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Research paper

The SSU rRNA secondary structures of the Plagiorchiida species (Digenea), its applications in systematics and evolutionary inferences

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ABSTRACT

The small subunit ribosomal RNA (SSU rRNA) is widely used phylogenetic marker in broad groups of organisms and its secondary structure increasingly attracts the attention of researchers as supplementary tool in sequence alignment and advanced phylogenetic studies. Its comparative analysis provides a great contribution to evolutionary biology, allowing find out how the SSU rRNA secondary structure originated, developed and evolved. Herein, we provide the first data on the putative SSU rRNA secondary structures of the Plagiorchiida species. The structures were found to be quite conserved across broad range of species studied, well compatible with those of others eukaryotic SSU rRNA and possessed some peculiarities: cross-shaped structure of the ES6b, additional shortened ES6c2 helix, and elongated ES6a helix and h39 + ES9 region. The secondary structures of variable regions ES3 and ES7 appeared to be tissue-specific while ES6 and ES9 were specific at a family level allowing considering them as promising markers for digenean systematics. Their uniqueness more depends on the length than on the nucleotide diversity of primary sequences which evolutionary rates well differ. The findings have important implications for understanding rRNA evolution, developing molecular taxonomy and systematics of Plagiorchiida as well as for constructing new anthelmintic drugs.

1. Introduction

The platyhelminth class Trematoda Rudolphi, 1808 comprises two subclasses with largely different species diversity, Aspidogastrea with 80 species (external parasites) and Digenea with 18,000 species (internal parasites). Subclass Digenea consists of two orders: the small Diplostomida (with only suborder, 3 superfamilies) and the large Plagiorchiida (comprises 13 suborders, 19 superfamilies) (Olson et al., 2003; Kostadinova and Pérez-del-Olmo, 2014). Over 100 species of digeneans, mainly Plagiorchiida, have been recorded infecting hundreds of millions of people worldwide through a food (Gaevskaya, 2015). Some of them have important implications for human health being a reason of heavy diseases, disability and mortality (for example, fascioliasis, paragonimiasis, opisthorchiasis and clonorchiasis) resulted in significant public health problems and economic loss (Blair, 2014; Lewis and Tucker, 2014; Saijuntha et al., 2014; Chelomina, 2018). However, the food-borne diseases were neglected until recent years. High epidemiological and medical importance has stimulated the expansion of molecular-genetics and genomic studies aimed primarily at the development of methods for a quick and accurate diagnosis, and creation of the improved drugs for the treatment and prevention of diseases associated with fluke infestation.

The eukaryotic ribosomal DNA (rDNA) is a complexly organized locus of the most conservative and most used eukaryotic genes which play a basic role in ribogenesis and protein synthesis. rRNA gene sequences are widely used in phylogenetic studies of Trematoda and are largely represented in GenBank, however, there is a lack in complete sequences. Recently, the secondary structures became to use for supplementing of the primary sequences analysis (Yusupov et al., 2001; Alkemar and Nygard, 2003; Taylor et al., 2009; Arrigoni et al., 2017; Zhang et al., 2017). The ascertainment of phylogenetic relationships among digeneans is important for their taxonomic identification, diagnosis and treatment of parasitic diseases. However, in spite of the undoubted practical importance, the Plagiorchiida taxonomy has many gaps; the reason is a relatively small number of morphological traits for traditional analysis (Littlewood and Olson, 2001; Herrmann et al., 2014). The rRNA secondary structure can be determined by different methods such as thermodynamic predictions (Zuker, 2003), co-variation (Noller et al., 1981), and molecular interactions within 3D structures (Petrov et al., 2013). To day, the structures for SSU rRNA of Archaea (Mears et al., 2002), Bacteria (Mears et al., 2002), and Eucarya (De Rijk et al., 1992; Demeshkina et al., 2000; Doudna and Rath, 2002; Mears et al., 2002; Gillespie et al., 2006; Taylor et al., 2009; Armache et al., 2010; Zhao et al., 2013; Chelomina et al., 2016; Arrigoni et al.,

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Table 1
Sequences of the 18S rRNA genes incorporated in the molecular analyses.

Suborder	Family	Species	GenBank accession numbers	Authors/year
Echinostomata	Echinochasmidae	*Echinochasmus japonicus*	LT904764	This work
		E. milvi	LT904765	This work
	Echinostomatidae	*Euparyphium melis*	AY222131	Olson et al. (2003)
		Drepanocephalus spathans	JN993268	Griffin et al. (2012)
	Fasciolidae	**Isthmiophora hortensis*	AB189982	Sato and Suzuki (2006)
	Philophtalmidae	*Fasciolopsis buski*	L06668	Blair and Barker (1993)
	Psilostomatidae	*Fascioloides magna*	EF051080	Bildfell et al. (2007)
		Philophthalmus gralli	JX121231	Literak et al. (2013)
		Sphaeridiotrema monorchis	LT904763	This work
Haplosplanchnata	Haplosplanchnidae	**Haplosplanchnus pachysomus*	FJ211224	Blasco-Costa et al., unpublished
Opisthorchiata	Heterophyidae	**Euryhelmis costaricensis*	AB521797	Sato et al. (2010)
		Haplorchis pumilio	HM004195	Thaenkham et al. (2010)
		**H. taichui*	HM004213	Thaenkham et al. (2010)
		H. yokogawai	HM004207	Thaenkham et al. (2010)
		Metagonimus takahashii,	HQ832629	Pornruseetairatn et al. (2016)
		M. yokogawai	HQ832632	Pornruseetairatn et al. (2016)
		**Procerovum cheni*	HM004212	Thaenkham et al. (2010)
		**P. varium*	HM004200	Thaenkham et al. (2010)
		**Stellantchasmus falcatus*	HM004202	Thaenkham et al. (2010)
		**Amphimerus ovaliss*	AY222121	Olson et al. (2003)
		**Clonorchis sinensis*	AF217100	Lee and Huh (2004)
		Metorchis orientalis	JF314771	Zhu, unpublished
	Opisthorchiidae	*Opisthorchis viverrini*	JF823987	Thaenkham et al. (2012)
Pronocephalata	Notocotylidae	*Notocotylus intestinalis*	LT904762	This work
		N. pacifera	AY245765	Flowers et al., unpublished
Xiphidiata	Allocreadiidae	**Crepidostomum cooperi*	EF202097	Choudhury et al. (2007)
	Encyclometridae	**Polylekithum ictaluri*	EF202096	Choudhury et al. (2007)
	Haploporidae	**Dicrogaster contracta*	FJ211256	Blasco-Costa et al., unpublished
	Paragonimidae	**D. perpusilla*	FJ211230	Blasco-Costa et al. (2009)
	Troglotrematidae	**Forticulcita gibsoni*	FJ211226	Blasco-Costa et al., unpublished
		**Haploporus benedeni*	FJ211228	Blasco-Costa et al. (2009)
		**Lecithobotrys putrescens*	FJ211229	Blasco-Costa et al. (2009)
		**Ragaia lizae*	FJ211231	Blasco-Costa et al., unpublished
		**Saccocoelium brayi*	FJ211227	Blasco-Costa et al., unpublished
		**S. cephalic*	FJ211232	Blasco-Costa et al., unpublished
		**S. obesum*	FJ211253	Blasco-Costa et al., unpublished
		**S. tensum*	FJ211251	Blasco-Costa et al., unpublished
		Paragonimus heterotremus	LT855188	This work
		P. kellicotti	HQ900670	Fischer et al. (2011)
		P. iloktsuenensis	AY222141	Olson et al. (2003)
		P. vietnamiensis	LT855189	This work
		P. westermani	AJ287556	Littlewood and Olson (2001)
		Nanophyetus japonensis	LT796167	Voronova and Chelomina (2018)
		N. salmincola	AY222138	Olson et al. (2003)
		N. schikhobalowi	LT745948	Voronova et al. (2017)

