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INTERNATIONAL UNION FOR THE PROTECTION OF NEW VARIETIES OF PLANTS

Geneva

TECHNICAL WORKING PARTY FOR VEGETABLES

Forty-Eighth Session
Paestum, Italy, from June 23 to 27, 2014

ADDENDUM TO DOCUMENT TWV/48/2

MOLECULAR TECHNIQUES

Document prepared by the Netherlands

Disclaimer: this document does not represent UPOV policies or guidance

The annex to this document contains a copy of the presentation made by an expert from the Netherlands, at the forty-eighth session of the Technical Working Party for Vegetables (TWV), on “DNA in DUS examination for Registration and PBR/PVP”.

[Annex follows]

DNA in DUS examination for Registration and PBR/PVP

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Molecular markers in DUS

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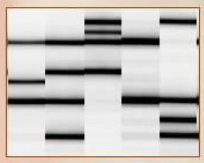

Use of DNA marker technology at Naktuinbouw

- According to the UPOV-BMT models
- As a supportive tool in addition to the traditional DUS examination

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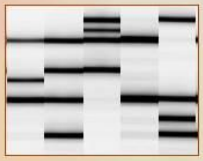
Molecular Markers in DUS



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- **UPOV-BMT**

The Working Group on Biochemical and Molecular Techniques and DNA-profiling in particular (BMT)

MODEL 1:	Characteristic-specific molecular markers
MODEL 2:	Combining phenotypic and molecular distances; management of variety collections
MODEL 3:	Calibrated molecular distances in the management of variety collections
MODEL 4:	Use of molecular marker characteristics

BMT MODEL: 1

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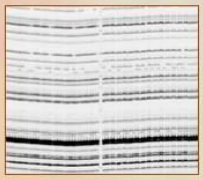
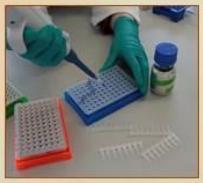
Model 1: Molecular Characteristics as Predictors of 'Traditional' Characteristics

a) Gene-specific markers for predicting individual phenotypic characteristics. (Reliable linkage between the marker and the expression of the characteristic required)
- e.g. disease resistance

b) Use of a set of molecular characteristics which can be used to reliably estimate traditional characteristics; e.g. *quantitative trait loci (QTL)*

BMT MODEL: 1 - strategy





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Model 1: Molecular Characteristics as Predictors of 'Traditional' Characteristics

Perform the validated Molecular Test

- PCR test performed on min. 20 individual plants. When resistance gene present and TQ resistant; enough proof – no bioassay needed.
- When resistance gene absent (susceptible allele present); bioassay will be performed.
- When PCR result and TQ do not match; bioassay.
- When there is any (other) doubt; bioassay.

MODEL 1a: overview of tests

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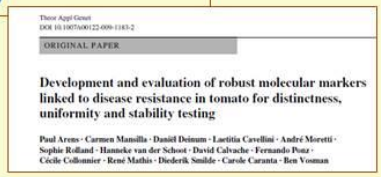
Disease resistance in tomato:

- ***Meloidogyne incognita***
  - MI1.2 (traditional PCR)
- ***Tomato Mosaic Virus (ToMV)***
  - Tm1 (traditional PCR)
  - Tm2 and Tm2^2 (tetra ARMS)
- ***Verticillium dahliae***
  - Ve1 and Ve2 (same locus) (tetra ARMS)
- ***Fusarium oxysporum f. sp. lycopersici***
  - I-2 gene (traditional PCR)



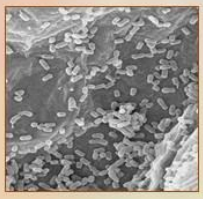
Theor Appl Genet
DOI 10.1007/s00122-009-1183-2

ORIGINAL PAPER

Development and evaluation of robust molecular markers linked to disease resistance in tomato for distinctness, uniformity and stability testing

Paul Arens · Carmen Mansilla · Daniël Deinum · Laetitia Cavellini · André Moretti · Sophie Rolland · Hanneke van der Schoot · David Calvache · Fernando Pons · Cécile Collonnier · René Mathis · Diederik Smilde · Carole Caranta · Ben Vosman

