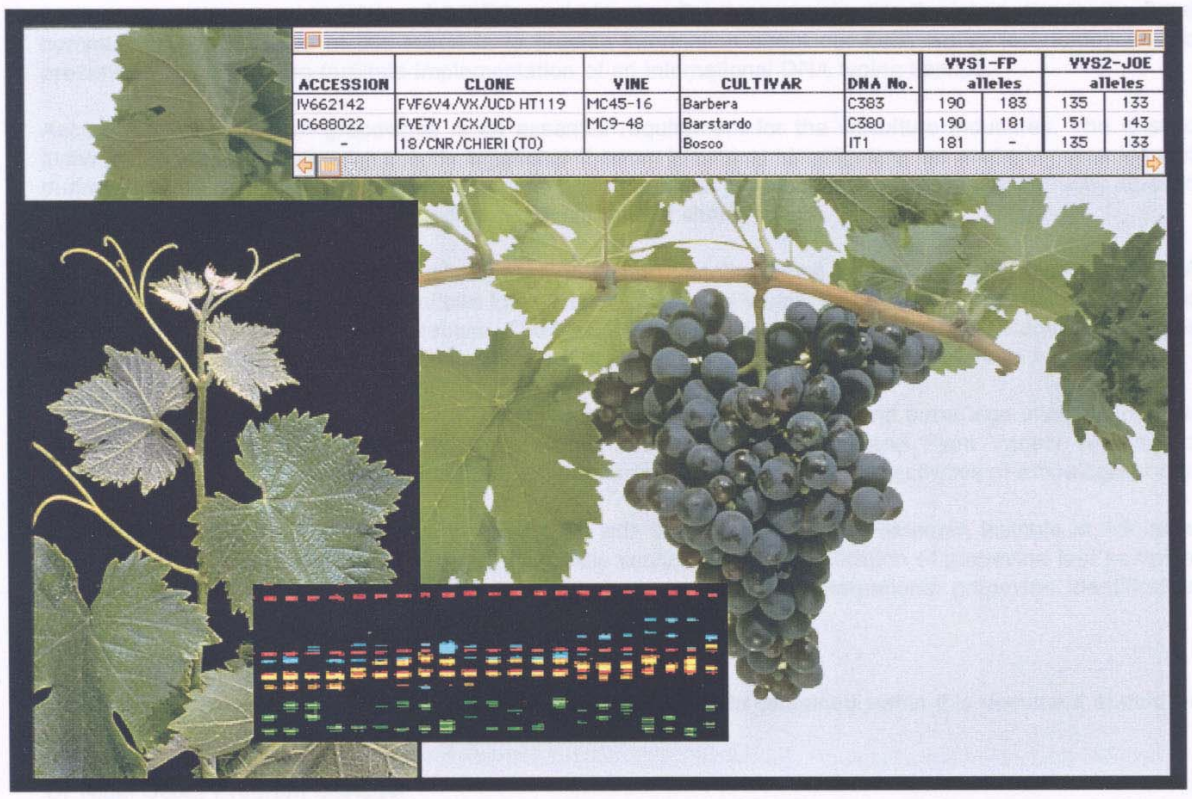
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DNA Typing of Grapevines

a universal technology for identification, cultivar description and evaluation of genetic relatedness.



Division of Horticulture
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May, 1994

DNA Typing of Grapevines:

a universal technology for identification, cultivar description and evaluation of genetic relatedness.

Summary

The CSIRO Division of Horticulture has developed a reliable DNA typing system for grapevines using superior genetic marker technology, which is protected by an international patent. Research and production engineering on the system is now complete, and CSIRO would like to offer the technology to the international viticultural community. The purpose of this report is to provide information about our DNA typing technology and to present an opportunity to facilitate implementation of an international DNA typing service.

Accurate identification of grapevines is an essential requirement for the viticulture industries. The cost to individual growers and wineries can be significant if errors in cultivar identification are made in nurseries and during vineyard establishment prior to commercial wine and fruit production. Distribution of incorrectly labelled wine or fruit can affect reputations and compromise market share.

