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CHROMOSOMAL ANALYSIS OF *BOTHRIOCEPHALUS ACHEILOGNATHI* YAMAGUTI, 1934 (CESTODA: PSEUDOPHYLLIDEA) IN JAMMU AND KASHMIR

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ABSTRACT
The diploid complements of mitotic metaphase plates of *Bothriocephalus acheilognathi* (Yamaguti), a specific parasite of *Schizothorax* spp. were studied using conventional Giesma staining and karyometric analysis. *Bothriocephalus acheilognathi* showed a karyotype with 2n = 14 chromosomes which are all biarmed metacentric, meta-submetacentric and submetacentric. The chromosomes are small – their total length ranges from 2 to 8.3 µm. For Karyotyping, chromosomes were cut out of the photomicrographs and paired on the basis of size and centromere position. Relative lengths of chromosomes were calculated by the division of the individual chromosome length by the total haploid length. Centromeric indices (Ci) were determined by the division of the length. Measurements are based on all chromosomes from 10 metaphase spreads for each species.

KEY WORDS: *Bothriocephalus acheilognathi*, chromosomes, Giesma staining, *Schizothorax* spp.

INTRODUCTION

Bothriocephalus acheilognathi which was initially called *Bothriocephalus gowkongensis* Yeh, 1955 (now a synonym of *B. acheilognathi* Yamaguti, 1934), was first found in the 1950s as an important pathogen in grass carp *Ctenopharyngodon idella* (Cuvier and Valenciennes), the major fish species in aquaculture in China (Liao and Shih, 1956). With the subsequent finding of bothriocephalid cestodes in grass carp and other cyprinid fish during the 1960s and 70s in other countries, several new taxa were erected in the genus, and, consequently, disagreement over the identity of these cestodes in cyprinid fishes was inevitable (Pool and Chubb, 1985). In a scanning electron microscope study, Pool and Chubb (1985) compared the scoleces of described species of *Bothriocephalus* Rudolphi, 1808 parasitic in cyprinid fishes, and they concluded that only one species, *B. acheilognathi* Yamaguti, 1934, occurred in this group of fishes. Recent surveys have shown that some non-cyprinid fishes also harbor pseudophyllidean cestodes which have been designated as *B. acheilognathi*, and that the geographical distribution of this cestode is still increasing (Hoole, 1994; Font and Tate, 1994; Dove *et al.*, 1997; Brouder and Hoffnagle, 1997; Scholz, 1997; Nie *et al.*, 2000), implying that the parasite may have a high potential to colonize both new definitive hosts and new localities (Kennedy, 1994). It would, therefore, be interesting to use modern molecular tools to investigate the evolutionary variation in this parasite during colonization and to elucidate the relationships of bothriocephalid cestodes parasitizing cyprinid fishes.

Molecular genetic approaches have been used in investigations into helminth systematics and phylogeny, and some parameters, such as internal transcribed spacer of ribosomal DNA (ITS rDNA), have proved valuable for determining the phylogenetic relationships of closely-related species (Bowles *et al.*, 1995). By examining the ITS rDNA sequences and DNA/DNA hybridization, Verneau *et al.* (1997) confirmed that *B. scorpii* (Muller, 1776) constituted a complex of sibling biological species, and considered that the isolation and differentiation of the species in the complex occurred recently and over a short time span. In 1998, Liao and Lun (1998) used random amplified polymorphic DNA (RAPD) analysis to compare *Bothriocephalus* in grass carp, common carp *Cyprinus carpio* L. and another cyprinid, *Opsariichthys bidens* Gunther, and suggested that *B. opsariichthydis* Yamaguti, 1934 should be considered a valid species. More recently, Feng and Liao (2000), also using the RAPD technique, found high genetic diversity among bothriocephalid cestodes in both grass carp and common carp collected in different localities in China.

