

Identification of *Anisakidae* Larvae at the species level by PCR/RFLP

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1. AIM AND FIELD OF APPLICATION

To identify the species or genotype of single *Anisakidae* larvae preserved in ethanol by a PCR/RFLP analysis. This method can be applied to larvae collected from human biopsies or from tissues of animal origin.

2. PRINCIPLE OF THE METHOD

The PCR is a molecular biology technique that allows for the amplification of specific nucleic acid fragments, of which the initial and terminal nucleotide sequences are known (oligonucleotide pair). If a species (or genotype) has its own characteristic DNA portion, due to its composition and/or dimension, it is possible to choose an oligonucleotide pair allowing for its amplification. The PCR amplification is characterized by a high sensitivity and specificity.

It is possible to combine the “standard PCR” with the “Restriction Length Fragment Polymorphism” (RLFP), that means the analysis of DNA restriction fragments. The technique allow to distinguish PCR fragments of comparable length by enzymatic digestion with one or more endonucleases, enzymes able to cut DNA by recognition of short and specific oligonucleotide sequences. In our case it is possible to amplify the same portion of DNA from different species and then distinguish them based on the size of restriction DNA fragments.

The larval stage of nematodes of the *Anisakidae* family parasitize fishes, cephalopods and shrimps and the *Anisakidae* adult worm parasitize fishes, marine birds and mammals. The larvae of the genus *Anisakis* and *Pseudoterranova* are responsible, if ingested by humans, of the disease known as anisakiasis. Larvae obtained from human patients or infected hosts, can be morphologically identified, however larvae of the genus *Anisakis* and *Pseudoterranova* cannot be distinguished. Molecular methods based on the PCFR/RFLP allow the identification at the species level of single larvae of *Anisakis* spp. and *Pseudoterranova* spp., in particular seven species of *Anisakis*, *A. simplex* sensu-stricto (ss), *A. pegreffii*, *A. simplex* C, *A. ziphidarium*, *A. physeteris*, *A. typica*, *Anisakis* sp. A and *Pseudoterranova* spp (*P. decipiens* o *P. krabbei* o *P. bulbosa*). The species of *Anisakis*, *Pseudoterranova*, *Contracaecum* and *Hysterotilacium* differ in the composition and/or size in the DNA sequence of the locus ITS (Internal Transcribed Spacer), allowing the unambiguous identification of all epidemiologically relevant anisakidae.

The size of the fragments, expressed in nucleotide base pair number, produced by the amplification are shown in Table A. In Table B are reported the ITS digestion fragments obtained by enzymatic digestion, with the specified enzymes.

Table A - Size of the ITS fragments (expressed in base pairs) for each species

<i>A. pegreffii</i>	<i>A. simplex</i> s.s.	<i>A. simplex/pegreffii</i> hybrid	<i>A. simplex</i> C	<i>A. ziphidarium</i>	<i>A. physeteris</i>	<i>A. typica</i>	<i>A. sp.A</i>	<i>Pseudoterranova</i> spp. (<i>P. decipiens</i> s.s.)	<i>Hysterotilacium</i> spp (<i>H. aduncum</i>)	<i>C. rudolphii</i> (A, B, C)	<i>C. osculatum</i>
951	951	951	953	931	898	956	926	906	1031	978	964

Table B - Size of the expected size of ITS digestion fragments for each species (base pairs), for each enzyme, distinguished by electrophoresis

Species	PCR fragments of ITS amplicon obtained by restriction enzyme	
	<i>HinfI</i>	<i>HhaI</i>
<i>A. pegreffii</i>	34, 67, 235, 284, 331	419, 532
<i>A. simplex</i> ss	34, 67, 235, 615	419, 532
<i>A. simplex/pegreffii</i> ibrido	34, 67, 235, 284, 331, 615	419, 532
<i>A. simplex</i> C	34, 67, 237, 615	142, 279, 532
<i>A. ziphidarium</i>	34, 273, 292, 332	413, 518
<i>A. physeteris</i>	34, 241, 263, 360	385, 513
<i>A. typica</i>	34, 328, 594	103, 153, 180, 212, 308
<i>A. sp A</i>	34, 269, 288, 335	137, 272, 517
<i>Pseudoterranova</i> s.p.p. (<i>P. decipiens</i> s.s.)	35, 179, 693	413, 493
<i>Hysterotilacium</i> spp (<i>H. aduncum</i>)	348, 683	48, 74, 149, 184, 269, 307
<i>Contracaecum rudolphii</i> (A, B, C)	3, 49, 89, 320, 492	70, 126, 143, 240, 374
<i>Contracaecum osculatum</i>	463-501	212, 375, 377