The system provides objective identification of grapevine varieties using a rapid chemical test of the DNA extracted from vine tissue. The test allows identification of any vine variety that has previously been recognised and registered on the electronic database. The test measures and records the size of individual DNA alleles found in a number of defined genetic loci that are distributed between all *Vitis* varieties tested so far.

We anticipate that the system can be reliably used for cultivar identification and parentage analysis in order to assist with asset identification, legal protection through Plant Patents and Plant Variety Rights and population studies. The system is intended to complement and enlarge on the activities of ampelographers.

Currently the Division of Horticulture is negotiating with the Australian Wine Research Institute in Adelaide, South Australia with a view to providing a diagnostic service for the identification of grapevine leaf samples. Successful negotiations would eventually see the development of an international grapevine identification service.

Expressions of interest concerning this project and the proposals contained within this document should be directed to: -

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Contents

Summary ---- 1

The Technology ---- 5

Applications ---- 9

A Proposal for a DNA Typing Service for Grapevine Varieties ---- 13

Appendices ---- 15

References ---- 17

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The Technology

The Genetic Markers
CSIRO has developed a reliable DNA typing system for grapevines using superior genetic marker technology. The system provides objective identification of grapevine varieties using sequence-tagged microsatellite sites (STMSs), Polymerase Chain Reaction (PCR) technology and the tagging of the products with dyes to produce a machine readable result. The test allows identification of any vine variety that has previously been recognised and registered on the electronic database. It measures and records the size of individual DNA alleles found in a number of defined genetic loci that are distributed between all *Vitis* varieties tested so far.

Of the large number of different types of genetic markers available, SMTSs have proved to be the superior markers for use with grapevines. They have the following advantages:

- they detect high levels of DNA polymorphisms in grapevine
- they show a co-dominant mode of inheritance, i.e. they obey simple mendelian inheritance
- they are easily scored for machine storage
- data can be reliably transferred between users
- they allow simple computerised data interpretation, producing a clear, speedy result

The simple inheritance of STMS markers, the rapidity of PCR analysis, the ease of scoring alleles, and the capacity to automate the system are advantages lacking in the RFLP and RAPD marker systems we have previously investigated. Single locus markers simplify the storage of data in an electronic database. Automation of the system has allowed absolute base pair sizes to be assigned to STMS alleles rather than arbitrary alphabet letters as previously assigned.

Each STMS marker chosen detects mendelian inherited co-dominant alleles at a single locus in the grapevine genome. Allele polymorphisms are detected as different lengths of the microsatellite when separated on polyacrylamide gels. Many of these STMS loci are present not only in *Vitis vinifera* but also in other *Vitis* species including the American species used throughout the world as rootstocks. The STMS loci appear to be genetically stable with a very low frequency of somatic mutation during vegetative propagation of cultivars.

This marker type is very versatile and can be used for cultivar identification, determination of parentage, population genetics and genetic and physical gene mapping.

The Database of Cultivars

The existing database has been built up from established mother vines obtained from the accredited *Vitis* collections in Australia located at the CSIRO Division of Horticulture's Merbein research station in Victoria and the South Australian Department of Primary Industry's Advisory Centre collection at Nuriootpa in South Australia.

All genotype data entered into the database was produced using standardised procedures and automated equipment. Computer collection and analysis of the raw data by the GENESCAN software permits simple electronic transfer of allelic data into the database. The database has search and sort commands which allow fast cultivar comparisons to be made. The format of the database is shown in Table 1.

The database has two major divisions -on the left side is information about the cultivar and on the right side are the loci that have been used for DNA typing. The data consists of:

- an accession (quarantine importation) number if known
- the clonal source of the material
- the location of the single vine from which leaves for DNA extraction were collected
- the cultivar name
- the DNA number used for the PCR reaction.

The DNA number identifies the DNA sample used and the addition of the letter C (for CSIRO) allows identification of the user who entered the data. The numeric DNA typing data is the length of the alleles in base pairs (bp). In the database each cultivar has been DNA typed at the same 7 loci. By combining the genotypes across these loci it is possible to distinguish each cultivar by its unique combined genotype.

