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BSI Standards Publication

Molecular biomarker analysis — SSR analysis of sunflower

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National foreword

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TECHNICAL REPORT

ISO/TR 17622

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Molecular biomarker analysis — SSR analysis of sunflower

Analyse moléculaire de biomarqueurs — Méthode d'analyse SSR sur le tournesol

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Foreword

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For an explanation on the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the WTO principles in the Technical Barriers to Trade (TBT) see the following URL: Foreword - Supplementary information.

The committee responsible for this document is ISO/TC 34, *Food products*, Subcommittee SC 16, *Horizontal methods for molecular biomarker analysis*.

Introduction

Varietal identification testing requires high-quality markers, which are able to provide reproducible data using a variety of equipment, chemistries, and reagents. Accordingly, this Technical Report only addresses specific amplification methods for sunflower.

The aims of this Technical Report are to provide a list of simple sequence repeat (SSR) markers and methods of analysis for sunflower. The set of SSR markers was established based on expert advice from molecular biologists using lists of publicly-available markers (for ORS markers: Tang et al., 2002: TAG 105:1124-1136 and for SSL markers: GIE Cartisol – Paris – France), and then validated through an intralaboratory study at GEVES (Laboratoire BioGEVES, Domaine du Magneraud, CS40052, 17700 SURGERES). The method is applied in officially testing hybrid conformity as part of the process of registering sunflower varieties in the French national varieties catalogue.

This document is linked to ISO 13495 where the different steps towards method validation are listed, and acceptance criteria are defined.

TECHNICAL REPORT **ISO/TR 17622:2015(E)**

Molecular biomarker analysis — SSR analysis of sunflower

1 Scope

The methods and SSR markers included in this Technical Report can be used for testing hybrid conformity and other applications such as molecular fingerprinting of varieties and checking variety identity.

2 Principle

SSR analysis is based on the amplification and visualization of the polymorphism caused by variation in the number of repeats in a sequence motif that is two to five base-pairs in length, also known as a microsatellite. SSR analysis consists of the following steps: sample preparation, DNA extraction, PCR amplification, separation and detection of the PCR products.

3 Consumables and equipment

— 96-well or 384-well microplate

— PCR reagents (DNA polymerase, buffer, $MgCl_2$, dNTP, primers, etc.)

— Capillary electrophoresis reagents

— Mixer/grinding mill

— Microplate centrifuge

— Adjustable-volume micropipettes

— Micro-centrifuge for microtubes

— Capillary electrophoresis system with fluorescence detection

— Thermocycler

4 Procedure

4.1 Sample preparation

For each sample, either individual seeds or seed mixes depending on the context are ground using a suitable mill (such as an IKA A10 or a Retsch MM301[1)]).

1) IKA A10 and Retsch MM301 are examples of suitable products available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of the product named. Equivalent products may be used if they can be shown to lead to the same results.

4.2 DNA Extraction and Quantification

a) Obtain an aliquot of each homogenously ground sample; the amount required will depend upon the extraction protocol employed.

b) Extract DNA using in house protocol or equivalent

NOTE Collaborative study has been carried out with QIAGEN DNeasy® 96 Plant Kit[2)].

c) The laboratory will validate that the quantity of DNA extracted is appropriated to ensure a reliable result.

4.3 PCR amplification

Conditions optimized for ABI 9700 thermocycler.

a) Mix preparation (see Table 1).

Table 1 — Mix preparation

	Concentration	Volume for 1X
H_2O		3,125 µl
Buffer 10X	1X	1 µl
dNTP (10 mmol/l)	125 µmol/l	0,125 µl
$MgCl_2$ (25 mmol/l)	3 mmol/l	1,2 µl
Taq DNA polymerase (5 U/µl)	0,25 U	0,05 µl
Forward primer (10 µmol/l)	0,25 µmol/l	0,25 µl
Reverse primer (10 µmol/l)	0,25 µmol/l	0,25 µl
Vol 1x mix		***6* µl**
DNA (2,5 ng/µl)		4 µl
Final PCR vol		***10 µl***

b) Amplification conditions (see Table 2).

A touchdown (TD) program is used: the hybridization temperature is lowered from 64 °C to 55 °C in decrements of 1 °C per cycle.

Table 2 — Amplification conditions

	10 cycles			30 cycles				
94 °C	94 °C	TD		94 °C				
10:00	0:30	*	72 °C	0:30		72 °C	72 °C	
		64 °C	0: 30		55 °C	0:30	10:00	10 °C
		0:30			0:30			∞
NOTE Units for times in Table 2 are in “minutes: seconds”.								

2) QIAGEN DNeasy® 96 Plant Kit is an example of suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of the product named. Equivalent products may be used if they can be shown to lead to the same results.

5 Established list of SSR markers for sunflower hybrid conformity testing

5.1 Characteristics of the SSRs

Data obtained with a 3130 Genetic Analyser[3)] (Applied Biosystems).

See Table 3.

