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This report represents a summary of the presentations and discussions at the workshop and must not be considered as a official position of the European Commission

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1. Glossary

Alleles: are the different alternative DNA sequences that can be present at the same physical location in the genome. Different alleles may or may not result in different phenotypic traits.

Chromatography is the collective term for analytical methods that separate the different chemicals present in a complex sample. The sample is dissolved in a mobile phase and injected onto the head of the chromatographic column. The column is coated with a microscopic layer of a specific liquid or polymer, also called stationary phase. The different chemicals in the sample will exit the column at different rates depending on their various chemical and physical properties and their interaction with the specific column coating. At the end of the column the chemical are detected and identified. The mobile phase can be gaseous, in **gas chromatography (GC),** or liquid, in **liquid chromatography (LC).**

DNA chip also called **array:** consist of a collection of thousand microscopic spots on a solid surface, such as glass or plastic. Each spots contains a specific short DNA sequence, called probe.

DNA microarray: is a multiplex technique that uses a DNA chip to accomplish many genetic tests in parallel. This technique is based on the hybridization of specific DNA fragments (also called targets) present in the test sample with the probes on the DNA chip. The labelling of the DNA in the test sample, usually with fluorescent labels, allows visualization of the target-probe hybridizations.

Gene flow: is the transfer of alleles or genes from one population to another. For plants gene flow can occur by pollen that are transported by animals or wind.

Genotype: describes the genetic information present in the DNA sequence of an organism. A genotype is typically focused on the differences of a certain individual within a group or a species.

High throughput analysis methods are designed to analyse large amount of samples in a time- and cost-efficient manner

Hybrid: is a variety that originates from cross-breeding of different varieties.

Insertion-deletion polymorphism (Indel): is a variation in the DNA sequence caused by the presence or absence of one or several succeeding nucleotides. For example, the following variation can be observed in two DNA sequences obtained from different individuals (or from paired chromosomes of a single individual)

AAGC**CTAA**TCG and
AAGCTCG,

This Indel variation consists of 4 nucleotides. Indel variations can only have two alleles: presence or absence of the involved nucleotides.

Landrace: are domesticated varieties that are adapted to the environment in which they grow. Landrace often develop naturally with minimal assistance or guidance from humans using traditional breeding methods.

Mass spectrometry (MS): is an analytical technique used to elucidate the chemical structures of molecules, such as small organic molecules, amino acids and peptides. The MS method starts with the ionization of the molecules. The resulting ions will pass through electric and magnetic fields and their speed and moving direction will depend on their mass-to-charge ratio. At the end the separated ions will be detected.

Phenotype: is the collective term for all observable characteristics or traits of an organism, including its morphology, development, biochemical and physiological properties, and behaviour. The phenotype of an organism results from the expression of its genotype as well as the influence of environmental factors and possible interactions between the two.

Polymerase chain reaction (PCR): is an analytical technique to amplify a single or few copies of a specific DNA fragment by several orders of magnitude to generating thousands to millions of copies.

Standard operating procedure (SOP): is a written document with a detailed description of all steps in a measurement procedure. Implementation of these steps without any deviation or modification is required to guarantee the expected outcome.

Simple sequence repeat (SSR) also called **microsatellite:** is a repetition of a short DNA sequence (usually 2 to 4 nucleotides long) that appears in tandem. The number of repetitions varies. For example, the following variation can be observed in DNA sequences obtained from different individuals:

CAGCAGCAG	3 repeats,
CAGCAGCAGCAGCAG	5 repeats and
CAGCAGCAGCAGCAGCAG	6 repeats

Each fragment with a different number of repeats is called an allele and most SSR have more than two alleles.

Single Nucleotide Polymorphism (SNP): is DNA sequence variation occurring at a specific position in the genome. The nucleotide, A, T, C, or G, present at this position differs. For example, the following variation can be observed in two DNA sequences obtained from different individuals

AAGC**C**TA and
AAGC**T**TA,

In this case we say that this SNP has two alleles: C and T. Almost all common SNPs have only two alleles.

SNP array or SNP chip: a type of DNA microarray that allows the identification of the alleles for thousands of SNPs in a test sample. The probes present on these chips are allele specific oligonucleotides.

Ring trail: is a study organised to evaluate or validate a certain method or material. Several different laboratories are included in these ring trails.

Qualitative authentication methods: allow the identification of the different varieties present in a certain sample. However, the quantity of each variety within the sample can not be determined using a qualitative method.

Quantitative authentication methods: allow the identification and quantification of the different varieties present in a certain sample.

2. List of acronyms

DArT	Diversity arrays technology
DG AGRI	Directorate General for Agriculture and Rural Development
DG RTD	Directorate General for Research
DNA	Deoxyribonucleic acid.
EC	European Commission
EU	European Union
FSA	Food Standards Agency
GC	Gas chromatography
Indel	Insertion-deletion polymorphism
IRGC	International Rice Genebank Collection
IRMM	Institute for Reference Materials and Measurements
IRRI	International Rice Research Institute
JRC	Joint Research Centre
LC	Liquid chromatography
MS	Mass spectrometry
PCR	Polymerase chain reaction
SNP	Single nucleotide polymorphism
SSR	Simple sequence repeat
SOP	Standard operating procedure
UPOV	International union for the protection of new varieties

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3. Executive summary of the plant authentication workshop

3.1. Background

Basmati rice represents a small but superior group of rice which is distinguished by quality traits like aroma and grain length. In the European Union (EU) the highly valued Basmati accounts for 30% of the total rice import, i.e. around 300 000 tons per year. According to two agreements concluded in August 2004 between the EU and India and Pakistan, nine Basmati rice varieties can be imported as brown rice into the EU at a zero import duty. The agreements also foresee the creation of a Community control system based on DNA analysis to monitor the rice imports. The control system should be developed by the EU in active collaboration with India and Pakistan.

3.2. Purpose

This workshop was organized by the Institute for Reference Materials and Measurements (IRMM) of the Joint Research Centre (JRC) on behalf of the Directorate General for Agriculture and Rural Development (DG AGRI). The aim of this workshop was to consult with experts in the relevant special scientific fields about the different aspects which should be considered in the development and validation of methods for the authentication of eligible varieties of Basmati rice in imports. Another aim was to get an update of the status (development stage and validation status) of different methodological approaches which might be used for plant and more in particular Basmati rice authentication. Therefore recognized experts from different relevant scientific areas and with particular specialisation (plant breeding, genetics, metabolomics and authentication) were invited to participate.

3.3. Organisation of the workshop

In total 14 experts from institutions in the EU Member States and 17 experts from outside the EU were considered for being invited to the workshop. After consultation between IRMM, DG AGRI and Directorate General for Research (DG RTD) 13 experts from the EU Member States and 17 experts from outside the EU were contacted by IRMM to check for their availability and preparedness to contribute to the workshop. For those supposed to give a presentation the topic was pre-defined and the content was coordinated with other presentations to reduce redundancies and to make sure that all relevant aspects would be addressed.

Finally 10 experts from EU Member States (Germany, Portugal, Spain, The Netherlands and United Kingdom) and 10 from institutions outside the EU (Australia, India, Mexico, Pakistan, Peru, Philippines) could attend and contribute to the workshop, which took place on 21 and 22 September at the IRMM in Geel, Belgium.

The scientific programme of the workshop comprised three sessions with scientific presentations and following discussions and a wrap up session.

The first session focussed on the context and general validation requirements for gene-based authentication methods, including presentations on the definition and maintenance of plant varieties according to the International Union for the Protection of New Varieties (UPOV) guidelines, a presentation on a case study on the authentication of maize landraces as well as an overview presentation on the genetic diversity of rice, the population structure and the potential gene flow.

The second session provided insight into the varietal and genetic diversity of rice in India and Pakistan. The different aspects were covered by three presenters from the two countries.

