

**Marker-Trait Association
for Herbage Quality in
Perennial Ryegrass**

Association Mapping Panel (AMP)

- Diverse germplasm - 192 genotypes
- 3 ecotypes - 64 genotypes for each ecotype
 - Zürich Uplands
 - Kangaroo Valley
 - Victorian
- Two cultivars - 48 genotypes for each cultivar
 - Tolosa
 - Bronsyn

Trial and Phenotyping

- Trial with diverse germplasm and 3 ecotypes in Hamilton in two environments (glass house and shade house)
- Trial with 2 cultivars in New Zealand
- Plant samples collected at reproductive and vegetative stages
- Individual samples freeze dried, ground and NIRS

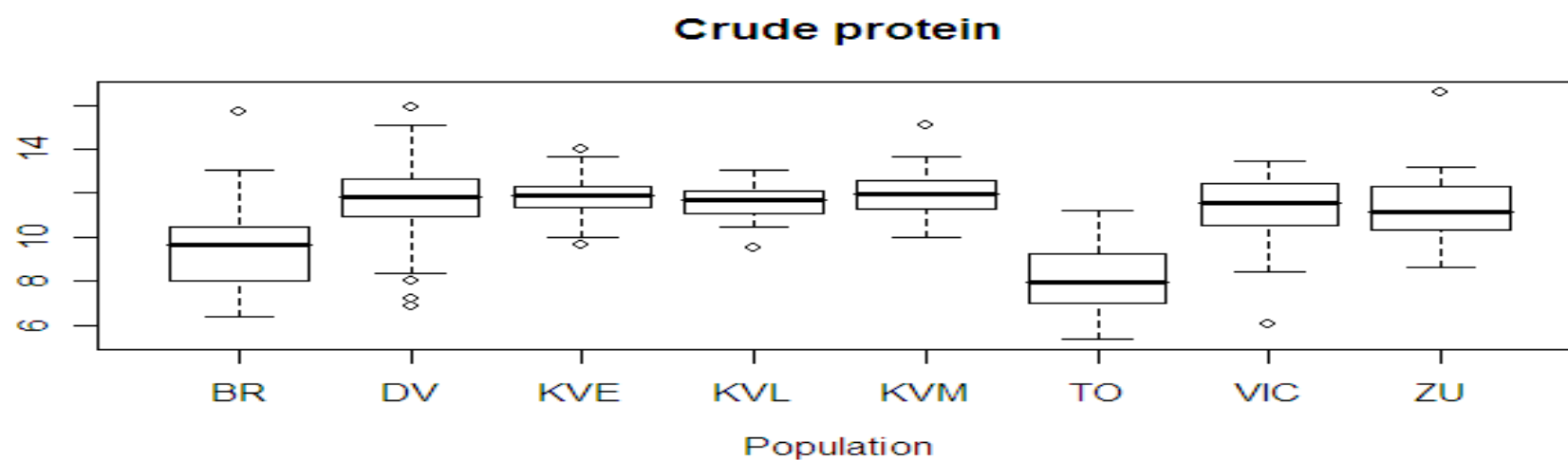
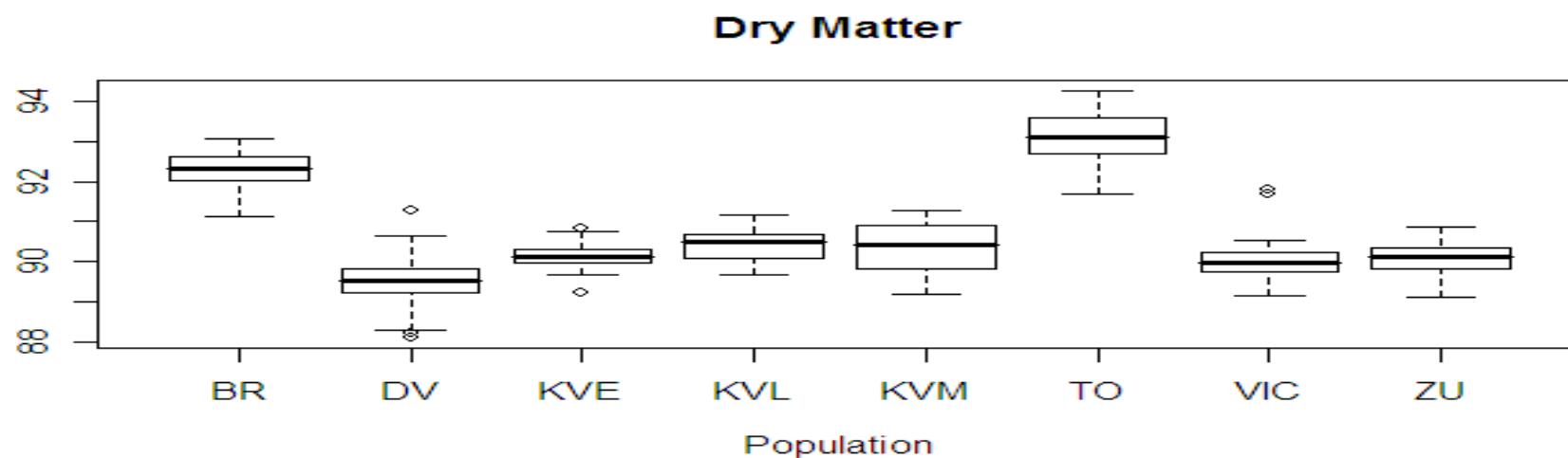
Trial and Phenotyping

- Traits
 - Dry Matter (DM)
 - Crude Protein (CP)
 - Acid Detergent Fibre (ADF)
 - Neutral Detergent Fibre (NDF)
 - *In vivo* Dry Matter Digestibility (IVVDM)
 - Water Soluble Carbohydrates (WSC)
 - Ash
 - Starch

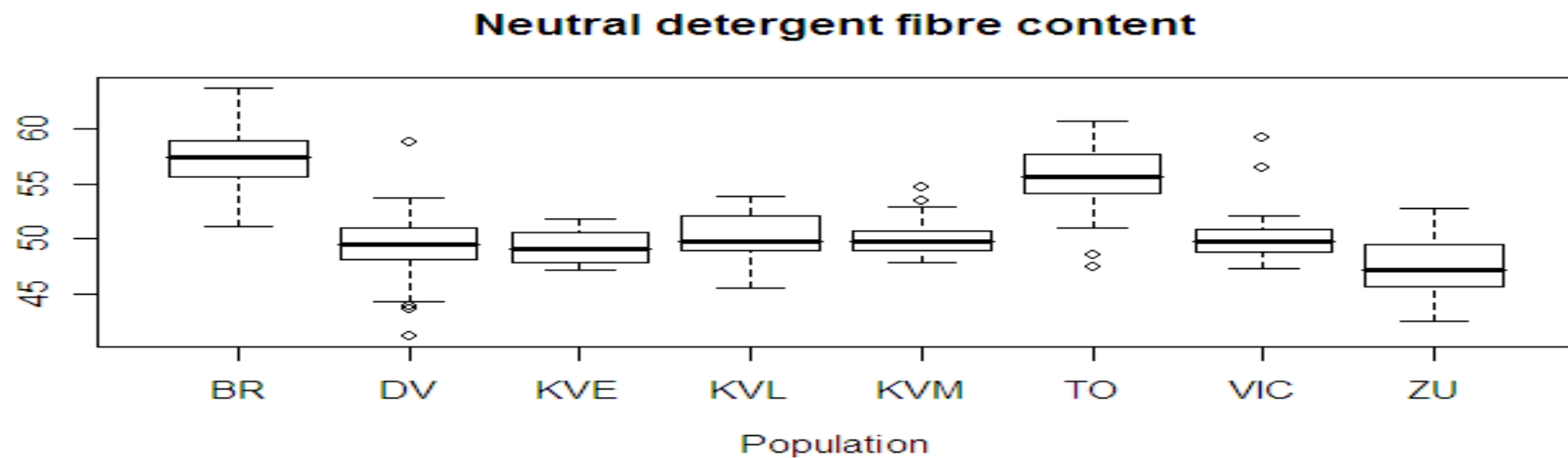
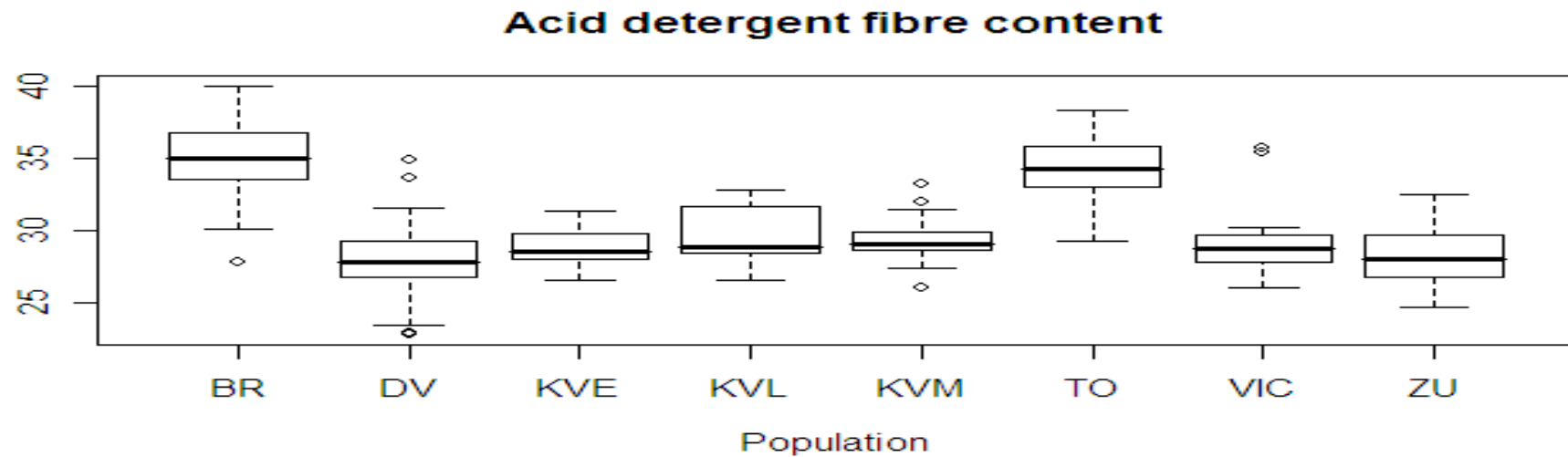
Genotyping