MODEL 1a: predictor for resistance



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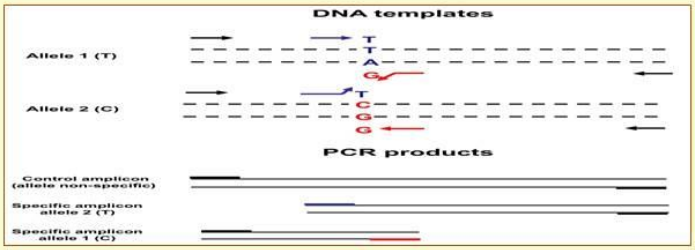
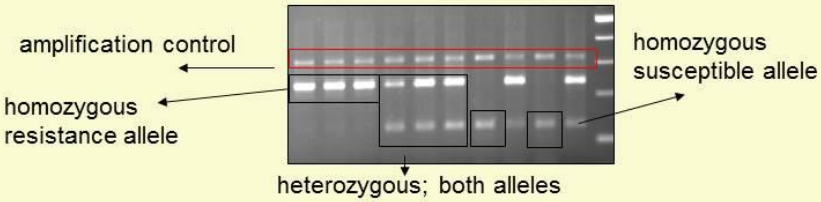
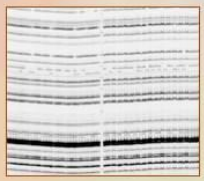
Example: Amplification Refractory Mutation System (ARMS) for the detection of Ve-1/Ve-2 and Tm2/Tm2^2





MODEL 1a: correlation



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Verticillium dahliae Ve1 and Ve2 genes

Total # varieties	Correlation PCR vs Bioassay
94	98%
2	Ve1-ve2 new haplotype = intermediate resistance

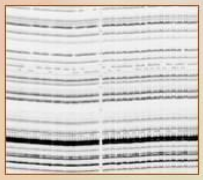
Discovery of combination of new alleles (=haplotype) that might explain newly observed intermediate resistance levels for Verticillium.

Meloidogyne incognita MI1.2 gene

Total # varieties	Correlation PCR vs Bioassay
130	99%
1 resistant fragment	Susceptible in bioassay

This candidate variety also had intermediate resistance levels for Ve.
This application was not registrated. Not DUS.

MODEL 1a: correlation



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Tomato Mosaic Virus (ToMV) Tm2 and $Tm2^2$

Total # varieties	Correlation PCR vs TQ and Bioassay
100	100%

Fusarium oxysporum f. sp. lycopersici I-2 gene

Total # varieties	Correlation PCR vs TQ and Bioassay
100	100%

MODEL 1a: overview of tests

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Disease resistance in tomato:

- ***Tomato Spotted Wilt Virus (TSWV)***
  - Sw-5 (TaqMan PCR)
- ***Tomato Yellow Leaf Curl Virus (TyLCV)***
  - Ty-1 / Ty-3 (Melt Curve analysis)

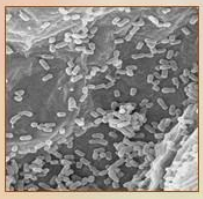
TSWV reference:
Dianese E.C., Fonseca M.E.N., Goldbach R., Kormelink R., Inoue-Nagata A.K., Resende R.O., Boiteux L.S.(2009) Development of a locus-specific, co-dominant SCAR marker for assisted-selection of the *SW-5* (*Tospovirus* resistance) gene cluster in a wide range of tomato accessions. Mol Breeding (2010) 25:133-142.

TyLCV reference:

Verlaan M.G.: The Tomato Yellow Leaf Curl Virus Resistance Gene Ty-1 and TY-3 are allelic and Code for DFGD-Class RNA Dependent RNA Polymerases. PLOS Genetics March 2013 Volume 9 issue 3.