The third session focussed on rice authentication methods and their respective degree of validation. The presentations described the utility and validation status of single nucleotide polymorphisms (SNP), simple sequence repeats (SSR) and insertion-deletion polymorphisms (InDel) based methods, as well as of metabolomics and diversity arrays technology (DArT) based approaches.

Besides the reports of the rapporteurs of the three preceding sessions the fourth session consisted of a presentation on practical requirements for variety authentication methods from the point of view of customs laboratories and an open discussion for the wrap up of the workshop.

3.4. Topical summary of the presentations and sessions

3.4.1. Definition of a plant variety

The concept of a plant variety can have a different meaning for taxonomists, farmers, authorities and breeders. Mr Salaices presented on behalf of UPOV the definitions and conditions for listing of new varieties for their protection.

A variety is the lowest rank of plant grouping within a single botanical taxon. According to the International Convention for the Protection of New Varieties of Plants, a variety is defined by the expression of several characteristics resulting from a given genotype or a combination of genotypes. At least one of these characteristics discriminates the variety from all the existing varieties. The expression of the characteristics is uniform within all plants of the variety and remains unchanged after propagation at a specific environment. A unique genetic profile alone, without expression of a distinct phenotype, can not define a variety.

The maintenance of an approved variety is the responsibility of the breeder. When requested by the authority, the breeder should provide all information and material required for verification of the maintenance.

UPOV has developed guidelines for the examination of varieties which are available on their website: www.upov.int. The technical guideline document for rice varieties (TG/16/8) is listing 64 characteristics.

A breeder's right for the protection of a variety has to be cancelled if the variety is no longer uniform or stable (as it is claimed by some Indian parties for the evolved Basmati variety CSR-30) or because the breeder does not enable the maintenance of the variety.

Gene flow is a relevant parameter in plant breeding and for the maintenance of varieties. Mr McNally confirmed that the gene flow risk is low for rice varieties since outcrossing occurs within some tens of meters only.

3.4.2. Landraces

Mr Taba presented a case study on the Bolita maize landrace grown in certain areas of Mexico. Such landraces can be preserved for up to 30-60 years on farm level. Amongst 170 accessions from 15 villages all belonging to the Bolita landrace, different trait clusters could be observed. Pronounced differences in phenotype could be observed between accessions although belonging to the same landrace. Although the genotype of the accessions has not been characterised, the differences in phenotype lead to the expectation that there will be also significant differences in genotype.

One accession has been selected and registered as a variety. The example showed that the trait and genetic heterogeneity of landraces poses a significant challenge if distinction from other varieties or landraces would be based on the phenotype and/or genotype.

3.4.3. The varietal and genetic diversity of rice in the Indian subcontinent

The validation requirements for a plant authentication method largely depend on the practices concerning seed and grain production and the varietal diversity present in the cultivated area.

These topics were discussed in the presentations of experts from the International Rice Research Institute (IRRI) in The Philippines (Mr McNally), the Birsa Agricultural University in India (Mr Singh), the Indian Agricultural Research Institute (Mr Mohapatra) and the National Institute for Genomics and Advanced Biotechnology in Pakistan (Mr Zafar)

Rice belongs to the Oryza genus and is characterised by an extended biodiversity. This diversity has evolved over thousands of years, as farmers selected different types depending on the local cultivation practices and needs. Rice was domesticated by at least three independent invents. The domestication in West Africa created the Oryza glaberrima species. The Oryza sativa species were domesticated twice: the Indica varieties have their origin in South Asia while the Japonica varieties originate from East Asia.

The rice varieties grown on the Indian subcontinent consist of wild varieties, landraces, improved cultivars and hybrids. Landraces or traditional varieties developed naturally with minimal assistance or guidance from humans using traditional breeding methods. In general these traditional varieties are low yielding but they have a high yield stability and are more resistant to biotic and abiotic stress. The seed production is not controlled and genetic analysis of a landrace from the farmer's field, presented by Mr Mohapatra, reveals a composite genetic structure. According to statements made during the workshop there are almost no landraces cultivated in Pakistan while the number of landraces varieties cultivated in India today is around 100 to 150. These landraces are usually scented. For each landrace the cultivated area is around 1000 to 2000 hectare.

Improved cultivars are derived from intensive breeding programs performed at public and private institutes. For each generation the plants were selected for the desired phenotype and the resulting varieties are pure inbred lines. Total control of the seed production assures the genetic uniformity within these commercial cultivars. Intensive breeding programs performed by several public and private institutes lead to the constant release of new varieties. Mr Mohapatra estimates that during the period between 1969 and 2005 at least 732 new varieties were released in India. These high-yielding new cultivars have rapidly replaced low-yielding landraces. According to estimates of the MS Swaminathan Research Foundation (as presented by Mr McNally) up to 400 000 varieties were grown in India in the past and still about 100.000 exist. These numbers indicate the huge genetic diversity of rice but also the ambiguities in the definition of a rice variety. Mr Singh and Mr Mohapatra stated that currently in total about 900 rice varieties are released in the different states of India.

Many centers in the world are engaged in the documentation and conservation of the rice biodiversity by setting up germplasm collections. The International Rice Genebank Collection (IRGC) at IRRI is the world's most comprehensive collection. The IRGC contains 108 000 registered accessions originating from 117 countries. Within India and Pakistan several institutes also preserve a collection; the National Bureau of Plant Genetic Resources in New Delhi has a collection of 87 000 accessions and the collection of the Genetic Resource Preservation and Research Laboratories in Islamabad contains 2092 samples. Profound characterization of these collections has not yet been performed and they all contain duplicates. None of these collections represents completely the biodiversity of rice.

3.4.4. Basmati rice

3.4.4.1. Characteristics of Basmati rice

Basmati rice is premium aromatic rice and its high value stems from unique quality characteristics. The grains are aromatic in both raw and cooked state. The length of the uncooked grains is at least 6.5 mm and during the cooking process the grains duplicate in length without expanding in width. The cooking and eating quality of Basmati is largely determined by the starch properties: amylase content, gel consistency and the gelatinization temperature.

The geographical region in which Basmati rice is cultivated is restricted to the northern and north-western part of the Indian Subcontinent due to the climate conditions. This area encompasses the regions Punjab, Haryana and Uttar Pradesh located both in India and Pakistan.

The traditional Basmati varieties are mostly tall and low yielding varieties which were improved by traditional breeding. Modern breeding programs have produced evolved Basmati varieties that do not fit into the traditional group because they are derived from crosses between traditional Basmati varieties and other rice varieties. Many farmers prefer to grow these modern cultivars because of their higher yield (like Pusa 1121) or their resistance to biotic or abiotic stress (like CSR-30 because of its salt tolerance). The eating quality of the evolved Basmati varieties is comparable with the traditional varieties. Currently, Pusa 1121 and CSR-30 are the most cultivated rice varieties in the Basmati region.

3.4.4.2. Genetic diversity of Basmati rice

Mr Mohapatra presented a study performed with 55 SSR markers to examine the genetic differentiation of 23 aromatic rice types including several Basmati varieties. Polymorphism was observed for 41 out of the 55 investigated SSR markers. On the basis of the 55 SSR markers all tested varieties could be differentiated from each other with a low probability of identical match by chance. Remarkably, this study also showed genetic distinctness between three lines, i.e. Taraori Basmati, Karnal Local and HBC19, which are considered as one variety by the European Commission (EC) legislation, consequently indicating genetic heterogeneity within the 'Taraori variety'.

Uniformity and stability of 12 rice varieties, including two Basmati's, were also tested by using individual plants with diverse seed origin. The distinct molecular composition of Pusa Basmati 1 showed to be uniform and stable. However, some genetic variation could be observed within the Basmati 370 indicating problems in the maintenance of this variety.