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

MATERIALS AND METHODS

Specimens of *Bothriocephalus acheilognathi* of different sizes and maturity used for karyological analysis were obtained by the dissection of *Schizothorax* species, caught in River Jhelum of Kashmir. Whole living specimens were placed in physiological saline (0.65 NaCl) containing Colchicine (0.01 %) for 3-4 hours at room temperature, then transferred into distilled water for about 1 hour for hypotony and fixed in Ethanol-Glacial Acetic Acid (3: 1), with two changes, 15 minutes each. Spread preparation of mitotic and meiotic chromosomes was made as described by

Frydrychova and Marec (2002). Small portions (medullary parenchyma with testes) of fixed worms were transferred into drop of 60 % acetic acid on a slide and torn into fine pieces with the help of tungsten needles. Then the slide was placed on a heating plate at 45°C and the drop of cell suspension was slowly drawn along the slide until it evaporated. Slides were dehydrated in an ethanol series (70 %, 80 % and 100 %, 1 minute each) and stored at -20°C until use. Slides were stained with 4 % Giesma solution (pH 6.8 in phosphate buffer) for 30 minutes, rinsed in tap water and allowed to dry. The best chromosome plates were photographed and used for morphological studies. For Karyotyping, chromosomes were cut out of the photomicrographs and paired on the basis of size and centromere position. Relative lengths of chromosomes were calculated by the division of the individual chromosome length by the total haploid length. Centromeric indices (Ci) were determined by the division of the length. Measurements are based on all chromosomes from 10 metaphase spreads for each species. The terminology relating to centromere position follows that of Levan *et al.*, (1964). A chromosome is metacentric (m) if the Ci falls in the range 37.5 – 50.0, sub metacentric (sm) if 25.0 – 37.5, sub telocentric (st) if 12.5 – 25.0 and acrocentric (a) if < 12.5. When the centromere position was on the borderline between two categories, both are listed. Data were analyzed by Students t-test and in all cases were normally distributed. Results were considered significant when $P < 0.05$.

RESULTS

The results are based on observations of 140 mitotic metaphases of dividing embryonal and/or gametogonial cells occurring in 2 gravid strobila. Cell suspensions made from 14 other strobila did not contain mitotic cells, although juvenile, mature, and gravid worms were studied. The diploid number 2n = 14 chromosomes (Fig. 1) was found in 82% of cells, whereas 8.8 % of cells were aneuploid (2n = 11, 13). In all, 1 cell contained 16 chromosomes; some other plates containing fewer chromosomes (12 or 13) were very probably artificially incomplete. Overall, 6 meiotic spermatocytes showing 7 bivalents during diplotene confirmed the modal diploid number of 14 chromosomes (7 pairs).

The somatic complement of this species revealed a diploid number of 2n=14 (Figure 2a) comprising 08 metacentric pairs, 2 meta-sub metacentric pairs and 4 sub metacentric pairs of chromosomes and a fundamental arm number (FN) = 56 (Figure 2b). The chromosomes range in length between 2 µm to 8.3 µm. The total length of the haploid complement equals 48.0 µm. Arm ratio of the complement ranges between 1- 2.12 and the centromeric index ranges between 32- 50 (Table 1).

On the basis of absolute length and centromeric position the chromosomes have been arranged in order of decreasing length in an ideogram (Figure 2).

Karyotype Formula:

(K) 2n=14= 08m+2m-sm+4sm

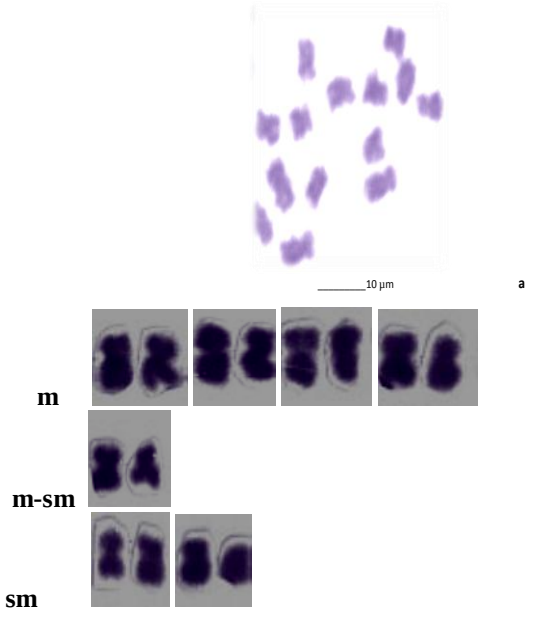

Figure 1a. Chromosome preparation of *Bothriocephalus acheilognathi*
1b. Karyotype constructed from mitotic cells of *Bothriocephalus acheilognathi* stained with Giemsa (m= metacentric., m-sm= meta- submetacentric., sm= submetacentric)

Table 1. Chromosome morphometry of *Bothriocephalus acheilognathi* (m=metacentric, m-sm=meta-sub metacentric and sm=sub metacentric).

