CYTOGENETIC STUDIES OF *PSEUDOBARBUS* AND SELECTED *BARBUS* (PISCES: CYPRINIDAE) OF SOUTHERN AFRICA.

THESIS

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Pseudobarbus burchelli A. Smith (1841)

A redfin minnow

This thesis is dedicated to Ba and Bapuji
(Savita and Thakor Naran)

ABSTRACT

The aim of this study was to explore the karyology of temperate southern African barbine species in order to determine the extent and significance of polyploidy within the species. The study presents an optimised *in vivo* karyological protocol for the small barbines. The analysis of chromosomal data was explored using two approaches; measured (quantitative) and visual (qualitative).

The karyology of 16 species of *Barbus* and six species of *Pseudobarbus* (Pisces: Cyprinidae) is reported. The study represents an almost complete (22/23 species) karyological survey of the temperate barbines. Chromosome number, arm numbers (NF) are recorded and karyotype morphology described for all species. Nucleolar organiser regions (NOR)s of 18 species have been examined by silver staining. Seven different AgNOR phenotypes occur among the species examined.

Chromosome complements of the different species indicate at least three ploidy levels; diploid, tetraploid and hexaploid. Higher ploidy levels are represented in 78% (18/23) of species within the temperate fauna. This finding is remarkable, in the light of global proportions of cyprinid karyotypes, and provides an opportunity to shed further light on the evolution of polyploidy in barbine cyprinids.

Results show that morphologically defined species complexes have distinct karyotypes, and most probably form cohesive phyletic clades.

A phylogenetic tree was constructed using traditional morphological characters and tested against karyological data. The results suggests that similar ploidy states do not necessarily indicate close relationship between species. This supports a hypothesis of polyphyly for the African barbine cyprinids and also provides independent support for species complexes such as the "chubbyhead barb group" and the *Pseudobarbus* lineage.

The broader implications of karyological findings are discussed within the context of African barbines and recommendations for further cytogenetic research are provided.

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SL - standard length

a - acrocentric chromosomes

m - metacentric chromosomes

sm-st/sm+st - submetacentric and subtelocentric chromosomes

NF - total number of chromosome arms

NF1 - 2(**m+sm**)+**st+a**

NF2 - 2(**m+sm+st**)+**a**

AgNOR - silver stained Nucleolar Organiser Region

large:**s**mall;**l:s** - largest chromosome to smallest chromosome ratio

L/S arm-ratio - ratio of long arm ÷ short arm (*sensu* Levan *et al.* 1964).

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CHAPTER 1: INTRODUCTION

The cyprinids are a major component of the freshwater ichthyofauna of Africa (Skelton *et al.* 1991, Skelton 1994). The most speciose genus within the family is *Barbus*, with approximately 300 species in Africa, and comprising 85% of the fauna in southern Africa (Skelton 1993, 1994). This diverse group of African *Barbus* has been divided into two groups, based on morphological criteria, large and small to medium (Boulenger 1911, Banister 1973). The large *Barbus* are characterised by adult size of ≥200mm standard length and have parallel scale striae. The small and medium *Barbus* are characterised by adult size ≤200mm standard length and with radiate scale striae. The latter group comprise 75-80% of the African species and are in turn subdivided into categories based on the nature of the primary dorsal fin ray: flexible, soft rayed minnows; spinefin barbs and sawfin barbs (Jubb 1967). These parameters have served to organise the genus into broad species groups or lineages. However, many taxonomic and systematic problems within these heterogeneous barbine groups remain unresolved.

Cytogenetic analysis and genetic studies have increasingly been used to resolve phylogenetic relationships in many taxa. Karyological diversity within several vertebrate assemblages such as marine mammals (Árnason 1980), bats (Baker & Bickham 1980), neotropical turtles (Bickham & Baker 1976), mole rats (Nevo *et al.* 1995), frogs (Bogart 1979, Channing & Bogart 1996), and snakes (Baker *et al.* 1972, Robert *et al.* 1972, Bogart 1979) has been used to explore the evolutionary history of the taxa. The karyotype of a species has become a valuable tool for resolving many systematic, phylogenetic, taxonomic and evolutionary problems in fishes. Among some fish taxa, diploid numbers and karyotype morphologies are constant and are thought to represent a more conservative type of evolution. For example, the neotropical Cichlidae where the diploid number is 48, and the karyotype is made up of predominantly subtelocentric-acrocentric chromosomes (Feldberg & Bertollo 1985). Other families presenting very similar chromosome numbers and karyotypes include Anastomidae, Curimatidae, Chilodontidae (in Feldberg *et al.* 1992).

In other fish taxa a diverse array of chromosome numbers have been found, and several are polyploid taxa. Ancient taxa such as sturgeons (Acipenceriformes), paddlefish (Poliodontidae) and lungfish (Protopteridae) show evidence of tetraploidy (Dingerkus & Howell 1976, Schultz 1979, Vervoort 1980). Occurrence of polyploidy in salmonids and thymallids (Hartley 1987, Rab *et al.* 1994a), catostomids (Uyeno & Smith 1972), cobitids (Kobayashi 1976), poecilids (Sola *et al.* 1990) callichthyids (Oliveira *et al.* 1992) and cyprinids (Buth *et al.* 1991, Klinkhardt *et al.* 1995) have been reported.

Cyprinids have a variable chromosome complement involving several degrees of polyploidy. Buth *et al.* (1991), estimated that there are at least 52 polyploid cyprinid species and subspecies that generally include three ploidy levels; triploids 2n=70, tetraploids 2n=100 and hexaploids 2n=150.

Genetic (allozyme) and karyological studies on the cyprinids, particularly Eurasian species, are contributing to resolving some of the phylogenetic problems (Yu *et al.* 1987, Agnèse *et al.* 1990b,

Berrebi *et al.* 1990, Collares-Pereira & Madeira 1990, Rab & Collares-Pereira 1995). Karyological data have been used to unite putative monophyletic groups within cyprinids (Arai 1982, Zan *et al.* 1986, Yu *et al.* 1987). Polyploidy has been used to indicate lineages within the family.

Currently, there are two theories proposed to account for the ploidy levels found within the family. Ohno (1970a&b), proposed that the ancestral group has a diploid complement of about 50 and the derived group has a tetraploid complement of about 100. This hypothesis is based on the fact that 74% of the cyprinids examined have modal diploid numbers of about 50. Higher ploidy levels are proposed to occur via duplication of the original chromosome set.

Collares-Pereira & Coelho (1989) proposed an alternative theory suggesting that the ancestral condition has a high diploid chromosome number of about 100 and that this has been reduced through successive fusion events to about 50. These fusion events probably occurred independently among several lineages and the other ploidy levels arose secondarily by polyploidy of the previously diploid karyotypes. Karyology is therefore contributing to an active debate on the evolution of this large family.

Since the first report of hexaploid karyotypes amongst southern African *Barbus* by Oellermann (1988) and Oellermann & Skelton (1990), three recent findings of hexaploidy (amongst large species) have been reported (Golubstov & Kryzanov 1993, Guégan *et al.* 1995, Rab *et al.* 1995). Tetraploidy has been reported for *Barbus* sensu stricto, and is also thought to include other North African *Barbus* which are shown to be hexaploid species (Doadrio 1990, Guégan *et al.* 1995, Rab *et al.* 1995). These investigations indicate the importance of karyological analyses for interpreting phylogenetic problems in this diverse group.

To date, only a few southern African *Barbus* species have been karyotyped. Karyotypes of only six large *Barbus* species have been published (Oellermann & Skelton 1990). Preliminary modal chromosome numbers of two *Labeo*, one *Varicorhinus* and seven *Barbus* species are known (Oellermann 1988). Naran (1992) has shown high chromosomes numbers of evolutionary tetraploid origin in four *Pseudobarbus* species and diploid origin in four *Barbus* species. The existence of multiple genomes in several cyprinid taxa suggests that the occurrence of polyploidization in the family may represent a complex situation (Rab & Collares-Pereira 1995). These few studies clearly indicate that several ploidy levels are represented within the southern African barbines. Further investigations could be useful by providing an independent means of interpreting phylogenetic relationships.

The southern African ichthyofauna have been biogeographically grouped into temperate (Cape and/or Karroid) and tropical (Zambezian) regions (Skelton 1993) (Fig. 1.1). Cyprinids comprise 81% or 27 out of 33 species within the temperate region. The majority (23 species) are barbine, organised into the distinctive species groups; *Barbus*, *Labeo* and *Pseudobarbus* (Skelton *et al.* 1991, Skelton 1994).

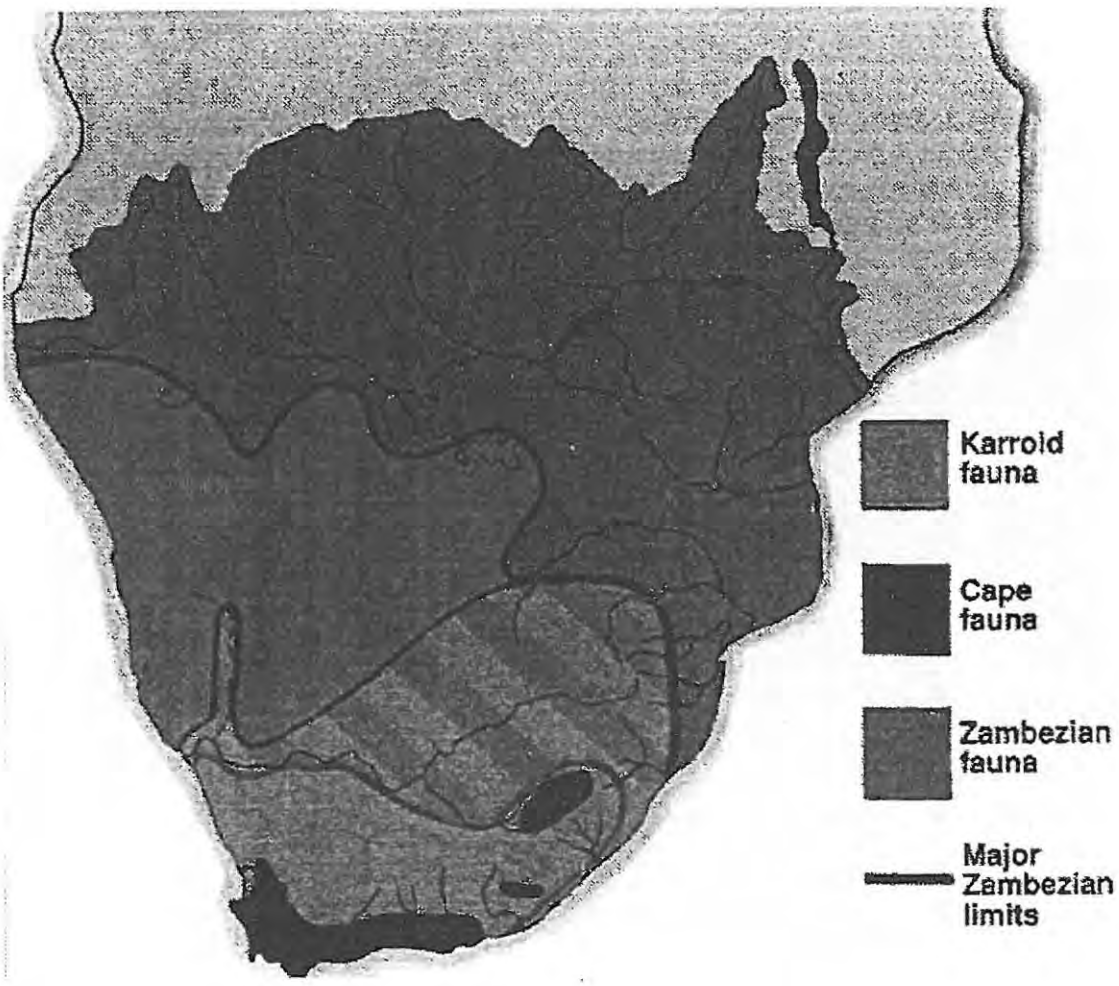


Figure 1.1 Biogeographic zones of southern African ichthyofauna (from Skelton 1993).

The genus *Pseudobarbus*, commonly known as redfin minnows, is a distinct southern African lineage which was separated from *Barbus* by Skelton (1980, 1988). The seven species of *Pseudobarbus* are characterised by a suite of morphological and osteological features which indicated these formed a monophyletic lineage (Skelton 1980, 1988). The sister group relationship of the flexible rayed, *Pseudobarbus* is not yet established, but, was suggested by Skelton (1980, 1988) to include a group of small, flexible rayed species known as the *B. anoplus*-complex. Furthermore, the relationship of two serrated rayed species, *B. calidus* and *B. erubescens*, each with similar colour patterns (red fins) to the *Pseudobarbus*, was undetermined. Subsequently, using detailed comparative (morphological and osteological) analysis both serrated rayed species were excluded from the *Pseudobarbus* group (Skelton 1980, 1988). Skelton (1980), suggested that possible candidates for sister group relationships with the serrated rayed redfins are three other serrated rayed species *B. hospes*, *B. trevelyani* and *B. argenteus* (Skelton 1980).

Preliminary karyological findings reported by Oellermann (1988), indicated that three of the

Pseudobarbus species have high chromosome numbers and this finding provides an excellent opportunity to test the monophyly of the *Pseudobarbus* group and to examine the role of polyploidy in the southern African barbines.

Obtaining good reliable metaphase spreads for karyological interpretation was one of the major problems indicated by Oellermann & Skelton (1990). Accordingly, an initial objective of this study was to optimise a working technique for small barbine fishes.

The main aims of this study were:

1) to describe the karyotypes of the *Pseudobarbus* and other southern African barbines;

2) to compare and seek shared derived characters that can be used to test the following hypotheses:

i) *Pseudobarbus* is a monophyletic lineage;

ii) sister outgroup is the *B. anoplus*-complex; and

iii) *B. calidus* and *B. erubescens* are not members of the *Pseudobarbus*-lineage, and are possibly more closely related to *B. hospes*, *B. trevelyani* and *B. argenteus*.

The interpretations of species relationships in this study are according to Hennigian (cladistic) principles, where shared derived characters (synapomorphies) defining a group are considered most important and relationships are displayed using cladograms. The species within a monophyletic group are related by synapomorphic characters.

The structure of this thesis is as follows:

In chapter 2 and 3 the methods used for karyotyping *Pseudobarbus* and small *Barbus* are described and the method used to analyse the karyotype is discussed. The karyotypes of the *Pseudobarbus* species are explored in chapter 4, and karyotypes of their hypothesised (flexible rayed) sister group, *B. anoplus*-complex is examined in chapter 5. A heterogenous outgroup of serrated rayed minnows are investigated karyologically in chapter 6. The silver stained nucleolar organizer region (AgNOR) are described in chapter 7. Karyological data are interpreted using a morphologically based cladogram in chapter 8. A general discussion with conclusions, incorporating other karyologically based ideas and trends within the African *Barbus* species are explored in chapter 9.

CHAPTER 2: MATERIALS AND METHODS

INTRODUCTION

Phylogenetic studies on cyprinids including other taxa have increasingly involved modern cytogenetic techniques (Klinkhardt *et al.* 1995, Rab & Collares-Pereira 1995). Several recent reviews on the methods used to prepare fish chromosomes are available (Gold *et al.* 1990, Ozouf-Costaz 1987, Thorngaard & Disney 1990, Al-Sabti 1991, Foresti *et al.* 1992a, Foresti *et al.* 1992b). Cyprinid chromosomes are generally small and numerous (Amemiya & Gold 1986, Rab & Collares-Pereira 1995) and it is difficult to obtain the necessary quantity of quality metaphase cells to adequately analyse the chromosome complements. Attempts have been made to standardise the protocol for fish karyotyping. However, optimal treatment can depend on the several factors such as the nature of the taxon (freshwater, marine, migratory, temperate, tropical or cold water), season, sexual maturity, growth rate, age, feeding, health condition and, undoubtably, on individual response to such treatment (Foresti *et al.* 1992b). Often, difficulties with fish chromosomes studies are technical in nature which can only be overcome with experimentation.

Many of the species examined in this study are small (>150mm SL, mass range=1.0-5.0g) so that certain tissues obtained from these specimens are also limited. As considerable effort was spent to optimise the karyological method for species used in this study, an outline of the basic protocol for preparing the chromosomes is presented in this chapter.

The study of the detailed structure of chromosomes is an active area of research. According to the scaffold model (Paulson & Laemmli 1977, in Sumner 1990), DNA, the main component of chromosomes, is thought to be arranged in large loops of chromatin fibres surrounding a scaffold itself. It is also thought that the scaffold may assume a spiral form (Sumner 1990). The chromosomes contract during metaphase thereby making them visible under the light microscope.

Numerous, relatively small sized chromosomes with a gradual centromere shift from median to terminal position are characteristic of barbine species examined in this study. These features contribute to difficulties in clearly determining chromosome categories (Buth *et al.* 1991, Rab & Collares-Pereira 1995). Another feature of karyotype data is that studies have different informative values when obtained from using different techniques (Rab & Collares-Pereira 1995), and interpretation of chromosome category numbers should be evaluated critically.

Chromosome categories can be determined using measured or visual grouping techniques. Both techniques were explored in order to establish a suitable means of quantitatively describing the karyotypes of the species.

Species selected for karyological analysis

Three barbine species groups were analysed. The seven known members of the genus *Pseudobarbus* were the prime target species chosen for this study. The analysis was extended to the proposed closest sister group *viz.* the *B. anoplus*-complex which comprises four species (Skelton 1980). Several other southern African barbines were karyologically screened in pilot studies and selected on the basis of their ploidy level for comparison with *Pseudobarbus*. The species analysed in this study are listed according to Skelton (1993) (Table 2.1).

Collection of fish

Karyological analysis is carried out using live animals and specimens were collected by electroshocking or seining and kept in aquaria at the J.L.B. Smith Institute of Ichthyology until required. Collection sites, locality and sources for the samples used are listed in Table 2.1. All karyotyped specimens are housed at the J.L.B. Smith Institute of Ichthyology as RUSI voucher samples (Table 2.1).

Fish treatment

Fish chromosomes are studied microscopically after extraction from mitotically-inhibited metaphase cells. Colchicine is the most frequently used mitotic inhibitor and brings about a change in the colloidal state of the cytoplasm by increasing the fluidity of the nuclear substance and causes spindle disturbance (Sharma & Sharma 1972).

Several protocols for fish karyology have been described ranging from *in vivo* to short and long term *in vitro* preparations (Hartley & Horne 1985, Gold *et al.* 1990, Foresti *et al.* 1992a). An *in vivo* method was used for all chromosome isolation in this study. Chromosomes were prepared according to the protocol presented by Collares-Pereira at the fish cytogenetic workshop held in Concarneau in 1992 (Foresti *et al.* 1992a). Modifications made to optimise the conditions are discussed in each section.

Many of the specimens analysed in this study were mature adults, (>150mm SL), past their active growth phase and this probably contributed to a relatively poor yields of metaphase cells. Several methods have been developed to increase active cell division for increasing chromosome yields. Examples of methods for enhancing the number of actively dividing cells in kidney tissue include yeast infection (Bertollo 1986, in Foresti *et al.* 1992a), phytohemaglutinin (Gold *et al.* 1990), horse serum (Ojima & Kurishita 1980, in Foresti *et al.* 1992a), CoCl3 treatment (Cucchi & Baruffaldi 1979) or protozoa infestation (Foresti *et al.* 1992a). Generally, the reaction is based on an immune response where the antigen triggers cell division, which, in turn, results in an increased yield of metaphase cells.

Table 2.1 Southern African barbine fishes karyotyped in this study. (* indicates tropical species, # indicates species analysed by Oellermann 1988, ## indicates species analysed by Naran 1992). Species are listed according to Skelton (1993).

Species	RUSI	Co-ordinates	Source	Locality
Barbus amatolicus	52690	31°19'00"S 27°51'00"E	W. Haselau	(ex Bashee system), E. Cape.
B. anoplus	52693	25°38'20"S 30°40'48"E	D. Naran & I. R. Bills	Palmiet R, E. Cape.
B. anoplus	52692	33°15'30"S 26°15'30"E	N.P.E. James & P. H. Skelton	Elands R, Incomati system, Mpumalanga.
B. gurneyi	52698	29°38'45"S 30°25'00"E	M. Coke	Karkloof R, Mgeni system, Kwazulu-Natal.
B. motebensis	52702	25°47'00"S 26°22'00"E	P. H. Skelton *et al.*	Marico R, Limpopo system, North West Province.
B. brevipinnis	52695	25°49'00"S 26°03'00"E	P. H. Skelton *et al.*	Molopo Oog, Molopo R, North West Province.
B. pallidus##	52703	28°01'00"S 30°23'00"E	M. Coke	Buffalo River, Tugela system, Kwazulu-Natal.
*B. argenteus**	52694	25°36'30"S 30°40'30"E	N.P.E. James & D. Naran	Elands R, Incomati system, Mpumalanga.
*B. eutaenia**	52697	24°58'00"S 31°30'00"E	P. H. Skelton *et al.*	Sabi R, Incomati system, Mpumalanga.
*B. paludinosus**	52704	25°53'00"S 26°01'00"E	P. H. Skelton *et al.*	Molopo Oog, Molopo R, North West Province.
B. calidus	52696	32°43'15"S 19°04'05"E	S. Thorne	Noordhoeks R, Olifants system, W. Cape.
B. erubescens	51376	32°40'35"S 19°16'05"E	I.R. Bills & M. Mariott	Twee R, Olifants system, W. Cape.
B. hospes	52699	28°28'36"S 16°41'48"E	P. H. Skelton	Orange R, N. Cape.
B. trevelyani	52706	32°45'00"S 27°15'00"E	W. Haselau	Buffalo R, E. Cape.
B. andrewi	52691	33°57'00"S 18°55'00"E	W. Haselau	(ex Breë R), W. Cape.
*B. trimaculatus**	52707	26°12'00"S 26°10'00"E	D. Naran *et al.*	Mooi R, Vaal-Orange system, North West Province.

Table 2.1 Southern African barbine fishes karyotyped in this study (* indicates tropical species, # indicates species analysed by Oellermann 1988, ## indicates species analysed by Naran 1992) cont.

Species	RUSI	Co-ordinates	Source	Locality
Pseudobarbus afer	52709	-	J. Cambray	Bezuidenhouts R, Swartkops system, E. Cape.
P. afer	52710	25°18'00"S 33°41'00"E	D. Naran *et al.*	Blindekloof R, Swartkops system, E. Cape
P. asper	52712	33°28'00"S 24°42'00"E	D. Naran *et al.*	Groot R, Gamtoos system, E. Cape.
P. burchelli	52713	33°34'08"S 19°08'21"E	S. Thorne	Wolwekloof R, Bainskloof, W. Cape.
P. burchelli	52714	33°33'19"S 19°09'00"E	S. Thorne	Steenboks R, Breë system, W. Cape.
P. burgi	52715	33°50'27"S 19°07'15"E	S. Thorne	Wemmers R, W. Cape.
P. phlegethon	52716	32°47'52"S 19°07'15"E	S. Thorne	Thee R, Olifants system, W. Cape.
P. tenuis	52717	33°22'00"S 22°10'48"E	P. H. Skelton *et al.*	Grobbelaars R, Gourits system, W. Cape.
P. quathlambae#	29009	29°32'03"S 29°15'15"E	P. H. Skelton	Sani R, Orange system, Lesotho.

Pretreatment of fish

Initial results using only colchicine treatment yielded very low quantities of metaphase cells. Thus, later in the study, some specimens were pre-stimulated using a freshly prepared yeast solution. The solution was prepared using 0.5 g of yeast and 0.5 g of sucrose, diluted in 7ml of distilled water and incubated at 37°C for 15 minutes. The solution was cooled before use. A volume of 0.1 ml/g of the yeast solution was injected into the fish. The fish were placed in an aquarium at 23-24°C for up to 48 hours. Smaller specimens were processed after 24 hours, since their survival rates decreased after 24 hours.

Fish mortality was less when the yeast treatment was performed on anaesthetised fish. Fish were anaesthetised with a 0.01% vol/vol solution of L-phenoxyethanol.

Isolation of mitotic chromosomes

Preparation of cells

1. A 0.01% wt/vol of a colchicine solution was injected into the peritoneal cavity of the fish at a dosage of 0.1 ml per gram wet weight.
2. The specimens were kept in a well-aerated aquarium at a temperature of 2-3°C above that of the holding tank. It was found that specimens kept at an elevated temperature for a week prior to the experiment gave a higher yield of metaphase cells, especially during the winter months when room temperatures dropped.
3. Specimens were sacrificed two hours after the colchicine treatment, using an overdose of L-phenoxyethanol, (5% vol/vol). The kidneys, gonads and occasionally gills were used for obtaining chromosomes (see * page 10, for previously used technique).
4. The tissue was placed in a hypotonic solution of 0.4% wt/vol potassium hydroxide for 30 minutes. During the first 10 minutes the tissue was macerated over a 0.05mm mesh-size grid. The resultant cell suspension was filtered through the grid several times to remove large pieces of tissue debris. The cell suspension was placed into a centrifuge tube and spun at 1000 rpm for 10 minutes.

Fixation is one of the critical stages in the procedure. The purpose of fixation is to halt cell division without causing any distortion of the components to be studied. Thus, the fixation process should not only increase the visibility of the chromosome structure, but should also clarify the details of chromosome morphology, such as primary and secondary constrictions and heterochromatic regions. Acetic acid is a powerful precipitant of nucleic acid (Baker 1958, in Sumner 1990), and has little effect on other cytoplasmic proteins, leaving cells in a soft condition.

5. After discarding the supernatant, the pellet was resuspended in freshly prepared fixative: Carnoy solution (3 methanol: 1 acetic acid), and then centrifuged at 800 rpm for 5 minutes. The fixation procedure was repeated twice. To prevent cell shock the fixative was mixed gently with the pellet.

6. A final cell suspension was prepared by adding a few drops of fresh Carnoy solution to the pellet.

*** In previous studies (Oellermann 1988, Oellermann & Skelton 1990, Naran 1992), fish were incubated for 3 hours, with no yeast prestimulation (Table 2.2). Gill tissue was removed, hypotonized for 30 minute, and fixed in the same manner as mentioned above. However, a cell suspension of macerated gill tissue was prepared by mechanically teasing the gills with a pair of forceps in a 50% acetic acid solution. The cell suspension was not centrifuged and as a result contained much of the cell debris. The slides were prepared by dropping the cell suspension onto cleaned and labled slides and retracting the fluid back into the pasteur pipette, leaving a ring of cells on the slide. Slides were allowed to air dry and were stained in 5% Giemsa solution for 20 minutes.**

Table 2.2 Comparison of chromosome isolation techniques from an earlier and later study.

Action	1992	1994-1996
Prestimulation	none	yeast (24-48hrs)
Colchicine incubation	3 hours	1.5-2 hours
Tissue	gills	kidney
Maceration of tissue	forceps	grid sieve
Cell suspension	not filtered	filtered
Centrifugation	no	yes
Slide preparation	drop & retract	drop on condensate

Slide preparation

7. Slides were cleaned in 70% ethanol and labelled. A cleaned slide was placed over boiling water until a thin film of condensate had formed. Two-three drops of the cell suspension were dropped onto the slide. The slide was then air-dried on a slide rack and stained in a 4-6% cool Giemsa solution for 5 minutes. The Giemsa stain was prepared using heated distilled water and filtered through number 1 Whatman paper to remove undissolved crystals.

Isolation of meiotic chromosomes

The protocol for isolating meiotic chromosomes from male and female gonads was the same as that for kidney and gill tissue. Better slide preparations were obtained from tissue kept in hypotonic solution for 50 minutes. The gonads were fixed intact in Carnoy solution and the tissue was macerated

using the 0.5 mm mesh grid. The resultant cell suspension was dropped onto clean slides.

Karyotype examination

1. The slide preparations were scanned using one of the following compound light microscopes: Nikon Optiphot, Olympus BX40 or Olympus BX50.
2. The metaphase chromosome spreads were examined under oil immersion (100 x objective and 10 x eyepiece). Chromosome counts were made using the following three procedures:
i. The light source was redirected to a video recorder (Panasonic digital WVCP 410) from the compound microscope, from there an enlarged image was projected onto a video monitor (Panasonic WV CM-1000). The monitor screen was covered with a clear plastic sheet onto which the chromosomes were traced using a water soluble pen.
ii. Photomicrographs were taken using either Agfapan APX 25 or Agfa XRG 200 film, in a Nikon M35-s or Olympus PM-C35DX camera mounted onto the compound microscope. The photographs were enlarged (usually x5) and the counts made.
iii. In some instances metaphase complements were determined using the negatives of the photomicrographs. An enlarged picture was projected onto a white screen from where the chromosome outlines were traced and scored.

Chromosome analysis

Karyotype morphology

The chromosome morphology of a mitotic complement can be defined by at least 3 features: chromosome lengths (size), centromere position and morphological features such as number and position of satellites. All these features were considered when examining the metaphase spreads.

The position of the centromere is the basis for the classification of chromosomes, (Table 2.3) (Levan *et al.* 1964, Denton 1973, see chapter 3). Chromosomes can be visually assessed and categorised as mentioned below.

Table 2.3 Chromosome categories based on the position of the centromere (from Denton 1973).

Centromere position	Chromosome classification	
median	**m**	biarm
submedian	**sm**	"
subterminal	**st**	biarm/uniarm
terminal	**a/t**	uniarm

Levan *et al.* (1964) proposed a system of chromosome categories based on the ratio of the length of the long arm divided by the length of the short arm. Although, this system has been followed by several authors working with cyprinid chromosomes (Gold 1979, Rab 1981, Suzuki & Taki 1981, 1986, Collares-Pereira & Madeira 1990), these numerical designated ratios were not suitable for the *Barbus* species examined in this study. It was found that chromosome categories as defined by arm ratios, particularly in the **sm** and **st** groups, were too specific. This is (further) investigated in chapter 3, where results pertaining to this problem and the ensuing discussions are presented.

Chromosome analysis used in this study

A minimum number of 10 spreads was analysed per specimen and up to 35 spreads per species were examined from the slides to obtain the frequency distribution of the chromosome number. Based on this a modal value of the total chromosome number and chromosome complement for each species was obtained. In some cases only 1-2 specimens of a species were available for analysis. Although, cytogenetic determinations have been determined from low numbers of specimens (Rab pers. comm.), results of such studies needs to be further confirmed with larger samples.

The karyotype was prepared from the best photomicrographs having the modal chromosome values. The chromosomes were cut-out and paired according to size, shape and centromere position in order to prepare a photokaryotype.

Components of a karyotype are important in providing additional characters with which to analyse chromosomal data. Species analysed by different researchers were shown to have different karyotype determinations, some of which are now thought to be artifacts of the technique (Rab & Collares-Pereira 1995). However, in recent years, due to several advances in protocol of chromosome preparations, the results and analysis of different researchers are becoming increasingly comparable.

Several authors have grouped chromosomes only as biarm component and uniarm component (Golubstov & Kryzanov 1993, Oellermann & Skelton 1990) or three categories (**m**, **s** and **a**) (Guégan *et al.* 1995, Agnèse *et al.* 1990a, Collares-Pereira 1989, Magtoon & Arai 1993). Many species, including cyprinid chromosomes, have been grouped into four categories (**m**, **sm**, **st**, **a**) *sensu* Levan *et al.* (1964), (Suzuki & Taki 1981, Magtoon & Arai 1993).

Metaphase spreads are visually examined for karyotype analysis in this study. In order to determine the karyotypes a selection of good metaphase spreads were analysed and mean, standard deviation (sd), modal values and range of the chromosomes in the particular categories were determined. Attempts were made to separate the **sm** and **st** chromosomes to allow karyotype comparison with published data. The chromosomes categories in this study follow the format in Table 2.3, and are reported as **m**=metacentric chromosomes, **a**=acrocentric chromosomes. The **sm**=submetacentric and **st**=subtelocentric chromosomes are represented as a combined category; **sm+st**.

Large and small: chromosome determinations

The longest and smallest chromosome pairs, particularly in the **sm+st** categories, were distinct features of the metaphase cell that could be identified. In order to quantify these distinct chromosomes, their lengths were measured using precision Helios callipers and expressed as a ratio of **l**arge:**s**mall chromosome lengths (**l:s**). This value is relative to the metaphase cells as they are subject to various factors which affect the chromosome contraction and resolution. Measurements were taken from selected metaphase spreads.

In the photokaryotypes chromosomes are arranged in decreasing size order within the chromosome categories.

NF: total number of chromosome arms

Further analysis of the karyotype is possible by examining the total number of chromosome arms.

Chromosome structural changes such as pericentric inversions, translocations, heterochromatin additions/deletions, that do not change chromosome numbers have been inferred in cyprinids on the basis of reported differences in fundamental chromosome arm numbers (Cataudella *et al.* 1977, Gold 1979, Gold *et al.* 1981, Zan *et al.* 1986).

The number of major chromosome arms derived from uniarmed and biarmed chromosomes is referred to as the "*nombre fondamental*" (NF). The NF number was first described and used by Matthey (1945, in White 1977), to follow some of the chromosomal rearrangements within a taxon.

In the literature, there is a variation in terminology and in the method for determining NF values. Some authors use Fundamental Number (FN) as the abbreviation (Oellermann 1988, Oellermann & Skelton 1990, Golubstov & Kryzanov 1993).

The variation in NF determination is as a result interpreting **st** chromosomes as either biarmed or uniarmed. Collares-Pereira & Madeira (1990) have classified cyprinid karyotypes as **m**, **s** (submetacentric) and **a** chromosomes, where NF=2(**m**+**s**)+**a**. A similar format has been interpreted by Valenta *et al.* (1979) and Gold *et al.* (1981). Magtoon & Arai (1989) and Khuda-Bukhsh *et al.* (1986), have four categories of **m**, **sm**, **st** and **a** or **t** for cyprinid chromosomes and NF=2(**m**+**sm**)+**st**+**a**.

In some cases the resolution of chromosomes allow biarm components (**m** & **sm** chromosomes) and uniarm components (**st** & **a** chromosomes) only. As has been the case by Oellermann & Skelton (1990) and Golubstov and Kryzanov (1993), here the NF=2(biarm)+(uniarm). Magtoon & Arai (1993) report several studies where a second type of NF value (NF2) has been calculated that includes **st** as biarmed chromosomes, *viz.* NF2= 2(**m**+**sm**+**st**)+(**a**) where **sm**, **st** and **m** are biarmed chromosomes and **a** are uniarmed chromosomes.

Thus, the Fundamental Number can be calculated using the formula NF1 = 2(**m**+**sm**)+(**st**+**a**) where **sm** and **m**=biarmed chromosomes and **st** and **a**=uniarmed chromosomes. Although both

formulae are used in this study, results from the latter calculation were more applicable as many of the **st** chromosomes have a second arm.

Meiotic chromosome analysis

Observation of meiotic chromosomes is useful for determining the type of polyploidization that occurred in the evolution of the species (Rab pers. comm.). Chiasma formation during the reduction chromosomal division can be observed in gonadal cells, where sister chromatids pair for bivalents, tetravalents or multivalents. The presence of tetravalent (and multivalent) conformations is an indication of autopolyploidy, while the absence of such assemblages is an indication of allopolyploidy or remoteness of autopolyploidy. Since tetraploidy has been inferred in some of the species examined in this study, it was decided to examine the meiotic cells to determine how this may have occurred.

Meiotic preparations were obtained from testes by following the same protocol as discussed previously. A minimum of 10 meiotic chromosome spreads were analysed per specimen. Fourteen species were examined and are listed in each chapter. The chromosomal elements were scored on the basis of absence or presence of bivalents, tetravalents or multivalents.

DISCUSSION

A comparison of current results with previous results (Oellermann 1988, Naran 1992) using different techniques, highlights the value and need for continued technical refinement in cytogenetic studies.

Field studies of two redfin minnows (*P. afer* and *P. asper*) showed that most growth occurred during the summer months (when water temperatures were elevated) and growth slowed down when winter temperatures were low (Cambray 1992). Temperature affects growth of fish and thereby increases the turnover of cells. It was found that higher yields of metaphase cells were obtained when fish were kept at 23-24°C, i.e. 2-3°C above ambient for a week before colchicine injection.

Chromosome fixation using Carnoy solution is a sensitive and delicate procedure. It was found that adding the acetic acid fixative to the cell suspension, and gently flushing the solution with a pasteur pipette produced good chromosome yield.

Exposure to colchicine for three hours in the protocol using gill tissue resulted in highly contracted metaphase chromosomes, which were difficult to categorise and as a result grouped into two categories, biarmed and uniarmed categories.

The refinement procedures of, prestimulation with yeast, shorter colchicine incubation, concentration of cell suspension through centrifugal action and better techniques of slide preparation employed with the kidney tissue resulted in superior chromosome preparations.

Maceration of the kidney tissue using a mesh grid resulted in fewer cells being ruptured causing chromosomes to remain in the cells; it was found that the total chromosome counts were less variable

using this method. The cell suspension was centrifuged and much of the cellular debris was removed, thus resulting in uncluttered slide preparations. Using the condensation technique, to drop the cell suspension onto a slide, also resulted fairly homogeneous chromosome spreading. The overall result was that the kidney tissue method gave less variable chromosome counts and well resolved karyotypes.

CONCLUSIONS

High mitotic divisions and well-spread metaphasic chromosomes were reliably and regularly obtained using *in vivo* preparations for the small barbine species examined in this study. The improved quality of the chromosomal preparations were indicated by the low variability of chromosomal counts and very few instances of cell overlapping. Karyotype morphologies of all species yielded good data for comparative analyses.

CHAPTER 3: ASSESSMENT OF THE TECHNIQUE USED TO DESCRIBE CYPRINID CHROMOSOMES

INTRODUCTION

An increase in human chromosomal research in the 1960's-1970's resulted in the development of an objective method of chromosomal analysis. Levan *et al.* (1964) proposed a method where the measured chromosome groups were based on the centromere position, by calculating the ratio of long arm to short arm (L/S), or as the difference between long arm and short arm. Using arm ratios, four chromosome groups were recognised. Metacentric chromosomes (**m**) are chromosomes where the arms are equal, the centromere position is median and L/S ratio ranges from 0.1-1.7. Submetacentric chromosomes (**sm**), where one set of chromosome arms are longer than the other, the centromere position is submedian and L/S ratio ranges from 1.7-3.0. Subtelocentric chromosomes (**st**), where one set of chromosome arms are much shorter than the other, the centromere position close to the terminal and L/S ratio ranges from 3.0-7.0. Acrocentric chromosomes (**a**), where the centromere position is terminal, the range is 7.0-∞.

Chromosome categories based on these L/S arm-ratios have been used extensively in several taxa; fish (including cyprinids) (Feldberg *et al.* 1992, Rab 1981, Suzuki & Taki 1981, 1986, Collares-Pereira & Madeira 1990, Amemiya & Gold 1990), frogs (Alpagut & Falakali 1995), snakes (Baker *et al.* 1972) and mole-rats (Nevo *et al.* 1995).

The main aim in this chapter was to determine whether measured chromosomes and visually assessed chromosomes are comparable with each other. The null hypothesis was that visual chromosome categories are the same as measured chromosome categories. An alternative hypothesis was that visual chromosome categories are not the same as measured chromosome categories. Accordingly, the key questions are:

i) how many chromosome categories are there in the barbine metaphase cells;

ii) are the number of chromosomes in each category comparable to the chromosome categories obtained in the next cell?

Analysis of chromosome measurements considered the following factors:

1) numbers of chromosomes in each category as determined by Long arm/Short arm-ratios (L/S arm-ratios) according to Levan *et al.* (1964), were determined;

2) frequency analysis of chromosome ratios was also explored to see how chromosomes were distributed within the L/S arm-ratio categories as proposed by Levan *et al.* (1964);

3) thereafter the analysis considered whether chromosome lengths could be used to categorise chromosomes.

An alternative means of determining chromosome categories is by visual chromosome assessments (Rab pers. comm.).

4) a chi-squared analysis was used to evaluate differences between measured and visually grouped

chromosomes.

L/S arm-ratios analysis

Materials and methods

Chromosome measurements were performed on at least four and a maximum of nine sets of good quality metaphase chromosomes, using Helios precision callipers. The L/S-arm-ratios and chromosome lengths of the measured chromosomes were calculated and then assigned to Levan *et al.* (1964) categories.

Three groups of species were chosen for this exercise, on the basis of total chromosome numbers or ploidy levels. The groups include species with chromosome numbers of 50, 100 and 150 or diploid, tetraploid and hexaploid, respectively.

Two levels of comparisons were considered, within a specimen (intraspecimen), to see whether metaphase chromosomes differed from one cell to another, and between specimens of a species (intraspecific) to examine whether the chromosome categories differed from one individual to the next.

Diploid (2n=50)

Metaphase chromosomes spreads of *B. amatolicus* and *B. anoplus* from two locations, Elands River and Palmiet River, were measured and the long arm vs short arm ratios (L/S arm-ratio) were calculated.

Tetraploid (2n=100)

Metaphase chromosome sets of *B. calidus* were measured and L/S arm-ratios calculated.

Hexaploid (2n=150)

Metaphase chromosomes of *B. polylepis* were measured and L/S arm-ratios calculated.

Results

Measured chromosome analysis show that all four chromosome categories are present in the three groups of species examined (Tables 3.1, 3.2 & 3.3). Complete sets of L/S arm-ratio results for the measured chromosomes are shown in Appendix 1.

Intraspecimen comparison

Diploid

Within the chromosome complement of the different samples and different species, the largest chromosomes are the **a** chromosomes (43-50%) and the smallest chromosome category comprises the **st** chromosomes (4.0-1.6%) (Table 3.1). There is an almost equal number of biarmed (**m&sm**) and uniarmed chromosomes (**st&a**) in the complement. In five metaphase spreads examined for *B. anoplus* (Elands), chromosome numbers as determined by L/S arm-ratios are different, even within the same category (Table 3.1, Fig. 3.1). Comparison of four metaphase cells from *B. anoplus* (Palmiet) and four metaphase cells from *B. amatolicus* show the same result as above i.e. the proportions of the number of chromosomes within the four categories in both species are different from each other (Table 3.1, Fig. 3.1).

Table 3.1 Chromosome L/S arm-ratio categories determined according to Levan *et al.* (1964) for *B. anoplus*-complex species (2n=50). (E)=Elands River, (P)=Palmiet River, n=number of metaphase cells examined, size=size of chromosomes in each category, numbers=numbers of chromosomes in each category.

CHROMOSOME CATEGORIES	SIZE µm Mean (Range)	NUMBERS Mean (Range)	SIZE µm Mean (Range)	NUMBERS Mean (Range)
	INTRASPECIMEN		INTRASPECIFIC	
B.anoplus (E)		n=5		n=7
m	1.19 (0.96-1.39)	9.0 (8-10)	1.22 (0.98-1.55)	7.9 (5-10)
sm	1.28 (0.86-2.10)	15.5 (14-16)	1.31 (0.96-1.99)	16.6 (14-21)
st	1.16 (1.15-1.16)	1.0 (0-2)	0.86 (0.82-0.89)	1.0 (0-3)
a	1.24 (0.87-1.52)	24.8 (23-26)	1.25 (0.96-1.52)	24.9 (23-26)
B. anoplus (P)		n=4		n=9
m	1.99 (1.69-2.28)	9.8 (6-14)	1.91 (1.55-2.26)	8.3 (3-14)
sm	2.18 (1.50-3.21)	14.2 (12-16)	2.06 (1.48-2.98)	15.7 (12-19)
st	0.80 (0.78-0.85)	0.4 (0-1)	0.80 (0.78-0.85)	0.8 (0-3)
a	1.81 (1.34-2.25)	23.8 (21-25)	1.76 (1.35-2.19)	23.9 (21-26)
B. amatolicus		n=4		n=6
m	2.29 (1.64-3.76)	13.8 (8-18)	2.23 (1.56-3.69)	12.8 (8-18)
sm	2.00 (1.43-2.90)	12.8 (10-18)	2.05 (1.45-305)	13.5 (10-18)
st	1.81 (1.75-1.88)	2.0 (1-5)	2.01 (1.96-2.06)	1.8 (1-5)
a	1.77 (1.39-2.13)	21.3 (20-22)	1.79 (1.41-2.17)	21.5 (20-23)

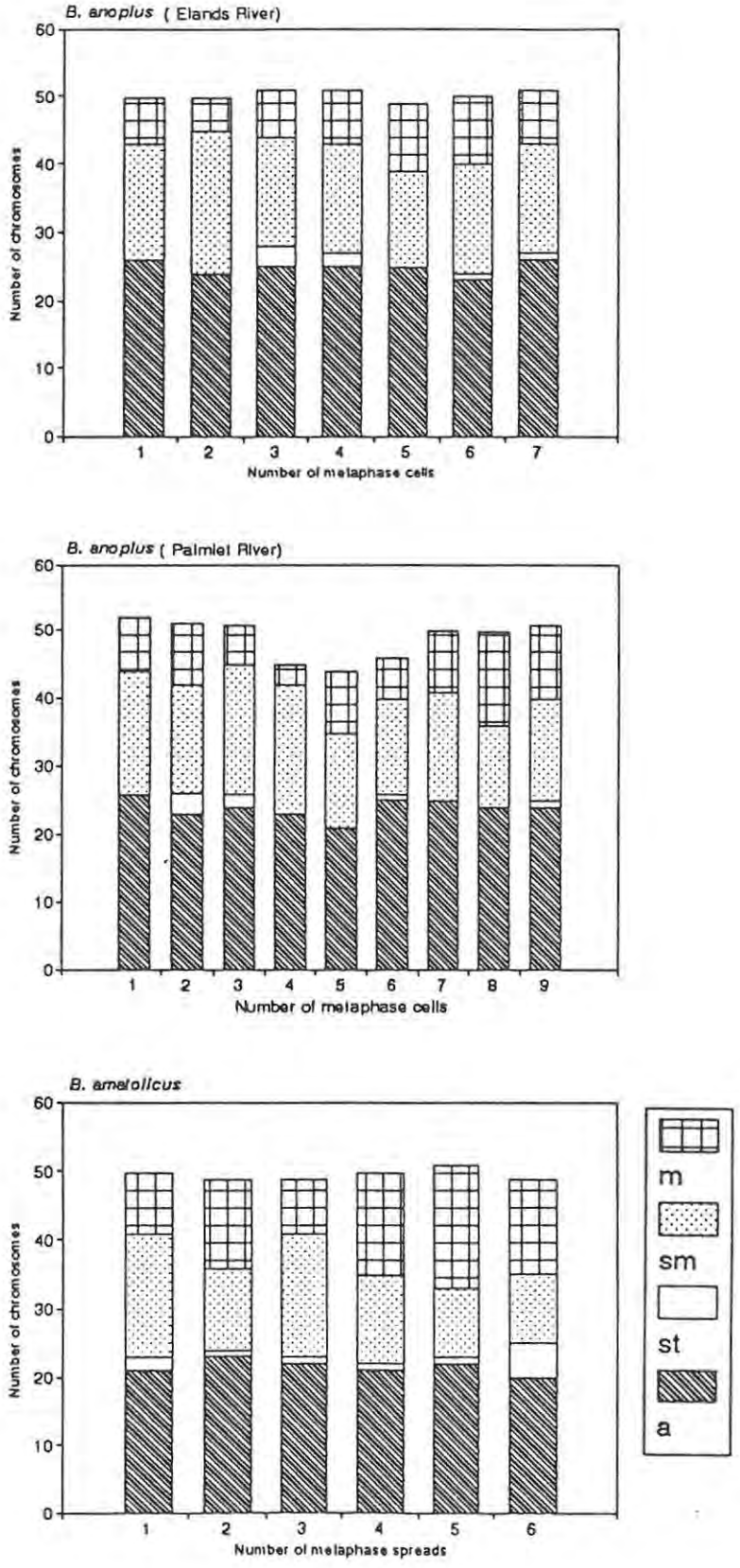


Figure 3.1. Intraspecific and intraspecimen comparison of chromosome L/S arm-ratio categories as determined by Levan *et al.* (1964) in *B. anoplus* (Elands River), *B. anoplus* (Palmiet River), *B. amatolicus* all with 2n=50, Appendix 1. Bar represents one metaphase spread.

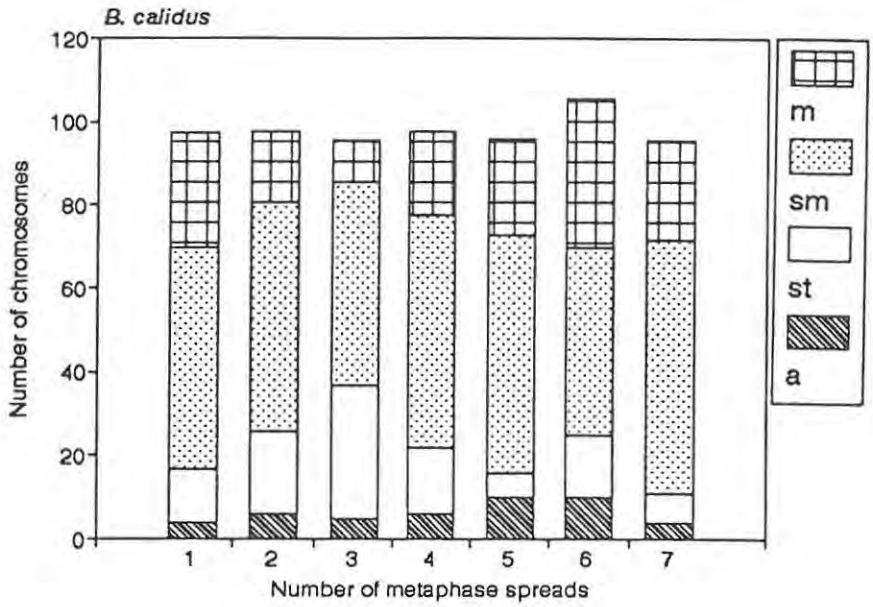


Figure 3.2 Intraspecific and intraspecimen comparison of chromosome L/S arm-ratio categories as determined by Levan *et al.* (1964) in *B. calidus* (2n=100), Appendix 1. Bar represents one metaphase spread.

Tetraploid

In all the cells examined there are more biarmed chromosomes than uniarm chromosome. The **sm** chromosome category has the highest number of chromosomes (54.2%), followed by **m** chromosomes (24.2%) (Table 3.2). All four **m, sm, st & a** chromosome categories show large variations in range especially in the **m** and **sm** chromosome categories. This variation in range indicates that chromosome numbers in the four L/S arm-ratio determined categories are not consistent even within the same specimen (Table 3.2, Fig. 3.2).

Table 3.2 Chromosome L/S arm-ratio categories determined according to Levan *et al.* (1964) for *B. calidus* (2n=100), n=number of metaphase cells examined, size=size of chromosomes in each category, numbers=numbers of chromosomes in each category.

CHROMOSOME CATEGORIES	SIZE µm Mean (range)	NUMBERS Mean (range)	SIZE µm Mean (range)	NUMBERS Mean (range)
	INTRASPECIMEN		INTRASPECIFIC	
B. calidus		n=4		n=7
m	2.08 (1.55-2.84)	23.3 (20-26)	2.12 (1.54-2.96)	21.1 (10-28)
sm	2.12 (1.43-3.21)	54.8 (45-61)	2.13 (1.41-3.23)	53.7 (45-61)
st	2.16 (1.65-2.84)	11.0 (6-16)	2.11 (1.58-2.80)	15.6 (6-32)
a	1.80 (1.18-2.30)	7.5 (4-10)	1.85 (1.41-2.22)	6.4 (4-10)

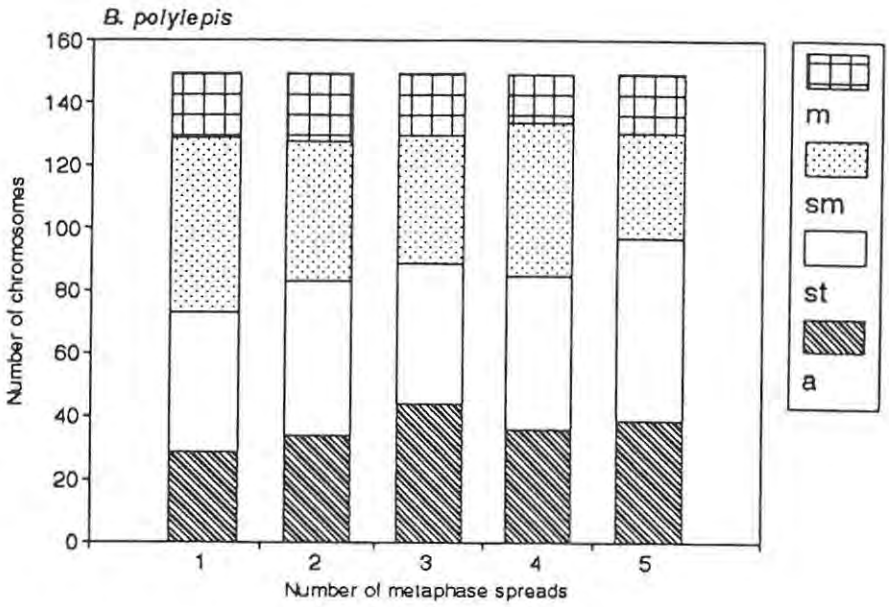


Figure 3.3 Intraspecimen and intraspecimen comparison of chromosome L/S arm-ratio categories as determined by Levan *et al.* (1964) in *B. polylepis* (2n=150), Appendix 1. Bar represents one metaphase spread.

Hexaploid

The largest number of chromosomes was recorded in the **st** (32.6%) and **sm** (30.2%) categories in the complement (Table 3.3). L/S-arm-ratio chromosome categories show that there was a higher proportion of biarm chromosomes compared to the uniarm chromosomes in the metaphase cells examined. *B. polylepis* metaphase cells also show that the total number of chromosomes in each category varies in range (Table 3.3, Fig. 3.3).

Table 3.3 Chromosome L/S arm-ratio categories determined according to Levan *et al.* (1964) for *B. polylepis* (2n=150), n=number of metaphase cells examined, size=size of chromosomes, numbers=numbers of chromosomes in each category.

CHROMOSOME CATEGORIES	SIZE µm Mean (Range)	NUMBERS Mean (Range)
	INTRASPECIMEN	
B. polylepis		n=5
m	1.67 (1.41-2.62)	19.6 (16-22)
sm	1.82 (1.39-3.48)	45.0 (34-56)
st	2.14 (1.17-3.30)	49.0 (40-58)
a	2.02 (1.35-2.62)	36.4 (29-44)

Discussion

The L/S arm-ratio analysis showed that the number of chromosomes allocated to the four categories within a metaphase spread within the same specimen varies in all four species of the three groups. The L/S arm-ratio determined **sm** and **st** chromosome categories have a larger range variation in the species with 100 and 150 chromosomes, compared to species with 50 chromosomes. This is possibly because those two chromosome categories are numerically dominant in the latter two species. Such variation between cells within a specimen make it difficult to characterise the species karyotype using the measured chromosome approach. In the intraspecimen comparisons, the L/S arm-ratio determined chromosome categories gives inconsistent results even when good photokaryotypes are measured albeit only a small sample. This applies to all three groups of species examined.

Intraspecific comparison

Materials and methods

Metaphase spreads from different specimens, within the three groups as mentioned above, were measured to ascertain chromosomal categories. Chromosomes from three specimens of *B. amatolicus*, two and three specimens each of *B. anoplus* samples were measured from the Palmiet and Elands Rivers respectively (Table 3.1). Three specimens of *B. calidus* were measured (Table 3.2). Only one specimen of *B. polylepis* was analysed, so it is not possible to examine intraspecific variation for the hexaploid group.

Results

Diploid

Comparison of intraspecific chromosome measurements show that L/S arm-ratio chromosome categories remain variable for three specimens each of *B. anoplus* and *B. amatolicus* (Table 3.1, Fig. 3.1).

Tetraploid

Comparison of chromosome categories obtained using the L/S arm-ratios show that there is variation in the L/S arm-ratio categories, **m**, **sm** and **st** (Table 3.2, Fig. 3.2). The variations are indicative of unclear chromosomal assignment into a particular category using the method as proposed by Levan *et al.* (1964).

Discussion

The chromosome categories are relatively well defined in species with 2n=50, with respect to numbers of chromosomes in a particular category. With only 50 chromosomes in the metaphase spreads, the chromosomes are probably better resolved compared to the species with 100 or 150 chromosomes.

Both intraspecimen and intraspecific level comparison shows that the chromosome categories obtained using L/S arm-ratios are not consistent even within the same specimen or within the same species. Similar results with measured chromosomes were observed by Rab (pers. comm.).

The conclusion to be reached from these results is that chromosomal categories proposed by Levan *et al.* (1964) may be too discretely defined for interpreting cyprinid chromosomes.

Inherent in the protocol for chromosome isolation are other factors affecting chromosome condensation such as the concentration and temporal exposure to colchicine (Rab and Collares-Pereira 1995). There has been great variability of chromosome preparations fixed with acetic acid (Sumner 1990). These factors contribute to the size and shape variation of the chromosomes from one metaphase cell to another. Factors which may affect chromosomal arm contraction using the protocol followed in this study, may be attributed to one of the following reasons:

a) the stage at which the cell division is arrested by colchicine treatment (e.g. prometaphase, late metaphase);

b) dosage and duration of colchicine exposure;

c) hypotonic penetration and fixative penetration into the cell. Despite these influences on the chromosomes the categories should remain distinct so that species karyotypes can be determined from cells that are all treated in the same way. The Levan *et al.* (1964) chromosome categories are determined as ratios, the results should therefore be comparable, at all levels.

Frequency analysis of measured chromosomes

Materials and methods

The L/S arm-ratio chromosomes were spread along a ratio gradient in order to determine the distribution of chromosomes within the categories.

Results

Chromosomes are distributed within all four categories, indicating that the centromere position outlined by Levan *et al.* (1964), is present in these karyotypes. Results for all three groups of species are presented in Figs. 3.4-3.6.

Diploid

The **m** chromosomes range from an L/S arm-ratio of 1.0-1.70, with a peak of chromosomes with L/S arm-ratio of 1.4 & 1.7. The **sm** chromosomes are distributed along the entire L/S arm-ratio range, with peaks at L/S arm-ratio of 1.71, 1.9 & 1.1, for *B. anoplus* and *B. amatolicus*. There are a few **sm** chromosomes distributed near the **st** chromosomes (Fig. 3.4). The **a** chromosome distributions for *B. amatolicus* have a wider spectrum compared to *B. anoplus* (Fig. 3.4).

Tetraploid

The **m** chromosomes are distributed mainly from L/S arm-ratio of 1-1.7, with 19.5% of the chromosomes at L/S arm-ratio of 1.6 (Fig. 3.5). The **sm** chromosomes are distributed along the entire range, with a 14.7% of the chromosomes close to the **st** category of L/S arm-ratio 2.8-3.0. The majority of the chromosomes (61%) in the **st** category are close to the **sm** L/S arm-ratio of 3.1-3.5 (Fig. 3.5).

Hexaploid

The *B. polylepis* chromosomes are similar in their frequency distribution to the *B. calidus* above, in that **m** chromosomes are found within the 1-1.7 ratios, and the **sm** chromosomes are found throughout the range (Fig. 3.6). The *B. polylepis* karyotype is different in that the **st** chromosomes are also found throughout the range. 18.1% of the **sm** chromosomes are close to the **st** category and 32.8% of the chromosomes are near the **sm** category. The **a** chromosomes have a wide spectrum of distribution (Fig. 3.6)

Discussion

This analysis shows that for two species with high chromosome numbers (*B. calidus* and *B. polylepis*) many of the chromosomes (51-76%) are close to the **sm+st** boundary. The chromosomes are very small (0.5-4μm), so even with the best photokaryotypes, a slight error in measuring the chromosome arms, and assessing the centromere position, may result in chromosomes falling into either **sm** or **st** L/S arm-ratio categories. A solution to this problem would be to combine the two chromosome categories **sm** and **st** and this has been done in this study.

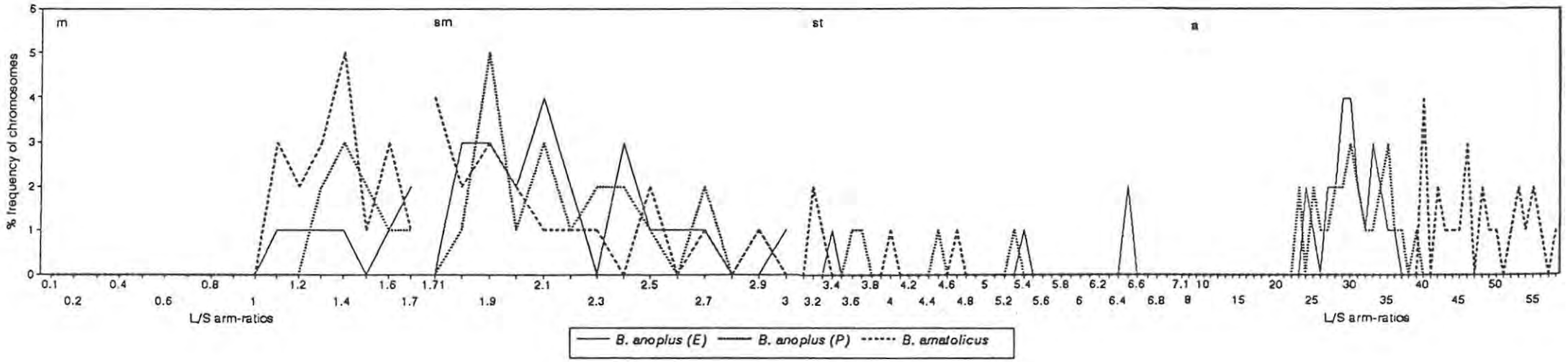


Figure 3.4 Frequency distribution of the four chromosome categories (**m**, **sm**, **st**, **a**) as determined by L/S arm-ratio Levan *et al.* (1964) in *B. anoplus* (Elands river), *B. anoplus* (Palmiet river), *B. amatolicus* all with 2n=50.

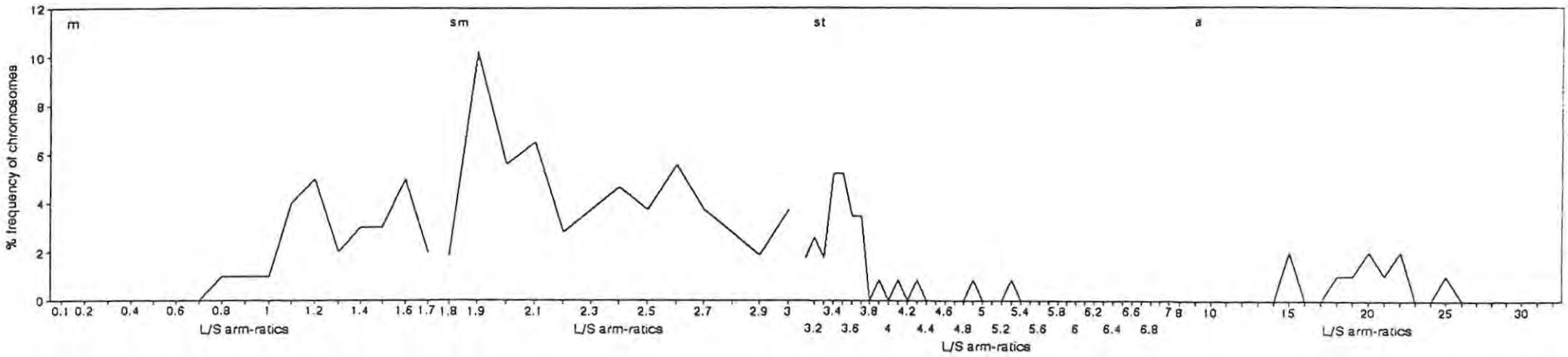


Figure 3.5 Frequency distribution of the four chromosome categories (**m**, **sm**, **st**, **a**) as determined by L/S arm-ratio Levan *et al.* (1964) in *B. calidus* (2n=100).

