FISH and DAPI staining of the synaptonemal complex of the Nile tilapia (*Oreochromis niloticus*) allow orientation of the unpaired region of bivalent 1 observed during early pachytene

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Abstract

Bivalent 1 of the synaptonemal complex (SC) in XY male *Oreochromis niloticus* shows an unpaired terminal region in early pachytene. This appears to be related to recombination suppression around a sex determination locus. To allow more detailed analysis of this, and unpaired regions in the karyotype of other *Oreochromis* species, we developed techniques for FISH on SC preparations, combined with DAPI staining. DAPI staining identified presumptive centromeres in SC bivalents, which appeared to correspond to the positions observed in the mitotic karyotype (the kinetochores could only be identified sporadically in silver stained EM SC images). Furthermore, two BAC clones containing *Dmo* (*dmrt4*) and *OniY*227 markers that hybridize to known positions in chromosome pair 1 in mitotic spreads (near the centromere, FLpter 0.25, and the putative sex determination locus, FLpter 0.57, respectively) were used as FISH probes on SCs to verify that the presumptive centromere identified by DAPI staining was located in the expected position. Visualization of both the centromere and FISH signals on bivalent 1 allowed the unpaired region to be positioned at Flpter 0.80 to 1.00, demonstrating that the unpaired region is located in the distal part of the long arm(s). Finally, differences between mitotic and meiotic measurements are discussed.

Keywords: cytogenetics, fish, fluorescence in situ hybridization (FISH), sex chromosomes, synaptonemal complex (SC)

Introduction

Tilapias of the genus *Oreochromis* (Cichlidae) show chromosome pairing anomalies in early pachytene in the heterogametic sex (males in *O. niloticus* and *O. mossambicus*, females in *O. aureus*) that appear to be associated with the differentiation of sex chromosomes (Foresti et al., 1993; Carrasco et al., 1999; Campos-Ramos et al., 2001; Griffin et al., 2002; Campos-Ramos et al., 2003). The exact relationship between these unpaired regions and sex determining loci in this genus is not clear: for example, a terminal region of the largest bivalent shows delayed pairing in XY *O. niloticus* but not in XX or YY genotypes (Carrasco et al., 1999), however sex-linked LG1 markers in this species have been mapped by FISH onto a small pair of chromosomes (Lee et al., 2003; Mota-Velasco, unpublished observations; Cnaani et al., 2008). Unpairing in both the largest bivalent and a small bivalent have been observed in WZ *O. aureus* at pachytene (Campos-Ramos et al., 2001), and linkage studies suggest two unlinked genes affect sex determination in this species, with the dominant one (WZ/ZZ, in LG3) mapping to the largest pair of chromosomes (Lee et al., 2004; Cnaani et al., 2008).

The kinetochore (centromere) has been visualised in some TEM synaptonemal complex preparations in *O. niloticus* (Carrasco et al., 1999), allowing orientation of the unpaired region with respect to the chromosome, but this has not been achieved consistently. In this study, we set out to develop a technique that would allow both identification of the centromere and FISH on pachytene stage chromosomes, using male *O. niloticus*, with the objective of being able to simultaneously visualise unpaired

regions, centromeres and FISH markers at pachytene, to further the study of the role of delayed meiotic pairing in the evolution of sex determination in this genus.

Materials and Methods

Experimental fish

Phenotypic male and female *O. niloticus* (originating from Lake Manzallah, Egypt) used in this experiment were held at the Institute of Aquaculture, University of Stirling.

Preparation of SC spreads

The preparation of SC spreads from testes was carried out according to the protocol described by Foresti et al. (1993) and Campos-Ramos et al. (2001) with slight modifications. Fish were killed by immersion in anaesthetic (0.01% benzocaine solution) followed by destruction of the brain before dissection. Testes were first placed in 15 ml containers with Hank's saline solution (Sigma-Aldrich) at 4°C. The gonads were then placed in Petri dishes and minced with razorblades. The resulting cell suspension was transferred to 1.5 ml centrifuge tubes. The cell suspensions were centrifuged at 100 x g for 2 min to remove debris and then the supernatant was transferred to another tube and centrifuged again at the same speed for another 8 minutes. The tiny pellet formed was taken out carefully in 20 µl and pipetted into another tube containing 40 µl of 0.2 M sucrose and 60 µl of 0.2% Triton X (each buffered to pH 8.5 with 0.01 M sodium tetraborate) then shaken gently. After 10 min the cells were fixed with 90 µl of 4% paraformaldehyde (buffered to pH 8.5 with 0.2 M sodium tetraborate). After 90 min incubation at room temperature, about 90 µl of the fixed cell suspension was pipetted onto a microscope slide and air-dried horizontally for about 2 hours in a fume cupboard.

Slides were washed first by immersion in distilled water and then with 95% ethanol before final air-drying.

Mitotic spreads

O. niloticus metaphase spreads were prepared from peripheral blood leukocytes isolated from centrifuged whole blood and incubated for 3 days at 30°C in PB-max karyotyping medium (Gibco BRL, Paisley, UK). Cells were arrested at metaphase by addition of colcemid (to a final concentration of 0.1 µg ml^{-1}) to the culture medium and incubated at 30°C for 1h. The cells were then incubated in a hypotonic solution (0.075 M KCl) for 15 min and fixed in three changes of 3:1 methanol: acetic acid. Cell suspensions were dropped onto ethanol-cleaned glass slides.