- 60 SSR loci evenly distributed across genome
 - Rigorous QA for single locus character
- 141 SNP in 20 candidate genes

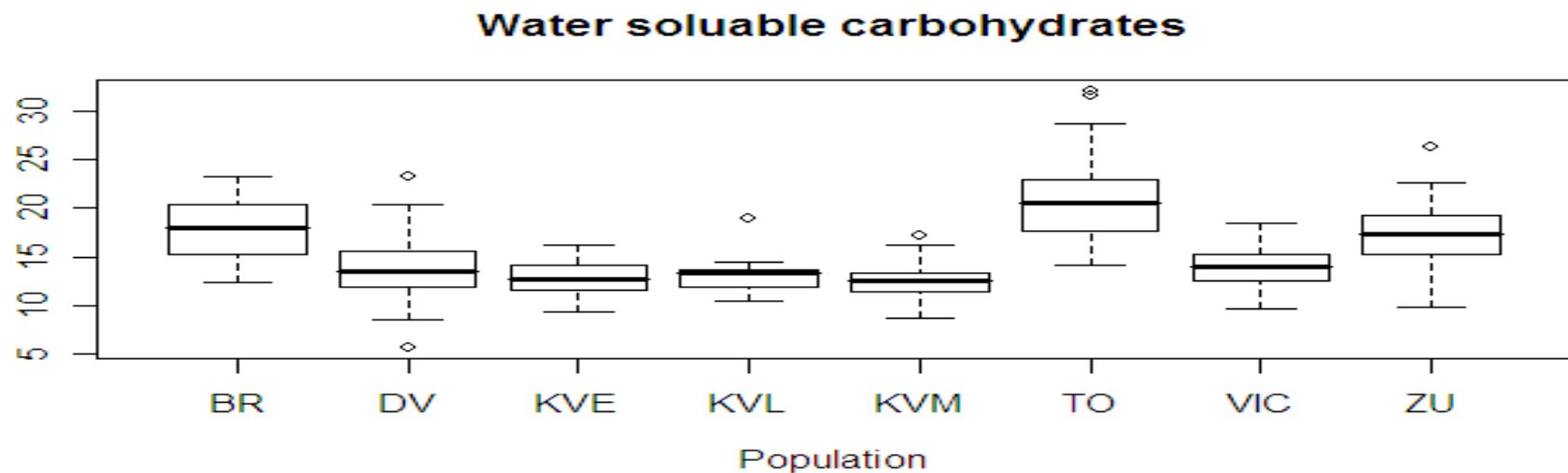
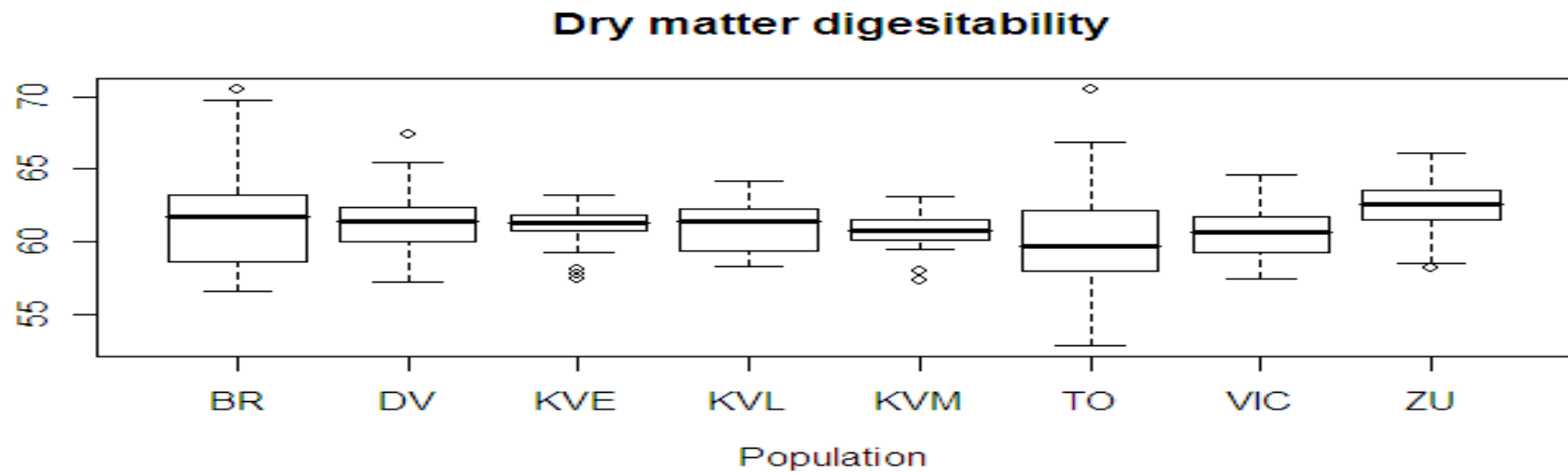
Descriptive Statistics (Reproductive)



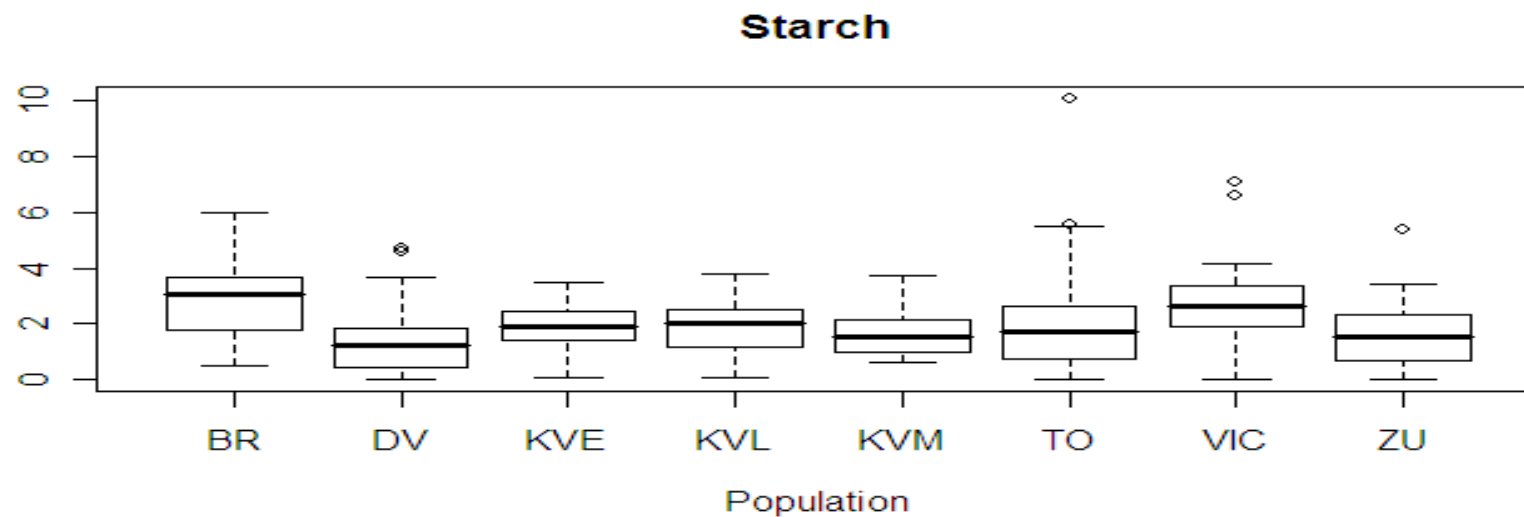
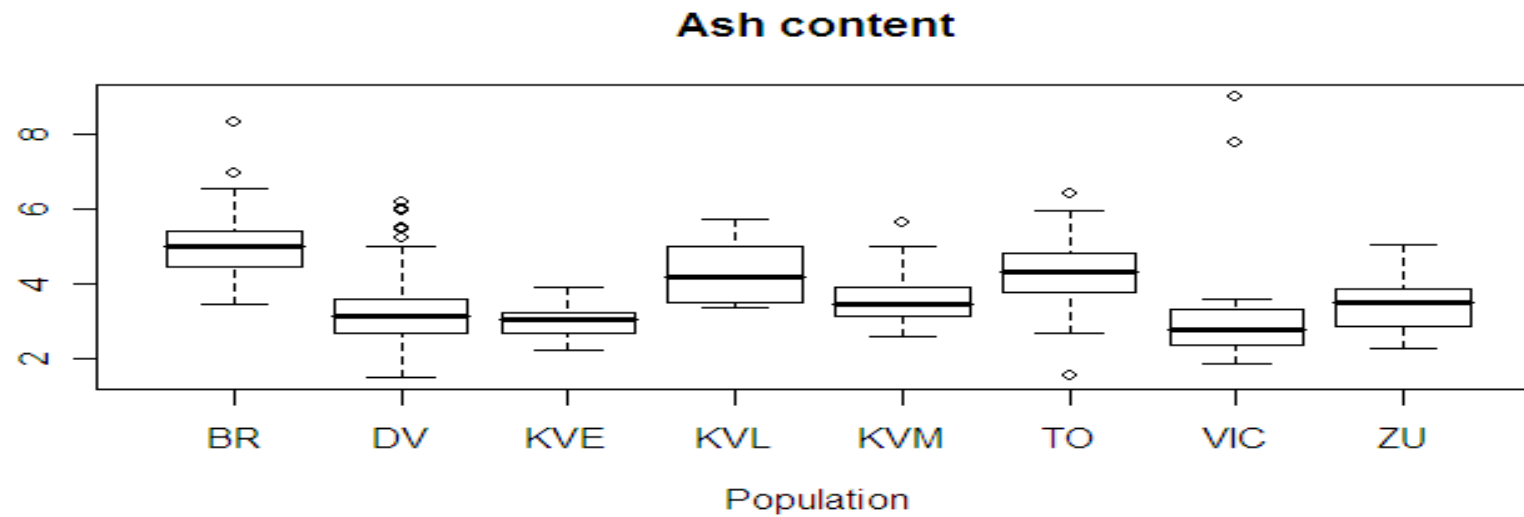
Descriptive Statistics (Reproductive)



