Vitis vinifera ssp. *sylvestris* (Gmeli) Hegi Populations in Southern Portugal: Assessing the Genetic Diversity for its Future Management and Conservation

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Abstract: A survey of *Vitis vinifera* ssp. *sylvestris* (Gmeli) Hegi in Portugal has revealed the existence of wild-vine populations that occur only in riparian wood habitats on river margins, as is the case for other European populations. The genetic diversity of four populations has been evaluated using nuclear and chloropastidial microsatellites as molecular markers. An analysis of molecular variance (AMOVA), showed that most of the genetic diversity was attributable to differences among individuals within populations. Only 7% of the total variance was attributable among populations; suggesting the existence of a low level of population differentiation. Chloroplastidial microsatellites revealed the expected situation for the Iberian Peninsula, (i.e. the presence of only chlorotypes A and B; with chlorotype A as the most frequent within the wild-vine populations). The diversity obtained is a starting point for the management and conservation of wild-vines *in situ* and *ex situ*. Several measurements have to be taken to maintain their natural habitat, and in order to preserve its diversity.

Keywords: genetic diversity; management and conservation; nuclear and chloroplastidial microsatellites; *Vitis vinifera* ssp. *sylvestris*

A survey of *Vitis vinifera* ssp. *sylvestris* (Gmeli) Hegi in Portugal has revealed the existence of wild-vine populations, only in riparian wood habitats on river margins, as is the case of the other European populations (Arnold *et al*. 1998). All of these populations are located in the Tagus River basin and in the Alentejo region (Cunha *et al.* 2004). A selection of four of these wild-vine populations, in four distinct hydrological basins, has been characterised morphologically (Cunha *et al*. 2007, 2009), as well as its sanitary status assessed (Santos *et al.* 2003). The aim of this work was to measure the present genetic diversity of these populations, using nuclear and chloroplastidial microsatellites as molecular markers.

Young leaves from fifty-three plants (male and female) were collected in the wild-vine populations near Alcácer do Sal (12), Castelo Branco (11), Montemor-o-Novo (22) and Portel (8). In order to obtain the maximum variability and no redundant genotypes, sampling was done by choosing the most morphologically different plants. The DNA was extracted following the protocol from Thomas *et al.* (1993). The primers sequences, and the protocol used for DNA analysis with six nuclear microsatellites, were described in Almadanim *et al.* (2007); the protocol for the four chloroplastidial microsatellites was described in Cunha *et al.* (2009). PowerMarker v3.23 software (Liu 2002) was used to calculate the average number of alleles

per locus (Na), the observed heterozygosity (Ho), the expected heterozygosity or gene diversity (He), and the Polymorphism Information Content (PIC) for each nuclear microsatellite locus.

An analysis of molecular variance (AMOVA) was used to determine the distribution of genetic variation, both within and among populations within a region, using GENALEX software (Peakall & Smouse 2006). The significance of the fixation index (Fst) was tested non-parametrically by 1000 permutations.

A total of fifty three alleles were observed across the six nuclear markers in the wild-vine samples. The number of alleles per locus ranged from seven (VRZag 62) to eleven (VVS2), with a mean value of 8.8 alleles per locus. For all loci, most alleles varied in steps over two nucleotides; only loci VVS2 showed alleles that differed from the others with two nucleotides. The allelic frequencies for each locus were generally high (over two, three, or four alleles), with an overall frequency value greater than 0.30. Allelic frequency values ranged from 0.006 (alleles present in single plants) to 0.750. Overall observed heterozygosity values per marker ranged from 0.509 to 0.736, with a mean value of 0.656. The expected heterozygosity values were similar as the observed heterozygosity values; ranging from 0.588 to 0.801, with a mean value of 0.676. All nuclear microsatellite loci scores in this study were polymorphic, displaying values of PIC from 0.569 to 0.778.

The AMOVA analyses (Table 1) of the genetic distances between populations, defined by geographic hydrological basins, indicated that 93% of the genetic variation was attributable to differences among individuals within populations, with only 7% of the total variance being attributeable among populations; suggesting the existence of a low level of population differentiation. The fixation index, Fst, (also known as Wright's F-statistics) measures the decline in heterozygosity due to subdivision within a population. The fixation index ranges from 0 (indicating no differentiation between the overall population and its subpopulations) to a theoretical maximum of 1; although in practice, the observed fixation index is much less than 1, even in highly differentiated populations. The Fst index for *Vitis vinifera* ssp. *sylvestris* populations, based on hydrological basins of permutation in the AMOVA, was estimated at 0.071. The low Fst index for ssp. *sylvestris* suggests that heterozygosity may be highly maintained, primarily through random crosses.

The percentage of variance within the populations (93%), shows that the method followed for collecting the samples assures the maximum variability; and it is to be used when collecting samples in new surveys, or for selecting *ex situ* cuttings collections.

The chloroplastidial microsatellites revealed the expected situation for the Iberian Peninsula (i.e. the presence of only chlorotypes A and B; with chlorotype A as the most frequent (66%) within the wild-vine populations). The distribution of chlorotypes in the four Southern Portuguese populations is heterogeneous. The Montemor-o-Novo population has only chlorotype A. Alcácer do Sal, Castelo Branco and Portel populations have both chlorotypes, but with different distributions of chlorotype B of 91.6%, 18%, and 62.5%, respectively.

The diversity obtained is a starting point for the management and conservation of wild-vines' *in situ* and *ex situ* conservation. Several measurements must be taken for the management of the natural habitat, and to preserve its diversity. The present overall diversity can be safeguarded in an *ex situ* collection already underway at Quinta da

Table1. Summary of AMOVA of *Vitis vinifera* ssp. *sylvestris* on four distinct hydrological basins from Southern Portugal

Variance component	Degrees of freedom	Sum of squares	Variance components	Percentage of variation
Among populations	3	17.03	0.15	7.00
Within populations	102	198.05	1.94	93.00
Total	105	215.08	2.09	
Fixation index (Fst)		0.071 ($P < 0.001$)		

Almoinha, Dois Portos (PRT051). For the *in situ* conservation, guidelines should be established for the maintenance of small river margins, in order to prevent loss of diversity due to either natural hazards or human interventions.

Aknowledgements. This work was supported by a FCT-PARIPIPI-Project A from Portugal and GrapeGen06 EU Project. J. Cunha is supported by a Ph.D. grant (SFRH/BD/16226/2004) from Portugal.

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