Physical mapping of BAC clones on SCs and mitotic spreads

Two BAC clones that had been shown to consistently hybridize to chromosome 1, containing an AFLP marker *OniY*227 and *dmrt4* (*Dmo*) respectively, were chosen as FISH probes (Ezaz et al., 2004; Lee et al., 2005). The position of *Oni*Y227 in the linkage map was determined by comparative physical mapping of this and BAC clones containing a series of LG3 markers (not shown). These BAC clones were from the *O. niloticus* BAC library held at the Institute of Aquaculture, University of Stirling (HCGS-02TI; well IDs 72B20 and 60B8 respectively). DNA was prepared using a commercial plasmid preparation kit (Qiagen, Crawley, UK). For one hybridization, 10-12 µL of a 100 ng/mL solution of each probe were labelled by Bio-Nick Translation Mix or DIG-Nick Translation Mix (Roche) following the manufacturer's recommendations. Then 10 µl of

labelled probe solution (50 ng/µL stock solution) were combined with 50 fold excess of degenerate tilapia genomic DNA (50 µL of DOP-PCR Blocking DNA), desiccated and resuspended in 20 µl of hybridization solution (containing dextran sulfate and formamide, Sigma-Aldrich product no. H7782). Probes were denatured at 75°C for 5 min.

Slide metaphase and SC spreads were dehydrated by passage through an alcohol series (70%, 80% and 95% ethanol), denatured in 70% formamide/2XSSC at 70°C for 1min 10 sec, chilled in ice-cold 70% ethanol for 3 min, dehydrated by passage through a second alcohol series (80%, 90% and 100% ethanol) and air dried. The probes were dropped on the slides preparations, covered with coverslips and incubated overnight at 37°C in a moist chamber. After hybridization, slides were washed three times in 50% formamide/2XSSC at 42°C, twice in 0.2XSSC at 42°C and once in 4XSSC/0.05% Tween-20 at room temperature. Slides were then incubated for 40 min at room temperature in 3% BSA/4XSSC/0.05 % Tween-20. Hybridization of the labeled probes was detected by incubation of the slides at 37°C for 30 min in 1% BSA/4XSSC/0.05% Tween-20 containing Cy3-conjugated streptavidin (Amersham Pharmacia Biotech) and antidigoxygenin-fluorescein (FITC)-labeled antibody fragments (Roche). After detection, slides were washed twice in 4XSSC/0.05% Tween-20 at 42°C and finally in distilled water before air dry at room temperature in the dark.

Image processing and measurement

After slides were air dried, chromosomes were counterstained with 4', 6-diamidino-2-phenyloindole (DAPI) (Vectashield). Cy3, FITC and DAPI signals were captured separately as 8-bit black-white images, which were pseudo-colored, enhanced and

overlaid using a Cytovision image analysis system (Applied Imagine). Mitotic and meiotic chromosomes, paired and unpaired regions, and distances between hybridization signals were measured in micrometers (µm) by using the free software, ImageJ (http://rsb.info.nih.gov/ij/index.html) and the measurements from the mitotic and meiotic spreads were converted to Flpter. The Flpter distances from the marker *OniY*227, the gene *dmrt4* (*Dmo*), the centromere and the branch point of the unpaired region from chromosome 1, hybridised onto meiotic chromosomes, were converted into cM distances for a direct comparison with the LG3 map, using the formula:

cM= 95 (1 -Flpter)

Where 95 is the total length in cM of the LG3 map (Lee et al., 2005). The comparison of the cytogenetic markers of the bivalent 1 and the respective linkage group (LG) was supposed to identify any contraction or expansion of the LG relative to the physical distances between the mentioned markers.

Results

Morphology of mitotic chromosomes and SCs after DAPI staining

The diploid chromosome number in the Nile tilapia mitotic spreads examined was 44, and 22 bivalents were observed in the meiotic spreads, as previously reported (Majumdar and McAndrew, 1986; Foresti et al., 1993; Carrasco et al., 1999). The Nile tilapia karyotype consists of only subtelocentric chromosomes and chromosome 1 is the longest and most easy recognizable chromosome. Both mitotic and meiotic spreads stained with DAPI provided banding patterns corresponding to chromosomal regions showing different chromatin composition. In almost all analysed male mitotic cells (210 chromosome spreads from five different males), it was possible to observe different patterns of DAPI staining between the two copies of chromosome 1, that enabled us to distinguish chromosome 1 type A and chromosome 1 type B (Fig. 1). Both of these displayed terminally located DAPI negative bands in the q arm, however this region was longer in type A. DAPI staining of mitotic chromosomes from females (45 spreads from three different fish) showed two copies of type B chromosome 1 in each case.

Small discrete DAPI positive sites corresponding to the pericentromeric regions were observed on most of the bivalents at pachytene stage (arrowheads in Figs. 2 and 3b). Such sites were not seen in mitotic metaphase spreads.

