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Ribosomal ITS2 structure in *Caryophyllaeus laticeps* and *Caryophyllaeus brachycollis* (Cestoda: Caryophyllidea), parasites of cyprinid fish

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Summary

Ribosomal internal transcribed spacer 2 (ITS2) structure was studied in monozoic tapeworms *Caryophyllaeus laticeps* (Pallas, 1781) from freshwater bream *Abramis brama* (L.) and white-eyed bream *Ballerus sapa* (Pallas) and *Caryophyllaeus brachycollis* Janiszewska, 1951 from *A. brama* and Mediterranean barbel *Barbus meridionalis* (Risso). Homologous intragenomic ITS2 structure (474 bp) was determined for *C. brachycollis* from both fish hosts and for *C. laticeps* from *A. brama* (486 bp). Contrary to this, divergent intragenomic ITS2 copies (ITS2 paralogues) were detected in *C. laticeps* from *B. sapa*. They were mostly induced by different numbers of short repetitive motif $(TA)_n$ within the sequences, allowing their assortment into two ITS2 variants (457 and 467 bp). Current data represent first information on ITS2 structure/ITS paralogues in the caryophyllidean family Caryophyllaeidae with focus on their applicability in the molecular taxonomy of the genus *Caryophyllaeus*.

Keywords: monozoic tapeworms; internal transcribed spacer; intragenomic ITS variants

Introduction

Ribosomal spacers are rapidly evolving DNA regions with high frequency of nucleotide mutations and thus they serve as frequent species-specific molecular markers (Hillis & Dixon, 1991). Contrary to this general statement, intragenomic internal transcribed spacer variants (ITS paralogues) have recently been detected in several caryophyllidean (monozoic) species of the family Lytocestidae. ITS2 paralogues were confirmed in all species of the genus *Atractolytocestus*; Slovak, American and British populations of *A. huronensis* (Králová-Hromadová *et al*., 2010; Bazsalovicsová *et al*., 2011), in *A. sagittatus* from Japan (Bazsalovicsová *et al*., 2012) and *A. tenuicollis* from China (Králová-Hromadová *et al*., 2013). Divergent intragenomic ITS2 copies were also revealed in *Caryophyllaeides fennica* (Orosová *et al*., 2010) and in three out of six studied species of the genus *Khawia*; *K. japonensis* from Japan, *K. saurogobii* from China and in Slovak, Japanese and Chinese populations of *K. sinensis* (Králová-Hromadová *et al*., 2012).
The family Caryophyllaeidae is the most specious family possessing about 20 genera, including the type genus *Caryophyllaeus* Gmelin, 1790, where three species are recognized; *Caryophyllaeus laticeps* (Pallas, 1781), *Caryophyllaeus brachycollis* Janiszewska, 1951, and *Caryophyllaeus fimbriceps* Annenkova-Chlopina, 1919 (Protasova *et al*., 1990). Whereas *C. laticeps* infects a wide spectrum of cyprinid fish, especially breams (*Abramis, Ballerus* and *Blicca*) and has a large distribution area covering Europe and Palaearctic Asia (Protasova *et al*., 1990), *C. brachycollis* is a typical parasite of barbels (*Barbus*) and chub (*Squalius cephalus*), and occurs mainly in central and eastern Europe and the western part of Russia (Protasova *et al*., 1990).
The aim of the current work was to provide the pilot data on molecular structure of ribosomal ITS2 in *C. laticeps* and *C. brachycollis*, with focus on an assessment of its applicability in molecular taxonomy.

Material and methods

Tapeworms studied were obtained by dissection of fish from eastern Slovakia (see Table 1) and were fixed in ethanol (95 – 99 %) for DNA analyses. Genomic DNA was isolated using the QIAamp® DNA Kit (QIAGEN, Hilden, Germany). For PCR amplification, a total volume of amplification mixture was 20 µl and contained 10 – 20 ng of genomic DNA, 20 pmol of each of the forward and reverse primers, 0.2 mM of each of the deoxynucleotide triphosphate (Fermentas UAB, Vilnius, Lithuania), 0.5 U of *Taq* DNA polymerase (Promega, Madison, Wisconsin, USA) with corresponding reaction buffer and 1.5 mM $MgCl_2$. For amplification of complete ITS2 spacer, the 5.8S-2 (5´-

gtcgatgaagagcgcagc-3´; Królová-Hromadová *et al*., 2003) and ITS-2 (5´-aggaggcgaatcactat-3; Cunningham, 1997) primers with annealing positions in the 5.8S and LSU rDNA, respectively, were applied. The PCR amplifications were run as follows: 5 min at 94 °C as an initial step; then 30 cycles of 1 min at 94 °C, 1 min at 55 °C, and 2 min at 72 °C. The final step was 10 min at 72 °C. The PCR products were loaded on the 1 % agarose gel and purified using the Wizard PCR purification Kit (Promega). Purified amplicons from two *C. laticeps* individuals (CL1 – host *B. sapa*, CL2 – host *A. brama*) and two *C. brachycollis* specimens (CB1 – host *B. meridionalis*, CB2 – host *A. brama*), were cloned into the pGEM®-T Easy vector (Promega) following the manufacturer's protocol. Five recombinant clones (CL1/1-5, CL2/1-5, CB1/1-5, CB2/1-5) from each individual were purified with the Plasmid miniprep kit (Genomed, Löhne, Germany) and sequenced using universal primers T7 and Sp6. Sequencing was performed using automatic genetic analyzer Applied Biosystems 3130xl (Applied Biosystems, Foster City, California, USA) and BigDye Terminator v3.1 Cycle sequencing kit (Applied Biosystems). The sequence alignment was performed using ClustalW (Thompson *et al*., 1994).

