ONLINE RESOURCES

Genetic variability in *Sambucus nigra* L. clones : a preliminary molecular approach

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Introduction

Sambucus nigra L. is an economically important species that has been widely used in different industries: medicine, pharmacology and cosmetics, among others (Valles *et al.* 2004). In Portugal, producers from the Varosa Valley (a region were *S. nigra* is an important crop species) are interested in increasing the production and quality of elderberries. According to the Agricultural Cooperative of Varosa Valley (Tarouca, Portugal), its clonal field of *S. nigra* resulted from stems of different mother-trees harvested in distinct geographical regions in the north of Portugal, where it has been cultivated by local producers. Previous morphological, yield and production evaluations revealed that clones from the Ucanha region showed higher yield and production rates of elderberries (Braga *et al*. 2002). Additionally, these clones also present different morphological characters compared to others.

The goals of the present study were to perform a first and preliminary molecular characterization of 30 *S. nigra* clones from different mother-trees and distinct regions of the Varosa Valley using three molecular marker systems: inter-simple sequence repeat (ISSR), internal transcribed spacer (ITS) polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP) and simple sequence repeat (SSR).

Materials and methods

Plant material and extraction of genomic DNA

In the present study, we used foliar samples of 30 clones (table 1) collected in the clonal field of the Agriculture Cooperative of Varosa Valley (Tarouca, Portugal).

This clonal field arose from cutting propagation using stems of different mother-plants that were collected in five regions of the Varosa Valley: Cimbres (C), Dalvares (D), Ferreirim (F), Salzedas (S) and Ucanha (U) (table 1). Each mother-plant gave rise to 14 clones that constitute a section within the clonal field (table 1).

The foliar samples were immediately frozen in liquid nitrogen after their collection, and further stored at −80°C until genomic DNA extraction. The isolation of nuclear DNA was performed with the NucleoSpin® Plant II kit (Macherey-Nagel, Germany), following the manufacturer's instructions.

Amplification of ISSR regions

For ISSR amplifications, we tested 11 primers from the set 100/9 (University of British Columbia): 807 [$(AG)_8$T]; 808 [$(AG)_8$C]; 810 [$(GA)_8$T]; 811 [$(GA)_8$C]; 812 [$(GA)_8$A]; 818 [$(CA)_8$G]; 823 [$(TC)_8$C]; 826 [$(AC)_8$C]; 834 [$(AG)_8$YT]; 841 [$(GA)_8$YC] and 880 [$(GGAGA)_3$], being Y = C, T. The amplification conditions and result detection were performed as described by Carvalho *et al*. (2005).

ITS PCR-RFLP technique

The ITS1-5.8S-ITS2 rDNA region was amplified using the primers ITS-4 (5′-TCCTCCGCTTATTGATATGC-3′) and the modification of the ITS1 (White *et al*. 1990), (5′-GTCCACTGAACCTTATCATTTAG-3′) (see Urbatsch *et al*. 2000).

The amplification conditions and result detection were as described by Carvalho *et al*. (2009a). The ITS amplified products were digested with seven restriction endonucleases: *Alu*I, *Taq*I, *Hpa*II, *Msp*I, *Eco*RI, *Sau*I96 and *Rsa*I, according to the manufacturer's instructions (Fermentas, Opelstrasse,

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Table 1. *Sambucus nigra* clones used in the present study and geographical region of their mother plants.

*Clone code	Geographical region of their mother plants
C1:2, C1:3, C1:4, C2:2, C2:3, C2:4	Cimbres (Armamar)
D1:2, D2:2, D3:3	Dalvares (Tarouca)
F1:1, F1:2, F1:4, F2:2, F2:3, F2:4	Ferreirim (Lamego)
S1:2, S1:3, S2:2, S2:3, S2:4	Salzedas (Tarouca)
U1:2, U1:3, U1:4, U2:2, U2:4, U3:2, U3:3, U3:4, U4:4, U4:6	Ucanha (Tarouca)

*Clone code: letter, initial of the geographical region of the mother plant; first number, section number in the clonal field; second number, number of the clone.

