more easly identifiable. FISH experiments with the X-chromosome painting probes in *Myzus persicae* showed signal previously scattered on the sex chromosomes but also on telomeric ends of all autosomes. Further, after long hybridization time, preferentially the highly repeat sequences hybridized, for example, to telomeric and subtelomeric sequences that contain tandemly repeated DNA sequences as observed in plants (Siroky et al., 1999). A possible explanation of this result could be the DNA composition of the *Myzus persicae* X chromosome. In the other words, the X chromosomes do not contain sufficient amount of chromosome-specific repeats required to obtain a highly specific

painting with the X-chromosome derived probes; it contains probably a significant amount of ubiquitous repetitive sequences common to all chromosomes and unique sequences which are not a good target for FISH with fluorochrome-labelled probes. An simila result has been obtained in pyralid moths, *P. interpunctella* and *G. mellonella* (Vítkova et.al., 2004), where the species-specific W-probes failed to stain several regions on the W chromosomes especially the ends. The results indicate that in these species the DNA sequences of terminal segments considerably differ from the main mass of the W chromosome. Nevertheless, W-chromosome paining probes developed in several Lepidoptera, showed strong hybridization signals along most of the W chromosome length, obviously due to the accumulation of both W-specific and common repeats (Fuková et al., 2007; Vítková et al., 2007; Yoshido et al., 2013). However, all attempts to develop a Z-chromosome or autosome painting probes in several tortricid moths failed as the probes had a very low specificity and showed scattered hybridization signals on all chromosomes (Šíchová, 2009, 2011; F. Marec, personal communication). I encountered a similar problem in aphids. Like it happened in the lepidopteran Z chromosomes, the X chromosomes in *Myzus persicae* were either weakly or not stained but the majority of chromosome ends were intensely stained with the X-probe. The insufficient specificity of the X-probe is consistent with recent findings on the distribution of repetitive DNAs in holokinetic chromosomes of aphids. According to Mandrioli et.al. (2012), highly repetitive sequences, which are generally characteristic for centromeres in organisms with monocentric chromosomes, are often found scattered among chromosomes in species with holokinetic chromosomes. Also the amount and distribution of heterochromatin differs in taxa possessing holokinetic chromosomes, which have usualy poor amount of heterochromatin and if present, it shows a telomeric and, sometimes, intercalary localization in the chromosomes. Therefore, the possibility to generate a chromosomes specific probe in aphids is limited. The strong clustered hybridization signals of the X-probe in some intherphase nuclei of *Myzus persicae* may represent the X-linked rDNA. In most eukaryotic organisms the rRNA genes are organized in tandem arrays clustered at one or several chromosomal sites. Protein-protein interactions between heterochromatin-binding proteins are likely to contribute to clustering of rDNA repeats from different chromosomes and, consequently, to the construction of the nucleolar compartment. However, I observed multiple hybridization signals in the majority of interphase nuclei of *M. persicae*. These signals most probably correspond the repetitive sequences of telomeric and subtelomeric heterochromatin according to the previously reported distribution of telomeres in the aphid nucleus (Monti et al., 2011). In conclusion, these FISH experiments

demonstrates that the X-probe prepared by laser microdissection and WGA4/WGA3 amplification works and hybridizes with the *Myzus persicae* DNA, but it is not sufficiently specific to be used for as a whole chromosome painting probe for the study of X-chromosome fragmentation. In order to improve the specificity of the X-probe in *Myzus persicae* and suppress telomeric signals, it might be useful to use a competitor DNA (i.e., blocking DNA), composed, for example, of unlabelled subtelomeric and telomeric sequences. This is feasible because this telomeric regions of *Myzus persicae* chromosomes have been molecularly described (Monti et.al., 2011). Indeed, for the overwhelming majority of the species including, for example, mammals, plants and moths, a relatively large amount of species-specific genomic competitor DNA were used in FISH experiments, in order to suppress unspecific hybridization of the probe to the autosomes (Hobza et al., 2004; Ried et al., 1998; Vítkova et Al., 2004).

