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Detection and identification of *Meloidogyne enterolobii* in complex DNA backgrounds using LNA-probe based real-time PCR assays

(Euphresco II project: *Meloidogyne enterolobii)*

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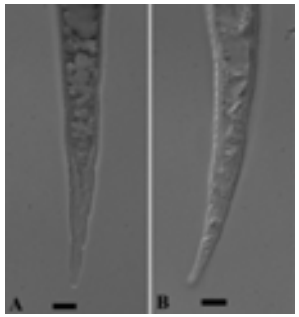

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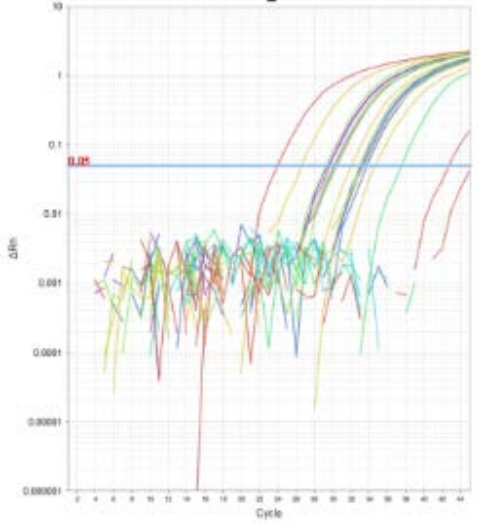
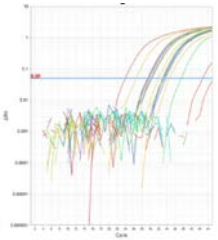
Outline



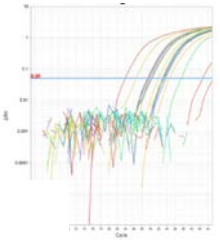
- Aim of the study
- *Meloidogyne enterolobii*
- qPCR & LNA probes
- Method development
- In-house validation (analytical specificity and sensitivity, Ct-cut off values, repeatability, selectivity, reproducibility)
- Test performance study
- Summary & Outlook

Aim



- Develop a fast, reliable DNA-based method (qPCR assays) to detect and identify *M. enterolobii*
- Develop an assay that can detect one individual (L_2) in a sample (complex DNA-background)
- Assay should perform equally on different platforms and with different chemistry; by any lab offering molecular diagnostic analyses
- Simple lysis buffer protocol; no additional purification steps (Holterman et al., 2009)

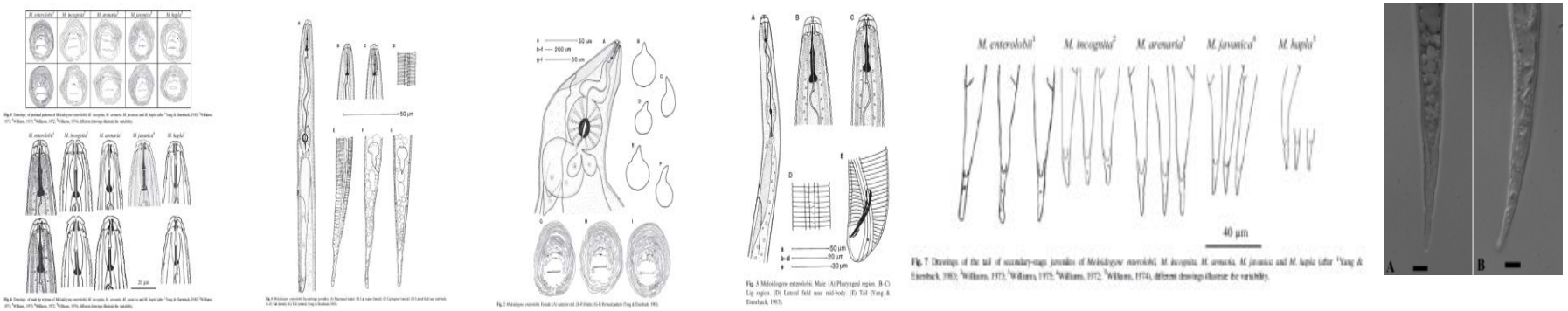
Aim



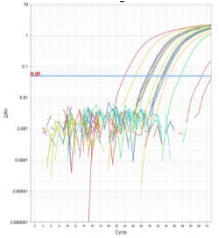
- In-house validation: Optimize primers and probes conc.; Tm; find LODs, i.e. cut-off Ct values for different chemistries and platforms
- Proof analytical specificity and sensitivity (aim for a limit of detection of finding one L_2 in a susp. obtained 100 ml soil or DNA-background from 1000 soil nematodes)
- Conduct test performance study