Germany). The ITS PCR-RFLP products were visualized after electrophoresis on 2% agarose gels stained with ethidium bromide.

Amplification of SSR regions

To amplify the SSR regions, we tested eight SSR primers that were specifically developed for *S. nigra*: Sn002; Sn003; Sn010; Sn016; Sn017; Sn019; Sn023 and Sn025 (see Clarke and Tobutt 2006). Each SSR amplification reaction (final volume of 12.5 μL) was constituted by: 10 ng/μL of genomic DNA, 25 pmol of each primer, 1.5 mM $MgCl_2$ (Fermentas), 1× $(NH4)_2SO_4$ reaction buffer (Fermentas), and 0.3 units of *Taq* DNA polymerase (Fermentas).

The SSR amplifications occurred on a T-Professional thermocycler (Biometra, Goettingen, Germany) under the

Table 2. Molecular data produced among the 30 clones by ISSR, ITS PCR-RFLP and SSR.

Molecular marker	Primer	T_a (°C)	NAB	NPB	%*P*
ISSR	807	45	5	2	40.00
	808	47	5	2	40.00
	810	45	10	3	30.00
	811	47	12	8	66.67
	812	45	11	5	45.45
	818	47	8	8	100
	823	47	7	5	71.43
	826	47	9	7	77.78
	834	48	10	9	90.00
	841	50	8	5	62.50
	880	45	12	6	50.00
Total			97	60	61.86%
ITS PCR-RFLP	Enzyme		Pattern	Monomorphic bands (bp)	Polymorphic bands (bp)
	*Hpa*II		*A*	750, 550, 220	None
			B	750, 550, 220	600
	*Msp*I		*C*	750, 550, 220	None
			D	750, 550, 220	600
	*Sau*I96		*E*	600, 200	None
			F	600, 200	750
	*Taq*I		*G*	275, 150, 100, 75, 50	None
			H	275, 150, 100, 75, 50	350
			I	275, 150, 100, 75, 50	400, 350
			J	275, 150, 100, 75, 50	690, 400, 350
SSR	Pair of primers		NAB	NPB	%*P*
	Sn002		3	2	66.6
	Sn003		3	2	66.6
	Sn010		5	4	80
	Sn016		2	1	50
	Sn017		2	1	50
	Sn019		3	1	33.3
Total			18	11	61.1%

T_a, annealing temperature; NAB, number of amplified ISSR bands; NPB, number of polymorphic ISSR bands; %*P*, percentage of polymorphism.

following conditions: an initial denaturation at 94°C for 3 min, followed by 34 cycles of denaturation at 94°C for 30 s, annealing of the primers at 50°C for 90 s and extension at 72°C for 1 min. The final extension was performed at 72°C during 20 min.

The SSR amplified products were resolved after electrophoresis on 2% agarose gels prepared with UltraPure Agarose 1000 (Invitrogen, Barcelona, Spain), under a constant voltage of 90 V. The gels were further stained with ethidium bromide.

Statistical analysis

For the three marker systems used here, the amplification was repeated when a band polymorphic between

