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Isolation of a new germ line-specific repetitive DNA family in *Acricotopus* by microdissection of polytenized germ line-limited chromosome sections from a permanent larval salivary gland preparation

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Abstract. In *Acricotopus lucidus* (Diptera, Chironomidae) the germ line-limited chromosomes (=Ks) have developed from the soma chromosomes (=Ss) by endoreduplication, rearrangements and accumulation of germ line-specific repetitive sequences. For molecular analysis of specific small K sections, microdissection of metaphasic Ks generally yields very limited amounts of DNA. In this study, K-specific DNA was microdissected from defined polytenized K sections of X-ray induced K-S-rearrangements of permanent salivary gland chromosome preparations and was then amplified by DOP-PCR. A new germ line-specific tandem repetitive DNA family was isolated by this way from a heterochromatic K segment, characterized and localized on the Ks by FISH. The repetitive elements are related to sequences of earlier described K-specific tandem repetitive DNA families in *A. lucidus*, but are located mainly in terminal heterochromatin bands of the two largest Ks and only to a limited degree in the paracentromeric K heterochromatin. This demonstrates that a collection of permanent preparations of K-S-rearrangements with polytenized heterochromatic and S-homologous K sections of *A. lucidus* can be used as a source for obtaining K sequences of defined K parts to investigate the molecular evolution of the Ks.

Introduction

The genetic phenomenon of a chromosomal germ line-soma differentiation with the occurrence of additional chromosomes, present only in germ line cells, was established in the dipteran families Cecidomyiidae, Chironomidae and Sciaridae, and also in some hagfish species (Vertebrata, Cyclostomata) (Kohno et al., 1986; Tobler, 1986; Kubota et al., 1993; Goday and Esteban, 2001; Redi et al., 2001). In the Orthocladiinae, a subfamily of the Chironomidae, the germ line-limited chromosomes (=Ks; K being derived from "Keimbahn") and the soma chromosomes (Ss) pass through a complex chromosome cycle with interesting genetic features as elimination mitoses and monopolar movements of chromosome complements (Bauer and Beermann, 1952; Bauer, 1970). In the last few years, chromosome painting analyses with S-specific probes, and the isolation and localization of tandem repetitive DNA families clearly demonstrated that in the orthocladiid *Acricotopus lucidus* the Ks have developed from the Ss by duplication, rearrangments and accumulation of germ line-specific repetitive sequences (Staiber et al., 1997; Staiber and Schiffkowski, 2000; Staiber and Wahl, 2002). First indications that the Ks are composed of S-homologous sections and heterochromatic segments resulted from X-ray induced K-S-rearrangments analysed in the polytene larval salivary gland chromosomes (Staiber and Thudium, 1986; Staiber, 1991).

Only very limited amounts of DNA can be obtained by microdissecting defined small sections of metaphasic Ks. Therefore, a different strategy was applied by using the collection of permanent preparations of polytenized K parts from the X-ray experiments for isolation and characterization of K-derived DNA sequences by microdissection of defined K sections, DOP-PCR (Telenius et al., 1992) and cloning. In the present study, a new germ line-specific repetitive DNA family was identified and localized on the Ks by FISH. The new DNA family exhibits partial homologies to earlier isolated germ line-specific highly repeated DNA sequence families of *Acricotopus* (Staiber et al., 1997). Therefore, the collection of permanent preparations of X-ray induced polytene K-S-rearrangements can be used for obtaining DNA from small defined K sections, e.g. for comparing homologous sequences of Ks and Ss to analyse the molecular evolution of the Ks.

Materials and methods

Sample preparation and microdissection

A permanent preparation of polytene salivary gland chromosomes of *Acricotopus lucidus* (Diptera, Chironomidae) from April 1980 carrying an X-ray induced rearrangment between a germ line-limited chromosome and a somatic one was used for microdissection (Staiber and Thudium, 1986). For chromosome preparation larval salivary glands had been fixed in ethanol/glacial acetic acid (3/1, v/v; 2h), stained with acetic-carmine (12 h) and acetic-lactic orcein (1.5 h) and squashed in a drop of the latter. Preparations had been dehydrated in 100% ethanol and embedded in Euparal (Chroma, Stuttgart). The coverglass was carefully removed

under a stereomicroscope using a scalpel and the mounting medium was extracted from the preparation by immersing the slide with several changes in absolute ethanol for 12 h. The air-dried preparation was stored in a dustfree chamber and used for microdissection within one day. Three heterochromatic chromosome segments of the X-ray induced rearrangment shown in Fig. 1B were collected for probe generation. Chromosome microdissection and treatment of isolated chromatin with proteinase K was performed as described in Staiber and Schiffkowski (2000). Spermatocyte preparations were made via the dry ice method from prepupal testes previously treated with 0.5% sodium citrate for 20 min and fixed in ethanol:acetic acid (3:1, v/v) for 2h.

