Comparative karyological analysis of three species of *Bothriocephalus* Rudolphi 1808 (Cestoda:Pseudophyllidea) from *Schizothorax* species of Kashmir valley

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Abstract Karyotypes of three species of *Bothriocephalus* were studied using conventional Giemsa staining and comparative karyometric analysis. *Bothriocephalus claviceps* and *Bothriocephalus gregarius* showed a karyotype with $2n = 14$ chromosomes. All chromosomes of *B. claviceps* were biarmed, metacentric or meta-submetacentric. The two first pairs of homologues were markedly larger than the remaining elements and represented 48.62 % of the total chromosome length. The karyotype of *B. gregarius* had similar metric values for the chromosomes, but clearly differed in the chromosome morphology of pairs 5, 6 and 7, which had terminally located centromeres. The diploid number $2n = 12$ was found in *B. scorpii*. The karyotype consists of five pairs of metacentric and one pair of submeta–metacentric chromosomes. The possible pathways of karyotype evolution within *Bothriocephalus* spp. and their phylogenetic relations with the other karyologically studied groups in the order Pseudophyllidea are discussed.

Keywords *Bothriocephalus* · Giemsa staining · Karyotype · Centromere

Introduction

Two tapeworms of the large cosmopolitan genus *Bothriocephalus* Rudolphi 1808 are common parasites of marine

and freshwater fish hosts (Yamaguti 1959; Protasova 1977). The type species *Bothriocephalus scorpii*, which was originally reported from the *Schizothorax labiatus* in Kashmir, has subsequently been observed in more than 50 teleostean species from all of the seas and oceans of the Northern Hemisphere (Protasova 1977). Observations made on the morphology of *B. scorpii* from littoral fishes in Britain revealed differences between the specimens from different host species, but the observed variation was regarded as intraspecific (Jones 1975). However, the adult stages of cestodes tend to exhibit a fairly high degree of host specificity, and molecular techniques (isoenzymes, RAPD, sequencing and DNA–DNA hybridisation) applied on *Bothriocephalus* species complexes revealed that the original species *B. scorpii* constituted a complex of biological species highly specific for their host species (Renaud et al. 1983, 1986; Renaud and Gabrion 1984; Verneau et al. 1997a, b).

As with molecular data, cytogenetic information can reveal differences and similarities that may not be obvious at the morphological level. White (1978) estimated that more than 90 % of all speciation events are accompanied by karyotypic change. If this is correct, then chromosomal studies should be widely applicable to the problems of sorting groups of morphologically similar (sibling) species. Chromosomes are studied as a morphological manifestation of the genome in terms of their microscopically visible size, shape and number, and karyology represents a qualitative approach to phylogeny. To what extent, the karyotypic features can be suitable for phylogenetic inference? Patterns of chromosomal divergence within a group may not necessarily parallel those of morphological features (Gold 1980; Baker and Bickham 1980), but most often species related from a morphological point of view show karyological affinities. If karyotypic features are plotted over a phylogenetic tree based on molecular or

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morphological data, the processes involving chromosome evolution might be clarified.

The objective of the present study is to describe the karyotypes of three *Bothriocephalus* species: *Bothriocephalus claviceps*, *B. scorpii* and *Bothriocephalus* (supra sp. *scorpii*) *gregarius*, Renaud et al. (1983). Next, these results will be compared with published data obtained by molecular studies on *Bothriocephalus* spp. and with cytogenetic information available for other pseudophyllidean species. In addition, similarities and differences among the karyotypes will be related to evolutionary relationships within the Pseudophyllidea. The karyotypes of all three species are described for the first time.

Materials and methods

Schizothorax species were caught in Dal of Kashmir, with the help of local fisherman and were brought into Parasitology Research Laboratory, Department of Zoology, University of Kashmir, Srinagar. Specimens of *B. claviceps* of different sizes and maturity were obtained by the dissection of *Schizothorax niger*, whereas mature specimens of *B. gregarius* and *B. scorpii* used for karyological analysis were collected from the intestines of *S. curvifrons* and *S. labiatus* respectively.