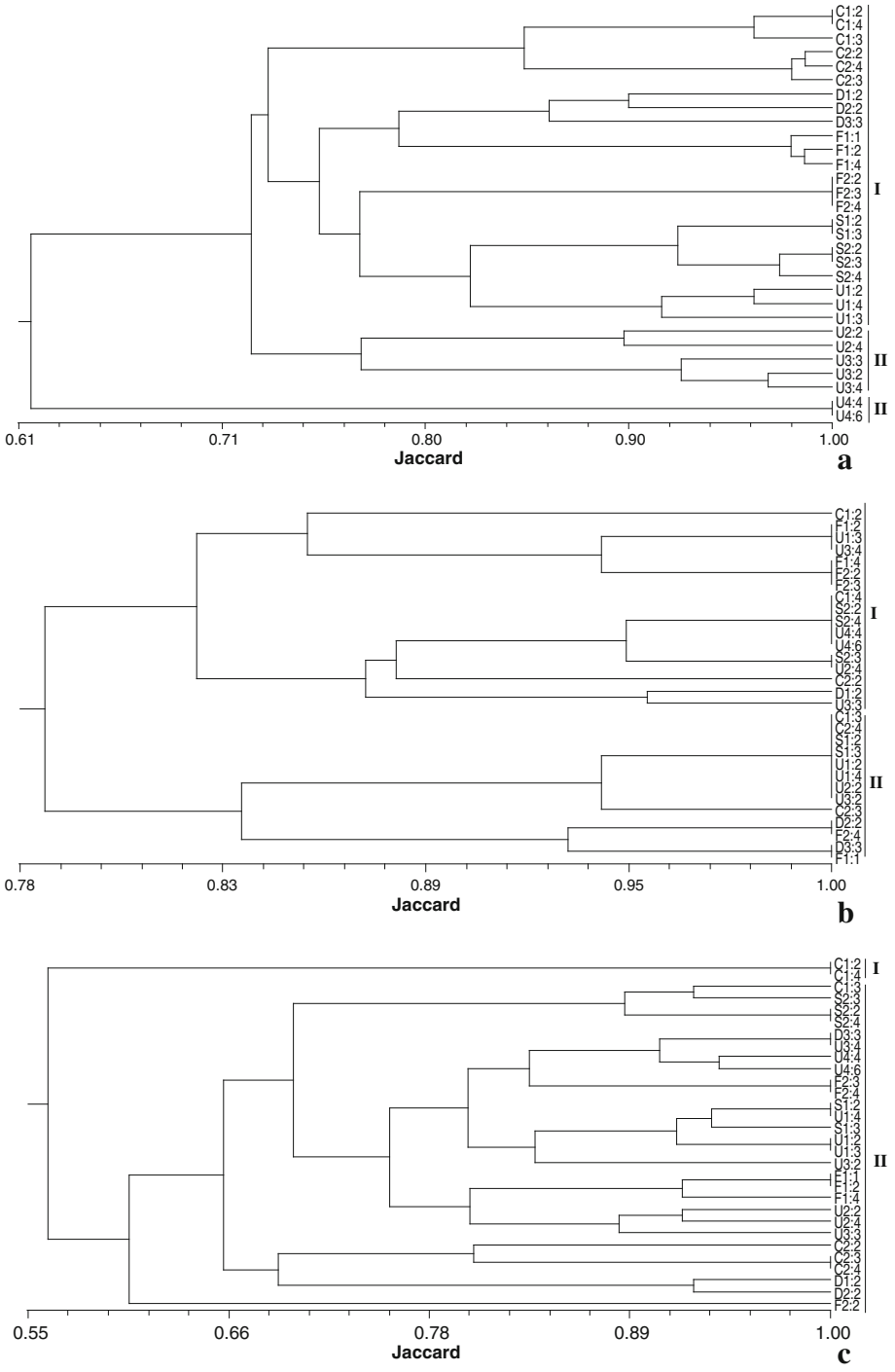

Figure 1. UPGMA dendrograms of genetic similarity among 30 *S. nigra* clones based on the pool data of: a) ISSRs; b) ITS PCR-RFLPs; and c) SSRs, using Jaccard's coefficient (Jaccard 1908).

clones was observed, first with the same DNA, and then with DNA from a different extraction from the same clone.

Despite the fact of being codominants, the ITS PCR-RFLP and SSR markers were analysed as dominant ISSRs, by a presence (1) / absence (0) analysis.

For each marker, the reactions were repeated several times, and only reproducible bands were considered for the construction of binary matrices. Those matrices were further used for the elaboration of unweighted pair group method with arithmetic mean (UPGMA) dendrograms of genetic similarity with the software NTSYS pc2.02 (Rohlf 1998), to evaluate the genetic relationships among 30 clones studied here. Principal coordinates analysis (PCoA) was performed using the software GenALEx 6 (Peakall and Smouse 2006).

