Mode of action, origin and structure of the Paternal Sex Ratio chromosome in the parasitoid wasp *Trichogramma kaykai*

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Contents

Abstract

Selfish genetic elements are defined as genetic elements that have a replication advantage relative to the rest of the genome. They are ubiquitous in nature and were extensively reported for almost all species studied so far. A special type of selfish genetic element, the sex ratio distorter, is most frequent in arthropods and changes the offspring sex ratio of its host. An example of an extremely selfish male biasing sex ratio distorter is the paternal sex ratio (PSR) chromosome in the parasitoid wasps *Trichogramma kaykai* and *Nasonia vitripennis*. These wasps have an arrhenotokous sex determination in which fertilized diploid eggs develop into females and males develop from unfertilized haploid eggs. Only part of the male wasps contains this additional B chromosome, which upon fertilization eliminates the paternal genome, while keeping itself and the maternal chromosomes intact. The resulting haploid embryo develops into a B chromosome-carrying male. This extremely selfish B chromosome was first discovered in *N. vitripennis*. The recent discovery of a second PSR chromosome in the unrelated wasp *T. kaykai* provided an opportunity for a comparative study on PSR chromosomes. In this study I determined the mechanism, the origin and structure of the PSR chromosome in *T. kaykai* and compared my results with previous studies on the PSR chromosome in *N. vitripennis*.

The mode of action of the *Trichogramma* PSR chromosome was revealed by examining microscopic preparations of freshly fertilized eggs. This chromosome modifies the paternal genome into a dense chromatin mass at the beginning of the first mitotic division, while the PSR chromosome itself escapes its own destructive effect and continues embryo development with the maternal chromosomes. Comparing the modes of action of the *Trichogramma* and *Nasonia* PSR chromosomes suggests that both systems are identical, except for the diameter of the paternal chromatin mass (PCM) and the occurrence of PCM-associated nuclei. However, their molecular mechanism remains unknown. Furthermore, both PSR chromosomes share the main structural characteristics of B chromosomes: They are much smaller than the normal chromosomes and contain high amounts of transposable elements and tandem repetitive DNA. B chromosomes are thought to accumulate transposable elements because they do not recombine meiotically with the normal chromosomes. Repeats have proven to be an important factor for the transmission efficiency of the *Nasonia* PSR chromosomes and are also thought to correlate with the B chromosome size in general. However, none of the DNA sequences found on the *Trichogramma* PSR chromosome were found on the *Nasonia* PSR chromosome or visa versa. About two thirds of the *Trichogramma* PSR chromosome comprises tandem arrays of 45S ribosomal DNA (rDNA), while on the *Nasonia* PSR chromosome three B chromosome specific repeat families are located and one repeat family that is also present on the *Nasonia* genome. 45S rDNA consists of three conserved genes essential for protein processing separated by three spacer sequences that are hypervariable between species but conserved within species. These spacer sequences are therefore often used for taxonomic purposes. One of the spacer sequences of the 45S rDNA on the *Trichogramma* PSR chromosome, i.e. ITS2, contains at least five different sequences that resemble either the ITS2 of *T. kaykai* or the related *T. oleae*. We

therefore concluded that this B chromosome originated from *T. oleae* or a *T. oleae*-like species. Retrotransposon analysis revealed that the *Nasonia* PSR chromosome most likely originated from the *Nasonia* related wasp genus *Trichomalopsis*. Though both PSR chromosomes have a similar mode of action, the absence of any sequence homology between both chromosomes implies different PSR chromosome ancestors. This again makes it less likely that the molecular mechanism of paternal genome loss is identical. Future studies should focus on comparing the molecular mode of action and DNA sequence homology of both PSR chromosomes and revealing the incidence of more PSR chromosomes in other haplo-diploid organisms. This will not only provide more knowledge on the mechanism of early embryogenesis and in particular on the paternal chromosome processing following fertilization, but also on the origin and evolution of PSR chromosomes. Extending our knowledge on PSR chromosomes is expected most useful for the control of pest insects with haplo-diploid sex determination systems like the Argentine ant. Without females such insect populations will quickly perish.

Chapter 1

General Introduction

In this thesis I describe the paternal sex ratio (PSR) chromosome in the parasitoid wasp *Trichogramma kaykai* which causes paternal genome loss in fertilized eggs of its host. In this chapter all aspects of this unique chromosome that combines properties of a selfish genetic element, a sex ratio distorter and a B chromosome are introduced.

1 Parasitic elements

1.1 Selfish genetic elements

Most nuclear genes have a normal Mendelian inheritance through which two alleles at a locus have an equal chance of ending up in a particular gamete. Exceptions to this rule are 'selfish' genetic elements (SGEs) that manipulate their transmission in such a way that they end up in more than half of the gametes. SGEs are defined as elements that have a replication advantage relative to the rest of the genome and may have a neutral or detrimental effect on the individual's survival or reproduction (Werren et al. 1988). Examples of SGEs are transposons, (post-)segregation distorters, B chromosomes, cytoplasmic microorganisms, meiotic drive chromosomes and homing endonucleases. SGEs are not only diverse, but can also occur in very large numbers in the host genome, such as transposable elements which comprise 15% of the *Drosophila* genome and over 50% of the maize genome (SanMiguel et al. 1996; Biemont & Cizeron 1999). Besides affecting genome organization, SGEs are also thought to influence evolutionary processes, including genome evolution, sex determination, host speciation, and even may give rise to host extinction (Hurst & Werren 2001). An example of the involvement of SGEs in genome evolution is the suppression of transposon activity and their resulting deleterious mutations by methylation of cytosine residues in the genomes of vertebrates and various plant species (Hurst 2001). This transposon-silencing mechanism is thought to have triggered genome wide evolution of methylation sensitive and insensitive genes (Bestor & Tycko 1996; Yoder et al. 1997). Another example is the cytoplasmic incompatibility (CI) inducing bacterium *Wolbachia pipientis*, which is thought to play a role in speciation, because it induces reproductive isolation between closely related host species, thus allowing divergence to continue and species to evolve (Breeuwer & Werren 1990; Shoemaker et al. 1999).

SGEs not only provide fundamental knowledge on the origin, organization and evolution of genomes and other traits, but can also be exploited for introducing genes from one species to the other and even into distantly related organisms. Examples of such SGEs are P elements in *Drosophila* and mariner transposable elements (Robertson & Lampe 1995; Boeke 2002).

One of the most selfish DNA elements described so far is a B chromosome that completely eliminates the genome of its host while transmitting itself to the next generation (Werren & Stouthamer 2003). This so-called paternal sex ratio (PSR) chromosome has, until now, only been found in part of the male population of the parasitoid wasps *Trichogramma kaykai* and *Nasonia vitripennis*, where it distorts the sex ratio in favour of males.

1.2 Sex ratio distorters

Sex ratio distorters (SRDs) are selfish genetic elements that are easily detected, because they produce an unusual offspring sex ratio in their host. They change their host's sex ratio in favour of the sex with the highest SRD transmission efficiency. The group of nuclear SRDs include meiotic drive chromosomes and B chromosomes, whereas cytoplasmic SRDs generally reside in organelles and microorganisms (Stouthamer et al. 2002). Many SRDs are found in insects (Werren et al. 1995; Stouthamer 1997; Arakaki et al. 2001), but have also been reported in mites (Weeks & Breeuwer 2001) and isopods (Bouchon et al. 1998) and even in nematodes (Vandekerckhove et al. 2000), plants (Taylor 1999) and vertebrates (Cockburn et al. 2002).

Female biasing SRDs, such as microorganisms and organelles, are common and transmit predominantly from mother to offspring through the cytoplasm (Stouthamer et al. 2002). An extreme example is the parthenogenesis inducing (PI) bacterium *Wolbachia pipientis*, which causes virgin female wasps to produce only daughters (Stouthamer & Kazmer 1994; Stouthamer 1997). Other effects of maternally transmitted SRDs are feminization (Bouchon et al. 1998), maternal sex ratio (Skinner 1982) and male killing (Hurst 1991).

The far more exceptional paternally transmitted SRDs are mainly located in the nuclear genome of their host. Examples include meiotic drive sex chromosomes and PSR chromosomes (Jaenike 2001; Werren & Stouthamer 2003). PSR chromosomes are supernumerary chromosomes that transmit through sperm and manipulate the haplo-diploid sex determination system of their host to produce males from fertilized eggs by eliminating the complete paternal chromosome set without destroying itself. The resulting haploid egg develops into a male with the PSR chromosome, instead of a diploid female (Werren et al. 1987; Stouthamer et al. 2001).

SRDs are selfish elements that not only provide insight into evolutionary aspects of their host (Hurst 1995; Werren & Beukeboom 1998; Hurst & Werren 2001), but are also valuable for understanding early developmental processes of their host (Tram & Sullivan 2002). They may also be helpful for biological control of pest species, either directly as a mechanism to suppress pest populations or to support natural enemies, or indirectly as vehicle for moving desirable genes across species boundaries (Stouthamer & Luck 1993; Werren & Stouthamer 2003).

2 Paternal genome loss

Paternal genome loss (PGL) involves the transition of an initially diploid genome to a haploid genome. PGL has been described for several organisms, though it is most frequent in organ-

isms in which sex is determined by the number of chromosome sets (Nur 1980). In this type of sex determination females are diploid with two chromosome sets, while males have only one chromosome set and are haploid. In theory, a haploid organism can arise either from the paternal or maternal genome or from a combination of both, but until now only haploid offspring with maternally derived DNA has been found. Arrhenotoky is a sex determination system in which males arise from unfertilized and females from fertilized eggs and haploid offspring therefore only contains maternal DNA. However, this system does not involve PGL, because unfertilized eggs never obtain the paternal genome. Four different types of PGL can be distinguished, which differ in timing and cause of PGL.

1) Pseudo-arrhenotoky or parahaploidy is a sex determining system involving PGL in some insects and mites (Nur 1980). All offspring results from fertilized eggs, but (in)complete mitotic PGL during embryo or larval development gives rise to haploid embryos which develop into males, while females develop from diploid embryos without PGL. Complete PGL occurs in some scale insects (Nur 1980), the coffee berry borer (Brun et al. 1995) and phytoseiid mites (Schulten 1985), while in other scale insects the paternal genome is not lost but only inactivated by facultative heterochromatinization (Brown & Nur 1964; Brown 1966; Nur 1980).

2) Complete PGL during first mitosis in fertilized eggs is caused by PSR chromosomes in *T. kaykai* (Stouthamer et al. 2001; Van Vugt et al. 2003) and *N. vitripennis* (Werren et al. 1987; Reed & Werren 1995), the PSR-like element in *Encarsia pergandiella* (Hunter et al. 1993) and the CI inducing *Wolbachia* in arthropods (Stouthamer et al. 1999). These reproductive parasites eliminate the paternal genome at the beginning of first mitosis in fertilized eggs, causing the originally diploid eggs to develop into haploid males. This type of PGL has some similarity with pseudo-arrhenotoky, since both types cause initially diploid embryos to develop into haploid male progeny. However, timing and mechanism of PGL are completely different. While 'PGL during first mitosis' is caused by a reproductive parasite and considered not essential for the survival of the host, the PGL in pseudo-arrhenotoky occurs during embryo or larval development and is the only method to generate males in these arthropods.

3) In complete meiotic PGL all chromosomes of the father are lost during meiosis and only chromosomes of maternal original contribute to gametes and offspring. This type of PGL is found in male meiosis I of sciarid flies (Fuge 1997; Goday & Esteban 2001; Goday & Ruiz 2002) and in male and female meiosis of some scale insects (Nur 1980). In sciarid flies the paternal X chromosome is not eliminated in male meiosis and sperm cells therefore have a single chromosome set originating from the mother plus two X chromosomes. Sex in these flies is determined by the loss of one or two paternal X chromosomes during early embryogenesis, resulting in female (XX) or male (XO) progeny, respectively.

4) Finally, genome incompatibility between two closely related eukaryote species may result in hybrid offspring in which the genome from the father is gradually eliminated over multiple mitotic divisions in the early embryo. This complete mitotic PGL during early embryo development causes the resulting haploid organism to perish. Examples in plants are wheat-pearl millet hybrids with a gradual PGL within twenty days after fertilization by formation of micronuclei at interphase (D. Gerland, IPK Gatersleben, personal communication) and barley

hybrids with a gradual PGL within five days in which individual paternal chromosomes are excluded from mitotic metaphase or anaphase (Bennet et al. 1976; Finch 1983). A comparable genome loss was observed in Masu salmon-rainbow trout hybrids with a gradual PGL from fertilization till blastocyst stage (Fujiwara et al. 1997).

3 B chromosomes

A chromosomes form the indispensable part of the genome, whereas B chromosomes are extra chromosomes that are not essential for their host's survival, can in most cases not recombine with the A chromosomes and follow their own evolutionary trajectory (Beukeboom 1994b). B chromosomes occur in a variable number of individuals in a population or species (Jones & Rees 1982) and are thought to occur in 10-15% of all plant and animal species (Jones 1985). In most cases they are smaller and more heterochromatic than the A chromosomes (Beukeboom 1994a; Jones & Houben 2003). They can accumulate by replication advantages, such as meiotic drive or pollen mitosis non-disjunction (Jones 1991), and can be lost without such mechanisms (Pardo et al. 1994; Perrot-Minnot & Werren 2001). B chromosomes generally impose no or only very weak phenotypic effects (Jones & Rees 1982; Camacho et al. 2000). Clear examples of exceptions are the B chromosomes in *Allium schoenoprasum*, which have a beneficial effect on seed germination of these plants (Plowman & Bougourd 1994) and the PSR chromosomes in *T. kaykai* and *N. vitripennis*, which have a strong effect on the host sex ratio (Werren & Stouthamer 2003).

The traditional view that B chromosomes are derived from autosomal chromosomes or sex chromosomes, is still generally accepted (Jones & Rees 1982). How they originate is more controversial. They are thought to originate 1) from extra chromosomes in aneuploid genomes or 2) from centric fragments resulting from genome incompatibility or cytoplasmic incompatibility or 3) through fusion of chromosomes or chromosome fragments (Breeuwer & Werren 1993; Camacho et al. 2000; Jones & Houben 2003). Most B chromosomes originate from intraspecific hybridisation, i.e. they arose from chromosomes or chromosome fragments from their host (Camacho et al. 2000). Some B chromosomes, however, contain non-host DNA and either originated during interspecific hybridisation (Schartl et al. 1995; Perfectti & Werren 2001) or originated intraspecifically and introgressed to a related species.

Most B chromosomes are rich in repetitive DNA, which is either similar to sequences on normal chromosomes or is specific for the B chromosome (López-León et al. 1994; Wilkes et al. 1995; Camacho et al. 2000; Dhar et al. 2002). A large number of B chromosomes have 45S ribosomal DNA (rDNA) (Green 1990; Jones 1995), but the ribosomal genes of this repeat are not necessarily transcribed (López-León et al. 1991; Stitou et al. 2000; Szczerbal & Switonski 2003). A number of explanations for the presence of 45S rDNA on B chromosomes have been postulated (Schubert & Wobus 1985; Beukeboom 1994a; Dubcovsky & Dvorak 1995): 1) its usually (sub)distal position could make 45S rDNA more susceptible to chromosome breakage and due to the generally short distance to the centromere this breakage may easily include sequences

with centromere activity, 2) the homologous chromosome region of the nuclear organiser region which harbours the 45S rDNA tandem array remains in most species unpaired with no or hardly any crossing-overs, which may cause 45S rDNA to be meiotically isolated, 3) the transposable 45S rDNA sequences may easily be transferred to an existing B chromosome. Other types of DNA that occur at high frequency on B chromosomes are transposable elements. B chromosomes are thought to accumulate this type of dispersed repeat, because transposable elements accumulate in regions with a low recombination frequency (Camacho et al. 1991). Repeats and transposons can be used to identify the origin of B chromosomes. Examples for such application are the repeats on the B-chromosomes in the plant *Brachicome dichromosomatica* and the NATE transposon on the PSR chromosome in *N. vitripennis* (McAllister 1995; McAllister & Werren 1997; Houben et al. 1999).

4 Biology of the hosts of PSR chromosomes

PSR chromosomes have so far only been discovered in the parasitoid wasps *T. kaykai* and *N. vitripennis* (Figure 1a) (Werren et al. 1981; Nur et al. 1988; Stouthamer et al. 2001; Werren & Stouthamer 2003). Both wasps belong to the superfamily Chalcidoidea. *Trichogramma* species (Trichogrammatidae) are minute (ca. 0.5 mm) egg parasitoids of mostly butterflies and moths, while *Nasonia* species (Pteromalidae) measure approximately 3 mm in size and parasitize pupae of blowflies and fleshflies (Whiting 1967; Pinto 1999). Whereas *N. vitripennis* is found worldwide in bird nests and carcasses, *T. kaykai* is endemic to the Mojave Desert, USA, where it parasitizes the eggs of the butterfly *Apodemia mormo deserti* (Lepidoptera, Lycaenidae, Figure 1b) (Pinto et al. 1997). *A. mormo deserti* females deposit their eggs on several plant species of the genus *Eriogonum* (Polygonacae) (Pratt & Ballmer 1991), such as the desert trumpet, *Eriogonum inflatum*. This species is found throughout the Mojave Desert mostly near washes, roadsides and on foothills (Figure 1c) (Munz 1959). *T. kaykai* wasps are obtained by collecting parasitized *A. mormo deserti* butterfly eggs on *Eriogonum inflatum* plants from March till June. In the laboratory cultures of *T. kaykai* are maintained on eggs of the mealmoth *Ephestia kuehniella*.

Figure 1.1 (a) *Trichogramma kaykai* female parasitizing a *Mamestra brassicae* egg in the laboratory (photo Duotone, Wageningen); (b) *Apodemia mormo deserti* butterfly (photo Marc Maas); (c) *Eriogonum inflatum* plant in Last Chance Canyon, Kern Co., California, USA (photo Richard Stouthamer). For colour photo: see page 117.

Trichogramma and *Nasonia* have an arrhenotokous sex determination system, which means that unfertilized (haploid) eggs develop into males, while females develop from fertilized (diploid) eggs. Both wasp species are gregarious and have partial inbreeding. As a consequence a female biased sex ratio is favoured. Mated females store sperm in the spermatheca and regulate the release of sperm during oviposition. By fertilizing more than 50% of the eggs a female-biased offspring sex ratio is obtained.

In addition to the male biasing PSR chromosome, *N. vitripennis* and *T. kaykai* have sex ratio distorters that manipulate the offspring sex ratio in favour of females. *T. kaykai* has the PI bacterium *Wolbachia pipientis* (Stouthamer & Kazmer 1994). This bacterium disrupts the first anaphase spindle in unfertilized eggs, causing initially haploid eggs to develop into diploid females with only maternal genetic material. In addition to the PSR chromosome the following two sex ratio distorters are found in *N. vitripennis*: 'son-killer', which is a maternally inherited bacterium that causes mortality of unfertilized male eggs (Skinner 1985; Werren et al. 1986) and 'maternal sex ratio', which is a cytoplasmically inherited factor that causes inseminated female wasps to produce nearly 100% female offspring (Skinner 1982).

5 Paternal sex ratio chromosome

Upon fertilization with PSR chromosome containing sperm the complete paternal genome, except the PSR chromosome, is eliminated and the egg develops into a haploid male with the genome of maternal origin and only the PSR chromosome of paternal origin (Werren et al. 1981; Werren et al. 1987; Stouthamer et al. 2001). PSR chromosomes thus cause initially diploid eggs to develop into haploid male individuals and therefore will only occur in arrhenotokous species. The advantage for PSR chromosomes to transmit through males is that their transmission rate in the aberrant male meiosis is twice as high as through females in which normal meiosis reduces transmission to only 50% of the daughter cells (Hogge & King 1975).

The *Nasonia* PSR chromosome causes condensation of the paternal genome at the beginning of the first mitotic division in fertilized eggs (Werren et al. 1987). Subsequently, the paternal chromosomes cannot participate in any mitotic division and are eventually lost. The molecular mechanism of genome loss that PSR chromosomes exert on the paternal A chromosomes is still unknown. The origin of PSR chromosomes is not well understood either. The *Nasonia* PSR chromosome is assumed to have been associated with the closely related genus *Trichomalopsis* (McAllister & Werren 1997). This was determined by comparing retrotransposons on the PSR chromosome with retrotransposons in the genome of *Trichomalopsis*.

6 Thesis outline

The PSR chromosome was first discovered in the parasitoid wasp *N. vitripennis*. The recent discovery of a PSR chromosome in a second wasp species, i.e. *T. kaykai*, provides an opportu-

nity for a comparative study on different aspects of these selfish chromosomes. The aim of this study is to determine the mode of action, the origin and structure of the PSR chromosome in *T. kaykai*. I will compare the *Trichogramma* and *Nasonia* PSR chromosome systems and determine whether they have a similar mode of action and origin. This will provide knowledge on the evolution of PSR chromosomes and may shed light on specific aspects of early embryogenesis and in particular the mechanism of paternal chromosome processing following fertilization.

In **Chapter 2** I review the relevant aspects of both PSR chromosomes in *T. kaykai* and *N. vitripennis*. Their discovery, population dynamics, mode of action, origin and structure are discussed.

In **Chapter 3** the mode of action of the *Trichogramma* PSR chromosome is described by comparing the early embryogenesis of DAPI-stained eggs fertilized with PSR sperm with that of eggs fertilized with normal sperm. The PSR chromosome condenses the paternal genome into a dense chromatin mass at the beginning of the first mitosis, causing the paternal chromosomes to become excluded from any further development of the embryo. The PSR chromosome itself escapes from the effect it imposes on the paternal genome and becomes incorporated into the maternal nucleus. I discuss the very similar mode of action of the *Trichogramma* and *Nasonia* PSR chromosome.

Chapter 4 deals with the large tandem repeat of 45S rDNA on the *Trichogramma* PSR chromosome. This repeat contains five different spacer sequences, which in part resemble the spacer sequence of the related species *Trichogramma oleae*. The *Trichogramma* PSR chromosome is therefore thought to have originated from *T. oleae* or a related species.

In **Chapter 5** I focus on the multiple spacer sequences on the *Trichogramma* PSR chromosome by examining whether the 45S rDNA sequences on this chromosome are transcribed. Furthermore, the size of the *Trichogramma* PSR chromosome is estimated and the occurrence of repetitive sequences other than 45S rDNA on the genome of *T. kaykai* and its PSR chromosome is established.

In **Chapter 6** I describe how *Trichogramma* PSR chromosome specific sequences were detected with AFLP fingerprinting and used for a comparative study on PSR+ and PSR- individuals. Two retrotransposons are discovered. Internal primers designed for a third AFLP fragment can be used to screen for the PSR trait in male wasps.

In **Chapter 7** a final discussion of all results on the mode of action, origin and structure of the *Trichogramma* PSR chromosome is given, and a comparison is made with the *Nasonia* PSR chromosome. Finally, I make suggestions for future research and discuss the significance of the PSR chromosome for insect pest control.

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Chapter 2

Paternal Sex Ratio chromosomes in parasitoid wasps: an overview of the ins and outs of these extremely selfish B chromosomes

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1 Discovery of PSR factors

The paternal sex ratio (PSR) trait was first discovered in 1979 by Werren and Skinner while attempting to select for genetic variability in the offspring sex ratio of the parasitoids wasp *Nasonia vitripennis* (Werren et al. 1981). This trait caused the production of only male offspring and was therefore initially named daughterless (DL). In 1986 it was renamed to paternal sex ratio factor (Werren & Van den Assem 1986). Further analysis revealed that this all-male trait was paternally inherited, which was surprising because males in haplo-diploid sex determination systems normally do not transmit genetic material to male offspring (Werren et al. 1981). Although fertilization and double mating experiments demonstrated transmission of the PSR factor through sperm, chromosome analysis showed that male offspring carrying the PSR factor was haploid, suggesting that one of the chromosome sets was lost after fertilization. This unique phenomenon of PSR transmission to male offspring without inheriting the paternal genome proved that this all-male trait was controlled by an additional genomic element (Werren & Van den Assem 1986). Subsequent chromosome studies showed that sperm from PSR-carrying males entered the egg and that the paternal chromosomes were degraded into a dense chromatin mass at the onset of first mitotic division (Werren et al. 1987). Finally, in 1988 an additional small chromosome was detected exclusively in males with the PSR trait (Nur et al. 1988). Apparently, this PSR chromosome somehow escapes from the destructive effect it exerts on the rest of the paternal genome.

A second PSR factor has been described in the autoparasitoid wasp *Encarsia pergandiella* (Hunter et al. 1993). Wasps of this species oviposit fertilized female eggs in whitefly nymphs and unfertilized male eggs in immature parasitoids which are often females of their own species. Consequently males develop as hyperparasitoids and normally this is the only way they can develop, because unfertilized eggs oviposited in whiteflies die. In contrast, Hunter et al.

(1993) discovered a population in Ithaca, New York, in which some males emerged as primary parasitoids from whiteflies. Cytogenetic studies revealed that these primary males emerged from fertilized eggs in which the paternal genome was lost just after fertilization, thus converting fertilized eggs into haploid males. Since primary males only inherited chromosomes from their mother, this paternally inherited PSR trait is thought to be transmitted by an external genomic element. The nature of this PSR factor is not known. No extra chromosome could be detected.

The third case of a PSR factor was discovered in *Trichogramma kaykai* (Stouthamer et al. 2001). This wasp species was subject to extensive field and laboratory experiments with the goal to determine why the infection frequency with the parthenogenesis inducing (PI) *Wolbachia* in field populations remained relatively constant at around 10% of all the females. The PI *Wolbachia* causes infected females to produce daughters from both unfertilized and fertilized eggs. In unfertilized eggs PI *Wolbachia* disrupts the first mitotic anaphase, resulting in diploid and thus female wasps (Stouthamer & Kazmer 1994). In fertilized eggs PI *Wolbachia* does not seem to have any effect and fertilization causes eggs to develop into females. Modelling had shown that several factors, including inefficient vertical transmission of PI *Wolbachia* and PI *Wolbachia* suppressor genes, could contribute to the observed low infection frequency in natural populations. In these models it was assumed that homozygosity for suppressor genes would cause an infected female to become uninfected. An extensive search for these suppressor genes failed to show any evidence for them in field populations. Next, the influence of a PSR factor on the PI *Wolbachia* infection frequency was simulated. These models showed that PSR factors could maintain the PI *Wolbachia* infection at low levels. Therefore in the field season of 1997 an attempt was made to find evidence for PSR factors in *T. kaykai* (Stouthamer et al. 2001). In that season males of all-male broods were mated with females infected with PI *Wolbachia*. If these fathers were normal, such crosses would result in all-female offspring. However, part of the offspring of some fathers was male. This male producing trait was found to be inherited from father to son and the presence of a PSR factor was established. Determination of the chromosome number in PSR carrying males and normal males showed the presence of an extra chromosome in PSR males, similar to the B chromosome found in *N. vitripennis* (Stouthamer et al. 2001). Additional microsatellite analysis revealed that PSR males only inherited the microsatellites from their mother, indicating that the paternal genome was lost after fertilization, which was again consistent with the mode of action of the PSR chromosome in *N. vitripennis* (Stouthamer et al. 2001).

In all three species described above a deliberate search for sex ratio distorters led to the discovery of various female and male biasing factors. This led Stouthamer et al. (2001) to speculate that PSR factors must be more common than assumed thus far. PSR factors initiate an easily recognizable phenotype, though this is similar to that displayed by virgin females: the production of all-male offspring. All-male broods are known from many species of parasitoid wasps and their high frequency in some field populations are thought to be associated with virginity of the mothers (Godfray 1990). It is obvious that the all-male broods in such populations need to be studied to exclude the possibility of PSR factors. Only a single study has been

conducted to find PSR factors in a species with a mating structure that makes it vulnerable to invasion by PSR factors. In this study, however, no evidence was found for PSR (Henter 2004).

2 Population dynamics of PSR

2.1 Distribution and transmission efficiency of PSR

Trichogramma kaykai is only found in the Mojave Desert (Pinto 1999). Of the 4806 *T. kaykai* broods collected between 1997 and 2001 at 18 different locations in the Mojave Desert on average 2.6% carried males with the PSR chromosome (Stouthamer et al. 2001; Huigens 2003). Approximately 10% of all *T. kaykai* males carried the PSR trait (Stouthamer et al. 2001).

Under laboratory conditions the PSR transmission efficiency in *T. kaykai* is 100%, which means that all offspring resulting from eggs fertilized with sperm from PSR males is male and carries the PSR chromosome (Jeong 2004). Crossing experiments by mating females of recipient species with males carrying PSR showed that the *Trichogramma* PSR can be interspecifically transferred to the closely related species *Trichogramma deion* and *T. pretiosum*, and also to more distantly related species, such as *T. platneri* and *T. sibericum* (Jeong 2004; Stouthamer & Van Vugt, unpublished). However, the PSR chromosome has never been found in field populations of *T. deion* (Huigens 2003). This is even more surprising because *T. deion* and *T. kaykai* are sympatric and can sometimes even emerge from the same host (Huigens 2003). It is postulated that the PSR chromosome is occasionally transferred to *T. deion*, but that the PSR chromosome cannot persist in this species. This could be due to the mating structure of *T. deion* or the lower transmission efficiency of PSR in this species (Jeong 2004). PI *Wolbachia* is thought to cause additional mitotic instability of the PSR chromosome in this species, resulting in absence of PSR in sperm (Jeong 2004).

Although *N. vitripennis* has a worldwide distribution, its PSR chromosome has so far only been found in an area of 150 x 350 km in the Great Basin Area in the USA, covering northern Utah, south-eastern Idaho and western Wyoming (Skinner 1983; Beukeboom & Werren 2000). Between 1988 and 1991 0-6% of the females in the field had mated with PSR males (Beukeboom & Werren 2000). The PSR transmission efficiency in *N. vitripennis* was very high. In the laboratory more than 90% of PSR males transmitted the PSR chromosome to all their offspring (Werren & Van den Assem 1986). The remaining PSR males also produced some female offspring that did not carry the PSR chromosome, indicating that only part of the sperm cells of these PSR males contain the PSR chromosome (Beukeboom & Werren 1993a). Like in *T. deion*, mitotic instability of the *Nasonia* PSR is thought to be influenced by *Wolbachia* infection, though in *Nasonia* this is a cytoplasmic incompatibility (CI) inducing bacterium and not a PI *Wolbachia* (Ryan et al. 1985; Werren 1991).

N. vitripennis has two sibling species: *Nasonia giraulti*, which only occurs in eastern North America, and *Nasonia longicornis*, which is distributed in the western part of North America (Darling & Werren 1990). All three *Nasonia* species are reproductively isolated from each oth-

er, both through genetic incompatibility and infection with different strains of CI *Wolbachia* (Breeuwer & Werren 1995; Bordenstein & Werren 1998). When *N. vitripennis* males are cured from their *Wolbachia* infection, they can easily transmit the PSR chromosome to *N. giraulti* and *N. longicornis*, where it is maintained over multiple generations at a transmission efficiency comparable to that in *N. vitripennis* (Dobson & Tanouye 1998a; Beukeboom & Werren 2000). However, the PSR chromosome has never been found in *N. longicornis* and *N. giraulti*, even though *N. longicornis* occurs in sympatry with PSR-carrying *N. vitripennis* wasps and once even originated from the same patch from which PSR-mated *N. vitripennis* females were collected (Beukeboom & Werren 2000). Most likely, the *Nasonia* PSR chromosome cannot overcome the reproductive isolation between the three sibling species.

Both PSR chromosomes appear to be restricted to single wasp species and single geographical areas in North America, in spite of their high transmission efficiency to sympatric sibling species. *Wolbachia* infections seem to play a role in the species restriction of PSR. Examples of other factors that may create a species barrier for PSR chromosomes are different courtship behaviour and genetic incompatibility (Van den Assem & Werren 1994). In addition PSR chromosomes may not remain in recipient species because of host fitness effects, population structure, mating structure or the presence of other sex ratio distorters (Van den Assem & Werren 1994; Dobson & Tanouye 1998a; Huigens 2003; Jeong 2004).

