Morphological description and phylogenetic position of xiphidiate cercaria of *Prosthogonimus pellucidus* (Trematoda: Digenea)

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ABSTRACT: Xiphidiocercariae is a non-taxonomic group of digenean larvae. These small cercariae are characterized by complex morphology, which complicates their accurate identification. Recently, we have devoted a series of papers to the least studied groups of xiphidiocercariae — *Cercariae virgulae* and *Cercariae microcotylae*. Here we present the morphological description and molecular phylogenetic analysis of the new microcotylous cercaria. We have identified morphological features that allow us to distinguish this larva from all previously described cercariae. This species lacks virgula organ and possesses three pairs of penetration glands and Y-shaped excretory bladder. Molecular phylogenetic analysis performed with 28S rDNA and *cox*1 mtDNA sequences made it possible to confirm the taxonomic position of this cercaria within the family Prosthogonimidae, and to determine its species identity as one of the least common adult digenean *Prosthogonimus pellucidus.*
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KEY WORDS: Xiphidiocercariae, stylet cercaria, Plagiorchiida, penetration glands, Prosthogonimidae.

Морфологическое описание и филогенетическое положение ксифидиатной церкарии *Prosthogonimus pellucidus* (Trematoda: Digenea)

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РЕЗЮМЕ: Стилетные церкарии — это сборная группа морфологически сходных церкарий. Ранее нами были изучены две группы ксифидиатных церкарий — *Cercariae virgulae* и *Cercariae microcotylae*. В данной работе мы представляем результаты морфологического описания и молекулярно-филогенетического анализа новой микрокотилидной церкарии. Мы выявили морфологические особенности, позволяющие отличить ее от всех ранее описанных церкарий. Этот вид обладает тремя парами желез проникновения, Y-образным экскреторным пузырем и лишен виргулы. Молекулярно-филогенетический анализ, проведенный с использованием последовательностей 28S рДНК и *cox*1 мтДНК, позволил подтвердить таксономическую принадлежность этой церкарии к семейству Prosthogonimidae и определить ее видовую идентичность с одним из наименее распространенных видов трематод — *Prosthogonimus pellucidus*.
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КЛЮЧЕВЫЕ СЛОВА: Xiphidiocercariae, Plagiorchiida, железы проникновения, Prosthogonimidae.

Introduction

Xiphidiocercariae are free-swimming stylet larvae of digenean hermaphroditic generation. The essential role of these cercariae is to find and infect a second intermediate host (Galaktionov, Dobrovolskij, 2003). Morphological studies of xiphidiocercariae are obstructed due to the small size, short lifespan during microscopy (3 to 5 minutes in average), and high motility of these larvae. Stylet cercariae from subgroups *Cercariae virgulae* and *Cercariae microcotylae* are larvae of adult digeneans that parasitize birds and mammals (prevalently bats). When direct parasitological dissections of definitive hosts are impossible due to their conservation status or ethical reasons, studying the biodiversity of cercariae is the only way to gain insight into the digenean fauna of a geographic region. After the molecular methods had become routine in zoology, studies of the biodiversity of cercariae advanced to a new level. During the long-term studies of cercarial fauna in the North-West region of Russia, we described several new morphological types of virgulate and microcotylous xiphidiocercariae, which reliably correspond to a current taxa: Lecithodendriidae, Pleurogenidae, Microphallidae, and Prosthogonimidae (Shchenkov *et al*., 2019). New important data on the morphology of cercariae of Phaneropsolidae were generated by Dellagnola *et al*. (2019). These recent data replaced the previous concept of diversity of virgulate and microcotylous cercariae elaborated by M. Lühe (Lühe, 1909). Although interest in investigations of cercariae still remains strong, studies on their biodiversity are far from over.

Here we provide a detailed morphological description and molecular phylogenetic analysis of microcotylous cercaria of *Prosthogonimus pellucidus* from freshwater snail *Bythinia tentaculata* Linnaeus, 1758 (Caenogastropoda: Bythiniidae).