Amplification and labeling of probes, fluorescence in situ hybridization (FISH)

The DOP PCR Master-Kit (Roche) using Taq DNA polymerase and a degenerate primer 5'-CCG ACT CGA GNN NNN NAT GTG G-3' (Telenius et al., 1992) was applied for amplification the DNA of the microdissected chromosome sections according to the supplier's protocol. Labeling of probes with biotin-16-dUTP, digoxigenin-11-dUTP and fluorescein-12-dUTP (Roche) by PCR or by nick translation, and FISH and painting procedures with hybridization, post-hybridization washing and detection was done according to the specifications in Staiber and Schiffkowsky (2000).

For rehybridization of the painted metaphase I preparations, coverslips and antifading solution were removed by soaking the slides in 4xSSC, 0.2% Tween 20 and further washed for 60 min at room temperature. The preparations were then dehydrated in ethanol (70%, 80%, 100%), fixed in ethanol:acetic acid (3/1, v/v; 10 min), immersed twice in 100% ethanol and air-dried (Müller et al., 2002). Hybridized probes were removed during the denaturation step in 0.07 N NaOH (3 min). The new DNA probe was pretreated, hybridized and detected as the probes above. Chromosomes were counterstained with 2.5 µg/ml propidium iodide in fluorescence antifade solution Vectashield (Vector Laboratories). Metaphase images were captured using a Biorad confocal laser scanning microscope (MC 1024) attached to an epifluorescence microscope (Axioscope, Zeiss) and processed with a Corel Draw software package.

Isolation of genomic DNA

Germline DNA was extracted together with somatic DNA from abdomen of frozen male imagines of *Acricotopus lucidus* using a QIAamp DNA Mini kit (Qiagen).

Amplification and cloning of repetitive sequences

The amplification of AlKeRe3 sequences from germ line DNA was accomplished via PCR using the proofreading "ProofStart" DNA polymerase (Qiagen) and the primer pair 5'-TGG TAT CAA ATT GTA GGT ATT G-3' and 5'-TTT TGA GCG GTC TAC GAC GGT-3' deduced from the nucleotide sequence of AlKeRe3-9/2 (Fig. 3B). The DNA fragments resulting from DOP-PCR of the heterochromatic polytenized K segments and the AlKeRe3 sequences from PCR with ProofStart DNA polymerase (Qiagen) after adding 3' A-overhangs, were purified and ligated into

the pCRII-TOPO plasmid vector (TOPO TA Cloning kit, Invitrogen), and transformed into the *Escherichia coli* stain TOP10F' (Invitrogen). Recombinant clones were screened by electrophoresis after miniprep isolation (QIAprep Spin Miniprep Kit) and the nucleotide sequences of insert DNAs were determined (GENterprise, Mainz). Sequences were compared using the HUSAR computer program package of the DKFZ (Heidelberg).

Results

A permanent preparation of polytene salivary gland chromosomes of an X-ray induced reciprocal translocation between soma chromosome SIII and a germ line limited chromosome (=K) of *A. lucidus* made in 1980 (Staiber and Thudium, 1986) was used in this study for obtaining K chromatin by microdissection. The K section is translocated to the long arm of SIII (=III/6) (Fig. 1A) and is composed of a section with a banding pattern homologous to a section of the short arm of SII (=II/3) and of a large terminal heterochromatic segment (Fig. 1B). In some of the polytene nuclei, like that in Figs. 1A, B, the banded K section (116 bands) is paired with its homologous section in SII forming together an enlarged Balbiani ring BR2. In other nuclei of the preparation, the K section is not paired and expresses an additional BR2 (data not shown). This indicates, that the BR2 gene locus of the S-homologous K section can be activated.