The sequences included only in the ES6 analysis are indicated by stars.

2017; Zhang et al., 2017) were analyzed. Simultaneously solving the taxonomic problems, it is possible to find out how the SSU rRNA secondary structure develops and evolves (Zrzavy et al., 1998; Cannone et al., 2002; Tafforeau et al., 2013). Nevertheless, no studies devoted to modeling the secondary structures of the SSU rRNA for Plagiorchiida were reported until present.

Despite common establishing patterns of the secondary structures, within the SSU rRNA, the regions of different degree of variability were identified. The conservative sequences forming the catalytic core necessary for the ribosome functioning ('minimal ribosome'), as well as their spatial organization is almost identical in all organisms studied to date, and mutations in these regions may be lethal (Velichutina et al., 2000). Base substitutions can be localized to conserved core rRNA sequences, indicating that the ribosome may undergo conformational changes during evolution (Zrzavy et al., 1998; Velichutina et al., 2000; Johnston, 2006; Mallatt et al., 2010). The main variability of the SSU rRNA is accumulated in expansion segments (ESs) organized together with the conserved ones in most eukaryotes, however, little is known about the precise role of the variable regions (De Rijk et al., 1992; Gerbi, 1996). In the tertiary conformation of the molecule, these areas are located on the periphery of the region surrounding the tRNA interacting with mRNA without affecting the catalytic core (Mears et al., 2002). Most authors associate this feature by the ancient origin of the protein synthesis machinery (Hofacker, 2003; Lee and Gutell, 2004).

ES6 is an extensive region that occupies a central position on the 80S subunits models, commonly recognized as the most variable, and probably possessing the best ability for taxa identification (Zhao et al., 2013). ES6 structures are always unique, vary greatly in length and bear numerous substitutions that have a crucial influence on the conformation. In this regard, a precise number of hairpins separating from the central multiple loops was not identified. Based on the comparative analysis of the gene sequences for more than 6000 species of eukaryotes, it was concluded that the ES6 secondary structure remains similar in most multicellular organisms and that it likely consists of five hairpins and one inner helix (Alkemar and Nygard, 2003). In the 3D structure for the 80S ribosome of wheat *Triticum aestivum* ES6 was surrounded by the protein densities facilitating its folding (Armache et al., 2010). A number of researchers indicate the presence of the specific motifs in the ES3 and ES6 regions participating in formation the quaternary structure of the SSU rRNA molecule (Alkemar and Nygard, 2003; Gillespie et al., 2005; Holzer et al., 2007). They identified

consensus sequences for wheat (A^{216}AGGCUGAC^{224} in ES3 and G^{839} UUGGCCUU^{847} in ES6) and mice (A^{231}AAACCAAC^{239} in ES3 and G^{893} UUGGUUUU^{901} in ES6). For all other Metazoa, an analysis of the complementarities of the homologous sequences of the genes showed that binding sites in ES3 and ES6 are quite conservative. The main differences from the consensus motives (AAAACCAAU for ES3 and GUUGGUUUU for ES6) were observed in Cnidaria, Myxozoa, Nematoda, and Porifera. For most of these organisms, the maximum length of the expected sites of interaction was limited to eight base pairs. In total, changes in the secondary structures organization of the SSU rRNA molecule are hierarchical. The first level of variability distinguishes representatives of the major kingdoms (De Rijk et al., 1992). The second level is specific for types and classes of organisms (i.e. yeast, insects, worms, mammals), with maintaining the general principle of folding for the most helices (Gutell et al., 1986; Woese, 1987; Cannone et al., 2002; Gillespie et al., 2006; Zhao et al., 2013). The third type includes ESs with a specific structural variability characterizing orders and families of organisms (Matejusova and Cunningham, 2004; Gillespie et al., 2006; Zhao et al., 2013).

In this paper, we provide the first analysis of the 18S rRNA secondary structures for the Plagiorchiida species, including those especially pathogenic for humans and animals (the causative agents of clonorchiasis, opisthorchiasis and paragonimiasis). Using the SSU rRNA gene sequences obtained in this study and derived from the GeneBank for a broad diversity of taxa we modeled the secondary structures of eleven variable regions. We tested the correspondence of the 18S rRNA secondary structures of the flukes to other eukaryotes (i); evaluated the variability of these structures between different Plagiorchiida species (ii); explored how the primary nucleotide sequence affects its secondary structure (iii); assessed the suitability of the variable domains for trematode's systematics (iv); and we also tried to elucidate a possible relation between type of tissue where parasite inhabits and on the SSU RNA secondary structure (v).

2. Materials and methods

2.1. Parasite samples

Specimens of *Echinochasmus japonicus*, *E. milvi*, *Paragonimus heterotremus*, *P. vietnamiensis*, *Sphaeridiotrema monorchis* and *Notocotylus intestinalis* adult flukes were kindly provided by Dr. V.V. Besprozvannykh (Federal Scientific Centre of the East Asia Terrestrial Biodiversity, Far Eastern Branch, Russian Academy of Sciences, Vladivostok, Russia).

2.2. DNA extraction, gene amplification and sequencing

DNA was extracted from individual adult trematodes fixed in 70% ethanol using HotSHOT technique (Hot Sodium Hydroxide and Tris) (Truett et al., 2000). The complete 18S rDNA was amplified by polymerase chain reaction (PCR) using Thermo Scientific PCR Master Mix (2×) (California, USA) according to the manufacturer's instructions. Primers used for PCR-amplification of the respective 18S rDNA are detailed as follows: 373c (5′-GATTCCGGAGAGGGAGCCT-3′), 570c (5′-GTAATTCCAGCTCCAATAGC-3′), 892c (5′-GTCAGAGGTGAAATTCT TGG-3′), 1262c (5′-GTGGTGCATGGCCGTTCTTA-3′), 1/F (5′- CACACC GCCCGTCG -3′) (all Forward); 373 (5′-AGGCTCCCTCTCCGGAATC-3′), 570 (5′- GCTATTGGAGCTGGAATTAC -3′), 1137 (5′-GTGCCCTTCCGT CAAT-3′), 1262 (5′- GAACGGCCATGCACCAC-3′) (all Reverse) (Krieger et al., 2006). The thermal gradient of this marker started with an initial denaturation at 94 °C (30 s), annealing at 58 °C (30 s), extension at 72 °C (4 min) 35 cycles and final extension at 72 °C (11 min). PCR products were purified using ExoSAP-IT PCR Product Cleanup Reagent (Thermo Scientific, USA), then sequenced in both directions using PCR primer set utilizing ABI BigDye chemistry (Applied Biosystems, USA), and run on an ABI Prism 3130 automated capillary sequencers (Applied Biosystems). Contiguous sequences were assembled using Mega 5.1 (Tamura et al., 2007), and submitted to GenBank.