2.2 Persistence of PSR

How can PSR chromosomes persist in natural populations of *N. vitripennis* and *T. kaykai*? Factors that influence the persistence of PSR in natural populations are: egg fertilization frequency, mating and population structure, maternal sex ratio factors, percentage of females that reproduce as virgins and transmission efficiency of PSR. The dynamics in arrhenotokous populations as modelled by Skinner (1987) shows key features of the PSR dynamics. Assuming random mating the frequency that PSR can attain is a function of only the egg fertilization rate. If x is the egg fertilization rate, then the equilibrium PSR frequency among males is given by:

$$PSR_{eq} = (2x - 1)/x \qquad (1)$$

This model shows: 1) PSR will attain an equilibrium frequency, 2) the PSR frequency is a function of the fertilization proportion; the higher the fertilization percentage the higher the PSR frequency among males, 3) if the fertilization proportion of eggs is lower than 50%, PSR cannot persist in the population. Another effect of the PSR trait is the reduction of the population growth rate, because some eggs destined to become females in populations lacking PSR become males in populations with PSR. This could in principle lead to the extinction of the population, if PSR attains a high frequency in the population.

In general, random mating is not found in populations with a female biased sex ratio. Female biased sex ratios are associated with a mating structure that involves the mating between

brothers and sisters, i.e. sibmating. Assuming that females in such a population mate only once and sibmating occurs with a frequency of s, the PSR frequency among males becomes:

$$PSR_{eq} = (2x - xs - 1)/(x - s) \quad (2)$$

Figure 1 displays the PSR frequency among males in a population as a function of the sibmating frequency and the egg fertilization proportion. This figure shows a negative correlation between the sibmating frequency and the parameter space where PSR can exist in the population. In addition, when the population has a random mating structure and there is no sibmating, this figure gives the relationship between fertilization rate and PSR frequency (formula 1).

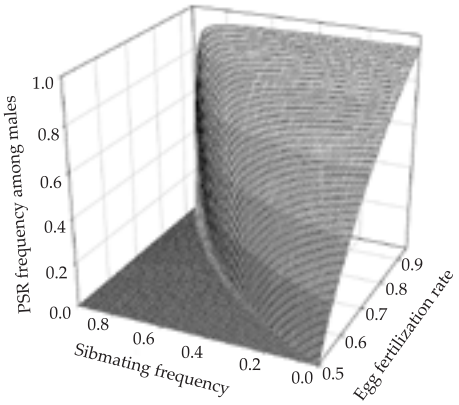

Figure 1: The PSR equilibrium frequency among males for an arrhenotokous population where a fraction of the females sibmate as a function of the egg fertilization rate.

Both *N. vitripennis* and *T. kaykai* have a highly subdivided population structure in which hosts occur in temporary patches lasting one generation and mating occurs just after the wasps emerge from their hosts, resulting in sibmating (Werren 1983). In *T. kaykai* approximately 70% of the females mate with their brother upon emergence (Huigens 2003). Inseminated females then disperse in search of new hosts. In a highly subdivided population only few females will parasitize a new host patch. The *Nasonia* PSR chromosome cannot be maintained in populations with less than three foundress females per host patch (Beukeboom & Werren 1992; Werren & Beukeboom 1993). At higher foundress numbers, the equilibrium frequency is strongly influenced by the fertilization proportion of the eggs (Beukeboom & Werren 1992; Werren & Beukeboom 1993).

In *Nasonia* populations the fertilization frequency is increased by the presence of maternal sex ratio (MSR), which is a cytoplasmically inherited factor of unknown origin that causes inseminated females to fertilize nearly 100% of their eggs (Skinner 1982). In general, MSR will increase the PSR frequency in a population because more eggs are fertilized (Werren & Beukeboom 1993). However, a high PSR frequency leads to a negative effect on MSR if only three or less foundress females parasitize each host patch, because nearly all mates available

for females are PSR males and thus the number of female offspring with MSR is reduced (Beukeboom & Werren 1992; Werren & Beukeboom 1993). A relatively small sample from the field did not show the expected correlation between MSR and PSR frequencies (Beukeboom & Werren 2000). More thorough examination of PSR and MSR interaction in field populations is needed to be conclusive in this matter.

PI *Wolbachia* in *T. kaykai* females is a female biasing sex ratio distorter which is important for maintaining PSR in this wasp species (Stouthamer et al. 2001). Only when mated with a PSR male an infected female produces male offspring, because then fertilized eggs develop into males and females emerge from unfertilized eggs. In this way PSR male offspring can mate with their infected sisters emerging from the same brood whereas in uninfected populations PSR males would emerge with only brothers. The percentage of infected *T. kaykai* females in the field ranges from 4 to 26%. Even without infection PSR should be able to maintain itself in *T. kaykai*, because of the relative low frequency of sibmating and the highly female biased sex ratio in this species (Stouthamer et al. 2001).

The PSR persistence is also influenced by the fraction of females that remain virgin. If v is the proportion of females that remain virgin then the PSR equilibrium frequency becomes:

$$PSR_{eq} = (2x (1 - v) -1)/(x (1 - v)) \quad (3)$$

Figure 2 shows the relationship between the frequencies of female virginity, egg fertilization and the PSR equilibrium. The higher the percentage of virgin females the smaller the parameter space is where PSR can persist in the population.

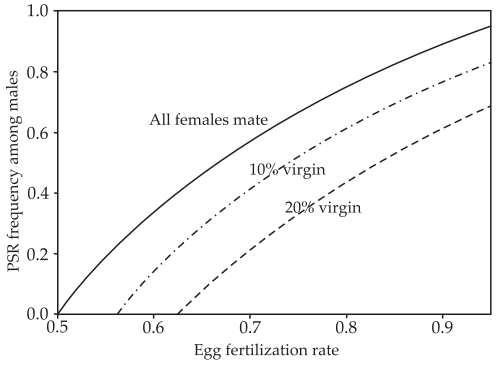

Figure 2: The PSR equilibrium frequency in a randomly mating arrhenotokous population where either: all females mate, 10% remain virgins or 20% remain virgins.

Similarly, the PSR persistence will be influenced by the transmission fidelity of PSR. If we assume that 1 - d is the fraction of eggs that have been fertilized with PSR sperm and do not become PSR males but normal females, then the PSR equilibrium frequency in an arrhenotokous population is given by:

$$PSR_{eq} = (x + dx - 1)/(dx) \quad (4)$$

Figure 3 illustrates this equilibrium and makes clear that a lower PSR transmission efficiency results in a lower PSR equilibrium frequency and a smaller window in which PSR can persist in the population. All these variables clearly show that the existence of PSR in populations is rather precarious and depends on 1) an egg fertilization rate that is higher than 50%, 2) a mating structure that allows for a substantial rate of mating between non-sibs, 3) the presence of a female biasing sex ratio distorter, 4) a low proportion of virgin females, and 5) an efficient transmission of PSR.

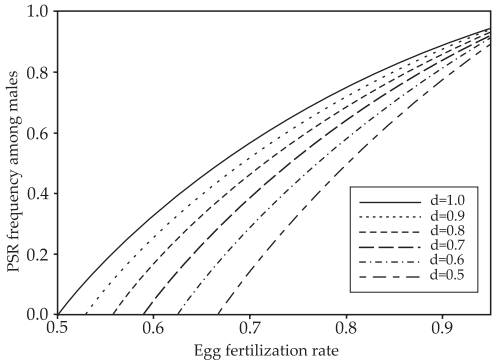

Figure 3: The PSR equilibrium frequency among males in a randomly mating arrhenotokous population where all females mate, but transmission efficiency of PSR varies from 100% (d = 1) to 50% (d = 0.5).

3 Mode of action of PSR

PSR chromosomes cause initially diploid eggs to develop into haploid males carrying PSR (Werren et al. 1981; Stouthamer et al. 2001). Microsatellite analysis in *T. kaykai* revealed that PSR male offspring always has maternal microsatellite markers and therefore has lost the paternal genome (Stouthamer et al. 2001). Mutant marker inheritance in *Nasonia* showed a similar result; all male progeny from crosses with scarlet-eyed females and PSR males had scarlet eyes and all mutant markers for five linkage groups representing each of the five chromosomes were maternally inherited by PSR male offspring (Werren et al. 1981; Werren et al. 1987).

Chromosome studies revealed that sperm of PSR males enters the egg followed by condensation of the paternal genome into a dense chromatin mass at the beginning of the first mitosis (Figure 4) (Werren et al. 1987; Van Vugt et al. 2003). This paternal chromatin mass (PCM) does not participate in any mitotic division, though it is often associated with one of the maternal nuclei (Reed & Werren 1995; Van Vugt et al. 2003). After several nuclear divisions the PCM is lost from the embryo in *Nasonia* (Reed & Werren 1995). PSR chromosomes are extremely selfish genetic elements because every generation they destroy the complete chromosome set that they were associated with. It is generally believed that PSR chromosomes benefit from transmission through male wasps. In females they would have to pass meiosis and at best transmit to 50% of the offspring, whereas in males, which have germ cells generated by abortive non-reductive meiosis, the transmission rate can be hundred percent (Hogge & King 1975).

Non-functional PSR chromosomes in *Nasonia* were transmitted by females, but to only 5-10% of their eggs (Beukeboom & Werren 1993b).

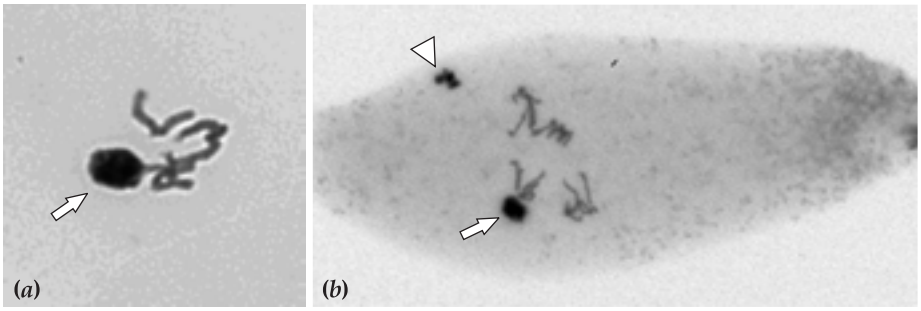

Figure 4: DAPI-stained *T. kaykai* eggs fertilized with sperm containing the PSR chromosome: (a) Metaphase of first nuclear division. (b) Metaphase of third nuclear division. The arrows point to the PCM. The arrowhead points to the polar body, which is the leftover of the maternal meiosis.

How PSR chromosomes cause the paternal chromosomes to condense at the beginning of the first mitosis is still not known. *Nasonia* crossing experiments of diploid males carrying PSR with diploid females demonstrate PSR deletes all autosomes of its host, irrespective of their number (Dobson & Tanouye 1998b). Deletion mapping of the *Nasonia* PSR chromosome positioned the region responsible for paternal genome loss at either end of both chromosome arms (McAllister et al. 2004). This could mean that DNA sequences located in these regions are responsible for the PSR effect, or that a minimum number of repetitive DNA sequences on the PSR chromosome is needed to cause this effect. The PSR chromosome is thought either to disrupt the paternal nucleus formation in the embryo or to influence genome processing during spermatogenesis causing it to be improperly processed during paternal pronucleus formation (Werren 1991; Beukeboom & Werren 1993b; Van Vugt et al. 2003). An effect very similar to that of the PSR chromosome is initiated by the maternal effect mutant *sésame* (*ssm*) in *Drosophila* (Loppin et al. 2000). This mutant prevents the maternal histones from incorporating the male pronucleus, thereby disrupting the paternal genome decondensation (Loppin et al. 2001). Similarly, the PSR chromosome could prevent replacement of the male-specific histones by maternal histones in the paternal pronucleus. Three models are proposed for the PSR mediated paternal genome loss. The 'imprinting' model hypothesizes that a product encoded by PSR imprints the paternal A chromosomes during spermatogenesis (Werren 1991; Beukeboom & Werren 1993b). This imprinted status interferes with the normal processing of the paternal chromosomes after fertilization. A second hypothesis assumes a 'sink' of repetitive sequences on the PSR chromosome binds a product needed for processing of the paternal chromosomes either during spermatogenesis or pronucleus formation (Werren 1991; Beukeboom & Werren 1993b). A third theory conjectures that a product encoded by PSR disrupts or delays the signalling of normal male chromosome processing, either during spermatogenesis or pronucleus formation (Tram & Sullivan 2002).

PSR chromosomes are able to initiate breakdown of the genome they have been associated

with, while somehow remaining immune for their own action. How this effect is established and how they associate themselves with the maternal chromosome set is still unknown. Interestingly, deletion mapping of the *Nasonia* PSR demonstrated that the ability to cause paternal genome loss and the ability of the PSR chromosome to escape this effect are located in different regions on the PSR chromosome (McAllister et al. 2004). Although both PSR chromosomes in *T. kaykai* and *N. vitripennis* appear to have a similar mode of action, it remains unknown whether the molecular mechanism of both PSR chromosomes is also the same.

4 PSR structure and origin

PSR chromosomes are much smaller than the A chromosomes and can therefore easily be recognized in the metaphase cell complement (Nur et al. 1988; Stouthamer et al. 2001). The PSR chromosome in *Nasonia* is submetacentric and represents 5.7% of the haploid genome with approximately 21 Mbp, based on the haploid genome size estimation of 340 Mbp (Rasch et al. 1975; Reed 1993). The PSR chromosome in *T. kaykai* has about 9 Mbp and covers 3.9% of the 216 Mbp haploid genome (Chapter 5). The *Trichogramma* PSR chromosome is thus about twice as small as the *Nasonia* PSR chromosome.

B chromosomes consist to a greater part of repetitive DNA sequences (Camacho et al. 2000). PSR2, PSR18 and PSR22 are repeat families specific for the *Nasonia* PSR chromosome, while a fourth repeat sequence, NV79, also occurs on the A chromosomes (Eickbush et al. 1992). These repetitive sequences have proven to be an important factor for PSR chromosome transmission efficiency and possibly even its size (Beukeboom et al. 1992; Reed et al. 1994). The only tandem repeat found on the *Trichogramma* PSR chromosome is 45S ribosomal DNA (rDNA) (Chapter 4 & 5). This ubiquitous ribosomal repeat occurs in tandem arrays of several hundreds of copies in insect genomes (Long & Dawid 1980) and contains three ribosomal genes, separated by three spacer regions. The presence of 45S rDNA on B chromosomes is not uncommon (Green 1990; Jones 1995) and its occurrence on the *Trichogramma* PSR chromosome is thought to serve a similar function as the repeats on the *Nasonia* PSR chromosome (Chapter 5).

The origin of the *Trichogramma* PSR chromosome was revealed by examining the ITS2 spacer sequences of the 45S rDNA repeat on this chromosome. These sequences resemble both *T. kaykai* and *T. oleae* ITS2 and this PSR chromosome is therefore thought to have originated from *T. oleae* or a *T. oleae*-like species (Chapter 4). Although *T. kaykai* and *T. oleae* are allopatric, they belong to the same species complex (Voegelé & Pointel 1979; Pinto et al. 1986; Pinto et al. 1991; Pinto et al. 1993; Pinto et al. 1997; Schilthuizen & Stouthamer 1997; Stouthamer et al. 1999; Stouthamer et al. 2001). A species close to *T. oleae* is found near Mt. Shasta in Northern California (Pinto 1999) and may have been the source for this PSR chromosome. McAllister et al. (1995) discovered a retrotransposon on the *Nasonia* PSR chromosome that resembles retrotransposons in the genome of the related wasp *Trichomalopsis* (McAllister & Werren 1997). This PSR chromosome is therefore thought to originate from this wasp genus. Attempts to cross *Trichomalopsis* sp. with *N. vitripennis* in the laboratory have been unsuccessful (McAllister &

Werren 1997). A direct PSR transmission between both wasp genera therefore seems unlikely, though the presence of a functional PSR chromosome in *Trichomalopsis* may have helped to overcome genome incompatibility between both wasps. A second possibility is that another wasp served as PSR-host species in between *Trichomalopsis* and *N. vitripennis*, which is supported by the fact that the PSR chromosome can easily be transferred to the other two *Nasonia* species, *N. longicornis* and *N. giraulti* (Dobson & Tanouye 1998a).

In spite of their identical mode of action, no common DNA sequences have been found so far on both PSR chromosomes. This suggests that either they derived from the same PSR ancestor, were distributed to different arrhenotokous hosts and changed significantly over time, or they originated independently and have a similar phenotypic effect on their hosts. In support of this latter hypothesis are the experiments of Perfectti & Werren (2001) that show that chromosome fragments created in incompatible crosses rapidly evolve traits that enhance their transmission through males. Ultimate persistence of such fragments would require these fragments to obtain a PSR trait that enables them to avoid female meiosis.

Concluding remarks

Even though a lot has been learned in the last 20 years about PSR chromosomes in populations of *N. vitripennis* and *T. kaykai*, large gaps in our knowledge remain to be filled. How common are PSR chromosomes in other species of haplo-diploid arthropods? It is unlikely that such chromosomes are limited to only these species. Discovering more examples of PSR chromosomes will provide more insight on their origin and evolution, for instance on how easy it is to generate these chromosomes *de novo*. Chromosomal fragments with PSR-like traits are generated relatively frequent in incompatible crosses in *Nasonia* (Perfectti & Werren 2001), but it is questionable as to whether this is a common phenomenon in incompatible crosses of other species. And if such chromosome fragments are generated, how can they become true PSR chromosomes? This latter question might be answered by determining what genes or sequences are responsible for the PSR effect. Although a lot of theories have been proposed to account for the molecular basis of PSR, we do not have direct evidence for their validity. Are the large number of repetitive sequences found both on the *Nasonia* and the *Trichogramma* PSR chromosome at all related to the PSR phenotype or are they simply important for the PSR transmission (Beukeboom et al. 1992; Reed et al. 1994; McAllister et al. 2004)? Deletion mapping of the *Trichogramma* PSR chromosome may provide additional information on this matter. Also, a more complete overview of the sequences found on the different PSR chromosomes is needed to reveal the molecular mechanism of PSR chromosomes. Isolation of both chromosomes, for example by microdissection, and generating and comparing their libraries may reveal sequences that both chromosomes have in common, which may lead to the PSR mechanism. Finally, little has been done to determine if PSR of either *Nasonia* or *Trichogramma* would induce the PSR phenotype in phylogenetically more distant species. Through microinjection (Sawa 1991) PSR carrying sperm could be introduced in the eggs of other species to

determine if it would induce the same phenotype. If so, the PSR chromosome could be applied for the control of pestiferous species with a haplo-diploid sex determination system, potential targets could include species such as the worldwide pest the Argentine Ant (*Iridomyrmex humilis*) (Werren & Stouthamer 2003).

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Chapter 3

The paternal sex ratio chromosome in the parasitic wasp *Trichogramma kaykai* condenses the paternal chromosomes into a dense chromatin mass

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Abstract

A recently discovered B chromosome in the parasitoid wasp *Trichogramma kaykai* was found to transmit through males only. Shortly after fertilisation this chromosome eliminates the paternal chromosome set leaving the maternal chromosomes and itself intact. Consequently, the sex ratio in these wasps is changed in favour of males by modifying fertilized diploid eggs into male haploid offspring. In this study we show that in fertilized eggs this paternal sex ratio (PSR) chromosome condenses the paternal chromosomes into a so-called paternal chromatin mass (PCM) in the first mitosis. During this process the PSR chromosome is morphologically not affected and is incorporated into the nucleus containing the maternal chromosomes. In the first five mitotic divisions, 67% of the PCMs is associated with one of the nuclei in the embryo. Furthermore, in embryos with an unassociated PCM all nuclei are at the same mitotic stage, while 68% of the PCM-associated nuclei is at a different mitotic phase than the other nuclei in the embryo. Our observations reveal an obvious similarity of the mode of action of the PSR chromosome in *T. kaykai* with that of the PSR-induced paternal genome loss in the unrelated wasp *Nasonia vitripennis*.

Keywords

Paternal sex ratio, PSR, *Trichogramma kaykai*, B chromosome, paternal chromatin mass, embryogenesis

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Introduction

Selfish genetic elements, like B chromosomes, enhance their own transmission at the expense of the rest of their host's genome and are thought to have a strong influence on evolution of the host species (Werren et al. 1988; Stouthamer et al. 2002). An easily detectable group of selfish genetic elements are sex ratio distorters (SRDs), which cause unusual offspring sex ratios in their hosts. The most prevalent cases known so far are female biasing SRDs, which transmit only from mother to offspring through the egg cytoplasm. Examples of such SRDs include organelles and heritable bacteria (Stouthamer et al. 2002). One of the most extreme female biasing SRDs is the parthenogenesis inducing (PI) bacterium *Wolbachia* found in many wasps and in a few thrips and mite species (Stouthamer et al. 1999). Virgin females infected with PI *Wolbachia* produce only daughters and males are no longer needed (Stouthamer & Kazmer 1994; Stouthamer 1997). Male biasing SRDs are rare and are generally located on nuclear chromosomes of the organism. Examples include meiotic drive sex chromosomes and B chromosomes (Stouthamer et al. 2002).

SRDs have been studied extensively in parasitic wasps (Skinner 1982; Werren et al. 1986; Werren et al. 1987; Stouthamer & Kazmer 1994; Hunter & Woolley 2001; Stouthamer et al. 2001). Wasps have a haplo-diploid sex determination, in which haploid eggs become males, while diploid eggs develop into females. Distortion of the sex ratio is induced by mortality of male (haploid) offspring (Werren et al. 1986), by manipulating female wasps to change their egg fertilization frequency (Skinner 1982) or by changing the number of chromosome sets in the first mitotic division of eggs (Werren et al. 1987; Stouthamer & Kazmer 1994; Stouthamer et al. 2001). Upon mating a female stores sperm in her spermatheca and either fertilizes the egg (diploid, daughter) or leaves the egg unfertilized (haploid, son). Presence of a female biasing SRD such as the PI *Wolbachia* induces doubling of the maternal chromosome set in unfertilized eggs at the first mitosis, resulting in a homozygous daughter instead of a son (Stouthamer & Kazmer 1994).

An example of a male biasing SRD is the paternal sex ratio (PSR) chromosome of the wasp *Nasonia vitripennis* (Nur et al. 1988). This small B chromosome causes destruction of the entire paternal chromosome set in eggs fertilized with sperm carrying the PSR chromosome resulting in eggs containing the maternal set plus the B chromosome of the father, which then develop into males carrying the PSR chromosome (Werren et al. 1987). Enhancing its transmission by eliminating all host chromosomes each generation and thereby causing its host to become genetically extinct has made the PSR chromosome the most selfish genetic element known so far (Werren et al. 1988). This male biasing SRD was first discovered in *N. vitripennis* (Nur et al. 1988) and was long considered an interesting, yet unusual example of a SRD. However, the recent discovery of a second case of a PSR chromosome in the distantly related parasitoid wasp *Trichogramma kaykai* raises the possibility that these factors may be more common than thought previously (Stouthamer et al. 2001).

Our observations of the PSR chromosome in *Trichogramma* suggest close resemblance with the PSR system in *Nasonia*. In both species the PSR is controlled by a small B chromosome that

takes up about 5% of the genome and eliminates the paternal chromosome set in a similar way (Werren et al. 1987; Reed 1993; Stouthamer et al. 2001). In *Nasonia* the PSR chromosome eliminates the paternal chromosomes by condensation of the genome into a dense mass at first mitosis in PSR fertilized eggs. This so-called paternal chromatin mass (PCM, Reed & Werren 1995) cannot take part in any subsequent nuclear division and is lost after a few mitotic divisions (Werren et al. 1987). How the PSR chromosome condenses the paternal chromosomes while escaping its own action is not known. We also do not know how the PSR chromosome of *T. kaykai* eliminates the paternal chromosome set. Does it use the same mechanism of destruction as the one in *N. vitripennis*? And if so, do the PSR chromosomes of *Trichogramma* and *Nasonia* originate from the same ancestral species or did they originate independently? Here we report on the cytological mode of action of the PSR chromosome in *T. kaykai*. This mechanism of paternal genome elimination during early embryonic development in *T. kaykai* is strikingly similar to the PSR mechanism in *N. vitripennis*, though some differences were found.

Material and Methods

Trichogramma source and maintenance

The *T. kaykai* strains came from the Mojave Desert in California, where they oviposit in eggs of the lyceanid butterfly *Apodemia mormo deserti* or the Desert Metalmark (Pinto et al. 1997). Strain LC19-1 is an isofemale line that originated with a single PI *Wolbachia* infected *T. kaykai* female collected in 1995 in Last Chance Canyon, Kern Co, CA. The *Wolbachia* free strain LC19-1 Tetra originated from the LC19-1 strain by treating it with the antibiotic tetracycline more than 50 generations earlier. Strain P1-A PSR LC19-1 containing the PSR chromosome was maintained by adding infected LC19-1 females to the culture strain. The PSR chromosome in this strain originated from a *T. kaykai* male that was collected in 1997 near Danby, San Bernardino Co, CA. The wasps were maintained in glass test tubes (150x16 mm) in a rearing chamber at 23°C ± 1°C with light period 18:6, light : dark. Freshly emerged wasps were provided with egg-sheets containing irradiated *Ephestia kuehniella* eggs (supplied by Koppert bv, Berkel & Rodenrijs, The Netherlands) and a small amount of honey.

Egg and slide preparation

Between 80 and 200 newly emerged wasps were allowed to parasitize about ten eggs of *Mamestra brassicae* for 15 min. After removing the wasps from the eggs with a soft brush, the *M. brassicae* eggs were dissected after various time intervals in a drop of 70% acetic acid (AA) on a grease free microscope slide using micro-dissection needles. The *M. brassicae* chorions were peeled off and the yolk released. The *Trichogramma* eggs were spread over the slide by slowly moving the drop of AA for three minutes using micro-dissection needles. Preparations were fixed in Carnoy's fixative (100% ethanol: chloroform: 70% AA = 6:3:1) and finally air-dried and stored at 4°C.

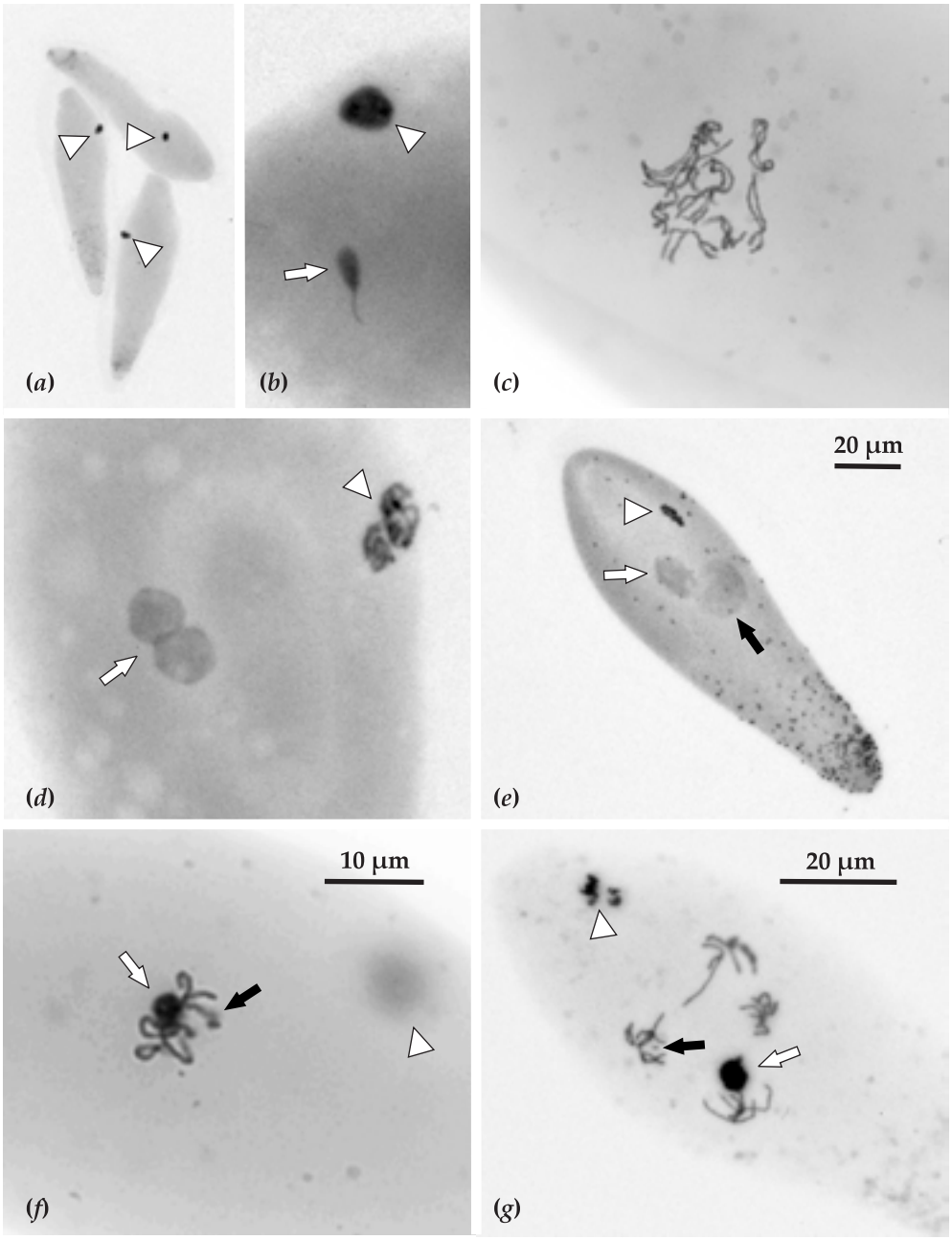
We studied 113 slides, each of them prepared with *Trichogramma* eggs from about ten parasitized *M. brassicae* eggs. Of these slides 79 were prepared using *Trichogramma* eggs fertilized with PSR sperm and 34 were prepared using eggs fertilized with normal sperm. To determine the timing of embryonic events after oviposition, the wasps were allowed to parasitize the eggs for 15 min and the *M. brassicae* eggs were either directly used to prepare slides or incubated for 15, 30, 60, 90, 120, 150 or 180 min.

Air-dried slides were stained with 12 µl DAPI in VectaShield mounting medium (Vector Laboratories, Burlingame, CA, USA) (100 ng DAPI/ml) and studied under a fluorescence microscope. Images were photographed on 400 ISO negative films and scanned at 1000 dpi. The images were then inverted and processed with Adobe Photoshop 6.0 (Adobe Systems Incorporated).

Results

DAPI penetrates both chorion and vitelline membrane of AA fixed *Trichogramma* eggs within minutes and produces well-differentiated images of chromosomes, nuclei and *Wolbachia* bacteria. The latter can be seen as 0.2-0.5 µm fluorescing particles located mostly at the posterior end of the egg (Figure 1e). Although flattened in the microscopic preparation, eggs still retained their three-dimensional information, which could best be visualized with a 100x high numeric aperture objective. In this way the position and appearance of each structure in the egg was established. Number and developmental stage of the nuclei determined the embryonic stage of each PSR fertilized egg. Figure 2 shows a schematic time scale of the early embryonic events up to the fifth mitosis in eggs fertilized with PSR sperm. Because the wasps were allowed to parasitize the *M. brassicae* eggs during 15 minutes, the time scale in figure 2 is an estimation of the embryonic events in time with a range of 15 minutes.

