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A method to detect the parasitic nematodes from the family Anisakidae, in *Sardina pilchardus*, using specific primers of 18 S DNA gene

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Abstract Anisakiasis is the infestation of man by the third larval stage of the Anisakidae family parasites, caused by the consumption of raw or undercooked seafood. The hosts of the third larvae parasite are marine fishes, among which *Sardina pilchardus* is of high commercial interest in many countries. So, the detection of anisakid larvae in fisheries products (fresh and processed) is of great interest to a rapid and feasible diagnosis of parasitism. Currently, there are different types of parasite detection methods but they are all difficult and tedious. The aim of this work was to detect, using DNA-based techniques, the presence of anisakid larvae in the entire fish host. Three pairs of primers were designed in the 18 S DNA gene taking into account the maximum matching between the anisakid species sequences and the minimum matching concerning for both the fish species and some other groups of organisms that parasitize fish (non-anisakid nematodes, trematodes, and cestodes). Experimental mixtures of parasite and fish host were made and the sensitivity of anisakid DNA detection tested by PCR. All the above primers and two others combinations of them were specific for the nematode group within the studied species, and the sensitivity of detection was on the order of 1 part of nematode to 100 000 parts of fish (anisakid weight:fish sample fresh weight = 1:~100 000).

Keywords Anisakid · Detection · 18 S DNA · PCR · Sequencing

Introduction

Anisakis simplex, *Pseudoterranova decipiens*, *Contracaecum osculatum*, and *Hysterothylacium aduncum* are anisakid nematodes (roundworms) that have been responsible of human infections (anisakiasis or anisakidosis) caused by the consumption of raw, undercooked [1] not adequately salted, pickled, or smoked seafood [2]. About 12 000 documented cases of anisakiasis in humans were already reported [3].

The symptoms of anisakiasis are the same of an acute appendicitis, peritonitis, gastric ulcer or tumour, cholecystitis, diverticulitis, cancer of the pancreas, or those of Crohn's disease [4, 5]. Symptoms occur from as little as an hour to about 2 weeks after consumption of raw or undercooked seafood. One nematode is the usual number recovered from a sick patient [6].

To identify anisakiasis as the correct diagnosis, it is necessary to detect anisakid across several histological preparation of tumour or affected intestinal sub mucous [7].

The therapeutic recommendations to avoid severe infections from anisakid are: the prior deep-freezing of the fish in order to kill them, and/or the avoidance of raw or undercooked fish ingestion [8].

In the case of light salted (8–9%) and ripened fish, some authors [9] showed that the maximum survival time of *A. simplex* in these conditions is about 5–6 weeks. Therefore, the ripening time for an 8–9% of salt must be longer than six weeks. In some countries (such as Spain and France), which have a long tradition of eating anchovies in vinegar (pickled anchovies), the deadline for the consumption of such a product must be greater than 60 days after processing [10].

However, these methods do not protect the consumers from possible allergic reactions caused by the ingestion of parasite antigen [11]. It was observed that some patients, without foregoing or associated digestive complaints,

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exhibited episodes of urticaria [12] angioedema or severe anaphylactic reactions [13] linked to the ingestion of fish or seafood, that originally seemed to have no apparent relation with the ingested foodstuff (cooked or not).

In fact, multiple allergic hypersensitivity reactions to the fish parasite *A. simplex* have been documented and reported since the 1990s in subjects sensitized to it. Episodes of occupational conjunctivitis and asthma [14, 15] and rheumatological symptoms [16] have also been recently reported to be directly associated with the sensitization of the subject to this parasite. Some collagenolytic enzymes produced in the parasite's esophagus might be responsible, not only for the attachment of the parasite to the gastric mucous, but also for the allergic manifestations in sensitized individuals [17].

In vitro tests reveal the ability of a number of *A. simplex* allergens to resist heat [18]; so, prevention against possible allergic hazard due to the ingestion of dead parasites could not be done through the current fish salting and marinating techniques and also through heat treatment [10].

The detection of anisakid larvae in fisheries products (fresh and processed) is of great interest to a rapid and feasible diagnosis of parasitism. Currently, there are different types of parasite detection methods depending on fish or human. The methods applied in fish are digestion, sedimentation, identification and fixation [19], or candling and observation under U.V. [20]. In anisakiasis, the immunological detection [21] and the biopsy histological identification [7] are currently used.