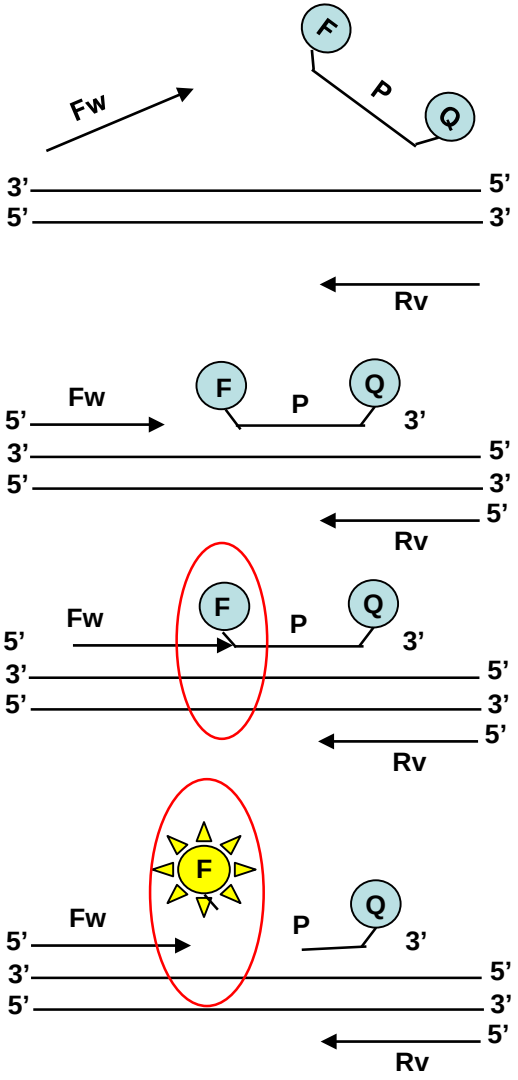
Introduction – *Meloidogyne enterolobii*

- Very agressive and virulent tropical root-knot nematode species
- recommended for regulation as quarantine species (EPPO A2 list)
- Pathways exist (interceptions)
- *Meloidogyne* spp. are difficult to ID based on morphology

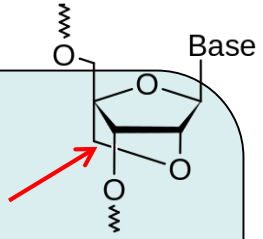


qPCR using LNA probes





1. Before PCR-cycle: probe intact, F-emission suppressed by Q; denaturation DNA 95°C; TaqPol starts activity
2. Annealing: Primer & probe anneal to DNA
3. Extension & Elongation: Taq Pol inserts nucleotides in DNA and amplifies PCR product in each cycle; simultaneous destruction of probe by TaqPol 5'→3' exonuklease (DNAse) activity
4. Emission of fluorescence, measurable in positive samples (presence of DNA-targets, Ct-values)



<u>Specific features of LNA probes!</u>

- 5'-FAM and dark quencher (NFQ)-3'
- No background fluorescence such as TAMRA
- **Much shorter than normal TaqMan & MGB probes (8-9 nt)**
- Locked nucleic acids are DNA nucleotide analogues with higher affinity to target DNA
- Higher specificity and higher detection rate of short target DNA sequences
- **Roche Universal Probe Library (Probefinder Software)**

qPCR development for *M. enterolobii*

DNA-Barcoding for species identification (DNA Fingerprints)

(Kiewnick et al., 2014. EJPP)

