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Molecular Systematics and Evolution of Basal Cestode Lineages

Ph.D. Thesis

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Annotation

Molecular phylogenetic techniques have entered the field of cestode systematics more than fifteen years ago and since then have significantly contributed to the understanding of their interrelationships and taxonomy. However, more than a few blind spots remain to be clarified throughout the cestode evolutionary tree. This dissertation represents an attempt to shed more light on several of these spots, aimed preferentially on the basal groups of tapeworms. The first part of the thesis focuses on the development of novel molecular markers and the evaluation of the utility of the conventionally used targets for studies of inter- and intraphyletic relationships of cestode orders. The second part is formed by three lower level taxonomical studies in which molecular phylogeny plays a complementary but indispensable role. The third part presents an optimization of a particular molecular method for diagnostics of human cestode infections.

Declaration [in Czech]

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RNDr. Jan Brabec

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List of original papers and author's contribution

The thesis is based on the following papers:

I. **Brabec**, **J**., Scholz, T., Králová-Hromadová, I., Bazsalovicsová, E., Olson, P.D. 2012. Substitution saturation and nuclear paralogs of commonly employed phylogenetic markers in the Caryophyllidea, an unusual group of non-segmented tapeworms (Platyhelminthes). *International Journal for Parasitology* 42: 259–267. (IF_{2010} = 3.822)

   Jan Brabec conceived the study, collected nuclear sequence data, characterized paralogous sequences of the mitochondrial genes, performed analyses of the data, and wrote the manuscript.

II. Wicht, B., Yanagida, T., Scholz, T., Ito, A., Jiménez, J.A., **Brabec**, **J**. 2010. Multiplex PCR for differential identification of broad tapeworms (Cestoda: *Diphyllobothrium*) infecting humans. *Journal of Clinical Microbiology* 48: 3111–3116. (IF_{2010} = 4.220)

   Jan Brabec helped with the design of the study, performed multiplex PCR experiments and obtained sequences of a part of the studied parasites, and helped draft the manuscript.

III. Scholz, T., **Brabec**, **J**., Králová-Hromadová, I., Oros, M., Bazsalovicsová, E., Ermolenko, A., Hanzelová, V. 2011. Revision of *Khawia* (Cestoda: Caryophyllidea), parasites of cyprinid fish, including a key to their identification and molecular phylogeny. *Folia Parasitologica* 58: 197–223. (IF_{2010} = 1.533)

   Jan Brabec was responsible for the collection of the nuclear sequence data, performed phylogenetic analyses, and partially contributed to the writing and revision of the manuscript.

IV. Kuchta, R., Burianová, A., Jirků, M., de Chambrier, A., Oros, M., **Brabec**, **J**., Scholz, T. 2012. Bothriocephalidean tapeworms (Cestoda) of freshwater fish in Africa, including erection of *Kirstenella* n. gen. and description of *Tetracampos martinae* n. sp. *Zootaxa* 3309: 1–35. (IF_{2010} = 0.853)

   Jan Brabec characterized and phylogenetically analyzed the sequences of studied parasites, and partially contributed to the writing and revision of the manuscript.

V. Oros, M., Ash, A., **Brabec**, **J**., Kar, P.K., Scholz, T. 2012. A new monozoic tapeworm, *Lobulovarium longiovatum* n. g., n. sp. (Cestoda: Caryophyllidea), from barbs *Puntius* spp. (Teleostei: Cyprinidae) in the Indomalayan region. *Systematic Parasitology* (in press). (IF_{2010} = 1.056)

   Jan Brabec characterized the sequences, performed the phylogenetic analysis, and partially contributed to the writing of the manuscript.

The senior and corresponding authors of the manuscripts included in this thesis, hereby confirm that Jan Brabec contributed significantly to these publications, according to the statements above.

Roman Kuchta   Mikuláš Oros   Tomáš Scholz   Barbara Wicht

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Preface

When I look back at the original goal of my PhD thesis, developed in late spring 2006, I would now consider it more of a slippery attempt to bring more light into one of the still-resisting areas of tapeworm molecular systematics—the interrelation-ships of the eight or nine groups of these parasites that have been, traditionally and in agreement with current molecular phylogenetic studies, considered basal to the rest (and decisive majority) of tapeworms. The original strategy intended to gather enough data that would contain a signal capable of resolving this phylogenetic question was quite elegant and, for me personally, unique and very tempting. The very beginning of the twenty-first century still counted as a period that widely and almost exclusively employed Sanger sequencing for obtaining data for molecular phylogenetic studies and the advent and spread of the next generation sequencing techniques was yet to come. At least in our latitudes. The idea was based on the recently discovered genetic feature of tapeworms to *trans*-splice part of their nascent mRNAs. During this form of RNA maturation, a short unique spliced-leader sequence is added to the 5' end of each of the processed mRNAs. In flatworms, this phenomenon was first described in the human-infecting digenean parasite *Schistosoma*, and a few years later in other human parasites—tapeworms *Echinococcus* and *Taenia*. It complements the traditional model of mRNA synthesis, and according to some estimates, accounts for about one-third of the total mRNA processing in the named parasitic flatworms.

Keeping this in mind, we speculated that if we were able to characterize the spliced-leader sequence from representatives of each of the basal tapeworm groups, we would be able to relatively easily amplify and sequence that specific part of their transcriptomes. Subsequently, we would search them for homologous factors and use these to formulate phylogenetic hypotheses. Moreover, using the *trans*-spliced factors for resolving the basal phylogeny of cestodes would be just one of the two birds to kill with one stone. A far bigger potential of the data would lie in their possible applicability for answering the main question presented by the existence of the spliced-leader *trans*-splicing process in parasitic flatworms—the purpose and function of this mechanism. While there have been hypotheses formulated for some of the other groups of eukaryotic organisms that employ *trans*-splicing (e.g. kinetoplastid flagellates and nematodes), no-one came up with a solid key explaining its function in parasitic flatworms. Unfortunately, I have never been able to take this project through to its successful end in a form of a publication, so I summarize the work done in one of the chapters of this thesis.

Since the possible outcomes of the main part of my PhD project were highly uncertain, and following up my interest in molecular systematics of fish tapeworms from my undergraduate and graduate studies, I also continued to participate in several projects carried out in the Laboratory of Helminthology at the Institute of Parasitology

which I was part of. Most of this work involved myself as a molecular phylogeneticist in charge of evaluating the systematic status and phylogenetic relationships of the cestode taxa studied based on various molecular markers. There are three papers published, primarily aiming at morphological description or re-description of cestode taxa—thus considered as classical alpha-taxonomy works—that form a part of this thesis, and that involved my contribution to some extent. I was also given an opportunity to participate in a more applicable project dealing with molecular diagnostics of cestode infections in clinical praxis. More specifically, we tried to develop a quick, cheap, and simple protocol for differential diagnosis of human-infecting *Diphyllobothrium* cestode species. An article summarizing the results of this project also constitutes a part of this dissertation.

Finally, as a "by-product" of my participation in a complex taxonomical revision of the cestodes of the order Caryophyllidea—a long-term project that again involved myself as a person responsible for collecting sequence data and carrying out phylogenetic analyses, I found out that some of the caryophyllidean tapeworms bear multiple haplotypes of mitochondrial genes. Since this finding represents a non-negligible source of error for phylogenetic studies, and based on our findings, most probably translates as a proof of the presence of nuclear mitochondrial pseudogenes (numts—a phenomenon known from a number of organisms but not yet encountered in any of the cestode groups), we put together an article describing these results that turned out as the keystone paper for this dissertation.

Introduction

The development of modern molecular biological techniques, most markedly the polymerase chain reaction and rapid DNA sequencing methods, has affected our understanding of the phylogenetic relationships and evolution of the organisms inhabiting the Earth. Together with the newly developed opinions on the systematic theory, a completely new field of molecular phylogenetics has evolved and spread during the last several decades through various biological disciplines, with systematic biology in their forefront. Following the development of the chain-termination method of DNA sequencing by Sanger (Sanger et al., 1977), its preferred use and further technical improvements (such as the fluorescent dye labeling and automated signal detection), the first automated DNA sequencers came to the light of a day in late eighties (Smith et al., 1986) and became a concern of commercial production. Over the turn of the century, the demand for a high-throughput and low-cost sequencing technique gradually increased eventually leading to development of the next-generation sequencing methods (e.g. Mardis, 2008). Such methods then allowed for characterization of a considerably greater number of nucleotides (at the magnitudes of genomes) in fraction of time when compared to conventional Sanger's method. As of the second decade of the twenty-first century, phylogenetic discipline entered a new era of research that is based on analyses of large multi-locus sequence data sets covering whole genomes or transcriptomes—the era of phylogenomics. However, conventional sequencing methods remain widely used especially in majority of works that rely on a limited number of predetermined molecular loci to be characterized.

Flatworm evolutionary history

Platyhelminthes

Tapeworms have been traditionally recognized as a highly specialized group of flatworms (phylum Platyhelminthes), a group composed of an enormous diversity of bilaterally symmetrical, dorsoventrally flattened animals lacking anus and body cavity (acoelomates). According to the estimates, the group consists of more than 20,000 described species, probably still a fraction of the total number of the extant species (Caira and Littlewood, 2001). Given their soft bodies without any protective covering, they inhabit various but at least to some extent moist or aquatic environments, displaying the entire scale of life strategies from free-living species through commensalists to obligate parasites. Traditional views on animal systematics placed Platyhelminthes as the most basal group of bilaterally symmetrical animals (Bilateria), the ancestral archetypes from which one of the two cornerstone groups of the Bilateria, the Protostomia, originated (Littlewood et al., 2004). The division of the Bilateria into

the two groups termed Protostomia and Deuterostomia, coined by Grobben (1908), is based on a mosaic of features, and is currently preferred over the other variations proposed by other authors in the history. The Protostomia/Deuterostomia names are, however, a bit unfortunate. They were meant to reflect the different fates of the blastopore that should become either mouth in protostomes or anus in deuterostomes, but in fact the ontogenetic fate of blastopore is highly variable within the bilaterian phyla (Halanych, 2004; Nielsen, 2012).

It has been the simple body plan and the absence of body cavity that once had placed flatworms at the same base of Bilateria and allowed them to occupy that position for over a hundred years. The breakthrough came during the nineties with the first phylogenetic analyses based on sequences of ribosomal RNA coding genes (rDNA), the small subunit rDNA being the first of them (e.g., Adoutte et al., 1999; Halanych, 2004). The initial molecular-based works quickly redivided existing and morphologically well-characterized animal phyla into three fundamental groups that form Bilateria, among other things supporting the long-recognized groups Protostomia and Deuterostomia, and further subdividing Protostomia into Lophotrochozoa (Halanych et al., 1995) and Ecdysozoa (Aguinaldo et al., 1997). Platyhelminthes were found to be members of the Lophotrochozoa (Balavoine, 1997; Carranza et al., 1997), and the former hypothesis where platyhelminthes shared a monophyletic lineage with the acoelomorph flatworms (i.e. Acoela and Nemertodermatida) as originally thought was abandoned (Ruiz-Trillo et al., 1999, 2002; Jondelius et al., 2002; Telford et al., 2003). Since then, flatworm phylogenies have progressed significantly, from targeted-gene approaches and limited number of taxa analyzed to phylogenomic analyses based on hundreds of genes and/or hundreds of taxa. To summarize the prevailing evidence from recent molecular analyses, we are now confident of the Lophotrochozoa monophyly (e.g., Dunn et al., 2008; Hejnol et al., 2009; Mallatt et al., 2010) and the exclusion of both acoelomorph groups from the remaining Platyhelminthes (Philippe et al., 2007, 2011; Mallatt et al., 2010). On the other hand, the interrelationships among lophotrochozoan groups, especially the phylogenetic position of the phyla Platyhelminthes, Gastrotricha, and Gnathifera that in part of the analyses (e.g., Passamaneck and Halanych, 2006; Giribet et al., 2009) form a monophyletic clade Platyzoa (Cavalier-Smith, 1998), remain to be resolved confidently (Dunn et al., 2008; Edgecombe et al., 2011).