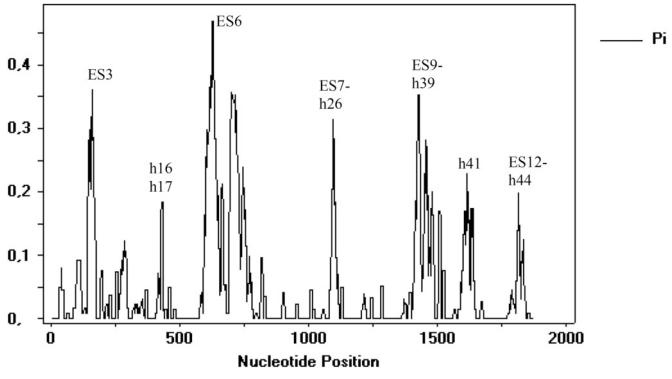

Fig. 1. The distribution pattern of nucleotide diversity (Pi) along the SSU rRNA gene sequences of the Plagiorchiida species with localization of the secondary structure's elements as computed using the sliding-window option.

2.3. Statistical analyses of genetic data

The newly-generated sequences and matching sequences of different Plagiorchiida taxa from other studies deposited in GenBank (Table 1) were aligned using the Clustal W program (Thompson et al., 1994).

The distribution of nucleotide diversity (p) along the gene sequence (a “sliding-window” approach; window length of 10 sites and step size of 1 sites), nucleotide composition and uncorrected pairwise genetic distances between sequences were estimated using MEGA v. 5.1 (Tamura et al., 2007). The variance analyses were performed (under a license agreement) in Tibco STATISTICA v. 13.3 (https://www.tibco.com/products/data-science). For each variable region, nucleotide substitution rates were estimated by the ratio of both Nv:Nc and Nv:Nt, where Nv, Nc and Nt stand for a number of variable sites, a number of conservative sites and total number of sites compared, respectively (Ki, 2012).

2.4. Prediction of ribosomal RNA secondary structures

Published model for the SSU rRNA secondary structure of wheat *Triticum aestivum* (highly compatible with similar structures of Drosophila and humans) based on cryoelectron microscopy data (Armache et al., 2010) was used as a reference in our study. Secondary structure was predicted separately for each molecular segment and for each rRNA sequence. Primary sequences were used as an input for the thermodynamically stable RNA secondary structure prediction in MFOLD software version 3.2 (http://mfold.rna.albany.edu), recommended for most RNA folding, using free energy folding algorithms (Zuker, 2003). The method is based on predicting thermodynamically stable RNA molecules (linear or circular) by calculating free energies of all possible secondary structures and retaining one of lowest energy (i.e. the most stable); the quality of structure predictions decreases with increasing sequence length. The core algorithm predicts a minimum free energy (DG) and also minimum free energies for folding that must contain any particular base pair; the free energy parameters are fixed at 37 °C (the studied species parasitize warm-blooded animals). This version uses free energy data from Mathews et al. (1999). All five expansion segments (ES3, ES6, ES7, ES9, and ES12) following the ES nomenclature of Gerbi (1996) and six hairpins (h16, h17, h26, h39, h41 and h44) of the small subunit rRNA were identified and modeled for 25 studied species of Plagiorchiida, except ES6 obtained for 45 species (described in Results). In most cases, the online algorithm suggested only one structure; in cases when two structures were predicted, the structure with either the minimum free energy or with the best compatibility to similar sequences (all homologous sequences should form a common structure) was selected. Structures displayed using