Different studies had reported that products of many individual X-linked genes are found in equal amounts in males and females, whether assayed by their genetic (Muller, 1950) or enzymatic function (Lucchesi & Manning, 1987), or by direct measurement of protein (Korge, 1975) or transcript levels (Birchler et al., 1982; Ganguly et al., 1985). In species that have two sexes, a single genome encodes two morphs, as each sex can be thought of as a distinct morph. This means that the same set of genes are differentially expressed in the different sexes. Many questions emanate from this statement. What proportion of genes contributes to sexual dimorphism? How do they contribute to sexual dimorphism? How is sex-biased expression achieved? Which sex and what tissues contribute the most to sex-biased expression? (Miguel Gallach et.al, 2011). Understanding how sexual dimorphism occurs in aphids and what are the evolutionary forces driving sexual antagonism represent to date a molecular mechanism unknown probably for their complicated life cycle. Moreover, the identification of sex-specific genes is rather difficult because the pea aphid genome lacks a fine-scale linkage map then, without this resource, it has been impossible to assign individual genomic scaffolds to chromosomes (Bickel et al., 2013). So, I have chosen three predicted X-linked genes that have not been still assigned on this sexual chromosomes but currently, I have strong evidence that they are on it following several bio-informatic analysis and also since a bright band has been obtained after PCR amplification using *A. pisum* DNA obtained by X chromosomes microdissection. In this study I have beginning to analyse the forces driving X chromosome evolution in aphids male and female and whether dosage compensation is present in this study. Thanks to Real time, I measured the expression levels of predicted genes in each sex in order to verify if genes were differentially expressed. To confirm whether sex-linked genes of the aphids are

dosage compensated or not, I investigated three different genes Fibronectin, RPP15 and RAD50 which have been predicted to the X chromosome as candidate genes to test for dosage compensation. Several information about these genes arise from biological models, for instance mammals or yeast. Indeed, literature data explain that RRP15 gene encodes a protein that co-purifies with human nucleoli (De Marchis et al., 2005). Similar protein in budding yeast is a component of pre-60S ribosomal particles, and it is required for the early maturation steps of the 60S subunit. Furthermore, it seem to be required for the nuclear and/or nucleolar export of pre-60S particles (De Marchis et al, 2005). Instead, genetic screening based mainly on sensitivity to ionizing radiation, have identified a large number of loci which are required for the repair of DNA breaks and it has been shown that many of these genes are required for efficient *mating-type switching* and also for mitotic and meiotic recombination (Chiruvella et al., 2013). These genes, including RAD50, are also known as DNA repair agents. Moreover many studies of the mouse homolog of Rad50 suggest it is essential for cell growth and viability. Indirect evidences, in aphids, suggest the possibility that recombination may also be present during mitotic divisions that occur in the parthenogenetic generations at specific sites (possibly hot spot of recombination), as described in *A pisum* in which the presence of this kind of hot spots has been hypotesized within the cluster of genes rDNA (Mandrioli et al., 1999b). In this conection, the presence of RAD50 gene in aphids is lagerly expected. As a consequence of aphid sex determination, males are X0, so that, in absence of dosage compensation, real time analysis on X-linked gene expression in males should theoretically result in a 0.5 fold ratio compared to females. On the contrary values of fold changes (mean ± SD) in Table 3.25, suggest an over-expression in male compared to oviparous females for the target genes, especially for fibronectin in *A. pisum*. Noteworthy diverse magnitudes in the process of compensation dosage, suggest that other sex-linked factors, still unknown, may affect the expression level of the target genes, beyond the mere X chromosome number. All these results suggest that sex-linked genes are dosage compensated as described in *Drosophila melanogaster* (Miguel Gallach et.al 2011). A significantly different proportion of male genes located on the X chromosomes compared with female ones could be at the basis of this phenomenon (Vicoso and Charlesworth 2009). These observations provide evidence that male-biased genes are an important source of evolutionary change, are under strong selective pressures, and contribute greatly to sexual dimorphism (Gallach et.al, 2011).

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