Scientific Papers. Series B, Horticulture. Vol. LXVII, No. 1, 2023
Print ISSN 2285-5653, CD-ROM ISSN 2285-5661, Online ISSN 2286-1580, ISSN-L 2285-5653

MORPHOMETRIC ANALYSIS AND ASSESSMENT OF GENETIC DIVERSITY OF WILLOW (*SALIX* SP.) GENOTYPES USING SCoT MOLECULAR MARKERS

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Abstract

In this study, eleven willow genotypes from local populations and foreign varieties grown in an experimental field were morphologically and genetically evaluated to establish their genetic diversity. The results of this study show that the local clonal selections Caracal 101-103 recorded the best results for most of the analysed morphological characters. Out of 25 SCoT primers tested for genetic diversity analysis, 12 primers generated 113 polymorphic bands. The lowest number (8) was obtained with primers SCoT1, SCoT3, SCoT21 and SCoT23. The highest number (13) of polymorphic bands was amplified with the primer SCoT13. The percentage of polymorphism ranged from 72.72% (SCoT 1 and SCoT 21) to 100.0% (SCoT 13) with a mean value of 83.69%. The UPGMA dendrogram grouped willow genotypes into two main clusters. The results of the cluster analysis were then confirmed by the PCoA analysis which explained 61.76 % of the total variation. In conclusion, this study provides valuable data regarding the genetic diversity of willow genotypes that can be selected and used in future breeding programs.

Key words*: local genotypes, cultivars, morphometry, DNA-markers, genetic diversity*

INTRODUCTION

The genus *Salix* L., is the largest genus in the Salicaceae family, with about 450 species distributed worldwide (Argus, 1997). Furthermore, in Europe *Salix* is considered the largest genus of the woody plant, with approximately 65 described species (Marinček et al., 2023). The high genetic diversity of willow species and their high capacity for vegetative propagation reveal that *Salix* species represent an important crop for woody biomass and renewable fuel production (Horn et al., 2011; Serapiglia et al., 2013; Rönnberg-Wästljung et al., 2022).

In addition to its role as a source of woody biomass, *Salix* sp. plays an important role in the phytoremediation process, contributing to carbon storage, the absorption of heavy metals from the soil, as well as flood mitigation (Weih & Nordh, 2002). Moreover, *Salix* sp. can grow on marginal or less favourable land, thereby reducing land use competition between food and energy crops. The willow trees contribute to the global reduction of natural pollution by presenting a positive CO_2 balance and diversifying the rural and urban landscape (Scordia et al., 2022; Gonzalez- Garcia et al., 2012; Karp et al., 2011). In Romania, as in other European countries, there are many natural hybrids of *Salix* sp., which can be selected and cultivated in Short Rotation Coppice (SRC) crops for biomass production (Botu et al., 2012a; Botu et al., 2010).

In this context, large-scale collection, morphometric evaluation and selection of valuable willow individuals from inter- and intraspecific hybridizations represented important classical breeding activities (Botu et al., 2012b; Botu et al., 2013). Nonetheless, the assessment of genetic diversity based on morphological traits is time-consuming and may be influenced by environmental conditions (Mullis & Falcona, 1987). Conversely, the evaluation of the genetic diversity of willow species by DNA-based molecular markers has been important for the development of non-conventional breeding strategies and their

effective implementation for the improvement of willow genotypes in less time (Barker et al., 1999; Daneshvand et al., 2015; Sulima et al., 2017). In this regard, several molecular markers have been used in *Salix* sp. to assess genetic diversity, such as: AFLP (Amplified Fragment Length Polymorphism) (Barker et al., 1999); DArT (Diversity Arrays Technology) (Przyborowski et al., 2013), RAPD (Random Amplification of Polymorphic DNA) (Lin et al., 1994; Sulima et al., 2009; Przyborowski & Sulima, 2010; Sharma et al., 2022), ISSR (Inter-Simple Sequence Repeat) (Van Puyvelde & Triest, 2007; Corneanu et al., 2016; Ghaidaminiharouni et al., 2017, Sharma et al., 2022) and SSR (Simple Sequence Repeat) markers (Barker et al., 2003; Singh et al., 2013).

