

## 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” (RFLP), 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 PCR/RFLP allow the identification at the species level of single larvae of *Anisakis* spp. and *Pseudoterranova* spp., in particular 7 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. krabbeii* o *P. bulbosa*). The species of *Anisakis* and *Pseudoterranova* 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 genotype	<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>Hysterothylacium</i> spp ( <i>H. auctum</i> )	<i>Contraecaecum rudolphii</i> (A, B,e C)
951	951	951	953	931	898	956	926	906	1031	978

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

Restriction enzymes	PCR fragments of ITS amplicon	
	<i>Hinfl</i>	<i>Hhal</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> hybrid genotype	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>Contraecaecum rudolphii</i> (A, B, C)	3, 49, 89, 320, 492	70, 126, 143, 240, 374

Using the PCR/RFLP technique based on the size of the ITS digestion fragments with the *Hinfl* enzyme it is possible to distinguish larvae of the genus *Anisakis*, *A. pegreffii*, *A. simplex/pegreffii* hybrid genotype, *A. ziphidarium*, *A. physeteris*, *A. typica* and *A. shupakovi* from *A. simplex* C, *A. simplex* sensu-stricto, *Pseudoterranova*, *Contraecaecum* and *Hysterotilacium*. *A. simplex* C can be distinguished from *A. simplex* s.s. by ITS digestion fragments with *Hhal* enzyme

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#### 4. Definitions

**ITS** (Internal Transcribed Spacer 1), interspaced sequence the nuclear ribosomal gene including the ITS-1, the 5.8S gene, 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.

**Reference larvae**, larvae of *Anisakis pegreffii* in ethanol, supplied by Dipartimento di Scienze di Sanità Pubblica "G. Sanarelli", Università Sapienza, Roma

**Reference DNA**, purified DNA from larvae of *Anisakis pegreffii*, supplied by Dipartimento di Scienze di Sanità Pubblica "G. Sanarelli", Università Sapienza, Roma

**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 efficacy of the PCR

**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 1µg of DNA.

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

## 5. Devices/instruments

- 5.1 Stereo microscope, magnification 60÷100x
- 5.2 Bench top refrigerated centrifuge for 1.5 mL tubes, minimum 10,000xg
- 5.3 Freezer ≤-15°C
- 5.4 Thermoblock with vibration, temperature range 25÷100°C
- 5.5 Magnetic separation stand
- 5.6 PCR thermocycler
- 5.7 Refrigerator, temperature range +1÷+8°C
- 5.8 Horizontal electrophoretic apparatus
- 5.9 Analytical balance, readability 0.1 g
- 5.10 UV transilluminator
- 5.11 Digital imaging system
- 5.12 Adjustable volume pipettes, volume range: 1-10 µL, 2-20 µL, 20-100 µL, 50-200 µL, 200-1000 µL
- 5.13 Analytical grade water system production, resistivity ≥ 18 Mohm/cm
- 5.14 Vortex
- 5.15 Orbital shaker

## 6. Reagents and chemicals

- 6.1 **Incubation buffer.** Commercial solution: DNA IQ™ System kit, Promega, code DC6701 or DC6700. Once prepared, label the solution with “IB+”. Store according to the manufacturer’s recommendations. Aliquotes of PK and DTT should be stored at -20°C, whereas incubation buffer should be stored at +4°C.
- 6.2 **Lysis buffer.** Commercial solution: Tissue and Hair Extraction Kit, Promega, code DC6740. Once prepared, label the solution with “LB+”. Store according to the manufacturer’s recommendations. Aliquotes of DTT should be stored at -20°C.
- 6.3 **Paramagnetic resin.** Commercial suspension: DNA IQ™ System kit, Promega, code DC6701 or DC6700. Store at room temperature.
- 6.4 **Washing buffer.** Commercial solution: Tissue and Hair Extraction Kit, Promega, code DC6740. Once prepared, label the solution with “WB+”. Store at room temperature.
- 6.5 **Eluting buffer.** Commercial solution: Tissue and Hair Extraction Kit, Promega, code DC6740. Store at room temperature.
- 6.6 **2xPCR master mix.** Store according to the manufacturer’s recommendations, even after aliquots are prepared.

- 6.7 SetA.** The oligonucleotide mixture (6.8) used for the PCR; the mixture is obtained combining an equal volume of the 2 oligonucleotides NC5 and NC2 (6.8) in TE 0.1x (6.17); the final concentration corresponds to 20 pmol/μL; 100μL aliquots are prepared and stored frozen up to 10 years.
- 6.8 Oligonucleotides.** Commercial preparation (*Table C*); the lyophilized products is reconstituted with TE 0.1x, according to the manufacturer's recommendations, at a concentration of 100 pmol/μL; the lyophilized product can be stored frozen for up to 20 years; the reconstituted product can be stored frozen up to 10 years.

