Euphresco

Network for phytosanitary research coordination and funding

Euphresco

Final Report

Project title (Acronym)
Use of barcoding, from theory to practice (PRACTIBAR)

Project duration:

Start date:	2017-05-01
End date:	2019-06-01

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Contents

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2. Short project report

2.1. Short executive summary

DNA barcoding is increasingly used as a diagnostic tool in phytosanitary laboratories. The EPPO Standard PM7/129 'DNA barcoding as an identification tool for a number of regulated pests' was published on 2016-11-28. The document provides guidelines on the use of the DNA barcoding protocols in support of the identification of a number of regulated pests and invasive plant species comparing DNA barcode regions with those deposited in publicly available sequence databases. The outcome of DNA barcoding tests can be negatively affected by the incompleteness of databases, incorrectly identified species in databases, the amplification of pseudogenes or nuclear mitochondrial DNA segment (NUMTs) and introgression or hybridization events. For that reason, the analysis of sequence data should be performed by proficient operators. The main goal of the project was to raise awareness on barcoding as a generic method for pest identification and to train experts of the competent organisations.

2.2. Project aims

DNA barcoding is increasingly used as a diagnostic tool in phytosanitary laboratories. DNA barcoding protocols for selected European Union (EU)-regulated arthropods, bacteria, fungi, nematodes and phytoplasmas were developed within the EU project 'Quarantine organisms Barcoding of Life (QBOL)'. A test performance study (TPS) was set up in 2011 to validate the use of the developed protocols as diagnostic tools and to identify possible difficulties in the use of the protocols and databases (e.g. Q-bank). TPS data showed that the developed tests were very robust and produced highly reproducible results. Suggestions for additional work were considered in the framework of the Euphresco project 'DNA Barcoding - Optimizing and validating DNA barcoding protocols for plant pests'. An international test performance study (TPS) was organised to generate data on diagnostic sensitivity and robustness. Overall diagnostic sensitivity obtained when analysing the sample identification results provided by TPS participants was 87%. Analysis of the TPS data showed that neither the amplification/sequencing of loci, nor the creation of consensus sequences, negatively influenced the diagnostic sensitivity. The interpretation of analysis results had the biggest influence on the diagnostic sensitivity, and the majority of incorrectly identified samples were the result of conservative identification (i.e. identification on a higher taxonomical level) showing that some participants did not feel confident in assigning a lower taxon level to the sample. Re-analysis of the consensus sequence data provided by TPS participants showed that an overall diagnostic sensitivity of 99% could be obtained.

The EPPO Standard PM7/129 'DNA barcoding as an identification tool for a number of regulated pests' was published on 2016-11-28. The document provides guidelines on the use of the DNA barcoding protocols in support of the identification of a number of regulated pests and invasive plant species comparing DNA barcode regions with those deposited in publicly available sequence databases.

The outcome of DNA barcoding tests can be negatively affected by the incompleteness of databases, incorrectly identified species in databases, the amplification of pseudogenes or

nuclear mitochondrial DNA segment (NUMTs) and introgression or hybridization events. For that reason, the analysis of sequence data should be performed by proficient operators.

The main goal of the project was to raise awareness on barcoding as a generic method for pest identification and to train experts of the competent organisations, as highlighted at the end of the Euphresco project 'Update and validation of DNA Barcoding protocols by end-users' (final report available from Zenodo) and as recommended by the EPPO Panel on Diagnostics and Quality Assurance in 2016-01-19/21. Workshops and proficiency tests (PT) were organised and training material developed.

2.3. Description of the main activities

Two main activities were organised during the project: trainings and proficiency tests.
Training: the Dutch NPPO and the University of Guelph organised four trainings, as follows:

- Training 1, organised on 2017-10-09/13 in Paris, France;
- Training 2, organised on 2017-12-05/08 in Wageningen, the Netherlands;
- Training 3, organised on 2018-10-09/12 in Paris, France;
- Training 4, organised on 2018-12-11/14 in Wageningen, the Netherlands;

The workshops allowed participants to be informed of the most recent advancements on the DNA barcoding method and to receive training on a number of tools for data analysis: sequencing analysis software that allow assembly of raw sequence data (e.g. Geneious), and online databases (Q-bank, NCBI, BOLD). Raw sequence data covered examples from arthropods, bacteria, fungi, invasive plants, nematodes, phytoplasmas, viruses and viroids, depending on the needs and wishes of the workshops' participants.

The course was intended for technicians and researchers who want to use, or are using sequencing analysis, in a phytosanitary diagnostic framework. Participants had at least two years of experience with molecular biological tests, and basic knowledge of Sanger sequencing in certain cases.
In order to ensure maximum exchange between the trainers (Adriana Radulovici from the University of Guelph and Bart van de Vossenberg, Marcel Westenberg and Eveline Metz-Verschure from the Dutch National Plant Protection Organization) the number of participants was limited to 16 per training session. Experts from the following countries participated in the trainings (Fig. 1.):

- Training 1: Croatia, Czech Republic, Denmark, France, Germany, Greece, Ireland, Latvia, Portugal, Romania, Serbia, Slovenia
- Training 2: Austria, Bosnia and Herzegovina, Croatia, Germany, Greece, Ireland, Italy, Republic of Korea, Slovenia, Switzerland, Switzerland, Turkey, United Kingdom
- Training 3: Belgium, Bulgaria, Finland, France, Germany, Greece, Israel, Italy, Republic of Korea, Mexico, Peru, Slovenia,
- Training 4: Belgium, Germany, Ireland, Italy, the Netherlands, Poland, Portugal, Spain, Turkey, United Kingdom
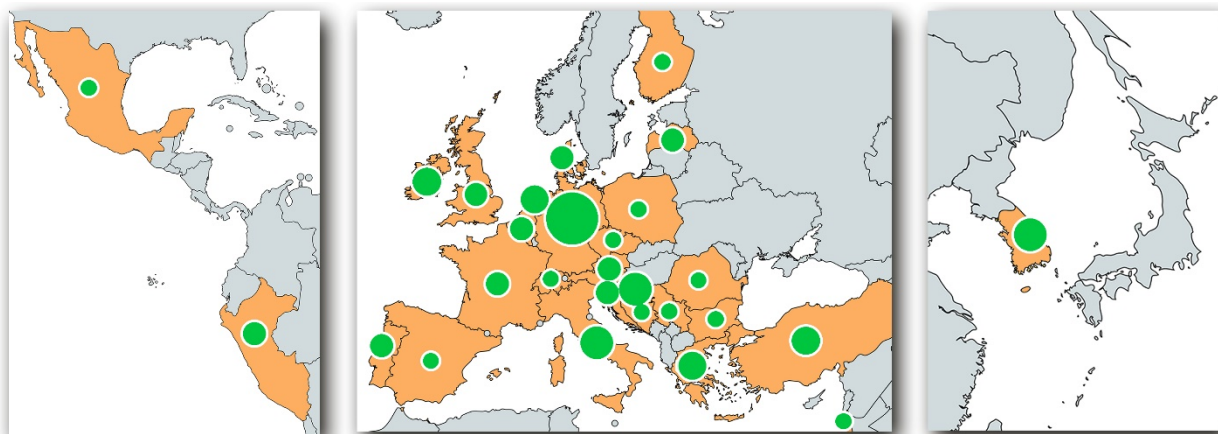
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Figure 1. Countries represented during the Practibar training courses. The green bubble sizes indicate the number of participants from a particular country ranging from 1 to 10 participants.