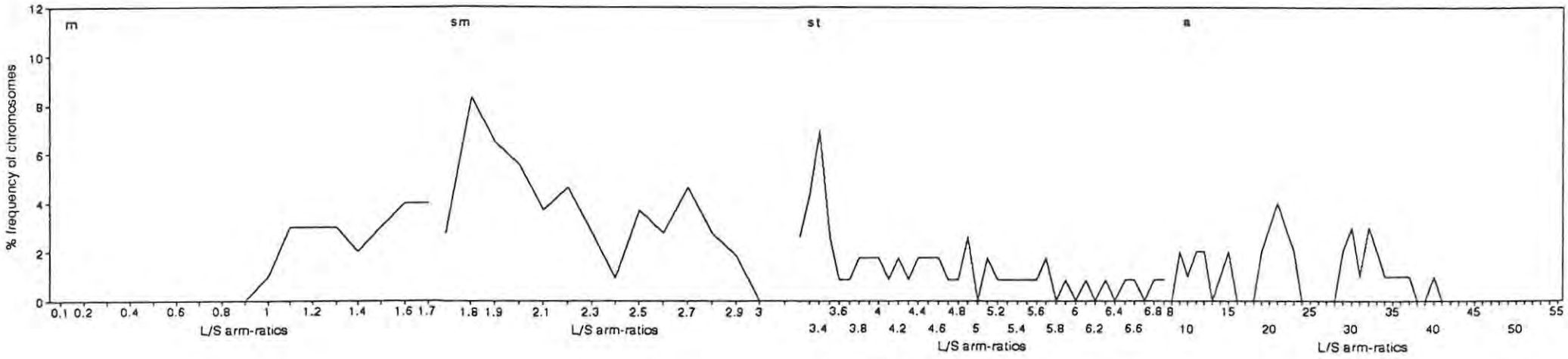


Figure 3.6 Frequency distribution of the four chromosome categories (**m**, **sm**, **st**, **a**) as determined by L/S arm-ratio Levan *et. al.* (1964) in *B. polylepis* (2n=150).

Profiles of chromosome sizes against number of chromosomes

Chromosome sizes were analysed to determine whether the Levan *et al.* (1964) L/S arm-ratio chromosome categories have discrete chromosomes sizes, for if they do the chromosome sizes could be used to group the chromosomes.

Materials and methods

Lengths of the long arm and short arms of the measured chromosomes were added together to obtain a value for the chromosome length, these values were adjusted to the microscopic and photographic magnifications and represented as micrometres.

Results

Figs. 3.7-3.9 show means plots of all the chromosomes in the metaphase karyotype for species with 2n=50, 100 & 150, respectively. Each bar in the graphs represents paired chromosome values which have been cumulated within each chromosome category, as a result the chromosome numbers are higher than the diploid values of the species (Appendix 1). They are arranged in a decreasing order of the Levan *et al.* (1964) type L/S arm-ratio, average length versus chromosome number.

Diploid

Chromosome size profiles, within the L/S arm-ratio categories for *B. anoplus* and *B. amatolicus* (2n=50) show that in all four categories there is a gradual change in chromosome size from large to small. However, only two pairs are distinct within the **sm** chromosome category; a large chromosome pair and a small chromosome pair. In all other chromosome categories, chromosome length changes gradually from 0.8μm to 2.3μm (Fig. 3.7).

This indicates that many of the chromosomes in the categories are not discrete with respect to chromosome size.

The plot also shows that the uniarm chromosomes are as numerous as the biarmed chromosomes for these species.

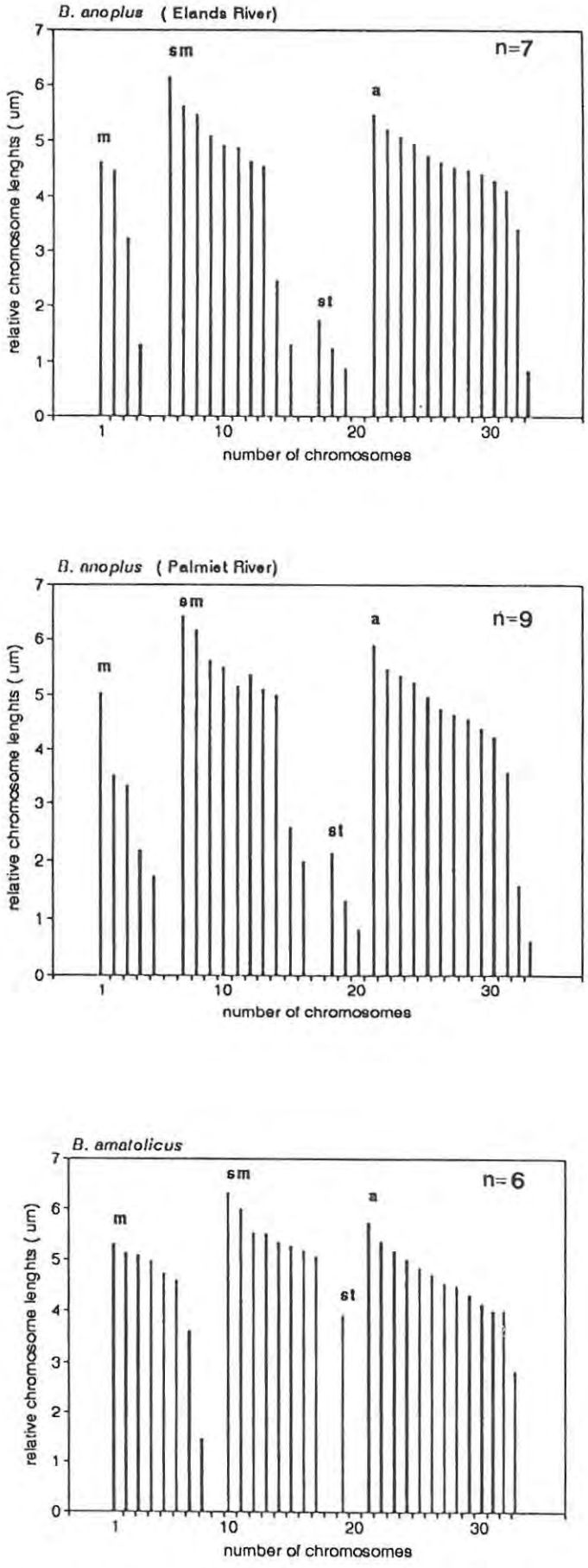


Figure 3.7 Chromosome sizes within the four chromosome categories (**m**, **sm**, **st**, **a**) as determined by L/S arm-ratio Levan *et al.* (1964) in *B. anoplus* (Elands river), *B. anoplus* (Palmiet river), *B. amatolicus* all with 2n=50, Appendix 1.

Tetraploid

B. calidus (2n=100) has a major proportion of the metaphase complement composed of biarmed chromosomes (Fig. 3.8). The largest chromosomes are found in the **st** and **sm** category and the smallest chromosomes are found in the **sm** category (Fig. 3.8). In all four categories the chromosomes size changes gradually from 1.8μm to 2.2μm, and the majority of chromosome size range from 2.1μm to 2.2μm.

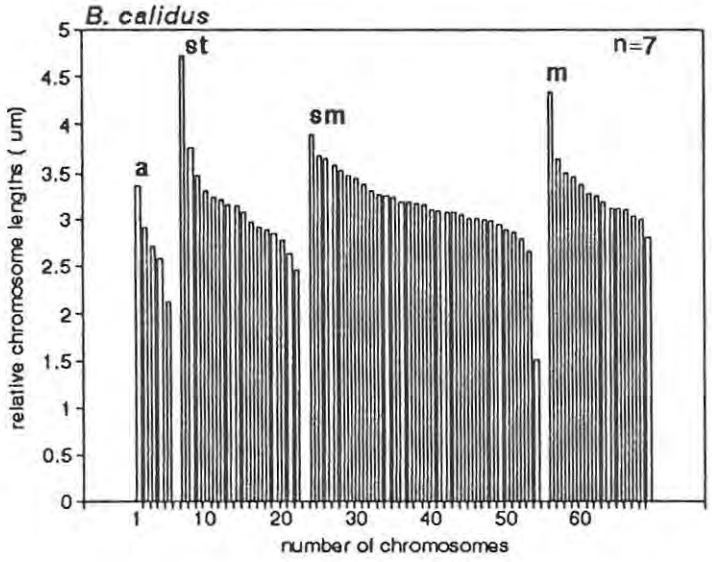


Figure 3.8 Chromosome sizes within the four chromosome categories (**m**, **sm**, **st**, **a**) as determined by L/S arm-ratio Levan *et al.* (1964) in *B. calidus* (2n=100), Appendix 1.

Hexaploid

B. polylepis has approximately 30% of karyotype composed of **a** chromosomes and the remaining 70% are **st, sm** and **m** chromosomes. The largest and smallest chromosomes are found in the **st** and **sm** chromosome category 3 to 3.4μm and ±1μm respectively (Fig. 3.9). In all four categories the chromosomes size changes gradually from (1 to 3.3μm), and the majority of chromosomes are 1-2μm in length.

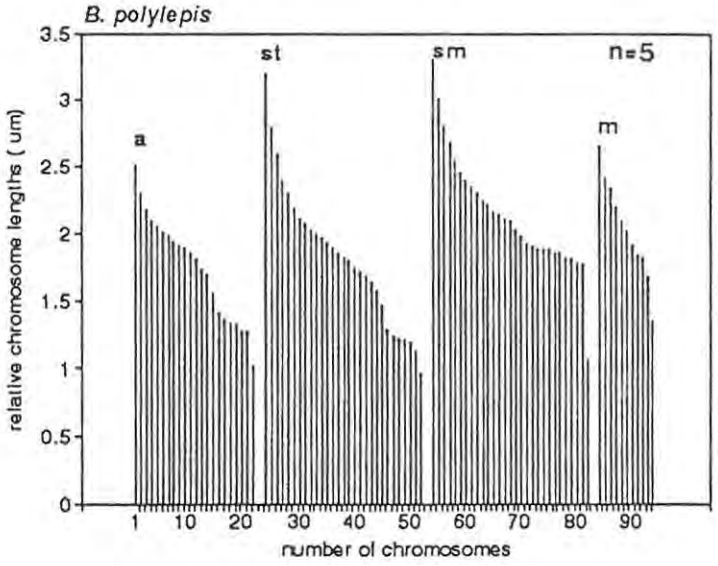


Figure 3.9 Chromosome sizes within the four chromosome categories (**m, sm, st, a**) as determined by L/S arm-ratio Levan *et al.* (1964) in *B. polylepis* (2n=150), Appendix 1.

Discussion

Two types of chromosomal morphology are apparent from the above analyses (Fig. 3.7-3.9). In the 2n=50 species there are similar numbers of uniarm and biarmed chromosomes while in the polyploid species *B. calidus* and *B. polylepis* there is a dominant biarm component in the metaphase karyotype. Generally, the chromosomes in all four categories gradually decrease in size and many chromosomes are therefore similar in size. This fact illustrates the difficulty in assigning chromosomes into discrete groups using size as a criterion. Furthermore it illustrates the difficulty in using a measuring method to classify chromosomes as the majority of chromosomes (in the **sm** and **st**) are only slightly different from one another.

A comparison of classifying chromosomes by measuring (quantitatively) and visually (qualitatively)

The first two analyses have shown that chromosome categories as determined by arm-length are variable in all three groups of species examined. The chromosome length analysis has indicated that many of the chromosomes change gradually in size. This is a possible reason why the chromosomes categories are not always consistently defined.

Materials and methods

Chromosomes of the same metaphase cells which were measured for the L/S arm-ratios, were assessed visually and grouped into four categories on the basis of centromere position, according to Levan *et al.* (1964) categories. To counter subjective chromosome categorising, chromosomes were scored several times over a period of a week and the metaphase spreads randomised.
The null hypothesis tested was that measured chromosome categories are the same as visually determined categories.

Results

Diploid

A comparison of metaphase spreads of *B. anoplus* and *B. amatolicus* shows that visually assessed chromosomes in all four categories have low ranges (Table 3.4). The **a** and **m** chromosome number remains clearly defined in all metaphase cells of both species examined and there are slight variations (range) within the **sm** and **st** chromosomes, indicating that the **sm+st** chromosomes are sometimes misallocated (Table 3.4). The first pair of **sm** chromosomes is distinctly longer in both species.

Table 3.4 Visual assessment of metaphase spread photomicrographs of the three species groups 2n=50, 2n=100, 2n=150 showing modal chromosome numbers and their ranges (). Both intraspecimen comparisons and interspecific comparisons are represented, (E)= Elands River, (P)=Palmiet River.

CHROMOSOME CATEGORIES	*B. anoplus* (E) 2n=50	*B. anoplus* (P) 2n=50	*B. amatolicus* 2n=50	*B. calidus* 2n=100	*B. polylepis* 2n=150
Intraspecimen comparison	n=5	n=4	n=4	n=4	n=5
m	6 (6)	6 (6)	6 (6-8)	16 (16)	16 (14-16)
sm	18 (18)	18 (18)	20 (16-20)	54 (50-54)	34 (30-40)
st	2 (2)	2 (2-4)	4 (4-6)	26 (24-28)	52 (44-56)
a	24 (22-24)	24 (22-24)	20 (20-22)	4 (4-6)	48 (36-48)
Intraspecific comparison	n=7	n=9	n=6	n=7	-
m	6 (6)	6 (6)	6 (6-8)	16 (14-16)	
sm	18 (18)	18 (18-19)	20 (15-20)	54 (50-54)	
st	2 (1-2)	2 (1-4)	4 (4-6)	26 (24-30)	
a	24 (22-24)	24 (22-24)	20 (20-22)	4 (4-6)	

Tetraploid

The **a** and **m** chromosome categories are clearly grouped as shown by low ranges (Table 3.4), there is a large range variation in **sm** and **st** chromosome categories of visually assessed metaphase chromosomes (Table 3.4). The **sm** and **st** chromosomes are the most numerous comprising 80% of the metaphase complement. Chromosomes sizes in these two categories range gradually from large to small.

Hexaploid

In *B. polylepis*, there is large variation in range in the number of **sm** and **st** chromosomes when compared to the **a** and **m** chromosomes (Table 3.4). As compared to the diploid and tetraploid groups there is more variation in the chromosome categories of the hexaploid. However this may be a multiplier effect, i.e. because there are three times as many chromosomes in the complement compared to species with 50 chromosomes (Table 3.4).

Discussion

In the three ploidy groups at the intraspecimen level and at intraspecific level (for diploid and tetraploid species) results are comparable, and indicate a small range variation. This analysis shows that careful visual assessment of well spread metaphase complements yield results which are reproducible at both intraspecific level and intraspecimen level.

Diploid

Visually assessed metaphase chromosomes at both intraspecimen level and intraspecific level show small variations in range for *B. anoplus* and *B. amatolicus* (Table 3.4). The biarmed chromosomes (**sm** and **st**) are miscategorized and this results in the range variation.

Tetraploid

The results of chromosome numbers in two categories **m** and **a** are fairly well defined and comparable from one cell to the next, whereas the chromosomes numbers in the **sm** and **st** categories are more difficult to represent.

Hexaploid

The **m** chromosomes numbers are generally well defined as shown by the range variation in this categories (Table 3.4). **sm** and **st** chromosomes are more variable with the result that these chromosome numbers in the categories are not always clearly distinguished (Table 3.4).

Discussion

Overall comparison of the visually assessed chromosome values, show that the **m** and **a** categories are clearly defined and are comparable at intraspecimen and intraspecific level. However, both **sm** and **st** chromosome categories in species with 100 and 150 chromosomes are more difficult to separate. This is mainly because the position of the chromosome centromere changes gradually and there is also a gradual change in chromosome size. Misallocation of **st** and **sm** chromosomes is encountered even with good metaphase spreads. It is therefore a better solution to recognise only one joint category, namely **sm+st**.

Chi-squared test to evaluate differences between measured and visually grouped chromosomes

Materials and methods

The number of chromosomes in the each category determined by visual and measured methods were compared using the chi-squared test. In the formula, measured chromosomes values were considered the expected values as they are more precise.

Result and Discussion

A chi-squared test was performed on the three groups of species, to compare the number of chromosomes in a particular category, using measured and visual chromosome assessments. In the case of diploid species the test reveals that there is no significant difference between the outcome of chromosome categories by measuring or visual determination. **m** chromosomes within *B. anoplus* (Palmiet) and **m** chromosomes in *B. amatolicus* are not significantly different ($p<0.01$) (Table 3.5). This suggests that either method could be employed to assess the chromosome categories of these species.

Table 3.5 Chi-squared comparison of measured chromosome L/S arm-ratio categories and visually grouped chromosomes for species with diploid (2n=50), tetraploid (2n=100) and hexaploid (2n=150) karyotypes. $p<0.01$. (S=Significant difference, NS= No significant difference), (E)= Elands River, (P)=Palmiet River.

Chromosome types	*B. anoplus* (P)	*B. anoplus* (E)	*B. amatolicus*	*B. calidus*	*B. polylepis*
	2n=50	2n=50	2n=50	2n=100	2n=150
m	NS	NS	NS	S	NS
sm	NS	NS	NS	NS	S
st	NS	NS	S	S	NS
a	S	NS	S	S	S

The chi-squared test for the tetraploid and hexaploid species is inconclusive. Many of the chromosome categories indicate that the L/S arm-ratio and visual chromosomes are significantly different at $p=0.05$. However, no trends are obvious, and chromosomes in all four categories show significant and non-significant differences. Further work is required on classifying polyploid cyprinid chromosomes before a suitable method can be established.

GENERAL DISCUSSION

There is a perceived problem with chromosome categories determination of **sm** and **st** with polyploid (2n=100 and 2n=150) species. Cyprinid chromosomes cannot easily be categorised using Levan *et al.* (1964), because of the inherent problem of gradual centromere change and gradual size change. However, some chromosomes in the **sm** and **st** categories are defined using Levan *et al.* (1964) but there are many that are not clearly defined. Apart from the factors mention in this chapter (pages 22-23) this problem is magnified due to small chromosome sizes. The centromere position with the small cyprinid chromosomes changes gradually. This is a major problem in determining **sm** and **st** categories. It is therefore suggested that the **sm** and **st** categories be combined.

Statistical comparison of measured and visually assessed data indicates that the results are comparable in species with low chromosome numbers. However, the visually obtained data show a consistent assessment from one metaphase spread to the next as compared to the measured chromosome data.

In this study, an alternative method to visually assess the chromosomes into the **m**, **sm+st** and **a** chromosomes categories has been used.

CONCLUSIONS

Classification based of L/S arm-ratios of polyploid cyprinid chromosomes needs to be further investigated.

Since visual chromosome categories were, in some cases, not significantly different to the measured chromosome values, results obtained in this study can be compared to published studies.

CHAPTER 4: THE REDFIN MINNOWS *PSEUDOBARBUS*

INTRODUCTION

The genus *Pseudobarbus* is a distinct group of seven species commonly referred to as redfin minnows. These species are an integral part of the temperate ichthyofauna of southern Africa (Skelton 1980, 1994). Six species are distributed within the rivers of the Cape Fold Mountains from Sundays River in the east to the Olifants River system in the west. One species is found in the highland tributaries of the Orange River in Lesotho (Skelton 1986, 1994). The habitat of most of the *Pseudobarbus* species is in clear waters of low or moderate gradient mountain streams, pools or sheltered parts of the rivers and riffles of streams (Skelton 1993). The substratum is mainly bedrock, boulders, rocks or soft-bottomed. A few species are found also in the mainstream stretches (*P. burchelli*), in vegetated pools (*P. phlegethon*) and standing pools and turbid waters (*P. asper*) (Cambray & Stuart 1985, Cambray 1992). The waters of many of the Cape Fold Mountain streams where the redfin minnows are found are oligotrophic (pH=4-5.5, Jubb 1967, pH=5.0-5.9, Cambray 1992, TDS=44-159 mg/l, Cambray 1992) and are peat stained.

Pseudobarbus species are a component of the small cyprinids in southern Africa, where adults are generally less than 150 mm SL in size (Skelton 1980, 1993). Features which the *Pseudobarbus* share with other small minnows include a flexible dorsal ray, radial scale striations and squamation pattern. Presence of subterminal to inferior mouths and long convoluted intestines of the *Pseudobarbus* species confirm that their diet comprises mostly small bottom dwelling invertebrates, algae and detritus (Skelton 1980, 1993, Cambray 1992).

They breed over an extended period, coinciding with summer months (September to February) (Cambray 1992, Rall 1993). The eggs are slightly adhesive which is advantageous as *Pseudobarbus* species breed in riffles (Cambray & Meyer 1988, Cambray 1991). Summer breeding coincides with lower water flows, increased water clarity, higher temperature and abundant food resources for the larvae and juveniles (Cambray 1992). Growth is rapid in the first year, when approximately 33-44% of the final fork length is reached in *P. burchelli* (Cambray & Stuart 1985). Comparative studies on the sister species *P. afer* and *P. asper* have shown that they have evolved different reproductive strategies in response to different environments (Cambray 1992).

Although it is difficult to clearly distinguish the sexes during the non-breeding seasons, sexual dimorphism and dichromatism is encountered (Skelton 1980, 1993). Prominent conical tubercles on the head, snout and fins of some species, brighter fin colour and darkening of the body pigmentation mark the onset of sexual maturity in males of this genus. In some species, females also have head tubercles and red(der) fins. In male *Pseudobarbus*, pectoral fins are more rounded and pelvic fins are longer than in females (Skelton 1980). In all seven species females are larger than males (Skelton 1980, Cambray 1990). In three species (*P. burchelli, P. asper* and *P. afer*) studied males live longer than females (Cambray & Stuart 1985, Cambray 1990).

Five of the seven *Pseudobarbus* species are listed in the Red Data Book-Fishes (Skelton 1987), indicating their threatened conservation status. The degree of endemicity and naturally restricted geographic ranges of the species are factors for their threatened status, as many are site restricted or restricted to a single river system (Skelton 1993). Main threats include water abstraction, habitat degradation, and impacts of introduced predatory fish species such as *Micropterus spp.* and *Oncorhynchus mykiss* (Cambray & Stuart 1985, Skelton 1993). With increased demands on the water resources in South Africa as the pace of development escalates, the biodiversity of the river systems are severely impacted upon, by water abstraction, damming projects, and pollution. These small minnows species can be used as indicators of disturbances in the aquatic environment. However, very little is known about many of the species. Details of their interactions is crucial for developing informed conservation strategies for these species, and their habitat.

In recent years there has been an increased focus on investigating the biology and ecology of the small minnow species (Cambray 1989, 1992).

Ecology of only four of the *Pseudobarbus* species have been investigated, namely *P. burchelli* (Cambray & Stuart 1985), *P. afer* and *P. asper* (Cambray 1992) and *P. quathlambae* (Rall 1993). Diverse studies include age and growth, larval development, comparative neuroecology, microwear and distribution of tubercles, detailed structure of egg envelopes, and the evolutionary importance of egg size and egg number (Cambray 1991, 1992, Cambray & Hecht 1995). The above discussion outlines the current general biological information available for the *Pseudobarbus*, and serves as background information for the fish examined in this study.

The *Pseudobarbus* species are the only southern Africa cyprinid group whose phylogenetic relationships have been explored (Skelton 1980, 1988). Using traditional taxonomic parameters (morphometric, meristic and osteology data) Skelton (1980, 1988), showed that the flexible rayed red-fined minnows were unique and morphologically distinct from other *Barbus* species. The seven extant members are distinguished by a suite of unique characters which include bright red fin bases, prominent nuptial tubercles on the head, scales and fins, distinctive pharyngeal teeth (present in 2-3 rows) and various osteological characters (Skelton 1980, 1988). On the basis of shared derived characters (synapomorphy) the genus *Pseudobarbus* was proposed.

The phylogeny of the *Pseudobarbus* lineage established by Skelton (1980, 1988) forms the basis upon which the karyological study is conducted. In this chapter, the aim is to describe the karyotype of each of the seven *Pseudobarbus* species. Interspecies relationships as indicated by Skelton's (1980) morphological studies are also examined with karyological data. Analysis of chromosomal data provides additional characters which are used to test the hypothesis of a monophyletic origin of *Pseudobarbus* species. This process is taken further in chapters 4 and 5 where outgroup comparisons are made with several *Barbus* species. The interspecies relationships as proposed by Skelton (1980) are also tested using cytogenetic data.

This chapter addresses the following questions:

1) What are the karyotypes of the *Pseudobarbus* species? and do all the members of this group share common cytogenetic characters?

2) What inferences can be made of the interrelationships of *Pseudobarbus* species using karyological data?

MATERIAL AND METHODS

Seven species were karyotyped, number and sex of species examined in this chapter are listed in Table 4.1, other details (location & RUSI number) are provided in Table 2.1. Chromosomal data were obtained following methods given in chapter 2.

Table 4.1 The number and sex of specimens used in karyological analysis. Be=Bezuidenhouts River, Bl=Blindekloof River, B=Bainskloof, S=Swellendam, n=number of specimens examined, **=metaphase cells photographed by Oellermann pers. comm.

Species		n	♂	♀	un/juv
P. afer	Bez	6	2	3	1
P. afer	Bl	3	2	1	
P. asper		9	7	2	
P. burchelli	B	5	3	1	1
P. burchelli	S	4	2	1	1
P. burgi		4	3	1	
P. phlegethon		5	2	2	1
P. tenuis		5	3	1	1
*P. quathlambae***		2	1	1	

The karyology of *P. quathlambae* was determined from chromosome photographs taken by Oellermann in 1988. The chromosomes spreads were obtained according to the method by Oellermann & Skelton (1990) using gill tissue.

The chromosomes within the described categories are arranged in decreasing size in the photokaryotypes. Chromosome morphology descriptions are based mainly on visually assessed metaphase spreads. The description of the large and small chromosomes is expressed as a ratio of the **l**argest:**s**mallest (**l**:**s**) relative to the largest chromosome; chromosome lengths were calculated from one good representative metaphase spread.

RESULTS

Table 4.1 lists the species analysed in this chapter. Individual species photokaryotype results are presented in Figs. 4.2-4.7 & Tables 4.2-4.8. Comparison of males and females specimens of only *P. afer* are presented, and the karyotypes show no gender differences. Results of chromosome morphology is depicted in Tables 4.11 & 4.12 and Figs. 4.2-4.8 & 4.9. Karyotypes of seven redfin species are presented in Tables 4.10-4.12.

P. afer

Specimens from two populations of *P. afer* within the Swartkops River system were analysed. Two male and one female specimens from Blindekloof River and two males, three females and one juvenile from the Bezuidenhouts River were analysed (Tables 2.1 & 4.1). Accounts of male and female specimens are separated to demonstrate that no karyotypic differences were detected in either sex.

Blindekloof River

Forty-nine metaphase spreads were examined to obtain the modal chromosome number of 100 chromosomes (Fig. 4.1). Mean and modal values from 40 photokaryotypes of both male and female specimens have biarm component comprising 12**m** chromosomes, and 42**sm**+36**st** chromosomes, the uniarm component comprises 10**a** chromosomes (Table 4.2).

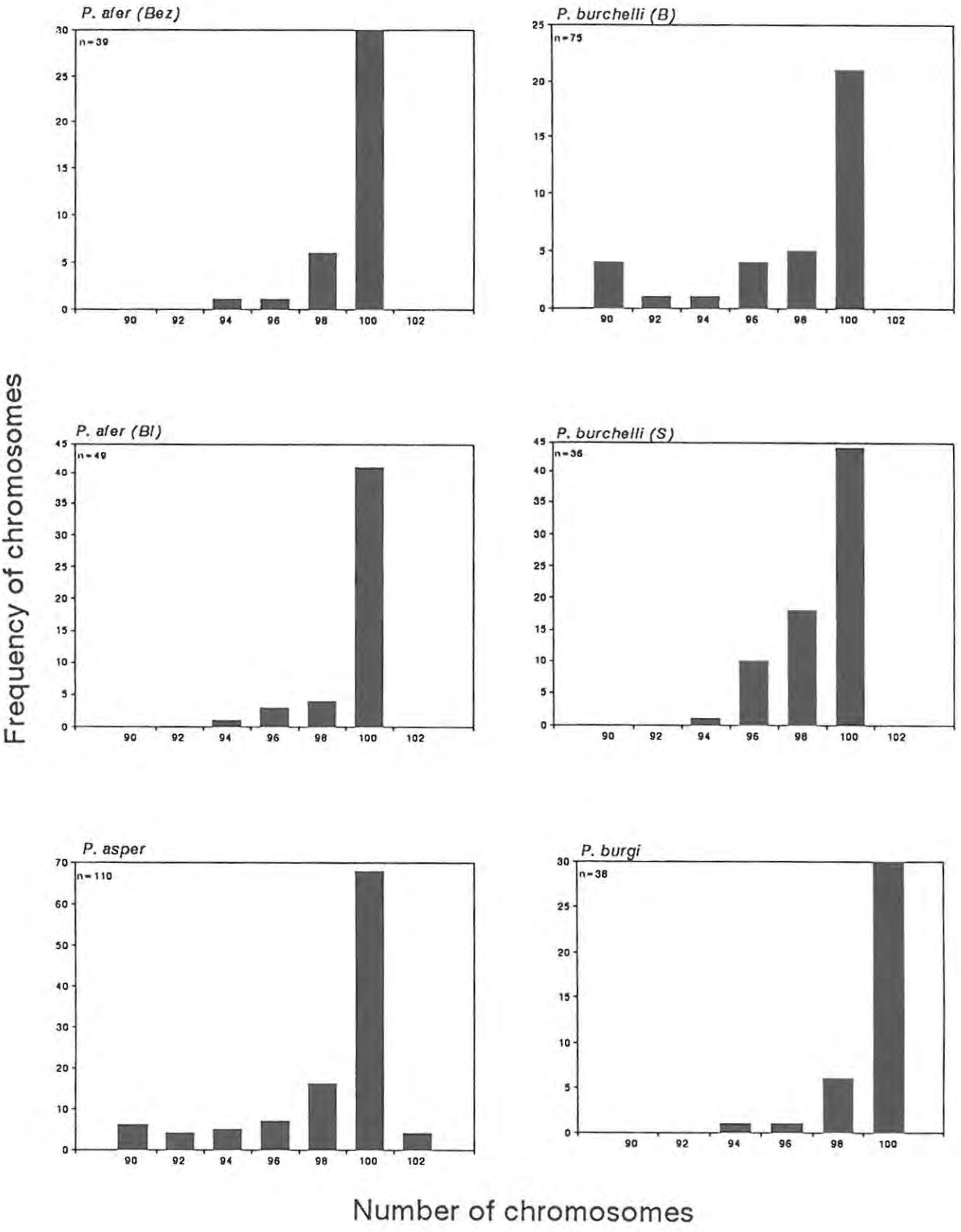


Figure 4.1 Percentage frequency distribution of chromosome numbers for seven *Pseudobarbus* species. Bez=Bezuidenhouts River, Bl=Blindekloof River, B=Bainskloof, S=Swellendam.

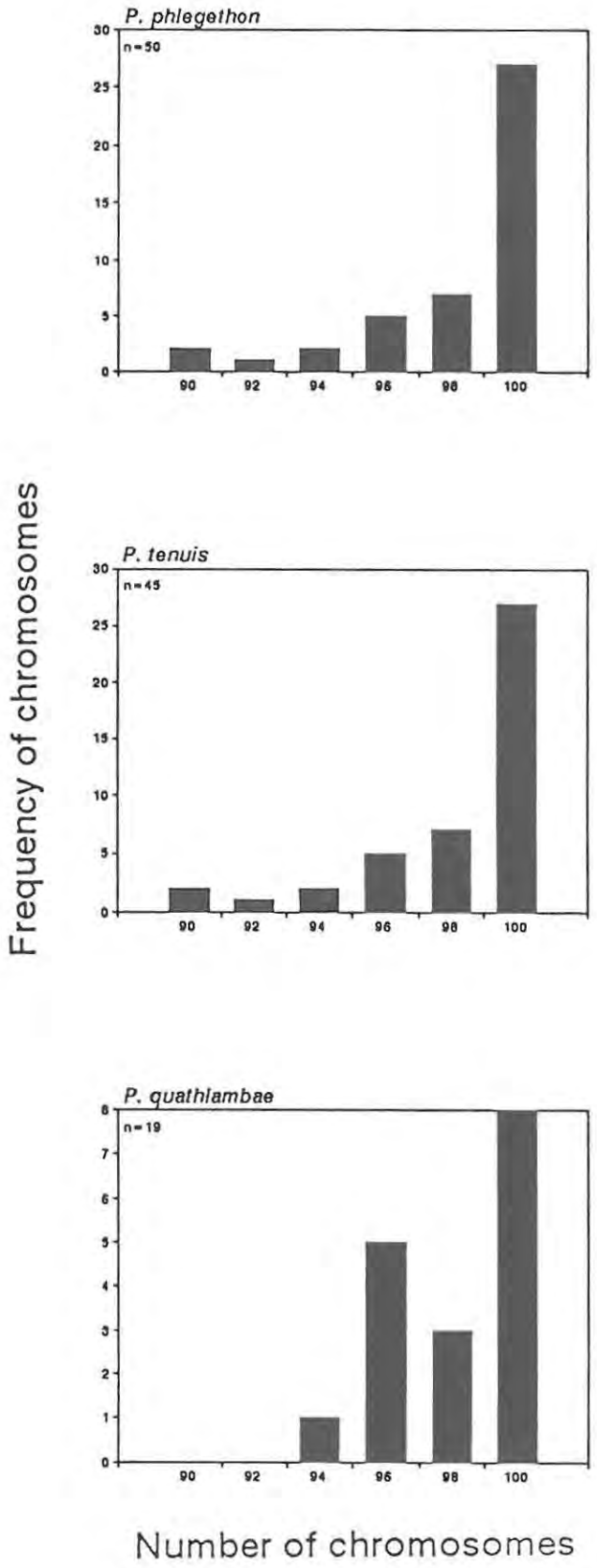


Figure 4.1 Percentage frequency distribution of chromosome numbers for seven *Pseudobarbus* species. (cont.)

Table 4.2 A detailed description of the photokaryotypes showing mean values, standard deviation (sd), modal values and range of number of chromosome components of *P. afer*, from the Blindekloof River. n=number of metaphase cells examined.

		Chromosome categories		
		m	**sm+st**	**a**
♂,n=21	Mean	11.9	41.2+37.1	9.1
	sd	0.7	1.7+1.3	1.0
	Mode	12	42+36	10
	Range	10-14	36-44+34-40	8-10
♀,n=19	Mean	11.6	41.0+36.3	10.4
	sd	0.8	2.0+1.2	1.1
	Mode	12	42+36	10
	Range	10-12	36-44+34-40	10-14

Chromosome morphology: No heteromorphic chromosomes were detected for either sex (Fig. 4.2a&b). Within all four chromosome categories there is a visual change in size from large to small chromosomes; the **m** and **a** chromosomes are all similar in size within each category; all the **sm+st** chromosomes gradually decrease in size, with a gradual change in centromere position; however, three pairs are noticeably larger within the **sm+st** chromosomes category (**l:s**=2.7,2.4&2.3); two pairs are noticeably smaller within the **sm+st** chromosomes category (Tables 4.11, 4.12 and Figs. 4.2a&b, 4.9).

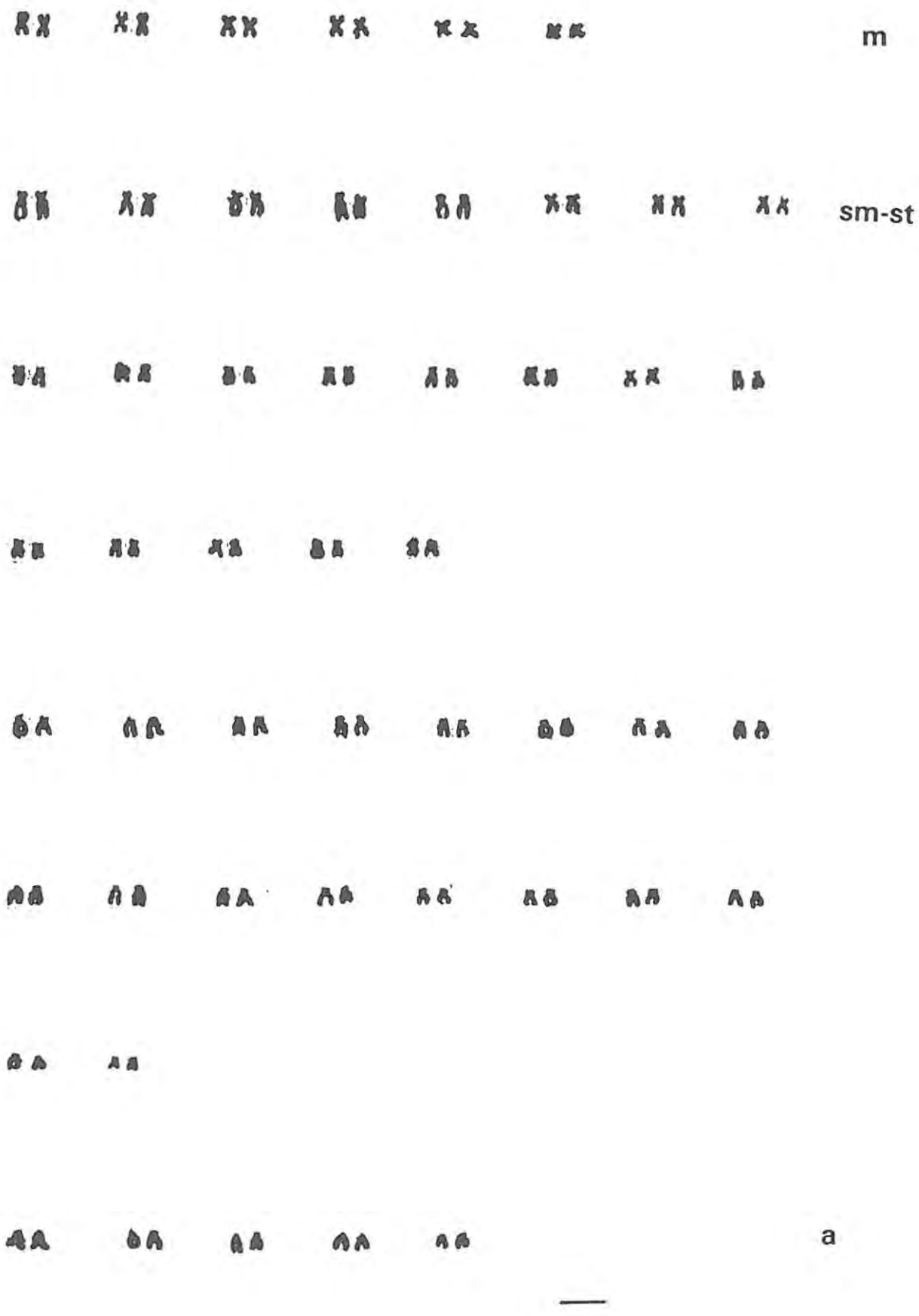


Figure 4.2a Photokaryotype of a male *P. afer* (Blindekloof River) with chromosome formula 2n=12**m**+42**sm**+36**st**+10**a**=100. Scale bar = 5µm.

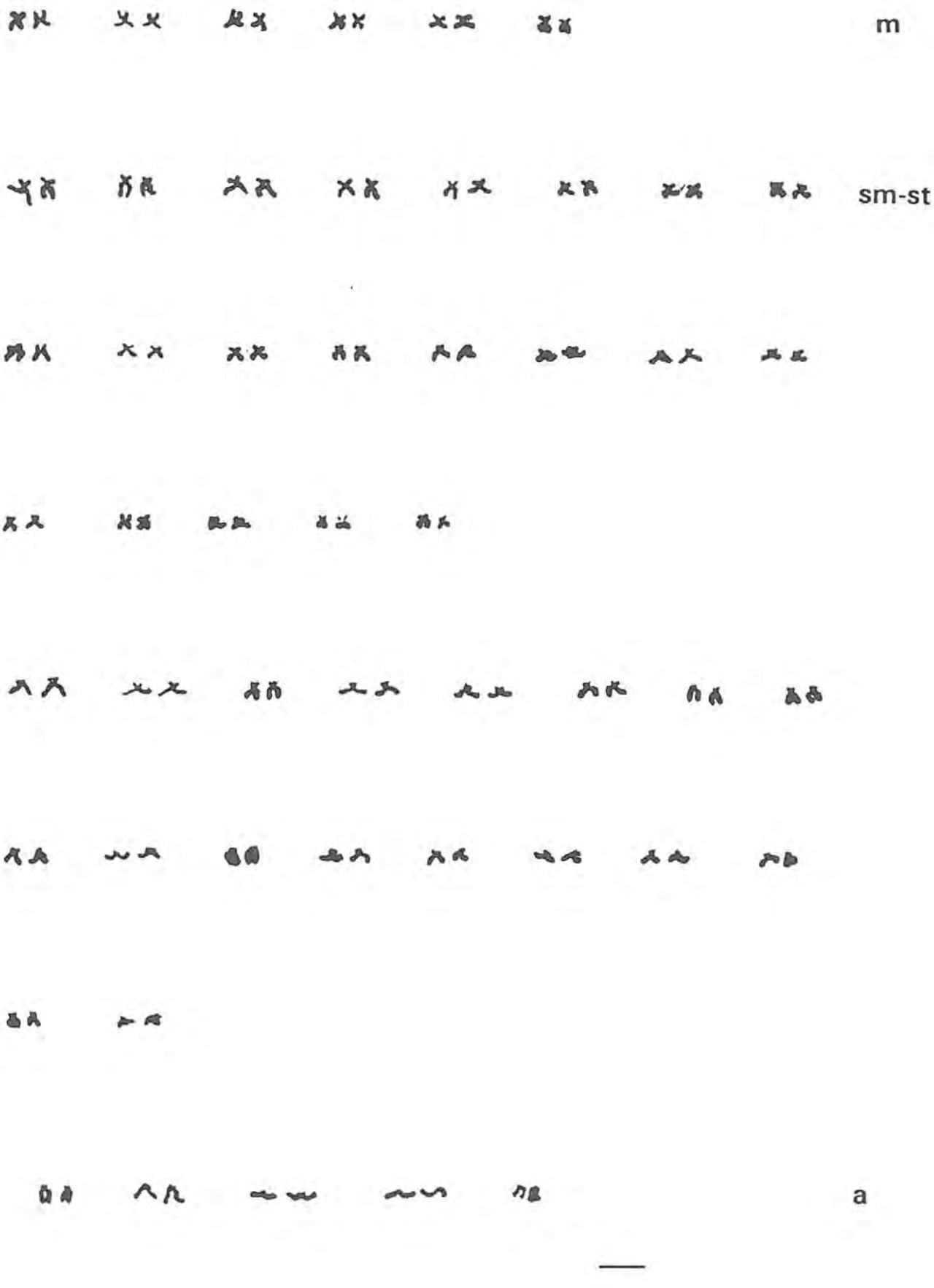


Figure 4.2b Photokaryotype of female *P. afer* (Blindekloof River) with chromosome formula 2n=12**m**+42**sm**+36**st**+10**a**=100. Scale bar = 5µm.

Bezuidenhouts River

A total of 39 metaphase spreads were analysed. The most frequent chromosome number is 100 (Fig. 4.1). Analysis of 20 photokaryotypes show that the modal biarm component consists of 12**m** chromosomes, 40**sm**+38**st** chromosomes, the modal uniarm component comprises and 10**a** chromosomes (Table 4.3).

Table 4.3. A detailed description of the photokaryotypes showing mean values, standard deviation (sd), modal values and range of number of chromosome components of *P. afer* from the Bezuidenhouts River. n=number of metaphase cells examined.

		Chromosome categories		
		m	**sm+st**	**a**
♂&♀,n=20	Mean	12.0	40.3+37.0	9.8
	sd	1.0	0.7+1.0	0.7
	Mode	12	40+38	10
	Range	10-14	40-42+36-38	8-10

Chromosome morphology: No heteromorphic chromosomes within any of the four chromosome categories were found in either sex (Fig. 4.3). The **m** and **a** chromosomes are all similar in size; three **sm+st** chromosome pairs are the largest chromosome pairs (**l:s**=2.1,2.1&1.8), and two pairs are the smallest in this category; the remaining chromosomes gradually decreasing in size (Fig. 4.3).

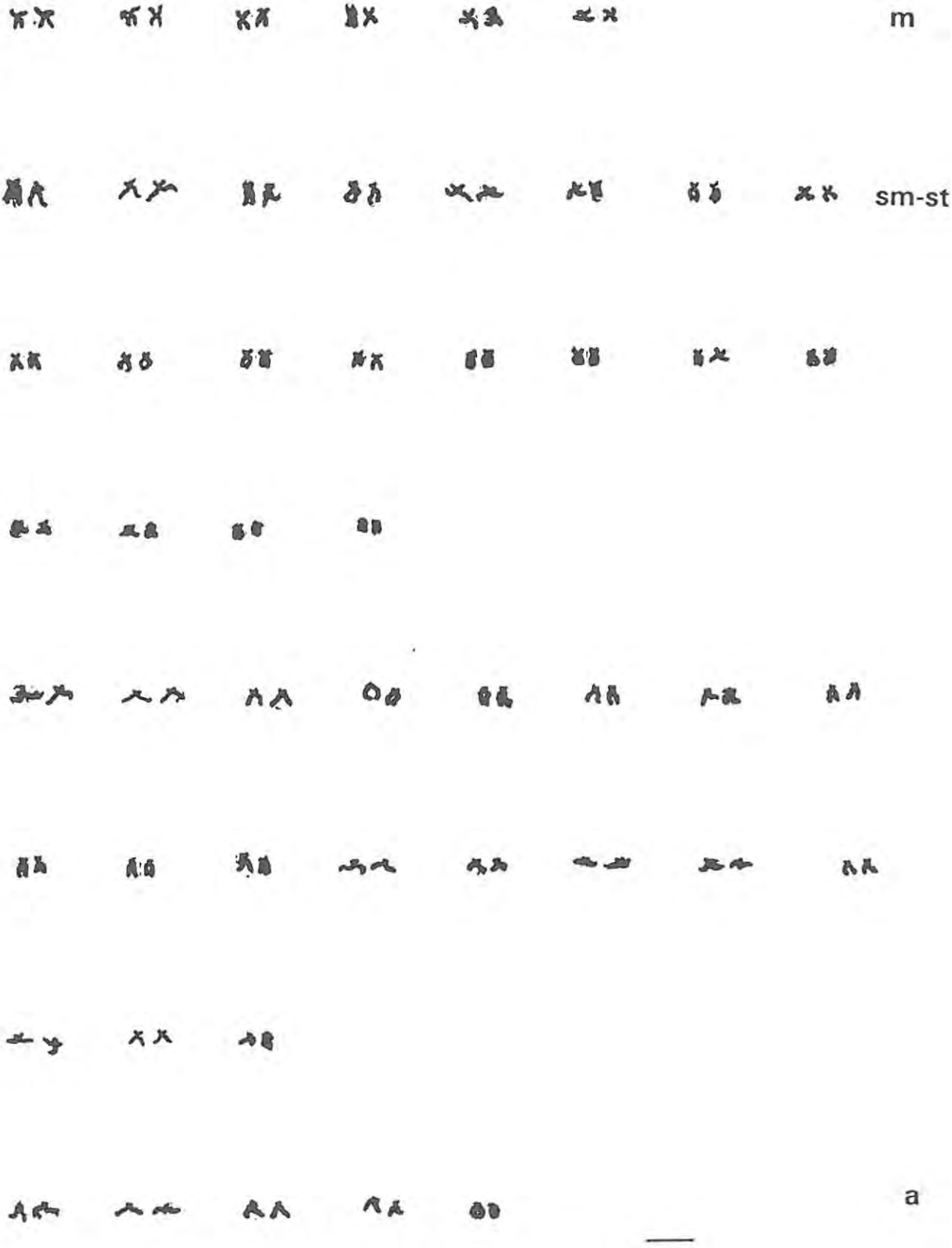


Figure 4.3 Photokaryotype of a male *P. afer* (Bezuidenhouts River) with chromosome formula 2n=12**m**+40**sm**+38**st**+10**a**=100. Scale bar = 5µm.

P. asper

Seven males and two females of *P. asper* from the Groot River, Gamtoos System were karyotyped (Table 4.1). In 110 metaphase cells the modal number is 100 (Fig. 4.1). Analysis of 24 male and female photokaryotypes shows that the modal biarm component comprises 14**m** chromosomes and 46**sm**+32**st** chromosomes (Table 4.4). The modal uniarm component comprises 8**a** chromosomes (Table 4.4 & Table 4.11). A pair of **st** chromosomes appears to have an achromatic region close to the centromere. However, this was not consistently observed in all the metaphases spreads and may represent a staining artifact.

Table 4.4. A detailed description of the photokaryotypes showing mean values, standard deviation (sd), modal values and range of number of chromosome components of *P. asper*. n=number of metaphase cells examined.

		Chromosome categories		
		m	**sm+st**	**a**
♂&♀,n=24	Mean	13.3	43.9+33.6	8.4
	sd	1.4	4.2+3.1	1.0
	Mode	14	46+32	8
	Range	10-14	32-48+32-4	8-12

Chromosome morphology: No heteromorphic chromosomes were identified in either male or female metaphase spreads (Fig. 4.4). There is a gradual shift in chromosome size and centromere position within each of the four categories; the **sm+st** chromosomes shows the largest range variation in centromere position and chromosome size; there are two pairs of small chromosomes in the **sm+st** category as well as three elongated **sm+st** chromosome pairs (**l:s**=3.7,3.6&3.4); the **m** and **a** chromosomes are very similar in size range (Tables 4.11, 4.12 and Figs. 4.4, 4.9).

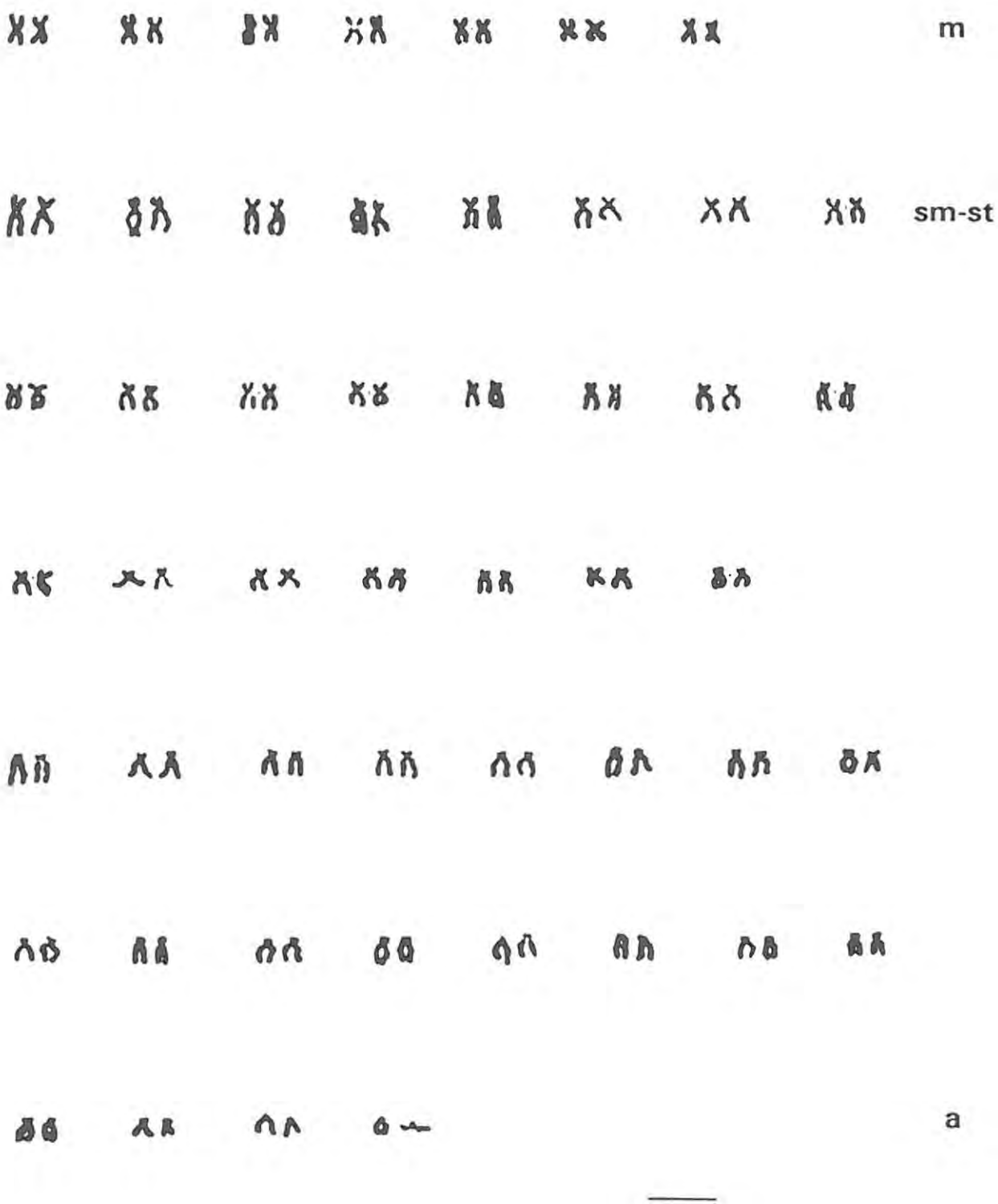


Fig. 4.4 Photokaryotype of a male *P. asper* (Groot River) with chromosome formula 2n=14**m**+46**sm**+32**st**+8**a**=100. Scale bar = 5µm.

P. burchelli

One female and three males, as well as one juvenile specimen from the Steenboks River, Bainskloof and two males, one female and one juvenile specimen whose sex was undetermined, from the Leeu River, Swellendam were analysed karyologically (Table 4.1).

Bainskloof

In 75 metaphase spreads, the modal chromosome number is 100 (Fig. 4.1). Mean and modal values from 28 photokaryotype of males and females shows that the biarm component comprises 10**m** chromosomes, 42**sm**+34**st** chromosomes (Table 4.5). The modal uniarm component consists of 14**a** chromosomes (Table 4.5).

Table 4.5. A detailed description of the photokaryotypes showing mean values, standard deviation (sd), modal values and range of number of chromosome components of *P. burchelli*. n=number of metaphase cells examined.

		Chromosome categories		
Bainskloof		**m**	**sm+st**	**a**
♂&♀,n=28	Mean	10.1	41.1+34.2	14.2
	sd	0.9	1.4+0.8	1.6
	Mode	10	42+34	14
	Range	8-12	38-42+32-36	10-18
Swellendam				
♂&♀,n=19	Mean	10.5	40.9+35.1	13.2
	sd	1.4	2.3+1.8	1.8
	Mode	10	40+36	14
	Range	8-14	36-46+32-38	10-16

Chromosome morphology: The largest size range variation is within the **sm+st** chromosome categories; there are three pairs of distinctly small and two pairs of distinctly large chromosomes within the **sm+st** chromosomes category; chromosomes within the **m** and **a** category are all similar to each other in size. Chromosome sizes were determined from visual karyotype assesment for this species.

Swellendam

A total of 36 metaphase cells were analysed. The modal chromosome number is 100 (Fig. 4.1). Analysis of 19 photokaryotypes shows the modal biarm component comprises 10**m** chromosomes, 40**sm**+36**st** chromosomes (Table 4.5). The modal uniarm component comprises 14**a** chromosomes (Table 4.5).

Chromosome morphology: The **sm**+**st** chromosomes are the most numerous, two pairs of **sm**+**st** chromosomes are notably elongated (**l:s**=7.7&7.0) and three pairs of **sm**+**st** chromosomes are noticeably small compared to the rest of the chromosomes in this category which show a gradation in size from large to small; the **m** and **a** chromosomes change slightly in size and the chromosomes are similar in size within each category (Tables 4.11, 4.12 and Figs. 4.5, 4.9). Neither male nor female karyotypes have heteromorphic chromosome pairs.

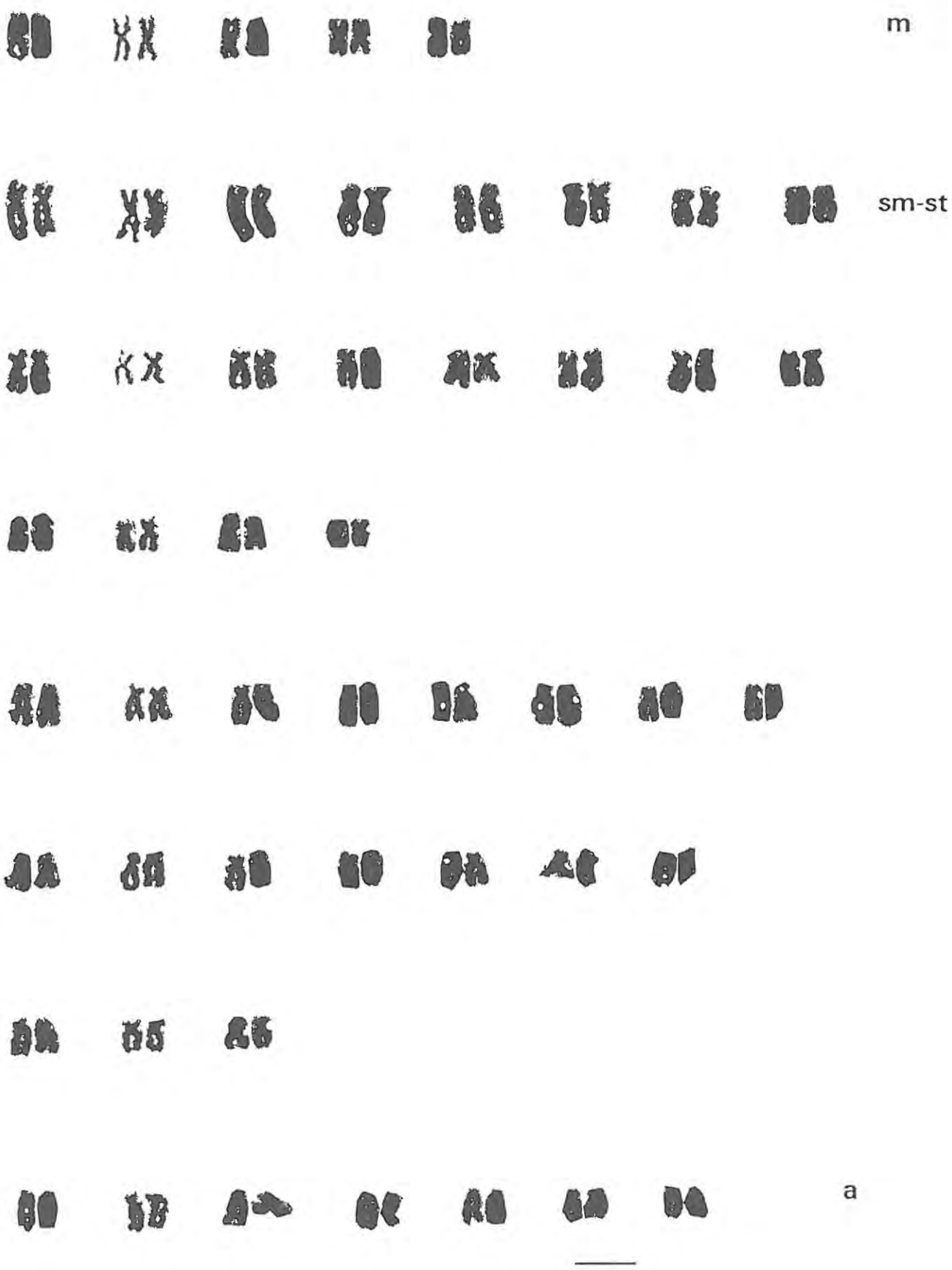


Figure 4.5 Photokaryotype of a female *P. burchelli* (Swellendam) with chromosome formula 2n=10**m**+40**sm**+36**st**+14**a**=100. Scale bar = 5μm.

P. burgi

Three males and one female of *P. burgi*, from the Wemmershoek River, Berg River System were karyotyped (Table 4.1). The karyotypes of *P. burgi* were determined from metaphase spreads that were more contracted than the other species. As a result the chromosomes were very small which could contribute to incorrectly classifying the chromosomes. Analysis of 38 metaphase cells showed 100 to be the modal chromosome number (Fig. 4.1). Mean and modal analysis of 12 photokaryotypes shows the biarm component to be composed of 14**m** chromosomes and 36**sm**+38**st** chromosomes (Table 4.6). The modal uniarm component comprises 12**a** chromosomes (Table 4.6).

Table 4.6. A detailed description of the photokaryotypes showing mean values, standard deviation (sd), modal values and range of number of chromosome components of *P. burgi*. n=number of metaphase cells examined.

		Chromosome categories		
		m	**sm+st**	**a**
♂&♀,n=12	Mean	12.7	35.2+40.0	12.0
	sd	2.2	1.5+3.6	0.8
	Mode	14	36+38	12
	Range	8-14	32-36+38-48	10-14

Chromosome morphology: Both the **m** and **a** chromosomes are composed of very similar sized elements; the **sm+st** chromosomes have a wide size range with a gradual change in centromere position; within the **sm+st** chromosomes two pairs are distinctly smaller and three pairs are distinctly larger (**l:s**=3.7,3.6&3.5) than the rest of the chromosomes in this category (Tables 4.12, 4.11, Figs. 4.6, 4.9). No heteromorphic chromosome pairs were identified in either male or female metaphase spreads (Fig. 4.6).

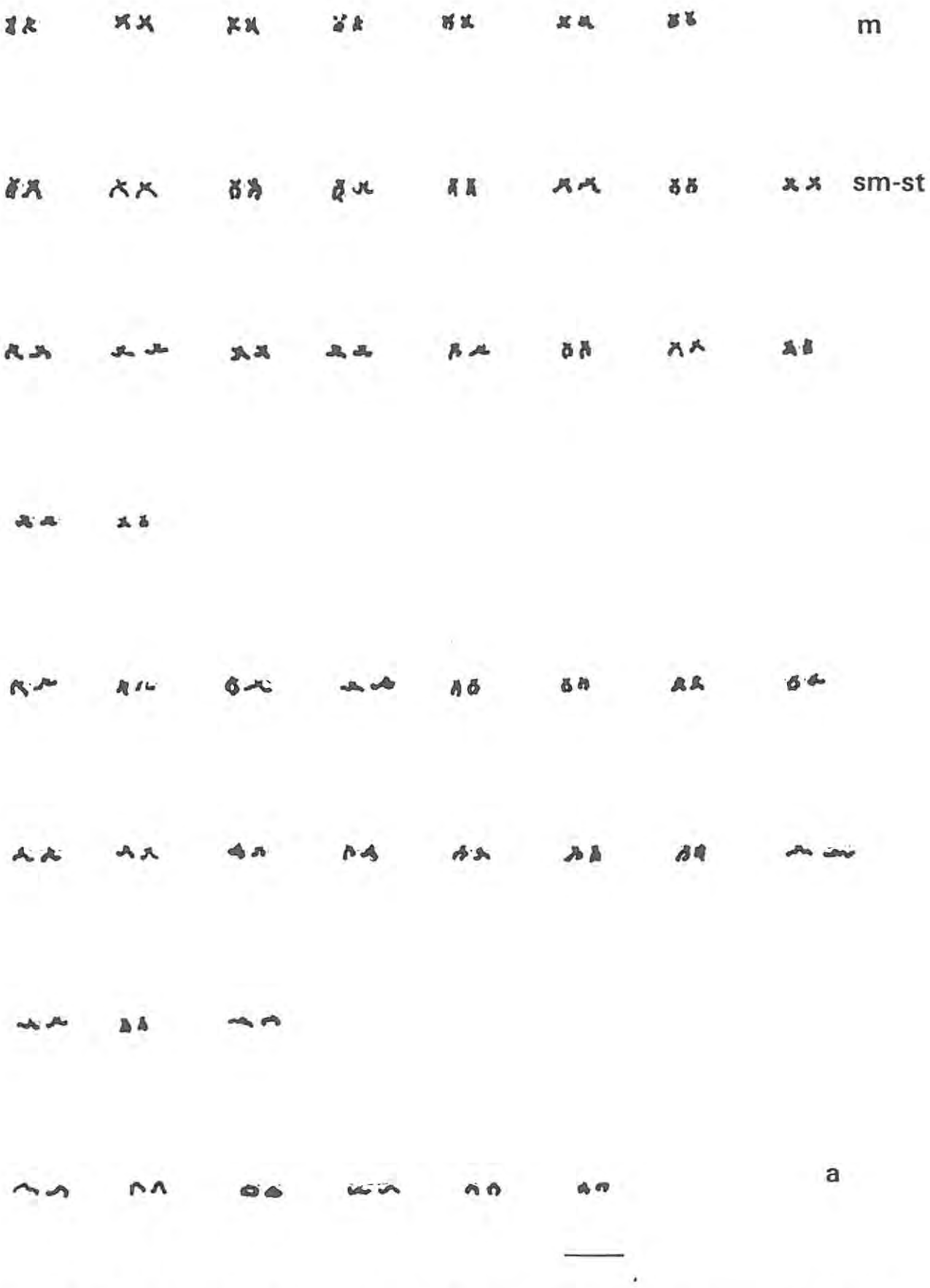


Figure 4.6 Photokaryotype of male *P. burgi* (Wemmers River) with chromosome formula 2n=14**m**+36**sm**+38**st**+12**a**=100. Scale bar = 5µm.