Descriptive Statistics (Reproductive)



Descriptive Statistics (Reproductive)

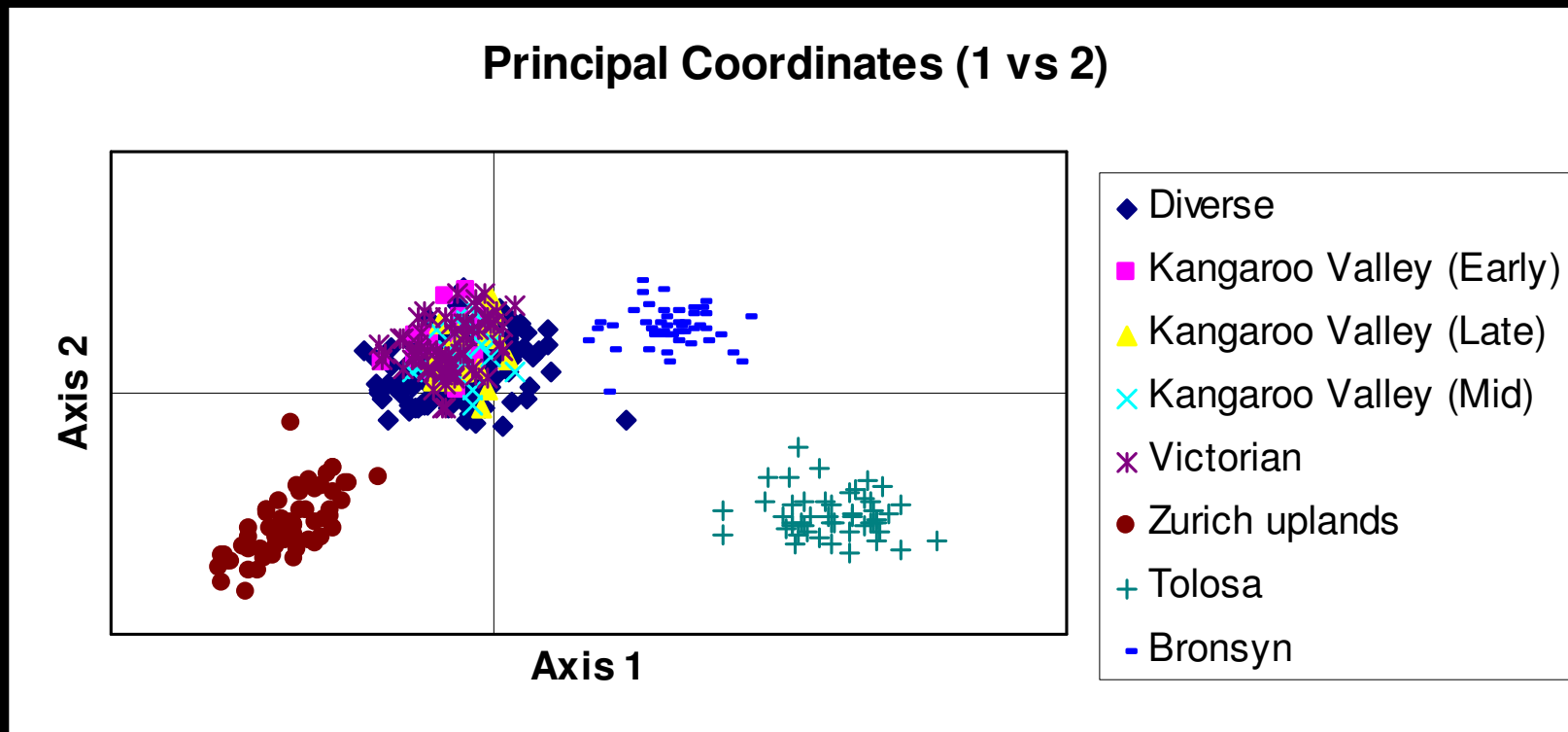


Correlation Coefficients Vegetative (Glasshouse)

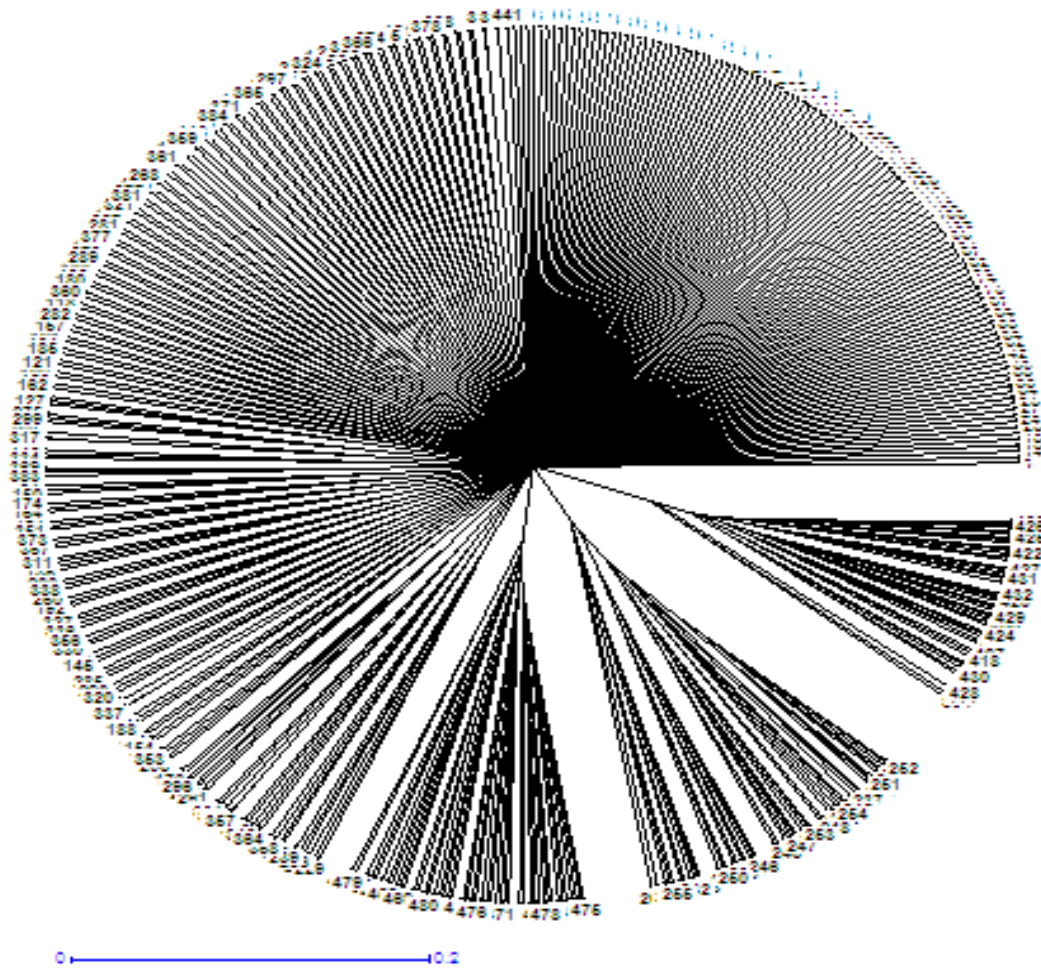
| | ADF | Ash | CP | DM | IVVDMD | NDF | Starch | WSC |
|--------|--------|--------|--------|-------|--------|--------|--------|-----|
| ADF | 1 | | | | | | | |
| Ash | 0.582 | 1 | | | | | | |
| CP | -0.715 | -0.271 | 1 | | | | | |
| DM | 0.157 | 0.373 | 0.275 | 1 | | | | |
| IVVDMD | -0.687 | -0.376 | 0.825 | 0.296 | 1 | | | |
| NDF | 0.901 | 0.462 | -0.775 | 0.05 | -0.814 | 1 | | |
| Starch | 0.379 | 0.354 | -0.303 | 0.163 | -0.283 | 0.285 | 1 | |
| WSC | -0.213 | 0.001 | -0.185 | 0.001 | 0.128 | -0.271 | 0.079 | 1 |

