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INTERNATIONAL UNION FOR THE PROTECTION OF NEW VARIETIES OF PLANTS

Geneva

OECD/UPOV/ISTA JOINT WORKSHOP ON MOLECULAR TECHNIQUES

Seoul, Republic of Korea, November 12, 2014

DNA-BASED METHODS FOR VARIETY TESTING

Document prepared by the International Seed Testing Association (ISTA)

Disclaimer: this document does not represent UPOV policies or guidance

The Annex to this document contains a copy of a presentation "DNA-Based Methods for Variety Testing" made at the OECD/UPOV/ISTA Joint Workshop on Molecular Techniques.

Benjamin Kaufman, Secretary General, The International Seed Testing Association (ISTA)

[Annex follows]





To be presented:

- Intro to ISTA
- ISTA Variety TCOM, DNA Working Group Updates (Rice, Wheat)
- What is Coming Next
- A call for Collaboration




The International Seed Testing Association (ISTA)



An international, non-profit association of seed testing laboratories and individual seed professionals.

ISTA operates under the governance of the government of member countries and distinct economies

 INTERNATIONAL SEED TESTING ASSOCIATION (ISTA)
<http://www.seedtest.org>



Following the ISTA Articles the Objectives of the Association are:

- a) **To develop, adopt and publish standard procedures for sampling and testing seeds, and promote uniform application of these procedures**
- b) **To actively promote research in all areas of seed science and technology**





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ISTA Technical Committees:

- ADVANCED TECHNOLOGIES
- BULKING AND SAMPLING
- FLOWER SEED
- FOREST TREE AND SHRUB SEED
- GERMINATION
- GMO
- MOISTURE
- NOMENCLATURE
- PROFICIENCY TEST
- PURITY
- RULES
- SEED HEALTH
- STATISTICS
- SEED STORAGE
- TETRAZOLIUM
- VARIETY
- VIGOUR

The block lists the various technical committees of ISTA. Three book covers are shown to illustrate the scope of their work:

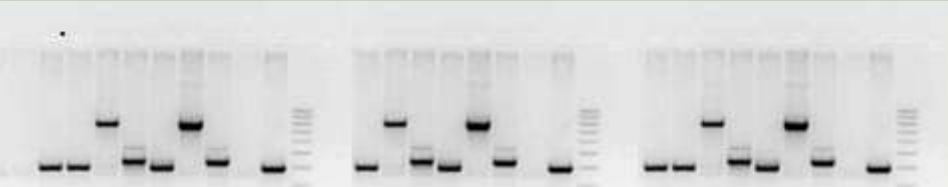
- ISTA Handbook on Seed Sampling:** A book with a brown cover showing sampling tools.
- ISTA Handbook on Sampling & Certification:** A book with a green cover showing laboratory equipment.
- ISTA Handbook on Purity Seed Testing:** A book with a purple cover showing seed samples.

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ISTA Variety Committee

The DNA Working Group was established in 2009. Working on development of variety characterization using DNA markers of:

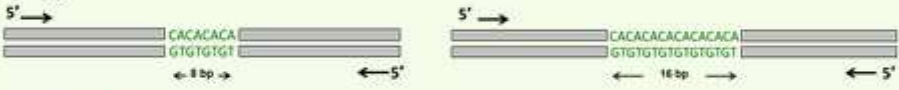
Maize	Soybean
Wheat	Rice



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Experimental Design

The markers:
Simple Sequence Repeats (SSRs, Microsatellites):
Screening large number of polymorphic evenly distributed throughout the genome markers.



The germplasm:
A variable collection of varieties representing (as much as possible) the genetic width of the crop

The Target:
The smallest subset of markers that provides a diagnostic combination (pattern) that uniquely identifies every variety in the collection in a reliable, repeatable and reproducible way.



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Summary of Resulting Varietal ID Marker Sets:

Working group	Number of selected SSR	Number of varieties tested	Total number of laboratories involved
Soybean	11	74	5
Wheat	8	84	5
Maize	12	72	9
Rice	15	192	5

Date - Cheryl Dollard

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Summary of Resulting Varietal ID Marker Sets Available now



- Suitable sets of SSRs for maize, soybean, wheat, rice able to discriminate a number of popular varieties has been identified.
- For some crops a different number of SSRs could be selected in order to identify a larger set of varieties.
- Different visualization systems give the same bands patterns (even if allele sizes are slightly different).