Material and methods

Host snails *Bythinia tentaculata* were collected in the Kristatel'ka River (Peterhof, St. Petersburg). Collections were made during different seasons of 2018–2022. Snails were kept in separate dishes and checked for invasion. Cercariae emerging from infected snails were placed on glass slides in a small water drop and studied *in vivo* with Leica DM2500 (Wetzlar, Germany) and LOMO MBR-1 (St. Petersburg, Russia) microscopes. All measurements were taken on cercariae fixed in 4% $AgNO_3$.

Samples for DNA isolation were fixed with 96% ethanol. Total DNA was isolated from the single

specimens using a ZymoBead Genomic DNA Kit™ (Irvine, California, USA) or Chelex-100 with Proteinase-K. Forward primer LSU-5 (5'-TAG GTC GAC CCG CTG AAY TTA AGC A-3'), and reverse primer 1500R (5'-GCT ATC CTG AGG GAA ACT TCG-3') (Littlewood, 1994; Snyder, Tkach, 2001) were used to amplify partial 28S rDNA gene, and JB3 (5'-TTT TTT GGG CAT CCT GAG GTT TAT-3'), JB4.5 (5'-TAA AGA AAG AAC ATA ATG AAA ATG-3') (Bowles *et al*., 1992) were used to amplify partial *cox*1 mtDNA gene. Polymerase chain reactions were performed in a total volume of 20 µl (11.5 µl H2O, 2.5 µl Taq buffer, 2 µl dNTP's at a concentration of 10 pM, 0.5 µl of each primer at a concentration of 10 pM, 1 µl of Syntol Taq polymerase, 1 µl of the DNA template). The thermal cycler parameters were as follows: initial denaturation at 95 °C (3 min); denaturation 20 s, 95 °C; annealing 20 s at 56 °C for LSU5/1500R primers, elongation 120 s at 72 °C; annealing 20 s at 48.9 °C for JB3/JB4.5 primers, elongation 50 s at 72 °C; final extension 5 min at 72 °C for both primer pairs, 35 cycles. Amplicons were purified using a Cleanup Mini Purification Kit™ (Evrogen, Moscow, Russia). All amplicons were sequenced using the equipment of the Research Park of St. Petersburg State University (Centre for Molecular and Cell Technologies). Sequences from both forward and reverse primers were assembled using Chromas Pro 1.7.4 (Technelysium Pty Ltd).

The general alignment of partial 28S rDNA and *cox*1 gene sequences was generated with "MUSCLE" algorithm (Edgar, 2004), and trimmed manually in SeaView v. 4 software (Gouy *et al*., 2010). The final length of alignment was 1258 bp for partial 28S rDNA gene sequence and 277 bp for *cox*1 gene. Full list of sequences used in phylogenetic reconstructions is provided in Supplementary Table S1.

The evolutionary model for Maximum likelihood estimation and Bayesian inference analysis was estimated with MrModeltest v. 2.4 (Nylander, 2004). The best fitted model was GTR + G + I in both cases. Maximum likelihood analysis was performed through the CIPRES portal (Miller *et al*., 2010) with non-parametric bootstrap with 1000 pseudoreplicates. Bayesian analysis was performed using MrBayes v. 3.2.7a at CIPRES portal for 15,000,000 generations. The quality of the chains was estimated using built-in MrBayes tools and additionally estimated with Tracer v. 1.6 package (Rambaut *et al*., 2018). Based on the estimates by Tracer, the first 50,000 generations were discarded for burn-in. Methodics were equal for both genes.

The *p*-distances were calculated based on partial *cox*1 mtDNA gene sequences with MEGA11 software (Tamura *et al*., 2021) with standard parameters. In our analysis, we included *Collyriclum faba* as reference species after Heneberg *et al*. (2015) to estimate genetic differences to relatively distant digenean taxa. A list of GenBank numbers of sequences used is given in Supplementary Table S1. For phylogenetic reconstruction, *C. faba* was used as an outgroup after Heneberg *et al*. (2015).

Results and Discussion

Morphological description

Cercaria of *Prosthogonimus pellucidus* (Fig. 1)

Type locality: Kristatel'ka River, Petergof, St. Petersburg, (59°53′30.5″N, 29°50′05.9″E).

First intermediate host: *Bythinia tentaculata* Linnaeus, 1758 (Caenogastropoda: Bythiniidae).

Accessions: partial 28S rDNA gene sequence GenBank No MT216312; partial *cox*1 mtDNA gene sequence GenBank No ON237641.