These methods are difficult and tedious. The immunological detection method, besides the referred constraints, is not always reliable when taking into account the cross-reaction problems with others parasites such as *Ascaris* spp. [22]. In the case of the biopsy histological identification, the larva must be completely developed for a correct morphological identification.

All these things show that there is a great need for a methodology that allows a rapid and unambiguous identification of these parasites, both in host fish and in anisakiasis patients.

The different genetic techniques developed in the last 40 years that were based on allozymes and DNA study, when applied to parasitism, revealed to be of great efficiency. So, the sibylline species of *A. simplex* [23] were discriminated by working with allozymes markers. Later, some authors applied the same technique to study the sibylline Anisakidae species of *C. osculatum*. Two sibylline species were distinguished [24] through the application of RFLP (restriction fragments length polymorphism) technique to the genomic DNA of *P. decipiens* and a procedure, based on differential amplifications of the D3 region of the 28S ribosomal gene allowed the identification of two species of the salmon parasite nematode of genus *Philomena* (*P. oncorhynchi* and *P. agubernaculum*) [25].

Due to the high economical importance of the *S. pilchardus* to the Portuguese people and their food habits tradition (to eat sardine, grilled with viscera, in an under-cooked way), this species was used as the experimental model. On the other hand, the percentage of infestation

found, in this species, in a Port Vendres harbor (Mediterranean sea), in 2002, was much higher—33% (personal observation) than the prevalence of 5% infected hosts with anisakid reported in [26] from all the sardine retrieved in the Milan's fish market.

So, the aim of our work is both to design pairs of primers that will amplify preferentially anisakid species and to know the sensitivity level of PCR detection of them from experimental mixtures of fish and parasites.

Material and methods

Fish sampling

The fish samples were caught in the Mediterranean Sea and immediately frozen after fishing in order to prevent migratory activity of the larvae from the viscera to the muscle region [27]. The frozen fish was dissected and the viscera taken out for microscope stereoscopic observation. The remainder was cut in fine slices and observed under UV light with the objective of detecting anisakid by fluorescence [21].

Parasite sampling

The larval anisakid used for study was retrieved from the sample collection of ethanol-preserved cists and larvae at the IPIMAR Institute (Lisbon, Portugal). Anisakid species were identified through the following morphological characters: the shape of the tail, the presence, length and position of the anterior intestinal caecum and posterior ventricular appendix [28, 29]. The four common morphological anisakid species were found—*A. simplex*, *P. decipiens*, *C. osculatum*, and *H. aduncum* [30].

Isolation of genomic DNA

Genomic DNA of anisakid and fish were extracted with QIAamp DNA Mini Kit (Qiagen). The DNA of non-anisakid nematodes and the trematodes-studied species was obtained from the Laboratoire de Biologie Animale (Perpignan, France).

Experimental dilutions of parasite DNA

Experimental dilutions of DNA extracted from a larva of *C. osculatum* (112 ng/ μ l), were made in order to know the amount needed to perform PCR from mixtures of DNA. The starting amounts of DNA (15 μ l) were 2240, 224, 22.4, 2.24, 1.12, and 0.448 ng, respectively. The PCR amplifications, with amounts in the order of 10-fold more (*C. osculatum* = 1021 ng/ μ l), were migrated in electrophoresis agarose gel stained with ethidium bromide and the intensity of the

bands observed. They were all sharp, so the amplification of experimental dilutions remained positive down to 1:5000. The anisakid amplified DNA remains detectable when the PCR starts with 0.448 ng DNA. As the correspondent band was still very sharp, amounts under 0.448 ng were assayed and experimental detections were achieved down to 0.1 ng.

Experimental mixtures of nematodes and eviscerated fish

The “experimental infestation” was performed by cutting, after weighting, both the ethanol preserved anisakid (*A. simplex* and *C. osculatum*) and the eviscerated fresh fish into small pieces that were latter carefully mixed. To reduce mass sample, posterior lyophilisation in a Maxi dry lyo equipment and boiling in a water-bath at 100 °C were applied as alternative methods (Table 1).