Physical mapping of the selected clones and the orientation of the unpaired region

Hybridization with the *OniY*227 probe indicated its interstitial location on the q arm of bivalent 1 as well as mitotic chromosome 1, while the *dmrt4* probe was assigned to the

same arm but the signal was closer to the centromere (Figs. 3 and 4). Both probes were always confined to the fully paired region of bivalent 1, however *OniY*227 was shown to be located close to the boundary of the unpaired region of this bivalent (Fig. 4). The DAPI positive pericentromeric region was located near to the opposite end of the bivalent to the unpaired region.

The average length of bivalent 1 in its fully paired state was 17.4 µm (n = 16, from four males, range 13.6 – 22.1 µm) and the average length of bivalent 1 where partial unpairing was observed was 19.4 µm (n = 5, from two males, range 14.3 – 21.6 µm), considerably longer than the average length of the mitotic chromosome 1 (6.0 µm: n = 30, from 4 males, range 3.8 - 10.2 µm) (Table 1 and 2). The length of the unpaired region on bivalent 1 differed between five analyzed cells (branch point at Flpter 0.79-0.93) from two different males (Table 3). The two arms of the same unpaired region sometimes differed in length, however this was inconsistent and the sample size was small.

The relative distances (Flpter) from the p terminus of the centromere and FISH signals for meiotic and mitotic stages are shown in Table 1. The centromere and FISH signals showed greater variation in estimated position in the mitotic preparations than in the meiotic preparations, probably due to the smaller size of the mitotic chromosomes.

Fig. 5 shows a physical map of this chromosome, using the present data converted into cM based on the estimated size of LG3. It was observed that the relative physical distances of *OniY*227 and *dmrt4* converted into cM (33cM and 74 cM, respectively) did not correspond closely to the distances from LG3 (22 cM and 54 cM, respectively) (Table 4).

Discussion

The Nile tilapia mitotic chromosomes and SC spreads were stained with DAPI fluorochrome which is specific for double stranded DNA, interacting with DNA by at least two different mechanisms. In regions where three or four AT base pairs are located in tandem, DAPI binds to the minor groove of the DNA and this results in a highly fluorescent compound. DAPI also intercalates between bases, which results in non-fluorescent compounds. The latter reaction is energetically favoured in GC-rich areas (Kapuscinski, 1995).

Discrete small DAPI positive sites corresponding to pericentromeric regions were shown on the Nile tilapia SCs (Figs. 2 and 3). This suggested that these chromosomal regions are rich in relatively small AT-rich clusters, visible only on extended loops of DNA. DAPI-positive regions in pericentromeric positions have been previously identified on mitotic chromosomes in several fish species and the intensity and number of the signals vary from few and very weak (Ocalewicz, 2005) to very strong and localized on most (Ocalewicz et al., 2004) or all of the chromosomes (Fischer et al., 2000). Moreover, DAPI positive signals can be seen in interstitial positions on fish chromosomes (Ocalewicz et al., 2007). This is the first report of identification of AT-rich clusters on fish SCs. In mouse SCs, DAPI positive staining of the AT-rich chromosomal segments is used as a marker of centromere position (Anderson et al., 1999; Froenicke et al., 2002). Neither pericentromeric heterochromatin, euchromatin nor telomeres were readily differentiated after DAPI staining in tomato (*Solanum lycopersicum*) SCs. However, some bivalents possess single regions which do not fluoresce – DAPI negative bands (Peterson et al., 1999). In the clam (*Dosinia exoleta*), ribosomal regions are associated

with the terminal, DAPI-negative chromatin of one bivalent (Hurtado and Pasantes, 2005). Moreover, DAPI banding has been used as a part of an approach aiming at identification of all chromosome arms in common shrew SCs. This method, combined with immunostaining, revealed characteristic DAPI positive and negative bands of the bivalents (Belenogova et al., 2006).

The unpaired region on the Nile tilapia bivalent 1 was visible after DAPI staining and was located at the opposite end to the DAPI bright band showing the centromere, while the two FISH probes (*OniY*227 and *dmrt4*) provided additional landmarks on this bivalent (Figs. 3 and 4). This confirms the orientation of the unpaired region in this bivalent, and DAPI staining appears to be more consistent in showing the centromeres than silver staining (Carrasco et al., 1999). The length of the unpaired region differs depending on the stage of meiosis (Griffin et al., 2002); we were able to observe fully paired copies of bivalent 1 and also the bivalent with an unpaired region of up to 20 % of its total length (Fig. 2). In early pachytene when the bivalent is longer and the unpaired region is visible, the DAPI positive pericentromeric bands are less visible, probably because the bivalent is less condensed.

Hybridization signals from the *OniY*227 probe were seen close to the beginning of the unpaired region. The second probe, *dmrt4*, was mapped close to the centromere in the long arm (Fig. 3). When comparing the meiotic map from the present results with LG3 (Lee et al., 2005) it was shown that while the total lengths are fairly similar (85 cM + p arm and 95 cM respectively: Fig. 5), recombination appears to be elevated around the centromere and suppressed in the distal region of the q arm, which corresponds to the region of delayed pairing in meiosis. A comparison of markers mapped to mitotic

chromosome pair 1 and LG3 gave similar results (Cnaani et al., 2008). However, physical mapping of markers from the end of LG3 that is located close to the q arm telomere (GM 354 and GM 204) shows a clear gap between these markers and the end of the chromosome arm, suggesting that LG3 does not span the entire length of the q arm of chromosome 1 (Mota Velasco, unpublished observations; Cnaani et al. 2008).