Trichogramma eggs display a characteristic pear-like shape with narrowing posterior end and measure approximately 150 µm in length (Figure 1a). Just after oviposition the maternal nucleus is located in the periphery of the anterior pole of the egg (Figure 1a). The chromosomes of the maternal nucleus are arrested at meiotic anaphase I (Stouthamer & Kazmer 1994), but meiosis completes shortly after oviposition (Figure 1c), resulting in the formation of a double polar body (PB) and maternal pronucleus. The PB shows a characteristic bipartite and granular morphology and is located in the periphery of the anterior pole of the egg (Figure 1d-1j). The female pronucleus is spherical with a diameter of approximately 20 µm (Figure 1d, 1e). Sperm cells with a comma shaped structure and long tail can be seen in the egg just after oviposition (Figure 1b). Eggs contain only one sperm cell, which is usually seen in the anterior pole. Once the sperm cell reaches the vicinity of the maternal pronucleus, it sheds its tail, decondenses partially and adopts a more spherical shape (Figure 1b). Just before the maternal and normal paternal pronuclei enter first mitosis, they appear round and swollen (Figure 1d). Subsequently, their chromosomes condense, align in the equatorial plane at metaphase and next disjoin their chromatids, producing two daughter nuclei, each containing ten chromosomes.


(a)
(b)
(c)
(d)
20 μm
(e)
10 μm
(f)
20 μm
(g)

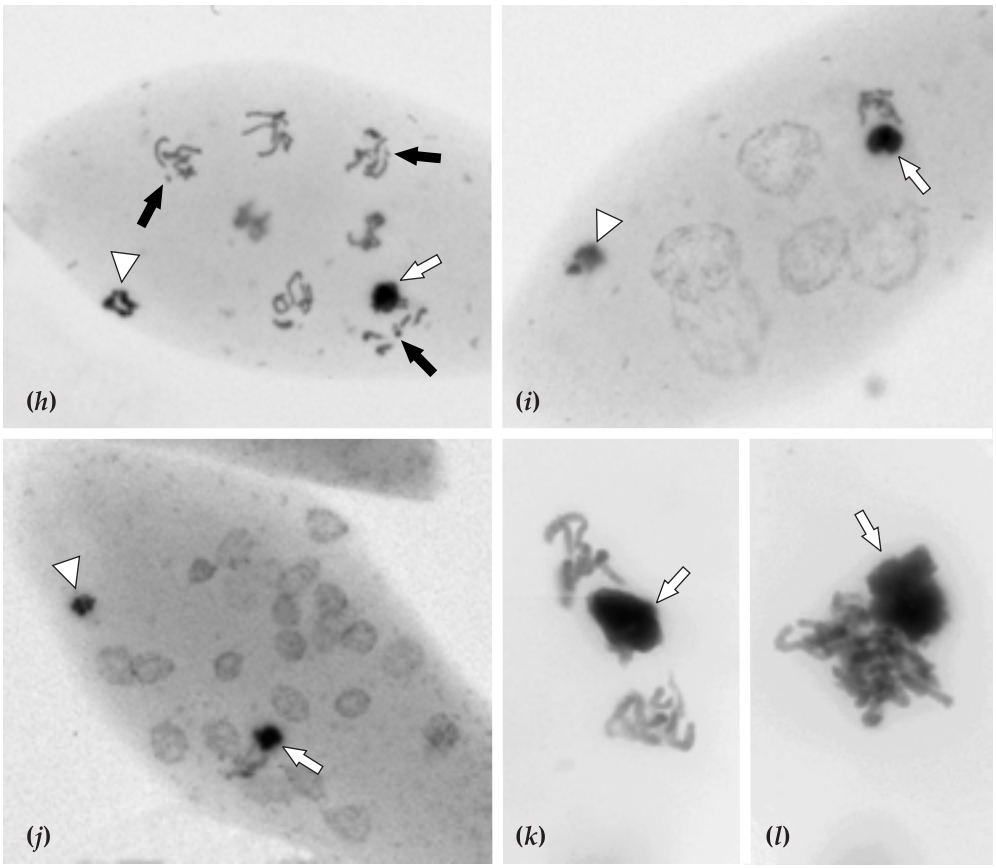


Figure 1: Early embryonic development of *Trichogramma* eggs. (a) Diploid maternal nucleus (arrowheads) before meiosis. (b) A sperm cell (arrow) approaching the diploid maternal nucleus (arrowhead). (c) Meiotic anaphase I of the maternal nucleus. (d-j) The polar body is indicated with an arrowhead. (d) Egg fertilized with normal (i.e. non-PSR) sperm. The paternal and maternal pronuclei lay close to each other and are perfectly round (arrow). (e) Egg fertilized with PSR sperm. The paternal pronucleus (white arrow) has a comma shape, while the maternal pronucleus (black arrow) is perfectly round. The small black dots at the posterior pole of the egg are *Wolbachia* bacteria. (f-l) White arrow indicates PCM. (f) PSR fertilized egg at metaphase of first mitosis. The PCM is formed and the maternal chromosomes (black arrow) are condensed into metaphase chromosomes. (g) PSR fertilized egg at anaphase of second mitosis. The PCM is closely located next to one of the dividing nuclei. The black arrow points to a probable PSR chromosome in one of the nuclei. (h) PSR fertilized egg at anaphase of third mitosis. The black arrows point to probable PSR chromosomes in the nuclei. (i) PSR fertilized egg at interphase of third mitosis. Most nuclei are at interphase, while the nucleus that is located next to the PCM is at metaphase. (j) PSR fertilized egg at interphase of fifth mitosis. Most nuclei are at interphase, while the nucleus that is located next to the PCM is at metaphase. (k) Example of PCM located in between two nuclei. (l) Example of PCM with chromatin clump formation.

Up to the formation of the maternal pronucleus the initial development of PSR fertilized eggs is identical to that of eggs fertilized with normal sperm as described above. However, a paternal pronucleus containing the PSR chromosome develops differently from a normal pa-

ternal pronucleus. A PSR-containing pronucleus is brighter and more elongated than a normal pronucleus, suggesting that it is more condensed than a pronucleus without the PSR chromosome (Fig 1e). Then, while the maternal pronucleus enters first mitosis, the paternal pronucleus condenses into a densely stained chromatin mass, called paternal chromatin mass (PCM, Figure 1f). Each egg contained a single PCM that is easily recognizable at all mitotic stages and is distinguishable from the PB by its central position in the egg and its non-granular structure and even brighter fluorescence. At first mitosis the globular or oval PCM measures 3-4 μm in diameter (Figure 1f). At the second mitotic cycle the PCM expands to approximately 6 μm with about the same fluorescence intensity, whereas its shape becomes irregular and rough-edged (Figure 1g).

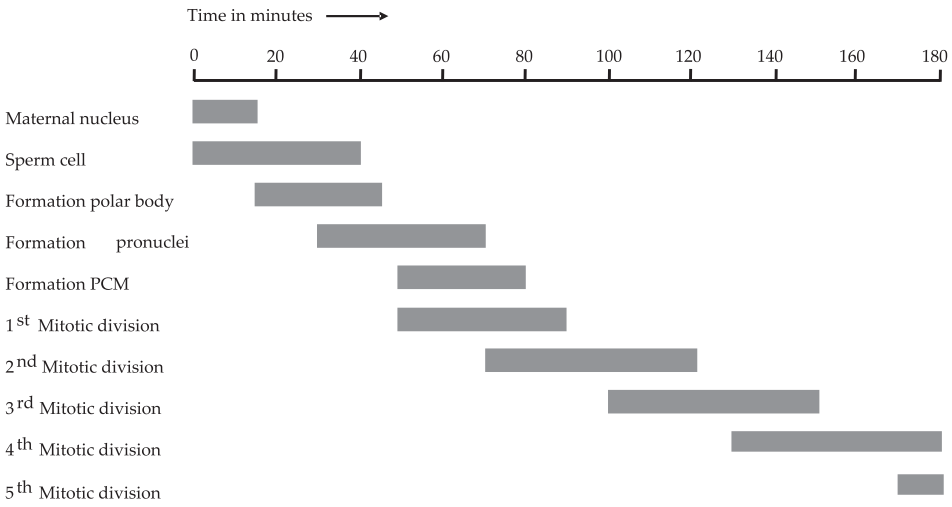


Figure 2: Time schedule of early embryonic events in *Trichogramma* eggs fertilized with PSR sperm. The horizontal bar indicates the time in minutes after oviposition. For each embryonic event the time after oviposition that this event occurred is indicated. The time the wasps were allowed to parasitize the eggs and the time the eggs were incubated without wasps before they were squashed on the slides is used as the total time after oviposition.

The position and effect of the PCM in relation to the other nuclei in the egg is shown in Table 1. In 30% of the PCM-containing eggs the PCM is positioned in between two dividing nuclei (Figure 1k). This phenomenon occurred significantly more often in eggs with an even number of nuclei ($P = 0.01$, $X = 0.05$, T-test). In 67% of all PCM-containing eggs the PCM was closer than half its diameter from a nearby nucleus or even attached to one (Figure 1g-1j). This association was not correlated with the age of the embryo (number of nuclei in the egg) or with an even or odd number of nuclei present. Of all PCM-associated nuclei 68% were at a different mitotic phase than the other nuclei (Figure 1h-1j), generally at late prophase or metaphase. In eggs with an unassociated PCM nuclei were always at the same mitotic stage. In 18% of the eggs without a PCM ($n = 242$, i.e. unfertilized eggs and eggs fertilized with normal sperm) some nuclei were at a different mitotic phase than the others, which is far less than the 45% of eggs

with a PCM. Furthermore, we observed that 15% of the PCM-associated nuclei consist of several stacked nuclei (Figure 1l, data not shown). The aforementioned phenomena were seen in all first five mitotic divisions of the embryo. At later stages with many nuclei in the embryo it was no longer possible to detect the PCM unequivocally.

The PSR chromosome is first observed in one of the nuclei of the second mitosis (Figure 1g). In a number of nuclei in the third mitosis the small B chromosome can also be clearly seen (Figure 1h). Both infected and uninfected females were crossed with PSR chromosome carrying males. No cytological differences were found between dividing nuclei and PCM formation in uninfected and infected eggs fertilized with PSR sperm.

Table 1. Position and effect of the PCM in relation to the number of nuclei in the egg. *The PCM is associated with one of the nuclei if the distance to it is smaller than half the diameter of the PCM. **The PCM has influence on an associated nucleus if this nucleus is at a different mitotic phase than the other nuclei.

Number of nuclei per egg (#)	Number of eggs (#)	PCM in between two nuclei		PCM associated*		PCM influence**	
		(#)	(%)	(#)	(%)	(#)	(%)
1	31	0	0	22	71	3	10
2	44	35	80	36	82	3	7
3	11	1	9	10	91	6	55
4	45	23	51	35	78	6	13
5	14	0	0	11	79	12	86
6	32	17	53	22	69	18	56
7	46	5	11	39	85	33	72
8	14	2	14	1	7	6	43
9	9	1	11	8	89	7	78
10	6	3	50	3	50	4	67
11	10	1	10	8	80	7	70
12	21	2	10	6	29	7	33
13	10	2	20	9	90	4	40
14	11	2	18	4	36	9	82
15	3	1	33	2	67	3	100
16	9	1	11	0	0	8	89
17	4	2	50	3	75	4	100
18	2	1	50	2	100	1	50
19	1	0	0	0	0	0	0
20	9	1	11	3	33	7	78
21	0	0	-	0	-	0	-
22	6	2	33	1	17	4	67
23	0	0	-	0	-	0	-
24	3	2	67	3	100	2	67
Total	**341**	**104**	**30**	**228**	**67**	**154**	**45**

Discussion

Our observations of *T. kaykai* eggs fertilized with PSR containing sperm have clearly indicated 1) clumping of the paternal chromosomes into a dense chromatin mass (PCM) at first mitosis and 2) exclusion of the paternal chromosome set from any mitotic division (Stouthamer et al. 2001). Paternal chromosomes are thus eliminated and embryos develop into males carrying the PSR chromosome with the genome of their mother. The PCM formation takes place at the beginning of first mitosis, somewhere between 50 and 80 minutes after oviposition (Figure 2) and remains clearly visible during all stages of nuclear division for at least five mitotic cycles (Figure 1 and 2).

The PSR chromosomes of *Trichogramma* and *Nasonia* show a striking resemblance in mode of action during early embryonic development. The distinct morphology of the paternal pronucleus, the formation of the PCM at first mitosis and the position and effect of the PCM at the first few mitotic divisions are similar in both wasp species (Werren et al. 1987; Reed & Werren 1995).

The most obvious effect of the PSR chromosome is the formation of the PCM, although the first effect of this B chromosome is the distinct morphology of the paternal pronucleus. In both wasps the PSR containing paternal nucleus is more elongated, compared to the globular shape of the maternal and the paternal pronucleus lacking the PSR chromosome (Reed & Werren 1995). During formation of a normal paternal pronucleus the sperm cell migrates to the maternal nucleus concomitant with the disappearance of tail, cell membrane and nuclear envelope, decondensation of its chromatin, replacement of the sperm-specific histones with embryonic histones, reshaping of a nuclear envelope and duplication of its chromosomes (Fitch et al. 1998). In many organisms the formation of the paternal pronucleus involves both paternal and maternal genomes (Fitch et al. 1998). Tram & Sullivan (2002) argued that timing plays a crucial role in this process in order to synchronize the behavior of paternal and maternal components. A slight delay in development of the male pronucleus compared to the female pronucleus may result in paternal genome loss (PGL) at first mitosis. An example of this is the PGL in uninfected eggs fertilized with CI *Wolbachia* infected sperm in *N. vitripennis*, resulting from a delay in nuclear envelope breakdown of the paternal pronucleus (Tram & Sullivan 2002).

The unusual shape of the paternal pronucleus followed by the PCM formation in PSR fertilized eggs of *Trichogramma* and *Nasonia* suggests that the PSR chromosome somehow interferes with either proper processing of the paternal chromosome set or the timing of this process (Reed & Werren 1995; Tram & Sullivan 2002). The PSR chromosome can interfere by either producing or removing a product involved in embryogenesis (Beukeboom & Werren 1993). If the PSR chromosome produces an interfering product, it requires unique DNA that encodes for a gene. This product could imprint the paternal chromosomes except for itself or it could be an enzyme or RNA product that disturbs an embryonic process. If the PSR chromosome removes an essential cellular compound, like a tubulin-associated protein, it requires specific repetitive or single copy DNA sequences on its chromosome. Interestingly, in *Nasonia* all PSR specific repeats contain two highly conserved palindromic sequences, which may act as binding sites

for proteins (Eickbush et al. 1992; Beukeboom & Werren 1993). Immunolabelling of metaphase spindles in *Nasonia* showed that the PCM is still attached to the spindle apparatus at the first mitosis (Dobson & Tanouye 1996). Together with the observations in *Trichogramma* and *Nasonia* that a great number of PCMs has a weak association with one of the nuclei (Reed & Werren 1995), it is likely that the paternal chromosomes still bind to spindle tubuli. Therefore the PSR chromosome probably does not interfere with spindle tubuli attachment, neither by acting as a 'sink' for tubuli binding nor by blocking the tubuli binding sites on the paternal chromosomes. The fact that the sperm chromatin is decondensed to some extent (the pronucleus is larger and less brightly stained than sperm nucleus) suggests that the paternal chromosomes are not blocked from signals that decondense the chromatin. This also means that the signals needed for this process are present in the cytoplasm. Moreover, the correct mitosis following the formation of the maternal pronucleus also proves that cytoplasmic signaling of pronuclei formation and chromosome condensation occurs. The PSR chromosome more likely acts directly on the paternal chromosomes by somehow altering them, rather than interfering with processes in the cytoplasm (Werren 1991). In doing so it could either make them not susceptible for proper processing (Werren 1991), or cause a delay in any of the early embryonic processes (Tram & Sullivan 2002). Knowledge about the effect of the PSR chromosome may lead to a greater understanding of important biological processes such as embryogenesis, nuclear division, chromosome condensation and separation and possibly DNA/RNA imprinting.

In the aforementioned hypotheses on the mode of action of the PSR chromosome, we did not consider that PSR escapes from its destructive method of chromosome elimination and is taken up by the maternal chromosome set. The mechanism by which this small chromosome protects itself from the influence exerted on the rest of the paternal chromosomes is not known. Escape only seems possible when PSR is not affected by its own effect. The PSR chromosome could for example imprint only the paternal A chromosomes (Dobson & Tanouye 1996) and/or it could escape the self-made proteins that influence the paternal chromosomes using an incorporated self defense mechanism, such as no receptor sequence or proteins.

Once the PCM is formed and the maternal nucleus containing the PSR chromosome starts dividing in the developing embryo, the PCM in *T. kaykai* is in 30% of the eggs located between two dividing nuclei. The fact that the PCM is significantly more often located between two nuclei in eggs with an even number of nuclei in the embryo is not easily explained. One possibility is that the PCM somehow influences the synchronization of nuclei division, resulting in an even number of nuclei in the egg. In 68% of the eggs in which the PCM in *T. kaykai* is associated with one of the nuclei, it also seems to delay this nucleus by keeping it longer at late prophase or metaphase. The same product that keeps the PCM in its condensed state might cause both PCM-association and the resulting delay of nuclear development. In *N. vitripennis* PCM-associated nuclei were only found when sperm from PSR males, infected with cytoplasmic incompatibility inducing *Wolbachia*, fertilized eggs from females that were not infected with *Wolbachia* (Reed & Werren 1995). In *T. kaykai* it was found independent of the presence of PI-*Wolbachia*. Whether or not *Wolbachia* has any influence on the PSR-induced PGL in *T. kaykai* is not known, though both infected and uninfected eggs fertilized with PSR sperm from in-

fected males show no cytological difference with respect to PGL.

In *Trichogramma* the diameter of the PCM doubles from approximately 3 μm at first mitosis to 6 μm at later mitotic divisions and visual observation showed no change in its fluorescence intensity between first and later mitotic divisions. The PCM in *Nasonia* apparently does not increase in size during the first few divisions, although the doubled fluorescence intensity at first mitosis (Reed & Werren 1995) suggests that the PCM in *Nasonia* doubles in size at the beginning of the first mitosis, while the PCM in *Trichogramma* does so between the first and second mitosis. The possibility exists that the PCM in *Trichogramma* also doubles in size at PCM formation and again doubles in size between first and second mitosis, because only the diameter and not the fluorescence intensity of the PCM is measured. This possible size difference of the PCM between *Trichogramma* and *Nasonia* can only be explained by a possible difference in effect of the PSR chromosome on the host genome.

The earliest observation of the PSR chromosome itself was in one of the nuclei in the second mitosis (Figure 2g). Although we could not detect this chromosome in all nuclei of the embryo, it is likely that the PSR chromosome is present in all nuclei and simply cannot always be seen. However, it cannot be excluded that the PSR chromosome is present in only part of the nuclei, for example because of non-disjunction at anaphase.

McAllister and Werren (1997) suggested that the PSR chromosome of *Nasonia* originated from an interspecific cross between wasps of the genus *Trichomalopsis* and *Nasonia*. Its origin in *Trichogramma* is not yet known. Since supernumerary chromosomes are easily established in *Nasonia* (Perfectti & Werren 2001), it is very likely that the two PSR chromosomes have an independent origin. This again suggests that the PSR chromosome manipulates a fundamental process that causes PGL through PCM formation. The ubiquity of the effect of the PSR chromosome is also indicated by the similar phenotype that the *Nasonia* PSR chromosome has when introduced into the species *N. giraulti* and *N. longicornis*. Similarly, the PSR chromosome of *T. kaykai* also functions in other *Trichogramma* species (Van Vugt and Stouthamer, unpublished). We expect that more cases of PSR chromosomes will be discovered and comparative research on these PSR chromosomes should make it possible to discover the exact cytological and genetic manipulations exerted by these PSR chromosomes.

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Chapter 4

The origin of a selfish B chromosome triggering paternal sex ratio (PSR) in the parasitoid wasp *Trichogramma kaykai*

Joke J.F.A. van Vugt, Hans de Jong & Richard Stouthamer

Abstract

This study uses molecular and cytogenetic methods to determine the origin of a B chromosome in some males of the wasp *Trichogramma kaykai*. This so called paternal sex ratio (PSR) chromosome is an extremely selfish genetic element that transmits only through sperm and shortly after fertilization triggers degeneration of the paternal genome, while keeping itself intact. The resulting embryos develop into haploid B chromosome-carrying males. Another PSR chromosome is found in the unrelated wasp *Nasonia vitripennis* and its origin was traced by transposon similarity to the genus *Trichomalopsis*. To determine whether both PSR chromosomes have a similar origin we aimed at revealing the origin of the PSR chromosome in *T. kaykai*. Using fluorescent *in situ* hybridisation we discovered a major satellite repeat on the PSR chromosome, the 45S ribosomal DNA. Analysis of the internal transcribed spacer 2 (ITS2) of this repeat showed the presence of multiple ITS2 sequences on the PSR chromosome resembling either the ITS2 of *Trichogramma oleae* or of *T. kaykai*. We therefore conclude that the *Trichogramma* PSR chromosome originates from *T. oleae* or a *T. oleae*-like species. Our results are consistent with different origins for the PSR chromosomes in *Trichogramma* and *Nasonia*.

Keywords

Trichogramma kaykai, PSR chromosome, B chromosome, chromosome origin, ITS2, rDNA

This chapter is submitted

Introduction

Sex determination in the insect order Hymenoptera is largely based on ploidy level. In general diploid female zygotes develop from fertilized eggs and haploid male zygotes from unfertilized eggs. A large number of heritable factors are known that manipulate this sex determination system to favour their own transmission. These sex ratio distorters can be roughly categorized into two classes: one class where the sex ratio distorter is transmitted through females and causes a female biased sex ratio and a second class where the transmitting sex is male resulting in a male biased sex ratio (Werren 1987). A well-known female biasing sex ratio distorter is the parthenogenesis-inducing bacterium *Wolbachia pipientis* (Stouthamer et al. 1999a). This bacterium is only transmitted cytoplasmically and causes abortion of the first mitotic anaphase in unfertilized eggs, resulting in diploid eggs that develop into females (Stouthamer & Kazmer 1994). An example of a male biasing sex ratio distorter that reduces the ploidy level of fertilized eggs is the paternal sex ratio (PSR) chromosome (Werren et al. 1987). This B chromosome only occurs in males, transmits via sperm and shortly after fertilization, during the first mitotic division in the egg, it condenses the paternal genome into a dense chromatin mass, somehow escaping from condensation itself (Nur et al. 1988; Van Vugt et al. 2003). The egg thus develops into a haploid male with the genome from the mother and the B chromosome from the father. PSR chromosomes have been called extremely selfish because every generation they destroy the complete chromosome set that allowed them to enter the next generation (Werren et al. 1988). The advantage for the PSR chromosome to transmit through male wasps is that its transmission rate is two times higher through males than through females. In males, which have nonreductive abortive meiosis the transmission rate can be hundred percent, while in females with normal meiosis it would at best transmit to 50% of the offspring.

So far, B chromosomes conferring paternal sex ratios have only been found in the parasitoid wasps *Nasonia vitripennis* and *Trichogramma kaykai* (Werren & Stouthamer 2003), both belonging to the superfamily Chalcidoidea. Species of the genus *Trichogramma* (Trichogrammatidae) are egg parasitoids of mainly butterfly and moth eggs, while *Nasonia* species (Pteromalidae) are pupal parasitoids of flies (Whiting 1967; Pinto 1999). Although both wasps are not closely related, their B chromosomes appear to have identical modes of action (Nur et al. 1988; Van Vugt et al. 2003), suggesting either a common B chromosome ancestor or an independent B chromosome origin with similar molecular mechanisms for paternal genome loss.

B chromosomes have been studied extensively in plants and grasshoppers. They originate either from autosomal chromosomes (e.g. *Zea mays* (Stark et al. 1996), *Crepis capillaris* (Jamilena et al. 1994)) or from sex chromosomes (e.g. *Eyprepocnemis plorans* (López-León et al. 1994), starting as aneuploid chromosomes or as centric fragments caused by cytoplasmic or genome incompatibility (Breeuwer & Werren 1993). Most B chromosomes arise from chromosomes of their host and consequently have an intraspecific origin (Camacho et al. 2000). Some B chromosomes, however, carry non-host DNA and either originated during an interspecific hybridisation (Schartl et al. 1995; Perfectti & Werren 2001) or may have been transferred interspecifically from the host of origin to a closely related species.

The origin of the PSR chromosome in *N. vitripennis* was elucidated by transposon analysis. Long terminal repeat (LTR) containing retrotransposons in this chromosome resemble those of *Trichomalopsis* (Chalcidoidea; Pteromalidae) (McAllister 1995; McAllister & Werren 1997), suggesting either interspecific transfer of the PSR chromosome from this closely related wasp species to *N. vitripennis*, or PSR chromosome origin during interspecific hybridisation of both species. Attempts to cross *Trichomalopsis* sp. with *N. vitripennis* in the laboratory have been unsuccessful, because females of either genus are not receptive (McAllister & Werren 1997). This reduces the chance of direct PSR chromosome transmission between both wasps. However, in the rare event of interspecific hybridization cytoplasmic or genomic incompatibility may have caused a potential PSR chromosome to originate. Other possibilities are that an existing PSR chromosome in *Trichomalopsis* has helped to overcome genome incompatibility between both wasps, or another wasp could have acted as intermediate species between *Trichomalopsis* and *N. vitripennis*. This is supported by the fact that the *Nasonia* PSR chromosome can easily be transferred to both *Nasonia longicornis* and *Nasonia giraulti*, indicating this chromosome can cross species boundaries (Dobson & Tanouye 1998).

The origin of the PSR chromosome in *Trichogramma* is still not established. Considering the interspecific origin or transfer of the *Nasonia* PSR chromosome (McAllister 1995; McAllister & Werren 1997) and our observations of PSR chromosome transfer between closely related *Trichogramma* species (Van Vugt & Stouthamer, unpublished), we hypothesize that the *Trichogramma* PSR chromosome originated in a related wasp species. In this study we clarified the origin of the *Trichogramma* PSR chromosome using cytogenetic techniques and DNA sequence comparisons. Fluorescent *in situ* hybridisation (FISH) revealed the presence of 45S ribosomal DNA on the *Trichogramma* PSR chromosome. This satellite repeat occurs in tandem arrays of a few hundreds of copies in insect genomes (Long & Dawid 1980) and contains the 18S, 5.8S and 28S ribosomal genes, separated by the IGS, ITS1 and ITS2 spacer regions (Figure 1). The spacer sequences of this repeat vary considerably between species and are relatively conserved within species and are therefore favourable targets for species discrimination. In *Trichogramma* the internal transcribed spacer 2 (ITS2) sequence is used to discriminate closely related species (Stouthamer et al. 1999b). Here, we analyzed the ITS2 sequences on the *Trichogramma* PSR chromosome and discovered that this B chromosome originated in a closely related *Trichogramma* species.

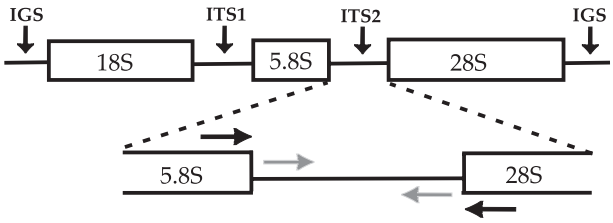

Figure 1: Schematic representation of the 45S ribosomal DNA complex in insects. IGS = intergenic spacer, ITS = internal transcribed spacer. Black arrows indicate the approximate location of the general ITS2 primers. Grey arrows indicate the approximate location of the specific ITS2 primers.

Material and Methods

Trichogramma and *Nasonia* source and maintenance

All *Trichogramma* strains used in this study were collected in the Mojave Desert of California. Strain LC19-1 is a *Wolbachia* infected *T. kaykai* strain, established in 1995 from a single female from Last Chance Canyon, Kern Co, CA and strain SW436-1 is a *Wolbachia* infected *T. deion* strain established in 1996 from a single female from Sidewinder Mountains, San Bernardino Co, CA. The *Trichogramma* PSR chromosome originated from a *T. kaykai* male that was collected in 1997 near Danby, San Bernardino Co, CA. The *T. kaykai* strain P1A PSR LC19-1 carrying this PSR chromosome was maintained by adding infected LC19-1 females to the culture strain. Infected eggs fertilized with PSR sperm develop into PSR carrying males, while unfertilized infected eggs develop into females. In this way all males in the population carry the PSR chromosome, while the females can maintain the population. The PSR chromosome was introgressed to *T. deion* in the lab by crossing a male from strain P1A PSR LC19-1 with a female from strain SW436-1. We maintained the PSR chromosome in the *T. deion* strain by adding infected SW436-1 females to the culture.

The *Nasonia vitripennis* LABII strains, one with and one without the PSR chromosome, originated from the laboratory of Dr. J. H. Werren (University of Rochester, New York, USA), but were received from Dr. L. W. Beukeboom (Rijksuniversiteit Groningen, The Netherlands). The *Nasonia* strain with PSR chromosome was maintained by crossing PSR males with virgin females from the LABII strain without PSR chromosome.

All wasps were kept in glass vials, 150 x 15 mm for *Trichogramma*, 150 x 22 mm for *Nasonia* without PSR and 75 x 10 mm for *Nasonia* with PSR, in a rearing chamber at 23°C ± 1°C with a light-dark period of 18 and 6 hours. Freshly emerged *Trichogramma* wasps were provided with egg-sheets containing *Mamestra brassicae* eggs or irradiated *Ephestia kuehniella* eggs and a small amount of honey as a food source. Freshly emerged *Nasonia* wasps were provided with *Protocalliphora* pupae.

Chromosome spreading preparation, probe labelling and FISH

Three to five days old *Trichogramma* larvae were dissected from *M. brassicae* eggs and fixed in freshly prepared ethanol: acetic acid (3:1) for 3 min at 20°C. Larvae were macerated in 60% acetic acid and cells were softened using a 60°C heating plate for 1 min. Finally, cells were spread with drops of ethanol acetic acid fixative around and on top of the cell mixture and the preparation was left to dry.

Probes for FISH were obtained from 45S rDNA and ITS2 sequences. For labelling the 45S rDNA probe we used the standard nick translation protocol (Roche) with biotin-16-dUTP (Boehringer) on the pTa71 plasmid, containing wheat 45S rDNA (Gerlach & Bedbrook 1979). The ITS2 probe was made by PCR labelling with biotin-16-dUTP using 1 µl Chelex extraction (see below) from a *T. kaykai* female as template. The primers used for this PCR labelling are the

general *Trichogramma* ITS2 primers described in Table 1. Both probes were dissolved in 50% deionized formamide in 2x SSC (3 M NaCl; 0.3 M tri-sodium citrate; pH 7.0), 50 mM sodium phosphate (pH 7.0) and 10% (w/v) dextran sulphate.