It should be noted that the samples analysed in the frame of the study originated only from two seed banks, thus potentially leading to an underestimation of the genetic heterogeneity of the varieties harvested at the farm level.

3.4.4.3. Economical aspects of Basmati rice

Together India and Pakistan export around 2.5 million ton of Basmati rice per year of which around 300,000 tons is imported in the EU. The significant price difference between Basmati rice and other inferior long-grain varieties and the difficulty in differentiating them abet fraudulent traders to adulterate traditional Basmati. The different starch properties of the adulterants may affect the cooking and eating quality of the mixture.

The agreements between the EU, India and Pakistan determine the nine varieties which benefit from the zero import duty if imported as brown rice. Table 1 lists these nine eligible varieties. During several presentations indications were given about which varieties are cultivated and suspicions were expressed which varieties may be used as unidentifiable adulterants in imported material. Table 2 lists the potential adulterants mentioned during the presentations. The varieties listed in table 3 are the traditional and improved varieties cultivated in Pakistan. Depending on the dimensions of the rice kernels some of these varieties could occur as adulterants in Basmati rice samples

Ms Fitzgerald was pointing to inconsistencies between the fraction of Pusa 1121 and CSR-30 grown, the available surface area for Basmati cultivation, the average yield for traditional Basmati varieties and the volume of eligible Basmati varieties exported to the EU, suggesting that a significant fraction of the Basmati exported to the EU is not, as declared, traditional Basmati but evolved Basmati's like CSR-30 and/or Pusa 1121.

Table 1: The eligible Basmati rice varieties.

These basmati rice varieties that can be imported as brown rice into the EU at a zero import duty according to two agreements concluded in August 2004 between the EU and India and Pakistan.

Variety	Origin
Basmati 370	India and Pakistan
Basmati 386	India
Basmati 217	India
Type 3 (Dehradun)	India
Taraori Basmati (HBC-19)	India
Ranbir Basmati	India
Pusa Basmati	India and Pakistan
Super Basmati	India and Pakistan
Kernel (Basmati)	Pakistan

Table 2: Potential adulterants mentioned in presentations

This list is based on the presentations of Ms Steele, Ms Fitzgerald, Mr Singh and Mr Zafar. Mr Zafar referred to the publication of Giraud and Pirzada, "Where is Basmati coming from?, A global trade-related overview", 2009 and varieties mentioned in this publication were also included. The list is most likely incomplete.

Variety	Origin
Non-eligible fragrant varieties	
Basmati 198	Pakistan
Basmati 385	Pakistan
Haryana Basmati (HKR228/IET10367)	India
Mahi Suganda	India
Punjab Basmati (Bauni Basmati)	India
Kasturi Basmati	India
Basmati 2000	India
Shaheen Basmati	Pakistan
Mugad Sugandha	India
Superfine	
Pusa Sugandha 1	India
Pusa Sugandha 2	India
Pusa Sugandha 3	India
CSR-30 (Yamini)	India
Supra	
Pusa 1121 (Pusa Sugandha)	India
Vasumati	India
Basmati C-622	Pakistan
Kashmir Basmati	Pakistan
Rachna Basmati	Pakistan
Non-fragrant varieties	
Sherbati	India
Pak 386	Pakistan
IR64	India
PR106	India
PR11	India
IR6	Pakistan
KS282	Pakistan
Pak 177	Pakistan
New potential adulterants	
Improved Pusa Basmati 1 (IET 18990)	India
Bhogavari	India
Geetanjali	India
Malviya Basmati -1	India
Sugandha 1	Nepal

Table 3: Traditional and improved varieties cultivated in Pakistan

This table is based on the article Rabbani et al., 2008, Electronic J. Biotech (11) 3. This publication was mentioned in the presentation of Mr Zafar. Depending on the dimensions of the rice kernels some of these varieties could occur as adulterants in Basmati rice samples.

Variety	Varietal group
Basmati-370	Aromatic
Basmati-385	Aromatic
Basmati-2000	Aromatic
Basmati-C622	Aromatic
Basmati –Pak	Aromatic
Jajai-77	Aromatic
Kashmir-Basmati	Aromatic
Khushboo-95	Aromatic
Lateefy	Aromatic
Rachna-Basmati	Aromatic
Shaheen-Basmati	Aromatic
Sonahri-sugdasi	Aromatic
Sugdasi-bengalo	Aromatic
Sugdasi-ratria	Aromatic
Sugasi-sadagulab	Aromatic
Super-Basmati	Aromatic
Dilrosh-97	Non-aromatic
DR-82	Non-aromatic
DR-83	Non-aromatic
DR-92	Non-aromatic
Fakhre-Malakand	Non-aromatic
IR6	Non-aromatic
IR36	Non-aromatic
Jhona-349	Non-aromatic
Kangni-27	Non-aromatic
Kanwal-95	Non-aromatic
KS-282	Non-aromatic
NIAB-IR9	Non-aromatic
Pak23710	Non-aromatic
Pakhal	Non-aromatic
Palman-suffaid	Non-aromatic
Sada-Hayat	Non-aromatic
Sarshar	Non-aromatic
Sathra	Non-aromatic
Shadab	Non-aromatic
Shua-92	Non-aromatic
Swat-1	Non-aromatic
Swat-2	Non-aromatic
Kinmaze	Japonica
Nipponbare	Japonica

3.4.5. Rice authentication methods

The starting point for developing a method for authentication of eligible Basmati rice is a comprehensive set of authenticated samples of each relevant variety (including the eligible varieties as well as all potential adulterants). For each variety several accessions should be collected to test the presence of genetic variation within one variety. If there are genetic differences between two accessions carrying the same variety name a designated authority should identify the 'true to type' variety.

The next step in the process is the selection of a technique suitable for plant authentication. The presentations on the second day of the workshop were focussing on the different techniques, both genetic and non-genetic, which in principle can be used for this purpose.

3.4.5.1. Simple sequence repeats and Indel markers

To date SSR markers, also called microsatellites, are the most widely used marker system for plant variety characterisation. SSR markers are tandem repetitions of small sequences with a length of 2 to 4 base pairs. Each SSR marker usually has multiple alleles determined by different numbers of repeats. This multiallelic nature makes SSR markers highly informative. The analysis of the markers is based on polymerase chain reaction (PCR) followed by fragment length analysis. Accurate analysis of the small length differences between two alleles requires specialised equipment like polyacrylamide gels or DNA sequencers. The complex pattern obtained for some markers complicates the interpretation of the results and makes automated analysis difficult. The use of an internal length standard is recommended to allow comparison of results between different labs or experiments. SSR markers are not completely stable and mutations from one allele to another can occur during propagation.

Insertion-deletion polymorphisms or Indel markers can be used as a more user-friendly system for public laboratories. These Indel markers have only two alleles depending on the presence or absence of the insertion sequence. The differences between the fragment sizes of both alleles are usually around 25 to 50 base pairs. The markers can be analysed with separation systems with lower resolution, i.e. less specialized and less expensive techniques. However, the markers are di-allelic and less informative then the SSR markers.

Ms Steele and her colleagues have developed two methods for qualitative variety testing, one based on 8 SSR and one based on 9 Indel markers. The performances of these methods were tested in ring trials. The results showed that these methods can be used to detect adulterations in Basmati samples which contain one predominant variety. Nonetheless these methods also have several important limitations. For example, the non-eligible evolved Basmati variety CSR-30 could not be distinguished for the eligible varieties. Additional inclusion of 50 SSR markers linked to salt tolerance traits were tested in 2007 but none could be found which would allow distinction of CSR-30 from eligible Basmati's. Additional research is required to identify markers that allow the differentiation. It should be noted that one of the invited experts stated that in the experts' laboratory a marker set (coded, identity not revealed) could be identified that allows to distinguish CSR-30 from eligible Basmati's.