P. phlegethon

Two males, two females and one specimen of undetermined sex, of *P. phlegethon* from the Thee River were karyotyped (Table 4.1). Fifty metaphase cells shows a modal chromosome number of 100 chromosomes (Fig. 4.1). Mean and modal values of 19 photokaryotypes indicate that the biarm component comprises 14**m** chromosomes 40**sm**+38**st** chromosomes (Table 4.7). The modal uniarm component comprises 8**a** chromosomes (Table 4.7).

Table 4.7. A detailed description of the photokaryotypes showing mean values, standard deviation (sd), modal values and range of number of chromosome components of *P. phlegethon*. n=number of metaphase cells examined.

		Chromosome categories		
		m	**sm+st**	**a**
♂&♀,n=19	Mean	13.6	40.4+37.6	8.0
	sd	1.0	2.0+2.1	0.6
	Mode	14	40+38	8
	Range	10-14	38-48+30-42	8-10

Chromosome morphology: The **m** and **a** chromosomes are similar in size to each other in the category; there are numerous **sm+st** chromosomes; three pairs of **sm+st** chromosomes are the largest in the complement (**l:s**=7.8,7.1,6.7), and three pairs are notably the smallest in the complement (Tables 4.11, 4.12, Figs. 4.7, 4.9). Karyotypes from male and female specimens showed no heteromorphic chromosomes (Fig. 4.7).

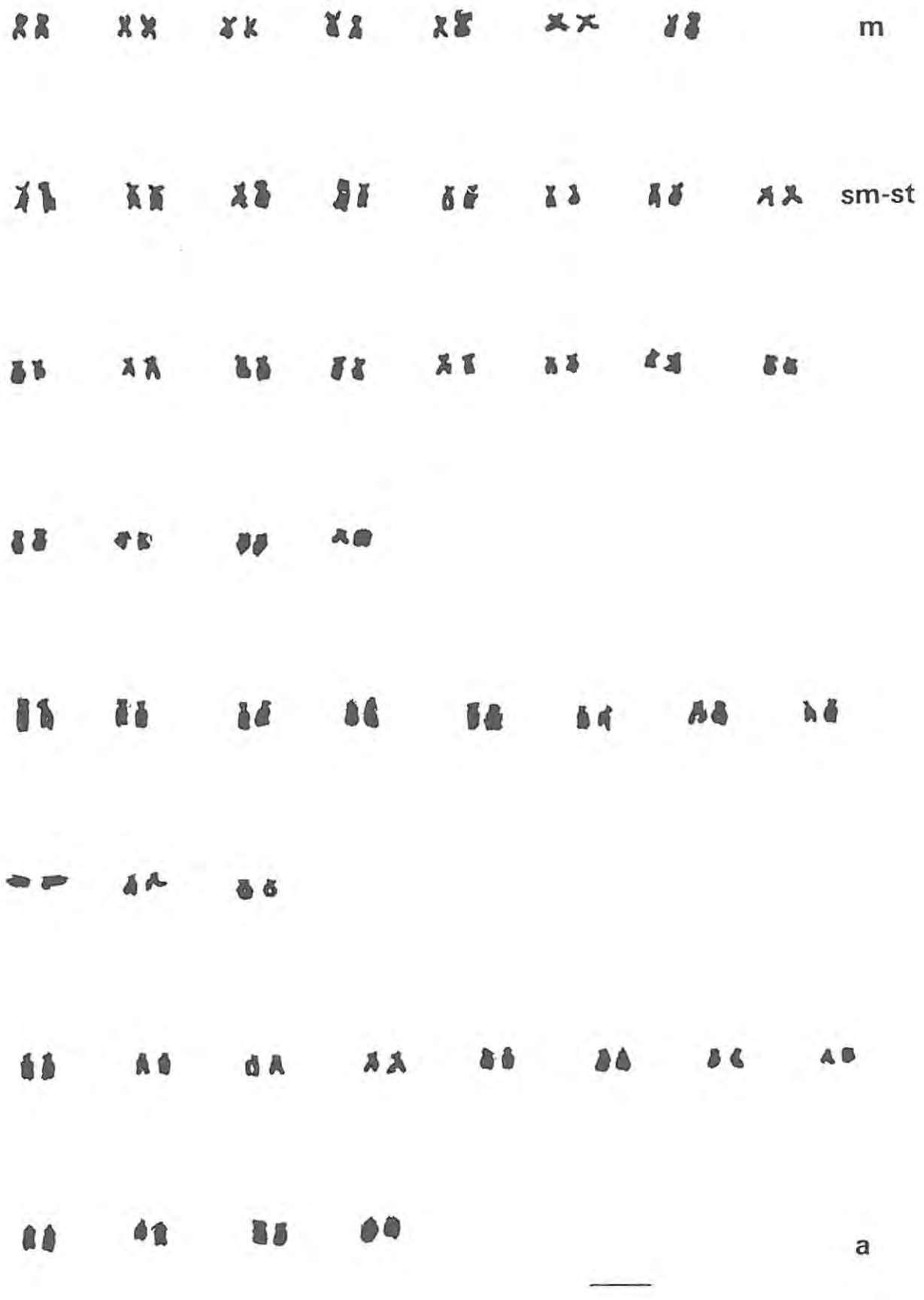


Figure 4.7 Photokaryotype of a male *P. phlegethon* (Thee River) with chromosome formula 2n=14**m**+40**sm**+38**st**+8**a**=100. Scale bar = 5µm.

P. tenuis

Three males, one female and one specimen of undetermined sex of *P. tenuis*, all from the Grobbelaars River (Table 4.1) were analysed. In 45 metaphase cells the modal chromosome number was 100 chromosomes (Fig. 4.1). Mean and modal analysis of 17 photokaryotypes showed that the biarm component comprises 14**m** chromosomes, 40**sm**+32**st** chromosomes, the uniarm component comprises 14**a** chromosomes (Table 4.8).

Table 4.8. A detailed description of the photokaryotypes showing mean values, standard deviation (sd), modal values and range of number of chromosome components of ♂ & ♀ *P. tenuis*. n=number of metaphase cells examined.

		Chromosome categories		
		m	**sm+st**	**a**
♂&♀,n=17	Mean	13.5	39.2+32.9	13.8
	sd	0.8	1.8+1.4	0.9
	Mode	14	40+32	14
	Range	12-14	34-42+32-36	12-16

Chromosome morphology: There is only a slight size variation within the **m** and **a** chromosomes, with the result that the chromosomes are very similar in size; there is a large range of sizes within the **sm+st** chromosomes; with three distinctly large **sm+st** chromosomes pairs in the complement (**l:s**=6.8,6.0&5.8) and a notably small chromosome pair in the complement (Tables 4.11, 4.12, Figs. 4.8, 4.9). No heteromorphic chromosomes were detected in either the male or female karyotypes (Fig. 4.8).

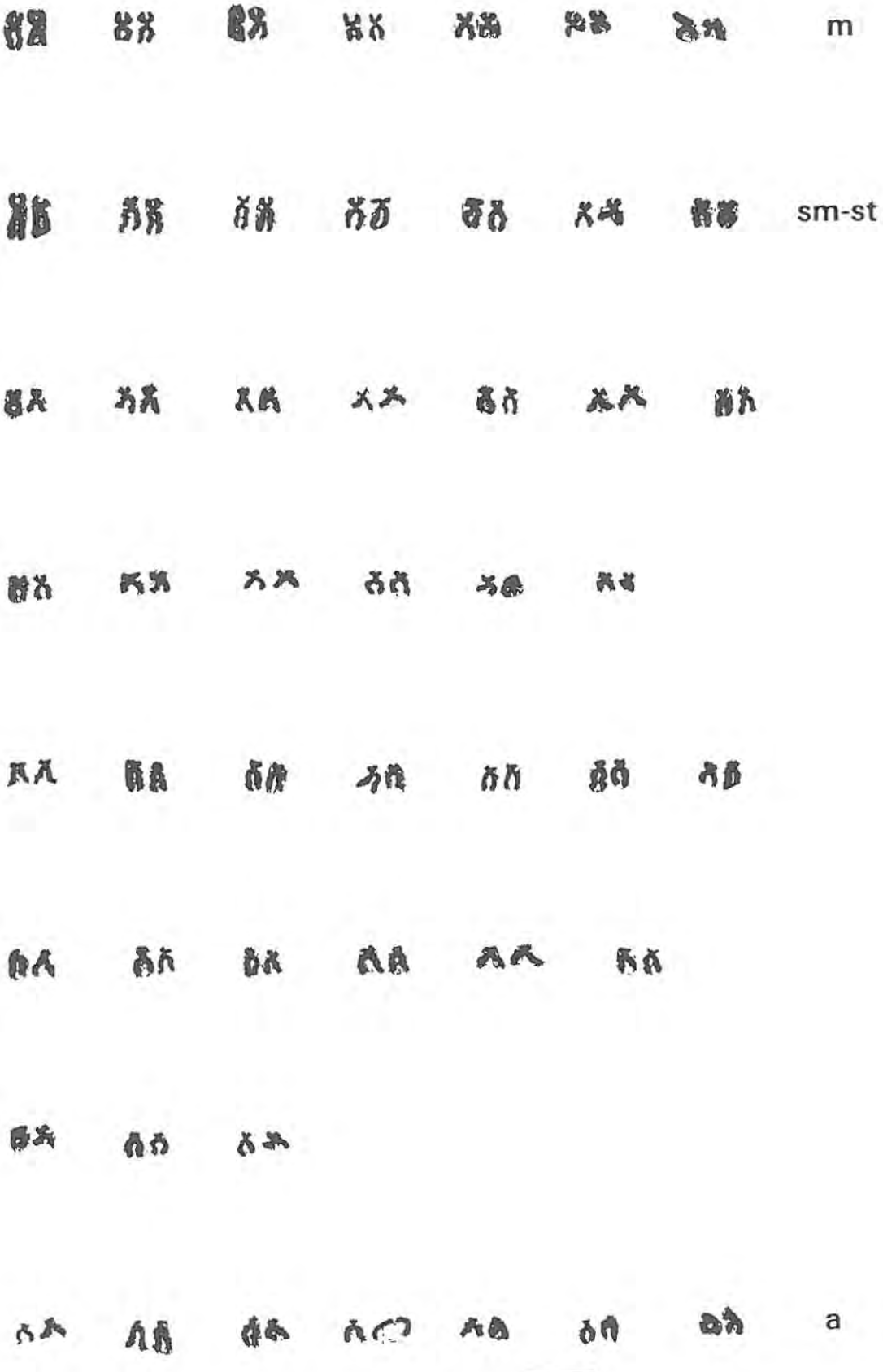


Figure 4.8 Photokaryotype of a female *P. tenuis* (Grobbelaars River) with chromosome formula 2n=14**m**+40**sm**+32**st**+14**a**=100. Scale bar = 5µm.

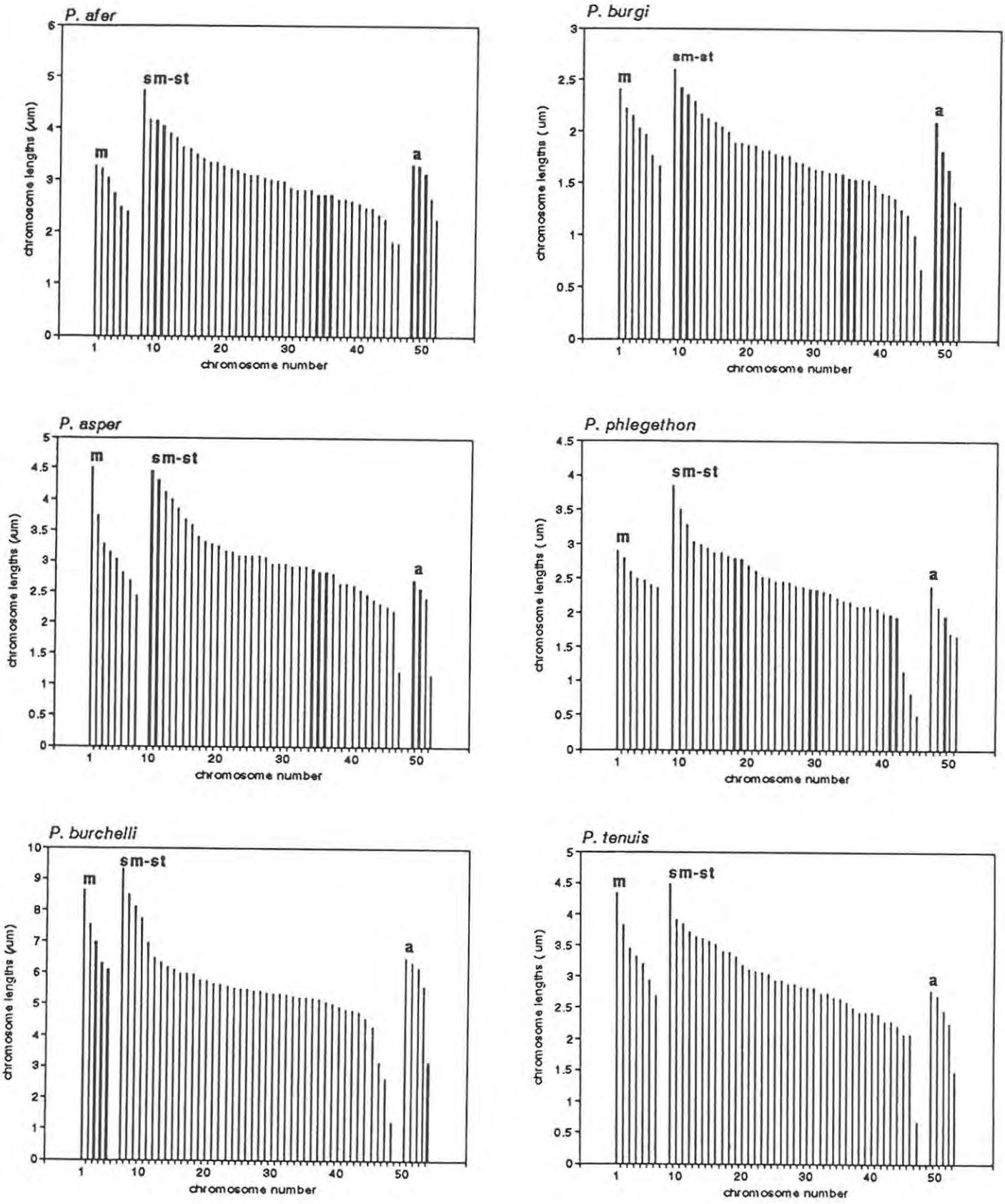


Figure 4.9 Average chromosome lengths (µm) in the **m**,**sm+st** and **a** chromosome categories of six *Pseudobarbus* species.

P. quathlambae

A total of 19 metaphase spreads from both male and female specimens were examined. The modal chromosome number ranged from 96-100 chromosomes (Fig. 4.1). Due to prolonged colchicine treatment (3-4 hours as compared to 2 hours in this study), the chromosomes were highly spiralized. There are 8**m** chromosomes, 34**sm**+36**st** chromosomes and 20**a** chromosomes (Table 4.9). This karyotype should be considered a preliminary result.

Table 4.9. Detailed description of the photokaryotypes showing mean values, standard deviation (sd), modal values and range of number of chromosome components of ♂ & ♀ *P. quathlambae***. n=number of metaphase cells examined, **=metaphase cells photographed by Oellermann pers. comm.

		Chromosome categories		
		m	**sm+st**	**a**
♂&♀,n=5	Mean	8.0	34.0+36.4	21.6
	sd	0	0+2.1	2.1
	Mode	8	34+36/38	20
	Range	8	34+33-39	19-25

Chromosome morphology: Chromosomes in the **m** and **a** categories appear to be similar in size, while the **sm+st** appear to have a wide range of chromosomes gradually decreasing in size. The chromosome size asessments were made visually from photomicrographs of the metaphase cells Heteromorphic chromosome pairs were not identified in either the male or female photokaryotypes.

NF: total number of chromosome arms

The two NF values for the redfin species in Table 4.10 show that the values range from 150-160, a range of 10 arms in NF1, and from 180-192, a range of 12 arms in NF2. The total chromosome number however is the same for all seven of the *Pseudobarbus* species.

Table 4.10 Total number of arms for seven *Pseudobarbus* species analysed using two different calculations, NF1=2(**m+sm**)+(**st+a**) and NF2=2(**m+sm+st**)+(**a**), Bez=Bezuidenhouts River, Bl=Blindekloof River, B=Bainskloof, S=Swellendam, **=metaphase cells photographed by Oellermann pers. comm.

Species		NF1	NF2
P. afer	Bl	154	190
P. afer	Bez	152	190
P. asper		160	192
P. burchelli	B	152	186
P. burchelli	S	150	186
P. burgi		150	188
P. phlegethon		154	192
P. tenuis		154	186
*P. quathlambae***		140/142	176/180

Meiotic chromosomes

The meiotic spreads were scanned for an indication of bivalent, tetravalent and multivalent chromosome pairs. In all male *Pseudobarbus* species examined no apparent tetravalent chromosome pairs were observed in the diplotene stage of first meiotic prophase (Fig. 4.10) . Both hypermodal and hypomodal counts of "chromosomal elements" were observed from the slides prepared with the testes material.

Slides were prepared using a smear technique, where the tissue was brushed over the surface of the slide. Chromosomal losses could be as a result of this smear technique where the probability of overlapping and or loss of chromosomal elements is increased. Hypermodal values may represent cells where chromatid separations have occurred or where chromosomal elements of two or more cells close together have become intermingled. In some cases the high chromosome values may be due to colchicine induction of polyploidy (Denton 1973).

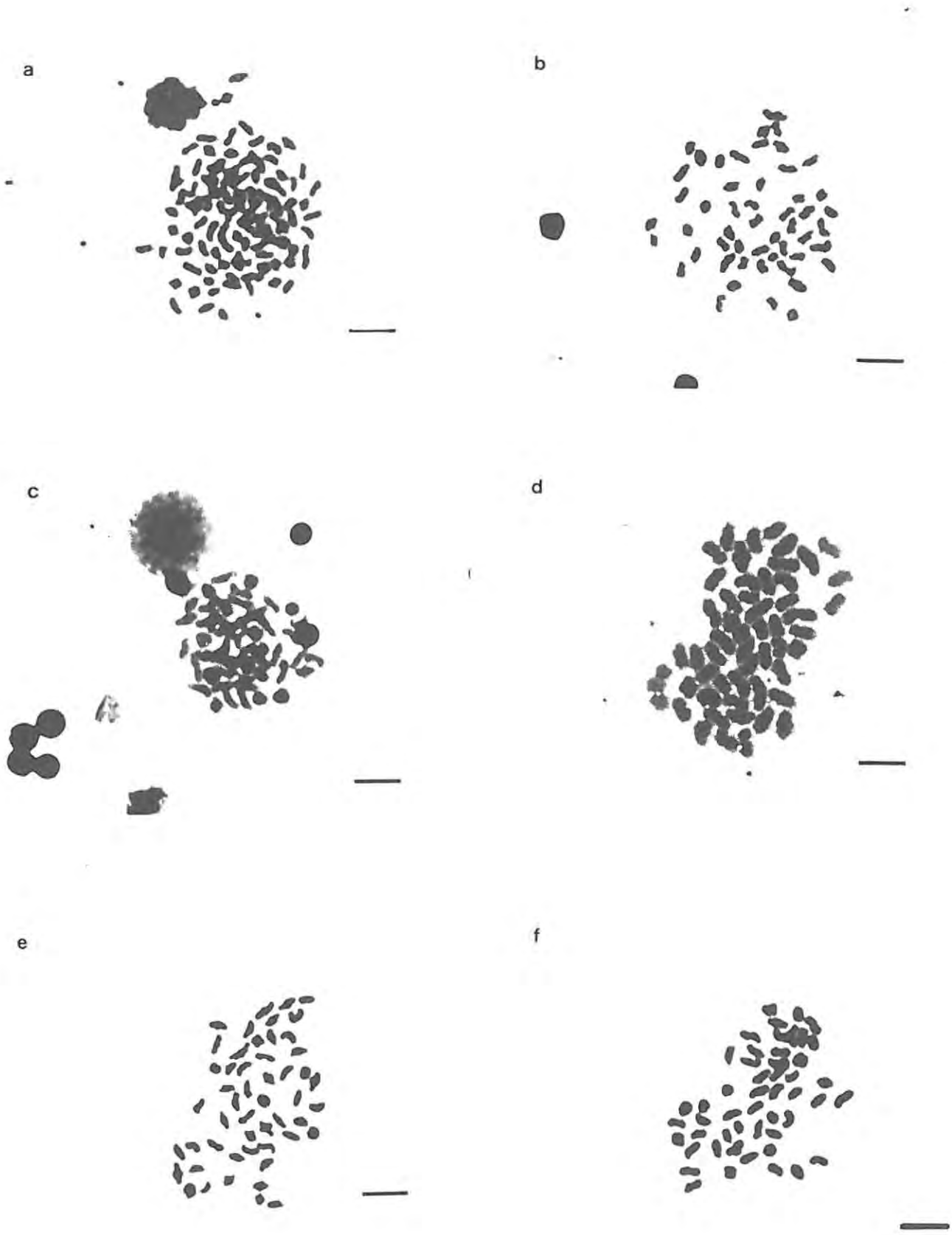


Figure 4.10 Meiotic spreads showing bivalent chromosomes in six *Pseudobarbus* species. a) *P. afer*, b) *P. asper*, c) *P. burchelli*, d) *P. burgi*, e) *P. phlegethon* and f) *P. tenuis*. Scale bar = 5µm.

Summary of characteristics of *Pseudobarbus* karyotypes

Good karyological preparations allows for detailed chromosomal descriptions into three categories **m**, **sm+st** and **a**. Characteristic features of the *Pseudobarbus* karyotypes are summarised as follows:

1. The majority of chromosomes are within the **sm+st** categories, with a range of **sm**(18-23)+**st**(16-19) pairs, comprising 32-46% of the karyotype (Table 4.11).

2. The **m** chromosomes range from five to seven pairs representing 10-14% of the karyotype. An exception is *P. quathlambae* with four **m** chromosome pairs (Table 4.11).

3. There are fewer **a** chromosomes (Table 4.11), 4-7 pairs are clear **a** chromosomes i.e. the centromere is terminal. 12-14% of the karyotype is represented by **a** chromosomes. The exception is *P. quathlambae* which has 10 pairs of **a** chromosomes. However, in this species the minute second arm of the **a** chromosomes are probably not apparent due to the contracted state of chromosomes.

4. The karyotype can be described as asymmetric because there are more biarmed chromosomes than uniarm chromosomes.

5. All seven species have at least two (or three) pairs of elongated **sm+st** chromosomes. These are the largest elements in the complement.

6. There are at least one and up to three pairs of **sm+st** chromosomes which are the smallest in the complement (Figs. 4.2-4.8 & Table 4.12).

7. Neither the **m** nor **a** chromosomes vary much in size from the first to the last pair (Figs. 3.2-3.8 & Table 4.12).

8. No difference between male and female karyotypes was apparent and no heteromorphic chromosomes were detected (Figs. 4.2a&b).

9. All species have high NF2 values ranging from 180-192, indicating the presence of many biarmed chromosomes (Table 4.10).

10. No tetravalent or multivalents are evident in the meiotic chromosome preparation (Fig. 4.10).

Table 4.11 Modal values of karyotype components from kidney cells of seven small flexible rayed *Pseudobarbus* species. Bez=Bezuidenhouts River, Bl=Blindekloof River, B=Bainskloof, S=Swellendam, n=number of metaphase cells examined, **=metaphase cells photographed by Oellermann pers. comm. Marker chromosomes: **sm**-l=number of submetacentric long chromosome pairs.

		Chromosome categories in pairs				
Species		n	**m**	**sm+st**	**a**	**sm**-l
P. afer	Bl	40	6	21+18	5	3
P. afer	Bez	20	6	20+19	5	3
P. asper		24	7	23+16	4	3
P. burchelli	B	28	5	21+17	7	2
P. burchelli	S	19	5	20+18	7	2
P. burgi		12	7	18+19	6	3
P. phlegethon		19	7	20+19	4	3
P. tenuis		17	7	20+16	7	2-3
*P.quathlambae***		5	4	17+18/19	10	-

Table 4.12 The size range (µm) of chromosomes in the three categories based on photokaryotypes of six *Pseudobarbus* species. Bez=Bezuidenhouts River, S=Swellendam.

Species		**m**	**sm+st**	**a**
P. afer	Bez	4.1-2.4	4.7-1.8+4.2-1.8	3.3-2.3
P. asper		4.5-2.2	4.5-1.2+4.1-2.4	2.7-1.2
P. burchelli	S	8.6-2.6	9.3-1.2+6.5-3.1	5.6-3.2
P. burgi		2.4-1.3	2.4-0.8+2.5-1.4	2.1-0.7
P. phlegethon		3.8-1.1	3.5-0.5-3.3-0.8	2.1-1.6
P. tenuis		4.3-2.1	4.5-0.6+3.7-2.3	3.3-1.5

DISCUSSION

Determination of chromosomal numbers

Variation in chromosomal numbers indicated by the standard deviation of the mean can be found on slides prepared from the same specimen (Fig. 4.1 & Tables 4.2-4.9). Such variations in intraspecimen chromosomal numbers may be artefactual, and possibly due to the technical features

(Gui *et al.* 1986).

However, variation in chromosome number, between specimens, may also reveal karyotypic diversification which has occurred between allopatric populations. Intraspecific, or populational differences, have been reported for *Diptychus gymnogaster (oschanini)* and *D. g. microcephalus* (Mazik *et al.* 1989) and in *Rutilus alburnoides* "complex" (Collares-Pereira 1985). Karyotypic variation has been observed at subspecies level within *Barbus bynni bynni* and *Barbus bynni occidentalis* (Guégan *et al.* 1995). Golubstov & Kryzanov (1993) reported geographical differences in karyotypes in the African *Barbus intermedius*.

Frequency distribution of chromosome numbers for *P. afer* from two different sites in one river system (Bezuidenhouts River and Blindekloof River) shows that 16% to 21% of the cells examined have hypomodal count (Fig. 4.1). Examination of cell spreads with hypomodal counts showed no morphological changes in the karyotypes such as particularly larger or smaller chromosomes which would indicate chromosomal rearrangements. The hypomodal values have been attributed to overlapping of chromosomes or loss of chromosomes and miscounting when chromosomes are obscured by cell debris (Collares-Pereira 1985, Rab & Roth 1989).

Only 1.4% of the cells analysed in the remaining *Pseudobarbus* species have hypermodal chromosomal counts. Hypermodal counts have been attributed to breakages or premature chromatid separation and a consequence of adjacent cell fusion (Collares-Pereira 1985). Very few observations were made where there were two or three metaphase cells close to each other suggesting that good cell separation was obtained using the grid maceration method (see chapter 2). It also indicates that, in a majority of cases, the cell membrane remained intact.

Variation in the chromosome numbers of *Pseudobarbus* species examined do not indicate inherent karyological differences between species. It does however, indicate that the quality of the chromosome preparations were satisfactory and gave reliable results for chromosome numbers.

Photokaryotype determination

General variations in uniarm-biarm determinations

In most of the *Pseudobarbus* karyotypes examined the mean and modal values are comparable (Tables 4.2-4.9). Differential contraction of the metaphase chromosomes within the many cells, of a single preparation, affects chromosome resolution. Results indicate that misallocations of all chromosome categories does take place when examining photokaryotypes. The photokaryotype determination for *Pseudobarbus* indicates an acceptable level of variation and can be presented for further analysis.

Intraspecific comparisons

Gender

Bertollo *et al.* (1986) show cytological evidence for sex determination within the freshwater fish families Erythrinidae, Anostomidae and Parodontidae, where species have total chromosome number ranging from 2n=42 to 2n=54. Multiple heteromorphic sex chromosomes have been detected in *Coregonus sardinella*, males 2n=81 and females 2n=80; and that the sex chromosomes involves **m** and **sm** chromosomes (Frolov 1990, in Rab and Jankun 1992).

Intraspecific level differences between males and females such as heteromorphic pairs have been reported for the cyprinid species *Garra lamta*, 2n=50 (Khuda-Bukhsh *et al.* 1986). The female has a heteromorphic pair, comprising a large **m** chromosome and a small **m** chromosome, while the male is homomorphic with one pair of small **m** chromosomes. No tetraploid cyprinid species with heteromorphic sex chromosomes have been found to date (Cataudella *et al.* 1977, Yu *et al.* 1987, Collares-Pereira & Madeira 1990, Rab & Collares-Pereira 1995).

Examination of the male and female photokaryotypes of the seven *Pseudobarbus* species shows that there are no heteromorphic chromosomes apparent in either sex. However, due to the presence of numerous small chromosomes in which the centromere position changes gradually, differences in male and female karyotypes cannot be ruled out, particularly if the (heterogeneous) sex chromosomes are made of pair(s) where the centromere positions is slightly different. Sex chromosome pairs which are at an early stage of differentiation may be demonstrated when chromosome banding techniques are used (Amemiya & Gold 1987).

The dichromatic and dimorphic morphological sexual differences found in *Pseudobarbus* male and female species are not represented in the karyotypes using a conventional stain.

Geographic differences

In Sub-Saharan Africa the *Barbus* species are typically Zambezian (tropical) or temperate in distribution (Fig. 1.1) (Skelton *et al.* 1991, Skelton 1993). Within temperate southern Africa they are further subdivided into Karroid and Cape faunas (Fig. 1.1) (Skelton 1986, 1993). Several of the species are distributed over entire river systems, so that variation may exist between populations, and may be detected by karyological means.

Barbus bocagei, a widely distributed Iberian species, shows two karyotypic forms. A population inhabiting large basins had a karyotype of 14**m**;48**s**;36**a** and in populations inhabiting smaller basins, the karyotype was 12**m**;52**s**;36**a** (Collares-Pereira & Madeira 1990). The large basin population has an extra pair of **m** chromosomes compared to the small basin population. Such intraspecific variation may reflect a process of karyotypic diversification in the species (Bertollo *et al.* 1986, Mazik *et al.* 1989). In such cases the differences in karyotypes can be used to trace chromosomal rearrangements.

Generally the *Pseudobarbus* species are confined to a single drainage system and/or their associated drainages within the coastal rivers of the Cape Fold mountains. *P. afer* is an exception in having a wide distribution. Two samples each of *P. afer* and *P. burchelli* (which has a relatively restricted distribution) were analysed. The karyotypes of each population within a species were similar. *P. afer* from Blindekloof River has 42**sm**+36**st** chromosomes and Bezuidenhouts River has 40**sm**+38**st** chromosomes (Tables 4.2 & 4.11). Metaphase spreads of *P. burchelli* from Bainskloof had 42**sm**+34**st** chromosomes while those from Swellendam had 40**sm**+36**st** chromosomes (Tables 4.5 & 4.11). In both karyotypes chromosome in the **sm+st** category are variable. However, because of the gradual change in centromere positions, it is difficult to precisely categorise the **sm** and **st** chromosomes, and the results are not considered conclusive at this stage.

The results obtained in this study *Pseudobarbus* species indicate that there are no heteromorphic chromosomes in either sex and there is no marked intraspecific difference within species examined. Similar reports of low intraspecific variation of the chromosomal number were given for the Ethiopian and West African hexaploid *Barbus* species (Golubstov and Kryzanov 1993, Guégan *et al.* 1995). The indications from African species with high chromosome numbers are different from their European counterparts in having conservative karyotypes.

Interspecific comparisons

Chromosome numbers

Species of *Barbus* (*sensu stricto* and *sensu lato*), have been reported to have chromosome numbers ranging from 96-100 (Sofradziji & Berberovic 1973, Cataudella *et al.* 1977, Hafez *et al.* 1978, Vujosevic *et al.* 1983, Oellermann 1988, Collares-Pereira & Madeira 1990). This study confirms the data reported by Oellermann (1988) and expands the list of species with a range of total chromosome number of 96-100 to include all seven redfin species. Oellermann (1988) and Skelton & Naran (1994) indicated that the redfins have modal chromosome number of 96-100. However, improved technical preparations and photographic resolution of the metaphase spreads, have resulted in clearer interpretation of the chromosome number and karyotypes (see chapter 2). The modal chromosome value for all *Pseudobarbus* species is 100. No losses of chromosomes are noted in the seven species analysed in this chapter, so, it is unlikely that chromosomal changes have not involved Robertsonian translocations.

Ploidy levels

In cyprinids, species exhibiting modal total chromosome numbers of 96, 98, 100 and up to 102 have been considered to be evolutionary tetraploid (Suzuki & Taki 1981, Yu *et al.* 1987, Mazik *et al.* 1989, Buth *et al.* 1991, Rab & Collares-Pereira 1995). Other cyprinids of evolutionary tetraploid origin are found in the genera *Aulopyge*, *Barbodes*, *Carassius*, *Cyprinus*, *Diptychus*, *Puntius*, *Schyzothorax*,

Sinocyclothorax and *Tor* have 2n=100-104 chromosomes (Suzuki & Taki 1986, Rab & Collares-Pereira 1995, Klinkhardt *et al.* 1995).

With the exception of *Diptychus* (6 species analysed) and *Sinocyclocheilus* (3 species analysed) and *Tor* (7 of the 8 species have 2n=100), none of the above listed genera are exclusively tetraploid (Mazik *et al.* 1981, Yu *et al.* 1987, Klinkhardt *et al.* 1995).

The high modal chromosome number equivalent to double the diploid chromosome number recorded for most cyprinids suggests that the *Pseudobarbus* species may have a tetraploid evolutionary origin. In studies reporting high ploidy levels, the authors have suggested that the chromosomes are arranged in triplets or hextets to reflect the ploidy level (Collares-Pereira 1985, Guégan *et al.* 1995), indicating the multiple event on the karyotype. However, no tetravalent chromosome sets are clearly identifiable in the *Pseudobarbus* karyotypes obtained with conventional Giemsa staining (Figs. 4.2-4.8). It has been suggested that the tetraploid genome is susceptible to functional diploidization (Ohno 1970b, Allendorf & Thorngaard 1985). In terms of karyotype morphology it may mean that the homologous pairs undergo independent rearrangements and are therefore no longer homologous quadruplets. *Pseudobarbus* karyotypes may have undergone similar chromosomal rearrangements so that tetravalent chromosome sets are no longer apparent.

Morphology of karyotypes

All seven *Pseudobarbus* species karyotypes are asymmetrical with a larger biarm (**m**, **sm**+**st**) component than uniarm (**a**) component (Figs. 4.2-4.8 & 4.9). This contrasts with Iberian *Barbus* karyotypes, which are dominated by a large uniarm component; 28-50**a** chromosomes (Collares-Pereira & Madeira 1990).

Cyprinid chromosomes are characterised by relatively small chromosomes (1.5-3µm: Buth *et al.* 1991, Rab & Collares-Pereira 1995), with the centromere position ranging gradually from median to nearly terminal (0.5-4.2µm) (Figs. 4.9 & Table 4.11).

Although definitive homologous chromosomal comparison with conventionally stained chromosomes is not possible with the present data, interspecific comparisons of broad chromosomal categories (**m**, **sm**+**st**, and **a**) of the standard karyotypes are possible (Figs. 4.2-4.8 and Table 4.11).

P. quathlambae has the lowest number of **m** chromosomes comprising 8% of the karyotype. *P. burchelli* is next with 10% followed by *P. afer* which has 12% of their respective karyotypes composed of **m** chromosomes. Four species, *P. asper, P. burgi, P. tenuis* and *P. phlegethon*, all have 14% of their respective karyotypes comprised of **m** chromosomes (Figs. 4.2-4.8 & Table 4.11). Only two species, *P. phlegethon* and *P. asper* with high **m** chromosomes have low **a** chromosome values (Table 4.11). All other species have 10-14% of **a** chromosomes except, *P. quathlambae* which has 20% of its karyotype comprised of **a** chromosomes.

Within the categories **a** and **m** chromosomes are fairly homogeneous in size between all *Pseudobarbus* species as seen in the karyotypes (Figs. 4.2-4.8 & 4.9, Tables 4.12).

All the *Pseudobarbus* have more than 40% of their karyotype comprising of **sm+st** chromosomes. As is typical of cyprinid chromosomes the **sm+st** components range gradually in dimensions from large to small (Mazik *et al.* 1989, Collares-Pereira & Madeira 1990). This makes the comparison of these categories between species very difficult (Rab & Collares-Pereira 1995).

The karyotype components, **m**, **sm+st** and **a** chromosomes, vary only from 1-2 pairs among the seven species of *Pseudobarbus*. The biarmed chromosomes (**m**, **sm+st**), show a progressive increase of a pair from *P. quathlambae* and *P. tenuis* which have the lowest number (72) to *P. afer*, *P. asper* and *P. phlegethon* which have the highest number (78) (Table 4.11). The uniarmed chromosomes (**a**) follow a corresponding reverse sequence. These "small" chromosomal changes among species are difficult to interpret as homologous chromosome pairs cannot be identified with confidence. However, these results indicate that in the evolution of the *Pseudobarbus* species, chromosomal rearrangement involving translocation of arms (pericentric inversions, deletion and addition) may have been significant.

The general *Pseudobarbus* karyotype pattern is one with a high proportion of biarmed chromosomes and fewer uniarmed chromosomes. The Eurasian species also have asymmetric karyotypes. However, the tetraploid *Barbus* karyotypes only have 10-30% of the karyotype composed of biarmed (**m-sm**) and a major component composed of uniarmed (**a-st**) chromosomes (Cataudella *et al.* 1977, Collares-Pereira & Madeira 1990).

A further feature of the *Pseudobarbus* karyotype is the relatively elongated and small chromosomes. The elongated (2-3 pairs) and small (1-2 pairs) in the **sm+st** chromosome categories are distinct compared to the rest of the chromosomes in the category (Fig. 4.9, Table 4.11). They are easily identified in a metaphase spread and may therefore be considered as marker elements for the *Pseudobarbus* (Figs. 4.2-4.9). In *Barbus meridionalis* and *Barbus plebejus* marker elements have been identified, as a pair of large **m** and **s** chromosomes (Cataudella *et al.* 1977). No marker chromosomes have been identified in the *B. barbus* (Hafez *et al.* 1978) or the five Iberian *Barbus* analysed by Collares-Pereira & Madeira (1990).

The distinctly large and small chromosomes may be as a result of chromosome translocations, which have occurred within the karyotypes of the species. Although, it is not possible to identify homologous chromosomes, the large and small chromosomes show an interesting pattern.

P. burgi and *P. phlegethon* have 3 large and 3 small chromosomes indicating that chromosomal rearrangements probably did not result in chromosomal losses. *P. burchelli* has 2 large and 3 small chromosomes indicating that there may be chromosomal arm losses. Three species (*P. afer*, *P. asper* and *P. tenuis*) have 3 large and 2 small chromosome pairs. *P. afer* and *P. asper* have been identified as sister species (Skelton 1980, Cambray 1992). *P. asper* and *P. tenuis* are found sympatrically in the Gouritz river system. *P. afer* and *P. tenuis* are found sympatrically in the Keurbooms river system. The similarity in marker chromosomes in the species could represent a shared character, which is phylogenetically useful for interpreting interspecies relationships.

At gross chromosomal level, these patterns are indicated as potential characters, useful for

phylogenetic analysis. But, the karyotypes need to be examined using chromosome banding techniques so that, chromosomal rearrangements can be exposed.

NF: total number of chromosome arms

Cyprinids have a wide range of NF (Klinkhardt *et al.* 1995, Rab & Collares-Pereira 1995). Several Eurasian tetraploid cyprinid species have low NF values indicating higher proportions of uniarmed chromosomes. Generally, the NF values for the tetraploid cyprinids ranges from 134-180 (Appendix 2). The lowest NF value is reported for *Tor putitora* (Khuda-Bukhsh 1982). NF values of five Iberian *Barbus* species range from 154-172, where NF=(**m+s**)+**a** (Collares-Pereira & Madeira 1990). The NF value for *Barbus meridionalis meridionalis* is 174 (Rab *et al.* 1993, in Rab *et al.* 1995).

These values are comparable to the NF1 values for *Pseudobarbus*. However, the NF2 values (when **st** chromosomes are considered as biarmed) are indicative of higher arm numbers and are not comparable to any of the Iberian *Barbus*. It could indicate that the genus *Pseudobarbus* is karyologically distinct from the Iberian *Barbus* species. As shown with chromosome morphology, *Pseudobarbus* NF2 values change very subtly between five species. There is loss of "two chromosomal arms" sequentially in *P. asper*, *P. afer*, *P. burgi*, *P. burchelli* and *P. tenuis*. Alternatively, this could be interpreted as a sequential gain in the reverse order.

The data provides evidence for chromosomal rearrangements without chromosomal losses in the evolution of the *Pseudobarbus*. However, the present data do not allow one to comment on whether the chromosomal arm rearrangements occurred in the sequence given above because homologous chromosomes cannot be recognised.

Meiotic chromosomes

Very few studies of meiotic chromosomes of European cyprinid species are available. All of those published concern species with 2n=50, where only bivalent meiotic elements have been found (Rab & Collares-Pereira 1995). Amongst the Asian cyprinids, no meiotic chromosomes have been reported (Suzuki & Taki 1981, 1986, Yu *et al.* 1987, Magtocn & Arai 1993). To date, only one polyploid African cyprinid species has been investigated. Chromosome preparations from testes of *B. intermedius* (2n=150) showed the presence of only bivalents (Golubstov & Kryzanov, 1993). The absence of multivalents in the meiotic metaphase was considered indirect evidence of the remoteness of polyploidization (Golubstov & Kryzanov, 1993). Similar preparations in this study for *Pseudobarbus* species (2n=100) indicate that the meiotic chromosome complement comprises mainly of bivalent elements (Fig. 4.10). Although some of the chromosomal elements were contracted, no indication of tetravalent chromosomes was evident in the species examined.

A distinction is made between the mode of origin of polyploid genomes. If both chromosome

sets in a tetraploid have been derived from a single species, they are referred to as autopolyploids, whereas if they have originated from two (or more) species and have a hybrid origin they are allopolyploid. Allopolyploid origin has been suggested in the formation of some species (Ohno 1970b), as evidenced by bivalent formation at meiosis. The elements of the diploid complement of each parental genome segregate normally. Since there are multiple copies of the same chromosomes from one genome in autopolyploids, tetravalent and multivalent elements are observed (King 1991). Multivalents have been observed in the salmonids (Allendorf & Thorngaard 1985, Hartley 1987), and in tree frogs (Gerhardt *et al.* 1994, Bogart pers. comm.), where the polyploidy was derived through autotetraploidy.

The absence of tetravalent or multivalents in the meiotic spreads of six *Pseudobarbus* suggests that the polyploidy event was either remote or may have been may have been derived through allopolyploidy. Polyploid origin of the *Pseudobarbus*, may be investigated by chromosomal banding and DNA sequencing studies (see general discussion, chapter 8).

CONCLUSIONS

Description of karyotypes of all seven *Pseudobarbus* species indicate that they are a distinct group. The same diploid chromosome number and the general similarity in karyotype morphology of the *Pseudobarbus* species at conventional karyological level supports taxonomic affinity and may indicate a common ancestral origin. However the monophyletic hypothesis can be tested only when compared to karyotypes of hypothesised sister group species (chapter 4 & 5).

Interspecific differences in the karyotypes of the seven species show trends such as increase in biarmed chromosome and consequently an increase in arm numbers from *P. quathlambae* to *P. asper*. The large and small chromosomes also indicate a pattern in three sympatric species. All *Pseudobarbus* species examined have 2-3 pairs of relatively elongated **sm+st** chromosomes and also 1-3 pair of small **sm-st** chromosomes. The evolution of species within this group has possibly occurred through translocation and rearrangement of the chromosomes.

Comment on the interrelationships among the *Pseudobarbus* species is not possible as homologous chromosome changes have not been detected. Conventionally stained chromosomes data are useful for identifying species lineages or species complexes. However, a higher degree of resolution is required to examine interspecific level relationships.

CHAPTER 5: THE SMALL, FLEXIBLE-RAYED *BARBUS* SPECIES

INTRODUCTION

Almost half (42.5% or 23/54) of all southern African barbine cyprinids have a flexible dorsal spine and are referred to as the soft-rayed *Barbus* (Jubb 1967, Skelton 1993). A minority of these species form part of the temperate karroid fauna (Skelton 1986, 1993). Adults of this group have a SL≤150 mm and characteristically have radially striated scales (Skelton *et al.* 1991). Amongst these there is a distinctive group of four species (possibly a lineage), referred to as the *Barbus anoplus*-complex, or the "chubby-head group" (Skelton 1994). The species comprising the complex are *Barbus amatolicus, Barbus anoplus, Barbus gurneyi* and *Barbus motebensis* (Skelton 1990). This group is identified by a suite of morphological characters and pigmentation patterns (Skelton 1993). All four species are endemic to the cooler waters of the Orange-Vaal river systems and the adjacent river systems (Jubb 1967, Cambray 1982, Skelton 1993).

B. anoplus is one of the few species for which there are published accounts of biology including early life history (Cambray 1982, 1983). Factors that have contributed to the success to the widespread distribution of *B. anoplus* include life history strategies such as early maturity, rapid growth in first year, high fecundity, and short generation time. The species is a multiple spawner with some individuals breeding over several reproductive seasons (Cambray 1982).

Basic biological data such as age, growth, population dynamics, spawning seasons and habitat, behaviour, diet and early ontogeny are lacking for the majority of small cyprinids in Africa (Cambray 1992).

Males of the *B. anoplus*-complex show sexually dimorphic and dichromatic features such as nuptial tubercles on the head, fins and body (Cambray 1982, Skelton 1993).

Studies to date have not explored the basis of the similarity of the four species beyond the similarities in colour and morphology. Recently, genetic studies of four small *Barbus* species suggest differences at population level (Engelbrecht & van der Bank 1994, 1996). However, these investigations need to be expanded. Cytogenetic investigation of the group provides an opportunity to explore the integrity of this group.

For further comparison of karyological data two other soft-rayed, small minnows *B. brevipinnis* and *Barbus pallidus* not associated with the *B. anoplus*-complex have been included in this karyological analysis.

Skelton (1980, 1988) suggested, on the basis of common morphological similarities (seven dorsal branched rays, more than 30 lateral line scales, 14-18 rows of caudal peduncle scales and more than 15 radial striae on scales), that the four flexible rayed species of the *B. anoplus*-complex comprise a natural group. This is the first hypothesis that is tested.

The distribution patterns of *B. anoplus*-complex and the *Pseudobarbus* groups are to a large extent allopatric and complementary with one marginal overlap (Skelton 1988). Characters such as the

development of conical tubercles, squamation, osteology (epineurals, supraneurals and the shape of the metapterygoids) were considered shared derived characters (synapomorphies) between the redfins and the chubby-head group. Skelton (1980, 1988) suggested that the *B. anoplus*-complex is the sister group to the *Pseudobarbus* lineage. It is therefore important for the purpose of phylogenetic reconstruction to investigate their karyology and compare it to that of the *Pseudobarbus*.

There are two hypotheses to consider:

i) that *B. anoplus*, *B. amatolicus*, *B. gurneyi* and *B. motebensis* are a monophyletic lineage

ii) and that this lineage is the sister group to *Pseudobarbus*.

In this chapter the karyology of the *B. anoplus*-complex and other small flexible rayed *Barbus* species are considered in order to test these hypotheses. Essentially three questions are being asked.

i) What are the karyotypes of the *B. anoplus* complex species?

ii) Do these karyological data support the concept of interrelationships between these species within the *B. anoplus*-complex ? and

iii) Is there karyological evidence to support the hypothesis of the *B. anoplus*-complex as a outgroup of the *Pseudobarbus*?

MATERIALS AND METHODS

Six small flexible rayed species are examined (Table 5.1). Karyotype data for *B. pallidus* were obtained using gill tissue, and with colchicine exposure of three hours, in a previous study by Naran (1992).

Total chromosome numbers were obtained from several sources; photographs, photo-negatives, video-monitor counts and from slides (chapter 2). Only photographs were used to determine mean, standard deviations and modal values for the chromosome categories of the karyotype. The modal values of chromosome categories were used for the species karyotypes.

The chromosome morphology descriptions are based largely on visually assessed metaphase spreads. Chromosomes from *B. anoplus* and *B. amatolicus* were measured to assess the large and small (**l:s**) chromosomes in the complement. Only selected chromosomes from *B. gurneyi* and *B. motebensis* were measured to assess the **l:s** chromosome ratios.

Table 5.1 Number and sex of specimens used for karyological analysis. P=Palmiet River, E=Elands River, un/juv=undetermined or juveniles. n=number of specimens examined.

SPECIES		n	male	female	un/juv
B. anoplus	(P)	3	1		2
B. anoplus	(E)	9	4	3	2
B. amatolicus		7	3	3	1
B. motebensis		7	5	2	
B. gurneyi		6	4	2	
B. brevipinnis		5		2	3

RESULTS

The photokaryotype results of males and females from the *B. anoplus*-complex species are separated to account for heteromorphic chromosomes differences.

B. anoplus

Specimens from the Elands River, Incomati system in the Eastern Transvaal, and from the Palmiet River, Swartkops River system in the Eastern Cape, were karyotyped (Table 2.1).

Elands River

Four males and three females, including two juvenile specimens of *B. anoplus* were karyotyped (Table 5.1). Modal values of 74 metaphase cells were analysed and showed that there are 50 chromosomes in the complement (Fig. 5.1). Mean and modal values of chromosomes as determined from 27 male and female photokaryotypes show that the uniarm chromosome component consists of 24**a** chromosomes, and the biarm chromosomes component consists of 6**m** and 18**sm**+2**st** chromosomes (Table 5.2).

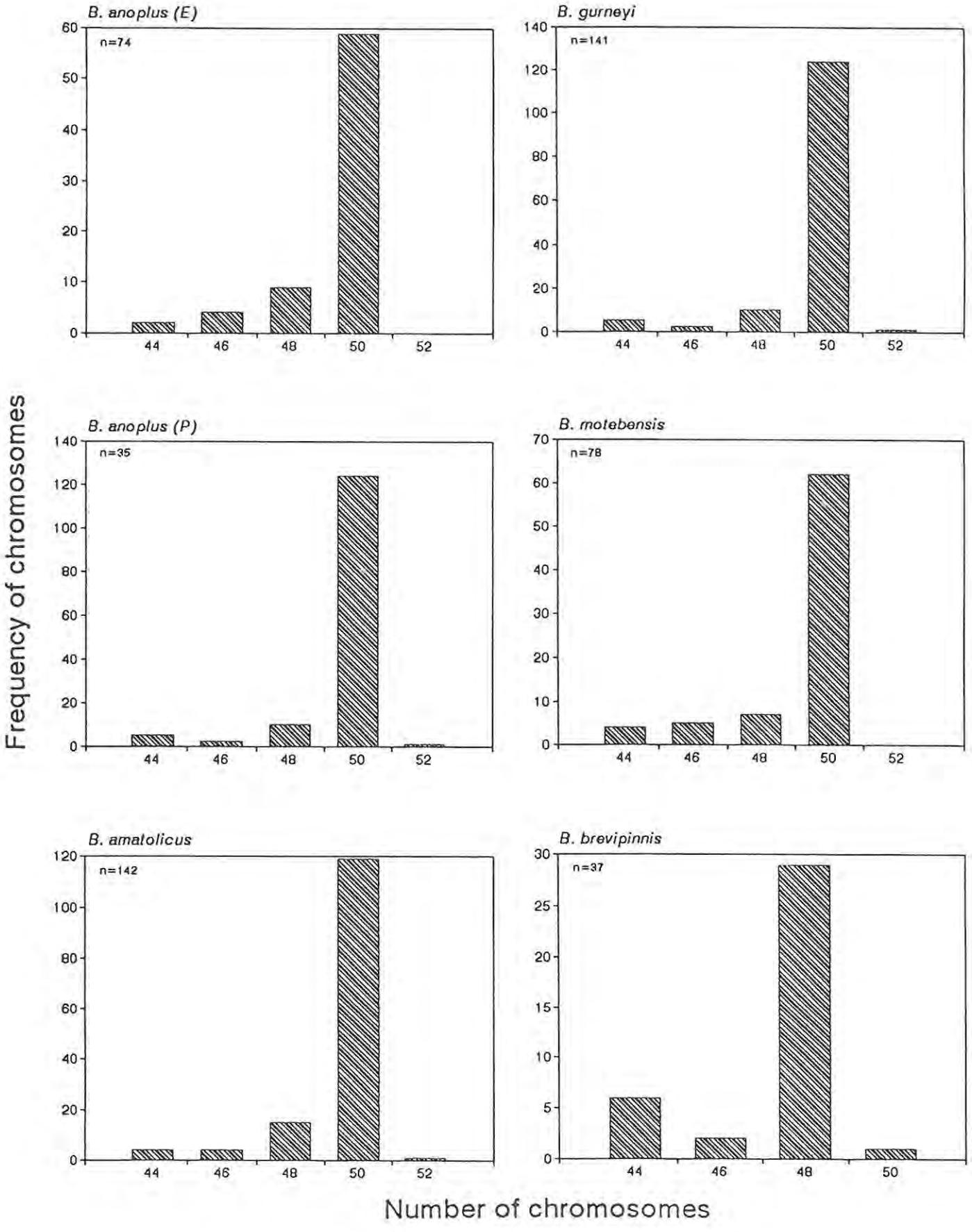


Figure 5.1 Percentage frequency distribution of chromosome numbers for five, small flexible rayed *Barbus* species examined in this chapter.

Table 5.2 Detailed description of the photokaryotypes showing mean, standard deviation (sd), modal values and ranges of number of chromosomes for *B. anoplus* from the Elands River. n=number of metaphase cells examined.

		Chromosome categories		
		m	**sm+st**	**a**
♂, n=14	Mean	5.7	20.5	23.8
	sd	0.7	1.2	0.5
	Mode	7	19	24
	Range	4-6	20-24	22-24
♀, n=13	Mean	4.8	21.4	23.7
	sd	0.8	1.2	0.7
	Mode	6	20	24
	Range	4-6	20-24	22-24

Chromosome morphology: Amongst the male photokaryotypes, a heteromorphic chromosome pair comprising of an **m** chromosome and an **sm** chromosome is observed (Fig. 5.2a). The female photokaryotypes show no heteromorphic chromosome pair (Fig. 5.2b).

The metaphase spreads of both sexes include one pair of **sm+st** chromosomes with elongated arms when compared to the rest of the biarm chromosomes complement (**l:s**=2.0); all **a** chromosomes are very similar in size; there is a gradual shift from large to small within both the **m** chromosomes and **sm+st** chromosomes (Figs. 5.2a&b, 3.7, Table 5.9).

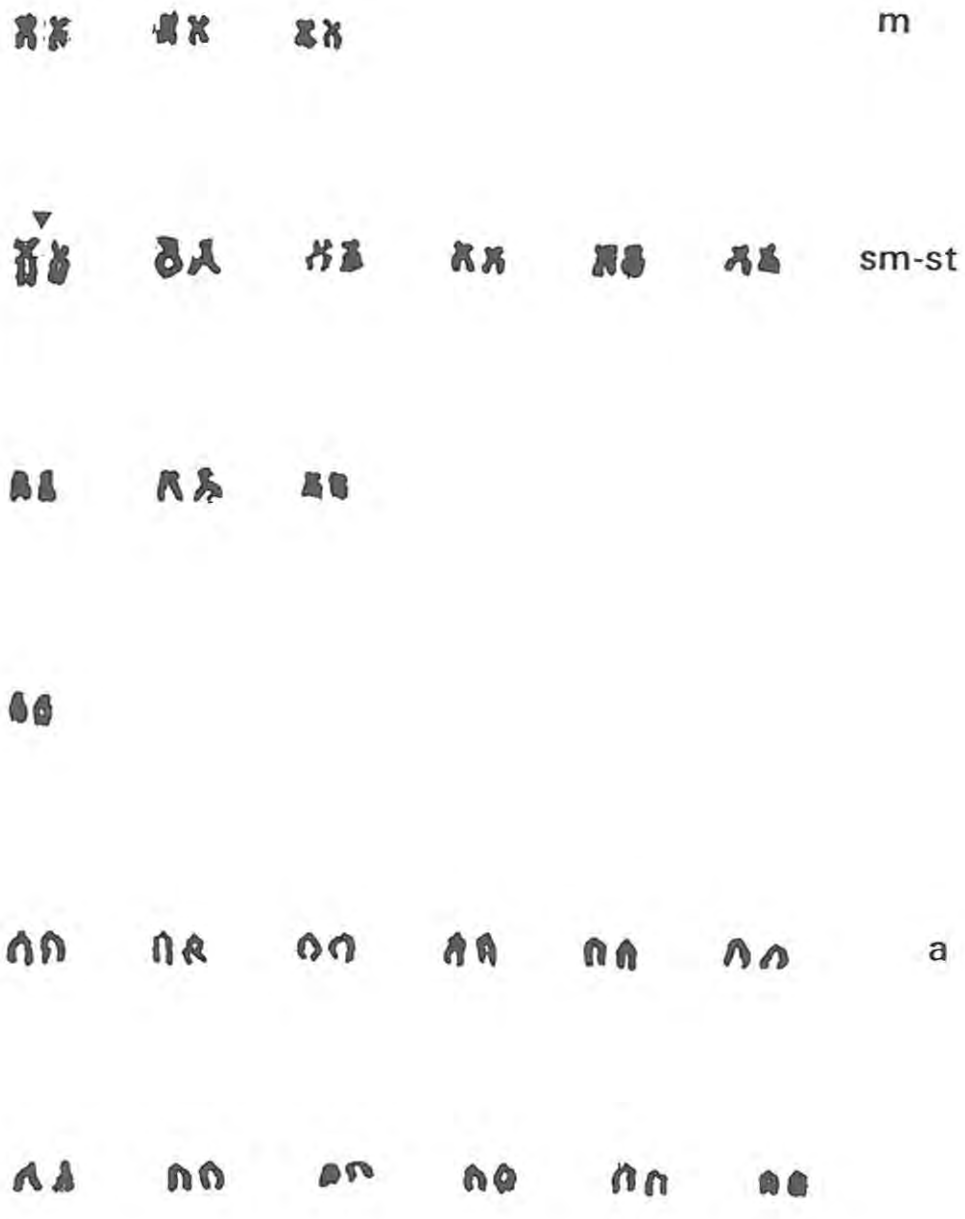


Figure 5.2a Photokaryotype of a male *B. anoplus* (Elands River) with chromosome formula 2n=7**m**+17**sm**+2**st**+24**a**=50. Arrowhead indicates heteromorphic chromosome pair. Scale bar = 5µm.

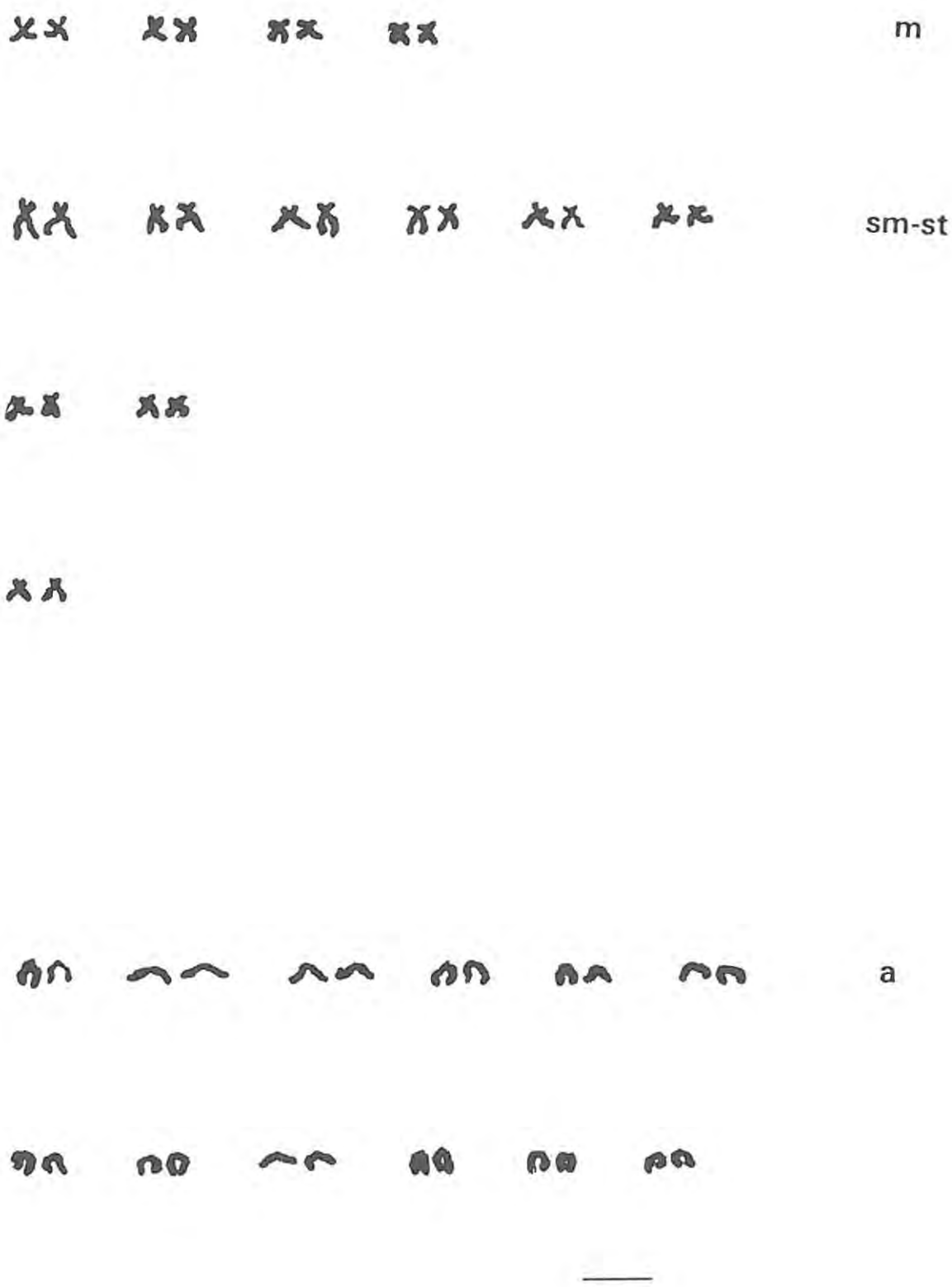


Figure 5.2b Photokaryotype of female *B. anoplus* (Elands River) with chromosome formula 2n=6**m**+18**sm**+2**st**+24**a**=50. Scale bar = 5µm.