The FISH protocol was based on the protocol of Zhong et al. (1996). First, the slides were air-dried for half an hour at 65°C or O/N at 37°C. Then, they were treated with 0.1 mg/ml RNAse A in 2x SSC for 1 hour at 37°C, washed three times for 5 min in 2x SSC and once for 2 min in 0.01 M HCl and treated again with 5 µg/ml pepsin in 0.01 M HCl for 10 min at 37°C. The slides were incubated for 2 min in distilled water, twice for 5 min in 2x SSC and then 10 min in 1% formaldehyde in PBS buffer (1% formaldehyde; 10 mM sodium phosphate; 140 mM NaCl; 50 mM MgCl2; pH 7.0). We washed the slides three times in 2x SSC, dehydrated them in 70%, 90% and 100% ethanol for 3 min each and air-dried them. The 20 µl probe mixtures were boiled for 10 min, chilled on ice, added to the pretreated chromosome preparations and covered with a 24x32 mm coverslip. The chromosomes and probe DNA were denatured at 80°C for 2.5 min and incubated for at least 16 hours at 37°C. We washed the slides three times in 2x SSC, three times 5 min in 50% formamide in 2x SSC (v/v) at 42°C, once for 5 min in 2x SSC and once for 5 min in 4T buffer (4x SSC; 0.5% (v/v) Tween 20). The preparations were incubated in TNB buffer (0.5% (w/v) blocking reagent (Roche); 0.1 M TrisHCl; 0.15 M NaCl; pH 7.5) for 30 min at 37°C and for 1 hour at 37°C in 4 mg/l streptavidin-Cy3 in TNB buffer. The slides were washed once for 5 min in 4T buffer, twice for 5 min in TNT buffer (0.1 M TrisHCl; 0.15 M NaCl; 0.5% Tween 20 (v/v); pH 7.5) and incubated them for 1 hour at 37°C in 20 mg/l biotinylated anti-streptavidin in TNB buffer. Subsequently, we incubated the slides three times for 5 min in TNT buffer, for 1 hour at 37°C in 4 mg/l streptavidin-Cy3 in TNB buffer and twice for 5 min in 2x SSC. Finally, the slides were dehydrated with 70%, 90% and 100% ethanol for 3 min each, air-dried and counterstained with 12 µl of 5 µg/ml DAPI in VectaShield (Vector Laboratories). The chromosome preparations were examined under a Zeiss Axioplan 2 Photomicroscope equipped with epifluorescence illumination, filter sets for DAPI, FITC and Texas-Red fluorescence. Selected images were captured by a Photometrics Sensys 1,305×1,024 pixel CCD camera and processed with Genus Image Analysis Workstation software (Applied Imaging Corporation). We sharpened the DAPI images with a 5x5 Hi-Gauss high pass spatial filter to accentuate minor details and heterochromatin differentiation of the chromosomes. Fluorescence images were pseudocoloured and further improved for optimal brightness and contrast with Adobe Photoshop image processing software. The chromosomes were linearized using a special algorithm designed by Dr. G. van der Heijden (Biometris, Plant Research International, Wageningen University) for the DIPimage toolbox (Dr. Lucas van Vliet, Technical University Delft, v. 1.5.0) in Matlab (The MathWorks, Inc., v. 6.1).

Molecular techniques

We isolated genomic DNA from individual wasps frozen at -80°C and ground with a sterile glass rod in a 0.5 ml tube, after which 50 µl 5% Chelex 100 (Bio-Rad Laboratories) and 2 µl Proteinase K (20 mg/ml) were added and the tube was incubated at 56°C for at least 6 hours

followed by 10 min at 95°C. After centrifugation 1 µl DNA extract was used in a PCR, together with 2.5 µl PCR buffer, 0.5 µl dNTP mix (10 mM each), 0.5 µl forward and reverse primer (25 pmol/µl) and 0.07 µl SuperTaq polymerase (Enzyme Technologies Ltd.) with a total reaction volume of 25 µl. The primers and their corresponding PCR programs are shown in Table 1. From each PCR product 10 µl was run on 1.5 % agarose gel. PCR products were excised from gel and the DNA was extracted from the agarose with the QIAEX II gel extraction kit (QIAGEN). Subsequently, the purified DNA was cloned into the pGEM-T vector (Invitrogen) and transformed into XL-2 Blue cells (Stratagene). The plasmid insert was sequenced using standard plasmid primers. We aligned the obtained sequences in Seqman (DNASTAR Inc.) and analysed all unknown ITS2 sequences in a BLASTN search (NCBI) with expect value 10, word size 11 and a low complexity filter. Restriction enzyme digestion was performed with 5 U restriction enzyme and 1.5 µl reaction buffer on 10 µl PCR product in a total volume of 15 µl for 1 hour at 37°C. The digested product was run together with undigested product on a 1.5 % agarose gel.

Table 1. ITS2 primer combinations and their PCR programs. F = forward primer, R = reverse primer

Primer combination	Primer sequences	PCR program
General *Trichogramma*	F = tgtcaactgcaggacacatg R = gtcttgcctgctctgag	3 min 94°C; 33 x [40 sec 94°C; 40 sec 58°C; 45 sec 72°C]; 5 min 72°C
General *Nasonia*	F = tgtcaactgcaggacacatg R = atgcttaaatttaggggta	3 min 94°C; 33 x [40 sec 94°C; 45 sec 53°C; 45 sec 72°C]; 5 min 72°C
PT1	F = acccgactgctctctcgcaagag R = agccagctataaaatagcgcgcg	3 min 94°C; 35 x [1 min 94°C; 1 min 55°C; 1 min 72°C]; 5 min 72°C
PT2	F = taaaaacgaactctctcgcaagag R = agccagctataaaatagcgcgcg	3 min 94°C; 40 x [1 min 94°C; 1 min 64°C; 1 min 72°C]; 5 min 72°C
PT2-kk	F = taaaaacgaactctctcgcaagag R = gtcttgcctgctctgag	3 min 94°C; 35 x [1 min 94°C; 1 min 57°C; 1 min 72°C]; 5 min 72°C
PT3	F = ctctctaccacctatctcttgc R = attcgagctggccaataacacgc	3 min 94°C; 40 x [1 min 94°C; 1 min 64°C; 1 min 72°C]; 5 min 72°C
PN	F = atatctctcttcttctggggagg R = acgagtatataatatatcaaatat	3 min 94°C; 35 x [1 min 94°C; 1 min 53°C; 1 min 72°C]; 5 min 72°C
T. kaykai specific	F = atctcttgctgctctcgagagg R = cacacgataatgataaacacgcg	3 min 94°C; 35 x [1 min 94°C; 1 min 53°C; 1 min 72°C]; 5 min 72°C
T. deion specific	F = tctgggcgctcgtgtcgctatc R = ggccattatttataaaaaatagcgcgc	3 min 94°C; 35 x [1 min 94°C; 1 min 55°C; 1 min 72°C]; 5 min 72°C
Nasonia specific	F = gtcttttctctctctttcaagcg R = atacgagtatataatatatgaaaaat	3 min 94°C; 35 x [1 min 94°C; 1 min 55°C; 1 min 72°C]; 5 min 72°C

Results

45S rDNA and multiple ITS2 sequences on the PSR chromosome

FISH with the wheat 45S rDNA probe clearly shows large fluorescent signals at the ends of the short arms of the A chromosomes one and four, as well as a large signal on the PSR chro-

mosome, covering about two thirds of this chromosome (Figure 2a). The *T. kaykai* ITS2 probe hybridizes to the same chromosome positions as wheat 45S rDNA (Figure 2b). This does not necessarily mean *T. kaykai* ITS2 is present on the PSR chromosome. In the same way that conserved parts of wheat 45S rDNA hybridize to conserved parts of *Trichogramma* 45S rDNA, *T. kaykai* ITS2 may hybridize to an ITS2 sequence resembling this sequence.

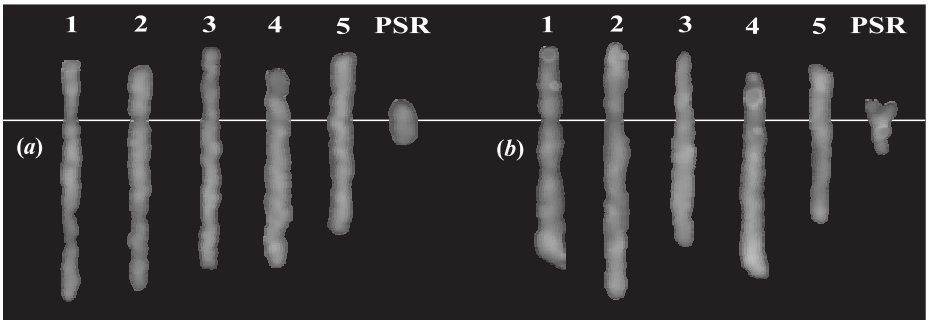

Figure 2: Karyogram of *Trichogramma kaykai*: (a) labelled with 45S rDNA from wheat; (b) labelled with ITS2 from *Trichogramma kaykai*. For colour graph: see page 117.

To determine what ITS2 sequence is on the PSR chromosome, we performed a PCR with the general ITS2 primers (Figure 1 and Table 1). In principle, a single PCR product is produced, because each species has only one ITS2 sequence. This is true for *Trichogramma* DNA without PSR, but *Trichogramma* DNA with PSR has two PCR products (Figure 3). The extra PCR products of approximately 500 bp and 580 bp on *T. kaykai* and *T. deion* DNA with PSR, respectively, suggest the PSR chromosome comprises at least two ITS2 sequences of different length. *Nasonia* DNA with and without PSR show a single PCR product of similar length, indicating no ITS2 sequence of a different length than the *Nasonia* ITS2 is present on the *Nasonia* PSR chromosome (Figure 3).

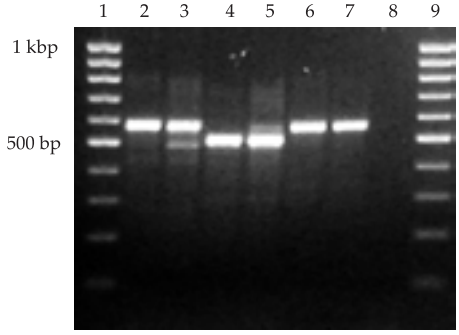

Figure 3: PCR products obtained with the general ITS2 primers on DNA of *T. kaykai*, *T. deion* and *N. vitripennis*, all with and without PSR chromosome: Lane 1 and 9) 100 bp marker; Lane 2) product of *T. kaykai* DNA; Lane 3) product of *T. kaykai* DNA with PSR chromosome; Lane 4) product of *T. deion* DNA; Lane 5) product of *T. deion* DNA with PSR chromosome; Lane 6) product of *N. vitripennis* DNA; Lane 7) product of *N. vitripennis* DNA with PSR chromosome; Lane 8) negative control with water as template. *Trichogramma* and *Nasonia* DNA was amplified with their respective general primers.

All PCR products in Figure 3 were cloned and at least ten plasmid clones were sequenced from each PCR product (Table 2). The sequences of the PCR products of *T. kaykai*, *T. deion* and *N. vitripennis* without PSR are identical to the ITS2 sequences of *T. kaykai*, *T. deion* and *N. vitripennis*, respectively (Figure 4, 5 and Table 2). The extra 500 bp PCR product of *T. kaykai* with PSR consists of four previously unknown ITS2 sequences, which we named PT1, PT2, PT2-kk and PT3 with accession numbers AY845190, AY845191, AY845192 and AY845189, respectively (Figure 4 and Table 2). PT2-kk is an ITS2 sequence of which one third resembles PT2 and the other two third resembles *T. kaykai* ITS2. The extra 580 bp PCR product of *T. deion* with PSR is identical to *T. kaykai* ITS2, while the 510 bp PCR product of *T. deion* with PSR contains two sequences, namely *T. deion* ITS2 and PT2-kk (Table 2). Sequencing of the PCR product of *N. vitripennis* with PSR resulted in two ITS2 sequences, the *N. vitripennis* ITS2 and an unknown ITS2 sequence, which we named PN (accession number AY845188, Figure 5 and Table 2).

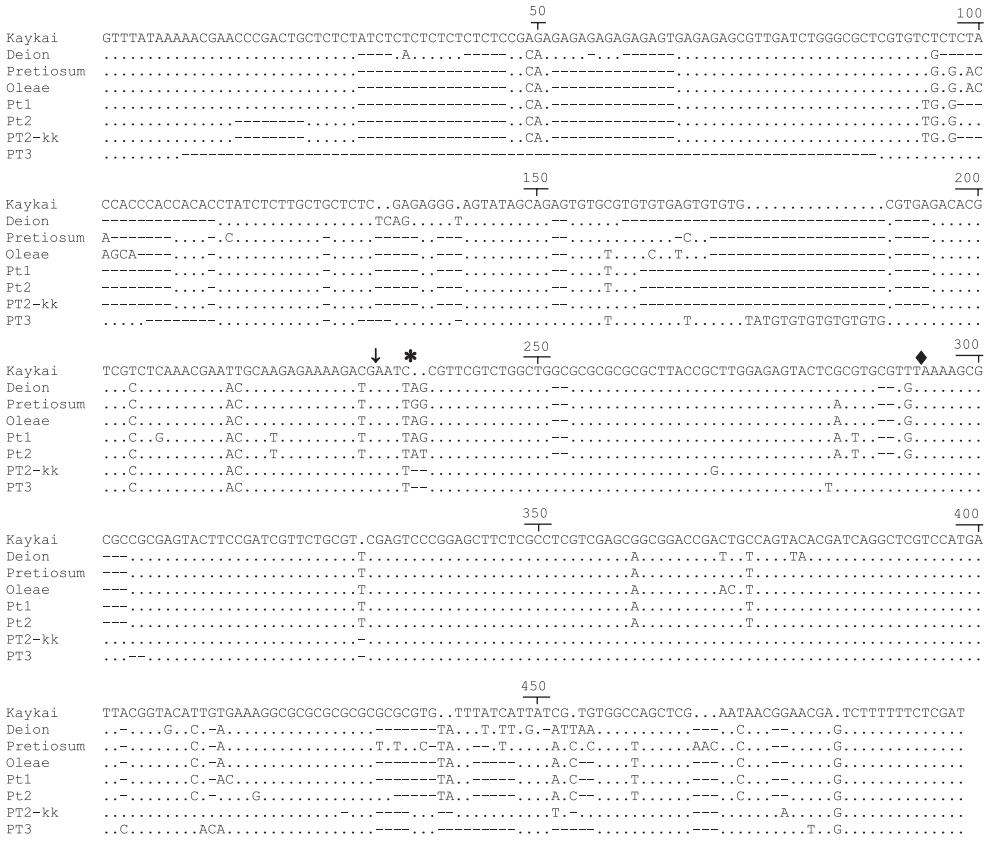

Figure 4: Alignment of *Trichogramma* ITS2 sequences. Identities are denoted by dots. A dash denotes a gapped position. The *T. pretiosum* ITS2 sequence is from strain PRV4 from Riverside, CA (accession number U76226, Pinto et al. 1997). The *T. oleae* ITS2 sequence is from strain 'Tunisia' (accession number U74601, Schilthuizen & Stouthamer 1997). Restriction enzyme digestion sites: diamond is *Dra*I (TTT|AAA), arrow is *Eco*RI (G|AATTC), star is *Xmn*I (GAANN|NNTTC).

Table 2. Number and insert sequence of plasmid clones of all isolated ITS2 PCR products. *T. kaykai* ITS2 is abbreviated as Kk. *N. vitripennis* ITS2 is abbreviated as N. PSR minus template is abbreviated as -. PSR plus template is abbreviated as +.

Template	PCR product	ITS2 insert sequence							
		Kk	Deion	PT1	PT2	PT2-kk	PT3	N	PN
Kaykai -	580 bp	10	-	-	-	-	-	-	-
Kaykai +	580 bp	10	-	-	-	-	-	-	-
	500 bp	-	23	2	2	1	-	-	-
Deion -	510 bp	-	10	-	-	-	-	-	-
Deion +	580 bp	10	-	-	-	-	-	-	-
	510 bp	-	9	-	-	1	-	-	-
Nasonia -	580 bp	-	-	-	-	-	-	10	-
Nasonia +	580 bp	-	-	-	-	-	-	9	1

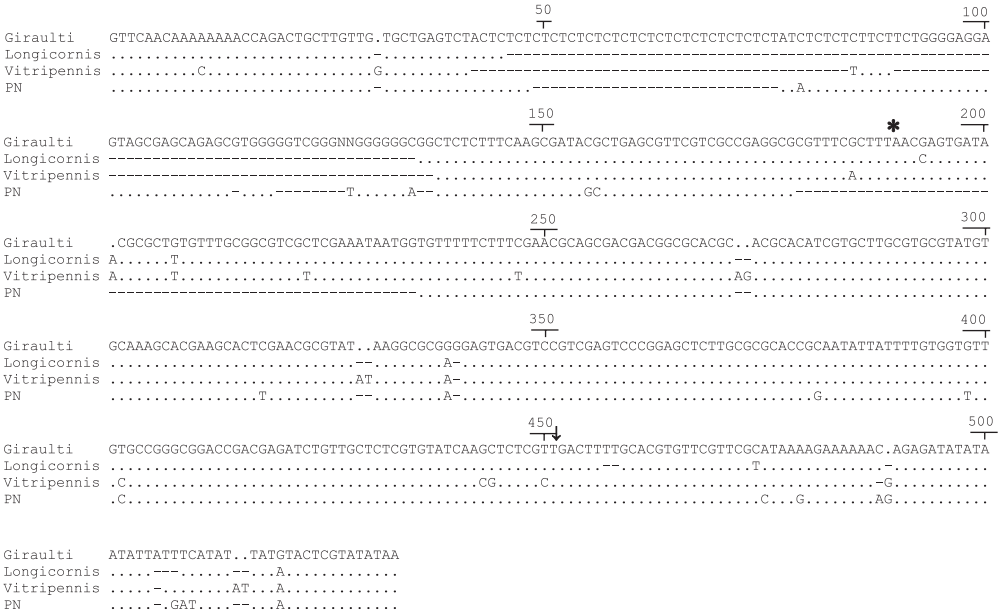

Figure 5: Alignment of *Nasonia* ITS2 sequences. Identities are denoted by dots. A dash denotes a gapped position. The *N. vitripennis*, *N. giraulti* and *N. longicornis* ITS2 sequences are from Campbell et al. 1993 and have accession numbers U02959, U02956 and U02953, respectively. Restriction enzyme digestion sites: arrow is *Hinc*II (GT(T/C)|(A/G)AC), star is *Mse*I (T|TAA).

BLASTN search with PT1, PT2, PT3 and PN in the GenBank database of NCBI reveals PT1 and PT2 resemble *T. oleae* ITS2 most (expect value e^{-154} and e^{-125}, respectively), PT3 resembles *T. kaykai* ITS2 most ($6e^{-79}$) and PN resembles *N. giraulti* ITS2 most (e^{-112}). DNA sequence homologies for PT1, PT2, PT2-kk, PT3 and PN with closely related ITS2 sequences were calculated with two different formulas (Table 3 and 4). The difference between both formulas is the gap

value. The first formula weights a gap by its size in bp, while in the second formula each gap independent of its size is weighted as one. Using either formula PT1 and PT2 have the highest similarity with *T. oleae* ITS2 and PT3 resembles *T. kaykai* ITS2 most. PN resembles *N. giraulti* ITS2 most when each gap values its size in bp and it resembles *N. longicornis* ITS2 most when the gap values one.

Table 3. DNA sequence homology of unknown ITS2 sequences: Every gap values its size in bp. Percentage of DNA sequence homology of PT1, PT2, PT2-kk, PT3 and PN with closely related *Trichogramma* and *Nasonia* ITS2 sequences calculated with the formula: % homology = # similar bp / (# similar bp + # gaps bp + # mismatches bp) * 100.

Name ITS2	Found in PSR species	% Homology				
		T. kaykai	***T. deion***	***T. oleae***	***T. pretiosum***	***T. nr. pretiosum***
PT1	*T. kaykai*	74.2	87.3	94.5	91.7	91.4
PT2	*T. kaykai*	72.9	84.2	91.8	89.7	90.0
PT2-kk	*T. kaykai* *T. deion*	80.6	80.7	84.3	84.9	83.8
PT3	*T. kaykai*	71.6	64.1	70.6	67.8	68.7

Name ITS2	Found in PSR species	% Homology		
		N. vitripennis	***N. giraulti***	***N. longicornis***
PN	*N. vitripennis*	71.5	78.1	74.2

Table 4. DNA sequence homology of unknown ITS2 sequences: Every gap values 1. Percentage DNA sequence homology of PT1, PT2, PT2-kk, PT3 and PN with closely related *Trichogramma* and *Nasonia* ITS2 sequences calculated with the formula: % homology = # similar bp / (# similar bp + # gaps + # mismatches bp) * 100.

Name ITS2	Found in PSR species	% Homology				
		T. kaykai	***T. deion***	***T. oleae***	***T. pretiosum***	***T. nr. pretiosum***
PT1	*T. kaykai*	88.5	94.1	97.7	96.2	97.1
PT2	*T. kaykai*	89.0	92.6	96.8	95.3	96.3
PT2-kk	*T. kaykai* *T. deion*	94.3	89.2	91.9	91.3	91.0
PT3	*T. kaykai*	94.1	88.5	90.4	88.0	87.8

Name ITS2	Found in PSR species	% Homology		
		N. vitripennis	***N. giraulti***	***N. longicornis***
PN	*N. vitripennis*	93.7	94.3	95.0

Are the new ITS2 sequences specific for the PSR chromosome?

We designed specific primers for each ITS2 sequence to determine whether the unknown ITS2 sequences are located on the PSR chromosomes (Table 1). The *T. kaykai* specific ITS2 primers not only amplify PCR products on *T. kaykai* DNA with and without PSR, but also on *T. deion*

DNA with PSR. Sequencing of all these products shows the *T. kaykai* ITS2 sequence and we can therefore conclude that the PSR chromosome has *T. kaykai* ITS2. The *T. deion* and *N. vitripennis* specific ITS2 primers only generate a product on *T. deion* and *N. vitripennis* DNA, respectively.

The ITS2 primers unique for PT1 and PT2 amplify a product on *Trichogramma* DNA with PSR and not on *Trichogramma* DNA without PSR or on *Nasonia* DNA (Figure 6). Sequencing of these DNA fragments confirms they are PT1 and PT2. We therefore conclude that PT1 and PT2 are located on the *Trichogramma* PSR chromosome only.

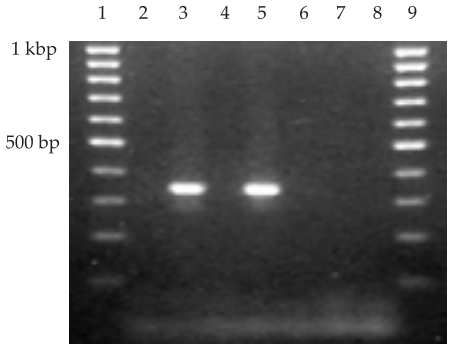

Figure 6: PCR products obtained with the PT2 specific ITS2 primers on DNA of *T. kaykai*, *T. deion* and *N. vitripennis*, all with and without the PSR chromosome. Lane 1 and 9) 100 bp marker; Lane 2) product of *T. kaykai* DNA; Lane 3) product of *T. kaykai* DNA with PSR chromosome; Lane 4) product of *T. deion* DNA; Lane 5) product of *T. deion* DNA with PSR chromosome; Lane 6) product of *N. vitripennis* DNA; Lane 7) product of *N. vitripennis* DNA with PSR chromosome; Lane 8) negative control with water as template.

The PT2-kk specific primers only produce bands on *T. kaykai* and *T. deion* DNA with PSR and on *T. deion* DNA without PSR, which would imply the presence of PT2-kk on the PSR chromosome and the *T. deion* genome. However, sequencing of the products on *T. deion* DNA with and without PSR resulted only in the *T. deion* ITS2 sequence. Furthermore, restriction enzyme digestion with *Dra*I, which digests PT2-kk and not *T. deion* ITS2, shows the products on *T. deion* DNA are not digested and the product on *T. kaykai* DNA with PSR is digested. Apparently, the PT2 forward and the *T. kaykai* reverse primer are not specific for the PT2-kk sequence, because they also amplify *T. deion* ITS2. Still we can conclude from the results on *T. kaykai* DNA with PSR that PT2-kk is present on the *Trichogramma* PSR chromosome.

The PT3 specific primers generate clear PCR products on *T. kaykai* DNA with and without PSR and on *T. deion* DNA with PSR, while a weak product which varies in size is observed on *T. deion* without PSR and no product on *Nasonia* DNA. These results suggest PT3 is present on both the PSR chromosome and the *T. kaykai* genome. Sequencing of PCR products from *T. kaykai* DNA with and without PSR and from *T. deion* DNA with PSR reveals the PT3 sequence. We were not able to sequence the PCR product from *T. deion* DNA without PSR, because the product was too weak and the size of the product varied too much. Restriction enzyme digestion of the *T. kaykai* PCR products with and without PSR with *Eco*RI and *Xmn*I results in the PT3 digestion pattern for both products. These results prove that PT3 is present on both the

PSR chromosome and the genome of *T. kaykai*.

The primers unique for PN amplify products on *Nasonia* DNA with and without PSR, suggesting PN is present on the genome of *N. vitripennis*. Sequencing and restriction enzyme digestion with *Hinc*II and *Mse*I confirm that both products are PN sequences (Figure 5). However, repeated PCR experiments from multiple DNA isolations show the product on template DNA with PSR is always stronger than on template without PSR, suggesting that PN is also present on the PSR chromosome.

Discussion

Extensive studies on rDNA in B chromosomes have demonstrated that a large number of B chromosomes contain 45S rDNA cistrons (López-Léon et al. 1994; Jones 1995; Donald et al. 1997; Cabrero et al. 1999; López-Léon et al. 1999; Stitou et al. 2000; Dhar et al. 2002; Szczerbal & Switonski 2003). There are a number of possible explanations for this phenomenon: 1) 45S rDNA is more prone to chromosome breakage, possibly because of its location usually at the end of a chromosome, 2) 45S rDNA may be susceptible to meiotic isolation, because it has little or no crossing-overs, disjoins later in anaphase I and has a different timing of expression than the rest of the genome, 3) B chromosomes without 45S rDNA may arise, but then easily obtain hyper transposable rDNA sequences (Beukeboom 1994).

So far, from only one B chromosome with 45S rDNA the ITS2 sequence was examined and it differed in only 2 bp (about 0.9 %) from the ITS2 sequence on its host genome (Donald et al. 1997). Here we show that the PSR chromosome in *T. kaykai* has at least four ITS2 sequences that are not only very different from the host ITS2, but also from any ITS2 sequence. All four ITS2 sequences have at least 2 to 6% DNA sequence difference with the ITS2 sequence they resemble most, when calculated with the 'gap values one' formula. These numbers even increase to 5 and 28% when calculated with the 'gap values its size in bp' formula. These data imply that the ITS2 sequences on the PSR chromosome either change very fast or that they had very long time to change.

The presence of PT1 and PT2 on the PSR chromosome suggests that these ITS2 sequences originated from *T. oleae*. *T. kaykai* and *T. oleae* both belong to the *T. deion/T. pretiosum* species complex (Pinto et al. 1986; Pinto et al. 1993; Pinto et al. 1997; Pinto 1999; Stouthamer et al. 1999b). Geographically, however, *T. oleae* and *T. kaykai* are very distant from each other. *T. oleae* has so far only been reported in Tunisia, former Yugoslavia and France (Voegelé & Pointel 1979; Schilthuizen & Stouthamer 1997), while *T. kaykai* has only been reported in the Mojave Desert in California (Pinto et al. 1997). *T. pretiosum* overlaps geographically with *T. kaykai* (Pinto et al. 1986; Pinto et al. 1993; Pinto et al. 1997) and its ITS2 resembles PT1 and PT2 second most. Interestingly, the ITS2 sequence of *T. nr. pretiosum* resembles *T. oleae* ITS2 more than it resembles *T. pretiosum* ITS2 and *T. nr. pretiosum* is in Mt. Shasta, California, much closer to *T. kaykai* than *T. oleae* is to *T. kaykai* (Stouthamer et al. 1999b). If the PSR chromosome originated in *T. nr. pretiosum*, PT1 and PT2, originally being *T. nr. pretiosum* ITS2, have evolved in such a way that

they resemble *T. oleae* ITS2 more than they resemble *T. nr. pretiosum* ITS2. Another possibility for the presence of PT1 and PT2 is that neither *T. oleae* nor *T. nr. pretiosum* donated their ITS2 sequence to the PSR chromosome, but an as yet unknown *Trichogramma* species was involved in the origin of the PSR chromosome. In favour of this latter theory is the fact that ITS2 sequences of more than 54 *Trichogramma* species are available in GenBank and also 18 ITS2 sequences of unknown *Trichogramma* species, but more than 190 *Trichogramma* species are described (Pinto 1999) and more species remain to be discovered. The original host of the *Trichogramma* PSR chromosome can still be undiscovered.

B chromosomes originate either through intra- or interspecific hybridization (Camacho et al. 2000). If the PSR chromosome originated in intraspecific hybridization, it must have originated in a *T. oleae*-like species. Later the PSR chromosome would then have been transferred from this species to *T. kaykai* via an interspecific cross. If so, the presence of PT2-kk, PT3 and *T. kaykai* ITS2 on the PSR chromosome indicates that this chromosome has obtained or is still obtaining parts of the genomic DNA from *T. kaykai*. The PSR chromosome could also have originated in an interspecific cross between *T. kaykai* and a *T. oleae*-like species. Then both *T. kaykai* rDNA and *T. oleae*-like rDNA could have contributed to the origin of the B chromosome, resulting in a B chromosome with ITS2 of both species. Even in an interspecific cross, however, it appears more likely that only the *T. oleae*-like species contributed to the B chromosome origin, because the autosomes of *T. kaykai* were likely to remain intact.

In general only one ITS2 sequence is present on a chromosome and usually also in a complete genome, caused by intra- and interlocus homogenisation, respectively (Dover 1982; Elder & Turner 1995). This means that even when two types of ITS2 are united in one genome, eventually one type will replace the other by means of molecular drive mechanisms, such as biased gene conversion and unequal crossing over (Dover 1982; Hillis et al. 1991). However, on the PSR chromosome at least five ITS2 sequences are found, i.e. PT1, PT2, PT2-kk, PT3 and *T. kaykai* ITS2, and there might be even more present. Does this mean the PSR chromosome is still in the process of homogenisation to a single ITS2 sequence or is there simply no ITS2 homogenisation on this chromosome? The fact that PT2-kk consists of both PT2 and *T. kaykai* ITS2 and the presence of multiple ITS2 sequences informs us that DNA rearrangements and changes occur on the PSR chromosome. A possible explanation is that these rearrangements and changes occur faster than homogenisation. Possible absence of homogenisation can be explained when the rDNA on the B chromosome is inactive, as shown in *Nicotiana tabacum* where homogenisation of rDNA only occurs when the rDNA is active during interphase (Lim et al. 2000). On the other hand, intact *T. kaykai* ITS2 on the PSR chromosome indicates that it recently obtained this sequence from the autosomes or experiences interlocus homogenisation, suggesting this ITS2 on the PSR chromosome is transcribed. If homogenisation only occurs in active rDNA and is lost in inactive rDNA, the rDNA on the PSR chromosome should partly be active and partly inactive.

Whether ITS2 is present on the PSR chromosome of *Nasonia* remains unverified. The newly discovered PN ITS2 appears to be present on the *N. vitripennis* genome, but resembles *N. giraulti* and *N. longicornis* ITS2 more. In contrast, the PN ITS2 gives a consistently stronger PCR

product on DNA with the PSR chromosome than without, which indicates PN ITS2 is also present on the *Nasonia* PSR chromosome. Future FISH studies with 45S rDNA and *Nasonia* or PN ITS2 as probe may reveal whether the *Nasonia* PSR chromosome has 45S rDNA.

The ITS2 sequences on the PSR chromosomes of *Trichogramma* do not resemble *Nasonia* ITS2. Also the *Nasonia* PSR specific NATE transposon and repetitive sequences PSR2, PSR18, PSR22 and PSR79 are not located on the *Trichogramma* PSR chromosome (unpublished data). From this we conclude that both PSR chromosomes have a very different structure and therefore a different origin. We cannot exclude, however, that both PSR chromosomes have altered so much over time that their DNA sequence homology has reduced dramatically. If both PSR chromosomes originated independent, the probability of discovering more PSR chromosomes in other haplo-diploid organisms diminishes. Also the modes of paternal genome loss of both B chromosomes are less likely to be identical, because the chance of two independently derived systems to originate from exactly the same genetic material is very small. On the other hand, the independent origin of two B chromosomes with a similar mode of paternal genome loss suggests either the existence of a simple molecular mechanism, like the involvement of only a single gene or chromosome part, or the existence of multiple possibilities to achieve paternal genome loss during the first mitotic division in the fertilized egg. Future studies should focus on the discovery of the exact molecular mechanisms of both PSR chromosomes. This knowledge can be important to control haplo-diploid pest organisms using male-biased sex ratio distortion.

