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DEVELOPMENT OF AN INTERNATIONAL SEED TESTING ASSOCIATION (ISTA) DNA-BASED APPROACH FOR TESTING VARIETY IDENTITY

Document prepared by experts from ISTA

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ANNEX

INTERNATIONAL SEED TESTING ASSOCIATION (ISTA)

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Development of an ISTA DNA-Based Approach for Testing Variety Identity

DEVELOPMENT OF AN INTERNATIONAL SEED TESTING ASSOCIATION (ISTA) DNA-BASED APPROACH FOR TESTING VARIETY IDENTITY

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Introduction

Many seed testing laboratories are receiving increasing numbers of requests to apply new technologies based on molecular markers for variety identification and genetic purity tests. For this reason, the International Seed Testing Association DNA working group was established to develop a common approach for DNA-based variety verification capable of providing repeatable and reproducible results among ISTA laboratories.

Importance Of Molecular Markers For Seed Testing

The ability of DNA-based markers to efficiently discriminate between closely related varieties has been reported. They are a step forward for variety characterization and verification compared to protein-based methods because they often reveal greater variation and are independent from the environment.

Among the different marker types, microsatellites were chosen as the most appropriate. The value of microsatellite markers for identification and genetic relationship studies in different crops arise from their multi-allelic nature, co-dominant transmission, relative abundance and extensive genome coverage. This methodology has been particularly useful for identification of genotypes and quantification of genetic diversity in a broad number of species such as potatoes, tomatoes, soybeans, rice, wheat, sunflower, sorghum, corn and many others.

Microsatellites, also known as Simple Sequence Repeats (SSR), are short tandemly repeated sequences of di-, tri-, tetra- or penta-nucleotide units, which are found scattered throughout the genomes of most eukaryotic species, making them an interesting tool for variety verification.

*Taiwan Province of China is considered as "Separate Customs Territory of Taiwan, Penghu, Kinmen and Matsu" under the rules of the International Seed Testing Association (ISTA).

The strategy for incorporating DNA-based tests into the ISTA Rules.

For each species, the strategy was to select a single set of markers that may be used worldwide. These sets of markers were selected for each crop from larger sets of markers proposed by crop experts and were evaluated on a diverse set of varieties from various regions through a series of collaborative tests (CTs). These CTs were performed initially by laboratories having experience with each crop and/or technique. Laboratories were free to choose the DNA extraction and PCR protocols and visualization system. This gave robustness to the study. Repeatability and reproducibility for each marker were assessed.

In the future, for their accreditation, laboratories will be required to perform a Proficiency Test. The way in which this will be carried out has not yet been decided in detail.

Species selection:

During the ISTA Congress held in Foz do Iguaçu in 2007 the ISTA DNA experts gathered to decide upon the strategy to follow to incorporate DNA-based methods into the ISTA Rules. There, it was decided that crops representing the most important ones in terms of the cultivated area and production would be chosen for these tests. The selected crops were: maize, wheat, rice and soybean. An expert was chosen to lead each crop working group.

Marker selection:

The markers used during the first CT were selected by each crop leader based on their experience. The subsequent selection of markers per crop was based on the performance of the markers. SSRs that did not provide consistent results among laboratories during the first CT were withdrawn from the marker set and replacements were selected. During the second and third CTs the same set of SSR for some crops, and modified marker sets for others, were used on a larger number of varieties.

The aims of the Comparative Tests (CTs)

The aim of the first comparative test was to provide lists of DNA-based markers which could be used to discriminate varieties of *Zea mays* (maize), *Oryza sativa* (rice), *Triticum durum* and *Triticum aestivum* (wheat) as well as *Glycine max* (soybean). The aim was also to compare results between participant laboratories, and evaluate whether it is possible to obtain the same band patterns and allele sizes even when using different reactants, equipment and working protocols. Varieties and markers were the same for all participating laboratories for each crop. The aim of the second CT was to verify if the marker set was polymorphic enough to provide unique DNA-based patterns for a larger variety set, and also to select more suitable SSRs. A third CT was run to further expand the range of varieties tested for identification by the SSR markers validated during the first and second CTs; and to include additional laboratories that would run the SSRs and varieties tested during the second CT.

Summary of results for all 3 rounds for all species

Results of these CTs have been very good for all crops.