In the recent past, SCoT markers have been employed in many commercially important and underutilized plant species for a variety of applications, including genetic diversity analysis, interspecific and intergeneric genetic relationships, cultivar identification, mapping analysis, and differential expression of the genes (Rai, 2023).

According to Collard & Mackill (2009), SCoT markers represent a simple, cost-effective, highly polymorphic, and reproducible molecular marker system. Their polymorphism is due to their ability to target the region flanking the start codon, which is considered a highly conserved region in plant genes (Collard & Mackill, 2009).

Thus, the aim of this study was to assess the genetic relationship of eleven willow genotypes using SCoT molecular markers and their morphological characteristics.

MATERIALS AND METHODS

Plant material and measurements of growth parameters

The plant material was represented by the following local clones named Caracal 101, Caracal 102, and Caracal 103 from *Salix* spp. (natural hybrids); Caracal 104 from *S. babylonica* and Caracal 105 from *S. m. tortuosa*, respectively. These clones were selected from an area of the lower course of the Olt River, from the south of Slatina to the confluence with the Danube River.

In addition, four international willow cultivars with complex genetic background such as: Inger (*S triandra* × *S. viminalis*), Terra nova [(*S. viminalis* × *S. triandra*) x *S. miyabeana*], Tora (*S. schwerinii* × *S. viminalis*) and Tordis [(*Salix schwerinii* × *S. viminalis)* × *S. viminalis*] and two other cultivars named Jorr (from *Salix viminalis*) and Lădești 1 (a clonal selection of *Salix* spp. from Romania) were used in this study for genetic diversity evaluation.

A field experiment was organized in Berindei locality (N44°6'44.385; E 24°20'46.714"). The experimental conditions were: brown forest-type soil with pH = 6.0 (0-30 cm) and an average value of recorded precipitation (1100mm/year) in the period 2019 - 2021.The willow individuals in the plots were unirrigated. The field trial was organized in a completed randomized block design (CRBD) with three replications, a single-row system and 15 individuals/ plot. Row spacing was 1.5 m and 1.0 m between individuals/row.

In the second year after planting, the following morphological characteristics were measured in 15 individuals of each genotype: the average number of shoots per plant, the average height of the shoots (cm), and the average diameter of the shoots (mm). To analyse the leaf characteristics of each genotype, 10 leaves/ individual were randomly collected and measured using a portable leaf area meter (LI-3100C, LI-CORGmbH, Germany). The following biometric parameters were recorded: length (cm), width (cm), perimeter (cm), and leaf area (cm^2). All analysed characteristics were subsequently processed as mean values.

Genetic analysis using SCoT markers

The collected leaves from each genotype were dried, ground into a fine powder (TissueLyser II, Qiagen, Germany) and kept at 4°C until the genetic analyses were carried out.

Total genomic DNA was isolated from 0.1 g of dried powder using a protocol based on the CTAB (cetyltrime-thylammonium bromide) method as published by (Lodhi et al., 1994) and improved by (Pop et al., 2003) and (Bodea et al., 2016).

The DNA purity and concentration were determined with a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific,

Waltham, MA, USA). Prior to performing the PCR (polymerase chain reaction) reactions, all DNA samples were diluted to 50 ng/µL, using sterile double distilled water.

For the SCoT analysis, the PCR amplification reactions were performed using the protocol described by (Collard & Mackill, 2009). The reaction mixture (a total volume of 15 µL) consisted of 50 ng/ µL of gDNA, distilled H_2O for the PCR reactions, 5X GoTaq Flexi Green buffer (Promega, Madison, WA, USA), 1.5 mM $MgCl_2$ (Promega, Madison, WA, USA), 0.2 mM of dNTP mix (Promega, Madison, WA, USA), 1 µM SCoT primer (GeneriBiotech, Hradec Králové, Czechia), and 1U of GoTaq polymerase (Promega, Madison, WA, USA).

