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**PHYLOGENETIC RELATIONSHIPS OF *MESOCESTOIDES*
VAILLANT, 1863 TETRATHYRIDIA FROM SMALL MAMMALS
OF EASTERN RUSSIA AND ALASKA BASED ON *18S rRNA* GENE**

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A fragment of 875 bp of the *18S rRNA* gene was studied in 12 samples of *Mesocestoides* tetrathyridia from small mammals of 7 species collected in geographically distant localities. Five haplotypes were identified, differing from each other by 27 nucleotide substitutions in 24 sites. It has been found that the *18S rRNA* haplotypes belong to two genetically distinct haplogroups. Molecular diversity indices were calculated for each of them. The conducted analysis allowed to suggest the following: 1) *Mesocestoides* sp. haplogroups A and B belong to two different species, but do not belong to any of the confirmed species of the genus; 2) the deletion of bp 729–747 and the insertion at site 761 of guanine can be regarded as a genetic marker for the species *Mesocestoides litteratus*.

Keywords: *Mesocestoides*, tetrathyridium, insectivores, rodents, *18S rRNA*, phylogenetic analysis, genetic marker

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The metacestode stage (tetrathyridium) of cestodes of the genus *Mesocestoides*, widespread parasites of predatory mammals, has no clear morphological characteristics that diagnostics usually relies on (in particular, there is no proboscis) (Kozlov, 1978; Dokuchaev, Gulyaev, 2004; Konyaev et al., 2011; Zaleśny, Hildebrand, 2012; Tokiwa et al., 2014).

It is not uncommon to find larval stages of *Mesocestoides* sp. in rodents, or *M. lineatus* in carnivorous mammals in the East of Russia (Gubanov, 1964; Gubanov, Fedorov, 1970; Domnich, 1985; Odnokurtsev, 2015). In addition, *M. kirbyi* Chandler, 1944 (Domnich, Obushenkov, 1983) and *M. paucitesticulus* (Konyaev et al., 2011) were recorded in this territory, and tetrathyridia that may belong to *M. kirbyi*, or *M. perlatus* (Goeze, 1782) were found in shrews from the Northern Okhotsk region and Chukotka (Dokuchaev, Gulyaev, 2004).

The first attempt to study the *Mesocestoides* tetrathyridia from Magadan region performed by molecular genetic methods (Pospekhova et al., 2018) had made it possible to outline some phylogenetic relationships within the genus. The subsequent work, based on the variability of the *12S rRNA* gene fragment in *Mesocestoides* sp. (Pospekhova et al., 2023) suggested that none of the studied samples of tetrathyridia from insectivores and rodents belong to any of the confirmed species of the genus, namely *M. lineatus* (Goeze, 1782), *M. litteratus* (Batsch, 1786), *M. canislagopodis* (Rudolphi, 1810) (Krabbe 1865), *M. corti* (Hoepli 1925) (= *M. vogae*) (Etges 1991)) и *M. melesi* Yanchev and Petrov 1985 (Yanchev, 1986; Gubány, Eszterbauer, 1998; Nickisch-Rosenegk et al., 1999; Padgett, Boyce, 2005; Literák et al., 2006; Hřčková et al., 2011; Skírnisson et al., 2016; Bajer et al., 2020).

The purpose of this work was to determine the nucleotide sequences of the *18S rRNA* nuclear gene fragment in tetrathyridia from 7 species of small mammals in the East of Russia and Alaska (USA) and to perform a preliminary analysis of the relationships between the haplotypes of the studied *Mesocestoides* with each other, and also with the data available in GenBank.

MATERIALS AND METHODS

Tetrathyridia samples were obtained as a result of theriological studies of rodents and insectivores conducted by N.E. Dokuchaev in 2002–2019; small mammal collection sites are shown in Fig. 1 and Table 1.



Figure 1. Collection sites for intermediate hosts of *Mesocestoides*. The numbers correspond to the sample numbers from Table 1.

