# A microsatellite-based system for the identification and legal protection of grapevine varieties

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## *Vitis vinifera* L.

- Woody, asexually multiplied
- Very old culture-very high number of varieties
- Two different markets: wine and table
- Microsatellites broadly used for characterization

## Sequence-Tagged Microsatellite Sites GENRES 081

- Ring test: 10 laboratories, 6 loci, 1 DNA / variety
- Proposal of a standard set of alleles / varieties. (This et al. TAG 2004)
- New OIV descriptors soon

LINKED
VVMD7-ZAG62
VVMD27-ZAG79

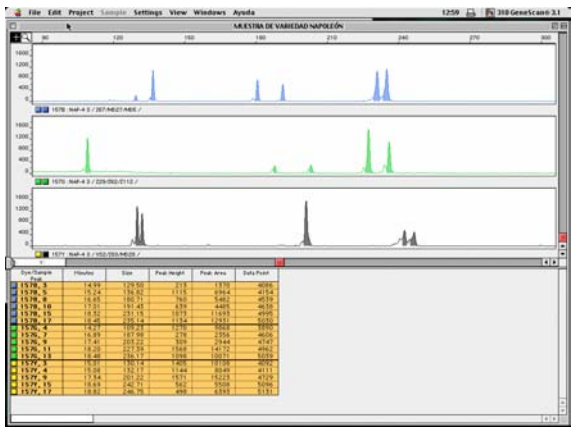
## Selection of Microsatellite Markers. Criteria

- Availability (public)
- Map position (genetically independent)
- Polymorphism (high)
- Range of amplification (that allow multiplexing)
- 'Quality' (ease of amplification and scoring, absence of known null alleles, absence of alleles differing only in 1 bp)

## Selection of Microsatellite Markers

| | | |
|---|---|---|
| ZAG67 | **VVMD5** | **VVMD27** |
| ZAG29 | **ZAG62** | ZAG112 |
| **VVS2** | ZAG83 | VVMD28 |

Multiplex PCR of 9 microsatellites



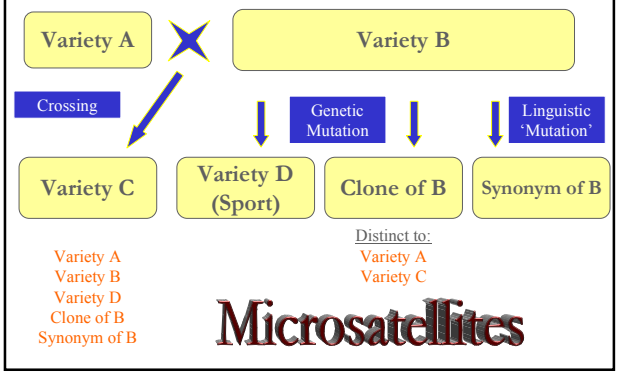
## Characterization of grapevines with microsatellites

System of 9 microsatellites used in:

- Study of a grapevine collection: About 1,000 accessions (2 plants / accession)
- Study of the Uniformity and Stability: More than 3,200 plants of 19 varieties

## Role of microsatellites in variety identification

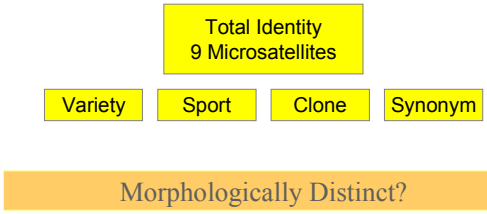
## Varieties, clones & synonyms




## Characterization of grapevine varieties with 9 microsatellites

- Study of 980 accessions
- 479,710 pair wise comparisons
- Literature / More microsatellites
- Conclusions:
  - All varieties arising from different embryos have been distinguished (2 or more alleles)
  - Only 5 microsatellites are enough
  - Clones and sports cannot be distinguished

## Role of microsatellites in Variety Identification

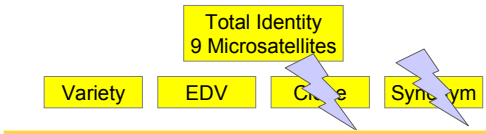



## Role of microsatellites in legal Identification

- Non protected varieties:
  - Mistakes and frauds from nurseries
  - Authorized by Origin Denomination Councils
  - List of commercial varieties
- Protected varieties:
  - Identification of illegal plants
  - Identification of EDVs

## Role of microsatellites in legal Identification

Grapevine protected variety




Morphologically Distinct?