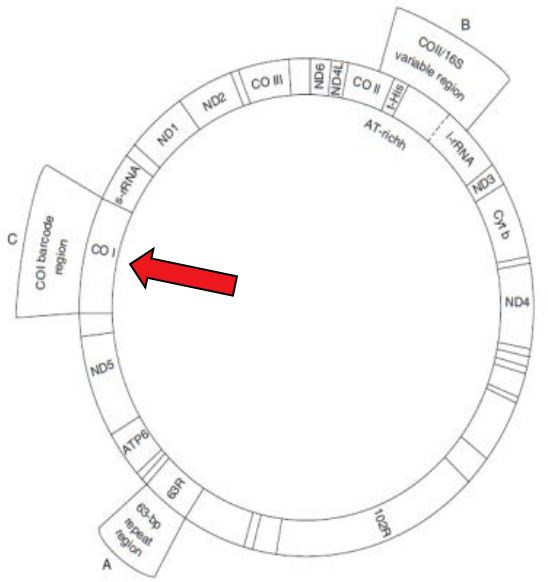
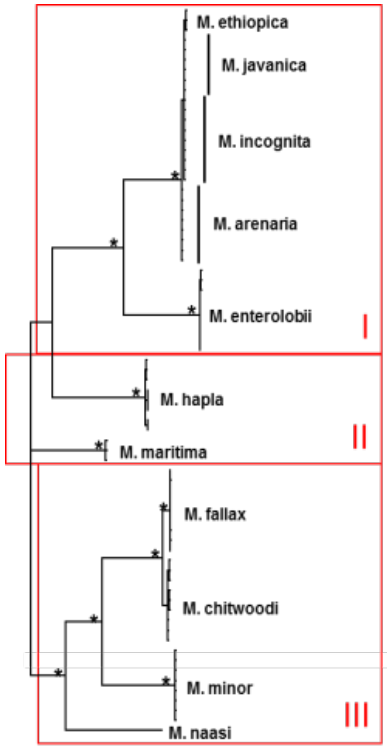


Fig. 4.1. *Meloidogyne* mitochondrial genome structure, showing regions used for diagnostic

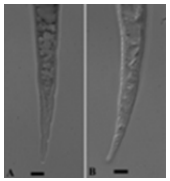
(Blok & Powers, 2009)

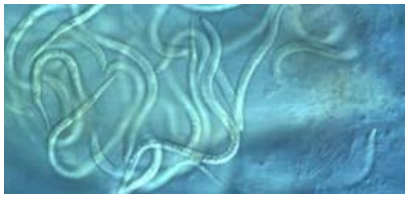


COI: cytochromoxidase subunit I gene (mitochondria; less variable coding region) Good discriminatory power between species; exception: some tropical *Meloidogyne spp*.

Little to no variation within species

qPCR development for *M. enterolobii*





IGS2: high intra-individual variation (variable non-coding region)

(Kiewnick et al, 2014. EJPP)

Results of in-house validation (analytical specificity)

Nematode	Number of populations/Isolates	Source/Reference collection	IGS2	COI
M. enterolobii	16	CH, F, NL, USA	+	+
M. incognita	6	CH, D, NL	-	-
M. hapla	2	CH, NL	-	-
M. fallax	1	CH	-	-
M. arenaria	2	CH, NL	-	-
M. javanica	2	NL	-	-
M. ethiopica	1	SI	-	-
M. chitwoodi	1	D	-	-
M. graminicola	1	D	-	-
Bursaphelenchus xylophilus	1		-	-
Bursaphelenchus mucronatus	1		-	-
Nacobus abberrans	1	F	-	-
Globodera rostochiensis	1	CH	-	-

No cross-reactions with 8 non-target *Meloidogyne* species including close relatives and 4 other genera. All *M. enterolobii* populations (16) reacted **highly specific** with developed assays (no difference between platforms used, assays were highly repeatable and reproducible)!

Results of in-house validation (analytical sensitivity)

Performance characteristics of qPCR assays on different real-time PCR platforms using standard curves based on dilutions of *Meloidogyne enterolobii* juveniles L2 (STD 174-176) crushed in 200 µl lysis buffer.

			Dynamic range [a] (L2 per reaction)		Linear regression [b]			Limit of detection (LOD) [c]		
qPCR Assay	Number of runs	Real-time platforms	Low limit	High limit	Slope *(k)*	R^2	*E (%)*	Nematode juveniles per rxn	Average C_T	C_T SD
COI	7	ABI 7500 Fast	0.003	0.3	-3.16	0.982	107.4	0.0003	35.2	1.06
	6	Roche LC480	0.003	0.3	-3.41	0.985	96.5	0.0003	36.0	1.21
IGS2	8	ABI 7500 Fast	0.003	0.3	-3.50	0.994	93.4	0.0003	35.2	0.73
	5	Roche LC480	0.003	0.3	-3.42	0.992	96.2	0.0003	35.6	0.10

[a] The range of concentrations for which C_T values were in linear relationship with logarithms of concentrations (determined by exploring slope values across sections of C_T values x $\log_{10}$ L2 per reaction).