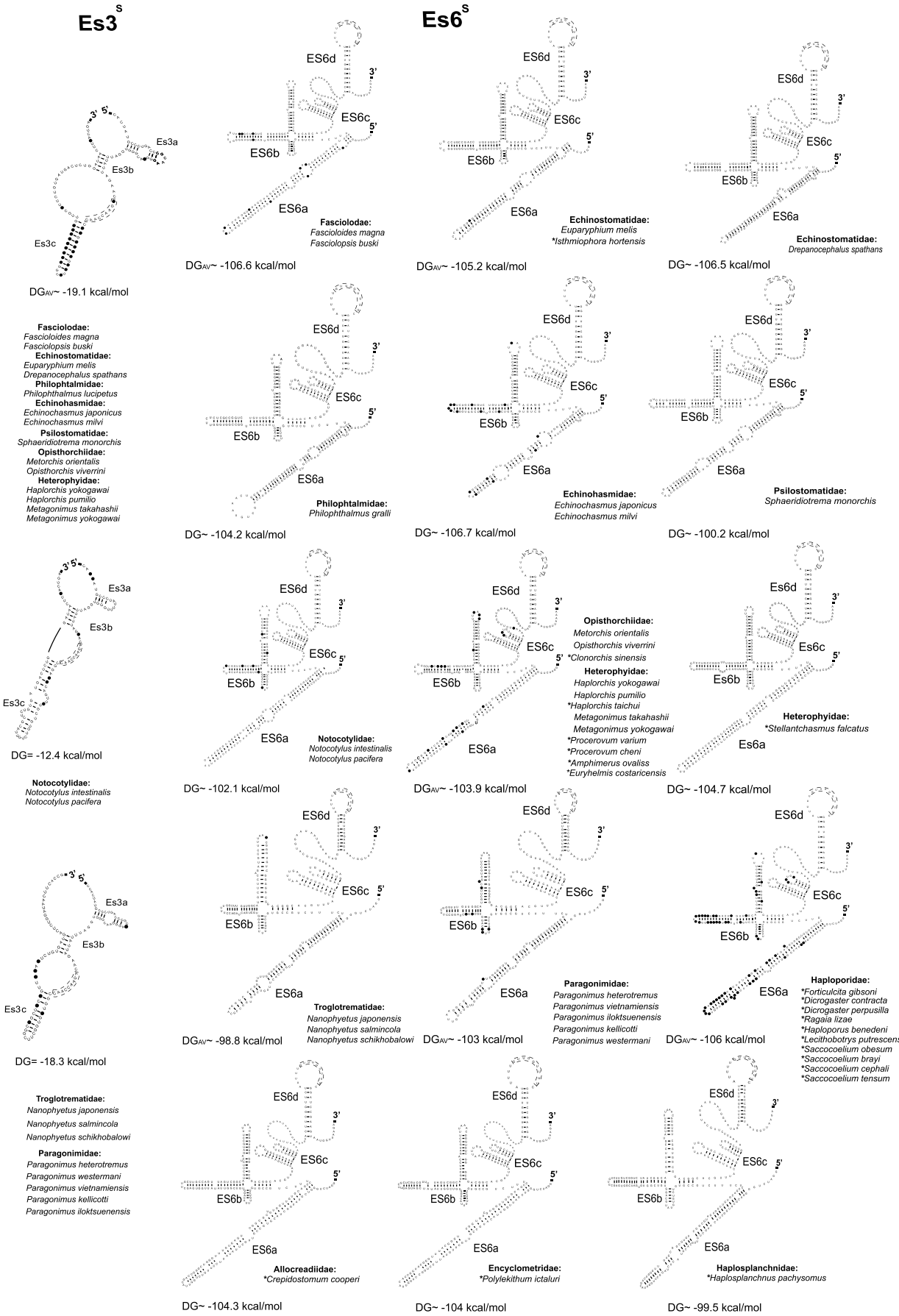

Fig. 2. De novo modeled in Mfold the putative secondary structures of ESs and helices for the Plagiorchiida species. The ES3 and ES6 interaction sites of rRNA are in dashed frame. The substitutions marked with filled black circles. DG is the quantity of energy required for formation of the secondary structures.

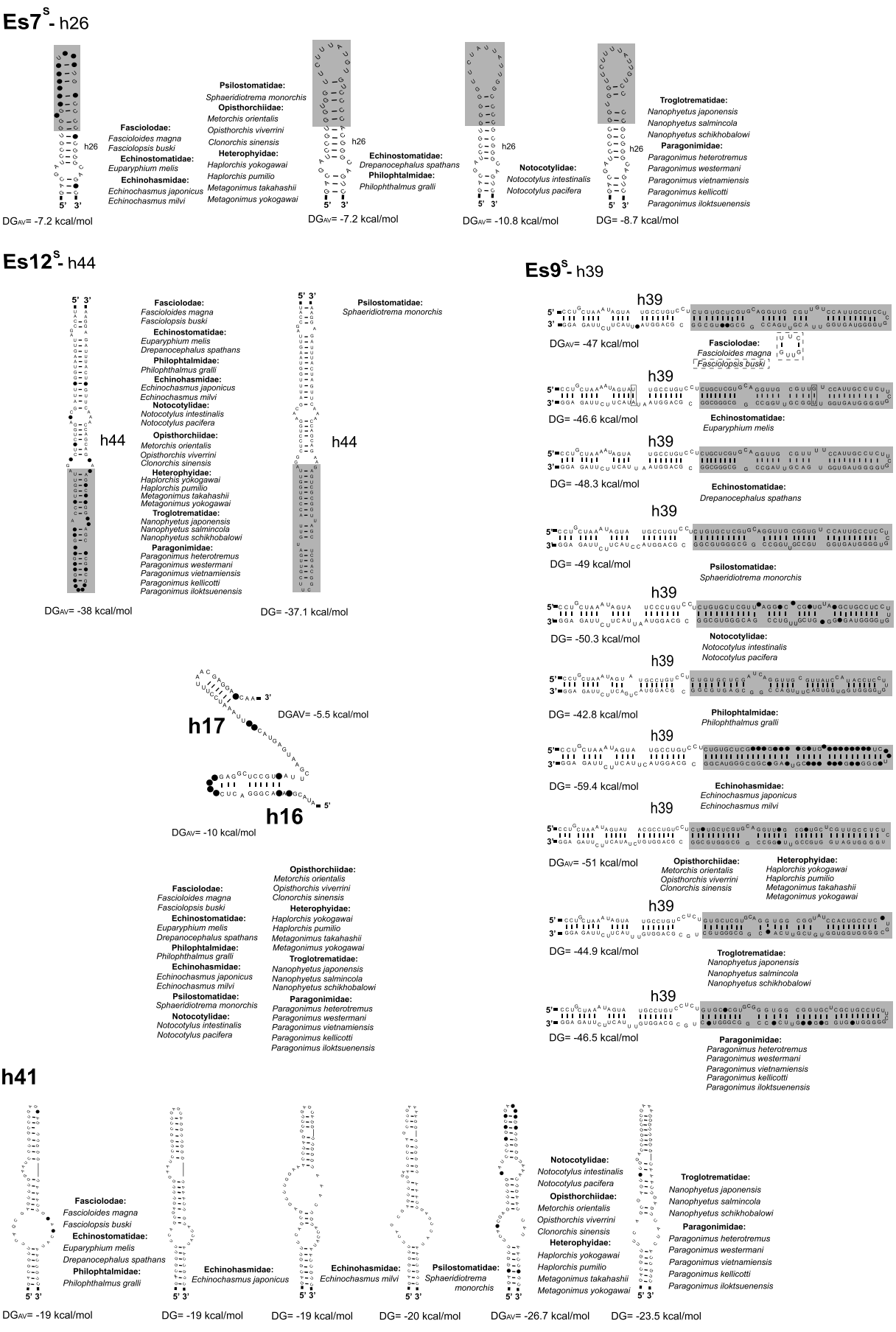

Fig. 2. (*continued*)

CorelDRAW X6 and the method of visual comparison of compensatory substitutions, in general, interspecific variable sites marked black circles (Kjer, 1995).

3. Results

3.1. Primary structure variations of ESs and helices

The distribution pattern of nucleotide diversity along the 18S rDNA sequences (Fig. 1) revealed high nucleotide polymorphism in variable regions both helices and ESs, while the core part of the molecule exhibited low variability. Based on these data, the secondary structures of expansion segments (ES3, ES6, ES7, ES9, and ES12) with helices adjacent to them (h26, h39, and h44), as well as with separate hairpins h16, h17 and h41 of SSU rRNA were modeled.

Table 2 presents a brief description of these regions. Their length among the analyzed species varies widely: from 35 (h16) to 327 (ES6) bp. However, the size of each domain is either in a narrow range of values, or it is constant. The minimum proportion of the helical regions was about 38% (ES3), and the maximum - 86% (ES9). The GC content differed depending on the species studied and the rRNA domain, and generally varied from 33% to 63%. The nucleotide differences of variable regions between two separate species were 1.8–12.3%. The nucleotide substitution rates (by *Nv:Nc* and *Nv:Nt* ratios) of the regions varied substantially with the highest and lowest values obtained for ES9-h39 and h17, respectively.