Reversal of the burden of proof

## Role of microsatellites in legal Identification

Forensic approach:

- Database of a Reference collection
- Analysis of microsatellites in the sample
- Mathematical analysis of the match

## Likelihood ratio

$$LR = \frac{P(R/V)}{P(R/NV)}$$

P(R/V): probability of the match if the sample belongs to the variety.

P(R/NV): probability of the match if the sample does NOT belong to the variety.

## Role of microsatellites in legal Identification

- 6 microsatellites. LR = 1 in 87.000
- 9 microsatellites. LR = 1 in 156.000.000

## Role of microsatellites in variety identification

Conclusion

The system of 9 microsatellites is suitable for:

- Variety identification
- Legal identification

*Legal Protection for Vitis vinifera* L.

## Possible roles of microsatellites in the legal Protection

- Characterization of the reference collection
- DUS tests: Rapid screening against the reference collection (previous to the morphological exam?)
- Faster resolution to the objections to applications

## Possible role of microsatellites in the legal Protection

Evaluation of microsatellite markers for:

- Distinctness
- Uniformity
- Stability

## Possible role of microsatellites in the legal Protection: Distinctness

Difference:

- Consistent
- Clear

→ MINIMUM DISTANCE

## Possible role of microsatellites in the legal Protection: Distinctness

MINIMUM DISTANCE →

- Lowest number of different alleles between varieties
- Highest number of different alleles within varieties

## Possible role of microsatellites in the legal Protection: Distinctness

- Lowest number of different alleles between varieties

parents, progenies, half-sibs, full-sibs, etc.

479,710 pair wise comparisons

2 cases with a difference of 2 alleles

## Possible role of microsatellites in the legal Protection: Distinctness

- Highest number of different alleles within varieties

Pinot meunier (1 allele in VVS2, chimerism) Franks et al. 2002

- Greco di Tufo (1 allele in VVS2) Crespan 2004
- Chasselas (1 allele in ZAG83)
- Merlot (possible chimerism in VVMD27)
- Cardinal (possible chimerism in VVMD5)

1 allele

## Possible role of microsatellites in the legal Protection: Distinctness

- Lowest number of different alleles between varieties
- Highest number of different alleles within varieties

2
1

MINIMUM DISTANCE

2 alleles

## Possible role of microsatellites in the legal Protection: Distinctness

2 or more different alleles
9 Microsatellites

Distinct Variety

## Possible role of microsatellites in the legal Protection: Distinctness

No different allele
9 Microsatellites

Morphologically Distinct?

## Possible role of microsatellites in the legal Protection: Distinctness

1 different allele
9 Microsatellites

Analyze more microsatellites

## Possible role of microsatellites in the legal Protection

Evaluation of microsatellite markers for:

- Distinctness
- Uniformity
- Stability

## Evaluation of 9 microsatellites for Uniformity and Stability

Problems for breeders

- Additional efforts during breeding
- Protection reduced (mutations)

## Evaluation of 9 microsatellites for Uniformity and Stability

**Project VIN01-025 (2002/2004)**

- 9 microsatellites and 19 varieties (wine and table)
- Plant material from Europe, Africa and America
- About 3,200 plants

## Evaluation of 9 microsatellites for Uniformity and Stability

- Uniformity:
  - 3 plantations / variety
  - 50 plants / plantation
- Present DUS tests in grapevine: 10 plants (1 off-type allowed)

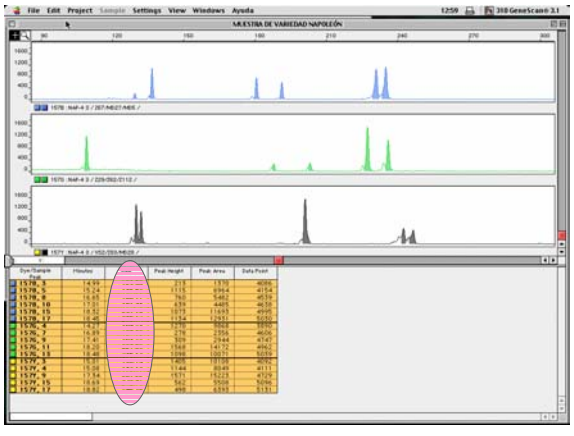
## Evaluation of 9 microsatellites for Uniformity and Stability

