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INTERNATIONAL UNION FOR THE PROTECTION OF NEW VARIETIES OF PLANTS
GENEVA

WORKING GROUP ON BIOCHEMICAL AND MOLECULR TECHNIQUES, AND DNA-PROFILING IN PARTICULAR

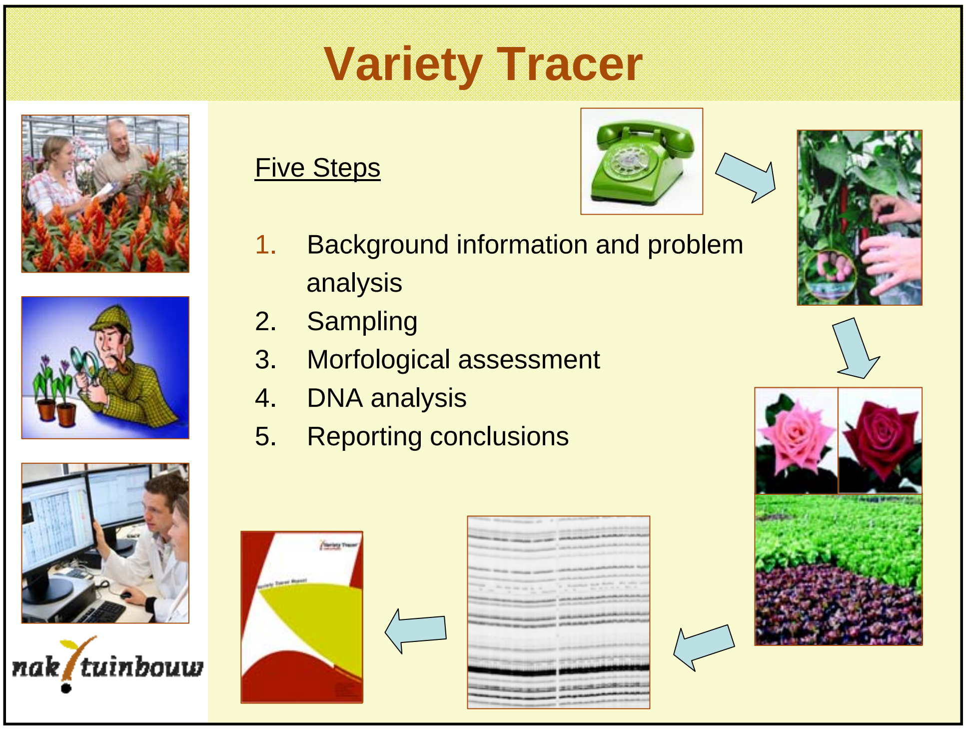
Thirteenth Session
Brasilia, November 22 to 24, 2011

ADDENDUM

DEVELOPMENTS CONCERNING THE VARIETY TRACER PROCEDURE

Document prepared by an expert from the Netherlands

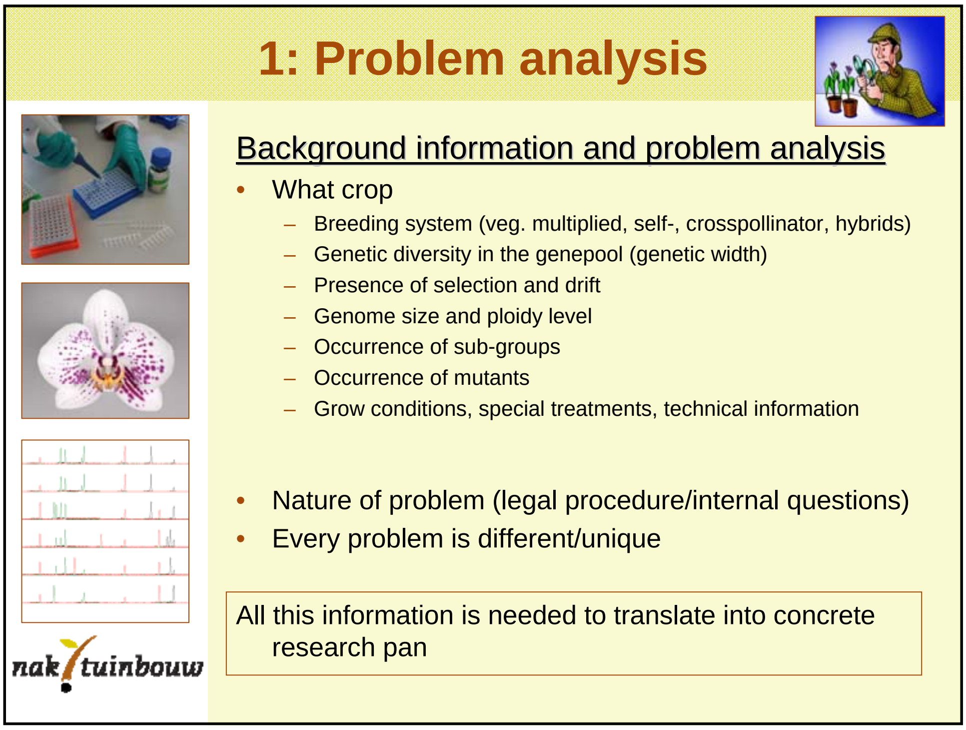
Developments concerning the Variety Tracer Procedure



