

# 34TH WORLD CONGRESS OF VINE AND WINE

Porto, PORTUGAL

20 –27th June 2011

## GENETICS IN THE VINEYARD

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### Summary

The increase of genetic studies in the vineyard is very important for the knowledge of the vegetative material and its behaviour in the field.

With this knowledge, we may understand its identity and genetic relationships.

In what concerns the origin of varieties, the more recent studies show that the origin of the species *Vitis vinifera* is in the Near East, but that the Iberian Peninsula was taken as an area of refuge of this species during the quaternary Glaciations, becoming a domestication center for this species. The wild plants from the Iberian Peninsula have their own genetic characteristics that integrate the autochthonous varieties cultivated in the Iberian Peninsula.

Concerning the identification of varieties, it is possible, using a group of microsatellites selected for their polymorphism, to identify the different varieties in culture. Currently, a database is being created, the European Vitis Database, where, in the future, the microsatellite profiles of all varieties will be recorded and available for public consultation.

The genetic expression of the variety and its relationship with the environment, is another objective of the genetic studies in the vineyard. Currently, it is possible to know which genes are expressed, in the different stages of the response of the variety to the environment, enabling to predict the cultural behaviour of varieties.

Finally, genome sequencing of the *Vitis vinifera* has allowed the study of molecular markers linked to phenotypic characteristics, enabling, in the future, the assisted breeding and selection.

How useful is genetics in the vineyard?

With genetics we can identify varieties, sometimes clones, understand their origin, identify the expression of the genes when they are under environmental conditions and identify molecular markers that can be used to assisted breeding.

The French-Italian Public Consortium released the reference genome sequence for grapevine by sequencing the near homozygous Pinot Noir line (PN40024) in 2007 (French–Italian Public Consortium, 2007). This has made it possible to quickly generate genetic analysis tools such as molecular markers based on sequence comparisons between variants and the reference sequence. In the same year, Velasco *et al.* (2007) sequenced the heterozygous Pinot Noir, providing valuable information on single nucleotide polymorphisms (SNPs) at the genome level.

## 1. ORIGIN OF VARIETIES

The archaeological record suggests that the *Vitis* genus have first appeared about 65 million years Before Present (BP), and the cultivation of the domesticated grape, *Vitis vinifera* subsp. *vinifera*, began 6 000 – 8 000 years Before Present in Transcaucasia, from its wild progenitor, where its greatest genetic diversity is found and where very early archaeological evidence, including grape pips and artefacts of a ‘wine culture’, have been excavated (McGovern, 2003).

Whether from Transcaucasia or the nearby Taurus or Zagros Mountains, it is hypothesized that this wine culture spread southwards and eventually westwards around the Mediterranean basin, together with the transplantation of cultivated grape cuttings. However, the existence of morphological and molecular differentiation between cultivars from eastern and western ends of the modern distribution of the Eurasian grape suggests the existence of different genetic contribution from local *sylvestris* populations or multilocal selection and domestication of *sylvestris* genotypes (Grassi *et al.*, 2003). To study this issue, Arroyo-García *et al.* (2006) analyzed chlorotype variation and distribution in 1 201 samples of *sylvestris* and *vinifera* genotypes from the whole area of the species’ distribution and studied their genetic relationships. They used nine polymorphic chloroplast microsatellite loci and have identified eight different chlorotypes. From these eight chlorotypes (A to H), chlorotype A is very prevalent in European *sylvestris* populations, but not found in the Near East. The chlorotype B, when compared with the other chlorotypes occupies a central position, and did not show a clear eastern or western pattern, occurring across the whole distribution area at low frequency, suggesting

that it could be an ancestral *V. vinifera* chlorotype. Chlorotypes C, D and G are frequent in Near Eastern populations but were not found farther west. Over 70% of the Iberian Peninsula cultivars display chlorotypes that are only compatible with the possibility of having derived from western *sylvestris* populations.

