Original Research Article

Analysis of morphological variability in five spontaneous Populations of *Rubus ulmifolius* Schott in Tunisia

Abstract

Aim : To study the morphological variability within and among *Rubus ulmifolius* Schott populations, a comparative analysis was undertaken in five wild populations grown under different climatic conditions in Tunisia.

Study Design : Morphological characterization using 10 quantitative traits concerning the morpho-metrical aspect of the shrub, the leaves and the inflorescences of 5 spontaneous populations of *Rubus ulmifolius* Schott.

Place and duration of Study: The provinces of Beja, Bizerte and Jendouba, Tunisia in June 2011.

Methodology: Morphological traits were measured and evaluated separately on a representative population of shrub, leaf and inflorescence. For each site, we made fifteen individual measurements. A sample of one shrub, twenty adult leaves and four inflorescences per plant were evaluated and measured. Samples were collected from fifteen mother shrubs per population, that were separated from each other by more than 20 m to minimize the risk of sampling closely related individuals.

Results : The ANOVA analysis as well as mean comparison of the morphological traits revealed a significant ($P < 0.01$) diversity for the majority of examined descriptors except, the number of flowers NFL (P=0,142) and the number of leaflets NF (P=0,119) which were not significantly different within and among the populations. Furthermore, in the multivariate analysis, the populations were separated into three different groups through the discriminating variables: height of the shrub, leaf dimensions, weight of dry matter, height and weight of the inflorescence.

Conclusion : The morphological variability exhibited by the Tunisian populations of *Rubus ulmifolius* Schott may be interpreted as relevant to the ecological plasticity and the physiological mechanisms. If *Rubus* morphology is partially due to environment, the divergences observed between these genetically differentiated populations suggest that the genetic systems that involve these phenotypes are under selection in the concerned environments. So adaptive morphological changes observed in

these populations reveal probably the progress of evolutionary phenomena within *Rubus ulmifolius* Schott.

Keywords: *Morphological variability, blackberry, Rubus, ANOVA, PCA.*

1. Introduction

The genus *Rubus* contains a large number of highly variably and heterogeneous species, which occur in all parts of the world except dessert regions. The genus has been divided into 12 subgenera of which only a few species have been domesticated ([1]; [2]). The largest subgenus Rubus, the blackberries, is further subdivided into 12 sections. Exotic species of *Rubus* have been introduced to Australia from Europe, North America and Asia [3]. *Rubus ulmifolius* Schott. Rosaceae commonly known as elm-leaf blackberry in English and zarzamora in Spanish is native to Europe and North Africa. This region represents rich biological diversity, particularly in edible fruits including blackberry. According to [4] variability in fruit characteristics of primitive varieties is partially due to their genetic makeup and is also influenced by environmental factors prevailing in the region. [5] intended for the management and utilization of genetic resources of wild raspberry and reported that wild raspberry represents high level of genetic variation among accessions for morphological traits. Previous study carried out by [6] and [7] on the genetic diversity of Colombian blackberries identified high phenotypic and molecular plasticity in the R. glaucus species. A study of morphological and genetic variation within the *Rubus fruticosus agg.* revealed at least fifteen species, the commonest and most widespread of which is R. anglocandicans, known previously as *R. procerus* or *R. discolor*. DNA phenotyping was used to confirm identification in doubtful cases and to support taxonomic determination based on morphology [8].

Studies on the genetic diversity of *Rubus* have been carried out in temperate species, such as *Rubus idaeus* ([9]; [10]; [11]) and *Rubus occidentalis* [12], and Asian species [13]. These genetic diversity and transferability of Rubus Microsatellite Markers to South American *Rubus* Species 153 works used RAPD, RFLP, and SCAR markers as well as SSR [14]. These plants were also submitted to morphological, agronomic, and molecular characterizations using AFLP and SSR molecular markers [7].

R. ulmifolius (*Rubus ulmifolius)* have been used in traditional medicine for their beneficial effects. Blackberry leaves have been used for their anti-inflammatory, antiviral and antimicrobial properties as well as their antiproliferative activity against cancer cells [15].