The agenda of the meetings is presented in Appendix I.

Participants of the training courses were expected to participate in a proficiency test. During the Proficiency Test, trace (*.ab1) files were provided for the participants to analyse them. Participants had the possibility to indicate for which groups of organisms they wanted to participate (arthropods, bacteria, fungi, invasive plants, nematodes, phytoplasmas, viruses and viroids). Trace data of one or more loci were provided for four samples per organism group. Participants were requested to assemble, analyse and report the data based on the EPPO DNA barcoding standard. The performance of the individual participants was communicated to the experts that participated in the proficiency test, and observed pitfalls and suggestions on how to improve results were shared with all participants anonymously.

2.4. Main results

At the end of each training session, participants were asked to fill-in a questionnaire to determine several success criteria:

- personal objectives met;
- quality of organization;
- quality of program;
- quality of documentation;
- number of sessions;
- content of sessions;
- relevance of sessions;

The individual criteria were scored on a scale 1 – 4 (poor – very good) and resulted in final scores ranging from 3.85 to 3.98.

To measure the impact of the training courses, a proficiency test (PT) was planned at the end of each training course. Of the 64 plant health experts that participated in the Practibar training course, 38 participated in the proficiency test. Participants received randomised anonymised participant identification numbers (Lab01-Lab38). Of the organism groups for which the

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opportunity to participate was offered, insects were requested most frequently followed by fungi and bacteria (Fig. 2).

For which organism group would you like to participate (3 samples per organism group)

38 responses
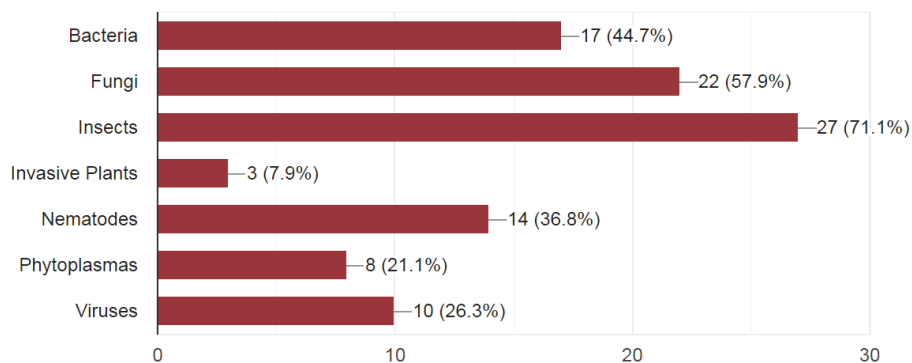
Figure 2. Organism groups for which participation was requested in the Practibar PT.

When looking at all participant-organism group combinations, Sanger Sequence data of 243 samples were analysed. The overall diagnostic sensitivity was 95%, this means that for 230 datasets the species identification was made at the correct taxonomical level. This is a major improvement compared to the success rate obtained in the Euphresco DNA barcoding TPS (87% overall success rate). Correct consensus sequence preparation improved after the Practibar training compared to the Euphresco DNA barcoding TPS (70% compared to 28%). Almost half of the 243 reports were scored "good" (48%), whereas 41% scored "fair", and 10% scored "poor" (see proficiency test report in the Appendices). However, areas for improvement were identified such as better describing analysis results and linking them to screenshots of the analysis output (e.g. tree views). A full report with the PT set-up and results is available in the Appendices.

2.5. Conclusions and recommendations to policy makers

The proficiency test results showed that there is a clear improvement of the overall correctness and quality of Sanger sequence-based identification after training. Given the interest raised, and the wishes expressed during the evaluation, training sessions such as the Practibar training course could be organized more frequently. Also, several participants expressed the wish to receive similar trainings of sequence analyses based on High-Throughput Sequence (HTS) data. Proficiency tests are a good way to monitor the impact that training has on the performance of individual laboratories. However, analysing all sequences and the reports is very time consuming. It is recommended to have a more qualitative analysis of sequence data-based results for future PTs. Participants that would like to follow-up on the individual results could then contact the PT provider.

2.6. Benefits from trans-national cooperation

Euphresco is a platform to ease research collaboration and favour knowledge exchange, in order to better use national resources available and to build/strengthen capacity. The Practibar project is a perfect example of the Euphresco philosophy. Scientists from different countries worldwide were able to benefit from the knowledge of experts from the University of Guelph and the Dutch National Plant Protection Organization; the two organisations are on the forefront respectively in the use of DNA-Barcoding as a taxonomic method for biodiversity and as a diagnostic method for pest identification. Trainees were able to improve their skills and can now use DNA barcoding in their daily activities and can raise awareness on the potential of the method in their countries. Below, the feedback of selected trainees is provided:

Ida Bartolini, Senasa, Peru: During the course offered in October 2018 by the Euphresco Practibar project I had the opportunity to learn many aspects of the method with special emphasis on the approach to selecting primers and especially the tips and recommendations to be taken in account during the bioinformatic analysis of the sequences obtained, the assembly of sequences to achieve quality consensus, the multi-locus analysis and the interpretation of results in the three best known databases: BLAST, BOLD Systems and Qbank. Other aspect I appreciated were the training on the formats for reporting results, literature and the possibility to exchange experiences with scientists from other countries. The training was very enriching, and I congratulate the initiative because it has allowed my group at SENASA to strengthen activities of the insect identification project with DNA Barcoding. SENASA has a database of 2600 records and has organized some events such as a workshop course and a theoretical course in 2018 and a seminar in 2019. At the moment we are using DNA barcoding for insect identification but for the next year it is planned to use the method for the identification of phytoplasms, bacteria and fungi. It is worth mentioning that in Peru there are other institutions that use DNA Barcoding, linked to the PeBOL (Peruvian Barcode of Life) http://pebol.org/ .