Comparison of bivalent no 1 and mitotic chromosome no 1 showed that the SC bivalent can be more than 3 times longer than the corresponding condensed chromosomes at the mitotic metaphase stage. This together with the facts that in SC spreads chromatin extends as a diffuse cloud around each proteinaceous structure and each bivalent shows four closely associated copies of each locus, while there are only two such copies on a metaphase chromosome, make the extended DNA loops from SCs a better substrate for FISH probes than the DNA in the metaphase chromosomes (Solari and Dresser, 1995). Such an approach for the physical mapping of closely associated loci seems to be very useful in fish with very small chromosomes.

Several lines of research have produced evidence that chromosome pair 1 in *O. niloticus* has characteristics of sex chromosomes. The region of delayed pairing seen in bivalent 1 is only observed in XY (but not XX or YY) genotypes (Carrasco et al., 1999; Griffin et al., 2002). Such behaviour is characteristic of differentiated sex chromosomes in fish (Penman and Piferrer, 2008) and other vertebrates (Solari 1994). The terminal part of the q arm which shows this delayed pairing is characterised by an accumulation of heterochromatin and the presence of transposable elements such as SINE, Ron-1 and CiLINE2 sequences (Oliveira et al., 1999; Harvey et al., 2003), often associated with regions of suppressed recombination in sex chromosomes. There are differences in

relative hybridization of microdissected chromosome 1 probes to XX, XY and YY karyotypes (Harvey et al., 2002) and differences in distribution of chromosome 1 repetitive DNA elements between the X and Y chromosomes (Harvey et al., 2003; Ferreira and Martins, 2008), and in the present study we observed differences in DAPI staining of the two copies of chromosome 1 in XY individuals. However, markers in LG1 have been shown to be strongly associated with sex in *O. niloticus* (Lee et al., 2003; Cnaani et al., 2008) and LG1 maps to a small pair of chromosomes, while LG3 (strongly associated with sex in *O. aureus* but not in *O. niloticus*) maps onto chromosome pair 1 (Lee et al., 2004; Cnaani et al., 2008, Fig. 4). The sex determining locus in LG1 also has an influence on sex in *O. aureus*, epistatic to that of the locus in LG3 (Lee et al., 2004; Cnaani et al., 2008). While the small pair of chromosomes corresponding to LG1 can be identified by FISH using LG1 marker probes (Cnaani et al., 2008), it is not possible to distinguish it from most of the other small chromosome pairs by conventional cytogenetic techniques.

Nagl et al. (2001) suggest that tilapiines diverged from haplochromines >8 million years ago, and that much of the divergence within the tilapiines occurred approximately 1.1 to 6 m years ago. Phylogenetic analysis suggests that there have been repeated transitions between LG3 and LG1 as sex chromosomes in tilapia, and it has been suggested that the sex determining locus in LG3 (chromosome pair 1) is the ancestral sex determining gene in this group (Cnaani et al., 2008). Chromosome pair 1 has more of the characteristics of a sex chromosome pair (a broad region of recombination suppression as well as the accumulation of repetitive elements and other features described above) than the chromosome pair carrying the proposed newer sex determination locus in LG1.

However, there is currently no explanation for the paradox that chromosome pair 1 still shows several features implicating it as a sex chromosome pair (e.g. unpaired region in early pachytene in XY individuals) in a species (*O. niloticus*) where it does not apparently determine sex.

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Table 1. The average, maximum and minimum distances in Fractional Length from the p terminus (Flpter) to the centromere, *dmrt4* and *OniY*227 in male *O. niloticus*.

	SC spreads			**Mitotic spreads**		
	Flpter *OniY*227	**Flpter *dmrt4***	**Flpter CEN**	**Flpter *OniY*227**	**Flpter *dmrt4***	**Flpter CEN**
Average	0.65	0.22	0.11	0.57	0.25	0.14
Maximum	0.72	0.25	0.13	0.65	0.36	0.24
Minimum	0.60	0.18	0.09	0.51	0.19	0.06
Range	0.12	0.07	0.05	0.14	0.18	0.17

CEN= Centromere

Table 2. The estimated positions (mean and range, in Flpter proportions) of the centromere and the two FISH signals in chromosome 1 of *O. niloticus*, estimated from bivalent 1 of the synaptonemal complex or from mitotic chromosome preparations (based on four males in each case).

	SC bivalent 1	**Mitotic chromosome 1**
Centromere	0.11 (0.09 – 0.13, n = 15)	0.14 (0.06 – 0.24, n = 30)
dmrt4	0.22 (0.18 – 0.25, n = 10)	0.25 (0.19 – 0.36, n = 13)
*OniY*227	0.65 (0.60 – 0.72, n = 15)	0.57 (0.51 – 0.65, n = 20)

Table 3. The length of the unpaired region on the bivalent of chromosome 1 in XY male *O. niloticus*. The measurements were made from the p terminus (Flpter) observed. Serial number 1, 3, 4 and 5 represent observations from XY male A and serial number 2 from XY male B.