*Table C – Oligonucleotides present in the setA (6.7 e 6.8), their codes and amplified nucleotide sequences.*

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

- 6.9 Loading buffer 6x.** Commercial product allowing DNA molecule electrophoresis to be performed. Store according to the manufacturer's recommendations.
- 6.10 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.
- 6.11 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
- 6.12 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.
- 6.13 Ethidium bromide solution.** Commercial product 10 mg/L. For the working condition, dilute 1:100,000; i.e. for 100 mL solution, add 1.0 μL. Store in the dark at room temperature according to manufacturer's recommendations
- NOTE: Ethidium bromide is potentially mutagenous, carcinogenic and teratogenic; wear disposable gloves and handle the solution containing this substance very carefully.
- 6.14 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.
- 6.15 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.
- 6.16 TE 1x solution.** Commercial product 10mM Tris-HCl (pH 8,0), 1mM EDTA-Na<sub>2</sub>, pH 7.9–8.1 at 25°C. Store refrigerated according to manufacturer's recommendations
- 6.17 TE 0.1x solution.** TE 1x diluted solution. For 100 mL preparation: take 10 mL from 1x solution and add 90 mL water. Filter with 0.22 μm filters and prepare aliquots of 10 mL. Store frozen for up to 24 months.
- 6.18 Milli-Q grade water.** Resistivity ≥ 18 Mohm/cm
- 6.19 Reference Larvae.** Larvae of *A. pegreffi* stored in ethanol (95-99%) supplied by Dipartimento di Scienze di Sanità Pubblica "G. Sanarelli", Università Sapienza, Roma. Store frozen for up to 10 years.
- 6.20 Reference DNA.** Genomic DNA purified from a reference larva. Reference DNA (1ng/μL) is supplied by Dipartimento di Scienze di Sanità Pubblica "G. Sanarelli", Università Sapienza, Roma, and prepared according to the protocols described in Sambrook et al. (1989). Store frozen for up to 10 years.



- 6.21 Restriction enzymes HinfI and HhaI.** Commercial products suitable for DNA enzymatic digestion. (i.e. New England Biolabs, HinfI, cod. R0155L; HhaI, cod. R0139L). Store refrigerated 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
<b>HinfI</b>	5'...G <sup>↓</sup> ANTC...3' 3'...CTNAG...5'
<b>HhaI</b>	5'...GCGC <sup>↓</sup> ...3' 3'... <sup>↓</sup> CGCG...5'

- 6.22 Restriction enzyme buffers.** Commercial products suitable for DNA enzymatic digestion with defined pH and saline concentration. The buffers are commonly sell together with the corresponding restriction enzyme. 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. If the condition are not suitable the test will not be performed.
- The ethanol containing the larvae is transferred into a Petri dish and observed under the stereo microscope. A maximum of 3 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. Under these conditions, larvae can be stored for the DNA extraction for up to 10 years.

### 7.2 Method

#### 7.2.1 DNA extraction from one single larva

- 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+ (6.1) and LB+ (6.2) 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) Add 100 µL of IB+ (6.1).
  - c) 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+ (6.2).
  - 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+ (6.2) 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 (6.4) 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+ (6.4) 3 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 (6.5) 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. Under these conditions, it can be stored for up to 10 years.

**7.2.2 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 (6.19) as positive control and water (6.17) 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 µL PCR tubes.
- c) Prepare a 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 (6.6)	25 µL
H <sub>2</sub> O	22 µL
SetA (6.7)	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

- i) At the end of the amplification phase, centrifuge the tubes at maximum speed for a few sec.
- l) Add 5.0 µL of loading buffer 6x (6.9).



- m) Vortex and centrifuge the tubes at maximum speed for a few sec.
- n) Keep tubes on ice or refrigerated until starting electrophoresis.

### 7.2.3 Result display

- a) Assemble the electrophoresis apparatus according to the manufacturer's recommendations. For the gel preparation, use a comb suited for the number of samples.
- b) Add 1 gr agarose (6.10) in 100 mL TAE 1x (6.12) in a glass beaker.
- c) Gently resuspend the powder by rotation.
- d) Boil the agarose suspension for 30 sec. If the solution is not homogeneous, continue to boil for another 30 sec.
- e) Restore with water the volume lost by boiling.
- f) Allow the agarose solution to cool.
- g) Before it solidifies (at about 47°C), add 1.0 µL of ethidium bromide solution (6.13).
- h) Shake gently to dissolve uniformly the ethidium bromide and pour the agarose in the gel tray previously prepared (point "a").
- i) Wait for the gel to solidify, which requires at least 30 min.
- j) Place the tray with the gel in the electrophoresis apparatus.
- k) Cover the gel with TAE 1x buffer (6.12) and gently pull out the comb.
- l) The first or last well are loaded with 15 µL of the L1000 solution (6.15).
- m) Load in each well 10 µL of the amplification product (point 7.2.1 "n"), respecting the progressive numbering of the tubes (point 7.2.1 "b").
- n) Connect the electrophoresis apparatus with the power supply and set 10 V/cm of gel.
- o) Run the gel for about 30 min or until the fastest dye, contained in the loading buffer (6.9), reaches a distance of 1 cm from the gel border.
- p) 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.
- q) At the end of the run, transfer the gel to the imaging system and print the result.

