UPOV

International Union for the Protection of New Varieties of Plants

E

Technical Working Party for Agricultural Crops

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TWA/48/8

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VARIETY TESTING IN ITALIAN PROTOCOLS – MICROSATELLITE FINGERPRINTING

Document prepared by an expert from Italy

The annex to this document contains a copy of a presentation on “Variety testing in Italian protocols – microsatellite fingerprinting”, to be made at the forty-eighth session of the TWA.

[Annex follows]

VARIETY TESTING IN ITALIAN PROTOCOLS

MICROSATELLITE FINGERPRINTING

Technical Working Party for Agricultural Crops

TWA / 48

16-20 September 2019 MONTEVIDEO, URUGUAY

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AGRICOLTURAL RESEARCER COUNCIL
RESEARCH CENTER FOR PLANT PROTECTION AND CERTIFICATION

Microsatellite Fingerprinting

Summary:

- Microsatellite fingerprinting
- CREA DC protocols
    - Soybean
    - Rice
    - Khorasan wheat
- Other purposes

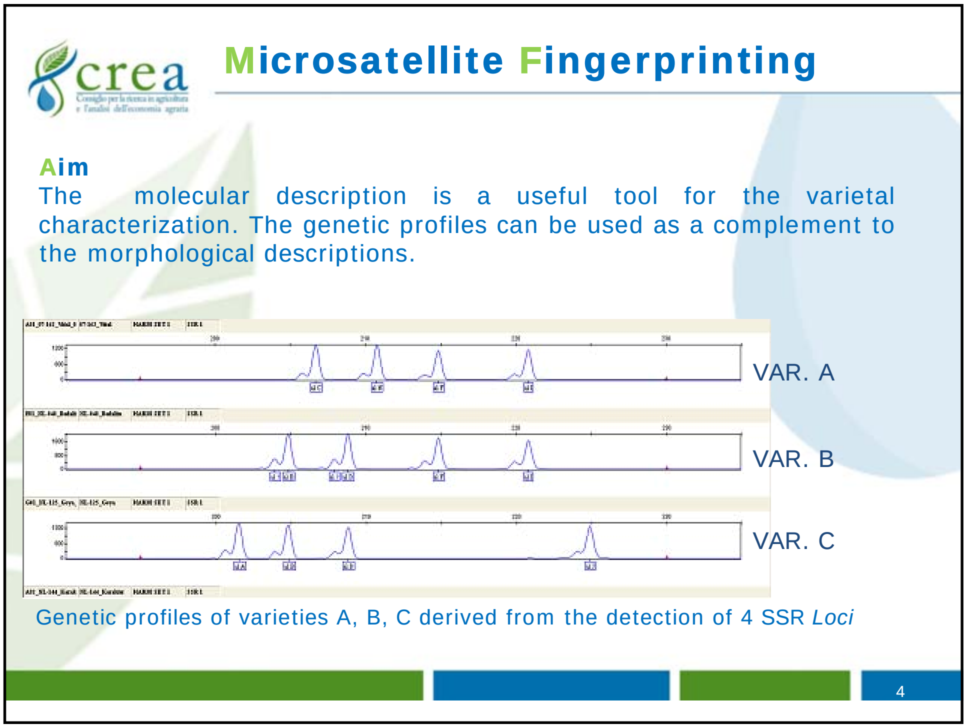
Microsatellite Fingerprinting

CREA-DC protocols are based on Microsatellites, also know as **SSR** (*Simple Sequence Repeats*).

The **SSR** are short sequence of DNA consisting of multiple repetitions of a set of two to nine base pairs, used as a genetic marker when individuals differ in the number of repetitions.
The sequences closed to SSR are usually highly conserved, consequently it is possible to select specific primers for the amplification of the fragment of interest by **PCR** (*Polymerase Chain Reaction*).

Codominant alleles are identified by the fragment length expressed in number of base pair .

The amplification of **SSR** and their separation by electrophoresis, will give origin to a typical profile for each individual/variety.



Microsatellite Fingerprinting

Aim
The molecular description is a useful tool for the varietal characterization. The genetic profiles can be used as a complement to the morphological descriptions.