The PCR temperature cycling conditions were: (a) 1 cycle of 5 min at 94°C for initial denaturation, (b) 35 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min and elongation at 72°C for 2 min, and (c) the final elongation step of 7 min at 72°C.

The PCR amplifications were repeated twice for each SCoT primer to ensure the reproducibility of the results. Separation of the PCR amplified products was carried out by electrophoresis on 1.4% agarose gels (Promega, Madison, WA, USA) stained with RedSafeTM Nucleic Acid staining solution (iNtRON Biotech, Seoul, South Korea) in 1X TBE (Tris Borate-EDTA buffer), at 110 V and 136 mA for 2.5-3 h. The electrophoretic profiles were visualized under UV (ultraviolet) in UVP Biospectrum AC Imaging System (Upland, CA, USA). The list of SCoT primers used in this study is shown in Table 1.

Table 1. The list of SCoT primers used

Primer name	The 3'-5'nucleotide sequence of the primer
SCoT 1	CAACAATGGCTACCACCA
SCoT 2	CAACAATGGCTACCACCC
SCoT 3	CAACAATGGCTACCACCG
SCoT 5	CAACAATGGCTACCACGA
SCoT 6	CAACAATGGCTACCACGC
SCoT 7	CAACAATGGCTACCACGG
SCoT 8	CAACAATGGCTACCACGT
SCoT 9	CAACAATGGCTACCAGCA
SCoT 10	CAACAATGGCTACCAGCC
SCoT 12	ACGACATGGCGACCAACG
SCoT 16	ACCATGGCTACCACCGAC
SCoT 18	ACCATGGCTACCACCGCC

Statistical analysis

One-way ANOVA was performed followed by Tukey's HSD test ($P \leq 0.05$) to determine the statistically significant differences between the means of the analysed morphological traits. Values shown are means ± SE. PAST software (PAle-ontological STatistics Version 4.11, Natural History Museum, Norway) was used to assess the genetic relationships between analysed genotypes based on SCoT molecular markers.

RESULTS AND DISCUSSIONS

Morphological characterization is important in plant breeding, and knowledge of phenotypic trait variations provides valuable information on the genetic variation of traits under improvement (Tharakan et al., 2005).

In this study, the average number of shoots/individual varied between 1.60 (Tordis cv.) and 2.80 in the clonal selection Caracal 101 (Figure 1).

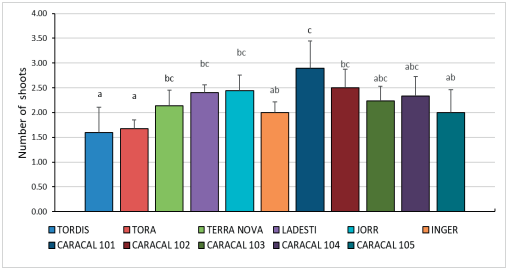

Figure 1. The average number of shoots recorded in eleven willow genotypes

Regarding the average shoot height (Figure 2), it can be seen that the longest shoots were obtained at genotype Caracal 102 (146.41 cm), followed by Caracal 103 (122.89 cm) and Caracal 101 (115.5 cm) with non-statistical differences between their recorded values. The shortest shoots were recorded at Tordis (62 cm) and Lădești 1 (58 cm) practically with a height halved compared to the genotypes named Caracal 101, 102, and 103. An explanation of these results is due to the particularities of vegetative development of the analysed genotypes. Tharakan et al. (2005) also reported in a comparative study of 30 *Salix* spp. clones differences in their growth characteristics.