Variety Tracer

Five Steps

1. Background information and problem analysis
2. Sampling
3. Morfological assessment
4. DNA analysis
5. Reporting conclusions

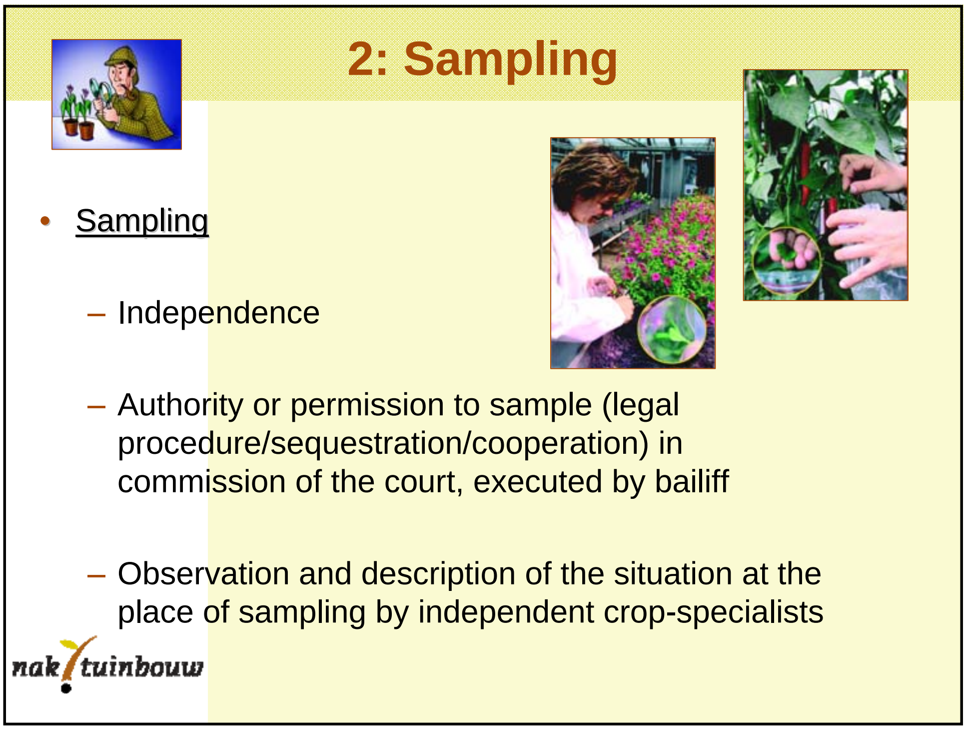


1: Problem analysis

Background information and problem analysis

- What crop
  - Breeding system (veg. multiplied, self-, crosspollinator, hybrids)
  - Genetic diversity in the genepool (genetic width)
  - Presence of selection and drift
  - Genome size and ploidy level
  - Occurrence of sub-groups
  - Occurrence of mutants
  - Grow conditions, special treatments, technical information
- Nature of problem (legal procedure/internal questions)
- Every problem is different/unique

All this information is needed to translate into concrete research pan



2: Sampling

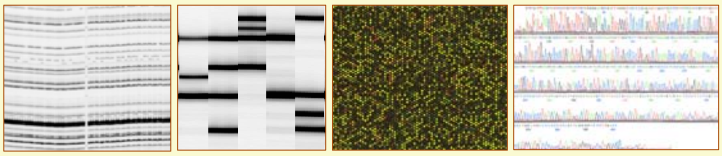
- Sampling
  - Independence
  - Authority or permission to sample (legal procedure/sequestration/cooperation) in commission of the court, executed by bailiff
  - Observation and description of the situation at the place of sampling by independent crop-specialists

3: Morphological assessment

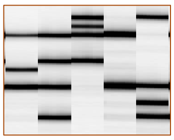
- Morphological assessment
  - Side by side comparison
  - Identical growing conditions
  - Descriptions according to the relevant UPOV/CPVO guideline/protocols
  - Comparison with original variety description

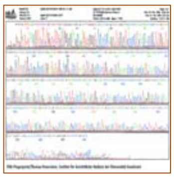
4: Genetic Conformity

- DNA analysis
  - Choice of DNA fingerprint technique (AFLP/SSR/other)
  - Availability of crop-specific markers in public domain
  - Availability of databases



5: Reporting





- <u>Reporting and conclusions</u>
  - Statistal analysis of results (for binary data such as presence or absence of molecular markers use SM, Jaccard, Dice)
  - Comparing and combining the morphological observations and the DNA analysis (complemantary, synergistic)

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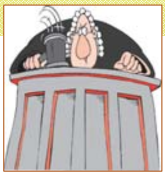
First EDV Court Case



- Decision of Court of Appeal (NL)
  December 2009

Blancanieves:

Million Stars:

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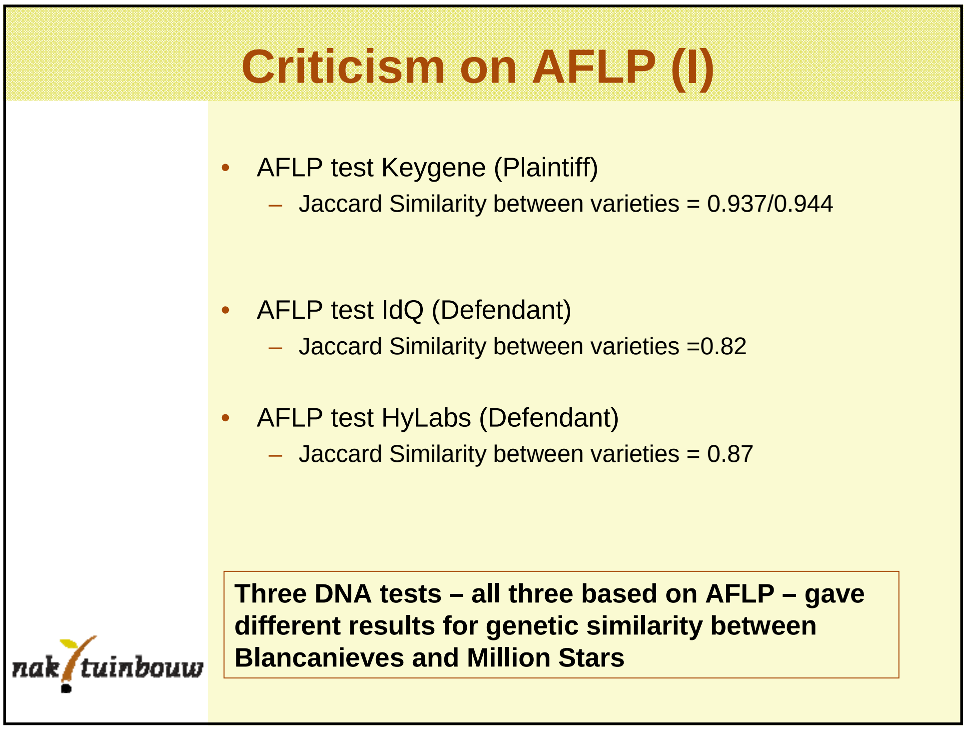
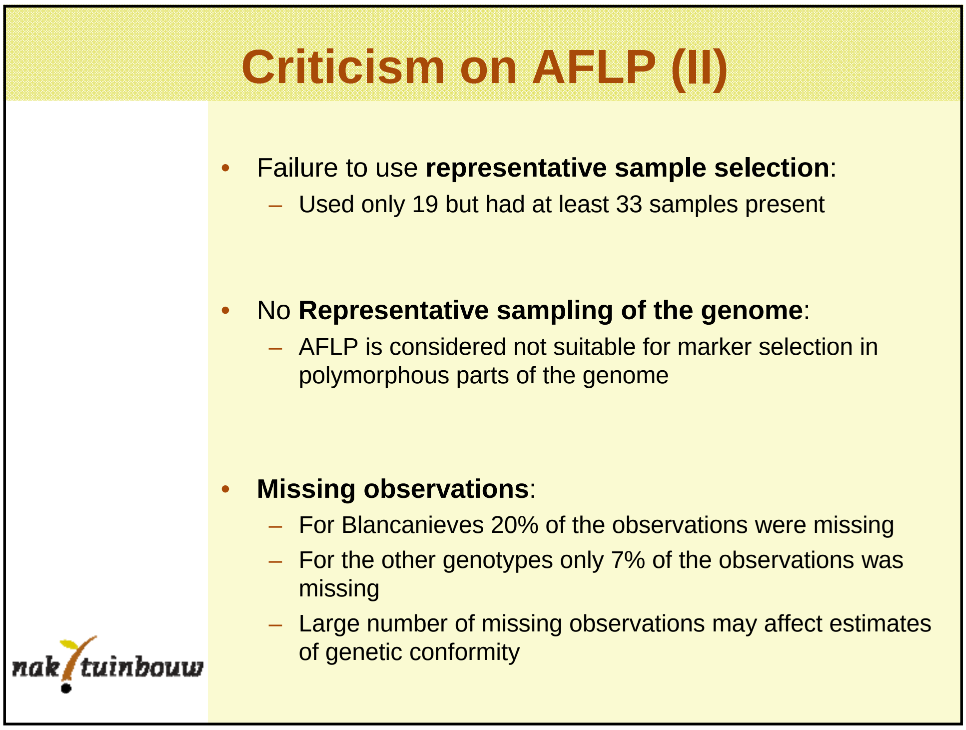


Criticism on AFLP (I)