Living specimens were incubated for 3–4 h in 0.01 % colchicines in physiological solution (0.65 %) at room temperature in order to arrest the cell division and were then transferred to distilled water for 1 h for hypotony (Frydrychova and Marec 2002). Fixation was in a freshly prepared mixture of ethanol and glacial acetic acid (3:1). Specimens were kept refrigerated until they could be processed in the laboratory. Slides were made from cell-suspensions using an air-drying technique (Petkeviciute and Ieshko 1991), stained with 4 % Giemsa, pH 6.8, rinsed in tap water and allowed to dry. The slides were placed on a heating plate at 45 °C and a drop of cell suspension was slowly drawn along the slide until it evaporated. Slides were dehydrated in an ethanol series (30, 50, 70, 90 and 100 %, 5 min each) and stored at –20 °C until use. Slides were stained with 4 % Giemsa solution (pH 6.8) in phosphate buffer for 30 min, rinsed in tap water and allowed to dry. The best chromosome plates were photographed and used for morphological studies.

Stable mitotic metaphases were photographed with Leica DM LS2 trinocular photomicroscope with a 100× objective under oil immersion using Mikrat-300 film. For karyotyping, chromosomes were cut out of the photographs and paired on the basis of size and centromeric position. Means and standard deviations of the absolute length in micrometres, relative length ($100 \times$ absolute chromosome pair length/total length of haploid complement) and the

centromeric index ($100 \times$ length of short arm/total length of chromosome) were calculated for each chromosome pair. The centromere position on the chromosomes was classified according to the nomenclature of Levan et al. (1964). A chromosome is metacentric (m) if the ci falls in the range of 37.5–50.0, submetacentric (sm) if 25.0–37.5, subtelocentric (st) if 12.5–25.0 and acrocentric (a) if <12.5. When a centromere position was on the borderline between two categories, the confidence limits of the means were calculated and two chromosome categories are reported. Data were analysed by the Student's *t* test. Results were considered significant when $P < 0.05$.

Results

Analysis of 10 mitotic metaphase spreads from seven specimens showed that the modal diploid complement of *B. claviceps* contains 14 chromosomes ($2n = 14$). The percentage of aneuploid cells ($2n = 11, 13$) was 8.8. The karyotype (Fig. 1a, c) included six metacentric and one meta–submetacentric chromosome pair (No. 6). Two first pairs of metacentric elements are distinctly larger than the remaining chromosomes and contributed 48.62 % to the total chromosome length. The karyotype formula of *B. claviceps* can be summarised as $2n = 14 = 12m + 2m/sm$. A summary of the results obtained after measuring the Giemsa-stained chromosomes of seven complete metaphase plates is given in Table 1. The chromosomes are middle sized; the largest measured 6.63 µm and the smallest were 1.77 µm. The total chromosome length of the haploid complement was 24.74 µm. The homologues of pairs 4 and 5 could not be distinguished clearly. There are no statistically significant differences in their sizes and centromeric indexes.

Examination of 78 mitotic metaphase plates from eight specimens of *B. gregarius* revealed the same modal diploid number, $2n = 14$ (Fig. 1b, d). Eight (10.3 %) aneuploid spreads displaying a chromosome number lower than the mode ($2n = 12, 13$) were also encountered. These might be related to technical shortcomings. Three (3.9 %) tetraploid cells ($4n = 28$) were found.

According to the centromere position, chromosome pairs 1, 2, 3 and 4 are considered metacentric; pairs 5, 6 and 7 are acrocentric. The karyotype formula can be summarised as $2n = 14 = 8m + 6a$. The largest chromosomes measured 7.22 µm, while the shortest were 2.19 µm long (Table 2). The mean total chromosome length of the haploid complement was 27.73 µm. It is notable that the individual identification of the homologues of pairs 5 and 6 was difficult due to the similarities in size and shape. Comparing the relative lengths and centromeric

Fig. 1 a–d Mitotic chromosomes of *Bothriocephalus* spp. a, c Mitotic metaphase and karyotype of *B. claviceps*. b, d Mitotic metaphase and karyotype of *B. gregarius*. Bar 10 μ m

indices of the individual chromosome pairs of *B. claviceps* with those of *B. gregarius*, the most distinct interspecific differences were observed in the centromeric index values of chromosome pairs 5, 6 and 7. In *B. gregarius* these chromosome pairs are acrocentric, while in the karyotype of *B. claviceps* they are biarmed.

All specimens of *B. scorpii* studied had 12 chromosomes in their modal diploid sets (Fig. 2). The results are based on the observation of 170 mitotic metaphases from 14 mature specimens. The modal diploid number, $2n = 12$, was found in 84.7 % of cells; 25 cells (14.7 %) were aneuploid ($2n = 10, 11$) and one cell (0.6 %) was tetraploid ($4n = 24$).