Aleksandra Bulajic, University of Belgrade, Serbia: Participating in PRACTIBAR to obtain the practical training for the analysis of the sequences of various quarantine organisms was a unique and important opportunity not only for me but for everyday practice in the Laboratory of Phytopathology, at Faculty of Agriculture-University of Belgrade. Engaged in Plant Quarantine official activities with the authorization of the Ministry of Agriculture of the Republic of Serbia, we have been working on the detection of various plant pathogenic viruses, fungi and fungi-like organism for over 10 years. The work-flow for the detection and identification of quarantine organisms followed in our lab had been developed over the years based on literature and experience. After my training in barcoding, our routine protocol for sequence analyses was upgraded by changing the system for data organization, by including Bold systems and Q-bank data bases in everyday queries during the sequence analyses, and above all by implementing the questionnaire for the documentation of the whole process of sequences analysis. The knowledge gained during the training was also disseminated by training two PhD students in my laboratory and was presented at a regional scientific Symposium (Bulajić *et al*., 2018. Utvrđivanje karantinskog statusa *Phytophthora ramorum* in Serbia. Proceedings from XV Symposia on Plant Protection in Bosnia and Herzegovina, Sarajevo, Bosnia and Herzegovina, pp. 17-18).

3. Publications

3.1. Article(s) for publication in the EPPO Bulletin
None.

3.2. Article for publication in the EPPO Reporting Service
None.

3.3. Article(s) for publication in other journals
None.

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4. Open Euphresco data

Video-tutorials of the training sessions were produced and published on YouTube:

Euphresco Practibar Workshop: from genome to barcode
The video-tutorial is accessible from the url below:
https://www.youtube.com/watch?v=kax_zl2IQiM&list=PLoVf4Pt04Db53pUVTI8qwcWkWgUgg46gm&index=2&t=0s
The video gives basic background information on genomes and the use of DNA barcoding as a tool in support in species identification.

Euphresco Practibar Workshop: use of EPPO Standard
The video-tutorial is accessible from the url below:
https://www.youtube.com/watch?v=v7nWT4fprVg&list=PLoVf4Pt04Db53pUVTI8qwcWkWgUgg46gm&index=3&t=0s
The video describes the EPPO DNA Barcoding standard in detail and explains the different elements in the document, such as primer tables, decision scheme, controls and sequence analysis guidance.

Euphresco Practibar Workshop: DNA barcoding wet lab
The video-tutorial is accessible from the url below:
https://www.youtube.com/watch?v=kGodtgHmK3Q&list=PLoVf4Pt04Db53pUVTI8qwcWkWgUgg46gm&index=4&t=0s
The video describes the web lab steps that need to be followed to get a better understanding on how raw Sanger sequence data is obtained.

Euphresco Practibar Workshop: assembly of Sanger sequence data
The video-tutorial is accessible from the url below:
https://www.youtube.com/watch?v=2OpylrF2N_Y&list=PLoVf4Pt04Db53pUVTI8qwcWkWgUgg46gm&index=5&t=0s
The video describes best practices for generating consensus sequences from raw Sanger sequence data.

Euphresco Practibar Workshop: barcoding workflow
The video-tutorial is accessible from the url below:
https://www.youtube.com/watch?v=zri9OX4rzr0&list=PLoVf4Pt04Db53pUVTI8qwcWkWgUgg46gm&index=6&t=0s
The video briefly explains what is a standardized workflow for DNA barcoding, from tissue to DNA sequence. The main components presented were: front-end (pre-processing) including collection of organisms, databasing, imaging, and tissue sample; molecular processing with all the steps (DNA extraction, amplification and sequencing of the standard barcode regions in animals, plants and fungi); bioinformatics (post-processing) which includes sequence editing and validation, comparison with the online public databases and interpretation of results.

Euphresco Practibar Workshop: influence of PCR on sequencing
The video-tutorial is accessible from the url below:
https://www.youtube.com/watch?v=iOBj7dGlDIs&list=PLoVf4Pt04Db53pUVTI8qwcWkWgUgg46gm&index=7&t=0s
The tutorial describes how wet lab activities can influence your sequence data quality, and how they can affect downstream analyses.

Euphresco Practibar Workshop: Barcode of Life Data Systems (BOLD)
The video-tutorial is accessible from the url below:

https://www.youtube.com/watch?v=neIQ3KNBXN8&list=PLoVf4Pt04Db53pUVTI8qwcWkWgUgg46gm&index=8&t=0s

The largest molecular database dedicated to DNA barcoding data and metadata is the Barcode of Life Data Systems (BOLD) hosted by the University of Guelph (Canada).This video is presenting briefly the main components of BOLD, the steps required to upload, validate and analyse data in BOLD, and the data life cycle for barcode data.

Euphresco Practibar Workshop: online resources for DNA analysis and interpreting Blast results

The video-tutorial is accessible from the url below:

https://www.youtube.com/watch?v=nJy_U7vyRRY&list=PLoVf4Pt04Db53pUVTI8qwcWkWgUgg46gm&index=9&t=0s

The video provides an introduction to the online resources that can be used to analyse consensus sequences, the analysis tools that can be used, and how to interpret Blast results and tree views.

Euphresco Practibar Workshop: multilocus introduction

The video-tutorial is accessible from the url below:

https://www.youtube.com/watch?v=h2MAfvzpkYI&list=PLoVf4Pt04Db53pUVTI8qwcWkWgUgg46gm&index=10&t=0s

This video introduces how to use multiple loci in a single analysis; Sometimes, a single barcode locus is not enough.