The naming of individual loci, i.e. VVS1-FP, reflects the cultivar from which the original microsatellite locus was cloned and sequenced, in this case V. vinifera cv 'Sultana', the arbitrary locus number 1, and the dye attached to the locus specific primer which is FluorePrime. Grapevine PCR products are automatically sized by the GENESCAN software.

The database at present consists of more than 80 cultivars, subdivided into four groups representing red wine cultivars, white wine cultivars, multipurpose and table grape cultivars, and rootstock cultivars. Table 1 includes a few from each group. Genetic differences between cultivars were observed for alleles at these loci. For example, Merlot had alleles of 190 and 181 bp at the VVS1-FP locus and 151 and 139 bp at the VVS2-JOE locus while Pinot Noir was genetically different at these two loci with values of 190 and 183 bp at the VVS1-FP locus and 151 and 137 bp at the VVS2-JOE locus. All cultivars that had a single allelic band were given a genotype like the VVS1-FP locus of Shiraz (181 :-). The (-) symbol makes no assumptions about the homozygosity or otherwise of the genotype and it may actually represent the other homozygous allele, a null allele, or an allele that was missed during the DNA typing procedure.

Nearly all the cultivars typed so far had either two alleles (heterozygous state) or one allele. However, some cultivars had no alleles for the two separate STMS loci, VVS5-FP and VVS19-JOE, and were given a null: null genotype. Most rootstock cultivars (non V. vinifera species) display a null: null genotype at the VVS19-JOE locus making it less informative for rootstocks. A null allele could occur when the DNA representing the locus is present but its amplification by PCR is prevented by DNA mutations (point mutations, DNA deletions or insertions) at or between the priming sites or the DNA representing the locus is not present in the cultivars genome. The presence of null alleles in the grapevine population for the STMS loci VVS5-FP and VVS19- JOE, and possibly other loci, indicates that cultivars displaying one allele at an STMS site may not be homozygous but may be heterozygous with a null allele.

Future Developments

At the moment, fresh grapevine leaf material is the preferred tissue for DNA typing, although fresh root pr stem tissue can be used. The possibility of using free run juice is under investigation.

The database is in a flat file format allowing simple search and spot procedures. An international version is being developed that combines both DNA typing and ampelographic information.