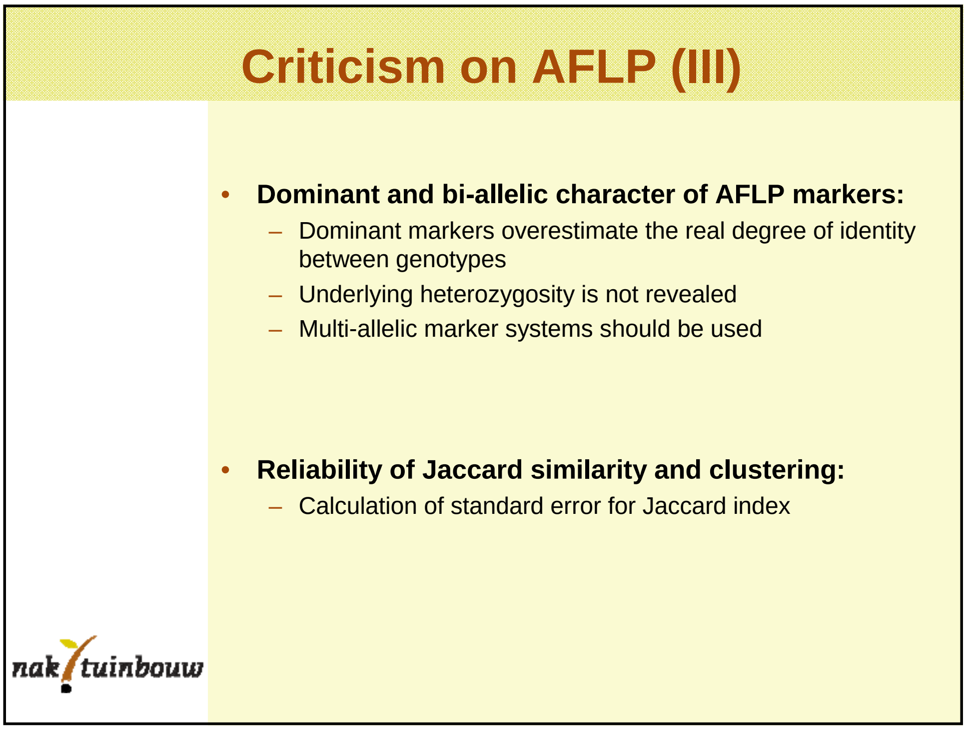
- AFLP test Keygene (Plaintiff)
  - Jaccard Similarity between varieties = 0.937/0.944
- AFLP test IdQ (Defendant)
  - Jaccard Similarity between varieties =0.82
- AFLP test HyLabs (Defendant)
  - Jaccard Similarity between varieties = 0.87

Three DNA tests – all three based on AFLP – gave different results for genetic similarity between Blancanieves and Million Stars



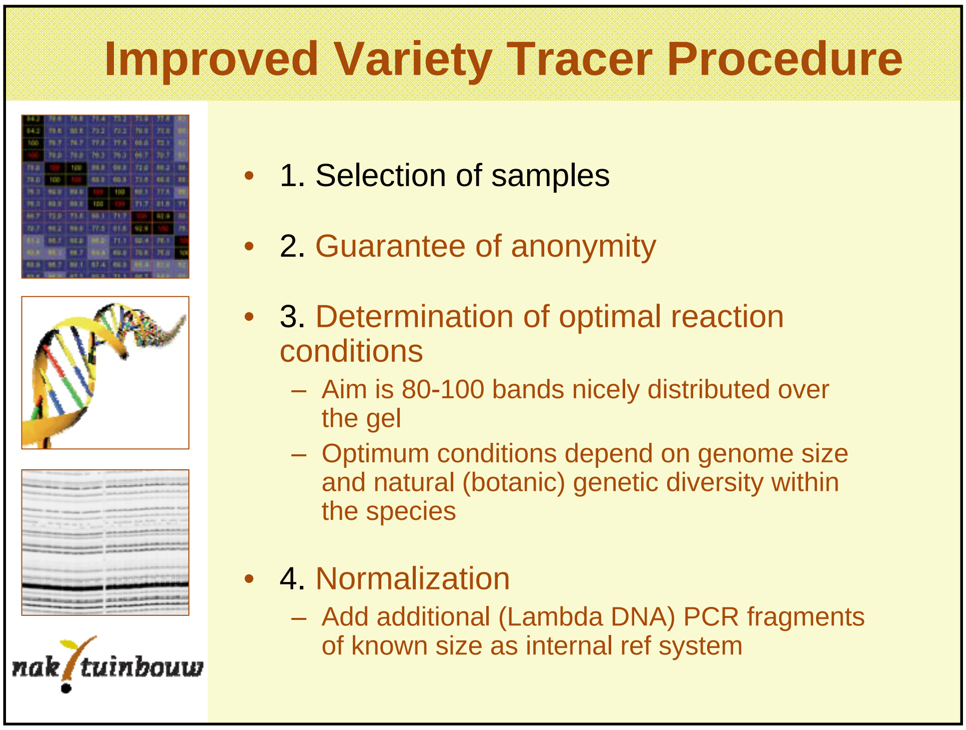
Criticism on AFLP (II)

- Failure to use **representative sample selection**:
  - Used only 19 but had at least 33 samples present
- No **Representative sampling of the genome**:
  - AFLP is considered not suitable for marker selection in polymorphous parts of the genome
- **Missing observations**:
  - For Blancanieves 20% of the observations were missing
  - For the other genotypes only 7% of the observations was missing
  - Large number of missing observations may affect estimates of genetic conformity