After the definite exclusion of the acoelomorph flatworms, Platyhelminthes consist of two well-supported clades—basally placed Catenulida and Rhabditophora that encompass all the remaining flatworms lineages including a paraphyletic assemblage of mainly free-living flatworms that were once classified as Turbellaria (this group also involved catenulid and acoelomorph flatworms) and the Neodermata, a historically well-recognized group of obligate parasites formed by tapeworms (Cestoda), flukes (Trematoda), and monogeneans (Monogenea). Catenulida was historically classified as

the basal clade of Platyhelminthes by Ehlers (1984), but this position was not resolved with confidence in the first molecular phylogenetic works based on rDNA data (e.g., Carranza et al., 1997; Littlewood et al., 1999a, 1999b; Telford et al., 2003). Catenulida in fact represent a rather poorly known group sharing only a limited number of synapomorphies with Rhabditophora (Littlewood, 2006), as also using different mitochondrial genetic code (Telford et al., 2000) than Rhabditophora that was more reliably recognized as their sister lineage only recently (Larsson and Jondelius, 2008; Paps et al., 2009).

Neodermata

Molecular phylogenetic works studying interrelationships of Platyhelminthes (e.g., Baverstock et al., 1991; Blair, 1993, Littlewood et al., 1999b; Litvaitis and Rohde, 1999; Littlewood and Olson, 2001; Lockyer et al., 2003) have consistently shown that Neodermata form the most derived clade within rhabditophorans. This agrees with the original cladistic system of Platyhelminthes as proposed by Ehlers (1984) who also established the term Neodermata, and also with the following morphological studies carried out on the group (e.g., Brooks and McLennan, 1993; Littlewood et al., 1999a, Zamparo et al., 2001). While being confident about the derived position and monophyletic status of the lineage of neodermatan parasites, we continue to know very little about the cladogenesis of the rest of the non-neodermatan rhabditophoran clades despite a number of morphology- and molecular-based attempts (Littlewood, 2006). Thus we can only guess which of these clades forms a sister lineage to the parasitic Neodermata, even if the most recent molecular-based studies (Littlewood and Olson, 2001; Lockyer et al., 2003) indicate that it might be a diversified group of several rhabditophoran lineages including Tricladida, Rhabdocoela, Fecampiida and Prolecithophora.

Interrelationships of the clades forming Neodermata (i.e. Cestoda, Monogenea and Trematoda) have been considered analogously problematic, mainly due to disparate views of the phylogenetic status of Monogenea. According to some (e.g. Boeger and Kritsky, 2001), monogeneans represent a monophyletic group consisting of two lineages —Monopisthocotylea and Polyopisthocotylea. Other regard them paraphyletic, both Monopisthocotylea and Polyopisthocotylea being independent and thus Monogenea forming an artificial lineage (Justine, 1998; Euzet and Combes, 2003). While some molecular studies (Littlewood and Olson, 2001) supported the existence of Cercomeromorphae (clade formed by Monogenea and Cestoda—conception based on a common presence of posterior extension of the body equipped with 6–16 hooklets) which would argue for monophyly of Monogenea, others have suggested a closer relationship between the Cestoda and Trematoda (e.g. Litvaitis and Rohde, 1999) along with the paraphyly of monogeneans (Lockyer et al., 2003). Recently, this problem seems

to have come to a conclusion offered by the mitochondrial protein-coding genes data that support earlier results based on almost complete rDNA data (Lockyer et al., 2003), rejecting monogenean monophyly and placing the epithelial feeding monopisthocotyleans at the base of the Neodermata and the blood-feeding polyopisthocotyleans as a sister group to the remaining neodermatans (Perkins et al., 2010).

As already mentioned above, parasitic flatworms jointly form a well-defined group termed Neodermata that encompasses four major groups: Cestoda (tapeworms), Trematoda (flukes), and monopisthocotylean and polyopisthocotylean "Monogenea." The monophyly of Neodermata is supported by several morphological features. The most distinctive of those features is surely the one that gave the group its name—the characteristics of the body surface. Neodermatan epidermis is a single layer of multiciliated cells (whose cilia lack the caudal rootlet) that is limited to the free-living larval stages only. During the maturation, as the parasite enters the next developmental stage, this outer epidermal layer is shed and replaced with syncytial neodermis that is formed from neoblasts—a separate population of stem cells (Reuter and Kreshchenko, 2004). But unlike in their rhabditophoran relatives where neoblasts gradually replace cells lost to normal physiological turnover during growth, in Neodermata the change of epidermis happens at one time, usually when the larval stage enters the first host. The neodermal cells which are situated below the basal lamina and outer muscle layers, generate cytoplasmic extensions that grow and fuse underneath the epidermal cells while the neodermal cell bodies with nuclei (called cytons) stay below the basal lamina.

The other apomorphy of Neodermata is the way their protonephridia are organized. The flame bulbs are formed by two cells, each cell having a weir circle. One circle is formed from longitudinal cytoplasmic rods of the flame bulb terminal cell while the second one is formed from longitudinal cytoplasmic rods of the first canal cell, and both together surround the cilia. Also the flagella of sperm are organized differently—neodermatan sperm axonemes are incorporated into the sperm body by proximo-distal fusion rather than remaining separate from the main sperm body cytoplasm (Littlewood et al., 2004). This is not meant to be a comprehensive list of neodermatan apomorphies. Other morphological features of the group were evaluated in the works of Ehlers (1984, 1985) and Littlewood et al. (1999a). Beside those, Joffe and Kornakova (2001) proposed to support the monophyly of Neodermata by the presence of two long and one short insertions in their small subunit rDNA sequences.

Cestoda

Cestodes represent a highly specific group within Neodermata that is characterized by several striking morphological features, the most obvious one being the evolutionary loss of digestive tract throughout all developmental stages. As adults, they reside in intestines of vertebrates, an environment extremely rich in nutrients that they

intake through the outermost layer of their bodies—the syncytial neodermis called tegument. Neodermis of tapeworms bear microtriches, distinct elaborations of various shapes whose nomenclature was standardized by Chervy (2009) and which contribute to nutrient absorption. Life-cycles of tapeworms typically include two or three hosts, the first being an arthropod that gets infected by the first larval stage called the oncosphere that possesses 6 or 10 larval hooks. The following developmental stages differ significantly among individual tapeworm groups and include various larval (metacestode) forms, their nomenclature can be found in Chervy (2002).

The body of cestodes consists of scolex, the most anterior part of the body that cestodes use to attach to the intestinal wall of the host, and strobila that refers to the rest of the body posterior to scolex. Morphology of the scolex is literally a parade of evolutionary designs and many groups display a scolex of a particularly modified shape that had, or still does serve as an important taxonomical character. Many groups have a scolex which bears four muscular membrane-bound structures known as acetabula. Acetabula can have a form of extensively prolonged bothridia that basically comprise the entire scolex, or the form of sessile suckers. Bothridia of some cestode groups are placed on stalks, can be fused in pairs, display myriads of shapes, being subdivided into various loculi, bear hooks or anterior muscular pad equipped with accessory sucker. Cestode groups that do not have acetabula either possess shallow, weakly muscular suctorial grooves called bothria which, unlike the acetabulates, lack the bounding membrane, or they are referred to as monobothriate. These bear a simple scolex with a single terminal funnel or shallow depressions called the loculi. Currently, the terms acetabulate and bothriate (Caira et al., 1999) are the preferred substitutes for the terms tetrafossate and difossate (i.e. having four-part and two-part scolices, respectively) that were formerly used in literature. To make the morphological variations of scolex even richer, some cestodes have their scolices divided into two different parts (the posterior part being called metascolex), or they have apex of the scolex modified into an apical organ that can be shaped as a retractable rostellum that can be further armed with hooks, subdivided into tentacles, or completely substituted by four armed retractable tentacles.

Posterior to the scolex, there is a germinative zone, from which a strobila originates. In most of the cestodes, strobila has a form of a ribbon-like structure consisting of several to countless segments (proglottids). This is not, however, a true segmentation but rather a chain repetition of one segment throughout the body. Each proglottid contains a set (or up to 14 sets) of male and female reproductive organs, and this organization of the body bearing multiple genital organs is referred as polyzoic. However, monozoic tapeworm groups with an unsegmented strobila also exist. As the proglottids are being produced in the germinative zone which is next to scolex throughout the life of the tapeworm, the youngest proglottids are found in the anterior part of the strobila and get mature and eventually gravid as they depart toward the

posterior end of the body. Here the proglottids either degenerate (anapolytic) or detach from the strobila at a given moment of maturity (apolytic).

With approximately 740 described genera and more than 5,000 recognized species, cestodes represent the second largest group of the Neodermata and, in fact, of the Platyhelminthes as a whole. Currently, there is a total of 18 valid cestode orders *sensu* Khalil et al. (1994), Olson et al. (2001), Caira et al. (2005), Kuchta et al. (2008), and Healy et al. (2009). Table 1 shows a complete list of the currently known tapeworm lineages considered to represent orders with a number of genera accommodated within them and the morphological and biological hallmarks of each group. The current state of knowledge suggests that tapeworms primarily evolved as parasites of fishes (Hoberg et al., 1999), and subsequently colonized all major vertebrate groups with the greatest diversification among tetrapods (i.e., the Cyclophyllidea).

Table 1. List of currently recognized major cestode orders and their characteristics (listed alphabetically).

Taxon	No. Genera[a]	Scolex Morphology	Habitat/Definitive Host Group
Cestodaria		Monozoic, lack scolex	
Amphilinidea	3 (8)	May have small sucker-like organ at anterior end of their leaf-like body	Cosmopolitan, body cavity of chondrosteans, freshwater teleosts and turtles, some marine teleosts
Gyrocotylidea	2 (16)	Muscular sucker-like attachment organ at anterior end, rosette-like adhesive organ at posterior end of body	Cosmopolitan, spiral intestine of mainly holocephalans, also described from sharks
Eucestoda		Generally polyzoic, ribbon-like body and distinct scolex	
Bothriocephalidea	46 (133)	Scolex with pair of bothria, may be absent or replaced by pseudoscolex or scolex deformatus, occasionally with hooks	Cosmopolitan, marine and fresh-water teleosts, some in paddle-fish, bichirs, sturgeons, salamanders
Caryophyllidea	49 (~150)	Monozoic, scolex afossate/fossate with 1–3 pairs of shallow structures (loculi, bothria)	Cosmopolitan excluding South America, freshwater cypriniform and siluriform teleosts
Cathetocephalidea	2 (4)	Scolex laterally expanded fleshy organ consisting of an apical pad, a band bearing numerous papillae, and a rugose base	Tropical and subtropical waters, carcharhiniform sharks
Cyclophyllidea	380–400 (>3000)	Scolex with 4 suckers, with or without rostellar apparatus	Cosmopolitan, tetrapods, mostly birds and mammals
Diphyllidea	2 (39)	Scolex consisting of 2 bothria and a cephalic peduncle with or without spines	Cosmopolitan, elasmobranchs
Diphyllobothriidea	16 (80)	Scolex unarmed, with pair of bothria	Cosmopolitan, marine mammals, also birds, reptiles, amphibians
Haplobothriidea	1 (2)	Primary scolex with 4 tentacles, strobila with segmented regions at intervals that separate off to become a secondary strobila, anterior segment of secondary strobila modified as flattened scolex, with 4 shallow indentations around central dome	North America, bowfin
Lecanicephalidea	22 (65)	Scolex with 4 sucker-like or bothridiate acetabula, diverse structure of apical organ	Tropical and subtropical waters, elasmobranchs (rays, some sharks)
Litobothriidea	1 (8)	Scolex with single apical sucker, consisting of 3–5 muscular pseudosegments	Tropical and subtropical waters, lamniform sharks
Nippotaeniidea	2 (~6)	Scolex with single apical sucker	Old World, freshwater fishes