Body 99–122 (116) µm long, 42–73 (58) µm wide (Fig. 1A). Tail 22–34 (26) µm long. Oral sucker 29–37 (31) µm in diameter. Ventral sucker 18–25 (21) µm in diameter, post-equatorial, well developed. Ventral/oral sucker ratio: 0.67:1. Stylet 14–19 (15) µm long (Fig. 1B). Spines in body and tail tegument not detected. Oesophagus narrow, other details not detected. Virgula absent. Penetration glands arranged in two adjacent rhomboid groups, three glands in each group: one large posterior pair with thin-granulated cytoplasm and two small submedian with hyalinized cytoplasm. Outlets of penetration glands opening near stylet tip and shoulders. Excretory bladder Y-shaped, walls thin. Shape of excretory bladder changes during systole and period, when it is fulfilled (Fig. 1C). Small loops of main excretory tubes located behind penetration glands. Excretory formula 2[(2+2+2)+(2+2+2)]=24. Parenchyma without fat droplets.

Remarks. Cercaria of *P. pellucidus* is morphologically close to cercariae of Prosthogonimidae by stylet and body shape, the shape of the excretory bladder, and excretory formula. However, it clearly differs from the previously described species of this family by the presence of only three pairs of penetration glands. According to gross morphology, cercaria of *P. pellucidus* is similar to *Cercaria cristatella* D except for the number of penetration glands (three pairs in cercaria of *P. pellucidus vs* four pairs in *C. cristatella* D). The body of cercaria of *P. pellucidus* is shorter and narrower than the body of *C. cristatella* D, but their tails are equal. Oral sucker of cercaria of *P. pellucidus* is smaller; the ventral sucker is larger than in *C. cristatella* D. The length of the stylet has no significant differences.

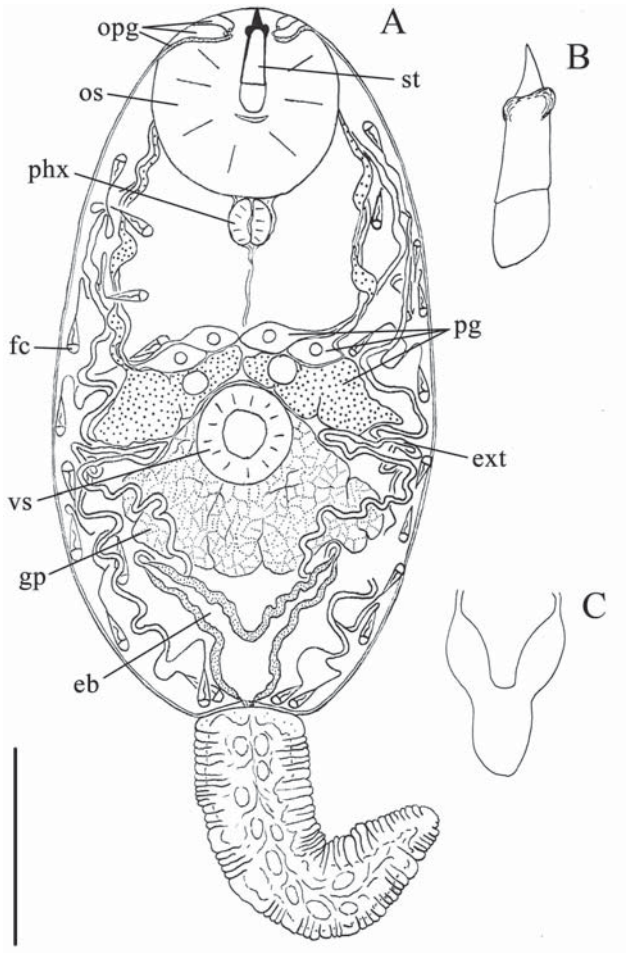

Fig. 1. General morphology of cercaria of *Prosthogonimus pellucidus*. A — general morphology (*in vivo*); B — stylet, lateral view; C — excretory bladder during the systole.
Abbreviations: eb — excretory bladder; ext — main excretory tube; fc — flame cell; gp — genital primordium; opg — outlets of penetration glands; os — oral sucker; pg — penetration glands; phx — pharynx; st —stylet; vs — ventral sucker. Scale bar: A — 30 μm; B, C — not to scale.
Рис. 1. Общая морфология церкарии *Prosthogonimus pellucidus*. A — общая морфология (*in vivo*); B — стилет, вид сбоку; C — мочевой пузырь во время систолы.
Обозначения: eb — мочевой пузырь; ext — главный собирательный канал; fc — циртоцит; gp — половой зачаток; opg — выводные протоки желез проникновения; os — ротовая присоска; pg — железы проникновения; phx — глотка; st — стилет; vs — брюшная присоска. Масштаб: A — 30 μm; B, C — не в масштабе.