These results suggest the existence of at least two important origins for the cultivated germplasm, one in the Near East and another in the western Mediterranean region, the latter of which gave rise to many of the current Western European cultivars..

The same conclusion is indicated by **Myles *et al.* (2011)**, using a SNP set of 9 000 SNP genotyping array – the Vitis9kSNP array. Relatedness among diverse geographically sample of *vinifera* and *sylvestris* provides strong support for the origin of *vinifera* in the Near East: All *vinifera* populations (west, central and east) are genetically closer to eastern *sylvestris* than to western *sylvestris* (Table 1). After domestication, grape growing and winemaking expanded westward, reaching Western Europe by 2 800 years BP (McGovern, 2003). They have also found that haplotype diversity in western *vinifera* is slightly reduced compared with eastern *vinifera*, suggesting that the grape experienced a modest reduction in genetic diversity as it was brought to Western Europe. However, the western *vinifera* is more closely related to western *sylvestris* than are other *vinifera* populations, what is consistent with gene flow between wild and cultivated grapes in Western Europe (Table 1).

**Table 1. Population pairwise Fst estimates**

	<i>Sylvestris</i> west	<i>Sylvestris</i> east	<i>Vinifera</i> west	<i>Vinifera</i> central	<i>Vinifera</i> east
<i>Sylvestris</i> west	—				
<i>Sylvestris</i> east	0.154	—			
<i>Vinifera</i> west	0.120	0.051	—		
<i>Vinifera</i> central	0.168	0.046	0.020	—	
<i>Vinifera</i> east	0.202	0.035	0.051	0.031	—

Geographic regions are defined as follows: “east” includes locations east of Istanbul, Turkey; “west” includes locations west of Slovenia, including Austria; and “central” refers to locations between them.

**Myles *et al.* (2011)**

**Myles *et al.*, (2011)** also verify that the *vinifera* pedigree structure is the result of a limited number of crosses made among elite cultivars that survived and were sometimes vegetatively propagated for centuries. Table grapes have more first-degree relationships than wine grapes, and the high degree of connectivity among table grapes suggests that there was more intense breeding in table grapes than in wine grapes.

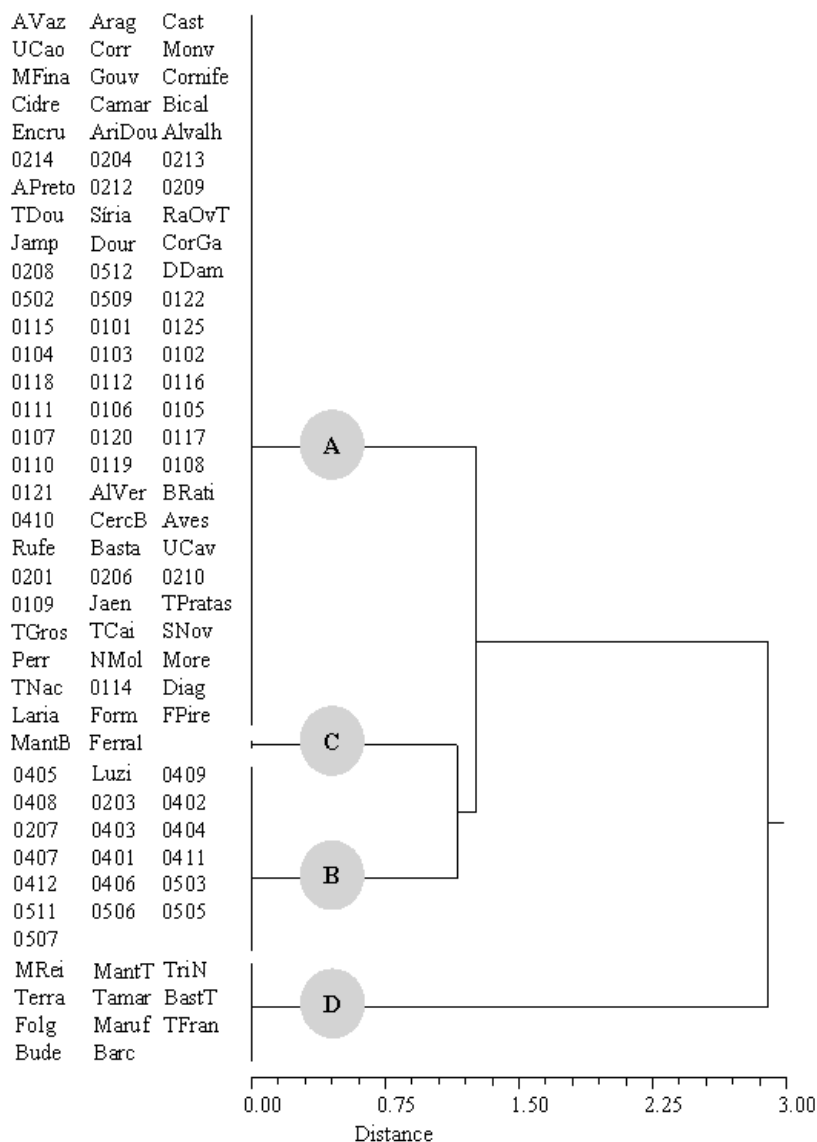
**Cunha (2009)**, using three, from the nine polymorphic chloroplast microsatellite loci used by **Arroyo-García *et al.* (2006)**, identified four different chlorotypes in 53 *sylvestris* plants