Results and discussion

All molecular marker systems revealed genetic variation among the 30 clones evaluated (table 2). Despite the fact of being clones, considerable total mean values of polymorphism were assessed with each molecular marker system (table 2). ISSRs presented the highest percentage of polymorphism (61.86%), followed by the SSRs (61.1%) and the

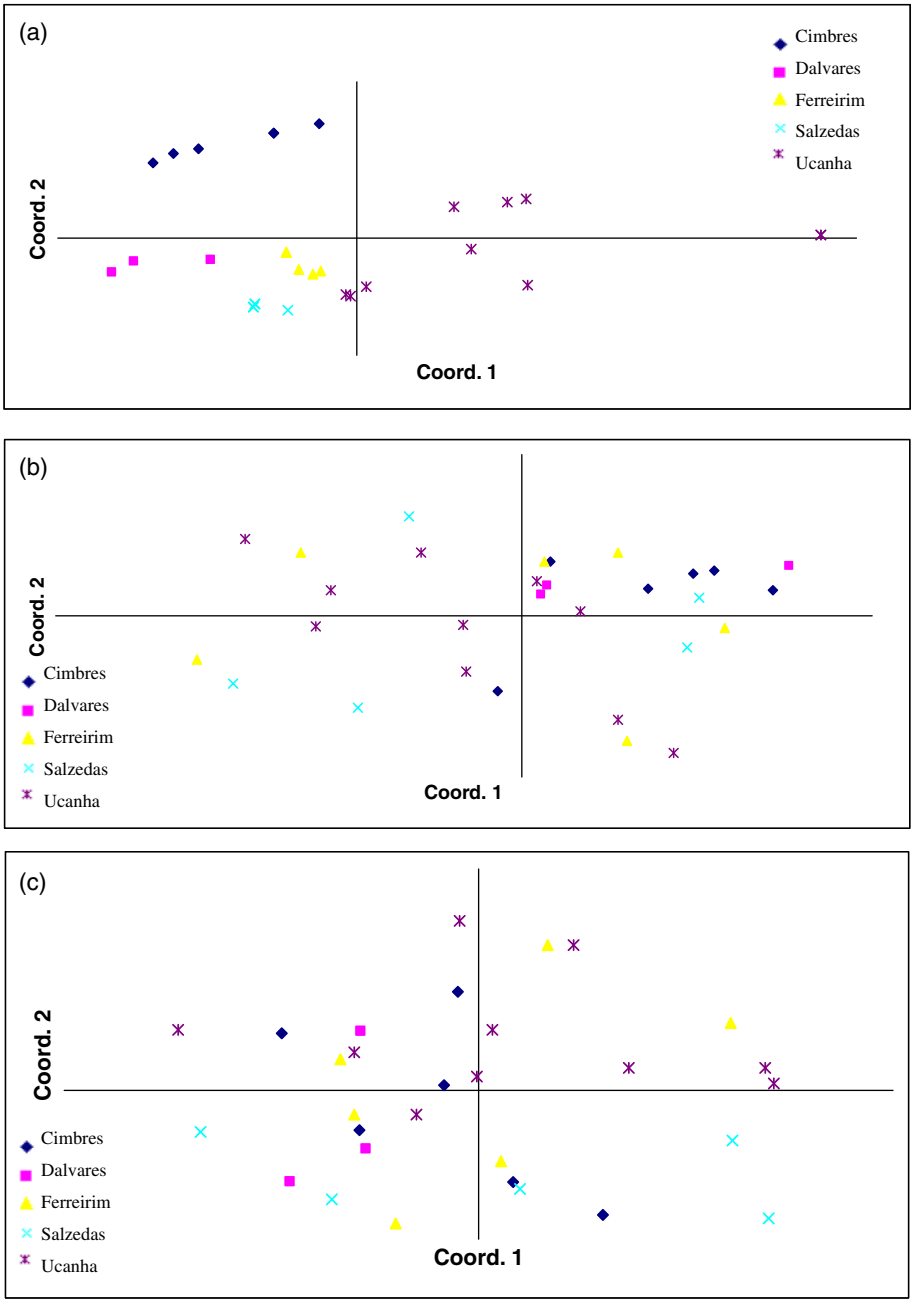

Figure 2. Principal coordinates analysis based on the: a) ISSR; b) ITS PCR-RFL P; and c) SSR data.

