European Union Reference Laboratory for Parasites (EURL-P; food safety)

DEPARTMENT INFECTIOUS DISEASES

Unit of Foodborne and Neglected Parasitic Diseases

10th Proficiency Test on "Molecular identification of Anisakid nematodes at the species level" – PT07

XXI Workshop of National Reference Laboratories for Parasites
Rome, 28th-29th October 2026

Marco Lalle, Antonio Di Grazia, **Francesco Celani**

Aim of the Proficiency Test

Evaluation of laboratories competence in molecular identification of anisakidae nematodes species



The PT is accredited according to the ISO 17043

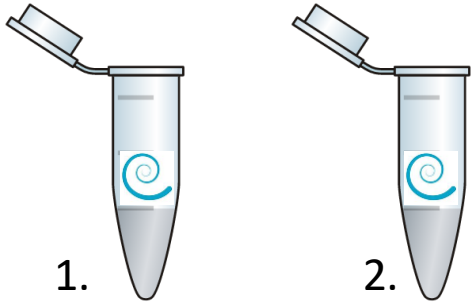
Timeline

Call for participation	27th January
Deadline for subscription	20th February
Pt sending	16th March
Deadline for submitting results	17th April
Final report published on the EURLP webpage	29th May



PT panel

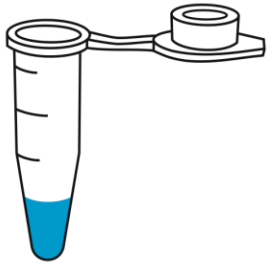
The single PT panel consisted of **4** items:



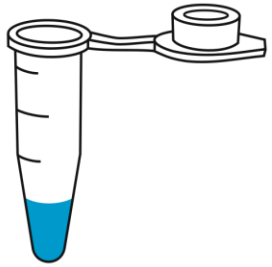
1. *Anisakis pegreffii*

2. *Anisakis simplex s.s.*

L3 larva fragments



3. *Hysterotilacium spp*



4. *A. pegreffii/A. berlandi (former A. simplex C) hybrid genotype*

Extracted DNA



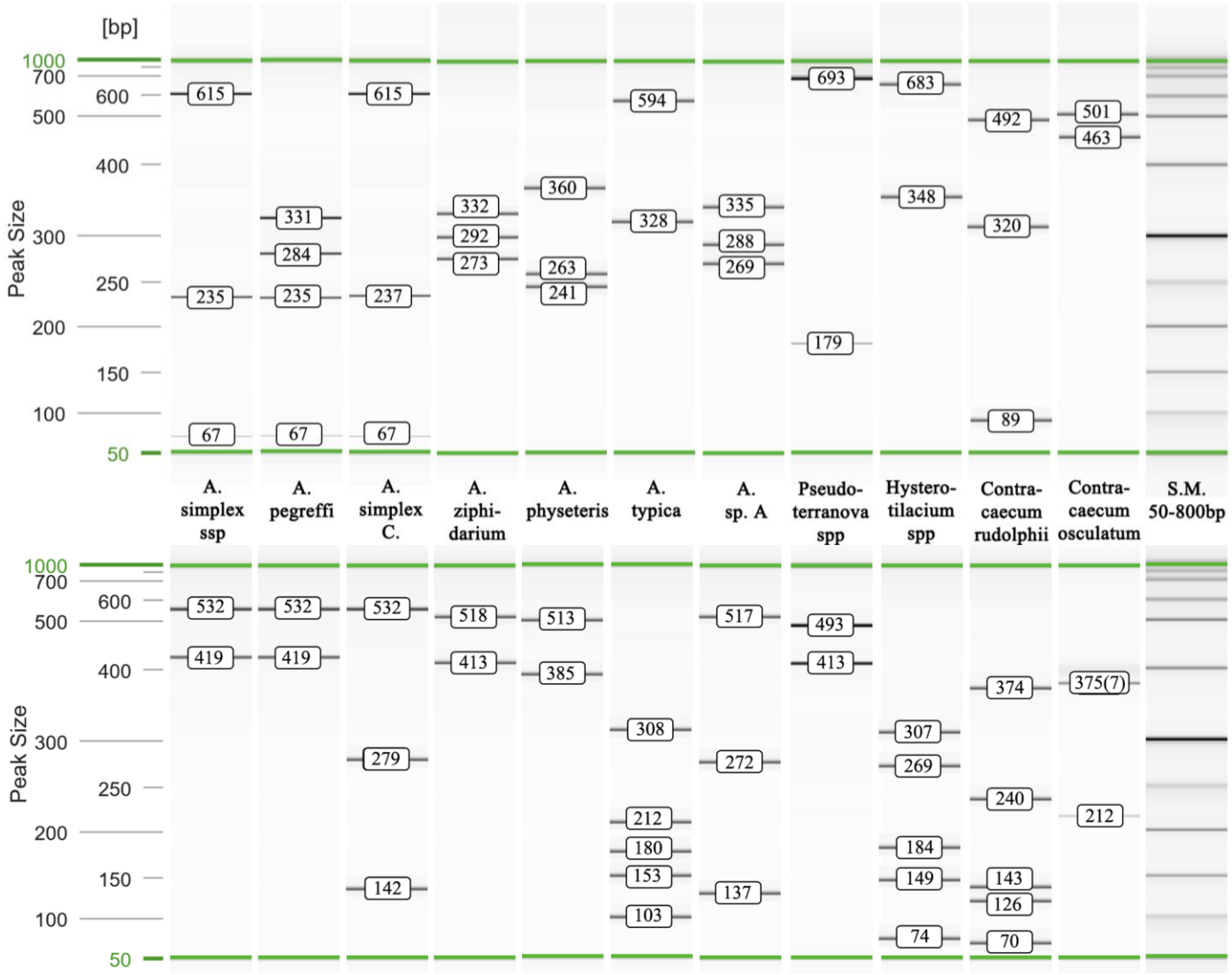
All larvae have been previously identified at species level by analysing one of their fragments by the EURLP method “Identification at species level of parasites of the family *Anisakidae* by PCR/RFLP”, MI04, same for DNA.