The choice of *Rubus ulmifolius* Schott for the investigations done here was dictated by the fact that it has aerial part of well-recognised structure, characteristic for the Rosa genus. In the Rosa genus taxonomy, it is considered as a “good species” with distinct diagnostic features ([16], [17]; [18] ; [19]).

The objective of this study was to evaluate the morphological diversity within and among natural populations of *Rubus ulmifolius* Schott populations in North West of Tunisia using 10 quantitative morphological traits.

2. Materials and methods

2.1. Plant material and study area

Fig. 1 shows the areas where the samples collected in June 2011. The survey area covers latitudes 36° to 37° N and longitudes 8° to 9° E and includes the provinces of Béja, Bizerte and Jendouba. Traits were measured and evaluated separately on a representative population of shrub, leaf and inflorescence. For each site, we made fifteen individual measurements. A sample of one shrub, twenty adult leaves and four inflorescences per plant were evaluated and measured. Samples were collected from fifteen mother shrubs per population, that were separated from each other by more than 20 m to minimize the risk of sampling closely related individuals. Location information of the sampled stands is provided in Table 1. All traits were taken from healthy and undamaged shrubs.

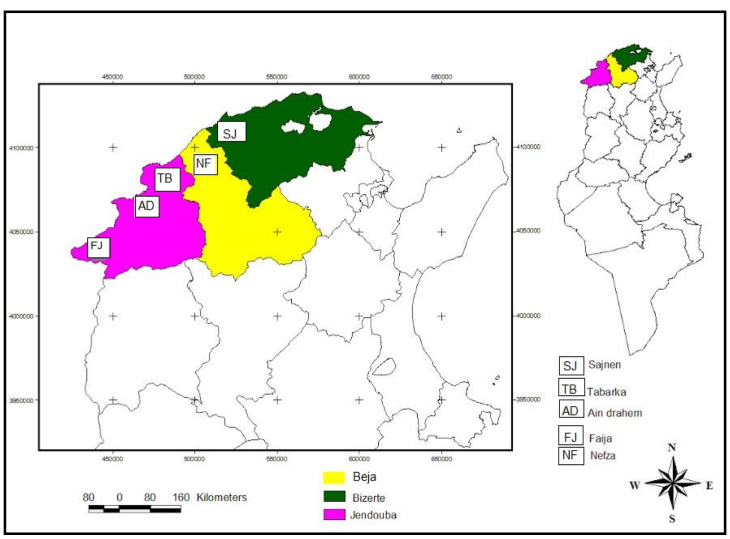

Fig. 1. Geographic location of prospected localities for morphological variability of *Rubus ulmifolius* Schott from North Tunisia

2.2. Pheno-morphological measurement

The pheno-morphological characterization within and among the analyzed populations was assessed using ten quantitative traits. Measured traits included Shrub height (H), Dry matter weight (PMS), average number of flowers per inflorescence (NFL), average Inflorescence height (HF), average Inflorescence weight (PF), average numbers of Leaflets per leaf (NF), average length of Leaf blade (LF), average Leaf blade width (LAF), average petiole length (LP), average petiole width (LAP). Leaves and inflorescences descriptors in each genotype were measured on twenty leaves and four inflorescences randomly collected from the same shrub (Table 2).

Table 1. Administrative data and GPS sites studied.

Name of site	Delegation	Acronym	Governorate	GPS coordinates	Altitude (m)	Bioclimatic stage
Ras Errajel	Tabarka	TB	Jendouba	36° 56' **N**, 8° 52' **E**	179	Subhumid
Babbouch	Aïn Drahem	AD	Jendouba	36° 42' **N**, 8° 40' **E**	670	humid
Bellif	Nefza	NF	Béja	37° 02' **N**, 9° 03' **E**	67	Subhumid
Sidi Mechreg	Sejnene	SJ	Bizerte	37° 07' **N**, 9° 06' **E**	90	Subhumid
Faïja	Ghardimaou	FJ	Jendouba	36° 49' **N**, 8° 30' **E**	910	Subhumid