Redesigning Rice SSR markers

Goals:

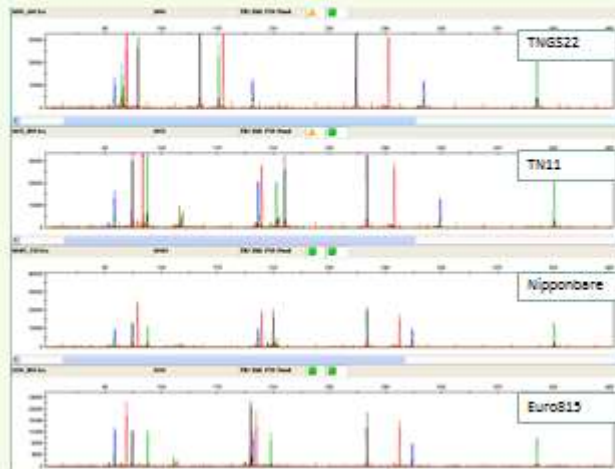
Use markers with 4 bp repeats instead of the previously used 3 bp repeats in order to:

- Reduce the stutter ratio which is higher for 3 bp repeats and sometimes will cause difficulties for distinguishing homo- and heterozygotes.
- Reduce the mutation rates which is likely to be higher for repeats with smaller unit.
- Increase the range of platforms that may be used for electrophoresis.

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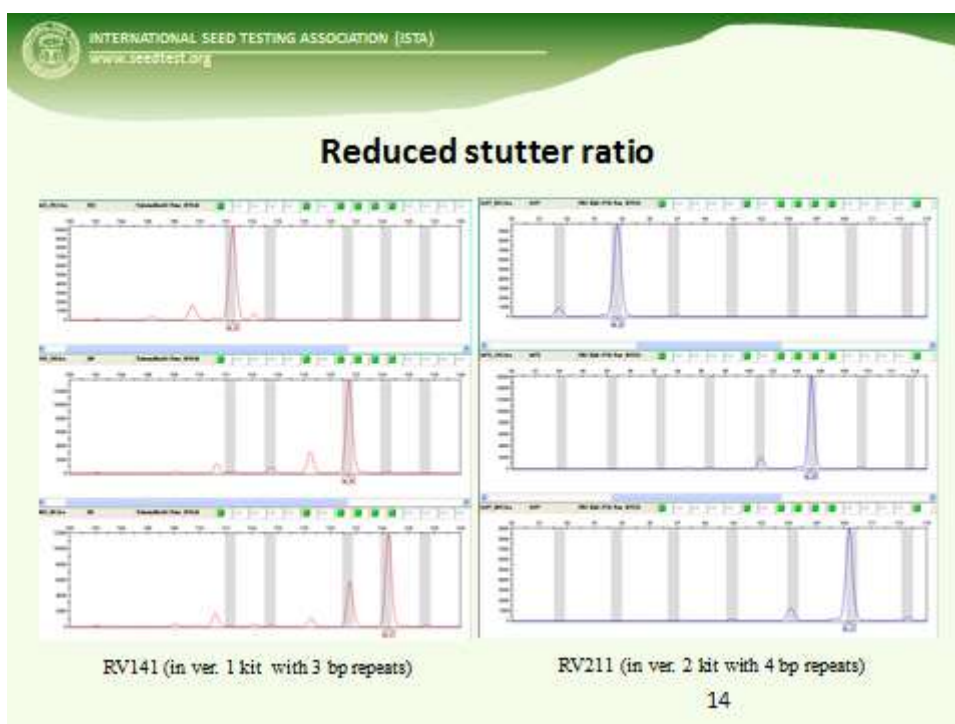


Redesigned Rice SSR marker set



Marker	Chr.	Labeled
RV211	4	6-FAM
RV212	12	6-FAM
RV213	9	6-FAM
RV221	8	VIC
RV222	1	VIC
RV223	7	VIC
RV231	5	NED
RV232	2	NED
RV233	11	NED
RV241	10	PET
RV242	6	PET
RV243	3	PET

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SSR kit evaluation

Tested over 109 national varieties and 189 accessions from 4 countries.