Table 1. Database examples of grapevine cultivars DNA typed

ACCESSION	CLONE	VINE	CULTIVAR*	DNA No.	VVS1-FP alleles		VVS2-JOE alleles		VVS3-JOE alleles		VVS4-FP alleles		VVS5-FP alleles		VVS19-JOE alleles		VVS29-TAM alleles	
AS702271	SA 1654-NURIOOTPA	HR11:V29	Shiraz	C24	181	-	133	-	222	216	173	168	121	-	203	186	179	171
IV692217	FVG9V3/VX/UCD	MC22-48	Cabernet Sauvignon	C198	181	-	151	139	222	216	175	168	123	103	-	-	181	179
IS642051	FVD5V12A/VX/UCD	HR11:V71	Pinot Noir	C27	190	183	151	137	222	216	173	168	146	121	186	168	179	171
IS672093	NFD3V14/VX/UCD	HR9:V62	Merlot	C17	190	181	151	139	222	216	175	168	146	121	186	-	181	175
-	PENFOLD 1958	HR13:V41	Cabernet Franc	C58	181	-	147	139	216	-	175	167	123	121	163	-	181	175
IC798307	284/CX/ANTAV	MC41-36	Gamay	C381	190	183	137	133	222	216	173	168	146	92	203	168	179	171
IS758129	FVI10V5/CX/UCD	HR10:V65	Chardonnay	C13	190	183	143	137	222	216	173	168	146	90	203	186	179	171
-	CX/VASSAL	MC15-26	Merlot Blanc	C241	190	181	151	-	222	-	169	168	121	110	203	186	179	175
IS642052	FVC3V15/VX/UCD	VCW3:V35	Traminer	C30	190	162	151	-	222	216	175	168	146	103	192	168	171	-
IS748150	1969/CX/URBANA	VCW3:V20	Villard Blanc	C31	194	191	143	133	216	214	175	167	110	92	163	-	181	175
-	SA 82-NURIOOTPA	HR9:V53	Semillon	C23	190	181	133	-	222	216	168	-	146	110	192	-	171	-
IV712308	FVF6V13/VX/UCD	MC45-36	Chenin Blanc	C388	181	162	151	133	222	216	173	168	146	101	168	-	179	171
-	CX/VASSAL	MC15-25	Crouchen	C385	190	181	151	133	222	216	168	159	103	90	192	-	181	171
IC728192	FV13V15/CX/UCD	MH87-40	Djandjal Kara,	C156	182	181	155	126	222	216	175	-	118	90	190	-	171	-
IV662140	FVB9V5/VX/UCD	MH85-38	Emperor	C276	181	-	135	133	216	-	175	167	118	90	168	-	171	-
IC748236	FVK5V8/CX/UCD	MH93-10	Flame Seedless	C364	181	-	151	133	222	216	175	-	118	-	186	-	179	171
IC688042	FVE3V8/CX/UCD	MC71-46	Muscat Hamburg	C355	190	181	149	135	222	-	175	168	110	-	168	-	179	171
IV692214	FVE11V5/VX/UCD	MH90-46	Waltham Cross	C154	188	181	135	133	222	216	175	169	146	110	-	-	171	-
-	UNSELECTED	MC54-67	Zante Currant	C137	188	181	143	137	222	216	175	169	146	144	190	-	171	-
-	EX/RUTHERGLEN	MD6p5V1	99 Richter	C411	195	194	149	137	218	214	185	177	110	97	-	-	189	175
IS742165	FVA5V11/VX/UCD	HR16:V50	Rupestris du Lot	C64	194	-	137	-	214	-	185	-	97	-	-	-	189	175
IC808338	13-45-5/CX/GEISENHEIM	MD5p6V3	5BB Kober	C422	195	194	149	141	218	214	185	177	110	103	-	-	175	-
IV662133	FVA3V13/VX/UCD	MD4p13V1	5A Teleki	C409	195	194	149	141	218	214	185	177	110	103	-	-	175	-
IC808344	10-48-49/CX/GEISENHEIM	MD5p8V4	5C Teleki	C419	195	183	149	145	218	214	187	177	110	103	-	-	175	-
IC808341	94/CX/INRA	MD4p11V3	SO 4	C417	195	183	147	145	218	214	185	175	110	103	-	-	175	-

Applications

Cultivar Identification and Statistical Comparisons
The information in the database can be used to objectively investigate cultivar identification errors that may have occurred as a result of mislabelling, multiple naming of a single cultivar, or incorrect ampelographic identification.

In the viticulture industry, both in Australia and world-wide, many instances exist where incorrect identification of grapevine cultivars has led to errors in cultivar names and mixed plantings. In Australia, confusion between Merlot and Cabernet Franc has resulted in mixed commercial plantings. Misidentification has resulted in instances where both Chen in Blanc and Crouchen have been wrongly labelled as Semillon. Incorrect identification of the rootstock Teleki 5C as S04 at the Foundation Plant Materials Services at the University of California has recently been uncovered. The data in Table 1 shows that the cultivars involved in the above misidentification cases are all objectively identified and distinguished by their unique DNA profile. For example, the DNA typing results for Merlot and Cabernet Franc show that they differ genetically at 6 of the 7 loci tested.

Of more than 80 cultivars DNA typed, all are genetically different except for the rootstocks 5A Teleki and 5BB Kober (Table 1). Both rootstocks as well as 5C Teleki are believed to have originated from a large batch of seed given to S. Teleki in 1896. The seed was thought to be a mixture of *Vitis berlandieri*, *Vitis berlandieri* X *V. riparia*, *Vitis berlandieri* X *V. rupestris*, *Vitis berlandieri* X V. *vinifera*, and V. *vinifera*. From 40,000 seedlings Teleki made selections including 5A and 5C and also sent some plants to F. Kober who made other selections including 5BB Kober. Statistics are often used in such cases to determine the probability of such a match between 5A Teleki and 5BB Kober occurring by chance. If the probability is low then an informed decision can be made to assume that the two matched cultivars are in fact the same cultivar.