Identification at species level of parasites of the family Anisakidae by EURLP method PCR/RFLP

1) PCR for ITS target (~900bp) on extracted DNA

2)

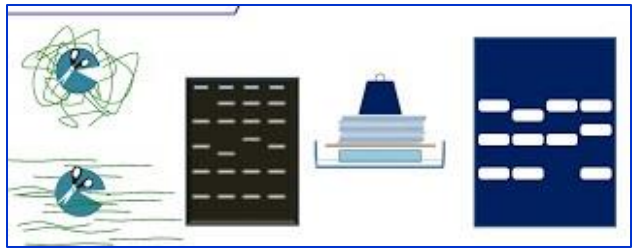
Restriction Enzyme	Target sequence
HinfI	5′...G▼ANTC...3′ 3′...CTNA▲G...5′
HhaI	5′...GCG▼C...3′ 3′...C▲GCG...5′



Molecular identification

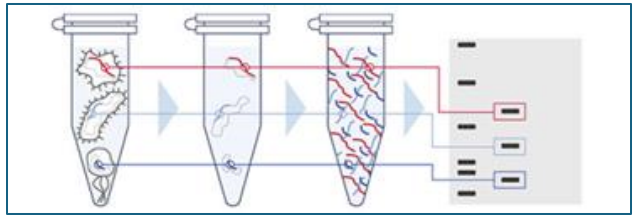
Recommended methods:

A) "Identification at species level of parasites of the family Anisakidae by PCR/RFLP"
MI04



A. simplex ss.
A. pegreffi
A. simplex/pegreffi hybrid
A. simplex C (A. berlandi)
A. ziphidarium
A. physeteris
A. typica
A. sp. A
Pseudoterranova spp
Hysterotilacium spp
Contracaecum rudolphii (A, B, C)   ***Contracaecum osculatum***

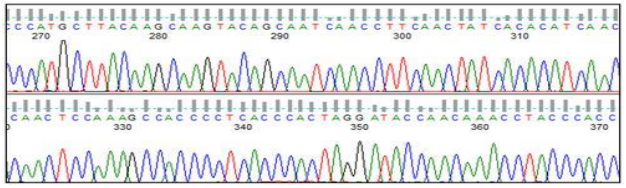
B) "Identification of Anisakidae Larvae at the species level by multiplex PCR"
MI10



A. pegreffii
A. simplex s.l.   *(incl. A. simplex/pegreffii hybrid)*
A. physeteris   *(incl. A. brevispiculata and A. paggiae)*
A. typica
Pseudoterranova spp
Hysterotilacium spp (H. aduncum)
Contracaecum rudolphii (A, B, C)   ***Contracaecum osculatum***

OR:

C) Any other suitable molecular method performed by the participant laboratory (i.e. PCR and sequencing)