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Appendix I Training agenda

Day 1 From 09:00 to 17:00	▪ Welcome and introduction ▪ From genome to DNA barcoding ▪ EPPO standard PM7/129(1) DNA barcoding ▪ DNA barcoding wet lab ▪ Assembly of Sanger sequence data
Day 2 From 09:00 to 17:00	▪ How PCR results can influence the downstream analysis ▪ Online resources used for data-analysis (NCBI, BOLD, Q-bank) ▪ Interpreting blastn and blastx results ▪ Is one genetic locus enough? Introduction to multi locus sequence analysis ▪ Sequencing analysis and quality assurance
Day 3 From 09:00 to 17:00	▪ Creating sequencing analysis reports ▪ Practical sessions
Day 4 From 09:00 to 15:00	▪ Tough cases ▪ Any other business ▪ Evaluation
	End of the meeting

Additional Information needed for sequence analysis of three different virus sequences provided for the Practibar Proficiency test

Sample 1 Tospoviruses

1.   General information

1.1   Protocols for the identification of selected regulated tospoviruses using conventional RT-PCR followed with Sanger Sequencing analysis. Based of the N protein the different tospoviruses will be grouped in five different clades. (Figure 1)

1.2   Protocol has been implemented at the National Plant Protection Organization (NPPO-NL)

1.3   The untranslated region (UTR) and nucleoprotein (N) gene test described in section 1.2.1. is used for species detection and identification of selected tospoviruses (American clade 1)

1.4   Primer sequences, amplicon sizes and thermocycler settings are provided in the test specific sections. HPLC purified primers should be ordered to avoid non-specific PCR amplification.

1.5   Reaction mixes are based on the OneStep RT-PCR enzym-mix (Qiagen)

1.6   Molecular grade water (MGW) is used to set up reaction mixes; this should be purified (deionised or distilled), sterile (autoclaved or 0.45 µM filtered) and nuclease free.

1.7   Amplification is performed in a Peltier-type thermocycler with heated lid, e.g. C1000 (BioRad).
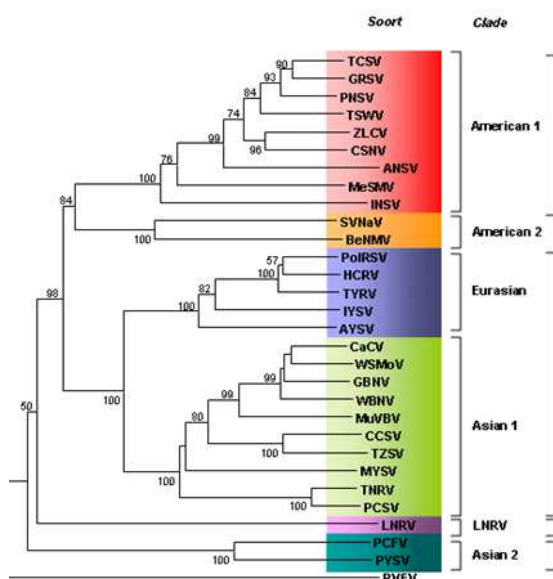
Fig 1: Five different clades of Tospoviruses

2. Methods
2.1   RT-PCR of the 3' untranslated region (UTR) and nucleoprotein (N) gene of American clade 1 tospovirus

2.1.1   PCR-sequencing of 750 bp (amplicon size incl. primers) of the 3'UTR and nucleoprotein N gene of the American clade 1 Tospovirus is adapted from Hassani-Mehraban *et al.,* 2014.

2.1.2   Primer sequences are described in the table below.

Primer name	Primer sequence (5'- 3' orientation)	Primer used for	
		PCR	Sequencing
AM1-FW	GGG GGA TCC AGA GCA ATT GTG TC	X	X
AM1-RV	CTT TGC TTT TCA GCA CAG TGC A	X	X

2.1.3   Master mixes are prepared according to the table below.

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water	N.A.	16.0	N.A.
OneStep RT-buffer (Qiagen)*	5 x	5.0	1x
dNTP's (Qiagen)	10 mM each	1.0	0.4 mM
AM1-FW	10 µM	0.5	0.2 µM
AM1-RV	10 µM	0.5	0.2 µM
OneStep RT-PCR enzyme mix (Qiagen)	5U/µl	1.0	1U
Subtotal		24.0	
RNA extract		1.0	
Total		25.0	

* or adequate PCR master mixes containing a polymerase with proof reading activity

2.1.4   Thermocycler profile: 30 min 50 °C, 15 min 95°C, 35x (30 sec 94°C, 30 sec 50°C, 1 min 72°C), 5 min 72°C, ∞ 20°C
2.1.5   Cycle sequencing reactions are performed using the obtained PCR products with primers used for amplification in separate reactions.
2.1.6   The amplified locus is a mix of coding and non-coding region. Translation tables do not apply.

Sample 2 Pospiviroids (PSTVd, TCDVd, MPVd en TPMVd)

1.   General information
1.1   Protocols for the identification of selected regulated pospiviroids van PSTVd, TCDVd, MPVd en TPMVd using conventional RT-PCR followed with Sanger Sequencing analysis. Based of the complete genome these different pospiviroids will be detected and identified.
1.2   Protocol has been implemented at the National Plant Protection Organization (NPPO-NL)
1.3   The test described in section 2.2.1. is used for species detection and identification of selected pospiviroids.
1.4   Primer sequences, amplicon sizes and thermocycler settings are provided in the test specific sections. HPLC purified primers should be ordered to avoid non-specific PCR amplification.
1.5   Reaction mixes are based on the OneStep RT-PCR enzym-mix (Qiagen)
1.6   Molecular grade water (MGW) is used to set up reaction mixes; this should be purified (deionised or distilled), sterile (autoclaved or 0.45 µM filtered) and nuclease free.
1.7   Amplification is performed in a Peltier-type thermocycler with heated lid, e.g. C1000 (BioRad).

2.   Methods
2.1   RT-PCR of the complete genome of the genus pospiviroids (PSTVd, TCDVd and (T)PMVd)
2.1.1   PCR-sequencing of 360 bp (amplicon size incl. primers) of the complete genome of pospiviroids PSTVd, TCDVd, MPVd and TPMVd is adapted from Shamloul *et al.,* 1997.
2.1.2   Primer sequences are described in the table below.

Primer name	Primer sequence (5'- 3' orientation)	Primer used for	
		PCR	Sequencing
3H1	ATC CCC GGG GAA ACC TGG AGC GAA C	X	X
2H1	CCC TGA AGC GCT CCT CCG AG	X	X

2.1.3   Master mixes are prepared according to the table below.

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water	N.A.	15.0	N.A.
OneStep RT-buffer (Qiagen)*	5 x	5.0	1x
dNTP's (Qiagen)	10 mM each	1.0	0.4 mM
3H1	10 µM	1.0	0.4 µM
2H1	10 µM	1.0	0.4 µM
OneStep RT-PCR enzyme mix (Qiagen)	5U/µl	1.0	1U
Subtotal		24.0	
RNA extract		1.0	
Total		25.0	

* or adequate PCR master mixes containing a polymerase with proof reading activity

2.1.4   Thermocycler profile: 30 min 50°C, 15 min 95°C, 40x (30 sec 94°C, 30 sec 63°C, 1 min 72°C), 10 min 72°C, ∞ 20°C

2.1.5   Cycle sequencing reactions are performed using the obtained PCR products with primers used for amplification in separate reactions.

