Chromosoma (2005) 114: 410–419
DOI 10.1007/s00412-005-0026-4

RESEARCH ARTICLE

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NOR activity and repeat sequences of the paternal sex ratio chromosome of the parasitoid wasp *Trichogramma kaykai*

Received: 12 July 2005 / Revised: 11 August 2005 / Accepted: 23 August 2005 / Published online: 23 September 2005

Abstract Part of the male population of the wasp *Trichogramma kaykai* carries a B chromosome that manipulates its host sex ratio in favour of males. The only known repeat on this paternal sex ratio (PSR) chromosome is the 45S rDNA, which includes here five different internal transcribed spacer 2 (ITS2) sequences. In this report, we describe that only part of these ITS2 sequences is transcribed. The absence of transcription of some ITS2 sequences might explain the presence of multiple ITS2 sequences on the PSR chromosome since homogenization of rDNA spacers is thought to occur only in transcribed regions. Analysis of the only other known tandem repeat in *Trichogramma*, the *Eco*RI repeat, showed that it is absent from the PSR chromosome, and that the *T. kaykai Eco*RI repeat has 98 and 77% DNA sequence homology with the *T. deion* and *T. brassicae Eco*RI repeats, respectively. The size of the PSR chromosome measures 9 Mbp and is equal to 3.9% of the haploid *T. kaykai* genome. Finally, fluorescent in situ hybridization with a pool of high and moderate repetitive *T. kaykai* DNA (C_0t-50) revealed only a very few major tandem repeats on the *Trichogramma* genome and only 45S rDNA on the PSR chromosome.

Communicated by P. Shaw

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Introduction

The paternal sex ratio (PSR) chromosome in the arrhenotokous wasp *Trichogramma kaykai* is a B chromosome only present in some 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 non-reductive abortive meiosis (Hogge and 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 and 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 rDNA that covers two thirds of this chromosome (Van Vugt 2005). 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 and Turner 1995). Analysis of one of the spacers, the internal transcribed spacer 2 (ITS2), revealed the presence of the *T. kaykai* ITS2 and four additional ITS2 sequences on the PSR chromosome that were named PT1, PT2, PT2-kk and PT3 (Van Vugt 2005). PT1 and PT2 resemble the ITS2 of *Trichogramma oleae* the most. *T. oleae* belongs, just like *T. kaykai*, to the *Trichogramma pretiosum*/*T. deion* species complex (Pinto et al. 1986, 1991, 1993, 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, although intact *T. kaykai* ITS2 on the PSR chromosome implies that the interlocus homogenization still occurs. Lim et al. (2000) found that the transcription of 45S rDNA resulted in the homogenization of the rDNA sequences. Here we determine whether 45S rDNA on the PSR chromosome is transcribed using reverse transcriptase–PCR on samples with and without PSR chromosome, with primers specific for each ITS2 sequence and using silver staining of metaphase chromosomes.

Except for the presence of 45S rDNA, little is known about the size and the content of the *Trichogramma* PSR chromosome. By measuring the chromosome lengths of a single metaphase complement, the size of the PSR chromosome was 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 is 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 is 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). 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). Landais et al. (2000) argue 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 existence of the *Eco*RI repeat in *T. kaykai* and *T. deion* and on the PSR chromosome is examined. We studied the possible occurrence of other major repeats in *T. kaykai* and its PSR chromosome using fluorescent in situ hybridization (FISH) with C_0t-50 DNA as probe. This probe contains the fraction of pooled repetitive DNA sequences obtained from reannealing single-stranded genomic DNA (Britten and Kohne 1968; Awgulewitsch and Bünemann 1986; Chang 2004).

Materials and methods

Insects

The origins of the *Wolbachia*-infected *T. kaykai* line LC19-1 and the PSR chromosome are described by Van Vugt et al. (2003). *T. deion* line SW436-1 was initiated with a single *Wolbachia*-infected female from Sidewinder Mountains, San Bernardino County, CA, in 1996. The PSR chromosome was introduced in the *T. kaykai* and *T. deion* isofemale lines in the lab. In the field, the *Trichogramma* PSR chromosome can only be found in *T. kaykai*. 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 was received from J. C. Monje (University of Hohenheim, Germany). Wasps were reared in 150×15-mm glass vials on UV-irradiated *Ephestia kuehniella* eggs (Koppert B.V.) and *Mamestra brassicae* eggs at 23±1°C with a light–dark period of 18 and 6 h.

Drosophila 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±1°C with a light–dark period of 16 and 8 h.