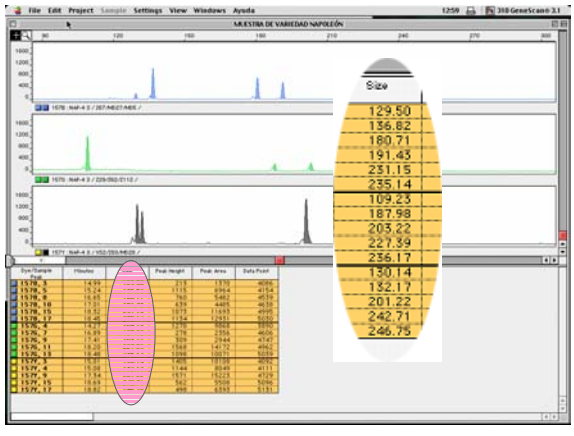
- Stability:
  - 10 plantations / variety
  - 5 plants / plantation
- Different plantations are probably in different cycles of propagation

## Evaluation of 9 microsatellites for Uniformity and Stability

Expected Results

- Lack of uniformity or stability: Different alleles between plants of the same variety
- Uniformity and stability: the same allele for all the plants of the same variety





## Analysis of GENESCAN values for Sugraone

| | Z67 | | MD27 | | MD5 | | Z29 | | Z62 | |
|---|---|---|---|---|---|---|---|---|---|---|
| AVERAGE | 123,55 | 136,81 | 176,69 | 178,77 | 222,68 | 232,74 | 109,09 | 111,04 | 185,69 | 187,81 |
| DIFFERENCE (max-min) | 0,30 | 0,30 | 0,47 | 0,51 | 0,47 | 0,90 | 0,67 | 0,61 | 0,48 | 0,50 |
| STANDARD DEVIATION | 0,0664 | 0,0632 | 0,094 | 0,1154 | 0,08 | 0,1813 | 0,1438 | 0,1369 | 0,1093 | 0,1149 |

| | Z112 | | VS2 | | Z83 | | MD28 | |
|---|---|---|---|---|---|---|---|---|
| AVERAGE | 232,18 | 0 | 115,41 | 131,99 | 189,59 | 195,33 | 246,45 | 0 |
| DIFFERENCE (max-min) | 0,82 | 0 | 0,56 | 0,46 | 0,41 | 0,28 | 0,96 | 0 |
| STANDARD DEVIATION | 0,0770 | 0 | 0,1090 | 0,0863 | 0,072 | 0,0518 | 0,1703 | 0 |

## Analysis of GENESCAN values for Cabernet sauvignon

| | Z67 | | MD27 | | MD5 | | Z29 | | Z62 | |
|---|---|---|---|---|---|---|---|---|---|---|
| AVERAGE | 123,36 | 136,72 | 172,55 | 186,24 | 228,17 | 236,26 | 108,64 | 0 | 187,67 | 193,45 |
| DIFFERENCE (max-min) | 0,65 | 0,48 | 0,99 | 0,85 | | | 0,98 | 0 | 0,92 | 0,80 |
| STANDARD DEVIATION | 0,1206 | 0,1074 | 0,2490 | 0,2057 | 0,4694 | 0,5282 | 0,2504 | 0 | 0,2290 | 0,1753 |

| | Z112 | | VS2 | | Z83 | | MD28 | |
|---|---|---|---|---|---|---|---|---|
| AVERAGE | 227,37 | 232,14 | 135,78 | 148,96 | 201,33 | 0 | 232,45 | 234,44 |
| DIFFERENCE (max-min) | 0,56 | 0,56 | | | 0,38 | 0 | 0,98 | 0,93 |
| STANDARD DEVIATION | 0,0951 | 0,1082 | 0,2134 | 0,247 | 0,0573 | 0 | 0,2467 | 0,2435 |

