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Molecular evolution of homologous gene sequences in germ line-limited and somatic chromosomes of *Acricotopus*

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Abstract

The origin of the germ line-limited chromosomes (Ks) as descendants of somatic chromosomes (Ss) and their structural evolution was recently elucidated in the chironomid *Acricotopus*. The Ks consist of large S-homologous sections and of heterochromatic segments containing germ line-specific highly repetitive DNA sequences. Less is known about the molecular evolution and features of the sequences in the S-homologous K sections. More information about this was received by comparing homologous gene sequences of Ks and Ss. Genes for 5.8S, 18S, 28S and 5S ribosomal RNA were choosen for the comparison, and therefore isolated first by PCR from somatic DNA of *Acricotopus* and sequenced. Specific K DNA was collected by microdissection of monopolar moving K complements from differential gonial mitoses and was then amplified by DOP-PCR. With the sequence data of the somatic rDNAs the homologous 5.8S and 5S rDNA sequences were isolated by PCR from the DOP-PCR sequence pool of the Ks. In addition, a number of K DOP-PCR sequences were directly cloned and analysed. One K clone contained a section of a putative N-acetyltransferase gene. Compared with its homolog from the Ss, the sequence exhibited only few nucleotide substitutions (99.2% sequence identity). The same was true for the 5.8S and 5S sequences from Ss and Ks (97.5-100% identity). This supports the idea that the S-homologous K sequences may be conserved and do not evolve independently from their somatic homologs. Possible mechanisms effecting such conservation of S-derived sequences in the Ks are discussed.

Key words*:* Microdissection, DOP-PCR, germ line-limited chromosomes, molecular evolution

Introduction

Germ line and somatic cells that differ in number and composition of their chromosomes were detected in members of the Orthocladiinae, a subfamily of the Chironomidae (Bauer and Beermann 1952). The same phenomenon was also found in the dipteran families Cecidomyiidae and Sciaridae, and in lower vertebrates, in some hagfish species (Cyclostomata) (White 1973; Kohno et al. 1986; Kubota et al. 1993; Goday and Esteban 2001). In the chironomid *Acricotopus lucidus* these germ line-limited chromosomes (=Ks; n = 6-16) together with the somatic chromosomes (=Ss; n = 3) pass through a complex chromosome cycle exhibiting genetic specialities as elimination mitoses and monopolar movements of chromosome complements (White 1973; Redi et al. 2001). For regular development of germ cells the presence of the Ks is indispensable as demonstrated in the cecidomiid *Wachtliella* (Geyer-Duszynska 1966).

The study of X-ray induced K-S-rearrangements on polytene salivary gland chromosomes of *Acricotopus* showed for the first time that Ks are composed of S-homologous sections and heterochromatic segments (Staiber and Thudium 1986; Staiber 1991a). Microdissection of such heterochromatic K segments and painting analyses of Ks in gonial metaphases with specific probes of the three Ss clearly demonstrated that the different K types have developed from the Ss by endoreduplication, rearrangements and accumulation of germ line-specific repetitive sequences (Staiber et al. 1997; Staiber and Schiffkowski 2000; Staiber 2002).

Pairing-like associations of homologous sections of Ks and Ss were observed in rare cases in a painting analysis of spermatocyte metaphase I configurations using S probes (Staiber and Wahl 2002). This suggests that recombination events between Ss and Ks might be possible, thereby effecting a conservation of S-homologous DNA sequences in Ks. This calls into question the molecular evolution of S-derived sequences in Ks.

The aim of the present study was to characterize and compare homologous sequences of Ks and Ss. First, small amounts of K-specific DNA were collected by microdissection of some monopolar moving K sets from differential gonial mitoses and amplified by DOP-PCR. Ribosomal RNA gene sequences were then isolated from somatic DNA and compared with homologous K sequences amplified from the K DOP-PCR sequence pool.

Materials and methods

Chromosome preparation, microdissection and DOP-PCR

Squash preparations of gonial and differential mitoses of *Acricotopus lucidus* were made via the dry ice method from testes of young fourth instar larvae (5 mm; shortly after molting) previously treated with hypotonic 0.5% sodium citrate solution (20 min) and fixed in ethanol - acetic acid (3:1) for 2h. Microdissection of monopolar moving K sets of differential mitoses (Figs. 1C and 1D) was carried out using mechanical Leitz micromanipulator and glass needles produced with a horizontal pipet puller (Bachofer, Reutlingen, Germany) and a De Fonbrune microforge (Beaudouin, Paris, France).

Treatment of dissected Ks with proteinase and DOP-PCR amplification of K DNA using a Roche DOP-PCR Master Kit with Taq DNA-Polymerase (8 cycles with a low annealing temperature (94°C for 1 min, 30°C for 1 min 20 s with a 4 min transition from 30°C to 72°C, and 72°C for 3 min), followed by 30 cycles of 94 °C for 1 min, 62°C for 1 min, and 72°C for 3 min adding 5 s at each cycle) were performed as described earlier (Staiber and Schiffkowski 2000).

Salivary glands prepared from prepupae were immediatly fixed in ethanol acetic acid (2h), squashed in 45% acetic acid and frozen on dry ice. After removing the coverslip polytene chromosomes were dehydrated in an ethanol series and air-dried.

Isolation of genomic DNA

Somatic DNA was extracted from thoraces of frozen male imagines of *Acricotopus lucidus* using a QIAamp DNA Mini kit (Qiagen, Hilden, Germany).

PCR and cloning of sequences

A section of a 5S rDNA array containing a 5S rRNA coding region and two non-transcribed spacer sequences (NTSs) was isolated by PCR from somatic DNA of *Acricotopus* using two internal complementary 5S rDNA primers, 5‘-CGCTTGGGAACACCGCGTGTTGTTG-3‘ and 5‘-GTCCCCCATCTAAGTACTAACCGC-3‘, derived from the *Drosophila melanogaster* 5S rDNA sequence (Samson and Wegnez 1988). The PCR was performed in 45 cycles of 94°C for 45 s, 50°C for 30 s, 72°C for 1 min 30 s with HotStar Taq DNA polymerase (Qiagen).