Patent: http://www.google.com/patents/WO2012125025A1?cl=en

MODEL 1a: predictor for resistance



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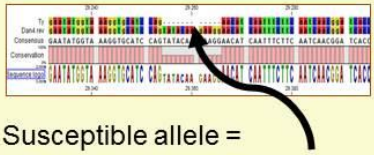
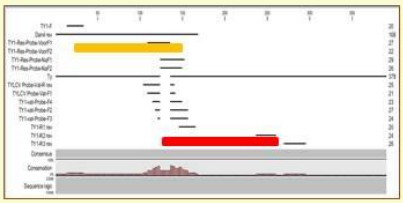
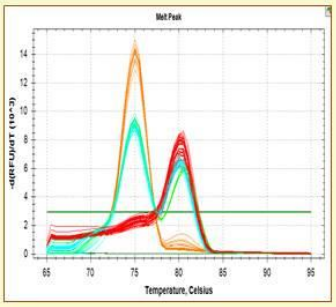
Example: melt curve analysis for TyLCV



Susceptible allele = deletion of 12 base pairs



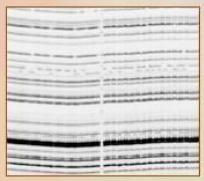


PCR product for resistant allel melts at 75°C (yellow peak)

PCR product for susceptible allel melts at 80°C (red peak)

In a heterozygous variety both peaks are visuable (blue)

MODEL 1a: correlation



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TSWV Sw-5

Total # varieties	Correlation PCR vs TQ
89	100%

TyLCV Ty-1/Ty-3

Total # varieties	Correlation PCR vs TQ
15	100%

Bioassay is not (yet) possible.

MODEL 1a: overview of tests

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Cytoplasmatic Male Sterility (CMS) in *Brassicaceae*

- Broccoli
- Cauliflower
- Red Cabbage
- White Cabbage
- Kohlrabi
- Savoy Cabbage
- Brussels Sprout
- Curly Kale

Ogura-type cytoplasmatic male-sterility from Japanese radish (covered by confidentiality clause)

CMS marker is located in *orf138*

Fertile varieties do not have *orf138*

MODEL 1a: overview of tests

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Cytoplasmatic Male Sterility (CMS) in *Brassicaceae*

Seeds are sown on wet filter paper. After one week seedlings are harvested.

For fertile varieties: 5 pools of 5 individuals are sampled and analysed by TaqMan PCR.

For sterile varieties: min. 20 individual plants were sampled and analysed by TaqMan PCR.

A

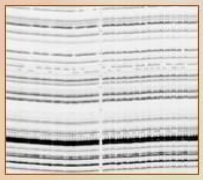
	Cq waarde FAM	Cq waarde VIC	Oordeel
A-01	19,24	19,49	Steriel
A-02	19,95	20,04	Steriel
A-03	19,17	19,26	Steriel
A-04	19,89	19,27	Steriel
A-05	19,5	19,41	Steriel
A-06	19,41	19,59	Steriel
A-07	19,76	19,88	Steriel
A-08	19,39	19,26	Steriel
A-09	19,45	19,42	Steriel
A-10	19,46	19,63	Steriel

E

	Cq waarde FAM	Cq waarde VIC	Oordeel
E-01	N/A	24,3	Fertiel
E-02	N/A	24,01	Fertiel
E-03	N/A	24,14	Fertiel
E-04	N/A	24,16	Fertiel
E-05	N/A	23,7	Fertiel
E-06	N/A	23,78	Fertiel
E-07	N/A	24,07	Fertiel
E-08	N/A	24,14	Fertiel
E-09	N/A	24,37	Fertiel
E-10	N/A	23,73	Fertiel

Internal DNA control NAD5

MODEL 1a: correlation CMS



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Results 2013:

Gewascode	RvP-nummer	Box nummer	Positie in box	Getoetste Individuen	Opgave bedrijf/TQ	Oordeel resultaten Merkertoets
KSP	KSP00254	KSP-1	A1 - B12	24	Steriel	Steriel
KSP	KSP00255	KSP-1	C1 - D12	24	Steriel	Steriel
KSP	KSP00256	KSP-1	E1 - F12	24	Steriel	Steriel
KSP	KSP00257	KSP-1	G1 - H12	24	Steriel	Steriel
KSP	KSP00258	KSP-2	A1 - B12	24	Fertiel	Fertiel
KSP	KSP00259	KSP-2	C1 - D12	24	Fertiel	Fertiel
KSP	KSP00260	KSP-2	E1 - F12	24	Fertiel	Fertiel
KSP	KSP00261	KSP-2	G1 - H12	24	Fertiel	Fertiel
KSP	KSP00262	KSP-3	A1 - B12	24	Fertiel	Fertiel
KSP	KSP00265	KSP-3	G1 - H12	24	Fertiel	Fertiel
KBL	KBL00817	KBL-1	A1 - B12	24	Fertiel	Fertiel
KWI	KWI00638	KWI-1	C1 - D12	24	Fertiel	Fertiel
KWI	KWI00639	KWI-1	E1 - F12	24	Steriel	Steriel
KWI	KWI00640	KWI-1	G1 - H12	24	Steriel	Steriel
KWI	KWI00641	KWI-2	A1 - B12	24	Steriel	Steriel
KWI	KWI00642	KWI-2	C1 - D12	24	Steriel	Steriel
KWI	KWI00643	KWI-2	G1-G9 H1-H9	18	Fertiel	Fertiel
KWI	KWI00644	KWI-3	A1-A11 B1-B11	22	Steriel	Steriel
KWI	KWI00646	KWI-3	C1 - D12	24	Steriel	Fertiel
KWI	KWI00647	KWI-3	G1 - H12	24	Fertiel	Fertiel
KRO	KRO00107	KRO-1	A1 - B12	24	Steriel	Steriel
KRO	KRO00108	KRO-1	C1 - D12	24	Steriel	Steriel
KRO	KRO00109	KRO-1	E1 - F12	24	Steriel	Steriel
KRO	KRO00110	KRO-1	G1 - H12	24	Steriel	Steriel
KSA	KSA00157	KSA+KBR	A1 - B12	24	Fertiel	Fertiel
KSA	KSA00163	KSA+KBR	C1 - D12	24	Fertiel	Fertiel
KBR?		KSA+KBR	E1 - F12	24	Fertiel	Fertiel
KBR?		KSA+KBR	G1 - H12	24	Steriel	Steriel

From 165 applications (2011-2014) in all *Brassicaceae* tested only one PCR result not in accordance with TQ/Bioassay (99,4%).

Different source of sterility (not OGURA-type)

BMT MODEL: 2

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- **Model 2:** Combining phenotypic and molecular distances and in the management of variety collections
- Overall genetic assessment in relation to overall morphological assessment

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BMT MODEL: 2

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Combining phenotypic and molecular distances
management of variety collections
model system POTATO

AIM:
- Better seletion of relevant references in field trial
- Exclude non-relevant reference varieties from field trial
- Reduce the number of reference varieties in the field trial
- Reduce the duration of the DUS test
- Reduce costs

MODEL 2: Phenotypic distance

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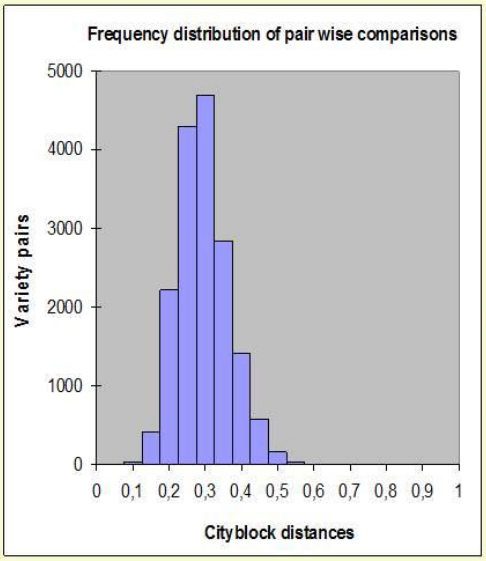

- 183 varieties
- 16.653 combinations
- Lightsprout and field characteristics
- Only quantitative characteristics
- Cityblock distance: sum of all differences

MODEL 2: Genotypic distance

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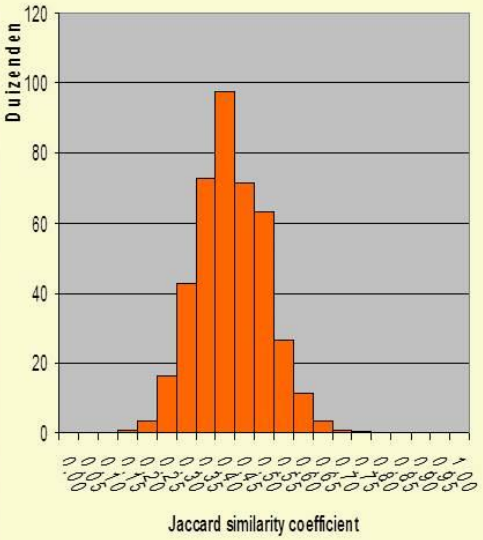