and 57 *vinifera* varieties from Portugal. These chlorotypes correspond to chlorotypes A, B, C and D of **Arroyo-García et al.** (2006). The results of this study show that the chlorotype A is the most common in all Portuguese analyzed plants. Only the variety Luzídio belongs to the chlorotype B, but 34% of wild grapevines belong to this group. This chlorotype in Spain has an exclusively southern distribution (Arroyo-García et al., 2006), but in Portugal seems to be more widely distributed. The chlorotype C is represented in only 1.8% of the samples. The chlorotype D is represented in 10% of all samples and is present on ‘Malvasia Rei’, ‘Manteúdo Tinto’, ‘Trincadeira’, ‘Tinta de Lisboa’, ‘Folgazão’, ‘Marufo’, ‘Touriga Franca’, ‘Barcelo’, ‘Budelho’, ‘Tamarez’ and ‘Terrantez’. The existence of these chlorotypes C and D, typical eastern chlorotypes (Arroyo-García et al., 2006), suggests the presence of remote eastern germplasm in Portugal (Fig.1).

With genetics we can also identify the origin of some varieties obtained by outcrossing or to confirm the parentage of new varieties obtained by human breeding.

**Lopes et al. (1999)**, for example, identify the Portuguese variety ‘Boal Ratinho’ as an offspring from ‘Malvasia Fina’ and ‘Síria’.

In what concerns the confirmation of parentage, **Lopes et al. (2006)** confirm that ‘Lusitano’ result from a cross between ‘Castelão’ with ‘Alicante Henri Bouschet’, as the Portuguese breeder Leão Ferreira de Almeida indicated, although he call these varieties by ‘Castelão Francês’ and ‘Tintinha’.



**Figure 1: Chlorotypes from all samples. UPGMA method using the correlation matrix (cophenetic correlation coefficient  $r = 0.987$ ) (Cunha, 2009)**