Serial number	SC full paired length (µm)	Unpairing origin 1st arm (µm)	Unpairing origin 2nd arm (µm)	SC Total length average (µm)	Flpter 1st arm	Flpter 2nd arm	Flpter Average	Range	Proportion Unpaired (%)
1	21.63	3.66	5.53	26.22	0.83	0.74	0.79	0.09	21.24
2	14.31	2.52	2.60	16.87	0.82	0.82	0.82	0.01	17.90
3	17.48	1.30	1.30	18.78	0.93	0.93	0.93	0.00	7.44
4	17.97	0.73	1.63	19.15	0.96	0.91	0.93	0.05	6.56
5	15.04	1.14	1.14	16.18	0.92	0.92	0.92	0.00	7.57

Table 4. Conversion to centimorgans (cM) of Flpter positions of minimum and maximum unpairing, *Oni*Y227, *dmrt4* and centromere, measured from bivalent 1 of SC meiotic spreads in XY male *O. niloticus* during pachytene stage.

Feature	Conversion of position (cM)
Minimum unpairing	7
Maximum unpairing	19
*OniY*227	33
dmrt4	74
Centromere	85

Figure captions

Figure 1. a) Graphic representation illustrating the difference in morphology between type A and B of homologous mitotic chromosome 1, showing brighter areas of high heretochromatin accumulation under DAPI stain and location in Flpter of markers observed by FISH; **b, c)** DAPI staining performed on the Nile tilapia female (XX) and male (XY) mitotic chromosome 1 respectively; **d, e)** enlarged images of chromosome 1 from Fig. 1b and c respectively. Arrows in Figs 1b and c indicate centromeres recognised by DAPI negative areas on the chromosome 1A and 1B. Dotted circles in Fig.1 b, c, d and e indicate the telomeric region on chromosome 1 where the main differences between A and B are observed. Scale bar = 5μm.

Figure 2. Nile Tilapia DAPI-stained meiotic spread (top) and karyotype (bottom) in black and white contrast, indicating pericentromeric positions by arrowheads. Scale bar = 5μm.

Figure 3. FISH with *OniY*227 (green) and *dmrt4* (pink) performed on the Nile tilapia male XY mitotic (**a**) and meiotic (**b**) spreads counterstained with DAPI. Arrows in Fig. 3a indicate centromeric constriction on the mitotic chromosomes 1, and arrowheads (where possible) in 3b indicate DAPI-bands on pericentromeric regions. Scale bar = 5μm.

Figure 4. The Nile tilapia male XY meiotic spreads showing a maximum (**a**) and minimum (**b**) unpaired region, also showing enlarged bivalents to the right. Marker *OniY*227 was hybridised by FISH and chromosomes counterstained by DAPI, enabling identification of pericentromeric regions (indicated by arrowheads where possible). Scale bar = 5μm.

Figure 5. Ideogram from chromosome 1 and LG3 (from Lee et al., 2005, with permission), compared after conversion of Flpter to cM. Early unpaired region during pachytene, sex-linked marker (*OniY*227), gene *dmrt4* and the centromere are indicated on the ideogram (left) also displaying ranges of measures. *OniY*227 and *dmrt4* are anchored to LG3, as indicated by a dotted line.

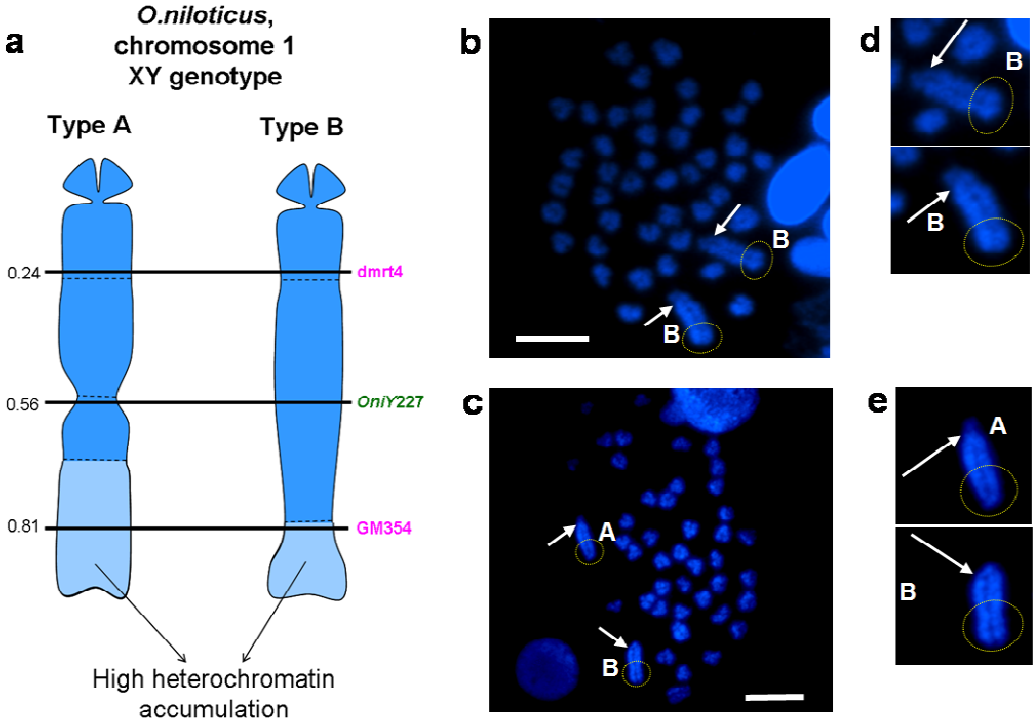
a
O.niloticus,
chromosome 1
XY genotype
Type A
Type B
0.24
dmrt4
0.56
OniY227
0.81
GM354
High heterochromatin
accumulation
b
B
B
c
A
B
d
B
B
e
A
B