Genetic profiles of varieties A, B, C derived from the detection of 4 SSR *Loci*

Variety Registration - Soybean

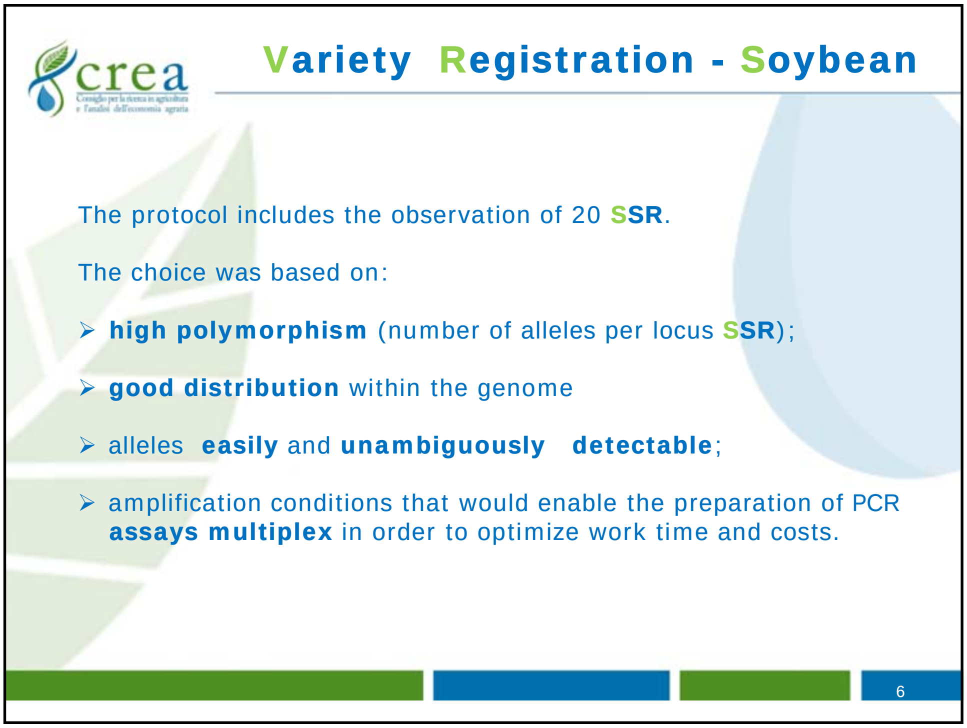
Protocol

For a final judgement on uniformity, distinctness and stability

- ✓ **UPOV TG/80/6 01 April 1998**
- ✓ **National guidelines** http://scs.entecra.it/prove%20iscrizioni/criteri/soia/Criteri-Soia-GU-50-1-03-2017-DM-13%20GENNAIO%202017.pdf

must be followed.

Photo by CREA-DC

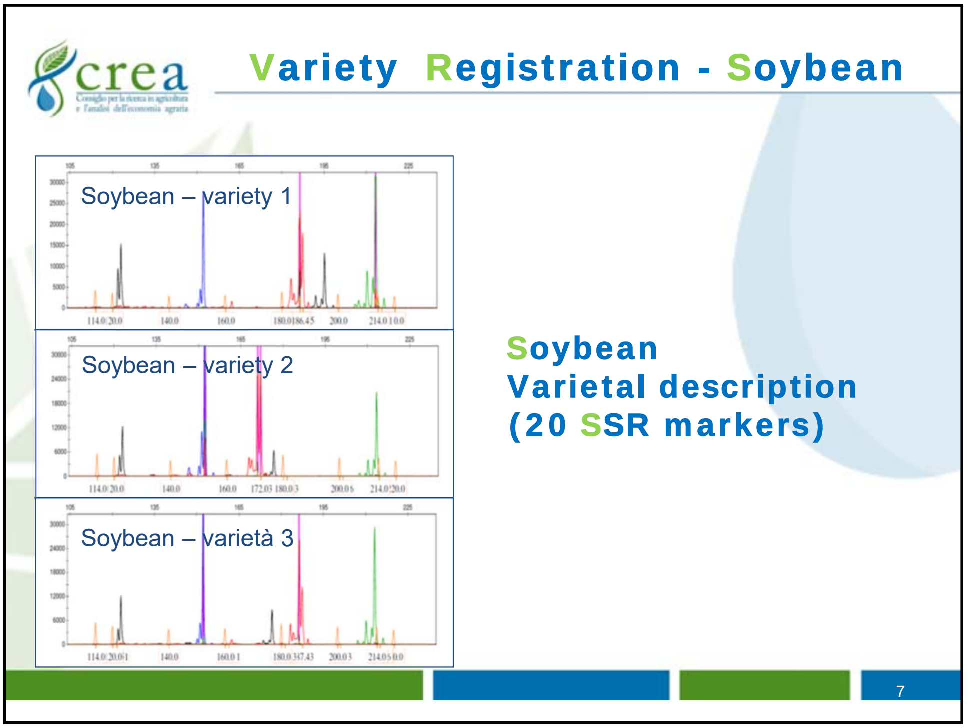


Variety Registration - Soybean

The protocol includes the observation of 20 **SSR**.