Table 1 Measurements (mean $\pm$ SD) and classification of chromosomes of *Bothriocephalus claviceps* (from seven metaphase plates)

Chromosome number	Absolute length (μ m)	Relative length (%)	Centromeric index	Classification
1	6.63 ± 0.98	26.77 ± 1.43	44.31 ± 1.71	Metacentric
2	5.42 ± 0.82	21.85 ± 0.89	45.67 ± 1.38	Metacentric
3	3.45 ± 0.44	13.95 ± 0.56	46.51 ± 1.54	Metacentric
4	2.93 ± 0.37	11.87 ± 0.89	44.46 ± 1.29	Metacentric
5	2.68 ± 0.30	10.87 ± 0.49	44.68 ± 2.30	Metacentric
6	1.86 ± 0.22	7.52 ± 0.35	37.69 ± 3.74	Metacentric–submetacentric
7	1.77 ± 0.22	7.15 ± 0.27	43.79 ± 2.98	Metacentric

Table 2 Measurements (mean $\pm$ SD) and classification of chromosomes of *Bothriocephalus gregarius* (from eight metaphase plates)

Chromosome number	Absolute length (μ m)	Relative length (%)	Centromeric index	Classification
1	7.22 ± 1.35	26.09 ± 2.06	46.13 ± 1.31	Metacentric
2	5.73 ± 1.08	20.70 ± 1.05	42.24 ± 3.66	Metacentric
3	4.04 ± 0.79	14.57 ± 0.81	37.84 ± 4.61	Metacentric
4	3.51 ± 0.77	12.54 ± 0.88	39.30 ± 1.59	Metacentric
5	2.62 ± 0.49	9.48 ± 0.76	5.43 ± 2.38	Acrocentric
6	2.42 ± 0.53	8.70 ± 0.37	5.74 ± 3.38	Acrocentric
7	2.19 ± 0.53	7.83 ± 0.58	3.14 ± 2.88	Acrocentric

Measurements of absolute length give values from 3.25–6.50 μ m (Table 3). The total length of the haploid genome reaches 26.83 μ m. The karyotype is composed exclusively of biarmed elements. Chromosomes 1, 2, 3, 4, and 6 are metacentric; pair 5 represents an intermediate between the submeta- and metacentric type of structure. Thus, the formula of the karyotype is $2n = 12 = 10 m + 2 sm/m$.

In order to better visualise the existing differences in chromosome morphology, ideograms were constructed using the centromere indexes and relative length values (Fig. 3).

Discussion

This survey on the karyotypes of three congeneric species revealed interspecific differences in diploid numbers and chromosome morphology. Karyotypes of *B. claviceps* and *B. gregarius* both possess 14 chromosomes and are similar in their ranges of chromosome size but clearly differ in the position of the centromere on the three last chromosome pairs (5, 6 and 7). These differences, involving the value of centromeric indices, may be the result of pericentric inversions (Nedeva and Mutafova 1988). Thus, even in a study such as the present one based on conventional techniques of staining, it has been possible to discriminate between species having identical diploid numbers.

Study of the karyotype of *B. scorpii* showed a reduced chromosome number, $2n = 12$ and a fairly symmetric complement composed exclusively of biarmed chromosomes.

Fig. 2 Mitotic metaphase and karyotype of *Bothriocephalus scorpii*. Bar 10 μ m

This chromosome number, $n = 6$, $2n = 12$, was revealed for *B. scorpii*, the parasite of *Myoxocephalus* sp., by Bazitov (1978). The chromosomes were counted in primary spermatocytes in histological sections. However, chromosome numbers obtained from meiotic divisions observed in histological sections are questionable, but re-examination based on the air-drying method confirmed the previous result.

The total haploid complement length (TCL) of *B. scorpii* does not differ significantly from that of the two other two species. Since TCL is generally correlated with genome size (Cavalier-Smith 1985), we can assume that no significant differences in nuclear DNA content occur among the species of *Bothriocephalus* studied. Thus, the chromosome complement of *B. scorpii* with $2n = 12$ presumably arose from a karyotype with 14 chromosomes by the centromeric fusion of two small acrocentric chromosomes (such acrocentric chromosomes were revealed in the karyotype of *B. gregarius*), resulting in the formation of a large metacentric chromosome pair, most likely pair 3 (according to comparative analysis of chromosome lengths). Further study of chromosome constitutions with banding techniques should be useful to answer the question of which pairs of chromosomes were involved in this rearrangement.