Other limitations of the SSR based methods are difficulties or even the inability to analyse samples consisting of a mixture of different approved and non-approved varieties. The sample set of potential adulterants used to develop these methods was incomplete and last updated in 2006. These methods are not validated for distinguishing the new potential adulterants.

For the SSR and InDel based methods quantitative analysis protocols were developed, however, the SSR based method is having difficulties with complex mixtures consisting of more than two varieties.

3.4.5.2. Single nucleotide polymorphisms

A SNP is a DNA sequence variation occurring at a single nucleotide. SNPs have several advantages in comparison to the SSR makers. The typical characteristics of a variety, like aroma and grain length, may result from SNPs located within genes. These SNPs are especially interesting for authentication purposes. The SNPs are more stable during propagation. Their di-allelic nature makes SNPs less informative and the analysis of more SNPs is required. However, the interpretation of the results is straightforward allowing automatisation and high-throughput analysis. SNP arrays can be used to analyze thousands of markers in one experiment. The Cornell University (represented by Ms Tung) and IRRI (represented by Mr McNally) are involved in the development of SNPs arrays for rice. A low resolution DNA chip containing 1536 SNPs is currently available and two larger chips containing 44 000 and 600 000 SNPs are under construction. The drawbacks of the SNP array technology are the requirement for specialized equipment and the inability to quantify the level of contamination. For the determination of the fraction of varieties in mixtures quantitative PCR methods would have to be developed.

3.4.5.3. Diversity arrays technology

The DArT technology was developed by the company Diversity Arrays Technology Pty Ltd represented by Mr Killian. This technology is based on the analysis several thousand markers distributed over the whole genome including different types of polymorphisms like SNPs, SSR and Indels. These markers are included in a DArT arrays and specialized reading instruments and advanced IT technology are needed for the collection and interpretation of the results. Quantification of the different varieties present in a mixed sample is possible with the DArT technology. A DArT array for rice has been developed and used to study seed purity and genetic diversity. For the authentication of Basmati and quantification of adulterants the DArT technology would have to be adapted and validated.

3.4.5.4. Analysis of the specific Basmati characteristics

This method analyses the characteristics of Basmati rice by combining genetic markers, morphological assessment during plant growth and quality analysis of grains. The included genetic markers are located within genes determining amylase content, gel consistency, gelatinization temperature and the fragrance of the rice. Ms Fitzgerald and her colleagues tested this method on the total collection of putative aromatic rice germplasm present in the IRGC collection (i.e. 500 accessions) including the eligible Basmati varieties and several potential adulterants.

The results of these experiments showed that several of the samples labelled Basmati did not fulfil the quality criteria. They are classified as "Fake" Basmati. The opposite was also true; some non-eligible varieties like Pusa1121 and CSR-30 could not be distinguished from the eligible varieties. The origin of these "look alike" samples was not limited to India and Pakistan; they also came from Nepal, Iran and Bangladesh. This experiment shows that there is an incomplete concurrence between the characteristics defining Basmati rice and the list of nine approved varieties. A method exclusively based on the typical Basmati characteristics is therefore not suitable for authentication of the eligible Basmati rice varieties.

3.4.5.5. The metabolomics technology

The so called metabolomics method is a non-genetic approach focused on the signal pattern of small metabolite molecules. The group of metabolites is typically analysed by gas chromatography or liquid chromatography combined with mass spectrometry (GC-MS and LC-MS). The resulting chromatogram shows a complex peak pattern and specialized informatics tools are required for interpretation of the metabolite profile. These profiles are a rich source of biochemical markers and minor differences in the origin or storage conditions of the samples can be detected. The metabolomics methods are already used for adulteration and authentication analysis of several food products including olive oil, wine and tea. Mr Hall from the Plant Research International collaborates in the METAPHORE project. Within this project the headspace analysis of volatile components of boiled rice has started. Samples from different varieties and origin were obtained including fresh and stored Basmati samples from Pakistan. Unfortunately no samples could be obtained from India. The preliminary results showed many differences between Basmati and other scented rice varieties. Within the group of Basmati's a distinction could be made between the different varieties and between fresh and stored samples.

3.4.6. Practical requirements for plant authentication methods

The Community control system has to enable the Member States of the EU to characterise the quality of the imported Basmati shipments. The custom laboratories would perform these measurements in daily practice. The organisers of this workshop invited Ms Peelen from the Dutch Customs Laboratory to present their point of view on the requirements for a plant authentication method.

A suitable method for plant authentication should fulfil the following requirements:

- validated for its purpose by different laboratories (in a ring trial.)
- availability of clear protocols and standard operating procedures (SOPs)
- capacity in relation to the amount of expected samples
- analysis within acceptable time limits and costs

The availability of a suitable method alone is not sufficient for the implementation of the control system. The effect of sample collection for the commercial imports should be investigated and described in protocols. The costs of the equipment necessary to perform the experiment should be acceptable. The personnel who will perform the analysis needs to receive the appropriate training. Finally the quality of the analysis should be monitored in each laboratory. This requires the availability of reference materials for calibration and quality control distributed by a designated authority. The organisation of proficiency tests will also contribute to the overall performance of the testing laboratories.

The Dutch Customs Laboratory has implemented the method developed and validated by the British Food Standards Agency (FSA) for the authentication of Basmati rice. This method is based on the analysis of 8 SSR markers and Ms Peelen reports the following observations:

- Some PCR (method) failures
- Some SSR markers are difficult to score
- Interpretation problems when analysing bulk samples compared to individual grain samples
- The ability to quantify depends on the adulterant
- Although only using 8 SSR markers the method was considered to be time consuming
- Strange alleles were pointing to the presence of unidentifiable new adulterants.

3.4.7. Practicality and investment considerations for a Basmati rice authentication method

This chapter summarises the time and investments required to develop and implement a method for Basmati authentication based on one of the presented techniques. This information was collected from the different presentations and the consecutive discussions.

The development of method for Basmati authentication starts with the selection of the markers which are suitable for distinguishing the involved rice varieties. Afterwards the reference samples of each involved rice variety will be analysed to determine the variety profiles. The time and investments required for the method development depends on the necessary equipment and the development stage of the technique. The techniques presented during the workshop are at different stages of the developmental process.

After the development and validation phase, the authentication method for basmati rice needs to be implemented for daily use in several customs or other testing laboratories. Aspects typically considered at this stage are the initial investments for the required equipment and the practically, robustness, costs and analysis time in daily practice.

3.4.7.1. Method based on SSR markers and Indel markers

SSR or Indel markers have already been used by several research groups for rice authentication in qualitative and quantitative tests. The extended amount of data available can be used as a basis for the selection of suitable markers. The type and number of markers required to distinguish varieties depends on the specific requirements for the method, i.e. which varieties should be distinguished from others. The time and the investment required of the selection of the markers and the set up of the database is difficult to predict and no estimations were included in the presentations.

The implementation of a SSR based method requires an investment on specialized equipment like polyacrylamide gels or DNA sequencers. The analysis of Indel markers, as presented by Ms. Steele, requires less expensive equipment. Some laboratories have already implemented the authentication method developed and validated by FSA which is based on 8 SSR markers. The costs of this method in daily practice are around 400 euro per sample. The Dutch customs laboratory considers the FSA method based on 8 SSR markers to be time consuming due to PCR failures and difficulties with the interpretation of results.

3.4.7.2. Method based on SNP markers

At this moment SNP markers are mainly used for association mapping of certain phenotypical characteristics. It is unclear which of these SNPs are also suitable for authentication purposes and Ms Tung indicated the need for additional research. Afterwards, the selected SNPs can be included in a custom made SNP array. The development of a SNP based authentication method may require larger investments and more time than a SSR based method, but no figures were presented during the workshop. There is currently no SNP based method available which allows quantification of the basmati varieties. New quantitative PCR methods, which require additional equipment, would have to be developed.