## Differences in Cabernet sauvignon

Differences in VVMD5 and VVS2 :

**¿Mutation or technical artefact?**

Mutation
Technical Artefact

Lack of stability
'+A' Peaks (Taq)
Electrophoresis

## Evaluation of 9 microsatellites for Uniformity and Stability

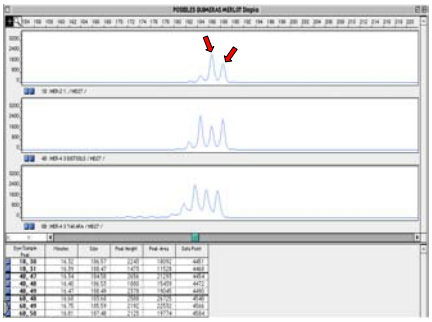
Results

- Mostly the same allele for all the plants of the same variety

BUT

- Presence of putative chimeras

## Evaluation of 9 microsatellites for Uniformity and Stability



## Evaluation of 9 microsatellites for Uniformity and Stability

Results

- Presence of putative chimeras
  - Cardinal-VVMD5 (2 / 191 plants)
  - Merlot-VVMD27 (49 / 189 plants):
    - Lack of uniformity and stability
    - Apparently not correlated with morphology

## Evaluation of 9 microsatellites for Uniformity and Stability

Results

Nº genotypes analyzed: 29.356
Nº genotypes correct: 29.305 (99.8%)

## Evaluation of 9 microsatellites for Uniformity and Stability

Conclusions

- The use of these microsatellites would not
  - reduce the protection
  - imply additional efforts for breeders

## A microsatellite-based system for the identification and legal protection of grapevine varieties

A microsatellite system suitable for

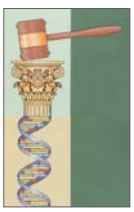
- Identification:
  - complete matching means identity (or EDV)
- Legal Protection:
  - Distinctness: Minimum distance: 2 alleles
  - Uniformity and stability: no reduction of protection; no additional efforts for breeders

| Carattere: Caractère: Merkmal: Characteristic: Carácter: | Marcatore SSR VVMD27 Marqueur SSR VVMD27 SSR-Marker VVMD27 SSR-marker VVMD27 Marcador SSR VVMD27 | Code No OIV |
|---|---|---|

Primer sequence: VVMD27a: GTA CCA GAT CTG AAT ACA TCC GTA AGT
VVMD27b: ACG GGT ATA GAG CAA ACG GTG T

| Distanza relativa espressa in paia di basi dall' allele di dimensione n / Distance de paires de bases relative à la taille de l' allèle n / Relativer Basenpaarenabstand zur Allelgröße n / Relative base pair distance to allele size n / Distancia relativa en pares de bases al alelo de grandeza n | Varietà di riferimento / Exemples de variétés / Beispielssorten / Example varieties / Ejemplos de variedades | | Ulteriori varietà di riferimento D'autres exemples de variétés Weitere Beispielssorten Further example varieties Otros ejemplos de variedades |
|---|---|---|---|
| | Annotazione (Codice dell'allele di riferimento) / Notation (code de variété) / Bonitur (Sortencode) / Notes (Variety code) / Notación (Codigo de variedad) | | |
| n | CS 1 | Cabernet Sauvignon N: 1 | Mancin N: 1, Agiorgitiko N: 1 |
| n + 2 | | | |
| n + 4 | MU 1 | Muscat à petits grains blancs B: 1 | Jacquez N: 1, Mourvedre N: 1 |
| n + 6 | CF 1 | Cabernet franc N: 1 | Portugieser N: 1, Sultanina B: 1 |
| n + 8 | FE 1 | Fercal N: 1 | Veltliner Rot RG: 1, Mavrodaphni N: 1 |
| n + 10 | PI 1 | Pinot N,G,B: 1 | Barbera N: 1 |
| n + 11 | GO 1 | Goethe 9: 1 | |
| n + 12 | VA 1 | Vialla N: 1 | |

## A microsatellite-based system for the identification and legal protection of grapevine varieties



Thank you for your attention