Locus	No. of alleles	PIC value				
		Korea	Japan	Taiwan	Italy	Accessions
RV211	12	0.48	0.42	0.67	0.60	0.83
RV212	3	0	0	0.33	0.50	0.66
RV213	12	0.38	0.32	0.63	0.70	0.85
RV221	10	0	0.42	0.49	0.66	0.80
RV222	13	0.58	0.40	0.57	0.74	0.84
RV223	4	0	0	0.32	0.49	0.63
RV231	5	0	0	0.32	0.58	0.55
RV232	14	0.66	0.66	0.68	0.69	0.81
RV233	4	0.64	0.42	0.42	0.53	0.58
RV241	9	0	0.32	0.53	0.43	0.72
RV242	10	0.50	0.42	0.45	0.34	0.74
RV243	9	0.48	0.46	0.48	0.66	0.72
Probability of identity		4.4×10^{-2}	5.8×10^{-2}	2.1×10^{-2}	2.0×10^{-2}	7.5×10^{-2}

Probability of Identity, P(ID): The probability that two individuals drawn at random from a population will have the same genotype at multiple loci.
Assuming inbreeding coefficient = 1 for rice varieties, overall P(ID) for unrelated random pairs of varieties is $P_{rel} = \prod (1 - PIC_i)$

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Wheat Comparative Tests (CTs)

Four labs completed the first two CTs using various separation systems including Li-Cor 4200 and 4300, ABI 3130xl, and silver staining of polyacrylamide gels.

CT1 examined:

- 8 multiplexed microsatellite markers
- 8 varieties (2 Canadian, 2 Brazilian, 4 Italian)
- 12 kernels per variety

Following CT1, two markers that appeared complex in some varieties were replaced with two other markers.

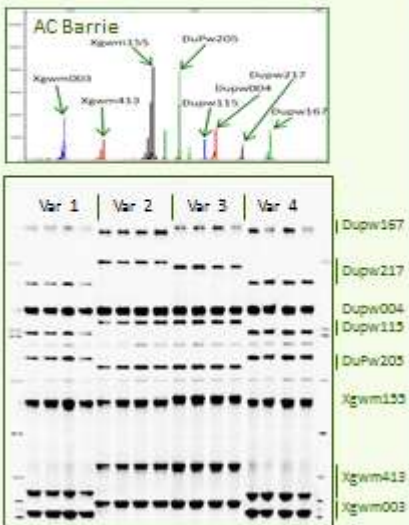
CT2 examined:

- Revised set of 8 multiplexed markers
- 16 additional varieties
- 6 kernels per variety and two 10-kernel bulks

The revised marker multiplex had improved performance.

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Wheat Comparative Test 3



Objectives & results:

to assess effectiveness of the revised marker set in a larger number of varieties

Additional varieties tested by 3 labs:
Canada, 24; France, 24; Italy, 12.

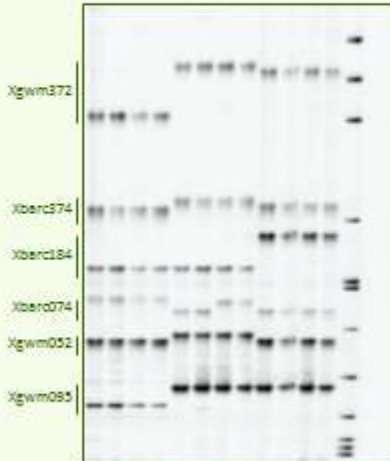
Of 84 varieties examined in the first CTs, all but two pairs of Canadian varieties three Italian durum varieties were distinguishable using the revised 8-marker set.

to invite other labs to perform analyses on CT2 varieties

Three new labs from Austria, Argentina and Canada participated.

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Wheat Comparative Test 4



Objectives & results:

To examine a second, set of 6 multiplexed microsatellites

three Canadian labs using Li-Cor 4300 and ABI 3130xl separation systems.