Evaluation criteria

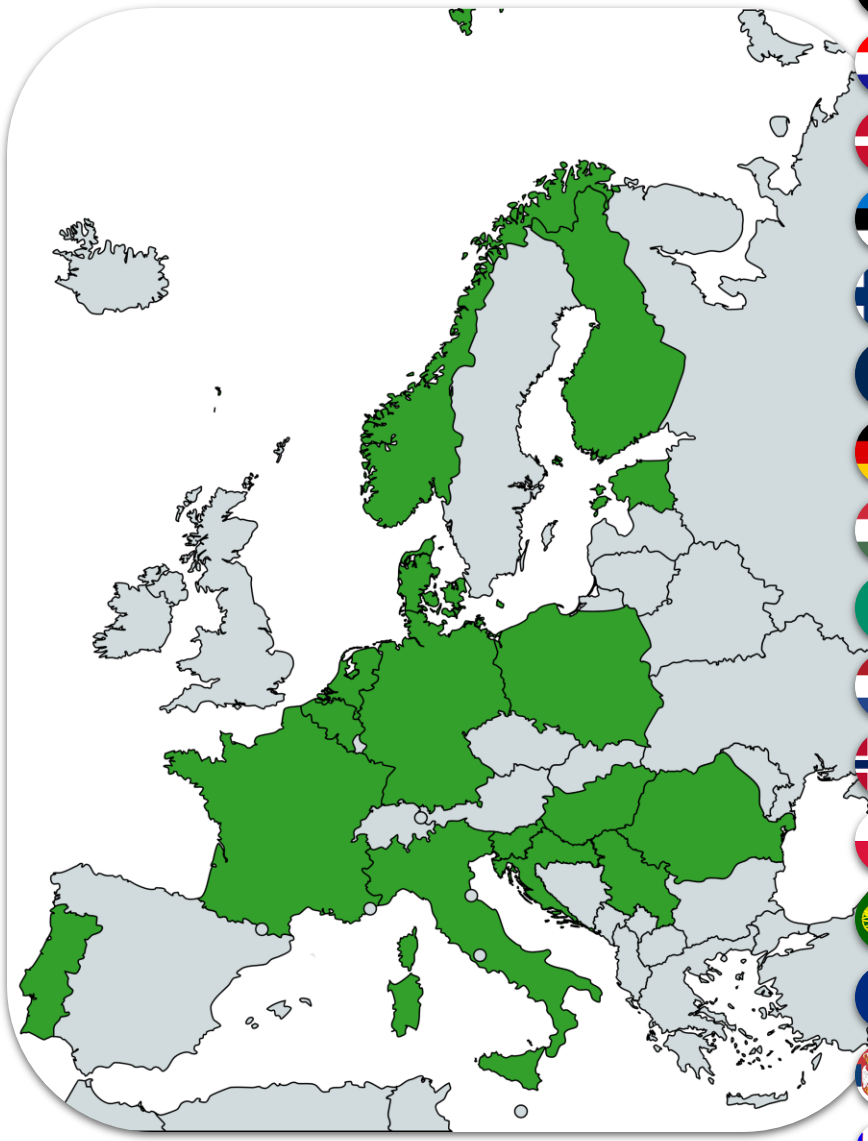
The PT evaluation is only **qualitative** and no statistical analysis of the results is applied.

For each PT item the result is **“correct”** or **“incorrect”** according to the correct or incorrect identification

- **The PT is considered “positive” if no “incorrect” results were obtained**
- **The PT is considered “negative” if at least one “incorrect” result was obtained**

PT participants

16 participants in 2026



- Belgium, NRLP ITPM
- Croatia, Veterinary Institute
- Denmark, NRL SSI
- Estonia, NRL LABRIS
- Finland, NRLP FINPAR
- France, NRL ANSES
- Germany, NRLP, Max Rubner-Institut
- Hungary, Food Chain Safety Laboratory Directorate
- Italy, LNR IZS Sicilia
- Netherlands, NRL RIVM
- Norway, NRL Institute of Marine Research
- Poland, National Veterinary Research Institute in Pulawy
- Portugal, INIAV I.P
- Romania, Institute For Hygiene And Veterinary Public Health
- Institute of Veterinary Medicine of Serbia
- Slovenia, NRL University of Ljubljana, Veterinary Faculty

Results-Methods

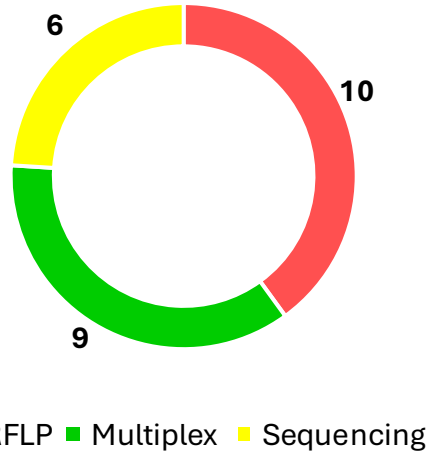


Participation

- **16/16** labs sent the results

Methods

- **5** EURL-P method 2 (Multiplex PCR)
- **4** EURL-P method 1 (PCR-RFLP) [1]
- **4** EURL-P method 1; EURLP method 2 [2]
- **2** EURLP method 1 PCR plus sequencing
- **1** In house method (PCR COX2 and sequencing)