- 900 varieties under which the 183
- 404100 combinations
- Tubers from several origins investigated by two labs (CPVO project)
- 9 SSR markers, randomly divided over the chromosomes
- Jaccard similarity:

Combination/Calibration of mol. distance

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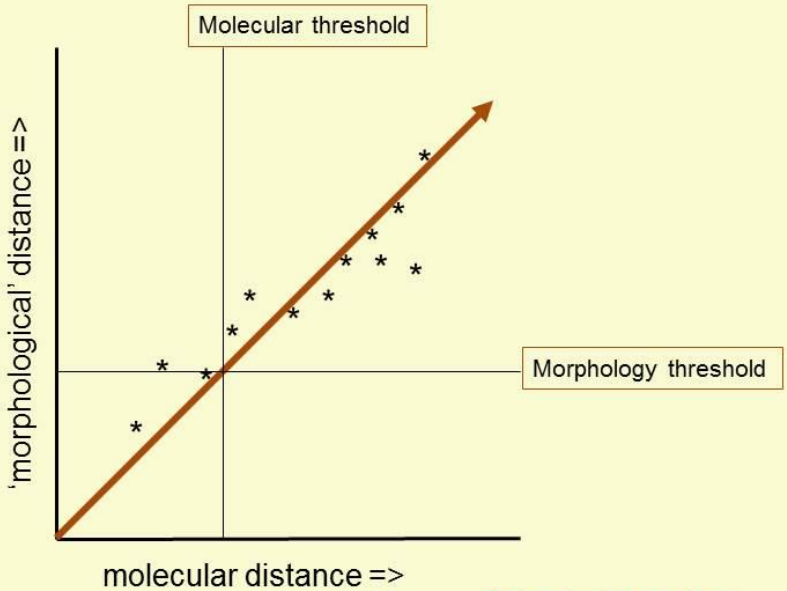

ideal situation

Combination/Calibration of mol. distance

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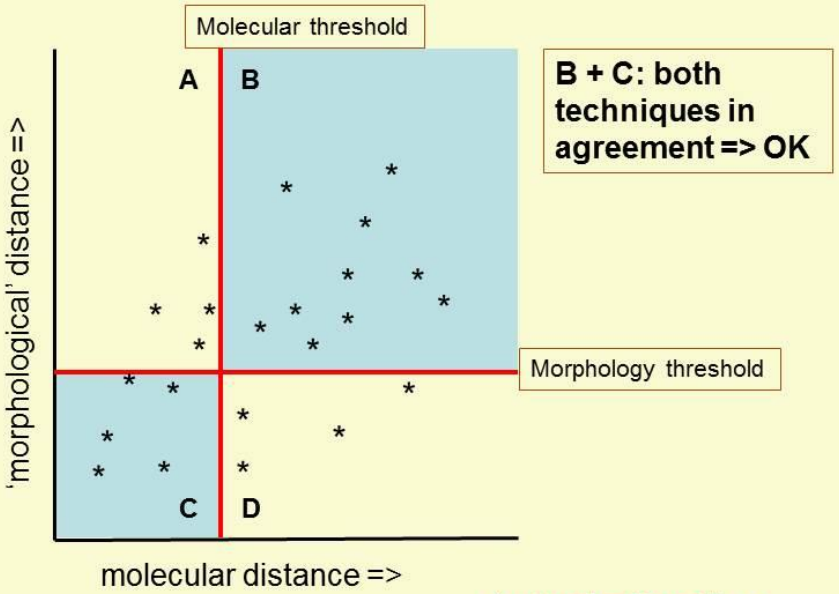