6 Canadian varieties (those included previously in CT1 and CT2)

analyses indicated that at least two of the previously indistinguishable pairs of varieties were distinguishable using the new set.

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“Uniformity in Seed Testing” Challenges to Standardization

- ISTA laboratories have different equipment, use different reagents and protocols.
- Available technologies progress quickly.

How can we guarantee both uniformity and standardization?





SEMI Performance based approach – DNA methods

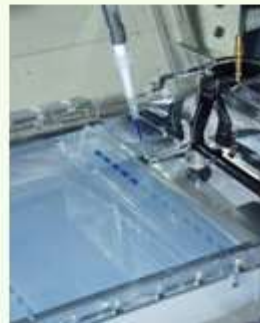
- Laboratories will use their own, validated, method of choice (Including: DNA extraction, quantification, PCRs and data collection).
- The marker sets will be “**prescribed**”. That is the ISTA recommended Variety Identification set.
- If these markers are not enough for obtaining a unique identification pattern per variety, **laboratories will be free to add as many markers of the same type as they need.**
- The recommended marker set per crop will be evolving constantly; markers will be added as needed to describe changing genetic base.

Tuesday 17- Cheryl Dollard



What is Next?

- **Proficiency Tests**
- **Single Nucleotide Polymorphisms**
- **More Crops**
- **Different applications of DNA markers**
- **Collaboration**





Proficiency Tests (PTs)



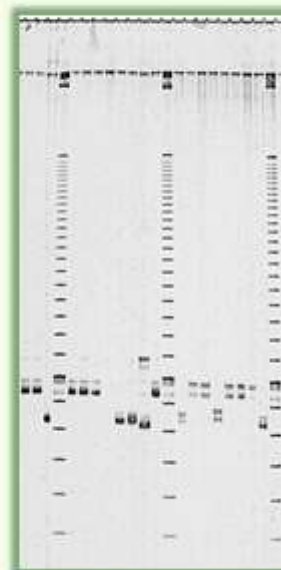
The PT program would aim to:

- assist laboratories in developing testing capacity for varietal testing using molecular markers
- will help evaluate the reproducibility of the results provided by the method selected by a laboratory
- evaluate the performance of laboratories
- help incorporate DNA based methods in the ISTA Rules
- The PTs would provide the basis for accrediting ISTA laboratories for variety identification tests using DNA-based methods.

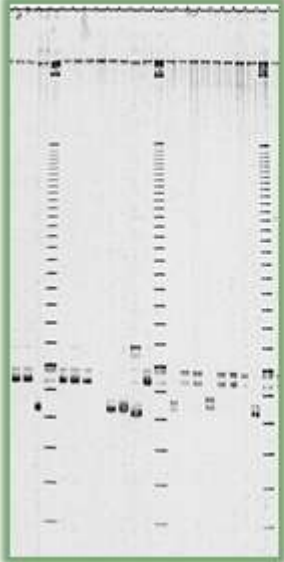


A PT should include:

- A "blind test", unknown varieties;
- Two known varieties as controls (with description of the expected allele profiles)
- provide the alleles recognition key.



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The PT program needs:

- a set of varieties to be used as reference materials (RMs);
- RMs should represent the allele variability within the species
- RMs should be pure seed (e.g. basic/pre-basic seed);
- RMs distributed to the laboratories may be represented by devitalized seeds or flour.

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Challenges:

Collecting RMs

Organization/logistics of the PTs

“Stumbling rocks”:

- identifying RMs suppliers (pure seed);
- financing RMs supply;
- storing RMs over several years;
- Delivering the PTs, e.g. preparing the PT samples, sending to the participating labs.




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Single Nucleotide Polymorphisms (SNPs)



The Variety Technical Committee in its last meeting during The ISTA 2014 annual meeting have decided that the DNA Molecular Markers Working Groups will initiate projects aiming at developing SNP panels For varietal ID.