1) Two labs in combination with sequencing
2) One lab in combination with PCR COX1 and sequencing

Results- Detection

Laboratory code	N° of samples correctly identified		N° of samples NOT correctly identified		Method applied	Final evaluation
	Larvae	DNA	Larvae	DNA		
AMM01	2	2	0	0	In house method (PCR Cox2 and sequencing)	POSITIVE
AMM02	2	0	0	2	EURLP method MI-10 (multiplex PCR)	NEGATIVE
AMM03	2	1	0	1	EURLP method MI-10 (multiplex PCR)	NEGATIVE
AMM04	2	0	0	2	EURLP method MI-10 (multiplex PCR)	NEGATIVE
AMM05	2	1	0	1	EURLP method MI-04 (PCR_RFLP); + sequencing	NEGATIVE
AMM06	2	1	0	1	In-house method according the EURLP method (PCR-RFLP) + sequencing	NEGATIVE
AMM07	2	2	0	0	EURLP method MI-04 (PCR_RFLP)	POSITIVE
AMM08	1	1	1	1	EURLP method MI-04 (PCR_RFLP); EURLP method MI-10 (multiplex PCR); In house method (CO1 plus Sanger sequencing);	NEGATIVE
AMM09	2	2	0	0	EURLP method MI-04 (PCR_RFLP); EURLP method MI-10 (multiplex PCR);	POSITIVE
AMM10	2	1	0	1	In house method POS CRENA 02 Rev. 11 (PCR-RFLP) and POS CRENA 11 Rev. 2 (PCR-Multiplex)	NEGATIVE
AMM11	2	2	0	0	EURLP method MI-04 (PCR_RFLP); EURLP method MI-10 (multiplex PCR);	POSITIVE
AMM12	2	2	0	0	EURLP method MI-04 (PCR_RFLP);	POSITIVE
AMM13	2	1	0	1	EURLP method MI-10 (multiplex PCR)	NEGATIVE
AMM14	2	1	0	1	EURLP method MI-10 (multiplex PCR)	NEGATIVE
AMM15	2	1	0	1	Published method (PCR and sequencing)	NEGATIVE
AMM16	2	1	0	1	EURLP method MI-04 (PCR_RFLP); Published method; EURLP method and Sequencing	NEGATIVE

1 Missed identification
+
1 Wrong identification

1 or 2 Wrong identification

1 Missed identification

5/16 (**31%**) of the reporting laboratories passed the PT

Results- Follow Up

Analysis of the cause of failure was provided by 10 labs using the online follow-up form