Table 1. *Mesocestoides* tetrathyridia specimens used in this work

Sample number	Haplotypes	Host	Location	GenBank number
11	Mes18S_1	<i>Clethrionomys rutilus</i>	Kulu settlement, Magadan region	MK634547
12	Mes18S_1	<i>Sorex isodon</i>	Bolshoy Shantar Island, Khabarovsk Territory	MK634546
13	Mes18S_1	<i>Craseomys rufocanus</i>	Bolshoy Shantar Island, Khabarovsk Territory	MK634544
28	Mes18S_4	<i>Craseomys rufocanus</i>	Umara riwer floodplain, Magadan region	MN031874
47	Mes18S_3	<i>Micromys minutus</i>	Georgievka village Khabarovsk Territory	OP161928
48	Mes18S_1	<i>Craseomys rufocanus</i>	Umara riwer floodplain, Magadan region	OP161977
49	Mes18S_5	<i>Sorex cinereus</i>	Fairbanks, Alaska, USA	OP161923
50	Mes18S_1	<i>Sorex caecutiens</i>	«Contact» station, Magadan region	OP161924
51	Mes18S_2	<i>Craseomys rufocanus</i>	Nedorazumenia island, Magadan region	OP161926
52	Mes18S_1	<i>Sorex caecutiens</i>	Yakutsk, Republic of Sakha (Yakutia)	OP161922
53	Mes18S_1	<i>Sorex tundrensis</i>	Yakutsk, Republic of Sakha (Yakutia)	OP161927
54	Mes18S_2	<i>Sorex caecutiens</i>	Duckcha river floodplane, Magadan region	OP161921

Notes. Gathering locations, sample numbers and GenBank numbers are shown.

The work used samples of tetrathyridia from 4 species of shrews of the order Eulipotyphla (Insectivores), and three species of rodents (Rodentia). The localization of tetrathyridia is somewhat different in different hosts. According to our observations, in shrews they are more often located in the liver and the large lymphoid organ; in rodents, in the body cavity.

Some *Mesocestoides* host names that we previously contributed to GenBank (*Myodes rutilus* and *M. rufocanus*) do not correspond to the current classification system, thus, in the text we use the names *Clethrionomys rutilus* and *Craseomys rufocanus*, respectively (Kryštufek, Shenbrot, 2022).

Before DNA extraction from alcohol-fixed material, tetrathyridia with tissue localization were freed from cysts and washed in alcohol of the same concentration.

Isolation and purification of total DNA was carried out using the phenol-chloroform method (Sambrook et al., 1989). Amplification of 875 base pairs (bp) (131–1005 bp from the beginning of the gene) of the *18S rRNA* gene fragment was carried out using newly designed primers Micr18SL61 gcc ttg ata cgg tga aac cgc gaa tgg (61–89 bp from the start of the gene) and Micr18SR14028 caa tct gtc aat cct cat agt gtc cgg cc (1428–1456 bp from the start of the gene). Polymerase chain reaction conditions followed the protocol of Literak et al., 2004: denaturing step 94°C – 5 min; then 40 cycles: 94°C – 1 min, 52°C – 1 min, 72°C – 2 min; final stage 72°C – 7 min. The amplified sections of nuclear DNA were purified and prepared for sequencing according to standard methods

using the Diatom™ DNA Clean-Up reagent kit from Isogen Laboratory. The structure of the nucleotide sequence of the *18S rRNA* gene was determined from 131 bp from the beginning of the gene using the Micr18SL61 primer according to the standard method using Big Dye Terminator DNA cyclic sequencing kits (Applied Biosystems, v. 3.1) and an ABI Prism 3500xL genetic analyzer (Applied Biosystems, USA). The *18S rRNA* gene fragment was mapped relative to the complete nucleotide sequence of this gene in *Mesocestoides corti* GenBank No. AF286984 (Olson et al., 2001). Haplotypes of the *18S rRNA* gene fragment of the studied samples of *Mesocestoides* sp. were assigned the abbreviation Mes18S.

For phylogenetic analysis of the obtained nucleotide sequences of the *18S rRNA* gene, information about the corresponding fragment of this gene from samples belonging to the genus *Mesocestoides* was taken from GenBank (Table 2).

Table 2. Nucleotide sequences of *18S rRNA* taken for comparison from GenBank

Cestode species	GenBank number, author	Country	Host
<i>M. corti</i>	GU442130 (Piseddu et al., unpublished)	Italy	<i>Canis familiaris</i>
<i>M. corti</i>	AF286984 (Olson et al., 2001)	Switzerland	laboratory mouse
<i>M. litteratus</i>	JN088190 (Zalesny, Hildebrand, 2012)	Poland	<i>Myodes glareolus</i>
<i>M. litteratus</i>	MN512711 (Bayer et al., 2020)	Poland	<i>Vulpes vulpes</i>
<i>M. litteratus</i>	MN512709 (Bayer et al., 2020)	Poland	<i>Vulpes vulpes</i>
<i>M. melesi</i>	MN401346 (Bayer et al., 2020)	Poland	<i>Meles meles</i>
<i>M. melesi</i>	MN401347 (Bayer et al., 2020)	Poland	<i>Myodes glareolus</i>
<i>Mesocestoides</i> sp.	EF095248 (Waeschenbach et al., 2007)	Bulgaria	<i>Apodemus agrarius</i>
<i>Mesocestoides</i> sp.	AF119678 (Crosbie et al., 2000)	USA	<i>Canis latrans</i>