At present we advocate a conservative approach for calculating the probability of a match for grapevines that does not depend on theoretical assumptions or an understanding of grapevine population genetics, as knowledge in this area is scarce. In this approach the frequency of a genotype is simply 1/n where n is the number of typed cultivars in the database. The probability of 5A Teleki and 5BB Kober having the same genotype simply due to chance was 1.25×10^{-2}. Though this calculation was conservative, reliability will increase as more cultivars are added to the database.

The present database shows that even genetically close cultivars, siblings, parents and progeny, can be identified by their unique DNA profiles. This system may sufficiently distinguish individual cultivars in a large database of 5,000-8,000 individuals, covering most of the grapevine cultivars that are presently grown world- wide. Seven loci were used to DNA type cultivars but the number can be increased for more discriminating power in a larger database.

Parentage Analysis
The mendelian inheritance of STMS alleles permits genetic examination of pedigrees. Table 2 lists a number of crosses that have been investigated. Tarrango, a progeny of Touriga X Sultana, shows the expected genotype across all loci having inherited one allele from both parents at each locus. The same result is seen for the crosses that produced Ruby Seedless, Marroo Seedless and Sultana Moscata. A three generation passage of genetic material, STMS alleles as well as surrounding DNA, is observed at the Marroo Seedless locus, VVS2-JOE, with the 151 bp allele originating from Sultana.

Aurelia and Műller- Thurgau are two cultivars whose genotypes do not agree with the suggested parentage. In the case of Aurelia, at the locus VVS5-FP, a 92 bp allele is contributed by Villard Blanc but the 101 bp allele is not present in the Chaouch genotype (148 and 110 bp). If there have been no errors in cultivar identification and labelling then this discrepancy would be due to a recent mutation at the locus changing the length of the microsatellite repeat and therefore the allele length. Mutations at microsatellite loci that change the length of the microsatellite repeat have been observed at a low frequency (4.5×10^{-4} -1.6×10^{-6}) in humans as a result of meiosis and it may be that the VVS5-FP 101 bp allele of Aurelia is the result of such a mutation.

Műller- Thurgau has a combined genotype across the STMS loci that clearly indicate that Sylvaner is not a parent. Four of the loci show that Sylvaner has no allele in common with Műller- Thurgau. This high genetic difference prevents considering mutations during meiosis as a possible explanation for the genetic difference. The other explanation, which cannot be ruled out, is that the plant analysed as Sylvaner is not a clone of Sylvaner but has been wrongly identified at some stage during its propagation and world-wide distribution. Műller- Thurgau originated from a cross made in 1891 and today is a major cultivar grown in Germany. Although it is considered to be a cross of Riesling and Sylvaner another opinion is that it may be the result of a Riesling X Riesling cross. An examination of the genetic data in Table 2 shows that Riesling is one parent of Műller- Thurgau but could not be both parents. Questions raised about the correct identity of the cultivars 5A Teleki, 5BB Kober, Műller- Thurgau and Sylvaner warrant further study, including DNA typing other clones of these cultivars obtained from different sources.