Population Structure: PCoA

| Axis | 1 | 2 | 3 |
|--------------|-------|-------|-------|
| % variation | 34.49 | 22.57 | 17.60 |
| % Cumulative | 34.49 | 57.06 | 74.66 |



Population Structure : Clustering

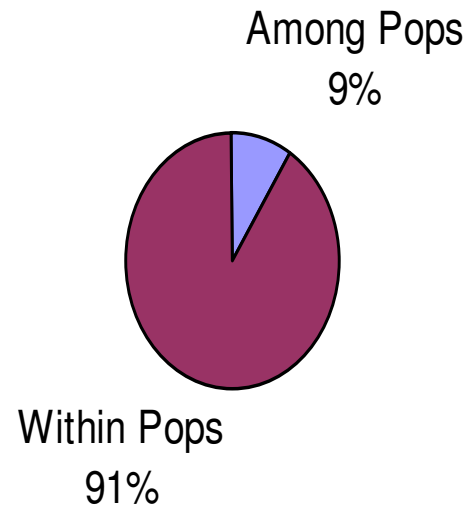


Clockwise from
big cluster,

1. Diverse
germplasm +
Kangaroo Valley
+ Victorian
2. Tolosa
3. Zürich Uplands
4. Bronsyn

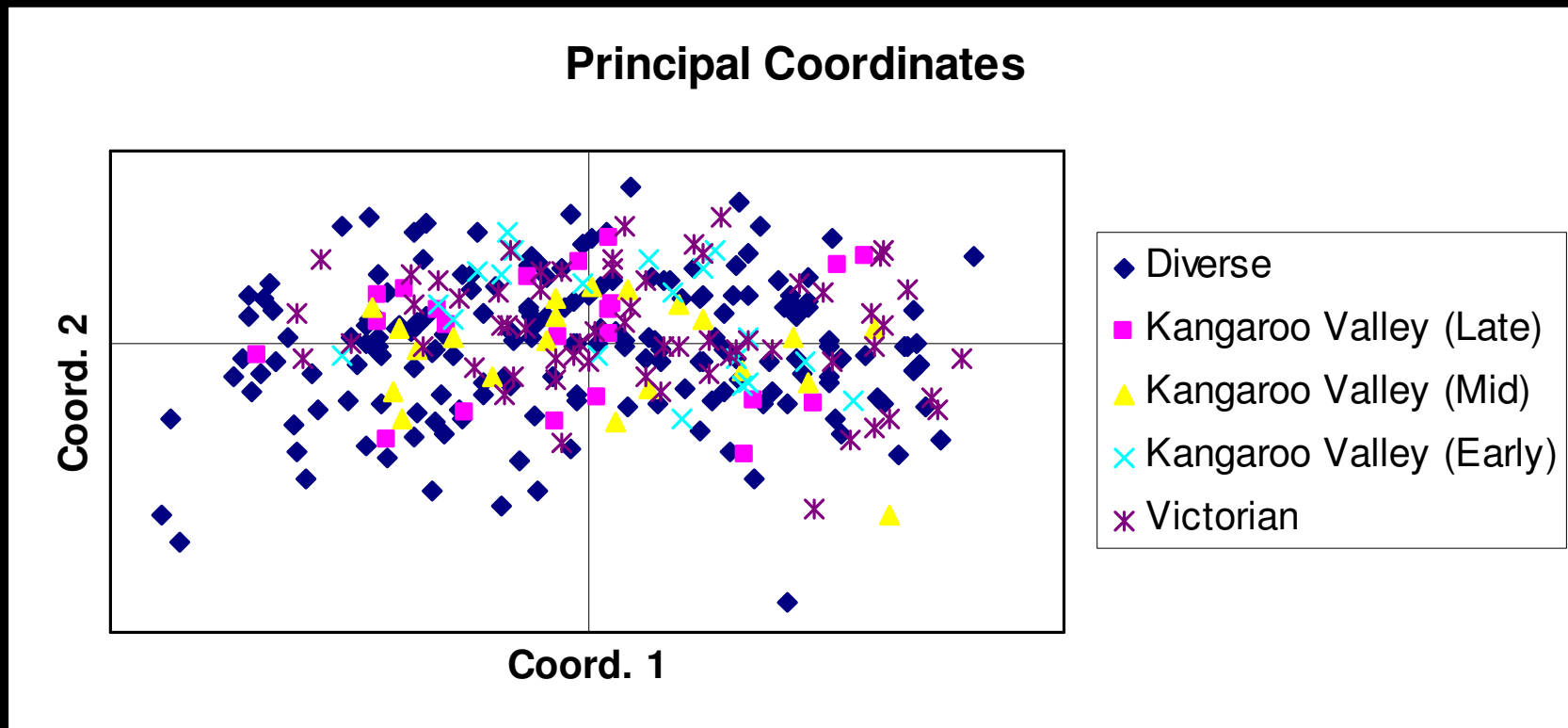
Population Structure: AMOVA

Percentages of Molecular Variance

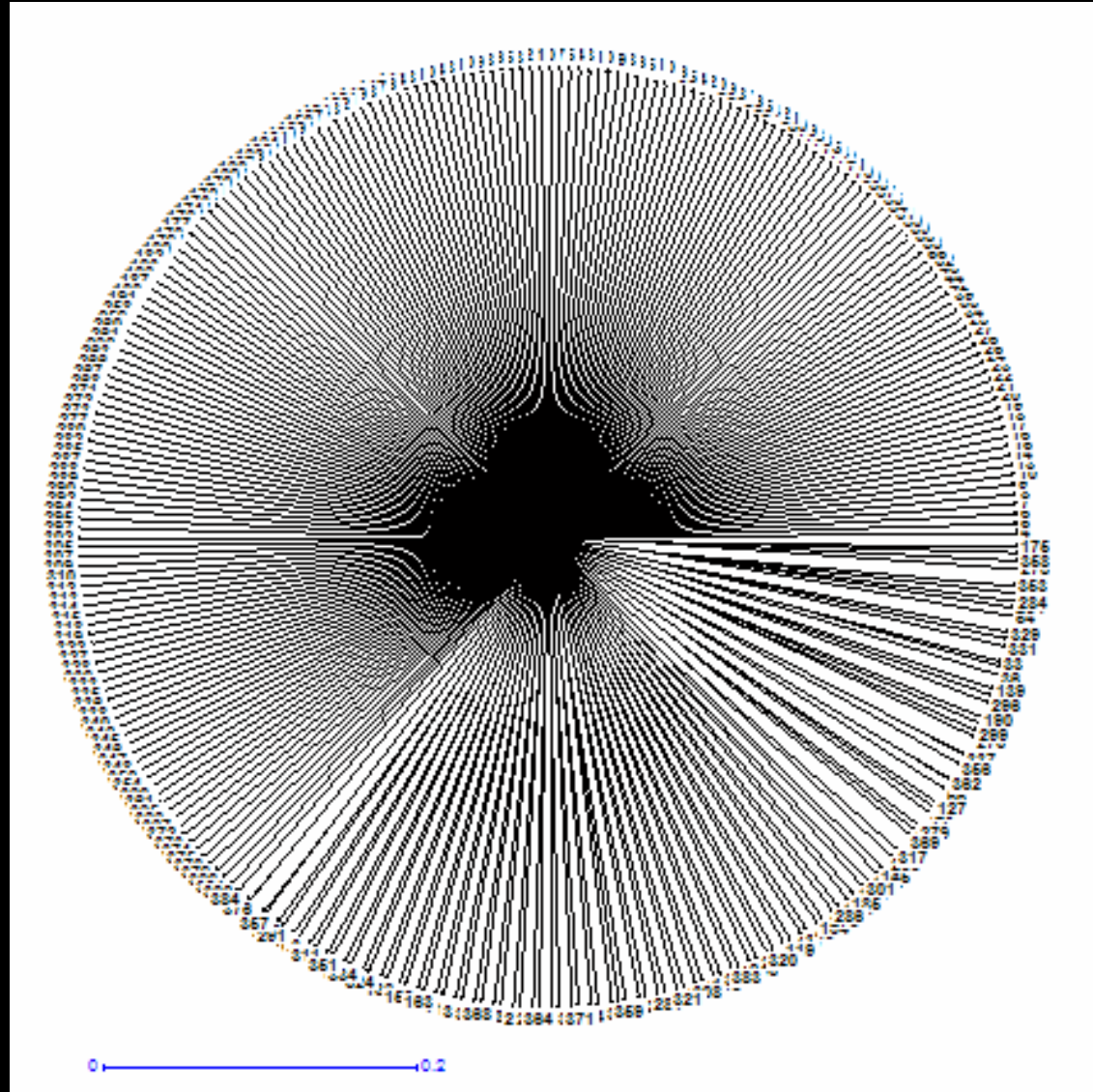


Population Structure: PCoA Meta-Population

| Axis | 1 | 2 | 3 |
|--------------|-------|-------|-------|
| % variation | 21.79 | 18.88 | 16.51 |
| % cumulative | 21.79 | 40.67 | 57.17 |