Genomic DNA isolation

We isolated genomic DNA from approximately 500 *Trichogramma* wasps, either with or without PSR chromosome. The wasps were frozen alive at −80°C and ground with a pestle in a 1.5-ml tube, after which a 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 h at 37°C. Subsequently, the suspension was extracted twice with phenol and once with chloroform/isoamyl al-

cohol (24:1). DNA was precipitated with two volumes of ice-cold 100% ethanol. The precipitated DNA was washed once with ice-cold 70% ethanol, air-dried and dissolved in 50 µl TE (pH 8.0).

RNA isolation 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 by incubating the mixture for 10 min at 20°C. After centrifugation for 15 min at 14,000 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 by incubating for 20 min at 37°C. After the 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 ice-cold 100% 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 perform reverse transcriptase–PCR 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, the solution was chilled shortly on ice, and 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. The mixture was incubated for 10 min at 25°C, 50 min at 37°C, followed by 15 min at 70°C. Finally, PCR was performed on 1 µl cDNA with the specific ITS2 primers using the cycle conditions as described in Van Vugt (2005) and the PCR protocol according to Huigens et al. (2004).

PCR, cloning and sequencing

PCR was performed on 50 ng genomic DNA according to Huigens et al. (2004) with the *Eco*RI repeat forward (5′-CCAAGTGTCGTGTAAAAATT-3′) and reverse (5′-ATT CTAGACCGGACAAAAGT-3′) primers (Landais et al. 2000). The cycle program was 3 min at 94°C, 30 cycles (1 min at 94°C; 1 min at 53°C; 1 min at 72°C), 5 min at 72°C. PCR products were extracted from agarose gel (QIAEX II gel extraction kit from Qiagen), subsequently cloned into the pGEM-T vector (Invitrogen) and transformed into XL-2 Blue cells (Stratagene). The plasmid insert was sequenced using standard plasmid primers. The sequences were aligned with the program Seqman (DNASTAR Inc.) and analysed in a BLASTN and BLASTX search (National Center for Biotechnology Information).

Southern blotting and hybridization

We digested 5 µg genomic DNA with 10 U *Eco*RI for 1 h 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 mJ 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 h at 65°C and hybridized for at least 16 h at 65°C in 15-ml Church buffer with 300 ng digoxigenin-labelled *T. kaykai* or *T. brassicae Eco*RI repeat. The probes were labelled with digoxigenin by PCR as described above, but with 10 ng plasmid insert as template and partly replacing the deoxythymidine triphosphate by digoxigenin-labelled deoxyuridine triphosphate (dUTP). The stringency washes following hybridization were twice 5 min 2× SSC, 0.1% SDS at 20°C and twice 5 min 0.5× SSC, 0.1% SDS at 65°C. Digoxigenin-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 h to an XAR-5 film (Amersham Biosciences), developed and scanned.

Flow cytometry

Trichogramma wasps and *Drosophila* flies were sedated with CO_2. 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 [3-(N-morpholino)propanesulfonic acid]; 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 wavelength of 488 nm and a band-pass filter of 585 nm (564–606 nm). For each measurement, between 5,000 and 13,000 nuclei were analysed. Data were analysed 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 and 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 0.3 M NaCl was sheared to fragments of 0.5–1 kbp by sonification, denatured for 10 min at 95°C, kept on ice for 10 s and incubated at 62.4°C for 37 h 40 min to slowly reanneal 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 the remaining double-stranded DNA fragments (C_0t-50) was extracted with 1 volume of chloroform/isoamyl alcohol (24:1), precipitated with 2 volumes of ice-cold 100% ethanol, and the DNA pellet was air-dried and dissolved in 20 µl distilled water.

Chromosome preparation, probe labelling and FISH

Three- to five-day-old *Trichogramma* larvae were dissected from *M. brassicae* eggs and were 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.

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. kaykaiEco*RI repeat as insert, respectively. The pTa71 plasmid with the wheat 45S rDNA insert (Gerlach and Bedbrook 1979) was labelled according to the standard nick translation protocol (Roche) with digoxigenin-11-dUTP (Boehringer).