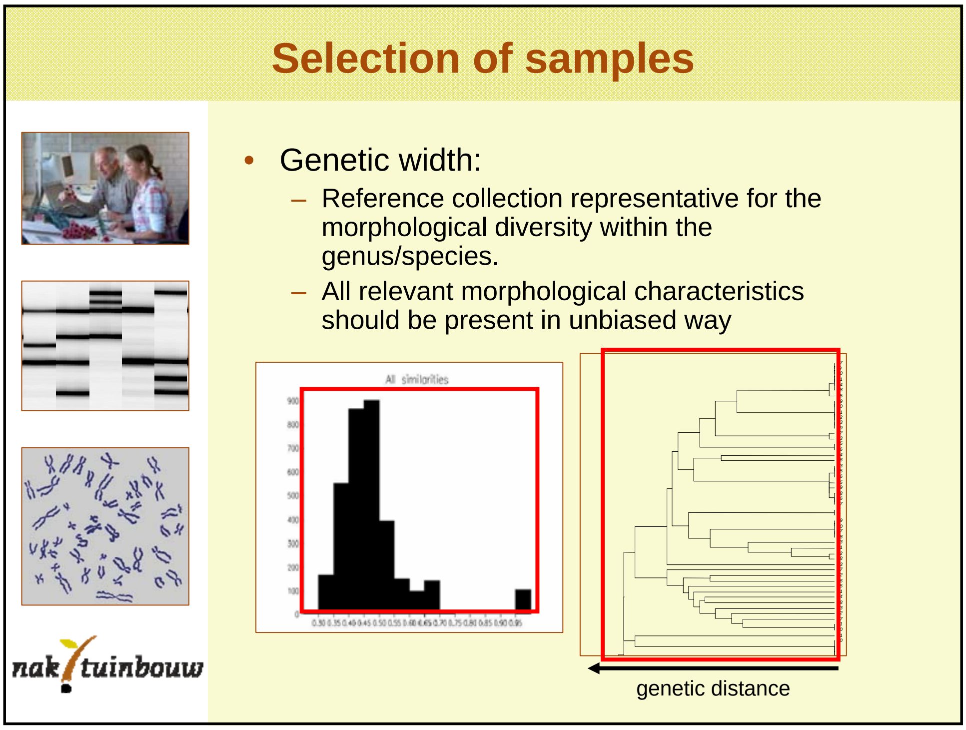
Criticism on AFLP (III)

- **Dominant and bi-allelic character of AFLP markers:**
  - Dominant markers overestimate the real degree of identity between genotypes
  - Underlying heterozygosity is not revealed
  - Multi-allelic marker systems should be used
- **Reliability of Jaccard similarity and clustering:**
  - Calculation of standard error for Jaccard index



Improved Variety Tracer Procedure

- 1. Selection of samples
- 2. Guarantee of anonymity
- 3. Determination of optimal reaction conditions
  - Aim is 80-100 bands nicely distributed over the gel
  - Optimum conditions depend on genome size and natural (botanic) genetic diversity within the species
- 4. Normalization
  - Add additional (Lambda DNA) PCR fragments of known size as internal ref system



Selection of samples

- Genetic width:
  - Reference collection representative for the morphological diversity within the genus/species.
  - All relevant morphological characteristics should be present in unbiased way

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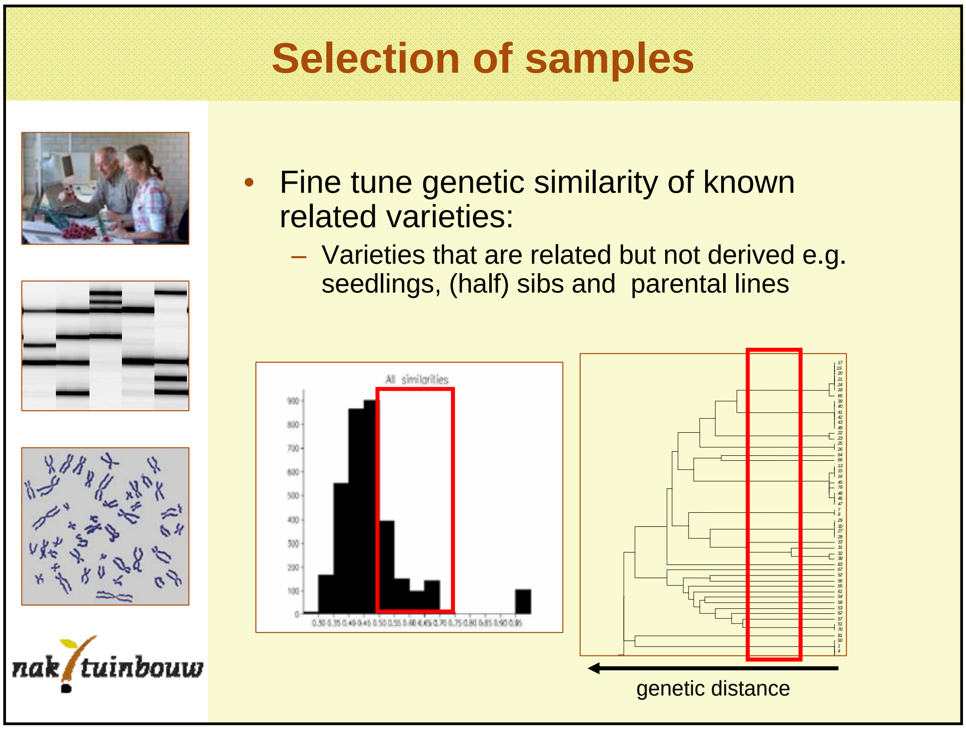
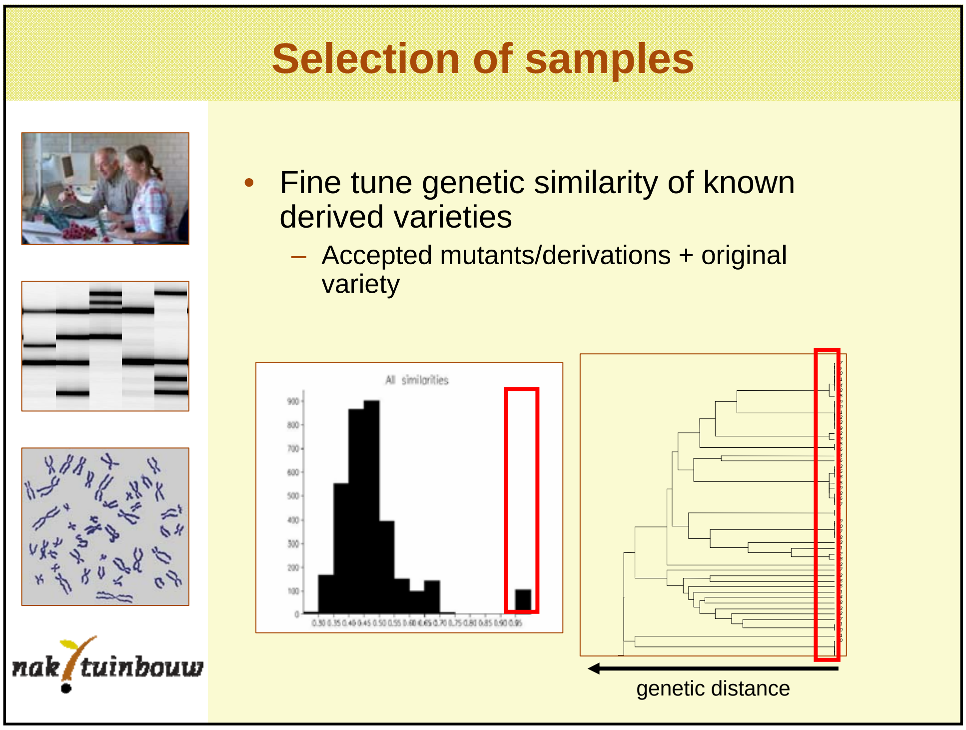
Reference collections