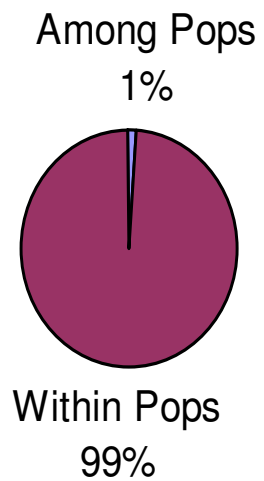


Population Structure - Meta-Population



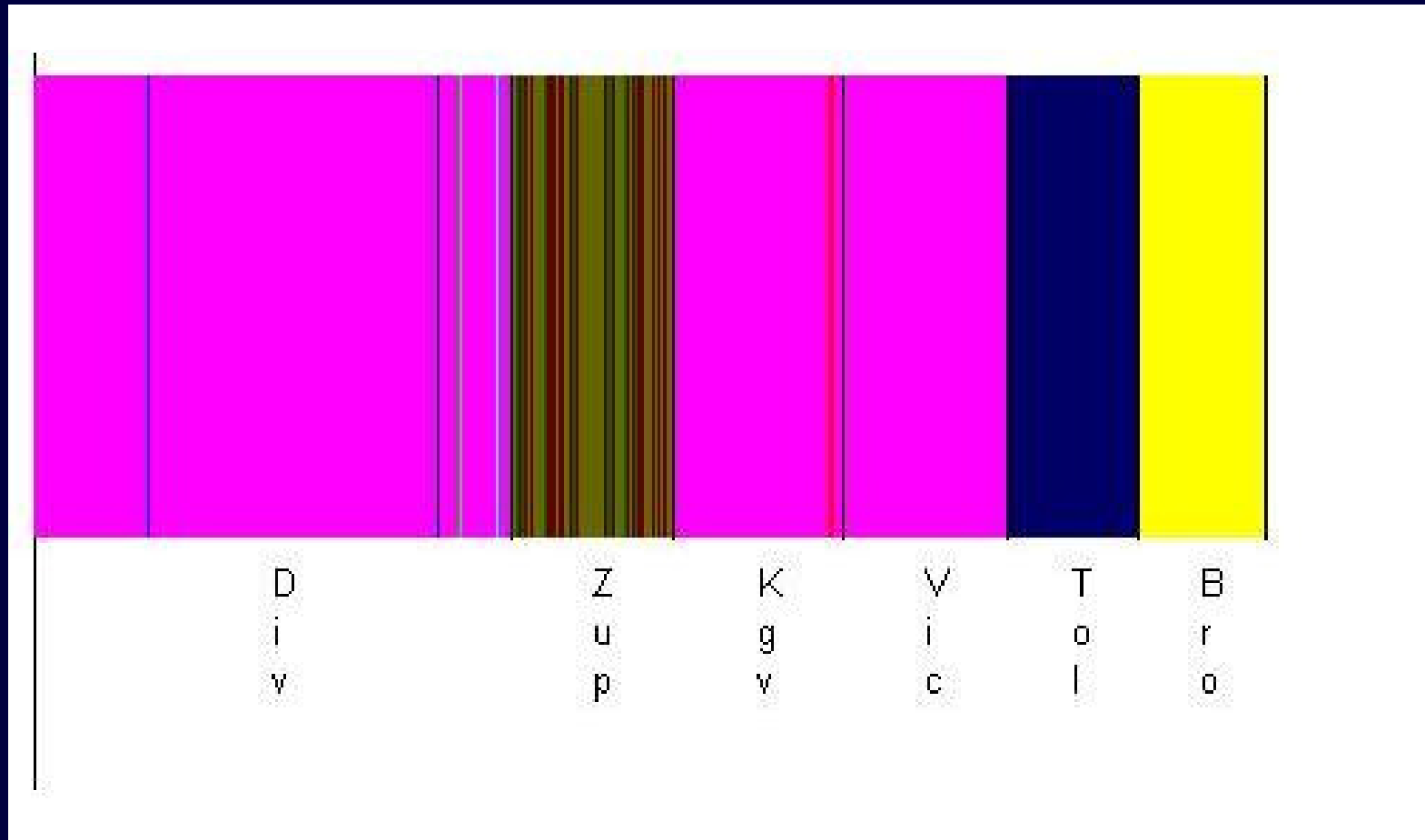
Meta-Population: AMOVA

Percentages of Molecular Variance



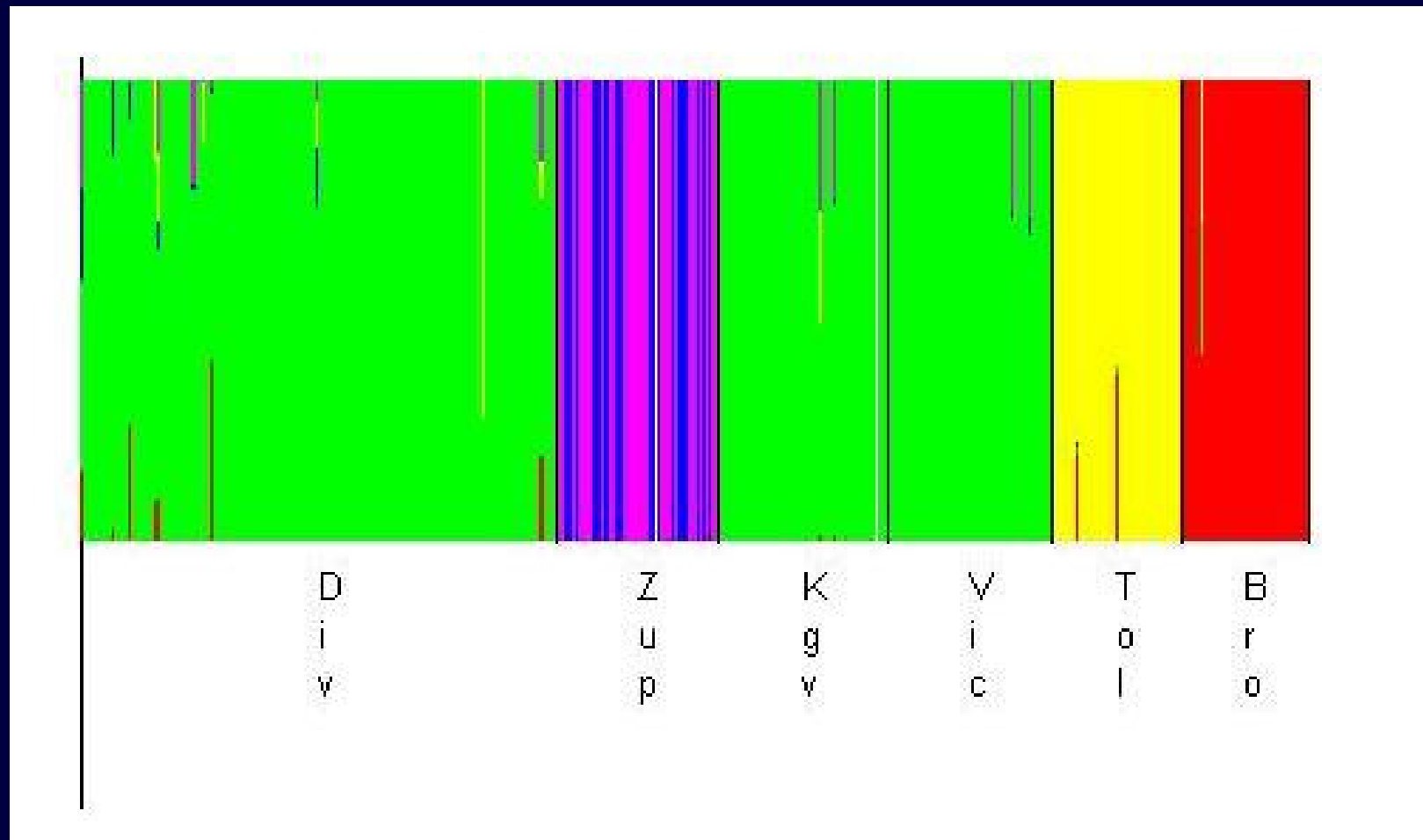
Population Structure

Parametric clustering



Population Structure

Parametric clustering



Population Structure

Parametric clustering

| Population | Meta | 2 | Bronsyn | 4 | 5 | 6 | Zurich uplands | Zurich uplands | Zurich uplands | Tolosa |
|-----------------|------|---|---------|---|---|---|----------------|----------------|----------------|--------|
| Bronsyn | | | 48 | | | | | | | |
| Tolosa | | | | | | | | | | 48 |
| Diverse | 174 | 1 | | 1 | 1 | | | | | 1 |
| Kangaroo Valley | 63 | | | | | | | | | |
| Victorian | 60 | | | | | 2 | | | | |
| Zurich uplands | | | | | | | 21 | 31 | 8 | |

Population Structure: Summary

- Four populations: Zürich uplands, Bronsyn, Tolosa and a meta-population (the diverse population, Kangaroo Valley [late], Kangaroo Valley [early], Kangaroo Valley [mid] and Victorian
- Meta-population has very little sub-structure

Genetic Relatedness Using 52 SSR Loci

- Relationship matrix between individuals, A

$$r_l = \frac{S_{xy} - s}{1 - s} \qquad s = \sum_{i=1}^a p_i^2$$

- r_l = relationship between individuals x and y and locus /
- S_{xy} = similarity index between individuals x and y
- s = similarity as a result of chance alone