The 5.8S rDNA somatic sequences were isolated by amplification of the section between the 18S and 28S rDNAs containing the genes for mature 5.8S rDNA and two internal transcribed spacers (ITS1, ITS2). The primer combinations, 5‘-TAACAAGGTTTCCGTAGGTG-3‘ and either 5‘-TCCCATATGAGTTGAGGTTG-3‘ or 5‘-ATTAATATGCTTAAATTCAG-3‘, and Taq DNA Polymerase (Roche) were used in 45 cycles of 94°C for 1 min, 50°C for 1 min, 72°C for 3 min adding 2 s to each cycle.

The section of the putative N-acetyltransferase gene from somatic DNA of *Acricotopus* were amplified with the primers 5‘-GGCCTCAATTATCATACGTCGC-3‘ and 5‘-TGGCGTAGAG GTTGAGTGCAGC-3‘ in 45 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min 30 s using ProofStart DNA Polymerase (Qiagen). The primer data are derived from the N-acetyltransferase gene section cloned from the K DOP-PCR pool (see below).

Homologous 5S and 5.8S rDNA sequences of the Ks were amplified from the K DOP-PCR sequence pool with the primer combinations 5‘-GTCAACGACCATGCCATGTTG-3‘ and 5‘-ATGCCAACGGCACGTAGCG-3‘ (5S rDNA), and 5‘-AACCCTAGACAGAGGA-TCAC-3‘ and 5‘-CACTCAACCATATGTACC-3‘ (5.8S rDNA) in 45 or 48 cycles of 94°C for 1 min, 50°C or 57.5°C for 30 s, 72°C for 30 s adding 2 s at each cycle with Taq polymerase (Roche,).

Some sequences of the K DOP-PCR sequence pool were directly and randomly cloned. One of the clones contained a section of a putative N-acetyltransferase gene.

The PCR amplicons were ligated into the pCRII-TOPO plasmid vector (TOPO TA Cloning kit, Invitrogen, Carlsbad, Calif.), and transformed into *Eschericia coli* TOP10F‘ (Invitrogen). The inserts were sequenced in both orientations (Genterprise, Mainz). The HUSAR computer program package of the DKFZ (Heidelberg), and the EMBL and FlyBase databases were used for analysing the sequences.

Labeling of probes and fluorescence in situ hybridization (FISH)

Labeling of probes via PCR or nick translation with biotin-16-dUTP, digoxigenin-11-dUTP and fluoresein-12-dUTP (Roche), FISH and painting procedures with post-hybridization washing and detection were performed as described in Staiber and Schiffkowski (2000). Digital images were taken with a Biorad laser scanning microscope (MC1024) and processed with the Corel Draw software package.

Results

Painting of spermatogonial metaphases with chromosome-specific probes of the three Ss (Fig. 1A) and subsequent DAPI staining revealing a reverse C-banding pattern (Fig. 1B) clearly demonstrates that the Ks of *Acricotopus* consist of large S-homologous sections and of paracentromeric and terminal heterochromatic segments. During evolution Ks most probably have developed from Ss by endoreduplication, rearrangements and accumulation of germ line-specific tandem repetitive DNA sequences (Staiber and Schiffkowski 2000; Staiber 2002).

In addition to the structural evolution of the Ks, the molecular evolution of the S-homologous sequences of the Ks is also of interest. To investigate this specific K DNA is necessary. The method of choice for collecting Ks is chromosome microdissection. Here one can use a genetic speciality in the K-S chromosome cycle of the Orthocladiinae (White 1973). A brief spatial separation of the Ks from the Ss takes place during the last gonial mitosis prior to meiosis, the so-called differential mitosis. The Ks move undivided to only one of the cell poles, while the paired Ss first remain in the equatorial plane (Fig. 1C). Only when the Ks reach the pole do the S sister chromatids separate and behave as in a regular mitosis. This temporal shift in anaphasic movement between Ks and Ss allows for the collection of a larger number of pure Ks by microdissection. Figures 1C and 1D show the microdissection of the K complements of two differential mitoses.

A total of six monopolar moving K complements each consisting of 9-10 Ks were collected from a spermatogonial squash preparation. After proteinase digest the K DNA was amplified by DOP-PCR (Telenius et al. 1992). The length of the randomly amplified sequences representing the microdissected K DNA ranged from 150 bp to 2000 bp. To examine to what extent sequences of the S-homologous K sections are present in the DOP-PCR probe an aliquot of the K amplicons were labeled with digoxigenin in a second PCR and hybridised onto polytene salivary gland Ss (Fig. 1E). In polytene S sets the hybridization signals were found to be almost