B + C: both techniques in agreement => OK

Actual situation

Combination/Calibration of mol. distance

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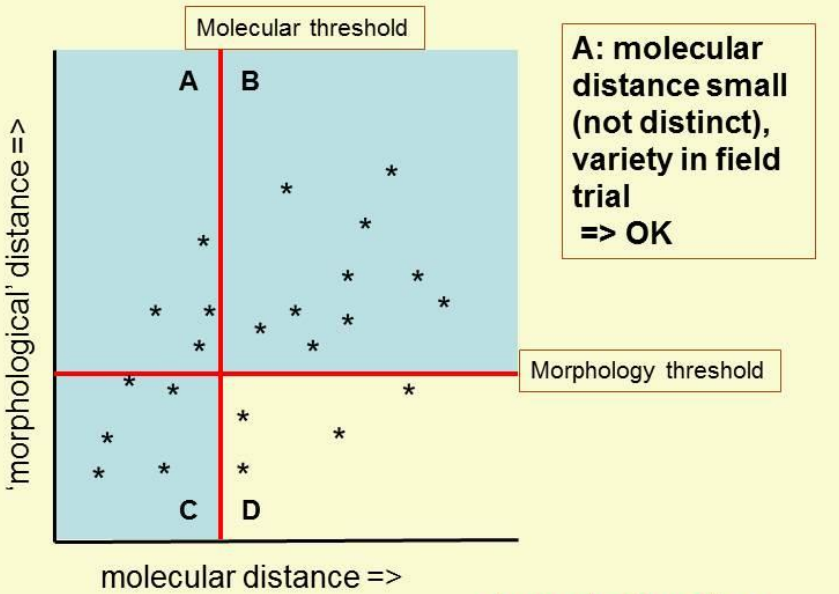

A: molecular distance small (not distinct), variety in field trial => OK

Actual situation

Combination/Calibration of mol. distance

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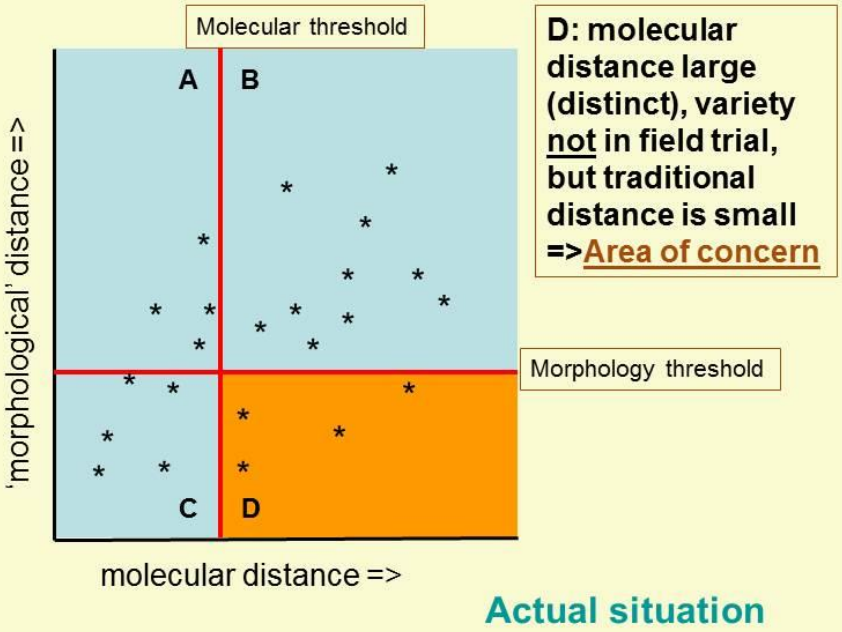

D: molecular distance large (distinct), variety not in field trial, but traditional distance is small =>Area of concern