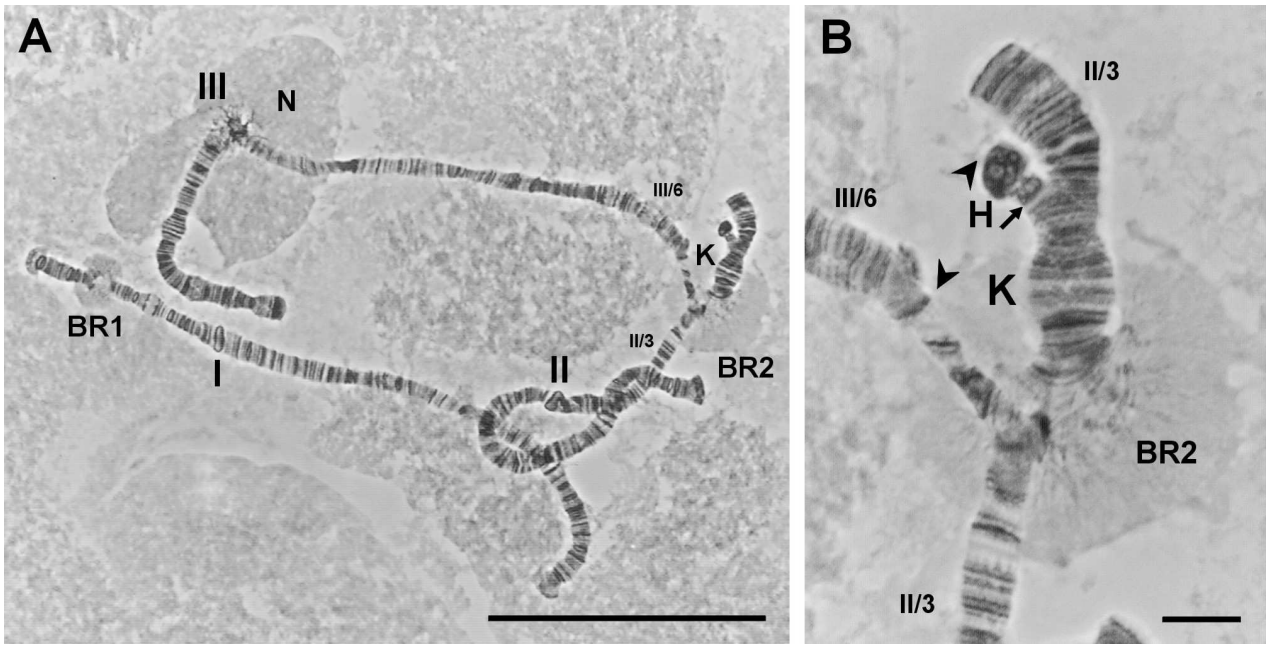

Fig. 1. X-ray induced rearrangement between a germ-line limited (K) and a soma (S) chromosome in *Acricotopus*. (**A**) Salivary gland S set (n=3) with a polytenized K section translocated to the long arm of SIII (III/6). **(B)** The K section (division between the arrowheads) is composed of a terminal heterochromatic segment (H, part between arrow and arrowhead), isolated by microdissection and amplified by DOP-PCR in this study, and a section with S-homologous banding pattern. The latter is paired with the homologous section of the short arm of SII (II/3) together expressing an enlarged Balbiani ring BR2. N, nucleolus. I-III, soma chromosome SI-SIII. Bars represent (**A**) 100µm and (**B**) 10µm

After removing the coverglass and extracting the mounting medium from the preparation three terminal heterochromatic blocks from polytenized K sections were microdissected, collected and digested with proteinase K, and their DNA was amplified by DOP-PCR. An aliquot of the resulting DNA sequence pool was labeled with digoxigenin und hybridized onto spermatocyte metaphases I preparations (Fig. 2A). The metaphases showed very strong fluorescence signals on two of the three terminal heterochromatin bands in the long arm of germ line limited chromosomes K1 (Fig. 2A) and on both terminal heterochromatin bands of K2 (not shown; K2 is not present in the K complement of the animal of Fig. 2A; the K complements can vary in composition and number of Ks between different animals; Staiber, 1988) and clear signals on the