The analysis of SNP arrays requires specialized and relatively expensive equipment. The combination of SNP arrays with specialized software for interpretation of the results allows high throughput analysis. No estimations of the analysis costs under routine conditions were made available.

3.4.7.3. Method based on of the DArT technology

The DArT technology has been used to study seed purity and genetic diversity of rice samples. This technology was presented as being capable of qualitative variety testing and quantification of the contamination level. It is difficult to predict the efforts required to implement this technique for the authentication of Basmati rice.

Mr Kilian referred to the difficulties in estimating the costs for routine analysis in the absence of a validated method and numbers on expected testing volumes and testing laboratories.

3.4.7.4. Method based on of metabolomics

Metabolomics is a new and promising approach however the presentation indicated that the method is at a premature level. The presenter did not feel in the position to make a reasonable cost estimation.

3.5. Final discussion

Basmati rice is considered to be a superior rice type because of its unique quality characteristics. The high price offered for Basmati abets fraudulent trades in adulterating Basmati samples with inferior long grain varieties. A Community control systems which monitors the rice imports in the EU is required to protect the right of the EU costumers. The farmers in India and Pakistan growing the Basmati varieties will also benefit from this system.

The development and full validation of a Basmati authentication protocol requires two essential elements: a comprehensive set of authenticated samples of all eligible rice varieties as well as all potential adulterants and a plant authentication technique/method suitable for daily practice in customs and other testing laboratories.

Access to a sufficiently comprehensive set of authenticated was seen as the major limiting factor in the development of an authentication method. There was unanimous agreement between the participants of this workshop that the set of authenticated samples needs to be as broad as possible. Without a complete set of authenticated samples, validation attempts will stay rudimentary and consequently ambiguity in decision making will persist.

The continuous release of new varieties could increase the number of potential adulterants and regular updates and verifications of the validity of the methods are required. The research

groups working on authentication method development and laboratories where authentication methods are applied in routine expressed their concerns that current validations are based on outdated sample sets and that they fall behind developments as indicated by the presence of unidentifiable adulterants in routine samples.

The second limiting factor for the development of a validated Basmati authentication protocol is the availability of a suitable and convenient technique/method. During this workshop several promising techniques have been presented but at this moment none of them covers all the required aspects (table 4). Four essential requirements have to be met in the development of a suitable technology/method:

- Practicality for routine use by the testing laboratories. The method should ideally be easy to use, robust, fast and cheap;
- Capacity to analyse complex mixtures containing different approved and non-approved varieties;
- Quantification of the adulteration with an acceptable measurement uncertainty. Via the design of the validation exercise it should be made sure that the uncertainty estimation is realistic. Relatively often the uncertainty of results of quantitative methods is underestimated because not all parameters influencing the quantification are being looked at during the validation exercise;
- Ability to distinguish the eligible Basmati varieties from all potential adulterants. This requirement can only be fulfilled and the suitability of the method can only be validated if the complete set of authenticated samples is available.

In other words a method can only be considered as suitable if it is able to distinguish the eligible varieties from all potential adulterants, i.e. if it performs as required with the comprehensive set of authenticated samples.

The eventually growing number of potential adulterants will complicate the maintenance of a suitable method. A method that was validated with an earlier reference set of varieties might not be able to distinguish all new adulterants.

There was agreement that there is still a way to go to have a sufficiently reliable method in hands. The development of methods should continue. Technologies are developing fast but the decision to go for a particular method for routine use cannot be postponed until the final and most suited method may become available. The approach based on SSR markers has already been investigated by different research groups and the knowledge about its use for rice authentication purposes is currently the most extensive. An authentication method based on SSR markers is therefore suggested as the most promising option for the near future. All workshop participants were asked whether they agreed. None of the experts objected and one explicitly confirmed.

The general sense of Council Decisions 2004/617/EC and 2004/618/EC was questioned in terms of its socio-economic impact and overall goals. The question was raised why farmers are invited to grow low-yield varieties if they can cultivate higher-yield varieties producing a Basmati rice product of similar quality and high market price. The extension of the list of eligible Basmati varieties with high-yield and high-quality evolved Basmati rice varieties has been suggested in this context, which would in fact increase rice production, without detectable loss of quality, and would increase farmer's income.

Table 4: Summary of the techniques presented during the workshop

Technique	Advantages	Disadvantages	Developmental phase	Validation
SSR markers	Highly informative for individual markers Extended amount of data available Quantification of adulterants possible	Unstable: high mutation rate Interpretation of results can be difficult Specialized equipment required (presentation Ms. Steele) Quantification methods show difficulties with complex mixtures	Both qualitative and quantitative authentication methods developed	Only validated with incomplete sets of authenticated samples. Problems to distinguish CSR-30 Quantitative methods require more comprehensive validation
Indel markers	Results can be easier interpreted Less specialized equipment required Quantification of adulterants possible	Less discriminative power than SSR markers Quantification methods show difficulties with complex mixtures	Both qualitative and quantitative authentication methods developed	Only validated with incomplete sets of authenticated samples. Problems to distinguish CSR-30 Quantitative methods require more comprehensive validation
SNP markers	Stable: low mutation rate Results easier for interpretation Automatisation allows high throughput analysis	Less informative for individual markers Higher costs to develop Specialized equipment required No technique available yet for quantification	SNP arrays in development Not yet adapted for rice authentication purposes	Not completed
DArT	Analysis of different marker types High throughput analysis Quantification of adulterants was claimed to be possible	Specialized equipment required	Specific array for rice authentication needs to be developed	Not available
Metabolomics	Rich source of biochemical markers.	Sensitive to growing conditions, storage of samples etc.	Premature phase substantial research required	Not available

3.6. Summary of the workshop

This summary was distributed to all invited experts for their comments. Three experts reacted and the comments in agreement with the presentations and in the additional information provided during the workshop were included.

- None of the institutions working on rice authentication methods development appears to have access to a sufficiently comprehensive sample set (reference samples). There was general agreement that access to a comprehensive sample set is an absolute prerequisite for adequate method validation.
- Varieties are typically defined via the phenotype rather than the genotype. During validation of genotype based methods the genetic homogeneity within varieties would have to be looked at, preferably at the level of the end product (i.e. the harvested material).
- Landraces pose a particular challenge since even the phenotype is not necessarily uniform as demonstrated with an example on maize.
- In India about 150 rice landraces exist, which are mainly falling into the aromatic rice group (both short- and long-grain types).
- In India about 900 varieties are currently released. The number of new varieties released in India for commercial cultivation is about 20-30 per year. In principle any of them could compromise the validity of a DNA based authentication method and continuous follow up of the validity of the authentication method would be required.
- In principle there are several technical approaches for a qualitative method which differ in practicality, costs and their potential to be used for quantitative applications.
- None of the suggested methods has so far been fully validated with a sufficiently comprehensive and up to date sample set and for the particular purpose of distinguishing eligible Basmati varieties according to Council Decisions 2004/617/EC and 2004/618/EC from other rice varieties.
- It was confirmed that Pusa 1121 and CSR-30 are widely cultivated varieties in North-West India and that CSR-30 can be distinguished via the phenotype (salt tolerance) from its parent variety Taraori (HBC-19) Basmati. Some selected SSR markers have been claimed to distinguish CSR-30 from its Taraori (HBC-19) parent, whereas other working groups have failed to identify suitable markers (up to 130 used).
- DArT has not yet been adapted to and validated for Basmati authentication and it is difficult to predict the effort required to implement it in routine use at testing facilities, even though the results for other applications on rice and other plant species look promising.
- SNP based methods for the distinction of varieties (qualitative) are being developed. For quantification quantitative PCR methods would have to be developed.
- Metabolomics approaches for rice authentication are at a premature stage and require still very substantial research and validation efforts.
- Indel based methods can be carried out in a user friendly format. But the Indel methods have a lower power of distinction of different varieties compared to SSR based methods.
- SSR based methods were considered as the most straightforward option for the near future. All workshop participants were asked whether they agreed. None of the experts objected and one explicitly confirmed. In the UK an SSR based method is applied for policing the rice marketing. Distinction of a larger number (not specified during the workshop) of varieties was claimed to be possible using up to 55 selected SSR markers.