Proteocephalidea	54 (~400)	Scolex with 4 suckers, with or without metascolex, may have an apical organ	Cosmopolitan, freshwater teleosts, some reptiles, amphibians, mammal
Rhinebothriidea	13[b]	Scolex with 4 unarmed bothridia on stalks	Cosmopolitan, batoids
Spathebothriidea	5 (4–7)	Scolex undifferentiated or in form of weakly muscular apical organ	Northern hemisphere, chondrosteans and teleosts
Tetrabothriidea	6 (~65)	Scolex acetabulate, 4 muscular bothridia	Cosmopolitan (mainly polar regions), marine homeotherms, seabirds, mammals
Tetraphyllidea	64 (>400)	Scolex with 4 widely variable muscular bothridia (sessile or stalked, with or without hooks	Cosmopolitan, elasmobranchs, holocephalan
Trypanorhyncha	66 (254)	Scolex with 4 retractable armed tentacles at its apex and 2 or 4 bothria	Cosmopolitan, elasmobranchs

[a] Genera and species (parenthesized) count taken from http://sites.google.com/site/tapewormpbi/home, unless stated otherwise; [b] Healy et al. (2009)

Molecular systematics of the Cestoda

Early systematic treatments

Phylogenetic relationships of cestode orders had been always problematic and remained so until the last decade of the twentieth century. From the historical point of view, there have been the following attempts to explain genealogical affinities of the main tapeworm lineages: Lönnberg (1897), Fuhrmann (1931), Baer (1950), Euzet (1959, 1974), Freeman (1973), Dubinina (1980), Euzet et al. (1981), Brooks et al. (1991), and Brooks and McLennan (1993). When compared, these studies lacked general agreement on various aspects of cestode systematics, and many of the minor cestode orders have not been universally recognized throughout those works (Hoberg et al., 1997). The disagreement stemmed mainly from a phenomenon that Mariaux (1996) called "personal authority systematics," a phenomenon based on an imbalanced personal emphasis assigned to different criteria, such as the homology and evolutionary importance of morphological structures. Diverse hypotheses then resulted in recognition of 9–19 cestode orders according to various authorities (Mariaux, 1996; Hoberg et al., 1997). The first study that attempted to phylogenetically evaluate the relationships of tapeworms was that of Brooks et al. (1991). However, the hypotheses (based on a suite of putatively homological morphological and ontogenetic characters from literature) presented in their study remained controversial (only 5 of the currently 12 accepted orders were recognized). These authors were the first to apply cladistic methods, and their paper along with Brooks and McLennan's (1993) study formed a basis of future phylogenetic studies.

Diagnostic Keys to cestode genera of Khalil et al. (1994) represent the most recent consensus achieved among specialists in the systematics and phylogeny of tapeworms (earlier systematic treatments of the group were only those of Yamaguti, 1959 and

Schmidt, 1986). The work of Khalil et al. (1994) was principally based on reexamination of type species and reevaluation of morphological characters, not simply on compiling data from literature. Since the main objective of these Keys was to provide an up-to-date key for identification of cestodes to the family and generic level, they lacked the phylogenetic approach, and thus did not reflect evolutionary relationships among the groups. As a result, the Keys significantly reappraised the cestode taxonomy and defined 14 major orders of the Cestoda formed by Amphilinidea, Gyrocotylidea, and Eucestoda. The wide acceptance of the Keys by the community of cestode parasitologists consequently enabled the following studies, both morphological and molecular, aimed at elucidating higher lever relationships to be more meaningful in comparing alternative hypotheses.

The paper of Hoberg et al. (1997) has greatly advanced the understanding of tapeworm systematics and evolution. Based on a comparative analysis of morphology and ontogeny and employing the orders recognized by Khalil et al. (1994) as a basis for the terminal taxa, it brought the first complex cladistic study of the major lineages of cestodes. The analysis of 49 characters by maximum parsimony supported, apart from other things, the monophyly of the higher tapeworms (Eucestoda) as previous studies suggested (e.g., Ehlers, 1984; Brooks et al., 1991; Justine, 1991; Brooks and McLennan, 1993). It also argued for the basal position of caryophyllideans, thus considering monozoy to represent the ancestral state, as claimed by Dubinina (1980). Difossate tapeworms with bilateral symmetry and bothria (Pseudophyllidea) appeared to be the most primitive among polyzoic cestodes. According to Hoberg et al. (1997), subsequent evolution then led to consecutive origin of difossate forms with bothria, followed by tetrafossate forms with bothria and tetrafossates with bothridia and suckers or suckers only. This contrasted with the view of Brooks et al. (1991) who interpreted both the difossate and tetrafossate scolex conditions as apomorphic characters which had arisen independently from the plesiomorphic condition of a single apical sucker. In addition, a study of Hoberg et al. (1997) indicated possible paraphyly of the Tetraphyllidea with the Onchobothriidae being basal to the Phyllobothriidae.

Interrelationships of the major cestode lineages

The paper by Hoberg et al. (1997) proposed a hypothesis for tapeworm relationships open for future testing, modifications and refinements. It was in the first half of the nineties that molecular data proved to be significantly helpful in resolving systematic problems in parasitic flatworms (e.g., Baverstock et al., 1991; Blair 1993; Rohde et al., 1993). Due to a number of advantages, such as universal distribution among organisms and variable rate of evolution along the molecule, the ssrDNA happened to play a pivotal role in the young field of molecular phylogenetics. Consequently, this molecule became increasingly often applied to inferring phylogenies

across a diversity of organisms, including parasitic flatworms. The first phylogenetic study of cestode ordinal relationships was based on partial sequences of ssrDNA (Mariaux, 1998), and promoted an independent corroboration of the main conclusions of Hoberg et al. (1997). Although the majority of the cestode orders *sensu* Khalil et al. (1994) were represented by a single specimen or even missed from the data set (gyrocotylideans, lecanicephalideans, haplobothriideans), the monophyly of the Eucestoda, basal position of monozoic caryophyllideans (followed by monofossate spathebothriideans), relative primitiveness of difossate lineages, and monophyly of tetrafossates were all supported. On the other hand, the interrelationships of the basal difossate groups (e.g., Diphyllidea, Pseudophyllidea, and Trypanorhyncha) was not clearly resolved, and the Pseudophyllidea and Tetraphyllidea were found to be paraphyletic.

The second attempt to overcome the limitations of morphological characters was that of Olson and Caira (1999). Their study differed in the representation of taxa and in the gene regions analyzed (complete ssrDNA and partial elongation factor-1α). Although the tree topologies resulting from the different data parts was not entirely consistent, generally the same patterns of cestode relationships as those of Mariaux (1998) were observed. Along with Mariaux's (1998) main observations, tetraphyllideans were found to be paraphyletic, and the Litobothriidea—considered a tetraphyllidean group by Brooks et al. (1991) and Euzet (1994) formed a distinct clade. Both studies thus produced a generally congruent, reasonably solid, and morphologically independent foundation of the phylogenetic hypothesis for the group based on molecular data (Mariaux and Olson, 2001).

In the next years, Kodedová et al. (2000) enlarged the ssrDNA data set of Olson and Caira (1999) by several representatives of Pseudophyllidea and Proteocephalidea, further supporting the paraphyletic status of pseudophyllideans. Similarly, Hoberg et al. (2001) added a few new ssrDNA sequences to the data of Mariaux (1998), combined it with the morphological character matrix, and as in the previous studies this added the overall support to the conclusions of Hoberg et al. (1997), Mariaux (1998), Olson and Caira (1999), and Kodedová et al. (2000).

In 2001, the most complex study dealing with cestode phylogeny so far has been published (Olson et al., 2001). It contained analyses of complete ssrDNA, partial (D1–D3) lsrDNA, and a reevaluated suite of morphological characters from Hoberg et al. (2001) and Justine (2001) for more than a doubled number of tapeworm taxa. The D1–D3 lsrDNA data were found to be more variable and thus informative than the ssrDNA, and were more consistent with inferences from morphology, although nodal support was generally weak for most basal nodes. Otherwise, the conclusions of this study generally supported the results of those mentioned above. Difossates were basal to all tetrafossate orders, the latter forming a strongly supported clade. As also detected by the

earlier works, orders Pseudophyllidea, Tetraphyllidea, and Trypanorhyncha were consistently found to be paraphyletic among all the molecular data parts, but due to the weak nodal support, no formal revision of the classification was made.

Half a decade later, analysis of a further expanded rDNA data set of cestodes was published by Waeschenbach et al. (2007). While limited to key representatives of cestode orders, the data set was enlarged for the remaining, in cestodes still largely uncharacterized, parts of the lsrDNA beyond the first three variable domains of the molecule. Combining them with the complete ssrDNA, Waeschenbach et al. (2007) achieved a more stable phylogenetic inference of cestode relations within analyses compared to the inferences based on ssrDNA alone, or ssrDNA and (D1–D3) lsrDNA combined. However, adding further statistical support was mainly limited to the nodes already generally resolved, at least to some extent, by the earlier studies (e.g., the paraphyly of the Pseudophyllidea and Tetraphyllidea, sister-group status of the Spathebothriidea to the rest of eucestodes). In other words, nodes obtaining relatively high support in earlier works got even higher support, and the poorly resolved nodes remained statistically doubtable, even if found continuously on the trees. Thus, the most interesting finding of the work is the suggestion that the D1–D3 lsrDNA data are too variable to infer cestode phylogenies at this level, and so bring more instability to the topologies instead of improving them when combined with the ssrDNA.

Subclass level molecular phylogenies

The results of the above-mentioned molecular studies have pointed at some conspicuous discrepancies between the molecular-based hypothesis and the currently valid systematics represented by the Keys to cestode parasites by Khalil et al. (1994). Therefore, it came as no surprise, when the first follow-up studies aimed at a more detailed testing of particular parts of cestode phylogeny started to appear. The common attributes of those papers were generally two: significantly extended data collection of the ingroup taxa of the clade in question and a phylogenetic analysis specifically aimed at testing the ingroup intra-relationships as well as the affinities of its subgroups to other close lineages. The first paper on this topic was that of Caira et al. (2005) which formally established a new order named Cathetocephalidea on the basis of a suite of distinct morphological characters of two genera composing the group (one newly described). A morphological description of the new species together with the analysis of partial ssrDNA and lsrDNA sequences supported the previous suspicion of Schmidt and Beveridge (1990) and Caira et al. (1999, 2001) that the genus *Cathetocephalus* (now with the newly described genus *Sanguilevator*) should be recognized in a distinct order of cestodes and not as a lineage of the otherwise obviously paraphyletic tetraphyllideans.

The second paper that led into the recognition of new orders was that of Brabec et al. (2006), although the formal description of the newly proposed orders came in the

later study of Kuchta et al. (2008). Brabec et al. (2006) tested the hypothesis of the possible paraphyly of pseudophyllidean tapeworms as pointed by previous studies of Mariaux (1998), Kodedová et al. (2000), and Olson et al. (2001). Their analyses of complete ssrDNA and partial lsrDNA sequences of a number of pseudophyllideans showed that the traditional order is artificially formed by two distinct clades for which the names Bothriocephalidea and Diphyllobothriidea were proposed (Kuchta et al., 2008). In agreement with the former molecular studies, Diphyllobothriidea formed a well-supported and relatively basal clade along with the Haplobothriidea, while Bothriocephalidea consistently appeared as a more derived sister group to the otherwise strongly supported clade of tetrafossate tapeworm lineages.