2.1.6   The amplified locus exists of several (nearly) conserved regions: the conserved terminal right domain (RD), the terminal conserved region (TCR) and the upper - and lower central conserved region (CCR).   Translation tables do not apply.

2.1.7   The primersequences of the complete genome of pospiviroids does not to be trimmed. Also the starting position of the sequence has to be adapted manually. In general the start position of pospiviroids starts with CGG. The sequence ends with TTCCT or CCCT. The upper CCR is around nucleotide position 90: CCCCGGGG

Sample 3 Begomovirus

1.   General information

1.1   Protocol for the detection and identification of begomoviruses using conventional PCR followed with Sanger Sequencing analysis. Based of a part of the DNA-A molecule begomoviruses will be detected and identified.

1.2   Protocol has been implemented at the National Plant Protection Organization (NPPO-NL)

1.3   The test described in section 3.2.1. is used for detection and identification of begomoviruses

1.4   Primer sequences, amplicon sizes and thermocycler settings are provided in the test specific sections. HPLC purified primers should be ordered to avoid non-specific PCR amplification.

1.5   Reaction mixes are based on the GoTaq DNA polymerase mix (Promega)

1.6   Molecular grade water (MGW) is used to set up reaction mixes; this should be purified (deionised or distilled), sterile (autoclaved or 0.45 µM filtered) and nuclease free.

1.7   Amplification is performed in a Peltier-type thermocycler with heated lid, e.g. C1000 (BioRad).

2.   Methods
2.1   PCR the detection and identification of the genus begomovirus

2.1.1   PCR-sequencing of 550 bp (amplicon size incl. primers) of the DNA-A molecule of begomoviruses is adapted from Wyatt *et al.* 1996.

2.1.2   Primer sequences are described in the table below.

Primer name	Primer sequence (5'- 3' orientation)	Primer used for	
		PCR	Sequencing
AV494	GCC YAT RTA YAG RAA GCC MAG	X	X
AC1048	GGR TTD GAR GCA TGH GTA CAT G	X	X

2.1.3   Master mixes are prepared according to the table below.

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water	N.A.	11.3	N.A.
Colorless GoTaq Flexi buffer (Promega)*	5 x	5.0	1x
MgCl2 (Promega)	25 mM	2.0	2 mM
dNTP's (Promega)	10 mM each	0.5	0.2 mM
AV494	10 µM	2.0	0.8 µM
AC1048	10 µM	2.0	0.8 µM
GoTaq DNA polymerase (Promega)	5U/µl	0.2	1U
Subtotal		23.0	
DNA extract		2.0	
Total		25.0	

* or adequate PCR master mixes containing a polymerase with proof reading activity

2.1.4   Thermocycler profile: 2 min 94°C, 10x (15 sec 94°C, 20 sec 65°C (touch down 1ºC/ cycle), 30 sec 72°C, 30x (15 sec 94°C, 20 sec 55°C, 30 sec 72°C), 10 min 72°C, ∞ 20°C

2.1.5   Cycle sequencing reactions are performed using the obtained PCR products with primers used for amplification in separate reactions.

2.1.6   The amplified locus exists of the movement protein (AV2) and coat protein (AV1).

Date: LABID: 1st Assessor:

Create table 1 for each loci

Consensus sequence information

Table 1

1	LIMS number and/or collection number	
2	Name locus	
3	Properties locus	☐ Coding ☐ non-Coding ☐ mix coding and non-Coding
4	Amplification primers used	/
5	Cycle sequence primers used	☐ same as amplification primers ☐ other:
6	Sequencing performed	☐ In-house ☐ external company (*)
7	n cycle sequence reactions performed: n chromatograms in consensus	: (* when not 1:1)
8a	Assembly method	☐ *De novo* assembly ☐ Reference assembly (go to 8b)
8a	Reference sequence used (collection or NCBI number)	
9	Untemplated –dA and amplification primers trimmed?	☐ yes ☐ no (*)
10	Obtained consensus length:expected consensus length	bp : bp (* when not 1:1)
11	% High-quality (HQ) bases (Phred score >40)	%
12	Orientation and start position consensus sequence correct?	☐ yes ☐ no(*)

* Provide detailed explanation below

Detailed information:

Analysis sources used, analysis settings and results

Table 2

Source	Analysis information	Parameters	Explanation, reference to analysis results and conclusion per database$
NCBI	Database used	☐ nucleotide collection (nr/nt) ☐ other (#)	
	Program used	☐ Megablast ☐ blastn ☐ other (#)	
	Tree method	☐ Fast Minimum Evolution ☐ NJ	
	Organism (optional)	☐ not used ☐ used (#)	
	Exclude (optional)	☐ not used ☐ used (#)	
BOLD	Database used	☐ COI ☐ ITS ☐ rbcL & matK	
	Subset COI database	☐ All ☐ Species level ☐ Public record	
	"Search result" BOLD	When applicable (#)	
Q-bank	Analysis method	☐ Single locus* ☐ Multi locus (#)	
	Settings modified	☐ no ☐ yes (#)	
	Tree method	When applicable (#)	
Other	When applicable provide details	Dataset	

\#   Provide details in the last column of the table.

*   Turn non-redundant GenBank option off.

$ e.g. number of nucleotides in analysis, % overlap, % similarity with 1st and/or specific match, E-value, clustering with taxon Z

Conclusion

e.g. Based on the analysis of xxx nt of locus X and xxx nt of locus Y in databases A, B and C we conclude that sample xxx is possibly/likely/very likely (not) taxon Z.

Note: Assessors of sequence data and the analysis thereof can use different semi-quantifiable levels of certainty when drawing conclusions, such as *possibly/likely/very likely*. The level of certainty is based on the experience of the assessor with a certain pest (in combination with locus/loci used, host and origin), the quality of raw sequence data, the quality of the consensus sequence, blast and clustering results and the availability of sequence data in data sources consulted. It has to be noted that, using identical sequences for the analysis, the level of certainty can differ between assessors. Verification of the Sanger sequence analysis by a second assessor safeguards the drawing of conclusions that are as accurate as possible.

Analysis results and other supportive information
For example, consensus sequence(s) and print screens of BLAST hit tables, tree views, alignment views, etc. with reference to Table 2 that lead to conclusions per database and to the general conclusion.