Collaboration with other international organisations can be a vital catalyst of these efforts

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DNA based methods WG: new crops

- » *Pisum sativum* (peas)
- » *Hordeum vulgare* (barley)
- » *Sorghum spp.* (sorghum)
- » Sorghum: (SSR and SNPs)
- » Cucurbits: (SSRs)


Work in progress

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Other Varietal identification applications for DNA markers:

DNA based diagnostic markers for detecting annual ryegrass contamination in perennial ryegrass

Lolium multiflorum



Lolium perenne



Other such needs\developments:

- Identification of Johnson grass in Sorghum
- Wild spp. in cultivated sunflower

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The Marker

Specific Sequence amplification:

- functional DNA Markers residing within or linked to a DNA sequence that is involved in determining a key differentiating trait: an InDel in the vernalization gene LPVRN2 that is associated with annuality;

A project in collaboration between International Seed Federation (ISF), Biodiagnostics and ISTA is in design preparing the validation study.

- Or unique, diagnostic sequences of no clear functional context.


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ISTA and UPOV

- synchronize the efforts in order to promote and facilitate the use of DNA based methods in variety testing for the different purposes;
- co-operate in the aim to define agreed key points, hopefully (a) unique protocol/s (e.g. definition of a minimum agreed set of markers for each species);
- share information, save time and resources.


For example:

- The ISF/American Seed testing Association (ASTA) published 3072 (2x1536) set of SNPs in the frame work of...

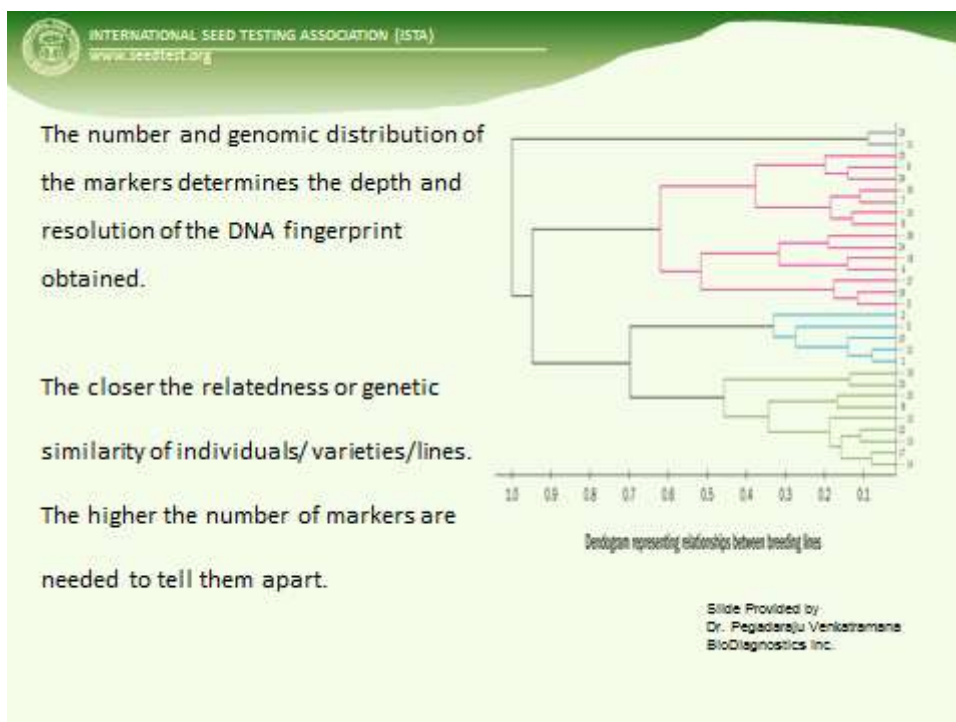
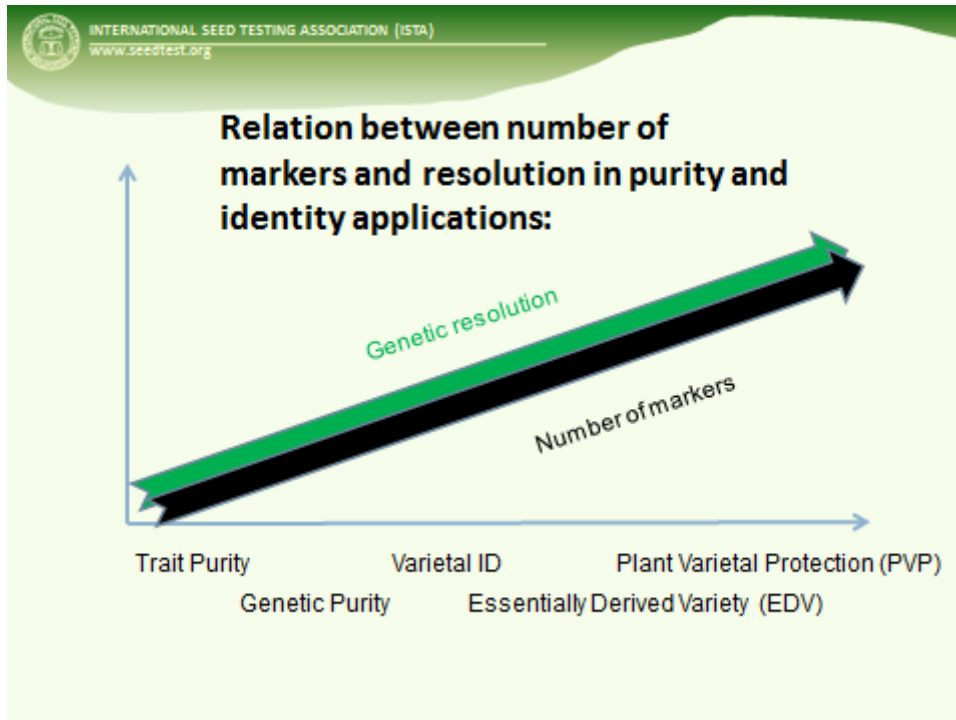