3.2. Secondary structures of ESs

ES3 consists of two branched loops and two essential hairpin helices ES3a and ES3c, and also ES3b connecting the large internal loop and ES3c with the junction. There are three types of design patterns for the region (Fig. 2). The first is identified for the majority of studied families (the Echinostomata and Opisthorchiata suborders), while the second one was specific only for Notocotylidae (the Pronocephalata suborder), and the third shared two families, Troglotrematidae and Paragonimidae (the Xiphidiata suborder). The main differences within these models were the presence or absence of internal loops in the ES3a and ES3c hairpins, size of helices and configuration of the central internal loop, especially in species of the Notocotylidae family, where it was bulged, asymmetrical and shifted to one side. Most of the nucleotide differences were concentrated in ES3c.

ES6 comprises five variable hairpin helices (subdomains): ES6a, ES6b, ES6c1, ES6c2, and ES6d. Based on the analysis of multiple ES6 alignments of the studied flukes, the region can be divided into two parts: variable on the 5′- end (ES6a and ES6b), and conservative on the 3′- end (ES6c1, ES6c2 and ES6d). Conformations of the subdomains were very similar and differed among themselves by the presence of internal and bulge loops inside the hairpins and a number of base pairs included in the hairpin loops.

There are 15 types of design patterns for ES6 (Fig. 2), family specific for a few exceptions. In the family Echinostomatidae, the internal loops of ES6a structures for *Drepanocephalus spathans* were greater then for *Euparyphium melis* and *Isthmiophora hortensis*. Besides, the Heterophyidae and Opisthorchiidae families, shared a common pattern, except for *Stellantchasmus falcatus* (Heterophyidae) possessed unique ES6 organization. Other models of the secondary structures were shown according to the principle of one pattern per one family: Allocreadiidae, Echinohasmidae, Encyclometridae, Fasciolidae, Haploporidae, Haplosplanchnidae, Notocotylidae, Philophtalmidae, Psilostomatidae, and Troglotrematidae.

ES6a is a simple hairpin included on the average about 90 nucleotides; most substitutions were distributed throughout the entire length of the hairpin. The number of base pairs making up the terminal loops is low and *Philophthalmus gralli* (the only among analyzed species which parasitizes in the eyes) had the largest hairpin loop. A multibranched loop is localized in the center of ES6b (average size 115 nucleotides) and connected three hairpins of different lengths, forming a large asymmetric cross-shaped structure. Almost all species in the center of the apical region of ES6b contain a single-nucleotide bulge loop, with the exception of *Notocotylus* and *Stellantchasmus falcatus* with all paired bases or an additional loop, respectively. ES6c subdomains are represented in the form of hairpins of different lengths (26 and 17 nucleotides). ES6d was quite conserved; it consistently contained 36 nucleotides and there was no variation in its secondary structure. ES6c and ES6d are connected by conservative 28–29 nucleotide unpaired region, most likely in the form of a loop.

ES7 is the smallest variable region, 21 nucleotides in length (Fig. 2), and helix 26 connected to the domain contains a fixed number of nucleotides – 20, and one internal loop is invariably localized inside this helix. There are four types of design patterns for h26-ES7. Hairpin loop was composed of three nucleotides for the majority of the studied families, whereas for the families Echinostomatidae (only *Drepanocephalus spathans*) with Philophtalmidae, Notocotylidae, and Troglotrematidae with Paragonimidae it was expanded and varied in size: 9, 12 and 13 nucleotides, respectively.

ES9 includes on the average 70 nucleotides and separated from h39 by the internal loop composed of four or eight nucleotides (Fig. 2). The conformation of h39 was identical among the species; ten base pairs at the proximal and distal ends of the region were conserved, while the major part of substitutions was concentrated in the ES9 hairpin. In total, h39-ES9 contains three to six internal loops, one-two bulge loops, and two-three single-nucleotide bulge loops. From them only two with some differences were identified for the family Echinostomatidae: the first was presented in *Euparyphium melis*, and the second was in *Drepanocephalus spathans*. The structure of *Euparyphium melis* contains additional paired nucleotides (A-U and G-U) with their localization in h39 and the ES9 apex.

ES12 is highly conserved in trematodes; it composed approximately of 40 nucleotides and connected with helix 44. The conformation of h44 was also very similar throughout the Plagiorchiida species, included three internal loops where the last one formed the ES12 hairpin. There are two types of design patterns for the h44-ES12 (Fig. 2). In most flukes, this domain has the configuration of hairpin with two internal loops: single-nucleotide bulge loop and small internal loop of three nucleotides. The exception was the structure for *S. monorchis* in which ES12 possessed two identical single-nucleotide bulge loops. Most of nucleotide substitutions that distinguished species of trematode families were concentrated in the ES12 apex.

Table 2
Sequence characteristics of different 18S rDNA regions.

Region	*L, bp*	*Nm*	*Nl*	*D*	*Lh*, %	*GC*, %	*Nv:Nc*	*Nv:Nt*
ES3	102–113	3	2	0.101	38–67	51.3	0.53	0.35
h16	35	1	1	0.072	50	47.3	0.25	0.20
h17	36	1	1	0.018	34	33.3	0.09	0.08
ES6	311–327	10	9	0.100	60–65	50.6	0.51	0.34
ES7-h26	41	4	1	0.122	59–78	62.8	0.46	0.32
ES9-h39	114–119	10	6	0.123	74–86	59.6	0.69	0.40
h41	63–64	6	2	0.109	59–73	52.3	0.47	0.31
ES12-h44	105	2	1	0.038	72–76	49.1	0.25	0.21

L, bp – length in base pairs; *Nm* – number of models; *Nl* – number of lengths; *D* - nucleotide differences; *Lh* – portion of helices; *GC* – mean G + C content; *Nv* – number of variable sites; *Nc* – number of conserved sites; *Nt* – total number of sites compared.

3.3. Secondary structures of separate helices

The h16 and h17 conformations across Plagiorchiida species were completely identical (Fig. 2), however, the nucleotide divergence in helix 16 was about four times higher than that of h17 (Table 2). Both