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Chapter 5

Size and repeat sequence characterization of the paternal sex ratio chromosome in the parasitoid wasp *Trichogramma kaykai*

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Abstract

Part of the male population of the wasp *Trichogramma kaykai* carries a B chromosome, which manipulates its host sex ratio in favour of males. Little is known about the size and DNA sequences of this so-called paternal sex ratio (PSR) chromosome. The only repeat on this chromosome described so far is 45S ribosomal DNA, which includes five different ITS2 sequences, i.e. *T. kaykai* ITS2, PT1, PT2, PT2-kk and PT3. Here, we determined the rDNA transcription using silver staining of metaphase chromosomes and reverse transcriptase PCR. Primers specific for each ITS2 sequence confirmed transcription of the *T. kaykai* ITS2 on the PSR chromosome, whereas PT3 was only very weakly transcribed and the other ITS2 sequences showed no transcription at all. Partial absence of transcription might explain the presence of multiple ITS2 sequences on the PSR chromosome, since homogenization of spacer sequences in rDNA is thought to occur only in transcribed regions. We also estimated the size of the PSR chromosome using relative chromosome lengths and fluorescence intensity measurements together with the estimated *T. kaykai* genome size using flow cytometry. The PSR chromosome measures 3.9% of the haploid *T. kaykai* genome, which represents ca. 9 Mbp. We furthermore characterized the *Trichogramma* specific *Eco*RI repeat which was discovered in *Trichogramma brassicae*. The *Eco*RI repeats in *T. kaykai* and *T. deion* have only 77% and 75% DNA sequence homology with the *Eco*RI repeat in *T. brassicae*. No *Eco*RI repeat is present on the PSR chromosome. Finally, FISH with a pool of high and moderate repetitive *T. kaykai* DNA (C_ot-50) revealed only very few major tandem repeats on the *T. kaykai* chromosomes and only 45S rDNA on the PSR chromosome.

Keywords

PSR, B chromosome, *Trichogramma kaykai*, repetitive sequences, rDNA transcription, *Eco*RI repeat, C_ot DNA

Introduction

The paternal sex ratio (PSR) chromosome in the arrhenotokous wasp *Trichogramma kaykai* is a B chromosome only present in male wasps. Upon fertilization it eliminates the complete paternal genome, except itself, and so manipulates eggs to develop into males, instead of females (Stouthamer et al. 2001; Van Vugt et al. 2003). Accordingly, this selfish chromosome can persist in the male gender, thereby circumventing female gametogenesis in which it would have a two times lower transmission rate than in spermatogenesis with a nonreductive abortive meiosis (Hogge & King 1975). Avoiding normal meiosis is the only characteristic that the PSR chromosome does not share with other B chromosomes, which do pass meiosis though usually in a non-Mendelian manner. Features that the PSR chromosome has in common with B chromosomes are its presence in only some individuals of the host population, its aberrant size and different evolutionary pathway compared to the normal chromosomes and the occurrence of a large amount of repetitive DNA.

Many B chromosomes are rich in repetitive DNA that varies considerably in repeat type and copy number (Eickbush et al. 1992; Cuadrado & Jouve 1994; López-León et al. 1994; Wilkes et al. 1995; Camacho et al. 2000; Dhar et al. 2002; Puertas 2002) and can originate from the A chromosomes or is exclusive for the B chromosome. The repeat families PSR2, PSR18 and PSR22 on the PSR chromosome in *Nasonia vitripennis* are examples of B chromosome-specific repeats (Eickbush et al. 1992). Repeats may form a significant part of a B chromosome or even the exclusive component, like the pSsP216 repeat in B chromosomes of *Drosophila subsilvestris* (Gutknecht et al. 1995). Sometimes, B chromosomes contain much larger amounts of repetitive DNA when compared to the genome of origin, thus suggesting substantial amplification of repeats on the B chromosome (Dhar et al. 2002). Repeat amplification is suggested to be a mechanism through which an initially small chromosome fragment becomes a stable member of the genome, resulting in the formation of a B chromosome (Reed et al. 1994; Camacho et al. 2000).

Like many other B chromosomes, the PSR chromosome in *T. kaykai* contains 45S ribosomal DNA (Chapter 4). This large satellite repeat consists of three ribosomal genes separated by spacers. Concerted evolution causes the different copies of the spacer sequences to be more similar within species than among species, making them suitable for taxonomic studies (Dover 1982; Elder & Turner 1995). Analysis of the internal transcribed spacer 2 (ITS2) revealed the presence of the *T. kaykai* ITS2 and four additional ITS2 sequences on the PSR chromosome which were named PT1, PT2, PT2-kk and PT3 (Chapter 4). PT1 and PT2 resemble the ITS2 of *Trichogramma oleae* most. *T. oleae* belongs, just like *T. kaykai*, to the *Trichogramma pretiosum/T. deion* species complex (Pinto et al. 1986; Pinto et al. 1991; Pinto et al. 1993; Pinto et al. 1997; Stouthamer et al. 1999). PT3 is most similar to the *T. kaykai* ITS2 and is also present on the *T. kaykai* genome. PT2-kk is a combination of *T. kaykai* ITS2 and PT2. Usually only one ITS2 sequence is present on a chromosome and in general also in a complete genome by intra- and interlocus homogenization, respectively. The existence of multiple ITS2 sequences on the PSR chromosome suggests the absence of homogenization, though intact *T. kaykai* ITS2 on the PSR chromosome implies

interlocus homogenization still occurs. Lim et al. (2000) found that transcription of 45S rDNA resulted in homogenization of the rDNA sequences. Here we determine whether the 45S rDNA on the PSR chromosome is transcribed. This is done with reverse transcriptase PCR on cDNA with and without PSR chromosome using primers specific for *T. kaykai* and *T. deion* ITS2, PT1, PT2, PT2-kk and PT3 and with silver staining of metaphase chromosomes.

Except for the presence of 45S rDNA little is known about the size and content of the *Trichogramma* PSR chromosome. By measuring the chromosome lengths of a single metaphase complement the size of the PSR chromosome is estimated at 4.4% of the haploid *T. kaykai* genome and measures approximately 2 μm in length (Stouthamer et al. 2001). In this study a more accurate estimation of the PSR chromosome percentage per haploid genome is performed by measuring chromosome lengths and fluorescence intensity of metaphase chromosomes in multiple nuclei. The number of nucleotides on the PSR chromosome will be determined by combining the assessed PSR percentage with our estimation of the *T. kaykai* genome size using flow cytometry of adult wasp nuclei. Additionally, the PSR chromosome will be examined for the presence of other satellite repeats, which may help in determining the origin and evolution of this chromosome. We studied a *Trichogramma*-specific *Eco*RI satellite repeat with a repeat unit size of 385 bp, that was discovered in *Trichogramma brassicae* where it comprises about 16% of the genome (Landais et al. 2000). Dot-blot hybridisation and PCR with primers specific for the *T. brassicae Eco*RI repeat showed that other *Trichogramma* species, *T. cacoeciae*, *T. evanescens*, *T. vogelei*, *T. daumalae* and *T. semblidis*, also have a monomer or multimer of this repeat. Landais et al. (2000) argued that related species share a number of low-copy satellite sequences, some of which could be amplified into a major satellite family independently in each species. Here, the possible presence of the *Eco*RI repeat in *T. kaykai* and *T. deion* and on the PSR chromosome is examined, using both molecular and cytogenetic techniques. We studied the possible existence of other major repeats in *Trichogramma* and its PSR chromosome using fluorescent *in situ* hybridisation (FISH) with C_0t-50 DNA as probe. This probe is the fraction of pooled repetitive DNA sequences obtained from reannealing single stranded genomic DNA (Britten & Kohne 1968; Awgulewitsch & Bünemann 1986; Chang 2004).

Material and Methods

Insects

T. kaykai line LC19-1 and *T. deion* line SW436-1 were collected from the Mojave Desert, California, and were each initiated with a single *Wolbachia* infected female from Last Chance Canyon, Kern County, CA, in 1995 and Sidewinder Mountains, San Bernardino County, CA, in 1996, respectively. The PSR chromosome came from a *T. kaykai* male collected in 1997 near Danby, San Bernardino County, CA, and was maintained in the *T. kaykai* and *T. deion* isofemale lines. The uninfected *T. brassicae* isofemale line was collected from a cabbage field near Wageningen, The Netherlands (Van Rijswijk 2000). *T. cacoeciae* originated from a single female collected in the

Planken Wambuis nature reservation in Ede, The Netherlands (Van Rijswijk 2000). *Wolbachia* infected *T. pretiosum* was collected in Penipe, Chimborazo province, Ecuador in 1997 and were received from J. C. Monje (University of Hohenheim, Germany). Wasps were reared in 150 x 15 mm glass vials on UV-irradiated *Ephestia kuehniella* eggs (Koppert B.V.) and *Mamestra brassicae* eggs at 23°C ± 1°C with a light-dark period of 18 and 6 hours.

D. melanogaster line '95/'98 originated from wild individuals collected in an orchard in Wageningen, The Netherlands, in 1995 and 1998. *D. melanogaster* flies were reared on a standard medium of yeast, sugar and agar at 23°C ± 1°C with light-dark period of 16 and 8 hours.

Genomic DNA isolation

We isolated genomic DNA from approximately 500 *Trichogramma* wasps, either with or without PSR chromosome of line P1A. The wasps were frozen alive at -80°C and ground with a pestle in a 1.5 ml tube, after which 500 µl Bender buffer (0.1 M NaCl; 0.2 M Sucrose; 0.1 M Tris-HCl; 50 mM EDTA; 0.5% SDS), 50 µg proteinase K and 10 µg RNase A were added and the mixture was incubated for at least 16 hours at 37°C. Subsequently, the suspension was extracted twice with phenol and once with chloroform : isoamylalcohol (24:1). DNA was precipitated using two volumes of ice cold 100% ethanol after which the tube was incubated for half an hour at -20°C. The precipitated DNA was washed once with ice cold 70% ethanol and the DNA pellet was air-dried and dissolved in 50 µl TE (10 mM Tris-HCl; 1 mM EDTA; pH 8.0).

RNA isolation, cDNA generation and reverse transcriptase PCR

About 500 *Trichogramma* wasps were frozen and ground as described above. We added 500 µl RNA Wiz buffer (Ambion) and incubated the mixture for 5 min at 20°C. The RNA extraction was performed with 100 µl chloroform. Next, the RNA was precipitated by adding 250 µl RNase free water and 500 µl isopropanol and incubating the mixture for 10 min at 20°C. After centrifugation for 15 min at 14000 rpm at 4°C the RNA pellet was washed with 500 µl 75% ethanol, air-dried and dissolved in 50 µl RNase free water. The DNA and RNase were removed by adding 1 µl 1 M Tris-HCl (pH 7.5), 2.5 µl 0.1 M $MgCl_2$, 0.25 µl 0.1 M DTT, 50 U RNase OUT (Invitrogen) and 20 U DNase to 20 µl RNA solution and incubating for 20 min at 37°C. After addition of 150 µl RNase free water the RNA was chloroform extracted and subsequently precipitated with 0.1 volume 3 M NaAc and 2.5 volumes 100% ice cold ethanol. The RNA pellet was washed with ice cold 75% ethanol, air-dried and dissolved in 20 µl RNase free water. From this RNA solution we used 18 µl to generate cDNA by adding 3 µl RNase free water, 0.5 µl random primers (3 µg/µl) and 2 µl 10 mM dNTP. After 5 min incubation at 65°C we chilled the solution shortly on ice. Then, 8 µl first strand buffer, 4 µl 0.1 M DTT, 80 U RNase OUT and 400 U M-MLV reverse transcriptase (Invitrogen) were added and incubation of the mixture was performed for 10 min at 25°C, 50 min at 37°C, followed by 15 min at 70°C. Reverse transcriptase PCR was performed on 1 µl cDNA with the specific ITS2 primers and with the PCR protocol and cycle conditions as described in chapter 4.

PCR, cloning and sequencing

PCR was performed on 50 ng genomic DNA with 2.5 µl PCR buffer, 20 pmol dNTP mix (5 pmol each), 12.5 pmol *Eco*RI repeat forward (5'-CCAAGTGTCGTGTAAAAATT-3'), 12.5 pmol *Eco*RI repeat reverse (5'-ATTCTAGACCGGACAAAAGT-3') primer (Landais et al. 2000) and 0.35 U SuperTaq polymerase (Enzyme Technologies Ltd.) in a total reaction volume of 25 µl with cycle program: 3 min 94°C, 30 cycles [1 min 94°C; 1 min 53°C; 1 min 72°C], 5 min 72°C. We ran 10 µl of each PCR product on a 1.5 % agarose gel. PCR products that had to be sequenced were extracted from the agarose with the QIAEX II gel extraction kit (QIAGEN). Subsequently, the purified DNA was cloned into the pGEM-T vector (Invitrogen) and transformed into XL-2 Blue cells (Stratagene). The plasmid insert was sequenced using standard plasmid primers. We aligned the sequences with the program Seqman (DNASTAR Inc.) and analysed the sequences in a BLASTN and BLASTX search (NCBI) with expect value 10, word size 11 and a low complexity filter

Southern blotting and hybridization

We digested 5 µg genomic DNA with 10 U *Eco*RI for one hour at 37°C and added 1 µl 0.5 M EDTA and incubated for 10 min at 65°C to arrest the enzyme digestion. As positive control we used 10 pg unlabelled *T. kaykai* and *T. brassicae Eco*RI repeat PCR products. DNA samples were run on a 0.6% agarose gel, pretreated and transferred to a 0.45 µm Hybond-N+ filter (Amersham Pharmacia Biotech) as described by Sambrook et al. (1989). We cross-linked the DNA to the filter with 120 mJoules UV light using a 2400 UV Stratalinker. The filter was prehybridized in 75 ml Church buffer (0.5 M $NaHPO_4$ (pH 7.2); 1 mM EDTA; 7% SDS) for 1 hour at 65°C and hybridized for at least 16 hours at 65 °C in 15 ml Church buffer with 300 ng digoxigenine labelled *T. kaykai* or *T. brassicae Eco*RI repeat. The probes were labelled with digoxigenine by PCR as described above, but with 10 ng plasmid insert as template and partly replacing the dTTP by digoxigenine labelled dUTP. The stringency washes following hybridization were twice 5 min 2x SSC, 0.1% SDS at 20°C and twice 5 min 0.5x SSC, 0.1% SDS at 65°C. Digoxigenine labelled DNA was detected with the DIG Luminescent Detection kit (Boehringer Mannheim) according to the manufacturer's protocol. Finally, the filter was exposed for 1 hour to an XAR-5 film (Amersham Biosciences), developed and scanned.

Flow cytometry

Trichogramma wasps and *Drosophila* flies were sedated with CO_2 and the heads of *Drosophila* females were cut off. Two female *Drosophila* heads and/or five intact *Trichogramma* wasps were added to 1 ml Galbraith buffer (21 mM $MgCl_2$; 30 mM tri-Sodium citrate dihydrate; 20 mM MOPS; 0.1% Triton X-100; 1 mg/l RNase A; pH 7.2) in a 2 ml Dounce Homogenizer (Nutacon B.V.) on ice and pulverized by stroking 15 times with an A pestle. The buffer was subsequently filtered through a 50 µm nylon mesh. We adjusted the volume to 1 ml with Galbraith buffer and added 50 µl Propidium Iodide (1 mg/ml). Flow cytometric analyses were performed on

a FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA) equipped with an excitation wave length of 488 nm and a band pass filter of 585 nm (564 to 606 nm) for Propidium Iodide fluorescence. For each measurement between 5000 and 13000 nuclei were analysed. Data were analyzed with the program CELLQuest version 3.1f (Becton Dickinson) using a linear scale. The coefficient of variation (cv) of each measurement was calculated by CELLQuest version 3.1f and is based on the standard deviation (s) in channel units and the mean number of measured nuclei (x): cv (%) = 100*s/x. The coefficient of variation reflects the imprecision of isolation, staining and measurement and is in general flow cytometric experiments ca. 3% (Marie & Brown 1993).

Isolation of C_0t fractions

C_0t fractions of *Trichogramma* genomic DNA were prepared according to Chang (2004). Approximately 10 µg genomic DNA with a concentration of 125 ng/µl in 0.3M NaCl was sheared to fragments of 0.5 to 1 kbp by sonification, denatured for 10 min at 95°C, kept on ice for 10 sec and incubated at 62.4°C for 37 hours and 40 min to slowly re-anneal the genomic DNA, as defined by Peterson et al. (1998). The remaining single stranded DNA was digested with S1 nuclease (Fermentas, final concentration 1 U/µg) for 90 min at 37°C. The pool of remaining double stranded DNA fragments (C_0t-50) was extracted with 1 volume chloroform : isoamylalcohol (24:1), precipitated with 2 volumes ice cold 100% ethanol and the DNA pellet was air-dried and dissolved in 20 µl distilled water.

Chromosome preparation, probe labelling and FISH

Chromosome preparation and FISH were performed as described in chapter 4. For labelling the C_0t fraction of genomic DNA and the *Eco*RI repeat we used the standard nick translation protocol (Roche) with biotin-16-dUTP (Boehringer) on 10 µl C_0t DNA and 1 µg plasmid DNA with the *T. kaykai Eco*RI repeat as insert, respectively. The pTa71 plasmid with the wheat 45S rDNA insert (Gerlach & Bedbrook 1979) was labelled according to the standard nick translation protocol (Roche) with digoxigenin-11-dUTP (Boehringer). The chromosome preparations were analysed and photographed as described in chapter 4. The chromosomes were linearized using a special algorithm designed by Dr. G. van der Heijden (Biometris, Plant Research International, Wageningen University) for the DIPimage toolbox (Dr. Lucas van Vliet, Technical University Delft, v. 1.5.0) in Matlab (The MathWorks, Inc., v. 6.1).

Silver staining

On top of dry chromosome preparations from *T. kaykai* PSR males (prepared as described in chapter 4) we placed ca. 250 µl freshly prepared 50% w/v $AgNO_3$ in distilled water. We covered the slides with 24x50 mm wet 300 µm mesh nylon membrane (Nybolt) and incubated them for 3 to 4 hours at 65°C in a moistened Petri-dish. After incubation, slides were washed 4 times 5 min in water. The nylon membrane was removed during the first wash. After air-drying, 12 µl

Entellan-Neu solution (Merck) and a 24x50 mm cover slip were placed onto the slides and they were allowed to dry for 24 hours. Subsequently, they were analyzed using a Zeiss Axioplan 2 bright-field microscope. Selected images were captured by a Photometrics Sensys 1,305 x 1,024 pixel CCD camera and processed with Genus Image Analysis Workstation software (Applied Imaging Corporation). Images were further improved for optimal brightness and contrast with Adobe Photoshop image processing software.

Chromosome size estimation by DAPI fluorescence and length measurements

Captured DAPI stained metaphase nuclei (see chapter 4) of *T. kaykai* with PSR were selected in which the chromosomes had similar fluorescence intensity and no overlap. The percentage of fluorescence intensity of the PSR chromosome was determined with the computer program Image Pro Plus v. 5.1 (MediaCybernetics). The relative PSR chromosome length was measured with the computer application MicroMeasure v. 3.3 (Reeves & Tear 2000).

Results

PSR chromosome size

Table 1 shows the flow cytometric genome size estimates of Propidium Iodide stained nuclei of *T. kaykai* with and without PSR. *Drosophila melanogaster* line '95/'98 with a genome size of 350 Mbp (2C value) was used as reference to establish the genome size of *T. kaykai* (Bennet et al. 2003). The genome size of *T. kaykai* females was estimated at 432 Mbp ± 10 Mbp, while the PSR *T. kaykai* male comprised 220 Mbp ± 7 Mbp. The slight difference between the PSR male and haploid female genome size gives a size estimation for the PSR chromosome of about 4 Mbp (220-(432/2)), which is 1.8% of the haploid genome (4*100/220).

Table 1. Genome size estimation of *T. kaykai* with and without PSR chromosome.

Organism	Ploidy level	Channel (#)	Coefficient of variation (%)	Genome size (Mbp)
D. melanogaster	2C	239	2.7	350
T. kaykai	2C	295	2.2	432 ± 10
T. kaykai + PSR	1C	150	3.1	220 ± 7

We also estimated the PSR chromosome size by measuring DAPI fluorescence intensity and chromosome lengths of metaphase complements in which all chromosomes were separated and clearly distinguishable. Three DAPI fluorescence intensity measurements gave an average PSR chromosome size of 4.0% ± 0.5% of the haploid *T. kaykai* genome (ranging from 3.5% to 4.4%). Twelve chromosome length measurements gave an average PSR chromosome size of 3.9% ± 1.0% of the haploid *T. kaykai* genome (ranging from 2.7% to 5.6%). Combining the values of both measurements gives an average PSR chromosome size of 3.9% ± 0.9%. Together with

the haploid *T. kaykai* genome size of 216 Mbp, these values estimate the PSR chromosome size at 9 Mbp ± 2 Mbp (216*3.9/96.1).

*Eco*RI repeat

The presence of the *T. brassicae Eco*RI repeat in other *Trichogramma* species was tested in a PCR assay using the *T. brassicae Eco*RI repeat primers on 50 ng genomic DNA of *T. kaykai* and *T. deion*, both with and without PSR, *T. cacoeciae*, *T. pretiosum* and *T. brassicae* (Figure 1). These primers amplify 314 bp of the 385 bp repeat sequence in *T. brassicae* (Landais et al. 2000). A 314 bp PCR product is amplified from all DNA templates, confirming the presence of the *Eco*RI repeat in all tested *Trichogramma* species. Except for *T. cacoeciae*, also the dimer of 699 bp (385 + 314 bp) can be seen in all species, whereas the *T. brassicae* PCR product is particularly strong compared to the other products and displays a ladder pattern up to the tetramer. In *T. deion* not only the mono-, di- and trimer can be distinguished, but also a second repeat with a repeat unit size of approximately 400 bp.

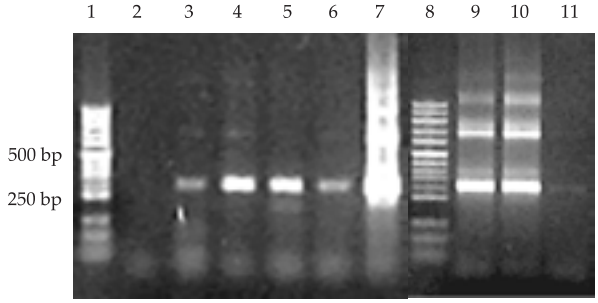

Figure 1: PCR products obtained with the *T. brassicae Eco*RI repeat primers on DNA of *T. kaykai* with and without PSR chromosome, *T. deion* with and without PSR chromosome, *T. cacoeciae*, *T. pretiosum* and *T. brassicae*: Lane 1 and 8) BIOzym Low Ladder, 100 bp marker above 500 bp and 50 bp marker below 500 bp; Lane 2 and 11) negative control with water as template and both *Eco*RI primers; Lane 3) product of *T. kaykai* DNA; Lane 4) product of *T. kaykai* DNA with PSR chromosome; Lane 5) product of *T. cacoeciae* DNA; Lane 6) product of *T. pretiosum* DNA; Lane 7) product of *T. brassicae* DNA; Lane 9) product of *T. deion* DNA; Lane 10) product of *T. deion* DNA with PSR chromosome. The very weak band pattern in lane 11 results from spilling of PCR product from lane 10 into lane 11 during loading of the samples on agarose gel.

The 314 bp monomers of *T. kaykai* and *T. deion*, both with and without PSR, and *T. brassicae* were cloned and three clones of each PCR product were sequenced (Figure 2). The 400 bp PCR product of *T. deion* was too weak to be cloned and sequenced. Re-amplification of isolated 400 bp product resulted in the 314 bp product which possibly reflects co-isolation of the 314 bp product. The consensus sequence of the *T. brassicae Eco*RI repeat is identical to that of the *T. brassicae Eco*RI repeat published by Landais et al. (2000) (accession number AF093063). The six clones of *T. kaykai* with and without PSR revealed a single *Eco*RI repeat sequence, which is different from the *T. brassicae* repeat, whereas in *T. deion* with and without PSR we obtained two *Eco*RI repeat sequences, both different from the *T. brassicae* repeat, of which one is identical to

the *T. kaykai Eco*RI repeat (2 clones) and the other is named the *T. deion Eco*RI repeat (4 clones). BLASTN and BLASTX search with all *Eco*RI repeats did not reveal any sequence homology except with the *T. brassicae Eco*RI repeat. Comparison of the 274 bp in between the primers shows that the *T. kaykai* and *T. deion Eco*RI repeats have 77% and 75% DNA sequence homology with the *T. brassicae Eco*RI repeat, respectively. The *T. kaykai* and *T. deion Eco*RI repeats differ only 2% from each other in these 274 bp. The primers themselves are not included when calculating the sequence homology, because they may have misprimed on the *T. kaykai* and *T. deion Eco*RI sequence.

```
                                                  50                                                  100
Brassicae  CCAAGTGTCGTGTAAAAATTTCAAATCGATCCCGCAAACCCTCGAAAAGTTATACCCGTTTAAAGTCACTTTTTGGCCCAAAAATGCCACATTAA.TAAG
Kaykai     ........................G......G........G.TC........C.GA.TCG.....................T....G..CCA..A.G..
Deion      ........................G......G........G.TC........C.GA.TCG.............A........T....G..CCA..A.G..
                                                  150                                                 200
Brassicae  GTTATCTTTGGTTCTAACGGTCGGATTGACTCGAAAATTTTTATGCACGTCTAGAATTGGATAATTTATGACCTTACAACTTGATCGAAAGGTTACAAAT
Kaykai     C......CG......GGT.....A.A.....T........C.......AC........T.............T.G...........A...C.....G...
Deion      C......CG......GGT.....A.A.....T........C..G....AC........T.............T.G...........A...C...TTG...
                                                  250                                                 300
Brassicae  AAAACTCTGAAAACTTTTCACGGTCATCGTGACTTTTCGAAAT.AAAAGTAATTTCATCTTTTGCGGTCTTCGTGACACTTCCGGCTGGTGATACAACTT
Kaykai     .............T..............TCT.......T..T.T.TT.T.....-..A..G.G.T...T....AAG..AG.T..CA....A...-.....
Deion      .............T..............TCT.......TT.T.T.TT.T.....-..A.CG.G.T...T....AAG..AG.T..CA....A...-.....

Brassicae  TTGTCCGGTCTAGAAT
Kaykai     ................
Deion      ................
```

Figure 2: Alignment of *Eco*RI repeat sequences of *T. brassicae*, *T. kaykai* and *T. deion*. Identities are denoted by dots. A dash denotes a gapped position.

Analysis of the Southern blot hybridization using the *T. brassicae Eco*RI repeat as probe shows a clear 385 bp ladder on *T. brassicae* DNA and *T. kaykai* DNA with and without PSR, while *T. deion* DNA with and without PSR presents a weak 385 bp repeat and a strong repeat of ca. 500 bp (Figure 3a, b). With the *T. kaykai Eco*RI repeat as probe a similar result is observed, but this Southern shows a much stronger signal on *T. deion* DNA, while only weak signal is detected on the *T. brassicae* mono- and dimer (Figure 3c, d). The presence of the *Eco*RI repeat in *T. kaykai* is not as profuse as in *T. deion*. Furthermore, the signal of the 500 bp repeat in *T. deion* is stronger than the 385 bp repeat signal, indicating that the 500 bp *T. deion* repeat is more abundant than its 385 bp repeat. Finally, no difference in amount or size of the *Eco*RI repeat is observed between DNA with and without PSR chromosome.

FISH with the *T. kaykai Eco*RI repeat and 45S rDNA as probes on metaphase chromosomes of *T. kaykai* with PSR revealed the presence of two *Eco*RI repeat loci on the genome of *T. kaykai* (Figure 4a). They are located at the ends of the long arms of chromosomes 1 and 4, the same chromosomes where the 45S rDNA is located on. We did not observe any *Eco*RI repeat signal on the PSR chromosome.

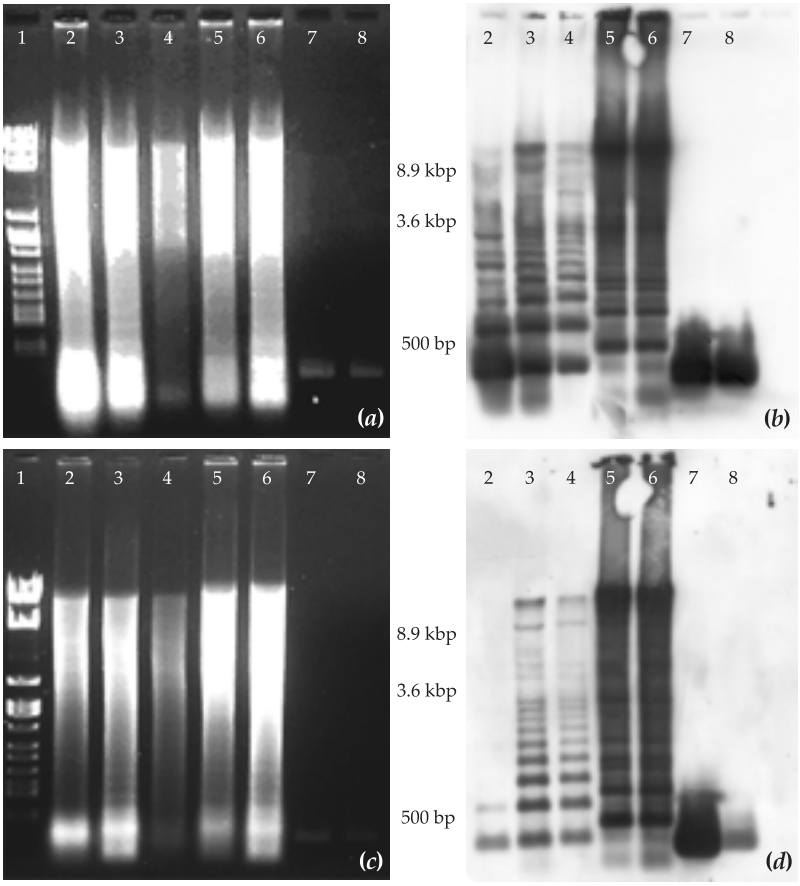

Figure 3: Southern hybridization of the *T. brassicae* and *T. kaykai Eco*RI repeats on partial *Eco*RI digested genomic DNA: (a) and (c) are the agarose gels of Southern hybridizations (b) and (d), respectively; (b) Southern hybridization with 300 ng digoxigenine-labelled *T. brassicae Eco*RI repeat; (d) Southern hybridization with 300 ng digoxigenine-labelled *T. kaykai Eco*RI repeat. Lane 1) Lambda *Eco*RI/*Bam*HI/*Hind*III ladder; Lane 2) *T. brassicae* DNA; Lane 3) *T. kaykai* DNA; Lane 4) *T. kaykai* DNA with PSR chromosome; Lane 5) *T. deion* DNA; Lane 6) *T. deion* DNA with PSR chromosome; Lane 7) positive control: 100 ng PCR product of *Eco*RI primers on *T. kaykai* DNA with PSR chromosome; Lane 8) positive control: 100 ng PCR product of *Eco*RI primers on *T. brassicae* DNA.

FISH with C_0t DNA

Hybridization of *T. kaykai* metaphase chromosomes with the C_0t-50 probe revealed strong signals on the 45S rDNA sites on chromosomes 1, 4 and the PSR chromosome (Figure 4b) and the *Eco*RI repeat signal on the long arm of chromosome 4. We also detected one large signal and few minor signals on chromosome 2, but the known *Eco*RI site on chromosome 1 did not reveal any fluorescence of the C_0t-50 probe. Finally, the end of the short arm of the PSR chromosome also contains a C_0t-50 signal, which might be similar to the ITS2 signal (Fig. 2b in chapter 4).