Cultivar Name	Abbreviation	Cultivar Name	Abbreviation	Cultivar Name	Abbreviation
Alfrocheiro N	APreto (D)	Dedo de Dama B	Ddam (A)	Molar N	RaOvT (BI)
Alva Verdial B	AlVer (BI)	Diagalves B	Diag (A)	Monvedro N	Monv (D)
Alvarelhão N	Alvalh (D)	Douradinha B	Dour (D)	Moreto N	More (A)
Antão Vaz B	AVaz (A)	Encruzado B	Encru (D)	Negra Mole N	NMol (A)
Aragonez N	Arag (A)	Fernão Pires B	FPire (A)	Perrum B	Perr (A)
Arinto do Interior B	AriDou (D)	Ferral R	Ferral (BI)	Rufete N	Rufe (BI)
Aveso B	Aves (D)	Folgasão roxo R	Folg (BI)	Samarrinho B	Samarr (D)
Barcelo B	Barc (D)	Formosa B	Form (A)	Seara Nova B	SNov (A)
Bastardo N	Basta (D)	Gouveio B	Gouv (D)	Siria B	Siria (BI)
Bastardo N	BastT (BI)	Grossa N	Gros (A)	Tamarez B	Tamar (D)
Bical B	Bical (D)	Jaen N	Jaen (D)	Terrantez B	Terra (D)
Boal Ratinho B	BRati (BI)	Jampal B	Jamp (BI)	Tinta Caiada N	TCai (A)
Camarate N	Camar (D)	Larião B	Laria (A)	Touriga Franca N	TFran (BI)
Castelão N	Cast (A)	Luzídio B	Luzi (D)	Touriga Nacional N	TNac (D)
Cerceal Branco B	CercB (D)	Malvasia Fina B	MFina (D)	Tourigo do Douro	TDou (BI)
Cidreiro N	Cidre (D)	Malvasia Rei B	MRei (A)	Trincadeira das Pratas B	TPratas (A)
Coração de Galo N	CorGa (D)	Manteúdo B	MantB (A)	Trincadeira N	TriN (A)
Cornifeito N	Cornife (D)	Manteúdo Preto N	MantT (A)	Uva Cão B	UCao (D)
Corropio N	Corr (A)	Marufo N	Maruf (BI)	Uva Cavaco B	UCav (BI)

## 2. VARIETY IDENTIFICATION

Genetics is a tool for the identification of grapevine varieties and can be a tool for germplasm collection management.

The process of vegetative propagation used to multiply grapevine varieties produces, in most cases, clones genetically identical to the parental plant. Nevertheless, spontaneous somatic mutations can occur in the regenerative cells that give rise to the clones, leading to consider varieties as populations of clones that conform to a panel of phenotypic traits. Currently, the SSRs molecular markers are used to identify varieties and the SNPs began to be used.

On specific conditions, we can also identify some clones or clusters of clones in the same variety.

The use of microsatellites to identify varieties is so important that the International Organization to Vine and Wine (OIV) named six microsatellites to identify the *Vitis vinifera* ssp. *vinifera*.

In Portugal, the 313 grapevine cultivars officially authorized for wine production (Portaria nº 428/2000, Diário da República nº 163, 17th July 2000) and existing in the Portuguese Ampelographic Collection, at INIA-Dois Portos, were characterized with these six nuclear microsatellites (VVMD5, VVMD7, VVMD27, VrZAG62, VrZAG79 and VVS2). These microsatellites loci were found to be highly informative and therefore constitute a useful marker set for the discrimination of the targeted cultivars. This study allowed the detection of 244 distinct genotypes as well as the identification of synonyms in 40 cultivars. The remaining 29 cultivars are distributed in 11 distinct sets, where each set displays identical SSRs profiles, however, these should be considered as distinct cultivars considering that these differ in berry colour. Taken together, the results of this work will contribute to improve the management of the Portuguese Grapevine National Collection as well as provide good technical support for the updating of the list of cultivars officially authorized for wine production in Portugal (Portaria nº 428/2000). Furthermore, it will also be useful for the control of plant material in the future (**Veloso et al., 2010**).

The use of SSR markers well scattered across the 19 grape chromosomes is very important to have a real representation of the genome variability.