[b] Linear regression of all positive samples in a plot of C_T values against logarithmic number of *M. enterolobii* larvae: *k* = slope of the linear regression line, R^2 = average square regression coefficient; *E* = efficiency of amplification.

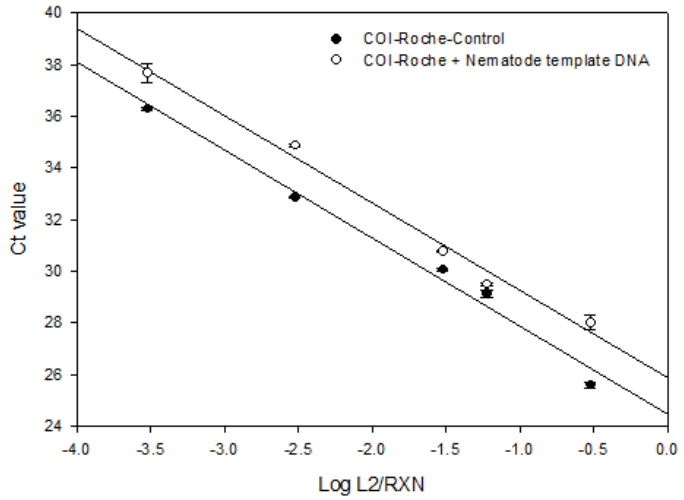
[c] LOD = limit of detection, for the purpose of this study defined as concentration at which at least two of the triplicate reactions were positive; i.e., detecting fewer than 0.0003 L2 per reaction.

Highly sensitive; 1 juvenile (L2) in suspension obtained from 100 ml soil (1 in 1000); LOD cut off COI qPCR: 35.2 on ABI; 36.0 on LC480

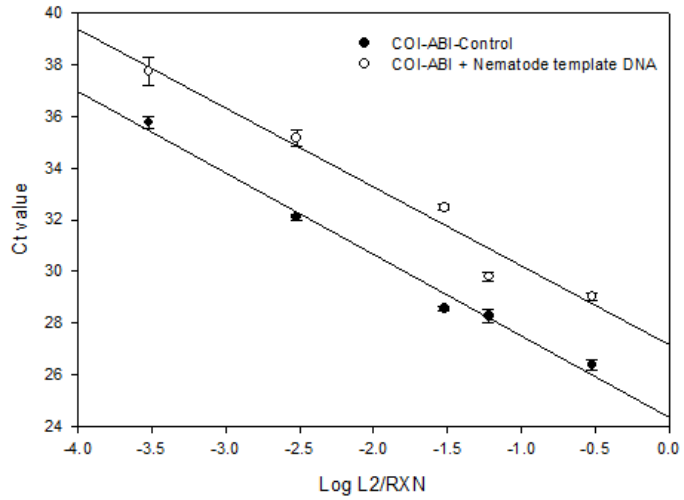
Results of in-house validation (selectivity I)

- **<u>Selectivity (PM 7/98; 2010 EPPO Bulletin 40, 5-22)</u>**: Not relevant for nematodes identification as they are previously isolated from the matrix. If test is used as a detection test, insensitivity of test to variation of matrix (here different amount of background nematodes) should be determined.
- Comparison of standard curves of pure *M. enterolobii* DNA with spiked SC (100/1000 background nematodes/200µl lysis buffer) using COI probe on two platforms