The choice was based on:

- ➢ **high polymorphism** (number of alleles per locus **SSR**);
- ➢ **good distribution** within the genome
- ➢ alleles **easily** and **unambiguously detectable**;
- ➢ amplification conditions that would enable the preparation of PCR **assays multiplex** in order to optimize work time and costs.



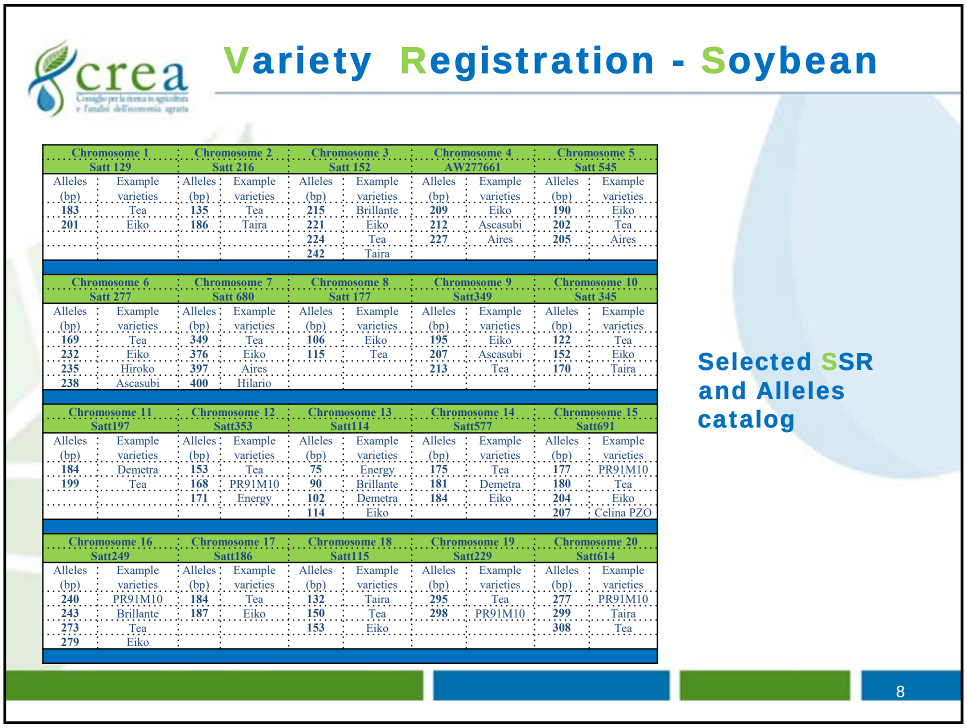
Variety Registration - Soybean

Soybean – variety 1

Soybean – variety 2

Soybean – varietà 3

Soybean
Varietal description
(20 SSR markers)



Variety Registration - Soybean

Chromosome 1 Satt 129		Chromosome 2 Satt 216		Chromosome 3 Satt 152		Chromosome 4 AW277661		Chromosome 5 Satt 545	
Alleles (bp)	Example varieties	Alleles (bp)	Example varieties	Alleles (bp)	Example varieties	Alleles (bp)	Example varieties	Alleles (bp)	Example varieties
183	Tea	**135**	Tea	**215**	Brillante	**209**	Eiko	**190**	Eiko
201	Eiko	**186**	Taira	**221**	Eiko	**212**	Ascasubi	**202**	Tea
				224	Tea	**227**	Aires	**205**	Aires
				242	Taira				

Chromosome 6 Satt 277		Chromosome 7 Satt 680		Chromosome 8 Satt 177		Chromosome 9 Satt349		Chromosome 10 Satt 345	
Alleles (bp)	Example varieties	Alleles (bp)	Example varieties	Alleles (bp)	Example varieties	Alleles (bp)	Example varieties	Alleles (bp)	Example varieties
169	Tea	**349**	Tea	**106**	Eiko	**195**	Eiko	**122**	Tea
232	Eiko	**376**	Eiko	**115**	Tea	**207**	Ascasubi	**152**	Eiko
235	Hiroko	**397**	Aires			**213**	Tea	**170**	Taira
238	Ascasubi	**400**	Hilario						