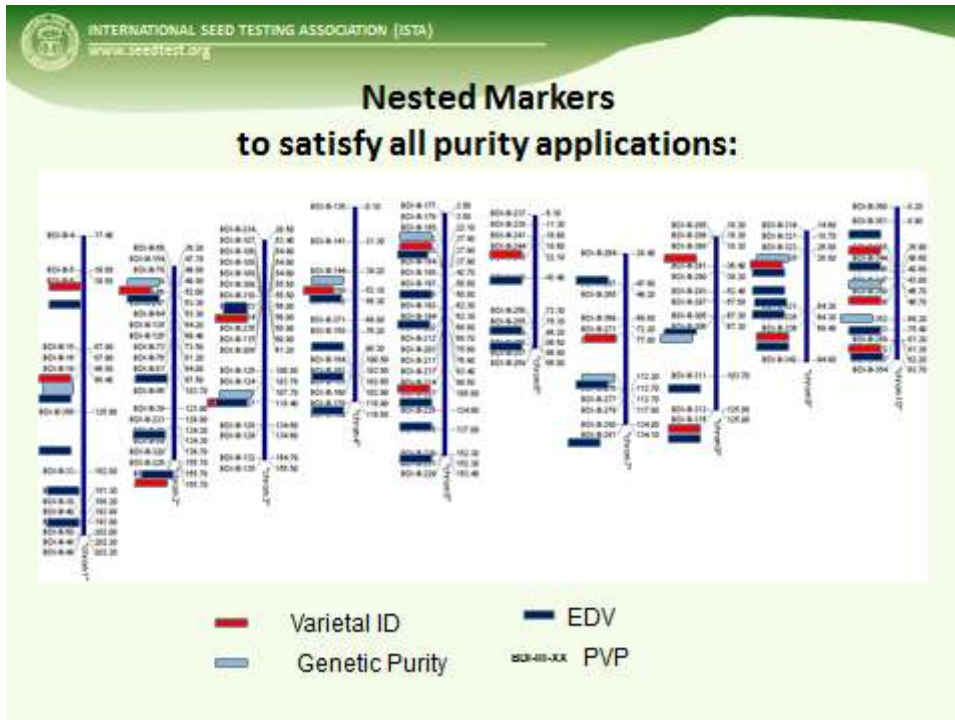
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Nested Molecular Markers for the testing of:



- Trait Purity
- Genetic Purity
- Varietal Identification
- Essentially Derived Varieties (EDV)
- Plant Varietal Protection





Collaboration needs more than wishes and agreements:

**A structure,
work plan,
resources,
concrete actions & assignments**

It is our role to have this started...



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