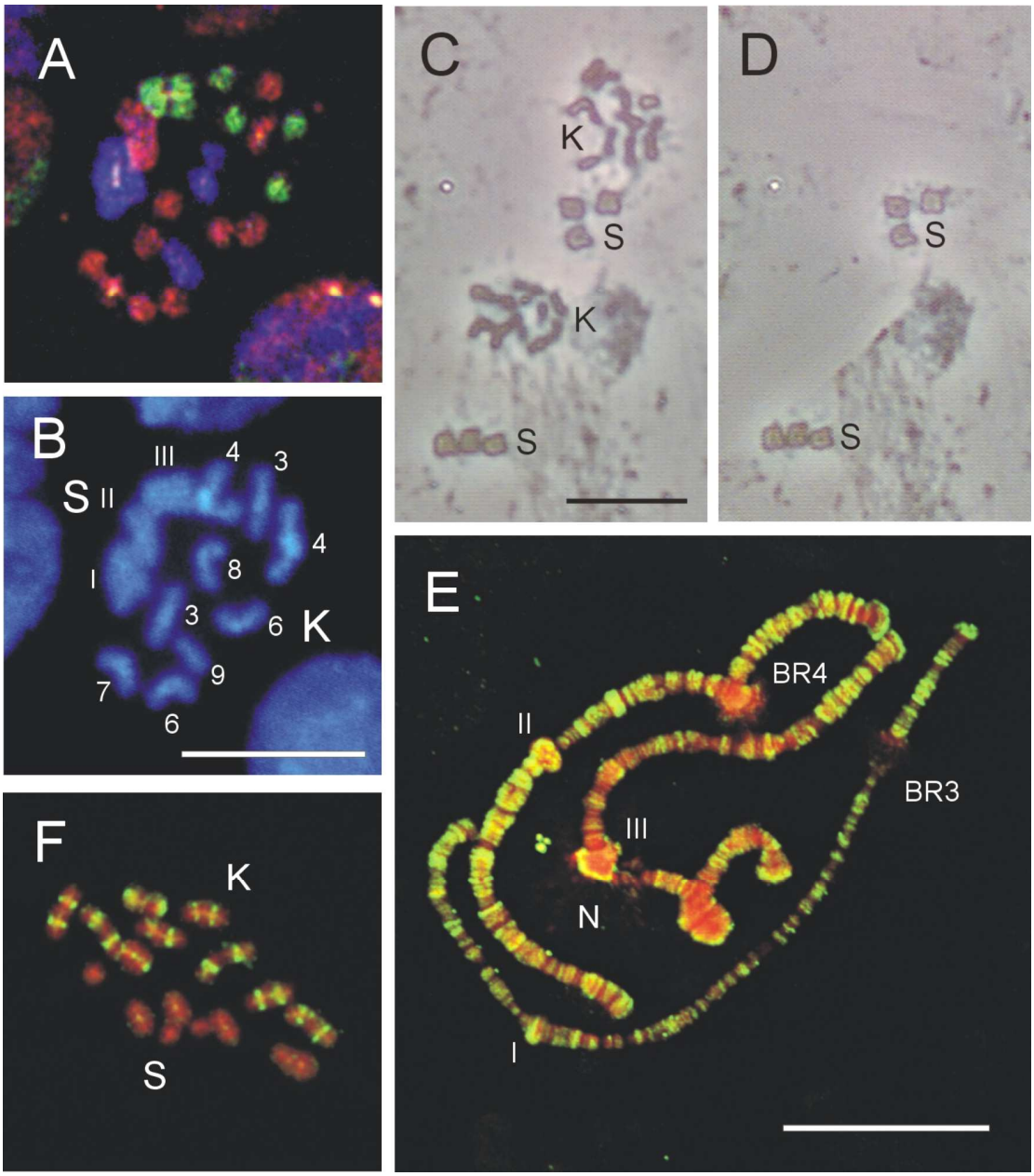

Fig. 1. (**A**) Visualization of the S-homologous sections of the Ks by three colour FISH using chromosome-specific probes of the somatic chromosomes SI (blue), SII (red) and SIII (green) to a metaphase of a spermatogonial mitosis of *Acricotopus*. (**B**) Metaphase in (A) counterstained with DAPI. The homologous Ss (2n = 6; I-III) are paired. The nine Ks are labeled with arabic numbers. (C, D) Microdissection of the K complements of two differential mitoses. Differential mitoses are the last gonial mitoses prior to meiosis and proceed unequally. (**C**) All Ks move unseparated to the upper cell poles, while the Ss still remain in the equatorial plane. (**D**) The Ks were selectively microdissected with fine glass needles, collected, digested with proteinase and their DNA was amplified by DOP-PCR. (**E**) FISH with the digoxigenated DOP-PCR probe of the microdissected Ks onto a salivary gland chromosome S set. Hybridization signals are almost evenly distributed over all sections of the three polytene Ss. (**F**) FISH with the same probe onto a spermatocyte metaphase I revealed strong signals preferably on the paracentromeric heterochromatin bands of the K bivalents resulting from the germ line-specific highly repeated AlKeRe DNA family located there. BR, Balbiani Ring. N, nucleolus. Bars represent 10 µm (**B**, **C**) and 50 µm (**E**).

evenly distributed over the three Ss indicating that the DOP-PCR probe contains a large plurality of sequences having homologous counterparts in all S sections. FISH with the same probe to spermatocyte metaphases I (Fig. 1F) produced strong signals on all paracentromeric hetero-

chromatin bands of the Ks resulting from the germ line-specific highly repetitive AlKeRe sequence families located there (Staiber 2002).