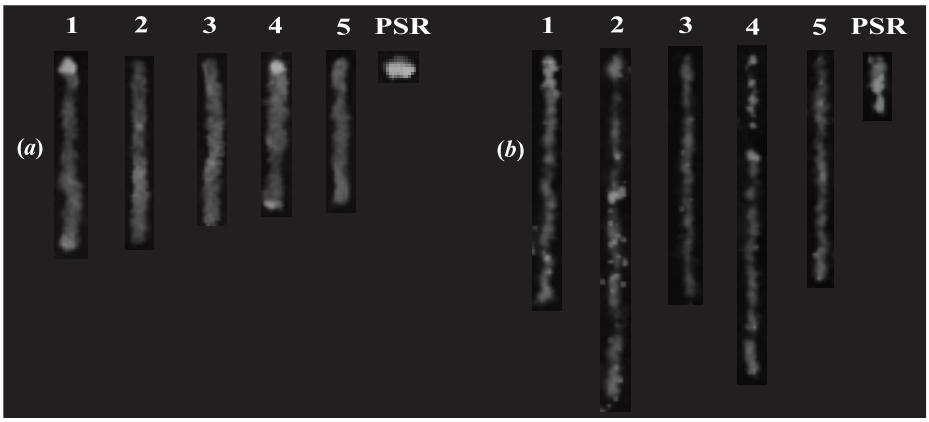

Figure 4: Karyogram of *Trichogramma kaykai*: (a) labelled with 45S rDNA from wheat and with the *T. kaykai* *Eco*RI repeat; (b) labelled with C_ot-50 from *Trichogramma kaykai*. For colour graph: see page 117.

45S rDNA transcription on PSR chromosome

Reverse transcriptase PCR was performed on cDNA of *T. kaykai* and *T. deion*, both with and without PSR, using the specific ITS2 primers for the PT1, PT2, PT2-kk, PT3, *T. kaykai* and *T. deion* ITS2 sequences as described in chapter 4 (Figure 5 and Table 2). *T. kaykai* specific ITS2 primers generated PCR products on *T. kaykai* cDNA with and without PSR and on *T. deion* cDNA with PSR (Figure 5 and Table 2). Presence of the latter product demonstrates that the *T. kaykai* ITS2 on the PSR chromosome is transcribed. *T. deion* specific ITS2 primers amplify a PCR product on *T. deion* cDNA with and without PSR (Figure 5 and Table 2). PT1, PT2 and PT2-kk are not transcribed on the PSR chromosome, since no PCR product was amplified with the primers specific for these ITS2 sequences (Table 2). In only two out of six PCR experiments a weak PT3 PCR product was detected in both *T. kaykai* and *T. deion* cDNA with PSR, while in the other four experiments no PT3 could be detected at all (Table 2), suggesting PT3 is only weakly transcribed on the PSR chromosome.

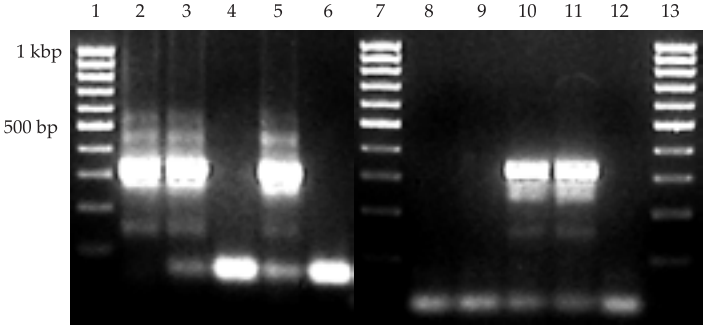

Figure 5: Reverse transcriptase PCR products obtained with the *T. kaykai* and *T. deion* specific ITS2 primers on cDNA of *T. kaykai* and *T. deion*, both with and without PSR: Lane 1, 7 and 13) 100 bp ladder; Lane 2 to 6) product from *T. kaykai* specific ITS2 primers; Lane 8 to 12) product from *T. deion* specific ITS2 primers; Lane 2 and 8) *T. kaykai* cDNA; Lane 3 and 9) *T. kaykai* with PSR cDNA; Lane 4 and 10) *T. deion* cDNA; Lane 5 and 11) *T. deion* with PSR cDNA; Lane 6 and 12) negative control with only water.

Table 2. Reverse transcriptase PCR with specific ITS2 primers on cDNA of *T. kaykai* and *T. deion*, both with and without PSR. Presence of the right PCR product is denoted as +. Absence of PCR product is denoted as -. +/- means only little PCR product of the right size is present in two out of six PCR experiments. *T. kaykai* specific ITS2 primers are abbreviated with Kk.

cDNA	Specific ITS2 primers					
	Kk	Deion	PT1	PT2	PT2-kk	PT3
Kaykai	+	-	-	-	-	-
Kaykai PSR	+	-	-	-	-	+/-
Deion	-	+	-	-	-	-
Deion PSR	+	+	-	-	-	+/-

We performed silver staining on metaphase chromosomes of *T. kaykai* with PSR to demonstrate whether the 45S rDNA on the PSR chromosome is transcribed. Figure 6 shows metaphase chromosomes in which a greater part of the PSR chromosome displays a dark silver staining, indicating ribosomal transcription of this region. Two more silver stained foci are observed, which are likely the NORs on chromosomes 1 and 4.

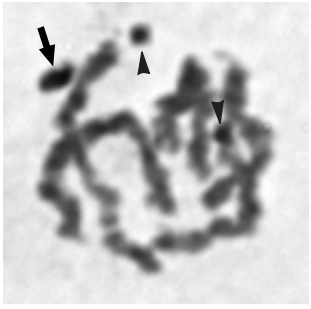

Figure 6: Silver staining of metaphase chromosomes of *T. kaykai* with PSR. Arrow indicates PSR chromosome. Arrowheads indicate the NORs of chromosome 1 and 4.

Discussion

Genome size

We established the size of the PSR chromosome in *T. kaykai* using the relative chromosome length and DAPI fluorescence of a number of selected metaphase complements. Both methods revealed a relative length for this PSR chromosome of 3.9% ± 0.9% of the haploid genome, which equals 9 Mbp ± 2 Mbp. This PSR chromosome is more than two times smaller then the 21 Mbp *Nasonia* PSR chromosome (Rasch et al. 1975; Reed 1993). Surprisingly, estimation of the PSR chromosome size by comparing the flow cytometric data of diploid females and haploid PSR males showed this chromosome has only 4 Mbp. It is questionable whether the ratio of Propidium Iodide fluorescence and DNA concentration is sufficiently linear over the measured channel range, which could result in an underestimation of the PSR chromosome

size following flow cytometric data. Another possible explanation for the two fold difference in PSR chromosome size could be that the PSR chromosome is not present in all nuclei of the male wasp. Because the PSR chromosome is very small, the two flow cytometric peaks resulting from nuclei with and without PSR chromosome would overlap and form one peak, which would subsequently have a lower channel number than the peak from nuclei with PSR chromosome.

The diploid genome size of *T. brassicae* is with 491 Mbp (Dr. J. S. Johnston, personal communication) 13.7% larger than the *T. kaykai* genome. Apparently the genome sizes of *Trichogramma* species are quite different. However, part of these differences can be attributed to variation in the environmental conditions during the experiments (Nardon et al. 2003). Significant variation in genome size was observed in *D. melanogaster* when the insects were reared at various temperatures and humidities (Nardon et al. 2003). Also the age of the insects and the temperature of the nuclei solution prior to flow cytometry resulted in a variable genome size in *Drosophila* (Nardon et al. 2003). Older insects had a smaller genome size, while a low humidity during insect rearing increased the genome size significantly.

Occurrence of tandem repeats

PCR with *T. brassicae*-specific *Eco*RI primers showed *Eco*RI repeat sequences in all our *Trichogramma* species and so confirm the results of *Trichogramma* species examined by Landais et al. (2000). The *Eco*RI repeat is not restricted to the *T. brassicae* genome, though the relative weak PCR product on DNA of the *Trichogramma* species other than *T. brassicae* demonstrates these *Eco*RI repeats either are less abundant in the genome, as suggested by Landais et al. (2000), or have a somewhat different sequence than the *T. brassicae Eco*RI repeat. Sequence data of the *T. kaykai* and *T. deion Eco*RI PCR products confirm the latter hypothesis, if we assume that the sequence differences in between the primers are also found in the sequences where the primers anneal. When we compared the 274 bp in between the primers, the *T. brassicae Eco*RI repeat sequence showed only 77% and 75% DNA sequence homology with the *T. kaykai* and *T. deion Eco*RI repeats, respectively. That PCR amplification of only the monomers is caused by a difference in *Eco*RI DNA sequence between the *Trichogramma* species and not only by a less abundant presence in the genome, is also supported by the Southern hybridisation which suggests the *Eco*RI repeat is more abundant in the *T. kaykai* genome than in the *T. brassicae* genome, while it seems even more excessive in the *T. deion* genome. However, more research is needed to confirm this, for example with real-time PCR. If the *Eco*RI repeat is at least as abundant in the *T. kaykai* genome as in the *T. brassicae* genome, it would comprise about 16% of the genome, according to Landais et al. (2000), which is about 80% of one *Trichogramma* autosome. However, our FISH study showed that the *Eco*RI repeat in *T. kaykai* is located on the distal parts of the long arms of chromosomes 1 and 4 and together cover at most 10% of the smallest A chromosome, while no signal was seen on the PSR chromosome. This discrepancy between our results and those of Landais et al. (2000) can only be explained if we assume that greater part of the *Eco*RI repeat in *T. kaykai* is dispersed in the genome in target domains too small to

be detected by FISH. However, Southern analysis does not support this theory, since practically all *Eco*RI repeat signal is visible as a ladder pattern, which is tandem repetitive DNA. We therefore think that the *T. kaykai Eco*RI repeat comprises at most 2% of the genome. Landais et al. (2000) estimated the relative abundance of the *Eco*RI repeat by comparing dot-blot hybridisations of different dilutions of *Eco*RI repeat monomers and genomic DNA. A difference in the membrane cross-link capacity between intact genomic DNA and the short monomer sequences may have resulted in an overestimation of the *Eco*RI repeat in the genome.

Like the *T. brassicae Eco*RI repeat the *T. kaykai* and *T. deion Eco*RI repeats do not show any sequence homology with any other sequence known in the GenBank database, nor does the DNA sequence show long open reading frames (Landais et al. 2000). Though in some organisms transcription of satellite DNA has been detected, the function of satellite DNA has never been demonstrated (Renault et al. 1999).

The presence of the *T. kaykai Eco*RI repeat in both *T. deion* and *T. kaykai* could either be a result of phylogenetic constraint of this repeat or could be due to hybridization between *T. kaykai* and *T. deion* resulting from incomplete reproductive isolation (Jeong 2004). Our Southern blot hybridizations demonstrate the existence of a 385 bp and a 500 bp repeat in *T. deion* of which the 500 bp repeat is more abundant. This 500 bp repeat is very likely the same as the 400 bp *T. deion* repeat obtained by PCR. The difference between both repeats could be due to the fact that the PCR amplifies only part of the repeat unit. The non-amplified part of the 500 bp repeat (approximately 100 bp) seems to be larger than the non-amplified region of the 385 bp repeat (71 bp). Since in *T. kaykai* only one *Eco*RI repeat is obtained with a 385 bp monomer, it seems likely that the *T. deion Eco*RI repeat in *T. deion* corresponds to the 500 bp repeat and the 385 bp repeat in *T. deion* to the *T. kaykai Eco*RI repeat.

In search for other large tandem repeats on the *T. kaykai* chromosomes, we isolated C_0t DNA and used it as probe in FISH to visualize all high-copy sequences, including rDNAs, telomeres and other unknown tandem repeats (Peterson et al. 2002; Chang 2004). FISH of metaphase chromosomes with the C_0t-50 probe showed only few major satellite repeats in the genome of *Trichogramma* and the PSR chromosome. The 45S rDNA is most common and is located on the PSR chromosome and the short arms of chromosome 1 and 4. No telomere signals were detected. The only other large tandem repeat is situated on chromosome 2 and may correspond to the 5S rDNA satellite repeat (unpublished results). The *Eco*RI repeat signal on the long arm of chromosome 4 is very weak, while no C_0t repeat signal was observed on the position of the *Eco*RI repeat on chromosome 1. The absence of many large satellite repeats and the rather homogenous DAPI fluorescence along the chromosomes suggest that the genome consists mostly of dispersed repeats. Future FISH studies with C_0t-100 or C_0t-200 fractions may reveal the positions of smaller tandem repeats as well as various classes of abundant dispersed repeats.

45S rDNA transcription on PSR chromosome

Both reverse transcriptase PCR and silver staining showed that the 45S rDNA on the PSR chromosome is transcribed. However, in some studies silver staining was considered a disputable method of finding active NORs. Although a vast number of papers on silver staining of active NORs may suggest its ubiquitous application for transcribed rDNA regions, others claim that not all of the signals correspond to 45S rDNA (Dobigny et al. 2002). We know, however, that large part of the PSR chromosome contains 45S rDNA (Chapter 4) and we have therefore no reason to doubt the interpretation of our silver staining results. The reverse transcriptase PCR with PSR chromosome specific ITS2 primers shows that not all ITS2 sequences on the PSR chromosome are transcribed, indicating this NOR is only partly active. *T. kaykai* ITS2 on the PSR chromosome is transcribed with a similar intensity as the ITS2 on the *T. kaykai* and *T. deion* genomes, while PT3 is transcribed at a very low level and PT1, PT2 and PT2-kk are not transcribed at all. In *Nicotiana tabacum* interlocus homogenization of rDNA only occurs when the rDNA is transcribed during interphase (Lim et al. 2000). They hypothesize that active rDNA decondenses, is undermethylated and is associated with the nucleolus, so a homogenization mechanism like gene conversion can easily occur, while this can not in inactive rDNA. Our results fit with this hypothesis. The transcribed *T. kaykai* ITS2 on the PSR chromosome is maintained by interlocus homogenization with the ITS2 on the *T. kaykai* genome, while the presence of multiple inactive ITS2 sequences on the PSR chromosome demonstrates the absence of homogenization among these sequences. However, the discovery of many identical copies of the inactive PT1 ITS2 (Chapter 4) suggests that homogenization of this ITS2 sequences still occurs. If no such homogenization would occur we would expect many differences among the copies of PT1. Alternatively, the appearance of many identical PT1 copies on the PSR chromosome may also result from a recent loss of PT1 homogenization, for example because of a recent transfer of the PSR chromosome to *T. kaykai*. Prolonged maintenance of the PSR chromosome in *T. deion* and observation of its ITS2 sequences might give us some idea about the process and speed of concerted evolution in this chromosome.

Conclusions

45S rDNA is the only large tandem repetitive sequence on the PSR chromosome in *T. kaykai*. This repeat covers about two thirds of this chromosome and is partial transcribed. The part of the PSR chromosome without 45S rDNA probably consists of unique and low repetitive DNA. Unlike *Trichogramma*, the *Nasonia* PSR chromosome has B chromosome specific repeat families, i.e. PSR2, PSR18 and PSR22 (Eickbush et al. 1992), but does not seem to have B chromosome specific ITS2 sequences and we are not even certain whether it contains 45S rDNA. The repetitive sequences on the *Nasonia* PSR chromosome have proved to be an important factor for the transmission efficiency of this chromosome and they may also correspond to its size (Beukeboom et al. 1992; Reed et al. 1994). Reduction of the repetitive sequences caused the *Nasonia* PSR chromosome to be mitotically unstable (Beukeboom et al. 1992). We think that the

large 45S rDNA repeat on the *Trichogramma* PSR chromosome serves a similar function as the B chromosome specific repeats on the *Nasonia* PSR and is important for its size and transmission efficiency in *T. kaykai*, though future studies, for example on deletion mapping of the *Trichogramma* B chromosome, should confirm these hypotheses.

Acknowledgements

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Chapter 6

AFLP analysis of the paternal sex ratio chromosome in the parasitoid wasp *Trichogramma kaykai*

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Abstract

The parasitoid wasp *Trichogramma kaykai* has a B chromosome conferring paternal genome loss during early embryogenesis, resulting in male offspring and hence a paternal sex ratio in this wasp. However, the molecular mode of action of this paternal sex ratio (PSR) chromosome is unknown. Here we show the results of an AFLP analysis on genomic *Trichogramma* DNA with and without PSR chromosome to obtain B chromosome specific sequences. These sequences will reveal more of the B chromosome content and can possibly lead us to genes or sequences involved in the mechanism of paternal genome loss. Based on their length and appearance fourteen PSR chromosome specific bands were analysed of which seven were successfully sequenced. One of them was identified as part of the reverse transcriptase gene of a non-LTR retrotransposon and one other resembled part of the *gag* sequence of a LTR retrotransposon. Internal primers designed from a third AFLP fragment allow PCR amplification of a PSR chromosome specific marker, which can be used to screen for the PSR trait in male wasps. However, this sequence was lost in nine out of ten PSR wasp lines reared on *T. kaykai* line LC19-1, while the PSR line reared on *T. deion* and one PSR line reared on *T. kaykai* LC19-1 still had this sequence. Apparently, line LC19-1 is involved in the loss of PT-AFLP 2 from the PSR chromosome. Southern analysis of this fragment revealed it is dispersed repetitively present in the *T. kaykai* genome and the PSR chromosome, which suggests the PT-AFLP 2 sequence is also part of a retrotransposon.

Keywords

Trichogramma kaykai, PSR chromosome, AFLP, retrotransposons

Introduction

A B chromosome present in some males of the arrhenotokous parasitoid *Trichogramma kaykai* initiates the loss of all paternal chromosomes, except itself, during the first mitotic division in eggs fertilized with sperm containing this B chromosome (Stouthamer et al. 2001; Van Vugt et al. 2003). Such eggs then develop into haploid males carrying this paternal sex ratio (PSR) chromosome. Another PSR chromosome with a similar mode of action is described for the parasitoid wasp *Nasonia vitripennis* (Nur et al. 1988). The still enigmatic mechanism of PSR chromosomes includes the condensation of the paternal genome into a chromatin mass while the B chromosomes remain unaffected. Most likely they somehow disrupt the paternal pronucleus formation in the zygote or affect the paternal genome processing during spermatogenesis making it subject to specific degradation (Werren 1991; Beukeboom & Werren 1993; Van Vugt et al. 2003). It can either do so by transcribing one or more genes or by acting as a sink for signal elements necessary for the proper processing of the paternal genome. The 'sink' theory suggests repetitive sequences on the PSR chromosome are responsible for the improper processing of the male genome (Eickbush et al. 1992; Beukeboom & Werren 1993). Although a previous study has shown that two thirds of the *Trichogramma* PSR chromosome is covered by a long tandem array of 45S ribosomal DNA (Chapter 4), it is highly unlikely that these sequences are involved in the PSR mechanism. In addition, we have no direct indication that 45S rDNA is present on the PSR chromosome of *Nasonia* (Chapter 4). No other repeats were found on the *Trichogramma* PSR chromosome to support the 'sink' theory (Chapter 5).

It seems plausible that the mode of action of the PSR chromosome is the consequence of B chromosome specific DNA sequences, either genes or repetitive sequences. Screening of a *Nasonia* genomic library with PSR, revealed 36 out of 6000 clones hybridized to PSR male DNA and not to control male DNA (Nur et al. 1988). In this way several PSR specific repeats with palindromic sequences were discovered, though no direct evidence exists that these repeats are involved in the PSR trait (Eickbush et al. 1992; Beukeboom & Werren 1993). Beside generating B specific sublibraries from complete genomic libraries other techniques to obtain B chromosome specific sequences involve subtraction libraries (Jamilena et al. 1994), microdissection followed by DOP-PCR (Jamilena et al. 1995; Houben et al. 1996; Sharbel et al. 1998; Cheng & Lin 2003), RAPD (Qi et al. 2002a), AFLP (Qi et al. 2002b; Ziegler et al. 2003; Peng et al. 2005) and flow sorting (Kubalakova et al. 2003), all in combination with PCR, Southern blotting or FISH to confirm the B specificity of the acquired sequences.

Here we aim at obtaining *Trichogramma* PSR chromosome specific sequences based on differential AFLP fingerprinting of genomic DNA with and without PSR. The PSR chromosome specific fragments obtained in this way were analysed for genes or sequences possibly involved in the mode of action of the PSR chromosome. PSR chromosome specific markers are also useful to easily determine whether a male wasp contains the PSR trait, which up to now was performed in laborious crossing experiments.

Material and Methods

Trichogramma wasps

The *T. kaykai* line LC19-1 and *T. deion* line SW436-1 originate from the Mojave Desert, California, and were initiated by a single *Wolbachia* infected female from Last Chance Canyon, Kern County, CA in 1995 and Sidewinder Mountains, San Bernardino County, CA, in 1996, respectively. The PSR chromosomes were collected from different locations in the Mojave Desert (Table 1). The wasps were maintained in 150 x 16 mm glass tubes in a rearing chamber at 23°C ± 1°C with a light-dark period of 18 and 6 hours. Freshly emerged wasps were provided with egg-sheets containing irradiated *Ephestia kuehniella* eggs (supplied by Koppert bv, Barrel & Rodenrijs, The Netherlands) and a small amount of honey.

Table 1. PSR chromosome containing *Trichogramma* lines are listed with the year and location of their collection in the Mojave Desert in California and the isofemale line that was used to maintain the PSR line in.

Name of PSR line	Year	Location	Isofemale line
B44	1996	Barstow-Daggett, San Bernardino Co.	*T. kaykai* (since 1996) *T. deion* (since 2004)
P1A	1997	Danby, San Bernardino Co.	*T. kaykai* (since 1997) *T. deion* (since 2001)
P21E3	1997	Last Chance Canyon, Kern Co.	*T. kaykai*
P33C3	1997	Last Chance Canyon, Kern Co.	*T. kaykai* (since 1997) *T. deion* (since 2004)
P37B1	1997	Yucca Valley, San Bernardino Co.	*T. kaykai*
P38B2	1997	Palomar Road, San Bernardino Co.	*T. kaykai* (since 1997) *T. deion* (since 2004)
IB15	1998	Ibex Hills, San Bernardino Co.	*T. kaykai*
PN243	1998	Pannamint Valley, Kern Co.	*T. kaykai*
PN260	1998	Pannamint Valley, Kern Co.	*T. deion*
RR32A	1998	Rasor Road, San Bernardino Co.	*T. kaykai* (since 1998) *T. deion* (since 2004)
SV21A	1998	Silurian Valley, San Bernardino Co.	*T. kaykai*

AFLP fingerprinting

Genomic DNA was isolated from about 500 *T. kaykai* wasps with and without PSR chromosome from line P1A as described in chapter 5 of this thesis. An AFLP fingerprint using several primer combinations was performed to identify fragment differences between DNA with and without PSR chromosome from line P1A. AFLP analysis was essentially performed as described by (Vos et al. 1995). Restriction and ligation were performed simultaneously using 250 ng genomic DNA and *Eco*RI/*Mse*I or *Pst*I/*Mse*I restriction enzymes and adapters. The *Eco*RI and *Mse*I adapters are described in Vos et al. (1995). The structure of the *Pst*I adapter is:

5′-CTC GTA GAC TGC GTA CAT GCA-3′
3′-CAT CTG ACG CAT GT-5′

5 µl of 10 times diluted restriction-ligation products were used in the pre-amplifications with the E01/M02 and P00/M02 primers (Table 2). Selective amplifications were performed using 5 µl 50 times diluted pre-amplification products and *Mse*I+2 primers M15 to M18 either in combination with IRDyeTM-700 labelled *Eco*RI+3 primers E32 and E34 to E40 or IRDyeTM-700 labelled *Pst*I+2 primers P12 to P14, P16, P18, P20, P22 and P26 (Table 2). Amplification products were analysed on a LI-COR IR2 automated sequencer (Westburg) according to the manufacturer's protocol. Approximately 0.5 µl of each denatured amplification product containing loading dye mixture was loaded onto a 5.5% denaturing polyacrylamide gel (Ready-to-use Gel Matrix, KBplus, Westburg) and separated for 2.5 hours at 40 W, 1500 V, 25 mA and 55°C.

Table 2. Primers used in AFLP experiment with the extra selective nucleotides added to the 3′ end of the adapter primer. (*) is the *Eco*RI adapter primer (Vos et al. 1995). (#) is the *Pst*I adapter primer 5′-GACTGCG-TACATGCAG-3′. (+) is the *Mse*I adapter primer (Vos et al. 1995).

***Eco*RI primers**		***Pst*I primers**		***Mse*I primers**	
Primer name	**Selective nucleotides**	**Primer name**	**Selective nucleotides**	**Primer name**	**Selective nucleotides**
E01	*A	P00	#	M02	+C
E32	*AAC	P12	#AC	M15	+CA
E34	*AAT	P13	#AG	M16	+CC
E35	*ACA	P14	#AT	M17	+CG
E36	*ACC	P16	#CC	M18	+CT
E37	*ACG	P18	#CT		
E38	*ACT	P20	#GC		
E39	*AGA	P22	#GT		
E40	*AGC	P26	#TT		

Isolation and sequencing of AFLP fragments

AFLP fragments selected for sequencing were amplified with specific *Mse*I primers that we designed by determining the third, fourth and fifth selective nucleotide following the *Mse*I restriction site according to the protocol described by Brugmans et al. (2003), with the following modifications. We first performed a non-labelled selective pre-amplification on 5 µl 50 times diluted pre-amplified DNA with and without PSR from the AFLP experiment described above using the eight *Pst*I/*Mse*I and *Eco*RI/*Mse*I primer combinations mentioned in the first column of Table 3. 10 µl of the selective pre-amplification product was examined on a 1% agarose gel. Thereafter, a selective amplification was performed using the *Mse*I+3 and the degenerate *Mse*I+4 and +5 primers shown in Table 4 and IRDyeTM-700 labelled *Pst*I+2 or *Eco*RI+3 primers on 3 µl 100 times diluted selective pre-amplified DNA. Amplified products were analysed on a LI-COR IR2 automated sequencer (Westburg) as described above.

Table 3. Designing of specific *Mse*I+5 primers for 14 PSR chromosome specific AFLP fragments. (+) is the *Mse*I adapter primer (Vos et al. 1995).

Primers	Fragment size (bp)	*Mse*I+3 primers	Designed *Mse*I+5 primers
P14/M17	190	M55 to M58	+CG CCC
E32/M18	150	M59 to M62	+CT ATA
E32/M18	450	M59 to M62	+CT TGA +CT TGC
E34/M17	170	M55 to M58	+CG GTG
E35/M18	90	M59 to M62	+CT AGA +CT AGC
E35/M18	120	M59 to M62	+CT AGT
E35/M18	170	M59 to M62	+CT AGT
E36/M17	150	M55 to M58	+CG GCT
E37/M15	410	M47 to M50	+CT ATG
E37/M18	109	M59 to M62	+CT TTG
E37/M18	110	M59 to M62	+CT GGC
E37/M18	150	M59 to M62	+CT GCA
E38/M18	90	M59 to M62	+CT TGC
E38/M18	130	M59 to M62	+CT GAA

Table 4. Primers used to reveal the third, fourth and fifth nucleotide following the *Mse*I restriction site of the PSR chromosome specific fragments. (+) is the *Mse*I adapter primer (Vos et al. 1995).

*Mse*I+3 primers		*Mse*I+4 & *Mse*I+5 primers	
Primer name	Selective nucleotides	Primer name	Selective nucleotides
M47	+CAA	M+3A	+NNNA
M48	+CAC	M+3C	+NNNC
M49	+CAG	M+3G	+NNNG
M50	+CAT	M+3T	+NNNT
M55	+CGA	M+4A	+NNNNA
M56	+CGC	M+4C	+NNNNC
M57	+CGG	M+4G	+NNNNG
M58	+CGT	M+4T	+NNNNT
M59	+CTA		
M60	+CTC		
M61	+CTG		
M62	+CTT		

The resulting selective *Mse*I+5 primers for each of the 14 AFLP fragments that were to be sequenced (Table 3) were used in combination with IRDyeTM-700 labelled *Eco*RI+3 or *Pst*I+2

primers and 3 µl 50 times diluted selective pre-amplified DNA in an AFLP experiment. After separation (as described above), the desired fragments were isolated from polyacrylamide gel (Brugmans et al. 2003) and sequenced directly (BaseClear, Leiden, The Netherlands) using the same primers as used for the amplification of the products. BLASTN and BLASTX search (NCBI) was executed on all AFLP sequences with expect value 10, word size 11 and a low complexity filter.

Internal primer gradient PCR on AFLP fragments

Internal primers were designed for the sequenced AFLP fragments, which were subsequently tested at different annealing temperatures in a gradient PCR on genomic *T. kaykai* and *T. deion* DNA with and without PSR chromosome from line P1A. The gradient PCR was performed on 5 ng genomic DNA with 2.5 µl PCR buffer, 0.5 µl dNTP mix (10 mM each), 0.5 µl forward and reverse primer (25 pmol/µl) and 0.07 µl SuperTaq polymerase (Enzyme Technologies Ltd.) with a total reaction volume of 25 µl with cycle program: 4 min 94°C, 40 cycles [1 min 94°C; 1 min 50°C ± 10°C; 1 min 72°C], 5 min 72°C. From each PCR product 10 µl was run on a 1.5 % agarose gel. PCR products were extracted from agarose with the QIAEX II gel extraction kit (QIAGEN). Subsequently, the purified DNA was cloned into the pGEM-T vector (Invitrogen) and transformed into XL-2 Blue cells (Stratagene). The plasmid insert was sequenced using standard plasmid primers. Aligning of the obtained sequences was done in Seqman (DNASTAR Inc.).

DNA isolation and PCR amplification of AFLP fragments on individual wasps

Individual *Trichogramma* wasps were frozen at –80°C and ground with a sterile glass rod in a 0.5 ml tube. Directly, 50 µl 5% Chelex 100 (Bio-Rad Laboratories) and 2 µl Proteinase K (20 mg/ml) were added and the tube was incubated at 56°C for at least 6 hours followed by 10 minutes at 95°C. After centrifugation 1 µl DNA extract was used in a PCR as described above with cycle program: 4 min 94°C, 40 cycles [1 min 94°C; 1 min 62°C; 1 min 72°C], 5 min 72°C. PCR products were visualized on a 1.5% agarose gel and extracted from agarose, cloned and sequenced as described above.

Nested anchor PCR

To acquire the DNA sequences flanking the seven sequenced AFLP fragments, anchor PCR was performed using the Genome Walker Kit (Clontech) based on the method of Siebert et al. (1995) and Brugmans et al. (2003). Internal nested primers were designed for all seven AFLP fragments (Brugmans et al. 2003). We used *Dra*I, *Eco*RV, *Hinc*II, *Hpa*I and *Sma*I restriction enzymes individually for restriction and ligation of the Genome Walker adapters on genomic DNA with and without PSR chromosome from line P1A. Subsequently, PCR was performed on 2 µl undiluted restriction-ligation product using 30 ng of the first internal primer, 30 ng of the first Genome Walker adapter primer, 0.4 µl SuperTaq polymerase (Enzyme Technologies Ltd.), 0.2 mM dNTP and 1x PCR buffer in a total volume of 20 µl with a touchdown cycle

program: 4 min 94°C, 12 cycles [30 sec 94°C; 30 sec 65-56°C; 2.5 min 72°C], 28 cycles [30 sec 94°C; 30 sec 56°C; 2.5 min 72°C], 5 min 72°C. On 5 µl 100 times diluted amplification product of the first PCR a nested PCR was performed with the second internal primer and the second adapter primer using the same material and conditions as described for the first PCR. From this second PCR 10 µl of each product was run on 1.5 % agarose gel. Amplified products were extracted from agarose, cloned and sequenced as described above.