ITS PCR-RFLPs (50%) (table 2). The high percentage of polymorphism assessed by the ISSR and SSR markers could be due to the highly repetitive DNA that constitutes these genome regions, which are prompt to spontaneous mutations and fast evolution rates. On other hand, the genetic variation observed among the clones could be a consequence of the intuitive selection of the farmers performed in their mother plants during years in this restricted geographical area.

The lower percentage of polymorphism revealed by the ITS PCR-RFL markers could be explained by the high rate of concerted evolution within the ITS rDNA region which provide the homogenization of their sequences and number of tandem repeats within each individual genome. Each enzyme produced two to four ITS PCR-RFLP patterns among the 30 clones, characterized by two to five monomorphic bands and the absence or presence of one to three additional polymorphic bands (table 2). Except for *Taq*I, the sum of the monomorphic bands produced by each enzyme exceeded the length of 750 bp, indicating the presence of different ITS types per individual genome. The ITS variants detected here reflected intraindividual ITS variation. As far as we know, this is the first study reporting ITS length variants in *S. nigra*.

Despite the use of specific SSR primers for *S. nigra*, only six of them produced amplification and allowed the detection of polymorphism among the 30 clones studied here (table 2). The highest numbers of amplified and polymorphic SSR bands were achieved by the primer Sn010 (table 2), similarly to what was reported by Clarke and Tobutt (2006). However, these authors obtained a higher number of alleles per primer, probably because they used a different methodology, including labelled forward primers and capillary electrophoresis for the detection of results, and different accessions of *S. nigra*.

In general, all molecular marker systems used here reflected and confirmed the occurrence of genetic variation among clones belonging to the same and to different field sections (data not shown). To evaluate the genetic relationships among 30 clones, we constructed three UPGMA dendrograms based on the molecular data achieved with each marker system (figure 1).

Only the ISSR markers clustered the clones by section and geographical region (figure 1a). These markers already proved to be specific enough for DNA fingerprinting (Carvalho *et al*. 2005), and establishment of taxonomic relationships (Carvalho *et al*. 2009b) in different plant genera. Besides, they have been successful in the detection of clonal variation in other vegetatively propagated species (Gemas *et al*. 2002; Moncada *et al*. 2006).

Although the ITS PCR-RFLP (figure 1b) and the SSR markers (figure 1c) failed to cluster the clones by section or geographical region, nuclear ITS sequences were previously useful in the establishment of phylogenetic relationships among different *Sambucus* species (Eriksson and Donoghue 1997), and DNA fingerprinting of *Sambucus* interspecific hybrids (Simonovik *et al*. 2007).

To confirm the clustering method used for the dendrograms construction, we performed PCoAs for each marker system (figure 2). The PCoAs revealed that only ISSRs grouped together the clones from the same geographical region (figure 2a). The ITS PCR-RFLP and SSR markers were both chaotic in the clustering of clones by this criterion (figure 2, b&c). Regarding the percentage of total variation explained by the first three axes, we noticed that the ISSRs explained the highest value (73.91%), followed by the ITS PCR-RFLP (62.79%) and the SSR markers (62.23%).

Our molecular and statistical data indicated that the ISSRs are the recommended markers for future studies in *S. nigra* clones. However, the further molecular characterizations should include a higher number of clones from each section and region, and will aim the establishment of correlations among specific ISSR bands and production characteristics.

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