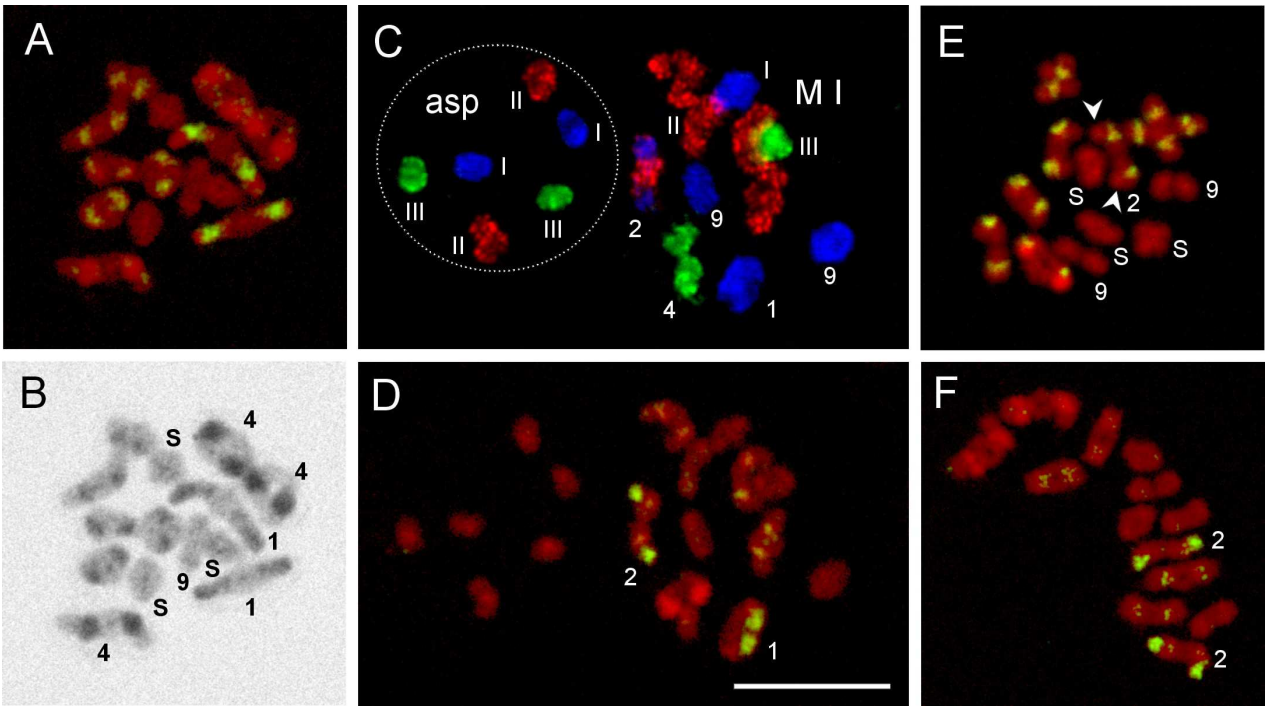

Fig. 2. Chromosomal localization of K heterochromatin derived probes by FISH. (**A**) Hybridization of a digoxigenated DOP-PCR probe from the microdissected heterochromatic segment of the polytenized K section in Fig. 1B onto a meiotic metaphase I of *Acricotopus*. Clear fluorescent signals are visible on terminal heterochromatin bands of two K1 bivalents and on the paracentromeric heterochromatin bands of most Ks, but not on the three S bivalents and the K9 bivalent. Chromosomes are counterstained with propidium iodide. (**B**) Digital inversion of the propidium iodide fluorescence in **A** resembling a C-banding pattern. (**C**) Three-color painting of a spermatocyte metaphase I (MI) and the related aberrant spermatocyte (asp; 2n = 6 Ss) using probes of SI (blue), SII (red) and SIII (green) visualizing the Ss and the S-homologous sections in the K bivalents. The SII-painted Ks cannot be distinguished with certainty from each other in meiotic stages. (**D**) Rehybridization onto the metaphases in **C** using digoxigenin-labeled AlKeRe3-9/1 and -9/2 repeats (=clone 9 insert) of the AlKeRe3 family as probe. Clear signals appear on the terminal heterochromatin bands of K1 and K2 and weak signals on the paracentromeric heterochromatin of several Ks, but not on the Ss of the aberrant spermatocyte and not on the S and K9 bivalents of the metaphase I. Bar represents 10 µm. (**E**) FISH with a probe of the earlier isolated germ line-specific AlKeRe1 family onto a metaphase I results in strong signals only on the paracentromeric heterochromatin bands of all Ks, except K9. The terminal bands of the K2s (arrowheads) are not labeled. (**F**) Hybridization of a digoxigenated probe of a 76 bp section of the AlKeRe3-17 sequence showing low similarity to the AlKeRe1 and AlKeRe2 sequences onto a metaphase I. Strong fluorescent signals are present on the terminal heterochromatin of the K2s but weak signals also appear on the paracentromeric heterochromatin bands of some Ks.

paracentromeric heterochromatin bands of all Ks, except K9 (Fig. 2A). No signals were detected on the three S bivalents not even with electronic long time-accumulation of fluorescence emission on the laser scanning microscope. For orientation, the digital inversion of the propidium iodide fluorescence of Fig. 2A resembling a C-banding pattern is shown in Fig. 2B.