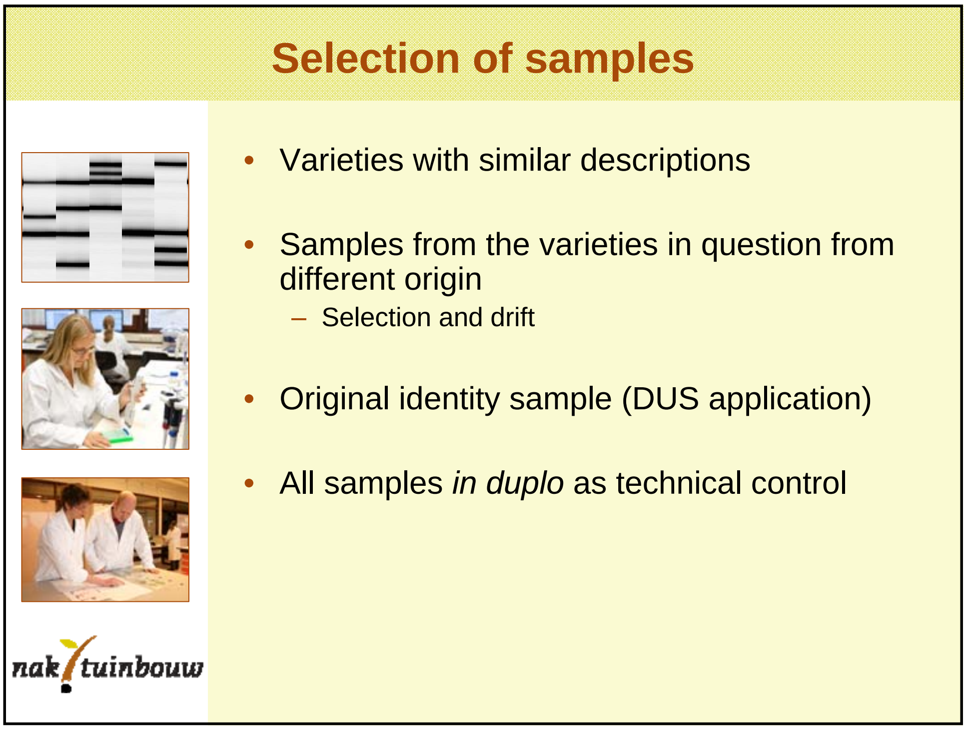
Selection of samples

- Fine tune genetic similarity of known related varieties:
  - Varieties that are related but not derived e.g. seedlings, (half) sibs and parental lines