Figure 1

Figure 2

Figure 3

…

Euphresco Practibar proficiency test report

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Introduction

DNA barcoding is increasingly used as a diagnostic tool in phytosanitary laboratories. DNA barcoding protocols for selected EU-regulated arthropods, bacteria, fungi, invasive plants, nematodes and phytoplasmas were developed and validated within the EU QBOL and Euphresco DNA Barcoding projects [1, 2]. The international comparison studies organized under both projects showed that the interpretation of analysis results had the biggest (negative) influence on the diagnostic sensitivity of the DNA barcoding tests. Re-analysis of the consensus sequence data provided by TPS participants showed that an overall diagnostic sensitivity of 99% could be obtained [2].

The need for training experts of the competent organizations was highlighted in the recommendations given at the end of the Euphresco project and by the EPPO Panel on Diagnostics and Quality Assurance. To address this need, a four-day training course was developed under the Euphresco Practibar project. Central in the training course was the EPPO diagnostic standard on DNA barcoding for selected EU-regulated pests [3], and participants received theoretical background information on DNA barcoding which was put to practice in hands-on sessions. The importance of the use of controls, general quality assurance and standardized reporting were highlighted.

Four training courses were organized in 2017 and 2018: two at the EPPO headquarters in Paris, France; and two at the Dutch National Plant Protection Organization in Wageningen, the Netherlands. In total, 64 plant health experts, with different levels of experience in regard to DNA barcoding, participated in the training courses, representing 29 countries and four continents (Fig. 1).
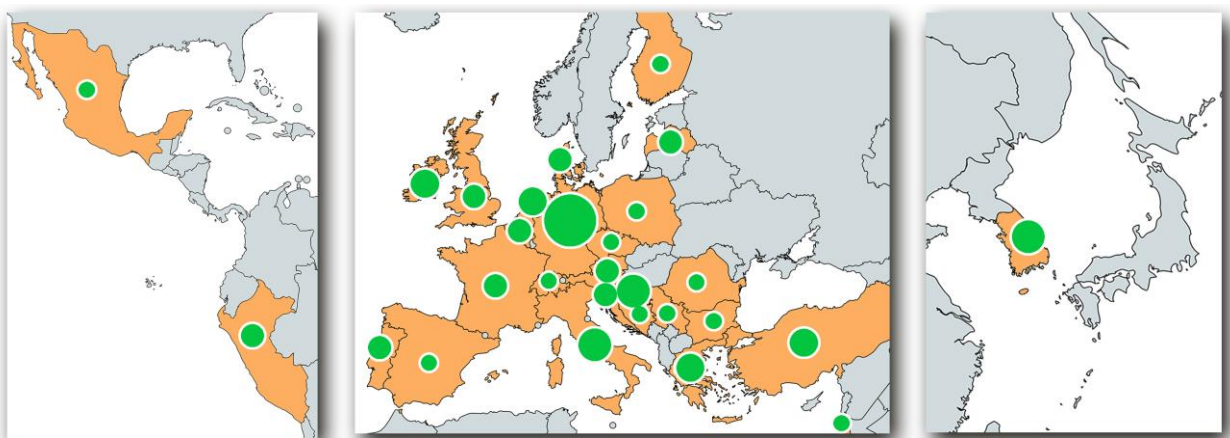
Figure 1. Countries represented during the Practibar training courses. The green bubble sizes indicate the number of participants from a particular country ranging from 1 to 10 participants.

To measure the impact of the training courses, a proficiency test (PT) was planned at the end of each training course. Participation to the PT for at least one organism group was initially regarded as mandatory. Due to circumstances beyond our control it was not possible to organize the PT in time. Instead, the PT was organized after completion of the last training course and participants could choose if they were willing to take part in the PT.

Material and Methods

Call for Proficiency Test participation Online registration forms were send to the 64 plant health experts that participated in the 2017 and 2018 Practibar training courses. Participants could register for one or more of the seven organism groups included: bacteria, fungi, insects, invasive plants, nematodes, phytoplasmas and viruses. Participants were informed they would receive Sanger sequence data for three samples for each of the organism group they expressed interest. This data had to be analysed following EPPO standard PM7/129(1) as shown during the Practibar course. The invitations were sent at the end of March 2019, and partners had two weeks to register their interest. Participants that registered for participation were randomized and a lab-ID was assigned.

Selection of samples and acceptance controls Per organism group, Sanger sequence data of three samples were selected from the NPPO-NL database. This data was generated from biological material intercepted during routine inspections using EPPO PM7/129(1). Data was re-analyzed to ensure that the specimen could be identified at the required taxonomical level. Table 1 gives an overview of the species and sequence data selected in the PT.

Sanger sequence data was exported from the NPPO-NL Geneious database as *.ab1 files and renamed to indicate the organism group, sample, test in PM7/129(1) used to generate the data, and sequencing primer used to generate the data. For instance, *BAC01_222_Reverse 358-339.ab1* for the sequence data generated with sequencing primer Reverse 358-339 for the amplicon obtained with test 2.2. of appendix 2 of the EPPO standard PM7/129(1) (16S rDNA) for bacterial sample 1 (*Ralstonia solanacearum sensu lato*).

Proficiency test and reporting Per organism group, PT participants received an email containing the Sanger sequence data and reporting information. When participants registered for more than one organism group, you received a separate email for each of the organism groups. The Sanger sequence data included belonged to three different samples which had to be analyzed individually. For the analysis of the samples, the guidelines of in EPPO PM7/129(1) "DNA barcoding as an identification tool for a number of regulated pests" had to be followed. For the virological samples, short test descriptions were provided following the format of EPPO standard PM7/129(1) as viruses are not included in the barcoding standard (S-file 1). Analysis results had to be documented using a standardized form (S-file 2). Apart for the reporting form, the generated consensus sequences had to be provided to the PT organizer, and participants had to complete an online form (table 2) to allow further analyses. Participants had 2 months to analyze and report their samples.

Table 1. Overview of samples included in the Practibar PT.