Another attempt to reassess the ordinal level composition of cestodes focused on a subgroup of apparently polyphyletic tetraphyllidean cestodes, more specifically on the traditionally recognized phyllobothriid subfamily Rhinebothriinae. Healy et al. (2009) analyzed complete ssrDNA along with D1–D3 lsrDNA of several tens of candidate species to conclude that the majority of the taxa assigned to the Rhinebothriinae by Euzet (1994) creates a monophyletic clade, distinct from the rest of tetraphyllideans. The clade was named Rhinebothriidea and was mostly found to form one of the basal lineages of acetabulate cestodes, a place also occupied by the orders Cathetocephalidea, Lecanicephalidea, and Litobothriidea as detected by some of the earlier works (e.g., Olson and Caira, 1999; Olson et al., 2001; Caira et al., 2005).

Palm et al. (2009) and Olson et al. (2010) almost simultaneously presented a pair of phylogenetic studies evaluating relationships of cestodes of the order Trypanorhyncha. The monophyly of this numerous group of parasites of essentially all known lineages of elasmobranchs has remained generally unsupported over the earlier molecular-based studies (Olson and Caira, 1999; Olson et al., 2001), and a lack of support has been also the reality for their phylogenetic position among other bothriate tapeworm groups. While none of the two studies included representatives of the other phylogenetically close genera (except for the Diphyllidea that served as an outgroup), monophyly of Trypanorhyncha remained untested. Both Palm et al. (2009) and Olson et al. (2010) also used the molecular markers of choice in cestode phylogenetic studies—complete ssrDNA in combination with partial lsrDNA. On their basis, both studies accordingly revealed a division of the order into two fundamental groups that follows their primary host associations. Olson et al. (2010) then coined the names for these two clades, Trypanobatoidea for primary parasites of rays and Trypanoselachoida for shark parasites.

Litobothriidea represents the last of currently recognized cestode orders that was not considered an order in the Key to cestode parasites (Khalil et al., 1994). This group had been recognized as an order earlier in the history by Dailey (1969), however, Euzet (1994) considered it rather a distinct family of the Tetraphyllidea. Molecular

phylogenetic analyses (Olson and Caira, 1999; Olson et al., 2001; Waeschenbach et al., 2007) with the exception of the work of Olson et al. (1999) then fully supported its ordinal status, mostly placing it as a basal lineage of the acetabulate groups. Although the litobothriideans have never seen their formal resurrection to the rank of order, this status is currently widely accepted among cestodologists.

Molecular phylogeny of cestodes in 2012

It has been fifteen years since we first got the opportunity to see how molecular data influence our view of the interrelationships of the major cestode lineages. Over these fifteen years, we have witnessed the development of those views stemming from an iterative enrichment of the data analyzed, either for newly sequenced cestode taxa or extended lengths of the rDNA molecules. If we look back to review the achievements in the field, we can summarize that cestode systematics have not gone through any striking discoveries since the first molecular-based hypothesis has been formulated by the works of Mariaux (1998), Olson and Caira (1999), and Olson et al. (2001). In other words, what now appears as a strongly supported node on the cestode tree have already been found supported ten years ago, and vice versa. Among the solid findings of fifteen years of cestode molecular systematics belong:

Monophyly of Cestoda
Monophyly of Eucestoda
Monophyly of the clade of acetabulates (i.e., Lecanicephalidea, Litobothriidea, Rhinebothriidea, paraphyletic Tetraphyllidea including Proteocephalidea, clade of "higher" acetabulates)
Monophyly of the clade of higher acetabulates (i.e., Cyclophyllidea, Nippotaeniidea, Mesocestoididae, Tetrabothriidea)
Validity of bothriate orders Caryophyllidea, Bothriocephalidea, Diphyllidea, Diphyllobothriidea, and Spathebothriidea
Sister-group relationship of Haplobothriidea and Diphyllobothriidea
Validity of acetabulate orders Cyclophyllidea, Lecanicephalidea, Litobothriidea, Nippotaeniidea, Rhinebothriidea, and Tetrabothriidea
Monophyly of Proteocephalidea
Trypanorhyncha split into two fundamental lineages with various divergence rates

Only most recently has the process of inferring cestode phylogenies advanced from being based exclusively on nuclear rDNA genes to utilization of another source of sequential data from a different genomic locus (the only historical exception being the ~900bp long fragment of elongation factor–1α gene used by Olson and Caira, 1999). Waeschenbach et al. (2012) have technically extended the data set of Waeschenbach et al. (2007) by a more than 4,000bp long part of mitochondrial genome spanning across

two and one complete protein-coding and mitochondrial ribosomal genes, respectively. The trees reconstructed from their mitochondrial data set then allowed for a direct confrontation of rDNA based hypothesis with a new one based on a distinct pool of data. Mitochondrial analyses resolved all the traditionally recognized cestode clades listed above and further added an independent support for some other continuously found but only weakly supported lineages (e.g., monophyly of Trypanorhyncha, validity and branching pattern of Lecanicephalidea, Litobothriidea, and Rhinebothriidea). On the other hand, mitochondrial data revealed some innovative patterns not ordinarily recovered by rDNA data including: a sister-group relationship of Caryophyllidea to the clade of Spathebothriidea and the rest of eucestodes (hypothesis assuming the body plan of eucestode last common ancestor was monozoic); Diphyllidea not being sister lineage of Trypanorhyncha; primitive position of Amphilinidea relative to Gyrocotylidea; and the higher acetabulates not forming a group within the paraphyletic Tetraphyllidea. Altogether, the work of Waeschenbach et al. (2012) represents most significant advancement in molecular cestode systematics over the last more than ten years and assumes that alternative hypotheses of cestode phylogeny might arise in the future.

Aims and summary of PhD thesis

It has been fifteen years since the molecular phylogenetic techniques entered the field of cestode systematics and allowed unbiased testing of the traditionally held views of the group taxonomy. The development of currently valid views of the cestode phylogeny has been rather iterative, following the path of a progressive addition of molecular data and increasing confidence and statistical support for the hypotheses obtained. Over the fifteen years, molecular data have become an indispensable source of information that could be employed across the entire scale of taxonomical levels—from order to strains. However, fifteen years have been only enough to realize, what challenges we are going to face first. One of those is definitely the problem of unresolved interrelationships of basal cestode lineages which proved to be impossible to solve using only the widely exploited small and large subunit nuclear ribosomal RNA gene data (ssrDNA, lsrDNA).

ssrDNA and lsrDNA has been utilized in phylogenetic studies of cestodes from its beginning, and one of the results based largely on their analysis is the current topology of the cestode tree. However, the nature of the rDNA molecules is to mutate at incrementally different speed among their variable and conserved domains from which the molecules consist. The consequences of this feature for cestode phylogeny are the statistically relatively strongly supported branches of the tree that delineate most notably the individual cestode orders, main lineages within them, or the fundamental groupings of them, e.g. the acetabulate clade. On the other hand, interrelationships of these branches remain highly dubious, simply because the signal at these phylogenetic levels cannot be detected, if present at all.

The original goal of this doctoral thesis was to resolve the problem of basal cestode orders by obtaining bigger amount of data by targeting a specific subpopulation of cestode mRNAs—the spliced leader (SL) *trans*-spliced genes. SL *trans*-splicing is a process in which a short unique SL sequence is appended to the 5' end of a nascent mRNA to eventually form its first 5' exon. By using a primer against this sequence in combination with the oligo-d(T) primer against the 3' polyA-tail of mRNAs, we planned to obtain a population of protein-coding genes free of hyper-abundant ribosomal proteins and polyA-containing mitochondrial transcript "contaminations." To do that, we first needed to characterize the SL exon from each of the cestode lineages we wanted to study. By combining the knowledge of the primary SL sequence structure of the evolutionary derived tapeworms *Echinococcus* and *Taenia* and primitive flukes *Schistosoma* and *Fasciola* along with the knowledge of some peculiar characteristics of the SL *trans*-spliced transcripts, we characterized the SL gene from total of three representatives of basal cestode lineages. The methodology and summary of the preliminary results are outlined in a chapter of this thesis entitled "Characterization of spliced leader genes from basal cestodes." The efforts to accomplish the original goals of

the project have been hold up when the timetable designated to characterize the SL was markedly exceeded without obtaining a complete set of SLs necessary to conclude the project. Furthermore, the financial benefits of this type of study were found to be outperformed by a lower price of such a project undertaken using the next generation sequencing techniques. However, a further experimental work employing the data summarized here is being currently underway and includes the next generation sequencing of the SL *trans*-spliced transcripts to provide a new wealth of data to shed more light onto the molecular mechanisms and purposes of SL *trans*-splicing in cestodes.

The papers that form the remaining parts of this thesis could be generally sorted into three groups. The first paper listed as **Paper I** and entitled "Substitution saturation and nuclear paralogs of commonly employed phylogenetic markers in the Caryophyllidea, an unusual group of non-segmented tapeworms (Platyhelminthes)" falls into a category of "molecular phylogenetic studies" and represents one of the most relevant projects in which I participated during my postgraduate studies. This project aims at a multidisciplinary taxonomic re-description of the probably most primitive order of cestodes—the Caryophyllidea. This paper represents a pivotal study to optimize the methodological approach to inferring molecular phylogenies of this group. While evaluating the phylogenetic utility of several popularly used molecular markers (i.e., the rDNA and mitochondrial protein-coding data), we have detected and confirmed the presence of multiple sequence variants of a ~1500bp long part of mitochondrial genome in several representatives of the order Caryophyllidea. In this paper, we describe and discuss the consequences of this phenomenon for the inferences of caryophyllidean phylogenies, and also analyze the phylogenetic utility of the other molecular markers studied (i.e. the rDNA data).

During the PhD studies, I was given an opportunity to significantly contribute to the project that escapes the otherwise systematical focus of the remaining papers presented here, and rather aims at "development of a diagnostic method of cestode infections" for the use in clinical praxis. In this project, whose outcomes are described in **Paper II**, we developed a quick, cheap, and straightforward protocol based on multiplex PCR for differential diagnosis of human-infecting *Diphyllobothrium* cestode species. From the medical perspective, there are four important *Diphyllobothrium* species that infect humans, and that are being repeatedly reported as infective agents of an elevated number of residents in developed countries. While number of these cases represents a locally acquired infection by an allochtonous parasite species, and while those four species are hard or even impossible to differentiate morphologically (especially when the diagnostics is limited to parasite eggs), it calls for an increased awareness of these parasites from the epidemiological standpoint and a development of straightforward method to be used by technicians in parasitological diagnostic laboratories.

The third category of papers published within the scope of my PhD studies primarily represents morphological descriptions and re-descriptions of cestode taxa that systematically fall among the basal orders Caryophyllidea (**Paper III** and **Paper V**) and Bothriocephalidea (**Paper IV**). Those truly extensive multidisciplinary works are considered classic alpha-taxonomy works and my contribution to these was solely limited to the characterization of the molecular markers of choice and their phylogenetic analyses, including formulations of phylogenetic hypotheses and molecular data implications for the systematics of the tapeworm taxa studied.

Spliced leader *trans*-splicing in parasitic flatworms

Spliced leader (SL) *trans*-splicing is an RNA maturation process that adds a short, identical SL exon to the 5' end of various precursor messenger RNA (pre-mRNA) molecules that are transcribed from an independent genomic locus. The SL exon is transferred from a 5' end of a specialized non-coding SL RNA molecule (the remaining 3' part, after the SL exon transfer, is called the SL intron) that is transcribed from a gene organized on chromosomal DNA in tandem repeats. The length of the SL exon as well as the length of the longer donor SL RNA differs to some extent among different organisms. In platyhelminth flatworms, documented lengths of SL exon span from 34 to 51 nucleotides and SL RNA reaches lengths of 93 to 110 nucleotides, depending on the organism (Rajkovic et al., 1990; Davis et al., 1994; Davis, 1997; Brehm et al., 2000, 2002). The SL RNA molecule has, among eukaryotes, several unique characteristics: It bears a hypermodified 5'-cap structure (either a 2,2,7-trimethylguanosine (TMG) cap in metazoans or a 7-monomethyl cap 4 in kinetoplastids); it lacks the polyadenylated tail; and it shares similarities with the uridine-rich Sm-binding class of small nuclear RNAs (snRNAs; U1, U2, U4, U5 and U6 snRNA) by bearing a potential Sm-binding site—a nucleotide sequence that promotes attachment of the Sm proteins to RNA. snRNAs play an essential role in the conventional *cis*-splicing machinery, and form rich secondary structures of a conserved number of stem-loops. The difference between snRNAs and SL RNAs is the presence of 5' splice site signal in the SL RNA that denotes the division between the 3' end of SL exon and the 5' end of SL intron. The 5' splice site is always found within a base-paired region of the stem-loop secondary structure (Davis, 1996; Lasda and Blumenthal, 2011).