Palmiet River

Two juveniles and a male specimen of *B. anoplus* were karyotyped (Table 5.1). The frequency analysis of 35 metaphase cells shows that the total chromosome number of this species is 50 (Fig. 5.1). Mean and modal values of chromosomes as determined from 20 photokaryotypes of both sexes, indicate that the uniarm chromosome component consists 24a chromosomes (Table 5.3). The biarm chromosome component consists of 6**m** chromosomes, 18**sm**+2**st** chromosomes (Table 5.3).

Table 5.3 Detailed description of the photokaryotypes showing mean, standard deviation (sd), modal value and range of number of chromosomes for *B. anoplus* from the Palmiet River. n=number of metaphase cells examined.

		Chromosome categories		
		m	**sm+st**	**a**
♂, n=10	Mean	4.6	20.6	23.6
	sd	0.9	1.7	1.2
	Mode	7	19	24
	Range	4-6	17-22	20-24
♀,n=10	Mean	4.4	21.6	24.0
	sd	0.8	1.7	0.9
	Mode	6	20	24
	Range	4-6	17-22	22-26

Chromosome morphology: The male photokaryotypes include a pair of heteromorphic chromosomes comprised of one **m** chromosome and a **sm** chromosome (Table 5.3). There is no indication of a heteromorphic pair in the metaphase spreads of the juveniles, suggesting that they are females (Fig. 5.3). One pair of **sm+st** chromosomes is relatively elongated and can be easily recognised within metaphase chromosomes, (**l:s**=2.1); all the **a** chromosomes are similar in size; the **sm+st** chromosomes and **m** chromosomes range in size from large to small (Figs. 5.3, 3.7, Table 5.9).

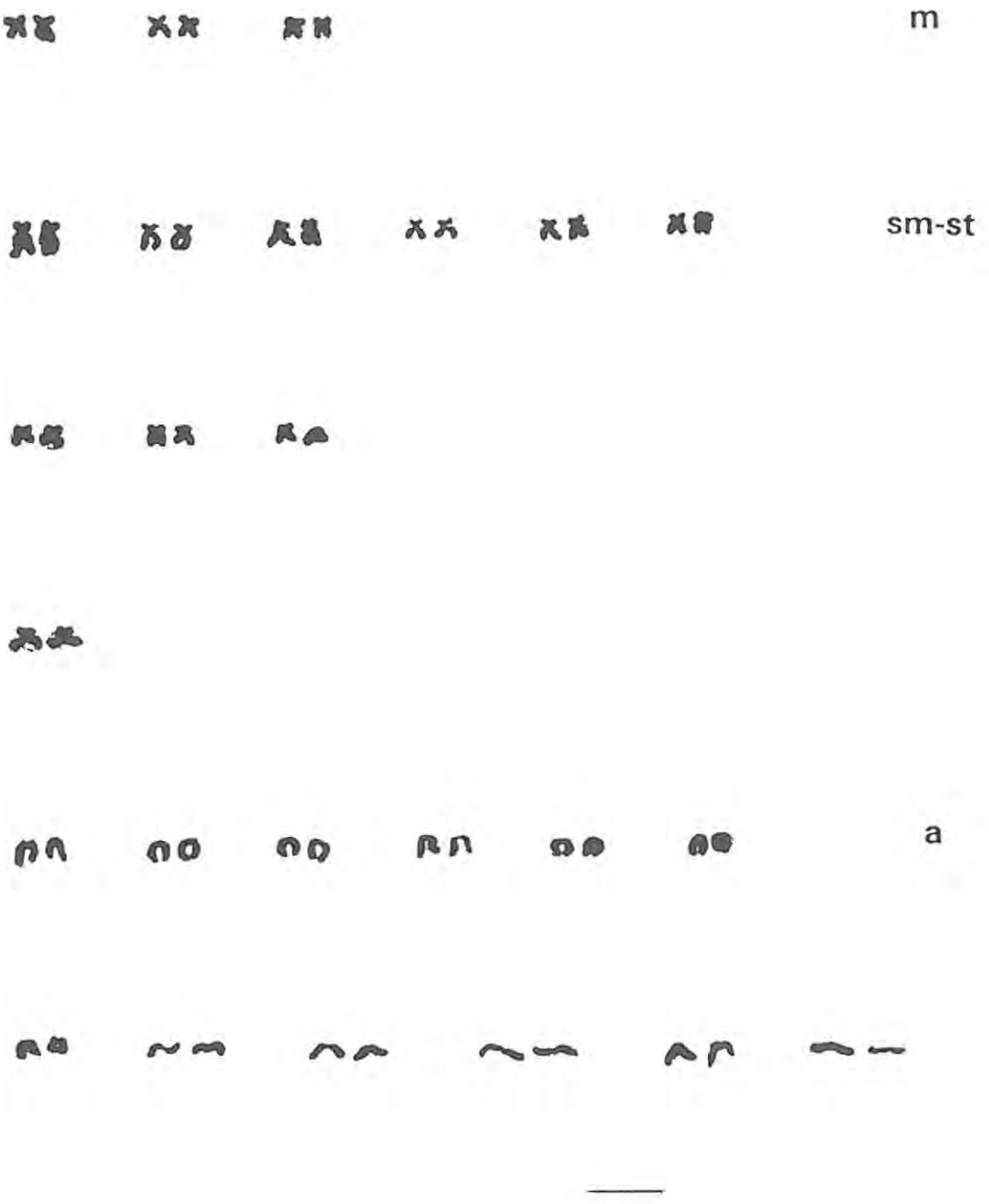


Figure 5.3 Photokaryotype of a female *B. anoplus* (Palmiet River) with chromosome formula 2n=6**m**+18**sm**+2**st**+24**a**=50. Scale bar = 5μm.

B. amatolicus

Three males and three females, including one specimen of undetermined sex of *B. amatolicus* were analysed (Table 5.1). 142 metaphase cells were analysed, and showed that the modal chromosome number is 50 (Fig. 5.1). Mean and modal values of chromosomes as determined from examination of 26 photokaryotypes shows that the uniarm chromosomes component comprises 20**a** chromosomes and the biarm chromosomes component comprises 18**sm**+4**st** chromosomes and 8**m** chromosomes (Table 5.4).

Table 5.4 Detailed description of the photokaryotypes showing mean, standard deviation (sd), modal value and range of number of chromosomes for *B. amatolicus*. n=number of metaphase cells examined.

		Chromosome categories		
		m	**sm+st**	**a**
♂, n=14	Mean	8.4	21.2	20.4
	sd	1.0	0.9	0.7
	Mode	9	21	20
	Range	6-9	20-24	20-24
♀, n=12	Mean	7.5	22.0	20.5
	sd	1.2	1.2	1.2
	Mode	8	22	20
	Range	4-8	20-24	18-22

Chromosome morphology: The male photokaryotypes includes a pair of heteromorphic chromosomes, comprising one **m** chromosomes and one **sm** chromosome (Fig. 5.4). Heteromorphic chromosomes have not been detected in the female photokaryotypes (Table 5.4). Chromosomes sizes range from large to small within the **sm+st** chromosomes and **m** chromosomes categories; all **a** chromosomes are similar in size; a relatively elongated pair of **sm+st** chromosomes is present in the complement (**l:s**=3.4) (Figs. 5.4, 3.7, Table 5.9).

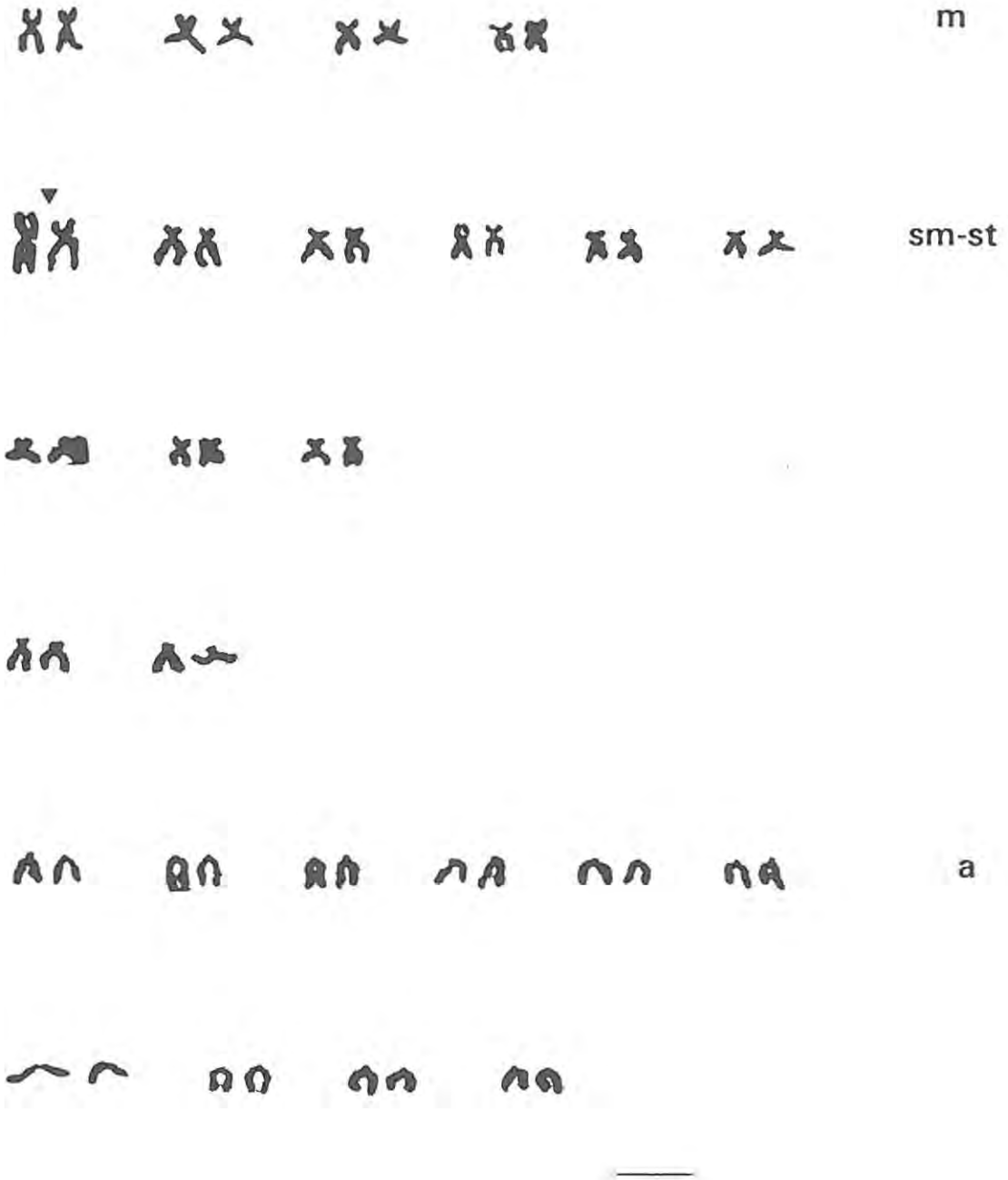


Figure 5.4 Photokaryotype of a male *B. amatolicus* (Bashee system) with chromosome formula 2n=9**m**+17**sm**+4**st**+20**a**=50. Arrowhead indicates heteromorphic chromosome pair. Scale bar = 5μm.

B. gurneyi

The chromosomes of four males and two females were analysed (Table 5.1). Out of 141 metaphase cells analysed the modal chromosome number was 50 (Fig. 5.1). Mean and modal values of chromosomes, determined from 25 photokaryotypes, show that the uniarm chromosomes comprised 22**a** chromosomes and the biarm chromosomes comprised 18**sm+4st** chromosomes and 6**m** chromosomes (Table 5.5).

Table 5.5 Detailed description of the photokaryotypes showing mean, standard deviation (sd), modal value and range of number of chromosomes for *B. gurneyi*. n=number of metaphase cells examined.

		Chromosome categories		
		m	**sm+st**	**a**
♂, n=15	Mean	5.0	21.4	22.7
	sd	1.0	2.4	0.9
	Mode	7	21	22
	Range	4-7	16-24	22-24
♀, n=10	Mean	4.5	21.8	23.8
	sd	0.8	1.2	0.6
	Mode	6	22	22
	Range	4-6	20-24	22-24

Chromosome morphology: There is a heteromorphic pair, one **m** chromosome and a **sm** chromosome in the male metaphase spreads (Table 5.5). Female karyotypes do not feature a heteromorphic pair; all the **a** chromosomes are similar in size while chromosomes within the **sm+st** and **m** categories range from large to small (Fig. 5.5 & Table 5.9). There is a pair of elongated **sm+st** chromosomes within the karyotype (**l:s**=3.2) (Fig. 5.5).

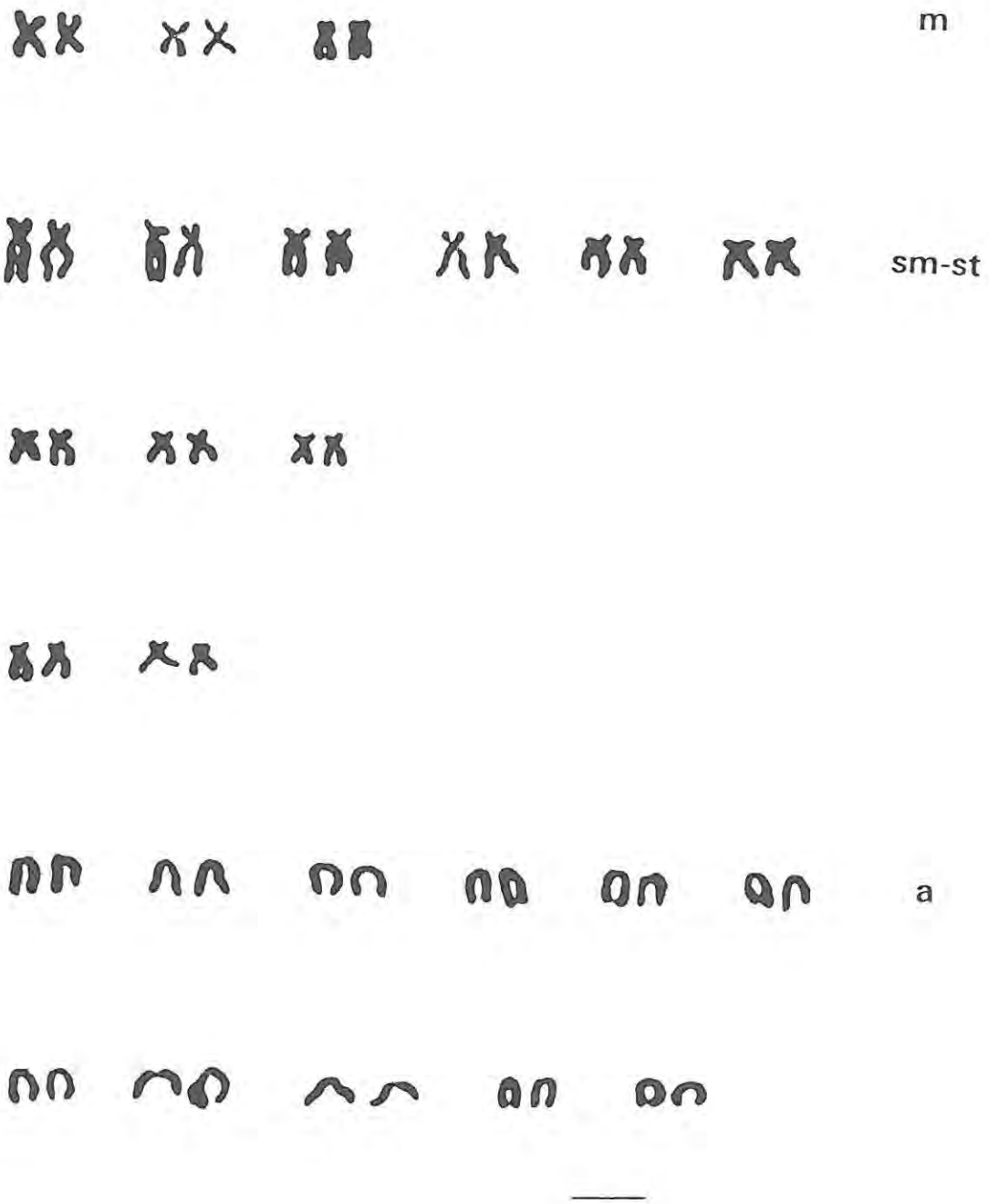


Figure 5.5 Photokaryotype of a female *B. gurneyi* (Karkloof River) with chromosome formula 2n=6**m**+18**sm**+4**st**+22**a**=50. Scale bar = 5µm.

B. motebensis

Five males and two females were analysed (Table 5.1). Modal values of 78 metaphase spreads were examined to show the chromosome number for this species is 50 (Fig. 5.1). Mean and modal values of chromosomes as determined from 19 photokaryotypes indicate that the uniarm chromosomes are comprised 24**a** chromosomes and the biarm chromosomes consists of 16**sm**+2**st** chromosomes and 8**m** chromosomes (Table 5.6).

Table 5.6 Detailed description of the photokaryotypes showing mean, standard deviation (sd), modal value and range of number of chromosomes for *B. motebensis*. n=number of metaphase cells examined.

		Chromosome categories		
		m	**sm+st**	**a**
♂, n=10	Mean	6.4	21.6	21.8
	sd	0.9	1.5	1.6
	Mode	9	17	24
	Range	2-6	19-24	20-24
♀, n=9	Mean	5.8	21.0	23.3
	sd	1.2	1.0	1.6
	Mode	8	18	24
	Range	4-8	18-22	20-26

Chromosome morphology: Males have a pair of heteromorphic chromosomes, consisting of a **m** and a **sm** chromosome (Table 5.6), but these are not present in female photokaryotypes (Fig. 5.6). There is a range of sizes from large to small within the **sm-st** and **m** chromosome categories, while the **a** chromosomes are all similar in size (Fig. 5.6, Table 5.9). There is one pair of relatively elongated **sm+st** chromosomes in the karyotype (**l:s**=2.0) (Fig. 5.6).

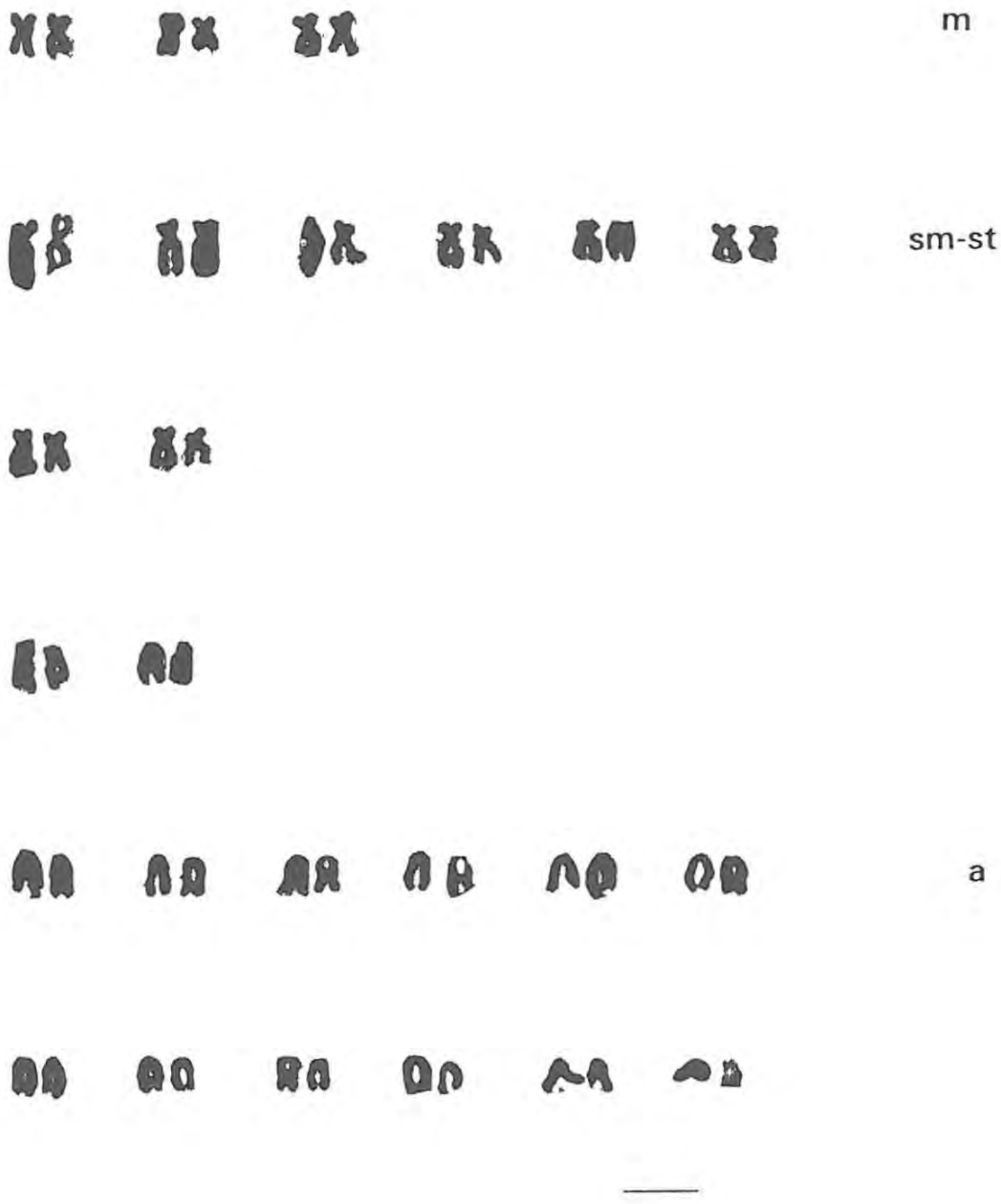


Figure 5.6 Photokaryotype of female *B. motebensis* (Marico River) with chromosome formula 2n=8**m**+16**sm**+2**st**+24**a**=50. Scale bar = 5µm.

B. brevipinnis

Two female specimens and three juvenile specimens of *Barbus brevipinnis* were examined karyologically (Table 5.1). Modal chromosome number taken from 37 metaphase cells is 48 (Fig. 5.1). The karyotype of *B. brevipinnis* is dominated by biarm elements and differs from the previous four species in this respect. Mean and modal values of chromosomes as determined from 14 photokaryotypes showed that the biarm chromosomes component consists of 20**sm**+6**st** chromosomes and 18**m** chromosomes while the uniarm chromosomes component comprises 4**a** chromosomes (Table 5.7).

Table 5.7 Detailed description of photokaryotypes showing mean, standard deviation (sd), modal value and range of number of chromosomes for *B. brevipinnis*. n=number of metaphase cells examined., no heteromorphic chromosome pairs were detected in the ♂&♀ metaphase cells.

		Chromosome categories		
		m	**sm+st**	**a**
♂&♀, n=14	Mean	17.3	20.2+6.6	3.4
	sd	1.0	2.3+0.9	0.9
	Mode	18	20+6	4
	Range	16-18	18-28	2-4

Chromosome morphology: No heteromorphic pairs of chromosomes were detected in either male or female karyotypes (Fig. 5.7). There is a size range from large to small in all the chromosome categories (Fig. 5.7). There are two pairs of relatively elongated chromosomes; the largest chromosomes is an **sm+st** chromosome pair and the second largest chromosomes is a **m** chromosomes pair (Fig. 5.7).

Figure 5.7 Photokaryotype of female *B. brevipinnis* (Molopo River) with chromosome formula 2n=18**m**+20**sm**+6**st**+4**a**=50. Scale bar = 5μm.

B. pallidus

The chromosomes were highly spiralised and the karyotype data presented is therefore considered preliminary. Five male specimens and one female specimen of *B. pallidus*, from Buffalo River, Tugela system, were analysed. Out of 26 metaphase spreads the most frequent chromosome number is 50 (Appendix 2). Preliminary assessment reveals that the karyotype is similar to the *B. anoplus*-complex. The biarm chromosome component comprises 6**m** chromosomes, 12**sm**+8**st** chromosomes and the uniarm component comprises 22 **a** chromosomes (Appendix 2).

Chromosome morphology: No apparent heteromorphic pairs of chromosomes were detected in either male or female karyotypes. The **m** chromosomes all are similar in size. There is a size range from large to small in **sm**+**st** and **a** chromosome categories. The first **sm**+**st** chromosome pair is relatively elongated.

NF: total number of chromosome arms

B. anoplus, *B. gurneyi* and *B. motebensis* have the NF1 values of 74 while *B. amatolicus* has two extra arms, NF1=76 (Table 5.8). The other flexible rayed species, *B. brevipinnis* has NF1=86, and *B. pallidus* has NF1=70 (Table 5.8). NF2 values (considering **st** chromosomes as biarmed) of the five soft-rayed minnows show that *B. brevipinnis* still has the highest number of arms, NF2=92 (Table 5.8). Members of the chubby-head group have NF2 values within a narrow range from 76 to 80 (Table 5.8).

Table 5.8 Total number of arms for the six soft rayed *Barbus* species analysed using two different calculations NF1=2(**m**+**sm**)+(**st**+**a**) and NF2=2(**m**+**sm**+**st**)+(**a**), E= Elands River, P=Palmiet River.

SPECIES		NF1	NF2
B. anoplus	(E)	74	76
B. anoplus	(P)	74	76
B. amatolicus		76	80
B. gurneyi		74	76
B. motebensis		74	76
B. brevipinnis		86	92
B. pallidus		70	78

Meiotic chromosomes

Meiotic spreads from gonadal tissue of five species showed bivalents and unsynapsed univalents (Fig. 5.8).

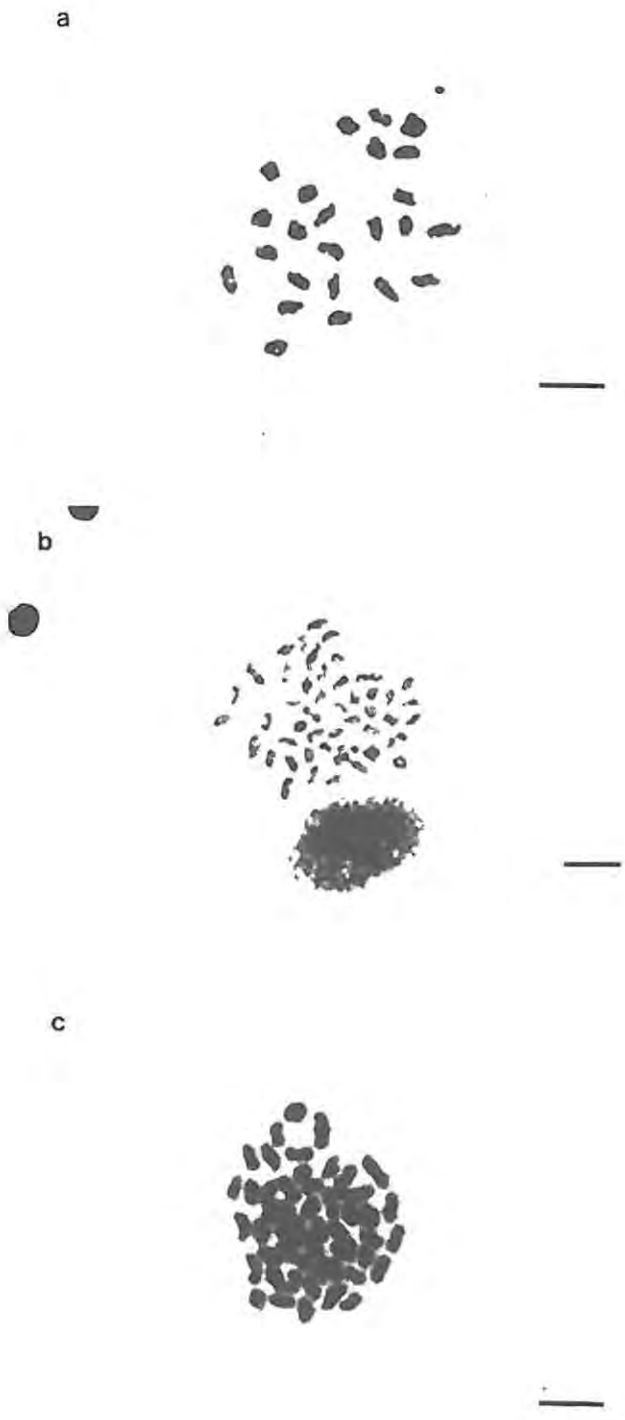


Figure 5.8 Meiotic spreads showing bivalent and univalent chromosomes in three *Barbus* species analysed in this chapter. a) *B. anoplus*, b) *B. amatolicus*, c) *B. gurneyi*. Scale bar = 5µm.

Summary of characteristics of flexible rayed *Barbus* species karyotypes

Features that characterise the karyotypes of the soft-rayed minnows; *B. anoplus*-complex, *B. pallidus* and *B. brevipinnis* can be summarised as follows,

1. Species of the *B. anoplus*-complex and *B. pallidus* have a total of 50 chromosomes (2n=50) while, *B. brevipinnis* has a total of 48 chromosomes (2n=48) (Table 5.10).

2. Males of the *B. anoplus*-complex have a heteromorphic chromosome pair (Figs. 5.2a&5.4).

3. The karyotype is considered symmetrical because of the presence equal numbers of uniarm (20-24) chromosomes and biarm (26-30) chromosomes for the members of the *B. anoplus*-complex, and *B. pallidus* (Table 5.10). *B. brevipinnis* has a high biarm chromosome component and a low uniarm chromosome component and the karyotype is considered asymmetrical.

4. The total arm number, NF1 ranges from 70-76 for the species with 2n=50 and 86 for *B. brevipinnis*; NF2 ranges from 76-80 for the former group and 92 for the latter species (Table 5.8).

5. The sizes of biarmed (**m**, **sm+st**) chromosomes range from large to small. The **a** chromosomes are all similar to each other in size within the *B. anoplus*-complex, as well as for *B. pallidus* and *B. brevipinnis* (Figs. 5.2-5.7&3.7, Table 5.9, Appendix 2).

6. Two elongated pairs of **sm** chromosomes are present in the karyotypes of the four species comprising the *B. anoplus*-complex (Figs. 5.2-5.6), *B. brevipinnis* has two pairs of elongated chromosomes, one **m** and the other **sm**. No marker chromosomes could be distinguished from *B. pallidus* as the metaphase spreads were highly contracted.

7. A pair of elongated chromosomes form the heteromorphic pair within the male specimens of *B. anoplus*-complex. Neither *B. brevipinnis* nor *B. pallidus* have detectable heteromorphic pairs.

Table 5.9 The size range (μm) of chromosome within each chromosome category for five small flexible rayed *Barbus* species. E= Elands River, P=Palmiet River. n=number of metaphase cells examined.

				Chromosome categories	
SPECIES		n	**m**	**sm+st**	**a**
B. anoplus	(E)	7	1.3-2.3	1.4-2.3:1.2-1.8	1.5-2.2
B. anoplus	(P)	9	1.3-2.3	1.5-3.1:1.7-2.1	1.7-2.3
B. amatolicus		7	1.9-3.0	1.3-4.4:1.7-2.1	1.4-2.2
B. motebensis		2	1.9-2.4	1.7-3.5:1.6-2.1	1.5-2.0
B. gurneyi		2	1.4-1.9	1.3-3.0:1.7-2.0	1.4-1.9

Table 5.10 Modal values of chromosome categories from kidney cells of five small flexible rayed *Barbus* species. E= Elands River, P=Palmiet River. n=number of metaphase cells examined.

				Chromosome categories	
SPECIES		n	**m**	**sm+st**	**a**
B. anoplus	(E)	27	6	18+2	24
B. anoplus	(P)	20	6	18+2	24
B. amatolicus		26	8	18+4	20
B. gurneyi		25	6	18+4	22
B. motebensis		19	8	16+2	24
B. brevipinnis		14	18	20+6	4

DISCUSSION

General determination of chromosome numbers, biarm chromosomes and uniarm chromosomes within the karyotype are considered here.

Determination of chromosome number

Frequency distribution of chromosome numbers of *B. anoplus* from the Palmiet River and the Elands River show that only 4.3% and 2.0% respectively of the metaphase cells examined, were hypomodal (Fig. 5.1). Hypomodal cells comprise less than 5% in the other species examined. With the

exception of *B. amatolicus*, none of the species had hypermodal cells. In all five species, no distinctly large, small or additional chromosome components were observed in the metaphase spreads and all the chromosomes resemble the "normal" karyotype of the species. This indicates that chromosomal rearrangements are not responsible for the hypomodal values. Therefore it is reasonable to assume that the hypomodal counts were artifacts of preparation. The low percentage of hypomodal counts also indicates that the cells remained intact. The absence of hypermodal values is indicative of good cell distribution, and this resulted in overall good chromosomal preparations and low variability in chromosome numbers.

Differences in chromosome condition are attributed to technical factors and have been discussed in chapters 2 & 3.

Intraspecific comparisons

Gender

Relatively few fish species have been reported with different male and female karyotypes (Porto *et al.* 1992). Within the cyprinids, the majority of studies show no difference in male-female karyotypes (Klinkhardt *et al.* 1995, Rab & Collares-Pereira 1995). Chromosomal heteromorphy has not been reported previously for African *Barbus* with 2n=48-50 (Golubstov & Kryzanov 1993, Guégan *et al.* 1995, Rab *et al.* 1995).

The chromosome pairs in the *B. anoplus*-complex species are matched together on the basis of morphology and size (Fig. 5.2a, 5.4). In the female metaphase spreads the **m** and **sm+st** chromosomes were all easily matched into homologous pairs (Figs. 5.2b, 5.3, 5.5 & 5.6), whereas the male metaphase spreads had morphologically heteromorphic chromosomes; although the size of the chromosomes being matched were similar, they differed in centromere position (Figs. 5.2a, 5.4). This heteromorphic pair comprised one **m** and one **sm** (Levan *et al.* 1964, type l/s=1.1 and l/s= 2.1 respectively).

Geographic differences

Most studies have not investigated variation at chromosome level throughout the distribution range of a particular species (Rab & Collares-Pereira 1995). To date, 12 African *Barbus*, with a total number of 48-50 chromosomes, have been examined. Most of the analysis have been from specimens from a single locality, and no intra and/or interpopulation variation have been noted (Rab *et al.* 1995). *B. anoplus* is a widely distributed species, extending south of the Limpopo River system (Skelton 1993). This is in contrast to other *Barbus* species examined in this chapter which generally have narrow distribution range (Skelton 1993).

B. anoplus from the Elands River, Incomati River System and from the Palmiet River Swartkops

River System (Table 2.1) were karyotyped in this study and have 2n=50 (Tables 5.2,5.3&5.8, Fig. 5.2). These results differ from Oellermann (1988), who obtained 2n=48, from a population of *B. anoplus* from the Dorps River, Limpopo River System. The difference in chromosome number may be indicative of intraspecific level variation in *B. anoplus* karyotypes. However, these difference in chromosome number may be more likely due to the technical differences in preparations of the chromosome spreads (see chapter 2).

Interspecies comparisons

Chromosome numbers

With the exception of African cyprinids species, the karyology of cyprinid fishes is well studied (Rab & Collares-Pereira 1995). A large number of cyprinid species (64.8%) have a chromosome number of 50, while fewer species (16.1%) have 48 chromosomes, and the remainder of the species (7.8%) have 100 and 150 chromosomes (Buth *et al.* 1991, Klinkhardt *et al.* 1995). Only one North American cyprinid has 48 chromosomes while the remainder have 50 chromosomes (Buth *et al.* 1991). Within karyotyped African small *Barbus*, three species have 48 chromosomes and the remaining nine species have 50 chromosomes in the complement (Rab 1981, Oellermann 1988, Golubstov & Kryzanov 1993, Rab *et al.* 1995, Appendix 2).

The karyological results of this study indicates that all four species within the *B. anoplus*-complex and *B. pallidus* have a total chromosome number of 50 (Fig. 5.1). *B brevipinnis* is the only species in this group studied that consistently has a total chromosome number of 48 (Fig. 5.1).

With the present data, and considering only the temperate southern African diploid fauna, there is one species with 48 chromosomes and other species with 50 chromosomes.

Ploidy levels

A diploid state is represented by species having total chromosome numbers ranging from 42 to 52 (Buth *et al.* 1991). Diploidy is the most common ploidy level within cyprinid species karyotyped to date (Buth *et al.* 1991, Yu *et al.* 1987, Rab & Collares-Pereira 1995, Klinkhardt *et al.* 1995). The majority of the cyprinids species assayed have 2n=48-50; represented by 80.9% in North America and Asia (Yu *et al.* 1987, Buth *et al.* 1991) and 79% in Europe (Rab & Collares-Pereira 1995).

Cyprinids are a major component (447 species) of the African freshwater ichthyofauna and are represented by three subfamilies, *viz.* cyprinines, bariliines and leuciscines (Skelton *et al.* 1991). Only a few (25 species) of the African *Barbus* species have been karyotyped (Appendix 2). Of the presently

available data on African *Barbus* 50-52% have 2n=48-50 (Rab 1981, Oellermann 1988, Golubstov and Kryzanov 1993, Rab *et al.* 1995). All six species analysed in this chapter were found to be diploid.

Morphology of karyotypes

Salmonid karyotypes are morphologically grouped into group A (more **a** chromosomes than **m** chromosomes) and group B (more **m** chromosomes than **a** chromosomes) (Hartley 1987). Rab & Collares-Pereira (1995) have categorised various European cyprinid karyotypes. For example, the karyotype morphology of European leuciscine cyprinids (2n=50) comprises 6-8**m** pairs, 12-14**sm+st** pairs and very few 2-4**st-a** pairs of chromosomes, while gobionine cyprinids (2n=50) comprises 12**m** pairs, 12**sm+st** pairs and only one **a** pair (Rab & Collares-Pereira 1995). The majority (94%) of North American cyprinid species (mainly *Notropis* 2n=50) have relatively low numbers (0-2 pairs) of **a** chromosomes and higher numbers of biarmed chromosomes (Gold *et al.* 1981, Gold & Amemiya 1986, Amemiya *et al.* 1992). In Asia, *Puntius* and related genera with 2n=50 have species characterised by 10-12**m+sm** and 13-15**st-a** chromosomes (i.e. almost equal numbers of biarm and uniarm chromosomes), including species with low (5-8) uniarm chromosomes pairs (Magtoon & Arai 1989, 1993, Yu *et al.* 1987, Klinkhardt *et al.* 1995). Thus, many cyprinids species have been broadly grouped according to the proportions of chromosome in each category. However, the phylogenetic implications of these cytogenetic characteristics remains obscure.

The karyotype pattern shown by the few African *Barbus* assayed thus far is skewed, and is composed of few uniarmed: **a** and **st** (2-16) chromosomes and a larger number of biarmed chromosomes (Rab *et al.* 1995). The karyotype of *B. brevipinnis* is similar to the asymmetric karyotype of the small *Barbus* with 2n=48-50 species from Angola and Guinea (Appendix 2) (Rab 1981, Rab *et al.* 1995) and some of the *Puntius* in Asia (Magtoon & Arai 1989, 1993). *B. brevipinnis*, including species with 2n=50 from Angola and Ethiopia (Appendix 2) are a part of the Zambezian fauna of Africa.

Another, karyotype pattern is also apparent in a few of the small *Barbus* species analysed in this study. Four species of the *B.anoplus*-complex and *B. pallidus* (Table 5.10, Figs. 5.2-5.6) have symmetrical karyotypes.

Within the biarm chromosome categories of the *B. anoplus*-complex, there are fewer **m** chromosomes than **sm+st** chromosomes. Again this is with the exception of *B. brevipinnis* where the biarm chromosomes comprise almost equal numbers of **m** chromosomes and **sm+st** chromosomes. It is possible that a trend is emerging for the small *Barbus* karyotypes of southern Africa and Africa (species with symmetric or asymmetric karyotypes), however more species need to be analysed to determine the full extent and significance of these data.

The largest element in the leuciscine karyotype is an **a** chromosome (Rab & Collares-Pereira 1995). Three large *Barbus* species from Guinea have an elongated pair of **m** chromosomes (Rab *et al.* 1995). These elongated chromosome pairs have been considered as 'marker' elements. An elongated or long pair of **sm+st** chromosomes is found in the metaphase cells of all the four members

of the *B. anoplus*-complex. *B. brevipinnis* is unique among the flexible rayed *Barbus* species analysed in this chapter in having two pairs of elongated chromosomes namely **m** and **sm+st**. No marker elements were apparent in the metaphase spreads of *B. pallidus* due to the contracted nature of the chromosomes. These marker (elongated) chromosomes may be used to identify species groups within the genus.

Acrocentric chromosomes of the *B. anoplus*-complex are of a similar size, and only the **sm+st** chromosomes range in size from large to small. Within the *B. brevipinnis* karyotype, the **m**, **sm+st** chromosome categories show a range of size from large to small and the **a** chromosomes are of a similar size. Although chromosome rearrangements can only be determined accurately from banding data, chromosome categories with a range in size may be areas where these chromosomal rearrangements have occurred. It can be speculated that *B. brevipinnis* represents a species where large chromosomes are the result of Robertsonian translocations where two pairs of chromosomes were lost.

NF: total number of chromosome arms

Chromosomal rearrangements in the form of pericentric inversions and Robertsonian translocations have occurred in the evolution of modern salmonids (Hartley 1987). Such structural rearrangements have been determined by considering the arm numbers within the karyotypes of (related) species. High NF1 values of the small *Barbus* from Ethiopia and Guinea have involved arm numbers that range from 92 to 100 (Golubstov & Kryzanov 1993, Rab *et al.* 1995). These data reflect the high number of biarmed chromosomes in the karyotypes. *B. brevipinnis* has high NF1 and NF2 values (86-92) compared to the four species of the *B. anoplus*-complex where NF1 and NF2 values range from 74-80, which reflect the presence of uniarmed chromosomes (Table 5.8). Although, allozyme analysis has shown that the three "tubercled" species *B. motebensis*, *B. amatolicus* and *B. gurneyi* are closer to each other than *B. anoplus* (Engelbrecht & van der Bank 1996) no such segregation is apparent when examining the NF1 and NF2 values.

Heteromorphic chromosomes (?sex chromosomes)

About 10% of teleost species karyologically studied show heteromorphic sex chromosomes (Yu *et al.* 1987, Morescalchi 1993). Heteromorphic chromosomes have been reported in species in the orders Siluriformes (Li *et al.* 1985, in Yu *et al.* 1987), Perciformes (Unpub data, in Yu *et al.* 1987) and Clupeiformes (Hong *et al.* 1984, in Yu *et al.* 1987). Chromosomal heterogamety has been reported in several South American freshwater fishes, namely Erythrinidae, Anostomidae and Parodontidae, where sex chromosome mechanism ranges from XX-XY, ZZ-ZW and multiple sex chromosomes (Bertollo *et al.* 1983). *Garra lamta* has a pair of heteromorphic **m** chromosomes in the female and the homomorphic

m chromosomes comprises the male complement, the heteromorphic pairs are considered as sex chromosomes (Khuda-Bukhsh *et al.* 1986). Heteromorphic chromosomes comprising unpairable chromosomes in a few populations European cyprinids have been reported (Chiarelli *et al.* 1969, Fontana *et al.* 1970, in Rab & Collares-Pereira 1995). However, such odd chromosome pairs are considered to represent karyotype variability, such as size polymorphism or technical misidentification of small cyprinid chromosomes (Rab & Collares-Pereira 1995).

This is the first account of heteromorphic chromosomes present within a *Barbus* karyotype. In the four species comprising the *B. anoplus*-complex, a pair of equally long chromosomes were observed to be heteromorphic, comprised of one **m** chromosome and one **sm+st** chromosome in all the male metaphase cells. Sexual dimorphism is well defined in the *B. anoplus*-complex where males develop keratinized tubercles on the head snout or pelvic fins, have larger fins and exhibit breeding colours. The presence of the heteromorphic pair in only male specimens, which show sexually dichromatic and dimorphic characters suggest that the chromosome pair may be the sex chromosomes. This heteromorphism was observed in the male metaphase cells of *B. anoplus*, *B. amatolicus*, *B. motebensis* and *B. gurneyi*. The absence of heteromorphic pairs in *B. brevipinnis* and *B. pallidus* may indicate that the process is still in an early stage of differentiation in these two species (Gold *et al.* 1981).

CONCLUSIONS

Based on the results presented here, all four species of the *B. anoplus*-complex karyotypes are distinct in comparison to the other small, flexible rayed species studied.

A distinct chromosome number and karyotype pattern separated *B. brevipinnis* from members of the *B. anoplus*-complex as well as *B. pallidus*. However, neither *B. brevipinnis* or *B. pallidus* are member of the *B. anoplus*-complex. The former has 2n=48 and a karyotype morphology where there are only 2 pairs of uniarm chromosomes, two pairs of elongated chromosomes and the latter have 2n=50, and approximately half the karyotype is composed of uniarmed chromosomes and have only one pair of elongated chromosomes.

The *B. anoplus*-complex differ from *B. brevipinnis* in having a large (almost 50%) **a** chromosome component and chromosomal heteromorphism in males.

Within the six flexible rayed, diploid species examined in this chapter, two groups are apparent based on chromosome morphology. Firstly, *B. brevipinnis* which has very few uniarmed chromosomes and is a component of the tropical fauna and secondly, the *B. anoplus*-complex species, along with *B. pallidus* which have an equal biarm and uniarmed chromosomes in the karyotype and are a component of the temperate fauna.

Although *B. pallidus* shows closer affinity to the *B. anoplus*-complex than to *B. brevipinnis*, better karyotype resolution is needed to comment further on this pattern.

The hypothesis that the four species of the *B. anoplus* complex comprise a monophyletic group is supported by their similar karyology.

In general cyprinid chromosomes, including the *Barbus* examined in this study, have a karyotype morphology which is distinct for a species group as defined by morphological characters. Within these groups however, a particular karyotype is highly conservative, with apparent lack of chromosomal arm rearrangement. In the salmonids arm rearrangements are observed and have been used to define relationships and propose a phylogeny (Hartley 1987).

The second hypothesis is that of the sister group relationship of *Pseudobarbus* and the *B. anoplus*-complex. Tetraploidy, resulting from a diploid ancestor is suggested by species having multiple number of chromosomes (Ohno 1970a&b, Rab pers. comm.).

Support of a polyploid origin for *Pseudobarbus* would be that the *B. anoplus*-complex has exactly half the chromosome number as the *Pseudobarbus* species. No chromosomes appear to have been lost in the tetraploidization event and no chromosome quadrivalents have been observed in the meiotic spreads of the *Pseudobarbus*. Tetraploidy resulting from allopolyploidization (species hybridization) rather than autoteraploidization (genome doubling) is suggested from two points of observations.

Tetraploidization of the (ancestral) *B. anoplus* without an increase in chromosomal number, and some level of chromosomal rearrangement would result in a karyotype with a fairly high number of uniarms, different to that observed in the *Pseudobarbus* species. It is more likely that the diploid ancestor had a karyotype morphology with a high number of biarm chromosomes and a low number of uniarm chromosomes, like that of *B. brevipinnis*.

Karyological data does not support the relationship of sister group of the *B. anoplus*-complex to *Pseudobarbus*.

Although, conventionally stained chromosomal data can be used to support morphologically determined species groups and lineages the data are not sufficiently detailed to provide interspecies resolution.

CHAPTER 6: THE SERRATED RAYED MINNOWS

INTRODUCTION

The spinefin, sawfin and serrated rayed minnows are other distinct groups of *Barbus* species distributed in southern Africa and comprise a third of a total of 54 *Barbus* in the region (Skelton 1993). Characteristically, these *Barbus* have a stout and/or serrated dorsal fin ray and radially striated scales. With the exception of the two sawfin species, *Barbus serra* and *Barbus andrewi*, most of the species are considered to be small or moderate sized minnows (SL≤150mm) (Skelton 1980, 1993). The serrated rayed barbines are a heterogeneous assemblage. Nine species analysed in this chapter represent a morphologically heterogeneous group, and are not considered a monophyletic group (Skelton 1994, Skelton pers. comm.).

Distributions of these fishes in the temperate and subtropical regions are well documented (Skelton 1994). General biology, habitat and conservation status of many of the serrated *Barbus* species are known. However, in most cases there is very little information regarding their life history requirements, population dynamics and age and growth (Cambray 1992).

General biology and distributional surveys for *Barbus hospes* and *Barbus trevelyani* have been conducted (Bok & Heard 1982, Hay pers. comm.) and impact studies of the effect of damming have been done (Bok & Heard 1982). Cambray (1985) investigated the larval development of *B. trevelyani*. A study on the biology and conservation status of *Barbus erubescens* is presently being carried out (Marriott pers. comm.).

Due to their large size (SL=500-600mm), both *B. serra* and *B. andrewi* are angling targets (Skelton 1993). A combination of environmental threats have resulted in a marked decline in their numbers, and both are listed in the Red Data Book-Fishes (Skelton 1987). Attempts to artificially propagate both species for restocking in suitable rivers have contributed towards an understanding of their spawning behaviour and breeding biology (Bok & Heard 1982). However, detailed ecological and biological investigations are required to develop suitable conservation strategies for the management of these species (Skelton 1987).

Traditional taxonomic characters have been used to separate this heterogenous group of *Barbus* into species complexes or lineages (Jubb 1967, Skelton 1988, 1994). Two distinct groups have been identified: (a) four smaller species with radial striated scales and serrated dorsal spine namely; *Barbus calidus*, *B. erubescens*, *B. trevelyani* and *B. hospes* and (b) the two large, serrated rayed species namely; *B. serra* and *B. andrewi*.

Skelton (1980) considered that *B. calidus* and *B. erubescens* are sister species on the basis of the following synapomorphies: red fins, unbranched dorsal fin ray, six or seven branched anal rays, dorsal fin origin placed posterior to origin of pelvic fin base, high average vertebral counts, high average predorsal fin and vertebral counts. Both, *B. calidus* and *B. erubescens* resemble the *Pseudobarbus* in having red fin coloration. However, Skelton (1980) considered this feature homoplaseous and

discounted both species as the sister outgroup to the *Pseudobarbus* lineage.

The affinities of *B. calidus* and *B. erubescens* with other serrated species with seven branched dorsal fin rays (*B. trevelyani* and *B. hospes*) is not clear but, all these species have been included for cytogenetic analysis in this study.

Two species within this group, *B. trevelyani* and *B. serra* were reported by Oellermann (1988) to have high chromosome numbers, 96 and 102 respectively (Appendix 2). These preliminary data indicate that tetraploidy may be a feature of at least three lineages of southern African barbines.

Both the question of the relationships of *B. calidus* and *B. erubescens* to the *Pseudobarbus* lineage, and the relationships of *B. calidus* and *B. erubescens* to *B. trevelyani* and *B. hospes* are examined using karyological data.

Additional serrated rayed barbine minnows were karyotyped in order to provide an expanded, comparative base of karyological analysis within the southern African *Barbus* species. These are *Barbus eutaenia*, *Barbus paludinosus* and *Barbus argenteus*. *Barbus trimaculatus* is the only spinefin barbine examined.

Key questions to be answered in this chapter are:

1. Are the serrated-rayed *Barbus* a polyphyletic group?
2. Are *B. calidus* and *B.erubescens*, sister species?
3. How are *B. trevelyani* and *B. hospes* related to *B. calidus* and *B. erubescens*? If at all.
4. Are any of these species related to the other redfinned *Pseudobarbus* species ?

Examining the karyotypes of this heterogeneous group of *Barbus* provides additional independent characters which may allow a more refined phylogeny to be constructed.

The discussion in this chapter is of a different format compared to the preceding two chapters, in that karyological findings will be discussed considering ploidy levels and the species and/or sister species groupings.

MATERIAL AND METHODS

Nine species were karyotyped for this chapter, following methods outlined in chapter 2, numbers and sex of specimens are listed in Table 6.1.

Chromosome morphology from photokaryotypes of *B. eutaenia* is not presented in the usual detail because the chromosome slide preparations had deteriorated before being examined.

Measured chromosome lengths of the species were determined from selected chromosomes in the metaphase spreads.

Table 6.1 Number and sex of nine serrated rayed *Barbus* species used for karyological analysis. n= number of specimens examined.

Species	n	♂	♀	Un/juv
B. argenteus	10	5	3	2
B. paludinosus	3	2	1	
B. eutaenia	2	1	1	
B. trimaculatus	15	4	6	5
B. calidus	7	4	3	
B. erubescens	3			3
B. hospes	1	1		
B. trevelyani	7	4	3	
B. andrewi	1		1	

RESULTS

Mean, standard deviation (sd), modal values and range of chromosomes components are reported in tables 6.3-6.11. A summary of the chromosome numbers and karyotypes are presented in tables 6.2, 6.13-6.14 and figures 6.1-6.9.

B. argenteus

Five males, three females and two specimens whose sex was undetermined, from Elands River were analysed (Tables 2.1 & 6.1). The modal chromosome number, determined from 74 metaphase cells, is 50 (Table 6.2). Mean and modal values, determined from 13 photokaryotypes, shows that the biarm component comprises 12**m**, 22**sm**+12**st** chromosomes, and the uniarm component consists of 4**a** chromosomes (Tables 6.3, 6.14).

Table 6.2 Distribution of chromosome number counts from kidney cells of nine serrated rayed *Barbus* species. n=number of metaphase cells examined.

		Diploid chromosome numbers							
Species	n	38	40	42	44	46	48	50	52
B. argenteus	74			4	4	2	3	59	1
B. paludinosus	40				1	1	2	36	
B. eutaenia	12						1	11	
B. trimaculatus	86				3	4	76	3	
		Diploid chromosome number							
		88	90	92	94	96	98	100	102
B. calidus	104	9	3	1	4	18	25	44	4
B. erubescens	30	2			1	2	1	24	
B. hospes	35					25	4	6	
B. trevelyani	61	2				12	10	57	
B. andrewi	29		1			2	2	24	

Table 6.3 Detailed description of the photokaryotypes showing mean standard deviation (sd), modal values and ranges of number of chromosomes for *B. argenteus* (Elands River). n=number of metaphase cells examined.

	Chromosome categories		
n=13	**m**	**sm+st**	**a**
Mean	11.8	33.7	4.5
sd	0.53	0.72	1.15
Mode	12	22+12	4
Range	10-13	32-34	4-8

Chromosome morphology: No heteromorphic pairs of chromosomes were detected in the metaphase spreads of either males or females (Fig. 6.1). In the **m** and **sm+st** categories, chromosome sizes are not uniform they range in size from large to small, two pairs of **sm+st** chromosomes are relatively elongated (**l:s**=2.5&2.0) and one pair of chromosomes are notably the smallest in that category; one pair of **m** chromosomes is also relatively elongated (**l:s**=2.5) compared to the remainder and both **a** chromosomes are similar in size (Fig. 6.1, Table 6.12).

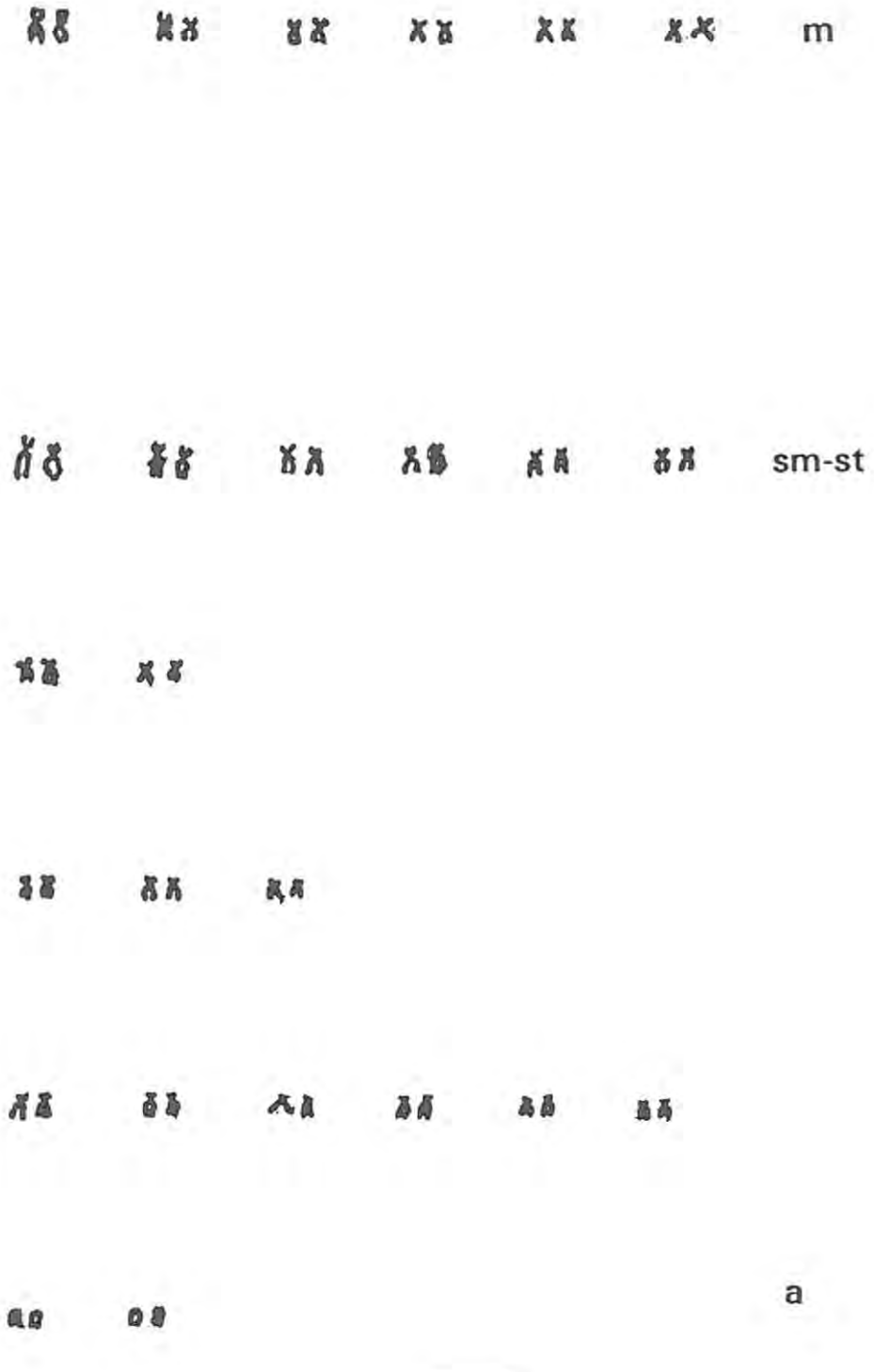


Figure 6.1 Photokaryotype of a male *B. argenteus* (Elands River) with chromosome formula 2n=12**m**+22**sm**+12**st**+4**a**=50. Scale bar = 5µm.

B. paludinosus

Two males and one female from the Molopo River were karyotyped (Tables 2.1 & 6.1). The modal chromosome number is 50 (Table 6.2). Mean and modal values from seven photokaryotypes indicate that the biarm components comprises 10**m** and 28**sm**+6**st** chromosomes (Tables 6.4, 6.14). There are 6**a** chromosomes.

Table 6.4 Detailed description of the photokaryotypes showing mean, standard deviation (sd), modal values and ranges of number of chromosomes for *B. paludinosus* (Molopo River). n=number of metaphase cells examined.

	Chromosome categories		
n=7	**m**	**sm+st**	**a**
Mean	8	34.2	7.14
sd	0	2.24	1.8
Mode	10	28+6	6
Range	8-10	32-38	4-10

Chromosome morphology: Neither males nor female have heteromorphic chromosomes (Fig. 6.2). Two pairs of **sm+st** chromosomes are relatively more elongated than the rest of the complement and one pair is the shortest in the complement; the largest differential in chromosome size is noted in the **sm+st** category; the **m** and **a** chromosomes sizes changes very slightly from the first to the last pair (Fig. 6.2, Table 6.12).

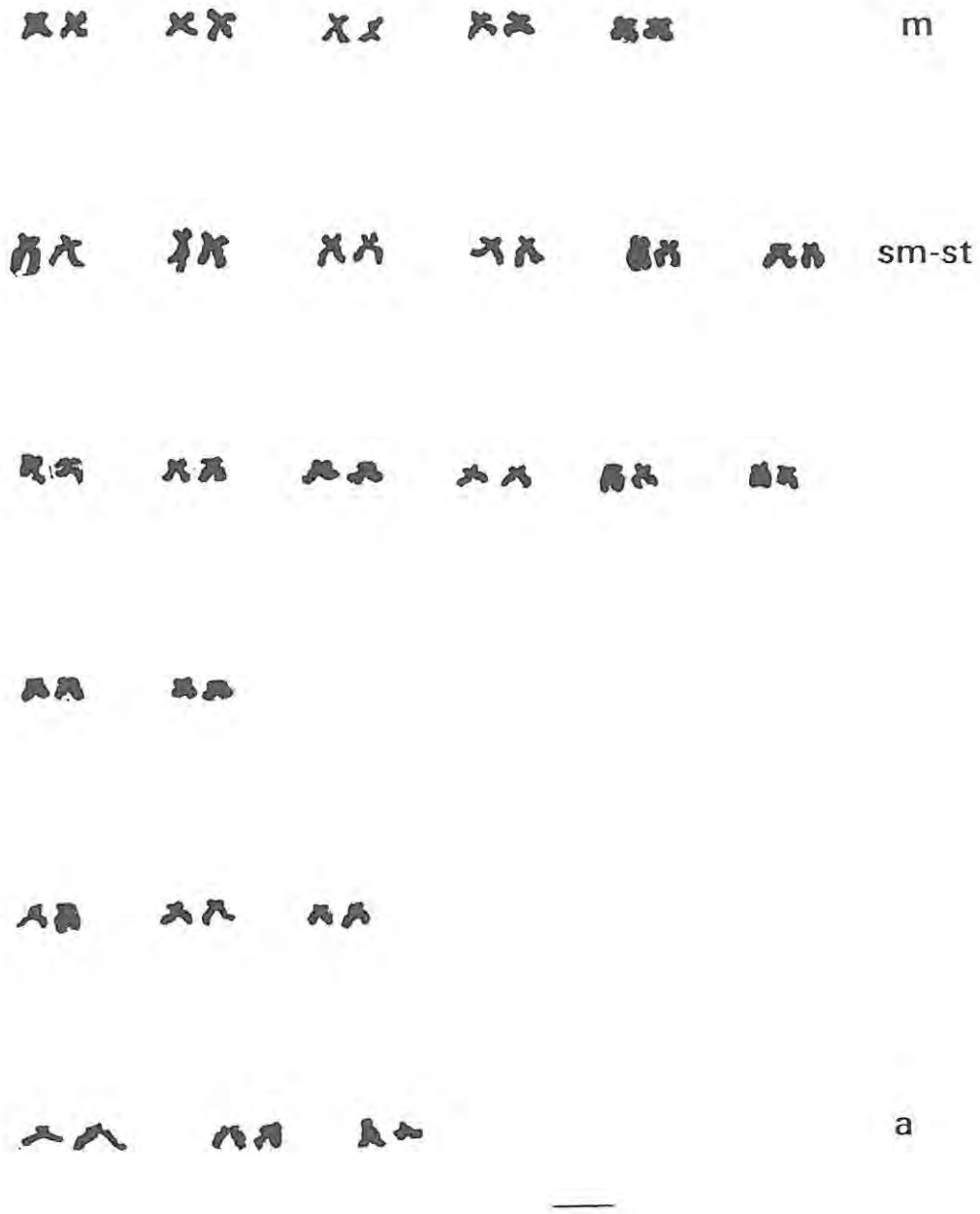


Figure 6.2 Photokaryotype of a male *B. paludinosus* (Molopo River) with chromosome formula 2n=10**m**+28**sm**+6**st**+6**a**=50. Scale bar = 5μm.

B. eutaenia

One male and one female from the Sabi River were analysed (Tables 2.1 & 6.1). The modal chromosome number determined from 12 metaphase cells is 50 (Table 6.2). There are 32 biarm components and 18 uniarm components (Tables 6.5 & 6.14). The detailed composition are not presented as no clear photomicrographs were made. The **a** category comprised uniarmed chromosomes, this is considered a preliminary interpretation of the karyotype.