Table 1 summarizes the origin of the participating laboratories for the CTs, the crops included in this study and the different visualization methods used by different laboratories.

Table 1: list of participating laboratories and different visualization methods used by each laboratory over 3 test rounds.

Crop group	Participating laboratories per CT			Visualization methods used
	CT1	CT2	CT3	
Wheat	Canada x2 France Italy	Canada x2 France Italy	Austria Argentina Canada x3 France Italy	Licor 4200 and 4300, ABI 3130xl and Silver stain
Rice	Canada Italy Taiwan Province of China	Canada Italy Taiwan Province of China	Canada India Italy USA x2 Taiwan Province of China	ABI3100 and 3130, Licor 4300 and Agarose
Soy	Argentina Brazil Canada	Argentina Brazil Canada USA	Argentina x3 Brazil Canada USA	Silver stain and ABI 3100
Maize	Argentina Brazil Canada France	Argentina Brazil Canada France	Brazil Canada China France x2 Italy USA x2	ABI3130xl, Silver stain, ABI 3100 and Silver Stain

A summary of the results obtained for each is below:

Wheat

SSR markers assessed in collaborative trials

Table 2

CT1	CT2	CT3	CT4 (*)	Status (CT1 to 3)
DuPw004	DuPw004	DuPw004		Good
DuPw115	DuPw115	DuPw115		Good
DuPw167	DuPw167	DuPw167		Good
DuPw205	DuPw205	DuPw205		Good
DuPw217	DuPw217	DuPw217		Good
Xgwm003	Xgwm003	Xgwm003		Good
Xgwm099				May be complex in hexaploid varieties
Xgwm526				May be complex in hexaploid varieties
	Xgwm155	Xgwm155		Good
	Xgwm413	Xgwm413		Good
			Xbarc074	Will be evaluated after CT4
			Xbarc184	Will be evaluated after CT4
			Xbarc347	Will be evaluated after CT4
			Xgwm052	Will be evaluated after CT4
			Xgwm095	Will be evaluated after CT4
			Xgwm372	Will be evaluated after CT4

(*) CT not yet completed

All markers assessed within a collaborative trial may be combined in a single multiplex PCR.

In CT1, eight varieties were included from Brazil (2), Canada (2) and Italy (4). In CT2, four varieties were added from each of Brazil, Canada, France and Italy, giving a total of 24 varieties. In CT3, new participants examined these same 24 varieties while the original participants each examined larger numbers of varieties specific to their countries; 24 each from France and Canada and 12 from Italy. In total 84 wheat varieties have been examined. Of these, all but one pair of Italian varieties and two pairs of Canadian varieties could be identified using the specified markers. The objective of CT4 is to test an additional set of six multiplexed markers which may enhance discrimination among varieties.

Rice

SSR markers tested and validated for all 3 rounds

Table 3

CT1	CT2	CT3	Status
		RM1	Will be evaluated after CT4
		RM19	Will be evaluated after CT4
RM70	RM70	RM70	Will be evaluated after CT4
	RM101		
RM105	RM105		
	RM151		
		RM154	Will be evaluated after CT4
RM159	RM159		
		RM171	Will be evaluated after CT4
RM215	RM215		
		RM237	Will be evaluated after CT4
	RM264		
RM266	RM266		
RM276	RM276		
	RM287	RM287	Will be evaluated after CT4
		RM307	Will be evaluated after CT4
		RM316	Will be evaluated after CT4
RM333	RM333	RM333	Will be evaluated after CT4
		RM334	Will be evaluated after CT4
	RM347		
		RM413	Will be evaluated after CT4
		RM447	Will be evaluated after CT4
		RM510	Will be evaluated after CT4
		RM514	Will be evaluated after CT4
RM567	RM567		

In CT 1 and 2, participating laboratories were able to demonstrate usefulness of microsatellite markers in rice variety testing and established DNA extraction, PCR setup and visualization approaches that could produce consistent results under variable conditions. The third CT was set up to evaluate 12 markers selected from a core set of rice SSRs previously shown to be discriminatory on a large set of Asian varieties. Six laboratories participated, including laboratories from Taiwan Province of China, Canada, Italy, India and the USA on varieties from Taiwan Province of China, India and Italy. Based on these results, some minor adjustments to the conditions have been made to improve performance and a forth CT is in preparation, which will determine if these markers are

suitable to identify varieties from more countries. An additional 26 Italian varieties have been obtained for this work, and the group is working to establish contacts with other Asian countries to try to secure additional varieties. The countries of key interest include China, India, Indonesia, Japan, Republic of Korea, Philippines, Thailand, and Viet Nam.

