UPOV

International Union for the Protection of New Varieties of Plants

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Technical Working Party on Testing Methods and Techniques **TWM/3/25**

Third Session
Beijing, China, April 28 to May 1, 2025

Original: English
Date: April 24, 2025

ISTA UPDATE ON THE USE OF TECHNIQUES FOR VARIETY IDENTIFICATION AND VERIFICATION

Document prepared by an expert from the International Seed Testing Association (ISTA)

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1. During the meeting, we are going to present an update on the activities of the ISTA Variety Committee (VARCOM) in regards of the use of molecular markers for variety identification, single trait determination and the use of image analysis.

2. Together with the statistical committee, we discussed the use of a new methodology for data analysis and interpretation of comparative tests (CT) results. This will provide a more realistic analysis of DNA data coming from CT and will unify criteria with other committees that produce and analyze CT data.

3. During the last year, ISTA VARCOM continued the work on the development and validation of a single trait test for the determination of adventitious presence of annual rye grass varieties (ARG) in perennial varieties (PRG) by means of real time and digital PCR. Some results will be presented.

4. Variety identification is one of the main topics for the committee and the use of the most advanced technologies is a challenge. Validation of methods using image analysis was never done before in ISTA and now is a key point. Some draft analysis coming from the second data set produced, will be presented.

5. Finally we will show some advances on the DNA handbook.

6. The annex to this document contains a copy of a presentation “ISTA update on the use of techniques for variety identification and verification”, to be made by an expert from the International Seed Testing Association (ISTA), at the third session of the TWM.

[Annex follows]

ISTA update on the use of techniques for variety identification and verification

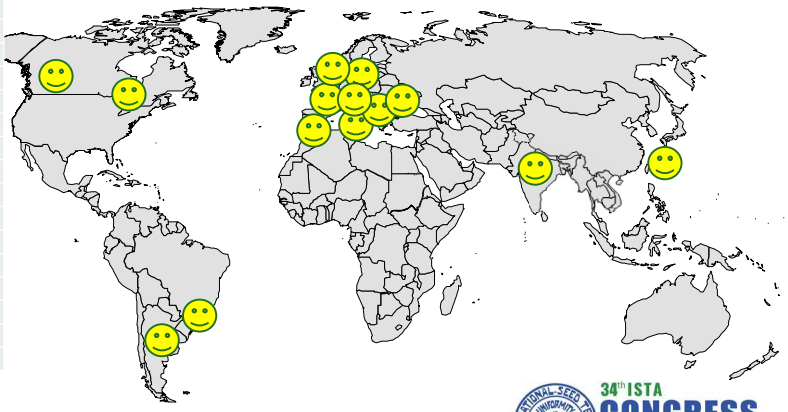
Ana Vicario – VARCOM Chair
TWM - UPOV
April, 2025

34th ISTA CONGRESS CHRISTCHURCH NEW ZEALAND 05-09 May 2025

1

ISTA VARCOM members

	COMMITTEE MEMBERSHIP LIST	Active since
1	Chair: Ana Laura Vicario	2007
2	Vice-Chair: Marie-Claude Gagnon	2020
3	Sean Walkowiak	2022
4	Chiara Delogu	2007
5	Anne Bernole	2015
6	Kae-Kang Hwu	2007
7	Kunusoth Keshavulu	2007
8	Ksenija Taski-Ajdukovic	2007
9	Mariana Menoni	2021
10	Ksenia Markovic	2007
11	Berta Killermann	2007
12	Ana Patricia Fernandez Getino	2021
13	Lorella Andreani	2023
14	Umashankar Bellan	2023
15	Beni Kaufman	2024



Chapter 8 “species and variety verification”
The aim of the committee is to determine the extent that the submitted sample conforms to the species or variety as requested by the applicant, using other methods than those specified in Chapter 3.

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2

Agenda

New statistical method for DNA data analysis

Update on the development of new markers for detection of annual types in perennial rye grass varieties

Use of neuronal networks for variety identification

DNA-based markers handbook

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3

New statistical method for DNA data analysis

Together with the statistical committee we are discussing the application of a new methodology for CT data analysis and interpretation.

This will provide a more realistic analysis of DNA data coming from comparative tests (CT) and will unify criteria with other committees that produce and analyze CT data.

We will introduce two concepts:

- **Accordance** (equivalent to repeatability) when multiple seeds per variety are included in the CT, defined as the **proportion of identical results** across replicates for a **specific lab, variety, and marker**.
- **Concordance** (equivalent to reproducibility), defined as the **proportion of matching results** across all pairwise comparisons of **laboratories for a given marker and variety**.

Discussion on these topcis are ongoing

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4

Update on the development on new markers for detection of annual types in perennial RG

Collaborative work
Project leader: Giovanny López (ATC)
Collaboration with Shaun Bushman from USDA who developed the markers, Daniel Curry from Oregon State University who provided seeds samples for the test and technical support, and Ingo Lenk from DLF providing technical support.

Markers development
Markers selection
KASP tests
Real time PCR tests

Digital PCR test – results

Project with the support of ISTA
Tests run at USDA-ARS Forage and Range Research Laboratory by Sean Bushman and his team

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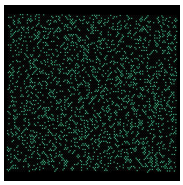
5

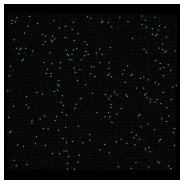
Update on the development on new markers for detection of annual types in perennial RG

Real time PCR was often, but not always, able to distinguish ARG contamination in PRG

So we tried dPCR

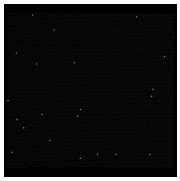


FAM



SUN (HEX)

An ARG entry shows this pattern.
Many FAM amplifications
Few SUN amplifications.