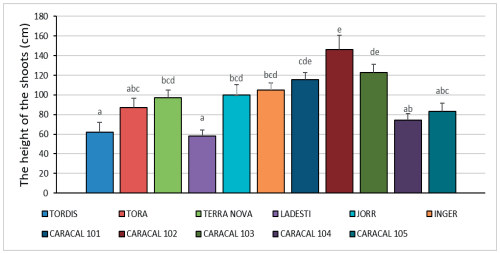

Figure 2. The average shoot height recorded in eleven willow genotypes

Similar to shoot height, the genotypes Caracal 101, 102, and 103 presented the best results in terms of the average of the diameter of shoots (7.46 mm; 8.48 mm, and 7.84 mm, respectively). The lowest mean value of diameter was recorded in Tordis (4.59 mm) and Caracal 104 (4.77 mm) genotypes as shown in Figure 3. It should be noted that Tordis cv. originating from Northern Europe (Sweden) had the weakest morphological development in the environmental conditions of the experimental field organized in Berindei, Romania.

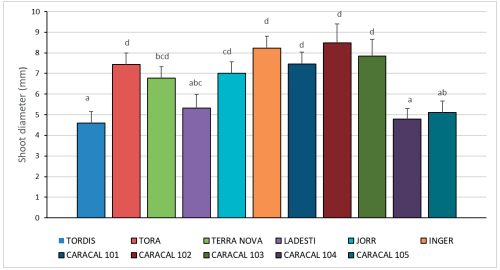

Figure 3. The average shoot diameter recorded in eleven willow genotypes

Leaf morphometry

The longest leaves were recorded in the clonal selection Caracal 102 with 9.94 cm and Inger 10.03 but with statistically non-significant differences. The leaves with the smallest length were measured at Caracal 104 (5.58 cm) followed by Caracal 101 (5.88 cm).

It should be noted that the highest mean value of the leaf width (1.94 cm) was recorded in the Lădești 1 genotype, while the lower value was recorded in Caracal 104 (0.77 cm) (Figure 4).

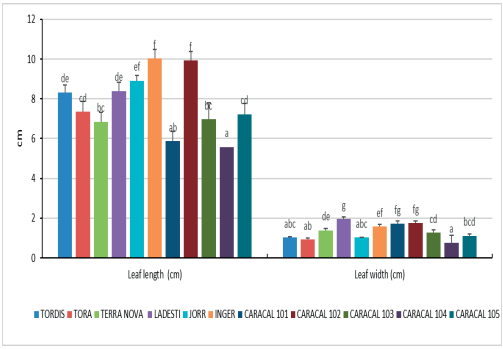

Figure 4. The average leaf length and leaf width (cm) recorded in eleven willow genotypes

The smallest leaf surface (2.83 cm^2) and the smallest leaf perimeter (11.85 cm) were recorded in the Caracal 104 genotype and the best results were recorded in the Caracal 102 both in terms of surface (11.23 cm) as well as the perimeter of the leaf (20.76 cm) (Figure 5).

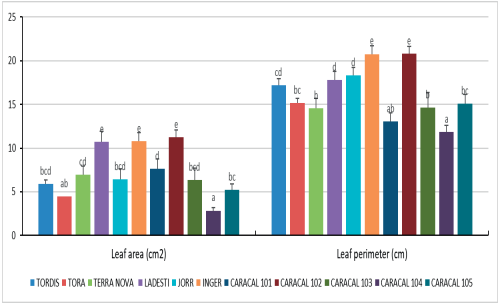

Figure 5. The average leaf area (cm^2) and leaf perimeter (cm) recorded in eleven willow genotypes

After conducting a comparative study of the leaves of the selected clones it was determined that the leaves of the different clones differ in size. In this study, after conducting the comparative analysis of the leaf morphological parameters it can be stated that the leaves of the clones differ in size. Nevertheless, it has been observed that leaf size was also influenced by environmental conditions not only by the biological characteristics of the genotype and its provenance. Our results are in agreement with those reported by Norieka R. (2015) who evaluated the biological characteristics of purple willow (*Salix purpurea* L.) clones and leaf morphometry under experimental short-rotation culture conditions and concluded that

environmental conditions influenced the size of willow leaves.

Genetic analysis based on SCoT molecular markers

In Romania, the efforts to improve willow biomass through breeding are currently hampered by the limited information available on genetic diversity and genetic relationships within and between species, clones and hybrids in the gene pool (Corneanu et al., 2016). On the other hand, according to Przyborowski & Sulima (2010) willow hybridisation occurs commonly in nature and the relatedness of many clones is unclear. Thus, molecular biology techniques were applied to analyse the genetic diversity of willow genotypes (Hanley & Carp, 2013).