Roche LC 480



ABI

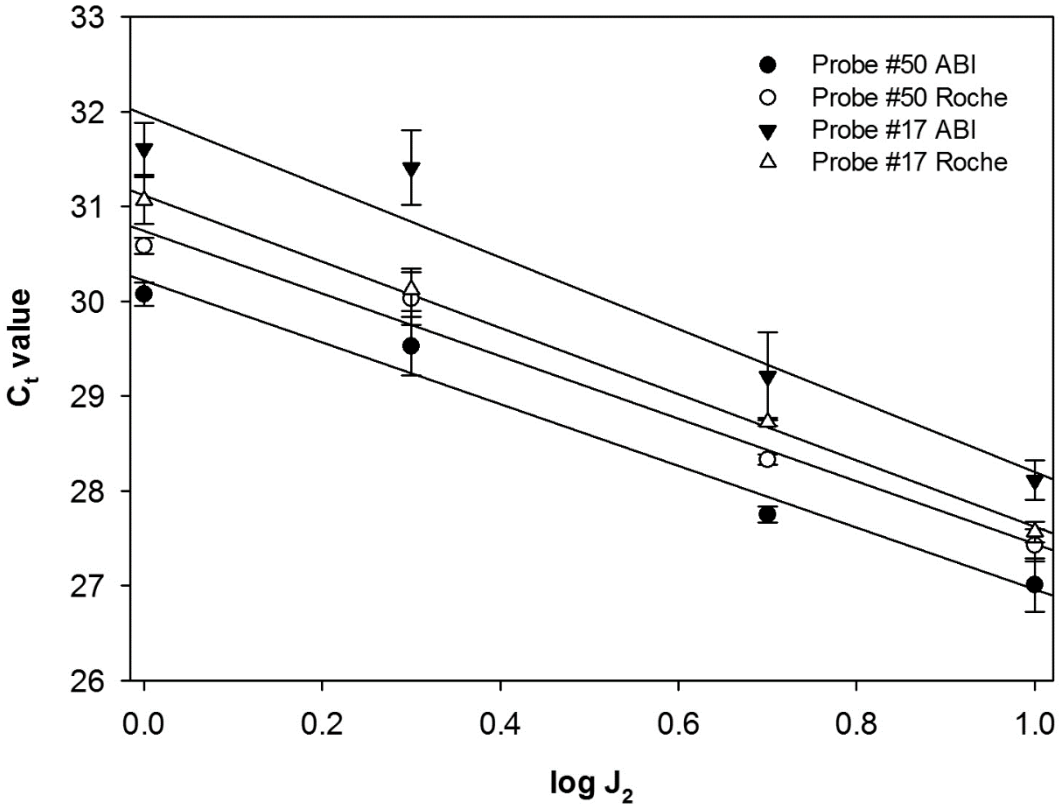


No qPCR inhibition. Slight shift of Ct-values; Slope of regression not affected (validity of COI qPCR test!)

Results of in-house validation (selectivity II)

Selectivity (EPPO Bulletin 40, 5-22):

- If test is used as a detection test, insensitivity of test to variation of matrix (here 1000 background nematodes + diff amounts of L2 juveniles added) should be determined.



M. enterolobii was correctly detected in nematode suspensions containing DNA of 1000 other soil nematodes

Results of in-house validation (selectivity III)

- Detection of *M. enterolobii* in spiked/non-spiked Bonsai soil and root samples with different qPCR probes and platforms/chemistries
- Samples contained low to moderate numbers (0 to >40) of other *Meloidogyne* species (*M. incognita, M. javanica*) in the background
- Spiked samples = 1 Me L_2 was added before DNA extraction using Lysis buffer

Results of in-house validation (selectivity III)

- All bonsai samples (soil & roots) were negative for Me.
- Spiked Bonsai samples (soil & roots) are positive for Me
- Test is non-selective, i. e. insensitive to matrix variation (soil, roots)

	Non-spiked suspension							Spiked suspension						
	Soil 1	Soil 2	Soil 3	Roots 1	Roots 2	Roots 3	PIC (H2O + 2-3 L2)	Soil 1	Soil 2	Soil 3	Roots 1	Roots 2	Roots 3	Control (H2O + 1 L2)
IGS2 ABI	38.23	38.26	37.83	38.36	38.89	36.63	25.17	29.00	30.04	29.84	30.00	29.86	30.22	29.08
IGS2 Roche	35.99	36.09	36.84	36.81	35.60	34.92	25.13	28.98	29.86	29.73	29.99	29.94	30.11	29.20
COI ABI	40.00	40.00	39.96	39.78	40.00	39.22	26.86	30.83	31.25	31.08	30.90	30.92	31.33	30.82
COI Roche	37.20	38.20	39.72	37.99	38.36	36.66	25.48	32.69	31.22	31.04	31.27	32.63	31.49	30.57

Numbers in table are averaged Ct-values (n=6)