Following the lyophilisation and boiling methods, two extraction methods were applied—CTAB 2% and Qiagen

- Lyophilisation/CTAB extractions—the lyophilised mixtures were homogenized in a mortar, sub sampled in 2.0–4.0 g, placed in each centrifuge tube and subjected to CTAB extraction.
- Boiling/CTAB extraction—the frozen mixtures, after being cut into small pieces, was boiled during 10 min and centrifuged for a further 10 min at 13 500 rpm. The supernatant was then divided into sub-samples with 10 ml and subjected to the CTAB treatment.
- Qiagen extraction—25 mg of frozen/ethanol preserved/lyophilised material were used for extraction.

Extracted DNA quantification was done in an Eppendorf BioPhotometer.

Primers

A search was made in the National Center for Biotechnology Information (NCBI) database to find a set of primers that would present the maximum matching between the anisakid species and the minimum matching with both the fish host and the DNA from other groups of animals that can parasitize fish (trematodes, cestodes, and other nematodes).

An analysis of sequences from several nuclear genome regions (such as those encoding 18S ribosomal RNA, 28S ribosomal RNA, the internal transcribed spacer (ITS1), the internal transcribed spacer (ITS2)) and from mitochondrial DNA regions (such as those encoding 5.8S ribosomal RNA, cytochrome *b* gene, cytochrome *c* oxidase subunit 1 (COI), cytochrome oxidase subunit 2 (COX-2), small and large ribosomal RNA subunits)), was performed. The conservative 18S ribosomal RNA gene was chosen because it was the one presenting the largest number of sequences (for all the studied groups (Table 2)) and with the characteristics mentioned above.

Three pairs of PCR primers were designed from an alignment of 18S sequences covering four species of anisakid, fourteen other non-anisakid nematodes, three cestodes, three trematodes, and three marine fishes (Table 2). The amplification of short sequences was preferred to allow the possibly of working with DNA that was more or less degraded due to bad preservation conditions or fish food processing.

Table 1 Mixture samples, aliquots studied, extraction methods and sensitivity of each aliquot

Sardine	Fww (g)	Sww (g)	Sdw (g)	Anisakidae	Aw (g)	Aliquots	Qiagen Extraction	CTAB Extraction	Aw:Sww ratio	Aw:Fww ratio
S1	16.73	16.73	4.82	1 <i>A. simplex</i> larvae	0.0074	1	Lyophilisate	Lyophilisate	1 for 2 261	1 for 2 261
S2	16.08	16.08	4.80	3 <i>A. simplex</i> larvae	0.0275	1	Lyophilisate	Lyophilisate	1 for 585	1 for 585
S3	14.03	14.03	3.77	5 <i>A. simplex</i> larvae	0.0477	1	Lyophilisate	Lyophilisate	1 for 294	1 for 294
S4	30.73	8.53 9.62	2.83 3.36	3 <i>A. simplex</i> larvae	0.0044	2	Lyophilisate Lyophilisate	Lyophilisate Lyophilisate	1 for 2 186	1 for 4 125
S5	32.83	9.57 11.73	3.59 3.88	2 kysts	0.0051	2	Lyophilisate Lyophilisate	Lyophilisate Lyophilisate	1 for 2 300	1 for 4 176
S6	36.30	36.30		1 kyst + 4 <i>C. osculatum</i> larvae	0.0055	1		Boiling/Supernatant	1 for 6 600	1 for 6 600
S8	44.40	18.53 16.80	4.20 6.60							
S9	39.03	16.96 15.19	5.63 4.74							
S10	35.75	13.31 16.08	6.16 6.37							
Total			96.87	1 <i>C. osculatum</i>	0.0010	11	Lyophilisate		1 for 96 870	1 for 96 870

ASP: *Anisakis* sp.; HF: *Hysterothylacium fortalezae*; HP: *Hysterothylacium pelagicum*; HR: *Hysterothylacium reliquens*; CM: *Contracaecum multipapillatum*; PM: *P. decipiens*; HS: *Homo sapiens*

Table 2 Species retrieved from NCBI database and aligned to design set of primers