Table 6.5 Photokaryotypes showing mean, standard deviation (sd), modal values and ranges of number of chromosomes for *B. eutaenia* (Sabi River). n=number of metaphase cells examined.

	Chromosome categories	
n=4	**m+sm**	**a**
Mean	32	16.5
sd	0.0	0.86
Mode	32	16
Range	32	16-18

B. trimaculatus

Fifteen males and females from Mooi River were analysed (Tables 2.1 & 6.1). The modal chromosome number determined from 86 metaphase cells is 48 (Table 6.2). Mean and modal values from 13 photokaryotypes show that the biarm component comprises 8**m**, 24**sm**+16**st** chromosomes, there are no **a** chromosomes (Tables 6.6 & 6.14).

Table 6.6 Detailed description of the photokaryotypes showing mean, standard deviation (sd), modal values and ranges of number of chromosomes for *B. trimaculatus* (Mooi River). n=number of metaphase cells examined.

	Chromosome categories		
n=13	**m**	**sm+st**	**a**
Mean	7.53	40.3	-
sd	0.84	0.92	-
Mode	8	24+16	-
Range	6-8	39-42	-

Chromosome morphology: Chromosome size in all the chromosome categories are not uniform and show a range in from large to small (Fig. 6.3, Table 6.12). There are 3 pairs of large chromosomes; one pair of **m** chromosomes and two pairs of **sm+st** chromosome are distinctly elongated, one pair **sm+st** is noticeably the smallest in the complement (Fig. 6.3, Table 6.12); neither males nor females have heteromorphic chromosomes.

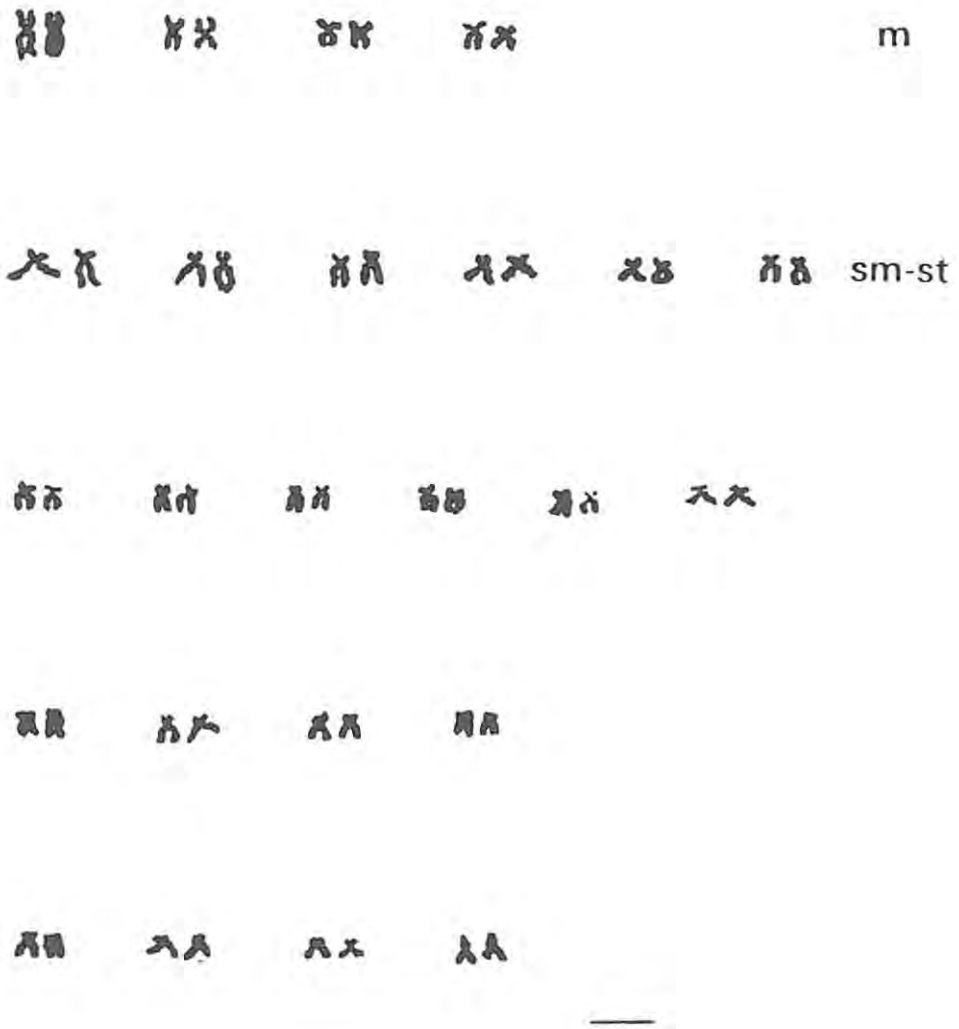


Figure 6.3 Photokaryotype of female *B. trimaculatus* (Mooi River) with chromosome formula 2n=8**m**+24**sm**+16**st**=48. Scale bar = 5µm.

B. calidus

Four males and three females from the Noordhoeks River were analysed (Tables 2.1 & 6.1). The modal chromosome number determined from 104 metaphase cells is 100 (Table 6.2). Mean and modal values from 12 photokaryotypes indicate that the biarm component comprises 14**m** and 54**sm**+26**st** chromosomes and the uniarm consists of 6**a** chromosomes (Tables 6.7 & 6.14).

Table 6.7 Detailed description of the photokaryotypes showing mean, standard deviation (sd), modal values and ranges of number of chromosomes for *B. calidus* (Noordhoeks River). n=number of metaphase cells examined.

	Chromosome categories		
♂&♀,n=12	**m**	**sm+st**	**a**
Mean	13.7	80.3	5.83
sd	2.9	4.7	2.1
Mode	14	54+26	6
Range	8-16	76-88	4-10

Chromosome morphology: Neither males nor females have heteromorphic chromosomes (Fig. 6.4). There are four large chromosomes; three pairs of **sm+st** chromosome are relatively elongated (**l:s**=2.6,2.4&1.9), and one pair of **m** chromosomes are elongated (**l:s**=1.6) compared to the remainder of the complement; two pairs of **sm+st** chromosomes are the smallest; the **sm+st** chromosome category show the largest change in size within the complement; the **m** and **a** chromosomes change very slightly in size, and thus appear similar in size (Figs. 6.4, 3.12 & Table 6.12).

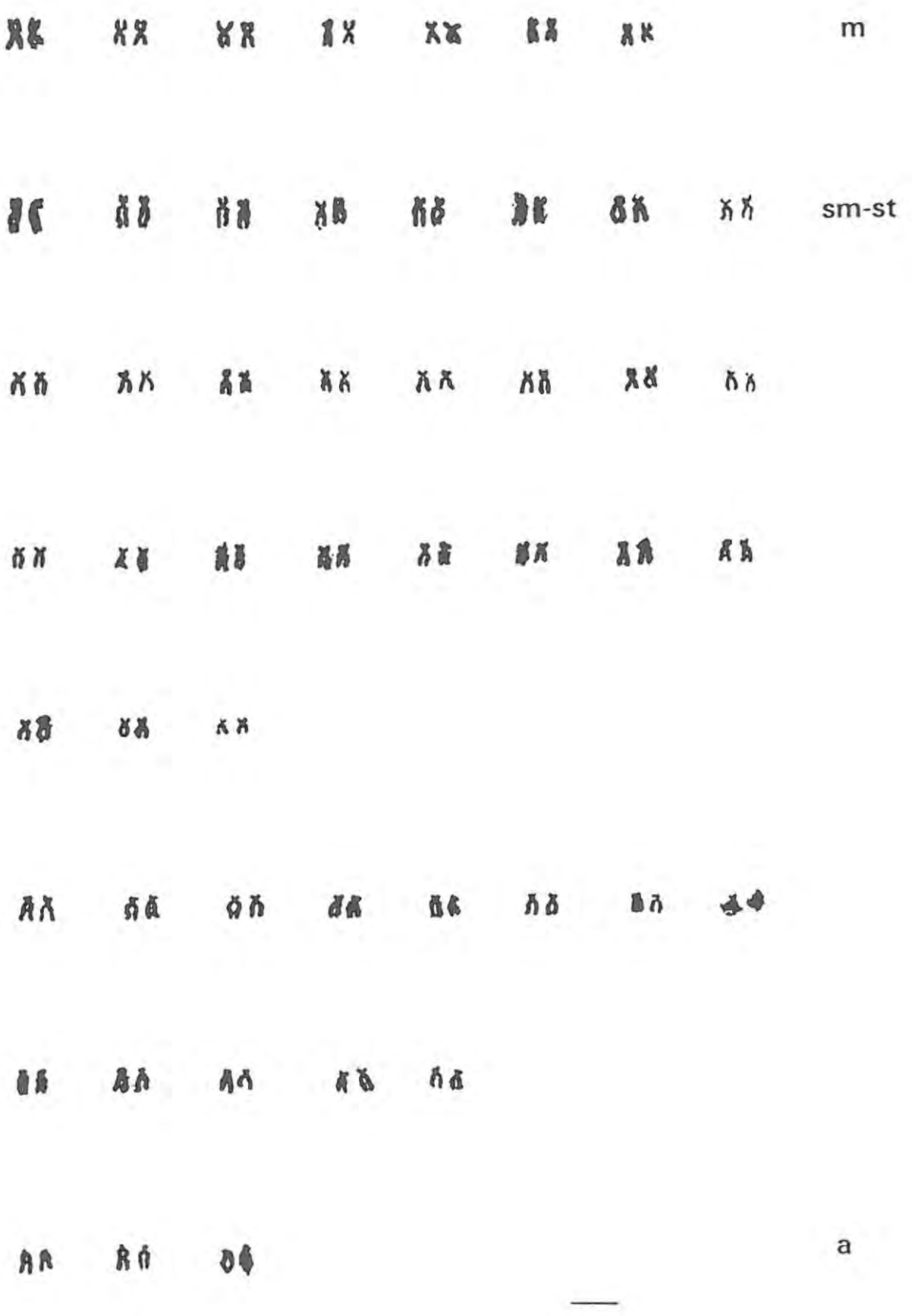


Figure 6.4 Photokaryotype of a male *B. calidus* (Noordhoeks River) with chromosome formula 2n=14**m**+54**sm**+26**st**+6**a**=100. Scale bar = 5µm.

B. erubescens

Three juveniles from the Noordhoeks River were analysed (Tables 2.1 & 6.1). The modal chromosome number is 100 (Table 6.2). Mean and modal values from five photokaryotype shows that the biarm component comprises 14**m** and 34**sm**+44**st** chromosomes and the uniarm component consists of 8**a** chromosomes (Tables 6.8 & 6.14).

Table 6.8 Detailed description of the photokaryotypes showing mean, standard deviation (sd), modal values and ranges of number of chromosomes for *B. erubescens* (Twee River). n=number of metaphase cells examined.

	Chromosome categories		
n=5	**m**	**sm+st**	**a**
Mean	11.2	79.6	9.2
sd	0.98	1.5	1.0
Mode	14	34+44	8
Range	10-12	78-82	8-10

Chromosome morphology: No heteromorphic chromosomes were observed in the metaphase spreads of this species (Fig. 6.5). Chromosomes in the **m** and **a** categories change slightly in size within the complement; there is a range of size in the **sm+st** category of chromosomes, with two pairs of **sm+st** chromosomes relatively elongated than the rest of the complement (**l:s**=1.8&1.7); and one pair of elongated **m** chromosomes (**l:s**=1.5); there are two pairs of **sm+st** chromosomes which are the smallest in this category (Fig. 6.5 & Table 6.12).

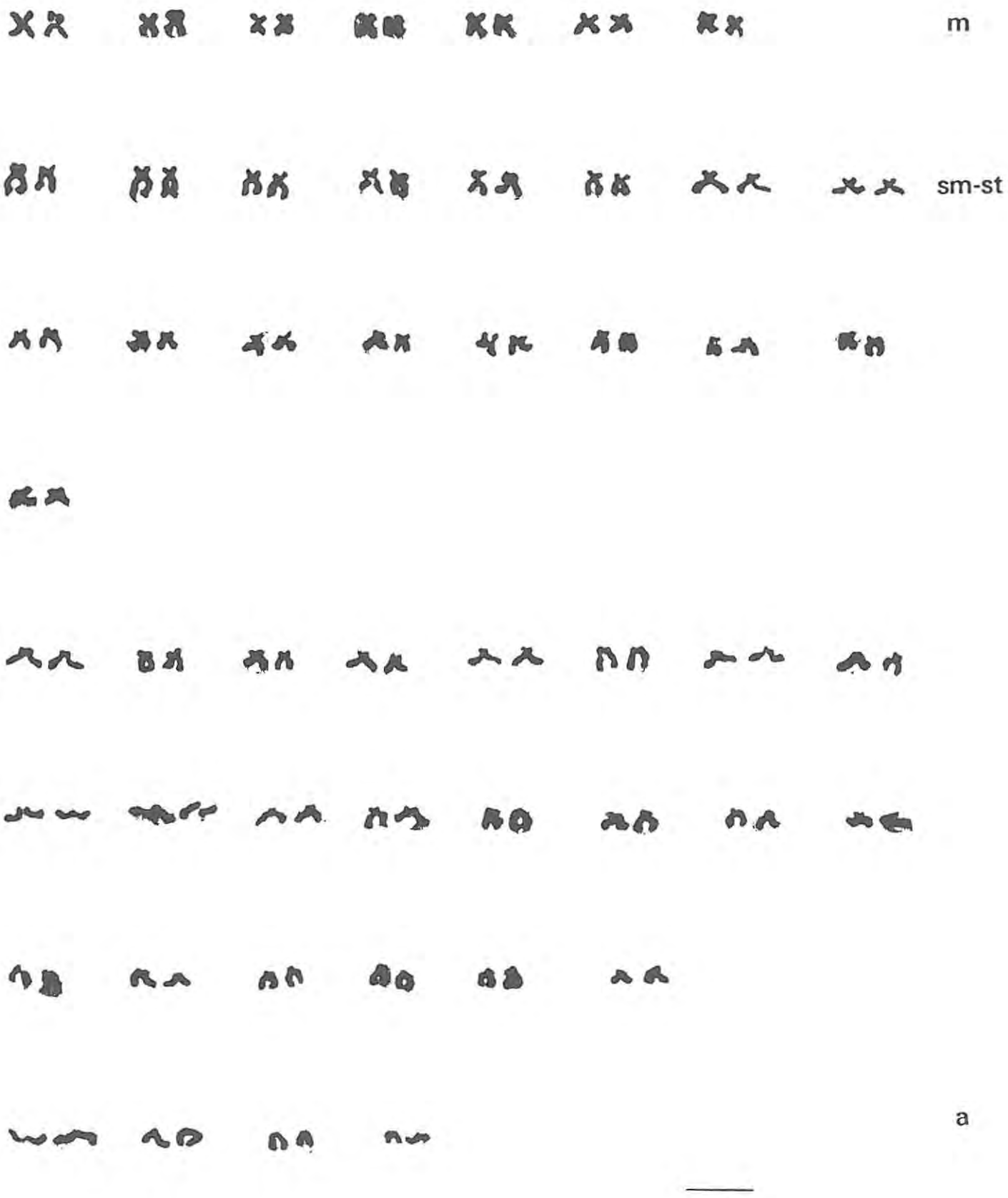


Figure 6.5 Photokaryotype of a male *B. erubescens* (Twee River) with chromosome formula 2n=14**m**+34**sm**+44**st**+8**a**=100. Scale bar = 5μm.

B. hospes

A single male specimen was analysed from the lower Orange River. The most frequent chromosome number from 35 metaphase cells examined is 96 with a slightly lower frequency of occurrence for 98 and 100 chromosomes (Table 6.2). Only one specimen was examined. At least 17% of the metaphase cells have 100 chromosomes, this is similar to the modal chromosome number of other tetraploids examined in this study, I therefore consider that *B. hospes* has 100 chromosomes. Mean and modal values from four photokaryotypes shows that the uniarm component consists of 24-26**a** chromosomes and the biarm component consists of 14**m** and 44**sm**+16**st** chromosomes (Tables 6.9 & 6.14).

Table 6.9 Detailed number of photokaryotype showing mean, standard deviation (sd), modal values and ranges of number of chromosomes for *B. hospes* (Orange River). n=number of metaphase cells examined.

	Chromosome categories		
n=4	**m**	**sm+st**	**a**
Mean	12.5	60.0	26.0
sd	2.6	3.2	2.5
Mode	14	44+16	24/26
range	8-14	56-64	24-26

Chromosome morphology: No heteromorphic chromosome pair was observed in the male karyotype (Fig. 6.6). The photokaryotype reveals that there is a pair of **a** chromosomes which is distinctly longer than the rest of the uniarm chromosomes (**l:s**=2.0) and one pair is distinctly the smallest in the complement; two pair of **sm+st** chromosomes are also elongated compared to the rest of the complement (**l:s**=2.2&1.7), and one pair of **sm+st** chromosomes is the smallest; the remaining **sm+st** and **a** chromosomes all gradually decrease in size (Fig. 6.6 & Table 6.12).

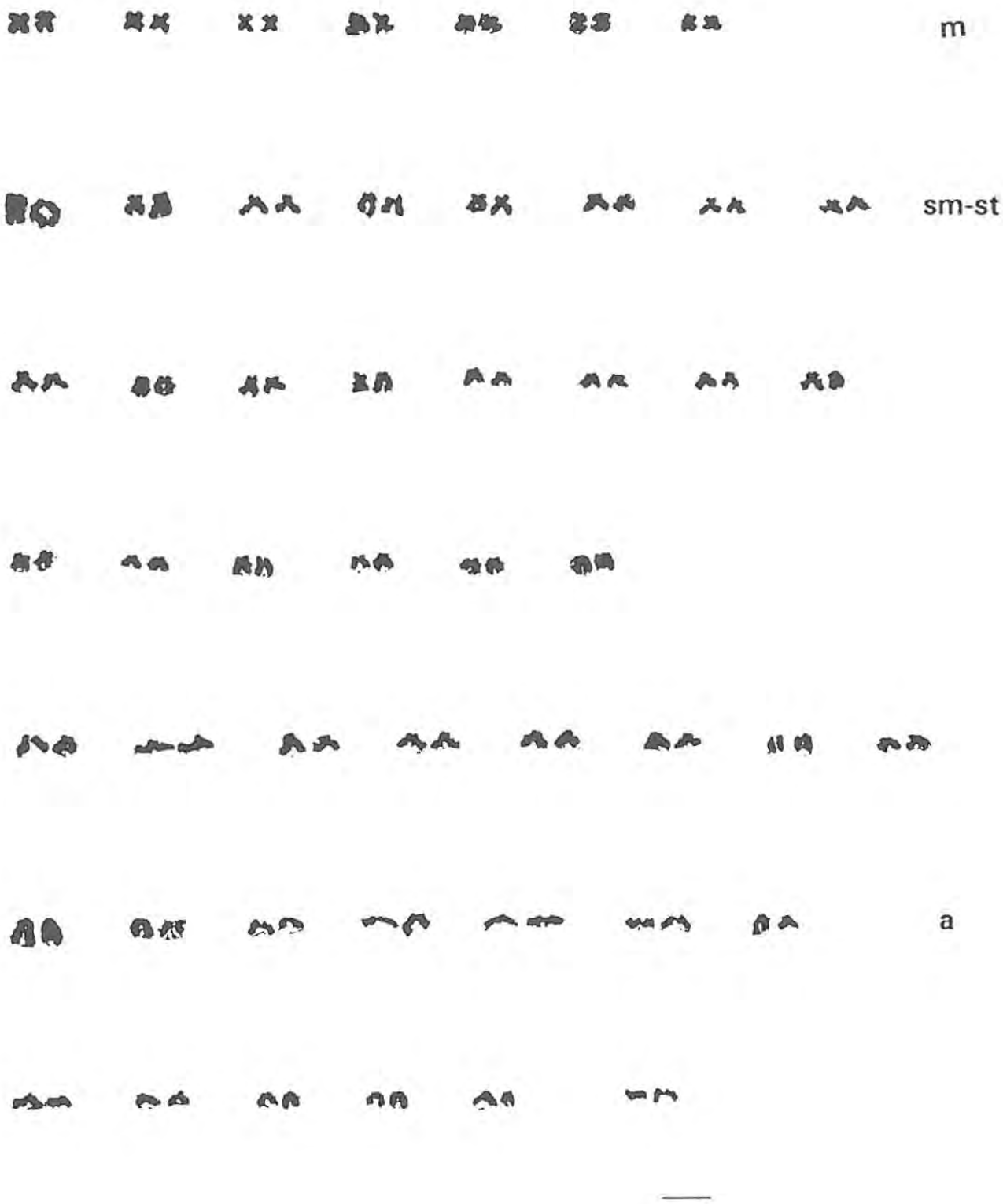


Figure 6.6 Photokaryotype of a male *B. hospes* (Orange River) with chromosome formula 2n=14**m**+44**sm**+16**st**+26**a**=100. Scale bar = 5µm.

B. trevelyani

Four males and three females from the Buffalo River were analysed (Tables 2.1 & 6.1). The modal chromosome number determined from 61 metaphase cells is 100 (Table 6.2). Mean and modal values from 11 photokaryotypes shows that the biarm component comprises 14**m** and 46**sm**+30**st** chromosomes the uniarm component consists of 10**a** chromosomes (Tables 6.10 & 6.14).

Table 6.10 Detailed description of the photokaryotypes showing mean, standard deviation (sd), modal values and ranges of number of chromosomes for *B. trevelyani* (Buffalo River). n=number of metaphase cells examined.

	Chromosome categories		
♂&♀,n=11	**m**	**sm+st**	**a**
Mean	12.6	78	9.1
sd	2.3	2.6	2.3
Mode	14	46+30	10
range	8-16	72-82	6-14

Chromosome morphology: No heteromorphic chromosomes were apparent in the male and female karyotypes examined (Fig. 6.7). All the **m** and **a** chromosomes appear to be similar in size to each other within those categories; the size of **sm+st** chromosomes changes gradually; three pairs of **sm+st** and two **m** chromosome are elongated (**l:s**=4.5,4.3) and (**l:s**=2.1) respectively; two pairs of **sm+st** are the smallest in the complement (Fig. 6.7 & Table 6.12).

Figure 6.7 Photokaryotype of male *B. trevelyani* (Buffalo River) with chromosome formula 2n=14**m**+46**sm**+30**st**+10**a**=100. Scale bar = 5µm.

B. andrewi

One male specimen from the Breë River was analysed (Tables 2.1 & 6.1). The modal chromosome number determined from 29 metaphase cells is 100 (Table 6.2). Examination of 15 photokaryotypes indicate that the biarm comprises 16**m** and 36**sm**+42**st** chromosomes and the uniarm consists of 6**a** chromosomes (Tables 6.11 & 6.14).

Table 6.11 Detailed description of the photokaryotype of showing mean, standard deviation (sd), modal values and ranges of number of chromosomes for *B. andrewi* (Breë River). n=number of metaphase cells examined.

	Chromosome categories		
n=12	**m**	**sm+st**	**a**
Mean	15.1	79.0	5.9
sd	1.9	1.9	1.6
Mode	16	36+42	6
range	10-16	76-84	4-10

Chromosome morphology: No heteromorphic chromosome pairs were observed in the metaphase spreads (Fig. 6.8). There are six pairs of elongated chromosomes; two pairs of **m** chromosomes (**l:s**=2.7&2.6) and four pairs of **sm+st** chromosomes (**l:s**=3.4,3.0,2.8,2.3) respectively; one pair in the **sm+st** category are the smallest in the complement; the remaining chromosomes in the **m**, **sm+st** categories change gradually in size from large to small; the **a** chromosomes also change in size (2.7-1.5µm) (Fig. 6.8 & Table 6.12).

Table 6.12 The size range (μm) of selected chromosome pairs within the chromosome categories of serrated rayed *Barbus*. (n=1, except *B. calidus*, n=9).

	Chromosome categories		
Species	**m**	**sm+st**	**a**
B. argenteus	2.4-1.3	2.9-1.2:1.9-1.4	135-130
B. paludinosus	2.5-1.9	3.6-1.9:2.3-1.9	2.5-2.1
B. trimaculatus	3.1-1.6	2.4-1.3:1.6-1.4	-
B. calidus	3.2-2.4	3.8-1.9:3.1-2.3	2.8-2.6
B. erubescens	2.6-2.4	3.7-2.5:2.1-1.6	1.9-1.2
B. hospes	2.4-1.8	3.5-2.2:2.1-1.6	2.9-1.8
B. trevelyani	3.6-2.4	3.7-2.5:2.7-2.4	3.0-2.1
B. andrewi	3.2-2.1	3.1-1.8:3.7-2.4	2.5-2.1

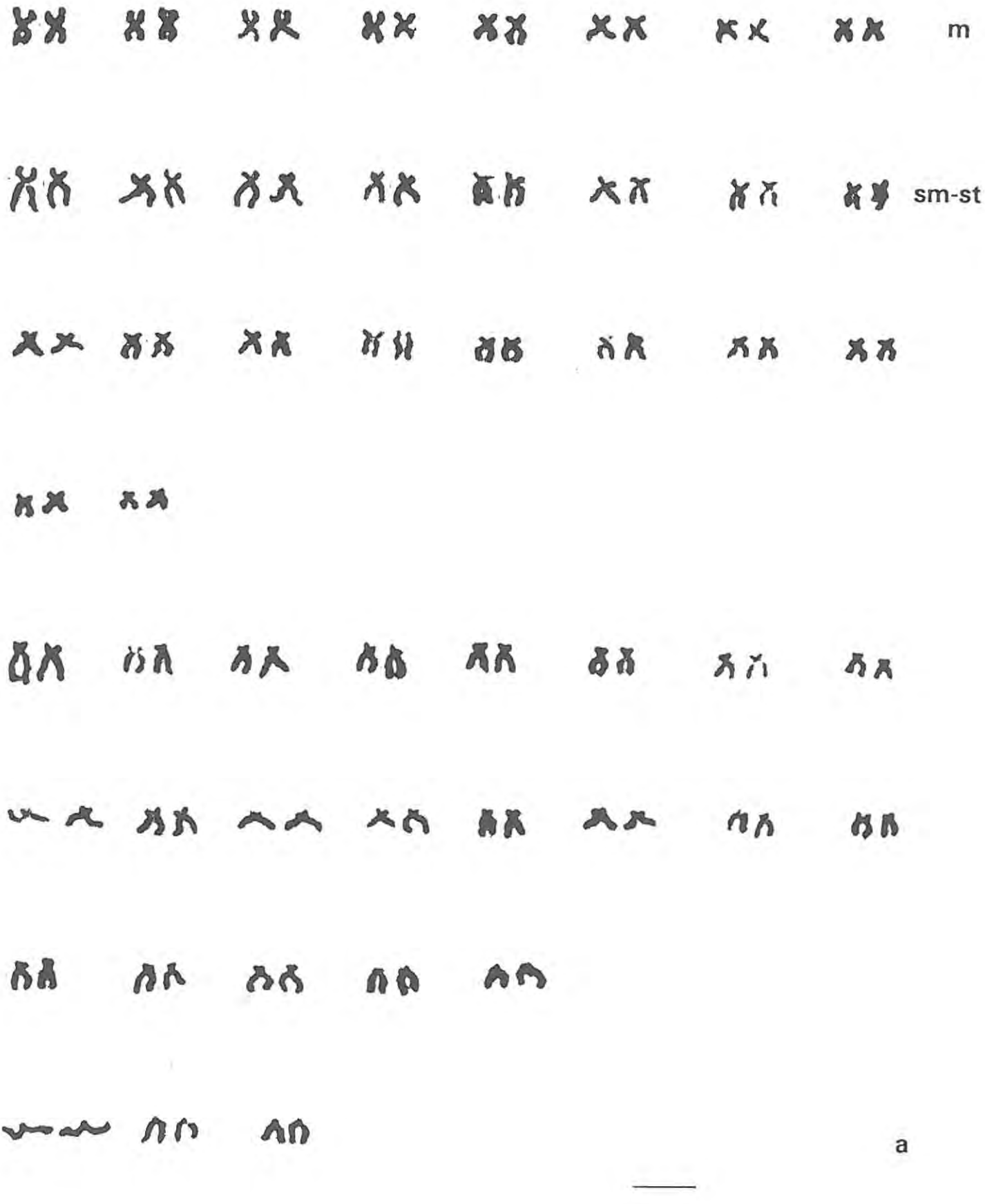


Figure 6.8 Photokaryotype of a male *B. andrewi* (Breë River) with chromosome formula 2n=16**m**+36**sm**+42**st**+8**a**=100. Scale bar = 5µm.

NF: total number of chromosome arms

The arm numbers for all species in this group are high, indicating the dominance of biarmed chromosomes in the karyotype. NF1 values for the *Barbus* with 2n=48-50, range from 74-84, and NF2 values range from 92-100 (Table 6.13). For the *Barbus* species with a chromosome complement of 2n=96-100, NF1 values range from 148-170, and NF2 range from 178-196 (Table 6.13).

Table 6.13 Total number of arms for nine serrated rayed *Barbus* analysed using different calculations NF1=2(**m+sm**)+(**st+a**) and NF1=2(**m+sm+st**)+(**a**).

Species	NF1	NF2
B. argenteus	84	96
B. paludinosus	88	94
B. eutaenia	74	-
B. trimaculatus	80	96
B. calidus	168	194
B. erubescens	148	192
B. hospes	156/158	172/174
B. trevelyani	160	190
B. andrewi	152	194

Meiotic chromosomes

No tetravalents were found in the metaphase cells prepared from gonadal material of five species with 2n=96-100 (Fig. 6.9).

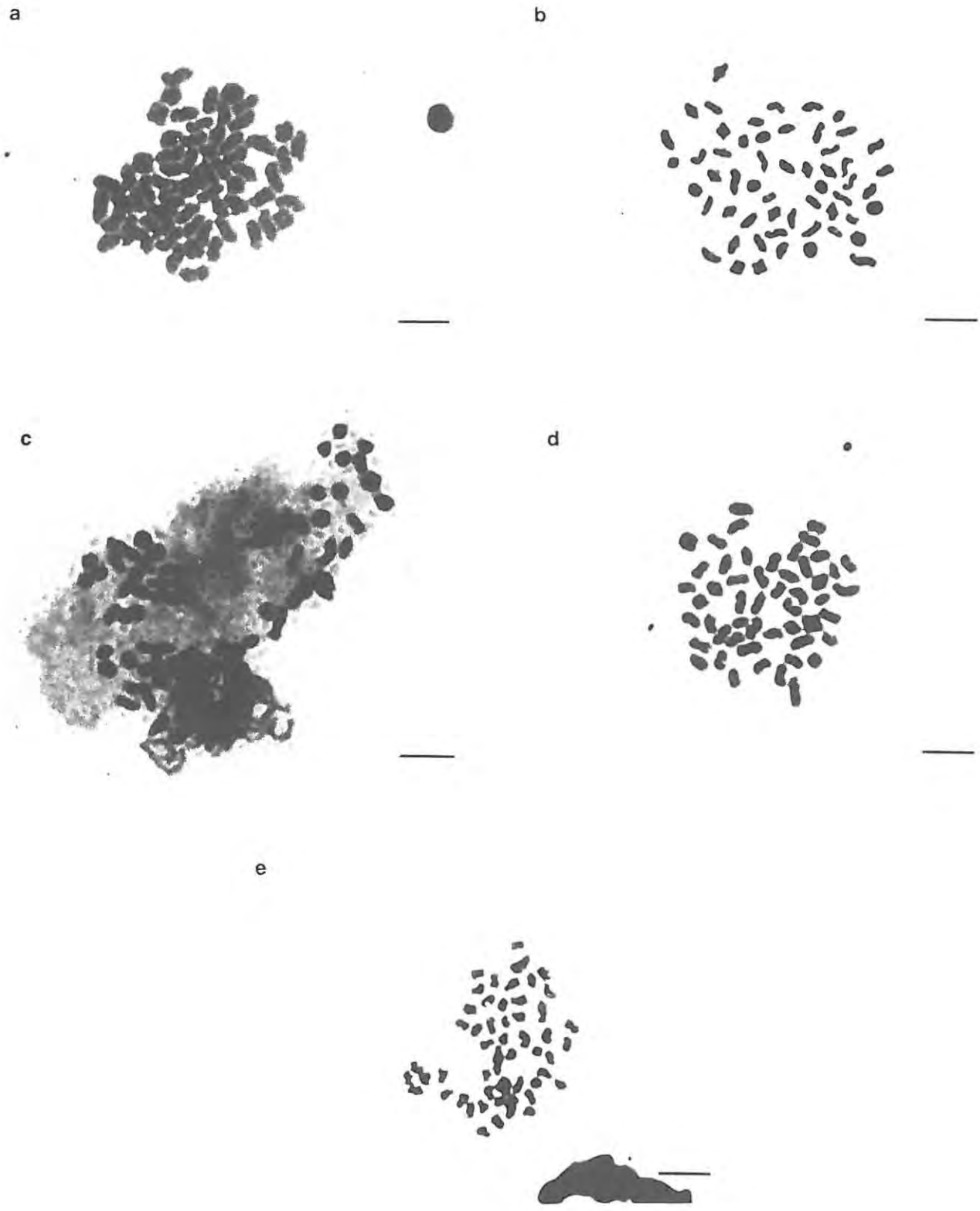


Figure 6.9 Meiotic spreads showing bivalent chromosomes in five *Barbus* species. a) *B. calidus*, b) *B. erubescens*, c) *B. hospes*, d) *B. trevelyani* and e) *B. andrewi*. Scale bar = 5µm.

Summary of characteristics of nine serrated rayed *Barbus* species karyotypes

1. Two ploidy level groups are present i.e., four species with 2n=48-50 and five species with 2n=96-100 (Table 6.2, 6.14).

2. Biarmed chromosomes are dominant; **m**, **sm+st** comprising 94-100% of the 2n=48-50 karyotype and comprising 74-92% of the 2n=100 karyotype (Table 6.14).

3. The **m** chromosomes comprises 16-24% of the karyotype of the former group, and 14-16% of the karyotype of the latter group (Table 6.14).

4. The **a** chromosomes comprises 6-26% of the karyotype of the species with 2n=100, and 8-12% of the karyotype of species with 2n=50 (Table 6.14).

5. Species have large marker chromosomes. In most species these marker chromosomes are biarmed (**m**, **sm+st**), except for *B. hospes* which is the only species with a pair of elongated **a** chromosomes (Figs. 6.1-6.8).

6. No heteromorphic chromosomes were detected (Figs. 6.1-6.2, 6.4-6.8).

7. The NF values of all species are high and this is indicative of the high biarmed chromosomes in the karyotypes (Table 6.13).

8. Chromosomes in the three categories are not uniform in size and range from large to small (Table 6.12, Fig. 3.8).

9. In the 2n=100 species no quadrivalent pairs are evident in the meiotic chromosome preparations (Fig. 6.9).

Table 6.14 Modal values of chromosome categories from kidney cells of nine serrated rayed *Barbus*. Elongated chromosomes: **a**-l=acrocentric-long, **m**, **sm+st**-l=submetacentric-long, *preliminary karyotype description.

	Chromosome categories				
Species	**m**	**sm+st**	**a**	**a**-l	**m,sm+st**-l
B. argenteus	12	22+12	4	-	3
B. paludinosus	10	28+6	6	-	2
B. eutaenia	32*		10*	-	?
B. trimaculatus	8	24+16		-	3
B. calidus	14	54+26	6	-	4
B. erubescens	14	34+44	8	-	3
B. hospes	14	44+16	24/26	1	3
B. trevelyani	14	46+30	10	-	5
B. andrewi	16	36+42	6	-	6

DISCUSSION

General determination of chromosome numbers, biarm-uniarm components with the karyotypes are considered in the following section.

Determination of chromosome numbers

Species with fewer chromosomes are easier to enumerate compared with species having high chromosome numbers. This is seen in the standard deviation values (Tables 6.3-6.6). A greater variation in chromosome numbers is apparent with species having higher chromosome numbers (Tables 6.7-6.11). Hypermodal and/or hypomodal counts were encountered in the photokaryotype analysis in all species analysed in this chapter. Comparison of modal metaphase cells with the hypo/hypermodal metaphase cells revealed no chromosome morphological differences which would indicate chromosomal rearrangements. Moreover, variation in chromosomal elements were observed in preparations within a specimen. This indicates that variations in chromosome determination were due to technical factors, rather than an inherent characteristic of karyotypes observed.

Intraspecies comparisons

Gender

Breeding males of *B. erubescens* and *B. calidus* turn red at the base of their fins; both males and females develop small tubercles on the head and back; *B. trevelyani* and *B. andrewi* both male and female develop tubercles on head and males of the former species turn golden, *B. paludinosus* are females usually larger than males (Skelton 1993). However, sexually dimorphic and dichromatic features in the morphologies of male and/or female species are not clearly ascertained for most species (Skelton pers. comm.). None of the species analysed in this chapter have heteromorphic chromosomes in either the male or females karyotypes (Figs. 6.1-6.9).

Geographic differences

B. paludinosus is one of the few small minnows that has a wide distribution, extending from the coastal rivers of East Africa south to Vungu River, Natal, and from the southern Zaire tributaries to the Quanza River in Angola and to the Orange River (Skelton 1993). Results of two studies show that *B. paludinosus* has a chromosome number of 50 (Golubstov & Kryzanov 1993 & this study). However, the karyotype of species from the Molopo river (this study) is different to the species analysed from Ethiopia (Golubstov & Kryzanov 1993, Appendix 2), in having two fewer biarmed chromosomes and two additional uniarmed chromosomes. This difference in chromosomes may represent a geographical variation in the karyotype of this widespread species or may indicate a species level difference. It must also be noted that the chromosomes were obtained from different tissues in each case, namely kidney tissue was used in the present study whereas thymus tissue was used in the Ethiopian study. In addition it is possible that the determination of chromosome categories may differ between authors (see chapters 2,3).

B. argenteus and *B. trimaculatus* have a divided distribution (Skelton 1993) and only specimens from the Incomati system have been karyotyped in this study. A comparison with the karyotypes of species from the West coast rivers, Cuanza and Cunene would be valuable in understanding the intraspecies relationship of the disjunct distribution of these species.

The five species (2n=100) are either site restricted (*B. erubescens*) or restricted to a single river system (*B. trevelyani*) (Skelton 1993). Karyotypes of these species were derived from individuals. So, it is not possible to explore changes in the karyotypes at population level in this study. Karyotypes of species determined from individuals taken from different localities are required to make assessments of population level variation.

Interspecific comparisons

Closely related diploid and tetraploid species are reported in *Cobitis biwae* (Kobayashi 1976) and *Corydoras* (Dunham *et al.* 1980). A few genera of cyprinids have both diploid and tetraploid species. Three out of the five genera of the subfamily Barbinae (*Barbus*, *Barbodes* and *Tor*) are known to have both 48-50 and 96-100 chromosome numbers in China (Yu *et al.* 1987, Rab & Collares-Pereira 1995). *Puntius*, a widely distributed Asian cyprinid genus (Talwar & Jhingran 1991), *Percocypris* and *Tor* (Arai 1982, Khuda-Bukhsh *et al.* 1986, Yu *et al.* 1987, Klinkhardt *et al.* 1995) have species with both 50 and 100 chromosome numbers amongst its members.

Both diploid and tetraploid levels are present within the serrated rayed *Barbus* of southern Africa. The common cyprinid diploid complement represented by 50 chromosomes is found in three unrelated species, and one spinefin species is represented by 48 chromosomes. The tetraploid complement represented by 100 chromosomes is found in six unrelated species examined (Tables 6.1 & 6.14, Appendix 2).

The presence of 96-100 chromosomes in four small, serrated rayed *Barbus* represent the first report of tetraploidy in small *Barbus* of southern Africa and Africa. However, the majority of African *Barbus* have yet to be karyotyped, so the significance of tetraploidy in smaller barbines is not yet clear.

The different ploidy levels within these endemic species provides a further opportunity to examine the systematics and phylogenetic relationships of the group(s).

For the interspecific discussion, the *Barbus* examined are separated into the diploid and tetraploid groups. Chromosome numbers, ploidy level and karyotype morphology of species are further discussed within species and/or sister-species subdivisions.

Interspecific comparison: sawfin and spinefin barbs (2n=48-50)

Chromosome numbers & ploidy levels

Three unrelated sawfin barbs *B. argenteus*, *B. eutaenia* and *B. paludinosus* have 50 chromosomes (Table 6.1). Of the two members of the spinefin species in southern Africa only one, *B. trimaculatus*, is examined in this study. It has 48 chromosomes (Table 6.1). Chromosome number of 48 is found in 16-33% of the karyotyped cyprinids (Yu *et al.* 1987, Buth *et al.* 1991), and it is relatively unusual among the karyotyped southern African barbines.

Morphology of karyotypes

All three diploid species *B. argenteus*, *B. paludinosus* and *B. trimaculatus*, have a larger biarmed chromosome component compared to uniarmed chromosomes (Figs. 6.1-6.3 & Table 6.14). The biarmed component is mainly made of **sm+st** chromosomes, a smaller proportion comprises **m** chromosomes. *B. argenteus* and *B. paludinosus* differ in the number of **a** chromosomes and in the number of **m** chromosomes (Figs. 6.1, 6.2 & Table 6.14).

B. trimaculatus is distinct in having only **m** and **sm+st** chromosomes, there are no uniarmed **a** chromosomes in the karyotype (Fig. 6.3 & Table 6.6). Three other African *Barbus* species are similar to *B. trimaculatus* in having 2n=48 and no **a** chromosomes in the karyotype (Rab 1981, Rab *et al.* 1995, Appendix 2). However, unlike *B. trimaculatus*, the two species have a flexible dorsal ray (Skelton pers. comm.), and may represent separate lineages.

B. eutaenia is distinct among the species discussed above, in having the highest number of uniarmed chromosomes. Due to the poor quality of photomicrographs, further resolution of the karyotype is not possible.

These karyotypes are distinct from the diploid *B. anoplus*-complex and *B. pallidus* examined in chapter 3, which have symmetric karyotypes, of almost equal uniarm and biarm chromosomes.

Asymmetric karyotypes are represented in five *Barbus* species (2n=50), from Ethiopia (Golubstov & Kryzanov 1993, Appendix 2) and three *Barbus* species (2n=48-50) from Guinea (Rab *et al.* 1995, Appendix 2).

All four 2n=48-50 species analysed in this chapter are components of the Zambezian ichthyofauna of southern Africa. In contrast, the species comprising the *B. anoplus*-complex, which are a component of the temperate Cape-Karriod ichthyofauna (Skelton 1994). Although, only 9% of the southern African, subtropical species (out of 54 species) have been karyotyped in this study, a higher biarm component in diploid karyotypes is a characteristic feature of these tropical *Barbus* (Figs. 6.1-6.3, Table 6.14). When considering the Ethiopian and Guinean karyotypes, of species with 2n=48-50, asymmetric karyotypes are well represented.

Unlike the "*Notropis*" group from North America, representing 90% of the North American cyprinids, where conventionally stained karyotypes are homogeneous (Amemiya & Gold 1990), southern African *Barbus* species examined so far have karyotypes that seem to follow broad morphologically defined parameters.

The karyological data suggest that the three species *B. trimaculatus*, *B. argenteus* and *B. paludinosus* are distinct from each other but the present data set is too limited for phylogenetic inferences among this diverse assemblage.

NF: total number of chromosome arms

The NF1 values for species with 2n=48-50 from Ethiopia and Guinea ranges from 84-100 (Rab 1981, Golubstov & Kryzanov 1993, Rab *et al.* 1995, Appendix 2), with the exception of *B. kerstenii*, the high value indicates the presence of a high biarmed chromosomes in the karyotype.

The serrated rayed species with 2n=48-50 have higher arm numbers (NF1 & NF2) compared with the flexible rayed species of the *B. anoplus*-complex and an unrelated *B. pallidus* analysed in chapter 4. This is indicative of the large biarm component in the karyotypes (Table 6.13). The high NF1 & NF2 values of the species analysed in this chapter are comparable to the species analysed in the three studies of African *Barbus* mentioned above. These results indicate interesting features which need to be explored for their cytosystematic and phylogenetic significance.

Interspecific comparison: sawfin barbs (2n=100)

Chromosome numbers & ploidy levels

Five serrated rayed *Barbus* species examined in this chapter have chromosome numbers of 2n=100, resembling tetraploid karyotypes.

Two sister species, *B. calidus* and *B. erubescens* have a chromosome number of 100. Both species have a low number of **a** chromosomes, comprising 6-8% the karyotypes. An unrelated species, *B. trevelyani* (2n=100) also has 8% of its karyotype comprised of **a** chromosomes. All three species have karyotypes consisting of 14% of **m** chromosomes (Table 6.14). However, the *B. trevelyani* karyotype is slightly different. It has 4-5 pairs of relatively elongated biarmed chromosomes and two pairs of small biarmed chromosomes (Fig. 6.8, Table 6.14).

Although, the numbers of uniarmed and biarmed chromosomes are comparable in these tetraploid species within the serrated-rayed group, *B. hospes* has the highest number of **a** chromosomes comprising 24-26% of the karyotype and this is unusual (Fig. 6.6, Table 6.14). In all the species except *B. hospes*, the **a** chromosomes appear similar in size, *B. hospes* has a pair of **a** chromosomes that is elongated. This is also a unique character.

One pair of **sm** chromosomes displayed in the *B. hospes* karyotype appears to be heteromorphic. However this was not observed in the other metaphase spreads examined, so this may be an artifact caused by preparing slides from previously fixed gill tissue (Fig. 6.6).

Two species, *B. andrewi* and *B. serra*, have high chromosome numbers, the former has chromosome number of 100 (this study) and the latter a chromosome number of 106 (Oellermann 1988, Appendix 2). *B. andrewi* is distinct in having the lowest number of **a** chromosomes, namely 6% of the karyotype, and the highest number of **m** chromosomes, namely 16% of the karyotype (Fig. 6.9, Table 6.14). *B. andrewi*'s karyotype pattern is also different in having six large biarmed chromosomes

and only one small biarmed chromosome (Fig. 6.9). *B. andrewi* and *B. trevelyani* have relatively elongated **sm** chromosomes compared to the sister species pair of *B. calidus* and *B. erubescens*.

The feature of high chromosome number is shared with several European *Barbus* species considered as *sensu stricto* (Guégan *et al.* 1995). Five Iberian *Barbus* species and three other species including *B. barbus*, a Centro-European species also have chromosome number of 100 (Collares-Pereira & Madeira 1990, Appendix 2). Since, there are *Barbus* in southern African waters with a total chromosome number of 100, they are compared with these European *Barbus* karyotypes.

Morphology of karyotypes

All five of the tetraploid species analysed show asymmetric karyotypes, where the karyotypes have a higher biarm component than uniarm components. In all five species the **m** chromosomes change very slightly in size and as a result, they all appear similar in size (Figs. 6.4-6.9). The most numerous **sm+st** chromosomes show a definite change from large to small chromosomes. Generally the smallest chromosomes are **sm+st** chromosomes. The first 2-4 pairs of **sm+st** and the first pair of **m** chromosomes of *B. calidus*, *B. erubescens*, *B. trevelyani* and *B. andrewi* are the largest (Figs. 6.4-6.9). Marker chromosomes in the **m** and **s** chromosome categories were reported for *B. meridionalis* and *B. plebejus* (Cataudella *et al.* 1977). However, no marker chromosomes were identified amongst the Iberian *Barbus* species (Collares-Pereira & Madeira 1990). Differences in the marker chromosomes among the heterogeneous tetraploid *Barbus* may indicate the different mechanism in chromosomal rearrangements within the population. Such diffrences could be expored using chromosomal banding techniques and may be phylogenetically informative.

NF: total number of chromosome arms

European *Barbus* with a 2n=100 chromosome complement have an NF1 value ranging from 142-178 (Cataudella *et al.* 1977, Hafez *et al.* 1978, Valenta *et al.* 1979, Collares-Pereira & Madeira 1990, Appendix 2). The southern African *Barbus* (2n=100) have NF1 values ranging from 156-170. Only one species *B. hospes* has a relatively low value of 156 for NF1, indicating relatively numerous **a** chromosomes.

Considering the **st** chromosomes as biarmed, NF2 values of the species range is 172-194. The southern African tetraploid *Barbus* have a lower uniarmed component, or a high biarmed component in the karyotype. Once again this contrasts with the European species where the karyotypes have high uniarm components (Collares-Pereira & Madeira 1990).

Meiotic chromosomes

Within *Barbus* (2n=100) there are no reported morphologically differentiated chromosomes (Cataudella *et al.* 1977, Hafez *et al.* 1978). Meiotic chromosome of the four tetraploid species examined in this chapter show only bivalent chromosomal elements (Table 6.14 & Fig. 6.9). These results are similar to that for the *Pseudobarbus* (chapter 3). The absence of tetravalents may indicate the remoteness of the polyploidy event(s) (Golubstov & Kryzanov 1993) or may suggest an allopolyploidy origin of the tetraploids.

CONCLUSIONS

The karyotype data supports the hypothesis that the serrated-spined *Barbus* are a polyphyletic group. Based on chromosome number and ploidy level at least three groups are evident 2n=48,50 & 100.

Only 16% of the 37 Zambezian *Barbus* species have been analysed in this chapter. All species artificially grouped together as having a tetraploid karyotype have the same total number of chromosomes, and similar chromosomal morphology, i.e. very few **a** chromosomes are present in the karyotype. The species grouped together as having a diploid karyotype all have the same total number of chromosomes with the exception of *B. trimaculatus* (2n=48). They all have a similar chromosome morphology, with very few **a** chromosomes. In common with numerous other cyprinid species (Klinkhardt *et al.* 1995), none of the species examined in this chapter show heteromorphic chromosomal pairs.

B. trimaculatus karyotype is distinct in not having any **a** chromosomes and 2-3 long **sm** chromosomes. *B.trimaculatus* can therefore be clearly distinguished from the other 2n=50 karyotypes examined in this study.

Both the diploid and tetraploid groups have elongated and short pairs of biarmed chromosomes.

The diploid species *B. argenteus*, *B. paludinosus* and *B. trimaculatus* have 2-3 pairs of elongated **sm+st** chromosome pairs. Only the former two has 1-2 pairs of small chromosomes. *B. trimaculatus* has no distinctly small chromosomes, indicating that translocation of chromosome arms may have resulted in the formation of the elongated pairs and with a subsequent loss of a pair of chromosomes.

The chromosome involved in translocation may be different in *B. argenteus* and *B. paludinosus*, because of the difference in large and small chromosomes. However, since homologous pairs cannot be identified at this stage, conclusive interpretation of chromosomal rearrangements can not be made with the conventionally stained chromosomes.

All the tetraploid species examined in this chapter have 3-6 pairs of elongated chromosomes and 1-2 small chromosomes. *B. hospes* is unique amongst these species, in having a pair of elongated **a** chromosomes, and not having an elongated **m** chromosome pair.

The two sister species *B. calidus* and *B. erubescens* have the same number of large and small chromosome pairs. *B. calidus-erubescens* have similar karyotype morphology which is distinct from *B. andrewi*, *B. trevelyani* and *B. hospes*. This is support for the hypothesis that *B. calidus* and *B. erubescens* are sister species.

The above five *Barbus* species and *Pseudobarbus* (chapter 4) have the same number of chromosomes, 2n=100. All these species are considered to have a tetraploid karyotype. The karyotype morphology is similar in both groups, namely fewer uniarmed chromosomes contributing to an asymmetric karyotype. The *Pseudobarbus* species also have the **sm+st** chromosome elongated and this is also found in all five tetraploid *Barbus* examined in this chapter. However, the tetraploid *Barbus* have a greater number of elongated chromosomes.

Gross characteristics of the karyotypes (2n=100, asymmetry) of the tetraploid species suggests that there has been a single tetraploidy event. Following on from this idea *B. andrewi*, *B. trevelyani*, *B. hospes*, *B. calidus*, *B. erubescens* and *Pseudobarbus* would form a monophyletic group. This relationship is not supported by morphological data (Skelton 1980, 1988) and the idea needs to be explored using a cladistic approach (see chapter 8).

Using conventionally stained karyotypes provides a means of comparing species groups but it does not provide resolution of species within a group. The data can only be used in conjunction with more distinct karyotype markers (such as AgNOR, G and/or C-banding) or morphological characters in a phylogenetic analysis to explore the relationships of these species and the redfin species (chapter 8).

CHAPTER 7: CHROMOSOME BANDING: NUCLEOLAR ORGANISER REGION (NOR)

INTRODUCTION

Cyprinid chromosomes are small structures (average cyprinid chromosome size is ± 4-6µm in length), and the centromere position changes gradually from a median to terminal position (Buth *et al.* 1991, Rab & Collares-Pereira 1995). Distinct marker chromosomes with satellites or achromatic regions are not always identified in the conventionally prepared chromosome spreads. Consequently, very few distinguishing, homologous features have been found that can be used for comparative studies.

The uniformity of chromosome morphology within the southern African *Barbus* and *Pseudobarbus* species (chapters 4-6) groups, is a general feature of cyprinids (Rab & Collares-Pereira 1995). Chromosomal structural changes (e.g. pericentric inversions, translocations, heterochromatin addition/deletions) that do not involve a change in chromosome number have been inferred in cyprinids on the basis of fundamental arm number changes (Cataudella *et al.* 1977, Gold *et al.* 1979). The analysis of *Pseudobarbus* (chapter 3) and the flexible and serrated rayed *Barbus* (chapters 4&5) karyotypes indicate also that changes have not largely involved Robertsonian translocations.

Arm rearrangements leading to chromosomal change have occurred during cyprinid evolution and evidence for this has been indirectly provided from studies employing banding (Gold & Amemiya 1986, Amemiya & Gold 1988). Specific staining procedures are available which attempt to identify homologous chromosomes by chromosome banding. The banding patterns are generally horizontal markings on the chromosomes (Sumner 1990).

The silver staining of the Nucleolar Organiser Region (NOR) on chromosomes is a banding method that has been used extensively with fish chromosomes compared to C-banding, G-banding and RE-banding.

The NORs are chromosomal sites, intimately related to interphase nucleolus formation and contain the main genes (18s and 28s) for ribosomal RNA (rRNA). The rRNA molecules are synthesised and processed in pre-ribosomes in the nucleoli, and ultimately become part of the mature ribosomes in the cytoplasm (Sumner 1990). The rRNA is the most common class of RNA in the cell and is required in large quantities, the rRNA genes are therefore present in multiple copies in all eukaryotes that have been studied (Sumner 1990).

NORs are generally detected by a silver nitrate staining method (Howell & Black 1980), and/or by using G-C specific fluorochromes namely; chromomycin A3 (Schmid 1982 in Amemiya *et al.* 1992) or mythramycin M (Mayr *et al.* 1987).

NORs are located on specific chromosomes and can therefore be used to identify homologous pairs within a metaphase spread.

Intra and interspecific NOR variations have been reported, and they provided additional cytogenetic characters for phylogenetic analysis in fishes. Phylogenetic hypotheses based on NOR

variation have been inferred in salmonids (Phillips *et al.* 1989), North American cyprinids (Gold & Amemiya 1986, Gold *et al.* 1988, Amemiya & Gold 1990, Amemiya *et al.* 1992) and Asian cyprinids (Magtoon & Arai 1993).

Ag-stained chromosomal NORs correspond to Ag-stained nuclei NORs, and have been used to determine ploidy levels, especially in experimentally induced polyploids (Phillips *et al.* 1989, Flajšhans *et al.* 1992). Whilst, this implies that the number of NORs correspond to the ploidy level, there are a few complications to the practical application of this approach (Rab, in Foresti *et al.* 1992a). In order to support the ploidy determination from metaphase chromosome counts, ploidy levels of *Pseudobarbus* and *Barbus* species were explored by examining silver stained interphase nuclei.

An independent study, to determine ploidy levels using erythrocyte diameter was also carried out (Appendix 5).

The primary aim in this chapter was to investigate the variability of NORs in the *Pseudobarbus* and *Barbus* species. Accordingly, it was necessary to identify AgNOR bearing chromosomes in the diploid and tetraploid species and to examine interspecific and generic level differences for phylogenetic purposes.

METHODS AND MATERIALS

Eighteen species were analysed for AgNOR chromosome markers.

Silver staining (AgNOR) of chromosomes

The silver staining technique of Howell and Black (1980), modified by Gold and Ellison (1983) and (Gold pers. comm.), was used to identify NOR bearing chromosomes. A summary of the method used is as follows:

Prepared slides were kept in a slide holder for two to three days to allow complete dehydration. They were then treated with silver staining solution, as outlined below.

1. Two drops of a freshly prepared, 50% wt/vol silver nitrate solution and four drops of a 2% wt/vol gelatin solution were pipetted onto a slide, and the solutions were mixed with a glass rod.
2. The slides were covered with a coverslip and placed onto a slide heater, stabilised at 40°C, for 8-12 minutes. Temperature and time are both critical components for clearly resolving NOR differentiation of the chromosomes (Rab pers. comm.). Various temperatures (from 40-70°C) and time exposures (from 4-16 minutes) for the silver staining reaction were tested to ascertain an optimum reaction time for the species examined. It was found that chromosomes were reliably stained at a temperature of 40°C for a period ranging from 10-12 minutes.
3. Once the silver solution turned deep golden to brown, the coverslip was rinsed off with distilled water. The slide was then placed in a 5% wt/vol solution of sodium thiosulphate for four minutes to stop the

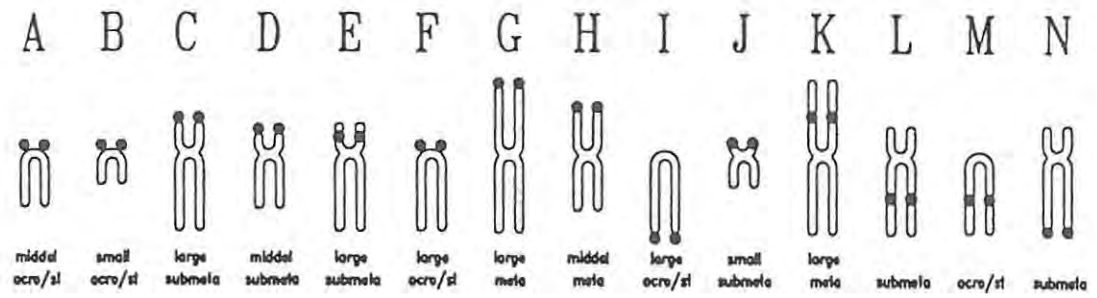
reduction of silver salts. Thereafter, the slide was rinsed with distilled water.

4. The slide was counter-stained with a 3% vol/vol Giemsa solution for 5 minutes and allowed to air dry.

5. Slides were then scanned under a compound microscope and suitable metaphase spreads were photographed.

The variation of AgNOR position on the chromosomes were recorded and the NOR bearing chromosomes categorised according to a format of letter designated phenotypes depicted by Amemiya & Gold (1988) and Klinkhardt *et al.* (1995) (Table 7.1). Characterisation of AgNOR phenotypes was based on the position of NOR on the chromosome (terminal or interstitial), centromere position of the chromosome (**m**, **sm+st** & **a**) and the relative size of the chromosome within the complement. Chromosomes size was assessed visually. A minimum of 10 well spread AgNOR metaphase spreads were examined per specimen.

Table 7.1 (from, Amemiya & Gold 1988, Klinkhardt *et al.* 1996). A diagrammatic representation of silver stained NOR chromosome phenotypes. AgNORs positions are indicated by darkened spots. AgNOR phenotypes: A, B, F, NOR is terminal on the short arms of small, medium or large **a** chromosome; I, NOR is terminal on the long arms of a large **a** chromosome; M, NOR is interstitial on the long arms of a large **a** chromosome; C, D, J, NOR is terminal on the short arms of small, medium or large **sm** chromosome; N, NOR is terminal on the long arms of a large **sm** chromosome; E, NOR is interstitial on the short arms of a large **sm** chromosome: G, NOR is terminal on the arms of a large **m** chromosome; K, NOR is interstitial on the arms of a large **m** chromosome. **sm+st** category used in this study corresponds to the **sm** category listed above.



Determination of ploidy level by Ag-staining of cells

The number of AgNORs on cells on the slides were scored to assess the ploidy level for seventeen *Pseudobarbus* and *Barbus* species.

RESULTS

The AgNOR analysed species are separated into three categories corresponding to the species groups in the chapters 3-5 (Tables 7.2-7.4).

Flexible rayed *Pseudobarbus* species

All six species of *Pseudobarbus* examined show a single pair of AgNOR bearing chromosomes (Table 7.2). The AgNOR is terminal on the short arms of medium to small sized **sm+st** chromosomes, corresponding to D phenotype (Table 7.2 & Fig. 7.1). Intraspecific level heteromorphic AgNORs were consistently found in all species (Table 7.2, Fig. 7.1).

Ag-staining of the nuclei show that 55-67% of the cells have one nuclei, and 23-32% of the cells have two nuclei, in six species of *Pseudobarbus* (Table 7.5).

Table 7.2 AgNOR chromosome phenotypes of six *Pseudobarbus* species. AgNOR phenotype classification after Amemiya & Gold (1988) and Klinkhardt *et al.* (1995). Bez=Bezuidenhouts River, Bl=Blindekloof River, B=Bainskloof, S=Swellendam, n=number of metaphase cells examined, N=number of specimens examined, *=heteromorphic AgNOR.

Species		N	n	Chromosome pairs	AgNOR position PHENOTYPE	
P. afer	Bez	4	20	1	D	J*
P. afer	Bl	3	15	1	D	J*
P. asper		3	22	1	D	I*
P. burchelli	B	2	26	1	D	N*
P. burchelli	S	2	14	1	D	L*
P. burgi		2	10	1	-	-
P. phlegethon		4	50	1	D	L*
P. tenuis		2	26	1	D	L*
P. quathlambae#		-		-		-

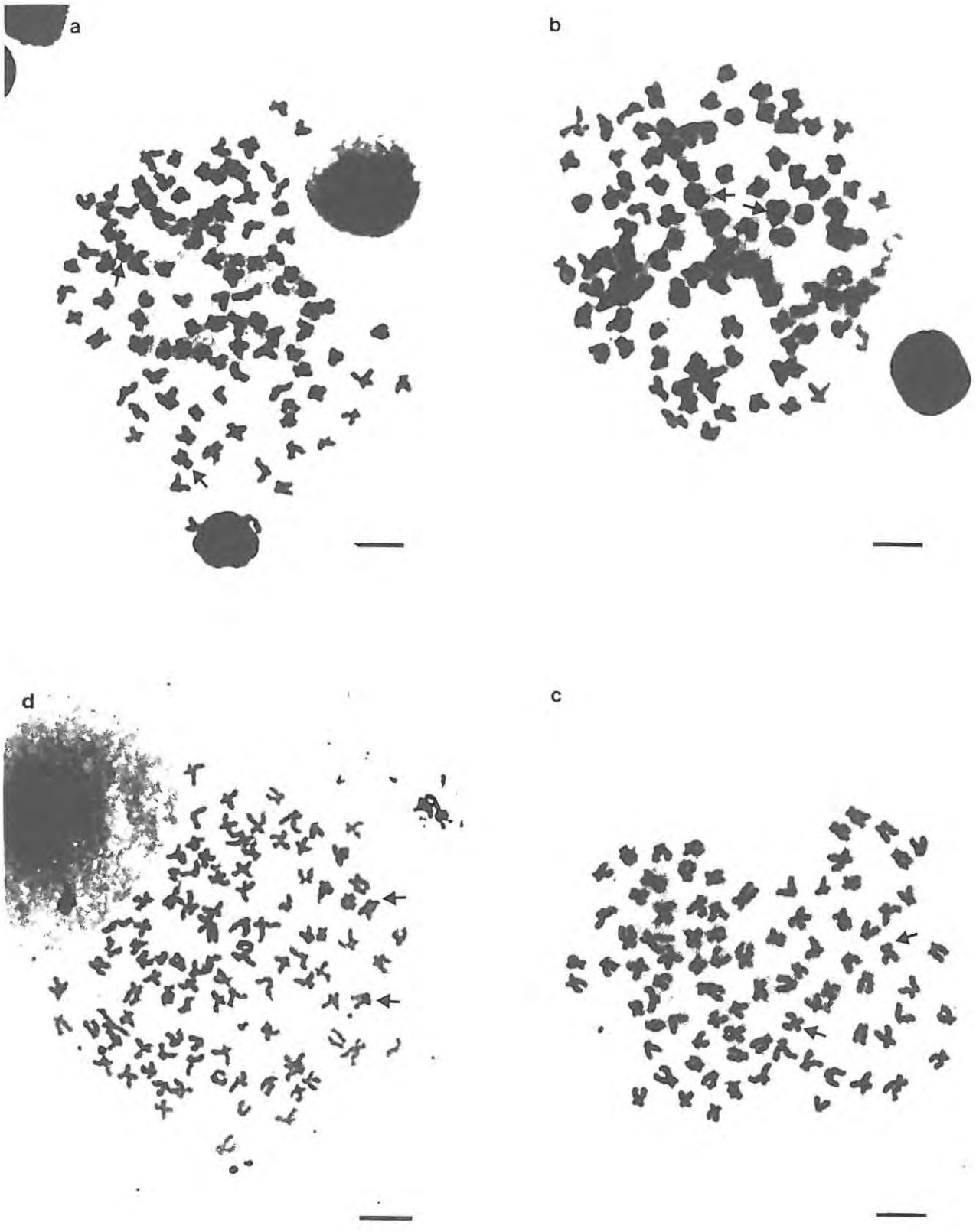


Figure 7.1 AgNOR metaphase chromosome spreads counter stained with Giemsa, from a) *P. afer*, b) *P. asper*, c) *P. burchelli and* d) *P. tenuis*, NOR phenotype D. Arrows indicate the AgNOR position. Scale bar = 5µm.