The mechanism of SL *trans*-splicing is almost identical to the mechanism of conventional *cis*-splicing (Lasda and Blumenthal, 2011). There are three fundamental signals that serve in intron-recognition by the spliceosome: (i) the 5' splice site (donor site—at the 5' end of an intron), (ii) the polypyrimidine tract/3' splice site (acceptor site —at the 3' end of an intron), and (iii) a branch site—upstream of the 3' splice site (Black, 2003). Splicing begins when the U1 snRNA recognizes and binds the 5' splice site and thus triggers a precise cascade of interactions of other factors with the rest of the splicing signals, eventually leading to the formation of a spliceosome (Wahl et al., 2009). SL *trans*-splicing recognizes the same intron splice site signals and follows the same path except for the fact that it joints two independently transcribed pre-mRNAs, and thus the pre-mRNA lacks the 5' splice site—it will be donated by SL RNA molecule. The mechanism how the SL RNA containing the 5' splice site gets to the proximity of the 3' splice site of the pre-mRNA remains, however, so far unanswered. Another unique feature of the SL *trans*-splicing is the fact that it does not require, for *cis*-splicing essential, U1 snRNP to initiate the *trans*-splicing cascade (Lasda and Blumenthal, 2011). This has been so far documented only in nematodes (e.g. Nilsen, 1993), but it might be

also true for other taxa as well. After the spliceosome catalyzes two *trans*-esterification reactions, two products are formed—a mature *trans*-spliced mRNA, and a residue of the two introns, joined at the branch point and forming a Y-structure that gets degraded afterwards (Lasda and Blumenthal, 2011).

SL *trans*-splicing has been described to occur in a number of eukaryotic organisms spanning several kingdoms of life. Originally, the phenomenon was found in kinetoplastids (e.g., Sutton and Boothroyd, 1986; Liang et al., 2003; Tessier et al., 1991), nematodes (e.g., Krause and Hirsh, 1987; Huang and Hirsh, 1989; Nilsen et al., 1989; Pettitt et al., 2008), and parasitic flatworms (e.g., Rajkovic et al., 1990; Brehm et al., 2000). Later on, the process was discovered in a myriad of organisms as cnidarians (Stover and Steele, 2001), urochordates (e.g. Vandenberghe et al., 2001), rotifers (Pouchkina-Stantcheva and Tunnacliffe, 2005), dinoflagellates (Zhang et al., 2007), or chaetognathes (Marlétaz et al., 2008). On the other hand, SL *trans*-splicing was searched for, but evidence for its presence was never found, in some of the genetically well-studied groups such as vertebrates, insects, plants, or fungi. This findings were recently confirmed by an exhaustive bioinformatic search undertaken throughout the databases of metazoan expressed sequence tags. Screening the 5' ends of mRNAs for conserved patterns revealed a further presence of SL in amphipod and copepod crustaceans, ctenophores, and hexactinellid sponges and at the same time did not show its existence in some close relatives of these primitive metazoans that SL *trans*-splice (Derelle et al., 2010; Douris et al., 2010). Moreover, a striking difference among groups of organisms can be also found considering the portion of transcripts that undergo *trans*-splicing. In kinetoplastids, for example, 100% of transcripts is processed by SL *trans*-splicing (Agabian, 1990) while in some copepods it seems to account for less than 1% of mRNA processing (Douris et al., 2010). Altogether, the findings in the field over the last twenty-five years suggest that both numbers of *trans*-splicing and non-*trans*-splicing organisms are going to increase along with the expansion of organisms with characterized genomes (Hastings, 2005).

Knowing the distribution of the SL *trans*-splicing across the eukaryotic organisms, it is currently impossible to judge how the phenomenon originated during the evolution. According to the two antagonistic hypotheses, SL *trans*-splicing either represents an old mechanism of mRNA processing that originated early in the evolution of eukaryotes, and was subsequently lost in number of lineages, or it is a process that evolved independently on multiple occasions and should be considered a homoplasy. So far, the evidence keeps both of the explanations viable (e.g., Nilsen, 2001; Hastings, 2005; Douris et al., 2010). Based on the shared features of the spliceosome of the extant eukaryotes and their obvious similarities, the ancestral eukaryote most likely disposed of a functional *cis*-splicing spliceosome (Roy and Irima, 2009). Also, the signals that control *cis*- and *trans*-splicing are mostly shared by both machineries, and SL snRNPs

and Sm-class snRNP—an essential components of the spliceosome—share conserved patterns. Studies have even shown that it is possible to make non-*trans*-splicing organisms (mammalian cells) *trans*-splice accurately, once they are supplemented with the gene encoding the SL RNA, even from completely unrelated organisms such as kinetoplastids (Bruzik and Maniatis, 1992). This confirms the hypothesis of conservation of the *cis*- and *trans*-splicing machineries and the likely potential of *cis*-splicing organisms to relatively easily acquire SL *trans*-splicing. On the other hand, existence of the SL *trans*-splicing in diverse groups of eukaryotic lineages while sharing some striking characteristics (e.g., the hypermethylated 5' cap, Sm-binding domain, 5' splice site placed within the stem region of the secondary RNA structure, relatively short length of the SL exon—Davis, 1996; Lasda and Blumenthal, 2011), would rather speak for a common origin of the phenomenon and its later loss in groups in which we nowadays find no evidence of *trans*-splicing. However, since there seems to be such a small step from converting a Sm-class snRNA to a SL snRNA, those joint characteristics could be considered to originate convergently from a common universally distributed precursor—the snRNAs. At the same time, the consequences of a potential loss of SL *trans*-splicing are likely to be only hardly compatible with life. Given the facts that, firstly, the SL *trans*-splicing leaves space for accumulation of mutations in the 5' untranscribed region of the SL *trans*-spliced genes and, secondly, it is often coupled with the presence of operons (evolution of which was probably allowed by the presence of SL *trans*-splicing mechanism—Blumenthal, 2004), loss of the SL *trans*-splicing would result in the death of an organism.

Characterization of spliced leader genes from basal cestodes

The SL *trans*-splicing of flatworms displays some specific characteristics unusual to other groups of organisms. Comparison of known SLs of flatworms have shown that SL varies significantly in the sequence primary structure, length, and predicted secondary structure suggesting that neither one of those features is essential for functionality of the process in flatworms (Davis, 1997). The variability of flatworm SL RNA sequence also includes some unusual characteristics of the Sm-binding site situated within the SL intron, and differing from the other flatworm snRNA Sm-binding sites. The consensus of the known SL RNA Sm-binding domain sequence of flatworms is $VRU_{1-4}MU_3GR$, with the insertion of the C or A nucleotide in the middle of the U chain being the most distinct feature (Davis, 1997). However, despite being variable among various flatworm lineages, SL exon always terminates with a highly conserved AUG sequence which might, and evidence supports it, later serve as a translation initiator methionine of the *trans*-spliced mRNAs in a part of the mRNA population (Davis et al., 1995; Davis 1997; Brehm et al., 2000, 2002; Cheng et al., 2006).

After the SL *trans*-splicing has been discovered in the first representative of the flatworms—fluke *Schistosoma mansoni* (Rajkovic et al., 1990), several approaches have been utilized to explore the potential distribution of the phenomenon across the flatworms, and to characterize their SLs. The first attempt employed the presumption that if there is a relatively small fraction of mRNAs undergoing SL *trans*-splicing in schistosomes (Davis et al., 1995), there might also be a conserved gene expression pattern ensuring that homologous genes are processed the same way in a group of related organisms. Truly, this idea appeared to be valid when a particular gene that is SL *trans*-spliced in *Schistosoma* was found to receive SL through *trans*-splicing in several other flukes (*Fasciola*, *Haematolechus*, *Stephanostomum*) or polycladid rhabditophoran *Stylochus* (Davis et al., 1994; Davis, 1997). However, this approach failed to detect SL *trans*-splicing in the other flatworm lineages including cestodes and a representative of monogenea as well as some other metazoan groups. Even subsequent Northern hybridizations using probes based on a conserved part of the SL RNA (spanning the 3' end of SL exon and the splice donor site) under lowly stringent conditions have not indicated presence of SL RNA with a similar sequence motif in these organisms (Davis, 1997).

SL *trans*-splicing in cestodes, specifically in the fox and dog tapeworms *Echinococcus multilocularis* and *E. granulosus* was, contrary to previous non-success, described by simultaneous finding of two different mRNA transcripts of the same gene, one of them obtaining short 5' exon through SL *trans*-splicing (Brehm et al., 2000). The identity of the SL was then confirmed by providing evidence that the SL exon is present on 5' end of various transcripts. It is donated by a 104bp long, non-polyadenylated, TMG-capped RNA which has common characteristics with other flatworm SLs. The full-length SL gene was also characterized from its tandem repetitions on chromosomal DNA. Two years later, Brehm et al. (2002) characterized the SL gene from another closely related cestode *Taenia solium* that along with the previous study suggested that SL *trans*-splicing represents a molecular process common to the cestodes.

Experimental procedure

Based on the fact that the SL *trans*-splicing was described in a group of highly evolved tapeworms (*Echinococcus*, *Taenia*) on one side and in the relatively primitive flukes (e.g. *Schistosoma*) on the other, assumption that the SL *trans*-splicing is universally distributed across the two sister groups of flatworm parasites was formed and ready to be tested on a suite of specimens representing basal cestode lineages. Table 2 lists the material collected and used in the current study which represents majority of known basal cestode lineages. Specimens were preserved either using absolute ethanol as a preservative for DNA isolations or the RNAlater (Qiagen) at −80 °C for the total RNA isolation.

Table 2. List of taxa analyzed within this study.