Table 2. Grapevine parentage analysis

ACCESSION	CLONE	VINE	CULTIVAR	DNA No.	VVS1-FP alleles	VVS2-JOE alleles	VVS3-JOE alleles	VVS4-FP alleles	VVS5-FP alleles	VVS19-JOE alleles	VVS29-TAM alleles
IC698118	FVE6V12/CX/UCD HT83	MC10-33	Touriga*	C157	183 181	151 143	222 -	175 169	121 109	192 188	171 -
AC758261	MH47-40/C/MERBEIN	MG73-12	Tarrango	C373	181 -	151 143	222 216	175 -	121 101	192 186	179 171
AS648160	H5/C/MERBEIN	HR 14:V68	Sultana	C29	188 181	151 145	222 216	175 -	118 101	201 186	179 171
IC698202	1969/CX/URBANA	MH87-32	Carolina Blackrose	C408	191 181	135 -	216 -	175 168	110 92	- -	175 171
-	MS23-7/C/MERBEIN	MA9-7	Marroo Seedless	C280	181 -	151 135	216 -	175 168	110 -	168 -	171 -
IV692223	FVB13V15/VX/UCD	MH92-26	Ruby Seedless	C275	181 -	151 133	222 216	175 -	118 -	201 168	179 171
IV662140	FVB9V5/VX/UCD	MH85-38	Emperor	C276	181 -	135 133	216 -	175 167	118 90	168 -	171 -
IV692223	FVB13V15/VX/UCD	MH92-26	Ruby Seedless	C275	181 -	151 133	222 216	175 -	118 -	201 168	179 171
IC658015	1965/CX/CONEGLIANO	MH92-41	Sultana Moscata	C277	188 181	151 149	222 216	175 168	118 -	201 -	179 171
IS758041	FVB6V8/CX/UCD	VCW 3:V38	Muscat Gordo Blanco	C59	181 180	149 133	222 -	175 168	- -	168 -	179 171
IC658015	1965/CX/CONEGLIANO	MH92-41	Sultana Moscata	C277	188 181	151 149	222 216	175 168	118 -	201 -	179 171
AS648160	H5/C/MERBEIN	HR 14:V68	Sultana	C29	188 181	151 145	222 216	175 -	118 101	201 186	179 171
IS748150	1969/CX/URBANA	VCW 3:V20	Villard Blanc	C31	194 191	143 133	216 214	175 167	110 92	163 -	181 175
-	UNSELECTED	MC74-8	Aurelia	C353	191 181	135 133	222 216	175 -	101 92	163 -	175 -
-	UNSELECTED	MH89-32	Chaouch	C136	181 180	151 135	222 214	175 -	148 110	190 -	175 171
IS750012	239/NX/GEISENHEIM	HR 9:V23	Riesling	C21	190 -	151 143	222 216	168 -	103 90	203 194	179 171
IV712316	FVH10V9/VX/UCD	MC42-48	Müller-Thurgau	C402	190 183	151 143	222 -	173 168	110 90	203 186	179 -
-	FVC6V13/VX/UCD 1962	MC36-1	Sylvaner	C392	190 180	153 151	222 -	168 -	146 125	168 -	179 171

* Crosses are arranged with the female (Touriga) first, progeny (Tarrango) second, and male parent (Sultana) last. Inherited alleles are outlined.
Note that the genotypes maintain the database format of an '-' allele which can represent a homozygous allele or a null allele.
In most cases it is obvious from the parentage whether the '-' represents homozygosity for a particular allele, and homozygosity is assumed for most situations.

A Proposal for a DNA Typing Service for Grapevine Varieties

The Australian National Vine Improvement Association has expressed interest in using this technology to complement ampelography in certifying their initial plantings and mother vine plantations. Individual wine makers have indicated that they believe it will be necessary to use this technology to allow robust identification of vines in their own vineyards and subsequently in all vineyards from which they source their material. This will be part of the methodology of providing an audit trail to satisfy ISO 9000 and export requirements. The need to begin the introduction of this technology is predicted to occur in about 2-3 years from now.

International interest in the technology is also high but the commercial applications are not yet determined. The technology is such that it could be practical to provide a world wide service from an Adelaide base. Options are:

- providing or selling the primers to overseas groups together with the technology
- receiving PCR reactions from overseas groups, analysing them with GENESCAN in Adelaide and returning the completed analysis and result
- receiving the frozen grapevine material and doing the complete procedure in Adelaide

An internationally accessible and useful database would need data from grapevine varieties not commonly used in Australia. The development and maintenance of an international database will require collaboration with overseas groups for analyses of material and entry of new material in to the reference database.

It is possible that other crops could also be analysed in this way. The CSIRO Division of Horticulture has developed a similar system for use in the hop industry and it is likely that the hop industry will be looking for a suitable analytical service on both an Australian and international basis. Technically there will be no difference between analyses for hops and grapevines and this would allow expansion of the service.

We are currently negotiating with the Australian Wine Research Institute to set up a facility for DNA typing of grapevines on a national and eventually an international basis using the technology developed by the CSIRO Division of Horticulture.