Fig. 1

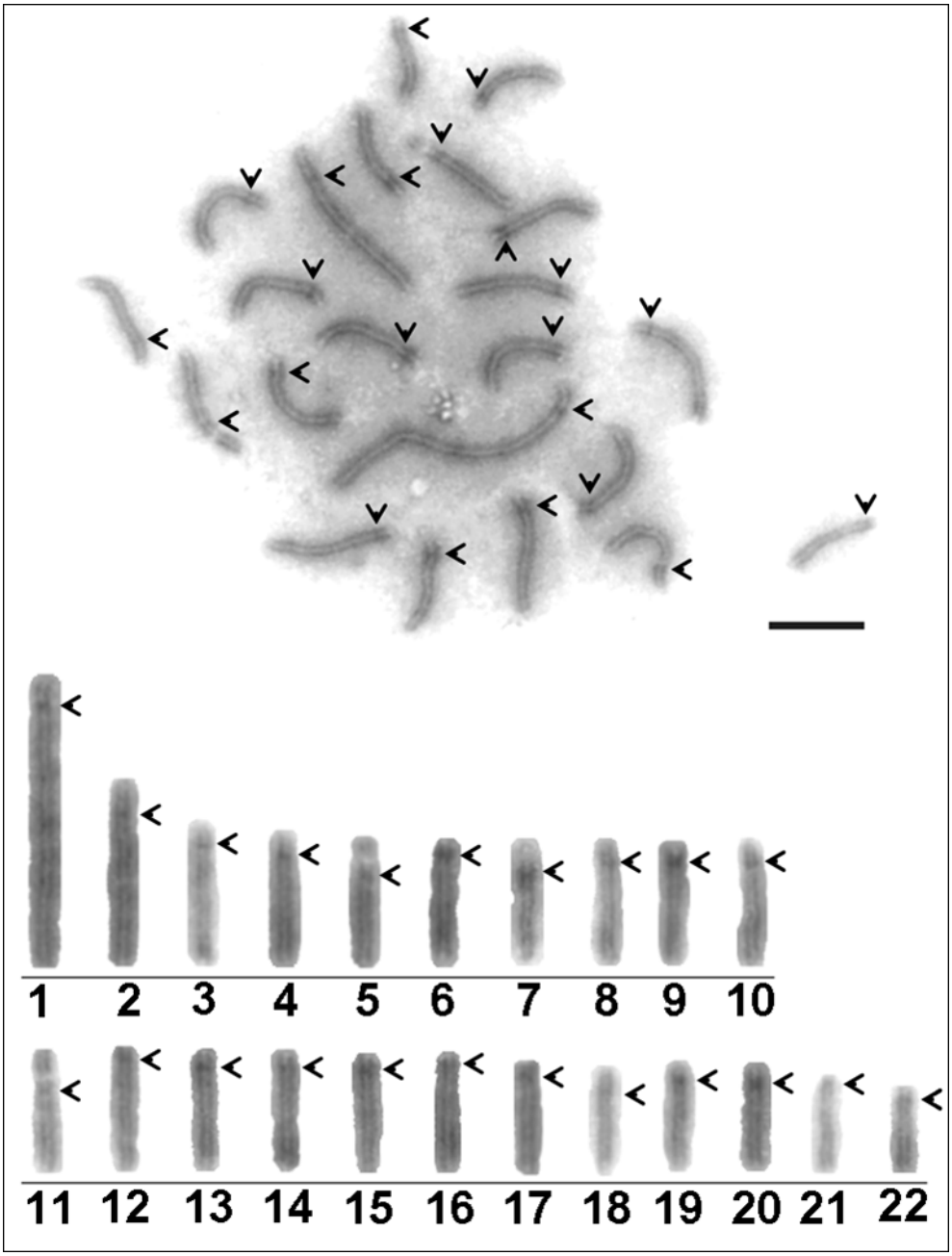

Fig. 2

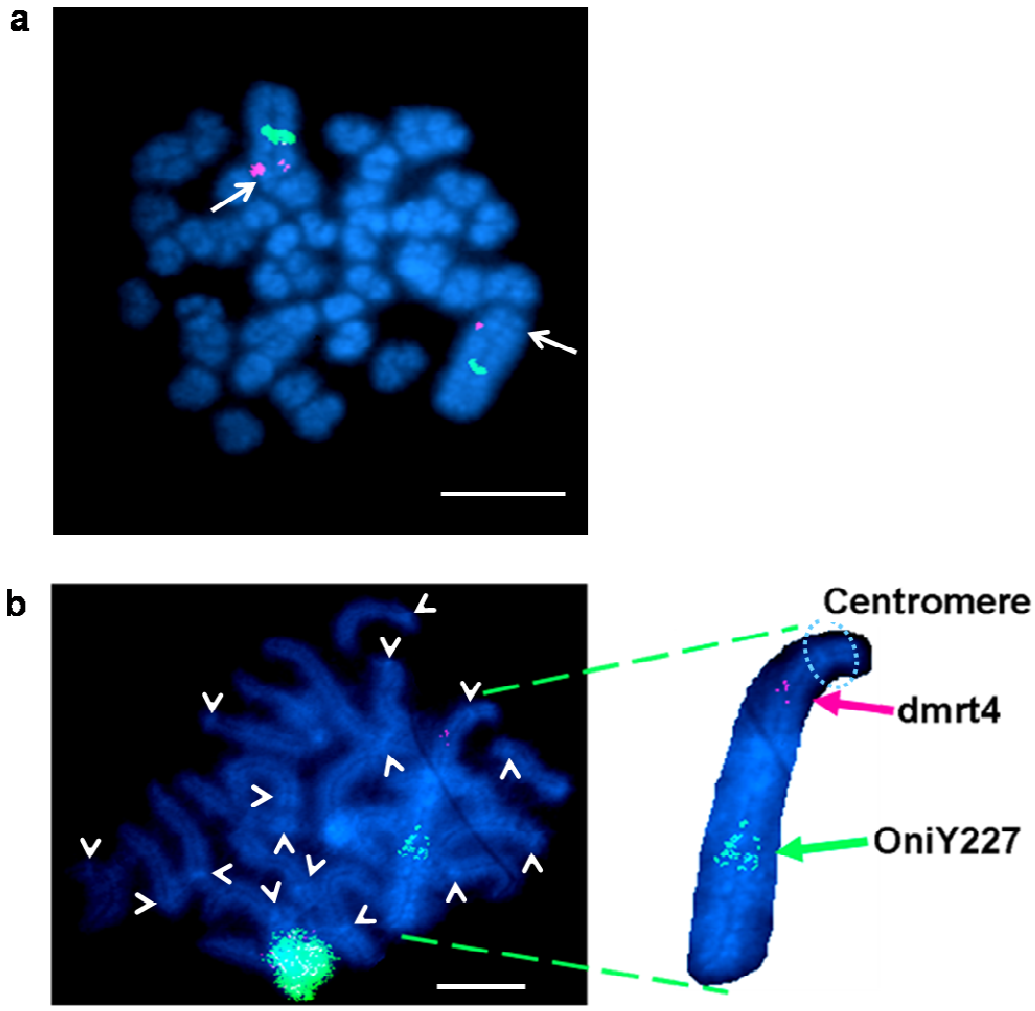

Fig. 3