2.3. Quantitative trait measurement

Seventy five shrubs were selected for the five populations for measurement of plant height (m), using a measuring tape. Twenty leaves were selected randomly from the one hundred leaves collected per shrub for measurement of leaf dimensions (cm, mm), by use of a vernier caliper. Four randomly selected inflorescences per shrub for the five populations for measurement of inflorescence height (cm) and weight (g). Weight of dry matter (PMS) (g) was also determined, using a weighing balance to the nearest 0.001g. Number of leaflets and flowers, respectively, in each leaf and inflorescence measured was counted. Seventy five shrubs were identified based in the height morpho-metric traits of mature plant. Each provenance consisted of fifteen mother-shrub, twenty leaves and four inflorescences per shrub. List of quantitative traits used as descriptors is provided in Table 2.

Table 2. List of shrub, leaf and inflorescence quantitative traits used as descriptors.

Characters	**Acronym**
Shrub height (m)	H
Leaf blade length (cm)	LF
Leaf blade width (cm)	LAF
Petiole length (mm)	LP
Petiole width (mm)	LAP
Leaflets number	NF
Inflorescence height (cm)	HF
Flowers number	NFL
Inflorescence weight (g)	PF
Dry matter weight (g)	PMS

2.4. Statistical Analyses

2.4.1. Analysis of variance and mean comparisons

The (ANOVA) method was used to determine the effect of site on all morphological traits. Differences between mean values were compared using the Duncan multiple range test (at 5% level).

2.4.2. Principal component analysis

Principal component analysis (PCA) is carried out in order to discriminate between populations on the basis of linear combinations of descriptors.

2.4.3. Canonical discriminant analysis

In canonical discriminant analysis, a multivariate statistical technique, all independent variables are considered simultaneously in the differentiation of populations. The resulting differentiation of populations is more distinct compared with univariate analysis. Therefore, canonical discriminant analysis determines the linear combinations of independent variables which best discriminates among the groups [20]. Then, to study the structuring of variability in *R. ulmifolius*, a canonical discriminant analysis, based on [21], was carried out on all individuals, representing five populations (Aïn drahem, Faïja, Nefsa, Sajnen and Tabarka, respectively, designated as AD, FJ, NF, SJ,TB). Canonical discriminant analysis was used to perform graphical representation of the five populations of north Tunisia on a two dimensional graph.

All analyses were carried out using the XLSTAT 2015 program on all individuals, for the seventy five shrubs selected in the five provenances.

3. Results

In spite of the observed variation, the ANOVA revealed statistically-significant differences between the populations for the majority of morphological traits (*P* < 0.01) except the number of leaflets (NF) and flowers (NFL) (Table 3).

Table 3. Descriptive statistics of characters measured in 75 Shrubs within 5 populations of *R. ulmifolius* [mean character values, degree of freedom and the associated F and P values, for the significance of the differences.

Variable	Mean	df	F	P
(H) (m)	3,153	4	10,403	0,000***
(HF) (cm)	12,010	4	6,546	0,000***
(LAF) (cm)	12,017	4	12,145	0,000***
(LF) (cm)	10,590	4	9,493	0,000***
(LAP) (mm)	2,694	4	15,622	0,000***
(LP) (mm)	3,762	4	6,498	0,000***
(NF)	4,573	4	1,906	0,119*
(NFL)	25,773	4	1,784	0,142*
(PF) (g)	3,217	4	5,322	0,001***
(PMS) (g)	0,396	4	33,157	0,000***

***: highly significant (P ≤ 0.01); *: not significant.

The comparison of means (Tables 3, 4) reveals that the population FJ showed the highest values for: height of the shrub (H) (3.67 m), width of the leaf blade (LAF) (13.88 cm), length of the leaf blade (LF)

(12.20 cm), width of petiole (LAP) (3.62 mm) and length of petiole (LP) (4.47 mm). Population AD showed the highest mean values for the height of inflorescence (HF) (14.78 cm). Population TB showed the highest mean values for the weight of inflorescence (PF) (4.61 g). The tow Population NF and SJ showed the highest mean values for the weight of dry matter (PMS) (0.44 g).