Phylum	Class	Order	Family	Genus	Specie	Databases ref	bp number
Fish	Osteichthyes	Clupeiformes	Clupeidae	Clupea	<i>C. harengus</i>	X98845	1791
		Salmoniformes	Salmonidae	Salmo	<i>S. trutta</i>	X98839	1781
		Perciformes	Sparidae	Pagrus	<i>P. major</i>	AB028214	1390
Platyhelminthes	Trematoda	Strigeidida	Fellodistomidae	Tergestia	<i>T. laticollis</i>	AJ287580	1932
		Azygiida	Syncoeliidae	Copiatestes	<i>C. filiferus</i>	AJ287490	1780
		Plagiorchiida	Monorchidae	Ancylocoelium	<i>A. typicum</i>	AJ287474	1925
	Cestoda	Eucestoda	Bothriocephalidae	Bothriocephalus	<i>B. sp</i>	AF267289	2126
					<i>B. scorpii</i>	AJ228776	2096
Nematoda	Enoplea	Pseudophyllida	Diphyllobothriidae	Diphyllobothrium	<i>D. stemmacephalum</i>	AF124459	2163
		Mermithida	Mermithidae	Mermis	<i>M. nigrescens</i>	AF036641	1749
		Enoplida	Enoplidae	Enoplus	<i>E. meridionalis</i>	Y16914	1852
		Trichocephalida	Trichuridae	Trichuris	<i>T. muris</i>	AF036637	1845
	Chromadore	Rhabditida	Rhabditidae	Pellioditis	<i>P. mediterranea</i>	AF083020	1707
			Diploscapteridae	Diploscapter	<i>D. sp</i>	AF083009	1734
			Heterorhabditidae	Heterorhabditis	<i>H. hepialus</i>	AF083004	1734
		Tylenchida	Heteroderidae	Meloidogyne	<i>M. incognita</i>	U81578	1706
			Aphelenchoididae	Bursaphelenchus	<i>B. sp</i>	AF037369	1705
		Ascaridida	Anisakidae	Anisakis	<i>A. sp</i>	U94365	1755
				Contracaecum	<i>C. multipapillatum</i>	U94370	1754
				Pseudoterranova	<i>P. decipiens</i>	U94380	1755
				Hysterothylacium	<i>H. fortalezae</i>	U94374	1753
				Ascaris	<i>A. sp</i>	M58348	1115
					<i>A. suum</i>	U94367	1754
					<i>A. lumbricoides</i>	U94366	1154
		Chromadorida	Chromadoridae	Chromadoropsis	<i>C. vivipara</i>	AF047891	1742
		Monhysterida	Xyalidae	Daptonema	<i>D. procerus</i>	AF047889	1748
		Diplogasterida	Diplogasteridae	Diplogaster	<i>D. lethieri</i>	AF036643	1744
		Spirurida	Philometridae	Philonema	<i>P. sp</i>	U81574	1749

Fwdw: Fish dry wet weight; Swdw: Sample dry wet weight; Sdww: Sample wet dry weight; Adw: Anisakidae dry weight

- ANIF 5'-AGCCTACTAAATAGTCATCGGATAA-3'
- ANIR 5'-ATTAAGGCGGTTTCCACCAAAGA-3'
- ANI1F 5'-GCGCCTCAGGACTTGGTCCGCCC-3'
- ANI1R 5'-TGAAGGCAACGTAGGGAAAACCAACA G-3'
- ANI2F 5'-AGCGTTCCGTCGGTGGTAAATACG-3'
- ANI2R 5'-TACCTTTCGGCAATGGTTACAGCA-3'

The designed primers should amplify fragments of 81 bp (ANI1F/ANI1R), 373 bp (ANIF/ANIR) and 466 bp (ANI2F/ANI2R) and some of their combinations should amplify fragments of 170 (ANIF/ANI2R) and 1096 bp (ANI1F/ANIR). All of them were tested on the four morphological species of anisakid, three fish species, two trematode species, and six non-anisakid nematodes from the Laboratoire de Biologie Animale work collection.

PCR conditions

Amplification reaction: 10 mM-tris HCl (pH 8.4); 50 mM KCl; 3.0 mM MgCl₂; 200 µM of each dNTP; 0.32 µM of each primer and 2 units of *Taq* polymerase (Promega) in a total volume of 50 µl. The amount of eluted/diluted DNA used varied between 1 and 30 µl. The PCR were performed in a PTC-100 Thermocycler (MJResearch) following the conditions below.