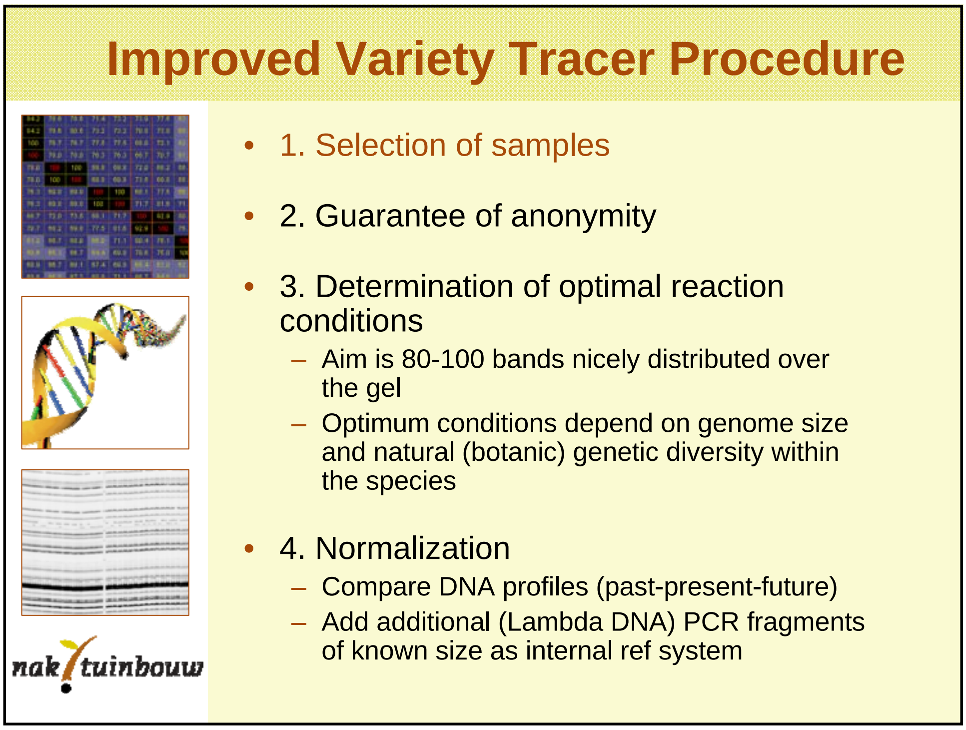
Selection of samples

- Fine tune genetic similarity of known derived varieties
  - Accepted mutants/derivations + original variety



Selection of samples

- Varieties with similar descriptions
- Samples from the varieties in question from different origin
  - Selection and drift
- Original identity sample (DUS application)
- All samples *in duplo* as technical control



Improved Variety Tracer Procedure

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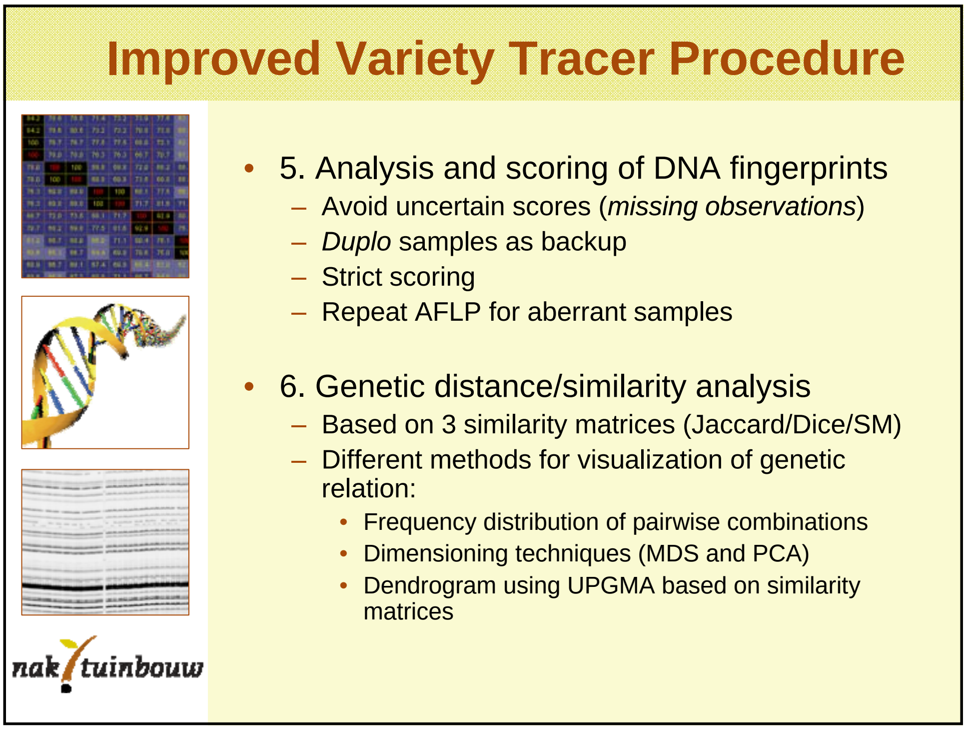
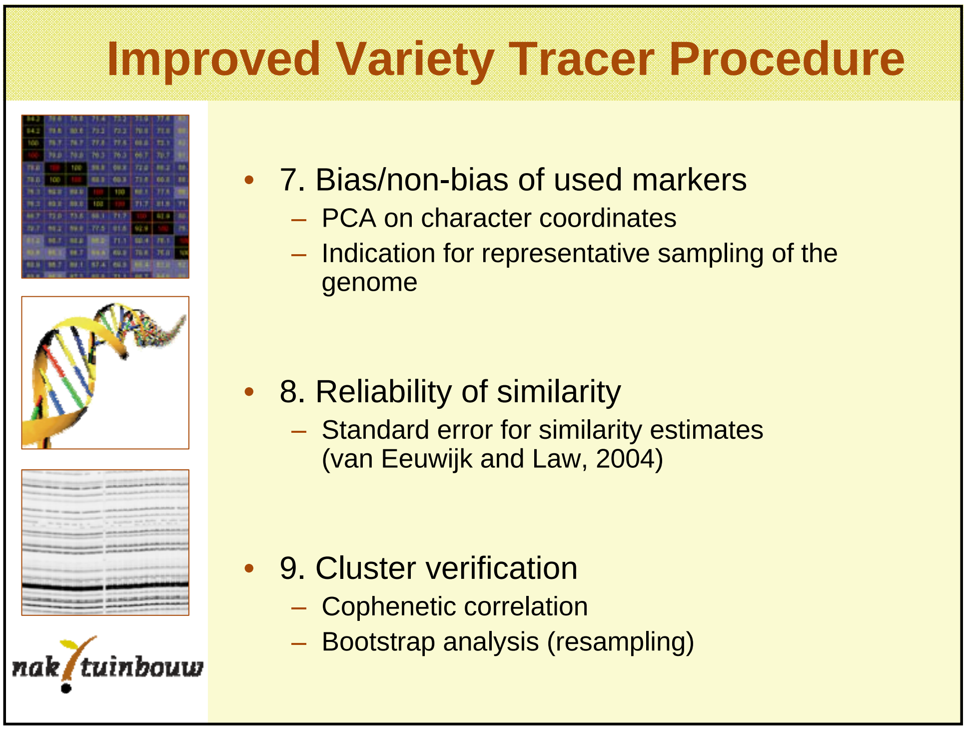
- 3. Determination of optimal reaction conditions
  - Aim is 80-100 bands nicely distributed over the gel
  - Optimum conditions depend on genome size and natural (botanic) genetic diversity within the species
- 4. Normalization
  - Compare DNA profiles (past-present-future)
  - Add additional (Lambda DNA) PCR fragments of known size as internal ref system