Group	#	NPPO-NL ID	Scientific name	Locus/loci (EPPO PM7/129(1) appendix & test)
Bacteria	1	PD2762	*Ralstonia solanacearum sensu lato*	16S rDNA (2.2.2) *mutS* (2.2.4)
	2	PD406	*Clavibacter michiganesis* subsp. *sepedonicus*	16S rDNA (2.2.2) *gyrB* (2.2.3)
	3	PD992	*Xanthomonas axonopodis* pv *dieffenbachiae*	16S rDNA (2.2.2) *gyrB* (2.2.5) *AvrBs2* (2.2.6)
Fungi	1	PD89-1016-4	*Stagonosporopsis chrysanthemi*	ITS (3.2.3) *act* (3.2.6)
	2	PD013-04088118	*Ceratocystis fagacearum*	ITS (3.2.3)
	3	PD06-03209311	*Phytophthora kernoviae*	ITS (3.2.3) *cox1* (3.2.7)
Insects	1	4367125	*Spodoptera exigua*	*cox1* (1.2.2)
	2	CMV3	*Aedes atropalpus*	*cox1* (1.2.2)
	3	6252890	*Monochamus galloprovincialis*	*cox1* (1.2.2)
Invasive Plants	1	6147492	*Ludwigia grandiflora*	rbcL (4.2.2) trnH-psbA (4.2.3)
	2	6147396	*Hydrocotyle ranunculoides*	rbcL (4.2.2) trnH-psbA (4.2.3)
	3	6618418	*Myriophyllum hetrerophyllum*	rbcL (4.2.2) trnH-psbA (4.2.3)
Nematodes	1	5048291	*Bursaphelenchus xylophilus*	18S rDNA (5.2.2) 28S rDNA (5.2.3) *cox1* (5.2.4)
	2	4088257A	*Aphelenchoides ritzemabozi*	18S rDNA (5.2.2) *cox1* (5.2.4)
	3	Globodera tabacum 2012	*Globodera tabacum*	18S rDNA (5.2.2) 28S rDNA (5.2.3) *cox1* (5.2.4)
Phytoplasmas	1	4344310	*Candidatus* Phytoplasma phoenicium	*tuf* (6.2.2) 16S rDNA (6.2.3)
	2	5446042	*Candidatus* Phytoplasma solani	*tuf* (6.2.2) 16S rDNA (6.2.3)
	3	4226277	*Candidatus* Phytoplasma trifolii	*tuf* (6.2.2) 16S rDNA (6.2.3)
Viruses and viroids	1	2560570	Tomato spotted wilt virus TSWV	3'UTR - N protein[a]
	2	32760557	Potato Spindle Tuber Viroid PSTVd	Complete genome[a]
	3	5703784	Tomato leaf curl new delhi virus TolCNDV	Coat protein[a]

a. Viral protocol are not part of the EPPO standard and are provided as supplementary file (S-file 1)

Table 2. Online reporting form questions

Generic questions	Did you use PM7/129 (1) DNA barcoding as an identification tool for number of regulated pests for the analysis of the samples?
	Which software analysis package did you use to generate consensus sequences?
	How did you experience the data analysis of the [ORGANISM GROUP] samples?
	Do you have any additional remarks or suggestions regarding the PT?
Specific questions for locus/loci analyzed	Could you create a consensus sequence for locus [LOCUS]?
	What was the consensus sequence length in base pairs (bp) for locus [LOCUS]?
	Which online databases did you use to draw a final conclusion?
	Taxonomical level at which an identification could be made.
	State possible identity of the specimen (scientific name at which a identity could be made reliably).
	Copy and paste your conclusion as stated in the reporting form.

Analysis of PT results Participants returned one or more consensus sequence(s) per diagnostic sample to the PT organizer. All consensus sequences were aligned to the reference sequence previously defined by the organizer. Consensus sequences produced by the PT participants were checked on correct length (primer trimming), orientation, calling of ambiguous sites and overall sequence similarity. Sequence analysis reports were inspected to determine if all required information was recorded on the analysis report. Sequencing analysis reports were ranked as good, fair or poor using the following criteria:
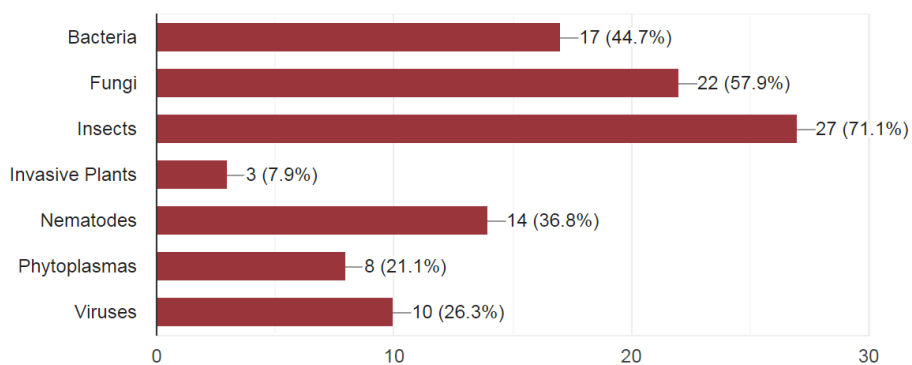
Good   The conclusion was correct and all analysis steps and analyses output have been thoroughly documented allowing a second assessor to draw the same conclusion as the PT participant

Fair   A correct conclusion was provided, but from the information provided did not allow (complete) reproduction of the provided conclusion

Poor   The conclusion was incomplete and/or no information was provided to assess the reliability of the analysis performed.

Results

Of the 64 plant health experts that participated in the Practibar training course 38 participated in the proficiency test. Participants received randomized anonymized participant identification numbers (Lab01-Lab38). Of the organism groups for which the opportunity to participate was offered, insects were requested most frequently followed by fungi and bacteria (Fig. 2)

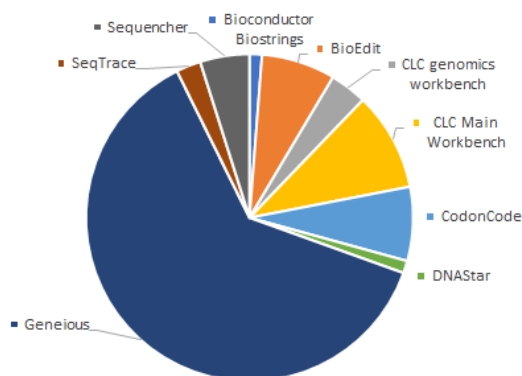## For which organism group would you like to participate (3 samples per organism group)

38 responses

Organism	Responses
Bacteria	17 (44.7%)
Fungi	22 (57.9%)
Insects	27 (71.1%)
Invasive Plants	3 (7.9%)
Nematodes	14 (36.8%)
Phytoplasmas	8 (21.1%)
Viruses	10 (26.3%)

Figure 2. Organism groups for which participation was requested in the Practbar PT.