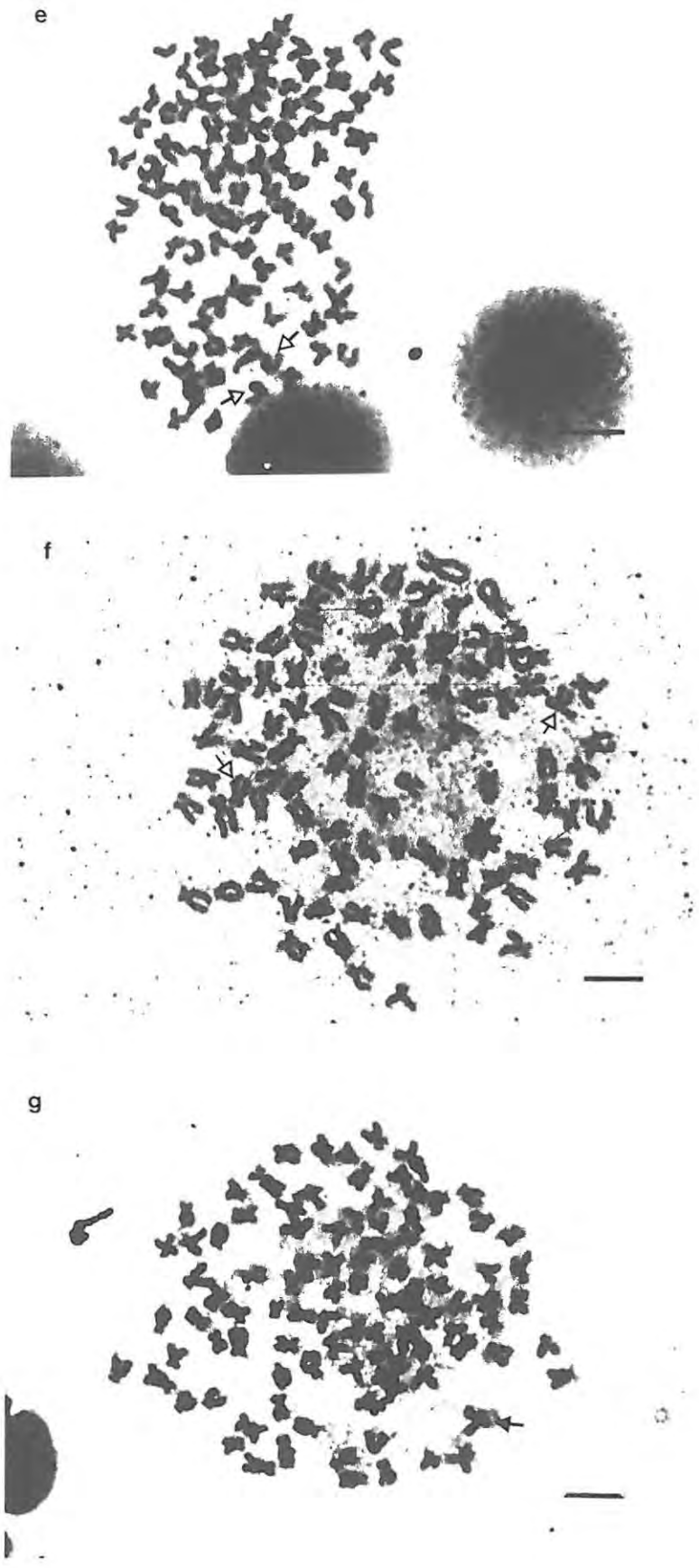


Figure 7.1 cont. AgNOR stained metaphase chromosomes spread counter stained with Giemsa, from e) *P. asper*, clear arrows indicate heteromorphic chromosomes: phenotype I; f) *P. burchelli* clear arrows indicate heteromorphic chromosomes: phenotype L and g) *P. tenuis*, arrows indicate only one pair with AgNOR. Scale bar = 5µm.

Flexible rayed species (*B. anoplus*-complex)

Four species within the *B. anoplus*-complex have a single pair of AgNOR chromosomes (Table 7.3). The AgNOR is terminal on the large to medium sized **a** chromosomes, I phenotype (Table 7.3 & Fig. 7.2). Variation in AgNOR position on the **a** chromosome in both *B. amatolicus*, *B. motebensis* and *B. gurneyi* showed that the AgNOR was present only on one arm (Fig. 7.3). A few metaphase spreads of *B. anoplus*-complex had the AgNOR terminally, on the short arms of a **sm**+**st** chromosomes (Fig. 7.3). *B. brevipinnis* has one pair of AgNORs which is terminal on the short arms of medium to small sized **m** chromosomes, corresponding to H phenotype (Fig. 7.4). *B. pallidus* was not examined.

The five flexible rayed species analysed show a dominant number of cells with only one nuclei. 86-90% of the cells have a single nuclei, 1-11% of the cells examined have 2-4 nuclei (Table 7.5).

Table 7.3 AgNOR chromosome phenotypes of the five flexible rayed *Barbus* species. AgNOR phenotypes according to classification by Amemiya & Gold (1988) and Klinkhardt *et al.* (1995). E=Elands River, P=Palmiet River, n=number of metaphase cells examined, N=number of specimens examined. *=heteromorphic AgNOR.

Species		N	n	Chromosome pairs	AgNOR position PHENOTYPE	
B. anoplus	E	4	26	1	I	D*,L*
B. anoplus	P	2	16	1	I	D*
B. amatolicus		5	46	1	I	D*
B. motebensis		3	39	1	I	D*,M*
B. gurneyi		2	15	1	I	D*,M*
B. brevipinnis		2	10	1	H	N*

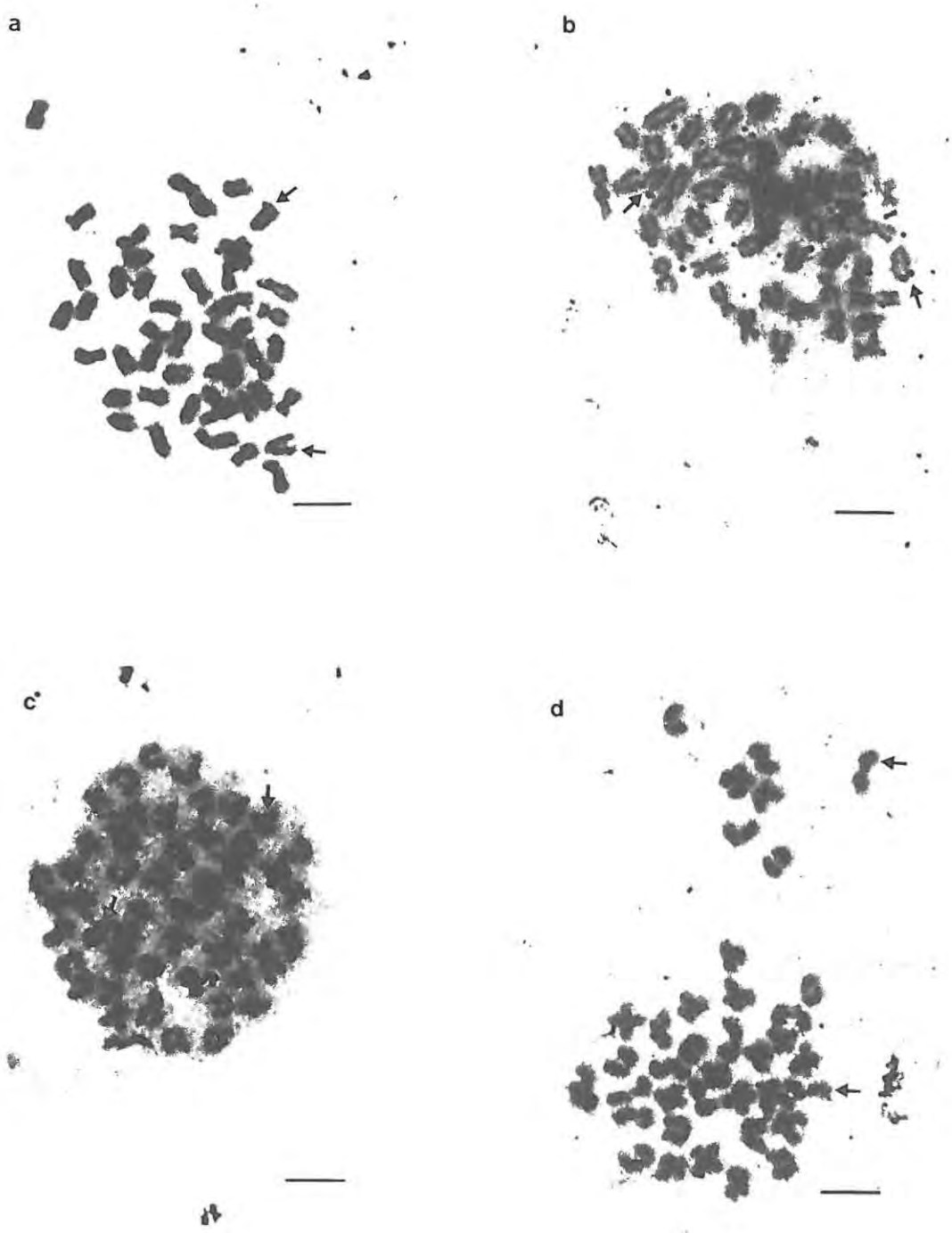


Figure 7.2 AgNOR metaphase chromosome spreads counter stained with Giemsa, a) *B. anoplus*, b) *B. amatolicus*, c) *B. gurneyi*, clear arrows indicate heteromorphic chromosomes: phenotype D, d) *B. motebensis*. Filled arrows indicate AgNOR phenotype I. Scale bar = 5µm.

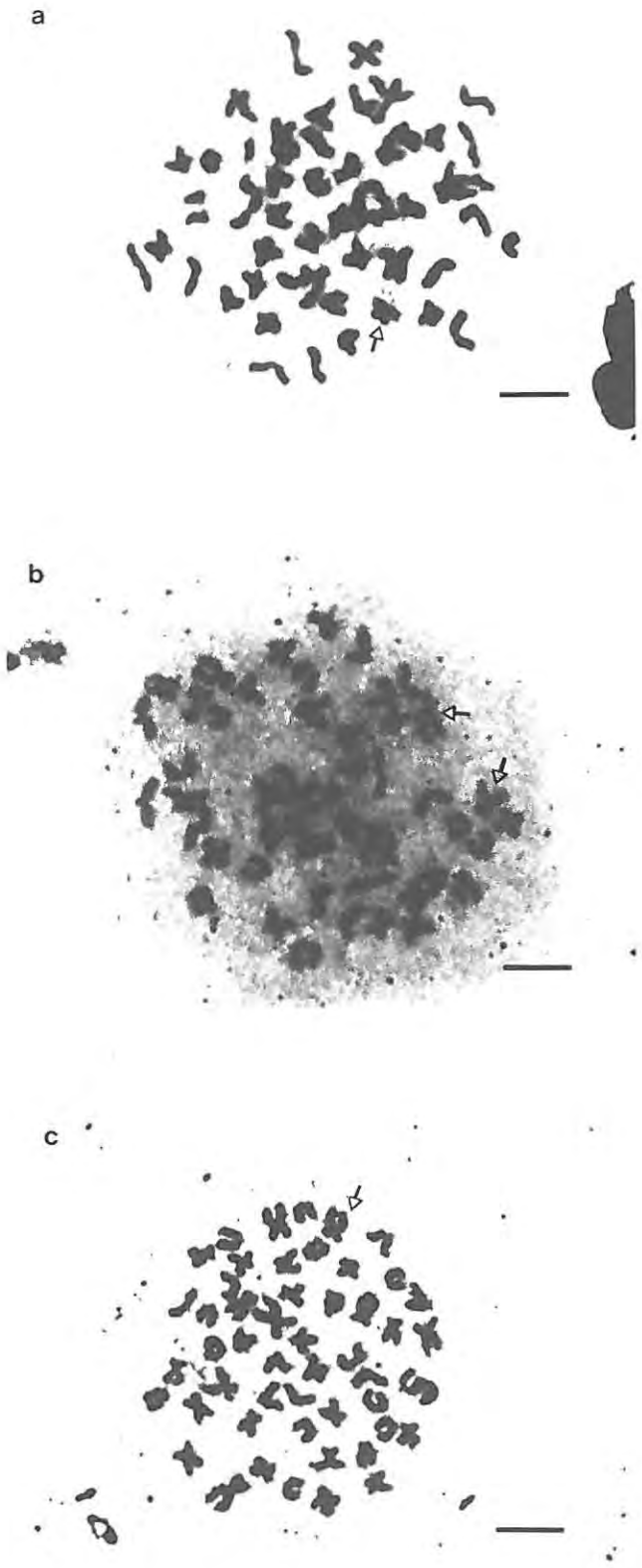


Figure 7.3 AgNOR stained metaphase chromosomes spread counter stained with Giemsa, from a) *B. amatolicus*, heteromorphic chromosome: phenotype D; b) *B. gurneyi* and c) *B. anoplus* clear arrows indicate heteromorphic chromosomes: phenotype D&L. Scale bar = 5μm.

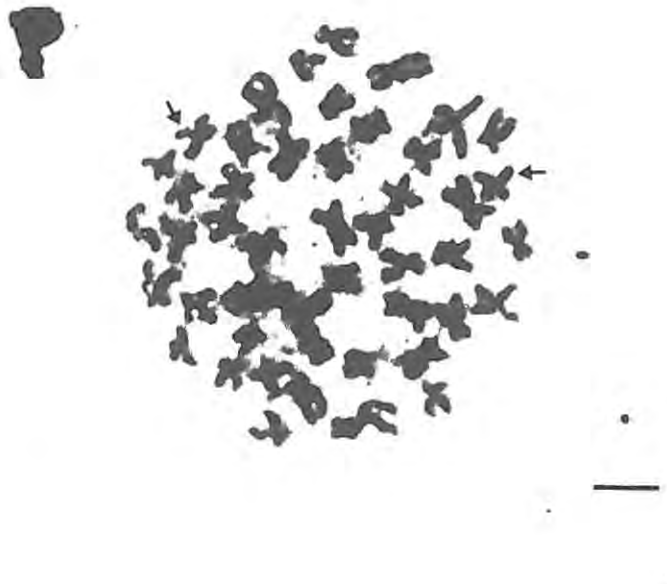


Figure 7.4 AgNOR stained metaphase chromosomes spread counter stained with Giemsa, from *B. brevipinnis*, NOR phenotype H. Arrows indicate the AgNOR position. Scale bar = 5µm.

Serrated rayed *Barbus* (tetraploid and diploid) species

A heterogeneous group of six serrated rayed species were analysed for AgNOR chromosomes (Table 7.4). Among the diploid species *B. argenteus* has two heteromorphic AgNOR chromosomes; one AgNOR pair is terminal on the short arms of a medium to small **sm+st** chromosome pair (D phenotype) (Fig. 7.5), and the other AgNOR is on the long arm of **sm+st** chromosomes (L or N phenotype) (Table 7.4). *B. trimaculatus* has one pair of AgNOR chromosomes on the short arm of a large to medium pair of **sm+st** chromosome (D phenotype) and the other AgNOR is on the long arm of **sm+st** chromosomes (L or N phenotype) (Fig. 7.5). Clear AgNOR results were not obtained for *B. eutaenia* and *B. paludinosus*.

All four tetraploid species have similar AgNOR bearing chromosomes (Table 7.4). *B. calidus* and *B. erubescens* each have one pair of AgNOR chromosomes. A AgNOR pair on the long arms of medium to small **sm+st** chromosomes, N phenotype is evident in *B. erubescens* (Table 7.4, Fig. 7.6). In *B. calidus*, the AgNOR pair was located interstitially on a medium **m** chromosome (Table 7.4, Fig. 7.6). A few metaphase spreads of *B. trevelyani* with a single AgNORs on the long arms of **sm+st**

chromosomes were observed corresponding to N phenotype (Table 7.4, Fig. 7.7). *B. andrewi* is the only tetraploid species with 2 pairs of AgNOR chromosome phenotypes (Table 7.4), the AgNORs are located terminally on a small and a medium sized **sm+st** chromosomes, D & J phenotypes (Fig. 7.8). AgNOR results for *B. hospes* are considered preliminary as NORs were not consistently observed in all metaphase spreads. Two AgNOR bearing chromosomes; terminal on the long arm of a pair of medium sized **sm+st** chromosomes (N phenotype) and another on a pair of medium sized **m** chromosomes (H phenotype) (Table 7.4). The latter has only been observed in a few of the metaphase spreads.

Three diploid serrated rayed species examined show that 89-91% of the cells have only one nucleus, and within five tetraploid species examined, *B. hospes* has a higher number of cells with two nuclei when compared to the other four species examined (Table 7.5).

Table 7.4 AgNOR chromosome phenotypes of the nine serrated rayed *Barbus* species. AgNOR phenotypes according to classification by Amemiya & Gold (1988) and Klinkhardt *et al.* (1995). n=number of metaphase cells examined, N=number of specimens examined.
*=heteromorphic AgNOR.

Species	N	n	Chromosome pairs	AgNOR position PHENOTYPE	
B. argenteus	3	15	1	D	L*,N*
B. paludinosus			unclear AgNOR		-
B. eutaenia			unclear AgNOR		-
B. trimaculatus	2	22	1	D	A*,N*,I*
B. calidus	3	18	2?	DorC	?H*,L*
B. erubescens	2	15	1	N	D*,L*
B. hospes	1	10	1	N/H	D*
B. trevelyani	4	38	2?	N	L*,N*
B. andrewi	1	18	2	D&J	-

Table 7.5 Percentage distribution frequency of nucleoli in silver stained interphase cells of southern African barbine cyprinids. * denotes tropical species, n=number of cells.

SPECIES	n	% FREQUENCY			
		1	2	3	4
Pseudobarbus afer	249	67	28	4	1
P. asper	115	76	30	7	2
P. burchelli	102	59	23	13	6
P. burgi	91	66	30	3	1
P. phlegethon	118	59	31	8	3
P. tenuis	128	55	32	10	3
Barbus anoplus	113	90	9	1	
B. amatolicus	102	88	9	3	
B. gurneyi	106	90	9	2	
B. motebensis	109	88	11	1	
*B. brevipinnis**	113	86	11	3	1
*B. argenteus**	123	91	7	27	
*B. paludinosus**	108	90	7	4	
*B. trimaculatus**	108	89	7	5	
B. calidus	109	53	34	7	6
B. hospes	132	14	64	10	12
B. trevelyani	111	49	33	12	6

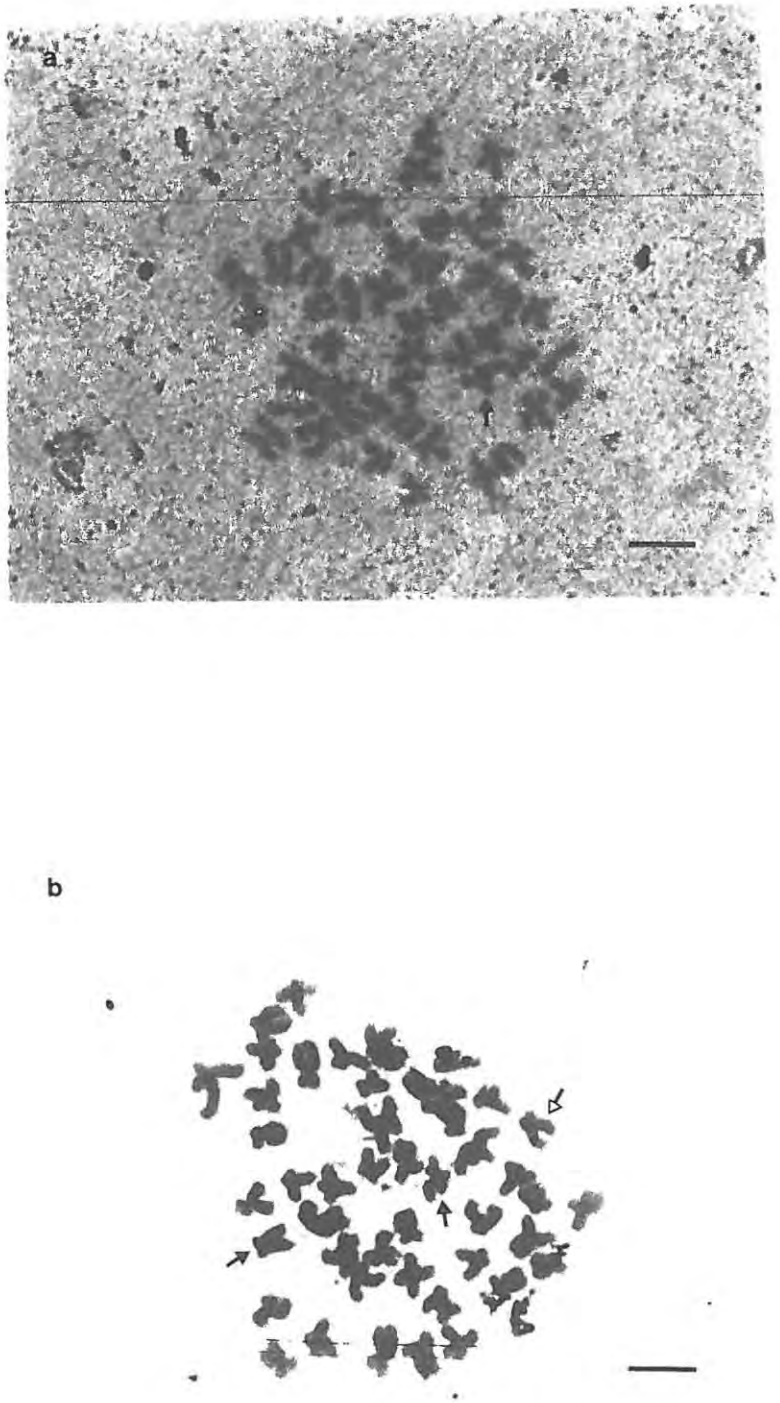


Figure 7.5 AgNOR stained metaphase chromosomes spread counter stained with Giemsa, from a) *B. argenteus* and b) *B. trimaculatus*, clear arrows NOR phenotype L/N and filled arrows indicate phenotype D. Scale bar = 5μm.

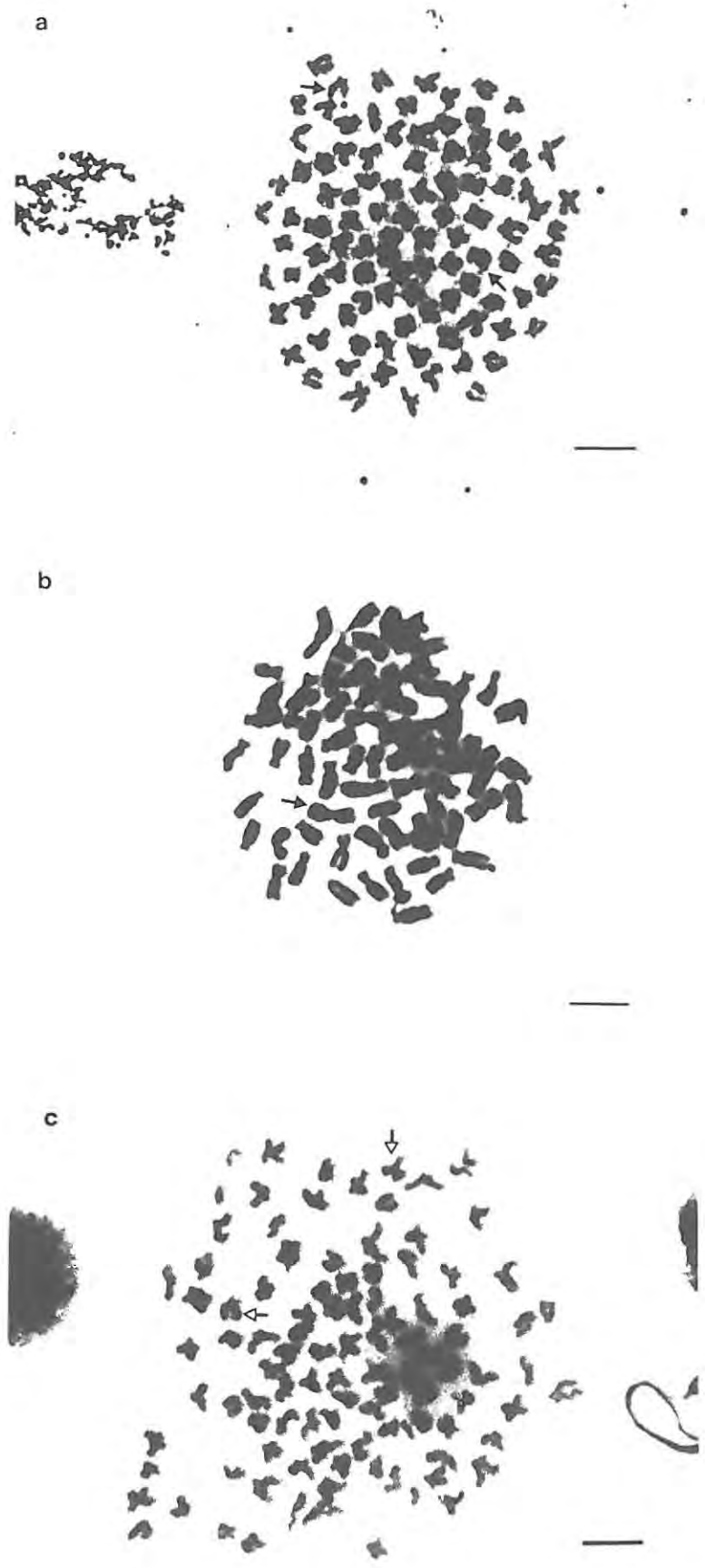


Figure 7.6 AgNOR stained metaphase chromosomes spread from counter stained with Giemsa, from a) *B. erubescens*, phenotype N and b) *B. calidus*, phenotype H. Arrows indicate the AgNOR position, c) *B. calidus* clear arrows indicate heteromorphic chromosomes: phenotype N. Scale bar = 5μm.

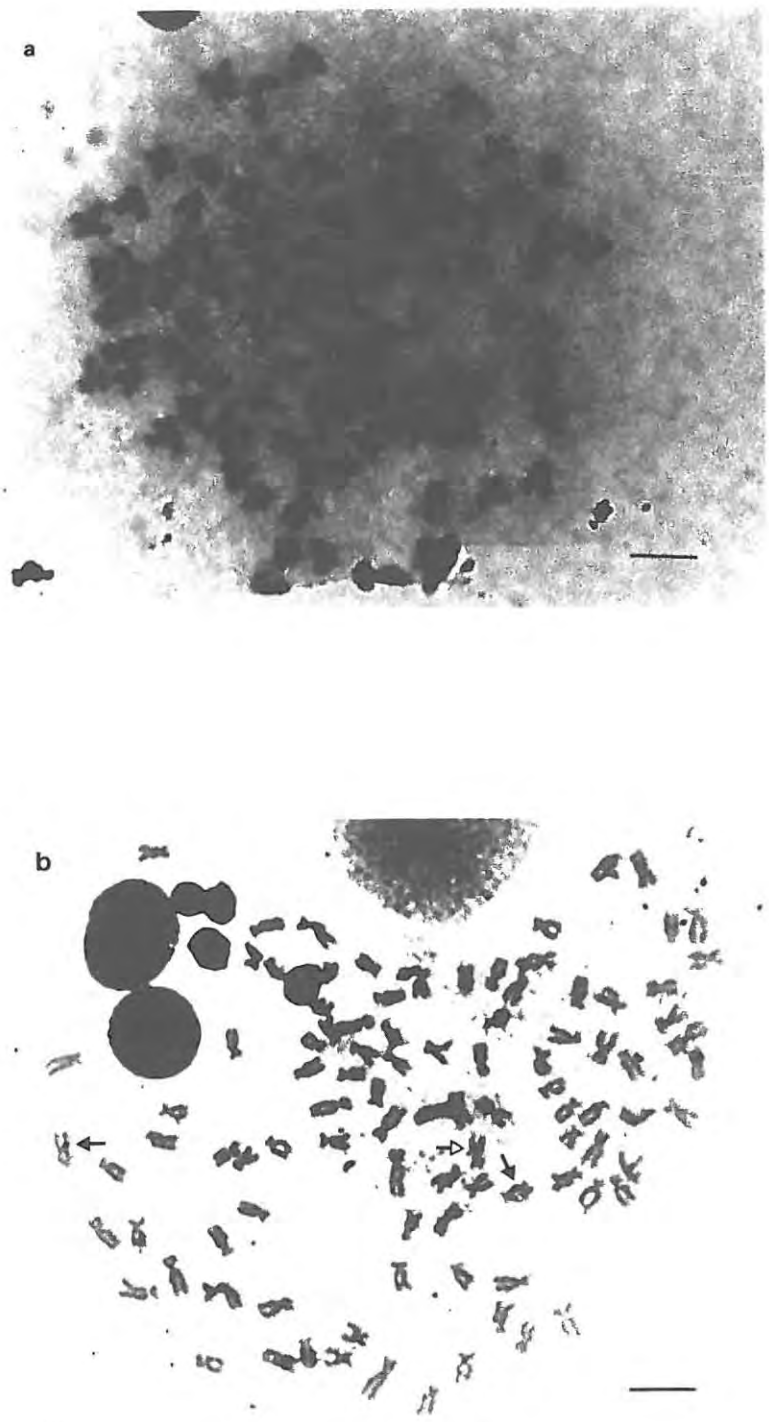


Figure 7.7 AgNOR stained metaphase chromosomes spread counter stained with Giemsa, from a) & b) *B. trevelyani*. Filled arrows indicate the AgNOR position, phenotype L, and clear arrows indicate heteromorphism. Scale bar = 5μm.

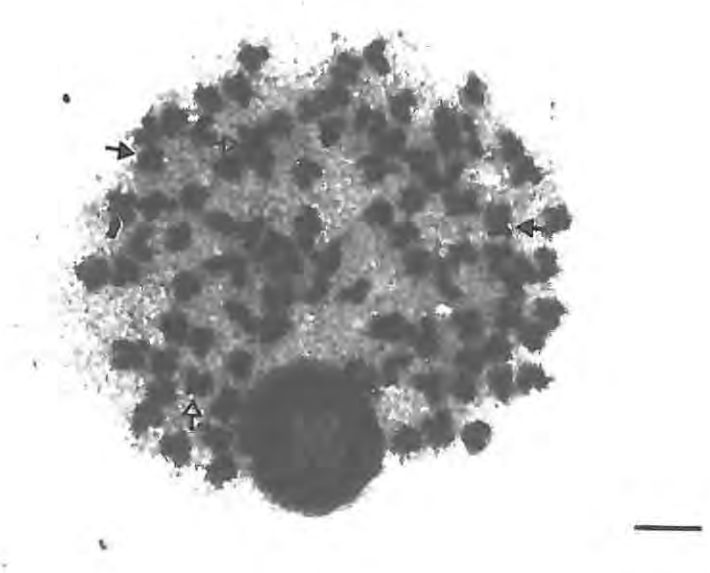


Figure 7.8 AgNOR stained metaphase chromosomes spread counter stained with Giemsa, from *B. andrewi.* Filled arrows indicate the AgNOR phenotype D, clear arrows indicate the AgNOR phenotype J. Scale bar = 5μm.

DISCUSSION

Variation of AgNOR chromosomes

Cytogenetic studies on the AgNORs of fishes have focused almost exclusively on NOR variation within and amongst species (Gold & Amemiya 1986, Amemiya & Gold 1988). Multiple NORs within diploid cyprinid species at intraspecific levels were reported, where four to six AgNORs were detected within the complement (Gold & Amemiya 1986, Amemiya & Gold 1988, Magtoon & Arai 1993). Other variation on AgNOR include AgNOR deletion, AgNOR activity heteromorphism and size heteromorphism (Gold & Amemiya 1986, Amemiya & Gold 1988).

In all the species examined in this study, intraspecific variation in AgNOR chromosomes is observed, with the exception of *B. andrewi* (Table 7.2-7.4). A high degree of heteromorphy in AgNOR

chromosomes at intraspecies level was encountered in species of the *B. anoplus*-complex and the tetraploid *Barbus*, where up to four phenotypes were recorded (Table 7.3, 7.4). Whereas all other species only 1-2 heteromorphic AgNOR phenotypes were apparent (Table 7.2-7.4).

These variations in AgNOR can be attributed to the use of silver staining. Silver does not stain NORs which were genetically inactive in the preceding interphase, nor does it stain NORs on chromosomes in late prophase I to metaphase II (Howell 1982, in Amemiya & Gold 1986). Multiple NOR phenotypes which have been detected may represent influences of such factors (Table 7.2). The detection of a single AgNOR on only one homologue has been reported for several species, e.g. *Leporinus reinhardti* (Characiformes, Anastomidae) (Galleti Jr 1984 in Feldberg *et al.* 1992), *Chaetobranchopsis australe* (Perciformes, Cichlidae) (Feldberg & Bertollo 1985) and *Curimata vittata* (Characiformes, Curimatidae) (Feldberg *et al.* 1992). The authors suggest that this may represent variation in the transcriptional activity of the rDNA cistrons, which is determined by the protein needs of the cell.

Some of the AgNOR heteromorphism observed in the metaphase chromosomes may be an artefact of the technique (Gold pers. comm.) and represent coincidental silver deposits on the chromosome arms as a result of overstaining with silver or nonspecificity of the silver stain.

Furthermore, the frequency of intraspecific AgNOR heteromorphism among fishes is high (Amemiya & Gold 1986, Moreira-Filho *et al.* 1984). The results of this analysis are therefore considered preliminary, and need to be further investigated using fluorochromes; CMA or Mithramycin A3 which stains NORs regardless of previous genetic activity (Gold & Amemiya 1986). A few interphase cells with multiple nuclei were detected indicating that silver deposits could have been arbitrary on some cells.

Determination of ploidy level nuclei show that the majority of the *Pseudobarbus* and *Barbus* species, considered as evolutionary tetraploids, have a single nucleus. This may indicate a diploidized state within species with an evolutionary tetraploid history. Conversely, the presence of two NOR sites may count as evidence against polyploidy origin (*sensu* Collares-Pereira & Coelho 1989). However, both hypotheses can be tested using a cladistic approach (chapter 8).

Description of AgNOR within cyprinids

A large number of cyprinid species analysed have terminal NORs located on the short arms of biarmed chromosomes. This is also the most common NOR position among fishes (Klinkhardt *et al.* 1995). Most (72%) of the North American cyprinids analysed have a single pair of NOR bearing chromosomes and only a few (28%) have multiple NORs (Zoch & Gold 1988, Amemiya *et al.* 1992). A large proportion of the NORs are located terminally on the short arms of biarmed chromosomes. Of the Asian cyprinid species analysed 64% possess a single NOR chromosome pair and all the species have NORs located terminally on short arms (Takai & Ojima 1986, Magtoon & Arai 1993). With a few exceptions, in most of the European cyprinids (mainly leuciscines) the NORs are located terminally on small **st-a** chromosomes pairs (Rab & Collares-Pereira 1995). Most of the species analysed in this

study have AgNORs situated terminally on the short arms of biarmed chromosomes (Tables 7.2-7.4). NOR sites, housing the genes encoding 18 and 28s RNA are important in protein synthesis (Sumner 1990). The presence of AgNOR on similar regions (i.e. terminal), on the chromosomes of the majority of cyprinids may indicate that NORs are highly conserved and are under high selective pressure. In terms of phylogenetics AgNORs may reveal deeper (generic level) patterns and can be investigated further using chromosome banding techniques to access homology within the barbines.

Number of AgNOR bearing chromosomes

Six NOR sites have been reported for a European tetraploid *Barbus meridionalis* and up to eight NORs have been detected in an African hexaploid *Barbus* (Rab *et al.* 1993, in Rab *et al.* 1995). Three west African *Barbus* (2n=50), have a single pair of AgNOR chromosomes (Rab *et al.* 1995).

The majority of tetraploid *Pseudobarbus* and *Barbus* species examined were found to have a single pair of AgNOR bearing chromosomes. Only one species, *B. andrewi* has two pairs of AgNOR chromosomes (Tables 7.2-7.4). *B. trevelyani* has a relatively high number of cells with a second pair of AgNORs. With the exception of the four diploid species of the *B. anoplus*-complex, which have the AgNORs predominantly on the **a** chromosomes, the AgNOR is found mainly on short arms of **sm+st** chromosomes. This indicates that the four species of the *B. anoplus*-complex are distinct from the remaining *Barbus* species analysed in this chapter.

Specific differences in chromosomal NOR size have been reported by Gold (1984), however no size differences in AgNOR were apparent in the species examined in this study.

Patterns of interspecific AgNOR variation among southern African barbines

This is the first study in which AgNOR banding has been explored within the southern African cyprinids. A total of seven different AgNOR phenotypes occur among the seventeen species analysed (Tables 7.2-7.4). The seven AgNOR phenotypes are distributed on a minimum of eight different chromosomes (Tables 7.2-7.4).

Most of the AgNOR positions on the chromosomes are terminal. Only two AgNOR phenotype position are interstitial (L and M phenotypes, Table 7.2-7.3, Figs. 7.1, 7.6), and these occur in the *B. anoplus*-complex species, *Pseudobarbus* and *B. argenteus*. These interstitial AgNORs may be features representing paracentric inversions and sites of chromosomal translocations.

Within the *Pseudobarbus*, the AgNOR phenotype D is dominant in all eight species analysed, with a variation of other phenotypes represented in biarmed chromosomes. The homogeneity in the D phenotype could indicate a plesiomorphic character, shared by the entire group. The presence of one pair of AgNOR bearing chromosomes may be indicative of the diploidized nature of the genome (*sensu*

Ohno 1970a).

AgNOR phenotypes within the *B. anoplus*-complex are represented mainly on the uniarmed chromosomes (Table 7.3). The AgNOR phenotype data thus provides another chromosomal character indicating the uniqueness of these four species. However, metaphase chromosomes with D and L AgNOR phenotypes were also observed and may be artefactual, this situation requires further investigation. *B. brevipinnis* is unique in having an H AgNOR phenotype. However the present data do not allow determination of its relationship to other *Barbus* species.

A majority of the serrated rayed *Barbus* species examined have the AgNORs on the biarmed chromosomes (Table 7.4). Within the tetraploid species the AgNOR phenotypes are generally different to the *Pseudobarbus* group, indicating a different pattern of chromosomal rearrangements. The sister species pair, *B. calidus* and *B. erubescens* have similar AgNOR phenotypes (Table 7.3).. Results for *B. trevelyani* are not clear with several metaphase chromosomes showing two pairs of AgNOR chromosomes; and this anomaly needs to be investigated further.

B. andrewi is unique within the set of species investigated in having two pairs of AgNOR chromosomes. This might be indicative of a relatively recent tetraploidy compared to the *Pseudobarbus* species which generally have only one AgNOR pair. It may also indicate an undiploidized site with respect to the NOR coding rRNAs.

A problem in using chromosomal NORs for systematic or phylogenetic inference stems from the difficulty in establishing chromosomal homologies among taxa. NOR banding alone does not provide the resolution necessary for determining whether a given NOR phenotype found in different species represents the same homologous character. The present data are not sufficiently refined to follow chromosomal rearrangements within the species groups and to examine interspecific relationships. For example the D phenotype is found within the *Pseudobarbus*, *B. calidus* and *B. andrewi* and it cannot be ascertained from NOR banding alone whether these are homologous chromosomes.

The AgNOR data suggest that there may have been separate tetraploidy events within the tetraploid barbines in southern Africa. These results supports the morphologically based species-assemblage separation as given by Skelton (1994).

Ploidy level by Ag-staining of cells

Determination of ploidy levels of the *Pseudobarbus* and *Barbus* displayed in Table 7.5, by examining the number of Ag-stained NORs of interphase cells.

The nuclear AgNOR counts were more successful than the AgNOR deposition on metaphase chromosomes. Sumner (1990) has observed that the amount of silver staining of interphase nucleoli is much greater than that of the metaphase chromosomes in the same preparation. He suggests that this may be taken to reflect the suppression of transcription at mitosis.

The ploidy level indications from interphase cell nuclei counts support the ploidy determination

from metaphase chromosome counts in the *Barbus* species with 2n=50. The varied ploidy determinations of interphase nuclei in the tetraploid species of *Barbus* and *Pseudobarbus* may be an indication of difference in the origin of tetraploidy in the species.
, ploidy determination in the tetraploid species of *Barbus* and *Pseudobarbus* may be an indication of difference in the origin of tetraploidy in the species.

The cytometry results (Appendix 5) corresponds with the ploidy level determination of the metaphase chromosome counts and of the Ag-stained nuclei. The results indicate that the cell nucleus diameter and volume of different species from each ploidy group are similar, and the expected increase of diameter and volume with increasing ploidy level occurs. In general terms tetraploid species show 2 fold (approximately 1.8 times) increase in cell nuclear diameter and the hexaploid species a 3 fold increase on the diploid state.

Although, this information is additional confirmation of the ploidy levels found in the southern African barbines, further support for tetraploidy can be expected from DNA sequencing studies.

CONCLUSIONS

Although, silver staining does not reveal all sites on the chromosomes that contain rDNA genes (Sumner 1990), and this makes for incomplete AgNOR characterisation of phenotypes of species, different AgNOR patterns are found amongst the heterogeneous assemblage of species examined in this chapter.

AgNOR phenotype for *Pseudobarbus* and *B. anoplus*-complex are identified as distinct for these two groups. The variation in AgNOR phenotypes among the tetraploid species suggests separate origin, and support morphologically determined assemblages (*sensu* Skelton 1994). However, the data are preliminary and need to be examined using alternative staining protocols.

The present AgNOR phenotypes do not allow intraspecific level comparison as homologous chromosomes pairs between species are not clearly identified. C and G banding provides detailed chromosome comparison and when employed in conjunction with NOR stains may allow intraspecific level comparison.

CHAPTER 8: PHYLOGENETIC RELATIONSHIPS OF *BARBUS* AND *PSEUDOBARBUS* OF SOUTHERN AFRICA USING KARYOLOGY DATA

INTRODUCTION

Cladistic analysis is a rigorous method of establishing species relationships. The approach in such an analysis is to examine shared, derived characters (synapomorphies) against a (putative) outgroup to generate the most parsimonious tree.

Phylogenetic analyses based on chromosomal data have been considered in several fish taxa: catostomids (Uyeno & Smith 1972), salmonids (Hartley 1987), notothenids (Ozouf-Costaz 1987), cyprinids (Yu *et al.* 1987, Amemiya *et al.* 1992), silurids (Rab *et al.* 1994b) and esocids (Crossman & Rab 1996).

As mentioned in the main introduction (chapter 1), *Barbus* is a dominant fish genus in southern Africa. Relationships of the southern African barbines are not well established (Skelton 1994), but as they are all "*Barbus*" *sensu lato* there is an expectation of some degree of relationship. A cladogram has been established for the *Pseudobarbus* lineage (Skelton 1980, 1988), and this provides a starting point to test the phylogenetic significance of karyological data.

The application of karyotypic data to systematics, i.e. taxonomy and phylogenetic analysis, is based on the premise that the karyotype comprises a heritable character (or character set) in each species (Chiarelli & Capanna 1973). Since karyotypes generally undergo specific patterns of chromosomal rearrangement within different evolutionary lineages (White 1977, Chiarelli & Capanna 1973), karyotypic differences between species should also be useful in phylogenetic inference.

At least three ploidy levels exist amongst the barbine cyprinids of southern Africa: tetraploids - chapters 3 & 5, diploids - chapters 4 & 5 and hexaploids (Oellermann 1988, Oellermann & Skelton 1990, Appendices 2,4).

Two theories have been proposed to account for ploidy-level differences in cyprinids. Ohno (1970a&b) hypothesised that the ancestral chromosome number is 50, because 2n=50 is common. Polyploidy on this basis is therefore considered a derived state. This argument relies on the premise that common is equivalent to primitive, which may be misleading and is considered faulty.

An alternate hypothesis proposes that the ancestral cyprinid condition is 2n=100 and that 2n=50 is a derived state (Collares-Pereira & Coelho 1989, Collares-Pereira, unpublished manuscript). In some species polyploidy (tetraploidy) may have been secondarily rederived (and in effect represent a character reversal).

The aim of this chapter is to use karyological data to test and interpret a morphologically-derived cladogram of relationships of *Pseudobarbus* and two outgroup species. One involved the outgroup suggested by Skelton (1980) and the other involved a heterogeneous group of tetraploid *Barbus* species.

I was interested in comparing all the tetraploid species (*B. calidus*, *B. erubescens*, *B. trevelyani*, *B. hospes* and *B.andrewi*) identified in this study with *Pseudobarbus*. The outgroup relationship of *B.*

calidus and *B. erubescens* was particularly relevant as both species have been excluded from the *Pseudobarbus* lineage (Skelton 1980; chapter 6).

The origin of tetraploidy within the southern African barbines is also examined in the context of the hypothesised phylogeny.

MATERIALS AND METHODS

Morphological, osteological and meristic characters from Skelton (1980) were used to construct a computer-generated cladogram for *Pseudobarbus* and outgroup species. A data matrix consisting of 44 characters and nineteen taxa (Table 8.1) was analysed with the program HENNIG-86 version 1.5 (Farris 1988). These characters were re-examined before the analysis and some were refined for the purpose of tree building (Skelton 1980, Skelton pers. comm.). A list of characters and the refined polarity for the character transformations used to construct the trees are listed in Appendix 4. The program resolves character conflicts on the basis of strict or Wagner parsimony (Farris 1988). Polarity of characters was established according to the outgroup criterion (Nixon & Carpenter 1993). *Barbus motebensis* of the *B. anoplus*-complex was designated as the outgroup (Skelton 1980).

Table 8.1 Morphological, meristic and osteological data matrix for *Pseudobarbus*, *B. anoplus*-complex and tetraploid *Barbus* species (from Skelton 1980 & Appendix 4).

B. anoplus	2001220000000000200000000111000110001010000010
B. amatolicus	2001120000000001200000000111000110001010000010
B. gurneyi	2000020000000001200000000111000110001111000020
*B. motebensis**	2000020000000001200000000111000110001111000020
P. afer	2011110010000001200002101121000210010000022110
P. asper	2011121110010001200003101121000210010100002110
P. burchelli	2010000010010101200002101121000210000010022110
P. burgi	2010100010000001200003100121000210000020022110
P. phlegethon	2011210010000001101002102121000211000010022110
P. tenuis	2011210010000001202001001121010111111000122210
P. quathlambae	2012221210000001202100023021010110110201222210
B. calidus	2010000201122012010010022000201000001010000011
B. erubescens	2010200101121222012010021000201000001210000011
B. hospes	1002000110202000010000012000001110000000022?02
B. trevelyani	1001200012201002010001110000100210001200220000
B. andrewi	0000012100223212010001120000101210000100111100
B. serra	0000012100223202010000130000101210000100111100

* indicates designated outgroup.

Data were analysed using a branch breaking (bb) option to generate the maximum number of most parsimonious trees.

Chromosomal data were not included in the tree building, to avoid circularity of argument. These data were examined independently to investigate how each character fitted the tree. The chromosomal characters are presented in chapters 4-7 and include chromosome number, karyotype components: **m**, **sm-st**, **a** chromosomes, NF values, marker chromosomes (Tables 4.11, 5.10, 6.14) and AgNOR chromosomes (Tables 7.1-7.3).

RESULTS

Six equally parsimonious trees were generated involving the three species groups; *Pseudobarbus*, *B. anoplus*-complex and a heterogeneous group of tetraploid *Barbus* (bb length=147, ci=48, ri=69; Fig. 8.1). In all six trees, morphologically-determined sister species, including *B. calidus-erubescens*, *B. andrewi-serra*, *P. burchelli-burgi* and *B. tenuis-quathlambae* are consistent and therefore considered well resolved (Fig. 8.1). A Nelsen consensus tree was generated and used to interpret the karyological data (Fig. 8.2). The relationship of the sister species *P. afer-asper* and *P. phlegethon* however, is not well resolved. The unresolved relationships are evident in the polytomies of the strict consensus tree (Fig. 8.2).

The *B. anoplus*-complex species, *Pseudobarbus* and the "heterogeneous group" of tetraploid *Barbus* were well separated into three clades (Figs. 8.1 & 8.2). Furthermore, the relationships of the heterogeneous/unrelated *Barbus* clade is well resolved compared to *Pseudobarbus* clade.

Based on the above phylogeny, the entire tetraploid *Barbus* clade can be considered as the sister group of *Pseudobarbus*.

Using chromosome number, the species groups are also well defined. *B. anoplus*-complex species all have 2n=50 and both *Pseudobarbus* and *Barbus* species have 2n=100. All trees indicate that the tetraploidy event is synapomorphic for both the *Pseudobarbus* and *Barbus* species. This scheme suggests that there was only one tetraploidy event in the *Pseudobarbus* and *Barbus* clades.

Characters which are synapomorphic for all four species of the *B. anoplus*-complex include symmetrical karyotypes, heteromorphic sex chromosomes, low NF values indicating the large number of uniarms (chapter 5) and AgNOR phenotype I (Table 7.2), are characteristic of this clade and further indicate the monophyly of this group.

Pseudobarbus and tetraploid *Barbus* species are characterised by asymmetrical karyotypes and high NF values, indicating high numbers of biarmed chromosomes. The species of tetraploid *Barbus* clade have 6-8 **a** chromosomes (with the exception of *B. hospes,* which may represent an autapomorphy, or reversal to the ancestral condition *sensu* Ohno 1970a), and *Pseudobarbus* have 8-20 **a** chromosomes. The two branches may be separated on the basis low **a** chromosome numbers (Tables 4.11 & 6.14). Chromosomal characters conform with the two branches as determined on morphological grounds.

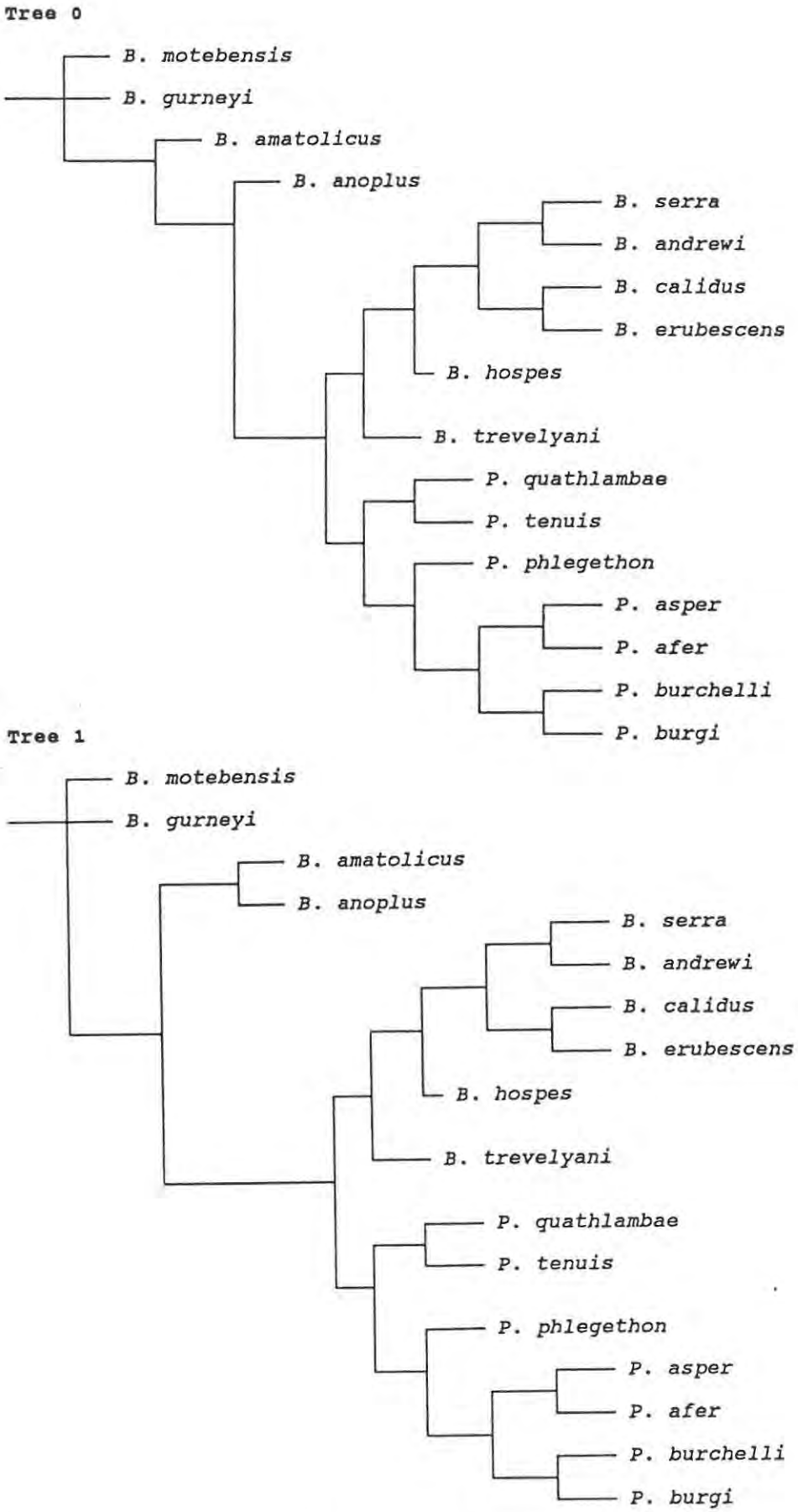


Figure 8.1 Phylogenetic hypotheses of relationships of some southern African barbines based on data from Skelton (1980, Appendix 4). Trees were generated using the Hennig86 program (Farris 1988).

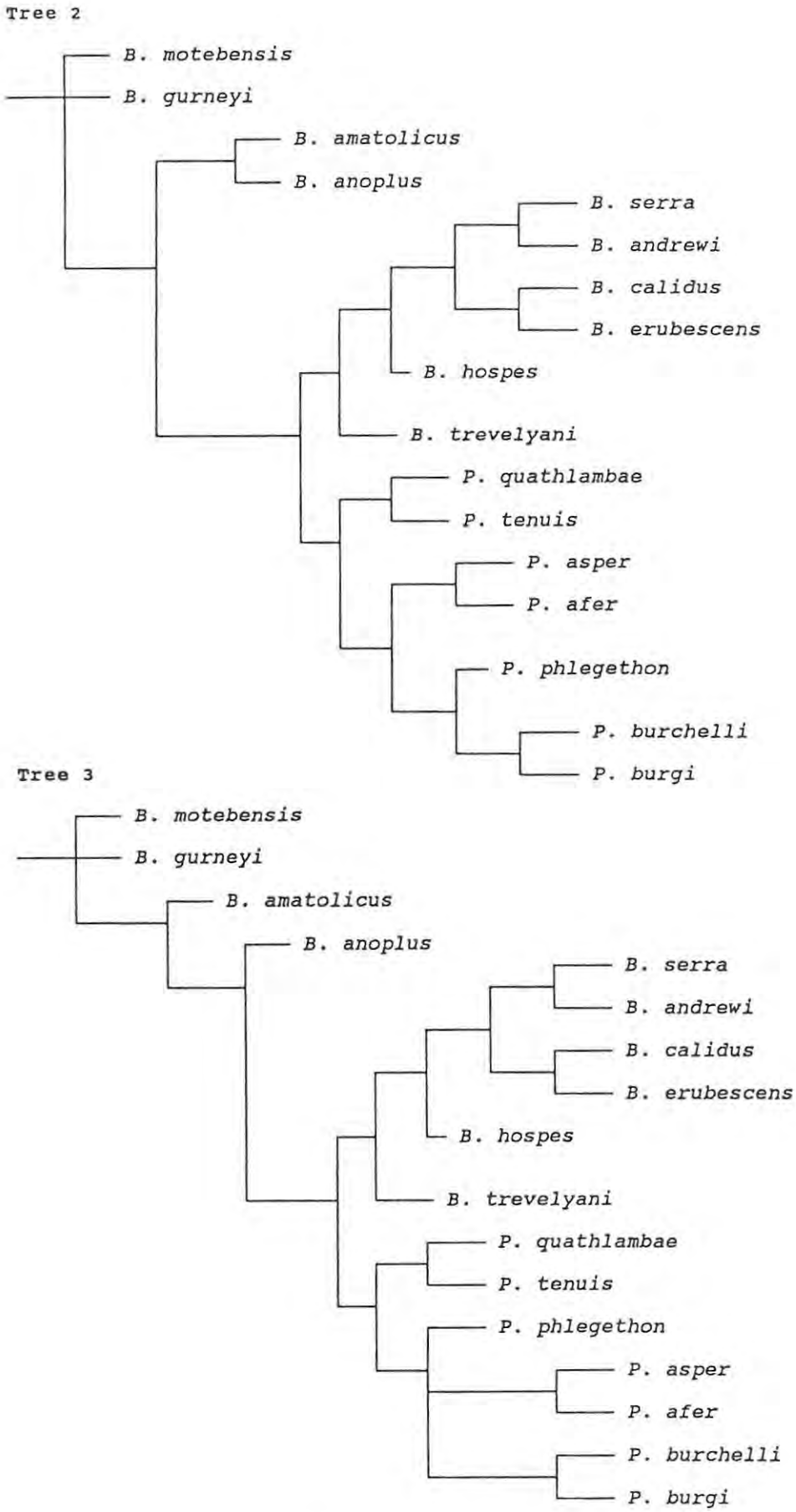


Figure 8.1 cont. Phylogenetic hypotheses of relationships of some southern African barbines based on data from Skelton (1980, Appendix 4). Trees were generated using the Hennig86 program (Farris 1988).

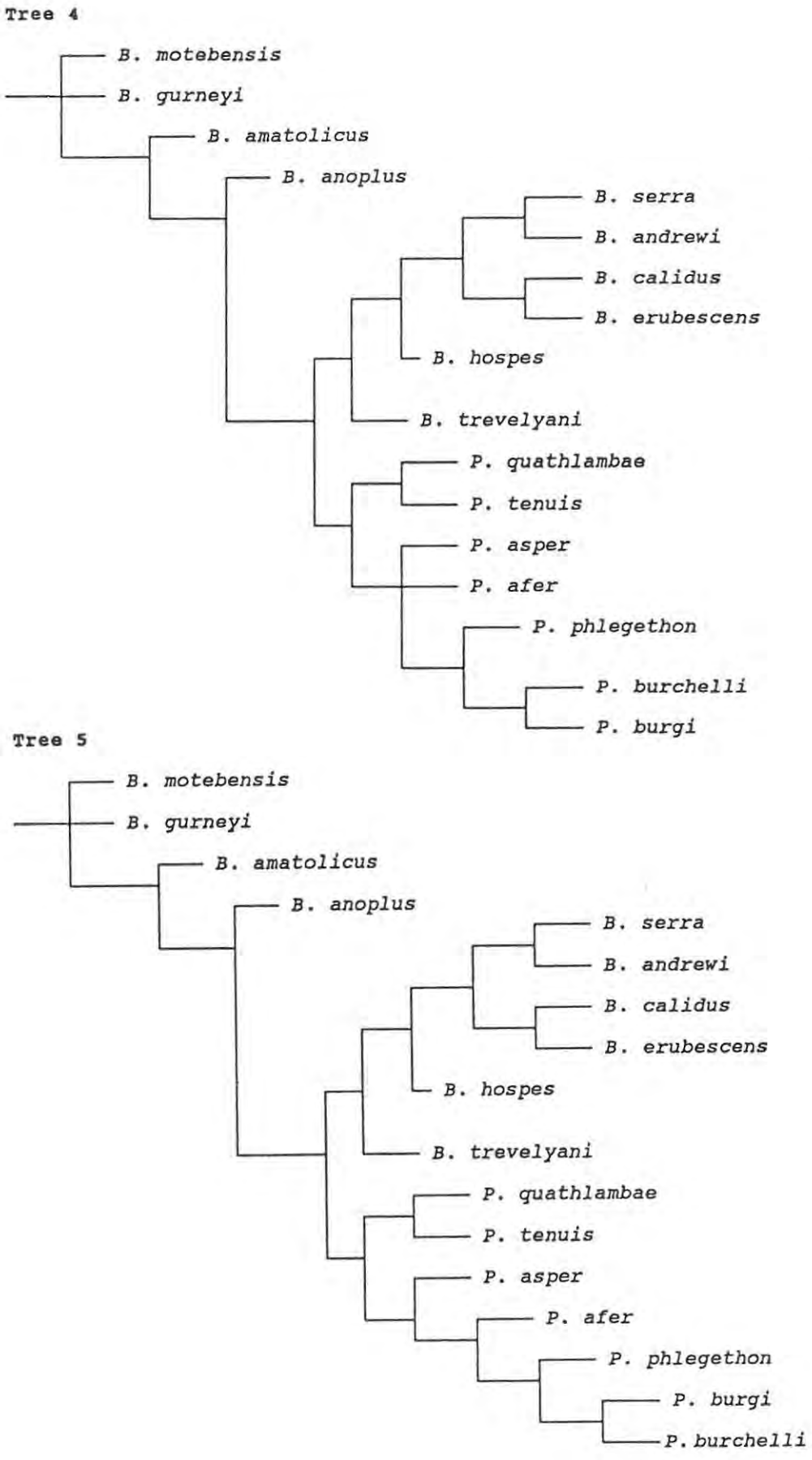


Figure 8.1 cont. Phylogenetic hypotheses of relationships of some southern African barbines based on data from Skelton (1980, Appendix 4). Trees were generated using the Hennig86 program (Farris 1988).