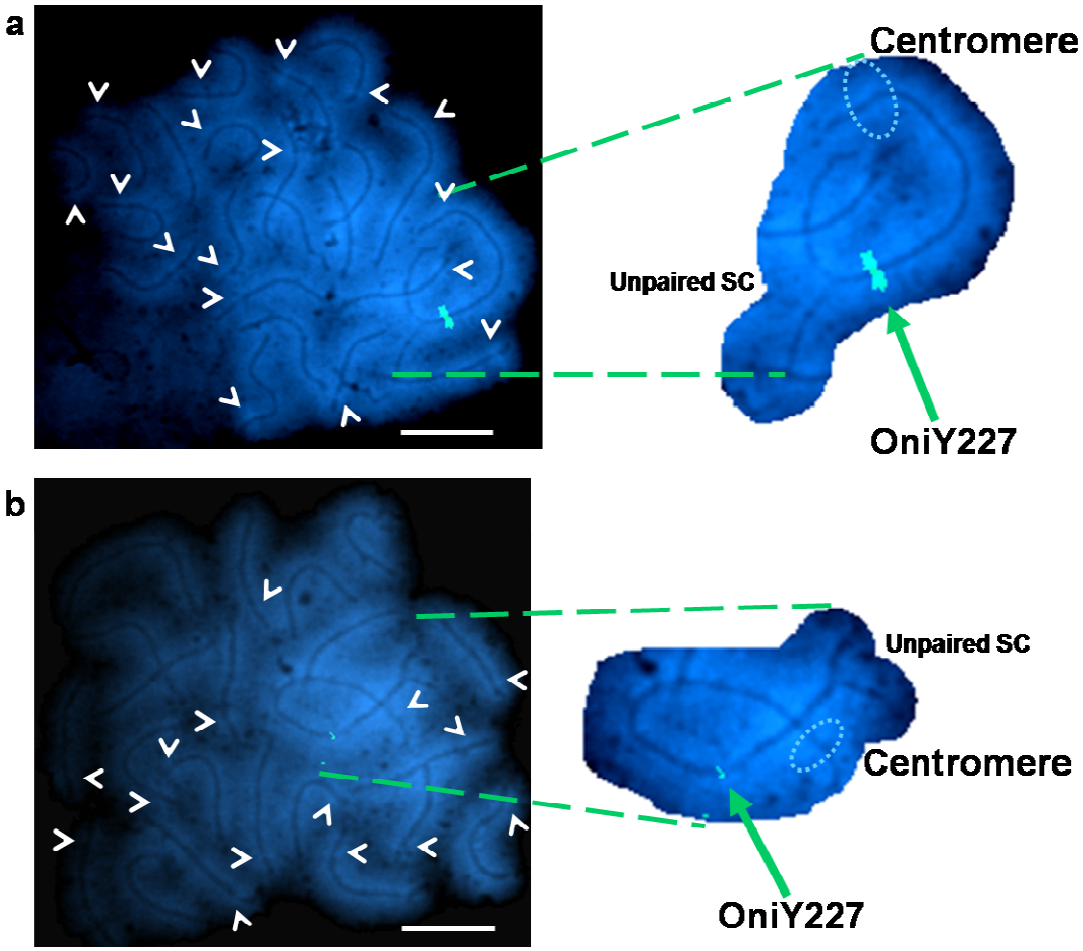
a
Centromere
Unpaired SC
OniY227
b
Unpaired SC
Centromere
OniY227