Surprisingly, cloning and sequencing of amplificates of the DOP-PCR sequence pool revealed sequences of human origin. The degenerate primer used in the DOP-PCR was most probably contaminated with human DNA. Therefore, to select those K-heterochromatin-derived sequences of *A. lucid*us, that hybridized on the K1s in Fig. 2A, a total of ten K1-bivalents of the preparation of Fig. 2A showing strong fluorescence signals were microdissected and collected. A regular PCR using degenerate primers at an annealing temperature of 62°C was performed without any further treatment of the dissected chromosomes. FISH with the resulting PCR products labeled with digoxigenin produced the same hybridization pattern as in Fig. 2A. Following this, the PCR products of the ten microdissected K1 bivalents were ligated into a TOPO-vector via TA-cloning, transformed into *E. coli*, analysed by electrophoresis and sequenced. Four clones exhibited inserts containing about 1 1/7 (clones 2, 6 and 17) and about 2 1/7 repeat units (clone 9) of a new 164-bp tandem repetitive DNA family. Fig. 3A shows the insert sequence of clone 6. On both ends, the insert exhibits different primers of the degenerate primer sequence pool (underlined). The primers differ from each other in the sequence of the degenerate hexanucleotide cassette (see dotted line). The repeat unit starts with the last three nucleotides TGG of the primer followed after 164 nucleotides by a 25 nucleotide section of the next repeat, which ends with CCA in the complementary sequence of the reverse primer.

The first germ line-specific highly repeated DNA family isolated in *A. lucidus* was named AlKe family (from ***A***. ***l****ucidus* and "**Ke**imbahn", Staiber et al., 1997). Two subfamilies has been differentiated, subfamily A, repeat units 183-184 bp, and subfamily B, repeat units 175-178 bp, being nonhomologous in a section of about 75 bp. For a clear differentiation of repetitive K sequences, subfamily A will now be designated as AlKeRe1 DNA family and subfamily B as AlKeRe2 family (from ***A***. ***l****ucidus* and "**Ke**imbahn", **R**epetitive **e**lement). The new germ line-specific DNA family isolated and characterized in this study will be classified as AlKeRe3 family. The sequences of the repeat units of clones 2, 6, 9 and 17, named AlKeRe3-2, -6, -9/1 and -9/2, and –17, are shown in Fig. 3B.

For an exact localization of the new repetitive DNA family on the Ks, spermatocyte preparations were first hybridized with specific painting probes of the three Ss (Fig. 2C) and were then rehybridized with the digoxigenin-labeled clone 9 insert sequence (Fig. 2D). Figs. 2C and D show a spermatocyte metaphase I with K and S bivalents and the metaphase of the related aberrant spermatocyte (asp) containing only unpaired Ss. Both types of spermatocytes are the products of the last inequal gonial mitosis before meiosis, the so-called differential mitosis, resulting in the regular spermatocyte receiving two S sets and all the Ks, and the aberrant spermatocyte receiving one only two Ss sets (2n =6) (Bauer and Beermann, 1952; White, 1973).

A

Clone 6 insert

```
5'-CCGACTCGAGTGCGGGATG

1       10         20         30         40         50         60
TGGTATCAAA TTGTAGATAT TGCCAAGATG CGTCGATTGA TATATAACAA CATGGGTTTA
       70         80         90        100        110        120
AAGTAAGGTT TATAGGGAAA AAATCAAATA TATAGAAAAA TATATGTATA TTTTATATAT
      130        140        150        160
AAATAGCAAT AAATCCTGAA AAAACCGTCG TAGACCGCTC AAAA
1       10         20
TGGTATCAAA TTGTAGGTAT TGCCA

CATCCCAGACTCGAGTCGG-3'  227 bp
```