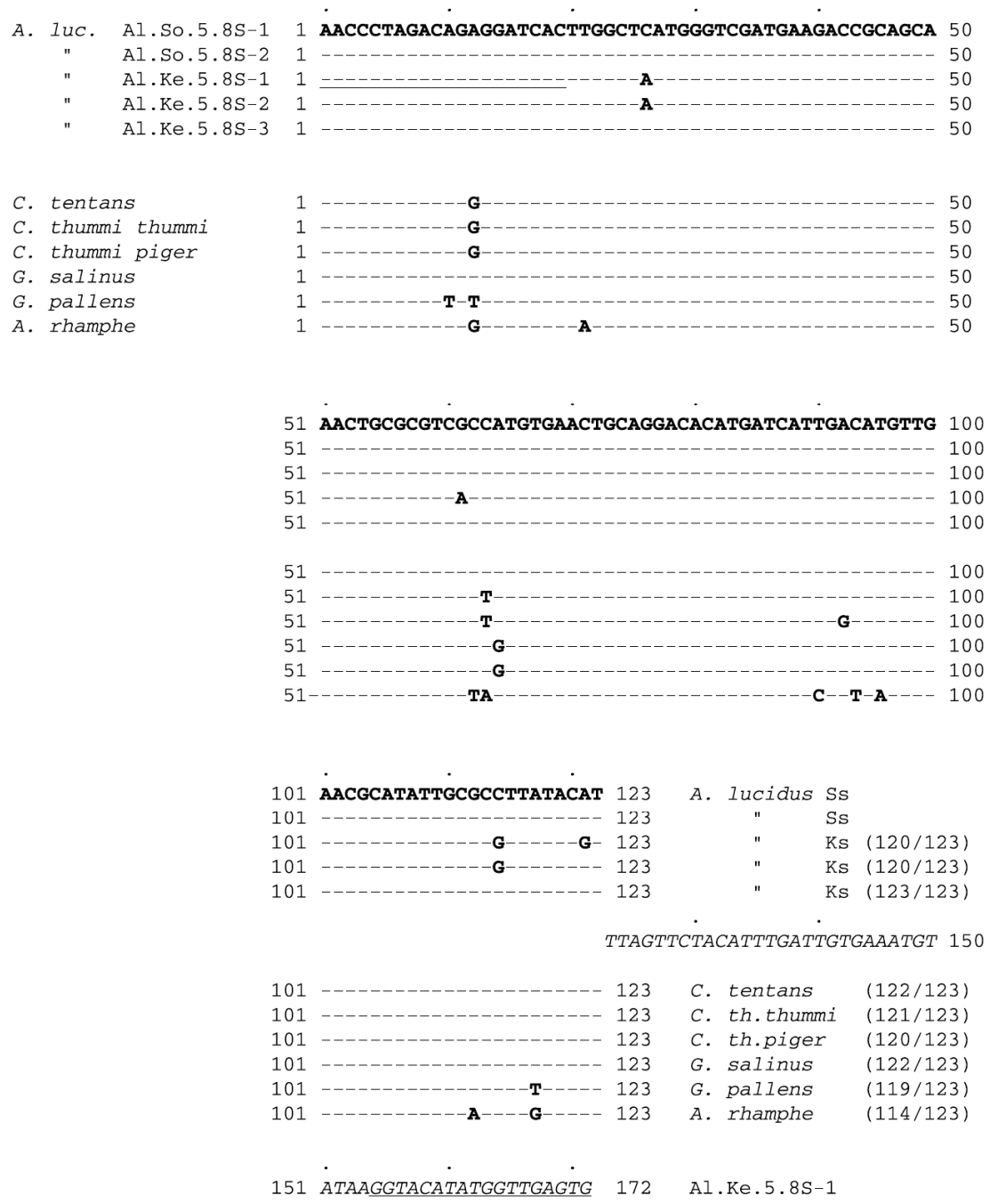

Fig. 2. Comparison of 5.8S rDNA sequences from Ss (=soma; Al.So.5.8S-1 and -2) and Ks (Al.Ke.5.8S-1 to -3) of *Acricotopus lucidus*, and from soma of the chironomids *Chironomus tentans*, *Chironomus thummi thummi*, *Chironomus thummi piger, Glyptotendipes salinus* and *Glyptotendipes pallens* (accession nos.: X99212, Aj296820, Aj296808, Aj296802, Aj296798), and *Ablabesmyia rhamphe* (Miller et al. 1997; accession no.: U48384). The two somatic sequences of *Acricotopus* derive from two different PCR and cloning experiments of the section between 18S and 28S rDNA containing the 5.8S rDNA. The K sequences are from one PCR. The primers for amplification of the K 5.8S rDNA from the K DOP-PCR sequence pool are underlined. The sequence from positions 124 to 172 (italics) represents a short section of the flanking ITS2 (31 bp) plus the S-derived primer sequence (clone Al.Ke.5.8S-1). Dashes (-) indicate nucleotides identical to those of somatic 5.8S rDNA of *Acricotopus*, base substitutions are indicated by the respective bases. The sequence data of the 5.8S rDNA of *Acricotopus* (Al.So.5.8S-1) are available from the EMBL database under accession no. AJ586563.

Only very faint fluorescence signals are present on the Ss and the S-homologous sections in the chromosome arms of the Ks. The hybridization can be seen more clearly with increasing electronic accumulation of the signals (not shown).