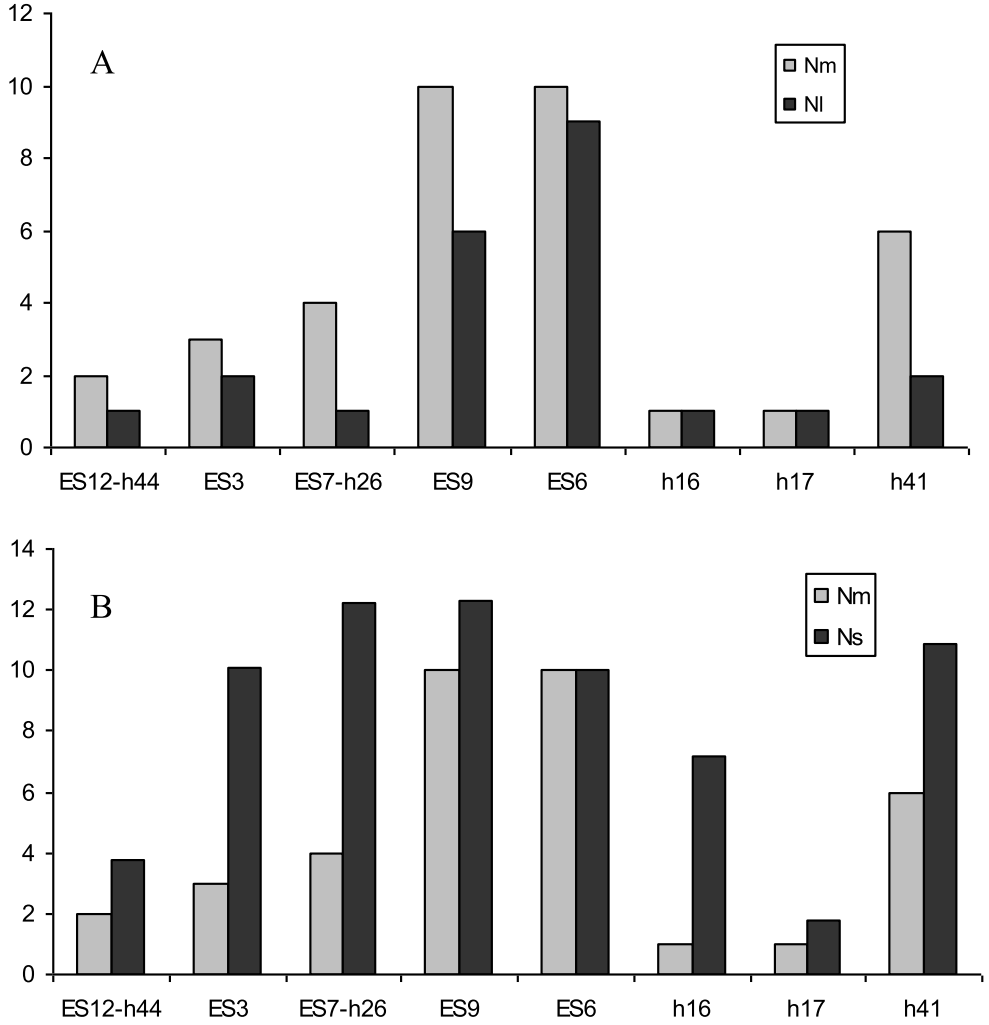

Fig. 3. The number of the secondary structure models with the number of lengths (A) and nucleotide differences (B) for each variable region of the SSU rRNA in the Plagiorchiida species. *Nm* – number of models, *Nl* – number of lengths, *Ns* – nucleotide differences.

helices were terminated by small loops; they much expanded by extended exterior loops formed at the 5′ - ends.

The h41 was formed by 63 or 64 nucleotides. There are six types of design patterns for this helix (Fig. 2). All models differed from each other by lengths, conformation, and localization of internal loops. Three helices were found to be species-specific. In *E. milvi*, due to the insertion of adenine at position 27 of the 5′ - end of the helix, the structure of h41 differs from other species by the presence of two internal loops disposed through a pair of bases. In the related species *E. japonicus*, the bulge loop was sufficiently removed from the internal loop, both loops were small, and there was a single-nucleotide bulge loop between them. This structure was very similar to that of Fasciolidae, Echinostomatodae and Philophtalmidae with more expanded distal loop (13 vs. 8 bases). In helix 41 of *S. monorchis*, in addition to the internal and bulge loops, a single nucleotide loop was identified. In the families Troglotrematidae and Paragonimidae, three loops were localized inside the helix: two neighboring internal loops (3 and 8 nucleotides) and a bulge loop of three nucleotides.

3.4. Nucleotide variability and variations of the secondary structures

The secondary structures of h16 and h17 were estimated to be unaltered. The secondary structure variations of remain regions increased in the series ES12, ES3, ES7, h41, ES6 and ES9 in direct proportion to nucleotide diversity ($r = 0.672$, $p < 0.05$) and especially to length variability ($r = 0.897$, $p < 0.05$) of the region (Fig. 3). The dependence is maintained, the larger the domain dimension, the greater the number of model variants that can be obtained for it. This trend is well traced in representatives of the family Echinostomatidae: *Euparyphium melis* and *Drepanocephalus spathans* in ES9 (Fig. 2), where the difference in domain length, i.e. insertion of two pairs of nucleotides into the structure of *Euparyphium melis*, entailed a change in the conformation of the structure, the acquisition of its uniqueness, and an increase in the free folding energy (from −48.3 kcal/mol in *D. spathans* to −46.6 kcal/mol in *E. melis*). Most of the structures are common for a number of species; the largest number of unique structures is found for the *Notocotylus* species.

3.5. Secondary structure of the complete SSU rRNA molecule

The inferred secondary structure for the entire SSU rRNA (Fig. 4) emerged to be quite conserved across Plagiorchiida species even in the most variable ESs. These structures posses a number of peculiarities: a cross-shaped structure of the ES6b (i), additional shortened ES6c2 helix (ii), and both elongated ES6a helix (iii) and h39 + ES9 region (iv). Despite the high stability, there are small changes discretely localized in subdomains; some of them estimated to be specific at different taxonomic levels (suborder, family or species). The secondary structures of separate domains can be not only taxon specific (for example, ES6 and ES9 are specific to the Plagiorchiida families) but also specific to tissue