The dendrogram of *18S rRNA* gene haplotypes was constructed using the maximum likelihood (ML) method based on the Kimura biparametric distance model selected using the Bayesian information criterion. The stability of branch nodes was assessed using the bootstrap method (500 iterations).

Some of the data taken for comparison in GenBank had identical sequences; to simplify the structure of the ML-phylogenetic tree, we included in the analysis only one sequence from a number of identical ones.

The nucleotide sequence of the corresponding fragment of the *18S rRNA* gene GQ260092 of *Echinococcus granulosus* (Jia, Yan, 2009, unpublished) was used as an outgroup.

Genetic data analysis was carried out using MEGA software packages 10.0.2.74 (Tamura et al., 2013) and ARLEQUIN ver. 3.5 (Excoffier et al., 2005).

RESULTS AND DISCUSSION

Characteristics of nucleotide sequences of Mes18S_1-Mes18S_5 haplotypes of the *18S rRNA* gene fragment of *Mesocestoides* sp.

The 875 bp fragment of the *18S rRNA* gene was sequenced for twelve samples of *Mesocestoides* sp. (Table 1). Five Mes18S haplotypes were identified. Among them, we detected 27 nucleotide substitutions (ns) at 24 sites (Fig. 2).

		2222444456	6777777888	8888	
		3344025514	7123444114	4448	
		0779912782	3191259175	6784	HAPLOGROUP
MK634544	Mes18S_1	AAATGGGGCG	AGATTATGTG	TCAG	A
OP161926	Mes18S_2	CGGC....A.	G.GCCGC.CA	CTG.	B
OP161928	Mes18S_3C	A
MN031874	Mes18S_4CTAA.A	.T.....A..	...T	A
OP161923	Mes18S_5	CGGCT...A.	GCGCCGC.CA	CTG.	B

Figure 2. Haplotypes Mes18S_1– Mes18S_5 of the *18S rRNA* gene fragment of *Mesocestoides* sp. Substitution sites are shown relative to the Mes18S_1 nucleotide sequence from the beginning of the *18S rRNA* gene.

The Mes18s_1 haplotype included 7 nucleotide sequences (MK634544, MK634546, MK634547, OP161922, OP161924, OP161927, OP161977) and Mes18s_2 included two sequences (OP161921 and OP161926), however, to simplify the analysis we used only one of identical sequences (for Mes18s_1 – MK634544, for Mes18s_2 – OP161926).

The number and type of ns indicate the presence of two haplogroups within the studied nucleotide sequences groups A (haplotypes Mes18S_1, Mes18S_3, Mes18S_4) and B (Mes18S_2, Mes18S_5). The presence of two haplogroups is a consequence of the high level of polymorphism in the nucleotide sequence of the *18S rRNA* gene fragment in the general sample of *Mesocestoides* spp. In the haplogroup A, 8 polymorphic sites were identified (Fig. 2, Table 3). Mes18S_1 variant was predominant among the identified haplotypes, both within haplogroup A and in the general sample. It was found in samples from hosts belonging to different systematic groups (*Clethrionomys rutilus*, *Craseomys rufocanus*, *Sorex caecutiens*, *S.isodon*, *S.tundrensis*) with different levels of metabolism, and collected in geographically distant localities (Magadan region, Khabarovsk region and Yakutia) (Fig. 1, Table 1).

Haplogroup B is represented by two haplotypes Mes18S_2 and Mes18S_5, differing from each other by two transversions. Haplotype Mes18S_2 was the second in the proportion of identified nucleotide sequences; it was found in specimens parasitizing *C. rufocanus* and *S. caecutiens* from Magadan region. Haplotype Mes18S_5 was found in a specimen from alaskan *S. cinereus* (Table 1).

The total number of polymorphic sites in haplogroups A and B was 24. Taking into account all mutations in both haplogroups, haplogroup A differed from haplogroup B by 21 transitions and 6 transversions (Fig. 2).