- The quantitative potential of SSR based methods has so far been evaluated under idealised conditions and with a circular calibration (calibration was done with the same material composing the unknowns to be quantified). Significant problems related to the identification and quantification of mixtures consisting of more than two varieties have been reported when using SSR based methods.
- Some experts questioned the general sense of Council Decisions 2004/617/EC and 2004/618/EC in terms of its socio-economic impact and overall goals. An extension of the list of eligible varieties with high-yield and high-quality Basmati rice varieties has also been suggested.

4. Agenda of the workshop

Monday 21 September 2009

Session 1: Context and general validation requirements for gene based authentication methods

Chairman: Mr A. Herrero, rapporteur: Mr M. Ghislain

09:00-09:30	Opening of the workshop *Mr H. Versteijlen, political context 15 min; Mr A. Herrero, scientific context 15 min*
09:30-10:15	Definition of a plant variety, its maintenance and monitoring of its properties *Mr L. Salaices - Spanish Plant Variety Office*
10:15-10:45	Coffee break
10:45-11:30	A case of maize landrace authentication process in Mexico *Mr S. Taba – International Maize and Wheat Improvement Center*
11:30-12:45	Rice genetic diversity, population structure and gene flow *Mr K.L. McNally – IRRI*
12:45-14:00	Lunch break

Session 2: Knowledge on varietal and genetic diversity of rice in India and Pakistan

Chairman: Mr A. Maquet, rapporteur: Mr L. Salaices

14:00-15:00	Varietal diversity of rice in India *Mr B.N. Singh - Birsa Agricultural University*
15:00-15:30	Genetic diversity of rice in India *Mr T. Mohapatra - Indian Agricultural Research Institute*
15:30-16:00	Coffee break
16:00-16:45	Genetic diversity of rice in Pakistan *Mr Y. Zafar - National Institute for Genomics and Advanced Biotechnology*
16:45-18:00	Discussion
18:00	Closure day 1

Tuesday 22 September 2009

Session 3: Rice authentication methods and degree of validation
Chairman: Mr K.L. McNally, rapporteur: Mr S. Garrett

09:00-09:45 Development of SNP genotyping assays and applications in rice authentication
Ms C. W. Tung - Cornell University

09:45-10:15 SNPs in grain quality genes and their use for genotyping, conclusion for rice authentication.
Ms M. Fitzgerald - IRRI

10:15-10:45 Coffee break

10:45-11:30 Rice authentication using SSR and Indel markers
Ms K.A. Steele - Bangor University

11:30-12:00 The potential of the DArT technology for rice authentication
Mr A. Kilian - Diversity Arrays Technology Pty Ltd

12:00-12:30 Non-DNA based methods for rice variety authentication, including metabolomics
Mr R. Hall - Plant Research International

12:30-13:30 Lunch break

Session 4: Wrap-up of the workshop
Chairman: Mr. B. Buffaria, rapporteur: Mr. H. Emons

13:30-14:00 Practical requirements for variety authentication methods
Ms T. Peelen - Dutch Customs Laboratory

14:00-15:00 Résumé from the three session rapporteurs

15:00 -15:30 Coffee break

15:30-17:00 Open discussion and wrap-up

17:00 Closure workshop

5. Participants list to the workshop

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6. Detailed description of the consecutive discussions

Session 1: Context and general validation requirements for gene based authentication methods

Mr L. Salaices: Spanish Plant Variety Office

Title: Definition of a plant variety, its maintenance and monitoring of its properties.

Answer to the question concerning the membership of India and Pakistan to UPOV:

India and Pakistan are currently not a member of UPOV and most of the Basmati varieties are not registered by UPOV. However, the Indian government has created a special law to register and protect the rice varieties. Pakistan is developing a similar law.

Answer to the question concerning the possibility to register varieties based on genetic differences alone:

It is not possible to base a variety registration only on genetic characteristics without morphological differences. DNA genotyping can be used as a tool for variety characterisation but not as a criterion.

Answer to the question concerning the consequences of instability of a variety:

The stability of a variety is the responsibility of the breeder. When a variety is no longer stable, the registration must be cancelled. If the variety has evolved into a new variety, the breeder should make a new request.

Mr S. Taba: International Maize and Wheat Improvement Center

Title: A case of maize landrace authentication process in Mexico

Answer to the question concerning the genetic diversity of the landrace:

The division of the 170 accessories was only based on the phenotypes, the genetic diversity was not investigated but it is expected to be large

Mr K.L. McNally: IRRI

Title: Rice Genetic Diversity, Gene Flow, and Population Structure

Additional information to the slides

Related to the slide about gene flow: The presence of gene flow within one gene pool is quite rare and gene flow between the gene pools is even rarer. The risk of cross breeding between Basmati and wild varieties is therefore low.

Answer to a question of concerning the estimated total number of rice varieties:

The MS Swaminathan Research Foundation estimated the total number of rice varieties world wide at 500,000. The term variety was considered in a loose meaning. Wild rices, traditional varieties, landraces, and commercial varieties were included.

Answer to the question about including landraces in the validation process of methods and handling possible polymorphisms:

The accessions present in the IRGC database are usually quite pure lines. If a landrace showed phenotypical diversity, it was divided in different accessories numbers. A large number of accessions were included in the studies using single seeds, consequently multiple accessions of landraces were involved.

The traditional Basmati varieties are not really landraces. Although they were not systematically breed like the evolved varieties, they are improved by traditional breeding systems.

SNPs analysis of the varieties showed heterogeneity within some varieties but most varieties were homogeneous. The varieties where analysed through single seed decent. Currently, more than 2000 varieties, respectively accessions, are genotyped using 600,000 SNPs.

Answer to the question concerning the smaller genetic diversity observed for aromatic varieties:

The smaller diversity of the aromatic varieties compared to Japonica and Indica varieties' is probably caused by the sample size. The group of aromatic rice varieties is smaller.

Session 2: Knowledge on varietal and genetic diversity of rice in India and Pakistan

Mr B.N. Singh - Birsa Agricultural University

Title: Varietal diversity of rice in India

Answer to the questions concerning the characteristics of Pusa1121:

Pusa1121 meets all the characteristics of Basmati rice including elongation and aroma. However, it is not considered to be a real Basmati variety because none of the parents (only one grand parent) is a traditional Basmati variety. Pusa1121 is not included in the list of eligible varieties determined by the EC legislation. The variety is mainly exported to the Middle East (80%) and to Europe and the USA (together 20%).

Answer to the question concerning the total number of varieties in India and the number of landraces:

In India, 900 varieties are released by "All India Coordinated Research Projects" across different states. Most of the landraces are only maintained in seed banks. Today, 100 to 150 landraces are still grown on the field. Most of them are scented and around 1000 to 2000 hectare is grown for each variety.

Answer to the question concerning the effect of cultivation region on the quality of Basmati rice:

The region of cultivation has an important effect on the growth and fragrance of Basmati rice. The northern regions, in particular the Punjab area, provide the ideal climate conditions resulting in a better aroma.

Answer to the question concerning the number of crops harvested per year:

The Basmati varieties deliver only one crop per year. The more early maturing rice is banned by the Indian government because they require too much water. All the export quantities mentioned on the slides are polished rice quantities.

Mr T. Mohapatra - Indian Agricultural Research Institute

Title: Genetic diversity of rice in India

Answer to the question concerning the number of aromatic rice varieties:

There are less then 1000 aromatic varieties and about 100 of them are Basmati-like types.