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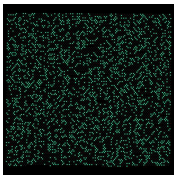

SUN (HEX)

A PRG entry shows this pattern.
Few FAM amplifications.
Many SUN amplifications.

Instead of creating a threshold where PCR becomes detectable (like real-time PCR), digital PCR conducts 8,500 mini PCR reactions in each well and counts "+" or "-".

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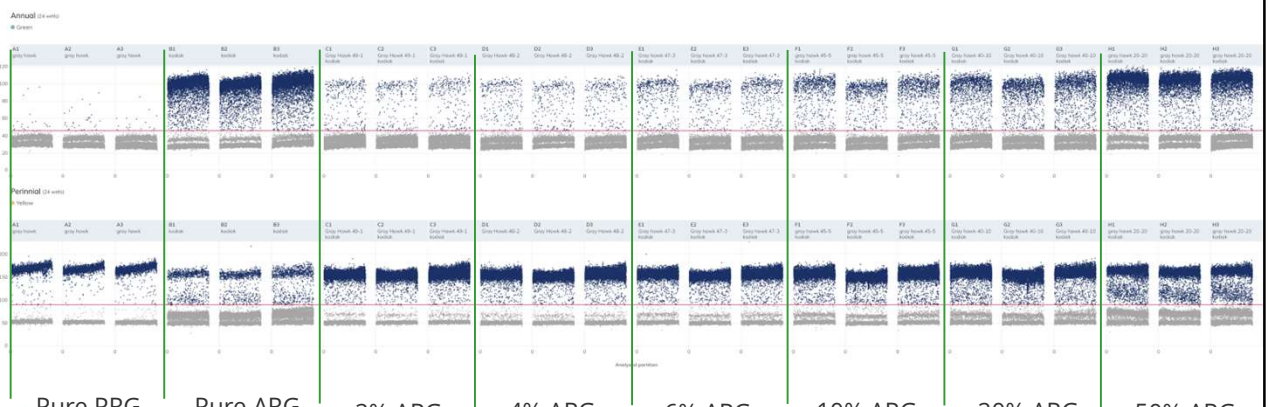
6

Update on the development on new markers for detection of annual types in perennial RG



3 candidate markers tested
2 gave good performance
1 example is shown here

spike test, triplicates, 50-seed bulk DNA extractions

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7

Update on the development on new markers for detection of annual types in perennial RG

Some conclusions

- ARG vs PRG comparisons are accurate and consistent with all three probe sets.
- The digital PCR instrument is more quantitative and sensitive than real-time PCR.
- Digital PCR runs like a real-time PCR instrument – so about 3 hours + about 30 minutes for the data analysis once the PCR is completed.
- The upstream DNA extractions and combining samples always takes the most time, whatever the PCR method used.
- dPCR has acceptable throughput: either 24 or 96 samples can be run on each plate in some instruments.
- Unlike qPCR instruments, most seed labs do not yet have dPCR instruments.
- Price per sample, consumables (plates, master mixes, probes, plastic) is quite acceptable.
- We will keep on using this instrument for the validation

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8

Use of neuronal networks for variety identification

Second data set produced

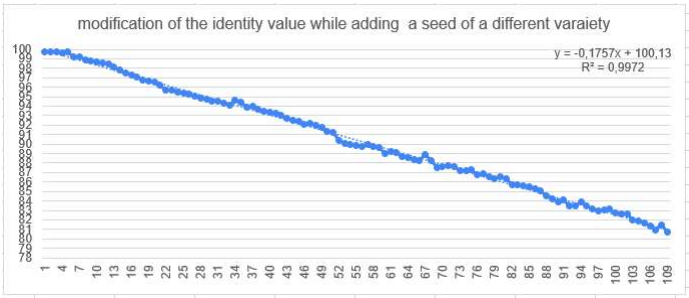
The expected variation in the identification value is the impact of 1 different seed in the grid. This theoretical vale is 0.185 (1 seed/542 wells).

The value obtained for this second data set was 0.176 calculated based on the experiment.

For the first data set the experimental vale was 0.188.

STACOM is now working with this second data set produced in a different facility in the aim to evaluate if the data sets produced give equivalent results.

Next steps in regards of the validation of this new technology will be discussed after data analysis



9

DNA-based markers handbook

6 members in the working group

Kae-Kang Hwu
Ksenija Taski Ajdukovic
Lorella Andreani
Chiara Delogu
Marie-Claude Gagnon
Sean Walkowiak
Ana Vicario

Regular meetings

Discussion with the STACOM
And with the Accreditation Department

Heavy technical discussion within the group

HANDBOOK ON DNA BASED TESTS – Table of Contents

10

DNA-based markers handbook

Heavy technical discussion within the group

- Comparative Tests (CTs) organization
- Crop leader, markers and varieties selection
- Considerations on the number of varieties to be used
- Considerations on the sample size (single or bulk)
- Considerations in relation on markers performance
- Number of markers
- Repeatability of the markers / variety
- Reproducibility of the test results
- Statistical approaches for results analysis (including : evaluation of performance method and proficiency tests
- Evaluation of markers performance, when it is a good marker
- Evaluation of laboratory performance
- Reference material collection (RMC)
- ISTA accreditation: evaluation of performance method and proficiency tests
- Statistical approaches for results analysis
- ISTA accreditation for DNA based testing
- Preparation for the audit

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11

THANKS FOR YOUR ATTENTION

Engage to the Variety Committee contacting the ISTA Secretariat
ista.office@ista.ch

34th ISTA CONGRESS CHRISTCHURCH NEW ZEALAND 05-09 May 2025

12

[End of Annex and of document]