**Laucou et al., (2011)** analyzed 4 370 accessions of the INRA grape repository at Vassal, mostly cultivars of *Vitis vinifera* subsp. *vinifera* (3 727), but also accessions of *V. vinifera* subsp. *sylvestris* (80), interspecific hybrids (364), and rootstocks (199). The analysis

revealed 2 836 SSR single profiles: 2 323 *vinifera* cultivars, 72 wild individuals (*sylvestris*), 306 interspecific hybrids, and 135 rootstocks, corresponding to 2 739 different cultivars in all.

The mean genetic diversity (GDI) on the INRA grape repository was 0,797 and the level of heterozygosity was 0,76. Interspecific hybrids and rootstocks were more heterozygous and more diverse (GDI = 0.839 and 0.865, respectively) than *V. vinifera* cultivars (GDI = 0.769), *Vitis vinifera* subsp. *sylvestris* being the least divergent with GDI = 0.708.

Slight clonal polymorphism was detected. The limit between clonal variation and cultivar polymorphism was set at four allelic differences out of 40.

The discrimination between some cultivars and clones was possible with the twenty SSR markers used on this study. The molecular differences observed in this analysis were homozygote versus heterozygote differences (*eg* Grolleau noir is 360/360 whereas Grolleau gris is 360/371 at the VVIV67 locus) or size shifts in 1 allele (Pinot noir is 135/149 whereas Meunier is 126/135 at the VVS2 locus; Chasselas blanc is 163/180 whereas Chasselas Muscat is 180/192 at the VVIP31 locus; Carignan noir is 315/324 whereas Carignan blanc is 315/326 at the VVIP60 locus).

These Authors also propose the use of a set of nine SSR markers, that distinguish 99.8% of the analyzed accessions, as suitable for routine characterization and will be valuable for germplasm management. These nine SSR markers are VVMD5, VVMD27, VVMD7, VVMD25, VVIh54, VVIP60, VVIN16, VVIb01, VVIq52. However, these nine SSR markers are different from the nine SSR markers used by GRAPEGEN06 project: VVMD5, VVMD7, VVMD25, VVMD27, VVMD28, VVMD32, VVS2, VrZAG62, VrZAG79, that are used to establish the European *Vitis* Database.

Nevertheless, **Myles *et al.* (2011)** developed a 9K genotyping SNP array on *Vitis* species, which could be used to rapidly genotype thousands of cultivars at a lower cost.

Although SSR markers are appropriate for cultivar identification, considering the low level of diversity between clones revealed with this technique (2% of the plants, 138 alleles concerned), SSR markers are definitely not well suited to clonal identification, as already mentioned (Imazio *et al.*, 2002; Cipriani *et al.*, 2010). Clonal variation associated with color differences can also be differentiated using the *VvMybA1* gene, a transcription factor involved in the qualitative and quantitative control of color (This *et al.*, 2007), as recently carried out on a few dozen cultivars (Giannetto *et al.*, 2008). Similarly, genes involved in the aroma traits (Duchêne *et al.*, 2009) could also be used to distinguish aromatic mutants. Other methods based on transposable elements (Pelsy, 2007), DNA methylation

(Schellenbaum *et al.*, 2008), copy number variation (CNV), grape genome sequencing (French–Italian Public Consortium, 2007), or next generation sequencing (NGS) could also be used.

**Pelsy *et al.* (2010)**, studied the clonal variability within seven French wine grape variety collections: ‘Cabernet Franc’, ‘Cabernet Sauvignon’, ‘Chenin’, ‘Grolleau’, ‘Pinot Noir’, ‘Riesling’, ‘Savagnins’; using SSRs and comparing a total number of 344 accessions of certified clones and introductions preserved in French repositories. Ten accessions resulted in being either selfprogeny, possible offspring of the expected variety or misclassified varieties. Out of the 334 remaining accessions, 83 displayed genotypes different from the varietal reference, i.e., the microsatellite profile shared by the larger number of accessions. They showed a similarity value ranging from 0.923 to 0.992, and thus were considered as polymorphic monozygotic clones. The fraction of polymorphic clones ranged from 2 to 75% depending on the variety and the set of markers, the widest clonal diversity being observed within the Savagnin. Among the 83 polymorphic clones, 29 had unique genotype making them distinguishable; others were classified in 21 groups sharing the same genotype. All microsatellite markers were not equally efficient to show diversity within clone collections and a standard set of five microsatellite markers (VMC3a9, VMC5g7, VVS2, VVMD30, and VVMD 32) relevant to reveal clonal polymorphism is proposed.