Answer to the questions concerning the cultivation of landraces:

In India some farmers still grow landraces and there is no control on the seed production. Farmers keep a part of their harvest as bulk and use them as seed for the next year. These landraces are mostly grown in non-Basmati areas, i.e. outside Punjab.

Answer to the question of concerning possible differences in the rice diversity pattern depending on the used markers

The diversity pattern obtained with SNPs is largely corresponding to the pattern obtained with SSR markers.

Answer to question concerning the used SNPs:

The number of SNPs tested was 384 and the SNPs were distributed over the whole genome. The SNPs were selected from a database and they were not directly related to the aroma genes.

Mr Y. Zafar - National Institute for Genomics and Advanced Biotechnology

Title: Genetic diversity of rice in Pakistan

Additional information to the slides:

The main Basmati variety cultivated in Pakistan is Super Basmati; 90 % of the rice fields within the Basmati GI area are used for its cultivation.

Answer to the question concerning the statement that 44.2 % of the Basmati rice is grown from certified seeds:

These data were obtained of a presentation at the International Association of Agricultural Economists Conference. Some of the data from this presentation can also be found in the paper from G. Giraud and S.W. H. Pirzada "Where is Basmati rice coming from? A global Trade-related overview"

Answer to the question concerning the cultivation of landraces in Pakistan:

The Pakistani farmers do no longer cultivate landraces. The seeds of improved varieties are provided by 480 private companies.

Answer to the question concerning the export of Basmati rice from Pakistan:

The amount of Basmati rice exported by Pakistan is 1.27 million ton milled rice. This corresponds to 2.1 million ton paddy rice. Super Basmati is the main Basmati variety that is exported to the EU. The second and third variety are Kernel Basmati and Basmati 370.

Answer concerning the list within the EC legislation:

The development of new varieties is currently not reflected in an expansion of this list. This is a controversial issue since there is no common definition for the varieties present on the list. There is no consensus about the criteria for varieties to be included on the list and this has a negative effect on the rice evolution in Pakistan.

Answer to the question concerning the method used to test the commercial samples:

The method of the FSA was used to test the purity of the commercial Basmati samples. The samples were rejected if the contamination with non-eligible varieties was >10%. Many samples were rejected but we can not provide the exact number. In the past he used a test based on morphological characteristics and then about 16% of the samples were rejected. In the year 2006-2007 more tests were performed because each sample was tested twice; before and after shipment.

Closure of day 1: discussion

Answer to the question concerning the rice varieties tested for uniformity and stability in the study presented by Mr Mohapatra

The uniformity and stability of 12 varieties, including 2 Basmati varieties and 10 non-Basmati, was tested using 55 SSR markers. The samples were obtained from 2 different seed collections. Pusa Basmati 1 showed good uniformity but Basmati 370 showed some variations. These variations can be due to problems with the maintenance of the Basmati 370 variety. The 10 non-Basmati varieties, which were not specified, showed also good uniformity except for one.

Comment concerning the breeding process of different Basmati varieties

Basmati 370 was obtained from a selection of local Basmati collections in 1933. The modern Basmati varieties like Pusa1121 were created by several breeding steps. During each step the plants were checked for quality and selections were made. The source material was always purified. The modern varieties are therefore more inform and stable.

Answer to the question concerning the origin of the samples of the rice varieties that were tested for uniformity and stability in the study presented by Mr Mohapatra

Different generations were analysed and from each generation 10 plants were tested. These experiments were done on single seeds not on grain pools. Further experiments are indeed required.

Answer to the question concerning the observation of artefacts in the SSR markers analysis used to test uniformity and stability.

The analysis was done with high resolution agarose. This technique has a lower resolution then currently used automatic fragment analysis systems. Therefore, artefacts like stutter effects could not be observed.

Comment:
The authentication of the final product should also be tested. The purity of the seeds at higher breeding level does not always guaranty the purity of the end product.

Comment
Some of the milled final products has been tested and the development on these methods is currently going on.

Comment
The expert has implemented the rice authentication method that was developed and validated by FSA. Testing of clean samples shows that most varieties can be identified. But complex mixtures are very difficult to analyse and the present varieties can not be distinguished.

Comment
On the second day of the workshop there will be a presentation about a technique that will allow the analysis of complex mixtures.

Answer to the question concerning the import of Basmati varieties that are not listed in the EC legislation:

These Basmati varieties can also be imported into the EU however they can not be exempted from import duties.

Comment:
The Basmati varieties that are not listed in the EC legislation are mostly exported to third parties.

Comment:
The mixing of Basmati rice with non-Basmati occurs indeed and is often done by the traders. This mixing affects the export and testing should definitely be performed by specialized institutions. But the main question is which technique should be used for this purpose? The method using the 55 SSR markers was only used in research on pure samples. This method still needs to be tested on commercial and possibly mixed samples.

Session 3: Rice authentication methods and degree of validation

Ms C. W. Tung: Cornell University

Title: Development of SNP genotyping assays and applications in rice authentication

Answer to a question concerning the minimum coverage required to develop a SNP array for authentication purposes:

The development requires resequencing with a minimum coverage of 7X-8X. Comment of IRMM: Coverage of 7X means that the area around the SNP needs to be resequenced completely 7 times.

Comment:
The identification of minor allele SNPs will require a higher coverage of about 20X

Answer of to the question concerning to validation process of the SNP arrays:

All SNPs were chosen from the Nipponbare high accuracy genome sequence. These SNPs are analyze correctly in at least 99.99% of the analyses. The development of this array requires a full set of reference samples for all varieties involved.

Answer to the question concerning the required start material for a SNP array:

There is no additional PCR step required before the hybridization on the SNP array; 250 ng of extracted DNA can be directly added to the array.

Ms M. Fitzgerald - IRRI

Title: Basmati and quality and questions

Answer to the question of concerning the possible loss of genetic diversity caused by cultivating only high-yielding varieties:

When farmers only cultivate the high-yielding evolved Basmati varieties, the gene diversity of the landraces can be maintained in a gene bank. It will still be possible to adapt the high-yielding varieties by introducing resistant genes from the traditional varieties.

Comment:
The plan to adapt the list included in the EC legislation looks very simple but will be more complex in reality. The current EU list is only applicable for brown rice not for white.

Answer about the effect of cultivation of high-yielding Basmati on the market price of Basmati rice:

The cultivation of high yielding varieties will increase the amount of Basmati rice available on the market. The value of Basmati rice however is unlikely to be affected. The high value stems from the good quality, not from the availability.

Comment:
There are several problems with the testing of Basmati varieties using the FSA method. Therefore a proficiency test has been started to evaluate the lab performance. Labs that would like to participate can contact Ms Hird.

Comment:
The traditional Basmati varieties are low yielding and improvement was required. Pusa 1121 was obtained by the selection of genes for grain length and elongation. During the breeding process the Basmati traits were conserved. Therefore it might be necessary to redefine the criteria for Basmati varieties and include Pusa1121.

Farmers are growing Pusa 1121 because they obtain a higher yield and an increased income. 80% of this rice is exported to the Middle East. Europe can support this evolution by adapting the list within the EC legislation. But farmers will still grow Pusa1121, even when it is not included in the list, because they receive a good price for it.

Answer to the question concerning the location where the 500 investigated rice varieties were grown:

The 500 putative aromatic rice varieties tested for Basmati characteristics, including sensory and cooking properties, were grown at IRRI in The Philippines.

Comment:
Climate conditions have an effect on several characteristics including aroma and can influence the observed similarities/ differences.