Pair No.	Length of short arm	Length of long arm	Total length (µm) L+S	Arm ratio (L/S)	Centromeric index (ci)	Category
1	3.9	4.2	8.1	1.07	48.14	m
2	3.7	3.7	7.4	1.00	50.00	m
3	3.2	3.7	6.9	1.15	46.37	m
4	1	1	2	1.00	50.00	m
5	3.2	5.1	8.3	1.59	38.55	m-Sm
6	2.9	5.4	8.3	1.86	34.9	Sm
7	2.5	5.3	7.8	2.12	32.00	Sm

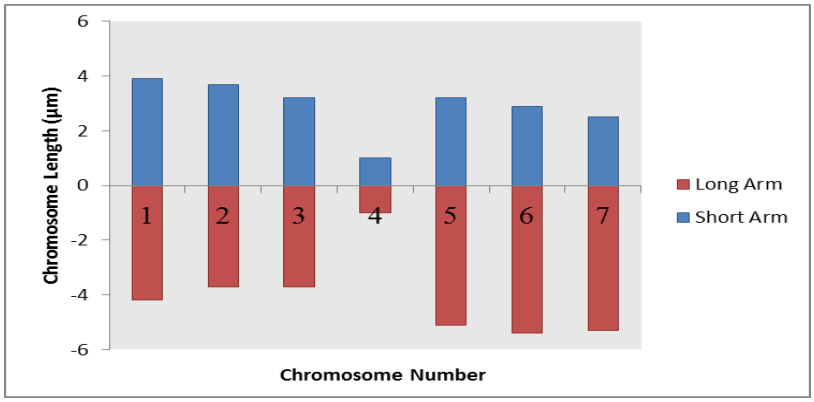

Figure 2. Haploid ideogram of *Bothriocephalus acheilognathi*.

DISCUSSION

Bothriocephalus acheilognathi is a specific parasite of *Schizothorax* species. The classification of bothriocephalids parasitizing cyprinid fishes was very confused until Pool & Chubb (1985) and Pool (1987) suggested that there existed only one species, *B. acheilognathi*, with numerous synonyms. *B. acheilognathi* has a typical spherical or heart shaped scolex with short, deep bothria directed anterolaterally which makes it easy to differentiate from its congeners. *B. acheilognathi* is unusual in its extreme morphological variability world-wide geographical distribution and extraordinarily wide spectrum of definitive host species. It was expected that a karyology study of *Bothriocephalus acheilognathi* might provide some data that would be useful for the assessment of possible relationships of this tapeworm to other Pseudophyllidean genera and families. The results of the present study, however, show that the chromosomal characteristics of *Bothriocephalus acheilognathi* differ considerably from those of the most closely related Pseudophyllidean species that have thus far been studied cytogenetically. *Eubothrium rugosum*, a member of the previously recognized family Amphicotylidae (syn. Triaenophoridae according to Bray *et al*. 1994), is characterized by smaller (absolute length up to 8.8 µm), more (2n = 16), and morphologically different chromosomes (Petkeviciute and Kuperman 1991) as compared with those of *B. acheilognathi*. Similarly, the karyotype of *B. acheilognathi* differs significantly from that of *Triaenophorus nodulosus* and *T. crassus* (Petkeviciute and Ieshko 1991). Both *Triaenophorus* species have middle-sized, predominantly biarmed chromosomes. *T. nodulosus* differs considerably from *B. acheilognathi* in chromosome number (2n = 26); *T. crassus* has diploid number (2n = 18), and the micromorphology of its chromosome pairs is distinctly different.

The diploid value of 2n=14 was also obtained by Nedeva and Mutafova (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 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 2.71µ m (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.

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 biarmed 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 acrocentric 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 specialized 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 diphyllobothriids form a distinct clade within the Pseudophyllidea (Mariaux 1998; Bray *et al*. 1999; Kodedova *et al*. 2000). All of the diphyllobothriids studied (*Diphyllobothrium*, *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 (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 metacentric 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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