Organization of TPS

- Lessons learned from previous TPS: **keep it simple**; only compare few methods, provide most of the material used
- 7 labs/EU partners participated
- **DNA extraction** methods: use **only one**: available lysis buffer protocol
- Participants were allowed to use own extraction method in comparison with this **simple lysis buffer**
- **One qPCR** method based on COI developed by Agroscope on platforms commonly used by the partners

Organization of TPS:

- 18 samples per set provided to 7 participating labs (some labs got two sets upon request, e.g. for optional additional test)
- Distribution of samples by express courier (1-2 day delivery)
- Analyses of samples: nematode suspensions directly, if possible otherwise **store frozen** or at 4°C

Organization of TPS

Sample codes	Tube content	
1	Soil A only	Detection
2	Soil A + 2 L2	Detection
3	Soil A + 10 L2	Detection
4	Soil B only	Detection
5	Soil C only	Detection
6	Lysis buffer	Identification
7	L. buffer + 2 L2	Identification
8	L. buffer + 10 L2	Identification
9	Me DNA 100x dil	Linearity
10	Me DNA 1000x	Linearity
11	Me DNA 10000x	Linearity
12	Me DNA 100000x	Linearity
13	*M. hapla* DNA	Specificity
14	*M. graminicola* DNA	Specificity
15	*Nacobus abberrans* DNA	Specificity
16	*Globodera rostochiensis* DNA	Specificity
17	*M. chitwoodi* DNA	Specificity
18	*M. fallax* DNA	Specificity

Samples 1-8: Detection & ID of Me in nematode suspensions from soil; 500 individuals per 1.5 ml H_2O spiked with Me juveniles (L2);

DNA extraction to be performed with simple lysis buffer (including β-mercaptoethanol and proteinase K)

Samples 9-18:
Linearity & Specificity on DNA provided by
Agroscope (test organizer)

Agroscope

Results TPS:

qPCR performance criteria according to PM 7/98

	Performance criteria	Calculation	Detection	Specificity	Identification
M. enterolobii	Negative agreement (NA[a])	Number of negative samples×8 tests	24	56	8
	Positive agreement (PA[b])	Number of positive samples×8 tests	16	32	16
	Negative deviation (ND[c])	Number of negative deviations×8 tests	0	0	0
	Positive deviation (PD[d])	Number of positive deviations×8 tests	0	0	0
	Sensitivity (SE in %)	PA/N+[e]	100	100	100
	Specificity (SP in %)	NA/N-[f]	100	100	100
	Accuracy (%)	[(PA/N+) + (NA/N-)]/2	100	100	100

Braun-Kiewnick et al., 2015 EJPP

Assessment of TPS (stability)

- Related to target samples for detection (nem. susp. samples 1-5)
- Overall excellent stability over period of 6-8 wks; only few variations noticed
- Fresh extraction and short storage (<5 d) at 4°C yields good DNA quality for qPCR (very similar Ct-values; diff. 1-2)
- Longer storage at -18/20°C works also well (mostly done by labs, that could not handle samples directly)
- **Storage of nem susp at 4-6°C for longer periods before DNA extraction not recommended, yields lower DNA content or decrease in quality (higher Ct-values >2-3)**

Summary *M. enterolobii* qPCR assays

- **Species-specific** qPCR method for the detection/quantification and identification of *M. enterolobii* from soil or plant roots.
- **Highly sensitive** (1 L_2 in a background of 1000 soil nematodes; quantification possible if necessary
- **Highly repeatable** (level of agreement between reps of samples tested under same conditions; very small STDs)
- **Highly reproducible** (provides consistent results even when tested under diff. conditions (time, equipment)

Summary TPS

- Lessons learned from previous TPS: **keep it simple**, test only single parameters at once, not too many methods: **Only 1 DNA extraction method (simple lysis buffer) & one qPCR method**
- Improvement of reaction conditions (better efficiency/sensitivity) by providing most of the solutions needed (buffers, primers, etc.)
- Keep in mind different equipment and test robustness under different settings (define cut off before launching TPS)
- **No real difficulties encountered during TPS**

Outlook

- Publication of method and TPS
- Revision of EPPO Standard protocol for diagnostics of *M. enterolobii* (PM 7/103) under way

Thanks to:

Juerg Frey, Martijn Holterman,
Andrea Braun-Kiewnick
TPS Participants

and you for your attention!