L1ANIS protocol: Initial denaturation at 95 °C for 5 min; 40 cycles of 65 °C for 1 min (annealing), 72 °C for 1 min

(extension), 94 °C for 45 s (denaturation); 65 °C for 1 min (annealing), and a final extension step at 72 °C for 10 min.

ANIS65 protocol: the same as L1ANIS but with 26 repeated cycles instead of 40.

A negative control reaction (without DNA) was always performed each reaction.

Size of PCR products was checked using a 100 bp DNA Ladder (Promega) on 1.0–2.5% agarose gels, stained with ethidium bromide and digitalized in an U.V.P. BioDoc-It System.

Sequencing

The amplified DNA, after extraction and purification from agarose bands using the Concert Nucleic Acid Purification System (GibcoBRL) was sequenced.

Cycle sequencing reaction: 3.2 pmol of primer (1.6 µl); 4.0 µl of BigDye (BigDye Terminator Cycle Sequencing v2.0 Ready Reaction); 10.4 µl of amplified DNA and 4.0 µl of H₂O milli-Q in a total volume of 20 µl.

Sequencing conditions: 95 °C for 5 min (denaturation); 60 cycles of 95 °C for 30 s (denaturation), 50 °C for 20 s (annealing), 60 °C for 4 min 30 s (extension); 15 °C for 10 h. The primers used were the same used in the PCR.

The reaction products were run in an Automated 373 ABI PRISM Sequencer (Perkin-Elmer) and sequences were read and corrected with the Sequencer Software.

Results and discussion

Best conditions of DNA extraction

All the procedures used for DNA extractions gave DNA of good quality for amplifications.

The Qiagen method, when applied to lyophilised samples, is the best method when considering both the amount of sample needed (25 mg) and the quality of the DNA. However, as the total sample can not be processed using this technique, its success only depends on positive results. If the obtained results are always negative, an extra CTAB extraction of the whole sample must be made.

The CTAB technique, when applied to the lyophilised sample, permits the study of the total original material. So, when working with aliquots of 4 g, no problem arise from the size of the sample. The detection process is applied to the aliquots in a sequential way. One positive detection is enough to consider the product contaminated. However, the lyophilisation of such amount of tissue takes about 3 days

to finish, so a rapid answer from the contamination process is not possible.

The CTAB method, when applied to the boiled sample, is better than the lyophilisation/CTAB method because boiling also permits the study of the total original material, needs a smaller number of aliquots of the sample and takes only 10 min to obtain. However, this technique does not allow the preliminary extraction of the DNA with the used type of Qiagen kit used.

Primers designing

The results of these PCR, containing different pair of primers, are presented in Table 3.

All the primers worked properly with anisakid DNA but not with fish and trematodes DNA. The pairs of primers ANI1F/ANI1R and ANIF/ANI2R are the least specific because they amplified five non-anisakid nematodes. The other pairs of primers only amplified *Nemelix dakeri* (terrestrial nematode). This species amplifies each time the process is performed and works with all primers, prob-

Table 3 Studied species

	81 pb ANI' F-ANI' R	170 pb ANI F-ANI2 R	373 pb ANI F-ANI R	466 pb ANI2 F-ANI2 R	1096 pb ANI' F-ANI R
Anisakidae					
<i>Anisakis simplex</i>	+	+	+	+	+
<i>Contracaecum maculatus</i>	+	+	+	+	+
<i>Pseudoterranova decipiens</i>	+	+	+	+	+
<i>Hysterothylacium aduncus</i>	+	+	+	+	+
Other nematodes					
<i>Anguillicolla crassus</i>	±	+	—	—	—
<i>Rhabditis terricola</i>	±	—	—	—	—
<i>Steinernema carpocapsae</i>	—	+	—	—	—
<i>Nemelix dakeri</i>	+	+	+	+	+
<i>Romanomermis culicivorax</i>	±	+	—	—	—
<i>Caenorhabditis elegans</i>	±	+	—	—	—
Trematodes					
<i>Dactylogyrus ramulosus</i>	—	—	—	—	—
<i>Schistosoma mansoni</i>	—	—	—	—	—
Fish					
<i>Sardina pilchardus</i> —7 individuals	—	—	—	—	—
<i>Merluccius merluccius</i>	—	—	—	—	—
<i>Sarda sarda</i>	—	—	—	—	—

(+): good amplification; (±): weak amplification; (—): no amplification

ably due to its phylogenetic position in clade III of Secernentea [31] like anisakid species (Ascaridida). However, *A. crassus* is in the same condition and only amplified with ANI1F/ANI1R and ANIF/ANI2R. In any case these amplifications of non-anisakid nematodes will pose no problem concerning the proposition of this work because even though there are approximately 500,000 species of nematodes, only a dozen or so commonly infect humans [32] and only a few of these have marine fish as hosts.