Molecular data

We will not discuss phylogenetic relationships among all microphalloid species, but within Prosthogonimidae family in details. *Prosthogonimus rarus* is close to *P. ovatus* with full support. The newly obtained sequence of 28S rDNA gene of cercaria of *P. pellucidus* forms a sister clade to *P. cuneatus* with full support. Altogether, prosthogonimids are the sister clade to other microphalloid species except for *Pachypsolus irroratus*, which is basal to other studied taxa.

According to newly obtained phylogenetic tree (Fig. 2, unedited Bayesian tree available as Supplementary Data S1), Microphallidae is sister group to Lecithodendriidae, Phaneropsolidae, and Stomylotrematidae families. Stomylotrematidae is the sis-

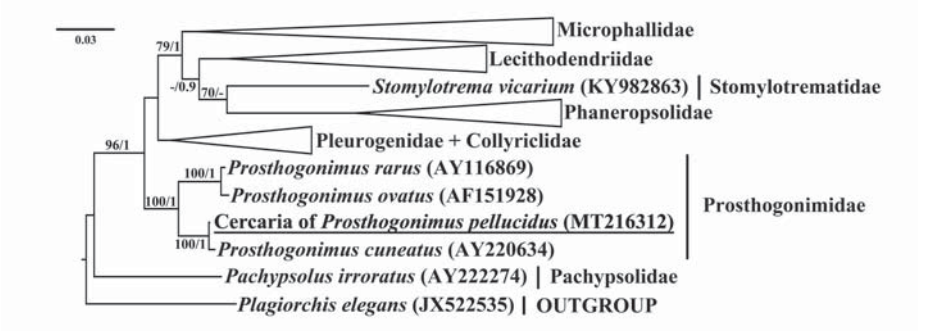

Fig.2. Molecular phylogenetic relationships of cercaria of *Prosthogonimus pellucidus* with other microphalloid species based on the partial 28S rDNA sequence. Node support: ML/BI, only ML≥50 and BI≥90 are shown.
Рис. 2. Реконструкция молекулярно-филогенетических отношений церкарии *Prosthogonimus pellucidus* с другими видами Microphalloidea, построенная на основе частичной последовательности 28S рДНК. Узловые поддержки: ML/BI, показаны только значения ML≥50 и BI≥90.

ter clade to Phaneropsolidae in both ML and BI analysis. Pleurogenidae and Collyriclidae are sister taxa to all previously listed ones.

In *cox*1-based phylogenetic tree (Fig. 3), cercaria of *P. pellucidus* is clustered together with adult specimens. All specimens of *P. pellucidus* form a sister clade to *P. cuneatus*. Other two sister clades are formed by *P. rarus* and *P. ovatus*. Our data are fully support the results of Heneberg *et al*. (2015) obtained for the same molecular marker.

On average, the *p*-distance calculated on partial *cox*1 mtDNA sequences of four prosthogonimid species under consideration and between each of them and *Collyriclum faba* is 0.15. Average difference of cercaria of *P. pellucidus* from *P. ovatus, P. cuneatus, P. rarus,* and *C. faba* is 0.13. The most explicit *p*-distance occurred between cercaria of *P. pellucidus* and *C. faba*: 0.15. The *p*-distance between cercaria of *P. pellucidus* and its adult is 0.01, making this the smallest rate observed (Table 1).