Species	Classification	Preserved in	Locality of Origin and Date
Amphilina foliacea	Amphilinidea	RNAlater/−80 °C	Hungary, 2007
Caryophyllaeus laticeps	Caryophyllidea	RNAlater/−80 °C	Slovakia, 2008
Cyathocephalus sp.	Spathebothriidea	RNAlater/−80 °C	Italy, 2007
Didymobothrium rudolphii	Spathebothriidea	RNAlater/−80 °C	Portugal, 2006
Gilquinia squali	Trypanorhyncha	RNAlater/−80 °C	USA, 2009
Khawia sinensis	Caryophyllidea	RNAlater/−80 °C	Slovakia, 2008
Ligula intestinalis	Diphyllobothriidea	RNAlater/−80 °C	Czech Republic, 2007
Neobothriocephalus sp.	Bothriocephalidea	RNAlater/−80 °C	Peru, 2006
Spathebothrium simplex	Spathebothriidea	RNAlater/−80 °C	USA, 2009
Triaenophorus nodulosus	Bothriocephalidea	RNAlater/−80 °C	Czech Republic, 2008
Amphilina foliacea	Amphilinidea	EtOH/4 °C	Russia, 2006
Caryophyllaeus laticeps	Caryophyllidea	EtOH/4 °C	Russia, 2004
Cyathocephalus truncatus	Spathebothriidea	EtOH/4 °C	Russia, 2004
Didymobothrium rudolphii	Spathebothriidea	EtOH/4 °C	Portugal, 2005
Ditrachybothrium macrocephalum	Diphyllidea	EtOH/4 °C	United Kingdom, 2002
Grillotia pristiophori	Trypanorhyncha	EtOH/4 °C	Australia, 2001
Gymnorhynchus isuri	Trypanorhyncha	EtOH/4 °C	USA, 2003
Gyrocotyle sp.	Gyrocotylidea	EtOH/4 °C	North Atlantic, 2001
Ligula colymbi	Diphyllobothriidea	EtOH/4 °C	Czech Republic, 2001
Marsipometra hastata	Bothriocephalidea	EtOH/4 °C	USA, 2002
Monobothrioides sp.	Caryophyllidea	EtOH/4 °C	Sudan, 2006
Nybelinia scoliodoni	Trypanorhyncha	EtOH/4 °C	Australia, 2001
Sphyriocephalus sp.	Trypanorhyncha	EtOH/4 °C	Atlantic Ocean, 2002

Initially, we chose a degenerative PCR approach using the “Easy gene walking” technique (Harrison et al., 1997) and chromosomal DNA as template to test if it would be possible to amplify the SL gene using primers directed against the conserved regions of the known flatworm SL RNAs. Two nested forward primers, CestSL1 and CestSLnest, were used in two subsequent PCR runs in combination with two sets of reverse “easy gene walking primers”: one of the T7NHSP, T7NALUR, or T7NSAU primers in the first run, and the TOPO-T7 in the second run (see Table 3 for primer characteristics). PCR cycles were as follows: first run: 35 cycles of 94 °C/40 sec, 53 °C/2 min, 72 °C/2 min; second run: 1 cycle of 94 °C/2 min, 50 °C/2 min, 72 °C/3 min followed by 35 cycles of 94 °C/40 sec, 56 °C/1 min, 72 °C/2 min. PCR products were gel-purified and cloned into *E. coli*. A putative ~800bp long fragment of the SL gene repeat amplified from *Ligula* sp. chromosomal DNA using T7NSAU primer was identified and used to design a specific pair of primers (LigSLfor and LigSLrew) directed toward each other for amplification of the whole SL gene tandem repeats. 30 PCR cycles of 94 °C/40 sec, 52 °C/40 sec, 72 °C/5 min then resulted in the amplification of 2483bp PCR product in which one putative SL RNA gene was found.

To confirm the identity of the *Ligula* SL exon, we prepared a SL-cDNA library following the protocol described in Fernández and Maizels (2009) which comprised the following steps (only alternative steps to the protocol are detailed): total RNA isolation using TRI Reagent (Molecular Research Center) according to the manufacturer's protocol; first-strand cDNA synthesis using the SuperScript II reverse transcriptase (Invitrogen) and the CD3-RT oligo-d(T) primer; PCR amplification of SL-cDNAs using 5 U of *Taq* DNA polymerase (TaKaRa), primers LigulaSL-lib and CD3, employing the cycling protocol of 16–30 cycles of 94 °C/1 min, 53 °C/1 min, 72 °C/5 min; gel-separation and purification of a ~500–1500bp excised fragment using the QIAquick kit (Qiagen) according to the manufacturer's protocol; cloning the insert into *E. coli* using the p-Gem-T Easy vector (Promega); and sequencing ~10 randomly chosen colonies using universal vector primers. Sequences obtained were assembled and screened for LigulaSL-lib primer which has been found at the positions 1–21 of the insert followed by a common 4bp sequence TATG that represents the highly conserved termination sequence of the SL exon of flatworms.

Table 3. List of primers used.

Name	Sequence	Purpose
CestSL1	NKTGYATGGTRAGWAYC	Easy gene walking (based on flatworm 3' end of SL exon and 5' end of SL intron)
CestSLnest	KTGYATGGTRAGWAYCG	Easy gene walking (based on flatworm 3' end of SL exon and 5' end of SL intron)
T7NHSP	CACTATAGGGCGANNNCATG	Easy gene walking
T7NSAU	CACTATAGGGCGANNNGATC	Easy gene walking
T7NALUR	CACTATAGGCGCANNNTGCA	Easy gene walking
TOPO-T7	TACGACTCACTATAGGGCGA	Easy gene walking
LigSLfor	GTATGGTGAGTACCGACGTGGC	*Ligula* SL gene tandem repeat amplification
LigSLrew	CACGTCGGTACTCACCATACAC	*Ligula* SL gene tandem repeat amplification
LigulaSL-lib	CGGTATTTTACCTACCCAGTG	Amplification of *Ligula* SL cDNA library
CaryoSL-lib	CGGTTCTTACTTTACTTGTTG	Amplification of *Caryophyllaeus* SL cDNA library
TriaeSL-lib	AGGTCTATACTATACTCATTG	Amplification of *Triaenophorus* SL cDNA library
TapeSL1for	CACCGTTAMWCGGTNYTT	Degenerative PCR to amplify cestode SL gene tandem repeat
TapeSL1rew	AARNACCGWKTAACGGTG	Degenerative PCR to amplify cestode SL gene tandem repeat
TapeSL-SLRNAfor	TGTATGGTGAGTAYCGA	Degenerative PCR to amplify cestode SL gene tandem repeat
TapeSL-SLRNArew	TCGRTACTCACCATACA	Degenerative PCR to amplify cestode SL gene tandem repeat
TapeSLRNAfor	TTTGGCTGGTCCKTCRRGG	Degenerative PCR to amplify cestode SL gene tandem repeat
TapeSLRNArew	CCYYGAMGGACCAGCCAAA	Degenerative PCR to amplify cestode SL gene tandem repeat
Caryo-SL5	AGAACCGTGTAACGGT	*Caryophyllaeus* SL gene tandem repeat amplification
Caryo-SL3	TCTTACTTTACTTGTTGTATG	*Caryophyllaeus* SL gene tandem repeat amplification
Triae-SL5	TAGACCTAGTTATAGGT	*Triaenophorus* SL gene tandem repeat amplification
Triae-SL3	TACTATACTCATTGTATG	*Triaenophorus* SL gene tandem repeat amplification

Following the characterization of the *Ligula* SL gene sequence, we made an effort designing a set of degenerated primers (TapeSL1for, TapeSL1rew, TapeSL-SLRNAfor, TapeSL-SLRNArew, TapeSLRNAfor, TapeSLRNArew) that we used to amplify potential

SL genes from the chromosomal DNA of representatives of all basal cestode taxa (see Table 3 for taxa stored in EtOH). However, while using myriads of combinations of PCR cycling conditions to obtain a number of variously sized PCR products, we never confirmed single one of them to include a potential SL gene.

As a complementary alternative to the chromosomal DNA based approach to characterize the SL genes of basal cestodes described above, we also used the total RNA as a starting template to employ a methodology of enriching cDNA libraries for the TMG cap-bearing population of mRNAs. It was described by Fernández and Maizels (2002) that the TMG cap of the SL *trans*-spliced mRNAs instead of being cleaved analogously to the 7-methylguanosine (7MG) cap of conventional mRNAs by the enzyme tobacco acid pyrophosphatase (TAP) is left intact. We treated the total RNA isolated as described above first with the TAP enzyme and afterwards with Terminator 5'-P-dependent Exonuclease (both from Epicentre Biotechnologies) according to manufacturer's instructions. The first of the enzymes cleaves the 7MG cap of the conventional mRNAs to leave them with a 5'-monophosphorylated terminus. The second enzyme is a 5'–3' exonuclease that digest everything with a 5'-monophosphate and thus, applied in combination with TAP on the total RNA, would leave only the TMG-capped SL-bearing mRNAs intact. After the enzyme treatment, RNA was reverse-transcribed and PCR amplified using the SMART PCR cDNA Synthesis Kit along with the Advantage 2 PCR kit (both Clonetech) according to manufacturer's instructions and cloned into *E. coli* using the p-Gem-T Easy vector (Promega). From the constructed cDNA libraries, several tens of clones have been sequenced and screened for the presence of a common sequence motif at their 5' end.

Total RNAs of several cestode representatives were processed using this protocol and a putative SL exon was detected in cases of two organisms—*Caryophyllaeus* and *Triaenophorus*. To verify the identity of the putative SL exon sequences, degenerated primers were designed and used as described above in the case of *Ligula* SL gene amplification. Primer pairs used were Caryo-SL5 and Caryo-SL3, to amplify *Caryophyllaeus* SL gene, and Triae-SL5 and Triae-SL3 in case of *Triaenophorus* (Table 3). This way, a single amplicon was obtained in each of the reactions, the PCR products were cloned, sequenced, and a 34bp and 35bp long SL exons were found within each of a 1521bp and 1436bp long fragments of *Caryophyllaeus* and *Triaenophorus* SL tandem repeats, respectively.

As in the case of *Ligula*, specific SL exon primers CaryoSL-lib and TriaeSL-lib were designed and used to construct the SL-cDNA libraries following the protocol by Fernández and Maizels (2009) with the minor alternations described above. Also in this cases, authenticity of the SL RNAs of *Caryophyllaeus* and *Triaenophorus* have been confirmed by the presence of a 21bp long primer sequence used in the PCR amplification of the SL-cDNA libraries that was followed, without exception, by a

conserved TATG sequence marking the 3' end of the SL exon. All the three newly characterized sequences of the SL RNAs were aligned with the rest of the currently known neodermatan SL RNA sequences to compare the relative sequence conservation of the SL RNA molecule. The minimum free energy folding patterns of the newly described SL RNAs were predicted using the RNAfold algorithm and consistence with the secondary structure predictions of the already known cestode SL RNAs was checked.

Results and discussion

The characterization of three new SL gene sequences representing distinct cestode orders that have not been sequenced yet are presented here along with a preliminary analysis of their primary and secondary sequence structure and their homologies to the already known SL RNA genes of their close relatives. According to the current knowledge, the cestodes *Caryophyllaeus*, *Ligula*, and *Triaenophorus* represent relatively primitive cestode lineages when compared to the highly phylogenetically derived *Echinococcus* and *Taenia*. This makes them a suitable model for obtaining a better insight into the evolution of SL RNA sequence in the parasitic flatworms.

The essential characteristics of the three sequences are summarized in the Table 4 and Figure 1 and 2. All the three SL RNA sequences display typical flatworm SL properties including high sequence homology with the rest of the flatworm SL RNA sequences limited to the region close to the splice donor site (splice donor site is situated at the position 38 of the alignment depicted in Figure 1) and a region of the putative Sm-binding site (cestode alignment region 81–89). All of them bear among flatworms universally conserved AUG codon sequence which is found at the 3' end of the SL exon. This codon sequence can provide the translation initiator methionine to the fraction of the SL *trans*-spliced mRNAs as detected by some studies (Brehm et al., 2000). The sequence of the putative Sm-binding site shows a perfect consensual structure with the rest of the flatworms with one striking difference observed in *Triaenophorus* whose 3' half of the domain contains a string of adenines instead of the VRU_{1-4} consensus. Otherwise, all three new cestodes have a typical flatworm adenine/cytosine insertion in the middle of the Sm domain that in flatworm U snRNAs contains a string of five uridines and which was hypothesized to have some effect on the SL interactions with Sm proteins (Davis, 1997).

Table 4. Primary sequence structure characteristics of *Caryophyllaeus*, *Ligula*, and *Triaenophorus*.

	SL exon length	SL RNA length [a]	SL gene tandem repeat
Caryophyllaeus laticeps	34	88	1521
Ligula intestinalis	35	89	2483
Triaenophorus nodulosus	35	90	1436

[a] The 3' end of the SL RNA molecule has been determined according to the alignment with the SL RNA's 3' end of *Echinococcus* and *Taenia*.