Table 4. Descriptive statistics (mean) for 8 morphological traits measured in 5 populations of *R. ulmifolius*.

Populations	H	HF	LAF	LF	LAP	LP	PF	PMS
Aïn drahem	2,79 **b**	14,78 **a**	10,41 **b**	9,29 **c**	2,36 **b**	3,17 **b**	3,13 **b**	0,33 **b**
Faïja	3,67 **a**	10,52 **c**	13,88 **a**	12,20 **a**	3,62 **a**	4,47 **a**	2,51 **b**	0,43 **a**
Nefsa	2,55 **b**	9,91 **c**	10,48 **b**	9,35 **c**	2,36 **b**	3,19 **b**	2,40 **b**	0,44 **a**
Sajnen	3,45 **a**	12,04 **bc**	12,73 **a**	11,12 **ab**	2,56 **b**	4,11 **a**	3,43 **b**	0,44 **a**
Tabarka	3,30 **a**	12,79 **ab**	12,59 **a**	10,99 **b**	2,56 **b**	3,87 **a**	4,61 **a**	0,34 **b**

Means followed by different letters (a–c) are significantly different according to the Duncan test ($P < 0.05$).

This study also revealed high, significant and positive correlation coefficients between the width (LAF) and length of the leaf blade(LF) (r = 0.942); the number of flowers (NFL) and weight of inflorescence (PF) (r = 0.869); the number of leaflets (NF) and the width of the leaf blade (LAF) (r = 0.403); the length of the leaf blade (LF) and the width of petiole (LAP) (r = 0.635); the length of petiole (LP) and the height of the shrub (H) (r = 0.556); the number of leaflets (NF) and the width of petiole (LAP) (r = 0.478); the height of inflorescence (HF) and the number of flowers (NFL) (r = 0.672); the width of the leaf blade (LAF) and the width of petiole (LAP) (r = 0.710); the number of leaflets (NF) and the length of petiole (LP) (r = 0.417); the height (HF) and weight of inflorescence (PF) (r = 0.680); the width of the leaf blade (LAF) and the length of petiole (LP) (r = 0.726); the height of the shrub (H) and the width of petiole (LAP) (r = 0.444); length of the leaf blade (LF) and the length of petiole (LP) (r =

0.709); the height of the shrub (H) and the width of the leaf blade(LAF) (r = 0.717); the width (LAP) and length of petiole (LP) (r = 0.622); the height of the shrub (H) and length of the leaf blade (LF) (r = 0.727). Significantly and positively correlation coefficients were observed between the number of leaflets (NF) and the length of the leaf blade (LF) (r = 0.330); the height of the shrub (H) and the number of leaflets (NF) (r = 0.356). While the weight of the dry matter (PMS) is not correlated with any of the other characters (Table 5).

Table 5. Pearson's correlation among ten traits in a collection of 5 Tunisian *R. ulmifolius* populations.

Trait	NF	LAF	LF	LAP	LP	H	HF	NFL	PF	PMS
NF	**1**									
LAF	0,403**	**1**								
LF	0,330*	0,942**	**1**							
LAP	0,478**	0,710**	0,635**	**1**						
LP	0,417**	0,726**	0,709**	0,622**	**1**					
H	0,356*	0,717**	0,727**	0,444**	0,556**	**1**				
HF	-0,074	0,039	0,064	-0,013	0,069	0,073	**1**			
NFL	-0,138	-0,039	-0,063	-0,044	-0,014	0,005	0,672**	**1**		
PF	-0,076	0,098	0,062	-0,050	0,052	0,103	0,680**	0,869**	**1**	
PMS	0,086	0,085	0,074	0,198	0,120	0,109	-0,333	-0,083	-0,207	**1**

* Significant ($P < 0.05$); ** highly significant ($P \leq 0.01$).

Table 6 shows relative and percent proportions of the total variance for each of the first three principal components, the calculated eigen values and the coefficient of correlations between the principal components (axis 1, axis 2 and axis 3) and the original variables; these coefficients indicate the contribution of each trait to the formation of axis 1, axis 2 and axis 3.