Chromosome 11 Satt197		Chromosome 12 Satt353		Chromosome 13 Satt114		Chromosome 14 Satt577		Chromosome 15 Satt691	
Alleles (bp)	Example varieties	Alleles (bp)	Example varieties	Alleles (bp)	Example varieties	Alleles (bp)	Example varieties	Alleles (bp)	Example varieties
184	Demetra	**153**	Tea	**75**	Energy	**175**	Tea	**177**	PR91M10
199	Tea	**168**	PR91M10	**90**	Brillante	**181**	Demetra	**180**	Tea
		171	Energy	**102**	Demetra	**184**	Eiko	**204**	Eiko
				114	Eiko			**207**	Celina PZO

Chromosome 16 Satt249		Chromosome 17 Satt186		Chromosome 18 Satt115		Chromosome 19 Satt229		Chromosome 20 Satt614	
Alleles (bp)	Example varieties	Alleles (bp)	Example varieties	Alleles (bp)	Example varieties	Alleles (bp)	Example varieties	Alleles (bp)	Example varieties
240	PR91M10	**184**	Tea	**132**	Taira	**295**	Tea	**277**	PR91M10
243	Brillante	**187**	Eiko	**150**	Tea	**298**	PR91M10	**299**	Taira
273	Tea			**153**	Eiko			**308**	Tea
279	Eiko								

Selected SSR
and Alleles
catalog

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Variety Registration - Soybean

Working Sample and Uniformity Threshold

For the varietal characterization 16 single seeds are tested.

If only one seed is different (off-type) 8 more seeds will be analized.

A variety is considered non-homogeneous if 2 off-type are retrieved out of 24 seeds.

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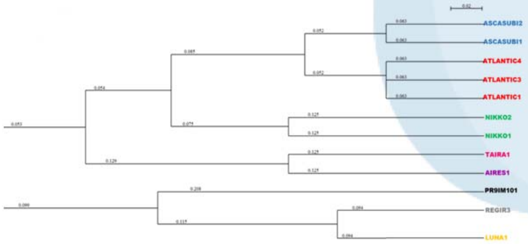
Variety Registration - Soybean

Evaluation of the results

The results are recorded on an electronic sheet and elaborated by a specific software[1] for the evaluation of genetic distance.



The molecular fingerprinting will be used to complete the varietal descriptions.

Literature

1= Felsenstein, J. 1989. PHYLIP - Phylogeny Inference Package (Version 3.2). Cladistics 5: 164-166.

Variety Registration - Rice

Protocol

For a final judgement on uniformity, distinctness and stability

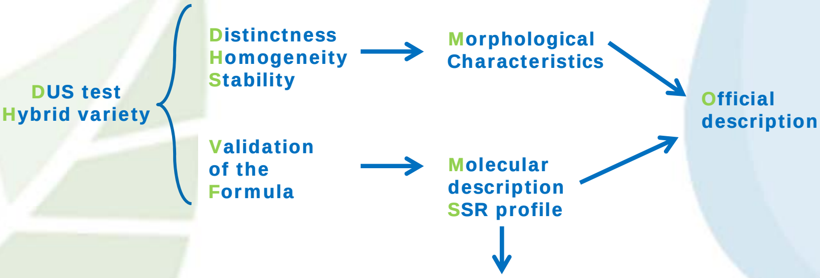
- ✓ **CPVO / TP-016 / 3 01 October 2015**
- ✓ **National guidelines**
  http://scs.entecra.it/prove%20iscrizioni/criteri/riso/Criteri-Riso-GU-91-18-4-2014.pdf

Must be followed.


Photo by Alessandra Sommovigo

Variety Registration - Rice

Validation of the Formula of the hybrid variety



DUS test
Hybrid variety

Distinctness
Homogeneity
Stability
→ **Morphological Characteristics** → **Official description**

Validation of the Formula
→ **Molecular description**
SSR profile → **Official description**

Microsatellites profile

Codominant
Expression independent of the environment
Good level of polymorphism

From a methodological point of view

High repeatability and scorability
Quick results
Molecular profiles amenable to database

Variety Registration - Rice

Molecular profile- Aims

- **Validation of the hybrid formula:**
  taking advantage of the codominant nature of SSR markers it is possible, through the description of parental lines, to infer the hybrid theoretical formula and compare it with that actually found in the hybrid variety in registration
- **Homogeneity *evaluation:***
  the detection of the level and type of non-homogeneity (presence of different alleles at the same locus) in the pure lines will allow to explain the possible variability detected in the hybrid and will therefore be useful for the validation of the formula
- **Database:**
  The molecular fingerprinting will be used to complete the varietal descriptions