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4. DEFINITIONS

ITS (Internal Transcribed Spacer), interspaced sequence the nuclear ribosomal gene including the ITS-1, the 5.8S gene and ITS-2 gene with the addition of 70 bp of the 28S gene

Oligonucleotide, short sequence (15/30 nucleotide bases) used to amplify a DNA specific fragment

SetA, mix of 2 oligonucleotide base pairs amplifying ITS from each species.

Positive control for the DNA extraction, a reference larva analysed in the same working session of test samples, to verify the efficacy of the DNA extraction session

DNA/larva, DNA extracted from a single larva

Positive control for the amplification, a reference DNA; this control is used in the amplification session to verify the efficacy of the PCR

Negative control for the amplification, reagent grade water; this control is used in the amplification session to verify the absence of contamination in the PCR reaction

PCR, Polymerase Chain Reaction

Restriction Enzyme. Restriction endonucleases are enzymes of bacterial origin able to cut DNA at specific site, that are sequences of 4-8 nucleotides different for each enzyme, allowing the DNA fragmentation in a reproducible and specific manner. Endonucleases cut inside to the DNA chain. Enzyme concentration is measured as “enzymatic units” (U). In this case 1U correspond to the amount of enzyme needed to completely digest 1 µg of DNA.

The definitions and terminology used in the ISO 22174 standard are applied in the present protocol.

5. DEVICES

Stereo microscope, magnification 60÷100x

Bench top centrifuge for 1.5 mL tubes, minimum 10,000xg

Freezer ≤-15°C

Thermoblock with vibration, temperature range 25÷100°C

Magnetic separation stand

PCR thermocycler

Refrigerator, temperature range 1-8°C

Qiaxcel, capillary electrophoresis system.

Alternative equipment to perform horizontal electrophoresis:

Horizontal electrophoretic apparatus

Analytical balance, readability 0.1 g

UV transilluminator

Digital imaging system

Adjustable volume pipettes, volume range: 1-10 µL, 2-20 µL, 20-100 µL, 50-200 µL, 200-1000 µL

Analytical grade water system production

Vortex

Orbital shaker

6. REAGENTS AND CHEMICALS

6.1 Reagents and chemicals extraction protocol A

Dithiothreitol (DTT), commercial product 1M. Store refrigerated according to manufacturer's recommendations.

Proteinase K (PK), commercial product 18mg/ml. Store refrigerated according to manufacturer's recommendations.

Store frozen ($T \leq -15^{\circ}\text{C}$) PK and DTT and/or their aliquots.

Incubation buffer IB+. Commercial solution: Tissue and Hair Extraction Kit, Promega. Once prepared, label the solution with "IB+". Store according to the manufacturer's recommendations. Store incubation buffer in a refrigerator (T range $1 \div 8^{\circ}\text{C}$).

Lysis buffer LB+. Commercial solution: DNA IQ™ System kit, Promega. Once prepared, label the solution with "LB+". Store according to the manufacturer's recommendations. Store lysis buffer at room temperature.

Paramagnetic resin. Commercial suspension: DNA IQ™ System kit, Promega. Store at room temperature.

Washing buffer. Commercial solution: Tissue and Hair Extraction Kit, Promega. Once prepared, label the solution with "WB+". Store at room temperature.

Eluting buffer. Commercial solution: Tissue and Hair Extraction Kit, Promega. Store at room temperature.

6.2 Reagents and chemicals extraction protocol B

Phosphate-buffered saline (PBS),

KH ₂ PO ₄	0.34 g
Na ₂ HPO ₄	1.21 g
NaCl	8.0 g
distilled and deionized water	up to 1000 mL

Add the components specified above in about 750 mL of distilled and deionized water. Stir with magnetic stirrer until completely dissolved. Check the pH (7.3 ± 0.2) and make up to volume. Store at room temperature. Stability: 6 months.

Dithiothreitol (DTT), commercial product 1M. Store refrigerated according to manufacturer's recommendations.

Proteinase K (PK), commercial product 18mg/ml. Store refrigerated according to manufacturer's recommendations.

Store frozen ($T \leq -15^{\circ}\text{C}$) PK and DTT and/or their aliquots.