Appendix I

DNA Typing Technology and Automation
Amplification of STMS alleles by PCR (polymerase chain reaction) has a number of advantages that includes the capability to analyse nanogram quantities of grapevine DNA and rapid manual or automated amplification and analysis of the STMS alleles. To ensure sensitivity and ease of automated or manual analysis DNA primers for each STMS were designed so that all amplified allele fragments would be larger than 80 base pairs (bp) but less than 250 bp. This size range was chosen to allow resolution of 1-2 bp differences in normal sequencing gels.

Maximum automation of the DNA typing procedure with present technology has been achieved by combining PCR with an automated gel analysis system (ABI) running GENESCAN software. This software permits a computer to collect the data from the gel during a run and automatically analyse the results after collection is complete. Non-radioactive detection of STMS alleles is done with the use of fluorescently labelled STMS primers. From a 20 µL PCR reaction less than 0.5 µL is loaded onto the gel for analysis leaving the remainder of the sample to be kept in the freezer for long-term reference. The dyes are extremely stable with samples still useable after more than one year. The cover illustration features a computerised image of a gel where alleles from different loci have a different colour due to the different coloured dye on the primers. Different coloured primers permit a number of loci to be run in the same lane along with the size standard which allows the computer to automatically size the different alleles.

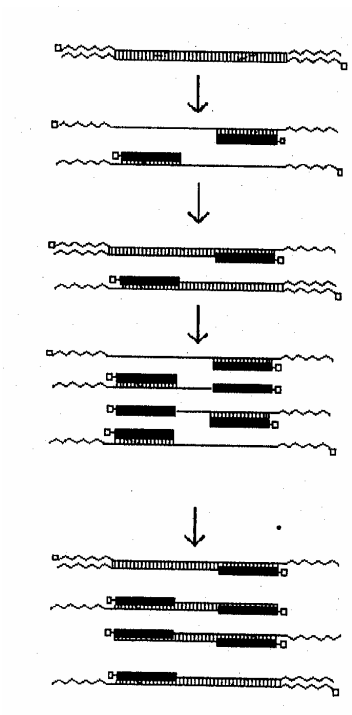
By using a single DNA polymerase, Taq DNA polymerase, and standardising dyes to particular STMS primers the system used in this study has proven to be robust for more than one year. High reproducibility of size calling for replicate samples run on different gels (+/- 0.05 to 0.38 bp) was observed as well as a high level of accuracy for sizing common alleles between different cultivars on the same gel (+/- 0.0 to 0.4 bp) and over different gels (+/- 0.16 to 0.83 bp). In practical terms this means it is not necessary to run cultivar samples side by side on the same gel every time cultivars are compared. In most cases the results collected from a single gel are sufficient for entry into the database for subsequent comparisons.

Appendix II

The Polymerase Chain Reaction (PCR)
The Polymerase Chain Reaction provides a method by which a million or more copies *of* a short region of DNA can be made in a process similar to the manner in which it is done naturally in the cell. With PCR amplification, very small samples *of* tissue -theoretically even a single cell -can be used to study DNA.

In the PCR process, two short oligonucleotide primers are bound to the opposite strands *of* a target DNA segment in positions flanking the region to be amplified. The PCR is basically a three step process. In Step 1, the double stranded DNA sample is dissociated into single strands by incubation at high temperature, typically 94°C. In Step 2, the temperature is lowered to allow the oligonucleotide primers to bind to their complementary sequences in the DNA to be amplified. In Step 3, a DNA polymerase extends the primers from each *of* the two primer-binding sites across the region between them with the target sequence as template.

There is in principle a doubling *of* the sequence in each cycle. The primers become physically incorporated into the amplified products. Both amplification and genetic typing can be completed in a day. An extended discussion of the way in which the PCR is used for detection *of* genetic variation can be found in *PCR Technology: principles and applications for DNA amplification,* listed at the back of this report.



Step 1 Small sample *of* DNA sequence *of* interest is heated with selected primers and DNA polymerase

Step 2 DNA is denatured and primers attach to sites on either end of sequence of interest

Step 3 Primer extension - DNA polymerase completes a new copy of the sequence *of* interest between the primers

Process begins again - DNA is denatured with heat and primers bind to selected sites flanking the sequence of interest

DNA polymerase completes new copies of the sequence of interest. Approximately 30 cycles are required to produce a yield of about 10^6-10^7 copies of the target sequence.

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