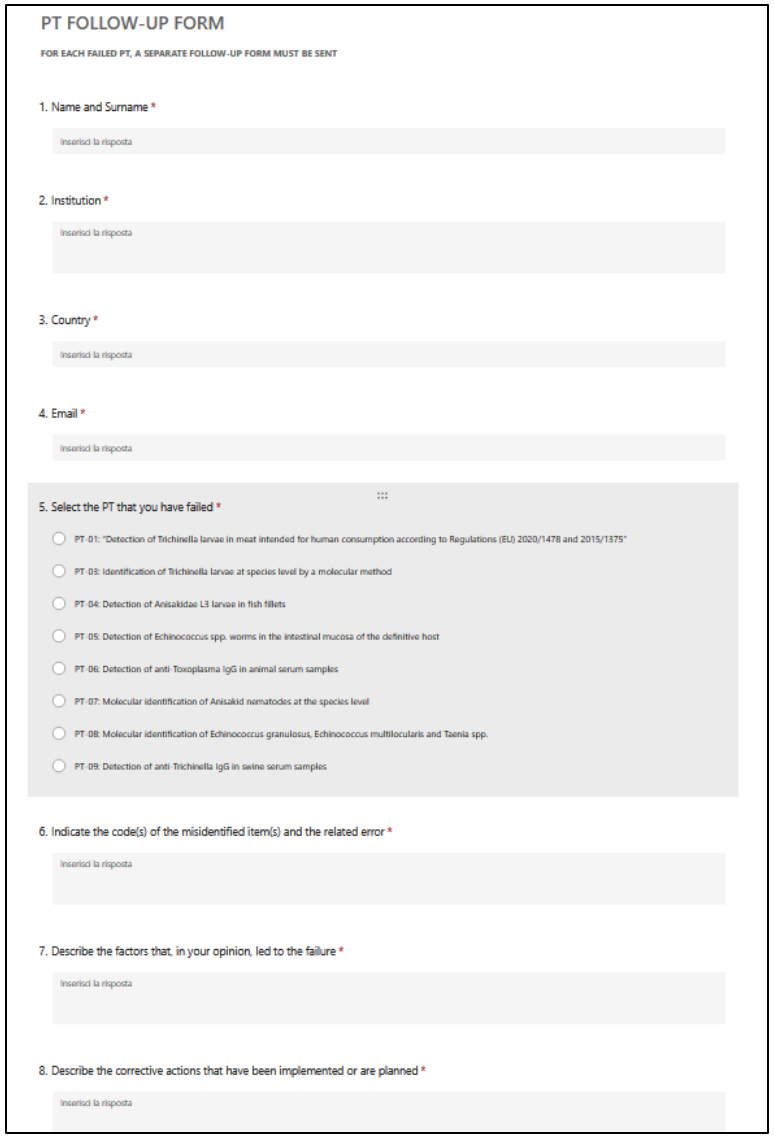
AMM16 No feedback was received *

Problems reported from the other participants

- Incorrect interpretation of the PCR fragments obtained
- Non optimal execution of PCR / Inexperience with this PT
- Presence of an additional band with the multiplex method
- Errors during the analysis of the sequences

* PT failure could be due to:

- Errors or inappropriate changes made by the operator during the DNA extraction or PCR amplification steps.
- Use of unsuitable reagents during the DNA extraction or PCR amplification steps.



PT FOLLOW-UP FORM

FOR EACH FAILED PT, A SEPARATE FOLLOW-UP FORM MUST BE SENT

1. Name and Surname *

Inserisci la risposta

2. Institution *

Inserisci la risposta

3. Country *

Inserisci la risposta

4. Email *

Inserisci la risposta

5. Select the PT that you have failed *

- PT-01: "Detection of Trichinella larvae in meat intended for human consumption according to Regulations (EU) 2020/1478 and 2015/1375"
- PT-03: Identification of Trichinella larvae at species level by a molecular method
- PT-04: Detection of Anisakidae L3 larvae in fish fillets
- PT-05: Detection of Echinococcus spp. worms in the intestinal mucosa of the definitive host
- PT-06: Detection of anti-Toxoplasma IgG in animal serum samples
- PT-07: Molecular identification of Anisakid nematodes at the species level
- PT-08: Molecular identification of Echinococcus granulosus, Echinococcus multilocularis and Taenia spp.
- PT-09: Detection of anti-Trichinella IgG in swine serum samples

6. Indicate the code(s) of the misidentified item(s) and the related error *

Inserisci la risposta

7. Describe the factors that, in your opinion, led to the failure *

Inserisci la risposta

8. Describe the corrective actions that have been implemented or are planned *

Inserisci la risposta

A. pegreffii/A. berlandi (former *A. simplex C*) hybrid genotype

ITS sequence

>Sample_**AM1023**_ITS_consensus

GTAGGTGAACCTGCGGAAGGATCATTATCGAGCGAATCCAAAACGAACGAAAAAGTCTCCCAACGTGCATACCTTCCATTTGCATGTTGTTGTGAGCC
ACATGGAAACTCGTACACACGTGGTGGCAGCCGTCTGCTGTGCTTTTTTTAGGCAGACAATGGCTTACGAGTGGCCGTGTGCTTGTTGAACAACGGTG
ACCAATTTGGCGTCTACGCCGTATCTAGCTTCTGCCTGGACCGTCAGTTGCGATGAAAGATGCGGAGAAAGTTCCTTTGTTTTGGCTGCTAATCATCATT
GATGAGCAGCAGCTTAAGGCAGAGTYGAGCAGACTTAATGAGCCACGCTAGGTGGCCGCCAAAACCCAAAACACAACCGGTCTATTTGACATTGTTA
TTTCATTGTATGTGTTGAAAATGTACAAATCTTGGCGGTGGATCACTCGGTTCGTGGATCGATGAAGAACGCAGCCAGCTGCGATAAATAGTGCGAATTG
CAGACACATTGAGCACTAAGAATTCGAACGCACATTGCGCTATCGGGTTCATTCCCGATGGCACGTCTGGCTGAGGGTCGAATTACGGTGAACTGTCT
TCACGGTCTTTCTGGACTGTGAAGCATTCGGCAAGCAATTGCTGTTGTGTTGTTGGTGATTCTATCATGGACAATATGACGMGCGGTTCCTTGCTTAGTG
ATGAYAAAAGAAGACGTCAACACCGAATCTACTATACTACTAATACTAGTATATAGGTGAGGTGCTTTTGGTGGTCACAAAAGTGACAAGTATGCCATTTC
ATAGGGGCAACAACCAGCATACGTGATAAGTTGGCTGGTTGATGAAACGGCAACGGAATGACGGACGTCTATGTGATCAAAAATGATACTATTTGACCT
CAGCTCAGTCGTGATTACCCGCTGAATTTAAGCATATAATTAAGCGGAGGAAAAGAAACTAAA