Southern blotting and hybridization

We performed the southern blot and hybridization as described in chapter 5 of this thesis with the following modifications: 1) Genomic DNA was digested with *Eco*RI or *Eco*RI and *Mse*I, 10 U each; 2) As positive control we used 10 pg unlabelled 128 bp PT-AFLP2 PCR product; 3) The (pre- and post-)hybridization temperature was 60°C; 4) We used 300 ng digoxigenine-labelled 128 bp PT-AFLP2 PCR product as probe; 5) The filter was exposed for 2 hours to an XAR-5 film (Amersham Biosciences), developed and scanned.

Results

PSR chromosome specific AFLP fragments

The DNA fragments of 32 *Eco*RI+3/*Mse*I+2 selective primer combinations and 32 *Pst*I+2/*Mse*I+2 selective primer combinations were assessed (Table 2). Each primer combination yielded approximately 50-80 fragments of 40-500 bp, resulting in around 4200 fragments. The *Pst*I+2/*Mse*I+2 and *Eco*RI+3/*Mse*I+2 primer combinations produced ten and twenty-two fragments unique to DNA with PSR, respectively (Figure 1). We selected fourteen clearly visible PSR chromosome specific DNA fragments from eight primer combinations for AFLP mediated mini-sequencing (Table 3). To avoid co-isolation of background amplification products, more specific *Mse*I primers were designed for each of the fourteen AFLP fragments by determining the third, fourth and fifth selective nucleotide following the *Mse*I restriction site (Table 3 and 4). An extra nucleotide at the 3′ end of one of the primers reduces the background amplification product with ¾ at equal GCAT ratio. Three extra nucleotides on either 3′ end of the primers therefore reduce the background product with 63/64 at equal GCAT ratio. The developed fragment specific *Mse*I+5 primers were used in an AFLP fingerprint together with the fluorescently labelled *Eco*RI+3 or *Pst*I+2 primers. Out of fourteen PSR chromosome specific AFLP fragments for which we designed a *Mse*I+5 primer, seven AFLP fragments were sequenced successfully by this method and named PT-AFLP 1 to PT-AFLP 7 (Table 5). 'PT' in PT-AFLP stands for 'PSR *Trichogramma*'. The other seven AFLP fragments were not amplified by the specific *Mse*I+5 primers in combination with the *Eco*RI+3 or *Pst*I+2 primers. The size of the seven successfully sequenced fragments varied between 77 and 147 bp. BLASTN and BLASTX search with all seven sequences in the GenBank database of NCBI revealed no DNA or amino acid sequence homology with any sequence in the database.

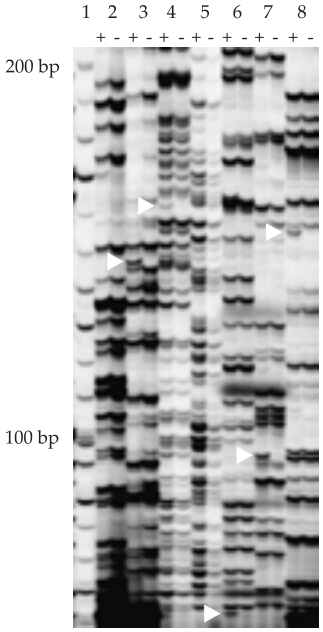

Figure 1: Part of an *Eco*RI+3/*Mse*I+2 AFLP gel. Lane 1 is a 10 bp marker. Lanes 2 to 8 each have two lanes, (+) and (-). The (+) lane is from template with PSR chromosome. The (-) lane is from template without PSR chromosome. Lanes 2) E32/M17 primer set; Lanes 3) E32/M18 primer set; Lanes 4) E34/M17 primer set; Lanes 5) E34/M18 primer set; Lanes 6) E35/M17 primer set; Lanes 7) E35/M18 primer set; Lanes 8) E36/M17 primer set. White arrow heads indicate AFLP bands in (+) lanes that are absent in (-) lanes.

Table 5. Summery of the isolated and sequenced AFLP fragments. (+) is the *Mse*I adapter primer (Vos et al. 1995). (*) is the *Eco*RI adapter primer (Vos et al. 1995).

AFLP fragment	*Mse*I & *Eco*RI primers	Fragment size	Accession number
PT-AFLP 1	+CTATA *AAC	123 bp	AY927999
PT-AFLP 2	+CGGTG *AAT	138 bp	AY928001
PT-AFLP 3	+CTAGT *ACA	147 bp	AY928003
PT-AFLP 4	+CGGCT *ACC	131 bp	AY928005
PT-AFLP 5	+CTTTG *ACG	87 bp	AY928006
PT-AFLP 6	+CTGGC *ACG	88 bp	AY928009
PT-AFLP 7	+CTTGC *ACT	77 bp	AY927998

PSR specificity of the AFLP fragments

We designed internal primers for each of the sequenced AFLP fragments to establish whether the PSR specificity of the AFLP fragments depends merely on the flanking restriction sites and/or the two to three adjacent nucleotides or whether the complete sequence of the AFLP fragments is unique to the PSR chromosome. These primers were subsequently tested in a temperature gradient PCR on DNA of individual wasps with and without PSR chromosome from the P1A line. Except for the PT-AFLP 2 fragment, all the internal primer pairs amplified PCR product of the expected length on DNA with and without PSR. A PSR specific PCR pattern was observed for the PT-AFLP 2 fragment with primers 5'-ATGACAATTCCGAATATG-TAACC-3' and 5'-ACGGTGATACGTAGCGAGAA-3' (Figure 2). These primers were tested on at least 25 wasps with and 25 wasps without PSR chromosome of line P1A. The 128 bp band of PT-AFLP 2 is only observed on template DNA with PSR, though a 260 bp product is detected on *T. kaykai* DNA without PSR.

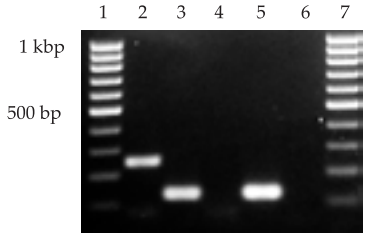

Figure 2: PCR products obtained with PT-AFLP 2 primers on *T. kaykai* and *T. deion* DNA, both with and without PSR chromosome from line P1A: Lane 1 and 7) 100 bp ladder; Lane 2) *T. kaykai*; Lane 3) *T. kaykai* with PSR; Lane 4) *T. deion*; Lane 5) *T. deion* with PSR; Lane 6) negative control with only water.

The 260 and 128 bp products were isolated from agarose gel and sequenced. Alignment of both sequences revealed that the approximate 260 bp product counts 257 bp and resembles the 128 bp PT-AFLP 2 sequence, except for a 129 bp insertion between bp 99 and 100 of the 128 bp sequence (Figure 3). This 129 bp insertion does not resemble PT-AFLP 2. BLASTN and BLASTX search with the 257 bp PT-AFLP 2 sequence in the GenBank database revealed no DNA or amino acid sequence homology with any sequence in the database.

```
                                                      50                                                  100
PT-AFLP 2 kk ATGACAATTCCGAATATGTAACCGAAATTATCCTAGGACTTCTAAAACGAAAAGTAGTTTTGACGCCTATCACAAAGCTTCCTCTATGAGGAAGCAATAT
PT-AFLP 2    ....................................................................................................A.-
                                                     150                                                  200
PT-AFLP 2 kk AAGAATTGCTTGATATATACAATACAAAAAAGTGAAAAAAAATTTTTTCAAAAAAAAAAATTTAACAACTTGAAAATAAAGCATAAGTTTTTAACGCTTC
PT-AFLP 2    ----------------------------------------------------------------------------------------------------
                                                     250
PT-AFLP 2 kk TTACGACACACAAATCCTTCAAAACAAGCCGAACTACTTCTCGCTACGTATCACCGT
PT-AFLP 2    ----------------------------..A...A.....................
```

Figure 3: Alignment of the 128 bp and 257 bp PT-AFLP 2 sequences. Identities are denoted by dots. A dash denotes a gapped position. PT-AFLP 2 KK is the 257 bp PCR product from *T. kaykai* DNA without PSR. PT-AFLP 2 is the 128 bp PCR product from *T. kaykai* and *T. deion* DNA with PSR chromosome from line P1A.

The PSR chromosome specificity of the 128 bp PT-AFLP2 sequence was further examined by performing PCR with the PT-AFLP 2 primers on individual wasps from fifteen wasp lines with PSR chromosomes obtained from nine different locations in the Mojave Desert (Figure 4 and Table 1). For each line at least ten individual wasps were assessed. All lines except P1A from Danby and PN260 from Pannamint Valley show the 257 bp PT-AFLP 2 sequence, suggesting that either the PSR chromosomes of these lines have the 257 bp sequence, or the 128 bp PT-AFLP 2 sequence is absent from the PSR chromosomes of these lines and the 257 bp products originate from the *T. kaykai* genome. The latter is confirmed by the result from the *T. deion* lines with PSR P33C3, RR32A and B44, where no PCR product is observed, indicating the absence of the 128 bp PT-AFLP 2 sequence from the PSR chromosomes in these lines (Figure 4).

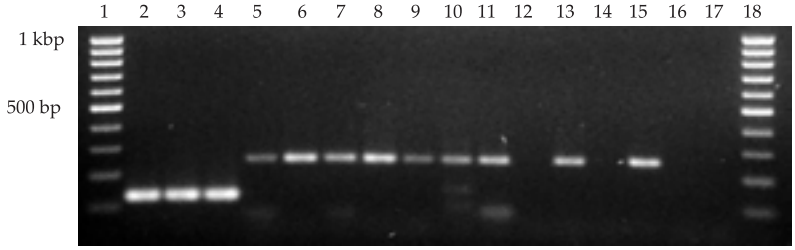

Figure 4: PCR products obtained with PT-AFLP 2 primers on 15 wasp lines with PSR chromosomes collected from 9 different locations in the Mojave Desert in California: Lane 1 and 18) 100 bp ladder; Lane 2) P1A *T. kaykai*; Lane 3) P1A *T. deion*; Lane 4) PN260 *T. deion*; Lane 5) PN243 *T. kaykai*; Lane 6) IB15 *T. kaykai*; Lane 7) SV21A *T. kaykai*; Lane 8) P21E3 *T. kaykai*; Lane 9) P37B1 *T. kaykai*; Lane 10) P38B2 *T. kaykai*; Lane 11) P33C3 *T. kaykai*; Lane 12) P33C3 *T. deion*; Lane 13) RR32A *T. kaykai*; Lane 14) RR32A *T. deion*; Lane 15) B44 *T. kaykai*; Lane 16) B44 *T. deion*; Lane 17) negative control with only water.

Nonetheless, PCR on wasps collected in the Mojave Desert and preserved in 90% ethanol showed that PSR chromosomes from Pannamint Valley (PN260 and PN337 from 1998, PY124 from 2000), Ibex Hills (IB172 from 1998), Last Chance Canyon (LY39 from 2000) and Rasor Road (RR32A from 1998) had the 128 bp product, demonstrating that this sequence was present on the PSR chromosomes when collected from the Mojave Desert and was lost from the PSR chromosomes in the *T. kaykai* LC19-1 wasp lines reared in the lab (Figure 5). The presence of the 128 bp PT-AFLP 2 product in ethanol-preserved wasps PN260 and RR32A and in line PN260 reared on *T. deion* and the absence of this sequence in line RR32A reared first on *T. kaykai* LC19-1 and later on *T. deion* clearly shows that the 128 bp PT-AFLP 2 sequence was lost during the rearing on *T. kaykai* LC19-1.

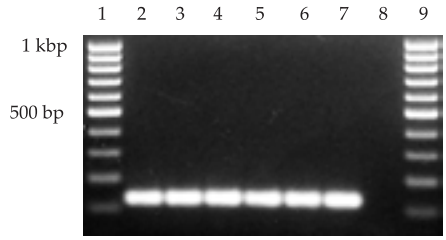

Figure 5: PCR products obtained with PT-AFLP 2 primers from alcohol preserved wasps with PSR chromosomes collected from 4 different locations in the Mojave Desert in California: Lane 1 and 9) 100 bp ladder; Lane 2) PN260 from 1998; Lane 3) PN337 from 1998; Lane 4) PY124 from 2000; Lane 5) IB172 from 1998; Lane 6) LY39 from 2000; Lane 7) RR32A from 1998; Lane 8) negative control with only water.

Nature of the AFLP fragments

The fragments PT-AFLP 1 to 7 were too short to properly analyse their sequence. We therefore tried to obtain the sequences adjacent to these AFLP fragments by nested anchor PCR (Siebert et al. 1995; Brugmans et al. 2003). We designed internal nested primers for each AFLP fragment and used these primers with adapter primers in a nested PCR on genomic DNA with and without PSR of the P1A line digested individually with five restriction enzymes and ligated with adapters (Figure 6). DNA fragments from the second PCR present in PSR+ DNA and absent in PSR- DNA were sequenced. We acquired sequences flanking the *Eco*RI side of PT-AFLP 1, PT-AFLP 2, PT-AFLP 3 and on both sides of PT-AFLP 5 (Table 6).

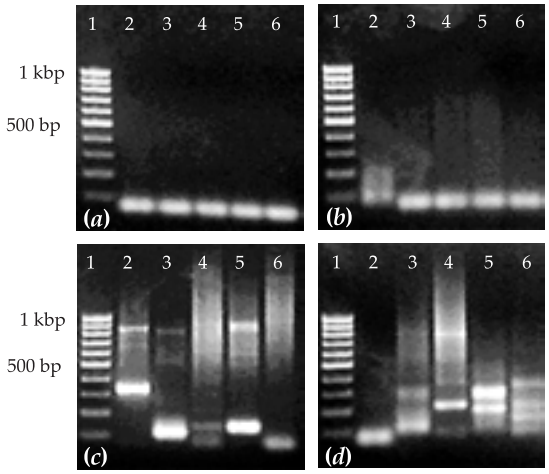

Figure 6: PCR products obtained from nested anchor PCR with internal nested PT-AFLP 1 primers to obtain the sequence upstream of the PT-AFLP 1 fragment: (a) First PCR on *T. kaykai* DNA with P1A PSR; (b) First PCR on *T. kaykai* DNA without PSR; (c) Second PCR on *T. kaykai* DNA with P1A PSR; (d) Second PCR on *T. kaykai* DNA without PSR: Lane 1) 100 bp ladder; Lane 2) template DNA digested with *Dra*I; Lane 3) template DNA digested with *Eco*RV; Lane 4) template DNA digested with *Hinc*II; Lane 5) template DNA digested with *Hpa*I; Lane 6) template DNA digested with *Sma*I.

Table 6. Sequence elongation of 4 AFLP fragments with internal nested primers. Both the first and second internal primer are shown. The bp number in the sequence elongation column excludes the bp number of the original PT-AFLP sequence.

AFLP fragment	Nested primers	Sequence elongation	Accession number
PT-AFLP 1	1st: 5′-TATCTGTAGAACGCGGTCGTTG-3′ 2nd: 5′-CGTTGACTAAAGTGCATTATGC-3	*Eco*RI side 203 bp	AY928000
PT-AFLP 2	1st: 5′-ACGGTGATACGTAGCGAGAAG-3′ 2nd: 5′-TAGAGGAAGCTTTGTGATAGGC-3	*Eco*RI side 235 bp	AY928002
PT-AFLP 3	1st: 5′-TATAATGGAACGACACCTACAGC-3′ 2nd: 5′-ATTCAGACATCGGAAACAGCGG-3	*Eco*RI side 200 bp	AY928004
PT-AFLP 5	1st: 5′-TATAATGGAACGACACCTACAGC-3′ 2nd: 5′-ATTCAGACATCGGAAACAGCGG-3	*Eco*RI side 792	AY928008
PT-AFLP 5	1st: 5′-GAATTCACGTTTTCCTGTACCTT-3′ 2nd: 5′-GCCTGTGTGATGAGAGGATTCA-3	*Mse*I side 277 bp	AY928007

The 326 bp PT-AFLP 1 sequence is part of a reverse transcriptase gene from a non-LTR retrotransposon resembling the Juan family of the jockey clade of LINE retrotransposons (expect value e^{-11}) (accession number M95171, Mouchès et al. 1992). The 373 bp PT-AFLP 2 and the 1156 bp PT-AFLP 5 sequences showed no DNA or amino acid sequence homology with sequences in the GenBank database. In the sequence flanking PT-AFLP 2 a CA-microsatellite is located with 33 repeat units. The 347 bp PT-AFLP 3 sequence resembles part of the *gag* gene of the *mag* retrotransposon in the silkworm *Bombyx mori*, which is placed within the gypsy group of LTR retrotransposons (expect value $9e^{-26}$) (accession number X17219, Michaille et al. 1990; Garel et al. 1994).

PT-AFLP 2 was further examined by Southern blot analysis. A dispersed pattern of strong bands in a strongly smeared background is observed when *Eco*RI digested *T. kaykai* DNA with and without PSR was hybridized with the 128 bp PT-AFLP 2 fragment (Figure 7). No difference is observed between *T. kaykai* DNA with or without PSR of line P1A, which may be due to the abundant presence of PT-AFLP 2 or a PT-AFLP 2-like sequence in the *T. kaykai* genome and the fact that the PSR chromosome only contributes to 3.9% of the genome (Chapter 5). When hybridizing PT-AFLP 2 to *Eco*RI digested *T. deion* DNA with and without PSR of line P1A a clear distinct pattern is observed with a light smear on *T. deion* DNA without PSR and approximately 11 discrete signals in a lightly smeared background on *T. deion* DNA with PSR. Insufficient transfer of small DNA fragments to the nylon membrane may have caused the *Eco*RI and *Mse*I digested DNA to have a less clear banding pattern. The only difference between PSR+ and PSR- DNA digested with *Eco*RI and *Mse*I is the faint 128 bp band observed in *T. deion* DNA with PSR which is absent in *T. deion* DNA without PSR.

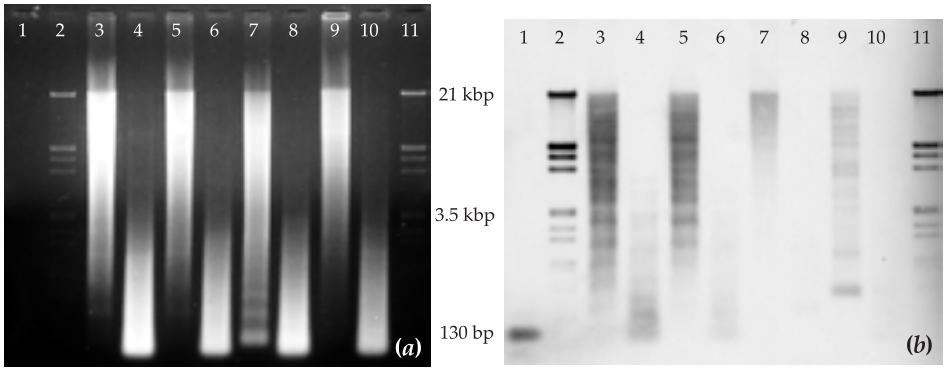

Figure 7: Agarose gel and Southern blot of the 128 bp PT-AFLP2 fragment: (a) Agarose gel with *Eco*RI or *Eco*RI and *Mse*I digested genomic DNA; (b) Southern blot of agarose gel hybridized with 300 ng digoxigenine-labelled PT-AFLP2; Lane 1) positive control: 100 ng PCR product of 128 bp PT-AFLP2; Lane 2 and 11) digoxigenine labelled DNA marker (Roche); Lane 3) *T. kaykai* DNA digested with *Eco*RI; Lane 4) *T. kaykai* DNA digested with *Eco*RI and *Mse*I; Lane 5) *T. kaykai* DNA with P1A PSR digested with *Eco*RI; Lane 6) *T. kaykai* DNA with P1A PSR digested with *Eco*RI and *Mse*I; Lane 7) *T. deion* DNA digested with *Eco*RI; Lane 8) *T. deion* DNA digested with *Eco*RI and *Mse*I; Lane 9) *T. deion* DNA with P1A PSR digested with *Eco*RI; Lane 10) *T. deion* DNA with P1A PSR digested with *Eco*RI and *Mse*I.

Discussion

PSR chromosome specific AFLP fragments

The AFLP fingerprinting technique produced approximately as many DNA fragments with the *Pst*I+2/*Mse*I+2 primer combinations as with the *Eco*RI+3/*Mse*I+2 primer combinations, even though the *Pst*I primers had only two selective nucleotides and the *Eco*RI primers had three selective nucleotides. Apparently, in the *T. kaykai* genome the restriction frequency of *Pst*I is less than that of *Eco*RI. In some plants the cytosine methylation percentage is more than 30% and *Pst*I can be methylation sensitive (Adams 1996; Vuylsteke et al. 1999). However, with only 0-3% of their cytosines methylated insect genomes are highly undermethylated compared to other organisms (Adams 1996; Field et al. 2004). The *Pst*I restriction sites in insects are therefore most likely not methylated and are normally digested. The difference between the restriction frequency of *Pst*I and *Eco*RI in *T. kaykai* is likely due to a difference in the occurrence frequency of both restriction sites in the *Trichogramma* genome. This again can not be caused by a difference in bp number that both enzymes recognize, because they both recognize a six bp sequence. Since the *Pst*I recognition sequence (CTGCAG) has a higher GC-content than the *Eco*RI sequence (GAATTC), the presence of more *Eco*RI than *Pst*I restriction sites in the *Trichogramma* genome can be explained if the genome is more AT-rich than GC-rich. Other insect genomes have a GC-content of less than 50%, for example *D. melanogaster* has a GC-content of 42.5% and *Anopheles gambiae* 44.7% (Jabbari & Bernardi 2004). *Trichogramma* probably also has a GC-ratio lower than 50%, which would explain the difference in restriction site number of

*Eco*RI and *Pst*I.

In order to maintain a PSR line, which contains many males, we added approximately every second generation wasps of the same line without PSR, which have only females, to the PSR line. The PSR chromosome is thus the only difference between both lines and we therefore assume that the extra DNA fragments in DNA with PSR arise from this extra chromosome. Only 32 (0.8%) out of 4200 DNA fragments are PSR specific. This is less than expected, knowing the PSR chromosome covers approximately 3.9% of the genome (Chapter 5) and assuming the sequence distribution on the PSR chromosome represents that of the complete genome. However, FISH results show that two third of the PSR chromosome consists of a large satellite repeat, the 45S rDNA (Chapter 4). Most of this repeat is identical to the 45S rDNA on the *T. kaykai* genome, which would explain why less than 3.9% of the DNA fragments in the AFLP fingerprint are PSR chromosome specific.

PSR specificity of the AFLP fragments

We performed a temperature gradient PCR with internal primers specific for each fragment to determine whether the complete sequence of the seven sequenced fragments is unique to the PSR chromosome or whether the PSR specificity of these fragments results from the flanking restriction sites or the two or three adjacent nucleotides. Only PT-AFLP 2 internal primers were successful in amplifying PSR specific PCR product of the expected 128 bp. The internal primers unique for the other six fragments amplified PCR product of the expected size on DNA with and without PSR, which implies the PSR specificity of these fragments is based on the flanking restriction sites or the adjacent two or three nucleotides and the overall sequences are present on both the *T. kaykai* genome and the PSR chromosome. The discovery of the 257 bp PCR product on *T. kaykai* DNA without PSR which is identical to the 128 bp PT-AFLP 2 sequence except for a 129 bp insert between bp 99 and 100 of the 128 bp sequence implies the PSR chromosome obtained this sequence from the *T. kaykai* genome and some time during its evolution deleted the 129 bp between bp 99 and 100. All male wasps carrying PSR collected from nine different locations in the Mojave Desert have the 128 bp PT-AFLP 2 sequence, while it is absent in nine out of ten PSR wasps lines reared on *T. kaykai* LC19-1 in the lab. Only the PSR line reared on *T. deion* from the moment it was collected in the desert, PN260, and one PSR line reared on *T. kaykai* LC19-1, P1A, still have the 128 bp sequence. Apparently, *T. kaykai* LC19-1 is involved in the loss of the 129 bp insert on the PSR chromosome. We can not explain, however, why the P1A PSR line reared on *T. kaykai* LC19-1 still has the 128 bp PT-AFLP 2 sequence.

The PT-AFLP 2 primers can successfully be used to determine whether a male wasp contains the PSR chromosome (Jeong 2004). Amplification of a 128 bp product demonstrates the wasp has the PSR chromosome, while a 257 bp product or no product proves the absence of the B chromosome. However, care should be taken with PSR lines reared on *T. kaykai* LC19-1. This line has proved to be able to remove the 128 bp sequence from the PSR chromosome, causing the 128 bp product to be absent while the PSR chromosome is still present. Future studies should focus on revealing what kind of sequence PT-AFLP 2 is and why this sequence can

be removed from the PSR chromosome in line LC19-1. Furthermore, beside PT-AFLP 2 other molecular markers are needed to confirm the presence or absence of the PSR chromosome in genomic DNA. For that purpose the primers specific for the ITS2 sequences on the PSR chromosome, PT1 and PT2, can be used (Chapter 4).

Nature of the AFLP fragments

The seven sequenced AFLP fragments were too small to result in any sequence homology with the sequences in the GenBank database of NCBI. We obtained flanking sequences of four of the seven fragments. Two of these fragments, PT-AFLP 1 and 3, are part of conserved regions in retrotransposons. The jockey clade of LINE retrotransposons that PT-AFLP 1 partly resembles is abundant in insects, like fruit flies, mosquitoes, silkworms and honey bees (Mouchès et al. 1992; Kimura et al. 1993). Also PT-AFLP 3 resembles part of an insect retrotransposon and is categorized within the gypsy group of LTR retrotransposons (Michaille et al. 1990; Garel et al. 1994). The presence of transposons on the PSR chromosome is not surprising, because it is suggested that B chromosomes accumulate mobile elements (Camacho et al. 2000). A clear example is the retrotransposon NATE on the PSR chromosome of *N. vitripennis* (McAllister 1995; McAllister & Werren 1997). Obtaining flanking sequences of PT-AFLP 5 did not result in any significant sequence homology with the sequences in the GenBank database. The largest putative open reading frame found in this sequence is 264 bp. In future studies southern analysis and FISH with PT-AFLP 5 and the other PT-AFLP fragments will reveal more about the nature of these fragments.

Southern analysis revealed the presence of PT-AFLP 2 or a PT-AFLP 2-like sequence on the *T. kaykai* genome and on the PSR chromosome. The dispersed repetitive nature of this sequence together with the absence of sequence homology with any known DNA or amino acid sequence suggests PT-AFLP 2 may be a non-conserved part of a retrotransposon. To be conclusive we should sequence more flanking region of PT-AFLP 2. The Southern patterns of AFLP sequences from other organisms, like those of the B chromosome in maize, are similar to the Southern pattern of PT-AFLP 2, because they also show a number of discrete bands with a smeared background (Peng et al. 2005). Analysis of maize B chromosome specific sequences obtained by AFLP and microdissection resulted in remarkably similar results, consistent with our findings: the B chromosome specific sequences are dispersed repetitive, possess a high degree of homology to the A chromosomes and have no similarity to any published gene other than fragments of retrotransposons (Cheng & Lin 2003; Peng et al. 2005). The PT1, PT2 and PT2-kk ITS2 sequences on the PSR chromosome were not revealed in our AFLP experiment, even though these sequences are repetitive and PSR-specific. This could be due to the fact we did not examine all PSR specific AFLP fragments. Furthermore, restriction of the PSR-specific ITS2 sequences with *Eco*RI, *Mse*I and *Pst*I may not result in an AFLP polymorphism with the sequences on the *T. kaykai* genome. The PSR chromosome specific ITS2 sequences PT-AFLP 2 and probably also the other PT-AFLP sequences can be helpful in future studies to obtain more unique sequences on the PSR chromosome, which can be done by screening a genomic

library of *T. kaykai* and/or *T. deion* with PSR using these sequences. PSR chromosome specific sequences obtained in this way may again be helpful to reveal the mode of action of the PSR chromosome, for example by discovering putative genes or by use of the PSR sublibrary in deletion mapping (Beukeboom & Werren 1993; McAllister et al. 2004).

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Chapter 7

General discussion

B chromosomes have fascinated scientists for many years, as is evident from the large number of studies on these special chromosomes (Jones 1995). What makes them so intriguing are their aberrant molecular organisation and skewed segregation behaviour compared to the A chromosomes. Most B chromosomes are considered parasitic and selfish, because they can lower the fitness of their host or host population and do not obey the Mendelian laws of inheritance with transmission rates exceeding those of normal chromosomes (Nur 1977; Jones & Rees 1982; Jones 1985; Jones 1991). They also follow their own evolutionary pathway, because they do not recombine with any of the A chromosomes, which has remarkable consequences for their content and appearance (Camacho et al. 2000). These characteristics make B chromosomes ideal candidates to study chromosome evolution.

In this thesis I focused on a special case of B chromosomes, the PSR chromosomes. These chromosomes induce, even for B chromosomes, an extreme effect on their hosts. Every generation they eliminate the complete genome that they were associated with and at the same time reach a transmission efficiency of nearly 100% in male wasps (Werren & Van den Assem 1986; Stouthamer et al. 2001; Jeong 2004). As a consequence DNA sequences from the PSR chromosome cannot become part of the host genome, though the host genome can provide the PSR chromosome with new genetic material. The PSR chromosome was first discovered in *Nasonia vitripennis* in which it has been studied extensively (Werren 1991; Werren & Stouthamer 2003). The discovery of a second PSR chromosome in the unrelated wasp *Trichogramma kaykai* provided an opportunity to compare the mode of action and content of both B chromosomes and examine the origin and evolution of these selfish chromosomes. In the previous chapters I described the mode of action, the structure and origin of the PSR chromosome in *T. kaykai*. Here I will summarize my results of the PSR system of *T. kaykai* and compare them with the findings of the *Nasonia* PSR chromosome. Finally, I will also discuss some possibilities for future research.

1 Mode of action of PSR

In chapter 3 I have described how the *Trichogramma* PSR chromosome eliminates one complete genome in eggs fertilized with PSR sperm, which is a process very similar to that of the *Nasonia* PSR chromosome (Reed & Werren 1995; Dobson & Tanouye 1996; Van Vugt et al. 2003). The first visible PSR effect is the incomplete decondensation of the paternal pronucleus, followed by condensation of the male genome into a dense chromatin mass at the beginning of the first mitosis in fertilized eggs (Reed & Werren 1995; Dobson & Tanouye 1996; Van Vugt et

al. 2003). Exactly how and when the PSR chromosome exerts its effect on the paternal pronucleus formation is still unknown. The PSR chromosome can affect the male DNA either during spermatogenesis or pronucleus formation by 1) imprinting the paternal A chromosomes (Werren 1991; Beukeboom & Werren 1993), for example by histone modification, such as the facultative heterochromatin in mealybugs (Bongiorni & Prantera 2003) or RNA coating, such as the X chromosome inactivation in humans (Brown et al. 1991; Clemson et al. 1996), 2) acting as 'sink' for products needed for the processing of the paternal chromosomes (Werren 1991; Beukeboom & Werren 1993), or 3) disrupting or delaying the signalling of normal male chromosome processing, such as the paternal genome loss by the cytoplasmic incompatibility (CI) inducing *Wolbachia* in *Nasonia* (Tram & Sullivan 2002). A *Drosophila* mutant with a cytological effect similar to the PSR chromosome is the maternal effect mutant *sésame* (*ssm*) (Loppin et al. 2000). This recessive mutant prevents the maternal histones from incorporating the male pronucleus, thereby causing an incomplete decondensation of the paternal pronucleus followed by compression of the nucleus into a chromatin mass prior to the first mitotic division (Loppin et al. 2001). Similarly, the PSR chromosome could prevent replacement of the male-specific histones for maternal histones in the paternal pronucleus. This could be done by: 1) preventing the male-specific histones to separate from the paternal genome, 2) preventing the maternal histones from entering the paternal pronucleus, or by 3) acting as a sink for the maternal histones. The CI *Wolbachia* bacterium in *N. vitripennis* demonstrates that paternal genome loss during first mitosis can also be the consequence of a delay in nuclear envelope breakdown of the male pronucleus (Tram & Sullivan 2002).