We used the FISH protocol of Zhong et al. (1996) with the following modifications. Instead of the first prewash in 2× SSC, the slides were air-dried for half an hour at 65°C. The chromosomes and probe DNA were denatured at 80°C for 2.5 min. All post-washes in 0.1× SSC followed by 2× SSC were skipped. 4T and TNT (1 M Tris HCl, pH 7.5; 1.5 M NaCl; 0.5% v/v Tween 20) buffers were replaced by TN buffer containing 1 M Tris–HCl and 1.5 M NaCl (pH 7.5). Instead of 4M buffer, TN buffer containing 0.5% blocking reagent (Roche) was used. Biotin-labelled probes were detected with 0.4 µg streptavidin-Cy3 followed by 2 µg biotinylated antistreptavidin and 0.4 µg streptavidin-Cy3. Finally, the slides were counterstained with 12 µl of 5 µg/ml 4′-6-diamidino-2-phenylindole (DAPI) in Vecta-Shield (Vector Laboratories) and were examined under a Zeiss Axioplan 2 Photomicroscope. Images were captured by a Photometrics Sensys 1,305×1,024-pixel CCD camera, processed with Genus Image Analysis Workstation software (Applied Imaging) and improved for optimal brightness and contrast with the Adobe Photoshop software. The chromosomes were linearized using an algorithm designed by Dr. G. van der Heijden (Biometris, PRI, Wageningen University).

Silver staining

On top of dry chromosome preparations from *T. kaykai* PSR males (prepared as described above), we placed ca. 250 µl of freshly prepared 50% w/v $AgNO_3$ in distilled water. The slides were covered with 24×50 mm wet 300-µm mesh nylon membrane (Nybolt) and incubated for 3–4 h at 65°C in a moistened Petri dish. After incubation, the slides were washed four 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 24×50-mm coverslip were placed onto the slides, and they were allowed to dry for 24 h. Subsequently, they were analysed using a Zeiss Axioplan 2 bright-field microscope. Selected images were captured by a Photometrics Sensys 1,305×1,024-pixel CCD camera and processed with the Genus Image Analysis Workstation software (Applied Imaging). Images were further improved for optimal brightness and contrast with the Adobe Photoshop image processing software.

Chromosome size estimation by DAPI fluorescence and length measurements

Captured DAPI-stained metaphase nuclei 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 and 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. *D. 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).

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

Organism	Ploidy level	Channel (no.)	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

The genome size of *T. kaykai* females was estimated at 432±10 Mbp, whereas PSR *T. kaykai* males comprised 220±7 Mbp. The slight difference between the genome size of PSR males and haploid females 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).

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±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* a nd *T. deion*, both with and without PSR, *T. cacoeciae*, *T. pretiosum* and *T. brassicae*. The expected 314-bp PCR product (Landais et al. 2000) 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.

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 (Fig. 1). The 400-bp product of *T. deion* was too weak to be cloned and sequenced. Reamplification of the 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 (two clones) and the other is named the *T. deion Eco*RI repeat (four clones). BLASTN and BLASTX search with all newly discovered sequences 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 by 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.

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, whereas *T. deion* DNA with and without PSR presents a weak 385-bp repeat and a strong repeat of ca. 500 bp (Fig. 2a,b). With the *T. kaykai Eco*RI repeat as probe, a similar result is observed, but this Southern blot analysis shows a much stronger signal on *T. deion* DNA, whereas only a weak signal is detected on the *T. brassicae* monomer and dimer (Fig. 2c,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

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                                                               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      ................
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Fig. 1 Alignment of *Eco*RI repeat sequences of *T. brassicae*, *T. kaykai* (accession number DQ119056) and *T. deion* (accession number DQ119057). Identities are denoted by *dots*. A *dash* denotes a gapped position