IUPAC Code:

Y = T or C

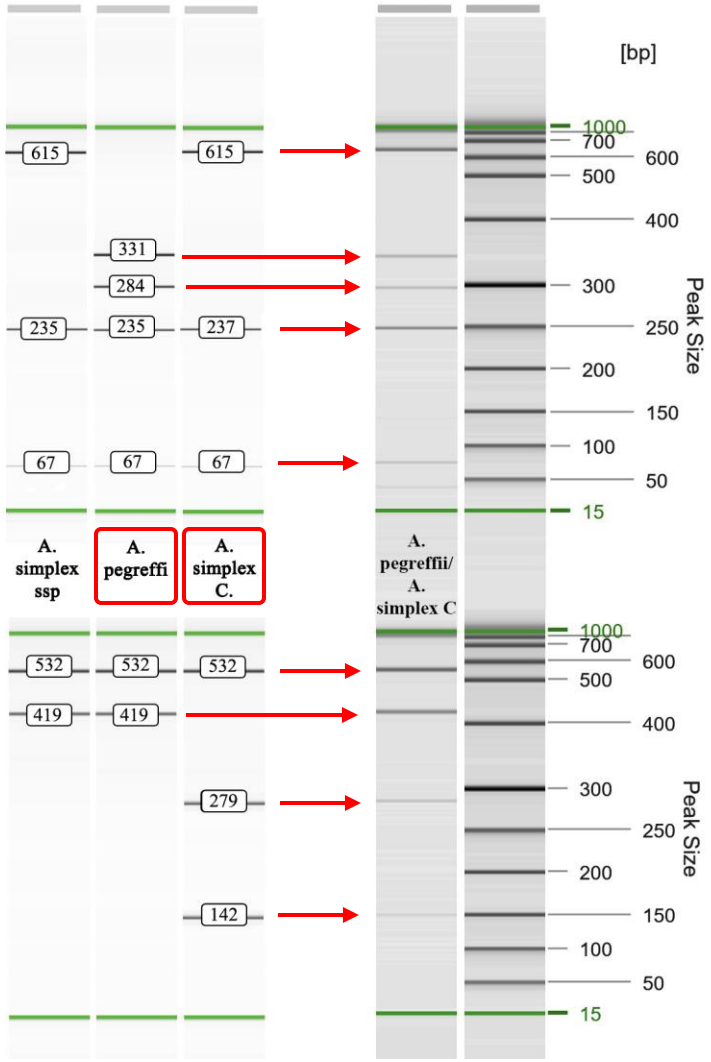
M = A or C

GAnTC -> *HinfI* : 615bp + 331bp + 248bp + 240bp + 67bp + 34bp

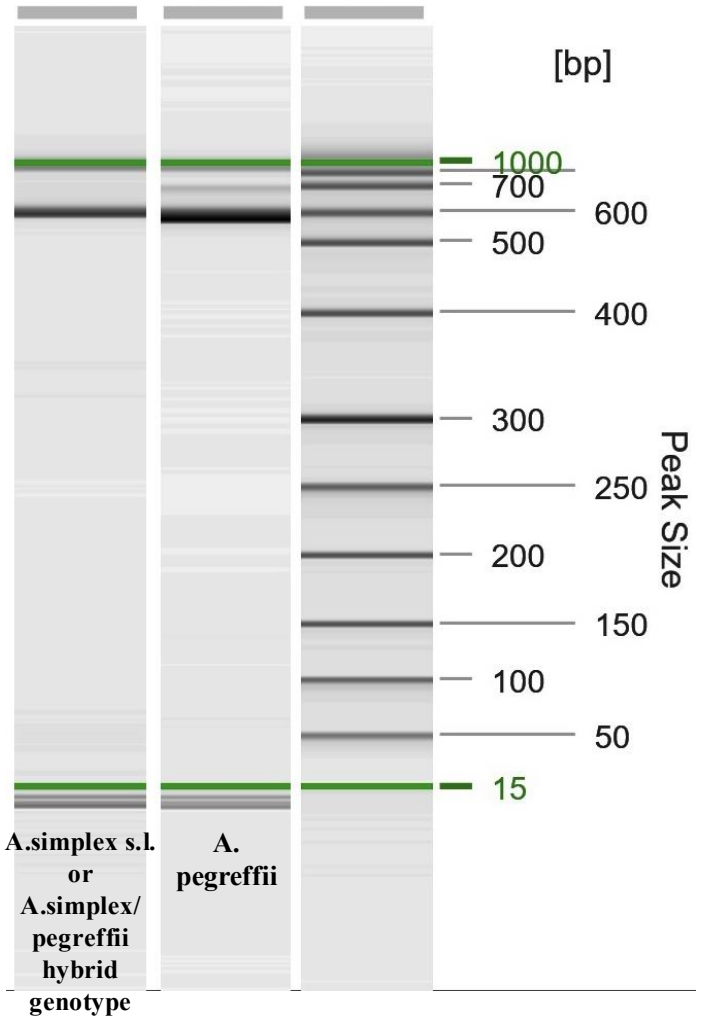
GCGC -> *HhaI* : 532bp + 424bp + 282bp + 142bp