Nucleotide diversity (π) and the average number of pairwise differences between haplotypes (P_i) are higher in haplogroup A than in haplogroup B. At the same time, haplotype diversity (h) is higher in group B (Table 3).

The genetic distance (pairwise F_{st}) between haplogroups A and B calculated by the pairwise differentiation method is 0.89583. The degree of genetic differences reliability ($p_{Fst} = 0.00901 \pm 0.0091$) is less than 0.05, which indicates the genetic isolation of the nucleotide sequences of these haplogroups.

Table 3. Characteristics of Mes18s_1–Mes18s_5 haplotypes of the 18S rRNA gene fragment of *Mesocestoides* sp.

Haplogroup	Haplotype	Sample share		N	n/m	k	Substitution share		Molecular diversity indices		
		Haplotype	Haplogroup				Transition	Transversion	$\pi \pm sd$	$h \pm sd$	$Pi \pm sd$
A	Mes18s_1	0.5833	0.7500	9	10/8	3	0.500	0.500	0.0023 \pm 0.0016	0.4167 \pm 0.1907	1.9722 \pm 1.2272
	Mes18s_3	0.0833									
	Mes18s_4	0.0833									
B	Mes18s_2	0.1667	0.2500	3	2/2	2	0	1.00	0.0015 \pm 0.0016	0.6667 \pm 0.3143	1.3333 \pm 1.0983
	Mes18s_5	0.0833									
	Substitutions between haplogroups A and B	–									
General sample		–	–	–	27/24	–	0.7779	0.2222	–	–	–
		–	–	12	27/24	5	0.7779	0.2222	0.0095 \pm 0.0053	0.6667 \pm 0.1409	8.3333 \pm 4.1537

Notes. N – sample size, n – number of substitutions, m – number of polymorphic sites, k – number of haplotypes in the sample, π – nucleotide diversity, h – haplotype diversity, Pi – average number of pairwise differences between haplotypes, sd – standard deviation, “–” – data is not calculated.

Phylogenetic relationships among *Mesocestoides*

Phylogenetic relationships within *Mesocestoides* spp. are presented in Fig. 3.

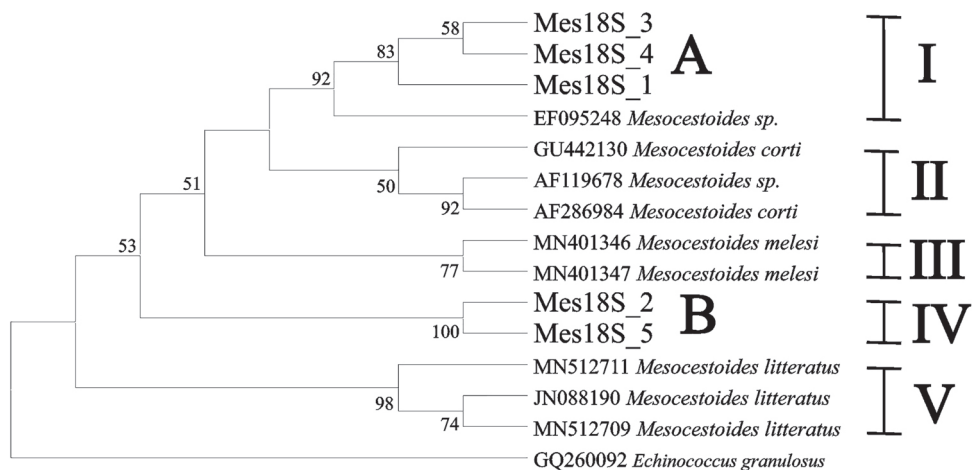


Figure 3. ML-phylogenetic tree constructed based on data on the variability of the nucleotide sequence of the studied haplotypes of the *18S rRNA* gene fragment of *Mesocestoides* sp. and data from GenBank. The nodes indicate bootstrap indices ($\geq 50\%$).

Five clades have been identified in the ML-phylogenetic tree of *Mesocestoides* spp. Clade I includes nucleotide sequences of the haplogroup A and EF095248 *Mesocestoides* sp. (Waeschenbach et al., 2007). Clade II contains the nucleotide sequences of *M. corti*: GU442130 (Piseddu et al., unpublished), AF286984 (Olson et al., 2001) and *Mesocestoides* sp. AF119678 (Crosbie et al., 2000). It can be assumed that the nucleotide sequences of the *18S rRNA* gene fragment of the same *Mesocestoides* species representatives have a sufficiently similar structure to form subclades with statistically significant bootstrap indices on the branches of the ML phylogenetic tree. Therefore, it is possible, that AF119678 *Mesocestoides* sp. (Crosbie et al., 2000), which forms clade II together with AF286984 and GU442130, belongs to the species *Mesocestoides corti*. In addition, haplotype MK239661 (Heneberg et al., 2019) of *Mesocestoides* sp. has the same nucleotide sequence with GU442130, and AF119678 *Mesocestoides* sp. is identical to AF286984, AF119688-AF119690, which also allows us to classify these samples of *Mesocestoides* sp. as *M. corti*.