Previously, cercariae of *P. pellucidus* were partially studied during experimental reconstruction of its life cycle (Krasnolobova, 1960, 1962). According to the brief description provided by Krasnolobova (1960), cercaria of *P. pellucidus* has relatively larger body sizes (body length is 141 μm *vs* 116 μm in the present description, but body width is 53 μm *vs* 58 μm, respectively; oral sucker is 28 μm *vs* 31 μm, length of stylet is 13 μm *vs* 15 μm, ventral sucker is 25 μm *vs* 21 μm). Since Krasnolobova (1960, 1962) did not provide any data on the measurement procedure, the differences between our data and previously obtained ones could be explained by methodological reasons. The most significant discrepancy between the two descriptions is a number of penetration glands (three pairs in the present description *vs* four pairs in *P. pellucidus* sensu Krasnolobova, 1960). As long as we used vital staining to clarify all controversial points and reproduced descriptions independently from several authors, we consider the newly obtained data to be preferable. Krasnolobova (1960, 1962) did not provide any details on excretory system organization in cercaria of *P. pellucidus*.

In our previous paper, we summarized the data on virgulate and microcotylous xiphidiocercariae (Shchenkov *et al*., 2019). The representatives of Prosthogonimidae family possess cercariae with Y-shaped excretory bladder and four pairs of penetration glands. Although cercaria of *P. pellucidus* has almost the full set of characters common for "Prosthogonimidae" subtype, this species differs sharply from the previously described larvae in the smaller number of penetration glands.

Molecular and morphological data clearly established the phylogenetic position of cercaria of *P. pellucidus* among representatives of Prosthogonimidae family. Due to this fact, it is possible now to spread the characteristics of "Prosthogonimidae" subtype: cercariae belonging to this group have four or three pairs of penetration glands. In total, the phylogenetic tree obtained in this study is in agreement with previous ones (Shchenkov *et al*., 2019; Sokolov *et al*., 2020; Dellagnola *et al*., 2022; Fernandes *et al*., 2022). It is important to note, that the phylogenetic position of cercaria of *P. pellucidus* distinguished by 28S rDNA-based analysis duplicate phylogenetic position of adults of *P. pellucidus* revealed on ITS2, *cox*1 mtDNA, and ND1 sequences (Heneberg *et al*., 2015). Unfortunately, there are no sequences of 28S rDNA available for *P. pellucidus* to date. However, in our analysis differences between mitochondrial gene sequences of cercariae and adults of *P. pellucidus* were less than one percent which indicates their species identity (see, for example, Vilas *et al*, 2005; Králova-Hromdova *et al*., 2008; Tatonova *et al*.,