RLT, lysis buffer, commercial solution Qiagen. Commercial solution, store according to the manufacturer's recommendations.

Lysis solution, mix 16 µL PK 18 mg/mL, 8 µL DTT 1M and 56 µL PBS.

MagAttract Suspension G, commercial suspension, Qiagen, containing paramagnetic resin. Store according to the manufacturer's recommendations.

Soluzione RLT, prepare, according manufacturer's recommendations, by mixing 200 μL RLT and 5 μL MagAttract Suspension G

AW1, washing buffer, Qiagen. Commercial solution, store according to the manufacturer's recommendations.

RPE, washing buffer, Qiagen. Commercial solution, store according to the manufacturer's recommendations.

AE, elution buffer, Qiagen. Commercial solution, store according to the manufacturer's recommendations.

6.3 Reagents and chemicals for following steps

2xPCR master mix. Store according to the manufacturer's recommendations, even after aliquots are prepared.

SetA. The oligonucleotide mixture used for the PCR; the mixture is obtained combining an equal volume of the oligonucleotides NC5 and NC2 8 diluted to 20 pmol/ μL in reagent grade water or Milli Q. The final concentration of each oligonucleotide corresponds to 10 pmol/ μL ; 100 μL aliquots are prepared and stored frozen ($T \leq -15^\circ\text{C}$) up to 10 years.

Oligonucleotides. Commercial preparation (*Table C*); the lyophilized products is reconstituted with reagent grade water or Milli Q, according to the manufacturer's recommendations, at a concentration of 100 pmol/ μL ; the lyophilized product can be stored frozen ($T \leq -15^\circ\text{C}$) for up to 20 years; the reconstituted product can be stored frozen ($T \leq -15^\circ\text{C}$) up to 10 years.

Table C – Oligonucleotides present in the setA, their codes and amplified nucleotide sequences.

Oligonucleotide sequences	Code	Amplified gene
5'-GTAGGTGAACCTGCGGAAGGATCATT-3' 5'-TTAGTTTCTTTTCTCCGCT-3'	NC5 NC2	ITS

QIAxcel DNA High Resolution kit. Commercial product to be used with QIAxcel. It contains high-resolution gel cartridge and buffers for sample preparation and analysis. Store according to manufacturer's recommendations

Alignment marker 15-600 bp (Alignment marker with 15 bp and 600 bp fragments). Commercial product to be used with QIAxcel. Store according to manufacturer's recommendations.

Alignment marker 15-1000 bp (Alignment marker with 15 bp and 1 kb fragments). Commercial product to be used with QIAxcel. Store according to manufacturer's recommendations.

DNA size marker. Commercial product to be used with QIAxcel. Store according to manufacturer's recommendations.

Milli-Q grade water.

Reference Larvae. Larvae, or its portion, of Anisakidae characterized at species/genotype level stored in ethanol (95-99%). Store frozen ($T \leq -15^\circ\text{C}$) for up to 10 years.

Reference DNA. Genomic DNA purified from a reference larva. Store frozen ($T \leq -15^\circ\text{C}$) for up to 10 years.

Restriction enzymes HinfI and HhaI. Commercial products suitable for DNA enzymatic digestion. (i.e. New England Biolabs, HinfI, cod. R0155L; HhaI, cod. R0139L). Store according to manufacturer's recommendations. The oligonucleotide sequence recognized by each enzyme is reported in *Table D*.

Table D - Oligonucleotide sequence recognized by the reported enzyme

Restriction Enzyme-	Target sequence
HinfI/	5'...GANTC...3' 3'...CTNAG...5'

HhaI	$ \begin{array}{c} 5' \dots \text{GCGC} \dots 3' \\ 3' \dots \text{CGCG} \dots 5' \end{array} $
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Restriction enzyme buffers. Commercial products suitable for DNA enzymatic digestion with defined pH and saline concentration. The buffers are commonly sold together with the corresponding restriction enzyme. Store according to manufacturer's recommendations.

Reagents reported below are required only in case agarose gel is used

Loading buffer. Commercial product allowing DNA molecule electrophoresis to be performed. Store according to the manufacturer's recommendations.

Agarose and high resolution agarose. Commercial products suitable for performing DNA molecule electrophoresis. The high resolution agarose is suitable for the analysis of small DNA fragments (25-700 bp), improving their separation in gel electrophoresis. Store at room temperature for up to 24 months.