Analytical Models

| Model | Marker | Population | Plant (Relationship) |
|---------|--------|------------|-------------------------|
| Fixed | Fixed | no | no |
| PFixed | Fixed | Fixed | no |
| PRandom | Fixed | Random | no |
| Mixed | Fixed | no | yes |
| PMixed | Fixed | Random | yes |

Marker-Trait Association

Results are from the analysis using

- Meta-population
- Traits measured at the reproductive stage

Haplotype analysis was done only for the four full length genes, i.e. *LpFT1*, *LpFT3*, *LpCCR1* and *LpCAD2*

Dry Matter

| Marker | Gene | Model | P |
|--------------------|---------|-------|-------|
| LpCAD2-2779r | CAD2 | MIXED | 0.021 |
| LpCAD2-2812f | CAD2 | MIXED | 0.021 |
| LpCAD2-4666f (r) | CAD2 | FIXED | 0.013 |
| LpCAD2-4666f (r) | CAD2 | MIXED | 0.004 |
| LpCCoNA157f | CCoAOMT | FIXED | 0.014 |
| LpCCoNA157f | CCoAOMT | MIXED | 0.015 |
| LpCCR1AU10212r | CCR1 | FIXED | 0.044 |
| LpCCR1AU10212r | CCR1 | MIXED | 0.005 |
| LpCCR1AU10292r | CCR1 | FIXED | 0.028 |
| LpCCR1AU10292r | CCR1 | MIXED | 0.002 |
| LpCCR1AU11057f | CCR1 | FIXED | 0.049 |
| LpCCR1AU11136f | CCR1 | MIXED | 0.015 |
| LpCCR1NA10132f | CCR1 | FIXED | 0.013 |
| LpCCR1NA10132f | CCR1 | MIXED | 0.003 |
| LpCCR1NA10243f | CCR1 | FIXED | 0.046 |
| LpCCR1NA10243f | CCR1 | MIXED | 0.004 |
| LpCCR1NA8589f | CCR1 | FIXED | 0.000 |
| LpCWInvAU136f | CWInv | FIXED | 0.048 |
| LpCWInvAU136f | CWInv | MIXED | 0.035 |
| LpFT1NA4009f | FT1 | MIXED | 0.046 |
| LpFT3AU2684f | FT3 | MIXED | 0.029 |
| LpFT3AU3726f | FT3 | FIXED | 0.023 |
| LpFT3bp3159f | FT3 | MIXED | 0.040 |
| LpOMT3AU729r | OMT3 | FIXED | 0.010 |
| LpSPSfNA256r | SPS | MIXED | 0.050 |

Crude Protein

| Marker | Gene | Model | P |
|----------------|-------|-------|-------|
| LpCAD2-2779r | CAD2 | MIXED | 0.020 |
| LpCAD2-2812f | CAD2 | FIXED | 0.005 |
| LpCAD2-5523r | CAD2 | MIXED | 0.033 |
| LpCCR1-7705f | CCR1 | MIXED | 0.013 |
| LpCCR1AU10212r | CCR1 | MIXED | 0.014 |
| LpCCR1AU10292r | CCR1 | FIXED | 0.040 |
| LpCCR1AU10292r | CCR1 | MIXED | 0.016 |
| LpCCR1AU11018r | CCR1 | FIXED | 0.032 |
| LpCCR1AU11018r | CCR1 | MIXED | 0.018 |
| LpCCR1AU11481r | CCR1 | FIXED | 0.020 |
| LpCCR1AU11481r | CCR1 | MIXED | 0.004 |
| LpCCR1NA10132f | CCR1 | FIXED | 0.002 |
| LpCCR1NA10132f | CCR1 | MIXED | 0.007 |
| LpCCR1NA10243f | CCR1 | FIXED | 0.008 |
| LpCCR1NA10243f | CCR1 | MIXED | 0.035 |
| LpCCR1NA8719f | CCR1 | MIXED | 0.049 |
| LpCCR1NA8773r | CCR1 | FIXED | 0.013 |
| LpCCR1NA9916f | CCR1 | FIXED | 0.046 |
| LpCCR1NA9916f | CCR1 | MIXED | 0.020 |
| LpCCRBNA419r | CCRB | MIXED | 0.024 |
| LpCWInvAU136f | CWInv | FIXED | 0.009 |
| LpFT1AU9765r | FT1 | MIXED | 0.049 |

Acid Detergent Fibre

| MARKER | GENE | MODEL | P |
|---------------|-------------|--------------|----------|
| Lp4CLJaNA323r | 4CLJa | FIXED | 0.048 |
| LpCCoNA100r | CCoAOMT | FIXED | 0.004 |
| LpCCoNA100r | CCoAOMT | MIXED | 0.001 |
| LpCCoNA157f | CCoAOMT | MIXED | 0.015 |
| LpCCR1-7705f | CCR1 | FIXED | 0.018 |
| LpCCR1-7705f | CCR1 | MIXED | 0.012 |
| LpCCR1NA8589f | CCR1 | FIXED | 0.028 |
| LpCCR1NA8719f | CCR1 | FIXED | 0.012 |
| LpCCR1NA8719f | CCR1 | MIXED | 0.010 |
| LpCCRbNA337f | CCRb | MIXED | 0.047 |
| LpCCRbNA419r | CCRb | MIXED | 0.006 |
| LpFT1AU4883r | FT1 | FIXED | 0.043 |
| LpFT1NA3938f | FT1 | MIXED | 0.030 |
| LpFT1NA4009f | FT1 | MIXED | 0.020 |
| LpFT3AU2987f | FT3 | FIXED | 0.048 |
| LpFT3bp3159f | FT3 | FIXED | 0.006 |
| LpSPSfAU160r | SPS | FIXED | 0.026 |

Neutral Detergent Fibre

| Marker | Gene | Model | P |
|-----------------|-----------|-------|-------|
| CADlike12NA528f | CADLike12 | MIXED | 0.026 |
| Lp4CLJaNA323r | 4CLJa | FIXED | 0.037 |
| LpCAD2-4976f | CAD2 | FIXED | 0.001 |
| LpCAD2-4976f | CAD2 | MIXED | 0.005 |
| LpCAD2-5756f | CAD2 | FIXED | 0.033 |
| LpCAD2-5756f | CAD2 | MIXED | 0.038 |
| LpCCoNA100r | CCoAOMT | FIXED | 0.016 |
| LpCCoNA100r | CCoAOMT | MIXED | 0.003 |
| LpCCR1-7705f | CCR1 | FIXED | 0.026 |
| LpCCR1-7705f | CCR1 | MIXED | 0.008 |
| LpCCR1NA8773r | CCR1 | MIXED | 0.048 |
| LpFT1AU3788r | FT1 | MIXED | 0.024 |
| LpFT1NA3938f | FT1 | MIXED | 0.015 |
| LpFT1NA4009f | FT1 | MIXED | 0.018 |
| LpFT3bp3159f | FT3 | FIXED | 0.005 |
| LpFT3NA4512f | FT3 | FIXED | 0.022 |
| LpFT3NA4512f | FT3 | MIXED | 0.045 |
| LpSFTaNA500f | SFTa | FIXED | 0.031 |
| LpSPSfAU160r | SPS | MIXED | 0.018 |

Dry Matter Digestibility

| Marker | Gene | Model | P |
|-------------------|-----------|-------|-------|
| LpCAD2-4976f | CAD2 | FIXED | 0.002 |
| LpCAD2-4976f | CAD2 | MIXED | 0.001 |
| LpCADlike09NA168f | CADLike09 | MIXED | 0.044 |
| LpCADlike09NA260f | CADLike09 | MIXED | 0.026 |
| LpCCoNA100r | CCoAOMT | FIXED | 0.028 |
| LpCELLNA668f | CELL | FIXED | 0.005 |
| LpFT1AU4883r | FT1 | FIXED | 0.003 |
| LpFT1AU9158f | FT1 | MIXED | 0.034 |
| LpFT3AU2987f | FT3 | FIXED | 0.013 |
| LpFT3AU2987f | FT3 | MIXED | 0.020 |
| LpFT3bp3159f | FT3 | FIXED | 0.012 |

Water Soluble Carbohydrates

| Marker | Gene | Model | P |
|-------------------|-----------|-------|-------|
| LpCAD2-2812f | CAD2 | FIXED | 0.012 |
| LpCAD2-2812f | CAD2 | MIXED | 0.033 |
| LpCADlike09NA260f | CADLike09 | FIXED | 0.021 |
| LpCADlike09NA260f | CADLike09 | MIXED | 0.032 |
| LpCCR1AU11481r | CCR1 | FIXED | 0.015 |
| LpCCR1AU11481r | CCR1 | MIXED | 0.012 |
| LpCCR1NA10132f | CCR1 | FIXED | 0.013 |
| LpCCR1NA10132f | CCR1 | MIXED | 0.009 |
| LpCWInvAU136f | CWInv | FIXED | 0.013 |
| LpFT3AU3726f | FT3 | FIXED | 0.032 |
| LpOMT3AU729r | OMT3 | FIXED | 0.015 |
| LpSPSfNA100f | SPS | FIXED | 0.039 |
| LpSPSfNA100f | SPS | MIXED | 0.034 |
| LpSPSfNA244f | SPS | FIXED | 0.047 |
| LpSPSfNA244f | SPS | MIXED | 0.049 |
| LpSPSfNA256r | SPS | FIXED | 0.011 |
| LpSPSfNA256r | SPS | MIXED | 0.006 |
| LpSPSfNA292f | SPS | MIXED | 0.047 |
| LpSPSfNA303r | SPS | FIXED | 0.027 |
| LpSPSfNA303r | SPS | MIXED | 0.021 |