The results of the present study show that SCoT markers were suitable to assess the genetic relationships between eleven willow genotypes. Out of the 25 primers screened for their ability to amplify the DNA samples from *Salix* spp. genotypes, twelve revealed reproducible and consistent results. The levels of polymorphism detected with selected SCoT primers are presented in Table 2.

Table2. The level of polymorphism detected with SCoT primers in eleven *Salix* spp. genotypes

Primer name	Size of bands (bp)	NPB	NTB	PPB (%)
SCoT 1	450-1750	8	11	72.72
SCoT 2	400-1500	10	12	83.33
SCoT 3	250-1550	8	10	80.00
SCoT 6	350-2300	10	11	90.90
SCoT 12	250-1700	10	12	83.33
SCoT 13	300-2000	13	13	100.00
SCoT 16	250-2000	10	13	76.92
SCoT 18	350-1750	11	12	91.66
SCoT 19	350-2000	10	11	90.90
SCoT 21	300-1500	8	11	72.72
SCoT 22	600-2500	9	11	81.81
SCoT 23	450-1550	8	10	80.00
Total		113	137	
Mean		9.41	11.41	83.69

The twelve SCoT primers amplified 137 reproducible fragments ranging from 250 to 2500 bp, out of which 113 bands were polymorphic bands (9.41/primer). The number of polymorphic bands for each primer ranged from 8 to 13. The highest number of polymorphic bands (13) was generated by SCoT 13 (Figure 6). The lowest number of amplified polymorphic bands (8) was obtained with the primers SCoT 1, SCoT 3, SCoT 21 and SCoT 23.

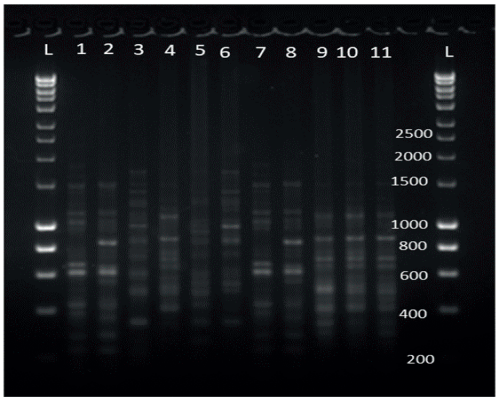

Figure 6. The genetic profiles of 11 genotypes with primer SCoT 13: 1-Caracal 104; 2-Caracal 105; 3-Lădești 1; 4-Jorr; 5-Inger; 6-Terra nova; 7-Tora; 8-Tordis; 9-Caracal 101; 10-Caracal 102; 11-Caracal 103

The percentage of polymorphism (no. of polymorphic bands/ no. of total bands x 100) ranged from 72.72% (SCoT 1 and SCoT 21) to 100.0% (SCoT 13) with a mean value of 83.69%.

In the present study, the genetic diversity between eleven willow genotypes was also assessed by multivariate analysis.

The UPGMA dendrogram, built based on Euclidean distances, grouped the willow genotypes into two main clusters as shown in Figure 7. The first main cluster included two sub-clusters: the first grouped Caracal 105, Caracal 104, Tordis, and Tora; the second sub-cluster grouped the genotypes named Caracal 101-103. In addition, the second main cluster included Jorr, Inger, Terra nova, and Lădești 1, respectively. These clustering patterns suggest that local Caracal 104 and 105 clonal selections differ at the molecular level and confirm their morphological differences due to the species of origin.

For Caracal 101-103 genotypes, phenotypic identification was not possible, being probably interspecific hybrids. In a previous study on the assessment of molecular polymorphism in *Salix* spp. accessions from Romania, also Corneanu et al. (2016) identified a high level of

polymorphism both among different accessions of a species and between species.
In this study, the results of the cluster analysis were then confirmed by the Principal Coordinate Analysis (PCoA) which explained 61.76 % of the total variation (Figure 8).