A

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  1  CGCTTGGGAA CACCGCGTGT TGTTGGCATC TTACTTTTTG CAAAATTCTC
 51  TTAAAAATAT TATTTTTGTC AGATGATTGG AGAACAGGAG GAGGGGGTCA
101  ACACTTAAAT TAAAAATAAA AACATGATTT AACTTTTCAT ATCAAAAGTG  NTS
151  TTTTAATATT TTTTTACAAT CAAAAGCAAT GAATGAAATG AATAAAAATA
201  GTTGAACGAT ATTGAACATG ATGAACATTT TGTGAGAGAA CTTTTGTCAA
251  CGACCATGCC ATGTTGAAAA CACCGGTTCT CGTCCGATCA CCGAAGTTAA
301  GCAACATCGG GCGTAGTTAG TACTTAGATG GGTGACCGCT TGGGAACGCT  5S
351  ACGTGCCGTT GGCATCTTAC TTTTTGCAAA ATTCTCTTAA AAATATTATT
401  TTTGTCAGAT GATTGGAGAA CAGGAGGAGG GGGTCAACAC TTAAATTAAA
451  AATAAAAACA TGATTTAACT TTTCATATCA AAAGTGTTTT AATATTTTTT
501  TACAATCAAA AGCAATGAAT GAAATGAATA AAAATAGTTG AACGATATTG  NTS
551  AACATGATGA ACATTTTGTG AGAGAACTTT TGTCAACGAC CATACCATGT
601  TGAAAACACC GGTTCTCGTC CGATCACCGA AGTTAAGCAA CATCGGGCGC
651  GGTTAGTACT TAGATGGGGG AC     Al.So.5S-20
```

B

A. lucidus

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             1                                                  50
soma         GTCAACGACC ATGCCATGTT GAAAACACCG GTTCTCGTCC GATCACCGAA
germ line    ---------- ---------- ---------- ---------- ----------

            51                                                 100
            GTTAAGCAAC ATCGGGCGTA GTTAGTACTT AGATGGGTGA CCGCTTGGGA
            ---------- ---------- ---------- ---------- ----------

            101              120
            ACGCTACGTG CCGTTGGCAT    Al.So.5S-20; 5S rDNA
            ---------- ----------    Al.Ke.5S-1, Al.Ke.5S-2, Al.Ke.5S-3
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Fig. 3. (**A**) Section of a 5S rDNA array from the Ss of *Acricotopus lucidus* (clone Al.So.5S-20; accession no. AJ586564) composed of one 120 bp coding region (fat letters in the middle) flanked by two identical non-transcribed spacer sequences (NTS, 216 bp; italics) and parts of 5S rDNA sequences at both ends (fat letters). The section was isolated from somatic DNA by PCR using internal opposite directed primers (underlined) derived from *Drosophila melanogaster* 5S rDNA. (**B**) Comparison of the somatic 5S rDNA of (A) with 5S rDNA sequences of the Ks isolated from the K DOP-PCR sequence pool by PCR (primers are underlined). The somatic 5S rDNA and the three K 5S rDNAs (Al.Ke.5S-1 to -3) are identical.

Members of the tandemly repetitive organized ribosomal RNA genes, such as the 5.8S, 18S, 28S and 5S rRNA genes, are conserved in evolution and show high similarity among eukaryotic species, and are therefore used to uncover phylogenetic relationships between species (Wägele and Rödding 1998).

In this study, 18S, 5.8S, 28S and 5S rRNA gene sequences were isolated from somatic DNA of *Acricotopus* by PCR using primers derived from the homologous genes of *Drosophila melanogaster* (5S, 5.8S, 18S rDNA) and *Chironomus tentans* (28S rDNA), and then cloned and sequenced. The sequences of the somatic 5.8S and 5S rDNA are shown in Figs. 2 and 3. The sequence data are available under the accession numbers AJ586561 (18S rDNA), AJ586562 (28S rDNA), AJ586563 (ITS1, 5.8S rDNA, ITS2) and AJ586564 (5S rDNA, NTS) in the EMBL/Genbank/DDBJ databases.

A digoxigenated probe of all sequences of a rDNA transcription unit covering the 18S, ITS1, 5.8S, ITS2 and 28S rDNA sequences (a total of 5,67 kb) was used to identify and localize rDNA loci of the Ks on spermatocyte metaphases I by FISH (Fig. 4A-4D). In addition to signals on the regular NORs on the SIIIs (see also the nucleolus in the short arm of the polytene SIII adjacent to the centomeric heterochromatin block in Fig. 1E) clear hybidization signals were found on K4s (Fig. 4A). The FISH analysis also uncovered the presence of two different subtypes of K4s in the K complement (Figs. 4B and 4C): one with two signals, a very strong and a weak signal (arrowheads in the insert in Fig. 4B and in Fig. 4D) on both sides of the central heterochromatic block, and the other subtype with only one weak signal adjacent to one side of the heterochromatin. The latter signal is only clearly seen after strong electronic accumulation of the fluorescence (arrows in the insert in Fig. 4B and in Fig. 4D). The stronger fluorescence signal on K4 has nearly the same intensity as that of the NOR locus on SIII. The presence of K4 in the microdissected K complements of the differential mitoses in Fig. 1C could be verified by its characteristic shape and size. In a former G-banding study of spermatogonial mitoses the K4 type, that has developed from SIII during evolution (Staiber and Schiffkowski 2000), was present in 1-5 copies in 30 of 31 larval K complements analysed (Staiber 1988).

Using the 5S rDNA and the NTS as probe (see Al.So.5S-20 in Fig. 3A), the 5S rDNA locus in the Ss of *Acricotopus* was localized on polytene salivary gland chromosomes in the long arm of SII in the band 4E5 (Fig. 4E; chromosome maps in Staiber and Behnke 1985). That a section containing the 4E5 band and therefore the 5S rDNA locus is present in the Ks was demonstrated on X-ray translocated polytenized K sections (S-homologous K section 4B97-4E19/21/23 in Staiber 1991a). The translocated section derived either from K3, K6 or K7 that are painted by the SII probe (see red painting in Fig. 1A).