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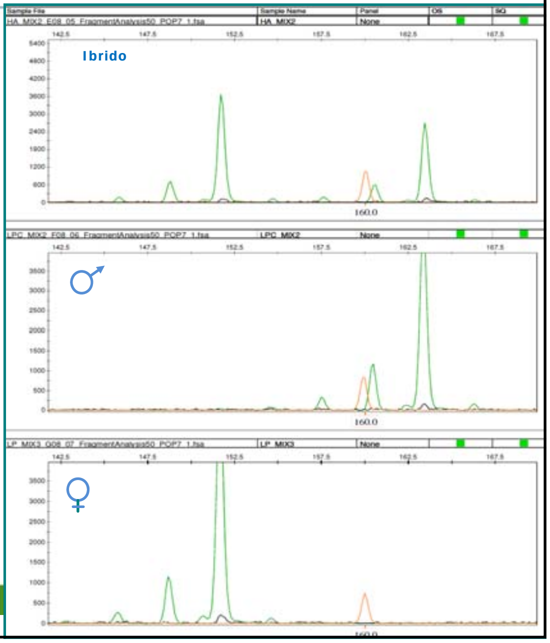
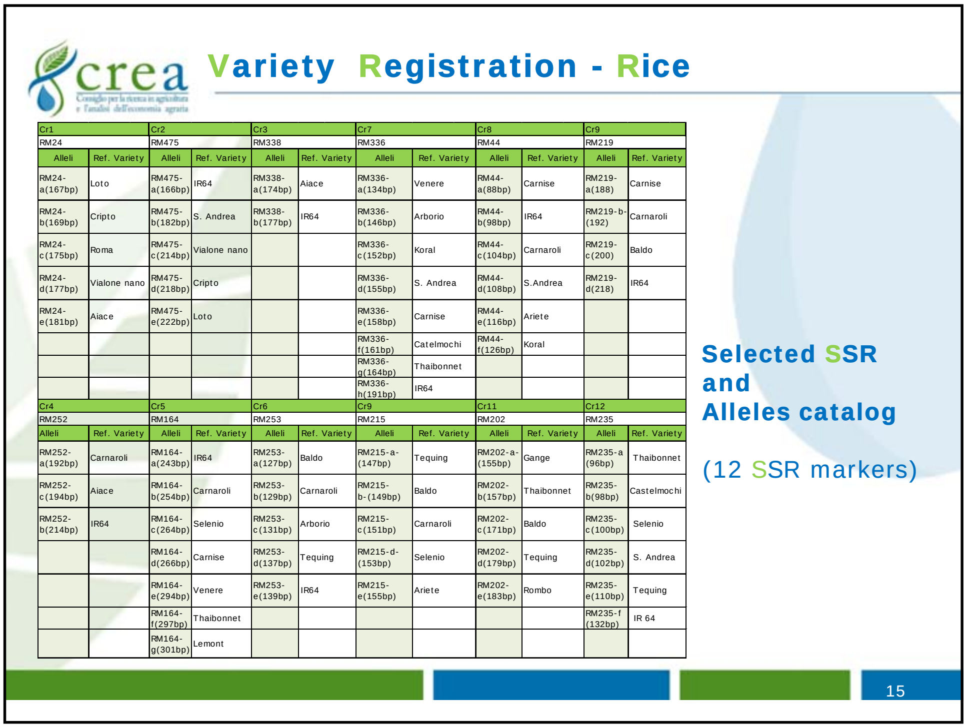
Variety Registration - Rice