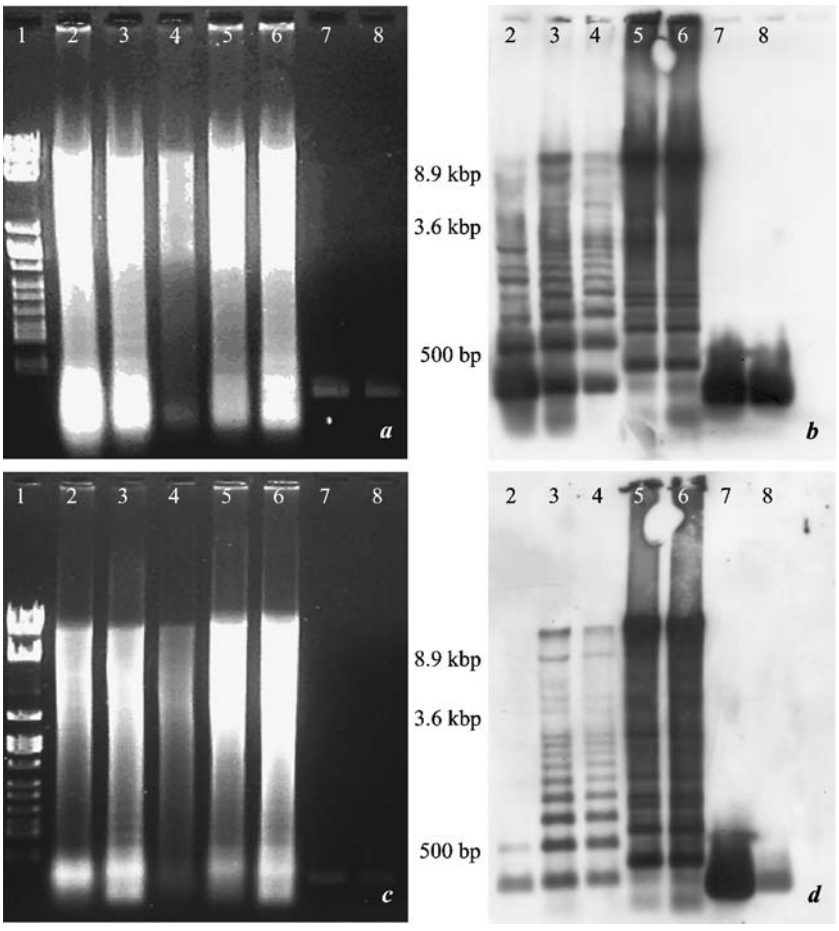

Fig. 2 Southern hybridization of the *T. brassicae* and *T. kaykai Eco*RI repeats on *Eco*RI-digested genomic DNA: **a**, **c** agarose gels of Southern hybridizations (**b**, **d**), respectively. **b** Southern hybridization with 300 ng digoxigenin-labelled *T. brassicae Eco*RI repeat; **d** Southern hybridization with 300 ng digoxigenin-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

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* (Fig. 3a). They are located at the ends of the long arms of chromosomes 1 and 4, the same chromosomes where the 45S rDNA is located. We did not observe any *Eco*RI repeat signal on the PSR chromosome.

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 and 4 and the PSR chromosome (Fig. 3b) and the *Eco*RI repeat signal on the long arm of chromosome 4. We also detected one large signal and a 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 (Van Vugt 2005).

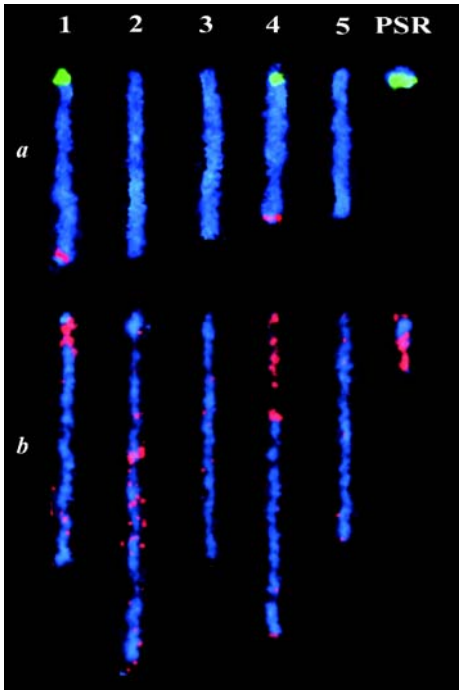

Fig. 3 Karyogram of *T. kaykai*: **a** labelled with 45S rDNA from wheat (*green*) and with the *T. kaykai Eco*RI repeat (*red*); **b** labelled with C_0t-50 from *T. kaykai*

Table 2 Reverse transcriptase–PCR with specific ITS2 primers on cDNA of *T. kaykai* and *T. deion*, both with and without PSR

cDNA	Specific ITS2 primers					
	Kk	Deion	PT1	PT2	PT2-kk	PT3
T. kaykai	+	−	−	−	−	−
T. kaykai PSR	+	−	−	−	−	±
T. deion	−	+	−	−	−	−
T. deion PSR	+	+	−	−	−	±

The presence of the right PCR product is denoted as +. The absence of PCR product is denoted as −. +/− means only little PCR product of the right size is present in two of six PCR experiments. *T. kaykai*-specific ITS2 primers are denoted as Kk

45S rDNA transcription on PSR chromosome

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 (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. Since no *T. kaykai* ITS2 is present on the *T. deion* genome (Van Vugt 2005), the presence of the latter product demonstrates that the *T. kaykai* ITS2 on the PSR chromosome is transcribed in a *T. deion* background. *T. deion*-specific ITS2 primers amplify a PCR product on *T. deion* cDNA with and without PSR. 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. In only two of six PCR experiments, a weak PT3 PCR product was detected in both *T. kaykai* and *T. deion* cDNA with PSR, whereas in the other four experiments, no PT3 could be detected at all, suggesting that PT3 is only weakly transcribed on the PSR chromosome.