A. pegreffii/A. berlandi (ex *A. simplex C*) hybrid genotype

MI-04 PCR-RFLP (ITS)



MI-10 PCR-MULTIPLEX (ITS)

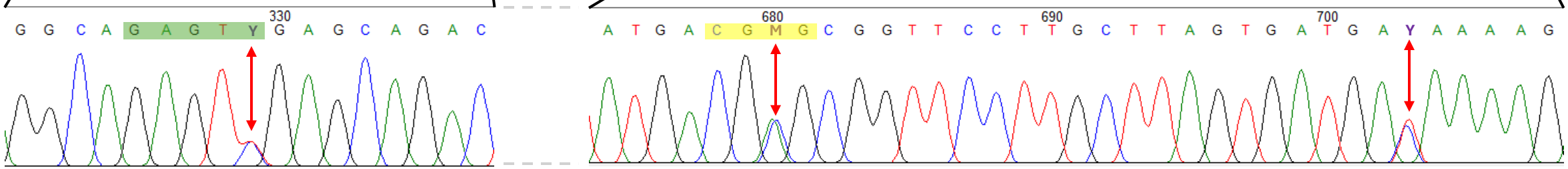


A. pegreffii/A. berlandi (ex *A. simplex C*) hybrid genotype

PCR + Sequence (ITS)