Rice varietal registration

Hybrid formula validation





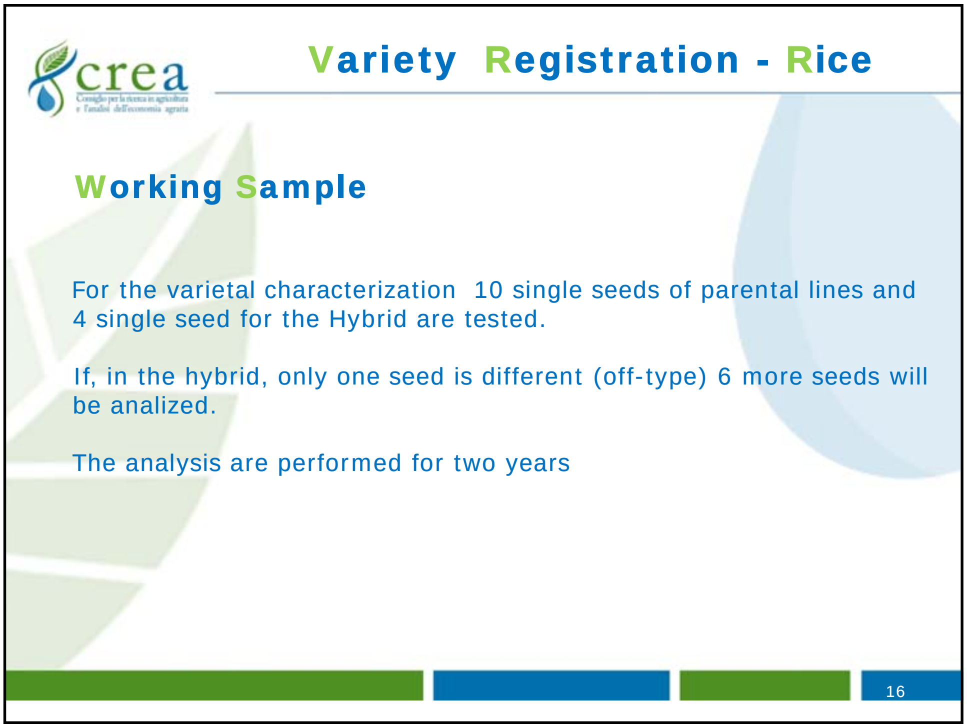
Variety Registration - Rice

Cr1		Cr2		Cr3		Cr7		Cr8		Cr9	
RM24		RM475		RM338		RM336		RM44		RM219	
Alleli	Ref. Variety	Alleli	Ref. Variety	Alleli	Ref. Variety	Alleli	Ref. Variety	Alleli	Ref. Variety	Alleli	Ref. Variety
RM24-a(167bp)	Loto	RM475-a(166bp)	IR64	RM338-a(174bp)	Aiace	RM336-a(134bp)	Venere	RM44-a(88bp)	Carnise	RM219-a(188)	Carnise
RM24-b(169bp)	Cripto	RM475-b(182bp)	S. Andrea	RM338-b(177bp)	IR64	RM336-b(146bp)	Arborio	RM44-b(98bp)	IR64	RM219-b-(192)	Carnaroli
RM24-c(175bp)	Roma	RM475-c(214bp)	Vialone nano			RM336-c(152bp)	Koral	RM44-c(104bp)	Carnaroli	RM219-c(200)	Baldo
RM24-d(177bp)	Vialone nano	RM475-d(218bp)	Cripto			RM336-d(155bp)	S. Andrea	RM44-d(108bp)	S.Andrea	RM219-d(218)	IR64
RM24-e(181bp)	Aiace	RM475-e(222bp)	Loto			RM336-e(158bp)	Carnise	RM44-e(116bp)	Ariete		
						RM336-f(161bp)	Catelmochi	RM44-f(126bp)	Koral		
						RM336-g(164bp)	Thaibonnet				
						RM336-h(191bp)	IR64				

Cr4		Cr5		Cr6		Cr9		Cr11		Cr12	
RM252		RM164		RM253		RM215		RM202		RM235	
Alleli	Ref. Variety	Alleli	Ref. Variety	Alleli	Ref. Variety	Alleli	Ref. Variety	Alleli	Ref. Variety	Alleli	Ref. Variety
RM252-a(192bp)	Carnaroli	RM164-a(243bp)	IR64	RM253-a(127bp)	Baldo	RM215-a-(147bp)	Tequing	RM202-a-(155bp)	Gange	RM235-a(96bp)	Thaibonnet
RM252-c(194bp)	Aiace	RM164-b(254bp)	Carnaroli	RM253-b(129bp)	Carnaroli	RM215-b-(149bp)	Baldo	RM202-b(157bp)	Thaibonnet	RM235-b(98bp)	Castelmochi
RM252-b(214bp)	IR64	RM164-c(264bp)	Selenio	RM253-c(131bp)	Arborio	RM215-c(151bp)	Carnaroli	RM202-c(171bp)	Baldo	RM235-c(100bp)	Selenio
		RM164-d(266bp)	Carnise	RM253-d(137bp)	Tequing	RM215-d-(153bp)	Selenio	RM202-d(179bp)	Tequing	RM235-d(102bp)	S. Andrea
		RM164-e(294bp)	Venere	RM253-e(139bp)	IR64	RM215-e(155bp)	Ariete	RM202-e(183bp)	Rombo	RM235-e(110bp)	Tequing
		RM164-f(297bp)	Thaibonnet							RM235-f(132bp)	IR 64
		RM164-g(301bp)	Lemont								