B

```
consensus            10         20         30         40         50         60
sequence: 5'-TGGTATCAAA TTGTAGGTAT TGCCAAGACG CGTCGATTGA TATATAACAA CATGGGTTTA

AlKeRe3-2    ---------- ------A--- --------T- ---A------ ---------- ----------
       -6    ---------- ------A--- --------T- ---------- ---------- ----------
       -9/1  ---------- ------A--- --------T- ---------- ----..---- ----------
       -9/2  ---------- ---------- --------T- ---------- ---------- ----------
       -17   ---------- ------A--- --------T- ---A------ ---------- ----------
AlKeRe3-1.1  ---------- ---------- ---------- ---------- -G---T---- ----------
       -1.2  ---------- ---------- --T------- ---------- -G-----TGT T---T--CA-
       -1.7  ---------- ---------- --T------- ---------- -G-----T-T --------A-
       -1.11 ---------- ---------- --T------- --------A- -G-------- --A-------
       -1.12 ---------- ---------- ---------- -A-------- -G-------- ----------
       -1.17 ---------- ---------- ---------- ---------- -------TT- ---------C

consensus            70         80         90        100        110        120
sequence:    AAGTAAGGTT TATAGGGTAA AAATCAAATA TATAGAAAAA TATATGTATA TTTTATATAT

AlKeRe3-2    ---------- ---G---A-- ---------- ---G------ ---------- ----------
       -6    ---------- -------A-- ---------- ---------- ---------- ----------
       -9/1  ---------- -------A-- ---------- ---------- ---------- ----------
       -9/2  ---------- -------A-- ---------- ---------- ---------- ----------
       -17   ---------- -------A-- ---------- ---G------ ---------- ----------
AlKeRe3-1.1  --AC------ G--------- ---------- ---------- ---------- ----------
       -1.2  .-AC------ ---------- ----T----- ---------- ---------- ----------
       -1.7  G-AC------ ---------- ----T----- ---------- ---------- ----------
       -1.11 ---------- ---------- ---------- ---------- ---------- ----------
       -1.12 --AC-G---- ---------- ---------- -----G---- ---------- ----------
       -1.17 --AC------ -CA------- ---------- ---------- ---------- ----------

consensus          130        140        150        160
sequence:    AAATAGCAAT AAATCCTGAA AAAACCGTCG TAGACCGCTC AAAA-3'   bp

AlKeRe3-2    ---------- ---------- ---------- ---------- ----     164
       -6    ---------- ---------- ---------- ---------- ----     164
       -9/1  ---------- ---------- ---------- ---------- ----     162
       -9/2  ---------- ---------- ---------- ---------- ----     164
       -17   ---------- ---------- ---------- ---------- ----     164
AlKeRe3-1.1  ---------- ---------- ---------- ---------- ----     164
       -1.2  ---------- ---------- ---------- ---------- ----     163
       -1.7  ---------- ---------- ---------- ---------- ----     164
       -1.11 ---------- ---------- ---------- ---------- ----     164
       -1.12 ---------- ---------- ---------- ---------- ----     164
       -1.17 ---------- ---------- ---------- ---------- ----     164
```

Fig. 3. (**A**) DOP-PCR insert sequence of clone 6 derived from the K heterochromatin block of Fig. 1B containing one repeat (164 nucleotides) of the new germ line-specific AlKeRe3 family (=AlKeRe3-6

sequence) and 25 nucleotides of the next repeat unit. The DOP-primer are underlined, the different hexanucleotide cassettes are dotted (see text). (**B)** The consensus nucleotide sequence (top) and five AlKeRe3-repeat units derived from DOP-PCR clones of the K heterochromatin block (=AlKeRe3-2 to AlKeRe3-17) and six repeat units (=AlKeRe3-1.1 to AlKeRe3-1.17) amplified directly from genomic germ line-soma DNA of *A. lucidus* using a proofreading DNA-polymerase and a specific primer combination (underlined in the consensus sequence). AlKeRe3-9/1 and -9/2 are repeat units of a dimer. Dashes (-) represent nucleotides identical to those of the consensus sequence, base substitutions are indicated by the respective bases and (**.**) represent alignment gaps. The sequence data of AlKeRe3-9/2 are available from the EMBL/GenBank/DDBJ databases under accession number AJ515919.

The three color FISH in Fig. 2C using painting probes of SI (blue), SII (red) and SIII (green), clearly identify the Ss and the S-homologous sections in the Ks indicating by that the derivation of each of the Ks from the corresponding S. While the Ss form bivalents in metaphases I, the Ss are unpaired in metaphases of aberrant spermatocytes. The rehybridization with the clone 9 insert, as a probe (Fig. 2D), resulted in very strong signals on the terminal heterochromatin bands in the long arms of the K1s and the K2s, weak signals on the paracentromeric heterochromatin bands of all Ks, except K9, and no signals on the S bivalents of the metaphase I and on the Ss of the aberrant spermatocyte. The K2 bivalent can be easily recognized in the paint of Fig. 3C, because the long arms of the K2s are painted by the SI probe (blue) and the short arms by the SII probe (red). The short arms are connected by a chiasma.

The Taq DNA polymerase used for the amplification of the microdissected K heterochromatin by DOP-PCR has no proofreading activity. To reduce the rate of possible replication errors and misincorporated bases per PCR product, some repeat units of the AlKeRe3 family were directly amplified by PCR from *Acricotopus* genomic germ line DNA using a proofreading DNA polymerase and a primer pair determined from the sequence of the second repeat unit in clone 9 (AlKeRe-9/2, EMBL/GenBank accession No. AJ515919), and were then cloned and sequenced. Six different AlKeRe3 repeat units designated as AlKeRe3-1.1, -1.2, -1.7, -1.11, - 1.12 and -1.17 shown in Fig. 3B were isolated. The consensus sequence of all AlKeRe3 repeat units is given on the top. The primers for the isolation of the AlKeRe3-1.1 to AlKeRe3-1.17 sequences are underlined. The sequences AlKeRe3-2 to AlKeRe3-17 derived from the K heterochromatin of the K-S-rearrangement from 1980 exhibit only two bases, in position 29 (T) and 78 (A), which are not found in one of the sequences isolated directly from *A. lucidus* germ line DNA.