Clade III is formed by the nucleotide sequences of *M. melesi*. The MN401346 variant is identical to MN401345 and MN512707 (Bayer et al., 2020). Haplotype MN401347 differs from these nucleotide sequences by four transitions and five transversions.

Haplotypes of haplogroup B form clade IV with a bootstrap index of 100%. This indicates the genetic isolation of samples Mes18S_2 and Mes18S_5 from other nucleotide sequences. It should be emphasized that haplogroup B contains samples from remote habitats - from the vicinity of Magadan and Fairbanks (USA).

Information about 16 nucleotide sequences of the *18S rRNA* gene of the species *Mesocestoides litteratus* was taken from the electronic database for phylogenetic analysis. These sequences form clade V, represented on the ML phylogenetic tree by three haplotypes. When comparing all analyzed nucleotide sequences in the MEGA software package, it was found that only representatives of *M. litteratus* have a deletion of 729–747 bp and an insertion of guanine at the 761 site. The results obtained suggest that the 729–747 bp segment of the rRNA nucleotide sequence does not significantly affect either the structure or functional activity of ribosomes. Perhaps the identified deletion and insertion can claim to be a genetic marker for the species *M. litteratus*.

The location of haplogroups A and B on the ML-phylogenetic tree and the significant difference between the nucleotide sequences of the haplotypes of these phylogroups allow us to assume that *Mesocestoides* sp., having haplotypes Mes18S_1, Mes18S_3 and Mes18S_4, belong to one species, and Mes18S_2 and Mes18S_5 to another.

As long as the *Mesocestoides* samples taken from GenBank correspond to the declared species, the data obtained as a result of genetic analysis (nucleotide substitutions, molecular diversity indices, topology of subclades on the ML phylogenetic tree) indicate that the tetrathyridia *Mesocestoides* sp. from our study do not belong to the species *M. corti*, *M. melesi* and *M. litteratus*. For two other confirmed species (*M. canislagopodis* and *M. lineatus*), there is no comparative material on *18S rRNA* in GenBank, however, taking into account the results of our previous works (Pospekhova et al., 2018, 2023), there is a high probability that the studied samples of tetrathyridia from intermediate hosts of Eastern Russia and Alaska (USA) do not belong to any of the five confirmed *Mesocestoides* species.

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ETHICS APPROVAL AND CONSENT TO PARTICIPATE

No humans or live animals were used in this work. The work used alcohol-fixed samples from the helminthological collection of our institute.

CONFLICT OF INTEREST

The authors of this work declare that they have no conflicts of interest.

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ФИЛОГЕНЕТИЧЕСКИЕ ОТНОШЕНИЯ ТЕТРАТИРИДИЕВ *MESOCESTOIDES* VAILLANT, 1863 ОТ МИКРОМАМАЛИЙ ВОСТОКА РОССИИ И АЛЯСКИ НА ОСНОВЕ 18S *pPHK* ГЕНА

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Ключевые слова: *Mesocestoides*, тетратиридий, насекомоядные, грызуны, 18S *pPHK*, филогенетический анализ, генетический маркер

Исследован фрагмент 875 пар нуклеотидов гена 18S *pPHK* у 12 образцов тетратириди-ев *Mesocestoides* от мелких млекопитающих 7 видов, собранных в географически удаленных локальностях. Определено пять гаплотипов, различающихся между собой 27 нуклеотидными заменами в 24 сайтах. Установлена принадлежность гаплотипов 18S *pPHK* к двум генетически различным гаплогруппам. Для каждой из них рассчитаны индексы молекулярного разнообразия. Проведённый анализ позволил предположить: 1) *Mesocestoides* sp. гаплогрупп А и Б относятся к двум разным видам, но не принадлежат ни к одному из подтверждённых видов рода; 2) делеция 729-747 пн и вставка в сайте 761 гуанина могут служить генетическим маркером вида *Mesocestoides litteratus*.