To reveal the molecular mechanism of PSR chromosomes, future studies should focus on examining the different aspects of the paternal pronucleus formation, i.e. nuclear envelope breakdown, histone replacement, decondensation process and DNA replication, to investigate in what phase of the pronucleus formation the PSR chromosome acts. In this way the possible products or even genes involved may be identified. The pronucleus formation can be examined by immunostaining newly fertilized eggs with antibodies against lamin (nuclear envelope protein), histones and DNA polymerase. Furthermore, differences in the degree of DNA methylation and histone methylation and acetylation may show whether the male DNA is delayed in chromosome (de)condensation (Goday & Ruiz 2002; Taverna et al. 2002), while differences in histone phosphorylation may show whether the male genome starts to degenerate prior to mitosis (Houben et al. 1999). In such immunolabelling experiments it is important to compare the effect of the *Nasonia* and *Trichogramma* PSR chromosome. Even though microscopic observations show both PSR chromosomes have a similar mechanism, the *sésame* mutant in *Drosophila* and the CI *Wolbachia* in *Nasonia* prove that a similar mode of paternal genome loss does not necessarily involve the same molecular mechanism. Understanding the PSR mechanism will provide insight in the normal mechanism of early embryogenesis and in particular the mechanism of paternal chromosome processing following fertilization.

The second major question on the mode of action of the PSR chromosome is when and how the PSR chromosome itself escapes from the specific modification and degradation of the paternal chromosomes. Our observations and those in *Nasonia* only revealed that it becomes

associated with the maternal chromosomes (Reed & Werren 1995; Dobson & Tanouye 1996; Van Vugt et al. 2003). This process most likely occurs just prior to or during the first mitotic metaphase, when both nuclei are physically close and their chromatin condensed. The PSR chromosome might avoid its destructive effect for example by imprinting only the paternal A chromosomes or by using a self-defence mechanism, such as no receptor sequences or proteins (Dobson & Tanouye 1996). To identify the position and movement of PSR chromosomes during embryo development *in situ* hybridization can be performed during the immunostaining experiments described above with labelled PSR-specific high-copy DNA sequences. The *Nasonia* PSR chromosome can be hybridized with the PSR specific repeat sequences, PSR2, PSR18 and PSR22 (Nur et al. 1988; Eickbush et al. 1992). The *Trichogramma* PSR chromosome can be labelled with 45S rDNA (Chapter 4). Even though this high-copy sequence is not only positioned on the PSR chromosome, but also on two of the A chromosomes, possible differences in immunolabelling between the three loci can be visualized.

2 PSR structure

The PSR chromosome in *Trichogramma* measures 3.9% of the haploid genome and counts approximately 9 Mbp (Chapter 5). In comparison, the *Nasonia* PSR chromosome is more than two times larger with 21 Mbp and accounts for 5.7% of the haploid genome (Rasch et al. 1975; Reed 1993). About two thirds of the *Trichogramma* PSR chromosome comprises a single satellite repeat, the 45S ribosomal DNA (Chapter 4). Neither the *Trichogramma*-specific *Eco*RI repeat nor any other large repeat was found on this chromosome (Chapter 5). The 45S rDNA repeat on this B chromosome has ITS2 sequences that originated from two related *Trichogramma* species and only the part that originates from its current host *T. kaykai* is transcribed while the other part, originating from another *Trichogramma* species, is inactive (Chapter 4 and 5). The presence of original *T. kaykai* ITS2 on the PSR chromosome suggests that this transcribed part has interlocus homogenization with the 45S rDNA on the A chromosomes of *T. kaykai* (Chapter 5). AFLP analysis revealed few additional sequences on the *Trichogramma* PSR chromosome, which were identified as parts of retrotransposons, one LTR and one non-LTR transposon (Chapter 6). Southern analysis of a third AFLP sequence, PT-AFLP 2, revealed a dispersed repetitive pattern both on the PSR chromosome and the *T. kaykai* genome, suggesting that this sequence is also a transposable element (Chapter 6). The *Nasonia* PSR chromosome also contains a LTR retrotransposon, which was named NATE (McAllister 1995). Unlike the retrotransposons found on the *Trichogramma* PSR chromosome, NATE is found on the genome of the sibling species *N. giraulti* and *N. longicornis* and not on the A chromosomes of the host itself (McAllister 1995). Beside a retrotransposon the *Nasonia* PSR chromosome also has four repeat families, i.e. PSR2, PSR18, PSR22 and NV79 (Eickbush et al. 1992). NV79 is also positioned on the genome of all three *Nasonia* species, while the other three repeat families are unique for the PSR chromosome. Though both PSR chromosomes have a high amount of repetitive DNA, including retrotransposons, they do not seem to share any sequence homology.

3 PSR origin

Chapter 4 describes my study on the origin of the *Trichogramma* PSR chromosome based on one of the spacer sequence of its 45S rDNA repeat, the ITS2 sequence. Five ITS2 sequences were found, the *T. kaykai* ITS2 and four unknown ITS2 sequences, which I named PT1, PT2, PT2-kk and PT3. PT2-kk consists partly of PT2 and partly of the *T. kaykai* ITS2. PT3 resembles the *T. kaykai* ITS2 most, while PT1 and PT2 are more similar to the *T. oleae* ITS2. We therefore conclude that the PSR chromosome in *T. kaykai* originated from *T. oleae* or a close relative of this species. Both species belong to the same species complex together with five other *Trichogramma* species (Stouthamer et al. 1999). However, *T. oleae* occurs in former Yugoslavia, France and Tunisia and so is geographically isolated from the other species of this complex, which are found in the south-western part of North America and *T. pretiosum* also throughout America, Hawaii and Australia (Voegelé & Pointel 1979; Pinto et al. 1986; Pinto et al. 1991; Pinto et al. 1993; Pinto et al. 1997; Schilthuizen & Stouthamer 1997; Pinto 1999). A species close to *T. oleae*, *T. near pretiosum*, is found near Mt. Shasta in Northern California (Pinto 1999), and seems a more likely source for the *Trichogramma* PSR chromosome.

The origin of the *Nasonia* PSR chromosome was elucidated by the discovery of the NATE retrotransposon on this B chromosome, which resembles a retrotransposon in the related wasp *Trichomalopsis* (McAllister 1995; McAllister & Werren 1997). The NV79 repeat also occurs in *Trichomalopsis* species and the *Nasonia* PSR chromosome is therefore thought to originate from this wasp species (Eickbush et al. 1992). So far, the only sequence *N. vitripennis* and its PSR chromosome seem to have in common is the NV79 repeat, which not necessarily has to originate from *N. vitripennis*, because this repeat also occurs on the genome of the two other *Nasonia* species and *Trichomalopsis*. This implies a relatively recent transfer of the PSR chromosome to *N. vitripennis*. In contrast, most of the sequences on the *Trichogramma* PSR chromosome identified so far are also present on the A chromosomes of *T. kaykai*, which suggest that this B chromosome has a longer history with its current host.

My study on the origin of the *Trichogramma* PSR chromosome strongly favours the hypothesis that this PSR chromosome originated from a closely related wasp species, which agrees with the theory on the *Nasonia* PSR chromosome origin. The absence of any sequence homology between both chromosomes implies a different PSR chromosome ancestor. We cannot exclude however that both chromosomes do have a common ancestor and changed dramatically during evolutionary divergence over time in different hosts. This would result in a completely different DNA content for both chromosomes, except maybe for the sequences responsible for their mode of action. To discover whether both B chromosomes originated independently or from a single ancestor we should compare their DNA sequence homology by creating specific libraries for each chromosome, for example by microdissection or by subtraction hybridization techniques. Furthermore, determining their molecular mechanism of paternal genome loss may also tell more about the origin of PSR chromosomes. A different PSR mechanism would prove that both chromosomes have different ancestors. Finally, research on the occurrence of PSR chromosomes in other haplo-diploid species will reveal whether more PSR chromosomes

exist and may shed more light on their origin.

4 Conclusions

Even though the *Nasonia* and *Trichogramma* PSR chromosomes seem to have a similar mode of action, their different DNA content suggests a different origin. If they originated independently it may be less likely that their molecular mechanism of paternal genome loss is identical. However, these results do not necessarily mean that no other PSR chromosomes exist in other haplo-diploid organisms. In laboratory experiments with *Nasonia* chromosome fragments were generated from incompatible crosses and quickly obtained traits that enhanced their transmission through males (Perfectti & Werren 2001). This suggests that chromosome fragments can also be generated in natural populations. If the PSR trait involves a simple molecular mechanism, such chromosome fragments may easily acquire the PSR trait and evolve into PSR chromosomes. In addition, PSR chromosomes can relatively easy overcome species boundaries and can spread within a population when the right conditions are met, though it may be difficult for PSR chromosomes to maintain themselves in a new host species (Dobson & Tanouye 1998; Jeong 2004; Van Vugt & Stouthamer, unpublished data).

Their easy intra- and interspecific transfer and their ability to modify the sex of fertilized eggs make PSR chromosomes attractive candidates for biological control of haplo-diploid pest insects, like whiteflies, stinging wasps, spider mites, thrips and ants (Werren & Stouthamer 2003). They can do so either directly by suppressing the haplo-diploid pest species or indirectly by moving desirable genes or sequences across species boundaries. PSR chromosomes may be introduced to target species by microinjection of sperm into eggs. However, the success of introducing *Trichogramma* or *Nasonia* PSR chromosomes in pest insects for biological control purposes is still speculative. Understanding the PSR mechanism and what genes or sequences are involved may ultimately lead to the possibility of introducing the PSR trait into a pest species in a different way, for example by creating PSR chromosomes *de novo* in pest species or manipulating existing B chromosomes to become PSR chromosomes.

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Samenvatting

Dit proefschrift gaat over het Paternal Sex Ratio (PSR) chromosoom in de sluipwesp *Trichogramma kaykai*. In tegenstelling tot wat veel mensen denken, doen sluipwespen mensen geen pijn en zijn ze juist erg nuttig. *Trichogramma* wordt bijvoorbeeld gebruikt in de gewasbescherming ter bestrijding van vlindereieren. Een ander misverstand over sluipwespen is dat ze net zo groot zijn als gewone wespen. Dit gaat op voor een aantal sluipwespen, maar *Trichogramma* is met zijn 0,5 mm nog maar net met het blote oog waarneembaar. Het PSR-chromosoom in *T. kaykai* is met zijn 2 µm helaas niet met het blote oog waarneembaar. Het had mijn onderzoek een stuk gemakkelijker gemaakt als dat wel zo was geweest. Dit PSR-chromosoom is zowel een 'zelfzuchtig genetisch element' als een 'geslachtsverhoudingverst oorder' als een 'B-chromosoom'. Zelfzuchtige genetische elementen komen voor in het DNA van alle organismen en worden gedefinieerd als DNA fragmenten met een replicatie voordeel ten opzichte van de rest van het DNA. Een speciaal soort zelfzuchtig genetisch element is de geslachtsverhoudingverstoorder. Geslachtsverhoudingverstoorders komen het meest voor in geleedpotigen en kunnen gemakkelijk worden herkend omdat ze de geslachtsverhouding van de nakomelingen van hun gastheer veranderen door of meer dochters of meer zonen dan normaal te produceren. Een extreem voorbeeld hiervan is het PSR-chromosoom die voor meer dan normale hoeveelheden zonen zorgt, wat al aangegeven wordt door zijn naam die letterlijk vertaald 'mannelijke geslachtsverstoorder' betekend. Dit chromosoom komt niet alleen voor in *T. kaykai* maar ook in de niet-verwante sluipwesp *Nasonia vitripennis*. Deze sluipwespen hebben een geslachtsbepaling waarbij bevruchte eieren met twee sets chromosomen zich tot vrouwtjes ontwikkelen en mannetjes ontstaan uit onbevruchte eieren met maar één chromosomenset. Het PSR-chromosoom is een B-chromosoom, wat betekent dat het een extra chromosoom is dat niet in alle individuen van een populatie of soort voorkomt. Het PSR-chromosoom komt alleen in sommige mannetjes van beide sluipwesp soorten voor en elimineert vlak na de bevruchting de vaderlijke chromosomenset, terwijl het zelf behouden blijft. Het resulterende embryo heeft slechts één chromosomenset, namelijk die van de moeder, en het PSR-chromosoom en ontwikkelt zich in een mannetje met het PSR-chromosoom. Dit extreem zelfzuchtige B-chromosoom wordt dus alleen van vader op zoons overgedragen en werd voor het eerst ontdekt in *N. vitripennis*. De recente ontdekking van een tweede PSR-chromosoom in *T. kaykai* bood de mogelijkheid om een vergelijkende studie te doen aan PSR-chromosomen. In mijn onderzoek heb ik de werking, de herkomst en de structuur van het PSR-chromosoom in *T. kaykai* bepaalt en mijn resultaten vergeleken met de reeds bekende resultaten van het *Nasonia* PSR-chromosoom.

De werking van het *Trichogramma* PSR-chromosoom werd achterhaald door preparaten van pasbevruchte eitjes met een microscoop te onderzoeken. Het *Trichogramma* PSR-chromosoom condenseert de vaderlijke chromosomen in een dichte chromatine massa aan het begin van de eerste mitotische kerndeling in het bevruchte ei, terwijl het zelf ontkomt aan zijn eigen destructieve effect en deel gaat uitmaken van de overgebleven chromosomen

van maternale herkomst. In dit opzicht zijn de werking van het *Trichogramma* en *Nasonia* PSR-chromosoom identiek, maar hun precieze werking op moleculair niveau is onbekend en kunnen verschillend zijn. De algemene structuur van beide PSR-chromosomen is ook hetzelfde en is vergelijkbaar met die van B-chromosomen in het algemeen: Ze zijn kleiner dan de normale chromosomen en bevatten transposon elementen en een grote hoeveelheid tandem repetitief DNA. Een transposon element is ook een zelfzuchtig genetisch element, maar dan één die zichzelf kan kopiëren en zich op een andere plek in het DNA weer kan invoegen. Tandem repetitief DNA is DNA waarvan de sequentie direct achter elkaar een groot aantal keer herhaald wordt. Geen van de DNA sequenties op het *Trichogramma* PSR-chromosoom komt echter overeen met die op het *Nasonia* PSR-chromosoom of omgekeerd. Ongeveer tweederde van het *Trichogramma* PSR-chromosoom bestaat uit tandem repetitief 45S ribosoomaal DNA (rDNA) wat ook op de normale chromosomen voorkomt, terwijl op het *Nasonia* PSR-chromosoom drie PSR-chromosoomspecifieke repeat families zijn gevonden en één repeat familie die ook op de normale chromosomen van *Nasonia* voorkomt. 45S rDNA is een bijzonder soort tandem repeat, omdat het i.t.t. andere tandem repeats genen bevat. Deze genen zijn essentieel zijn voor eiwitproductie en komen voor in alle organismen. Tussen deze genen liggen spacers die hypervariabel zijn tussen soorten maar geconserveerd zijn binnen een soort en daarom vaak gebruikt worden om nauw verwante soorten uit elkaar te houden. Eén van de spacer sequenties van het 45S rDNA op het *Trichogramma* PSR-chromosoom, ITS2, bevat ten minste vijf verschillende spacer sequenties die of lijken op de ITS2 sequentie van *T. kaykai* of op die van de verwante soort *T. oleae*. Dit B-chromosoom lijkt daarom afkomstig te zijn van *T. oleae* of een *T. oleae*-achtige soort. Retrotransposon analyse bracht eerder al aan het licht dat het *Nasonia* PSR-chromosoom waarschijnlijk afkomstig is van het aan *Nasonia* verwante sluipwesp genus *Trichomalopsis*. Ondanks het feit dat beide PSR-chromosomen dezelfde werking lijken te hebben, impliceert de afwezigheid van enige sequentie homologie tussen beide chromosomen de afwezigheid van een gemeenschappelijke voorouder. Hierdoor is het minder waarschijnlijk dat ze een identiek moleculair mechanisme hebben waarmee ze de vaderlijke chromosomen elimineren. Toekomstig onderzoek zou zich moeten richten op het vergelijken van de moleculaire werking en de DNA sequentie homologie van beide PSR-chromosomen. Ook het bestaan van meer PSR-chromosomen in andere organismen met hetzelfde geslachtsbepalingsysteem moet nader onderzocht worden. Hierdoor zullen we niet alleen meer kennis opdoen over het mechanisme achter de vroege gastheer embryogenese en die van de vaderlijke gastheer chromosomen in het bijzonder, maar ook over de herkomst en evolutie van PSR-chromosomen. Meer kennis over PSR-chromosomen kan mogelijk leiden tot het gebruik ervan bij de plaaginsecten bestrijding zoals de Argentijnse mier. Zonder vrouwtjes zullen dergelijke insectenpopulaties snel uitsterven.

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First of all, I would like to send my utmost gratitude to my co-supervisors Dr. Hans de Jong and Dr. Richard Stouthamer. Hans, your excellent knowledge on the field of chromosomes and your enthusiasm for writing scientific English and producing digital scientific artwork, has been a great inspiration and an enormous help for me. I greatly enjoyed working in your cytogenetics team with our nine o'clock Friday morning meetings and also joining you on long-distance car trips to Germany, once to Würzburg with Olga and Song-Bin and once to Gatersleben. That was great! Richard, your true expertise and interest in *Trichogramma* and its PSR-chromosome has not only helped me getting started very quickly as a young apprentice in the field, but also kept me going in times of great need during my four years of the PhD study. I also want to express my gratitude for your warm hospitality during my two visits to Riverside and your hometown Redlands, where I took great pleasure in seeing my first Cirque du Soleil show. Furthermore, you gave me the opportunity to search for *Trichogramma* wasps in the Mojave Desert, which has been an absolutely wonderful experience!

Not only have I been blessed with my two co-supervisors, but also with my two promoters, Prof. Joop van Lenteren and Prof. Rolf Hoekstra. I want to express my sincere thanks for your quick and always positive responses to all my attacks with tasks like judging my thesis, summaries, propositions, etc. Because of his expertise on the PSR chromosome in *Nasonia*, Prof. Leo Beukeboom has also contributed substantially to my PhD thesis. Thank you very much for all our nice discussions on PSR chromosomes, for inviting me to the wonderful *Nasonia* meeting at Schiermonnikoog and providing me very kindly with *Nasonia* wasps. Unfortunately, I will not continue to unravel the mechanism of PSR chromosomes in the near future, though I for sure not exclude the possibility that I will someday. I want to acknowledge Prof. Marcel Dicke for his support and involvement as head of the laboratory of Entomology.

All my chromosome preparations and FISH studies would not have succeeded without the help of Jannie, who taught me absolutely everything in these matters. We together managed to prepare the tiny chromosomes of those incredibly minute wasps. I very much enjoyed our spontaneous chats about small and big things during work and life. Besides teaching me how to 'FISH', you also taught me how to bake terrific cakes and even how to knit socks, which both I enjoy now very much! You are almost like a second mum to me. The first time I set foot on long-distance ice-skates was with Bert. Together with Theo you showed me how to ice-skate, which resulted in our many visits to the skating rink in Nijmegen. Unfortunately, our dream of a tour on natural ice has not yet come true in the years that we have been ice-skating together, but who knows what the future will bring. In my first half year at laboratory of Entomology

Bertha instructed me about everything in the molecular biology of *Trichogramma*. Later on we also tried to make a genomic library of *Trichogramma*, which unfortunately was not successful as we had hoped for. Bertha, I am very grateful for all your help and I deeply admire how you manage with what you are going through right now. My roommate Patrick was always the first one to learn about everything that happened to me in the past years and I am glad he wanted to listen to all my adventures, both glorious and disastrous. Together we performed flow cytometry and southern blots and in our free time we played Kolonisten van Katan with Sander and Wieteke. How nice that was! With Peter I shared many diners at Donatello's and I truly enjoyed our endless and often even philosophical talks. His eternal generosity provided me among others with many beautiful barn stones. My warmest gratitude for that! I also want to memorize here the former research group of Richard with Fabrice, Gilsang, Ties, Raul and Emmanuel. We had many nice working discussions and I am still grateful for the refreshing statistics course. Song-Bin, always with your good humour and easy smile, thank you for sharing your knowledge on the isolation of C_0t-fractions with me. We really miss you in the lab! Of course, I could not have managed without the help of my students. Merijn, Andrea, Arnaud and Silvester, I enjoyed working with you very much! Also the insect rearing would have become most disastrous without a backup *Trichogramma* rearing system provided by Leo, André and Frans. Thank you so much! I also want to thank all secretaries at Entomology and Genetics, Sabine, Trees and Aafke, for handling all kinds of nasty paper work and financial businesses.

Besides working at Entomology and Genetics, I also spend much time in the Microscopy Centre to work with their fluorescence microscopes, which was excellently hosted by Boudewijn with his incredible knowledge on computers and microscopes, while Marijke and her team of the Molecular Biology lab sequenced my many plasmid clones. The group of Pim Lindhout at Plant Breeding and later on Ron and Bart from EasyGene taught me the ins and outs of the AFLP technique and beyond: Reuze bedankt hiervoor! The most beautiful photos that have ever been made of *Trichogramma* were made by Adriaan of Plant Cell Biology with the scanning electron microscope. Simply incredible!

After working hours I spent pretty much of my social life with my colleagues. With the many PhD students from Entomology (okay, too many to mention them all) I did things like ice-skating, with Jetske, Remco and Tjeerd, quiz-nights at café Tuck, with Sander and Wieteke, and the happy beer hours on an empty stomach. The feestcie is doing a great job in organizing many nice activities, like Sinterklaas (who got me three times in four years!) and game nights. Also with the Cytogenetics group I enjoyed many fantastic social activities, like eating Friday lunch soups, bowling and eating pancakes with Hans, Jannie, Bert, Song-Bin, Lak, Radim, Penka and Henny. I greatly enjoyed every moment of these events!

And then of course, there is my family! Lieve pap and mam, thank you so much for making this all possible and for your great support and confidence, even in times that I was not as social as I would have liked to be. You always provide me a listening ear and stimulate me to do the things I like most. Mieke and Barry, you made me aunt twice during my PhD thesis, which truly enriched my life enormously. I for sure would not mind becoming aunt for a third time!

Hanneke, your friendship and involvement mean very much to me. Once you mentioned you were thinking about getting your doctor's degree as well, but after you have seen me struggling at the end of my thesis I am not sure whether you still want to, do you?

And last but definitely not least: Otto. Your love and deep involvement kept me strong and going. You shared my disasters and together we celebrated my successes. Without you I would not have managed to do all this. You are truly my first, my last, my everything…

Curriculum Vitae

On Thursday August 17th 1978 I, Joke Johanne Frederike Auguste van Vugt, was born at Meeuwensingel 23 in Rozenburg, Zuid-Holland, The Netherlands. After spending a wonderful youth in Rozenburg and all summer holidays on Terschelling I went in 1990 to pre-academic school (VWO) at CSG Westland-Zuid in Vlaardingen, which was later renamed in CSG Aquamarijn. All my life I have been interested in the how and why of life, so in 1996 I decided to study biology in Wageningen. Molecular & Cell biology soon fascinated me most because it triggered the most how and why questions that could not easily be answered. My first major was at the Laboratory of Virology in Wageningen where I attempted to identify the genes required for *Spodoptera exigua* multicapsid nucleopolyhedrovirus (SeMNPV) DNA replication. I succeeded to individually transform and express all required genes in *S. exigua* cells, though transforming all genes together did not result in the expected viral infection of these cells. My research on viruses was continued in Denmark at the Danish Veterinary Institute for Virus Research (SVIV) at Lindholm, where I discovered that the chance of recombination between North-American and European strains of the porcine reproductive and respiratory syndrome virus (PRRSV) in pigs is close to zero. With a minor topic on the detection of microsatellites on tomato chromosomes by fluorescent *in situ* hybridisation at the Laboratory of Genetics in Wageningen I first met Dr. Hans de Jong who later coached me during my PhD research. In August 2000 I obtained my Master of Science degree in Biology *cum laude*. In November of the same year I started my PhD project at the Laboratory of Entomology and Genetics of the Wageningen University. This research was funded by NWO-ALW and the result of this research is described in this thesis. Dr. Hans de Jong and Dr. Rolf Hoekstra supervised me at the Laboratory of Genetics, whereas at the Laboratory of Entomology I was supported by Dr. Richard Stouthamer and Dr. Joop van Lenteren, though Dr. Richard Stouthamer moved from Wageningen to the Department of Entomology of the University of California in Riverside, USA. This year I will start a post-doc project on chromatin dynamics in yeast with Dr. Colin Logie at the Department of Molecular Biology at the Nijmegen Centre of Molecular Life Sciences (NCMLS).

List of Publications

Van Vugt, J. J. F. A., Storgaard, T., Oleksiewicz, M. B. & Botner, A. 2001 High frequency of RNA recombination in porcine reproductive and respiratory syndrome virus occurs preferentially between parental sequences with high similarity. *Journal of General Virology* **82**, 2615-2620.

Van Vugt, J. F. A., Salverda, M., De Jong, H. & Stouthamer, R. 2003 The paternal sex ratio chromosome in the parasitic wasp *Trichogramma kaykai* condenses the paternal chromosomes into a dense chromatin mass. *Genome* **46**, 580-587.

Chang, S.-B., Yang, T. J., *Van Vugt, J. J. F. A.*, Vosman, B., Kuipers, A., Wennekes, J., Wing, R., Jacobsen, E. & De Jong, H. 2004 FISH mapping and molecular organization of the major repetitive DNA classes of tomato. In thesis by Chang, S.-B. 2004 *Cytogenetic and molecular studies on tomato chromosomes using diploid tomato and tomato monosomic additions in tetraploid potato*, Wageningen University, The Netherlands.

Van Vugt, J. J. F. A., De Jong, H. & Stouthamer, R. (submitted) The origin of a selfish B chromosome triggering paternal sex ratio (PSR) in the parasitic wasp *Trichogramma kaykai*.

Van Vugt, J. J. F. A. & Stouthamer R. (to be submitted) Paternal Sex Ratio chromosomes in parasitoid wasps: an overview of the ins and outs of these extremely selfish B chromosomes. In *Insect Symbiosis*, Volume II (ed. K. Bourtzis & T.A. Miller), CRC Press, Boca Raton.

Van Vugt, J. J. F. A., De Nooijer, S., Stouthamer, R. & De Jong, H. (to be submitted) Size and repeat sequence characterization of the paternal sex ratio chromosome in the parasitoid wasp *Trichogramma kaykai*.

Van Vugt, J. J. F. A., Van der Hulst, R. G. M., Pruijssers, A. J. P., Verbaarschot, P., Stouthamer, R. & De Jong, H. (to be submitted) AFLP analysis of the paternal sex ratio chromosome in the parasitoid wasp *Trichogramma kaykai*.

Colour graphs

Figure 1.1 (a) *Trichogramma kaykai* female parasitizing a *Mamestra brassicae* egg in the laboratory (photo Duotone, Wageningen); (b) *Apodemia mormo deserti* butterfly (photo Marc Maas); (c) *Eriogonum inflatum* plant in Last Chance Canyon, Kern Co., California, USA (photo Richard Stouthamer).

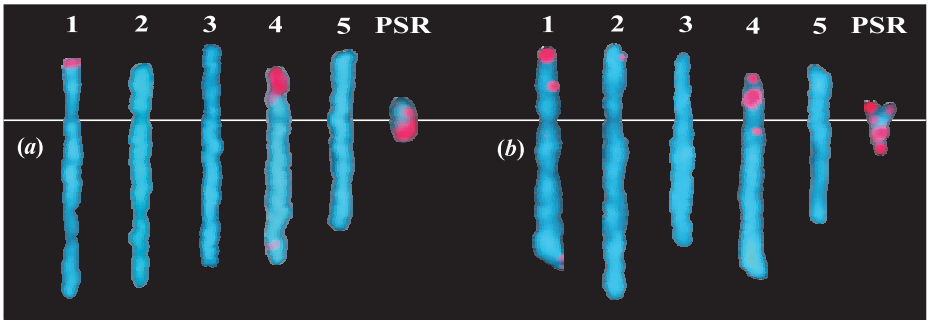

Figure 2: Karyogram of *Trichogramma kaykai*: (a) labelled with 45S rDNA from wheat (red); (b) labelled with ITS2 from *Trichogramma kaykai* (red).

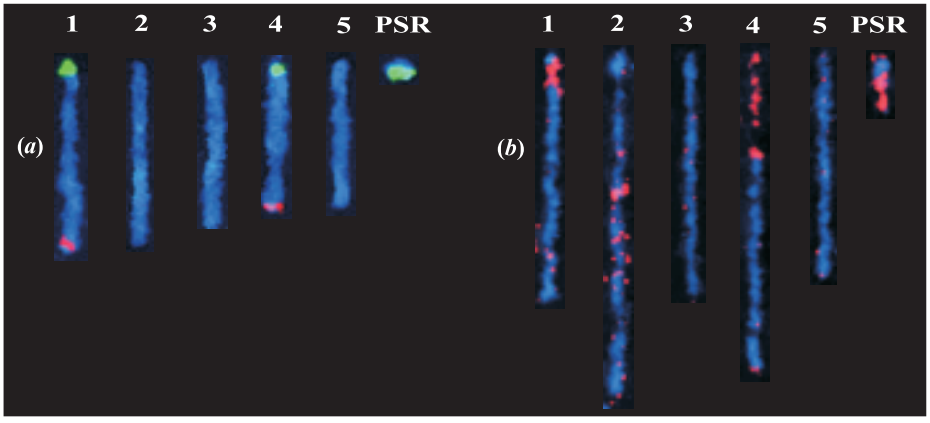

Figure 4: Karyogram of *Trichogramma kaykai*: (a) labelled with 45S rDNA from wheat (green) and with the *T. kaykai Eco*RI repeat (red); (b) labelled with C_ot-50 from *Trichogramma kaykai* (red).

Education Statement of the Graduate School Experimental Plant Sciences

1) Start-up phase

First presentation of my project	February 2001
Weekly research discussions	2001-2005
Writing a book chapter in "Insect Symbiosis, Volume II"	2005
Subtotal Start-up phase	**7.0 credits***

2) Scientific Exposure

EPS PhD student days	2001/2002/2003
- Poster presentations	2002/2003
EPS theme symposia	2001/2002/2003
- Oral presentation	2003
Seminars, workshops & symposia	
- Seminar series "Frontiers in plant development"	2001/2002
International conferences	
- 14th International Chromosome Conference, Würzburg, Germany	2001
- 2nd International B chromosome Conference, Bubion, Spain	2004
- 15th International Chromosome Conference, London, England	2004
National meetings	
- Nederlandse Entomologendag, Amsterdam	2001/2002
- International Nasonia meeting, Schiermonnikoog	2003
- FEBS workshop nuclear architecture, Wageningen	2004
Excursions	
- Collection *Trichogramma* wasps in the Mojave Desert, California, USA	2001/2002/2003
Subtotal Scientific Exposure	**11.0 credits***

3) In-Depth studies

EPS course or other PhD course	
- Springschool "Bio-informatics"	2001
Subtotal In-Depth studies	**3.0 credits***

4) Personal Development

Skill training courses	
- Guide to digital scientific artwork	2001
- Teaching and supervising thesis students	2001
Subtotal Personal Development	**2.0 credits***
Total number of credit points	**23 credits***

* A credit point represents a normal study load of 40 hours

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