TAE solution 50x. Commercial product (2M Tris-acetate, 50mM EDTA, pH 8.2–8.4 at 25°C). Store at room temperature according to manufacturer's recommendations

TAE solution 1x. 1000 mL preparation: take 20 mL of the 50x solution and bring to 1000 mL with water. Store at room temperature for up to 1 month.

DNA intercalating agent. Commercial product able to fit in the DNA double helix, used to view the amplification product on agarose gel. Store according to the manufacturer's recommendations.

L50. Commercial product containing markers for DNA molecular weight multiple of 50 bp. All commercial products containing molecules of multiples of 50 bp within the 50-1000 bp range can be used. Store refrigerated according to manufacturer's recommendations.

L1000. Commercial product containing markers for DNA molecular weight up to 1000 bp. All commercial products containing molecules of within the 50-1000 bp range can be used. Store refrigerated according to manufacturer's recommendations.

7. PROCEDURE

7.1 Sample preparation

- Test samples are inspected to verify the presence of larvae and the preservation conditions.
- The ethanol containing the larvae is transferred into a Petri dish and observed under the stereo microscope. A maximum of three larvae are collected and placed in 1.5 mL conical tubes, one larva in each tube. Excess ethanol is removed and the minimum volume is left.
- Spin tubes containing larvae at maximum speed for a few seconds.
- Store the tubes frozen ($T \leq -15^{\circ}\text{C}$). Under these conditions, larvae can be stored for the DNA extraction for up to 10 years.

7.2 Method

Perform DNA extraction with one of the two following alternative protocols, reported as **Protocol A** and **Protocol B**

7.2.1 DNA extraction from one single larva (Protocol A)

If not otherwise specified, the procedure is carried out at room temperature.

Each working session requires the DNA extraction of a reference larva identified as "positive control for the extraction".

Before starting the procedure, prepare a sufficient volume of the IB+ and LB+ solutions according to the manufacturer's recommendations.

- Centrifuge the tubes containing the larvae to be identified at maximum speed for a few seconds.
- Add 100 μL of IB+.
- Incubate at 55°C for 30-60 min in the thermoblock. During incubation, shake at 1,400 vibrations/min.

- d) Centrifuge, as in point “a”.
- e) Add 200 µL of LB+.
- f) Add 10 µL of paramagnetic resin (6.3) after resuspending it by vortexing.
- g) Incubate for 5-10 min at 25°C in the thermoblock. During incubation, shake at 1,400 vibrations/min.
- h) Place the tubes in the magnetic separation stand and wait for 30-60 sec, so that the paramagnetic resin particles are blocked by the magnetic field on the tube wall.
- i) Discard the liquid phase by aspirating, avoiding the dislodging of the resin particles.
- j) Add 100µL of LB+ and resuspend the resin particles by vortexing.
- k) Place the tubes in the magnetic separation stand, as in point “h”.
- l) Discard the liquid phase by aspirating.
- m) Add 100 µL of WB+ 1x and resuspend the resin particles by vortexing.
- n) Place the tubes in the magnetic separation stand, as in point “h”.
- o) Discard the liquid phase by aspirating.
- p) Repeat the washing step, from “m” to “o”, with WB+ three times.
- q) After the last washing, leave the tubes open to let the resin particles dry for 15-20 min.
- r) Add 100µL of the eluting buffer and gently resuspend the resin particles, do not vortex.
- s) Incubate at 65°C for 5 min. During incubation, shake at 1,400 vibrations/min.
- t) Place the tubes in the magnetic separation stand, as in point “h”.
- u) Collect the liquid phase (about 90-100 µL) and transfer it to a 1.5 mL tube.
- v) The resulting extract is defined as “DNA/larva” and stored frozen ($T \leq -15^{\circ}\text{C}$). Under these conditions, it can be stored for up to 10 years.

7.2.2 DNA extraction from one single larva (Protocol B)

If not otherwise specified, the procedure is carried out at room temperature.

Each working session requires the DNA extraction of a reference larva identified as “positive control for the extraction”.

Before starting the procedure, prepare a sufficient volume of the IB+ and LB+ solutions according to the manufacturer’s recommendations.