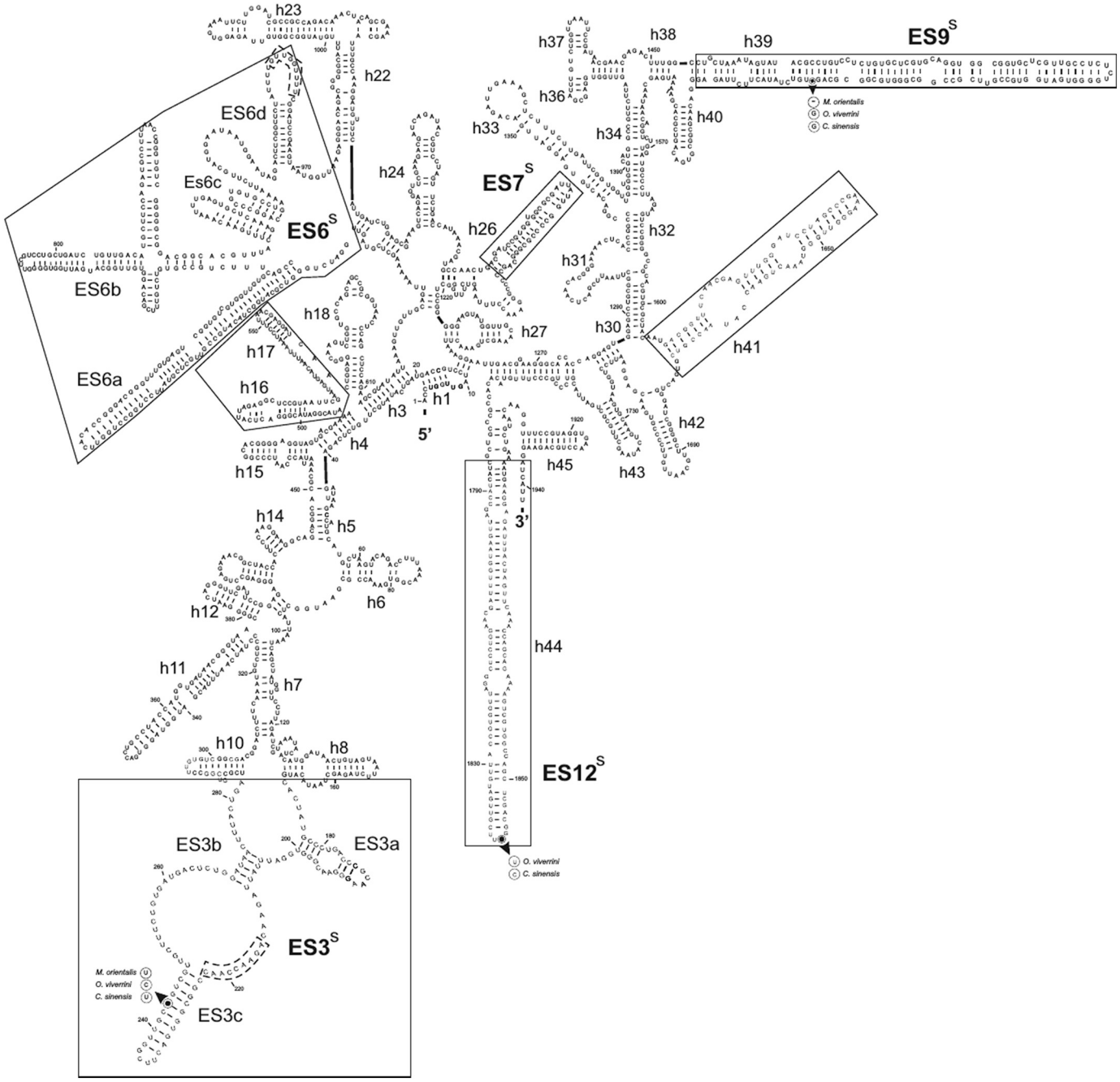

Fig. 4. The putative scheme of the 18S rRNA secondary structure for the Plagiorchiida species from the Opisthorchiidae family based on the data for the *Triticum aestivum* SSU rRNA (Armache et al., 2010). De novo modeled expansion segments and helices are in the frames. The nucleotide substitutions between *Metorchis orientalis*, *Clonorchis sinensis* and *Opisthorchis viverrini* are indicated in callouts.

type of host where parasite habitat (ES3 and ES7).

4. Discussion

4.1. Peculiarities within the SSU rRNA secondary structures in plagiorchiidans

In order to evaluate the variability and reveal possible peculiarities of the complete SSU rRNA secondary structure of plagiorchiidans we compared them to each other and with those published for different organisms.

The inferred secondary structures of the entire SSU rRNA of the Plagiorchiida species demonstrate well compatibility with those of others eukaryotic SSU rRNA and a lack of unusual structural features that may questioning the functionality of the molecular model (Armache et al., 2010; Yu et al., 2013; Petrov et al., 2014; Chelomina et al., 2016). The comparative analysis showed that the SSU rRNA secondary structure of the flukes possess some peculiarities mainly related to ES6 including additional shortened ES6c2 helix. The existence of the ES6c2 is inconsistent with the comparative data analyses of other eukaryotes, however, does not contradicts the generally accepted opinion about the variability of ES6. Perhaps, ES6c2 is an additional segment similar to that of ES6e in yeast (Taylor et al., 2009; Armache et al., 2010) and separation of ES6c in plagiorchiidans is essential for their SSU rRNA's folding.

In Plagiorchiida, the secondary structures of variable regions of the

SSU rRNA for the entire time of the divergent evolution did not change their general architecture, including significant variation in length. It can be associated with the unique complex digenean life-cycle (acquisition of a vertebrate definitive host, alternation of sexual and asexual reproductive generations, asexual generations within the first intermediate host – typically mollusc, free-swimming cercaria with a tail; cited by Kostadinova and Pérez-del-Olmo, 2014) because the increased length and complexity of the variable regions are related to the complex biology (Gillespie et al., 2006) and invertebrate host switching is strongly associated with structural changes in the SSU rRNA (Gillespie et al., 2005; Holzer et al., 2007). The SSU rRNA secondary structures also found to be extremely conservative within Scleractinian corals (Arrigoni et al., 2017) while they were much more variable in myxozoans (Holzer et al., 2007), Acari (Zhao et al., 2013) and Cestoda (Zhang et al., 2017).

Although the rRNA structures were estimated to be quite conservative across species studied variable regions differed among themselves by their diversity and specificity. The most variable regions ES6 and ES9 exhibited family specificity. In other group of worms - cestodes, the V4 (ES6) secondary structure was variable within family (Diphyllobothriidae) and specific to single genus or group of genera (Zhang et al., 2017). Zhao and colleagues (Zhao et al., 2013) studied the SSU rRNA of parasitic ticks, compared their secondary structures with those of other species, and found that helix E23 _(ES6) 5 exists in representatives of the Pterygota but absent in Acari. Researchers of Acari noted that the conformation of the Demodicidae ES9 was slightly variable, and h41 was more important for taxonomy (Gillespie et al., 2006; Zhao et al., 2013). ES9 structures in Hemiptera, on the contrary, were variable, but the authors considered them only together with h41, in which four functional substitutions differed Hemiptera from Coleoptera although did not influence the final folding (Ouvrard et al., 2000).

A significant part of the nucleotide differences among plagiorchiidans resulted in alterations of the SSU rRNA secondary structures were localized in ES3c and ES7. As a result, hepatic and pulmonary trematodes posses different ES3c and ES7 conformations, while intestinal flukes shared all of them. It is well agreeing with data that rRNA (ITS2) secondary structure of mobilid ciliates is related to the parasitic location (gill, urceolariid or diverse) (Du et al., 2018). Interesting that in insects, it was noted that ES3 models of Hemiptera (most feed on plants, some are parasitic, others are predators) could not be reconstructed in concordance with those of Coleoptera (phytophages, detritophages, predators, coprophages, necrophages) due to the variability of ES3c (Ouvrard et al., 2000). Similarly, in Trombidiformes (parasites of plants, animals and human), a new helix E10_1c in ES3 starting from strictly conservative five nucleotides was detected in mites of the Tetranychoidae family (herbivorous mites), but was absent in Demodicidae (mammalian parasites) (Zhao et al., 2013).