The first three principal components explain 75, 96% of the total variance; in particular Axis 1 contributing with 40.27%, Axis 2 with 25.93% and Axis 3 with 9.74%. Axis 1 is mostly positively correlated with leaf dimensions, the number of leaflets and the height of the shrub; Axis 2 is mostly positively correlated with parameters of inflorescence; Axis 3 is positively correlated especially with dry matter.

Table 6. Correlation between the first three principal components (axis) and original variables for collection of the 5 Tunisian populations of *Rubus ulmifolius*.

Components	**Axis 1**	**Axis 2**	**Axis 3**
Eigen values	4,028	2,594	0,975
Proportion of total variance (%)	40,278	25,937	9,747
Cumulative (%)	40,278	66,215	75,961
Variables defining the axis of PCA and their correlations	NF(0,56), LAF(0,94), LF(0,91), LAP(0,80), LP(0,83), H(0,78)	HF(0,86), PF(0,92), NFL(0,90)	PMS(0,90)

The first three components scores account for 57.06 %; 20.28 % and 16.10 % of the total variation, respectively (Table 7). The first canonical discriminant root was strong enough to separate populations. Axis 1 was highly correlated with LAF, LF, LAP, HF and PMS, axis 2 with H, LP and PE. According to Figure 2, the projections of five populations on the plane formed by the two components F1 and F2 show that the populations AD, NF and SJ form a single group while the populations TB and FJ form two different groupings.

Table7. Canonical discriminant analysis of the 5 Tunisian populations of *Rubus ulmifolius*.

Canonical axis	**Axis 1**	**Axis 2**	**Axis 3**
Eigen values	3,006	1,068	0,848
discrimination (%)	57,064	20,283	16,103
Cumulative (%)	57,064	77,347	93,449
Variables defining the axis and their correlations	LAF(0,43), LF (0,41), LAP(0,48), HF(-0,53),PMS(0,87)	LP(-0,50), H(-0,66), PF(-0,44)	---

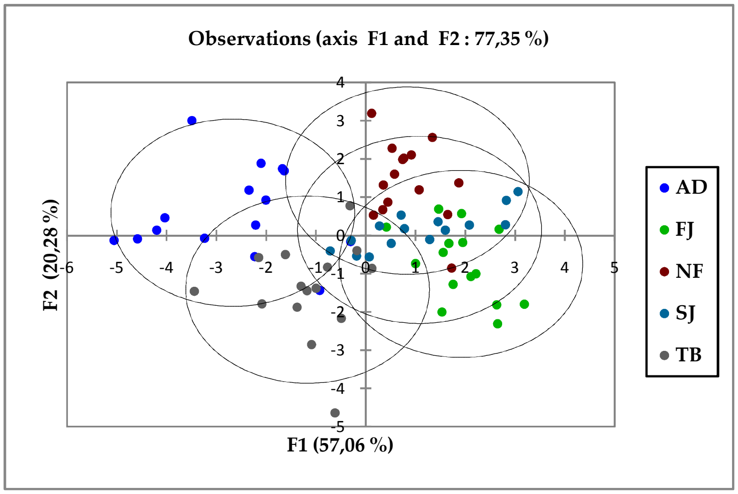

Fig. 2. Projection of the *R. ulmifolius* population onto the plane defined by the first two canonical discriminant functions (F1 and F2) performed

4. Discussion

Many parameters affect growth and health condition of *R. ulmifolius.* These are physical factors (climate, soils, water balance and topography), biological factors (diseases and pests, especially insect) and traditional constraints (human land use systems). The study of *R. ulmifolius* morpho-metrics traits of the natural populations is often considered to be useful step in the study of the genetic variability. This would involve identifying different provenances representing homogenous environmental zones within which selections are deployed and used in further breeding. Phenotypic variation is determined by genotype and environment interactions and is assumed to be the expression of genotypic variation when environmental conditions are controlled ([22]; [23]; [24]; [25]).