### 7.2.4 Result Interpretation

- The size of the amplification bands revealed by the electrophoresis is evaluated by their comparison with the reference molecular weight L1000 (6.15) and with the positive control of extraction and amplification. The visual evaluation is considered sufficient and adequate (see *Table A*).
- The amplification test is considered valid if:
  - a) the amplification of the positive control shows an amplification product as in *Table A*;
  - b) the amplification of the negative control does not show any amplification product or, eventually, only bands related to unused oligonucleotides and/or primer dimer;
  - c) the positive control of the extraction product shows an amplification product as in *Table A*;

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 be processed further and the identification will not be possible.

### 7.2.5 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 (6.21; 6.22) 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.
  - a) Thaw PCR products and 10X restriction enzyme buffers.
  - b) Mark with a progressive number an adequate number of 1.5 mL tubes.
  - c) Prepare an adequate cumulative volume of the enzymatic digestion mix for each restriction enzyme. Evaluate 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 (6.23)	1.5 μL
Restriction enzyme (6.22)	5U (0.25-0.5 μL)
PCR product	10 μL
H <sub>2</sub> O	3-3.25 μL
Total	15 μL

- d) Mix each enzymatic digestion mix by vortexing and centrifuge at maximum speed for a few sec.
- e) Transfer 5 μL of the cumulative amplification mix to each tube (point "b").
- f) Add 10 μL of the PCR product to be tested to each tube.
- g) Close the tubes, mix by vortexing and centrifuge at maximum speed for a few sec.
- h) Incubate the tubes at 37°C for 2 h in the thermoblock (5.2) without shaking.
- i) At the end of the amplification phase, centrifuge the tubes at maximum speed for a few sec.
- l) Add 3 μL of loading buffer 6x (6.9).
- m) Vortex and centrifuge the tubes at maximum speed for a few sec.
- n) Keep tubes on ice or refrigerated until starting electrophoresis.

#### 7.2.6 *Result display*

- a) Assemble the electrophoresis apparatus according to the manufacturer's recommendations. For the gel preparation, use a comb suited for the number of samples.
- b) Add 3 gr agarose (6.10) in 100 mL TAE 1x (6.12) in a glass beaker.
- c) Gently resuspend the powder by rotation and leave at 4°C in the refrigerator for 30 min.
- d) Boil the agarose suspension for 30 sec. If the solution is not homogeneous, continue to boil for another 30 sec.
- e) Restore with water the volume lost by boiling.
- f) Allow the agarose solution to cool.
- g) Shake gently to dissolve uniformly the ethidium bromide and pour the agarose in the gel tray previously prepared (point "a").
- h) Wait for the gel to solidify, which requires at least 30 min.
- i) Place the tray with the gel in the electrophoresis apparatus.
- l) Cover the gel with TAE 1x buffer (6.12) and gently pull out the comb.
- m) The first or last well are loaded with 15 μL of the L50 solution (6.14).
- n) Load in each well the enzymatic digestion product (point 7.2.1 "n"), respecting the progressive

numbering of the tubes (point 7.2.1 “b”).

- o) Connect the electrophoresis apparatus with the power supply and set 10 V/cm of gel.
- p) Run the gel for about 30 min or until the fastest dye, contained in the loading buffer (6.9), reaches a distance of 1 cm from the gel border.
- q) Stain the DNA by transferring the gel in 400 ml of ddH<sub>2</sub>O with 4 µL of ethidium bromide solution (6.13). Gently shake the gel on a orbital shaker (5.16).
- r) After 30 min, remove the staining solution and replace it with 400 ml of ddH<sub>2</sub>O. Gently shake the gel on a orbital shaker (5.16) for 15 min. Repeat the washing step.
- s) After 30 min, place the gel under UV illumination 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.
- t) At the end of the run, transfer the gel to the imaging system and print the result.

### 7.2.7 Result Interpretation

The size of the amplification bands revealed by the electrophoresis is evaluated by their comparison with the reference molecular weight L50 (6.14) and with the positive control of enzymatic digestion. The visual evaluation is considered sufficient and adequate (see *Table A*).

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.

## 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 *Hysterotilacium* 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).

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 considered "impossible".

### 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.