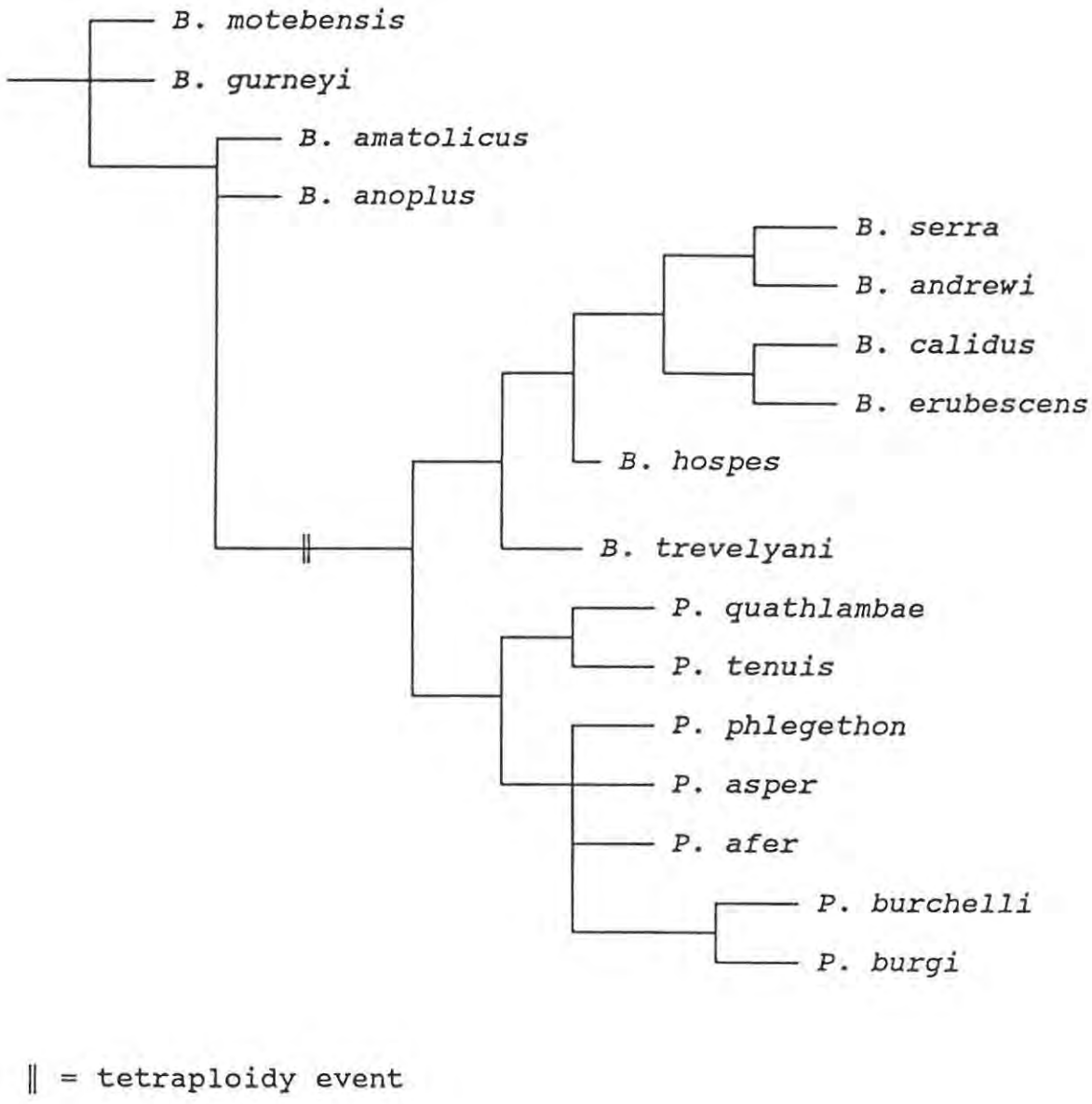


Figure 8.2. A Nelsen consensus tree for the southern African barbines showing the hypothesised tetraploidy event.

Species within the tetraploid *Barbus* clade are distinct on the basis of karyotype characters (chapter 6). For instance, all five species in the *Barbus* clade have a higher number of large chromosomes compared to the *Pseudobarbus*. An exception among all tetraploid species, *B. hospes* has **a** and **sm-st** chromosomes as marker pairs. D phenotype is represented in most of the *Pseudobarbus* species (Table 7.1). This phenotype is also recorded for *B. calidus*, *B. erubescens*, *B. trevelyani* and *B. andrewi*. However, AgNOR data are considered preliminary, as they are not sufficiently resolved for all the species considered in this discussion and the phylogenetic significance of this character is therefore not clear. The conventional karyotype data are too crude to resolve the relationships among species, but does support distinct lineage/clade level separation. This indicates that chromosomal data are useful for deeper phylogenetic resolution among the southern African barbines.

DISCUSSION

Considering *B. anoplus* as the outgroup, the sister group to the *Pseudobarbus* clade is the tetraploid *Barbus* clade. Based on morphological and osteological criteria these species were not considered to be related to the redfins (Skelton 1980). This indicates the preliminary nature of the present phylogenetic analysis with a limited number of species.

The *B. anoplus*-complex species have a symmetrical distribution of uniarm and biarmed chromosomes whereas all the tetraploids have an asymmetrical distribution of uniarm-biarm chromosomes.

Tetraploidy involves a doubling of the genome set, either through allo- or autotetraploidy. If the symmetric karyotype of the *B. anoplus*-complex undergoes autotetraploidy the ancestral tetraploids would have symmetrical karyotypes. No chromosomes were lost in the tetraploidy process as indicated by 2n=100. Rearrangements of a minimum of 10-20 chromosomes through acentric translation, centromeric shifts and pericentric/paracentric inversions would result in asymmetrical karyotypes found in the tetraploid species. This scheme suggests a non-parsimonious route resulting in an asymmetric karyotype and is therefore discarded.

Alternatively, the ancestral tetraploidy event could have been a fusion of two different genomes (allopolyploidy), where one is represented by an asymmetric karyotype and the other represented by a symmetric karyotype. Diploid species, both with flexible and serrated rays have been investigated karyologically *B. brevipinnis*, *B. pallidus* (chapter 5) and *B. argenteus*, *B. eutaenia B. trimaculatus* (chapter 6) all have asymmetrical karyotypes. The morphological and osteological characters of these species need to be studied so that they can be incorporated in the phylogenetic analysis and their relationship to the tetraploid group(s) can be examined further.

Although interspecific level comparisons using the present conventional karyotype data are not possible, AgNOR results show that only *B. andrewi* has two pairs of NOR-bearing chromosomes compared to one pair in most of the other tetraploid species. This may be evidence of incomplete

diploidization of the NOR site, i.e. incomplete diploidization of the rRNA genome (Rab *et al.* 1993, in Rab *et al.* 1995). It may also be an indication of a tetraploidy event which may have been a separate event from the single event suggested by the tree (Fig. 8.2).

The results indicate that chromosomal data are useful for examining phylogenetic hypotheses and the integrity of species complexes. However, the results also indicated that phylogenetic relationships are not fully resolved and that the analysis needs to be expanded to include other barbine species.

The present analysis does not allow clear phylogenetic level discussion on the 2n=50 as plesiomorphic *sensu* (Ohno 1970a) or 2n=100 as plesiomorphic *sensu* (Collares-Pereira & Coelho 1989, Collares-Pereira, unpublished manuscript) as the outgroup represented in the analysis were designated. However, it could be speculated that the plesiomorphic ploidy level could be an species with 2n=50 (Ohno 1970) as there are small barbines with such a complement. But, the phylogenetic analysis has to be expanded to include other species for consideration.

CONCLUSIONS

This analysis of the *Pseudobarbus* phylogenetic hypothesis, using independent chromosomal data has lead to an reconsideration or re-examination of outgroup sister species.

Chromosomal data in conjunction with traditional morphological osteological indicates a way forward for resolving interrelationships of lineages, and thereby contribute to the resolution of the genus.

The phylogenetic analysis supports the integrity of the *Pseudobarbus* clade, but the outgroup hypothesis needs to be investigated further. As the integrity of the sister group hinges crucially on the choice of outgroup, the phylogenetic analysis needs to be expanded to include other barbines, unrelated to the three clades presently analysed. At present the species relationships are not clearly resolved using either morphological or karyological data.

CHAPTER 9: GENERAL DISCUSSION

Barbus, *Labeo*, *Pseudobarbus* and *Varicorhinus* are some of the cyprinid genera represented in southern African ichthyofauna (Skelton *et al.* 1991). The genus *Barbus* comprises the dominant group, 70% of the fauna and the next largest is *Labeo* comprising 12% of the fauna (Skelton 1994).

The karyology of 25 southern African *Barbus* and *Pseudobarbus* species have been explored in this study. This represents 95% (22/23) of the temperate (Cape and Karroid) fauna and 11% (6/54) of the subtropical (Zambezian) fauna (Skelton 1993, 1994).

Although African fish cytogenetics is relatively poorly explored, standard karyotypes of numerous cyprinid subfamilies, worldwide, have been reported (Suzuki & Taki 1986, Khuda-Bukhsh *et al.* 1986, Yu *et al.* 1987, Al-Sabti 1987, Mazik *et al.* 1989, Arai & Magtoon 1991, Amemiya *et al.* 1992, Magtoon & Arai 1993, Rab & Collares-Pereira 1995, Rab *et al.* 1995). About 420-450 cyprinid species have been karyotyped (Klinkhardt *et al.* 1995, Rab pers. comm.). The majority of species have 2n=48-50 (Buth *et al.* 1991). According to Collares-Pereira & Coelho (1989) and Buth *et al.* (1991) only 8-10% of cyprinids have chromosome numbers resembling evolutionary tetraploids (ranging from 96-102). Approximately, 4% of the cyprinids have chromosome numbers resembling evolutionary hexaploids (ranging from 148-150).

Chromosome numbers of southern African and African barbines

Cytogenetic studies on the African *Barbus* have been explored only recently (Rab 1981, Oellermann 1988, Oellermann & Skelton 1990, Golubstov & Kryzanov 1993, Guégan *et al.* 1995, Rab *et al.* 1995). Of the species presented in these papers 11 species (39.3%) have 136-150 chromosomes, four species (14.3%) have 96-102 chromosomes and 13 (46.5%) have 48-50 chromosomes (Appendix 2).

The above mentioned numbers are obviously biased, because of the species targeted for each of the analyses. Nonetheless, it would appear that southern African, and African large barbines represent a heterogenous group of species with a high range of ploidy variation.

This study increases the number of southern African species karyotyped. A significant finding is that 78% (18/23) of temperate barbine species have high chromosome numbers resembling tetraploid or hexaploid complements. All seven *Pseudobarbus* species (*30.0% ; chapter 4) and six serrated rayed *Barbus* species (* 26.1%[1] ; Oellermann 1988, Appendix 2) analysed in this study have high chromosome number resembling tetraploid karyotypes including five hexaploid species (*21.7% ; Oellermann & Skelton 1990, Skelton & Naran 1994, Appendices 2,4).

The prevalence of polyploidy in this fauna (77.8% of fauna) is remarkable, but because the karyology of African barbine fauna has not been extensively explored, the high ploidy situation may not

[1] (* values represent % of temperate fauna).

be as extraordinary in African barbines as it presently appears.

Chromosome number, ploidy level and morphology

Species separated on the basis of size and scale striae (*sensu* Boulenger 1911 and Banister 1973) are distinct with respect to chromosome number. All the large *Barbus*, characterised by parallel striae are hexaploid (Oellermann & Skelton 1990, Skelton & Naran 1994, Appendices 2,4). The small-moderate sized barbines characterised by radial scale striae, and which have been classified on the state of the fourth dorsal ray, have a range of ploidy levels (Table 9.1). Within the serrated rayed species two groups are apparent: *B. argenteus*, *B. eutaenia* and *B. paludinosus* 2n=50; and *B. calidus*, *B. erubescens*, *B. trevelyani*, *B. hospes*, *B. andrewi* and *B. serra* 2n=100 (Table 9.1). Within the flexible rayed species two groups are apparent: *B. anoplus*-complex species have 2n=50; and *B. brevipinnis* 2n=48 (Table 9.1).

Table 9.1 The condition of fourth dorsal ray within radially striated barbines, *Pseudobarbus* = seven redfin species.

4th ray	FLEXIBLE			SERRATED		SPINE
2n=	100	50	48	100	50	48
	A	*S*	*A*	*A*	*A*	*A*
Taxa:	*Pseudobarbus*	*B. anoplus*	*B. brevipinnis*	*B. calidus* & *B. erubescens*	*B. paludinosus*	*B. trimaculatus*
				B. trevelyani	*B. argenteus*	
				B. hospes	*B. eutaenia*	
				B. andrewi & *B. serra*		
Fauna:	TEMPERATE		ZAMBEZIAN	TEMPERATE	ZAMBEZIAN	

A = indicates asymmetric karyotype, S = indicates symmetric karyotype.

A review of nine other African small barbine species are shown to have 2n=48-50 (Rab *et al.* 1995). Both serrated rayed species (three species have 2n=50) and flexible rayed species (two have 2n=50 and three have 2n=48) are represented in the study mention above (Skelton pers. comm.). From the karyological data it seems that the state of the 4th dorsal ray (simple/serrated) may have had multiple origins or reversals. In other words the flexible and serrated rayed barbines are both polyphyletic.

The relevance of karyotype distribution of a few 2n=48 compared to many 2n=50 is unclear at present (Table 9.1), and needs to be explored further to interpret its systematic implication in the barbine fishes.

Karyology of cyprinids groups

Karyotypes of southern African barbine cyprinids, which have diploid, tetraploid and hexaploid karyotypes, resemble other cyprinid karyotypes in having small chromosomes with centromere positions ranging gradually from a median to terminal position.

North America conventional karyotype studies, show fairly homogeneous karyotype patterns among the species assemblages (Buth *et al.* 1991, Amemiya *et al.* 1992). Karyotypes have been used to identify species assemblages. Magtoon & Arai (1989) reviewed Asian cyprinids in the genus *Puntius sensu lato* and recognised four groups within the karyotyped species: (1) 2n=48, and low NF values 52-54; (2) 2n=50 and NF=more than 82; (3) "Capoeta" species 2n=50 and NF=54-58; (4) "Capoeta" species 2n=50 and NF=82-98. Karyological studies of the European cyprinids show similar resolution of karyotype based assemblages (Rab & Collares-Pereira 1995).

In the present study gross karyotype patterns have, in some instances, confirmed species groups established on morphological characters (chapters 4-7). Species within an assemblage or lineage are characterised by similar chromosome features such as chromosome number, ploidy level, karyotype morphology and AgNOR phenotypes.

Including the results of Oellermann & Skelton (1990) and (Appendix 2), with the present study, a minimum of seven groups can be identified; (1) *Pseudobarbus*, 2n=100, high NF values, (2) *B. anoplus*-complex, 2n=50, low NF values, symmetric karyotypes and heteromorphic chromosomes, (3) *B. brevipinnis* 2n=48, high NF values, asymmetric karyotype, (4) *B. trimaculatus*, 2n=48, no **a** chromosomes, (5) *B. argenteus* and *B. paludinosus* 2n=50, high NF values, (6) up to four morphologically defined lineages represented *Barbus* 2n=100, high NF values, variable number of marker chromosomes and AgNOR phenotypes (7) yellowfishes (including *B. marequensis*), 2n=150, high NF values. In order to assess the integrity and phylogenetic relationships among these karyological groups, they should be examined using a cladistic approach i.e. parsimony analysis on the basis of synapomorphies.

The karyotype patterns of southern African tetraploid *Pseudobarbus* and *Barbus* species are distinct with fewer (4-20) uniarm components and fewer (2-4) AgNOR chromosomes when compared to the European tetraploid *Barbus* species that have a relatively higher (28-46) uniarm components and one species has six NOR chromosomes (Collares-Pereira & Madeira 1990, Klinkhardt *et al.* 1995, Rab *et al.* 1993, in Rab *et al.* 1995). Biochemical (allozyme) (Agnèse *et al.* 1990b, Berrebi *et al.* 1990) and karyological studies (Golubstov & Kryzanov 1993, Rab *et al.* 1995 and Guégan *et al.* 1995) have been used to propose that the *Barbus sensu stricto* encompasses North African and Iberian-European species with 2n=100-150 (Guégan *et al.* 1995, Rab *et al.* 1995 and Machordom *et al.* 1995). A comparative investigation of the tetraploids would be an interesting, and may contribute to the *sensu strict/sensu lato* barbine separation.

Karyotypes of Zambezian (Tropical) and Temperate fish fauna

Within the small barbines characterised by 2n=48-50, two karyotype morphologies are apparent, which coincides with the biogeographic ichthyofaunal distribution. Four species of the *B.anoplus*-complex and *B. pallidus* of the temperate fauna have symmetrical karyotypes whereas four species with Zambezian affinities have asymmetrical karyotypes (Table 9.1). Species with Zambezian affinities, analysed from Ethiopia, Guinea and Angola, all have asymmetric karyotypes (Rab 1981, Golubstov & Kryzanov 1993, Rab *et al.* 1995, Appendix 2). This aspect needs to be investigated further and might confirm the idea that the temperate barbines are distinct from other tropical species (*sensu* Skelton 1986, Skelton pers. comm.)

Phylogeny

In chapter 8, phylogenetic trees based on cladistic principles were erected using morphological data and compared to the chromosome characteristics of *Pseudobarbus*, *B. anoplus*-complex and the tetraploid *Barbus.* Very few studies have examined the intraspecific relationships of African *Barbus* species using a cladistic approach.

The phylogenetic analysis supports the monophyly of *Pseudobarbus* and *B. anoplus*-complex, and suggests that all the tetraploid *Barbus* form a distinct clade. Based on karyological data, the sister group relation of *Pseudobarbus* to *B. anoplus* is not supported, but the tetraploid *Barbus* are suggested as the sister group.

Only the two outgroups, *B. anoplus*-complex and the tetraploid *Barbus*, were considered in this analysis. In order to resolve the identity of the sister group the analysis needs to include other outgroup taxa, such as *B. brevipinnis*, *B. argenteus*, the hexaploid *Barbus*, and other cyprinids such as *Labeo*, *Varicorhinus* or *Opsaridium*.

This study shows that interspecific level comparison is not possible with conventional karyotype data. In part the problem is the identification of homologous chromosome pairs for comparative purposes. Attempts to use a simple AgNOR marker yielded inconclusive results because of the inherent features of this method. The problem may resolved by using selective chromosome banding techniques, especially of the marker chromosomes. The study does indicate that the karyological data may be useful for generic level resolution within the barbines.

Another, problem is that compared to traditional morphological analyses, conventional karyotype analysis of barbines do not yield high numbers of characters for phylogenetic analyses. However, phylogenetic analysis using both morphological and karyological data, as done in this study, can provide alternative perspectives.

NF - Karyotype evolution

It has been suggested that the African (small) *Barbus* may be close relatives to the diploid southern Asian *Puntius* (Golubstov & Kryzanov 1993, Rab *et al.* 1995). The chromosomal evolution in the genus *Puntius* may be associated with chromosome rearrangements involving a decrease in NF value, whilst retaining the diploid chromosome number 2n=50 (Suzuki & Taki 1986). When using a similar approach to compare the southern African *Barbus* karyotypes; *B. brevipinnis*, *B. paludinosus*, *B. argenteus* including *B. trimaculatus* have high NF values (chapters 5 & 6), whilst members of the *B. anoplus*-complex have lower NF values (chapters 5). However, the relationships between small, serrated and flexible rayed barbine species are as yet not resolved and any evolutionary interpretation based on NF values would be premature.

Sex and polyploidy

Complex reproductive mechanisms in polyploid frogs have been recorded; parthenogenetic tetraploids, gynogenetic triploids, unisexual reproduction, natural hybrids between diploid-tetraploids (Gerhardt *et al.* 1994, Bogart pers. comm.).

Polyploidy as shown in cyprinids is complex and may indicate different origins due to the diverse reproductive mechanisms found in the taxa (Rab & Collares-Pereira 1995). Variant sex reproduction mechanisms include: triploid-diploid complexes in the European genus *Leusciscus* (Collares-Pereira, 1985); triploidy in evolutionary tetraploid species (eg *Carassius auratus*) (in Rab & Collares-Pereira 1995); spontaneous triploidy occurring in both evolutionary diploid-tetraploid and hexaploid populations (Rab & Collares-Pereira 1995); normal bisexual reproduction in evolutionary hexaploids (Oellermann & Skelton 1990, Golubstov & Kryzanov 1993, Rab *et al.* 1995); ancestral tetraploidy represented in many cyprinines, barbines and schizothoracins (Rab & Collares-Pereira 1995).

Within *Pseudobarbus* and *Barbus* species reproduction is via normal sexual reproduction (Skelton pers. comm.). No evidence of variant reproductive forms have been detected in the species examined in this study, indicating that the tetraploid species reproduce via a normal bisexual means. This may imply that the tetraploidy is very ancient. Furthermore, remoteness of the polyploidy event is indicated by the presence of bivalents in the meiotic chromosomes. A similar conclusion was reached for tetraploid schizothoracins (Mazik *et al.* 1989) and hexaploid *Barbus* (Golubstov & Kryzanov 1993).

Karyotypic evolution: origin of polyploidy

The origin of the *Pseudobarbus* and *Barbus* tetraploids is still unclear. Comparison of the karyotype morphology of the proposed ancestors (symmetric) and the tetraploid groups (asymmetric)

indicate that the sister group species need to be reviewed and the phylogenetic analysis needs to be extended to other species.

Within the temperate ichthyofauna examined in this study 13 species are tetraploid (Oellermann 1988, Oellermann & Skelton 1990, this study). Among the few (17 species) Zambezian species analysed there have been no tetraploids recorded (Rab 1981, Golubstov & Kryzanov 1993, Guégan *et al.* 1995, Rab *et al.* 1995).

Three species, *B. paludinosus*, *B. trimaculatus* and *B. brevipinnis* are most probably members of species complexes, and have a widespread distribution in Africa (Skelton *et al.* 1991, Bills pers. comm.). It is reasonable to speculate that the karyotypes of the species within these complexes will be similar to the three analysed in this study, *viz.* 2n=50 with an asymmetric karyotype. Based on this pattern of tetraploid and diploid separation (where 56.1% of fauna are tetraploid), it is most probable that the tetraploidy event/origin occurred in a temperate ancestor of the *Pseudobarbus* and *Barbus*. Ploidy level changes may be induced by environmental factors (Yu *et al.* 1987). It is feasible that natural polyploidy could have been induced in the region of faulting and upliftment where natural temperature fluctuations are expected to have occured in the environment. The Cape Fold mountains underwent upliftment and faulting during the breakup of Gondwanaland. The *Pseudobarbus* are endemic to some of the drainage systems of these mountains. It is speculated that the polyploid event occurred in southern Africa fauna. The nature of the tetraploidy via allo or autotetraploidy is more difficult to interpret with the present data.

Fish size and ploidy level

Based on chromosomal data Golubstov & Kryzanov (1993), observed a difference in the large and small *Barbus* ploidy levels, where large *Barbus* (including *Varicorhinus*) have high ploidy levels represented by a hexaploid karyotype and small *Barbus* are represented by a diploid chromosome number. Considering further the chromosomal data presented by Oellermann & Skelton (1990) and reports of high ploidy levels revealed by allozyme data (Agnèse *et al.* 1990b, Berrebi *et al.* 1990), they proposed that the common ancestry of the large and small *Barbus* depends on finding an intermediate tetraploid species in Africa. The results in this study reveal at least two (possibly three) barbine groups which are tetraploid (*Pseudobarbus*, *B.calidus-B. erubescens*, *B. andrewi-B. serra*). Although this is an interesting situation, it is not within the scope of this study to examine the phylogenetic implication of the southern African hexaploids (Appendix 2), Ethiopian hexaploids and the tetraploids (chapters 4 & 6).

The results of this study also indicate that higher ploidy levels are also represented in small barbines.

FURTHER RESEARCH

The interpretation of traditional taxonomic characters with cytogenetic characters, as explored in this study, suggest interesting and useful possibilities for phylogenetic relationships of barbine species. Investigations at both levels need to be expanded to allow for a more thorough comparative analysis of species relationships.

The cytological investigation needs to incorporate specific NOR banding protocols to establish the range of NOR phenotypes within the *Barbus* and *Pseudobarbus*. Banding (G and C) methods which identifies homologous chromosome pairs and thereby allows for a more rigorous analysis of karyological data for would be useful for phylogenetic interpretations.

Further cytological studies should also consider examining species with a wide distribution and/or disjunct distribution, from different location for intraspecies variations.

The phylogenetic analysis of the redfin and tetraploid barbines and its polyploid origin is as yet not fully resolved and represents an exciting challenge to be solved.

There is no distinct reduction in chromosome numbers for indication of the Robertsonian translocation. In the species with same chromosome numbers pericentric inversions, translocations after the tetraploid event, seems to have happened. Changes in the chromosomes can only be exposed using banding studies or DNA sequencing. This will allow a better understanding of how evolution has taken place in these species. An alternative database using DNA sequencing information would also allow another dimension of species level comparisons to be made.

CONCLUSIONS

This study represents an important step forward for the karyology of southern African barbine cyprinids.

Within the barbines examined at least three ploidy levels are shown, diploid, tetraploid and hexaploid. These karyological ploidy level differences have been shown to have important implications to the taxonomy and systematics of the genus.

Southern African barbines have very interesting karyotypes which can contribute to an understanding of species-complexes and their phylogenetic relationships. The small barbines have been separated on the basis of the nature of dorsal fin ray, although this division is not supported by karyological data. However, a suite of morphological features characterising species lineages, are well supported by karyological data. For instance the monophyletic clades of *B. anoplus*-complex and *Pseudobarbus* are supported by synapomorphic chromosomal characters.

Improved karyological techniques have greatly enhanced the information derived from standard karyotypes in this study. The use of kidney tissue, prestimulated with yeast, and exposed to only one and a half to two hours with colchicine yielded high numbers of metaphase cells from the small barbine

fishes. The slide preparations were improved considerably by centrifuging the cell suspension as much of the cellular debris was removed.

Visual analysis of metaphase photomicrographs yielded reliable karyotype information and allowed general comparison of species karyotypes. However, the problems of classifying polyploid cyprinid chromosomes, particularly (**sm-st**) categories were encountered, and this aspect need to be examined more thoroughly.

Chromosomal analysis of all seven *Pseudobarbus* species have been shown to have high chromosome numbers, indicating the evolutionary tetraploid state of the lineage. Comparative phylogenetic analysis with proposed outgroups supports the monophyletic hypothesis for the lineage by Skelton (1980). However, the refinement of interspecific level relationships are not possible with the present data set from conventionally stained chromosomes.

The phylogenetic analysis presented in this study indicates that tetraploidy may be a derived feature in two lineages from a diploid ancestor. The tetraploidy event may be remote (ancient) in these taxa. However, other cyprinid taxa need to be incorporated in the phylogenetic analysis to establish sister group relationships.

Similarity of the karyological features of the four species comprising the *B. anoplus*-complex clearly supports the morphological basis of that assemblage. Using karyological data *B. brevipinnis* is shown as a distinct flexible rayed small minnow, from the *B. anoplus*-complex. *B. pallidus* data indicates a closer affinity of the species to *B. anoplus*-complex. However, the results presented are preliminary and this affinity needs to be reinvestigated.

Karyological data suggests that the *B. anoplus*-complex species are not a likely sister group to the *Pseudobarbus*. Rather the data indicates that the search be extended to incorporate other cyprinid species.

Using karyological information the heterogeneous group of serrated rayed *Barbus* show two ploidy levels, 2n=48-50 and 2n=100. All the species analysed have asymmetric karyotype morphologies. However, there are distinct karyotype features that are peculiar to the species. Some of the barbine species have extensive distribution and are part of species assemblages. Karyotype data may be useful to identify such species assemblages. The karyotype features of the tetraploid five *Barbus* from conventionally stained chromosomes are similar and are therefore difficult to use for interpreting intraspecies relationships. The karyological examination needs to be extended to include more species.

Morphologically based phylogenetic analysis indicates that the tetraploid species are derived from a diploid ancestor. The phylogenetic analysis also indicates that the *Barbus* tetraploid clade is more closely related to the *Pseudobarbus* than *B. anoplus*-complex species. The interpretations from this data is that karyological data is providing alternative ideas for species relationships which were based on morphological and osteological parameter. However, the resolution is restricted, and both the phylogenetic and karyological investigations need to be extended to include other species.

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CHAPTER 11: APPENDICES
Appendix 1

Chromosome measurement for *B. anoplus* (Elands River). (r) = L/S arm-ratio values and (l) = chromosome length (l) calculated according to Levan *et al.* (1964), N=number of chromosomes, A-G= indicates metaphase cells n=7, CHR= chromosome categories.

	CHR	A		B		C		D		E		F		G	
n		l	r	l	r	l	r	l	r	l	r	l	r	l	r
1	a	3.6	35	3.4	33	4	39	3.2	31	3.7	36	6.1	60	3	29
2		3.55	34.5	3.25	31.5	3.9	38	3.1	30	3.5	34	5.9	58	2.9	28
3		3.3	32	3.25	31.5	3.9	38	3	29	3.45	33.5	5.7	56	2.9	28
4		3.3	32	3.2	31	3.8	37	2.95	28.5	3.35	32.5	5.4	53	2.8	27
5		3.25	31.5	3.15	30.5	3.8	37	2.9	28	3.25	31.5	5.3	52	2.8	27
6		3.2	31	3.1	30	3.7	36	2.8	27	3.25	31.5	5.3	52	2.8	27
7		3.2	31	3.1	30	3.7	36	2.8	27	3.2	31	5.3	52	2.75	26.5
8		3.2	31	2.9	28	3.6	35	2.75	26.5	3.2	31	5.2	51	2.7	26
9		3.1	30	2.85	27.5	3.6	35	2.7	26	3.2	31	5.2	51	2.7	26
10		3	29	2.8	27	3.55	34.5	2.7	26	3.1	30	5.1	50	2.7	26
11		3	29	2.8	27	3.45	33.5	2.65	25.5	3.1	30	5.1	50	2.6	25
12		2.95	28.5	2.8	27	3.45	33.5	2.65	25.5	3.1	30	4.9	48	2.6	25
13		2.9	28	2.8	27	3.4	33	2.65	25.5	3.05	29.5	4.9	48	2.45	23.5
14		2.85	27.5	2.8	27	3.4	33	2.6	25	3	29	4.9	48	2.45	23.5
15		2.85	27.5	2.75	26.5	3.4	33	2.6	25	3	29	4.7	46	2.35	22.5
16		2.8	27	2.75	26.5	3.4	33	2.6	25	3	29	4.7	46	2.3	22
17		2.8	27	2.7	26	3.35	32.5	2.6	25	3	29	4.7	46	2.3	22
18		2.8	27	2.7	26	3.3	32	2.55	24.5	2.9	28	4.7	46	2.15	20.5
19		2.8	27	2.7	26	3.1	30	2.5	24	2.9	28	4.6	45	2.1	20

		A		B		C		D		E		F		G	
n		l	r	l	r	l	r	l	r	l	r	l	r	l	r
20		2.75	26.5	2.65	25.5	3.1	30	2.45	23.5	2.9	28	4.6	45	2.1	20
21		2.7	26	2.6	25	3	29	2.4	23	2.8	27	4.4	43	1.9	18
22		2.7	26	2.45	23.5	3	29	2.4	23	2.8	27	4.4	43		
23		2.7	26	2.3	22	2.8	27	2.35	22.5			4.2	41		
24		2.6	25	2.3	22			2.35	22.5			4.1	40		
25		2.5	24					2.1	20			3.45	33.5		
26	st	3.4	3.86			3.9	6.8			2.25	3.5	4.5	4		
27						3.75	6.5								
28						3	6.5								
29						2.6	3.33								
30	sm	3.9	2.9	4.25	2.86	3.2	3	2.05	2.73	3.4	2.78	4.35	2.63	4	2.64
31		4.35	2.48	3.25	2.61	3.4	2.78	3.7	2.7	2.6	2.71	6.3	2.32	3.4	2.4
32		3.1	2.44	3.6	2.6	3.7	2.7	2.5	2.57	3.1	2.44	6.2	2.1	3	2.33
33		3	2.33	3.4	2.4	3.3	2.67	2.65	2.53	4.1	2.42	4.6	2.07	3.65	2.32
34		2.9	2.22	2.7	2.38	3.8	2.45	2.1	2.5	3.7	2.36	4.6	2.07	1.6	2.2
35		2.9	2.22	3	2.33	3.1	2.44	2.4	2.43	2.35	2.36	4.85	2.03	4.3	2.19
36		2.9	2.22	4.3	2.31	5.75	2.38	2.7	2.38	1.65	2.3	8.4	2	3	2
37		3.2	2.2	3.95	2.16	3.8	2.17	3.6	2.27	2.45	2.06	6.3	2	2.65	1.94
38		3.15	2.15	2.8	2.11	3.7	2.08	2.45	2.27	2.65	1.94	4.7	1.94	3.15	1.86
39		2.8	2.11	3.4	2.09	4	2.08	3.7	2.08	2.9	1.9	4.1	1.93	2	1.86
40		2.8	2.11	3.45	2	4.6	2.07	2.45	2.06	2.6	1.89	3.8	1.92	2.8	1.8
41		3.1	2.1	3	2	4.9	2.06	2.1	2	2.6	1.89	4.6	1.88	2.8	1.8

		A		B		C		D		E		F		G	
n		l	r	l	r	l	r	l	r	l	r	l	r	l	r
42		3.4	2.09	2.7	2	3.9	2	2.35	1.76	3.4	1.83	5.1	1.83	1.95	1.79
43		3	2	3	2	3.2	1.91	2.9	1.76	3	1.73	4.2	1.8	1.9	1.71
44		2.9	1.9	3.2	1.91	3.2	1.91			3	1.73	3.9	1.79		
45		3.1	1.82	3.05	1.9	2.55	1.83			2.85	1.71	3.3	1.75		
46		2.85	1.71	2.9	1.9										
47				3.25	1.83										
48				2.5	1.78										
49				2.75	1.75										
50				3	1.73										
51	m	2.4	1.67	2.7	1.7	2.7	1.7	2.7	1.7	4.6	1.71	4.5	1.65	2.15	1.69
52		2.6	1.6	2.6	1.6	3.95	1.63	2.4	1.67	2.6	1.6	4.2	1.63	3.05	1.65
53		2.3	1.56	2.9	1.42	3.3	1.54	2.9	1.64	2.3	1.56	5.4	1.57	2.6	1.6
54		2.8	1.55	3.3	1.36	3.4	1.27	2.1	1.63	2.15	1.53	5.1	1.32	2.45	1.58
55		2.45	1.45	2.3	1.3	3.95	1.14	2.45	1.45	2.65	1.52	5	1.27	2.15	1.53
56		3.1	1.21			2.65	1.12	2.2	1.44	3.75	1.34	5.1	1.13	2.75	1.2
57		4.4	1.1			3.55	0.97	2.05	1.41	3.15	1.33	3.9	1.05	2.4	1.18
58								2.6	1.36	2.1	1.33	5.6	1	2.1	1.1
59								3.75	1.21	2.3	1.09			3.35	1.03
60								2.4	1.18	2.65	1.04				

Appendix 1

Chromosome measurement for *B. anoplus* (Palmiet River) (r) = L/S arm-ratio values and (l) = chromosome length (l) calculated according to Levan *et al.* (1964), N=number of chromosomes, A-I= indicates metaphase cells n=9, CHR= chromosome categories.

	CHR	A		B		C		D		E		F		G		H		I	
n		l	r	l	r	l	r	l	r	l	r	l	r	l	r	l	r	l	r
1	a	4.9	48	4.9	48	3.4	33	3.4	33	3.2	31	3.7	36	3.6	35	4	39	4.7	
2		4.2	41	4.6	45	3.3	32	3.2	31	3.1	30	3.65	35.5	3.3	32	3.6	35	4.5	46
3		4.1	40	4.5	44	3.3	32	3.2	31	3.1	30	3.5	34	3.3	32	3.45	33.5	4.4	44
4		4	39	4.5	44	3.2	31	3.1	30	3	29	3.4	33	3.3	32	3.4	33	4.25	43
5		4	39	4.35	42.5	3.1	30	3.1	30	3	29	3.4	33	3.3	32	3.4	33	4.2	41.5
6		3.9	38	4.1	40	3.1	30	3.1	30	3	29	3.4	33	3.25	31.5	3.35	32.5	4.2	41
7		3.9	38	4.1	40	3.1	30	3	29	2.95	28.5	3.4	33	3.2	31	3.3	32	4.1	41
8		3.9	38	3.9	38	3	29	2.9	28	2.9	28	3.4	33	3.2	31	3.15	30.5	4	40
9		3.8	37	3.8	37	3	29	2.9	28	2.9	28	3.25	31.5	3.1	30	3.1	30	3.9	39
10		3.7	36	3.75	36.5	2.9	28	2.9	28	2.9	28	3.15	30.5	3.05	29.5	3	29	3.85	38
11		3.7	36	3.7	36	2.9	28	2.8	27	2.8	27	3.1	30	3	29	2.9	28	3.7	37.5
12		3.6	35	3.65	35.5	2.9	28	2.8	27	2.8	27	3.1	30	2.95	28.5	2.8	27	3.7	36
13		3.6	35	3.6	35	2.8	27	2.7	26	2.7	26	3.1	30	2.9	28	2.8	27	3.6	36
14		3.6	35	3.6	35	2.8	27	2.7	26	2.7	26	3.05	29.5	2.85	27.5	2.8	27	3.55	35
15		3.5	34	3.6	35	2.8	27	2.7	26	2.7	26	3	29	2.85	27.5	2.8	27	3.55	34.5
16		3.45	33.5	3.6	35	2.8	27	2.7	26	2.6	25	2.9	28	2.8	27	2.75	26.5	3.55	34.5
17		3.4	33	3.55	34.5	2.75	26.5	2.5	24	2.6	25	2.8	27	2.8	27	2.75	26.5	3.4	34.5
18		3.3	32	3.4	33	2.75	26.5	2.5	24	2.6	25	2.7	26	2.8	27	2.7	26	3.4	33
19		3.3	32	3.3	32	2.7	26	2.4	23	2.5	24	2.6	25	2.7	26	2.65	25.5	3.4	33
20		3.2	31	3.2	31	2.7	26	2.4	23	2.4	23	2.6	25	2.6	25	2.6	25	3.35	33

	CHR	A		B		C		D		E		F		G		H		I	
n		l	r	l	r	l	r	l	r	l	r	l	r	l	r	l	r	l	r
21		3.15	30.5	3.1	30	2.6	25	2.4	23	2.4	23	2.6	25	2.4	23	2.5	24	2.85	32.5
22		3.1	30	3.05	29.5	2.5	24	2.3	22	2.2	21	2.6	25			2.5	24	2.8	27.5
23		3	29	3	29	2.4	23	2.2	21	2.1	20	2.45	23.5					2.6	27
24		3	29	2.9	28	2.2	21			1.95	18.5	2.35	22.5						25
25				2.85	27.5							2.35	22.5						
26	st	5.5	3.23					2.3	3.6	2.6	4.2	4.6	3.18	3.05	6.63	2.75	5.88		
27										2.7	3.5			3.05	5.1	2.45	3.9		
28														3.6	4.14	2.45	3.08		
29														3.2	3.57				
30	sm	3.5	2.89	5.85	2.25	3	2.75	2.2	2.67	2.35	2.62	3.5	2.89	3.75	2.75	4.4	3	5.25	2.89
31		5	2.85	3.85	2.08	2.4	2.43	3.6	2.6	4.45	2.42	3.85	2.5	2.6	2.71	4.4	2.67	4.75	2.8
32		5.5	2.67	4.6	2.07	5.4	2.18	3.2	2.56	2.7	2.38	3.75	2.41	3.7	2.7	3.25	2.61	6.45	2.79
33		3.7	2.36	5.7	2	3.8	2.17	2.8	2.5	3	2.33	3.4	2.4	4.95	2.67	3.4	2.4	5.35	2.45
34		3.6	2.27	4.2	2	3.4	2.09	2.25	2.46	3.65	2.32	3.4	2.4	4.95	2.41	3.7	2.36	4.1	2.42
35		4.75	2.17	3.2	1.91	4.85	2.03	4.5	2.46	2.65	2.31	3.35	2.35	2.7	2.38	2.9	2.22	3.7	2.36
36		4.1	2.15	5.5	1.89	3.9	2	2.4	2.43	2.3	2.29	4.9	2.27	3.6	2.27	2.8	2.11	3.7	2.36
37		4	2.08	3.3	1.87	4.5	2	2.9	2.22	3.45	2.29	3.75	2.26	3.9	2.25	2.45	2.06	3.9	2.25
38		5.35	2.06	6.6	1.87	2.45	1.88	3.2	2.2	2.75	2.24	2.9	2.22	3.05	2.21	3.35	2.05	3.8	2.17
39		6.3	2	4.5	1.81	3.15	1.86	2.5	2.13	2.5	2.13	3.1	2.1	3.3	2.14	4.2	2	4.4	2.14
40		3.5	1.92	4.2	1.8	3.7	1.85	2.4	2	3.1	2.1	3.9	2	4	2.08	2.4	2	3.55	2.09
41		4	1.86	5.15	1.78	2.8	1.8	4.8	2	3.75	2	5.1	2	2.75	2.06	3.6	2	3.7	1.96
42		3.85	1.75	3.85	1.75			3.5	1.92	2.55	2	3.2	1.91	2.9	1.9	3	2	3.5	1.92
43		5.05	1.73	4.4	1.75			2.8	1.8	2.1	2	3.1	1.82	4.05	1.89	3.05	1.9	5.5	1.89
44				4.1	1.73			2.8	1.8	2.6	1.89	2.8	1.8	2.8	1.8	2.9	1.9	4.9	1.88

	CHR	A		B		C		D		E		F		G		H		I	
n		l	r	l	r	l	r	l	r	l	r	l	r	l	r	l	r	l	r
45				3.8	1.71					3.3	1.75	3	1.73	3.35	1.79	2.9	1.9	4	1.86
46																2.6	1.89	4.2	1.8
47																3.1	1.82	3.9	1.79
48																2.75	1.75	5.2	1.74
49	m	3.25	1.71	3.7	1.64	2.9	1.64	2.7	1.7	2.7	1.7	2.95	1.68	2.9	1.64	3.4	1.62	4.5	1.5
50		4.1	1.56	4.6	1.56	2.6	1.6	2.9	1.64	2.1	1.63	2.95	1.46	3.15	1.63	2.8	1.55	3.95	1.47
51		4.5	1.5	4.95	1.54	2.3	1.56	4.1	1.56	2.4	1.4	4.2	1.4	2.6	1.6	3.4	1.43	4.4	1.2
52		4.1	1.05	3.8	1.53	3.35	1.48	2.05	1.56	1.95	1.29	3.05	1.35	3.6	1.57	1.9	1.11		
53		2.4	1	4	1.5	3.4	1.43	2.5	1.5	2.7	1.25	3.5	1.33	3.45	1.56	4	1.11		
54		4.5	0.5	4.8	1.18	2.9	1.42	3	1.5	2	1.22	3.5	1.33	3	1.5	3.75	1.08		
55				4.6	1.09	2.85	1.38	2	1.5	2.2	1.2	3	1.14	3.05	1.44				
56				4.5	1.05	3	1.31	3.7	1.47	2.9	1.07	3.55	1.09	3.25	1.41				
57				4.9	0.69	2.75	1.2	3.45	1.3					3.85	1.41				
58						3	1.14	3.4	1.27										
59						3.45	1.09	3.6	1.12										
60						3.3	1.06												
61						2.85	1.04												
62						3.6	0.85												
63																			

Appendix 1

Chromosome measurement for *B. amatolicus*. (r) = L/S arm-ratio values and (l) = chromosome length (l) calculated according to Levan *et al.* (1964), N=number of chromosomes, A-F indicates metaphase cells n=6 CHR= chromosome categories.

N	CHR	A		B		C		D		E		F		mean	
		l	r	l	r	l	r	l	r	l	r	l	r	l	r
1	a	1.82	38	2.71	75	2.28	48	2.1	58	3.22	68	2.21	61	20.4	5.57
2		1.82	38	2.57	71	2.14	45	2.1	58	3.03	64	2.19	60.5	19.8	5.83
3		1.82	38	2.53	70	2.14	45	2.07	57	2.89	61	2.17	60	19.5	5.64
4		1.75	36.5	2.46	68	2.14	45	2.03	56	2.84	60	2.17	60	19.5	5.56
5		1.72	36	2.42	67	2.14	45	2	55	2.84	60	2.17	60	19.3	5.5
6		1.68	35	2.42	67	2.14	45	1.96	54	2.7	57	2.07	57	18.9	5.4
7		1.63	34	2.37	65.5	2.1	44	1.94	53.5	2.7	57	2	55	18.6	5.33
8		1.63	34	2.25	62	2.05	43	1.93	53	2.7	57	2	55	18.3	5.24
9		1.61	33.5	2.21	61	1.96	41	1.89	52	2.56	54	1.93	53	17.8	5.09
10		1.59	33	2.17	60	1.96	41	1.89	52	2.54	53.5	1.85	51	17.4	4.99
11		1.59	33	2.14	59	1.86	39	1.82	50	2.47	52	1.85	51	17.1	4.88
12		1.59	33	1.98	54.5	1.86	39	1.78	49	2.45	51.5	1.82	50	16.8	4.81
13		1.59	33	1.96	54	1.82	38	1.75	48	2.42	51	1.82	50	16.4	4.69
14		1.59	33	1.96	54	1.82	38	1.75	48	2.42	51	1.82	50	16.3	4.67
15		1.56	32.5	1.93	53	1.77	37	1.71	47	2.42	51	1.78	49	16.2	4.64
16		1.54	32	1.93	53	1.77	37	1.68	46	2.31	48.5	1.78	49	15.8	4.53
17		1.49	31	1.93	53	1.68	35	1.68	46	2.26	47.5	1.71	47	15.7	4.49
18		1.49	31	1.93	53	1.68	35	1.64	45	2.19	46	1.64	45	15.3	4.38
19		1.45	30	1.89	52	1.61	33.5	1.53	42	2.14	45	1.53	42	15	4.27

N	CHR	A		B		C		D		E		F		mean	
		l	r	l	r	l	r	l	r	l	r	l	r	l	r
20		1.35	28	1.82	50	1.59	33	1.5	41	2.03	42.5	1.53	42	14.9	4.08
21		1.35	28	1.75	48	1.52	31.5	1.44	39.5	2.03	42.5	0	0	14.2	4.02
22		0	0	1.73	47.5	1.49	31	0	0	1.91	40	0	0	14.2	4.08
23		0	0	1.53	42	0	0	0	0	0	0	0	0	14	4.03
24	st	1.68	5	3.17	3.24	1.86	3.44	1.8	3.04	2.59	3.44	2.32	5.5	1.66	5.6
25		1.63	4	0	0	0	0	0	0	0	0	1.8	5.31	1.41	4.28
26	sm	1.63	2.89	3.24	2.79	2.59	2.96	2.14	2.53	2.98	3	2.39	2.94	1.02	6.21
27		1.96	2.82	2.89	2.68	1.63	2.89	2.39	2.19	2.66	2.56	2.39	2.72	0.94	5.82
28		1.82	2.55	1.57	2.67	1.96	2.82	1.98	2.17	3.31	2.38	1.46	2.42	0.89	4.88
29		3.12	2.53	2.35	2.67	1.35	2.63	2.42	2.09	3.22	2.29	2.42	2.4	0.87	6.12
30		2.52	2.38	2.39	2.53	3.17	2.4	1.68	1.94	2.38	2.19	1.71	2.2	0.81	5.58
31		1.49	2.2	3.6	2.16	2.14	2.29	2.34	1.91	2.84	2.05	1.93	2.18	0.76	5.99
32		1.63	2.18	2.21	2.1	2.75	2.28	1.55	1.9	3.26	1.98	3.74	2.09	0.74	6.24
33		1.45	2.1	2.28	1.91	2.38	2.19	1.75	1.88	2.24	1.82	1.96	2.06	0.71	4.97
34		1.4	2	2.53	1.84	2.52	2.18	1.84	1.86	2.98	1.78	1.96	1.75	0.68	5.43
35		1.54	2	2.32	1.83	2.19	2.13	1.57	1.84	2.77	1.77	2.14	1.73	0.67	5.14
36		1.77	1.92	2.3	1.74	2.56	2.06	1.68	1.76	0	0	0	0	0.67	4.6
37		2	1.87	2.14	1.73	1.61	2	1.55	1.72	0	0	0	0	0.65	4.07
38		1.93	1.86	0	0	2.05	1.93	2.03	1.71	0	0	0	0	0.63	4.75
39		1.59	1.83	0	0	1.35	1.9	0	0	0	0	0	0	0.61	5
40		1.45	1.82	0	0	2.14	1.88	0	0	0	0	0	0	0.61	4.33
41		1.96	1.8	0	0	1.72	1.85	0	0	0	0	0	0	0.59	4.8

N	CHR	A		B		C		D		E		F		mean	
		l	r	l	r	l	r	l	r	l	r	l	r	l	r
42		1.54	1.75	0	0	3.82	1.83	0	0	0	0	0	0	0.56	7.35
43		2.66	1.71	0	0	1.96	1.8	0	0	0	0	0	0	0.32	2.1
44	m	1.86	1.67	2	1.67	2.24	1.67	2.89	1.61	3.08	1.64	1.6	1.65	0.58	5.61
45		2.47	1.65	1.96	1.62	2.42	1.48	1.98	1.58	4.38	1.61	1.59	1.62	0.56	6.11
46		1.52	1.6	2.6	1.61	1.96	1.47	1.75	1.58	3.5	1.59	2.03	1.48	0.55	5.44
47		1.77	1.53	3.57	1.56	4.9	1.44	1.69	1.5	5.03	1.57	3.1	1.42	0.54	8.63
48		1.86	1.5	1.64	1.56	3.5	1.27	1.78	1.5	2.42	1.48	1.89	1.41	0.51	5.81
49		2.38	1.43	2.39	1.48	2.19	1.14	1.85	1.48	2.75	1.46	2.32	1.32	0.5	7
50		3.54	1.3	2.28	1.46	2.52	1.08	2.25	1.42	2.75	1.46	2.32	1.32	0.5	5.08
51		1.17	1.27	1.82	1.43	2.63	1.05	2.32	1.41	3.4	1.43	2.78	1.23	0.49	5.95
52		1.82	1.17	1.96	1.29	0	0	2.64	1.39	2.52	1.35	2.14	1.22	0.49	6.15
53		0	0	1.89	1.21	0	0	3.32	1.33	3.01	1.35	2.01	1.22	0.48	5.09
54		0	0	2.42	1.13	0	0	1.85	1.26	2.38	1.32	4.14	1.19	0.46	5.25
55		0	0	1.64	1.09	0	0	2.25	1.25	2.8	1.22	2.16	1.12	0.45	6.23
56		0	0	1.96	1.08	0	0	2.46	1.12	3.17	1.19	3.33	1.08	0.43	7.63
57		0	0	0	0	0	0	2.67	1.08	3.73	1.11	2.07	1.07	0.41	5.93
58		0	0	0	0	0	0	1.87	1.06	2.42	1.08	0	0	0.4	5.67
59		0	0	0	0	0	0	0	0	2.45	1.06	0	0	0.39	6.08
60		0	0	0	0	0	0	0	0	3.45	1.06	0	0	0.38	7.45
61		0	0	0	0	0	0	0	0	3.45	1.06	0	0	0.38	6.33

Appendix 1

Chromosome measurement for *B. calidus*. (r) = L/S arm-ratio values and (l) = chromosome length (l) calculated according to Levan *et al.* (1964), N=number of chromosomes, A-G= indicates metaphase cells n=7, CHR= chromosome categories.

N	CHR	A		B		C		D		E		F		G		mean	
		l	r	l	r	l	r	L	r	l	r	l	r	l	r	l	r
1	**a**	2.15	35.5	1.88	31.0	1.76	17.6	2.8	17	2.8	17	1.64	16.4	2.5	15.2	1.76	29.1
2		2.12	35.0	2.42	40.0	2	20	3.0	18.2	2.8	17	1.55	15.5	3.2	19.4	1.94	31.9
3		2.3	38.0	2.12	35.0	0.79	7.88	2.9	17.6	4.5	27	2	20	3.3	20	1.95	32.2
4		1.97	7.1	1.94	32.0	1.73	17.3	3.5	20.9	2.5	14.8	1.7	17	3.2	19.4	1.84	26.7
5				2.12	35.0	1.52	15.2	3.0	17.9	2.7	16.4			2.9	17.6	1.76	29.1
6				2.39	7.8	1.7	17	3.0	18.2	1.1	6.67					1.64	19.2
7						2.06	20.6			4.5	27.3					2.39	39.5
8						1.82	18.2			0.9	5.45					1.18	19.5
9						0.88	8.79			3.6	21.8					1.53	25.3
10						1.97	19.7			3.2	19.4					1.95	32.3
11	**st**	2.58	5.1	2.18	5.0	1.7	2.22	2.7	2.67	3.3	3.39	1.88	2.53	3.0	5.35	1.97	5.2
12		2.09	3.9	2.3	4.8	2.55	2.22	2.7	2.61	3.3	3.39	2.21	2.16	3.3	5.02	2.1	4.9
13		2.36	3.9	2.15	4.5	1.79	2.14	3.4	2.52	2.2	2.73	2.61	2.14	2.8	3.56	1.99	4.3
14		2.3	3.8	2.76	4.4	2.42	2.09	2.9	2.32	3.2	2.63	1.64	2.12	3.2	3.27	2.11	4.1
15		2.55	3.7	2.12	3.7	1.58	2.02	3.8	2.27	3.3	2.47	1.85	2.03	3.0	3.03	2.03	3.8
16		2.18	3.5	2.48	3.6	3.03	1.92	2.8	2.22	2.9	2.32	2	1.89	3.0	3.03	2.14	3.7
17		2.36	3.3	2.18	3.5			2.3	2.18	2.4	2.3	2	1.89	3.5	2.93	1.92	3.7
18		2.36	3.3	2.36	3.3			2.8	2.17	2.7	2.07			2.9	2.91	1.95	3.7
19		2.61	3.3	2.36	3.3			3.2	2.16	3.5	2.05			3.8	2.68	2.27	3.6

N	CHR	A		B		C		D		E		F		G		mean	
		l	r	l	r	l	r	L	r	l	r	l	r	l	r	l	r
20		1.91	3.2	2.61	3.3			3.2	2.16	3.8	1.95			3.5	2.61	2.17	3.5
21		2	3.1	2.85	3.3			4.0	2.09	4.2	1.94			3.2	2.58	2.35	3.5
22		2.24	3.1	2.58	3.3			3.5	2.05	3.8	1.92			2.9	2.53	2.19	3.4
23		1.97	3.1	3.03	3.2			3.7	2.03	3.3	1.89			3.1	2.47	2.22	3.4
24				2.73	3.1			2.2	2	3.7	1.89			2.5	2.42	1.95	3.4
25				2.09	3.1			3.0	1.99	4.5	1.87			3.0	2.42	2.11	3.4
26				2.33	3.1			3.4	1.97					3.0	2.37	2.06	3.4
27								3.4	1.93					3.8	2.27	2.17	3.5
28								3.5	1.89					2.8	2.22	1.91	3.4
29								3.7	1.89					2.1	2.22	1.76	3.4
30								3.7	1.89					2.6	2.2	1.89	3.4
31														3.5	2.18	2.09	3.6
32														3.2	2.16	1.94	3.6
33														5.0	2.15	3.03	3.5
34														4.0	2.09	2.42	3.4
35														3.1	2.08	1.88	3.4
36														3.0	1.99	1.82	3.3
37														2.8	1.96	1.67	3.2
38														4.2	1.94	2.55	3.2
39														3.6	1.93	2.15	3.2
40														2.9	1.9	1.76	3.1
41														5.3	1.86	3.21	3.1

N	CHR	A		B		C		D		E		F		G		mean	
		l	r	l	r	l	r	L	r	l	r	l	r	l	r	l	r
43	sm	2.85	2.9	2.58	3.0	1.58	1.82	5.1	1.84	4.4	1.79	2.18	1.82	3.6	1.82	2.44	3.0
44		2.58	2.9	2.94	3.0	1.45	1.82	3.6	1.82	3.5	1.75	1.91	1.78	3.4	1.78	2.17	3.0
45		2.64	2.8	2.42	3.0	2.67	1.82	3.6	1.82	3.3	1.71	2.61	1.76	3.2	1.78	2.34	2.9
46		2.48	2.7	3.03	3.0	2.15	1.78	3.6	1.82	1.9	1.7	2.36	1.76	3.2	1.78	2.18	2.9
47		2.67	2.7	1.94	3.0	2.12	1.75	3.0	1.82	3.0	1.67	2.06	1.68	2.6	1.77	2	2.9
48		1.97	2.6	2.67	3.0	1.97	1.71	3.0	1.78	3.8	1.67	1.94	1.68	2.7	1.73	2.03	2.8
49		3.82	2.6	2.61	2.9	1.73	1.7	3.5	1.75	3.3	1.62	1.36	1.67	3.5	1.72	2.25	2.8
50		2.18	2.6	2.61	2.9	1.91	1.64	3.5	1.75	4.0	1.6	2.27	1.67	2.9	1.7	2.18	2.8
51		3	2.5	2.36	2.9	2.79	1.62	5.1	1.75	3.2	1.55	1.91	1.64	3.4	1.68	2.45	2.7
52		2.97	2.5	2.12	2.9	1.67	1.62	3.1	1.7	4.6	1.54	1.76	1.59	3.0	1.67	2.14	2.7
53		2.55	2.5	2.21	2.8	2.18	1.58	2.9	1.7	3.5	1.52	1.85	1.57	3.0	1.67	2.06	2.7
54		2.33	2.5	2.55	2.8	2.61	1.57	3.6	1.69	2.8	1.52	2.36	1.54	3.0	1.67	2.22	2.6
55		2.3	2.5	2.3	2.8	3.67	1.55	4.0	1.67	3.8	1.49	2.12	1.52	2.6	1.65	2.38	2.6
56		2.06	2.4	2.3	2.8	2.36	1.54	3.0	1.67	3.1	1.48	1.7	1.52	3.2	1.64	2	2.6
57		3.09	2.4	2.06	2.8	2.36	1.54	3.0	1.67	3.1	1.48	1.7	1.52	1.9	1.64	2	2.6
58		3.27	2.4	3.39	2.7	2.36	1.54	4.9	1.66	3.3	1.47	1.79	1.5	4.8	1.63	2.66	2.6
59		2.24	2.4	2.24	2.7	1.91	1.52	4.1	1.65	3.8	1.46	2.3	1.49	2.4	1.59	2.13	2.5
60		2.42	2.3	2.24	2.7	1.7	1.52	3.4	1.65	2.7	1.44	1.88	1.48	4.3	1.57	2.07	2.5
61		2.42	2.3	2.24	2.7	2.73	1.49	4.8	1.63	3.4	1.42	1.88	1.48	4.3	1.57	2.4	2.5
62		1.91	2.3	2.45	2.7	2.09	1.48	3.3	1.62	3.0	1.41	2.39	1.48	2.9	1.55	2.06	2.5
63		2.61	2.3	2.21	2.7	3.09	1.45	3.7	1.61	3.3	1.39	2.45	1.44	4.5	1.55	2.47	2.5
64		3.48	2.3	3.09	2.6	2.64	1.42	2.5	1.56	3.6	1.38	2.33	1.42	3.2	1.55	2.45	2.4
65		2.27	2.3	3.52	2.6	3.03	1.41	3.6	1.55	2.8	1.35	2.42	1.41	3.2	1.55	2.43	2.4

N	CHR	A		B		C		D		E		F		G		mean	
		l	r	l	r	l	r	L	r	l	r	l	r	l	r	l	r
66		1.58	2.3	2.18	2.6	1.61	1.4	4.6	1.54	2.9	1.35	1.61	1.4	3.5	1.52	1.95	2.4
67		1.58	2.3	2.18	2.6	2	1.39	3.5	1.52	2.9	1.35	2.27	1.37	2.1	1.52	1.88	2.4
68		2.15	2.2	2.12	2.5	2.18	1.38	4.9	1.52	2.9	1.35	1.58	1.36	3.5	1.52	2.13	2.4
69		1.94	2.2	2.55	2.5	2.36	1.36	3.5	1.52	2.9	1.35	1.76	1.35	3.8	1.49	2.11	2.3
70		1.94	2.2	2.64	2.5	2.06	1.36	4.9	1.49	2.9	1.35	2.58	1.3	3.1	1.48	2.26	2.3
71		2.61	2.2	2.06	2.4	2.33	1.34	3.1	1.48	4.0	1.31	2.85	1.29	3.4	1.45	2.31	2.3
72		2.21	2.2	2.24	2.4	1.94	1.33	3.1	1.48	2.4	1.29	1.7	1.28	2.4	1.43	1.83	2.3
73		2.3	2.2	2.61	2.3	1.94	1.33	3.8	1.46	3.1	1.27	1.88	1.27	2.3	1.39	2.04	2.2
74		2.3	2.2	2.18	2.3	1.94	1.33	3.8	1.46	3.1	1.27	2.24	1.26	2.6	1.36	2.06	2.2
75		2.06	2.1	2.18	2.3	1.73	1.31	3.1	1.45	3.1	1.27	1.85	1.24	3.1	1.34	1.91	2.2
76		2.61	2.1	2.36	2.3	1.73	1.31	3.0	1.41	3.4	1.27	1.82	1.21	3.4	1.27	2.06	2.1
77		3.33	2.1	2.36	2.3	1.7	1.28	2.7	1.4	3.7	1.26	1.64	1.21	3.4	1.27	2.13	2.1
78		2.03	2.0	2.06	2.2	1.88	1.27	3.8	1.4	2.7	1.21	1.64	1.21	4.1	1.21	2	2.1
79		2.12	2.0	1.76	2.2	1.97	1.27	3.8	1.4	3.0	1.21	2.45	1.21	3.0	1.21	2.03	2.1
80		1.64	2.0	2.3	2.2	2.42	1.26	3.3	1.39	2.6	1.21	2.09	1.21	2.6	1.21	1.94	2.1
81		2	2.0	3.55	2.2	2.12	1.24	3.3	1.39	4.4	1.17	2.36	1.21	3.0	1.21	2.36	2.1
82		2.73	2.0	2.24	2.1	2.21	1.24	2.9	1.35	4.1	1.17	1.36	1.21	3.3	1.21	2.11	2.0
83		2.36	2.0	2.52	2.1	2.18	1.21	3.9	1.34	4.1	1.17	2.73	1.21	2.7	1.18	2.32	2.0
84		2.15	2.0	2.39	2.0	2	1.21	3.5	1.32	3.8	1.17	1.73	1.21	3.5	1.16	2.12	2.0
85		3.21	1.9	1.82	2.0	1.82	1.21	3.5	1.32	2.0	1.13	1.64	1.21	2.6	1.14	1.91	2.0
86		2.58	1.9	1.82	2.0	1.64	1.21	3.2	1.3	3.0	1.13	1.64	1.21	4.3	1.13	2	2.0
87		2.21	1.9	1.82	2.0	1.97	1.18	3.2	1.3	3.6	1.07	2.15	1.19	3.6	1.12	2.06	1.9
88		2.09	1.9	2.45	2.0	2.12	1.16	3.7	1.26			1.79	1.18	3.4	1.11	2.13	1.9

N	CHR	A		B		C		D		E		F		G		mean	
		l	r	l	r	l	r	L	r	l	r	l	r	l	r	l	r
89		2.61	1.9	2.73	2.0	1.94	1.16	2.5	1.25			1.79	1.18	2.5	1.08	2.01	1.9
90		2.61	1.9	2.27	2.0	2.79	1.14	3.3	1.21			1.94	1.16	3.3	1.06	2.27	1.9
91		2.58	1.8	2.52	2.0	2.09	1.14	3.0	1.18			1.94	1.16	2.8	1.06	2.1	1.9
92		2.58	1.8	2.15	2.0	1.97	1.11	2.6	1.14			1.76	1.15	0.0	0	2.01	1.9
93		2.48	1.8	2.39	1.9	1.88	1.1	2.6	1.14			1.76	1.15	0.0	0	2.02	1.9
94		2.12	1.8	2.3	1.9	2.21	1.1	4.3	1.13			1.67	1.15	0.0	0	2.18	1.9
95		2.5	1.8	2.61	1.9	2.12	1.09	3.4	1.11			1.67	1.15	0.0	0	2.19	1.8
96				2.61	1.9	1.7	1.09	4.2	1.09			1.39	1.14	0.0	0	2.06	1.8
97				2.03	1.8	2.03	1.09	2.8	1.06			2	1.13	0.0	0	1.93	1.8
98				2.85	1.8	2.36	1.08					1.73	1.12	0.0	0	2.31	1.8
99						1.52	1.08					1.97	1.11	0.0	0	1.74	1.8
100												1.97	1.11	0.0	0	1.97	1.8
101												1.79	1.1	0.0	0	1.79	1.8
102												1.52	1.08	0.0	0	1.52	1.8
103												2.18	1.07	0.0	0	2.18	1.8
104	m	2.48	1.7	2.21	1.7	1.91	1.05	4.3	1.02	3.0	1.05	2.15	1.05	2.2	1.02	2.07	1.7
105		2.48	1.7	2.24	1.6	2.48	1.05	5.9	1.02	2.4	1.01	2.21	1.03	2.7	1	2.29	1.7
106		2.06	1.7	1.91	1.6	1.82	1.05	3.2	1.01	3.1	1	1.64	1.03	3.0	0.79	1.86	1.6
107		2.06	1.7	2.85	1.6	2.12	1.03	2.4	1.01	2.9	0.99	1.64	1.03	4.3	0.7	2.07	1.6
108		2.3	1.7	2.52	1.6	2	0.99	2.4	1.01	2.4	0.98	1.64	1.03	3.2	0.69	1.9	1.6
109		2.42	1.7	2.18	1.6	1.76	0.99	4.5	1	2.8	0.94	1.64	1.03	3.7	0.66	2.09	1.6
110		2.58	1.7	2.18	1.6	2.55	0.98	3.1	0.96	3.0	0.91	2.12	1.03	2.5	0.63	2.09	1.5
111		2.15	1.6	2.48	1.6	2.21	0.97	3.8	0.93	3.4	0.9	1.3	1.02	2.5	0.63	2	1.5

N	CHR	A		B		C		D		E		F		G		mean	
		l	r	l	r	l	r	L	r	l	r	l	r	l	r	l	r
112		2.55	1.6	2.3	1.5	2.36	0.97	2.9	0.9	2.7	0.88	2.58	1	3.4	0.62	2.17	1.5
113		2.58	1.5	3.33	1.5	2.18	0.95	3.7	0.89	2.2	0.88	1.67	0.98	3.1	0.25	2.17	1.4
114		2.73	1.5	2.58	1.4	2	0.93	3.3	0.88	3.4	0.87	2	0.93			2.23	1.5
115		2.55	1.5	2.88	1.3	1.97	0.91	3.9	0.83	2.1	0.86	2.06	0.87			2.18	1.4
116		2.39	1.5	2.73	1.3	2.03	0.79	4.0	0.82	4.1	0.86	1.91	0.86			2.33	1.4
117		2.36	1.4	2.21	1.2	2.18	0.76	3.5	0.72	4.1	0.84	1.45	0.85			2.13	1.3
118		2.55	1.3	2.24	1.2	2	0.73	3.5	0.64	2.6	0.83	1.88	0.84			2.06	1.3
119		2.91	1.3	1.94	1.1	2.79	0.72	3.7	0.64	3.9	0.78	1.85	0.82			2.35	1.2
120		2.79	1.2	2.85	1.1	2.09	0.7	2.1	0.64	2.9	0.78	1.82	0.79			2.09	1.2
121		2.09	1.2	3.09	1.1	1.82	0.69			2.9	0.78	1.36	0.76			2.02	1.2
122		2.45	1.1	1.97	1.0	2.18	0.68			3.1	0.76	1.61	0.68			2.01	1.1
123		2.52	1.1	2.58	1.0	1.88	0.65			3.6	0.76	1.91	0.67			2.21	1.1
124		2.18	1.1			2.24	0.64			3.3	0.73	2.79	0.66			2.3	1.1
125		3.15	1.1			2.15	0.62			3.5	0.72	2.27	0.66			2.42	1.1
126		2.12	1.1			2.76	0.62			3.1	0.65	1.76	0.65			2.13	1.1
127		2.36	1.1							3.1	0.63	2.03	0.62			2.08	1.0
128		3.09	1.0							3.0	0.61					2.45	1.0
129		2.76	1.0							3.1	0.29					2.32	0.7
130		2.76	1.0													2.76	1.0
131		2.97	0.8													2.97	0.8

Appendix 1

Chromosome measurement for *B. polylepis*. (r) = L/S arm-ratio values and (l) = chromosome length (l) calculated according to Levan *et al.* (1964), N=number of chromosomes, A-E= indicates metaphase cells n=5, CHR= chromosome categories.