The diploid value of $2n = 14$ was also obtained by Nedeva and Nedkova (1988) for *Bothriocephalus acheilognathi* Yamaguti, 1934, a parasite of *Cyprinus carpio*. The karyotype resembles that of *B. claviceps* in all chromosomes, which are metacentric. Differences exist in the centromeric index values of the corresponding chromosome pairs, especially pairs 3 and 6. Part of the small

Table 3 Measurements (mean $\pm$ SD) and classification of chromosomes of *Bothriocephalus scorpii* (from ten metaphase plates)

Chromosome number	Absolute length (μ m)	Relative length (%)	Centromeric index	Classification
1	6.02 $\pm$ 0.85	24.30 $\pm$ 1.30	44.20 $\pm$ 1.77	Metacentric
2	5.16 $\pm$ 0.84	19.21 $\pm$ 0.88	42.34 $\pm$ 1.76	Metacentric
3	4.36 $\pm$ 0.74	16.21 $\pm$ 0.77	38.69 $\pm$ 3.60	Metacentric
4	3.95 $\pm$ 0.60	14.54 $\pm$ 0.89	38.16 $\pm$ 3.47	Metacentric
5	3.61 $\pm$ 0.44	13.50 $\pm$ 0.65	35.21 $\pm$ 3.34	Submetacentric–metacentric
6	3.25 $\pm$ 0.55	12.07 $\pm$ 0.67	41.91 $\pm$ 1.58	Metacentric

Fig. 3 Idiogram of *B. claviceps* **a**, *B. gregarius* **b** and *B. scorpii* **c**

differences in chromosomal shapes has possibly arisen from different chromosome condensation rather than from structural change. Chromosomes of *Bothriocephalus acheilognathi* were found to be considerably smaller than those of *B. claviceps*, with absolute length values ranging from 1.30 to 2.71 μm (Nedeva and Mutafova 1988). However, differences in the absolute length of chromosomes may be partially accounted for by differences in slide preparation technique and comparisons of chromosome morphologies are best made on the results obtained in single laboratories using standardized techniques.

It is notable that the genetic distances estimated from DNA/DNA hybridisation experiments and sequencing of rDNA revealed low levels of divergence among seven marine species of *Bothriocephalus*, including five species previously known under the name *B. scorpii* (Verneau et al. 1997a, b). In these studies, *B. claviceps*, the only fresh water species included, was widely divergent from all of the other bothriocephalids studied. The molecular data showed that the differentiation of the *B. scorpii* sibling species complex occurred recently and over a short time (Verneau et al. 1997b). Nevertheless, it was accompanied by significant karyotypic changes between two of the species studied, *B. scorpii* and *B. gregarius*. These results could be additional supporting evidence for the validity of new species. It is to be supposed that the karyotypic analysis of the other related species could reveal pathways of karyotype evolution and speciation in the group.

Recent phylogenetic analysis of tapeworms based on complete sequences of the 18S rRNA genes confirmed the monophyletic status of the genus *Bothriocephalus* and revealed the closer relationship of freshwater species to each other than to the marine cestode *B. scorpii* (Kodedova et al. 2000). Comparative karyological data are in agreement with the molecular findings: according to the structure of karyotypes, the freshwater species *B. claviceps* and *B. acheilognathi* are more closely related to each other than to the marine species *B. gregarius* and *B. scorpii*.

Chromosomal data for pseudophyllideans are very limited. Only 15 species have been studied karyologically so far. Since the last review (Petkeviciute 1996), three additional species, the representatives of the previously recognised family Amphicotylidae (syn. *Triaenophoridae* according to Bray et al. 1994) were studied (see Spakulova and Sotnikova 1999; Petkeviciute and Bondarenko 2001). Most pseudophyllideans exhibit a chromosome number of $2n = 13$ or $2n = 16$. *Bothriocephalus* spp. possess lower numbers: $2n = 12$ and $2n = 14$, but chromosome morphology follows the trend seen in other pseudophyllidean cestodes in which more than half of the mitotic chromosomes are meta- and submetacentrics.