In Fig. 4, the AlKeRe3-17 sequence (164 bp) is compared with the reverse and complementary sequences of AlKeRe1-1 (185 bp) and AlKeRe2-6 (175 bp) from Staiber et al. (1997; designated as AlKe1 and AlKe6). To get optimal alignment with the AlKeRe3-17 sequence (Clustal W program; Thompson et al., 1994), the section with the bases 1 to 40 of the latter sequences were moved to their ends (see numbers below the sequences). The comparison of the AlKeRe3-17 sequence with the rearranged sequences of AlKeRe1-1 and AlKeRe2-6 using the HUSAR program 'bestfit' resulted in 68.9% and 70.5% identity.

The AlKeRe1 and AlKeRe2 families hybridize only in the paracentromeric heterochromatin bands of the Ks (Staiber et al., 1997) as demonstrated by the strong signals in the FISH of Fig. 2E using labeled AlKeRe1 sequences as a probe. The terminal heterochromatin bands of the K2s

are not labeled (see arrowheads). Because of the partial homology between the AlKeRe3 sequence and the AlKeRe1 and AlKeRe2 sequences it might be possible, that the fluorescence signals on the paracentromeric heterochromatin bands of the Ks in the FISH in Fig. 2D, in which whole AlKeRe3 repeats were labeled and used as probe, result from hybridization of this partial homologous sections onto AlKeRe1 and AlKeRe2 elements. This raises the question, whether the AlKeRe3 DNA family is present only in the terminal heterochromatin bands of K1 and K2, and not in the paracentromeric heterochromatin. To investigate this, a digoxigenated probe was made by PCR from a 76 bp section of AlKeRe3-17 (position 49 to 124), showing lower similarity to the AlKeRe1-1 (43.4%) and AlKeRe2-6 (48.7%) sequences than the total AlKeRe3-17 sequence.

```
           1  →
AlKeRe3-17   TGGTATCAAA TTGTAGATAT TGCCAAGATG CGTAGATTGA TATATAAC..
AlKeRe1-1    ---------- ----T-C-GG --------C- -A-C------ --------TG
AlKeRe2-6    ------G--- ----T-C-GG -A------C- -A-C------ -----G-TT.
           41 →
                    |→
AlKeRe3-17   .........A ACATGGGTTT A.AAGTAAGG TTTATAGGGA AAAAATCA.A
AlKeRe1-1    AGTATATCA- -CACCAA-C- G.-TA--TCT -CATAG---T C-C---G-GA
AlKeRe2-6    ....TGATG- -TT-TA--T- GT-CT--TTT G--G--AA-T TC----T-..

                                                        ←|
AlKeRe3-17   ATATATGGAA AAA.......  TATATGTATA TTTTATATAT AAATAGCAAT
AlKeRe1-1    -TT-T-CC-- ---GTGCCAT -T-G-----T ---C-C--TA TGT-GA----
AlKeRe2-6    .T-G-AAA-- TC-ACTTTTT -T-G-----. ---C-C--TA TGT-GG----
                                                      183|1 →
                                                      175|1 →
AlKeRe3-17   AAATCCTGAA AAAACCGTCG TAGACCGCTC AAAA  164 bp
AlKeRe1-1    ----T--ATC -C--A----C ----G----- ----  183 bp
AlKeRe2-6    ----T--ATC -C--A----C ----G----- ----  175 bp
                                          40
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Fig. 4. The nucleotide sequences of AlKeRe3-17 and the reverse and complementary sequences of AlKeRe1-1 and AlKeRe2-6, were aligned by Clustal W (Thompson et al., 1994). To obtain optimal alignment the sections of the first forty nucleotides of the latter sequences were shifted to their ends (see numbers below the sequences). Primers for the amplification of a 76 bp fragment of AlKeRe3-17 are underlined (see text).

The primers used are marked with a dotted line in Fig. 4. Weak hybridization signals on the paracentromeric heterochromatin bands of the Ks in the FISH of Fig. 2F using this probe, demonstrate that AlKeRe3 sequences are present to a limited extent in these bands. The vast majority of the AlKeRe3 repeated elements is mainly concentrated in the terminal heterochromatin bands of K1 and K2.

Discussion

Chromosome microdissection is an established technique for generation of painting probes of regular chromosomes or rearranged complex marker chromosomes from human or animal metaphase spreads and of probes of nuclei from tumor tissue sections for comparative genomic