Results and discussion

Homologous intragenomic ITS2 structure was detected in *C. laticeps* from *A. brama* and *C. brachycollis* from both fish hosts. All five clones of each of the three individuals possessed identical structure and no intragenomic variation was observed. The length of the ITS2 spacer was 474 bp in both *C. brachycollis* individuals, and 486 bp in *C. laticeps* (Table 1). Contrary to this, divergent intragenomic ITS2 copies were detected in *C. laticeps* from white-eyed bream *B. sapa*. ITS2 polymorphism was mostly induced by three single nucleotide polymorphisms (SNPs) and by different numbers of short repetitive motif $(TA)_n$ within the sequences, allowing their assortment into two ITS2 variants. The repeat was present either in $(TA)_2$ (457 bp; variant 1; detected in three clones) or in $(TA)_7$ (467 bp; variant 2; present in two recombinant clones). The sequence identity between both ITS2 variants was 99.1 %. The intraspecific polymorphism observed within both tapeworm species was very low. In the ITS2 structure of *C. brachycollis* from two host species, the pairwise sequence identity was 99.3 %, the intraspecific variation was caused by three nucleotide substitutions. In *C. laticeps,* the intraspecific differences between tapeworms from two different hosts were slightly higher due to intragenomic sequence variants detected in *C. laticeps* from *B. sapa*; the pairwise sequence identity in this tapeworm was 96.7 – 96.9 %. The overall interspecific ITS2 similarity between both *Caryophyllaeus* species ranged between 84.3 – 86.0 %.

For molecular taxonomy, an adequate knowledge of the intraspecific variability has to be available before genetic differences between species are compared. An occurrence of ITS paralogues and their potential use and reliability in taxonomy, diagnostics and phylogeny may be questioned. In general, they can be applied as molecular markers, but several factors have to be considered when envisaging them (for review see Feliner & Rosselló, 2007). Despite occurrence of ITS2 paralogues in *C. laticeps* from *B. sapa*, intraspecific sequence identities in *C. laticeps* (96 – 99 %) and *C. brachycollis* (99 %) were significantly higher than interspecific pairwise sequence identity (84 – 86 %). Similar results were obtained in species of the genera *Atractolytocestus* and *Khawia* (Lytocestidae) in which the structure and distribution of various ITS2 variants and sequence types were generally well fixed within the species and differed significantly from the other congeners (Królová-Hromadová *et al*., 2012, 2013; Bazsalovicsová *et al*., 2012).

Caryophyllidean cestodes are unique among the Eucestoda in the possession of monozoic body without internal and external segmentation, with only one set of reproductive organs (Mackiewicz, 1994). Besides unique morphology, these cestodes possess also specific molecular and genetic features, such as ITS paralogues, triploidy, multiple ribosomal loci (Królová-Hromadová *et al*., 2010) as well as nuclear copies of mitochondrial DNA (NUMTs) (Brabec *et al*., 2012). The presence of NUMTs was also revealed in *C. laticeps* and *C. brachycollis* specimens from freshwater bream and the following combinations were found in the analysed specimens: (i) NUMTs/ homologous ITS2 (*C. laticeps* from *A. brama* and *C. brachycollis* from *A. brama*); (ii) homologous mtDNA/ ITS2 paralogues (*C. laticeps* from *B. sapa*); and (iii) homologous mtDNA/ homologous ITS2 (*C. brachycollis* from *B. meridionalis*) (Brabec *et al*., 2012; current data). This indicates that development of intragenomic divergence in ribosomal spacers and mitochondrial genes could be a result of independent evolutionary events in *Caryophyllaeus* tapeworms.

Table 1. Details on *Caryophyllaeus laticeps and Caryophyllaeus brachycollis* tapeworms and their ITS2 structure

***Caryophyllaeus* species**	**Fish host**	**Locality**	**ITS2 structure**	**ITS2 length**	**GenBank Accessions**
C. laticeps	white-eyed bream *Ballerus sapa*	Latorica River, Slovakia	paralogous 2 ITS2 variants	457 bp 467 bp	KF700252 KF700251
C. laticeps	freshwater bream *Abramis brama*	Tisa River, Slovakia	homologous	486 bp	KF700250
C. brachycollis	freshwater bream *A. brama*	Tisa River, Slovakia	homologous	474 bp	KF700249
C. brachycollis	Mediterranean barbel *Barbus meridionalis*	Myslava stream, Slovakia	homologous	474 bp	KF700248

Detection of ITS paralogues exclusively in *C. laticeps* from white-eyed bream, but not from freshwater bream nor in *C. brachycollis* from both fish hosts, i.e. freshwater bream and Mediterranean barbel, is surprising. However, it corresponds to the fact that cestodes from *B. sapa* possess two morphological characters (unusually large vitelline follicles and their complete absence alongside the cirrus-sac and preovarian uterine loops), in which they markedly differ from conspecific specimens from the type host, *A. brama*, and other fish hosts (unpublished data). An application of other genetically more informative molecular markers, such as multiple-copy nuclear genes (short tandem repeats, microsatellites) might provide additional information about character of morphological and molecular distinctiveness and possible speciation processes in *C. laticeps* from *B. sapa*.

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