Interspecific comparisons of the chromosome morphology of congeneric species of pseudophyllidean cestodes

have so far been performed in the genera *Diphyllbothrium*, *Triaenophorus* and *Eubothrium* (Wikgren and Gustafsson 1965; Petkeviciute and Ieshko 1991; Petkeviciute and Bondarenko 2001). Three species of the genus *Diphyllbothrium*: *D. latum*, *D. osmeri* (= *ditremum*) and *D. dendriticum*, studied using the squash method, showed $2n = 18$ with no differences in karyotypes, which were composed of seven pairs of metacentric and two pairs of submetacentric chromosomes (Wikgren and Gustafsson 1965). However, a more recent study of *D. ditremum* using an air-dried method revealed a somewhat different karyotype with the formula $2n = 18 = 10 \text{ m} + 2 \text{ m/sm} + 2 \text{ sm/m} + 2 \text{ sm} + 2 \text{ sm/st}$ (Petkeviciute 1992). Since the air-dried preparations usually contain less contracted chromosomes, their morphology is more distinct and, possibly, the re-examination of *Diphyllbothrium* spp. would reveal species specific characters in chromosome morphology.

Two species of the genus *Triaenophorus* were found to have different chromosome sets, differing significantly both in the number of chromosomes: $2n = 26$ in *Triaenophorus nodulosus* and $2n = 18$ in *Triaenophorus crassus*, and in their morphology (Petkeviciute and Ieshko 1991). Three species of *Eubothrium* showed the same diploid number, $2n = 16$, but exhibited clear interspecific differences in the centromeric index values of the corresponding chromosomes (Petkeviciute and Bondarenko 2001).

In general, comparative analyses revealed different types of chromosome rearrangements among pseudophyllidean species having a comparatively close phylogenetic relationship. One type was characterised by a chromosome rearrangement accompanied by drastic changes in chromosome number and karyotype. Chromosome rearrangements among *Triaenophorus* spp. is the only example of this type of karyotypic evolution. The other type was characterised by chromosome rearrangements usually not accompanied by any change in chromosome number. Karyological evolution basically involves the position of the centromere, presumably due to the pericentric inversions of different entities. This type of differentiation of chromosome sets is evident from the comparative analysis of the congeneric species of *Eubothrium*. The small number of chromosomes ($2n = 12$, $2n = 14$) together with the predominant occurrence of bi-armed chromosomes in karyotypes of *Bothriocephalus* spp. indicates that the fusion of chromosomes has played some role in evolution. The fixation of centromeric fusions in natural populations often encounters minimal meiotic problems due to the ability of trivalents to segregate normally (Baker and Bickham 1986). Cytogenetic theory leads to the prediction that centromeric fusion would be among the most common types of chromosomal rearrangement incorporated in evolution, but no cases of speciation by centromeric fusions (or fissions) have been revealed among

pseudophyllidean cestodes to date. In many systematic groups, chromosome sets containing many acrocentrics are considered primitive; evolutionarily younger species tend to have symmetrical karyotypes with few, mostly metacentric chromosomes (White 1978; Grossman and Cain 1981; Birstein 1987). Thus karyological data provides evidence to indicate that the Bothriocephalidae is a highly specialised family. The reduction of the chromosome numbers could be an evolutionary tendency in the order Pseudophyllidea. However, knowledge of pseudophyllidean chromosomes, based on recent techniques, is still very fragmentary and extensive application of karyotype data to phylogenetic problems must await further studies on additional species in various families. Yet the available data indicate certain points of interest. Karyological results are entirely congruent with present phylogenies based on molecular and morphological studies in that the diphyllbothriids form a distinct clade within the Pseudophyllidea (Mariaux 1998; Bray et al. 1999; Kodedova et al. 2000). All of the diphyllbothriids studied (*Diphyllbothrium*, *Ligula*, *Schistocephalus*, *Spirometra*) show close karyological affinities in having the same chromosome number ($2n = 18$ or $3n = 27$); only minor differences exist in the relative lengths of the corresponding chromosomes (for a review see Petkeviciute 1996). The karyotypes of species of the genus *Bothriocephalus* and *Eubothrium*, differing in chromosome number, resemble each other in having the two first pairs of metacentric chromosomes markedly larger than the remaining elements. These two pairs of large metacentrics are most stable in all of the karyotypes studied so far and may show phylogenetic affinities among diversified families. The karyological relations between *Bothriocephalus* and *Triaenophorus* are obscure. Many more analyses at a deeper level would be necessary in order to provide more certain conclusions on this subject.

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