CONCLUSIONS

In this study, the identification of the local clonal selections Caracal 101-103 that presented the best results regarding most of the analyzed morphological parameters represents promising results regarding their use in future willow breeding programs. Cultivation and evaluation of these local clones for several years under experimental field conditions will represent an important objective in promoting these genotypes for biomass production. To our knowledge, this is the first report highlighting the genetic diversity of *Salix* spp. using SCoT markers.

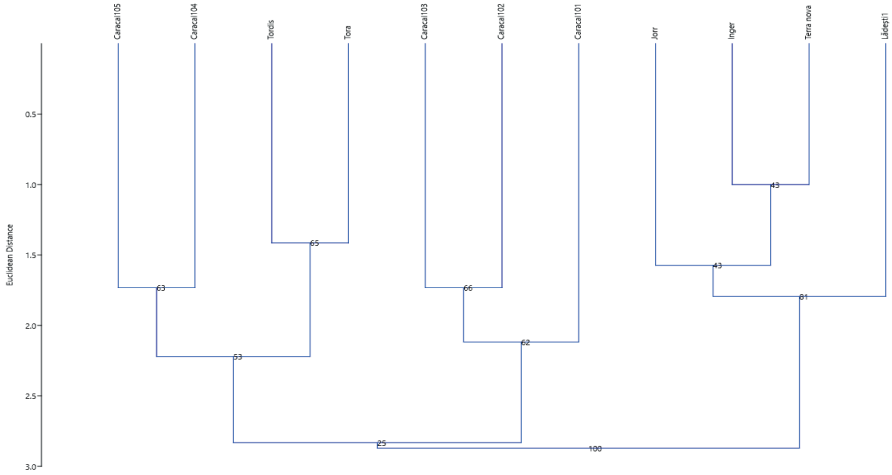

Figure7. UPGMA dendrogram generated by SCoT markers, showing the relationships between eleven willow genotypes and based on Euclidean's distance index. Numbers on the branches show bootstrap values, computed from 9999 replications

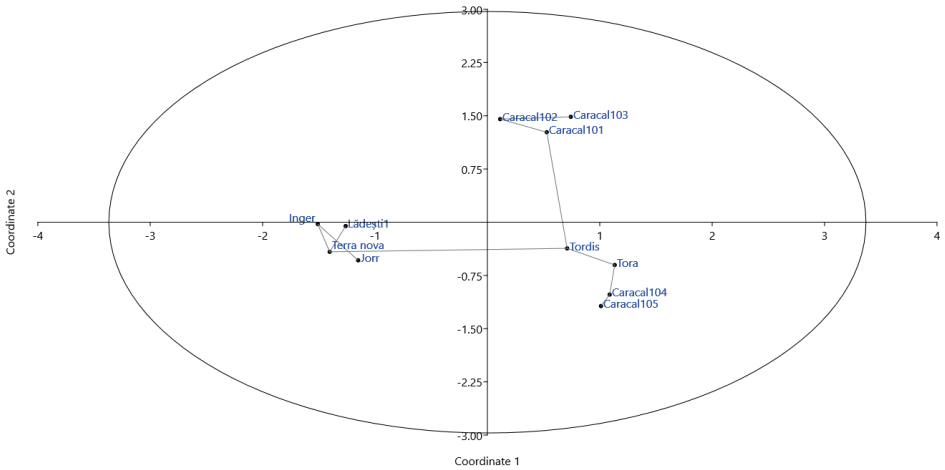

Figure 8. PCoA plot showing the relationships between eleven willow genotypes based on Euclidean distance index

ACKNOWLEDGEMENTS

This research was funded by a Ph.D. Project of the University of Craiova, Faculty of Horticulture and a grant of the Romanian National Authority for Scientific Research and Innovation, CNCS/CCCDI—UEFISCDI, project number PN-III-P2-2.1-PTE-2019-0670, within PNCDI III.

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