- a) Centrifuge the tubes containing the larvae to be identified at maximum speed for a few seconds.
- b) Transfer one larva or a fraction of larva in a tube.
- c) Add 80 µL of lysis solution.
- d) Incubate at 55°C for 30-40 min in the thermoblock. During incubation, shake at 900 vibrations/min.
- e) Add 200 µL of RLT solution added with 5 µL of paramagnetic resin.
- f) Incubate for 3 min at room temperature. During incubation, shake at 1,400 vibrations/min.
- g) Place the tubes in the magnetic separation stand and wait for 30-60 sec, so that the paramagnetic resin particles are blocked by the magnetic field on the tube wall.
- h) Discard the liquid phase by aspirating, avoiding the dislodging of the resin particles.
- i) Add 100 µL of AW1 and resuspend the resin particles by vortexing.
- j) Incubate for 1 min at room temperature. During incubation, shake at 1,400 vibrations/min.
- k) Discard the liquid phase by aspirating.
- l) Add 100 µL of RPE and resuspend the resin particles by vortexing.
- m) Incubate for 1 min at room temperature. During incubation, shake at 1,400 vibrations/min.
- n) Place the tubes in the magnetic separation stand, as in point “g”.
- o) Discard the liquid phase by aspirating.
- p) Repeat the washing step, from “l” to “o”.
- q) Add 80 µL of the AE buffer and gently resuspend the resin particles, do not vortex.

- r) Incubate 3 min at room temperature. During incubation, shake at 1,400 vibrations/min.
- s) Place the tubes in the magnetic separation stand, as in point "g".
- t) Collect the liquid phase (larval DNA) and transfer it to a new 1.5 mL tube.
- u) The resulting extract is defined as "larval DNA" and stored frozen ($T \leq -15^{\circ}\text{C}$). Under these conditions, it can be stored for up to 10 years.

7.2.3 PCR amplification

Unless otherwise clearly stated, store tubes on ice; use tips with barrier and wear disposable gloves.

At each working session, use a positive and a negative amplification control. Use reference DNA as positive control and water as negative control.

- a) Thaw DNA/larva, 2x PCR MasterMix, SetA, and positive amplification controls.
- b) Mark with a progressive number an adequate number of 0.2 mL PCR tubes.
- c) Prepare an adequate cumulative volume of the amplification mix. Evaluate the volume on the basis of a single sample amplification mix (*Table E*) and of the total number of samples plus two (1 for the positive amplification control and 1 for the negative one).

Table E – single sample amplification mix: components and volumes

2x PCR MasterMix	25 μL
H ₂ O	22 μL
SetA	1 μL
Total	48 μL

- d) Mix the amplification mix by vortexing and centrifuge at maximum speed for a few sec.
- e) Transfer 48 μL of the cumulative amplification mix to each PCR tube (point "b").
- f) Add 2 μL of the larval DNA to be tested to each tube.
- g) Close the tubes, mix by vortexing and centrifuge at maximum speed for a few sec.
- h) Start the amplifying cycle (*Table F*) on the thermocycler device; wait for the temperature to reach 95°C and insert tubes in the thermocycler by pausing the instrument.

Table F – amplification cycle

Pre-denaturation #	2 min/ 95°C
Amplification	30 s/ 95°C 30 s/ 55°C 75 s/ 72°C
Number of cycles	35
Final extension	7 min/ 72°C

pre-denaturation length may vary according to the Master Mix manufacturer's recommendations

- i) At the end of the amplification phase, centrifuge the tubes at maximum speed for a few sec.
- l) Keep tubes on ice or refrigerated until starting electrophoresis.

7.2.4 Display of amplification results

- a) Switch on the Qiaxcel instrument and the relative Qiaxcel ScreenGel management software on the PC;
- b) access the "Process Profile" panel; indicate under "Cartridge Type" the option "DNA HighRes"; indicate the desired profile and Experiment Directory;
- c) move the tube tray to the "Access Position" by selecting the "Load Position" item from the "Status Information" panel;
- d) insert in MARKER1 position the 12 tubes containing at least 10 μL of the chosen "Alignment Marker" (6.10); then return the tray to the initial position by selecting the "Park Position" from the "Status

Information" panel;