	CHR	A		B		C		D		E	
n		l	r	l	r	l	r	l	r	l	r
1	a	2.4	65.0	2.1	33.0	1.9	31.0	2.4	38.0	1.9	30.0
2		2.2	60.0	1.9	31.0	1.9	30.0	2.1	33.0	1.8	29.0
3		2.0	54.0	1.9	30.5	1.9	30.0	2.0	32.0	1.8	29.0
4		1.9	53.0	1.8	29.5	1.9	30.0	1.9	30.0	1.8	28.0
5		1.8	50.0	1.8	28.5	1.9	30.0	1.8	29.0	1.8	28.0
6		1.8	50.0	1.8	28.0	1.8	29.0	1.8	29.0	1.8	28.0
7		1.7	48.0	1.8	28.0	1.8	29.0	1.8	29.0	1.8	28.0
8		1.7	48.0	1.7	26.5	1.8	28.5	1.8	29.0	1.8	28.0
9		1.7	48.0	1.6	26.0	1.8	28.0	1.8	29.0	1.7	27.0
10		1.7	47.0	1.6	26.0	1.8	28.0	1.8	28.0	1.7	27.0
11		1.7	45.5	1.6	25.5	1.8	28.0	1.8	28.0	1.7	27.0
12		1.5	40.0	1.6	25.5	1.8	28.0	1.8	28.0	1.7	26.5
13		1.5	40.0	1.6	25.0	1.8	28.0	1.8	28.0	1.6	25.5
14		1.5	40.0	1.6	25.0	1.7	27.0	1.8	28.0	1.6	25.5
15		1.4	38.0	1.6	25.0	1.7	27.0	1.8	28.0	1.6	25.0
16		1.4	38.0	1.6	25.0	1.7	27.0	1.7	27.5	1.6	25.0
17		1.9	10.7	1.6	25.0	1.7	26.5	1.6	26.0	1.6	25.0
18		1.6	10.5	1.5	24.5	1.7	26.5	1.6	26.0	1.5	24.0
19		1.9	9.4	1.5	24.5	1.7	26.5	1.6	25.0	1.5	24.0
20		1.9	9.4	1.5	24.5	1.6	25.5	1.6	25.0	1.5	24.0

n	CHR	A		B		C		D		E	
		l	r	l	r	l	r	l	r	l	r
21		1.8	9.2	1.5	24.0	1.6	25.0	1.5	24.0	1.5	24.0
22		1.8	9.0	1.5	24.0	1.6	25.0	1.5	24.0	1.5	23.0
23		1.7	8.7	1.5	24.0	1.6	25.0	1.5	24.0	1.4	22.0
24		1.7	8.6	1.5	23.0	1.6	25.0	1.5	23.5	1.4	22.0
25		1.6	8.0	1.4	22.5	1.6	25.0	1.5	23.0	1.3	21.0
26		2.2	7.9	1.4	22.0	1.5	24.5	1.5	23.0	1.3	21.0
27		1.9	7.8	1.4	21.5	1.5	24.0	1.4	22.5	1.3	21.0
28		2.1	7.4	1.3	21.0	1.5	24.0	1.3	21.0	1.3	20.0
29		2.1	7.3	1.3	21.0	1.5	23.5	1.3	21.0	1.3	20.0
30				1.3	20.0	1.4	22.5	1.3	20.0	1.2	19.0
31				1.9	9.7	1.4	22.0	1.3	9.5	1.2	18.0
32				1.8	9.0	1.3	21.0	1.9	9.3	1.1	17.0
33				1.6	8.0	1.3	21.0	2.3	8.5	1.0	16.0
34				2.0	7.3	1.3	21.0	1.6	8.0	2.4	9.0
35						1.2	19.0	2.7	7.8	1.7	8.3
36						1.5	15.3	1.7	7.1	1.6	8.0
37						0.8	12.5			1.6	7.7
38						1.4	10.8			1.5	7.3
39						1.9	9.3			2.0	7.1
40						1.4	8.4				
41						1.6	8.0				
42						1.6	8.0				
43						1.6	7.8				
44						1.6	7.8				

n	CHR	A		B		C		D		E	
		l	r	l	r	l	r	l	r	l	r
45	st	1.7	6.8	1.5	7.0	1.5	7.0	1.4	6.7	1.5	7.0
46		1.6	6.7	1.4	6.8	1.5	7.0	1.8	6.5	1.5	7.0
47		1.6	6.3	1.4	6.7	1.5	7.0	2.1	6.0	2.3	6.7
48		2.0	6.1	1.8	6.5	1.7	7.0	1.7	6.0	1.4	6.7
49		1.9	6.0	1.8	6.3	2.4	7.0	1.2	5.7	0.9	6.5
50		2.1	5.9	1.7	5.9	1.9	7.0	1.8	5.1	1.3	6.3
51		2.0	5.9	1.6	5.6	2.4	6.8	1.5	5.0	1.3	6.2
52		1.8	5.7	1.6	5.5	1.3	6.3	1.5	5.0	1.3	6.0
53		1.4	5.7	1.5	5.3	1.5	6.3	1.2	4.9	1.3	6.0
54		1.6	5.6	1.9	5.2	1.7	6.1	1.8	4.8	2.5	6.0
55		1.9	5.5	1.8	5.0	1.7	6.1	1.8	4.8	1.7	6.0
56		2.3	5.4	1.5	5.0	1.7	6.0	1.4	4.8	1.6	5.5
57		1.8	5.3	1.4	4.9	1.2	5.8	1.8	4.5	1.6	5.5
58		1.8	5.3	2.1	4.8	2.0	5.7	1.3	4.5	1.2	5.3
59		2.0	5.1	1.8	4.8	1.2	5.7	2.3	4.4	1.2	5.3
60		1.9	5.1	1.8	4.8	2.0	5.6	1.2	4.4	1.5	5.3
61		2.0	4.7	1.8	4.8	1.4	5.6	1.6	4.4	1.5	5.0
62		3.2	4.7	2.5	4.8	1.4	5.4	1.6	4.4	1.1	5.0
63		1.8	4.7	1.4	4.8	1.5	5.4	1.3	4.4	1.8	4.9
64		1.2	4.6	1.7	4.7	1.5	5.4	1.9	4.3	1.4	4.9
65		2.1	4.6	1.7	4.7	1.5	5.0	1.3	4.3	1.8	4.8
66		1.8	4.6	2.0	4.6	1.2	4.9	1.6	4.2	1.8	4.8
67		1.7	4.5	1.7	4.5	1.8	4.8	1.6	4.2	1.4	4.8
68		2.5	4.3	2.5	4.5	1.6	4.4	1.6	4.2	1.4	4.8

	CHR	A		B		C		D		E	
n		l	r	l	r	l	r	l	r	l	r
69		1.5	4.3	1.5	4.4	1.6	4.4	1.6	4.2	1.0	4.7
70		1.9	4.2	1.6	4.4	1.6	4.3	1.9	4.2	1.7	4.6
71		2.3	4.2	1.3	4.4	1.6	4.3	1.9	4.2	1.3	4.5
72		1.4	4.0	1.3	4.4	0.9	4.2	2.2	4.1	1.6	4.4
73		1.9	4.0	1.9	4.3	1.5	4.0	1.5	4.1	1.9	4.3
74		1.8	4.0	1.6	4.2	1.5	4.0	1.5	4.1	1.9	4.3
75		1.7	3.9	1.6	4.2	2.4	3.9	1.2	4.0	1.6	4.3
76		2.3	3.8	1.6	4.2	1.6	3.8	1.5	4.0	1.6	4.2
77		1.7	3.7	1.2	4.1	1.7	3.7	0.9	4.0	1.6	4.2
78		3.0	3.7	2.8	4.1	1.6	3.5	1.8	4.0	2.1	4.0
79		1.7	3.7	1.8	3.9	1.9	3.5	0.9	4.0	1.5	4.0
80		2.0	3.6	1.8	3.9	1.3	3.4	1.5	4.0	1.8	3.8
81		2.7	3.5	1.3	3.8	1.3	3.4	1.5	4.0	1.5	3.8
82		2.5	3.5	1.2	3.8	2.7	3.3	1.5	3.8	1.5	3.8
83		1.9	3.4	2.3	3.8	1.0	3.3	2.2	3.7	1.5	3.8
84		2.4	3.3	2.4	3.7	1.7	3.2	1.3	3.4	1.5	3.8
85		1.7	3.3	1.4	3.7	1.3	3.2	2.7	3.4	2.3	3.8
86		2.0	3.3	2.2	3.5	1.3	3.2	1.6	3.3	1.7	3.7
87		1.9	3.2	1.3	3.4	1.5	3.2	2.1	3.3	2.1	3.6
88		2.2	3.1	1.1	3.4	1.8	3.1	1.5	3.2	1.4	3.6
89				2.8	3.2	3.0	3.1	1.5	3.2	1.4	3.6
90				2.8	3.2			1.5	3.2	1.9	3.6
91				1.5	3.2			1.5	3.2	1.9	3.6
92				1.8	3.1			1.8	3.1	1.9	3.6

n	CHR	A		B		C		D		E	
		l	r	l	r	l	r	i	r	l	r
93				1.8	3.1			1.4	3.1	1.6	3.5
94										1.4	3.5
95										1.3	3.4
96										1.3	3.4
97										1.3	3.4
98										1.5	3.4
99										1.4	3.3
100										2.5	3.2
101										1.8	3.1
102										2.0	3.1
103	sm	1.9	3.0	1.3	3.0	1.5	3.0	1.7	3.0	1.2	3.0
104		1.8	3.0	2.6	3.0	1.5	3.0	1.7	3.0	1.9	3.0
105		1.8	3.0	1.5	2.9	1.9	2.9	1.5	3.0	2.2	2.9
106		1.5	2.8	2.4	2.9	2.1	2.9	1.6	3.0	1.6	2.9
107		1.4	2.8	1.6	2.9	1.6	2.8	1.9	3.0	1.4	2.8
108		1.6	2.8	1.5	2.8	2.3	2.8	1.4	2.8	2.8	2.8
109		2.6	2.8	1.4	2.8	1.9	2.7	2.8	2.8	2.5	2.8
110		3.2	2.8	1.7	2.8	1.9	2.7	2.7	2.8	1.8	2.8
111		1.9	2.8	1.7	2.8	1.5	2.6	1.8	2.8	2.1	2.8
112		1.9	2.8	2.1	2.8	2.4	2.6	1.8	2.8	3.9	2.8
113		1.8	2.7	2.3	2.8	1.8	2.6	2.5	2.7	2.5	2.7
114		2.9	2.7	2.5	2.7	2.2	2.6	1.6	2.7	1.6	2.7
115		1.5	2.7	1.3	2.7	2.2	2.6	1.3	2.7	1.7	2.7
116		1.3	2.7	1.5	2.6	1.9	2.6	1.3	2.7	1.5	2.6

	CHR	A		B		C		D		E	
n		l	r	l	r	l	r	l	r	l	r
117		2.5	2.6	2.1	2.5	1.3	2.5	2.1	2.6	1.3	2.5
118		2.1	2.6	1.5	2.5	1.3	2.5	1.8	2.6	1.3	2.5
119		1.6	2.6	1.6	2.5	1.8	2.5	2.6	2.6	1.7	2.5
120		1.5	2.6	1.7	2.4	1.9	2.4	1.9	2.6	1.7	2.5
121		1.8	2.6	1.5	2.4	1.9	2.4	1.5	2.4	2.1	2.5
122		1.7	2.5	2.2	2.4	1.8	2.3	1.5	2.4	1.4	2.3
123		2.0	2.5	2.2	2.4	2.5	2.3	2.2	2.4	1.6	2.3
124		2.0	2.5	1.7	2.4	2.7	2.3	2.5	2.3	1.9	2.2
125		2.0	2.4	1.8	2.3	1.6	2.3	1.6	2.3	2.2	2.2
126		1.6	2.4	1.8	2.3	1.8	2.2	2.6	2.3	1.7	2.1
127		1.5	2.4	2.4	2.3	2.3	2.2	2.0	2.3	1.9	2.1
128		2.5	2.4	1.6	2.3	1.5	2.2	2.4	2.3	1.9	2.1
129		2.6	2.4	2.0	2.3	1.9	2.2	1.8	2.2	1.7	2.1
130		1.3	2.4	1.6	2.3	1.3	2.1	1.8	2.2	1.6	2.0
131		1.4	2.3	1.6	2.3	1.3	2.1	2.6	2.2	1.5	2.0
132		1.6	2.3	1.4	2.2	1.7	2.1	2.1	2.2	2.6	1.9
133		1.9	2.3	1.9	2.2	2.3	2.1	1.5	2.1	1.6	1.9
134		1.8	2.3	1.3	2.1	1.6	2.0	1.5	2.1	1.4	1.9
135		2.9	2.3	1.6	2.1	1.6	2.0	2.1	2.1	1.4	1.9
136		2.2	2.2	1.7	2.1	2.4	2.0	1.5	2.0	1.4	1.8
137		1.5	2.2	1.8	2.1	1.6	1.9	1.1	2.0		
138		1.2	2.2	2.0	2.0	1.8	1.9	1.8	2.0		
139		1.4	2.2	1.4	2.0	1.4	1.9	1.6	2.0		
140		2.2	2.2	1.6	2.0	1.9	1.8	1.8	2.0		

	CHR	A		B		C		D		E	
n		l	r	l	r	l	r	l	r	l	r
141		2.2	2.2	2.4	2.0	1.9	1.8	1.4	2.0		
142		1.5	2.1	1.9	1.9	2.7	1.8	1.8	1.9		
143		1.7	2.1	1.9	1.9	2.5	1.7	1.4	1.9		
144		1.8	2.1	1.8	1.9			1.4	1.9		
145		1.9	2.1	1.6	1.9			2.2	1.8		
146		2.4	2.0	1.7	1.9			1.0	1.8		
147		2.0	2.0	1.7	1.8			1.9	1.8		
148		2.9	2.0					1.7	1.8		
149		1.9	1.9					1.9	1.7		
150		2.1	1.9					1.8	1.7		
151		2.1	1.9					1.7	1.7		
152		1.6	1.9								
153		1.8	1.9								
154		2.5	1.9								
155		2.0	1.9								
156		1.9	1.8								
157		2.2	1.8								
158		1.8	1.8								
159	m	1.8	1.7	2.6	1.7	1.7	1.7	1.6	1.7	1.5	1.7
160		1.8	1.7	2.0	1.7	1.5	1.7	1.6	1.7	1.5	1.7
161		2.0	1.6	2.4	1.7	2.2	1.6	1.3	1.6	1.8	1.6
162		1.7	1.6	1.8	1.6	2.1	1.6	2.1	1.6	1.8	1.6
163		1.9	1.5	1.4	1.6	1.9	1.6	1.6	1.6	1.6	1.6
164		1.9	1.5	1.7	1.5	1.8	1.6	1.5	1.5	1.9	1.6

	CHR	A		B		C		D		E	
n		l	r	l	r	l	r	l	r	l	r
165		1.8	1.4	1.7	1.5	1.2	1.6	1.5	1.5	1.8	1.5
166		1.7	1.4	1.7	1.5	1.9	1.5	1.2	1.5	1.3	1.4
167		2.1	1.4	2.0	1.5	1.8	1.4	2.0	1.4	1.8	1.4
168		1.4	1.4	1.3	1.5	1.2	1.4	2.2	1.4	1.5	1.4
169		2.5	1.4	1.7	1.5	2.5	1.4	1.7	1.3	1.3	1.4
170		1.9	1.4	1.8	1.4	1.6	1.4	1.7	1.3	1.6	1.4
171		2.3	1.3	1.8	1.4	2.1	1.3	2.4	1.3	2.0	1.4
172		1.8	1.2	1.6	1.3	1.8	1.3	1.9	1.2	1.8	1.3
173		2.2	1.2	1.9	1.3	1.9	1.3	2.0	1.2	2.1	1.3
174		2.4	1.2	1.7	1.3	2.0	1.2	2.0	1.0	1.1	1.2
175		2.2	1.1	1.5	1.3	2.2	1.1			1.9	1.1
176		2.2	1.1	1.8	1.3	1.6	1.1			2.0	1.1
177		2.0	1.1	1.7	1.1	1.6	1.1			2.0	1.1
178		1.8	1.0	2.0	1.1	0.9	1.1				
179		1.9	0.7	2.1	1.1						
180				1.9	1.0						

Appendix 2

A list of karyotypes of barbines. (m = indicates metacentric chromosomes, sm = indicates submetacentric chromosomes, st = indicates subtelocentric chromosomes, a = indicates acrocentric chromosomes).

Species	2n	Karyotype	NF	References
Barbus serra	104	not given		Oellermann 1988
B. trevelyani	96	not given		Oellermann 1988
B. anoplus	48	not given		Oellermann 1988
B. argenteus	52	not given		Oellermann 1988
B. trimaculatus	46/48	not given		Oellermann 1988
B. marequensis	134	not given		Oellermann 1988
Pseudobarbus afer	96	not given		Oellermann 1988
P. burgi	96	not given		Oellermann 1988
P. quathlambae	96	not given		Oellermann pers. comm.
B. capensis	150	29msm 46sta	208	Oellermann & Skelton 1990
B. kimberleyensis	148	28msm 46sta	204	Oellermann & Skelton 1990
B. natalensis	150	25msm 50sta	200	Oellermann & Skelton 1990
B. polylepis	150	28msm 47sta	196	Oellermann & Skelton 1990
B. aeneus	148	24msm 50sta	180	Oellermann & Skelton 1990
B. pallidus	50	26msm 24sts	76	Naran 1992
B. fasciolatus	48	16m 8sm	96	Rab 1981
B. holotaenia	50	12m 13sm	100	Rab 1981
B. anema	50	21msm 4a	92	Golubstov & Kryzanov 1993
B. kerstenii	50	17msm 8a	84	Golubstov & Kryzanov 1993
B. paludinosus	50	23msm 2a	96	Golubstov & Kryzanov 1993
Barbus sp.1	50	22msm 3a	94	Golubstov & Kryzanov 1993
Barbus sp.2	50	22msm 3a	94	Golubstov & Kryzanov 1993
Barbus sp.3	50	24msm 1a	98	Golubstov & Kryzanov 1993
B. bynni	150	35msm 40sta	220	Golubstov & Kryzanov 1993
B. intermedius	150	25msm 50sta	200	Golubstov & Kryzanov 1993
B. intermedius	150	25msm 50sta	200	Golubstov & Kryzanov 1993
B. ethiopicus	150	20msm 55sta	190	Golubstov & Kryzanov 1993
B. bynni occidentalis	148	not given		Guégan *et al.* 1995
B. petitjeani	148	36m 90sm-st 24a	186	Guégan *et al.* 1995
B. wurtzi	150	not given		Guégan *et al.* 1995

B. ablabes	50	9m 15sm 1sta	96	Rab *et al.* 1995
B. bigornei	48	9m15sm	98	Rab *et al.* 1995
B. macrops	50	7m 14sm 4sta	92	Rab *et al.* 1995
B. meridionalis	100	11m 10sm 6st 23a	142	Collares-Pereira & Madeira 1990
B. plebejus	100	6m 26sm 18sta	164	Collares-Pereira & Madeira 1990
B. barbus	100	24msm 50sta	160	Collares-Pereira & Madeira 1990
B. bocagei	100	8m 24sm 18sta	164	Collares-Pereira & Madeira 1990
B. bocagei	100	6m 26sm 18sta	164	Collares-Pereira & Madeira 1990
B. microcephalus	100	9m 25sm 16sta	168	Collares-Pereira & Madeira
B. meridionalis	100	11m 10sm 6st 23a	142	Cataudella *et al.* 1977
B. plebejus	100	13m 9sm 9st 19a	144	Cataudella *et al.* 1977
B. barbus	100	13m 9sm 9st 19a	144	Cataudella *et al.* 1977
B. meridionalis	100	14m 23sm 2st 11a	174	Valenta *et al.* 1979
B. barbus	100	14m 23sm 2st 11a	174	Valenta *et al.* 1979
B. barbus	100	6m 24sm 20a	160	Hafez *et al.* 1978
B. brachycephalus	100	12m 38smsta		Vasil'ev 1985, in Klinkhardt *et al.* 1995
B. comiza	100	6m 30sm 14sta	172	Collares-Pereira & Madeira 1990
B. sclateri	100	5m 22sm 23sta	154	Collares-Pereira & Madeira 1990
B. steindachneri	100	5m 24sm 21sta	158	Collares-Pereira & Madeira 1990

Appendix 3: LIST OF CHARACTERS USED IN PHYLOGENETIC ANALYSIS

#1. Standard length
1. large (>150 mm)
2. medium (50-150 mm)
3. small (<50 mm)

#2. Scale striations
1. radiate
2. parallel

#3. Redfins
1. absent
2. present

#4. Barbels
1. two
2. single
3. none

#5. Barbel develop
1. well developed
2. single well dev
3. short

#6. Scale radii
1. up to 10
2. 10-15
3. above 15

#7. Scale count(lateral line-scales)
1. 30-36
2. 37-40
3. above 40

#8. Scale count(caudal peduncle scales)
1. 10-15
2. 16-20
3. more than 32

#9. Pelvic auxilary scale
1. present
2. absent

#10. Breast scales size
1. reduced
2. moderately reduced
3. normal

#11. Breast scale state
1. deeply embedded
2. embedded
3. not embedded

#12. Unbranched dorsal ray
1. iii
2. iii-iv
3. iv

#13. Dorsal ray development
1. simple
2. partial serrations
3. serrated
4. bony serrated
5. spine

#14. Number of branched dorsal rays
1. 7
2. 7-8
3. 8
4. 8-10

#15. Number of branched anal rays
1. 5
2. 6
3. 7

#16. Tubercles
1. absent male and female
2. present male
3. present male and female

#17. Conical tubercles
1. no tubercles
2. few tubercles
3. conical

#18. Erupted tubercles
1. absent
2. present

#19. Pharyngeal teeth, row 1
1. 2
2. 1
3. 0

#20. Pharyngeal teeth, row 3
1. 5
2. 4

#21. Pharyngeal teeth form
1. slender
2. broad

#22. Intestine ratio = SL : intestine length
1. 0.5-1
2. 1.5-2.4
3. 2.5-3
4. above 3.5

#23. Intestine shape
1. s flexured
2. involuted

#24. Vertebrae
1. 34-37
2. 38-39
3. above 40

#25. Predorsal vertebrae
1. 0-12
2. 13
3. 14
4. 15

#26. Preanal vertebrae
1. 21 and more
2. 18-20

#27. Supraneurals
1. present well developed
2. reduced
3. absent

#28. Intermuscular bones (Axial skeleton)
1. well developed
2. reduced

#29. Frontal supraethmoids
1. non overlap (abut)
2. inter digitate
3. overlap

#30. Ossification of supraethmoids
1. ossified
2. reduced ossification

#31. Pterosphenoids
1. divided
2. joined

#32. Exoccipital border
1. no flange
2. slender flange
3. broad flange

#33. Exoccipital presence
1. process
2. no process

#34. Neurocranium shape
1. moderately deep and broad
2. shallow deep and narrow

#35. Supraorbitals canal
1. present
2. absent

#36. Supraorbital form
1. stout
2. slender

#37. Infraorbital lachrymal
1. low peak
2. high peak

#38. Dermospenotic
1. small
2. medium
3. large

#39. Supraopercle canal
1. present
2. absent

#40. Opercle
1. moderate
2. broad

#41. Premaxilla
1. slender and long
2. intermed
3. deep and short

#42. Maxilla
1. slender
2. intermed
3. deep

#43. Metapteragoid
1. concave
2. convex
3. convex deep notched

#44. Intercalars
1. present
2. vestigial
3. absent

#45. Weberian crest formation
1. irregular and simple
2. expanded distally
3. expanded anteriorly

APPENDIX 4: THE LARGE YELLOWFISH *BARBUS*

INTRODUCTION

Several of the large *Barbus* species, commonly known as the yellowfishes, were amongst the first southern African *Barbus* investigated karyologically (Oellermann 1988, Oellermann & Skelton 1990). All five members (*B. aeneus*, *B. capensis*, *B. kimberleyensis*, *B. natalensis* and *B. polylepis*) of the small-scaled group were found to have a high ploidy level (2n=148-150) (Oellermann & Skelton 1990), and one species of the large-scaled group (*Barbus marequensis*) also has a high ploidy level (2n=136) (Oellermann 1988). High ploidy levels are considered to represent evolutionary hexaploidy. The authors suggest that within high ploidy levels support a monophyletic lineage for these large *Barbus* (Oellermann & Skelton 1990).

The finding of evolutionary hexaploidy among the large *Barbus* stimulated karyological investigation of African *Barbus*. Four large *Barbus* species from Ethiopia, have high chromosome numbers represented by 2n=150 (Golubstov & Kryzanov 1993). Two large *Barbus* from West Africa have chromosome numbers ranging from 2n=148-150 (Guégan *et al.* 1995, Appendix 2). Guégan *et al.* 1995, further suggest that hexaploidy is probably a feature in the large African *Barbus* which are pan African in distribution.

In all three studies the respective authors have interpreted their data of high chromosome numbers as evidence of an hexaploid evolutionary ancestry within all the large African *Barbus*.

At least three ploidy levels have been reported within *Barbus*, *Diptychus* and *Puntius* species (Klinkhardt *et al.* 1995). Ploidy levels changes within the family Cyprinidae, including the most specious African genus *Barbus*, has been considered a complex phenomenon (Rab & Collares-Pereira 1995). The various levels of polyploidy may represent different kinds of origins and can therefore be regarded to be phylogenetically informative.

Oellermann & Skelton (1990) mention that their data, due to technical problems, may have been misinterpreted with regard to chromosome morphology. The technique used in the present study provides clearer chromosome resolution than the earlier technique in this laboratory, so that, a review of the karyotype of these large *Barbus* species is possible here (see chapters 2, 3).

A proposal that the hexaploid origin of large *Barbus* is a result of allopolyploidy or autopolyploidy has been presented (Oellermann & Skelton 1990, Guégan *et al.* 1995). This study also explores species "groups" of *Barbus* and *Pseudobarbus* having evolutionary tetraploid karyotypes. Comparative phylogenetic and karyological analysis of these species may contribute to suggesting possible outgroup candidates for the allo-hexaploid ancestor. Hence, we may be able to comment on hexaploid origin in the large *Barbus* of southern Africa.

METHODS

One specimen each of *B. capensis* and *B. polylepis*, and five specimens of *B. marequensis* were karyotyped following the protocol outlined in chapter 2. The karyotype morphology was visually assessed. All karyotyped specimens are housed at the JLB Smith Institute as RUSI voucher samples (Table 4.1)

Table 4.1 The number and sex of specimens used in karyological analysis, n=total number of specimens examined.

Species	n	♂	♀	RUSI	Source
B. capensis	1	1		52705	P. Skelton & N.P.E. James
B. polylepis	1		1	53163	R. Bills
B. marequensis	5	4	1	52700	D. Naran *et al.*

RESULTS

B. capensis

One male specimen from the Rondegat River, Olifants system (Table 4.1 was analysed. Modal chromosome number determined from 17 metaphase is 150 (Table 4.2). The biarm comprises 16**m** chromosomes, 58**sm**+42**st** chromosomes and the uniarm comprises 34**a** chromosomes (Table 4.3).

Table 4.2 Frequency distribution of chromosome counts from kidney cells of three large *Barbus* species, n=total number of metaphase cells examined.

Species	n	Chromosome count											
		<140	1	2	3	4	5	6	7	8	9	150	>151
B. capensis	17	3			1				1	1	2	9	
B. polylepis	15	2				1				1		11	
B. marequensis	35	6						1				27	1

Table 4.3 Detailed description of photokaryotype showing mean values, standard deviations (sd), modal values and range of number of chromosome components of *B. capensis*, n= number of metaphase cells examined.

	Chromosome categories		
♂,n=6	**m**	**sm+st**	**a**
Mean	15.7	101.7	34.7
sd	0.9	1.4	0.9
Mode	16	58+42	34
Range	14-18	100-104	34-36

Chromosome morphology: No heteromorphic chromosomes were observed in the metaphase spreads (Fig 4.1). Chromosome in all categories ranged in size from large to small (Fig 4.1).

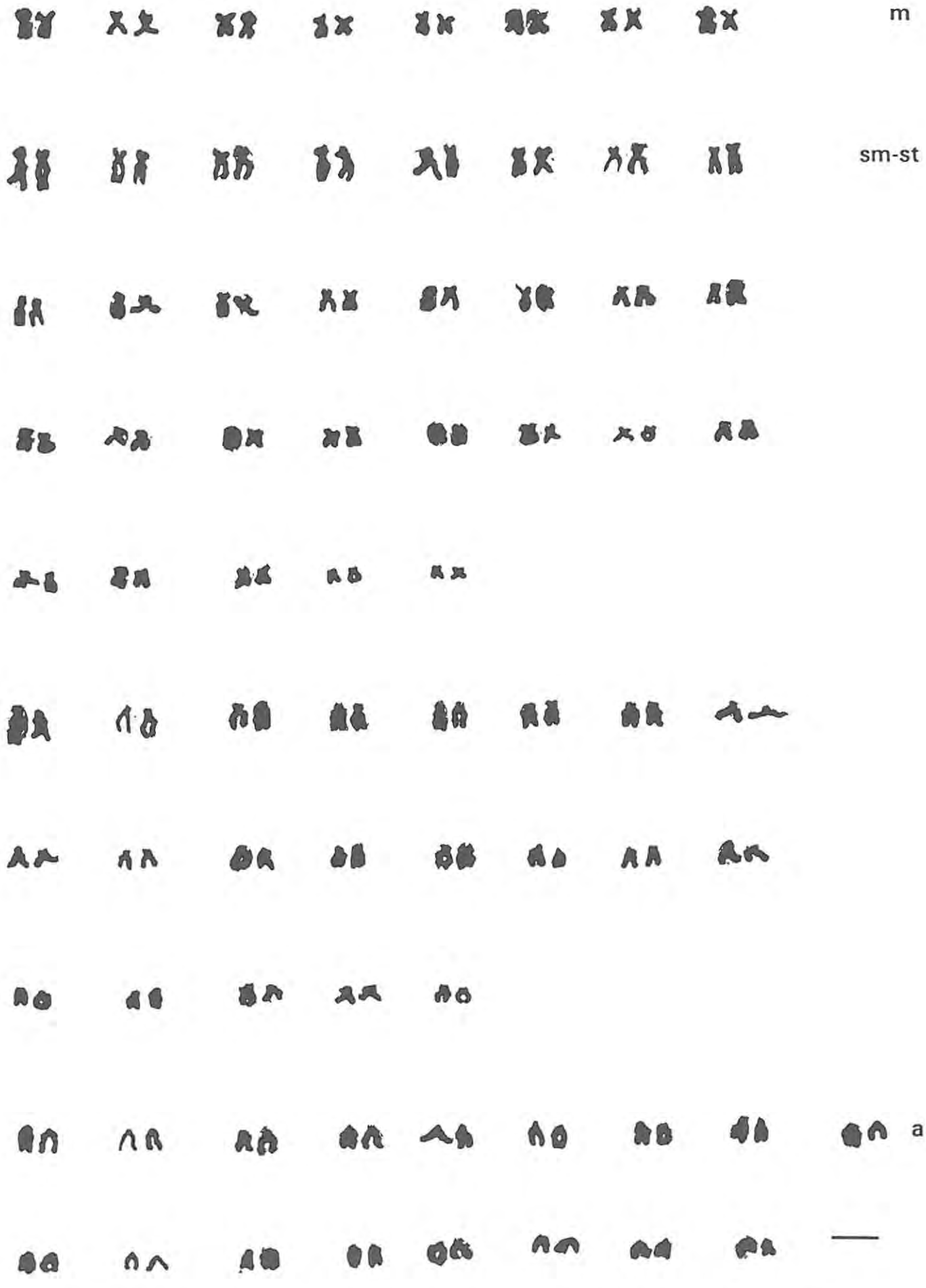

Figure 4.1 Photokaryotype of male *B. capensis* (Rondegat River) with chromosome formula 2n=16**m**+58**sm**+42**st**+34**a**=150. Scale bar = 5µm.

B. polylepis

One female specimen of *B. polylepis* from the Elands River, Incomati system (Table 4.1) was analysed. The modal chromosome number is 150 (Table 4.2). The modal values of 9 photokaryotypes indicate that the biarm component comprises 18**m** chromosomes, 60**sm**+42**st** component chromosome and the uniarm comprises 30**a** chromosomes (Table 4.4).

Table 4.4 Detailed description of photokaryotype showing mean values, standard deviations (sd), modal values and range of number of chromosome components of *B. polylepis*, n= number of metaphase cells examined.

	Chromosome categories		
♀,n=9	**m**	**sm+st**	**a**
Mean	18.3	98.6	33.1
sd	2.2	4.6	6.1
Mode	18	60+42	30
Range	14-20	88-104	30-48

Chromosome morphology: Chromosome in all categories ranged in size from large to small, no heteromorphic chromosomes were observed in the metaphase spreads of this specimen (Fig 4.2).

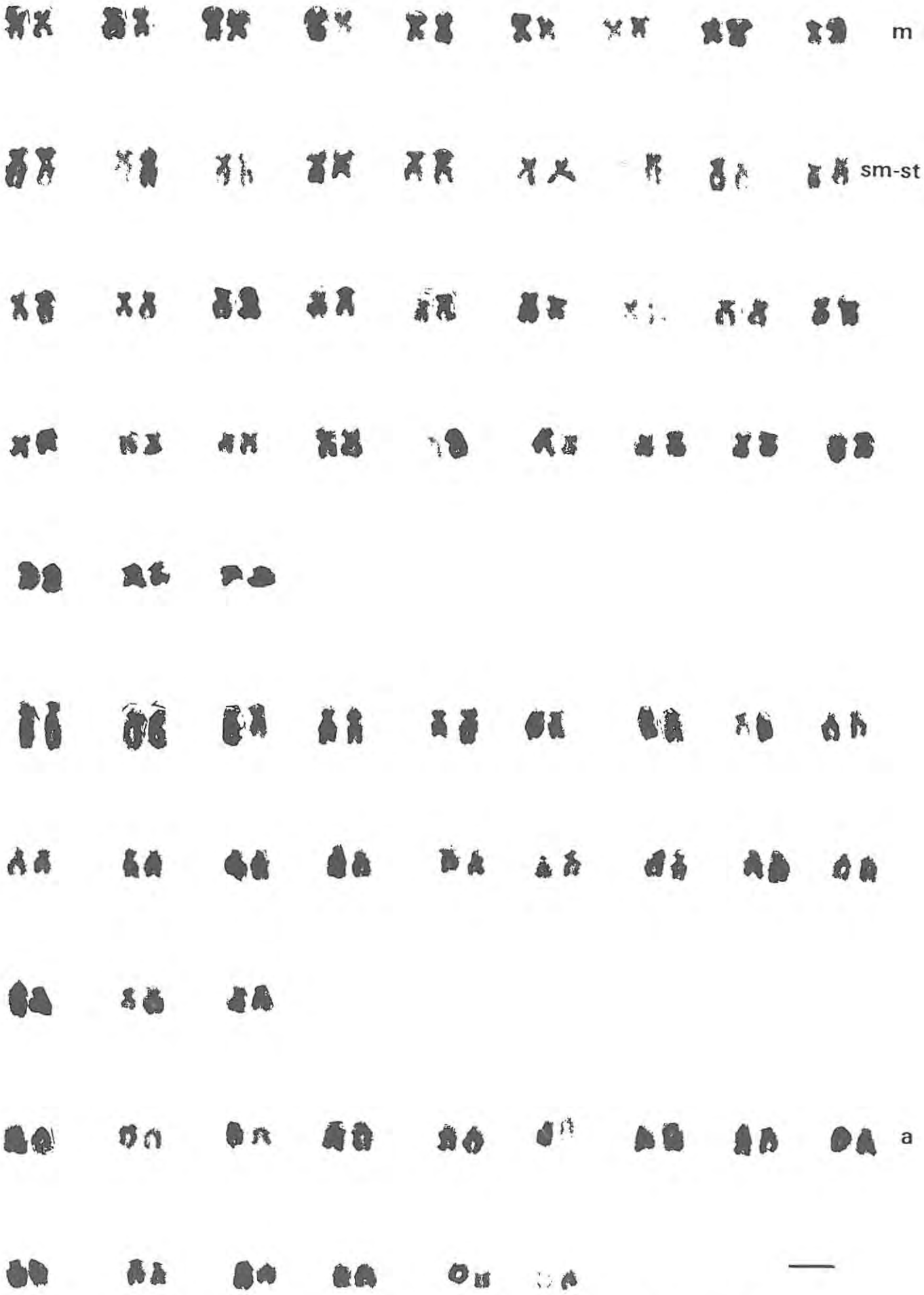

Figure 4.2 Photokaryotype of a male *B. polylepis* (Elands River) with chromosome formula 2n=18**m**+60**sm**+42**st**+30**a**=150. Scale bar = 5µm.

B. marequensis

The karyological analysis included four males and one female specimen collected from the type locality at Marico River of the Limpopo system (Table 4.1). Analysis of 35 metaphase cells shows the modal chromosome number is 150 (Table 4.2). The biarm component comprises 26**m** chromosomes, 44**sm**+42**st** chromosomes and the uniarm component comprises 38**a** chromosomes (Table 4.5).

Table 4.5 Detailed description of photokaryotypes showing mean values, standard deviations (sd), modal values and range of number of chromosome components of *B. marequensis*. n=number of metaphase cells examined.

	Chromosome categories		
♂&♀,n=9	**m**	**sm+st**	**a**
Mean	24.2	87.4	38.2
sd	3.9	2.9	1.1
Mode	26	44+42	38
Range	16-28	84-94	36-40

Chromosome morphology: No heteromorphic chromosomes were apparent in the five specimens examined, and chromosome in all categories ranged in size from large to small (Figure 4.3).

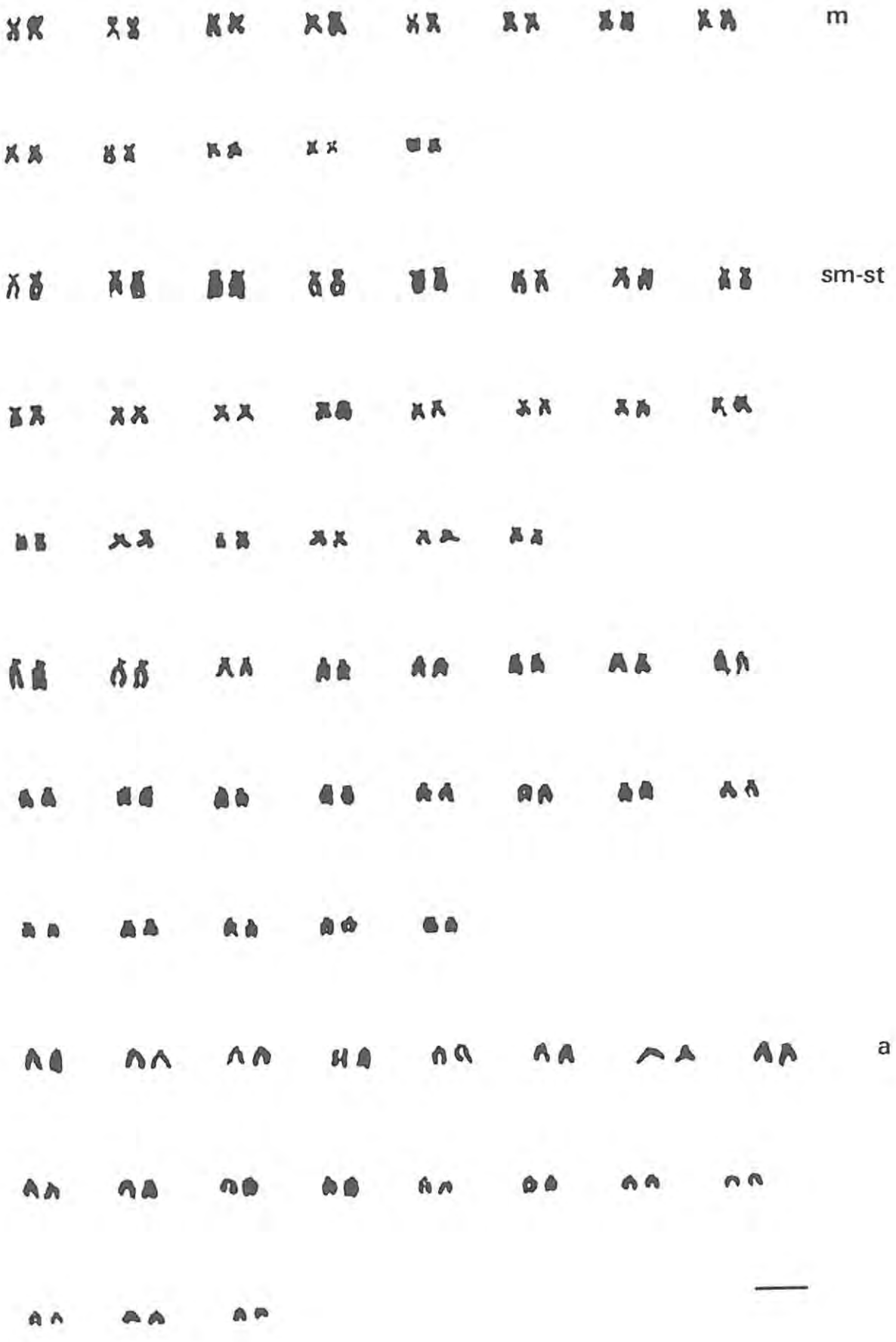

Figure 4.3 Photokaryotype of a female *B. marequensis* (Marico River) with chromosome formula 2n=26**m**+44**sm**+42**st**+38**a**=150. Scale bar = 5µm.

NF: total number of chromosome arms

NF1 values range from *B. polylepis* (214) to *B. marequensis* (220). High NF2 values range from 262-270 indicating the presence of a high proportion of biarmed chromosomes (Table 4.6)

Table 4.6 Total number of arms for three large yellowfish *Barbus* species analysed using two different calculations, NF1= 2(**m+sm**)+**st+a**, NF2= 2(**m+sm+st**)+**a**.

Species	n	NF1	NF2
B. capensis	6	224	266
B. polylepis	9	228	270
B. marequensis	9	220	262

Meiotic chromosomes

Meiotic chromosomes spreads from gonadal material of *B. capensis* and *B. marequensis* indicated the presence of only bivalent chromosomes (Fig 4.4).

Figure 4.4 Meiotic spreads showing bivalent chromosomes in *B. marequensis*. Scale bar = 5µm.

Summary of characteristics of large *Barbus* karyotypes

1. The majority of chromosomes are within the **sm+st** categories, with a range of **sm**(44-60)+**st**(42) pairs comprising 57-68% of the karyotype (Table 4.7).

2. The **m** chromosomes range from 16-26 pairs comprising 11-17% of the karyotype, *B. marequensis* has the highest number of **m** chromosomes (Table 4.7).

3. There are fewer **a** chromosomes comprising 20-25% of the karyotype.

4. The karyotype can be described as asymmetrical because of the dominant proportion of biarmed components compared to uniarmed components (Table 4.6).

5. No heteromorphic chromosomes were detected in the species examined (Fig. 4.1-4.3).

6. There were no apparent tetravalents in the meiotic chromosome spreads (Fig. 4.4).

Table 4.5 Modal values of karyotype components from kidney cells of three large yellowfish *Barbus* species.

Species	**m**	**sm+st**	**a**
B. capensis	16	58+42	34
B. polylepis	18	60+42	30
B. marequensis	26	44+42	38

DISCUSSION

Determination of chromosome number

Frequency distribution of chromosome numbers indicate that 20-35% of the metaphase cells examined are hypomodal (Table 4.2). The lower chromosomal counts are mainly due to chromosome overlapping and losses, as no evidence of chromosomal rearrangements was observed in the metaphase cells. The variation in chromosomal numbers is a technical artefact and is discussed in chapter 2.

Photokaryotype determination

There is variation in the numbers of chromosomes in a particular category in all three species examined as shown by the standard deviation values. The variation is mainly attributed to chromosome misallocation, which in turn is due to both duration and dose of colchicine treatment (see chapter 2,3).

Intraspecific comparisons

Gender

No heteromorphic chromosomes have as yet been reported for the hexaploid *Barbus* species (Oellermann & Skelton 1990, Golubstov & Kryzanov 1993, Guégan *et al.* 1995). Morphological parameters of yellowfishes showed an almost identical mean plot (r squared coefficient =1, x coefficient =0.99) for both male and female fish (Oellermann 1988). Suggesting that males are similar to females in appearance. Chromosome morphology, determined from photokaryotypes of male and female, *B. marequensis* reveals no heteromorphic pairs of chromosomes. However, heteromorphism in chromosome pairs may be masked by the multiple chromosomes introduced by polyploidy. These are possibly not detected using conventional staining.

Geographical differences

Variation in chromosome number and karyotypes of specimens obtained from different geographical locations have been reported within cyprinids of tetraploid and hexaploid composition (Mazik *et al.* 1989, Golubstov & Kryzanov 1993). Guégan *et al.* (1995) report a difference in chromosome number, at subspecies level of *B. bynni bynni* (2n=150) and *B. bynni occidentalis* (2n=148) from the Nile basin and Senegal basin respectively. Differences in chromosome morphology for *B. intermedius* (2n=150) from two localities has been reported (Golubstov & Kryzanov 1993).

The present study confirms the presence of high chromosome numbers of 150 reported by Oellermann & Skelton (1990), for both *B. capensis* and *B. polylepis*. A different chromosome number is found from that reported by Oellermann (1988) for *B. marequensis* (Appendix 2).

The specimens of *B. marequensis* were obtained from two separate river systems, the Marico River (this study) and the Blyde River (Oellermann 1988), widely separated tributaries of the Limpopo River system. *B. marequensis* is widely distributed from the middle and lower Zambezi to the Phongola systems (Skelton 1993). The different chromosome numbers in the two populations examined (2n=150, this study and 2n=134, Oellermann 1988), may be an indication of interpopulation variability within the species, and needs further investigation.

However, the differences in chromosome numbers from the two studies could also be attributed to the techniques used in both studies (chapter 2).

The yellowfishes are "extremely variable in shape and appearance, even within the same population" (Skelton 1993). *B. aeneus* and *B. capensis* sometimes have thickened lips which facilitates feeding off stony substrates; in the Elands river population of *B. polylepis* has a range of mouth forms, from thickened "rubberlip" type to where the broad lower lip has a sharp cutting edge (Gaigher 1975).

The range in phenotypes may be a result of the multiple chromosomes introduced by polyploidy, and may be evident in the karyotypes in these species. *B. polylepis* and *B. capensis* have relatively restricted distributions (Skelton 1993). Specimens of *B. capensis* in this study and Oellermann's (1988) study were collected from the same river system, but, from different localities; the Noordhoeks River (this study) and Clanwilliam hatchery, Olifants River system (Oellermann 1988). The specimens of *B. polylepis* were obtained from two separate river systems, the Incomati River (this study) and the Olifants River, Limpopo system (Oellermann 1988). Chromosome morphology of *B. capensis* and *B. polylepis* as presented in this study differs from that presented by Oellermann (1988), where the karyotype is asymmetrical, having a smaller biarm component (38.7-37.3% of the karyotype) than uniarm component (Appendix 2). The karyotypes in this study are asymmetric, with the biarm component dominant, comprising 77-80% of the karyotype (Table 4.7). This difference may indicate intraspecific variation. The results presented above are a comparison of species collected from only two localities and the variation in karyotypes need to be investigated further. This difference may also be as a result of the quality of metaphase spreads which contributes to different interpretation of the chromosome types (Rab & Collares-Pereira 1995).

Interspecies comparisons

Chromosome number and ploidy levels

Chromosome numbers ranging from 147-196 are found in several cyprinids. *Carassius auratus gibelio*, a European cyprinid, has chromosome counts of 98, 150, 158 and 160 (Rab & Collares-Pereira 1995). *Cyprinus carpio* is another European cyprinid with total chromosome counts of 147, 150 and 196 (Al-Sabti 1991). Eight Asian cyprinid species of the genus *Schizothorax* have chromosome counts of 148 (Zan *et al.* 1986). Chromosome numbers of 2n=148-150 have been reported in the African cyprinid genera *Varicorhinus* and large *Barbus* (Oellermann & Skelton 1990, Golubstov & Kryzanov 1993, Guégan *et al.* 1995). Two species of *V. nelspruitensis* (2n=138), Oellermann & Skelton (1990), and *V. beso* (2n=150) Golubstov & Kryzanov (1993) have been reported.

These high chromosome number are thought to represent hexaploidy and represent changes from 2n=48-50 and/or 2n=96-100 through polyploidization (Golubstov & Kryzanov 1993).

Hexaploid karyotypes of the southern African species have been taken as evidence of monophyly for the yellowfish group (Oellermann & Skelton 1990). However, with the findings of more large *Barbus* with hexaploid karyotype the monophyly may include other African large *Barbus* (Golubstov & Kryzanov 1993).

Skelton (1986) suggested that the most probable outgroup relationships with large *Barbus* may be species from east and central Africa or with Indian or southern Asian species (Skelton 1986). A phylogenetic comparison of these large *Barbus* species may provide further resolution to the taxonomy and systematics of the large genus.

Morphology of karyotypes

Schizothorax chromosomes have been grouped into three chromosome categories, **m**, **sm** and **st-a** (Zan *et al.* 1986, Yu *et al.* 1987). The reported karyotypes show that there are between 48-50**m** (32.4-33.8% of the total chromosome number) chromosomes, and 28 & 38**sm** chromosomes (18.9-25.7% of the total chromosome number). The uniarm chromosomes make up 44.5-52.7% (70-78 **st-a** chromosomes) of the karyotype (Klinkhardt *et al.* 1995). *Varicorhinus beso* has 44% of the total chromosome number comprised of **m-sm** chromosomes and 56% of **a** chromosomes; no **st** chromosomes are reported (Golubstov and Kryzanov 1993). The above descriptions indicate a more or less symmetric karyotype in both *Schizothorax* and *Varicorhinus*. The three southern African large *Barbus* species analysed show a typically cyprinid karyotype, where there is a gradual change in chromosomes size and centromere position from median to near terminal. All categories of chromosomes (**m**, **sm+st**, and **a**) are represented within the large *Barbus* karyotype analysed in this study (Figs 4.1, 4.2 & 4.3). There are approximately 16-26 pairs of **m** chromosomes (10-17% of the karyotype) present in the southern African *Barbus*, and approximately 30-38 **a** chromosomes (20-25% of the karyotype (Tables 4.5-4.5). The majority of the karyotype comprises **sm+st** chromosomes (57-68%) (Tables 4.5-4.5).

The karyotype of *B. marequensis* can not be compared to Oellermann's (1988) result, as he only listed a chromosome number and did not present a karyotype. However, the photokaryotype reveals that *B. marequensis* has a slightly higher number of **m** chromosomes (17% of the karyotype) than the *B. capensis* and *B. marequensis* (10-12% of the karyotype) and fewer **sm+st** chromosomes (57% of the karyotype).

The division of large-scaled/small-scaled groupings of the southern African large *Barbus* may be supported by major cytogenetic differences.

B. petitjeani has the highest number of **m** chromosomes yet reported within African large *Barbus* (24%) of the karyotype and there are 24 **a** chromosomes (16%) in its karyotype (Guégan *et al.* 1995, Appendix 2). The **m** and **sm** chromosomes of the Ethiopian hexaploid *Barbus* which comprise 26-60% of the karyotype, while the **a** chromosomes comprise 40-73% of the karyotype (Golubstov & Kryzanov 1993, Appendix 2).

The three southern African large *Barbus* have an asymmetrical karyotype with smaller uniarm component and a larger biarm component (Figs. 4.1, 4.2 & 4.3). In this respect they are similar to the west African species *B. petitjeani* but differ from the three large Ethiopian *Barbus*.

The common ancestor of the southern African yellowfish was proposed to have entered

southern Africa in the mid-Pliocene (Skelton 1980), this idea has been reviewed by Skelton (1993, 1994). Gill parasite studies of *B. petitjeani* has shown that it harbours very primitive parasites, in accordance with this finding it is thought that the species is primitive (Guégan *et al.* 1995). The similarity in the karyotype composition may indicate that the west African large *Barbus* may be the sister group to the southern African large *Barbus*, however, further data needs to be analysed to establish the validity of this relationship.

The west African species, *B. petitjeani* karyotype has been arranged in homomorphic hextets (Guégan *et al.* 1995). However, due to the gradual change in chromosome size and centromere position (see chapter 2), no clear hextets are apparent in the three southern African species analysed in this study.

In all three species the largest variation in chromosome size are apparent in the **sm+st** categories from the first to the last pair and with very little size differences (visually) in the **m** and **a** chromosome from the first to the last pair (Figs 4.1, 4.2 & 4.3).

NF: total number of chromosome arms

Hexaploid karyotype (2n=148) is presented in the genus *Schizothorax*, where NF values range from 216-226, indicating dominant presence of uniarm chromosomes (Zan *et al.* 1986). NF1 values for the three large Ethiopian *Barbus* range from 190-240, indicating that there are few biarm components in the karyotype. Considering only **m** chromosomes as biarmed, NF1 value for *B. petitjeani* is 186 (Guégan *et al.* 1995). *B. polylepis* has the highest NF1 value (228) of all the southern African large *Barbus*. *B. capensis* has a value of 224 followed by *B. marequensis* which has a value of 220. These values indicate that the southern African *Barbus* have higher numbers of biarms compared to the Ethiopian *Barbus*. A similar pattern is found between the European tetraploid *Barbus* (Collares-Pereira & Madeira 1990) and African tetraploid *Barbus* (chapters 3 & 5).

When considering the **st** chromosomes as biarmed (referred to as NF2), as done by Magtoon & Arai (1993). *B. capensis* has a value of 266 and *B. polylepis* which has a value of 270 has the highest number of arms. *B. petitjeani* has a value of 276. These values indicate, that the uniarm component is reduced in the southern African and West African large *Barbus*. The uniarm component of A chromosomes of the Ethiopian large *Barbus* ranges from 60-110, indicating a quite different pattern (Golubstov & Kryzanov 1993).

Meiotic chromosomes

Golubstov & Kryzanov (1993), observed exclusively bivalents in meiotic spreads from testis preparations of *B. intermedius*. A similar absence of multivalents within a *B. capensis* and *B. marequensis* testis preparation were observed. Golubstov & Kryzanov (1993) consider the absence of

multivalent chromosomes as and indication of the "remoteness of polyploidic event" in the hexaploid *Barbus* (as cited in Vasil'ev 1985). A similar condition to *B. intermedius* may apply to the southern African hexaploid species.

The polyploidy event may have occurred very early in the ancestry of this group and there has been sufficient time for diploidization.

Mode of reproduction

In the polyploid cyprinid species a range of reproduction modes have been reported (Rab & Collares-Pereira 1995). Polyploid species in *Rutilus* show gynogenetic mode of reproduction (Collares-Pereira 1989). In the hexa- and octaploid forms of *Carassius*, *Carassioides, Cyprinus* and *Procypris* there is no indication of normal bisexual reproduction (Fan & Liu 1990,in Golubstov & Kryzanov 1993). *Carasius auratus gibelio*, hexaploids which are produced by unisexual mode of reproduction has been reported (Rab & Collares-Pereira 1995). Within the hexaploid forms of *Carasius auratus* and schizotoracins there is evidence of normal bisexual reproduction (Rab & Collares-Pereira 1995).
Within the large *Barbus* of southern Africa and Ethiopia the mode of reproduction is also normally bisexual (Oellermann & Skelton 1990). The parthenogenic (gynogenetic) forms may be considered to be intermediate stages in the formation of sexually reproducing polyploids (Schultz 1979). The normal bisexual condition could be indicative of a derived ancestry of the southern African and Ethiopian large *Barbus* species.

Origin of hexaploidy

Available karyological data show that the African genus *Barbus* have three ploidy levels; diploid, tetraploid and hexaploid (Oellermann & Skelton 1990, Golubstov & Kryzanov 1993, Guégan *et al.* 1995 & present study). The most probable pathway of the origin of hexaploidy is via a tetraploid stage (Golubstov & Kryzanov 1993). Furthermore, Golubstov & Kryzanov (1993) suggest that the small and large *Barbus* relationship depends on discovering an intermediate tetraploid form(s) in Africa. They propose that the large *Barbus* of Africa have their ancestral link with a tetraploid *Barbus sensu stricto* in Europe or with the Asian genus *Tor* and a hexaploid event occurred before the largescale dispersal of *Barbus* over African. Until now (chapter 5), no large tetraploid *Barbus* species had been found, so, it was thought that ancestral group was probably beyond African limits (Golubstov & Kryzanov 1993). The presence of these tetraploids provides an opportunity to investigate their relationship to the large hexaploid species.

CONCLUSIONS

Although large *Barbus* species have the chromosome numbers of 2n=150, there are differences in the karyotypes of the species. Within the three southern African species, *B. marequensis* has a relatively high proportion of **m** and **a** chromosomes than *B. capensis* and *B. polylepis*.

The southern African species appear more similar to *B. petitjeani*, a West African species, than they are to the three Ethiopian species.

The monophyly of southern African large *Barbus* based on hexaploidy needs to be investigated further with other large *Barbus* species.

Appendix 5

CYTOMETRY : A separate analysis using erythrocyte diameter/volume was conducted to examine ploidy levels in southern African barbines

It has been shown by Benfey *et al.* (1984) that blood cell sizing is an effective method to screen for induced polyploidy in salmonid fishes. In order to test this for natural cyprinid polyploids I, with assistance of Dr Gert Steyn of the Zoology Department, Rand Afrikaans University, Johannesburg measured erythrocyte nucleus volume and diameter using a Coulter Multisizer. Fish were anaesthetized in a weak solution (0.01ml) of L-phenoxyethanol and blood samples were drawn into a heparinized microcapilary tube from a superficial scratch in the gular region. The blood sample was diluted with a Isoton II acid-free balanced electrolyte solution and treated with Zap-Oglobin lysing agent to remove the erythrocyte membrane. The Coulter Multisizer was calibrated with a 10.3µm diameter latex particle calibration standard.

RESULTS & DISCUSSION: The blood cell nuclear diameters and volumes of species analysed are given in Table 5.1

Table 5.1 Summary of erythrocyte nucleus cytometry of certain southern African barbine cyprinids. Values are the means of median measures of combined samples N=number of specimens, n=number of cells. * indicates tropical species.

SPECIES	N	n	Diameter (µm)	Volume (µm³)
Pseudobarbus afer	3	135292		13.58
P. burchelli	4	130861	3.06	15.07
P. burgi	2	128924	3.08	15.29
P. phlegethon	2	123178	3.08	15.34
P. tenuis	2	132591	3.09	15.51
Barbus anoplus	2	117816		8.02
B. amatolicus	2	131138		8.02
B. gurneyi	2	127710	2.52	8.40
B. motebensis	2	96780	2.57	8.85
*B. brevipinnis**	2	123581	2.49	8.11
*B. paludinosus**	2	134650	2.51	8.26
*B. trimaculatus**	1	136589		6.5
B. trevelyani	3	108441		13.00

The results indicate that the cell nucleus diameter and volume of different species from each ploidy group are similar and the expected increase of diameter and volume with increasing ploidy level occurs. In general terms tetraploid species show 2 fold (approximately a 1.8 times) increase in cell nuclear diameter and the hexaploid species a 3 fold increase on the diploid state. At this stage standards have not yet been established to determine genome size in different species.

CONCLUSION : These preliminary cytometry results confirm the ploidy levels determined from chromosome numbers.