Ash

| Marker | Gene | Model | P |
|------------------|---------|-------|-------|
| LpCAD2-4976f | CAD2 | FIXED | 0.026 |
| LpCCoNA157f | CCoAOMT | FIXED | 0.007 |
| LpCCoNA157f | CCoAOMT | MIXED | 0.006 |
| LpCCoNA316f | CCoAOMT | FIXED | 0.032 |
| LpCCR1-11856r | CCR1 | FIXED | 0.001 |
| LpCCR1-7708f | CCR1 | FIXED | 0.012 |
| LpCCR1-7743f | CCR1 | FIXED | 0.012 |
| LpCCR1AU11136f | CCR1 | FIXED | 0.038 |
| LpCCR1NA8719f | CCR1 | MIXED | 0.034 |
| LpCCR1NA8773r | CCR1 | FIXED | 0.033 |
| LpCCR1NA8773r | CCR1 | MIXED | 0.037 |
| LpCELLNA668f | CELL | FIXED | 0.017 |
| LpCELLNA668f | CELL | MIXED | 0.033 |
| LpFT3NA4512f | FT3 | FIXED | 0.013 |
| LpINVaAU206.116r | INVa | FIXED | 0.013 |
| LpINVaAU206.211r | INVa | MIXED | 0.047 |
| LpOMT3AU729r | OMT3 | FIXED | 0.008 |
| LpOMT3AU729r | OMT3 | MIXED | 0.001 |
| LpSFTaAU602r | SFTa | FIXED | 0.001 |
| LpSPSfAU115r | SPS | MIXED | 0.035 |
| LpSPSfAU160r | SPS | MIXED | 0.002 |
| LpSPSfNA292f | SPS | MIXED | 0.043 |

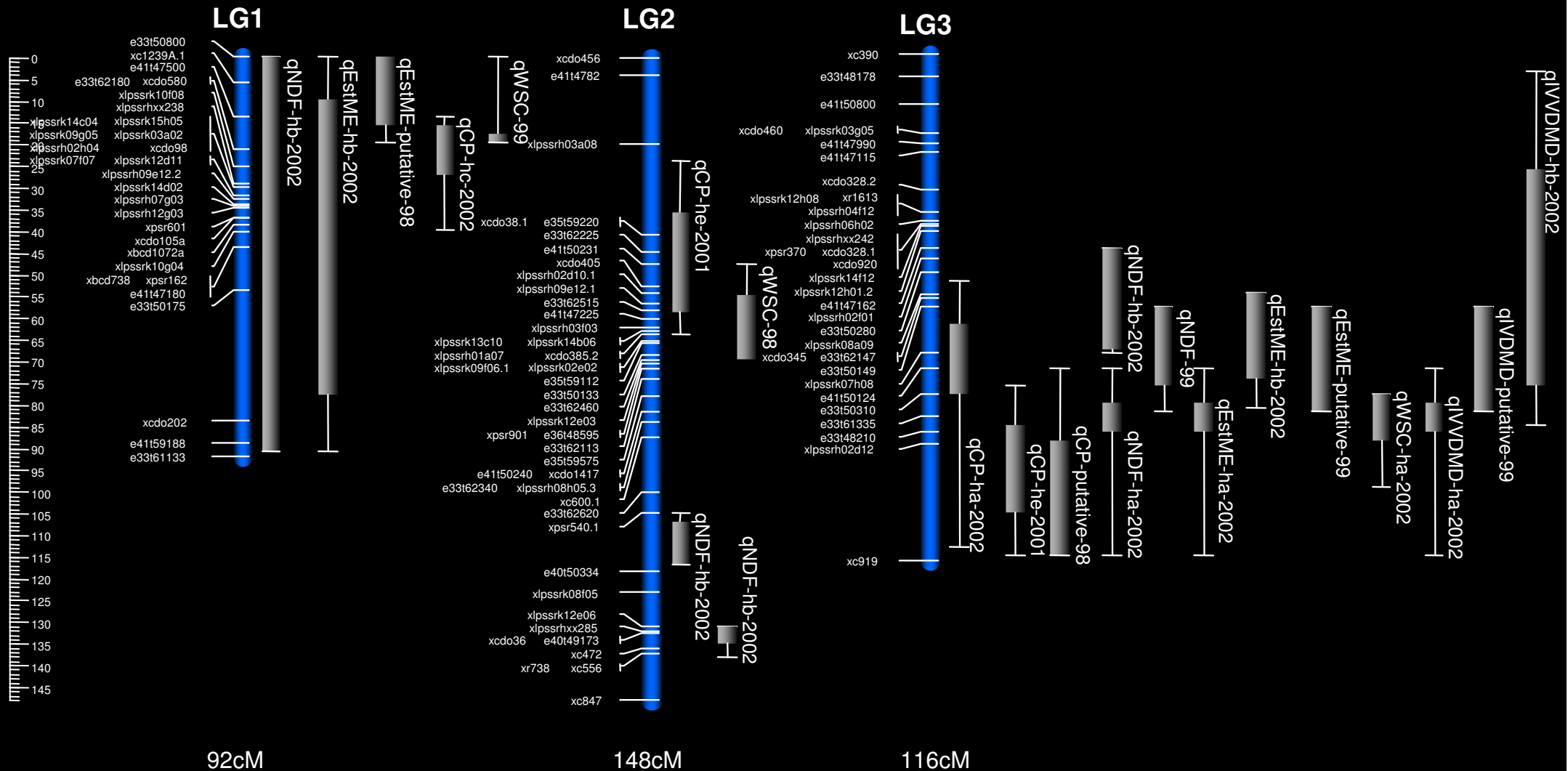
Starch

| Marker | Gene | Model | P |
|-----------------|---------|-------|-------|
| Lp4CLJaAU523f | 4CLJa | MIXED | 0.046 |
| LpCCoNA157f | CCoAOMT | FIXED | 0.023 |
| LpCCoNA157f | CCoAOMT | MIXED | 0.010 |
| LpCCR1-7705f | CCR1 | FIXED | 0.023 |
| LpCCR1-7705f | CCR1 | MIXED | 0.022 |
| LpCCR1AU11541f | CCR1 | FIXED | 0.041 |
| LpCCR1AU11541f | CCR1 | MIXED | 0.032 |
| LpCCR1NA8589f | CCR1 | FIXED | 0.033 |
| LpCCR1NA8589f | CCR1 | MIXED | 0.050 |
| LpCCR1NA8719f | CCR1 | FIXED | 0.034 |
| LpCCR1NA8719f | CCR1 | MIXED | 0.048 |
| LpCCR1NA9916f | CCR1 | MIXED | 0.038 |
| LpCELLNA563r | CELL | FIXED | 0.033 |
| LpCWInvBoth101r | CWInv | MIXED | 0.044 |
| LpFFTaNA128f | FFTa | MIXED | 0.028 |
| LpFT1AU3788r | FT1 | FIXED | 0.004 |
| LpFT1AU3788r | FT1 | MIXED | 0.037 |
| LpFT1AU8723f | FT1 | FIXED | 0.024 |
| LpFT1NA3938f | FT1 | FIXED | 0.041 |
| LpSPSfAU160r | SPS | FIXED | 0.001 |
| LpSPSfAU160r | SPS | MIXED | 0.013 |
| LpSSaNA61.82f | Ssa | FIXED | 0.040 |
| LpSSaNA61.82f | Ssa | MIXED | 0.027 |