hybridization (CGH) (Carter, 1994; Sharma and Sharma, 2001; Teixeira, 2002). In *A. lucidus*, a probe of a specific K type was made by microdissection of ten K4s of freshly prepared spermatogonial metaphases for reverse painting onto polytene salivary gland Ss to identify the S-homologous sections of K4 (Staiber and Schiffkowski, 2000). Microdissection of defined small sections of metaphasic Ks results in only very little amounts of DNA. Polytenized parts of Ks of *A. lucidus* are available in a collection of permanent Euparal-embedded salivary gland chromosome preparations of X-ray induced K-S-rearrangements (Staiber, 1991). In recent studies microdissection from archival formalin fixed and paraffin-embedded sections and DOP-PCR were successfully combined to analyse chromosomal imbalances in carcinoma biopsies or aneuploidy in archival pregnancy-loss tissues by CGH (Verhagen et al., 2000; Bell et al., 2001; Marchio et al., 2001 Verhagen et al., 2000). In the present study, it was demonstrated that it is possible to isolate and amplify K-derived DNA from permanent preparations of polytene K-S-rearrangements with subsequent cloning, sequencing and chromosomal localization of the cloned sequences.

The five repeat units AlKeRe3-2 to –17 (Fig. 3B) received from the heterochromatic segment of the polytenized K section via DOP-PCR amplification are more homogeneous in their nucleotide sequences, than the sequences AlKeRe3-1.1 to -1.17 (Fig. 3B). The somewhat higher variability of the latter sequences may result from the fact, that they were isolated from genomic germ line DNA, and so the different repeat units may be derived from the paracentromeric K heterochromatin as well as from the terminal heterochromatin bands of K1 and K2. In contrast, the first sequences are derived from tandem repetitive arrangments in a defined heterochromatin block, in which homogenization processes like gene conversion and unequal crossing over proposed for tandem repeated sequences may have an effect (Elder and Turner, 1995; Li, 1997).

The similarities between the three germ line-specific tandem repetitive DNA families isolated in *A. lucidus* indicate, that the sequences are related and most probably descend from a common ancestor sequence. One can develop the idea, that the evolution of the K-specific repetitive sequences took place in subsequent steps: First, the accumulation of an ancestor germ line-specific repetitive element forming the paracentromeric K heterochromatin bands characteristic for all Ks, except K9, then the differentiation into diverse families within the paracentromeric bands, i.e. into AlKeRe1, AlKeRe2 and AlKeRe3, and finally the translocation of repetitive elements of the AlKeRe3 family into terminal regions of some chromosomes with subsequent rapid lateral amplification of repeat units, visible then as terminal heterochromatin bands, such as in K1 and K2 (Walker, 1971). As reported earlier, the exceptional germ line-limited chromosome K9 is most probably an evolutionarily younger K, which shows a specific S-like pairing behavior with its homologs in gonial mitoses and a delayed monopolar movement against the other Ks in differential mitosis (Staiber, 1987). As demonstrated by FISH, K9 contains in its centromere, as the only K, elements of the tandem repetitive AlSo DNA family, that is characteristic for the centromeres of the Ss (Staiber et al., 1997).

A similar evolution of germ line-restricted tandem repetitive DNA sequences into diverse related families with different chromosomal distribution on the germ line-limited chromosomes was established in the hagfish *Eptatretus cirrhatus* (Goto et al., 1998). The authors summarize their findings in stating that "the eliminated chromosomes in hagfish are mosaics of highly repeated, germ-line restricted families of DNA sequences".

The activation of the Balbiani ring BR2-locus in the polytenized K section (seen in the enlarged BR2 in Fig. 1A, B) indicates that there might be mechanisms in *A. lucidus*, which conserve S-homologous sequences in the Ks. The pairing behavior of Ks and Ss in male meiosis of *A. lucidus,* studied in a painting analysis, suggests that such a conservation might result from the occurrence of rare recombination events between homologous sections of Ss and Ks in meiotic prophase (Staiber and Wahl, 2002).

To my knowledge, the isolation of the repetitive AlKeRe3 family in this study is the first successful amplification of microdissected DNA from a carmine-stained and Euparal-mounted permanent cytogenetic preparation. This allows one to microdissect the homologous bands or small sections from polytenized K parts and from polytene salivary gland Ss, and then to amplify with specific primer combinations the homologous gene sequences of Ks and Ss, i.e. 5S-rRNA genes (localized in the band 4E5 of SII; Staiber unpublished) or histone genes (located in the bands 1B27/29 of SI; chromosome maps in Staiber and Behnke, 1985), with the aim of analyzing the molecular evolution of the S-homologous sequences in the Ks.

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