Among the other features of the three new SLs sequences that are also commonly found among other flatworms belong the unified prediction of their SL RNA secondary structure. Only *Schistosoma* (Rajkovic et al., 1990) does not display the otherwise continuously observed three-stem-loop structure with the 5' splice donor site situated within the base-paired region of the first stem. Also, the Sm-binding region, when constrained to be single-stranded, is continuously situated between the second and third stem structure.

Amplification of the SL RNA gene from the chromosomal DNA using primers oriented in the opposing directions confirmed that the SL RNA gene is encoded in tandem repeats as the rest of the flatworm SL genes known. *Ligula* might represent the most extensive SL repeat structure discovered in flatworm parasites since the longest had been *Taenia* with a 2040bp repetition (Brehm et al., 2002). The last, and most probably the most important, proof of the identity of the newly described SL RNA genes is the confirmation of their functional activity done by screening the SL-cDNA libraries. The common presence of the SL-specific primer sequence used to amplify the libraries followed by the remaining quartet of the conserved SL exon TATG nucleotides indicated that the SL exon is being objectively *trans*-spliced to a subpopulation of nascent mRNA. However, the question to what extend do the newly characterized tapeworms employ SL *trans*-splicing remains untested by this study. In fact, by simply counting the questions that might (and will) arise from the fact that the new tapeworm SLs are being characterized, I conclude that the current status of this study raises more questions then answers.

Figure 1. Alignment of the currently known SL RNA genes of parasitic flatworms with the newly characterized sequences of *Caryophyllaeus*, *Ligula*, and *Triaenophorus* and the predicted secondary structures constrained to keep the putative Sm-binding domain (alignments positions 81–89) of the SL RNA single stranded. In case of *Ligula*, two alternative secondary structures were predicted.

Caryophyllaeus
ACCGTTACACGGTTCTTACTTTACTTGTTGTATGGTGAGTATCGTATGACTTRTGTCATAAATAGTATTTGGCTGGTCCGAAAGGGCCTAGAACAA
ATGAAATAAATAATTGTGACTGTTAGAATCGCTTACGATTGTTTGATTGTACACAAATTCCTATAGATAAATAAATTTATCCACACATCTTTCATTGCCATGCC
TGAAAATGTAAGTAACTAGAACGAGGTGAACCTCAATCCAAACATATATACATTTTTTGAGTCAATTTGCAATTATGTCTGAGCTACTAACTGTAAGAACCT
GATGCACTATACACTTCCGGCAAGGTCTAAGTCGATTTAACTCTACGAGGACTACAATTTAGCTTCAGTGAAGTTAAGCCATGCGACCTCTAACGTCCGAG
TTTATCACGTTATATAGTGCACCATTAGGGCACCCAATATCTGCAAGTTAATCCAACACAACTCTTTTTAACGTCACCACTACTAAATTACACAATGCTTATA
ATTAGATTAATCTCAACGTTGTATATACCATGACCTAAGTCAATTAAATTGAGCCTCAAACTAGGACCGAACAGGTCTTCGAACCATTGATCATATGAAGAT
GATATAGATCTATCACATACAGAAGACTGGTGTATATTTAAATCAAATTTTCCAATTAGTCACAGTCCTACATTGTAAAAAGTTTATTTAGTAGTCGCGAAAG
TAATCCATTATCATAGGATACTGCCTAGATTCAGTACAACAATTAATTTATTATTCAATCATTTTAARACGCTGCATATTAGTTCATCAACTGCAGTCAACTTG
TTATCCATCATATATTGGTTATGTTATCTTAAATGAGTAAGTTTATTAGCTACTGACTGATGGTTATACTGCGATAAATTTCAGTGTAGTCTGGATCTTAACCTA
TGYCAAAGCTGATGAGCTCCAGATTGTTTCCGCCACGAGAATCTGGTAACATAACCGACACATAGCGCTCTTATCCTCTCAGCCACCGATGCACGACTTCG
ATATKGGGAATATTATGTGTAAAACGTGCGAGTTCCAATTATTCGAAAAATATATTCCAAAGTCCACGCCGGGCTTTTAACTCTGAGTAGTTAGAAACGCTA
GCAGATAATCTGATAATACGTTTTGTACATACATGTATATTCTTCCATATCACTGTGTGCACTGCATGTATGGCCATGCTTATGAAAKGTTCAATTTTCTTCGA
AATAGACTGATTTATTCATGTATGAAATACCGATCGCATTATATTACAATAAATTTATAAATTATACCCATACTACTTATTAAATTAGTGAGTAGATTTAATTCAG
CTGACGACTACAGACGAAATATGGAGAAAAGTGCAATGTTCGATACAAAAATGGATGAAATGTATATCGGAGATTTTAATTTATATTAAAGAGGGTCAATT
ACCACGAATGTTGAATTATTTATTAAATTTATTTTTACTGGGCAACTAATGATATTATAGGGTTATTTACAAGTTGCAGGTTTCTTT
Ligula
CACCGTTACACGGTATTTTACCTACCCAGTGTATGGTGAGTACCGACGTGGCTTGTCCATGAAAAGTCTTTGGCTGGTCCGTCAGGGCCTAGATAT
GAAATATAGAGGAAGCGATCAGTGCAACAGGAGATTCATTGTCCTCATGTTTCCGATTCCGTCGGAGATCCTTCATCTGAGACATGCGCAGATCTCAGGC
CTTCCTATGATAAATGATTCCCACGGAATCCAAAACGTCAGAACAATAAAACTCTTGTTCCAGTGATCACTTCTGCTTACTGATACTCACATATTTAATTGG
ATTGGCAATATTTCATAATGTTAGCGAGGTGTGCATTGCCAATACATTGCTCGGCCGTACTGCAATGCGCGCCAGCATGTACATTCCGGTTAGGGCTTCCCT
CTCTCCTTCCAACAGCCTCCGCATGTTTGTCACCTAGATATCATTTCTGCTTCTGTGAGAACATGAAAATGTCTATGCAAGAAAAGCACATAGAGTTGGTA
CAGCTACGGCTACTTTAAATCATCCTCTTTGTCAGCGAGTGAGAACGAAGTGCCAACAACCACTTCCATATGTTGAGACAGGTTCTGATTTGGCTCCAGA
AGTGCATAAGGGTGCTGTGTGGAGTGTAGCAATACAAATCCCGTCAGTCTCCTTTCTGTGATGGTTTGCAACCTTGTCGCAGTCACTGCACACTGTCACCT
CTGAGACGAACTTACCGTATTTCACATGAAGGACGGTAGTTTGGCTGTCTTGCTAAAATGCCTCTACTTTTCGCCCATCTACCACGCAGCATCAATTCTGG
AGATGATGCTGTCCGTTAAACAGCGGCATCACTCTGAGGTTTCTGAAACGGACTACTACGTTAAGTTGACAGCCACTAATCGCAAGAAGTGCCTTCTTCT
GGTGTGGAATGCAGCTTGGAAGGGTCTCACCCTTGAGGCAGGGAATCGCACGAGAAGAACCACAGCGAAGTAAATAAGGCTTCTCTCGTGATAGTTCT
AATGTCTGGTTTTATATCACCCCTCGAGGACGGATGCGAGGATACNCCGGGCATCAGTCATCGGGACTGAACTCCCCACTGCACACTTCTTCAACCGCCT
GATCGAGTCAGGATATAGGACCCGTAGATTTCAACGGTAATGCCGTCCTCTCAGGGTGGCTTGTTGTTGAGCAGACTTTGGATCGAGTCGGTAACTTCTG
CTTCAGAAGGTGTGCGAGGCTTAGATGGGTATGCATGGCCTGAAGGGCAATTCAACTCGGCTGCAGAGGAGAGTGAGTGTGAGATTGTGATGAACTTT
TCAAGAAGTGCTCATGCCATCGCTCCACTCCGATCGAGGTTTTAACAATGAATATGTGATAAGTATCAAAAACAGAGTCCTTCAGTGCAGAGGGATTACCA
CTGACTTTACGGTAGGTTCTCCGACTGTCACTAGCGTCTATGGCCTACTCCATGTGGGTCCCTATTTCAGCTCGACAAATCTCAAGAGCCTATCTTCGACTA
CTACACACACCTGGTCCTGCAACAATGGGTCAGGATCTTTCACAAAGATTATATTTCCAAACACCCAGAAACACACACACACACACACACACATGCACA
CACATAAAGCATAAGACGCCGTAGTCTATTGTCATTGTAGGGACTTTTCTTAGGTTTCGCCGGATCATACCTGACCTAAGCCAACGTATGCCTTCAGGAGC
ACTCCTCATGTCAGCATGCAGGGTAATCAGCGAAAAAGCACATTATGATTTGCCAAGTAAAGATGCGGAGTAGATTTCTCACGCAACCCATGGCAGAGG
GATGGAATGAGCTAGGAAGCTGTAGCAAGGTCAACGATGCTAAAGCCAATGAGGAGAAAGTTGGATAGCTTCATATAGTCCTCACTTTGTAAGGATATAC
AGAAGTCGAGCTGAAGACAGCATGAAATCCAAAGAAGGCATTGAAGCATTTTGTCCATTGCCATACGCACACTGCATCATGCAAGACGAAATGTCCGAT
GGAAAAGGTGTCAAAGATTGCACTAAGCCTCCCACCCCTCCCCTCTACTGGTGGGCTTCTGATGGTTTTGGCGTTGGAAATGTTGAAGAAGGACTGGAC
TGGTCTATCCCTCAAAATGTCCATGGTCATCGCACAATAGCCCGCTTATCCTGGGCTGGGAGGACACATAGTGGTCTGCGTGTCCTACTTAACTGAGAGGT
CTGTACGTGTGTCGGCAAGTAAGGTGTTCTCGGAGCACCAGCGGTGCAAATATGCATGTACTACTGAATGAAATTAAGTAAGTACGCCAGAGATCTAAAG
CCATTAATGCAAAAGTGCTATGAAAATGGGAAAGACTTCACAAGCGTTGAGCAAGCGGTAGTATTGCAGAAAAGATGTATTGGACGTTTGTGCACATAA
GGTAGGCATTGATACTTAGGAAAAATCCTATGCAATGAGGCAAGGAGATAGACTTATGTGGGTCAAGAAATGTGTGTCCAAGGT
Triaenophorus
ACCTATAACTAGGTCTATACTATACTCATTGTATGGTAAGTATCTAGGTGACTTCGGTCACTGTAAAAATTTGGCTGGTCCTTGCGGGCCTAGAATT
GTAAAAAAGTTTGTCGATGAATGGTTTAAATGTGAAATGCTTCACGTTGTAATGTTCCTTCGATTCACAATTTAGTGTAAAAAGCCCTTTAAAAACTCGAA
CGAGTGTTCCATCACATATTGTCAAATGTCGACTCGCAGCAGTCATTCTTCCGATCGGCTTCTTCCTCGCATGTTTAAATAGCTATGGAGTACGCAACACGA
ATTCGCCATACTTGTGCAATTTTCCCTGTAGCCTTTCCGTGACAGTGAGTTAGAGAGAGTTTCATGTGTTCCTGTGGAAGCGGCGATCTCGATGACCAGTT
GCCATTTCATTGGGATCCACCATGGAGATCGCAGAAGAAGTTGCCGTCGAGTAGTCCAGGGTAACTTACTTATATCGTTAACATTTTCGTATTAAATGATGA
TAAAGGTTTAGTTTAACTTTCTTTTTGAAATTTGTGGACCCATCTCTGTTAATTTGTTTTTATCTCCATATCCGTCTAATCATAGATGGTTCGTGCATTACTTTT
TTAGTCTAGCCTGCTATTTTTTGGAACTAAGATTTTGCAATATTTAGCCGATATGGTTTCATTAACAATATTGATACTGCCTGTGAAACTGGTGCAGTGAAAC
AAATGTTTAGGTGACTCTACTTAAACCATCGTTGGTGCTGCAATAAACATAAAGAAAATCGGATTTACCTTACTATTTATCTGCTTTCATTTAGTATTACTCT
GAAAATTAATGTGACATTTTATATTGAACTTACTATTTCTTTATCCATACTGTATATTGCATTTTGGCTTTCCTTTTCATTATTTACGCATACGAGACTGAGTAG
GCATGATTTGTCAATGTTATGTAACTTCCAGCGTTTCATTTTTTTAGCATTGTTCACTCGATGACCATATCAAGTGATTTGCAAACGACGCCTGTATAAATCG
TGCTGAAAAGAAAAAATTCAATTCAATTCAAGAGGATACTTGAAGAAAATTGAAAATTCAGTTTTTGTCATCTTCAAGTTGACGTGTGATTGTTAAATGATT
GAAATGTGATGTCCCATTTCTCTGTTTGCGCTGTAATTTCCCCAACTGGACTGTTTTTCTCGTTATCAATTATTGTGTATTATTTTATACTACTTAAATAATTAG
ATCAAAAACAAGCAGATAAGTTCGAAAAGCTTTAAAATGAAACAGTGGTTTGTTATTTTGTAACAATAATAAATAACAATATTGCATGATGATTATAAAAAC
AGAAAGATAATGGGAAATTGTTAAAATAATTATTAAATTATTTCTTTTAAAATGTTGGACAGTGGTAAATGATGAATGGGTGATGAGGCAAGATGCGTTT
GGGTT

Figure 2. Complete sequence of the SL gene chromosomal tandem repeat of the newly described SL genes of *Caryophyllaeus*, *Ligula*, and *Triaenophorus* with SL exon (underlined) and putative SL RNA gene sequence (bold) highlighted.