Soybean

SSR markers tested and validated for all 3 rounds

Table 4

CT1	CT2	CT3	CT4 (*)	Status (CT1 to 3)
ATT-177	ATT-177	ATT-177		Very good
ATT-094	ATT-094	ATT-094		Good
ATT-233	ATT-233	ATT-233		Good
ATT-307	ATT-307	ATT-307		Good
ATT-105	ATT-105			Not good
ATT-114	ATT-114			Not good
ATT-216	ATT-216			Not good
ATT-231	ATT-231			Not good
AT_-001				Not good
ATT-353				Not good
ATT-446				Not good
ATT-534				Not good
ATT-577				Not good
	ATT-030	ATT-030		Very good
	ATT-181	ATT-181		Very good
	ATT-311	ATT-311		Good
	ATT-449	ATT-449		Good
		ATT-070		Not good
		ATT-147	ATT-147	Will be evaluated after CT4
		ATT-180	ATT-180	Will be evaluated after CT4
		ATT-191		
		ATT-373	ATT-373	Will be evaluated after CT4
		ATT-703		
			ATT-080	Will be evaluated after CT4
			ATT-352	Will be evaluated after CT4
			ATT-540	Will be evaluated after CT4
			ATT-600	Will be evaluated after CT4
			ATT-728	Will be evaluated after CT4

(*) not finished yet.

The status "good" and "very good" of the marker means that repeatable and reproducible results were obtained for that marker during 2-3 rounds of comparative tests.

For the first CT, 8 soybean varieties, 4 from Brazil and 4 from Argentina were analyzed using 12 SSR markers (see Table 4). For the second CT, 16 additional soybean varieties, 4 from Brazil, 4 from Canada and 8 from Argentina were analyzed making a total of 24 varieties. Twelve SSRs were tested, 4 new and 8 from the previous CT (see Table 4). During the third round, 14 SSRs were tested: 4 were tested for the third time, 4 for the second time and 6 were new ones. The 24 varieties used for this analysis were from: Brazil (8), Argentina (12) and Canada (4). A forth CT is in progress to validate new SSR markers by comparing results (reproducibility and repeatability of the markers) among and within participating laboratories. Each laboratory will use their own

methodology for obtaining the results. Three SSR markers were already run during the third comparative test and 5 are new ones. These markers are being tested on a set of 24 varieties from Brazil (8), Canada (4) and Argentina (12).

Maize

The maize group has established an SSR set that is ready to be applied for the organization of a Performance Test. For the Performance Test it is necessary to have a core set of varieties to be used as reference material (RM). These varieties will be tested using the markers already selected during the previous comparative tests.

CT1	CT2	CT3	Status (CT1 to 3)
Phi 109275	Phi 109275	Phi 109275	Very good
Phi083	Phi083	Phi083	Good
Phi 102228	Phi 102228	Phi 102228	Very good
Phi015	Phi015	Phi015	Good
Umc 1545	Umc 1545	Umc 1545	Very good
Umc 1061	Umc 1061	Umc 1061	Very good
Phi 032	Phi 032		Good
Umc 1122			Good
Phi 093			Good
Phi 452693			Not good
Umc 1153			Not good
Umc 1152			Not good
	Umc 1448	Umc 1448	Good
	Umc 1117	Umc 1117	Good
	Umc 1133	Umc1133	Very good
	Bnlg 1782		Good
	Umc 1792		Not good
		Phi 233376	Good
		Bnlg 1129	Not good
		Umc 1478	Good

Further steps

- Once the marker sets have been established, a defined set of "reference varieties" will be identified for each crop that can used by laboratories that wish to establish protocols for variety verification.

- Following this, the group will work to define a strategy together with ISTA's Statistics Committee for the accreditation of laboratories performing DNA-based variety verification tests. This means specification of how the proficiency testings (PTs) will be carried out and consideration of reference materials required for such tests.
- Finally, a proficiency testing (PT) program will be established in the future to assist laboratories develop testing capacity for varietal verification for these crops using molecular markers and to promote use of these internationally standardized methods.

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[End of Annex and of document]