We performed silver staining on metaphase chromosomes of *T. kaykai* with PSR to demonstrate whether the 45S rDNA on the PSR chromosome is transcribed. Interphase nuclei showed silver deposits of one large compound signal or two and three nucleolar organizer regions (NORs) (Fig. 4a) up to a larger number of NORs in endopolyploid nuclei. Silver staining in the (pro)metaphase chromosomes shows that a greater part of the PSR chromosome displays a dark brown 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 (Fig. 4b).

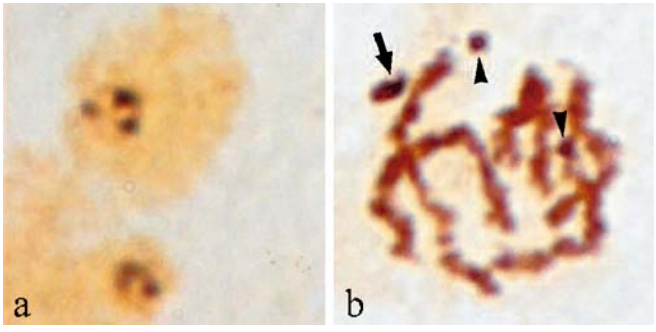

Fig. 4 Silver staining of interphase nuclei and metaphase chromosomes of *T. kaykai* with PSR. **a** Two interphase nuclei displaying small nucleoli and three dark-brown silver deposits at the NORs. **b** The (pro)metaphase complement shows three chromosomes with a silver staining at one chromosome arm. The *arrow* indicates PSR chromosome. *Arrowheads* indicate the NORs of chromosomes 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. This revealed a relative PSR chromosome length 3.9±0.9% of the haploid genome, which is equal to 9± 2 Mbp. This chromosome is more than two times smaller than the 21-Mbp *Nasonia* PSR chromosome (Rasch et al. 1975; Reed 1993). Surprisingly, the estimation of the PSR chromosome size by comparing the flow cytometric data of diploid females and haploid PSR males showed that 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 twofold 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 491 Mbp (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, whereas 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 relatively weak PCR product on the DNA of the *Trichogramma* species other than *T. brassicae* demonstrates that 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. 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 blot hybridization that suggests that the *Eco*RI repeat is more abundant in the *T. kaykai* genome than in the *T. brassicae* genome, whereas 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 autosome. This discrepancy between our results and those of Landais et al. (2000) can only be explained if we assume that the 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 blot 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 hybridizations 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. deionEco*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, the 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- 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.

Since no FISH signal was seen with the *Eco*RI repeat probe on the PSR chromosome, this repeat cannot be of use in determining the origin and evolution of the PSR chromosome. 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 a few major satellite repeats in the genome of *Trichogramma* and the PSR chromosome. The 45S rDNA is the most common and is located on the PSR chromosome and the short arms of chromosomes 1 and 4. No telomere signals were detected. The only other large tandem repeat is situated on chromosome 2 and likely corresponds to the 5S rDNA satellite repeat (unpublished results). 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 both in a *T. kaykai* (silver staining) and a *T. deion* background (PCR). 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 a large part of the PSR chromosome contains 45S rDNA, 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 that this NOR is only partly active. *T. kaykai* ITS2 on the PSR chromosome is in the *T. deion* background transcribed with a comparable intensity as the ITS2 on the *T. kaykai* and *T. deion* genomes since the respective PCR products appear equally strong, whereas 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, whereas this cannot occur 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, whereas 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 (Van Vugt 2005) suggests that the homogenization of these 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 a better indication of 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 partially 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 (Van Vugt 2005) and displays no NOR activity (Reed 1993). The repetitive sequences on the *Nasonia* PSR chromosome have proven to be an important factor for the transmission efficiency of this chromosome, and they may also be responsible for 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 45S rDNA 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, e.g., on deletion mapping of the *Trichogramma* B chromosome, might test these hypotheses.

Acknowledgements We thank Dr. J. S. Johnston from Texas A&M University, USA, and Mr. Patrick Verbaarschot for their help on estimating the genome size. We also thank Mrs. Jannie Wennekes for her help on the FISH of the *Eco*RI repeat and Ms. Penka Pavlova for measuring the fluorescence intensity of metaphase chromosomes. We also acknowledge the Dutch Science Foundation (NWO-ALW 810.34.006) for their financial support.

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