Actual situation

MODEL 2: Combination Morph/Mol. distances

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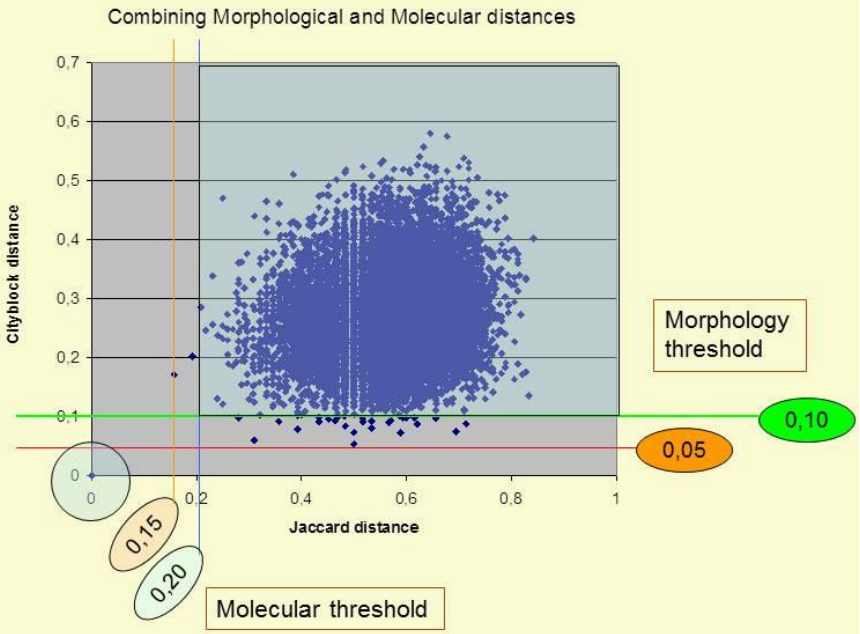
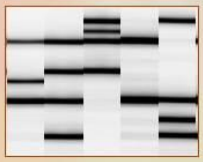


Improved DUS system POTATO



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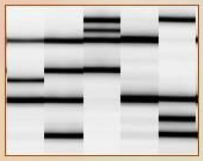
DUS test based on morphology ***and*** DNA:

- **First year:**
  - start of season: description lightsprout (compared with photo database) and DNA profiling
  - Growing season: morphological description
- **End first year:**
  - DUS decision based on morphological distance and genetic distance (distinct plus thresholds)
- **Second year test for candidate varieties only when:**
  - Candidate/reference pair below 'distinct plus' threshold for morphological distance (cityblock distance < 0,1)
  - Candidate/reference pair below 'distinct plus' threshold for genetic distance (Jaccard < 0,2).

Improved DUS system LETTUCE?



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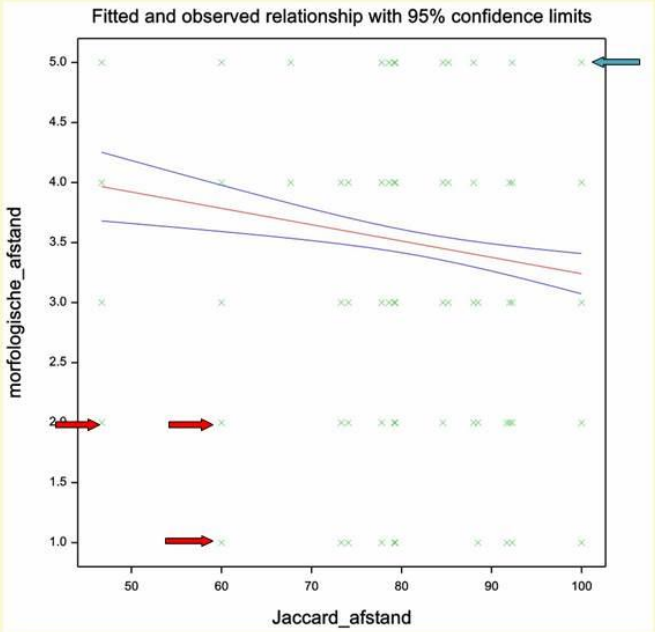

MODEL 2: Combination Morph/Mol. distances

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Advantages :

- A hugh collection of varieties in *common knowledge* in a DNA database, not a living collection.
- Increased reliability for a candidate variety to be (or not to be) distinct from all that is known.
- Exclude or include reference varieties based on their DNA profiles.
- In most cases the duration of DUS trial can be reduced (cost reduction).
- Spin-off: use DNA database for other purposes than DUS.

BMT MODEL: 3

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- **Model 3: Calibrated molecular distances in the management of variety collections.**