Selected SSR and Alleles catalog

(12 SSR markers)



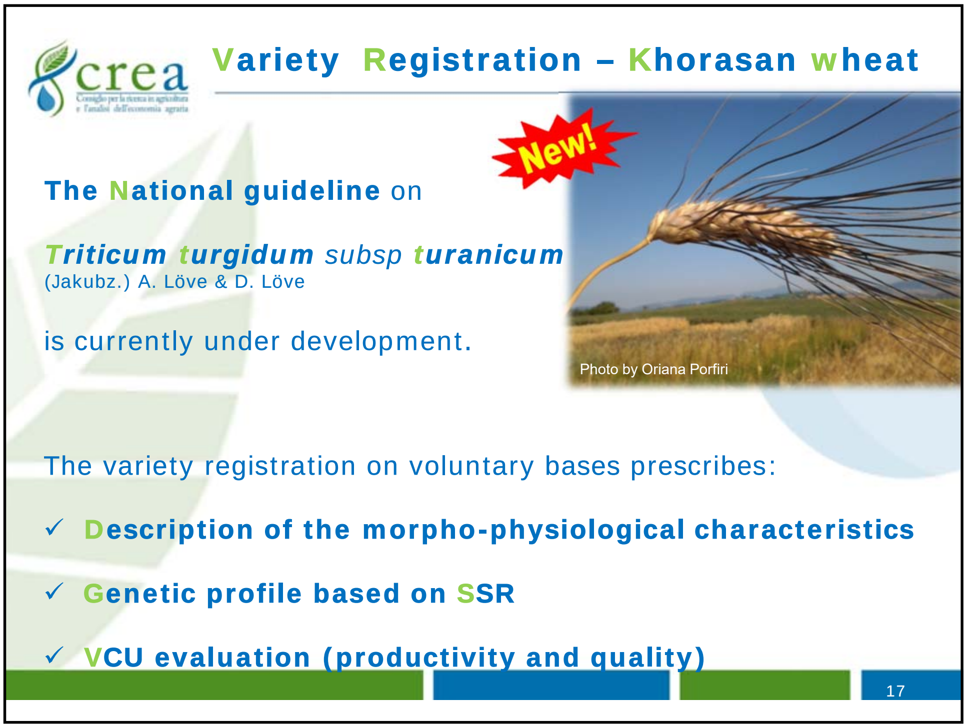
Variety Registration - Rice

Working Sample

For the varietal characterization 10 single seeds of parental lines and 4 single seed for the Hybrid are tested.

If, in the hybrid, only one seed is different (off-type) 6 more seeds will be analized.

The analysis are performed for two years



Variety Registration – Khorasan wheat

New!

The National guideline on

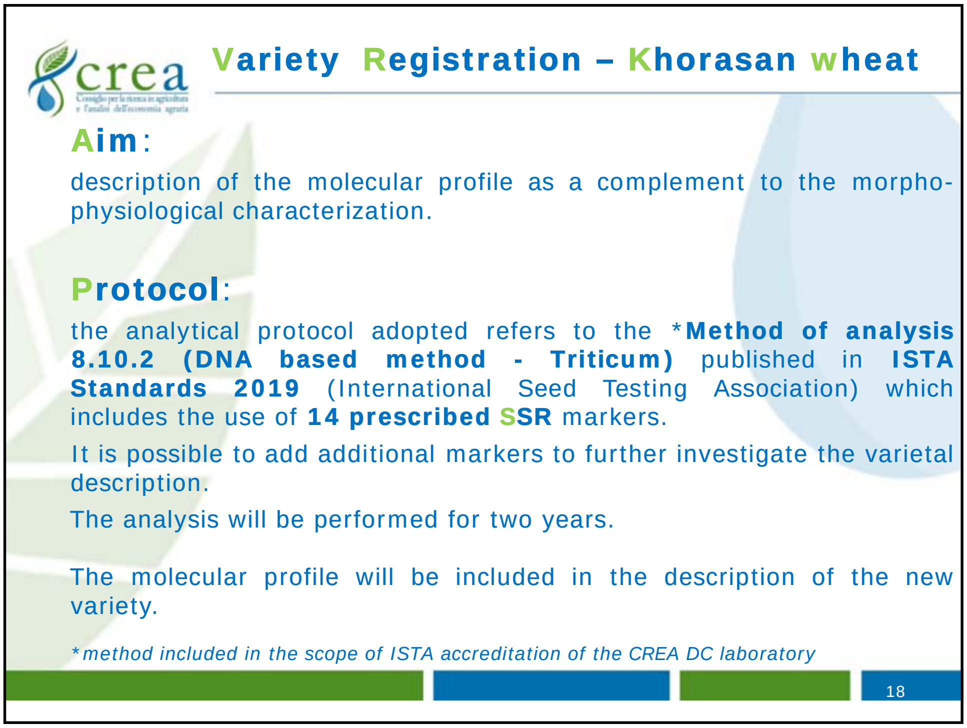
Triticum turgidum *subsp* ***turanicum***
(Jakubz.) A. Löve & D. Löve