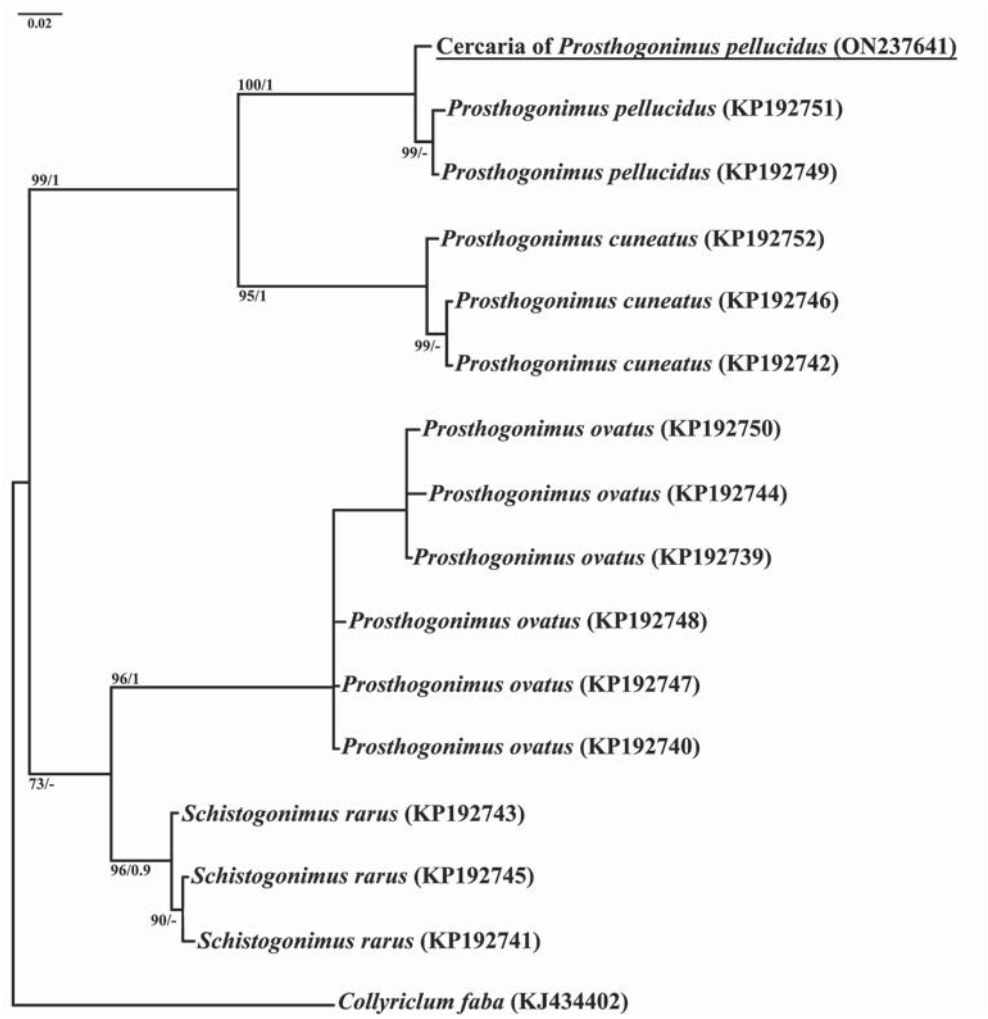

Fig. 3. Molecular phylogenetic relationships of cercaria of *Prosthogonimus pellucidus* with other prosthogonimids based on the *cox*1 mtDNA gene sequence. Node support: ML/BI, only ML≥50 and BI≥90 are shown.

Рис. 3. Реконструкция молекулярно-филогенетических отношений церкарии *Prosthogonimus pellucidus* с другими видами Prosthogonimidae, построенная на основе последовательности *cox*1 гена митохондриальной ДНК. Узловые поддержки: ML/BI, показаны только значения ML≥50 и BI≥90.

2013; Waki *et al*., 2018). Thus, our data complement the information about the life cycle of *P. pellucidus* and the morphology of its larvae.

According to Heneberg *et al*. (2015), *P. pellucidus* is the least common species of the *Prosthogonimus* in Central Europe where it parasitizes ducks and geese. Several species of potential definitive hosts (*Anas platyrhynchos,* sometimes *A. nyroca*) are common in a studied geographic region (own observations), although there are no data on their infection by prosthogonimids.

Newly obtained morphological data on cercaria of *P. pellucidus* support the current view on the possible way of evolution of Xiphidiocercariae associated with their juvenilization (Galaktionov, Dobrovolskij, 2003; Shchenkov *et al*., 2019). The reduction of penetration glands quantity reflects their oligomerization as one of the important evolutionary trend characteristics of this diverse group of cercariae. This trend is also clearly observed in cercariae of other digeneans, especially in furcocercariae (Ginetsinskaya, 1968; Galaktionov, Dobrovolskij, 2003).

Table 1. *p*-distances calculated in MEGA11 software based on *cox*1 mtDNA sequences of cercaria of *Prosthogonimus pellucidus* and closely related digenean species.
Таблица 1. *p*-дистанции, рассчитанные с помощью пакета программного обеспечения MEGA11, на основе частичных последовательностей гена *cox*1 церкарии *Prosthogonimus pellucidus* и близкородственных видов трематод.

Species	Cercaria of *P. pellucidus*	*P. pellucidus*	*P. cuneatus*	*P. rarus*	*P. ovatus*
Prosthogonimus pellucidus	0.01				
Prosthogonimus cuneatus	0.11	0.11			
Prosthogonimus rarus	0.13	0.15	0.17		
Prosthogonimus ovatus	0.14	0.15	0.18	0.09	
Collyriclum faba	0.15	0.18	0.18	0.13	0.14

Compliance with ethical standards

CONFLICTS OF INTEREST: The authors declare they have no conflict of interest.

Supplementary data. The following materials are available online.

Table S1. Species used for molecular analysis. GenBank accession numbers and references are given. Sequences obtained for this study are highlighted in bold.

Data S1. Unedited Bayesian phylogenetic tree based on a 28S rDNA marker.

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