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Research articles

Paper I

Brabec, **J.**, Scholz, T., Králová-Hromadová, I., Bazsalovicsová, E., Olson, P.D. 2012. Substitution saturation and nuclear paralogs of commonly employed phylogenetic markers in the Caryophyllidea, an unusual group of non-segmented tapeworms (Platyhelminthes). *International Journal for Parasitology* 42: 259–267.

ABSTRACT

Caryophyllidean cestodes (Platyhelminthes) represent an unusual group of tapeworms lacking serially repeated body parts that potentially diverged from the common ancestor of the Eucestoda prior to the evolution of segmentation. Here we evaluate the utility of two nuclear and two mitochondrial molecular markers (ssrDNA and lsrDNA, *nad*3 and *cox*1) for use in circumscribing generic boundaries and estimating interrelationships in the group. We show that these commonly employed markers do not contain sufficient signal to infer well-supported phylogenetic estimates due to substitution saturation. Moreover, we detected multiple *trn*K + *nad*3 + *trn*S + *trn*W + *cox*1 haplotypes within individuals, indicating a history of gene exchange between the mitochondrial and nuclear genomes. The presence of such nuclear paralogs (i.e. numts), to our knowledge described here in cestodes for the first time, together with the results of phylogenetic, saturation and split-decomposition analyses all suggest that finding informative markers for estimating caryophyllidean evolution is unusually problematic in comparison to other major lineages of tapeworms.

Paper II

Wicht, B., Yanagida, T., Scholz, T., Ito, A., Jiménez, J.A., **Brabec**, **J.** 2010. Multiplex PCR for differential identification of broad tapeworms (Cestoda: Diphyllobothrium) infecting humans. *Journal of Clinical Microbiology* 48: 3111–3116.

ABSTRACT

The specific identification of broad tapeworms (genus *Diphyllobothrium*) infecting humans is very difficult to perform by morphological observation. Molecular analysis by PCR and sequencing represents the only reliable tool to date to identify these parasites to the species level. Due to the recent spread of human diphyllobothriosis in several countries, a correct diagnosis has become crucial to better understand the distribution and the life cycle of human-infecting species as well as to prevent the introduction of parasites to disease-free water systems. Nevertheless, PCR and sequencing, although highly precise, are too complicated, long, and expensive to be employed in medical laboratories for routine diagnostics. In the present study we optimized a cheap and rapid molecular test for the differential identification of the most common *Diphyllobothrium* species infecting humans (*D. latum*, *D. dendriticum*, *D. nihonkaiense*, and *D. pacificum*), based on a multiplex PCR with the cytochrome c oxidase subunit 1 gene of mitochondrial DNA.

Paper III

Scholz, T., **Brabec**, **J.**, Králová-Hromadová, I., Oros, M., Bazsalovicsová, E., Ermolenko, A., Hanzelová, V. 2011. Revision of *Khawia* (Cestoda: Caryophyllidea), parasites of cyprinid fish, including a key to their identification and molecular phylogeny. *Folia Parasitologica* 58: 197–223.

ABSTRACT

Monozoic cestodes of the genus *Khawia* Hsü, 1935 (Caryophyllidea: Lytocestidae), parasites of cyprinid fish in Europe, Asia, Africa and North America, are revised on the basis of taxonomic evaluation of extensive materials, including recently collected specimens of most species. This evaluation has made it possible to critically assess the validity of all 17 nominal species of the genus and to provide redescriptions of the following seven species considered to be valid: *Khawia sinensis* Hsü, 1935 (type species); *K. armeniaca* (Cholodkovsky, 1915); *K. baltica* Szidat, 1941; *K. japonensis* (Yamaguti, 1934); *K. parva* (Zmeev, 1936); *K. rossittensis* (Szidat, 1937); and *K. saurogobii* Xi, Oros, Wang, Wu, Gao et Nie, 2009. Several new synonyms are proposed: *Khawia barbi* Rahemo et Mohammad, 2002 and *K. lutei* Al-Kalak et Rahemo, 2003 are synonymized with *K. armeniaca*; *K. coregoni* Kritscher, 1990 with *Caryophyllaeus laticeps* (Pallas, 1781) (family Caryophyllaeidae); *K. cyprini* Li, 1964 and *K. iowensis* Calentine et Ulmer, 1961 with *K. japonensis*; *K. dubia* (Szidat, 1937) (syn. *Bothrioscolex dubius* Szidat, 1937) with *K. rossittensis*; and *Tsengia neimongkuensis* Li, 1964 and *T. xiamenensis* Liu, Yang et Lin, 1995 with *K. sinensis*. *Khawia prussica* (Szidat, 1937) (syn. *Bothrioscolex prussicus* Szidat, 1937) is considered to be *species incertae sedis*, but its morphology indicates it may belong to *Caryophyllaeus* Gmelin, 1790 (Caryophyllaeidae). The molecular analysis of all seven valid species, based on comparison of sequences of two nuclear ribosomal and two mitochondrial genes, has shown that the species form three major groups clustered according to their fish hosts. Five species from common and crucian carp and goldfish were grouped together, whereas *K. armeniaca* from barbels (Barbinae) and *K. baltica* from tench (*Tinca*) formed separate clades. In contrast, geographical distribution does not seem to play a crucial role in grouping of individual taxa. A phylogenetic tree based on morphological characters was incongruent with that inferred from molecular data, which indicates that some morphological traits may be homoplastic. A key to identification of all species of *Khawia* based on morphological characteristics is provided.

Paper IV

Kuchta, R., Burianová, A., Jirků, M., de Chambrier, A., Oros, M., **Brabec**, **J.**, Scholz, T. 2012. Bothriocephalidean tapeworms (Cestoda) of freshwater fish in Africa, including erection of *Kirstenella* n. gen. and description of *Tetracampos martinae* n. sp. *Zootaxa* 3309: 1–35.

ABSTRACT

A survey of bothriocephalidean tapeworms (Cestoda) parasitizing African freshwater fish is provided. Based on critical evaluation of type specimens and extensive, newly collected material, only the following seven species, instead of 19 taxa listed in the literature, are considered to be valid and their redescriptions are provided: *Bothriocephalus acheilognathi* Yamaguti, 1934 (with 3 synonyms from Africa); *Bothriocephalus claviceps* (Goeze, 1782) (marginally in Africa); *Ichthybothrium ichthybori* Khalil, 1971; *Kirstenella gordoni* (Woodland, 1937) n. comb. (1 synonym); *Polyonchobothrium polypteri* (Leydig, 1853) (4 synonyms); and *Tetracampos ciliotheca* Wedl, 1861 (4 synonyms). In addition, *Tetracampos martinae* Kuchta n. sp. is proposed for tapeworms from the catfish *Bagrus meridionalis* from Lake Malawi. The new species differs from *T. ciliotheca* in a much larger body (19 cm versus 3 cm), dorsoventally flattened strobila and numerous (39 versus 25–35) and longer apical hooks (up to 98 μm versus less than 50 μm). *Kirstenella* Kuchta n. gen. is proposed to accommodate *Senga gordoni* Woodland, 1937 as its type species. The new genus is distinguished from other genera of the Bothriocephalidae by the presence of an apical disc armed with two lateral semicircles of large hooks, cortical vitelline follicles and large-sized cirrus-sac. All valid species were recollected. Bothriocephalidean cestodes are widely distributed throughout Africa, but only two species, *B. acheilognathi* and *T. ciliotheca*, occur in other continents. All but one species (*B. acheilognathi*) exhibit narrow host specificity, being limited either to one host species (*K. gordoni* in *Heterobranchus bidorsalis* and *T. martinae* in *Bagrus meridionalis*) or one host genus (*I. ichthybori* in *Ichthyborus* spp., *P. polypteri* in *Polypterus* spp. and *T. ciliotheca* in *Clarias* spp.). Molecular data based on partial sequences of the large subunit rDNA (lsrDNA) show monophyletic position of all African taxa analysed (*B. acheilognathi*, *I. ichthybori*, *K. gordoni*, *P. polypteri* and *T. ciliotheca*).

Paper V

Oros, M., Ash, A., **Brabec**, **J.**, Kar, P.K., Scholz, T. 2012. A new monozoic tapeworm, *Lobulovarium longiovatum* n. g., n. sp. (Cestoda: Caryophyllidea), from barbs *Puntius* spp. (Teleostei: Cyprinidae) in the Indomalayan region. *Systematic Parasitology* (in press).

ABSTRACT

A new caryophyllidean cestode is described from barbs *Puntius* spp. (Cypriniformes: Cyprinidae), with *P. sophore* (Hamilton) as its type-host, in the Ganges and Brahmaputra river basins in India and Bangladesh, and a new genus, *Lobulovarium* n. g., is proposed to accommodate it. The genus belongs to the Lytocestidae because its vitelline follicles are situated in the cortex. It is typified by: (i) a peculiar ovary, which is roughly H-shaped, but with asymmetrical, irregular lobes on its ventral and dorsal sides; (ii) an extensive vitellarium formed by numerous vitelline follicles scattered throughout the cortex; (iii) a long, conical postovarian part of the body with numerous vitelline follicles; (iv) a broadly digitate scolex with a slightly protrusible central cone; (v) a single gonopore (male and female genital ducts open via a single pore and a common genital atrium is absent); and (vi) a small number of testes (< 60). Molecular data (partial sequences of the lsrDNA) indicate that *Lobulovarium longiovatum* n. sp. belongs among the most basal caryophyllidean cestodes, being unrelated to species from siluriform catfishes in the Indomalayan region. *Paracaryophyllaeus osteobramensis* (Gupta & Sinha, 1984) Hafeezullah, 1993 (syn. *Pliovitellaria osteobramensis* Gupta & Sinha, 1984) from another cyprinid fish, *Osteobrama cotio* (Hamilton), in Uttar Pradesh, India, is tentatively transferred to *Lobulovarium* as *L. osteobramense* (Gupta & Sinha, 1984) n. comb. It differs from *L. longiovatum* by having much smaller eggs (length <50 μm versus >90 μm in *L. longiovatum*), which are spherical (length/width ratio 1:1 versus 2.5–3:1 in the new species), and the presence of vitelline follicles alongside the ovarian lobes (almost completely absent in *L. longiovatum*).