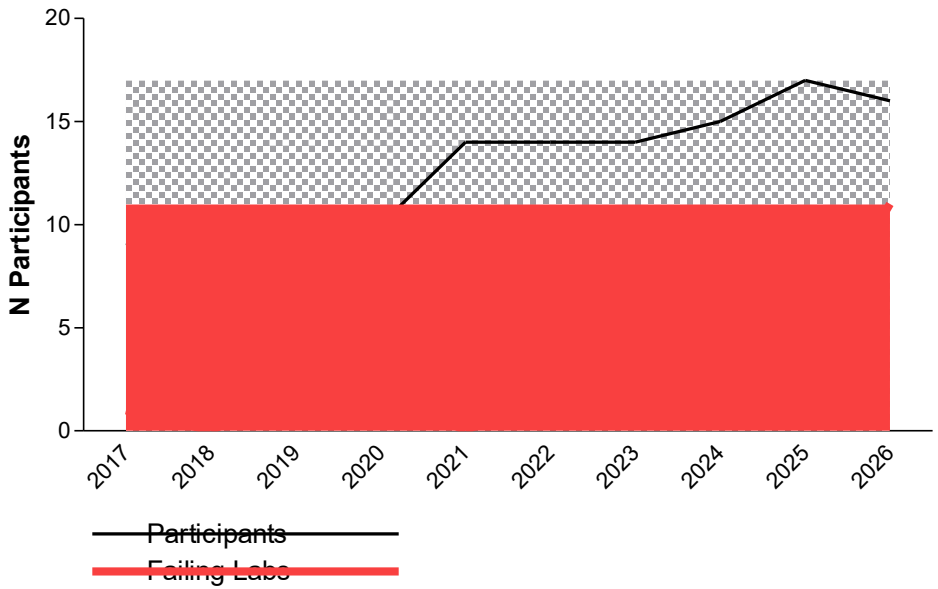
>ITS_consensus

GTAGGTGAACCTGCGGAAGGATCATTATCGAGCGAATCCAAAACGAACGAAAAAGTCTCCCAACGTGCATACCTTCCATTTGCATGTTGTTGTGAGCCAC
ATGGAAACTCGTACACACGTGGTGGCAGCCGTCTGCTGTGCTTTTTTTAGGCAGACAATGGCTTACGAGTGGCCGTGTGCTTGTTGAACAACGGTGACCA
ATTTGGCGTCTACGCCGTATCTAGCTTCTGCCTGGACCGTCAGTTGCGATGAAAGATGCGGAGAAAGTTCCTTTGTTTTGGCTGCTAATCATCATTGATGAG
CAGCAGCTTAAGGCAGAGTYGAGCAGACTTAATGAGCCACGCTAGGTGGCCGCCAAAACCCAAAACACAACCGGTCTATTTGACATTGTTATTTCATTGT
ATGTGTTGAAAATGTACAAATCTTGGCGGTGGATCACTCGGTTCGTGGATCGATGAAGAACGCAGCCAGCTGCGATAAATAGTGCGAATTGCAGACACATT
GAGCACTAAGAATTCGAACGCACATTGCGCTATCGGGTTCATTCCCGATGGCACGTCTGGCTGAGGGTCGAATTACGGTGAACTGTCTTCACGGTCTTTC
TGGACTGTGAAGCATTCGGCAAGCAATTGCTGTTGTGTTGTTGGTGATTCTATCATGGACAATATGACGMGCGGTTCCTTGCTTAGTGATGAYAAAAGAAGA
CGTCAACACCGAATCTACTATACTACTAATACTAGTATATAGGTGAGGTGCTTTTGGTGGTCACAAAAGTGACAAGTATGCCATTTCATAGGGGCAACAACCA
GCATACGTGATAAGTTGGCTGGTTGATGAAACGGCAACGGAATGACGGACGTCTATGTGATCAAAAATGATACTATTTGACCTCAGCTCAGTCGTGATTACC
CGCTGAATTTAAGCATATAATTAAGCGGAGGAAAAGAAACTAAA



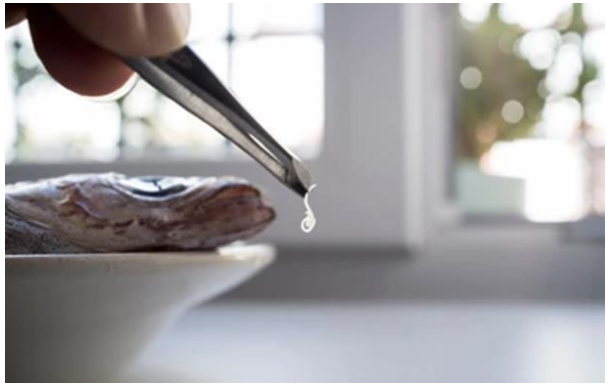
PT trend

Laboratory code 2026	PT year									
	2017	2018	2019	2020	2021	2022	2023	2024	2025	2026
AMM01	NP	NP	POS	NP	POS	POS	POS	POS	POS	POS
AMM02	NP	NP	NP	NP	NP	NP	NP	NP	POS	NEG
AMM03	POS	POS	NEG	POS	POS	POS	POS	POS	POS	NEG
AMM04	NP	POS	NP	NP	NP	NP	NEG	POS	POS	NEG
AMM05	POS	POS	POS	NEG	POS	POS	NEG	POS	NEG	NEG
AMM06	NP	NP	NP	POS	POS	POS	NEG	POS	NEG	NEG
AMM07	POS	POS	POS	POS	POS	POS	POS	POS	NP	POS
AMM08	NP	POS	NP	NEG	POS	NEG	NEG	POS	POS	NEG
AMM09	POS	NP	NP	NP	POS	POS	NEG	POS	POS	POS
AMM10	POS	POS	POS	POS	POS	POS	POS	NEG	POS	NEG
AMM11	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS
AMM12	POS	POS	POS	POS	POS	POS	NP	POS	NEG	POS
AMM13	POS	POS	POS	POS	POS	POS	POS	POS	POS	NEG
AMM14	NP	NP	NP	NP	POS	POS	POS	POS	POS	NEG
AMM15	NP	NP	NP	NP	NP	NP	NP	NP	POS	NEG
AMM16	NP	NP	NP	NP	NP	POS	NEG	POS	POS	NEG



- 20 Laboratories enrolled in 2017-2026
- 5 Laboratories always participating
- 1 Laboratory always participating with a positive evaluation in every edition

Conclusions



- 5/16 (**31%**) reporting laboratories passed the PT.
- The PCR-RFLP and Multiplex PCR remain the preferred methods but sequencing, directly or in confirmation of other methods, is also frequently performed.
- Method of choice and PT performance seems not correlated.
- More challenging items can help to enhance the participants performance.

Thanks

See you in 2027!



More info: marco.lalle@iss.it