According to our data, ES12-h44 is the most conserved variable segment of the plagiorchiidans. Indeed, the interaction sites of two eukaryotic ribosomal subunits on h44 (the bridge between rpS17e (40S) and rpS24e (60S)) are classified as areas with a low degree of variability. It is also known that ES12 adjacent to h44 in different animal species are relatively variable in nucleotide sequences, but with the common pattern of the secondary structures (Johnston, 2006; Armache et al., 2010; Mallatt et al., 2010). In this regard, it is extremely interesting to find out why there are some variations in the ES12-h44 secondary structure.

4.2. Specific motifs of the SSU rRNA molecule

What is a possible functional significance of pecularities revealed in the SSU rRNA structures of the flukes? In Plagiorchiida, the ES6 interaction site consisting from nine nucleotides (G^{926}UUGGUUUU^{934}) is not different from consensus sequence. However, the ES3 site was modified both at the beginning and at the end: C^{212}AACCAAGA^{119}. As a result, the interaction sites of ES3 and ES6 can include only seven nucleotides, of which only six are complementary, (i.e. could form the helix) compared to 7-9 bp in other eukaryotes. These nucleotide pairs (AACCAA) are the same in different groups of organisms such as mammals, myxozoans, and fungi (Alkemar and Nygard, 2003; Gillespie et al., 2005; Holzer et al., 2007). Although all the reconstructions support an interaction between the ES3 and ES6 hairpins, its precise significance has not been clear yet. However, since function of many RNA types is defined by the formation of intramolecular base pairs, the rRNA sequence variation may alter the structure and function of the ribosome and its interplay with components of a cellular milieu (Parks et al., 2018).

An important role in many basic cellular activities plays RNA-RNA interactions. We found that the numerous substitutions inside the ES9 hairpin strongly affect the number of its loops and make the secondary structure to be family specific. Probably, the appearance of such types of variability was necessary requirement for evolution since structured loops are acknowledge to be the most common functional motif of natural RNAs often playing a major role in ensuring the specificity of RNA-RNA interactions (Battle and Doudna, 2002).In plagiorchiidans, we identified tissue specific secondary structure for ES3 and ES7, also characterised by increased GC-content. These observations are associated with ability of rRNA to bind with numerous extra-ribosomal factors required to synthesize proteins, at that some types of interaction are determined by the primary sequences of molecules, while others depend on their secondary structures (Parks et al., 2018). Additionally, it was reported that the initiation factors (IFs) of translation in eukaryotes are presumably associated with ES7 (Taylor et al., 2009). Consequently, ES7 can play a role in the recognition or stabilization of some specific eukaryotic IFs providing adaptation of parasites to their location in the host's organism.

4.3. The SSU rRNA secondary structures and Plagiorchiida systematics

The taxonomic classification and phylogenetic relations of the Plagiorchiida species remain to be controversial on a number of issues and require clarification in spite of a number of published comprehensive studies (Tompkins et al., 2010; Lawton et al., 2011; Tkach et al., 2016). Our data provide important information that the ES6 and ES9 secondary structures are family specific. Following examples support this inference and allow considering these structures as promising molecular markers at a family level useful for solving the systematic problems in Plagiorchiida.

The Echinostomatoidea Looss, 1899 is a large, heterogeneous and globally distributed group of cosmopolitan trematodes. In larvae stages, they parasitize in different groups of mollusks, polycheta and amphibia; adults inhabit in vertebrate species of all classes especially in birds (Tkach et al., 2016). The problems of the species recognition and phylogenetic relationships within Echinostomatoidea Looss, 1899 exist long ago (Tkach et al., 2016). According to data obtained in this study, the genera *Euparyphium* and *Drepanocephalus* should be considered as sufficiently distant taxa, because of a high divergence of their 18S rDNA primary structures and different configurations of their ES6S and ES9S regions, implying species affiliation to different families. In recently reported comprehensive phylogeny for the Echinostomatoidea superfamily based on the partial 28S rDNA sequences these species were also clear separated into two well differentiated phylogenetic branches although the authors consider them to be members of the same family.

The next example concerns the superfamily Opisthorchioidea Looss, 1899 comprising families Opisthorchiidae and Heterophyidae, similar in morphology and life cycles. In larvae stages, they parasitize in Gastropods and fishes; adults inhabit piscivorous teleosts, reptiles, birds and mammals. For this superfamily, the uncertainty of relations on the different taxonomic levels has been repeatedly noted (Olson et al., 2003; Scholz, 2008; Thaenkham et al., 2011). Based on the phylogenetic analysis inferred from the rDNA sequences, Thaenkham et al.

(2011) made conclusion about paraphyletic relationships between Opisthorchiidae and Heterophyidae that implies their inseparability. This idea obtained an unexpected support in studying the fauna of cetaceans' flukes (Fraija-Fernandez et al., 2015). Our results also correspond to these data, since they demonstrate for both families the unity of ES6 and ES9 structures.

However, ES6S secondary structure for *Stellantchasmus falcatus* was revealed to be different from those of other species from this superfamily. Such a molecular feature of the species fits perfectly into the existing viewpoint on the morphology and phylogeny of the Heterophyidae family. For the first time, Pearson (2008) drew attention to the fact that the level of subfamilies in Heterophyidae should be revised by using the molecular studies (i). Subfamily Haplorchiinae is well separated from others in the family due to characters of the ventrogenital complex (ii), and the phylogenetic tree identified *S. falcatus* as the first species split off a common ancestor of Haplorchiinae (iii) (Thaenkham et al., 2010). Altogether, it means that taxonomy of the superfamily Opisthorchioidea should be revised. Our data on ES6S which, as we believe, determine the boundaries of the family, point to an appropriateness of Opisthorchiidae and Heterophyidae fusion into one family in the rank of subfamilies. Additionally, they imply elevating rank of the subfamily Haplorchiinae with *S. falcatus* to the family status that for today does not seem indisputable.

Finally, the example from the Xiphidiata suborder, the lagest among Plagiorchiida. Based on phylogenetic analysis of the 28S rDNA gene sequences from different Trematoda families Curran et al. (2006) demonstrated that *Polylekithum ictaluri* is most close to Encyclometridae but not to Allocreadiidae as previously reported. Indeed, we obtained different ES6 and ES9 secondary structures for *Polylekithum ictaluri* and *Crepidostomum cooperi* (Allocreadiidae) confirming this inference.

5. Conclusion

To date, no group of parasitic worms has spread into the animal's body as widely as Plagiorchiida, and perhaps no other group of parasitic worms has such numerous and complex adaptations to live in so diverse environments. The rRNA structures, obtained in this study, clearly illustrate the acquisition of novel and subtle adaptabilities of the flukes during their evolutionary development and simultaneous maintenance of the overall trematode-specific architectonics of the molecule, thus demonstrating the virtuosic ability of parasites to adapt and a high flexibility of the molecule which is known for its evolutionary conservatism. The findings have important implications for epidemiology and medicine, including the development of new anthelmintic drugs as well as for understanding the rRNA molecule evolution, and development the molecular taxonomy and systematics for the Plagiorchiida species.

Declaration of Competing Interest

None.

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