From the sequence data of the somatic rDNAs some primer combinations were derived and used to isolate sections of rDNAs from the K DOP-PCR sequence pool via PCR. Figure 2 presents a comparison of 5.8S rDNA sequences (123 bp) from the Ss (Al.So.5.8S-1 and Al.So.5.8S-2) and from the Ks (Al.Ke.5.8S-1, Al.Ke.5.8S-2, Al.Ke.5.8S-3) of *Acricotopus lucidus*, and from six other chironomid species *Chironomus tentans*, *Chironomus thummi* subsp.

thummi, Chironomus thummi subsp. *piger, Glyptotendipes salinus, Glyptotendipes pallens* (accession nos.: X99212, Aj296820, Aj296808, Aj296802, Aj296798), and *Ablabesmyia rhamphe* (accession no.: U48384; Miller et al. 1997) having no germ line-limited chromosomes.

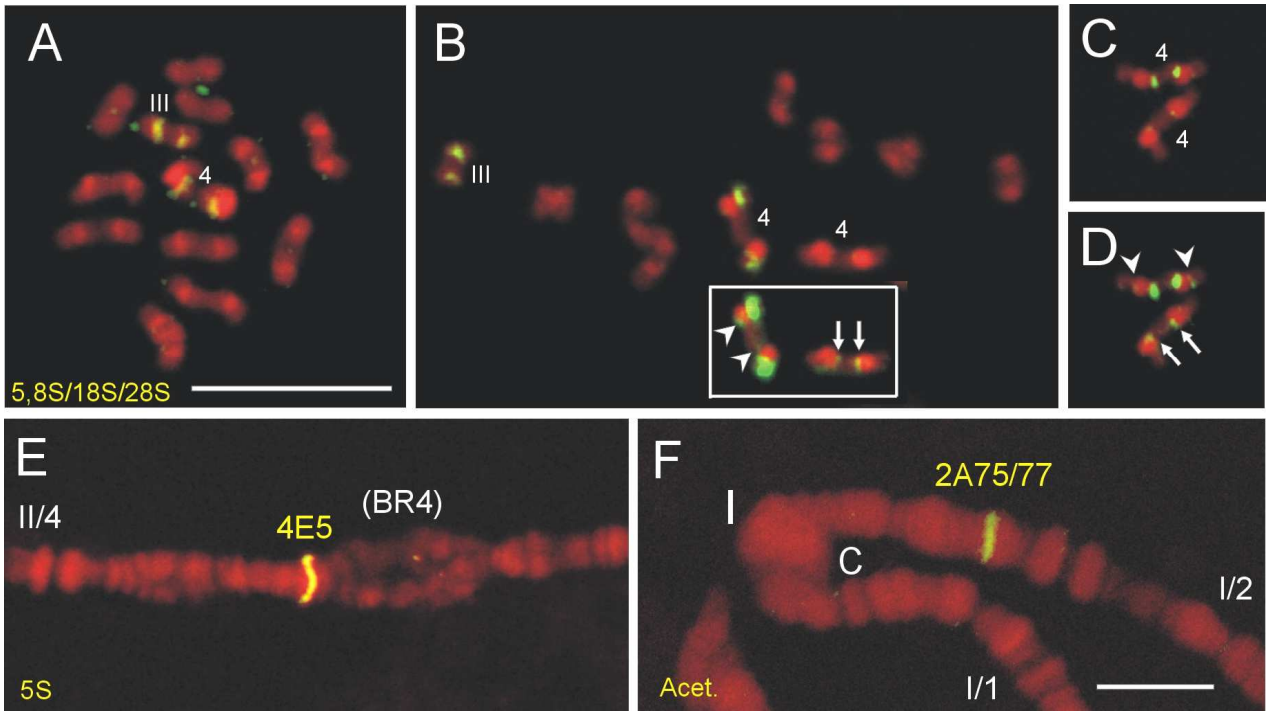

Fig. 4 (**A**-**D**) Localization of rDNA loci on Ks and Ss of spermatocyte metaphases I by FISH using a probe containing digoxigenated 5.8S, 18S and 28S rDNA sequences of *Acricotopus*. Chromosomes are counterstained with propidium iodide. (**A**) Hybridization signals occur on the NORs of the SIII bivalent and on the K4s. (**B**) A metaphase I presenting two K4 bivalents with very different signal intensities. The *insert* shows both K4 subtypes with increased electronic accumulation of the fluorescein signals. Strong and weak signals (*arrowheads*) appear on both sides of the central heterochromatin blocks (intense red propidium iodide fluorescence) on the left K4s. On the right K4s only weak signals are present (arrows). (**C**, **D**) Both K4 subtypes from another metaphase clearly show the different hybridization patterns. (**E**) FISH with a 5S rDNA probe of *Acricotopus* onto polytene salivary gland chromosomes localizes the only 5S rDNA locus of the Ss in band 4E5 in the long arm of SII (II/4). (BR4) = regressed Balbiani ring 4. (**F**) FISH with a probe of the K-derived N-acetyltransferase gene sequence (clone Al.Ke.AT-3.3) detects the N-acetyltransferase locus in the right arm (I/2) of the polytene SI in bands 2A75/77 (Staiber and Behnke, 1985) near the heterochromatic centromere region (*C*). Bars represent 10 µm (**A**, **F**).

The two 5.8S rDNA sequences of the Ss were isolated from somatic DNA in different PCR amplifications, in which the intermediate section between the 18S and 28S rDNAs, that contains the 5.8S rDNA and two internal transcribed spacer sequences (ITS1 and ITS2), was amplified (accession no. AJ586563). The 5.8S rDNA sequences (123 bp) of the Ks are parts of 172 bp PCR fragments received in one PCR from the K DOP-PCR sequence pool using primer sequences derived from 5.8S rDNA-ITS2 somaic sequence (see Fig. 2; primers are underlined). The sequence from position 124 to 172 (italics in Fig. 2) shows a short section of the flanking ITS2 (31 nucleotides) plus the S-derived primer sequence of clone Al.Ke.5.8S-1. Until now no other spacer sequences could be isolated from the DOP-PCR sequence pool. Both somatic 5.8S rDNA sequences of *Acricotopus* and one of the K sequences (Al.Ke.5.8S-3) are identical.

A

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                .         .         .         .         .
Al.Ke.AT-3.3     1 CCGACTCGAGCTGCGAATGTGGTTACAAAGTAATCTTAACAGTTCAATGA 50

                .         .         .         .         .
Al.Ke.AT-3.3    51 CTCATAAGACCTTTTTTCAAATTCAAGGCCTCAATTATCATACGTCGCAG 100
Al.So.AT-4                                 GGCCTCAATTATCATACGTCGC--

                .         .         .         .         .
               101 AAGACGATAAAGGAAATATTGTTGGATATGTTCTAGCGAAAATGGAGGAG 150
                   --------------------------------------------------

                .         .         .         .         .
               150 CCTGAGCCAGGAGAGGAGAGCAAGCATGGGCATATCACTTCGTTAGCCGT 200
                   --------------------------------------------------

                .         .         .         .         .
               201 AAAAAGACCGTTCCGTTGTCTTGGTCTCGCTCAAAAACTCATGAATCAGG 250
                   ----------------C---------------------------------

                .         .         .         .         .
               251 CTTCCTATGCCATGGTCGAATGCTTCGATGCACAATATGTGAGCCTTCAT 300
                   -----------------------------------------T------

                .         .         .         .         .
  Al.Ke.AT-3.3 301 GTCCGCAAAAGCAATCGTGCTGCACTCAACCTCTACGCCACATGAACAAC 350
  Al.So.AT-4       ------------------GCTGCACTCAACCTCTACGCCA    264 bp

                .
  Al.Ke.AT-3.3 351 TCGAGTCGG   359 bp
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B

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                .         .         .         .         .
Al.So.AT-4       1 PQLSYVAEDDKGNIVGYVLAKMEEPEPGEESKHGHITSLAVKRPFRRLGL 50
Al.Ke.AT-3.3       ------------------------------------------------C---
A. gambiae         --V-----------------------------T-----------SY-----
D. melanogaster    -------V----A--------------N---R-----------SS-----

                .         .         .         .
              51 AQKLMNQASYAMVECFDAQYVSLHVRKSNRAALNLYA
                 ------------------------------------   (86/87)
                 ---------K------N-------------------T   (80/87)
                 ---------Q------N-------------------T   (79/87)
```