The general linear model (ANOVA) revealed significant inter-population differences for the majority of examined traits ($P < 0.01$) except the number of flowers (NFL) ($P = 0.142$) and the number of leaflets (NF) ($P = 0.119$). The comparison of means reveals that the population FJ showed the highest values for: height of the shrub (H) (3.67 m), width of the leaf blade (LAF) (13.88 cm), length of the leaf blade (LF) (12.20 cm), width of petiole (LAP) (3.62 mm) and length of petiole (LP) (4.47 mm).

Principal component analysis (PCA) shows that first three principal components explain 75.96% of the total variance. Axis 1 is mostly positively correlated with leaf dimensions, the number of leaflets and the height of the shrub; Axis 2 is mostly positively correlated with parameters of inflorescence; Axis 3 is positively correlated especially with dry matter.

The canonical variates were used to cluster the populations into three groups on the basis of the differentiating traits. Canonical discriminant analysis was useful in identifying the genetic variation and the traits that better describe the variation among *R. ulmifolius* populations. The results revealed that shrub, leaf and inflorescences characters except, the number of flowers (NFL) and leaflets (NF) were prevalent in the first and the second discriminant functions, and contributed most to the total variation (77.34 %).

However, the divergence between sites was relatively weak, reflecting their similar climatic conditions. Topography of the location and variation in climatic conditions (rainfall, temperatures, soil type…) might explain the differences in morphological traits between populations.

These results are in agreement with the findings of [26] who recorded maximum height in cultivated raspberry plants growing in rich organic soil. Further, the number of inflorescences and flowers, plant shape, dry matter accumulation and plant size also depend upon light penetration into the plant ([26]; [27]). Wild blackberry plants studied, also exhibited diversity in foliar dimensions among the locations. This variability in leaf size is consistence with the findings of [28] who observed remarkable diversity in leaves of Rosaceous fruits. This diversity might be due to environmental as well as genetic factors and other salient features of the area. Also Concerning quantitative characteristics of wild raspberry (*Rubus idaeus L.*), significant differences were found in plant height, leaf length and width and number of braches per plant among the samples collected from three different locations of Azad Jammu and Kashmir (Pakistan). This diversity might be due prevailing soil and climatic conditions and topography of the location [29].

[30] and [31] reported that morphological and physiological properties of most woody plants are affected by different abiotic factors, in particular over altitudinal gradients.

5. Conclusion

In this study, morpho-metric traits were used for characterization of genetic diversity within and among natural populations of *R. ulmifolius.* The information obtained would be used for germplasm conservation, management and selection for domestication and improved shrub regeneration. We have observed significant variations within shrub in provenance in all traits (P < 0.01), except the number of flowers (NFL) (P = 0.142) and the number of leaflets (NF) (P = 0.119). Intra and inter-provenance variability were of the same order; therefore, it was very difficult to characterize provenances according to their geographic origin. This diversity is explained by the hybridization, apomixis and polyploidy in this species which makes very difficult a breeding program based in morphological markers. The logical reason for its diversity is also prevailing climatic conditions like temperature, sunlight, rainfall, snowfall and hailstorms.

In order to make *R. ulmifolius* rural in Tunisia a commercially practicable business, there is urgent need to accomplish molecular genetic diversity assessment of the species throughout its natural habitats in the country. The hyper-variability of molecular markers constitutes great advantages for these types of studies. Further research including available ISSR (inter simple sequence repeats) and microsatellites marker would hopefully provide additional information.

The surveyed ecosystems in North Tunisia undergo a very strong regression due mainly to anthropogenic pressures of many small communities surrounding these natural stands. Our investigation on the morphological characterization of *R. ulmifolius* demonstrated a large diversity and allowed us to assess the phenotypic biodiversity of this local species. These findings suggest the importance of preserving the genetic resources of wild pear in this area in order to establish an ex situ collection; and could be a starting point for further studies, using for instance molecular markers with the aim of implementing a conservation strategy that prevents genetic erosion, and consequently the reduction of the biodiversity of this species in North Tunisia. Furthermore, this study allowed us to validate the morpho-metrical approach as a tool for selection of genotypes for afforestation. The evaluation of local populations has important implications for genetic improvement of *R. ulmifolius* in term of predicting ability or useful agronomic traits.

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