AIM:
Predict morphological distance based on calibrated molecular distance

MODEL 3: Calibration of molecular distance

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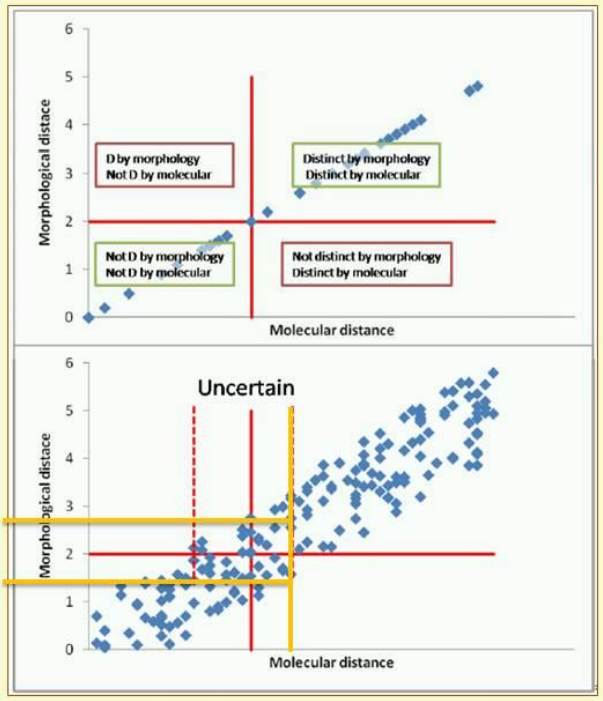

Currently under study

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Molecular Characteristics as Predictors of 'Traditional' Characteristics

- Markers linked to disease resistance genes in tomato
- Markers for CMS in cabbage

Combination/Calibration of threshold levels for the management of reference collections

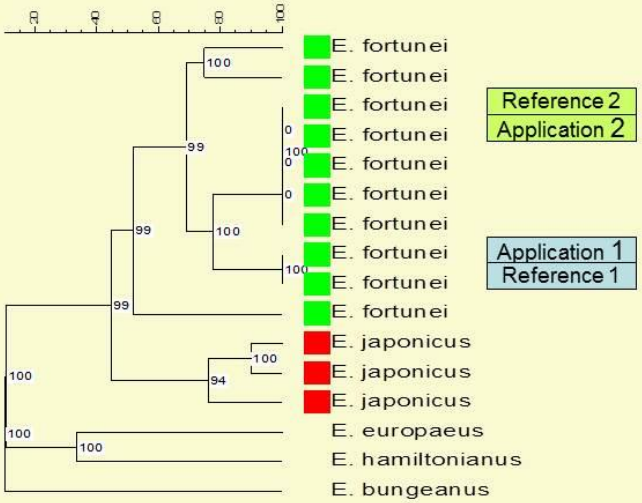
- Potato (in cooperation with SASA Scotland)
- Lettuce (in cooperation with France)
- Phalaenopsis (in cooperation with Taiwan)
- *Lolium perenne* (in cooperation with ILVO, Australia and North Ireland)

DUS Support with DNA at Naktuinbouw

Example 1

nak tuinbouw

Investigate two *Euonymus* candidate varieties with their reference varieties because of doubt on distinctness



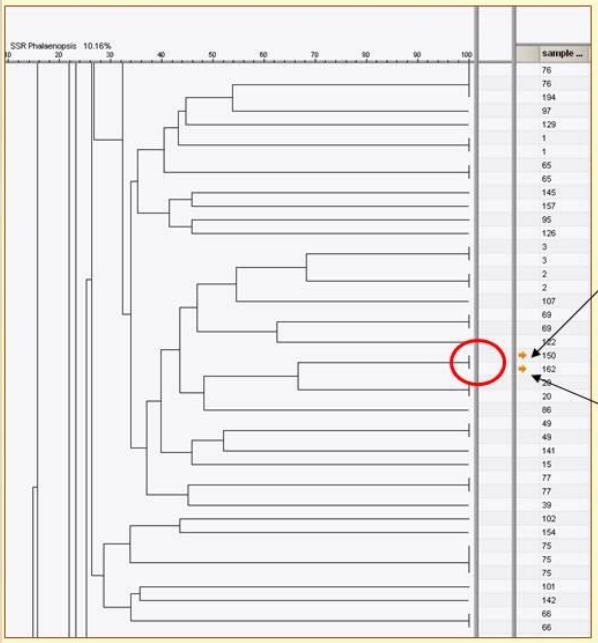
Differences on morphology resulted from physiology differences, different treatments and age differences of propagating material

DUS Support with DNA at Naktuinbouw

Example 2

nak tuinbouw

Investigate two applications to each other and the database



Identical profiles *Doritanopsis*

and

Phalaenopsis Candidate varieties ???

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