Comment:
The reason of the EC policy is to protect the rights of the EU customers. The policy in agriculture used to be supply driven but maybe it is time the move away from this approach. In 2003 negotiations were started with India and Pakistan. This was part of the development aid policy of the EC. An economic analysis of who benefit from this policy did not provide a clear answer. The Basmati rice which is imported duty free represents only less then 10% of the total rice import. The political question is not the main topic of this workshop.

The main question is: Do we need to continue the development of a new method for rice authentication and how? Is it possible to develop a precise and feasible method within 1 to 3 years?

Ms K.A. Steele: Bangor University

Title: Rice Authentication using SSR and InDel markers

Additional information to the slides:

The data in this presentation were obtained in the period between 2003 and 2007. Some aspects can be somehow outdated.

Answer to the question concerning the observed decrease in adulteration of rice samples:

In the last years a decrease in the number of adulterated samples was observed. This can be caused by the appearance of new undetectable varieties. The method used for sampling is also very important. The samples used for these studies were quite small so the whole bag was mixed and a random sample was taken. For large batches the sampling is more complex and guidelines are developed and transmitted to the testing labs.

Answer to the question concerning the uncertainty of the quantitative methods:

For the quantitative test an uncertainty of 6% was obtained. The calculation of this uncertainty was done by the statisticians of FSA and Ms Steele was not sufficiently familiar with the calculations to be able to specify the components which were included.

Comment:

The uncertainty of 6% was obtained in a ring trail of 11 labs that all used the same standard which was of the same origin as the unknowns. In practice and because of the absence of a common international standard labs would use different standards and therefore an uncertainty of 6 % will be an underestimation.

Comment:

This can lead to a multiplication with a factor of 5, i.e. about 30%. This uncertainty has been estimated for other quantitative DNA methods.

Mr A. Kilian: Diversity Arrays Technology Pty Ltd

Title: Potential of Diversity Arrays Technology (DArT) for rice authentication

Answer to the question concerning the detection limit of the method:

The detection limit of an array depends on the number of fragments available of the array. An increase of the number of fragments will improve the sensitivity of the method.

Mr R. Hall - Plant Research International

Title: Metabolomics as a tool for plant authentication?

Answer to the question concerning the effect of the growing conditions

The growing conditions have an important impact on the results of the metabolomics analysis. This is a very sensitive technique and harmonisation is important.

Answer to the question concerning the robustness of the metabolics technique:

The robustness of the technique was tested in a ring trail. All labs used exactly the same protocols and material. The metabolic analyses performed with nuclear magnetic resonance spectroscopy showed to be robust. Some variations were observed in the results of the GC-MS and LC-MS experiments and the protocols need to be optimised to improve reproducibility.

Comment:
Most labs are more confident with these chemical analyses than with genetic analysis. So there might be a future for this technique.

Session 4: Wrap-up of the workshop

Ms Peelen: Dutch Customs Laboratory

Title: Practical requirements for plant authentication methods

No questions were asked after this presentation.

Résumé from the three session rapporteurs followed by the open discussion

Comment:
For the development and validation of a method for the authentication of Basmati rice, there are several important requirements:

- a reliable method
- a good standard protocol
- a good reference sample set including both approved and adulterating varieties
- correct application by the customs labs (proficiency tests)
- regular (yearly?) and complete update of all new varieties appearing

Comment:

The authentication is essential for each self respecting country. The work for the development of a method has already started and should be continued. The technical evolution of available techniques is going fast but we can not wait for the development of a final method. For the moment SSR markers are the best choice. The current available marker sets are not able to distinguish all varieties so new markers should be identified. The markers search can be focussed on the regions surrounding genes that contribute to the phenotype characteristics of Basmati rice. The development of the method will require a considerable amount of time.

The method developed by FSA is currently available but this method can not distinguish all varieties involved. The creation of a marker database with reference samples of all the important varieties is the essential first step. The creation of this database requires openness of all the parties involved.

There are also two other problems to consider. First, even when the good SSR markers are known, the analysis of complex mixtures will still be difficult. Some of the Basmati varieties are grown outside the GI area and the quality of the final product is affected. This can not be tested with genetic markers.

Comment:

Pakistan and the EU have already collaborated on different areas and one of them is rice. Pakistan has already complied with the suggestions that were made in previous meetings and it will also cooperate in the outcome of this workshop.

Comment:

The method developed for the authentication of Basmati rice will also have its use for breeding processes. The Indian and Pakistani breeders can use these typing technologies for characterising and enhancing the varieties.

Comment:

This workshop has led to a fruitful discussion and has been very useful to gather information. But the final question remains: Do we have an approach to develop a method which is sufficient validated and flexible for the constant update with new varieties? Based on the data presented here, I think we can conclude that there is still a long way to go.

Comment:

I share this opinion. How long will it take to develop a protocol that can be used by all the Member States? One should take into account that most commercial samples are mixtures (sometimes only with approved varieties) and that the technique should be suitable for use in custom laboratories.

Comment:

The technique should not only be qualitative but also quantitative.

Comment:

Different methods have been presented during this workshop and they all have their potential. But none of these techniques can be used to develop a validated method as long as there is no full set of reference samples available. Nobody seems to have this collection for the moment. India and Pakistan should provide all the information about potential adulterants and make sure that the necessary samples will be made available.

Another approach could be to use the cooking quality as a test.

What about the CSR-30 variety which can not be distinguished using the SSR markers method of FSA?

Comment:

We have identified a marker which might be able to distinguish CSR-30 from the other Basmati varieties. But we have also no knowledge on the new varieties which can be adulterants. Our database is falling behind all the time.

Comment:

Pakistan has been collaborating and has send samples of all requested varieties except for two. These two varieties are only grown in a limited amount and are used for local consumption.

Comment:

The proficiency test will be performed before the end of this year. Suggestions about which samples should be tested are always welcome.

Comment:

The presentations and the discussions of yesterday and today make it clear that the major limiting factor for the development of a method is not the available techniques but the access to a clear and complete reference sample set. The purity of these samples should be very high and an independent facility should deliver these services. This is very important to ask ourselves why all these efforts are done.

Comment:

I agree on the importance of the reference samples. The method should be developed in this way that it can distinguish all the varieties. The varieties on the list within the EC legislation are a political issue, not so important for the development of the method. If this method can not be developed we might indeed switch back to the cooking method.

Comment:

How can you develop a method without the list and is this list still useful? What was the original purpose of this legalisation? Was it designed to provide some compensation to the farmers that are growing the low-yielding Basmati varieties? The evolved Basmati varieties are higher yielding with the same quality so compensation might no longer be necessary.

Comment:

There might indeed be a need for redefining the list but this is not the topic of this workshop. The list is not directly related to the development of a method. The rice markets are changing quickly and there are other countries developing aromatic rice which are not falling into the Basmati category. Therefore there will be more competition within the aromatic rice group and this legislation will help to protect some Basmati varieties. The legislation requires the support by scientific tools.

Comment:

A recommendation for the collection of reference materials would be more appropriate.

Comment:

It is desirable to use a sufficiently broad reference sample set. But we should determine what is sufficiently broad. Inclusion of all the commercial varieties may not be enough. Varieties that are currently not commercialised can become adulterants in the future. Therefore the number of required reference samples will be large.

Comment:

One should realise that during trade the names of the varieties are not important. Trade is based on quality not on the variety name.

Comment:

Do we all agree that the list should be as broad as possible?

Comment:

I agree, but we can not go further than the current available standards.

Comment:

How is the authentication of champagne done? Can we use a similar approach for the Basmati rice?

Comment:

The system for champagne is based on the region of origin. This system is sufficient but it took more then ten years to implement.

Comment:

Basmati is only grown in a certain region since the aroma is region specific.

Comment:

The importance of GI is also included in the agreement and we can develop a complementary tool to analyse this.

Comment:

GI tools are currently developed for wheat and honey and this can also be done for rice. But also for this technique a good reference sample database is essential.

Mr Emons closes this workshop by thanking all the participants.