When working with the ANIS1F/ANIS1R primers that amplify a very small fragment (81 bp) some unexpected bands appear in the amplifying position of anisakid in the agarose gel due to remainder amplifying RNA (not destroyed by Rnase) and primer-dimer formations. These bands can lead to false positive assignment if not correctly observed. By running PCR fragments in 2.5% (or higher) agarose at 50 V until half migration and 100 V after, the obtained separation will allow the discrimination of these bands from nematode amplifications.

Sensitivity of the method

In mixtures, the “anisakid weight:sample fresh weight” ratio was always considered in order to start the PCR with sufficient amounts (≈ 0.1 ng) of DNA to detect the anisakid fraction (from 5 to 30 μ l). These ratios (sensitivity levels), in experimental mixtures, varied between 1:294 ratio and 1:96 870 ratio. So, the maximum sensitivity attained in experimental mixtures (nematodes + eviscerated fish) was in the order of 1 part of nematode DNA to $\approx 100\,000$ parts of fish DNA.

When working with aliquot sub-samples, there was always the possibility of detecting even one parasite in the whole fish, whatever its total weight (Table 1).

The ANIS65 protocol amplified the ethanol-preserved anisakid (larvae and cists) using all set of primers without problems. On the other hand, depending on the minimal quantity of anisakid DNA in mixtures, this protocol proved to be insufficient. When using the L1ANIS protocol some of the mixtures amplified. When the starting amount needed a ≈ 100 -fold amplification, PCR from PCR was done with the PCR DNA products that did not previously amplify (not-detected) by using the ANIS65 protocol. In this case almost all the contaminated samples amplified.

Sequencing

Some of the amplified fragments were sequenced and compared with the sequences in the NCBI databases in order to confirm the identification accuracy of this method. All the sequences were correctly assigned to the anisakid species that were used in each mixture.

Conclusions

It is possible to detect nematode parasite in fish or processed fisheries products by using either the three different pairs of designed primers or some combinations of

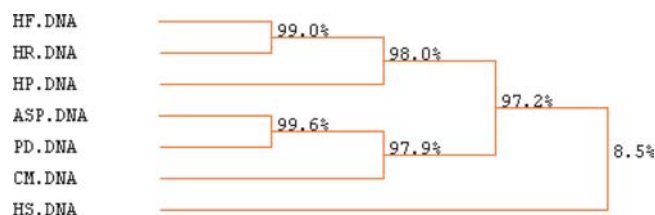


Fig. 1 Phylogenetic tree of multiple alignment (Higgins and Sharp, 1988), in the 18S nuclear DNA region, of six Anisakidae species sequences and *Homo sapiens* sequence

them. The best set of primers to be used depends on the quality of the sampled DNA. Therefore, all the primers can be used in fresh/frozen/ethanol-preserved fish. When working with processed fish products, that have been subjected to conditions that degrade DNA (such as thermal or pressure treatments) the ANI1F/ANIR and ANIF/ANI2R primers must be exclusively used, due to the small size of the amplified fragments (81 and 170 bp), respectively.

The PCR protocols worked well alone or associated. When DNA from negative results (not-detected DNA) obtained with L1ANIS PCR was submitted to a second PCR with ANIS65 protocol all the contaminated samples amplified. This happens because ethidium bromide staining has a limited level of detection in agarose, other methods like acrilamide gels and silver staining were not used because they were too expensive for routine work. With agarose method we detected DNA from anisakidae in a mixture nematode + fish in the proportion of 1: $\approx 100\,000$.

The possibility of nematode detection in biopsies from anisakiasis patients could not be tested, but regarding the great divergence (Fig. 1) found between *Homo sapiens* 18S gene and Anisakidae species 18S gene, in databases, the detection should be feasible.

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