Fig. 4

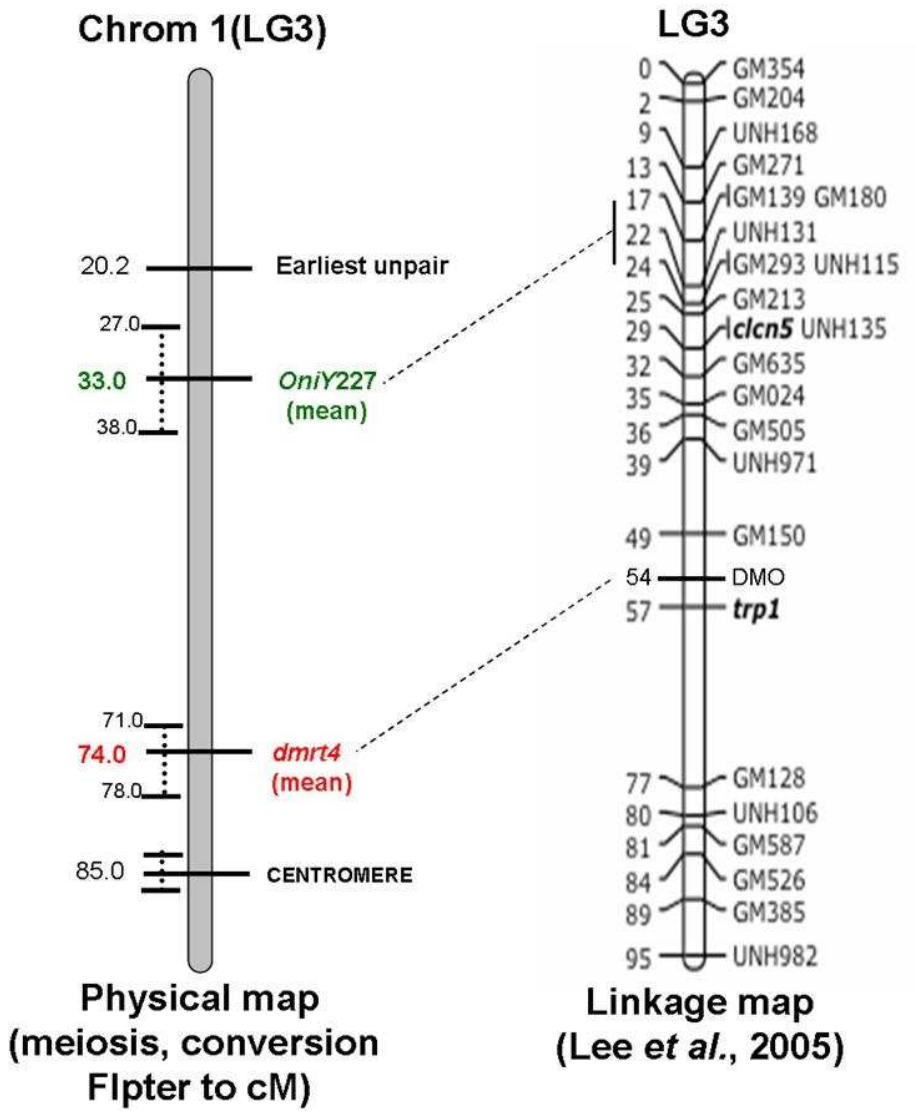
Chrom 1(LG3)
20.2
Earliest unpair
27.0
33.0
OniY227
(mean)
38.0
71.0
74.0
dmrt4
(mean)
78.0
85.0
CENTROMERE
Physical map
(meiosis, conversion
FIpter to cM)
LG3
0 GM354
2 GM204
9 UNH168
13 GM271
17 GM139 GM180
22 UNH131
24 GM293 UNH115
25 GM213
29 clcn5 UNH135
32 GM635
35 GM024
36 GM505
39 UNH971
49 GM150
54 DMO
57 trp1
77 GM128
80 UNH106
81 GM587
84 GM526
89 GM385
95 UNH982
Linkage map
(Lee et al., 2005)

Fig. 5