is currently under development.

Photo by Oriana Porfiri

The variety registration on voluntary bases prescribes:

- ✓ **Description of the morpho-physiological characteristics**
- ✓ **Genetic profile based on SSR**
- ✓ **VCU evaluation (productivity and quality)**



Variety Registration – Khorasan wheat

Aim:

description of the molecular profile as a complement to the morpho-physiological characterization.

Protocol:

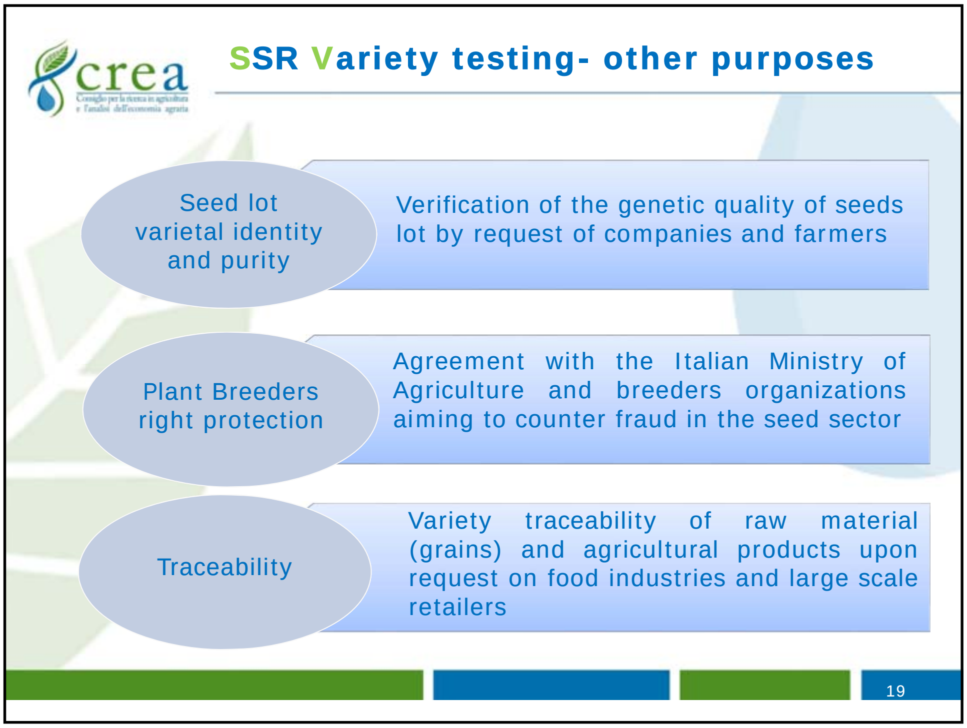
the analytical protocol adopted refers to the ***Method of analysis 8.10.2 (DNA based method - Triticum)** published in **ISTA Standards 2019** (International Seed Testing Association) which includes the use of **14 prescribed SSR** markers.

It is possible to add additional markers to further investigate the varietal description.

The analysis will be performed for two years.

The molecular profile will be included in the description of the new variety.

**method included in the scope of ISTA accreditation of the CREA DC laboratory*



SSR Variety testing- other purposes

Seed lot varietal identity and purity	Verification of the genetic quality of seeds lot by request of companies and farmers
Plant Breeders right protection	Agreement with the Italian Ministry of Agriculture and breeders organizations aiming to counter fraud in the seed sector
Traceability	Variety traceability of raw material (grains) and agricultural products upon request on food industries and large scale retailers

Thank you for your attention

[End of Annex and of document]