## 2.1 Core Collections

Genetics can be a tool for the establishment of *Vitis vinifera* core collections.

The usefulness of core collections is due to their ability to capture the diversity of the whole species.

The first high quality draft of the grape genome sequence has already been published (**French–Italian Public Consortium, 2007**). This is a critical step in accessing all the genes of this species and increases the chances of exploiting the natural genetic diversity through association genetics. However, the basic knowledge of the extent of allelic variation within the species is still not sufficient. Towards this goal, **Le Cunff et al. (2008)** propose the constitution of a nested genetic core collections (G-cores) with the aim of to capture the simple sequence repeat (SSR) diversity of the cultivated grape (*Vitis vinifera* L. subsp. *vinifera*) from the world's largest germplasm collection (Domaine de Vassal, INRA Hérault, France), containing 2262 unique genotypes.

They selected sub-samples of 12, 24, 48 and 92 varieties of *V. vinifera* L., based on their genotypes for 20 SSR markers using the M-strategy, and they verify that these samples represent respectively 58%, 73%, 83% and 100% of total SSR diversity.

They conclude that the final G-48 core is highly non-redundant and highly diverse.

When they built the G-48 core, and considering the rapid evolution of SSR markers, they assumed that the alleles present in two or less cultivars in the collection did not adequately represent gene diversity and they were thus removed. Only one additional SNP was revealed in the G-92 sample compared to the G-48. On one hand, the gain in the unlinked diversity was high in the G-48, probably due to the decrease in redundancy compared to the Vassal collection. On the other hand, when compared to a random sampling, the gain was much higher using the M-method.

### 3. GENETIC EXPRESSION

The obtaining of the grape genome sequence by the **French–Italian Public Consortium (2007)** is a major step to the knowledge of the genetic activity responsible for the phenotypic response of varieties in different situations of culture. For example, gene expression against biotic and abiotic stress, synthesis and accumulation of anthocyanins, sugars, flavors, etc.

In what concerns the biosynthetic pathway of anthocyanins, Sparvoli *et al.* (1994) characterized in grapevine, the genes coding enzymes involved in the biosynthetic pathway of anthocyanins: phenylalanine ammonia lyase [PAL], chalcone synthase [CHS], chalcone isomerase [CHI], flavanone-3-hydroxylase [F3H], dihydroflavonol 4-reductase [DFR], leucoanthocyanidin dioxygenase [LDOX], and UDP glucose-flavonoid 3-o-glucosyl transferase [UGT].

**Boss *et al.* (1996)** determined the expression of these seven genes of the anthocyanin biosynthetic pathway and they verified that the onset of anthocyanin synthesis in ripening grape berry skins coincides with a coordinated increase in expression of a number of genes in the anthocyanin biosynthetic pathway, suggesting the involvement of regulatory genes.

Using the GeneChip Affymetrix for *Vitis vinifera* L, **Cardoso (2011)** compared two clones with high total skin anthocyanins concentration and two clones with low total skin anthocyanins concentration from Aragonez cultivar. 24 genes, 2 genes encoding flavonoid metabolism enzyme and 22 transcription factors comprising members of the *Myb*, *Myc*, zinc finger, homeodomain and WRKY families, have variation on gene expression level.

With these results, candidate genes for association mapping can be selected and correlated with the most important candidate genes, we can select molecular markers and use them for assisted breeding.