Fig. 5. (**A**) Section of a putative N-terminal acetyltransferase gene of the Ks cloned from the K DOP-PCR sequence pool (Al.Ke.AT-3.3; 359 bp) compared with a homologous section from S DNA isolated by PCR (Al.So.AT-4; 264 bp) using primers (double underlined) derived from the K sequence. The degenerated oligonucleotide primer (DOP) sequences at the ends of AlKe.AT-3.3 are single underlined (constant sequences in fat letters). (**B**) Alignment of the translated amino acid sequences of Al.So.AT-4 and of Al.Ke.AT-3.3 (position 79 to 339 in (**A**), open reading frame, 87 amino acids) from *Acricotopus* with homologous sequences from *Anopheles gambiae* (BX010882) and *Drosophila melanogaster* (CG11989). Amino acid substitutions are indicated.

Each of the two other K sequences (Al.Ke.5.8S-1; Al.Ke.5.8S-2) differ in three positions from the somatic sequences (sequence similarity 97.5%). Two nucleotide substitutions are present in both K sequences. The *Acricotopus* somatic 5.8S rDNA sequence differ from that of the other chironomid species in one to nine positions and vice versa (Fig. 2).

With internal opposite directed primers derived from 5S rDNA of *Drosophila melanogaster* a section of a 5S rDNA tandem array shown in Fig. 3A was isolated from *Acricotopus* somatic DNA covering an NTS, a 5S rRNA coding region (120 bp; fat letters), a second NTS and parts of 5S rDNA sequences (fat letters) on both ends. The two 216 bp NTS sequences are identical. Then, a PCR using end primers derived from the *Acricotopus* 120 bp somatic 5S rDNA resulted in a successful amplification of 120 bp 5S rDNA sequences from the K DOP-PCR sequence pool. The primers are underlined in Fig. 3B. The control PCR without template DNA showed no amplification of any sequence. The three 5S rDNA elements from the Ks were isolated from the same PCR (Al.Ke.5S-1 to –3; Fig. 3B) and were as identical among themselves as they were to the somatic 5S rDNA sequence. It cannot be excluded that the three K 5S rDNA sequences could be derived from the same template.

An alternative to the above experiments is to clone first gene sequences of the K DOP-PCR pool and then isolate homologs from the Ss. The clones from the K DOP-PCR sequence pool had 3‘-CCGACTCGAGNNNNNNATGTGG-5‘ degenerated primer sequences on both ends. One K DOP-PCR clone (Al.Ke.AT-3.3; 359 bp) shown in Fig. 5A contained a sequence, which exhibited in a 69% nucleotide similarity in a FlyBase BLAST search over a 323 bp section (position 18 to 340) to a *Drosophila melanogaster* peptide α-*N*-acetyltransferase gene. Then, with a primer pair derived from this K sequence defining a 264 bp section, a homologous section was amplified from somatic DNA (Al.So.AT-4, accession no. AJ586565; Fig. 5A). Only two nucleotide substitutions (transitions; T-C, C-T) were detected between the S and K sequences (99.2% identity). When comparing the translated amino acid sequences (open reading frame starts 5‘ in the third position of the 264 bp section with CCT), then only one of the 87 amino acids is different (Fig. 5B). When comparing the somatic amino acid sequence of *Acricotopus* with those translated from the homologous nucleotide sequences of *Anopheles gambiae* (BX010882) and *Drosophila melanogaster* (CG11989) then 80 of 87 and 79 of 87 amino acids are identical. A BLAST search with the somatic amino acid sequence of *Acricotopus* in SWISS-PROT resulted in homologies to the human N-terminal acetyltransferase complex ard1 subunit (accession no. P41227; 65 of 87 amino acids are identical). FISH with the K clone Al.Ke.AT-3.3 as probe onto polytene salivary gland Ss of *Acricotopus* localized the putative N-terminal acetyltransferase ard1 subunit gene in the long arm of SI (Fig. 4F) in the bands 2A75/77 (chromosome maps in Staiber and Behnke 1985).

Discussion

The descent of the germ line-limited chromosomes (=Ks) from the somatic chromosomes (=Ss) and their structural evolution in *Acricotopus* was elucidated in a series of X-raying

experiments and painting studies using chromosome-specific probes of the three Ss (Staiber 1991a; Staiber and Schiffkowski 2000; Staiber and Wahl 2002). The Ks are composed of both large S-homologous sections and paracentromeric and terminal heterochromatic segments containing germ line-specific highly repetitive DNA sequences (Staiber 2002). Until now, not much is known about the molecular evolution of the S-homologous sequences in the Ks.