- e) starting from the "A" row, position the samples to analyze (minimum volume 10 µL) in rows of 12 tubes. If the samples to be analyzed are not enough to complete the row of 12, add the required number of tubes containing the QX DNA dilution buffer (minimum volume 10 µL) supplied with the QIAxcel DNA High Resolution kit.
- f) for each analysis session (which may include up to a maximum of 8 runs of 12 samples), include a tube containing the DNA size marker.
- g) in "Run Parameters" set the option 0M500 under "Method"; select the runs on the virtual plate in the "Sample Row Selection" side panel.
- h) in "Sample Selection" set the run parameters as follows: "Plate ID": PCR + data "Alignment Marker": 15bp - 500bp. In "Sample Information" enter the names of the samples in the corresponding boxes.
- i) in "Run Check" check that all the selected rows are occupied by tubes containing PCR products to be analyzed or QX DNA dilution buffer and that the alignment Marker has been loaded, then select the appropriate boxes; finally select "Run".
- j) visualize the results by selecting the "Absolute migration time" mode from the "Image options" menu and process the data with the "Start analysis" command.
- k) scroll through the electropherogram of each sample to check peaks above the highest band of the alignment marker.
- l) print the results to archive.
- m) at the end of the run, close the program and turn off the instrument.

Alternative protocol to perform horizontal electrophoresis:

- i) Assemble the electrophoresis apparatus according to the manufacturer's recommendations. For the gel preparation, use a comb suited for the number of samples.
- ii) Weigh 1 gram of agarose in 100 mL TAE 1x in a glass beaker.
- iii) Gently resuspend the powder by rotation.
- iv) Boil the agarose suspension for 30 sec. If the solution is not homogeneous, continue to boil for another 30 sec.
- v) Restore with water the volume lost by boiling.
- vi) Allow the agarose solution to cool.
- vii) Before it solidifies (at about 47°C), add DNA intercalating agent according to the manufacturer's instructions.
- viii) Shake gently to dissolve uniformly the DNA intercalating agent and pour the agarose in the gel tray previously prepared (point "a").
- ix) Wait for the gel to solidify, which requires at least 30 min.
- x) Place the tray with the gel in the electrophoresis apparatus.
- xi) Cover the gel with TAE 1x buffer and gently pull out the comb.
- xii) The first or last well are loaded with 15 µL of the L1000 solution.
- xiii) Load in each well 10 µL of the amplification product (point 7.2.1 "n"), adding in each tube the loading buffer, if not present in the PCR master mix.
- xiv) Connect the electrophoresis apparatus with the power supply and set 10 V/cm of gel.
- xv) Run the gel for about 30 min or until the fastest dye, contained in the loading buffer, reaches a distance of 1 cm from the gel border.
- xvi) After 30 min, switch off the power supply, place the gel under UV illumination, or imaging system, and check the band separation. The electrophoresis run is adequate if it is possible to easily distinguish all bands of molecular weight marker ranging from 50 to 1000 bp. If the separation is incomplete, continue the run.
- xvii) At the end of the run, transfer the gel to the imaging system and print the result.

7.2.5 Result interpretation

The amplification test is considered valid if:

- the amplification of the positive control shows an amplification product as in *Table A*;
- the amplification of the negative control does not show any amplification product or, eventually, only bands related to unused oligonucleotides and/or primer dimer;
- the positive control of the extraction product shows an amplification product as in *Table A*;
- the test sample shows an amplification product between 800 and 1000 bp (as in *Table A*);

Proceed to the next step of the protocol if the amplification products are between 800 and 1000 bp

7.2.6 Enzymatic DNA digestion with endonucleases

Unless otherwise clearly stated, store tubes on ice; use tips with barrier and wear disposable gloves.

At each working session independent digestions with HinfI and HhaI enzymes are performed. The proper digestion will be checked by contemporary digestion of a positive control represented by the PCR amplification product of the reference DNA.

The procedure uses restriction enzymes at the initial concentration of 10-20 U/μl and 10x concentrated restriction enzyme buffers. In case of different concentration adjust the protocol according to the manufacturer's instruction.

- Thaw PCR products and 10X restriction enzyme buffers.
- Mark with a progressive number an adequate number of 0.2 mL tubes.
- Prepare an adequate cumulative volume of the enzymatic digestion mix for each restriction enzyme. Calculate the volume on the basis of a single sample enzymatic digestion mix (*Table G*) and of the total number of samples plus the positive control.