Table 3 — Characteristics of the SSRs

No.	SSR	Linkage group	Number of alleles recorded	Range of estimated allele sizes (bp)	Nei's diversity index[a]
1	ORS309	4	2	121 – 131	0,48
2	SSL003	14	6	118 – 142	0,70
3	ORS342	2	5	307 – 345	0,42
4	ORS547	5	7	178 – 191	0,68
5	ORS613	10	8	201 – 230	0,62
6	SSL171	—	6	129 – 162	0,62
7	ORS432	3	3	160 – 164	0,52
8	ORS510	9	3	248 – 259	0,37
9	ORS605	1	8	174 – 203	0,66
10	ORS329	8	2	231 – 236	0,41
11	ORS621	11	7	232 – 250	0,63
12	SSL283	—	4	130 – 141	0,76
13	ORS307	14	4	109 – 137	0,53
14	ORS811	17	3	106 – 155	0,62
15	ORS502	12	5	92 – 165	0,38
16	ORS407	16	4	426 – 447	0,43

[a] Values for the Nei's diversity index were obtained on 124 male and female lines.

NOTE Source is Zhang et al., 2005 [2].

3) 3130 Genetic Analyzer is an example of suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of the product named. Equivalent products may be used if they can be shown to lead to the same results.

5.2 SSR primer sequences

See Table 4.

Table 4 — SSR primer sequences

No.	SSR	Forward primer sequence (5'–3')	Reverse primer sequence (5'–3')
1	ORS309	CATTTGGATGGAGCCACTTT	GATGAAGATGGGGAATTTGTG
2	SSL003	CACAACCTTTTCTTCTGCTTCC	GAGTCTCATTTGAGCCCACC
3	ORS342	TGTTCATCAGGTTTGTCTCCA	CACCAGCATAGCCATTCAAA
4	ORS547	TTGTCTTCATCTGCGTGTGA	TTGCTGTTGTTGATCGGTGT
5	ORS613	GTAAACCCTAGGTCAATTTGCAG	ATCTCCGGAAAACATTCTCG
6	SSL171	TCTGAACTGGAGGATGGGAC	TGCAAAGAAGAAGAAGTGGAGA
7	ORS432	TGGACCAGTCGTAATCTTTGC	AAACGCATGCAAATGAGGAT
8	ORS510	CATCGCGTCCCTCTCTCTAA	CCAACCATCACAGCAATCAG
9	ORS605	CGCGTGATGTGACGATTATT	ACGGAGCAAAGTTTCGAGGT
10	ORS329	CATCCTCCTCACCAACCAGA	GGGAAATCTTCTAAACGGTATGG
11	ORS621	CGCCTTATGCTGAGAGGAAA	CCTGAAGCGAAGAAGAATCG
12	SSL283	TTCCCAGTTGATTCCCTTTG	GAGCATTGGAGGCCAATAAG
13	ORS307	CAGTTCCCTGAAACCAATTCA	GCAGTAGAAGATGACGGGATG
14	ORS811	CCTTCTCCTCAATCTTTGGCTA	AGGAATGAAATGGGTGTGTGT
15	ORS502	ATCCCAACAGACGCCATTAT	AACATTGGAGGGAGCCAATA
16	ORS407	TGGCTAGGATTGCTTCATCA	TTTGCTTGCGCTTCTTACCT

5.3 Observed SSR profiles of sunflower lines

See Table 5.

Table 5 — SSR profiles observed for ten sunflower lines during intralaboratory validation

		Name of the line									
No.	**SSR**	**RHA274**	**RHA377**	**RHA801**	**PAC2**	**RHA266**	**HA89**	**H52**	**HA372**	**HA383**	**HA821**
1	ORS309	131	131	131	131	131	121	121	131	121	131
2	SSL003	121/139	121	121	121	139	139	139	134	134	134
3	ORS342	337	337	337	328	337	340	337	307	337	337
4	ORS547	184	178	184	178	178	178	188	188	178	178
5	ORS613	201/226	201	201	230	226	211	226	226	226	226
6	SSL171	158	129/158	158	129	129	158	129	149	158	129
7	ORS432	164	164	164	162	164	164	162	162	164	160
8	ORS510	248/257	257	253	248	257	257	257	257	248	257
9	ORS605	197	199	197	197	197	197	199	203	189	189
10	ORS329	231	236	236	231	236	236	236	236	236	236
11	ORS621	240	239	239	235	233	250	235	235	250	235
12	SSL283	130	130	130	133	130	130	130	130	130	139
13	ORS307	135	112/135	112	112	112	135	112	112	112	112
14	ORS811	110	155	110	155	110	155	106	106	155	106
15	ORS502	116	116	116	116	116	92	116	116	116	116
16	ORS407	426	443	426	426	441	443	null allele	441	443	441

Molecular profiles of ten sunflower lines are reported as estimated fragment sizes in bp for each of 16 SSR markers. For a given SSR in a line, a single fragment size indicates the marker is monomorphic within the line while two fragment sizes indicate that the marker is polymorphic within the line. The "null allele" entry indicates the absence of a PCR product in the repeatability and reproducibility tests.

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[1] Tang S., Yu J.K., Slabaugh M.B., Sintani D.K., Knapp S.J. Simple Sequence Repeat Map of the sunflower genome. TAG. 2002, **105** pp. 1124–1136

[2] Zhang L.S., Le Clerc V., Li S., Zhang D. Establishment of an effective set of simple sequence repeat markers for sunflower variety identification and diversity assessment. Can. J. Bot. 2005, **83** pp. 66–72

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