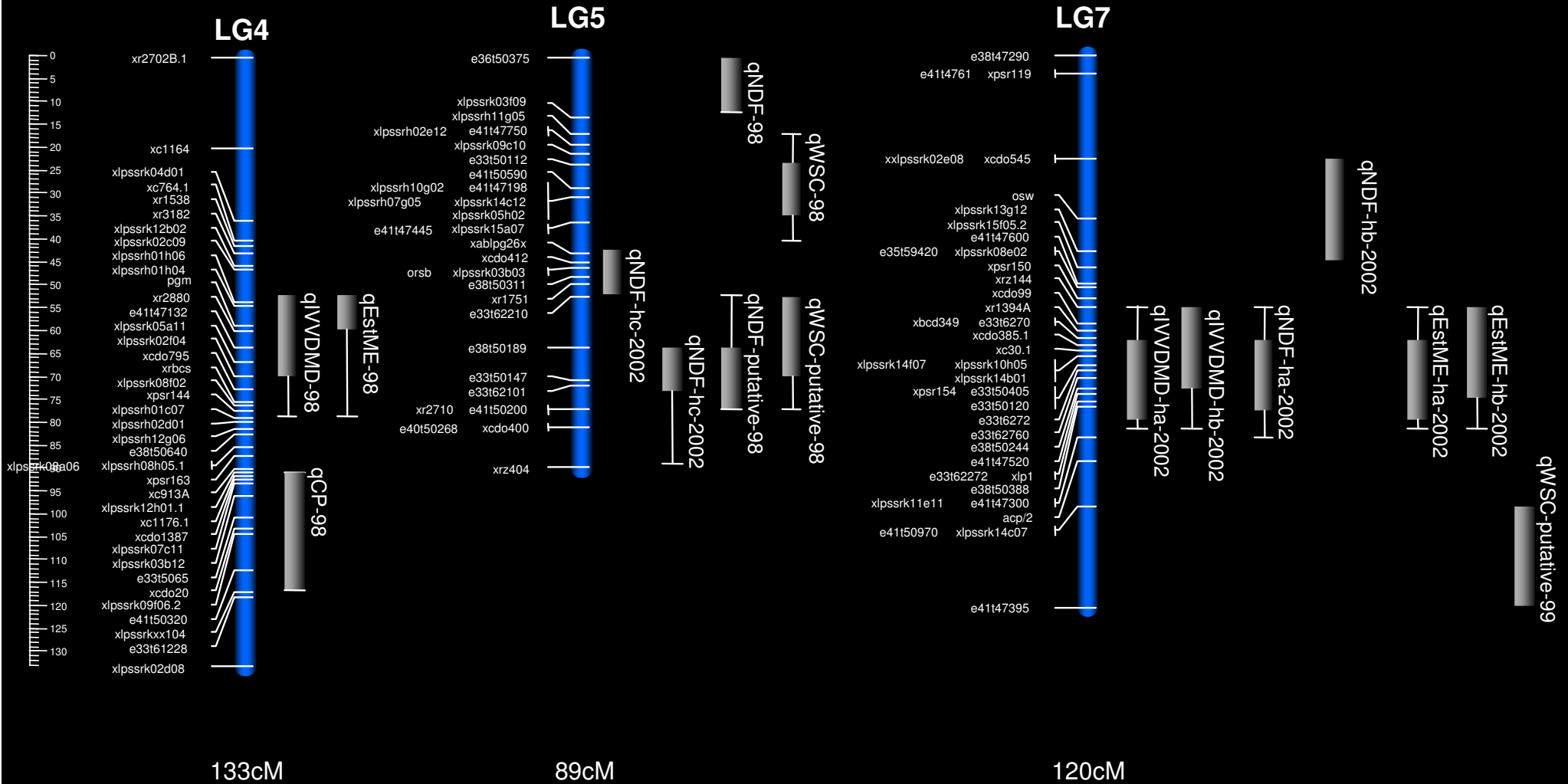
Haplotype Association

| Trait | <i>LpFT1</i> | <i>LpFT3</i> | <i>LpCAD2</i> | <i>LpCCR1</i> |
|--------|--------------|--------------|---------------|---------------|
| DM | 0.155282 | 0.044087 | 1.99E-04 | 0.000261 |
| CP | 0.922616 | 0.399567 | 0.473694 | 0.006289 |
| ADF | 0.33244 | 0.617087 | 0.226567 | 0.07567 |
| NDF | 0.554726 | 0.533667 | 0.643339 | 0.591198 |
| IWDMD | 0.229145 | 0.704101 | 0.056386 | 0.234244 |
| WSC | 0.311147 | 0.734463 | 0.398643 | 0.110255 |
| ASH | 0.770347 | 0.92017 | 0.543101 | 0.994479 |
| STARCH | 0.025189 | 0.021606 | 0.332218 | 0.015541 |

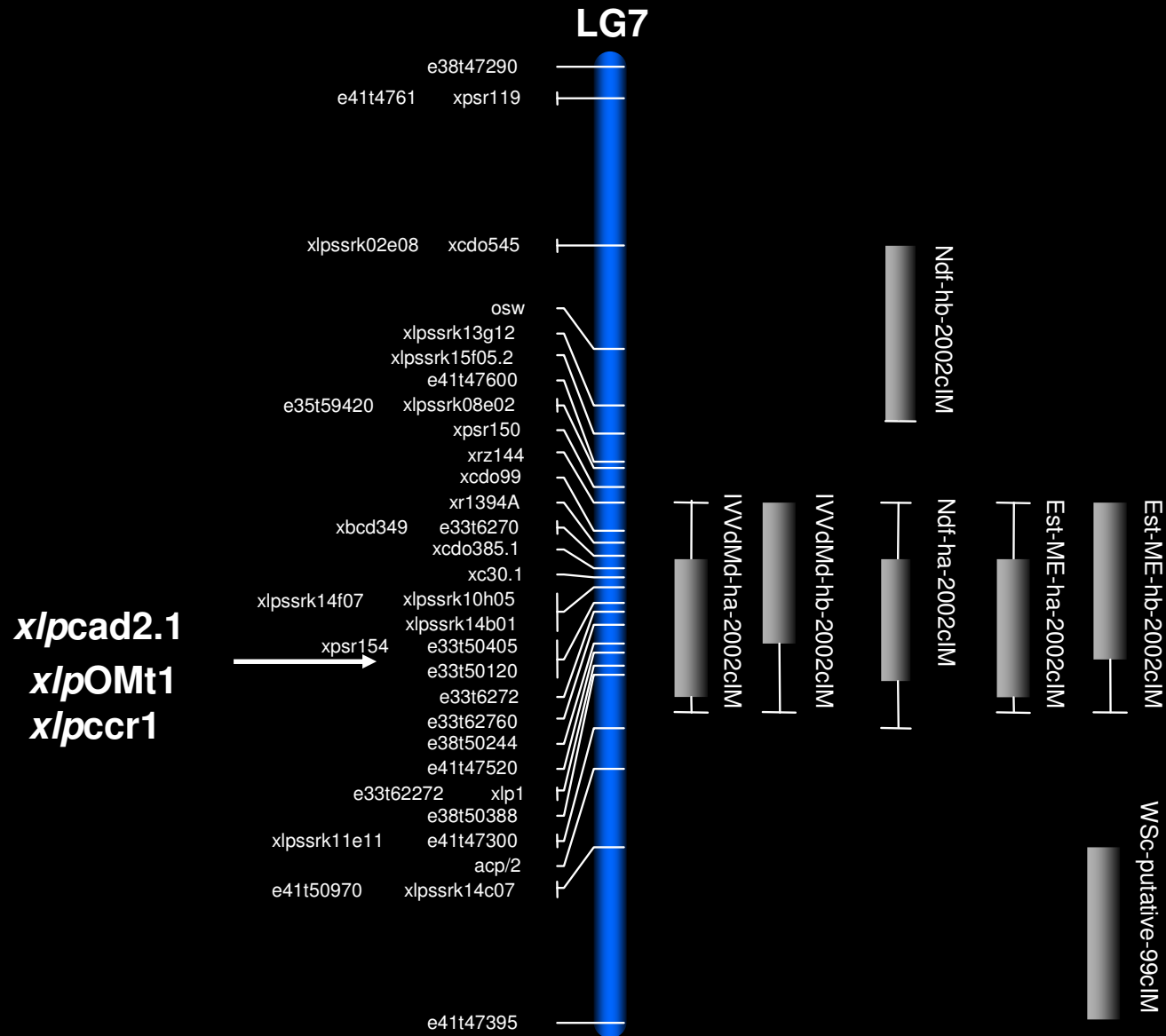
QTL identified in the P150/112 Reference Mapping Population using NIRS-Calibrated Estimates



QTL identified in the P150/112 Reference Mapping Population using NIRS-Calibrated Estimates



LG7 Map from p150/112 Population: Co-location of QTL with Candidate Gene Markers



'Moving-Window' Haplotyping

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 1 | 2 | 3 | | | | | | |
| 2 | | 2 | 3 | 4 | | | | | |
| 3 | | | 3 | 4 | 5 | | | | |
| 4 | | | | 4 | 5 | 6 | | | |
| 5 | | | | | 5 | 6 | 7 | | |
| 6 | | | | | | 6 | 7 | 8 | |
| 7 | | | | | | | 7 | 8 | 9 |



| | | |
|---|---|---|
| 0 | 0 | 0 |
| 0 | 0 | 1 |
| 0 | 1 | 0 |
| 0 | 1 | 1 |
| 1 | 0 | 0 |
| 1 | 0 | 1 |
| 1 | 1 | 0 |
| 1 | 1 | 1 |



Association

Summary of Observations

- Strong associations were identified between some of the candidate genes and all of the quality traits using single marker analysis
 - Correspondence with
 - Pre-existing genetic analysis
 - Knowledge of background biology
- Preliminary full-length gene haplotype analysis gave disappointing results.
- The 'moving-window' analysis is very promising
- Population structure represents a real problem if association mapping is conducted using selected materials (cultivars)
- For many of the candidate genes, SNP genotypes are population specific
 - hence not possible to test whether these SNPs contribute to the phenotypic difference

Further Analysis

- Haplotype analysis
 - Haplotype analysis using maximum likelihood based 'moving-window' approach using different window sizes
 - Analysis based on haplogroups
- Analysis using traits that are different among populations as covariates to further control background effect
- Compare the differences using various genetic relatedness analyses
- Prediction of target genotypes for selection

Further Actions

- Validation of identified associations
 - Phenotyping of the meta-population in field trial
 - Six clonal replicates per plant
 - Latinized row-column design
 - Genotyping plants in the meta-population to reduce missing marker information
- Increasing number of unlinked markers (SSR or SNP) for control of possible fine-scale population structure

Further Actions

- Test identified associations in major cultivars used in Australia
 - Frequencies of desirable alleles within and across cultivars
 - Phenotyping of individuals selected on basis of marker genotypes
 - Association analysis