Table G – Enzymatic digestion mix for a single sample: components and volumes

10x buffer	1.5 μL
Restriction enzyme	5U (0.25-0.5 μL)
H ₂ O	3-3.25 μL
Total	5 μL

- Mix each enzymatic digestion mix by vortexing and centrifuge at maximum speed for a few sec.
- Transfer 5 μL of the cumulative amplification mix to each tube (point "b").
- Add to each tube 10 μL of the PCR product to be tested.
- Close the tubes, mix by vortexing and centrifuge at maximum speed for a few sec.
- Incubate the tubes at 37°C for 2 hours in the thermoblock without shaking.
- At the end of the restriction phase, centrifuge the tubes at maximum speed for a few sec.
- Keep tubes on ice or refrigerated until starting electrophoresis.

7.2.7 Result display

- Follow paragraph 7.2.4 from point a) to j) using the marker "Alignment Marker" 15bp – 1000bp
- Visualize the results by selecting the "Absolute migration time" mode from the "Image options" menu and process the data with the "Start analysis" command
- Scroll through the electropherogram of each sample to check and identify the peaks relating to the alignment markers
- Process again the data with the "Start analysis" command in "Relative migration time" mode by selecting the corresponding option
- Print the results

vi) At the end of the run, close the program and turn off the instrument

Alternative protocol to perform horizontal electrophoresis, refer to paragraph 7.2.3 from point i) to point xvii).

Point ii) 3 grams of agarose high resolution are used.

7.2.8 Result interpretation

The size of the amplification bands revealed by the electrophoresis is evaluated by their comparison with the reference molecular weight DNA size marker” o di L50 and with the positive control of enzymatic digestion.

The enzymatic digestion test is considered valid if the digestion of the positive control shows a profile of bands product in accordance with *Table B*

The species identification is made after enzymatic digestion of the amplified fragments comparing the size of the band(s) produced by the sample(s) with those shown in *Table B*.

In case the sample shows an unexpected band, the sample will not processed further and the identification will not be possible, the result of the test is expressed as “undeterminable species”.

8. RESULTS

The results are expressed as follows:

If the digestion profile with *HinfI* is 34, 67, 235, 615bp and with *HhaI* is 419, 532bp, the sample is identified as *A. simplex* ss.

If the digestion profile with *HinfI* is 34, 67, 235, 284, 331bp and with *HhaI* is 419, 532bp, the sample is identified as *A. pegreffi*.

If the digestion profile with *HinfI* is 34, 67, 235, 284, 331, 615bp and with *HhaI* is 419, 532bp, the sample is identified as *A. simplex/pegreffi* hybrid genotype.

If the digestion profile with *HinfI* is 34, 67, 237, 615bp and with *HhaI* is 142, 279, 532bp, the sample is identified as *A. simplex C*.

If the digestion profile with *HinfI* is 34, 273, 292, 332bp and with *HhaI* is 413, 518bp, the sample is identified as *A. ziphidarium*.

If the digestion profile with *HinfI* is 34, 241, 263, 360bp and with *HhaI* is 385, 513bp, the sample is identified as *A. physeteris*.

If the digestion profile with *HinfI* is 34, 328, 594bp and with *HhaI* is 103, 153, 180, 212, 308bp, the sample is identified as *A. typica*.

If the digestion profile with *HinfI* is 34, 269, 288, 335bp and with *HhaI* is 137, 272, 517bp, the sample is identified as *A. sp. A*.

If the digestion profile with *HinfI* is 35, 179, 693bp and with *HhaI* is 413, 493bp, the sample is identified as *Pseudoterranova* spp.

If the digestion profile with *HinfI* is 348, 683bp and with *HhaI* is 48, 74, 149, 184, 269, 307bp, the sample is identified as *Hysterothylacium* spp.

If the digestion profile with *HinfI* is 3, 49, 89, 320, 492bp and with *HhaI* is 70, 126, 143, 240, 374bp, the sample is identified as *Contracaecum rudolphii* (A, B, C).

If the digestion profile with *HinfI* is 463, 501bp and with *HhaI* is 212, 375, 377 bp, the sample is identified as *Contracaecum osculatum*.

In case the digestion test was valid but the sample displays a profile of bands not comparable with those reported in *Table B*, identification at the species level will be not possible, the result of the test is expressed as “undeterminable species”.

9. CHARACTERISTICS OF THE METHOD

This method has been characterised in terms of repeatability and specificity. The results of the validation

protocol are used to confirm that the method is suitable for the expected aim and are reported in the validation report, which can be received upon request.

10. SAFETY MEASURES

This method has to be carried out only by authorized personnel. The operator should wear individual protection devices during the test performance. For general safety measures, refer to the CDC guidelines.