Generic questions Participants reported back per organism group, and participants indicated that in all but one participant-organism group combination (99%) the EPPO DNA barcoding standard was used when analyzing the provided Sanger sequence data. Participants used a wide range of sequencing analysis tools when analyzing their data, and in general analysis of sequence data was regarded easy to rather difficult (Fig. 3A and B)

Figure 3. Generic questions PT organization. **A** Sanger sequence analysis software used, and **B** difficulty experienced.

Below, several useful additional remarks made by the participants are shown:

- "I believe the EPPO Standard and Practibar guidance for virus datasheet would both benefit by having gene diagrams of where the primers target. This would be especially useful for coding regions but even non-coding regions are targeted for specific reasons and a diagram would help the user to understand why these are/aren't arbitrary and assess whether these would be useful for their specific needs when implementing barcoding."
- "One difficulty encountered with the analysis of the [Phytoplasma] results is the fact that NCBI has for many entries only common names of the organisms instead of the latin names. E.g. 'Cantharus roseus' Naxos yellows phytoplasma instead of 'Candidatus Phytoplasma phoenicium'. Thus, more databases (e.g. global EPPO) and literature must be checked to determine the different hits."
- "It would be useful to have comments in the EPPO standard PM7/129 (1), on a comparison between the DNA barcoding protocol for the phytoplasmas and certain commonly used PCR approaches … recommended in certain other EPPO standards (e.g. for 'Ca. Phytoplasma pyri')."
- "Documenting and reporting of Sanger sequence analyses is very time-consuming"

Success rates participants When regarding all participant-organism group combinations, Sanger Sequence data of 243 samples were analyzed. The overall success rate was 95%, this means that for 230 datasets the correct species identification was made at the taxonomical level indicated in table 1. Below, graphical representations are provided for the participant-sample combinations for each organism group. Green circles indicate a species identification at the correct taxonomical level, whereas red indicates an incorrect identification. Incorrect identifications are mainly caused by conservative identification of the specimen (e.g. identification at genus level when species level was possible).

Fungi

	Sample 1	Sample 2	Sample 3		Sample 1	Sample 2	Sample 3
LAB01	🟢	🟢	🟢	LAB20	🟢	🟢	🟢
LAB02	🟢	🟢	🟢	LAB22	🟢	🟢	🟢
LAB04	🟢	🟢	🟢	LAB24	🟢	🟢	🟢
LAB05	🟢	🟢	🟢	LAB25	🟢	🟢	🟢
LAB09	🔴	🟢	🟢	LAB26	🟢	🟢	🟢
LAB11	🟢	🟢	🟢	LAB27	🟢	🟢	🟢
LAB13	🟢	🟢	🟢	LAB33	🟢	🟢	🟢
LAB17	🟢	🟢	🟢	LAB34	🟢	🟢	🟢
LAB18	🟢	🟢	🟢	LAB37	🟢	🟢	🟢

Insects

	Sample 1	Sample 2	Sample 3		Sample 1	Sample 2	Sample 3		Sample 1	Sample 2	Sample 3
LAB01	🟢	🟢	🟢	LAB11	🟢	🟢	🟢	LAB27	🟢	🟢	🟢
LAB02	🟢	🟢	🟢	LAB13	🟢	🟢	🟢	LAB29	🟢	🟢	🟢
LAB03	🟢	🟢	🟢	LAB16	🟢	🟢	🟢	LAB32	🟢	🟢	🟢
LAB06	🟢	🟢	🟢	LAB17	🟢	🟢	🟢	LAB33	🟢	🟢	🟢
LAB07	🟢	🟢	🟢	LAB18	🟢	🟢	🟢	LAB34	🟢	🟢	🟢
LAB08	🟢	🟢	🟢	LAB20	🟢	🟢	🟢	LAB35	🟢	🟢	🟢
LAB09	🟢	🟢	🟢	LAB25	🟢	🟢	🟢	LAB37	🟢	🟢	🟢
LAB10	🟢	🟢	🔴	LAB26	🟢	🟢	🟢				

Invasive Plants

	Sample 1	Sample 2	Sample 3
LAB03	🟢	🟢	🟢
LAB34	🟢	🟢	🟢
LAB37	🟢	🟢	🟢

Phytoplasmas

	Sample 1	Sample 2	Sample 3
LAB06	🟢	🟢	🟢
LAB12	🟢	🟢	🟢
LAB25	🟢	🟢	🟢
LAB27	🟢	🟢	🟢
LAB34	🟢	🟢	🟢
LAB36	🟢	🟢	🟢

Nematology

	Sample 1	Sample 2	Sample 3
LAB01	🟢	🟢	🔴
LAB04	🟢	🟢	🔴
LAB08	🔴	🟢	🔴
LAB11	🟢	🟢	🟢
LAB18	🟢	🟢	🟢
LAB29	🟢	🟢	🟢
LAB30	🟢	🟢	🔴
LAB31	🟢	🟢	🔴
LAB34	🟢	🟢	🔴

Virology

	Sample 1	Sample 2	Sample 3
LAB03	🟢	🟢	🟢
LAB09	🟢	🟢	🟢
LAB11	🟢	🟢	🟢
LAB16	🟢	🟢	🟢
LAB24	🟢	🟢	🟢
LAB25	🟢	🟢	🟢
LAB30	🟢	🟢	🟢
LAB34	🟢	🟢	🟢
LAB36	🟢	🟢	🟢

Assessment of results In total, participants generated 446 consensus sequences, and 243 analysis reports. All consensus sequences were visually assessed to determine if the correct sequence was obtained, if amplification primers were trimmed correctly, and if the obtained consensus sequences were in the correct orientation. In 67% of all consensus sequences, primers were trimmed correctly, the consensus sequence and orientation were correct. Sequencing analysis reports were ranked as good, fair or poor. Almost half of the reports scored "good" (48%), whereas 41% scored "fair", and 10% scored "poor". In general, improvements could be made by better describing analysis results and linking them to screenshots of the analysis output (e.g. tree views).

Literature

1. van de Vossenberg, B.T.L.H., M. Westenberg, and P.J.M. Bonants, *DNA barcoding as an identification tool for selected EU-regulated plant pests: an international collaborative test performance study among 14 laboratories.* EPPO Bulletin, 2013. **43**(2): p. 216-228.
2. van de Vossenberg, B.T.L.H., J. Hodgetts, and M. Maes, *EUPHRESCO DNA Barcoding - Optimizing and validating DNA barcoding protocols for plant pests.* Online publication, 2016.
3. EPPO/OEPP, *PM 7/129 (1) DNA barcoding as an identification tool for a number of regulated pests.* EPPO Bulletin, 2016. **46**(3): p. 501-537.