Therefore, the aim of the present study was to compare homologous gene sequences of Ks and Ss to receive more information about the molecular features and evolution of the S-homologous K sequences. Sequences of the ribosomal RNA genes were chosen for the present analysis. They belong to the class of evolutionarily conserved genes (Gerbi 1985). The rDNA sequences are frequently used for studies of molecular evolution, e.g. to infer phylogenetic relationships in pro- and eukaryotes (Hillis and Dixon 1991; Olsen and Woese 1993; Wägele and Rödding 1998). Arrays of rDNA repeat units occur at one or several sites within and among chromosomes, the arranged 18S, 5.8S and 28S rDNAs are located in the nucleolar organizing regions and the 5S rDNA is separated in other loci. Individually repeating units do not evolve independently, but in concert (Dover et al. 1982; Drouin and Moniz de Sa 1995). Concerted evolution seems primarly to be driven by unequal exchanges between arrays (Williams et al. 1989; Hillis and Dixon 1991; Lyckegaard and Clark 1991), but also gene conversion may promote concerted evolution (Curtis and Bender 1991). This leads to a homogenization of repeats within a multigene family (Dover et al. 1982; Polanco et al. 1998; Gonzalez and Sylvester 2001).

First in this study, 5.8S, 18S, 28S and 5S rRNA genes were isolated from DNA of the Ss of *Acricotopus* by PCR and sequenced. Then, using these sequence data for primer generation, homologous sections were amplified from a DOP-PCR sequence pool of the DNA of microdissected Ks. The 120 bp 5S rDNA gene sequences isolated from Ss and Ks were identical. The sequences of the 123 bp 5.8S rDNAs from Ss and Ks were identical or differed only in three nucleotide positions. A part of a N-acetyltransferase gene from the Ks differed in a 264 bp section from the homologous gene of the Ss by one synonymous plus one non-synonymous nucleotide substitution.

The nucleotide sequence of rRNAs define the secondary conformation, where pairing is essential to maintain structures. Natural selection on the conformation preserves structural and sequence features, as shown in Fig. 2 with the 5.8S rDNAs from different chironomid species. The few nucleotide substitutions between the funtional gene sequences of the Ss and homologous sequences in the Ks may indicate that the K sequences are conserved.

First indications that S-homologous K gene sequences might be conserved and can still be activated came from the expression of an additional Balbiani ring BR2 on an X-ray translocated and polytenised K section in the larval salivary gland chromosomes (Staiber and Thudium 1986). That this polytenized section really derived from a K was recently demonstrated by microdissection of heterochromatic sections from this K part with subsequent amplification and cloning of a germ line-specific repetitive sequence family. This family could be localized by FISH only on distinct sections of the Ks (Staiber 2002).

There may be different processes, that could explain the high degree of identity of homologous S and K sequences. For example, (***i***) the occurrence of crossing over and recombination events between homologous sections of Ss and Ks in meiotic prophase, (***ii***) a permanent process of formation of new Ks from the Ss, e.g. by rearrangements, which continues permanently, or, (***iii***) exchange of genetic information between nonhomologous chromosomes, as detected for ribosomal genes of nonhomologous chromosomes in human and apes (Arnheim et al. 1980; 1982; Krystal et al. 1981; Gonzalez and Sylvester 2001).

The painting pattern of the metaphasic Ks in Fig. 1A produced by probes of the three Ss and the hybridization pattern of the polytene S set in Fig. 1E resulting from the DOP-PCR probe of the microdissected Ks indicates that Ks and Ss feature equivalent gene repertoires.

In a recent painting study of male meiosis of *Acricotopus* some pairing-like associations between Ss and Ks were observed indicating that crossing over and recombination between homologous sections of Ss and Ks might be possible (Staiber and Wahl 2002). Experiments to analyse meiotic pairing behavior of Ks in *Acricotopus* using X-ray induced marker Ks demonstrated that in male meiosis the vast majority of K bivalents are autobivalents (Staiber 1991b). Autobivalents are formed by the former sister chromatids of the non-separated monopolar moving Ks in differential mitosis, thus they are composed of identical Ks. Here recombination by crossing over events has no genetic effect. However, in some cases bivalent formation and chiasmata were also observed among homologous Ks, the marker K and the unchanged homolog. This leads to recombination within a K type. Recombination within a K type also takes place by quadrivalent formation, frequently observed at K4, resulting from crossing overs and chiasmata formation between homologous and identical Ks (Staiber and Wahl 2002). In the latter study, chiasma formation was observed in rare cases between different K types that derived from the same S type and so having homologous sections. Both processes would distribute recombied S-derived sections within the Ks and would effect a conservation and homogenization of the S-homologous sections in the Ks. Consequently, S-homologous sequences in the Ks would not evolve independently from their somatic homologs.

The somewhat larger difference of up to three nucleotide substitutions in the K 5.8S rDNA sequences compared to the S sequence, may be the result off a reduced rate of recombination events between rDNA loci on K4s caused by the repressing influence of the adjacent large heterochromatin block (Figs. 4A-4D) in recombination. But it can also not be excluded, that PCR errors are responsible for one or more of the substitutions in the K rDNA sequences isolated, because these sequences were amplified from a DOP-PCR sequence pool that itself represents amplicons (38 cycles with Taq polymerase) of microdissected K DNA. If this is true, the identity between S and K sequences would be still higher. Until now, no formation of an additional nucleolus to the regular SIII nucleolus, which would indicate that the rDNA arrays on K4 are expressed, could be detected in spermatogonial interphase cells using silver staining (data not shown).

For parasitic nematodes, exhibiting the phenomenon of chromatin elimination in the course of germ line-soma differentiation, Müller and Tobler (2000) presented the hypothesis that an ancestor underwent a partial duplication of its genome. The duplication of certain genes was advantagous for the production of large quantities of eggs. But, the duplication of large portions of the genome can produce genetic imbalances and be fatal for the organism. Therefore, the mechanism of elimination of duplicated genes evolved to restore the balance in the soma. One central point in this view is that genes in the duplicated germ line-limited part of the genome are functional and are expressed during germ cell development

The results of the present study support the idea, that the S-homologous sequences in the Ks of *Acricotopus* do not evolve independently from their homologs in the Ss, and that there are processes in effect that lead to the homogenization and conservation of such sequences.

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