The development of genetic expression studies also led to the emergence of genechip expression arrays provide the most exhaustive coverage of model systems genomes, including *Vitis*, mainly after *Vitis* genome sequence became available in 2007. Assuming that real biology happens at the transcript level, though transcriptional profiling is the most convenient approach to focus on responsive transcriptional states of cells, tissues and organs, namely to study the regulatory pathways triggered in *Vitis* leaves by abiotic stresses.

In Portugal, the abiotic stress gene expression signature of wine making grapevine varieties through microarray transcriptomic analysis of the leaves under realistic multiple stress conditions are being studied.

Plants under normal environmental conditions are exposed to multiple abiotic stresses due to large fluctuations in temperature, irradiance and water availability. Nevertheless, experimental research on the effects of abiotic stress over plant physiology and production has mainly focused individual stresses. Field observations reproduce the effects of simultaneous occurrence of multiple stresses, integrate their variation in extent and intensity and indicate the acclimation responses. The response of plants to multiple abiotic stresses is not predictable by single factor analyses because its simultaneous occurrence may lead to synergistic or antagonistic effects. Transcriptomic responses to multistress conditions can be unique and cannot be extrapolated from the response to each one applied individually.

The aim is understand the functional genomics of grapevine leaves under field multiple stress conditions: drought, high temperature and high irradiance. This objective can be attained (1) assessing the expression of grapevine genes by microarray analysis 23K Affymetrix Genechip 23K (corresponding to 30% of the genome, custom made to J.M. Zapater) in leaf samples from plants under multiple stresses (MSs) in realistic field conditions and plants in greenhouse under controlled individual abiotic stresses (ISs) – water shortage (WS) or high temperature (HS) or excessive light (LS) or combination of the ISs; (2) validating the expression of genes significantly responsive to ISs or MSs by relative quantification through quantitative real-time (qRT) RT-PCR after genomewide identification of genes with stable expression to be used as reference genes; (3) to predict promoter sequences of responsive genes; (4) designing a gene array with a low number of high specific sequences to test grapevine varieties for the response to multiple environmental stresses.

#### **4. ASSISTANT BREEDING**

The creation of new varieties of vine is obtained by means of sexual enhancement.

These studies usually aim at obtaining new varieties with characteristics adapted to market needs, whether in terms of earliness for table grapes, of the levels of anthocyanins in red grapevine varieties, or of specific flavours for white grapevine varieties.

The selection of these varieties is time consuming because of the waiting time for production, sometimes in lasting long in order to make the necessary assessments.

This whole process can be shortened using the determination of molecular markers for the traits under study.

We are faced with the assistant breeding or improvement seen with molecular markers.

The anthocyanin content and the regulation of anthocyanin biosynthesis is a trait of major interest in *Vitis vinifera* L. Anthocyanin accumulation in berry skin determines their colour and other organoleptic characteristics of grapes and wine, and has an important beneficial effect on human health.

Different authors have studied the genes interfering in the control of the total skin anthocyanin and on specific types of anthocyanins.

**Silvana Cardoso (2011)** found three SNPs associated with total skin anthocyanin concentration. The SNP s36 from *MYC<sub>B</sub>*, the SNP s68 from *MYBCC* and the SNP s90 from *MYB11*, three genes coding transcription factors. This study was carrying out on 149 red and rose cultivars from the INIA grape repository at Dois Portos.

## ACKNOWLEDGMENTS.

We thank to

Pedro Fevereiro (ITQB), Silvana Cardoso (ITQB), Jorge Cunha (INRB) for comments on drafts of the manuscript.

Sara Amâncio, Margarida Rocheta, Luisa Carvalho, Lucas Coito, Manuela Chaves (CBAA, Instituto Superior de Agronomia, Lisboa), research team to the project VITMULTISTRESS - Gene expression in grapevine (*Vitis vinifera* L) under combined abiotic stress.

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