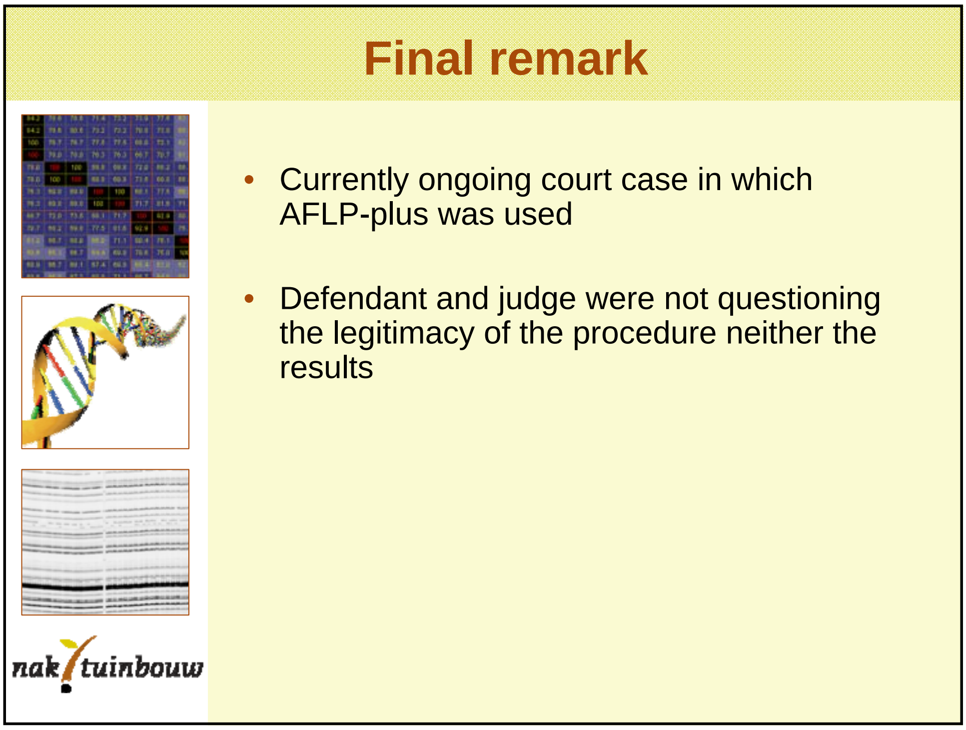
Improved Variety Tracer Procedure

- 5. Analysis and scoring of DNA fingerprints
  - Avoid uncertain scores (*missing observations*)
  - *Duplo* samples as backup
  - Strict scoring
  - Repeat AFLP for aberrant samples
- 6. Genetic distance/similarity analysis
  - Based on 3 similarity matrices (Jaccard/Dice/SM)
  - Different methods for visualization of genetic relation:
    - Frequency distribution of pairwise combinations
    - Dimensioning techniques (MDS and PCA)
    - Dendrogram using UPGMA based on similarity matrices



Improved Variety Tracer Procedure

- 7. Bias/non-bias of used markers
  - PCA on character coordinates
  - Indication for representative sampling of the genome
- 8. Reliability of similarity
  - Standard error for similarity estimates (van Eeuwijk and Law, 2004)
- 9. Cluster verification
  - Cophenetic correlation
  - Bootstrap analysis (resampling)



Final remark

- Currently ongoing court case in which AFLP-plus was used
- Defendant and judge were not questioning the legitimacy of the procedure neither the results

Quality in Horticulture

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